Biol Trace Elem Res (2013) 151:68–74
DOI 10.1007/s12011-012-9527-1

Effects of *Astragalus membranaceus* with Supplemental Calcium on Bone Mineral Density and Bone Metabolism in Calcium-Deficient Ovariectomized Rats

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Received: 26 July 2012 / Accepted: 11 October 2012 / Published online: 9 November 2012

Abstract It has been reported that *Astragalus membranaceus*, an Asian traditional herb, has an estrogenic effect in vitro. To examine the possible role of *A. membranaceus* extract with supplemental calcium (Ca) on bone status in calcium-deficient (LCa) ovariectomized (OVX) rats, a total of 48 female rats were divided into six groups: (1) normal control, (2) sham operation with LCa (sham-LCa), (3) OVX with LCa (OVX-LCa), (4) *A. membranaceus* supplementation with OVX-LCa (OVX-MLCa), (5) Ca supplementation with OVX (OVX-Ca), and (6) *A. membranaceus* and Ca supplementation with OVX (OVX-MCa). *A. membranaceus* ethanol extract (500 mg/kg BW) and/or Ca (800 mg/kg BW) were administered orally for 8 weeks along with a Ca-deficient diet. Results revealed that Ca supplementation with or without *A. membranaceus* extract significantly improved bone mineral density, biomechanical strength, and ash weight of the femur and tibia in OVX rats. High Ca with *A. membranaceus* combination supplementation significantly increased the ash weight of the femur and tibia and decreased urinary Ca excretion compared with supplementation of Ca alone. Uterine weight was not changed by *A. membranaceus* administration in OVX rats. These results suggest that *A. membranaceus* extract combined with supplemental Ca may be more protective against the Ca loss of bone than *A. membranaceus* or supplementation of Ca alone in calcium-insufficient postmenopausal women.

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Keywords *Astragalus membranaceus* · Supplemental calcium · Bone mineral density · Bone metabolism · Calcium-deficient · OVX rats

Introduction

Osteoporosis is a significant public health problem, particularly among postmenopausal women. Menopause accelerates bone turnover through an imbalance between bone formation and bone resorption [1]. Also, estrogen deficiency decreases intestinal calcium (Ca) absorption and body Ca availability [2]. Therefore, the intake of the proper amount of Ca and increase of Ca bioavailability are important for the prevention of osteoporosis. In Korea, the Ministry of Health and Welfare recommends that female Koreans over 50 years old have a Ca intake of 650~700 mg/day [3]. Considering the recommended adequate intake (AI) of Ca for Americans (1,200 mg) [4], this is not a high level; however, the average Ca intake of Koreans is less than this recommendation [5]. Especially in the age of postmenopause, the percentage of subjects consuming less than the estimated average requirement of Ca for Koreans was 86.1 % for women aged 50–64 years and 62.0 % for women aged over 65 years [5]. Also, a multi-ethnic study reported that only 16.4 % of four ethnicities of middle-aged and older Americans met the AI for Ca [6]. Hence, Ca supplementation should be considered for individuals who fail to meet the AI for Ca, and perimenopausal and postmenopausal women to prevent and treat postmenopausal osteoporosis [7, 8]. Along with Ca supplementation, hormone replacement therapy (HRT) has been commonly prescribed to postmenopausal women with low bone mass or bone fracture history [9]. The protective effects of HRT alone on attenuating postmenopausal bone loss and reducing the risk of bone fractures are enhanced

when combined with Ca supplementation [10]. But it has been suggested that HRT increases the risk of breast cancer, stroke, myocardial infarction, and thromboembolic events [11]. Consequently, there is an urgent need to examine the alternative treatments that can be combined with Ca supplementation to lower the risk of osteoporosis and related fracture among postmenopausal women.

There is an increasing interest in plant-derived compounds which have an estrogenic effect because of their similar structure to estrogen and their ability to bind to estrogen receptors [12]. Breitman and colleagues reported that Ca supplement combined with isoflavones provided greater protection against the loss of bone mass and strength after ovariectomy compared with Ca supplement alone [9]. The estrogen-like activity of these compounds may provide an alternative strategy to replace or augment the HRT [9].

