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Increased activity of osteocyte autophagy in ovariectomized rats and its correlation with oxidative stress status and bone loss

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ABSTRACT

Objectives: The objectives of the present study were to investigate ovariectomy on autophagy level in the bone and to examine whether autophagy level is associated with bone loss and oxidative stress status.
Methods: 36 female Sprague–Dawley rats were randomly divided into sham-operated (Sham), and ovariectomized (OVX) rats treated either with vehicle or 17-β-estradiol. At the end of the six-week treatment, bone mineral density (BMD) and bone micro-architecture in proximal tibias were assessed by micro-CT. Serum 17β-estradiol (E2) level were measured. Total antioxidant capacity (T-AOC), superoxide dismutase (SOD) activity, catalase (CAT) activity in proximal tibia was also determined. The osteocyte autophagy in proximal tibias was detected respectively by Transmission Electron Microscopy (TEM), immunofluorescent histochemistry (IH), realtime-PCR and Western blot. In addition, the spearman correlation between bone mass, oxidative stress status, serum E2 and autophagy were analyzed.
Results: Ovariectomy increased Atg5, LC3, and Beclin1 mRNA and proteins expressions while decreased p62 expression. Ovariectomy also declined the activities of T-AOC, CAT, and SOD. Treatment with E2 prevented the reduction in bone mass as well as restored the autophagy level. Furthermore, LC3-II expression was inversely correlated with T-AOC, CAT, and SOD activities. A significant inverse correlation between LC3-II expression and BV/TV, Tb.N, BMD in proximal tibias was found.
Conclusions: Ovariectomy induced oxidative stress, autophagy and bone loss. Autophagy of osteocyte was inversely correlated with oxidative stress status and bone loss.

1. Introduction

Postmenopausal osteoporosis, characterized by low bone mass, altered bone microarchitecture and increased risk of fracture, was one of the most prevalent degenerative diseases affecting women [1]. Osteocyte, differentiated osteoblasts, embedded in bone matrix, was the most abundant of bone cells and played a significant role in the maintenance and integrity of bone [2–4]. Previous studies had linked osteocyte dysfunction to the initiation of the bone remodeling process [5,6]. Subsequently, several researchers confirmed that bone loss was due in part to osteocyte apoptosis following estrogen depletion [7,8].

Oxidative stress, resulting from excessive generation of reactive oxygen species, could damage all components of the cell [9]. At present, the positive correlation was observed between oxidative stress and osteoporotic status [10,11]. More importantly, oxidative stress was positively correlated with bone loss in osteoporosis patients [12,13]. Evidences mainly obtained from the above studies provided a paradigm shift from the "estrogen-centric" account of the pathogenesis to one that osteocyte dysfunction and oxidative stress was also protagonists in the developing postmenopausal osteoporosis. However, the exact mechanisms by which estrogen loss led to osteocyte dysfunction and oxidative stress in bone was incompletely understood.

Autophagy, an important response mechanism under different kinds of cellular stress, was a cellular protective mechanism as well as another way of programmed cell death in addition to apoptosis. Previous studies suggested that autophagy existed in osteocyte [14]. Furthermore, autophagic receptor, NBR1, had an important effect on the activity and differentiation of osteoblasts [15,16]. Subsequently, researchers found that autophagy played as a protective role when osteocyte was stressed by hypoxia or glucocorticoid in vitro [14,17]. At present, it is generally accepted that reactive oxygen species induces autophagy [18,19], and autophagy, in turn, serves to reduce oxidative damage in several pathological conditions [20,21]. However, it remains unclear whether autophagy of osteocyte was correlated with oxidative stress and bone loss in the mode of ovariectomized rat.

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Based on the above observations that oxidative stress was associated with bone loss, and autophagy might resist oxidative stress injury. We hypothesized bone loss induced by ovariectomy might be attributed to the increased oxidative stress status and autophagy of osteocyte might involve in estrogen-deficiency bone loss. To prove our hypothesis, OVX rats were used to mimic the development of estrogen deficiency-induced osteoporosis [22]. Autophagy level and oxidative stress status in the proximal tibia in OVX rats were assessed. We also examined whether autophagy level was correlated with serum estrogen, bone mass, bone microarchitecture and antioxidative biomarkers in the proximal tibia. Given that autophagy could exert a critical influence on oxidative stress injury, it might be beneficial to investigate the potential for therapeutic targeting of autophagic processes.

