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Protective effect of steroidal saponins from rhizome of *Anemarrhena asphodeloides* on ovariectomy-induced bone loss in rats[1]

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Key words

Anemarrhena asphodeloides; ovariectomy; osteoporosis; serum; bone mineral density; histochemistry

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Abstract

Aim: To investigate the protective effect of steroidal saponins from *Anemarrhena asphodeloides* (ATS) on ovariectomy (OVX)-induced bone loss. **Methods:** Sprague-Dawley rats were divided into sham and OVX groups. The OVX rats were treated with vehicle, nylestriol or steroidal saponins extract for 12 weeks. Serum calcium, phosphorus, estradiol (E_2), osteocalcin concentration and serum alkaline phosphatase activity were measured. Bone density was assayed by dual-energy X-ray absorptiometry. The undecalcified longitudinal proximal tibial metaphysical (PTM) sections were cut and stained for histomorphometric analysis of the bone. **Results:** In OVX rats, alkaline phosphatase activities in serum were markedly increased and concentrations of osteocalcin were decreased by ATS treatment, which had no influence on the body weight. Meanwhile, atrophy of the uterus and descent of bone mineral density (BMD) was suppressed by treatment with ATS. In addition, ATS completely corrected the decreased the concentration of calcium and E_2 in serum observed in OVX rats. Histological results showed ATS prevented decreases in trabecular thickness and increases in trabecular separation of proximal tibia metaphysis (PTM) in OVX rats. However, it did not alter osteoclast number in OVX rats. Moreover, ATS (300 mg/kg) had a remarkable effect on promoting bone formation action in OVX rats. Nylestriol treatment decreased the bone formation rate and mineral apposition rate. **Conclusion:** An adequate supply of steroidal saponins of *Anemarrhena asphodeloide*s prevented OVX-induced bone loss in rats through the promotion of bone formation but not the inhibition of bone resorption.

Introduction

Osteoporosis is a chronic, progressive disease of the skeleton characterized by bone fragility caused by a reduction in bone mass and possibly alteration in bone architecture, which leads to a propensity to fracture with minimum trauma[1]. Osteoporosis associated with ovarian hormone deficiency following menopause is by far the most common cause of age-related bone loss[2]. Menopause results in elevated bone turnover, an imbalance between bone formation and bone resorption, and net bone loss. Postmenopausal osteoporosis has become a major problem with significant morbidity and mortality[3].

The design of anti-osteoporotic drugs are based on the processes of bone remodeling. Some agents are aimed at preventing bone resorption (estrogen, calcitonin, bisphosphonates, calcium, vitamin D, raloxifene) and other agents mainly stimulate bone formation (fluoride, anabolic steroids)[4]. Among these, estrogen replacement therapy (ERT) used to be a popular regime for prevention and treatment of postmenopausal osteoporosis. However, recent investigation suggests that ERT is associated with an increased risk of breast, ovarian and endometrial cancer[5,6]. In addition, anti-osteoporotic drugs are too expensive to benefit the ordinary

people in developing or even developed countries. Thus, it is imperative to discover alternative approaches for managing osteoporosis.

Anemarrhena asphodeloides Bunge (*Liliaceae*) is a perennial herb and widely grows in most parts of China. The *Anemarrhena asphodeloides* rhizomes have been demonstrated not only to have anti-diabetic activity, platelet aggregation inhibitory activity, diuretic activity, molluscicidal activity, anti-fungal activity and anti-yeast activity, but also to have inhibiting effects on cyclic AMP phosphodiesterase[7–11]. In traditional Chinese medicine, the *Anemarrhena asphodeloides* rhizomes are used for the treatment of lung disease, fever, diabetes and constipation[12]. Analysis of chemical compositions of the rhizomes of *Anemarrhena asphodeloides* found that steroidal saponins (such as timosaponins AI, AII, AIII, and AIV, and timosaponins BI, and BII, *etc*) xanthone C-glyco-sides, polysaccharides, and norlignans were the major active compounds; and the content of steroidal saponins are more than 5%[13–16]. The steroidal saponins from plants are the main raw material of the sex hormone and their structures are somewhat similar to that of mammalian estrogens. The activities of chemical compounds are closely associated with their structures. Thus we inferred that the steroidal saponins from the rhizomes of *Anemarrhena asphodeloides* might have the activity of estrogens associated with the estrogen receptor dependent pathway and antiosteoporotic properties.

