

4-Hexylresorcinol Inhibits Osteoclastogenesis by Suppressing NF- κ B Signaling Pathway and Reverses Bone Loss in Ovariectomized Mice

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Research

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Abstract

Background: 4-Hexylresorcinol is a small organic compound that is widely used as additive antiseptic and antioxidant. In the present study, we investigated its role in osteoclastogenesis.

Methods: Bone marrow-derived macrophages were used to test the role of 4-Hexylresorcinol on osteogenesis. Ovariectomy model was created to examine the effect of 4-Hexylresorcinol *in vivo*.

Results: In this study 4-Hexylresorcinol effectively suppressed RANKL-induced osteoclastogenesis in a dose-dependent manner. It also significantly suppressed the expression of osteoclast-specific markers including tartrate-resistant acid phosphatase, cathepsin K, nuclear factor of activated T-cells cytoplasmic 1 and c-Fos. Furthermore, it inhibited osteoclastogenesis by inhibiting activation of NF- κ B signaling pathway. Consistent with *in vitro* results, 4-Hexylresorcinol effectively ameliorated ovariectomy surgery induced bone loss and profoundly reduced osteoclast number *in vivo*.

Conclusions: Overall, our results suggested that 4-Hexylresorcinol may be a novel therapeutic approach for osteoporosis treatment.

Introduction

Osteoporosis is the most common disease which mainly affects elderly people and post-menopause women [1]. It is characterized by low bone mineral density and an increased risk of fracture [2] which often are associated with increased mortality and significant morbidity, including enhanced risk of pain[3] and disability[4]. Thus, there is great need for us to investigate the cause of the disease. As previous studies have showed osteoporosis was a disease of imbalance of osteoblast (OB) and osteoclast (OC)[5], that is generally attributed to decreased OBs and increased OCs. OCs are giant bone-resorbing multinucleated cells that originates from nonnuclear-macrophages. Throughout life, bone undergoes continuous remodeling through concerted bone matrix formation and mineralization (anabolic process) and mineralized bone matrix degradation (catabolic process) by OCs[6]. In osteoclastogenesis, M-CSF and RANKL are essential for OC differentiation and also stimulate the function and survival of mature osteoclasts [7, 8]. Thus, disruption of the interaction of RANKL and RANK using human monoclonal antibody Denosumab, prevents OCs formation, activity and survival [9-11]. However, new therapeutic strategies about the intervention of osteoclastogenesis have not been extensively studied. Antiresorptive drugs include estrogens (with or without progesterone), bisphosphonates (alendronate, risedronate, ibandronate, and zoledronic acid), an estrogen agonist/antagonist (raloxifene), and salmon calcitonin. Teriparatide and recombinant human parathyroid hormone are the only approved anabolic agents [12].

In the present study, we analyzed the role of 4-Hexylresorcinol (4HR)- a small organic compound that is an additive antiseptic and antioxidant[13]. It has been widely used as an antimicrobial in cosmetics and antiseptics [14]. It was shown that 4HR accelerates orthodontic tooth movement and increases the expression level of bone turnover markers in ovariectomized rats, indicating a role of 4HR in bone remodeling [15]. However, its role in osteoclastogenesis has not been fully elucidated. In our study, we

found 4HR significantly decreased osteoclastogenesis in BMMs with reduced TRAP, Cathepsin K, NFAT c1 and c-fos level. Furthermore, 4HR significantly decreased RANKL induced NF- κ B signaling pathway and reverses bone loss in ovariectomized mice. Our results revealed a potential role of 4HR in regulating OC function and treating osteoporosis.

Material And Methods

1. Chemicals and Reagents

Anti-actin antibody, 4HR, TRAP staining kit, and other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti TRAP, NFAT c1 and Cathepsin K antibodies were from Proteintech Group (Wuhan, China). Phosphorylated-IKK β (pIKK β) and total IKK β antibodies were from Cell Signaling Technology (Beverly, MA). Recombinant soluble M-CSF and RANKL were obtained from PeproTech (Rocky Hill, NJ). Cell counting kit-8 (CCK-8) kit was purchased from Wuhan Boster Biological Technology Company (Wuhan, China). Serum cross-linked C-telopeptide of type I collagen (P1NP) and C-terminal telopeptide of type1 collagen (CTX1) were from Cloud-Clone (Katy, TX, USA).

2. Animals and OVX surgery

All animal work was approved by Tongji Medical College, Huazhong University of Science and Technology. C57BL/6J mice used in the study were purchased from Experimental Animal Center of Tongji Medical College. All mice were kept under standard laboratory conditions with 12 h light and 12 h dark cycle, water and food ad libitum. All animal experiments were performed according to the guidelines of the Care and Use of Laboratory Animals by the National Institute of Health, China.

For OVX surgery and drug treatment, 4 months old female C57BL/6J were used, mice were anaesthetized by 2% isoflurane, and the ovaries were bilaterally removed according to established protocol[16] except the sham-operated mice. OVX mice received equal volume of DMSO intraperitoneally injection daily for 60 days after surgery. Another OVX group received 1mg/kg 4HR intraperitoneally injection daily for 60 days after OVX surgery (OVX+4HR). Mice were sacrificed after the final administration, the tibias of the all the mice were collected and scanned using microCT.

3. Bone specimen collection and microCT scanning

The tibias were dissected and fixed in neutral buffered formalin for 48 hrs. After fixation, the tibias were scanned using Viva CT 40 (Scanco Medical, Switzerland) at 15 μ m resolution and 70kVP and 112 μ A x-ray energy. The parameters of BV/TV (Bone volume per tissue volume), Tb.N (Trabecular Number), Tb. Th (Trabecular Thickness), Tb. Sp (Trabecular Separation) were collected and analyzed from each sample.

4. Histology

After microCT scanning, the tibias were decalcified in 10% EDTA for 4 weeks. Before sectioning, the tissues were paraffin embedded. Sections were cut at 5 μ m thickness and then stained with hematoxylin

and eosin (H&E). TRAP staining was performed using 387A kit (Sigma-Aldrich, St. Louis, MO, USA) following a standard protocol.

