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Abstract Milk has more beneficial effects on bone health than other food sources. Recent in vitro and in vivo studies have shown that milk whey protein, especially its basic protein fraction (milk basic protein, MBP), contains several components capable of promoting bone formation and inhibiting bone resorption. The object of this study was to examine the effect of MBP on the bone metabolism of healthy menopausal women. Thirty-two healthy menopausal women were randomly assigned to treatment with either placebo or MBP (40 mg per day) for 6 months. The bone mineral density (BMD) of the lumbar vertebrae L2-L4 of each subject was measured by dual-energy X-ray absorptiometry (DXA) at 0 and 6 months of treatment. Serum and urine indices of bone metabolism were measured at 0, 3 and 6 months. Twenty-seven subjects who completed the study in accordance with the protocol were included in the analysis. The mean rate of gain of lumbar BMD in the MBP group (1.21%) was significantly higher than in the placebo group (−0.66%, $P = 0.046$). When compared with the placebo group, urinary cross-linked N-telopeptides of type-I collagen (NTx) were significantly decreased in the MBP group at 6 months, but no significant difference in serum osteocalcin was observed between the two groups. The urinary NTx excretion was found to be related to serum osteocalcin in the MBP group at 3 and 6 months, indicating that MBP maintained the balance of bone remodeling. These results suggested that MBP supplementation was effective in preventing bone loss in menopausal women and that this improvement in BMD may be primarily mediated through the inhibition of bone resorption while maintaining the balance of bone remodeling by MBP supplementation.

Keywords Bone mineral density · Healthy adult women · Menopause · Milk basic protein

S. Aoe
Department of Home Economics, Otsuma Women's University, 12 Sanban-cho Chiyoda-ku, 102-8357 Tokyo, Japan

T. Koyama
Koyama Takao Clinic, 6-14-2 Ginza, Chuo-ku, 140-0061 Tokyo, Japan

Y. Toba (✉)
Functional Products Business Development, Snow Brand Milk Products Co. Ltd., 13 Honshio-cho Shinjuku-ku, 160-8575 Tokyo, Japan
E-mail: y-toba@snowbrand.co.jp
Tel.: +81-3-32262391
Fax: +81-3-32262123

A. Itabashi
Central Clinical Laboratory, Saitama Medical School, 38 Morohongo Moroyama Iruma, 350-0495 Saitama, Japan

Y. Takada
Product Planning Department, Snow Brand Milk Products Co. Ltd., 1-1-2 Minamidai Kawagoe-shi, Saitama, Japan

Introduction

Osteoporosis is characterized by enhanced bone fragility, resulting in an increased risk of fracture, and is usually defined as a reduction in bone mineral density (BMD) [1]. In elderly women, bone resorption is markedly increased, with a rapid decrease in bone mass. The rate of bone loss is high during menopause and increases the risk of osteoporosis and fracture. Optimal management of osteoporosis consists of maximizing peak bone mass in early adulthood and preventing rapid bone loss at menopause [2]. Bone formation and resorption are continuous processes that maintain the integrity of bone tissue. Bone tissue consists of a wide variety of cells of bone-forming and bone-resorbing cell lineage. Osteoblasts and osteoclasts are especially important for bone remodeling. While bone formation and bone resorption are balanced in young women, bone resorption exceeds bone formation with the onset of menopause and with aging. Internationally, the agents most widely used for the treatment of postmenopausal osteoporosis are calcium, estrogen, calcitonin, fluoride, calcitriol and

bisphosphonate. In general, these agents can be divided into two categories according to whether they stimulate bone formation or inhibit bone resorption [3]. Agents with the dual effects of increasing bone formation and suppressing bone resorption are desirable to improve unbalanced bone metabolism. Bone remodeling by formation and resorption is a continuous process. Nutrition can be an important factor because the administration of dietary components that contain several factors related to bone metabolism might affect both bone formation and resorption and is relatively safe and inexpensive. In the near future, fortification of some nutrients may improve bone health.

