RESEARCH ARTICLE

Protective effects of bovine colostrum acid proteins on bone loss of ovariectomized rats and the ingredients identification

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Scope: Milk basic proteins and bovine colostrum extracts have preventive effects on osteoporosis. However, the effects of bovine colostrum acidic proteins (BCAP) on properties of bone have not been reported. This study investigated the effect of BCAP on the prevention of bone loss in ovariectomized (OVX) rats.

Methods and results: Forty-eight 3-month old female Sprague–Dawley rats were OVX and another 12 rats underwent a sham operation (Sham). The OVX rats were randomly separated into four groups, *i.e.* OVX control, OVX plus 2 mg/day BCAP, OVX plus 10 mg/day BCAP, and OVX plus 50 mg/day BCAP, and were gavaged once *per* day for 12 wk. The effects on bone mineral content, bone mineral density, microarchitecture and biomechanical properties were determined. The bioactive ingredients in BCAP were isolated and identified. Results showed that BCAP increased the bone mineral content and bone mineral density of the femur in a dose-dependent manner. Scanning electron microscope observation and mechanical testing further confirmed the positive effects of BCAP. These positive effects attribute to the fact that osteopontin, lactoferrin, epidermal growth factor and insulin-like growth factor-2 are the dominant proteins in BCAP.

Conclusions: BCAP (2–50 mg/day) could prevent osteoporosis caused by bone loss in OVX rats.

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1 Introduction

Osteoporosis is one of the most critical global health disorders associated with advancing age, particularly among postmenopausal women. The menopause results in an imbalance between bone formation and resorption, and a decreased bone mineral density (BMD). Osteoporosis is caused mainly by the decline of BMD [1, 2]. Bioactive components from milk may directly affect bone metabolism, because milk has an important role in the growth of newborn animals. Milk basic protein (MBP) comprises a group of bioactive proteins from bovine whey [3, 4]. Some studies suggest that MBP suppresses osteoclast-mediated bone resorption, increases

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Abbreviations: **BCAP,** bovine colostrum acid proteins; **BCE,** bovine colostrum extract; **BMC,** bone mineral content; **BMD,** bone mineral density; **E2,** estradiol; **EGF,** epidermal growth factor; **FSH,** follicle-stimulating hormone; **IGF-1,** insulin-like growth factors 1; **IGF-2,** insulin-like growth factors 2; **LF,** lactoferrin; **LH,** luteinizing; **MBP,** milk basic protein; **OPN,** osteopontin; **OVX,** ovariectomized (ovariectomy); **SEM,** scanning electron microscope; **%Tb.Ar,** trabecular area percentage; **Tb.N,** trabecular number; **Tb.Sp,** trabecular separation; **Tb.Th,** trabecular thickness; ***W/L,*** femur weight/femur length; **WAF,** acidic protein fraction from whey

BMD, and prevents bone loss caused by ovariectomy (OVX) [4–6].

Bovine colostrum contains higher levels of immunoglobulins and growth factors compared with mature milk [7, 8]. Colostrum provides remarkable skeletal repair and growth capabilities. Colostrum is the only natural source of transforming growth factors α and β, and insulin-like growth factors 1 and 2 (IGF-1 and IGF-2). These growth factors have significant cartilage repair characteristics [9]. Influences of bovine colostrum extract (BCE) on the proliferation of osteoblasts and bone development of fetal rats have been determined by Yao *et al.* [10]. The effect of BCE was greater than that of calcium, though the calcium dose in BCE group was lower than that of control. With respect to the proliferation of neonatal rat calvaria osteoblast cells in the medium treated with BCE, the growth rates were stimulated in the range of 0.002–20 mg/mL. It was also reported that acidic protein fractions from bovine mature milk whey (WAF) protected against bone loss and maintained BMD. WAF had positive effects on bone stiffness and resistance against breaking in OVX rats [11]. In these respects, acidic protein fractions from bovine colostrum may have more advantages on reducing the perceived risk of osteoporosis in older women by attenuating the rapid menopausal bone loss. The nutritional and physiological safety of colostrum was evaluated by Davis *et al.* and the study gives some confidence that no adverse effects would be expected in normal healthy subjects [8].

The bioactivity of colostrum has received widespread attention and there were many studies on the health functions of colostrums proteins [5, 9, 12, 13]. However, the detailed effects of bovine colostrum acid proteins (BCAP) on bone properties have not been extensively investigated. Therefore, the objective of this study was to investigate the effects of BCAP on the prevention of bone loss in aged OVX rats, isolate, and analyze the bioactive ingredients in BCAP.

