Preventive Effect of β-Alanyl-L-histidinato Zinc on the Deterioration of Bone Metabolism in Ovariectomized Rats

Yoshihide SEGAWA,[a] Naoki TSUZUIKE,[a] Eijiro TAGASHIRA,[a] and Masayoshi YAMAGUCHI*,[b]

Department of Pharmacology, Central Research Laboratories, Zeria Pharmaceutical Co., Ltd.,[a] 2512–1 Oshikiri, Osato-gun, Saitama 360–01, Japan and Laboratory of Metabolism and Endocrinology, University of Shizuoka,[b] 52–1 Yada, Shizuoka city 422, Japan. Received November 5, 1992

The preventive effect of β-alanyl-L-histidinato zinc (AHZ) on the deterioration of bone metabolism was investigated in the femoral diaphysis of ovariectomized rats. AHZ (10, 30 and 100 mg/kg body weight/d) was orally administered to ovariectomized rats for 6 weeks. Ovariectomy produced a significant decrease in estradiol, calcitonin, calcium and inorganic phosphorus concentrations in the serum as compared with those from sham-operated rats. The dose of 30 and 100 mg AHZ/kg prevented any decrease in serum inorganic phosphorus concentration caused by ovariectomy. Alkaline phosphatase activity, deoxyribonucleic acid (DNA) and calcium contents in the femoral diaphysis of ovariectomized rats significantly decreased in comparison with those from sham-operated rats. These decreases were completely prevented by the dose of AHZ (10, 30 and 100 mg/kg). Electron microscopical analysis showed a rough alteration of bone matrix in the femoral diaphysis of ovariectomized rats. This alteration was clearly modified by the doses of AHZ (10, 30 and 100 mg/kg). Also, dosages of AHZ (30 and 100 mg/kg) restored the atrophy of osteoblasts and cartilage cells caused by ovariectomy. The present study suggests that oral administration of AHZ can prevent the deterioration of bone metabolism by ovariectomy. AHZ may have a therapeutic role in the treatment of osteoporosis.

Keywords bone metabolism; β-alanyl-L-histidinato zinc; zinc; osteoporosis; ovariectomized rat

Introduction

It is well known that zinc is essential for growth in humans and many animals.[1] Bone has one of the highest zinc concentrations of all tissues. Bone growth retardation is a common finding in various conditions associated with zinc deficiency.[2,3] Recently, it has been demonstrated that zinc plays a role as an activator in the stimulation of bone formation and calcification *in vivo*[4] and *in vitro*[5]; the metal stimulates bone protein synthesis, which is a cellular mechanism.[6] More recently, it has been reported that β-alanyl-L-histidinato zinc (AHZ), a new zinc cmpound, can stimulate bone growth and calcification in weanling[7] and elderly[8] rats, and that the effect of AHZ on bone metabolism is more potent than that of zinc sulfate *in vivo*[7] and *in vitro*.[9] The effect of AHZ in preventing bone disorder, however, has not been fully clarified.

On the other hand, it is established that a lack of estrogen induces osteoporosis in humans and in rats.[10–12] Ovariectomy causes a lack of estrogen. Therefore, the present study was undertaken to clarify whether AHZ can prevent the disorder of bone metabolism in ovariectomized rats. It was found that oral administration of AHZ prevents the revelation of bone disorder in ovariectomized rats. The present study suggests a therapeutic role for AHZ in the treatment of osteoporosis.

Materials and Methods

Animals and Drugs Female Wistar rats (conventional) weighing 100—120 g (5 weeks old) were obtained from Japan SLC (Hamamatsu, Japan). 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. Rats were divided into 5 groups of 6 rats. Animals in group 1 were given a sham-ovariectomy and animals in groups 2 to 5 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 6 weeks. During this time, 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 from Zeria Pharmaceutical Co. (Tokyo, Japan), 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 6 weeks. The animals were killed 24 h after the last administration of AHZ. Control rats (ovariectomized) received the vehicle solution orally.

