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ORIGINAL PAPER

Ameliorative effects of *Vaccaria segetalis* extract on osteopenia in ovariectomized rats

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Abstract The purpose of this study was to determine the ameliorative effects of a crude extract of *Vaccaria segetalis* (Neck.) Garcke (Caryophyllaceae) (VSE) on osteopenia in ovariectomized (OVX) rats over 12 weeks. Rats were divided into the sham and OVX groups. The OVX rats were allowed to lose bone for 6 weeks. At 6 weeks post-OVX, the OVX rats were divided into four groups treated with water, 17β-estradiol (30 μg/kg, daily subcutaneous injection), or VSE (0.5 or 1.0 g/kg, daily, orally) for 6 weeks. In OVX rats, the increases of serum total cholesterol were significantly decreased by VSE or 17β-estradiol treatment. There were decreases in bone density and calcium content, including the left femur and the fourth lumbar vertebra, when compared with the sham control rats. Treatment with 17β-estradiol or VSE ameliorated these changes induced by OVX. In addition, ovariectomy increased urinary deoxypyridinoline (DPD) amounts ($P < 0.001$). The increases were suppressed by 17β-estradiol and 0.5 or 1.0 g/kg VSE ($P < 0.01$, $P < 0.05$, $P < 0.01$, respectively). Our results demonstrated that VSE ameliorates ovariectomy-induced osteopenia by inhibition of bone resorption.

Keywords *Vaccaria segetalis* · Ovariectomy · Osteopenia

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Introduction

The plant *Vaccaria segetalis* (Neck.) Garcke (Caryophyllaceae) is an annual herb widely distributed in Asia, Europe, and other parts of the world. The seeds of this plant, called *Wang Bu Liu Xing* in traditional Chinese medicine, have been prescribed frequently to cure diseases in women after childbirth owing to their capacity to activate blood flow and promote milk secretion. *Wang Bu Liu Xing* is also used in the treatment of amenorrhea and breast infections [1].

Previous studies have shown that the ethanol extract of *Vaccaria segetalis* seeds contains many compounds including triterpenoid saponins, glycosides, and cyclic peptides [2–7]. Moreover, some of the isolated nonsteroidal cyclic hexa-and pentapeptides (segetalin A, B, G, and H) exhibit estrogen-like activity [2, 4].

In previous work, a methanol extract (MeOH Ext) and ethyl acetate extract (AcOEt Ext) of *Vaccaria segetalis* seeds and a cyclic peptide from the extracts were bioassayed for their female sex hormone-like activities in rats. When MeOH Ext, AcOEt Ext, and segetalis A and B were administrated to rats for 14 consecutive days, the weight of the uterus increased dose dependently [2]. However, that study was restricted to the normal animal model for a short period.

Nevertheless, studies in appropriate animal models will provide additional insights into the physiological effects of specific susceptibility. Ovariectomy-induced bone loss in

the rat and postmenopausal bone loss share many similar characteristics, and similar skeletal response to therapy with 17β-estradiol [8]. Standard dose estrogens reduce bone turnover markers by 40–50%. Low dose oral estrogen [9] or transdermal estradiol [10] suppress bone turnover by about 25–30%, whereas ultralow estrogen dosage reduces turnover by about 20% [9, 11]. These similarities are strong evidence that the ovariectomized (OVX) rat bone loss model is suitable for studying postmenopausal bone loss in women [12].

Thus, the present study was designed to develop the animal model that resembles disease course in order to examine the ameliorating effect of VSE on bone loss due to OVX. In order to probe one of the possible mechanisms of VSE-induced amelioration of bone loss, we also examined the effect of VSE on the levels of serum alkaline phosphatase and urinary deoxypyridinoline.

Materials and methods

Preparation of the plant extract

Dry seeds of *Vaccaria segetalis* (Neck.) Gracke (Caryophyllaceae) were purchased from the local market in August 2007. All materials were sorted and identified by Associate Professor Chao-Lin Kuo and voucher specimens (CMCP 1560) were deposited in the China Medical University, Taichung, Taiwan.