2 Materials and methods

2.1 Preparation of BCAP

BCAP was obtained from fresh bovine colostrums (the milk produced for the first 24 h in lactation) milking from farms in Hohhot. After refrigerated rapidly, the milk was stored at 4°C and processed within 12 h. The colostrum was defatted by centrifugation (15 000 × *g*, 20 min, 4°C), sterilized by 0.22 μm ceramic membrane filtration (GEAFiltration, Global Engineering Alliance, Bochum, Germany) (30°C, 6 bar), concentrated by ultrafiltration membrane with 5000 Da nominal molecular weight limit (15°C) and the pH was then adjusted to 4.6 using 0.1 mol/L hydrochloric acid (HCl). The precipitated casein was removed by centrifugation (15 000 × *g*, 20 min, 10°C). After the pH adjusted to 6.9 using sodium hydroxide (0.1 mol/L), the sequential whey was concentrated by ultrafiltration membrane with 1000 Da nominal molecular weight limit (15°C) and loaded onto a column that had been packed with anion exchange resin, $DEAE_{52}$ cellulose (GE Healthcare, Uppsala, Sweden). The column was sufficiently washed with deionized water (pH 6.9) and the bound protein was eluted with 1 mol/L NaCl solution (pH 6.9) with a flow rate of 2 mL/min at 4°C, the pH was adjusted using HCl (0.1 mol/L). BCAP was then obtained by lyophilization and stored at −20°C, after dialysis of the eluted fraction in a cellulose membrane tube (Millipore, Billerica, MA, USA).

Table 1. Dietary composition (g/kg)

Group	Sham/OVX-C	OVX-2	OVX-10	OVX-50
Sodium caseinate	150	149.9	149.5	147.5
BCAP	0	2 mg/day[a)]	10 mg/day[a)]	50 mg/day[a)]
Cystine	2.7	2.7	2.7	2.7
Glycine	3.3	3.3	3.3	3.3
Methionine	1.5	1.5	1.5	1.5
Glutamine	7	7	7	7
Cellulose	50	50	50	50
Vitamin mix[b)]	50	50	50	50
Mineral mix[b)]	50	50	50	50
Corn oil	50	50	50	50
$CaCO_3$	12.5	12.5	12.5	12.5
Starch	623	623	623	623
Total	1000	1000	1000	1000

a) To make the diet fair for each group, the gavage dosage of BCAP plus the content of sodium caseinate in groups OVX-2, OVX-10, and OVX-50 was approximately equal to the content of sodium caseinate in groups Sham and OVX-C. The mean food intake over the trial was 20 g/day *per* animal.

b) Formulated by Crop & Food Research, New Zealand, according to the National Research Council (1995) nutrient requirements for laboratory animals (AIN 93 M). Sham, Sham operation; OVX-C, OVX model control; OVX-2, OVX rats gavaged with BCAP at 2 mg/day; OVX-10, OVX rats gavaged with BCAP at 10 mg/day; OVX-50, OVX rats gavaged with BCAP at 50 mg/day.

2.2 Animals and diets

Sixty female Sprague–Dawley rats (Body weight 195 ± 15 g; 3 months old) were supplied by the Experimental Animals Center of Harbin Medical University in China. Guidelines for the care and use of animals were followed and all procedures were approved by the Ethical Committee of Harbin Medical University ([2001]-545). Forty-eight rats were ovariectomized (OVX) and the other 12 underwent a sham operation (Sham). The animals were fed a modified AIN-76 diet [14, 15] (control diet in Table 1) for a 3-wk recovery period after the operation. Afterward, the OVX rats were randomly separated into four groups, *i.e.* OVX-control group (OVX-C), OVX plus 2 mg/day BCAP (OVX-2), OVX plus 10 mg/day BCAP (OVX-10), and OVX plus 50 mg/day BCAP (OVX-50). The rats were gavaged once *per* day for 12 wk. The dose of the fraction was selected based on the previously published studies on the MBP [4] and WAF [16]. The animals were housed separately in shoebox cages, and kept in a temperature (22 ± 2°C) and light-controlled (12 h day/night cycle) room in the Small Animal Production Unit at Harbin Medical University. These animals had access *ad libitum* to deionized water. The rats were fed a casein-based semi-synthetic diet for 12 wk. The sham and the OVX control groups received the base diet without BCAP added. Body weight was recorded once a week and food intake monitored daily. Food intake was adjusted weekly according to the sham group's body weight to prevent excessive body weight gain in the OVX groups. At the end of the experiment, ten rats in each group were deprived of food overnight and sacrificed. The femurs of both sides were separated and the muscular and fat tissues along with the connective tissues were removed.

