European Review for Medical and Pharmacological Sciences 2019; 23: 6352-6359

Resveratrol alleviates osteoporosis through improving the osteogenic differentiation of bone marrow mesenchymal stem cells

X.-H. CHEN[1], Z.-G. SHI[2], H.-B. LIN[1], F. WU[3], F. ZHENG[1], C.-F. WU[1], M.-W. HUANG[3]

[1]Department of Orthopedics, the Affiliated Hospital of Putian University; Affiliated Hospital of Putian University Teaching Hospital of Fujian Medical University; Affiliated Putian Hospital of Southern Medical University; Putian, China
[2]Department of Anesthesia, Beijing Chest Hospital, Capital Medical University, Beijing, China
[3]Department of Rehabilitation Medicine, the Affiliated Hospital of Putian University; Affiliated Hospital of Putian University Teaching Hospital of Fujian Medical University; Affiliated Putian Hospital of Southern Medical University; Putian, China

Xuanhuang Chen and Zhiguo Shi were contributed equally to this work

Abstract. – OBJECTIVE: To investigate the protective effect of Resveratrol (RES) on TNF-α-induced inhibition of osteogenic differentiation, thus alleviating the progression of osteoporosis (OP).

MATERIALS AND METHODS: OP model in rats was first conducted by performing ovariectomy (OVX). Rats were randomly divided into sham group, OVX group, and RES+OVX group. Body weight of each rat was regularly recorded every week. Bone mineral density (BMD) of rat femoral metaphysis was measured by micro-CT. Changes in radial degrees and loads of rat femora were examined through three-point bending experiments. Relative levels of OCN and Runx2 in each group were determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Alkaline phosphatase (ALP) activity and calcification ability were assessed through ALP staining and alizarin red staining, respectively. Bone mesenchymal stem cells (BMSCs) were extracted from healthy rats and divided into control group, Tumor necrosis factor-α (TNF-α) group, RES group, and TNF-α+RES group based on different treatments. Relative levels of OCN and Runx2, ALP activity, and calcification ability in each group were detected in the same way. Finally, protein levels of NF-κB and β-catenin in BMSCs were determined.

RESULTS: Rats in each group gained body weight during the experimental period, especially those in OVX group and RES+OVX group. No significant difference in the body weight was found between OVX group and RES+OVX group. BMD in rat femora of RES+OVX group was higher than in OVX group but lower than sham group. Elastic/max radial degree and elastic/max load of femora were markedly reduced in OVX group compared to RES+OVX group. Relative levels of OCN and Runx2, ALP activity and calcification ability decreased in OVX group relative to sham group, which were partially reversed by RES treatment. After osteogenic differentiation in BMSCs induced with TNF-α, viability and calcification ability were markedly reduced and were upregulated by RES treatment. Moreover, RES treatment enhanced the downregulated levels of OCN and Runx2 in BMSCs undergoing TNF-α induction. Upregulated protein levels of nuclear factor kappa-B (NF-κB) and β-catenin in TNF-α-induced BMSCs were downregulated by RES treatment.

CONCLUSIONS: The inhibited osteogenic differentiation of BMSCs undergoing TNF-α induction is improved by resveratrol treatment, which contributes to alleviate the progression of osteoporosis.

Key Words:
Osteoporosis, Resveratrol, TNF-α.

Introduction

Osteoporosis (OP) is a systemic bone disease where decreased bone mass and degenerated bone microstructure increase bone fragility and fracture risk[1]. The incidence of OP increases with the aging of the population and severely affects the quality of life and physical health of the elderly. Drug treatment is preferred for OP patients. Nevertheless, these anti-OP drugs have many adverse

Corresponding Author: Haibin Lin, MM; e-mail: 1552279981@qq.com

events. For example, calcitonin clinically leads to nausea and vomiting, loss of appetite, dizziness, fatigue and flush fever. Withdrawal of bisphosphonate results in the elevation of bone degradation rate and loss of bone mass[2]. It is important to develop effective drugs for the prevention and treatment of OP.

Resveratrol (RES) is a natural plant antitoxin found in a wide variety of plants, including grapes, blueberries, cranberries, mulberry, nuts, and chocolate. RES structurally consists of two phenolic rings with cis-and trans-isomers. One of the ortho-position is substituted by a dihydric hydroxyl group, and the other is substituted by a hydroxyl group[3,4]. RES has a protective effect on OP through the promotion of bone formation[4]. Due to a reduction of the ovarian function, postmenopausal women present reduced estrogen secretion, accelerated bone turnover, and imbalanced bone metabolism. These populations are prone to suffer from OP because of bone mass reduction and bone fragility increase. RES has an estrogen-like effect, which can enhance estrogen levels in postmenopausal women, thereby maintaining the balance of bone formation and bone resorption[5].

