BioFactors 22 (2004) 5–19
IOS Press

Studies on action of menaquinone-7 in regulation of bone metabolism and its preventive role of osteoporosis

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Abstract. The effect of menaquinone-7 (MK-7) on bone components and bone resorbing factors induced-bone resorption using the femoral-diaphyseal and –metaphyseal tissues obtained from elderly female rats *in vitro* were examined. Calcium content, alkaline phosphatase activity and deoxyribonucleic acid (DNA) in the diaphyseal and metaphyseal tissues in elderly females rats were significantly decreased as compared with that of young rats, indicating that aging causes a deterioration of bone formation. The presence of MK-7 (10^{-6}-10^{-5} M) caused a significant prevention of reduction of biochemical components. On the other hand, the bone-resorbing factor, parathyroid hormone (1–34) (PTH; 10^{-7} M) and prostaglandin E_2 (PGE_2; 10^{-5} M) caused a significant decrease in calcium content in the diaphyseal and metaphyseal tissues. This decreases was completely inhibited in the presence of MK-7 (10^{-7}-10^{-5} M). In addition, MK-7 (10^{-7}-10^{-5} M) completely prevented the PTH (10^{-7} M) or PGE_2 (10^{-5} M) induced increases in medium glucose consumption and lactic acid production by bone tissues, Furthermore, the effect of the prolonged intake of dietary MK-7 on bone loss in ovariectomized rats was investigated. As a result, it was found that the intake of experimental diets containing the fermented soybean (natto) with supplemental MK-7 caused significant elevations of MK-7 and γ-carboxylated osteocalcin concentration, a bio marker of bone formation, in the serum of both ovariectomized rats and normal subjects, suggesting that MK-7 may play an important role in the prevention of age-related bone loss.

Keywords: Menaquinone-7 (MK-7), vitamin K_2, bone formation, bone resorption, osteocalcin, osteoporosis

1. Introduction

Bone loss with increasing age induces osteoporosis [1–3]. This loss may be due to induced bone resorption and decreased bone formation. A decrease in bone mass leads to bone fracture. Osteoporosis is widely recognized as a major public health problem [4]. Postmenopausal osteoporosis is resulted from estrogen deficiency. This is partly involved in the deterioration of bone metabolism with increasing age. Pharmacological and nutritional factors are important in preventing age-related bone loss.

There is growing evidence that vitamin K, which is a nutritional factor, may play a role in the regulation of bone metabolism. Vitamin K_2 is essential for γ-carboxylation of osteocalcin. A bone matrix protein contains γ-carboxyglutamic acid, which is synthesized in osteoblast of bone tissues [5–8]. Menaquinone-7 (MK-7) with seven isoprene units, one of analog of vitamin K_2, is abundant in fermented soybean (natto).

However, whether MK-7 has a direct anabolic effect on bone metabolism *in vitro* has not been fully clarified.

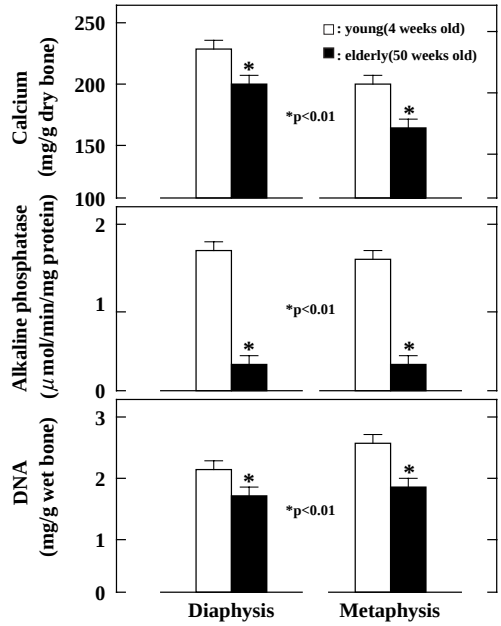

Fig. 1. Alteration in calcium content, alkaline phosphatase activity and DNA content in the femoral-diaphyseal and -metaphyseal tissues obtained from elderly female rats.

The present study was first undertaken to determine whether MK-7 has a stimulatory effect on bone formation and an inhibitory effect on bone-resorbing factors-induced bone resorption in elderly female rats *in vitro*. We found that MK-7 could increase biochemical components and it was also found that MK-7 could inhibit the parathyroid hormone (1–34) (PTH) or prostaglandin E_2 (PGE_2) -induced bone resorption in bone culture of female aged rat femoral tissues *in vitro*, suggesting that MK-7 can inhibit bone resorption in the femoral-diaphyseal and -metaphyseal tissues obtained from elderly female rats in which bone formation may be deteriorated with aging. Furthermore, the present study was undertaken to determine whether ovariectomy (OVX)-induced bone loss is prevented by the prolonged intake of diets containing natto with MK-7 of comparatively lower levels. We found that the feeding of dietary MK-7, which was added at 1.5-and 2.0-fold to the usual MK-7-containing natto, for 5 months, prevented bone loss in OVX rats. Moreover, the present study was undertaken to determine whether the dietary intake of fermented soybean (natto) with reinforced MK-7 content increases the serum concentration of MK-7 and γ-carboxylated osteocalcin in healthy humans.

2. Change in biochemical components in the femoral tissues from elderly rats

Femoral-diaphyseal and -metaphyseal tissues were obtained from young (4 weeks old) or elderly (50 weeks old) female rats. Bone tissues were cultured for 48 hr in a Dulbecco's MEM (serum free) without MK-7. Calcium content, alkaline phosphatase activity, and deoxyribonucleic acid (DNA) content in the femoral-diaphyseal and -metaphyseal tissues obtained from elderly rats was significantly lowered as compared with those obtained from young rats (Fig. 1). Increasing age caused a significant decrease in bone biochemical components, indicating that aging induced a deterioration of bone formation.

