Original Article
Vitamin B5 inhibit RANKL induced osteoclastogenesis and ovariectomy induced osteoporosis by scavenging ROS generation

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Abstract: B vitamins are a class of water-soluble vitamins that play important roles in cell metabolism. The participation of B vitamins in bone health has been recognized for decades. Pantothenic acid (vitamin B5) is mainly known for its wide variety of sources. However, the potential role of pantothenic acid in bone health and metabolism is still unclear. In this study, we found pantothenic acid has a dual effect on RANKL-induced osteoclastogenesis. Tartrate-resistant acid phosphatase (TRAP) stain shows that osteoclastogenesis was remarkably induced in a lower dosage of pantothenic acid (< 200 mM) and significantly inhibited while the pantothenic acid concentration increases to a certain extent (> 500 mM). We further confirmed this dual effect of pantothenic acid in osteoclastogenesis by detecting osteoclast formation and bone resorption using focal adhesion stain and pit formation, respectively. Mechanistically, we found phosphatidylinositol 3 kinase-protein kinase B (PI3K-Akt) pathway was activated in pre-osteoclasts (pOCs) after cultured with lower dosage of pantothenic acid; while the ROS generation was eliminated with upregulation of forkhead box O1 (FoxO1), forkhead box O2 (FoxO2) and NF-E2-related factor 2 (Nrf2) in pOCs after cultured with higher dosage of pantothenic acid. Finally, we used ovariectomized (OVX) mice to explore the potential role of pantothenic acid rich dietary in regulating bone metabolism *in vivo*, the result shows that pantothenic acid rich dietary can protect bone loss from estrogen deficiency. In brief, our study identified a new understanding of pantothenic acid in regulating osteoclastogenesis, revealed a therapeutic potential of pantothenic acid in prevention of bone loss related disorders.

Keywords: Osteoclast, osteoclastogenesis, vitamin B5, ROS, osteoporosis

Introduction

Bone homeostasis is maintained by a persistent and coordinated process between osteoclast bone resorption and osteoblast bone formation [1]. In this process, the two most pivotal cell types are osteoclast and osteoblast. Osteoblasts are derived from mesenchymal stem cells (MSCs) and responsible for bone formation and mineralization. Osteoclasts, also known as bone resorbing cells, are derived from hematopoietic stem cells (HSCs) or monocytes/macrophage progenitor cells and in charge of bone resorption [2, 3]. Collaboration between these two types of cells orchestrated and stabilized the process of bone remodeling [4]. Mature osteoclasts (mOCs) are bone-specific polykaryons. Two of the most crucial regulating factors are receptor activator of nuclear factor

κB ligand (RANKL) and macrophage-colony stimulating factor (M-CSF) [5]. M-CSF contributes to proliferation, differentiation and survival of pOC at the early stage of osteoclastogenesis [6]. Binding of RANKL to its receptor RANK drives the differentiation of pOC into mOC and maintains the survival and functions of mOC [7]. Disorders of osteoclast differentiation and function can cause a series of bone diseases including osteoporosis, pycnodysostosis, and Paget's disease [8, 9].

Reactive oxygen species (ROS), known as its cytotoxicity and injury, plays important roles in regulating the molecular signaling of osteoclast differentiation [10, 11]. Study shows that intracellular ROS regulates osteoclast differentiation by regulating RANK signaling pathway [12, 13]. Moreover, mitochondrial derived genera-

tion of ROS and H_2O_2 have also been shown critical in RANKL induced osteoclastogenesis [14, 15].

Vitamin B5, also known as pantothenic acid, is a member of the B vitamins which generally are a series of co-enzyme factors that participating in the energy-generating metabolic pathways for carbohydrates, fats and proteins in multiple organ systems [16]. Vitamin B5 has been shown to have the ability of regulating body immunity via increasing the generation of inflammatory cytokines in epithelial cells [17]. Besides, Vitamin B5 can also promote the development and maintain the function of nervous system by regulating the energy production and fat metabolism [18]. As a nutrient, Vitamin B5 is generally used in medicine, food additives and feed additives, which is an indispensable trace substance for human and animal to maintain basic physiological functions through regulation of metabolism. In bone, Vitamin B9 (folate) and Vitamin B12 (cobalamin) are demonstrated to have close relationships with bone health and fracture risk by involving in the remethylation of homocysteine metabolism [19-22]. However, the function of Vitamin B5 in bone and the specific mechanism of affecting osteoclastogenesis has not been investigated and remains unknown. Therefore, we assume that Vitamin B5 has strong regulatory effects on osteoclastogenesis and bone homeostasis, which have not been previously investigated.

In this study, we investigated the effects of pantothenic acid in RANKL induced osteoclastogenesis. Pantothenic acid showed a dual effect in RANKL induced osteoclastogenesis and pantothenic acid rich dietary showed a protective effect against ovaryecotomy (OVX) induced bone loss. These findings raise the possibility that pantothenic acid has an important role in regulating bone health and metabolism, which has not been studied and mentioned before.

Materials and methods

Regents

α-MEM medium and fetal bovine serum (FBS) were purchased from Gibco (life technologies, Carlsbad, CA, USA). RAW 264.7 cells were kindly provided by Stem Cell Bank (Chinese Academy of Sciences, China). Penicillin-streptomycin solution and alpha minimal essential medium (α-MEM) were obtained from Hyclone (Thermo

Scientific, USA). BMMs were induced by RANKL (50 ng/mL) and M-CSF (50 ng/mL) for 72 h to acquire mature osteoclasts. Pantothenic acid was purchased from Aladin (Beijing, China). Antibodies against NFATc1, c-FOS, Ctsk, OSCAR, DC-STAMP, p-p38, p38, p-ERK, ERK, p-JNK, JNK, tublin FoxO1, FoxO2, Nrf2, BLIMP1, p-PI-3K, PI3K, p-Akt, Akt and GAPDH used in Immunoblotting were all purchased from Bioss (Beijing, China). Focal Adhesion Staining Kit and TRAP stain kit were obtained from Sigma (St. Louis, USA).

