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# Oral administration of egg white ovotransferrin prevents osteoporosis in ovariectomized rats

Nan Shang[a,b,c], Xiaoyu Bao[a], Michael Doschak[d], Jianping Wu[a,*]

[a] Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton T6G 2P5, Canada
[b] College of Engineering, China Agricultural University, Beijing 100083, China
[c] Key Laboratory of Precision Nutrition and Food Quality, Department of Nutrition and Healthy, China Agricultural University, Beijing 100193, China
[d] Faculty of Pharmacy & Pharmaceutical Sciences, University of Alberta, Edmonton T6G 2H5, Canada

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ABSTRACT

Ovotransferrin, an iron-binding glycoprotein, accounting for approximately 12% of egg white protein, is a member of transferrin family. Our previous studies showed that ovotransferrin stimulates the proliferation and differentiation of osteoblasts, while inhibits osteoclastogenesis and resorption activity. The work aims to study the efficacy of orally administered ovotransferrin on the prevention of osteoporosis using ovariectomized (OVX) Sprague-Dawley rats. Oral administration of ovotransferrin showed no negative effect on body weight, food intake and organ weight. After 12-week treatment, feeding ovotransferrin at a dose of 1% (1 g ovotransferrin/100 g diet) prevented OVX-induced bone loss and maintained relatively high bone mineral density and integrated bone microarchitecture. The serum concentration of biomarkers indicating bone formation was increased in ovotransferrin administration groups, while the bone resorption biomarkers were decreased. Ovotransferrin feeding also decreased the production of serum cytokine TNF-α and IL-6, which are two stimulators for osteoclast differentiation. In addition to its direct regulatory role on bone turnover, ovotransferrin supplementation might benefit osteoporosis prevention by inhibiting adipogenesis, and regulating immune response. Our results suggested the potential application of ovotransferrin as a functional food ingredient on the prevention of osteoporosis.



## 1. Introduction

Osteoporosis is a multifactorial systemic skeletal disease, characterized by decreased bone mineral density and micro-architectural deterioration of bone tissue, resulting in increased bone fragility and susceptibility to fracture[1]. As an aging-related disease, osteoporosis is a major public health concern. Healthy bone requires continuous remodeling which is pivotal for bone density maintenance[2]; however, the normal bone turnover cycle can be impaired and result in bone loss. Several factors are associated with osteoporosis such as decreased level of sex hormones, e.g. estrogen, testosterone[3-4] and growth factors, e.g. insulin-like growth factor, fibroblast growth factor[5-6], drug side effects, e.g. glucocorticoid-induced osteoporosis[7], lack of essential nutrients, e.g. mineral, vitamin[8], as well as acquired habit, e.g. smoking and alcohol intake[9]. Although both men and women will suffer from bone loss during their life, women are more susceptible to osteoporosis and osteoporotic fractures, especially postmenopausal women[2].

Current osteoporosis therapies rely on either anti-resorptive agents like bisphosphonates, selective estrogen receptor modulator (SERMs), denosumab, or an anabolic agent like parathyroid hormone (PTH) analogs[1]. However, these therapies are associated with side effects such as gastrointestinal toxicity[10], and increased risk of cancer, strokes and cardiovascular system diseases[11]. The

* Corresponding author at: Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton T6G 2P5, Canada.
*E-mail address*: jwu3@ualberta.ca (J.P. Wu)
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presence of numerous side effects has prompted further research to explore alternatives, especially food derived bioactive components. For example, the potential of soy isoflavones in the prevention and treatment of osteoporosis has been demonstrated in a number of studies[12-14], including epidemiologic studies[15-16]; lactoferrin, an iron binding protein from milk, has gained attention for its ability to prevent osteoporosis by stimulating bone formation while inhibiting bone resorption[17-18]. Moreover, lactoferrin is also able to benefit for bone health by promoting the differentiation of pluripotent mesenchymal cells into osteoblastic and chondroblastic lineage[19] and inhibiting the production of inflammatory cytokines, such as IL-6 and TNF-α[20], suggesting a multifunction of bioactive proteins in regulating bone health.

Egg white ovotransferrin, a 76 kDa iron-binding glycoprotein and a member of the transferrin family, is a key player of innate immunity in poultry[21]. As a major egg white protein, ovotransferrin has been widely studied for its multiple biological functions such as immunomodulatory, anti-inflammatory activity and antioxidative activities[22]. Our recent studies showed that ovotransferrin not only stimulates the proliferation and differentiation of osteoblasts, but also inhibits osteoclastogenesis and resorption activity[23-24]. These results suggested the potential of ovotransferrin in preventing osteoporosis. However, the efficacy of orally administered ovotransferrin on bone health has not been studied. Although orally administered proteins are likely digested in the gastrointestinal tract and thus may lose their biological activity, oral uptake of ovotransferrin has been found to exhibit similar physiologic effects *in vivo* as displayed *in vitro*, such as its anti-inflammatory activity[25]. The objectives of this study were 1) to evaluate the effect of oral administration of ovotransferrin on osteoporosis prevention using ovariectomized (OVX) rats; 2) to investigate the regulatory role of ovotransferrin on bone metabolism by measuring the expression of bone formation/resorption biomarkers (representing the bone remodeling process), and the production of osteoclastogenesis associated cytokines; 3) to investigate the auxiliary functions of ovotransferrin on bone health management including the regulatory role on adipogenesis, phenotypes of immune cells in bone marrow cells (representing local immunity) and spleen (representing systemic immunity).

## 2. Materials and methods

### 2.1 Chemicals

Alendronate sodium, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), bovine serum albumin (BSA) and penicillin-streptomycin were purchased from Sigma-Aldrich (Oakville, ON, Canada). Egg white ovotransferrin with a purity ≥ 75% was prepared from egg white according to previous method[26]. The Krebs-Ringer-HEPES (KRH) buffer and ACK lysis buffer was prepared according to the previous study[27]. Fluorescent pre-labeled monoclonal antibodies CD3, CD4, CD80, CD28 were purchased from eBioscience (San Diego, CA, USA), antibodies CD8, CD25, CD71, OX62, CD11b/c, CD51 were purchased from Biolegend (Vindeland, ON, Canada), and antibodies RANK, OX12 and OX6 were purchased from BD Biosciences Pharmingen (Mississauga, ON, Canada). AIN-93M purified animal diets and casein were purchased from Envigo (Madison, WI, USA).

### 2.2 Experimental protocol

This experiment protocol was approved by the University of Alberta Animal Welfare Committee (Protocol AUP00001960) in accordance with the guidelines issued by the Canadian Council on Animal Care and also adhered to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health. The experiment was carried out using 12-weeks-old virgin female Sprague-Dawley rats weighing (292 ± 20) g obtained from Charles River (Senneville, QC, Canada), in which 40 rats were ovariectomized (OVX) and 8 rats were sham-operated (Sham). Upon arrival, 48 rats were housed at University of Alberta animal facility with a 12:12 h cycle of light: dark in a humidity and temperature controlled (60% relative humidity (RH), and 23 °C) environment and maintained on diet and water *ad libitum*. In addition to the sham group ($n$ = 8), 40 OVX rats were randomly divided into 5 groups ($n$ = 8 per group); 1) negative control (NC), 2) positive control (PC, injected alendronate sodium 3 times/week), 3) high ovotransferrin treatment (HOvt, 1 g ovotransferrin/100 g diet, 1%), 4) medium ovotransferrin treatment (MOvt, 0.2 g ovotransferrin + 0.8 g casein/100 diet, 0.2%), 5) low ovotransferrin treatment (LOvt, 0.04 g ovotransferrin + 0.96 g casein/100 g diet, 0.04%). Sham, NC and PC groups were provided with a normal diet supplemented with 1% casein to make isonitrogenous. After 12 weeks, rats were sacrificed, and tissues were collected.

