Alteration in Bone Metabolism with Increasing Age: Effects of Zinc and Vitamin D₃ in Aged Rats

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The alteration in bone metabolism with increasing age was investigated in the femoral diaphysis of male rats. Calcium content was highest in the bone from 3-week-old rats (491 ± 13 mg/g bone ash), falling gradually with age to 357 ± 7 and 306 ± 9 mg/g bone ash in 28- and 52-week-old rats, respectively. Bone zinc content increased until rats were 3 weeks of age, and thereafter remained constant. Deoxyribonucleic acid (DNA) content was highest in the bone from 1-week-old rats, and it decreased markedly with increasing age. Alkaline phosphatase and acid phosphatase activities increased up to 3 weeks, then subsequently declined with age. Thus, the retardation of bone metabolism was induced by ageing. When zinc sulfate (5.0, 10.0 and 20.0 mg Zn/kg body weight) was administered orally for 3 d to 28-week-old rats, alkaline phosphatase activity and calcium content in the femoral diaphysis was elevated markedly by all doses. The oral administration of vitamin D₃ (2.0 and 20 μ g/kg) for 3 d in 28-week-old rats did not produce an appreciable increase in bone alkaline phosphatase activity or calcium content, while 1,25-dihydroxyvitamin D₃ (1.5 μ g/kg) caused a significant increase in those biochemical indices. These results indicate that zinc and 1,25-dihydroxyvitamin D₃ play a role as activators in bone metabolism of ageing rats.

Keywords — bone metabolism; ageing; rat femur; calcium; alkaline phosphatase; zinc; DNA; 1,25-dihydroxyvitamin D₃

Introduction

It has been reported that intestinal absorption of calcium decreases with increasing age.^{1,2)} This decrease results from the decline of serum levels of 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], which promotes intestinal calcium absorption, with increasing age.^{1,3)} 1,25(OH)₂D₃ is produced by 1-hydroxylase in the mitochondria of kidney.¹⁾ This enzyme activity is lowered by ageing.⁴⁾ It is suggested, as a result, that increasing age induces the decrease of calcium content in bone. Also, with increasing age, the biological fate of parathyroid hormone which stimulates bone resorption is extended,⁵⁾ and this brings on an elevation of serum levels of the hormone.⁶⁾ Additionally, the secretion of calcitonin, which inhibits bone resorption, decreases with age.⁷⁾ Such alterations of calcium-regulating hormones with age may result in less calcium content in bone.

Alterations in the function of bone cells with increasing age, however, are not precisely known. The present study was therefore undertaken to clarify whether bone metabolism is al-

tered with the passage of time. The results suggested the retardation of bone metabolism in aged rats. It was also found that this retardation is partly restored by the administration of zinc and 1,25(OH)₂D₃, which stimulate bone formation.^{8–10)}

Materials and Methods

Pregnant female Wistar rats (conventional) were obtained from Shizuoka Laboratory Animal Center, Hamamatsu, Japan. The animals were fed commercial laboratory chow (solid) containing 57.5% carbohydrate, 1.1% Ca, 1.1% P, and 0.012% Zn at a room temperature of 25 °C, and distilled water freely. After delivery, baby rats were lactated from the mother rats. The weanling rats were divided into male and female groups, and bred separately. The rats were sacrificed at 1-, 2-, 3-, 5-, 7-, 11-, 28-, and 52-week-old.

In separate experiments, 28-week-old male rats received oral administration of zinc sulfate solution by a stomach tube for 3 d. Zinc sulfate was dissolved in distilled water to the concentra-

tion of 2.5–10.0 mg as Zn/ml, and these solutions were given to rats as 5.0, 10.0 and 20.0 mg Zn/kg of body weight. The rats were killed at 24 h after the last administration of zinc solution. Control rats received oral administration of distilled water.

Vitamin D₃ (purchased from Wako Pure Chemical Co., Ltd., Osaka, Japan) or 1,25 (OH)₂D₃ (given by Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) was administered orally in doses of 2.0 and 20 µg, or 0.5 and 1.5 µg in 0.5 ml of propylene glycol per kg body weight, respectively. Vitamin D₃ metabolites were given 2 h after zinc administration, 3 times at 24-h intervals. The rats were sacrificed 24 h after the last administration of zinc. Control rats received oral administration of propylene glycol (0.5 ml/kg).