2. Materials and methods

2.1. Study design

36 virgin, female Sprague–Dawley rats aged six-week ($n = 12$ for each group) were randomly divided into sham-operation (Sham), and ovariectomized rats treated with vehicle (OVX + veh) or 10 μg/kg/day 17-β-estradiol (OVX + E2) [23,24]. OVX was performed under anesthesia with 10% chloral hydrate according to our previous method [25]. The rats were killed at the end of the 6-week surgery. Bone mass and bone micro-architecture in the proximal tibias were measured by Micro-CT. The osteocyte autophagy in proximal tibias was detected by TEM, IH staining, realtime-PCR and Western blot, respectively. The protocol for animal experimentation was previously approved by the Animal Research Committeen of the university, and all subsequent animal experiments adhered to the "Guidelines for Animal Experimentation".

2.2. Specimen processing

After administration with E2 for 6 weeks, all animal were sacrificed by an overdose of 10% chloral hydrate. Blood samples were obtained from the abdominal aorta for E2 level assay. The left proximal tibias of rats were removed with a razor blade to clean off adherent soft tissue and cartilage and the bone marrow was obtained from the tibial canal with a syringe and collected in a 15 mL centrifuge tube, stored at −80 °C for Realtime PCR, Western-blot analysis and oxidative status tests. The whole tibias were obtained from the right tibia cleaned off adherent soft tissue and cartilage.

2.3. Bone measurement with Micro-CT

The right tibias were scanned with high-resolution micro-computed tomography system (GE Locus SP) in a standard resolution mode. Measurements of bone were taken from a 15 mm^2 area in the central region beginning 0.2 mm distal to the growth plate to assess bone mass, density, and micro-architecture of trabecular. Briefly, bones were scanned at a resolution of 16 μm. The volume of interest (VOI), $29 \times 29 \times 29\ \mu m^3$, was selected using a semi-automatic contouring method. Series of planar transverse gray-value images were generated. All images were used to reconstruct three-dimensional images. Software in the Micro-CT system was used to compute parameters within the VOI: bone mineral density (BMD), bone volume over total volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular separation (Tb.Sp).

2.4. Measurement of T-AOC, CAT, and SOD activity

The bone tissues in the proximal tibia (100 mg) were homogenized in cold saline. The homogenate was centrifuged at 1000*g* for 10 min. The T-AOC, CAT, and SOD activities were determined using kits from Beyotime (Jiangsu, China) according to the manufacturer's protocol.

2.5. Serum E2 assay

Blood samples were centrifuged at 3000*g* for 15 min at 4 °C, and the supernatants were used as serum samples. Serum E2 level (Shanghai Institute of Biological Product, Shanghai, China) was determined using commercially available ELISA kits according to the manufacturer's protocol.

2.6. TEM assay

Specimens from the proximal tibias were fixed in 2% glutaraldehyde for 24 h and demineralized in 5% EDTA for 2 weeks. Then the specimens were cut into 1 mm × 1 mm × $1mm^3$. After rinsing in 0.1 M PBS for 3 days, specimens were postfixed with 1% OsO_4 and embedded in epoxy resin. Before thin slides were cut and stained with uranyl acetate, the specimens were polymerized at 60 °C for a week followed by lead citrate. Finally, the ultrathin slides were observed in a TEM (Phillips CM-80, Eindhoven, Netherlands).

2.7. IH staining

The proximal tibias were fixed in 4% paraformaldehyde for 48 h at 4 °C. Sagittal tissue sections (4 μm thick) were prepared for IH staining following decalcified in 15% ethylenediaminetetraacetic acid (EDTA) at room temperature for 1 week. Sections were incubated with LC3-II antibody (1:100, CST Inc., Danvers, USA) overnight at 4 °C. Finally the slides were photographed with a fluorescence microscope and the expression of LC3-II was quantified by using the Image-Pro-Plus 6.0 analytic system (IPP 6.0, Media Cybernetics, Inc.).

2.8. Real-time RT-PCR analysis

Total RNA of proximal tibias (50 mg from the proximal tibia epiphyseal) was extracted using trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. The expressions of Atg5, p62 LC3, Beclin1, and β-actin genes were measured by real-time PCR using SYBR Premix Ex Taq (TaKaRa Biotechnology Co., Ltd, Dalian, China) and an ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA) with the following sequences of primers in Table 1. All primers were designed and synthesized by TaKaRa Biotechnology Co., Ltd (Dalian, China). Each gene was normalized to β-actin by relative

Table 1
Primer sequences used for real-time PCR.

