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Oil from Antarctic krill (*Euphausia superba*) facilitates bone formation in dexamethasone-treated mice

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Abstract Glucocorticoids are the leading cause of secondary osteoporosis. In the current study, the in vivo effects of Antarctic krill (*Euphausia superba*) oil (AKO) on dexamethasone-treated mice were investigated. Results showed that AKO significantly prevents bone loss, as evidenced by improved bone mineral density, biomechanical strength, and cancellous bone microstructure. Fluorescence double-labeling of femur showed that AKO induces new bone formation. Toluidine blue staining of marrow cavity indicated that AKO increases the number of trabecula, and decreases the generation of adipose cells. Runt-related transcription factor 2 (Runx2) and Peroxisome proliferator-activated receptor γ (PPARγ) are the switches for osteogenic and adipogenic differentiation of bone marrow mesenchymal stem cells, respectively. AKO significantly promoted the expression of Runx2 protein, and reduced PPARγ expression in bone tissue. Furthermore, AKO increased the mRNA expression of osteogenesis-related genes and decreased the expression of adipogenesis-related genes. In conclusion, AKO improved glucocorticoid-induced osteoporosis via promoting bone formation.

Keywords Antarctic krill oil · Dexamethasone · Bone formation · Runt-related transcription factor 2 · Peroxisome proliferator-activated receptor γ

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Introduction

Glucocorticoids (GCs) are widely applied clinically as immune-modulators for treating a variety of chronic diseases, such as rheumatoid arthritis, polymyalgia rheumatica and inflammatory bowel disease. However, long-term use of GCs leads to some adverse effects, including a high incidence of osteoporosis (Pasqualetti et al., 2015). GCs are the leading cause of secondary osteoporosis. They have been found to induce the destruction of bone microstructure, thereby accelerating bone loss and increasing the risk of fracture (Reid et al., 2009; van Staa et al., 2005).

GCs inhibit bone formation through their direct and indirect effects on osteoblasts, osteoclasts, and osteocytes (Weinstein et al., 2010). Loss of vitality and differentiation capacity of osteogenic cells is the principal cause of GC-induced bone degeneration. Osteoblasts are differentiated from bone marrow mesenchymal stem cells (BMSCs). GCs stimulate BMSCs differentiation into adipocytes instead of osteoblasts, resulting in the decreased osteogenesis (Li et al., 2013; Zhou et al., 2014).

Since the existing drugs for treating osteoporosis have adverse effects, it necessary to extract novel alternatives from natural products. Omega-3 polyunsaturated fatty acids (ω-3PUFAs) are a group of essential fatty acids, and they play important roles in the protection of bone metabolism (Bonnet and Ferrari, 2011). There are several biological pathways whereby ω-3 PUFAs regulate bone formation and resorption. First, ω-3 PUFAs could change the fatty acid composition of membrane phospholipids, specifically the biosynthesis of pro-inflammatory prostaglandin E2 which promotes bone resorption (Blackwell et al., 2010; Fetterman and Zdanowicz, 2009). In addition, the suppression of the production of other inflammatory cytokines, such as TNF-α, IL-1, and IL-6, helps ω-3

PUFAs to inhibit bone resorption and prevent bone loss (Appleton et al., 2011). Second, ω-3 PUFAs could promote bone formation by preventing the creation of products that inhibit osteoblastogenesis, such as lipid peroxide (Poulsen et al., 2007). Both ω-6 and ω-3 PUFAs can act on precursor cells of osteoblasts and adipocytes, but ω-3 PUFAs do not exhibit a strong adipogenic effect, thus, stimulating osteoblast differentiation (Casado-Diaz et al., 2013).

Antarctic krill (*Euphausia superba*) has an estimated biomass of 379 million tonnes, and it attracts more and more attention due to its high nutritional content (Atkinson et al., 2009). Two major ω-3PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoicacid (DHA), are abundant in the oil of Antarctic krill (Phleger et al., 2002). In the current study, oil was extracted from Antarctic krill, and its anti-osteoporotic activity was investigated on dexamethasone-treated mice for the first time.

