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Fructose 1,6-bisphosphate inhibits osteoclastogenesis by attenuating RANKL-induced NF-κB/NFATc-1

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Abstract

Background Although some glycolytic intermediates have been shown to modulate several cell type formation and activation, the functional role of fructose 1,6-bisphosphate (FBP) on osteoclastogenesis is still unknown.

Methods Osteoclastogenesis was evaluated on bone marrow preosteoclasts cultured with M-CSF − 30 ng/ml, RANKL − 10 ng/ml, and two concentrations of FBP (100 and 300 μM). TRAP-positive stained cells were counted, and osteoclastogenic marker genes expression were evaluated by qPCR. Osteoclasts resorption capacity was evaluated by the expression of specific enzymes and capacity to resorb a mineralized matrix. The NF-κB activation was detected using RAW 264.7, stably expressing luciferase on the NF-κB responsive promoter.

Results We show that FBP, the product of the first stage of glycolysis, inhibited RANKL-induced osteoclasts differentiation and TRAP activity. The treatment of preosteoclasts with FBP attenuated osteoclast fusion and formation, without affecting cell viability. Moreover, the inhibition of several osteoclastogenic marker genes expression (TRAP, OSCAR, DC-STAMP, Integrin αv, NFATc1) by FBP correlates with a reduction of mineralized matrix resorption capacity. The mechanism underlying FBP-inhibition of osteoclastogenesis involves NF-κB/NFATc1 signaling pathway inhibition.

Conclusion Altogether these data show a protective role of a natural glycolytic intermediate in bone homeostasis that may have therapeutic benefit for osteolytic diseases.

Keywords Bone remodeling · Osteoclasts · NFATc1 · Fructose 1,6-bisphosphate

Introduction

The bone is a specialized rigid tissue that is continuously being remodeled in the growing and adult skeleton, in response to mechanical and other stimuli. The active remodeling process of bone is orchestrated mainly by osteoclasts and osteoblasts [1]. Several pathological conditions can break the dynamic balance of bone homeostasis leading to an imbalanced activity of osteoclast or osteoblasts. Excessive bone resorption is associated with several skeletal diseases including osteoporosis, periodontal disease, rheumatoid arthritis, multiple myeloma and metastatic cancers [2, 3]. The osteoclasts are multinucleated giant cells that differentiate from cells of hematopoietic myeloid lineage under the presence of two critical factors: the macrophage–monocyte colony-stimulating factor (M-CSF) and the receptor activator of NF-kB ligand (RANKL) [4, 5]. RANKL binding RANK activates NF-κB signaling in osteoclasts precursors cells which is essential for the initial induction of nuclear factor of activated T cells, cytoplasmic 1 protein (NFATc1). NFATc1 induces the expression of crucial genes for osteoclasts differentiation and function such as tartrate-resistant acid phosphatase (TRAP), cathepsin K, calcitonin receptor (CTR), and integrin β3 [6].

Although the osteoclasts differentiation process includes several steps, the correct sequence of events and activators involved in their formation are not completely clear. As a giant multinucleated cell, osteoclasts undergo a metabolic adaptation to the increased demand for adenosine

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triphosphate (ATP) during the differentiation process that involves proliferation, migration, and fusion. Moreover, mature bone-resorbing osteoclasts also request significantly high-energy during the sealing zone formation and activation of its resorptive machinery by secreting protons and collagenolytic enzymes [7, 8]. Therefore, an extensive metabolic reconfiguration occurs during osteoclasts differentiation and activation course [9, 10].

Glucose is the major extracellular energy source for most mammalian cells activity. Additionally, the previous study has shown evidence demonstrating that glycolytic metabolism was stimulated during differentiation, since they detected increased consumption of glucose and lactate production, increased expression of both glucose transporter 1 and glycolytic enzyme genes during osteoclastogenesis [11, 12]. Moreover, in addition to glucose, another bioenergetic substrate such as pyruvate is also increased during osteoclast differentiation [9]. Apart from its well-known role as an energetic source, the glycolysis provides living cells with intermediates and substrates for other metabolic pathways [13]. However, little is known about the role of those intermediates on osteoclastogenesis.