Historically, cow's milk has been widely consumed because of its excellent nutritional value. In particular, milk is a good source of bioavailable calcium compared with other food sources. Recent studies have demonstrated that milk whey protein plays a functional role in bone remodeling [4, 5]. In these reports, the active components responsible for promotion of bone formation and suppression of bone resorption were found to be in its basic protein fraction (milk basic protein, MBP). Our in vivo study showed that milk whey protein and fractionated whey protein enhanced femoral bone strength in young ovariectomized rats [6, 7]. We also showed that MBP prevented bone loss in aged ovariectomized rats, used as a model of osteoporosis [8]. Because MBP clearly reduced urinary excretion of deoxypyridinoline (a biochemical marker of bone resorption) in the animal study, we hypothesized that MBP suppressed osteoclast-mediated bone resorption [8].

We tried to examine the effect of the daily intake of MBP on BMD and bone metabolism in healthy humans [9, 10, 11]. MBP suppressed bone resorption and increased BMD in human studies. From the standpoint of the prevention of osteoporosis, we examined the effect of MBP on lumbar BMD and biochemical markers of bone metabolism in healthy adult women at the onset of menopause.

Materials and methods

Subjects

We invited applications from women 40 years of age and older (generally considered to be the menopausal age group) through direct mailings and from women attending presentations about this study. Thirty-two healthy women at the onset of menopause [mean age (±SD), 50.5±3.0 years] were recruited. Written informed consent was obtained from each subject. The protocol was approved by the ethical committee of the participating institution. All subjects had a moderate level of physical activity—they did clerical work, operated machines, met people and did housework. Exclusion criteria included heart disease, renal disease, liver disease, osteoporosis, hormone replacement therapy and other medications known to affect bone metabolism. Women were also excluded if they could not drink the test beverage for more than 4 consecutive days, or for more than 10 days per month, or did not record their food intake.

Study design and supplements

In this 6-month double-blind, placebo-controlled trial, the volunteers were randomly assigned at a ratio of 1:1 to either the placebo or the MBP group with stratification based on their body weight, height, body mass index and BMD, according to a computer-generated list of random numbers. Seventeen women received the experimental beverage containing 40 mg of MBP, and the other 15 women received a matching placebo beverage. Each beverage contained lactic acid, sweetener and flavoring as masking ingredients in 50 ml of water. MBP was prepared from fresh skimmed milk. This was loaded onto a column that had been packed with cation exchange resin, sulfonated chitopearl (Fuji-bouseki, Tokyo, Japan). The column was washed with deionized water and the bound proteins were eluted with 1 M sodium chloride. MBP was obtained by freeze-drying after dialysis of the eluted fraction in a cellulose membrane tube (Sanko-juntaku, Tokyo, Japan). Protein concentration of the MBP was 98%, and the MBP fraction contained several 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 to avoid taking supplemental minerals and vitamins throughout the 6 months of the study. Each woman came to the clinic every 3 months for evaluation. At each 3-month visit, urine and blood measurements were performed for each subject. At baseline and at the 6-month evaluation, they were also measured for lumbar BMD (L2-L4). During the study period, a prospective standardized 3-day food record was completed by each subject at 3 and 6 months. The nutrient content of their diet was quantified using a computer program based on the Standard Tables of Food Composition [12].

Status of subjects and compliance

During the 6-month study period, two women in the placebo group and three women in the MBP group dropped out of the study because they did not drink the beverage for several days or did not keep a food record. No bloating, diarrhea or allergies were reported in either group. We conducted a per protocol analysis of the 27 subjects who completed the study according to the protocol; this was not an intention to treat analysis.

Analytic methods

Bone mineral density (BMD) of the lumbar spine (L2-L4) was measured by dual-energy X-ray absorptiometry

using a DPX-NT scanner (Lunar, Madison, Wis.). The coefficients of variation for the measurements were 2.0%. A phantom consisting of bone ash embedded in a 12-cm block was scanned every day as a control; the BMD of the phantom remained unchanged throughout the study.

