Raloxifene improves TNF-α-induced osteogenic differentiation inhibition of bone marrow mesenchymal stem cells and alleviates osteoporosis

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Abstract. Effect of raloxifene (RLF) on the improvement of inhibited osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) resulted from tumor necrosis factor-α (TNF-α) induction, thus alleviating the progression of osteoporosis (OP), was investigated. An in vivo OP rat model was constructed by performing the procedures of ovariec-
tomy (OVX). Rats were randomly divided into sham group, OVX group and RLF+OVX group. BMSCs were extracted from healthy rats, and randomly divided into control group, TNF-α group, RLF group and TNF-α+RLF group. Viability and cellular calcification ability in each group were detected. The relative levels of osteocalcin (OCN), Runx2 and NF-κB in cells with different treatments were determined. The body weight of rats in the OVX group and RLF+OVX group gradually increased compared with that in the sham group on the 8th week. No significant difference in body weight was observed between the rats of the OVX group and RLF+OVX group. Bone metabolism index (BMD) in the rats of the RLF+OVX group was higher than that of the OVX group, and lower compared with that of the sham group. Compared with the sham group, the elastic/max radial degree and elastic/max load of femora were reduced in the OVX group and RLF+OVX group, especially in the OVX group. The relative levels of OCN and Runx2, as well as the ALP activity and calcification ability, were decreased in the OVX group compared with the sham group, and the effect was partially reversed by the RLF treatment. After osteogenic differentiation of BMSCs, the viability and calcification ability were markedly reduced in TNF-α group, which was reversed by RLF treatment. Moreover, TNF-α induction downregulated the relative levels of OCN and Runx2, and RLF treatment could enhance their levels. The upregulated NF-κB protein level, induced by TNF-α, was reduced after RLF treatment. TNF-α induction inhibits osteo-
genic differentiation of BMSCs, which could be remarkably alleviated by RLF. It is suggested that RLF contributes to the alleviation of OP progression.

Introduction

Osteoporosis (OP) is a systemic bone disease manifested as low bone mass, destruction of bone microstructure, increased bone fragility and fracture risk (1). The prevalence of OP is on the rise due to the aging of population. It has been reported that in 2010 in China, the prevalence of OP in females >70 years of age was 40.0-59.3%, and in males was 14.2-18.9% (2). OP-induced pain and fracture severely affect life quality and pose economic burden on the elderly (3). It is of significance to focus on the prevention and treatment of OP.

The prevention and treatment strategies of OP are comprehensive, including lifestyle adjustment, calcium and vitamin D supplementation, and application of anti-OP drugs. Estrogen replacement therapy (ERT) can prevent bone loss caused by estrogen deficiency; however, ERT increases the risks of uterine and breast cancer (4). Selective estrogen receptor modulators (SERMs) have been developed as tissue-specific estrogen agonists that are applied for the treatment of postmenopausal OP (5). Raloxifene (RLF) is a second-generation SERM for treating postmenopausal OP which does not have the adverse effects of ERT (6). As an estrogen agonist on bone and several other tissues, RLF suppresses bone loss and reduces fracture risks. In addition, RLF reduces the susceptibility to uterine
cancer as an estrogen antagonist (7). Currently, RLF has been utilized in the clinical treatment of OP (8); however, the specific pharmacological role of RLF remains to be further explored.

Tumor necrosis factor-α (TNF-α) is a cytokine produced by activated macrophages/monocytes that exerts a crucial role in osteogenic differentiation of stem cells (9). A great number of studies have demonstrated the involvement of TNF-α in mediating multiple pathways related to osteogenic differentiation, such as Wnt, Smads and NF-kB pathways (10-12). Nevertheless, controversies exist regarding the factors that determine the promotive or inhibitory role of TNF-α in osteogenic differentiation (13). The aim of the present study was to investigate the effect of RLF on TNF-α-induced inhibition of osteogenic differentiation and the potential mechanism, and the conclusions of the study may provide new insights for the clinical treatment of OP.

