

# Resistant dextrin protects against pathological bone loss in ovariectomized rats and inhibits RANKL-induced osteoclastogenesis

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**Summary.** Background. Osteoporosis is a common disease in postmenopausal women characterized by systemic bone mass loss, microstructure fragility and increased incidence of fractures. Resistant dextrin (RD) is a soluble fiber with beneficial metabolic effects. However, the beneficial effect of RD in osteoporosis remains to be determined.

**Methods.** In this study, we investigated the effect of dietary RD supplement on osteoporosis in ovariectomized (OVX) rats. Both the control (sham) and OVX group rats were gavaged with RD (10 g/kg/d) or equal amount of saline for 12 weeks, and histological and biomechanical analyses were conducted to evaluate bone microstructure and strength. Furthermore, we also evaluated the effects of RD on osteoclastogenesis in bone marrow macrophages (BMMs) by detecting the expression of osteoclast-related genes using qRT-PCR and Western blot analysis.

**Results.** The results showed that in OVX rats the bone strength and microstructure characteristics were significantly improved with RD supplement for 12 weeks. Additionally, the mRNA and protein expression of osteoclast markers, such as CTSK, NF- $\kappa$ B and NFATC1, were significantly down-regulated in BMMs isolated from RD supplement group. RD also suppressed RANKL-induced osteoclastogenesis in BMMs.

**Conclusion.** These findings suggest that RD ameliorates osteoporosis in OVX rats by inhibiting osteoclast differentiation. RD suppresses RANKL-induced osteoclastogenesis possibly through modulating Akt and NF- $\kappa$ B signaling pathways. These data indicate that a dietary supplement of RD might serve as an intervention strategy for menopausal osteoporosis.

**Key words:** Resistant dextrin, Ovariectomized osteoporosis, Ovariectomized, Short-chain fatty acids, Osteoclastogenesis

## Introduction

Osteoporosis is the most common bone disease in postmenopausal women and is characterized by bone mass loss, abnormal bone micro-structure and an increased risk of fractures (Liu et al., 2018). Osteoporosis and the associated fractures pose major health threats to the quality of life of postmenopausal women worldwide (Compston et al., 2019). Postmenopausal osteoporosis (PMOP) is a bone homeostasis disorder caused by chronic estrogen deficiency, which leads to excessive bone resorption and impaired bone formation (Baczyk et al., 2012). According to statistical estimation, half of women aged over 50 are prone to osteoporosis-related fractures, and the risk of death from hip fracture is comparable to the death rate of breast cancer and four times higher than that of death from endometrial cancer (Sanders and Geraci, 2013). PMOP is the result of reduced ovarian function and a drop in estrogen level, therefore estrogen supplementation is widely used as a traditional treatment strategy. However, recent studies have shown that hormone therapies are not effective at preventing fractures and might increase the risk of some types of cancers, including breast and endometrial cancer (Eastell and Szulc, 2017). Therefore, the development of alternative therapeutic strategies with more effective outcomes and fewer side effects is crucial to ameliorate PMOP.

Resistant dextrin (RD) is a type of soluble prebiotic fiber extracted from wheat or corn starch, which is prepared by a highly controlled gelatinization process of partial hydrolysis and repolymerization (Pasma et al., 2006). RD has the features of better gastrointestinal

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tolerance compared to other dietary fibers, with beneficial effects in attenuating nonalcoholic fatty liver disease, improving insulin resistance, and reducing the risk of type 2 diabetes (Aliasgharzadeh et al., 2015; Hu et al., 2020). Emerging evidence suggests that a high-fiber diet is beneficial for bone health. For example, Lucas et al. suggest that a diet enriched with short-chain fatty acids (SCFAs) and a high-fiber diet can prevent postmenopausal and inflammation-induced bone loss (Lucas et al., 2018). A recent study reported that resistant starch can attenuate bone loss in ovariectomized mice by regulating the intestinal microbiota and bone-marrow inflammation (Tousen et al., 2019). However, although there are some reports regarding the beneficial effect of RD on osteoporosis, whether RD can ameliorate postmenopausal osteoporosis and the underlying mechanisms have not yet been elucidated. In this study, we evaluated the effects of dietary RD on the expression of bone metabolic markers, the features of bone biomechanics, and bone microstructures in ovariectomized (OVX) rats. Besides, the effect of RD on RANKL-induced osteoclastogenesis of bone marrow macrophages (BMMs) and the potential signaling pathways involved were explored.

## Materials and methods

### *Animal model and RD treatment*

Female Sprague-Dawley (SD) rats (Vital River Corporation, Beijing, China) at 16 weeks old were housed in a contained animal house under the conditions of a 12h light/dark cycle, 50% humidity and a temperature of 23°C. The rats were allowed to access food and water freely. After 2 weeks' feeding, rats with similar body weight were selected and randomly divided into four groups: the sham group (sham operation), the OVX group (with ovariectomization), the sham plus dietary RD (sham+RD) group and the OVX plus dietary RD (OVX+RD) group (n=10 per group). After surgery, rats in sham+RD and OVX+RD groups were fed with 10g/kg/day-RD (Honsear Co., Ltd., Guangzhou, China). 10g RD was dissolved in 10mL normal saline and the solution was gavaged in rats for 12 weeks. The sham and OVX groups were gavaged with the same amount of saline for 12 weeks. Twelve weeks after RD feeding, the rats were sacrificed and the samples of serum and bones were collected for subsequent analysis.

All animal experiments were conducted according to the guidelines for the use and care of laboratory animals, and were approved by the Animal Study Committee at Rizhao People's Hospital (Rizhao, China). The approval number of the above animal experiments is 11400700254579.

### *Serum biochemical analysis*

ELISA kits were used to determine the

concentrations of procollagen type 1 N-terminal propeptide (PINP), osteocalcin (OCN), tartrate-resistant acid phosphatase (TRAP), C-telopeptide of type 1 collagen (CTX), interleukin-1 $\beta$  (IL-1 $\beta$ ), and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in serum samples of each experimental group. The kits used for each measurement include: PINP determination kit (No. Csb-e12774r), CTX determination kit (No. Csb-e12776r, purchased from Cusabio, Wuhan, China), rat IL-1 $\beta$  ELISA Kit (Icosar Cat No. Er008-96) and rat TNF- $\alpha$  ELISA kit (Icosar Cat No. Er006-96). According to the manufacturer's instructions, 100  $\mu$ m serum samples were added into the antibody-coated plate wells, followed by incubation with HRP labeled avidin. After washing, color was developed by incubating samples with TMB for 30 min, and color development was stopped with TMB stop solution. The OD value (450 nm absorbance) was measured with a microplate reader. The concentrations of PINP, CTX, IL-1 $\beta$  and TNF- $\alpha$  were calculated based on the linear regression of the standards in each kit.

### *Bone histology analysis*

The left tibias from the rats were dissected and fixed in 4% paraformaldehyde (Sigma-Aldrich) for 48h. Decalcification was performed in 10% of ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich) for 4 weeks. Afterward, bone samples were dehydrated in an increasing gradient of ethanol and embedded in paraffin. A longitudinal section (5  $\mu$ m thick) was generated by microtome and was stained with hematoxylin and eosin (Sigma-Aldrich). Randomly selected visual fields of the bone sections were observed under light microscope (Axiovert 100M Zeiss, Zeppelinstrasse, Germany) at 100 $\times$ magnification. The number of trabecular bones in the images was determined by a licensed pathologist.

### *Bone biomechanical analysis*

The right tibias from the rats were isolated and packed with saline-soaked gauze to maintain humidity, and then stored at -20°C. The three-point bending test was carried out on the right femurs using an electro-force dynamic mechanical testing system (Bose ElectroForce 3230, USA) to assess bone strength. Prior to the measurement, the bones were placed in the instrument, and the head of the bone was positioned in the center of the two bearings to ensure that the axis of bending and the long axis of loading were in the same direction. A loading force was applied at 5-6N with a constant deformation rate of 2 mm/min until a fracture occurred, and the yield and fracture parameters were determined. After data recording, a load-deformation curve was generated and bone biomechanical parameters, including the maximum load, maximum fracture load, stiffness, energy absorption, elastic modulus, and maximum strength, were calculated.

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### Micro-CT analysis

Micro-CT was performed to analyze the bone mass and trabecular bone microarchitecture using a Skyscan 1176  $\mu$ CT scanner (Bruker, Belgium), according to the previous report (Minematsu et al., 2017). The left femurs from the mice were isolated, fixed in 4% paraformaldehyde, and maintained in 75% of ethanol. The femurs were then scanned with the following parameters: a scanning accuracy of 17.93  $\mu$ m, a scanning rotation angle of 180°, a 1 mm A1 filter, a scanning voltage of 70kV, a current of 278  $\mu$ A, and an exposure time of 450 ms; the scans were then processed into 2D images. All 3D image manipulations and analyses were performed using MicroView, v.2.1 software (GE Healthcare, USA). The following trabecular parameters were analyzed: trabecular volume bone mineral density (Tb.vBMD), trabecular bone volume/total volume (Tb.BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and structure model index (SMI).

