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Original Article

Correlation between autophagy of osteoblasts and oxidative stress of osteoporosis rats

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Abstract: The proliferation, apoptosis and autophagy of osteoblast are correlated with osteoporosis (OP), during which oxidative stress is involved. Oxidative stress can induce cell autophagy injury. This study established an OP rat model, in which osteoblast autophagy was investigated along with oxidative stress. Female SD rats (6 weeks old) were randomly assigned into sham, model and estrogen (E2) groups (N=20). OP model was established by bilateral ovariectomy, followed by E2 (10 μg/kg/d) for 6 weeks. Rats were sacrificed to test micro-structure and volume of bone tissues. HE staining and transmission electron microscopy were used to observe morphology and autophagosome. ELISA was used to quantify serum level of osteocalcin (OCN), type I collagen carboxyl terminal peptide (CTX), E2, superoxide dismutase (SOD), reactive oxygen species (ROS) and catalase (CAT). Western blot was used to quantify Beclin-1, light chain 3 of microtubule related protein (LC3-II), mammalian target of rapamycin (mTOR), and phosphatidyl inositol-3-hydroxykinase (PI3K), in order to analyze the relationship between autophagy and oxidative stress. OP model had elevated autophagosome number, OCN, CTX, ROS, Beclin-1 and LC3-II expression, plus depressed E2, CAT, SOD, mTOR and PI3K ($P<0.05$). E2 treatment improved OP disease, decreased autophagosome number, OCN, CTX, ROS Beclin-1 and LC3-II, and elevated E2, CAT, SOD, mTOR and PI3K levels ($P<0.05$). Autophagy of osteoblast was negatively correlated with bone volume and anti-oxidative enzyme, and was positively correlated with bone trabecular distance. Autophagy of osteoblast is correlated with bone microstructure, volume and oxidative stress, and it plays a role in OP pathology.

Keywords: Osteoporosis, oxidative stress, autophagy, BECN-1

Introduction

Osteoporosis is one systemic metabolic bone disease, featured with injury of microstructure of bone tissues and depressed volume. Due to elevated bone plasticity, patients are predisposed for bone fracture, with elevated incidence by years with aged population [1, 2]. Various factors including drugs, hormones, nutrients and aging can cause imbalance of bone absorption/formation. Studies showed that the over-activation of osteoclasts and abnormal function of osteoblasts play an important role in the occurrence and progression of OP [3, 4]. Serum levels of leptin and protectin are abnormal in OP patients. Menopaused OP individuals had decreased activity of osteoblasts, whose reactivity for leptin and estrogen was down-regulated [5, 6]. Reactive oxygen species (ROS) can induce oxidative stress for disrupting cellular dysfunction. Study showed the important role of oxidative stress in pathological processes of various OP subtypes. Bone tissue ROS is elevated to various extents in glucocorticoid, aging and menopaused subtypes of OP patients. Oxidative stress can facilitate apoptosis and inhibit differentiation of osteoblast, with functional pathways correlated with apoptotic and cellular autophagy signal pathways of endoplasmic reticulum and mitochondria [7]. Currently the role of osteoblast autophagy in oxidative stress has not been fully illustrated. Oxidative stress can induce various autophagy injuries, as proliferation, apoptosis, autophagy of osteoblasts are closely correlated with OP progress [8]. Certain stress factors such as high glucose and glucocorticoid can cause autophagy of bone tissues. Under these stress environments, autophagy inhibits oxidative stress injury, thus exerting certain cell protective roles. The overrun of autophagy, however, may lead to apoptosis [9]. Autophagy of cells

inside brain tissues can facilitate the clearance of oxidative reagent and maintain cellular functions. The over-reaction of myocardial cell oxidative stress disrupts cellular autophagy level, activates NF-κB pathway to facilitate cardiac cell apoptosis, decreases cellular oxidative stress level, thus partially restoring cellular autophagy functions [10]. The conditional knockout of osteoblast autophagy gene Atg7 can elevate cellular oxidative stress level, inhibit cell autophagy level, decrease osteoblast activity, thus causing aging OP [11]. Currently few studies have been performed about the autophagy effect of oxidative stress injury. This study thus established rat PO model via bilateral ovariectomy, and observed autophagy in bone tissues, to investigate the correlation between autophagy and oxidative stress.