5. *In vitro* osteoclastogenesis

The tibia and femur were dissected from 6-8 week C57BL/6J mice and BMMs were flushed from the tibia and femur (N=3 each group, male mice were used). Then the cells were centrifuged and plated onto 100 mm tissue culture dish containing 10% FBS and 10 ng/ml M-CSF. The next day, non-adherent bone marrow progenitor cells (monocyte-macrophage) were collected. The BMMs were cultured in α -MEM plus 10%FBS supplemented with 10 ng/ml M-CSF. For osteoclastogenesis, BMMs were cultured in α -MEM containing 10% FBS in the presence of 10 ng/ml M-CSF and 100 ng/ml RANKL for 5 days. Mature osteoclasts number and area in each well were quantified using imageJ. RNA was extracted using Trizol reagent from 3 days of osteoclastogenesis BMMs. The total RNA was reverse transcribed to cDNA for RT-qPCR. The primers were designed as below, NFATc1, forward primer, 5'-TCTTCCG AGTTCACATCCC-3' and reverse primer, 5'-GACAGCACCATCTTCTTCC-3'; TRAP, forward primer, 5'-cagcagccaaggaggactac-3', reverse primer, 5'-acatagcccacaccgttctc-3'; Cathepsin K forward primer, 5'-ccagtgggagctatggaaga-3', reverse primer, 5'-tggtcatggccagttcata-3', and those genes mRNA levels were normalized by mouse β -actin, forward primer, 5'-tgttaccaactgggacgaca-3', reverse primer, 5'-ggggtgtgaaggctctcaaa-3'. The relative mRNA levels of target genes were calculated by the comparative CT method [17].

6. Western-blot analysis

Protein extracts were prepared in radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitor and phosphatase inhibitors. The proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis 10% gel and then transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA). Membranes were blocked with 5% BSA in Tris-buffered saline Tween 20 (TBST) for 60 min and then incubated with the indicated primary antibodies overnight. Then the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies. Finally, the membranes were visualized using electrochemiluminescence reagents and the band densities were quantified using Image Lab 5.1 software (Bio-Rad, Hercules, CA) and normalized to actin.

7. Luciferase assay

HEK293T cells were transfected with NF- κ B -luciferase reporter plasmid together with control vector (Promega, USA) as reference controls using Lipofectamine 3000 (Invitrogen, USA). 48 hours later, cells were subject to different treatments. The luciferase activity measurement was performed as described in Dual luciferase reporter assay kit (Promega, USA).

8. Analysis of serum biomarkers

Serum P1NP, an indicator of bone formation, and CTXI level, an indicator of bone resorption, were measured using an ELISA kit (Cloud-Clone, Katy, TX, USA) followed the instructed methods [18].

9. Statistical analysis

Data were shown as mean \pm SD and were analyzed using SPSS software (SPSS Inc, Chicago, IL, USA). One-way ANOVA followed by LSD's post hoc tests were used to test the differences among groups. Student t test was applied to compare difference between two groups. The levels of significance are presented as $*p<0.05$, $**p<0.01$, and $***p<0.001$.

Data availability

All data supporting the conclusions are provided in the manuscript.

Results

1, 4-Hexylresorcinol reversed RANKL induced osteoclastogenesis. Osteoclastogenesis is a multistep process including OC proliferation, commitment, fusion and activation triggered by RANKL and RANK binding [19], with the formation of giant multi-nucleated merged cells. To test the role of 4-Hexylresorcinol in osteoclastogenesis, we first isolated BMMs from long bones of the mice and measure the cell activity using CCK-8 assay. Results showed no significant difference was observed when BMMs were exposed to different concentrations of 4HR (0, 5, 10, 20 μ g/ml, Fig 1, A), indicating no obvious cytotoxicity for the drug treatment. To mimic osteoclastogenesis process in vitro, we then treated BMMs using RANKL together with M-CSF to induce osteoclastogenesis, with or without 4HR pre-incubations. Surprisingly, 4HR reduced multinucleated TRAP positive cell number and area in a dose-dependent manner (0, 5, 10, 20 μ g/ml, Fig 1, B, C, D). The mRNA level of osteoclastogenesis related gene such as TRAP, Cathepsin K, NFAT c1 and C-fos, were up-regulated by RANKL during osteoclastogenesis, however, were significantly reversed by different concentrations of 4HR pre-incubation, suggesting the protective effect of 4HR (Fig 1, E, F, G, H). To further verify the effects of 4HR in osteoclastogenesis, we tested the protein level of those genes, results showed 20 μ g/ml 4HR exposure remarkably reduced TRAP, NFAT c1 and Cathepsin K protein level in osteoclastogenesis process (Fig 2, A, B). 3. 4HR suppresses RANKL-induced activation of NF- κ B pathway. NF- κ B play an important role in OC induced osteoporosis and osteoclastogenesis [20, 21]. To explore the molecular basis of 4HR in regulating the activity of NF- κ B pathway, we first examined the effect of 4HR treatment on NF- κ B reporter assay in 293T cell line. After transfection, we treat the cells with RANKL with or without 4-HR pre-incubation, results showed a profound inhibition of NF- κ B transactivation by 20 μ g/ml 4HR pre-incubation (Fig 2, C). We further treated BMMs with M-CSF and RANKL in the presence of 20 μ g/ml 4HR for 0, 15, 30, and 60 min, the active form of NF- κ B, p-IKK β was decreased after 20 μ g/ml 4HR pre-treatment (Fig 2, D, E). Those results strongly suggested 4HR suppressed osteoclastogenesis by inhibiting NF- κ B pathway. 3. 4HR treatment are resistant to ovariectomy-induced bone loss in vivo. To test the role of 4HR in osteoporosis in vivo, we created ovariectomy model in 4 months old female mice. Mice were divided into sham-operated, OVX, and OVX with 4HR (OVX+4HR) injection groups. After OVX surgery, mice in OVX group received equal amount of solvent daily, OVX +4HR group received 1mg/kg 4HR daily. 2 months later, mice were sacrificed and the tibias of each mouse were collected and subjected to microCT scanning. Our data showed, OVX surgery

decreased bone mass significantly in proximal tibia with reduced BV/TV, Tb.N, Tb. Th and increased Tb, Sp compared to sham operated mice, however, mice with 4HR administration remarkably reversed OVX induced bone loss, as shown by increased BV/TV, Tb.N, Tb. Th, and decreased Tb. Sp compared to OVX group. These results confirmed us the role of 4HR in osteoporosis in vivo (Fig 3, A , B , C , D , E). We next examined the bone mass using H&E staining in paraffin-embedded bone slides. As expected, we found OVX surgery reduced trabecular bone mass in proximal tibia as less trabecular bone was seen in the image. 4HR application, however, rescued the bone loss profoundly compared to OVX mice (Fig 4, A). To measure the OC number in vivo, TRAP staining was performed. OVX surgery increased TRAP positive cell number in proximal tibia area but 4HR treatment reduced TRAP positive cell number (Fig 4, B, C), which is consistent with our in vitro experiments and microCT data. We then measured P1NP and CTX1 level in the serum, which are important bone formation marker and bone resorption markers [22, 23]. As expected, the OVX mice showed aN increased CTX1 level compared to Sham-operated mice but no difference in PTNP level. 4-HR treated mice, however, exhibited a lower level of CTX1, no difference was seen in P1NP level (Fig 4, D, E).