Tumor necrosis factor-α (TNF-α) is a cytokine produced by the activation of macrophages and monocytes. Its precursor is composed of 233 amino acids and contains a signal peptide consisting of 76 amino acid residues. The mature TNF-α is formed after the excision of the signal peptide, which consists of 157 amino acid residues with a molecular weight of 17 kDa. TNF-α maps to chromosome 6p21.3 and spans four exons and three introns, which is closely linked to the tissue-compatibility complex gene cluster[6]. It is reported that TNF-α can affect the protein content and amino acid transport in skeletal muscle cells, and accelerate proliferation and differentiation of multiple types of cells[7,8]. The promotive or inhibitory effect of TNF-α on osteogenic differentiation of stem cells remains to be controversial[9]. In this research, we aim to uncover the role of RES in TNF-α-induced inhibition of osteogenic differentiation. Our results may provide novel ideas for clinical prevention and treatment of OP.

Materials and Methods

Animal Procedures

Rats were randomly divided into sham group, OVX group, and OVX+RES group. OVX procedures were performed as follows: rats were anesthetized by peritoneal administration of 3 mL/kg of 10% chloral hydrate. After routine skin-disinfection, a longitudinal incision at 1.5 cm near the spine was cut to expose the abdominal cavity. Both sides of ovaries were resected and fallopian tubes were ligated. The incision was sutured and disinfected using 75% ethanol and 5% iodine tincture. Rats in sham group were subjected to abdominal cavity exposure and removal of some fat tissues. Intramuscular injection of 40 KU/ (kg·d) penicillin was performed for postoperative anti-infection for three days. Body weight of each rat was weekly measured and recorded. This investigation was approved by the Animal Ethics Committee of Putian University Animal Center

RES Administration

Two weeks after OVX procedures, rats were in active growth without any deaths. Rats in OVX group and RES+OVX group were subjected to intragastric administration of 0.2 μM RES once, while those in sham group were administrated with the same volume of saline.

BMSCs Extraction and Culture

Rats were sacrificed by cervical dislocation, 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 with Dulbecco's Modified Eagle's Medium (DMEM; HyClone, South Logan, UT, USA), and the fluids were inoculated in a culture bottle. Half of the medium was replaced at three days and completely replaced at five days. BMSCs were cultured in DMEM containing 10% fetal bovine serum (FBS; HyClone, South Logan, UT, USA).

In vitro Osteogenic Differentiation

BMSCs were cultured in a 25 mL culture bottle at 1×10^7cells/L. Osteogenic induction medium (DMEM containing 10% FBS, 10 nmol/L dexamethasone, 10 mmol/L β-glycerophosphate, 50 μg/ml ascorbic acid, 1% L-glucose and 1% penicillin-streptomycin) was applied for inducing *in vitro* osteogenic differentiation of BMSCs. Half of the medium was replaced on the other day. BMSCs were passaged every 7 days.

Micro-CT

Rat femoral metaphysis and attached surrounding soft tissues were removed, fixed in 4% paraformaldehyde solution, and scanned with SCANCO Medical micro-CT (SCANCO Medical AG,

Zurich, Switzerland). Bone histomorphology indicators were determined and analyzed using Image Processing Language.

Biomechanical Examinations

A three-point bending experiment was conducted to record elastic/max radial degree and elastic/max load of rat *femora*. Briefly, right femora were placed on the Instron Material Mechanics Testing Device. At the middle position of femora, a persistent test velocity of 10 mm/min was loaded until femoral fracture. Data were recorded and finally analyzed to obtain the max load.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA in BMSCs was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and subjected to reverse transcription. The extracted complementary deoxyribonucleic acid (cDNA) was applied for PCR using the SYBR Green method (TaKaRa, Otsu, Shiga, Japan). Primer sequences were as follows: OCN forward, 5′-GCCCTGACTGCATTCTGCCTCT-3′ and reverse, 5′-TCACCACCTTACTGCCCTCCTG-3′; Runx2 forward, 5′-GGACCGACACAGCCATATAAA-3′ and reverse, 5′-GCCTCATTCCCTAACCTGAAA-3′; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward, 5′-GCAAGGATACTGAGAGCAAGAG-3′ and reverse, 5′-GGATGGAATTGTGAGGGAGATG-3′.

Determination of 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, Shanghai, China).

