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Protective effect of porcine placenta in a menopausal ovariectomized mouse

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Abstract

Menopause is a significant physiological phase that occurs as women's ovaries stop producing ovum and the production of estrogen declines. Human placenta and some amino acids are known to improve menopausal symptoms. In this study, we investigated that porcine placenta extract (PPE) and arginine (Arg), a main amino acid of PPE, would have estrogenic activities in ovariectomized (OVX) mice as a menopause mouse model, human breast cancer cell line (MCF-7) cells, and human osteoblast cell line (MG-63) cells. PPE or Arg significantly inhibited the body weight and increased the vagina weight compared to the OVX mice. PPE or Arg ameliorated the vaginal atrophy in the OVX mice. The levels of 17β-estradiol and the activities of alkaline phosphatase (ALP) were significantly increased by PPE or Arg in the serum of OVX mice. Trabecular bone parameters such as bone mineral density and porosity were also improved by PPE or Arg in the OVX mice. In the MCF-7 and MG-63 cells, PPE or Arg significantly increased the cell proliferation, estrogen receptor β mRNA expression, and estrogen-response elements luciferase activity. Finally, PPE or Arg increased the activations of ALP and extracellular signal-regulated kinase 1/2 in the MG-63 cells. These results indicate that PPE or Arg would have estrogenic and osteoblastic activity. Therefore, PPE or Arg may be useful as new pharmacological tools for treating menopausal symptoms including osteoporosis.

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Introduction

Menopause is a natural biological phase that occurs in every woman's life. The menopause leads to typical menopausal symptoms such as insomnia, vaginal dryness, hot flushes, or night sweats (Dennerstein *et al.* 2000). However, postmenopausal status is associated with an increased risk of immune disorders such as rheumatoid arthritis (D'Amelio 2013) or metabolic syndromes such as obesity due to estrogen deficiency (Kaaja 2008). A systemic skeletal disease, osteoporosis, characterized by microarchitectural deterioration of bone tissue with a consequent increase in bone fragility and low bone mass, also resulted from menopause or estrogen deficiency (Shuid *et al.* 2011). Estrogen is a well-known regulator of bone metabolism, post-menopausal osteoporosis, and the morbidity which are major health problems in elderly women (Melton *et al.* 2004). Hormone replacement therapy (HRT) has been used to reduce these symptoms and to protect women against estrogen deficiency. However, HRT was reported to increase the risk of breast cancer (Lagro-Janssen *et al.* 2010).

Some specific types of amino acids are known to improve menopausal symptoms. In menopause, arginine (Arg), a kind of semi-essential amino acids, contributes to decreasing hot flushes or endothelial dysfunctions resulting from a nitric oxide synthase insufficiency (Tuomikoski *et al.* 2012). Also, lysine, a kind of essential amino acids, can support this effect of Arg and is increasingly recommended for menopausal women who are at risk of osteoporosis because of boosting the absorption of calcium in bones (Civitelli *et al.* 1992). As these amino acids are not produced by the human body or not produced sufficiently, they have to be ingested from food. Placenta is a reservoir of a large number of bioactive molecules such as hormones, proteins, and amino acids. Human placenta has traditionally been used to improve menopausal symptoms in Korea (Kong & Park 2012). Porcine placenta extract (PPE) was reported to have anti-oxidant (Choi *et al.* 2014) or immune activity-enhancing

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Table 1 Amino acid analysis of PPE.

Test subject	Result (mg/100 g)
Free amino acid (arginine)	1668.57
Free amino acid (cystine)	88.38
Free amino acid (tyrosine)	932.21
Free amino acid (threonine)	951.54
Free amino acid (alanine)	1445.69
Free amino acid (proline)	609.90
Free amino acid (lysine)	1788.53
Free amino acid (histidine)	991.48
Free amino acid (isoleucine)	935.58
Free amino acid (leucine)	2452.40
Free amino acid (methionine)	476.66
Free amino acid (phenylalanine)	1309.27
Free amino acid (tryptophane)	377.40
Free amino acid (valine)	1397.35
Free amino acid (glutamic acid)	1270.14
Free amino acid (aspartic acid)	965.80
Free amino acid (serine)	978.91
Free amino acid (glycine)	549.83

effect (Lee *et al.* 2013). In addition, PPE showed neuroprotective and cognition-enhancing effects in a murine menopause model (Takuma *et al.* 2012). PPE is an abundance of various amino acids (Table 1).

We hypothesized that PPE consisting of various amino acids would regulate various menopausal symptoms. Our present study thus aimed at clarifying the effect of PPE and Arg, a main amino acid of PPE, in an ovariectomized (OVX) menopausal animal model (Yamazaki & Yamaguchi 1989), estrogen receptor (ER)-positive human breast cancer cell line (MCF-7) cells (Dickson *et al.* 1986), and ER-positive and osteoblast-like human osteosarcoma cell line (MG-63) cells (Dohi *et al.* 2008).