Ovariectomy induced bone loss in the rat and postmenopausal bone loss share many similar characteristics, and similar skeletal responses to therapy with 17β-estradiol[17]. These similarities are strong evidence that the ovariectomized (OVX) rat bone loss model is suitable for studying bone loss in postmenopausal women[18]. The purpose of this study was to evaluate whether the steroidal saponins from the rhizomes of *Anemarrhena asphodeloides* are effective in ameliorating bone loss due to OVX and, if so, whether they function in a manner similar to estrogen.

Materials and methods

Drugs and reagents Nylestriol was purchased from Shanghai Hualian Pharmaceutical. The reagent kits for measurement of calcium, inorganic phosphorus and alkaline phosphatase activity in serum were obtained from Fortune Biomedical Engineering (Shanghai, China). RIA kits for measurement of estradiol (E_2) and osteocalcin (also called Bone Gla Protein, BGP) levels were purchased from the Atomic Energy Institute of China. Goldner's staining reagents were purchased from Sigma Chemical(St Louis,MO,USA). Methyl methacrylate, dibutyl phthalate, benzoyl peroxide and other reagents were domestic analytical grade.

Preparation of steroidal saponins Rhizomes of *Anemarrhena asphodeloides* were collected in a valley located in Bozhou, (Anhui, China) in September 2004 and identified by Prof Han-chen ZHENG. A reference specimen (voucher No 20040903) was deposited in the Herbarium of the Second Military Medical University, China.

The rhizomes of *Anemarrhena asphodeloides Bunge* were ground into powder. A total of 10 kg of the powder was added to a container and extracted by percolation once with a 200 L volume of 30% aqueous ethanol. The ethanolic extracts were filtered and concentrated under vacuum to a volume of 15 L. The soluble extracts were chromatographed on macroporous resin (D101, Zheng Tian-cheng Chemical Company, Tianjin, China), eluted with water, 20% and 50% ethanol successively. The elutes of 50% ethanol were concentrated to remove solvent and obtain dried powders. These dried powders were the total steroidal saponin of the rhizomes of *Anemarrhena asphodeloides* (ATS). Analysis of the chemical compositions of ATS found that timosaponin BII, E1, B, and A-III were the major active components. The chemical compositions were analyzed by HPLC-ELSD. Total saponin content was 77% of ATS.

Animals and experimental protocol Sixty female Sprague-Dawley rats, 12 weeks of age, were purchased (SLACOM Experimental Animal Company of Shanghai, China) and acclimated to conditions for 1 week before the experiment. The experimental animals were housed in an air-conditioned room with 12 h/12 h light-dark illumination cycles at constant temperature 24±0.5 °C and humidity (45%–50%). Food and drinking water were supplied *ad libitum*. The rats were weighed every week during the experiments.

Ten rats were sham-operated and treated with vehicle (deionized water) as aging control (sham+Veh). The remaining rats were bilaterally ovariectomized and randomly divided into five groups with 10 per group. They were treated with vehicle (water), nylestriol[12] (1 mg/kg, ig, weekly) or ATS (50, 150, and 300 $mg \cdot kg^{-1} \cdot d^{-1}$, ig) for 12 weeks. Rats received treatments *po* starting from one day after surgery. For *in vivo* fluorochrome labels, tetracycline (20 mg/kg) and calcein (10 mg/kg) were injected into the rats 14 d, 13 d, 4 d, and 3 d before death. Success of ovariectomy was confirmed at necropsy by failure to detect ovarian tissue and by observation of marked atrophy of uterine horns. At the end of the treatment, the blood samples from all the groups were withdrawn by the eye vein method to assess biochemical parameters. The uteruses were removed and immediately weighed. This experiment was approved by the Bioethic Committee of

the Second Military Medical University, and the procedures of the experiment were strictly in accordance with generally accepted international rules and regulations.

Bone mineral density (BMD) assay The femurs were cleaned off adhering soft tissues, and then enclosed with gauze saturated by PBS and stored in a freezer at -80 ºC. The bone mineral density was determined by dual-energy X-ray absorptiometry (LUNAR, USA) using the small animal scan mode. The coefficients of variation (CV) of inter-observed and intra-observed BMD measurement of femurs were 0.68% and 1.16%, respectively.

