Anabolic Effect of Zinc-Containing Marine Alga (*Fucus*) Extract on Bone Components in Rat Femoral Tissues *in vitro* and *in vivo*

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The effect of zinc-containing marine algae on bone components in the femoral tissues of rats *in vitro* and *in vivo* was investigated. Marine alga (*fucus*, *Phaeophyceae*) contained a comparatively higher concentration of zinc (3.16%). Water-extracted marine alga solution was prepared at concentrations of 10^{-7} to 10^{-5} M zinc. The femoral-diaphyseal and -metaphyseal tissues obtained from weanling rats were cultured for 24 h in Dulbecco's modified Eagle's medium containing either vehicle or zinc-containing marine alga water extract (10^{-7} to 10^{-5} M zinc) *in vitro*. The presence of zinc-containing marine alga extract caused a significant increase of calcium content, alkaline phosphatase activity, and deoxyribonucleic acid (DNA) content in the femoral-diaphyseal and metaphyseal tissues. This anabolic effect was completely blocked by the presence of a chelator of zinc ion or an inhibitor of protein synthesis. Moreover, when weanling or elderly rats received daily an oral administration of zinc-containing marine alga suspension (5.0 mg zinc/100 g body weight) for 8 or 10 days, respectively, the femoral-diaphyseal and -metaphyseal components were significantly elevated. This administration produced a significant increase in the serum and femoral zinc concentrations of elderly rats (50 weeks old). The present study demonstrates that zinc-containing marine alga materials have an anabolic effect on bone metabolism *in vitro* and *in vivo*, suggesting a useful role for zinc-containing food on the prevention of bone loss with increasing age.

Keywords: zinc, bone metabolism, zinc-containing marine alga, *fucus*, bone anabolic effect, aging, rat femur

Zinc is known to be an essential element for the growth of humans and other animals (Prasad *et al.*, 1961; Burch *et al.*, 1975). Zinc is required for the growth, development, and maintenance of healthy bones. Bone growth retardation is a common finding in various conditions associated with zinc deficiency (Hsieh & Navia, 1980; Oner *et al.*, 1984). Zinc plays a physiologically important role in bone tissues. In recent years, there is growing evidence that zinc has a role in the regulation of bone metabolism; the metal has been demonstrated to stimulate bone formation and to inhibit bone resorption (Yamaguchi, 1998).

On the other hand, osteoporotic patients have been shown to have lower levels of skeletal zinc than controls (Reginster *et al.*, 1988). In postmenopausal women, urinary zinc has been suggested as a marker of bone resorption, since women with osteoporosis excreted more than 800 μ g zinc/g creatinine in urine (Herzberg *et al.*, 1990). Zinc supplementation could have beneficial effects on bone density.

Recently, it has been demonstrated that zinc compounds have a preventive effect on bone loss in ovariectomized rats as an animal model of osteoporosis (Yamaguchi, 1995a; 1995b), suggesting a pharmacological usefulness of zinc in the prevention of bone loss due to osteoporosis. However, whether or not a supplement of zinc-containing foods can have a preventive effect on osteoporosis with increasing age has not yet been shown. Marine algae (*fucus*, *Phaeophyceae*) contain a comparatively higher concentration of zinc, and may be used as a supplemental zinc nutrient. Therefore, the

present study was undertaken to clarify the effect of marine algae (*fucus*) on bone metabolism *in vitro* and *in vivo*.

Materials and Methods

Chemicals Dulbecco's modified Eagle's medium (high glucose, 4.5 g/dl) and a penicillin-streptomycin solution (5000 U/ml penicillin; 5000 μ g/ml streptomycin) were purchased from Gibco Laboratories (Grand Island, N.Y.). Bovine serum albumin (fraction V), dipicolinate (2,6-pyridinedicarboxylic acid; neutralized with sodium hydroxide prior to use), and cycloheximide were purchased from Sigma Chemical Co. (St. Louis, Mo.). Zinc sulfate and other chemicals were reagent grade from Wako Pure Chemical Industries (Osaka).

Marine alga extracts The powder of marine alga (*fucus*) (Benfact Co., Tokyo) contained 3.16% of zinc, but amount of lead, copper, selenium, vanadium, chromium, nickel, and cobalt were negligible. Mineral components in marine alga powder were 0.73% calcium and 0.07% phosphorus. Marine alga powder (206.9 mg) was dissolved in distilled water (10 ml), mixed vigorously for 1 h at room temperature, and filtered. The concentration of zinc in the filtrate was 49.5 μ g/ml (7.6×10^{-4} M); the metal was determined by atomic absorption spectrophotometry. Before its use in bone culture experiments, marine alga extract was aseptically filtered through a membrane filter (0.22 μ m).