Materials and methods

Experimental animals. Fifty-four female Sprague-Dawley (SD) rats, 12-weeks old and weighing 238.4±14 g, were provided by the Shanghai SIPPR-Bk Lab Animal Co., Ltd. Rats were randomly divided into the sham group, ovariec-tomy (OVX) group and OVX+RLF group, with 18 rats in each group. The rats in the OVX+RLF group were administered with 0.2 µM RLF. The body weight of each rat was recorded every week. Rats were housed in a temperature controlled room (21±2˚C) on a 12:12-h light/dark cycle (lights on at 06:00), and all rats had free access to water and food. The study was approved by the Animal Ethics Committee of Beihang University Animal Center (Beijing, China).

Preparation of OVX procedure. Rats were anesthetized by peritoneal administration of 40 mg/kg pentobarbital sodium. After shaving and skin disinfection, a 2-cm longitudinal incision at 1 cm near the spine and 2 cm above posterior iliac crest was made. The abdominal cavity was exposed to resect ovaries. The wound was sutured in layers. Postoperative peri-operative administration of 1 mg/kg gentamicin was applied. The rats peritoneal administration of 40 mg/kg. The rats were sacrificed by activated macrophages/monocytes that exerts a crucial role in osteogenic differentiation of stem cells (9). A great number of studies have demonstrated the involvement of TNF-α in mediating multiple pathways related to osteogenic differentiation, such as Wnt, Smads and NF-kB pathways (10-12). Nevertheless, controversies exist regarding the factors that determine the promotive or inhibitory role of TNF-α in osteogenic differentiation (13). The aim of the present study was to investigate the effect of RLF on TNF-α-induced inhibition of osteogenic differentiation and the potential mechanism, and the conclusions of the study may provide new insights for the clinical treatment of OP.

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MicroCT. After rats were sacrificed, the femoral metaphysis and attached surrounding soft tissues were removed, fixed in 4% paraformaldehyde solution, and scanned with SCANCO medical microCT (SCANCO Medical AG). Bone histomor-phology indicators were determined and analyzed using Image Processing Language (version X; Adobe Systems, Inc.).

Biomechanical examinations. Three-point bending exami-nation was conducted to conduct elastic/max radial degree and elastic/max load of rat femora. Briefly, the right femora were placed on the Instron Material Mechanics Testing Device (Instron). At the middle position of femora, a persistent test velocity of 1 mm/min was loaded until femoral fracture. Data were recorded and analyzed to obtain the max load.

Isolation and culture of bone marrow mesenchymal stem cells (BMSCs). Rats were sacrificed by cervical disloca-tion after being anesthetized with pentobarbital sodium via peritoneal administration at a dose of 40 mg/kg. The rats were immersed in 75% ethanol for 5 min and isolated for femora and humeri. Rat bones were washed and the epiphysis of long bones was removed. Marrow cavity was repeatedly washed by Dulbecco's modified Eagle's medium (DMEM), and the fluids were inoculated in a 25-ml culture bottle. After incubation for 48 h, the un-adherent cells were washed. Cell passage was performed until 80-90% confluence. The fourth generation BMSCs were harvested and divided into control group, TNF-α group, RLF group and TNF-α+RLF group.

Osteogenic differentiation. Fourth generation BMSCs were cultured in a 25-ml culture bottle at 1x10⁵ cells/l. BMSCs were subjected to osteogenic differentiation in DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 10 mmol/l dexamethasone, 10 mmol/l β-glycerophosphate, 50 µg/ml ascorbic acid, 1% L-glucose and 1% penicillin-streptomycin. Osteogenic induction medium was replaced every 2 days.

