Histomorphological Confirmation of the Preventive Effect of β-Alanyl-L-histidinato Zinc on Bone Loss in Ovariectomized Rats

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The effect of β-alanyl-L-histidinato zinc (AHZ), in which zinc is chelated to β-alanyl-L-histidine, on bone loss was investigated in the femur of ovariectomized rats. AHZ (10, 30 and 100 mg/kg/d) was orally administered to ovariectomized rats for 3 months. Ovariectomy significantly decreased the estradiol concentration in the serum as compared with that from sham-operated rats. This decrease was not altered by the dose of AHZ. The bone ash weight and mineral density in the femur of ovariectomized rats significantly decreased in comparison with those from sham-operated rats. Moreover, the trabecular bone at the femoral metaphysis was clearly decreased by ovariectomy. The decreases in the femoral ash content and mineral density and the metaphyseal trabecular bone were clearly prevented by the tested doses of AHZ (10, 30 and 100 mg/kg/d). The present finding with histomorphological study further supports the view that the administration of AHZ can prevent bone loss by ovariectomy.

Keywords osteoporosis; β-alanyl-L-histidinato zinc; ovariectomy; rat femur

Zinc has been demonstrated to serve a wide variety of functions in the mammalian system, and is essential for growth in humans and many animals. Bone growth retardation is a common finding in various conditions associated with zinc deficiency. It has been demonstrated that zinc has a stimulatory effect on bone formation and mineralization in vivo and in vitro, the metal stimulates bone protein synthesis which is a cellular mechanism. Thus, zinc plays a physiologic role as an activator in the regulation of bone formation. β-Alanyl-L-histidinato zinc (AHZ), in which zinc is chelated to β-alanyl-L-histidine, is a new zinc peptide, and it has been demonstrated that AHZ can stimulate bone formation and calcification in vivo and in vitro, and that the compound has a more intensive effect on bone formation than zinc sulfate. AHZ has a potent stimulating effect on the proliferation and differentiation of osteoblastic cells in vitro, and this effect is dependent on protein synthesis.

AHZ may play a therapeutic role in bone metabolism disorders, although this has not been fully shown. More recently, it has been reported that AHZ has a preventive effect on osteopenia in rats with skeletal unloading, feeding low-calcium and vitamin D-deficiency diet, hydrocortisone and adjuvant arthritis. It is also known that ovarian hormone deficiency at menopause stimulates bone loss. The present investigation was undertaken to clarify the preventive effect of the prolonged administration of AHZ on bone loss in ovariectomized rats by histomorphological studies. It was found that AHZ prevents bone loss in the femur of ovariectomized rats.

MATERIALS AND METHODS

Administration Procedures Female Wistar rats (conventional) weighing 100—120 g (5-weeks-old) were obtained from Japan SLC (Hamamatsu). The animals were fed commercial laboratory chow (solid) containing 1.1% Ca, 1.1% P, and 0.012% Zn, at a room temperature of 25°C, with free access to distilled water. At 6-weeks of age, rats were divided into 5 groups of 10 rats. Animals in group 1 were given a sham-ovariectomy, and animals in groups 2 to 5 were given a bilateral ovariectomy under ether anesthesia. In the sham-operated animals, both ovaries were handled, but not removed. All animals were fed matched amounts of the chow described above for 3 weeks. From 9 weeks of age, the rats in groups 3, 4 and 5 received orally 10, 30 and 100 mg AHZ/kg of body weight per day. AHZ (z-103), which was supplied by Zeria Pharmaceutical Co. (Tokyo), was dissolved in 1 n HCl and adjusted to pH 7.0 with 1 n NaOH to the concentrations used: 1.0, 3.0 and 10.0 mg/ml (2.2, 6.6 and 22.0 mg Zn/ml). These solutions (1.0 ml/100 g body weight) were orally administered to the rats using a stomach tube for 3 months. The animals were killed 24 h after the last administration of AHZ. Normal (sham-operated) and control (ovariectomized) rats received the vehicle solution orally.

Analytical Procedures The rats were bled by cardiac puncture under light anesthesia with ether, and the blood and femurs were removed immediately. Blood samples were centrifuged 30 min after collection. The serum was separated and analyzed immediately. Serum estradiol was assayed by a double-antibody method of enzyme-linked immunosorbent assay using KIT (Cayman Chemical Company, Ann Arbor MI, U.S.A.).

Femurs were removed after bleeding and soaked in ice-cold 0.25M sucrose solution. The right femurs were cleaned of soft tissue, and the bones were dried for 12 h at 110°C to measure the mineral density. Mineral density was measured in the total, distal (epiphysis and metaphysis) and middle (diaphysis) sections of the femur using a dual X-ray bone densitometer (XR-26; Norland Co., Ltd., U.S.A.). After the measurement, the femurs were ashed for 17 h at 640°C and weighed to determine

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the zinc and other contents of the ashes. Bone zinc content was measured by atomic absorption spectrophotometry after dissolution with 1.0N HCl.\textsuperscript{7}

The excised left femurs were fixed in 10% formalin and in 0.5% cyanuric chloride. The fixed materials were decalcified with neutralized ethylenediaminetetraacetic acid (EDTA) and embedded in paraffin. Histological sections from the middle of the shanks were cut perpendicularly to the femoral axis, and stained with hematoxylin and eosin for identification of ostoid tissue, as described by Yoshiki.\textsuperscript{17}