### 2.3 Sample collection

Rats were weighed every 2 days and food intake was measured 3 times per week. Blood, urine, and fecal samples were collected every 4 weeks and stored at –80 °C. After 12 weeks of treatment, the rats were placed in metabolic cage for urine collection. Blood was collected immediately after euthanasia, and centrifuged to prepare plasma. Tissues were collected, weighted and stored at –80 °C until analysis.

### 2.4 Micro-computerized tomography analysis and bone mineral density determination

Analysis of bone mineral density was performed by micro-computerized tomography analysis (micro-CT) using Skyscan 1076 (Kontich BE, Belgium) at 18 μm resolution with 70 kV, 100 μA with a 1.0 mm Aluminum filter. Briefly, inhalant isofluorane was used for animal anaesthesia by gradually increasing the concentration of isofluorane from 0.5% to 2% in 100% oxygen. Then animal was placed on the bed of micro-CT scanner face down and the right leg was straightened and immobilized with tapes. Gently pushed the bed into the scanner and performed a Scout Scan of the animal to get the images of tibia. Projected images were reconstructed using vendor-supplied software Nrecon 1.6.1.5 (SkyScan NV, Kontich BE) with images oriented in axial plane of section. A circular region of interest (ROI) relative to the size of the tibia bone was used to sample a standardized amount of trabecular bone in the vertebral body in order to conduct standard histomorphometric analysis. The tibia growth plate was utilized as anatomical landmarks in order to consistently segment and sample the same region of trabecular bone from all samples. Vendor morphometric software CTAn 1.10.0.1

(SkyScan, Kontich BE) was then used to analyze tibia trabecular microarchitecture parameters (percentage bone volume, trabecular thickness, trabecular numbers and trabecular separation) and tissue mineral density against the vendor-supplied bone phantoms of known mineral density (0.25 and 0.75 g/cm$^3$) to calibrate BMD values from Hounsfield units.

### 2.5 Bone biochemical markers and cytokine measurement

Serum and urinary calcium were measured in triplicate with a calcium assay kit (Abcam, Toronto, ON, Canada) and urinary creatinine was measured in triplicate with a creatinine assay kit (Abcam, Toronto, ON, Canada). Serum osteocalcin, parathyroid hormone (PTH), and urinary pyridinoline (PYD) were measured in duplicate with ELISA kit (Quidel, San Diego, CA, USA). Serum bone alkaline phosphatase (BALP) and procollagen I intact N-terminal (PINP) were measured in duplicate with ELISA kit (Abbexa, Houston, TA, USA). Serum TNF-α and IL-6 were measured in triplicate with ELISA kit (Abcam, Toronto, ON, Canada).

### 2.6 Isolation of spleen and bone marrow cells

At necropsy, spleens were removed and placed in sterile Krebs-Ringer-HEPES (KRH) buffer (pH 7.4) supplemented with 0.5% BSA. Spleen cells were extracted by push spleen through the cell strainers (40 μm) using barrel of sterile syringe. Isolated spleen cells were treated with ACK lysis buffer to remove excess red blood cells for 5 min on ice. Cells were washed and re-suspended in the KRH buffer and counted on a haemocytometer. The extracted spleen cells were used for phenotype determination.

Bone marrow was harvested by inserting a syringe needle (23 gauge) into one end of the femur and flushed with DMEM containing 10% FBS and 5% pen-strep. Marrow cells were centrifuged and treated with ACK lysis buffer for 5 min at room temperature, then washed and centrifuged. Cells were re-suspended in fresh DMEM and the total counting was determined with a haemocytometer. The extracted bone marrow cells were used for immune cells phenotype determination and osteoclast differentiation. Leftover cell pellets were transferred to 1.5 mL microcentrifuge tubes, rinsed with PBS and storage at –80 °C for qRT-PCR and western blot analysis.

### 2.7 Bone marrow cells and spleen cells phenotype analysis

Freshly isolated immune cells from both bone marrow and spleen were analyzed using a direct four-color labelled immunofluorescence assay. Cells were incubated with pre-labelled monoclonal antibodies to identify and quantify the phenotypes. The 96 well V-bottom plate was pre-coated with 200 μL of 4% FBS in PBS for 30 min at room temperature. 20–40 μL ($1 \times 10^5$–$4 \times 10^5$ cells/well) bone marrow cells or spleen cells were added into V-bottom plate and stained with antibodies according to manufacturer's recommendations. After 30 min incubation in dark, cells were fixed in 4% paraformaldehyde, and the proportion of positive cells for each marker was determined according to the relative fluorescence intensity by flow cytometry Canto II (BD Biosciences, Mississauga, ON, Canada) using the FlowJo software (FlowJo LLC, Ashland, OR, USA).

### 2.8 Osteoclast differentiation and resorptive activity analysis

Bone marrow cells were seeded in 24-well plate at a density of $1 \times 10^4$–$5 \times 10^4$ cells/well and cultured with DMEM containing 10% FBS, 5% pen-strep and 10 ng/mL M-CSF for 2 days. Then, the culture medium was replaced with DMEM containing 10 ng/mL M-CSF and 100 ng/mL RANKL to stimulate osteoclastogenesis. Osteoclasts were successfully differentiated by incubation with M-CSF and RANKL after 4 days. TRAP-staining kit was used to confirm and count the multinucleated osteoclast-like cells according to the manufacturer's instruction. Simply, cells were fixed in 4% paraformaldehyde for 1 h in 4 °C and then stained with TRAP staining solution (0.1 mg/mL naphthol AS-MX phosphate and 0.3 mg/mL Fast Red Violet LB staining). Cells were observed with a light microscope under the 10× lense (Olympus IX83, Richmond Hill, ON, Canada) and images were captured by Metamorphy (Olympus, Richmond Hill, ON, Canada). Any TRAP-positive multinucleated cells containing three or more nuclei were counted.

### 2.9 RNA extraction and qRT-PCR

Total RNA was extracted from the cells using TRIzol® reagent following the manufacturer's instruction, and cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen, Burlington, ON, Canada) according to the manufacturer's instruction. Real-time PCR was performed using Fast SYBR Green PCR Master Mix (Applied Biosystem, Burlington, ON, Canada) and an ABI 7300 Sequencing Detection System (Applied Biosystem, Burlington, ON, Canada). The amplification conditions were as follow: initial denaturation at 95 °C for 10 min, followed by 35 cycles of 10 s at 95 °C, 15 s at 60 °C, and 10 s at 72 °C.

