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Effect of Ibandronate On MAPK Pathway and Autophagy in Osteoporosis

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Abstract

Aim: To investigate the role of ibandronate in osteoporosis (OP) and its association with mitogen-activated protein kinase (MAPK) pathway.

Methods: MC3T3-E1 cells were assigned into control group (CG), model group (MG) and intervention group (IG). The MG and IG were treated with dexamethasone (DEX) to establish cell models of OP, and IG was treated with ibandronate additionally. Cell apoptosis and viability were tested with flow cytometry and, cell counting kit-8 (CCK-8) respectively. Western Blotting (WB) was employed to quantify receptor activator of NF-κB ligand (RANKL), osteoprotegerin (OPG), P38MAPK, phosphorylated-P38MAPK (p-P38MAPK), Beclin-1 and light chain 3-II (LC3-II).

Results: MG presented remarkably decreased cell viability and protein levels of OPG, p-P38MAPK, Beclin-1, LC3-II than CG, as well as increased apoptosis and RANKL. Whereas results were quite the opposite in IG: Cell viability and protein levels of OPG, p-P38MAPK, Beclin-1, LC3-II in IG were remarkably higher, and the apoptosis and RANKL were remarkably lower than those in MG.

Conclusion: Ibandronate enhances the viability and inhibits the apoptosis of osteoblasts by activating the phosphorylation of P38MAPK and upregulating autophagy, thereby relieving OP.

Keywords: ibandronate; osteoporosis; MAPK pathway; autophagy

Introduction

Osteoporosis (OP), a prevalent chronic metabolic bone disease that easily induces fractures, is a public health issue worldwide(Kanis et al.2019). Patients with OP suffer from damaged bone microstructure, reduced bone mass, and weakened osteoblast function, leading to the activation of osteoclasts and the enhancement of their viability, resulting in increased bone fragility and fracture susceptibility (Peck et al.1993；Faienza and Chiarito.2018). The incidence of OP in China is rising, with the total incidence of ~27.96%, and females are more susceptible than males (25.41 % *vs.* 15.33 %)(Wang et al.2009; Chen and Li.2016). The elderly are also vulnerable to OP, which may cause disability and even death(Bystrytska et al.2020).

Bisphosphates are a class of bone resorption inhibitors that effectively inhibit the viability of osteoclasts and improve OP (Tang et al.2020). As one of bisphosphonates, ibandronate has faster onset, better analgesic effect and fewer adverse reactions(Maier et al.2016; Wilson and Martin.2019), but the specific intervention mechanism of ibandronate remains poorly explored.

Mitogen-activated protein kinase (MAPK) pathway is essential in signaling transduction system that mediates cell reactions. It is phosphorylated and activated after being stimulated by signal molecules, and activated MAPK participates in multiple physiological processes (Gurgel et al.2019; Weng and Tu.2019; Li and Zhou.2016). MAPK pathway regulates OP pathogenesis and improves treatment efficacy(Wang et al.2020; Pan and Tong.2018). Exosomes from bone marrow mesenchymal stem cells simulate osteoblast proliferation and alleviate OP via increasing the protein levels related to MAPK pathway(Zhao et al.2018). Du et al. mentioned that ibandronate inhibits p38, c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK)1/2 and other MAPK pathway related proteins in vascular smooth muscle cells (Du et al.2014). However, the role of ibandronate in MAPK pathway in OP has been rarely reported.

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Therefore, this study aims to provide reference for the treatment of OP by evaluating the role of ibandronate in MAPK pathway.

Methods

Reagents and Instruments

MC3T3-E1 cells (Be Na Biology, BNCC284107); phosphate buffer saline (PBS), Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), Penicillin-Streptomycin (Gibco, USA, 10566024, 10010010023, 2640044, 15070063); radioimmunoprecipitation assay (RIPA) buffer, bicinchoninic acid (BCA) kit, electrochemiluminescence (ECL) kit (Thermo Scientific); goat anti-mouse (IgG) secondary antibodies against osteoprotegerin (OPG), P38, phosphorylated-P38 (p-P38), Beclin-1, light chain 3-II (LC3-II), receptor activator of NF-κB ligand (RANKL) (Thermo Scientific, USA); Annexin V/PI apoptosis kit (Yeasen Biotechnology Co., Ltd., 40302ES20); flow cytometer (BD, USA, FACS Canto II).