The femur was removed immediately after bleeding and soaked in ice-cold 0.25 M sucrose solution. The femur was cleaned of soft tissue and marrow, and the diaphysis and epiphysis (containing metaphyseal tissue) were separated and weighed. The right femoral-diaphyseal tissues were immersed in 3.0 ml of ice-cold 6.5 mM barbital buffer (pH 7.4), cut into small pieces, homogenized in a Potter-Elvehjem homogenizer with a Teflon pestle, and disrupted for 60 s with an ultrasonic device. The supernatant centrifuged at $600 \times g$ for 5 min was used for the measurement of enzyme activity. The enzyme assay described below was carried out under optimal conditions. Alkaline phosphatase and acid phosphatase activities were determined by the method of Walter and Schutt.¹¹⁾ The enzyme activity was expressed as µmol of *p*-nitrophenol liberated per min per mg protein. Protein concentration was determined by the method of Lowry *et al.*¹²⁾

To measure deoxyribonucleic acid (DNA) content in the femoral tissue, the diaphyseal fragments of the right 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.¹³⁾ After alkali extraction, the samples were centrifuged at $10000 \times g$ for 5 min, and the supernatant was collected. DNA content in the supernatant was determined by the method of Ceriotti¹⁴⁾ and expressed as the amount of DNA (mg)/g wet weight of bone tissues.

The left femoral-diaphyseal fragments were ashed for 24 h at 640 °C, weighed, and then dissolved in 6 N HCl solution.⁸⁾ Calcium was determined by atomic absorption spectrophotometry. Bone calcium content was expressed as mg of calcium per g bone ash.

Zinc content in the femoral diaphysis was determined by atomic absorption spectrophotometry after digestion with nitric acid.⁸⁾ Bone zinc content was expressed as µg of zinc per g wet bone tissue.

Data are expressed as the mean $\pm$ S.E.M. Statistical differences were analyzed using Student's *t*-test. *p* values of less than 0.05 were considered to indicate statistically significant differences.

Results

The effect of age on calcium content in the femoral diaphysis of male rats is shown in Fig. 1. Bone calcium content was highest in the 3-week-old rats (491 ± 13 mg/g bone ash) and declined gradually with age to 357 ± 7 and 306 ± 9 mg/g bone ash in 28- and 52-week-old rats, respectively. The calcium content in 11-, 28-, and 52-week-old animals was significantly less ($p < 0.01$) than the value in the young rats (3-week-old). Further, the calcium content in the

Fig. 1. Alteration of Calcium Content in the Femoral Diaphysis of Male Rats with Increasing Age

Rats were sacrificed each week. Each point represents the mean of five animals. Vertical lines give the S.E.M.

a) $p < 0.01$, as compared with the value of 3-week-old rats.

Fig. 2. Alteration of Zinc Content in the Femoral Diaphysis of Male Rats with Age

Rats were sacrificed each week. Each point represents the mean of five animals. Vertical lines give the S.E.M.

52-week-old rats was decreased significantly relative to that in the mature adult (11-week-old; $p < 0.01$).

Zinc, an essential metal, localized largely in bone tissues.⁸⁾ The alteration of zinc content in the femoral diaphysis of male rats with increasing age is shown in Fig. 2. Zinc content was $53.4 \pm 13.5 \mu\text{g/g}$ wet bone in 1-week-old rats and increased markedly ($p < 0.01$) with age to 156.9

Fig. 4. Alteration of Alkaline Phosphatase Activity in the Femoral Diaphysis of Male Rats with Age

Rats were sacrificed each week. Each point represents the mean of five animals. Vertical lines give the S.E.M.

a) $p < 0.01$, as compared with the value of 3-week-old rats.

$\pm 7.7 \mu\text{g/g}$ wet bone in 3-week-old animals. The value did not alter significantly with age.

The alteration of DNA content in the femoral diaphysis of male rats with increasing age is shown in Fig. 3. DNA content was highest in 1-week-old rats ($5.450 \pm 0.174 \text{ mg/g}$ wet bone), and thereafter decreased with time. The DNA content ($0.389 \pm 0.042 \text{ mg/g}$ wet bone) in 28-week-old rats was markedly less ($p < 0.01$)

Fig. 3. Alteration of DNA Content in the Femoral Diaphysis of Male Rats with Age

Rats were sacrificed each week. Each point represents the mean of five animals. Vertical lines give the S.E.M.

a) $p < 0.01$, as compared with the value of 3-week-old rats.

Fig. 5. Alteration of Acid Phosphatase Activity in the Femoral Diaphysis of Male Rats with Age

Rats were sacrificed each week. Each point represents the mean of five animals. Vertical lines give the S.E.M.

a) $p < 0.01$, as compared with the value of 3-week-old rats.

