Anti-osteoporotic effect of *Salvia miltiorrhiza* extracts

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The preventive effect of Salvia miltiorrhiza extracts (SMEs) on the progress of bone loss induced by ovariectomy (OVX) was studied in rats. We measured body weight and bone histomorphometry in sham, OVX or SMEs-administered OVX rats. From light microscopic analyses, a porous or erosive appearances were observed on the surface of trabecular bone of tibia in OVX rats, whereas those of the same bone in sham rats and in SMEs-administered rats were composed of fine particles. The trabecular bone area and trabecular thickness in OVX rats decreased by 50% from those in sham rats, these decreases were completely inhibited by administration of SMEs for 7 weeks. In this study, the mechanical strength in femur neck was significantly enhanced by the treatment of SMEs for 7 weeks. In OVX rats, free T3 was normal in all cases, whereas free T4 was significantly increased. Although there was no difference between OVX and SMEs-administered rats in T3 level, we have found significant difference between them in T4 level. These results strongly suggest that SMEs are effective in preventing the development of bone loss induced by OVX in rats.

Key words : Ovariectomy (OVX); Salvia miltiorrhiza extracts (SMEs); osteoclast; osteoblast; Serum biochemistry; Bone histomorphometry

Introduction

Bone mass decreases during aging, and low bone mass is the major determinant of all osteoporotic features. The dramatic increase in bone turnover rate and imbalance of bone formation and bone resorption in the first years after the cessation of ovarian function are responsible for the high rate of postmenopausal bone loss in older women. Estrogen therapy is effective in preventing postmenopausal bone loss. In elderly women, in whom bone loss persists as shown by high rates of bone formation and bone resorption, estrogen replacement therapy may increase bone mass[1].

The ovariectomized (OVX) rat is a good animal model of estrogen-deficiency-induced osteopenia. Some age-related changes documented in humans, such as the appearance of porosites on the endosteal surface of bone and a loss of trabecular bone, also occur in old rats[2,3].

The annual herb, Salvia miltiorrhiza, is distributed from China and Korea. Salvia miltiorrhiza is a traditional Chinese medicine, which has been well documented for its anti-cancer effects[4]. The anti-fibrotic effects of extract form Salvia miltiorrhiza (Labiatae) was recently studied[5]. Salvia miltiorrhiza-induced inhibitory effect on cell hyperplasia by ballon angioplasty was also recently studied in rats[6]. Therefore, it is resonable that extracts of Salvia miltiorrhiza extracts be investigated for antiosteoporotic activity. In the present study, we have shown that Salvia miltiorrhiza extracts prevented the progression of bone loss induced by OVX in rats.

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Materials and Methods

1. Preparation of Salvia miltiorrhiza extracts

Salvia miltiorrhiza extracts were prepared by dissolving natural substances with distilled water at 60℃ for 3 hrs. The extract was filtered with 0.45 mm filters, lyophilized and kept at 4℃. These materials were obtained from Oriental Medicine Hospital, Wonkwang University (Iksan, South Korea) and identified by J. Kim, School of Oriental Medicine, Wonkwang University. A voucher specimen (No.99-02-0009) was deposited at the Herbarium at the College of Pharmacy, Wonkwang University.

2. Experimental animals

Thirty female Sprague-Dawley rats weighting 200-300 g were purchased from Dae Han Experimental Animal Center (Eumsung, Chungbuk, Korea), and the animals were maintained under constant temperature (25±2℃) and

humidity (55%±5%), and under 12 h/12 h light-dark cycles. The rats were housed individually in standard cages and provided with a commercial standard diet containing 1.2% calcium and 0.8% phosphorus.

At 15 weeks of age, animals were either OVX or Sham-operated (Sham). Bilateral ovariectomies were performed under anesthesia with sodium nembutal administered intraperitoneally. Sham operations were performed by exteriorizing the ovaries. OVX rats were divided into two groups based on treatment with either Salvia miltiorrhiza exracts or physiological saline. After 7 weeks of treatment, blood collected and stored at -70℃ until biochemical determinations (see below). The subsequent day, the rats were sacrificed. Both femora and tibia were dissected. The right femurs were cleaned from adherent muscles, placed in sterile saline, and stored for 8-16 h at 4℃. Left femurs of 12 and 8 randomly selected animals per group were fixed with Burkhart fixative and used further for histomorphometrical analysis (see below). All procedures using animals were carried out in accordance with the guidelines presented in the Guidline Principles for the Care and Use of Animals in the Field of Physiological Sciences, published by the Physiological Society of Korea, and were approved by the National Institute of Fitness and Sports Animal Care Committee.