Gene	Sequence (sense, antisense: 5′-3′)	Size (bp)
LC3	GAGTGGAAGATGTCCGGCTC CCAGGAGGAAGAAGGCTTGG	201
Beclin1	AGCACGCCATGTATAGCAAAGA GGAAGAGGGAAAGGACAGCAT	119
p62	GCTGCTCTCTTCAGGCTTACAG CCTGCTTCACAGTAGACGAAAG	206
Atg5	TGAAGGAAGTTGTCTGGATAGCTCA AAGTCTGTCCTTCCGCAGTC	136
Dmp1	CCTGTCGCCAGATACCAA TCCCGGCACTCTTAGAGA	184
Beta-actin	CACCCGCGAGTACAACCTTC CCCATACCCACCATCACACC	207

quantification. The $2^{-\Delta\Delta CT}$ (cycle threshold) method was used to calculate relative gene expression levels as previously described.

2.9. Western blot analysis

Total protein of proximal tibias was extracted using Western & IP Cell lysis Kit (Beyotime, Jangsu, China) and transferred to polyvinylidene difluoride (PVDF) membrane. The PVDF membranes were incubated with specific antibody and detected using the ECL detection kit (Beyotime, Jiangsu, China). Protein expression was quantified by densitometry analysis using the Image Lab version 2.1 (Bio-Rad) software.

2.10. Statistical analysis

All data were reported as means ± standard deviation (SD) for at least three independent experiments. Differences in the variables of LC3-II, BMD, and Micro-CT among the three groups were analyzed by one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test or the Student Newman Keuls Test using the statistics package SPSS 11.5 (SPSS, Chicago, IL, USA). Spearman correlation coefficients were used to analyze the relationship between parameters of autophagy level in tibia proximal with the serum estrogen, oxidative stress status, BMD and microstructure of tibia proximal. Differences were considered significant for $P < 0.05$ and highly significant for $P < 0.01$.

3. Results

3.1. E2. therapy reversed the increased autophagy in osteocyte caused by ovariectomy

To investigate whether the portion of the total protein or mRNA in our sample was mainly derived from osteocytes, we compared the amount of an osteocyte-specific gene (Dmp1) in bone of proximal tibias, bone marrow, and the whole tibias. Real-time PCR (Fig. 1A) and Western blot analysis (Fig. 1B) demonstrated that the expression of Dmp1 in the bone of proximal tibias was much higher than in the bone marrow or the whole tibias. These results proved that the total proteins or mRNAs extracted from the bone of proximal tibias predominantly came from osteocytes instead of osteoblasts, bone marrow cells or other cells.

Next, the hallmarkers of autophagy in the bone of proximal tibias, such as LC3-II/I, p62, Atg 5 and Beclin1, were assessed by real-time PCR and Western blot [26]. Realtime PCR results showed that the mRNA expression of Atg 5, LC3 and Beclin1 increased in the OVX + veh group, while E2 therapy significantly restored the expression levels of Atg 5, LC3 and Beclin1 (Fig. 1C). Accordingly, the mRNA expression of p62 decreased in the OVX + veh group, but no significant difference was observed among three groups. Consistently, as shown in Fig. 1D, the protein expression of Atg 5, LC3 and Beclin1 in the OVX + veh group detected by Western-blot was 4.2-fold, 2.65-fold and 3.05-fold higher than in Sham group, while there was a decrease of p62 expression in the OVX + veh group. However, no significant difference was found between the OVX + E2 and Sham groups. To further locate the autophagy cells in the proximal tibia, Immunofluorescence staining was applied. The results indicated that the expression of LC3-II was major in the cytoplasm of osteocyte (Fig. 2A), and had the same trend with the Western blot's results. In addition, since autophagy was characterized morphologically by the accumulation of acidic vesicular (AVs) [32], AVs of osteocyte in the proximal tibias was detected by TEM. The typical AVs shown in Fig. 2B, the number of AVs in the OVX + veh group were more than that in the Sham and OVX + E2 group. The results above indicated that the number of osteocyte autophagy in proximal tibias increased in the OVX + veh group.

