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Drynaria fortunei J. Sm. improves the bone mass of ovariectomized rats through osteocalcin-involved endochondral ossification

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ABSTRACT

Aim of this study: Our previous study showed that *Drynaria fortunei* J. Sm. (Kunze), a traditional Chinese medical herb, can promote osteoblast differentiation and maturation. This study was further aimed to confirm the traditional effects of Kunze on the bone mass of ovariectomized rats.
Materials and methods: Female Wistar rats were given an ovariectomy and then administered the water extract of Kunze (WEK). Systemic and tissue toxicities of WEK were assessed. A biomechanical test, bone mineral contents, and bone histomorphometry were analyzed to determine the effects of the WEK on the bone mass. Levels of osteocalcin (OCN) in bone tissues were determined by immunohistochemistry and immunoblotting. The effects of naringin, one of the bioactive compounds of the WEK, on the bone mass were evaluated.
Results: A bilateral ovariectomy in rats caused a time-dependent decrease in levels of serum 17β-estradiol. Exposure of ovariectomized rats to the WEK at 0.5 and 1 g/kg body weight/day for 1, 2, 3, and 6 months did not induce systemic or tissue toxicities. Biomechanical testing and a bone mineral content analysis showed that the ovariectomy decreased the bone torsion force and bone ash in time-dependent manners. In comparison, after exposure to the WEK, the ovariectomy-induced reductions in the bone torsion force and bone ash were significantly alleviated. In parallel, results of a bone histomorphometric assay further revealed that the ovariectomy caused significant diminution in the production of prehypertrophic chondrocytes and trabecular bone but enhanced hypertrophic chondrocyte numbers in the growth plate. However, exposure to the WEK lowered ovariectomy-induced changes in these cellular events. As to the mechanism, the WEK increased OCN biosynthesis in bone tissues of ovariectomized rats. Administration of naringin to ovariectomized rats caused significant amelioration of the bone strength, bone mineral contents, and trabecular bone amounts.
Conclusions: This study shows that the WEK can translationally promote the bone mass in ovariectomized rats through stimulating OCN-involved endochondral ossification.
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1. Introduction

Osteoporosis, a progressive bone disease, is characterized by a decrease in the bone mass and density which can induce bones to weaken and be more likely to break (Adams, 2012). In the clinic, osteoporosis is also called as a silent disease because it proceeds without pain or symptoms until bone fracture occurs (Bradbury et al., 2012). Bone fractures typically linked to osteoporosis are very dangerous and can cause patients to suffer permanent disabilities or even death (Edwards et al., 2012). A variety of risk factors contribute to the causes of osteoporosis, including low levels of sex hormones, inadequate calcium intake, heredity, chronic diseases, lifestyle habits, medication use, and aging (Rubin et al., 2013). Among these, sex hormones are reported to play the most crucial roles in regulating bone remodeling and maintaining bone mass. However, post-menopausal hormone

Abbreviations: ALT, aminotransferase; AST, aspartate aminotransferase; BUN, bilirubin; CRE, creatinine; LDH, lactate dehydrogenase; OCN, osteocalcin; OPN, osteopontin; UA, uric acid; WEK, water extracts of Kunze

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therapy for osteoporosis may increase the risk of coronary heart disease, stroke, and breast cancer (Rozenberg et al., 2013). Hence, discovering hormone analogs such as phytoestrogen would be helpful in developing de novo therapeutic strategies for osteoporosis.

The bone structure is maintained through a dynamic balance between osteoblast-mediated bone formation and osteoclast-mediated bone resorption (Seeman and Delmas, 2006). In skeletal development and bone healing, bone is formed through two essential processes, namely endochondral ossification and intramembranous ossification (Shore and Kaplan, 2010). In endochondral ossification, bone is formed with hyaline cartilage as the model (Hojo et al., 2010). In comparison, cartilage is not present in the course of intramembranous ossification (Shore and Kaplan, 2010). Miscellaneous stages are involved in endochondral ossification, including collar formation, cavity formation, vascular invasion, elongation, and epiphyseal ossification (Hojo et al., 2010). During the process of endochondral ossification, chondrocytes differentiated from stem cells first proliferate and then differentiate into hypertrophic chondrocytes (Mackie et al., 2011). Lastly, hypertrophic chondrocytes are gradually replaced by bone, leading to an increase in trabecular bone. As a result, osteoblasts participate in endochondral ossification by colonizing regions of the cartilage (Aubin, 1998; Medici and Olsen, 2012). A complicated network of differentiation proteins is involved in regulating osteoblast-mediated osteogenesis (Stein et al., 1996; Vandenput and Ohlsson, 2009). The process of endochondral bone development needs to be evaluated to discover innovative biomaterials that can prevent or treat osteoporosis.

