Whey Protein Concentrate Hydrolysate Prevents Bone Loss in Ovariectomized Rats

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ABSTRACT Milk is known as a safe food and contains easily absorbable minerals and proteins, including whey protein, which has demonstrated antiosteoporotic effects on ovariectomized rats. This study evaluated the antiosteoporotic effect of whey protein concentrate hydrolysate (WPCH) digested with fungal protease and whey protein concentrate (WPC). Two experiments were conducted to determine (1) efficacy of WPCH and WPC and (2) dose-dependent impact of WPCH in ovariectomized rats (10 weeks old). In Experiment I, ovariectomized rats ($n=45$) were allotted into three dietary treatments of 10 g/kg diet of WPC, 10 g/kg diet of WPCH, and a control diet. In Experiment II, ovariectomized rats ($n=60$) were fed four different diets (0, 10, 20, and 40 g/kg of WPCH). In both experiments, sham-operated rats ($n=15$) were also fed a control diet containing the same amount of amino acids and minerals as dietary treatments. After 6 weeks, dietary WPCH prevented loss of bone, physical properties, mineral density, and mineral content, and improved breaking strength of femurs, with similar effect to WPC. The bone resorption enzyme activity (tartrate resistance acid phosphatase) in tibia epiphysis decreased in response to WPCH supplementation, while bone formation enzyme activity (alkaline phosphatase) was unaffected by ovariectomy and dietary treatment. Bone properties and strength increased as the dietary WPCH level increased (10 and 20 g/kg), but there was no difference between the 20 and 40 g/kg treatment. WPCH and WPC supplementation ameliorated bone loss induced by ovariectomy in rats.

KEY WORDS: • bone physical properties • bone strength • osteoporosis • ovariectomy • whey protein hydrolysate

INTRODUCTION

OSTEOPOROSIS IS A bone disease in which bone strength is reduced and risk of fracture is increased. Osteoporosis in women is widely recognized as a major public health problem.¹

Postmenopausal osteoporosis is a heterogeneous disorder characterized by a progressive loss of bone tissue after natural or surgical menopause.² Estrogen deficiency affects bone resorption of osteoclasts by mediated proosteoclastogenic factors (interleukin [IL]-1, IL-6, tumor necrosis factors, macrophage colony-stimulating factor, and prostaglandin E2 [PGE2]) and antiosteoclastogenic factors (IL-1 γ , osteoprotegerin, and transforming growth factor [TGF] β).^{3–6} Postmenopausal osteoporosis is treated with anabolic (para-thyroid hormone) and antiresorptive (estrogen, calcitonin, and bisphosphonates) agents.^{7–12} However, undesirable side effects have been associated with these treatments, including gastrointestinal upset, nausea, headaches, and breast cancer.¹²

Milk is known as a safe food. Compared to nondairy food, milk is a good source of easily absorbable minerals and proteins.^{13–15} Milk is generally consumed in its natural form or as a processed product, such as cream, milk powder,

butter cheese, yogurt, casein, and whey protein. Whey protein is an industrially obtained by-product of the cheese and casein manufacturing process. Highly active molecules such as epidermal growth factor,¹⁶ colony-stimulating factor,¹⁷ TGF α , β ,^{18,19} insulin-like growth factor,²⁰ and fibroblast growth factor²¹ have been isolated from whey protein. These protein components can stimulate growth or differentiation of various cell lines in culture, including bone cells.^{22,23} Several studies have reported that whey protein stimulated the proliferation and differentiation of osteoblastic cells²³ and suppressed osteoclastic cells formation and bone resorption.²⁴ In ovariectomized rats, whey protein supplementation was found to enhance bone strength by augmenting collagen.²⁴

Whey protein is widely used as a functional ingredient in food products. Unfortunately, β -lactoglobulin, the major whey protein, is known to have allergic reactions,²⁵ but this antigenicity could be reduced enzymatic digestion.²⁶ Also, modification of whey proteins using high pressure, heat treatment, and enzymatic hydrolysis can alter its structure and functionality.^{27,28} Properties of protein hydrolysate are affected by type of enzyme, degree of hydrolysis, and substrate pretreatment.²⁹

This study examined the effect of whey protein concentrate hydrolysate (WPCH) on bone loss in ovariectomized rats and compared the effects of WPCH to whey protein concentrate (WPC), and determined the effect of dosage

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using various concentrations of WPC. Bone breaking force, bone mineral density (BMD), bone mineral content (BMC), bone physical properties, and bone resorption and formation enzyme activities of rats treated with WPC and WPC were evaluated.

MATERIALS AND METHODS

Materials

All chemicals, amino acids and reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of WPC

One hundred grams of WPC (Alacen 878; New Zealand Milk Products, Inc., Wellington, New Zealand) was dissolved in 500 mL of distilled water and 10 mL of protease from *Aspergillus oryzae* (Flavourzyme® 500; Sigma-Aldrich) mixed and then diluted to 1000 mL with distilled water. The solution was incubated in a water-bath with agitation (60 RPM) at 50°C for 120 min, then heated to 85°C for 5 min to inactivate protease activity, and cooled in ice-cold water and then freeze-dried. Freeze-dried samples were ground into a fine powder with a mortar and pestle. Incubation conditions were determined by measuring the degree of hydrolysis (data not shown).

