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Review

Biological Significance of Milk Basic Protein (MBP) for Bone Health

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The social phenomenon of the growing number of elderly patients with bone disease or fractures attributable to osteoporosis is closely associated with an increase in the number of bedridden elderly, and is a causative factor in the rise of the total cost of national health care. We hypothesized that the excellent effects of cow's milk on bone health might not be limited to an abundance of calcium, and investigated substances in milk that regulate bone remodeling to enhance bone mineral density. We successfully identified the "milk basic protein" (MBP) component of milk, which has a direct effect on the strengthening on bones. MBP stimulated proliferation of osteoblasts involved in bone formation and collagen production and also suppressed the activity of osteoclasts involved in bone resorption. The supplementation of some daily foods with MBP has made it possible to maintain properly balanced bone remodeling and to increase bone mineral density in human volunteers. MBP is expected to be a functional component in helping to maintain human bone health.

Keywords: milk basic protein, MBP, bone mineral density, bone formation, bone remodeling, osteoporosis, FOSHU

I. Introduction: Mystery of Milk

We have long been working to fathom the mysteries of milk, focusing on cow's milk, which is essential to keep people healthy, and mother's milk, which is the only nutrient able to support a baby's development. Animal (mammalian) babies can grow very fast on mother's milk alone during the several months after birth. While animals feed on various foods, milk is the only nutrient in the world that must be ingested. This means that milk contains components that are critical and ideal for human life. Interestingly, human milk is known to contain more whey than cow's milk. This indicates a natural law by which the components in whey are vital to human beings. Whey contains not only nutritionally important components but also various physiologically active substances (functional components). Milk basic protein (MBP), which we discuss here, is one of these active substances.

II. Bone Health and Milk

In our aging society, the number of elderly bedridden people due to lifestyle-related diseases who need nursing care is rapidly growing and is becoming a social problem (Ray *et al.,* 1997; Looker *et al.,* 1995; Hui *et al.,* 1988). In a survey on the causes of the requirements for nursing care, bone disease from fractures and falls were reported to be placed third, after cerebral stroke and senility. Japanese patients with osteoporosis numbered over 10 million in 2000, and were estimated to reach 16 million if potential patients who had just begun to lose bone mass were included (Sone and Fukunaga, 2004). Moreover, patients with osteoporosis are now estimated to total 200 million worldwide (Lin and Lane, 2004). As mentioned above, an issue common to aging societies, including not only Japan but also Western countries, is to decrease the number of osteoporotic patients by improving eating habits. At present, women account for 80% of patients with osteoporosis, but men are equally at risk when they are over 80 years of age (Peate, 2004; Ebeling, 1998). Osteoporosis is attributed to aging, but its occurrence can be delayed by changing dietary habits (Wei *et al.,* 2003). In particular, daily intake of calcium is critical in suppressing the development of the disease (Dawson-Hughes, 1999; McKane *et al.,* 1996), although the current daily calcium intake in Japan is not sufficient to reach the level of the Recommended Dietary Allowances for Japanese. In addition, it has also become an issue that young women lose bone mineral density because of excessive and imbalanced dieting (Flynn, 2003). In our society, where the population is aging rapidly, it is presumed that bone-related diseases will further increase, suggesting that, from the viewpoint of dietary life, it is more important to pay attention to bone health in peoples' earlier years so as to maintain a healthy and active daily life (Silverman *et al.,* 2001; Prince, 1997).

Bone tissue, which appears similar to a lump of calcium, has an internal network of vessels and, similar to other organs, is actively remodeled (Bryant *et al.,* 2002; Pittenger *et al.,* 1999). Present inside the bones are osteoblasts, osteoclasts, and osteocytes, which mutually interact to control bone remodeling (Boyle *et al.,* 2003; Erlebacher *et al.,* 1995; Aarden *et al.,* 1994). Namely, bone formation by osteoblasts (Fig.1), which produce the bone

