Original Research

Effect of the methanol extract of the Lion's Mane mushroom, Hericium erinaceus, on bone metabolism in ovariectomized rats

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Summary

Purpose: Osteoporosis is a major health concern in postmenopausal women. The aim of the study was to investigate the effect of *Hericium erinaceus* (*H. erinaceus*), also known as the Lion's Mane mushroom, consumption on bone metabolism in ovariectomized (Ovx) rats. *Materials and Methods*: Rats in the Baseline group were sacrificed immediately, whereas rats in the Sham group underwent sham surgery, and those in the Ovx and Ovx +HE groups underwent bilateral Ovx. A diet containing the methanol extract of *H. erinaceus* was given to the Ovx+HE group for 13 weeks. *Results*: There was no significant difference in the femoral bone mass between the Ovx and Ovx+HE groups. In the histomorphometric study of the proximal tibial metaphysis, the osteoblast surface, mineralizing surface, and fat cell number were lower in the Ovx+HE group than those in the Ovx group. *Conclusion*: The present findings suggest that *H. erinaceus* does not prevent but rather accelerates bone loss in Ovx rats.

Key words: Adipocyte; Bone histomorphometry; Bone mineral density; Menopause; Mushroom; Rat.

Introduction

Osteoporosis is a systemic skeletal disorder that results from an imbalance in bone remodeling, leading to a reduction in bone strength, with microarchitectural disruption, and skeletal fragility, increasing fracture susceptibility [1]. Although osteoporosis is a multifactorial disorder, estrogen deficiency after menopause plays an important role in the pathogenesis of rapid bone loss, making women more prone to osteoporosis than men [2]. Although anti-osteoporotic drugs, such as estrogen, bisphosphonates, selective estrogen receptor modulators (SERMs), teriparatide, and denosumab [3] may help prevent bone loss and fracture after menopause, lifestyle and diets rich in calcium and vitamin D should be mainstay strategies for preserving bone mass after menopause [4].

Mushrooms have been considered functional foods as well as valuable sources of natural medicines and neutraceuticals, and a large variety of mushrooms have been utilized traditionally in many cultures for the maintenance of health, as well as in the prevention and treatment of diseases [5, 6]. A number of biologically active compounds, such as polysaccharides, amino acids, and trace elements, have been recognized in different mushroom species. In addition, mushrooms exposed to sunlight or ultraviolet radiation are an excellent source of dietary vitamin D_2 (ergocalciferol) because they contain a high concentration of the vitamin D precursor, provitamin D_2 [7, 8]. Although studies that investigated the effects of mushrooms on bone metabolism are limited, some studies have suggested that certain mushroom species have anti-osteoporotic effects [9-16].

Hericium erinaceus (*H. erinaceus*), also known as the Lion's Mane or Yamabushitake mushroom, has been used as a food source and an herbal medicine for centuries in Asian countries. Various studies have demonstrated that *H. erinaceus* may exhibit several bioactivities, such as antitumor, immunomodulatory, anti-gastric ulcer, neuroprotective, neuroregenerative, antioxidative, hepatoprotective anti-hyperlipidemic, anti-hyperglycemic, anti-fatigue, and anti-aging activities [17]. In addition, Nagano *et al.* [18] investigated the effect of *H. erinaceus* intake on menopause, depression, sleep quality and indefinite complaints, and showed that its intake may reduce depression and anxiety in menopausal women. Recently, Hiraki *et al.* [19] demonstrated that *H. erinaceus* may have anti-obesity effects in ovariectomized mice. Based on these findings, the present group hypothesized that the consumption of *H. erinaceus* may be helpful in preventing bone loss following menopause. Therefore, the aim of the present study was to investigate the effects of the methanol extract of *H. erinaceus* on bone mass and bone histomorphometry in ovariectomized (Ovx) rats. Osteoporosis is associated with in-
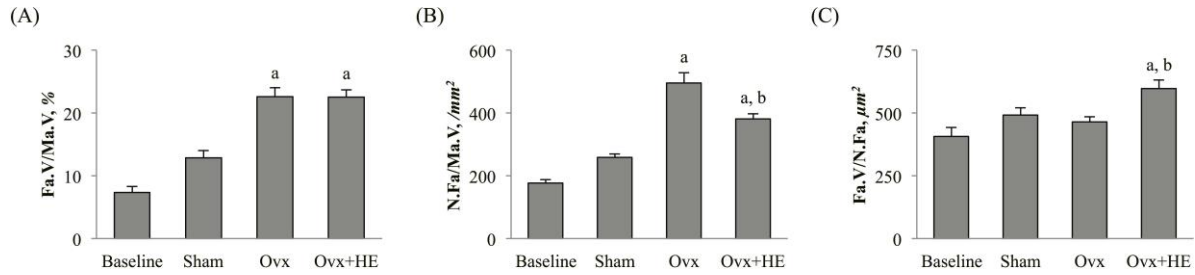
(A)   (B)   (C)

