Preventive Effect of Marine Alga *Sargassum Horneri* Extract on Bone Loss in Streptozotocin-Diabetic Rats *in Vivo*

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The effect of *Sargassum horneri* (*S. horneri*) extract on bone components in the femoral-diaphyseal and -metaphyseal tissues of streptozotocin (STZ)-diabetic rats was investigated. Rats received a single subcutaneous administration of STZ (6.0 mg/100 g body weight), and then the animals were orally administered water-solubilized extract (10 mg/100 g body weight) of *S. horneri* once daily for 14 or 21 days. The administration of STZ caused a significant decrease in body weight and a significant increase in serum glucose, triglyceride, and calcium levels, indicating a diabetic state. These alterations were significantly prevented by the administration of *S. horneri* extract for 14 or 21 days. The administration of *S. horneri* extract to normal rats for 14 or 21 days did not have a significant effect on body weight or serum glucose, triglyceride, and calcium levels. Calcium content, alkaline phosphatase activity, and DNA content in the femoral-diaphyseal and -metaphyseal tissues were significantly decreased in STZ-diabetic rats. These decreases were significantly prevented by the administration of *S. horneri* extract for 14 or 21 days. The administration of *S. horneri* extract to normal rats for 14 or 21 days caused a significant increase in calcium content, alkaline phosphatase activity, and DNA content in the femoral-diaphyseal and -metaphyseal tissues. Femoral-diaphyseal and -metaphyseal tissues obtained at 14 days after STZ administration were cultured for 48 hr in a medium containing either vehicle or *S. horneri* extract (10, 25, or 50 μg/ml of medium) *in vitro*. Calcium content and alkaline phosphatase activity in the femoral-diaphyseal and -metaphyseal tissues obtained from STZ-diabetic rats was significantly increased in the presence of *S. horneri* extract (10, 25, or 50 μg/ml). The present results demonstrate that the intake of *S. horneri* extract has a preventive effect on bone loss in STZ-diabetic rats.

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

INTRODUCTION

Bone loss occurs with increasing age.[1–3] Osteoporosis with an increase in bone loss 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. Pharmacological and nutritional factors may play a role in the prevention of bone loss with increasing age. Recent studies have shown that isoflavones, saponin and menaquinone-7 (an analogue of vitamin K_2), which are abundant in fermented soybean (*natto*), have a preventive effect on bone loss induced in ovariectomized rats as an animal model of osteoporosis.[5–11] These factors have been shown to stimulate osteoblastic bone formation and inhibit osteoclastic bone resorption.[11–14] Thus nutritional factors may be important in the prevention of bone loss with increasing age.

Further studies have shown that, among various marine algae, *Sargassum horneri* (*S. horneri*) extract has an anabolic effect on bone calcification in rat femoral tissues *in vivo* and *in vitro*.[15–18] *S. horneri* extract has been demonstrated to stimulate osteoblastic bone formation and inhibit osteoclastic bone resorption *in vitro* using rat femoral-diaphyseal and -metaphyseal tissues.[16,17] Thus the dietary intake of *S. horneri* extract may have a preventive effect on the decrease in bone mass with increasing age. However, whether the intake of *S. horneri* extract has a preventive effect on bone loss in a pathophysiologic state has not been clarified.

The present study was undertaken to determine

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the preventive effect of the intake of *S. horneri* extract on bone loss induced in the diabetic state.[19,20] We found that the intake of *S. horneri* extract can prevent bone loss in the femoral-diaphyseal (cortical bone) and -metaphyseal (trabecular bone) tissues of streptozotocin (STZ)-induced diabetic rats.

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), streptozotocin 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 Alga Extracts —— The marine alga *S. horneri* was seasonally gathered from the coast at Shimoda (Shizuoka prefecture, Japan), and was freeze-dried and powdered. The fresh marine alga gathered was homogenized in distilled water with a Physcotron homogenizer, and the homogenate was centrifuged at 5500 *g* in a refrigerated centrifuge for 10 min.[17] The 5500 *g* supernatant fraction was pooled for freeze-drying. Powder of the water-solubilized extract was dissolved in ice-cold distilled water to use in experiments.

Animals —— Male Wistar rats (conventional) weighing 90–100 g (4 weeks old) were obtained from Japan SLC (Hamamatsu, Japan). The animals were fed commercial laboratory chow (solid) containing 1.1% calcium and 1.1% phosphorus at room temperature of 25°C, with free access to distilled water.