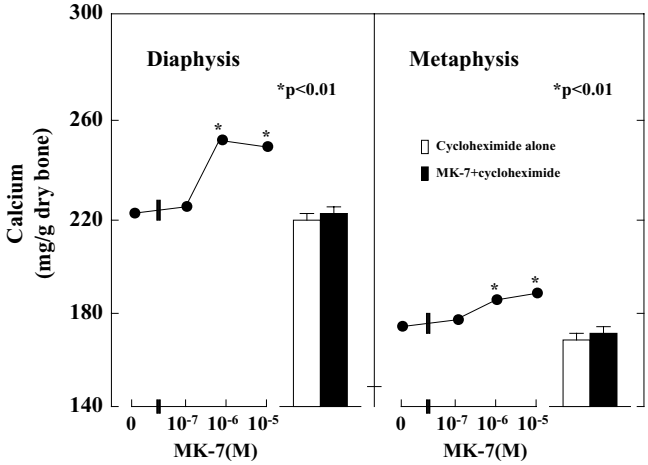

Fig. 2. Effect of MK-7 on calcium content in the femoral-diaphyseal and -metaphyseal tissues obtained from elderly female rats *in vitro*.

2.1. Effect of MK-7 on biochemical components in the femoral tissues from elderly rats

Femoral-diaphyseal and -metaphyseal tissues obtained from elderly female rats were cultured in a medium containing either vehicle or MK-7 (10^{-6}-10^{-5} M) *in vitro*. The presence of MK-7 (10^{-7} or 10^{-5} M) causes a significant increase in calcium content (Fig. 2), alkaline phosphatase activity (Fig. 3), and (DNA) content (Fig. 4) in the femoral-diaphyseal and -metaphyseal tissues. These bone components were not significantly altered in the presence of 10^{-7} M MK-7 (Figs 2–4). Cycloheximide is an inhibitor of protein synthesis in the translation process. The effect of MK-7 (10^{-5} M) in increasing calcium content, alkaline phosphatase activity, and DNA content in the femoral-diaphyseal and -metaphyseal tissues obtained from elderly female rats was completely abolished in the presence of cycloheximide (10^{-6} M) *in vitro* (Figs 2–4). The results indicate that the MK-7-induced increase in bone components may be related to a newly synthesized protein component.

2.2. Change in calcium content, glucose consumption and lactic acid production in bone tissues with aging

Femoral-diaphyseal and -metaphyseal tissues obtained from young (4 weeks old) or elderly (50 weeks old) were culture in a serum-free medium without MK-7. Bone calcium content was significantly decreased in the femoral-diaphyseal and -metaphyseal tissues obtained from elderly female rats as compared with those of young rats (Fig. 5). Medium glucose consumption and lactic acid production by the diaphyseal and metaphyseal tissues were significantly lowered in the bone tissues from elderly rats as compared with those from young rats (Fig. 5). Increasing age causes a significant decrease in calcium content, glucose consumption, and lactic acid production in the femoral tissues of female rats.

2.3. Effect of MK-7 on the bone-resorbing factor-reduced bone calcium content in vitro

The effect of MK-7 on the bone-resorbing factors-induced decrease in calcium content in the femoral tissues obtained from elderly female rats was examined *in vitro*. Femoral-diaphyseal or -metaphyseal tissues were cultured for 48 hr in a medium containing either vehicle or PTH (10^{-7} M), or PGE2

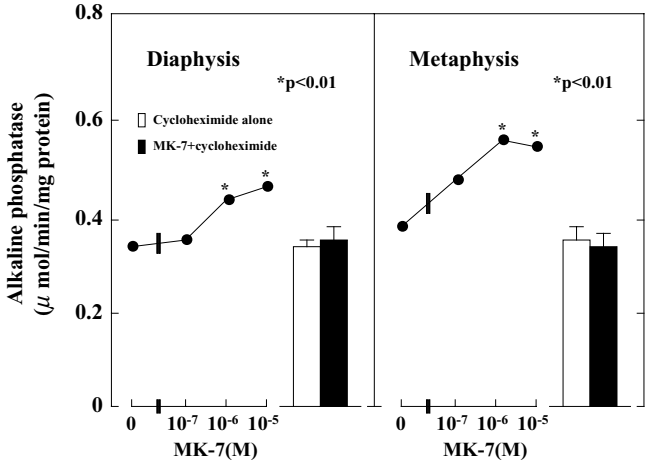

Fig. 3. Effects of MK-7 on alkaline phosphatase activity in the femoral-diaphyseal and -metaphyseal tissues obtained from elderly female rats *in vitro*.

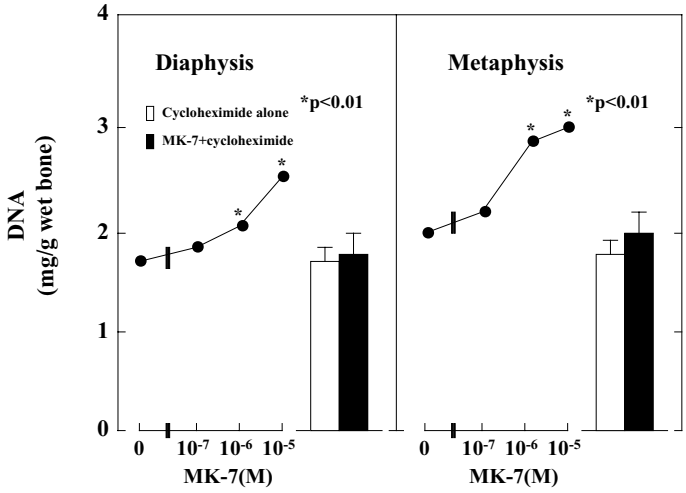

Fig. 4. Effect of MK-7 on DNA content in the femoral-diaphyseal and -metaphyseal tissues obtained from elderly female rats *in vitro*.

(10^{-5} M) in the absence of MK-7 (10^{-7}-10^{-5} M), as shown in Figs 6 and 7, respectively. Diaphyseal and metaphyseal calcium contents were significantly decreased in the presence of PTH (Fig. 6) or PGE2 (Fig. 7). These decreases were completely prevented by MK-7 (10^{-7}-10^{-5} M), as shown in Figs 6 and 7, respectively.