2.3 Hormone, length, and weight of femur

Blood (0.5 mL) was collected from the tail of each rat in OVX-C, OVX-2, OVX-10, and OVX-50 before OVX and 3 wk after OVX. Estradiol (E2), luteinizing (LH), and follicle-stimulating hormone (FSH) levels in the blood were determined using radioimmunoassay kits (Biosynthesis Biotechnology, Beijing, China). Bone weight was measured using an analytical balance (FA1004N, MoreChina, Shanghai, China), and bone length using a vernier caliper (Mitutoyo, Tianjin, China).

2.4 Bone ashing

The right femurs were thawed, scraped clean of remaining flesh, and dried overnight at 105°C. After being weighed, the femurs were ashed overnight at 660°C and weighed again.

2.5 BMD determination

The left femur was divided into three segments (proximal end, middle segment, and distal end) with a bone saw (Yongkang Tiange Electric, Guangzhou, China). The BMD of these three segments was measured with dual-energy X-ray absorptiometry, using a Dichroma Scan DCS-600 A (Aloka, Tokyo, Japan) with beam energies of 22 and 53 keV adapted for measuring small animals. The scanning speed was 10 mm/s.

2.6 Scanning electron microscope observation and bone histomorphometry

Distal ends of the left femurs were thoroughly cleaned with ethanol and isotonic salt solutions to remove the remaining soft tissues. These distal ends were dipped in 2.5% glutaraldehyde for 24 h, and immersed in 10% sodium hypochlorous for 6 h, then washed in ultrasonic cleaner (KQ5200DE, Kunshan Ultrasonic Instrument, Kunshan, China) for 20 min [17, 18]. Bones were then cut (using a Dremil tool with a small drill bit) to produce flat, smooth cross-sectional samples. These were then mounted on stubs and sputter coated with gold and palladium to obtain better scans at higher magnifications. The samples were placed in the chamber of the scanning electron microscope (SEM) (AIS2100, MIRERO, Seongnam, South Korea) and analyzed with an electron beam of energy that ranged from 12 to 20 keV. SEM images were taken with a secondary electron detector. An image-analysis system (VIDS, AMS, England) was used for bone histomorphometry. Trabecular number (Tb.N), thickness (Tb.Th), separation (Tb.Sp), and area percentage (%Tb.Ar) were calculated.

2.7 Biomechanical strength test

The remaining soft tissues were removed from the left femurs of rats in groups OVX-C and OVX-50 and a force was applied (at a speed of 5 mm/min) to the center of the femur, which was placed on two supports (three-point bending) [19]. The biomechanical strength properties were measured using a Twin Column Testing Machine (LR5KPlus, Lloyd, England). The biomechanical strength properties were measured using a Twin Column Testing Machine (LR5KPlus). The factors measured are defined as follows. Maximum load (Max-Load, N): Maximum load applied at fracture of the specimen, and achieved directly from the load-deformation curves. Strain at maximum load (Max-Strain, N/mm): It as expressed as millimeters deformation of specimen at maximum load *per* millimeter of original length of the specimen. This parameter is a measure of the compressibility of the tissue. Maximum stress (Max-Stress, N/mm^2): The maximum compressive load *per* unit cross-sectional area of the bone specimen. Maximum stiffness (Max-Stiffness, N/mm^2): The maximum slope of the stress–strain curve determined by a mathematical differentiation [20].

2.8 Isolation and identifications of proteins in BCAP

The lyophilized BCAP was dissolved in 0.2 mol/L PBS (pH 6.9). Fractions of BCAP were isolated consecutively by Sephadex G-100 column (1.5 × 35 cm) (GE Healthcare) and Econo–pac Q prepacked column (0.59 × 3.6 cm) (Bio-Rad, Beijing, China). Size exclusion chromatography was run with a buffer mobile phase (0.2 mol/L PBS, pH 6.9) at a flow rate of 0.5 mL/min. According to the purity requirement, the fraction from Sephadex G-100 was purified further by Econo–pac Q. Anion exchange chromatography was performed using 1.0 mol/L NaCl 0.2 mol/L PBS (pH 6.9) buffer as a mobile phase with a 0.1∼1.0 mol/L NaCl linear salt gradient at a flow rate of 0.8 mL/min. All chromatography was performed at 4°C with column effluent monitored at 280 nm. The eluted fraction was concentrated by ultrafiltration after which it was dialyzed exhaustively against ultra-pure water. Each purified protein fraction was identified by *N*-terminal amino acid sequence analysis technique.