Cell culture

MC3T3-E1 cells were grown in DMEM (10% FBS, 1% penicillin-streptomycin) at 37°C/5% CO_2, then cultured in DMEM (10% FBS, streptomycin (100μg/ mL), penicillin (100 U/mL), L- ascorbic acid (50 μg/ mL) and beta-glycerophosphate) to induce osteoblast differentiation. The control group (CG) received no treatment, whereas the model group (MG) and the intervention group (IG) were treated with dexamethasone (DEX, 1 μmol/ L) to establish OP models(Huang et al.2020), and the IG was additionally treated with ibandronate (50 μM) (Thaler et al.2013).

Western blotting (WB)

The cultured cells were subjected to RIPA lysis to isolate total protein, and the concentration was determined by BCA kit. After separation with 10% of sodium dodecylsulphate-polyacrylamide gel electrophoresi (SDS-PAGE), the protein with a concentration of 4 μg/μL was grafted to a polyvinylidene difluoride (PVDF) membrane, then dyed in Ponceau S staining solution, and washed in Phosphate Buffered Saline with Tween 20 (PBST) for 5 min. Afterwards, the membrane was sealed with 5% defatted milk powder for 2 h and cultured overnight with primary antibodies (1: 1000) at 4℃. Following removal of primary antibodies, secondary antibodies (1: 5000, HRP-conjugated) were added and cultured at 37℃ for 1 h. Next, the membrane was washed three times with PBS for 5 min each. Protein bands on the membrane were visualized in a darkroom with enhanced chemiluminescence (ECL) kit. The gray value was analyzed by Quantity One software after band scanning. Relative expression of target protein = gray value of target protein band / that of β-Actin band.

Cell viability test (CCK-8)

Cell viability was tested by CCK-8 assay. Cells ($4*10^6$) were grown in a 96-well plate for 48 h, 3 wells for each group. After adding 10 μL of CCK-8 reagent to each well, a 1-h incubation was performed at 37℃. The absorbance (A) value at 450 nm wavelength was measured under a microplate reader within 15 min.

Apoptosis test (Flow Cytometry)

Treated cells were trypsinized (0.25%), washed twice with PBS, added with 100μL of binding buffer, and prepared into suspension ($1*10^6$/mL). Afterwards, AnnexinV - FITC and PI were applied sequentially, and the cells were incubated for 5 min in dark at indoor temperature to determine the apoptosis with FC500MCL flow cytometer. The average value was obtained after three repeated experiments.

Statistical analysis

GraphPad 7 was employed for data processing and graph plotting. Measurement data distribution was identified by Kolmogorov-Smirnov (K-S) test, wherein normal-distributed data were displayed as ($\bar{x}$±sd). Inter-group comparison adopted independent samples *t* test, multi-group comparison adopted one-way analysis of variance (ANOVA) (denoted by *F*), post-hoc pairwise comparison adopted Fisher's least significant difference-t test (LSD-t test), comparison between multi-time points adopted repeated measures ANOVA (denoted *F*), followed by Bonferroni post-hoc test. Differences were statistically significant at $P < 0.05$.

Result

Ibandronate enhances cell viability and inhibits apoptosis

CCK-8 assay demonstrated that cell viability in MG and IG was lower that that in CG (P<0.05), and the viability in IG was higher than that in MG (P<0.05). Cell apoptosis in MG and IG was higher than that in CG (P<0.05), and the apoptosis in IG was lower than that in MG (P<0.05), as shown in Figure 1.

