Original Article
CoQ10 suppression of oxidative stress and cell senescence increases bone mass in orchiectomized mice

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Abstract: Numerous studies support the detrimental effects of oxidative stress and cell senescence on skeletal homeostasis. Coenzyme Q10 (CoQ10) acts as a scavenger for oxidative stress and protects mitochondrial activity from oxidative damage. However, it is unclear whether CoQ10 has a protective effect on osteoporosis caused by orchiectomy. To investigate suppression effect of antioxidant CoQ10 on osteoporosis in orchiectomized (ORX) mice, ORX mice were supplemented with/without CoQ10, and were compared with each other and with sham-operated mice. Our results showed that CoQ10 could prevent ORX-induced bone loss by inhibiting oxidative stress and cell senescence, subsequently promoting osteoblastic bone formation and inhibiting osteoclastic bone resorption. The results of this study not only reveal the mechanism of CoQ10 supplementation in anti-osteoporosis, but also provide experimental and theoretical basis for the clinical application of CoQ10 in the prevention of osteoporosis.

Keywords: ORX-induced bone loss, coenzyme Q10, oxidative stress, cell senescence

Introduction
Osteoporosis is a systemic metabolic bone disease characterized by microstructural destruction of bone tissue, marked reduction in bone volume, increased fragility of bone tissue and prone to osteoporotic fractures [1]. With the aging of the population and the increasing average life expectancy of men, osteoporosis in older men has become a global health issue that has attracted more and more attention. The number of fractures caused by osteoporosis in the elderly also increases with age. The risk of fracture in older men over 60 years old increases. And approximately 33.3% of hips (including femoral neck, intertrochanteric, femoral shaft, acetabulum) fractures occur in older men [2]. In the past two decades, despite the relevant research achieved, the pathogenic mechanisms of male osteoporosis remain controversial.

Androgen deficiency is currently considered to be an important risk factor for osteoporosis in older men, but its pathophysiological mechanism was not well defined. Androgen receptors (ARs) exist on the surface of osteoblast membranes and participate in a series of functions of osteoblasts, such as the formation of osteoblast proliferation factors, growth factors and collagen, osteocalcin and osteopontin. Androgen can regulate the ability of osteoblasts to secrete insulin-like growth factor I and the secretion of basic fibroblast growth factor (bFGF) which can be promoted by autocrine or paracrine [3]. Testosterone and dihydrotestosterone also inhibit the action of inflammatory cytokines and bone resorption [4].

ROS is a harmful by-product of the life cycle of oxygen-consuming organisms. Oxidative stress occurs when cells produce ROS levels higher than their ability to scavenge, thereby damaging proteins, lipids and deoxyribonucleic acid (DNA), leading to cell senescence or cell death. Studies have shown that oxidative stress can lead to aging, atherosclerosis [5], tumorigenesis [6] and osteoporosis [7]. Low concentration of ROS
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Coenzyme Q10 (CoQ10) is a fat-soluble steroid compound, which is widely present in all biofilms and participates in the process of respiratory chain electron transport, antioxidant, metabolic regulation and cell differentiation regulation [15, 16]. Coenzyme Q10 is a powerful antioxidant that scavenges free radicals which has been proven to effectively prevent the oxidation of proteins, lipids, and DNA damage [17]. It is reported that CoQ10 promoted osteoblast proliferation and differentiation and prevented osteoporosis by ovariectomy [18-20]. The study found that CoQ10 supplementation inhibits the reduction of testosterone caused by chemical reproductive toxicants by reducing the destruction of oxidants produced by chemical reproductive toxicants [21]. New functions of CoQ10 have been described in recent years, including anti-inflammatory effects, regulation of gene expression and stabilization of lipid bilayer membranes, which explains its involvement in aging and age-related diseases such as cardiovascular disease, renal failure and neurodegenerative disease [22]. However, it is unclear whether CoQ10 supplementation can reverse ORX induced bone loss.

We hypothesized that CoQ10 supplementation can rescue bone loss caused by androgen deficiency by inhibiting oxidative stress and cell senescence, promoting bone formation of osteoblasts, inhibiting bone resorption of osteoclasts. To answer this question, we established an orchitectomy (ORX) mouse model on both sides and then supplemented the ORX mice with CoQ10. The control mice were sham operated and fed with normal diet. We examined the alterations in bone microarchitecture, bone formation, bone turnover, oxidative stress and cell senescence to investigate whether CoQ10 can inhibit the ORX-induced bone loss by inhibiting oxidative stress and cell senescence.

