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ORIGINAL CONTRIBUTION

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Dietary calcium and vitamin D_2 supplementation with enhanced *Lentinula edodes* improves osteoporosis-like symptoms and induces duodenal and renal active calcium transport gene expression in mice

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Abstract The two main sources of vitamin D_3 are de novo synthesis induced by exposure to ultraviolet (UV) light from the sun, and diet. Vitamin D_3 deficiency causes rickets or osteoporosis. Oak mushrooms (*Lentinula edodes*) that are exposed to UV radiation contain enhanced vitamin D_2 and have much higher calcium content than unmodified (non-irradiated) mushrooms. Such modified edible mushrooms have been proposed as a natural alternative source of dietary vitamin D. In the current study, we have examined whether modified oak mushrooms could improve or prevent osteoporosis-like symptoms in mice fed with low calcium and vitamin D_3-deficient diet. Four-week-old male mice were fed low calcium, vitamin D_3-deficient diets supplemented with 5, 10, or 20% unmodified, calcium-enhanced, or calcium plus vitamin D_2-enhanced oak mushrooms for 4 weeks. To assess the effects of the supplemented diets, we evaluated femur density and length, bone histology, the expression of active calcium transport genes, and serum calcium levels. Mice fed with low calcium and vitamin D_3-deficient diet developed osteoporosis-like symptoms within 4 weeks. Femur density and tibia thickness were significantly higher in mice fed calcium plus vitamin D_2-enhanced mushrooms, and the expression of duodenal and renal calcium transport genes was significantly induced. These results indicate that in mice, vitamin D_2 and/or calcium derived from irradiated oak mushrooms may improve bone mineralization through a direct effect on the bone, and by inducing the expression of calcium-absorbing genes in the duodenum and kidney.

Key words *L. edodes* – active calcium transporting genes – osteoporosis – vitamin D_2

Introduction

Vitamin D is involved in the regulation of essential physiological functions, including skeletogenesis and the maintenance of mineral ion homeostasis [10, 16]. There are two main forms of vitamin D, vitamin D_3 or cholecalciferol, which is synthesized by the skin when it is exposed to sunlight or ultraviolet (UV) light, and vitamin D_2, or ergocalciferol, which is derived from irradiated plants or material of plant origin. While vitamin D deficiency has been associated with cancer,

heart disease, obesity, diabetes, and arthritis [21], the most common clinical manifestation of vitamin D deficiency is osteoporosis. Osteoporosis is a condition in which the density of the bone mass decreases over time, resulting in a thinning and weakening of the bone. It is caused by a variety of factors, including aging and calcium deficiency. In animal models, a low calcium diet and/or vitamin D_3 deficiency leads to the development of the radiological features of rickets and histological features of osteoporosis [26]. In the present study, the development of radiologic rickets and histologic osteomalacia in low calcium and vitamin D deficient dieted mice was taken to be indicative of "osteoporosis-like symptoms".

In the body, calcium ions are actively absorbed from the duodenum and then reabsorbed in kidney. The calcium active transport system involves three distinct steps: calcium influx, transfer through the cytosol, and extrusion into the bloodstream. In the active calcium transport organs, these steps are mediated by calcium entry channel proteins of the outer cell membrane, cytosolic buffering or transfer proteins, and excretory pump proteins [9, 31], respectively. Two highly selective calcium channels located in the apical cell membrane, transient receptor potential vanilloid 6 (TRPV6) and TRPV5, are the main portals of calcium-ion entry into cells. Calbindin-D9k (CaBP-9k) is an intracellular calcium ion-binding protein that is believed to participate in shuttling calcium ions from the apical to the basolateral membrane, where calcium ions are extruded [11].

In the current study, we have investigated the therapeutic potential of calcium and vitamin D_2 from modified oak mushrooms (*Lentinula edodes*; *L. edodes*) on bone mineralization in an animal model of diet-induced osteoporosis-like symptoms. Mice were fed a low calcium, vitamin D_3 deficient diet supplemented with increasing percentages of unmodified, calcium-enhanced, or calcium plus vitamin D_2-enhanced oak mushrooms powder during their growth stage (from 4 to 8 weeks of age). After 4 weeks, bone mineralization and the duodenal and renal expression of active calcium transport system were examined. We also examined the levels of serum calcium as a maker of calcium homeostasis.

