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Original Article

Suppression effect of N-acetylcysteine on bone loss in ovariectomized mice

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Abstract: Oxidative stress can trigger DNA damage response and activation of cellular senescence. Accumulating studies have demonstrated that senescent cells can produce senescence-associated secretory phenotype that leads to increased bone resorption and decreased bone formation. And elimination of senescent cells or inhibition of SASP secretion has been shown to prevent bone loss in mice. N-acetylcysteine (NAC) is a strong antioxidant. However, it is unclear whether reversed estrogen deficiency-induced bone loss by antioxidant NAC was associated with the inhibition of oxidative stress, DNA damage, osteocyte senescence and SASP. In this study, OVX mice were supplemented with/without E2 or NAC, and were compared with each other. Our results showed that oxidative stress, DNA damage, osteocyte senescence and the secretion of senescence-associated inflammatory cytokines were increased in OVX mice compared with sham-operated mice. However, these parameters were obviously rescued in OVX mice supplemented with E2 or NAC. Data from this study suggest that NAC can prevent OVX-induced bone loss by inhibiting oxidative stress, DNA damage, cell senescence and the secretion of the senescence-associated secretory phenotype.

Keywords: OVX-induced bone loss, N-acetylcysteine, oxidative stress, cell senescence

Introduction

Osteoporosis is a systemic skeletal disease characterized by decreased bone mass, poor bone strength and deterioration of bone microarchitectural changes, leading to an enhanced risk of fragility fractures, deformity and other complications that affect quality of life [1]. As the global population ages, the prevalence of osteoporosis and its consequences is increasing worldwide. Postmenopausal osteoporosis is a high prevalence form of primary osteoporosis where the pathogenesis is associated with decreased estrogen levels, which leads to enhanced bone remodelling, results in lower bone density and higher fracture rates [2, 3]. Despite the intense research and the relevant progress achieved, the pathogenic mechanisms of how estrogen deficiency causes bone loss remain controversial.

In the last two decades, increasing oxidative stress has been identified as a vital pathogenic mechanism of bone loss caused by estrogen deficiency [4]. Oxidative stress occurs as a result of an overproduction of ROS not balanced by an adequate level of antioxidants. The excess of ROS can inhibit osteoblastic bone formation and promote osteoclastic bone resorption [5, 6] while antioxidants contribute to activation of osteoblast differentiation, mineralization process and reduction of osteoclast activity [7, 8]. Multiple human reports demonstrated an inverse and positive association between peripheral markers of oxidative stress and bone mass density in postmenopausal osteoporosis [9, 10]. And numerous evidence from animal studies show that bilateral ovariectomy can induce a redox imbalance characterized by increased levels of lipoperoxidation markers, such as ROS and malonaldehyde

(MDA), and decreased activity of antioxidant enzyme es like glutathione peroxidase (GSH-px), glutathione reductase (GSR) and superoxide dismutase (SOD) [11-13].

Oxidative stress is an important cause of cell senescence. Oxidative stress can cause DNA damage such as DNA double-strand breaks, activate ATM kinase activity, promote phosphorylation of γ-H2AX, up-regulate p53/p21 activity and trigger cellular senescence [14]. It is reported that accumulation of DNA damage and cell senescence has been proposed to play a causal role in deregulation of bone homeostasis [15]. Bone marrow stromal cells from old mice exhibit elevated expression of senescence-associated secretory phenotype (SASP) genes, such as TNF-α, IL-1α, MMP-13, CXCL12, which can be greatly attenuated by the senolytic drug ABT263 [16, 17].

N-acetylcysteine (NAC) is one of the most commonly used antioxidant compounds frequently used to decrease the oxidative stress caused by excessive ROS. However, it is unclear whether reversed estrogen deficiency-induced bone loss by antioxidant NAC treatment was associated with the inhibition of DNA damage, cellular senescence and SASP secretion. To answer this question, we established a bilateral ovariectomy (OVX) mouse model which is a mature and widely used animal model in the study of postmenopausal osteoporosis and then supplementing the OVX mice with 17β-estradiol (E2) or NAC. The control mice underwent a sham operation and were fed normally. We examined the changes in bone microarchitecture, bone turnover, oxidative stress, DNA damage, cell senescence and SASP of these mice to investigate whether NAC can inhibit the estrogen deficiency-induced bone loss by inhibiting DNA damage, cell senescence and SASP secretion.

Materials and methods

Mice

Three-week-old female C57/BL6J mice were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). 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 four groups: 1) sham surgery; 2) OVX group; 3) OVX group injected with 17β-estradiol (E2) at a dose of 10 μg/kg body weight subcutaneously from 8-week-old to 16-week-old; 4) OVX group supplemented with 1 mg/ml N-acetylcysteine (NAC) in the drinking water from 3-week-old to 16-week-old. Mice were anesthetized with chloral hydrate and bilaterally ovariectomized (OVX) when they are eight weeks old. Sham operations were independently performed. All mice were sacrificed at 16 weeks of age, 8 weeks after surgery. This study was approved by the Institutional Animal Care and Use Committee of Nanjing Medical University.

X-ray and microcomputed tomography (micro-CT)

After sacrifice, the right femurs were removed and fixed in PLP fixative (2% paraformaldehyde containing 0.075 M lysine and 0.01 M sodium periodate). These samples were then scanned for X-ray and micro-CT. Radiographs were performed as described previously. Same samples were then scanned on a micro-CT scanner (Sky Scan 1072 Scanner) using energy of 100 kV, and 98 μA intensity. Three-dimensional (3D) images were generated using the 3D Creator software supplied with the instrument as described previously [18].