Conclusion

Taken together, these data suggested that 4-HR treatment ameliorates OVX-induced osteoporosis by inhibiting bone resorption without significantly affecting bone formation. Our study provide a novel reagent for treating osteoporosis.

Discussion

Osteoclastogenesis is regulated by complex signaling cascades that are triggered by RANKL. In this study, 4HR application decreased RANKL-induced osteoclastogenesis in a dose-dependent manner in BMMs. It significantly reduced TRAP positive cell number and area [24]. TRAP, Cathepsin K, NFAT c1 and C-fos are important osteoclastogenesis markers as they enhance the differentiation and function of OCs. In the present study, BMMs exposed by 4HR significantly reduced TRAP, Cathepsin K, NFAT c1 and C-fos level (**Fig, 1-2**). These findings were confirmed in both PCR and Western blot.

OC differentiation is regulated by many signaling pathways. Most importantly, NF- κ B is a vital downstream pathway in osteoclastogenesis. It was also reported NF- κ B contributes to OVX-induced bone loss [25]. In previous studies, 4HR has been reported to suppress NF- κ B signaling pathway [26, 27]. In our study, using NF- κ B reporting plasmid, we verified 4HR suppressed NF- κ B signaling induced by RANKL in BMMs. Furthermore, phosphorylated IKK β was decreased after 4HR treatment. Thus, 4HR may exert its anti-inflammatory effect in osteoclastogenesis.

OVX is a model results in decreased estrogen, and is a good way for model for osteoporosis research. The main characteristic is increased OC number and activity[28]. The administration of 4HR to OVX mice resulted in significantly less degrees of bone loss caused by OVX as evidenced by micoCT and histology (**Fig, 3-4**). As shown by elevated BV/TV, Tb. N, Tb. Th and decreased Tb. Sp in proximal tibia as well as

H&E staining. To confirm whether the protective effect was a result of interfering osteoclastogenesis, we performed TRAP staining to quantify TRAP positive cell number in each group. Our results showed indeed, the TRAP positive cells were increased in OVX mice compared to Sham operated mice, however, 4HR administration profoundly decreased TRAP positive cell number compare to OVX mice.

The limitation of our study is we were not sure if OBs also take a part in the 4HR's role in OVX model. As we all know, maintaining normal bone mass is a balance of OBs and OCs. The level of bone formation marker P1NP showed no difference among groups indicated 4HR may not involved in our model. Future study may extend the investigation to OBs.

Overall, our study found 4HR administration was beneficial for osteoporosis treatment by reducing osteoclastogenesis both *in vitro* and *in vivo*. This may provide therapeutic thoughts in clinic study and encourage us to discover more effective drugs in widely used chemical reagents to treat osteoporosis.

Abbreviations

4HR: 4-Hexylresorcinol

OC: Osteoclast

OB: Osteoblast

BMM: Bone marrow-derived macrophages

M-CSF: Macrophage colony stimulating factor

CCK-8: Cell counting kit-8

RANKL: Receptor activator of NF- κ B ligand;

TRAP: Tartrate-resistant acid phosphatase

NFATc1: Nuclear factor of activated T-cells cytoplasmic 1

OVX: Ovariectomy

BV/TV : Bone volume/Total volume

Tb.N: Trabecular Number

Tb. Th: Trabecular Thickness

Tb. Sp: Trabecular Separation

P1NP: C-telopeptide of type I collagen

CTX1 : C-terminal telopeptide of type1 collagen

Declarations

Ethics approval and consent to participate:

Not applicable

Consent for publication:

All the authors consent of the publication.

Availability of data and materials:

Not applicable.

Competing interests:

The authors have no competing interests.

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Author contributions:

M.L. designed the experiments. W.Y. performed most of the experiments; T.L contributed to the NF- κ B reporter assay; X.G. and Y.X contributed to the OVX surgery, W.Y. and M.L wrote the manuscript. All authors provided editorial comments.

