



Anti-osteoporosis activity of red yeast rice extract on ovariectomy-induced bone loss in rats

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ABSTRACT. Osteoporosis is the most common bone disease, affecting millions of people worldwide and leading to significant morbidity and high costs. Monacolin K, an extract of red yeast rice (RZR, *Hongqu*), plays important roles in the management of dyslipidemia, coronary heart disease, and diabetes. Our study aimed to investigate the protective effect of monacolin K on ovariectomy-induced bone loss in rats. Fifty female Sprague-Dawley rats were randomly divided into a sham-operated and five ovariectomized (OVX) groups: OVX with vehicle, OVX with fluvastatin, and OVX with RZR extract of three graded doses. Bone mineral density (BMD), biochemical markers, and cell viability were analyzed by dual

energy X-ray absorptiometry, enzyme-linked immunosorbent assay, and 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays. Gene expression was evaluated by real-time polymerase chain reaction amplification and western blot. Our results showed that administration of RYR extract markedly increased the bone mineral density in OVX rats. Moreover, RYR extract decreased the levels of bone turnover markers, including osteocalcin and tartrate resistant acid phosphatase activity. The MMT assay revealed that RYR extract treatment significantly improved the osteoblast viabilities in a dose-dependent manner ($P < 0.05$). At the molecular level, we further demonstrated that RYR extract enhanced the expression of Bmp2 and Bmp4 both at the mRNA and protein levels. Collectively, these data suggested RYR extract could protect against osteoporosis in ovariectomized rats, most likely through activation of BMP2/4 expression.

Key words: Osteoporosis; Red yeast rice; BMP-2; BMP-4

INTRODUCTION

Osteoporosis is one of the most common bone diseases resulting from low bone mass and structural deterioration of bone tissues (Burge et al., 2007). Nearly half of all women and one third of men will experience an osteoporotic fracture during their lifetime (Zhang et al., 2012). The overall prevalence of osteoporosis in the United Kingdom is approximately 55% among women aged 50 years or older, while in mainland China, the prevalence is 40.1% (Reginster et al., 2013). Fragility fractures resulting from osteoporosis are the main cause of disablement and death of aged women, bring enormous financial burdens, and are becoming a leading public health threat. Therefore, it is critical to explore new and effective drugs for the prevention and treatment of osteoporosis.

Traditional Chinese herbs are a significant source of drugs that serve as potential therapeutic compounds for treatment of disease, including osteoporosis. Red yeast rice (RYR, *Hongqu*) is a traditional Chinese food that is generally fermented and obtained from rice with *Monascus purpureus*, a specific strain of red yeast; it has also been used in traditional medicinal therapy in Asia for centuries (Yang and Mousa, 2012). Monacolin K, extracted from RYR, plays an important role in the management of dyslipidemia, coronary heart disease, diabetes, and osteoporosis (Liu et al., 2006). However, the protective effect of monacolin K on ovariectomy-induced bone loss in rats has not been elucidated. Therefore, in the present study, we aimed to investigate the effects of RYR extract (monacolin K) on preventing osteoporosis and ameliorating bone loss in ovariectomized rats.

MATERIAL AND METHODS

Animals

Female Sprague-Dawley rats were supplied by the Animal Experimental Center of Zhejiang University of Traditional Medicine, Hangzhou, China. Ovariectomy was performed under sterile conditions and general anesthesia with 10-15 mg/kg 2.5% barbital sodium when

the rats were 6 months old. The animals were then divided into six experimental groups, with 10 individuals per group. All the experimental procedures were approved by the Animal Care and Use Committee at Zhejiang University of Traditional Medicine.

Agents and animal treatments

RYR (monacolin K), provided by the Integrated Chinese and Western Medicine Hospital of Zhejiang Province, was identified morphologically and histologically according to a previous published study (Cho et al., 2010). RYR extract was dissolved in 75% ethanol, sonicated for 30 s, and evaporated in a waterbath at 60°C. Solutions were prepared at 0.1, 0.2, and 0.4 g/mL. Rats were treated with different concentrations of RYR extract (1.56, 3.12, or 6.24 g·kg body weight⁻¹·day⁻¹) and fluvastatin (5 mg·kg body weight⁻¹·day⁻¹) by oral gavage, while the rats in the control group were given saline (10 mL·kg body weight⁻¹·day⁻¹) (Wong and Rabie, 2008; Hanayama et al., 2009). Fluvastatin sodium was purchased from Novartis (Lescol; Beijing Novartis Pharma Co., Ltd., Beijing, China).