Animals and diets

Two different experiments were conducted with 10-week-old female Sprague-Dawley rats [SamTacN(SD)BR] from Samtako (Gyunggi, Korea). Sixty rats were subjected to either sham-operation (SHAM, $n=15$) or bilateral ovariectomy (OVX, $n=45$) in Experiment I, and 75 rats were used for sham-operation (SHAM, $n=15$) or bilateral ovariectomy (OVX, $n=60$) in Experiment II. After a 5-day recovery period, rats that had lower body weights than mean (standard deviation) were excluded from experiments (total $n=8$, SHAM=2 and OVX=6 in Experiment I; and total $n=10$, SHAM=2 and OVX=8 in Experiment II). In Experiment I, rats were allotted one of four dietary treatments using a randomized complete-block design. The positive control group (SHAM) and negative control group (OVX-C) were fed a NIM-31M diet supplemented with a 10 g/kg amino acids and minerals mixture (AMM) (Table 1). The other two OVX groups were fed diets containing WPC (10 g/kg) or WPC (10 g/kg). AMM was formulated to supply the same amount of amino acids and minerals as WPC. Rats were fed experimental diets for 6 weeks. In Experiment II, rats were allotted six different dietary treatments. The positive control group (SHAM) and negative control group (OVX-C) were fed a NIM-31M diet containing 40 g/kg AMM. The other four OVX groups were fed diets containing three different concentrations of WPC with AMM (10 g/kg WPC and 30 g/kg AMM, 20 g/kg WPC and 20 g/kg AMM, and 40 g/kg WPC). Rats were fed experimental diets for 6 weeks. During Experiment I and II, rats were housed in individual stainless steel cages and kept in a temperature ($22^{\circ}\text{C} \pm 2^{\circ}\text{C}$) and light (12 h day-night

TABLE 1. NUTRIENT COMPOSITIONS OF WHEY PROTEIN CONCENTRATE AND THE AMINO ACIDS AND MINERALS MIXTURE SUPPLEMENTED IN SHAM AND OVX TREATMENTS (G/KG)

	Whey protein concentrate	AA and mineral supplements
Amino acids	775	775
Essential amino acid		
Histidine	17	17
Isoleucine	45	45
Leucine	84	84
Lysine	75	75
Methionine	15	15
Phenylalanine	26	26
Threonine	56	56
Tryptophan	16	16
Valine	45	45
Nonessential amino acid		
Alanine	38	—
Arginine	16	—
Aspartic acid	84	—
Cystein	18	—
Glutamic acid ^a	130	396
Glycine	14	—
Proline	45	—
Serine	37	—
Tyrosine	14	—
Lactose	80	80
Ca	7.75	7.75
P	2	2
Fat	100	100
Cellulose	—	35.25

^aNonessential amino acids in the amino acid and mineral mixture were substituted with glutamic acid based on weight. The fat source was corn oil.

cycle) controlled room in the Small Animal Experimental Unit (Korea University, Seoul, Korea). Rats had free access to water and feed throughout the experiments. Body weight was measured weekly and feed intake measured daily. The average daily weight gain and average daily feed intake were calculated. All rats were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals and all procedures involving use of animals were approved by the Korea University Institutional Animal Care and Use Committee (KUIACUC-20130520-1).³⁰

Sample preparation

After 6 weeks, blood samples were collected by cardiocentesis under anesthesia (i.p. injection of 90 mg/kg·bw ketamine and 10 mg/kg·bw xylazine). Serum was recovered from collected blood using a SST® Vacutainer (BD Bioscience, San Diego, CA, USA), divided into aliquots, and stored at -70°C until analysis. Anesthetized rats were sacrificed by cervical dislocation and then the femurs and tibias were excised, remaining flesh was carefully scraped away, and femurs were stored at -70°C until analysis. Tibias were snap-frozen in liquid N_2 and stored at -70°C for further analysis. The uterus and horns were removed and weighed to confirm that ovariectomy was conducted successfully.

Analytical methods

Serum Ca and P concentration was determined with an Advia® 1650 chemistry system (Bayer, Fernwald, Germany). Both femurs were dried at 80°C for 2 days, after which dry weight was measured, and the length between the proximal end of intercondylar notch and the base of femoral neck was measured using a Vanier caliper. BMD and BMC of both femurs were measured by dual-energy X-ray absorptiometry (DEXA, XR-26 Mark II; Norland, Fort Atkinson, WI, USA), using the laboratory animal mode (beam energy; 22 keV, scanning speed; 20 mm/sec, and resolution; 0.5 × 1.0 mm). Bone breaking force of the femoral diaphysis was measured using a Texture analyzer (TA-XT2i; Stable Micro System, Godalming, Surrey, United Kingdom) with the three-point bending method.³¹ Right femurs were dry-ashed at 550°C overnight, after which ash weight was determined. Organic matter weight was calculated as the difference between the dry weight and ash weight. Proximal epiphysis of the left tibia were used to determine bone alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) activities as described by Janckila *et al.*³² Protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Activities of ALP and TRAP were calculated based on protein concentration.

Statistical analysis

The effect of dietary treatments was assessed by GLM procedure using SAS (version 8.1) software (SAS, Cary, NC, USA). To determine dose-dependent effects of WPC and WPC in Experiment II, linear and quadratic effects were determined. Significant differences between groups were determined by least significant difference tests with significance defined at $P < .05$.