TABLE I. Alteration of Bone Metabolism in the Femoral Diaphysis of Female Rats with Age

Bone component	Age (week)	
	3	28
Calcium (mg/g bone ash)	476.4 ± 10.4	454.3 ± 7.1
Zinc (μg/g wet bone)	211.6 ± 14.4	191.3 ± 5.1
DNA (mg/g wet bone)	1.797 ± 0.246	0.431 ± 0.042 ^{a)}
Alkaline phosphatase (μmol/min/mg protein)	2.594 ± 0.228	0.496 ± 0.057 ^{a)}
Acid phosphatase (nmol/min/mg protein)	277.5 ± 25.7	34.8 ± 7.7 ^{a)}

Each value is the mean ± S.E.M. of five rats. The rats were sacrificed at 3- and 28-week-old.

^{a)} $p < 0.01$, as compared with the control value.

TABLE II. Effect of Zinc Administration on Various Components in the Femoral Diaphysis of 28-Week-Old Male Rats

Bone component	Control	Zinc administration (mg/kg body weight)		
		5.0	10.0	20.0
Zinc (μg/g wet bone)	155.0 ± 8.9	161.7 ± 11.1	195.7 ± 17.7	214.3 ± 4.2 ^{a)}
Calcium (mg/g bone ash)	357.4 ± 6.6	393.3 ± 8.8 ^{a)}	432.3 ± 4.4 ^{a)}	453.3 ± 10.3 ^{a)}
DNA (mg/g wet bone)	0.389 ± 0.042	0.449 ± 0.090	0.431 ± 0.050	0.392 ± 0.085
Alkaline phosphatase (μmol/min/mg protein)	0.395 ± 0.062	0.739 ± 0.065 ^{a)}	0.685 ± 0.040 ^{a)}	0.693 ± 0.031 ^{a)}
Acid phosphatase (nmol/min/mg protein)	58.4 ± 8.6	59.9 ± 12.9	85.3 ± 10.2	83.2 ± 9.2

Each value is the mean ± S.E.M. of five rats. The rats received oral administration of zinc (5.0, 10.0 and 20.0 mg/kg body weight) for 3 d, and were sacrificed 24 h after the last administration.

^{a)} $p < 0.01$, as compared with the control value.

than the value in young rats (3-week-old).

The effect of age on alkaline phosphatase activity in the femoral diaphysis of male rats is shown in Fig. 4. The enzyme activity was 0.888 ± 0.052 μmol/min/mg protein in 1-week-old rats and increased with age, being highest in 3-week-old animals (1.864 ± 0.126 μmol/min/mg protein). Alkaline phosphatase activity decreased markedly with age.

Acid phosphatase activity in the femoral diaphysis of male rats increased gradually up to the age of 5 weeks, when it was 150.5 ± 9.9 nmol/min/mg protein (Fig. 5). With increasing age, it decreased significantly and the value in 28-week-old rats was 58.4 ± 8.6 nmol/min/mg protein.

The effect of age on bone metabolism in female rats is shown in Table I, where it was compared between weanling (3-week-old) and senescent rats (28-week-old). DNA content, al-

kaline phosphatase and acid phosphatase activities in the femoral diaphysis of 3-week-old rats had lowered markedly ($p < 0.01$) when they reached 28 weeks. The bone calcium content, however, was not decreased significantly at that time, although with prolonged age it might decrease further. Zinc content did not alter significantly with increasing age.

The alteration of bone metabolism in the femoral diaphysis of male 28-week-old rats orally administered zinc sulfate is shown in Table II. Doses of 5.0, 10.0 and 20.0 mg Zn/kg body weight were given for 3 d, and zinc content in the bone was raised with increasing doses until by 20.0 mg Zn/kg a significant increase was apparent. With all doses of zinc, calcium content alkaline phosphatase activity in the bone increased significantly while bone DNA content and acid phosphatase activity were not altered markedly by any administration of this metal.

