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Sheep bone collagen peptide ameliorates osteoporosis by regulating RANK/RANKL/OPG signal pathway

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Abstract

Sheep bone collagen peptide (SBCP) has attracted attention due to its potential effects on bone health. This study aims to explore the bone protective effect of SBCP in the presence of estrogen deficiency. Ovariectomized (OVX) female rats were given different dose rates (0, 0.68, 2.05, 3.40 g/kg BW) of SBCP for 8 consecutive weeks. At the end of the treatment, the serological indexes and tibial mineral content were measured. The microstructure and morphological changes of femur bones were observed by scanning electron microscopy (SEM) and hematoxylin and eosin (H & E) staining. Femur bones were harvested for determination of expression level of RANK, RANKL, OPG by molecular biological techniques. 3.40 g/kg BW SBCP treatment decreased the PINP and β-CTx levels but increased the tibial calcium and phosphorus contents significantly in estrogen deficient rats. Expression of RANK and RANKL decreased however expression of OPG increased in the bone of estrogen deficient rats which received 3.40 g/kg BW SBCP with greater effects than SBCP-M and SBCP-L treatments. SBCP helps to overcome the adverse effect of estrogen deficiency the bone and thus this nutriment could potentially be used for the treatment and prevention of osteoporosis in postmenopausal women.

Keywords: sheep bone collagen peptide; osteoporosis; RANK; RANKL; OPG.

Practical Application: In this work, we evaluated sheep bone collagen peptide could regulate the activity of osteoclasts through the RANK/RANKL/OPG signal pathway, improve the bone tissue structure and mineral content, and thus intervene the occurrence and development of osteoporosis caused by estrogen deficiency.

1 Introduction

Osteoporosis (OP) is a metabolic disease mainly characterized by deterioration of bone microstructure and reduction of bone density (Gamsjaeger et al., 2021; Langdahl, 2020), which is clinically manifested as easy fracture and limb pain (Miyamura et al., 2021). Epidemiological studies have found that the incidence of OP is increasing in older women, and the incidence rate of postmenopausal women is as high as over 60% (Dai et al., 2022; Xie et al., 2020), and the female patients increase at a rate of 20% every 10 years (Yu & Xia, 2019). OP has become a global and social health problem of great concern.

At present, many pharmacological compounds (such as strontium ranelate, teriparatide, raloxifene, alendronate, hormone melatonin, etc.) have been applied in osteoporosis treatment (Shanks et al., 2019). However, these medicines do have some limitations such as undesired side effects, drug resistances, and high prices (Hayes et al., 2021). Therefore, health professionals have paid much attention to both safety and economical food-derived compounds. Collagen is the most abundant protein in bone. A large number of studies have shown that collagen peptide, the hydrolysate of collagen protease, can improve bone metabolism, and has the advantages of wide source, high bioavailability, safety and no side effects (Cordeiro et al., 2020). It is a good natural foodborne component for the prevention of osteoporosis.

RANK/RANKL/OPG pathway is known to be important for the bone remodeling process (Varley et al., 2015). Binding of RANK by RANKL on osteoclasts would lead to increase in osteoclast activity and subsequently osteoclast-mediated bone resorption (Amin et al., 2020). On the other hand, suppression of RANKL binding to RANK by osteoprogerin (OPG) which is produced by osteoblasts (Brunetti et al., 2019) will lead to osteoclast inactivation, and thus reduces bone resorption. A series of hormone factors such as estrogen can act on the RANK/RANKL/OPG system through RANKL or OPG to regulate bone metabolism (Zhao et al., 2020). In view of its important role in the pathogenesis of OP, the signal pathway has become a significant target for drug design in the treatment of OP (Yasuda, 2021).

Sheep bone collagen peptide (SBCP) was proven to have the improvement effect on the bone of OVX rats in the previous studies. This study aims to explore the mechanism to scientifically justify the bone protective effect of SBCP and thus help to support the use of this nutriment in protecting the bone against detrimental effect of estrogen deficiency.