Materials and methods

Materials

We purchased DMEM and fetal bovine serum (FBS) from Gibco BRL; anti-extracellular signal-regulated kinase (ERK) and anti-phosphorylated ERK (pERK) antibodies from Santa Cruz Biotechnology.

Preparation of PPE, Arg, and 17β-estradiol

Lyophilized powder of porcine placenta was obtained from Cellamedic Korea Co. (Seoul, Republic of Korea), dissolved in distilled water (DW), filtered with 0.22 μm syringe filter, and prepared at doses of 0.1, 1, and 10 μg/ml according to a previous report (Han *et al.* 2013). Arg (Sigma Chemical Co.) was also dissolved in DW, filtered with 0.22 μm syringe filter, prepared at a dose of 10 μg/ml according to a previous report (Han *et al.* 2013). 17β-estradiol (E_2, Sigma Chemical Co.) was dissolved in dimethyl sulfoxide, filtered with 0.22 μm syringe filter, prepared at a dose of 100 nM according to a previous report (Choi *et al.* 2011).

Animals

Seven-week-old female BALB/cAnNTac mice were purchased from Dae-Han Experimental animal center (Eumsung, Chungbuk, Republic of Korea) possessing mice imported from Taconic Biosciences, Inc. (Hudson, NY, USA). The mice were acclimated for 7 days prior to experimentation. Animals were maintained at a temperature of 22±1 °C and a relative humidity of 55±10% under a 12 h light:12 h darkness cycle (light on at 0700 h and off at 1900 h) throughout the study. Any animals were not be used more than once. All protocols for animals were approved by the institutional animal care committee of Kyung Hee University (KHUASP (SE)-14-024).

Animal experiment protocol

Ovariectomy was done under general anesthesia. A bilateral dorsolateral incision was made below the skin and the underlying muscle was dissected to locate ovaries and fallopian tube. The tubes were ligated with a suture line and the ovaries were taken out. The skin and muscle were then sutured with an absorbable suture. Post surgery, povidone-iodine solution (Sungkwang Pham. Co., Ltd, Cheonan, Republic of Korea) was applied to OVX mice. After 3 weeks of recovery from surgery, the mice were randomly assigned to eight groups ($n=5$ per group): sham-operated mice, sham-operated mice treated with 10 mg/kg PPE, OVX mice, OVX mice treated with 10 mg/kg Arg, OVX mice treated with 100 nM E_2, and OVX mice treated with 0.1, 1, and 10 mg/kg PPE. PPE, Arg, or E_2 were orally administered daily for 8 weeks. The body weight of each mouse was measured once a week until the final day of administration. On the last day of the study, blood samples were collected from heart and then stored at −70 °C until examination. The vaginas and uteruses were dissected out and immediately weighed. The dissected bones were fixed with formaldehyde.

Analysis of serum

Blood was collected to analyze the levels of E_2, luteinizing hormone (LH), and alkaline phosphatase (ALP) with enzyme linked immunosorbent assay. The levels of E_2 (Invitrogen), LH (Abcam, Cambridge, UK), and ALP (Abcam) were analyzed according to the manufacturer's instructions.

Immunohistochemistry

Formaldehyde-fixed vaginal tissues were embedded in paraffin and cut into 4 μm-thick sections. After dewaxing and dehydration, the vaginal specimens are treated with 1% methylene blue for 45 min. The specimens can be examined after mounting.

Microcomputed tomography

Trabecular bone parameters were measured on a region 0.4–0.9 mm from growth plate. High-resolution microcomputed tomography (μCT) was used to provide the 2D and 3D information on bone geometry, as previously described (Kim *et al.* 2014).

Cell cultures

MCF-7 and MG-63 cells were purchased from Korean Cell Line Bank (KCLB, Seoul, Republic of Korea) and cultured in DMEM,

1% penicillin/streptomycin, and 10% FBS at 37 °C under 5% CO_2 in humidified air.

Bromodeoxyuridine assay

MCF-7 cells and MG-63 cells (1×10^4) were treated with PPE, Arg, or E_2 for 48 h. The proliferation was determined using a colorimetric immunoassay based on the mensuration of bromodeoxyuridine incorporated by DNA synthesis (Roche Diagnostics GmbH).

Quantitative real-time PCR

Total RNA was isolated from MCF-7 cells or MG-63 cells according to the manufacturer's protocol using an easy-BLUE RNA extraction kit (iNtRON Biotech, Seongnam, Republic of Korea). The concentration of total RNA in the final elutes was measured by Thermo Scientific (Wilmington, DE, USA) NanoDrop spectrophotometer. The cDNA synthesis was performed for 60 min at 42 °C and 5 min at 94 °C using a cDNA synthesis kit (Bioneer Corporation, Daejeon, Republic of Korea). Quantitative real-time PCR was performed with a SYBR Green master mix using an ABI StepOne real-time PCR System (Applied Biosystems). Primer sequences for the reference gene GAPDH, gene Ki-67, and gene ERβ were as follows: GAPDH (5′-TCG ACA GTC AGC CGC ATC TTC TTT-3′; 5′-ACC AAA TCC GTT GAC TCC GAC CTT-3′); Ki-67 (5′-ATA AAC ACC CCA ACA CAC ACA A-3′; 5′-GCC ACT TCT TCA TCC AGT TAC-3′); ERβ (5′-TTC CCA GCA ATG TCA CTA ACT T-3′; 5′-TTG AGG TTC CGC ATA CAG A-3′). We determined the expression ratios relative to the control sample and normalized them to GAPDH.