2.9 Statistical analysis

A total of 60 rats (12 *per* group) were used in the trial and ten rats selected randomly from each group were used for statistical analysis. Differences among the groups were determined by one-way ANOVA in the pattern of pairwise comparison. All statistical calculations were performed by the GLM procedure in the SAS Statistical Analysis Package (version 6; SAS Institute, Cary, NC). Significance was assigned at $p \leq 0.05$. Values and graphs are expressed and shown as means with their SD.

3 Results

3.1 Confirmation of ovariectomy

The hormone levels of rats before and after ovariectomy are summarized in Table 2. When compared with those before the operation, E2 content decreased after ovariectomy, whereas LH and FSH contents increased ($p < 0.05$). Moreover, there is no significant difference on contents of these hormones in OVX groups. These results indicate that the ovariectomy in groups OVX-C, OVX-2, OVX-10, and OVX-50 was successful [21].

3.2 Bone weight

As summarized in Table 3, there were no significant differences in final body weight between groups, although an increasing trend was observed as BCAP concentration increased. Femur weight/femur length (*W*/*L*) values of left

Table 2. Hormone levels of rats before and after ovariectomy (mean ± SD, $n = 10$ *per* group)

Group	E2 (pg/mL)		LH (IU/L)		FSH (IU/L)	
	Before	After	Before	After	Before	After
Sham	NM	NM	NM	NM	NM	NM
OVX-C	5.08 ± 0.32	2.40 ± 0.15[a)]	1.17 ± 0.09	2.14 ± 0.18[b)]	1.32 ± 0.08	6.30 ± 0.26[c)]
OVX-2	4.69 ± 0.29	2.37 ± 0.13[a)]	1.52 ± 0.09	2.43 ± 0.17[b)]	2.39 ± 0.11	7.02 ± 0.35[c)]
OVX-10	4.85 ± 0.26	2.38 ± 0.16[a)]	1.52 ± 0.10	2.45 ± 0.15[b)]	2.41 ± 0.13	7.11 ± 0.40[c)]
OVX-50	4.79 ± 0.30	2.33 ± 0.14[a)]	1.57 ± 0.08	2.49 ± 0.17[b)]	2.37 ± 0.13	7.08 ± 0.38[c)]

Sham, Sham operation; OVX-C, OVX model control; OVX-2, OVX rats gavaged with BCAP at 2 mg/day; OVX-10, OVX rats gavaged with BCAP at 10 mg/day; OVX-50, OVX rats gavaged with BCAP at 50 mg/day. NM, the value was not measured; Before, before ovariectomy; After, after ovariectomy.
a) There is a significant difference in E2 values between rats and OVX rats ($p < 0.05$).
b) There is a significant difference in LH values between rats and OVX rats ($p < 0.05$).
c) There is a significant difference in FSH values between rats and OVX rats ($p < 0.05$).

Table 3. Final body weight and *W*/*L*

Group	Left *W*/*L* (g/cm)	Right *W*/*L* (g/cm)	Body weight (g)
Sham	0.2224 ± 0.0113[b]	0.2219 ± 0.0093[b]	296.2 ± 19.3
OVX-C	0.2143 ± 0.0101[a]	0.2136 ± 0.0100[a]	312.28 ± 20.2
OVX-2	0.2316 ± 0.0128[b]	0.2313 ± 0.0125[b]	335.75 ± 15.32
OVX-10	0.2362 ± 0.0071[c]	0.2360 ± 0.0093[c]	340.69 ± 13.88
OVX-50	0.2481 ± 0.0136[d]	0.2466 ± 0.0164[d]	342.31 ± 25.11

Sham, Sham operation; OVX-C, OVX model control; OVX-2, OVX rats gavaged with BCAP at 2 mg/day; OVX-10, OVX rats gavaged with BCAP at 10 mg/day; OVX-50, OVX rats gavaged with BCAP at 50 mg/day. Value followed by different letters in the same column are significantly different ($p < 0.5$).