Materials and methods

Experimental animals and grouping

A total of 60 healthy male SD rats (6 weeks old, body weight 200~220 g) were provided by Laboratory Animal Center of Hunan Chinese Medicine University (Certificate No. SYXK-2013-0025). Animals were singly housed in an SPF grade facility with food and water *ad libitum*. All rats had no trauma or swelling of knee joints, with normal extension and retraction functions. Animals were randomly assigned into sham, model, solvent control and estrogen E2 groups (N=20).

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of the Second People's Hospital of Hunan Province.

Drugs and reagents

Hydrate chloral and paraformaldehyde were purchased from Kemiou Chemical (China). Horseradish peroxidase (HRP) labeled goat anti-rabbit secondary antibody was purchased CST (US). ELISA kit for osteocalcin (OCN), type I collagen carboxyl terminal peptide (CTX), E2, superoxide dismutase (SOD), reactive oxygen species (ROS) and catalase (CAT) were purchased from Jiancheng Bio (China). DAB staining kit was purchased from Golden Bridge Bio (China). Rabbit anti-Beclin-1, anti-light chain 3 (LC3-II) of microtubule related protein, anti-mammalian target of rapamycin (mTOR), and anti-phosphatidyl inositol-3-hydroxykinase (PI3K) antibody was purchased from Boster (China). Micro-CT (Locus SP, GE, US); Transmission electron microscopy (CM-80, Philips, Netherland).

Animal model preparation

After one week of acclimation, SD rats were prepared for OP model using bilateral ovariectomy as previously reported methods [12]. Animals were fasted for 8 hours before surgery, and were anesthetized using 10% hydrate chloral via intraperitoneal injection. A middle incision was made along the dorsal spine to remove bilateral ovarian tissues. In sham group, peripheral fat tissues were removed instead. After suture, penicillin was injected to prevent infection. General motility and wound healing conditions were observed after surgery. E2 (10 μg/kg/d) was given via oral intubation for 6 consecutive weeks. Equal volume of saline was given to control and sham groups.

ELISA for serum OCN, CTX, ROS, E2, CAT and SOD levels

5 ml venous blood samples were collected and centrifuged at 14,000 g (r=10 cm, 3500 r/min) for 15 min. Supernatants were saved and tested for OCN, CTX, ROS, E2, CAT and SOD levels using ELISA kit following manual instruction.

HE staining for morphology of bone tissues

6 weeks after surgery, rats were sacrificed and dissected. Samples of partial proximal right tibia were collected and immersed in paraformaldehyde. After paraffin embedding and sectioning (5 μm thickness), hematoxylin-eosin staining was performed. Tissue sections were observed under a light filed microscope.

Micro-CT examination of tibia microstructure and bone volume

6 weeks after surgery, rats were sacrificed and dissected. Samples of partial proximal right tibia were collected and scanned under micro-CT (16 μm resolution) to measure parameters of tibia microstructure including bone mineral density (BMD), bone trabecular volume/total volume (BV/TV), trabecular bone number (Tb.N), trabecular thickness (Tb.Th) and trabecular distance (Tb.Sp).

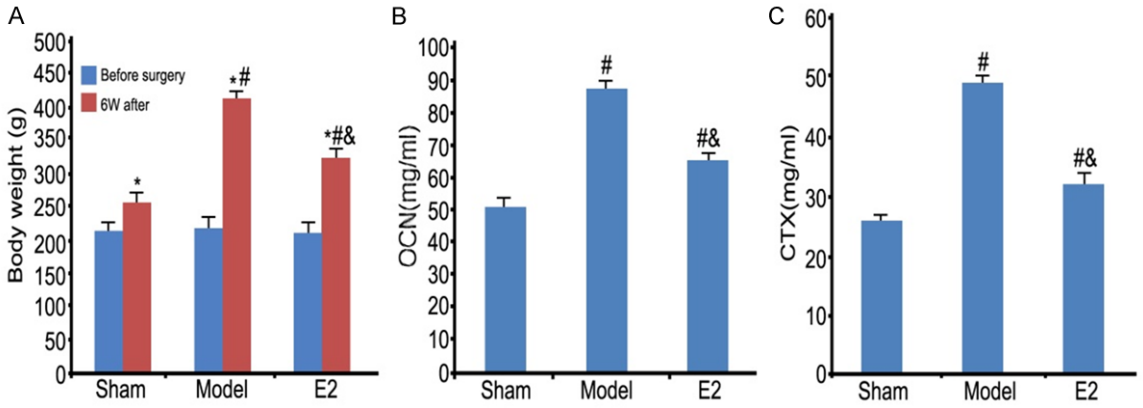

Figure 1. Body mass (A) and serum OCN (B), CTX (C) levels. *, P<0.05 compared to those before surgery; #, P<0.05 compared to sham group; &, P<0.05 compared to model group.

