Osteoporosis Prevention by Adlay (薏苡 Yì Yǐ: The Seeds of Coix Lachryma-Jobi L. var. ma-yuen Stapf) in a Mouse Model

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ABSTRACT

Osteoporosis is characterized by reduced bone mass and quality due to an imbalanced bone remodeling. A grass crop, adlay (Coix lachryma-jobi), is a kind of nourishing food, which has also been used in traditional Chinese medicine. In this study, we investigated the effect of adlay (C. lachryma-jobi L. var. ma-yuen Stapf) on osteoporosis using an ovariectomized mouse model. The adlay diet (10% and 30% adlay in mouse diet) or water extract of adlay (0.3 g/kg/day) was given to ovariectomized mice for 4 weeks. In some experiments, the primary rat osteoblast cells were used to test the possible mechanism of adlay on osteoporosis. The body weight was slightly increased and uterus weight was markedly decreased in ovariectomized mice, which were not affected by adlay treatment. Adlay diet (30%) and adlay extract feedings significantly reversed the decreased bone alkaline phosphatase activity and calcium contents and bone mineral density in ovariectomized mice. Moreover, adlay extracts increased the osteoblast cell proliferation in a dose-dependent manner. Adlay extracts also increased the protein expressions of proliferating cell nuclear antigen and phosphorylated extracellular signal-regulated kinase (ERK) 1/2 in osteoblast cells. ERK inhibitor PD98059 significantly reversed the increased osteoblast cell proliferation by adlay extracts. Taken together, these findings indicate that adlay effectively alleviates the osteoporotic status in ovariectomized mice. Adlay is capable of increasing the proliferation of osteoblast cells via an ERK-regulated signaling pathway. Adlay may be a helpful healthy food for osteoporosis prevention.

Key words: Adlay, Osteoblast cells, Osteoporosis, Proliferation

INTRODUCTION

Bone remodeling is known as an incorporated interaction between bone resorption and bone formation, which plays an important role in bone homeostasis. Osteoporosis is caused by a dysfunction of endocrine system in bone that results to the inability to maintain the bone homeostasis, leading to a decrease in bone mineral density (BMD), microarchitecture alteration, and eventually skeletal fractures. It has been analyzed that the worldwide annual costs of hip fracture in 1990 were estimated to be about US$ 34.8 billion and are expected to increase over the next 50 years, indicating that osteoporosis is a global public health concern and a great socioeconomic burden. An unbalanced bone remodeling process in which bone resorption is higher than bone formation triggers the decrease in BMD. Osteoporosis might occur
in all populations, although it is prevalent in white postmenopausal women.\cite{31} There are several therapeutic strategies for the treatment of osteoporosis, including the reduction of bone loss and the enhancement of bone mass; however, these anti-resorptive drugs (e.g. bisphosphonates) and anabolic drugs (e.g., parathyroid hormone) have their own limitations and safety issues.\cite{31}

A grass crop, adlay (Coix lachryma-jobi), is a kind of nourishing food, which has also been used in traditional Chinese medicine for a long time, for example, in the treatment for warts, chapped skin, rheumatism, female endocrine system, and neuralgia. The studies in recent decade have shown that adlay possesses the beneficial effects, including suppression of allergic reactions,\cite{32} influence on the growth of intestinal bacteria,\cite{33} inhibition of lymphoma cell proliferation,\cite{34} inhibition of nitric oxide and superoxide production from macrophages,\cite{35} anti-mutagenic activity,\cite{36} anti-proliferative and chemopreventive effects in lung cancer,\cite{37} hypolipidemic effect,\cite{38} anti-obesity effect,\cite{39} and hypoglycemic effect in streptozotocin-induced diabetic rat model.\cite{40} Our previous study has shown that water extracts of adlay seed effectively enhance the bone formation markers and reduce the bone resorption markers in the bone tissue culture models, suggesting that adlay may be a helpful healthy food for osteoporosis prevention.\cite{41} However, the in vivo effect of adlay on the bone system or osteoporosis remains to be clarified. In the present study, we investigated the in vivo effect of adlay (C. lachryma-jobi L. var. ma-yuen Stapf) on osteoporosis using an ovariectomized mouse model. The results showed that adlay effectively alleviates the osteoporotic status in ovariectomized mice.