Mice

Female C57BL/6 mice at four-week-old are provided by the animal center of Army Medical University. All experimental procedures were authorized by Army Medical University and were conducted on the basis of the instructions of care and use for laboratory animal. Every effort was made to reduce the number of tested animals and their suffering. Mice were randomly divided into four groups: sham operated mice treated with pantothenic acid deficient dietary (PADD) (n = 8), OVX mice treated with PADD (n = 8), sham operated mice treated with pantothenic acid rich dietary (PARD) (n = 8), OVX mice treated with PARD (n = 8). All mice were feed in pantothenic acid diets for four weeks. Concentration of pantothenic acid was calibrated and mice were weighed daily every day before administration. After four weeks treatment with pantothenic acid diets, cervical dislocation was performed to kill all treated mice 1 day after last administration. The femur of mice were removed and waited for further detection.

CCK-8 assay

RAW 264.7 cells and BMMs were cultured (2 × 10^3 per well) in 96-well plates overnight, respectively. Cells were induced with M-CSF (50 ng/mL) and RANKL (50 ng/mL) for 24 h or 72 h dissolved different dosages of pantothenic acid. According to the manufacturers' instructions, cell viability was detected by Cell Counting Kit-8 (CCK8, obtained from Dojindo) at 24 h and 72 h. Then 96-well plate reader at 450 nm was used to measure the absorbency of cells to evaluate the cell viability.

Apoptosis assay

Cell apoptosis was detected to evaluate the effects of several concentrations of pantothen-

Table 1. Primer sequences for qPCR

Genes	Forward	Reverse	Tm (°C)
Tm7sf4	5'-TTATGTGTTTCCACGAAGCCCTA-3'	5'-ACAGAAGAGAGCAGGGCAACG-3'	62
ATP6v0d2	5'-AGCAAAGAAGACAGGGAG-3'	5'-CAGCGTCAAACAAAGG-3'	60
Ctsk	5'-GCGGCATTACCAACAT-3'	5'-CTGGAAGCACCAACGA-3'	61
c-FOS	5'-AGGCAGAACCCTTTGA-3'	5'-GGTGACCACGGGAGTA-3'	60
TRAP	5'-AGACGAGGTTACGCTGTGC-3'	5'-TCGGGGACAATTCGGTAAACT-3'	62
NFATc1	5'-GAGGAGTTGGCTCAGTG-3'	5'-TAGCGTTCCGTTCGTT-3'	61
β-actin	5'-TCCCTGTATGCCTCTG-3'	5'-ATGTCACGCACGATTT-3'	61

ic acid on cell viability by flow cytometry analyses using a BD Accuri C6 flow cytometer. Cell apoptosis was determined using Annexin V/PI Apoptosis Detection Kit (KeyGEN, Biotech). After cells were stained with annexin V-FITC and PI, cell apoptosis analysis were performed on samples treated with various concentrations of pantothenic acid. Viable cells were negative in both annexin V and PI; early apoptotic were positive for annexin V and negative for PI; late apoptotic cells were both positive for annexin V and PI; necrotic cells were positive for PI and negative for annexin V.

Reactive oxygen species (ROS) assay

BMMs (5 × 10^3 cells/well in 96 well plates) were cultured in α-MEM medium (10% FBS, 1% Penicillin-streptomycin) dissolved M-CSF (50 ng/mL) and RANKL (50 ng/mL) for 24 h and each well was replaced with α-MEM medium. ROS positive control group was set after cells were treated with different dosages of pantothenic acid and level of intracellular ROS of each group was measured by 21, 71-dichlorofluorescein diacetate (DCFH), which can be oxidized into fluorescent DCF. After fixing, cells were washed by 1 × PBS and incubated using DCFH-DA (10 μM) avoid light for 30 minutes. Cells were counterstained using DAPI for better observation. After finding the ideal field of vision, images were taken using the fluorescence of DCF by fluorescence microscopy.

Tartrate-resistant acid phosphatase (TRAP) staining

BMMs were cultured in α-minimal essential medium (MEM) containing 10% FBS and 1% Penicillin-streptomycin solution with M-CSF (50 ng/mL) and RANKL (50 ng/mL) for 72 h to generate mature multinucleated osteoclasts. For TRAP stain, cells were cultured in a 96-well plate at a density of 5 × 10^3 cells/well. Cells were fixed in 4% paraformaldehyde for 20 min

after 72-h induction and then stained using a TRAP staining kit (Sigma, St. Louis, USA) according to the instructions. Then TRAP-positive cells and TRAP multinucleated cells containing three or more nuclei were counted, respectively.

Pit formation assay

BMMs were cultured in 96-well plates (Corning Osteo Assay Surface) (2 × 10^3 cells/well). Then cells were induced with RANKL (50 ng/ml) and M-CSF (50 ng/ml) for 72 h with different dosages of pantothenic acid. Bleach solution was added to 96-well osteo surface plates to remove cells. Detailed analysis of pit formation area was described in our previous study [23]. The percentage of resorption area on osteo surface and bone slice was quantified using image J software (ver. 1.47).

Real-time qPCR

Total RNA was isolated using Trizol reagent (Life Technologies).

Then single-stranded cDNA was prepared from 1 mg of total RNA using reverse transcriptase with oligo-dT primer (Promega). Two microlitres of each cDNA was subjected to PCR amplification using specific primers for Ctsk, NFAT2, c-fos, ATP6v0d2, Tm7sf4 and TRAP with detailed information in **Table 1**.