Materials and methods

Materials and reagents

Tetracycline and calcein were purchased from Sigma-Aldrich (St. Louis, MO, USA). Moloney murine leukemia virus reverse transcriptase (M-MLV), random primers, dNTPs, and PageRuler prestained protein ladder were purchased from TaKaRa Bio Inc (Otsu, Shiga, Japan). FastStart Universal SYBR Green Master (Rox) came from Roche (Basel, Switzerland). Primers for bone sialoprotein (BSP), type I procollagen (Col1a), APETALA2 (ap2) and fatty acid binding protein 4 (FABP4) were synthesized by GENEWIZ (Suzhou, China). Primary rabbit anti-Runt-related transcription factor 2 (Runx2, #12556) and peroxisome proliferator-activated receptor-γ (PPARγ, #2345) were from Cell Signaling (Beverly, MA, USA). The goat anti-rabbit IgG-HRP secondary antibody was from proteintech (Catalog: SA00001-2, Chicago, IL, USA).

Preparation of Antarctic krill oil (AKO)

Freeze-dried Antarctic krill (purchased from China national fisheries CORP, Beijing, China) powder was extracted using 95% ethanol at 45 °C for 6 h (1:12, m/v). Then the supernatant was filtered and concentrated to obtain crude extract of AKO. 20% chitosan was added in AKO, and agitated at 40 °C for 60 min to remove free fatty acids. 10% β-cyclodextrin was added in AKO, and agitated at 40 °C for 30 min to remove cholesterol. Ceramic membrane filtration and molecular distillation technologies were applied to remove other impurities, and finally obtained high-quality AKO. It contained approximately 46.1% of phospholipids, 35.2% of triglycerides, 2.2% of cholesterol and 12.6% of free fatty acids. There were 26.72% of EPA and 18.31% of DHA in AKO.

Animals and experimental design

This study was approved by the Ethical Committee of Experimental Animal Care at Ocean University of China (Certificate number: SYXK20120014). Ten-week-old female C57BL/6J mice (n = 40, 20 ± 2.0 g) were purchased from Vital River Laboratory Animal Center (Beijing, China; license ID: SCXK2012-0001). The animals were housed four per cage at 22–24 °C with a 12-h light/12-h dark cycle and given food and water ad libitum. Thirty mice were randomly divided into three groups (n = 10 per group): Model group, alendronate sodium (ALF) group as positive control and AKO group. They were subcutaneous injected with dexamethasone sodium phosphate (10 mg/kg body weight) three times a week to establish a glucocorticoid-induced osteoporosis model. The remaining 10 mice were subcutaneously injected with physiological saline, serving as a control group. The injection volume was 1 mL/100 g body weight. At the same time, the ALF and AKO groups were intragastrically treated with alfacalcidol (0.15 μg/kg body weight) and AKO (160 mg/kg body weight), respectively. The control and model groups were both intragastrically treated with physiological saline. Animals in each group were given intragastric administration of physiological saline or respective drugs (1 mL/100 g body weight) once a day. After 90 days of treatment, serum was collected to measure bone turnover indicators. Femur and tibia were isolated immediately after sacrifice, followed by an evaluation of bone mineral density (BMD), biomechanical properties, bone microstructure, and gene expression.

Measurement of serum biochemical indicators

The bone formation indicators including bone morphogenetic protein 2 (BMP2), carboxy-terminal propeptide of procollagen type I (PICP), tartrate resistant acid phosphatase (TRAP) and C-telopeptide of type 1 collagen (CTX-1) were measured in serum using corresponding commercial ELISA kits (R&D, Minneapolis, MN, USA).

BMD and biomechanical property testing

BMD of femur was determined using a GK99-UNI-GAMMA X-RAY PLUS Bone Densitometer (L'ACN, Napoli, Italy). Biomechanical property of tibia, including ultimate load and stiffness, were tested using a YLS-16A bone strength tester (Yiyan CORP, Jinan, China).

Histological analysis

The distal femur was fixed in 10% neutral formaldehyde for 24 h and decalcified in 10% ethylene diamine tetraacetic acid (EDTA) for 4 weeks. The decalcified tissue was then embedded in paraffin after dehydration with ascending grades of ethanol. The embedded samples were serially sectioned (4 μm thickness) and stained with hematoxylin and eosin (H&E) or toluidine blue. Finally, the tissue sections were observed and photographed using BH-2 microscope (Olympus, Tokyo, Japan) equipped with matching camera software.