Alizarin Red Staining

BMSCs were subjected to osteogenic differentiation for 7 days. Cells were washed with phosphate-buffered saline (PBS) twice, fixed in 4% paraformaldehyde (Beyotime, Shanghai, China) for 10 min and stained with 0.1% alizarin red staining (pH 4.1) for 10 min. Calcification nodules were observed and captured using an inverted microscope (magnification 200×).

Cell Counting Kit-8 (CCK-8)

BMSCs were seeded in the 96-well plate with 3×10^4 cells per well. Absorbance (A) at 450 nm was recorded at the established time points using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) to depict the viability curve.

Western Blot

Total protein was extracted from BMSCs using radioimmunoprecipitation assay (RIPA) and quantified by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). The protein sample was loaded for electrophoresis and transferred on a polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Membranes were blocked in 5% skim milk for 2 h and subjected to incubation with primary and secondary antibodies. Bands were exposed by enhanced chemiluminescence (ECL) and analyzed by Image Software (Version X; Media Cybernetics, Silver Springs, MD, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 (SPSS, Chicago, IL, USA) software was used for data analyses. Data were expressed as mean ± SD. Intergroup differences were analyzed by the *t*-test. $p<0.05$ was considered as statistically significant.

Results

RES Improved Body Weight, BMD, and Bone Biomechanics

During the whole experimental period, rats were regularly fattened in the three groups. Body weight in RES+OVX and OVX group was gradually elevated in the 8th week relative to that of sham group. We failed to obtain a significant difference in the body weight between RES+OVX and OVX group (Figure 1A). OVX procedures in rats decreased the BMD (bone metabolism index). Nevertheless, rat BMD was higher in RES+OVX group than in of OVX group (Figure 1B). Biomechanical parameters of femoral radial degrees and loads were examined. Elastic/max radial degrees and elastic/max loads markedly decreased after OVX procedures. RES treatment helped to improve biomechanical parameters (Figures 1C and 1D).

