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Original Research Article

Tricin protects rats from ovariectomized-induced osteoporosis by enhancing Wnt/β-catenin pathway

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Abstract

Purpose: *To investigate the effects of tricin on ovariectomized-induced osteoporosis, and unravel the underling mechanism of action.*
Methods: *An osteoporosis rat model was established by conducting ovariectomy (OVX). Changes in the microstructure of the trabecular bone were visualized using Hematoxylin and eosin (H&E) staining, and a three-point bending test was employed to assess the biomechanical stability of the femurs, after the administration of tricin (20 and 40 mg/kg). Subsequently, bone marrow mesenchymal stem cells (BMSCs) were isolated and treated with tricin (7 and 15 μM). Alizarin red staining was performed to assess mineralization, and Runt-related transcription factor 2 (RUNX2); osteocalcin (OCN) and collagen type I alpha 1 (Col1a1) were quantified using western blot analysis. The Wnt/β-catenin pathway related proteins, i.e., Wnt3a, β-catenin, glycogen synthase kinase-3 β (GSK-3 β) were determined.*
Results: *Ovariectomy induced thinner and discontinuous trabecular bone, with increased marrow cavities, while application of tricin significantly improved the density and regularity meshwork, but reduced marrow cavities. Tricin also enhanced biomechanical competence as seen in the upregulated maximum load, stiffness, young modulus and maximum stress compared with OVX group ($p < 0.01$). Furthermore, tricin increased calcification in BMSCs, and significantly upregulated the expressions of RUNX2, OCN and COL1A1 when compared with OVX group ($p < 0.01$). It promoted Wnt/β-catenin signaling by enhancing Wnt3a and β-catenin, while inhibiting GSK3β expression, compared with OVX group ($p < 0.05$ or $p < 0.01$).*
Conclusion: *Tricin exerts protective effects against OVX-induced osteoporosis by enhancing Wnt/β-catenin pathway. Thus, tricin is a potential therapeutic agent for the management of osteoporosis.*

Keywords: *Tricin, Osteoporosis, Ovariectomy, Wingless, INT-1 (Wnt)/β-catenin*

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INTRODUCTION

Osteoporosis is a chronic metabolic disease manifested as reduced bone mineral density, degeneration of bone tissue, and qualitative changes in bone microstructure, all of which result in the susceptibility to fracture. Osteoporosis is prevalent in elderly postmenopausal women due to estrogen deficiency (postmenopausal osteoporosis). In addition, it has been reported that osteoporotic fracture is related to the increased mortality

among children aged 5-10 [1]. Bone remodeling cycle is a closely coupled process, in which the rate of bone reabsorption is roughly the same as that of new bone formation. However, the imbalance between osteoclasts and osteoblasts will lead to osteoporosis. Trabecular bone has a larger surface area and the most active bone metabolism, and the remodeling of the trabecular bone is more dominant [2]. Therefore, this study focuses on the trabecular bone.

The Wnt/β-catenin pathway plays a pivotal role in differentiation and development, and its disorder is considered to be related to various diseases such as degenerative disorders and malignancies. Compelling evidence has demonstrated that the Wnt/β-catenin pathway, also known as a canonical Wnt pathway, affects bone modeling and bone remodeling, especially osteoblast differentiation [3]. A recent study have reported that triggering the Wnt / β-catenin pathway contributes to bone formation and attenuates osteoporosis [4]. An in-depth understanding of the Wnt / β-catenin signaling pathways facilitates the development of potential therapeutics for osteoporosis. Tricin is a flavonoid compound extracted from bamboo leaves, and from grains such as rice, wheat, corn. Tricin is a widely distributed flavone with many biological characteristics, especially in drug activity. A previous study revealed that Tricin inhibited inflammatory responses in colon carcinogenesis [5]. Meanwhile, is was reported that tricin could exert inhibitory effects on cancer development, and it was suggested that tricin-containing food contains promising supplements for colorectal cancer patients [6]. In addition, a study has confirmed that tricin promotes MSC proliferation and mineralization through the regulation of Wnt / β-catenin pathway [7]. Nevertheless, the role of Tricin in osteoporosis and its related mechanism are still unclear.

This study established an OVX model, and osteogenic differentiation was evaluated in the trabecular bone, followed by Wnt / β-catenin signaling assessment.

EXPERIMENTAL

Animals

A total of 48 female Sprague Dawley rats (12-week-old, weighing 200 ± 10 g) were obtained from Animal Experiment Center of China, Three Gorges University (Yichang, China). Animals were allowed free access to water and diet, and kept in pathogen-free conditions at 22 ± 3 °C and 65 % humidity, with 12-h light/12-h dark cycles. After acclimatizing to the prevailing conditions, the animals were assigned into 4 groups (n = 12/group): sham group, OVX group, OVX + 20 mg/kg of tricin group, and OVX + 40 mg/kg of tricin group. The animal experiments in this study were approved by the Ethics Committee of the Central Hospital of Enshi Tujia and Miao Autonomous Prefecture (Approval No. 2020-19), and conducted in accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines [8].

