# Enhancing Effect of Zinc and Vitamin $K_2$ (Menaquinone-7) on Bone Components in the Femoral Tissue of Female Elderly Rats

**Zhong Jie Ma,[a] Aki Igarashi,[a] Konosuke Yamakawa,[b] and Masayoshi Yamaguchi*,[a]**

*[a]Laboratory of Endocrinology and Molecular Metabolism, Graduate School of Nutritional Sciences, University of Shizuoka, 52–1 Yada, Shizuoka 422–8526, Japan and [b]Department of Health Foods and Fine Chemicals, Honen Corporation, 1–2–3 Ohtemachi, Chiyoda-ku, Tokyo 100–8150, Japan*

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The effect of zinc and vitamin $K_2$ (menaquinone-7) on bone components in the femoral tissue of female elderly rats was investigated. Rats were orally administrated either vehicle (distilled water), zinc sulfate (1.0 mg Zn/100 g body weight), menaquinone-7 (MK-7, 0.5 mg/100 g) or zinc (1.0 mg/100 g) plus MK-7 (0.5 mg/100 g) once a day for 7 days. Femoral dry weight was significantly increased by the administration of both zinc and MK-7, although a significant change was not seen by zinc or MK-7 alone. Calcium content in the femoral-diaphyseal and metaphyseal tissues was significantly increased by zinc administration. Such an increase was not found by MK-7. Bone calcium content was synergistically enhanced by the administration of both zinc and MK-7. Alkaline phosphatase activity and deoxyribonucleic acid (DNA) content in the diaphyseal and metaphyseal tissues were significantly increased by zinc or MK-7 administration; these increases were additively enhanced by both zinc and MK-7. Moreover, the intake of supplement containing both zinc (1.675 mg/100 g) and MK-7 (168.8 $\mu$g/100 g) once a day for 15 days caused a significant increase in femoral dry weight, alkaline phosphatase activity, DNA, calcium and zinc contents in the diaphyseal and metaphyseal tissues. This study demonstrates that the administration of both zinc and MK-7 can enhance additively or synergistically bone components in female elderly rats, suggesting a role in the prevention of osteoporosis with increasing age.

**Key words** —— zinc, vitamin $K_2$, menaquinone-7, bone metabolism, osteoporosis

## INTRODUCTION

Bone loss with increasing age induces osteoporosis.[1–3] This loss may be due to increased bone resorption and decreased bone formation. Osteoporosis with a decrease in bone mass is widely recognized as a major public health problem.[4] Nutritional factors may be important in the prevention of bone loss with increasing age. This, however, is poorly understood.

Pharmacological factors, which can stimulate bone formation, have not been fully clarified. Zinc, an essential trace element, has been demonstrated to have a potent stimulatory effect on bone formation.[5–9] Zinc can stimulate protein synthesis in osteoblastic cells and bone tissue culture systems *in vitro* by means of activating aminioacyl–tRNA synthetase.[10–12] The oral administration of a zinc compound can prevent bone loss in ovariectomized rats,[13,14] an animal model of osteoporosis.

Vitamin $K_2$ is suggested to play a role in preventing age-related bone loss. Vitamin $K_2$ is essential for the $\gamma$-carboxylation of osteocalcin, a bone matrix protein containing $\gamma$-carboxyglutamic acids, which is synthesized in osteoblasts of bone tissues.[15–17] Menaquinone-7 (MK-7) with seven isoprene units, an analog of vitamin $K_2$, is abundant in fermented soybean (natto).[18] An appropriate amount of MK-7 may be significant in preventing age-related bone loss, however, the biological effect of this substance has not yet been determined. It was recently demonstrated that the prolonged dietary intake of MK-7 has a preventive effect on bone loss induced by ovariectomy in rats.[18,19]

The combination of nutritional factors may have an additive or synergistic effect on bone loss with increasing age. It has been reported that MK-7 can