Reverse transcription-quantitative polymerase chain reac-tion (RT-qPCR). Total RNA was extracted from BMSCs using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and the concentration of total RNA was measured using an ultraviolet spectrophotometer (Hitachi, Ltd.). Total RNA was reverse transcribed into cDNA at 50˚C for 45 min by using PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd.), according to the manufacturer’s protocol. qPCR was subse-sequently performed using the SYBR-Green Master kit (Roche Diagnostics). The reaction system volume was 25 µl in total and the thermocycling conditions were: pre-denaturation at 95˚C for 5 min, denaturation at 95˚C for 30 sec, annealing at 60˚C for 45 sec, extension at 72˚C for 3 min, with 35 cycles, and then extension at 72˚C for 5 min. qPCR products were stored at 4˚C. The relative levels were quantitatively analyzed using the 2-∆∆CT method (14). GAPDH was used as internal reference. Primer sequences were as follows: osteocalcin (OCN) forward, 5’-GCCCTGACTGATTCTGCCTCT-3’ and reverse, 5’-TCACACCTTACTGCCCCTCTCTG-3’; Runx2 forward, 5’-GGACCGACACGACGCTATAAA-3’ and reverse, 5’-GCCTCATCCCTTAACCTGAAA-3’; GPDH forward, 5’-GCAAGGATACCTGAGACAGAG-3’ and reverse, 5’-GGATGGAATTGTGAGGGAGATG-3’.

Determination of alkaline phosphatase (ALP) activity. BMSCs were subjected to osteogenic differentiation for 7 days. After cell lysis and centrifugation, the supernatant was harvested to determine the absorbance at 520 nm. The relative ALP activity was calculated based on the protocols of ALP determination kit (Beyotime Institute of Biotechnology).

Alizarin red staining. BMSCs were subjected to osteogenic differentiation for 21 days. Cells were washed with PBS twice, fixed in 4% paraformaldehyde for 10 min at 37˚C and stained with 0.1% alizarin red (pH 4.1) for 10 min at 37˚C. Calcification nodules were observed and captured using a light inverted microscope (magnification, x200; BX-42, Olympus Corporation).

Cell Counting Kit-8 (CCK-8). BMSCs were seeded in a 96-well plate with 3x10⁴ cells/well. The absorbance at 450 nm
was recorded at the appointed time-points using the CCK-8 kit (Dojindo Molecular Technologies, Inc.) for depicting the viability curve.

Western blot analysis. Total protein was extracted from BMSCs using radioimmunoprecipitation assay and was quantified by bicinchoninic acid (both from Beyotime Institute of Biotechnology) method. A total of 30 µg of protein were loaded per lane for electrophoresis. The extracted proteins were separated using a 10% SDS-PAGE gel. After transferred onto PVDF membranes (EMD Millipore) the proteins were blocked with 5% skim milk at 20˚C for 2 h. The membranes were incubated with primary antibodies at 4˚C overnight and secondary antibodies at 20˚C for 2 h. Bands were exposed by enhanced chemiluminescence (ECL) detection kit (Amersham; GE Healthcare) and analyzed by ImageJ Software (version 1.38; National Institutes of Health). Rabbit polyclonal NF-κB antibody (dilution: 1:500; cat. no. ab8805), rabbit polyclonal GAPDH antibody (dilution: 1:500; cat. no. ab37168) and secondary goat anti-rabbit (HRP) IgG antibody (dilution: 1:2,000; cat. no. ab6721) were all purchased from Abcam.

Statistical analysis. SPSS 20.0 statistical software (IBM Corp.) was used for data analysis. Data were expressed as the mean ± standard deviation. Comparisons between multiple groups were made using one-way ANOVA followed by the Least Significant Difference post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

RLF influences body weight, bone metabolism index (BMD) and biomechanical parameters. Rat body weight was weekly recorded during the whole experiment. All rats in the three groups presented an increased trend in the body weight. Compared with the sham group, the body weight of the rats in the RLF+OVX and OVX groups was gradually elevated in the 8th week. However, no significant difference was observed in the body weight of rats between the RLF+OVX and OVX group (Fig. 1A). In the RLF+OVX group, BMD was higher compared with that in the OVX group and lower than that of the sham group (Fig. 1B). The biomechanical parameters of femoral radial degree and load were also examined. Compared with the sham group, the elastic radial degree/load and max radial degree/load were reduced in the OVX group. However, the RLF treatment markedly increased these biomechanical parameters (Fig. 1C and D).