Serum biochemical index assay Serum calcium (Ca), inorganic phosphorus (Pi) concentration and serum alkaline phosphatase (ALP) activity were measured on an automatic analyzer (Ciba-Corning 550, USA) using diagnostic reagent kit *in vitro* determination. The levels of E_2 and BGP were determined using a specific and sensitive double-antibody RIA kit on γ-ray counter (CAS-SN 695B, China).

Cancellous bone histomorphometric analysis The left proximal tibia metaphysis (PTM) were opened to expose the marrow cavity using an isomet low speed saw (Buechler, USA) and fixed in 10% phosphate buffer formalin for 24 h. They were then dehydrated in ethanol, defatted in xylene and embedded undecalcified in methyl methacrylate. The frontal sections were cut at 4-μm and 10-μm thickness with microtome (Leica RM 2155, Germany). The 4-μm section was stained with Goldner's Trichrome staining for static histomorphometric measurements, the unstained 10-μm sections were used for dynamic histomorphometric analyses.

Quantitative bone histomorphometric measurements were performed with a digitizing system consisting of a light and epifluorescent microscope. The system was coupled to an Apple Macintosh computer with a morphometry program, Stereology (KSS Computer Engineers, Magna, UT). The studied region of PTM was cancellous bone between 1 and 4 mm distal to the growth plate-epiphyseal junction. Ten animals from each group were studied and three sections per animal were used. 2–10 random fields were selected for each section. Positive fields were observed under a microscope. The CV of inter-observed and intra-observed histomorphometric analysis were 1.08% and 1.58%, respectively.

Statistical analysis The data was analyzed using one-way ANOVA followed by *post hoc* Sheffe's test using SPSS computer software Version 6.0. Level of significance was fixed at 0.05.

Results

Effects of OVX and drug treatment on body weights and uterine weights The six rats started with similar mean body weights. At 12 weeks post-OVX, body weights gained in OVX rats were significantly greater than in the sham group (Figure 1). Increases in the body weight of animals treated with ATS (OVX+ATS) were almost the same as those in OVX rats (in 11−12 weeks). With the administration of nylestriol, the increase was also similar to that of sham rats (Figure 1). The weights of uterus in OVX rats were decreased compared to that in the sham group (836±22 g *vs* 140±6 g, $P<0.01$, Figure 2). Administration of nylestriol increased the weight of the uterus in OVX rats. The weight of uterus in OVX +ATS group was slightly greater than that in the OVX group ($P<0.05$, Figure 2).

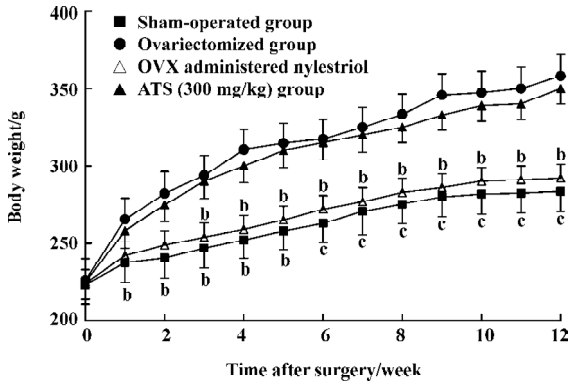

Figure 1. Changes in body weight in rats. As the changes of ATS (50 mg/kg) and ATS (150 mg/kg) group were the same as ATS (300 mg/kg) group, their curves were not shown. n=10. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* ovariectomized group at corresponding times.

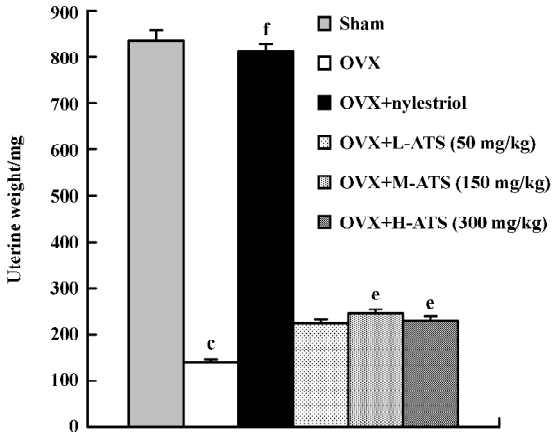

Figure 2. Effects of OVX and drug treatment on uterine weights of rats. n=10. Mean±SD. $^cP<0.01$ *vs* Sham rats. $^eP<0.05$, $^fP<0.01$ *vs* OVX rats.

