Nutrition 33 (2017) 42–51

Contents lists available at ScienceDirect

Nutrition

journal homepage: www.nutritionjrnl.com

Basic nutritional investigation

Noni leaf and black tea enhance bone regeneration in estrogen-deficient rats

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ARTICLE INFO

Article history:
Received 28 April 2016
Accepted 18 August 2016

Keywords:
Morinda citrifolia leaves
Bone regeneration
Epicatechin
Scopoletin
Black tea
Remifemin

ABSTRACT

Objective: Black tea and Nonileaf are among the dietary compounds that can benefit patients with bone resorption disorders. Their bone regeneration effects and their mechanisms were studied in estrogen-deficient rats.
Methods: Noni leaves (three doses) and black tea water extracts were fed to ovariectomized rats for 4 mo, and their effects (analyzed via mechanical measurements, micro-computed tomography scan, and reverse transcriptase polymerase chain reaction mRNA) were compared with Remifemin (a commercial phytoestrogen product from black cohosh).
Results: The water extracts (dose-dependently for noni leaves) increased bone regeneration biomarker (runt-related transcription factor 2, bone morphogenetic protein 2, osteoprotegerin, estrogen receptor 1 [ESR1], collagen type I alpha 1A) expressions and reduced the inflammatory biomarkers (interleukin-6, tumor necrosis factor-α, nuclear factor [NF]-κB, and receptor activator of NF-κB ligand) mRNA expressions/levels in the rats. The extracts also improved bone physical and mechanical properties. The extracts demonstrated bone regeneration through improving bone size and structure, bone mechanical properties (strength and flexibility), and bone mineralization and density.
Conclusions: The catechin-rich extract favored bone regeneration and suppressed bone resorption. The mechanisms involved enhancing osteoblast generation and survival, inhibiting osteoclast growth and activities, suppressing inflammation, improving bone collagen synthesis and upregulating ESR1 expression to augment phytoestrogenic effects. Estrogen deficiency bone loss and all extracts studied (best effect from Morinda leaf at 300 mg/kg body weight) mitigated the loss, indicating benefits for the aged and menopausal women.

Introduction

Dietary compounds that can promote bone formation are a good complementary therapy for patients with bone resorption disorders. Phytocompounds that suppress osteoclast bone resorption activities may help prevent and treat osteoporosis, Paget's disease, and bone-associated inflammation such as in rheumatoid arthritis or periodontal disease. High intake of black tea and particular classes of flavonoids were associated with lower risk for fracture-related hospitalizations in elderly women at high risk for fracture [1]. Black tea consumption increased serum estradiol and prevented bone loss in an estrogen-deficient rat model [2]. Bone strength and integrity rely on sustaining a subtle balance between bone resorption by osteoclasts and bone formation by osteoblasts. With age, diseases, or sedentary lifestyle this balance tends to favor bone resorption rather than bone formation, making bones brittle and increasing fracture risk [3].

Phytoestrogens such as isoflavones from soybeans have shown modest to no effect on bone health. National Institutes of Health-supported clinical trials have failed to demonstrate a bone-sparing effect of soy isoflavones. However, a clinical trial report indicated Remifemin (black cohosh *Actaea racemosa*

This study was supported by the Herbal Development Office, Ministry of Agriculture (Grant number: NH0513S009). The authors have no conflicts of interest to declare.
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http://dx.doi.org/10.1016/j.nut.2016.08.006
0899-9007/

commercial product) and conjugated estrogens have beneficial effects on bone metabolism. Black cohosh extract stimulated osteoblast activity, whereas conjugated estrogens inhibited osteoclast activity [4].

Noni *(Morinda citrifolia* L.) is traditionally used as a poultice for broken bones and sprains, deep cuts, bruises, sores, and wounds [5]. Noni fruit extract reportedly increased osteoblast activity, enhanced matrix mineralization, and restrained osteoclast activity [6]. However excessive intake of Noni fruit extract may cause liver toxicity [7]. The water extract of Noni leaf (a vegetable) promoted osteogenic differentiation and matrix mineralization in human periodontal ligament cells [8]. Additionally, Noni leaf has been shown to be nontoxic and to have therapeutic and nutritional properties such as anticancer, antiviral, antibacterial, antitubercular, antiinflammatory, analgesic, hypotensive, and immune-enhancing effects [9–11].