Analytical Procedures The rats were bled by cardiac puncture under light anesthesia with ether, and the blood and femur were removed immediately. Blood samples were centrifuged 30 min after collection. The serum was separated and analyzed immediately. Serum calcium was determined by the method of Willis.[13] Serum inorganic phosphorus and alkaline phosphatase activity were measured by the methods of Tassuky and Shon,[14] and Kind and King,[15] respectively. Serum estradiol, calcitonin and parathyroid hormone (44—68) were assayed by a double-antibody method of radioimmunoassay using RI KIT.

The femur was removed after bleeding and soaked in ice-cold 0.25 mol/l sucrose solution. The femur was cleaned of soft tissue and marrow, and the diaphysis and epiphysis (containing metaphyseal tissue) were separated and weighed. The femoral diaphyseal tissues were digested with nitric acid.[7] Zinc was determined by atomic absorption spectrophotometry. The bone zinc content was expressed as micrograms of zinc per gram of wet bone tissue.

The femoral diaphyseal tissues were washed for 24 h at 640 °C, weighed and then dissolved in 6.0 N HCl solution. Calcium was determined by atomic absorption spectrophotometry.[5] The calcium content in bone was expressed as milligrams per gram bone ash.

To measure the DNA content in the femoral tissues, the diaphyseal fragments of the left femur were shaken with 4.0 ml of ice-cold 0.1 N NaOH solution for 24 h after the homogenization of the bone tissue.[16] After alkali extraction, the samples were centrifuged at 10000 *g* for 5 min, and the supernatant was collected. The DNA content in the supernatant was determined by the method of Ceriotti[17] and expressed as the amount of DNA (mg) per gram wet weight of bone tissue.

The femoral diaphyseal tissues were immersed in 3.0 ml of ice-cold 6.5 mmol/l 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 centrifuged at 600 *g* for 5 min was used for the measurement of enzyme activity. The enzyme assay described below was carried out under optimal conditions. Alkaline phosphatase activity was determined by the method of Walter and Schutt.[18] The enzyme activity was expressed as units per milligram protein. The protein concentration was determined by the method of Lowry *et al.*[19]

Morphological Analysis The midshafts of the excised femurs were cut into 2 mm thick cross sections and used for electron microscopy. They were fixed in neutral buffered 2.5% glutaraldehyde and then decalcified

in 4% ethylenediaminetetraacetic acid (pH 7.2) at 4 °C. After being washed several times in phosphate buffer solution, they were postfixed in 1% osmium tetroxide, dehydrated in acetone, and embedded in Spurr's resin. Sections were stained with carbon and platinum and examined in an electron microscope.

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 bred for 6 weeks after ovariectomy. The body weight was not decreased by ovariectomy in comparison with that of sham-operated rats. The body weight was not altered by oral administration of AHZ (10, 30 and 100 mg/kg/d) for 6 weeks to ovariectomized rats (data not shown). The alteration of biochemical indices in the serum of rats is shown in Table I. Ovariectomy caused a significant decrease in serum estradiol, calcitonin, calcium and inorganic phosphorus concentrations in comparison with those of the sham-operated rats. Serum parathyroid hormone (44—68) concentration (less than 3 pg/ml) was not altered by ovariectomy (data not shown). When AHZ (10, 30 and 100 mg/kg/d) was orally administered to ovariectomized rats for 6 weeks, no ovariectomy-induced decrease in serum inorganic phosphorus concentration was seen. Meanwhile, the administration of AHZ did not produce a significant alteration of serum calcitonin, parathyroid hormone (44—68) or calcium concentrations, or alkaline phosphatase activity in comparison with those of the control (ovariectomized) rats.

