Total Flavonoid Fraction of Rhizoma Drynaria Improves Bone Properties in Ovariectomized Mice and Exerts Estrogen-Like Activities in Rat Osteoblast-Like (UMR-106) Cells

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ABSTRACT

Rhizoma Drynaria (RD) has been widely used for healing bone fractures or related diseases in traditional Chinese medicine. The present study was designed to determine if RD total flavonoids (RDTF) could exert estrogen-like protective actions on bone. Young C57/BL6J mice were ovariectomized (OVX) and treated orally with RDTF (0.087, 0.173 or 0.346 mg/g/day), 17β-estradiol (2μg/g/day) or its vehicle for 6 weeks. Bone mineral densities (BMD) was measured by peripheral quantitative computed tomography (pQCT). Rat osteoblast-like UMR-106 cells were co-incubated with ER antagonist ICI 182, 780 to determine if the effects of RDTF on osteoblastic functions were ER-dependent. The functional transactivation of ER-α and ER-β as well as ER-αphosphorylation by RDTF were also studied. RDTF increased trabecular-rich BMD at distal femur and lumbar spine in OVX mice. 0.173 mg/g/day was most effective in improving bone properties in OVX mice. The stimulatory effects of RDTF on osteoblastic functions could be abolished by co-incubation with ICI 182, 780 in UMR-106 cells. Transient transfection study indicated that RDTF dose-dependently stimulated ERE-dependent luciferase activity in UMR-106 cells *via* ER-α and ER-β. Moreover, 0.2 μg/mL of RDTF significantly induced ER-α phosphorylation at serine 118 in UMR-106 cells.

INTRODUCTION

Osteoporosis is a condition characterized by low bone mineral density and microarchitectural deterioration of bone tissue, resulting in an increase risk in fracture[(1)]. Increased risks of morbidity and mortality were found in osteoporotic patients suffering from hip and vertebral fractures[(2)]. Estrogen replacement therapy (ERT), a major regimen for the management of osteoporosis, was recently found to be associated with an increased risk in developing stroke and breast cancer in postmenopausal women[(3)]. Thus, affordable alternative approaches with minimal side effects, such as the use of traditional Chinese medicine (TCM), for the management of postmenopausal osteoporosis are worth exploring. This is particularly important for aging population in the developing countries as majority of the patients could not afford long term use of high cost medications.

One of the most frequently used herb in Chinese medicine for treatment of bone fractures is Rhizoma Drynaria. It is characterized as the dried rhizome of perennial pteridophyte Drynaria Fortunei (Kunze) J. Smith (Polypodiaceae). Its Chinese name is 'Gu Sui Bu', which means bone fractures healer. It is recognized as a 'kidney-tonifying' herb in the TCM formulae for treatment of bone related disorders such as osteoporosis and bone fractures for many years in China. The bone protective effects of RD have been reported previously[(4)]. Its crude extract has been shown to promote osteoblast differentiation and mineralization in pre-osteoblastic MC3T3-E1 cells through regulation of bone morphogenetic protein-2[(4)]. Wong *et al*. reported that RD crude extract exerted strong effects on bone formation in mice[(5)]. Jia *et al* reported that RD mimicked estrogen in maintaining normal trabecular structure and suppressing bone turnover rate of postmenopausal osteoporosis. Total flavonoid fraction of RD was previously demonstrated to exert strong anti-oxidative effect as well as osteoprotective effect[(6)]. RDTF was found to significantly restore OVX-induced bone loss in rats. In addition, it was found not only to suppress osteoclastic cell activity but also to promote osteoblastic cell proliferation *in vitro*[(6)]. However, the optimal dosage of RDTF for exerting bone protective effects *in vivo* has not been characterized.

The present study aimed to systematically characterize the dose-dependent effects of RDTF on bone

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properties in OVX mice, followed by the study of its effects on rat osteoblast-like UMR 106 cells. It is hope that the studies would provide evidence to support the use of Rhizoma Drynaria as alternatives for prevention and treatment of postmenopausal osteoporosis.

MATERIALS AND METHODS

I. *Preparation of Rhizoma Drynaria Total Flavonoid Extract*

Rhizomes of Drynaria fortune were collected in Guizhow province, China in 2005 and were identified by Professor Zhou Rong-Han (Chinese Pharmaceutical University, Nanjing, Jiansu, China). The total flavanoid fraction of rhizomes of D. fortune (50 kg) were preapared by extraction with 60% ethanol and chromatographed on a D-101 macroporous resin column (Φ25 × 150 cm) with gradients of water and 30, 50 and 95% alcohol. High performance of liquid chromatography (HPLC) was performed on a Shimadzu C_{18} column (Φ250 × 4.6 cm) in Agilent series 1100 (USA) to analyze the compounds in the total flavonoid extract of Rhizoma Drynaria.

