Food Sci. Technol. Res., **9** (1), 87–90, 2003

Note

Stimulatory Effect of Wasabi Leafstalk Extract (*Wasabia japonica* MATSUM.) on Bone Calcification: Interaction with Bone Anabolic Factors in Mouse Calvaria Tissue *in Vitro*

Toshihiro SUZUKI[1] and Masayoshi YAMAGUCHI[2]

[1]*Shizuoka Industrial Research Institute of Shizuoka Prefecture, 2078 Makigaya Shizuoka 421-1298, Japan*
[2]*Laboratory of Endocrinology and Molecular Metabolism, Graduate School of Nutritional Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422-5826, Japan*

Received July 17, 2002; Accepted November 16, 2002

The stimulatory effect of wasabi leafstalk extract (*Wasabia japonica* MATSUM.) on bone calcification was compared with the anabolic effect of insulin, insulin-like growth factor I (IGF-I), 17β-estradiol, and genistein. Mouse calvaria tissues were cultured for 48 h in a serum-free Dulbecco's modified Eagle's medium (high glucose, 4.5%). The presence of wasabi leafstalk extract (25 μg/ml) caused significant increases in bone calcium content. The combination of 10^{-10} M 17β-estradiol and 15 μg/ml wasabi leafstalk extract had an additive effect on bone calcium content, while the increasing effect of phyotoestrogen genistein (10^{-9} or 10^{-7} M) on this content was not significantly greater in the presence of the same amount of the extract. Insulin (10^{-8} or 10^{-7} M) had a significant effect on bone calcium content, and the combination of both 10^{-8} or 10^{-7} M insulin with 15 μg/ml wasabi leafstalk extract had an additive effect. Such an effect was not seen in the case of IGF-I (10^{-8} M), however which increased bone calcium content. The present study demonstrates that wasabi leafstalk extract has an enhancing effect on the anabolic action of 17β-estradiol or insulin, which regulates bone formation and calcification *in vitro*.

Keywords: wasabi leafstalk , bone calcification, 17β-estradiol, insulin, IGF-I, genistein

The decrease in bone mass with increasing age induces osteoporosis (Cooper & Melton, 1992). Bone loss is due to increased bone resorption and to decreased bone formation. Pharmacological and nutritional factors are needed to prevent bone loss with increasing age (Bonjour *et al*., 1996). Recently, isoflavone (genistein and daidzein) has been demonstrated to have a direct stimulatory effect on bone formation and an inhibitory effect on bone resorption in a tissue culture system *in vitro* (Yamaguchi, 2002 for a review). The combination of genistein and zinc has been shown to have a synergetic effect in the prevention of bone loss in aged rats (Gao & Yamaguchi, 1998). Thus, food and nutritional factors may play an important role in the prevention of osteoporosis with ageing, although this has not yet been fully confirmed.

We reported previously that wasabi leafstalk extract (*Wasabia japonica* MATSUM.) contains active components which stimulate bone calcification in a tissue culture system *in vitro* (Suzuki *et al*., 1997). The oral administration of wasabi leafstalk extract to rats has also been shown to have an anabolic effect on bone components *in vivo* (Suzuki & Yamaguchi, 1999a). The active component in this extract has been suggested to be a low molecular component (Suzuki & Yamaguchi, 1999b), and the extract may have a role as a food factor in preventing bone loss with ageing.

The present study was undertaken to determine whether wasabi leafstalk extract can modulate the action of bone-regulating factors which have a physiologically anabolic effect on bone calcification of mouse calvaria tissue *in vitro*. We found that the extract can enhance the anabolic effect of 17β-estradiol, genistein, and insulin on bone calcification.

E-mail: toshi@s-iri.pref.shizuoka.jp

Materials and Methods

Chemicals Dulbecco's modified Eagle's medium (high glucose, 4.5 g/100 ml) and penicillin-streptomycin solution (5000 U/ml penicillin; 5000 μg/ml streptomycin) were obtained from Gibco Laboratories (Grand Island, N.Y.). Bovine serum albumin (fraction V), 17β-estradiol , and genistein were obtained from Sigma Chemical (St. Louis, MO.). Other chemicals were reagent grade from Wako Pure Chemical Industries (Osaka).