*To whom correspondence should be addressed: Laboratory of Endocrinology and Molecular Metabolism, Graduate School of Nutritional Sciences, University of Shizuoka, 52–1 Yada, Shizuoka 422–8526, Japan. Tel.: +81-54-264-5580; Fax: +81-54-264-5580; E-mail: yamaguch@fnsl.u-shizuoka-ken.ac.jp

stimulate bone calcification in the femoral-metaphyseal tissues of normal and skeletal-unloaded rats *in vitro*, and that this effect is synergistically enhanced by zinc.[20]

This study, therefore, was undertaken to determine whether the combination of zinc and MK-7 has an additive or synergistic effect on bone components in female elderly rats. Such an effect has been found to be induced by the oral administration of zinc and MK-7 to aged rats.

## MATERIALS AND METHODS

**Animals** —— Female Wistar rats (conventional) weighing 210–240 g (50 weeks old) were obtained from Japan SLC (Hamamatsu, Japan). The animals were fed commercial laboratory chow (solid) containing 1.1% calcium and phosphorus at a room temperature of 25°C, with free access to distilled water.
**Administration Procedures** —— For group 1, distilled water (0.5 ml/100 g body weight) was orally administered. For group 2, zinc sulfate was dissolved in distilled water a concentration of 1.0 mg Zn per ml. For group 3, vitamin $K_2$ (menaquinone-7; MK-7) was dissolved in 20% ethanol solution (0.5 ml/100 g body weight) and was orally administered to rats through a stomach tube once a day for 7 days. For group 4, MK-7 (0.5 mg/100 g body weight) was orally administrated immediately after the oral administration of zinc (1.0 mg/100 g) to rats. The animals were sacrificed 24 hr after the last administration by cardiac puncture under light ether anesthesia, and the blood and femur were removed immediately.

In other experiments, aged rats (50 weeks old) were orally administered a suspension (0.25 ml/100 g body weight) of supplement containing zinc (1.675 mg/100 g body weight) and MK-7 (168.8 $\mu$g/100 g) through a stomach tube once a day for 15 days, and 24 hr after the last administration the animals were sacrificed by desanaguination. This suspension (Honen Corporation, Tokyo) contained 45 g of fermented soybean oil (including 1,500 ppm MK-7), 6.7 g of zinc yeast (including 10% Zn), 5 g of natural tocopherols (including 40% D-$\alpha$-tocopherol), 26.7 g of soybean oil, and 16.7 g bees wax per 100 ml.
**Analytical Procedures** —— Blood samples were centrifuged for 30 min after collection, and the serum was separated and analyzed immediately. Serum calcium and zinc were determined by atomic absorption spectrophotometry.[21] Serum inorganic phosphorus was measured by the method of Tausky and Shon.[22]

Femur was cleaned of soft tissue, and dried for 16 hr at 110°C and weighed. The femoral-diaphyseal and metaphyseal tissues were separated and weighted. These bone tissues were determined by atomic absorption spectrophotometry.[5] The calcium and zinc contents in bone tissues was expressed as milligram per gram of dry bone.

To assay bone alkaline phosphatase activity, the diaphyseal and metaphyseal tissues were immersed in 3.0 ml ice-cold 6.5 mM barbital butter (pH 7.4), cut into small pieces, homogenized with a Physcotron homogenizer, and disrupted for 60 sec with an ultrasonic device. The supernatant centrifuged at $600 \times g$ for 5 min was used to measure enzyme activity. Enzyme assay was carried out under optimal conditions. Alkaline phosphatase activity was determined by the method of Walter and Schutt.[23] Enzyme activity was expressed as $\mu$mol of *p*-nitrophenol liberated per min per mg protein. The protein concentration was determined by the method of Lowry *et al.*[24]

To measure bone DNA content, the diaphyseal and metaphyseal tissues were shaken with 4.0 ml of ice-cold 0.1 N NaOH solution for 24 hr after homogenization of the bone tissues.[25] After alkali extraction, the samples were centrifuged at $100 \times g$ for 5 min, and the supernatant was collected. DNA content in the supernatant was determined by the method of Ceriotti[26] and expressed as the amount of DNA (milligrams) / gram weight of bone tissue.
**Statistical Analysis** —— The significance of the difference between values was estimated by Student's *t*-test. *p*-Values of less than 0.05 were considered to indicate statistically significant differences. We also used a multiway analysis of variance (ANOVA) and Turky–Kramer multiple comparison test to compare the treatment groups.