II. *Animal Study*

Sixty one-month old female C57/BL6J mice from the Chinese University of Hong Kong were housed in environmentally controlled central animal facilities. The mice were either sham-operated or ovariectomized (OVX) at the age of one month. After recovering for two weeks, the mice were randomly selected and divided into 6 groups. One group was sham vehicle (2% Ethanol), the others were OVX vehicle; OVX 17β-estradiol (2μg/g/day) and 3 groups of OVX RD total flavonoid (RDTF) extract: Low dose: 0.087; Medium dose: 0.173; and High dose: 0.347 mg/g/day. Treatments were administrated orally to the 6 groups of animals for 6 weeks. Animals were pair-fed with diet containing 0.6% Ca and 0.65% P (TD 98005, Teklad, Madison, WI) and were allowed free access to water throughout the course of the studies as previously described[7]. The care and treatment protocol was approved by the Animal Ethics Committee of the Hong Kong Polytechnic University. Urine samples were collected one day before sacrifice. Blood samples, uteri and bone specimens (femur, tibia and lumbar spine) were collected at the end of the experiment.

III. *Assessment ofBone Propertiesby Peripheral Quantitative Computed Tomography (pQCT)*

Trabecular and cortical bone densities of left femur, tibia and lumbar spine region L1 were measured using StraTec XCT2000 machine (Norland Stratec Medizintechnik, GmbH, Birkenfeld, Germany). Mid-shaft and distal regions of femur and tibia were scanned. All scans were performed using the developed protocol designed for studying isolated small bones[8]. Total bone mineral densities (BMD), trabecular BMD, total cross-sectional area, trabecular cross-sectional area and stress-strain index (SSI) in the distal/proximal were determined.

IV. *Rat Osteoblastic UMR-106 Cell Culture Study*

UMR-106 cells (ATCC no. CRL-1661) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin 100 U/mL and streptomycin 100 μg/mL at 37°C in a humidified atmosphere of 95% air and 5% CO_2 as previously described[7]. After 48 h, the medium was changed to phenol-red free DMEM supplemented with 1% dextran-charcoal-stripped serum (sFBS) for 24 h. The cells were then treated with RDTF extract (0.02 to 0.2 μg/mL), 17β-estradiol (10 nM) or vehicle in the presence or absence of ICI 182, 780 (1 μM) for 48 h before treatment. Cell proliferation of UMR 106 cells were studied by using MTS (3-(4,5-dimethylthiazol -2-yl)-5-(3-carboxymethoxyphenyl) -2-(4-sulfophenyl)-2H tetrazolium) assay (Promega, #G3580). ALP activity was measured directly on the monolayer of cell cultures. 100 μL per well of PBS containing 10 mM *p*-nitrophenylphosphate (PNP) was added and shaken for 30 minutes at 37°C. The absorbance of colour change was measured at 405nm in a microplate reader. To normalize the result, Bradford protein assay was carried out and ALP activity was expressed in a unit of U/L/μg protein.

V. *Transient Transfection and Estrogen Receptors (ER) Mediated Luciferase Activity Assay*

The cells were transfected by Lipofectamine™ 2000 reagent as previously described[9]. ER-α, ER-β and ERE-containing luciferase reporter plasmid vERETkluc were kindly provided by Dr. Vincent Giguere (McGill University, Montreal, Quebec, Canada). After 6 hours of transfection, the cells were treated with vehicle, 17β-estradiol (10 nM) or RDTF extract (0.2 μg/mL) for 24 h. Cells were then lysed with passive lysis buffer and luciferase activity was measured using Dual Luciferase Reporter Assay System (Promega Corporation, Madison, MI, USA) and the signal was detected by TD-20/20 Luminometer (Turner Design, Sunnyvale, CA, USA). The estrogen promoter activity was expressed as *Firefly* luciferase values normalized by pRL-TK *Renilla* luciferase values.