Bone mineral density (BMD) analysis

The BMD of the right femora was measured by dual energy X-ray absorptiometry (GE Healthcare, Madison, WI, USA). The values were calculated automatically by the Encore 2006 software package (GE Healthcare).

Serum bone biomarkers assay

Tartrate-resistant acid phosphatase (TRAP) activity was determined using the microplate assay method. Serum was incubated for 30 min at 37°C with a substrate solution consisting of 7.6 mM 4-NPP in 100 mM sodium acetate buffer containing 50 mM sodium tartrate (pH 5.5). After incubation, 1 mM NaOH was added to stop the reaction and the absorbance at 405 nm was monitored to detect product formation. Serum osteocalcin, a bone turnover marker, was measured with an enzyme-linked immunosorbent assay (Biomedical Technology; Staughton, IN, USA).

Cell culture

Rat calvariae were removed, washed with sterile phosphate-buffered saline (PBS) solution, and digested in 0.2% collagenase A (Roche, Indianapolis, IN, USA) for 1 h at 37°C. Cells were cultured in 25-cm² flasks in minimum essential medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco) and antibiotics (100 U/mL penicillin-streptomycin). Cell culture medium was replaced every three days. When osteoblast cells reached 80% confluence, they were harvested using 0.25% trypsin-ethylene-diaminetetraacetic acid solution.

Cell proliferation assay

Cell proliferation was measured by a 3-(4,5-dimethylthiazol)-2,5-diphenylterazolium bromide (MTT) assay. Osteoblast cells were plated on 96-well plates at a density of 1 x 10⁴ cells/well. After 2-day culture, the cells were treated with RYR extract (0, 50, 100, and 150 μM)

for 48 h. The supernatant was then removed and 20 μ L 0.5% MTT (Sigma, St. Louis, MO, USA) was added to each well. The plates were read on an enzyme-linked immunosorbent assay reader (Multiskan EX; Thermo Electron Corporation, Waltham, MA, USA) at a wavelength of 490 nm.

Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted from cells using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). For cDNA synthesis, the template was reverse-transcribed using SuperScript II RNase H-reverse transcriptase and oligo(dT)25 as a primer (Invitrogen, Carlsbad, CA, USA). qPCR was carried out under the following conditions: an initial stage of 95°C for 30 s, then a two-step program of 95°C for 5 s, and 60°C for 31 s over 40 cycles. Amplifications were performed in triplicate. mRNA expression was determined with the $2^{-\Delta\Delta C_t}$ method. The relative target mRNA levels were analyzed with the ABI Prism 7300 software and normalized to that of the internal control Gapdh. Primers for each gene are shown in Table 1.

Table 1. Primers used in real time PCR experiments.

| Gene | Forward primer sequence (5'-3') | Reverse primer sequence (5'-3') |
|-------|---------------------------------|---------------------------------|
| BMP-2 | ACTTTTCTCGTTTGTGGAGC | GAACCCAGGTGTCTCCAAGA |
| BMP-4 | GGGCTTCCACCGAATAAACA | CGCAGGGCTCACATCAAA |
| GAPDH | GGTGCTGAGTATGTTGTGGAGTC | GCTGACAATCTTGAGGGAGTTG |

Western blotting

Protein was extracted from bone tissues with 100 μ L lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Igepal, 0.1% sodium dodecyl sulfate, 10 μ g/mL phenylmethylsulfonyl fluoride, 10 μ g/mL leupeptin, 10 μ g/mL pepstatin, and 10 μ g/mL heat-activated sodium orthovanadate). Cell lysates were harvested and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20, and incubated overnight at 4°C with sheep anti-BMP-2/4 antibody (1:1000) and Gapdh antibody (1:2000). After washing with PBS, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody, and immunoreactive bands were visualized using ECL detection reagents. GAPDH served as an internal control.

Data analysis

For statistical analysis, all the data are reported as means \pm standard deviation and treated for statistical analysis with the SPSS program (SPSS, Chicago, IL, USA). Comparison between groups was made using analysis of variance (ANOVA) and $P < 0.05$ was defined as statistical significance.