RESULTS

In Experiment I, body weight was significantly lower in SHAM group compared to OVX groups (Table 2). The difference in body weight was due to increased daily feed intake and feed efficiency by OVX groups ($P < .05$). Among OVX groups, dietary treatment had no effect on body weight, daily body weight gain, daily feed intake, and feed efficiency. Uteri weight of SHAM group was higher than those of OVX groups, which indicated success of ovariectomy. Serum Ca and P concentrations were not affected by ovariectomy and dietary treatments. Physical properties of femurs are summarized in Table 3. Femur length was unaffected by ovariectomy or dietary treatments. However, dry weight of the femurs was lower in the OVX-C group, and dry weight loss of the femurs was prevented by WPC or WPC supplementation ($P < .05$). OVX-C group showed about 12% decrease in femur ash weight compared with the SHAM group, and this decrease ameliorated by WPC or WPC supplementation ($P < .05$). Also, ovariectomy decreased femoral organic matter content (~8%), and WPC or WPC supplementation in OVX rats showed intermediate

TABLE 2. INITIAL AND FINAL BODY WEIGHT, BODY WEIGHT GAIN, FEED INTAKE, FEED EFFICIENCY, AND UTERI WEIGHT OF RATS FED EXPERIMENTAL DIETS FOR 6 WEEKS (EXPERIMENT I)

Treatment	SHAM	OVX-C	WPC	WPC	SEM
Initial weight (g)	243	248	245	246	3
Final weight (g)	301 ^a	350 ^b	348 ^b	340 ^b	4
Body weight gain (g/day)	1.4	2.4	2.5	2.2	0.1
Feed intake (g/day)	16.1 ^a	20.1 ^b	19.5 ^b	19.7 ^b	0.2
Feed efficiency (g/kg)	85 ^a	121 ^b	124 ^b	118 ^b	8
Uterus weight (mg)	708 ^b	143 ^a	151 ^a	148 ^a	52
Serum Ca (mg/dL)	9.89	10.05	9.89	9.94	0.08
Serum P (mg/dL)	7.24	7.61	7.64	7.75	0.07

All dietary supplementations were 10 g/kg of the basal diet. The effects of the supplements were analyzed using general linear model followed by least significant difference test.

^{ab}The different superscripts in the same row indicate significant differences ($P < .05$).

SHAM, dietary amino acids and minerals mixture supplementation without ovariectomy; OVX-C, dietary amino acids and minerals mixture supplementation with ovariectomy; WPC, dietary whey protein concentrate supplementation with ovariectomy; WPC, dietary whey protein concentrate hydrolysate supplementation with ovariectomy; SEM, standard errors of the means.

value between SHAM and OVX-C ($P < .05$). The ratio between ash and organic weight was significantly decreased by ovariectomy, but WPC or WPC supplementation did not completely prevent this decrease. This data indicated that WPC and WPC supplementation prevented bone loss induced by ovariectomy by sustaining both inorganic and organic bone material.

BMC (Fig. 1) of the OVX-C group was less than that of the SHAM group, which indicated that mineral loss in the femur occurred in response to ovariectomy, and decreases were attenuated by dietary supplementation with WPC and WPC ($P < .05$). BMD of the OVX-C group was significantly lower than the SHAM group, but did not differ from WPC and WPC groups. The greatest force was required for the SHAM group, followed by WPC, WPC, and OVX-C groups (Fig. 2). More force was required to break femurs in WPC group than the OVX-C group, but less force was required than the SHAM group ($P < .05$). There was no difference in bone breaking forces between WPC and

TABLE 3. PHYSICAL PROPERTIES OF FEMURS OF RATS FED EXPERIMENTAL DIETS FOR 6 WEEKS (EXPERIMENT I)

	SHAM	OVX-C	WPC	WPC	SEM
Length (mm)	34.2	34.1	34.1	34.6	0.1
Dry weight (mg)	458 ^a	414 ^b	444 ^a	440 ^a	7
Ash weight (mg)	289 ^a	255 ^c	278 ^b	275 ^b	4
Organic weight (mg)	169 ^a	159 ^b	166 ^a	165 ^a	2
Ash (%)	63.1 ^a	61.5 ^b	62.6 ^{ab}	62.5 ^{ab}	0.2
Ash per organic weight	1.71 ^a	1.60 ^b	1.67 ^{ab}	1.66 ^{ab}	0.1

All dietary supplementations were 10 g/kg of the basal diet. The effects of the supplements were analyzed using general linear model followed by least significant difference test.

^{ab}The different superscripts in the same row indicate significant differences ($P < .05$).

FIG. 1. Bone mineral content (BMC, solid bars) and bone mineral density (BMD, open bars) of femurs of rats fed experimental diets for 6 weeks (Experiment I). SHAM, dietary amino acids and minerals mixture supplementation without ovariectomy; OVX-C, dietary amino acids and minerals mixture supplementation with ovariectomy; WPC, dietary whey protein concentrate supplementation with ovariectomy; WPCCH, dietary whey protein concentrate hydrolysate supplementation with ovariectomy. Values are mean $\pm$ standard deviation (SD). All dietary supplementations were 10 g/kg of the basal diet. The effects of the supplements were analyzed using general linear model followed by least significant difference test. ^{ab}The different superscripts indicate significant differences ($P < .05$).