VI. *Immunoblotting*

Treated cells were harvested and lysed with passive lysis buffer (Promega, Madison, MI, USA) as previously described[9]. Protein concentrations were determined using Bradford assay. Equal amounts of proteins were separated by SDS-PAGE on a 10 % reducing gel at a constant voltage (200 V) for about an hour, and transblotted onto polyvinylidene fluoride fluoropolymer (PVDF) membranes (Immobilin-P, Millipore Corp., Ma, USA). The

blots were probed with monoclonal rabbit anti-human phosphor-ER-α at serine 118 residues (1 : 2000) or anti-human ER-α (1 : 3000), and followed by incubation with goat anti-rabbit conjugated with horseradish peroxidase (1 : 2000). The antigen-antibody complexes were then detected with enhanced chemiluminescence (ECL) reagent and visualized by the Lumi-Imager using Lumi-Analyst version 3.10 software (Roche, Mannheim, Germany).

VII. *Statistical Analysis*

The *in vivo* data were analyzed one-way ANOVA followed by Tukey's post test and the *in vitro* data were analyzed by the non-paired student's t-test between control group and each treatment group using Graphpad PRISM® software package. Results were expressed as Mean ± SEM (standard error mean). p-values < 0.05 were considered as significant.

RESULTS AND DISCUSSION

The present study evaluated the osteoprotective effects and mechanism of actions of the total flavonoid fraction of Rhizoma Drynariae in ovariectomized mice and in rat osteoblast-like UMR 106 cells. Our results clearly demonstrated that RDTF could suppress OVX-induced loss in bone mass in OVX mice. In particular, treatment of OVX mice with RDTF at 0.087, 0.173 or 0.346 mg/g/day significantly increased total BMD and trabecular BMD at both distal femur and proximal tibia. The increase was most significant with RDTF at 0.173 mg/g/day (medium dose) at distal femur as shown in Table 1.

Table 1. Effects of RD Total flavonoid extract on Total Bone Mineral Density of distal femur and proximal tibia in Sham and OVX mice.

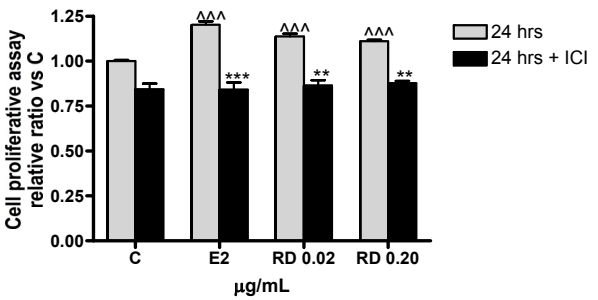
	Total BMD (mg/cm)	
	Distal Femur	Proximal Tibia
OVX (Veh)	335.2 ± 6.5 [a]	240.9 ± 5.9 [a]
E2	453.4 ± 8.5 [c]	325.5 ± 12.7 [c]
RD TF (Low)	377.4 ± 4.5 [b]	267.9 + 5.4 [b]
RD TF (Med)	395.6 ± 4.8 [b]	300.0 ± 4.1 [b]
RD TF (High)	394.3 ± 7.2 [b]	270.9 ± 11.9 [b]
Sham (Veh)	394.3 ± 7.2 [b]	283.7 ± 9.8 [b]

Results were analyzed by one-way ANOVA and expressed as mean ± SEM (n = 8) [a] $p < 0.05$ vs OVX (Veh), [b] $p < 0.05$ vs Sham Veh), [c] $p < 0.05$ vs OVX (Veh) and Sham (Veh).

According to the normal daily uptake of flavonoids in human (9 mg/kg/day), our dosages used was about 10, 20 and 40-fold higher than normal. Some studies in China talked about the acute and chronic effect of administrating RDTF in mice. Zhao *et al.*[10] found the LD_{50} was 66-fold higher from normal daily human uptake of total flavonoids in mice. Our dosages were regarded as safe to eat. Zhao *et al.*[11] also reported another publication about the chronic toxicology test. The experimental mice, which they were fed by RDTF in 60, 80 and 540-fold higher than normal, did not have obvious side-effect. After all, RDTF is regarded as safe to consume in terms of both short and prolong time frame.

Our study showed that RDTF mimicked estrogen in stimulating cell proliferation and ALP activity *via* estrogen receptor (ER) in UMR 106 cells. As shown in Figure 2A and Figure 2B, RDTF at both 0.02 and 0.2 mg/mL increased osteoblastic cell proliferation as well as alkaline phosphatase activities (osteoblastic differentiation) in UMR 106 cells, and the increase were abolished in the presence of ICI 182, 780, a specific estrogen receptor antagonist. These results suggest that the stimulatory effects of RDTF on osteoblastic fucntions in UMR 106 cells were estrogen receptor dependent. The results were in agreement with those reported by others[12, 13].

A.



B.

