Stimulatory Effect of *Sargassum Horneri* Extract on Bone Formation in Rat Femoral-Diaphyseal and -Metaphyseal Tissues *in Vitro*

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The effect of *Sargassum horneri* extract on bone formation in the femoral-diaphyseal and -metaphyseal tissue of rats *in vitro* was investigated. Femoral-diaphyseal and -metaphyseal tissues were cultured for 24 hr in Dulbecco's modified Eagle's medium containing either vehicle or a water-solubilized extract (10 and 25 μg/ml of medium) obtained from various marine algae (*S. horneri, S. ringgoldianum* Harvey, and *S. yamadae* Yoshida et T. Konno). Diaphyseal and metaphyseal calcium contents were significantly increased in the presence of *S. horneri* extract. Such an effect was not seen in the presence of *S. ringgoldianum* Harvey or *S. yamadae* Yoshida et T. Konno extract. Heat-treated *S. horneri* extracts (for 30 min at 80°C) did not have an anabolic effect on bone calcium content. Alkaline phosphatase activity and deoxyribonucleic acid (DNA) content in the femoral-diaphyseal and -metaphyseal tissues were significantly increased by culture with *S. horneri* extract (25 μg/ml of medium) for 24 hr. The effect of *S. horneri* extract in increasing calcium content, alkaline phosphatase activity, and DNA content in the femoral-diaphyseal and -metaphyseal tissues was completely abolished in the presence of cycloheximide (10^{-6} M), an inhibitor of protein synthesis. The present study demonstrates that, among marine algae which belong to *Sargassum*, *S. horneri* extract has a unique stimulatory effect on bone formation and calcification *in vitro*.

Key words —— bone metabolism, *Sargassum horneri*, marine algae, osteoporosis, rat femur

INTRODUCTION

Bone mass decreases with increasing age.[1–3] Osteoporosis with a decrease in bone mass is widely recognized as a major public health problem.[4] The most dramatic expression of this disease is represented by fractures of the proximal femur. Nutritional factors can help to prevent bone loss with increasing age.[5] These factors are poorly understood, however.

Recent studies have shown that isoflavones and saponins in soybean have an anabolic effect on bone metabolism.[6–11] Isoflavones, including genistein and daidzein, have been demonstrated to stimulate osteoblastic bone formation[12,13] and inhibit osteoclastic bone resorption,[14–16] thereby increasing bone mass.

Vitamin K_2 is suggested to play a role in preventing age-related bone loss. Vitamin K_2 is essential for the γ-carboxylation of osteocalcin, a bone matrix protein containing γ-carboxyglutamic acids, which is synthesized in the osteoblasts of bone tissue.[17] Menaquinone-7, an analogue of vitamin K_2, is abundant in fermented soybean (*natto*).[18] Menaquinone-7 has been shown to stimulate osteoblastic bone formation[19] and inhibit osteoclastic bone resorption.[20] The prolonged dietary intake of menaquinone-7 has a preventive effect on bone loss induced by ovariectomy in rats.[21] The intake of dietary menaquinone-7 in reinforced *natto* can stimulate γ-carboxylation of osteocalcin, which plays an important role in bone formation in normal individuals.[22]

Nutritional factors may be important in the prevention of bone loss with increasing age. The effect of marine algae on bone metabolism, however, has not yet been clarified. More recently, it has been reported that *Sargassum horneri* extract has an anabolic effect on bone calcification in rat femoral-metaphyseal tissues *in vivo* and *in vitro*.[23] The action of *S. horneri* extract on bone metabolism has not been fully clarified, however.

The present study, furthermore, was undertaken

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to determine the characterization of *S. horneri*'s effect on bone metabolism in rat femoral-diaphyseal and -metaphyseal tissues *in vitro*. Of various marine algae which belong to *Sargassum*, *S. horneri* extract was found to have a unique stimulatory effect on bone formation and calcification *in vitro*.

MATERIALS AND METHODS

Chemicals —— Dulbecco's modified Eagle's medium (MEM) (high glucose, 4.5 g/dl) and a penicillin-streptomycin solution (penicillin 5000 U/mg; streptomycin 5000 μg/ml) were purchased from Gibco Laboratories (Grand Islasnd, NY, U.S.A.). Bovine serum albumin (fraction V) and cycloheximide were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other chemicals were of reagent grade from Wako Pure Chemical Industries (Osaka, Japan).

