Consumption of vitamin D₂ enhanced mushrooms is associated with improved bone health

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Abstract

Mushrooms are the best nonanimal food source of vitamin D₂. Pulsed irradiation can enhance vitamin D₂ in mushrooms quickly. We investigated the effect of supplementing high vitamin D₂ *Pleurotus ferulae* mushrooms in a mouse model of osteoporosis. Thirty-two female C57BL/6JNarl mice were divided into four groups including sham, ovariectomized (OVX), OVX+nonpulsed mushroom (NPM) and OVX+pulsed mushroom (PM). After 23 weeks of treatment, serum samples were analyzed for osteoblast and osteoclast indicators, as well as metabolites using NMR spectroscopy. To examine bone density, femurs were analyzed using micro-computed tomography. The NPM and PM treatment mice showed increased bone density in comparison with OVX mice. In addition, the PM mice showed higher osteoblast and lower osteoclast indicators in comparison with OVX mice. Serum metabolomics analysis indicated several metabolites that were different in PM mice, some of which could be correlated with bone health. Taken together, these results suggest that pulsed irradiated mushrooms are able to increase bone density in osteoporotic mice possibly through enhanced bone metabolism. Further studies in humans are needed to show their efficacy in preventing osteoporosis.
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1. Introduction

Vitamin D plays an essential role in maintaining skeletal health through regulation of calcium and phosphate homeostasis [1,2]. Vitamin D deficiency causes rickets, osteomalacia, muscle weakness

Abbreviations: OVX, ovariectomized; NPM, nonpulsed mushroom; PM, pulsed mushroom; micro-CT, micro-computed tomography; ALP, alkaline phosphatase; PICP, procollagen I C-terminal propeptide; PYD, pyridinoline; NTXI, cross-linked N-telopeptide of type I collagen; Tb.BMD, trabecular bone mineral density; BV/TV, bone volume/tissue volume; BS/TV, bone surface/tissue volume; Tb.Th, trabecular thickness; Tb.N, trabecular number; Conn.Dn, connectivity density; BS/BV, bone surface/bone volume; Tb.Pf, trabecular pattern factor; EC, endosteal circumference; PC, periosteal circumference; PCA, principal component analysis.

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and osteoporosis and can increase the risk of fracture. Low vitamin D may cause increased bone resorption by secondary hyperparathyroidism and induce osteomalacia by raised serum levels of parathyroid hormone to produce phosphaturia and hypophosphatemia [2–4]. Epidemiological and prospective studies have related vitamin D deficiency with cancer, diabetes and infection, as well as cardiovascular, autoimmune and neurodegenerative diseases [1–5]. The prevalence of vitamin D deficiency was shown to be very high in children in industrialized Western countries during the first half of the 20th century due to decreased exposure to ultraviolet (UV) light, a major source of vitamin D. As a consequence, food and supplements were fortified with vitamin D. Recent changes in lifestyles and diets and an increase in the use of sunscreens have led to further deficiencies in this vitamin [3,4].

There are two major forms of vitamin D including vitamin D₂ (ergocalciferol) and vitamin D₃ (cholecalciferol). Vitamin D₂ is manufactured from ergosterol through UV irradiation in phytoplankton, invertebrates, yeast and fungi. Vitamin D₃ is synthesized from 7-dehydrocholesterol through UV irradiation in skin, although a small amount can also be obtained from the diet through ingestion of fatty fish, eggs and milk [1,3,5]. Vitamin D₃ is metabolized in the liver to 25-hydroxyvitamin D [25(OH)D] (which may be used to detect and

evaluate the vitamin D status in a subject) and further in the kidneys to its active form, 1,25-dihydroxyvitamin D [$1,25(\text{OH})_2\text{D}$] [3,6]. Vitamin D_2 is metabolized similarly to vitamin D_3 , being converted into $25(\text{OH})\text{D}$ that is detectable in the serum. Vitamin D_2 has been purported to be as effective as vitamin D_3 in adults [7,8]; however, some studies report that vitamin D_2 is less effective than vitamin D_3 in humans and should not be regarded as a nutrient suitable for supplementation [9,10]. These studies involved supplementing individuals with tablets containing either vitamin D_3 or vitamin D_2 , and to our knowledge, we have not seen studies that have looked at the ability of vitamin D_2 in a food product to affect health outcomes.