RESULTS

Effect of RYR extract on body weights and uterus index

Initial body weights showed no significant differences among the six groups ($P > 0.05$), but the body weight of OVX rats increased rapidly compared to the sham group. How-

ever, the final body weights in RYR extract-treated groups were significantly lower than those of the OVX group ($P < 0.05$) (Figure 1A). The uterus weight was significantly reduced in the OVX group compared to the sham group ($P < 0.05$). Fluvastatin administration prevented the loss of uterine weight compared to the OVX group, and RYR extract treatment produced a similar effect on uterine weight (Figure 1B).

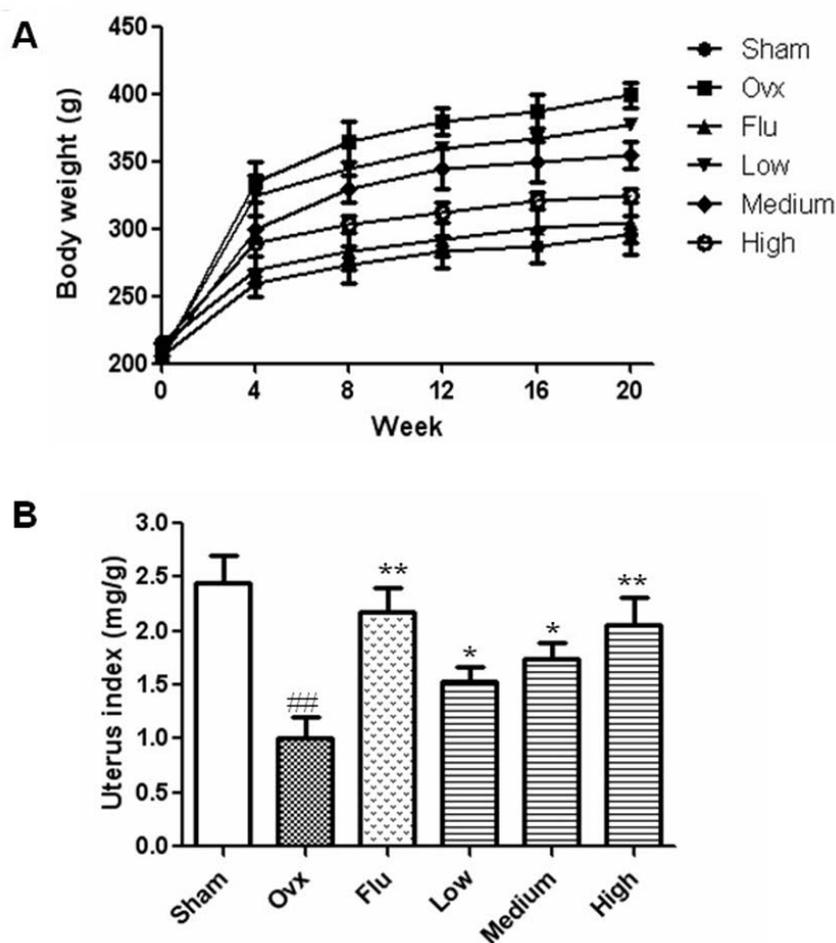


Figure 1. Effect of RYR extract on body weights and uterus index. Effects of RYR extract on (A) the body weight and (B) uterus index of OVX rats. * $P < 0.05$, ** $P < 0.01$ vs the Ovx group; ## $P < 0.01$ vs the sham group.

RYR extract treatment increased BMD

Ovariectomy significantly decreased the right femur BMD compared with the sham group ($P = 0.007$). However, administration of RYR extract significantly increased the right femur BMD compared with the OVX group ($P < 0.05$) (Figure 2). These findings suggested that the RYR extract predominantly protected against ovariectomy-induced bone loss.