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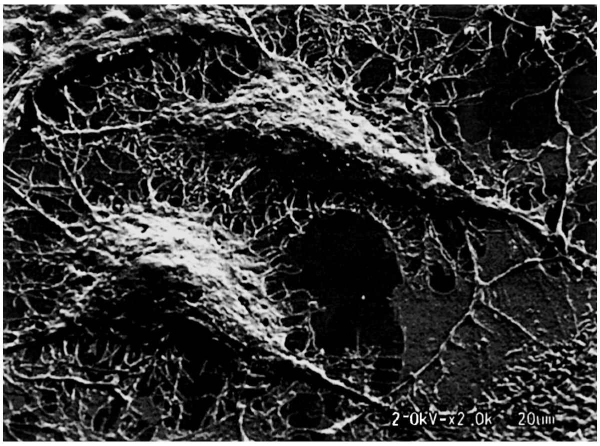

Fig. 1. Scanning electron micrograph of osteoblast.

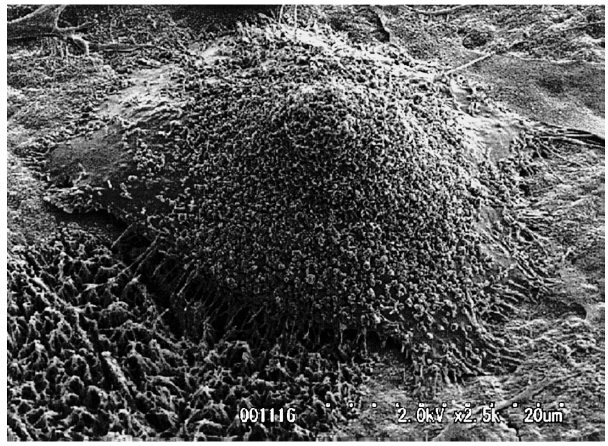

Fig. 2. Scanning electron micrograph of osteoclast.

matrix protein collagen to adsorb bone minerals such as calcium, and bone resorption by osteoclasts (Fig. 2), which dissolve collagen and calcium, typically occur in balance. It is an imbalance between bone formation and resorption that results in osteoporosis (Matsumoto, 2004).

It is well known that cow's milk is useful in keeping bones healthy, and the reason is because it contains high levels of calcium, a nutrient essential for bone formation (Kalkwarf *et al.,* 2003; Teegarden *et al.,* 1999; Sandler *et al.,* 1985). However, our company proposed the hypothesis that the excellent effects of cow's milk on bone health might not be limited to an abundance of calcium, and we have searched for more than 10 years for components that may enhance the strength of bones. In neonates, in whom the only source of nutrition is mother's milk, bones are actively remodeled, suggesting the possibility that mother's milk may contain some functional components other than calcium that affect bone metabolism. To maintain healthy bones, it is ideal not only to increase the intake of calcium as a material for bone formation but also to simultaneously assist the formation of bones and suppress loss of bone mass. Then, we searched for substances in milk that regulate bone remodeling to enhance bone mineral density and successfully identified the milk component MBP, which is directly effective in strengthening bones. We now consider MBP a component of milk that is essential to the maintenance of bone health. The bone-strengthening effects of MBP have been confirmed in MBP-intake studies in volunteers as well as in cell culture studies and animal experiments. These results suggest that the importance of milk in the maintenance of human health resides not only in calcium but also in MBP.

III. What is "MBP" ?

Milk basic protein is composed of natural proteins with basic isoelectric points that are present in trace amounts in whey. We can obtain MBP by processing skimmed milk or whey with cation-exchange resins. Skimmed milk was first fractionated into casein and whey, and the whey fraction was further fractionated into components of proteins and lactose, etc., to investigate their bone metabolism-regulating actions. The study results indicated that whey protein stimulates osteoblasts in the remodeling of bones and simultaneously suppresses the bone dissolving action of osteoclasts. Further studies focusing on the active components of whey proteins discovered that a protein aggregation in whey with an alkaline isoelectric point was MBP, which stimulated bone formation and simultaneously suppressed bone resorption. The study results eventually revealed that MBP stimulated the proliferation of osteoblasts involved in bone formation and collagen production as well as suppressed the activity of osteoclasts involved in bone resorption.