## RESULTS

### Effect of Zinc and MK-7 on Bone Components in Rats

Female elderly rats were orally administered either vehicle (distilled water), zinc sulfate (1.0 mg Zn/100 g body weight), menaquinone (MK-7; 0.5 mg/100 g) or zinc (1.0 mg/100 g) plus MK-7 (0.5 mg/100 g) once a day for 7 days. Body weight of rats was not significantly altered by the adminis-

**Table 1.** Change in Serum Components in Elderly Female Rats Orally Administered Zinc and MK-7

| Treatment | Calcium (mg/dl) | Inorganic phosphorus (mg/dl) | Zinc ($\mu$g/dl) |
|---|---|---|---|
| Control | 9.25 ± 0.12 | 4.55 ± 0.19 | 127.1 ± 6.6 |
| Zinc | 9.24 ± 0.15 | 4.50 ± 0.17 | 146.4 ± 4.2* |
| MK-7 | 9.27 ± 0.16 | 4.55 ± 0.19 | 126.3 ± 5.4 |
| Zinc + MK-7 | 9.30 ± 0.16 | 4.56 ± 0.19 | 146.8 ± 4.5* |

Rats were orally administrated either vehicle (distilled water), zinc sulfate (1 mg Zn/100 g body weight), MK-7 (0.5 mg/100 g) or zinc (1 mg/100 g) plus MK-7 (0.5 mg/100 g) once a day for 7 days, and 24 hr after the last administration they were sacrificed. Each value is the mean ± S.E.M. of five rats. *$p < 0.01$ compared with the control value.

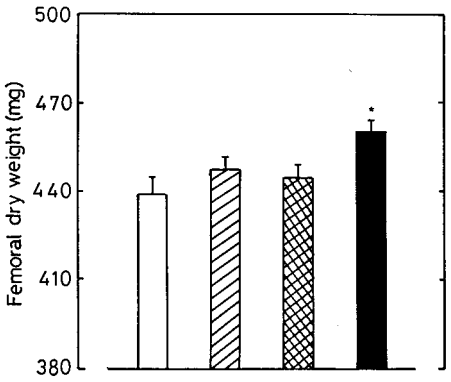


**Fig. 1.** Change in Dry Weight of the Femoral Tissue of Rats Administered Zinc and MK-7

Rats were orally administered either vehicle (distilled water), zinc sulfate (1 mg Zn/100 g body weight), MK-7 (0.5 mg/100 g) or zinc (1 mg/100 g) plus MK-7 (0.5 mg/100 g) once a day for 7 days. Animals were sacrificed 24 hr after the last administration. Each value is the mean ± S.E.M. of five rats. *$p < 0.01$, compared with the control value. White bar, control; hatched bar, zinc; double hatched bar, MK-7; black bar, zinc plus MK-7.

