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Original Article

Effect of TNF-α on osteoporosis by regulating the RANKL/OPG signaling pathway

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Abstract: Osteoporosis (OP) is a chronic systemic skeletal system disease characterized by decreased bone mass, reduced bone density, structural changes, and weakened biomechanical properties, which are prone to fracture. Tumor necrosis factor-α (TNF-α) can inhibit the osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs). However, the role of TNF-α in OP has not been elucidated. SD rats were randomly divided into 3 groups, including the OP rat model group that was prepared by ovariectomy; the sham operation group; and the treatment group that was administrated TNF-α neutralizing antibody. TNF-α expression was detected by enzyme-linked immunosorbent assay (ELISA). Bone density was tested by dual energy line bone densitometer. Serum alkaline phosphatase (BALP) and osteocalcin (OC) levels were detected by ELISA. Osteogenic factors Opn and Runx2 expression levels were determined by real-time PCR. Alkaline phosphatase (ALP) content was analyzed. RANK ligand (RANKL) and osteoprotegerin (OPG) protein expression in lumbar vertebrae was detected by Western blot. TNF-α expression increased, bone density reduced, serum BALP and OC levels elevated, Runx2 and Opn mRNA expression were downregulated, whereas ALP content declined, RANKL protein enhanced, and OPG protein was attenuated in the OP group compared with sham group ($P < 0.05$). TNF-α neutralizing antibody treatment inhibited TNF-α expression, elevated bone density, decreased serum BALP and OC expression, upregulated Runx2 and Opn mRNA expression, increased ALP content, attenuated RANKL protein expression, and enhanced OPG protein expression compared with the OP group ($P < 0.05$). TNF-α was increased in OP. Down-regulation of TNF-α promoted osteogenic differentiation and increased bone mineral density by regulating the RANKL/OPG pathway, thereby improving osteoporosis.

Keywords: Osteoporosis, TNF-α, RANKL, OPG, osteogenic differentiation

Introduction

Osteoporosis (OP) is a chronic systemic skeletal system disease, which is common in the elderly and menopausal women [1, 2]. With the natural loss of bone and menopause, taking drugs such as glucocorticoids, the incidence of OP is increasing. Currently, there are more than 200 million OP patients worldwide [3, 4]. Among the OP caused by many factors, postmenopausal women can have bone loss due to the decrease of hormone levels such as estrogen [5]. Postmenopausal osteoporosis accounts for a large proportion of OP [6]. OP is mainly characterized by decreased bone mass, reduced bone density, structural changes, and weakened biomechanical properties, which are prone to fracture [7]. OP not only causes orthopedic diseases, but also has adverse effects on the oral cavity, which can lead to serious oral problems, such as atrophy of the gums, alveolar bone absorption, and root exposure, resulting in a serious decline of the quality of life [8, 9]. The high disability rate and mortality rate of OP seriously affect the quality of life and bring a heavy psychological burden to patients [10]. With the advancement of medical technology, there are many methods for the diagnosis and treatment of OP. However, despite the symptomatic methods of inhibiting bone resorption, estrogen supplementation, and calcium supplementation, OP treatment efficacy is poor so far due to problems such as poor drug absorption and drug side effects [10].

TNF-α is an important cytokine that plays a crucial role in diseases, such as tumor and inflammation [11, 12]. It was showed that TNF-α can

cause stem cell dysfunction during chronic inflammation, and can inhibit osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) [13]. In gingival inflammation, an increase in TNF-α can cause excessive proliferation of stromal cells in the gingiva, which in turn induces inflammatory gingival hyperplasia [14, 15]. However, the effect of TNF-α in OP has not been elucidated. Therefore, this study analyzed the role of TNF-α in OP and related mechanisms by establishing an OP rat model.

Materials and methods

Experimental animals

Twenty healthy female Sprague-Dawley rats at 2 months old, SPF grade, and weighted 250 ± 20 g were purchased from the experimental animal center of Shandong University. The rats were fed in SPF animal experiment center with the temperature at 21 ± 1°C, relative humidity at 50-70%, and 12 hour day/night cycle. Animal experiments were performed in strict accordance with the experimental design by experienced technicians to minimize animal suffering. This study was approved by Ethics Committee in the First People's Hospital of Guiyang and all the enrolled objects had signed informed consent. Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Yucheng people's hospital, Yucheng, Shandong, China.