Animals Male Wistar weanling rats (4 weeks old; 5 animals) or elderly male rats (50 weeks old; 5 animals) were

obtained from Japan SLC (Hamamatsu). Five animals were put in a cage and fed a commercial laboratory pellet diet containing 57.4% carbohydrate, 1.1% of Ca, and 1.1% of P, and housed in a temperature (25°C)-controlled room with free access to distilled water.

Bone culture Weanling rats (5 in each group) were sacrificed by cardiac puncture under light anesthesia with ether. The femurs were removed aseptically after bleeding and soaked in ice-cold 0.25 M sucrose solution. The femur was removed from soft tissue and marrow, and the diaphysis and metaphysis were separated. Bone tissues were cut into small pieces. Bone fragments (17–23 mg wet weight) were cultured for 24 h in a 35-mm dish in 2.0 ml medium consisting of Dulbecco's modified Eagle's medium (high glucose, 4.5 g/dl) supplemented with 0.25% bovine serum albumin containing antibiotics (100 U penicillin and 100 µg streptomycin/ml of medium) (Yamaguchi *et al.*, 1987). Cultures were maintained at 37°C in a water-saturated atmosphere containing 5% CO₂ and 95% air. The culture medium contained zinc-containing marine alga extract (10⁻⁷, 10⁻⁶, and 10⁻⁵ M Zn) or zinc sulfate (10⁻⁵ M) in the absence or presence of cycloheximide (10⁻⁶ M) or dipicolinate (10⁻³ M).

Administration procedures The dried powder of marine alga (Hibamata) (79.2 and 158.3 mg) was suspended in distilled water (1.0 ml) to give the concentrations of 2.5 and 5.0 mg of Zn per ml. Weanling or elderly rats (5 in each group) received daily an oral administration of marine alga suspension (1.0 ml/100 g body weight) through a stomach tube for 8 or 10 days, respectively, and 24 h after the last administration the animals were sacrificed by bleeding. Five control rats received an oral administration of distilled water. In another experiment, zinc sulfate (5.0 mg Zn/ml/100 g body weight) was orally administered daily to 5 weanling rats through a stomach tube for 8 days. Finally, the blood and femurs were immediately obtained from all the rats.

Analytical procedures The bone tissues from the weanling and elderly rats were dried for 16 h at 110°C, weighed, and then hydrolyzed with nitric acid (2.0 ml) for 12 h at 120°C. Calcium and zinc were determined by atomic absorption spectrophotometry (Yamaguchi *et al.*, 1987). The calcium content was expressed as mg per g dry bone.

To assay alkaline phosphatase activity, the bone tissues were immersed in 3.0 ml of ice-cold 6.5 mM barbital buffer (pH 7.4), cut into small pieces, homogenized with a Physcotron homogenizer, and disrupted for 60 s with an ultrasonic device. The supernatant was centrifuged at 600×*g* for 5 min and used to measure the enzyme activity. Alkaline phosphatase activity was determined by the method of Walter and Schutt (1965) and expressed as µmol of *p*-nitrophenol liberated per min per mg protein. Protein concentration was determined by the method of Lowry *et al.* (1951).

To measure bone DNA content, the bone tissues were shaken with 4.0 ml of ice-cold 0.1 N NaOH solution for 24 h after their homogenization using the method described by Flanagan and Nichols (1962). After alkali extraction, the samples were centrifuged at 10,000×*g* for 5 min, and the supernatant was collected. DNA content in the supernatant was determined by the method of Ceriotti (1955) and expressed as the amount (mg) of DNA per g wet weight of

bone tissue.

Serum calcium was determined by the method of Willis (1960). Serum inorganic phosphorus was measured by the method of Taussky and Shon (1953). Serum zinc was determined by atomic absorption spectrophotometry (Yamaguchi *et al.*, 1987).

Statistical analysis The significance of the difference between values was estimated by the Student's *t*-test. A *p* value of less than 0.05 was considered to be a statistically significant differences. Also, we used a multi-way ANOVA with a Duncan's post hoc test to compare the tested groups.

