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Mushroom Extracts Decrease Bone Resorption and Improve Bone Formation

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ABSTRACT: Mushroom extracts have shown promising effects in the treatment of cancer and various chronic diseases. Osteoporosis is considered one of the most widespread chronic diseases, for which currently available therapies show mixed results. In this research we investigated the *in vitro* effects of water extracts of the culinary-medicinal mushrooms *Trametes versicolor*, *Grifola frondosa*, *Lentinus edodes*, and *Pleurotus ostreatus* on a MC3T3-E1 mouse osteoblast-like cell line, primary rat osteoblasts, and primary rat osteoclasts. In an animal osteoporosis model, rats were ovariectomized and then fed 2 mushroom blends of *G. frondosa* and *L. edodes* for 42 days. Bone loss was monitored using densitometry (dual-energy X-ray absorptiometry) and micro computed tomography. In the concentration test, mushroom extracts showed no toxic effect on MC3T3-E1 cells; a dose of 24 μg/mL showed the most proliferative effect. Mushroom extracts of *T. versicolor*, *G. frondosa*, and *L. edodes* inhibited osteoclast activity, whereas the extract of *L. edodes* increased osteoblast mineralization and the production of osteocalcin, a specific osteoblastic marker. In animals, mushroom extracts did not prevent trabecular bone loss in the long bones. However, we show for the first time that the treatment with a combination of extracts from *L. edodes* and *G. frondosa* significantly reduced trabecular bone loss at the lumbar spine. Inhibitory properties of extracts from *L. edodes* on osteoclasts and the promotion of osteoblasts *in vitro*, together with the potential to decrease lumbar spine bone loss in an animal osteoporosis model, indicate that medicinal mushroom extracts can be considered as a preventive treatment and/or a supplement to pharmacotherapy to enhance its effectiveness and ameliorate its harmful side effects.

KEY WORDS: biological drug, bone formation, bone resorption, *Grifola frondosa*, *Lentinus edodes*, mushroom β-glucan dietary supplement, osteogenic, postmenopausal osteoporosis

ABBREVIATIONS: **ALP**, alkaline phosphatase; **BMD**, bone mineral density; **BV/TV**, bone volume–to–tissue volume ratio; **CT**, computed tomography; **diH_2O**, deionized water; **DXA**, dual-energy x-ray absorptiometry; **FBS**, fetal bovine serum; **GF**, *Grifola frondosa*; **LE**, *Lentinus edodes*; **OVX**, ovariectomy; **PBS**, phosphate-buffered saline; **PO**, *Pleurotus ostreatus*; **TRAP**, tartrate-resistant acid phosphatase; **TV**, *Trametes versicolor*

I. INTRODUCTION

Chronic diseases such as heart diseases, stroke, various cancers, and diabetes have become the leading cause of mortality in the world.[1,2] Chronic disease is defined as a condition that persists for more than 3 months and results in lifelong disability, thus decreasing quality of life.[3] For thousands of years mushrooms have been valued both as food and medicine because they contain few calories, little fat, and large amounts of protein, vitamins, minerals, and fibers, as well as high-molecular-weight polysaccharides, polysaccharopeptides, and low-molecular-weight secondary metabolites that not only act as strong immunomodulators but are also capable of interfering with various cellular signalling pathways linked to cancer development and prevention. Taxa such as *Cordyceps* spp., *Fomes fomentarius*, *Fomitopsis officinalis*, *Ganoderma lucidum*, *Grifola frondosa* (GF), *Lentinus edodes* (LE), and *Piptoporus betulinus* have a long history of use, especially in Asian countries such as China, India, Japan, and Korea.[4,5] During the past 30 years there has been a renewed interest in studying their medicinal properties.[6] Certain mushroom extracts have shown promising effects in the treatment of cancers, diabetes, cardiovascular diseases,

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and various other diseases through their antiviral, antibacterial, anti-inflammatory, neuroprotective, and hepatoprotective effects.[7]

Osteoporosis is regarded as a major public health problem. Worldwide, 1 in 3 women and 1 in 5 men over age 50 will experience osteoporotic fractures.[8,9] Becuase of changes in population demographics, the number of men and women with osteoporosis in the European Union is expected to increase from 27.5 million in 2010 to 33.9 million in 2025, corresponding to an increase of 23%.[10] Osteoporosis is characterized by a reduction in bone strength, which leads to an increased risk of fractures, and is defined by a *T*-score of −2.5 or less. Vertebral and hip fractures are the main manifestations. This is a consequence of a disruption in bone remodeling, a lifelong process that consists of bone resorption, which is mediated by osteoclasts, and bone formation, which is mediated by osteoblasts. Risk factors for osteoporosis are estrogen deficiency in postmenopausal women, advanced age, low calcium intake, and inadequate physical activity. Recent evidence indicates that the long-term use of hormone replacement therapy, which is an established therapy for the prevention of postmenopausal bone loss, can lead to an increased risk of certain types of cancers such as breast, ovarian, and endometrial cancers.[11] This has also led to the further investigation of a number of herbs and mushrooms used in traditional Chinese medicine for treating fractures and joint diseases.[12] Among other, isolated 4 sterol-related compounds from the medicinal mushroom *Agrocybe chaxingu*, which suppress osteoclast formation, their structures were identified by spectroscopic analysis. This could indicate that such compounds have potential therapeutic effects in osteoporosis.[13] Recent *in vitro* studies have used cultured bone cells, pharmacologically relevant concentrations (<30 μg/mL) of water extracts. This was established by measuring mineralization and alkaline phosphatase (ALP) activity, which are used as indicators of osteoblastic cell maturation, to confirm the osteogenicity (i.e., bone-enhancing effects) of well-known medicinal mushrooms such as LE and GF.[14,15]