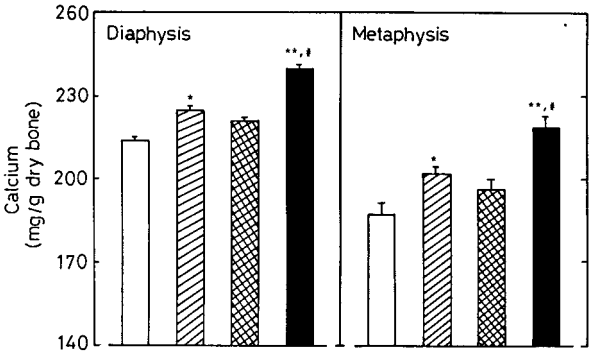


**Fig. 2.** Change in Calcium Content in the Femoral-Diaphyseal and Metaphyseal Tissue in Rats Administered Zinc and MK-7

Rats were orally administered zinc and MK-7 as described in Fig. 1. Each value is the mean ± S.E.M. of five rats. *$p < 0.05$ and **$p < 0.01$, compared with the control value. #$p < 0.01$, compared with the value for zinc or MK-7 alone. White bar, control; hatched bar, zinc; double hatched bar, MK-7; black bar, zinc plus MK-7.

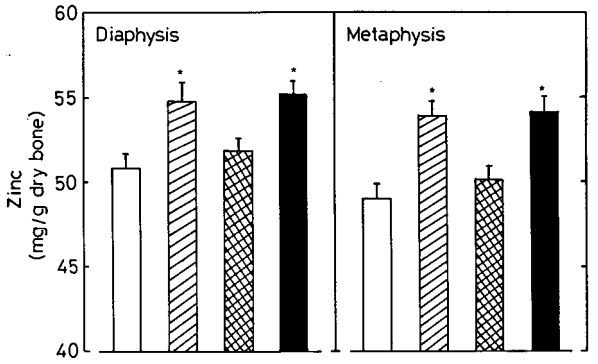


**Fig. 3.** Change in Zinc Content in the Femoral-Diaphyseal and Metaphyseal Tissue of Rats Administered Zinc and MK-7

Rats were orally administered zinc and MK-7 as described in Fig. 1. Each value is the mean ± S.E.M. of five rats. *$p < 0.05$, compared with the control value. White bar, control; hatched bar, zinc; double hatched bar, MK-7; black bar, zinc plus MK-7.

tration of zinc, MK-7, or zinc plus MK-7 (data not shown). Serum calcium and inorganic phosphorus concentrations were not significantly changed by those administration (Table 1). Serum zinc concentration was significantly raised by the administration of zinc or zinc plus MK-7 (Table 1).

The change in femoral dry weight of rats administered zinc and MK-7 is shown in Fig. 1. Femoral dry weight was significantly increased by the administration of both zinc and MK-7, although it was not raised by the administration of zinc or MK-7 alone.

The change in calcium content in the femoral-diaphyseal and metaphyseal tissues of rats administered zinc and MK-7 is shown in Fig. 2. Zinc administration caused a significant increase in calcium content in the femoral-diaphyseal and metaphyseal tissues. Such an increase was not seen by MK-7 administration. The diaphyseal and metaphyseal calcium contents were synergistically increased by the administration of both zinc and MK-7 as compared with that of zinc or MK-7 administration.

Zinc content in the femoral-diaphyseal and metaphyseal tissues was significantly increased by the administration of zinc or both zinc and MK-7 (Fig. 3). MK-7 administration did not cause a significant alteration in the femoral zinc content.

The change in alkaline phosphatase activity and DNA content in the femoral-diaphyseal and metaphyseal tissues of rats administrated zinc and MK-7 is shown in Figs. 4 and 5. Alkaline phosphatase activity and DNA content in the diaphyseal and meta-

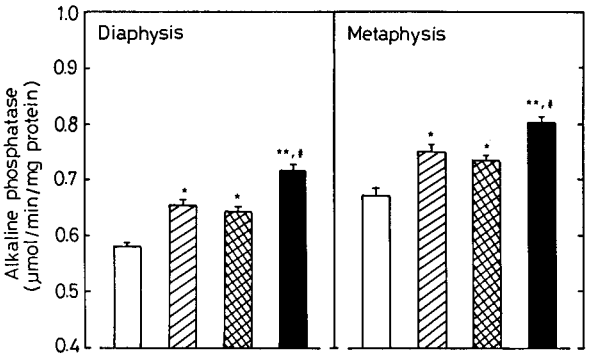


**Fig. 4.** Change in Alkaline Phosphatase Activity in the Femoral-Diaphyseal and Metaphyseal Tissue of Rats Administered Zinc and MK-7