2.4. Effect of MK-7 on the bone-resorbing factor-stimulated glucose consumption by bone tissues in vitro

The effect of MK-7 on the PTH or PGE2-induced stimulation of medium glucose consumption in the femoral-diaphyseal and -metaphyseal tissues obtained from elderly female rats is shown in Figs 8 and 9. The presence of PTH (10^{-7} M) or PGE_2 (10^{-5} M) caused a significant increase in medium glucose

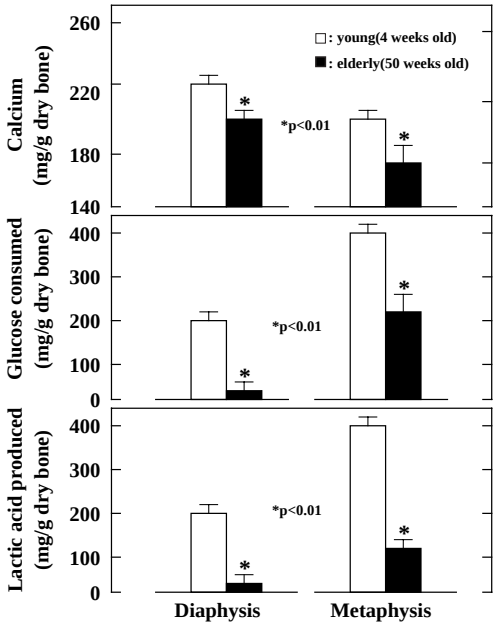

Fig. 5. Alteration in calcium content, glucose consumption and lactic acid production in the femoral-diaphyseal and -metaphyseal tissues of elderly female rats.

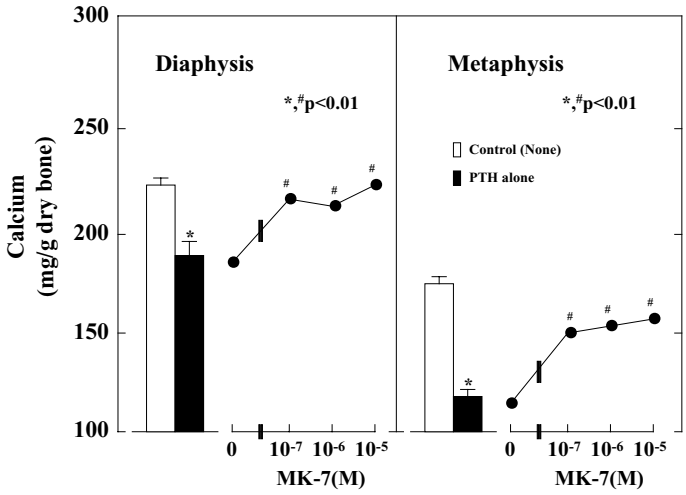

Fig. 6. Effect of MK-7 on PTH-decreased bone calcium content in the femoral-diaphyseal and -metaphyseal tissues of elderly female rats *in vitro.*

consumption by the femoral-diaphyseal and -metaphyseal tissues, when the bone tissues were culture for 48 hr. These increases were completely prevented in the presence of MK-7 (10^{-7}-10^{-5} M).

2.5. Effect of MK-7 on bone-resorbing factor-stimulated lactic acid production by bone tissues in vitro

The effect of MK-7 on the PTH- or PGE2-induced increase of lactic acid production in the femoral-diaphyseal and -metaphyseal tissues obtained from elderly female rats is shown in Figs 10 and 11.

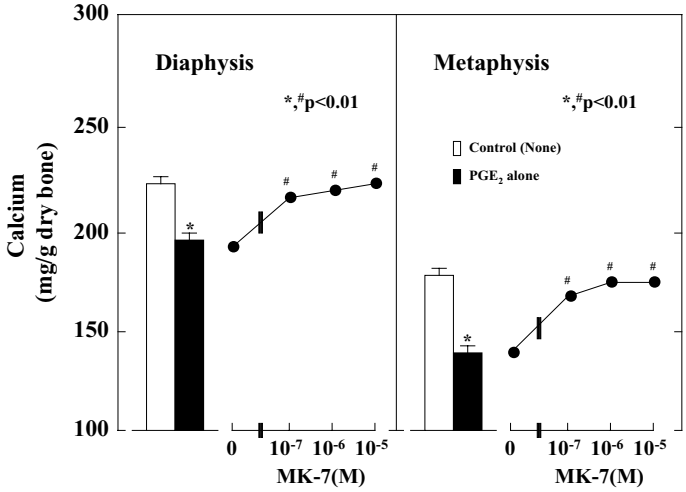

Fig. 7. Effect of MK-7 on PGE2-decreased bone calcium content in the femoral-diaphyseal and -metaphyseal tissues of elderly female rats *in vitro.*

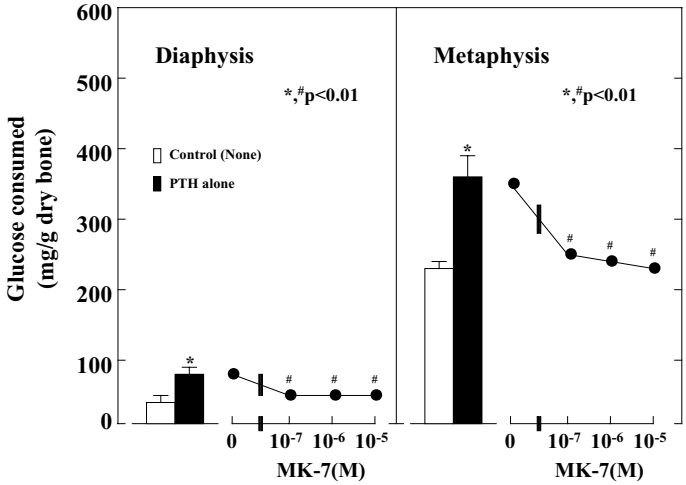

Fig. 8. Effect of MK-7 on PTH-stimulated glucose consumption by the femoral-diaphyseal and -metaphyseal tissues of elderly female rats *in vitro.*

Bone tissues were cultured for 48 hr. The production of lactic acid by the femoral-diaphyseal and -metaphyseal tissues was significantly increased in the presence of PTH (10^{-7}M) or PGE_2 (10^{-5} M), as shown in Figs 10 and 11, respectively. PTH- or PGE2-induced increase in bone lactic acid production was completely prevented in the presence of MK-7 (10^{-7}-10^{-5} M) (Figs 10 and 11).

