Resveratrol Alleviates Osteoporosis by Promoting Osteogenic Differentiation of Bone Marrow Mesenchymal Stem Cells via SITR1/PI3K/AKT Pathway

El Resveratrol Alivia la Osteoporosis al Promover la Diferenciación Osteogénica de las Células Madre Mesenquimales de la Médula Ósea a Través de la Vía SITR1/PI3K/AKT

Xu Han; Guo-feng Jia & Feng Zhu


SUMMARY: Senile osteoporosis is mainly caused by reduced osteoblast differentiation and has become the leading cause of fractures in the elderly worldwide. Natural organics are emerging as a potential option for the prevention and treatment of osteoporosis. This study was designed to study the effect of resveratrol on osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) in osteoporosis mice. A mouse model of osteoporosis was established by subcutaneous injection of dexamethasone and treated with resveratrol administered by gavage. In vivo and in vitro, we used western blot to detect protein expression, and evaluated osteogenic differentiation of BMSCs by detecting the expression of osteogenic differentiation related proteins, calcium deposition, ALP activity and osteocalcin content. Resveratrol treatment significantly increased the body weight of mice, the level of serum Ca²⁺, 25(OH)D and osteocalcin, ration of bone weight, bone volume/total volume, trabecular thickness, trabecular number, trabecular spacing and cortical thickness in osteoporosis mice. In BMSCs of osteoporosis mice, resveratrol treatment significantly increased the expression of Runx2, osterix (OSX) and osteocalcin (OCN) protein, the level of calcium deposition, ALP activity and osteocalcin content. In addition, resveratrol treatment also significantly increased the expression of SIRT1, p-PI3K / PI3K and p-AKT / AKT in BMSCs of osteoporosis mice. In vitro, resveratrol increased the expression of SIRT1, p-PI3K / PI3K and p-AKT / AKT, Runx2, OSX and OCN protein, the level of calcium deposition, ALP activity and osteocalcin content in BMSCs in a concentration-dependent manner, while SIRT1 knockdown significantly reversed the effect of resveratrol. Resveratrol can attenuate osteoporosis by promoting osteogenic differentiation of bone marrow mesenchymal stem cells, and the mechanism may be related to the regulation of SIRT1/PI3K/AKT pathway.

KEY WORDS: Resveratrol; Osteoporosis; Osteogenic differentiation; Bone marrow mesenchymal stem cells; SIRT1.

INTRODUCTION

Osteoporosis (OP) is a systemic metabolic disease characterized by decreased bone tissue mass, increased bone resorption, degeneration of bone microstructure, and increased bone fragility (Baccaro et al., 2015; Kanis et al., 2019). Osteoporosis is prone to osteoporotic fractures due to the decrease in bone strength, while the disability and mortality rate of osteoporotic fractures and their complications increase significantly, bringing great economic burden to families and society, and seriously affecting the quality of life (Kanis et al., 2020; Cheng et al., 2021). The causes of osteoporosis are complex, including decreased estrogen levels, aging due to aging, and excessive use of glucocorticoids (Wang et al., 2021; Wu et al., 2022). Although the pathogenesis of osteoporosis varies, they are mainly manifested as a breakdown of bone reconstitution balance due to decreased bone formation and increased bone resorption (Chen et al., 2018; Kim et al., 2020). Therefore, promoting bone formation and inhibiting bone resorption to replace lost bone tissue are key to the treatment of osteoporosis.

As the main source of osteoblasts, bone marrow mesenchymal stem cells (BMSCs) have weakened osteogenic differentiation and enhanced lipogenic differentiation ability, which is one of the important causes of osteoporosis (Zhao et al., 2018a; Yang et al., 2019). Bone

1 Department of Orthopedics, Affiliated Wuxi Fifth Hospital of Jiangnan University (The Fifth People's Hospital of Wuxi), Wuxi, Jiangsu 214100, China.
2 Department of Orthopedics, Suzhou Kowloon Hospital Shanghai Jiao Tong University School of Medicine. Suzhou, Jiangsu 215000, China.
3 Department of Orthopedics, The 904th Hospital of Joint Logistic Support Force of PLA. Wuxi, Jiangsu 214100, China.