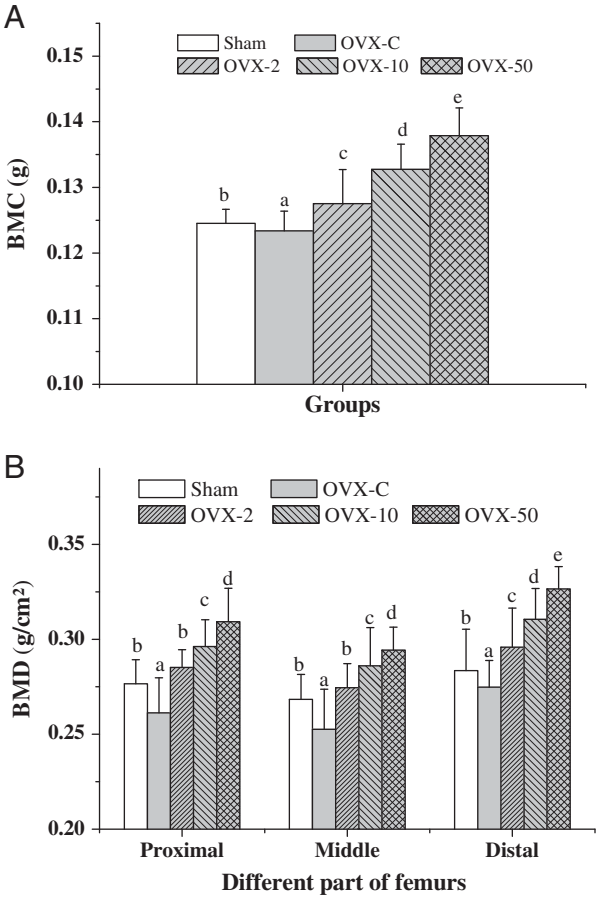

Figure 1. BMC (A) and BMD (B) of right femurs (mean ± SD, $n = 10$ *per* group). BMC was determined by the bone ash determination method. BMD was measured by dual-energy X-ray absorptiometry. Proximal: proximal end; Middle: middle segment; Distal: distal end. Sham, Sham operation; OVX-C, OVX model control; OVX-2, OVX rats gavaged with BCAP at 2 mg/day; OVX-10, OVX rats gavaged with BCAP at 10 mg/day; OVX-50, OVX rats gavaged with BCAP at 50 mg/day. Values followed by different letters are significantly different ($p < 0.05$).

and right femurs in group OVX-C were lower than those of Sham. *W*/*L* values (both left and right) for groups OVX-10 and OVX-50 were higher than those for groups Sham and OVX-C. *W*/*L* for OVX-2 was higher than that of Sham but was not significantly different from *W*/*L* for OVX-C. Meanwhile, *W*/*L* values were significantly different for groups OVX-2, OVX-10, and OVX-50 ($p < 0.05$). There are numerous factors known to affect *W*/*L*, such as the average cross-sectional area, the calcium content of the marrow, and the density of marrow. Hence, *W*/*L* is an indirect index of BMD.

3.3 Bone mineral content and BMD

Figure 1A shows that the bone mineral content (BMC) in the femurs of rats in OVX-C group was lower than that of Sham ($p < 0.05$), indicating successful ovariectomy. BMC of groups OVX-2, OVX-10, and OVX-50 was higher than for groups OVX-C and Sham ($p < 0.05$). This suggests that BCAP had a positive effect on the calcium metabolism of the femur in rats. BCAP alleviated or suppressed the declining calcium levels resulting from ovariectomy, and thus BCAP prevented the development of osteoporosis in OVX rats. Moreover, BMC value was significantly different among groups OVX-2, OVX-10, and OVX-50 ($p < 0.05$) in the order of OVX-50 > OVX-10 > OVX-2, and thus dose dependent. As shown in Fig. 1B, BMDs of the proximal end, middle segment, and distal end in OVX-C group were all significantly lower than those of Sham, which indicated bone loss owing to the lack of estrogen in OVX rats. This observation is consistent with the results of the BMC data. With respect to the proximal end and middle segment, the BMD of OVX-2 was significantly higher than that of OVX-C and similar to that of Sham ($p > 0.05$). On the contrary, the BMD of groups OVX-10 and OVX-50 was higher than that of groups Sham and OVX-C. For the distal end of the femur, the BMD of groups OVX-2, OVX-10, and OVX-50 was higher than those of groups Sham and OVX-C. OVX-50 had the highest BMD among all five groups and was markedly higher than those in groups OVX-2 and OVX-10. Moreover, BCAP exhibited stronger activity on BMD at the distal end of bone.