2.6. Alteration in serum MK-7 or MK-4 concentration of OVX rats fed the experimental diets containing natto

OVX did not cause a significant alteration in serum MK-7 concentration as compared with that of sham-operated rats (Fig. 12). Serum MK-7 concentration of OVX rats in OVX MK-7 $\times$ 1, OVX MK-7 $\times$ 1.5 and OVX MK-7 $\times$ 2 fed natto diets including MK-7 (9.4, 14.1, and 18.8 μg/100 g diet,

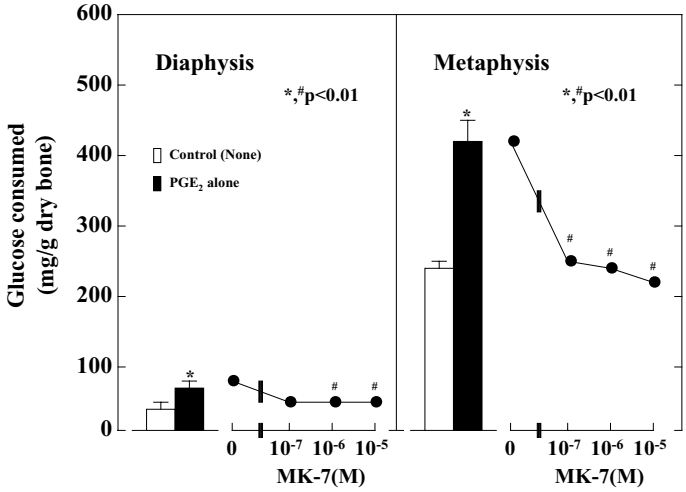

Fig. 9. Effect of MK-7 on PGE2-stimulated glucose consumption by the femoral-diaphyseal and -metaphyseal tissues of elderly female rats *in vitro*.

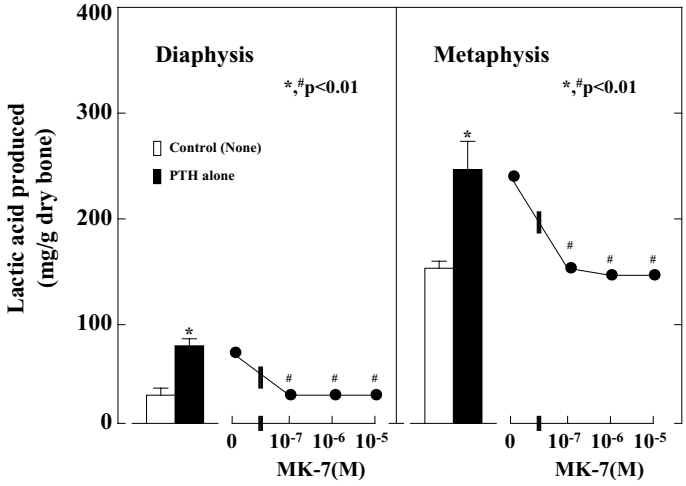

Fig. 10. Effect of MK-7 on PTH-stimulated lactic acid production by the femoral-diaphyseal and -metaphyseal tissues of elderly female rats *in vitro*.

respectively) was significantly increased as compared with those of sham-operated rats (Sham). Serum MK-4 concentration in OVX rats (OVX MK-7 $\times$ 2), where were fed natto diets with 2-fold MK-7 added (18.8 μg as total/ 100 g diets) of that in natto was significantly elevated as compared with that of sham-operated rats (Sham) or OVX rats (OVX). In the femur of OVX rats fed natto diets including MK-7 (9.4, 14.1 or 18.8 μg/100 g diets), MK-7 was not detected. Also, femoral MK-7 in sham-operated and OVX rats were not detected. In this case, however, femoral MK-4 concentration was significantly increased. This result indicates that MK-4 degraded from MK-7 is accumulated in bone tissues.

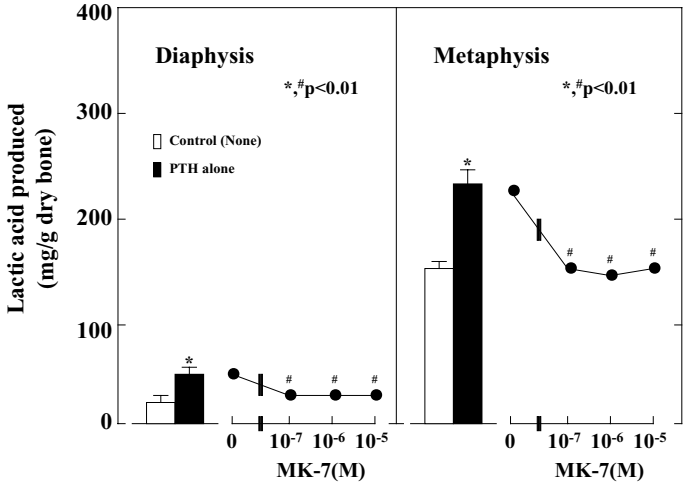

Fig. 11. Effect of MK-7 on PGE2-stimulated lactic acid production by the femoral-diaphyseal and -metaphyseal tissues of elderly female rats *in vitro*.

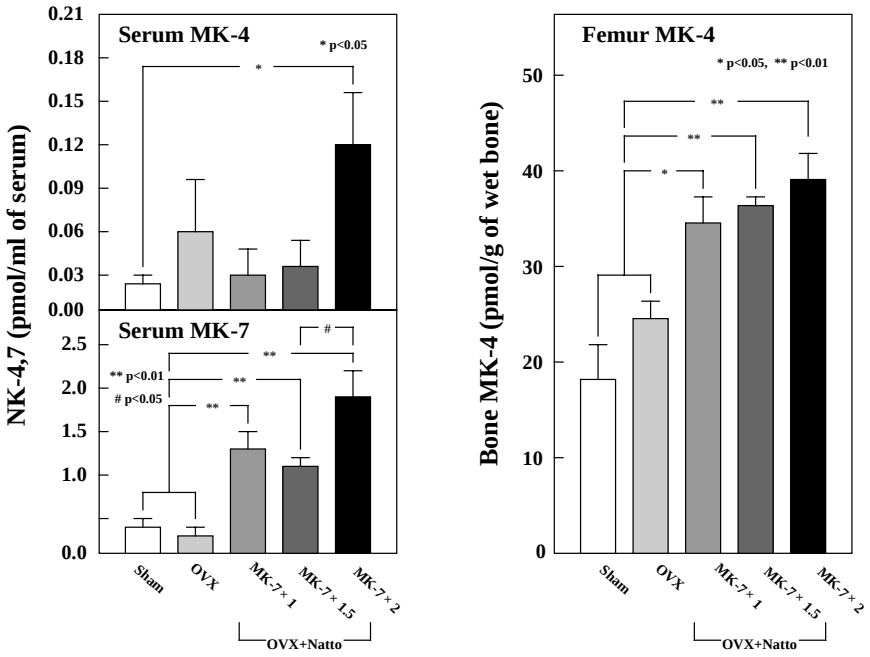

Fig. 12. Alteration in MK-4 and MK-7 concentrations in the serum (left) and femur (right) of rats fed diets containing natto with supplemental MK-7.