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2 Materials and methods

2.1 Animal preparations

Fifty 8 weeks old adult female Sprague-Dawley (SD) rats, weighing 203 ± 10 g, were housed in a standardized environment (12 h light: 12 h dark cycle, temperature 24 ± 2 °C). Rats were fed with chow diet and received distilled water ad libitum. After a week of acclimatization, 40 rats underwent bilateral ovariectomy under ketamine and 10 in the sham group underwent peri-ovary fat removal operations (Sayem et al., 2018). Seven days after ovariectomy, drug treatment was initiated, the ovary-free rats were randomly divided into 1 model group and 3 SBCP groups (n = 10). The SBCP (Inner Mongolia Taihao Biological Products Co., Ltd., China) dosages for the high- (SBCP-H), medium- (SBCP-M), and low-dose groups (SBCP-L) were 3.40, 2.05 and 0.68 g/kg BW respectively (Jin et al., 2018). The drugs were administered for 8weeks by using oral gavage tube. All animal procedures were approved by the ethical committee of animal research in the Shanxi Agricultural University, and complied with the requirements of the National Act on the use of experimental animals (China).

2.2 Determination of serum PINP and β-CTx

The procollagen type I N-terminal propeptide (PINP) and C-terminal cross-linking telopeptide of type I collagen (β-CTx) levels in serum were measured by using the corresponding commercial kits (Elabscience Biotechnology Co., Ltd., China) and the levels were expressed in pg/mL.

2.3 Histological changes in femur tissues

The femur bones were harvested and fixed with 4% paraformaldehyde, decalcified by EDTA, then the bone histomorphology was analyzed by the hematoxylin and eosin (H & E) staining method (Xia et al., 2015). Sections were viewed under light microscope (Olympus, Inc., Shinjuku, Japan) at 100 × magnification.

2.4 Observation of bone microstructure

The femur bones were fixed with glutaraldehyde and then decalcified with EDTA (Liu et al., 2017), the cross-section of the metaphysis was taken to make sections for SEM (JEM-6490LV, JEOL, Japan) analysis.

2.5 Determination of calcium and phosphorus content in tibia

Tibias treated with liquid nitrogen and stored in -80 °C were taken out and weighed. The tibias were ground to powder and lysed at the ratio of 2 mL/g protein lysate for 30 min, the supernatant was harvested by centrifugation at 3500 r/min for 15 min. The contents of calcium and phosphorus were detected at the wavelength of 600 nm and 340 nm respectively by A6 semi-automatic biochemical analyzer (Songshang Technology Co., Ltd. Beijing, China).

2.6 RNA extraction and quantitative real-time PCR (qRT-PCR)

Realtime PCR was performed according to the method as previously described (Trakunram et al., 2019). In brief, total RNA was extracted from 50 mg femur by using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Purity and concentration of RNA were assessed by 260/280 UV absorption ratio. Complementary DNA (cDNA) was then synthesized by using cDNA Synthesis kit (TaKaRa, Dalian, China). PCR was performed by using SYBR® Premix Ex Taq™ II Reagent (TaKaRa, Dalian, China) with primers listed in Table 1. All primers were designed by NCBI Primer-BLAST tool, synthesized by Shanghai Biotechnology Bioengineering Co., Ltd. For each cycle, 20-μL final volume including 10 μL of 2 × QuantiFast SYBR Green master mix (TaKaRa, Dalian, China), 3 μL of DNA, and 0.2 μM of each primer were used. Thermal cycling conditions were as follows: denaturation and activationof 1 cycle at 95 °C for 5 min, followed by 40 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 10 s. These were done by using StepOnePlus Real-time PCR system (Applied Biosystems® Inc., Foster City, CA, USA). β-actin was chosen as the reference gene to adjust the threshold cycle (Ct) values of the target genes. Data were analysed by using Comparative Ct ($2^{-\Delta\Delta Ct}$) method.

2.7 Statistical analysis

Data were shown as means ± standard deviation (S.D.). Comparisons among multiple groups were analyzed using ANOVA and graphed using IBM SPSS for Mac 26.0 software (IBM Corp., Armonk, NY, USA). Bonferroni correction was used for multiple comparisons in the pairwise analysis. Data mapping was done using GraphPad Prism 7 (GraphPad Software Inc., USA). A P value < 0.05 was considered statistically significant.

3 Results

3.1 Effect of SBCP on serum PINP and β-CTx levels

A significant increase in serum PINP (Figure 1A) and β-CTx (Figure 1B) levels was observed in Model group ($P < 0.05$) when compared to Sham group. However, there was a significant decrease in serum PINP levels in OVX rats following treatment with 3.40, 2.05, and 0.68 g/kg BW SBCP ($P < 0.05$ when compared to Model group). Following treatment with 3.40 and 2.05 g/kg BW SBCP, serum β-CTx levels were significantly lower when compared to Model group ($P < 0.05$). Treatment with 3.40 g/kg BW SBCP resulted in highest decrease in serum β-CTx level in OVX rats and β-CTx levels were almost similar to Sham group ($P > 0.05$). However, there was no significant difference in serum β-CTx between SBCP-L group and Model group ($P > 0.05$).

