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Effect of ethanol extract of *Lepidium meyenii* Walp. on osteoporosis in ovariectomized rat

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Abstract

Maca (*Lepidium meyenii* Walp.) is a cruciferous plant from the Andes of Peru. The root of Maca is traditionally employed for its supposed properties in aphrodisiacs and improving fertility, it also has been widely used to help alleviate the symptoms of menopause. The purpose of this study was to evaluate the effect of ethanol extract of Maca on postmenopausal osteoporosis in ovariectomized rats. Female Sprague-Dawley rats were divided into four groups: Sham-operated and ovariectomized groups were fed with equivolume of distilled water, and the remaining ovariectomized groups were orally administrated with ethanol extract of Maca at 0.096 and 0.24 g/kg for 28 weeks. The findings derived from the basis of bone mineral density, biomechanical, biochemical and histopathological parameters indicated that higher dose of ethanol extract of Maca was effective in the prevention of estrogen deficient bone loss.

Keywords: *Lepidium meyenii*; Osteoporosis; Bone mineral density; Biomechanics; Biochemical parameters; Ash calcium

1. Introduction

Postmenopausal osteoporosis is the most frequent metabolic bone disease, it is characterized by a rapid loss of mineralized bone tissue, disruption of trabecular architecture of the bone and changes in the crystalline properties of mineral deposits, which result in the structural failure (fracture) of sites rich in cancellous bone, such as the vertebrae, hip and distal forearm (Chestnut, 1995; Paschalis et al., 1997). Estrogen deficiency is considered as the major determinant of bone loss in postmenopausal women. Menopause results in elevated bone turnover, an imbalance between bone formation and bone resorption and net bone loss (Riggs and Melton, 1986). As the population ages, the incidence of hip fractures and costs for treatment will rise dramatically in the future, unless effective prophylactic measures are taken (Kannus et al., 1996).

Hormone replacement therapy (HRT) has proven efficacious in preventing bone loss and reducing the incidence of skeletal fractures in postmenopausal women (Turner et al., 1994). However, findings from the Women's Health Initiative Trial, suggest that long-term HRT use increases the risk of breast cancer, endometrial cancer, thromboembolic events and vaginal bleeding and it is a less desirable option for many women (Genant et al., 1998; Termine and Wong, 1998).

Maca (*Lepidium meyenii* Walp.) from the Brassicaceae family grows exclusively at altitudes over 4000 m at the Peruvian central Andes. The hypocotyl, edible part of the plant, has widely been used as a nutritional supplement and folk medicine to increase fertility and sexual function (Canales et al., 2000). Maca hypocotyl has been also used to treat women with menopausal symptoms including hot flushes, tender breasts, vaginal dryness, osteoporosis, etc. The Maca alkaloids, steroids, glucosinolates, isothiocyanates and macamides are probably responsible for its aphrodisiac, adaptogen, anabolic, immunostimulant and hormonal balance properties (Gonzales et al., 2003; Oshima et al., 2003; Valentova and Ulrichova, 2003). A number of studies showing the aphrodisiac effects of Maca both in animals and humans have appeared over the last few years (Zheng et al., 2000). But since now no studies have been carried out to evaluate whether Maca has an antiosteoporosis activity in rats. The present research was conducted to investigate the effect of ethanol extract of *Lepidium meyenii* on postmenopausal osteoporosis in ovariectomized (OVX) rats.

Abbreviations: OVX, ovariectomy; BMD, bone mineral density; HRT, hormone replacement therapy; LV, lumbar vertebrae; ALP, alkaline phosphatase

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2. Materials and methods

2.1. Preparation of ethanol extract of Lepidium meyenii

Lepidium meyenii pulverized root (standardized 0.6% macamides and macaenes) was obtained from Lima, Peru, through Chinese Maya Bioengineer Co., Hubei. The identity of the plant was authenticated by Irma Fernandez, Botanist, Department of Pharmaceutical Sciences, Universidad Peruana Cayetano Heredia. The voucher specimen (PA.247) was placed in School of Life Science & Technology, Huazhong University of Science & Technology. To prepared ethanol extract, the dried powder (400 g) were extracted with 95% ethanol (3 × 1500 mL) at room temperature for 2 days, the preparation was filtrated and evaporated using a rotary evaporator at 40 °C to get an extract at a yield of 19.2%, by weight of the starting materials. The extract was further diluted with distilled water to obtain different concentrations and kept at 4 °C.

