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on Bone Metabolism in Healthy Adult Women**

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Milk has more beneficial effects on bone health compared to other food sources. Recent *in vitro* and *in vivo* studies showed that milk whey protein, especially its basic protein fraction, contains several components capable of both promoting bone formation and inhibiting bone resorption. However, the effects of milk basic protein (MBP) on bone metabolism of humans are not known. The object of this study was to examine the effects of MBP on bone metabolism of healthy adult women. Thirty-three normal healthy women were randomly assigned to treatment with either placebo or MBP (40 mg per day) for six months. The bone mineral density (BMD) of the left calcaneus of each subject was measured at the beginning of the study and after six months of treatment, by dual-energy x-ray absorptiometry. Serum and urine indices of bone metabolism were measured at the base line, three-month intervals, and the end of the study. Daily intake of nutrients was monitored by a three-day food record made at three and six months. The mean (\pm SD) rate of left calcaneus BMD gain of women in the MBP group ($3.42 \pm 2.05\%$) was significantly higher than that of women in the placebo group ($2.01 \pm 1.75\%$, $P = 0.042$). As compared with the placebo group, urinary cross-linked N-telopeptides of type-I collagen/creatinine and deoxypyridinoline/creatinine were significantly decreased in the MBP group ($p < 0.05$), while no significant differences between the two groups were observed in serum osteocalcin and bone-specific alkaline phosphatase concentrations. A daily MBP supplementation of 40 mg in healthy adult women can significantly increase their BMD independent of dietary intake of minerals and vitamins. This increase in BMD might be primarily mediated through inhibition of osteoclast-mediated

bone resorption by the MBP supplementation.

Key words: milk basic protein; bone mineral density; bone resorption; healthy adult women

Like western Europe and the United States, Japan has developed into an aged society with a correspondingly high incidence of osteoporosis. Osteoporosis is characterized by increased bone fragility, resulting in an increased risk of fracture, and is usually defined as a reduction in bone mineral density (BMD).¹⁾ Optimal management of osteoporosis consists of maximizing peak bone mass in early adulthood and preventing rapid bone loss occurring after menopause for women.²⁾ Bone remodeling by formation and resorption is a continuous process, however the bone turnover rate is relatively slow. Therefore nutrition can be an important factor compared with drugs that have a rapid effect, because the administration of nutrients related to bone metabolism is relatively safe and inexpensive. In the near future, fortification of some physiologically functional components may help to improve bone health.

Historically, cow's milk has been consumed widely because of its excellent nutritional value. In particular, milk is a good source of bioavailable calcium compared with other food sources. Recent studies demonstrated that milk whey protein, a by-product of cheese or casein manufacturing, plays a functional role in bone remodeling.^{3,4)} In these reports, the active components responsible for promotion of bone formation and suppression of the bone resorption were characterized as in its basic protein fraction (milk basic protein, MBP). And, our *in vivo* study showed that the milk whey protein and fractionated

they protein increase femoral bone strength in young ovariectomized rats.^{5,6} We also showed that MBP prevented bone loss in aged ovariectomized rats as a suitable model of osteoporosis.⁷ Because MBP clearly reduced the urinary excretion level of deoxyypyridinoline (a biochemical marker of bone resorption) in the animal study, we suggested that MBP suppressed the osteoclast-mediated bone resorption.⁷

From the standpoint of the prevention of osteoporosis, we examined the effects of the MBP on BMD and biochemical markers of bone metabolism in healthy adult women.

Subjects and Methods

Subject. Thirty-three healthy women (mean [\pm SD] age, 28.8 ± 8.7) were recruited through direct mailings and attending presentations about this study in our institute. The protocol was approved by the ethical committee of the participating institution. Written informed consent was obtained from each subject. The level of physical activity of all subjects was moderate. Their work was clerical and they operated machines, met people, and did housework. Women were excluded from the study if they had used estrogen, glucocorticoids, or other medications known to affect bone metabolism for the last three years. The study complied with the code of ethics of the World Medical Association (Helsinki Declaration of 1964 as revised in 1989).