Table 1. Real time PCR (qRT-PCR) primers sequence.

Gene	Primer sequences	Product size (bp)
RANK	F 5'-TGGTTCACTGTTCCTAATCC-3' R 3'-CGTGAAACACTGGCTTAAAC-5'	100
RANKL	F 5'-AGGCTGGGCCAAGATCTCTA-3' R 3'-GATAGTCCGCAGGTACGCTC-5'	134
OPG	F 5'-TGTGAAAGCAGTGTGCAACG-3' R 3'-CCAGGCAAGCTCTCCATCAA-5'	83
β-actin	F 5'-CCTAAGGCCAACCGTGAAAAGA-3' R 3'-AGTGGTACGACCAGAGGCATA-5'	114

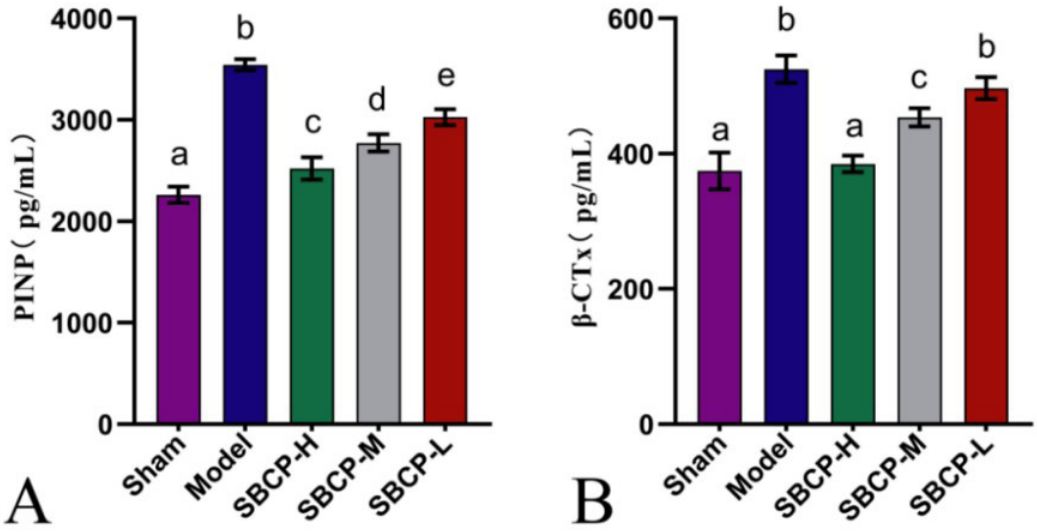

Figure 1. Effect of SBCP on serum (A) PINP (pg/mL) and (B) β-CTx (pg/mL) levels. Bar graphs that do not share the same symbol are significantly different ($P < 0.05$). Values are expressed as mean ± S.D (n = 10). Sham: sham operated. Model: ovariectomized control rats. SBCP-H: ovariectomized rats treated with 3.40 g/kg BW SBCP. BW: Body weight. SBCP-M: ovariectomized rats treated with 2.05 g/kg BW SBCP. SBCP-L: ovariectomized rats treated with 0.68 g/kg BW SBCP.

3.2 Histopathological changes in the femur bones

H & E staining has revealed a relatively greater destruction of the trabecular bone (TB) network in OVX rats (Figure 2). In sham operated rats, the trabecular networks appear normal. However, a relatively lesser destruction of the TB network was observed when OVX rats were given 3.40, 2.05, and 0.68 g/kg BW SBCP treatments. Compared with Model group, the improvement of bone microstructure in the SBCP-H group was the most obvious.

3.3 Effect of SBCP on the bones microstructure

In Sham group (Figure 3), the area of the bone marrow (BM) cavity was small, the TB structure was clear, closely arranged, thick and continuous. In Model group, changes such as thinning, tapering, breakage, and perforation made the bones structure lose its integrity. These changes contributed to an obviously increasing separation of inter-trabeculae. The number of TB in SBCP-H group and SBCP-M group was significantly increased compared with Model group. The thickness was more uniform, and the three-dimensional network structure was more complete.