Transient transfections and estrogen-response element luciferase reporter assay

The reporter gene estrogen-response element (ERE)-TATA-Luc was constructed using the enhanced luciferase reporter gene. We used Lipofectamine 2000 (Invitrogen) to transiently transfect pERE-TATA-Luc and pSV40-Luc reporter gene constructs into each MCF-7 cell and MG-63 cell. Next, the both of MCF-7 cells and MG-63 cells were treated with PPE, Arg, or E_2 for 48 h after transfection and then harvested. To measure the luciferase activity, we used a luminometer 1420 luminescence counter (Perkin Elmer Inc., Waltham, MA, USA) following the manufacturer's instructions. The relative luciferase activity was determined as the ratio of firefly to renilla luciferase activity.

ALP assay

For ALP assay, MG-63 cells were plated in 96-well (3×10^5) plates and incubated for 24 h. After induction, total ALP activity was analyzed using *p*-nitrophenylphosphate and quantified colorimetrically at 405 nm according to the manufacturer's instructions.

Western blot analysis

MG-63 cells were treated with PPE, Arg, or E_2 for 5 min and then harvested. MG-63 cells were lysed and separated through 10% sodium dodecyl sulfate-PAGE. After electrophoresis, the proteins were transferred to nitrocellulose membranes. Next, the membrane was blocked with 5% skim milk in PBS-tween-20 for 1 h at room temperature, incubated with anti-ERK or anti-pERK antibodies, and then incubated with secondary antibodies. Finally, the protein bands were visualized using an enhanced chemiluminesence assay (Amersham Co.) following the manufacturer's protocol.

Statistical analysis

A power analysis was performed to determine a suitable sample size. Using two independent sample *t*-tests, we calculated the sample sizes and G power. Sample size (five mice per group: type I error 0.05; power 98.22%) was determined in a pilot study. All data are representative of three independent experiments and represented as the mean ± S.E.M. The statistical values of the results were performed by an independent *t*-test, an ANOVA with a Tukey *post hoc* test, or an ANOVA with a least significant difference *post hoc* test. The results were considered significant at a value of $P<0.05$.

Results

Administration with PPE or Arg improved body and vagina weights in OVX mice

The menopause was characterized by increased body weight (Alvehus *et al.* 2012). First, we investigated the regulatory effect of body weight of PPE or Arg in the OVX mice. From 5 weeks of oral administration, the mean of body weight of the OVX group significantly started being increased, compared to the sham group. At three and 4 weeks, Arg significantly reduced the body weight compared to the OVX group ($P<0.05$; Fig. 1A). At the end of the study, PPE (10 mg/kg), Arg, or E_2 (a positive control) significantly inhibited the body weight compared to the OVX group ($P<0.05$; Fig. 1A). And PPE, Arg, or E_2 significantly increased the vagina weight decreased by ovariectomy ($P<0.05$; Fig. 1B). However, PPE or Arg did not significantly increase uterus weight (Fig. 1C). PPE (10 mg/kg) does not have an effect on the body, vagina, and uterus weights in sham-operated mice. Histological analysis of the vagina showed that vaginal epithelium of the OVX mice was atrophic with less cell layers composed of flattened cells with no cornification and thin epithelial thickness. However, multilayered epithelium layers with cornification and increased epithelial thickness were observed in the vaginal epithelium of the OVX mice treated with PPE, Arg, or E_2 (Fig. 1D upper panel). In addition, the size of vaginal cells from the OVX mice was smaller than those from the OVX mice treated with PPE, Arg, or E_2 (Fig. 1D lower panel).

Administration with PPE or Arg increased serum E_2 and ALP in OVX mice

The biology underlying the transition to menopause involves central neuroendocrine changes (Burger *et al.* 2002).