Treatments

The OVX model was established as previously reported [9]. A combination of Vetalar V™ (75 mg/kg) and Dormitor™ (1 mg/kg) was intraperitoneally injected as anaesthetics. A 2 cm single midline dorsal incision was made and the oviduct was ligatured, followed by the removal of the ovary. Following ovariectomy, the uterus and the remaining part of the oviduct were placed in the abdomen. The ovary was then placed in the abdomen in the sham group rats. Subsequently, tricin (20 and 40 mg/kg) was intragastrically administered daily for 12 weeks. Rats in the sham group and OVX group were administered equal volumes of distilled water. At the end of the experiment, the rats were sacrificed under 1 % sodium pentobarbital anesthesia (0.4mL / 100 g). The left femurs and were exercised and stored at −80 °C.

Cell culture

After the rats were sacrificed, they were placed in 75 % ethanol for 5 min and the right femurs were collected. After femurs being washed, the epiphysis was exercised, and the marrow cavity was repeatedly washed using Dulbecco's modified Eagle's medium. Then, the flushing fluids were placed into a 25-ml culture bottle, and incubated for 48 h. The BMSCs were collected at 4th generation and assigned to sham group, OVX group, 7 μM Tricin group, and 15 μM Tricin group.

Histological examination

Histological changes in the trabecular bone were determined using H&E staining. Femurs were fixed in 4 % paraformaldehyde for 4 days, decalcified for 5 weeks using 0.5 M EDTA (Bohu Biological Technology Co. LTD, Shanghai, China), dehydrated and embedded in paraffin. The 5-μm thick sections were prepared and subsequently stained with H&E and Toluidine blue. The microstructural changes were examined under Olympus BX53 fluorescence microscope (Tokyo, Japan).

Biomechanical assessment

A three-point bending test was performed to assess the biomechanical stability of the femurs. Prior to biomechanical assessment, the length of the femurs and midshaft diameter were determined using a caliper. The biomechanical stability of the femoral diaphysis was assessed at the speed of 2 mm/min. The load and displacement were recorded when the femurs samples were broken with the central loading point changing. Next, the maximum load, stiffness, maximum stress and Young's modulus are recorded based on the load deformation curve.

Alizarin red staining

Mineralization was measured using the use of alizarin red staining. BMSCs (1×10^5 cells / well) were placed in 24-well plates, incubated with 2 % alizarin red (pH 4.1) for 10 min and rinsed with distilled water. The images of calcium deposition were visualized using a phase-contrast microscopy.

Immunoblotting analysis

Total protein was isolated from BMSCs using RIPA (Beyotime, China), and the concentration was quantified via bicinchoninic acid (Beyotime, China). A total of 30 µg of protein were separated by 10 % SDS-PAGE. transferred onto PVDF membranes (Millipore, USA), and blocked with 5 % non-fat milk for 2 h. The membranes were incubated with primary antibodies against RUNX2 (1:1000, #ab236639), OCN (1:1000 diluted, #ab93876), Col1a1 (1:1000, #ab270993), Wnt3a (1:1000, #ab219412), β-catenin (1:5000, #ab32572), GSK3β (1:500, #ab93926) at 4 °C overnight.

These antibodies were bought from Abcam, USA. The secondary goat anti-rabbit (HRP) IgG antibody (1:5000, #ab97080, Abcam) was then incubated with the members at room temperature for 2 h. The bands were visualized using enhanced chemiluminescence and quantified using ImageJ Software (version 1.38, NIH).

Statistical analysis

The results are expressed as mean ± standard deviation (SD). One-way ANOVA, and Dunnett's multiple comparison post hoc tests were employed for the comparison between groups. A value of $p < 0.05$ was considered indicative of statistically significant difference.

RESULTS

Tricin improved the microstructure of the trabecular bone in OVX rats

Changes in the microstructure are responsible for the bone strength, which makes it easier to fracture. As displayed in Figure 1, the femurs of the animals in the sham group had a dense and regular reticular structure, while ovariectomy led to a thinner and discontinuous trabecular bone, and the marrow cavities were increased in OVX group. Application of tricin markedly improved the microstructure, as seen in the density and regularity meshwork, as well as the reduction in marrow cavities. Besides, a dose of 40 mg/kg of tricin improved trabecular bone microstructure better than tricin at a dose of 20 mg/kg.