Wasabi leafstalk extract Wasabi leafstalks (*Wasabia japonica* MATSUM.) were collected in May 2000 and used for the experiment. Fresh wasabi leafstalk (about 50 g) was homogenized for 3 min in 20% ethanol solution (150 ml), and the homogenate was centrifuged at 10000×*g* for 20 min. The resulting supernatant was filtered through filtration paper, and the filtration solution was then extracted 3 times with diethyl ether (about 150 ml). The ether phase was removed, and the resulting aqueous phase was freeze-dried. The powder was dissolved in distilled water. Before wasabi leafstalk extract was used in bone culture experiments, the extract solution was aseptically filtered through a membrane filter (0.22 μm).

Bone culture Male ddy mice (4 weeks old) were obtained from Japan SLC Inc. (Hamamatsu). These animals were fed commercial laboratory chow (solid; Oriental Yeast Co., Ltd., Tokyo) containing 57.4% carbohydrate, 1.1% Ca, and 1.1% P at a room temperature of 25°C and were given distilled water freely until sacrifice. The mice were sacrificed by decapitation under

light anesthesia with diethyl ether. The calvaria tissues were removed aseptically and cut along the sagittal suture into left and right halves. One-half of each calvarium served as a control for its paired, treated half. Calvaria fragments were cultured for 48 h in a 35-mm dish in 2.0 ml medium consisting of Dulbecco's modified Eagle's medium (high glucose, 4.5 g/100 ml) supplemented with 0.25% bovine serum albumin plus antibiotics (100 U penicillin and 100 μg streptomycin/ml of medium) (Yamaguchi *et al*., 1987). The experimental medium contained either vehicle or wasabi leafstalk extract (5, 12.5 or 25 μg/ml) in the absence or presence of 17β-estradiol (10^{-10}–10^{-8} M), insulin (10^{-9}–10^{-7} M), IGF-I (10^{-10}–10^{-8} M) or genistein (10^{-9}–10^{-7} M). Cultures were maintained at 37°C in a water-saturated atmosphere containing 5% CO_2 and 95% air.

Analytical procedures Calvaria tissues cultured were dried for 16 h at 110°C, weighed, and then digested with nitric acid (2.0 ml) for 12 h at 120°C. Calcium was determined by atomic absorption spectrophotometry (Yamaguchi *et al*., 1987), and the calcium content was calculated as mg/g dry bone. Data were represented as the percentage (%) of control (none) value.

Statistical analysis The significance of difference between values was estimated by Student's *t*-test. *p* Value of less than 0.05 was considered to show statistically significant differences. We also used a multi-way ANOVA with a Duncan's post hoc test to compare the treatment groups.

Results

The effect of increasing concentrations of wasabi leafstalk extract on calcium content in bone tissues cultured without or with 17β-estradiol is shown in Fig. 1. Calvaria tissues were cultured for 48 h in the medium either vehicle or wasabi leafstalk extract (5–25 μg/ml of medium) in the absence or presence of 17β-estradiol (10^{-9} M). Bone calcium content was significantly increased in the presence of 25 μg/ml of the extract. The effect of this amount of extract in increasing bone calcium content was not significantly enhanced in the presence of 17β-estradiol (10^{-9} M).

The effect of increasing concentrations of 17β-estradiol on calcium content in bone tissues cultured in the presence of wasabi leafstalk extract is shown in Fig. 2. Calvaria tissues were cultured for 48 h in the medium containing either vehicle or 17β-estradiol (10^{-10}–10^{-8} M) in the absence or presence of 15 μg/ml of extract. Bone calcium content was significantly increased in the presence of 17β-estradiol (10^{-10}–10^{-8} M). The anabolic effect of 17β-estradiol (10^{-10}–10^{-8} M) on bone calcium content was not significantly enhanced in the presence of 15 μg/ml of wasabi leafstalk extract. However, the combination of 10^{-10} M 17β-estradiol and 15 μg/ml of extract had an additive effect on bone calcium content as compared with the value of 10^{-10} M 17β-estradiol alone.