Histology

The right 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 EDTA glycerol solution for 5 to 7 days at 4°C. The decalcified tibias were dehydrated and embedded in paraffin, after which 5 μm sections were cut on a rotary microtome. The sections were stained histochemically for total collagen or tartrate resistant acid phosphatase (TRAP) activity as described previously [19] or immunohistochemically as described below.

Immunohistochemical staining

Immunohistochemical staining was carried out for phosphorylation of histone H2AX on Ser139 (γ-H2AX), β-galactosidase (β-gal), $p16^{INK4a}$, Interleukin (IL)-1α, IL-1β and MMP13, using the avidin-biotin-peroxidase complex technique with affinity-purified goat anti-rabbit γ-H2AX (Cell Signaling Technology, MA, USA), β-gal (Santa Cruz Biotechnology, Santa Cruz, CA, USA),

$p16^{INK4a}$ (Abcam, MA, USA), IL-1α (Abcam, MA, USA), IL-1β (Abcam, MA, USA) and MMP13 antibody (Abcam, MA, USA), following previously-described methods [20].

Detection of 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, then 106 cells per sample were incubated with 5 μL 2',7'-dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich, St. Louis, MO, USA) for 30 mins in the dark followed by incubation with 10% FBS for 20 mins at 37°C. ROS levels were calculated from mean fluorescence intensity (MFI) measured using a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Western blotting

Proteins were extracted from the left femurs of each group of mice. Immunoblotting was carried out as previously described [21]. Primary antibodies against SOD2 (Novus Biological, Centennial, CO, USA), γ-H2AX (Cell Signaling Technology, MA, USA), 8-OHdG (Novus Biological, Centennial, CO, USA), $p16^{INK4a}$ (Abcam, MA, USA), TNF-α (Abcam, MA, USA) and MMP3 (Abcam, MA, USA) were used. And β-tubulin (Bioworld Technology, St. Louis Park MN, USA) were used as loading control. Immunoreactive bands were visualized with ECL chemiluminescence (Amersham Biosciences, Chalfont St. Giles, UK) and analyzed by Scion Image Beta 4.02 (Scion, National Institutes of Health, Bethesda, MD, USA).

RNA isolation and quantitative real-time RT-PCR

RNA was isolated from left 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 [20]. 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.

Computer-assisted image analysis

After histochemical or immunohistochemical staining of sections from mice of each group, images of micrographs from single sections were digitally recorded using a rectangular template, and recordings were processed and analysed using Northern Eclipse image analysis software as described previously [20].

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 E2 or NAC on the bone mineral density and bone volume in ovariectomized mice

To examine the effect of E2 or NAC on the bone mineral density and bone mass in ovariectomized mice, eight-week-old mice were bilaterally ovariectomized (OVX) and supplemented with or without N-acetylcysteine (NAC) in the drinking water, or subcutaneously injected with 17β-estradiol (E2) at a dose of 10 μg/kg body weight. After 8 weeks, body weight and uterus weight were compared with each other. Results showed that, compared with sham-operated mice, the body weight was enhanced significantly in OVX mice which were rescued by NAC or E2 administration (**Figure 1A**). The uterus weight of the OVX mice was decreased compared to that of the sham-operated mice, and they were not altered by NAC supplementation. However, the supplementation of E2 significantly inhibited the reduction in uterus weight of OVX mice (**Figure 1B**). Then, we examined bone mineral density and bone volume parameters. We found that bone mineral density, total

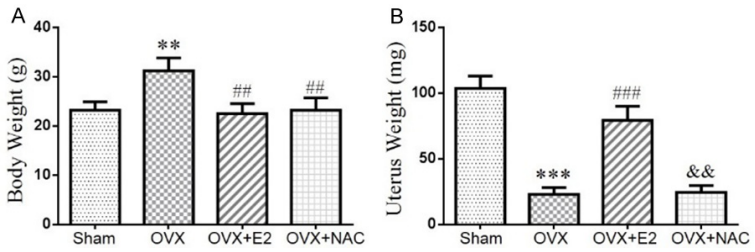

Figure 1. The effect of E2 or NAC on the body weight and uterus weight in ovariectomized mice. (A) Body weight and (B) uterus weight were measured before sacrificed at 16 weeks of age. Data are presented as the mean ± SEM of determinations, each data-point was the mean of five specimens. **P<0.01, ***P<0.001, versus sham mice. ##P<0.01, ###P<0.001 versus OVX mice. &&P<0.01 versus OVX+E2 mice.

collagen positive area, bone volume, trabecular number and thickness (**Figure 2A-I**) were all decreased markedly while trabecular separation was increased in OVX mice. However, both exogenous E2 and NAC supplementation could normalize these parameters. These results indicate that the role of NAC supplementation in preventing bone loss of OVX mice is comparable to E2 supplementation.

The effect of E2 or NAC on the osteoclastic bone resorption in ovariectomized mice

To determine the potential role of osteoclasts in the bone loss after OVX operation and increased bone mineral density following E2 or NAC supplementation, the parameters of osteoclastic bone resorption were assessed by histochemical staining for TRAP, image analysis and real-time RT-PCR. The number of osteoclasts (**Figure 3A**, **3B**), TRAP-positive osteoclast surface (**Figure 3C**) and RANKL/OPG ratio (**Figure 3D**) expression levels were increased in OVX mice compared with sham mice. However, these changes were largely rescued in OVX mice with E2 or NAC supplementation. Our data demonstrated that E2 or NAC supplementation could inhibit osteoclastic bone resorption in OVX mice.

