Spinosin Attenuates Osteoclastogenesis Through Suppressing NF- κ B by the Activation of Nrf2/HO-1 Signaling in Osteoporosis

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Research

Keywords: osteoporosis, osteoclastogenesis, Spinosin, Nrf2/HO-1 signaling, NF- κ B

Posted Date: January 29th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-154482/v1>

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Abstract

Objective

Osteoporosis is a prevalent metabolic skeletal disorder featured by microarchitecture bone injury and excessive osteoclastic activity. Here, we aimed to explore the effect of Spinosin on osteoclastogenesis of osteoporosis.

Design

The receptor activator of nuclear factor-kappaB ligand (RANKL)-induced osteoclastogenesis model was established in bone marrow macrophages (BMMs) *in vitro*. The ovariectomy (OVX)-induced bone loss mouse model was constructed *in vivo*, followed by micro-CT analysis, Histomorphometric analysis, Hematoxylin and Eosin (H&E) and TRAP staining.

Results

Our data showed that the treatment of Spinosin significantly inhibited the TRAP positive osteoclast and bone resorption induced by RANKL in the BMMs. Spinosin significantly reduced the expression of osteoclast-specific factors, including osteoclast stimulatory transmembrane protein (OC-STAMP), dendritic cell-specific transmembrane protein (DC-STAMP), cathepsin K (CTSK), TRAP, c-Fos and nuclear factor of activated T cells cytoplasm 1 (NFATc1) in the RANKL-treated BMMs. Mechanically, Spinosin was able to inactivate NF- κ B by stimulating Nrf2/HO-1 signaling in BMMs. The trabecular space (Tb.Sp), trabecular number (Tb.N), trabecular thickness (Tb.Th), and bone volume to total volume (BV/TV) were inhibited by OVX treatment, and Spinosin could reverse the effect in the bone resorption mouse model. The OVX-induced serum levels of tumor necrosis factor- α (TNF- α) and tartrate-resistant acid phosphatase 5 B (TRAcP5B) were blocked by Spinosin in the mice. Moreover, Spinosin was able to alleviate OVX-induced loss of femur bone and osteoclasts *in vivo*.

Conclusions

In conclusion, Spinosin attenuates osteoclastogenesis of osteoporosis through inhibiting NF- κ B by activating Nrf2/HO-1 expression. Spinosin may serve as the potential candidate for the treatment of osteoporosis.

1. Introduction

Osteoporosis serves as a prevalent skeletal disorder featured by the weak bone magnitude and microarchitecture bone injury, affecting 10 of thousands of post-menopausal women because of estrogen insufficiency (1, 2). A recent report shows that 65-years men and 55-years women and older possess a heightened fracture chance affected by osteoporosis (3). Osteoporosis fracture is correlated with enhanced death and cause an enormous financial burden of modern people and society (4), yet currently, rare clinical medications are accessible for osteoporosis (5–7). In recent years, targeting

osteoclastogenesis becomes an available healing approach to osteoporosis (8, 9). However, the popular anti-osteoporosis therapies, such as bisphosphate and denosumab, accompany the jaw necrosis risk, leading to application limitation (10, 11). Therefore, innovative alternative treatment candidates for osteoporosis that are able to regulate osteoclastogenesis through various mechanisms with advanced risk-benefit outlines are necessarily required.