The effect of genistein, a phytoestrogen, on calcium content in

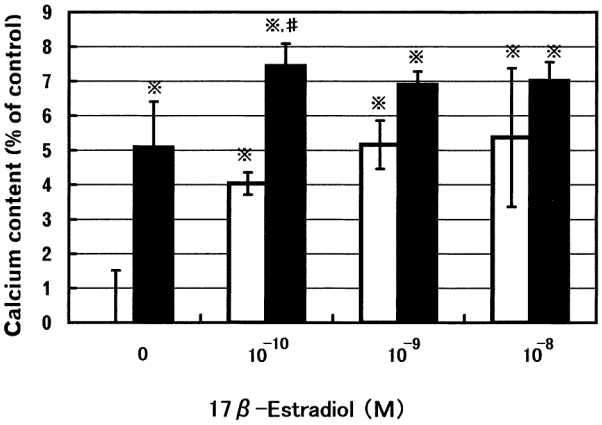

Fig. 2. Effect of increasing concentrations of 17β-estradiol on calcium content in mouse calvaria tissues cultured with wasabi leafstalk extract. Tissues were cultured for 48 h in medium containing either vehicle or 17-estradiol (10^{-10}–10^{-8} M) in the absence or presence of extract (15 μg/ml of medium). Each value represents the mean±SEM of five different animals. ※ $p<0.01$, compared with the control (none) value. # $p<0.01$, compared with the value of 17β-estradiol alone. □, 17β-Estradiol; ■, Wasabi leafstalk extract plus 17β-estradiol.

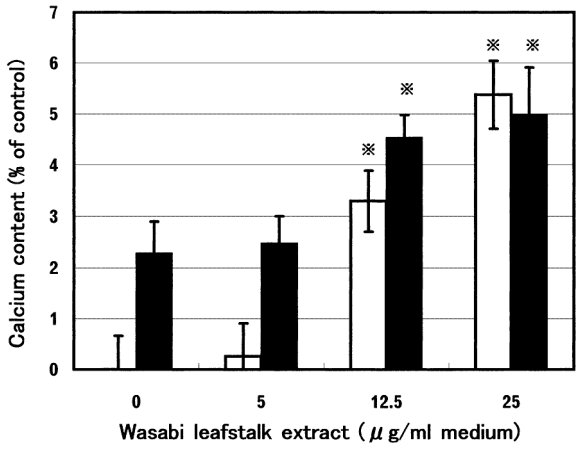

Fig. 1. Effect of increasing concentrations of wasabi leafstalk extract on calcium content in mouse calvaria tissues cultured with 17β-estradiol. Tissues were cultured for 48 h in medium containing either vehicle or extract (5, 17.5, or 25 μg/ml of medium) in the absence or presence of 17β-estradiol (10^{-9} M). Each value represents the mean±SEM of five different animals. ※ $p<0.01$, compared with the control (none) value. # $p<0.01$, compared with the value of 17β-estradiol alone without wasabi leafstalk extract addition. □, Wasabi leafstalk extract ; ■, 17β-Estradiol plus wasabi leafstalk extract.

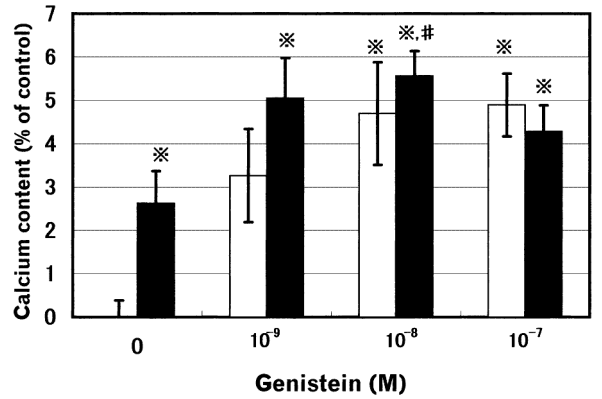

Fig. 3. Effect of genistein, a phytoestrogen, on calcium content in mouse calvaria tissues cultured with wasabi leafstalk extract. Tissues were cultured for 48 h in medium containing either vehicle or genistein (10^{-9}–10^{-7} M) in the absence or presence of extract (15 μg/ml). Each value represents the mean±SEM of five different animals. ※ $p<0.01$, compared with the control (none) value. # $p<0.01$, compared with the value of extract alone. □, Genistein; ■, Wasabi leafstalk extract plus genistein.

