Onion (Allium cepa L.) flavonoids extract ameliorates osteoporosis facilitating osteoblast proliferation and differentiation, and inhibiting RANKL-induced osteoclastogenesis

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Abstract

Osteoporosis, a bone metabolic disease, is a global chronic health problem. Flavonoids, a group of natural active compounds widely existing in vegetables, fruits, beans and cereals, were reported to be anti-osteoporosis. Onion is a common vegetable rich in flavonoids. In this study, bone mineral density (BMD) was increased significantly after taking onion flavonoids extract (OFE) orally in an ovariectomized (OVX)-induced rat model, the trabecular structure and serum biomarkers were both improved, with effects similar to estradiol. Compared with the OVX rats, the contents of E2, calcium and phosphorus in the serum of rats were enhanced, but ALP and Trap levels in serum were decreased. In addition, the activity of on bone health was assessed by human osteoblast-like cells MG-63 and osteoclast precursor Raw 264.7 cells in vitro as well. After treated with OFE, MG-63 cells proliferation and mineralization were promoted, and alkaline phosphatase (ALP) activity and mRNA expression of OPG/RANKL were increased significantly. Moreover, RANKL-induced osteoclastogenesis of Raw 264.7 cells and osteoclast activity were inhibited with decrease of tartrate-resistant acid phosphatase (TRAP) activity, and mRNA expression of remarkable enzymes was down-regulated in cells. Furthermore, it was demonstrated that OFE regulated activity of osteoblast mainly via the OPG/RANKL signaling pathway and osteoclastogenesis. The results provided evidence a new model of dietary supplements for preventing osteoporosis.

Introduction

Nowadays, osteoporosis as a common bone health problem worldwide, mainly occurs to the elderly and postmenopausal women [1], which is a metabolic bone disorder distinguished by destruction of the microstructure of bone tissue, bone loss and decreased bone strength [2]. It was investigated that the prevalence of osteoporosis over the age of 40 in China is 5% in men and 20.6% in women [3]. And patients with osteoporosis are at high risk for fractures, which puts a heavy burden on China's medical system along with huge costs [4].

Bone homeostasis is under constant bone remodeling, which depends on the balance between bone formation and bone resorption modulated by osteoclast and osteoblast, respectively [5]. The imbalance of these two processes eventually leads to osteoporosis [6]. The OPG-RANKL-RANK system has a decisive position in the development of osteoporosis. Receptor activator of nuclear factor-kappa B ligand (RANKL), secreted by osteoclasts, promotes osteoclastogenesis and bone resorption after binding to receptor activator of nuclear factor-kappa B (RANK), located on the surface of osteoclasts’ precursors [7]. Osteoprotegerin (OPG), a decoy receptor also derived from osteoblasts, can block the activation of osteoclast downstream pathways by competitively binding with RANKL [8]. Consequently, it is considered that the ratio of OPG to RANKL is a principal hint of bone resorption.

Vitamin D, hormones and bisphosphonates are common drugs to treat osteoporosis. However, these drugs have serious side effects, damaging gastrointestinal tract and increasing the risk of cancer [9]. For this reason, it is more essential to take precautions early time to alleviate the side effects and economic
burden for patients apart from treatments with drugs. Dietary supplements are becoming one of the most important means of prevention of osteoporosis in recent years [10].

Dietary flavonoids, a group of natural phenolic compounds present in daily consumed vegetables, fruits, and drinks [11], are well known for diverse biological effects. Notably, anti-osteoporosis is reported to be a remarkable biological activity of flavonoids. Previous studies showed that genistein supplements could improve bone mineral density (BMD) in postmenopausal women with osteopenia [12]. In addition, Epimedium-derived isomeric flavonoids exerted anti-osteoporosis activity targeting OPG/RANKL [13]. Onion, known as “the queen of vegetables”, has a large content of dietary flavonoids and good for health, due to its bioactivity like antioxidant [14] and anti-cancer [15]. Law et al reported that onion juice could improve antioxidant activities, thus enhancing BMD [16]. Despite these efforts, mechanism of onion anti-osteoporotic effect remains largely unexplored.

The chief goal of the present study is to consider whether onion flavonoids extract (OFE) could serve as a promising functional food in preventing osteoporosis and avoiding the stress on patients during illness and treatments.

Materials and Methods

The materials and methods section is described as supplementary material.

Results and Discussion

Effects of OFE on morphological parameters by micro-CT

To determine the anti-osteoporosis effects of OFE treatment, bone micro architecture was analyzed by micro-CT. As is shown in Fig. 1, it was calculated that BMD declined significantly in OVX groups \((p < 0.05)\), and the net-structure of trabecular was destroyed. Fortunately, this situation was reversed with the oral administration of OFE in a dose-dependent manner. BMD (Fig. 1C) in both OVX + M-OFE group and OVX + H-OFE group was recovered and Th.Sp (Fig. 1F) decreased. BV./TV. (Fig. 1B) and Th.N (Fig. 1D) were enhanced in OVX + H-OFE group significantly \((p < 0.05)\), which was the same as the ALN group. Bone mineral density was enhanced significantly by supplementary of OFE compared to OVX group.