Fifty kilograms of ground *Vaccaria segetalis* was soaked in 270 l methanol and extracted in a Waring blender (four times), followed by a 2-h reflux. The methanol extracts were filtered and concentrated under reduced pressure at 40°C to obtain a 1,912-g crude VSE extract in approximately 3.6% yield. The extract was stored at −20°C until use. The extract was diluted and adjusted as appropriate, then administered orally to rats at a dose of 0.5 g or 1.0 g/kg body weight, respectively. Control rats were administered water in a similar way.

A sample of crude VSE extract was partitioned between H_2O, EtOAc, and *n*-butanol. The *n*-butanol-soluble material was purified using a Diaion HP-20 column (H_2O–MeOH). The 80% MeOH-eluted fraction was chromatographed on ODS-MPLC and finally ODS-HPLC to afford two isolated peptides, segetalins G (0.001%) and H (0.002%), containing basic amino acids such as Arg and Lys (Fig. 1).

Animals

Female Wistar rats were obtained from the National Laboratory Animal Breeding and Research Center, National Science Council, and fed with laboratory diets (Fwu Sow Industry, Taichung) and tap water ad libitum. The experimental animals

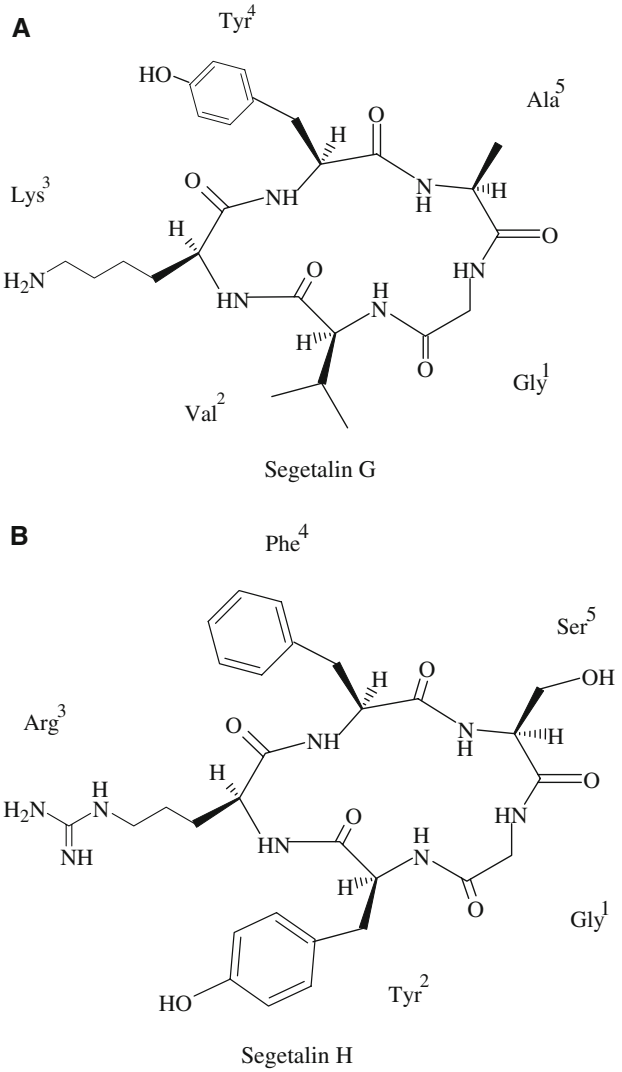

Fig. 1 Structure of segetalins G and H

were housed in an air-conditioned room at 23 ± 2°C with 12 h of light. At the beginning of the study, rats were 6 months old with an average weight of 320 ± 6.8 g. The rats were weighed every week during the experiments.

The rats were divided into five groups ($n = 9$): four OVX groups and one sham-operated group. The OVX rats were allowed to lose bone for 6 weeks. At 6 weeks post-OVX, the OVX rats were subdivided into four groups that received vehicle (water), 17β-estradiol (30 μg/kg, daily, subcutaneous injection), or VSE (0.5 or 1 g/kg, daily, orally) for 6 weeks, respectively. The sham-operated group was treated orally with vehicle. 17β-Estradiol was dissolved in a small volume of absolute alcohol, and the concentration was adjusted with sesame oil.