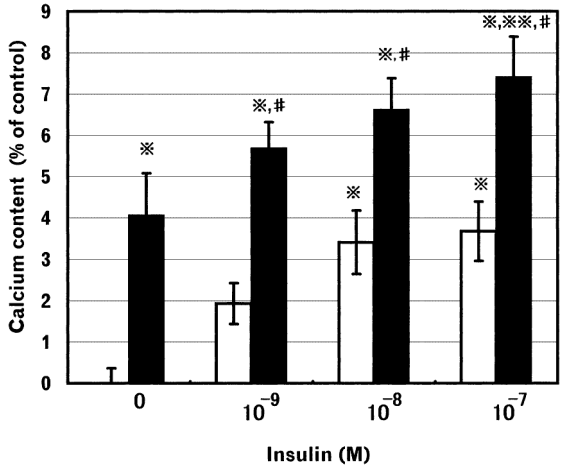

Fig. 4. Effect of insulin on calcium content in mouse calvaria tissues cultured with wasabi leafstalk extract. Tissues were cultured for 48 h in medium containing either vehicle or insulin (10^{-9}–10^{-7} M) in the absence or presence of extract (15 μg/ml) . Each value represents the mean±SEM of five different animals. ※ $p<0.01$, compared with the control (none) value. ※※ $p<0.01$, compared with the value of wasabi leafstalk extract alone. # $p<0.01$, compared with the value of insulin alone. □, Insulin; ■, Wasabi leafstalk extract plus insulin.

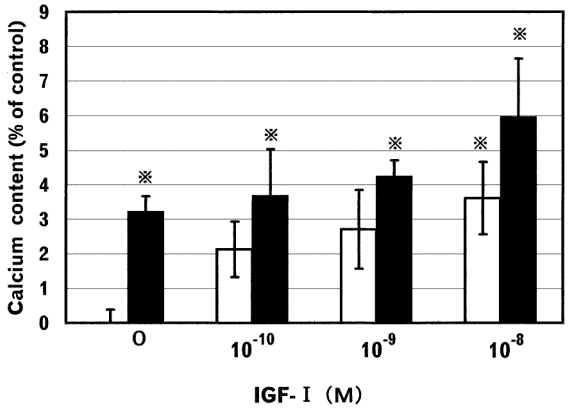

Fig. 5. Effect of IGF-I on calcium content in mouse calvaria tissues cultured with wasabi leafstalk extract. Tissues were cultured for 48 h in medium containing either vehicle or IGF-I in the absence or presence of extract (15 μg/ml). Each value represents the mean±SEM of five different animals. ※ $p<0.01$, compared with the control (none) value. □, IGF-I; ■, Wasabi leafstalk extract plus IGF-I.

bone tissues cultured in the presence of wasabi leafstalk extract is shown in Fig. 3. Calvaria tissues were cultured for 48 h in the medium containing either vehicle or genistein (10^{-7}–10^{-9} M) in the absence or presence of the extract (15 μg/ml). Bone calcium content was significantly increased in the presence of genistein (10^{-8}–10^{-7} M). The anabolic effect of 10^{-8} or 10^{-7} M genistein on bone calcium content was not significantly enhanced by the addition of 15 μg/ml of wasabi leafstalk extract.

The effect of insulin on calcium content in bone tissues cultured in the absence or presence of wasabi leafstalk extract is shown in Fig. 4. Bone calcium content was significantly increased in the presence of 10^{-8} or 10^{-7} M insulin. The effect of 15 μg/ml of wasabi leafstalk extract in increasing bone calcium content was not significantly enhanced in the presence of insulin (10^{-9}–10^{-7} M). However, the combination of 15 μg/ml of extract and insulin (10^{-9}–10^{-7} M) had an additive effect on this content as compared with the value of insulin alone.

The effect of IGF-I on calcium content in bone tissues cultured in the absence or presence of wasabi leafstalk extract is shown in Fig. 5. The presence of 10^{-8} M IGF-I caused a significant increase in bone calcium content, but this effect was not additively enhanced in the presence of 15 μg/ml of the extract.

Discussion

The decrease in bone mass with increasing age induces osteoporosis. Food and nutritional factors may play a role in the prevention of bone loss with aging (Bonjour *et al*., 1996). Isoflavones and menaquinone-7 (vitamin K_2) in fermented soybean (*natto*) have been demonstrated to have an anabolic effect on bone mass in rats (Yamaguchi, 2002; Yamaguchi *et al*., 2000). The intake of fermented soybean has been shown to increase serum levels of menaquinone-7 and γ-carboxylated osteocalcin in normal individuals (Tsukamoto *et al*., 2000), suggesting that the dietary intake of *natto* in daily life can stimulate bone mineralization.