Western blot assay

Cells were lysed in a lysis buffer containing 10 mM Tris, pH 7.2, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 1% Triton X-100, and 1% deoxycholic acid. For Western blots, 20 mg of protein samples were subjected to SDS-PAGE followed by transfer onto PVDF membranes. After blocking in 5% skim milk, membranes were incubated with rabbit antibodies against c-FOS, NFATc1, Ctsk, OSCAR, DC-STAMP, p-ERK, ERK, p-p38,
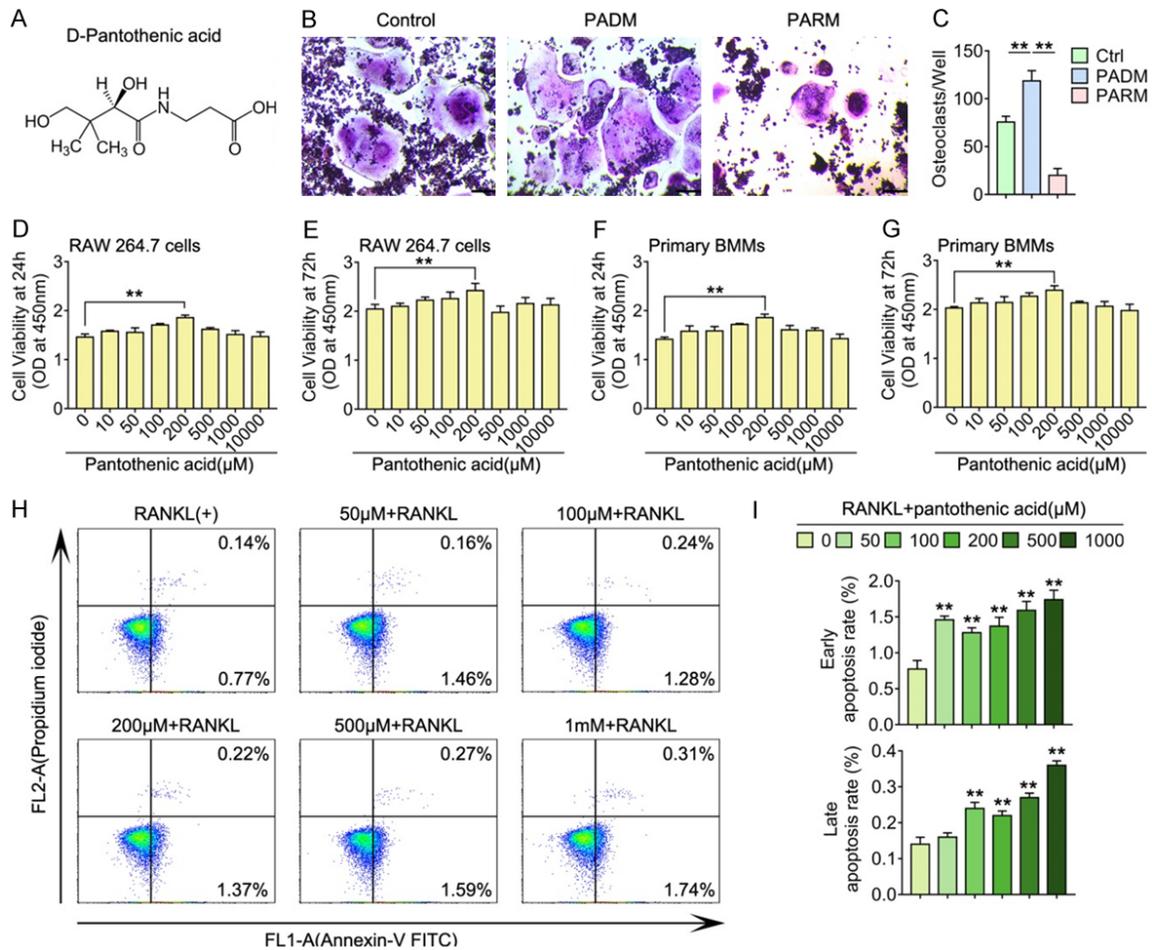
Figure 1. Comprehensive toxicity evaluation of pantothenic acid on receptor activator of nuclear factor κB ligand (RANKL)-induced osteoclastogenesis: (A) Chemical formula of pantothenic acid. (B) Representative TRAP stain images of osteoclasts cultured with complete media (Ctrl), pantothenic acid deficient medium (PADM) and pantothenic acid rich medium (TRM). Bar represents 200 µm. (C) Quantification of TRAP (+) cells with more than three nuclei in each well (96-well plate). (D-G) Cell counting kit-8 (CCK8) analysis of cell viability at 24 h or 72 h of Raw 264.7 cells and BMMs treated with eight different dosages of pantothenic acid (0 µM, 50 µM, 100 µM, 200 µM, 500 µM, 1000 µM and 10000 µM). (H) FCM analysis of cell apoptosis rate of BMMs treated with six different dosages of pantothenic acid (0 µM, 50 µM, 100 µM, 200 µM, 500 µM and 1000 µM). (I) Statistical analysis of early stage cell apoptosis rate and (H) late stage cell apoptosis rate of BMMs treated with six different dosages of pantothenic acid. Relative TRAP activity was measured by colorimetric analysis. The data in the figures represent the averages ± SD. Statistically significant differences between the treatment and control groups are indicated as *($P < 0.05$) or ** ($P < 0.01$).

p38, p-JNK, JNK, tublin, FoxO1, FoxO2, Nrf2, BLIMP1, p-PI3K, PI3K, p-Akt, Akt and GAPDH (1:1000, Bioss, Beijing, China) overnight at 4°C followed by an one hour incubation with secondary antibody (1:2000). Blots against GAPDH and β-actin served as loading control.