Traditional Chinese herbs have been applied thousands of years for the treatment of human diseases and have drawn growing attention in drug discovery investigation in recent years (12). Suanzaoren, also termed Semen Ziziphi Spinosae (SZS), is the *Ziziphus jujuba* Mill (13). Usually, SZS serves as a hypnotic-sedative plant, and Asian medication practitioners generally utilize it to prescribe anxiety and insomnia (14). SZS includes more than fifty active composites, such as C-glycoside flavonoids, cyclopeptide alkaloids, and saponins (15). Among them, a C-glycoside flavonoid called 2"- β -O-glucopyranosyl swertisin (Spinosin) is applied in Western countries as healthcare agents and exhibits biomedical activities, including hypnotic and anxiolytic impacts (16). Besides, Spinosin has been identified to enhance mice's cognitive accomplishment, attenuate memory deficiencies, and improve neurogenesis (17, 18). Recent research indicates that Spinosin displays neuroprotective influences in Alzheimer's disease mouse model (19). Moreover, Spinosin is able to stimulate Nrf2/HO-1 signaling to inhibit the aggregation and production of A β_{1-42} (20). Spinosin may present a potential role in the modulation of osteoporosis due to that Nrf2/HO-1 signaling performs a important function during osteoclastogenesis.

The development of osteoporosis and osteoclastogenesis is complicated and many crucial signaling pathways are involved in the processes. Oxidative stress is a crucial process correlated with osteoporosis's pathogenesis, and the oxidative stress progression-induced modulation of the Nrf2/HO-1/NF- κ B (p65) signaling has been extensively investigated in osteoporosis as well (21). More and more evidence shows that the accumulation of reactive oxygen species (ROS) causes oxidative stress at condition of medicine stimulation, illnesses, or aging, and followed by inhibiting the stimulation of Nrf/HO-1 expression and enhancing p65, leading to the progression and genesis of osteoporosis (22, 23). Accordingly, the agents that is able to activate Nrf/HO-1 signaling and thereby suppress NF- κ B signaling may be the agents for the osteoporosis therapy.

In this study, we were interested in the role of Spinosin in the pathogenesis of osteoporosis. We identified that Spinosin could inhibit RANKL-induced osteoclastogenesis *in vitro*. Spinosin was able to attenuate OVX-induced loss of femur bone and osteoclasts *in vivo*. Melancholically, Spinosin inhibiting NF- κ B by enhancing Nrf2 expression. We demonstrated a novel function of Spinosin in alleviating osteoclastogenesis of osteoporosis by suppressing NF- κ B through activating Nrf2/HO-1 signaling.

2. Materials And Methods

2.1. The isolation of bone marrow macrophages (BMMs)

BMMs were used to obtain the osteoclast progenitor cells. Shortly, BMMs were obtained from 8-10-week-old C57 mice's femurs by flushing the MEM (Gibco, USA) medium's bone marrow cavity. BMMs were cultured in MEM medium with 10% FBS- α -MEM (Gibco, USA). The unattached BMMs were gathered and cultured for three days in the MEM medium with M-CSF (25 ng/mL) and FBS (10%) to settle BMMs

2.2. Osteoclast differentiation

For the osteoclast differentiation, BMMs were collected in α -MEM medium with M-CSF (25 ng/mL), RANKL (100 ng/mL), and FBS (10%). The MC3T3-E1 cells were cultured in the α -MEM medium (Gibco, USA) at 37°C with 5% CO₂. For osteoblast differentiation, the cells were plated into the 24-well wells and incubated for 7 days or 21 days in the α -MEM comprising 10% FBS, ascorbic acid (50 μ g/ml, Sigma, USA). The osteoblast differentiation was analyzed by ALP staining and alizarin red staining, respectively. The siRNA of Nrf2 (5'-GUAAGAAGCCAGAUUUAAAdUdU-3') was obtained (RiboBio, China) The transfection in the cells was performed by Liposome 3000 (Invitrogen, USA) according to the manufacturer's instructions.

2.3. CCK-8 assay

The cell viability was analyzed by the CCK-8 assays. About 5×10^3 BMMs were plated in 96-well plates and incubated for 12 hours, followed by indicated treatment. The BMMs were incubated with a CCK-8 solution (KeyGEN Biotech, China) and culture for another 2 hours at 37°C. The cell viability was analyzed at 450nm absorbance by applying the ELISA browser (Bio-Tek EL 800, USA).