2.2. Animals

Forty 90-day-old female Sprague-Dawley rats were purchased from Tongji Medical College (Wuhan, China). After arriving at our institution, animals were housed under controlled conditions (room temperature 23 ± 2 °C; relative humidity 55 ± 5%; 12 h-light/12 h-dark cycle), and had free access to tap water and commercially available standard solid food containing 1.22% calcium and 0.43% phosphorus throughout the whole experimental period. All experimental designs and procedures had received approval from the Animal Ethics Committee of Huazhong University of Science & Technology.

2.3. Protocol

After 7 days of acclimation, the rats were ovariectomized or Sham operated. The rats were anesthetized with pentobarbital sodium (40 mg kg^{-1}, i.p.), the ovaries were removed bilaterally. Sham-operation was performed in the same manner but only exposing the ovaries. They were administrated with prophylactic penicillin (4000 IU kg^{-1}, i.p.) for 3 days. A week later, all the rats were randomly divided into four groups of ten animals. Sham and ovariectomized control (OVX) received the vehicle (equivalence of distilled water) through gastric tube. The remaining ovariectomized rats were administrated with ethanol extract of Maca by gavage at two doses of 0.096, or 0.24 g/kg body weight. The doses represented 0.5 g, 1.25 g of dried Maca root/kg body weight, respectively. The treatment continued for 28 weeks. Body weights of all animals were measured weekly. At the end of the 28-week trial, all the rats were deprived of food for one night (7:00 p.m. to 9:00 a.m.). On the next day, urine (0–24 h) was collected, then the animals were anesthetized with pentobarbital sodium (40 mg kg^{-1}, i.p.), and blood were taken from common carotid artery. The blood samples were centrifuged at 2500 rpm for 15 min to extract the serum and preserved (−20 °C) for analysis of calcium phosphorus osteocalcin and alkaline phosphatase. Soon after the collecting of urine and blood, some selected organs (liver, spleen, suprarenal gland, uterus) were carefully removed, cleaned and weighed. The lumbar vertebrae (LV1–LV6) and the femurs were isolated and stored at −80 °C until bone mineral density and biomechanical testing were performed.

2.3.1. Femur physical parameters

Fresh isolated left femurs were weighed using an electronic scale. Femur length, external diameter was measured using a digital slide calipers. Bone volume was measured by fluid displacement. Length was measured from the proximal tip of the femur head to the distal tip of the medial condyle. The external diameter was measured at the midshaft femur. After measuring the above parameters, the femurs were dried at 110 °C for 12 h, and weights of dried femurs were measured.

2.3.2. Bone mineral density

Bone mineral density of the right femur and the lumbar vertebra (LV1–LV6) was measured using dual-energy X-ray absorptiometry (PIXImus, Lunar Corp./GE, USA). Lumbar vertebra 1 through 6 (LV1–LV6) were measured intact. Each right femur and vertebra (LV1–LV6) was placed in the center of a plastic container, which contained 40 mL of distilled water to mimic soft tissue (Ward et al., 2001). BMD was automatically calculated (Nagy and Clair, 2000).

2.3.3. Biomechanical properties of femur

After measuring the bone mineral density, the right femurs were assessed for their biomechanical parameters by three point bending test using a MTS instrument (SGS-H, Shimadzu, Japan) (Peng et al., 1994). The femur was placed in the material testing machine with two supports separated by a distance of 24 mm, then the middle of the femur shaft was compressed by a rounded press at 5 mm/min until fracture occurred. The biomechanical properties that were determined included the following: max-load, max-stress, max-stroke, max-strain, elastic and energy.