### Serum SCFAs measurements

In this study, blood samples were collected from rats for SCFA (short chain fatty acid) analysis using chromatography-mass spectrometry (GC-MS), as described by a previous study (Zhang et al., 2019). The SCFAs including acetate, propionate, and butyrate were used as standards. Briefly, the following protocols were used: each serum sample (200  $\mu$ L) was mixed with 2 mL of ultrapure water and then centrifuged at 15,000 rpm for 15 min at 4°C. Then, 200  $\mu$ L of the supernatant was poured into a new 0.5 mL microtube that was pre-filled with 20  $\mu$ L of HCl to bring the pH of the solution to 2. Next, the supernatant (100  $\mu$ L) was collected, and 20  $\mu$ L of internal standard was added for GC-MS analyses (Thermo Fisher Scientific, Waltham, MA, USA). The chromatographic separation of SCFAs was performed using a free fatty acid phase (DB-FFAP, 30 m  $\times$  0.53 mm  $\times$  0.25  $\mu$ m) as follows: 50°C for 1 min, temperature increase at a rate of 20°C/min until 180°C, holding for 1 min, temperature increase at a rate of 20°C/min until 200°C, and final holding for 2 min. Helium (1 mL/min) was used as the carrier gas. The concentrations of SCFAs were quantified using the internal standard curve method.

### Bone marrow macrophages (BMMs) isolation, osteoclast induction and TRAP staining

BMMs were isolated from SD rats (8 weeks old, Vital River Corporation, Beijing, China) as described previously (Li et al. 2019). BMMs were seeded in 96-well plates and cultured with  $\alpha$ -MEM medium supplemented with 10% fetal bovine serum (FBS, Invitrogen, USA), 1% antibiotic penicillin-streptomycin and 20 ng/mL macrophage colony-stimulating factor (M-CSF; Sino Biological, Shanghai, China) and 50

ng/mL receptor activator of nuclear factor  $\kappa$ B (NF- $\kappa$ B) ligand (RANKL; R&D Systems). BMMs were treated with 100 ng/ml RD at the beginning of osteoclast differentiation induction. Osteoclast differentiation was evaluated by TRAP staining in fixed cells using a leukocyte acid phosphatase kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol. The number of osteoclasts, defined as TRAP positive cells with three or more nuclei, was counted under a light microscope.

### Cell proliferation assay

The effect of RD on cell proliferation was evaluated by a cell counting kit-8 (CCK-8, Dojindo, DJ657, Japan) according to the manufacturer's instructions. The BMMs were treated with different concentrations of RD (0, 100, and 200 ng/mL) for 12h, 24h, 48h, and 72h respectively. 10  $\mu$ L CCK8 reaction solution (Solarbio, CA1210, Beijing, China) was added to the cell culture at the indicated time point and incubated for 1 hour in a humidified cell culture incubator. Absorbance was measured at 450 nm on a microplate reader (spectra max plus 384, Jinan Dongdai Scientific Equipmet Co., Ltd., Jinan, China), and the absorbance values were compared with those of untreated controls.

### RNA extraction and real-time quantitative PCR (RT-PCR)

The total RNA was extracted from the right tibia samples and cultured BMMs using TRIzol (TakaRa, Dalian, China). The extracted total RNA was dissolved in DEPC water and its concentration was measured with NanoDorp. 5  $\mu$ g of total RNA was used for reverse-transcription into cDNA using the Prime Script®RT reagent kit (TakaRa, Dalian, China) according to the manufacturer's directions. Briefly, total RNA was prepared in 10 $\mu$ L reverse transcription reaction, followed

**Table 1.** Primer sequences used for the determination of gene expression.

Gene (Rats)	Primer sequence (5'-3')
Runx2	Forward CCTGAACTCAGCACCAAGTCCT
	Reverse TCAGAGGTGGCAGTGTCACTCA
Osx	Forward CTGGGAAAAGGAGGCACAAAGA
	Reverse GGGGAAAAGGGTGGGTAGTCATT
TRAP	Forward CACTCCCACCCCTGAGATTTGT
	Reverse CATCGTCTGCACGGTTCTG
CTSK	Forward GAAGAAGACTACCAGAAAGCAG
	Reverse TCCAGGTTATGGGCAGAGATT
NF $\kappa$ B	Forward AGGCTTCTGGGCCTTATGTG
	Reverse TGCTTCTCTGCCAGGAATAC
NFATC1	Forward TGGGAGATGGAAGCAAAGACTGA
	Reverse CATTGGCAGGAAGGTACGTGAA
$\beta$ -actin	Reverse TGCTATGTTGCCCTAGACTTCG
	Forward GTTGGCATAGAGGTCTTTACGG

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by incubation at 37°C for 30 min and 85°C for 5 min. Real-time PCR was performed on a LightCycler480 (Roche Diagnostics, Mannheim, Germany), using SYBR premix EX TAQ II kit (Takara, Dalian, China). PCR reaction was prepared according to the manufacturer's instructions, and the primers are listed in Table 1. The PCR conditions were as follows: one cycle at 93°C for 2 min (initial denaturation), followed by 40 cycles at 93°C for 1 min, 55°C for 1 min, 72°C for 1 min, with a final extension for 5 min at 72°C. Data analysis was performed using the  $2^{-\Delta\Delta CT}$  method, and  $\beta$ -actin was used as internal reference for normalization.

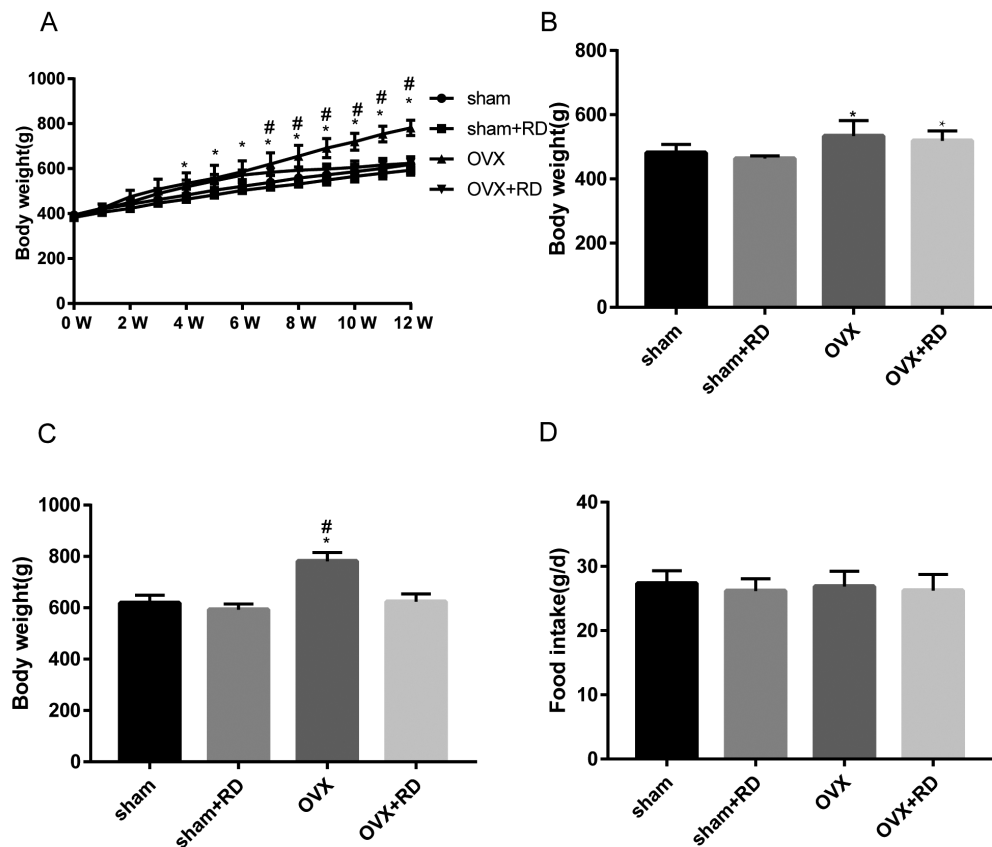
## Western blot analysis

The lumbar vertebrae were removed from the liquid nitrogen, quickly broken with a hammer, and ground in a mortar with liquid nitrogen. The total protein of the lumbar vertebrae and cultured BMMs were extracted using RIPA buffer according to the manufacturer's instructions (Shenergy Biocolor Bioscience & Technology CO., Shanghai, China). Cells suspended in ice-cold RIPA buffer were lysed on ice for 10 min and lysed cells were centrifuged at 14000 rpm for 10 min. The supernatant containing total protein lysate was quantified by a BCA Protein assay kit (Beyotime

Biotechnology, Shanghai, China). Proteins were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in 10% of polyacrylamide gels, and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk for 1 hour, the membrane was then incubated with primary antibodies overnight at 4°C. Then, the membrane was washed 3 times with TBST buffer and incubated with secondary antibodies for 1 hour at room temperature. The protein bands were visualized using an enhanced chemiluminescence kit (Santa Cruz, USA) and detected by Flurochem Q chemiluminescence imaging system (Alpha Innotech, USA). GAPDH was used as the loading control.