IV. Physiological Actions of MBP

1. Effects on proliferation and differentiation of osteoblasts

Mouse osteoblast cell lines, MC3T3-E1 and MG63, were used to study the effects of MBP on DNA synthesis and collagen production as a bone matrix. As shown in Fig. 3, MBP was found to stimulate proliferation of osteoblasts in a concentration dependent manner and production of PICP (procollagen I carboxy-terminal propeptide), which is an indicator of synthesis of collagen as a bone matrix.

2. Actions to suppress bone resorption by osteoclasts

The effects of MBP on the bone-resorbing action of osteoclasts were studied. Osteoclasts isolated from rabbit femurs were cultured on dentine slices in media containing or not containing MBP for 48 hrs, and the number of pits formed by osteoclasts indicating destruction of bone tissue was counted. As shown in Fig. 4, the number of pits formed by osteoclasts decreased depending on MBP concentration, suggesting that MBP was effective in suppressing the bone-resorbing action of osteoclasts.

3. Stimulation of bone formation in rats during the growth period

The bone mineral density and strength of femur were significantly increased in rats (female SD rats, 5-week-old) orally administered MBP for 4 weeks during the growth

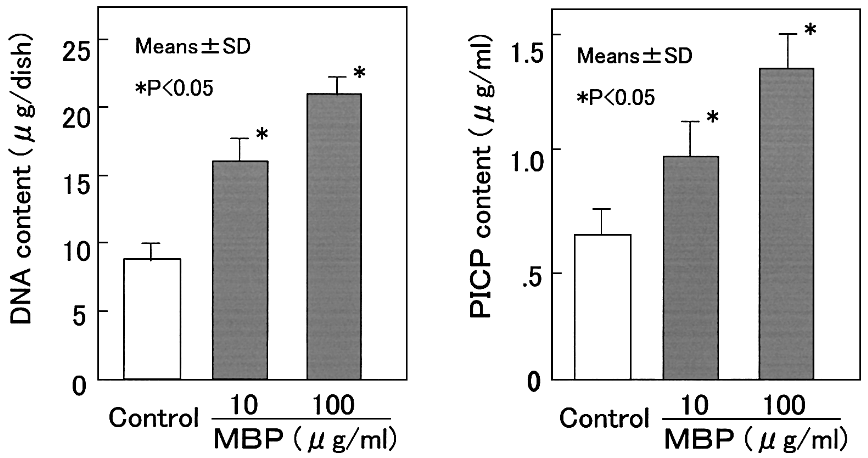

Fig. 3. Effects of MBP on DNA content and procollagen type 1 peptide formation of osteoblast.
DNA content was measured after 18 hrs of incubation in an assay with or without MBP. Procollagen type 1 peptide content was measured after 5 days of incubation in an assay with or without MBP.

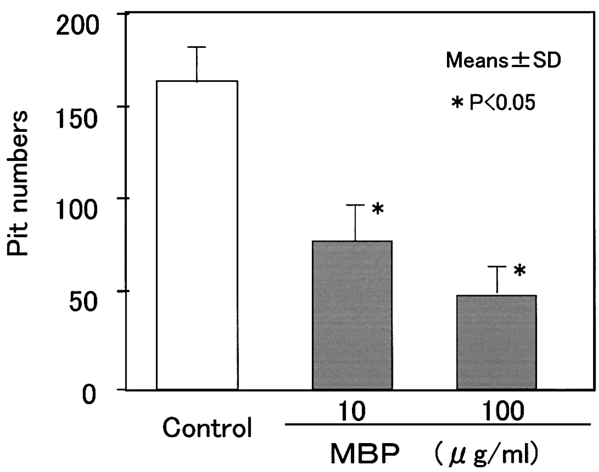

Fig. 4. Suppressive effect of MBP on pit formation by osteoclast.
The number of pit formed by the osteoclast on a dentine slice was counted after 48 hrs of incubation in an assay with or without MBP.

period. The activity of serum alkaline phosphatase (ALP), a bone formation marker, was significantly increased in rats fed MBP, suggesting that MBP accelerated bone formation. Another study on tibial morphology found that MBP administration increased cancellous bone and induced development of epiphysial plates. These results suggested that MBP accelerated bone formation during the growth period of animals.

