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The protective effects of triptolide on age-related bone loss in old male rats

Deqing Luo[a], Hongyue Ren[b], Huantang Zhang[c], Peng Zhang[d], Zunxian Huang[c], Hang Xian[a], Kejian Lian[a], Dasheng Lin[a,*]

[a] Orthopaedic Center of People's Liberation Army, The Affiliated Southeast Hospital of Xiamen University, Zhangzhou, 363000, Fujian, China
[b] Department of Pathology, The Affiliated Southeast Hospital of Xiamen University, Zhangzhou, 363000, Fujian, China
[c] Department of Orthopaedic Surgery, Quanzhou Orthopedic-Traumatological Hospital of Fujian Chinese Medical University, Quanzhou, 362000, Fujian, China
[d] Department of Orthopaedic Surgery, The First Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou, 310000, Zhejiang, China

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ABSTRACT

Background: Previous studies have showed that triptolide have a critical role in inhibiting osteoclast formation, bone resorption and attenuating regional osteoporosis. However, the protective role of triptolide on age-related bone loss has not been investigated. In the study, we assessed the effect of triptolide supplementation on bone microstructure and bone remolding in old male rat lumbars.

Methods: Fifty-two 22-month-old male Sprague-Dawley rats were randomly assigned to either triptolide treatment group or control group. Triptolide (15 μg/kg/d) or normal saline was administered to the rats of assigned group for 8 weeks. Lumbar bone mineral density (BMD) and bone microstructure were analyzed by micro-CT. Fluorochrome labeling of the bones was performed to measure the mineral apposition rate (MAR) and bone formation rate (BFR). Osteoclast number was also measured by TRAP staining. Plasma level of osteocalcin and tartrate-resistant acid phosphatase 5b (Tracp 5b) was also analyzed.

Results: Micro-CT results revealed that triptolide-treated rats had significant higher BMD, bone volume over total volume (BV/TV), trabecular thickness (Tb.Th), bone trabecular number (Tb.N), and lower trabecular separation (Tb.Sp) compared to the control group. Although fluorochrome labeling result showed no significant difference in MAR and BFR between the groups, triptolide decreased osteoclast number in vivo. In addition, a significant higher level of plasma Tracp 5b was observed in the triptolide-treated rats. Furthermore, triptolide also reduced the expression of receptor for activation of NF-κB ligand (RANKL) and increased osteoprotegerin (OPG) expression in the lumbars.

Conclusion: These results suggested that triptolide had a protective effect on age-related bone loss at least in part by reducing osteoclast number in elder rats. Therefore, triptolide might be a feasible therapeutic approach for senile osteoporosis.

1. Introduction

It is increasingly evident that senile osteoporosis is an age-related disorder characterized by bone loss and microstructural deterioration, which is the primary cause of osteoporotic fractures in the elderly [1,[2]]. Additionally, epidemiological study has demonstrated that the incidence of bone fracture increases with advancing age, which becoming a major public health problem since its association with medical costs and increased risk of mortality [3]. Bone homeostasis is maintained by a delicate balance between osteoclastic bone resorption and osteoblastic bone formation. Although it is clear that the balance between bone resorption and bone formation changes toward bone resorption with age, the underlying mechanisms are only partially understood [2]. Moreover, there is no standard therapeutic agent for the treatment of senile osteoporosis.

Tripterygium Wilfordii Hook F (TWHF) has been widely used in China for the treatment of various diseases, such as rheumatoid arthritis, nephritic syndrome, systemic lupus erythematosus and ankylosing spondylitis [4,5]. Triptolide, is a major component of TWHF, known for its anti-inflammatory and immunosuppressive activities [6,7]. Recently, triptolide has attracted the attention of researchers for its cardiovascular protective and anti-tumor properties [8,9]. In addition, triptolide also has attracted the attention of researchers for its potential beneficial effects on other pathological conditions. Interestingly, a recent study demonstrated that triptolide is able to inhibit osteoclast formation through suppressing RANKL-induced NF-κB activation which required for osteoclast development [10]. Besides, some studies have demonstrated the effect of triptolide on the prevention of regional osteoporosis induced by titanium particle in a rat model [11,12].

Based on the above observations, triptolide showed a potential

* Corresponding author.
E-mail address: linds@xmu.edu.cn (D. Lin).

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effect on bone loss disease. However, the potential therapeutic role of triptolide for the treatment of age-related bone loss has not been investigated. Therefore, we have conducted this in vivo study to investigate the effect of administration of triptolide on bone mass density, bone microstructure and bone turnover of old male rats.

2. Methods

2.1. Animal grouping and triptolide treatment

The experimental procedures were reviewed and approved by the Animal Care and Use Committee at Xiamen University and all rats were purchased from the animal laboratory of Xiamen University. Fifty-two 22-month-old male Sprague–Dawley rats, weighing approximately 520 g, were divided into two groups and were kept in rooms subjected to a 12-h light–dark cycle at the constant temperature of 23 °C. All rats were allowed free access to water and a standard rodent diet. Rats in experimental groups were administrated by intraperitoneal injection with triptolide at doses of 15 μg/kg/d for 8 weeks. While in the control groups, rats were treated with normal saline in a similar manner. At the end of treatment, all the mice were sacrificed.