The effect of E2 or NAC on redox balance in ovariectomized mice

To determine whether the effect of E2 or NAC on OVX-induced bone loss was associated with oxidative stress, we examined the ROS levels, the antioxidant enzyme activity, MDA content, the protein and gene expression levels of antioxidant enzymes in long bone. We found that ROS levels (**Figure 4A**, **4B**) and malondialdehyde (MDA) level (**Figure 4C**) were elevated significantly, while total antioxidant capacity (T-AOC) (**Figure 4D**), the protein (**Figure 4E**, **4F**) and gene (**Figure 4G**) expression level of superoxide dismutase 2 (SOD2) were reduced markedly in OVX mice compared with sham mice. However, these changes were largely rescued in OVX mice supplemented with E2 or NAC. These data indicated that the role of E2 or NAC supplementation in the prevention of OVX-induced bone loss may be interrelated with decreased oxidative stress.

The effect of E2 or NAC on DNA damage in ovariectomized mice

To investigate whether increased oxidative stress could induce DNA damage in OVX mice, the DNA damage markers, phosphorylation of histone H2AX on Ser139 (γ-H2AX) and 8-hydroxy-2'-deoxyguanosine (8-OHdG) were examined by western blot, real time RT-PCR or immunohistochemical staining in paraffin-embedded sections of long bone. Results showed that the percentages of osteocytes positive for γ-H2AX (**Figure 5A**, **5B**) and the protein (**Figure 5C-E**) and gene (**Figure 5F**, **5G**) expression levels of γ-H2AX, 8-OHdG were higher in OVX mice than that in sham-operated mice. However, these changes were down-regulated dramatically in OVX mice supplemented with E2 or NAC. These results indicated that increased oxidative stress in OVX mice could induce DNA damage whereas E2 or NAC supplementation could inhibit DNA damage.

The effect of E2 or NAC on osteocyte senescence in ovariectomized mice

DNA damage response can trigger the activation of cellular senescence. To determine the effect of E2 or NAC on osteocyte senescence in OVX mice, the cellular senescence markers, β-galactosidase (β-gal) and $p16^{INK4a}$, were examined by Western blot or immunohistochemical staining in paraffin-embedded sections of long bone. We found that the percentages of osteocytes positive for $p16^{INK4a}$