2.3.4. Femur ash weight and calcium content

The left dried femurs of rats were placed in tared fused silica crucibles, and ashed for 8 h at 750 °C. The ash weights were determined and the samples were suitably dissolved with aqua fortis and diluted with deionized water to estimate calcium content using Atomic Absorption Spectrophotometer (AA300, P.E. Co., USA).

2.3.5. Biochemical assays of serum and urine

The levels of total calcium and inorganic phosphorus in serum and urine were determined by colorimetry using commercially available test kits. Serum alkaline phosphatase activity was also measured by the same technique using commercially available diagnostic reagent kit (Burtis and Ashwood, 1986). Serum osteocalcin level was determined by radioimmunoassay using commercial kit (JiuDing Medical Bio-engineering Co., Tianjin, China) (Lee et al., 2000).

2.3.6. Histopathological evaluation

The lumbar vertebraes (LV2) were fixed in 10% formalin for 24 h, decalcified in 20% ethylene diamine tetra acetic acid (EDTA) for 7 days, and embedded into paraffin. The block was

Table 1
Effect of treatment of ethanol extract of *Lepidium meyenii* on body, uterine weights, femur physical parameters in OVX rats for 28 weeks ($n = 10$/group)

	Sham	OVX	Maca	
			0.096 g/kg	0.24 g/kg
Initial body weight (g)	192.2 ± 6.22	193.3 ± 17.8	201.2 ± 25.9	193.0 ± 12.1
Final body weight (g)	276.4 ± 26.6	282.8 ± 27.3	294.1 ± 32.4	274.7 ± 23.7
Uterine weight (mg/g BW)	2.536 ± 1.04	1.238 ± 0.87**	1.276 ± 0.85**	1.509 ± 0.67*
Femur				
Length (mm)	32.79 ± 0.44	32.76 ± 0.68	32.57 ± 1.07	32.80 ± 0.89
Diameter (mm)	4.169 ± 0.205	4.195 ± 0.161	4.111 ± 0.288	4.455 ± 0.317*,a
Wet weight (g)	0.869 ± 0.052	0.836 ± 0.061	0.847 ± 0.095	0.876 ± 0.063
Dry weight (g)	0.550 ± 0.016	0.529 ± 0.039	0.545 ± 0.052	0.544 ± 0.044
Volume (mL)	0.623 ± 0.049	0.589 ± 0.046	0.602 ± 0.098	0.638 ± 0.079

All values are expressed as mean ± S.D.
[a] $P < 0.05$ vs. OVX.
* $P < 0.05$ vs. Sham.
** $P < 0.01$ vs. Sham.

then cut into 4 μm slices along the sagittal plane passing through the transversalis axis of the lumbar vertebrae. The sections were stained with hematoxylin and eosin (HE), and examined for morphology under a light microscope (Bancroft and Cook, 1980).

3. Statistical analysis

Data were presented as mean ± S.D. and analyzed by one-way analysis of variance followed by Student's *t*-test for comparison of two groups using SPSS software Version 12.0. *P* values of less than 0.05 were considered statistically significant.

4. Results

4.1. Effect of Maca on body, organ weights and femur physical parameters in OVX rats

The body, uterine weights, femur physical parameters of all groups were presented in Table 1. Initial and final body weight showed no significant differences among the four groups, but body weight of OVX rats increased rapidly in the first month. Regarding organ weight, the wet weights of the uteri in the Sham group were significantly higher ($P < 0.01$ or $P < 0.05$) compared to OVX and Maca-treated groups while uterine weights were not different between OVX and Maca-treated groups. No significant differences were observed in the weights of liver, spleen, suprarenal gland among groups (data not shown). As for femur physical parameters, only femur diameter in Maca-treated group (0.24 g/kg) was remarkably larger than that in the OVX group ($P < 0.05$).