### 2.10 Western blot

Whole-cell protein extracts were prepared from the bone marrow cells. The cells were lysed in boiling hot Laemmle's buffer containing 50 μmol/L dithiothreitol (DTT) and 0.2% Triton-X-100 to prepare samples for Western blot as described previously[23]. The cell lysates were run in SDS-PAGE, blotted to nitrocellulose membranes and immunoblotted with antibodies. The concentration of each antibody was determined according to the manufacturer's instruction. The protein bands were detected by a Licor Odyssey BioImager and quantified by densitometry using corresponding software (Licor Biosciences, Lincoln, NB, USA). Each band was normalized to its corresponding band of a loading control. The results were expressed as percentage of the corresponding negative.

### 2.11 Statistical analysis

Data are presented as mean ± SEM (standard error of mean) of between 4 and 7 independent experiments. Data were analyzed using Two Way Analysis of Variance (ANOVA) with Tukey's multiple comparisons test or One Way Analysis of Variance (ANOVA) with Dunnett's post-hoc comparisons test. The PRISM 6 statistical software (GraphPad Software, San Diego, CA, USA) was used for the analyses. $P < 0.05$ was considered significant.

## 3. Results

### 3.1 Ovotransferrin administration showed no negative effect on body weight, food intake and tissue weight

All rats increased the body weight at the end of the experiment (Fig. 1A). OVX rats gained significantly higher weight than that of the Sham rats. High dose ovotransferrin, but not medium and low dosage, prevented the OVX-induced weight gain. No significant differences in food intake were observed among different dietary groups (Fig. 1B). Tissue weight was not affected by the treatments (Table 1). As expected, OVX caused atrophy of uterine tissue, indicating the success of the surgical procedure (Fig. 1C). However, treatment with ovotransferrin and drug alendronate sodium had no influence on the uterine weight.

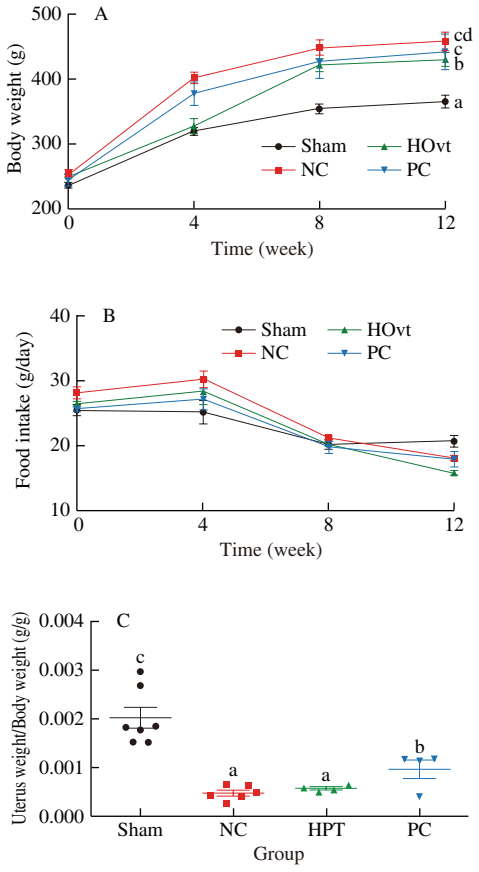


**Fig. 1** Effects of ovotransferrin diet on body weight, food intake, and uterus weight. (A) The change of body weight during 12-week treatment. (B) The change of food intake during 12-week treatment. (C) Uterus weight after 12 weeks. Data were expressed as mean ± SEM ($n$ = 8 per group). Means without a common letter indicated $P < 0.05$.

### 3.2 Ovotransferrin administration prevented the decrease of bone mineral density in OVX rats

OVX rats successfully developed osteoporosis with a significant decrease in tibia trabecular bone mineral density during the 12-week experiment (Fig. 2A), but did not affect the mineral density in tibia cortical bone (Figs. 2C and D). Over the 12-week treatment, both PC and HOvt groups significantly retarded the decline of trabecular bone mineral density compared to the negative control (NC) group (Figs. 2A and B), although the bone mineral density value was still less than Sham group. OVX rats supplemented with casein (NC) did not counteract OVX-induced bone mineral density loss, suggesting that the protection role of ovotransferrin is not due to the nutritional role of food protein. OVX rats treated with medium dose and low dose of ovotransferrin did not show prominent effects on preventing BMD loss (Fig. 2B).

### 3.3 Ovotransferrin administration preserved bone micro-architecture in OVX rats

In addition to improving bone mineral density, ovotransferrin also prevented the deterioration of bone micro-architecture in tibia trabecular bones caused by ovariectomy. Ovariectomy markedly reduced the percent bone volume, trabecular thickness, trabecular number, and increased the trabecular separation on tibia compared to the Sham rats (Figs. 3A-D), indicating a serious damage to bone microstructure and integrity. Ovotransferrin administration at high dose significantly preserved the OVX-induced decrease of percent bone volume, trabecular thickness, and trabecular number, but not the elevation of trabecular separation (Figs. 3A-D). Similar results were also observed in drug injection group (PC). Direct evidence was observed in the reconstructed three-dimensional trabecular images (Fig. 3E). The plate-like structure mostly resolved into a rod-like structure, suggesting significant loss of connecting rods in OVX rats (NC), whereas in the ovotransferrin group, this loss of trabecular bone mass and connectivity was prevented (Fig. 3E). Microstructure, which deteriorated substantially in OVX rats, was significantly improved by ovotransferrin administration.

### 3.4 Ovotransferrin administration regulated bone formation and bone resorption in OVX rats

To investigate the regulatory role of ovotransferrin on bone remodeling, serum and urine biomarkers, which indicate bone formation and bone resorption, were measured. Serum calcium ($Ca^{2+}$) was not affected (Table 2), while the urinary $Ca^{2+}$ was significantly increased in OVX rats compared to the Sham group. Interestingly,

**Table 1**
The tissue weight (mg) in all treatment groups ($n$ = 8).

| Tissue | Sham | NC | HOvt | MOvt | LOvt | PC |
|---|---|---|---|---|---|---|
| Heart | 1.63 ± 0.08 | 1.73 ± 0.19 | 1.62 ± 0.08 | 1.65 ± 0.16 | 1.63 ± 0.08 | 1.63 ± 0.04 |
| Liver | 13.42 ± 1.39 | 15.87 ± 3.27 | 12.92 ± 0.91 | 13.79 ± 2.94 | 12.21 ± 1.76 | 12.10 ± 1.22 |
| Kidney | 2.37 ± 0.21 | 2.46 ± 0.18 | 2.28 ± 0.23 | 2.47 ± 0.14 | 2.43 ± 0.14 | 2.32 ± 0.19 |
| Spleen | 0.67 ± 0.08 | 0.80 ± 0.44 | 0.69 ± 0.05 | 0.87 ± 0.38 | 0.64 ± 0.06 | 0.67 ± 0.06 |
| Uterus | 0.90 ± 0.42 | 0.22 ± 0.07 | 0.27 ± 0.03 | 0.40 ± 0.18 | 0.44 ± 0.14 | 0.43 ± 0.17 |

Note: Data were expressed as mean ± SEM.