Administration Procedures —— Streptozotocin was dissolved in 50 mM sodium citrate (pH 4.5) solution containing 150 mM NaCl.[21] The solution (6.0 mg/0.5 ml/100 g body weight) was subcutaneously administered in rats, and 14 or 21 days later the animals were killed by exsanguination. The water suspension (10 mg/ml/100 g body weight) of the powder of a water-solubilized extract of the marine alga *S. horneri* was orally administered to rats through a stomach tube once daily for 14 or 21 days. The *S. horneri* extract was orally administered 3 hr after the administration of streptozotocin (6.0 mg/100 g). Rats were killed 24 hr after the last administration of marine alga *S. horneri* extract, and the blood and femur were removed immediately.

Bone Culture —— The femurs were removed aseptically after exsanguination and 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. Femoral-diaphyseal and -metaphyseal tissues were then cultured in a 35-mm dish in 2.0 ml of medium consisting of Dulbecco's modified Eagle's medium (high glucose; 4.5%) supplemented with 0.25% bovine serum albumin (fraction V) plus antibiotics in the absence or presence of water-solubilized extracts (10, 25, and 50 μg/ml of medium) of *S. horneri*.[22] Cultures were maintained at 37°C in a water-saturated atmosphere containing 5% CO_2 and 95% air for 48 hr.

Analytical Procedures —— Blood samples obtained by cardiac puncture were centrifuged 30 min after collection, and the serum was separated. Serum was frozen at –80°C until assay. Serum glucose, triglyceride, calcium, and inorganic phosphorus concentrations were determined using an assay Kit (Wako Pure Chemical Industries).

The diaphyseal or metaphyseal tissues were dried for 16 hr at 110°C. Calcium was determined by atomic absorption spectrophotometry.[22] Calcium content in bone tissues was expressed as milligrams per gram of dry bone.

To assay alkaline phosphatase activity, the diaphyseal or 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. 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 micromol of *p*-nitrophenol liberated per minute per milligram of protein. Protein concentration was determined by the method of Lowry *et al*.[24]

To measure bone DNA content, the diaphyseal or metaphyseal tissues were shaken with 4.0 ml of ice-cold 0.1 N NaOH solution for 24 hr after the homogenization of the bone tissues.[25] After alkaline extraction, the samples were centrifuged at $1000 \times g$ for 5 min, and the supernatant was determined by the method of Ceriotti[26] 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

Table 1. Effect of *S. horneri* Extract Administration on the Change in Body Weight and Serum Glucose and Triglyceride Levels in STZ-diabetic Rats

Treatment	Body weight (g)	Serum level (mg/dl)	
		Glucose	Triglyceride
14 days			
Control	145.2 ± 4.9	138.5 ± 5.7	135.3 ± 14.3
S. horneri	152.5 ± 3.8	130.4 ± 6.1	131.5 ± 7.6
STZ	82.2 ± 2.8*	801.1 ± 24.3*	1660.5 ± 95.4*
STZ + *S. horneri*	105.1 ± 8.6*,#	467.5 ± 42.9*,#	838.1 ± 80.3*,#
21 days			
Control	177.2 ± 1.6	158.3 ± 2.5	171.0 ± 10.1
S. horneri	185.8 ± 3.2	160.1 ± 1.9	96.7 ± 2.1*
STZ	88.2 ± 2.3*	604.2 ± 15.7*	1385.3 ± 25.1*
STZ + *S. horneri*	102.5 ± 2.9*,#	462.3 ± 10.8*,#	1056.9 ± 43.7*,#

Rats received a single subcutaneous administration of STZ (6.0 mg/100 g body weight), and 3 hr later the animals were orally administered a water-solubilized extract (10 mg/ 100 g) of *S. horneri* once daily for 14 or 21 days. The animals were killed 24 hr after the last administration. Each value is the mean ± S.E.M. for six rats. *$p < 0.01$, compared with the control (none) value. #$p < 0.01$, compared with the control value from STZ treatment.

indicate statistically significant differences. We also used a multiple analysis of variance (ANOVA) and the Turkey-Kramer multiple-comparison test to compare the treatment groups.

RESULTS

Effect of *S. horneri* Extract Administration on Serum Biochemical Components in STZ-diabetic Rats *in Vivo*

Rats received a single subcutaneous administration of STZ (6.0 mg/100 g body weight), and the animals were orally administrated water-solubilized extract (10 mg 100 g body weight) of *S. horneri* once daily for 14 or 21 days. The changes in body weight and serum biochemical components are shown in Tables 1 and 2. The body weight of animals was significantly decreased 14 or 21 days after the administration of STZ. This reduction was significantly prevented by the administration of *S. horneri* extract for 14 or 21 days (Table 1).