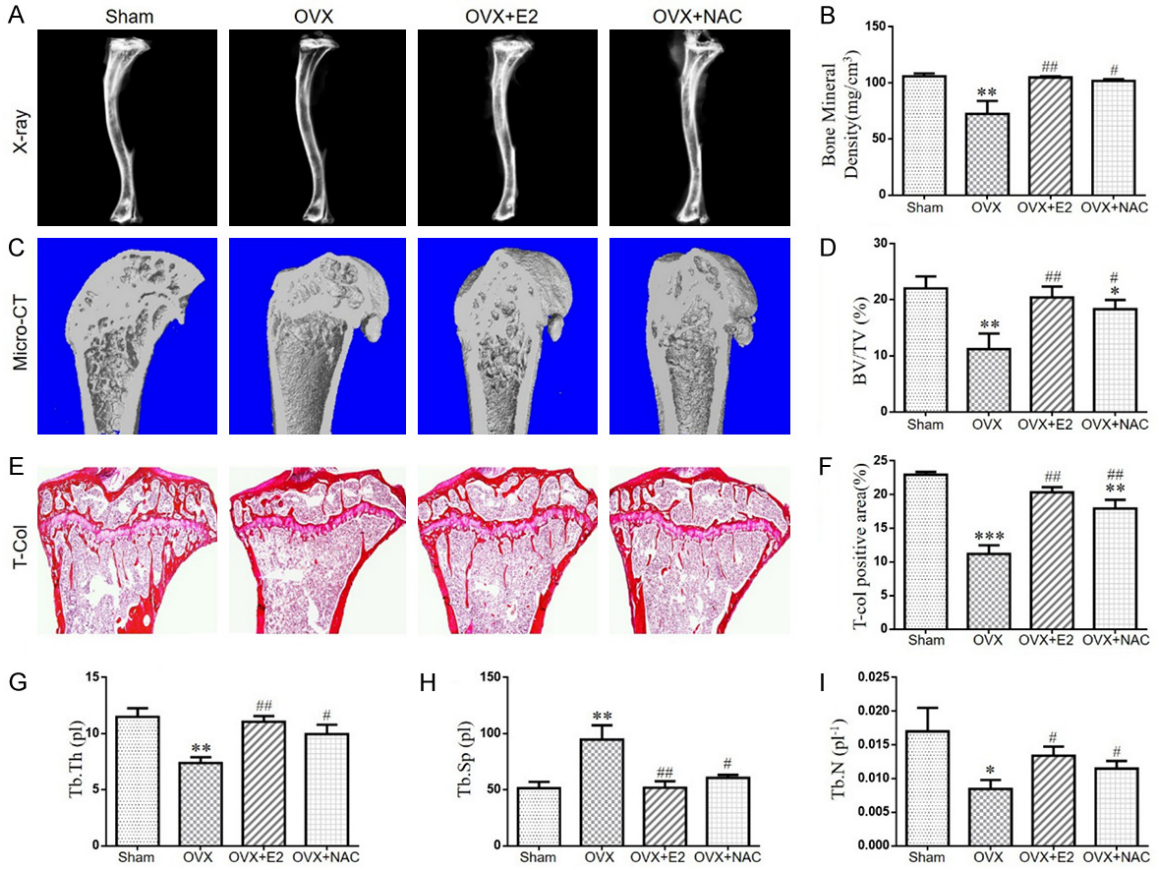

Figure 2. The effect of E2 or NAC on the bone mineral density and bone volume in ovariectomized mice. (A) Representative radiographs of tibias from 16-week-old mice of each group. (B) Bone mineral density analysis of the trabecular bone. (C) Representative Micro-CT-scanned and 3D reconstructed sections along the longitudinal direction of tibias. (D) Trabecular bone volume relative to tissue volume (BV/TV, %). (E) Representative micrographs of paraffin sections of tibias were stained histochemically for total collagen. (F) Total collagen positive area (%). (G) Thickness of trabecular bone (Tb.Th), (H) Trabecular separation (Tb.Sp), (I) Trabecular number (Tb.N) as analyzed with Micro-CT SkyscanCTAn software. 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 OVX mice. (E) Magnification, 50×.

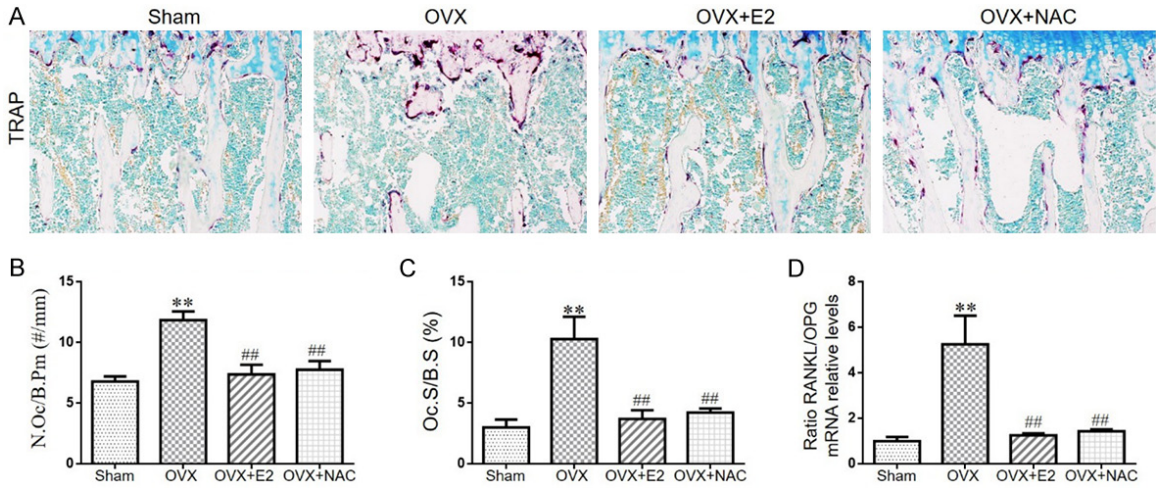

Figure 3. The effect of E2 or NAC on the osteoclastic bone resorption in ovariectomized mice. A. Representative photomicrographs of paraffin sections of tibias from 16-week-old mice of each group stained histochemically for TRAP.

B. The number of TRAP-positive osteoclasts (N.Oc) per mm bone perimeter (B.Pm) was measured and presented. C. Osteoclast surface relative to bone surface (Oc.S/B.S, %). Real-time RT-PCR was performed on bone tissue extracts from 16-week-old mice of each group. D. Gene expression of RANKL/OPG ratio are 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 mice. Data are presented as the mean ± SEM of determinations, each data point is the mean of five specimens. **P<0.01, versus sham mice. ##P<0.01 versus OVX mice. A. Magnification, 200×.