2.4. Tartrate-resistant acid phosphatase (TRAP) staining

Cells were cleaned using PBS and fixed for 5 minutes based on the paraformaldehyde. Cells were cultured at 37°C for 1 hour away from light in the reaction from Leukocyte Acid Phosphatase Assay Kit (Sigma) according to the manufacturer's guidance. Cells were distilled by water, and TRAP-positive cells comprising five or more nuclear were imaged by microscopy and calculated as mature osteoclasts. The quantification was performed by applying the ImageJ software.

2.5. Bone resorption assay

The bone resorption was analyzed by applying a pit formation assay. BMMs were plated in the 24-well plates. The cells were re-collected every three days with fresh α -MEM medium. After 5 days, the cells were treated with NH₄OH (1N, 5 minutes) to eliminate the attached BMMs. The bone resorption area was quantified by utilizing the imageJ software.

2.6. Quantitative reverse transcription-PCR (qRT-PCR)

The total RNAs were extracted by TRIZOL (Invitrogen, USA) from the tissues and cells. The first-strand cDNA was synthesized using Stand cDNA Synthesis Kit (Thermo, USA) as the manufacturer's instruction. The qRT-PCR was carried out by applying SYBR Real-time PCR I kit (Takara, Japan). The standard control for mRNA was GAPDH. Quantitative determination of the RNA levels was conducted by SYBR

GreenPremix Ex Taq™ II Kit (TaKaRa, Japan). The experiments were independently repeated at least three times. The primer sequences are as follows:

DC-STAMP Forward: 5'-AAAACCCTTGGGCTGTTCTT-3',

DC-STAMP Reverse: 5'-AATCATGGACGACTCCTTGG-3'

c-Fos Forward: 5'- CGGGTTTCAACGCCGACTA-3'

c-Fos Reverse: 5'- TGGCACTAGAGACGGACAGAT-3'

OC-STAMP Forward: 5'- CTGTAAACGAACTACTGACCCAGC-3'

OC-STAMP Reverse: 5'- CCAGGCTTAGGAAGACGAAGA-3'

NFATc1 Forward: 5'-CCGTTGCTTCCAGAAAATAACA-3'

NFATc1 Reverse: 5'-TGTGGGATGTGAACTCGGAA-3'

TRAP Forward: 5'-CTGGAGTGCACGATGCCAGCGACA-3'

TRAP Reverse: 5'-TCCGTGCTCGGCGATGGACCAGA-3'

CTSK Forward: 5'-CTTCCAATACGTGCAGCAGA-3'

CTSK Reverse: 5'-TCTTCAGGGCTTTCTCGTTC-3'

2.7. Western blot analysis

Total proteins were extracted using RIPA buffer (Millipore, USA). Nuclear and cytoplasmic proteins were extracted by applying the Nuclear Extraction Kit (Thermo, USA) and Cytoplasmic Extraction Kit (Thermo, USA), respectively. Protein concentrations were measured by using the BCA Protein Quantification Kit (Abbkine, USA). Same concentration of protein was divided by SDS-PAGE (12% polyacrylamide gels), transferred to PVDF membranes (Millipore, USA) in the subsequent step. The membranes were hindered with 5% milk and hatched overnight at 4°C with the primary antibodies for Nrf2 (1: 1000, CST, USA), HO-1 (1: 1000, Abcam, USA), IκBα (1: 1000, CST, USA), p65 (1: 1000, CST, USA), LaminB (1: 1000, CST, USA), NFATc1 (1: 1000, CST, USA), c-Fos (1: 1000, CST, USA), and β-actin (1: 1000, CST, USA), in which β-actin served as the control. Then, the corresponding second antibodies (1: 1000, Abcam, USA) were used for hatching the membranes 1 hour at room temperature, followed by the visualization by using an Odyssey CLx Infrared Imaging System.