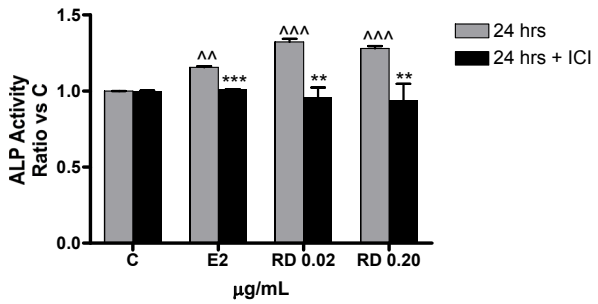


Figure 2. The effect of Rhizoma Drynaria total flavonoids on UMR106 a) cell proliferation and b) cell differentiation in the pres ence and absence of estrogen antagonists. ^^^ $p < 0.001$ vs C, ** and *** $p < 0.01$ and $p < 0.001$ vs corresponding dosage without blocker treatment.

Jeong *et al.*[12] demonstrated that crude extract of RD was shown to promote osteoblast differentiation and mineralization in pre-osteoblastic MC3T3-E1 cells through regulation of bone morphogenetic protein-2. Similarly, Zhang *et al.*[13] showed that serum from RDTF treated rats significantly promoted proliferation and differentiation of cultured osteoblasts extracted from newborn Sprague Dawley rats in a time dependent manner.

To determine if RDTF could activate estrogen receptor and activate estrogen response element (ERE)-

dependent transcription, UMR 106 cells were transfected with ERE-luciferase construct and co-transfected with plasmids that encode for either ERα or ERβ. As shown in Figure 3, RDTF at 0.2 μg/mL but not 0.02 μg/mL significantly induced ERE-dependent luciferase activities *via* ERβ in UMR 106 cells.

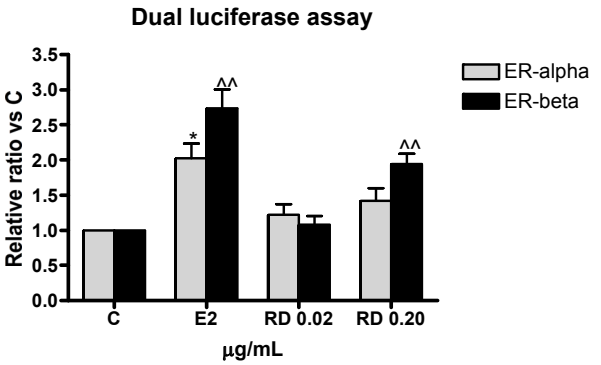


Figure 3. The effect of Rhizoma Drynaria total flavonoids on ER-α and ER-β-mediated ERE-dependent luciferase activity in UMR106 cells. * $p < 0.05$ vs C (ER-α) ^^ $p < 0.01$ vs C (ER-β).

Recent studies showed that ER could be activated in the absence of ligand binding by modulating various signal transduction pathways such as the mitogen-activated protein kinase (MAPK) mediated pathways[14-16]. Upon activation of unliganded ERα by MAPK cascade, ERα was shown to be phosphorylated at serine residue 118 (Ser118), 104 (Ser104) and 167 (Ser167) within the AF-1 domain of ERα[17]. To determine if RDTF activates ER ligand-independently, the ratio of phosphorylated ERα (Ser118) to total ERα expression in UMR 106 cells in response to RDTF treatment was studied. As shown in Figure 4, 0.2 μg/mL RDTF as well as 10^{-8}M E2 significantly induced ER phosphorylation at serine 118 ER and decreased the expression of ER in UMR 106 cells. ER phosphorylation at Ser118 was preferentially chosen as Ser118 is a highly conserved residue that represents the major site of phosphorylation in response to estradiol[18]. Our results clearly indicated that RDTF at 0.2 μg/mL could activate ERα phosphorylation in UMR 106 cells, suggesting that RDTF could also activate ER ligand-independently in osteoblastic cells.

The present study clearly demonstrated RDTF is effective in protecting against ovarietomy-induced bone loss in mice and exerts direct effects in regulating osteoblastic cell growth *via* the activation of ER. Our study clearly identified the optimal dose of RDTF for achieving bone protective effects in ovariectomized mice to be 0.173 mg/g/day and demonstrated that RDTF could activate ER in both ligand dependent as well as ligand-independent manner. Future studies will be needed to evaluate the clinical efficacy of RDTF for treatment of osteoporosis in postmenopausal women.