Materials and methods

Mice

C57/BL6J mice in this study were raised in SPF laboratory animal center of Nanjing Medical University. They were maintained in a virus- and parasite-free barrier facility and exposed to a 12-h light, 12-h dark cycle. All the mice were randomly divided into three groups (n=8 per group): 1) sham operation group, given normal diet; 2) ORX group, given normal diet; 3) ORX group supplemented with CoQ10 (50 mg/kg/day). Mice were anesthetized with chloral hydrate and orchiectomized (ORX) at both sides when eight weeks old. Sham operations were carried out independently. All mice were sacrificed at 48 weeks of age, 40 weeks after surgery. Long bones were harvested. This study was approved by the Institutional Animal Care and Use Committee of Nanjing Medical University.

X-ray photography, micro-computed tomography (micro-CT) scanning and 3D reconstruction test

The tibias from three groups of mice were fixed in 4% paraformaldehyde and then scanned for X-ray and micro-CT separately. The fixed tibia was radiographed using a Faxitron Model 805 radiographic detection system (Faxitron Contact, Germany; 22 kV and 4-minute exposure time) to observe changes in bone morphology and bone density. Take the fixed tibia for Micro-
CT scan (Sky Scan 1072 Scanner, voltage 100 kV, current 98 mA) and 3D reconstruction.

**Histology**

The tibias were removed and dissected free of soft tissue, fixed with 2% PLP fixative at 4°C and processed histologically. Then the tibias were decalcified in 14% EDTA glycerol solution for 5 to 7 days at 4°C. The decalcified tibias were dehydrated and embedded in paraffin for paraffin sections, after which 5 μm sections were cut on a rotary microtome. The sections were stained with hematoxylin and eosin (H&E) or tartrate resistant acid phosphatase (TRAP) activity as described previously [4] or immunohistochemically as described below. The images were collected with DP70 CCD (Olympus company of Japan). Image quantification was performed from Northern Eclipse software (Empix Imaging, USA).

**Immunohistochemical staining**

Immunohistochemical staining was carried out for type I collagen, β-galactosidase (β-gal) and p16INK4a using the avidin-biotin-peroxidase complex technique with affinity-purified goat anti-rabbit type I collagen antibody (Southern Biotechnology Associates, Birmingham, AL, USA), β-gal (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and p16INK4a (Abcam, MA, USA) following previously-described methods [4].

**RNA isolation and quantitative real-time RT-PCR**

RNA was isolated from tibias with TRizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. Reverse transcription reactions were performed with the SuperScript First-Strand Synthesis System (Invitrogen). To determine the relative expression of genes of interest, quantitative real-time RT-PCR was carried out in an Applied Biosystems Cycler with a SYBR Green PCR reagent kit. The PCR primers were used as described previously [4]. GAPDH was used as the internal control for each reaction. All primers were tested for their specificity by conventional PCR before being used for quantitative analysis by real-time RT-PCR. All PCRs were performed in triplicate. Results were analyzed with SDS 7300 software, and the relative amount of mRNA was calculated after normalization for GAPDH mRNA.

**Cell cultures**

Mouse bone marrow MSCs from the tibias were generated as described. Briefly, tibias were collected. Attached soft tissues were removed from the bones. All cells from tibias were obtained by digestion with 3 mg/mL collagenase type I (Sigma-Aldrich, St. Louis, MO, USA) and 4 mg/mL dispase II (Sigma-Aldrich) for 60 min at 37°C. Single-cell suspensions were obtained through 70-mm cell strainers and seeded into a clear dish as passage 1, then continually cultured for another 7 days to allow cell growth to confluence. The third passage MSCs were used for osteoblast induction plus Fast Blue TR/Naphthol AS-MX staining. For CFU-f and CFU-ALP assays, bone marrow cells were cultured in 12-well-plates at 1×10⁶ cells per well in α-Minimum Essential Medium (α-MEM) containing 10% FBS (Sciencell Research Laboratories, Carlsbad, CA, USA) with or without 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate for 12 days. At the end of the culture period, cells were stained with Fast Red TR/Naphthol AS-MX for ALP-positive colonies (CFU-ALP) and stained with Methylene blue for total colonies (CFU-f) as described [4].