Effect of OFE on the Histopathological analysis of bone tissues of OVX rats

To characterize the efficacy of OFE on trabecular structure, the histopathology of the femur was performed in OVX rats by HE staining. As shown in Fig. 1B, the femoral trabecular in OVX groups was disordered, and fracture of bone trabecular decreased significantly in number and increased in spacing with a lot of fat vacuoles compared with that of sham group. 8-week oral administration of OFE treatment, especially OVX + H-OFE group, significantly decreased the fat vacuoles and improved the structure of bone trabecular.
Effect of OFE on serum biochemical markers

Ovariectomy caused a sharp drop in estrogen levels in rats, a common osteoporosis model in vivo. After performed ovariectomy, the function of osteoclasts was active, and the function of osteoblasts was enhanced in compensatory, with bone showing a high transition state. As a result, compared with Sham group, serum estrogen (E2) decreased rapidly, serum ALP and Trap increased and serum calcium and phosphorus deficiency ($p < 0.05$, Fig. 2). After administration of OFE, the level of E2 (Fig. 2A), Ca (Fig. 2C) and P (Fig. 2D) enhanced significantly compared with the OVX group ($p < 0.05$). A significant reduction of ALP (Fig. 2B) and Trap (Fig. 2E) was observed in OVX + H-OFE group compared with the OVX group as well ($p < 0.05$). Surprisingly, OVX + H-OFE group performed as well as the OVX + ALN group, a bisphosphonate drug used in treating osteoporosis commonly. Based on the result, it is recommended for human dietary supplement 12 mg/kg onion flavonoids every day.

OFE promoted proliferation and differentiation of MG-63 cells

What’s more, it was proved that OFE exerted anti-osteoporosis by stimulating MG-63 cells proliferation and differentiation, and inhibiting osteoclastogenesis via OPG/RANKL signaling pathway. Osteoblasts mediate bone formation by proliferation, differentiation, and matrix mineralization. To ascertain effects of OFE on proliferation of MG-63 cells, MTT assay was conducted. MG-63 cells were exposed to various concentrations of OFE for 24 h. As shown in Fig. 3A, MG-63 cells proliferation was promoted significantly with OFE at the concentration of 25 and 50 µg/mL ($p < 0.01$).

In general, osteoblasts, proliferating late, begin to differentiate, and matrix maturation stage is involved in osteoblast differentiation. During the process of maturation, ALP, a biomarker of early differentiation, increases. ALP is a crucial enzyme during early differentiation period of osteoblast. Cells treated with OFE, ALP activity was increased significantly ($p < 0.01$), at 50 µg/mL OFE (Fig. 3B). The results showed that OFE promoted the differentiation of osteoblasts by increasing ALP activity.

Meanwhile, osteoblasts perform matrix mineralization during matrix maturation stage [17]. Calcium deposit is the mean factor of bone matrix mineralization. After induced by complete medium containing ascorbic acid and β-glycerolphosphate, both MG-63 cells of mineralization group and treatment group (12.5, 25, 50 and 100 µg/mL) formed opaque nodules (Fig. 3C), and opaque nodules were formed more in mineralization groups than control group. Quantitative analysis indicated that the combination of ARS and calcium was increased significantly after MG-63 cells treated with OFE (12.5, 25, 50 µg/mL), and as a result, formation of mineralized nodules was promoted (Fig. 3D).

Effect of OFE on OPG/RANKL pathway

OPG/RANKL/RANK signaling pathway plays a dominant role in interactions between osteoblast and osteoclast. Osteoclast activity is close to osteoblast activity via the production of OPG and RANKL. Normally, RANKL binds to its receptor, RANK, on the precursor osteoclasts and osteoclasts to activate the
function of osteoclasts. Nevertheless, OPG, secreted by osteoblasts, binds to RANKL competitively as a
decoy receptor [18]. As such, OPG/RANKL ratio indicates the state of bone resorption. It was shown that
increase of OPG/RANKL ratio weaken bone resorption in turn [19]. Therefore, expression of OPG/RANKL
in mRNA level was explored. As shown in Fig. 4, after treatment with OFE, expression of OPG/RANKL in
mRNA level was upregulated significantly ($p < 0.01$). It implied that OFE regulated activity of osteoblast
via OPG/RANKL signaling pathway. Thus, the molecular triad, OPG/RANKL, serve as a crucial target for
preventing osteoporosis [20]. Of course, OPG/RANKL signaling pathway is regulated by several other
pathways and mechanism of OFE still needs further exploration.

**OFE inhibited the differentiation of osteoclasts without
cytotoxicity**

To evaluate whether the inhibitory effect of OFE on osteoclastogenesis by OFE was attributed to its
potential toxicity, Raw 264.7 cells viability were detected using MTT assay treated with OFE at different
concentrations in the absence of RANKL. As expected, concentration of OFE below 50 µg/mL showed
non-toxic effect on cell viability (Fig. 5A), indicating that OFE suppressed osteoclastogenesis induced by
RANKL.