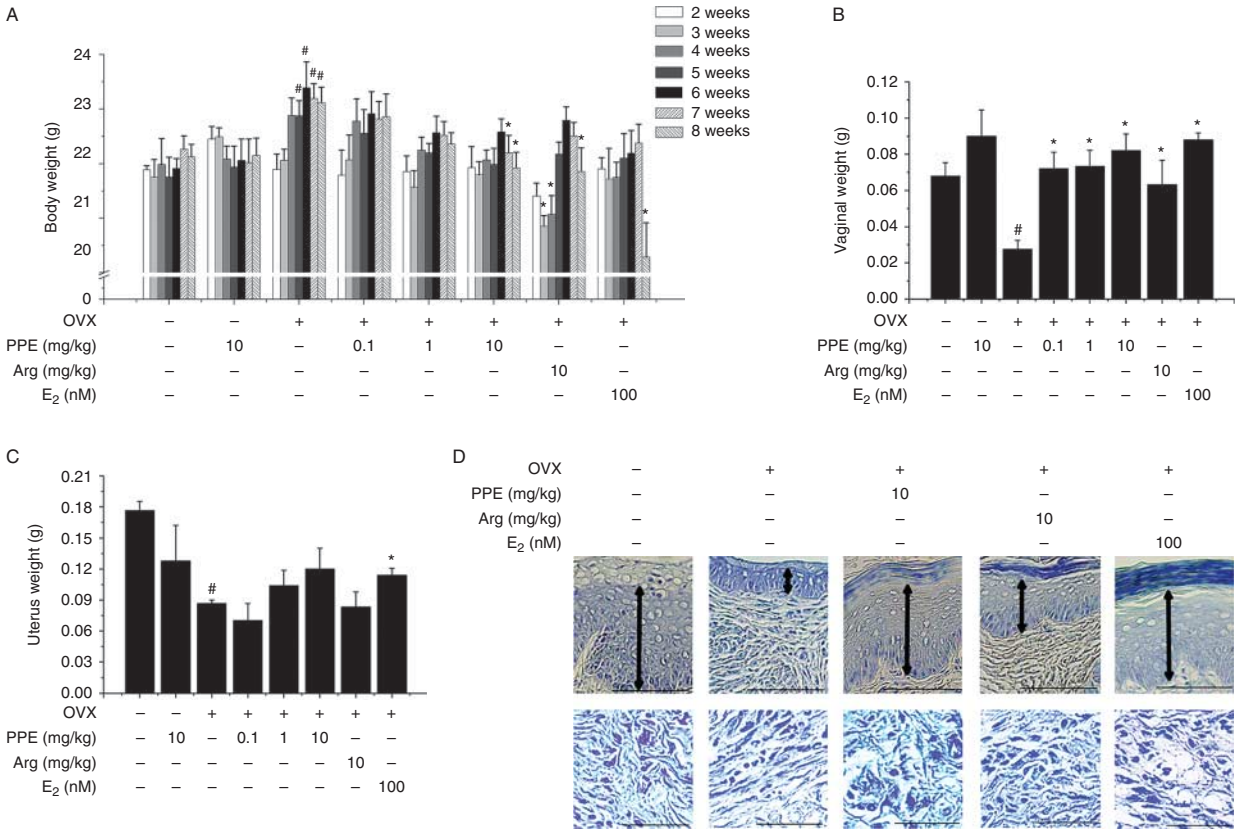

Figure 1 Administration with PPE or Arg improved body and vagina weights in the OVX mice. (A) Body weight was measured once a week for 8 weeks. The weights of (B) vagina and (C) uterus were measured at the end of the 8-week treatment period. $^{\#}P<0.05$; significantly different from the sham-operated mice. $^{*}P<0.05$; significantly different from the OVX mice. (D) Each vaginal section was stained with methylene blue. Representative photomicrographs were taken at 200× magnification for observing vaginal epithelium (upper; scale bar=150 μm) and 400× magnification for observing the size of cells (lower; scale bar=75 μm).

The hormone E_2 is decreased and LH is increased in the OVX mice (Veras *et al.* 2014). PPE (10 mg/kg) significantly increased the serum E_2 level in the OVX mice ($P<0.05$; Fig. 2A). However, PPE and Arg had no significant effect on the serum LH level (Fig. 2B). ALP is a marker of osteoblast differentiation and bone formation (Prins *et al.* 2014). Serum ALP level was significantly decreased in the OVX mice compared to the sham. However, PPE, Arg, or E_2 significantly increased the serum ALP level ($P<0.05$; Fig. 2C).

PPE or Arg enhanced bone formation in OVX mice

Estrogen deficiency plays a critical role in the development of osteoporosis in menopausal women (Manolagas & Jilka 1995). Thus, we performed μCT measurements to evaluate whether PPE or Arg could affect developmental bone formation in the OVX mice. PPE, Arg, or E_2-administered mice showed an increase in the bone mineral density (BMD) ($P<0.05$; Fig. 3A). Trabecular connectivity-representing parameters converted from the 3D μCT reconstruction of trabecular bone images, revealed an increase in the trabecular bone volume density (BV/TV) and a decrease in the total porosity at tibia by administration of PPE, Arg, or E_2 in the OVX mice ($P<0.05$; Fig. 3B, C and D).