Drynaria fortunei J. Sm. (Korean name; Kunze) is a variety of the traditional Chinese herb, Gusuibu, which is frequently used by Chinese people to prevent or treat bone-related diseases. Long et al. (2005) reported that the flavonoid fractions of Kunze can prevent nephrotoxicity and promote regeneration of kidney primary epithelial tubular cells. Previous studies further showed that the components of Kunze can stimulate proliferation of osteoblast-like UMR106 cells and induce bone formation in mice (Wong and Rabie, 2006; Wang et al., 2008). Our previous study showed that the water extract of Kunze (WEK) can protect rat calvarial osteoblasts from hydrogen peroxide-induced insults (Liu et al., 2001). Recently, we further showed the effects of the WEK on promoting osteoblast maturation by inducing differentiation-related gene expression and protecting against oxidative/nitrosative stress-induced apoptotic insults (Huang et al., 2010; Hsu et al., 2011). Osteocalcin (OCN) is an early osteoblast biosignature that participates in controlling osteoblast function and bone extracellular matrix mineralization (van Leeuwen et al., 2001). A previous study reported that 11 flavonoids were extracted from Kunze (Wang et al., 2008). In our lab, we demonstrated that naringin, one of these flavonoids, can induce OCN expression during osteoblast differentiation (Huang et al., 2010). Thus, this study was further designed to corroborate the translational effects of Kunze and naringin on improving bone mass using ovariectomized rats as our experimental model.

2. Materials and methods

2.1. Preparation of the WEK

Kunze (*D. fortunei*) was kindly provided by the Brion Research Institute, Sun Ten Group (Taipei, Taiwan). The herb was identified by institutional experts, and its chemical and physical characteristics were routinely analyzed (Huang et al., 2010). The WEK was prepared as previously described (Liu et al., 2001). The extract was stored at room temperature and protected from light and moisture as described previously (Hsu et al., 2011). Naringin was purchased from Sigma (St. Louis, MO, USA).

2.2. Animals

Female Wistar rats (200–250 g) were purchased from the Animal Center of the College of Medicine, National Taiwan University (Taipei, Taiwan). Before the experiments began, animals were allowed to acclimatize for 1 week in their animal quarters with air conditioning and an automatically controlled photoperiod of 12 h of light daily. All experimental procedures were performed according to the National Institutes of Health *Guidelines for the Use of Laboratory Animals* and approved by the Institutional Animal Care and Use Committee of Taipei Medical University (Taipei, Taiwan). Rats were randomly divided into 4 groups: a sham control, an ovariectomy, an ovariectomy with the WEK at 0.5 g/kg body weight, and an ovariectomy with the WEK at 1 g/kg body weight. Animals were allowed free access to rodent laboratory chow (Purina Mills, St. Louis, MO, USA).

2.3. Surgical procedures and drug treatment

Rats were anesthetized using ketamine (100 mg/kg) and xylazine hydrochloride (10 mg/kg) intramuscularly. A bilateral ovariectomy was conducted following a previous method (Brennan et al., 2009). A mid-ventral incision was made, and the bilateral ovaries and ovarian fat were removed. The ovaries were isolated by ligation of the most proximal portion of the oviduct before removal. In the sham-operated group, animals were subjected to the same procedure, but the ovaries were not removed. Animals were kept warm during the procedure and recovery. After surgery, rats were administered different doses of WEK by oral gavage for various time intervals.

2.4. Measurements of serum clinical parameters

Systemic toxicity of the WEK was assayed by measuring serum clinical parameters as described previously (Chen et al., 1998). Briefly, animals were sacrificed after drug treatment, and blood samples were collected for assessment of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), bilirubin (BUN), creatinine (CRE), and uric acid (UA).

2.5. Measurement of serum 17β-estradiol

After drug treatment, rats were sacrificed, and blood samples were collected. Serum fractions were prepared for analysis of 17β-estradiol. Levels of serum 17β-estradiol were assayed following the instructions of the Elecsys-Estradiol II enzyme-linked immunosorbent assay (Roche Diagnostics, Mannheim, Germany).

2.6. Hematoxylin and eosin (HE) staining

After drug treatment, animals were sacrificed. Livers and kidneys were removed and collected. Samples were fixed using phosphate-buffered 4% paraformaldehyde and processed for routine paraffin embedding. All tissue samples were sliced into 5-μm transverse sections and stained with HE. Specimens were observed and photographed using a light microscope.

2.7. Biomechanical testing

After drug treatment, animals were sacrificed, and femur tissues were removed and collected. Following collection of muscle and connective tissues, the femurs were examined by peripheral quantitative computed tomography using a standardized cantilever-bending technique as described previously (Wang et al., 2000). Briefly, distal condyles of the femurs were loaded in the antero-posterior direction at a constant rate of 1.0 mm/min until failure in a universal testing device (Avalon Technologies, Rochester, MI, USA).

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