**Detection of reactive oxygen species (ROS) levels**

The bone marrow cells from long bones were converted into single cell suspensions with syringe, then washed with cold PBS and resuspended in binding buffer. 5 ul of 2',7'-dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich, St. Louis, MO, USA) were added. The supernatant was discarded, 10% fetal bovine serum was added and incubated at 37°C, the supernatant was discarded and an appropriate amount of cold PBS was added. ROS levels were calculated from mean fluorescence intensity (MFI) measured using a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

**Biochemistry assays**

Serum total antioxidant activities (T-AOC) was measured with respective mice-specific kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). All measurements were performed according to the manufacturer’s instructions and all samples were assayed in duplicate within the same protocol.
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Statistical analysis

Statistical analysis was performed using SPSS software, version 16.0 (SPSS Inc., Chicago, IL, USA). Measured data are presented as mean ± SEM. Statistical comparisons were performed using a one-way ANOVA of qualitative data to compare differences between groups. Values of \( P < 0.05 \) were considered statistically significant.

Results

The effect of CoQ10 on ORX-induced bone loss

To determine whether CoQ10 supplementation can prevent ORX-induced bone loss, we used orchiectomized (ORX) mice as an animal model. The mice were then supplemented with or without CoQ10, and the bone phenotype from each group of mice was compared using X-ray and micro-CT, respectively. The results showed that the bone mineral density (Figure 1A-C), bone volume (Figure 1D), trabecular thickness (Figure 1E) and trabecular number (Figure 1F) of the ORX mice were reduced significantly compared with the sham control mice. However, these parameters were rescued in ORX mice administrated with CoQ10. These findings demonstrate that supplementation with CoQ10 can prevent ORX-induced bone loss.

Figure 1. The effect of CoQ10 on ORX-induced bone loss. Eight-week-old male C57/BL6J mice were randomly performed ORX or Sham surgery and euthanize after 40 weeks. (A) Representative radiographs of tibias. (B) Representative Micro-CT-scanned and 3D reconstructed sections along the longitudinal direction of tibias. Analysis of the distal femoral trabecular bone parameters by micro-CT, (C) Bone mineral density, (D) Trabecular bone volume relative to tissue volume (BV/TV, %), (E) Thickness of trabecular bone (Tb.Th), (F) Trabecular number (Tb.N), (G) Trabecular separation (Tb.Sp). Data are presented as the mean ± SEM of determinations, each data-point was the mean of five specimens. **P < 0.01, ***P < 0.001, versus sham mice; *P < 0.05, **P < 0.01, ***P < 0.001 versus ORX mice.
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The effect of CoQ10 on the osteoblastic bone formation in ORX mice

In order to clarify whether the bone loss in ORX mice rescued by CoQ10 supplementation was related to increased osteoblastic bone formation, we examined the parameters of osteoblastic bone formation by histochemistry, immunohistochemistry and real-time RT-PCR. We found that the number of osteoblasts (Figure 2A, 2B) and the type I collagen-positive area (Figure 2C, 2D) were reduced significantly in ORX mice compared with sham mice by staining with H&E, immunohistochemically for type I collagen and computer-assisted image analysis. And gene expression levels of OPG (Figure 2E), RUNX2 (Figure 2F) and OCN (Figure 2G) were all decreased in ORX mice compared with sham mice by real-time RT-PCR. However, these parameters were largely rescued in ORX mice with CoQ10 supplementation. These results demonstrated that CoQ10 could increase osteoblastic bone formation in ORX mice.

The effect of CoQ10 on the osteoclastic bone resorption in ORX mice

In order to determine whether the bone loss in ORX mice rescued by CoQ10 supplementation was related to reduced osteoclastic bone resorption, we examined the parameters of osteoclastic bone resorption by histochemical staining for TRAP, image analysis and real-time RT-PCR. Results showed that the number of osteoclasts (Figure 3A, 3B), TRAP-positive osteoclast surface (Figure 3C) and RANKL/OPG ratio (Figure 3D) expression levels were increased in ORX mice compared with sham mice. However, CoQ10 supplementation could rescue these changes. Our data demonstrated
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that CoQ10 could inhibit osteoclastic bone resorption in ORX mice.

The effect of CoQ10 on BM-MSCs proliferation and differentiation into osteoblasts

In order to investigate whether increased osteoblastic bone formation in ORX mice by CoQ10 supplementation was associated with the alterations of bone marrow mesenchymal stem cells (BM-MSCs) proliferation and differentiation, ALP+ CFU-F and total CFU-F colony formation were examined by total bone marrow cell cultures. Our results revealed that cells from ORX mice formed significantly lower numbers of ALP+ CFU-F (Figure 4A, 4C) and total CFU-F colonies (Figure 4B, 4D) than cells from sham mice. However, CoQ10 supplementation can increase the numbers of ALP+ CFU-F and total CFU-F colonies. These results indicated that CoQ10 supplementation can increase osteoblastic bone formation in ORX mice by stimulating the proliferation and differentiation of BM-MSCs.