RES Improved the Osteogenic Differentiation Ability of BMSCs

Relative levels of OCN and Runx2 were downregulated in OVX group and RES+OVX

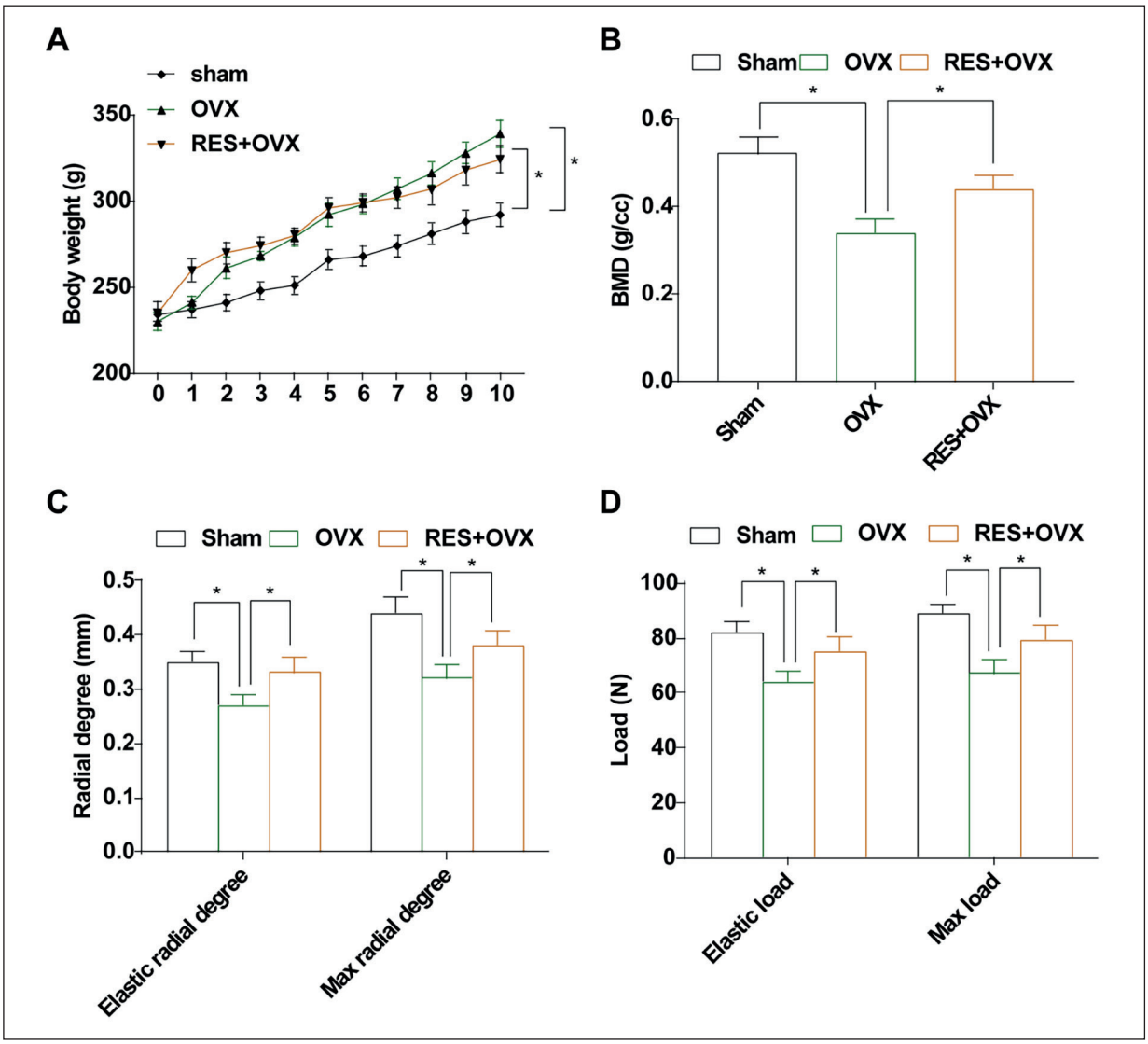

Figure 1. RES improved body weight, BMD and bone biomechanics. ***A,*** Weekly recorded body weight (g) in rats of sham group, OVX group, and RES+OVX group recorded every week. ***B,*** BMD (g/cc) in rats of sham group, OVX group, and RES+OVX group. ***C,*** Elastic/max radial degree (mm) in rats of sham group, OVX group, and RES+OVX group. ***D,*** Elastic/max load (N) in rats of sham group, OVX group, and RES+OVX group.

group compared to sham group. RES treatment could upregulate their levels but was still lower than the baseline levels (Figures 2A and 2B). ALP activity was markedly attenuated in rats undergoing OVX but was enhanced to some extent by RES treatment (Figure 2C). A similar trend was observed in assessing the calcification ability as Alizarin red staining revealed (Figure 2D). It is indicated that RES was capable of stimulating the osteogenic differentiation in OVX rats.

RES Improved Viability and Calcification Ability of BMSCs

Subsequently, we explored the *in vitro* role of RES in osteogenic differentiation of BMSCs. According to the different treatments, primary BMSCs were assigned into control group, TNF-α group, RES group, and TNF-α+RES group. TNF-α induction suppressed the viability of BMSCs, which was enhanced after RES treatment (Figure 3A). Both ALP and alizarin red staining revealed the attenuated ALP activity and calcifi-