4. Bone matrix-increasing and bone-strengthening actions in a rat model of bone resorption

Ovariecomized female rats (SD, 10-week-old) were fed a low calcium diet to reduce bone mass in a study of the effects of MBP on enhancement of bone mass and strength (Kato *et al.,* 2000). This animal model is presumed to resemble young women who practice undesirable dieting, which leads to calcium deficiency and hormone imbalance. As shown in Fig. 5, following a 3-week diet containing MBP, femoral strength was significantly increased in the ovariecomized rats. In addition, hydroxyproline and hydroxylysine, characteristic of matrix collagen and beneficial in making bones robust and pliable, were found significantly more after MBP administration. These results suggested that MBP increased bone matrixes such as collagen to enhance bone strength.

5. Bone mass reduction-suppressing action in a rat model of osteoporosis

A rat model of postmenopausal osteoporosis (SD, 55-week-old) was used to study whether MBP suppresses bone mass reduction (Toba *et al.,* 2000). The results showed that the intake of MBP significantly suppressed reduction in femoral bone mineral density (Fig. 6). Additionally, observation of non-decalcified tibial specimens confirmed that intake of MBP significantly suppressed reduction in cancellous bone (Fig. 7). Furthermore, urinary excretion of deoxypyridinoline (DPD), a degradation product of collagen as a bone matrix, was significantly lowered under MBP administration, suggesting that bone resorption was suppressed. Thus, MBP was suggested to be effective in suppressing bone mass reduction due to postmenopausal acceleration of bone resorption.

6. Bone formation-stimulating and bone resorption-suppressing actions in humans

An MBP intake study was conducted in healthy male adult volunteers who were given MBP-containing beverages (Toba *et al.,* 2001). Urine and blood samples were collected before and 16 days after initiation of beverage intake, to determine markers of bone metabolism. As shown in Fig. 8, the study results revealed that an intake of MBP significantly increased serum concentration of osteocalcin, a marker indicating bone formation, and significantly decreased urinary excretion of cross-linked N-teleopeptides of type-I collagen (NTx), a marker indicat-

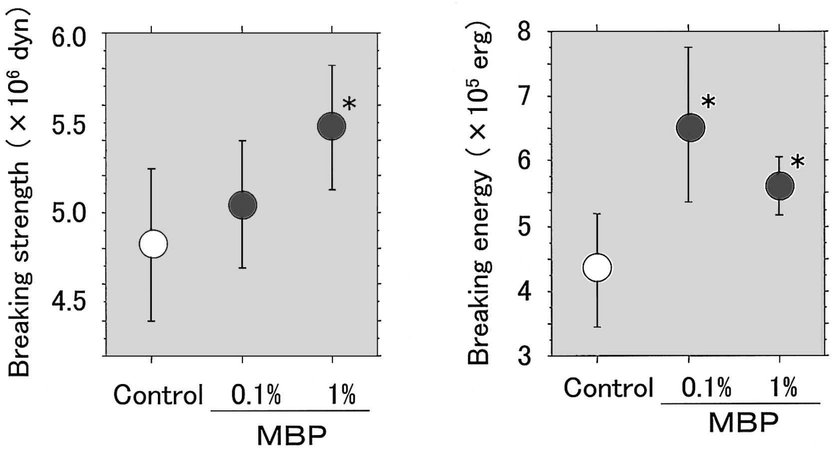

Fig. 5. Effect of MBP on breaking strength and breaking energy of the femur.
Ten-week-old ovariectomized rats were fed a low calcium diet containing 0%, 0.1% or 1% MBP for 3 weeks. Means±SD, * $p < 0.05$.