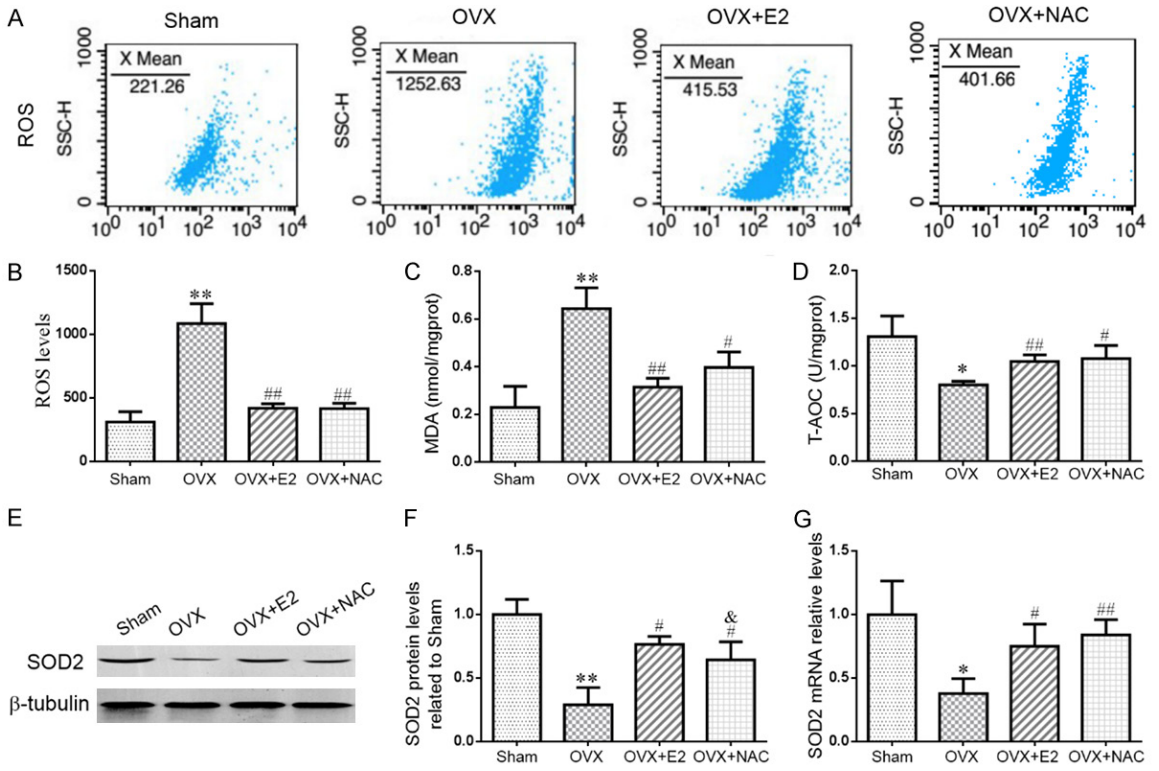

Figure 4. The effect of E2 or NAC on redox balance in ovariectomized mice. (A) Representative flow cytometric analysis of ROS levels of bone marrow cells from sham, OVX, OVX+E2 and OVX+NAC mice. (B) Relative fluorescence intensity (RFI) of ROS was calculated and expressed relative to the sham mice. Biochemistry analysis of bone tissue extracts from sham, OVX, OVX+E2 and OVX+NAC mice for the (C) malonaldehyde (MDA) content and (D) total antioxidant capacity (T-AOC). (E) Representative western blots of bone tissue extracts showing expression of superoxide dismutase 2 (SOD2); β-tubulin was used as loading control for the western blots in the sham, OVX, OVX+E2 and OVX+NAC mice. (F) SOD2 protein levels relative to β-tubulin protein levels were assessed by densitometric analysis and expressed relative to levels in sham-operated mice. (G) Real-time RT-PCR was performed on long bone extracts from 16-week-old mice of each group. Gene expression of SOD2 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 versus sham mice. #P<0.05, ##P<0.01 versus OVX mice. &P<0.05 versus OVX+E2 mice.

(**Figure 6A**, **6B**), β-gal (**Figure 6C**, **6D**) and the protein expression levels of $p16^{INK4a}$ (**Figure 6E**, **6F**) were elevated in OVX mice compared with sham-operated mice. However, these alterations were rescued markedly in OVX mice supplemented with E2 or NAC. Our results indicated that DNA damage could induce osteocyte senescence whereas E2 or NAC supplementation could prevent osteocyte senescence in OVX mice.

The effect of E2 or NAC on SASP in ovariectomized mice

Senescent cells can secrete a variety of cytokines, namely senescence-associated secretory phenotypes (SASP), including IL-1α, IL-1β, IL-6, IL-8, MMP-3, MMP-13, TNF-α and TGF-β. To verify if E2 or NAC supplementation could prevent SASP secretion in OVX mice, we examined the alterations of SASP by immunohisto-