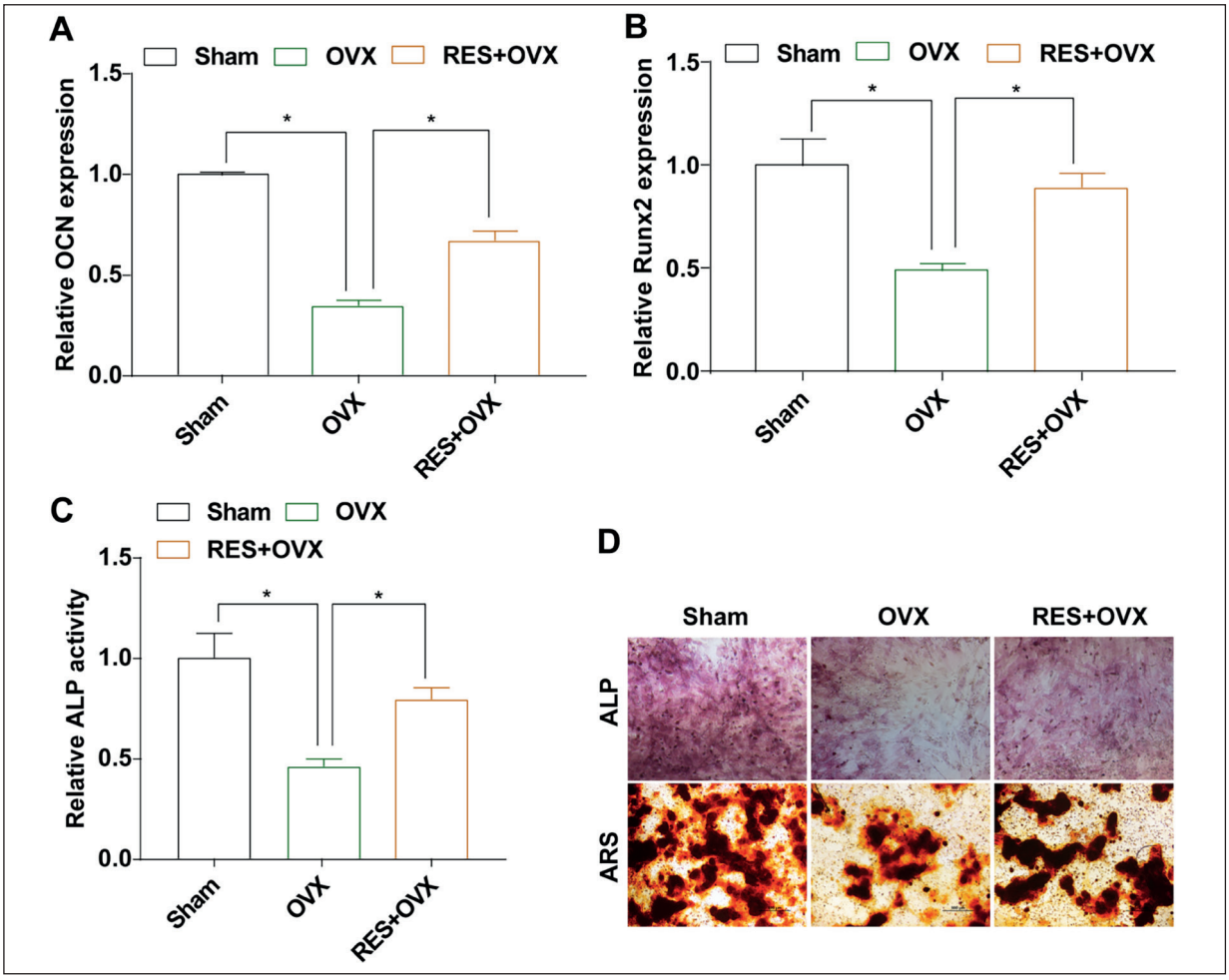

Figure 2. RES improved the osteogenic differentiation ability of BMSCs. ***A,*** The mRNA level of OCN in rats of sham group, OVX group, and RES+OVX group. ***B,*** The mRNA level of Runx2 in rats of sham group, OVX group, and RES+OVX group. ***C,*** Relative activity of ALP in rats of sham group, OVX group, and RES+OVX group. ***D,*** ALP staining and alizarin red staining of rat femora in sham group, OVX group, and RES+OVX group.

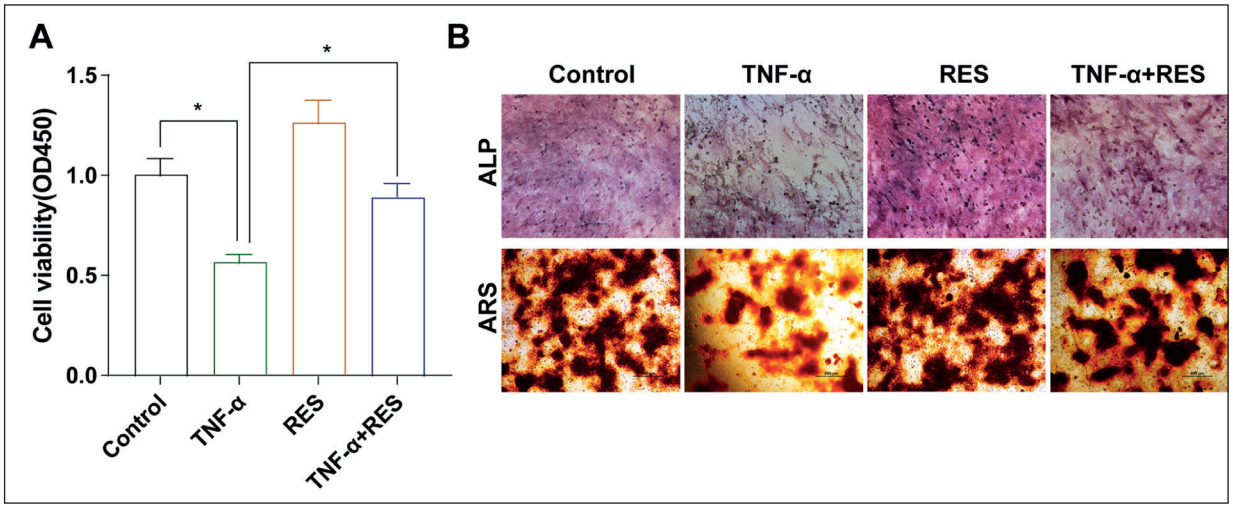

Figure 3. RES improved viability and calcification ability of BMSCs. ***A,*** BMSCs viability in control group, TNF-α group, RES group, and TNF-α+RES group. ***B,*** ALP staining and alizarin red staining of BMSCs in control group, TNF-α group, RES group, and TNF-α+RES group.

cation ability in BMSCs undergoing TNF-α induction. RES treatment markedly protected TNF-α-induced inhibition in ALP activity and calcification ability (Figure 3B).