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marrow mesenchymal stem cells are the only way to renew bone tissue in the body, so regulating the osteogenic differentiation of bone marrow mesenchymal stem cells will help the treatment of osteoporosis (Qi et al., 2017; Chen et al., 2021a). Resveratrol, a non-flavonoid polyphenol organic compound, is an antitoxin produced by many plants when stimulated. Recently, resveratrol has been found to promote osteogenic differentiation of mesenchymal stem cells, including synergistic BMP9 effects (Wang et al., 2022), regulation of miR-193a/SIRT7 axis (Song et al., 2022), and regulation of Wnt/β-Catenin pathway (Zhao et al., 2018b) and inhibition of endogenous reactive oxygen species production (Zhou et al., 2019). Study showed that SIRT1 has been found to be very important for bone growth and development in mice. SIRT1 conditional knockout mice not only greatly affect long bone development (Cohen-Kfir et al., 2011; Iyer et al., 2014), such as reduced bone mineral density, long bone length margin and reduced calcium nodules, but also accelerate the course of osteoarthritis in mice (Jeong et al., 2013; Matsuzaki et al., 2014). However, it is still unknown whether resveratrol can promote osteogenic differentiation of BMSCs by activating SIRT1, ultimately achieving the function of preventing or treating osteoporosis.

In this study, we first evaluated the effect of resveratrol treatment on osteoporosis mice and evaluated the effect of resveratrol treatment on osteogenic differentiation of BMSCs in osteoporosis mice. To verify the mechanism, we established SIRT1-knocked BMSCs in vitro and then investigated the effect of resveratrol on osteogenic differentiation of BMSCs.

**MATERIAL AND METHOD**

**Experimental animals and administration:** There are 30 male C57BL/6 mice (6 weeks old, 18-20 g) purchased from Beijing Weitong Lihua Experimental Animal Technology Co., Ltd, and they were kept in an environment with a temperature of 25 degrees Celsius and 50 % humidity for 1 week. Then, 30 mice were randomly divided into 3 groups (10 mice in each group), namely, control group, OP group and RES group. Mice in OP group and RES group were subcutaneously injected with 40 mg/kg dexamethasone once every day for 4 weeks to establish osteoporosis mice. And mice in control group were give equal amounts of normal saline during the construction of a mouse model of osteoporosis. For resveratrol (R5010, sigma) treatment, osteoporosis mice in RES group were treated with resveratrol (40 mg/kg body weight, Sigma-Aldrich, China) was performed intraperitoneally once every day for 8 weeks, and mice in control group and OP group were given equal amounts of normal saline.

**Detection of serum Ca2+, 25(OH)D, osteocalcin and DPD.** At the 4th and 8th weeks of resveratrol treatment, we collected mouse blood through the tail vein to isolate serum, and measured serum 25(OH)D (ab213966, abcam) and osteocalcin (ml026391, mlbio) levels using ELISA kit, and detected serum Ca2+using biochemical automatic analyzer (PUZS-600A/B, PERLONG). At the same time, we collected the urinary of mice, and determined deoxypyridinoline (DPD) using an ELISA kit (ml001904, mlbio).

**Bone microstructure analysis.** At the 8th week of resveratrol treatment, we firstly measured bone mineral density (BMD) of mice proximal tibia using an X-ray absorptiometry (NORLAND XR-46, Nanjing Norland International Trading Co., Ltd). And then a micro-CT (SKYSCAN 1276, BRUKER) was used to scan the proximal metaphysis of the tibia in mice and select the target area to reconstruct the three-dimensional bone result map for analyzing bone volume/total volume (BV/TV), trabecular thickness (Tb Th), trabecular number (Tb N), trabecular spacing (Tb sp) and cortical thickness.