Our study aims to evaluate the osteogenic properties of several well-known medicinal mushrooms *in vitro*, as well as to investigate *in vivo* the bone-maintaining properties from the most promising candidates. For this purpose, we tested 4 mushroom water extracts of *Trametes versicolor* (TV), GF, LE, and *Pleurotus ostreatus* (PO) *in vitro* on the MC3T3-E1 mouse osteoblast-like cell line, primary rat osteoblasts, and primary rat osteoclasts. Based on the findings obtained, we chose 2 proprietary extract blends of GF and LE for further *in vivo* testing to assess the bone-maintaining properties of these extracts under conditions of bone loss in ovariectomized rats.

II. MATERIALS AND METHODS

A. Mushroom Extract Preparation

We collected TV and GF from the wild (in the woods near Zagreb). PO was obtained from a local mushroom cultivator (OPG Vrban, Zagreb, Croatia), and LE was bought from a Chinese cultivator (Baixing Food Co.). Mushroom species of Croatian origin were dried in Dr Myko San's facilities, and LE was dried by the Chinese manufacturer. Single mushroom preparations of TV, GF, LE, and PO were prepared for this study. Generally, dried sporophores were extracted in boiling water for 24 hours using a special approach based on the modified traditional method of making medicinal decoctions in Far Eastern countries, avoiding any denaturation, homogenization, or fermentation procedures. The suspensions obtained were forced through a filter press to remove insoluble matter. Subseqeuent steps were ethanol precipitation and spray-drying to obtain powdered mushroom extracts.

Test mushroom extracts from LE and GF, LE/GF and GF/LE, were blends of the 2 medicinal mushrooms in different ratios. Mushroom extracts were dissolved in pure water in a concentration of 1.5 g/kg animal weight. For each animal, 1.5 mL of water was used to prepare a 2-mL extract suspension. Since the extracts do not fully dissolve in water, a magnetic stirrer was used to prevent precipitation of the extracts.

B. Cell Culture Materials

The *in vitro* studies used AlphaMEM (Sigma), fetal bovine serum (FBS; Sigma), antibiotic/antimicotic (Sigma), phosphate-buffered saline (PBS; Invitrogen), cell culture–treated plates (with 12, 24, 48, and 96 wells), receptor activator of nuclear factor-κB ligand (R&D Systems), macrophage colony-stimulating factor (R&D Systems), ascorbic acid (Sigma), β-glicerophosphate (Sigma), MTT tetrazolium dye (Sigma), and a tartrate-resistant acid phosphatase (TRAP) staining kit (387-A; Sigma).

C. Cell Culture

Primary rat osteoblasts and osteoclasts were differentiated from isolated mesenchymal cells from the femurs and tibias of 3-month-old female rats and cultured in AlphaMEM containing 10% FBS and 1% antibiotic/antimicotic at 37°C in a humidified atmosphere (5% carbon dioxide in air). For osteoclast differentiation, receptor activator of nuclear factor-κB ligand (20 ng/mL) and macrophage colony-stimulating factor (20 ng/mL) were added to the medium from day 1. For osteoblast differentiation, β-glicerophosphate (10 mmol/L) and ascorbic acid (50 μg/mL) were added to the medium on day 6 until termination of the culture on day 21. The medium was changed every 2 days. Osteoclasts were treated with mushroom extracts (25 μg/mL) on day 5 for 72 hours. Osteoblast treatment with mushroom extracts (25 μg/mL) started on day 7, and fresh extracts were added to the medium every 2 days.

D. RNA Isolation and Real-Time Polymerase Chain Reaction

Total RNA was isolated from primary cells at the end of the treatment period using TriReagent (Ambion) according to the manufacturer's instructions. Complementary DNA was generated by reverse transcription of 1 μg adjusted RNA using the SuperScript III First-Strand Synthesis System (Invitrogen), as indicated by the manufacturer's instructions. Expression of the genes of interest was measured using a SYBR Premix Ex Taq II (Takara) in a LightCycler instrument (Roche Diagnostics). Results are represented as the fold change of the control expression level. Osteocalcin was used as an osteoblastic marker (forward primer: AAGCCCAGCGACTCTGAGTCT, reverse primer: CCGGAGTCTATTCACCACCTTACT), whereas *Trap* was used as an osteoclastic marker (forward primer: ACGCCAATGACAAGAGGTTC, reverse primer: AGGTGATCATGGTTTCCAGC). β-Actin (forward primer: GCGCAAGTACTCTGTGTGGA, reverse primer: ACATCTGCTGGAAGGTGGAC) was used as a normalizer gene.