RES Mediated Osteogenesis-Related Genes Through NF-κB Pathway

QRT-PCR data showed that TNF-α induction downregulated OCN and Runx2 in BMSCs, which were partially reversed after RES treatment (Figures 4A and 4B). In TNF-α group, the protein level of NF-κB was upregulated but β-catenin was downregulated relative to control group. RES treatment reversed protein level changes in NF-κB and β-catenin (Figures 4C and 4D).

Discussion

OP leads to the reduction of bone mass, destruction of bone microstructure, bone fragility increases, and fracture risks[10]. There are typically no symptoms in the early-stage OP until fragility fractures occur in the hip, spine, proximal *humerus*, and distal forearms[11,12]. The relative number of osteoblasts greatly decreases due to attenuated proliferative ability, inhibited differentiation and calcification, and increased apoptotic rate of osteoblasts[13]. These cellular changes lead to bone loss and eventually turn out to OP. The capacity to stimulate the osteogenic differentiation of BMSCs is a major standard to

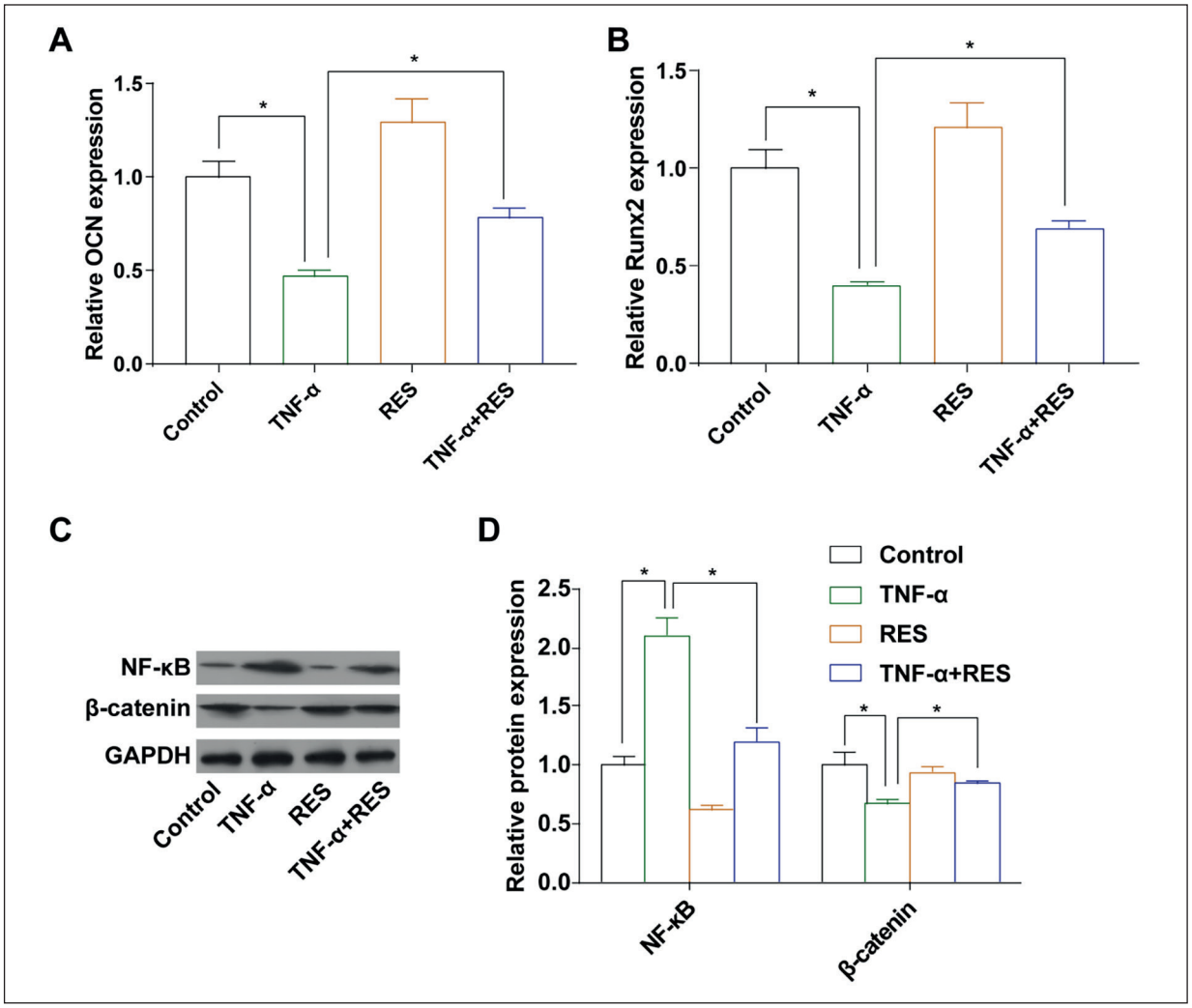

Figure 4. RES mediated osteogenesis-related genes through NF-κB pathway. ***A,*** The mRNA level of OCN in control group, TNF-α group, RES group, and TNF-α+RES group. ***B,*** The mRNA level of Runx2 in control group, TNF-α group, RES group, and TNF-α+RES group. ***C,*** Western blot analyses of NF-κB and β-catenin in control group, TNF-α group, RES group, and TNF-α+RES group. ***D,*** Protein levels of NF-κB and β-catenin in control group, TNF-α group, RES group, and TNF-α+RES group.

evaluate the efficacy of anti-OP drugs. RES has been well concerned due to its diverse biological functions, including anti-oxidation, anti-inflammation, anti-cancer, and anti-aging. It effectively inhibits NF-κB expression and inflammatory factors, thus exerting its pharmacological roles in functional disorder and tissue damage repair[14]. RES is also beneficial to bone formation. It inhibits osteoclast activity, stimulates osteogenic differentiation and bone loss in rats undergoing OVX[15-17]. Consistently, this work also identified the promotive role of RES in enhancing osteogenic differentiation of BMSCs.

TNF-α is a vital inflammatory factor involving in cell proliferation and differentiation[18]. A relevant study[19] showed upregulated TNF-α in patients with OP and chronic inflammation, indicating the involvement of TNF-α in bone metabolism[19].

NF-κB participates in the process of immune response, cell proliferation and apoptosis. Extracellular TNF-α and other inflammatory factors can stimulate the phosphorylation of NF-κB inhibitory protein. The tripolymer formed by the binding of NF-κB and inhibitory protein is degraded into a dipolymer through the activated ubiquitination pathway, thus translocating into the cell nucleus and mediating transcriptional regulation[20]. The bone morphogenetic protein induces osteogenic differentiation by regulating the Smad pathway and phosphorylation levels of downstream effectors[21]. Studies[22,23] have reported that RES can downregulate NF-κB-dependent pro-inflammatory factors, including iNOS, IL-6, and TNF-α. In this paper, TNF-α induction markedly suppressed the osteogenic differentiation of BMSCs, which was reversed by RES treatment through activation of the NF-κB pathway.