Marine Algae Extracts —— Marine algae (*S. horneri*, *S. ringgoldianum* Harvey, and *S. yamadae* Yoshida et T. Konno) were seasonally gathered from the coast at Shimoda (Shizuoka prefecture, Japan), then freeze-dried. The gathered fresh marine algae were homogenized in distilled water with a Physcotron homogenizer, and the homogenate was centrifuged at 5500 *g* in a refrigerated centrifuge for 10 min. The 5500 *g* supernatant fraction was pooled for freeze-drying. The powder of water-solubilized extract was dissolved in ice-cold distilled water for use in the experiments. In another experiment, the water-solubilized extract of *S. hoeneri* was treated at 80°C for 30 min in water bath.

Bone Culture —— The femurs were removed aseptically after bleeding, and were then soaked in ice-cold 0.25 M sucrose solution. The femur was cleaned of soft tissue and marrow, and the diaphysis and metaphysis (not containing epiphyseal tissue) were separated. The femoral-diaphyseal and -metaphyseal tissues were cut into small pieces. Diaphyseal or metaphyseal fragments were cultured for 24–72 hr in a 35 mm dish in 2.0 ml of medium consisting of Dulbecco's MEM (high glucose, 4.5 g/dl) supplemented with 0.25% bovine serum albumin plus antibiotics (penicillin 100 units and streptomycin 100 μg/ml of medium).[24] In our experiments, bone tissues were cultured in a medium containing either vehicle or water-solubilized marine algae extract. The concentration of calcium in the marine algae extracts was in the range of 0.05 to 1.0 μg/ml of medium. Cultures were maintained at 37°C in a water-saturated atmosphere containing 5% CO_2 and 95% air.

Analytical Procedures —— The diaphyseal and metaphyseal tissues were dried for 16 hr at 110°C. Calcium was determined by atomic absorption spectrophometry.[24,25] Calcium content in bone tissues was expressed as milligrams per gram of dry bone.

To assay alkaline phosphatase activity, the metaphyseal tissues were immersed in 3.0 ml of ice-cold barbital buffer 6.6 mM (pH 7.4), cut into small pieces, 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. An enzyme assay was carried out under optimal conditions. Alkaline phosphatase activity was determined by the method of Walter and Schutt.[26] Enzyme activity was expressed as micromol of *p*-nitrophenol liberated per minute per milligram of protein. Protein concentration was determined by the method of Lowry *et al*.[27]

To measure bone DNA content, the diaphyseal and metaphyseal tissues were shaken with 4.0 ml ice-cold 0.1 N NaOH solution for 24 hr after the homogenization of the bone tissues.[28] After alkali extraction, the samples were centrifuged at $1000 \times g$ for 5 min, and the supernatant was collected. DNA content in the supernatant was determined by the method of Ceriotti[29] and expressed as the amount of DNA (mg)/g wet weight of bone tissue.

Statistical Analysis —— The significance of difference between values was estimated by Student's *t*-test. *p*-Values of less than 0.05 were considered to indicate statistically significant differences.

RESULTS

Effect of *S. horneri* Extract on Bone Calcification *in Vitro*

The effect of increasing concentrations of a water-solubilized extract of *S. horneri* on calcium content in rat femoral-diaphyseal and -metaphyseal tissues *in vitro* is shown in Fig. 1. Bone tissues were cultured for 24 hr in the presence of *S. horneri* extract. Diaphyseal and metaphyseal calcium content was significantly increased in the presence of *S. horneri* extract (10, 25, or 50 μg/ml of medium).

The time course of *S. horneri* extract's effect on calcium content in rat femoral-diaphyseal and -metaphyseal tissues *in vitro* is shown in Fig. 2. Bone tissues were cultured for 24–72 hr in the presence of *S.*

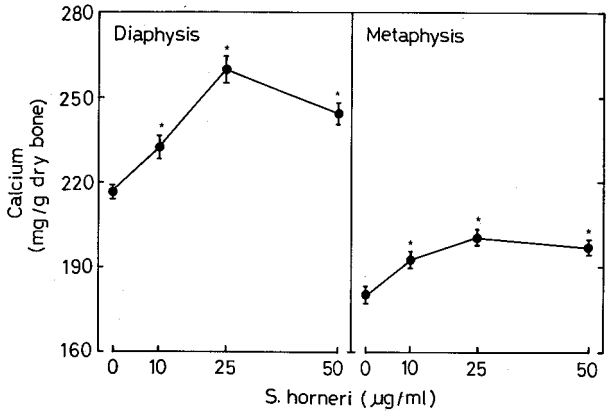

Fig. 1. Effect of Increasing Concentrations of *S. horneri* Extract on Calcium Content in Rat Femoral-Diaphyseal and -Metaphyseal Tissues *in Vitro*