Autophagosome observation by transmission electron microscopy

Proximal tibia tissues were fixed in glutaraldehyde, decalcified in 10% ethylenediaminetetraacetic acid (EDTA), and embedded in resin to prepare ultrathin slices (80 nm thickness). Uranyl acetate was used to stain tissues, whose inner structure was observed under transmission electron microscope to check the existence of autophagosome with dual membrane structures including undigested cytoplasm or organelles. The number of autophagosome, inner contents, integrity and initial/degraded phagocytic vesicles were recorded.

Immunofluorescence for measuring LC3B positive cells in proximal tibia

Bone fraction (1 mm^3) was collected from proximal tibia within 1~5 mm of epiphyseal plate, and was fixed in paraformaldehyde. Following decalcified in 10% EDTA, bone tissues were prepared into 5 μm slices from sagittal plane. Immunofluorescence was performed to observe under the light field microscopy. Positive expression of LC3B mainly locates within cytoplasm as shown by red fluorescence. 5 randomly selected slices were counted for LC3B-positive cell number and total cell number to calculate LC3B-positive percentage.

Western blotting for Beclin-1, LC3-II, mTOR and PI3K expression

Rat bone tissues were lysed in lysis buffer. Tissue homogenate was collected by centrifugation, and was quantified for protein contents using BCA kit. Protein samples were separated in SDS-PAGE, and transferred to PVDF membrane, which was blocked in blocking buffer for 1 h, followed by 4°C overnight incubation in primary antibody against Beclin-1 (1:200), LC3-II (1:400), mTOR (1:500), PI3K (1:500), PI3K (1:1000) or β-actin. On the next day, the membrane was washed in PBST for three times, following the addition of secondary antibody (1:2000) for 1 h incubation. ECL reagent was used to develop the membrane, which was exposed in dark room. Quantity One software was used to analyze protein bands, whose absorbent values were analyzed for relative expression level against those of internal reference proteins.

Statistical methods

SPSS20.0 software was used for statistical analysis. Measurement data were firstly tested for normal distribution, those fitted normal distribution were presented as mean ± standard deviation (SD). The comparison of means among multiple groups was done by one-way analysis of variance (ANOVA), followed by post-hoc LSD comparison between paired data. Spearman test was used for correlation analysis. A statistical significance was defined when P<0.05.

Results

Body weight and serum OCN, CTX levels

Compared to sham group, OP model rats had significantly elevated body mass, serum OCN and CTX levels (P<0.05). E2 treatment remarkably decreased body mass and serum OCN, CTX levels (P<0.05 compared to model group, **Figure 1**).