RANKL and OPG levels in each group

Protein levels of RANKL and OPG in each group were measured by WB. It turned out that RANKL level in DEX-treated MG and IG was higher, and OPG level was lower than that in CG (P<0.05). In IG treated with ibandronate, RANKL decreased and OPG increased (P<0.05), as shown in Figure 2.

Ibandronate activates phosphorylation of P38MAPK

There was no significant difference in P38MAPK among the three groups (P>0.05). After DEX treatment, the level in MG was down-regulated (P<0.05), while that in IG receiving ibandronate treatment was up-regulated, higher than CG and MG (P<0.05), as shown in Figure 3.

Ibandronate promotes autophagy

Levels of autophagy-related proteins (Beclin-1 and LC3-II) in MG were lower than those in CG (P<0.05), while the autophagy in IG was enhanced, and levels of Beclin-1 and LC3-II was upregulated (P<0.05), as shown in Figure 4.

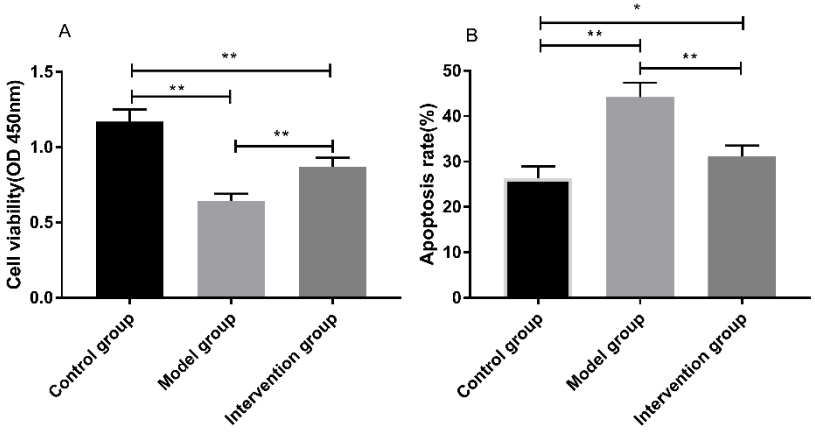

Figure 1. **Comparison of cell viability and apoptosis. A, Cell viability in CG is remarkably higher than that in MG and IG, and the viability in IG is remarkably higher than MG. B, Cell apoptosis in CG is remarkably lower than that in MG and IG, and the apoptosis in IG is remarkably lower than that in MG. * P<0.05, ** P<0.01.**
CG: control group, MG: model group, IG: intervention group

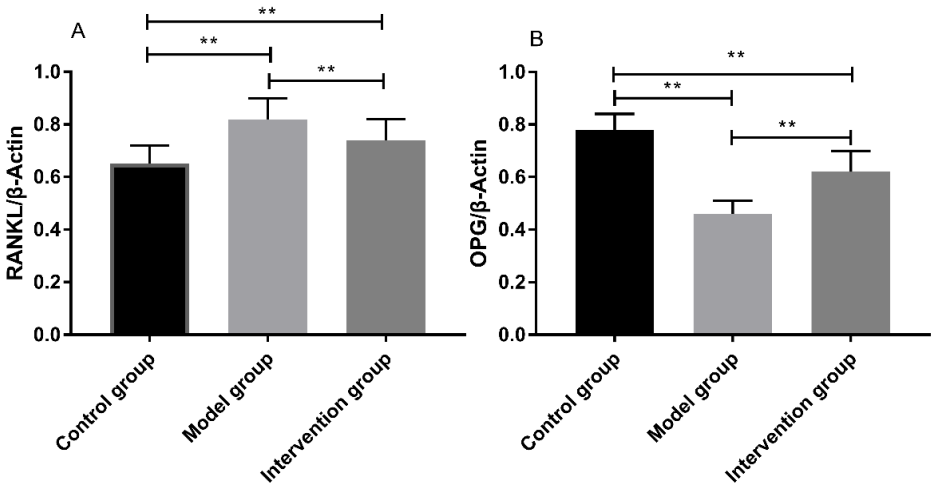

Figure 2. **RANKL and OPG levels in each group. A, Protein level of RANKL in CG is remarkably lower than that in MG and IG, and the IG is remarkably lower than the MG. B, Protein level of OPG in CG is significantly higher than that in MG and IG, and the IG is remarkably higher than the MG. **P<0.01.**
CG: control group, MG: model group, IG: intervention group