2.8. OVX-induced bone loss mouse model

The C57BL/6 mice (8 weeks, male) were randomly divided in three groups (n = 5), including control group, OVX treatment group, and OVX + Spinosin co-treatment group. In the control group, the mice were injected equal volume water; In the OVX treatment group, the mice were intraperitoneally injected with

streptozotocin (STZ, 50 mg/kg, 5 days, sigma, USA); In the OVX + Spinosin co-treatment group, the mice were intraperitoneally injected with OVX and orally administered with Spinosin-treated group (50 mg/kg/day). The mice were sacrificed after 9 day, and the tibias from the mice were subjected in high-resolution micro-CT analysis and Histomorphometric analysis. The levels of TRAcP5B and TNF- α were measure by ELISA assays. The loss of femur bone and osteoclasts was analyzed by Hematoxylin and Eosin (H&E) and TRAP staining. Animal care and method procedure were authorized by the Animal Ethics Committee of Linyi Central Hospital.

2.9. Statistical analysis

Data was presented as mean $\pm$ SD, and the statistical analysis was conducted by GraphPad prism 7. The unpaired Student's *t*-test was used to compare two groups, and the one-way ANOVA was utilized to compare among multiple groups. $P < 0.05$ were considered as statistically significant.

3. Results

3.1. Spinosin inhibits RANKL-induced osteoclastogenesis

To assess the potential cytotoxic effect of Spinosin (Fig. 1A) on BMMs, the BMMs were treated with Spinosin at the dose of 5, 10, 25, 50, 100, 200, and 400 μ M. CCK-8 assay showed that Spinosin treatment (≤ 100 μ M) failed to affect the cell viability of BMMs compared with the 0 μ M Spinosin, suggesting that Spinosin has no cytotoxic effect on BMMs at dose up to 100 μ M (Fig. 1B). Then, we selected the dose of 25, 50, and 100 μ M for examining the role of Spinosin in the modulation of RANKL-induced osteoclast formation. Our data showed that the treatment of Spinosin significantly inhibited the TRAP (+) osteoclasts in a dose-dependent manner in the RANKL-treated BMMs compared with the 0 μ M Spinosin ($P < 0.001$, Fig. 1C). Meanwhile, the resorption pit formation assay revealed that Spinosin treatment dose-dependently reduced RANKL-caused bone resorption compared with the 0 μ M Spinosin ($P < 0.001$, Fig. 1D). Taken together, these data indicate that Spinosin can inhibit RANKL-induced osteoclastogenesis *in vitro*.

3.2. Spinosin reduces the expression of osteoclast-specific genes in the RANKL-treated BMMs

Then, to validate the effect of Spinosin on osteoclastogenesis, the expression of osteoclast-specific genes, including nuclear factor of activated T cells, cytoplasm 1 (NFATc1), c-Fos, TRAP, cathepsin K (CTSK), dendritic cell-specific transmembrane protein (DC-STAMP), and osteoclast stimulatory transmembrane protein (OC-STAMP), was analyzed by qPCR. Our data showed that the treatment of Spinosin significantly reduced the expression of NFATc1, c-Fos, TRAP, CTSK, DC-STAMP, and OC-STAMP in a dose-dependent manner in the RANKL-treated BMMs compared with the 0 μ M Spinosin ($P < 0.001$, Fig. 2). Together, these results suggest that Spinosin inhibits the expression of osteoclast-specific genes in the RANKL-treated BMMs.

3.3. Spinosin fails to affect osteoblast differentiation.

Then, we further explored the effect of Spinosin on osteoblastic differentiation. To this end, the MC3T3-E1 cells were treated with Spinosin at indicated doses. CCK-8 assays showed that Spinosin did not affect the cell viability of MC3T3-E1 cells, suggesting that Spinosin has no cytotoxic effect on MC3T3-E1 cells ($P > 0.05$, Fig. 3A). ALP staining and Alizarin Red staining revealed that the osteoblastic differentiation of MC3T3-E1 cells was not affected by Spinosin treatment (Fig. 3B). Meanwhile, the mRNA expression levels of osteoblast differentiation markers, such as amphiphysin-like protein 1 (ALP1), runt-related transcription factor 2 (RUNX2), and osteocalcin (OCN), were not changed by Spinosin treatment in the cells ($P > 0.05$, Fig. 3C). Taken together, these results indicate that Spinosin fails to affect osteoblast differentiation.