Rats were orally administered zinc and MK-7 as described in Fig. 1. Each value is the mean ± S.E.M. of five rats. $*p < 0.05$ and $**p < 0.01$, compared with the control value. $^{\#}p < 0.025$, compared with the value for zinc or MK-7 alone. White bar, control; hatched bar, zinc; double hatched bar, MK-7; black bar, zinc plus MK-7.

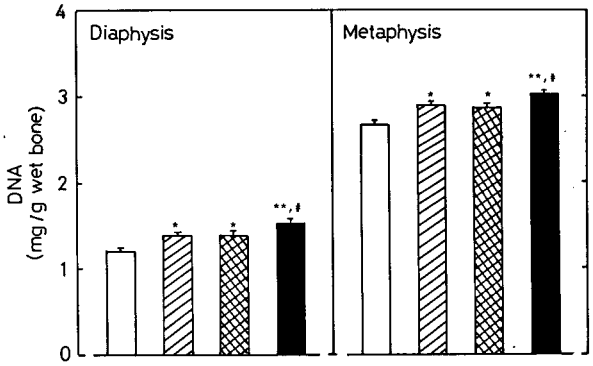


**Fig. 5.** Change in DNA Content in the Femoral-Diaphyseal and Metaphyseal Tissues of Rats Administered Zinc and MK-7

Rats were orally administered zinc and MK-7 as described in Fig. 1. Each value is the mean ± S.E.M. of five rats. $*p < 0.05$ and $**p < 0.01$, compared with the control value. $^{\#}p < 0.05$, compared with the value for zinc or MK-7 alone. White bar, control; hatched bar, zinc; double hatched bar, MK-7; black bar, zinc plus MK-7.

physeal tissues were significantly increased by the administration of zinc or MK-7. These increases were significantly enhanced by the administration of both zinc and MK-7. This enhancing effect was additive.

### Effect of Supplement Containing Zinc and MK-7 on Bone Components in Rats

Female elderly rats were orally administered supplement containing zinc (1.675 mg/100 g body weigh) and MK-7 (168.8 μg/100 g) once a day for 15 days. Serum calcium and inorganic phosphorus concentrations were not significantly altered by the intake of supplement, although serum zinc concentration was significantly increased by the intake of supplement (Table 2). Femoral dry weight was significantly increased by supplemental intake (Fig. 6A) Body weight of rats was not changed by supplemental intake (Fig. 6B).

**Table 2.** Change in Serum Components in Rats Orally Administered Supplement Containing Zinc and MK-7

| Treatment | Calcium (mg/dl) | Inorganic phosphorus (mg/dl) | Zinc (μg/dl) |
|---|---|---|---|
| Control | 9.28 ± 0.31 | 4.60 ± 0.27 | 125.8 ± 5.9 |
| Zinc + MK-7 | 9.28 ± 0.16 | 4.53 ± 0.20 | 149.8 ± 4.5* |

Rats were orally administrated either vehicle (distilled water) or supplement containing zinc (1.675 mg/100 g body weight) and MK-7 (168.8 μg/100 g) once a day for 15 days, and 24 hr after the last administration they were sacrificed. Each value is the mean ± S.E.M. of five rats. $*p < 0.01$ compared with the control value.

