Life Sciences 209 (2018) 140–148

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Cynomorium songaricum prevents bone resorption in ovariectomized rats through RANKL/RANK/TRAF6 mediated suppression of PI3K/AKT and NF-κB pathways

Xueqin Ma*,[1], Jingjing Liu[1], Lingling Yang, Bo Zhang, Yanhong Dong, Qipeng Zhao*

Department of Pharmaceutical analysis, School of Pharmacy, Key Laboratory of Hui Ethnic Medicine Modernization, Ministry of Education, Ningxia Medical University, 1160 Shenli Street, Yinchuan 750004, China

ARTICLE INFO

Keywords:
Cynomorium songaricum Rupr.
Ovariectomized rats
TRAF6
Bone formation
Bone resorption

ABSTRACT

Aim: *Cynomorium songaricum* Rupr., an edible and important Traditional Chinese medicine has long been used in folk for treatment of kidney deficiency, was chosen to estimate the antiosteoporotic activity and underlying molecular mechanism on rats induced by ovariectomy (OVX).

Main methods: 9 of 45 rats were underwent bilateral laparotomy without removing the ovaries as sham group, remains were underwent bilateral ovariectomy and equally randomized into four groups: with vehicle (0.5% CMC-Na) as model group, estradiol valerate (1 mg/kg body weight/day) as positive control, with 100 and 300 mg/kg body weight/day of ethanol extracts of *C. songaricum* extract (CSE) as low and high dosage groups, respectively.

Key findings: After 12 weeks of continues orally intervention, the decreases of bone mineral density, bone mineral content, tissue mineral content, as well as the increases of bone trabecular separation and bone resorption markers were significantly reversed by CSE in the OVX rats, and in particular, a contradictory phenomenon on calcium and phosphorus contents was observed and elucidated. Mechanistically, the expressions of tumor-necrosis factor receptor-associated factor 6 (TRAF 6), nuclear factor kappa B (RANK) and its ligand (RANKL), as well as the nuclear factor kappa B (NF-κB), phosphoinositide 3-kinase (PI3K) and protein kinase B (AKT) levels were significantly down-regulated by CSE intervention, whereas the osteoprotegerin (OPG) was significantly up-regulated by CSE as compared to the control.

Significance: Concisely, *C. songaricum* exhibited potential therapeutic effect on bone metabolism of ovariectomized rats, and this effect was possibly exerted by RANKL/RANK/TRAF6 mediated down-regulation of NF-κB and PI3K/AKT pathways.

1. Introduction

Osteoporosis, “a global perspective of a silent killer”, is characterized by low bone mass density (BMD) and micro-architectural deterioration deriving from an imbalance between osteoblast-mediated bone formation and osteoclast-mediated bone resorption, and finally caused osteoporotic fracture [1]. Osteoporosis is particularly common in postmenopausal women, whose estrogen deficiencies lead to high bone turnover and bone loss [2], and this fact has been confirmed by hormone replacement therapy (HRT) which is able to prevent postmenopausal osteoporosis by supplying the loss of estrogen at menopause. However, increased risk of endometrial cancer and coronary heart disease caused by HRT exceed its benefits [3] and limit its clinical applications. Therefore, more safe and effective natural extracts derived

Abbreviations: AKT, protein kinase B; ALP, alkaline phosphatase; BGP, bone Gla-protein; BMC, bone mineral content; BMD, bone mineral density; BVF, bone volume fraction; Ca, calcium; CSE, *Cynomorium songaricum* extract; DPD, deoxypyridinoline; EV, estradiol valerate; GSH, glutathione; HRT, hormone replacement therapy; MDA, malondialdehyde; NF-κB, nuclear factor kappa B; OPG, osteoprotegerin; OVX, ovariectomized; P, phosphorus; PI3K, phosphoinositide 3-kinase; RANK, nuclear factor kappa B; RANKL, receptor activator for nuclear factor kappa B Ligand; ROI, region of interest; SOD, superoxide dismutase; Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness; TCM, traditional Chinese medicine; TNF, tumor necrosis factor; TMC, tissue mineral content; TMD, tissue mineral density; TRAF6, tumor-necrosis factor receptor-associated factor 6; TRAP, tartrate-resistant acid phosphatase

* Corresponding authors.
E-mail addresses: maxueqin217@126.com (X. Ma), 25136659@qq.com (Q. Zhao).

