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Original Research Article

Effect of total flavonoids from *Drynaria* rhizome on bone loss in ovariectomized rats

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Abstract

Purpose: *To determine the potential effect of total flavonoids from Drynaria rhizome on bone loss in ovariectomized (OVX) rats.*
Methods: *The rats were divided into four groups: normal control, ovariectomized (OVX) control, and two Drynaria rhizome (DR) flavonoids treatments. Post-operation, osteoporotic OVX rats were given Drynaria rhizome total flavonoids for 3 months. Thereafter, the expressions of bone-related genes and biochemical indices were investigated in samples taken from rat serum and bone.*
Results: *Treatment with total flavonoids from Drynaria rhizome prevented bone mineral loss and improved some related biochemical indices associated with osteoporosis, namely, alkaline phosphatase (ALP), tartrate-resistant acid phosphatase (TRAP), bone gla protein (BGP) and estradiol (E2). Reverse transcription-polymerase chain reaction (RT-PCR) data showed that treatment with the total flavonoids significantly downregulated mRNA expression of Wnt10b, β-catenin, recombinant human bone morphogenetic protein-2 (BMP2) and BMP4 in OVX rats, but significantly reversed OVX-induced downregulation of dickkopf1 (Dkk1) mRNA expression.*
Conclusion: *These results indicate that total flavonoids from Drynaria rhizome exert anti-osteoporotic effects in rats through the WNT signaling and BMP-2 signaling pathways.*

Keywords: *Drynaria rhizome, Bone loss, Total flavonoids, Ovariectomized rats, BMP-2 signaling pathway*

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INTRODUCTION

Osteoporosis is a concomitant of old age and a source of serious debilitation due to increased incidence of bone breakages, deterioration in life quality or even mortality [1,2]. It is a disease of global concern which affects more than 200 million people yearly [3-5]. Studies have revealed that 30 % of females and 20 % of males above the age of 50 experience bone fractures [5]. Osteoporosis occurs mostly in women who have attained menopause, a condition termed postmenopausal osteoporosis.

Drynaria rhizome is a fern native to China. Its rhizomes are essential components of a popular

Traditional Chinese Medicine for treating osteoporosis and fractures [6]. Previous phytochemical investigations reported the isolation of some triterpenoids and flavonoids from Drynaria rhizome [7]. Recently, it was reported that naringin, one of the key phytochemicals present in Drynaria rhizome, improves bone quality in retinoic acid-induced osteoporosis [8], and in orchidectomized rat models [9]. These findings confirmed that naringin is one of the active principles that account for the osteo-protective effects of Drynaria rhizome. In addition, naringin markedly enhanced proliferation, total protein concentration and ALP activities of rat osteoblastic-like UMR 106 cells [10].

Studies on the phytochemical constituents of Drynaria rhizome have revealed that the predominant compounds are flavonoids, and researchers have focused on these with respect to their anti-osteoporotic properties [11]. The major flavonoids of Drynaria rhizome are neoeriocitrin and naringin [12].

In this study, the anti-osteoporotic effect Drynaria rhizome total flavonoids in rats, and the underlying mechanism, were investigated.

EXPERIMENTAL

Animals

Eight-week-old female Wistar rats weighing 250 – 300 g were stabilized and conditioned to laboratory environment for 7 days prior to commencement of the study. The experiments were conducted in accordance with the guidelines provided by the Animal Care and Welfare Committee [13] and approved by the Institutional Ethics Committee of DaLian Medical University (approval no. 20170628).

Osteoporotic rat model

Following 6 h fast, ovaries were excised from the rats under pentobarbital anesthesia (50 mg/kg *i.p.*). In normal control rats, oophorectomy was not done. At the end of the operation, the rats received gentamicin treatment intramuscularly for 3 days successively. After 16 weeks, the animals were grouped into 4, with 8 rats per group: sham control, oophorectomized control (oophorectomy without treatment); low-dose total flavonoids from Drynaria rhizome treatment (0.3 g/kg); and high dose total flavonoids from Drynaria rhizome treatment (0.5 g/kg) groups. Rats in the last two groups were given Drynaria rhizome total flavonoids for 3 months through the oral route.

Biochemical assays

Bone alkaline phosphatase (BALP) and TRAP were assayed with ELISA kits according to the protocol of the kit manufacturers.

Determination of bone mineral density (BMD)

Bone mineral density was assessed prior to operation (baseline) and post-treatment through femur bone scan with dual-energy X-ray absorptiometry (GE Lunar Prodigy, Chicago) at 1 mm/sec and 0.5 × 0.5 mm resolution. The mean of 3 repeated measurements was used in order to eliminate repositioning errors. Bone mineral density (BMD) was calculated and presented in mg/cm^2.

Determination of bone gla protein (BGP), estradiol (E2) and OPG

Serum estradiol, bone gla protein and OPG were determined with their respective ELISA kits (WuHan HuaMei Company, China), in line with the protocols specified in the kit manuals.