**Western blot analysis.** After different treatments, BMSCs are collected and then added to the RIPA lysis buffer to extract the total protein. Next, the total protein from BMSCs was analyzed using a 10 % SDS-PAGE (PN203, New Cell & Molecular Biotech Co., Ltd). After transferring, PVDF membranes (LC2002, ThermoFisher) was first blocked with 5 % skimmed milk powder, and then was probed with primary antibodies against Runx2 (1:1000, ab192256, ABCAM), Osteocalcin (OCN, 1:500, ab93876, ABCAM), osterix (OSX, 1:500, ab183910, ABCAM), SIRT1 (1:500, 8469, Cell Signaling Technology), p-PI3K (1:200, 17366, Cell Signaling Technology), PI3K (1:1000, 4292, Cell Signaling Technology), p-AKT (1:500, 4060, Cell Signaling Technology), AKT (1:1000, 9272, Cell Signaling Technology). Proteins were visualized with ECL solution (WBKLS0100, Beijing Xinjingke Biotechnologies Co., Ltd?China), followed by densitometry analysis using Imag J 3.0 (IBM, USA) and b-actin was loading as control.

**Calcium deposition, ALP activity and osteocalcin content.** On one hand, BMSCs from osteoporosis mice were directly lysed for detection, including used a mouse OC/BGP (Osteocalcin) ELISA Kit (D721126, Sangon Biotech) to analyze the content of osteocalcin, and used an ALP activity assay kit (ab267583, ABCAM) to detect ALP activity, and use the Calcium Assay Kit (S1063S, Beyotime) to measure calcium deposition. On the other hand, normal BMSCs were treated in osteogenic induction medium (OIM, PD-003, Procell) with resveratrol at different concentrations (0, 5, 15 and 25 mg/mL) cultivate for 21 days. And then we collected BMSCs to detect calcium deposition, ALP activity and osteocalcin content using the corresponding reagent kit.
Isolation, identification, and administration of BMSCs. After the mouse was sacrificed by cervical dislocation, we collected the bone marrow cavity rinse, filtered using a strainer to prepare a single-cell suspension (FSTR100, Betotime), and seed the cell suspension in a 100 mm cell culture dish (CCD06-100A, BIOLAND). BMSCs were cultured in DMEM medium with 10% fetal bovine serum (10091148, ThermoFisher scientific), and third generation BMSCs were used for identification by detecting cell surface markers using flow cytometry, such as CD29 (ab193591, abcam), CD90 (ab226, abcam), CD34 (ab187568, abcam) and CD45 (ab305209) using corresponding antibodies. Resveratrol was treated with BMSCs at concentrations of 0, 5, 15 and 25 mg/mL for 1, 3, 7 and 14 days.

Establishment of SIRT1 knockdown BMSCs. About 4×10⁶ BMSCs were seeded into 10 cm cell culture dishes. 24 h later, lentiviral particles targeting SIRT1 (si-SIRT1) were added according to the instructions (sc-40987, Santa Cruz Biotechnology), and negative control lentiviral particles (si-NC) were added into BMSCs as control. 24 h after lentivirus infection, we changed the medium of BMSCs. 72 h after lentivirus infection, we collected BMSCs to verify successful establishment of SIRT1 knockdown BMSCs cell lines via detecting SIRT1 protein expression by western blotting.

Statistical analysis. SPSS20.0 software (IBM, USA) was used to analyze data in this study. Differences between two groups were compared using unpaired t test, and difference between multiple groups were compared using one-way ANOVA with Tukey test as post hoc test. And P<0.05 indicated significant difference.

RESULTS

Resveratrol alleviates deterioration of bone microstructure in osteoporosis mice. At the 4th and 8th week of resveratrol treatment, we measured the body weight and bone metabolism related indicators of each group of mice, and found that the body weight (Fig. 1A), serum Ca²⁺ (Fig. 1B), serum 25 (OH) D (Fig. 1C), serum osteocalcin (Fig. 1D) in the osteoporosis (OP) group were all lower than that in control group and resveratrol treatment (RES) group, but the level of DPD in urine in OP group was significantly higher than that in control group and RES group (Fig. 1E). At the same time, we measured the weight of tibia and femur at 8th week of resveratrol treatment, and results showed that compared to control group, the weight of tibia and femur / body weight in OP group were significantly reduced, while the weight of tibia and femur / body weight in RES group were significantly higher than that in OP group (Fig. 1F).