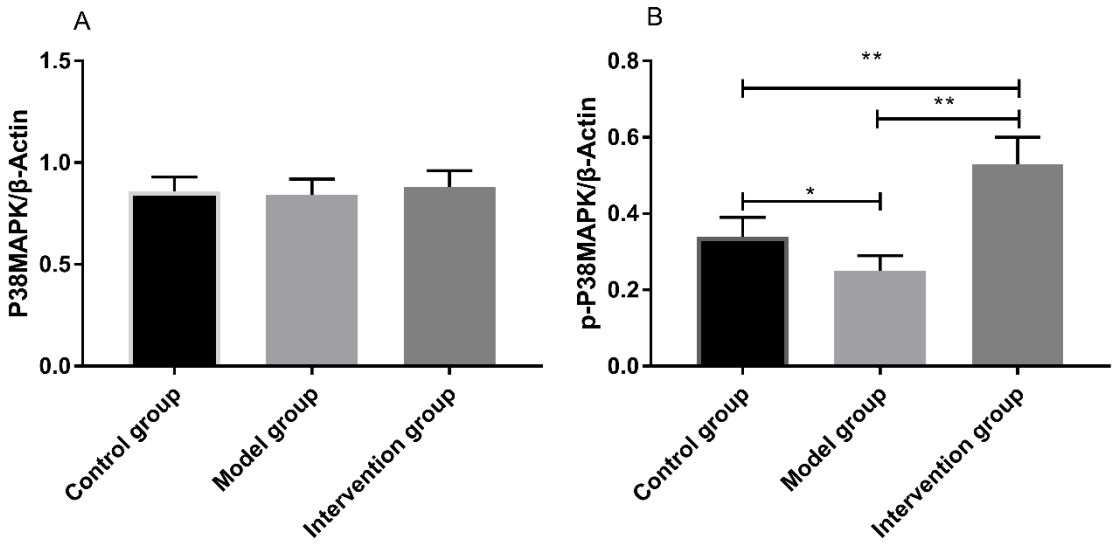

Figure 3. **Levels of P38MAPK and p-P38MAPK. A, there was no significant difference in P38MAPK among the three groups. B, p-P38MAPK in MG is remarkably lower than that in CG, and the IG is remarkably higher than the MG and CG. *P<0.05, ** P<0.01.**
CG: control group, MG: model group, IG: intervention group