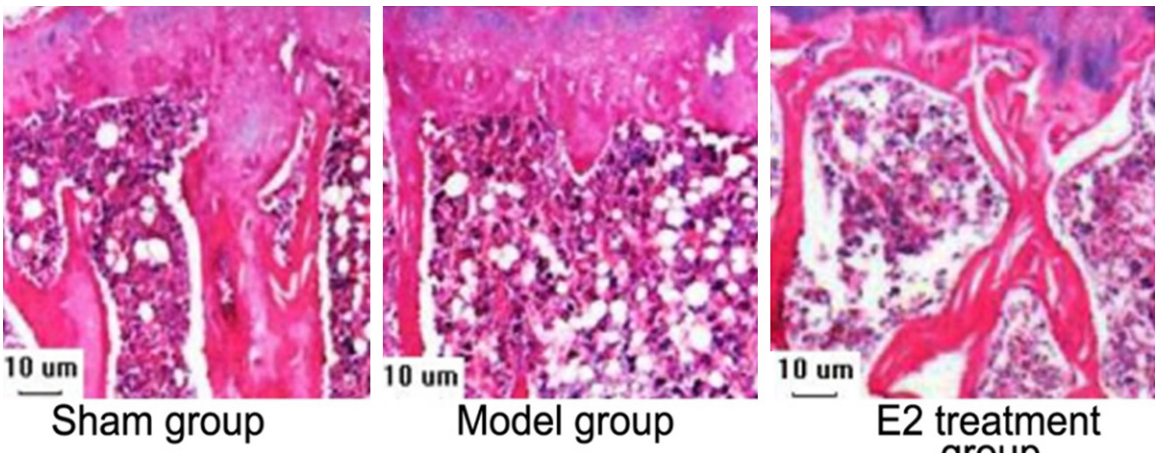

Figure 2. Morphology of bone tissues (HE, ×400).

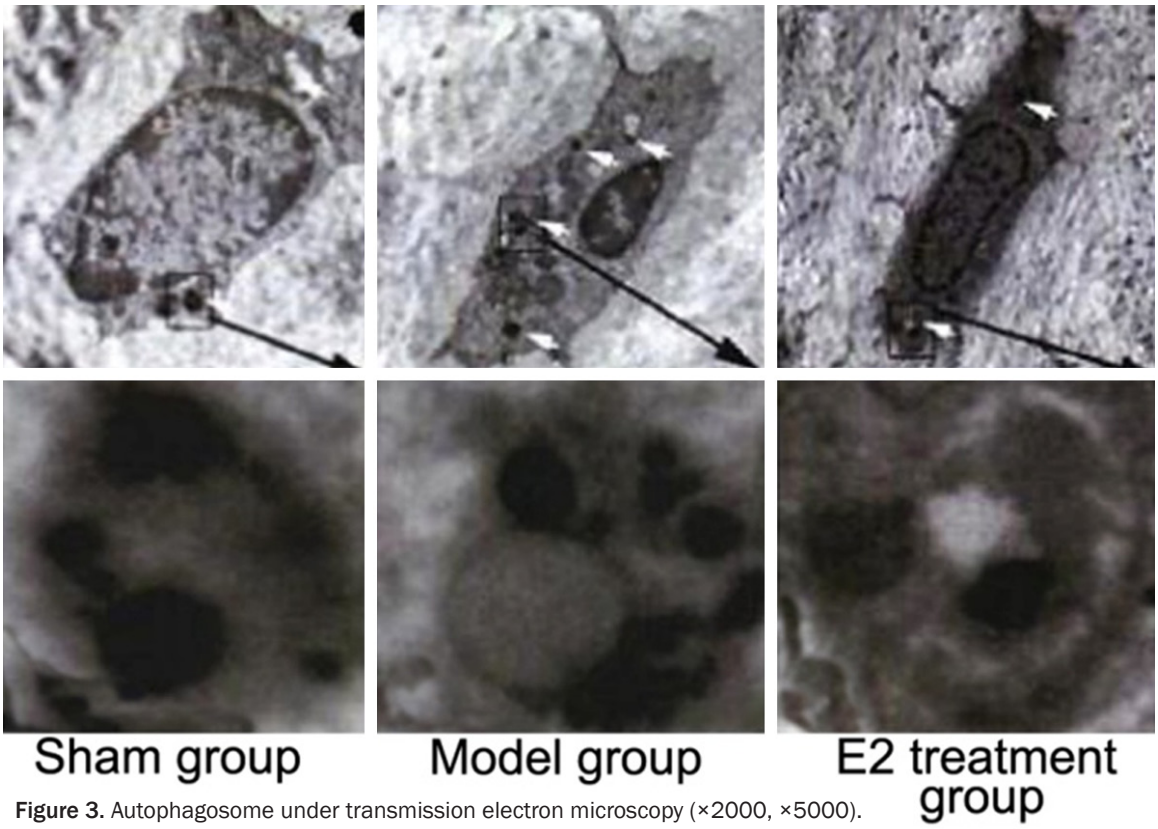

Figure 3. Autophagosome under transmission electron microscopy (×2000, ×5000).

Morphology of bone tissues

HE staining revealed complete growth plate, thick trabecular bone, regular cell arrangement, and complete structure. OP model rats had irregular arrangement of osteoblasts, incomplete structure, decreased cell number, dilated bone marrow cavity, thinned and incomplete growth plate, fine bone trabecula, showing signs of OP. E2 treated group had significantly improved OP signs (**Figure 2**).