A. membranaceus is one of the most widely used medicinal herbs in Asian traditional medicine and is known for containing cycloartane triterpene glycosides and flavonoids, particularly isoflavones, as its principal constituents [13–15]. *A. membranaceus* extract was reported to have an estrogenic effect [16], to inhibit osteoclast development in vitro [15], and to improve the MG-63 cell proliferation [17]. Also, an in vivo study using ovariectomized (OVX) rats showed that *A. membranaceus* extract administration inhibited tibia and lumbar bone loss [15]. Through some of these studies, it is suggested that *A. membranaceus* extract may have a synergic effect to improve bone mineral density, if it is combined with Ca supplement. However, it has not been established whether Ca and *A. membranaceus* extract supplementation by combination have a synergic effect on Ca and bone metabolism compared with each supplementation. Therefore, the present study was designed to examine the effect of the combination of Ca and *A. membranaceus* extract supplementation on bone metabolism, bone physical property, and bone mineral density in Ca-deficient OVX rats.

Materials and Methods

Animals and Study Protocols

A total of forty-eight 6-week-old Sprague Dawley female rats (8 normal, 32 OVX, and 8 sham operated) were purchased from Central Lab. Animal Inc. (Seoul, Korea). The animals were housed in a room maintained at 22±3 °C on 12-h light/12-h dark cycles. After a 2-week adaptation period, the OVX rats were separated into six experimental groups. The experimental group design was as follows: (1) normal control, (2) sham operation and Ca deficient (Sham-LCa), (3) OVX and Ca-deficient (OVX-LCa), (4) *A. membranaceus* supplementation with OVX-LCa (OVX-MLCa), (5) Ca supplementation with OVX (OVX-Ca), and (6) *A. membranaceus* and Ca supplementation with OVX (OVX-MCa). The basic diet was based on a Ca-deficient diet containing Ca 60 mg/kg except for the normal control group. The normal control group was provided the normal diet containing $CaCO_3$ 12 g/kg (Ca 0.5 % diet) based on an AIN-93 purified diet for laboratory rodents [18].

Ca and *A. membranaceus* extract were orally administrated daily, six times per week, to the experimental rats in demineralized water (Ca, 800 mg/kg body weight/day; *A. membranaceus* extract, 500 mg/kg body weight/day). Ca was purchased from Il-Yang Pharm. Co., Ltd (Gyeonggido, Korea). For extraction optimization, a package of Radix Astragali slices (*A. membranaceus*; KFDA standardized product) cultivated in Jecheon, Korea, and packaged under nitrogen was purchased from a local drugstore. Whole roots of *A. membranaceus* (3 years old) were powdered, and 1 kg of Radix Astragali powder was extracted with 10 L of 70 % EtOH under reflux for 24 h and then filtered; the filtrate evaporated to dryness in a vacuum.

The daily food intake and body weight of the rats were weighed on a weekly basis. The experiment was maintained for 8 weeks. At the termination of the experiment, animals were anesthetized with ether. Blood samples from the anesthetized rats were collected via the abdominal aorta and centrifuged at 500×*g*, 4 °C for 30 min. Blood samples were stored at −80 °C until the time of assay. The uterine, right femur, and tibia of each animal were dissected, cleaned, and weighed using an electric balance (AB204-S/F31, Mettler-Toledo International Inc.) The right femurs and tibias were stored at −80 °C until bone mineral density (BMD), strength, and ash contents were examined. All experimental procedures followed the guiding principles in *Guide for the Care and Use of Laboratory Animals* [19] and were approved by the Institutional Animal Care and Use Committee of Gachon University.

Serum Analysis

Serum alkaline phosphatase (ALP) was measured with a commercial kit using the Kind-King method (Asan Pharmaceutical Co. Ltd, Seoul, Korea), and the serum levels of osteocalcin (OC) were measured with an Glu-OC/Gla OC competitive ELISA kit (Takara, Shiga, Japan) by an ELISA reader (ELx808, BioTek Instruments Inc., Winooski, VT, USA). Urinary calcium levels were determined using a commercial kit (Qeantichrom calcium assay kit, Bioassay System, Hayward, USA).

Measurement of Bone Status

BMD for the right femur and tibia were measured by dual-energy X-ray absorptionmetry, a PIXImus2 (Lunar,

Madison, WI, USA) adapted for measuring small animals. Our percent coefficient of variation (%CV) for this measurement, determined from three measurements made in the femur and tibia of 15 rats at intervals of 2~3 days, were 0.51 and 0.96 %, respectively. The instrument was calibrated daily. The biomechanical property (bone strength) was assessed by a three-point bending test of the femoral bone and tibia (the center of the femur and tibia) using a material testing system (Instron, model 2242, Norwood, MA, USA) and software program (Bluehill2, ver 2.19; Instron). Prior to the three-point bending test, the femur and tibia were thawed in saline (0.9 % NaCl) at room temperature to protect hydration. The femur and tibia were placed posterior side down on two supporting bars positioned 15 mm apart. After being fitted with 500-N load cells, the crosshead loaded at a speed of 2 mm/min until bones were fractured. Bone strength (kilogram force, kilogram per square centimeter) and flexure extension were measured.