The alteration of zinc content in the femoral diaphysis of rats is shown in Fig. 1. Ovariectomy did not cause a significant alteration of bone zinc content in comparison with that of sham-operated rats. The dose of 100 mg AHZ/kg/d for 6 weeks caused a significant increase in zinc content in the femoral diaphysis of ovariectomized rats, while it was not increased by the doses of 10 and 30 mg/kg.

The alteration of calcium content in the femoral diaphysis of rats is shown in Fig. 2. Calcium content in the femoral diaphysis was significantly decreased by ovariectomy. This decrease was clearly prevented by the oral administration of AHZ (10, 30 and 100 mg/kg/d) for 6 weeks. The effect of 30 mg AHZ/kg was greater than that of 10 mg AHZ/kg.

The alteration of DNA content in the femoral diaphysis of rats is shown in Fig. 3. Ovariectomy caused a significant decrease of DNA content in the femoral diaphysis. This decrease was completely prevented by the oral administration of AHZ (10, 30 and 100 mg/kg/d).

Alkaline phosphatase activity in the femoral diaphysis significantly decreased in ovariectomized rats as compared with that of sham-operated rats (Fig. 4). Oral administration of AHZ (10, 30, and 100 mg/kg/d) clearly blocked the decrease in bone alkaline phosphatase activity caused by

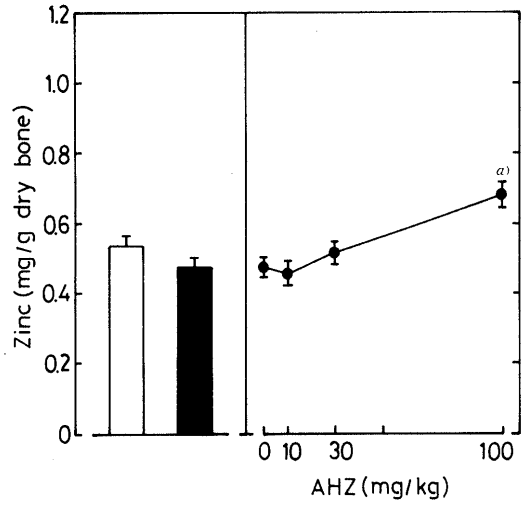

Fig. 1. Alteration of Zinc Content in the Femoral Diaphysis of Ovariectomized Rats Administered AHZ

Rats received oral administration of AHZ (10, 30 and 100 mg/kg/d) for 6 weeks. The rats were killed 24 h after the last administration of AHZ. Each value is the mean ± S.E.M. of 6 animals. *a*) $p<0.05$, as compared with the ovariectomized rats (control). □, sham-operated rats; ■, ovariectomized rats (control).

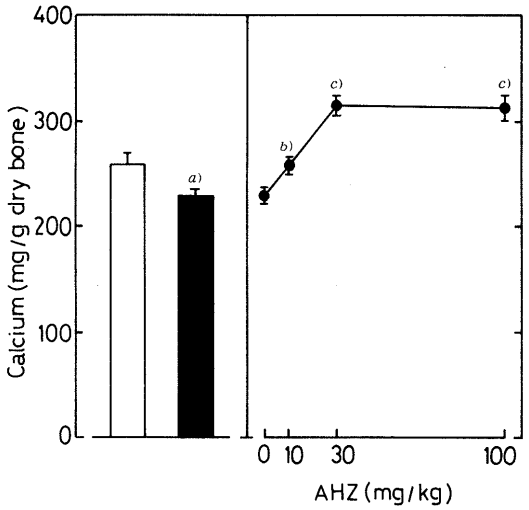

Fig. 2. Effect of AHZ on Calcium Content in the Femoral Diaphysis of Ovariectomized Rats

Rats received oral administration of AHZ (10, 30 and 100 mg/kg/d) for 6 weeks. The rats were killed 24 h after the last administration of AHZ. Each value is the mean ± S.E.M. of 6 animals. *a*) $p<0.05$, as compared with the sham-operated rats (normal). *b*) $p<0.05$, *c*) $p<0.01$, as compared with the ovariectomized rats (control). □, sham-operated rats; ■, ovariectomized rats (control).

