Selenium-Containing Protein from Selenium-Enriched Spirulina platensis Relieves Osteoporosis by Inhibiting Inflammatory Response, Osteoblast Inactivation, and Osteoclastogenesis

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1. Introduction

Osteoporosis (OP) is a systemic bone disease with low bone mass and destruction of the microscopic structure [1, 2]. Currently, more than 200 million people worldwide suffer from osteoporosis [3]. Postmenopausal osteoporosis in women and degenerative osteoporosis in the elderly are considered the two main primary osteoporosis. OP can increase bone fragility and lead to osteoporosis in the spine, hips, distal radius, and proximal humerus. Therefore, maintaining of bone homeostasis is of great significance. Nowadays, many antiosteoporosis drugs are used for anti-osteoporosis treatment, but the therapeutic effect is far ideal, and long-term usage will lead to sever complications. Therefore, developing novel safer agents for antiosteoporosis therapy is a topic of intense research.

Selenium (Se), the essential nutrient and trace element for human beings, possess multiple biological effects, such as...
antibacterial, anti-inflammatory, antioxidant, and immune modulation activities though its incorporation into proteins, such as glutathione peroxidase and thioredoxin reductases. There are 25 and known selenoproteins in humans [4, 5]. Particularly, glutathione peroxidases and thioredoxin reductases have an important role in maintaining the cellular redox homeostasis to combat free radicals-related cardio-vascular diseases, diabetes, and chronic inflammatory disease [6]. Additionally, Se is also considered as vital importance to bone-building mineral, and Se deficiency is the major causing reasons of Kaschin–Beck disease [7–9]. Evidence demonstrated that Se supplementation could maintain bone homeostasis through regulating the balance of osteoclasts-mediated resorption and osteoblasts-mediated bone formation [10–12] with the involvement of activating Wnt/β-catenin pathway [13]. These evidences suggested the promising prospective of developing Se-based agents for combatting OP. However, the biological activities of Se are highly related to its chemical forms, and Se level also dramatically affected the synthesis of selenoprotein [14]. Organic selenium and inorganic selenium usually show high toxicity towards human organs and cells, which usually exhibited pro-oxidant activity by inducing oxidative stress [15,16]. Selenium can be metabolized in cells and integrated into proteins. Selenocysteine was the most common Se form of existence in protein. Se in the form of selenium-containing proteins usually exhibited antioxidant activity via inhibiting oxidative stress [17, 18]. Although nanotechnology-mediated therapy reveals a promising method for Se in treating OP, a more acceptable method that could achieve large-scale production and possess clinical transformation potential is still needed.

In this study, Spirulina platensis (S. platensis), an edible microorganism that possess multiple nutrients and could be mass-produced, was used as Se “carrier” for the treatment of OP. Our results demonstrated that Se-SP from Se-enriched S. platensis could alleviate OP in ovariectomized mice model through inhibiting inflammatory response, osteoblast in-activation, and osteoclastogenesis.

2. Materials and Methods

2.1. Chemicals. MTT was obtained from Beyotime company (Shanghai, China). DMEM medium and fetal bovine serum (FBS) were purchased from Gibco (Rock-vill, MD, 313 USA). RANKL was obtained from R&D (Minneapolis, MN, USA). ALP staining kit, sodium selenite (Na2SeO3), and other agents were all purchased from Sigma. All antibodies were purchased from Cell Signaling Technology, Inc.

2.2. Culture of Se-Enriched S. platensis and Growth Measurement. Se-enriched S. platensis were obtained as previously reported methods [19]. Briefly, Spirulina platensis was cultured with Zarrouk medium (pH 9.0) at 30°C and received 14 hours of light every day. Selenium addition was added to the culture medium to increase gradually from 100 mg/L Na2SeO3 at day 7, 150 mg/L at day 8, and 200 mg/mL at day 9. After culturing for 11 days, Se-enriched S. platensis was harvested, lyophilized, and stored at −20°C. The growth of Se-enriched S. platensis was measured by biomass. Briefly, 10 mL Se-enriched S. platensis were filtered by a pre-weighed 0.22 µm filter paper. Filter was washed and dried at 80°C overnight, and then re-weighed. A hematocytometer was conducted to cell counting. Unit cell weight of the microalgae was calculated according to methods developed by Li et al. [20].

