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Phenytoin-Induced Bone Loss and Its Prevention with Alfacalcidol or Calcitriol in Growing Rats

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Abstract. Studies were carried out to determine the effects and mechanism of action of phenytoin on the bone metabolism in male rats. Administration of phenytoin, 20 mg/kg/day for 5 weeks, did not affect the growth curve. Biochemical data indicated that the serum osteocalcin, a marker of bone formation, was decreased significantly but there were no significant differences in the levels of serum calcium, pyridinoline, 25-hydroxyvitamin D_3 (25OHD) and parathyroid hormone (PTH) in the phenytoin-treated group compared with the vehicle-treated group. The values of bone mineral density (BMD) were decreased in all regions of bones tested (mandibular head, tibial metaphysis, tibial diaphysis, femoral metaphysis, and femoral diaphysis) in the phenytoin-treated group. In histomorphometric analysis, phenytoin decreased trabecular bone volume and trabecular thickness, and increased osteoclast numbers per area of bone surface in the secondary trabecular bone of the tibia. Additionally, there was no significant difference in osteoid thickness. Combined administration of either alfacalcidol or calcitriol with phenytoin for 5 weeks prevented the reduction of BMD induced by phenytoin. From these findings, it is unlikely that toxic effects on the growth curve caused the decreased BMD induced by phenytoin. It is also evident that repeated administration of phenytoin may cause osteopenia which may be due to bone loss by inhibiting bone formation and/or by accelerating bone resorption rather than osteoid accumulation. The bone loss is not rachitic because of the lack of increase in osteoid thickness. Moreover, combined administration of alfacalcidol or calcitriol with phenytoin showed a preventative effect against bone loss. The bone loss induced by phenytoin in this study may be a convenient model for further research into the problem of drug-induced osteopenia.

Key words: Phenytoin — Alfacalcidol — Osteocalcin — Bone mineral density — Osteopenia

In clinical practice, it is well known that antiepileptic drugs induce hypocalcemia, which is associated with bone fractures and osteoporosis [1–3] . For example, in a Japanese hospital and institution for the mentally handicapped [4], 15 of 21 patients taking long-term antiepileptic drugs showed bone fractures and osteoporosis, although their level of physical activity was reduced. The reason for the bone loss induced by multiple antiepileptic drugs was thought to be due to vitamin D deficiency, regarded as osteomalacia or rickets, after the classical proposal by Schmid [5] and Kruse [6]. Some data have been presented that support this proposal that antiepileptics decrease the circulating concentrations of 25-hydroxyvitamin D_3 (25OHD), an active vitamin D metabolite in epileptic patients [1, 3, 7, 8] and in vitamin D-deficient rats [9, 10].

Nevertheless, clinical investigations of large numbers of epileptic patients with long-term courses of antiepileptic drugs have demonstrated that both osteomalacia and rickets are relatively uncommon [11, 12]. Much recent evidence now suggests that bone loss induced by antiepileptics drugs is not simply explained by vitamin D deficiency [13–17] . In fact, Wark et al. [13] reported that therapeutic doses of diphenylhydantoin (5,5-diphenyl-2, 4-imidazolidinedione, phenytoin) without other antiepileptics do not have a clinically significant effect on plasma 25OHD. This was experimentally confirmed by Gascon-Barre et al. [14] using rats to determine if adequate vitamin D was obtained.

With regard to bone metabolism, the mechanisms of phenytoin-induced bone loss have not been conclusively demonstrated. Bone is lost when the rate of resorption exceeds the rate of bone formation and is gained when bone formation exceeds bone resorption [18].

Churesigaew et al. [19] examined the relationship between epilepsy and abnormal calcium metabolism in institutionalized mentally handicapped patients. Incidences of osteoporosis, hypocalcemia, and increased alkaline phosphatase (ALP) were found to be significantly increased in the epileptic group. The only significant difference between the osteoporotic and nonosteoporotic epileptic patients was an increase in ALP in the former [19]. Later, Takeshita et al. [16] found that the circulating levels of bone gamma-carboxyglutamic acid-containing protein (bone gla protein) (BGP) are increased in children receiving anticonvulsant

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therapy, suggesting a high rate of bone turnover due to anticonvulsant drug complications. More recently, Telci et al. [17] reported that only the resorption phase of bone turnover is affected during chronic antiepileptic drug use in epileptic patients. In contrast, it has been reported that phenytoin stimulates bone formation to increase bone volume *in vivo* and *in vitro* [20–22] , although bone loss was not explained by the results in which phenytoin might have a stimulatory action on bone formation. Thus, there is conflicting evidence concerning the effects of phenytoin on bone metabolism. In addition, experimental information regarding the effects of phenytoin on bone mineral density (BMD) is not available. We developed a measuring system for BMD using image analysis of soft X-ray microradiographs [23, 24].