TABLE I. Effect of AHZ on Biochemical Indices in the Serum of Ovariectomized Rats

Treatment (mg/kg/d)	Estradiol (pg/ml)	Calcitonin (pg/ml)	Calcium (mg/100 ml)	Inorganic phosphorus (mg/100 ml)	Alkaline phosphatase (U/100 ml)
Sham Normal	116.4±9.4	107.0±16.5	10.3±0.03	6.2±0.16	284.2± 3.4
Ovariectomy					
Control	40.1±4.2[b)]	60.0±2.7[b)]	10.1±0.07[a)]	5.4±0.12[a)]	293.6± 6.5
AHZ, 10	47.9±3.6[b)]	58.8±1.6[b)]	10.1±0.12	5.7±0.18	299.6± 7.1
AHZ, 30	40.3±9.2[b)]	45.5±2.5[b)]	10.0±0.08	6.2±0.24	291.8±14.6
AHZ, 100	53.1±4.3[b)]	47.0±2.5[b)]	10.2±0.06	6.2±0.19	296.8±10.1

Each value is the mean ± S.E.M. of 6 animals. Ovariectomized rats received oral administration of AHZ for 6 weeks. *a*) $p<0.05$, *b*) $p<0.01$, as compared with the sham (normal) rats.

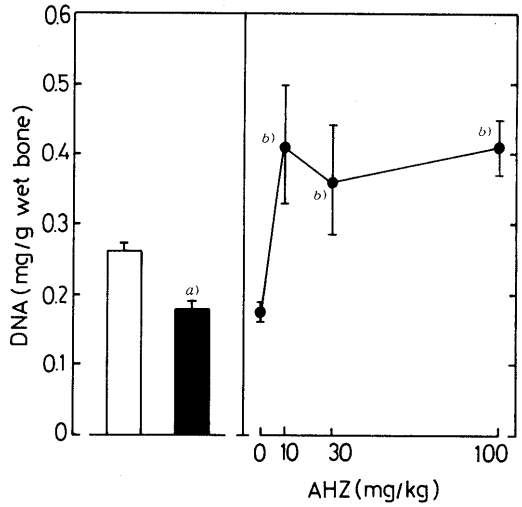

Fig. 3. Effect of AHZ on DNA Content in the Femoral Diaphysis of Ovariectomized Rats

Rats received oral administration of AHZ (10, 30 and 100 mg/kg/d) for 6 weeks. The rats were killed 24 h after the administration of AHZ. Each value is the mean ± S.E.M. of 6 animals. *a)* $p<0.05$, as compared with the sham-operated rats (normal). *b)* $p<0.01$, as compared with the ovariectomized rats (control). □, sham-operated rats; ■, ovariectomized rats (control).

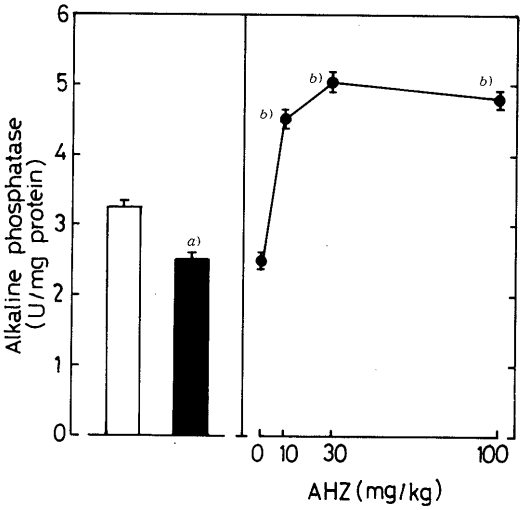

Fig. 4. Effect of AHZ on Alkaline Phosphatase Activity in the Femoral Diaphysis of Ovariectomized Rats

Rats received oral administration of AHZ (10, 30 and 100 mg/kg/d) for 6 weeks. The rats were killed 24 h after the last administration of AHZ. Each value is the mean ± S.E.M. of 6 animals. *a)* $p<0.05$, as compared with sham-operated rats (normal). *b)* $p<0.01$, as compared with the ovariectomized rats (control). □, sham-operated rats; ■, ovariectomized rats (control).

ovariectomy. The increase in bone alkaline phosphatase activity caused by the administration of AHZ (30 and 100 mg/kg) to ovariectomized rats was about 2-fold that of the control value.