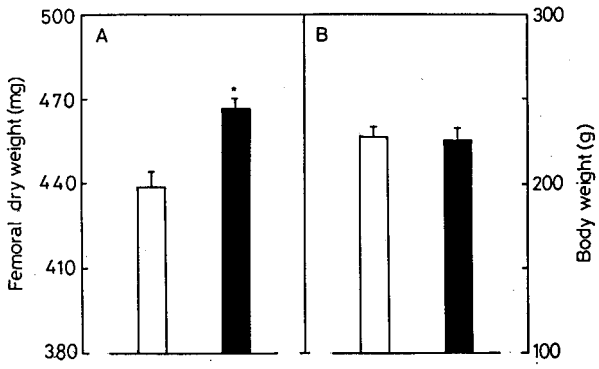


**Fig. 6.** Change in Dry Weight of the Femoral Tissues of Rats Administered Supplement-Containing Zinc and MK-7

Rats were orally administered supplement containing zinc (1.675 mg/100 g body weight) and MK-7 (168.8 μg/100 g) once a day for 15 days. Animals were sacrificed 24 hr after the last administration. The femoral dry weight (A) and body weight (B) were measured. Each value is the mean ± S.E.M. of five rats. $*p < 0.01$, compared with the control value. White bar, control; black bar, supplement containing zinc and MK-7.

The change in calcium and zinc contents, alkaline phosphatase activity, and DNA content in the femoral-diaphyseal and metaphyseal tissues of rats orally administered supplement containing zinc and MK-7 is shown in Table 3. These bone components were significantly increased by supplemental intake.

## DISCUSSION

Nutritional factors may be significant in preventing bone loss with increasing age. Zinc has been shown to stimulate bone formation and calcification.[5–9] The present study confirms that the oral ad-

**Table 3.** Change in Bone Components in the Femoral-Diaphyseal and Metaphyseal Tissue of Rats Orally Administered Supplement Containing Zinc and MK-7

| Treatment | Calcium (mg/g dry bone) | Zinc ($\mu$g/g dry bone) | Alkaline phosphatase ($\mu$mol/min/ mg protein) | DNA (mg/g wet bone) |
|---|---|---|---|---|
| Diaphysis | | | | |
| Control | 214.6 ± 4.2 | 50.8 ± 1.3 | 0.581 ± 0.017 | 1.218 ± 0.058 |
| Supplement | 235.9 ± 4.6* | 57.5 ± 1.3* | 0.709 ± 0.019* | 1.449 ± 0.037* |
| Metaphysis | | | | |
| Control | 187.2 ± 4.9 | 48.8 ± 1.3 | 0.669 ± 0.023 | 2.695 ± 0.047 |
| Supplement | 212.6 ± 5.3* | 57.0 ± 1.7* | 0.799 ± 0.018* | 2.932 ± 0.040* |

Rats were orally administrated either vehicle (distilled water) or supplement containing zinc (1.675 mg/100 g body weight) and MK-7 (168.8 $\mu$g/100 g) once a day for 15 days, and 24 hr after the last administration they were sacrificed. Each value is the mean ± S.E.M. of five rats. *$p < 0.01$ compared with the control value.

ministration of zinc has an anabolic effect on bone components in female elderly rats, suggesting a role in the prevention of bone loss with increasing age.

MK-7 has been demonstrated to stimulate bone mineralization in tissue culture system *in vitro*.[20] The present finding that the oral administration of MK-7 induced an increase in bone components in female elderly rats, supports the view that MK-7 may have a preventive role in bone loss of ovariectomized rats.[18,19] Thus, MK-7 has an anabolic effect on bone metabolism *in vitro* and *in vivo*.

The combination of zinc and MK-7 revealed a synergistic effect on the femoral dry weight and the calcium content in the femoral-diaphyseal and metaphyseal tissues of elderly rats. Zinc has been shown to increase the production of osteocalcin, which is involved in bone mineralization, in osteoblastic cells.[27] Vitamin $K_2$ is essential for the $\gamma$-carboxylation of osteocalcin, a bone matrix protein containing $\gamma$-carboxyglutamic acids, which is synthesized in osteoblasts of bone tissues.[15–17] MK-7 may stimulate the $\gamma$-carboxylation of osteocalcin in the femoral tissues of elderly rats. The combination of zinc and MK-7 may induce a synergistic effect on bone calcium content.