Fructose 1,6-bisphosphate (FBP), one endogenous intermediate of the glycolytic pathway, is a naturally occurring sugar produced by the phosphorylation of fructose 6-phosphate by phosphofructokinase-1. Interestingly, exogenously administered FBP confers a protective action in myocardial ischemia [14], skin [15] and neuronal damage [16]. More recently, interesting studies have shown that fructose 1,6-bisphosphate attenuates experimental arthritis symptoms [17] and bone loss induced by experimental ovariectomy [18]. Therefore, in the present study, we aimed to investigate the potential protective role of FBP on bone metabolism by investigating the effect of this glycolytic pathway intermediate on RANKL-induced osteoclastogenesis.

Materials and methods

Animals

Male C57/BL6 mice (5–7 weeks old) were obtained from the central animal facility of the University of Sao Paulo (USP-Ribeirao Preto, SP, Brazil). All protocols were conducted following ethical guidelines and approved by the Animal Welfare Committee of the School of Pharmaceutical Sciences of Ribeirao Preto - USP (protocol number: 15.1.750.60.0).

Osteoclastogenesis and cell culture

Bone marrow cells from C57BL/6 mice were flushed from tibia and femur with α-MEM (GIBCO, Invitrogen, Carlsbad, CA, USA). Cells were cultured for 3 days in Petri dishes (Corning, NY, USA) with α-MEM containing 10% Fetal Bovine Serum (FBS; Gibco™), 100 units/mL penicillin, 100 mg/mL streptomycin and 30 ng/mL of M-CSF (R&D Systems, Inc., Minneapolis, MN, USA). Nonadherent cells were washed out, and the adherent cells (preosteoclasts) were cultured in the osteoclastogenic medium: with M-CSF (30 ng/ml) and RANKL (10 ng/ml; R&D System, Inc.), in the presence of 100 and 300 μM of D-Fructose 1,6-bisphosphate (Sigma-Aldrich, St. Louis, MO, USA) based on our previous study [19].

Osteoclast formation assessment and bone resorption assay

Preosteoclasts were seeded on 96-well plates (2×10^4 cells/well) and cultured with the osteoclastogenic medium for 3 or 4 days. Cells were stained with TRAP Kit (Sigma 387-A kit; Sigma-Aldrich), and the number and area of TRAP-positive multinucleated cells (with three or more nuclei) were obtained using the image analyzing system *ImageJ*.

Preosteoclasts were seeded on 24-well culture plates (2×10^5 cells/well) and cultured for 3 days. Cell lysates were obtained with RIPA Buffer (Sigma-Aldrich), and the sample was incubated with the Tartrate-Resistant Acid Phosphatase solution (TRAP-Sigma 387-A kit) at 37 °C for 30 min. The TRAP activity was determined by measuring the absorbance of the cells at 580 nm in a microplate reader. Protein amounts were quantified by the BCA Protein Assay Kit (Pierce®, Thermo Scientific). Data are expressed as the mean optical density (O.D.)/protein.

Preosteoclasts were seeded on 96-well osteoassay surface plate (Corning, NY, USA) at a density of 2×10^4 cells/well and cultured with the osteoclastogenic medium for 96 h. After the culture period, cells were removed using 3% sodium hypochlorite (5 min). The resorption area was analyzed under a stereomicroscope (Leica Microsystems).

Cell viability assay

Preosteoclasts were seeded on 96-well culture plates (2×10^4 cells/well) and cultured with osteoclastogenic medium and D-Fructose 1,6-bisphosphate for 24 h. Then, the cells were washed with PBS and incubated for 3 h in a colorimetric substrate MTT (3-(4,5-dimethylthiazol-2-thiazolyl)-5-diphenyltetrazolium bromide; Sigma-Aldrich) at a final concentration of 5 mg/ml. Next, the cells were dissolved in Dimethyl Sulfoxide (DMSO), and the cell viability was determined by measuring the absorbance at 570 nm in a microplate reader. The cell viability was expressed as a percentage relative to the untreated control cells.