**MATERIALS AND METHODS**

**Plant materials**

Adlay samples, Taichung Shuenyo No. 4 of C. lachryma-jobi L. var. ma-yuen Stapf, were provided by a farmer (Taichung, Taiwan). The air-dried adlay seeds were dehulled and blended into powder, and then screened through a 20-mesh sieve. The dehulled adlay was added to mouse diet to prepare the adlay diet (10% and 30%). Moreover, the powder of adlay was further extracted as described previously.\cite{41} In brief, after methanolic extraction, the plant materials were continuously extracted with warm water at 50°C for 30 min, and then the plant material was filtered to obtain the warm water extract. The extracts were dried using a freeze-dryer (model SFD-25, Chang Juing Co., Kaohsiung, Taiwan). These extracts of dehulled adlay were stored at −20°C until use.

**Ovariectomized mouse model**

Adult female Imprinting Control Region (ICR) mice (20-25 g) were obtained from Laboratory Animal Center of the College of Medicine, National Taiwan University, Taipei, Taiwan. All animals were fed with standard Purina Rodent Chow 5001 (Labdiet®, Richmond, IN, USA) and distilled water ad libitum. The procedures of the animal study were approved by the Committee of Animal Study in College of Medicine, National Taiwan University (IACUC Approval no.: 20040139). The ovariectomy procedure and sham operation were performed in mice under anesthesia. At 4 weeks after ovariectomy, the mice of adlay treatment groups were fed with diets containing 10% and 30% adlay or adlay water extract (0.3 g/kg/day) for 4 weeks. The other ovariectomized mice and sham control mice were fed with standard diets containing no adlay. At the end of the program, the body weight and BMD were measured and then the mice were sacrificed by decapitation. The weights of the uteri were measured, and the femoral metaphyseal tissues were collected for the measurement of alkaline phosphatase (ALP) and calcium content.

**Cell cultures**

The primary osteoblast cells were isolated from the calvaria of fetal rats (18 days old) as described previously.\cite{42} In brief, the calvaria of fetal rats were dissected by aseptic technique under anesthesia and divided into small pieces. These pieces were treated with 0.1% collagenase solution for 10 min at 37°C. The collagenase digested pieces were filtered through a 70-μm nylon filter. Cells were grown on plastic cell culture dishes in 95% air –5% CO2 with Dulbecco’s modified Eagle’s medium, which was supplemented with 20 mM HEPES and 10% heat-inactivated fetal calf serum, 2 mM glutamine, penicillin (100 U/mL), and streptomycin (100 μg/mL) (pH 7.6). The characteristics of osteoblast cells were confirmed by morphology and ALP expression.

**Cell proliferation assay**

**MTS assay**

Cell proliferation was detected by a nonradioactive cell proliferation assay kit (CellTiter 9696™ AQueous, Promega, Madison, WI, USA), which contained a tetrazolium compound [3,4-(5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS)] and an electron-coupling reagent [phenazine methosulfate (PMS)]. Osteoblast cells (1 × 10^4 cells/mL) were incubated for 24 h with or without test materials, and then added at a concentration of 20 μL/well of combined MTS/PMS solution. Absorbance was measured at 490 nm using an enzyme-linked immunosorbent assay (ELISA) microplate reader.

**Proliferating cell nuclear antigen (PCNA) protein expression**

PCNA is a marker for cellular proliferation.\cite{43} Cells were treated with or without test materials for 24 h and analyzed by Western blotting with antibody specific for PCNA.

**Measurement of ALP activity**

The ALP activity in metaphyseal tissues was measured with a color reaction assay. The tissues were homogenized and centrifuged at 600 × g for 5 min, and then the supernatants were used for activity assay. After incubation with p-nitrophenylphosphate at 37°C for 10-30 min, the reaction was stopped with 0.1 N NaOH. The absorbance was read at 405 nm. Each value was normalized to the protein concentration.

**Measurement of calcium content**

The metaphyseal tissues were dissolved in nitric acid overnight, followed by 100 times dilution with distilled water. The calcium levels were determined by Raichem® colorimetric assay (Hemagen Diagnostics, San Diego, CA, USA).
BMD measurement

The BMD of anesthetized mice was measured with a dual-energy X-ray absorptiometer (DEXA, Norland Corporation, Fort Atkinson, WI, USA) using a mode adapted to the measurement of small subject.