Conclusions

We found that the inhibited osteogenic differentiation of BMSCs undergoing TNF-α induction is improved by Resveratrol treatment, which contributes to alleviate the progression of osteoporosis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

Natural Science Foundation of Fujian Province, Grant No: 2016J01604.

References

1) Lynn HS, Lau EM, Au B, Leung PC. Bone mineral density reference norms for Hong Kong Chinese. Osteoporos Int 2005; 16: 1663-1668.
2) Zhang SY, Gao F, Peng CG, Zheng CJ, Wu MF. MiR-485-5p promotes osteoporosis via targeting Osterix. Eur Rev Med Pharmacol Sci 2018; 22: 4792-4799.
3) Zarychta B, Gianopoulos CG, Pinkerton AA. Revised structure of trans-resveratrol: Implications for its proposed antioxidant mechanism. Bioorg Med Chem Lett 2016; 26: 1416-1418.
4) Tang F, Xie Y, Cao H, Yang H, Chen X, Xiao J. Fetal bovine serum influences the stability and bioactivity of resveratrol analogues: a polyphenol-protein interaction approach. Food Chem 2017; 219: 321-328.
5) Böttner M1, Christoffel J, Rimoldi G, Wuttke W. Effects of long-term treatment with resveratrol and subcutaneous and oral estradiol administration on the pituitary-thyroid-axis. Exp Clin Endocrinol Diabetes 2006; 114: 82-90.
6) Ding J, Ghali O, Lencel P, Broux O, Chauveau C, Devedjian JC, Hardouin P, Magne D. TNF-alpha and IL-1beta inhibit RUNX2 and collagen expression but increase alkaline phosphatase activity and mineralization in human mesenchymal stem cells. Life Sci 2009; 84: 499-504.
7) Tayek JA. Effects of tumor necrosis factor alpha on skeletal muscle amino acid metabolism studied in-vivo. J Am Coll Nutr 1996; 15: 164-168.
8) van Hall G. Cytokines: muscle protein and amino acid metabolism. Curr Opin Clin Nutr Metab Care 2012; 15: 85-91.
9) Gilbert L, He X, Farmer P, Rubin J, Drissi H, van Wijnen AJ, Lian JB, Stein GS, Nanes MS. Expression of the osteoblast differentiation factor RUNX2 (Cbfa1/AML3/Pebp2alpha A) is inhibited by tumor necrosis factor-alpha. J Biol Chem 2002; 277: 2695-2701.
10) Wright NC, Looker AC, Saag KG, Curtis JR, Delzell ES, Randall S, Dawson-Hughes B. The recent prevalence of osteoporosis and low bone mass in the United States based on bone mineral density at the femoral neck or lumbar spine. J Bone Miner Res 2014; 29: 2520-2526.
11) Watts NB, Lewiecki EM, Miller PD, Baim S. National Osteoporosis Foundation 2008 Clinician's Guide to Prevention and Treatment of Osteoporosis and the World Health Organization Fracture Risk Assessment Tool (FRAX): what they mean to the bone densitometrist and bone technologist. J Clin Densitom 2008; 11: 473-477.
12) Cosman F, de Beur SJ, LeBoff MS, Lewiecki EM, Tanner B, Randall S, Lindsay R. Erratum to: Clinician's guide to prevention and treatment of osteoporosis. Osteoporos Int 2015; 26: 2045-2047.
13) Armour KJ, Armour KE, van't Hof RJ, Reid DM, Wei XQ, Liew FY, Ralston SH. Activation of the