Main reagents and instruments

Sodium pentobarbital was purchased from Shanghai Zhaohui Pharmaceutical Co., Ltd. Western blot related chemical reagents were purchased from Beyotime. ECL reagents were purchased from Amersham Biosciences. Rabbit anti-mouse RANKL and OPG monoclonal antibodies and goat anti-rabbit horseradish peroxidase (HRP) IgG secondary antibody were purchased from Cell Signaling. TNF-α neutralizing antibody was purchased from Sigma. TNF-α, BALP, and OC ELISA kits were purchased from R&D. ALP active colorimetric quantitative detection kit was purchased from Nanjing Jiancheng Bioengineering Institute. RNA extraction kit and the reverse transcription kit were purchased from Axygen. Other commonly used reagents were purchased from Sangon. The ABI 7700 Fast Quantitative PCR Amplifier was purchased from ABI. The Labsystem Version 1.3.1 microplate reader was purchased from Bio-rad. The VD-650 clean bench was purchased from Suzhou Purification Equipment Factory. Medical surgical microscopy equipment was purchased from Suzhou Medical Instrument Factory. The dual-energy X-ray absorptiometry instrument was purchased from GE Corporation.

Methods

Animal modeling and grouping: Twenty healthy female SD rats were randomly divided into 3 groups with 10 in each group, including OP group that was established by ovariectomy, sham operation group, and the treatment group that was treated by 5 μg/kg TNF-α neutralizing antibody tail vein injection in OP rats.

Rat OP model establishment: According to the literature [16], rats were fixed on the operating table after intraperitoneal injection of 30 mg/kg sodium pentobarbital and sterilized by iodophor. A 0.5 cm longitudinal incision was made at the junction of the bilateral spine under the costal margin. The skin was separated and the back muscle was cut to expose the ovaries. The fat around the ovary was isolated, and the fallopian tubes and blood vessels were ligated to completely remove the ovary. The uterus was placed back to the abdominal cavity, and the incision was sutured. The surgical procedure of the sham operation group was the same as that of the OP group without removing the ovary. A conventional 200,000 IU/kg penicillin anti-inflammatory treatment was performed after surgery.

Sample collection and bone density analysis: After treatment, 5 ml of blood was collected from the tail vein and centrifuged at 3000 rpm for 15 minutes. The upper serum was aspirated and stored at 20°C for ELISA. The rats were sacrificed and the lumbar vertebrae were taken for subsequent experiments. Bone density measurements were used to detect changes in bone density.

ELISA: Blood was taken from the abdominal aorta and centrifuged at 2000 rpm for 10 minutes. The serum was aspirated and separated. The experimental procedure was followed according to the ELISA kit instructions. The linear regression equation of the standard curve was drawn according to the corresponding absorbance (A) value to calculate the sample concentration.

Real-time PCR: Total mRNA was extracted using Trizol reagent and reverse transcribed to

Table 1. Primer sequences

Gene	Forward 5'-3'	Reverse 5'-3'
GAPDH	AGTAGTCACCTGTTGCTGG	TAATACGGAGACCTGTCTGGT
Runx2	CATGGCGAAGGATGGA	TCAAAG CTCGCTGGTA
Opn	TCCAACATGACTAGGC GA	ACAACTCCCATGCGTCGAG

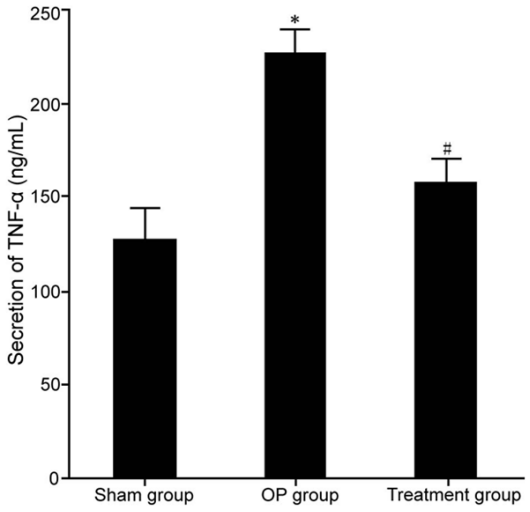

Figure 1. TNF-α expression in the OP rat. *P < 0.05, compared with sham group. #P < 0.05, compared with OP group.