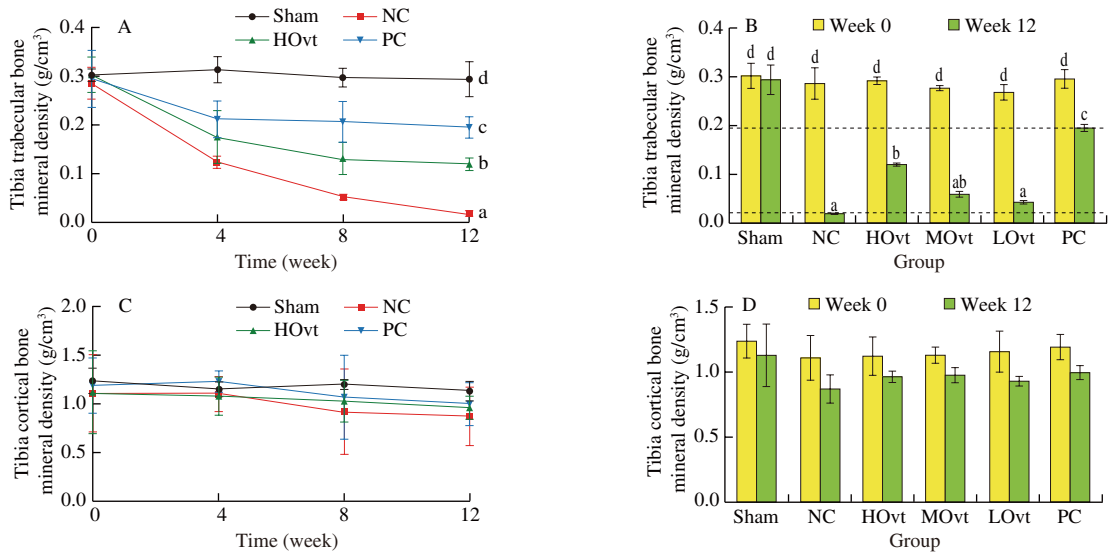


**Fig. 2** Effect of ovotransferrin administration on tibia trabecular bone mineral density and cortical bone mineral density. (A) The change of tibia trabecular bone mineral density during 12-week treatment, (B) The tibia trabecular bone mineral density in week 0 and week 12, (C) The change of tibia cortical bone mineral density during 12-week treatment, (D) The tibia cortical bone mineral density in week 0 and 12. Data were expressed as mean ± SEM ($n$ = 8 per group). Means without a common letter indicated $P < 0.05$.

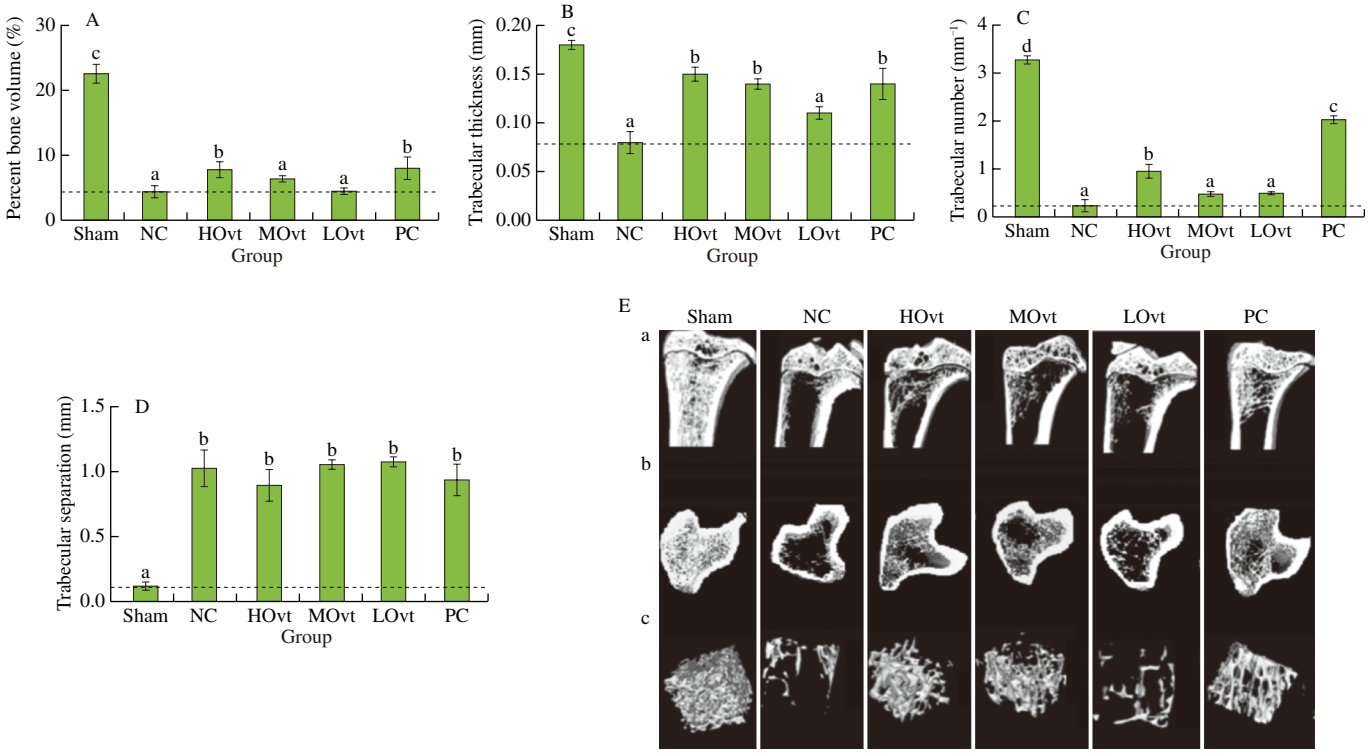


**Fig. 3** Effect of ovotransferrin administration on tibia trabecular micro-architecture. (A) The tibia trabecular percent bone volume in week 12, (B) The tibia trabecular thickness in week 12, (C) The tibia trabecular number in week 12, (D) The tibia trabecular separation in week 12, (E) Three-dimensional trabecular images of vertical section (a) and transversal section (b) of proximal tibia and the volume of interest of proximal tibia (c) in week 12. Data were expressed as mean ± SEM ($n$ = 8 per group). Means without a common letter indicated $P < 0.05$.

ovotransferrin administration attenuated the increase of urinary $Ca^{2+}$ in OVX rats. Additionally, the concentrations of serum bone alkaline phosphatase (BALP) and urinary pyridinoline (PYD), the markers of bone formation and bone resorption, respectively, were higher in the OVX rats (NC) than those of the Sham rats (Table 2). Due to the interplay between bone resorption and formation, the exceeding bone resorption caused by OVX also stimulates bone formation correspondingly. Both ovotransferrin administration and drug injection (PC) showed a suppression on serum BALP and urinary PYD, suggesting the inhibitory effects on OVX-induced overactive bone turnover and therefore help rehabilitate normal bone remodeling. The effect of ovotransferrin on preventing bone resorption was also evidenced by a decreased of serum osteocalcin level after ovotransferrin administration (Table 2). Generally, there is a two-fold rise in plasma osteocalcin concentration at the menopause, consistent with our results. Serum N-terminal propeptide of