2.7. Alteration in serum γ-carboxylated osteocalcin concentration of rats fed dietary MK-7

Serum osteocalcin concentration was significantly decreased by OVX. This reduction significantly prevented by the feeding of natto diets with supplementation of MK-7 (14.1 and 18.8 μg/100 g diets) (Fig. 13). Femoral dry weight was significantly decreased by OVX (Fig. 14). This decrease was significantly prevented by the feeding of natto diets including MK-7 (9.4, 14.1, or 18.8 μg/100 g diets);

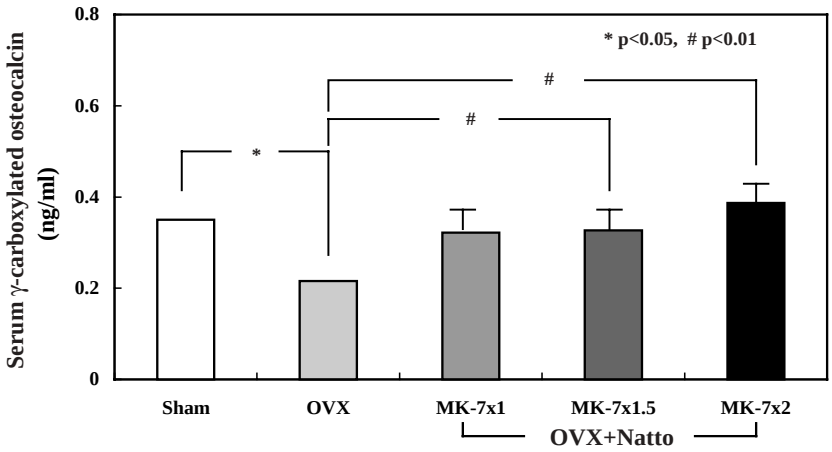

Fig. 13. Alteration in serum γ-carboxylated osteocalcin of rats fed diet containing natto.

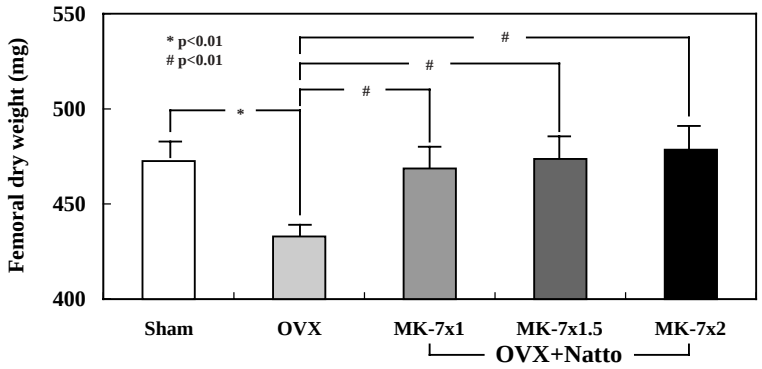

Fig. 14. Alteration in femoral dry weight of rats fed diets containing natto.

their femoral dry weight were restored to the level of sham-operated rats by the intake of dietary MK-7. Also, calcium content in the femoral-diaphyseal and metaphyseal tissues was significantly reduced by OVX (Fig. 15). Decrease in diaphyseal calcium content was significantly prevented by the feeding of natto diets including MK-7 (9.4, 14.1, or 18.8 μg/100 g diets). In the metaphyseal tissues, OVX-induced decrease in calcium content was not seen in rats fed with natto diets including MK-7.

2.8. Alteration in bone mineral density for the total of the femur

OVX caused a significant decrease in bone mineral density (Fig. 16). This decrease was significantly prevented by the feeding of natto diets including MK-7 (18.8 μg/100 g diets). A significant difference of bone mineral density was not seen between sham-operated and OVX rats fed with natto diets including MK-7 (18.8 μg/100 g diets).

2.9. Changes in serum MK-7 concentration in healthy men following the start of the intake of natto containing different contents of MK-7

The intake of regular natto containing 775 μg of MK-7 per 100 g (MK-7 $\times$ 1) for 1 and 7 days significantly raised serum MK-7 concentration (Fig. 17). Serum MK-7 concentration was enhanced by

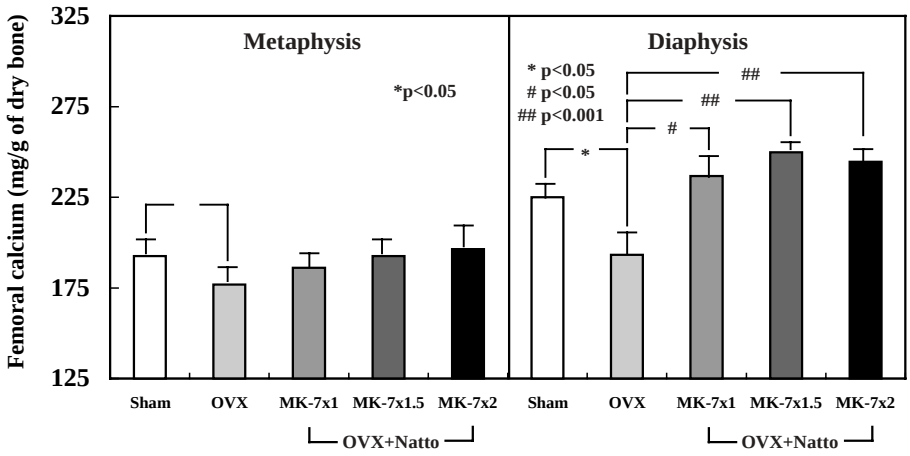

Fig. 15. Alteration in calcium content in the femoral-diaphyseal(right) and -metaphyseal (left) tissues of rats fed diets containing natto.

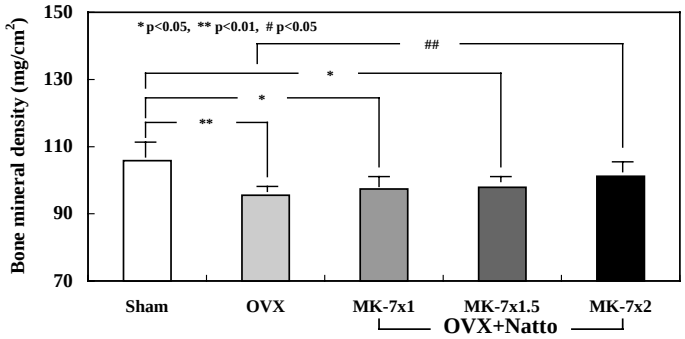

Fig. 16. Alteration in bone mineral content in the femur (total) of rats fed diets containing natto.

the dietary intake of natto containing 1298 or 1765 μg of MK-7 per 100 g (MK-7 $\times$ 1.5, MK-7 $\times$ 2) for 1 and 7 days, respectively. Serum vitamin K1 and MK-4 were not detected in subjects whose dietary intake was natto containing 775 to 1765 μg of MK-7 per 100 g (data not shown).