Mushrooms contain high levels of provitamin D_2 (ergosterol). Exposure of mushrooms to sun or artificial UV light can create high levels of vitamin D_2 . The vitamin D_2 produced in UV-irradiated mushrooms has been shown to be bioavailable as measured through increases in serum $25(\text{OH})\text{D}_2$ levels [11,12]. Irradiation of mushrooms by pulsed UV broad-spectrum light (100–800 nm) to deliver radiation in the form of high intensity pulses for a short time (Xenon Corporation, USA) has been shown to significantly increase the vitamin D_2 content of mushrooms [13]. The *Pleurotus* genus of mushrooms (oyster mushrooms) is edible fungi cultivated worldwide, especially in Southeast Asia, India, Europe and Africa [14]. We choose *Pleurotus ferulae* as the mushroom of choice to deliver vitamin D_2 to ovariectomized (OVX) mice, as this species produces the highest vitamin D_2 compared with other oyster mushrooms when irradiated. Vitamin D_2 from UVB-light-exposed edible mushrooms has been shown to be both bioavailable and safe in humans [15].

Bisphosphonates are widely used for the prevention and treatment of osteoporosis [16]. However, side effects including increased risk of fracture [17], osteonecrosis of the jaw [18], gastrointestinal upset, musculoskeletal pain, atrial fibrillation and esophageal cancer [19,20] make bisphosphonates undesirable for general use [21]. One alternative could be an alteration of the diet, such as with supplementation with UV-pulsed mushrooms (PM).

Herein, we describe the effect of dietary supplementation of vitamin D_2 enriched in mushrooms through UV irradiation on OVX mice, a model of osteoporosis. The effect of vitamin D_2 supplementation was assessed through bone density measurements using a micro-computed tomography (micro-CT), which was compared with indicators of osteoblast and osteoclast activity using ELISA, and serum metabolite profiles. We show that, compared with OVX mice, mice fed with UV-PM have higher bone density and an altered serum metabolite profile compared with OVX mice that may be associated with improved bone health.

2. Materials and Methods

2.1. Sample and preparation

P. ferulae, obtained from Q-Yo Bio-Technology Farm (Chunghua, Taiwan), was freeze-dried, ground and exposed to 60 pulses (equivalent to 18 s) of irradiation using a XENON RC-847 pulsed light system (Xenon Corporation, Woburn, MA, USA) employing a C-type lamp (190–700 nm) resulting in $65 \mu\text{g/g}$ dry weight of vitamin D_2 .

2.2. Animals and design

Thirty-two female C57BL/6JNarl mice aged 7 weeks were purchased from National Laboratory Animal Center (Taipei, Taiwan) and acclimated to conditions for 5 weeks before surgery. Animals were housed in an air-conditioned room with a 12-h light/dark illumination cycle at a constant temperature of $25 \pm 2^\circ\text{C}$ and humidity of $65 \pm 5\%$. The basal diet Lab 5001 (Purina Mills, St. Louis, MO, USA) and drinking water were supplied *ad libitum*. The mice were weighed on a weekly basis during the experimental period. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of National Chung Hsing University (IACUC approval number 99-62). At surgery, 12-week-old mice received an anesthetic cocktail injected intramuscularly that was composed of 0.03 ml Zoletil 50 and 0.01 ml 0.2% xylazine. Bilateral ovariectomy (OVX) was performed on 24 of the mice as described by Lasota [22]. Another eight mice were given sham surgery. The 24 OVX mice were randomly assigned to one of the following: OVX (no treatment) (9 mice); PM (receiving UV-PM) (8 mice); NPM (receiving non UV-PM) (7 mice). The vitamin D_2 group (PM mice) was gavaged with

UV-PM on a daily basis during the experimental period, the dose of which was based on the human adult equivalent dose of $5 \mu\text{g/day}$ [23]. The amount of nonpulsed mushroom (NPM) gavaged into the NPM group was the same as the PM group. Blood was collected from the cheeks of mice after 12 weeks of treatment to measure osteocalcin and alkaline phosphatase (ALP). After 23 weeks, mice were sacrificed by CO_2 asphyxiation, and blood was collected by cardiac puncture. Femur samples were collected by removing tissue and placing the bone in ethanol. Serum was collected by allowing the blood to clot for 15 min on ice, centrifuging at 5000 rpm for 15 min and collecting the top layer followed by storage at -80°C until ready for analysis.