RANKL-induced osteoclastogenesis from Raw 264.7 cells is a standard of osteoclast differentiation in
vitro. TRAP is an essential biomarker for bone resorption, whose activity was explored in the period of
osteoclastogenesis. Raw 264.7 cells were co-cultured by RANKL and OFE to determine the effect on
osteoclastogenesis. As Fig. 5B illustrated, TRAP activity decreased with 50 µg/mL OFE significantly ($p <
0.05$).

**Effect of OFE on expression of osteoclast-specific mRNA**

Osteoclasts are the only recognized, multinucleated cells responsible for bone resorption, derived from
mononuclear precursor cells of the monocyte–macrophage lineage [21]. Differentiation of osteoclast
precursors into osteoclasts results from the binding of RANKL, secreted by osteoblasts, to its receptor.
This increased the expression of the transcription factor, NFATc1, which plays an essential role in
activation of osteoclasts and bone resorption [22] and a pivotal gene during osteoclastogenesis.
Furthermore, NFATc1 can generate the mRNA expression of crucial enzymes, such as Ctsk, MMP-9, and
TRAP. These enzymes were involved in degradation of bone matrix and bone resorption, reflecting the
activity of osteoclasts. TRAP is an osteoclast-specific enzyme, marking the state of bone resorption and
quantification of osteoclast numbers [23]. Besides, Ctsk was involved in digestion of proteinaceous
matrix [24], especially breaking down type collagen which is a main composition of bone matrix.
Excessive osteoclast activity and osteoporosis go hand in hand. During differentiation, mRNA expression
levels of Trap, Ctsk, Mmp-9 and Nfatc-1 in cells treated with 50 and 25 µg/mL OFE were decreased
significantly compared to control ($p < 0.05$) (Fig. 5C).

**Conclusion**
Daily dietary supplement onion flavonoids could increase BMD and improve the bone microstructure significantly. MG-63 cells proliferation and mineralization were promoted and mRNA expression of OPG/RANKL were increased significantly by OFE. Meanwhile, RANKL-induced osteoclastogenesis of RAW264.7 cells were inhibited after treated with OFE. Consequently, onion would be recommended as a daily dietary supplement to prevent osteoporosis.

**Declarations**

**Ethics declarations**

**Ethical Approval and Consent to Participate**

All the animal experiments were conducted with the aproval of the Ethics Committee of Dalian University of Technology, China (No. DUTSBE230621-03). All animal experiments of the study were carried out in accordance with the European Community guidelines (Directive 2010/63/EU).

**Declaration of Interest Statement**

The authors declare no conflict of interest.

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**Data Availability**

All relevant data are within the manuscript and its supplementary material, and are also available from the corresponding author on reasonable request.

**Consent for Publication**

All authors agree to publish.

**CRediT authorship contribution statement**

**Danyang Zhang**  Investigation, Formal analysis, Writing - original draft. **Xiaoyu Wang**: Methodology, Investigation. **Kezhuo Sun**: Methodology. **Jianli Guo**: Formal analysis **Jia Zhao**: Investigation. **Yuesheng Dong**: Writing - review & editing. **Yongming Bao**: Conceptualization, Supervision, Funding acquisition, Writing - review & editing.

**References**


Tables

Table 1 Primer sequences used in the RT-PCR analysis
<table>
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<th>Target mRNA</th>
<th>Primer sequence (5'-3')</th>
<th>Fragment size (bp)</th>
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<td>F: GAAACGTTTCCTCCAAAGTACC</td>
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<td>R: CTGTCTGTGTAGTAGTGTCAG</td>
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<td>R: TGGTCCAGGGTTCTTACTCC</td>
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Figures
Figure 1

Effects of OFE on bone microstructure in OVX rats.

(A) 2D longitudinal section of distal femur. (B) Hematoxylin and eosin staining of distal femur. (C) BMD of the trabecular bone. (D) BV./TV. (E) Tb. N (F) Tb. Sp. The different letters represent significant differences between different groups (p < 0.05).
Figure 2

Effects of OFE on serum biomarkers in OVX rats.

(A) E2 (B) ALP (C) Ca (D) P (E) Trap. The different letters represent significant differences between different groups (p < 0.05).
Effects of OFE on MG-63 cells proliferation, ALP activity and calcium deposition.

(A) MTT assay. (B) ALP activity. (C) Cells were cultured for 21 days after mineralization induction and stained by ARS. (D) Quantitative analysis of relative calcium deposition. Data shown were the mean ± SD of three independent experiments. * p<0.05, ** p<0.01 compared with mineralization group.
Figure 4

Effects of OFE on mRNA expression of OPG/RANKL in MG-63 cells.

Data shown were the mean ± SD of three independent experiments. * p<0.05, ** p<0.01 compared with the control.
Effects of OFE on TRAP activity and mRNA expression of osteoclast markers.

(A) Effects of OFE on Raw 264.7 cells viability. (B) TRAP activity in Raw 264.7 cells, co-cultured with RANKL and different concentrations of OFE. (C) mRNA expression in Raw 264.7 cells, co-cultured with RANKL and different concentrations of OFE. Data shown are the mean ± SD of three independent experiments. *p<0.05, ** p<0.01 compared with the control.

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