Fig. 1. Effect of resveratrol on the general data of osteoporosis mice. At weeks 4 and 8 of resveratrol treatment, we measured the level of the body weight (A), serum Ca²⁺ (B), serum 25 (OH) D (C), serum osteocalcin (D) and urinary DPD (E) in osteoporosis mice and detected the weight of tibia and femur at 8th weeks (F). 5 mice in each group, and data were expressed as (mean ± standard deviation). ns P>0.05, * P<0.05, ** P<0.01 and ***P<0.001.
At the 8th week of resveratrol treatment, we measured the BMD of mice in each group and found that the BMD of mice in OP group were significantly lower than that in control group and RES group (Fig. 2A). At the same time, we also used the micro-CT to determine BV/TV (Fig. 2B), Tb Th (Fig. 2C), Tb N (Fig. 2D), Tb sp (Fig. 2E) and cortical thickness (Fig. 2F), and found that the BV/TV, Tb Th, Tb N and cortical thickness of mice in OP group were significantly lower than that in control group and RES group, while the Tb sp of mice in OP group were significantly lower than that in control group and RES group.

Resveratrol promotes osteogenic differentiation of BMSCs in osteoporosis mice. Firstly, we determined the expression of osteogenic differentiation related proteins in BMSCs at the 8th week of resveratrol treatment, such as Runx2, OSX and OCN (Fig. 3A), and we found that the expression of Runx2 (Fig. 3B), OSX (Figure 3C) and OCN (Fig. 3D) protein in BMSCs of mice in OP group were all significantly lower than that in control group and RES group. In addition, we cultured BMSCs of mice in each group in OIM medium for 21 days, and then collected the cell culture medium of BMSCs and BMSCs to analyze the osteogenic differentiation indicators. Results suggested that the level of calcium deposition (Fig. 3E), ALP activity (Fig. 3F) and osteocalcin content (Fig. 3G) in OP group during osteogenic differentiation induction of BMSCs were all significantly lower than that in control group and RES group.

Fig. 2. Resveratrol treatment attenuates deterioration of bone microstructure in osteoporosis mice. After 8 weeks of resveratrol treatment, we used a dual energy X-ray absorptiometry to measure Bone mineral density (BMD) (A), and micro-CT to measure bone volume/total volume (BV/TV) (B), trabecular thickness (Tb Th) (C), trabecular number (Tb N) (D), trabecular spacing (Tb sp) (E) and cortical thickness (F) in osteoporosis mice. 5 mice in each group, and data were expressed as (mean ± standard deviation). **P<0.01, ***P<0.001.

Fig. 3. Resveratrol treatment increases the expression of osteogenic differentiation markers in BMSCs of osteoporosis mice. After 8 weeks of resveratrol treatment, we euthanized mice to isolate BMSCs, and then detected the expression levels of osteogenic differentiation markers using western blot (A), such as Runx2 (B), osterix (OSX) (C), and osteocalcin (OCN) (D) in BMSCs. We also detected calcium deposition (E), ALP activity (F) and osteocalcin levels (G) in BMSCs after being induced osteogenic differentiation. 5 mice in each group, and data were expressed as (mean ± standard deviation). ***P<0.001.
Resveratrol activates SIRT1/PI3K/AKT pathway in BMSCs of osteoporosis mice. SIRT1 has been shown to be associated with bone regeneration in mice [17, 18]. Therefore, we evaluated resveratrol for changes in the SIRT1 protein and its downstream PI3K/AKT pathway in BMSCs of osteoporosis mice using western blot (Fig. 4A), and the grayscale analysis results of protein bands showed that the expression of SIRT1 (Fig. 4B), p-PI3K / PI3K (Fig. 4C) and p-AKT / AKT (Fig. 4D) protein in OP group all significantly lower than that in control group and RES group.