## Statistical analysis

The results were expressed as means  $\pm$  standard deviation (SD). The data were analyzed using SPSS 22.0 software, with the two-tailed unpaired Student's *t* test for the comparison between two groups and the one-way analysis of variance (ANOVA) test for multiple group comparison. Comparisons of data at multiple time points were examined using two-way ANOVA. A value of  $P < 0.05$  was considered significant.



**Fig. 1.** Resistant dextrin reduces the body weights of OVX rats but it has no effect on food intake. **A.** The body weights of rats in the four groups were monitored weekly after OVX surgery. The body weights of rats in the four groups at 4 (**B**) and 12 weeks (**C**) after surgical operation are shown. **D.** The weekly food intake of rats in the four experimental groups after surgery. The data are expressed as means  $\pm$  SD ( $n=10$ ). \* $P < 0.05$  compared with the sham group, # $P < 0.05$  compared with the OVX+RD group.



## Resistant dextrin prevents pathological bone loss

## Results

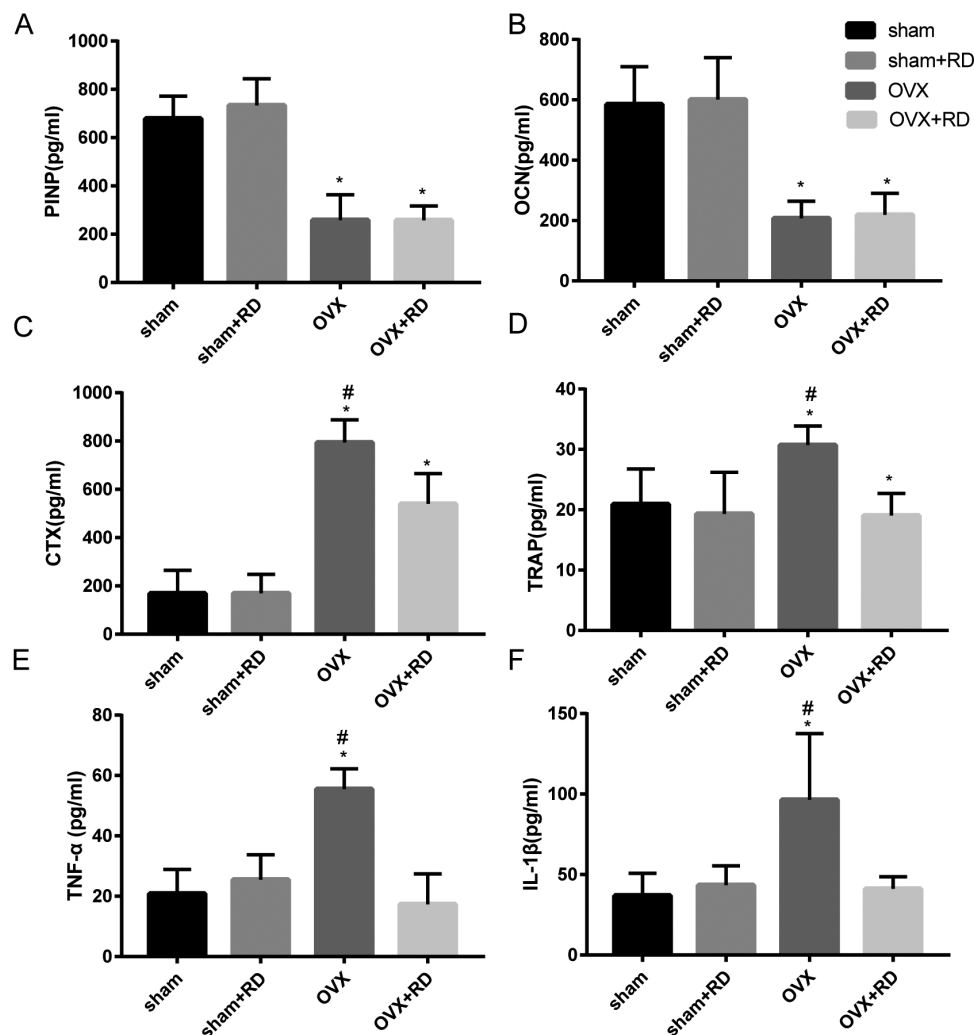
*Dietary RD reduces the weight of OVX rats but does not affect food intake*

In this study, the weights of the four groups of rats were recorded weekly after the sham operation or OVX procedures. The data showed that there was no significant difference in the weights of rats between the sham and sham+RD groups (Fig. 1A). Compared with the sham group, there was more weight gain in the OVX group from 4 weeks after surgery, the body weights of the OVX group were statistically higher than those of rats in the sham group. However, the dietary RD attenuated the body weight gain in the OVX+RD group (Fig. 1A,B). At 12 weeks after surgery, the body weights of rats in the OVX+RD group were significantly lower than those in the OVX group (Fig. 1C), while there was no significant difference between the sham+RD group

and sham group (Fig. 1A-C). We also measured the food intake in each group, which demonstrated that there was no detectable difference in weekly food intake among all groups (Fig. 1D). These data suggest that dietary RD reduces the weight gain in OVX rats without affecting food intake.

*RD decreases the expression of bone resorption markers and inflammatory biomarkers in OVX rats, but shows no effect on bone formation*

The bone formation markers, including P1NP (Procollagen Type I N Terminal Propeptide) and OCN (Osteocalcin), were measured in the serum samples of each group. OVX operation significantly reduced the levels of both P1NP and OCN when compared to the sham groups, but there was no detectable difference between OVX and the OVX+RD group (Fig. 2A,B). In contrast, the level of markers related to bone resorption



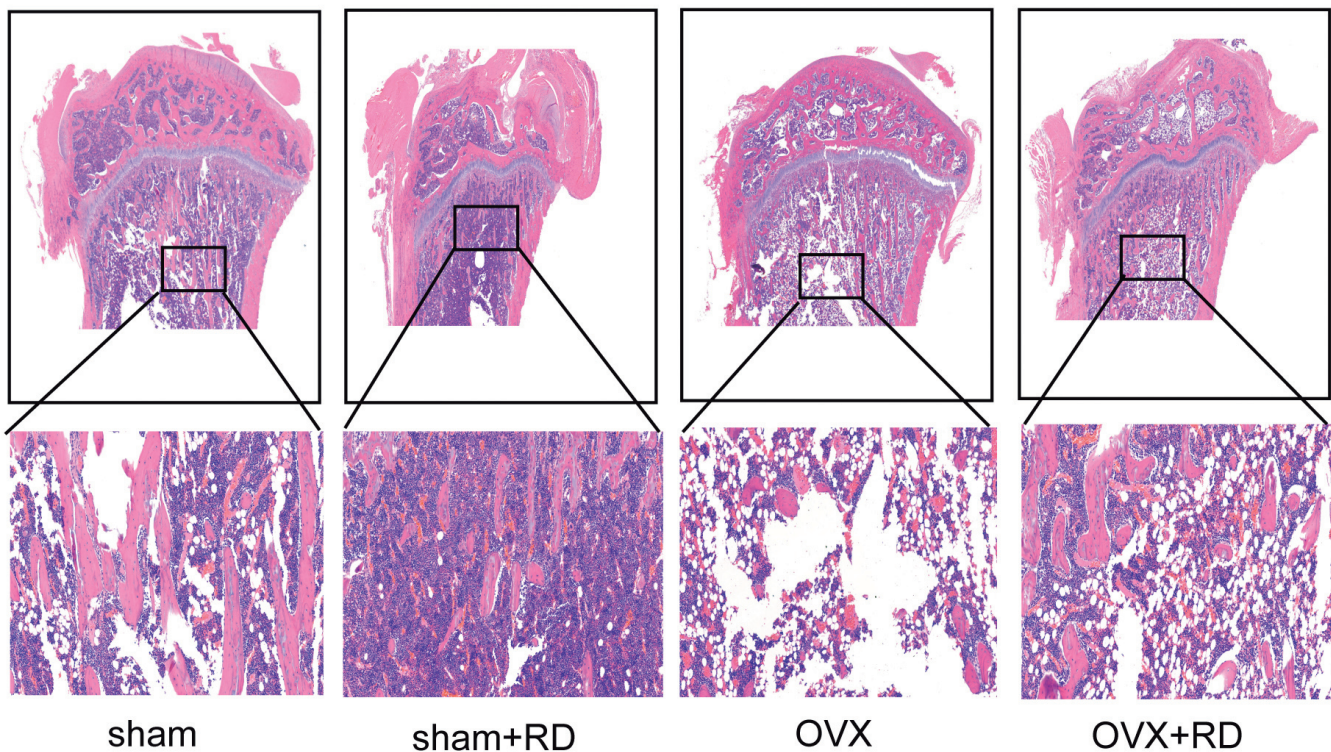
**Fig. 2.** Resistant dextrin decreases osteoclastic markers and inflammatory biomarkers but has no effect on osteoblastic markers in the OVX rats. Serum concentrations of P1NP (A), OCN (B), CTX (C), TRAP (D), TNF- $\alpha$  (E), and IL-1 $\beta$  (F) were measured by ELISA. The data are expressed as means  $\pm$  SD (n=10). \*P<0.05 compared with the sham group, #P<0.05 compared with the OVX+RD group.