Osteoporosis is defined as a condition whereby the bone mineral density (BMD) or mass is significantly below (>2.5 SD) the mean for normal young woman. Osteoporosis causes >8.9 million fractures annually [12], and affects >200 million women worldwide; approximately 10% aged 60, 20% aged 70, 40% aged 80, and 70% aged 90 [13]. Osteoporosis increases fracture risks and occurrence of physically debilitating injuries that affect physical and mental health. Physical activity and healthy diet (including calcium and vitamins) help ameliorate osteoporosis. There is good justification for finding vegetarian-compliant alternatives and complementary or preventive therapy for osteoporosis through functional food. The present study investigates the comparative efficacy and mechanisms by which *M. citrifolia* leaf, black tea, and Remifemin aqueous extracts enhanced bone regeneration in an estrogen-deficient osteoporosis rat model.

Methods

Plant material and aqueous extraction

M. citrifolia leaves (MCLs) were obtained from the Institute of Bioscience. A voucher specimen SK2322/14 was deposited at the Laboratory of Natural Products, Institute of Bioscience, Universiti Putra Malaysia. MCLs were oven dried at temperature of 60°C for 6 d. They were milled into fine powder and were extracted by boiling in distilled water (1:10, w/v) for 3 h. The resulting suspensions were filtered and evaporated to dryness at 60°C.

The black tea Lipton (Unilever Holdings Bhd, Kuala Lumpur, Malaysia) was used, and Remifemin (Schaper & Brummer GmbH & Co, Salzgitter, Germany) was purchased from the local pharmacy store. The 250 mg/kg black tea water extract and Remifemin were prepared daily [14].

The extracts (50 μL injection volume) were analyzed using Waters 2996, high-performance liquid chromatography (HPLC), equipped with an Atlantis C18 column (4.6 mm × 250 mm; 5 μm, Waters Corporation, Milford, MA, USA), with three solvents mobile phase system of A; acetonitrile (MeCN), B; methanol (MeOH), and C; 0.1 trifluoroacetic acid (TFA)% in H_2O (v/v); programmed in linear gradients as follows: 0 min, 10% A, 10% B, and 80% C; 15 min, 20% A, 20% B, and 60% C; 26 min, 40% A, 40% B, and 20% C; 28 to 39 min, 50% A, 50% B, and 0% C; and 40 to 45 min, 10% A, 10% B, and 80% C; and flow rate of 1.0 mL/min; monitored between 210 and 450 nm. Epicatechin, scopoletin, HPLC-grade MeOH, MeCN, and analytical grade TFA were from Merck (Darmstadt, Germany).

Fifty-six female Sprague-Dawley rats (3 mo old, weighing 250–300 g) were purchased from the Faculty of Veterinary Medicine, Universiti Putra Malaysia, and were acclimatized to laboratory conditions for 7 d before the experiment. Animals were housed in an environmentally controlled animal laboratory and maintained on a 12-h light/dark cycle at 25 ± 2°C. They were given standard pellet food (Teckland Diet) and water ad libitum throughout the experimental period. The experimental protocol was approved by the Institutional Animal Care and Use Committee, Universiti Putra Malaysia (REF: UPM/IACUC/AUP-R070/2013).

After the adaptation period, the rats were ovariectomized (OVX) or sham operated under anesthesia (12:80 mg/kg xylazine/ketamine, intraperioneally) whereby the ovaries were removed bilaterally. Sham-operated animals were operated in same manner, but the ovaries were only exposed. Four weeks after recovering from surgery, the estrogen-deficient rats were randomly divided into six groups: (1) control untreated OVX estrogen-deficient rats (OVX), distilled water administered, (2) OVX and treated with 100 mg/kg Remifemin, (3) OVX and treated with 250 mg/kg black tea (BT), (4) OVX and treated with 100 mg/kg Noni leaf extract (MCL100), (5) OVX and treated with 200 mg/kg Noni leaf extract (MCL200), and (6) OVX and treated with 300 mg/kg Noni leaf extract (MCL300). All samples were suspended in distilled water, and administered orally through an oral gavage. Rats in the sham groups were administered the same volume of distilled water. The treatments started 4 wk after the surgery and lasted for 16 wk. Rats' body weights were measured weekly.