Results

Effect of marine alga extract in bone tissue culture

The effect of marine alga extract added to the culture system at the concentrations of 10⁻⁷, 10⁻⁶, and 10⁻⁵ M zinc using the femoral-diaphyseal and -metaphyseal tissues obtained from weanling rats was examined. Calcium content in the diaphyseal tissues was significantly increased by the presence of marine alga extract (10⁻⁷ to 10⁻⁵ M), and that of the metaphyseal tissues was significantly elevated by the 10⁻⁵ M (Fig. 1). Alkaline phosphatase activity in both tissues was significantly increased by the presence of the extract (10⁻⁶ and 10⁻⁵ M zinc) (Fig. 2). DNA content in the diaphyseal tissues was also significantly elevated (10⁻⁷ and 10⁻⁶ M zinc), while the 10⁻⁵ M addition did not have such a significant effect (Fig. 3). DNA content in the metaphyseal tissues was significantly increased by the 10⁻⁵ M zinc-containing extract (Fig. 3). Thus, this extract had a direct anabolic effect on bone components in the femoral-diaphyseal and -metaphyseal tissues obtained from weanling rats.

The effect of marine alga extract (10⁻⁵ M zinc) and zinc sulfate (10⁻⁵ M) on calcium content and alkaline phosphatase activity in the femoral-diaphyseal and -metaphyseal tissues was compared, and the result is shown in Fig. 4. These components were significantly increased by the presence of both substances; the effects of the two zinc materials were

Fig. 1. Effect of marine alga extract on calcium content in the femoral-diaphyseal and -metaphyseal tissues from weanling rats *in vitro*. Bone tissues were cultured for 24 h in the medium containing either vehicle (distilled water) or marine alga extract (10⁻⁷ to 10⁻⁵ M zinc). Each value is the mean ± SEM of five bone tissues from different animals. **p* < 0.01, compared with the control value.

Fig. 2. Effect of marine alga extract on alkaline phosphatase activity in the femoral-diaphyseal and -metaphyseal tissues from weanling rats *in vitro*. Bone tissues were cultured for 24 h in the medium containing either vehicle (distilled water) or marine alga extract (10^{-7} to 10^{-5} M zinc). Each value is the mean $\pm$ SEM of five bone tissues from different animals. * $p < 0.01$, compared with the control value.

Fig. 3. Effect of marine alga extract on DNA content in the femoral-diaphyseal and -metaphyseal tissues from weanling rats *in vitro*. Bone tissues were cultured for 24 h in the medium containing either vehicle (distilled water) or marine alga extract (10^{-7} to 10^{-5} M zinc). Each value is the mean $\pm$ SEM of five bone tissues from different animals. * $p < 0.01$, compared with the control value.

equal.

To determine whether or not the effect of zinc-containing marine alga extract on calcium content and alkaline phosphatase activity in the femoral-diaphyseal and -metaphyseal tissues is zinc dependent, the present study used dipicolinate, a potent chelator of zinc ion. Zinc-containing extract-induced increase in the bone components was completely prevented by the presence of dipicolinate (10^{-3} M) (Fig. 5), indicating that zinc in the marine algae is an essential factor for the increases in bone calcium content and alkaline phosphatase activity.

The presence of cycloheximide (10^{-6} M), an inhibitor of protein synthesis, caused a complete inhibition of marine alga extract (10^{-5} M zinc)-induced increase in calcium content and

Fig. 4. Effect of marine alga extract or zinc sulfate on calcium content and alkaline phosphatase activity in the femoral-diaphyseal and -metaphyseal tissues from weanling rats *in vitro*. Bone tissues were cultured for 24 h in the medium containing either vehicle (distilled water), marine alga extract (10^{-5} M zinc) or zinc sulfate (10^{-5} M). Each value is the mean $\pm$ SEM of five bone tissues from different animals. * $p < 0.01$, compared with the control value. □, Control; ▨, marine alga extract; ■, zinc sulfate.

Fig. 5. Effect of dipicolinate on marine alga extract-increased calcium content and alkaline phosphatase activity in the femoral-diaphyseal and -metaphyseal tissues from weanling rats *in vitro*. Bone tissues were cultured for 24 h in the medium containing either vehicle (distilled water) or marine alga extract (10^{-5} M zinc) in the absence or presence of dipicolinate (10^{-3} M). Each value is the mean $\pm$ SEM of five bone tissues from different animals. * $p < 0.01$, compared with the control value. □, Control; ■, marine alga extract.

Fig. 6. Effect of cycloheximide on marine alga extract-increased calcium content and alkaline phosphatase activity in the femoral-diaphyseal and -metaphyseal tissues from weanling rats *in vitro*. Bone tissues were cultured for 24 h in the medium containing either vehicle (distilled water) or marine alga extract (10^{-5} M zinc) in the absence or presence of cycloheximide (10^{-6} M). Each value is the mean $\pm$ SEM of five bone tissues from different animals. * $p < 0.01$, compared with the control value. □, Control; ■, marine alga extract.