DNA according to the kit instructions. The primers were designed by Primer 6.0 Premier 6.0 software and synthesized by Invitrogen (**Table 1**). The PCR reaction was performed at 55°C for 1 minute, followed by 35 cycles of 92°C for 30 seconds, 58-60°C for 45 seconds, and 72°C for 35 seconds. The relative expression level was calculated by $2^{-\Delta Ct}$ method.

Western blot: BMSCs were added with lysis and quantified by BCA method. The isolated proteins were electrophoresed using 10% SDS-PAGE. The gel was transferred to PVDF membrane by semi-dry transfer method at 100 mA for 1.5 hours. After blocked for 1 hour, the membrane was incubated in RANKL and OPG primary antibodies (1:1000 and 1:800, respectively) at 4°C overnight. After incubated in secondary antibody (1:2000) avoid of light for 30 minutes, the membrane was imaged using chemiluminescence reagent for 1 minute and analyzed by image processing system software and Quantity one software. The experiment was repeated four times (n = 4).

ALP content detection: ALP content was determined according to the instructions of the ALP test kit. The supernatants of each group were collected and centrifuged at 1000 rpm for 10 minutes. Next, the cells were collected by adding Triton-X100. The optical density (OD) values of each group were measured at 520 nm to calculate the ALP content.

Statistical analysis

All data analyses were performed on SPSS 16.0 software. The enumeration data were compared by χ^2 test. The measurement data are presented as mean ± standard deviation and compared by t test or one-way ANOVA. P < 0.05 was considered as statistical difference.

Results

TNF-α expression in OP rat

Expression of TNF-α in serum of each group was detected by ELISA. Serum TNF-α expression in the OP group was significantly increased compared with the sham operation group (P < 0.05). TNF-α neutralizing antibody administration inhibited TNF-α expression compared with the OP group (P < 0.05) (**Figure 1**).

Bone mineral density changes

Bone mineral density changes in each group were analyzed using a bone density analyzer. It was showed that the bone density was markedly reduced in the OP group compared with the sham operation group (P < 0.05). TNF-α neutralizing antibody apparently declined bone density compared with OP group (P <0.05) (**Figure 2**).

ALP content changes

The content changes of ALP in each group of rats were examined. It was found that ALP was significantly declined in the OP group compared with the sham operation group (P < 0.05). TNF-α neutralizing antibody administration obviously promoted ALP secretion compared with OP group (P < 0.05) (**Figure 3**).

Serum BALP and OC expression changes

BALP and OC expression in the serum remodeling index of each group were analyzed by ELISA. It was revealed that serum BALP and OC expressions in OP group were markedly upregulated compared with the sham operation group (P <

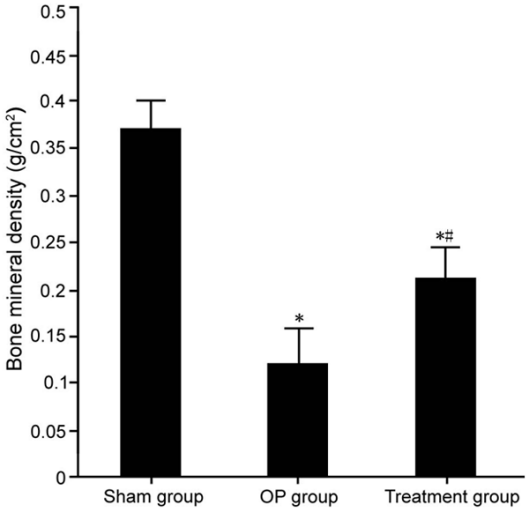

Figure 2. Bone mineral density changes. *P < 0.05, compared with sham group. #P < 0.05, compared with OP group.