WPCCH groups. Bone formation enzyme (ALP) and resorption enzyme (TRAP) activity from the tibia epiphysis is presented in Figure 3. Ovariectomy and dietary treatments had no effect on ALP activity; however, the TRAP activity changed significantly in response to ovariectomy and dietary treatments. TRAP activity was significantly higher in the OVX-C group than SHAM, WPC, and WPCCH groups.

FIG. 2. Bone breaking strength of femurs of rats fed experimental diets for 6 weeks (Experiment I). Values are mean $\pm$ SD. All dietary supplementations were 10 g/kg of the basal diet. The effects of the supplements were analyzed using general linear model followed by least significant difference test. ^{abc}The different superscripts indicate significant differences ($P < .05$).

FIG. 3. Alkaline phosphatase (ALP, solid bars) and tartrate-resistant acid phosphatase (TRAP, open bars) activities of proximal epiphyses of femur of rats fed experimental diets for 6 weeks (Experiment I). pNPP, para-nitrophenylphosphate. Values are mean $\pm$ SD. All dietary supplementations were 10 g/kg of the basal diet. The effects of the supplements were analyzed using general linear model followed by least significant difference test. ^{ab}The different superscripts indicate significant differences ($P < .05$).

These findings indicate that WPC or WPCCH supplementation decrease bone resorption activity induced by ovariectomy. These data suggest that WPC and WPCCH could prevent osteoporosis caused by estrogen deficiency and maintain physical bone strength.

In Experiment II, three levels (10, 20, and 40 g/kg of feed) of WPCCH supplementation were used to evaluate possible dose-dependent effects on bone loss in ovariectomized rats. Body weight significantly increased in OVX compared to SHAM by the end of 6 weeks (Table 4; $P < .05$). This increase was due to increased feed intake and improved feed efficiency, as found in Experiment I. Except bone length, bone physical properties were affected by WPCCH supplementation in OVX groups. Dry weight, ash weight, organic weight, ash percentage, and ash per organic material weight were significantly increased with WPCCH supplementation ($P < .05$). When WPCCH was introduced more than 20 g/kg feed, there were no differences from SHAM group in bone dry weight, ash weight, organic weight, ash percentage, and bone ash to organic matter ratio (Table 5). BMC was significantly reduced by ovariectomy, but were significantly increased in WPCCH2 (Fig. 4). BMD showed reduced density after ovariectomy (OVX-C), and quadratic effects were found as dietary WPCCH concentration was increased ($P < .05$). Bone breaking force also decreased with ovariectomy (Fig. 5). However, WPCCH improved strength as concentration increased and showed no statistical difference from SHAM when more than 20 g WPCCH/kg feed was supplemented. ALP activity of tibia epiphysis was unaffected by ovariectomy and dietary treatment, but TRAP activity increased with ovariectomy and decreased with WPCCH supplementation (Fig. 6).

TABLE 4. INITIAL AND FINAL BODY WEIGHT, BODY WEIGHT GAIN, FEED INTAKE, FEED EFFICIENCY, AND UTERI WEIGHT OF RATS IN EXPERIMENT II FOR 6 WEEKS

	SHAM	OVX-C	WPCH1	WPCH2	WPCH4	SEM	Probability	
							Linear	Quadratic
Initial weight (g)	242	246	239	241	235	1.24		
Final weight (g)	272 ^b	325 ^a	322 ^a	324 ^a	313 ^a	3.52	0.63	0.48
Body weight gain (g/day)	0.71 ^b	1.85 ^a	1.87 ^a	1.84 ^a	1.88 ^a	0.12	0.68	0.57
Feed intake (g/day)	15.7 ^b	20.6 ^a	21.1 ^a	21.1 ^a	20.1 ^a	1.14	0.83	0.74
Feed efficiency (g/kg)	45.1 ^b	89.9 ^a	88.4 ^a	87.0 ^a	93.3 ^a	2.48	0.61	0.54
Uterus weight (mg)	652 ^a	115 ^b	117 ^b	108 ^b	132 ^b	18	0.59	0.41
Blood Ca (mg/dL)	10.24	10.11	10.18	10.14	10.19	0.12	0.81	0.71
Blood P (mg/dL)	7.21	7.11	7.18	7.28	7.14	0.14	0.67	0.59

The effects of the supplements were analyzed using general linear model followed by least significant difference test ($n=13$). Linear indicates linear effects of dietary WPCH; quadratic indicates a quadratic effects of dietary WPCH.

^{ab}The different superscripts in the same row indicate significant differences ($P<.05$).

SHAM, 40 g/kg dietary amino acid and mineral mixture supplementation without ovariectomy; OVX-C, 40 g/kg dietary amino acid and mineral supplementation with ovariectomy; WPCH1, 10 g/kg dietary whey protein concentrate hydrolysate and 30 g/kg dietary amino acid and mineral mixture supplementation with ovariectomy; WPCH2, 20 g/kg dietary whey protein concentrate hydrolysate and 20 g/kg dietary amino acid and mineral mixture supplementation with ovariectomy; WPCH4, 40 g/kg dietary whey protein concentrate hydrolysate supplementation with ovariectomy.