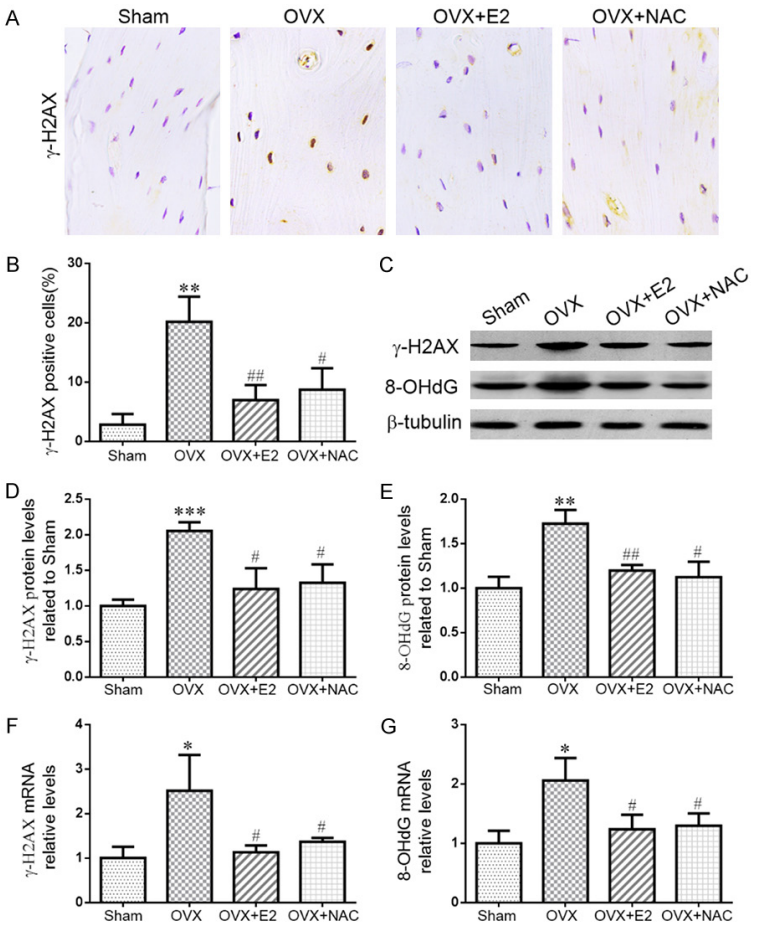

Figure 5. The effect of E2 or NAC on DNA damage in ovariectomized mice. (A) Representative micrographs of paraffin sections of tibias from 16-week-old mice of each group stained immunohistochemically for γ-H2AX. (B) The percentages of γ-H2AX-positive cells were determined by image analysis. (C) Representative western blots of bone tissue extracts showing expression of γ-H2AX and 8-OHdG; β-tubulin was used as loading control for the western blots in the sham, OVX, OVX+E2 and OVX+NAC groups. (D) γ-H2AX and (E) 8-OHdG protein levels relative to β-tubulin protein levels were assessed by densitometric analysis and expressed relative to levels in sham-operated mice. Real-time RT-PCR was performed on long bone extracts from 16-week-old mice of each group. Gene expression of (F) γ-H2AX and (G) 8-OHdG 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<0.01, ***P<0.001 versus sham mice. #P<0.05, ##P<0.01 versus OVX mice. (A) Magnification, 400×.

chemistry and western blotting. Compared with the sham-operated mice, the percentage of osteocytes positive for IL-1α (**Figure 7A**, **7B**), IL-1β (**Figure 7C**, **7D**), MMP13 (**Figure 7E**, **7F**) and the protein expression levels of TNF-α, MMP3 (**Figure 7G-I**) were all increased dramatically in OVX mice. However, all the alterations were markedly rescued in OVX mice by E2 or NAC supplementation. These results indicated that E2 or NAC supplementation could inhibit the production of SASP.

Discussion

Osteoporosis, characterized by a decrease in bone mineral density, deterioration of bone microstructure, the consequent increase in bone fragility and the risk of fracture, has become a global public health burden. Postmenopausal osteoporosis is a high prevalence form of osteoporosis, with an incidence of up to 75% [22]. In the absence of estrogen, upregulated formation and activation of osteoclasts increase bone resorption, resulting in a 3-5% reduction in bone mass per year after menopause. Drugs, such as bisphosphonates, parathyroid hormone-related peptides (PTH) and monoclonal antibodies against RANKL have been suggested for the treatment of postmenopausal osteoporosis to inhibit bone resorption or promote bone formation [23]. However, there are still many patients with osteoporosis who have not yet received appropriate treatment. Therefore, it is necessary to find better treatments for postmenopausal osteoporosis. In our current study, we used the OVX mouse model which is a mature and widely used animal model in the study of osteoporosis to investigate the possible mechanism of osteoporosis and the protective effect of NAC on the postmenopausal osteoporosis. Firstly, we showed that OVX mice exhibited estrogen deficiency, uterine atrophy and body weight gain while exogenous E2 supplementation can rescue serum E2 levels, uterine weight and body weight. At the same time, we found that antioxidant NAC supplementation limited the increase of the body weight and