E. MTT Test for Cell Toxicity

MC3T3-E1 cells were plated in a 96-well plate (8×10^3 cells/well) and cultured overnight in AlphaMEM containing 10% FBS and 1% antibiotic/antimicotic at 37°C in a humidified atmosphere of 5% carbon dioxide in air. Next day the medium was changed and the cells were treated for 48 hours with mushroom extract concentrations ranging from 1.8 to 120 μg/mL. Triton-X100 (1%) was used as the positive control. After 48 hours, the medium was changed to pure AlphaMEM, and 10 μL of MTT substrate (tetrazolium dye MTT; 5 mg/mL in PBS) was added to each well for 2 hours. The formazan crystals that formed were dissolved in 40 mmol/L HCl in isopropanol (100 μL per well) and the signal was read at 562 nm using a spectrophotometer.

F. von Kossa Staining Protocol

On day 21 of cultivation, the osteoblast cells were fixed for 10 minutes in absolute ethanol and washed once in deionized water (diH_2O). To each well was added 5% silver nitrate, and the cells were incubated for 1 hour under a 100-W lightbulb. The cells were washed in diH_2O and 5% sodium tiosulphate was added to each well for 5 minutes. The cells were then washed again, and nuclear fast red was added for another 5 minutes before the cells were washed and left to dry. Mineralized bone nodules were counted in each well.

G. TRAP Staining

For TRAP staining, the osteoclast cells were cultivated in a 12-well plate. On day 7, the medium was removed and the cells were fixed in 2.5% glutaraldehide (in PBS) for 20 minutes. After fixation, the cells were washed twice with diH_2O and incubated for 1 hour at 37°C in the dark, with 0.5 mL of chromogen added to each well. After incubation, the chromogen was removed and the cells were washed in water and left to dry before mature osteoclasts were counted under a microscope. Only the osteoclasts with 3 or more nuclei were counted.

H. Animal Model of Ovariectomy Induced Bone Loss

Female Sprague-Dawley rats, 16 weeks of age and weighing 300 g, were used in the experiment. Animals were operated on under general anesthesia using an intraperitoneal injection of ketamine (0.8 mL/kg) and diazepam (0.6 mL/kg). A small incision was made on the lower ventral side of the animal, through which the ovaries were clamped and excised. After the surgery, the incision was closed with 3 sutures and disinfected. Sham animals were opened and the ovaries were only touched by a sterile needle. Animals received analgesic therapy for 2 days following the ovariectomy (OVX).

I. Animal Treatment with Mushroom Extracts

After OVX the animals were randomly assigned into 4 test groups: (1) sham (n = 6), (2) OVX (OVX + vehicle; n = 8), (3) LE/GF (OVX + LE/GF extract; n = 8), and (4) GF/LE (OVX + GF/LE extract; n = 8). The animals were allowed to recuperate from the surgery for 2 days, after which the therapy started. The therapy was administered using an oral gavage 6 days/week. The mushroom extract suspension was drawn into a syringe and administered through the probe directly into the animal's stomach. The vehicle or extract suspension (2 mL) was given to animals, whereas sham animals were left untreated. Animals were maintained in standard laboratory conditions with a 12-hour light/12-hour dark cycle and had access to water and chow ad libitum.

On day 42, rats were killed using an intraperitoneal injection of ketamine (1 mL/kg) and an intrapulmonary injection of embutramide (1 mL/kg). Tissues were collected from the animals for the *ex vivo* analyses. Tissues were fixed in freshly prepared 4% buffered formaldehyde (Kemika).

J. Bone Assessment

Bone volume was analyzed by a Hologic QDR 4000 densitometry (dual-energy X-ray absorptiometry [DXA]) device on days 0, 21, and 35 after surgery. The whole-body scan mode was used to scan the rats, with concurrent analysis of bone mineral density (BMD) in the hind limbs and the lumbar spine.

K. Micro Computed Tomography Analyses

Micro computed tomography (CT) analysis was performed using a SkyScan 1076 micro CT device. Bone samples were scanned using 18-μm spatial resolution, an 0.5-mm aluminum filter, and 0.5 degrees of rotational step across 198 degrees; frame averaging was set at a value of 2. Data were reconstructed using NRecon software (SkyScan). Data were analyzed using CTAn software (SkyScan). Trabecular bone was analyzed at the distal femur and lumbar spine (the third lumbar vertebrae body [L3]).

L. Statistical Analysis

Results were displayed as mean ± standard deviation. Statistical analysis was performed using Statistica 10 software (StatSoft). All data were tested for normal distribution, and accordingly, 1-way analysis of variance with the Tukey post hoc test was done. Level of significance was set at $P < 0.05$.