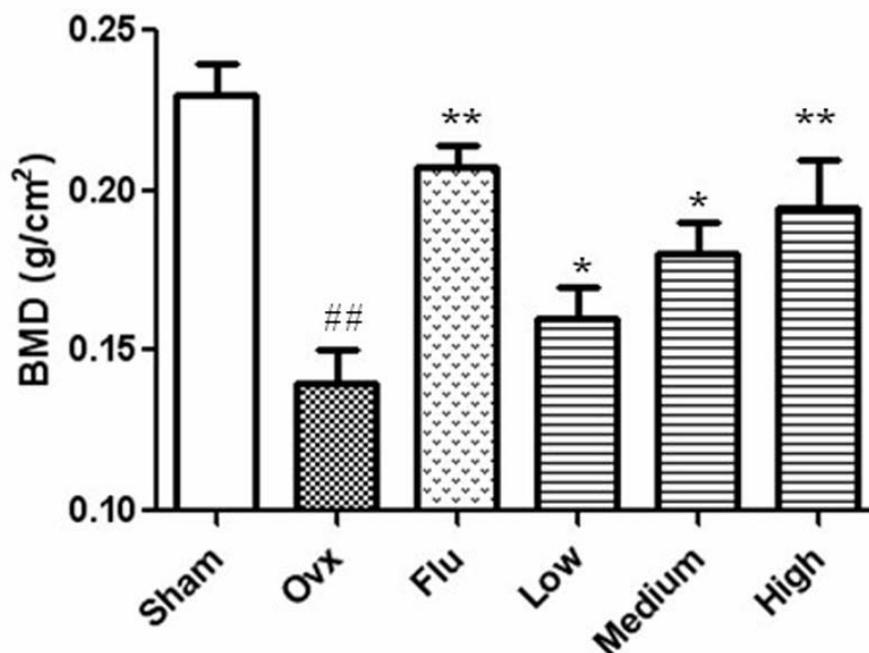


Figure 2. Effects of RYR extract on bone mineral density. Effects of RYR extract on the bone mineral density (BMD) in right femur of OVX rats evaluated by DXA. * $P < 0.05$, ** $P < 0.01$ vs the Ovx group; ## $P < 0.01$ vs the sham group.

Effects of RYR extract on biochemical bone turnover markers

To evaluate the effect of RYR extract on bone turnover in OVX rats, we measured the serum osteocalcin concentration and TRAP activity. We found that the serum osteocalcin concentration in OVX rats was significantly higher compared to the sham group ($P < 0.05$). However, RYR extract significantly reduced the serum osteocalcin concentration ($P < 0.05$) (Figure 3A). The TRAP activity of osteoclasts, an index of bone resorption, was much higher in OVX rats and RYR extract treatment reduced the TRAP activity (Figure 3B). Collectively, these results suggested that RYR extract decreased the bone turnover rate by suppressing osteoclast activity in OVX rats.

RYR extract promoted cell proliferation and activated BMP expression

The MTT assay revealed that RYR extract significantly improved osteoblast viability in a dose-dependent manner ($P < 0.05$) (Figure 4A). To determine the mechanism of RYR extract anti-osteoporosis activity, we examined its effect on the expression of BMP2 and BMP4. Results showed that RYR extract significantly enhanced mRNA (Figure 4B and C) and protein (Figure 4 D-F) expression of BMP2 and BMP4 in primary osteoblasts ($P < 0.05$). Taken together, these results demonstrated that RYR extract could regulate osteoblast proliferation through activation of BMP expression.

