An Extract of *Eisenia Bicyclis* Stimulates Mineralized Nodule Formation by Osteoblasts

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Abstract: Diet is an important factor maintaining bone health. Several studies have reported that extracts of brown marine alga containing polyphenols positively regulate bone metabolism. Therefore, we examined the effects of *Eisenia bicyclis* (Japanese name, Arame), an edible brown marine algae, on cell proliferation, alkaline phosphatase activity, collagenous and non-collagenous bone matrix protein expressions, and mineralized nodule formation using ROS17/2.8 cells as osteoblasts. A thermal extract derived from *E. bicyclis* was applied to a Diaion HP-20 column, and a portion of the 50% ethanol fraction was used as the *E. bicyclis* extract. The cells were cultured in the presence or absence of *E. bicyclis* extract, and cell proliferation and alkaline phosphatase activity were determined. The expressions of type I collagen, bone sialoprotein, and osteopontin were examined at the mRNA level using real-time PCR. Mineralized nodule formation was detected via alizarin red staining. Alkaline phosphatase activity and the expressions of type I collagen, bone sialoprotein, and osteopontin were increased in the presence of *E. bicyclis* extract, whereas cell proliferation was not affected. The *E. bicyclis* extract also increased mineralization. Together, these results indicate that the extract induces osteogenic function in osteoblasts.

Key words: Alkaline phosphatase, Bone matrix protein, Marine algae, Osteoblasts

Introduction

Bone mass and quality are maintained via remodeling, a process of formation and resorption. In adult bones, osteocytes are abundant, and the proportions of osteoblasts and osteoclasts are approximately 4–6% and 1–2%, respectively. Despite being less abundant, osteoblasts play a central role in bone remodeling. They have an osteogenic phenotype that release compounds such as alkaline phosphatases (ALPases) and bone matrix proteins, whereas osteoclasts secrete H⁺ and proteases that hydrolyze inorganic and organic components of bone into the resorptive microenvironment.

Most bone diseases stem from an imbalance in bone remodeling. When the resorption process predominates, bone becomes fragile, and the risks for osteoporosis and subsequent bone fracture increase. Although the major risk factors for osteoporosis are advanced age and postmenopause status in women, lifestyle is also associated with bone fragility. A study that evaluated bone mineral density (BMD) revealed that the odds ratio of osteoporosis or osteopenia with low BMD was significantly increased by smoking and was decreased by daily calcium consumption, even after adjusting for age and sex. A longitudinal study also reported that a healthy diet, abundant in vegetables and non-fried fish, was positively associated with retaining BMD. Thus, diet is an important factor in the maintenance of bone health.

Recently, *in vitro* and *in vivo* studies have reported that polyphenols and carotenoids derived from food, such as isoflavones in fermented soybeans, phlorotannins in brown algae, and β-cryptoxanthin in fruit and vegetables, positively regulate bone metabolism. In the present study, we cultured the ROS17/2.8 osteoblastic cell line with extracts of *E. bicyclis*, an edible brown marine algae that contains phlorotannins, and examined cell proliferation, ALPase activity, the mRNA expressions of bone matrix proteins, and *in vitro* mineralization. The extract facilitated osteogenic function in osteoblasts.

Materials and Methods

Preparation of the extract

*E. bicyclis* (Japanese name, Arame) samples were collected from along the coast of Oki Island in Shimane Prefecture, Japan. The algae were washed with filtered seawater and soaked in ion-exchanged water. Thermal extraction was carried out for 3 h at 95°C. The crude extract was applied to a Diaion HP-20 column (Mitsubishi Chemical Co., Ltd., Tokyo, Japan) and a portion of the 50% ethanol fraction was used as extract.

Preparation of the extract was conducted in the laboratory of the Department of Applied Science, Inabata Koryo Co., Ltd., Osaka, Japan.

Cell culture and proliferation

The rat osteosarcoma cell line ROS17/2.8 cells that were from...
Department of Biochemistry and maintained in the Department of Oral Health Sciences, Nihon University School of Dentistry, Tokyo, Japan(18) were used as a model of osteoblasts. The cells were placed in 96-well plates at a density of $5.0 \times 10^3\text{cells/cm}^2$ and left overnight to settle in α-minimal essential medium (MEM) (Gibco-BRL, Rockville, MD, USA) containing 10% (v/v) heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT, USA) and 1% (v/v) penicillin-streptomycin solution (Sigma-Aldrich, St. Louis, MO, USA) at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Then the cells were cultured in medium supplemented with 0, 0.1, 1, 10, or 100 ng/ml E. bicyclis extract for up to 7 days. The culture medium was changed every 2 days. Cell proliferation was determined using a cell-counting kit (Dojindo Molecular Technologies Inc., Kumamoto, Japan). Briefly, the culture medium was replaced with 100 μM α-MEM containing 10% cell-counting reagent, and then the plates were incubated for 1 h at 37°C. The absorbance at 450 nm of each well was measured using a microplate reader (Spectra Max 190, Molecular Devices LLC., San Jose, CA, USA).