Serum glucose and triglyceride levels were markedly elevated in STZ-administered rats, indicating that the administration induces a diabetic state. These increases were significantly prevented by the administration of *S. horneri* extract for 14 or 21 days (Table 1). The administration of *S. horneri* extract for 14 or 21 days did not have an effect on serum glucose levels in normal rats. Serum triglyceride levels in normal rats were significantly decreased by the administration of *S. horneri* extract for 21 days.

Table 2. Effect of *S. horneri* Extract Administration on the Change in Serum Calcium and Inorganic Phosphorus Levels in STZ-diabetic Rats

Treatment	Serum level (mg/dl)	
	Calcium	Inorganic phosphorus
14 days		
Control	10.41 ± 0.14	9.48 ± 0.29
S. horneri	10.11 ± 0.31	9.60 ± 0.30
STZ	12.57 ± 0.05*	7.93 ± 0.27*
STZ + *S. horneri*	11.76 ± 0.12*,#	8.78 ± 0.32
21 days		
Control	10.25 ± 0.21	8.50 ± 0.12
S. horneri	10.35 ± 0.23	8.82 ± 0.16
STZ	11.27 ± 0.09*	7.97 ± 0.28
STZ + *S. horneri*	10.83 ± 0.05*,#	8.54 ± 0.29

The procedure of administration is described in the legend to Table 1. Each value is the mean ± S.E.M. for six rats. *$p < 0.01$, compared with the control value of STZ treatment.

This effect was not seen with administration for 14 days.

The serum calcium level was significantly elevated 14 or 21 days after the administration of STZ (Table 2). These increases were significantly inhibited by the administration of *S. horneri* extract (10 mg/ 100 g) for 14 or 21 days. Serum inorganic phosphorus levels were significantly decreased 14 days after STZ administration (Table 2). This decrease was not seen 21 days after the administration of STZ. The administration of *S. horneri* extract to normal rats for 14 or 21 days had no effect on serum

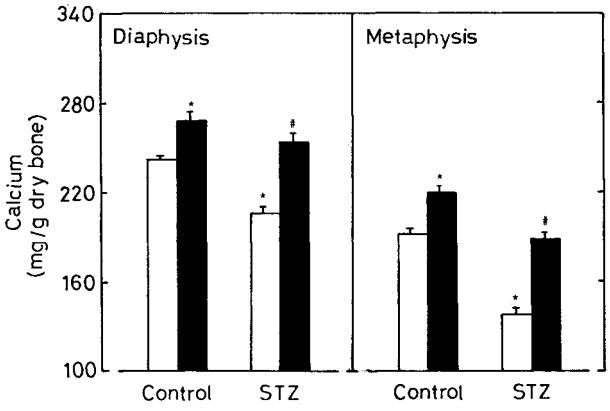

Fig. 1. Effect of *S. horneri* Extract Administration for 14 Days on Calcium Content in the Femoral-diaphyseal and -metaphyseal Tissues of STZ-diabetic rats *in Vivo*

The procedure of administration is described in the legend to Table 1. Rats were killed 14 days after STZ administration. Each value is the mean ± S.E.M. for six rats. $*p < 0.01$, compared with the control (none) value. $\#p < 0.01$, compared with the control value from STZ treatment. White bars, control; black bars, *S. horneri* extract.

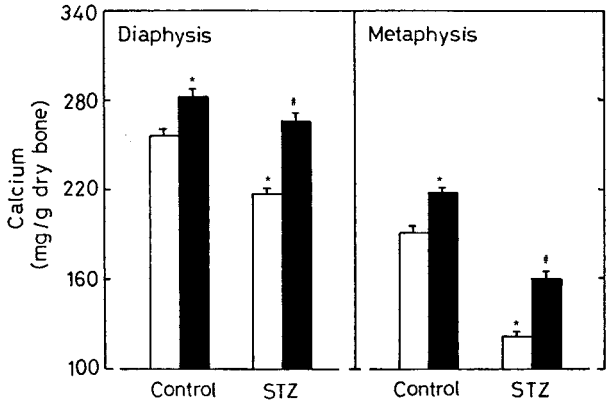

Fig. 2. Effect of *S. horneri* Extract Administration for 21 Days on Calcium Content in the Femoral-diaphyseal and -metaphyseal Tissues of STZ-diabetic Rats *in Vivo*

The procedure of administration was described in the legend to Table 1. Rats were killed 21 days after STZ administration. Each value is the mean ± S.E.M. for six rats. $*p < 0.01$, compared with the control (none) value. $\#p < 0.01$, compared with the control value from STZ treatment. White bars, control; black bars, *S. horneri* extract.