Transmission electron microscopy for observing cellular autophagosome

Under transmission electron microscopy, OP model rats had abundant circular materials inside cytoplasm of bone tissues. These particles had vesicles with dual membranes, which are features of autophagosome. The number of autophagosome in model group (18.5 ± 3.4%) was significantly higher than sham group (7.8 ± 1.3%). E2 treatment group had significantly

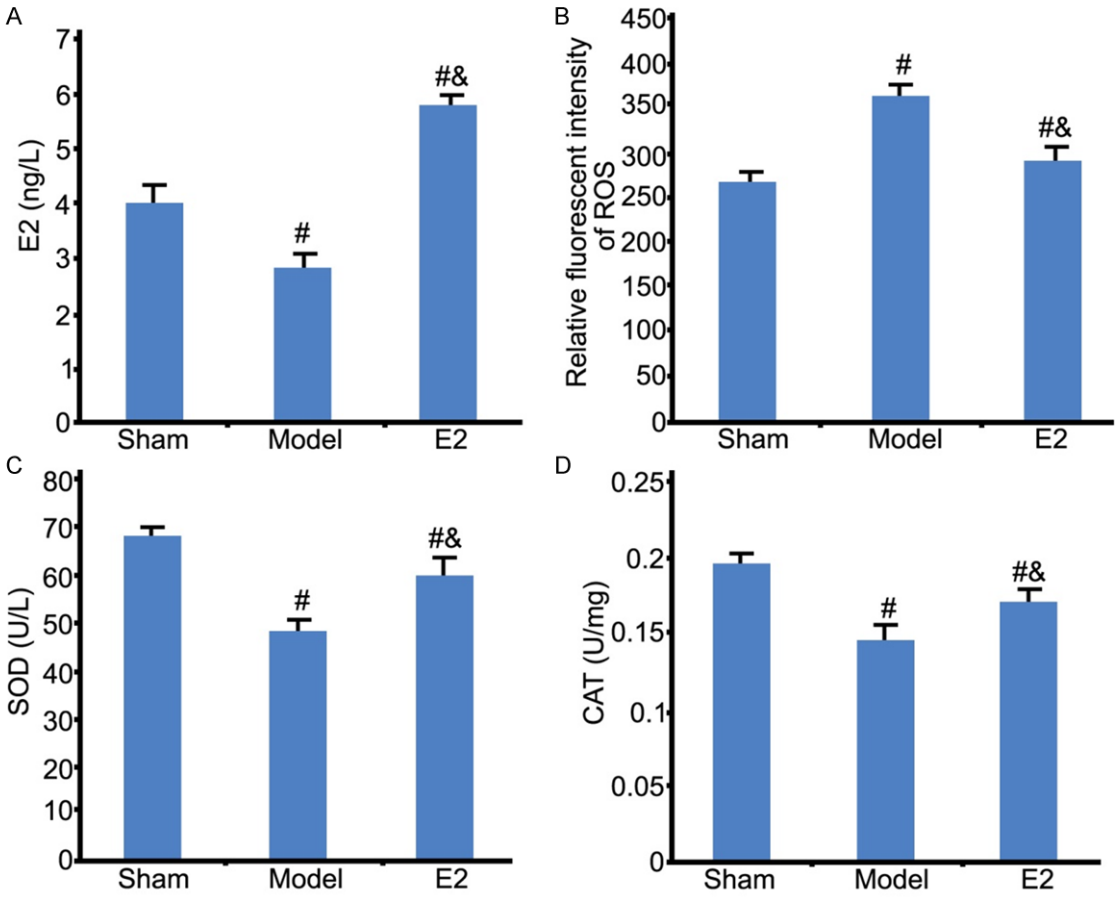

Figure 4. Serum ROS (A), E2 (B), CAT (C) and SOD (D) levels. #, P<0.05 compared to sham group; &, P<0.05 compared to model group.

lowered autophagosome number (10.3 ± 2.2%) compared to model group (P<0.05, **Figure 3**).

Serum ROS, E2, CAT and SOD levels

OP model rats had elevated ROS level, and decreased serum E2, CAT, SOD levels (P<0.05 compared to control group). E2 treatment decreased ROS levels, and increased E2, CAT and SOD levels (P<0.05, **Figure 4**).