Of various plant and food extracts, wasabi leafstalk extract was found to have a unique stimulatory effect on bone calcium content using mouse calvaria tissues *in vitro* (Suzuki *et al*., 1997). It has further been demonstrated that the oral administration of this extract to rats has an anabolic effect on bone metabolism *in vivo* (Suzuki & Yamaguchi, 1999a), suggesting the usefulness of this food component(s) in the preventing bone loss.

The present study was undertaken to determine whether wasabi leafstalk extract can modulate the effect of bone-regulating factors on bone calcification *in vitro*. The presence of 17β-estradiol (10^{-10}–10^{-8} M), phytoestrogen genistein (10^{-10}–10^{-7} M), insulin (10^{-10}–10^{-7} M), or IGF-I (10^{-8} M) in culture medium caused a significant increase of bone calcium content in mouse calvaria tissue culture *in vitro*. The effect of 17β-estradiol (10^{-10} M) or insulin (10^{-9}–10^{-7} M) in stimulating bone calcium content was found to be additively enhanced when combined with wasabi leafstalk extract (15 μg/ml). In addition, the effect of this extract in increasing bone calcium content was additively enhanced in the presence of genistein (10^{-8} M) or insulin (10^{-7} M) as compared with that of the extract alone. From these findings, it is speculated that the intake of wasabi leafstalk extract may modulate the anabolic effect of 17β-estradiol or insulin on bone formation and calcification. This may be a significant mechanism of metabolic regulation by food factors.

The mechanism by which wasabi leafstalk extract enhances 17β-estradiol or genistein-increased bone calcium content is unknown at present. The receptors of 17β-estradiol are located on the nucleus of osteoblastic cells which are related to bone formation (Gray *et al*., 1987; Eriksen *et al*., 1988). Genistein has been shown to bind to estrogen's receptors in osteoblastic cells (Kuiper *et al*., 1997). 17β-Estradiol or genistein can stimulate bone formation by increasing the protein synthesis in osteoblastic cells (Gray *et al*., 1987; Sugimoto & Yamaguchi, 2000). The active component in wasabi leafstalk extract presumably modulates the binding of 17β-estradiol or genistein to the estrogen receptors which are localized in the nucleus of osteoblastic cells in bone tissues. Moreover, it is possible that the extract may enhance transcription activity which is mediated through 17β-estradiol or genistein action in the nucleus of these cells.

Insulin or IGF-I has been shown to stimulate bone formation in osteoblastic cells (Levy *et al*., 1986; Kream *et al*., 1985; Hock *et al*., 1988; Schmid *et al*., 1989; Canalis *et al*., 1993). The receptors of insulin or IGF-I are located on the plasma membranes of these cells (Ituarte *et al*., 1989; Centrella *et al*., 1990), and the actions of these factors are mediated through signal transduction in osteoblastic cells (Saltiel *et al*., 1987; Canalis *et al*., 1993). Wasabi leafstalk extract enhanced the anabolic effect of insulin on bone calcification *in vitro*, while it had no effect of IGF-I.The active component in this extract thus may modulate the signaling processes of insulin action in osteoblastic cells.

The cellular mechanism by which the active component in wasabi leafstalk extract stimulates bone calcification remains to be elucidated. Stimulation by the extract on bone calcification was found to be completely prevented in the presence of cycloheximide, an inhibitor of protein synthesis, suggesting that the effect of the extract may be caused by newly synthesized protein components. It is speculated that the protein components, which are synthesized by stimulation of the extract, may be partly involved in the action of 17β-estradiol, genistein or insulin in osteoblastic cells.

In conclusion, it has been demonstrated that the effect of bone-regulating factors (17β-estradiol, genistein or insulin) in increasing bone calcium content in mouse calvaria tissues *in vitro* is enhanced by the active component in wasabi leafstalk extract. This finding may support the view that the dietary intake of the extracts may have a role in preventing the bone loss which often accompanies aging.

References

Bonjour, J.-P., Schurch, M.-A. and Rizzori, R. (1996). Nutritional aspects of hip fracture. *Bone*, **18**, 139S–144S.

Canalis, E., Dash, J., Gabbitas, B., Rydziel, S. and Vanghese, S. (1993). Growth factors regulate the synthesis of insulin-like growth factor-I in bone cell culture. *Endocrinology*, **133**, 33–38.

