

Effects of Colostrum Basic Protein from Colostrum Whey Protein: Increases in Osteoblast Proliferation and Bone Metabolism

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Abstract

Colostrum basic proteins (CBP) (MW 1~30 kDa) were isolated from bovine colostrum using a series of ultra-filtration processes and their effects on osteoblast cell proliferation and bone metabolism were investigated in cell line and animal models. Treatments with CBP (1, 10, 100 µg/mL) dose-dependently increased cell proliferation of osteoblastic MC3T3 cells. Alkaline phosphatase activity, a marker of osteoblastic phenotype, in the cells was also increased after treatments with CBP in a dose-dependent manner. Significant increases in bone density were observed in femur of ovariectomized rats which were fed a diet with 1% and 10% CBP, compared to rats fed a normal diet. These results suggest that CBP may increase bone mass and density and be useful for the prevention of bone-related diseases.

Key words: colostrum basic protein, osteoblast, bone density, alkaline phosphatase activity

INTRODUCTION

Recently, there has been increased research on bioactive compounds from natural foods by the pharmaceutical and functional food industries to meet the demands people who are interested in health prevention.

Milk is a rich biological fluid that contains physiologically active proteins to protect against infections (1-3). It contains numerous bioactive molecules associated with nutrition and regulation various metabolic processes (4-8). Human milk or bovine colostrum in place of serum in cell culture supports normal growth of various cell types, such as epithelial cells, fibroblasts, and smooth muscle cells (9-11). Physiologically active substances have been found in trace amounts at specific stages during lactation (12). Colostrum is considered to be a possible source of factors with anabolic effects on bone (13). It has twice the solids content and 5 times the protein of milk, but crude fat contents were 67% of that in milk. Colostrum has immune factors, physical factors, and cell division factors, etc. (14). Colostrum in mammals has growth factors including IGF-1 and TGF-β (MW. 5~10 kDa) and it has 10~500 times the growth factors of milk (15,16). The concentration of immune factors is also much higher in colostrums than in milk. Research (17) has shown that colostrum has powerful natural immune and growth factors that bring the body to a state of homeostasis. Colostrum helps to sup-

port healthy immunity, marrow, neuron, digestion, and muscle functions.

Deficiency of growth factors is associated with impaired bone metabolism such as age-related bone loss and osteoporosis. Lactoferrin, lactoperoxidase, and related peptides in colostrum are related to calcium metabolism. The main purpose of our paper was to investigate the effect of colostrum basic protein (CBP) on cellular growth and differentiation. Thus it was possible to further clarify the effect CBP on the differentiation and mineralization of osteoblast-like MC3T3-E1 cells. Moreover, we performed an *in vivo* study using ovariectomized rats to investigate whether CBP would prevent bone loss and bone resorption.

MATERIALS AND METHODS

Isolation of colostrum basic protein from bovine colostrum

A schematic of the procedure for preparation of colostrum basic protein (CBP) is shown in Fig. 1. Bovine colostrum was collected during the first 48 hours after calving from cows and centrifuged at 5,000 rpm for 30 min to remove the cream layer (18). The defatted bovine colostrum was acidified with 1 N HCl to pH 4.6, which resulted in the coagulation of casein. The coagulated casein was separated from the whey by centrifugation for 30 min at 5,000 rpm and then the casein was discarded.

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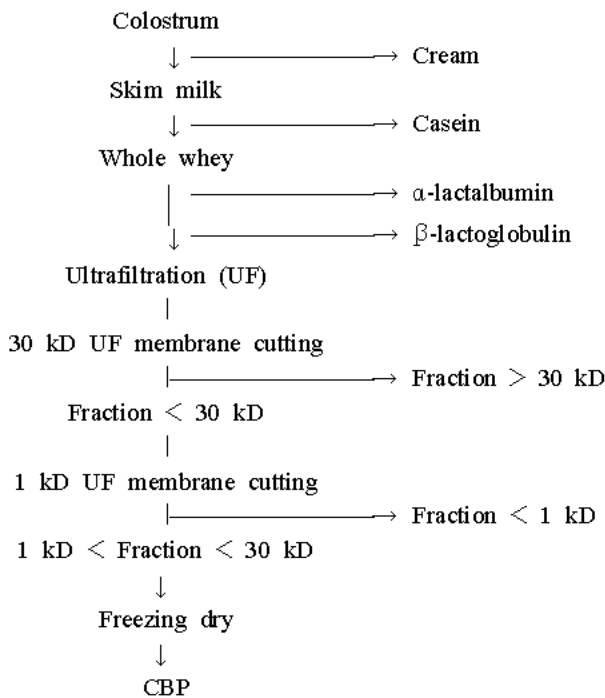


Fig. 1. Schematic procedure of isolating colostrum basic protein (CBP) from colostrums. CBP was centrifuged to separate skim milk and cream fractions. The skim milk was adjusted to pH 4.6, and casein aggregates were removed by centrifugation. The α - and β -lactoglobulin were removed by pH adjustment and centrifugation. The various fractions were obtained by ultrafiltrations.