Microstructure and volume of bone tissues

Parameters such as BMD, BV/TV, and Tb.N were significantly depressed in OP model rats, while Tb.S was remarkably elevated (P<0.05 compared to control group). E2 treatment increased BMD, BV/TV and Tb.N levels and decreased Tb.S remarkably (P<0.05 compared to model group). Therefore estrogen retreat after ovariectomy leads to injury of bone microstructure and lower bone volume (**Figure 5**).

Western blotting for bone expression of Beclin-1, LC3-II, mTOR and PI3K

OP model rats had elevated Beclin-1 and LC3-II expressions, plus depressed mTOR and PI3K expressions (P<0.05 compared to control group). E2 treated rats had lower Beclin-1 and LC3-II expression, plus elevated mTOR and PI3K expressions (P<0.05 compared to model group, **Figure 6**).

Correlation between autophagy of osteoblast and microstructure, bone volume and oxidative stress

Immunofluorescence revealed that LC3B positive rate of proximal tibia was negatively correlated with BV/TV, BMD and Tb.N values (r=-0.78, -0.64 and -0.80, P<0.05), and was positively correlated with Tb.S (r=0.73, P<0.05). LC3B positive rate was also positively correlat-

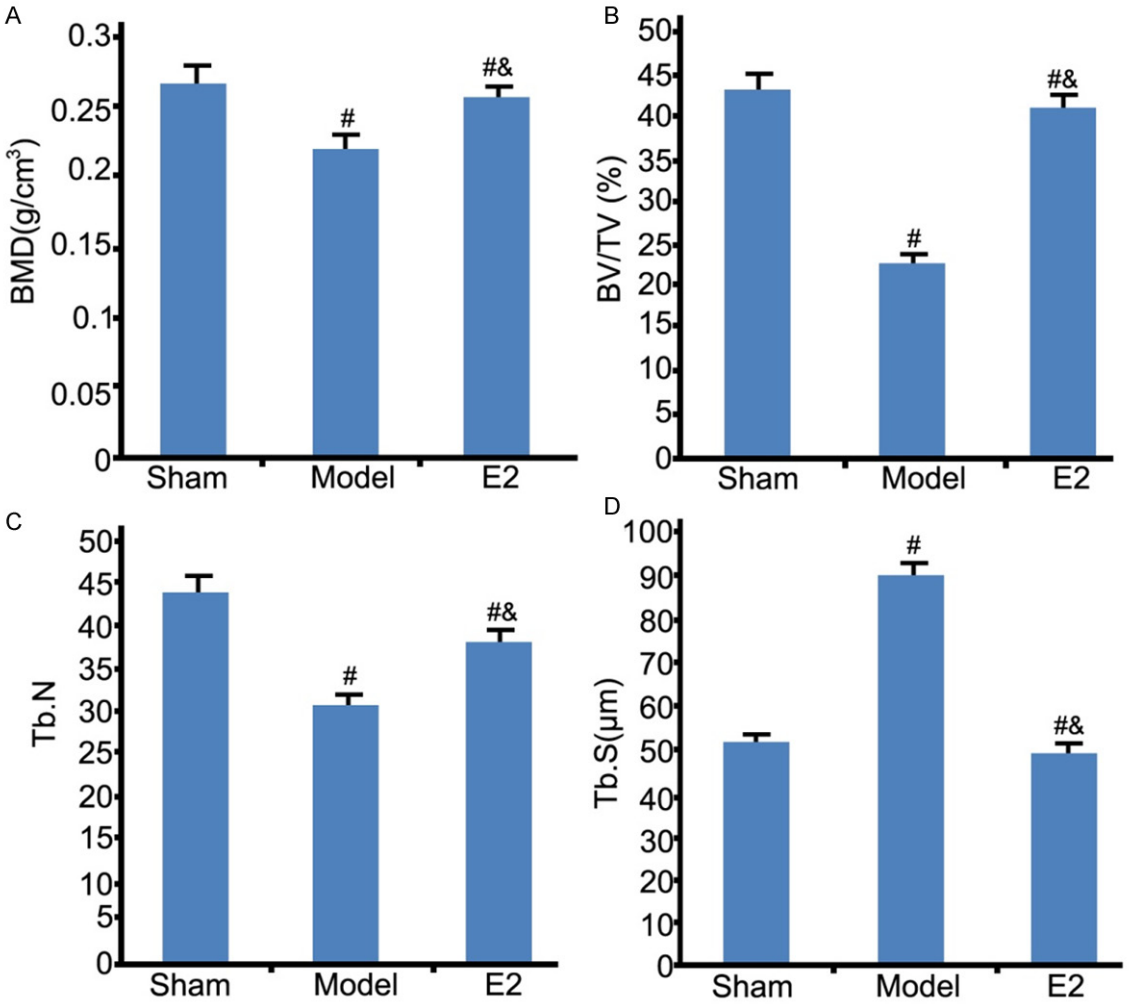

Figure 5. Bone microstructure and bone volume. #, P<0.05 compared to sham group; &, P<0.05 compared to model group. BMD, bone mineral density; BV/TV, bone trabecular volume/total volume; Tb.N, trabecular bone number; Tb.S trabecular distance.

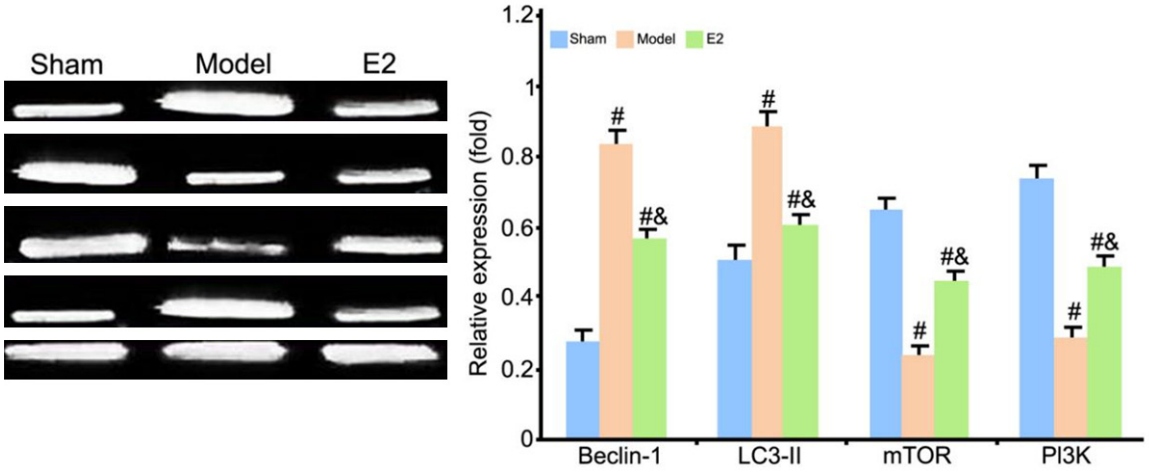

Figure 6. Western blot for bone expression of Beclin-1, LC3-II, mTOR and PI3K expression. #, P<0.05 compared to sham group; &, P<0.05 compared to model group.

Table 1. Correlation between osteoblast autophagy and bone volume and oxidative stress

Index		LC3B positive rate of autophagy cells	
		r	*P* value
Bone microstructure and volume	BV/TV	-0.78	<0.05
	BMD	-0.64	<0.05
	Tb.N	-0.80	<0.05
	Tb.S	0.73	<0.05
Oxidative stress	ROS	0.63	<0.05
	CAT	-0.68	<0.05
	SOD	-0.76	<0.05

ed with serum ROS (r=0.63, P<0.05) and was negatively correlated with E2, CAT and SOD levels (r=-0.41, -0.68 and -0.76, P<0.05, **Table 1**).