**ALPase activity**

The cells were plated into 96-well plates at a density of $5.0 \times 10^3\text{cells/cm}^2$ and cultured in medium with 0, 0.1, 1, 10, or 100 ng/ml extract for up to 7 days. A total of 200 μl enzyme assay solution (8 mM p-nitrophospho-nyl phosphate, 12 mM MgCl₂, 0.1 mM ZnCl₂, and 0.1 M glycine-NaOH buffer, pH 10.5) was added per well, and the plates were incubated for several minutes at 37°C. The enzyme reaction was terminated by adding 50 μl 0.2 M NaOH. The amount of p-nitrophospho-nyl released was determined by measuring the absorbance at 405 nm using a microplate reader. One unit of ALPase activity was defined as the amount required for release of 1.0 μM p-nitrophospho-nyl per minute. Enzyme activity was recorded in milliunits (mU)/10⁵ cells.

**Real-time PCR**

Cells were plated into 6-well plates at a density of $5.0 \times 10^3\text{cells/cm}^2$ and cultured in medium with 0, 10, or 100 ng/ml extract for up to 7 days. Total RNA including mRNA was eluted from cells harvested after 3 or 5 days incubation using NucleoSpin RNA (Takara Bio Inc., Shiga, Japan). cDNA synthesis was conducted using 1 μg RNA in 20 μl solution containing random primers, a dNTP mixture, and reverse transcriptase; 2 μl aliquots of the cDNA solution were subjected to quantitative real-time RT-PCR. Reactions were performed using a Thermal Cycler Dice Real Time System (Takara Bio) and analyzed using the instrument’s software. The reaction conditions such as temperature for denaturation, annealing, and extension were the same as those used in a previous study.(18)

![Figure 1. Effects of E. bicyclis extract on osteoblast proliferation.](image)

ROS17/2.8 cells were cultured with 0 (control), 0.1, 1, 10, or 100 ng/ml E. bicyclis extract for up to 7 days. The cells were quantified on days 3, 5, and 7 of culture. Each bar indicates the mean ± SD of three independent experiments. **p < 0.01 vs. the control.

**Figure 2. Effects of E. bicyclis extract on ALPase activity.** ROS17/2.8 cells were cultured with 0 (control), 0.1, 1, 10, or 100 ng/ml E. bicyclis extract, and ALPase activity was determined on days 3, 5, and 7 of culture. Each bar indicates the mean ± SD of three independent experiments. **p < 0.01**

**Formation of mineralized nodules**

The cells were plated into 24-well tissue culture plates at a density of $5.0 \times 10^3\text{cells/cm}^2$ and cultured in α-MEM with 50 mM β-glycerophosphate and 50 μg/ml ascorbic acid in the presence of α-MEM with 0, 10, or 100 ng/ml E. bicyclis extract for 10 days. The conditions of the cells and nodule formation were checked routinely by phase-contrast microscopy (Nicon DIAPHOTO phase contrast ELWD 0.3, Nikon Corp., Tokyo, Japan) at ×100 magnification. Mineralized nodules were detected by staining with alizarin red (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan), as described previously.(18)

**Statistical analyses**

Values are reported as the mean ± standard deviation (SD). Significant differences were determined using Bonferroni’s modification following analysis of variance. Differences with a value of $p < 0.05$ were considered statistically significant.

**Results**

**Effects of E. bicyclis extract on cellular proliferation and ALPase activity**

The cells were cultured in the absence (0 ng/ml; control) or presence (0.1, 1, 10, or 100 ng/ml) of E. bicyclis extract for 7 days, and cell proliferation (Fig. 1) and ALPase activity (Fig. 2) were determined on days 3, 5, and 7. The extract did not affect cell proliferation but ALPase
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Effects of E. bicyclis extract on type I collagen, bone sialoprotein, and osteopontin mRNA expression

The cells were cultured in the absence (0 ng/ml; control) or presence (10 or 100 ng/ml) of E. bicyclis extract for up to 5 days. The expressions of type I collagen, bone sialoprotein, and osteopontin at mRNA levels were determined by real-time PCR on days 3 and 5 of culture. Each bar indicates the mean ± SD of three independent experiments. *p < 0.05, **p < 0.01 vs. the control.