DISCUSSION

Ovariectomy closely mimics the postmenopausal state in that endogenous estrogen levels are significantly decreased and bone fragility increased, as a result of increased bone resorption.² An ovariectomized rat has been widely used for preclinical osteoporosis tests due to its similarities with humans.^{33,34} This study evaluated WPCH processed using fungal protease to determine whether it exerts antiosteoporotic effects in ovariectomized rats. Results indicated that dietary WPCH ameliorated some ovariectomy-induced bone loss by increasing the amount of organic and inorganic bone material. Results also indicated that dietary WPCH or WPC can prevent increased bone resorption induced by ovariectomy, suggesting that the rate of bone turnover is attenuated by dietary WPCH.

Ovariectomy is known to increase food intake, body weight, and adiposity as secondary effects of estrogen on

food intake via anorexigenic pathways,^{35–37} and increased body weight exerted positive effects on bone health through increased mechanical loading.^{38,39} Jiang *et al.*⁴⁰ reported no effects of ovariectomy-induced hyperphagia and weight gain on BMD and bone strength. This study did not use pair-feeding to maintain same body weight gain in SHAM and OVXs or observe a correlation between body weight and bone parameters, as data were not corrected for body weight.

Composite materials of bone are organic (primarily collagen type I) and inorganic (crystalline calcium hydroxyapatite).⁴¹ Changes in femoral physical properties, decreased dry weight and ash weight by ovariectomy were consistent with results from Kruger *et al.*⁴² However, they reported that inorganic material of the femur was changed by ovariectomy and dietary acidic and basic whey protein fractions treatment. Whey protein²⁴ and basic milk protein⁴³ have been shown to increase the level of organic material in bone, especially collagen-specific amino acids such as proline and

TABLE 5. PHYSICAL PROPERTIES OF FEMURS OF RATS FED DIFFERENT DIETS FOR 6 WEEKS (EXPERIMENT II)

	SHAM	OVX-C	WPCH1	WPCH2	WPCH4	SEM	Probability	
							Linear	Quadratic
Length (mm)	33.65	33.13	34.21	34.46	33.08	0.13	0.87	0.58
Dry weight (mg)	485 ^a	438 ^c	457 ^b	486 ^a	480 ^a	4	0.12	0.04
Ash weight (mg)	299 ^a	264 ^c	274 ^b	291 ^a	292 ^a	3	0.18	0.02
Organic weight (mg)	185 ^b	174 ^c	177 ^{bc}	193 ^a	182 ^b	2	0.24	0.14
Ash (%)	61.8 ^a	60.3 ^b	61.1 ^a	61.7 ^a	62.0 ^a	0.2	0.21	0.09
Ash per organic weight	1.62 ^a	1.51 ^b	1.60 ^a	1.64 ^a	1.68 ^a	0.01	0.18	0.02

The effects of the supplements were analyzed using general linear model followed by least significant difference test ($n=13$). Linear indicates linear effects of dietary WPCH; quadratic indicates a quadratic effects of dietary WPCH.

^{abc}The different superscripts in the same row indicate significant differences ($P<.05$).

SHAM, 40 g/kg dietary amino acid and mineral mixture supplementation without ovariectomy; OVX-C, 40 g/kg dietary amino acid and mineral supplementation with ovariectomy; WPCH1, 10 g/kg dietary whey protein concentrate hydrolysate and 30 g/kg dietary amino acid and mineral mixture supplementation with ovariectomy; WPCH2, 20 g/kg dietary whey protein concentrate hydrolysate and 20 g/kg dietary amino acid and mineral mixture supplementation with ovariectomy; WPCH4, 40 g/kg dietary whey protein concentrate hydrolysate supplementation with ovariectomy.

FIG. 4. BMC (solid bars) and BMD (open bars) of femurs of rats fed experimental diets for 6 weeks (Experiment II). SHAM, 40 g/kg dietary amino acids and minerals mixture supplementation without ovariectomy; OVX-C, 40 g/kg dietary amino acids and minerals mixture supplementation with ovariectomy; WPCH1, 10 g/kg dietary whey protein concentrate hydrolysate and 30 g/kg dietary amino acids and minerals mixture supplementation with ovariectomy; WPCH2, 20 g/kg dietary whey protein concentrate hydrolysate and 20 g/kg dietary amino acids and minerals mixture supplementation with ovariectomy; WPCH4, 40 g/kg dietary whey protein concentrate hydrolysate supplementation with ovariectomy. Values are mean $\pm$ SD. The effects of the supplements were analyzed using general linear model followed by least significant difference test. ^{ab}The different superscripts indicate significant differences ($P < .05$). ^QIndicates a quadratic effects of dietary WPC ($P < .05$).

FIG. 5. Bone breaking strength of femurs of rats fed experimental diets for 6 weeks (Experiment II). SHAM, 40 g/kg dietary amino acids and minerals mixture supplementation without ovariectomy; OVX-C, 40 g/kg dietary amino acids and minerals mixture supplementation with ovariectomy; WPCH1, 10 g/kg dietary whey protein concentrate hydrolysate and 30 g/kg dietary amino acids and minerals mixture supplementation with ovariectomy; WPCH2, 20 g/kg dietary whey protein concentrate hydrolysate and 20 g/kg dietary amino acids and minerals mixture supplementation with ovariectomy; WPCH4, 40 g/kg dietary whey protein concentrate hydrolysate supplementation with ovariectomy. Values are mean $\pm$ SD. The effects of the supplements were analyzed using general linear model followed by least significant difference test. ^{ab}The different superscripts indicate significant differences ($P < .05$). ^QIndicates a quadratic effects of dietary WPC ($P < .05$).