Blood was drawn after the subjects had fasted for at least 8 h. A second spontaneous urine sample was collected before breakfast. Aliquots of samples were frozen at –20°C until analysis. Osteocalcin was measured by an immunoradiometric assay (BGP IRMA, Mitsubishi Kagaku, Tokyo, Japan). Urinary cross-linked N-teleopeptides of type-I collagen (NTx) were measured by an enzyme-linked immunosorbent assay (Osteomark, Ostex International, Inc., Seattle, Wash.). The urinary biomarkers were adjusted for creatinine (Cr) excretion and expressed as per mmol Cr. The coefficients of variation for these assays ranged from 5.0 to 8.0%. All biochemical markers of bone metabolism were analyzed by Mitsubishi BML Inc. (Tokyo, Japan). Other blood and urine assays were analyzed using clinical analyzers.

Statistical analysis

Comparisons of baseline values between the study groups were made with the two-sample *t*-test. Regression analysis was used to examine the relationship of the BMD and serum and urinary bone biomarkers at baseline and after 3 or 6 months in the placebo and MBP groups. If no significant correlation was observed, a comparison between the study groups was made by a two-sample *t*-test at each period. If a significant correlation was observed, the value after 3 or 6 months as a response variable was predicted from the baseline value as a function of regressor variables by each regression line. With both intercepts of regression lines estimated as zero, the rate of change in value during 3 or 6 months was calculated, comparisons between the study groups were made with a two-sample *t*-test, and the adjusted value after 3 or 6 months was calculated with the baseline value as a covariate. When the intercept was not zero, the mean slopes were compared by analysis of covariance with the baseline value as a covariate. The results for serum and urinary bone biomarkers were also analyzed by repeated-measures analysis of variance (ANOVA) adjusted with degrees of freedom by Huynh and Fedlt [13]. Dietary records were analyzed by the Mann-Whitney test. Correlation coefficients between bone biomarkers and between gain of BMD and dietary intake of minerals or vitamins were also calculated. All calculations were performed using the GLM procedure in the SAS statistical analysis package [14]. 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 for any of the parameters of age, weight, height, body mass index or lumbar spine BMD.

Bone mineral density (BMD)

The initial mean values for lumbar BMD were similar in the two groups (Table 1). The changes in lumbar BMD during the study are shown in Table 2. The effect of MBP on BMD was estimated as the percentage of gain of BMD after 6 months of treatment. As shown in Fig. 1, the mean gain of lumbar BMD was significantly higher in the MBP group (1.21%) than in the placebo group (–0.66%, $P=0.046$).

Biochemistry

Biochemical indices of bone metabolism in the two groups are shown in Table 2. Biochemical indices of bone metabolism in the two groups were similar at baseline. No significant difference between the two groups was observed in serum osteocalcin, a bone formation marker, by repeated-measures analysis and covariance analysis with the baseline value as a covariate. However, repeated measure analysis showed that time-dependent change in urinary NTx excretion, a bone resorption marker, differed significantly between the two groups ($P=0.030$), and the mean was lower in the MBP group than in the placebo group at 6 months ($P=0.002$) by covariance analysis with the baseline value as a covariate. The relationship between serum osteocalcin concentration and urinary NTx excretion is shown in Table 2. Urinary NTx excretion was not found to be related to serum osteocalcin concentration in the placebo group at the start of the study, at 3 or at 6 months. The correlation coefficients between serum osteocalcin concentration and urinary NTx excretion in the placebo group at the start of the study, at 3 and 6 months were 0.436 ($P=0.137$), 0.447 ($P=0.125$) and 0.447 ($P=0.126$), respectively. The coefficient in the MBP group at the start of the study was 0.403 ($P=0.153$). Urinary NTx excretion was found to be related to serum osteocalcin in the MBP group at 3 and 6 months, with

Table 1 Baseline clinical characteristics of menopausal women given a placebo or MBP supplementation for 6 months*

Characteristic	Placebo	MBP
No. of subjects	13	14
Age (years)	51 ± 3	50 ± 3
Weight (g)	53 ± 6	53 ± 7
Height (m)	1.58 ± 0.57	1.55 ± 0.43
Body mass index**	21.4 ± 3.2	21.7 ± 2.6
Lumber (L2–4) spine BMD (g/cm^2)	1.07 ± 0.11	1.12 ± 0.09