To measure the ash contents of the right femur and tibia, the samples were dried at 110 °C for 6 h, and the dried bone samples were ashed at 600 °C for 24 h. After cooling down the completely ashed bones in a dessicator, the ash weight of the femur and tibia was measured using an electronic scale (AB204-S/F31, Mettler-Toledo International Inc.).

Statistical Analyses

Results are shown as means ± standard deviation. Statistical significance was determined with one-way analysis of variance (ANOVA) using the SAS™ (version 9.1; SAS Institute Inc., Cary, NC, USA). When the ANOVA indicated a significant difference among the means, the differences were further evaluated using Duncan's multiple range tests. The difference was considered significant when $p<0.05$.

Results

The initial and final body weight and weight gain of the experimental groups are shown in Table 1. The initial body weight of the normal and sham groups was significantly higher than that of the OVX groups. The final body weight of the OVX-LCa and OVX-MLCa groups was significantly higher than that of the normal and sham-LCa groups, but the final body weight of OVX-Ca and OVX-MCa groups was not significantly different from that of the normal group. The mean weekly weight gain was not significantly different among the experimental groups. The uterine weight and uterus index of the OVX groups were significantly lower than those of the normal and sham-LCa groups. *A. membranaceus* administration did not affect the uterine weight and uterus index in OVX rats (Table 2).

Table 1 Body weight of experimental rats

Group	Initial body weight (g)	Final body weight (g)	Weight gain (g/week)
Normal	168.75±8.34^{b}	281.12±13.23^{b}	14.05±1.79
Sham-LCa	163.13±7.03^{b}	257.88±6.45^{c}	11.84±1.16
OVX-LCa	193.75±7.44^{a}	308.37±17.63^{a}	14.33±2.00
OVX-MLCa	192.50±11.02^{a}	312.50±21.68^{a}	15.00±2.26
OVX-Ca	188.75±12.75^{a}	294.00±9.17^{ab}	13.16±1.70
OVX-MCa	191.88±14.13^{a}	290.87±36.36^{ab}	12.38±3.9
Significance	$p<0.001$	$p<0.001$	NS

Data are presented as mean±SD ($n=8$). Means with unlike letters in a column differ significantly (Duncan's multiple test)

Normal normal control, *Sham-LCa* sham and Ca-deficient diet, *OVX-LCa* OVX and Ca-deficient diet, *OVX-MLCa A. membranaceus* supplementation with OVX-LCa, *OVX-Ca* Ca supplementation with OVX, OVX-MCa *A. membranaceus* and Ca supplementation with OVX, *NS* not significant

Blood and urine bone metabolism-related markers are shown in Table 3. Serum ALP and OC (Gla and Glu) levels of the normal group were significantly lower than those of the other groups, and there was no significant difference among the OVX groups according to the supplement type. Ovariectomy and Ca-deficient diet significantly increased urinary Ca excretion, and *A. membranaceus* combined with Ca supplementation significantly reduced the urinary Ca excretion of the OVX groups.

Table 4 shows the wet and ash weights of the femur and tibia. The wet weights of the femur and tibia of the OVX-LCa and OVX-MLCa groups were significantly lower than those of the normal group. But the wet weights of the femur and tibia of the OVX-Ca and OVX-MCa groups were not

Table 2 Uterine weight and the uterus index of experimental rats

Group	Weight (g)	Index
Normal	0.56±0.14^{a}	0.20±0.05^{a}
Sham-LCa	0.54±0.17^{a}	0.21±0.06^{a}
OVX-LCa	0.08±0.01^{b}	0.02±0.00^{b}
OVX-MLCa	0.09±0.01^{b}	0.03±0.00^{b}
OVX-Ca	0.07±0.01^{b}	0.02±0.01^{b}
OVX-MCa	0.09±0.01^{b}	0.03±0.00^{b}
Significance	$p<0.001$	$p<0.001$

Data are presented as mean±SD ($n=8$). Means with unlike letters in a column differ significantly (Duncan's multiple test)