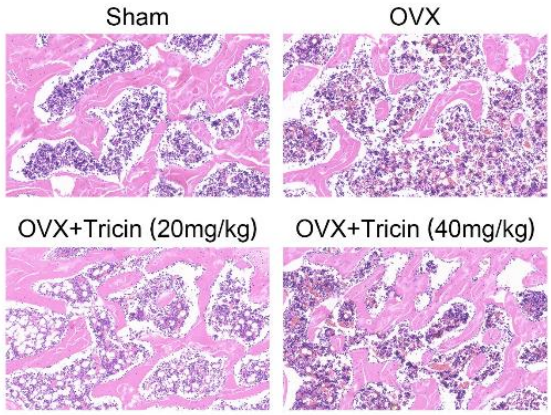

Figure 1: Tricin improved the microstructure of trabecular bone in OVX rats. Photomicrographs of H&E stained sections of sham, OVX, and tricin-treated rats (20 and 40 mg/kg)

Tricin enhanced biomechanical stability in OVX rats

Subsequently, the biomechanical parameters such as maximum load, stiffness, young modulus and maximum stress were evaluated in OVX and Tricin-treated rats. As shown in Figure 2, the levels of maximum load, stiffness, Young modulus and maximum stress were both significantly decreased after ovariectomy, when compared with the sham group; whilst, tricin administration significantly increased maximum load, stiffness, Young modulus and maximum stress ($p < 0.01$). In addition, 40 mg/kg of tricin showed a significant enhancement in biomechanical stability compared with tricin at a dose of 20 mg/kg.

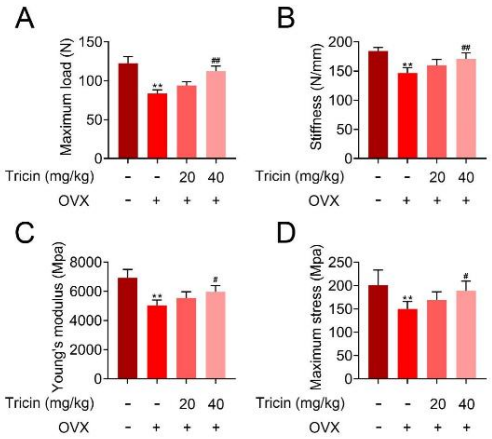

Figure 2: Tricin enhanced biomechanical competence in OVX rats. Biomechanical parameters such as maximum load (A), stiffness (B), Young modulus (C) and maximum stress (D) were quantified using 3 bending testing in OVX and Tricin-treated rats. $^{**}p$ < 0.01, *vs* sham group. $^{\#}p$ < 0.05, *vs* OVX group

Tricin promotes osteogenic differentiation in BMSCs

To investigate the effects of tricin on osteogenic differentiation, the alizarin red staining was performed in BMSCs. **Figure 3 A** showed that ovariectomy induced a significant decrease in calcium deposition, when compared with rats in sham group, while tricin at the dose of 7 and 15 μM visibly enhanced calcification. Immunoblotting analysis revealed that the protein levels of RUNX2, OCN and COL1A1 were significantly downregulated in OVX rats, when compared with sham group (p < 0.01). Meanwhile, administration of tricin upregulated RUNX2, OCN and COL1A1 levels with a dose-dependent manner, when compared with OVX group (p < 0.01).

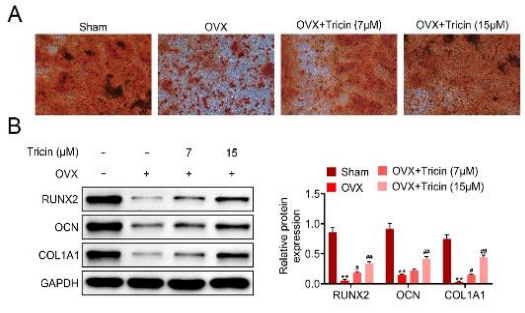

Figure 3: Tricin promoted osteogenic differentiation in BMSCs: A representative images of Alizarin red staining in Sham, OVX, 7 and 15 μM Tricin groups. B: The expression levels of RUNX2, OCN and COL1A1. $^{**}p$ < 0.01, versus sham group. $^{\#}p$ < 0.05, $^{\#\#}p$ < 0.01 versus OVX group

Tricin enhance Wnt/β-catenin signaling

Wnt / β-catenin signaling related genes such as Wnt3a, β-catenin and GSK3β were measured using western blot analysis. Figure 4 showed that ovariectomy led to significant reductions in levels of Wnt3a and β-catenin, but an increase in GSK3β when compared with sham group (p < 0.01). After treatment with 7 μM and 15 μM of Tricin, the levels of Wnt3a and β-catenin were significantly elevated, while the level of GSK3β was apparently decreased when compared with OVX group (p < 0.05 or p < 0.01). Tricin enhanced Wnt/β-catenin signaling.