4.2. Bone mineral density, femoral ash weight and calcium

The effects of ethanol extract of Maca on the BMD of the lumbar vertebrae and the midshaft femur, ash weight and calcium content in femoral ashes were presented in Table 2. The BMD of the lumbar vertebrae (LV1–6) was significantly lower in OVX group than in Sham group at 28 weeks after the operation. The BMD of the LV1–6 in the group treated with Maca at 0.24 g/kg increased significantly compared with that in OVX group, while treatment with lower dose of 0.096 g/kg did not increase the BMD of the LV1–6. On the other hand, the BMD of the midshaft femur did not differ among groups. Regarding the femur ash, extremely significant reduction in the femur ash weight, ash Ca was observed in OVX rats. Treatment with Maca (0.24 g/kg) significantly increased calcium content of femur compared with OVX group, but there was no effect on ash weight at two doses compared with OVX group.

4.3. Femur biomechanical measurements

No significant alteration was observed in femur biomechanical strength properties measured: max-load, max-stress, elastic, energy, either in Sham-operated rats,

Table 2
Effects of treatment of ethanol extract of *Lepidium meyenii* on BMD, ash weight and calcium content of femur in OVX rats for 28 weeks ($n = 10$/group)

	Sham	OVX	Maca	
			0.096 g/kg	0.24 g/kg
Midshaft femur BMD (g/cm^2)	0.257 ± 0.03	0.241 ± 0.02	0.245 ± 0.05	0.263 ± 0.07
Lumbar spine BMD (g/cm^2)	0.170 ± 0.03	0.138 ± 0.01**	0.154 ± 0.06	0.160 ± 0.03[a]
Ash weight (g)	0.320 ± 0.01	0.298 ± 0.02**	0.307 ± 0.03	0.316 ± 0.03
Ca (mg/g of dry ashes)	296.5 ± 29.1	266.8 ± 31.8*	293.0 ± 27.9	317.7 ± 53.1[a]

All values are expressed as mean ± S.D.
[a] $P < 0.05$ vs. OVX.
* $P < 0.05$ vs. Sham.
** $P < 0.01$ vs. Sham.

Table 3
Effect of ethanol extract of *Lepidium meyenii* on biomechanical parameters of femur in OVX rats for 28 weeks ($n = 10$/group)

	Sham	OVX	Maca	
			0.096 g/kg	0.24 g/kg
Max-load (N)	82.8 ± 0.6	82.5 ± 4.2	84.5 ± 5.4	86.1 ± 5.0
Max-stress (N/mm^2)	165.7 ± 1.15	165.0 ± 8.48	169.0 ± 10.80	172.3 ± 9.98
Max-stroke (mm)	0.900 ± 0.124	0.828 ± 0.045	0.840 ± 0.130	0.877 ± 0.204
Max-strain (%)	0.0338 ± 0.0046	0.0310 ± 0.0017	0.0315 ± 0.0048	0.0329 ± 0.0076
Elastic (N/mm^2)	614686 ± 78715	667291 ± 74192	683182 ± 86500	638206 ± 86354
Energy (J)	0.056 ± 0.010	0.059 ± 0.022	0.058 ± 0.006	0.057 ± 0.008

All values are expressed as mean ± S.D.

Table 4
Effect of treatment of ethanol extract of *Lepidium meyeni* on biochemical parameters in OVX rats for 28 weeks ($n = 10$/group)

	Sham	OVX	Maca	
			0.096 g/kg	0.24 g/kg
Alkaline phosphatase (King Å units)	14.40 ± 5.03	20.54 ± 7.50*	19.00 ± 5.82	17.02 ± 8.51
Serum calcium (mmol/L)	2.30 ± 0.29	2.40 ± 0.25	2.15 ± 0.16	2.49 ± 0.19
Serum phosphorus (mg/dL)	6.13 ± 1.11	6.02 ± 1.73	6.63 ± 1.73	7.44 ± 2.55
Serum osteocalcin (μg/L)	0.89 ± 0.21	1.76 ± 0.50**	1.86 ± 0.77**	1.38 ± 0.75
Urine calcium (mmol/L)	1.43 ± 0.46	1.72 ± 0.78	1.74 ± 0.50	1.67 ± 0.66
Urine phosphorus (mg/dL)	7.73 ± 3.94	11.60 ± 4.27*	10.20 ± 3.24	9.93 ± 3.71

All values are expressed as mean ± S.D.
* $P < 0.05$ vs. Sham.
** $P < 0.01$ vs. Sham.