3.4 Effect of SBCP on bone mineral content

The results (Figure 4) showed that compared with Sham group, the tibial calcium and phosphorus contents were significantly reduced in OVX rats ($P < 0.05$). After SBCP intervention, the tibial calcium and phosphorus contents of each dose group increased significantly ($P < 0.05$), and they were in a dose-dependent relationship. Treatment with 3.40 g/kg BW SBCP resulted in highest increase in calcium and phosphorus content in OVX rats.

3.5 Effect of SBCP on RANK, RANKL and OPG mRNA expression bones

Agarose gel electrophoresis (Figure 5) showed that each PCR product band was single, and no specific amplification was observed. The sizes of fluorescence quantitative PCR products were consistent with expectations, about 100 bp (RANK), 134 bp (RANKL), 83 bp (OPG) and 114 bp (β-actin), respectively. The homology between the sequencing results and the target sequences was more than 99.9%, indicating that the primers designed in the study were highly specific and reliable.

A significantly higher RANK (Figure 6A) and RANKL (Figure 6B) mRNA expression levels was observed in Model rats when compared to Sham control ($P < 0.05$). RANK and RANKL mRNA levels were significantly decreased when OVX rats were treated with 3.40, 2.05, and 0.68 g/kg BW SBCP when compared to Model group ($P < 0.05$). Moreover, with the increase of SBCP dose, the mRNA relative expression levels of RANK and RANKL were significantly decreased. However, there was no significant difference in RANK and RANKL mRNA expression levels between SBCP-H group and SBCP-M group ($P > 0.05$).

In contrast, the relative mRNA expression for OPG (Figure 6C) was lowest in Model group, but there was no significant difference from Sham group ($P > 0.05$). OPG mRNA levels were significantly increased when OVX rats were treated with 3.40, 2.05, and 0.68 g/kg BW SBCP when compared to Model group ($P < 0.05$). The RANKL/OPG ratio of Model group (Figure 6D) was significantly higher than Sham group ($P < 0.05$). However, no significant difference in RANKL/OPG ratio was observed between rats which received SBCP treatment and Sham group ($P > 0.05$).