Under these conditions, we conducted a study in growing male rats to determine the effects and mechanism of phenytoin action for 5 weeks on bone metabolism and, if present, whether phenytoin-induced osteopenia could be prevented by treatment with analogs of vitamin D.

Material and Methods

Animals

Male Wistar rats (SLC, Ltd., Shizuoka, Japan) weighing 80 ± 5 g were used. The animals were individually housed in wire-mesh cages (170 × 250 × 370 mm) in an air-conditioned room at constant temperature (22 ± 2°C) and humidity (60 ± 10%) on a 12 h/12 h light-dark cycle (light on at 7:30 a.m.). They were given laboratory chow (F-2®, SLC Co. Ltd., Shizuoka, Japan; Ca 0.74 g, P 0.65 g, vitamin D_3 200 IU/100 g) and deionized water *ad libitum.* The animals were treated humanely in compliance with the regulations of Tohoku University School of Medicine and Dentistry.

Grouping and Drug Treatments

Each group consisted of 10–11 animals, and each received a subcutaneous injection (s.c.) of the following drug schedule once a day (between 5:30 p.m. and 6:30 p.m.) for 5 successive weeks. Sodium phenytoin was suspended in 0.5% Tween80 solution. The concentration of calcitriol and alfacalcidol was adjusted by dilution in ethanol. Phenytoin was administered at 0.1 ml/100 g body weight, and alfacalcidol or calcitriol at 20 μl/100 g body weight.

Groups and drug schedule were as follows:

- Group A: 0.5% Tween80 (vehicle) (Wako Pure Chemicals Industries, Ltd., Osaka, Japan)-treated rats
- Group B: 20 mg/kg sodium phenytoin (Sigma Co. Ltd., NJ, USA)
- Group C: 20 mg/kg sodium phenytoin + 0.1 μg/kg 1 α $(OH)D_3$ (alfacalcidol) (Chugai Pharmaceutical Co. Ltd., Tokyo Japan)
- Group D: 20 mg/kg sodium phenytoin + 0.1 μg/kg 1 α ,25$(OH)_2D_3$ (calcitriol) (Solvay Duphar B.V., Weeps, the Netherlands)
- Group E: 0.1 μg/kg alfacalcidol Group F: 0.1 μg/kg calcitriol.

Measurement of Bone Mineral Density

Under pentobarbital anesthesia, the tibiae, femurs, and mandibulae were dissected and fixed in Karnovsky solution (pH 7.4). After removing the adhesive soft tissues, soft X-ray microradiographs of the bones were taken with a soft X-ray apparatus (Type-Softex, Softex Co. Ltd., Tokyo, Japan). A step-wedge made of synthetic hydroxyapatite plates (Mitsubishi Kasei Co. Ltd., Tokyo, Japan) of differing thickness was placed on the same radiographic films (soft X-ray film, Type FR, Fuji Photofilm, Tokyo, Japan) to serve as a standard for measuring the sample BMD. Each BMD was measured at the area shown in Figure 1, essentially according to the method of Shinoda et al. [23, 24]. In brief, BMD was determined by analyzing the grey levels of the target area in microradiographs with an image analyzer (Aspect, Mitani Corp., Fukui, Japan). A standardized relationship was established between the grey levels (0–255) and hydroxyapatite content/mm^2 by analyzing the image of the standard step-wedge. All of the images analyzed were fed into a video camera (TI-23A CCD, NEC Japan) at a magnification of 20. Since there was a linear relationship between the grey levels and the logarithm of the hydroxyapatite content under the conditions used for taking microradiographs (70–80 V, 1 mA for 40–60 sec), we were able to determine the BMD [23, 24].

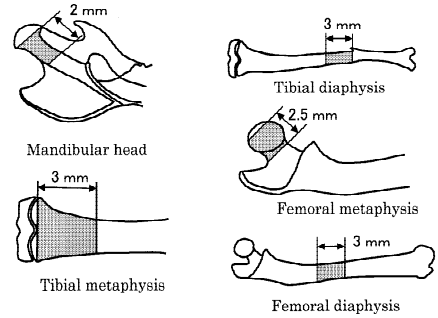

Fig. 1. Diagram of the sample areas used in the determination of BMD. The shaded parts of each bone were measured using image analysis of soft X-ray microradiographs.