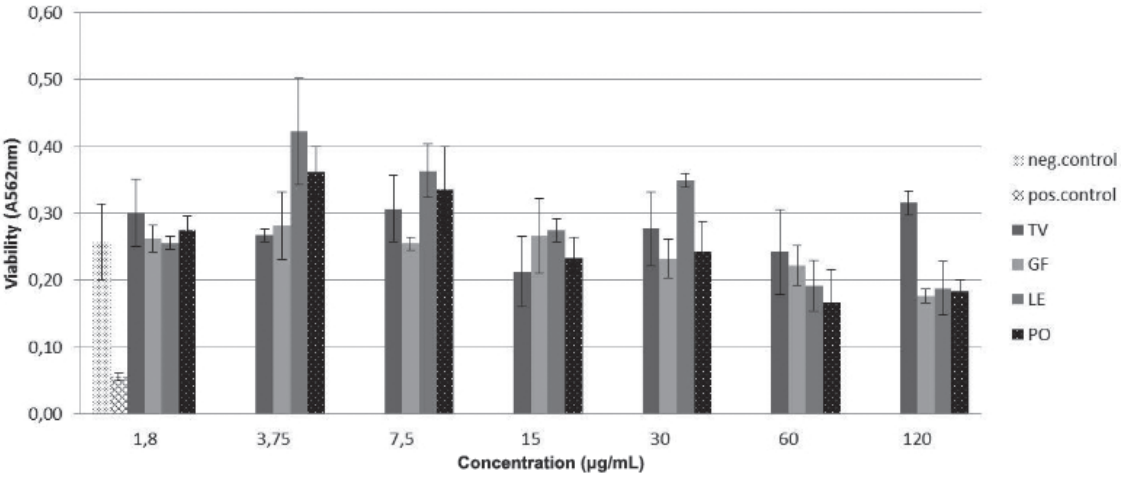

FIG. 1: Mushroom extracts show no toxic effects in the MC3T3-E1 cell line per the MTT assay. GF, *Grifola frondosa*; LE, *Lentinus edodes*; PO, *Pleurotus ostreatus*; TV, *Trametes versicolor*.

III. RESULTS

A. Testing of Mushroom Extracts' Toxicity Effects in MC3T3-E1 Cells

To demonstrate whether the tested extracts were toxic *in vitro*, we treated MC3T3-E1 cells with different doses of mushroom extracts. The MTT test showed that none of the tested concentrations (1.8–120 µg/mL) had toxic effects compared with the positive control (Fig. 1). Lower doses showed a more proliferative effect on cells, so a dose of 24 µg/mL was therefore chosen for further experiments.

B. Mushroom Extracts' Effect on Osteoclast Inhibition

To show the effects of the tested mushroom extracts on osteoclast activation and maturation, we treated primary rat osteoclasts with the chosen dose (24 µg/mL) of each extract for 72 hours. TV, GF, and LE extracts showed statistically significant inhibition of *Trap* expression; the LE extract was the most potent, with 49% lower *Trap* expression compared with the control cells (Fig. 2A). On the contrary, the PO extract showed a significant increase in *Trap* expression (111%). These results were confirmed with TRAP staining, where mature osteoclasts with 3 or more nuclei were counted and compared with the number in control cells (Fig. 2B). Extracts from TV, GF, and LE-treated wells showed 82%, 59%, and 56% of mature osteoclasts, respectively, compared with the control wells, whereas the extract PO wells showed 140%. These data suggest that GF and LE extracts significantly reduced mature osteoclast number and activity.

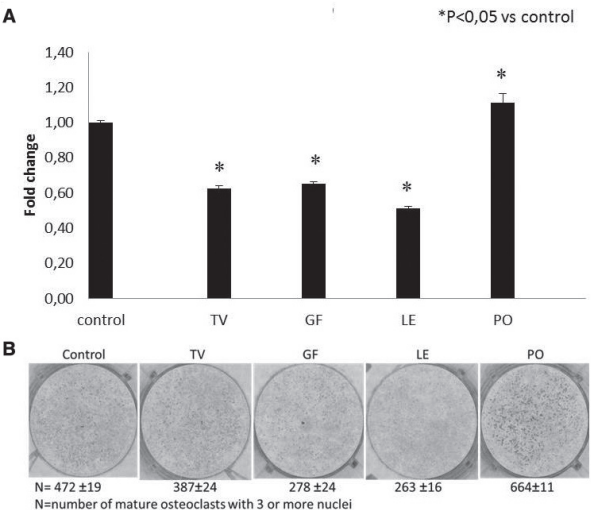

FIG. 2: (A) The effect of mushroom extracts on *Trap* gene expression in primary rat osteoclasts. (B) Tartarate-resistant acid phosphatase (TRAP) staining in primary rat osteoclasts. GF, *Grifola frondosa*; LE, *Lentinus edodes*; PO, *Pleurotus ostreatus*; TV, *Trametes versicolor.*