Surgery on animals was done under pentobarbital sodium (30 mg/kg, intraperitoneally) anesthesia. Bilateral ovariectomies were performed from a dorsal approach with a small midline dorsal skin incision. Success of

ovariectomy was confirmed at necropsy by failure to detect ovarian tissue and by observation of marked atrophy of uterine horns. The sham-operated rats were subject to sham surgery in which the ovaries were exposed but not removed.

Sixteen-hour urine samples were collected at 6 and 12 weeks after OVX. Blood samples were also collected at the same time. On the last day of the study, the animals were sacrificed under ether anesthesia. The femora and lumbar vertebra were dissected and stored in a freezer at −80°C until examinations. Several organs, including the uterus, vagina, and thymus, were dissected out and weighed immediately. All animals were treated humanely and the study protocols were in compliance with our institution's guidelines for the use of laboratory animals.

Biochemistry

Serum alkaline phosphatase, total cholesterol, serum calcium, and urinary calcium were determined on an automatic analyzer (Ciba-corning 550, USA) using Beckman's kits.

Urinary deoxypyridinoline (DPD) was assayed on an automated chemiluminescence system using Chiron diagnostic ACS: 180. The values of DPD in urine samples were expressed per millimole of urinary creatinine. Urinary creatinine content was determined enzymatically with an aliquot of each urine sample using a clinical chemistry analyzer.

Bone density

The right femur and fourth lumbar vertebra were cleaned of soft tissues. Bone volume and density were measured by using Archimedes' principle [12]. Briefly, each bone was put in an unstoppered vial filled with deionized water, and the vial was placed in a desiccator connected to a vacuum for 90 min. The desiccator was agitated periodically to ensure that all trapped air diffused out of the bone, at which time the bone was removed from the vial, blotted with gauze sponge, weighed, and returned to the vial containing deionized water. The bone was reweighed in a boat suspended, but completely immersed, in water previously equilibrated to room temperature, and the density (in grams per cubic centimeter of bone volume) was calculated. Before the density was measured as outlined above, the femur was cut at the mid-diaphysis and the marrow was washed out.

Bone calcium content

After the bone marrow had been carefully removed, the right femur and the fourth lumbar vertebra were desiccated in different baths of alcohol, and dried overnight at 100°C. The dry weight was then determined. Femur and the fourth lumbar vertebra were incinerated for 12 h at 1,000°C, and then the ash weight was determined. The samples were pulverized and divided into suitable aliquots for analysis.

The bone calcium content was determined by atomic absorptiometry (Z-8200; Hitachi, Ltd., Japan) using samples dissolved in a solution of 6 N HCl and 0.1 N HNO_3 (1:9), diluted with HNO_3, and containing 0.1% lanthanum chloride solution to prevent interference. The values were expressed as milligrams of calcium per cubic centimeter of bone volume.

Statistical analysis

Statistical significance was calculated by one-way analysis of variance coupled with the Dunnett's test. The statistically significant differences between the body weight, serum total cholesterol at 12 weeks post-OVX and 6 weeks post-OVX were tested by a paired *t* test. *P* values less than 0.05 were taken as significant.

Results

Body weight

The five rat groups started with similar mean body weights. At 6 weeks post-OVX, body weight gains in OVX rats were significantly greater than in sham rats (+25.1%). At 12 weeks post-OVX, both the OVX control group and OVX + VSE 0.5 g/kg group, but not OVX + 17β-estradiol and OVX + VSE 1.0 g/kg groups, had gained significantly more weight than at 6 weeks post-OVX (Table 1).