FIG. 6. ALP (solid bars) and TRAP (open bars) activities of proximal epiphyses of femur of rats fed experimental diets for 6 weeks (Experiment II). SHAM, 40 g/kg dietary amino acids and minerals mixture supplementation without ovariectomy; OVX-C, 40 g/kg dietary amino acids and minerals mixture supplementation with ovariectomy; WPCH1, 10 g/kg dietary whey protein concentrate hydrolysate and 30 g/kg dietary amino acids and minerals mixture supplementation with ovariectomy; WPCH2, 20 g/kg dietary whey protein concentrate hydrolysate and 20 g/kg dietary amino acids and minerals mixture supplementation with ovariectomy; WPCH4, 40 g/kg dietary whey protein concentrate hydrolysate supplementation with ovariectomy. Values are mean $\pm$ SD. The effects of the supplements were analyzed using general linear model followed by least significant difference test. ^{ab}The different superscripts indicate significant differences ($P < .05$). ^QIndicates a quadratic effects of dietary WPC ($P < .05$).

hydroxyproline, without changing dry weight or Ca and P content. However, our study found that both organic and inorganic materials of bone were reduced by ovariectomy and prevented by dietary WPC and WPCH supplementation (Table 3). Results suggest that WPCH and WPC supplementation could improve bone health by affecting both organic and inorganic parts of bone.

BMC of the OVX-C group was significantly lower than SHAM, WPC, and WPCH groups, indicating that more minerals were resorbed from the femurs of the OVX-C group (Fig. 1). Physical properties of the femur confirmed a decrease in mineral content (Table 3). BMD of the OVX-C group was also lower than other groups ($P < .05$), while WPC and WPCH groups did not differ from the SHAM group. Bone is a three-dimensional structure, with density obtained as volume. However, DEXA evaluated the three-dimensional structure in a two-dimensional fashion, which could lead to differences in results.⁴⁴ Bone is not a uniformly solid material, with the hard outer layer composed of compact bone tissue (cortical bone) and the interior (trabecular bone) is less compact than cortical bone.⁴⁴ Proximal femur is mainly trabecular bone, and the mid-shaft is cortical bone. Cortical bones have been shown to be unaffected by estrogen deficiency.⁴⁵ This may explain why there are fewer differences between BMD and BMC.

Bone breaking force is the maximum power required to break bone⁴⁶ and is related to BMC and protein.⁴⁷ Bone breaking force of femurs from the WPC group was not different from the SHAM group ($P < .05$). However, bone breaking strength of the WPC group was lower than that of SHAM group and higher than the OVX-C group ($P < .05$). WPC and WPC groups required similar force to break the femur, with the required force being numerically higher in the WPC group compared with the WPC group (Fig. 2). Results of previous studies indicated that an increased concentration of bone proteins could increase bone strength.^{31,43} Takada *et al.* examined the relationship between Ca concentration in the femur and bone strength, and reported that bone strength increased linearly as Ca content increased.³¹ Therefore, increased bone strength in WPC and WPC groups may be attributed to increased organic and inorganic material in the femur. When physical properties of the femur were evaluated, the WPC group was found to have a higher inorganic and organic material weight, which may have influenced bone strength.

Bone remodeling is a complex process that involves bone formation and resorption, and this remodeling processes is performed by specific bone cells, osteoblasts and osteoclasts. Formation of bone by osteoblasts is mediated by the bone formation enzyme, ALP,⁴⁸ while osteoclasts absorb bone via TRAP.⁴⁹ Activity of each enzyme was analyzed to determine which process was affected by WPC and WPC supplementation. Ovariectomy and dietary treatment only affected TRAP activity, and not ALP activity. In a previous study that implanted demineralized bone in growing rats fed WPC, supplemented diet showed increased ALP activity but TRAP activity was not changed.⁵⁰ Sinha *et al.*⁵¹ compared ALP and TRAP activity of demineralized and mineralized bone implants in young and old female rats and found that ALP activity was higher in demineralized bone implants and TRAP activity was higher in mineralized bone implants. Conflicting results may be attributed to differences in animal growth stage and BMC. Further, ALP has three different isoforms from the liver, intestine, and bone, and ALP circulates in body and reaches many organs. Therefore, ALP activity of the proximal epiphysis of femur may be comprised of different ALPs.

The majority of bone health parameters (bone physical properties, BMD, BMC, and bone strength) increased more in rats of Experiment II compared with rats of Experiment I, which may be due to the higher crude dietary protein concentration (~ 20 g/kg) with WPC supplementation compared to Experiment I. Dietary protein levels are known to be positively related to bone mass,^{52,53} and influence the production and action of IGF-I on bone anabolism.⁵⁴

Results of this study suggest that WPC digested by fungal protease and WPC ameliorate bone loss induced by ovariectomy. Strength and density of the femur increased in rats fed WPC or WPC for 6 weeks, due to increased organic and inorganic bone content. WPC and WPC affected bone resorption activity mediated by TRAP and may contain bioactive components that impact bone metabolism. However, the effect on bone formation was not investigated. Further study is needed to identify bioactive components that may be present in WPC or WPC digested by fungal protease.