*Values are means ± SD. There were no significant differences between the groups. **The weight in kilograms divided by the square of the height in meters

Table 2 The lumber L2-L4 BMD, biochemical indexes of bone metabolism (serum osteocalcin concentration and urinary NTx excretion) and relationship between osteocalcin and NTx in menopausal women during supplementation periods

	Placebo			MBP		
	Initial	3 months	6 months	Initial	3 months	6 months
L2–L4 BMD (g/cm^2)[1]	1.07 ± 0.11	–	1.09 ± 0.03	1.12 ± 0.09	–	1.11 ± 0.03
Serum osteocalcin (ng/day)[1]	6.09 ± 1.49	5.98 ± 0.37	5.82 ± 0.59	5.79 ± 1.18	5.96 ± 0.37	5.73 ± 0.59
Urinary NTx (nmol/mmol Cr)[1,§]	59.2 ± 17.9	59.2 ± 9.8	58.7 ± 8.3	56.4 ± 15.1	57.5 ± 9.8	47.3 ± 8.3*
Correlation coefficients between osteocalcin and NTx[2]	0.436	0.447	0.447	0.403	0.538**	0.739**

[1]Values are means ± SD at the initial or the adjusted means that was calculated with the initial value as a covariate ± SD at 3 or 6 months. *Indicates the significant differences between the groups within the same period ($P < 0.05$). §Indicates the significant differences by repeated-measures analysis ($P < 0.05$). [2]Values are the correlation coefficients between the serum osteocalcin concentration and the urinary NTx excretion. **Indicates the significant correlations ($P < 0.05$)

correlation coefficients of 0.538 ($P = 0.047$) and 0.739 ($P = 0.003$), respectively. 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 mean dietary intakes of energy, carbohydrate, protein, fat, calcium, phosphorus, magnesium, vitamin D, K and C are shown in Table 3. There was no significant difference in calcium, phosphorus, magnesium, vitamin D, vitamin K or vitamin C between the groups, except that the means of energy and carbohydrate in the MBP group at 3 months were temporarily and significantly lower than those in the placebo group ($P = 0.028$ and 0.024, respectively). There was no significant correlation between the gain of BMD and intake of any dietary minerals or vitamins in the placebo or MBP groups (data not shown).

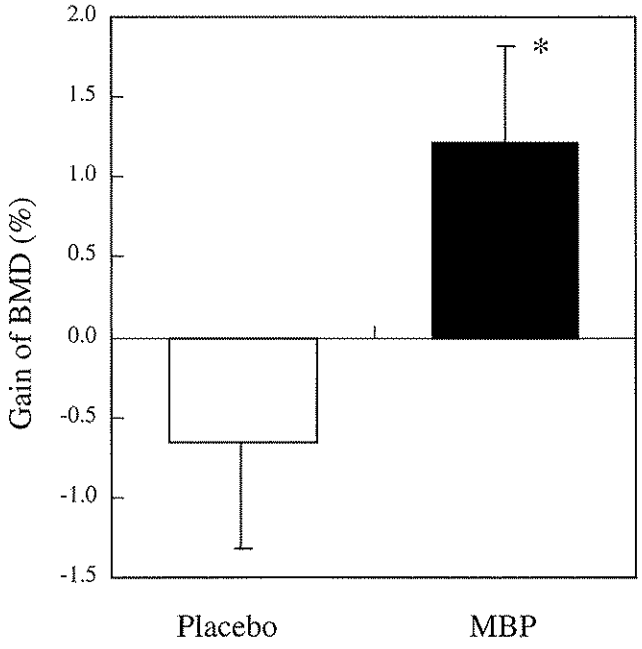

Fig. 1 BMD gain of lumber L2-L4 in healthy menopausal women given placebo or MBP supplementation for 6 months. *Error bars* represent SE. *Indicates the significant differences between the groups ($P < 0.05$)

Discussion

Women attain peak bone mass in their 30s [15]. When women are in their 50s, their bone mass decreases, particularly at the onset of menopause, and the rate of bone loss increases. In this study, we investigated the effect of MBP on healthy women at the onset of menopause. To prevent bone loss in menopausal women, it is important to maintain the bone mass as early as possible because bone loss due to estrogen deficiency occurs rapidly, and it is difficult to maintain BMD during the menopause. Although many long-term studies have demonstrated that calcium supplementation is effective in preventing bone loss [16, 17, 18], our study, for the first time provided evidence of the effect of MBP on bone metabolism in healthy adult menopausal women.