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Not applicable

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Figures

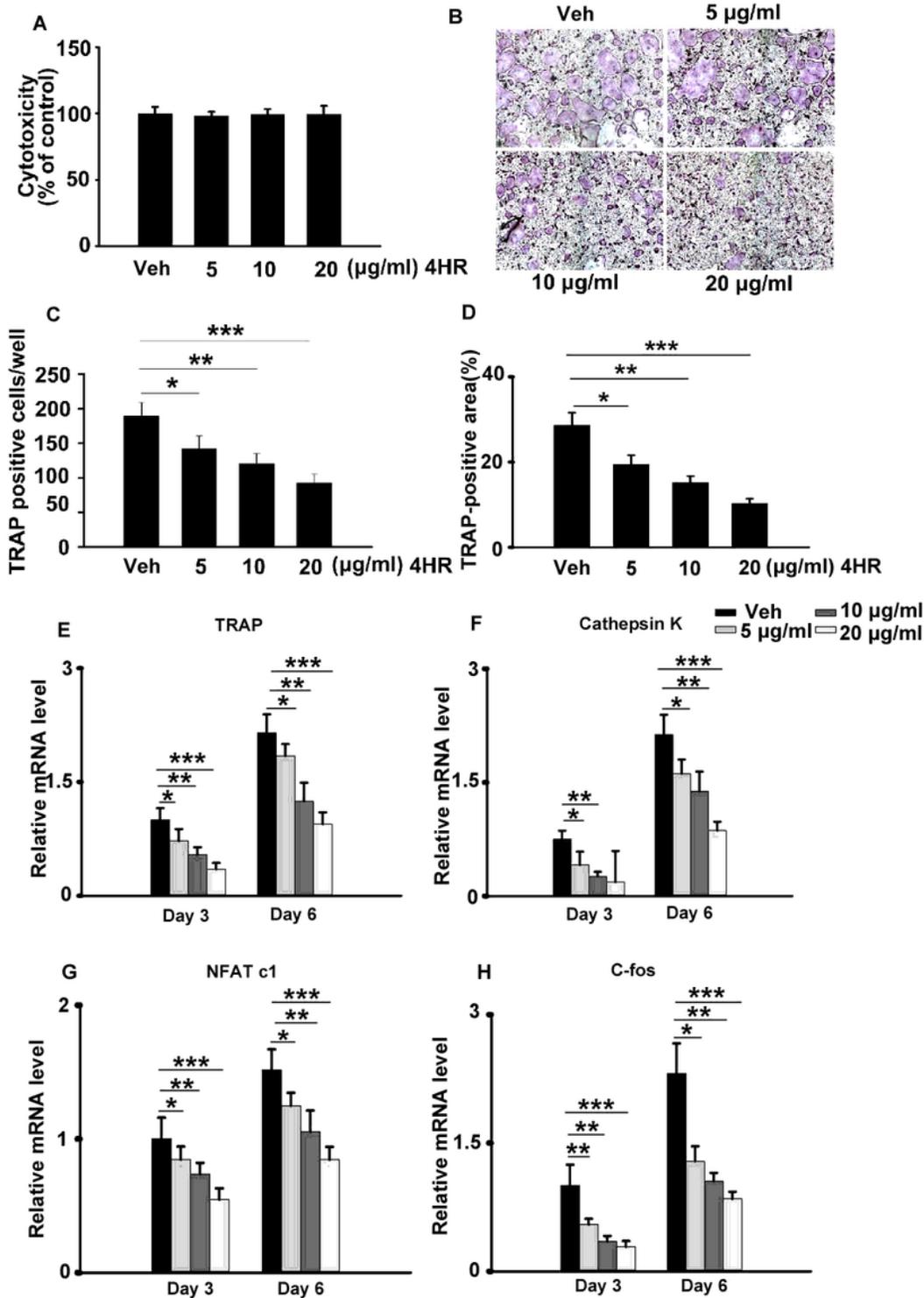


Figure 1

4HR inhibits RANKL-induced osteoclastogenesis in vitro. A, BMMs were treated with different concentrations of 4HR, (0, 5, 10, 20µg/ml) for 3 days in the presence of M-CSF. Cell proliferation was assessed using CCK-8 kit. No significant difference was observed among the groups. BMMs were seeded in 96-well plates and cultured with complete medium supplemented with 10 ng/ml M-CSF and 100 ng/ml RANKL. B, Representative TRAP staining images of 5 days of osteoclast differentiation with different

concentrations of 4HR (0, 5, 10, 20 μ g/ml) incubation, * p <0.05, ** p <0.01, *** p <0.001 versus BMMs with 0 μ g/ml 4HR treatment. C, TRAP-positive cells with three or more nuclei were quantified in each well * p <0.05, ** p <0.01, *** p <0.001 versus BMMs with 0 μ g/ml 4HR treatment. D, The percentage of the area of TRAP positive cells in each well was measured. BMMs were treated with different concentrations of 4HR (0, 5, 10, 20 μ g/ml) in the presence of 10 ng/ml M-CSF and 100 ng/ml RANKL, the mRNA level of osteoclastogenesis marker TRAP (E), Cathepsin K(F), NFAT c1(G) and c-fos(H) at day 3 of OC differentiation were shown in each group. * p <0.05, ** p <0.01, *** p <0.001 versus 0 μ g/ml 4HR treatment.