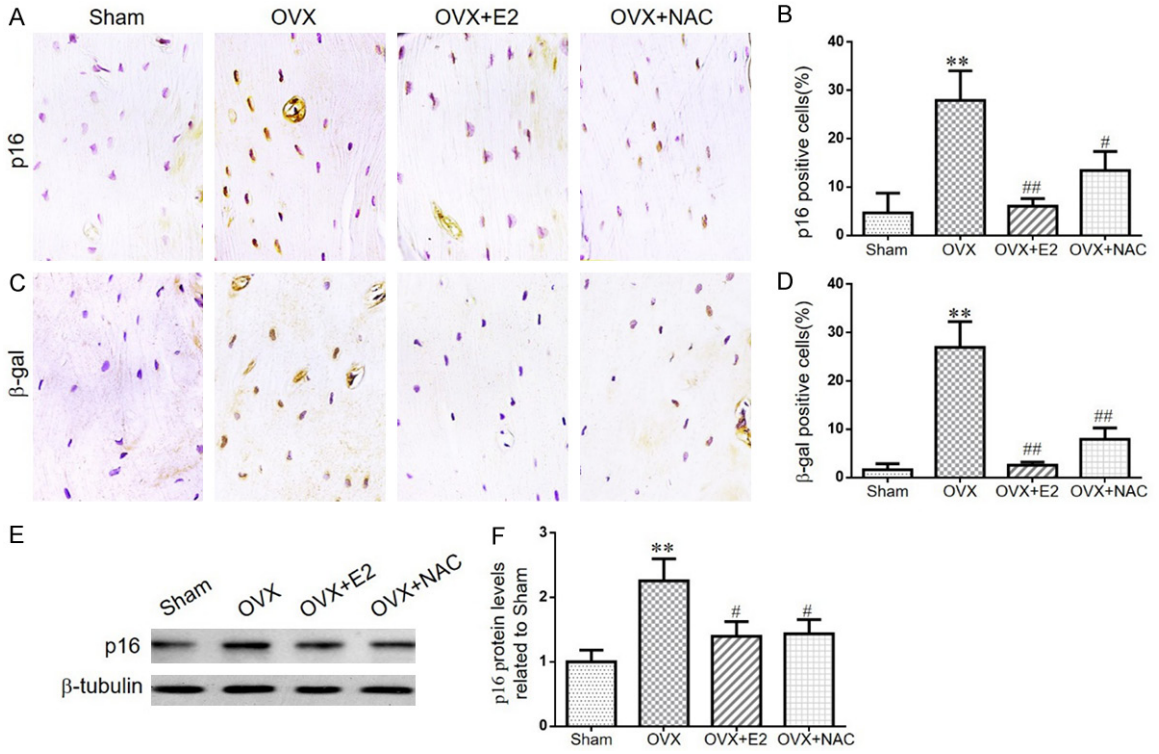

Figure 6. The effect of E2 or NAC on osteocyte senescence in ovariectomized mice. A. Representative micrographs of paraffin sections of tibias from 16-week-old mice of each group stained immunohistochemically for $p16^{INK4a}$. B. The percentages of $p16^{INK4a}$-positive cells were determined by image analysis. C. Representative micrographs of paraffin sections of tibias from 16-week-old mice of each group stained immunohistochemically for β-gal. D. The percentages of β-gal-positive cells were determined by image analysis. E. Representative western blots of bone tissue extracts showing expression of $p16^{INK4a}$; β-tubulin was used as loading control for the western blots in the sham, OVX, OVX+E2 and OVX+NAC groups. F. $p16^{INK4a}$ protein levels relative to β-tubulin protein levels were assessed by densitometric analysis 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<0.01 versus sham mice. #P<0.05, ##P<0.01 versus OVX mice. A, C. Magnification, 400×.

atrophy of the uterus, but did not alter serum E2 levels. Then, we followed the X-ray, Micro-CT scanning, three-dimensional (3D) reconstruction, total collagen staining and histomorphological parameters analysis to track changes in bone microarchitecture in each group of mice. We found that both E2 and NAC supplementation can save bone loss induced by OVX, which is consistent with previous studies.

Estrogen deficiency after menopause is recognized by increasing the formation of osteoclasts by providing a larger pool of osteoclast progenitors. The increased formation and activation of osteoclasts accelerates the area of resorption of the trabecular bone surface [24, 25]. Then, we evaluated the effects of OVX, E2 or NAC supplementation on the role of osteoclasts. We found that OVX accelerated osteoclastic bone resorption, whereas E2 or NAC can significantly inhibit the bone resorption of osteoclasts. Our results demonstrated that the administration of E2 or NAC in OVX mice prevented the destruction of the bone architecture by suppressing the increase in the osteoclast activity.

Although significant progress has been made in the mechanism how estrogen deficiency induces osteoporosis, it has been found that the underlying pathogenesis is complex [26]. One of the most interesting hypotheses is that these sex hormones protect bones through antioxidants that act as antioxidants [10]. Experimental studies demonstrated that oxidative stress is an important factor in this disorder of bone homeostasis [27-29]. In vitro and animal experiments showed that the oxidative stress is produced due to insufficient defense against reactive oxygen species (ROS) by the endogenous antioxidant defense system, which in turn stim-