Normal normal control, *Sham-LCa* sham and Ca-deficient diet, *OVX-LCa* OVX and Ca-deficient diet, *OVX-MLCa A. membranaceus* supplementation with OVX-LCa, *OVX-Ca* Ca supplementation with OVX, *OVX-MCa A. membranaceus* and Ca supplementation with OVX

Table 3 Serum ALP, serum OC, and urinary Ca levels of experimental rats

Group	ALP (U/L)	OC (Gla) (ng/ml)	OC (Glu) (ng/ml)	Urine Ca (mg/dl)
Normal	26.98±4.79^{b}	1.92713±0.00055^{d}	1.91631±0.00509^{b}	11.94±8.31^{d}
Sham-LCa	49.46±8.04^{a}	1.92806±0.00056^{c}	1.92508±0.00263^{a}	21.29±12.47^{c}
OVX-LCa	50.83±8.26^{a}	1.92886±0.00051^{ab}	1.92604±0.00094^{a}	34.61±4.97^{a}
OVX-MLCa	46.71±8.46^{a}	1.92896±0.00036^{a}	1.92578±0.00105^{a}	29.86±1.87^{ab}
OVX-Ca	50.96±3.22^{a}	1.92914±0.00050^{a}	1.92522±0.00086^{a}	34.20±5.19^{a}
OVX-MCa	45.56±7.78^{a}	1.92822±0.00118^{bc}	1.92523±0.00117^{a}	26.24±1.53^{bc}
Significance	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$

Data are presented as mean±SD ($n=8$). Means unlike letters in a column with differ significantly (Duncan's multiple test)

Normal normal control, *Sham-LCa* sham and Ca-deficient diet, *OVX-LCa* OVX and Ca-deficient diet, *OVX-MLCa A. membranaceus* supplementation with OVX-LCa, *OVX-Ca* Ca supplementation with OVX, *OVX-MCa A. membranaceus* and Ca supplementation with OVX

significantly different from those of the normal group. As shown in Table 4, the normal group had over twice the amount of femur and tibia ash contents as the OVX-LCa group. Each *A. membranaceus* or Ca supplementation increased the femur and tibia ash contents, and combined supplementation of *A. membranaceus* and Ca was most effective on improving the ash contents of the femur and tibia in Ca-deficient OVX rats. The OVX-MCa group's ash contents of femur and tibia were 60.8 and 37.0 % higher than those of the OVX-MLCa and 41.1 and 20.9 % higher than those of the OVX-Ca, respectively.

Table 5 shows the BMD of the femur and tibia of the experimental groups. Ca alone and Ca combined with *A. membranaceus* extract supplementation significantly increased the femur and tibia BMD in Ca-deficient OVX rats. The OVX-MCa group's BMDs of the femur and tibia were 45.7 and 50.0 % significantly higher than those of the OVX-MLCa. However, there was no significant difference in BMDs of the femur and tibia between the OVX-Ca and the OVX-MCa groups.

Bone strength of the femur and tibia was also significantly increased by Ca alone and Ca combined with *A. membranaceus* extract supplementation (Table 6). *A. membranaceus* extract alone supplementation did not increase the bone strength of the femur and tibia in Ca-deficient OVX rats.

Discussion

This study examined the effects of *A. membranaceus* in combination with supplemental Ca on BMD, biomechanical bone strength, and bone metabolism markers using the Ca-deficient OVX rat model. Ca supplementation with or without *A. membranaceus* extract significantly improved the

Table 4 Femur and tibia wet weight of experimental rats

Group	Femur		Tibia	
	Wet weight (g)	Ash weight (mg)	Wet weight (g)	Ash weight (mg)
Normal	0.78±0.05^{a}	336.30±29.50^{a}	0.57±0.03^{a}	254.46±22.66^{a}
Sham-LCa	0.68±0.09^{bc}	151.94±29.76^{c}	0.48±0.07^{b}	133.10±25.55^{ed}
OVX-LCa	0.66±0.05^{c}	120.94±18.73^{d}	0.47±0.07^{b}	120.94±18.73^{e}
OVX-MLCa	0.67±0.06^{bc}	143.41±20.03^{cd}	0.49±0.05^{b}	140.51±8.62^{cd}
OVX-Ca	0.73±0.05^{ab}	163.46±17.20^{c}	0.58±0.04^{a}	152.18±7.44^{d}
OVX-MCa	0.78±0.06^{a}	230.61±22.56^{b}	0.60±0.11^{a}	192.49±4.63^{b}
Significance	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$

Data are presented as mean±SD ($n=8$). Means with unlike letters in a column differ significantly (Duncan's multiple test)