Morphological alteration in the femoral diaphysis was examined. In the femoral diaphysis of ovariectomized rats, the bone matrix was roughly in comparison with that of sham-operated rats (data not shown). These morphological changes were clearly restored by the oral administration of AHZ (30 and 100 mg/kg/d), supporting that AHZ administration can restore bone disorder caused by ovariectomy.

Discussion

AHZ, in which zinc is chelated to β-alanyl-L-histidine, is a new zinc compound. Its molecular weight is 289.61. More recently, it has been demonstrated that AHZ can stimulate bone formation and mineralization in weanling and elderly rats.[7,8] At the same dosage as for zinc, the effect of AHZ on bone formation was more intensive than that of zinc sulfate.[7] A great increase in zinc accumulation in bone tissue was also caused by the administration of AHZ in comparison with zinc sulfate, suggesting that AHZ is absorbed easily from the intestine.[7] Moreover, it has been suggested that the zinc in AHZ may accumulate in bone cells without difficulty,[7] since the metal binds to the hydroxyapatite of bone tissue.[8] Thus, AHZ may have a potent nutritional and pharmacological use because zinc is an essential trace element for growth.[1] The therapeutic role of AHZ, however, has not been fully clarified. Therefore, the present study was undertaken to clarify the therapeutic effect of AHZ on bone disorder induced by ovariectomy.

It is established that a lack of estrogen induces osteoporosis in humans and in rats.[10-12] Ovariectomy causes a lack of estrogen,[12] and it induces the decline of calcitonin level.[20] In the present study, the decrease of estrogen and calcitonin concentrations in the serum of rats was seen at 6 weeks after ovariectomy. Moreover, the decrease of serum calcium and inorganic phosphorus concentrations was caused by ovariectomy. Serum parathyroid hormone level was not significantly altered by ovariectomy (data not shown). Oral administration of AHZ (10, 30, and 100 mg/kg/d) for 6 weeks did not prevent the decrease of serum estradiol and calcitonin levels by ovariectomy. However, AHZ administration completely prevented the revelation of bone disorder in ovariectomized rats, suggesting that the compound has a direct effect on bone metabolism in ovariectomized rats.

The disturbance of bone metabolism was observed in ovariectomized rats; it caused a significant decrease of alkaline phosphatase activity, DNA and calcium contents in the femoral diaphysis of rats. These decreases were completely prevented by the oral administration of AHZ (10, 30, and 100 mg/kg/d) for 6 weeks. A great increase in bone DNA content and alkaline phosphatase activity by AHZ administration to ovariectomized rats was seen in comparison with those of sham-operated rats. Thus, AHZ had a potent effect on bone metabolism in ovariectomized rats. Furthermore, the results of electron microscopical study showed that the oral administration of AHZ (10—100 mg/kg/d) prevented bone morphologic alteration caused by ovariectomy. These results clearly indicate that the oral administration of AHZ can block the deterioration of bone metabolism in ovariectomized rats. Presumably, AHZ has a therapeutic role for osteoporosis, since a lack of estrogen induces osteoporosis in humans and in rats.[10-12] The accumulation of zinc in the femoral diaphysis was not seen by the oral administration of AHZ (10 and 30 mg/kg/d) for 6 weeks, although the dose of 100 mg AHZ/kg caused a significant increase in bone zinc accumulation. Meanwhile, the doses of 10 and 30 mg AHZ/kg/d could stimulate bone metabolism deteriorated by ovariectomy. A comparative low dose of AHZ may be of great advantage as a therapeutic tool.