OVX rats or ovariectomized-Maca administered rats (Table 3).

4.4. Serum and urine biochemical analysis

The effect of ethanol extract of Maca on biochemical parameters in serum and urine were presented in Table 4. There were no significant differences in the serum calcium and inorganic phosphorus levels among any groups. OVX rats showed significant elevated levels of serum ALP and osteocalcin compared with Sham rats ($P < 0.05$, $P < 0.01$). Administration of Maca had no influence on serum ALP and osteocalcin. Urinary phosphorus level was significantly increased in OVX group compared with Sham group, and there was no alteration in urinary phos-

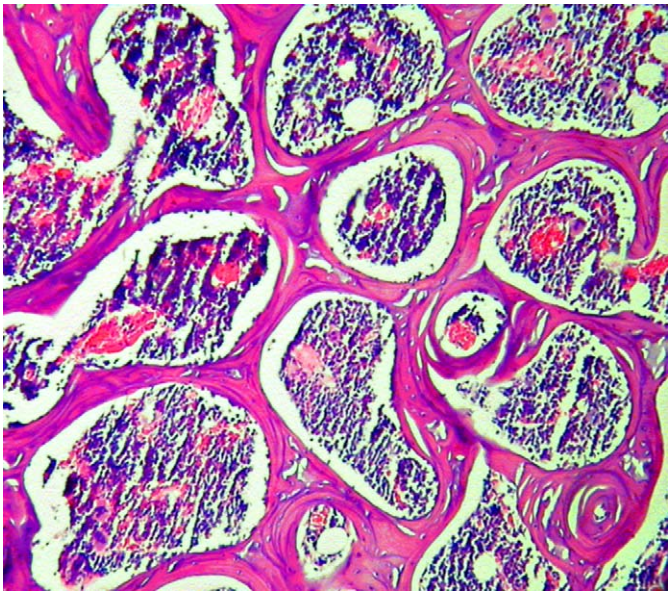

Fig. 1. Photomicrograph of lumbar vertebrae (L-2) of Sham group showing normal, dense and uniform trabecular (HE, 100×).

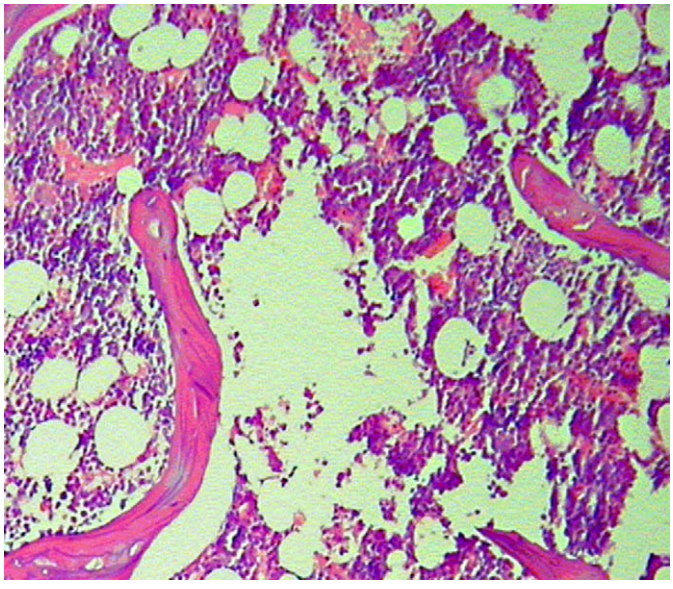

Fig. 2. Photomicrograph of lumbar vertebrae (L-2) of OVX group showing marked disruptive and sparse changes of trabecular bone (HE, 100×).