Organ weights

The weights of uterus and vagina in OVX rats were significantly decreased compared with sham rats ($P < 0.001$, $P < 0.001$, respectively). The weights of uterus and vagina were, respectively, as follows: 0.668 ± 0.137 and 0.269 ± 0.059 g for the sham group, 0.148 ± 0.011 g ($P < 0.001$) and 0.110 ± 0.050 g ($P < 0.001$) for the OVX control group. Administration of 17β-estradiol, but not VSE, increased in the weight of the uterus and vagina in OVX rats. The weights of uterus and vagina, respectively, were as follows: 0.843 ± 0.065 g ($P < 0.001$) and 0.240 ± 0.016 g ($P < 0.001$) for the OVX + 17β-estradiol group.

As shown in Table 2, there were no differences regarding ovariectomy in terms of thymus weight. The thymus weights of 17β-estradiol-treated rats were

Table 1 Effect of VSE or 17β-estradiol (E_2) on body weight and the weight of thymus in OVX rats

Drugs	Dose (mg/kg/day)	Body weight (g)		
		0 week	6 week	12 week
Sham	–	250.5 ± 10.8	317.8 ± 9.2	357.8 ± 14.0
OVX	–	246.5 ± 14.4	385.7 ± 7.0[a]	437.0 ± 14.1[a,b]
OVX + VSE	500	240.8 ± 8.8	415.3 ± 14.9[a]	440.0 ± 20.9[b]
	1,000	253.8 ± 9.9	394.4 ± 13.2[a]	412.3 ± 15.9
OVX + E_2	0.03	249.0 ± 12.6	394.3 ± 9.5[a]	410.1 ± 16.2

All values are mean ± SE

[a] $P < 0.01$ compared with the sham group

[b] $P < 0.05$ compared with the body weight at 6 weeks post-OVX by paired t test

Table 2 Effect of *Vaccaria segetalis* extract (VSE) or 17β-estradiol (E_2) on absolute weight of the uterus, vagina, and thymus in OVX rats

Drugs	Dose (mg/kg/day)	Weight		
		Uterus (g)	Vagina (g)	Thymus (g)
Sham	–	0.668 ± 0.137	0.269 ± 0.059	0.265 ± 0.022
OVX	–	0.148 ± 0.011[c]	0.110 ± 0.015[c]	0.335 ± 0.030
OVX + VSE	500	0.147 ± 0.011[c]	0.163 ± 0.013[a]	0.341 ± 0.022
	1,000	0.273 ± 0.104[b]	0.186 ± 0.032	0.390 ± 0.033[a]
OVX + E_2	0.03	0.843 ± 0.065[e]	0.240 ± 0.016[e]	0.227 ± 0.010[d]

All values are mean ± SE

[a] $P < 0.05$ compared with the sham group

[b] $P < 0.01$ compared with the sham group

[c] $P < 0.001$ compared with the sham group

[d] $P < 0.05$ compared with the OVX control group

[e] $P < 0.001$ compared with the OVX control group

significantly decreased compared with the OVX control rats ($P < 0.01$) (Table 2).

Serum total cholesterol

At 6 weeks post-OVX, the levels of serum total cholesterol in OVX rats were significantly greater than in sham rats (+37.1%). At 12 weeks post-OVX, the OVX + 17β-estradiol rats and the OVX + VSE 1.0 g/kg rats had significantly lower total serum cholesterol levels compared with the OVX control group ($P < 0.05$) (Table 3).

Urinary DPD, urinary calcium, and serum calcium

Ovariectomy induced a rise in the content of urinary DPD (+125.2%), and treatment with 17β-estradiol or 0.5 or 1.0 g/kg VSE lowered the content of urinary DPD compared with OVX control group ($P < 0.01$, $P < 0.05$, $P < 0.01$, respectively) (Table 3). Ovariectomy and treatment with 17β-estradiol or VSE had no effect on serum calcium. The serum calcium content of the sham group was 2.5 ± 0.2 nmol/l. Ovariectomy caused a decrease of urinary calcium and this was corrected by 17β-estradiol treatment. There were no differences between the VSE-treated group and OVX control group regarding the content of urinary calcium (Table 3).