## Resistant dextrin prevents pathological bone loss

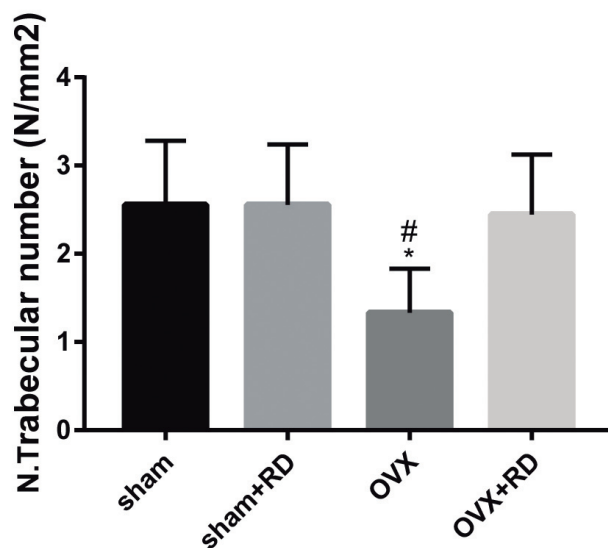
and inflammation, TRAP (tartrate-resistant acid phosphatase), CTX (C-telopeptide of type 1 collagen), TNF- $\alpha$  (tumor necrosis factor  $\alpha$ ) and IL-1 $\beta$  (Interleukin 1 $\beta$ ), were significantly higher in OVX groups than those in the serum of sham and sham+RD groups (Fig. 2C-F). Although there were no differences between the sham

and sham+RD groups, dietary RD significantly reduced the serum levels of bone resorption and inflammatory markers in the OVX+RD group when compared to the OVX group (Fig. 2C-F). These data suggest that dietary RD has beneficial effects in protecting against OVX-induced bone resorption and inflammation.

A



B



**Fig. 3.** Resistant dextrin improves the structure and number of trabecular bones in OVX rats. **A.** Representative images of trabecular bones in each experiment group are shown. **B.** The trabecular number (N. trabecular) per square millimeter was evaluated (n=5). \*P<0.05 compared with the sham group, #P<0.05 compared with the OVX+RD group. Scale bars: 500  $\mu$ m; 100  $\mu$ m

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## RD ameliorates the structure and number of trabecular bones in OVX rats

Bone histology analysis in the left tibia samples showed that the bone microstructure was damaged and the number of trabecular bones was significantly decreased in the OVX groups compared with the sham groups, and there was observable improvement in the microstructure and a significant increase in the number of trabecular bones in the OVX+RD group when compared with the OVX group (Fig. 3A,B). However, there were no significant differences between the sham and sham+RD groups (Fig. 3A,B). These data indicate that dietary RD protects against OVX-induced bone resorption.

## RD improves bone biomechanical properties in OVX rats

The femurs were subjected to the three-point bending test to examine the effect of RD on the biomechanical properties of the bones (Fig. 4). The biomechanical parameters including maximum load, maximum fracture load, stiffness, energy absorption, elastic modulus, and maximum strength were significantly impaired in OVX group when compared to the sham group (Fig. 4A-F). Consistent with the histological analysis, the deterioration of these bone biomechanics in OVX rats was significantly ameliorated in the OVX+RD rats, suggesting that dietary RD improves the bone mechanical properties after OVX operation (Fig. 4A-F).

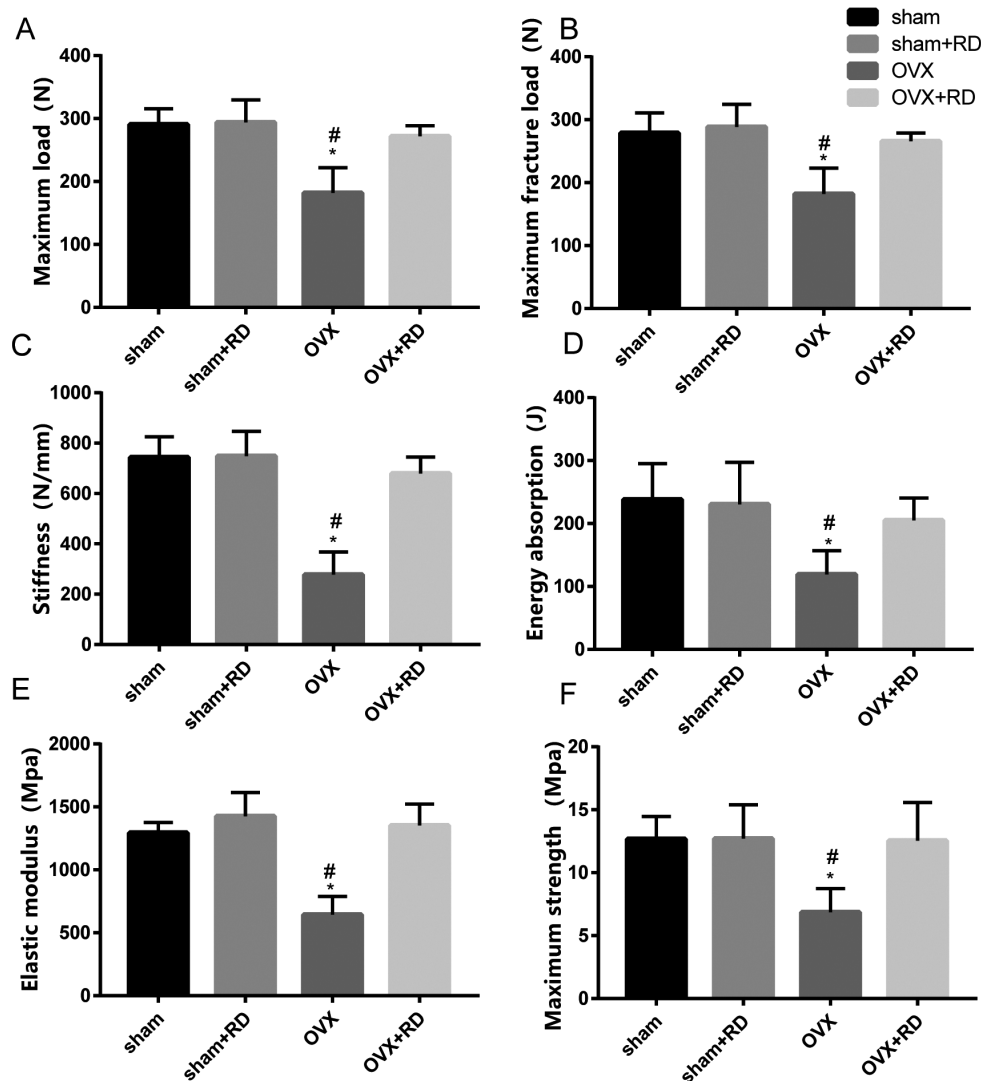
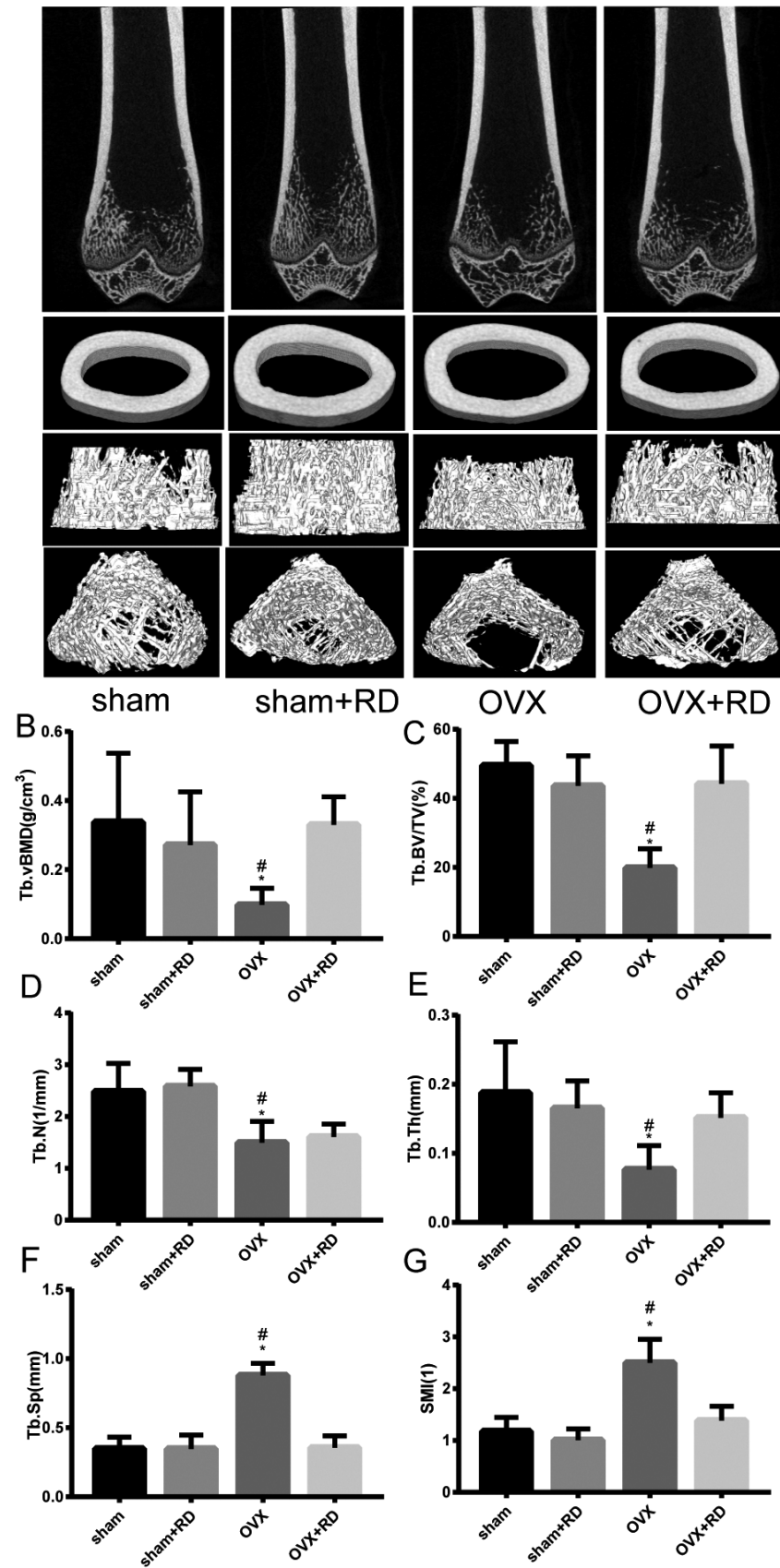