2.3. Extract of Se Containing Protein (Se-SP). The freeze-dried Spirulina platensis and Se containing S. platensis were resuspended with PBS (50 mM, pH 7.0) and subjected to ultrasonication (Sonic VEX 600 system, 200 W). After being sonicated for 3 minutes, the extracts were collected by centrifugation at 12000 rpm at 4°C for 30 min and total protein was collected and subjected to BCA analysis to determine the concentration of SP and Se-SP [21].

2.4. Cell Culture and Cell Viability. MC3T3-E1 cells were obtained from ATCC company (Manassas, USA) and cultured in DMEM medium (10% FBS and 1% penicillin-streptomycin) at 37°C and 5% CO2 atmosphere. After 24 h incubation, cells were plated in 96-well plates at a density of 1 × 104 cells/well. MC3T3-E1 cells were treated with different concentrations of SP and Se-SP for 48 h. Cells were added with 25 μL MTT solution and incubated for 5 hours; after dissolving with 150 μL DMSO solution, the OD values were recorded by microplate reader at 570 nm.

2.5. Alizarin Red Staining and Measurement of Calcium Content. MC3T3-E1 cells were treated with 5 and 10 μg/ml SP or Se-SP for 14 days in differentiation medium. Then, cells were fixed with 4% formaldehyde followed by incubation with 2% alizarin red (pH4.2) at room temperature for 5 min. After three washing, a phase contrast microscope was used to capture the calcification of MC3T3-E1 cells, and the absorbance of alizarin red was recorded at 420 nm after the elution with 10% formic acid. A calcium analysis kit was used to quantify the content of calcium according to the manufactory instructions by a O cresol phthalain complexion method.

2.6. ALP Staining and Measurement of ALP Activity. MC3T3-E1 cells after treatment of SP or Se-SP were fixed with 4% formaldehyde after indicated treatments and then subjected to ALP staining according to the experiment procedure of the ALP staining kit as previously described [22]. ALP activity evaluation was carried out using the following procedures: MC3T3-E1 cells (1.5 × 104 cells/mL) were seeded at 48 well plates overnight and then treated with indicated incubations. After that, 20 μL medium of each well was mixed with 100 μL working solution (0.5 mM Tris, 10 mM p-nitro phenyl phosphate, 2 mM MgCl2, 1 mM ZnCl2, pH 9.0) at 37°C for 30 min. Then, 80 μL 1 mM NaOH stop solution was added and the absorbance was recorded at 409 nm.
2.7. Western Blotting. MC3T3-E1 cells after were lysed with RIPA solution and the total protein was collected by centrifuging at 12000 rpm for 20 min at 4°C. After quantification by BCA assay, 40 μg protein of each treatment group was separated by SDS-PAGE electrophoresis. Then, proteins were transferred onto the PVDF membrane, blocked with 5% nonfatty milk, and incubated with primary antibodies overnight. After three washes with TBST, the membranes were incubated with HRP-linked antibodies for 2 h at room temperature and then the protein expression was visualized by an ECL. β-Actin was used for loading control.

2.8. In Vivo Model and Micro-CT. Female C57 mice (8-weeks, 12 mice/group) were given adaptive feeding for one week, and both ovaries were removed to establish the osteoporosis model. Then, mice were divided into four groups: sham group, ovariectomy group, SP-treated group (10 mg/kg), and Se-SP-treated group (10 mg/kg). Ovariectomy mice were given continued feeding for 2 months, and mice were given intraperitoneal injection with 10 mg/kg Se-SP or SP for another 2 months every other day. Then, micro-CT scanning was used to analyze the integrality of bone. Mice were euthanized and the femurs were collected and fixed with formalin (10%) at the end of the experiments. After decalcifying with EDTA for 3 weeks, the femurs were embedded in paraffin and a 6-μm-thick section was subjected to HE staining and IHC staining for morphological observation and expression. The bone integrity was reconstructed by NRecon before the CTAn software package. All in vivo experiments were done with the recommendations in the Guide for the Care and Use of Laboratory Animals published by the Shandong First Medical University.