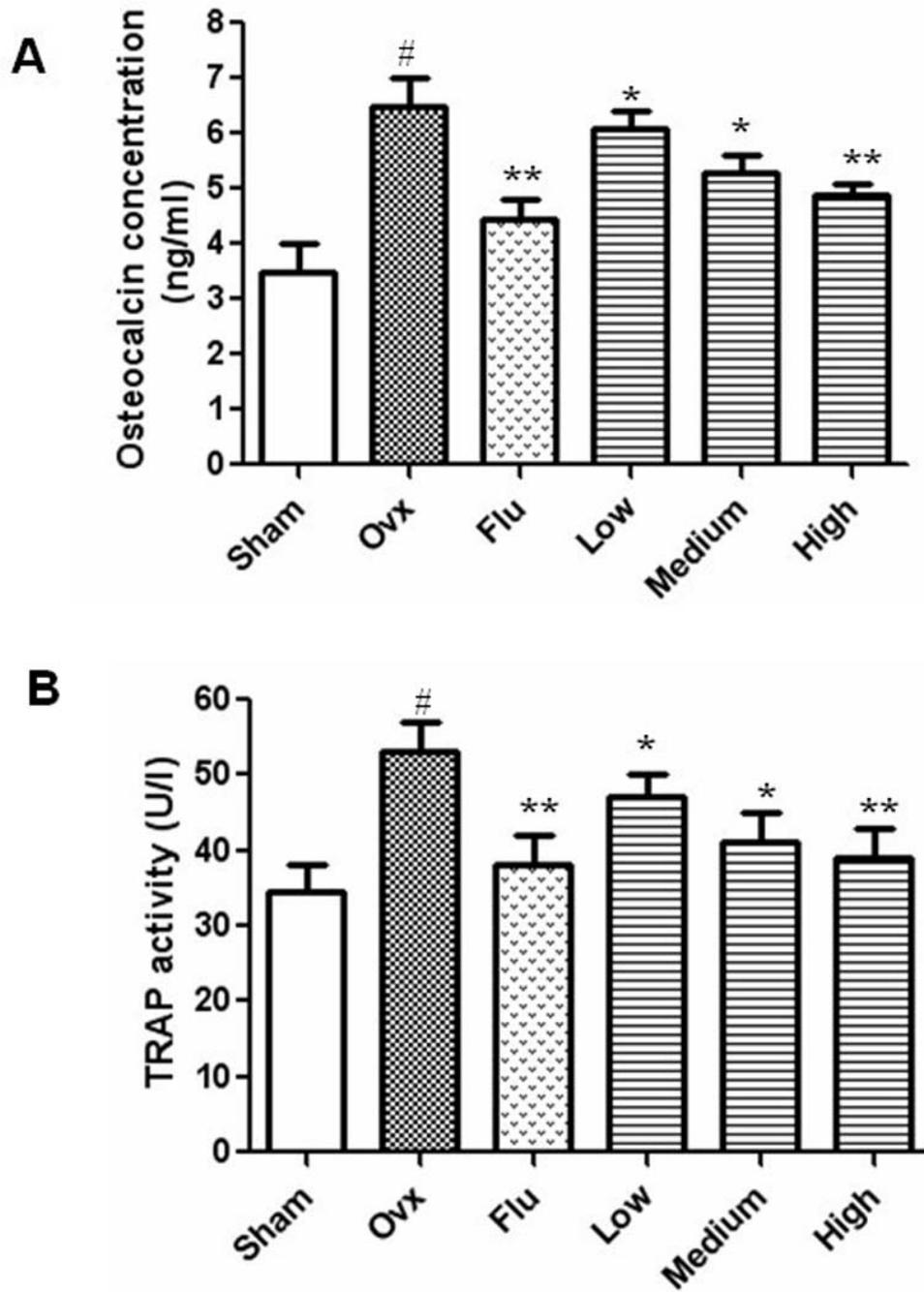


Figure 3. Effects of RYR extract treatment on biochemical bone turnover markers. Serum osteocalcin levels (A) and TRAP activity (B) were measured in sham-operated and ovariectomized (Ovx) rats receiving the indicated doses of RYR extract. * $P < 0.05$, ** $P < 0.01$ vs the Ovx group; # $P < 0.05$ vs the sham group.