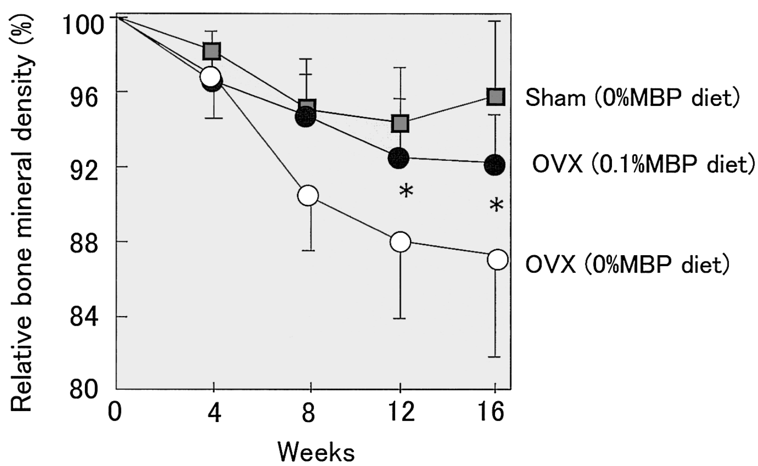

Fig. 6. Effect of MBP on decrease in bone mineral density of ovariectomized rats.
Fifty-five-week-old ovariectomized (OVX) and sham-operated rats were fed a low calcium diet with or without MBP for 17 weeks. Values are means with 95% confidence intervals. A significant difference (* $p < 0.05$) was compared between the OVX (0%MBP diet) group and the OVX (0.1%MBP diet) group.

ing bone resorption. In addition, there was a significant correlation between the serum concentration of osteocalcin and urinary excretion of NTx after initiation of MBP intake, indicating that bone formation was well balanced with bone resorption (Fig. 9). These results suggested that MBP regulated bone metabolism, maintaining a balance in bone remodeling.

7. Bone mineral density-increasing and bone metabolism-improving actions in humans

A 6-month MBP intake study was conducted in healthy female adult volunteers who were given MBP-containing beverages (Yamamura *et al.*, 2002). Participants were divided into MBP and placebo groups, to study under double-blind conditions. Measurements of bone metabolic markers revealed that urinary excretion of NTx, a bone resorption marker, was significantly lower 3 and 6 months after initiation of MBP intake. In addition, the increase in rate of bone mineral density at 6 months was significantly higher in the MBP group, as compared to the placebo group (Fig. 10). Conversely, analysis of records of food intake during the study period demonstrated no correlation between dietary intake of calcium, magnesium, and vitamins D and K and bone mineral density (Aoe *et al.*, 2001). The study results in human volunteers demonstrated that MBP increased bone mineral density.

8. Active components of MBP

MBP was further fractionated by various types of chromatography to probe for active components using osteoblast-proliferation activity as an indicator. The following substances were identified: Fragments 1・2 with MW 17 kDa of high molecular weight kininogen, a blood coagulating factor (Yamamura *et al.*, 2000), and high mobility group (HMG)-like protein with MW 10 kDa (Yamamura *et al.*, 1999). Furthermore, functional components were investigated using the bone resorption-suppressing activity in an isolated osteoclast culture system as an indicator, and a cystatin with MW 12 kDa was identified (Matsuoka *et al.,* 2002). Cystatin is produced by osteoblasts and inhibits cysteine protease, which is produced by osteoclasts and destroys bones, so that bone resorption is regulated (Johansson *et al.,* 2000; Lerner *et al.,* 1997). In addition, several growth factors in milk are well known to possess basic isoelectric points (Francis *et al.,* 1995). On the other hand, it has been reported in an *in vitro* cell culture study that lactoferrin, one of basic proteins in milk, stimulated the growth and differentiation of osteoblasts (Cornish *et al.,* 2004) and suppressed the differentiation and resorbing-activity of osteoclasts (Lorget *et al.,* 2002). Cornish *et al.* (2004) also reported that local injection of lactoferrin into mouse calvarium resulted in increased bone growth. However, in our *in vivo* study using a rat model of osteoporosis, it was confirmed that oral administration of MBP suppressed reduction in bone strength of the femur, whereas lactoferrin was not effective (Morita *et al.,* 2004). These results suggest that composite reactions of the various basic protein components contained in MBP comprehensively produce an improvement in bone metabolism and increase in bone mineral density.