µCT and histological analyses

Image of the whole femur was obtained using a Bruker MicroCT Skyscan 1272 system (Kontich, Belgium) with an isotropic voxel size of 10.0 µm. Scanning was performed in 4% paraformaldehyde using a 60 kV X-ray tube with an X-ray intensity of 166 µA and an exposure time of 1700 ms. Trabecular bones were thresholded at 86-255 (8-bit gray scale bitmap). µCT scans of the whole femur of mice were performed using isotropic voxel sizes of 148 µm. Reconstruction was accomplished by Nrecon (Ver. 1.6.10, Kontich, Belgium). 3D images were obtained from contoured 2D images by methods based on distance transformation of the gray scale original images (CTvox, Kontich, Belgium, Ver. 3.0.0). 3D and 2D analyses were performed using software CT Analyser (Kontich, Belgium, Ver. 1.15.4.0). All images presented are representative of the respective groups.
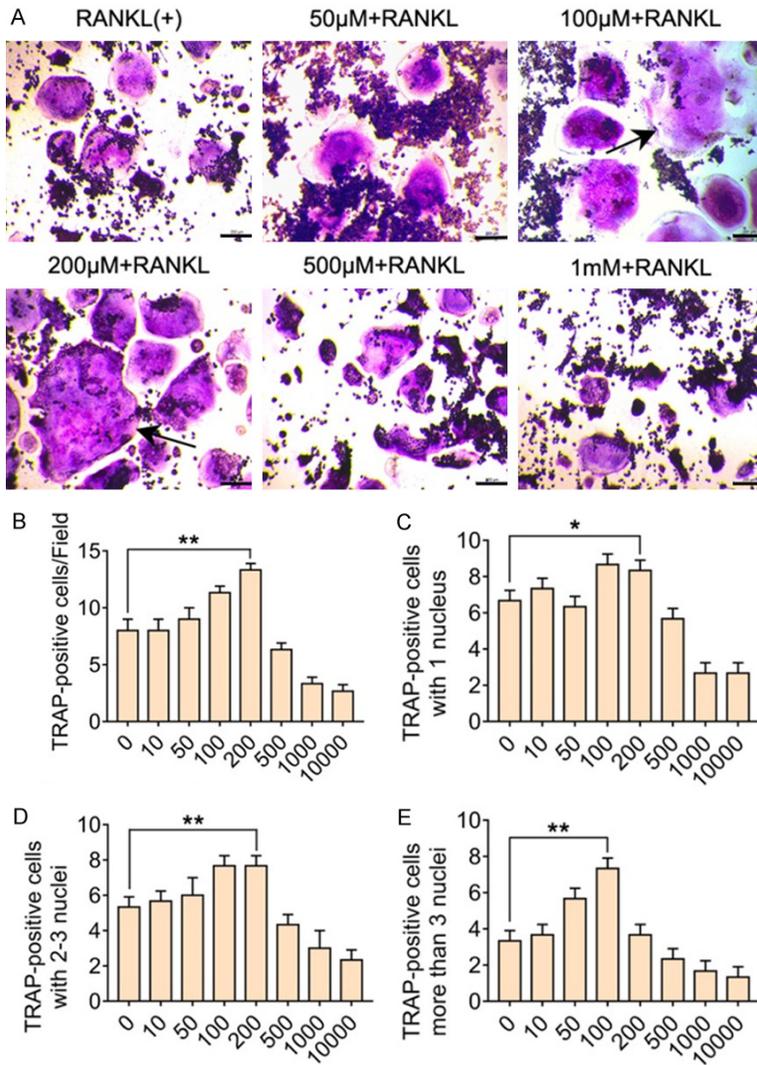
Figure 2. Pantothenic acid regulates RANKL induced osteoclastogenesis in a dose-dependent way. A. Representative TRAP stain images of osteoclasts treated with six different concentrations of pantothenic acid (0 μM, 50 μM, 100 μM, 200 μM, 500 μM and 1000 μM). Black arrows indicates osteoclasts with large volume and multiple nuclei. Bar represents 200 μm. B. Quantification analysis of TRAP (+) cells in each well (96 well plate) treated with different dosages of pantothenic acid. C. Quantification analysis of TRAP (+) cells with only one nucleus in each well treated with different dosages of pantothenic acid. D. Quantification analysis of TRAP (+) cells with 2-3 nuclei in each well treated with different dosages of pantothenic acid. E. Quantification analysis of TRAP (+) cells with more than three nuclei in each well treated with different dosages of pantothenic acid.

Statistical analysis

All data are representative of at least three experiments of similar results performed in triplicate, unless otherwise indicated. Data are expressed as mean ± S.D. One-way ANOVA, followed by Student-Newman-Keuls post hoc tests, was used to determine the significance of difference between results, with *$P < 0.05$, **$P < 0.01$ being regarded as significant.

Results

Pantothenic acid showed specific toxicity in osteoclasts

The chemical formula of pantothenic acid is shown (**Figure 1A**). To assess the effects of pantothenic acid in RANKL induced osteoclast differentiation, we compared osteoclastogenesis in complete media (Ctrl), pantothenic acid deficient media (PADM) and pantothenic acid rich media (PRDM) stimulated by the same dosage of RANKL (100 ng/ml) using TRAP stain (**Figure 1B**). The results showed that PADM significantly enhanced while PARM prominently reduced the osteoclast number per well compare with the control groups (**Figure 1C**). Then cell viability of RAW 264.7 cells and bone marrow macrophages (BMMs) treated with pantothenic acid was evaluated using CCK-8 (**Figure 1D-G**). The results displayed that pantothenic acid are nontoxic to either within the concentration of 10000 μM. Interestingly, pantothenic acid significantly increased cell viability of both cell types at 24 h and 72 h at the dosage of 200 μM. Flow cytometry (FCM) was then performed to assess the effects of pantothenic on cell apoptosis rate during osteoclastogenesis in BMMs (**Figure 1H**). The results showed that pantothenic acid increased both early and late cell apoptosis rate in osteoclasts in a dose dependent way (**Figure 1I**). We concluded that pantothenic acid was not toxic in macrophages/monocytes but had specific cytotoxicity in osteoclasts.