2.2. Micro-CT measurement

Eight rats of each group were used for the measurement of trabecular bone structure in the L4 vertebr by micro-CT (SkyScan 1176, Bruker-MicroCT, Kontich, Belgium). All the species were scanned under the identical conditions (resolution: 18 mm, Source Voltage: 65 KV, Source Current: 385 μA, Rotation Step: 0.7°). The whole vertebra body of L4 was scanned and the trabecular bone region was outlined for each micro-CT slice, excluding both the cranial and caudal endplate regions. Within these regions, trabecular bone was defined by the inside of the cortical bone on both sides of the vertebr. After scanning, the micro-CT image data was transferred to a workstation and analyzed by the software in the micro-CT system. The following parameters of the trabecular bone microarchitecture were evaluated, such as bone mineral density (BMD), bone volume over total volume (BV/TV), trabecular thickness (Tb.Th), bone trabecular number (Tb.N) and lower trabecular separation (Tb.Sp). The terminology used in this study has been recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research [13].

2.3. Fluorochrome double labeling

Eight rats of each group were injected with tetracycline HCl (30 mg/kg) at 14 days and 4 days before sacrifice to assess fluorochrome labeling of the bones. At termination, L4 lumbar vertebrae were excised and cleaned of soft tissue. The samples were fixed in10% neutral buffered formalin overnight, then were transferred to 70% ethanol, and then embedded undecalcified in methylmethacrylate. Samples were sliced into 10 μm sections and the distance between the middle of two fluorescein labels was measured under a fluorescent microscope (ZeISS Imager M1, Germany). Then, mineral apposition rate (MAR) which is the distance between the midpoints or between the corresponding edges of two consecutive labels, divided by the time between the midpoints of the labeling periods, was calculated. Bone formation rate (BFR) was also calculated.

2.4. TRAP staining

Samples from L4 lumbar were fixed in 10% neutral buffered formalin overnight. After decalcified in 10% ethylenediaminetetraacetic acid (EDTA) at room temperature for 2 week, the samples were dehydrated in an ascending series of ethanol and cleared in xylene, and longitudinally embedded in paraffin. Then, samples were sliced into 6 μm sections. Subsequently, sections were deparaffinized with xylene and rehydrated with graded concentrations of ethanol. Finally, the 6-μm-thick sections were stained with TRAP and counterstained with hematoxylin (Sigma, St. Louis, MO, USA) according to the manuscript. Multinucleated cells that were TRAP positive and adjacent to a trabecular bone surface were counted as osteoclasts.

2.5. Measurements of plasma osteocalcin and Tracp 5b

At sacrifice, blood samples were obtained by cardiac puncture. The plasma samples were obtained after the blood samples were centrifuged at 3000 rpm for 15 min at 4 °C. Plasma concentration of osteocalcin, a bone formation marker, was measured using a commercially available ELLSA kit (Takara, Co, Ltd.). TRAPTM enzyme immunoassay kit (IDS, Tyne & Wear, UK) were used to determine the plasma level of Tracp 5b, a marker of bone resorption. All the procedures of detection were conducted according to the manufacturer's instruction.

2.6. Quantitative real-time reverse transcription PCR assay (Q-PCR)

L4 lumbars were harvested and frozen immediately in liquid nitrogen just before rats being killed. Total RNA was extracted and reverse transcribed. The PCR primers are as follows: RANKL, forward primer CACTATTAATGCCACCGAC, reverse primer GGGTATGAGAAC TTGGGATT; OPG, forward primer GCTTGAAACATAGGAGCTG, reverse primer GTTTACTTTGGTGCCAGG; GAPDH, forward primer CACATGG CCTCCAAGGAGTAAG, reverse primer TGAGGGTCTCTCTCTTCCTCT TGT [14]. Q-PCR was performed using a fluorescent temperature cycler (LC480 Real Time PCR System, Roche Co., Ltd). GAPDH was used as an internal standard.

2.7. Western blot analysis

L4 lumbar were frozen in liquid nitrogen, and homogenized in RIPA buffer [10 mM Tris (pH7.4), 150 mM NaCl, 0.5% NP-40, 0.1%SDS, 0.1% deoxycholate, 1 mM PMSF, 2 mM sodium fluoride, and 1 mM sodium orthovanadate] supplemented with Protease Inhibitor Cocktail (Sigma, St. Louis, MO, USA) and PhosSTOP Phosphatase Inhibitor Cocktail (Roche Applied Science, Mannheim, Germany). Tissue lysates containing equal amounts of proteins were separated on 10% SDS-polyacrylamide gels and transferred onto PVDF membranes, which were blocked in 5% milk in PBST for 1h, with the primary indicated antibody then being incubated overnight at 4 °C. After three washes in PBST, the membrane was incubated with secondary antibodies, and the signal was finally visualized with an enhanced chemiluminescence (ECL) system. β-actin was used as an internal control.

2.8. Statistical analysis

All the results were expressed as mean ± SD and analyzed by SPSS 13.0 for windows. Each assay was repeated in at least three independent experiments. The difference between triptolide-treated group and control group was analyzed using two-tailed non-parametric Mann-Whitney test. Any value of P less than 0.05 were considered statistically significant.

3. Results

3.1. Triptolide reduces severity of experimental senile osteoporosis

BMD results measured by micro-CT revealed a higher BMD of L4 vertebra in triptolide-treated rats, with a significant difference ($P < 0.001$) (Fig. 1A and B). Consistent with these changes in BMD, microstructural results of the vertebra trabecular bone evaluated by micro-CT showed that triptolide-treated rats had a significant higher bone volume over total volume (BV/TV = 23.25 ± 2.23%) than controlled rats (BV/TV = 19.23 ± 1.62%). Consistently, the results shown a higher