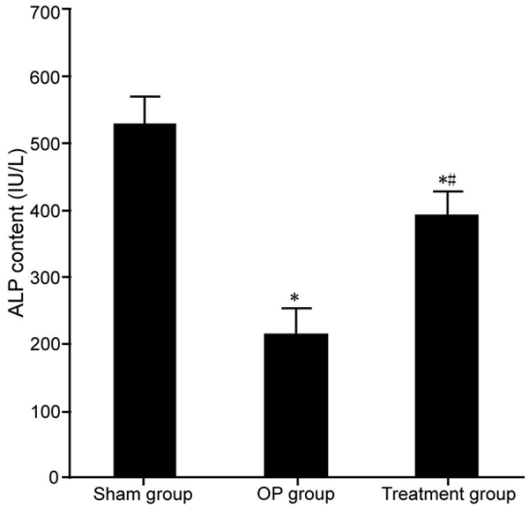

Figure 3. ALP content changes. *P < 0.05, compared with sham group. #P < 0.05, compared with OP group.

0.05). TNF-α neutralizing antibody administration apparently decreased serum BALP and OC expressions compared with OP group (P < 0.05) (**Figure 4**).

Runx2 and Opn expression changes

Real-time PCR was used to detect the effect of TNF-α on the expression of osteogenic genes Runx2 and Opn in OP rats. Expression of Runx2 and Opn genes were significantly downregulated in the OP group compared with the sham operation group (P < 0.05). RUNX2 and OPN

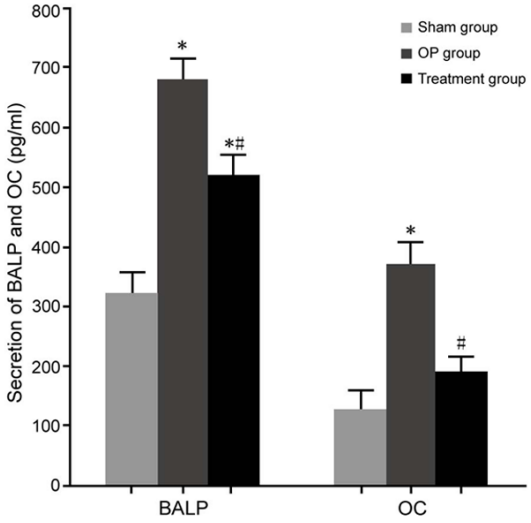

Figure 4. Serum BALP and OC expression changes. *P < 0.05, compared with the sham group. #P < 0.05, compared with the OP group.

mRNA expression was significantly enhanced after TNF-α neutralizing antibody administration (P < 0.05) (**Figure 5**).

RANKL/OPG pathway expression changes

Western blot was adopted to test expression of RANKL/OPG pathway in OP rats. RANKL protein expression was markedly upregulated, while OPG protein expression was apparently declined in the OP group compared with the sham operation group (P < 0.05). TNF-α neutralizing antibody administration decreased RANKL protein expression and enhanced OPG protein expression compared with OP group (P < 0.05) (**Figure 6**).

Discussion

During osteoporosis, a large number of osteoclasts cause bone resorption, thus breaking the dynamic balance between osteoclasts and osteoblasts, inhibiting osteoblast-mediated bone formation, resulting in increased bone resorption and bone mass reduction [17]. Postmenopausal osteoporosis accounts for a large proportion of osteoporosis. As estrogen levels decrease, bone resorption exceeds bone formation, resulting in decreased bone density and fracture [18]. Deficient estrogen can induce T-cells to secrete a large amount of inflammatory factor TNF-α, promote osteoclast activity, and inhibit osteoblast activity [19]. However, the exact role and mechanism of TNF-α in OP

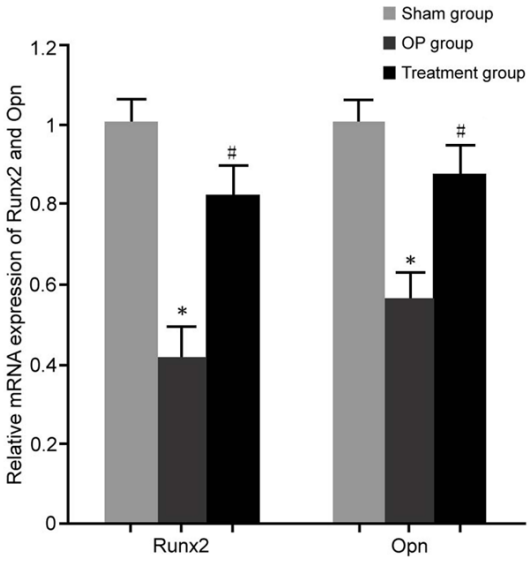

Figure 5. Runx2 and Opn gene expression changes. *P < 0.05, compared with the sham group. #P < 0.05, compared with the OP group.