Materials and methods

Generation of calcium and/or vitamin D_2-enhanced *L. edodes*

To generate vitamin D_2-enhanced oak mushrooms, the gill sides of plants were placed on shelves and exposed to 40 kJ/m^2 of ultraviolet-B (UV-B) radiation at a dose of 40 kJ/m^2 using a UV-B lamp (Vilber Lourmat, T-15M, 280–320 nm, France) in a UV chamber (Labcamp Co., Korea) at 25°C. The dose of UV-B light was measured with a UV Radiometer (CX-312, Vilber Lourmat, France). The irradiated samples were individually freeze-dried (TVTFD 10R, IlShin Co., Korea), immediately homogenized (HMC-150T, Hanil Co., Korea), and then stored at −20°C until analysis. The calcium-enhanced oak mushrooms were generated by adding 5.2% eggshell as a calcium source to sawdust source media.

Calcium and vitamin D_2 concentrations of the modified *L. edodes*

The vitamin D_2 content of the modified oak mushrooms (vitamin D_2/g mushroom dry weight) was determined as previously described, with minor modifications [23]. Freeze-dried mushroom powder (1.0 g) was placed in a 250 ml flask and mixed with 4.4 mg of butylated hydroxytoluene (BHT; an antioxidant), 40 ml of ethanol, and 25 ml of 50% potassium hydroxide. The mixture was shaken and then saponified under reflux at 85°C for 30 min. The mixture was immediately cooled to room temperature and then poured into a separating funnel. The mixture was extracted two times with 10 ml of deionized water and then 30 ml of *n*-hexane. The pooled organic layers were washed three times with deionized water until a neutral pH was reached. The organic layer was transferred into a flask, subjected to rotary evaporation to dryness at 50°C, and immediately re-dissolved in 2 ml of a solution of methanol:acetonitrile:isopropyl alcohol (3:1:4). The samples were passed through a 0.45 μm filter unit (PTFE, 13 mm, Whatman Ltd, UK), and 20 μl of the filtrate was subjected to HPLC. The Waters 1525 HPLC (Waters Corporation, USA) system was equipped with Waters 2487 Dual Absorbance UV detector (Waters, Milford, USA) and eluted through a reverse phase C18 column (Maxsil 5 C18, 150 × 4.6 mm, Phenomenex, USA) using acetonitrile:methanol (75:25) as the mobile phase at flow rate of 1 ml/min. The UV detection of the eluted was performed at UV 264 nm. Vitamin D_2 was identified by comparison to the retention times of a set of standards, and quantified using a calibration curve. In this study, the limit of detection (LOD) was 0.04 μg/ml, indicating that small amounts of vitamin D can be detected, at least in the mushroom samples, by the HPLC method. The limit of quantification (LOQ) was about 0.13 μg/mL (3.3 times of LOD) in this study.

The concentration of calcium in the oak mushroom preparations was measured using the Association of Official Agricultural Chemists (AOAC) method

[3]. Oak mushrooms were burned for 6 h at 600°C using an electric Thermolyne muffle furnace (Thermolyne #62700, USA) and the ash weight was determined. Mushroom ash was mixed with 10 ml of 6 N HCl and boiled for 30 min to remove organic material. The samples were filtered using Whatman filter paper (No. 541, UK) and then calcium content was measured using an atomic absorption flame emission spectrophotometer (AA-6701, Shimadzu Scientific Instruments, Inc., Japan).

Animals and treatments

Three-week-old male inbred strain of ICR mice were obtained from KOATECH (Pyeongtaek-si, Gyeonggi-do, Korea). All animals were housed in polycarbonate cages and acclimatized in an environmentally controlled room to 23 ± 2°C, and 50 ± 10% relative humidity, with frequent ventilation and a 12 h light/dark cycle prior to use. Fifty-five mice were randomly divided into 11 groups (n = 5 for each group in the same cage). Osteoporosis-induced animals were divided into ten groups by feeding with low calcium and vitamin D_3-deficient diet (AIN-76A purified rodent diet with 0.8% strontium, 0.02% calcium, and 0.35% phosphorus without vitamin D_3, #D10373A, Research Diets, Inc., Brunswick, NJ, USA) from 4-to 8-weeks of age. As a positive control, one group was fed a normal diet (AIN-76A purified rodent diet with 0.8% phosphorus and 1.1% calcium, DYET #113295, Dyet, Inc., Bethlehem, PA, USA) for the same period of time.

