Effects of Whey Protein on Calcium and Bone Metabolism in Ovariectomized Rats

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(Received September 18, 1996)

Summary We studied the effects of whey protein (WP) from cow's milk on calcium and bone metabolism in ovariectomized (OVX) rats. Six-week-old female Sprague-Dawley rats were ovariectomized and fed a low-calcium diet (0.03% Ca, 0.3% P) for 4 weeks. The OVX rats were divided into three groups and subjected to two experiments: Exp. 1, Cont group (20% casein, 0.3% Ca), WP (1%) group (19% casein, 1% whey protein, 0.3% Ca) and Low-Ca group (20% casein, 0.03% Ca); and Exp. 2, Cont group (20% casein, 0.3% Ca), WP (1%) group (19% casein, 1% whey protein, 0.3% Ca) and WP (2%) group (18% casein, 2% whey protein, 0.3% Ca). The rats were fed each experimental diet for 4 weeks. The final body weight, weight gain, food intake and food efficiency showed no significant difference between the Cont and WP (1%, 2%) groups in Exps. 1 and 2. There were no significant differences in the calcium balance, serum ALP or serum calcitonin levels between the Cont and WP groups in Exp. 1. But the breaking energies of the WP (1%, 2%) groups were higher than those of the Cont groups in Exps. 1 and 2. As for the amount of calcium, phosphorus and magnesium in the femur, there were no significant differences between the Cont and WP (1%, 2%) groups; however, the amounts of total amino acids in the femur of the WP (1%, 2%) groups were higher than those of the Cont groups in Exps. 1 and 2. The amounts of proline and hydroxyproline in the femur of the WP (1%, 2%) groups were also higher than those of the Cont groups in Exps. 1 and 2. These data indicate that the milk whey protein influence in OVX rats is an increase in bone proteins such as collagen and enhanced bone-breaking energy.

Key Words milk, whey protein, ovariectomized (OVX) rats, calcium metabolism, bone metabolism, calcium balance, bone mineral, breaking force, bone protein, hydroxyproline

There is enormous interest in research to prevent menopause-related bone loss (1,2). It is important to take sufficient dietary nutrients, especially calcium, for the prevention of bone loss. On the other hand, there is a need to develop food components that activate bone formation.

Milk is a good source for calcium absorption and bioavailability (3,4). However, milk is not only an important source of calcium, it is also an excellent source of other nutrients. Whey protein from milk contains excellent nutrients and is produced from whey using ultrafiltration, reverse osmosis, chromatography and dialysis to remove lactose and other components (5-8). Whey is obtained by adding an acid or rennet to milk or non-fat milk and removing the formed coagulate, which is usually produced as a by-product of cheese or casein manufacturing. One hundred milliliters of milk contains 0.55 g of whey protein, and the major whey proteins are α -lactalbumin (58%), β -lactoglobulin (20%), immunoglobulin (13%) and serum albumin (7%) (9). Milk is well known as a safe foodstuff which can be taken for a long time, and is beneficial to human health. Milk has a functional role in the growth of newborns, so milk whey protein may have components that affect bone metabolism. Previously, we found that milk whey protein from cow's milk stimulates the proliferation and differentiation of osteoblastic MC3T3-E1 cells (10,11). In this study, we therefore examined the effects of whey protein from cow's milk on calcium and bone metabolism using ovariectomized (OVX) rats.

MATERIALS AND METHODS

Animals and diet. Five-week-old female Sprague-Dawley rats (Charles River Inc., Japan) were housed in individual wire cages in a temperature- and humidity-controlled room ($23 \pm 1^\circ\text{C}$ and $60 \pm 5\%$ relative humidity, respectively) with a 12 h light/dark cycle. Under anesthesia, the 21 rats were ovariectomized as described by D'Amour et al. (12) at 6 weeks of age. The ovariectomized rats were fed a low-calcium (0.03%) diet ad libitum for 4 weeks. After 4 weeks of feeding, the OVX rats were separated into three experimental groups and subjected to two experiments: Exp. 1, Cont group (20% casein, 0.3% Ca), WP (1%) group (19% casein, 1% whey protein, 0.3% Ca) and Low-Ca group (20% casein, 0.03% Ca); and Exp. 2, Cont group (20% casein, 0.3% Ca), WP (1%) group (19% casein, 1% whey protein, 0.3% Ca) and WP (2%) group (18% casein, 2% whey protein, 0.3% Ca) of seven rats per group. The rats were fed each experimental diet for 4 weeks (Table 1). Whey protein from cow's milk was prepared from whey protein concentrate (WPC) by dialysis only. The WPC was purchased from the New Zealand Dairy Board (Wellington, New Zealand). All rats were given deionized water ad libitum. The phosphorus and magnesium contents were identical (Table 1). The amino acid compositions of protein in the diets are shown in Table 2.