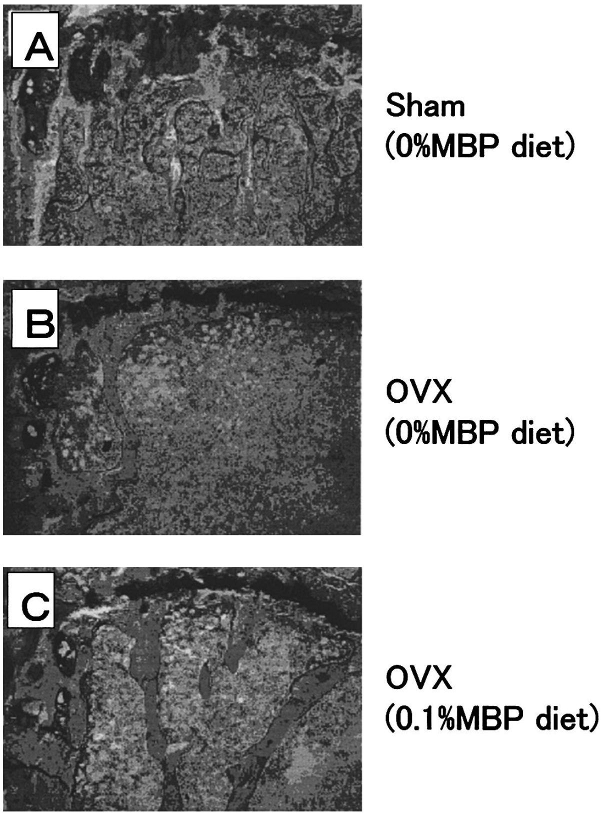

Fig. 7. Bone histology around the growth plate-metaphyseal junction in the proximal tibia from a sham-operated rat sham group (A), and from ovariectomized rats of the OVX-control group (B) and the OVX-0.1% MBP group (C) after the 17-week feeding trial.

V. Safety Evaluation of MBP

We performed safety evaluation studies in accordance with the "Ordinance on Standard of Conduct of Non-Clinical Studies of Drug Safety", from the Ministry of Health, Labor and Welfare Ordinance No. 21, Japan, March 26, 1997 and the "Guidelines for Designation of Food Additives and for Revision of Standards for Use of Food Additives", Notification No. 29 of the Environmental Health Bureau, the Ministry of Health, Labor and Welfare, Japan, March 22, 1996. In a single dose oral toxicity study, a 4-week oral repeated dose toxicity study, a 13-week oral repeated dose toxicity study, and a teratogenicity study of MBP, no change attributable to MBP was clinically observed in males or females of any dose group for body weight, food consumption, urinalysis, hematology, blood chemistry, ophthalmology, autopsy, organ weights and histopathology. In addition, mutagenic ac-