2.3. Histomorphometric analysis using micro-CT

Right femurs were analyzed using a high-performance *in vivo* micro-CT Skyscan 1076 (Skyscan, Kontich, Belgium), with image field at pixel size $9 \mu\text{m}$ to perform qualitative and quantitative analysis of bone. Three-dimensional (3D) images were reconstructed with software (CTVol, Skyscan). The distal femoral metaphysis was analyzed from a region that was 1.0 mm below the growth plate and 1.5 mm in length. The cortical bone of the femoral mid-diaphysis was analyzed at the region of 1.5 mm in the middle of the femur. For quantitative analysis, the software (CTAn, Skyscan) was used to obtain the following parameters within the region of interest: bone volume/tissue volume (BV/TV), bone surface/bone volume (BS/BV), bone surface/tissue volume (BS/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular pattern factor (Tb.Pf), connectivity density (Conn.Dn) and trabecular bone mineral density (Tb.BMD). The Conn.Dn was calculated using the Euler method [24]. Cortical bone parameters, including cortical bone thickness (thickness), endosteal circumference (EC), periosteal circumference (PC) and cross-sectional area (cross-sectional), were also measured.

2.4. Measurement of serum parameters

Serum concentrations of ALP, osteocalcin, procollagen I C-terminal propeptide (PICP), pyridinoline (PYD) and cross-linked N-telopeptide of type I collagen (NTX) were all measured using commercial kits according to manufacturer's instructions: ALP, ALP activity fluorometric assay kit (Biovision, Mountain View, CA, USA); osteocalcin, mouse osteocalcin EIA kit (Biomedical Technologies, Stoughton, MA, USA); PICP, ELISA kit for mouse PICP (USCIN Life Science, Wuhan, Hubei, China); PYD, mouse PYD ELISA kit (CUSABIO, Wuhan, Hubei, China); NTX, ELISA kit for mouse NTX (USCIN Life Science, Wuhan, Hubei, China). Calcium was determined with a calcium colorimetric assay kit (Biovision, Mountain View, CA, USA), and phosphate was measured with a phosphate colorimetric assay kit (Biovision, Mountain View, CA, USA).

2.5. NMR spectroscopy

Serum samples were prepared by thawing and centrifuging for 20 min at maximum speed at 4°C to remove particulate matter. Serum supernatant was subsequently filtered through Amicon 3000 molecular weight cutoff filters to remove protein and lipid particles. If needed, the serum filtrates were diluted with deionized water to a total volume of 207 μl and 23 μl of an internal standard (Chenomx Inc., Edmonton, Alberta, Canada) containing 4.86 mM 3-(trimethylsilyl)-1-propanesulfonic acid-d6 and 0.2% NaN_3 in 98% D_2O was

Fig. 1. Body weight of mice over 23 weeks. Body weight of sham ($n=8$), OVX ($n=9$), NPM ($n=7$) and PM ($n=8$) C57BL/6 mice over 23 weeks. Time 0 represents the time of surgical removal of ovaries or sham treatment. Analyzed by repeated measures two-way ANOVA followed by Tukey's multiple comparisons test. Each value is expressed as mean $\pm$ S.E.M.

Fig. 2. Food intake of mice. Average weekly food intake of sham, OVX, NPM and PM C57BL/6 mice during treatment weeks 8–23. Each value is expressed as mean $\pm$ S.D. * $P < .05$ versus OVX group by Student's t test. ** $P < .05$ versus PM group by Student's t test.

added. The pH value was adjusted to 6.8 for each sample by adding small amounts of 1 N HCl or NaOH. Of each sample, 180 μ l was transferred into 3 mm NMR tubes, and samples were stored at 4°C until NMR data acquisition (within 24 h of sample preparation). In cases where serum volume was less than 50 μ l, samples were not analyzed by NMR.