Fluorescence double-labeling

Mice were treated with tetracycline (20 mg/kg body weight) and calcein (20 mg/kg body weight) by intraperitoneal injection 13 and 3 days before sacrifice, respectively. The distal femur was collected and decalcified in 10% EDTA for 3 h. After dehydration in ascending grades of ethanol, tissue was embedded in paraffin and serially sectioned at 10 μm thickness. Tissue sections were dewaxed in xylene for 1 h, followed immediately by the addition of fluorescence quenching agent in a dropwise manner. Finally, tissue sections were analyzed and photographed under a Leica DM2500 fluorescence microscope (Scanco Medical AG, Bassersdorf, Switzerland).

Real time PCR

Total RNA from the proximal femur was extracted using TRIzol reagent, and 1 μg of RNA was reverse transcribed into cDNA using M-MLV. Real time PCR analysis was performed on a Multicolor Real-Time PCR Detection System (Bio-RAD, Hercules, CA, USA). Sequences of the primers used in this study are listed in Table S1. Each real time PCR reaction was assembled in a total volume of 25 μL containing 12.5 μL of FastStart Universal SYBR Green Master, 5 μL of cDNA template, 0.75 μL of forward primer (10 μM), 0.75 μL of reverse primer (10 μM), and 6 μL of sterile Milli-Q water. The reaction program was set as follows: denaturation at 95 °C for 10 min, 45 cycles of 15 s at 95 °C, 20 s at 60 °C, 30 s at 72 °C, and a final extension at 72 °C for 10 min. Standard curves were constructed from PCR reactions using fivefold serial dilutions of mixture samples. The relative mRNA expression was calculated as the ratio of signal intensity of the target genes to that of β-actin.

Western blot

The proximal femur was ground into powder using liquid nitrogen. Next, 0.1 g of the powder was lysed in Western and IP lysis buffer (Applygen, Beijing, China) and centrifuged to obtain liquid supernatant. The obtained proteins were separated on a 10% SDS-PAGE gel and then electrotransferred onto polyvinylidene fluoride membrane. The membrane was blocked with 5% bovine serum albumin and incubated with primary antibodies overnight at 4 °C. Subsequently, the membrane was incubated with horseradish peroxidase conjugated IgG secondary antibody at indoor temperature for 2 h. Protein bands were visualized using an ECL stain kit (Applygen, Beijing, China) and quantified with the Image J program.

Statistical analysis

All data were subject to one-way analysis of variance (ANOVA) using the SPSS statistical program (Version 17.0). Significant differences among treatments were compared by least-significance-difference (LSD) method multiple range test. The results were presented as mean ± standard deviation. Values of $p < 0.05$ were considered as significant.

Results and discussion

AKO improved bone turnover

In order to evaluate the changes of bone turnover, bone formation markers BMP2 and PICP, as well as resorption markers TRAP and CTX-1 were measured in serum. As shown in Table 1, the bone formation activity in the model group was distinctly decreased compared to the control group, whereas the resorption activity was increased ($p < 0.01$). This undesirable reduction of bone turnover was significantly reversed by AKO treatment. AKO increased BMP2 and PICP by 5.38 and 10.83%, respectively, and decreased TRAP and CTX-1 by 7.64 and 12.48%, respectively.

AKO inhibited bone loss and enhanced bone biomechanical strength

BMD is indicative of the bone loss status, thus, it is an effective means to diagnose osteoporosis (Cao et al., 2014). In this study, femur BMD was determined using dual energy X-ray absorptiometry (Fig. 1A). Results showed that dexamethasone administration resulted in a significant decrease of BMD in the model group compared to the control. However, the intervention with AKO clearly rescued the decreased BMD by 11.11%.