The effect of CoQ10 on redox balance in ORX mice

In order to clarify whether the effect of CoQ10 supplementation on ORX-induced bone loss was associated with the inhibition of oxidative stress, the ROS levels, the antioxidant enzyme activity and gene expression levels of antioxidant enzymes in long bone were measured. Results showed that ROS levels (Figure 5A, 5B) were increased significantly while total antioxidant capacity (T-AOC) (Figure 5C) and gene expression levels of Catalase (CAT) (Figure 5D), superoxide dismutase 1 (SOD1) (Figure 5E), superoxide dismutase 2 (SOD2) (Figure 5F), glutathione reductase (GSR) (Figure 5G) and peroxiredoxin 1 (Prdx1) (Figure 5H) were decreased markedly in ORX mice compared with sham mice. However, these changes were largely rescued in ORX mice supplemented with CoQ10. These data indicated that the role of CoQ10 supplementation in the prevention of ORX-induced bone loss may be associated with decreased oxidative stress.
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The effect of CoQ10 on cell senescence in ORX mice

In order to clarify whether ORX-induced bone loss prevented by CoQ10 supplementation was associated with decreased cell senescence, β-galactosidase (β-gal) and p16\textsuperscript{INK4a} were examined by real-time RT-PCR or immunohistochemical staining in paraffin-embedded sections of long bone. We found that the percentages of p16\textsuperscript{INK4a} (Figure 6A, 6C), β-gal (Figure 6B, 6D) positive cells and the gene expression levels of p16\textsuperscript{INK4a} (Figure 6E) were elevated in ORX mice compared with sham mice. However, these alterations were rescued markedly in ORX mice supplemented with CoQ10. Our results indicated that CoQ10 supplementation could prevent cell senescence in ORX mice.

Discussion

Osteoporosis is a disease that seriously harms human health. According to statistics, there will be 8.1 million new patients with bone fractures...
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due to osteoporosis from 2010 to 2050 [23]. In the past decade, male osteoporosis has increasingly become an important social health problem [24]. But there is only limited preventive and therapeutic drug for male osteoporosis. Therefore, it is necessary to elucidate its pathophysiological mechanism and develop new therapeutic drugs for osteoporosis in men. In our current study, we used the ORX mouse model to study the possible mechanism of osteoporosis in men and the effect of CoQ10 on the male osteoporosis. Firstly, we followed the X-ray, Micro-CT scanning and three-dimensional (3D) reconstruction to track changes of bone mass and bone mineral density in each group of mice. We found that ORX resulted in bone loss which is consistent with previous studies [25, 26]. Simultaneously, we confirmed that CoQ10 administration could rescue bone loss induced by ORX.

It is currently believed that androgen deficiency is the main risk factor for osteoporosis in men. On the one hand, androgen can directly bind to the androgen receptor in osteoblasts and exert the ability to stimulate the proliferation and dif-

Figure 5. The effect of CoQ10 on redox balance in ORX mice. (A) Representative flow cytometric analysis of ROS levels of bone marrow cells from 48-week-old mice of each group. (B) Relative fluorescence intensity (RFI) of ROS was calculated and expressed relative to the sham mice. (C) Biochemistry analysis of bone tissue extracts from 48-week-old mice of each group for the total antioxidant capacity (T-AOC). Real-time RT-PCR was performed on long bone extracts from 48-week-old mice of each group. Gene expression of (D) CAT, (E) SOD1, (F) SOD2, (G) GSR and (H) Prdx1 were shown. Messenger RNA expression, assessed by real-time RT-PCR analysis, was calculated as a ratio to the GAPDH mRNA level and expressed relative to levels in sham-operated mice. Data are presented as the mean ± SEM of determinations, each data-point was the mean of five specimens. *P < 0.05, versus sham mice; **P < 0.05, ###P < 0.01 versus ORX mice.
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Figure 6. The effect of CoQ10 on cell senescence in ORX mice. A. Representative micrographs of paraffin sections of tibias from 48-week-old mice of each group stained immunohistochemically for p16\textsuperscript{INK4a}. B. Representative micrographs of paraffin sections of tibias from 48-week-old mice of each group stained immunohistochemically for β-gal. C. The percentages of p16\textsuperscript{INK4a}-positive cells were determined by image analysis. D. The percentages of β-gal-positive cells were determined by image analysis. E. Real-time RT-PCR was performed on long bone extracts from 48-week-old mice of each group. Gene expression of p16\textsuperscript{INK4a} were shown. Messenger RNA expression, assessed by real-time RT-PCR analysis, was calculated as a ratio to the GAPDH mRNA level and expressed relative to levels in sham-operated mice. Data are presented as the mean ± SEM of determinations, each data-point was the mean of five specimens. *P < 0.05, **P < 0.01, ***P < 0.001, versus sham mice; #P < 0.05, ##P < 0.01 versus ORX mice.