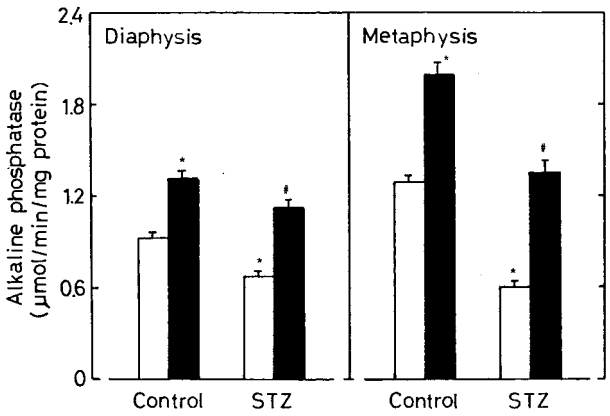

Fig. 3. Effect of *S. horneri* Extract Administration for 14 Days on Alkaline Phosphatase Activity in the Femoral-diaphyseal and -metaphyseal Tissues of STZ-diabetic Rats *in Vivo*

The procedure of administration is described in the legend to Table 1. Rats were killed 14 days after STZ administration. Each value is the mean ± S.E.M. for six rats. $*p < 0.01$, compared with the control (none) value. $\#p < 0.01$, compared with the control value from STZ treatment. White bars, control; black bars, *S. horneri* extract.

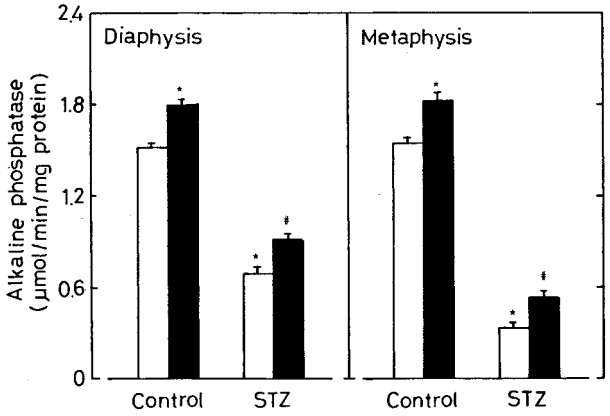

Fig. 4. Effect of *S. horneri* Extract Administration for 21 Days on Alkaline Phosphatase Activity in the Femoral-diaphyseal and -metaphyseal Tissues of STZ-diabetic Rats *in Vivo*

The procedure of administration is described in the legend to Table 1. Rats were killed 21 days after STZ administration. Each value is the mean ± S.E.M. for six rats. $*p < 0.01$, compared with the control (none) value. $\#p < 0.01$, compared with the control value from STZ treatment. White bars, control; black bars, *S. horneri* extract.

calcium and inorganic phosphorus levels.

Thus the oral administration of *S. horneri* extract to STZ-diabetic rats was found to have a significant preventive effect on the alteration in body weight and serum components in the diabetic state.

Effect of *S. horneri* Extract Administration on Bone Components in STZ-diabetic Rats *in Vivo*

The effect of *S. horneri* extract administration on bone components in the femoral-diaphyseal and -metaphyseal tissues of STZ-diabetic rats was examined. Calcium content (Figs. 1 and 2), alkaline phosphatase activity (Figs. 3 and 4), and DNA content (Figs. 5 and 6) in the femoral-diaphyseal and -metaphyseal tissues was significantly decreased 14 or 21 days after the administration of STZ (6.0 mg/100 g), respectively. These decreases were significantly prevented by the administration of *S. horneri*

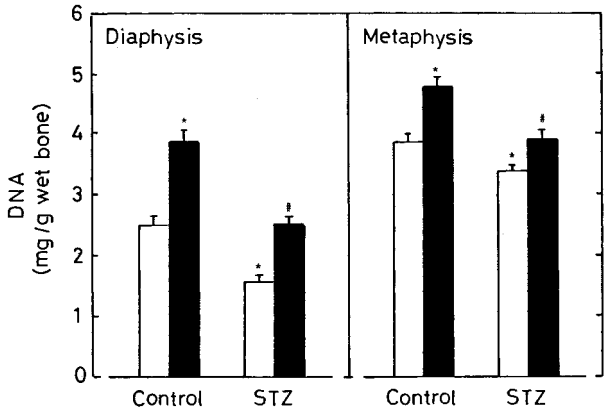

Fig. 5. Effect of *S. horneri* Extract Administration for 14 Days on DNA Content in the Femoral-diaphyseal and -metaphyseal Tissues of STZ-diabetic Rats *in Vivo*