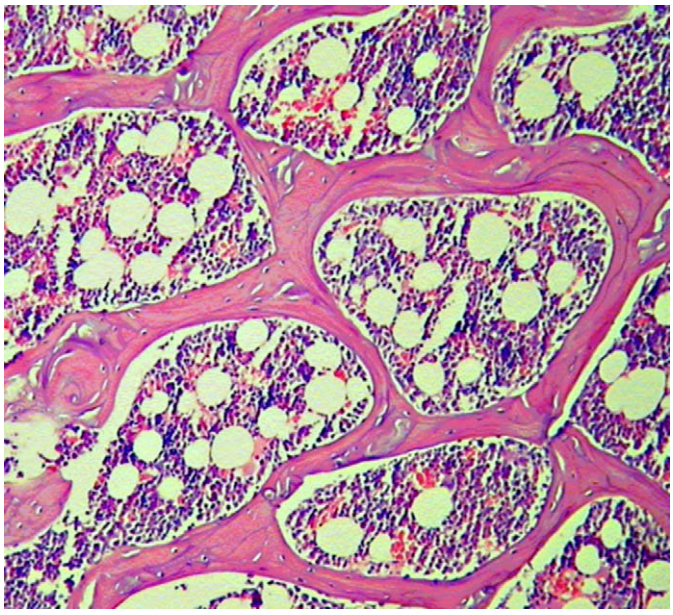

Fig. 3. Photomicrograph of lumbar vertebrae (L-2) of Maca-treated group (0.24 g/kg) showing restoration of normal architecture (HE, 100×).

phorus in Maca-treated animals. No significant changes of urine calcium were observed in any of the groups.

4.5. Lumbar vertebra histological analysis

Under the light microscope, the histology of the lumbar vertebra of Sham rats revealed normal size, shape, density and architecture of trabecular bone (Fig. 1); OVX group sections exhibited sparse, disrupted, spacing-enlarged and area-diminished trabecular bone (Fig. 2). There was significant restorative progress in the groups treated with Maca (Figs. 3 and 4).

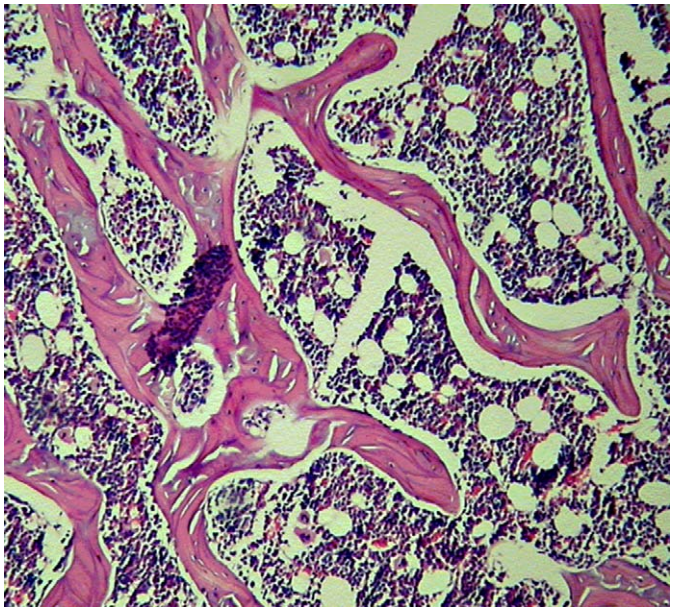

Fig. 4. Photomicrograph of lumbar vertebrae (L-2) of Maca-treated group (0.096 g/kg) showing partly trabecular restoration (HE, 100×).

5. Discussion

The present study is the first to evaluate the effect of ethanol extract of Maca on osteoporosis induced by ovariectomy. It is well known that estrogen deficiency is an important risk factor in the pathogenesis of osteoporosis. Ovariectomy in the rat results in an increase in bone turnover rate and significant loss of cancellous bone such as the proximal femur, vertebral bodies and the metaphysis of long bones (Omi and Ezawa, 1995). The micro architectural alteration in cancellous bone is similar to those observed in postmenopausal and age dependent (Bonjour et al., 1999). In our study, ovariectomy in 3-month-old rats for 28 weeks induced a decrease in lumbar vertebrae BMD, this indicated that trabecular bone loss had occurred. However, there were no alteration in the BMD and biomechanical properties of the midshaft femur among groups, the effect was due to structure difference between the lumbar vertebrae and the midshaft femur, moreover, higher Ca diet suppressed cortical bone loss in OVX rats. The results suggest that ovariectomy combined with normal Ca supplementation has less influence on cortical bone loss. With regard to bone metabolic markers, the serum ALP and osteocalcin levels associated with bone formation are increased in osteoporosis and other bone metabolic disorders (Swaminathan, 2001). Similar changes were observed in the present study. The unchanged levels of serum calcium and phosphorus in Sham and OVX group indicated that homeostatic mechanisms were able to maintain serum levels of these minerals despite ovariectomy. The urinary excretion of phosphorus was elevated, and urinary calcium didn't remarkably differ in the OVX group, this implied partial bone minerals loss in vivo.