Effects of OVX and drug treatment on BMD There were lower densities of the right femur in the OVX group when

compared with the sham group (0.269±0.011 g/cm^2 and 0.248±0.009 g/cm^2, P<0.05). This indicated that ovariectomy decreased the BMD of rats by 7.8%. Administration of nylestriol or ATS caused an increase of bone densities when compared with the OVX control group (from 0.248±0.009 g/cm^2 to 0.270±0.011 g/cm^2, P<0.05) (Figure 3).

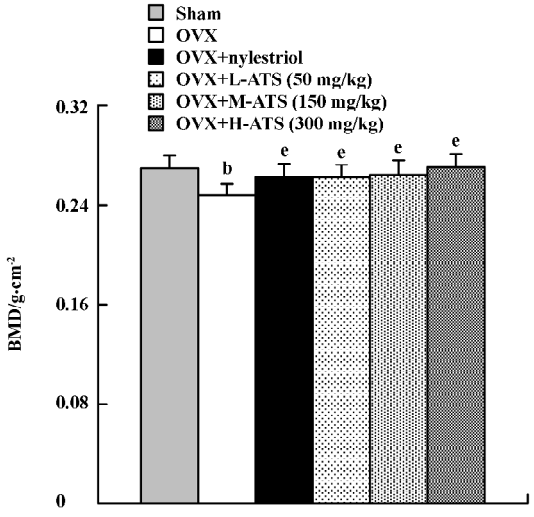

Figure 3. Effects of OVX and drug treatment on BMD of rats. n=10. Mean±SD. ^bP<0.05 *vs* Sham rats. ^eP<0.05 *vs* OVX rats.

Effects of OVX and drug treatment on serum parameters Ovariectomy induced a rise in serum ALP activity and BGP production, and the treatment with ATS decreased the BGP level, but increased the ALP activity compared with the OVX control group (P<0.05, Table 1). The contents of serum calcium and phosphorus were decreased in OVX rats compared with sham rats. Nylestriol recovered the level of serum calcium and phosphorus (P<0.05), while ATS could only increase the level of calcium, but could not improve the content of serum phosphorus. Ovariectomy induced a decrease in serum estradiol (from 25±2.1 ng/L to 14±1.5 ng/L, P<0.05), and treatment with H-ATS or nylestriol, improved the E_2 level compared with the OVX group (P<0.05, Table 1).

Effects of OVX and drug treatment on cancellous bone of PTM The proximal tibia sections from each experimental group were examined for any histological changes. Ovariectomy induced a marked decrease in the relative abundance of trabecular bone compared to sham animals (Figure 4B). OVX animals treated with ATS or nylestriol exhibited a lesser reduction in trabecular bone volume. Microscopic examination of the tibia of the sham group revealed normal size, shape and bone architecture with competent bone (Figure 4A). OVX Group sections exhibited disruptive and lytic changes and fibration matrix with osteodystrophy (Figure 4B). ATS or nylestriol showed significant restorative progress with mineralization along with fairly well-distributed osteocytes. Uniform trabeculae with variable dense matrix and shaft size were observed (Figure 4C, 4D).

This morphological observation was quantitated by histomorphometric analysis of longitudinal cross sections obtained from the proximal tibiae. A marked bone loss was observed in the OVX rats when compared with sham controls (Table 2). This bone loss was accompanied with a remarkable decrease in trabecular number (P<0.05) , trabecular thickness and bone area/tissue area (BA/TA), increase in trabecular separation (P<0.01 *vs* sham, Table 2), osteoclast number and bone formation parameters such as mineral apposition rate (MAR), bone formation rate (BFR)/bone surface (BS), and BFR/bone volume (BV) (P< 0.01 *vs* sham; Table 3). The nylestriol could partially prevent the bone loss at the PTM of OVX rats. In OVX rats treated with ATS, the trabecular thickness and BA/TA increased (P<0.05 *vs* OVX; Table 2), trabecular separation decreased (P<0.05 *vs* OVX; Table 2) and trabecular number was not different compared to OVX rats. The parameters of bone formation such as % label perimeter, BFR/BS, BFR/BV, and BFR/tissue volume (TV)

Table 1. Effect of ATS on serum biochemical markers. n=10. Mean±SD. ^bP<0.05, ^cP<0.01 *vs* Sham rats. ^eP<0.05, ^fP<0.01 *vs* OVX rats.