C. Mushroom Extracts' Effect on Osteoblast Differentiation and Activity

We treated primary rat osteoblasts for 3 weeks with the same concentration of each extract (24 µg/mL) and measured osteocalcin (*Oc*) expression by real-time quantitative reverse transcription polymerase chain reaction. While TV, GF, and PO extracts showed strong and significant inhibition of *Oc* expression, the LE extract significantly increased *Oc* expression in primary osteoblasts (Fig. 3A). These results were also confirmed by von Kossa staining, where the LE extract showed the highest number of mineralized osteoblast colonies compared with the control cells (155%). TV, GF, and PO extracts reduced the number of mineralized colonies (59%, 71%, and 27%, respectively; Fig. 3B). Altogether, these results suggest that the LE extract enhances osteoblast differentiation and activity.

D. BMD Analysis

DXA measurements revealed a BMD loss of 2.5% in rats following OVX. At the femur site, we noticed no significant effect of the mushroom extract therapy on maintaining bone volume (Fig. 4A). At the lumbar spine, there was also no observed effect of mushroom extract therapy on BMD. All groups had statistically significant bone loss on day 35 compared with the sham group (Fig. 4B).

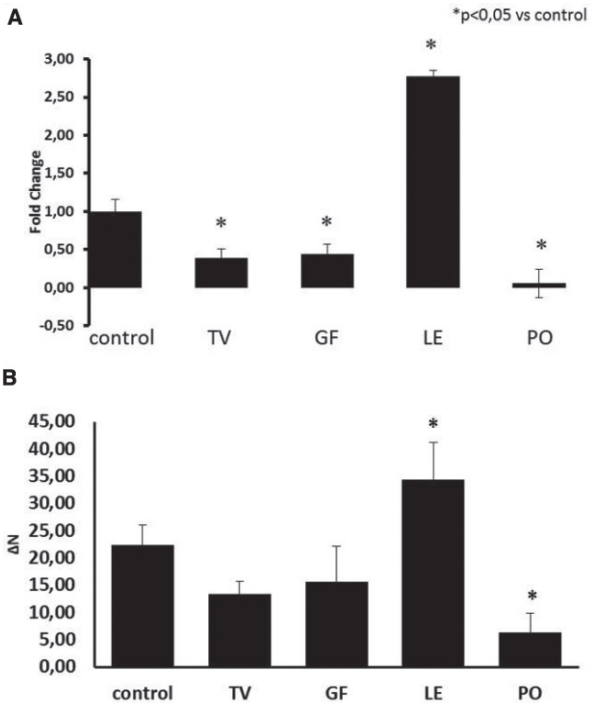

FIG. 3: (A) The effect of mushroom extracts on osteocalcin (OC) gene expression in primary rat osteoblasts. (B) Mineralization of osteoblast (OB) colonies after treatment with mushroom extracts. GF, *Grifola frondosa*; LE, *Lentinus edodes*; PO, *Pleurotus ostreatus*; TV, *Trametes versicolor*.

E. Trabecular Bone Loss at the Distal Femur

OVX-induced bone loss was noticeable in the femoral trabecular bone. All groups showed a significant reduction in bone volume (BV; calculated using the

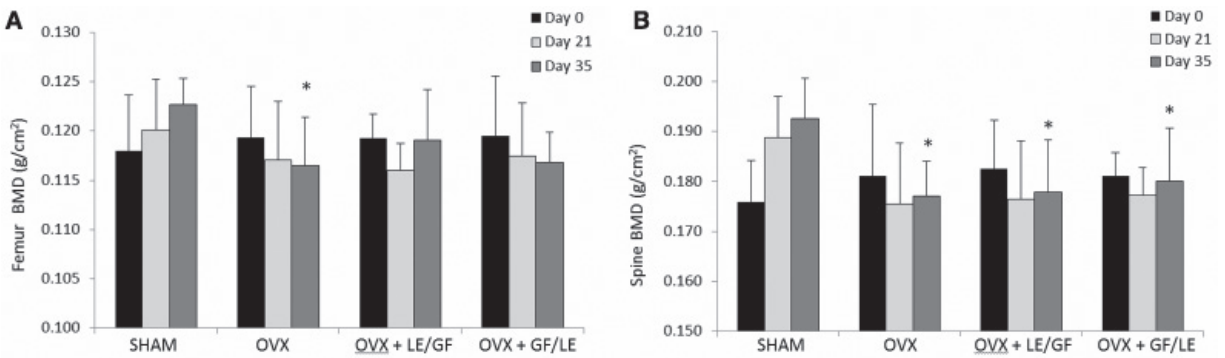

FIG. 4: Bone mineral density (BMD) analyses at the right hind limb (A) and the lumbar spine (B) before ovariectomy (OVX) and on days 21 and 35. *$P < 0.05$ vs. sham. GF, *Grifola frondosa*; LE, *Lentinus edodes*.