Discussion

Under physiological conditions, functions of osteoblasts and osteoclasts are kept at balance. Under pathological status, microenvironment of bones is altered by factors such as oxidative stress, inflammatory mediator and endocrine hormone, leading to imbalance of osteoblast/osteoclast and bone loss [13, 14]. Common pathological features of various OP subtypes are oxidative stress, for which cell autophagy exerts self-acclimation of environment. Various pathways underlie the regulation of autophagy on bone formation, as it can negatively regulate NK-κB pathway to facilitate BMSCs osteoclast differentiation, during which BMSCs autophagy level is elevated, thus inhibiting NK-κB activation of osteoblast for accelerating their mineralization and differentiation [15, 16]. Moreover, autophagy can facilitate cell proliferation and differentiation via activating Wnt/β-catenin signal pathway in osteoblast, as the knockout of Beclin-1 gene leads to depressed ability of mineralization and differentiation in osteoblasts [17, 18]. Under physiological condition, the body anti-oxidation system and production or ROS maintain at homeostatic status. In pathological conditions such as estrogen deficiency, body's anti-oxidative ability is down-regulated, producing abundant free radicals, leading to oxidative stress injury. The abolishment of estrogen causes oxidative stress, which is correlated with multiple factors including lowered body anti-oxidation function, and elevated inflammatory factor levels, both of which lead to decreased bone contents and abnormal bone metabolism [19, 20]. Estrogen can directly facilitate the transcription of anti-oxidative enzyme. Serum biochemical indexes for bone formation and bone absorption can reflect the degree of bone transformation. OCN is unaffected by bone absorptive factors, and can reflect bone cell activity and bone transformation rate, in conjunction with CTX as biomarker for bone absorption [21]. Results of this study showed that OP model rats generated by bilateral ovariectomy had significantly elevated ROS level, decreased E2, CAT and SOD activity, plus up-regulation of OCN and CTX levels. HE staining showed irregular arrangement of bone cells with incomplete structure, fewer cell number, dilated bone marrow cavity in model rats, which showed significant sings of OP. Estrogen treatment significantly improved OP symptoms, suggesting that estrogen deficiency caused insufficient anti-oxidative potency of bone tissues, accelerated bone transformation, disruption of normal function of osteoblasts, all of which cause OP.

LC3-II is one autophagy marker molecule, and mTOR is the major inhibitor signal for cell autophagy for receiving multiple signal stimuli such as oxidative stress and hormones, with two major active forms mTORC1 and mTORC2. mTORC1 can inhibit the formation of autophagosome via regulating downstream signal molecules S6K1. mTOR is under the regulation of multiple upstream stimulating factors such as MAPK and PI3K/AKT. AKT/mTOR signal pathway can regulate cell autophagosome functions [22]. Pluripotent protein Beclin-1 is the major regulator factor for autophagy, as its up-regulation elevates cell autophagy levels [9, 10]. Autophagy is closely correlated with oxidative stress. Some studies showed that autophagy can clear damaged mitochondria, activate Keap1/Nrf2-ARE signal pathway, facilitate free radical clearance, potentiate anti-oxidative function, alleviate oxidative stress injury of cells, and inhibit osteoblast apoptosis induced by stress factors such as glucocorticoid induction via alleviating the production of ROS in damaged mitochondria [22, 23]. *In vitro* study showed that H_2O_2 could activate AMPK pathway of MG63 cells to accelerate

cell autophagy level. Such elevated autophagy is correlated with AMPK-dependent inhibition of mTORC1 [22]. This study showed significant OP in model rats, whose increased autophagosome number, Beclin-1 and LC3-II expression, and decreased mTOR or PI3K expression, plus bone cell autophagy was negatively correlated with bone volume and anti-oxidative enzyme, and was positively correlated with trabecular distance. The deficiency of estrogen increased autophagy level of bone cells, along with up-regulation of Beclin-1 and LC3-II pathway. The down-regulation of PI3K/mTOR pathway suggested the participation of autophagy in OP pathological process. The negative regulation by mTOR is closely correlated with phosphorylated Atg 13-induced autophagosome formation, and acceleration of endoplasmic reticulum-ribosome adhesion for membrane formation of autophagosome. The inhibition of PI3K expression largely impedes downstream mTOR pathway. Estrogen treatment can reverse elevated autophagy level in bone cells. Such reversible bone cell autophagy is contrary to *in vitro* study in which estrogen could facilitate osteoblast autophagy for inhibition of cell apoptosis [11, 12]. Such inconsistency is probably correlated with multiple factors such as estrogen, inflammatory factors and endocrine hormones, all of which can affect autophagy level. Autophagy is one self-protective effect of cells against stress environment [23]. Inconsistent autophagy level may occur across *in vivo* and *in vitro* conditions. The detailed mechanism of autophagy on oxidative stress response requires further illustration.

Conclusion

Bone cell autophagy is correlated with microstructure of bones, bone volume and oxidative stress, all of which are involved in the pathological process of OP process.

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Disclosure of conflict of interest

None.

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