2.10. Changes in serum γ-carboxylated osteocalcin concentration following the start of the intake of natto

Serumγ-carboxylated osteocalcin concentration were not significantly elevated by the intake of natto containing 775 μg of MK-7 per 100 g for 7 days (Fig. 18). However, the dietary intake of natto with 1298 or 1765 μg of MK-7 per 100 g for 7 days significantly increased serumγ-carboxylated osteocalcin concentration (Fig. 18) and decreased serum undercarboxylated osteocalcin concentration (Fig. 19). Serum alkaline phosphatase activity and calcium concentration were constant the intake of natto with 775 or 1298 μg of MK-7 per 100 g for 7 days (Fig. 20). These serum markers were, however, significantly elevated by the intake of natto with 1765 μg of MK-7 per 100 g for 7 days (Figs 18–20). Serumγ-carboxylated osteocalcin concentration, alkaline phosphatase activity, and calcium concentration at baseline before dietary intake of MK-7 were not significantly changed as compared with those from dietary intake of natto containing 775 μg of MK-7 per 100 g.

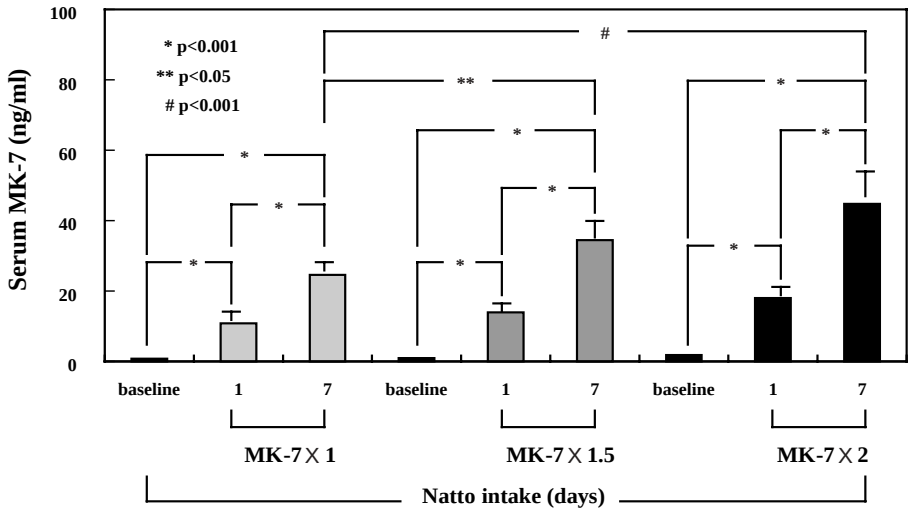

Fig. 17. Alteration in serum MK-7 concentration in dietary intake of natto containing different content of MK-7.

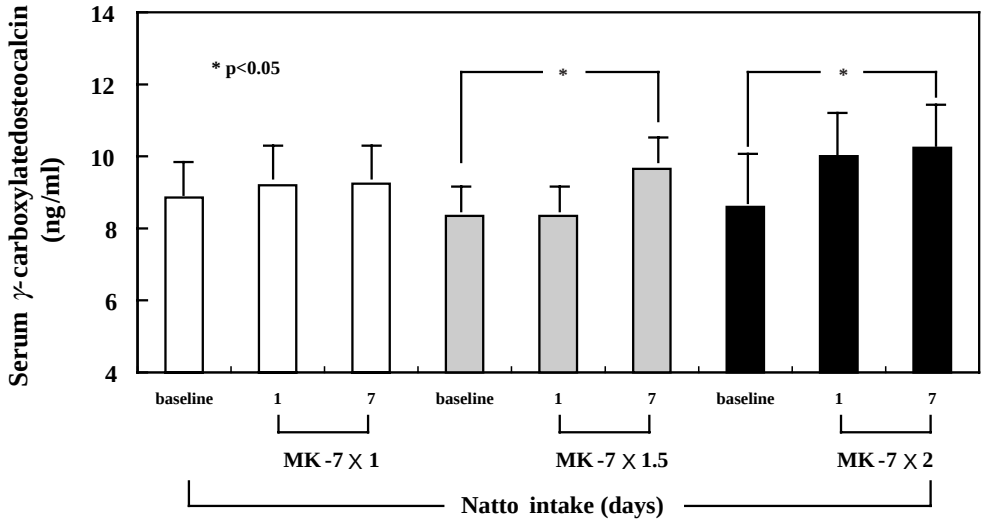

Fig. 18. Alteration in serum γ-carboxylated osteocalcin concentration in dietary intake of natto containing different content of MK-7.

2.11. Change in serum MK-7 concentration in normal individuals following the start of the intake of natto containing three different MK-7 contents

The intake of regular natto containing 865 μg of MK-7 per 100 g for 7, 10, and 14 days caused a significant elevation in serum MK-7 concentration (Fig. 21). Serum MK-7 concentration was significantly enhanced by the intake of natto containing either 1295 or 1730 μg of MK-7 per 100 g for 7, 10, and 14 days. Meanwhile, neither serum vitamin K1 nor MK-4 was detected in any subjects ingesting with any of three MK-7 amounts (data not shown). Comparison among three groups at each intake period containing either 865, 1298, and 1730 μg of MK-7 per 100 g showed significant differences among them at 7, 10, and 14 days after the intake. The increase in serum MK-7 concentration was observed especially