We have previously examined the effect of daily intake of MBP on bone mineral density (BMD) and bone metabolism in young healthy women [mean age (± SD), 28.8 ± 8.7]. The gain of calcaneus BMD in the MBP group was significantly higher than that in the placebo group, and the radial BMD value in the MBP group also increased significantly at 1/6 and 1/10 from the distal end of the radius [9, 11]. The present results for increase of BMD are consistent with this earlier study. In our previous animal study [8], we performed histological examination of the proximal tibia in ovariectomized rats. The results indicated that MBP reduced the loss of trabecular bone around the growth plate-metaphyseal junction in the proximal tibia. These results indicate that MBP is helpful in preventing trabecular bone loss. In this study, we measured the lumbar spine, which is one of the major measurement sites for osteoporosis, because we had targeted healthy menopausal women. Measurement of the femoral neck, which is one of the major fracture risk sites, will be performed when a study is conducted in older menopausal women.

Table 3 Dietary intake of minerals and vitamins related to bone metabolism in menopausal women during supplementation periods

	Placebo			MBP		
	Initial	3 months	6 months	Initial	3 months	6 months
Energy (kcal/day)	1,899 ± 315	1,888 ± 320	1,919 ± 398	1,818 ± 274	1,653 ± 190*	1,775 ± 249
Carbohydrate (g/day)	245 ± 53	243 ± 51	253 ± 44	229 ± 31	207 ± 24*	237 ± 41
Protein (g/day)	70 ± 10	73 ± 10	66 ± 11	69 ± 12	66 ± 9	67 ± 11
Fat (g/day)	66 ± 14	63 ± 13	64 ± 21	62 ± 17	56 ± 13	57 ± 14
Calcium (mg/day)	549 ± 169	504 ± 141	477 ± 164	529 ± 239	528 ± 208	505 ± 207
Phosphorus (mg/day)	1,058 ± 191	1,058 ± 167	986 ± 179	1,033 ± 258	1,029 ± 181	1,022 ± 189
Magnesium (mg/day)	266 ± 50	251 ± 54	241 ± 31	264 ± 51	254 ± 53	255 ± 54
Vitamin D (IU/day)	211 ± 116	385 ± 267	291 ± 212	339 ± 293	236 ± 183	280 ± 169
Vitamin K (μg/day)	307 ± 185	229 ± 126	190 ± 135	276 ± 137	294 ± 261	267 ± 239
Vitamin C (mg/day)	109 ± 45	94 ± 40	107 ± 68	94 ± 31	86 ± 30	74 ± 29

Values are means ± SD. *Indicates the significant differences between the groups within the same period ($P < 0.05$)