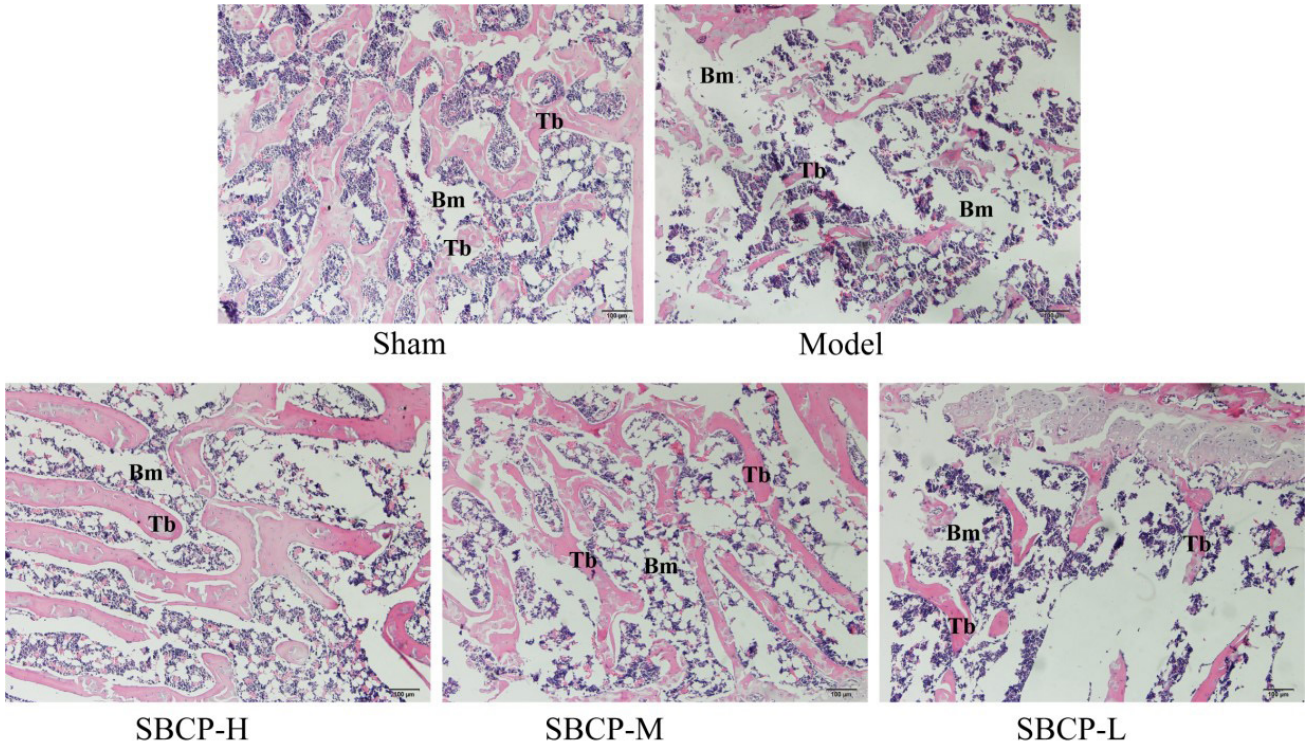

Figure 2. Histopathological images of H & E staining of the femur. Abbreviation: Bm = bone marrow; Tb = trabeculae bone. Scale bar = 100 μm. Images are taken at 400 × magnification. Sham: sham operated. Model: ovariectomized control rats. SBCP-H: ovariectomized rats treated with 3.40 g/kg BW SBCP. SBCP-M: ovariectomized rats treated with 2.05 g/kg BW SBCP. SBCP-L: ovariectomized rats treated with 0.68 g/kg BW SBCP.

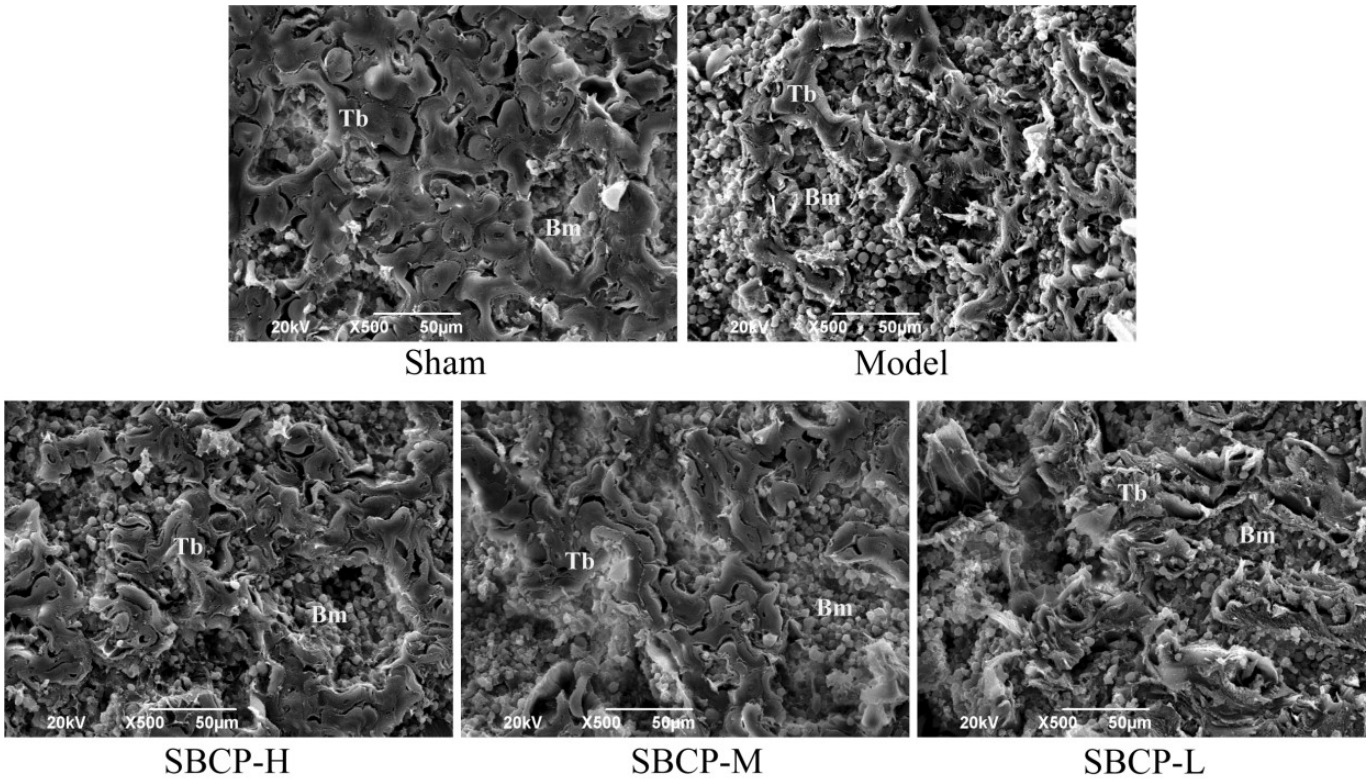

Figure 3. Images of femur bone under SEM. Abbreviation: Bm = bone marrow; Tb = trabeculae bone. Sham: sham operated. Model: ovariectomized control rats. SBCP-H: ovariectomized rats treated with 3.40 g/kg BW SBCP. SBCP-M: ovariectomized rats treated with 2.05 g/kg BW SBCP. SBCP-L: ovariectomized rats treated with 0.68 g/kg BW SBCP.