To assess the effect of calcium plus vitamin D_2-enhanced oak mushrooms on bone mineralization and the regulation of active calcium transport gene expression, the diets of nine of the low calcium, vitamin D_3-deficient groups were supplemented for 4 weeks with 5, 10, and 20% of unmodified, calcium-enhanced, or calcium plus vitamin D_2-enhanced oak mushroom powder. The average daily food intake of the mice was 2.7 ± 0.27 g, and of this amount, the animals whose diet was supplemented with 10% calcium plus vitamin D_2-enhanced oak mushroom powder received approximately 1 μg of vitamin D_2 [14]. All experimental and animal use procedures were approved by the Ethics Committee of the Chungbuk National University.

Bone mineral density and histomorphometry

Mice were euthanized at 8 weeks of age by carbon dioxide inhalation. The hind legs were removed and fixed in a 10% neutralized formalin solution for 3 days. The legs were then washed with water, and the skin and muscle were removed. The femur was dried over-night and bone mineral density was analyzed using a Direct View CR 500 (Eastman Kodak, Co., Rochester, NY, USA). The tibia was decalcified using Plank–Rycholo's solution, and the bone was embedded in paraffin, sectioned, and deparaffinized in xylene. The sections were hydrated in a series of solutions of descending ethanol concentration, then stained with hematoxylin and eosin, and subjected to histomorphometric analyses using light microscopy. Osteoid thickness was measured at the center point of the tibia.

Real-time PCR using TaqMan™ Probe

Total RNA was prepared from the duodenum and kidney using the TRIzol reagent (Invitrogen Life Technologies, Inc., Carlsbad, CA, USA). First stand complementary DNA (cDNA) was prepared by subjecting total RNA (1 μg) to reverse transcription using mMLV reverse transcriptase (Invitrogen Life Technologies, Inc.) and random primers (9-mer, TaKaRa Bio. Inc., Otsu, Shiga, Japan) [18, 20]. Real-time PCR was performed in a 20 μl reaction volume containing 10 μl of TaqMan Universal PCR Master Mix (Applied Biosystems, Foster, CA, USA), 1 μl of 20 × Assays-on-Demand™ Gene Expression Assay Mix (Applied Biosystems) (*TRPV6*, Mm00499069_m1; *TRPV5*, Mm01166029_m1; *CaBP-9k*, Mm00486654_m1; and *HPRT1*, Mm00446968_m1) and 5 μl of cDNA. Amplification was carried out using a 7,300 Real-Time PCR System (Applied Biosystems) and the following cycle parameters: initial denaturation at 50°C for 2 min; 90°C for 10 min; 40 cycles of denaturation at 95°C for 15 s; and annealing and extension at 60°C for 1 min. The expression levels were determined using RQ software (Applied Biosystems). The expression levels of *TRPV6*, *TRPV5*, and *CaBP-9k* were normalized to that of *HPRT1*.

Biochemical analysis

Blood was collected from each mouse into serum separator tubes and subjected to centrifugation at 3,000 rpm for 10 min. Serum calcium levels were determined using ASAN-Ca-Lq Reagents (ASAN-PHARM, Kyeonggido, Korea) according to manufacturer's instructions.

Data analysis

Data was analyzed by non-parametric one-way analysis of variance using the Kruskal Wallis test, followed by Dunnett's test for multiple comparisons to the negative control group. Data was ranked according to these tests. Statistical analysis was performed using

SPSS for Windows (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Results

Calcium and vitamin D_2 content of the modified oak mushrooms

The total calcium and vitamin D_2 content in unmodified, calcium-enhanced, or calcium plus vitamin D_2-enhanced oak mushrooms was measured. The levels of calcium ion were higher in calcium and calcium plus vitamin D_2-enhanced oak mushrooms (277.23 ± 18.32 and 354.30 ± 12.51 μg/g, respectively) than in unmodified oak mushrooms (206.43 ± 16.53 μg/g) as shown in Fig. 1 (left panel). The vitamin D_2 content of calcium plus vitamin D_2-enhanced mushrooms was 45.18 ± 0.99 μg/g, while it was nearly undetectable in the other types of oak mushroom (Fig. 1, right panel).

The effect of a modified oak mushroom-supplemented diet on osteoporosis-like symptoms

To determine the beneficial effect of modified *L. edodes* on osteoporosis-like symptoms in mice, animals in growth phase (4–8 weeks old) were fed low calcium, vitamin D_3-deficient diets supplemented with increasing amounts (5, 10, and 20%) of a oak mushroom powder (unmodified, calcium-enhanced, or calcium plus vitamin D_2-enhanced oak mushroom powder), or a normal diet as a positive control. Then, any resulting abnormalities in the fed mice were noted. Femur mineral densities were analyzed using computer radiography, as shown in Fig. 2. To deter-