Femoral-diaphyseal or -metaphyseal tissues obtained from normal rats were cultured for 24 hr in a medium containing either vehicle or water-solubilized extract (10, 25, or 50 μg/ml of medium) of *S. horneri*. Each value is the mean ± S.E.M. of six rats. $*p < 0.01$ compared with the control (none) value.

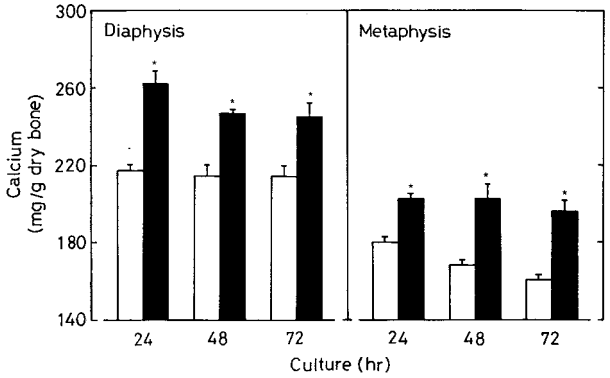

Fig. 2. Time Course of *S. horneri*'s effect on Calcium Content in Rat Femoral-Diaphyseal and -Metaphyseal Tissues *in Vitro*

Femoral-diaphyseal or -metaphyseal tissues obtained from normal rats were cultured for 24, 48, or 72 hr in a medium containing either vehicle or water-solubilized extract (25 μg/ml of medium) of *S. horneri*. Each value is the mean ± S.E.M. of six rats. $*p < 0.01$ compared with the control (none) value. White bars, control; black bars, *S. horneri* extract.

horneri extract (25 μg/ml of medium). Diaphyseal and metaphyseal calcium content was significantly increased by culture with *S. horneri* extract between 24 and 72 hr. Thus, the effect of *S. horneri* extract was maintained for a longer time.

The effect of a water-solubilized extract of *S. ringgoldanum* Harvey or *S. yamadae* Yoshida et T. Konnno extract on calcium content in rat femoral-diaphyseal and -metaphyseal tissues *in vitro* is shown in Fig. 3. These marine algae belong to *Sargassum*. Bone tissues were cultured for 24 hr in the presence

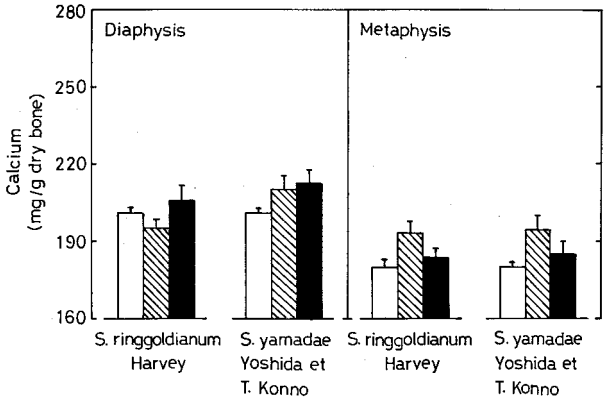

Fig. 3. Effect of Various Marine Algae on Calcium Content in Rat Femoral-Diaphyseal and -Metaphyseal Tissues *in Vitro*

Femoral-diaphyseal or -metaphyseal tissues obtained from normal rats were cultured for 24 hr in a medium containing either vehicle or water-solubilized extract (10 and 25 μg/ml of medium) of *S. ringgoldianum* Harvey or *S. yamadae* Yoshida et T. Konno. Each value is the mean ± S.E.M. of six rats. Data were not significant as compared with the control (none) value. White bars, control; hatched bars, 10 μg/ml; black bars, 25 μg/ml.

of *S. ringgoldianum* Harvey extract (25 μg/ml of medium) or *S. yamadae* Yoshida et T. Konno extract (25 μg/ml of medium). The anabolic effect on bone calcium content was not seen in the presence of these extracts.