3.4 Bone microarchitecture and histomorphometry

The cancellous microarchitecture of the distal femur of rats in group OVX-50 was investigated by SEM and contrasted with that of the rats in group OVX-C. SEM images at 50 × magnification are shown in Fig. 2A (OVX-C) and Fig. 2B (OVX-50). Results of bone histomorphometry are summarized in Table 4. Tb.N, Tb.Th, and %Tb.Ar of the femur in OVX-C were significantly lower than those in OVX-50. Moreover, the Tb.Sp in OVX-C was markedly larger than that in OVX-50. Some trabeculae were dissociated in the marrow cavity as a blind end and the cancellous microarchitecture was also destroyed. The surfaces of trabeculae were rough and hollow. A decrease in the number of trabecular nodes and increase in the number of free ends were found in OVX-C. For group OVX-50, the Tb.N was more apparent and the Tb.Th was uniform. The Tb.Sp was smaller than that in OVX-C. Moreover, there was a solid cancellous structure among the trabeculae, in which the trabeculae were columnar or lamella with smooth surfaces and radian. There were few free ends in group OVX-50.

We then observed the samples by SEM at 6000 × and the results are shown in Fig. 2C (OVX-C) and Fig. 2D (OVX-50). There were resorption covers with large scales on the trabecular surface of the femur and little quiescent bone surface in OVX-C. The bone resorption was severe in OVX-C. For example, there were many particle areas with different sizes and shapes

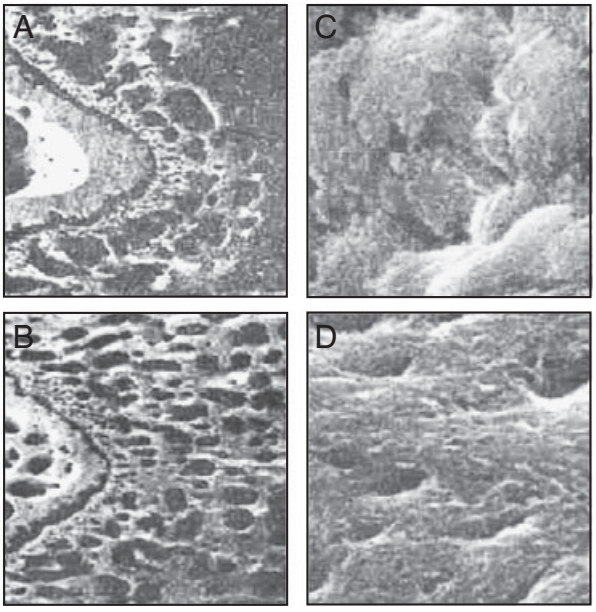

Figure 2. Observations of cancellous microstructure of the distal femur by SEM. The distal femur of rats in groups OVX-C (A and C) and OVX-50 (B and D). Magnification: 50 × (A and B), 6000 × (C and D). OVX-C, OVX model control; OVX-50, OVX rats gavaged with BCAP at 50 mg/day.

maximum-stress, and break-stress are material mechanical parameters. Results of the force test (Table 4) showed that both the structural and the material mechanical parameters of the femurs of rats in group OVX-50 were significantly higher than those in OVX-C. The Max-Load, Max-Strain, Max-Stress, and Max-Stiffness values of the diaphysis in group OVX-50 were 10.4, 13.33, 9.02, and 15.56% higher than the corresponding index in OVX-C, respectively. Therefore, the biomechanical properties were improved and the bone strength was enhanced in group OVX-50 *versus* OVX-C.

3.6 Composition of BCAP

After loading BCAP sample onto a Sephadex G-100 column, the elution profile showed three major peaks in Fig. 3A. Based on the purity requirement, the fraction corresponding to peak 3 was collected and subjected to Econo–pac Q column, with a typical elution profile revealing three major peaks in Fig. 3B. The proteins in peaks 1, 2, 3(B), and 3(C) rather than peak 3(A) were isolated and purified to homogeneity based on BCAP preparation and subsequent separation by size exclusion chromatography and anion exchange chromatography. According to the *N*-terminal