Calcium balance study. A two-part balance study was performed in the first and third weeks of the experimental periods. All rats were housed in metabolic

Table 1. Composition of diets (%).^{1,2}

	Group			
	Cont-OVX	WP (1%)-OVX	WP (2%)-OVX	Low-Ca-OVX
Casein	20.00	19.00	18.00	20.00
Whey protein ³	—	1.00	2.00	—
Sucrose	51.06	51.09	51.11	51.78
Corn starch	15.00	15.00	15.00	15.00
Cellulose	5.00	5.00	5.00	5.00
Corn oil	5.00	5.00	5.00	5.00
DL-Methionine	0.30	0.30	0.30	0.30
Choline chloride	0.20	0.20	0.20	0.20
Vitamin mix	1.00	1.00	1.00	1.00
CaCO ₃	0.75	0.74	0.73	0.08
KH ₂ PO ₄	1.32	1.31	1.30	1.32
NaCl	0.25	0.24	0.24	0.25
MgO	0.08	0.08	0.08	0.08
Basal mixture ⁴	0.04	0.04	0.04	0.04

¹ Basal composition of experimental diets was based on AIN-76 (American Institute of Nutrition) formulation.

² Total calcium, phosphorus, sodium, potassium and magnesium concentrations were 300, 300, 100, 380 and 50 mg/100 g, respectively.

³ One gram of whey protein contained calcium (2.9 mg), phosphorus (2.9 mg), sodium (2.4 mg), potassium (4.5 mg) and magnesium (0.4 mg).

⁴ Basal mixture composition was based on the AIN-76 Mineral Mix (MnCO₃: 12 mg, FeC₆H₅O₇·xH₂O: 21 mg, 70% ZnO: 5.6 mg, 55% CuCO₃: 1.1 mg, KIO₃: 0.035 mg, Na₂SeO₃·5H₂O: 0.035 mg, CrK(SO₄)₂·12H₂O: 1.9 mg).

cages for 5 days and subjected to a calcium balance study. During this period, food intake was monitored and feces and urine were collected.

The absorption and retention ratios of calcium were each determined by the following equations:

Apparent Ca absorption rate (%)

$$= (\text{Ca intake} - \text{Fecal Ca excretion}) / \text{Ca intake},$$

Ca Retention (mg/day)

$$= \text{Ca intake} - (\text{Fecal Ca excretion} + \text{Urinary Ca excretion})$$

and

$$\text{Ca Retention rate (\%)} = (\text{Retention} / \text{Ca intake}) \times 100.$$

Determination of calcium. Diets and feces were first dried and micropulverized. The micropulverized samples were then ashed at 550°C for 48 h. The ashed samples were dissolved in 1 N HNO₃ and diluted with distilled water. The amount of calcium in the diets, feces, and urine were determined by ICP-Atomic Emission Spectrometry (ST-3000, LEEMAN LABS; MA, USA).

Determination of breaking force of femur. Four weeks after the feeding of

Table 2. Amino acid composition of protein in diets (% of protein).

	Casein	WP	Group		
			Cont-OVX Low-Ca-OVX	WP (1%)-OVX	WP (2%)-OVX
Asp	6.84	10.34	6.84	7.02	7.19
Thr	4.11	7.16	4.11	4.26	4.42
Ser	5.72	5.39	5.72	5.70	5.69
Glu	19.99	16.19	19.99	19.80	19.61
Pro	10.94	6.79	10.94	10.73	10.53
Gly	1.88	1.94	1.88	1.88	1.89
Ala	3.01	5.36	3.01	3.13	3.25
Cys	0.06	0.33	0.06	0.07	0.09
Val	5.83	5.70	5.83	5.82	5.82
Met	2.79	2.17	2.79	2.76	2.73
Ile	4.49	6.02	4.49	4.57	4.64
Leu	8.87	10.81	8.87	8.97	9.06
Tyr	5.30	3.16	5.30	5.19	5.09
Phe	4.89	3.64	4.89	4.83	4.77
Lys	7.39	8.80	7.39	7.46	7.53
His	2.77	1.83	2.77	2.72	2.68
Trp	1.58	1.71	1.58	1.59	1.59
Arg	3.54	2.64	3.54	3.50	3.45