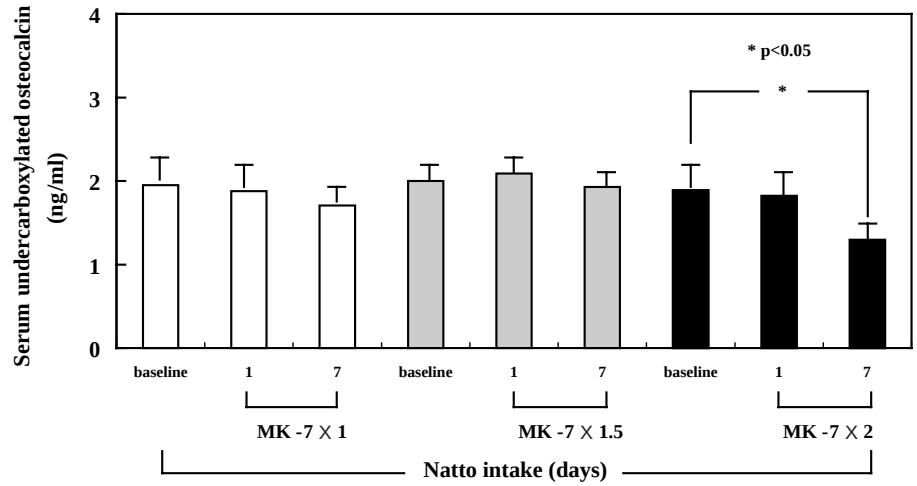

Fig. 19. Alteration in serum undercarboxylated osteocalcin concentration in dietary intake of natto containing different content of MK-7.

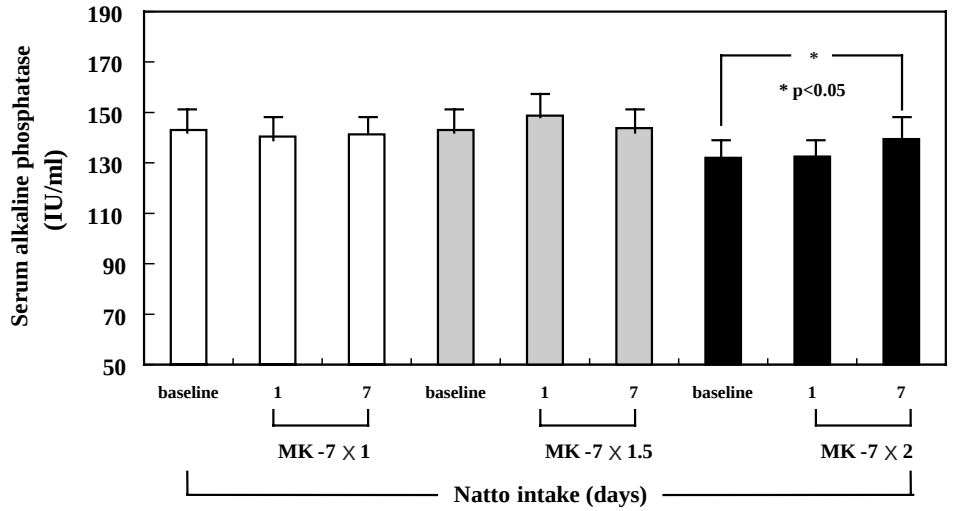

Fig. 20. Alteration in serum alkaline phosphatase activity in dietary intake of natto containing different content of MK-7.

by the intake of natto containing 1295 or 1730 μg of MK-7 per 100 g as compared with that of regular natto intake.

2.12. Change in serum γ-carboxylated osteocalcin concentration following natto intake

Regular natto did not cause a significant increase in serumγ-carboxylated osteocalcin concentration at 7, 10, and 14 days (Fig. 22). With respect to serum γ-carboxylated osteocalcin concentration, there was no significant difference among three groups at 7, 10 days. At 14 days, however, a significant difference was seen among groups. In addition, a significant rise in serumγ-carboxylated osteocalcin concentration was observed by the intake of natto containing either 1295 or 1730 μg of MK-7 per 100 g as compared with that of regular natto. Serum calcium and inorganic phosphorus concentration were significantly

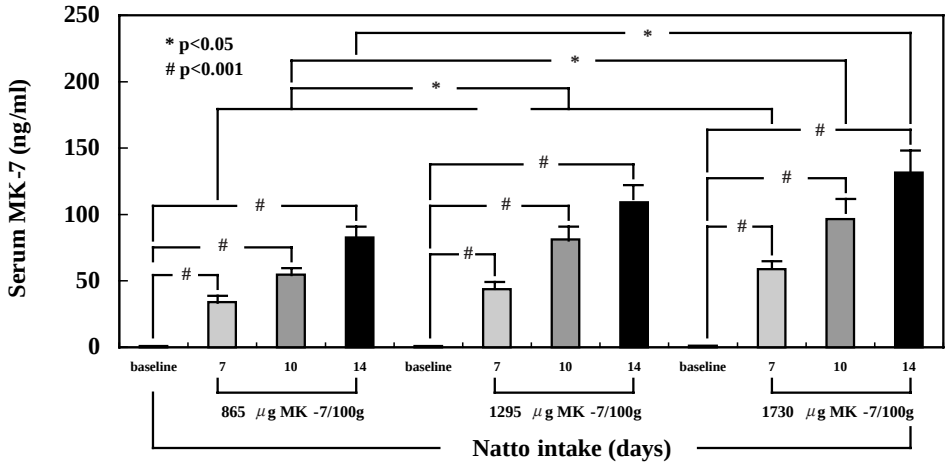

Fig. 21. Alteration in serum MK-7 concentration in dietary intake of natto containing different content of MK-7.

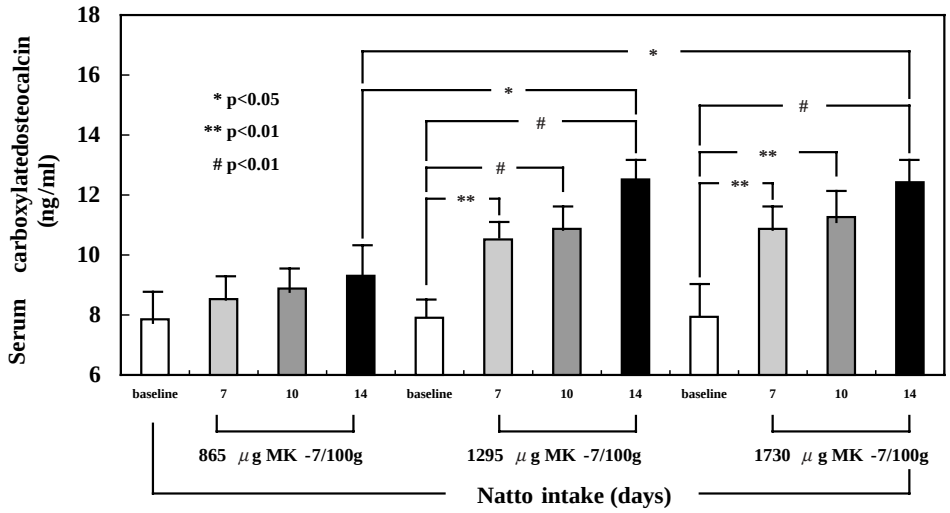

Fig. 22. Alteration in serum γ-carboxylated osteocalcin concentration in dietary intake of natto containing different content of MK-7.

elevated at 14 days after the intake of natto containing 1730 μg of MK-7 per 100 g, as compared with the value prior to ingestion (data not shown).