NMR spectra were acquired using the Bruker noesypr1d experiment on a Bruker Avance 600-MHz NMR spectrometer equipped with a SampleJet as previously

described [25]. Identification and quantification of metabolites were accomplished using Chenomx NMRSuite 7.6 (Chenomx Inc., Edmonton, Canada) [26]. Samples were corrected for dilution by multiplying by a correction factor calculated by the ratio of the final sample volume divided by the initial volume of serum.

2.6. Statistics

Unless otherwise indicated, all comparisons between the supplemented group and either the sham or OVX group was made using Student's t test. For body weight analysis, repeated measures two-way ANOVA was used followed by Tukey's multiple comparison test. Statistical significance was defined as $P < .05$. Data are expressed as mean $\pm$ S.D. (or S.E.M.). Data sets were analyzed for statistical significance using Prism GraphPad 6 software (GraphPad Software Inc., San Diego, CA, USA). Multivariate data analysis [principal component analysis (PCA)] was performed on $\log_{10}$ -transformed metabolite concentrations using SIMCA-P (version 13.0, Umetrics, Umeå, Sweden) as described in Slupsky [27].

3. Results

3.1. Body weight

Average body weight over 23 weeks of supplementation after ovariectomy is shown in Fig. 1. The OVX, NPM and PM mice had significantly higher body weights compared with sham from week 17 until the end of the experiment. Analysis by repeated measures two-way

Fig. 3. Micro-CT femur parameters. Bone characteristics of sham ($n=8$), OVX ($n=9$), NPM ($n=7$) and PM ($n=8$) C57BL/6 mice after treatment for 23 weeks. (A–H) Bone parameters of the distal femur analyzed by micro-CT. (A) Tb.BMD. (B) BV/TV. (C) BS/TV. (D) Tb.Th. (E) Tb.N. (F) Conn.Dn. (G) BS/BV. (H) Tb.Pf. (I–L) Cortical bone parameters of middle diaphysis analyzed by micro-CT. (I) Thickness, cortical bone thickness. (J) EC. (K) PC. (L) Cross-sectional, cross-sectional area. Each value is expressed as mean $\pm$ S.D. * $P < .05$ versus OVX group by Student's t test. ** $P < .05$ versus PM group by Student's t test.

Fig. 4. Micro-CT 3D images. Representative micro-CT 3D images of the distal femur region from (A) sham, (B) OVX, (C) NPM and (D) PM C57BL/6 mice after treatment for 23 weeks.

ANOVA revealed a significant effect of time ($P < .0001$) and treatment ($P = .04$) on each group as well as a significant interaction effect ($P = .008$). The major difference in weight was between sham and the OVX, NPM and PM mice, whereas no significant difference among OVX, NPM and PM mice was observed. Food intake is shown in Fig. 2. PM mice consumed significantly less chow than OVX, sham and NPM mice, while the sham, OVX and NPM mice had similar food intakes.

3.2. Micro-CT analysis and bone content

After 23 weeks of treatment, the parameters of trabecular bone in the distal femur and cortical bone in the middle diaphysis of the femur were analyzed by micro-CT and are shown in Fig. 3. Tb.BMD, BV/TV, BS/TV, Tb.Th, Tb.N and Conn.Dn measurements of PM mice were significantly higher than those of OVX mice, whereas the BS/BV and Tb.Pf measurements of PM mice were significantly lower than those of OVX mice. The NPM mice also showed significantly higher Tb.BMD, BV/TV and Tb.Th and significantly lower BS/BV compared with OVX mice. Comparison of the bone parameters between NPM and PM mice revealed that BS/BV and Tb.Pf of PM mice were significantly lower than those of NPM mice. Comparison of sham and OVX mice revealed no difference in the middle diaphysis of the femur. The EC, PC and cross-sectional area of the femoral mid-diaphysis of NPM mice were higher than those of OVX mice, but the femoral mid-diaphysis thickness showed no significant difference between any of the treatments. The 3D image of the distal femur with trabecula is shown in Fig. 4. The NPM and PM mice showed more trabeculae compared with sham and OVX mice indicating increased bone density. These results suggest that both NPM and PM appear to improve bone health in OVX mice.