RLF enhances the osteogenic differentiation ability of BMSCs. The relative levels of OCN and Runx2 were downregulated in
the OVX and RLF+OVX group, and especially in the OVX group (Fig. 2A and B). Similarly, the ALP activity was lower in rats undergoing OVX compared with that in rats of the sham group. RLF treatment enhanced ALP activity in OVX rats; however, ALP activity was still lower than that of the sham group (Fig. 2C). Alizarin red staining revealed a pronounced increase in the calcification ability of the RLF+OVX group, indicating the promotive effect of RLF on osteogenic differentiation (Fig. 2D).

**RLF improves viability and calcification ability of BMSCs.** To further uncover the *in vitro* effects of RLF on osteogenic differentiation of BMSCs, the primary BMSCs were divided into four groups based on different treatments. Cell viability markedly increased in the RLF group compared with the TNF-α group, and RLF treatment enhanced the calcification ability of BMSCs (Fig. 3).
decreased after TNF-\(\alpha\) induction in BMSCs. Conversely, RLF treatment could promote their viability. Notably, RLF treatment accelerated the viability in TNF-\(\alpha\)-induced BMSCs (Fig. 3A).

Furthermor
e, the RLF treatment stimulated the calcification ability in BMSCs; however, the calcification ability was markedly inhibited by TNF-\(\alpha\) induction (Fig. 3B).

RLF regulates osteogenesis-related genes and the NF-\(\kappa\)B pathway. TNF-\(\alpha\) induction downregulated OCN and Runx2 in BMSCs, which effect was partially reversed after the RLF treatment (Fig. 4A and B). The protein level of NF-\(\kappa\)B was determined in each group. After TNF-\(\alpha\) induction, the protein level of NF-\(\kappa\)B was remarkably upregulated, which was suppressed by the RLF treatment (Fig. 4C and D).

Discussion

Bones maintain structural stability through sustained bone remodeling and resorption. Bone tissues include osteoblasts, osteoclasts, and osteocytes. The osteogenic capacity of osteoblasts and the bone resorption function of osteoclasts maintain the balance of bone remodeling. Once the balance breaks, it leads to bone diseases, such as OP (14). OP is common in the elderly and enhances the fracture risks. It is estimated that 72% of females and 62% of males >50 years of age will suffer from OP or osteopenia in 2022 (15). OP-induced fractures pose a huge economic burden on the society (16).

ERT markedly alleviates bone mass reduction and reduces the incidence of fracture. However, several large-scale clinical trials have proposed that ERT increases the susceptibility to invasive breast cancer, coronary disease, stroke and pulmonary embolism (17). Selective estrogen receptor modulators (SERMs) have attracted attention because of their estrogen agonistic or antagonistic effects. RLF, the new generation of SERMs, has been applied as an ideal drug for postmenopausal OP (18).

Cytokines can stimulate the development of osteoclasts in the presence of stromal cells or osteoblasts, including IL-1\(\beta\), IL-1, IL-6 and TNF-\(\alpha\). It has been suggested that these cytokines may indirectly induce osteoclast formation through autocrine or paracrine modes (19). TNF is mainly synthesized by mononuclear macrophages, and can also be secreted by T cells, natural killer cells, chondrocytes, and osteoblasts (20).

Roggia et al (21) have pointed out the rapid bone loss in OVX mice, whereas TNF-\(\alpha\) deficiency mice do not present such a change, indicating the crucial role of TNF-\(\alpha\) in the development of OP induced by ovarian function decline. It has been reported that estrogens suppress the expression of TNF-\(\alpha\) in monocytes (22). Consistently, this study demonstrated the inhibitory effect of TNF-\(\alpha\) on osteogenic differentiation, which was reversed by RLF treatment through the NF-\(\kappa\)B pathway.

In conclusion, TNF-\(\alpha\) induction inhibits osteogenic differentiation of BMSCs, which could be remarkably alleviated by RLF. It is suggested that RLF contributes to the alleviation of OP progression.
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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors’ contributions

FY, JL and XN designed the study and performed the experiments. FY, YJ and QS established the animal model. CZ and CL acquired the data and were also involved in the conception of the study. WW, LD and SK analyzed the data. FY, JL and XN prepared the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Animal Ethics Committee of Beihang University Animal Center (Beijing, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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