The procedure of administration was described in the legend of Table 1. Rats were killed 14 days after STZ administration. Each value is the mean ± S.E.M. for six rats. *$p < 0.01$, compared with the control (none) value. #$p < 0.01$, compared with the control value from STZ treatment. White bars, control; black bars, *S. horneri* extract.

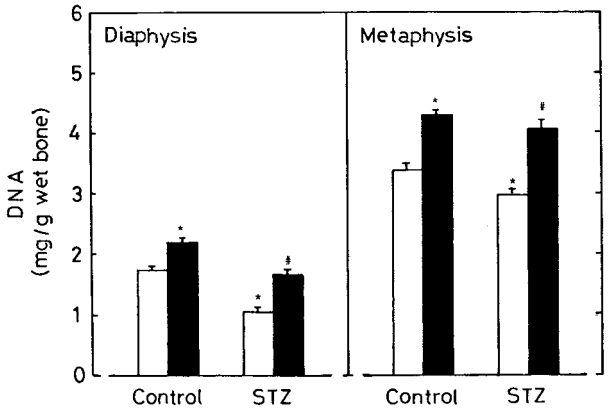

Fig. 6. Effect of *S. horneri* Extract Administration for 21 Days on DNA Content in the Femoral-diaphyseal and -metaphyseal Tissues of STZ-Diabetic Rats *in Vivo*

The procedure of administration is described in the legend to Table 1. Rats were killed 21 days after STZ administration. Each value is the mean ± S.E.M. for six rats. *$p < 0.01$, compared with the control (none) value. #$p < 0.01$, compared with the control value from STZ treatment. White bars, control; black bars, *S. horneri* extract.

extract (10 mg/100 g) for 14 or 21 days. The administration of *S. horneri* extract (10 mg/100 g) to normal rats for 14 or 21 days caused a significant increase in calcium content, alkaline phosphatase activity, and DNA content in the femoral-diaphyseal and -metaphyseal tissues (Figs. 1–6).

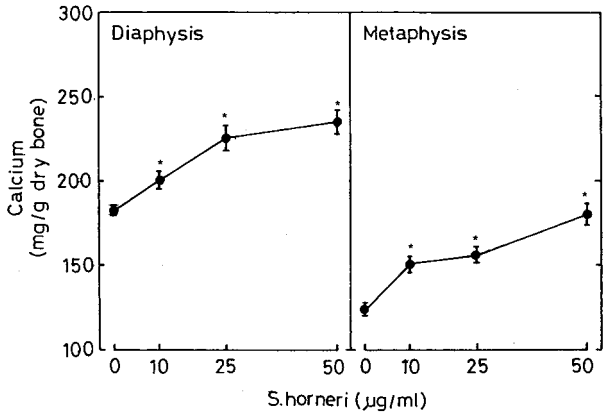

Fig. 7. Effect of *S. horneri* Extract on Calcium Content in the Femoral-diaphyseal and -metaphyseal Tissues Obtained from STZ-diabetic Rats *in Vitro*

Rats received a single subcutaneous administration of STZ (6.0 mg/ 100 g body weight), and 14 days later the animals were killed. Femoral-diaphyseal or -metaphyseal tissues obtained from STZ-diabetic 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. for six rats. *$p < 0.01$, compared with the control (none) value.

Effect of *S. horneri* Extract on Calcium Content and Alkaline Phoshatase Activity in the Femoral Tissues Obtained from STZ-diabetic Rats *in Vitro*

Femoral-diaphyseal and -metaphyseal tissues obtained 14 days after a single subcutaneous administration of STZ (6.0 mg/ 100 g) to normal rats were cultured in medium containing either vehicle or *S. horneri* extract (10, 25, or 50 μg/ml of medium) for 48 hr. Calcium content (Fig. 7) and alkaline phosphatase activity (Fig. 8) in the femoral-diaphyseal and -metaphyseal tissues obtained from STZ-diabetic rats were significantly increased in the presence of *S. horneri* extract (10, 25, or 50 μg/ml).