Alkaline phosphatase activity and DNA content in the femoral-diaphyseal and mataphyseal tissues were additively enhanced by the administration of both zinc and MK-7. Alkaline phosphatase is a marker enzyme of osteoblasts, and the enzyme participates in bone mineralization.[28] DNA content is an index of bone growth and the number of bone cells.[29] Both zinc and MK-7 may stimulate the proliferation and function of osteoblastic cells in bone tissues.

The intake of supplement containing zinc and MK-7 caused a significant increase in femoral dry weight, alkaline phosphatase activity, DNA, calcium and zinc contents in the femoral-diaphyseal and metaphyseal tissues of elderly rats, indicating that the intake can reveal an anabolic effect on bone metabolism. This finding suggests that the supplemental intake of zinc and MK-7 has a preventive effect on bone loss with ageing.

In conclusion, it has been demonstrated that the combined administration of zinc and MK-7 has a synergistic or additive enhancing effect on bone components in the femoral tissues of female elderly rats. Supplemental intake with zinc and MK-7 may be a useful tool in preventing osteoporosis with increasing age.

## REFERENCES


1) Nishimoto, S. K., Chang, C.-H., Gendler, E., Stryker, W. F. and Nimni, M. E. (1985) The effect of aging on bone formation in rats: biochemical and histological evidence for decreased bone formation capacity. *Calcif. Tissue Int.*, **37**, 617–624.
2) Schapira, C., Slinn, S., Sarid, M., Mokadi, S., Kabala, A. and Silbermann, M. (1995) Calcium and vitamin D enriched diets increase and preserve vertebral mineral content in aging laboratory rats. *Bone*, **16**, 575–582.
3) Wild, R. A., Buchaman, J. R., Myers, C. and Demers, L. M. (1987) Declining adrenal androgen: an association with bone loss in aging women. *Proc. Soc. Exp. Biol. Med.*, **186**, 335–360.
4) Bonjour, J.-P., Schurch, M.-A. and Rizzori R. (1996) nutritional aspects of hip fractures. *Bone*, **18**, 1395–1445.
5) Yamaguchi, M., Oishi, H. and Suketa Y. (1987)