Biochemical parameters in serum and urine are used clinically to assess the rate of bone formation and resorption. MBP contains active components to promote cell proliferation and collagen synthesis by osteoblasts [4]. We found two components with growth-promoting activity—one was a high mobility group-like protein [19] and the other was a kininogen fragment [20]. We also reported that MBP reduced urinary excretion levels of deoxypyridinoline by directly suppressing osteoclast-mediated bone resorption in aged ovariectomized rats [8]. These findings are consistent with our current knowledge of responses of osteoclasts studied in vitro [6, 8]. Cystatin purified from MBP suppressed osteoclast-mediated bone resorption [21]. It is also reported that recombinant cystatin C inhibits bone resorption in vitro [22, 23]. Thus, we speculate that milk cystatin is one of the active components of MBP related to bone resorption. We have also demonstrated that the active components responsible for suppression of bone resorption retain biological activity after gastrointestinal digestion and can be absorbed through the intestines by the everted gut-sac method [5]. Thus, the active components in MBP or partially digested MBP may be absorbed through the intestine and inhibit bone resorption directly by a physiological process. In the present study, there was no significant difference between the groups in serum osteocalcin concentrations, a bone formation marker. However, urinary cross-linked N-telopeptides of type I collagen, a bone resorption marker, were lower after 6 months of MBP supplementation 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 of deoxypyridinoline by directly suppressing osteoclast-mediated bone resorption in aged ovariectomized rats [8]. We also reported that MBP reduced urinary cross-linked N-telopeptides of type I collagen and deoxypyridinoline in healthy adult women [9]. These previous results are consistent with the present study of human menopause. In the present study, we have chosen urinary NTx as a bone resorption marker because NTx is reportedly more sensitive than deoxypyridinoline to a change in bone metabolism [24]. In our previous study, we also found that NTx was more sensitive than deoxypyridinoline to MBP supplementation [9]. On the other hand, we could not find a clear effect of MBP on bone formation judging from the data on osteocalcin. We did not find such an effect in our previous human study [9], although we found that MBP promoted bone formation in the in vitro study [4] and in the other human study [10] in which more MBP was ingested. As mentioned above, the effect of MBP on bone formation remains unclear. It is possible that MBP has stronger effects on bone resorption than on bone formation, that more MBP is needed to influence bone formation, and/or that further long-term ingestion of MBP is required to affect bone formation at the onset of menopause. Further studies are needed to determine the effect of MBP on bone formation. The yield of MBP from skimmed milk showed that approximately 800 ml of milk is equivalent to the 40 mg of MBP—40 mg of MBP contains at least 20 μg of cystatin. MBP is itself complex: it is a polyvalent fraction containing many factors other than milk cystatin. Its effect on bone health is likely to be more than can be accounted for by any single constituent, and the totality of MBP's effect may be more than the sum of the parts.

Efforts to treat bone diseases have primarily concentrated on the development of drugs to block bone resorption that decrease the formation or activity of osteoclasts. To prevent bone diseases in healthy menopausal women, it is probably unwise to strongly block bone resorption because this will unbalance bone remodeling. It is important to investigate whether MBP actually causes a loss of balance in bone remodeling because it has a suppressive effect on bone resorption. In the present study, the urinary NTx excretion (a biochemical marker of bone resorption) was not found to be related to the serum osteocalcin concentration (a biochemical marker of bone formation) in the placebo group at the start of the study, at 3 and 6 months. The correlation coefficient between urinary NTx excretion and serum osteocalcin concentration in the MBP group increased toward 1.0 with time, and the urinary NTx

excretion was found to be related to the serum osteocalcin concentration in the MBP group at 3 and 6 months. These results indicated that subjects who had a higher bone resorption activity also had a higher bone formation in the MBP group at 3 and 6 months. We consider that MBP maintains the balance of bone remodeling. This phenomenon also suggests that, while MBP suppressed bone resorption, it did not block bone resorption by remodeling. In our previous study, we found that urinary NTx excretion was related to serum osteocalcin concentration in healthy adult men after 16 days of ingesting 300 mg of MBP per day [10]. Our present study, where healthy menopausal women ingested 40 mg of MBP, was consistent with our previous study.

Our previous in vivo study showed that whey protein did not affect the calcium balance in rats [6, 7]. No significant correlation between the gain of BMD and dietary intake of any minerals or vitamins was detected in this study. Therefore, the gain of lumbar BMD with MBP supplementation was independent of the dietary intake of minerals and vitamins.

In conclusion, we report that MBP supplementation increased bone mineral density in healthy adult menopausal women, primarily by the inhibition of bone resorption, while maintaining the balance of bone remodeling. From this study, it appears that 40 mg/day of MBP supplementation could be effective for improving bone metabolism in the early stages of menopause. Further long-term and large-scale studies are needed to confirm the positive effects of MBP supplementation on the bone health of menopausal women. It may be useful to examine the effect of MBP supplementation in older postmenopausal women, as well as during the early menopause.

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