DISCUSSION

Food and nutritional factors may play a role in the prevention of bone loss with increasing age. More recent studies have shown that, of various marine algae, *S. horneri* extract has a specific anabolic effect on bone component.[15–18] The anabolic effect of *S. horneri* extract on bone calcium content may be the result of a stimulatory effect on bone formation and an inhibitory effect on bone resorption *in vitro*.[16,17] The prolonged oral administration of water-solubilized *S. horneri* extract to young and aged rats had an anabolic effect on femoral components *in vivo*,[18] suggesting that dietary supplementation

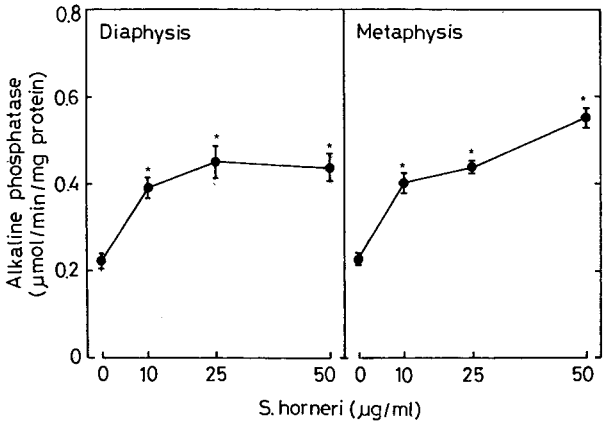

Fig. 8. Effect of *S. horneri* Extract on Alkaline Phosphatase Activity in the Femoral-diaphyseal and -metaphyseal Tissues Obtained from STZ-diabetic Rats *in Vitro*

Rats received a single subcutaneous administration of STZ (6.0 mg/100 g body weight), and 14 days later the animals were killed. Femoral-diaphyseal or -metaphyseal tissues obtained from STZ-diabetic 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. for six rats. $*p < 0.01$, compared with the control (none) value.

with *S. horneri* extract may prevent a decrease in bone mass with increasing age.

Diabetes has been shown to induce bone loss.[19,20,27] The oral administration of *S. horneri* extract to STZ-diabetic rats was found to have a preventive effect on bone loss with diabetes *in vivo*. This finding suggests that the dietary intake of *S. horneri* extract has a preventive effect on bone loss in the pathphysiologic state. When the femoral tissues obtained from STZ-diabetic rats were cultured in medium containing *S. horneri* extract solution, the femoral calcium content and alkaline phosphatase activity were significantly increased *in vitro*. Bone alkaline phosphatase is related to bone calcification.[28] *S. horneri* extract has been shown to stimulate bone formation[16] and to inhibit bone resorption[17] *in vitro*. Presumably, the preventive effect of *S. horneri* extract administration on diabetes-induced bone loss is related to a direct action of the active component of *S. horneri* extract.

The serum calcium level was found to increase in STZ-diabetic rats. Intestinal calcium absorption has been shown to be impaired in the diabetic state.[29,30] The increase in serum calcium concentration in STZ-diabetic rats may result from the release of calcium from bone tissues. The femoral calcium content was found to decrease markedly in STZ-diabetic rats. The oral administration of *S. horneri* extract to STZ-diabetic rats had a significant preventive effect on hypercalcemia with diabetic state. Presumably, the active component of *S. horneri* extract inhibits bone resorption, since the extract component has an inhibitory effect on bone resorption *in vitro*.[17]

Interestingly, the oral administration of *S. horneri* extract to STZ-diabetic rats had a significant preventive effect on the decrease in body weight and the increase in serum glucose and triglyceride levels induced in the diabetic state. This is a novel finding. The present result demonstrates that the intake of *S. horneri* extract has a partial restorative effect on serum biochemical finding with diabetes *in vivo*. Whether the active component of *S. horneri* extract in preventing bone loss induced with diabetes is identical to the component which can prevent an elevation of serum glucose and triglyceride levels with diabetes, is unknown.

The identification of active components in *S. horneri* extract remains to be elucidated. Heat-treated *S. horneri* extracts (30 min at 80°C) have been shown to counteract the stimulatory effect on bone formation *in vitro*.[16] 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*.[15] Further studies are in progress to identify the active component of *S. horneri* extract.

In conclusion, it has been shown that the oral administration of marine alga *S. horneri* extract to diabetic rats can prevent diabetes-induced bone loss, and that the administration has a partial prevent effect on the increase in serum glucose and triglyceride levels with diabetes *in vivo*.

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