RT-PCR

Bone cell total RNA extraction was done with TRIzol reagent (Invitrogen, USA), and 2 µg of the RNA extract was subjected to reverse-transcription to cDNA with RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA). Polymerase chain reaction was carried out using ABI 7300 RealTime PCR System. The sequences of the primers used are shown in Table 1. The PCR conditions were: initial denaturation at 95 °C for 10 min, then 40 cycles at 95 °C for 15 sec, and at 60 °C for 30 sec. Gene expressions relative to β-actin, were computed with the $2^{-\Delta\Delta Ct}$ procedure.

Table 1: Primer sequence of the genes

Wnt10b: Sense: 5′-GTAATCACGACATGGACTTTGGAG-3′, Antisense: 5′-GCACTTCCGCTTCAGGTTTT-3′
β-catenin: Sense: 5′-ATGCGGCTGCTGTTCTATTC-3′ Antisense: 5′-ACCAATGTCCAGTCCGAGAT-3′
BMP2: Sense: 5′-GGATTAGCAGGTCTTTGCACCA-3′, Antisense: 5′-GCTTGACGC TTTTCTCGTTTG-3′
BMP4: Sense: 5′-ACAATGTGACACGGTGGGAAAC-3′ Antisense: 5′-TGTGGGTGATGCTTGGGACTAC-3′
Dkk1: Sense: 5′-GCTCTGTCTGCCTCCGATCA-3 Antisense: 5′-GCCTTTCCTCCTGTGCTTGG-3′
GAPDH: Sense: 5′-AAGATGGTGAAGGTCGGTGT-3′ Antisense: 5′-CTTGCCGTGGTAGAGTCAT-3′

Statistical analysis

The results are presented as mean ± SEM. Statistical analysis was carried out with SPSS version 17.0. Groups were compared using one-way ANOVA with post hoc LSD test. Values of $p < 0.05$ were taken as indicative of statistical significance.

RESULTS

Serum ALP and TRAP

Oophorectomy led to significant increases in serum levels of ALP and TRAP, when compared with normal control ($p < 0.001$). However, the administration of total flavonoids from Drynaria rhizome (0.3 and 0.5 g/kg) dose-dependently and significantly decreased the ALP and TRAP, when compared to untreated OVX rats (Table 2).

Table 2: Effect of total flavonoids from Drynaria rhizome on ALP and TRAP levels (U/L)

Group	ALP	TRAP
Normal control	30.718±2.314	2.739±0.174
OVX control	49.619±2.715 **	4.528±0.228 *
OVX+DR1	40.628±3.061 #	3.914±0.184 ##
OVX+DR2	33.281±2.152 ##	3.051±0.263 ##

OVX+DR1: OVX + DR flavonoids (0.3 g/kg);
OVX+DR2: OVX + DR flavonoids (0.5 g/kg); *$P < 0.05$, relative to normal control; #$p < 0.05$, ##$p < 0.01$, relative to OVX control

Effect of Drynaria rhizome total flavonoids on BMD of OVX rats

Table 3 shows that oophorectomy brought about significant reduction in femoral BMD of OVX rats after 3 months, relative to normal control rats. However, after 3 months of administration of total flavonoids from Drynaria rhizome (0.3 and 0.5 g/kg), there were significant and dose-influenced enhancement of BMD in femurs of the OVX rats ($p < 0.05$, $p < 0.01$).

Table 3: Effect of total flavonoids from Drynaria rhizome on BMD levels

Group	Bone mineral density (g/cm^2)
Normal control	0.2389±0.0217
OVX control	0.1948±0.0131 **
OVX+DR1	0.2152±0.0184 #
OVX+DR2	0.2347±0.0175 ##

OVX+DR1: OVX + DR flavonoids (0.3 g/kg);
OVX+DR2: OVX + DR flavonoids (0.5 g/kg); *$P < 0.05$, relative to normal control; #$p < 0.05$, ##$p < 0.01$, relative to untreated OVX

Effect of total flavonoids from Drynaria rhizome on E2 and GBP

There were significantly higher levels of serum BGP in rats in untreated OVX group than in normal control rats. Significant reductions in serum E2 were seen in untreated OVX rats, when compared with normal control rats. However, serum BGP levels were significantly decreased by treatment with total flavonoids from Drynaria rhizome at both doses (0.3 and 0.5 g/kg), relative to untreated OVX group. In addition, treatment with the total flavonoids from Drynaria rhizome at both doses (0.3 and 0.5 g/kg) led to significant elevation of serum E2, when compared with the OVX control group. These results are presented on Table 4.