2.9. Immunofluorescence and TRACP Staining. A 4-μm-thick section of bone tissue was used for immunohistochemistry to analyze the expression of indicated proteins after the treatments. Briefly, the paraffin section was dewaxed and incubated with primary antibodies after antigen retrieval and 1% BSA blockage. After washing with TBS solution (0.025% Triton X-100) three times, the optimal dilution of the fluorescent-linked secondary antibody was added and incubated at room temperature for 1 h. A fluorescence microscope was used to detect the expression level of indicated proteins. TRACP staining was carried out according to the instructions of the TRACP staining kit. TRACP-positive cells were identified as red staining and containing three or more nuclei.

2.10. Statistical Analysis. Statistical analysis was performed using the SPSS17.0 software. The difference between two groups was conducted by Student’s t-test. The significance between three or more groups was conducted by one-way analysis of variance multiple comparisons. Bars in figures with different characters (A, B, C, or D) are statistically different at the $P<0.05$ level, which were achieved by multiple comparison between three or more groups.

3. Results

3.1. Extract of Se-SP and Cytotoxicity of MC3T3-E1. Selenium-enriched S. platensis was cultured in Zarrouk medium with the supplementation of Na2SeO3 with obvious heliciform appearance and characterized by bright red fluorescence under a fluorescence microscope (Figure 1A1–A3). Phycocyanin (PC) and allophycocyanin (APC) are the two main ingredients of S. platensis, and we found that the extracts of Se-SP displayed significant absorbance at 280 nm (Figure 1(b)), which may indicate the successful extraction SP-Se. The growth of Se-enriched S. platensis was measured by biomass at day-10, and the results indicated that the biomass concentration was about 3.52 g/L, the cell count was about $1.36\times10^{10}$ cells/L, and the cell weight was about $0.2\times10^{-9}$/cell. The method and result were both added into the revised manuscript. In order to evaluate the cytotoxicity of SP-Se on MC3T3-E1 cells, MTT assay was performed. As illustrated in Figure 1(c), no significant cell growth suppression effects were observed in SP-Se and SP treatment groups when compared with the untreated cells, which suggests the safety of Se-SP against MC3T3-E1 cells.

3.2. Se-SP Promoted Calcium Secretion of MC3T3-E1. Selenium and selenium proteins have been confirmed to display important roles in regulating the osteoporosis process [13]. In order to evaluate whether Se-SP could modulate osteoporosis, the osteoblastic cells MC3T3-E1 were used as a cell model to examine whether Se-SP could enhance the calcium secretion of MC3T3-E1. As shown in Figure 2(a), 10 μg/mL SP was able to induce the secretion of calcium in MC3T3-E1 cells. However, the supplementation of Se enhanced the modulation roles of SP in promoting calcium secretion in a dose-dependent manner as indicated by the obvious alizarin red staining. Furthermore, the absorbance of the dissolved eluted alizarin red and quantified calcium content in MC3T3-E1 cells also revealed that Se-SP dramatically facilitated the secretion of calcium than SP incubation (Figures 2(b) and 2(c)), which suggested the distinct role of Se in SP. Together, these results suggest that Se-SP could enhance the secretion of calcium in MC3T3-E1 cells.