3. Biochemical analysis

Serum calcium, phosphate, and alkaline phosphatase were determined by standard laboratory techniques. Serum free T4 (FT_4) and free T3 (FT_3) were measured by the Liso-Phase kits (Technogenetics, Milan, Italy) after chromatographic separation of the hormone by Sephadex LH-20 chromatography.

4. Bone histomorphometry

The left femurs were fixed for 24 h with Bürkhardt fixative, cut sagittally into 2 equal halves with a diamond saw (Buehler Isomet, low speed saw, Chicago, IL, USA), dehydrated with methanol and embedded in methylmetacrylate. These longitudinal sections of the proximal femur were cut with an AO Autocut/Jung 1150 microtome at 4 (m thickness. The sections with the widest marrow cavity near the central part of the femoral neck were selected for further histological processing and histomorphometric measurements. The 4 μm thick sections were stained according to the von Kossa method with a tetrachrome counterstain (Polysciences, Warrington, PA) for measurements of cancellous bone volume, osteoblast surface, and osteoclast surface.

All bone measurements were performed with the Bioquant Bone Morphometry System (R & M Biometrics Corp. Nashville, TN) as previously described[7]. Cancellous bone measurements were performed in the proximal femur in an area begining 1 mm distal to the growth plate-metaphyseal junction and extending further distally to the junction of the femoral neck and greater trochanter.

Cancellous bone volume as a percentage of bone tissue area and osteoblast and osteoclast surfaces as percentages of total cancellous perimeter were measured at a magnification of ×200. Trabecular number, width, and separation were calculated.

5. Measurement of mechanical strength of the femur neck

The femoral necks were fractured in a hydraulic testing device, using a loading rate of 0.095 radian/sec (5.43°/sec). First, the shafts were fractured 19 mm above the knee joint in three-point anterior bending. The necks were then fractured in combined bending and compression and bending, achieving highly reproducible and neck fractures, inititated fracture proximal and stop of the femoral neck and stretching downward in a straight line toward the laser trochanter in a shearing fashion. The load in the test apparatus was measured with a load cell connected to a microcomputer via amplifier. Load/deflection curves were recorded on-line using Work Bench Mac software (Strawberry Tree, Sunnyvale, CA)[8].

6. Statistical analysis

A software computer program (SAS, SAS Institute Inc. Cary, NC, USA) was used. Intergroup differences were analyzed by one-way analysis of variance (ANOVA), and Tukey's studentized Range test was used to compare pairs of means. These parametric statistical tests could be used because the data were normally distributed. Bone histomorphometry results were analyzed by nonparametric statistics using ANOVA and Wilcoxon tests.

Results

1. Effect of Salvia miltiorrhiza extracts on trabecular bone area

The different histological areas of the proximal tibia sections from sham, ovariectomy (OVX), OVX + Salvia miltiorrhiza extracts rats are depicted. As expected, the loss of trabeculsr bone was observed in ovariectomized rats and was most obvious in the central metaphysial region. The area partially filled with trabecular bone (the thick region) was observed only in the Salvia miltiorrhiza extracts-administered rats, which exhibited bone of a constant width. In the extracts-treated rats, the trabecular bone in the area between

the growth junction and the thick region was maintained compared with that of the sham rats. Conversely, the loss of trabecular bone in the area between the growth plate-metaphyseal junction and the thick region was remarkably pliable in the sham rats. The trabecular bone area (%) was measured (Table 1). As expected, the trabecular bone area was significantly lower in the OVX than in the sham group. However, the trabecular bone area in Salvia miltiorrhiza extracts-administered rats was maintained at the level observed in the sham rats.

Table 1. Effect of Salvia miltiorrhiza extracts (SM) on the trabecular bone area (%) of OVX rats.

Groups	No. of Animals	Trabecular bone area (%)
Sham	10	56.95±8.2
OVX	10	33.43±5.1***
OVX+SM	10	47.16±6.9#

Values are Mean±Standard Deviation. OVX: Ovariectomized. *: Statistical significance as compared with sham group (***: p(0.001). #: Statistical significance as compared with OVX group (#: p(0.05)

2. Effect of Salvia miltiorrhiza extracts on trabecular thickness and separation

At 7 weeks after operation, the trabecular thickness in OVX rats was significantly less than the value of sham. A decrease in trabecular thickness induced by OVX was inhibited by the administration of Salvia miltiorrhiza extracts for 7 weeks (Table 2). The trabecular separation in OVX rats was significantly inhibited by the administration of Salvia miltiorrhiza extracts for 7 weeks (Table 3). In addition, there was no significance of change in trabecular number between OVX and Salvia miltiorrhiza-administered OVX rats (OVX + Salvia miltiorrhiza) (data not shown).