...tion of osteoblasts; on the other hand, androgen is reduced to dihydrotestosterone (DHT) under the action of 5-α reductase and then combined with androgen receptor. In vitro experiments have shown that dihydrotestosterone has the strongest ability to bind to androgen receptors in osteocytes. Androgen can also be converted to estrogen by the action of aromatase, thereby exert the role of regulating bone metabolism [24]. Then, we evaluated the effects of ORX or CoQ10 supplementation on the role of osteoblasts or osteoclasts. We found that ORX not only inhibited osteoblastic bone formation but also accelerated osteoclastic bone resorption, whereas these changes were normalized in ORX mice by the supplementation of CoQ10. Our results suggest that the administration of CoQ10 in ORX mice prevented the bone loss by inhibiting osteoclastic bone resorption and stimulating osteoblastic bone formation.

Numerous studies suggested that oxidative stress played a central role in the onset and development of postmenopausal osteoporosis [27]. Then, we asked whether osteoporosis caused by ORX was associated with increased oxidative stress. There is clinical evidence showed that oxidative stress accelerated bone loss in man [28]. In agreement with previous
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Studies, our present findings showed that ORX could induce oxidative stress and reduced antioxidants levels which support that decreased osteoblast formation and increased osteoclast resorption by ORX may be partly associated to increased oxidative stress. CoQ10 is an antioxidant with free radical scavenging activity and cytoprotective action. The effects of CoQ10 in human disease have been extensively studied, revealing the protective effects of coenzyme Q10 in heart failure, cancer, muscular dystrophy and periodontal disease [29]. Then, we asked whether the inhibition of CoQ10 supplementation on ORX-induced bone loss was associated with the inhibition of oxidative stress. Previous study indicates that CoQ10 acts as an inhibitor of osteoclasts by down-regulating the production of reactive oxygen species (ROS) [18]. CoQ10 can enhance the proliferation of BMSCs and promote osteogenic differentiation in a dose-dependent manner [20]. CoQ10 is feasible due to its antioxidant activity, which can reduce age-related bone loss, probably due in part to the elimination of intracellular ROS to reduce osteoclasts [30]. In our current study, we gave the ORX mice with the antioxidant CoQ10 for 40 weeks and certified that CoQ10 supplementation effectively prevented ORX-induced bone loss by defending against oxidative stress which suggest that CoQ10 may have potential therapeutic significance in the treatment of male osteoporosis.

Increasing ROS levels have been demonstrated as potentially critical for induction and maintenance of cell senescence process [31]. In vitro studies reported that senescent-cell conditioned medium inhibited osteoblast mineralization and enhanced osteoclast-progenitor survival, leading to increased osteoclastogenesis which suggest that targeting senescent cells may have both anti-resorptive and anabolic effects on bone [14]. Then, we test the alterations of cell senescence in the tibias from each group of mice and found that ORX could induce cell senescence as shown increased the percentages of p16 and β-galactosidase positive cells, the mRNA expression levels of p16, whereas CoQ10 supplementation could inhibit cell senescence in ORX mice.

In conclusion, our results confirm that elevated levels of oxidative stress and cell senescence are important causes in ORX-induced osteoporosis, while antioxidant CoQ10 played a preventive and protective role in ORX-induced osteoporosis by promoting osteoblastic bone formation, inhibiting osteoclastic bone resorption and oxidative stress, and preventing cell senescence. Therefore, our findings provide new therapeutic targets for the prevention and treatment of osteoporosis caused by testosterone deficiency in elderly men or after surgery, and provide experimental evidence for the clinical application of CoQ10.

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Disclosure of conflict of interest

None.

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References

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