Serum alkaline phosphatase

At 12 weeks post-OVX, the levels of serum alkaline phosphatase in OVX groups were significantly higher than in the sham group ($P < 0.001$). At 12 weeks post-OVX, the OVX + 17β-estradiol group, but not the OVX + VSE groups, had a significantly lower level of serum alkaline phosphatase compared with the OVX group (Table 3).

Bone density and bone calcium content

There were significantly lower densities of the femur and the fourth lumbar vertebra in the OVX group compared with the sham group ($P < 0.001$, $P < 0.001$, respectively). Administration of 17β-estradiol and 1.0 g/kg VSE caused a significant increase of bone densities of the femur

Table 3 Effect of VSE or 17β-estradiol (E_2) on urinary deoxypyridinoline (DPD), serum alkaline phosphatase (ALP) content, and total serum cholesterol in OVX rats

Drugs	Dose (mg/kg/day)	Urine		Serum	
		DPD (nmol/mmol creatinine)	Calcium (μmol)	ALP (IU/l)	Cholesterol (mg/dl)
Sham	–	109.2 ± 8.5	22.1 ± 0.2	47.4 ± 8.7	74.2 ± 6.6
OVX	–	245.9 ± 25.9[b]	12.6 ± 0.3[a]	106.3 ± 9.6[b]	95.3 ± 5.5[a]
OVX + VSE	500	175.8 ± 8.4[c]	13.5 ± 0.8[a]	88.2 ± 7.2[a]	88.8 ± 1.5
	1,000	145.6 ± 19.2[d]	15.3 ± 0.9[a]	87.7 ± 12.4[a]	77.2 ± 4.1[c]
OVX + E_2	0.03	166.9 ± 7.0[d]	24.3 ± 0.5[c]	50.2 ± 8.3[e]	76.3 ± 4.4[c]

All values are mean ± SE

[a] $P < 0.05$ compared with the sham group

[b] $P < 0.001$ compared with the sham group

[c] $P < 0.05$ compared with the OVX control group

[d] $P < 0.01$ compared with the OVX control group

[e] $P < 0.001$ compared with the OVX control group

Table 4 Effect of VSE or 17β-estradiol (E_2) on bone densities and calcium contents of femur and fourth lumbar vertebra bone in OVX rats

Drugs	Dose (mg/kg/day)	Femoral bone		Fourth lumbar vertebra bone	
		Density (g/cm^3)	Ca content (mmol/cm^3)	Density (g/cm^3)	Ca content (mmol/cm^3)
Sham	–	1.30 ± 0.03	14.6 ± 0.3	1.26 ± 0.01	4.7 ± 0.3
OVX	–	1.20 ± 0.02[b]	13.0 ± 0.2[b]	1.11 ± 0.01[b]	3.3 ± 0.2[b]
OVX + VSE	500	1.22 ± 0.01[a]	13.2 ± 0.2[b]	1.16 ± 0.01[b]	3.8 ± 0.2
	1,000	1.26 ± 0.01[c]	14.0 ± 0.2[c]	1.16 ± 0.01[b,c]	3.9 ± 0.2
OVX + E2	0.03	1.27 ± 0.01[d]	14.1 ± 0.2[d]	1.28 ± 0.01[e]	4.7 ± 0.3[e]

All values are mean ± SE

[a] $P < 0.01$ compared with the sham group

[b] $P < 0.001$ compared with the sham group

[c] $P < 0.05$ compared with the OVX control group

[d] $P < 0.01$ compared with the OVX control group

[e] $P < 0.001$ compared with the OVX control group

compared with the OVX control group ($P < 0.01$, $P < 0.05$, respectively). Administration of 17β-estradiol and VSE caused a significant increase of bone densities of the fourth lumbar vertebra compared with the OVX control group ($P < 0.001$, $P < 0.05$, respectively) (Table 4).

Ovariectomy caused a significant decrease in calcium content of the femur and fourth lumbar vertebra. Administration of 17β-estradiol and OVX + VSE caused a significant increase in calcium content of the femur and fourth lumbar vertebra compared with the OVX control group (Table 4).