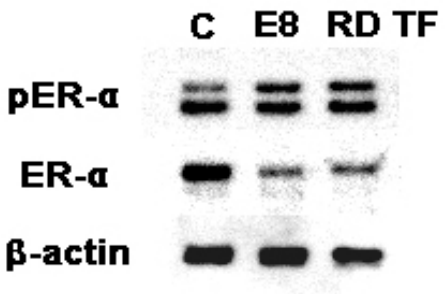


Figure 4. The effect of Rhizoma Drynaria total flavonoids on the expression of phosphorylation of ER-α in UMR106 cells.

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REFERENCES

1. Lau, E. M. and Cooper, C. 1996. The epidemiology of osteoporosis. The oriental perspective in a world context. Clin. Orthop. Relat. Res. 323: 65-74.
2. Gass, M. and Dawson-Hughes, B. 2006. Preventing osteoporosis-related fractures: an overview. Am. J. Med. 119: S3-S11.
3. Glasier, A. 2006. HRT and breast cancer. Womens Health Med. 13: 15-17.
4. Jeong, J. C., Lee, J. W., Yoon, C. H., Kim, H. M. and Kim, C. H. 2004. Drynariae Rhizoma promotes osteoblast differentiation and mineralization in MC3T3-E1 cells through regulation of bone morphogenetic protein-2, alkaline phosphatase, type I collagen and collagenase-1. Toxicol. In Vitro 18: 829-834
5. Wong, R. W. and Rabie, A. B. 2006. Systemic effect of crude extract from rhizome of Drynaria fortune on bone formation in mice. Phytother. Res. 20: 313-315.
6. Sun, J.. He, W. and Liu, K. 2008. The research of Drynaria total flavonoids and osteoporosis research. Chinese J. Osteoporosis 14: 763-766.
7. Xie, F., Wu, C. F., Lai, W. P., Yang, X. J., Cheung, P. Y., Yao, X. S., Leung, P. C. and Wong, M. S. 2005. The osteoprotective effect of Herba epimedii (HEP) extract in vivo and in vitro. Evid. Based Complement. Alternat. Med. 2: 353-361.
8. Gasser, J. 2002. Bone measurements by peripheral quantitative computed tomography in rodents. Methods Mol. Med. 80: 323-341.

9. Lau, W. S., Chan, R. Y., Guo, D. A. and Wong, M. S. 2008. Ginsenoside Rg1 exerts estrogen-like activities *via* ligand-independent activation of ERalpha pathway. J. Steroid Biochem. Mol. Biol. 108: 64-71.
10. Zhao, J., Xie, Y., Zhang, W., Wang, Z. and Liu, Y. 2005. Acute Toxicity Test of Total Flavonoids from Rhizome of Drynariae. Herald Med. 24: 12-14.
11. Zhao, H., Xie, Y., Zhang, W. J., Wang, G. Z. and Lir, Y. 2004. Chronic Toxicity Test of total flavnoids from Rhizoma Drynariae. J. Health Toxicol. 18: 301-302.
12. Jeong, J. C., Lee, J. W., Yoon, C. H., Kim, H. M. and Kim, C. H. 2004. Drynariae Rhizoma promotes osteoblast differentiation and mineralization in MC3T3-E1 cells through regulation of bone morphogenetic protein-2, alkaline phosphatase, type I collagen and collagenase-1. Toxicol. In Vitro 18: 829-834.
13. Zhang, J., Li, H. P., Yang, P. L., Liu, Y. H. and Yang, B. H. 2009. Effects of total flavonoids from Rhizoma Drynariae medicated serum on proliferation, differentiation, cell cycle and apoptosis of osteoblasts in vitro. Zhong Yao Cai 32: 1090-1093.
14. Lau, W. S., Chan, R. Y., Guo, D. A. and Wong, M. S. 2008. Ginsenoside Rg1 exerts estrogen-like activities *via* ligand-independent activation of ERalpha pathway. J. Steroid Biochem. Mol. Biol. 108: 64-71.
15. Bunone, G., Briand, P. A., Miksicek, R. J. and Picard, D. 1996. Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. EMBO J. 15: 2174-2183.
16. Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Gotoh, Y., Nishida, E., Kawashima, H., Metzger, D. and Chambon, P. 1995. Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. Science 270: 1491-1494.
17. Chen, D. S., Washbrook, E., Sarwar, N., Bates, G. J., Pace, P. E., Thirunuvakkarasu, V., Taylor, J., Epstein, R. J., Fuller-Pace, F. V., Egly, J. M., Coombes, R. C. and Ali, S. 2002. Phosphorylation of human estrogen receptor α at serine 118 by two distinct signal transduction pathways revealed by phosphorylation- specific antisera. Oncogene 21: 4921-4931.
18. Lannigan, D. A. 2003. Estrogen receptor phosphorylation. Steroids 68: 1-9.