Figure 1. — (A) Percent fat volume (Fa.V/Ma.V), (B) fat cell number (N.Fa/Ma.V, /mm^2), and (C) unit fat volume (Fa.V/N.Fa, μm^2) of the right femur measured at the proximal tibial metaphysis in female Wistar rats at the beginning of the study (Baseline) and in sham-operated (Sham) or ovariectomized rats fed the *H. erinaceus* diet (Ovx+HE) or control diet (Ovx). Data are expressed as the means ± standard error of the mean. [a]$P < 0.01$ compared with the Sham group, and [b]$P < 0.01$ compared with the Ovx group (one-way analysis of covariance with Tukey's honest significant difference test).

creased bone marrow fat [20]; thus, the effect of *H. erinaceus* on fat histomorphometry was also investigated.

Materials and Methods

The dried powder of fresh *H. erinaceus* used in the present study was a generous gift from Prof. S. Vikineswary Sabaratnam (University of Malaya, Kuala Lumpur, Malaysia). The powder (2 kg) was mixed with 8 L of 99.7% methanol and shaken for 24 hours at 50°C. The extracts were vacuum-filtered through filter paper, and the residues were extracted in 8 L methanol for another 24 hours before another vacuum filtration. These solutions were mixed and evaporated under vacuum, yielding an extract of 560 grams. The extract was stored at –20°C until use.

Forty-six female Wistar rats, aged eight weeks, were included. The animals were allowed to acclimate to the experimental room temperature (23 ± 2°C), humidity (55 ± 5%) and a 12-hour light-dark cycle and fed standard rodent pellet diet and water *ad libitum*. The experimental protocol was approved by the Animal Ethical Committee of the University of Shizuoka, Japan. The rats were divided into four groups based on weight: Baseline (n=6), Sham (n=10), Ovx (n=10) and Ovx+HE (n=10). At 12 weeks of age, rats in the Baseline groups were anesthetized by intraperitoneal injection of butorphanol tartrate 2.5 mg/kg body weight), medetomidine hydrochloride (0.375 mg/kg body weight) and midazolam (2 mg/kg body weight), and they were sacrificed immediately. This group provided the baseline data on skeletal mass, structure, and dynamics to permit determination of changes in skeletal tissue resulting from surgery and aging [21]. Under anesthesia, rats in the other groups were either bilaterally ovariectomized (Ovx and Ovx+HE groups) or sham-operated (sham group) from a dorsal approach. After surgery, rats in the sham and Ovx groups were continually given an MF diet, and rats in the Ovx+HE group were given a custom pellet diet containing methanol extract of *H. erinaceus* (10 g/kg). The body weight of each animal was recorded every week throughout the experiment, and the uterine weight was measured at necropsy as indices of the efficacy of the Ovx. Thirteen weeks after surgery, the animals in all three groups were anesthetized and sacrificed. To evaluate bone dynamics by histomorphometry, the rats were

given intraperitoneal injections of the fluorochrome markers calcein (10 mg/kg body weight) and tetracycline-HCl (25 mg/kg body weight) six and two days before necropsy, respectively. At necropsy, the uterus, tibias and femurs were collected. The right femurs were wrapped in saline-soaked gauze and stored at -20°C for subsequent measurement of bone density. The right tibiae were cleaned of soft tissue and fixed in 70% ethanol for bone histomorphometry.

The right femurs were completely thawed at room temperature. Bone mineral content (BMC) and bone mineral density (BMD) were measured and analyzed using small animal software and dual-energy X-ray absorptiometry, and three regions of the femur (proximal, middle, and distal thirds) were assigned for analysis to assess any regional differences in bone characteristics. The coefficients of variation for repeated scans on the same bone were < 1.0%.

The right tibiae were fixed with 70% ethanol at 4°C for two weeks. The proximal one-third of the tibiae were cut and stained using Villanueva bone stain powder for one week at room temperature, dehydrated in a graded series of ethanol, and embedded undecalcified in methyl methacrylate. Frontal sections (5 μm) were cut using a microtome and mounted on slides.