Pantothenic acid has a dual effect in regulating RANKL-induced osteoclastogenesis

BMMs were treated with RANKL (100 ng/ml) and M-CSF (50 ng/ml) for 3 days together with
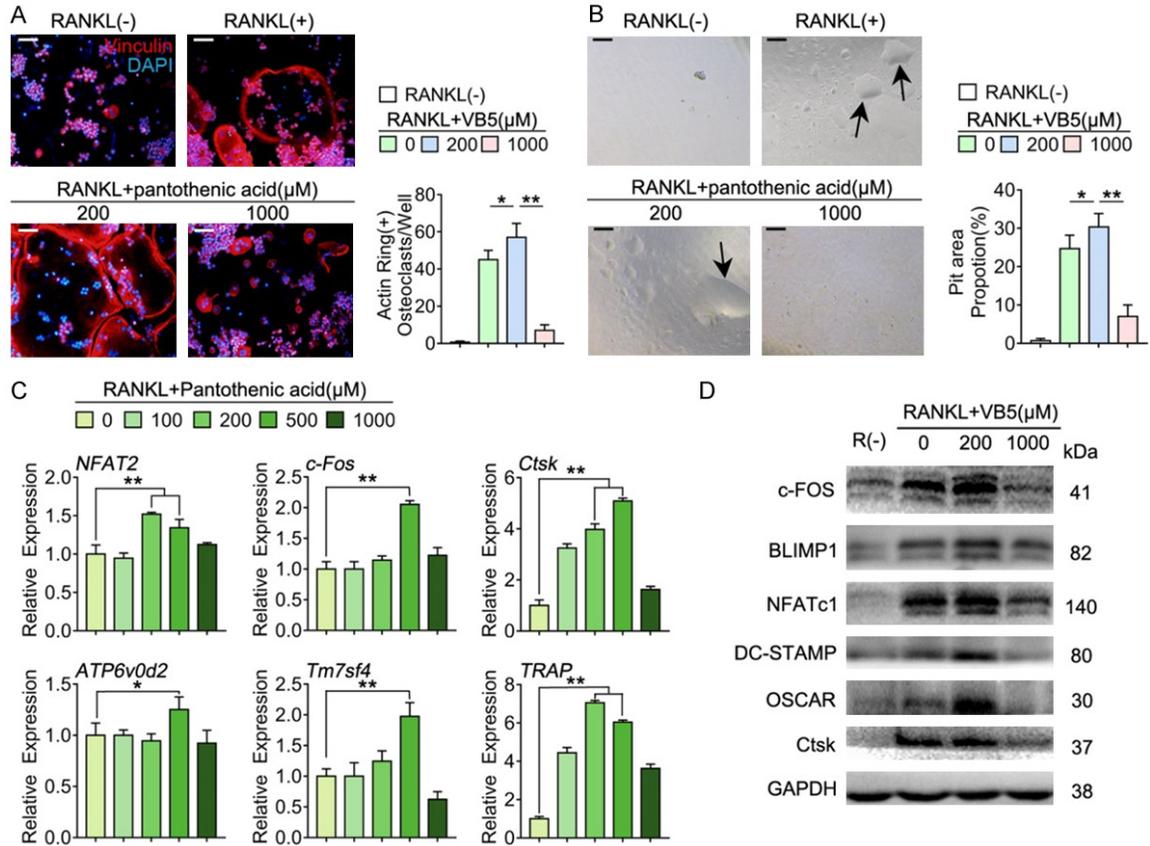
Figure 3. Pantothenic acid regulates RANKL induced osteoclastogenesis in a dose-dependent way. A. Representative images of focal and adhesion staining of BMMs stimulated with RANKL (50 ng/ml) with different dosage of pantothenic acid treatments (200 µM and 1000 µM) and quantification of actin ring (+) osteoclasts in each well. Immunofluorescence for nuclei using DAPI (blue) and vinculin using Vinculin Monoclonal Antibody (red) were shown. Bar represents 200 µm. B. Representative pit formation assay images of BMMs stimulated with RANKL (50 ng/ml) with different dosages of pantothenic acid (200 µM and 1000 µM) and quantification of pit area proportion. Black arrows indicates bone resorption pits. Bar represents 400 µm. C. Relative mRNA expression levels of *NFAT2, c-Fos, Ctsk, ATP6v0d2, Tm7sf4* and *TRAP* in osteoclasts treated with different dosages of pantothenic acid. D. Representative western blot images of c-FOS, BLIMP1, NFATc1, DC-STAMP, OSCAR, Ctsk and GAPDH in indicated groups. The data in the figures represent the averages ± SD. Statistically significant differences between the treatment and control groups are indicated as * ($P < 0.05$) or ** ($P < 0.01$).

pantothenic acid in different concentrations. Six different groups were set according to the screened concentrations of pantothenic acid treatment (0 µM, 50 µM, 100 µM, 200 µM, 500 µM, 1000 µM). TRAP stain was performed to evaluate the effects of pantothenic acid in osteoclast differentiation (**Figure 2A**). TRAP positive cells in each well (96-well plate) were counted in order to examine the effects of pantothenic acid on RANKL induced osteoclastogenesis. Based on the results, we found that pantothenic acid regulates RANKL induced osteoclastogenesis in a dose dependent way. Significantly, pantothenic acid has a facilitative effect on osteoclastogenesis at the concentration lower than 200 µM, and 200 µM of pantothenic acid enhances osteoclastogenesis markedly com-