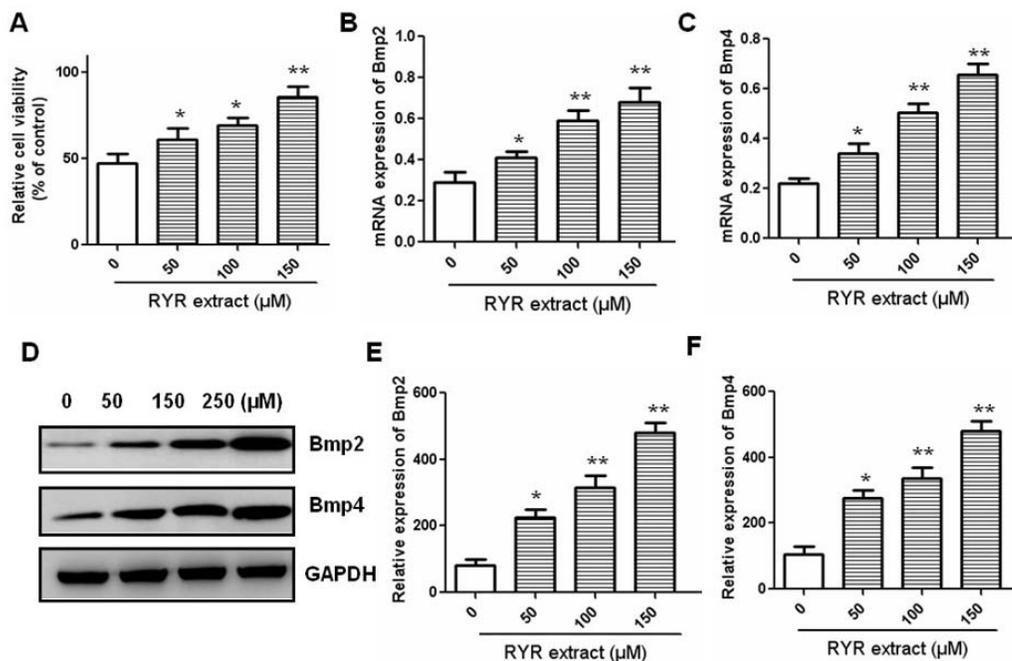


Figure 4. RYR extract promoted cell proliferation and activated BMP Signaling. MTT assay was used to measure the proliferative effect of RYR extract on primary osteoblasts (A). Real-time PCR was performed to measure the mRNA expression of Bmp 2 and Bmp 4 (B and C). Western blot was performed to determine the protein expression (D) and the expression of Bmp 2 (E) and Bmp 4 (F) were measured by relative band intensities. *P < 0.05, **P < 0.01 vs control group.

DISCUSSION

The aim of the present study was to evaluate the protective effects of RYR extract on osteoporosis induced by ovariectomy in rats. Our results showed that RYR extract treatment prevented osteoporosis and ameliorated bone loss in ovariectomized rats, most likely through activation of BMP2/4 expression.

Many studies have shown that the body weights in ovariectomized rats were significantly higher compared to those of sham-operated rats due to fat deposition caused by estrogen deficiency (Iwasa et al., 2011; Li et al., 2011). Increased body weight provides an additional stimulus for bone neoformation, serving as a partial protection against the osteopenia that occurs in long bones (Li et al., 2011). In the present study, the excess body weight gain was prevented by fluvastatin and RYR extract administration. Similar results were also observed on the uterine tissue growth in the OVX rats.

The integrity of the skeleton is maintained through a bone remodeling process that balances bone formation and bone resorption (Bartell et al., 2013; Tamma et al., 2013). Decreased bone mass is a major characteristic of osteoporosis and it is critical to monitor the BMD for the diagnosis and treatment of osteoporosis (Jager et al., 2011). In this study, the RYR extract treatment significantly increased the right femur BMD compared with the OVX group.

It is important to monitor the levels of circulating bone biochemical markers since

they can reveal the status of the bone remodeling process (Maimoun and Sultan, 2011; Biver et al., 2012). These markers include osteocalcin, an osteoblast-specific bone formation marker, and TRAP, an osteoclast-specific bone resorption marker (Habermann et al., 2007; Tang et al., 2011). Our study found that RYR extract decreased the bone turnover rate by suppressing osteoclast activity in OVX rats.

The MTT assay was performed to measure the proliferative effect of RYR extract on primary osteoblasts *in vitro*. We found that RYR extract dose-dependently improved osteoblast viabilities. In addition, we explored the underlying mechanism of the protective effect of RYR extract on osteoporosis. Bone morphogenetic proteins (BMPs) belong to a multigene family of osteoinductive growth factors that are potent stimulators of the proliferation of osteoblasts and chondroblasts as well as promoting fracture repair and bone regeneration (Gysin et al., 2002; Rundle et al., 2003; Haversath et al., 2012). Many BMPs have been identified, among which BMP2 and 4 have been demonstrated to be potent osteotropic factors that could promote bone formation *in vivo* and *in vitro* (Kawai et al., 2006; Kanakaris et al., 2009). Our study showed that RYR extract significantly enhanced the expression of BMP2 and BMP4 in primary osteoblasts ($P < 0.05$).

In conclusion, our study demonstrated, for the first time, that RYR extract plays a protective role on ovariectomy-induced bone loss in rats, most likely through activation of BMP2/4 expression.

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