The cancellous bone area of the proximal metaphysis was measured for the structural and dynamic histomorphometric indices at the secondary spongiosa located 0.3525-2.115 mm from the epiphyseal growth plate and 250 μm distal to the endocortical surface. Measurements were performed using a semiautomatic image analysis system and a fluorescence microscope set at a magnification of ×250. The histomorphometric nomenclature used in the present study is in accordance with the report of the American Society for Bone and Mineral Research Histomorphometry Nomenclature Committee [22]. In addition, fat histomorphometric parameters of the proximal metaphysis were measured as previously described by Tamura *et al.* [23]. Namely, the fat volume (Fa.V) is expressed as a percentage of the marrow volume (Ma.V) and calculated as the percent fat volume (Fa.V/Ma.V, %). The number of fat cells (N.Fa) per unit area (mm^2) of the marrow was determined manually and calculated as the fat cell number (N.Fa/Ma.V, /mm^2), and the volume of each fat cell was determined and calculated as the unit

Table 1. — *Bone mineral content and bone mineral density of the whole femur as well as the proximal, middle, and distal third of the femur in female Wistar rats at the beginning of the study (Baseline) and in sham-operated (Sham) or ovariec-tomized rats fed the H. erinaceus diet (Ovx+HE) or control diet (Ovx).*

		Baseline (n = 6)	Sham (n = 10)	Ovx (n = 10)	Ovx+HE (n = 10)
BMC, g					
	Whole	0.225 ± 0.004	0.284 ± 0.005	0.281 ± 0.008[b]	0.280 ± 0.004[a]
	Proximal third	0.083 ± 0.002	0.108 ± 0.002	0.103 ± 0.003[a]	0.104 ± 0.002[a]
	Middle third	0.050 ± 0.000	0.065 ± 0.002	0.070 ± 0.002	0.070 ± 0.000
	Distal third	0.093 ± 0.002	0.113 ± 0.003	0.110 ± 0.003[a]	0.111 ± 0.002[a]
BMD, g/cm^2					
	Whole	0.181 ± 0.002	0.202 ± 0.003	0.190 ± 0.002[b]	0.189 ± 0.002[b]
	Proximal third	0.182 ± 0.002	0.206 ± 0.003	0.187 ± 0.002[b]	0.190 ± 0.001[b]
	Middle third	0.149 ± 0.002	0.171 ± 0.002	0.171 ± 0.001	0.169 ± 0.002
	Distal third	0.204 ± 0.002	0.219 ± 0.003	0.206 ± 0.003[b]	0.200 ± 0.004[b]

Data are expressed as the means ± standard error of the mean. Significantly different from the Sham, [a] $p < 0.05$, [b] $p < 0.01$ (Sham vs. Ovx vs. Ovx+HE, analysis of co-variance with Tukey's honest significant difference test). BMC: bone mineral content; BMD: bone mineral density; Ovx: bilateral ovariectomy; HE: H. erinaceus.

Table 2. — *Structural and dynamic cancellous bone indices measured at the proximal tibial metaphysis in female Wistar rats at the beginning of the study (Baseline) and in sham-operated (Sham) or ovariectomized rats fed the H. erinaceus diet (Ovx+HE) or control diet (Ovx).*

		Baseline (n = 6)	Sham (n = 10)	Ovx (n = 10)	Ovx+HE (n = 10)
Structural indices					
	Bone volume, %	21.7 ± 0.9	29.7 ± 1.7	8.0 ± 0.8[b]	10.3 ± 0.8[b]
	Trabecular thickness, μm	58.6 ± 1.8	69.7 ± 3.0	61.8 ± 1.4[a]	65.9 ± 2.9
	Trabecular number, /mm	3.69 ± 0.09	4.25 ± 0.12	1.29 ± 0.10[b]	1.57 ± 0.11[b]
	Osteoid surface, %	23.1 ± 2.1	18.5 ± 2.1	31.8 ± 2.3[b]	27.6 ± 2.0[b]
	Osteoid volume, %	3.00 ± 0.34	2.11 ± 0.33	4.28 ± 0.49[b]	3.48 ± 0.46[a]
	Osteoblast surface, %	3.47 ± 0.44	1.13 ± 0.25	3.15 ± 0.65[b]	1.97 ± 0.49[a, d]
	Eroded surface, %	7.51 ± 1.02	3.32 ± 0.16	4.04 ± 0.35	4.17 ± 0.66
	Osteoclast surface, %	4.64 ± 0.69	0.65 ± 0.11	1.29 ± 0.12	1.64 ± 0.48
Dynamic indices					
	Single-labeled surface, %	37.4 ± 3.5	25.2 ± 1.5	25.7 ± 0.9	22.3 ± 0.9
	Double-labeled surface, %	19.3 ± 3.0	13.3 ± 1.3	21.6 ± 1.0[b]	19.0 ± 1.2[a]
	Mineralizing surface, %	38.0 ± 2.1	26.0 ± 1.4	34.4 ± 1.2[b]	30.1 ± 1.3[d]
	Mineral apposition rate, $\mu m/day$	2.05 ± 0.18	1.37 ± 0.03	1.40 ± 0.05	1.41 ± 0.05
	Bone formation rate, $mm^3/mm^2/year$	0.290 ± 0.042	0.131 ± 0.009	0.177 ± 0.010	0.156 ± 0.009
	Bone formation rate, %/year	1013 ± 153	382 ± 30	579 ± 44[a]	480 ± 37