experimental diets, the femoral bones were isolated, and the muscles and connective tissues carefully removed. The breaking strength and breaking energy of the femoral diaphysis (the center of the femur) was determined using a three-point bending rheolometer (RX-1600, Aitechno, Japan) by the method of Ezawa et al. (13). The way of measurement and determination of breaking strength and energy are shown in Fig. 1. The measurement conditions were as follows: plunger speed, 100 mm/min; and load range, 40.0 kg.

Femoral mineral (calcium, magnesium, phosphorus) and amino acid (hydroxyproline, proline) content. All femurs were lyophilized for 72 h. The right femurs were dissolved in 6 N HCl and hydrolysed at 120°C for 24 h. The samples were analyzed by an amino acid analyzer. The left femoral bones were ashed at 550°C for 48 h, dissolved in 1 N HNO₃ and analyzed by ICP-Atomic Emission Spectrometry.

Statistical analysis. Statistical analysis was done by analysis of variance and Tukey's multiple-range test. Differences in mean values between the groups were considered significant at $p < 0.05$.

RESULTS

The results of final body weight, body weight gain, food intake and food

Fig. 1. Measurement of breaking strength and breaking energy of femur by rheometer MAX (RX-1600).

Table 3. Effects of whey protein and low-calcium diets on body weight, food intake and food efficiency from 10 to 14 weeks.

	Exp. 1 group		
	Cont-OVX	WP (1%)-OVX	Low-Ca-OVX
Final weight (g)	377 ± 28.0	380 ± 18.5	368 ± 36.3
Body weight gain (g)	82.1 ± 15.8 ^a	85.6 ± 7.8 ^a	70.3 ± 14.9 ^b
Food intake (g)	586 ± 28.3	589 ± 30.3	570 ± 79.9
Food efficiency	0.14 ± 0.02	0.15 ± 0.01	0.13 ± 0.02
	Exp. 2 group		
	Cont-OVX	WP (1%)-OVX	WP (2%)-OVX
Final weight (g)	382 ± 25.2	385 ± 21.2	389 ± 24.3
Body weight gain (g)	80.7 ± 10.4	83.9 ± 8.6	87.1 ± 10.1
Food intake (g)	607 ± 41.2	611 ± 34.5	605 ± 32.7
Food efficiency	0.13 ± 0.02	0.14 ± 0.02	0.14 ± 0.02

Values are M ± SD ($n = 7$).

Different letters denote means that are significantly different ($p < 0.05$).

efficiency are shown in Table 3. The final body weight, weight gain, food intake and food efficiency were not significantly different between the Cont and WP (1%, 2%) groups in Exps. 1 and 2. The results of the balance study performed in the first and third weeks of the experimental period are shown in Table 4. Calcium absorption rates, calcium retention and retention rate at 13 weeks of age (beginning of the third week of the study period) decreased slightly compared with those measured at 11 weeks of age (end of the first week). However, the calcium balance of the WP groups showed no significant difference among the OVX groups in Exp. 1. The results of the serum ALP activity and serum calcitonin level are shown in Fig. 2. The serum ALP level of the Low-Ca group was higher than that of the Cont group,

Table 4. Effect of whey protein and low-calcium diets on apparent calcium absorption rate, calcium retention and retention rate in OVX rats.

	Group		
	Cont-OVX	WP (1%)-OVX	Low-Ca-OVX
Apparent Ca absorption rate (%)			
11 wk	78.6 $\pm$ 4.14 ^a	78.4 $\pm$ 3.67 ^a	95.5 $\pm$ 1.56 ^b
13 wk	66.1 $\pm$ 3.50 ^a	67.2 $\pm$ 2.80 ^a	94.8 $\pm$ 3.27 ^b
Ca retention (mg)			
11 wk	60.3 $\pm$ 3.18 ^a	61.3 $\pm$ 4.91 ^a	2.63 $\pm$ 0.45 ^b
13 wk	53.2 $\pm$ 2.88 ^a	55.4 $\pm$ 2.70 ^a	2.34 $\pm$ 0.26 ^b
Ca retention rate (%)			
11 wk	76.6 $\pm$ 3.70 ^a	76.2 $\pm$ 3.70 ^a	93.5 $\pm$ 1.48 ^b
13 wk	64.2 $\pm$ 3.58 ^a	65.4 $\pm$ 3.06 ^a	92.9 $\pm$ 3.32 ^b

Values are M $\pm$ SD ($n=7$).