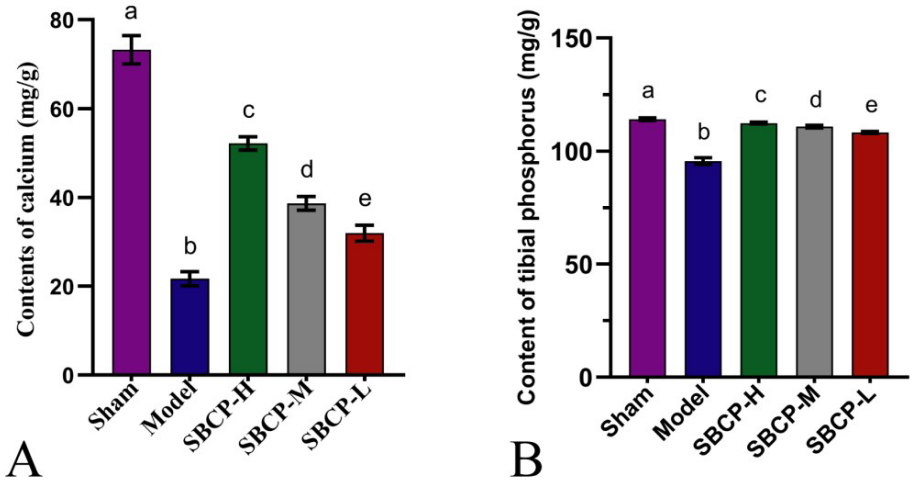

Figure 4. Effect of SBCP on tibial (A) calcium (mg/g) and (B) phosphorus (mg/g) contents. Bar graphs that do not share the same symbol are significantly different ($P < 0.05$). Values are expressed as mean ± S.D (n = 10). Sham: sham operated. Model: ovariectomized control rats. SBCP-H: ovariectomized rats treated with 3.40 g/kg BW SBCP. SBCP-M: ovariectomized rats treated with 2.05 g/kg BW SBCP. SBCP-L: ovariectomized rats treated with 0.68 g/kg BW SBCP.

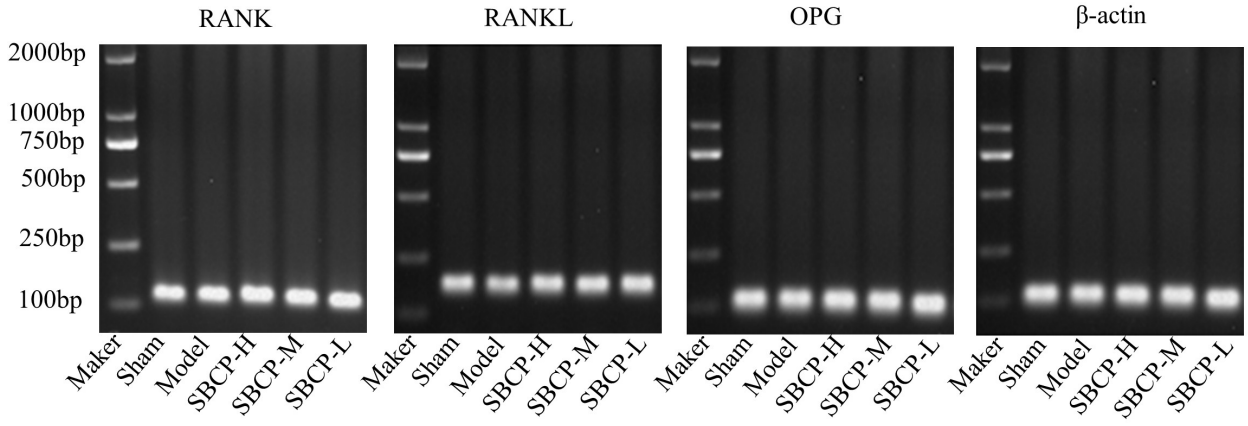

Figure 5. Agarose gel electrophoresis of qRT-PCR products of RANK, RANKL and OPG. Maker: DL2000 DNA marker. Sham: sham operated. Model: ovariectomized control rats. SBCP-H: ovariectomized rats treated with 3.40 g/kg BW SBCP. SBCP-M: ovariectomized rats treated with 2.05 g/kg BW SBCP. SBCP-L: ovariectomized rats treated with 0.68 g/kg BW SBCP.