Study design and supplements. In this six-month double blind, placebo-controlled trial, the volunteers were randomly assigned to either the placebo or the MBP group with stratification according to body weight, height, body mass index, and BMD. Seventeen women received the experimental beverage containing 40 mg of MBP and the other sixteen women received a matching placebo beverage. Each beverage contained lactic acid, sweetening, and flavor as masking ingredients in 50 ml of water. The MBP was prepared from acid whey. The whey was put onto a column that had been packed with 500 g of cation exchange resin. The column was sufficiently washed with deionized water and the bound proteins were eluted with 1 M sodium chloride. The MBP was obtained by freeze-drying after dialysis of the eluted fraction in a cellulose membrane tube (Sankojunyak, Tokyo, Japan). Absorption to and elution from cation exchange resin yielded the MBP fraction containing 1 to 2% whey protein. The protein concentration of the MBP was 98%, and the MBP fraction contained lactoferrin, lactoperoxidase, and other minor components including cystatin. Women in each group were instructed to drink one bottle (50 ml) of the beverage daily at any time. They were advised to maintain their usual diets and avoid taking supplemental minerals and vitamins on their own for

six months throughout the study. Each woman came to the institute every three months for evaluation. During each three months, each subject had urine and blood measurements. At baseline and six months evaluation, they were also measured for BMD. During the study period (Aug.-Jan.), a prospective standardized three-day food record was completed by each subject at three months (autumn) and six months (winter). The nutrient contents of the diets were calculated using a computer program based on the Standard Tables of Food Composition.⁸ Magnesium content of the diets was compensated for the similar food or another food composition table⁹ because the Standard Tables of Food Composition are not complete in terms of magnesium.

Status of subjects and compliance. During the six-months study period, none of the 33 women dropped out of the study. No bloating, diarrhea, or allergies were reported from either group. All subjects completed the study according to the protocol.

Analytic methods. BMD in the left calcaneus was measured by dual-energy x-ray absorptiometry with use of a DX 2000 scanner (Kyoto Daiichi Kagaku, Kyoto, Japan). The coefficients of variation for the measurements were 2.0 percent. The scans of the left calcaneus were performed in duplicate and the values were averaged. A phantom consisting of bone ash embedded in a 12-cm block was scanned every day as a control; the BMD of the phantom stayed unchanged throughout the study.

Blood was drawn between 9:00 a.m. and 11:00 a.m. after the subjects had fasted for at least eight hours. Second spontaneous urine was collected between 9:00 and 10:00 a.m. before breakfast. Portions of samples were frozen at -20°C until analysis. Serum bone-specific alkaline phosphatase was measured by an immunoselective enzyme assay (Alkphase-B, Metra Biosystems, Palo Alto, CA). Osteocalcin was measured by an immunoradiometric assay (BGP IRMA, Mitsubishi Kagaku, Tokyo, Japan). Urinary cross-linked N-teleopeptides of type-I collagen was measured by an enzyme-linked immunosorbent assay (Osteomark, Ostex International, Inc., Seattle, WA). Deoxyypyridinoline was measured by an enzyme-linked immunosorbent assay (Pyrlinks-D, Metra Biosystems, Palo Alto, CA). The urinary biomarkers were adjusted for creatinine (Cr) excretion and given as per mmol Cr. The coefficients of variation for these assays ranged from 5.0 percent to 8.0 percent. All biochemical markers of bone metabolism were analyzed by Mitsubishi Bio-Clinical Laboratories Inc. (Tokyo, Japan). Other blood and urine assays were analyzed by use of Clinical Analyzers (Model 7450 and 7070, respectively, Hitachi, Tokyo, Japan).

Statistical analysis. The results for serum and uri-

nary bone biomarkers were analyzed by repeated-measures analysis of variance adjusted with degrees of freedom by Huynh and Fedt.¹⁰ Regression analysis was used to examine the relationship between the BMD at baseline and after six months in the placebo and MBP groups, respectively. If the significant correlation was observed, the BMD after six months as a response variable was predicted from the baseline BMD as a function of regressor variables by each regression line. With the both intercepts of regression lines were estimated as zero, the rate of change in BMD during six months was calculated and comparisons between the study groups were made with two-sample *t*-test. When the intercept was not zero, the mean slopes were compared by analysis of covariance with baseline BMD as a covariate. Dietary records were analyzed by the Mann-Whitney U test. Other results were compared with the use of *t*-tests. Correlation coefficients between gain of BMD and dietary intake of minerals or vitamins were also calculated. All calculations were done using the GLM procedure in the SAS statistical analysis package.¹¹ All tests were two-tailed.

Results

Baseline characteristics of subjects.