PPE or Arg promoted estrogenic activity in MCF-7 cells and MG-63 cells

Estrogen regulates a number of physiological processes in ER-positive breast cancer cells, including proliferation (Hall *et al.* 2001). PPE, Arg, or E_2 significantly promoted the proliferation of MCF-7 cells ($P<0.05$; Fig. 4A). PPE, Arg, or E_2 significantly increased a marker of cell proliferation, Ki-67 mRNA expression ($P<0.05$; Fig. 4B). Estrogen exerts its effects by binding to ERs and modulating the transcription of target genes including growth factors (Heldring *et al.* 2007). The estrogen-ER dimer binds to ERE within regulatory regions of estrogen responsive genes (Legler *et al.* 1999). Thus, we investigated whether PPE or Arg would have estrogen function in MCF-7 cells. As indicated in Fig. 4C, PPE, Arg, or E_2 significantly increased the ERβ mRNA expression in MCF-7 cells ($P<0.05$). PPE, Arg, or E_2 also significantly increased the ERE-luc activity in MCF-7

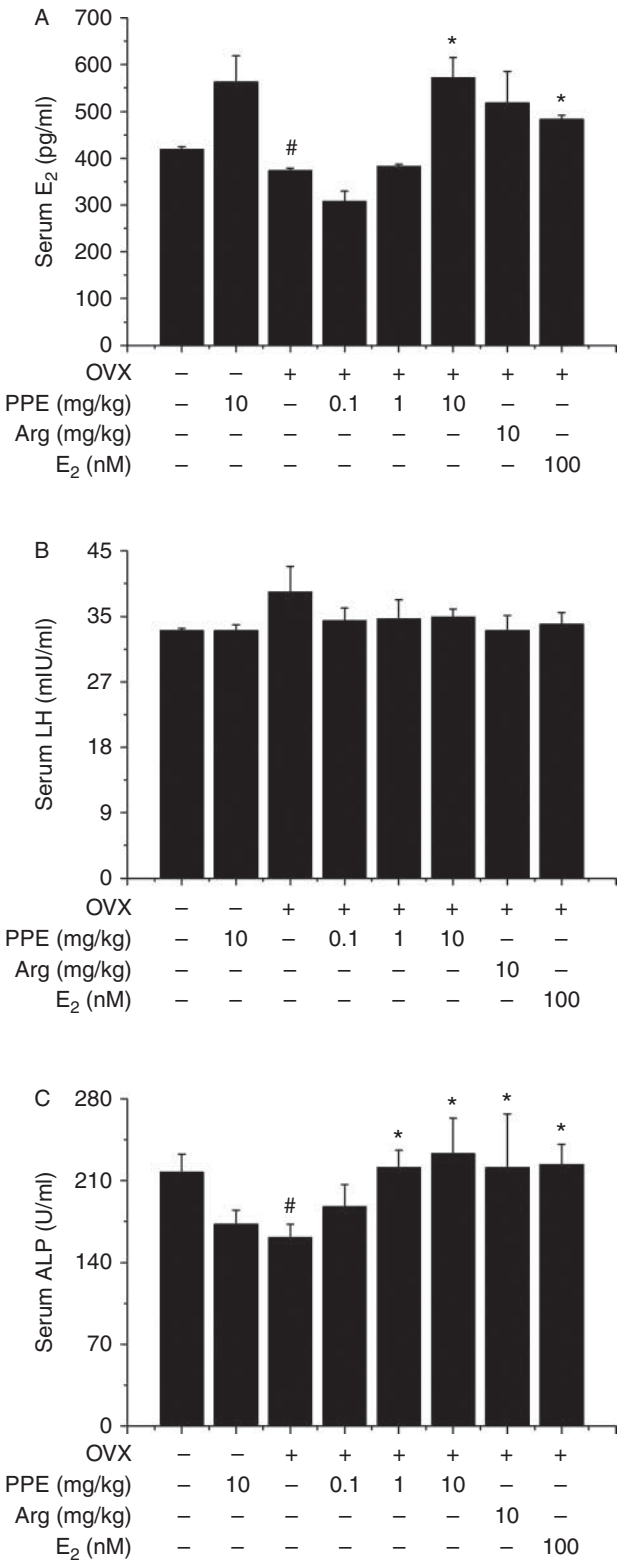

Figure 2 Administration with PPE or Arg increased serum E_2 and ALP in the OVX mice. Serum levels of (A) 17β-estradiol (E_2), (B) luteinizing hormone (LH), and (C) alkaline phosphatase (ALP) were measured at the end of the treatment period as described in materials and methods. $^{\#}P<0.05$; significantly different from the sham-operated mice. $^{*}P<0.05$; significantly different from the OVX mice.

cells ($P<0.05$; Fig. 4D). Further, we investigated that PPE or Arg would have estrogen-like effects in MG-63 cells. The proliferation was significantly promoted by PPE, Arg, or E_2 in the range of concentrations used ($P<0.05$; Fig. 5A). PPE, Arg, or E_2 significantly increased the ERβ mRNA expression ($P<0.05$; Fig. 5B). And PPE or Arg significantly increased the ERE-luc activity in MCF-7 cells ($P<0.05$; Fig. 5C).