4 Discussion

Bone metabolism biochemical markers are substances produced during bone conversion, which reflect the metabolic rate of bone, the bone resorption activity of osteoclasts (OC) and the bone formation activity of osteoblasts (OB). PINP and β-CTx are present in blood and serve as biochemical markers of bone metabolism to evaluate the occurrence of OP and the efficacy of drugs (Yoshimura et al., 2011). PINP reflects the activity of bone formation (Koivula et al., 2012), and β-CTx reflects the activity of bone resorption (Szulc et al., 2017), which are significantly increased in the high turnover OP model. The serum PINP and β-CTx levels in the model group of the study were significantly increased, indicating that the activity of bone formation and bone resorption were both enhanced, leading to the formation of high turnover bone and OP, which was consistent with the results of Cavalier et al. (2020). The levels of PINP and β-CTX in OVX rats were decreased by gavage of SBCP, suggesting that SBCP could inhibit the occurrence of OP caused by estrogen deficiency, and the inhibitory effect was more obvious as the dose of SBCP increased.

Ye et al. (2020) used ovariectomized rats and orally administered yak bone collagen peptides (YBCP) at a dose of 100, 200, 500 mg/kg BW for 12 weeks, respectively, and the improvement of bone mineral density, bone microstructure and

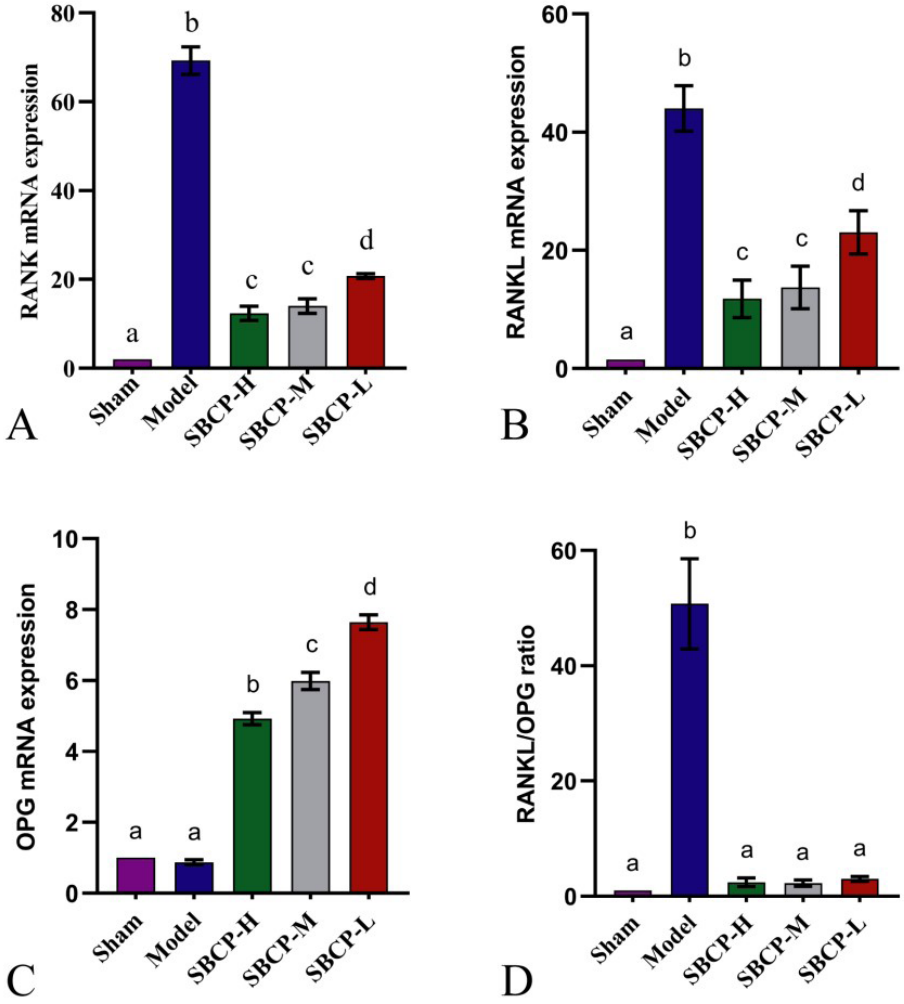

Figure 6. Effect of SBCP on RANK, RANKL and OPG mRNA expression levels in bones. Bar graphs show quantification of relative mRNA expression for (A) RANK, (B) RANKL, (C) OPG and (D) RANKL/OPG ratio in the bone in different treatment groups. Bar graphs that do not share the same symbol are significantly different ($P < 0.05$). Values are expressed as mean ± S.D (n = 10). Sham: sham operated. Model: ovariectomized control rats. SBCP-H: ovariectomized rats treated with 3.40 g/kg BW SBCP. SBCP-M: ovariectomized rats treated with 2.05 g/kg BW SBCP. SBCP-L: ovariectomized rats treated with 0.68 g/kg BW SBCP.

bone metabolism level in rats was observed, mostly in a dose-dependent manner. These results suggested that the intake of collagen peptides (CP) could effectively improve bone mineral density and microstructure of TB, improve bone biomechanical properties, and change the content and activity of biomarkers related to bone metabolism.

RANK/RANKL/OPG signaling pathway plays a very important role in the process of bone remodeling (Doustimotlagh et al., 2018; Kiesel & Kohl, 2016). Multiple studies have shown that CP can indirectly regulate osteoclasts through the regulation of this signaling pathway. The studies have found that when osteoporosis occurred, the expression of RANKL in the femur of rats was significantly increased, and the expression of OPG was significantly inhibited. In this study, it was found that the relative expression levels of RANK and RANKL in the model group were significantly increased compared with the sham group ($p < 0.01$), which was consistent with the research results of Han et al. (2009). After SBCP intervention, the relative expression levels