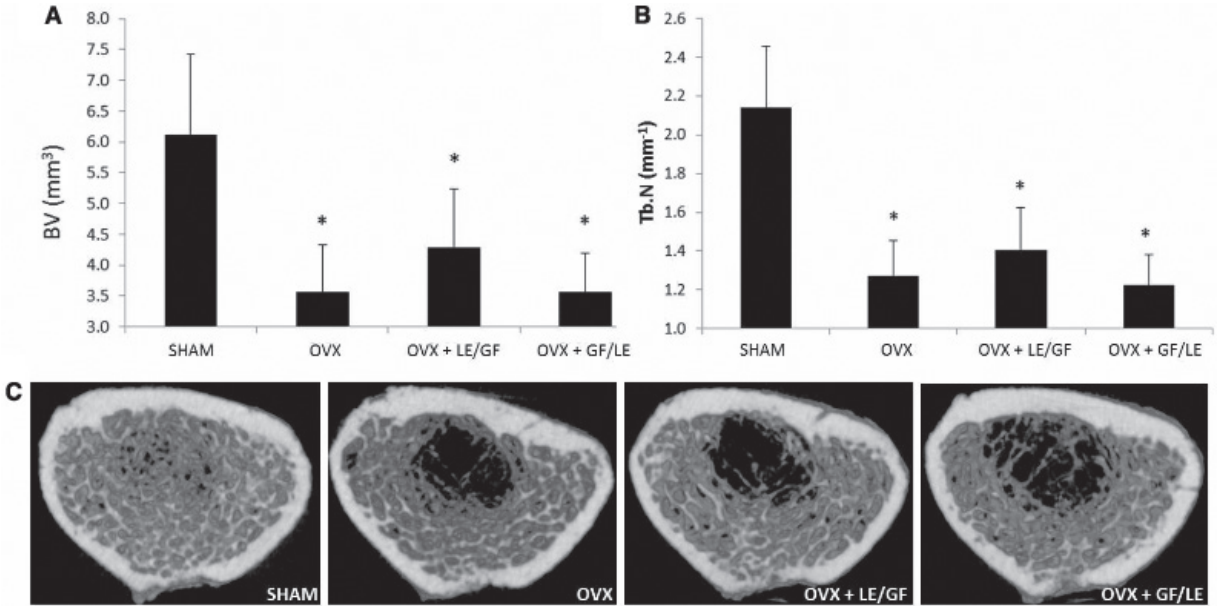

FIG. 5: Micro computed tomography analysis of the trabecular bone at the distal femur. The graphs show bone volume (BV; A) and trabecular number (Tb.N; B) parameters. (C) A 3-dimensional model of trabecular bone. **P* < 0.05 vs. sham. GF, *Grifola frondosa*; LE, *Lentinus edodes*; OVX, ovariectomy.

BV-to-tissue volume [TV] ratio) (Fig. 5A) and the number of trabeculae (Fig. 5B) compared with the sham group. Slightly lower loss of trabecular bone was observed in the group treated with the LE/GF extract, which mirrored the DXA analysis (Fig. 4A), but the statistical significance compared with the OVX group was not achieved, as can be seen on a 3-dimensional model (Fig. 5C).

F. Trabecular Bone Loss at the Lumbar Spine

Surprisingly, the LE/GF extract significantly decreased trabecular bone loss in the vertebral body, where the bone volume (BV-to-TV ratio) (Fig. 6A) and the number of trabeculae (Fig. 6B) were 17% higher compared with the OVX group. Treatment with the GF/LE extract showed a positive trend in decreasing bone loss but did not reach statistical significance compared with the OVX group, as can be seen in a 3-dimensional model (Fig. 6C).

IV. DISCUSSION

Osteoporosis is a disorder characterized by a reduced amount of bone tissue with an increased risk, or likelihood, of bone fracture.[16] A complex hormonal network influences osteoclast and osteoblast activity in maintaining normal bone. Disruption in this homeostasis causes osteoporosis, which is defined by a decrease in bone mass, a decrease in BMD, and changes in microarchitecture.[17] Uncoupled bone remodeling causes increased bone resorption and decreased bone formation, with a net bone loss. Reduced bone mass occurs as a result of improper accumulation of bone tissue during growth and maturation and/or increased bone loss in postmenopausal women and in men in their later years. Osteoporosis is the most common metabolic bone disease and a major cause of morbidity in adults. Histological characteristics of the disorder are thinned bone cortex and a reduced number and size of trabeculae in spongy bone. Treatment of osteoporosis includes prevention, estrogen, bisphosphonates, strontium ranelate, teriparatide, cathepsin K monoclonal antibody, calcium, and cholecalciferol supplementation.[18,19] Each of the therapies showed effectiveness in preventing bone loss and restoring bone mass but had adverse side effects or limited duration of action.