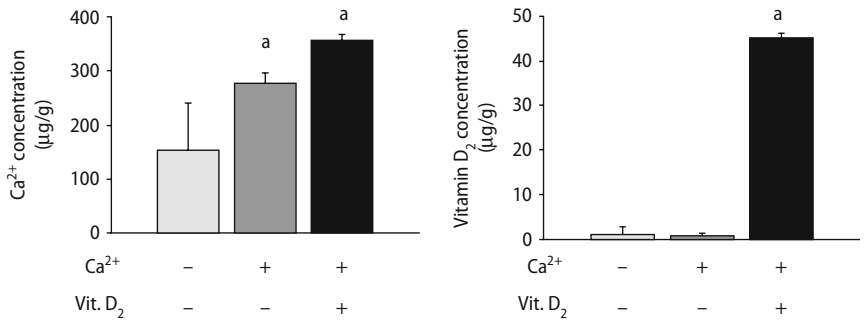


Fig. 1 Concentration of calcium and vitamin D_2 in unmodified, calcium-enhanced, and calcium plus vitamin D_2-enhanced oak mushrooms. – unmodified, + enhanced, *a* $P < 0.05$ versus unmodified oak mushrooms

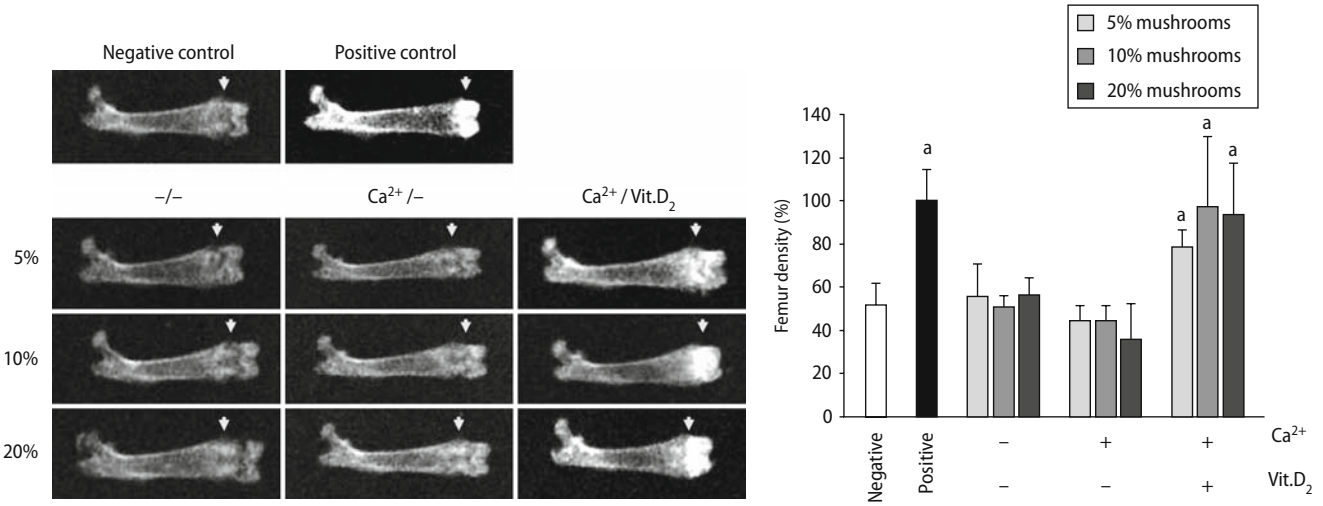


Fig. 2 The effect of enhanced oak mushrooms on osteoporosis-like symptoms was assessed using bone radiography. Femurs were obtained from mice and analyzed as described as "Materials and methods". Representative femurs from each of the indicated treatment groups are shown in the *left panel*; bone density of the proximal heads of all femurs ($n = 5$ per group) is presented in the *right panels*. Data represents the mean ± SEM of duplicate measurements of all samples, and is expressed as a percentage of the density of a normal femur. *Arrows* proximal head of the femur, *a* $P < 0.05$ versus the negative control group