**Statistical Methods** The significance of the 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**

Rats were fed for 3 weeks after ovariectomy, and then the animals were orally administered AHZ (10, 30 and 100 mg/kg/d) for 3 months. Ovariectomy caused a significant decrease in serum estradiol concentration in comparison with that of sham-operated rats; the value was reduced from 166.7 ± 26.9 to 49.4 ± 6.6 pg/ml (mean ± S.E.M. of 10 animals). The administration of AHZ (10—100 mg/kg/d) to ovariectomized rats did not cause an appreciable alteration of serum estradiol concentration (data not shown), but zinc content in the femur was clearly increased by the administration of AHZ to these animals (data not shown). The change in bone ash content (mainly carbon, calcium and phosphorus) of rat femur is shown in Fig. 1. Overall ash quantity was significantly decreased by ovariectomy (Fig. 1A). This decrease was completely prevented by the administration of AHZ (10—100 mg/kg/d) (Fig. 1B). Meanwhile, body weight of rats was increased by ovariectomy, and this increase too was clearly prevented by AHZ administration (data not shown). Thus, the preventive effect of AHZ on the decrease of bone ash content in the rat femur was not based on the change in body weight.

The alteration of mineral density in the femur of ovariectomized rats is shown in Table I. Ovariectomy caused a significant decrease in this density at the total, distal (epiphysis and metaphysis) and middle (diaphysis) sections. These decreases were clearly prevented by the administration of AHZ (10—100 mg/kg/d). Thus, AHZ was able to prevent bone loss in ovariectomized rats.

The effect of AHZ on histomorphological alteration in the femoral metaphysis of ovariectomized rats was histologically examined, because ovariectomy induced a comparatively greater decrease of the bone mineral density in the distal section of the femur. As shown in Fig. 2, the trabecular bone at femoral metaphysis was markedly decreased by ovariectomy. This decrease was largely prevented by the administration of AHZ (10—100 mg/kg/d). There was no difference between the number of metaphyseal trabecular bones in the sham-operated group and that in the AHZ (100 mg/kg/d) group. Many fine trabecular bones were seen at secondary spongiosam in the histology of the AHZ-administered group. AHZ administration caused an increase in osteoid tissue at primary and secondary spongiosam, indicating that osteoblastic bone formation may be stimulated by its administration. Thickness of the cortical bone at the femoral diaphysis was clearly thinned by ovariectomy, and this was appreciably prevented by the administration of AHZ (10—100 mg/kg/d) (data not shown).

**DISCUSSION**

A lack of estrogen induces osteoporosis in humans and in rats,\textsuperscript{15,16,18} and ovariectomy result in estrogen loss. Previous investigation showed that AHZ can completely block bone loss (calcium loss) in the femoral diaphysis of ovariectomized rats, when orally administered (10—100 mg/kg/d) for one month.\textsuperscript{19} This suggests that AHZ can prevent ovariectomy-induced osteoporosis. The present investigation was undertaken to clarify this preventive effect of AHZ administered over a prolonged period to rats ovariectomized 3 weeks earlier.

In this study, a remarkable decrease of estrogen concentration in rat serum was seen 3 months after ovariectomy. The oral administration of AHZ (10, 30 and 100 mg/kg/d) for 3 months did not influence the serum
estradiol level decreased by ovariectomy (data not shown). However, its administration completely prevented any reduction of ash content and mineral density in the femur, which is composed of trabecular and cortical bone tissues. This indicates that the compound has an anabolic effect on the femur of ovariectomized rats.

In the metaphysis (trabecular bone) of the femur, AHZ administration had a potent effect in preventing ovariectomy-induced bone loss; this was supported by the results of histomorphologic study. Marked decrease in the trabecular bone (secondary spongiosa) at the femoral metaphysis following ovariectomy, was clearly blocked by AHZ administration. Thus, the preventive effect of AHZ on trabecular bone loss was also demonstrated histomorphologically.

AHZ administration for 3 months can completely prevent reduction in mineral content in the cortical bone tissues of ovariectomized rats. 20 This was supported by the present result that the ovariectomy-induced decrease in the mineral density of femoral diaphysis is clearly restored by AHZ administration for this period. Thus, AHZ is shown to have a preventive effect on ovariectomy-induced bone loss in both trabecular and cortical bone tissues.

AHZ accumulates in bone tissues and may directly stimulate bone metabolism which has been deteriorated by ovariectomy, since it has a stimulatory effect on bone formation and calcification in the tissue culture system8 and on proliferation in osteoblastic cells (MC3T3-E1)9 in vitro. Presumably, AHZ directly stimulates bone formation and calcification in ovariectomized rats. It may also partly inhibit bone resorption in these rats, since it is known to have an inhibitory effect on bone resorption in tissue culture in vitro. 21

AHZ has a preventive effect on osteopenia associated with various conditions11-14; at the same dosage as zinc, its effect on bone formation was more intense than that of zinc sulfate. 7,8 Zinc accumulation in bone tissue was also increased more by the oral administration of AHZ than by zinc sulfate, suggesting that AHZ is absorbed
easily from the intestine. Moreover, it has been suggested that the zinc in AHZ may accumulate in bone cells (osteoblasts) without difficulty, since the metal binds to the hydroxyapatite of bone tissue. Thus, AHZ may have a potent nutritional and pharmacological use because zinc is an essential trace element for growth.

In conclusion, it has been demonstrated that AHZ can prevent histomorphologically bone loss in ovariectomized rats. This further supports the view that AHZ may have a therapeutic role in osteoporosis.

REFERENCES