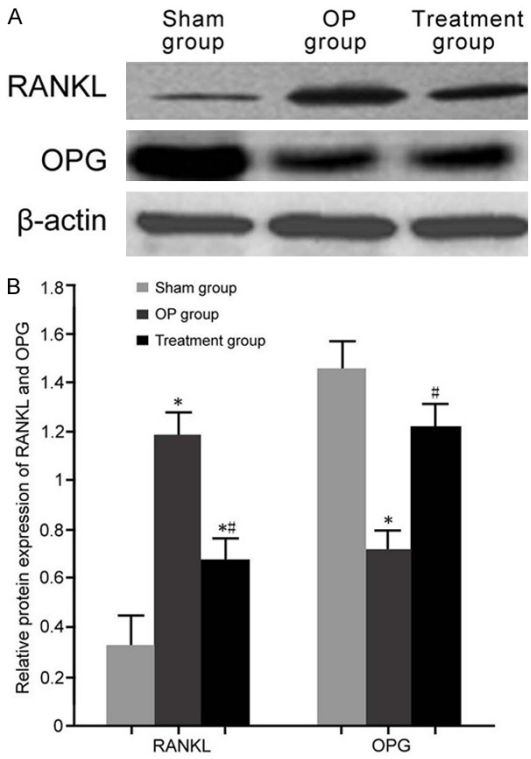

Figure 6. RANKL/OPG pathway expression changes. A. Western blot detection of RANKL/OPG pathway expression. B. RANKL/OPG pathway protein analysis. *P < 0.05, compared with the sham group. #P < 0.05, compared with the OP group.

remains poorly understood. Therefore, this study established an OP rat model by ovariectomy and confirmed that serum TNF-α expression was increased and bone density was decreased, supporting the role of TNF-α in the development and pathogenesis of OP. In addition, significantly reduced ALP activity was found. Furthermore, increased serum BALP and OC expression as well as decreased Runx2 and Opn mRNA levels declined in the OP rat, indicating that bone loss imbalance stimulates bone formation during remodeling process. A previous study showed that administration of TNF-α neutralizing antibody significantly restrained osteoclast activity to some extent [20]. Consistent with this, our present study demonstrated that injection of TNF-α neutralizing antibody increased bone mineral density, enhanced the ALP activity, downregulated serum BALP and OC expressions, as well as increased mRNA levels of osteogenic genes Runx2 and Opn, further supporting the pathogenic role of TNF-α in the pathogenesis of OP and indicating that therapeutic targeting TNF-α expression or function might improve the osteoporosis state of OP rats.

The RANKL/OPG pathway plays an important role in the bone formation microenvironment [21]. Since RANKL can be expressed in osteoblasts and bone marrow stromal cells, binding of RANKL and RANK can cause differentiation and activation of osteoclasts and inhibit apoptosis of osteoclasts. As a decoy receptor for RANKL, OPG can competitively bind to RANKL, thereby inhibiting the binding of RANKL and RANK, resulting in inhibition of osteoclast differentiation and function. Therefore, the RANKL/OPG pathway plays an important role in the dynamic balance of bone formation and resorption [22, 23]. In the present study, TNF-α secretion was increased, RANKL expression was upregulated, while OPG expression was reduced in OP rats. Inhibition of TNF-α expression by TNF-α neutralizing antibody in OP rats reduced RANKL protein level and enhanced OPG protein expression, suggesting that TNF-α might participate in the occurrence and development of OP possibly through RANKL/OPG pathway. However, the exact mechanism by how TNF-α is involved in the regulation of RANKL/OPG signaling pathway in the pathogenesis of OP remains unclear and requires further investigation. As the present study focuses on animal models, clinical samples are required to collect from patients with OP to confirm the role of TNF-α in OP development in the future.

Conclusion

TNF-α was increased in the OP. Down-regulation of TNF-α promoted osteogenic differentiation and increased bone mineral density by regulating RANKL/OPG pathway, thereby improving osteoporosis.

Disclosure of conflict of interest

None.

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