Normal normal control, *Sham-LCa* sham and Ca-deficient diet, *OVX-LCa* OVX and Ca-deficient diet, *OVX-MLCa A. membranaceus* supplementation with OVX-LCa, *OVX-Ca* Ca supplementation with OVX, *OVX-MCa A. membranaceus* and Ca supplementation with OVX

Table 5 Bone mineral density in femur and tibia of experimental rats

Group	Femur (mg/cm^2)	Tibia (mg/cm^2)
Normal	198.23±11.74^{a}	157.81±12.33^{a}
Sham-LCa	88.55±8.01^{c}	73.49±4.92^{c}
OVX-LCa	83.64±7.75^{c}	69.41±5.60^{c}
OVX-MLCa	91.70±11.36^{c}	75.59±9.02^{c}
OVX-Ca	135.94±1.62^{b}	113.80±2.06^{b}
OVX-MCa	133.58±7.95^{b}	113.35±5.21^{b}
Significance	$p<0.001$	$p<0.001$

Data are presented as mean±SD ($n=8$). Means with unlike letters in a column differ significantly (Duncan's multiple test)

Normal normal control, *Sham-LCa* sham and Ca-deficient diet, *OVX-LCa* OVX and Ca-deficient diet, *OVX-MLCa A. membranaceus* supplementation with OVX-LCa, *OVX-Ca* Ca supplementation with OVX, *OVX-MCa A. membranaceus* and Ca supplementation with OVX

Table 6 Bone strength in femur and tibia of experimental rats

Group	Femur (kgf)	Tibia (kgf)
Normal	13.82±1.83[a]	8.85±1.01[a]
Sham-LCa	3.29±0.49[c]	3.50±0.48[c]
OVX-LCa	3.16±0.42[c]	3.25±0.40[c]
OVX-MLCa	3.56±0.89[c]	3.96±1.32[c]
OVX-Ca	8.46±0.67[b]	7.58±0.67[b]
OVX-MCa	8.71±0.76[b]	6.92±1.04[b]
Significance	$p<0.001$	$p<0.001$

Data are presented as mean±SD ($n=8$). Means with unlike letters in a column differ significantly (Duncan's multiple test)

Normal normal control, *Sham-LCa* sham and Ca-deficient diet, *OVX-LCa* OVX and Ca-deficient diet, *OVX-MLCa A. membranaceus* supplementation with OVX-LCa, *OVX-Ca* Ca supplementation with OVX, *OVX-MCa A. membranaceus* and Ca supplementation with OVX

BMD, biomechanical strength, and ash weight of the femur and tibia in OVX rats. Although the rats receiving supplemental Ca with *A. membranaceus* did not show significantly different BMD and bone strength compared with the rats receiving Ca alone supplementation, this combination supplementation significantly increased the ash weight of the femur and tibia and decreased urinary Ca excretion compared with Ca alone supplementation. These comparison results between Ca alone supplementation and Ca with *A. membranaceus* extract combination reveal that *A. membranaceus* extract may provide a synergic effect on Ca supplementation, improving bone metabolism.

Several mechanisms could account for the protective effects of *A. membranaceus* and supplemental Ca on osteoporosis in Ca-deficient OVX rats. The principal constituent in *A. membranaceus* extract used in this study was isoflavone, specifically formononetin (Biochanin B, 7-hydroxy-4′-methoxyisoflavone) [17]. Based on previous research, ovariectomy reduces Ca absorption [20–22] and increases bone turnover and urinary Ca [23]. Therefore, supplemental Ca may help maintain a positive Ca balance. In addition, it is possible that estrogen-like chemicals such as isoflavones contained in *A. membranaceus* improve intestinal Ca absorption by an estrogen-like mechanism, such as administration of exogenous estrogen to ovariectomized rats, and enhance intestinal Ca absorption [24–26]. However, the mechanisms by which *A. membranaceus* increases intestinal Ca absorption have not been elucidated, and further investigation is required.