Discussion

Our results demonstrated that VSE ameliorated bone loss, but did not cause uterine hypertrophy in estrogen-deficient OVX rats.

Our data on body weight and serum total cholesterol supported observations from other investigators who reported that both gained body weight and elevated serum total cholesterol due to ovarian hormone deficiency are prevented by estrogen administration [12, 13]. Our results showed that VSE and 17β-estradiol treatment inhibited OVX-induced gained body weight and elevated serum total cholesterol.

According to previous evidence, rats in the OVX group had lower bone density and calcium content of the femur and the fourth lumbar vertebra, the loss of which was completely prevented in animals receiving 17β-estradiol [14–16]. In the present study, 17β-estradiol and VSE are also effective in preventing bone loss in the femur and the fourth lumbar vertebra.

Estrogen prevents bone loss due to ovarian hormone deficiency by a mechanism that involves the suppression of the rate of bone turnover that involves the depression of

both osteoclastic and osteoblastic bone formation [8]. Concentration of serum alkaline phosphatase, an index of bone formation, was significantly greater in the OVX group than in the sham-operated group [8, 17]. Administration of VSE did not decrease serum alkaline phosphatase in the OVX rats. Ovariectomy increased urinary DPD amounts (DPD is a marker of bone resorption [18]). Both VSE and 17β-estradiol decreased urinary DPD amounts. The results suggested that VSE ameliorated the bone loss due to OVX by inhibition of bone resorption, but not stimulation of bone formation.

Segetalins A and B exhibit estrogen-like activity and both peptides contain a common sequence, Trp-Ala-Gly-Val [2]. Previous study has shown that sequence homology among segetalins A, B, and G is recognized in Tyr-Ala-Gly-Val of segetalin G and in Trp-Ala-Gly-Val of segetalins A and B [4]. Furthermore, the sequence of Phe-Ser-Gly in segetalin H is partly similar to the above sequence in segetalins A, B, and G. The sequence homology and estrogen-like activity may be related each other [4].

According to the present results, we demonstrated that *V. segetalis* extract and 17β-estradiol exerted ameliorative effects on osteopenia in OVX rats. Since estrogen is reported to prevent bone loss [8], it is likely that the ameliorative effect of the former results from the content of segetalins G and H, which have sequence homology and exhibit estrogen-like activity; however, this hypothesis requires further investigation.

In this study and others [17, 19], treatment with 17β-estradiol completely corrected the decreased urinary calcium excretion observed in OVX rats. Our results showed that VSE has no effect on urinary calcium in OVX rats compared with the OVX control and sham group.

Estrogen is the drug of choice for preventing loss of bone in postmenopausal women [20]. However, estrogen therapy in postmenopausal osteoporosis increases the risk of endometrial cancer [16, 21]. Recently, a major research effort has been targeted at finding a drug that has the positive skeletal effects without the potentially negative effects on reproductive tissue [16]. Raloxifene, one example of a compound in this class, is a mixture of estrogen antagonist and agonist. Raloxifene has been shown to have the positive effects of estrogen on bone and serum total cholesterol [20] without causing uterine hypertrophy in OVX rats [22].

It is well known that estrogen increases the weight of the uterus and vagina [23]. The extract of *V. segatalis* also has reported estrogen-like activity and increased the weights of uterus and vagina [2, 4]. The present data showed that OVX decreased the weight of the uterus and vagina compared with the sham group. Administration of VSE did not cause uterine or vaginal hypertrophy and is effective in preventing bone loss in OVX rats. Our study result is not consistent with previous studies regarding uterus weight in different animal models. Thus, experimental conditions such as age, type, and metabolic status of the animal model, as well as the level and duration of VSE treatment may play an integral role in how VSE affects body composition. Our results indicated that VSE possessed estrogen agonist/antagonist character in OVX rats, suggesting that VSE may have a similar effect as raloxifene on bone loss and uterus weight.

The findings of the present study indicate that VSE may be useful for the treatment of osteoporosis. Further comprehensive chemical and pharmacological investigations will be needed to elucidate the exact mechanism causing this effect.

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