Resveratrol promotes osteogenic differentiation of BMSCs in vitro. To investigate the molecular mechanism by which resveratrol promotes osteogenic differentiation of BMSCs, we firstly prepared BMSCs from mice and harvested the third generation BMSCs for analysis using flow cytometry (Fig. 5A). Results showed that a total of 99.24 % cells were positive for CD29 and 97.83 % cells were positive for CD90, while only 0.98 % cells were positive for CD34 and 1.03 % were positive for CD45 (Fig. 5B). And then we cultured BMSCs in OIM medium with different concentration of resveratrol (0, 5, 15 and 25 mg/mL) for different days (1, 3, 7 and 14 days) to detect the activity of BMSCs using CCK-8 kit. We found that resveratrol increased cellular activity of BMSCs in a concentration-dependent manner (Fig. 5C).

Next, we analyzed the expression of osteogenic differentiation related proteins and the level of osteogenic differentiation indicators in BMSCs in vitro, and results showed that resveratrol increased the expression of Runx2, OSX and OCN protein in BMSCs in a
concentration-dependent manner (Fig. 6A-D). Moreover, results of osteogenic differentiation indicators indicated that resveratrol also increased the levels of calcium deposition (Fig. 6E), ALP activity (Fig. 6F) and osteocalcin content (Fig. 6G) in BMSCs in a concentration-dependent manner.

Resveratrol promotes osteogenic differentiation of BMSCs by activating SIRT1-mediated PI3K/AKT pathway. To verify that resveratrol promotes osteogenic differentiation of BMSCs in osteoporosis mice via SIRT1, we established SIRT1-knockdown BMSCs cell lines in vitro, and western blotting verified the successful establishment of SIRT1-knockdown BMSCs cell lines (Fig. 7A). Next, we used western blotting to assess SIRT/PI3K/AKT pathway in different BMSCs after being stimulated with resveratrol in OIM medium (Fig. 7B) and found that resveratrol significantly increased the expression of SIRT1 (Fig. 7C), p-PI3K/PI3K (Fig. 7D) and p-AKT/AKT (Fig. 7E) proteins in BMSCs, while si-SIRT1 significantly reversed the effect of resveratrol. In addition, we also found that the level of calcium deposition (Fig. 7F), ALP activity (Fig. 7G) and osteocalcin content (Fig. 7H) in RES group were significantly higher than that in Control group and RES + si-SIRT1 group.

**Fig. 6.** Resveratrol promotes osteogenic differentiation of BMSCs in vitro. After 21 days of incubation of BMSCs with different concentrations of resveratrol in OIM medium, we collected BMSCs to detect the expression of osteogenic differentiation markers using western blot (A), such as Runx2 (B), OSX (C), and OCN (D), and to measure the level of calcium deposition (E), ALP activity (F) and osteocalcin levels (G). 3 independent replicates per test and data were expressed as (mean ± standard deviation). Compared with 0 mg/mL RES, ns P>0.05, * P<0.05, ** P<0.01 and ***P<0.001.

**DISCUSSION**

Bones are organs with active metabolism, and constantly synthesize new bone through osteoblasts to replace damaged or old bone absorbed by osteoclasts, so that the body's skeletal system maintains normal function (Chen et al., 2018; Kim et al., 2020). However, the delicate balance between bone synthesis and bone breakdown and absorption disappears with aging or pathological destruction, leading to bone loss and osteoporosis (Hu et al., 2022; Phromnoi et al., 2022). Epidemiological statistics show that 20% of men and 50% of women will experience a decrease in bone density after the age of 50, and OP is one of the main causes of fractures in elderly patients (Lorentzon, 2019; Imamudeen et al., 2022). In China, there were about 2.69 million new cases of major osteoporotic fractures (hip, vertebral body, and wrist) in 2015, and about 4.83 million new cases are expected in 2035 and about 5.99 million new cases in 2050 (Wang et al., 2021; Wu et al., 2022). At present, most of the drug mechanisms of action for the treatment of osteoporosis are by inhibiting bone resorption and promoting bone formation, the former including calcitonin, bisphosphonates, estrogens, etc., and the latter including parathyroid hormone and its analogues, but its long-term application also brings many toxic side effects (Slupski et al., 2021; Reid & Billington, 2022). Therefore, the demand for
drugs that play a safer and more reliable effect in the treatment of osteoporosis is becoming more and more obvious.