Besides improving intestinal Ca absorption, there are some possible direct mechanisms that could account for the improving effects of *A. membranaceus* on bone metabolism. The phytoestrogens such as isoflavones may directly interact with estrogen receptors on bones and involve bone metabolism [27, 28]. For instance, it is reported that *A. membranaceus* extract inhibited osteoclast development in vitro and reduced the tibia and lumbar bone loss in vivo using OVX rats [15]. Also, our previous study showed that 70 % ethanol *A. membranaceus* extract showed strong proliferative activity on MG-63 cells, an osteoblast-like cell line [17]. From these reports, *A. membranaceus* seems to act directly on inhibiting osteoclasts and improving osteoblasts of the bone. Our study results partially support these direct mechanisms, as *A. membranaceus* with or without high Ca significantly decreased the urinary Ca excretion, one of the bone resorption markers. But supplementation of *A. membranaceus* and Ca, either combined or separately, did not affect the bone formation makers such as serum ALP and OC.

Kim and colleagues [15] reported that *A. membranaceus* extract administration inhibited tibia and lumbar bone loss in OVX rats. However, our study did not clearly show whether *A. membranaceus* extract alone supplementation without high Ca could improve bone mineral density, bone strength, and bone metabolism. There are some possible reasons that may explain why we did not observe a positive effect on bone metabolism with *A. membranaceus* extract alone as shown with Ca+*A. membranaceus* extract combination. The reasons include the Ca content of the basal diet and the age of the rats at the time of ovariectomy. The reported Ca levels of a low-Ca diet to induce osteoporosis or to study Ca supplemental effect in rats have been varied, showing a range of 0.02~0.2 % [29–33]. The diet Ca content used in this study was under 0.01 %, to induce certain osteoporosis. As shown in the BMD of the sham with low-Ca group, this low-Ca diet successfully induced osteoporosis without ovariectomy. However, in this severe Ca-deficient condition, it is hard to examine Ca metabolism improvement by *A. membranaceus* supplement, although *A. membranaceus* extract may indeed improve Ca metabolism.

Additionally, age at the time of ovariectomy could also contribute to *A. membranaceus* alone supplement not showing a significant effect on bone metabolism in Ca-deficient OVX rats. In this study, we used OVX rats that were 56–112 days old. But according to the previous research to investigate the effect of isoflavone supplementation on improving BMD or bone strength in OVX rats, isoflavone supplementation seems to be more effective in older OVX rats [9, 34–36]. It is possible that older rats may have had greater bone loss due to the natural aging process compared with younger rats [37] and therefore may have been more responsive to phytoestrogen intervention and its potential estrogen-like activity on bone [9].

Although *A. membranaceus* with supplemental Ca is purported to prevent bone loss in ovariectomized rats due to estrogenic effects, there is concern about whether it also has estrogenic effects on the uterus that may result in an increased risk of endometrial cancer [38]. We supplemented the *A.*

membranaceus extract at the dose of 500 mg/kg body weight in rats. According to the previous report, there was no toxic effect of *A. membranaceus* extract supplementation until the dose of 2 g/kg body weight in rats [39]. Also, findings from the present study indicate that *A. membranaceus* does not cause hypertrophy of the uterus. If we apply our results from experimental animals to humans, this supplemental amount can be converted to 30 g/day for an adult having 60 kg body weight. Therefore, it could easily be taken even in extract form by humans. However, further studies are needed before humans can benefit from the effect of *A. membranaceus* supplement on bone health.

Previous studies reported that ovariectomy results in weight gain caused by overeating [40], and there is also a report that ovariectomy decreases the secretion of estrogen, which induces weight gain without any change in the amount of food intake [41]. Also in our study, final body weight was the highest in the OVX group not fed supplemental Ca. However, rats supplemented with *A. membranaceus* in combination with high Ca had the lowest final body weight among the OVX groups. This result suggests that the high Ca intake may have regulated body weight gain. It has been reported that high dietary calcium inhibits lipogenesis, increases lipolysis, and increases thermogenesis, leading to a net reduction in fat mass [42, 43].

Our study has several limitations related to experimental design. First, we did not include the normal group with Ca-deficient diet as we thought that a comparison between the sham with the Ca-deficient diet group and the OVX with the Ca-deficient diet group could represent hormone deficient effects on bone metabolism. Second, as we mentioned earlier, the diet Ca content used in this study was very low, so it is hard to represent the common Ca-deficient condition in the normal human diet.

In conclusion, the findings from this study demonstrate that the combination of *A. membranaceus*, an herbal plant, and supplemental Ca is more protective against the bone Ca loss than Ca alone supplementation in postmenopausal woman with Ca-insufficient intake without hypertrophy of the uterus. Also, further investigation is required to determine the extent to which calcium and *A. membranaceus* preserve bone metabolism.

Acknowledgments This work was supported by a grant from the Next-Generation BioGreen 21 Program (no. PJ009049), Rural Development Administration, Republic of Korea.

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