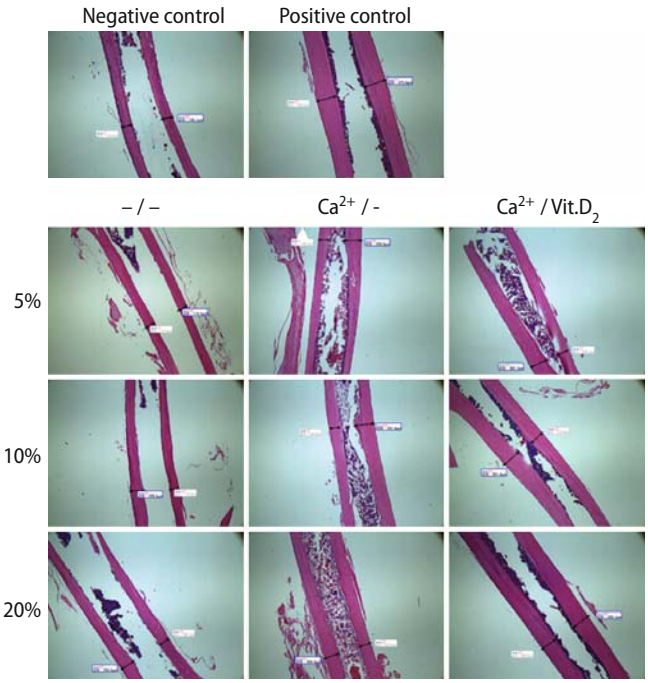
Table 1 Analysis of femur length in mice whose diets were supplemented with enhanced oak mushrooms

Negative control	Positive control	Intact *L. edodes*	Ca^{2+} enhanced *L. edodes*	Ca^{2+} and vit. D_2 enhanced *L. edodes*	Additional ratio in the diet (%)
1.35 ± 0.06	1.50 ± 0.07[a]	1.30 ± 0.01	1.28 ± 0.04	1.20 ± 0.06	5
		1.32 ± 0.04	1.30 ± 0.01	1.28 ± 0.05	10
		1.30 ± 0.08	1.32 ± 0.04	1.30 ± 0.01	20

[a] $P < 0.05$ versus negative control, unit; centimeters (cm)

mine the effect of calcium and/or vitamin D_2-enhanced mushrooms in more detail, distal femur head density was analyzed using Direct View CR 500 software (bar graph in Fig. 2). The density of the distal femur head of the positive control group was significantly higher than the negative control group. The bone densities of mice fed a diet of calcium and vitamin D_2-enhanced *L. edodes* were significantly higher than mice fed calcium-enhanced mushrooms, or the negative control group. When we analyzed femur length (Table 1), we found that mice fed a normal diet had significantly longer femurs than mice fed a low calcium, vitamin D_3-deficient diet, with or without supplementation. These results indicated that calcium plus vitamin D_2-enhanced mushrooms can increase bone density, but do not affect bone length.

We also analyzed the effect of modified oak mushrooms on osteoporosis-like symptoms in the mice by bone histomorphometry. Figure 3 shows representative tibia sections that were stained with eosin and hematoxylin. Osteoid thickness in the negative control group was significantly less than the positive control group, and thickness gradually increased with increasing percentage of calcium and/or vitamin D_2 enhanced *L. edodes* supplementation. The tibia thicknesses of all groups that were fed with low calcium and vitamin D_3-deficient diet were less than the positive control group. These results suggested that while dietary supplementation with modified oak mushrooms can overcome some osteoporosis-like symptoms in mice fed low calcium, vitamin D_3-deficient diets, it is not sufficient for healthy bone formation.



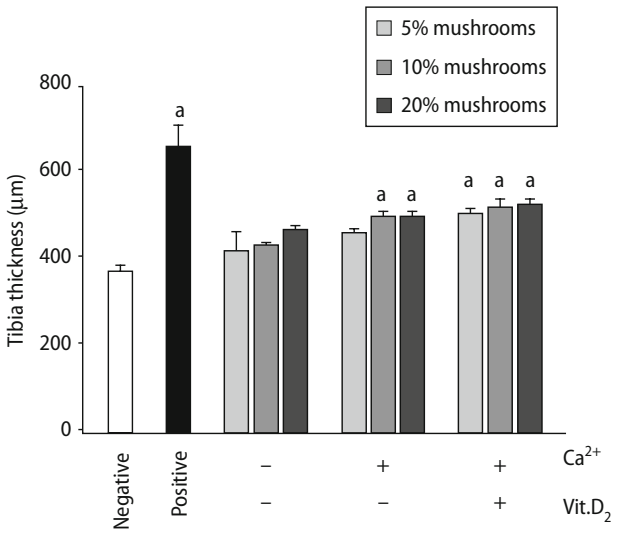


Fig. 3 The effect of enhanced oak mushrooms on osteoporosis-like symptoms was assessed using bone-histomorphometry. Tibias were removed from the mice and processed as described in "Materials and methods". Representative tibias from the indicated treatment groups are shown in the *left panel*; osteoid thickness of the middle of the bone for all tibias ($n = 5$ per group) is shown in the *right panel*. *Arrows* indicate the measured thickness, and the *space between the arrows* represents the bone marrow. Data represents the mean ± SEM of duplicate measurements of all samples. *a* $P < 0.05$ versus the negative control group