**Table 2**
Effects of ovotransferrin diet on the concentration of serum and urine bone remodeling biomarkers.

| Index | Sham | NC | HOvt | MOvt | LOvt | PC |
|---|---|---|---|---|---|---|
| **Serum** | | | | | | |
| $Ca^{2+}$ (μg/mL) | 2.37 ± 0.06[a] | 2.12 ± 0.06[a] | 2.12 ± 0.04[a] | 2.12 ± 0.03[a] | 2.12 ± 0.03[a] | 2.36 ± 0.43[a] |
| Osteocalcin (ng/mL) | 48.94 ± 28.48[a] | 81.14 ± 24.30[b] | 52.07 ± 15.99[a] | 61.24 ± 18.57[a] | 61.67 ± 16.23[a] | 43.34 ± 25.90[c] |
| BALP (ng/mL) | 3.24 ± 1.62[a] | 6.27 ± 1.39[b] | 2.15 ± 2.34[c] | 3.58 ± 1.69[a] | 4.10 ± 2.05[a] | 2.04 ± 1.29[c] |
| PINP (ng/mL) | 44.65 ± 7.74[a] | 21.53 ± 9.07[b] | 34.95 ± 6.26[c] | 25.55 ± 1.68[b] | 22.86 ± 8.58[b] | 36.79 ± 6.20[c] |
| PTH (pg/mL) | 327.94 ± 86.70[a] | 307.29 ± 162.46[a] | 474.10 ± 155.64[b] | 360.38 ± 73.38[a] | 347.20 ± 140.05[a] | 422.93 ± 158.46[b] |
| **Urine** | | | | | | |
| $Ca^{2+}$ (ng/mL)/Cr (mg/dL) | 12.1 ± 3.5[a] | 21.9 ± 2.5[b] | 14.7 ± 2.9[a] | 15.3 ± 1.4[a] | 18.8 ± 1.3[c] | N/A |
| PYD (pmol/mL)/Cr (mg/dL) | 40.7 ± 12.2[a] | 67.0 ± 39.3[b] | 45.4 ± 22.0[a] | 49.1 ± 25.8[a] | 49.9 ± 24.6[a] | N/A |

Note: Data were expressed as mean ± SD. Within a row, mean without a common letter indicated $P < 0.05$. N/A, not applicate.

type I collagen (PINP), a biomarker indicating collagen synthesis, was also evaluated as an indicator of bone formation. As expected, OVX showed significant inhibition in serum PINP, whereas ovotransferrin administration elevated the concentration. Serum PTH level in high dose ovotransferrin-treated OVX rats were significantly increased than other groups (Table 2). PTH is the only available anabolic drug used to stimulate bone formation in osteoporosis patients. The increased serum PTH level with ovotransferrin treatment may also suggest the possible ability of ovotransferrin in promoting bone formation.

### *3.5 Ovotransferrin inhibited the production of serum cytokines in OVX rats*

The bone resorption related cytokines were evaluated in this study. The serum TNF-α was significantly increased in the negative control group than the Sham group (Fig. 4A). Over 12-week of ovotransferrin administration, serum TNF-α level was significantly reduced compared to the negative control group, indicating that ovotransferrin is able to inhibit ovariectomy-caused TNF-α formation. Interestingly, ovariectomy did not cause a significant increase of IL-6. The serum IL-6 concentrations were similar in negative control (NC) and Sham groups; however, both high dose ovotransferrin treatment and drug intervention led to a lower level of serum IL-6 (Fig. 4B).

### *3.6 Osteoclast precursors derived from ovotransferrin-administrated OVX rats showed less differentiation ability and resorption activity*

To examine the effect of ovotransferrin on the differentiation ability of osteoclast precursors, bone marrow cells extracted from different animal groups were cultured in osteoclastogenic medium and the differentiated osteoclasts were counted with TRAP staining. After 6 days of incubation, osteoclasts were successfully differentiated from bone marrow cells (Fig. 5A). A greater number of osteoclasts were observed from bone marrow cells extracted from negative control group (NC), compared to the Sham group (Figs. 5A and B). Importantly, bone marrow cells extracted from the ovotransferrin administrated groups showed less mature osteoclasts, especially in the high dose group. Similar results were observed in drug injection group (PC). The absorption activity of differentiated osteoclasts was investigated using a calcium phosphate (CaP)-coated plate. Ovotransferrin administration not only reduced the number of differentiated osteoclasts, but also showed less absorptive activity compared to the negative group (NC) (Figs. 5C-E). Compared with the Sham rats, OVX rats showed higher osteoclastic resorption, whereas, ovotransferrin administration attenuated OVX-induced excessive osteoclastic resorption.

To understand the effect of ovotransferrin on bone marrow microenvironment, cathepsin K (CathK) and matrix metalloproteinase 9 (MMP9) were measured in bone marrow cells. Ovariectomy significantly increased the levels of CathK and MMP9 compared to the Sham group, while both ovotransferrin administration and drug injection reduced their expression (Fig. 6); only the HOvt group significantly decreased both CathK and MMP9 levels.

### *3.7 Ovotransferrin inhibited osteoclastogenesis by inhibiting the NF-κB pathway*

Our previously *in vitro* study showed that ovotransferrin prevented RANKL-induced osteoclastogenesis in RAW 264.7 cell by inhibiting NF-κB pathway[24]. This study further confirmed this finding in primary osteoclast precursors extracted from bone marrow. Ovotransferrin administration significantly inhibited the expression of

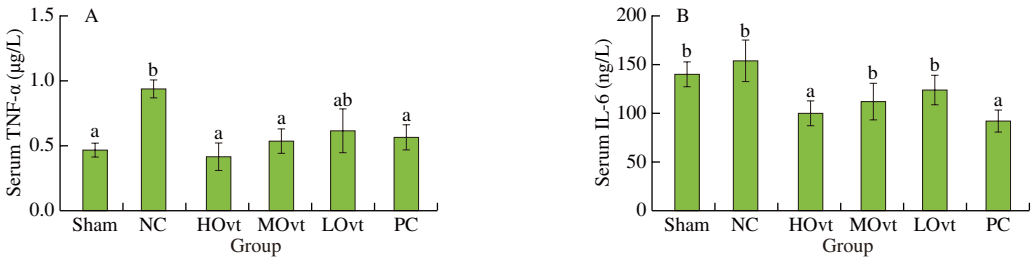


**Fig. 4** Effects of ovotransferrin administration on serum cytokine production. (A) The serum TNF-α concentration in week 12, (B) The serum IL-6 concentration in week 12. Data were expressed as mean ± SEM ($n = 8$ per group). Means without a common letter indicated $P < 0.05$.