Presently, we do not know the mechanism by which AHZ can stimulate bone metabolism in the femoral diaphysis of ovariectomized rats. However, it has been

demonstrated that AHZ (10^{-7}—10^{-4} M) can directly stimulate bone formation and calcification in bone tissue culture by using calvaria from weanling rats *in vitro*.[9] The bone response for AHZ required a newly synthesized bone protein; the compound markedly increased alkaline phosphatase activity and collagen content in bone tissue.[9] Such effect of AHZ was more potent than that of zinc sulfate.[9] Thus, AHZ had a direct stimulatory effect on bone formation and calcification *in vitro*. Therefore, AHZ may have a stimulatory effect on bone formation in ovariectomized rats. One cannot exclude the possibility, however, that AHZ partly inhibits bone resorption in ovariectomized rats, since it is known that AHZ has an inhibitory effect on bone resorption in tissue culture.[21]

In conclusion, it has been demonstrated that AHZ can prevent the revelation of the deterioration of bone formation and calcification in ovariectomized rats. The present study suggests that AHZ may have a therapeutic role in osteoporosis.

References

1) R. E. Burch, J. K. Hahn, and F. Sullivan, *Clin. Chem.* (N.Y.), **21**, 501 (1975).
2) H. S. Hsieh and J. M. Navia, *J. Nutr.*, **110**, 1581 (1980).
3) G. Oner, B. Bhagumick, and R. M. Bala, *Endocrinology*, **114**, 1860 (1984).
4) M. Yamaguchi and R. Yamaguchi, *Biochem. Pharmacol.*, **35**, 773 (1986).
5) M. Yamaguchi, H. Oishi, and Y. Suketa, *Biochem. Pharmacol.*, **36**, 4007 (1987).
6) M. Yamaguchi, H. Oishi, and Y. Suketa, *Biochem. Pharmacol.*, **37**, 4075 (1988).
7) M. Yamaguchi and K. Ozaki, *Res. Exp. Med.*, **190**, 105 (1990).
8) M. Yamaguchi and K. Ozaki, *Pharmacology*, **41**, 345 (1990).
9) M. Yamaguchi and H. Miwa, *Pharmacology*, **42**, 230 (1991).
10) J. C. Gallagher, B. L. Riggs, and H. F. DeLuca, *J. Clin. Endocrinol. Metab.*, **51**, 1359 (1980).
11) B. L. Riggs, J. Jowsey, P. J. Kelly, J. D. Jones, and F. T. Maher, *J. Clin. Invest.*, **48**, 1065 (1969).
12) G. R. Snow and C. Anderson, *Calcif. Tissue Int.*, **37**, 437 (1985).
13) J. B. Willis, *Nature* (London), **186**, 249 (1960).
14) H. H. Taussky and E. Shon, *J. Biol. Chem.*, **202**, 675 (1953).
15) P. R. N. Kind and E. J. King, *J. Clin. Pathol.*, **7**, 322 (1954).
16) B. Flanagan and G. Nichols, Jr., *J. Biol. Chem.*, **237**, 3686 (1962).
17) G. Ceriotti, *J. Biol. Chem.*, **214**, 39 (1955).
18) K. Walter and C. Schutt, "Methods of Enzymatic Analysis," Vol. 1—2, ed. by H. U. Bergmeyer, Academic Press, New York, 1974, pp. 856—860.
19) O. H. Lowry, N. J. Rosebrough, A. L. Fall, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
20) B. D. Catherwood, T. Onishi, and L. J. Deftos, *Calcif. Tissue Int.*, **35**, 502 (1983).
21) M. Yamaguchi, Y. Segawa, N. Shimokawa, N. Tsuzuike, and E. Tagashira, *Pharmacology*, **45**, 292 (1992).