paring with other dosages ($P < 0.01$). Inversely, pantothenic acid started to show an inhibitory effect when the concentration is above 500 µM. With pantothenic acid treatment of 1000 µM and 10000 µM, the inhibitory effect became significant (**Figure 2B-E**). We then selected two typical concentrations on the basis of TRAP experiment to evaluated mature osteoclast formation using immunostaining of F-actin revealing the actin ring formation (**Figure 3A**). Quantification analysis showed that pantothenic acid also has a dual effect on the number per well of osteoclast with actin ring (**Figure 3A**). Moreover, Bone resorption activity of osteoclasts was evaluated using pit formation assay on bovine bone slices, which also displays a prominent dual effect of pantothenic acid in osteoclast
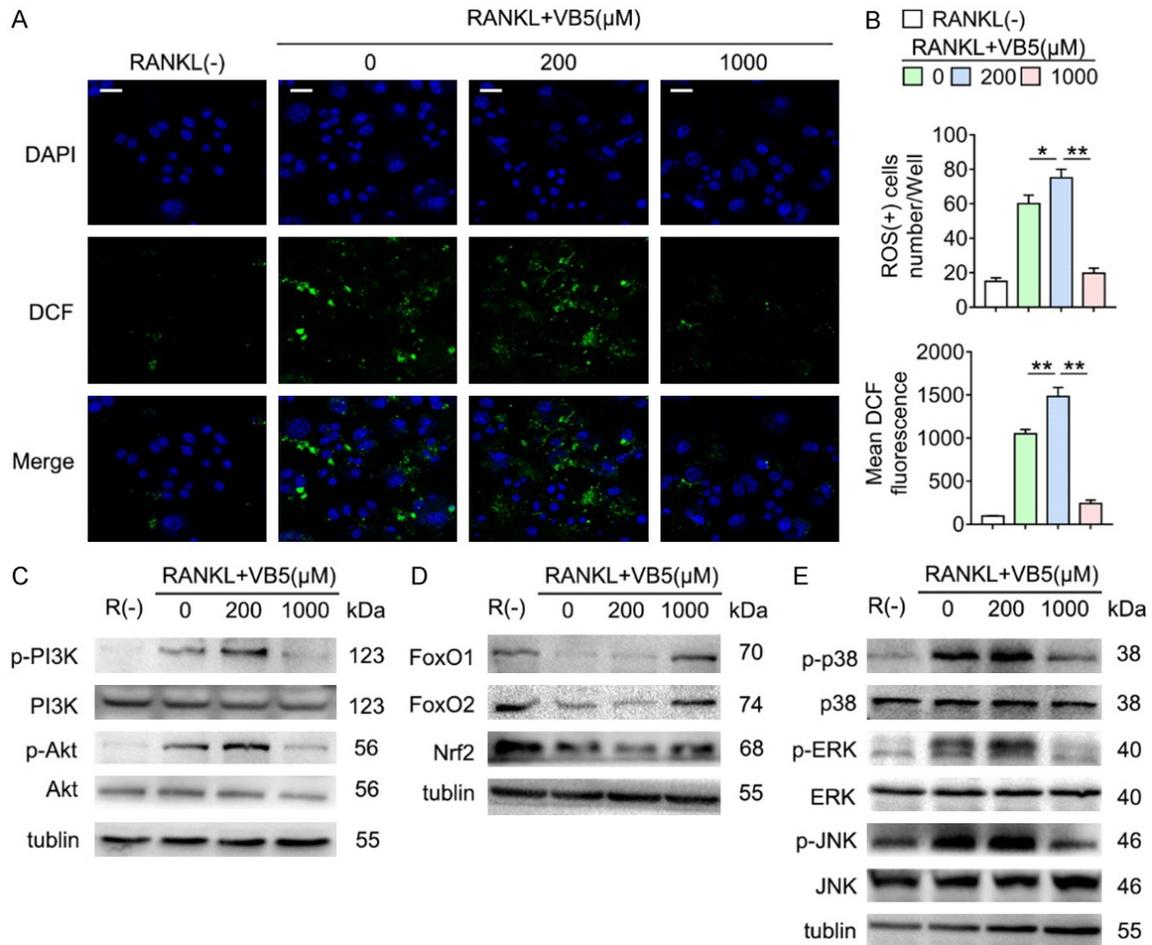
Figure 4. High concentration of pantothenic acid reduces RANKL induced ROS generation and inhibiting MAPK pathway. A. Representative images of DCFH stain of BMMs stimulated with RANKL treated with different concentrations of pantothenic acid (200 μM and 1000 μM). Immunofluorescence for nuclei using DAPI (blue) and ROS using DCFH-DA (green) were shown. Bar represents 40 μm. B. Quantification of ROS positive cells number and mean DCF fluorescence in each well. C. Representative western blot images of p-PI3K, PI3K, p-Akt, Akt and tublin in indicated groups. D. Representative western blot images of FoxO1, FoxO2, Nrf2 and tublin in indicated groups. E. Representative western blot images of p-p38, p38, p-ERK, ERK, p-JNK, JNK and tublin in indicated groups. The data in the figures represent the averages ± SD. Statistically significant differences between the treatment and control groups are indicated as * ($P < 0.05$) or ** ($P < 0.01$).

differentiation and function marked by quantification of pit area proportion (**Figure 3B**). In consistency, the expressions of osteoclast specific genes *NFAT2, c-Fos, Ctsk, ATP6v0d2, Tm7sf4* and *TRAP* were all biphasically regulated by pantothenic acid treatments as indicated (**Figure 3C**). Correspondingly, on the protein level, western blot results revealed a remarkable dual regulation in crucial osteoclast specific transcription factors (TFs) c-FOS, NFATc1 and BLIMP1. The protein levels of osteoclast marker Ctsk, OSCAR and DC-STAMP were also increased at a low concentration of pantothenic acid and reduced at a high pantothenic acid concentration (**Figures 3D**, S1). Besides, RT-qPCR analysis revealed that fusogenic genes *CD9, CD47,*

MITF and *OC-STAMP* were also biphasically regulated by different concentrations of pantothenic acid (Figure S2). Together, we showed that pantothenic acid has a dual effect on regulation in osteoclast differentiation, formation, function, specific TF and marker expressions.

Molecular mechanism of the dual effects of pantothenic acid in regulating osteoclastogenesis