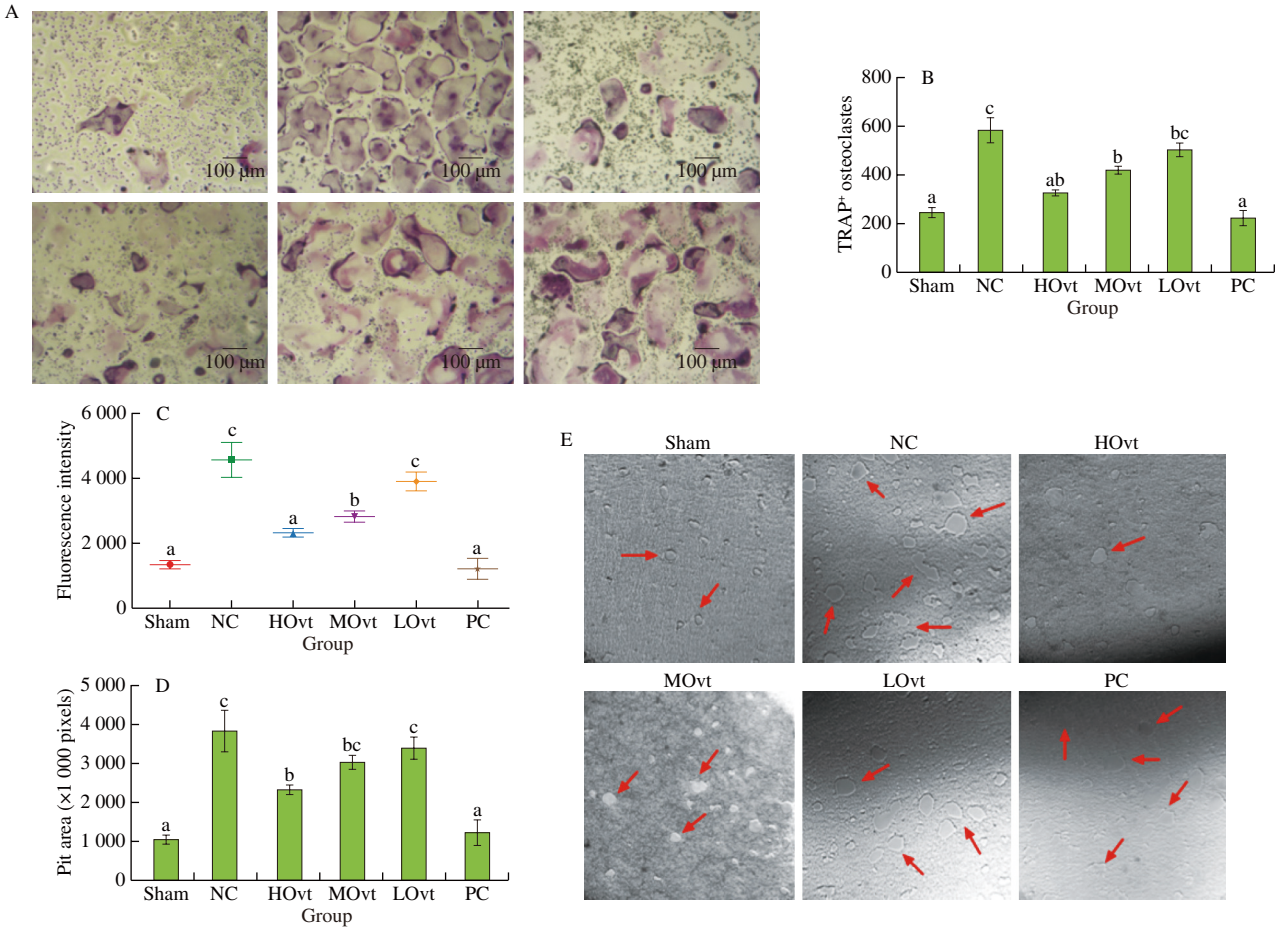


**Fig. 5** Effects of ovotransferrin administration on bone marrow cells-stimulated osteoclasts formation and resorption. Bone marrow cells were extracted from rat left femur and culture with 10 ng/mL M-CSF and 100 ng/mL RANKL for 6 days to stimulate osteoclasts. (A) The osteoclasts after TRAP staining, (B) The number of TRAP positive cells per well differentiated from different animal groups, (C) The osteoclastic resorption activity, (D) The osteoclastic resorbed pit area, (E) The images of osteoclastic resorbed area. Data were expressed as mean ± SEM ($n$ = 8 per group). Means without a common letter indicated $P < 0.05$.

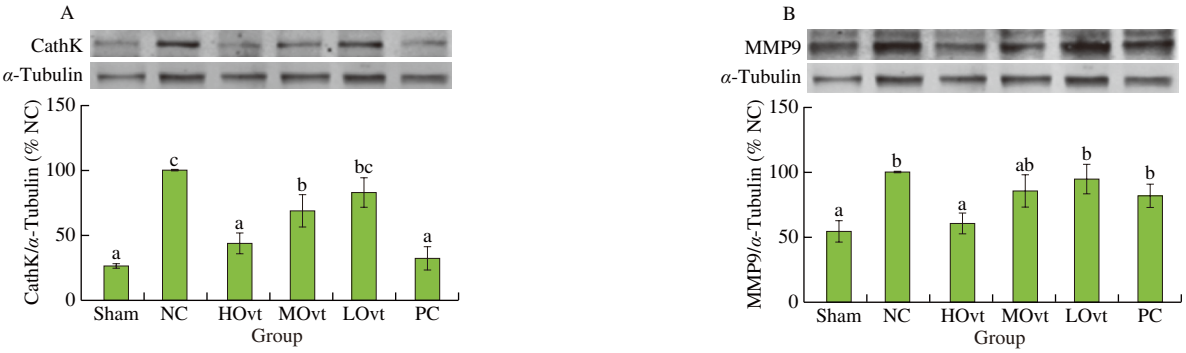


**Fig. 6** Effects of ovotransferrin administration on osteoclastogenesis-related protein expression in bone marrow. Bone marrow cells were extracted from rat left femur and whole proteins were extracted for Western blot analysis. (A) The protein expression of CathK, (B) The protein expression of MMP9. Data were expressed as mean ± SEM ($n$ = 8 per group). Means without a common letter indicated $P < 0.05$.

NF-κB p65 and IκBα (Fig. 7) in primary osteoclast precursors, which are two most important components involved in the NF-κB pathway.