ATP quantification

The concentration of intracellular ATP was measured in cell lysate using ATPlite Luminescence assay kit (PerkinElmer Inc., Waltham, MA, USA), following the manufacturer's instructions.

Lactate quantification

Lactate concentration was measured in cell culture supernatant using a colorimetric assay kit (Quibasa-Bioclin, Belo Horizonte, MG, Brazil), following the manufacturer's instructions.

Quantitative PCR analysis

Preosteoclasts were seeded on 24-well culture plate (2×10^5 cells/well) and cultured with the osteoclastogenic medium for 72 h. Total RNA was extracted from cells using the SV Total RNA Isolation System (Promega). cDNA was reverse-transcribed from 500 ng of total RNA using cDNA High-Capacity synthesis kit (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). Quantitative PCR (qPCR) was carried out on a StepOnePlus sequence detection system (Applied Biosystems, Carlsbad, CA, USA) using specific primers and TaqMan Universal master mix II (Applied Biosystems). Relative gene expression was calculated according to the $2^{-\Delta\Delta CT}$ method, using GAPDH as endogenous control. Two independent biological replicates were performed. Data are shown as fold increase over the control group without FBP. Specific primers for *Nfatc1* (Mm00479445_m1), *Acp5* (Mm00475698_m1), *Oscar* (Mm00558665_m1), *Ctsk* (Mm00484039_m1), *Dcstamp* (Mm04209236_m1), *Itgav* (Mm00434486_m1) and *Gapdh* (Mm99999915_g1) genes were employed (TaqMan® Gene Expression Assay).

Western Blot analysis

Preosteoclasts were seeded on 24-well culture plates and cultured with the osteoclastogenic medium for 72 h. After incubation, the cells were washed with PBS, and whole cell lysates were obtained with RIPA Buffer (Sigma-Aldrich) containing a cocktail of protease and phosphate inhibitors (Sigma-Aldrich). Protein samples (10 µg) were subjected to electrophoresis in 10% SDS–PAGE gels and transferred to PVDF membranes (Immobilon-P; MerckMillipore, Billerica, MA, USA). Membranes were incubated overnight at 4 °C with primary antibodies (anti-NFAT2 1:2000) and anti-Cathepsin K 1:3000 from abcam®, and anti-β-actin (as a loading control) 1:30,000 (Sigma-Aldrich) and with secondary anti-mouse (ab6728, abcam®) or anti-rabbit (ab6721, abcam®) antibody for 1 h. Protein bands were quantified by densitometer using the image analyzing system *ImageJ*. The target protein level was normalized to the β-actin.

Luciferase assay of NF-κB activity

Murine macrophage cell line RAW 264.7, stably expressing luciferase on the NF-κB responsive promoter (pNF-κB-Luc) was cultured in RPMI medium (Sigma) containing 10% FBS (v/v), penicillin (100 U/mL) and streptomycin (100 µg/mL), at 37 °C in a 5% CO_2. The RAW 264.7 cells (3×10^5 cells/well) were cultured in the presence of FBP (100 µM or 300 µM) for 6 h before stimulation with RANKL (100 ng/mL) for another 6 h. Intracellular contents were extracted in lysis buffer, and the luciferase activity was determined on a luminometer (FlexStation 3 Microplate Reader, California, USA) using the dual luciferase reporter assay system (Promega, Wisconsin, USA). Results are expressed as relative luciferase activity (fold difference compared to negative control).

Statistical analysis

All quantitative data are presented as the mean ± SD. Each set of independent experiments ($n = 3$–5 in each group) was performed two or three times, and results from one representative experiment are shown. Statistical analyses were performed using One-way ANOVA following by Bonferroni post-test for comparison of more than two groups (GraphPad Software, San Diego, CA, USA). *P* values less than 0.05 were considered significant.