Table 4: Effect of total flavonoids from Drynaria rhizome on BGP and E2 levels (µg/L)

Group	BGP	E2
Normal control	14.297±1.25	16.382±1.38
OVX control	35.821±2.62**	10.042±0.93**
OVX+DR1	27.206±1.96##	13.628±0.93##
OVX+DR2	16.825±1.18##	15.424±1.05##

OVX+DR1: OVX + DR flavonoids (0.3 g/kg);
OVX+DR2: OVX + DR flavonoids (0.5 g/kg); *$P < 0.05$, relative to normal control; ##p < 0.01, relative to OVX control

Effect of total flavonoids from Drynaria rhizome on expressions of bone-associated genes

Bilateral oophorectomy markedly enhanced the expressions of Wnt10b, β-catenin, BMP2, BMP4 mRNAs, while Dkk1 mRNA expression was markedly downregulated ($p < 0.01$), when compared to the normal control group (Table 5). As expected, treatment with total flavonoids from Drynaria rhizome (0.3 and 0.5 g/kg) significantly downregulated mRNA expressions of Wnt10b, β-catenin, BMP2, BMP4 in OVX rats, but significantly reversed OVX-induced downregulation of Dkk1 mRNA expression (Table 5).

DISCUSSION

The level of osteoclast isoenzyme of ALP is often employed as an index of degree of differentiation of osteoblasts. Indeed, TRAP serves as a specific parameter for functionality of osteoclasts [13]. The reduction in TRAP activity in rats treated with total flavonoids from Drynaria rhizome suggests that total flavonoids from Drynaria rhizome suppress the function of osteoclasts.

Table 5: Effect of total flavonoids from Drynaria rhizome on mRNA expressions of Wnt10b, β-catenin, BMP2, BMP4, and Dkk1

Group	Wnt10b	β-catenin	BMP2	BMP4	Dkk1
Normal control	1.00±0.00	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0
OVX control	1.7±0.1**	1.5±0.1**	1.6±0.1**	1.6±0.1**	0.6±0.0**
OVX+DR1	1.5±0.1##	1.5±0.1##	1.5±0.1##	1.3±0.1##	0.8±0.1##
OVX+DR2	1.2±0.1##	1.2±0.1##	1.3±0.1##	1.2±0.1#	1.2±0.1##

OVX+DR1: OVX + DR flavonoids (0.3 g/kg); OVX+DR2: OVX + DR flavonoids (0.5 g/kg); *$P < 0.05$, relative to normal control; ##$p < 0.01$, relative to untreated OVX group

In addition, the total flavonoids from Drynaria rhizome influenced serum ALP levels. These findings indicate that total flavonoids from Drynaria rhizome have obvious effect on osteoblast differentiation. Thus, total flavonoids from Drynaria rhizome may decrease bone loss.

Low levels of estrogens result in bone demineralization and osteoporosis [14]. In the present study, total flavonoids from Drynaria rhizome markedly blocked oophorectomy-induced reduction in E_2, indicating that the flavonoids possess phytoestrogenic potential. Serum BGP and OPG are used as markers of bone turnover [15]. In this study, ALP level (index of osteogenesis) was enhanced by treatment of OVX rats with total flavonoids from Drynaria rhizome. The results also showed that total flavonoids from Drynaria rhizome significantly decreased serum BGP in oophorectomized rats, suggesting that the flavonoids can increase osteogenesis while inhibiting osteoporosis. These findings explain the marked enhancement of bone mineral density of femur in the oophorectomized rats treated with total flavonoids from Drynaria rhizome.

The association of Wnt10b with various diseases has been extensively reviewed [16]. The Wnt-signaling route is a key factor involved in regulation of bone metabolism via enhancement of osteogenesis. When Wnt/β-catenin signal route is activated, precursor cells to osteoblasts proliferate and differentiate, and apoptotic changes in mature osteoblasts decrease, resulting in suppression of the osteoblast-induced inhibition of the ability of the osteoclasts to differentiate [17-19]. A large corpus of evidence have demonstrated that Dkk1 plays an important role in limb morphogenesis and head induction [20,21], and also in osteogenesis and bone disease. The BMPs facilitate osteogenesis in neonates. Studies on post-natal mice have shown that impairment of BMP function resulted in fragile bones and increased tendency to unprovoked fractures [22]. In the current study, total flavonoids from Drynaria rhizome significantly downregulated mRNA expressions of Wnt10b, β-catenin, BMP2 and BMP4 in oophorectomized rats, and markedly blocked OVX-associated downregulation of Dkk1 mRNA expression. The results indicate that the mechanism involved in the promotion of osteoblast proliferation in osteoporosis by total flavonoids from Drynaria rhizome is closely related to upregulation of mRNA expressions of Wnt10b, β-catenin, BMP2 and BMP4, and downregulation of Dkk1 mRNA expression. In addition, total flavonoids from Drynaria rhizome induce osteoblast proliferation possibly by regulating the relationship between classical WNT signaling pathway and BMP-2 signaling pathway.

CONCLUSION

The findings of this study suggest that total flavonoids from Drynaria rhizome decrease bone loss by decreasing ALP, TRAP and BGP levels, while increasing the production of BMD and E_2. Thus, these results demonstrate, for the first time to the best of our knoeledge, that the total flavonoids from Drynaria rhizome alleviate osteoporosis, in part, by regulating the relationship between classical WNT signaling and BMP-2 signaling pathways.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We affirm that this study was carried out by those whose names appear in this manuscript, and that all liabilities arising from claims in respect of the manuscript content will be the sole responsibility of the authors

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