Western blot analysis

The samples of cell lysates or nuclear extracts (30-50 µg) were subjected to electrophoresis on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels, and then electroblotted on poly-(vinylidene difluoride) membranes. After blocking, the blots were incubated with antibodies for PCNA (BD-Transduction Laboratories, NJ, USA), extracellular signal-regulated kinase (ERK) 1/2 and phosphorylated ERK, and C23 (Santa Cruz Biotechnology, Dallas, TX, USA) in phosphate-buffered saline within 0.1% Tween 20 for 1 h, followed by two 15-min washes. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies for 30 min. Enhanced chemiluminescence reagents (Amersham, Piscataway, NJ, USA) were employed to depict the protein bands on membranes.

Statistical analysis

The data are presented as means ± standard error. All analyses were performed by analysis of variance followed by a Fisher’s least significant difference test. A *P < 0.05 was considered statistically significant.

RESULTS

In vivo effects of adlay diet and adlay extract feedings on osteoporosis in an ovariectomized mouse model

The adlay diet (10% and 30% adlay in mouse diet) or water extract of adlay (0.3 g/kg/day) was given to ovariectomized mice for 4 weeks. As shown in Figure 1, the body weight was slightly increased and uterus weight was markedly decreased (*P < 0.05) in ovariectomized mice as compared with sham control mice. Both adlay diet and extract treatments did not affect these changes in ovariectomized mice. Moreover, decreases in bone ALP activity and calcium content [Figure 2] and BMD [Figure 3] were also observed in ovariectomized mice as compared with sham control mice (*P < 0.05). Both adlay diet (30%) and adlay extract feedings significantly reversed the decreased bone ALP activity and calcium content [Figure 2] and bone BMD [Figure 3] in ovariectomized mice (*P < 0.05).

In vitro effects of adlay extracts on the osteoblast cell proliferation

Next, we tried to investigate the possible mechanism of adlay on osteoporosis prevention using the primary rat osteoblast cells. As shown in Figure 4a, adlay extracts (0.1-1 µg/mL) significantly increased the osteoblast cell proliferation in a dose-dependent manner (*P < 0.05). Moreover, adlay extracts could also increase the protein expressions of PCNA (a marker for cellular proliferation) and phosphorylated ERK1/2 in osteoblast cells [Figure 4b]. To clarify the role of ERK1/2 in adlay extract-increased osteoblast cell proliferation, the ERK inhibitor PD98059 was used. As shown in Figure 5, PD98059 (10 µM) significantly reversed the increased osteoblast cell proliferation by adlay extracts (*P < 0.05).
In this study, the ovariectomized mice, an animal model of osteoporosis, were used to investigate the effect of adlay on bone metabolism. We found that adlay extract significantly increased the proliferation of osteoblasts and reduced bone loss in ovariectomized mice. This effect was mediated through the activation of ERK1/2 signaling, as demonstrated by increased phosphorylation of ERK1/2 and PCNA expression in cultured rat osteoblasts. ERK1/2 inhibitors, such as PD98059, significantly reversed the effect of adlay extract on osteoblast proliferation and bone formation.

**DISCUSSION**

The present study shows that adlay diet and adlay water extract feedings effectively alleviated the osteoporotic status in ovariectomized mice. Water extract of adlay seed significantly increased the osteoblast cell proliferation in vitro. We further found that ERK signaling is involved in the adlay water extract-increased osteoblast cell proliferation. These findings suggest that adlay is capable of alleviating the estrogen deficiency-induced osteoporosis in vivo through the stimulation of osteoblast-mediated bone formation.

Regarding adlay supplementation, previous studies have demonstrated that adlay has protective effects on bone metabolism. It has been shown to increase the osteoblast cell proliferation in vitro and in vivo. Adlay may be a helpful healthy food for osteoporosis prevention.

In conclusion, the present findings demonstrated for the first time that adlay possesses protective effect on altered bone metabolism in an ovariectomized mouse model. Adlay is capable of increasing the proliferation of osteoblast cells via an ERK-regulated signaling pathway. Adlay may be a helpful healthy food for osteoporosis prevention.
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REFERENCES

7. Seo WG, Pae HO, Chai KY, Yun YG, Kwon TH, Chung HT. Inhibitory effects of methanol extract of seeds of Job’s tears (Coix lachryma-jobi L. var. ma-yuen) on nitric oxide and superoxide production in RAW 264.7 macrophages. Immunopharmacol Immunotoxicol 2000;22:545-54.