In the lumbar vertebrae, the histopathological study revealed sparse, disrupted trabecular bone in OVX rats, and this disruption of the trabecular network may have led to the decrease in compressive bone strength of the lumbar vertebral. The restoration of trabecular network with less inter-trabecular spaces was observed in Maca-treated groups. Also, the present study showed that long-term ovariectomy in mature rats results in a reduction in femoral calcium content despite normal Ca diet, this means that Ca deposit in femur is decreased in OVX rats. However, the BMD of midshaft femur did not alter, this probably because of unequal distribution of Ca in femur. The BMD of the lumbar vertebra was increased by treatment with higher dose of Maca (0.24 g/kg), but the BMD in the midshaft femur showed no markedly change compared with OVX control. These observations indicate BMD-increasing effect of Maca is more obvious in cancellous bone-rich regions than in cortical bone-rich ones. Treatment with Maca (0.24 g/kg) decreased the reduction of femoral ash Ca to an appreciable level, ascertaining its favorable effect on Ca absorption. However, Maca protected against bone loss without significantly increasing uterine weights, this effect is different from HRT and has less risk of endometrial proliferation. In addition, Maca had no significant effect on bone metabolic markers in serum and other biochemical parameters. It is possible that Maca acts without the participation of bone metabolism mechanism.

In general, OVX rats gain weight due to ovariectomy-induced hyperphagia. However, in our study, final body weight of OVX rats was similar to that of Sham rats, a possible reason is that higher Ca diet may have regulated body weight gain. Recent reviews suggest that high dietary calcium inhibits lipogenesis, increases lipolysis, and increases thermogenesis, leading to a net reduction in fat mass (Zemel, 2002). Furthermore, 3-month-old Sham rats were in rapid growing period.

Maca had been reported earlier for its beneficial effects in menopause women (León, 1964). The mechanisms regulating endocrine are unclear at present, while some investigators suggest that Maca exerts hormonal balancing effect not through plant hormones or phytoestrogens, but through Maca alkaloids, which act on the hypothalamus-pituitary axis and the adrenals. Oshima et al. (2003) observed increased blood levels of testosterone and progesterone in mice fed Maca, which indicates a possible explanation for the steroid-like compounds improving osteoporosis in the OVX rats.

To our knowledge, the biological activity on bone of Maca may be due to one or more of the phytochemicals present in the extract. Maca has a lot of easily absorbable calcium, magnesium, and a fair amount of silica, which is useful in bone calcium loss in menopause women. It also contains alkaloids, steroids, glucosinolates, isothiocyanates and macamides (Dini et al., 1994; Muhammad et al., 2002). However, there is still unknown which particular ingredient has effect on the above variables studied. Previous publications have discovered some alkaloids derived from plant have antiosteoporosis activity, such as berberine and norzoanthamine (Li et al., 2003; Yamaguchi et al., 1999). More recently, the alkaloids components in Maca have been investigated actively (Zhao et al., 2005). However, their biological activity still remain unclear, whether Maca alkaloids have similar activity required further study.

In conclusion, our study showed that *Lepidium meyenii* improved the bone mass, restored trabecular network in lumbar vertebrae in OVX rats. This finding suggests that Maca is a potentially useful for postmenopausal osteoporosis, which occurs in women as a result of estrogen deficiency. Further studies are required to identify major phytochemicals in the ethanol extracts and its mechanism for postmenopausal osteoporosis.

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