Results

FBP inhibits osteoclast differentiation and function

To investigate the effect of FBP on osteoclastogenesis, we cultured bone marrow preosteoclasts with osteoclastogenic medium (M-CSF − 30 ng/ml and RANKL − 10 ng/ml) and two concentrations of FBP (100 and 300 µM). Osteoclasts, identified as TRAP-positive multinucleated cells stained purple-red, were quantified after 72 h of culture. Our data show that FBP suppressed RANKL-induced osteoclasts differentiation (Fig. 1a) as it reduced TRAP-positive cell number, in a dose-dependent manner (Fig. 1b). TRAP activity measured in the extract of mature osteoclasts (Fig. 1c) was markedly reduced, further elucidating the inhibitory effect of FBP on TRAP activity at the cellular level. We next examined the effect of FBP on the expression of osteoclastogenic marker genes by qPCR. We found that the mRNA expression of *Acp5*, a specific osteoclasts differentiation marker gene was significantly attenuated by 100 and 300 µM of

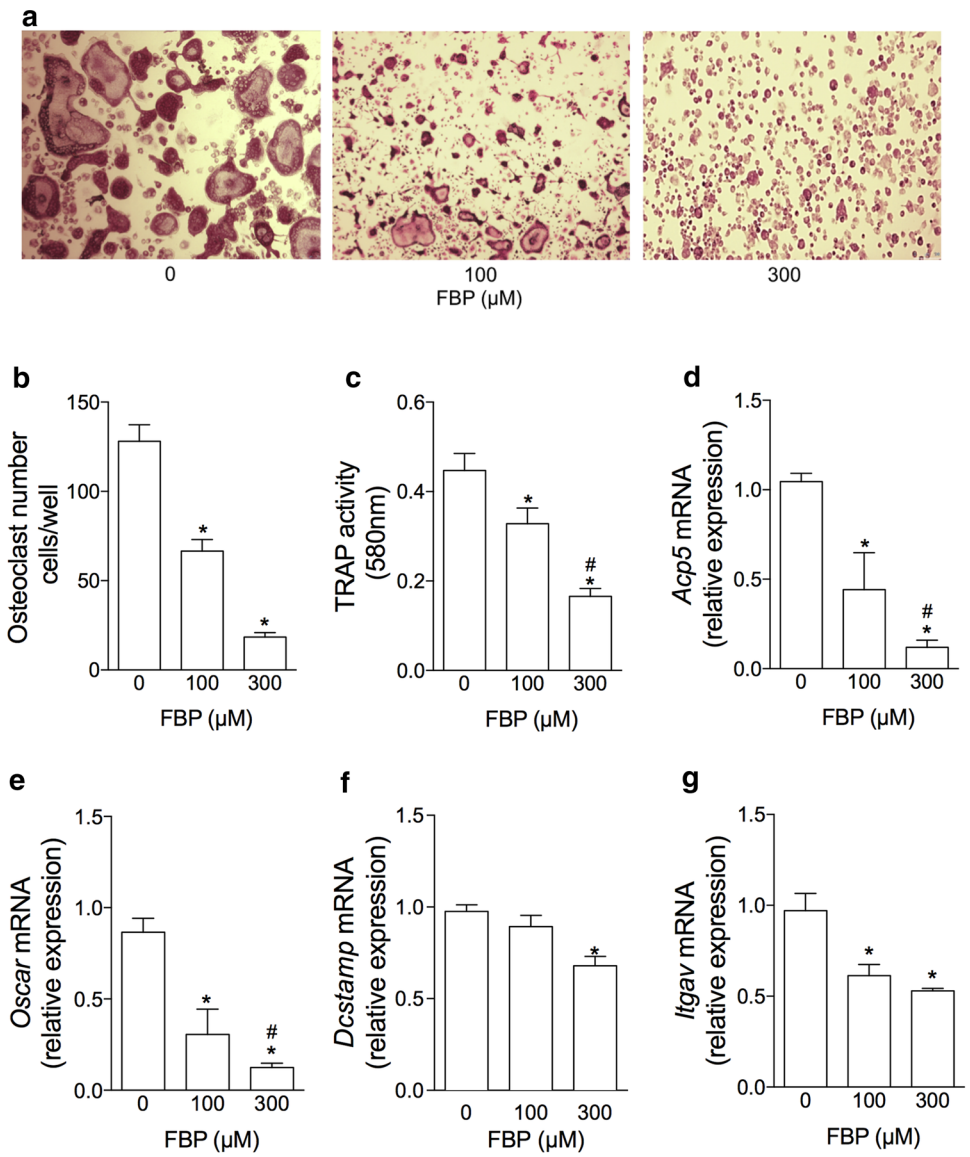

Fig. 1 Effect of Fructose 1,6-bisphosphate on RANKL-induced osteoclasts differentiation and expression of specific mRNA. BMMs were cultured with M-CSF (30 ng/mL) and RANKL (10 ng/mL) in the presence of 100 and 300 μM of FBP. RANKL-induced osteoclasts cultured for 96 h were stained as TRAP-positive cell (**a**). TRAP-positive cells with more than three nuclei were quantified (**b**). Effect of FBP (100 and 300 μM) on TRAP activity measured at 72 h of differentiation (**c**). Expression of osteoclastogenic markers genes *Acp5* (**d**), *Oscar* (**e**), *Dcstamp* (**f**) and *Itgav* (**g**) was evaluated by Real-Time PCR. The data shown are the mean ± SD of one representative ($n = 5–6$ per group) of three independent experiments. $^{*}P < 0.05$ versus cell without FBP. $^{\#}P < 0.05$ versus FBP 100 μM