The baseline clinical characteristics of the women are shown in Table 1. Overall, there was no significant difference between the MBP and placebo groups in any of the parameters of age, weight, height, body mass index, or BMD.

Bone mineral density (BMD)

The initial mean values for BMD at left calcaneus were similar in the two groups (Table 1). The changes in left calcaneus BMD during the study are shown in Fig. 1. Repeated-measurement analysis showed that BMD increased in both groups ($P < 0.0001$), but the increase was significantly greater in the MBP group than in the placebo group ($P = 0.0496$). Regression analysis showed that relationship between BMD at baseline and six months was linear and its intercept was zero in both groups, therefore the effect of MBP on the BMD was estimated as the percentage of gain of BMD after six months of treatment. As shown in Fig. 2, the mean (\pm SD) gain of left calcaneus BMD was significantly higher in the MBP group ($3.42 \pm 2.05\%$) than in the placebo group ($2.01 \pm 1.75\%$, $P = 0.042$).

Biochemistry

Biochemical indices of bone metabolism in the two groups were similar at baseline. Serum bone specific alkaline phosphatase and osteocalcin concentrations, bone formation markers, in both groups changed during the study period, $P = 0.0207$ and $P = 0.0001$, respectively, but no difference between the groups

Table 1. Baseline Clinical Characteristics of Healthy Adult Women Given Placebo or MBP Supplementation for Six Months*

Characteristic	Placebo Group	MBP Group
No. of subjects	16	17
Age (yr)	27 \pm 8	30 \pm 9
Weight (kg)	50 \pm 4	51 \pm 6
Height (m)	1.58 \pm 0.04	1.58 \pm 0.05
Body mass index [†]	20.0 \pm 2.0	20.5 \pm 2.4
Bone mineral density (g/cm ²)	0.84 \pm 0.07	0.83 \pm 0.09

* Values are means \pm SD. There were no significant differences between the groups.

[†] The weight in kilograms divided by the square of the height in meters.

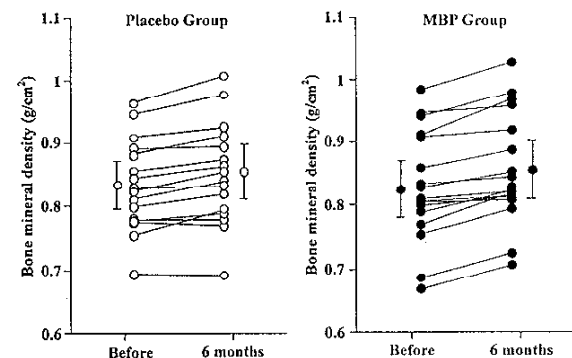


Fig. 1. Change in the Bone Mineral Density of Each Subject and Mean \pm 95% Confidence Intervals Given Placebo (○) or MBP Supplementation (●) for Six Months.

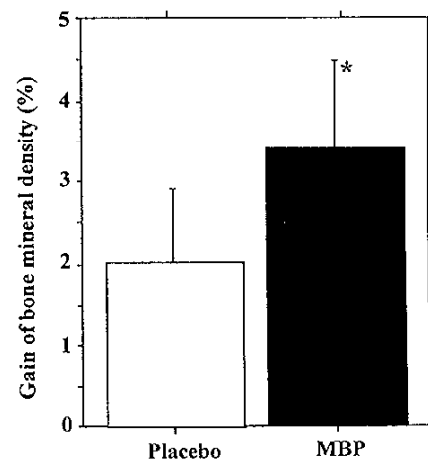


Fig. 2. Gain of Bone Mineral Density in Healthy Adult Women Given Placebo or MBP Supplementation for Six Months.

Error bars represent 95% confidence intervals. Significant differences between the groups are indicated by asterisks ($P < 0.05$).

was observed (Fig. 3). On the other hand, the mean urinary cross-linked N-telopeptides of type-I collagen excretion, a bone resorption marker, was lower in the MBP group than in the placebo group at both three and six months ($P = 0.0074$ and $P = 0.0244$,