Data are expressed as the means ± standard error of the mean. Significantly different from the Sham, [a] $p < 0.10$, [b] $p < 0.05$, [c] $p < 0.01$; significantly different from the Ovx, [d] $p < 0.10$ (Sham vs. Ovx vs. Ovx+HE, analysis of covariance with Tukey's honest significant difference test). Ovx: bilateral ovariectomy; HE: H. erinaceus.

fat volume (Fa.V/N.Fa, μm^2).

Statistical analysis was performed using JMP 9.02. The data are presented as the mean ± standard error of the mean (SEM). The effects of aging and surgery on body and uterine weights were compared among the Sham, Ovx and Ovx+HE groups by one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) test. The specific effects of Ovx and methanol extract of *erinaceus* on skeletal tissues among the three groups were analyzed by one-way analysis of covariance (ANCOVA) using body weight as the covariate [24, 25] followed by Tukey's HSD test. Statistical significance was considered at $p < 0.05$, but statistical trends ($p < 0.10$) are also indicated.

Results

The uterine weight of rats in the Ovx group was signifycantly lower than that of rats in the Baseline and Sham groups, suggesting that the Ovx was performed successf-

ully. The uterine weight of the rats in the Ovx+HE group was not significantly different from that in the Ovx group (data not shown). The body weight of rats in the Ovx and Ovx+HE groups increased after surgery, and there were significant differences between those and that in the Sham group from four weeks post-surgery to the end of experiment. However, there were no significant differences in body weight between the Ovx and Ovx+HE groups throughout the experiment (data not shown).

BMD and BMC in the whole femur, as well as in the proximal and distal third of the femur, which are sites that are high in cancellous bone, were significantly less in the Ovx group than those in the Sham group. However, there was no significant difference in BMD and BMC in the middle third of the femurs, which is a site that is high in cortical bone, between the Sham and Ovx group. The rats following a *H. erinaceus* diet (Ovx+HE group) presented

no significant differences in the BMC or BMD of the femur compared with the rats in the Ovx group, regardless of the femur site (Table 1).

Table 2 summarizes the structural and dynamic cancellous bone indices for the proximal tibial metaphysis. Ovariectomy resulted in significant decreases in bone volume, trabecular thickness and trabecular number (Sham *vs.* Ovx group). In addition, rats in the Ovx group presented a significantly higher osteoid surface, osteoid volume, osteoblast surface, double-labeled surface, mineralizing surface and bone formation rate, indices for bone formation, than those in the Sham group. Osteoblast surface and mineralizing surface of the rats in the Ovx+HE group tended to be lower than those in the Ovx group, although they were not statistically significant. Bone resorption indices, such as eroded surface and osteoclast surface, were not affected by feeding of the *H. erinaceus* diet.

The Fa.V/Ma.V and N.Fa/Ma.V of the Ovx and Ovx + HE groups were significantly higher than the Sham group. Although there was no significant difference in the Fa.V/Ma.V between the Ovx and the Ovx+HE groups, the N.Fa/Ma.V of the rats in the Ovx+HE group was significantly less than that of those in the Ovx group. As a result, the Fa.V/N.Fa of the Ovx+HE group was significantly higher than that in the Sham and Ovx groups.

Discussion

Homeostasis in bone is maintained by remodeling, a balance between resorption by osteoclasts and bone formation by osteoblasts. In postmenopausal women, estrogen deficiency causes a disproportionate increase in bone resorption compared to bone formation, which results in net bone loss [26, 27]. Therefore, one therapeutic strategy to prevent osteoporosis has focused on minimizing bone loss after menopause [28]. In the present study, Ovx rats administered the methanol extract of *H. erinaceus* (Ovx+HE group) did not have higher femur BMC or BMD according to the DXA measurements than rats in the Ovx group. The results from bone histomorphometry also revealed that such supplementation did not result in higher bone volume, trabecular thickness or trabecular number. However, bone formation indices, such as osteoblast surface and mineralizing surface, of rats in the Ovx+HE group tended to be lower than rats in the Ovx group. These results suggest that the consumption of *H. erinaceus* may not alleviate, but instead accelerate bone loss observed in postmenopausal women.