3.2. Correlation between autophagy level and BMD, microstructure in proximal tibias

A deteriorative microstructure was observed in the OVX + veh group as shown by a significant reduction in both trabecular

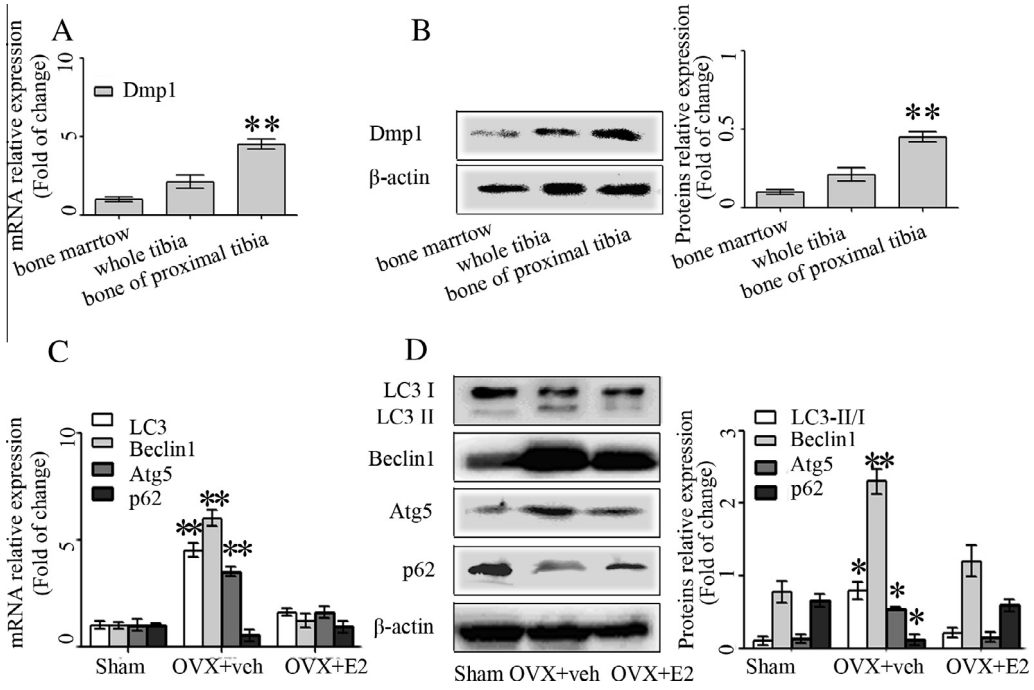

Fig. 1. Autophagy in osteocyte. (A) Real-time PCR for the mRNA expression and (B) Western blot for the protein expression of Dmp1 in the bone of proximal tibias, bone marrow and the whole tibias ($n = 12$). The expression of Dmp1 mRNA and protein in the bone of proximal tibias was significantly higher than in the bone marrow or the whole tibias. (C) Real-time PCR and (D) Western blot for the mRNA and proteins expressions of Atg 5, LC3, Beclin1 and p62 in the bone of proximal tibias. OVX increased expressions of Atg 5, LC3, Beclin1 while decreased p62. The protein expression levels were normalized against β-actin and data were presented as mean and SD from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus Sham.