Table 2 Serum calcium levels of mice whose diets were supplemented with enhanced oak mushrooms

Negative control	Positive control	Intact *L. edodes*	Ca^{2+} enhanced *L. edodes*	Ca^{2+} and vit. D enhanced *L. edodes*	Additional ratio in the diet (%)
4.9 ± 0.4	10.28 ± 1.2[a]	5.0 ± 0.1	6.5 ± 0.3	5.3 ± 1.1	5
		5.2 ± 0.8	5.8 ± 0.1	5.8 ± 0.6	10
		5.3 ± 1.1	6.7 ± 0.1	7.1 ± 0.1[a]	20

[a] $P < 0.05$ versus negative control, unit; mg/dl

We monitored serum calcium concentrations as a marker of calcium homeostasis. The serum calcium concentrations of the positive control group and the 20% supplementation group were higher than the negative control and the other supplementation groups (Table 2), which was consistent with the bone density data.

Effect of dietary supplementation with modified oak mushrooms on calcium processing gene expression

The addition of calcium and vitamin D_2-enhanced oak mushroom to low calcium and vitamin D_3-deficient diet appeared to mitigate some symptoms of diet-induced osteoporosis. To begin to understand the mechanism of the beneficial effect of modified oak mushrooms on osteoporosis, we examined the expression of duodenal and renal calcium absorbing genes using real-time PCR. The expression of duodenal *CaBP-9k* was similar in mice fed unmodified oak mushrooms, calcium-enhanced *L. edodes*, or the negative control diet (Fig. 4). By comparison, mice whose diets were supplemented with calcium and vitamin D_2-enhanced *L. edodes* exhibited a significant induction of duodenal *CaBP-9k* expression. The expression of duodenal *TRPV6* was also increased in the calcium and vitamin D_2-enhanced oak mushroom groups as compared to the other supplementation groups and the negative control group. In the kidney, *CaBP-9k*, *TRPV6*, and *TRPV5* were up-regulated in