Table 2. Effect of Salvia miltiorrhiza extracts(SM) on the trabecular thickness ((m) of OVX rats.

Groups	No. of Animals	Trabecular thickness ((m)
Sham	10	95.24±7.8
OVX	10	46.92±5.1***
OVX+SM	10	74.34±10.0###

Values are Mean±Standard Deviation. OVX: Ovariectomized. *: Statistical significance as compared with sham group (***: p(0.001). #: Statistical significance as compared with OVX group (###: p(0.01).

Table 3. Effect of Salvia miltiorrhiza extracts (SM) on the trabecular separation (μm) of OVX rats.

Groups	No. of Animals	Trabecular separation (μ m)
Sham	10	109.08±23.40
OVX	10	147.98±50.2***
OVX+SM	10	97.85±6.1***

Values are Mean±Standard Deviation. OVX: Ovariectomized. *: Statistical significance as compared with OVX group (***: p(0.001).

3. Effect of Salvia miltiorrhiza extracts on osteoclast number and osteoblast surface

The osteoclasts located on trabecular bone surfaces and the mean number of oseoclasts per unit area (mm2) of trabecular bone in the three experimental groups are shown in Table 4. The number of osteoclasts per bone area was remarkedly affected by OVX. However, there was no significant change of osteoclast numbers between in OVX rats and in OVX + Salvia miltiorrhiza rats. Although the surface of osteoblasts was significantly larger in OVX rats, the extracts-induced effect on osteoblast surface was not significant in these models (Table 4).

Table 4. Effect of Salvia miltiorrhiza extracts (SM) on the osteoclast number (No/mm) and surface (%) of OVX rats.

Groups	No. of Animals	Osteoclast number (No/mm)	Osteoclast surface (%)
Sham	10	0.58±0.20	7.20±1.6
OVX	10	1.63±0.50***	21.94±6.7***
OVX+SM	10	1.28±0.41	18.99±4.7

Values are Mean±Standard Deviation. OVX: Ovariectomized. *: Statistical significance as compared with sham group (***: p(0.001).

4. Effect of Salvia miltiorrhiza extracts on the mechanical strength of the femur neck

OVX decreased the ash weight of the femur neck at each follow-up timepoint after operation, the respective mean values being 33.86 N in the sham group and 29.89 N in the OVX group at 7 weeks after OVX. The maximum loads of femoral neck also decreased due to estrogen deficiency at each follow-up timepoint after operation (data not shown). The mechanical strength in femur neck was significantly enhanced by the treatment of Salvia miltiorrhiza extracts for 7 weeks (Table 5).

Table 5. Effect of Salvia miltiorrhiza extracts (SM) on the mechanical strength of the femur neck of OVX rats.

Groups	No. of Animals	Mechanical strength (N)
Sham	10	33.86±4.25
OVX	10	25.39±5.89*
OVX+SM	10	29.89±3.28#

Values are Mean±Standard Deviation. OVX: Ovariectomized. : Statistical significance as compared with sham group (*: p(0.05). Statistical significance as compared with OVX group (#: p(0.05).

5. Effect of Salvia miltiorrhiza extracts on serum biochemical levels

To assess the contribution of Salvia miltiorrhiza extracts to factors involved in antiosteoporotic reactions, we conducted the serum biochemical analysis. At 7 weeks, results of serum biochemical assessment showed that the serum level of alkaline phosphatase (ALP) or inorganic phosphorus (IP) was significantly higher in OVX rats, compared with sham rats. The increase of ALP was inhibited by the administration of Salvia miltiorrhiza extracts. However, the extracts had no regulatory effect on the change of IP in OVX rats. However, the levels of serum calcium were not affected by OVX or the administeration of Salvia miltiorrhiza extracts (Table. 6). The

data reported in Table 6 also refer to the serum T3 level and T4 level. Thyrotoxicosis is associated with increased bone turnover, resulting in bone loss. Mean T4 was significantly higher in OVX rats, although the amount of T3 was not changed in OVX rats compared to sham rats. When all available data on thyroid hormone measurements were evaluated, decreased level of T4 was found in Salvia miltiorrhiza extracts-administered OVX rats.

Table 6. Effect of Salvia miltiorrhiza extracts (SM) on serum biochemical levels of OVX rats.