PPE or Arg promoted ALP activity in MG-63 cells

ALP activity is up-regulated by E_2-ER complexes in MG-63 cells (Prouillet *et al.* 2004). Thus, we examined that PPE or Arg would modulate the ALP activity in MG-63 cells. As shown in Fig. 6A, PPE, Arg, or E_2 significantly increased the activity of ALP in MG-63 cells ($P<0.05$). ERK1/2 pathway affects the activity of ALP in MG-63 cells (Lai *et al.* 2001). Thus, we finally examined that PPE or Arg would induce the phosphorylation of ERK1/2. Expectedly, PPE, Arg, or E_2 induced the phosphorylation of ERK1/2 (Fig. 6B).

Discussion

HRT has beneficial effects protecting menopausal women against estrogen deficiency, but unfortunately it entails considerable risks. The risk of mortality or heart failure was significantly reduced in women receiving HRT after menopause without any noticeable increase in a risk of cancer (Schierbeck *et al.* 2012). However, Królik & Milnerowicz (2012) reported that HRT should always be preceded by a careful evaluation of individual conditions because HRT increases the risk of the formation of cancer of breast and uterus. This study aimed to clarify how treatment with PPE or Arg has estrogen-like functions in the OVX mice, MCF-7 cells, and MG-63 cells. The results clearly show that PPE or Arg increased the serum levels of E_2 and ALP, and ameliorated overweight and vaginal atrophy in the OVX mice. PPE or Arg increased the ER activation in MCF-7 cells and MG-63 cells. Moreover, PPE or Arg enhanced the bone formation in the OVX mice. PPE or Argm also augmented the ALP activity in MG-63 cells.

Weight gain and obesity were reported to be closely linked to menopausal transition (Al-Safi & Polotsky 2014). The estrogen deficiency is an important obesity-triggering factor in menopausal women (Clegg 2012). Also, estrogen deficiency can lead to symptoms of urogenital atrophy (Panay & Maamari 2012). Vaginal atrophy is associated with vaginal symptoms such as vaginal dryness, itching, and dysuria in postmenopausal women (Pastore *et al.* 2004). OVX caused vaginal atrophy and this vaginal atrophy resulted from the depletion of the estrogen supplied by the ovary. Vaginal atrophy also was reflected in decreased vaginal weights (Balakrishnan *et al.* 2014). Tibolone extensively used by

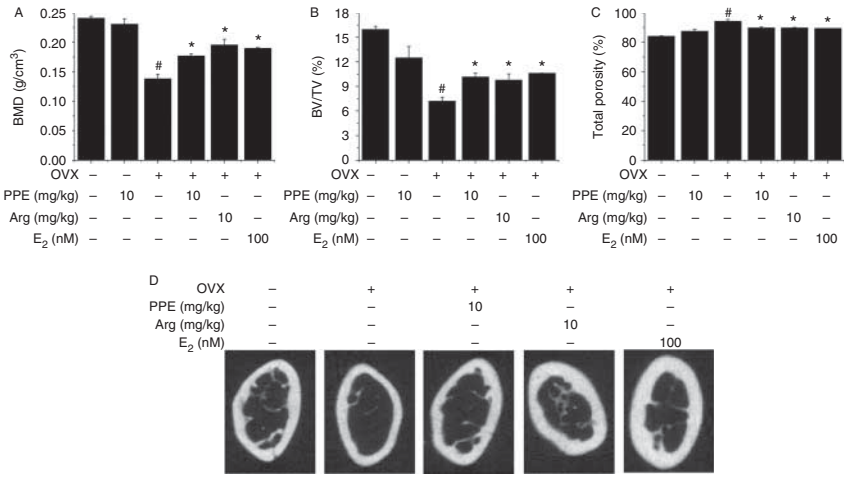

Figure 3 PPE or Arg enhanced bone formation in the OVX mice. (A) Bone mineral density (BMD) was determined using 2D μCT. (B and C) Quantified parameters of proximal tibia were determined using 3D μCT. BV/TV; trabecular bone volume density. (D) These images are representative cross-sectional μCT scannings of the tibia. $^{\#}P<0.05$; significantly different from the sham-operated mice. $^{*}P<0.05$; significantly different from the OVX mice.

postmenopausal women for the treatment of climacteric symptoms and prevention of osteoporosis has a suppressive effect on body weight (Dedeoğlu *et al*. 2009). Phytoestrogen-rich diet alleviates menopausal symptoms, increasing serum E_2 (Albertazzi *et al*. 1999) and vaginal cell maturation, and preventing vaginal atrophy in postmenopausal women (Chiechi *et al*. 2003). In this study, PPE and Arg restrained the OVX mice gain body weight and showed similar effect on body weight after 5 weeks. The various amino acids in PPE might affect the body weight of OVX mice. OVX-induced vaginal atrophy also was partially improved by the PPE or Arg treatment. PPE or Arg increased the serum E_2 level in the OVX mice. Thus, we assume that PPE or Arg would prevent from estrogen deficiency-induced weight changes and vaginal atrophy through increasing serum E_2 level.