Excessive bone loss leads to the increased bone fragility. In the current study, biomechanical strength of tibia, including ultimate load and stiffness, was determined by a

Table 1 Effects of AKO on bone turnover markers in serum

Group	BMP2 (ng/mL)	PICP (ng/mL)	CTX-1 (ng/mL)	TRAP (U/L)
Control	18.00 ± 0.48	17.88 ± 0.63	4.17 ± 0.17	30.56 ± 0.95
Model	16.00 ± 0.56^{aa}	14.87 ± 0.76^{aa}	5.85 ± 0.21^{aa}	36.53 ± 1.81^{aa}
ALF	16.84 ± 0.32^{aa,bb}	16.80 ± 0.79^{aa,bb}	4.94 ± 0.18^{aa,bb}	33.33 ± 1.71^{aa,bb}
AKO	16.86 ± 0.39^{aa,bb}	16.48 ± 0.56^{aa,bb}	5.12 ± 0.16^{aa,bb}	33.74 ± 1.63^{aa,bb}

Data are presented as the mean ± SD (n = 8 per group). Multiple comparisons were done using one-way ANOVA

$^{aa}p < 0.01$ versus control group

$^{bb}p < 0.01$ versus model group

AKO Antarctic krill (*Euphausia superba*) oil

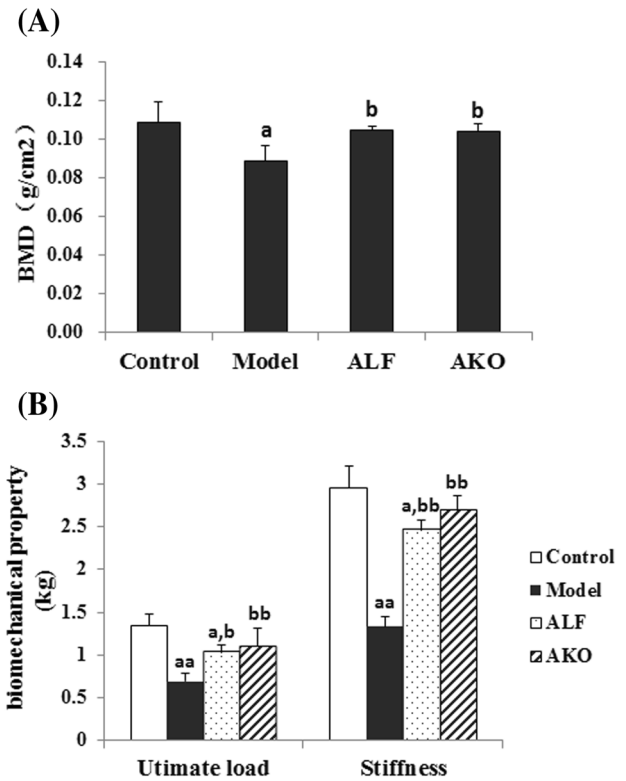

Fig. 1 Effects of AKO on BMD and bone biomechanical properties. (**A**) BMD of mice. Femurs BMD were determined by dual energy X-ray absorptiometry. (**B**) Bone biomechanical property of mice. A three-point bending test was applied to tibiae for the evaluation of bone stiffness and ultimate load. Data are presented as the mean ± SD (n = 8 per group). Multiple comparisons were done using one-way ANOVA. $^{a}p < 0.05$; $^{aa}p < 0.01$ versus control group; $^{b}p < 0.05$; $^{bb}p < 0.01$ versus model group

three-point bending test (Fig. 1B). Results indicated that the biomechanical strength in the model group was noticeably reduced compared to the control. Nevertheless, treatment with AKO significantly enhanced the biomechanical strength, as evidenced by increased ultimate load and stiffness by 59.42 and 102.26%, respectively.

AKO ameliorated cancellous bone microstructure

Bone histological analysis is commonly used to evaluate bone quality. Here, paraffin sections of the distal femur were stained with H&E stain to examine changes in cancellous microstructure. As shown in Fig. 2 that dexamethasone induced dramatic reduction of trabecular number and deterioration of trabecular structure when compared to the control. However, treatment with AKO remarkably recovered the undesirable changes in cancellous microstructure under growth plate.