Thus far, there are only a few studies that have investigated the effects of *H. erinaceus* on bone metabolism. The constituents of *H. erinaceus* include aromatic compounds, fatty acids, sterols and polysaccharides [29]. Luo and Chen [30] administered selenium-enriched polysac-charides extracted from *H. erinaceus* to rats in a D-galactose-induced osteoporosis model. They demonstrated that such administration helped prevent loss of BMD of the skull and mechanical properties of the femur. In a recent study by Li *et al.* [31], the investigators isolated five aromatic compounds and six sterols from the fruiting bodies of *H. erinaceus* and demonstrated that some of the aromatic compounds exhibited a cellular antioxidant activity and/or inhibited the receptor activator of nuclear factor kB (RANKL)-induced osteoclastic differentiation in vitro.

The reasons why the findings of the present study failed to corroborate the findings from previous studies are unknown. Recently, Li *et al.* [32] isolated 11 ergostane-type sterol fatty acids from the methanol extract of the dried fruiting bodies of *H. erinaceus*. They demonstrated that some compounds significantly activated peroxisome proliferator-activated receptors (PPARs) using a luciferase reporter assay. The investigators also reported that two compounds significantly activated the transcriptional activity of PPARα and PPARγ in a dose-dependent manner. PPARγ belongs to the PPAR family of transcriptional factors and nuclear receptors that regulates lipid biosynthesis, cell differentiation, and insulin sensitivity [33]. It has been reported that PPARγ signaling negatively regulates osteoblast function (bone formation) and bone mass [34-36] in vitro and in rodents. A randomized, double-blind, placebo-controlled study that investigated the effect of rosiglitazone, a PPARγ agonist, on bone metabolism in healthy postmenopausal women demonstrated that bone formation markers (serum procollagen type 1 N-terminal propeptide and osteocalcin) and total hip BMD was significantly decreased following 14-week administration of rosiglitazone, without affecting a bone resorption marker (serum β-C-terminal telopeptide of type I collagen) [37]. Thus, the authors speculate that the *H. erinaceus* extract might have diminished bone formation, possibly via PPARγ.

Changes in the number and size of bone marrow adipocytes are well-known to occur with aging and several clinical disorders of bone loss, such as osteoporosis [20]. Indeed, the present study demonstrated that the number of adipocytes (N.Fa/Ma.V) in the rats in the Ovx group was significantly higher than that in the rats in the Sham group, although there was no significant difference in the size of adipocytes (Fa.V/N.Fa). Although the mechanism and significance remain unknown, it is an interesting finding to note that the number of adipocytes of the rats in the Ovx+HE group was significantly lower than in the rats in the Ovx group, resulting in significantly higher adipocyte size in the Ovx+HE group. Recently, Hiraki *et al.* [19] investigated the effect of *H. erinaceus* on body fat in ovariec-tomized mice. The investigators found that feeding of *H. erinaceus*-containing diet to ovariectomized mice resulted in decreased parameters, such as visceral and subcutaneous fat, fatty tissue around kidney and abdomen, and plasma leptin levels, which show positive correlations with fat cell number [38], without affecting

body weight of the mice. Hiwatashi *et al.* [39] found that ethanol extract of *H. erinaceus* prevented obesity and hyperlipidemia in mice fed a high-fat diet and that these effects might be mediated by the modulation of lipid metabolic gene expression, at least in part, via activation of PPARα.

There are some limitations to note in the present study. First, because the *H. erinaceus* extract used in the present study was a crude extract, the authors cannot be certain of the bioactive components and their dose that resulted in observations in the present study. Second, osteoblasts and adipocytes share a common progenitor, the mesenchymal stem cell (MSC). Because this common origin leads to a competition between the alternative differentiation towards adipogenesis or osteogenesis [40], the results that *H. erinaceus* extract decreased the bone formation indices and adipocyte number seem to be contradictory. The authors cannot exclude the possibility that the observation simply resulted from cytotoxicity to MSCs caused by the extract.

In conclusion, feeding Ovx rats a methanol extract of *H. erinaceus* did not result in improvements in bone mass, but rather diminished bone formation, at least under the experimental conditions employed in the present study. Because it has been reported that *H. erinaceus* may have favorable effects on bone in only a few previous studies, further investigations are warranted to determine whether the consumption of *H. erinaceus* may be beneficial in mitigating bone loss in postmenopausal women.

Acknowledgements

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