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Beneficial effects of paeoniflorin on osteoporosis induced by high-carbohydrate, high-fat diet-associated hyperlipidemia *in vivo*

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ABSTRACT

Osteoporosis is linked to reduced bone mineral density (BMD) as a major risk factor for fragility fractures. Recent studies indicated an association between BMD and abnormally elevated lipid levels in blood as common indicators for hyperlipidemia. In this study, we assessed the protective effect of paeoniflorin, a phytochemical compound with multiple pharmacological activities, against hyperlipidemia-induced osteoporosis in rats fed a high-carbohydrate, high-fat diet (HCHF). The special diet-fed rats were subjected to an 8-week treatment with either paeoniflorin (20 mg/kg, daily) or vehicle. The control group received a normal diet during the entire study. At study conclusion, serum markers of lipid metabolism and bone turnover were measured. Bone strength was assessed by biomechanical testing, and femurs were scanned using micro-computed tomography to analyze trabecular and cortical bone structure. Interestingly, paeoniflorin controlled the serum lipid profile by significantly decreasing HCHF-induced high levels of total cholesterol, triglyceride, and low-density lipoprotein cholesterol. Paeoniflorin significantly improved trabecular and cortical parameters as well as femur length and width that were negatively affected by HCHF diet. Biomechanical strength testing showed that femurs of HCHF diet-fed rats endured significantly lower force but higher displacement and strain than those of control rats, whereas paeoniflorin reversed the negative effects. Moreover, paeoniflorin rescued osteoblast differentiation and cell spreading activities along with bone turnover markers. In conclusion, HCHF-induced hyperlipidemia caused adverse effects on the bone that were rescued by paeoniflorin treatment.

1. Introduction

Human lifespan extension is associated with risks of developing multiple diseases at an older age, such as osteoporosis. This disease is linked to reduced bone mineral density (BMD) and an elevated risk of fragility fractures [1], causing chronic pain, long-term disability, and even premature death. A high prevalence of osteoporosis combined with growing health care costs contribute to its significantly increased burden [2].

Hyperlipidemia with abnormally elevated serum levels of total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) was recently linked to low BMD, specifically in postmenopausal women [3]. Moreover, the low-BMD condition was replicated in laboratory rodents by feeding a high-fat diet [4]. Interestingly, multiple animal and clinical studies showed that cholesterol-lowering statins reduce osteoporosis-associated fracture risks [5], demonstrating the deteriorating effect of high cholesterol on bone health and providing new insights into potential osteoporosis treatments.

Paeoniflorin, a phytochemical isolated from peony root extracts, exhibits anti-inflammatory [6], antioxidant [7], antihyperlipidemic [7], and hepatoprotective activities [8], with low toxicity and few side effects [9]. Previously, we showed that modulation of NF-κB signaling by paeoniflorin reduced osteoclastogenesis but stimulated osteoblastogenesis, indicating its potential bone-protective effects in osteolytic diseases [10].

In this study, we hypothesized that paeoniflorin acts as a bifunctional drug in an animal model of hyperlipidemia-associated osteoporosis by simultaneously relieving hyperlipidemia and stimulating bone remodeling.

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2. Materials and methods

2.1. Materials

Paeoniflorin (C23H28O11; MW: 480.45; purity per HPLC: 98.78%) was purchased from Nanjing GOREN BIO Technology Co., Ltd. (Nanjing, China). Kits for measuring TC, triglycerides (TG), LDL-C, and high-density lipoprotein cholesterol (HDL-C), and kits for lysate alkaline phosphatase (ALP) and alizarin red analysis were obtained from Jian Cheng Biological Engineering Institute (Nanjing, China). The cell culture ALP kit was obtained from Sigma (St. Louis, MO). Dulbecco's modified Eagle's medium, fetal bovine serum, penicillin, and streptomycin were purchased from Hyclone through Thermo Fisher Scientific (Logan, UT, USA). ELISA systems kits for determining cross-linked C-terminal telopeptide of type 1 collagen (CTX-1), procollagen type 1 N-terminal propeptide (P1NP), and osteoprotegerin (OPG)/receptor activator of NF-κB ligand (RANKL) ratio were purchased from Immunodiagnostic Systems (Boldon, UK). Recombinant mouse bone morphogenetic protein-2 was purchased from Peprotech, Inc. (Rocky Hill, NJ, USA).