FBP (Fig. 1d). Similarly, the level of osteoclasts signature genes (*Oscar, Dcstamp, and Itgav*) were detected at a lower level in osteoclasts treated with FBP when compared to the untreated osteoclasts (Fig. 1e–g).

Since we determined that FBP promotes inhibition of osteoclasts formation, we next investigated the effect of FBP on resorption capacity. Consistent with the reduction in osteoclasts marker genes expression, osteoclasts treated with FBP expressed a significantly lower level of cathepsin K gene and protein (Fig. 2a, b). In line, our findings also demonstrate that osteoclasts cultured with FBP also exhibited reduced resorbed area in a mineralized matrix (hydroxyapatite-coated plates) (Fig. 2c, d). Together, all these data indicated that FBP, in a concentration-dependent manner, markedly downregulates RANKL-induced osteoclasts differentiation that resulted in reduced resorption.

FBP activates the preosteoclast glycolytic pathway

Having demonstrated that FBP inhibits osteoclasts formation, we next checked the effectivity of FBP on osteoclasts and cell viability. Our data show that FBP can activate glycolysis on preosteoclasts, as evidenced by the increased intracellular level of lactate and ATP triggered by FBP treatment (Fig. 3a, b). Moreover, to exclude the FBP-induced cytotoxicity during OC formation, MTT assay was performed. Interestingly, as shown in Fig. 3c, FBP did not show cytotoxicity against BMMs at any of the concentration used in this study, suggesting that FBP could inhibit OC formation without cytotoxicity in vitro.