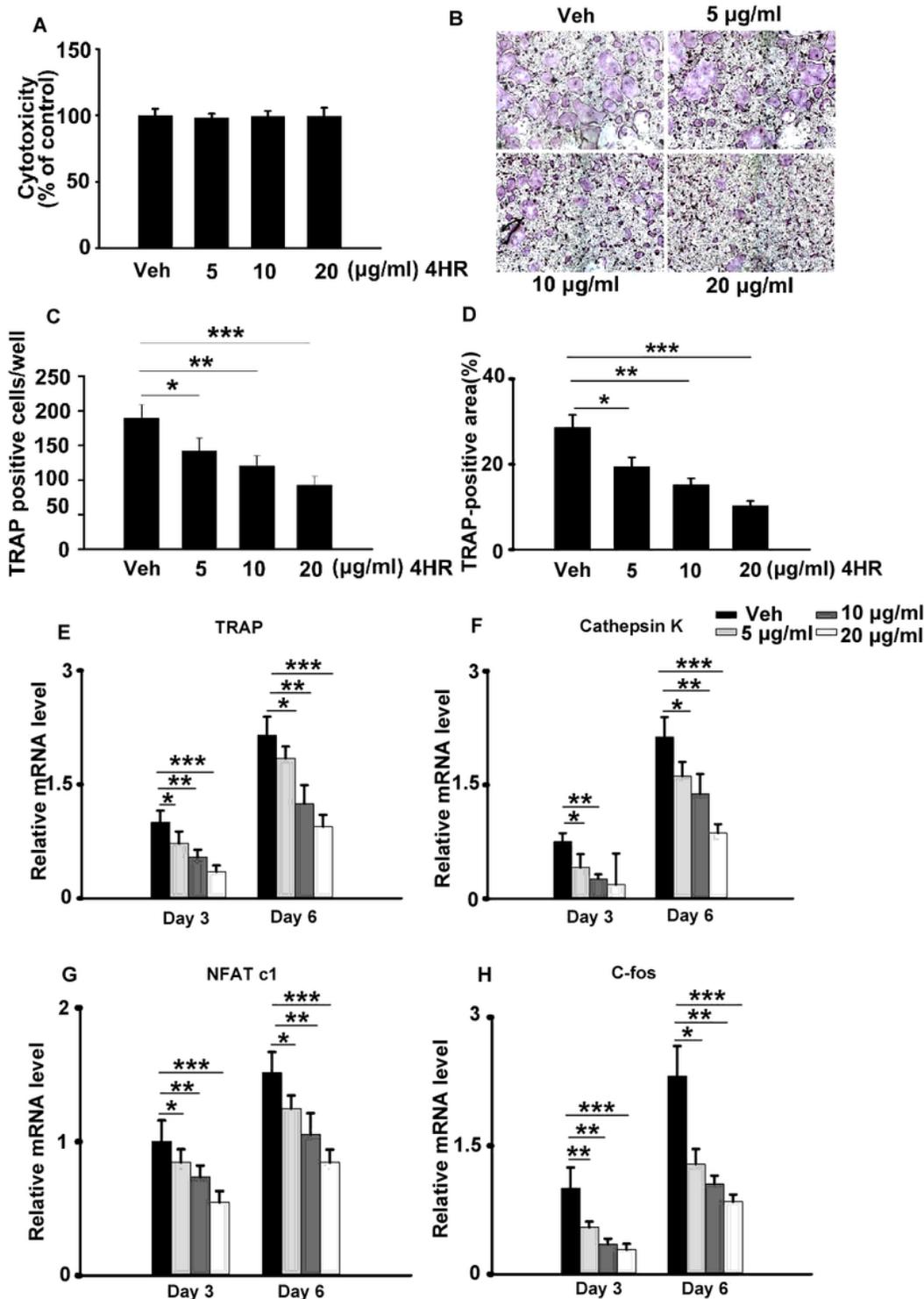


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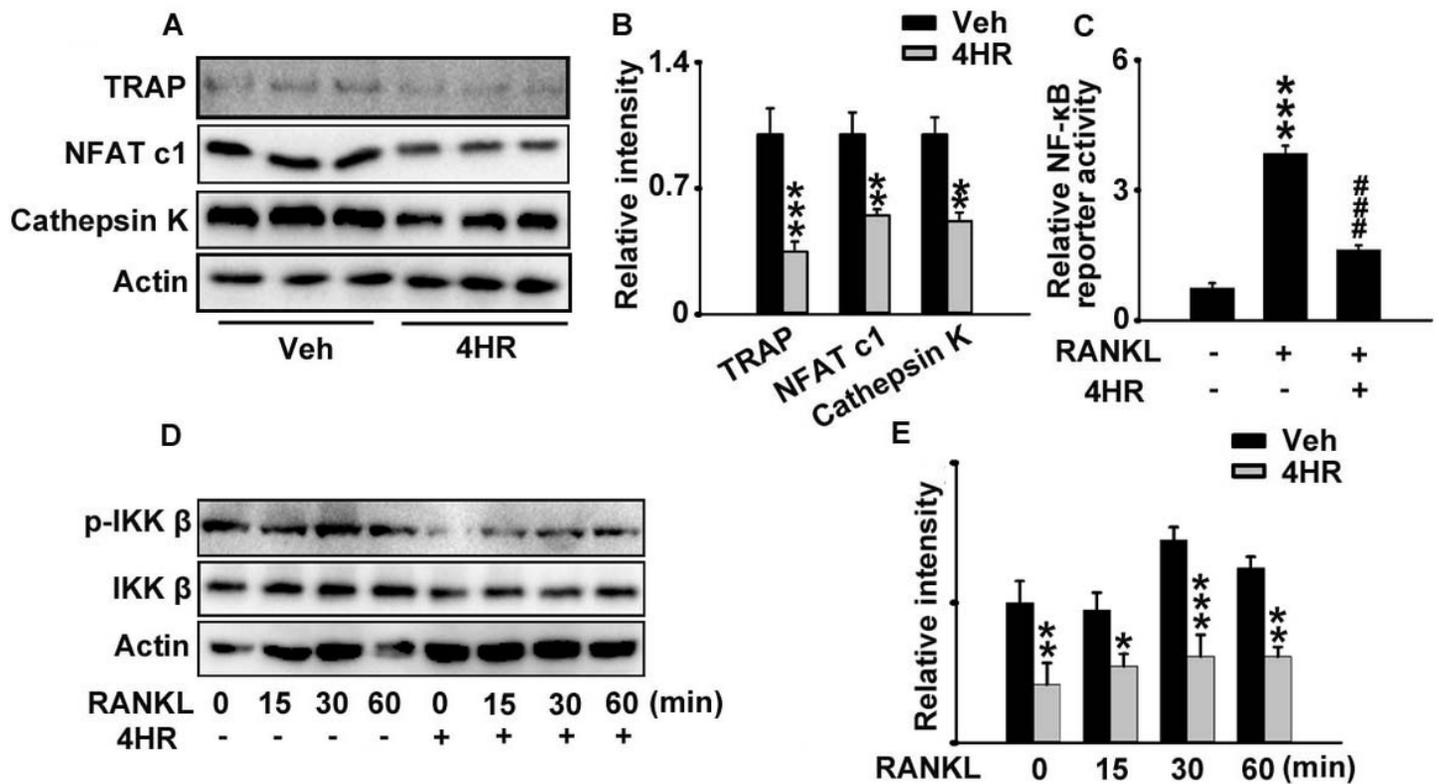


Figure 2

4HR suppresses RANKL-induced activation of NF- κ B pathway. A, Representative western blots images showed 4HR inhibited RANKL-induced protein level of TRAP, NFATc1 and Cathepsin K in BMMs after 20 μ g/ml 4HR treatment for 3 days in the presence of 10 ng/ml M-CSF and 100 ng/ml RANKL. B, Quantification of A. * p <0.05, ** p <0.01, *** p <0.001 versus BMMs without 4HR treatment (Vehicle, Veh). C, Luciferase assay of NF- κ B luciferase reporter in BMMs pre-treated with or without 4HR (20 μ g/ml) for 30 min in the presence of 10 ng/ml M-CSF and 100 ng/ml RANKL or not, *** p <0.001 versus BMMs without

RANKL or 4HR treatment. (Veh), ### $p < 0.001$ versus BMMs without 4HR treatment but with RANKL stimulation. D, Western blot analysis of lysate of RANKL incubation for indicated time points (0, 15, 30, 60 min) in BMMs with or without 4HR pre-incubation for 30 min (20 $\mu\text{g}/\text{ml}$). E, Quantification of D. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus BMMs without 4HR(Veh) treatment in different time points.