Histomorphometric Analysis

For histomorphometric analysis, we again housed rats that were treated following the same procedure described for groups A, B, C, and E. At the end of the experiment, the bones of these rats were perfused with 4% paraformaldehyde (Wako Pure Chemicals Industries, Ltd., Osaka, Japan) in 0.1 M phosphate buffer (pH 7.4) under pentobarbital anesthetic. After perfusion, the tibial metaphyses were excised for histomorphometric analysis. The areas 0.8 and 4.4 mm distal to the growth plate were examined by hematoxylin-eosin (H-E) staining, as shown in Figure 2. Before decalcification, some tibiae were treated with cyanuric chloride solution for 1 week to detect the osteoid matrix in the decalcified sections [26]. The tibiae were then decalcified in 10% EDTA at 4°C for 4 weeks. After decalcification, the tibiae on one side were dehydrated and embedded in paraffin. Sections were cut frontally at a thickness of 4 μm and stained with H-E for observation under a light microscope for qualitative changes in trabecular bone and osteoid matrix, or stained with a method for tartrate-resistant acid phosphatase (TRAP) and observed by light microscopy for the distribution of osteoclasts. Measurements and calculation-related parameters were as follows: total metaphyseal area (TV, mm^2)—metaphyseal area between 0.8 and 4.4 mm distal to the growth plate without cortices; trabecular bone area (BV, mm^2)—total area of trabecular bone within TV; trabecular bone surface (BS, mm)—length of the total perimeter of trabeculae; osteoclast number (Oc.N)—numbers of TRAP-positive cells situated on the bone surface within TV; bone volume (BV/TV, %)—BV/TV × 100; trabecular thickness (Tb. Th, μm)—2000/(BS/BV); trabecular number (Tb.N/mm) −BS/(2 × TV); trabecular separation (Tb.Sp, μm)—Tb.Th × (100/BV/TV −1); and osteoclast number per area bone surface (Oc.N/BS, /mm); and osteoid thickness (O.Th, μm). Chemicals for preparing buffer solutions were purchased from Wako Pure Chemicals Industries, Ltd., Osaka, Japan.

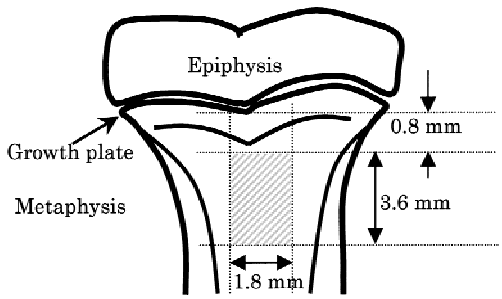

Fig. 2. Schematic representation of the sampling site for tibiae used for histomorphometric analysis. The shaded area (1.8 × 3.6 mm) 0.8 mm away from the growth plate was stained with hematoxylin-eosin (H-E) and is seen in Figure 6.

Measurement of Serum Bone Markers

The rats were anesthetized by intraperitoneal injection of 50 mg/kg sodium pentobarbital (Nembutal injection®, Abbott, North Chicago, USA). Blood samples were collected from the carotid arteries of the rats and centrifuged at 3000 rpm for 15 min. Each sample was stored in a refrigerator until used. The levels of serum calcium were measured by spectrophotometry (Hitachi 228 type, Tokyo, Japan) for absorbance at 570 nm using a commercially available kit (Calcium-C Test Wako®, Wako Pure Chemicals Industries, Ltd., Osaka, Japan); the kit is based on an *in vitroO*-cresolphtalein complexone (OCPC) method for quantitative determination of calcium in serum [25]. To clarify the effect of phenytoin administration on bone metabolism, the levels of serum osteocalcin, PTH, 25OHD, and serum pyridinoline were determined in the samples obtained from groups A, B, C, and E using an ELISA kit (osteocalcin rat ELISA system®, parathyroid hormone rat ELISA system®, Amersham Pharmacia Biotech K.K., Tokyo, Japan) and an EIA kit (25OHD, BIOMEDICA, Vienna, Austria, Serum Pyd®, Metra Biosystems, Inc., Mountain View, USA), respectively. Each sample was analyzed in duplicate.

Statistics

Statistical analysis was performed by analysis of variance followed by Williams and Wilcoxon's multiple comparison test, or the Duncan multiple comparison test.

Results

Effect of Phenytoin on Rat Growth Curves

At the beginning of the experimental period, the average body weight was 80 g in each group. Five weeks after the experiment, the average body weight was 229 g in group B, and there was no significant difference compared with the other five groups, as shown in Figure 3. In this period, there were no significant differences in bone length or volume among the six groups.