Different letters denote means that are significantly different ($p<0.05$).

Fig. 2. Effects of whey protein and low-calcium diets on serum ALP activity and plasma calcitonin level in OVX rats at 14 wk of age in Exp. 1. A, Serum ALP activity; B, plasma calcitonin level. The results are expressed as M $\pm$ SD ($n=7$). Different letters denote means that are significantly different ($p<0.05$).

while there was no significant difference between the Cont and WP groups in Exp. 1.

The results of breaking strength and breaking energy are shown in Figs. 3 and 4. There was no significant difference in breaking strength between the Cont and WP (1%, 2%) groups, but the breaking energies of the WP (1%, 2%) groups were higher than those of the Cont groups in Exps. 1 and 2. The results of the amounts of calcium, phosphorus and magnesium in the femur are shown in Table 5, and the results of the amounts of total amino acid in the femur are shown in Fig. 5. As for the amounts of calcium, phosphorus, and magnesium, there were no significant differences between the Cont and WP (1%, 2%) groups in Exps. 1 and 2.

Fig. 3. Effects of whey protein and low-calcium diets on breaking strength and breaking energy of femur in OVX rats at 14 wk of age in Exp. 1. A, Breaking strength; B, breaking energy. The results are expressed as $M \pm SD$ ($n=14$). Different letters denote means that are significantly different ($p < 0.05$).

Fig. 4. Effects of whey protein diets on breaking strength and breaking energy of femur in OVX rats at 14 wk of age in Exp. 2. A, Breaking strength; B, breaking energy. The results are expressed as $M \pm SD$ ($n=14$). Different letters denote means that are significantly different ($p < 0.05$).

Table 5. Effect of whey protein and low-calcium diets on the amounts of calcium, phosphorus and magnesium in the femur using OVX rats.

	Exp. 1 group		
	Cont-OVX	WP (1%)-OVX	Low-Ca-OVX
Femur calcium (mg)	123±11.2 ^a	124±14.4 ^a	60.9±7.14 ^b
Femur phosphorus (mg)	57.2±5.76 ^a	58.3±7.06 ^a	28.4±3.89 ^b
Femur magnesium (mg)	2.26±0.23 ^a	2.36±0.29 ^a	1.02±0.15 ^b

	Exp. 2 group		
	Cont-OVX	WP (1%)-OVX	WP (2%)-OVX
Femur calcium (mg)	120±13.2	123±10.9	125±11.3
Femur phosphorus (mg)	57.5±6.09	58.1±5.36	59.6±5.71
Femur magnesium (mg)	2.29±0.22	2.33±0.19	2.35±0.24

Values are $M \pm SD$ ($n = 7$).

Different letters denote means that are significantly different ($p < 0.05$).

Fig. 5. Effects of whey protein and low-calcium diets on total amino acids in the femur using OVX rats at 14 wk of age. A, Exp. 1; B, Exp. 2. The results are expressed as $M \pm SD$ ($n = 7$). Different letters denote means that are significantly different ($p < 0.05$).

However, the amounts of total amino acids in the femur of the WP (1%, 2%) groups were higher than those of the Cont groups in Exps. 1 and 2. The results of the amounts of proline and hydroxyproline in the femur are shown in Figs. 6 and 7. The amounts of proline and hydroxyproline of the WP (1%, 2%) groups were also higher than those of the Cont groups in Exps. 1 and 2.

DISCUSSION

In this study, we examined the effect of whey protein on calcium and bone

Fig. 6. Effects of whey protein and low-calcium diets on proline and hydroxyproline in the femur using OVX rats at 14 wk of age in Exp. 1. A, Femur proline; B, femur hydroxyproline. The results are expressed as $M \pm SD$ ($n=7$). Different letters denote means that are significantly different ($p < 0.05$).