To remove α -lactalbumin, pH of the whey was first adjusted to 5.5 with 1.5 M trisodium citric acid and then to 3.9 with 1.5 M citric acid. After pH adjustment, the whey was heated for 150 min at 35°C to precipitate α -lactalbumin and the precipitated α -lactalbumin was removed via centrifugation at 5,000 rpm for 30 min. To remove β -lactoglobulin, the supernatant was further adjusted to pH 4.5 with 1 N NaOH and then centrifuged at 18,000 rpm for 30 min. This final supernatant was further purified by sequential ultrafiltration to give CBP (19). The ultrafiltrations were conducted in a 25 mm diameter Amicon stirred ultrafiltration cell (model 8010, Amicon Corp., Beverly, MA) using 100 and 30 kDa molecular weight cut-off Biomax polyethersulfone membranes. The filtrates were then ultrafiltrated with a 1 kDa membrane. The residues were adjusted to pH 7.2 with 1N NaOH, centrifuged at 18,000 rpm for 30 min and re-ultrafiltrated with a 1 kDa membrane to remove small molecules such as salts and lactose. This final residue, designated colostrum basic protein, was freeze-dried and powdered.

SDS-Polyacrylamide gel electrophoresis analysis of purified CBP

To examine the purity of CBP isolated from bovine

colostrum, the purified CBP and bovine colostrum was analyzed using SDS-PAGE. The samples were separated in a 10% gel at 2~4 mA for 3 hours with bromophenol blue (BPB) as a tracer. After electrophoresis, the gel was stained with coomassie blue and destained with 7.5% acetic acid and 5% methanol solution. Prestained SDS-PAGE standard 97595 (cat. 161-0318, Bio-Rad, USA) was the molecular weight marker.

Cell culture and proliferation assay

Cell culture and proliferation assay was carried out using the method of Chung et al. (20), with slight modification. Osteoblast-like MC3T3-E1 cells (CRL-2593) were obtained from American Type Culture Collections (Manassas, VA) and maintained in α -MEM, Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 μ g/mL streptomycin, in a humidified atmosphere of 95% 5% CO₂ at 37°C.

For cell proliferation assay, cells were plated onto 96-well plates at a density of 1×10^4 cells/well in the presence of CBP (1, 10, and 100 μ g/mL). After 72 hours of incubation, MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was added to the media and incubated for 4 hours. Then, 20% sodium dodecylsulfate (SDS) solution was treated for 20 hours and the optical density was measured with spectrophotometer at 540 nm. The effect of CBP on cell proliferation was expressed as percent response compared to the control group.

Bone-specific alkaline phosphatase activity in osteoblasts

Alkaline phosphatase activity in osteoblasts was measured by Kurihara method (21). MC3T3-E1 cells were seeded onto 6-well plates at a density of 5×10^4 cells/2 mL/well and incubated with 1, 10, 100, and 1000 μ g/mL CBP for 72 hours. After incubation, cells were washed three times with phosphate-buffered saline (PBS) and collected with 0.2% Nonidet P-40. The collected cells were sonicated for 15 min and centrifuged at 10,000 g for 15 min. The supernatant was used for the measurement of bone-specific alkaline phosphatase activity using Metra BAP kit (Quidel Co., USA).

Animals and diet

Four-week-old female Sprague-Dawley rats were housed in individual cages in a temperature and humidity controlled room (23 \pm 1°C and 40~60% relative humidity) with a 12 hr light/dark cycle. All rats (30 rats) were given deionized water and diet *ad libitum* for a week. Under anesthesia with 100 mg/kg ketamine (Ketara) and

2% xylazine (Rompun 0.15 mL/kg), 25 rats were ovariectomized at 1 week-of-age as described by D'Amour et al. (22). The ovariectomized rats were fed a low calcium diet (#113095, Dyets Inc., USA) *ad libitum* for 5 weeks. After 5 weeks of feeding, the animals were separated into three experimental groups (control, 1% CBP, and 10% CBP) of 10 animals each. Each group of rats was fed the control diet (AIN-93 diet, Dyets Inc., USA), 1% CBP diet (no casein, 1% CBP), and 10% CBP diet (no casein, 10% CBP) for 3 weeks.