3.4. Spinosin suppresses NF- κ B signaling by activating Nrf2 pathway

Next, we further assessed the underlying mechanism of Spinosin-mediated osteoclastogenesis. Significantly, our data showed that the treatment of Spinosin enhanced the expression of nuclear factor E2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1) in the BMMS ($P < 0.001$, Fig. 4A and B). Moreover, the treatment of RANKL remarkably decreased the cytoplasm expression of inhibitor of nuclear factor kappa B alpha (I κ B α) and increased the nucleus accumulation of nuclear factor-kappa B (NF- κ B, p65), in which the treatment of Spinosin could reverse the effect in the BMMs ($P < 0.001$, Fig. 4C and D). Moreover, Western blot analysis demonstrated that the expression of nuclear Nrf2 and cytoplasm HO-1 was reduced, while the expression of nuclear p65 was enhanced by the Nrf2 shRNA in the Spinosin and RANKL co-treated BMMs compared with control shRNA ($P < 0.001$, Fig. 4E and F). Together, these results suggest that Spinosin is able to suppress NF- κ B signaling by activating Nrf2 pathway

3.5. Spinosin inhibits RANKL-induced expression of c-Fos and NFATc1

Given that c-Fos and NFATc1 serve as the master contributors to osteoclastogenesis, we further evaluated the effect of Spinosin on RANKL-mediated expression of c-Fos and NFATc1. Western blot analysis showed that the expression of c-Fos and NFATc1 was enhanced by the treatment of RANKL, in which the Spinosin treatment could reverse this effect ($P < 0.001$, Fig. 5).

3.6. Spinosin inhibits OVX-induced bone loss in vivo

To further examine the effect of Spinosin on osteoclastogenesis *in vivo*, the OVX-induced inflammatory bone resorption mouse model was established. The micro-CT analysis of the tibias showed that the OVX treatment stimulated the bone loss in the tibias while the treatment of Spinosin rescued the phenotype (Fig. 6A). Histomorphometric analysis revealed that the bone volume to total volume (BV/TV), trabecular

thickness (Tb.Th), and trabecular number (Tb.N), and trabecular space (Tb.Sp) were inhibited by OVX treatment, in which Spinosin could reverse the effect ($P < 0.001$, Fig. 6B). ELISA assays demonstrated that the serum levels of tumor necrosis factor- α (TNF- α) and tartrate-resistant acid phosphatase 5 B (TRAcP5B) was increased by OVX in the mice, but the treatment of Spinosin was able to reduce the phenotype ($P < 0.01$, Fig. 6C). Moreover, Hematoxylin and Eosin (H&E) and TRAP staining demonstrated that Spinosin was able to attenuate OVX-induced loss of femur bone and osteoclasts (Fig. 7A).

4. Discussion

Osteoporosis is featured as an osteoclast disorder stimulated by unnecessary osteolytic activation, affecting over two hundred million humans globally (2). Clinical anti-osteoporosis treatments principally comprise hormone-replacement therapy, denosumab, bisphosphonates, and parathyroid hormone. Simultaneously, long-term unfavorable effects, including thromboembolic disease, atypical fractures, and an enhanced risk of breast cancer, are common (5, 24). Furthermore, bisphosphonates and denosumab are well-known drugs that target osteoclasts, but they need to be injected (10). Therefore, the development of convenient and safe therapeutic strategies for the treatment of osteoporosis is urgent. Spinosin as a natural compound has presented anti-oxidative and anti-inflammatory potentials. In current study, we firstly identified that Spinosin induced an inhibitory effect on osteoclastogenesis of osteoporosis by repressing NF- κ B through stimulating Nrf2/HO-1 signaling.