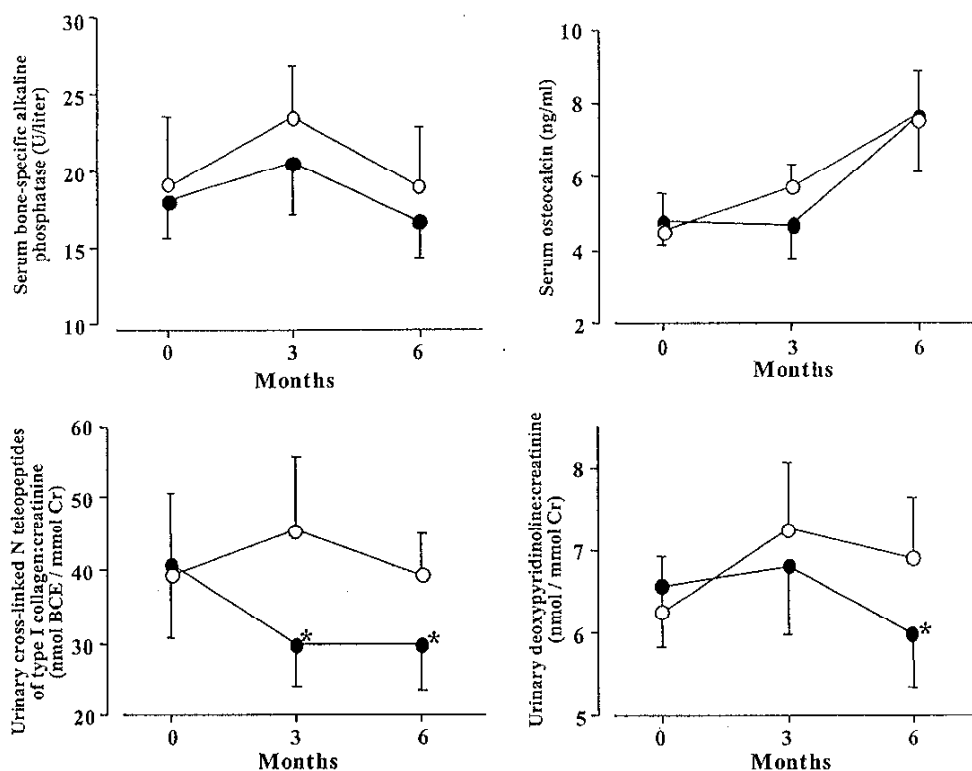


Fig. 3. Mean (\pm 95% Confidence Intervals) Biochemical Indexes of Bone Metabolism in Healthy Adult Women Given Placebo (\circ) or MBP Supplementation (\bullet) for Six Months.

Significant differences between the groups are indicated by asterisks ($P < 0.05$).

Table 2. Dietary Intake of Minerals and Vitamins related to Bone Metabolism in Healthy Adult Women During Supplementation Periods*

Characteristic	Placebo Group		MBP Group	
	3 Months (Autumn)	6 Months (Winter)	3 Months (Autumn)	6 Months (Winter)
Total Energy (kcal/day)	1808 (1264-2403)	1729 (935-2683)	1889 (1189-2991)	1815 (1325-2932)
Calcium (mg/day)	564 (346-1114)	542 (285-1107)	627 (293-1765)	544 (289-1334)
Phosphorus (mg/day)	941 (664-1552)	919 (615-1512)	1079 (662-1773)	1003 (541-1745)
Magnesium (mg/day)	214 (158-310)	197 (126-350)	224 (141-494)	217 (132-497)
Vitamin D (IU/day)	169 (41-459)	88 (23-424)	192 (16-547)	124 (6-559)
Vitamin K (μ g/day)	336 (32-1279)	290 (62-949)	290 (53-1285)	316 (49-1589)
Vitamin C (mg/day)	92 (32-202)	79 (37-190)	90 (45-250)	87 (35-308)

* Values are median and minimum-maximum values are shown in parentheses. There were no significant differences between the groups.

respectively). The mean urinary deoxypyridinoline excretion, another bone resorption marker, was also lower in the MBP group than in the placebo group at six months ($P = 0.0494$). Other biochemical results were normal and did not change in the two groups throughout the study period (data not shown).

Dietary minerals and vitamins intake

The median (minimum-maximum) dietary intake of calcium, phosphorus, magnesium, and vitamins D, K, and C is shown in Table 2. There were wide individual differences in the dietary intake. There was no significant difference in calcium, phosphorus,

magnesium, vitamin D, vitamin K and vitamin C between the groups by the Mann-Whitney U test.

Correlation coefficients between gain of BMD and dietary intake of minerals and vitamins are shown in Table 3. There was no significant correlation between gain of BMD and intake of any dietary minerals or vitamins in the placebo and MBP groups. These data suggest that a significant increase in BMD in the MBP group is independent of dietary intake of minerals (calcium, phosphorus, and magnesium) and vitamins (vitamins D, K, and C).