To understand the underlying molecular mechanism, we detected the influence of pantothenic acid in RANKL stimulated intracellular reactive oxygen species (ROS) accumulation using DCFH (**Figure 4A**). Quantification analysis
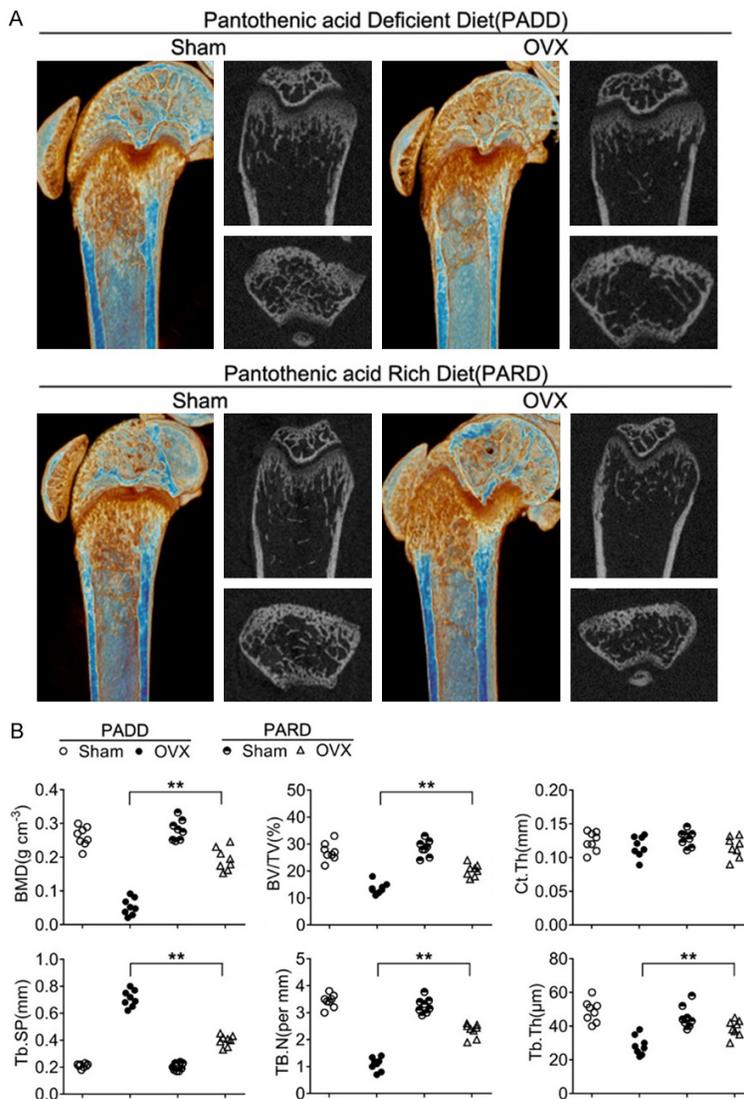
Figure 5. Pantothenic acid rich dietary alleviates OVX induced bone loss. A. Representative microCT longitudinal section images of femurs, cross-sectional view of the distal femurs and reconstructed trabecular structure of the region of interest (white solid box). Color scale bar represents the bone mineral density level. B. Quantitative microCT analysis of average femur length, distal femoral volumetric bone mineral density (BMD), trabecular bone volume fraction (BV/TV), trabecular separation (Tb.Sp), trabecular number (Tb.N), cortical thickness (Ct.Th) and trabecular thickness (Tb.Th) in each group. The data in the figures represent the averages ± SD. Statistically significant differences between the treatment and control groups are indicated as * ($P < 0.05$) or ** ($P < 0.01$).

thenic acid treatment at the concentration of 200 µM but were then suppressed at the concentration of 1000 µM marked by the phosphorylation of PI3K and Akt (**Figures 4C**, S3). Moreover, we further found that pantothenic acid up regulated the expressions of FoxO1, FoxO2 and Nrf2 at the concentration of 1000 µM but not at 200 µM, suggesting its acquirement of ROS scavenging activity at the higher dosage levels (**Figures 4D**, S3). We then investigated the MAPK signaling pathway using western blot. The results showed that both p38, ERK and JNK were activated by pantothenic acid at the concentration of 200 µM but were inhibited at the concentration of 1000 µM (**Figures 4E**, S3). Our results suggested that lower dosage of pantothenic acid (200 µM) increases osteoclastogenesis via activation of PI3K-Akt signaling while higher dosage of pantothenic acid (1000 µM) inhibits osteoclastogenesis via activating FoxO1, FoxO2 and Nrf2 and the following scavenging of RANKL induced ROS generation. This explained the dual effects of pantothenic acid in regulating RANKL induced osteoclastogenesis.

Pantothenic acid rich dietary alleviates bone loss in OVX mice

To investigate the *in vivo* effects of pantothenic acid in skeleton, we adopted the osteoporotic model of OVX mice.

revealed that both ROS positive cell number and mean DCF fluorescence were increased by pantothenic acid of 200 µM but were significantly brought down at the concentration of 1000 µM (**Figure 4B**). For better clarification, we first detected the PI3K-Akt signaling pathway influenced by pantothenic acid. Western blot analysis showed that PI3K-Akt signaling pathway was remarkable activated by panto-

Female mice were randomly arranged with pantothenic acid deficient dietary (PADD) or pantothenic acid rich dietary (PARD) one month after birth. Ovariectomy was performed at two months old followed by continuous dietary pattern before sacrifice at five months old. µCT was performed scanning the dissected femurs of sham operated mice or OVX mice from the PADD or PARD groups (**Figure 5A**). The results

showed that OVX mice in both PADD and PARD groups exhibited obvious bone loss compared with respective sham operated mice. However, quantification analysis revealed that OVX mice in the PARD group showed significantly increased bone mineral density (BMD), trabecular bone volume fraction (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th) and decreased trabecular separation (Tb.Sp) compared with the PADD group, suggesting a protective effect of pantothenic acid against OVX induced bone loss (**Figure 5B**). The results showed that dietary pantothenic acid is protective against estrogen deficiency induced bone loss.

Discussion

Recently, B vitamins have been reviewed for their possible roles in bone health in two reviews. Specific role of B2, B3, B6, folate, and B12 in bone physiology has been recognized. However, the potential role of pantothenic acid in bone metabolism and skeletal health remains unknown. It has been proved that increased levels of homocysteine induces osteoclast differentiation and bone resorption and inhibits bone formation [24, 25]. Animal studies have also revealed that lacks of folic acid, Vitamins B6 and B12 contributes to enhanced levels of Hcy, suggesting the regulation of osteoclast differentiation may be a possible way that B vitamins influence bone health [26].