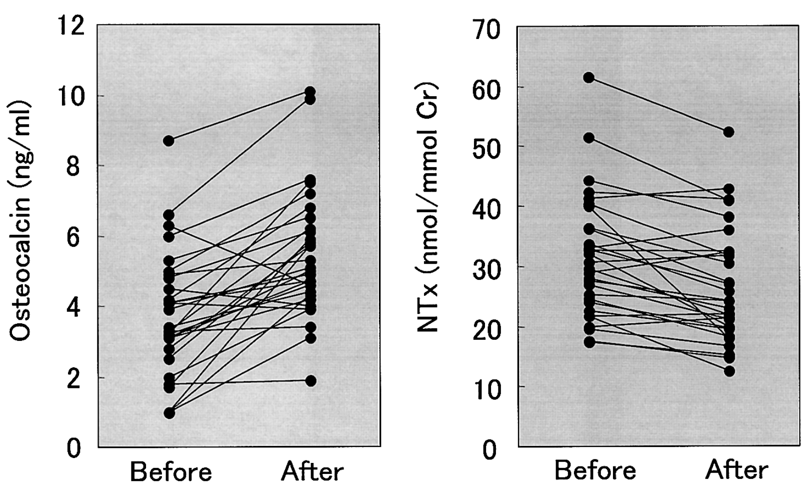

Fig. 8. Individual changes in serum osteocalcin concentrations (left) and urinary NTx excretion (right) before and after 16 days of ingesting an experimental beverage containing MBP. Increase in serum osteocalcin concentration was found in 28 of the 30 subjects, and decrease in urinary NTx excretion was in 24 of the 30 subjects.

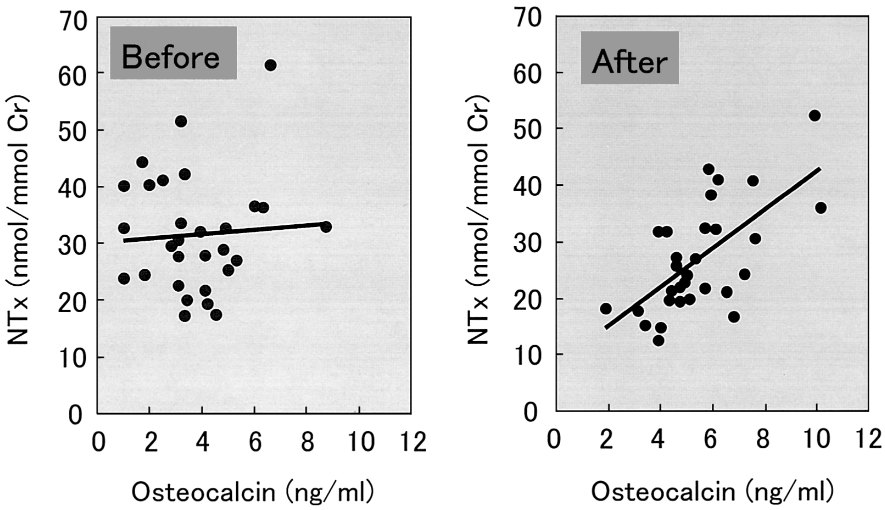

Fig. 9. Relationship between urinary NTx excretion and serum osteocalcin concentration before (left) and after 16 days (right) of ingesting an experimental beverage containing MBP. The correlation coefficient before ingestion was 0.0641 (P=0.7366), and after 16 days was 0.6457 (P<0.0001). Differences are considered significant if p<0.05.

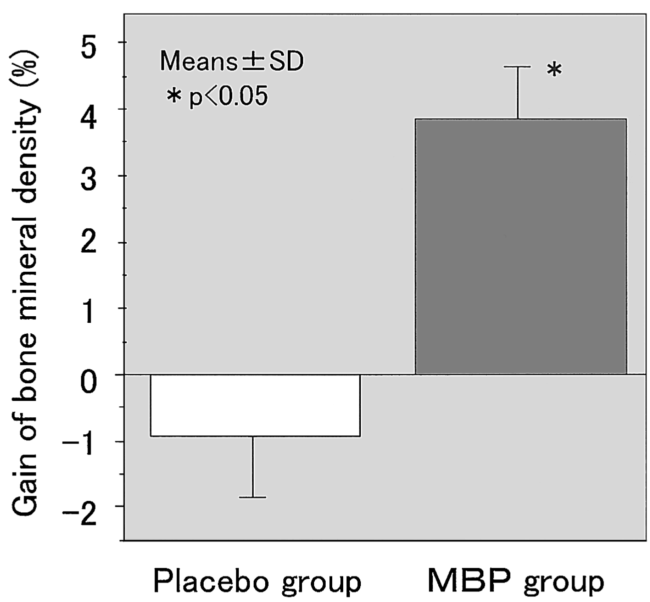

Fig. 10. Gain of bone mineral density in healthy adult women given placebo beverage or MBP beverage for six months.
The bone mineral density was measured at the 1/10 portion from the distal end of the radius. The gain of bone mineral density was significantly higher in the MBP group.

tivity of MBP was not observed in a reverse mutation-screening test using *Salmonella typhimurium*.