Fig. 4. Resistant dextrin improves the biomechanical properties in OVX rats. **A.** Maximum load. **B.** Maximum fracture load. **C.** Stiffness. **D.** Energy absorption. **E.** Elastic modulus. **F.** Maximum strength were measured in femur sample of each experimental group. The data are expressed as means  $\pm$  SD (n=10). \* $P < 0.05$  compared with the sham group, # $P < 0.05$  compared with the OVX+RD group.

A



**Fig. 5.** Resistant dextrin ameliorates the damage of trabecular bones after OVX. **A.** 2D-micro-CT and 3D-micro-CT images of femurs in different groups. **B.** Tb.vBMD, trabecular volume bone mineral density. **C.** Tb.BV/TV, trabecular bone volume/total volume. **D.** Tb.N, trabecular number. **E.** Tb.Th, trabecular thickness. **F.** Tb.Sp, trabecular separation. **G.** SMI, structure model index were measured in different groups. The data are expressed as means  $\pm$  SD (n=10). \*P<0.05 compared with the sham group, #P<0.05 compared with the OVX+RD group.



## Resistant dextrin prevents pathological bone loss

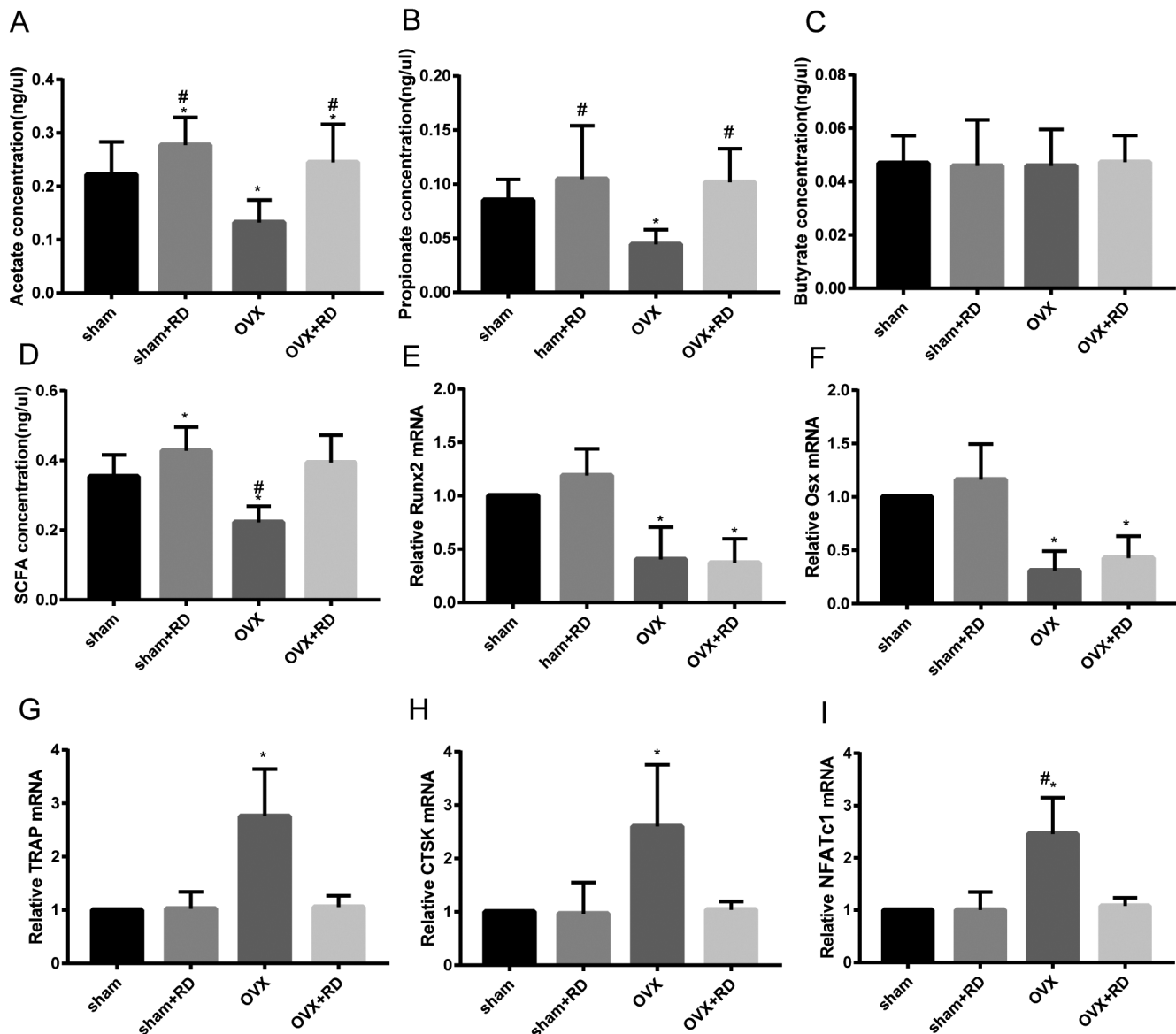
*RD improves trabecular bone damage regarding OVX-induced bone loss*

Micro-CT analysis revealed that dietary RD protected the rats against the OVX-induced loss of trabecular and cortical bones, which was evidenced by the 2D-micro-CT and 3D-micro-CT images (Fig. 5A). The quantitative analysis of trabecular bones are shown in Figures 5B-E, which summarize the common morphometric indexes derived from the 2D and 3D images. In the trabecular bone, compared with the sham group, the OVX group was characterized by a significant

decrease of Tb.vBMD, Tb.BV/TV, Tb.N, and Tb.Th. Reductions in these indexes were largely rescued in OVX+RD group. Conversely, OVX caused an increase in the Tb.Sp and SMI parameters, which were largely reduced with dietary RD treatment (Fig. 5F-G). These results suggest that dietary RD alleviates osteoporosis phenotypes in trabecular bone.