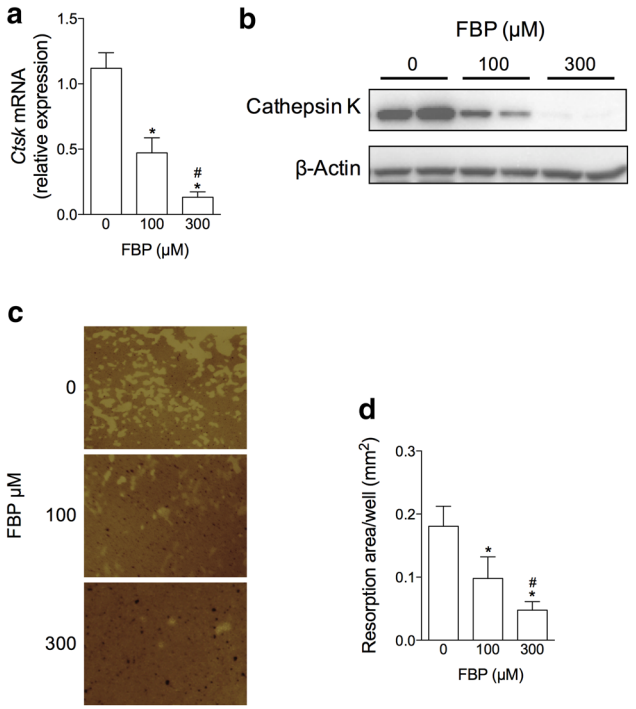

Fig. 2 Fructose 1,6-bisphosphate prevent osteoclasts pit formation. BMM were cultured with M-CSF (30 ng/ml) and RANKL (10 ng/ml) for 3 days in the presence of 0, 100 or 300 μM of the FBP. The gene expression (**a**) and protein (**b**) levels of cathepsin K were determined by Real-time PCR and Western Blot respectively. BMM were seeded in the hydroxyapatite-coated plate and cultured with osteoclastogenic medium with 100 or 300 μM of the FBP, and after 6 days, the resorption pits area was evaluated (**c**) and scored (**d**). The data shown are the mean ± SD ($n = 3$–5 per group), and graphics are representative of two or three independent experiments. *$P < 0.05$ versus cell without FBP. #$P < 0.05$ versus FBP 100 μM

FBP decreases the protein levels of NFATc1 and NF-κB activation

NFATc1 is considered a key transcription factor involved in osteoclasts differentiation and activation, thus to elucidate the molecular mechanisms underlying the inhibitory effect of FBP on osteoclastogenesis we assessed the NFATc1 expression and activation (Fig. 4a). Our data show that FBP attenuates RANKL-induced NFATc1 mRNA expression. Additionally, western blot analysis further revealed that FBP treatment decreased RANKL-mediated induction of NFATc1 protein, in a dose-dependent manner (Fig. 4b). Since the activation of RANK in preosteoclasts surface leads to NF-κB activation and transcription of downstream target genes, including NFATc1, we explored whether the mechanism underlying FBP inhibition of osteoclastogenesis involves direct inhibition of NFATc1 or upstream, targeting NF-κB on preosteoclasts. Our data show that FBP suppressed the RANKL-induced NF-κB reporter activity (4.3-fold) (Fig. 5).

Discussion

Mature osteoclasts request high levels of ATP for the biosynthesis of several components necessary for bone resorption. A previous study has shown that high levels of glucose increase osteoclast differentiation, whereas glucose deprivation inhibits the resorption capacity of these cells [12]. On the other hand, the literature highlighted the regulatory role of molecules involved in glycolysis and the energetic demands during osteoclastogenesis, for example, fructose 1,6-bisphosphate, one endogenous intermediate of the glycolytic pathway has been reported to exhibit a regulatory/

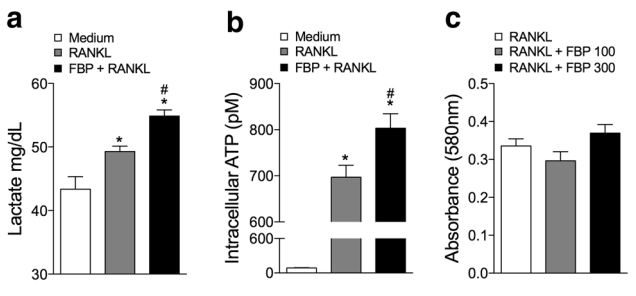

Fig. 3 Fructose 1,6-bisphosphate activate preosteoclasts without affecting cell viability. Osteoclasts were incubated with FBP (100 or 300 μM) for 6 h before stimulation with RANKL (100 ng/mL). After 3 h of RANKL stimulation, the level of lactate (**a**) in the supernatant and intracellular ATP (**b**) was measured. Osteoclasts were stimulated with 0, 100 or 300 μM of the FBP. Cell viability was identified in preosteoclasts by MTT after 24 h and of FBP stimulation (**c**). Graphic bars indicate the mean ± SD of one representative experiment ($n = 4$–5 per group). *$P < 0.05$ versus cell without FBP. #$P < 0.05$ versus FBP 100 μM