Multiple traditional natural compounds have been identified to modulate osteoporosis and osteoclastogenesis recent years. For instance, it has been reported that Kireneol represses RANKL-caused osteoclastogenesis and preserves ovariectomized-caused osteoporosis by inhibiting the Cav-1 signaling and Ca^{2+} /NFATc1 pathway (25). Cajaninstilbene acid decreases osteoporosis by reducing RANKL-related signaling and osteoclast formation (26). Tanshinone IIA relieves osteoclastogenesis by the inactivation of Akt and NF- κ B pathway in ovariectomized mice (27). Moreover, previous studies have identified the potential biomedical function of Spinosin. It has been found that Spinosin inhibits Alzheimer's disease-related synaptic disorder by regulating plasmin activity (28). Spinosin is able to activate Nrf2/HO-1 signaling to inhibit the aggregation and production of A β 1–42 (20). In this study, we identified that Spinosin could inhibit RANKL-induced osteoclastogenesis and the expression of osteoclast-specific genes in the RANKL-treated BMMs. Spinosin was able to attenuate OVX-induced loss of femur bone and osteoclasts *in vivo*. These data suggest that Spinosin can inhibit osteoclastogenesis, providing a novel function of Spinosin in the modulation of osteoporosis and enriching the understanding of the role of traditional natural compounds in osteoporosis.

The pathogenesis of osteoporosis is complicated and NF- κ B serves as a crucial regulator in osteoclastogenesis (25). RANKL leads to the I κ B α degradation and releases NF- κ B to the nuclear. I κ B suppresses the osteoclast formation by reducing the nucleus accumulation of NF- κ B, p65 phosphorylation and I κ B α degradation (25). Moreover, as a well-known suppressor of NF- κ B, the activation of Nrf/HO-1 signaling is able to inhibit NF- κ B activity (29). And the agents that stimulate Nrf/HO-1 signaling and inhibit NF- κ B present the potential to relieve osteoclasts and osteoclastogenesis.

For example, it has been reported that Hesperetin inhibits LPS-related bone loss and relieves RANKL-related osteoclastogenesis through regulating Nrf/HO-1/NF- κ B signaling (30). Lutein inhibits inflammation and oxidative stress by activating Nrf2 in the osteoporosis model (31). Glycyrrhizin represses oxidative stress and RANKL-caused osteoclastogenesis by suppressing MAPK and NF- κ B and stimulating AMPK/Nrf2 signaling (32). Stilbene glycoside reduces oxidative injury by Nrf2/HO-1/NF- κ B signaling in osteoblasts (33). Our mechanism investigation showed that Spinosin was able to suppress NF- κ B signaling by activating Nrf2 pathway in BMMs. It consistent with previous studies that targeting Nrf2/HO-1/NF- κ B signaling can alleviate osteoclastogenesis in osteoporosis. Our data provide the valuable information and mechanism that Nrf2/HO-1/NF- κ B signaling is involved in Spinosin-exerted biomedical activities.

Conclusion

In conclusion, we discovered that Spinosin attenuated osteoclastogenesis of osteoporosis by inhibiting NF- κ B through activating Nrf2/HO-1 signaling. Our finding provides new insights into the mechanism by which Spinosin modulates osteoporosis pathogenesis. Spinosin may serve as the potential candidate for the treatment of osteoporosis.

Declarations

Ethical Approval and Consent to participate

Informed consent was obtained from all individual participants included in the study. All producers were approved by Animal Ethics Committee of Linyi Central Hospital. Procedures operated in this research were completed in keeping with the standards set out in the Announcement of Helsinki and laboratory guidelines of research in China.

Consent to publish

Not applicable.

Availability of supporting data

The data that support the findings of this study are available on request from the corresponding author: * Yuna Zhang, Department of Pathology, Linyi Central Hospital, No.17 Jiankang Road, Yishui County, Linyi City, Shandong Province, 276400, PR. China.