Groups	No. of Animals	Calcium (mg/dl)	Phosphorus (mg/dl)
Sham	10	10.97±0.37	5.60±0.88
OVX	10	10.80±0.70	7.31±1.09**
OVX+SM	10	10.64±0.55	7.28±1.02
Alkaline Phosphatase (IU/L)		Triiodothyronine (T3; ng/ml)	Thyroxine (T4; g/dl)
122.50±29.24		1.10±0.19	1.02±0.31
324.60±43.05**		1.16±0.11	3.23±0.56***
214.14±80.28##		1.12±0.17	2.64±0.53#

Values are Mean±Standard Deviation. OVX: Ovariectomized. *: Statistical significance as compared with sham group (**: p<0.01; ***: p<0.001). #: Statistical significance as compared with OVX group (#: p<0.05; ##: p<0.01)

Discussion

In traditional medicine, there are many natural crude drugs that have the potential for use to treat bone diseases; however, not much laboratory work has been reported evaluating this possible use. In a search for natural crude drugs having inhibitory activity on bone resorption, we have screened a number of plants widely used in traditional medicine for their inhibitory activity on bone resorption induced by ovariectomy (OVX).

In this report, we studied the effects of Salvia miltiorrhiza extracts on trabecular bone volume. In the Salvia miltiorrhiza-treated rats, the trabecular bone in the area of the growth plate-metaphyseal junction was maintained compared with that of sham rats. Salvia miltiorrhiza -treated rats maintained their trabecular bone in the area between the growth plate-metaphyseal junction and the thick region, and resulted in maintaining the trabecular bone structure.

Humans and rats have many histomorphologic characteristics of the proximal femur in common[9,10]. Both exhibit similar changes in bone mass and structure at numerous skeletal locations following loss of estrogen. In this study, OVX promoted cancellous bone remodeling with an increase in bone resorption that exceeded the increase in bone formation resulting in a net loss of bone. This loss of bone was also accompanied by substantial changes in trabecular structure, as indicated by immunohistochemistry. In general, data collected on the rodent model of estrogen deficiency are comparable to other reports[11-13]. As shown in Table 5, changes in the maximum loads at the femoral neck revealed a deteriorating effect of OVX after 7 weeks after operation. The loss of trabecular bone breaking force was observed in OVX rats. In Salvia miltiorrhiza-administered rats, the trabecular bone force was maintained compared with that of sham rats. Next, the ovariectomized rats induced an increase in serum phosphorus level but calcium level does not change, as reported previously[14,15]. Kalu et al[16] have reported decreased serum calcium after OVX, however, this was detected only at a higher level of dietary calcium. There does not appear to be any evidence for a reduction in serum calcium prior to bone loss in the OVX rats. The increase in bone resorption does not therefore appear to occur in response to a detectable change in serum calcium. Serum albumin levels decrease rapidly after ovariectomy, possibly indicating a direct effect of estradiol on hepatic albumin production (data not shown). This change appears to be due to decreased albumin synthesis rather than albumin leakage from disfunctional liver cells[17]. In addition, we have found that serum phosphate (IP) was increased in OVX rats. However, Salvia miltiorrhiza extracts had no regulatory effect on the increased IP. The OVX-induced increase in IP was related to increased renal phosphate reabsorption and may have a direct effect of estrogen on specific receptors in the kidney[18]. In this study, we could not observe that the amount of IP is not different between OVX and Salvia miltiorrhiza -treated OVX rats (Table 6). Although IP is significantly related to the level of bone resorption, Salvia miltiorrhiza-induced regulatory effect on bone resorption is not due to IP.

Thyroid hormones play an important role in bone remodeling[19], and histomorphometric studies have shown that thyroid hormones stimulate osteoblastic and osteoclastic activities in cortical and trabecular bone[20]. Thyrotoxicosis is associated with increased bone turnover, and the resorption rate exceeds the formation rate, thus resulting in bone loss[8]. The OVX rats induced a significant increase in serum thyroxine (T4) levels. Our results also showed that a reduction of serum T4 levels in Salvia miltiorrhiza extracts administered rats. Considering that an increased rate of bone turnover was definite in subjects taking suppressive doses of T4, Salvia miltiorrhiza extracts-inhibited T4 levels suggests the oriental drug has a regulatory effect on bone turnover.

The data presented here support Salvia miltiorrhiza extracts-induced regulation on the maintenance of the normal bone remodeling in OVX-operated rats. The results obtained in the present study provide evidence that Salvia miltiorrhiza extracts importantly contributes to the prevention of bone loss at least in OVX rats. The studies on the isolation and

characterization of the active chemical constituents are in progress.

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