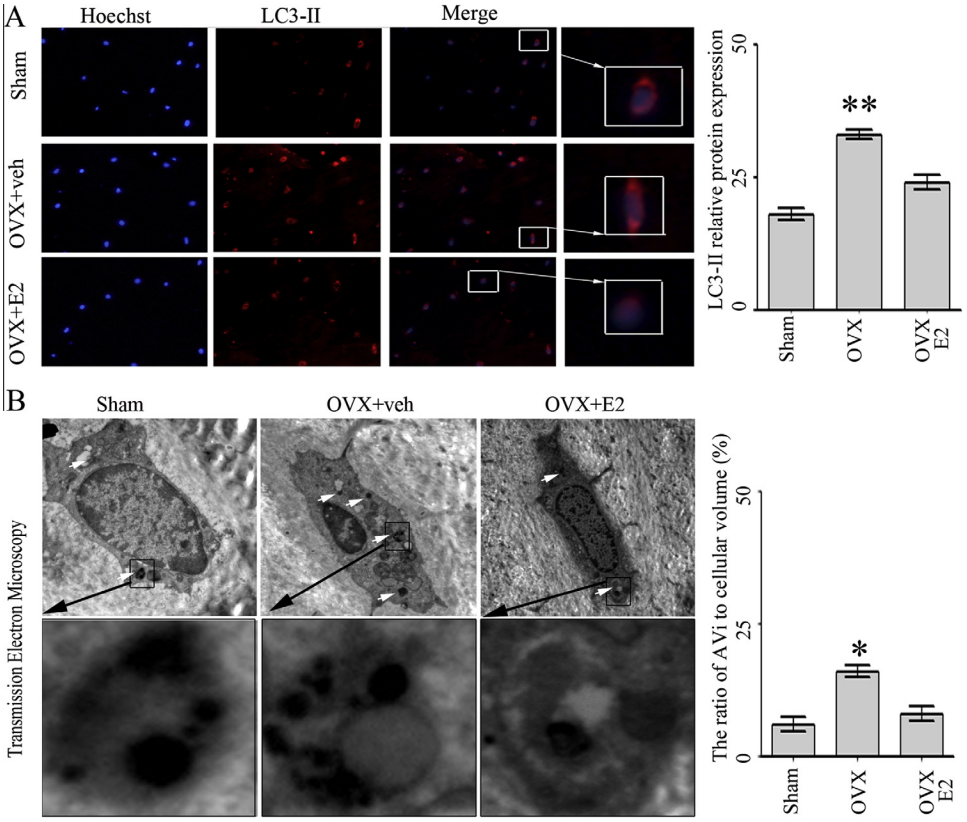

Fig. 2. Autophagy in osteocyte assessed by IH staining and TEM. (A) IH staining for LC3-II expression in the bone of proximal tibia. LC3-II was mainly in the cytoplasm of osteocyte and quantification results showed that the rate of LC3-II-positive osteocyte was higher in OVX group. The number was counted in five random fields of each chamber under the microscope (magnification, 200×). (B) The acidic vesicular (AVs) was displayed in the cytoplasm of osteocyte as the white arrow showed among three groups. The value of histogram was obtained by the volume of AVs occupied by cellular volume and the volume of AVs was counted in five random fields of each chamber under the microscope. The quantification results showed that there was much more AVs in OVX group (magnification 2000×, 5000×). $^{*}P < 0.05$, $^{**}P < 0.01$ versus Sham.

Table 2
Bone mass and microarchitectural parameters in proximal tibias.

	Sham + veh	OVX + veh	OVX + E2
BV/TV (%)	25.5 ± 2.3	15.9 ± 1.8*	20.7 ± 2.2
Tb.Th (μm)	40.8 ± 3.5	45.8 ± 5.1	43.19 ± 3.5
Tb.N (N/mm)	6.6 ± 1.2	4.3 ± 0.21**	6.15 ± 0.1
Tb.Sp (μm)	211.3 ± 24.1	315.2 ± 21.6	233.6 ± 20.0
BMD (mg/cc)	1006.5 ± 139.3	732.0 ± 126.1	960.5 ± 156.3

Data expressed as mean ± SD. BV/TV, cancellous bone volume; Tb.Th, trabecular thickness; Tb.N, trabecular number; Tb.Sp, trabecular separation; BMD, bone mineral density of proximal tibia.
* Significant difference between OVX + veh and Sham + veh ($P < 0.05$).
** Significant difference between OVX + veh and Sham + veh ($P < 0.01$).

volume (BV/TV) and trabecular number (Tb.N), and a significant increase in trabecular separation (Tb.S) (Table 2). The BMD of proximal tibias decreased significantly in OVX + veh group. However, E2 administration partly reversed this effect caused by ovariectomy because there was no significant difference in microstructure and BMD between Sham and OVX + E2 group. In addition, correlation was analyzed between osteocyte autophagy (LC3-II) and BMD, microstructure in proximal tibias. Spearman correlation analysis demonstrated that LC3-II positive cells rate was inversely correlated with the BMD ($r = -0.67$, $P < 0.01$), BV/TV ($r = -0.80$, $P < 0.01$), Tb.N ($r = -0.82$, $P < 0.01$) in proximal tibia and positively correlated with Tb.Sp ($r = 0.59$, $P < 0.01$). Correlation coefficients of spearman were displayed in the Fig. 3.