AKO induced new bone formation

Long-term treatment with glucocorticoids could directly induce the apoptosis of osteoblasts and osteocytes, impair the differentiation of osteoblasts, and prolong the life span of osteoclasts (Canalis, 2005; Weinstein et al., 2010; Yun et al., 2009). Deterioration of bone formation activity is a prominent reaction in glucocorticoid-induced osteoporosis

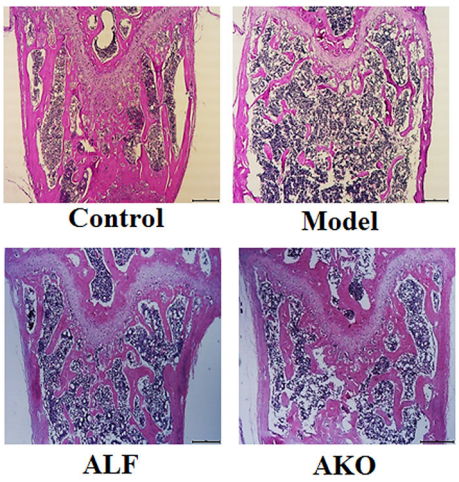

Fig. 2 Effects of AKO on cancellous bone microstructure. The distal femur was embedded in paraffin after 4 weeks of decalcification. Paraffin sections of 4 μm thickness were stained with H&E and analyzed using BH-2 microscope equipped with a matching camera software (n = 6, ×4 magnification)

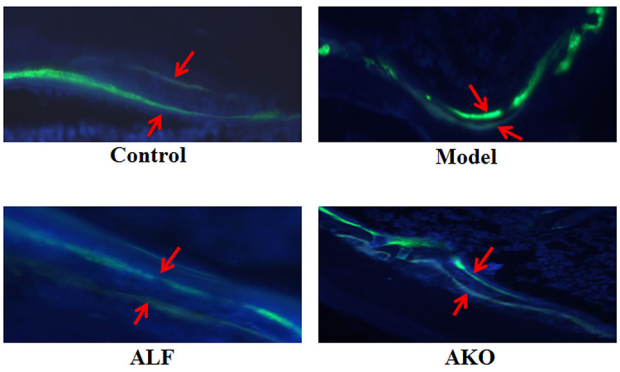

Fig. 3 Effects of AKO on new bone formation. Mice were treated with tetracycline (20 mg/kg body weight) and calcein (20 mg/kg body weight) by intraperitoneal injection 13 and 3 days before sacrifice, respectively. The distal femur was collected and decalcified in 10% EDTA for 3 h. The decalcified tissue was embedded in paraffin and serially sectioned at 10 μm thickness. After dewaxing with xylene and addition of fluorescence quenching agent, the tissue sections were immediately analyzed under a fluorescence microscope (n = 6, × 40 magnification)

(Wu et al., 2011). In the current study, tetracycline/calcein double labeling was performed to monitor dynamic bone formation in vivo (Fig. 3). Results showed that the distance between tetracycline and calcein labels in the model group was noticeably shorter than that in the control group, indicating that the rate of bone formation was reduced by dexamethasone. Treatment with AKO facilitated new bone formation, as evidenced by increased distance between both labels.

AKO facilitated the formation of trabecula and inhibited the generation of adipocyte in marrow cavity

Osteogenesis is mediated by recruiting BMSCs, and they can differentiate into osteoblasts. BMSCs exist mainly in bone marrow and subcutaneous fat, can differentiate into osteoblasts, adipocytes and chondrocytes (Fink et al., 2011; Mosna et al., 2010). Glucocorticoids disturb osteogenic differentiation capacity in bone tissue and stimulate BMSCs differentiation into adipose cells in marrow cavity (Rauch et al., 2010; Yao et al., 2008). Toluidine blue staining of bone marrow (Fig. 4A) showed that there were more adipocytes and fewer trabeculae in the model group compared to the control group. Correspondingly, the mRNA expressions of osteogenic marker genes BSP and Col1a were decreased, and the expression of adipogenic marker genes ap2 and FABP4 were increased in the model