### *3.8 Ovotransferrin administration modulated local immune function*

Ovotransferrin is considered as a member of innate immunity, which is closely related to osteoporosis in elderly population, thus, the effect of ovotransferrin administration on immune modulation was studied. Compared with the Sham group, OVX did not cause a significant change in spleenocyte phenotypes, but showed a significant effect on immune cells in the bone marrow compartment (Fig. 8). The percentage of total T cells ($CD3^+$) and T cytotoxic cells ($CD8^+$) in bone marrow were significantly decreased after ovariectomy, while ovotransferrin administration attenuated this decrease (Figs. 8A and B). Meanwhile, the population of T-cell subsets, including T-cell express IL-2 receptor ($CD25^+$), T-cell express co-stimulatory factors ($CD28^+$), T-cell express anti-integrin alpha ($CD51^+$), dendritic cells ($OX62^+$), and B-cells ($OX12^+$) was increased

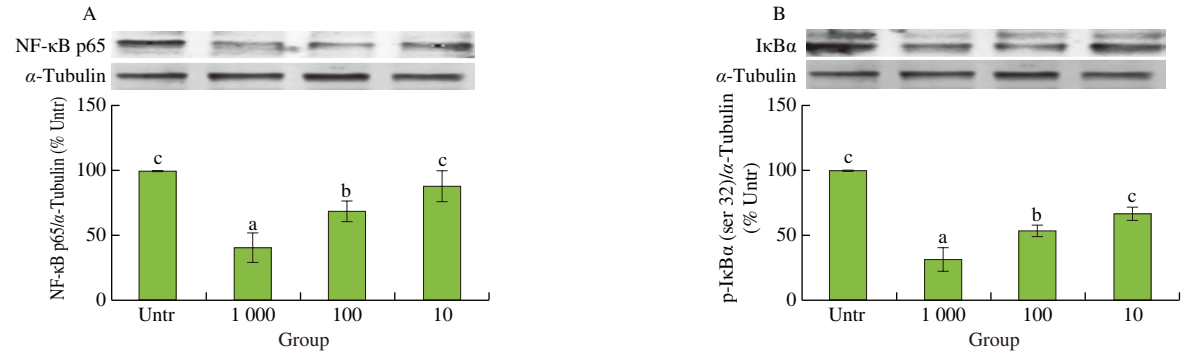


**Fig. 7** Effects of ovotransferrin administration on osteoclast differentiation. Bone marrow cells were extracted from left femur of Sham rats and cultured with 10 ng/mL M-CSF and 100 ng/mL and RANKL to stimulate osteoclasts. After 6 days, different concentrations of ovotransferrin were added and incubated for 24 h. Then cells were collected and protein was extracted for Western blot analysis. (A) The protein expression of NF-κB p65, (B) The protein expression of IκBα. Data were expressed as mean ± SEM ($n$ = 8 per group). Means without a common letter indicated $P < 0.05$. Untr, untreated.

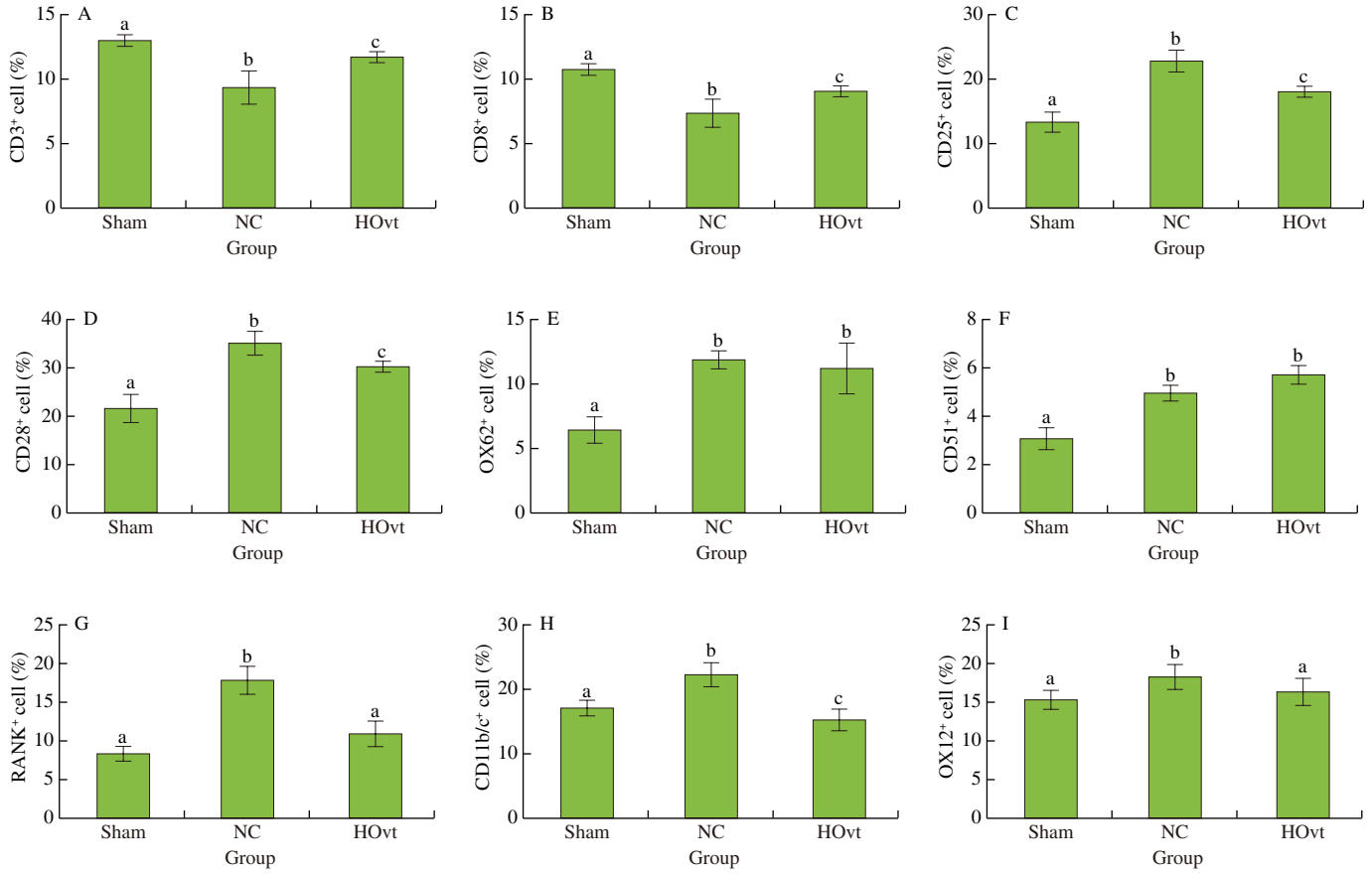


**Fig. 8** Phenotype analysis of freshly isolated immune cells. Values were percentage proportion of the total gate cells as determine by immunofluorescence. (A) The population of $CD3^+$ cells, (B) The population of $CD8^+$ cells, (C) The population of $CD25^+$ cells, (D) The population of $CD28^+$ cells, (E) The population of $OX62^+$ cells, (F) The population of $CD51^+$ cells, (G) The population of $RANK^+$ cells, (H) The population of $CD11b/c^+$ cells, (I) The population of $OX12^+$ cells. Data were expressed as mean ± SEM. Mean without a common letter indicated $P < 0.05$.

in OVX rats, while those of $CD25^+$, $CD28^+$ and $OX12^+$ cells were suppressed upon ovotransferrin administration (Figs. 8C, D and H). Especially the population of monocytes/macrophages ($CD11b/c^+$) and $RANK^+$ cells, which are the precursor cells of osteoclasts, were significantly increased in OVX rats, but were reduced at high dose ovotransferrin group (Figs. 8G and H). These results are consistent with our previous finding that fewer osteoclasts were differentiated from bone marrow cells derived from ovotransferrin treatment groups (Fig. 6).