*RD promotes the production of serum SCFAs in OVX rats and down-regulates osteoclast-related genes*

Next, we measured the serum level of SCFAs, and



**Fig. 6.** Resistant dextrin promotes serum SCFAs production and down-regulates the expression of osteoclast-related genes in OVX rats. Serum concentrations of acetate (A), propionate (B), and butyrate (C), and the total SCFA (D) were measured by GC-MS, and the relative mRNA expression of Runx2 (E), Osx (F), TRAP (G), CTSK (H), and NFATc1 (I) were measured by RT-qPCR. The data are expressed as means  $\pm$  SD (n=10). \* P<0.05 compared with the sham group, #P<0.05 compared with the OVX+RD group.

## Resistant dextrin prevents pathological bone loss

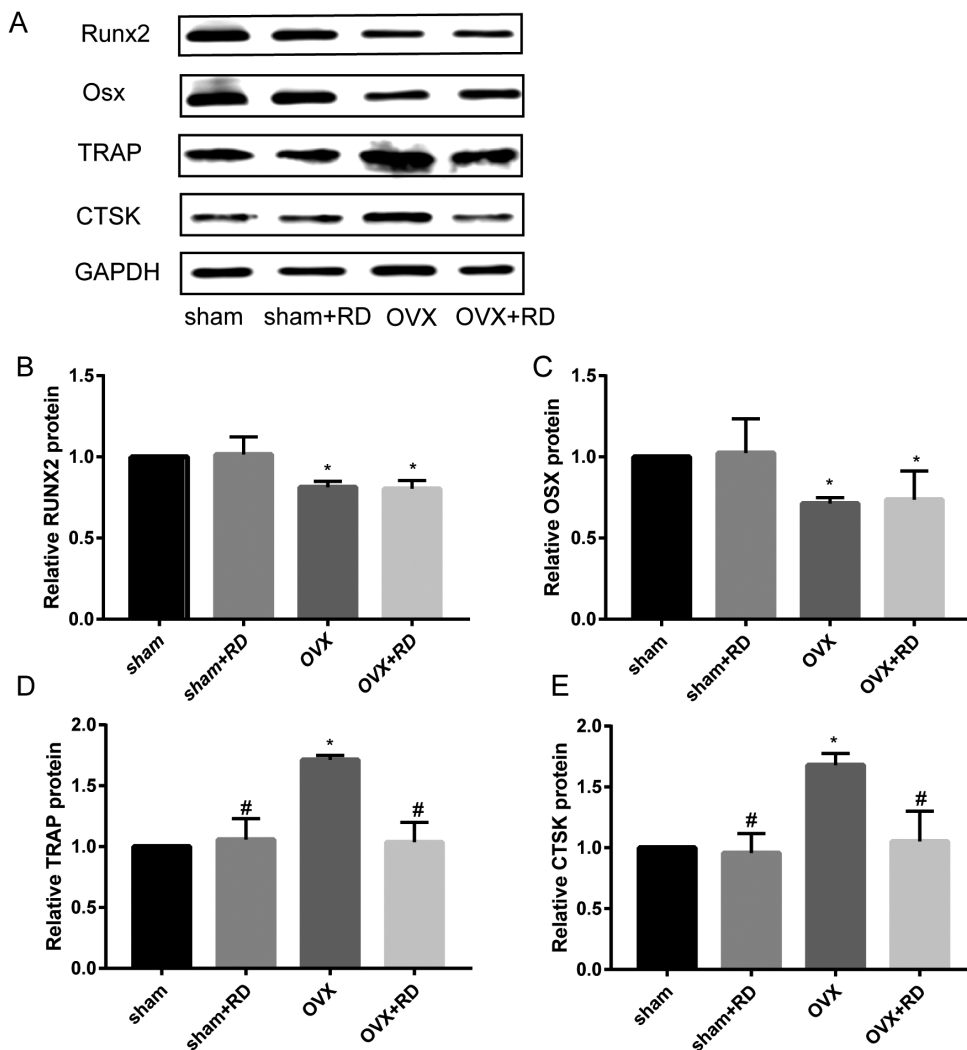
the data showed that there was a significant decrease of acetate, propionate and the total SCFAs in the OVX group when compared with the sham group, while RD promoted the production of serum SCFAs in OVX rats (Fig. 6A-D). It is noticeable that serum levels of SCFA production in the sham+RD group were also enhanced compared to the sham group, whereas there was no significant difference in the butyrate levels among the four groups (Fig. 6A-D).

Furthermore, the expression of osteoblast and osteoclast genes was measured in the right tibia samples. The mRNA levels of osteoblast-related genes, including Runt-related transcription factor 2 (Runx2) and Osterix (Osx), decreased significantly in the OVX group when compared with the sham group, and dietary RD did not affect their expression (Fig. 6E,F). On the contrary, osteoclast-related genes, including TRAP, cathepsin K (CTSK), and cytoplasmic 1 (NFATC1), were up-

regulated in the OVX group, while RD attenuated their up-regulation in the OVX+RD group (Fig. 6G-I). We further examined the protein expression levels of Runx2, Osx, TRAP, and CTSK in right tibia samples, and the results were consistent with the observation in the mRNA quantification (Fig. 7A-E). Together, these findings suggest that dietary RD diminishes bone loss and improves bone quality by inhibiting osteoclast generation.

*RD inhibits RANKL-induced osteoclastogenesis and suppresses osteoclast-related gene expression in BMMs*

To further validate whether RD interferes with osteoclast differentiation, we adopted a RANKL-induced osteoclastogenesis model in bone marrow macrophages (BMMs). First, the cell proliferation of RD-treated BMMs was measured using a CCK-8 kit, which showed



**Fig. 7.** Resistant dextrin down-regulates the protein levels of osteoclast-related genes in OVX rats. **A.** Western blot analyses of Runx2, Osx, TRAP, CTSK, and GAPDH proteins in the four groups. **B-E.** Western blot analyses of Runx2, Osx, TRAP, and CTSK proteins. The relative protein levels were quantified by densitometry and normalized to GAPDH. The data are expressed as means  $\pm$  SD (n=10). \*P<0.05 compared with the sham group, #P<0.05 compared with the OVX+RD group.

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that RD treatment did not affect cell proliferation in BMMs (Fig. 8A). BMMs were then stimulated with RANKL and MCSF in the presence or absence of RD for 7 days. TRAP staining showed that differentiated BMMs with the treatment of 100n g/mL RD showed significantly reduced size, and the number of TRAP-positive cells was also reduced (Fig. 8B-C). We also measured the mRNA and the protein levels of osteoclast-related genes (TRAP, CTSK) and two crucial transcription factors (NF- $\kappa$ B, NFATC1) that regulate the osteoclast differentiation. RANKL induction promoted both the mRNA and protein levels of TRAP, CTSK, NF- $\kappa$ B and NFATC1 genes (Fig. 9A-H). However, the presence of RD largely abrogated the induction effects of RANKL on the above genes (Fig. 9A-H). These results suggest that RD negatively regulates RANKL-induced osteoclast differentiation.