3.3. Se-SP Activated ALP Activity and Expression of MC3T3-E1. Alkaline phosphatase (ALP) plays a critical role in mineralizing the extracellular matrix, which is the process of depositing calcium and phosphate minerals to strengthen and harden the bone tissue. ALP is often used as a marker of osteoblastic activity, as it is produced by these cells during the process of bone formation. Therefore, next, we conducted an experiment to evaluate whether Se-SP could activate ALP. As illustrated in Figures 3(a) and 3(b), Se treatment significantly upregulated the ALP expression in MC3T3-E1 cells; however, Se introduction dramatically enhanced the expression level of ALP, which suggest that Se supplementation may augment the specific regulation properties of SP. In order to further examine the regulating
Figure 1: Culture of Se-SP and the cytotoxicity of Se-SP on MC3T3-E1 cells. (a) Culture of Se-SP. (A1) Photograph of Se-SP cultured in Erlenmeyer flasks. Representative phase contrast picture (A2) and fluorescence images of Se-SP (A3). (b) The ultraviolet absorption of the extracted protein from Se-SP. (c) Cytotoxicity effects of different concentrations of SP and Se-SP on MC3T3-E1 cells.

Figure 2: Se-SP enhanced the excretion of calcium in MC3T3-E1 cells. (a) Representative images of calcium staining in MC3T3-E1 cells after the treatment of SP and Se-SP. Quantification of the alizarin red absorbance (b) as well as calcium content (c) in cells treated with Se-SP and SP. Characters A, B, C, and D are considered statistically different between the groups.
activities of SP-Se on ALP, we also detected the expression of ALP after SP-Se treated MC3T3-E1 cells at different doses and incubation times. SP-Se upregulated the expression of ALP in a dose- and time-dependent manner (Figures 3(c) and 3(d)). Taken together, these results indicated that Se supplementation could enhance ALP expression induced by SP in MC3T3-E1 cells.

3.4. Se-SP Reversed Ovariectomy-Induced Bone Loss in Mice.
In order to further evaluate the regulation roles of SP-Se in osteoporosis, we established an ovariectomy mice model. After ovariectomy, mice were intragastrically administrated with 10 mg/kg SP-Se or SP daily for 14 consecutive days. At the end of the experiments, mice were euthanized and the femurs were collected and subjected to micro-CT analysis. As shown in Figure 4(a), a large area of bone erosion and obvious microstructure destruction of the tibias were observed in the ovariectomy mice model. SP treatment significantly inhibited bone loss in the ovariectomy mice model; however, the restraining effects of SP were dramatically enhanced by Se supplementation, as indicated by the upregulated BV, Tb.Th, Bv/TV, Tb.N, and BMD as well as the decreased Tb.Sp. For instance, the BMD value was increased to 0.941 ± 0.073 g/cm³ in the SP-Se treatment groups when compared to the OVX groups (0.470 ± 0.063 g/cm³). Tb.Sp value decreased from 0.435 ± 0.009 to 0.288 ± 0.001 mm when mice were treated with Se-SP (Figures 4(b)–4(g)). Together, these results demonstrate that Se-SP possesses superior inhibition effects against ovariectomy-induced bone loss.

3.5. Se-SP Inhibited Ovariectomy-Induced Osteoblast Inactivation in Mice. Evidence has confirmed that the imbalance of osteoblasts and osteoclasts contributed to bone density loss and osteoporosis [23]. The overactivated osteoclasts and inactivated osteoblasts may result in the coverage
of the absorption rate of bone tissue against the formation rate, which could thus result in osteoporosis. Therefore, we next detected the osteoblast activity in the Se-SP-treated mice. As shown in Figure 5(a), SP administration induced upregulation of the expression level of BMP2, RUNX, Col-I, and OCN as indicated by the enhanced fluorescence intensity. However, Se-SP treatment dramatically reversed the suppression effects of osteoblast activity induced by ovariectomy in mice as indicated by the significantly upregulated values (Figures 5(b)–5(e)), which suggest the specific
regulation properties of Se in SP. Together, these results demonstrate that Se-SP inhibits ovariectomy-induced osteoblast inactivation.