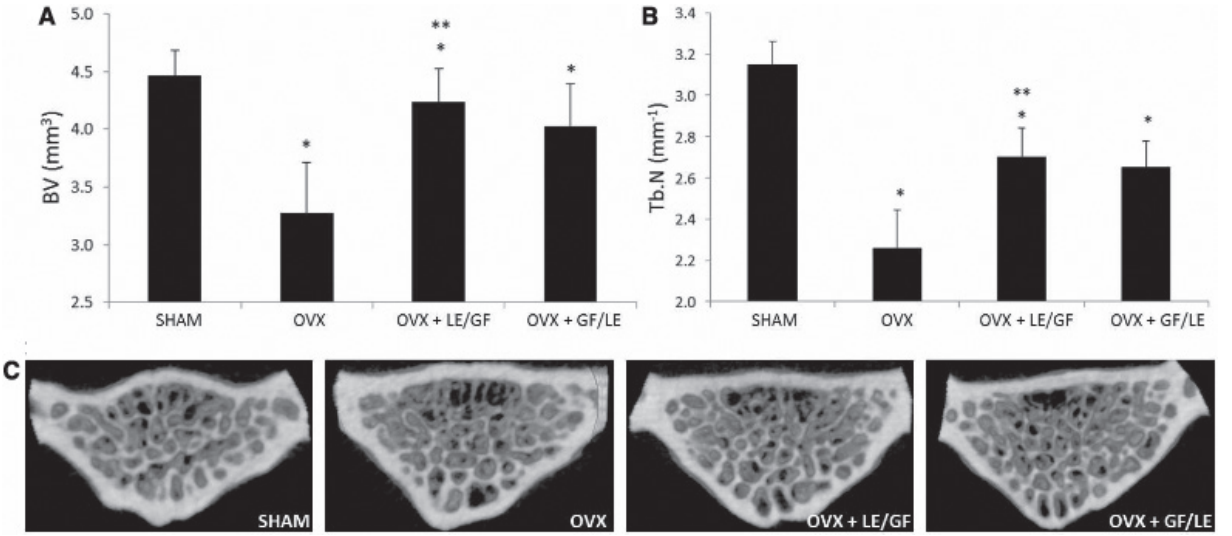

FIG. 6: Micro computed tomography analysis of the trabecular bone at the lumbar spine. The graphs show bone volume (BV; A) and trabecular number (Tb.N; B) parameters. (C) A 3-dimensional model of trabecular bone. **P* < 0.05 vs. sham; ***P* < 0.05 vs. ovariectomy (OVX). GF, *Grifola frondosa*; LE, *Lentinus edodes.*

The medicinal use of mushrooms has a long tradition, especially in Asian countries. To date, many biologically active compounds have been extracted from mushrooms, one of the most promising of which is the edible medicinal maitake mushroom (GF), which has been shown to have positive effects in treating diabetes and cancer.[15,20,21] Because of the possible relationship between diabetes and bone metabolism, it has also been studied for its possible antiosteoporotic effects *in vitro*, and it has been proved that it has a stimulating effect on human osteoblasts.[14] Another pronounced and well-studied edible medicinal mushroom, the shiitake (LE), which has strong antitumor, antiviral, and immunomodulating properties, has also been proven as a bone-inducing agent.[15] In this *in vitro* study the LE water extract showed a significant elevation of ALP activity and a 2-fold level of mineralization of human osteoblastic cell cultures; therefore we conclude that it deserves attention as a supportive dietary treatment in the case of diseases such as osteoporosis, osteopenia, and late complications of diabetes.

Since both *in vitro* studies demonstrated that GF and LE water extracts enhance ALP activity and bone mineralization at low doses, these effects cannot be attributed to the calcium content in these mushrooms, which is relatively low. The potentially existing compounds stimulating bone formation and bone mineralization, which remain to be identified, are probably from the broad group of mushroom polysaccharides and especially β-glucans. This hypothesis was actually confirmed by *in vitro* research that showed that polysaccharides ultrasonically extracted from the turkey tile medicinal mushroom (TV) affect osteoblast cell growth in a dose-dependent manner by stimulating ALP activity.[22] Another study confirmed that water extract of *Pleurotus citrinopileatus* also contains an osteoclast differentiation inhibitor, which was identified as a β-glucan polysaccharide.[23] On the other hand, different *in vitro* research has tried to elucidate the mechanisms by which some medicinal mushrooms could decrease bone resorption by osteoclasts and to identify the active compounds involved. The edible medicinal mushroom *Grifola gargal* contains 7 sterol compounds, called gargalols; 5 of them suppressed osteoclast formation without toxicity.[24] Two of 4 compounds isolated from the edible medicinal mushroom *Agrocybe chaxingu* also suppress the formation of osteoclasts.[25] Subsequent

research identified 4 additional substances that suppress osteoclast formation from the same mushroom species, so the authors concluded that this edible mushroom could be a promising functional food for postmenopausal women and aged people to improve and/or prevent osteoporosis.[13] Two sterols isolated from the edible medicinal mushroom *Leccinum extremiorientale* also showed inhibitory activity on osteoclast formation.[26]

Here we tested 4 different mushroom water extracts *in vitro* using primary rat bone cultures. We have shown that these extracts are not toxic to the cells, even in high doses. For 3 of the 4 tested extracts—TV, GF, and LE—we have shown a significant inhibition of primary osteoclast formation and maturation, proving the extracts' antiresorptive effects. However, TV and GF extracts inhibited osteoblast activity and maturation. What is interesting is that the LE extract showed simultaneous osteoblast-stimulating and osteoclast-inhibiting effects at a dose of only 24 µg/mL. To confirm these results, we have chosen to test the GF and LE extracts *in vivo*.