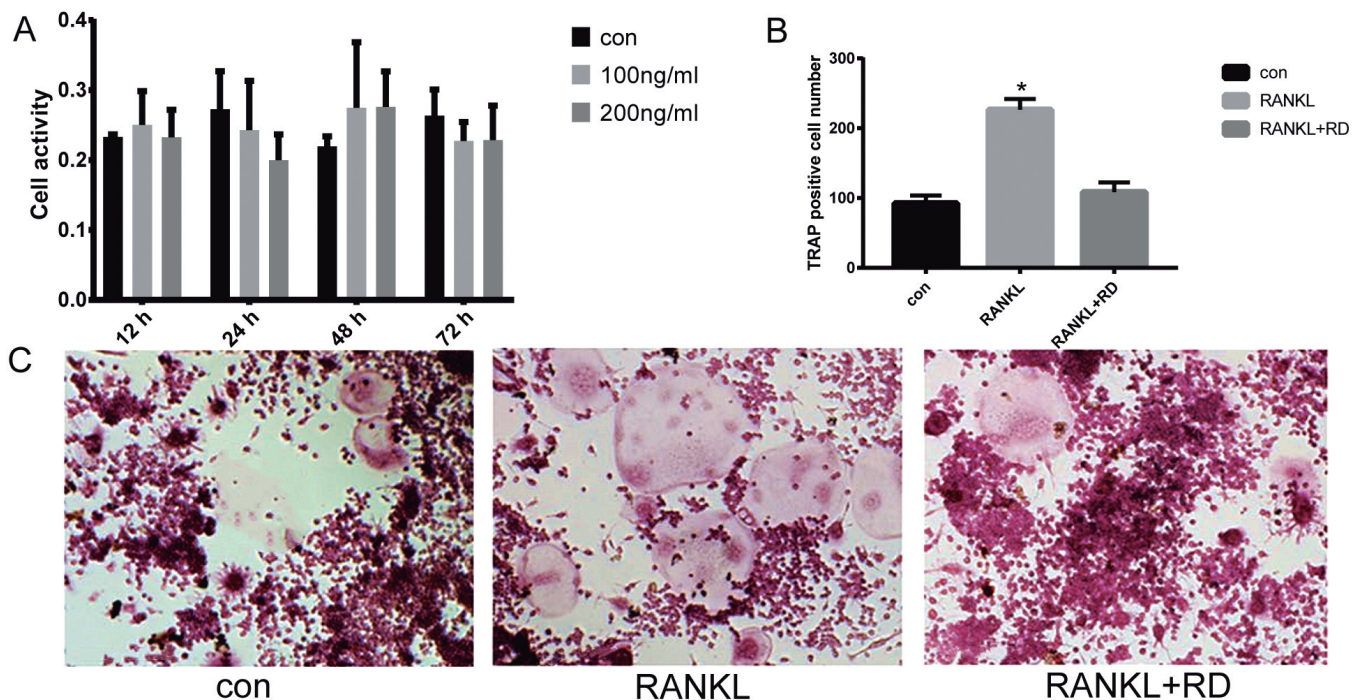
### *RD suppresses RANKL-induced Akt and NF- $\kappa$ B signaling pathway in vitro*

Previous studies have shown that the Akt pathway is activated during RANKL-induced osteoclastogenesis and the activation of the Akt signaling promotes osteoclast differentiation (Wong et al., 1999; Sugatani and Hruska, 2015). Thus, we investigated whether RD suppresses osteoclast differentiation by targeting the

activation of the Akt pathway. As expected, Akt phosphorylation was enhanced by RANKL and suppressed by RD, and RD treatment also suppressed the up-regulation of NF- $\kappa$ B and NFATC1 induced by RANKL (Fig. 10A-E). The inhibitory effect of RD was rescued by an Akt-specific activator SC79. These findings suggest that RD suppresses RANKL-induced Akt phosphorylation and the up-regulation of NF- $\kappa$ B pathway during osteoclast differentiation.

### Discussion

Osteoporosis is a prevalent disease occurring in postmenopausal women, which poses a great health threat, impairs physical disability and undermines quality of life. Calcium plus vitamin D, estrogen replacement therapy, calcitonin and etidronic acid can inhibit bone resorption and are commonly used as anti-osteoporosis therapies. However, hormone therapy is associated with risks such as cardiovascular disease, stroke, venous thrombosis, and breast cancer. Therefore, the development of novel interventions for osteoporosis is crucial to improve the life quality of postmenopausal women. Accumulating evidence indicates that high-fiber diets and dietary fiber supplementation have beneficial effects on bone metabolism, and dietary supplementation of soluble fibers are currently being considered as an



**Fig. 8.** Resistant dextrin inhibits RANKL-induced osteoclastogenesis in vitro. **A.** The cell proliferation of the BMMs treated with different concentrations of RD was determined by CCK-8 proliferation assay after 12h, 24h, 48h, and 72h respectively. **B.** BMMs were incubated with MCSF (20 ng/mL), RANKL (50 ng/mL) and treated with RD (100 ng/mL) for 7 days. The cells were fixed and stained with TRAP, TRAP-positive cells with multinucleated cells (nuclei $\geq$ 3) were quantified. **C.** Representative images showing TRAP-positive multinucleated cells. The data are expressed as means  $\pm$  SD (n=3). \*P<0.05 compared with the control group. Scale bars: 100  $\mu$ m.

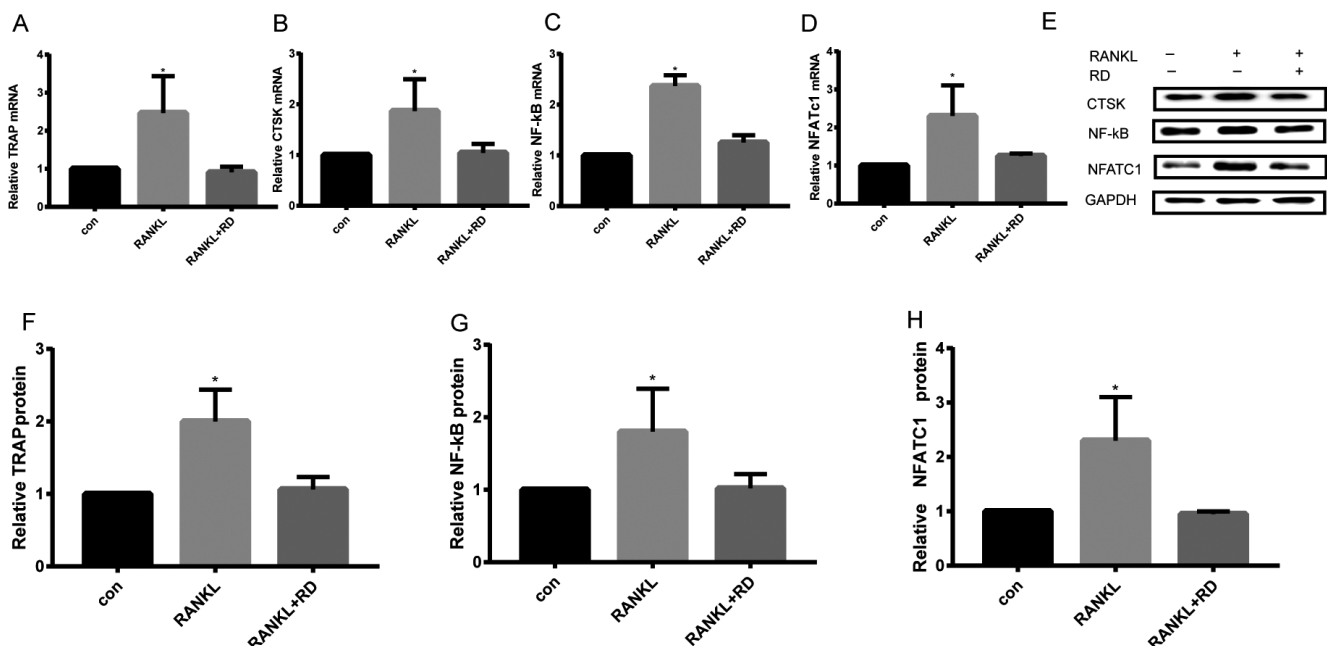
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alternative strategy to ameliorate osteoporosis (Muller et al., 2001; Bai et al., 2021). Previous studies reported that the supplementation of SCFAs is protective against pathological bone loss (Lucas et al., 2018). A combination of soya isoflavone and resistant starch also increases equol production and alters the intestinal microbiota and immune status in the bone marrow, which attenuates bone resorption in OVX mice (Tousen et al., 2016).

RD is a soluble fiber and the main fermentable source for SCFAs, with multiple beneficial effects on metabolic disorders. RD can improve insulin resistance induced by high fat diet and reduce liver fat deposition in mice (Aliasgharzadeh et al., 2015; Hu et al., 2020). The current study investigated the effects of dietary RD on osteoporosis in ovariectomized rats. We showed that dietary RD was able to decrease expression of the bone resorption markers and inflammatory cytokines in serum, improve bone biomechanical properties and microstructure, antagonize the bone loss in rats with OVX-induced osteoporosis. In addition, we demonstrated that RD inhibits osteoclastic differentiation by suppressing the Akt and NF- $\kappa$ B signaling pathways in vitro. Furthermore, we found that these effects are correlated with significant changes in serum SCFAs concentration. Our study suggests that dietary RD could modulate the serum SCFA concentration, suppress osteoclast differentiation and bone resorption in OVX rats.

Noticeably, the weight of the OVX rats significantly increased compared with sham group, which was suppressed by dietary RD, while dietary RD had no effect on the food intake in all groups. This is consistent with the findings of previous studies, which showed that RD can lead to weight loss in overweight people as a result of the improvement in insulin resistance (Li et al., 2010; Hu et al., 2020), and we suspect that the above effects may be associated with the modulation of SCFA metabolism by dietary RD in OVX rats.