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AUTHOR DISCLOSURE STATEMENT

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REFERENCES

1. Riggs BL, Melton LJ 3rd: Involutional osteoporosis. *N Engl J Med* 1986;314:1676–1686.
2. NIH Consensus Development Panel on Osteoporosis Prevention, Diagnosis, and Therapy: Osteoporosis prevention, diagnosis, and therapy. *JAMA* 2001;285:785–795.
3. Manolagas SC, Jilka RL: Bone marrow, cytokines, and bone remodeling. Emerging insights into the pathophysiology of osteoporosis. *N Engl J Med* 1995;332:305–311.
4. Pacifici R: Estrogen, cytokines, and pathogenesis of postmenopausal osteoporosis. *J Bone Miner Res* 1996;11:1043–1051.
5. Polan ML, Daniele A, Kuo A: Gonadal steroids modulate human monocyte interleukin-1 (IL-1) activity. *Fertil Steril* 1988;49:964–968.
6. Paech K, Webb P, Kuiper GG, *et al.*: Differential ligand activation of estrogen receptors ER α and ER β at AP1 sites. *Science* 1997;277:1508–1510.
7. Pottratz ST, Bellido T, Mocharla H, Crabb D, Manolagas SC: 17 β -Estradiol inhibits expression of human interleukin-6 promoter-reporter constructs by a receptor-dependent mechanism. *J Clin Invest* 1994;93:944–950.
8. Recker RR, Davies KM, Dowd RM, Heaney RP: The effect of low-dose continuous estrogen and progesterone therapy with calcium and vitamin D on bone in elderly women. A randomized, controlled trial. *Ann Intern Med* 1999;130:897–904.
9. Chesnut CH 3rd, Silverman S, Andriano K, *et al.*: A randomized trial of nasal spray salmon calcitonin in postmenopausal women with established osteoporosis: The prevent recurrence of osteoporotic fractures study. Proof Study Group. *Am J Med* 2000;109:267–276.
10. Dunn CJ, Goa KL: Risedronate: A review of its pharmacological properties and clinical use in resorptive bone disease. *Drugs* 2001;61:685–712.
11. Sharpe M, Noble S, Spencer CM: Alendronate: An update of its use in osteoporosis. *Drugs* 2001;61:999–1039.
12. Colditz GA, Hankinson SE, Hunter DJ, Willett WC, Manson JE, Stampfer MJ: The use of estrogens and progestins and the risk of breast cancer in postmenopausal women. *N Engl J Med* 1995;332:1589–1593.
13. Ponerros-Schneier AG, Erdman JW: Bioavailability of calcium from sesame seeds, almond powder, whole wheat bread, spinach and nonfat dry milk in rats. *J Food Sci* 1989;54:150–153.
14. NIH Consensus Conference: Optimal calcium intake. NIH consensus development panel on optimal calcium intake. *JAMA* 1994;272:1942–1948.
15. Kansal V, Chaudhary S: Biological availability of calcium, phosphorus and magnesium from dairy-products. *Milchwiss Milk Sci Int* 1982;37:261–263.
16. Yagi H, Suzuki S, Noji T, Nagashima K, Kuroume T: Epidermal growth factor in cow's milk and milk formulas. *Acta Paediatr Scand* 1986;75:233–235.
17. Sinha SK, Yunis AA: Isolation of colony stimulating factor from human milk. *Biochem Biophys Res Commun* 1983;114:797–803.