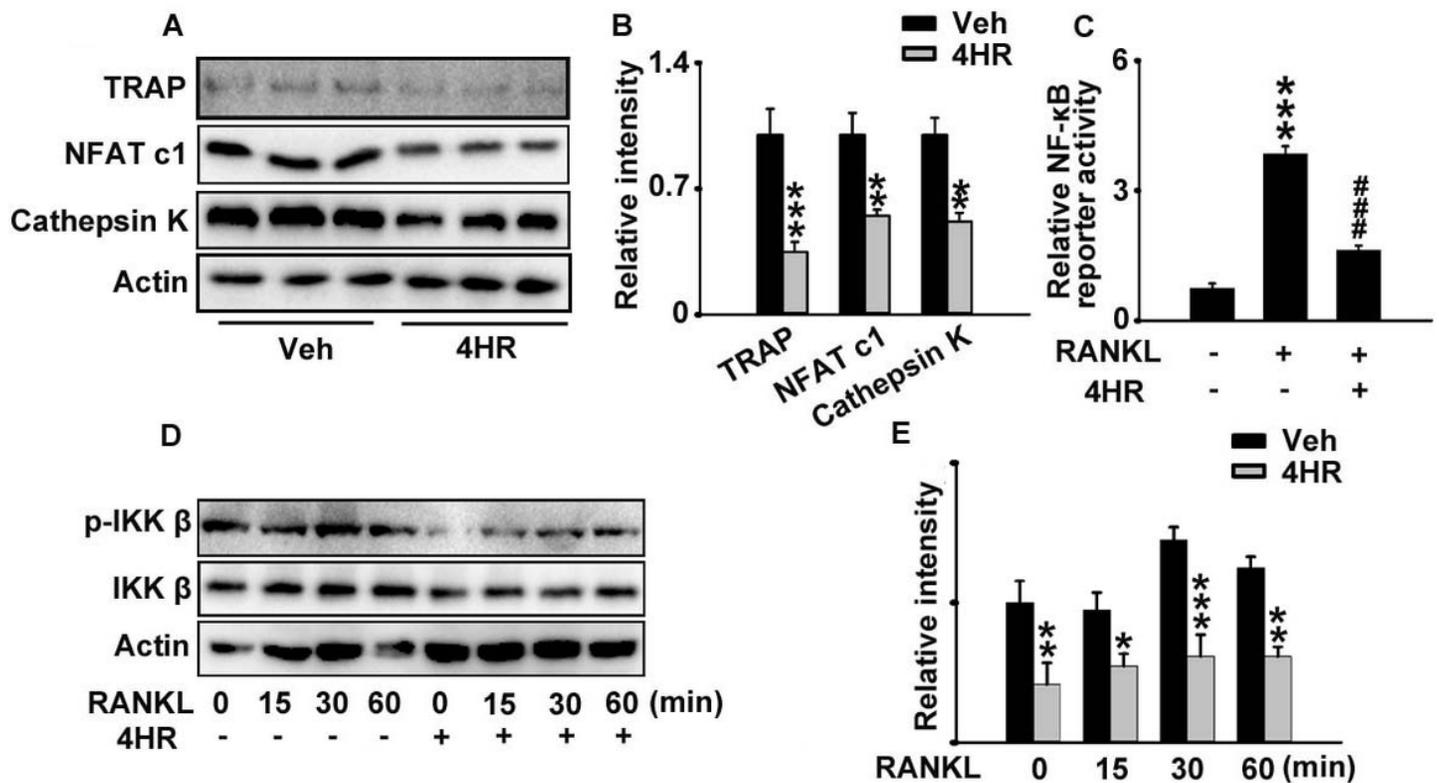


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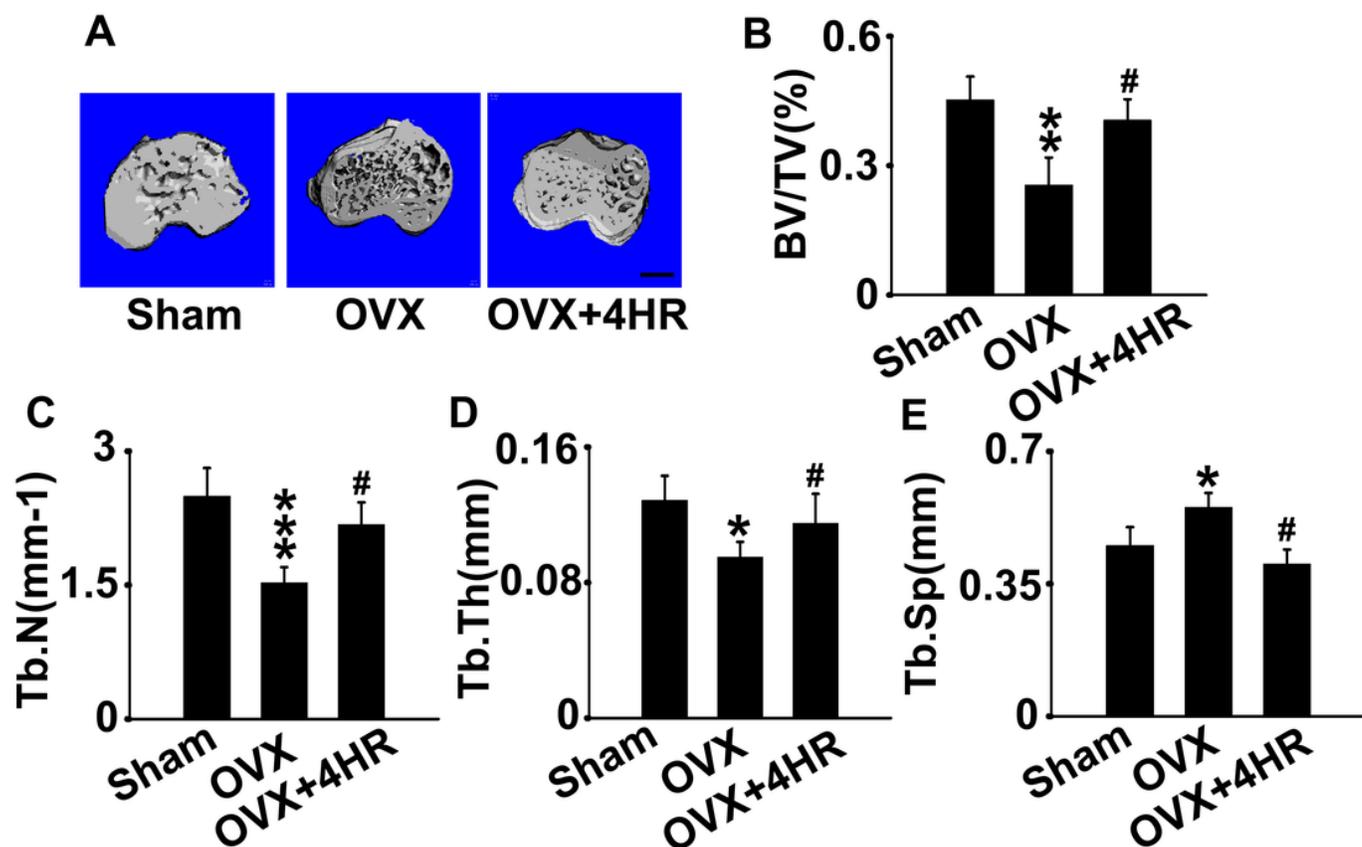


Figure 3

4HR treatment are resistant to ovariectomy-induced bone loss in vivo. A, MicroCT images of the proximal tibia at 60 days after ovariectomy surgery. Scale bar=1 mm. B, BV/TV ratio increased in the proximal tibia after 4HR administration for 60 days. C, Quantification of Tb.N in the proximal tibia showed significantly more Tb.N in mice treated with 4HR than OVX mice. D, Quantification of Tb.Th showed significantly more Tb.Th in 4HR treatment for 60 days after OVX surgery. E, Quantification of Tb.Sp in 4HR treatment for 60 days after OVX surgery. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus Sham-operated group. # $p < 0.05$ versus OVX group. N=6 each group.