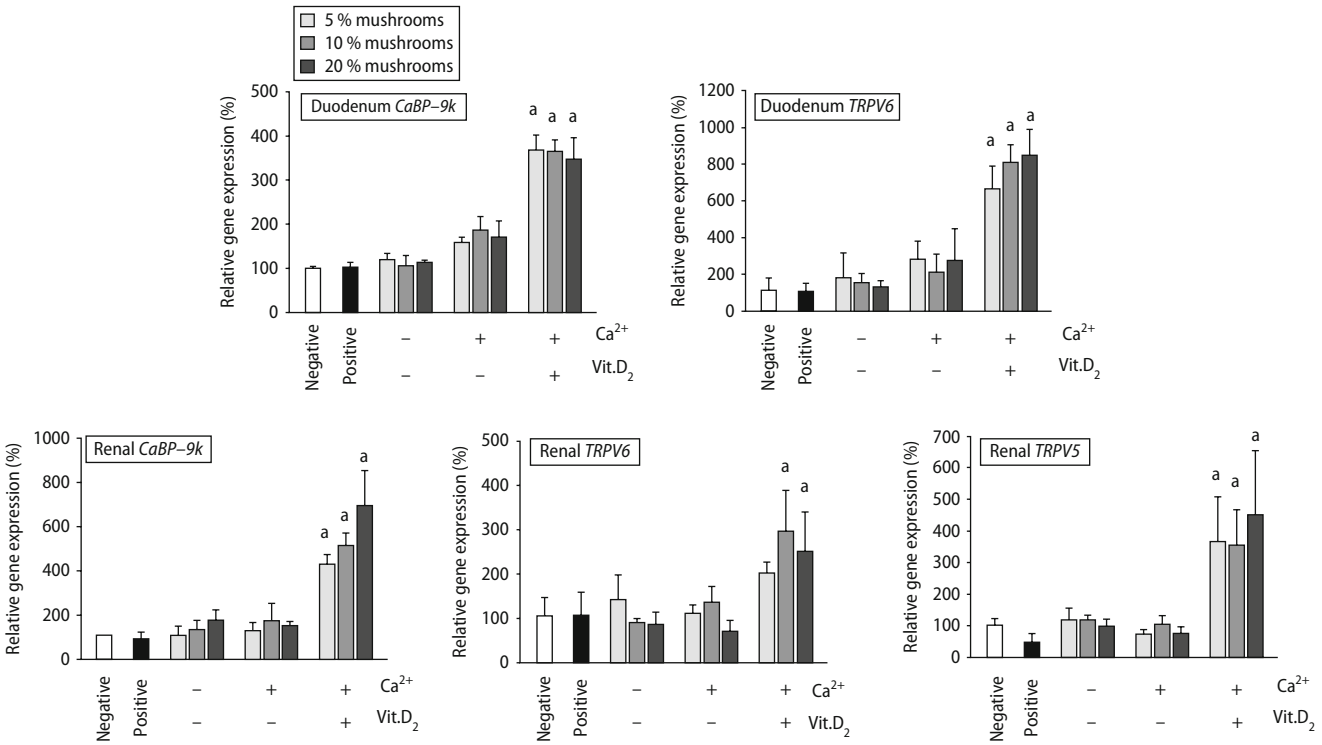


Fig. 4 The effect of enhanced oak mushrooms on the expression of active calcium transport genes. Duodenal and renal mRNAs were prepared from mice of the indicated treatment groups ($n = 5$ per group) as described in "Materials and methods". The relative expression levels of *CaBP-9k*, *TRPV5*, and *TRPV6* mRNAs were examined using real-time PCR. Data represents the mean ± SEM of duplicate measurements of all samples, and is expressed as a percentage of the expression of the internal control, *HPRT1*. *a* $P < 0.05$ versus the negative control group

mice that received calcium and vitamin D_2-enhanced *L. edodes* as compared the negative control group and the other oak mushroom groups. These distinct patterns of regulation of duodenal and renal calcium-processing gene expression suggested that the mechanism of prevention of osteoporosis-like symptoms by calcium and vitamin D_2-enhanced oak mushrooms involves the augmentation of total vitamin D levels in the body, and the induction of genes involved in the calcium active transport system.

Discussion

Vitamin D deficiency can occur as a result of premature or dysmature birth, heavily pigmented skin, low sunshine exposure, obesity, vitamin D malabsorption, and advanced age. The prevalence of vitamin D deficiency is high in elderly people as compared to adults, particularly among residents of homes for the elderly and nursing homes, and patients with hip fracture [21]. Severe vitamin D deficiency results in rickets and osteomalacia, while less severe vitamin D deficiency contributes to osteoporosis.

Nutritionally, mushrooms are low in calories and fat but rich in vitamins especially provitamin D "ergosterol" and minerals [27]. Ergosterol can be converted to ergocalciferol (vitamin D_2) by irradiation with sunlight or UV-B [22, 27]. UV light can be divided into three regions, UV-C (190–290 nm), UV-B (290–320 nm), and UV-B (290–320 nm). Among these, UV-B is the most efficient in converting ergosterol to vitamin D_2. In the human body, vitamin D_2 is not converted to vitamin D_3, the active form of vitamin D. Because mushrooms are a non-animal-based food containing vitamin D, they are a good source of natural vitamin D for vegetarians [22]. In our study, we used shiitake mushrooms as a vitamin D_2 source because these mushrooms are popular in Asian countries and the shape of the shiitake mushroom (*Lentinus edodes*) is suitable for irradiation.

Perera et al. previously reported that the irradiation of cultivated mushrooms induces a marked increase in the vitamin D_2 content of the mushroom, and that the mushrooms are well absorbed and metabolized in animals [14]. Furthermore, serum vitamin D_2 and calcium levels, and femur bone mineral density were increased in rats fed irradiated oak mushrooms [14]. In the current study, we prepared modified vitamin D_2-enhanced oak mushrooms using UV-B irradiation. The vitamin D_2-content of the modified oak mushrooms was over 45 μg/g, similar to what was previously reported [14], and the calcium concentration was two-times higher than that of unmodified *A. mella*. We then examined the ability of the modified oak mushrooms to prevent or improve osteoporosis-like symptoms in mice that were fed with low calcium and vitamin D_3-deficient diet. We demonstrated that bone density and tibia thickness increased in mice with increased levels of supplementation with calcium and vitamin D_2-enhanced oak mushrooms. However, while both calcium-enhanced oak mushrooms and calcium plus vitamin D_2-enhanced oak mushrooms increased tibia osteoid thickness, bone density in mice that were fed calcium-enhanced oak mushrooms was lower than mice that were fed calcium and vitamin D_2-enhanced oak mushrooms, or a normal diet. These results suggest that dietary supplementation with calcium-enhanced mushrooms, in the absence of vitamin D_2-enhanced mushrooms, is not sufficient to improve osteoporosis-like symptoms in mice. Thus, under the conditions of the current study, vitamin D_2 is an important risk reduction factor for the development of osteoporosis-like symptoms. Our results indicate that vitamin D_2 plus calcium-enhanced oak mushrooms may improve the osteoporosis-like symptoms induced by low calcium and vitamin D_3-deficient diet through the augmentation of vitamin D_2 levels in the body.