The extracts of *S. horneri* were heated with 80°C for 30 min. Bone tissues were cultured for 24 hr in the presence of heat-treated *S. horneri* extract (10 or 25 μg/ml of medium). The effect of *S. horneri* extract in increasing calcium content in the femoral-diaphyseal and -metaphyseal tissues completely disappeared (Fig. 4).

Effect of Cycloheximide on *S. horneri*-Increased Bone Components *in Vitro*

Rat femoral-diaphyseal and -metaphyseal tissues were cultured for 24 hr in a medium containing either vehicle or *S. horneri* extract (25 μg/ml of medium) in the absence or presence of cycloheximide (10^{-6} M), an inhibitor of protein synthesis at the translational process. The effect of *S. horneri* extract on increasing calcium content (Fig. 5), alkaline phosphatase activity (Fig. 6), and DNA content (Fig. 7) in the femoral-diaphyseal or -metaphyseal tissues was completely prevented in the presence of cycloheximide.

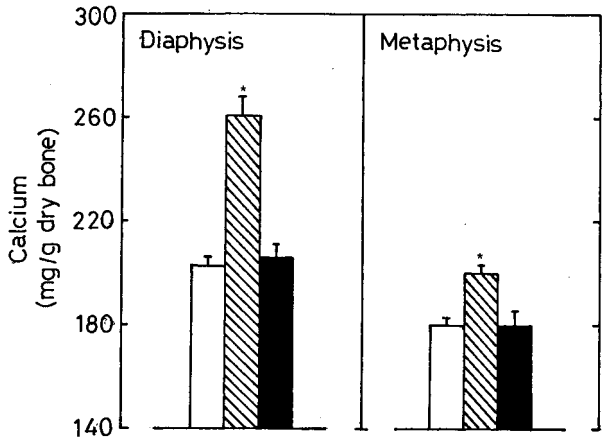

Fig. 4. Effect of Heat-Treated *S. horneri* Extracts on Calcium Content in Rat Femoral-Diaphyseal and -Metaphyseal Tissues *in Vitro*

Femoral-diaphyseal or -metaphyseal tissues were cultured for 24 hr in a medium containing either vehicle or water-solubilized extract (25 μg/ml of medium) with or without heat treatment of *S. horneri* extract. Each value is the mean ± S.E.M. of six rats. $*p < 0.01$ compared with the control (none) value. White bars, control (none); hatched bars, *S. horneri* extract without heat treatment; black bars, *S. horneri* extract with heat treatment.

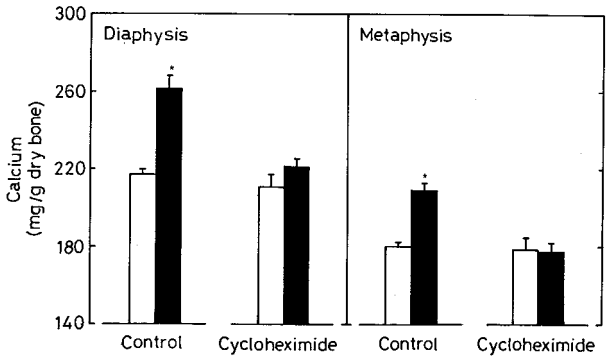

Fig. 5. Effect of Cycloheximide on *S. horneri*-Increased Calcium Content in Rat Femoral-Diaphyseal and -Metaphyseal Tissues *in Vitro*

Femoral-diaphyseal or -metaphyseal tissues were cultured for 24 hr in a medium containing either vehicle or water-solubilized extract (25 μg/ml of medium) of *S. horneri* in the absence or presence of cycloheximide (10^{-6} M). Each value is the mean ± S.E.M. of six rats. $*p < 0.01$ compared with the control (none) value. White bars, control; black bars, *S. horneri* extract.

DISCUSSION

Bone loss with increasing age induces osteoporosis.[1–5,30] Food and nutritional factors may play a role in the prevention of bone loss with aging. *S. horneri*, a marine algae, has been shown to have an anabolic effect on bone calcification in the femoral-metaphyseal tissues of rats *in vivo* and *in vitro*.[23] The present study, furthermore, demonstrates that *S. horneri*, among marine algae which belong to *Sargassum*, has a unique stimulatory effect on bone formation and calcification in rat femoral-diaphyseal and -metaphyseal tissues *in vitro*.