Table 3. Correlation Matrixes between Gain of Bone Mineral Density and Dietary Intake of Minerals and Vitamins Related to Bone Metabolism in Healthy Adult Women*

BMD gain vs.	Ca	P	Mg	V.D	V.K	V.C
<i>Autumn</i>						
Placebo	-0.259	-0.195	-0.169	-0.161	-0.492	-0.229
MBP	-0.259	-0.334	-0.266	-0.263	-0.259	-0.388
<i>Winter</i>						
Placebo	-0.012	-0.123	0.145	0.298	-0.250	-0.332
MBP	-0.038	-0.072	-0.196	0.218	-0.328	-0.402

* No significant correlation coefficient was observed.

Discussion

It is reported as the age to attain peak bone mass for the women in the thirties.¹²⁾ It is supposable that the observed increase of BMD in the placebo group was within an age-related increase. On the other hand, these results suggested that the MBP had the independent effect from the age-related increase on the BMD gain. We found that MBP could be one of the nutritional components that increase peak bone mass and reduce the future risk of osteoporosis for premenopausal women. The yield of the MBP from whey protein showed that approximately 400 to 800 ml of milk is equivalent to the 40 mg dose of the MBP. Although many long-term studies demonstrated that calcium supplementation is effective to prevent bone loss,¹³⁻¹⁵⁾ our study, for the first time, provided evidence of a direct effect of MBP on bone metabolism in healthy adult women.

Biochemical parameters in serum and urine are being used clinically to assess the rate of bone formation and resorption. It is reported that serum bone ALP, osteocalcin, and urinary deoxypyridinoline were more sensitive indicators of skeletal health.¹⁶⁾ Woitge *et al.* reported that the seasonal variability was most pronounced for serum bone-specific alkaline phosphatase as well as serum osteocalcin.¹⁷⁾ Douglas *et al.* also reported that bone-specific alkaline phosphatase activities in serum were higher in autumn than in spring.¹⁸⁾ It is therefore possible that the significant change of bone formation markers in both groups observed in this study might be at least partially attributable to seasonal variations. On the other hand, urinary cross-linked N-teleopeptides of type-I collagen and deoxypyridinoline excretion after six months of MBP supplementation were lower than at baseline, indicating that MBP supplementation led to a reduction in the rate of bone resorption. Previously, we reported that MBP clearly reduced urinary excretion level of deoxypyridinoline by directly suppresses osteoclast-mediated bone resorption in aged ovariectomized rats.⁷⁾ This result in animal study is consistent with that in this human study.

These findings are also consistent with our current knowledge of responses of osteoclasts studied *in*

vitro.^{4,7)} In our preliminary study, cystatin purified from the MBP suppressed osteoclast-mediated bone resorption (data not shown). It is also reported that recombinant cystatin C inhibits bone resorption *in vitro*.^{19,20)} Thus, it is speculated that one of the active components related to bone resorption in the MBP is milk cystatin. We also demonstrated that the active components, responsible for suppression of bone resorption, kept biological activity after gastro-intestinal digestion and can be absorbed through the intestines by everted gut-sac method.⁴⁾ Thus the active components in the MBP or partially digested MBP might be absorbed through the intestine and inhibit bone resorption directly by possible physiological process.

MBP also contains active components to promote cell proliferation and collagen synthesis of osteoblasts.³⁾ Two components that have growth-promoting activity in the MBP were characterized in our recent reports. One was a high mobility group-like protein.²¹⁾ The other was a kininogen fragment.²²⁾ In this study, however, serum bone-specific alkaline phosphatase and osteocalcin concentrations, bone formation markers, were not different between the placebo and MBP groups. We could not find the effect of MBP on bone formation clearly from bone formation markers.

Our previous *in vivo* study showed that the whey protein did not affect calcium balance in rats.^{5,6)} No significant correlation between gain of BMD and dietary intake of any minerals or vitamins was detected in this study. Therefore the gain of the left calcaneus BMD by the MBP supplementation was independent of dietary intake of minerals and vitamins.

In conclusion, we report that milk basic protein supplementation increased bone mineral density in healthy adult women primarily by inhibition of bone resorption. From this study, it appears that 40 mg/day of MBP supplementation could be effective for bone metabolism.

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