Table 1. Effect of oral administration of marine alga suspension or zinc sulfate on bone components in the femoral-diaphyseal and -metaphyseal tissues of weanling rats.

Treatment (mg/100g/day)	Calcium (mg/g dry bone)	Alkaline phosphatase (μ mol/min/mg protein)	DNA (mg/g wet bone)
Diaphysis			
Control	251.2 $\pm$ 4.47	2.45 $\pm$ 0.13	1.42 $\pm$ 0.07
Zinc sulfate (5.0 mg)	272.8 $\pm$ 3.99*	3.48 $\pm$ 0.28*	1.85 $\pm$ 0.12*
Marine alga (2.5 mg Zn)	237.2 $\pm$ 6.32	3.51 $\pm$ 0.10*	1.89 $\pm$ 0.16*
Marine alga (5.0 mg Zn)	302.2 $\pm$ 8.98*#	4.31 $\pm$ 0.29*	1.98 $\pm$ 0.16*
Metaphysis			
Control	231.6 $\pm$ 3.25	2.28 $\pm$ 0.07	2.57 $\pm$ 0.03
Zinc sulfate (5.0 mg)	235.6 $\pm$ 2.12	2.36 $\pm$ 0.04	3.57 $\pm$ 0.09*
Marine alga (2.5 mg Zn)	232.1 $\pm$ 1.76	2.51 $\pm$ 0.08	3.12 $\pm$ 0.04*
Marine alga (5.0 mg Zn)	246.2 $\pm$ 0.59*#	2.86 $\pm$ 0.08*#	3.48 $\pm$ 0.15*

Rats received an oral administration of zinc sulfate (5.0 mg Zn/100 g body weight) or marine alga suspension (2.5 and 5.0 mg Zn/100 g) daily for 8 days, and 24 h after the last administration the rats were sacrificed. Each value is the mean $\pm$ SEM of five rats. * $p < 0.01$, compared with the control value, # $p < 0.01$, compared with the value from zinc sulfate administration.

alkaline phosphatase activity in the femoral-diaphyseal and -metaphyseal tissues (Fig. 6).

Effect of oral administration of marine algae on bone components in rats Weanling rats received a daily oral administration of marine alga suspension (2.5 and 5.0 mg zinc/100 g body weight) or zinc sulfate (5.0 mg zinc/100 g) for 8 days. The administration of marine alga suspension did not cause a significant alteration in body weight; it was 126.6 ± 5.6 g in normal rats and 130.3 ± 6.9 g in marine alga

Table 2. Effect of oral administration of marine alga suspension on bone components in the femoral-diaphyseal and -metaphyseal tissue of elderly rats.

Treatment	Calcium (mg/g dry bone)	Alkaline phosphatase (μ mol/min/ mg protein)	DNA (mg/g wet bone)	Zn (μ g/g dry bone)
Diaphysis				
Control	192.8 $\pm$ 9.64	0.89 $\pm$ 0.09	0.62 $\pm$ 0.04	185.5 $\pm$ 15.2
Marine alga	250.2 $\pm$ 7.27*	1.15 $\pm$ 0.06*	2.08 $\pm$ 0.12*	396.4 $\pm$ 42.4*
Metaphysis				
Control	217.0 $\pm$ 3.29	0.79 $\pm$ 0.02	0.67 $\pm$ 0.08	435.6 $\pm$ 39.1
Marine alga	257.5 $\pm$ 4.49*	0.92 $\pm$ 0.04*	2.13 $\pm$ 0.06*	639.0 $\pm$ 40.2*

Rats received daily an oral administration of marine alga suspension (5.0 mg Zn/100 g body weight) for 10 days, and 24 h after the last administration the animals were sacrificed. Each value is the mean $\pm$ SEM of five rats. * $p < 0.01$, with the control value.

Table 3. Serum calcium, inorganic phosphorus, and zinc concentrations in elderly rats when orally administered marine alga suspension.

Treatment	Calcium (mg/dl)	Inorganic phosphorus (mg/dl)	Zinc (μ g/dl)
Control	9.91 $\pm$ 0.09	4.66 $\pm$ 0.32	160.3 $\pm$ 9.40
Marine alga	9.45 $\pm$ 0.20	5.30 $\pm$ 0.11	207.2 $\pm$ 6.28*

Rats received a daily oral administration of marine alga suspension (5.0 mg Zn/100 g) for 10 days, and 24 h after the last administration the animals were sacrificed. Each value is the mean $\pm$ SEM of five rats. * $p < 0.01$, compared with the control value.