While several indices of osteoporosis-like symptoms were improved in mice that were fed a diet supplemented with calcium and vitamin D_2-enhanced oak mushroom, femur length, serum calcium levels, and osteoid thickness in the mice was lower than in mice fed a normal diet. In the current study, while we did not measure serum vitamin D_2 levels in mice that were fed vitamin D_2-enhanced oak mushrooms, the improved bone thickness, femoral density, and serum calcium levels in these mice suggests that increased vitamin D levels due to the consumption of enhanced mushrooms conferred a beneficial effect on osteoporosis-like symptoms in these animals. However, the bioavailability of vitamin D_2 is much lower than the bioavailability of vitamin D_3 [4, 30]. This implies the existence of species-dependent differences in the availability of vitamin D_2 and vitamin D_3 [4]. Therefore, the results of the present study performed in mice may not necessarily apply to human.

In this study, we demonstrated that duodenal *TRPV6*, and renal *TRPV5* and *TRPV6* mRNAs were significantly induced in mice whose diets were supplemented with vitamin D_2 and calcium-enhanced oak mushrooms as compared to the other diet groups. TRPV5 and TRPV6 are calcium channels located in the apical membranes of intestinal and renal epithelial cells, and have been implicated in calcium uptake during trans-cellular calcium transport [8]. They are highly related proteins that are expressed primarily in cells involved in calcium absorption or re-absorption in the duodenum and kidney [29]. TRPV6 is distrib-

uted in the duodenum, jejunum, ileum, and kidney, and in exocrine tissues, such as the pancreas, prostate, mammalian gland, and sweat gland [8, 32, 35]. It has been shown that vitamin D induces the expression of *TRPV5* in rabbit renal epithelial cells, and *TRPV5* expression has been demonstrated in human, rat and mouse kidney [12]. Dietary calcium has also been shown to regulate duodenal and renal *TRPV6* mRNA expression [31]. Our results suggest that vitamin D_2 derived from enhanced mushrooms positively regulates the transcription of *TRPV5* and *TRPV6* to promote the entry of calcium ions into the cytoplasm of calcium absorbing cells.

We also demonstrated that duodenal and renal *CaBP-9k* mRNA levels were up-regulated by vitamin D_2 derived from enhanced oak mushrooms. CaBP-9k is a cytosolic protein that has a high affinity for calcium ions [6, 15]. In mammals, a number of tissues and organs, including the intestine, uterus, placenta, kidney, and bone, express *CaBP-9k* [1, 2, 5, 13, 17, 19, 24, 33, 36, 37]. In rodents, intestinal CaBP-9k is involved in intestinal calcium absorption and is regulated at the transcriptional and post-transcriptional levels by 1,25-$(OH)_2$D [7, 28, 34]. It has also been shown that the expression of duodenal *CaBP-9k* may be linked to 1,25-dihydroxycholecalciferol in humans [33]. Renal *CaBP-9k* is expressed at distal convoluted tubules, which are believed to facilitate calcium re-absorption [25]. Together with the results of *TRPV* gene expression analysis, these results indicate that vitamin D_2 derived from enhanced oak mushrooms is available in a bioactive form in mice. Furthermore, enhanced active calcium re-absorption in the kidney and duodenum due to increased transcriptional activity of the active calcium transport genes also partially explains the increased levels of serum calcium in mice fed the highest content of vitamin D_2 plus calcium-enhanced oak mushrooms.

In summary, the beneficial effect of vitamin D_2 and calcium-enhanced oak mushrooms on osteoporosis-like symptoms was evaluated by femur density and length, bone histology, serum calcium levels, and the mRNA levels of active calcium transport genes. Femur density, tibia thickness, serum calcium levels, and the expression of active calcium transport genes were significantly higher in mice fed with low calcium and vitamin D_3-deficient supplemented with vitamin D_2 plus calcium-enhanced oak mushrooms as compared to mice who did not receive supplementation. These results indicate that vitamin D_2 and/or calcium derived from mushrooms could improve bone mineralization directly, as well as calcium absorption in the duodenum and kidney.

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