At the end of 16 wk, all the rats were sacrificed under ether anesthesia. Blood samples were taken by cardiac puncture, then centrifuged at 2500*g* for 15 min to separate the serum and stored at −80°C for further analysis. The tibia and femurs were isolated and stored at −80°C for biomechanical and histopathological studies.

Bone physical parameters

Fresh isolated femur and tibia were weighed using an electronic scale. The thickness at epiphyseal regions and longitudinal length of right side of femur and tibia was measured using an electronic digital calliper. Bone mass density was compared for both the Archimede's method [15] and the manufacturer calibrated micro-computed tomography (micro-CT) software, for three- and two-dimensional (2D) bone mass density data respectively.

Bone mineral content (BMC; calcium, zinc, and magnesium) was determined by atomic absorption spectrophotometry (S Series GE712405 v1.30) [16]. The right tibiae were dried at 100°C for 24 h and placed in a muffle furnace at 550 to 600°C for another 24 h, and the ash weighed. The ash was pulverized into powder and hydrolyzed with 6M HNO_3 for mineral analysis measured at 4 sec (calcium: at 422.7 nm wavelength, 100% lamp current, 0.5 nm bandpass; zinc: 213.9 nm wavelength, 75% lamp current 7, 0.2 nm bandpass; magnesium: 285.2 nm wavelength, 75%, lamp current, 0.5 nm bandpass).

The biomechanical property was assessed by three-point bending test using the Instron (model 2242, Norwood, MA, USA) attached with a 500-N load cells, crosshead speed of 2 mm/min and the software program (Bluehill2, ver 2.19). Before mechanical testing, the left femurs were slowly thawed in saline (0.9% NaCl) at room temperature to prevent hydration. The femur was placed with the posterior side down on two supporting bars positioned 20 mm apart, and the maximum force required to fracture the bone, energy absorbed and bone flexibility was recorded from the load-deformation curve.

Micro-CT

Micro-CT scanning of the distal femur was conducted on SkyScan 1076 CT scanner system (SkyScan N.V., Kontich, Belgium). Images were obtained using 72 kV x-ray tube potential and 130 μA tube current, with pixel size of 18 μm. A stack of 2D x-ray shadow projections was reconstructed to obtain transverse images using NRecon software version 1.6.3.3 followed by the CTAn software version 1.11.0.0, for obtaining bone mass density, and bone morphometric parameters including bone area over total area (BA/TA), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and trabecular number (Tb.N). They were measured immediately below the growth plates as these are the most obvious regions for bone loss. During reconstruction, dynamic image range, postalignment value, beam hardening, and ring-artifact reduction were optimized for each experimental data set. Coronal, sagittal, and transverse 2D images of trabecular regions of interest were constructed using DataViewer software version 1.4.3.2 (SkyScan).

Serum biochemical markers

Serum calcium, phosphorus, and alkaline phosphatase (ALP) concentrations were measured using automatic biochemical analyzer. Serum interleukin (IL)-6, tumor necrosis factor (TNF)-α, receptor activator of nuclear factor (NF)-κB ligand (RANKL), osteoprotegerin (OPG), and osteocalcin (OC) concentration were determined using enzyme-linked immunosorbent assay kit (Cloud-Clone Corp, Houston, TX, USA), with <10% intraassay and <12% interassay variability according to the manufacturer.

Quantitative real-time polymerase chain reaction array

Tibia RNAs were isolated using a combination of Qiazol Lysis Reagent and RNeasy Mini Kit (Qiagen, Germany). The real-time polymerase chain reaction (RT-PCR) was performed using the Custom RT^2 Profiler PCR Array CAPR12761, plate format (12 genes × 8 rows) on Bio-Rad CFX96 with RT^2 SYBR green quantitative PCR (qPCR) master mix. Quantitative RT-PCR analysis was performed using the comparative threshold-cycle method to calculate fold-change in the gene expressions compared with control OVX estrogen-deficient rats. RT^2 Profiler PCR Array Data Analysis version 3.5 (SABiosciences; Fredrick, MD, USA), which normalized to *HSP90AB1* (NM_001004082) and *GAPDH* (NM_017008) as reference Housekeeping genes were used.