Groups	Calcium (mmol/L)	Phosphorus (mmol/L)	ALP (IU/L)	BGP (μg/L)	E_2 (ng/L)
Sham	3.6±0.5	2.50±0.02	92.1±26.1	2.7±0.1	25±2.1
OVX	2.6±0.2^b	1.62±0.01^b	124.0±9.3^b	6.4±0.2^b	14±1.5^b
OVX+nylestriol	3.4±0.4^e	2.29±0.02^e	105.4±19.1^e	4.2±0.4^e	21±2.0^e
OVX+50 mg/kg ATS	3.3±0.3^e	1.66±0.03	132±23.0^e	2.0±0.3^e	16±1.8
OVX+150 mg/kg ATS	3.3±0.3^e	1.65±0.02	143.1±28.8^e	2.2±0.3^e	17±1.6
OVX+300 mg/kg ATS	3.4±0.3^e	1.74±0.03	163.6±24.8^e	4.9±0.2^e	31±2.7^e

Table 2. Effect of ATS on static parameters of proximal tibial cancellous bone histomorphometry in rats induced by ovariectomy. n=10. Mean±SD. ^{b}P<0.05, ^{c}P<0.01 *vs* Sham rats. ^{e}P<0.05, ^{f}P<0.01 *vs* OVX rats.

Groups	Percent trabecular area/%	Trabecular thickness/μm	Trabecular number/mm^{-1}	Trabecular separation/μm
Sham	23.97±2.94	70.1±10.8	3.4±0.3	222.3±22.8
OVX	5.32±1.09^{c}	43.6±1.4^{b}	1.3±0.2^{c}	1267.4±127.6^{c}
OVX+nylestriol	13.57±2.27^{e}	57.4±4.8	2.4±0.3^{f}	372.5±52.1^{f}
OVX+50mg/kg ATS	6.93±1.98	64.6±8.6^{e}	1.1±0.2	701.8±87.7^{e}
OVX+150mg/kg ATS	7.05±3.33	58.8±5.0^{e}	1.2±0.5	792.5±111.4^{e}
OVX+300mg/kg ATS	12.17±1.59^{e}	69.3±9.9^{e}	1.2±0.6	768.5±95.1^{e}