E_2 is a key regulator of growth, differentiation, and function in target tissues, including reproductive tracts, mammary gland, and skeletal systems. The predominant biological effects of E_2 are mediated through intracellular receptors, ERα and ERβ (Giguère *et al*. 1998). E_2-ER complexes bind to ERE in target promoters leading to the regulation of gene transcription and subsequent tissue responses such as cell proliferation (Hall *et al*. 2001). E_2 responds in MCF-7 cells through higher ER expression

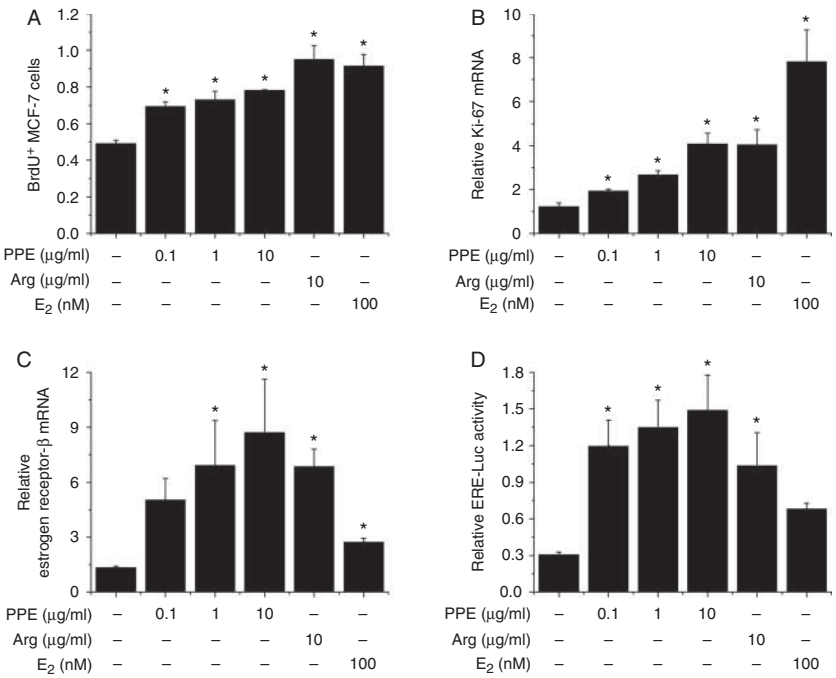

Figure 4 PPE or Arg promoted estrogenic activity in MCF-7 cells. (A) MCF-7 cells were treated with PPE, Arg, or E_2 for 48 h. Proliferation was measured with a BrdU incorporation assay. (B) MCF-7 cells were treated with PPE, Arg, or E_2 for 24 h. The Ki-67 mRNA expression was analyzed with the quantitative real-time PCR analysis. (C) MCF-7 cells were treated with PPE, Arg, or E_2 for 10 h. The ERβ mRNA expression was analyzed with the real-time quantitative PCR analysis. (D) MCF-7 cells were treated with PPE, Arg, or E_2 for 48 h. The ERE-luc activity was measured with a luciferase assay. $^{*}P<0.05$; significantly different from the untreated cells.

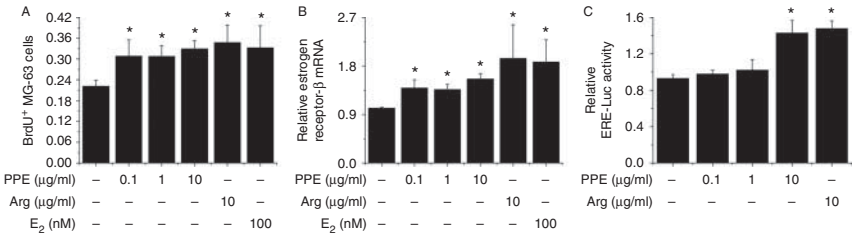

Figure 5 PPE or Arg promoted estrogenic activity in MG-63 cells. (A) MG-63 cells were treated with PPE, Arg, or E_2 for 48 h. Proliferation was measured with a BrdU incorporation assay. (B) MG-63 cells were treated with PPE, Arg, or E_2 for 10 h. The ERβ mRNA expression was analyzed with the quantitative real-time PCR analysis. (C) MG-63 cells were treated with PPE, Arg, or E_2 for 48 h. The ERE-luc activity was measured with a luciferase assay. *$P<0.05$; significantly different from untreated cells.

level (Gougelet *et al*. 2007). Thus, ER-positive MCF-7 cells were used to examine estrogenic activity of PPE or Arg. Quercetin exerted phytoestrogen-like activity, increasing proliferation and ERE activation, and activating ERβ in MCF-7 cells (van der Woude *et al*. 2005). Genistein also possess estrogen agonist activity in MCF-7 cells via an ER mechanism, as well as in the OVX mice (Hsieh *et al*. 1998). In this study, PPE or Arg also produced a robust stimulation of proliferation of MCF-7 cells as well as increased the ERβ mRNA expression and ERE activation. These results suggest that PPE or Arg would have an estrogenic effect via ERβ.