Stimulatory effect of zinc on bone formation in tissue culture. *Biochem. Pharmacol*., **36**, 4007–4012.
6) Yamaguchi, M. and Matsui R. (1989) Effect of dipicolinate, a chelator of zinc, on bone protein synthesis in tissue culture. The essential role of zinc. *Biochem. Pharmacol*., **38**, 4485–4489.
7) Hashizume, M. and Yamaguchi, M. (1993) Stimulatory effect of β-alanyl-L-histidinato zinc on cell proliferation is dependent on protein synthesis in osteoblastic MC3T3-E1 cells. *Mol. Cell. Biochem*., **122**, 59–64.
8) Yamaguchi, M., Kishi, S. and Hashizume M. (1994) Effect of zinc-chelating dipeptides on osteoblastic MC3T3-E1 cells: Activation of aminoacyl-tRNA synthetase. *Peptides*, **15**, 1367–1371.
9) Yamaguchi, M. and Matsui, T. (1994) Zinc enhancement of 17β-estradiol's anabolic effect in osteoblastic MC3T3-E1 cells. *Calcif. Tissue Int*., **60**, 527–532.
10) Kishi, S. and Yamaguchi, M. (1994) Inhibitory effect of zinc compounds on osteoclast-like cell formation in mouse marrow cultures. *Biochem. Pharmacol*., **48**, 1225–1230.
11) Yamaguchi, M. and Kishi, S. (1996) Zinc compounds inhibit osteoclast-like cell formation at the earlier stage of rat marrow culture but not osteoclast function. *Mol. Cell. Biochem*., **158**, 171–177.
12) Yamaguchi, M. (1998) Role of zinc in bone formation and bone resorption. *J. Trace Elem. Exp. Med*., **11**, 119–135.
13) Yamaguchi, M. and Kishi, S. (1997) Prolonged administration of β-alanyl-L-histidinato zinc prevents bone loss in ovariectomized rats. *Jpn. J. Pharmacol*., **63**, 203–207.
14) Kishi, S., Segawa, Y. and Yamaguchi, M. (1994) Histomorphological confirmation of the preventive effect of β-alanyl-L-histidinato zinc on bone loss in ovariectomized rats. *Biol. Pharm. Bull*., **17**, 862–865.
15) Hauschka, P. V., Lian, J. B., Gallop, P. M. (1975) Direct identification of the calcium-binding amino acid, γ-carboxyglutamate, in mineralized tissue. *Proc. Natl. Acad. Sci. U.S.A*., **72**, 3925–3929.
16) Hauschka, P. V. and Carr, S. A. (1982) Calcium-dependent α-helical structure in osteocalcin. *Biochemistry*, **21**, 2538–2547.
17) Price, P. A. (1985) Vitamin K-dependent formation of bone gla protein (osteocalcin) and its function. *Vitam. Horm*., **42**, 65–108.
18) Yamaguchi, M., Taguchi, H., Gao, Y. H. and Tsukamoto, Y. (1998) Effect of vitamin $K_2$ (menaquinone-7) in fermented soybean (natto) on bone loss in ovariectomized rats. *J. Bone Miner. Metab*., **17**, 23–29.
19) Yamaguchi, M., Kakuda, H., Gao, Y. H. and Tsukamoto, Y. (2000) Prolonged intake of fermented soybean (natto) diets containing vitamin $K_2$ (menaquinone-7) prevents bone loss in ovariectmized rats. *J. Bone Miner. Metab*., **18**, 71–76.
20) Ehara, Y., Takahashi, H., Hanahisa, Y. and Yamaguchi, M. (1996) Effect of vitamin $K_2$ (menaquinone-7) on bone metabolism in the femoral-metaphyseal tissues of normal and skeletal-unloaded rats: enhancement with zinc. *Res. Exp. Med*., **196**, 171–178.
21) Willis, J. B. (1960) Determination of calcium in blood serum by atomic absorption spectroscopy. *Nature* (London), **186**, 249–250.
22) Taussky, H. H. and Shon, E. (1953) A microcolorimetric method for the determination of inorganic phosphorus. *J. Biol. Chem*., **202**, 675–685.
23) Walter, K. and Schuut, C. (1974) Acid and alkaline phosphatase in serum. In *Methods of Enzymatic Analysis*, (Bergmeger, H. V., Ed.), Academic Press, New York, Vol. 1–2, pp. 856–860.
24) Lowry, O. H., Rosebrough, N. J., Farr. A. L. and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem*., **193**, 265–273.
25) Flanagan, B. and Nichols, G. Jr. (1962) Metabolic studies of bone in vitro. IV. Collagen biosynthesis by surviving bone fragments in vitro. *J. Biol. Chem*., **237**, 3786–3789.
26) Ceriotti, G. (1955) Determination of nucleic acids in animal tissues. *J. Biol. Chem*., **214**, 29–77.
27) Yamaguhci, M. and Hashizume M. (1994) Effect of β-alanyl-L-histidinato zinc on protein components in osteoblastic MC3T3-E1 cells: increase in osteocalcin, insulin-like growth factor-I and transforming growth factor-β. *Mol. Cell. Biochem*., **136**, 163–169.
28) Canalis, E., Centrella, M., Burch, W. and McCarthy, T. L. (1989) Insulin-like growth factor I mediates selective anabolic effects of parathyroid hormone in bone cultures. *J. Clin. Invest*., **83**, 60–65.
29) Lau, K.-H. W., Tanimoto, H. and Baylink, D. J. (1988) Vanadate stimulates bone cell proliferation and bone collagen synthesis in vitro. *Endocrinology*, **123**, 2858–2867.