2.2. Study design

Twenty-four male Wistar rats (body weight, 140−160 g) were obtained from Shanghai Laboratory Animal Research Center (Shanghai, China). Rats were housed in cages under standard conditions with *ad libitum* access to water and to the diets according to their treatment group. Rats were randomized into three groups (n = 8, per group): (i) normal diet (normal control); (ii) HCHF diet, mock treated (HCHF group); and (iii) HCHF diet, paeoniflorin treated (paeoniflorin group). The control group received standard diet, whereas both HCHF groups received standard chow supplemented with 2% cholesterol and 15% lard. After 8 weeks on diet, the paeoniflorin group rats received paeoniflorin by oral gavage at a daily dose of 20 mg/kg; the mock-treated HCHF rats received an equal volume of distilled water. After treatment for 8 weeks, left femurs were scanned using micro-computed tomography (μCT) for measuring bone mass. Right femurs were harvested, cleaned of soft tissue, preserved in gauze moistened with phosphate buffered saline (PBS), and stored at −70 °C for biomechanical strength test.

2.3. Determination of serum biochemistry

Serum levels of TC, TG, LDL-C, HDL-C, CTX-1, and P1NP, and OPG/RANKL ratio were determined using commercial kits according to instructions.

2.4. Evaluation of bone microarchitecture by μCT

High-resolution μCT scanning (Skyscan 1176; Skyscan; Aartselaar, Belgium) was performed for quantitative analyses of osteolysis in mouse femoral condyle at a resolution of 30 mm, 70 kV, 500 mA. For trabecular and cortical bone parameters, the volume of interest, 200 slices, was selected as reference to the proximal growth plate. The trabecular and the cortical region started at 1.0 mm and 5.0 mm, respectively, from the growth plate level, extending towards the distal end of the tibia. Trabecular bone measurements were connectivity density (Conn.D), structural model index (SMI), bone volume against tissue volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular separation (Tb.Sp). Cortical bone parameters included cortical thickness (Ct.Th), cortical bone area (Ct.Ar), and cortical area fraction (Ct.Ar/Tt.Ar).

2.5. Bone biomechanical strength test

Right femurs were prepared by thawing at room temperature, weighing, and measuring the length and width. Evaluation of bone biomechanical strength by a three-point bending test was conducted using a servohydraulic materials testing machine (MTS 858 Mini Bionix II; MTS Systems Corp., Minneapolis, MN, USA). Femurs were mounted on two supported jigs with a span of 10 mm. During the test, a roller stamp with load was driven down (speed 5 mm/min) to the femur midpoint on its anterior surface until fractured. The load-deformation curve was generated and analyzed using TestStar II software to calculate load, stress, displacement, and strain of the bone. Load-displacement and stress-strain graphs were plotted to compute stiffness and Young's Modulus of elasticity from the respective curve gradient.

2.6. Cell spreading

Cell spreading was monitored by visualizing filamentous actin of the cytoskeleton in bone marrow stromal cells (BMSC). After 24-h incubation, BMSC monolayers were gently washed three times with PBS to remove unattached cells. Cells were fixed in 4% paraformaldehyde for 15 min at room temperature and permeabilized with 0.1% Triton X-100 in PBS for 10 min. After washing three times with PBS, cells were stained with rhodamine-phalloidin (5 units/mL; Biotium, Hayward, CA, USA) for 30 min and washed again three times with PBS. Cytoskeleton analysis was performed by confocal laser scanning microscopy using a Leica Microsystems TCS SP2 (Heidelberg, Germany).

2.7. ALP analysis assays

BMSCs were obtained after 2-week incubation in osteoblast differentiation medium. Lysate ALP activity was assayed according to instructions of the respective kit, using *p*-nitrophenolphosphate as a substrate at pH 10.2 and measuring product formation as absorbance at 405 nm. The kit for ALP measurements used naphthol phosphate as a substrate and fast violet B for product visualization at pH 9.5.

2.8. Alizarin red S staining and mineralization

BMSCs were analyzed after 3-week incubation in osteoblast differentiation medium. Cells were fixed as described above and washed with distilled water before the 10-min incubation at room temperature in 2% alizarin red S at pH 4.2. Images were captured with a Nikon camera. For quantification, cells were incubated in 10% cetylpyridinium chloride for 20 min to elute the alizarin red S stain; 100 μL of each sample was transferred to fresh 96-well plates and read in a microplate reader at 485 nm.

2.9. Statistical analysis

Results are presented as the mean ± standard deviation (SD). To determine statistical differences, data were analyzed by one-way ANOVA followed by Dunnett post hoc test; $p < 0.05$ indicated statistical significance.

3. Results

3.1. Effect of paeoniflorin on serum lipids

In the HCHF diet group, abdominal circumference, TG, TC, and LDL-C significantly increased, whereas HDL-C significantly decreased, compared with the values in the normal control group