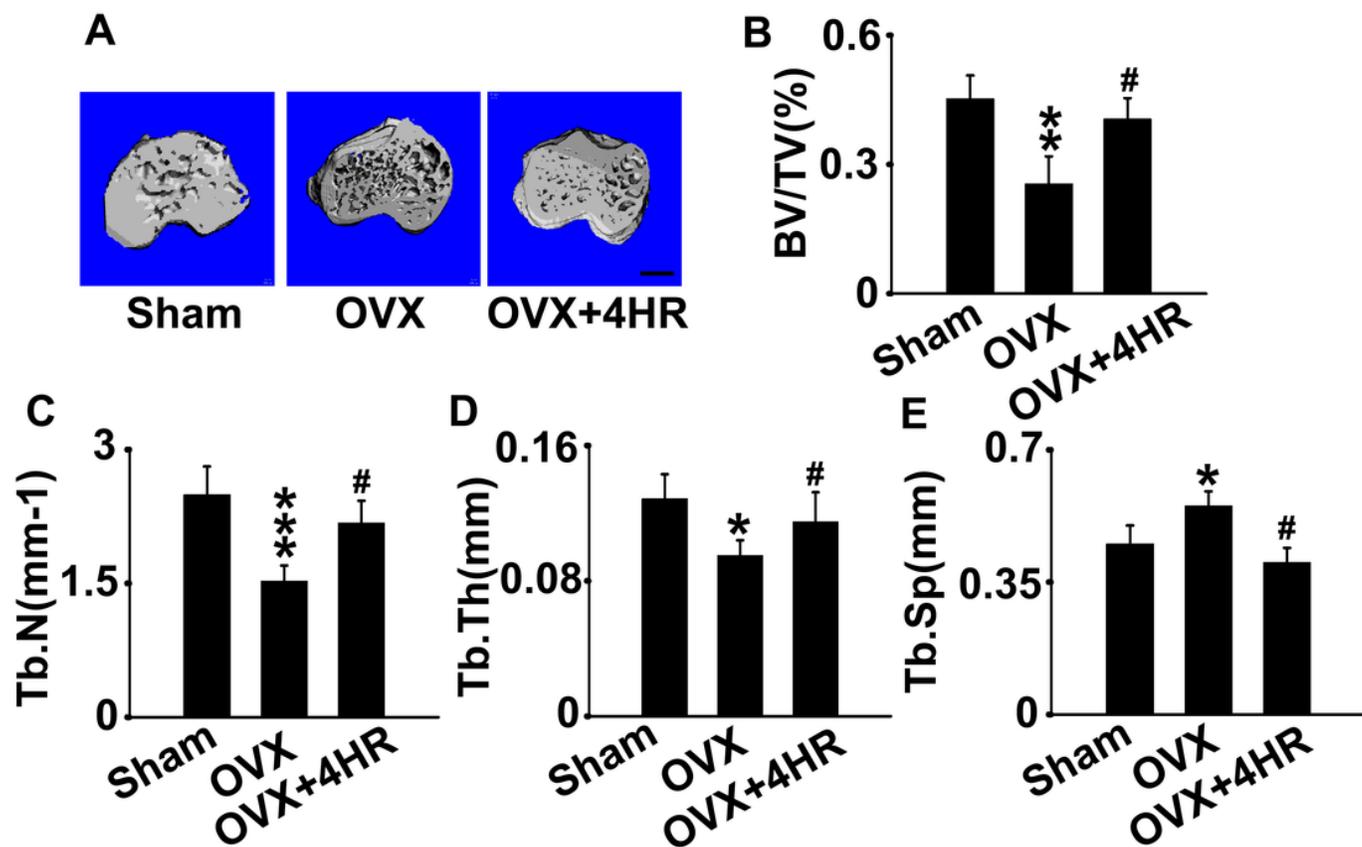


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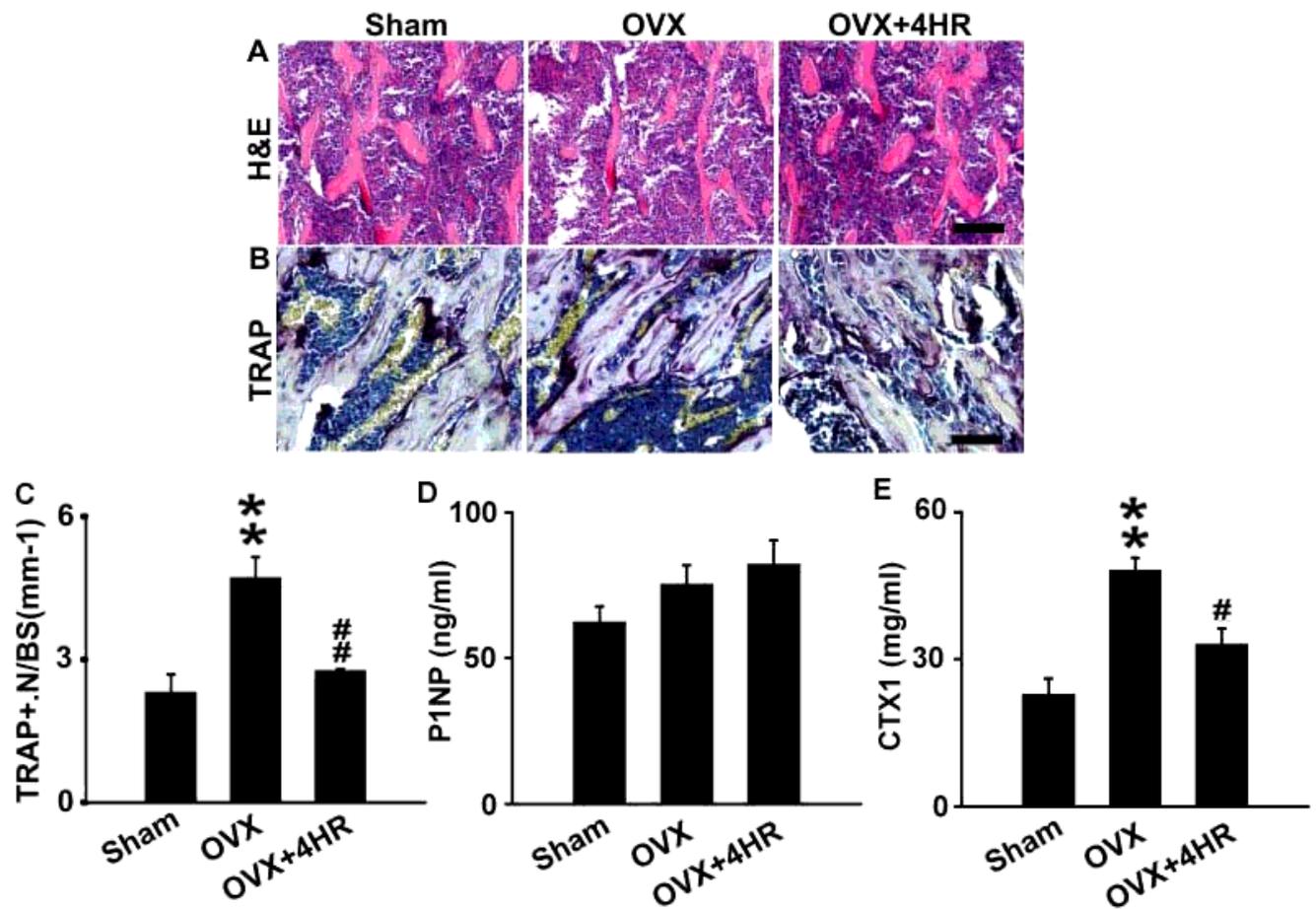


Figure 4

4HR administration increased bone mass and reduced OC number in vivo. (A) Representative images of H&E staining of proximal tibia after OVX surgery with or without 4HR administration in mice. Scale bar=25 μ m. (B) Representative images of TRAP staining