TABLE III. Effect of Vitamin D₃ on Various Components in the Femoral Diaphysis of 28-Week-Old Male Rats

Treatment	Alkaline phosphatase ($\mu\text{mol}/\text{min}/\text{mg}$ protein)	DNA (mg/g wet bone)	Calcium (mg/g bone ash)
None			
Control	0.322 ± 0.030	0.441 ± 0.016	364.9 ± 5.3
Vitamin D ₃ (2 $\mu\text{g}/\text{kg}$)	0.342 ± 0.014	0.438 ± 0.038	356.4 ± 9.7
Vitamin D ₃ (20 $\mu\text{g}/\text{kg}$)	0.307 ± 0.021	0.478 ± 0.011	369.2 ± 7.6
Zinc (10 mg/kg)			
Control	0.501 ± 0.022^a	0.438 ± 0.143	416.3 ± 9.9^a
Vitamin D ₃ (2 $\mu\text{g}/\text{kg}$)	0.379 ± 0.005	0.434 ± 0.030	423.4 ± 5.2^a
Vitamin D ₃ (20 $\mu\text{g}/\text{kg}$)	0.340 ± 0.012	0.485 ± 0.022	397.8 ± 5.3^a

Each value is the mean $\pm$ S.E.M. of five rats. The rats received oral administration of zinc (10 mg/kg), and 2 h later oral administration of vitamin D₃ (2 and 20 $\mu\text{g}/\text{kg}$) for 3 d. Animals were sacrificed 24 h after the last administration of zinc.

a) $p < 0.01$, as compared with the non-control value.

TABLE IV. Effect of 1,25-Dihydroxyvitamin D₃ [1,25(OH)₂D₃] on Various Components in the Femoral Diaphysis of 28-Week-Old Male Rats

Treatment	Alkaline phosphatase ($\mu\text{mol}/\text{min}/\text{mg}$ protein)	DNA (mg/g wet bone)	Calcium (mg/g bone ash)
None			
Control	0.343 ± 0.016	0.438 ± 0.020	359.1 ± 6.2
1,25(OH) ₂ D ₃ (0.5 $\mu\text{g}/\text{kg}$)	0.372 ± 0.019	0.397 ± 0.033	375.6 ± 8.1
1,25(OH) ₂ D ₃ (1.5 $\mu\text{g}/\text{kg}$)	0.448 ± 0.025^b	0.467 ± 0.046	383.4 ± 5.4^a
Zinc (10 mg/kg)			
Control	0.508 ± 0.019^b	0.422 ± 0.138	415.2 ± 10.1^b
1,25(OH) ₂ D ₃ (0.5 $\mu\text{g}/\text{kg}$)	0.517 ± 0.022^b	0.458 ± 0.138	411.4 ± 6.9^b
1,25(OH) ₂ D ₃ (1.5 $\mu\text{g}/\text{kg}$)	$0.619 \pm 0.029^{b,c}$	0.435 ± 0.036	430.2 ± 1.34^b

Each value is the mean $\pm$ S.E.M. of five rats. Animals received oral administration of zinc (10 mg/kg) and 2 h later oral administration of 1,25(OH)₂D₃ (0.5 and 1.5 $\mu\text{g}/\text{kg}$) for 3 d. Rats were sacrificed 24 h after the last administration of zinc.

a) $p < 0.05$ and b) $p < 0.01$, as compared with the non-control value. c) $p < 0.01$, as compared with the value of zinc alone or 1,25(OH)₂D₃ alone.

The effect of vitamin D₃ on bone metabolism in the femoral diaphysis of 28-week-old male rats is shown in Table III. The administration of Vitamin D₃ (2.0 and 20 $\mu\text{g}/\text{kg}$) did not produce a significant alteration in alkaline phosphatase activity, DNA content or calcium content in the bone. Also, administration of zinc (10 mg/kg) did not enhance the steroid effect on bone metabolism.

The effect of 1,25(OH)₂D₃ on bone metabolism in the femoral diaphysis of 28-week-old male rats is shown in Table IV. Administration of 1,25(OH)₂D₃ (1.5 $\mu\text{g}/\text{kg}$) caused a significant increase of bone alkaline phosphatase activity and calcium content, but not DNA content. At

the dose of 0.5 $\mu\text{g}/\text{kg}$ of the steroid, the effect was slight but not significant. The effect of 1,25(OH)₂D₃ (1.5 $\mu\text{g}/\text{kg}$) on bone alkaline phosphatase activity was additively raised by the administration of zinc (10 mg/kg), while the steroid effect on bone DNA content was not increased. Bone calcium content was slightly, but not significantly, increased by the administration of both 1,25(OH)₂D₃ and zinc.

Discussion

In the present study, it was found that bone metabolism in the femoral diaphysis of male and female rats fairly altered with increasing age.