Pantothenic acid (B5) is an important substrate for the synthesis of coenzyme A (CoA), deficiency pantothenic acid leads to blocked CoA synthesis [27]. Except for contributing to the synthesis of cholesterol, amino acids, phospholipids, and fatty acids, CoA also play an important role in regulating intracellular ROS [28]. Therefore, lack of pantothenic acid may be one of the important reasons for ROS accumulation. Interestingly enough, although ROS have certain damage to normal cells, it is a crucial factor that promote osteoclast differentiation and bone resorption [29, 30]. Recent study has also demonstrated that ROS accumulation in OC is vital for osteoclastogenesis and bone homeostasis [15]. In detail, MAP kinases including p38, ERK and JNK are crucial for osteoclast proliferation and differentiation, and both of these genes can be activated by ROS [13, 31]. On the other hand, several genes including FoxO1, FoxO3 and Nrf2 will be activated to transcript antioxidant enzymes [32, 33]. In this

study, we further detected the functions of pantothenic acid in osteoclastogenesis. We found that pantothenic acid reveals a dual effect on RANKL induced osteoclast differentiation in a dose-dependent way. Mechanistically, low concentration of pantothenic acid activates PI3K-Akt to support proliferation and differentiation of pOC; high concentration of pantothenic acid inhibits osteoclastogenesis by scavenging the generation of ROS.

In this study, we found pantothenic acid has a dual effect in osteoclast differentiation. Lower concentration of pantothenic acid induce osteoclast differentiation by stimulating PI3K-Akt pathway, while higher concentration of pantothenic acid suppress osteoclastogenesis by scavenging ROS generation, with upregulating the expressions of FoxO1, FoxO2 and Nrf2. Animal studies using ovariectomized (OVX) model suggested a potential role of pantothenic acid rich dietary in protecting bone loss against estrogen deficiency. In summary, this study recognized the potential role of VB5 in maintaining bone homeostasis and revealed a therapeutic potential of pantothenic acid diets for a preventive against osteoclast related disorders.

Acknowledgements

Disclosure of conflict of interest

None.

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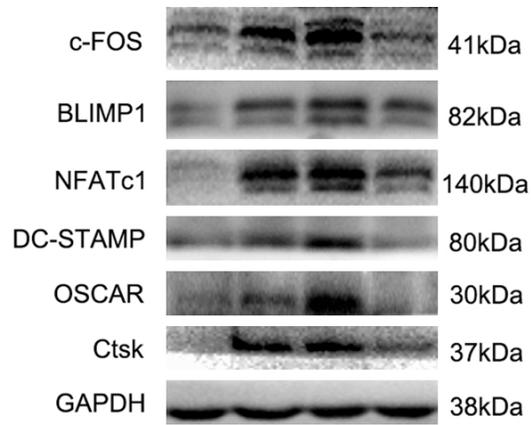
Figure S1. The western images of **Figure 3**.
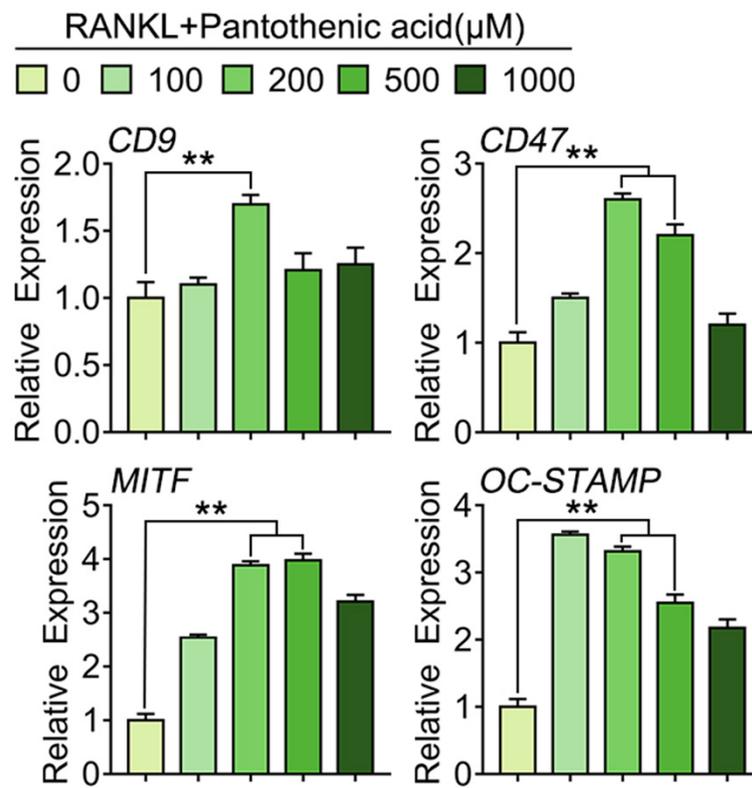
Figure S2. Relative mRNA expression levels of *CD9, CD47, MITF* and *OC-STAMP* in osteoclasts treated with different dosages of pantothenic acid.
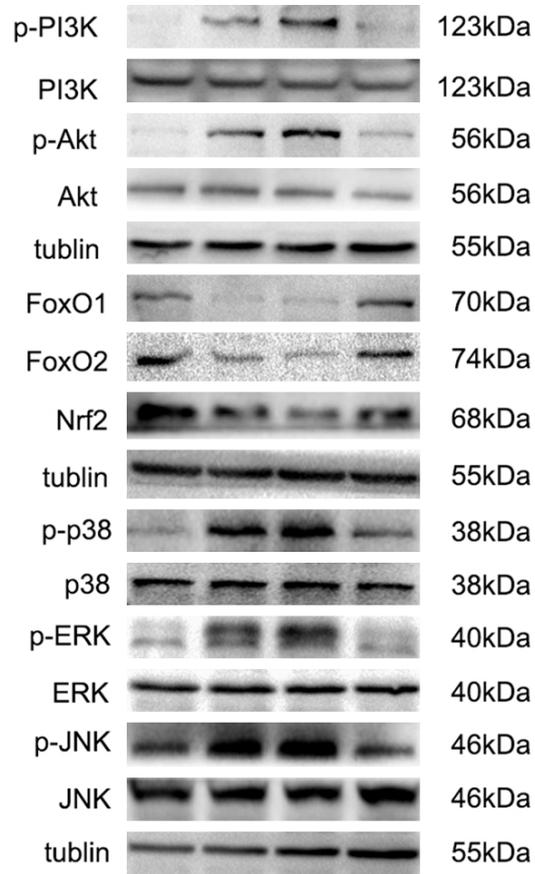
Figure S3. The western images of **Figure 4**.