In our *in vivo* research we showed for the first time that a medicinal mushroom extract can have a beneficial effect on trabecular bone in the lumbar spine in an animal model of osteoporosis.

The first line of bone health defense—before any pharmacotherapy—should be prevention with nutrition modification. On the other hand, the wide use of calcium supplementation has been questioned because of growing evidence of harmful cardiovascular and other side effects,[27,28] which increases the importance of using foods naturally rich in vitamin D and calcium. Edible mushroom species are useful for the prevention of osteoporotic bone fractures through nutrition because of their inhibitory activities on bone resorption. One study confirmed that 25 of 54 food ingredients tested had an inhibitory effect on bone resorption in rats, but it seems that the minimal inhibitory dose is 6.2 g fresh active items/kg body weight daily, divided among 5 servings.[29]

The application of edible medicinal mushrooms rich in vitamin D and calcium as foods, dietary supplements, and potential drugs has even greater importance in view of the fact that vitamin D and calcium deficiencies are connected with an increased risk of cancer. A number of animal and human studies have confirmed this correlation, which led to a conclusion that vitamin D and calcium have chemopreventive effects against breast and other human cancers. A broad spectrum of immunomodulatory and cytotoxic/cytostatic activities of different mushroom compounds inhibits and/or potentially reverses primary and metastatic tumor growth, which was detected in early research.[30] This inhibitory activity of certain mushroom species on the process of metastasis (including bone metastases) is confirmed through years of experience accumulated by our research group through treating human cancers with medicinal mushroom extracts, as well as in recent studies.[31–33]

In bone biology thus far, *in vitro* research of mushroom extracts has predominantly been performed,[14,15] and the effects in animals are not well known. To address this question we developed a rat osteoporosis model by performing OVX in mature females. The medicinal mushroom extracts were orally administered daily for the duration of the experiment. We showed that BMD, measured at the femur and lumbar spine, steadily declined in all groups that underwent OVX, as opposed to the sham group, in which animals gained bone mass over the course of 42 days. Detailed micro CT analysis revealed significant loss of trabecular bone at the distal femur after OVX when compared with the sham animals, matching results from previous studies[34,35]; treatment with mushroom extracts had no influence on preventing the bone loss at this site. Lumbar vertebral trabecular bone loss was also significant in the operated groups compared with the sham animals, but at this site the therapy with mushroom extract LE/GF significantly decreased bone loss when compared with the OVX group. Discrepancy between the femur and lumbar spine has been previously reported in patients with osteoporosis[36] and astronauts during long-duration spaceflight,[37] showing the different dynamics of bone loss at the 2 sites.

Reducing bone loss at the lumbar spine indicates that the appropriate dose and correct proportion of medicinal mushroom extracts can influence bone

mass in OVX-induced osteoporosis and can be considered as a supplement to pharmacotherapy to enhance its effectiveness and ameliorate harmful side effects.

IV. CONCLUSIONS AND FUTURE PERSPECTIVES

The results of our research confirmed the significant effects of medicinal mushroom extracts on bone resorption and bone formation *in vitro* and *in vivo*, which indicates the potential benefits of some medicinal mushroom extracts for the prevention of osteopenia and osteoporosis, as well as their clear osteogenic effects for therapeutic delay and alleviation of osteoporosis. Although of relatively short duration, *in vivo* research (6 weeks) displayed the significant influence of the tested mushroom extracts on bone remodeling; it could still be useful to perform additional research on the potential mid-term and long-term effects of these extracts (i.e., with a duration of 3, 6, and/or 12 months). Since all mammals share the same basic physiological mechanisms of bone remodeling, the results obtained from this rat animal model have sufficient allometric significance for establishing a strong correlate to humans. Despite this, future human clinical studies will be welcome.

The results of our research are especially promising because edible medicinal mushrooms are natural products, meaning that during long-term use the human organism is relatively better adapted to these natural substances compared with artificial/synthetic ones. Tested mushroom species are highly safe and harmless, which has been verified through millennial experiences in nutrition and traditional medicine(s) as well as in modern scientific research during the last 50 years. After all, this source of healthy food and natural medicine is (or at least can be) relatively available to a broader population.

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CONFLICT OF INTEREST

To avoid any doubt in objectivity, the coauthors affiliated with the Dr Myko San Company were not directly involved in any *in vitro* and *in vivo* measurements and have not influenced the results in any way.

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