The maintenance of normal bone mass depends on the dynamic balance between osteoblast-associated bone formation and osteoclast-associated bone resorption, which is implicated in the maintenance of bone homeostasis under different physiological conditions (Vellucci et al., 2018). The lack of estrogen secretion in postmenopausal women promotes the differentiation and the activity of osteoclasts, thus increasing bone absorption. There is also an increase in inflammatory cytokines, which also contributes to the progression of PMOP (Li et al., 2011). Pro-inflammatory cytokines such as TNF- $\alpha$  and IL-1 $\beta$  have been demonstrated to accelerate bone resorption, as seen in idiopathic osteoporosis and PMOP (Rifas, 1999). These two cytokines enhance bone resorption and inhibit bone formation via the activation of inflammatory signaling pathways (Patsch et al., 2011; Osta et al., 2014; Murakami et al., 2020). TNF- $\alpha$  gene knockout appears to protect against high-fat-diet induced bone loss by



**Fig. 9.** RD inhibits osteoclast-related gene expression in RANKL-induced BMMs. **A-D.** The mRNA expression of TRAP, CTSK, NF- $\kappa$ B and NFATC1 in BMMs treated with or without 100 ng/mL RD plus RANKL induction. **E.** Western blot analyses of CTSK, NF- $\kappa$ B and NFATC1 in BMMs. **F-H.** Western blot analyses of CTSK, NF- $\kappa$ B and NFATC1 proteins in BMMs. The relative protein levels were quantified by densitometry and normalized to GAPDH. Data are expressed as the mean  $\pm$  SD from three independent experiments. \* $P$ <0.05 compared with control group.



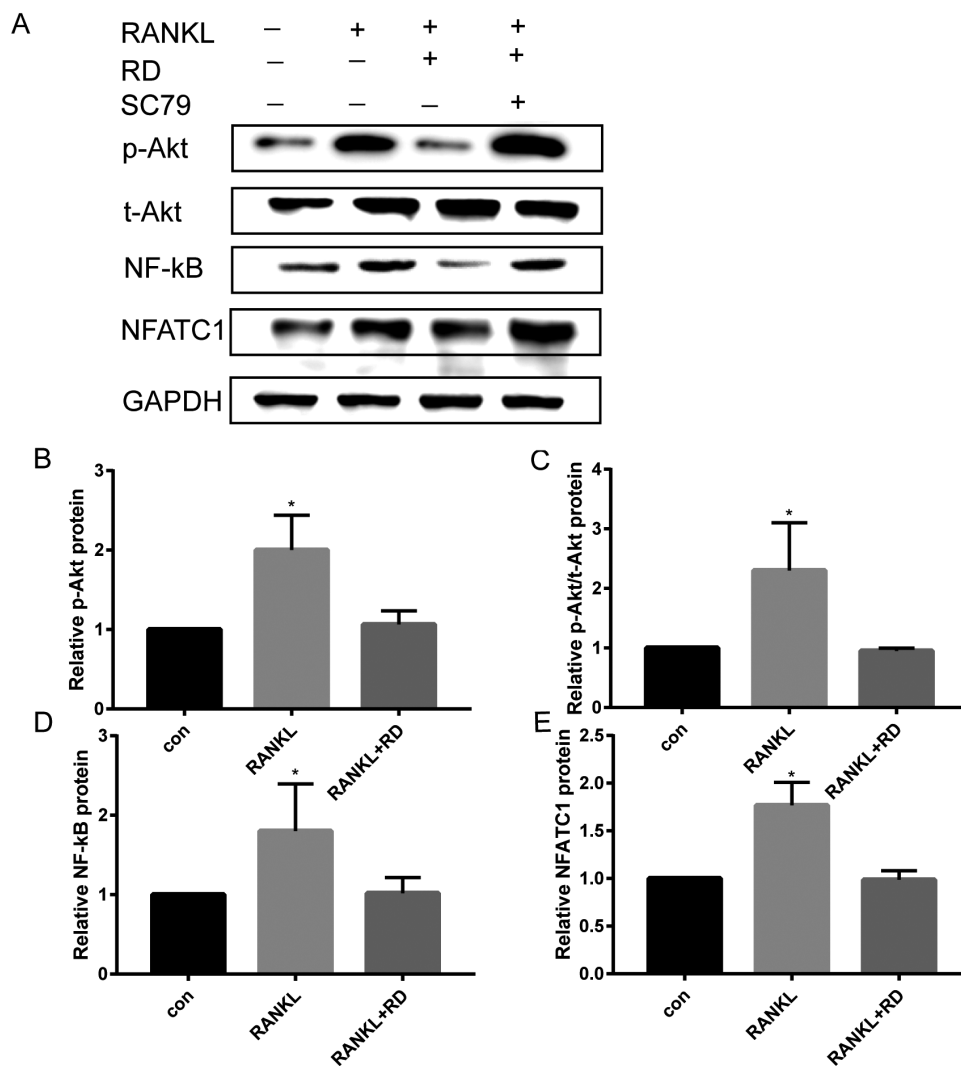
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suppressing bone resorption and enhancing osteoblastogenesis (Zhang et al., 2015). Our results also showed that dietary RD is able to suppress the level of serum inflammatory biomarkers and osteoclast markers, but had no effect on osteoblast markers in the OVX rats. These results suggest that RD treatment attenuates OVX-induced bone loss by suppressing the expression of OVX-induced inflammatory cytokines and bone resorption without affecting bone formation.

Previous reports suggest that SCFAs can influence bone homeostasis, thereby providing a mechanistic link between the gut microbiota and bone homeostasis (Weaver, 2015; Hernandez, 2017; Whisner and Castillo 2018). A recent clinical study showed that gut microbiota and metabolite alterations are associated with reduced bone mineral densities or bone metabolic indexes in postmenopausal osteoporosis (He et al., 2020). The protective effects of SCFAs on bone mass are associated with the inhibition of osteoclast

differentiation and bone resorption (Lucas et al., 2018). Consistently, our study showed that dietary RD significantly elevated the level of serum SCFAs in both the OVX and sham rats, including acetate, propionate and the total SCFAs. The results suggest that the beneficial effects of RD on bone metabolism may be correlated with the increased SCFAs levels.

A previous study reported that RANKL can induce the osteoclastic differentiation of BMMs by activating the NF- $\kappa$ B/NFATC1 pathway (Lacey et al., 1998). RANKL and MCSF can also promote osteoclast differentiation through activating the Akt pathway (Wong et al., 1999; Sugatani and Hruska, 2005). RANKL and its receptor RANK belong to the TNF family (Nakashima and Takayanagi, 2011) and are key factors involved in osteoclast differentiation from the progenitors (Theill et al., 2002). Furthermore, the binding of RANKL to RANK could activate a variety of downstream signal pathways, including Akt and NF- $\kappa$ B



**Fig. 10.** Resistant dextrin inhibits RANKL-induced osteoclastogenesis by suppressing the Akt and NF- $\kappa$ B signaling pathway in BMMs. BMMs were treated with or without 100 ng/mL RD plus RANKL, SC79 for 5 days. **A.** Western blot analyses of p-Akt, t-Akt, NF- $\kappa$ B and NFATC1 in BMMs. **B-E.** Western blot analysis of p-Akt, t-Akt, NF- $\kappa$ B and NFATC1 proteins in BMMs. The relative protein levels were quantified by densitometry and normalized to GAPDH. Data are expressed as the mean  $\pm$  SD from three independent experiments. \* $P$ <0.05 compared with control group.

pathways (He et al., 2012), and Akt and NF- $\kappa$ B signaling have been recognized as important contributors in osteoclastogenesis (Yang et al., 2019). In this study, we found that although RD had no effect on the proliferation of BMMs, RD inhibited the phosphorylation of Akt induced by RANKL during osteoclastic differentiation and down-regulated the expression of transcription factors NF- $\kappa$ B and NFATC1. Together, these data indicate that RD could inhibit osteoclast differentiation and osteoporosis possibly through the inhibition of Akt and NF- $\kappa$ B pathways in bone marrow cells.

In summary, our study demonstrated the beneficial effects of dietary RD against OVX-induced osteoporosis by decreasing bone resorption, inhibiting inflammatory cytokines, promoting SCFA production and bone biomechanics, and suppressing *in vitro* osteoclast differentiation by targeting the Akt and NF- $\kappa$ B signaling pathways. Our findings provide novel insights into the beneficial roles of RD on PMOP and highlight the importance of the supplementation of dietary fibers in PMOP intervention. The effects of dietary RD on osteoclast differentiation *in vivo* need to be further validated in a rat model. Because there are multiple signaling pathways involved in osteoporosis and osteoclast differentiation, a systematic approach such as RNA-seq analysis is needed to comprehensively profile the gene expression changes induced by RD in OVX rat.

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