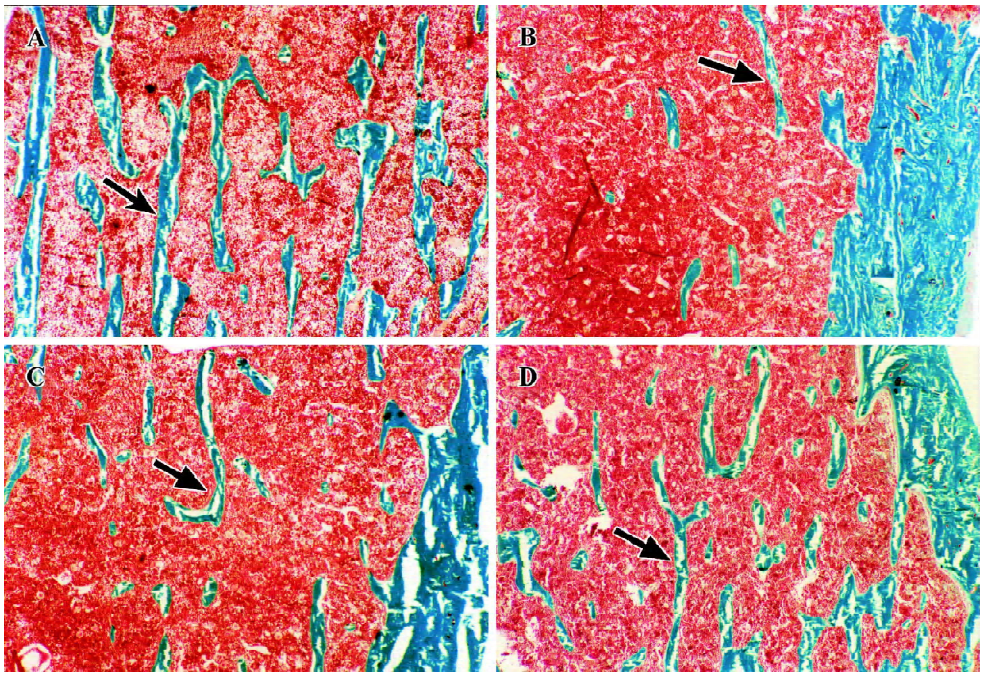

Figure 4. The representative image histological feature of PTM (n=10). (A) Photomicrograph of sham group showing normal, compact and uniform trabeculae with inter-trabecular spaces. (B) Photomicrograph of OVX group showing sparse, thinning of trabeculae with tendency for disappearance, loss of connectivity and widening of inter-trabecular spaces in an OVX rat. These trabeculae marked disruptive and lytic changes. (C) Photomicrograph of nylestriol group showing moderately thick elongated trabeculae and narrowed inter-trabecular spaces and showing restoration of normal architecture. (D) Photomicrograph of H-ATS group also showing complete restoration of normal architecture. ×40.

Table 3. Effect of ATS on dynamic parameters of proximal tibial cancellous bone histomorphometry in rats induced by ovariectomy. n=10. Mean±SD. ^{b}P<0.05, ^{c}P<0.01 *vs* Sham rats. ^{e}P< 0.05, ^{f}P<0.01 *vs* OVX rats.

	Osteoclast	% Label	Mineral rate/μm·d^{-1}	BFR/BS (μm/d×100)	BFR/BV (%/year)	BFR/TV (%/year)
Sham	3.5±1.8	22.9±5.10	0.78±0.08	15.7±4.1	141.7±54.1	32.9±8.59
OVX	11.9±1.5^{c}	33.8±5.9	1.2±0.08^{c}	37.4±2.5^{c}	402.6±44.9^{c}	24.7±6.8
OVX+nylestriol	10.2±2.7	24.1±3.7^{e}	0.76±0.13^{f}	18.2±4.0^{f}	194.2±48.9^{f}	26.6±8.8
OVX+50mg/kg ATS	9.9±1.6	26.9±3.8	1.20±0.13	33.6±7.9	320.1±67.4	21.3±4.0
OVX+150mg/kg ATS	14.2±1.3	26.8±1.9	1.16±0.19	32.9±3.1	334.1±59.9	22.1±8.0
OVX+300mg/kg ATS	11.5±1.8	45.5±2.9^{f}	1.28±0.11	55.0±1.3^{e}	517.5±33.9^{e}	42.6±3.6^{e}

were decreased at the dose of 50 mg/kg and 100 mg/kg ATS, whereas they were increased at the dose of 300 mg/kg (P<0.05 *vs* OVX, Table 3). However, ATS had no effect on osteoclast number in OVX rats.

Discussion

Our study clearly demonstrated the usefulness and beneficial effects of ATS in the prevention of bone loss induced by ovariectomy. ATS showed mild estrogenic action by slightly increasing the uterine weight (Figure 2) and E_2 level in serum in ovariectomized rats, and could increase the bone mineral density by promoting bone formation without a reduction in bone resorption.

OVX rats have been widely used as an animal model in the study of the prevention and treatment of postmenopausal osteoporosis. There are many observed similarities between ovariectomy-induced bone loss in rats and postmenopausal bone loss in humans such as increased bone turnover with resorption exceeding formation, and a significant loss of cancellous bone rather than cortical bone[17]. Furthermore, biochemical markers of bone turnover have been widely used as a research tool to measure the effect of drugs on bone remodeling. Serum BGP and ALP, two sensitive markers of bone formation, correlate with histomorphometric indices of bone formation[19]. Serum TRAP, a marker of bone resorption, positively correlates with histomorphometric indices of bone resorption. These serum parameters were increased in OVX rats.