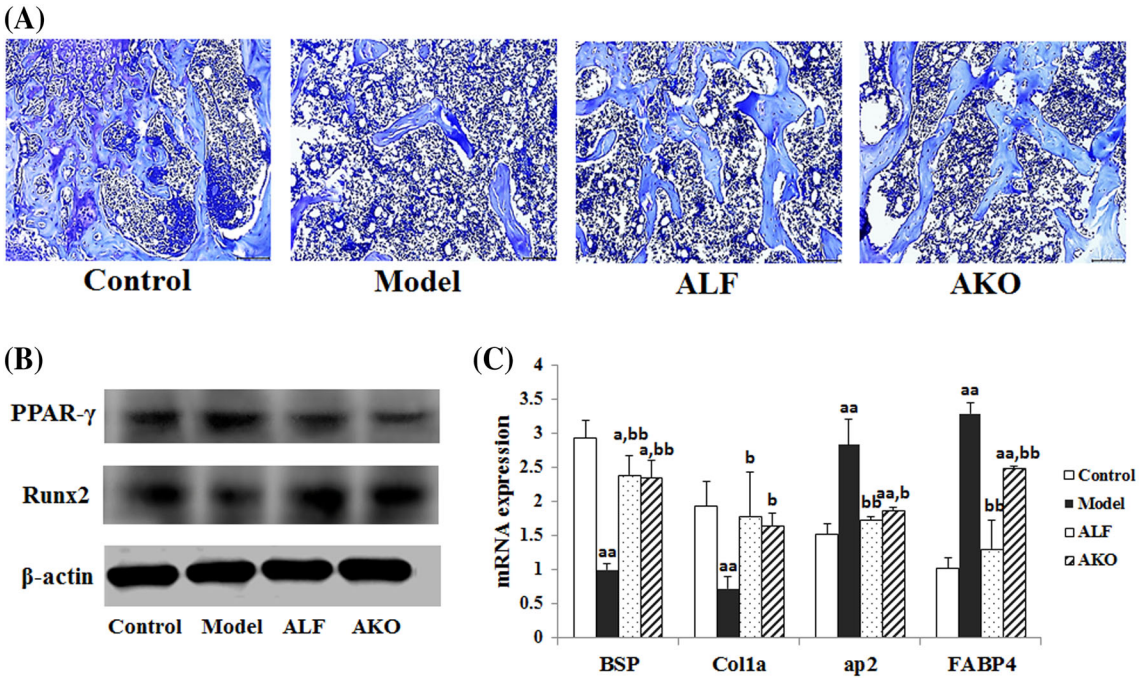

Fig. 4 Effects of AKO on directional differentiation of BMSCs in bone tissue. The distal femur was embedded in paraffin after 4 weeks of decalcification. Paraffin sections of 4 μm thickness were stained with toluidine blue and analyzed using BH-2 microscope equipped with a matching camera software (n = 6, × 10 magnification). The proximal femurs were used for real-time PCR and Western blot experiments. (**A**) Toluidine blue staining of marrow cavity in the femur. (**B**) The expressions of Runx2 and PPARγ proteins. (**C**) The mRNA expressions of BSP, Col1a, ap2, and FABP4. Data are presented as the mean ± SD (n = 6 per group). Multiple comparisons were done using one-way ANOVA. $^{a}p < 0.05$; $^{aa}p < 0.01$ versus control group; $^{b}p < 0.05$; $^{bb}p < 0.01$ versus model group

group (Fig. 4C). Nevertheless, AKO treatment remarkably increased the number of trabeculae in bone marrow. In addition, AKO decreased the mRNA expression of ap2 and FABP4 genes by 33.92 and 24.32%, respectively, and increased the expression of BSP and Col1a by 135.00 and 127.78%, respectively, when compared to the model group.

Osteogenic and adipogenic differentiation of MSCs is reversely regulated by critical differentiation regulators. Runx2 is the key transcription factor for BMSCs differentiation into osteoblasts, whereas PPARγ stimulates adipogenesis via inhibiting transcriptional activity of Runx2 (Jeon et al., 2003; Kawai and Rosen, 2010; Shi et al., 2000). The results in Fig. 4B showed that AKO significantly increased the expression of Runx2 protein but decreased PPARγ expression, suggesting that AKO promoted the directional osteoblastic differentiation of BMSCs in bone marrow.

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Compliance with ethical standards

Conflict of interest The authors declare no competing financial interest.

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