of RANK and RANKL in each dose group were significantly lower than those in the model group ($p < 0.01$), and decreased with the increase of SBCP dose, indicating that SBCP could inhibit the expression of RANK and RANKL. The study (Ye et al., 2020) showed that codfish CP could indirectly regulate the activity of osteoclasts by inhibiting the expression of RANKL in osteoblasts and promoting the expression of OPG, thus affecting bone metabolism, which was also consistent with the research results of Zhu et al. (2020).

In the dynamic equilibrium relationship between bone resorption and bone formation, the higher the RANKL/OPG ratio, the stronger the bone resorption activity. The RANKL/OPG ratio can directly affect the differentiation of OC and bone metabolism (He et al., 2017), and it is an important factor in

regulating the balance of bone resorption and bone formation (Stuss et al., 2013). In the study, when OP occurred, although the OPG level was basically unchanged, the ratio of RANKL/OPG increased significantly, while the ratio of each dose group of SBCP decreased. The results showed that SBCP inhibited the formation of OC and bone resorption activity by reducing the number of RANK receptors on the surface of OC precursor cells, inhibiting the secretion of ligand RANKL by OB and BMSCs, and enhancing the secretion of OPG by OB to prevent the occurrence of OP.

As the enzymatic hydrolysate of collagen, SBCP has greatly improved its bioavailability and is a natural foodborne ingredient. It has incomparable advantages over other kinds of drugs such as a wide range of sources, safety, no toxic side effects and so on (Schmidt et al., 2020). Therefore, it is expected to be promoted as a safe and effective dietary supplement to prevent osteoporosis. However, most of the existing studies only showed that CP mixtures could exert osteogenic activity, and the specific active components and key core sequences had not been isolated and identified, which also limited the precise research on its specific target, mechanism of action and dose-effect relationship (Liu & Li, 2021).This study showed that SBCP inhibited the formation of OC and bone resorption activity and prevented the occurrence of osteoporosis by reducing the number of RANK receptors on the surface of OC precursor cells, inhibiting the secretion of RANKL by OB and bone marrow stromal cells, and enhancing the secretion of OPG by OB, which provided a theoretical basis for the development of functional food for the prevention of osteoporosis by SBCP.

5 Conclusion

SBCP could regulate the activity of OC through the RANK/RANKL/OPG signal pathway, improve the bone tissue structure and mineral content, and thus intervene the occurrence and development of OP caused by estrogen deficiency.

Author contributions

Yixin Zhu and Shuxiu Jin conceived and designed the review/project/study. Zhuo Duan, Donghao Zhao and Linfeng Ma executed the experiment and analyzed the sera and tissue samples. Jing Chen and Tao Li analyzed the data. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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