[1] Both authors contributed equally to this work.

https://doi.org/10.1016/j.lfs.2018.08.008
Received 26 April 2018; Received in revised form 23 July 2018; Accepted 4 August 2018
Available online 06 August 2018

from daily edible medicinal herbs for the prevention and therapy of osteoporosis were highly warranted [4,5].

Bone resorption and remodeling is an intricately process, physiological step that requires the function of osteoclasts [6], and at the stage of menopause, the estrogen levels decrease sharply which leads to an increased bone resorption because of the enhanced osteoclastogenesis [7]. Osteoclastogenesis is inhibited when receptor activator of NF-kappa B (RANK) is activated by its ligand, RANKL; RANK then interacts with members of the family of TNF receptor-associated factors (TRAFs) that mediate activation of the phosphatidylinositol 3-kinase (PI3K) and NF-kappa B pathways [8]. Meanwhile, RANK could associate with several TRAFs to stimulate RANK transmission to the downstream NF-κB pathway, where emerging evidence points to a critical regulatory function for TRAF6 in RANKL/RANK-mediated signaling cascades. The binding of RANKL to RANK recruit TRAF6 and subsequently activate the differentiation of osteoclast, thus accelerate the process of bone resorption and finally lead to osteoporosis.

Cynomorium songaricum Rupr., an important natural health tonic and anti-aging remedy [9] which has been consumed over thousands of years in China, is used for safeguarding health and delaying the onset of senility. According to the Chinese pharmacopoeia [10], it is under the "pao" category [11], meaning supplementation, which is analogous to today's definition of functional foods. In addition, *C. songaricum* also has a long history use as a traditional medicine to treat various ailments such as impotence, kidney-yin deficiency, muscle weakness, lumbar debility, etc. [10]. Based on the theory of TCM, the bone resorption and formation is dominated by the kidney system, that is to say, the developments including the process of osteoporosis and osteoporotic fracture of the bone were depended on the kidney-essence, and this kidney-essence could nourish the bone, which means the kidney-essence could stimulate and strengthen the growth and restore of the skeleton [12], and vice versa, the deficiency of kidney-essence could influence the production of bone marrow, leading to flaccidity of skeleton. Therefore, we proposed that *C. songaricum* could prevent and treat osteoporosis for its efficacy of treating kidney deficiency. Our previous short communication reported the antiosteoporotic activity of *C. songaricum* by using a simple preliminary experiment [13]. Now, in the present study, we systematically estimated the therapeutic effect of *C. songaricum* on ovariectomized (OVX) rat model and the potential molecular mechanism was also evaluated.

2. Materials and methods

2.1. Plant identification and preparation

The stems of *C. songaricum* were collected from Ningxia province in August 2015 and identified by Prof. Lin Dong, school of pharmacy, Ningxia Medical University. A voucher specimen (#20150801) was conserved in Ningxia Hui Medicine Modern Engineering Research Center. Dry and powdered stems of *C. songaricum* (3.0 kg) were reflux-extracted 3 times with 80% ethanol (54 l, 3× 2 h), then the filtrates were concentrated under reduced pressure to afford the ethanol extract of *C. songaricum* (CSE), and the yield was 14%. A chemical profile of CSE was carried out on UPLC-TOF-MS (Fig. 1). For animal experiments, CSE was suspended in 0.5% CMC-Na solution and orally administered to rats at a volume of 1 ml/100 g body weight; for cell experiments, CSE was dissolved in DMSO solvent and diluted with α-MEM to obtain the concentration of 0.1, 1 and 10 mg/ml for cell analysis.