## 4. Discussion

The effect of egg white protein ovotransferrin on osteoporosis prevention was studied in OVX rats. OVX rat is a well-accepted osteoporosis model that mimics the estrogen deficiency-induced osteoporosis in postmenopausal women. In the study, adding ovotransferrin up to 1% (*m*/*m*) did not affect food intake, weight gain, and tissue weight, suggesting the safety of ovotransferrin. Our findings support that ovotransferrin at a dose of 1% (*m*/*m*) could

attenuate estrogen-dependent bone loss and micro-architecture deterioration in OVX rats. The mechanical properties of bone are influenced by bone mass as well as bone size, bone quality, and bone micro-architecture. In this study, ovotransferrin treatment resulted in an increase of percent bone volume consistent with the improvement in bone mineral density, suggesting ovotransferrin could prevent the loss of bone mass after ovariectomy. Ovotransferrin administration improved trabecular architecture as it increases trabecular architecture. In addition, ovotransferrin appears to partially restore trabecular connectivity by increasing trabecular numbers. Ovotransferrin showed more effective protection against trabecular loss. This study provides direct evidence that ovotransferrin can acts as an orally active agent to preserve bone mass and micro-architecture in a growing OVX rat model, further supporting the potential of ovotransferrin for the prevention of osteoporosis on the basis of our previous *in vitro* studies.

Ovariectomy leads to increased bone resorption and excessive bone loss. Interestingly, bone formation markers BALP and osteocalcin were elevated with a commensurate increase of the bone resorption marker PYD. This could be interpreted as a coupling between bone resorption and formation[28]. Accelerated bone resorption in ovariectomy also triggers a trivial stimulation on bone formation. Thus, the process is dominated by bone resorption. Administration of ovotransferrin, on the other hand, reduced BALP, osteocalcin and PYD, which suggested a role of ovotransferrin on bone remodeling. Moreover, ovotransferrin may function as a stimulator on bone formation as indicated by a substantial increase in collagen synthesis (PINP) after ovotransferrin administration. Furthermore, we measured two osteoclastogenic cytokines and found that ovotransferrin down regulated the production of serum TNF-α and IL-6 under the experimental conditions. TNF-α, along with IL-6, plays a critical causal role in the rapid bone loss associated with estrogen deficiency[29]. They promote bone resorption by affecting osteoclast differentiation and activity. Ovotransferrin was suggested as an innate immunity of laying hen eggs[30]; ovotransferrin and its cationic fragment OTAP-92 could enhance intestinal and systemic immune response[31-32]. Meanwhile, ovotransferrin hydrolysates and ovotransferrin-derived peptide showed anti-inflammatory activity by inhibiting the production of inflammatory cytokines, such as TNF-α and IL-6[1,33-34]. Our results suggested that ovotransferrin might inhibit bone resorption as an anti-inflammatory agent, thus improving bone health.

A further study on the effect of ovotransferrin on osteoclasts formation and resorption was investigated using freshly extracted bone marrow cells. Bone marrow cells derived from OVX rats developed more osteoclasts compared to the Sham rats, indicating estrogen deficiency could activate osteoclastogenesis and promote bone loss in OVX rats. Oral administration of ovotransferrin significantly ameliorated the osteoclast formation. Furthermore, ovotransferrin treatment also prevented OVX-induced excessive osteoclastic resorption. These might be partly due to the effects of ovotransferrin on regulating osteoclast precursors in bone marrow. In the study, ovotransferrin treatment significantly suppressed the levels of CathK and MMP9 in bone marrow cells. CathK is the key enzyme responsible for osteolysis of bone, which is also the only known mammalian protease capable of degrading both helical and non-helical regions of type I collagen[35]. Overexpression of CathK has been demonstrated to result in accelerated bone turnover[36-37]. Similarly, MMPs are a group of proteolytic enzymes involved in the degradation of extracellular matrix of various tissues including bone[38]. Among all, MMP9 is highly expressed at early stages of osteoclast development and in mature osteoclasts[39]. Increased MMP9 activity has been detected in human osteoclastomas and osteoclasts in Paget's disease[40]. It has also been shown that upregulation of MMP9 plays an important role in the pathogenesis of dental pulp inflammation and destruction[41]. Cytokines such as IL-1, TNF-α and granulocyte macrophage colony-stimulating factor (GM-CSF) have been shown to induce the upregulation of MMP9 and enhance the activity of MMP9, therefore contributing to bone degradation[38,42]. Thus, suppression of CathK and MMP9 expression in bone marrow cells might contribute to reduce osteoclastic bone resorption by preventing osteoclast formation and activity in OVX rats.

Our previous work showed that ovotransferrin was able to inhibit RANKL-induced osteoclastogenesis by regulating the activation of NF-κB pathway[24]. NF-κB is one of the important signaling pathways activated by the binding of RANKL to RANK. This binding triggers the translocation of NF-κB p65 into nucleus and foster the transcription of genes involved in osteoclast differentiation[43]. Using osteoclasts differentiated from bone marrow cells, ovotransferrin significantly inhibited RANKL-induced expression of NF-κB p65 and IκBα, indicating the involvement of NF-κB pathway in suppressing osteoclastogenesis. Inactivation of NF-κB interrupts osteoclastogenesis and osteoclasts function[44]. A2B adenosine receptor (A2BAR), a regulator of bone homeostasis, showed inhibition of NF-κB activation and suppressed the induction of osteoclast markers, including CathK and MMP9, which contribute to the decrease in osteoclast cell-cell fusion and bone resorption activity[44].

Immune system is involved in the pathogenesis of estrogen deficient osteoporosis[45]. Tight molecular and cellular links between immune activation and bone loss have been identified[46-47]. Here, we examined the phenotypic response of immune cells in spleen and bone marrow after treated with ovotransferrin. Although the phenotype changes were observed with ovotransferrin administration, especially in bone marrow (e.g. $CD3^+$, $CD8^+$ and $OX12^+$ population), it is not clear what role, if any, these changes plays in the regulation of bone metabolism. Previous studies suggested the involvement of $CD8^+$ T-cells and $OX12^+$ B-cells in regulating osteoclast differentiation by modulating the cytokines; however, the results remain controversial[48-51]. Although, further experiments are warranted to explore the relationship between ovotransferrin-induced immune modulation and bone metabolism in OVX rats, our findings provide new perspectives and means to investigate bone health promotion targeting to local bone marrow microenvironment.

## 5. Conclusion

In summary, for the first time we reported that oral administration of ovotransferrin, a major egg white protein and a member of transferrin family preserved OVX-induced loss of bone mineral density and deterioration of trabecular micro-architecture. Additionally, mechanism study showed that ovotransferrin administration suppressed the overactive bone remodeling by inhibiting osteoclastogenesis and osteoclastic bone resorption probably via NF-κB pathway, while also promoting bone formation. Ovotransferrin also modulated bone marrow immune function and therefore regulated local bone marrow microenvironment to rebuild

the bone remodeling balance. Our results suggested the potential application of ovotransferrin as a functional food ingredient on the prevention of osteoporosis.

## Declaration of conflict of interest

Nan Shang is an associate editor, and Jianping Wu is an editorial board member for *Food Science and Human Wellness* and were not involved in the editorial review or the decision to publish this article. All authors declare no conflict of interest.

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