inducible nitric oxide synthase pathway contributes to inflammation-induced osteoporosis by suppressing bone formation and causing osteoblast apoptosis. Arthritis Rheum 2001; 44: 2790-2796.

14) Seljak KB, Berginc K, Trontelj J, Zvonar A, Kristl A, Gašperlin M. A self-microemulsifying drug delivery system to overcome intestinal resveratrol toxicity and presystemic metabolism. J Pharm Sci 2014; 103: 3491-3500.

15) Zhang ZY, Teoh SH, Chong MS, Schantz JT, Fisk NM, Choolani MA, Chan J. Superior osteogenic capacity for bone tissue engineering of fetal compared with perinatal and adult mesenchymal stem cells. Stem Cells 2009; 27: 126-137.

16) Zaim M, Karaman S, Cetin G, Isik S. Donor age and long-term culture affect differentiation and proliferation of human bone marrow mesenchymal stem cells. Ann Hematol 2012; 91: 1175-1186.

17) Mareschi K, Ferrero I, Rustichelli D, Aschero S, Gammaitoni L, Aglietta M, Madon E, Fagioli F. Expansion of mesenchymal stem cells isolated from pediatric and adult donor bone marrow. J Cell Biochem 2006; 97: 744-754.

18) Raehtz S, Bierhalter H, Schoenherr D, Parameswaran N, McCabe LR. Estrogen deficiency exacerbates type 1 diabetes-induced bone TNF- α expression and osteoporosis in female mice. Endocrinology 2017; 158: 2086-2101.

19) Toussirot É, Aubin F. Paradoxical reactions under TNF- α blocking agents and other biological agents given for chronic immune-mediated diseases: an analytical and comprehensive overview. RMD Open 2016; 2: e000239.

20) Fu H, Li G, Liu C, Li J, Wang X, Cheng L, Liu T. Probucol prevents atrial remodeling by inhibiting oxidative stress and TNF-α/NF-κB/TGF-β signal transduction pathway in alloxan-induced diabetic rabbits. J Cardiovasc Electrophysiol 2015; 26: 211-222.

21) Nai A, Rubio A, Campanella A, Gourbeyre O, Artuso I, Bordini J, Gineste A, Latour C, Besson-Fournier C, Lin HY, Coppin H, Roth MP, Camaschella C, Silvestri L, Meynard D. Limiting hepatic Bmp-Smad signaling by matriptase-2 is required for erythropoietin-mediated hepcidin suppression in mice. Blood 2016; 127: 2327-2336.

22) Kueck A, Opipari AJ, Griffith KA, Tan L, Choi M, Huang J, Wahl H, Liu JR. Resveratrol inhibits glucose metabolism in human ovarian cancer cells. Gynecol Oncol 2007; 107: 450-457.

23) Kim BS, Kang HJ, Park JY, Lee J. Fucoidan promotes osteoblast differentiation via JNK- and ERK-dependent BMP2-Smad 1/5/8 signaling in human mesenchymal stem cells. Exp Mol Med 2015; 47: e128.