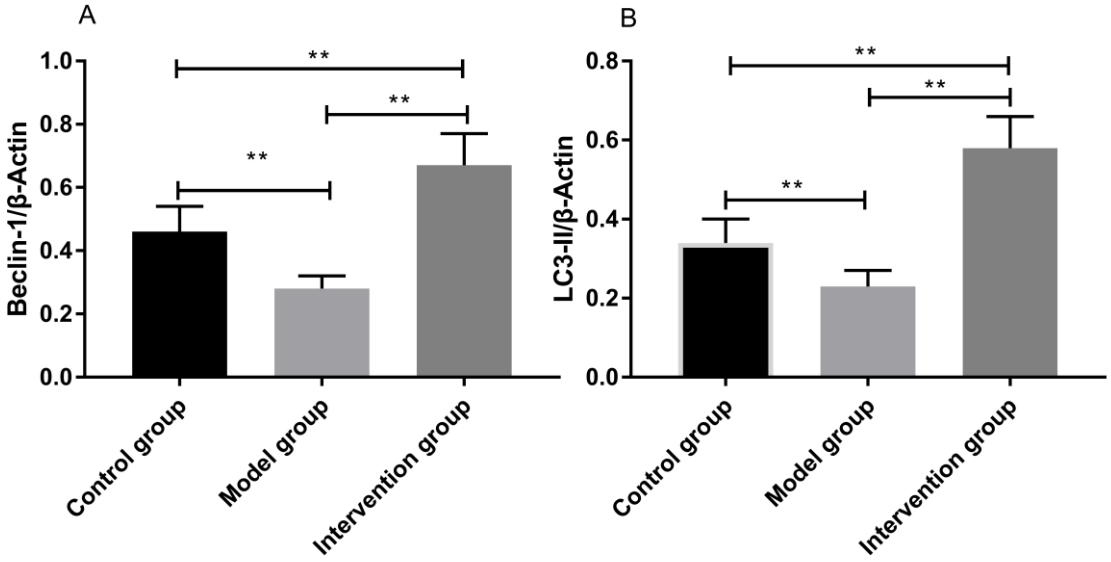

Figure 4. **Comparison of autophagy A, Beclin-1 level in MG is remarkably lower than that in CG, and IG is remarkably higher than CG and MG. B, LC3-II level in model group is remarkably lower than that in CG, and the IG is remarkably higher than the CG and MG. **P<0.01.**
CG: control group, MG: model group, IG: intervention group

Discussion

As the third generation of bisphosphonates, ibandronate has been reported to improve bone mineral density and reduce osteoclast viability, as well as enable osteoblasts to release factors that inhibit the activation and formation of osteoclasts, and block bone resorption(Soares et al.2016). In this study, the role of bone resorption inhibitor ibandronate in OP was evaluated.

MC3T3-E1 cells treated with DEX presented with reduced viability and increased apoptosis after induction of osteoblast differentiation. However,

through the intervention of ibandronate, the apoptosis was inhibited and the vitality was improved. RANKL and OPG are proteins related to osteoblasts and osteoclasts. OPG inhibits the formation and induces the apoptosis of osteoclasts, thereby inhibiting bone resorption and enhancing bone mineral density and bone strength. RANKL promotes bone resorption by prolonging the lifespan of osteoclasts and inhibiting their apoptosis (Xu et al.2013; Zhao and Wang 2020). In DEX-treated MC3T3-E1 cells, RANKL increased and OPG decreased. While in ibandronate-treated model cells, decreased RANKL and increased OPG were revealed, which indicates the effectiveness of ibandronate on repairing bone cells and promoting osteoblast differentiation. The elevated p-P38MAPK after ibandronate treatment suggested that ibandronate could induce the phosphorylation of P38MAPK.

Autophagy plays a pivotal role in growth, metabolism and regeneration of bones, and is activated during osteoblast differentiation, while OP inhibits the autophagy of cells (Bu et al.2019). There is evidence that bandronate affects autophagy related mechanisms (Ma et al.2018), so we suspected that autophagy may be involved in the regulation process of ibandronate on osteoblast differentiation. Therefore, we detected autophagy-related proteins Beclin-1 and LC3-II in this study. When autophagy is formed, LC3-I on the autophagosome membrane is converted into LC3-II, resulting in the increase iLC3-II/LC3-I, which is a marker for autophagy formation. Besides, Beclin-1 complex regulates autophagy and Beclin-1 plays a role in promoting autophagy(Liu et al.2016). Our study demonstrated that osteoblasts treated with DEX showed decreased LC3-II and Beclin-1, while those treated with ibandronate showed increased levels and enhanced autophagy. High dose of DEX accelerates the apoptosis of osteoblasts, whereas low dose may induce the activation of autophagy by up-regulating intracellular reactive oxygen species, leading to the enhancement of osteoblast viability(Zhang et al.2018).

There are still several limitations in this study. Firstly, the doses used are all based on previous studies, the effects of different doses are not discussed. Secondly, adverse reactions induced by ibandronate are not mentioned. More clinical trials will be carried out to supplement our results.

To sum up, ibandronate enhances the viability and inhibits the apoptosis of osteoblasts by activating phosphorylation of P38MAPK and upregulating autophagy, thereby relieving OP.

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