Effects of E. bicyclis extract on mineralized nodule formation

The cells were cultured in the absence (0 ng/ml; control) or presence (10 or 100 ng/ml) of E. bicyclis extract for 10 days, and mineralized nodule formation was observed via alizarin red staining (Fig. 4). The intensity of staining increased in cells cultured at 10 ng/ml but increased in those at 100 ng/ml. Type I collagen expression on day 5 and bone sialoprotein expression on day 3 were not affected by the extract.

Discussion

We examined the effects of an extract from E. bicyclis on osteogenesis in osteoblasts using ROS 17/2.8 cells, which have a high ability to form mineralized nodules. We first examined cell proliferation and ALPase activity. Cell proliferation was not affected by adding E. bicyclis extract in the concentration range of 0.1–100 ng/ml, whereas ALPase activity increased when incubated with 10 and 100 ng/ml extract. ALPase, which hydrolyzes the ester bond of organic phosphate compounds under alkaline conditions, plays an important role in bone calcification. ALPase hydrolyzes substances that inhibit calcification, such as pyrophosphate and adenosine triphosphate, and is also considered a potential Ca²⁺ carrier. We speculated that 10 and 100 ng/ml E. bicyclis extract...
might have an upregulatory effect on osteogenesis, and further studied the use of the extract at these concentrations.

Type I collagen is a major constituent of the organic phase of bone tissue, and plays a role in scaffolding during nucleation of hydroxyapatite crystals. Non-collagenous matrix proteins are also important in the organization of the collagen matrix and in regulating the formation and growth of hydroxyapatite crystals. Previous studies have reported that the expressions of bone matrix proteins in osteoblasts are affected by external factors, such as bacterial stimuli, nicotine, mechanical stress, and that these stimuli-induced changes in expression are associated with mineralized nodule formation in vitro. Hence, we examined the level of bone matrix protein expression, and found that 10 or 100 ng/ml E. bicyclus extract induced the expression of type I collagen, bone sialoprotein, and osteopontin mRNA. Moreover, alizarin red staining revealed that mineralized nodule formation was also increased by the extract. Bone sialoprotein anchors osteoblasts to the bone, and supports cell attachment and binding to calcium ion. Osteopontin, which contains potential phosphorylation sites, such as serine- and aspar-tate-rich motifs, is associated with both downregulation and upregulation during the calcification process in tissues. Phosphorylated osteopontin is considered an inhibitor of mineral deposition, whereas treatment of osteopontin with ALPase reduces the inhibitory effects of osteopontin on hydroxyapatite formation. Moreover, osteopontin can serve as an agent for intra-fibrillar mineralization in collagen. In the present study, mineralized nodule formation as well as ALPase activity and osteopontin expression increased in cells cultured with the extract. Hence, osteopontin might not be an inhibitor of mineral deposition in cells in which simultaneous increases in osteopontin expression and ALPase activity are induced by the extract.

Many studies have investigated the effects of substrates derived from food on bone metabolism and growth of hydroxyapatite crystals. Regarding marine algae, a few previous studies have reported their effects on osteogenesis in vitro. Ahn et al. and Karadeniz et al. examined the effects of extracts from E. cava on MT3T3-E1 murine osteoblastic cells, and found that ALPase activity and collagenous and non-collagenous protein expressions were significantly increased by treatment. They also isolated four kinds of phlorotannins (triphlorethol-A, eckol, dieckol and dioxinodehydroeckol) from crude extracts of E. cava, and confirmed that they also had osteogenic effects. In the present study, the E. bicyclus crude extract induced the expressions of collagenous and non-collagenous proteins, but its effects were not consistent; the upregulatory effects slightly favored the expression of bone sialoprotein over type I collagen, and the opposite effects were observed in osteopontin expression on day 3 using 10–100 ng/ml extract. A previous study reported that there were differences in the local and chemical distribution of phlorotannins between E. bicyclus and E. cava, and that these differences might influence the inconsistent effects of extracts on osteogenesis. In addition, it is also possible that a component with partially suppressive effects on bone matrix protein expression was present in the crude extract. Further study is needed to determine the active constituent(s) of the extracts that confer these effects on osteogenesis. Moreover, both osteoblastic bone formation and osteoelastic bone resorption contribute to bone remodeling, so a study that uses osteoclasts is also needed to clarify the effects of E. bicyclus on bone homeostasis.

In conclusion, the extract derived from E. bicyclus, an edible brown marine alga, increased mineralized nodule formation in ROS17/2.8 cells. It also upregulated ALPase activity and the expressions of bone matrix proteins, suggesting that these upregulatory effects might be positively associated with the mineralization process.

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Conflict of Interest

Torus Nakasugi is an employee of Inabata Koryo Co. Ltd. and the remaining authors have declared that no conflicts of interest exist.

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