3.3. Correlation between autophagy level and serum estrogen, antioxidative biomarkers in proximal tibias

As shown by Table 3, OVX was verified with a significant reduction in serum E2 level. Furthermore, ovariectomy decreased the levels of T-AOC, CAT, and SOD ($P < 0.05$ for CAT and SOD activity; $P < 0.01$ for T-AOC level versus Sham). However, OVX-induced reduction of antioxidative biomarkers was partly reversed by E2 therapy. In addition, correlation was analyzed between osteocyte autophagy (LC3-II) and serum E2, oxidative stress status in proximal tibias. As shown in Fig. 4, spearman correlation analysis demonstrated that LC3-II positive cells rate was inversely correlated with serum E2 ($r = -41$, $P = 0.05$), T-AOC activity ($r = -0.63$, $P < 0.01$), CAT activity ($r = -0.45$, $P = 0.03$), and SOD activity ($r = -0.78$, $P < 0.01$).

4. Discussion

Oxidative stress, characterized by increase of ROS, was implicated in the development of various bone loss [12,13]. Ovariectomy decreased the levels of T-AOC, SOD and CAT activities in our study. The reduction of antioxidant was possibly associated with several reasons; E2 deficiency, decrease of capability to scavenge free radicals, increase of interleukin-6 and Tumor Necrosis Factor alpha [27,28]. Autophagy in osteocyte, first reported by Zahm et al. [14], had a protective effects against oxidative stress through clearance of ROS [20,21]. Increased expressions of Atg5, p62, LC3 and Beclin1 in mRNA and protein levels were observed

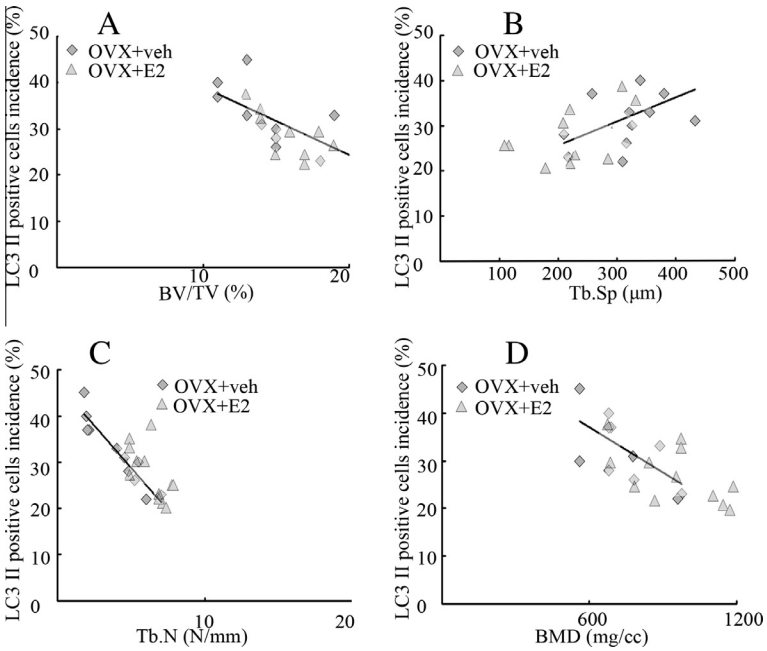

Fig. 3. Correlation between LC3-II level and BV/TV, Tb.Sp, Tb.S, BMD. Pearson's correlation was used to analyze the correlation between LC3-II positive cells and BV/TV (A), LC3-II positive cells and Tb.Sp (B), LC3-II positive cells and Tb.N (C), LC3-II positive cells and BMD (D).

Table 3
Oxidative stress status in proximal tibias.

	Sham + veh	OVX + veh	OVX + E2
Serum E2	32 ± 5.1	4.6 ± 1.5**	25 ± 4.9
T-AOC	10 ± 1.0	6.7 ± 2.5*	8.9 ± 1.5
CAT	10 ± 2.2	8.3 ± 2.1	9.5 ± 1.1
SOD	10 ± 0.9	7.2 ± 0.7*	9.6 ± 2.0

Data expressed as mean ± SD.
* Significant difference between OVX + veh and Sham + veh ($P < 0.05$).
** Significant difference between OVX + veh and Sham + veh ($P < 0.01$).

in the bone of proximal tibia after OVX. To further locate the increased autophagy, IH staining and TEM were used. The expressions of LC3-II were major in the cytoplasm of osteocyte evidenced by IH staining. Consistently, an increase of AVs in the cytoplasm of osteocyte was evidenced by TEM.