(5.0 mg zinc)-administered rats (mean $\pm$ SEM for five rats). Calcium content, alkaline phosphatase activity, and DNA content in the femoral-diaphyseal and -metaphyseal tissues were all significantly increased by the administration of zinc-containing marine alga suspension (5.0 mg zinc/100 g) (Table 1). A partial effect was seen by the dose of 2.5 mg zinc/100 g. Meanwhile, the administration of zinc sulfate (5.0 mg zinc/100 g) caused a significant increase in calcium content, alkaline phosphatase activity, and DNA content in the femoral-diaphyseal tissues, while only DNA content in the metaphyseal tissues was significantly raised (Table 1). Thus, marine alga suspension had a greater effect than zinc sulfate.

Elderly rats received daily an oral administration of marine alga suspension (5.0 mg zinc/100 g body weight) for 10 days. The administration caused a significant increase in calcium content, alkaline phosphatase activity, DNA and zinc contents in the femoral-diaphyseal and -metaphyseal tissues (Table 2). Calcium and inorganic phosphorus concentrations in the serum were not significantly changed by the oral administration of the same suspension to elderly rats, while serum zinc concentration was significantly elevated (Table 3).

Discussion

The effect of zinc-containing marine alga (fucus) extract on bone metabolism in rat femoral tissues *in vitro* and *in vivo* was investigated. The presence of water extracts from marine alga powder (containing 10^{-7} to 10^{-5} M zinc) in the culture medium caused a significant increase in bone components (calcium, alkaline phosphatase, and DNA) *in vitro*, indicating that the extract has an anabolic effect on bone metabolism.

Marine alga extract-induced increase in bone components (calcium content and alkaline phosphatase activity) was completely prevented by the presence of dipicolinate, a chelator of zinc ion (Pocker & Fong, 1980; Yamaguchi & Matsui, 1989). This result suggests that the anabolic effect caused by the extract relates to the zinc concentration in marine algae, and that the extract not containing zinc does not have the effect. At present, the chemical form of zinc in marine alga is unknown. The soluble zinc was about 8% of the total amount of zinc. Thus, most zinc may tightly bind to the algal components.

The increase in bone components caused by marine alga extract was completely blocked by the presence of cycloheximide, an inhibitor of protein synthesis, in the culture medium *in vitro*. The anabolic effect of the extract might be based on a newly synthesized protein component. Zinc is known to stimulate bone protein synthesis due to the activation of aminoacyl t-RNA synthetase in osteoblastic cells (Yamaguchi & Matsui, 1989; Yamaguchi *et al.*, 1994). Presumably, the anabolic effect of zinc-containing marine alga extract is caused by the stimulation of protein synthesis with zinc in osteoblastic cells of rat femoral tissues.

Examination of the *in vivo* effect of marine alga powder suspension on bone components in weanling and elderly rats showed that femoral components (calcium, alkaline phosphatase, and DNA) were significantly increased by an oral administration which included 2.5 and 5.0 mg zinc/ml to weanling rats for 8 days. Such effect was seen in the case of an oral administration of zinc sulfate (5.0 mg zinc/ml/100 g body weight) to these rats. However, the effect of marine alga suspension (5.0 mg zinc/100 g) was greater than that of zinc sulfate. The stimulation of β -alanyl-L-histidinato zinc, at the same dosage as zinc, on bone metabolism reportedly is more intensive than that of zinc sulfate in weanling rats *in vitro* (Yamaguchi & Ozaki, 1990).

It is known that bone mass decreases in both men and women with increasing age. This decrease may be due to increased bone resorption (Kalu *et al.*, 1989) or to decreased bone formation (Nishimoto *et al.*, 1989). In fact, the present study demonstrated that bone components (calcium and DNA contents, and alkaline phosphatase activity) in weanling rats markedly decreased with increasing age as seen in elderly rats. Bone components in elderly rats were significantly increased by an oral administration of marine alga suspension (5.0 mg zinc/100 g) to elderly rats for 10 days, suggesting that the intake of zinc-containing food has a preventive effect on bone loss with age. Serum zinc concentration and femoral zinc content were significantly elevated by the administration of marine alga suspension. An increase in serum zinc concentration after marine alga administration may cause zinc to accumulate into bone tissues. Marine alga suspension increased bone components *in vitro*. Presumably, zinc accumulated in the bone tissues directly stimulates bone formation. The supplementation of marine alga may be useful in preventing osteoporosis with increasing age.

In conclusion, the present study demonstrated that zinc-containing marine algae have an anabolic effect on bone components in the femoral tissues of rats *in vitro* and *in vivo*.

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