Centrella, M., McCarthy, T. L. and Canalis, E. (1990). Receptors for insulin-like growth factors-I and -II in osteoblast-enriched cultures from fetal rat bone. *Endocrinology*, **126**, 39–44.

Cooper, C. and Melton, J. III, (1992). Epidemiology of osteoporosis. *Trends Endocrinol. Metab*., **3**, 224–229.

Eriksen, E.F., Colvard, D.S., Berg, N.J., Graham, M.L., Mann, K.G., Spelsberg, T.C. and Riggs, B.L. (1988). Evidence of estrogen receptors in normal human osteoblast-like cells. *Science*, **241**, 84–86.

Gao, H.Y. and Yamaguchi, M. (1998). Zinc enhancement of genistein's anabolic effect on bone components in elderly rats. *Gen. Pharmac*., **31**, 199–202.

Gray, T.K., Flyynn, T.C., Gray, K.M. and Nabel, L.M. (1987). 17β-Estradiol acts directly on the clonal osteoblastic cell line. *Proc. Natl. Acad. Sci., USA*, **84**, 6267–6271.

Hock, J.M., Centrella, M. and Canalis, E. (1988). Insulin-like growth factor has independent effects on bone matrix formation and cell replication. *Endocrinology*, **122**, 254–260.

Ituarte, E.A., Ituarte, H.G., Iida-klein, A. and Hahn, T.J. (1989). Characterization of insulin binding in the UMR-106 rat osteoblastic osteosarcoma cell. *J. Bone Miner. Res*., **4**, 69–73.

Kream, B.E., Swith, M.D., Canalis, E. and Raise, L.G. (1985). Characterization of the effect of insulin on collagen synthesis in fetal rat bone. *Endocrinology*, **116**, 296–302.

Kuiper, G.G.J.M., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J., Nilsson, S. and Gustafsson, J.-A. (1997). Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptor α and β. *Endocrinology*, **138**, 863–870

Levy, J.R., Murray, E., Manolagas, S. and Olefsky, J.M. (1986). Demonstration of insulin receptors and modulation of alkaline phosphatase activity by insulin in rat osteoblastic cells. *Endocrinology*, **119**, 1786–1792.

Saltiel, A.R., Sherline, P. and Fox, J.A. (1987). Insulin-stimulated diacylglycerol production results from the hydrolysis of a novel phosphatidylinositol glycan. *J. Biol. Chem*., **262**, 1116–1121.

Schmid, C., Gulen, H.P., Rowe, D. and Froosch, E.R. (1989). Insulin-like growth factor I regulates type I procollagen mRNA steady state levels in bone of rats. *Endcrinology*, **125**, 1575–1580.

Sugimoto, E. and Yamaguchi, M. (2000). Anabolic effect of genistein in osteoblastic MC3T3-E1 cells. *Int. J. Mol. Med*., **5**, 515–520.

Suzuki, T., Nakayama, H. and Yamaguchi, M. (1997). Effect of wasabi leafstalk (*wasabia japonica* MATSUM.) extract on bone metabolism in mouse calvaria tissue culture. *Food Sci. Technol. Int. Tokyo*, **3**, 366–369.

Suzuki, T. and Yamaguchi, M. (1999a). Anabolic effect of wasabi leafstalk (*wasabia japonica* MATSUM.) extract on bone metabolism in rats. *Food Sci. Technol. Res*., **5**, 61–63.

Suzuki, T. and Yamaguchi, M. (1999b). Characterization of stimulatory effect of wasabi leafstalk (*wasabia japonica* MATSUM.) extract on bone calcification in mouse calvaria tissue culture. *Food Sci. Technol. Res*., **5**, 304–307.

Tsukamoto, Y., Ichise, H., Kakuda, H. and Yamaguchi, M. (2000). Intake of fermented soybean (natto) increases circulating vitamin K_2 (menaquinone-7) and -carboxylated osteocalcin concentration in normal individuals. *J. Bone Miner. Metab*., **18**, 216–222.

Yamaguchi, M., Oishi, H. and Suketa, Y. (1987). Stimulatory effect of zinc on bone formation in tissue culture. *Biochem. Pharmacol*., **36**, 4007–4012.

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 ovariectomized rats. *J. Bone Miner. Metab*., **18**, 71–76.

Yamaguchi, M. (2002). Isoflavone and bone metabolism; its cellular mechanism and preventive role in bone loss. *J. Health Sci*., **48**, 209–222.