VI. Application of MBP to Food Products

MBP, which is highly water-soluble and flavorless, can be easily blended into various types of food products. Although the active ingredients of MBP are proteins, the activities are not affected by heat or coexisting components and are resistant to the manufacturing processes for food production (homogenization, heating sterilization, and spray drying, etc.). Therefore, MBP could be applied to a wide variety of food products, including chilled foods, long shelf life foods, and foods distributed at ambient temperature, regardless of the type of food, fluid or solid.

At present, MBP is supplemented in various milk products, and enriched products, such as processed cheese, skim milk, follow-up milk, milk beverages, yoghurt, drinkable yoghurt, lactic acid bacteria beverage, ice cream, and milky beverages, etc., which are marketed both in Japan and abroad. Our company's product, "Mainichi Hone Kea MBP®", which is a Food for Specified Health Use (FOSHU), was permitted by the Ministry of Health, Labor and Welfare in February 2002, to be labeled in an advertisement with the statement, "This product contains MBP, which is effective in increasing bone mineral density, and is therefore a beverage suitable for persons who are concerned about their bone health." This is the only product that was approved for the label "increases bone mineral density" among foods specified for bone health use. "Mainichi Hone Kea MBP®" is served as a convenient drinking supplement and is widely distributed so that people can use the product even if they do not like cow's milk or milk products because of preference or due to lactose intolerance.

VII. Conclusion

In the 21st century, it is recognized to be more and more important to prevent various types of lifestyle-related diseases by having ordinary meals. In addition, the number of elderly people bedridden due to lifestyle-related diseases and in need of nursing care is a rapidly growing social problem (Ray *et al.*, 1997; Looker *et al.*, 1995; Hui *et al.*, 1988). A survey on the causes of the requirements for nursing care has reported that bone disease due to fracture or falls are placed third, after cerebral stroke and senility. In our rapidly aging society, it is presumed that bone-related diseases will continue to increase, suggesting that, from the viewpoint of dietary life, it is more important to pay attention to bone health in peoples' earlier years to maintain healthy and active daily lives (Silverman *et al.,* 2001).

The growing number of elderly patients with bone disease or fractures attributable to osteoporosis is a social phenomenon closely associated with an increase in the number of bedridden elderly (Melton *et al.,* 1992; Kanis *et al.,* 1994), and is a causative factor in the rise of the total cost of national health care (Kanis and Johnell, 1999; Simon and Mack, 2003). Until now, nutritional measures such as recommending an increase in calcium intake by administering calcium agents have been used to prevent such problems (Heaney, 2000). However, the supplementation of some daily foods with MBP has made it possible to maintain properly balanced bone remodeling. This will allow the utilization of MBP-containing foods to actively prevent occurrences of bone disorders in the elderly such as osteoporosis. Popularization of MBP-containing food products may help to curtail medical costs and also to exercise a major social influence over healthcare policy for the elderly. Additionally, reduction in bone mineral density often occurs not only in the elderly but also in young women, in particular, those on diets (Flynn, 2003). To maintain healthy bones in such young women and to reduce the number of patients with osteoporosis in the future, it is important for young people to take MBP-containing foods.

MBP contains components that not only stimulate the proliferation of osteoblasts but also components that suppress bone resorption by osteoclasts, helping bone formation and decreasing bone loss, thereby increasing bone mineral density and strengthening bones. MBP will inevitably increase bone formation and suppress bone resorption to a certain level, keeping bones healthy in a dual functional manner. In modern society, bone diseases attributable to lifestyle-related factors are growing, thus maintaining bone health by safe and reasonable improvement in bone metabolism through daily eating habits is recommended. MBP is a protein derived from milk, which human beings have experienced drinking since before the time of recorded history, when stockbreeding began, and is guaranteed to be completely safe. We hope that MBP will be used more and more widely as a functional component in helping to maintain human health.

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