3.6. Se-SP Suppressed Ovariectomy-Induced Osteoclastogenesis in Mice. Osteoclasts are primarily responsible for absorbing bone tissue. The overproduction of osteoclasts can lead to osteopenia, which affects the bone microstructure and reduce bone density, resulting in brittle bones. Inhibition of excessive osteoclast formation is an important strategy for osteoporosis therapy [23]. As depicted in Figure 6(a), an increased population of osteoclasts was observed in ovariectomy untreated mice; however, SP administration was found to inhibit the formation of osteoclasts as indicated by the decreased TRAcp and RANKL positive cells as well as the upregulation of OPG expression. Additionally, we also found that 10 mg/kg SP-Se treatment triggered more significant suppression effects on osteoclast formation than SP alone therapy. Furthermore, the quantification analysis of TRAcp, RANKL, and OPG expression (Figures 6(b)–6(d)) also confirmed the inhibition capacity of SP-Se on osteoclastogenesis. For example, the positive OPG expression level in the OVX groups was 21.262 ± 7.088 but SP-Se administration for 14 days significantly enhanced the expression and the value increased to 91.204 ± 7.828. Similarly, the RANKL expression level in the OVX was 100.00 ± 7.287 and dropped to 17.251 ± 9.196 in SP-Se treatment groups. Together, these results demonstrate that SP-Se could inhibit ovariectomy-induced osteoclastogenesis.

3.7. Se-SP Attenuated Ovariectomy-Induced Inflammatory Response in Mice. Inflammation is highly correlated with osteoporosis [24]. Chronic inflammation stimulates the secretion of inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6), which may promote the production and activity of

Figure 5: Se-SP inhibited osteoblast inactivation in ovariectomized mice. (a) Representative fluorescence images of the expression of BMP2, RUNX, Col-I, and OCN in the femur tissues. Quantification analysis of the expression of BMP2 (b), RUNX (c), Col-I (d), and OCN (e). Ovariectomy mice were treated with Se-SP and SP for 14 days, then mice were euthanized and the femurs were subjected to the analysis of osteoblast activation by staining with specific antibodies. Characters A, B, C, and D are represented as statistically different between the groups.
osteoclasts while inhibiting the function of osteoblasts, resulting in increased bone resorption and decreased bone formation. To further evaluate the regulation roles of SP-Se in osteoporosis, the expression level of IL-6, IL-10, IL-4, and IL-1β was examined by Western Blotting assay. As shown in Figure 7(a), the expression of the proinflammatory cytokines such as IL-6 and IL-1β were significantly inhibited by SP administration, meanwhile, the anti-inflammatory cytokines IL-10 and IL-4 expression was enhanced. Moreover, Se supplementation augmented the regulating properties of SP on these cytokines production, and these results were further confirmed by the quantification analysis. Collectively, these results suggest that SP-Se could balance the activation of osteoclasts and osteoblasts by regulating the inflammatory response.

4. Discussion

OP is one kind of bone disease that is characterized by reduced muscle power and an increased risk of fractures. Strategy that is based on bisphosphonates, estrogen/hormone re-placement therapy, and anti-RANKL antibody has been widely used for OP treatment [23–25]. However, the therapeutic efficiency is still limited. For instance, many

![Figure 6: Se-SP suppressed osteoclastogenesis in ovariectomized mice. (a) Images of osteoclasts that stained with TRAcP, OPG, and RANKL in different treatment groups. Quantification analysis of TRAcP + cells (b), OPG (c), and RANKL (d) expression level. Characters A, B, C, and D are represented as statistically different between the groups.](image)
antiosteoporosis drugs, especially bisphosphonates, can cause a range of side effects, such as gastrointestinal problems, musculoskeletal pain, and abnormal kidney function. Additionally, some of the antiosteoporosis drugs are costly and may not be affordable for all patients. This may limit treatment options and use for some patients. Therefore, searching for novel effective and safety agents for anti-OP therapy is of great interesting.