Blood sampling and osteocalcin contents in serum

After the feeding period, the rats were deprived of food overnight before being killed. A blood sample was obtained from each rat, set aside for 30 min, centrifuged for 20 min at 3,000 rpm (4°C), and the serum stored at -70°C. The osteocalcin contents in serum were measured with Rat-MID osteocalcin ELISA kit (Nordic Bioscience Diagnostics, Denmark).

Measurement of bone weight and density in femur

Femora were excised from rats, and the muscles, fats, and connective tissues were removed. These were dried in dry oven for 3 hours at 75°C, and the weight and length measured. Bone density of the femur was measured by dual-energy X-ray absorptionmetry (Norland XR series X-ray source 319A051, USA) (23,24).

Protein and ash contents in bone

Total crude protein contents were measured by the Micro-Kjeldahl (Kjeltec Analyzer unit 2300, Foos Tecator, Sweden) method, proline contents by an amino acid analyzer 430 (Sykam, German), and crude ash, calcium, and phosphorus contents by Inductively Coupled Plasma Emission Spectroscopy (Jobin Yvon 38 plus, France) (25).

Statistical analysis

Data from individual experiments were expressed as the mean \pm standard deviation. Statistical significances were analyzed using Student's t-test and the accepted level of significance was $p < 0.05$. Statistical analyses were performed using SAS software (26).

RESULTS AND DISCUSSION

CBP isolation and SDS-polyacrylamide gel electrophoresis

Colostrum basic protein (MW. 1 kDa ~ 30 kDa) was obtained from mammal colostrum, and the results are shown in Fig. 2. Protein patterns of isolated CBP were similar to colostrum protein, but SDS-PAGE revealed that CBP was more refined fraction of colostrums with lower molecular weights.

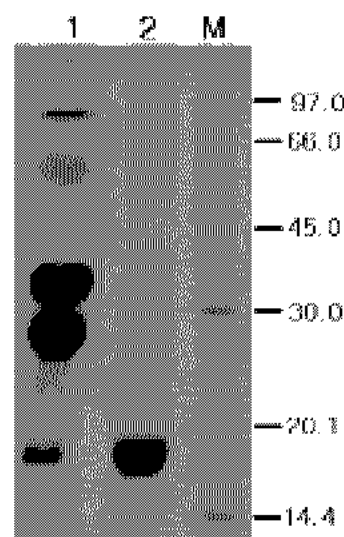


Fig. 2. The SDS-PAGE of CBP. Lane 1 is colostrums; lane 2 is a colostrum basic protein; lane 3 is a molecular weight marker (SDS-PAGE standard 97595).

Effect of CBP on proliferation of osteoblast

The osteoblast-like MC3T3-E1 cell is an established cell line used in many laboratories for osteoblast functional studies. MC3T3-E1 cells are pre-osteoblastic cells that undergo osteoblastic differentiation and mineralization when grown in the presence of β -GP and L-AA. It is known that proliferation, matrix maturation and mineralization are three sequential processes in the differentiation of osteoblasts.

In this study, we examined the effect of CBP on the proliferation of osteoblastic MC3T3-E1 cells. Experimental results are shown in Fig. 3. The proliferation of osteoblastic MC3T3-E1 cells was increased dose-dependently when cells were treated with CBP for 3 days

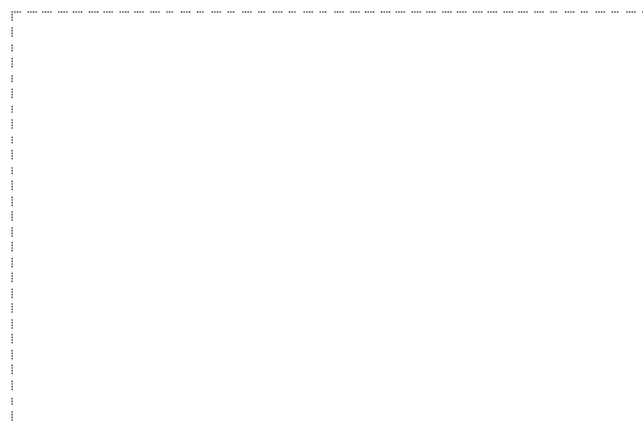


Fig. 3. The effect of CBP on proliferation of osteoblastic MC3T3-E1 cells for 72 hrs. The cells were incubated with CBP (1, 10, 100 μ g/mL) for 3 days. Cell proliferation was measured according to the procedure described in the Materials and Methods section. Each value is the mean \pm SD. There were no statistically significant effects ($p < 0.05$).

and the effect was up to 126.67% at 100 μg CBP/mL. Therefore, from our study we know that CBP potentiate osteoblast cell proliferation.