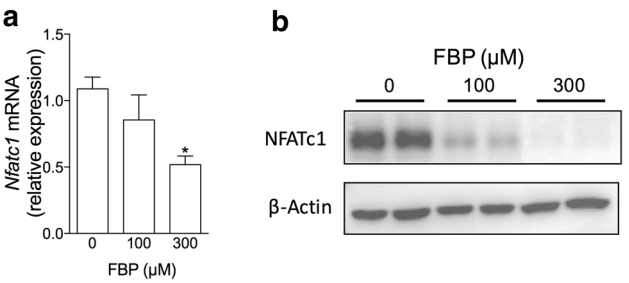

Fig. 4 Fructose 1,6-bisphosphate inhibits RANKL-induced expression of NFATc1. BMM were cultured with M-CSF (30 ng/ml) and RANKL (10 ng/ml) for 3 days in the presence of 0, 100 or 300 μM of the FBP. The gene expression (**a**) and protein (**b**) levels of NFATc1 were determined by real-time PCR and western blot. Graphic bars indicate the mean ± SD of one representative ($n = 4$–5 per group) of two independent experiments. The blot bands are a representative of 6 samples performed in two independent experiments ($n = 3$ each experiment). *$P < 0.05$ versus cell without FBP

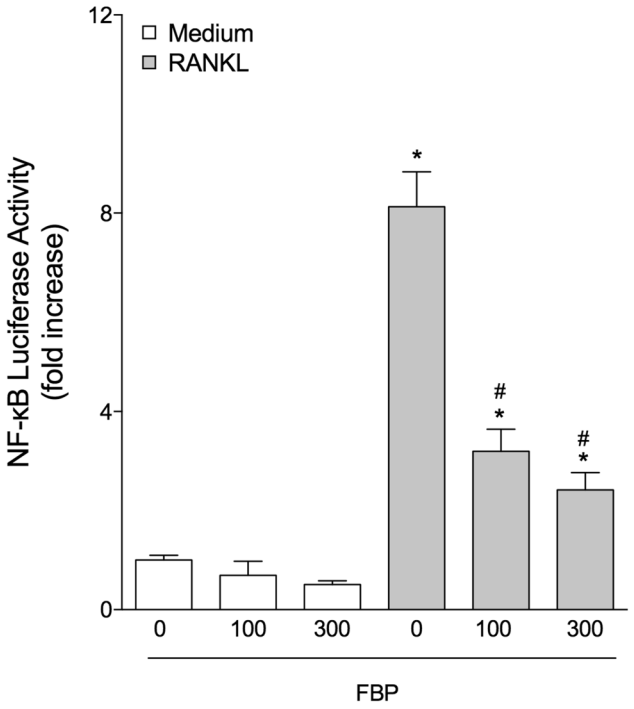

Fig. 5 Fructose 1,6-bisphosphate suppresses RANKL-induced NF-κB-dependent luciferase activity. RAW 264.7 expressing luciferase on the NF-κB responsive promoter (pNF-κB-Luc) were cultured with FBP (100 μM or 300 μM) for 6 h before stimulation with RANKL (100 ng/mL). The luciferase activity analysis was performed in culture treated with RANKL for 6 h. Results are expressed as relative luciferase activity. Bars represent fold difference compared to respective negative control. The data shown are the mean ± SD ($n = 5$ per group). $^{\#}P < 0.05$ versus negative control. $^{*}P < 0.05$ versus vehicle-treated RANKL-stimulated cells

protective activity in an inflammatory bone disorder. The combination of fructose 1,6-bisphosphate with strontium (FBP-Sr) appeared to be safe and effective for the treatment of postmenopausal osteoporosis in vivo [18]. Similarly, an exogenous administration of FBP markedly attenuated experimental arthritis by reducing the neutrophil infiltrate, articular hyperalgesia, and joint swelling [17]. Although diverse mechanisms explaining the FBP regulatory properties have been suggested, the FBP-induced protection mechanism on bone metabolism remains unclear.