Table 4. Bone histomorphometry and biomechanical properties of the femur

Group	Tb.N (♯/mm)	Tb.Th (μm)	Tb.Sp (μm)	%Tb.Ar (%)	Max-Load (N)	Max-Strain (N/mm)	Cross-sectional area (mm^2)	Max-stress (N/mm^2)	Max-Stiffness (N/mm^2)
OVX-C	5.17 ±0.32	50.69 ±6.21	142.31 ±10.16	58.64 ±2.35	120.67 ±8.87	0.030 ±0.002	6.960 ±0.508	18.29 ±1.65	5190.91 ±436.50
OVX-50	5.96 ±0.27*	73.72 ±5.49*	100.47 ±9.68*	88.26 ±3.20*	133.24 ±12.98*	0.034 ±0.002*	7.806 ±0.536*	19.94 ±1.41*	5998.55 ±482.38[a)]

OVX-C, OVX model control; OVX-50, OVX rats gavaged with BCAP at 50 mg/day.
a) Values are significantly different compared with values in OVX-C ($p < 0.05$).

on the trabecular surface. The particles were damaged collagen, whose normal microarchitecture was changed, loosened, and ruptured. Howship lacunae of different sizes and depths could be discerned on the trabeculae of OVX-C. Around the lacunae, the collagen fibril lamella, which normally covers the quiescent bone surface, was resorptive. On the contrary, there were many quiescent bone surfaces on the trabecular surface in group OVX-50, the venation of collagen fibers was clear and trim, and the collagen was arrayed tightly. The resorptive surfaces were decreased and the collagenous fibers were much more regular in group OVX-50 than in OVX-C.

3.5 Biomechanical properties

It is well known that maximum-load, break-load, and stiffness are structural mechanical parameters, and the elastic,

amino acid sequence analysis results (Table 5), the protein in peak 1 from Sephadex G-100 was identified as lactoferrin (LF) and the protein in peak 2 as osteopontin (OPN), the protein in peak 3(B) from Econo–pac Q as IGF-2 and the protein in peak 3(C) as epidermal growth factor (EGF). Moreover, peak 3(A) was a penetration peak under current elution conditions and a mixture dominated by peptides with low molecular weight (<10 kDa).

4 Discussion

It is generally known that the distal end of the femur consists of cancellous bone with trabecular bone, and the diaphysis of the femur mainly consists of cortical bone. The density of cortical bone changes more slowly than that of cancellous bone with the development of the femur and

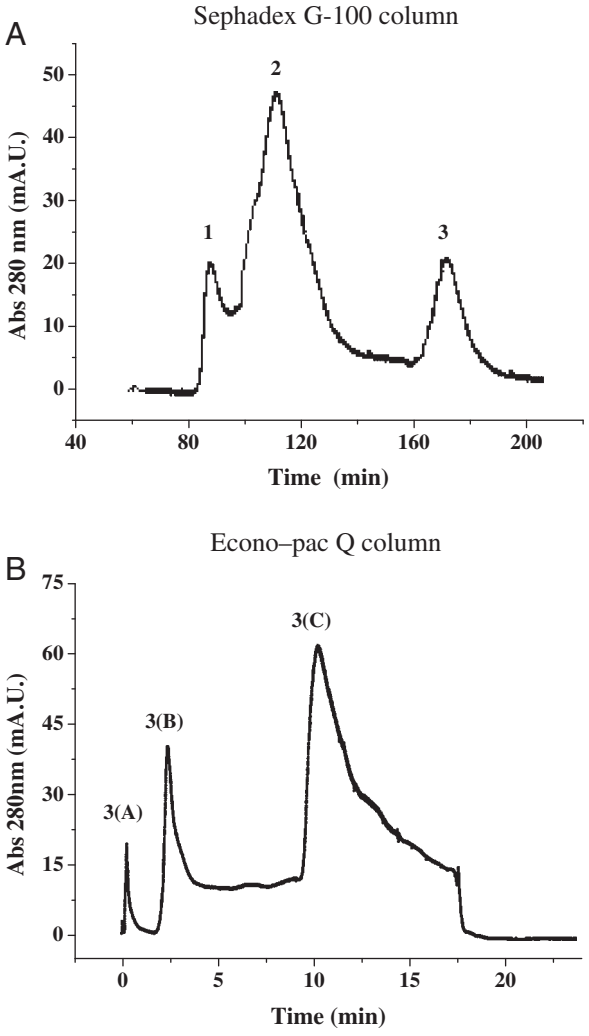

Figure 3. Purification on Sephadex G-100 and Econo–pac Q columns. (A): Sephadex G-100 column (1.5 × 35 cm), it was run with a buffer mobile phase (0.2 mol/L PBS, pH 6.9) at a flow rate of 0.5 mL/min; (B) Econo–pac Q column (0.59 × 3.6 cm), it was performed using 1.0 mol/L NaCl 0.2 mol/L PBS (pH 6.9) buffer as a mobile phase with a 0.1–1.0 mol/L NaCl linear salt gradient at a flow rate of 0.8 mL/min. All chromatography was performed at 4°C with column effluent monitored at 280 nm.