The osteoblast is a bone-forming cell derived from mesenchyme that deposits an eosinophilic osteoid matrix within collagenous tissue, which subsequently becomes mineralized. The osteoblast produces a subperiosteal collar of bone enclosing cartilaginous tissue in the center of the developing bone. Also, osteoblasts are active in collagen and mucopolysaccharide production in bone (27). Therefore, the effect of CBP on proliferation of osteoblast meant that collagen and bone formation ability was increased. According to Takada et al. (28), whey protein contains active components that can activate osteoblast cell proliferation and differentiation. Milk basic protein also contained active components to promote cell proliferation and collagen synthesis of osteoblasts.

Effect of CBP on alkaline phosphatase activity (ALP) in osteoblast

Alkaline phosphatase activity, as an indicator of osteoblastic differentiation (29,30), is a representative marker for the mature osteoblast. The osteoblast phenotypes are acquired in two stages. There is an initial period of proliferation and biosynthesis of the extracellular matrix, followed by a period of cell differentiation in two phases (31). In the first stage, the matrix matures and specific proteins associated with the bone cell phenotype such as ALP are detected. In the second stage, matrix becomes mineralized, and late markers such as osteocalcin are produced. Thus we examined cell proliferation and ALP activity to investigate the effect of CBP on the differentiation and mineralization of osteoblast-like cells.

Fig. 4 shows the effect of CBP on the differentiation of MC3T3-E1 cells by determining ALP activity in the cells. The result suggested that the effect of CBP on ALP activity was dose-dependent. CBP at the concentration of 1,000 μg CBP/mL increased the highest ALP activity in the MC3T3-E1 cells. Significant increases in ALP activity were observed at 100 μg CBP/mL and 1,000 μg CBP/mL. However, CBP had no effect in these cultures at the concentrations of 1 μg CBP/mL. These results indicate that although CBP affects the activity of osteoblastic cells, the concentration of CBP is an important factor for the activation of osteoblasts.

It is known that bone volume is maintained by two phases of bone remodeling, mainly bone formation by osteoblasts and bone resorption by osteoclasts. An imbalance between bone formation and bone resorption leads to metabolic bone diseases (32). We found that CBP was effective in increasing the osteoblast proliferation and ALP activity in osteoblasts.

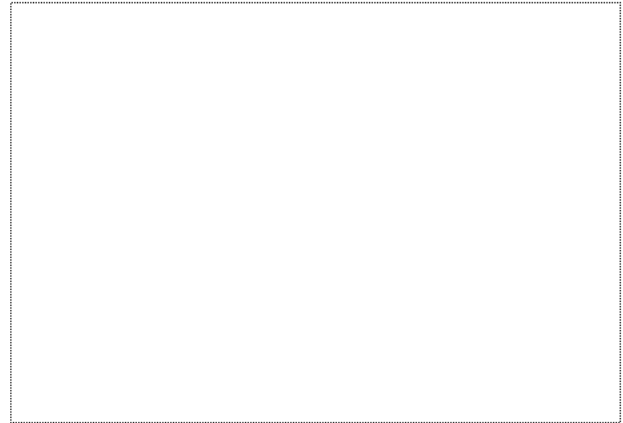


Fig. 4. Effect of CBP on alkaline phosphatase activity (ALP) in osteoblastic MC3T3-E1 cells for the proliferation period (72 hrs). The cells were incubated with CBP (1, 10, 100, 1000 $\mu\text{g}/\text{mL}$) for 3 days. The ALP activity was measured according to the procedure described in the Materials and Methods section. Each value is the mean \pm SD. *Significantly different from control ($p < 0.05$).

Effect of CBP on bone metabolism

Serum osteocalcin contents : We examined the effect of CBP on bone loss in aging 4~12 weeks ovx rats. Ovariectomy causes bone loss due to estrogen deficiency. Osteocalcin, bone gla-protein, is bound to hydroxyapatite and calcium in bone and is indirectly reflects osteoblast activity as a marker of bone formation (33,34).

The serum osteocalcin contents of the ovx-1% and 10% CBP groups were higher than that of the ovx-control group (Fig. 5). Osteocalcin content of the ovx-control group was 311.34 ng/mL, while those of the ovx-1% and 10% CBP groups were 317.33 and 317.48 ng/mL, respectively. However, the amount of CBP was not affected by osteocalcin contents in serum.