As a critical trace element, Se is mainly regulating biological function through its incorporation into proteins to form selenoproteins, which regulate redox homeostasis, inflammation, and signal transduction. The association of selenium with bone turnover and bone disease has been confirmed in various epidemiological studies [26, 27]. In this study, we make full use of Se containing proteins derived from Se-enriched *Spirulina platensis*, which is edible and possess multiple nutrients as well as could be mass-produced to combat OP progression in ovariectomized mice model. The therapeutic effects induced by Se-SP is very considerable as evidenced by the micro-CT characterization after Se-SP administration treatment, such as upregulation of BV, Tb.Th, Bv/TV, Tb.N, and BMD but downregulation of Tb.Sp. Our previous studies have confirmed that Se-containing proteins after Se incorporation showed enhanced the antioxidant and anti-inflammatory response activities [17, 28]. Hence, we speculated the possibility that Se incorporated into protein in the form of selenoamino acids, such as selenocysteine or selenomethionine, and enhanced the antioxidant and anti-inflammatory response by regulating multiple signals, which eventually alleviated ovariectomy-induced osteoporosis. These results demonstrated the promising application of Se-SP in OP treatment.

Osteoblasts are the cells responsible for forming bone tissue. They are active on the surface of bone and help bone grow, repair, and maintain bone density by depositing bone matrix (the structural framework of bone) and minerals. Osteoblasts synthesize collagen and other proteins that promote the formation of bone matrix and store minerals such as calcium and phosphorus in it. The activity of osteoblasts is called “bone resorption” and it plays a balancing role in bone growth, repair, and bone metabolic balance. Under normal physiological conditions, the activity of osteoblasts and osteoclasts is coordinated with each other to maintain bone homeostasis. However, the imbalance of osteoblasts and osteoclasts could lead to osteoporosis and fractures. Evidence has disclosed that Se may suppress ROS generation through selenoproteins such as GPX and TrxR to maintain bone homeostasis [13]. When trace for the reason...
of Se-SP could attenuate the progression of OP, we found that Se containing protein Se-SP administration dramatically inhibited osteo-clasts-mediated resorption but facilitate osteoblasts-mediated bone formation, which suggests that Se-SP alleviate bone lost through maintaining the balance of osteoblasts and osteoclasts. The underlining mechanisms may involve in the direct antioxidant properties of Se-SP or selenoprotein, which is derived from Se-SP metabolization into Se, thus modulating the expression of selenoprotein.

The inflammatory response may directly or indirectly contribute to the development of osteoporosis. For example, in chronic inflammatory diseases, the inflammatory process may release proinflammatory cytokines, which may affect bone metabolism, resulting in increased bone resorption, reduced bone formation, and ultimately osteoporosis. On the other hand, osteoporosis itself may trigger an inflammatory response. When bone tissue is destroyed or bone is damaged, the bone matrix and biochemical molecules released can activate the immune system, leading to an inflammatory response. This inflammatory response may further affect the destruction and repair process of bone tissue, forming a vicious cycle that leads to the aggravation of osteoporosis. We found that Se-SP treatment could restrict the inflame response in OP, which suggested that Se-SP may regulate the inflame relative signaling transduction such as TLR-mediated signaling pathway [29] and thus inhibit the inflammatory cytokines expression.

5. Conclusion

In conclusion, Se-SP was successfully extracted and prepared, and the antiosteoporosis effect and the underlying mechanism were investigated in MC3T3-E1cells and ovariectomized mice model. The results showed that Se-SP significantly promoted ALP activation and expression and enhanced excretion of calcium. Se-SP administration in vivo effectively alleviated ovariectomy-induced osteoporosis by activating osteoblast, inhibiting inflammatory cytokines, and blocking osteoclastogenesis through upregulating BMP2, RUNX2, COL-I, OCN, and OPG pression and down-regulating RANKL expression. Our finding validated that Se-SP administration may be a promising therapeutic strategy for osteoporosis.

Data Availability

The research data used in this study are not available.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors’ Contributions

YCP and XYF conceived and designed the experiments. YY, HY, XTF, QLS, JC, and YJH performed all experiments and analyzed the data. YY and HY wrote the paper. All authors revised and approved the final version of the manuscript.


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