3.3. Serum osteoblast and osteoclast activity indicators, and serum calcium and phosphate concentrations

The concentration of indicators of serum osteoblast and osteoclast activity (at 12 or 23 weeks), which provide information on bone formation and resorption, respectively, as well as serum calcium and phosphate concentrations at week 23 are shown in Fig. 5. Osteoblast indicators included osteocalcin, ALP and PICP. The osteocalcin concentration in PM mice was significantly higher than that in OVX

and NPM mice at week 12 indicating increased bone formation in PM-supplemented mice at this time point. While the ALP concentration of PM mice at week 12 was significantly higher than that of OVX mice, it was significantly lower at week 23.

Osteoclast indicators, which indicate increased bone resorption, included PYD and NTX (Fig. 5). Both PYD and NTX concentrations at 23 weeks were lower in PM mice compared with those in OVX mice suggesting decreased bone resorption in PM-supplemented mice. Serum calcium of PM mice was significantly lower than that of sham and NPM mice, whereas phosphate was not significantly different in the serum from mice from any of the treatment groups (Fig. 5).

3.4. Serum metabolomics

A PCA scores plot comparing metabolite concentrations obtained from the analysis of mouse serum after treatment for 23 weeks is shown in Fig. 6. Sham, OVX and NPM mice tended to cluster together and were distinct from PM mice (Fig. 6A). PCA of only PM vs. NPM revealed distinction (Fig. 6B), whereas no distinction among OVX, NPM or sham mice were observed (Fig. 6C) suggesting that the main difference in overall metabolism may be attributed to the effect of vitamin D₂.

Seventeen metabolites primarily responsible for the observed difference between OVX and PM mice at week 23 are summarized in Table 1. PM mice showed significantly higher concentrations of amino acids and metabolites associated with energy metabolism compared with OVX mice, as well as lower methanol concentrations. PM mice also showed significantly increased taurine concentrations compared with OVX mice. Eighteen metabolites primarily responsible for the observed difference between NPM and PM treatment groups at week 23 are summarized in Table 2 and showed significantly higher concentrations of amino acids and energy metabolites and lower 3-hydroxybutyrate and gut microbial related metabolites in PM mice compared with NPM mice.

4. Discussion

Postmenopausal osteoporosis is characterized by hormonal deprivation resulting in accelerated bone turnover and bone loss [28]. Low

Fig. 5. The concentrations of osteoblast and osteoclast activity indicators, calcium and phosphate in serum. Serum indicators of serum osteoblast and osteoclast activity, and serum calcium and phosphate concentration in sham ($n=8$), OVX ($n=8-9$), NPM ($n=7$) and PM C57BL/6 ($n=8$) mice after treatment for 12 (W12) or 23 (W23) weeks. (A–E) Osteoblast indicators. (A) W12 osteocalcin; (B) W23 osteocalcin; (C) W12 ALP; (D) W23 ALP; (E) PICP after 23 weeks treatment. (F and G) Osteoclast indicators. (F) PYD after 23 weeks treatment. (G) NTX after 23 weeks treatment. (H) Serum calcium concentration after 23 weeks treatment. (I) Serum phosphate concentration after 23 weeks treatment. Each value is expressed as mean $\pm$ S.D. * $P<0.05$ versus OVX group by Student's t test, ## $P<0.05$ versus PM group by Student's t test.

Fig. 6. Comparison of metabolic composition of serum. Comparison of serum metabolites of sham ($n=7$), OVX ($n=6$), NPM ($n=5$) and PM ($n=7$) C57BL/6 mice after 23 weeks treatment. (A) PCA of all groups ($R^2X=0.439$, $Q^2=0.187$). (B) PCA of NPM and PM mice only ($R^2X=0.548$, $Q^2=0.234$). (C) PCA ($R^2X=0.47$, $Q^2=0.204$) of sham, OVX and NPM mice.

levels of serum 25(OH)D are common in osteoporotic postmenopausal women [29]. The oral dose of vitamin D₂ required to rapidly achieve sufficient levels of 25(OH)D is much higher than the usual recommended vitamin D₃ dose in postmenopausal osteopenic/osteoporotic women [30]. However, vitamin D₂ is less stable than vitamin D₃ when provided as a tablet, and its effects have not been studied as a component of food. In this study, we evaluated the effect of pulsed enhanced vitamin D₂ *P. ferulae* on OVX mice, a classic model to study postmenopausal bone loss [31].