Fig. 7. Effects of whey protein diets on proline and hydroxyproline in the femur using OVX rats at 14 wk of age in Exp. 2. A, Femur proline; B, femur hydroxyproline. The results are expressed as $M \pm SD$ ($n=7$). Different letters denote means that are significantly different ($p < 0.05$).

metabolism using ovariectomized rats. The feeding of a normal diet to the ovariectomized rats that had been fed a low-calcium diet caused a 48% increase in bone mineral density within 30 days (13,14). The rats fed on the whey protein-supplemented diet showed a significant increase in bone strength and hydroxyproline and proline content. The results of this study have demonstrated that whey protein influences bone metabolism by increasing the amount of bone protein such as collagen and enhancing bone-breaking energy.

We first examined a calcium balance study and some serum parameters of the calcium metabolism because the enhancement of calcium absorption and calcium retention might cause an alternation of the bone calcium metabolism in rats,

resulting in an elevation of calcium content and eventually an increase in resistance against breakage (13–15). In the calcium balance study, we examined the apparent calcium absorption rate, calcium retention and calcium retention rate in the first and third weeks of experimental diet feeding. Previously, we reported the balance study of various calcium compounds using normal male rats. In spite of the lower calcium level (300 mg), calcium retention at 13 weeks of age in this study was higher than that of a previous study (43 mg/day; Ca 600 mg, P 460 mg) (16). The reason for this difference is thought to be that the demand for calcium was high because the osteoporotic rats recovered from the state of osteoporosis to the normal state immediately. However, the apparent calcium absorption rate, calcium retention and retention rate of the WP groups were not significantly different from the Cont groups. Moreover, the serum ALP levels of the Low-Ca groups were higher than those of the Cont groups. The high-risk state of osteoporotic rats given low-calcium diets is associated with the high serum ALP levels. The serum bone-type ALP levels in the OVX groups were increased, and this enzyme may be released in a large quantity from the osteoblast membrane into serum in OVX animals, similar to those in rickets or osteomalacia (17). These results show that this model is useful for evaluation of the calcium metabolism. However, there was no clear difference in serum ALP activity between the Cont and WP groups. We also measured plasma calcitonin because there was a correlation between plasma calcitonin and calcium retention (16). However, there was no difference in the calcitonin level between the Cont and WP groups.

However, the breaking energies of the femur were significantly higher in the WP (1%, 2%) groups as compared to those of the Cont groups in Exps. 1 and 2. Breaking strength is the maximum power that is required to break bone by the three-point bending method. On the other hand, breaking energy is an integration value of power that is required to make a break. It is thought that breaking strength is a momentary and maximum power, whereas breaking energy is the overall power that is required to make a break. That is, it is thought that breaking strength reflects both bone mineral and bone proteins such as collagen, whereas breaking energy mainly reflects bone proteins such as collagen. In this regard, it is thought that the enhancement of bone strength due to the intake of whey protein causes an alteration of bone metabolism, resulting in an elevation of bone protein. Mature bone matrix is a complex of highly mineralized tissue with a structural framework including bone proteins. Bone proteins consist of mainly collagen and a small number of non-collagenous proteins and glycoproteins. The amounts of total amino acids in the femur of the WP (1%, 2%) groups increased as compared to those of the Cont groups in Exps. 1 and 2. The amounts of proline and hydroxyproline in the femur of the WP (1%, 2%) groups also increased as compared to those of the Cont groups in Exps. 1 and 2. From these results, it is thought that the amounts of femur proline and hydroxyproline, which are typical amino acids in collagen, were increased by the intake of whey protein. An increase in the bone-breaking energy from whey protein might result from an increase in

bone proteins such as collagen, not the action of an increase in bone mineral.

In our previous study, whey protein stimulated the cell growth and promotion of hydroxyproline content in osteoblastic MC3T3-E1 cells (9). Using rat everted gut sac, the inner solution of a whey protein fraction, pepsin-digested and pepsin/pancreatin-digested fractions also stimulated cell growth in osteoblastic MC3T3-E1 cells (9). However, we could not demonstrate the relationship between these results *in vivo* and previous results *in vitro*. Further studies are required to determine the mechanism. In conclusion, it is suggested that whey protein has effect on bone metabolism.

We would like to express our appreciation to Dr. Ikuko Ezawa and Dr. Chie Igarashi for their helpful suggestions and support.

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