in proximal tibia in each group 60 days after OVX surgery. Scale bar=25 μ m. (C) Quantification of TRAP positive cells in proximal tibia 60 days after OVX surgery. (D-E) The serum level of P1NP and CTX1 were measured. **p<0.01 versus Sham-operated group. #, p<0.05 ##p<0.01 versus OVX group. N=6 each group.

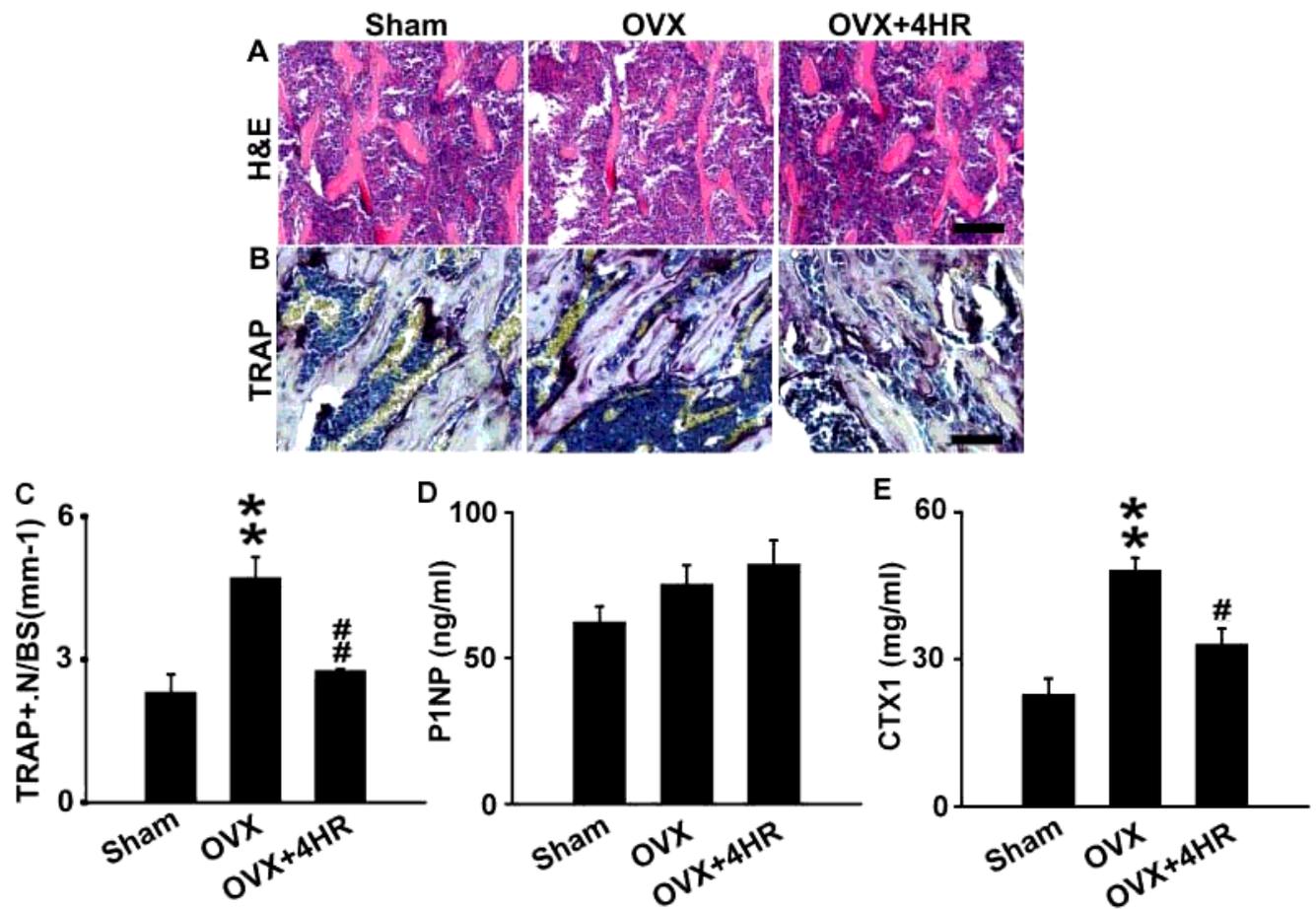


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