cannot be observed over a short period of time [22, 23]. Therefore, over the 12-wk experimental period, BMD of the femur distal end in the rats gavaged with BCAP changes more significantly contrasted with BMD of the proximal and middle segments.

Osteoporosis is associated with not only a decreased tissue amount in bone cross-section but also the damage to the microarchitecture with micro trauma of bone [24]. The microarchitecture and biomechanical properties of the femur provide more effective indexes of the extent of osteoporosis. They provide convincing clues to illustrate the compound's effect on osteoporosis in conjunction with the BMD of the femur. The results in this study indicated that

Table 5. *N*-terminal amino acids sequence of purified proteins

Proteins	*N*-terminal amino acids sequence (1–20)
Protein in peak 1	aprknvrwct isppegskch
Protein in peak 2	akdknqhsnl itxqensklx
Protein in peak 3(B)	mgitalafvl vllafasgks
Protein in peak 3(C)	angdfxdeds xlmggtxlsn

Peak 1 and peak 2 are elution peaks of Sephadex G-100 column; Peak 3(B) and peak 3(C) are elution peaks of Econo–pac Q columns.

BCAP can attenuate and prevent the negative effect of insufficient estrogen on bone properties. Moreover, BCAP at doses of 2–50 mg/day positively affects bone loss in OVX rats, which has the similar dosage level with the previous studies on BCE (0.002–20 mg/mL), MBP (2–20 mg/day), and WAF (60 mg/day) [4, 6, 10, 16].

Four kinds of dominant proteins/peptides in BCAP were identified. Among these proteins, OPN, EGF, and IGF-2 have an acidic p*I* value (4.5–6.5), respectively. They are bound proteins on anion exchange chromatography theoretically and actually. The occurrence of LF (p*I* value, 8.0–8.6) in BCAP can probably be accounted for protein –protein interactions. It has been demonstrated that LF can associate electrostatically with OPN [25, 26]. In addition, a group of proteins with low molecular weight (< 10 kDa) and alkaline p*I* value (>6.9) were found in BCAP, the authors presumed that these peptides are growth factors, such as IGF-1 or other functional peptides, immigrating with other proteins in BCAP in anion exchange chromatography. At present, the effects of these peptides on osteoporosis have been reported in many studies. OPN peptide is consistently observed in the acidic protein fraction of milk and is closely associated with bone development and maintenance [26, 27]. LF has been shown to have potent bone growth enhancement properties manifested through stimulation of the growth of osteoblasts and inhibition of osteoclasts [28]. Cornish *et al.* declared that LF can be used as a complement to various strategies in the prevention and treatment of osteoporosis [29]. EGF stimulates the proliferation of epidermal, epithelial, and embryonic cells and promotes wound healing and bone resorption. IGF-1 and IGF-2 have significant muscle and cartilage repair characteristics. They stimulate cellular growth, development, and proliferation [5, 9, 30].

In conclusion, the results of this study suggest that the BCAP may lead to a prevention or suspension of the bone loss associated with ovariectomy. Femoral BMD was significantly increased in the BCAP-fed rats after 12 wk of feeding. SEM observation and histomorphometry data indicated that BCAP maintains the normal microarchitecture of the femur in OVX rats. Biomechanical strength test confirmed the beneficial effects of BCAP on bone properties. BCAP predominantly contains bioactive

proteins (peptides) that act on bone, such as LF, OPN, EGF, and IGF-2.

BCAPs at the dose of 2–50 mg/day, approximately equates to an intake of 10–250 mg BCAP/kg/day, affect the bone properties in OVX rats. From a human dietary perspective, this would equate to an intake of 0.6–15 g BCAP/day for an average female weighing 60 kg.

In terms of dietary bone prophylaxis, this study demonstrated that the incorporation of some BCAP into the diet as a supplemental component would be beneficial to prevent osteoporosis. Further study of BCAP is of great significance in terms of preventing or delaying osteoporosis.

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