As expected, all OVX mice (OVX, NPM and PM mice) had higher body mass than sham mice after 23 weeks of treatment, which may be due to estrogen deficiency resulting in increased body fat deposition [32]. Nonetheless, supplementation with *P. ferulae* was shown to increase

bone density in PM and NPM mice compared with both OVX and sham mice. While all OVX mice had higher EC and PC than sham mice, those OVX mice supplemented with *P. ferulae* (NPM mice) showed higher EC and PC than nonsupplemented OVX mice or OVX mice supplemented with PM. These results may be suggestive of increases in bone resorption [33] and formation [34]; however, only the femur was analyzed. Interestingly, NPM mice did not show a difference in femur thickness compared with the other mice but did show increased cross-sectional area, consistent with increased bone density.

These results agree with a study in which OVX rats treated with 1 α (OH)D₂ had increased total bone mineral density and Tb.N in the tibia relative to OVX rats [35]. Higher Tb.BMD, BV/TV and Tb.Th and lower BS/BV in NPM mice compared with OVX mice are also in

Table 1
Comparison of average metabolite concentrations of sham, OVX, NPM and PM mice after treatment for 23 weeks.

Metabolite (μ M)		Sham	OVX	NPM	PM
Amino acids	Alanine	415.2 $\pm$ 68.9	374.7 $\pm$ 86.1	452.2 $\pm$ 70.0	512.8 $\pm$ 68.2 *
	Arginine	213.5 $\pm$ 43.6	172.4 $\pm$ 50.8	167.5 $\pm$ 35.0	241.6 $\pm$ 53.4 *
	Glutamate	91.4 $\pm$ 23.9	89.8 $\pm$ 22.3	88.9 $\pm$ 19.4	149.4 $\pm$ 21.5 **
	Glutamine	781.5 $\pm$ 154.6	721.8 $\pm$ 39.3	782.4 $\pm$ 138.3	919.5 $\pm$ 97.2 **
	Glycine	313.8 $\pm$ 41.1 *	249.8 $\pm$ 42.5	299.1 $\pm$ 27.1	350.8 $\pm$ 45.5 **
	Histidine	69.2 $\pm$ 13.2	73.4 $\pm$ 15.1	76.1 $\pm$ 14.1	92.3 $\pm$ 9.7 *
	Leucine	159.9 $\pm$ 29.8	161.3 $\pm$ 20.1	191.9 $\pm$ 47.5	196.0 $\pm$ 27.0 *
	Lysine	208.2 $\pm$ 28.4	221.3 $\pm$ 30.1	259.0 $\pm$ 55.7	279.6 $\pm$ 45.7 *
	Methionine	85.0 $\pm$ 18.8	79.6 $\pm$ 23.9	99.6 $\pm$ 30.1	110.2 $\pm$ 24.7 *
	Proline	133.2 $\pm$ 25.8	116.1 $\pm$ 31.5	131.3 $\pm$ 13.7	164.9 $\pm$ 20.7 *
	Serine	168.1 $\pm$ 33.1	164.8 $\pm$ 42.1	148.9 $\pm$ 11.1	216.1 $\pm$ 39.6 *
	Taurine	460.8 $\pm$ 95.1	467.6 $\pm$ 116.0	647.0 $\pm$ 125.0 *	792.2 $\pm$ 66.1 **
	Tryptophan	41.7 $\pm$ 5.3 *	53.3 $\pm$ 9.8	43.2 $\pm$ 10.9	35.5 $\pm$ 16.7 *
	Energy metabolism	139.0 $\pm$ 22.3	126.1 $\pm$ 23.0	148.3 $\pm$ 13.6	180.6 $\pm$ 28.5 **
Gut microbial	Lactate	6761.7 $\pm$ 966.5	7552.9 $\pm$ 1473.7	8242.4 $\pm$ 1213.0	10,336.7 $\pm$ 757.8 **
	Pyruvate	156.1 $\pm$ 10.0 *	192.8 $\pm$ 37.6	166.4 $\pm$ 11.9	283.1 $\pm$ 41.0 **
	Methanol	97.3 $\pm$ 42.4	70.3 $\pm$ 15.7	169.2 $\pm$ 65.1 *	37.6 $\pm$ 14.7 **

Each value is expressed as mean $\pm$ S.D. Only those metabolites that showed significant differences are shown.