3. Conclusion

The skeleton system plays a physiologically important role as the organ for body support. In bone tissues there are osteoblast cell, bone formation cell, and osteoclast cell, bone resorption cell. These cells was regulated by various hormones and cytokines, and they play a role in the regulation of bone remodeling for the resorption of old bone tissues and the formation of new bone tissues.

Bone mass may be decreased with aging. Especially, osteoporosis by the deficiency of estrogen after menopause causes drastically bone loss. The most dramatic expression of these diseases is represented

by fractures of the proximal femur. Malnutrition or under-nutrition is often observed in the elderly. Deficiency in nutrients appears to be implicated in the pathogenesis and consequences of hip fracture in the osteoporotic elderly. The remedy of osteoporosis causes the rise of medical treatment expense and consequently it becomes a serious public problem. The prevention is very important against osteoporosis and nutritional factor may play an important role but these factors have been poorly understood.

From the finding that vitamin K_2 in vitamin K , which was discovered as an essential factor for the blood coagulation, may have a relation to bone disease and a kind of vitamin K_2, menaquinone-4, has been applied clinically as a therapeutic medicine for osteoporosis. Most of the natural foods contains only poor amount of vitamin K_1 and vitamin K_2. However, the fermented soybean (natto) exceptionally contains a comparatively high amount of MK-7, and much attention has been paid to the role of MK-7 in the prevention of osteoporosis.

Therefore, this study was undertaken to clarify the regulatory function of MK-7 in bone metabolism and to determine its role in the prevention of osteoporosis. As the results, the following findings were discovered.

Firstly, the study was undertaken to determine whether MK-7 has a stimulatory effect on bone formation using the femoral-diaphyseal and -metaphyseal tissues obtained from elderly (50 weeks old) female rats *in vitro*. Calcium content, alkaline phosphatase activity and DNA in the diaphyseal and metaphyseal tissues obtained from elderly (50 weeks old) female rats were significantly decreased as compared with those of young (4 weeks old) rats, indicating that aging causes a deterioration of bone formation. The presence of MK-7 (10^{-6} or 10^{-5} M) caused a significant increase in biochemical components. The effect of MK-7 (10^{-5} M) in increasing calcium content, alkaline phosphatase activity and DNA content in the diaphyseal and metaphyseal tissues were completely abolished in the presence of cycloheximide (10^{-6} M), an inhibitor of protein synthesis *in vitro*. These findings demonstrate that MK-7 has a stimulatory effect on bone formation through the stimulation of protein synthesis.

Secondly, the study was undertake to determine the effect of MK-7 on bone-resorbing factor-induced bone resorption using the femoral-diaphyseal and -metaphyseal tissues obtained from elderly (50 weeks old) female rats *in vitro*. When the femoral-diaphyseal and -metaphyseal tissues were cultured in the presence of PTH (10^{-7} M) or PGE_2 (10^{-5} M), a significant decrease in calcium content in the diaphyseal and metaphyseal tissues were observed. In addition, PTH and PGE_2 caused a significant increase in medium glucose consumption and lactic acid production by bone tissues. MK-7 (10^{-7}-10^{-5} M) completely prevented the PTH (10^{-7} M) or PGE_2 (10^{-5} M)-induced increase in medium glucose consumption and lactic acid production by bone tissues. These results support the view that MK-7 has a direct inhibitory effect on the bone-resorbing factor induced bone resorption in bone culture.

In addition, the preventive effect of the prolonged (150 days) intake of the regular natto including 9.4 μg MK-7/100 g diets and the reinforced natto including 14.1 and 18.8 μg MK-7/100 g diets on bone loss in OVX rats was investigated. The feeding of natto diets containing MK-7 in OVX rats caused a significant increase in the serum MK-7 concentration. Serum γ-carboxylated osteocalcin concentration, femoral dry weight, femoral calcium content and bone mineral density were significantly decreased by OVX. These decreases were significantly prevented by the feeding of diets containing natto with 14.1 and 18.8 μg MK-7/100 g diets. This result demonstrates that the prolonged intake of natto diets containing 1.5 and 2-fold higher content of MK-7 (14.1 and 18.8 μg/100 g diets, respectively) as compared with that of regular natto may play a role in the prevention of bone loss caused by ovariectomy.

Moreover, the changes in circulating MK-7 and γ-carboxylated osteocalcin concentration in normal individuals with the dietary intake of the regular natto (775 μg MK-7/100 g) and reinforced natto with 1.5 and 2-fold higher content of MK-7 than that of regular natto were investigated. It was demonstrated

that the intake of reinforced natto containing 1.5 and 2-fold higher content of MK-7 than that of regular natto caused a significant increases of serum MK-7 and γ-carboxylated osteocalcin concentrations. Furthermore, it was confirmed that the dietary intake of 50 g of reinforced natto with 1.5 and 2-fold MK-7/100 g natto compared to that of regular natto for 14 days may cause significant increases of serum MK-7 and γ-carboxylated osteocalcin concentrations as compared with that of the intake of regular natto. The dietary intake of more than an appropriate amount of MK-7 may play a role in the prevention of age-related bone loss and aging-induced osteoporosis.

On the basis of these findings, the MK-7 reinforced natto was applied in order to obtain the permission as a Food for Specified Health Use from the Ministry of Health, Labour and Welfare, and it was approved as the first Food for the Specified Health Use, which can stimulate bone formation with the direct action on bone tissues.

This study provides a benefit finding in the prevention of osteoporosis, which is recognized as a major public health problem in accompanying with the advanced aged-society.

Acknowledgements

This study was undertaken in collaboration with the laboratory of Endocrinology and Molecular Metabolism, Graduate School of Nutritional Science, University of Shizuoka. I thank Professor Masayoshi Yamaguchi and his colleagues for their collaborations.

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