Nylestriol, like estriol, is structurally similar to mammalian estrogen 17β-estradiol. It has been widely used in clinical practice for antiosteoporosis and it is a type of hormone replacement therapy. Functionally, nylestriol has the same activities and mechanism as 17β-estradiol for osteoporosis. Nylestriol plays an important role in maintaining bone volume and improving bone microarchitecture by markedly inhibiting bone turnover and bone resorption. It is clear that nylestriol can prevent the bone loss of OVX-induced osteoporotic rats. Furthermore, nylestriol has seldom endometrial hyperplasia phenomena. Its therapeutic and preventive effect is better than estradiol used alone. Therefore, we applied nylestriol as the positive drug in our experiment.

A comparison of treatment with ATS to nylestriol shows many differences. One difference between ATS and nylestriol was their effects on body and uterine weight (Figure 1 and Figure 2). As reported in a previous study[18], nylestriol significantly suppressed the increased body weight of OVX rats and returned it to the sham levels, and significantly increased uterine weights compared to OVX rats. Currently estrogen is the drug of choice for preventing loss of bone in postmenopausal women. However, estrogen therapy in postmenopausal osteoporosis increases the risk of endometrial cancer. Recently, a major research effort has been targeted at finding a therapy that has the positive skeletal effects without the potentially negative effects on reproductive tissue. Raloxifene, one example of a compound in this class, is a mixture of estrogen antagonist/agonist. Raloxifene has been shown in the OVX rats to have the positive effects of estrogen on bone and serum total cholesterol without causing uterine hypertrophy[20]. Surprisingly, we found that ATS had no remarkable effect on body weight and uterine weight of OVX rats. This lack of uterotrophic activity could be beneficial in reducing the risk of endometrial, breast or ovarian cancer associated with estrogen treatment[21,22]. Another difference between ATS and nylestriol was their effects on the bone formation marker-serum BGP and ALP (Table 1) measured in this experiment. Nylestriol significantly decreased serum ALP and BGP level to sham levels, while ATS significantly increased the serum ALP level. Serum BGP and ALP levels most likely reflect a newly synthesized protein as well as that released from bone matrix during resorption. In the bone histomorphometric analysis, nylestriol could decrease the bone formation parameters and bone resorption parameters, whereas ATS decreased the bone formation parameters at the dose of 50 mg/kg and 100 mg/kg, increased it at the dose of 300 mg/kg, and did not alter the bone resorption parameters. Therefore, we thought that ATS might primarily affect the synthesis of a new protein without significantly affecting the loss of OC from bone matrix. The different effects of ATS and nylestriol indicate that their mechanisms of action may differ in relation to their skeletal effects. Although there is no significant difference between OVX and ATS in MAR. The parameters of bone formation such as % label perimeter, BFR/BS, BFR/BV, and BFR/TV were increased. The data in our present study indicated that ATS have direct effects on bones by promoting bone formation. Whether ATS does not work through the estrogen pathway needs to be further studied.

In the studies of prevention and treatment of osteoporosis, phytoestrogen has aroused general concern. Phytoestrogens are chemical compounds in higher plants with estrogen-like biological activity. The main types of phytoestrogens are isoflavones, flavonoids, coumestans and lignans. After consumption of isoflavones and lignans, heterocyclic phenols are formed, which in stereochemical structure are close to estrogen, and have the capacity to bind to the estrogen receptors[23]. In particular, isoflavones can enhance osteoblastic osteoprotegerin production, which in turn is known to block bone resorption (and osteoclast formation *in vitro*)[24]. It has been reported that steroidal saponin from *Dicorea spongiosa* has antiosteoporotic activity. These steroidal saponins prevent bone loss in ovariectomized rats by promoting proliferation, ALP activity of osteoblasts and in-

hibiting the TRAP activity of osteoclasts[25]. ATS is steroidal saponin extracted from *Anemarrhena asphodeloides*. However, it did not inhibit the bone resorption as did extract from *Dicorea spongiosa*. The difference in the antiosteoporotic effect of steroidal saponin from the rhizome of *Dicorea spongiosa* and *Anemarrhena asphodeloide*s warrants further study.

In conclusion, the findings of the present study indicate that ATS, similar to estrogen, could be just as effective as nylestriol administration in suppressing bone loss due to ovariectomy and that the main effects of ATS would be the promotion of bone formation and not the inhibition of bone resorption compared with nylestriol. Thus, the administration of ATS, instead of nylestriol, is a more useful treatment for bone loss caused by estrogen deficiency.

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