Resveratrol, also known as 3,4',5-trihydroxystilbene, is a classic natural polyphenol plant antitoxin, widely present in many plants and traditional Chinese medicine, with enhance autoimmunity, anti-inflammatory, antibacterial, antioxidant, cardiovascular protection, anti-tumor, and other effects (Tian & Liu, 2020; Zhou *et al*., 2021; Meng *et al*., 2021). Recently, resveratrol has increasingly attracted attention in the treatment of osteoporosis due to its multi-target, immediate availability, low cost, and low toxicity (Wang *et al*., 2022; Song *et al*., 2022). Study showed that osteoporosis caused by osteoclast and osteoblast imbalance is associated with osteogenic differentiation of BMSCs (Jiang *et al*., 2021; He *et al*., 2022). In this study, we treated osteoporosis mice by intraperitoneal injection of resveratrol, and found that resveratrol treatment could not only improve the content of bone metabolic indicators, but also help to alleviate the deterioration of bone microstructure in osteoporosis mice. Importantly, we also found that resveratrol treatment promoted the osteogenic differentiation of BMSCs in osteoporosis mice.

SIRT1 is the most widely studied member of the sirtuins family and has been shown to play an important role in the development of neurodegenerative diseases, diabetes, tumors, inflammation, and aging (Jiao & Gong, 2020; Iside *et al*., 2020; Yang *et al*., 2022). Previous studies have shown that SIRT1 conditional knockout not only reduces bone mineral density in mice, resulting in shorter long bone length and cortical bone thickness, but also leads to a decrease in bone calcium nodules and inhibits the formation of sclerosteostin (Cohen-Kfir *et al*., 2011; Iyer *et al*., 2014; Chen *et al*., 2021b). At the same time, the activation of SIRT1 is also thought to promote osteogenic differentiation of BMSCs by activating the SIRT1 mediated PI3K/AKT pathway.
mesenchymal stem cells, such as Lu et al. (2022), found that SRT2104, an activator of SIRT1, can promote osteogenic differentiation and angiogenesis of mesenchymal stem cells by activating the BMP/Smad and BMP/MAPK signaling pathways mediated by SIRT1. And Xu et al. (2021) found that thrombin-activated platelet-rich plasma enhanced osteogenic differentiation of human periodontal ligament stem cells by activating SIRT1-mediated autophagy. Our study also found that resveratrol treatment significantly increased the expression of SIRT1 in BMSCs in osteoporosis mice and knocking out SIRT1 in vitro significantly reversed the role of resveratrol in promoting bone differentiation of BMSCs. Therefore, our results indicated that resveratrol promoted the osteogenic differentiation of BMSCs by promoting SIRT1 expression, thereby weakening the disease progression in osteoporosis mice.

PI3K-AKT signaling pathway is one of the important signal transduction pathways in subcells, which can play a key role in inhibiting apoptosis and promoting proliferation and differentiation in cells by affecting the activation state of a variety of downstream effector molecules and play an important role in the occurrence and development of various human diseases (Yu & Cui, 2016; Xu et al., 2020).

In the present study, we found that resveratrol treatment could activate PI3K-AKT pathway in BMSCs of osteoporosis mice, while SIRT1 knockdown attenuated the function of resveratrol on the activation of the PI3K-AKT pathway in BMSCs, suggesting that resveratrol promotes osteogenic differentiation of BMSCs through the SIRT1-mediated PI3K/AKT pathway.

CONCLUSION

All in all, our results suggested that resveratrol can attenuate hormone-induced osteoporosis by promoting osteogenic differentiation of bone marrow mesenchymal stem cells, and the mechanism may be related to the regulation of SIRT1/PI3K/AKT pathway.

REFERENCES