2.2. Chemicals and solvents

Glutathione (GSH), superoxide dismutase (SOD) and malondialdehyde (MDA) reagent kits (Institute of Jianchen Biological Engineering, Nanjing, China); cathepsin K elisa kit (Biovision, American); bone gla-protein (BGP) and deoxypyridinoline (DPD) crosslinks elisa kits (Xinyu Biological Engineering Co. Ltd., Shanghai, China); estradiol valerate (elpharm Lille SAS, Paris, France); chloral hydrate (Sinopharm, China). Total protein extraction and BCA protein quantization kits (Ken Gen Biotech. Co. Ltd., Nanjing, China), primary antibodies of TRAF6, RANKL, RANK, NF-κB, PI3K, AKT, OPG and β-actin (Cell Signaling Technology, Beverly, MA, USA), secondary antibodies of horseradish peroxidase-conjugated goat anti-rabbit IgG (ZSGB-BIO, Beijing, China). DMSO and the other chemicals and biochemical agents used were of AR grade.

2.3. Animal experimental protocol

Animal experiments were carried out according to the guide for the care and use of laboratory animals and were approved by the Bioethics Committee of the Ningxia Medical University. Forty-five 3-month female Sprague-Dawley rats, with the initial body weight of 231.0 ± 24.7 g, were obtained from Ningxia Medical University. Every 4 or 5 rats were kept in one cage under an air-controlled condition and gave a standard laboratory diet and tap water. After acclimated with 7 days, all the rats were anesthetized with chloral hydrate (10%, i.p.) under aseptic conditions and underwent either bilateral laparotomy without removing the ovaries (SHAM, $n = 9$) or bilateral ovariectomy (OVX, $n = 36$). The 36 OVX rats were equally and randomly divided into four groups: orally treated with vehicle (0.5% CMC-Na) as model group (OVX); with estradiol valerate (1 mg/kg/day) as positive control (EV); with 300 and 100 mg/kg body weight/day of CSE as high (CSEH) and low (CSEL) dosage groups, respectively. The rats were weighed biweekly with the dose adjusted accordingly during the whole experimental period. After 12 weeks of continues orally administrated intervention, urine was collected from overnight (24 h) fasted rats by metabolic cages; blood was obtained and centrifuged at 4000 ×g for 10 min to afford serum; the femora, tibia and uterus were dissected. All the samples were stored at −80 °C until further analysis.

2.4. Bone mineral density and micro-CT analysis

The total bone mineral density (BMD, g/cm^2) of the right femur of each rat was measured by employing dual-energy X-ray absorptiometry (LUNAR, USA), and followed by the trabecular bone microarchitecture analysis by using a micro-CT system (eXplore Locus SP, GE) with the isotropic resolution set to 14 μm in all three spatial dimensions of the femur, and micview V2.1.2 micro-CT 3-D analysis software was used for scanned images reconstruction. The region of interest (ROI) of bone trabecular was selected according to the reference [14], and the bone morphometric parameters including bone volume fraction (BVF), tissue mineral density (TMD), tissue mineral content (TMC), bone mineral content (BMC), trabecular number (Tb. N), trabecular thickness (Tb. Th) and trabecular separation (Tb. Sp) were evaluated through analyzing the ROI by using ABA bone analysis software.

2.5. Biochemical parameters

The content of serum and urinary calcium (Ca), phosphorus (P) and alkaline phosphatase (ALP) was measured on an automatic analyzer (Ciba-Corning 550, USA); the levels of serum GSH, SOD, MDA, BGP, cathepsin K and urinary DPD were determined by corresponding reagent kits, and the activity of tartrate resistant acid phosphatase (TRAP) was analyzed according to the related literature [15].

2.6. Western blot

The osteoblastic cells were cultured according to the published method [15] and then treated with or without CSE (0.1 mg/ml as CSEL group, 1 mg/ml as CSEM group, 10 mg/ml as CSEH group, respectively) for 24 h, and subsequently analyzed in a buffer containing 20 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, protease and phosphatase inhibitors, the protein was successively extracted from the osteoblastic