Fig. 5. Change in serum osteocalcin by CBP intake contents. The ova rats were fed the control and CBP diet (1 and 10%) for 3 weeks. The serum osteocalcin contents were measured according to the procedure described in the Materials and Methods section. Each value is the mean \pm SD. (n=10). There were no statistically significant effects ($p < 0.05$).

Table 1. Effect of CBP on bone weight and density of ovx rats

	Bone weight (g/g bw)	Bone density (mg/cm ²)
Control	1.82 ± 0.54 ¹⁾	90.5 ± 0.01
CBP-1%	2.19 ± 0.31	95.9 ± 0.00*
CBP-10%	2.31 ± 0.27	97.4 ± 0.00*

The ovx rats were fed the control and CBP diet (1 and 10%) for 3 weeks. The bone weight and density were measured according to the procedure described in the Materials and Methods section.

¹⁾Each value is the mean ± SD (n=10).

*Significantly different from control (p<0.05).

Bone weight and density in femur of ovx rats : Femur bone weight and density of the ovx rats are shown in Table 1. The bone weight was increased in CBP groups. The bone weight of CBP-1% group was 20% higher compared with that of the control group even though the results were not statistically significant (p<0.05); the CBP-10% group was 27% higher than that of control group and was also not significantly different (p<0.05).

The bone density of femur was increased in CBP groups. The bone density was 90.5 mg/cm² in control, and 95.9 mg/cm² in CBP-1%, and 97.4 mg/cm² in CBP-10% group, which were significantly different between control group and CBP groups. These results indicate that CBP prevents the loss of bone density by ovariectomy. The CBP may be useful in the prevention of osteoporosis.

Milk basic protein (MBP) has also been shown to prevent bone loss in the ovx rats (35). According to Yasuhiro et al. (36), MBP supplementation (40 mg of MBP a day) increased bone mineral density. The increased level of bone mineral density might have been caused by the promoting effect of MBP on bone formation and by its suppressing effect on bone resorption. These results indicate that CBP also effects bone formation and resorption.

Protein and mineral contents in femur of ovx rats : The effect of CBP on protein and mineral contents in femur are shown in Table 2. Proline is main amino acid in collagen and an indicator of collagen metabolism. Protein contents of femur were slightly decreased with

CBP-1% group as 32.82 mg, but CBP-10% group had the highest protein contents. The proline contents were 1.08 mg for control, 1.03 mg for CBP-1%, and 1.25 mg for CBP-10%, but it was not significantly different between control and CBP groups.

Bone matrix is composed of mineral and other materials. Mineral is 60~66% of bone weight with moisture and protein weight contributing the remainder (37).

Ash contents in femur were slightly increased in the CBP group although it was not significantly different. Calcium contents in femur also showed a similar trend to ash contents. However, phosphorus contents were the highest in the CBP-1% group, and slightly lower with the CBP-10%, but both were significantly higher than the control group. We found that CBP was effective in increasing the bone matrix and bone proteins such as collagen in ovx rats, which suggests that the active component is absorbed by the small intestine.

The supplemental intake of food factors that increase bone mass may play a role in maintaining bone health and in prevention of bone loss with increasing age. CBP, which is a food factor, has been shown to have an anabolic effect on bone metabolism. In conclusion, it has been demonstrated that CBP can increase alkaline phosphatase activity and osteoblastic cells proliferation. Moreover, we found that CBP increased bone weight and density. We propose the possibility that the active component in CBP plays an important role in bone formation by activating osteoblasts. Therefore, CBP is one of the active components for prevention of bone disease and osteoporosis.

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Table 2. Effect of CBP on protein and mineral contents in femur of ovx rats

	Crude protein	Proline	Ash	Ca	P
Control	33.73 ± 1.38 ¹⁾	1.08 ± 0.17	54.96 ± 1.42	43.06 ± 1.28	9.80 ± 0.23
CBP-1%	32.82 ± 1.54	1.03 ± 0.06	56.18 ± 1.87	43.80 ± 1.29	10.54 ± 0.47
CBP-10%	33.97 ± 0.98	1.25 ± 0.07	56.01 ± 1.21	44.21 ± 0.77*	10.34 ± 0.41

The ovx rats were fed the control and CBP diet (1 and 10%) for 3 weeks. The protein and mineral contents in femur were measured according to the procedure described in the Materials and Methods section.

¹⁾Each value is the mean ± SD (n=10). *Significantly different from control (p<0.05).

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