Calcium content in the bone of male rats reduced significantly with age up to 28 weeks, while it did not decrease appreciably in female animals. Females may not undergo such calcium decline until they are older. The physiological mechanism of ageing as it affects the decline of bone calcium has not yet been fully defined. It is reported that the intestinal absorption of calcium decreases with age, and that this occurs in females at an advanced age^{2,15)}; this may, at least partly, lead to the decrease of bone calcium. The alteration of bone calcium content is related to the balance of bone resorption and bone formation, which is governed by calcium-regulating hormones. Disorder in secretion and metabolism of the hormones with ageing may also contribute to the reduction in bone calcium content.

With age, bone DNA content, alkaline phosphatase and acid phosphatase activities were reduced markedly in male and female rats, indicating that ageing may bring about a deterioration in bone cell function. The activity of acid phosphatase, a lysosomal enzyme, is elevated by stimulation of bone resorption due to the parathyroid hormone.¹⁶⁾ Alkaline phosphatase is implicated in bone mineralization.¹⁷⁾ DNA content in bone tissue represents a number of bone cells. The decrease in these bone components may cause the retardation of bone formation and mineralization.

Calcium content and alkaline phosphatase activity in the femoral diaphysis of 28-week-old male rats orally administered zinc sulfate were markedly increased in comparison with that of control rats, while bone DNA content and acid phosphatase activity were not raised significantly. The increase in bone calcium content may be linked to the elevation of alkaline phosphatase activity caused by zinc administration, since the enzyme plays a role in the promotion of bone mineralization.¹⁷⁾ Presently, the mechanism of zinc action in increasing bone alkaline phosphatase activity is not known. Alkaline phosphatase is a zinc-enzyme.¹⁸⁾ Bone alkaline phosphatase activity is increased by the presence of zinc in tissue culture *in vitro*.⁹⁾ This increase is blocked completely by cycloheximide, an inhibitor of protein synthesis, suggesting that zinc stimulates protein synthesis in bone cells.⁹⁾ The

increase in bone alkaline phosphatase activity caused by zinc administration may result from the increase of protein synthesis in bone cells. Thus, bone cells in aged rats may have responsibility for zinc given exogenously. Interestingly, zinc content in bone tissue did not decrease with increasing age. It is possible, however, that zinc concentration in bone cells may decrease in aged rats, although bone cellular zinc was not determined. Administration of zinc may cause an increase of the metal concentration in bone cells. From this, it is speculated that the increased cellular zinc may restore the deterioration of bone cell functions in aged rats; presumably, zinc plays a role as an activator of bone metabolism in such animals.

Zinc has a physiological role as an activator in bone growth and formation in weanling rats,⁸⁾ and zinc-deficiency prevents bone growth and formation.^{19,20)} The present finding, that zinc administration can induce bone mineralization in aged rats, suggests the further importance of this essential trace element in nutritional and pharmacological application.

The physiological expression of vitamin D₃ activity is exerted by the hormonal form vitamin D₃, 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃]; this hormonal form is produced in the kidney.¹⁾ More recently, it has been demonstrated that the production of 1,25(OH)₂D₃ is markedly decreased as rats age.⁴⁾ The oral administration of vitamin D₃ (5 — 20 µg/kg) to weanling rats can produce a significant increase of alkaline phosphatase activity in the femoral diaphysis;²¹⁾ however, the same doses to aged rats did not show an appreciable effect. This observation may further support the view that older rats experience a disorder of vitamin D₃ metabolism.⁴⁾ The oral administration of 1,25(OH)₂D₃ in older animals produced a significant increase in bone alkaline phosphatase activity and calcium content. This finding supports the view that the decrease of bone calcium content with age is, at least partly, caused by the decline of 1,25(OH)₂D₃ formation by ageing.

Previous investigation showed that zinc can enhance the effect of 1,25(OH)₂D₃ on bone metabolism in weanling rats.²²⁾ Also, in aged rats, zinc increased the effect of 1,25(OH)₂D₃

on bone alkaline phosphatase activity, and bone calcium content was slightly elevated. The combined effect of zinc and $1,25(\text{OH})_2\text{D}_3$ on bone alkaline phosphatase is completely prevented by treatment of protein inhibitor.^{21,22} Presumably, zinc and $1,25(\text{OH})_2\text{D}_3$ may increase bone protein synthesis in aged rats. This, however, remains to be elucidated.

In conclusion, the present investigation demonstrates that bone metabolism deteriorates with increasing age, and that the deterioration is restored by oral administration of zinc or/and $1,25(\text{OH})_2\text{D}_3$. Zinc and $1,25(\text{OH})_2\text{D}_3$ may thus be important as restorative factors for bone metabolism in ageing rats.

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