An increased autophagy level in osteoblast was observed in osteoblast-specific estrogen receptor knockout mice [29,30]. Consistent with the finding, we also showed that autophagy increased in osteocyte after ovariectomy. These data suggested that autophagy might be a responsive mechanism to acute inflammation and

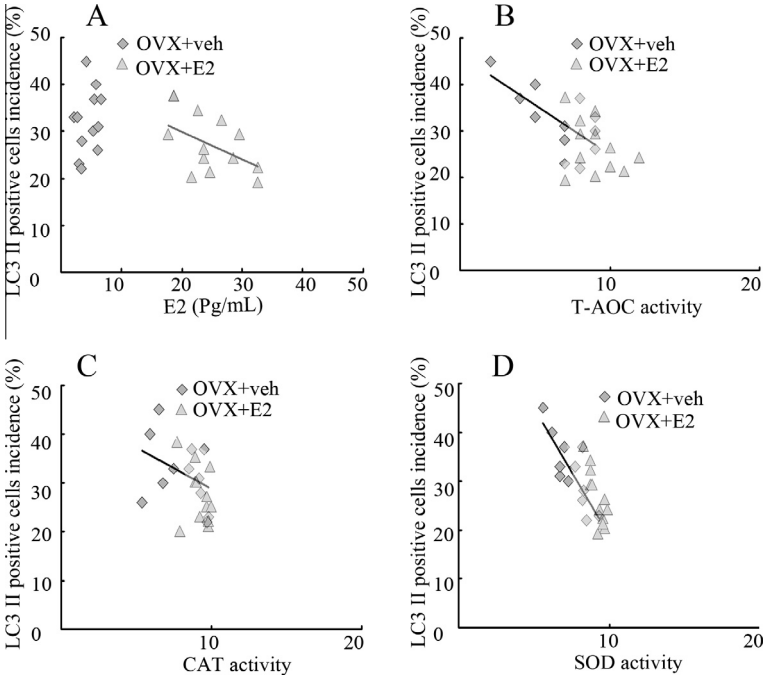

Fig. 4. Correlation between LC3-II level and E2 level, antioxidative biomarkers. Pearson's correlation was used to analyze the correlation between LC3-II positive cells and E2 levels (A), the correlation between LC3-II positive cells and T-AOC (B), the correlation between LC3-II positive cells and CAT activity (C), and the correlation between LC3-II positive cells and SOD activity (D).

stress under hormone deprivation or estrogen receptor deficiency condition. Furthermore, we found that autophagy level was inversely correlated with antioxidative biomarkers (T-AOC, SOD and CAT activities). Our results suggested that autophagy had a protective effect against oxidative stress, which was consistent with several studies [31,32]. The possible mechanism of antioxidative effect was that autophagy could remove oxidized proteins, reactive oxygen species and damaged mitochondrial [33]. In addition, our data demonstrated that autophagy level was inversely correlated with the BV/TV, Tb.N, and BMD. These findings were consistent with a previous study that autophagy showed significant association with distal radius BMD [34].

E2 was considered essential for women and men in maintaining healthy bone [27]. In our study, a decline of serum E2 was observed in ovariectomized rats. Furthermore, a decreased antioxidative biomarkers and increased autophagy level were observed in bone tissues of OVX rats while subcutaneous E2 treatment could partly reversed this effect. Consistently, a recent research found that uterine autophagy was induced by OVX and suppressed by E2 treatment [29]. Interestingly, these findings were contradicted with our previous result that E2 was a promoter for autophagy in vitro [35]. There were two possible explanations for the opposite results. One explanation was that autophagy was not only regulated by E2, but also by hormone-derived environment, cytokines, oxygen radical and so on. Another explanation was dual role of E2 in modulation of autophagy since autophagy could promote or inhibit apoptosis under different stimulus.

Long-term use of E2 had numerous adverse effects such as increased risks of breast cancer and thrombo embolic diseases [36,37]. Understanding the mechanism of E2-deficiency resulted in bone loss might help minimize these risks. Considering the notion that autophagy was correlated with oxidative stress status, bone loss and E2 levels, our findings encouraged further studies that use autophagy modulator as a strategy for postmenopausal osteoporosis therapy.

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Conflict of interest

The work described has not been submitted elsewhere, in whole or in part, and all the authors listed have approved the manuscript that is enclosed.

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