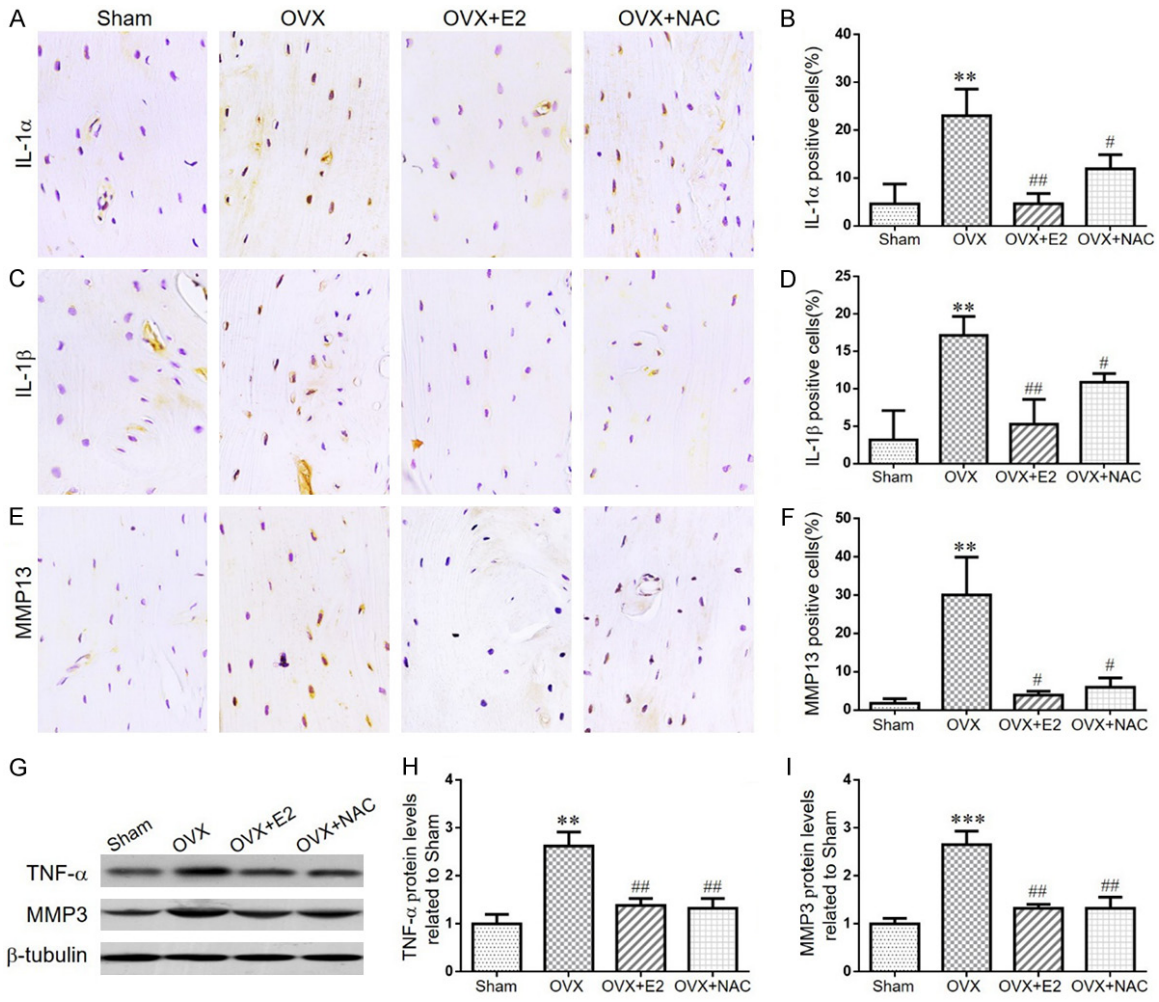

Figure 7. The effect of E2 or NAC on SASP in ovariectomized mice. (A) Representative micrographs of paraffin sections of tibias from 16-week-old mice of each group stained immunohistochemically for IL-1α. (B) The percentages of IL-1α-positive cells were determined by image analysis. (C) Representative micrographs of paraffin sections of tibias from 16-week-old mice of each group stained immunohistochemically for IL-1β. (D) The percentages of IL-1β-positive cells were determined by image analysis. (E) Representative micrographs of paraffin sections of tibias from 16-week-old mice of each group stained immunohistochemically for MMP13. (F) The percentages of MMP13-positive cells were determined by image analysis. (G) Representative western blots of bone tissue extracts showing expression of TNF-α, MMP3; β-tubulin was used as loading control for the western blots in the sham, OVX, OVX+E2 and OVX+NAC groups. (H) TNF-α and (I) MMP3 protein levels relative to β-tubulin protein levels were assessed by densitometric analysis 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<0.01 versus sham mice. #P<0.05, ##P<0.01 versus OVX mice. (A, C, E) Magnification, 400×.

ulates the formation and resorption activity of osteoclasts [30, 31]. Then, we examined in our study whether osteoporosis caused by OVX was associated with the imbalance between ROS production and endogenous antioxidant defense capacity. Consistent with previous studies, our current study confirmed that OVX could increase ROS generation and reduce antioxidants levels, thereby supporting OVX-induced osteoporosis that may be related with oxidative stress. And NAC or E2 supplementation could effectively defend against oxidative stress in OVX mice, supporting that antioxidants prevented OVX-induced bone loss partially through defense against oxidative stress.

Excessive accumulation of intracellular ROS can cause oxidative damage to lipids, proteins and DNA, leading to the production of oxidized biomolecule products, such as MDA, protein

carbonyl and 8-hydroxyguanine (8-OHG) [32, 33]. It has been reported that the accumulation of oxidative DNA damage contributes to age-related disorders [34]. Genetic mutations encoding proteins required for DNA damage have been observed to induce dysregulation of bone homeostasis, confirming that DNA damage plays a crucial role in bone defects [15, 35]. Our current results indicated that the DNA damage markers, γ-H2AX, and 8-OHdG, were increased in the osteocytes of OVX mice which suggested that increased oxidative stress can trigger DNA damage in the OVX mice whereas NAC could inhibit DNA damage in the estrogen deficiency-induced osteoporosis as effectively as the E2 supplementation.

Accumulation of DNA damage is the leading cause of senescence, which plays a fundamental role in the initiation of cellular senescence through the activation of cell cycle inhibitors, $p16^{INK4a}$ and $p21^{WAF1/CIP}$ [36]. Preclinical studies and supportive human data have shown an increase in senescent cells in the bone microenvironment with aging. Senescent cells can produce complex pro-inflammatory response called the senescence-associated secretory phenotype (SASP) that leads to increased bone resorption and decreased bone formation. Elimination of senescent cells or inhibition of SASP secretion has been shown to prevent age-related bone loss in mice [37-39]. Then, we asked if OVX-induced osteoporosis is associated with increased osteocyte senescence and SASP. To answer this question, the alterations of osteocyte senescence and SASP were examined. We demonstrated that OVX could induce osteocyte senescence and elevated SASP secretion as shown increased the expression levels of senescence marker, p16 and β-gal, and the secreted products of SASP, including IL-1α, IL-1β, MMP13, MMP3 and TNF-α, whereas E2 or NAC supplementation can efficiently suppress the expression of cell senescence markers and the development of the SASP. These results confirmed that E2 or NAC could exert an anti-osteoporosis role in OVX mice by inhibiting of senescent cells or SASP secretion.

Conclusively, the present work exhibited that supplementation of NAC may not only be useful to treat OVX-induced osteoporosis by simultaneously reducing osteoclastic bone resorption, oxidative stress and DNA damage, but also to concurrently prevent osteocyte senescence and SASP production by targeting a fundamental aging mechanism.

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Disclosure of conflict of interest

None.

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