18. Zwiebel JA, Bano M, Nexo E, Salomon DS, Kidwell WR: Partial purification of transforming growth factors from human milk. *Cancer Res* 1986;46:933–939.
19. Jin Y, Cox DA, Knecht R, Raschdorf F, Cerletti N: Separation, purification, and sequence identification of TGF- β 1 and TGF- β 2 from bovine milk. *J Protein Chem* 1991;10:565–575.
20. Ballard FJ, Nield MK, Francis GL, Dahlenburg GW, Wallace JC: The relationship between the insulin content and inhibitory effects of bovine colostrum on protein breakdown in cultured cells. *J Cell Physiol* 1982;110:249–254.
21. Rogers ML, Belford DA, Francis GL, Ballard FJ: Identification of fibroblast growth factors in bovine cheese whey. *J Dairy Res* 1995;62:501–507.
22. Tokuyama H, Tokuyama Y: Bovine colostric transforming growth factor- β -like peptide that induces growth inhibition and changes in morphology of human osteogenic sarcoma cells (MG-63). *Cell Biol Int Rep* 1989;13:251–258.
23. Takada Y, Aoe S, Kumegawa M: Whey protein stimulated the proliferation and differentiation of osteoblastic MC3T3-E1 cells. *Biochem Biophys Res Commun* 1996;223:445–449.
24. Takada Y, Matsuyama H, Kato K, *et al.*: Milk whey protein enhances the bone breaking force in ovariectomized rats. *Nutr Res* 1997;17:1709–1720.
25. Otani H: Antigenically reactive regions of bovine milk proteins. *Jpn Agric Res Q* 1987;21:135–142.
26. Nakamura T, Sado H, Sykunobe Y, Hirota T: Antigenicity of whey protein hydrolysates prepared by combination of two proteases. *Milchwissenschaft* 1993;48:667–670.
27. Chovert J, Briand L, Dufour E, Dib R, Dalgalarondo M, Haertle T: How to increase β -lactoglobulin susceptibility to peptic hydrolysis. *J Food Biochem* 1996;20:439–462.
28. Britten M, Giroux HJ, Gaudin V: Effect of pH during heat processing of partially hydrolyzed whey protein. *J Dairy Sci* 1994;77:676–684.
29. Adler-Nissen J: Proteases. In: *Enzymes in Food Processing*, (Nagodawithana T, Reed G, eds.). Academic Press, Inc., New York, NY, 1993, pp. 159–204.
30. Institute of Laboratory Animal Resources (US). Committee on Care, Use of Laboratory Animals, National Institutes of Health (US). Division of Research Resources: *Guide for the Care and Use of Laboratory Animals*. National Academies Press, Washington DC, 1996.
31. Takada Y, Kobayashi N, Matsuyama H, *et al.*: Whey protein suppresses the osteoclast-mediated bone resorption and osteoclast cell formation. *Int Dairy J* 1997;7:821–825.
32. Janckila AJ, Takahashi K, Sun SZ, Yam LT: Naphthol-ASBI phosphate as a preferred substrate for tartrate-resistant acid phosphatase isoform 5b. *J Bone Miner Res* 2007;16:788–793.
33. Egermann M, Goldhahn J, Schneider E: Animal models for fracture treatment in osteoporosis. *Osteoporos Int* 2005;16:S129–S138.
34. Bonjour JP, Ammann P, Rizzoli R: Importance of preclinical studies in the development of drugs for treatment of osteoporosis: A review related to the 1998 WHO guidelines. *Osteoporos Int* 1999;9:379–393.
35. Liang YQ, Akishita M, Kim S, *et al.*: Estrogen receptor beta is involved in the anorectic action of estrogen. *Int J Obes Relat Metab Disord* 2002;26:1103–1109.
36. Toth MJ, Poehlman ET, Matthews DE, Tchernof A, MacCoss MJ: Effects of estradiol and progesterone on body composition, protein synthesis, and lipoprotein lipase in rats. *Am J Physiol Endocrinol Metab* 2001;280:E496–E501.
37. Torto R, Boghossian S, Dube MG, Kalra PS, Kalra SP: Central leptin gene therapy blocks ovariectomy-induced adiposity. *Obesity (Silver Spring)* 2006;14:1312–1319.
38. Glauber HS, Vollmer WM, Nevitt MC, Ensrud KE, Orwoll ES: Body weight versus body fat distribution, adiposity, and frame size as predictors of bone density. *J Clin Endocrinol Metab* 1995;80:1118–1123.
39. Crepaldi G, Romanato G, Tonin P, Maggi S: Osteoporosis and body composition. *J Endocrinol Invest* 2007;30:42–47.
40. Jiang JM, Sacco SM, Ward WE: Ovariectomy-induced hyperphagia does not modulate bone mineral density or bone strength in rats. *J Nutr* 2008;138:2106–2110.
41. Seeman E: Modeling and remodeling: The cellular machinery responsible for the gain and loss of bone's material and structural strength. In: *Principles of Bone Biology*. (Bilezikian JP, Raisz LG, Martin TJ, eds.). Academic Press, San Diego, 2008, pp. 1–28.
42. Kruger MC, Poulsen RC, Schollum L, Haggarty N, Ram S, Palmano K: A comparison between acidic and basic protein fractions from whey or milk for reduction of bone loss in the ovariectomized rat. *Int Dairy J* 2006;16:1149–1156.
43. Kato K, Toba Y, Matsuyama H, *et al.*: Milk basic protein enhances the bone strength in ovariectomized rats. *J Food Biochem* 2000;24:467–476.
44. Gasser JA: Assessing bone quantity by pQCT. *Bone* 1995;17:145S–154S.
45. Han X, Xu Y, Wang J, *et al.*: Effects of cod bone gelatin on bone metabolism and bone microarchitecture in ovariectomized rats. *Bone* 2009;44:942–947.
46. Kruger MC, Plimmer GG, Schollum LM, Haggarty N, Ram S, Palmano K: The effect of whey acidic protein fractions on bone loss in the ovariectomized rat. *Br J Nutr* 2005;94:244–252.
47. Thompson DD, Simmons HA, Pirie CM, Ke HZ: FDA guidelines and animal models for osteoporosis. *Bone* 1995;17:S125–S133.
48. van Straalen JP, Sanders E, Prummel MF, Sanders GRB: Bone-alkaline phosphatase as indicator of bone formation. *Clin Chim Acta* 1991;201:27–33.
49. Burstone MS: Histochemical demonstration of acid phosphatase activity in osteoclasts. *J Histochem Cytochem* 1959;7:39–41.
50. Kelly O, Cusack S, Cashman KD: The effect of bovine whey protein on ectopic bone formation in young growing rats. *Br J Nutr* 2003;90:557–564.
51. Sinha R, Smith JC Jr., Soares JH Jr.: The effect of dietary calcium on bone metabolism in young and aged female rats using a short-term in vivo model. *J Nutr* 1988;118:1217–1222.
52. Cooper C, Atkinson EJ, Hensrud DD, *et al.*: Dietary protein intake and bone mass in women. *Calcif Tissue Int* 1996;58:320–325.
53. Geinzo G, Rapin CH, Rizzoli R, *et al.*: Relationship between bone mineral density and dietary intakes in the elderly. *Osteoporos Int* 1993;3:242–248.
54. Thissen JP, Ketelslegers JM, Underwood LE: Nutritional regulation of the insulin-like growth factors. *Endocr Rev* 1994;15:80–101.