In the present study, FBP significantly suppressed the RANKL-induced osteoclastogenesis, without causing a cytotoxic effect at the doses tested in this study. We show that FBP effectively inhibited multinucleated osteoclast formation, in a dose-dependent manner, which is demonstrated by the reduction of the number and area of TRAP-positive multinuclear cells. Confirming this data, we also showed that TRAP activity, a hallmark enzyme released by osteoclasts, was significantly reduced by FBP treatment. During osteoclastogenesis, RANKL binding to RANK induces a cascade of phosphorylation events that culminate with the expression of several osteoclast-related marker genes, illustrating each stage of differentiation [20]. Here, the treatment of preosteoclasts with FBP also reduced the expression of osteoclast-related marker genes such as NFATc1, TRAP (the hallmark of osteoclasts differentiation), DC-STAMP, integrin and OSCAR (markers related to the cell fusion). Thus, our data suggest that FBP inhibited RANKL-induced osteoclast differentiation by constraining the cell–cell fusion process.

Considering the unique morphology of multinucleated osteoclasts and their mission to resorb bone, by secreting acids and proteases, this cell type demand energy and a metabolic adaptation not only during the fusion stage, but also during their resorptive machinery activation [7]. Cathepsin K is the most abundant enzyme found in the resorption lacunae, together with TRAP and MMP-9 [21, 22]. Therefore, a decreased expression of CTK and TRAP, two essential enzymes for organic matrix dissolution, on FBP treated osteoclasts would impact on osteoclasts biological effect in bone resorption in vitro. Consistent with the reduction in osteoclasts formation, the bone resorption assay confirmed the reduced resorptive capacity of FBP-treated osteoclast, evidencing that FBP reduces osteoclastogenesis and consequently restrain osteoclast function.

Trying to go deep on the mechanism underlying the FBP protection on osteoclast formation and function, we evaluated the RANKL/RANK signaling pathway. It is known that the RANKL-RANK interaction results in the activation of osteoclastogenic transcription factors such as NF-κB in preosteoclasts, activator protein 1 (AP-1), and following nuclear factor of activated T cells 1 (NFATc1), the late induces the expression of osteoclastogenic markers genes [23, 24]. Thus, to explore the molecular mechanism underlying the inhibitory effect of FBP we evaluated the expression and induction of two molecules crucial for osteoclastogenesis NF-κB and NFATc1. Our data show that FBP inhibits osteoclast differentiation and function was associated with the down-regulation of NFATc1. Mainly, NFATc1 is known as the master regulator of osteoclastogenesis since it regulates several osteoclast-specific genes such as TRAP, cathepsin K, calcitonin receptor [23].

Moreover, NF-κB signaling is induced rapidly in preosteoclasts in response to RANKL, which is upstream NFATc1 expression and OC formation. Thus, to clarify whether FBP target is NFATc1 or upstream modulating NF-kB on preosteoclasts, we also checked the NF-kB activation under FBP. Our data show that FBP inhibits osteoclast differentiation by blockage of NF-κB in preosteoclasts and consequently NFATc1 transcriptional cascade.

In summary, this study highlights the importance of metabolic processes controlling osteoclastogenesis by providing new knowledge of the direct effect of FBP in osteoclasts formation and function. It further suggests that this natural glycolytic intermediate has a high potential to be included,

in the future, as a promising agent for the treatment of bone loss-associated diseases.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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