* $P<0.05$ compared with OVX group using Student's t test.

** $P<0.005$ compared with OVX group using Student's t test.

Table 2
Comparison of average metabolite concentrations of NPM and PM mice after treatment for 23 weeks.

Metabolite (μM)		NPM	PM
Amino acids	Arginine	167.5 $\pm$ 35.0	241.6 $\pm$ 53.4*
	Asparagine	49.6 $\pm$ 8.2	69.7 $\pm$ 16.5*
	Glutamate	88.9 $\pm$ 19.4	149.4 $\pm$ 21.5**
	Glycine	299.1 $\pm$ 27.1	350.8 $\pm$ 45.5*
	Histidine	76.1 $\pm$ 14.1	92.3 $\pm$ 9.7*
	Serine	148.9 $\pm$ 11.16	216.1 $\pm$ 39.0**
	Taurine	647.0 $\pm$ 125.0	792.2 $\pm$ 66.1*
	Proline	131.3 $\pm$ 13.7	164.9 $\pm$ 20.7*
	Creatine	148.3 $\pm$ 13.6	180.6 $\pm$ 28.5*
Energy metabolism	Creatinine	13.6 $\pm$ 2.1	17.1 $\pm$ 2.2*
	Lactate	8242.4 $\pm$ 1213.0	10,336.7 $\pm$ 757.8**
	Pyruvate	166.4 $\pm$ 11.9	283.1 $\pm$ 41.0**
Ketones	3-Hydroxybutyrate	118.6 $\pm$ 29.5	81.9 $\pm$ 20.6*
Gut microbial	Acetate	88.1 $\pm$ 24.6	43.3 $\pm$ 6.2**
	Methanol	169.2 $\pm$ 65.1	37.6 $\pm$ 14.7**
	Ethanol	150.4 $\pm$ 36.4	101.7 $\pm$ 18.4*
Others	Carnitine	50.1 $\pm$ 14.2	70.9 $\pm$ 10.8*
	Choline	48.3 $\pm$ 12.5	110.9 $\pm$ 42.3*

Each value is expressed as mean $\pm$ S.D. Only those metabolites that are significantly different are reported.

* $P < .05$ versus PM group by Student's t test.

** $P < .005$ versus PM group by Student's t test.

agreement with another study in which nonirradiated *Pleurotus* mushroom extracts were shown to alleviate the decrease in Tb.BMD in osteoporotic mice [36]. *P. ferulae* contains various phenolic and flavonoid compounds [37], as well as phytochemicals that have been shown to elicit a bone-protective effect [31,38,39].

Biochemical markers provide indirect information on bone resorption and formation [40]. Osteoblasts are bone-forming cells that synthesize and secrete numerous proteins, such as osteocalcin, ALP and PICP, which can be measured in serum as markers of osteoblast activity [41,42]. Osteocalcin, a small noncollagenous protein, is a unique and specific product of osteoblasts [28,42] that can be induced by 1,25(OH) $_2$ D [41]. Our results showed that the serum osteocalcin concentration in PM mice was higher than that in OVX mice and NPM mice, confirming that 1,25(OH) $_2$ D was responsible for the increase in serum osteocalcin. ALP is associated with osteoid formation and mineralization [43], but it is not as specific a marker of osteoblast activity as osteocalcin [42]. Our results showed that the ALP concentration of OVX mice was significantly higher than that of PM mice at week 23. Others have also shown that OVX rats have higher serum ALP than sham and zoledronate-treated (a bisphosphonate used for osteoporosis) rats [43]. One explanation for these observations may be those osteoblasts undergoing apoptosis release ALP into the blood [43,44]. Osteoporosis is also associated with bone collagen loss [45]. PICP, a marker of collagen synthesis, is released during the synthesis of osteocalcin and type I collagen [46]. The NPM-supplemented mice showed higher PICP than PM-treated mice, who remained similar to OVX mice, which may indicate that NPM-treated mice synthesize more collagen.