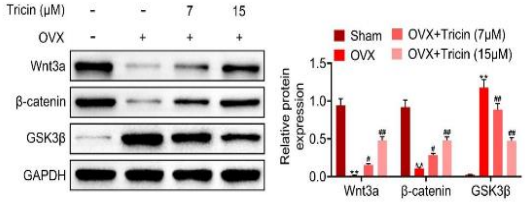

Figure 4: Tricin enhanced Wnt / β-catenin signaling. Western blot analysis was performed to determine the protein levels of Wnt3a, β-catenin and GSK3β in Sham, OVX, and tricin groups; $^{**}p$ < 0.01, versus sham group; $^{\#}p$ < 0.05, $^{\#\#}p$ < 0.01 versus OVX group

DISCUSSION

Sex hormones are essential in regulating peak bone mass and preventing bone loss. Thus decrease in estrogen will accelerate bone loss, and cause imbalance between bone reabsorption and bone formation, which leads to osteoporosis [10,11]. Post-menopausal women who lack oestrogen, especially those with osteoporosis, have worse bone quality and serious deterioration of microstructure. At present, ovariectomy in rodents is considered as the most widely used animal model for oestrogen depletion. Wang *et al* posited that ovariectomy induced disorders in trabecular bone microstructure, and the trabecular bone as well as cortical bone were thinner [12]. In this study, ovariectomy was performed in rats, and the OVX rats presented thinner and discontinuous trabecular bone, and the marrow cavities were decreased. However, the application of Tricin improved the density and regularity meshwork, and improved marrow cavities. Besides, tricin at a dose of 40 mg/kg showed a significant enhancement in trabecular bone microstructure.

As a consequence of the disparity in quantities of osteoblast and osteoclast, osteoporosis is associated with osteogenic differentiation. The RUNX2, OCN and COL1A1 are markers of osteogenic differentiation, and Xu *et al*

demonstrated that RUNX2, OCN and COL1A1 levels were elevated in mesenchymal stem cells with osteogenic differentiation [13]. A recent study revealed that overexpression of miR-7-5p promoted osteoblast differentiation and enhanced the expression of alkaline phosphatase (ALP), Runx2, Col1a1 and OCN [14]. Compelling evidence suggest that estrogen deficiency suppressed the expression of ALP and Runx2 at 2 and 7 days, and the alteration in Col1a1 was observed at day 14 under the condition of estrogen deficiency and daily mechanical challenge [15]. Wu and colleagues reported that OVX rats displayed reduced ALP, Runx2, Col1a1 and OCN, while the administration of cinnamaldehyde improved osteoblast differentiation by increasing the levels of ALP, Runx2, Col1a1 and OCN [16]. Consistent with these results, this study revealed that the levels of RUNX2, OCN and COL1A1 were significantly downregulated in OVX rats, while Tricin promoted osteogenic differentiation.

Wnt / β-catenin pathway is crucial in bone biology. In the canonical pathway, Wnt3a is known to bind to LRP5/6 receptor on the cell membrane and induce further accumulation of β-catenin in the cytoplasm, which is subsequently transferred to the nucleus. Wnt3a stabilizes β-catenin via the dissociation between β-catenin and GSK-3β, while GSK-3β inhibits the phosphorylation and degradation of β-catenin. In bone sarcoma cells, Wnt3a stimuli was proven to induce the accumulation of β-catenin in nucleus [17]. It has been reported that Ti particle activated GSK-3β but inhibited cytoplasmic and nuclear β-catenin expression, while administration of LiCl, a GSK-3β inhibitor, significantly increased β-catenin accumulation in the nucleus [18].

Suppression of the Wnt/β-catenin led to impaired osteogenic differentiation, while the application of lithium chloride provoked bone restoration in periapical bone tissues [19]. In the current study, results from immunoblotting demonstrated that ovariectomy led to reductions in the levels of Wnt3a and β-catenin, but an increase of GSK3β. Whist, Tricin significantly elevated the levels of Wnt3a and β-catenin, while the GSK3β levels were decreased. These findings suggest that Tricin attenuated OVX-induced osteoporosis by enhancing Wnt3a and β-catenin accumulation, but induced GSK-3β degradation.

CONCLUSION

The findings of this study show that tricin attenuates OVX-induced osteoporosis in rats via the promotion of Wnt/β-catenin pathway. These findings may be useful in the development of an alternative treatment for osteoporosis.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Rui Lei and Hong Du designed the experiments and carried them out. Lingbo Hu analyzed and interpreted the data, Xiaoyan Liu and Guogeng Sun prepared the manuscript with contributions from all co-authors. Rui Lei and Hong Du contributed equally to the work and should be considered as co-first authors.

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