There is a direct relationship between the estrogen deficiency and the development of osteoporosis in postmenopausal women (Roman-Blas *et al*. 2009). In osteoblasts, mitogen-activated protein kinase is also rapidly activated by E_2, which may be involved in the proliferative and bone protective effects (Kousteni *et al*. 2001). ALP activity is dependent upon both ERK pathway and ER activation in MG-63 cells (Prouillet *et al*. 2004). Estriol stimulated MG-63 cells proliferation via ER and prevented bone loss (Luo & Liao 2003). Equol, a phytoestrogen, promoted the bone formation increasing ALP activity and the MG-63 cells proliferation through ER activation (Wang *et al*. 2014). Phytoestrogens stimulated transcriptional activity of an ERE-luciferase reporter gene via ERβ and had osteogenic activity (Tang *et al*. 2008). Bone regeneration is regulated by a balance of biochemical and cellular events that stimulate osteoblasts to produce new extracellular collagen matrix. The collagen matrix is mineralized via ALP activity, inducing formation of calcium phosphate crystal seeds (Asawa *et al*. 2004). Han *et al*. (2007) reported that oyster shell increases the femur BMD and BV/TV in the OVX mice, indicating a potential for the cure of osteoporosis. Tibolone attenuated the development of osteoarthritis, concomitantly increasing serum ALP in the OVX-induced osteoporosis model (Yang *et al*. 2014). This study revealed that PPE or Arg are able to successfully reduce the estrogen deficiency-induced bone loss. PPE or Arg also markedly increased the proliferation, ALP activity, and ER activation in MG-63 cells. Many studies have reported that amino acids regulate menopausal symptoms. Lysine and Arg played a therapeutic role in osteoporosis and fracture healing (Fini *et al*. 2001). Glutamine and Arg ameliorated osteoporotic markers in OVX rats (Ahmed & Hamza 2009, Wang *et al*. 2006). Methionine was beneficial for the treatment of postmenopausal osteoporosis (Vijayan *et al*. 2014). Tryptophan mediated BMD and osteoblast function which may be important for bone health (Pernow *et al*. 2010). The combination ingestion of valine, proline, and isoleucine improved arterial compliance in postmenopausal women (Yoshizawa *et al*. 2009). In early postmenopausal women, phenylalanine also enhanced skeletal muscle and counteracted muscle loss (Holm *et al*. 2005). Thus, the osteogenic effect of PPE

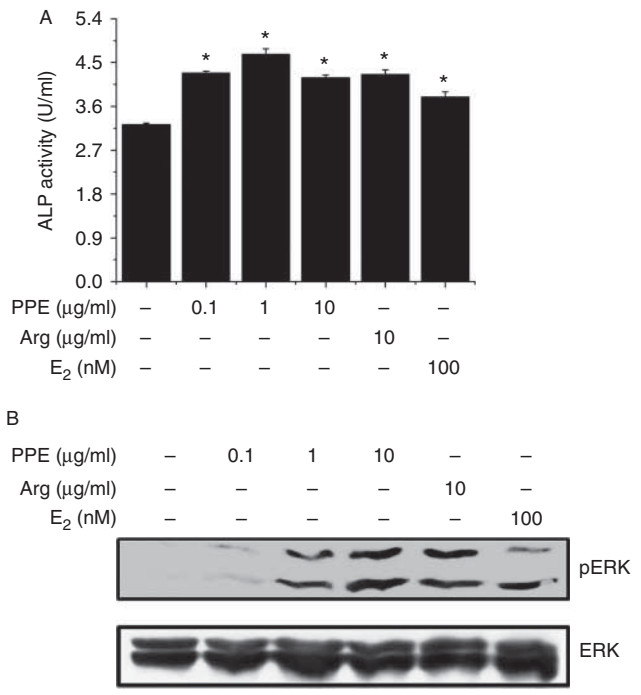

Figure 6 PPE or Arg promoted ALP activity in MG-63 cells. (A) MG-63 cells were treated with PPE, Arg, or E_2 for 48 h. The ALP activity was measured as described in the Materials and methods. (B) MG-63 cells were treated with PPE, Arg, or E_2 for 5 min. The levels were assessed by western blotting. *$P<0.05$; significantly different from untreated cells.

might derive from the amino acids. These results suggest that PPE or Arg have the potential to counteract the deleterious effects of estrogen deficiency on bone.

In conclusion, this study demonstrated that PPE or Arg ameliorate the overweight and vaginal atrophy by increasing the serum E_2 levels in OVX mice. PPE or Arg also improved bone loss by increasing serum levels of E_2 and ALP in the OVX mice. It is possible to assume that this positive effect of PPE or Arg against estrogen deficiency results from an increase in ER activation in the OVX mice. Overall, we suggest that PPE or Arg can be used to treat postmenopausal symptoms through estrogenic activity as a human placenta substitute. However, further studies about side effects of PPE or Arg are necessary to determine the clinical safety, considering those of HRT.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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