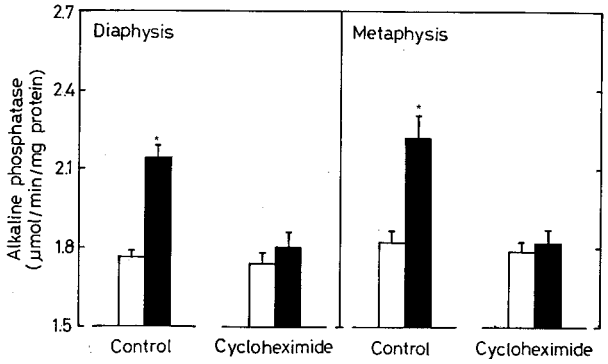

Fig. 6. Effect of Cyclohemide on *S. horneri*-Increased Alkaline Phosphatase Activity in Rat Femoral-Diaphyseal and -Metaphyseal Tissue *in Vitro*

Femoral-diaphyseal or -metaphyseal tissues were cultured for 24 hr in a medium containing either vehicle or water-solubilized extract (25 μg/ml of medium) of *S. horneri* in the absence or presence of cycloheximide (10^{-6} M). Each value is the mean ± S.E.M. of six rats. $*p < 0.01$ compared with the control (none) value. White bars, control; black bars, *S. horneri* extract.

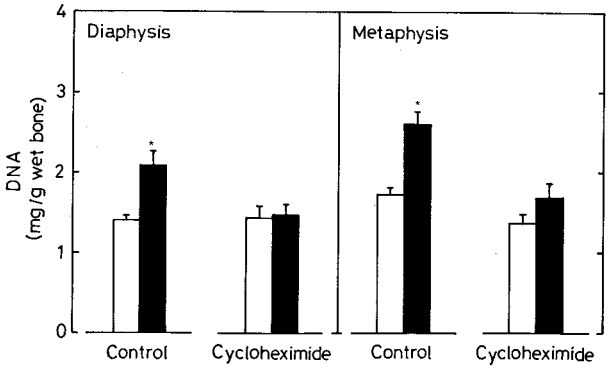

Fig. 7. Effect of Cycloheximide on *S. horneri*-Increased DNA Content in Rat Femoral-Diaphyseal and -Metaphyseal Tissues *in Vitro*

Femoral-diaphyseal or -metaphyseal tissues were cultured for 24 hr in a medium containing either vehicle or water-solubilized extract (25 μg/ml of medium) of *S. horneri* in the absence or presence of cycloheximide (10^{-6} M). Each value is the mean ± S.E.M. of six rats. $*p < 0.01$ compared with the control (none) value. White bars, control; black bars, *S. horneri* extract.

The effect of a water-solubilized extract of *S. horneri* in increasing bone calcification *in vitro* was maintained in culture for a long time, indicating the existence of potential components in the extract. When *S. horneri* extract was treated with heat at 80°C for 30 min, the stimulatory effect of *S. horneri* extract on bone calcification was not seen. The active components of a water-solubilized extract of *S.*

horneri are not related to trace elements. *S. horneri* extract solubilized with 20% ethanol had no effect on bone calcification *in vitro*.[23] The identification of active components remains to be elucidated.

The effect of *S. horneri* extract in increasing calcium content, alkaline phosphatase activity, and DNA content in rat femoral-diaphyseal and -metaphyseal tissues was completely prevented in the presence of cycloheximide, an inhibitor of protein synthesis at the translational process. This result suggests that an anabolic effect of *S. horneri* extract on bone metabolism is based on a newly synthesized protein component in bone tissues. The active components may stimulate the proliferation of osteoblastic cells in the femoral-diaphyseal and -metaphyseal tissues, since the extract could increase bone alkaline phosphatase activity and calcium content, which are markers of osteoblastic bone formation. Presumably, a water-solubilized extract of *S. horneri* can stimulate osteoblastic bone formation and calcification *in vitro*.

Food and nutritional factors which can stimulate bone formation and calcification are poorly understood. Prolonged intake of *S. horneri* extract may play a role in the prevention of bone loss with increasing age.

In conclusion, it has been demonstrated that *S. horneri* extract has a stimulatory effect on bone formation and calcification in rat femoral-diaphyseal and -metaphyseal tissues *in vitro*. Of marine algae which belong to *Sargassum*, the effect of *S. horneri* extract was unique.

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