Osteoclasts are bone-resorbing cells, and they are sensitive markers of bone resorption [41,42]. They are also better indicators than bone formation markers for evaluating the response to osteoporosis intervention in early postmenopausal women [47]. Serum NTX and PYD are osteoblast indicators and are products of type I collagen degradation [42,46]. PM mice showed lower PYD and NTX concentrations than OVX mice suggesting that pulsed *P. ferulae* may alleviate type I collagen breakdown. PM mice also showed significantly lower PYD than NPM mice, suggesting that vitamin D $_2$ is more active at inhibiting osteoclast activity than the mushrooms themselves. Taken together, these results suggest that mice provided the pulsed *P. ferulae* containing vitamin D $_2$ exhibited lower bone loss than those provided the nonpulsed *P. ferulae*.

Serum NMR metabolomics analysis indicated that several amino acids were elevated in mice fed pulsed *P. ferulae*, which included some associated with bone, such as taurine, arginine and lysine. Taurine has been reported to be expressed in osteoblasts to inhibit bone resorption, inhibit formation and survival of osteoclasts and bind divalent cations to play an important role in bone remodeling and skeletal development [48,49]. Taurine supplementation has been shown to increase bone mineral density in rats [50,51]. Furthermore, the pulsed *P. ferulae* treated mice had higher serum taurine concentrations than mice treated with alendronate [52], suggesting the pulsed *P. ferulae* may induce higher concentrations of metabolites related to bone health [52]. Arginine is involved in the synthesis of substrates (polyamines and proline) that are implicated in collagen synthesis [50,53,54]. Lysine affects the stimulation of Ca $^{2+}$ absorption and the cross-linking process of bone collagen [53,54]. Energy-metabolism-related metabolites such as creatine and lactate were also shown to be higher in pulsed *P. ferulae* supplemented mice. Creatine and lactate have been shown to be associated with bone health [55–58]. Creatine has been shown to stimulate bone growth and maintenance, as well as alleviate osteoporosis of postmenopausal women [55]. Creatine can also stimulate type I collagen synthesis and secretion [55]. It can become energy source for bone cells to survive, proliferate, differentiate and synthesize its extracellular matrix [55,56]. Lactate may also reduce bone loss, as an intermediate for the regulation of collagen biosynthesis during osteogenesis [57]. Indeed, in postmenopausal women, high bone mineral density is associated with higher serum lactate levels than low bone mineral density [57]. Although NPM- and PM-supplemented mice showed similar Tb.BMD, the PM mice had a slightly different metabolite profile compared with the NPM mice (Fig. 6B). Metabolites related to bone health including energy metabolites and amino acids, such as creatine, lactate, arginine and taurine, were higher in PM mice compared with NPM mice. Although our results showed the nonpulsed *P. ferulae* can improve bone health in OVX mice, the pulsed enhanced vitamin D $_2$ *P. ferulae* appears to have a stronger ability to improve bone health compared with nonpulsed *P. ferulae* in an OVX osteoporosis mouse model.

The winter season and northern latitudes, as well as modern lifestyles, diets and the use of sunscreens, have led to a high prevalence of low serum vitamin D in populations worldwide [3,4,58]. Our results show that pulsed enhanced vitamin D $_2$ *P. ferulae* can maintain bone health, alleviate the activity bone resorption markers and increase bone health-related metabolites in OVX mice through its action as both a mushroom (polyphenolics and fiber) and its vitamin D $_2$ content. Further studies are warranted to determine whether similar effects could be observed with just vitamin D $_2$ alone or whether a combined effect of the mushroom and the vitamin D $_2$ is important. Moreover, further studies in humans through supplementation of pulsed irradiated mushrooms as part of their diet are warranted to understand on a deeper level the health benefits of consuming UV-irradiated mushrooms.

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Conflict of interest

All authors have no conflicts of interests with respect to the data collected and procedures used within this study.

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