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The data are not publicly available due to their containing information that could compromise the privacy of research participants.

Competing interests

All other authors have no conflicts of interest.

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

Funding

Not Applicable.

Authors' contributions

Bo Liu, Yuna Zhang: study concepts, literature research, clinical studies, data analysis, experimental studies, manuscript writing and review, study design, literature research, experimental studies and manuscript editing, definition of intellectual content, data acquisition and statistical analysis.

Acknowledgements

Not Applicable.

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Figures

Figure 1

Spinosin inhibits RANKL-induced osteoclastogenesis. (A) The chemical structure of Spinosin was shown. (B) The cell viability was measured by CCK-8 assays in the BBMs, (C) The representative images of TRAP staining were shown in the indicated BBMs. The TRAP (+) osteoclasts were calculate. (D) The representative images of resorption pit formation assay BBMs were shown. The bone resorption area

was calculated by the ImageJ software. Data are presented as mean $\pm$ SD. Statistic significant differences were indicated: *** $P < 0.001$.

Figure 2

Spinosin reduces the expression of osteoclast-specific genes in the RANKL-treated BMMs. BMMs were treated 100 ng/mL RANKL, or co-treated with RANKL and Spinosin. The mRNA expression of NFATc1, c-

Fos, TRAP, CTSK, DC-STAMP, and OC-STAMP was analyzed by qPCR in the BMMs. Data are presented as mean $\pm$ SD. Statistic significant differences were indicated: *** $P < 0.001$.

Figure 3

Spinosin fails to affect osteoblast differentiation. (A) The cell viability of MC3T3-E1 cells was measured by CCK-8 assays. (B) The osteoblastic differentiation in MC3T3-E1 cells was analyzed by ALP staining

and Alizarin Red staining. (C) The mRNA expression of ALP1, RUNX2, and OCN was assessed by qPCR in the MC3T3-E1 cells. Data are presented as mean $\pm$ SD.

Figure 4

Spinosin suppresses NF- κ B signaling by activating Nrf2 pathway. (A and B) The protein expression of Nrf2, HO-1, and β -actin was measured by Western blot analysis in the BMMs. The result of Western blot analysis was analyzed by the ImageJ software. (C and D) The protein expression of I κ B α , p65, LaminB,

and β -actin was assessed by Western blot analysis in the BMMs. The result of Western blot analysis was analyzed by the ImageJ software. (E and F) The protein expression of Nrf2, p65, HO-1, and β -actin was tested by Western blot analysis in the BMMs. The result of Western blot analysis was analyzed by the ImageJ software. Data are presented as mean $\pm$ SD. Statistic significant differences were indicated: ** $P < 0.01$, *** $P < 0.001$.

Figure 5

Spinosin inhibits RANKL-induced expression of c-Fos and NFATc1. (A) The protein expression of NFATc1, c-Fos, and β -actin was measured by Western blot analysis in the BMMs. The result of Western blot analysis was analyzed by the ImageJ software. Data are presented as mean $\pm$ SD. Statistic significant differences were indicated: *** $P < 0.001$.

Figure 6

Spinosin inhibits OVX-induced bone loss in vivo. (A-C) The C57BL/6 mice were treated with OVX, or co-treated with OVX and Spinosin. (A) The representative images of micro-CT analysis in the mice were shown. (B) The V/TV, Tb.Th, Tb.N, and Tb.Sp was analyzed by Histomorphometric analysis in the mice. (C) The levels of TRAcP5B and TNF- α were measure by ELISA assays. Data are presented as mean $\pm$ SD. Statistic significant differences were indicated: ** P < 0.01, *** P < 0.001.

Figure 7

Spinosin attenuates RANKL-induced expression of c-Fos and NFATc1 femur bone in vivo. (A) The C57BL/6 mice were treated with OVX, or co-treated with OVX and Spinosin. The loss of femur bone and osteoclasts was analyzed by H&E and TRAP staining.