Effects of Phenytoin on Bone Mineral Density

The effects of phenytoin on BMD are shown in Figure 4. Five weeks after phenytoin treatment, each BMD value for the bones in group B was significantly decreased ($P < 0.05$) in the corresponding areas of group A. The rates of decrease were almost 7–9% in all areas.

Effect of Alfacalcidol or Calcitriol on the Decline of BMD Induced by Phenytoin

The effects of alfacalcidol or calcitriol on the decline of BMD induced by phenytoin are shown in Figure 5. Combined administration of either alfacalcidol or calcitriol with phenytoin prevented the decline of BMD induced by phenytoin. Regarding the prophylactic effects on the reduction of BMD induced by phenytoin, there was no significant difference between groups C and D in any of the areas measured.

Effect of Phenytoin Alone and in Combination with Alfacalcidol on Serum Bone Markers

The levels of serum calcium in Groups A, B, C, D, E, and F were 9.46 ± 0.19, 9.78 ± 0.21, 9.27 ± 0.22, 9.97 ± 0.14, 9.51 ± 0.19, and 9.48 ± 0.13 mg/dl (means ± SEM), respectively. There was no significant difference in serum calcium levels among the groups. The levels of serum osteocalcin, PTH, 25OHD, and pyridinoline are shown in Table 1. In the phenytoin-treated group, there was no significant difference in serum pyridinoline, PTH, or 25OHD, but serum osteocalcin, a parameter of bone formation, was decreased significantly ($P < 0.05$) compared with the vehicle-treated group. Administration of alfacalcidol with phenytoin decreased the levels of serum osteocalcin ($P < 0.05$), PTH ($P < 0.05$), and 25OHD ($P < 0.05$) compared with the vehicle-treated group.

Bone Histomorphometry

Figure 6 shows typical microphotographs of the proximal tibial metaphysis in Groups A, B, C, and E. Trabecular bone in Group B (phenytoin alone) was decreased compared with that in Group A (vehicle group). In the section of Group C (alfacalcidol with phenytoin), trabecular bone loss was not observed. The lower 4 sections of Figure 6 show micrographs of trabecular bone of sections treated with cyanuric chloride solution. A similar distribution of osteoid was observed in all groups. The static histomorphometric parameters are shown in Table 2. In Group B, BV/TV, Tb.Th, and Tb.N were decreased, and Tb.Sp and Oc.N/BS were increased compared with Group A. Combined administration of alfacalcidol with phenytoin did not decrease BT/TV and did not increase Oc.N/BS. There was no significant difference in O.Th among the groups.

Discussion

In this study, we found that treatment with phenytoin at 20

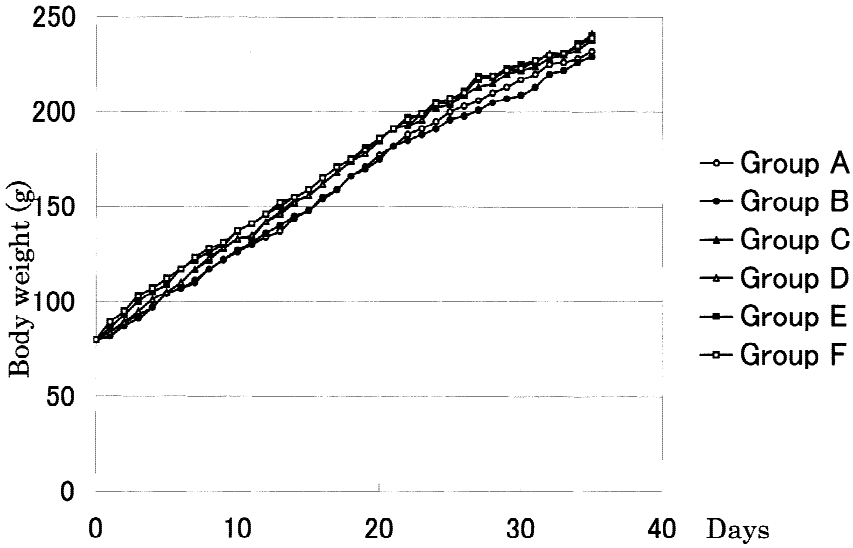

Fig. 3. Effects of phenytoin (20 mg/kg/day for 5 weeks) on rat growth curves. At the beginning of the experimental period, the average body weight was 80 g in each group. Five weeks after phenytoin treatment, the average body weight of group B was 229 g and there was no significant difference from the other five groups.

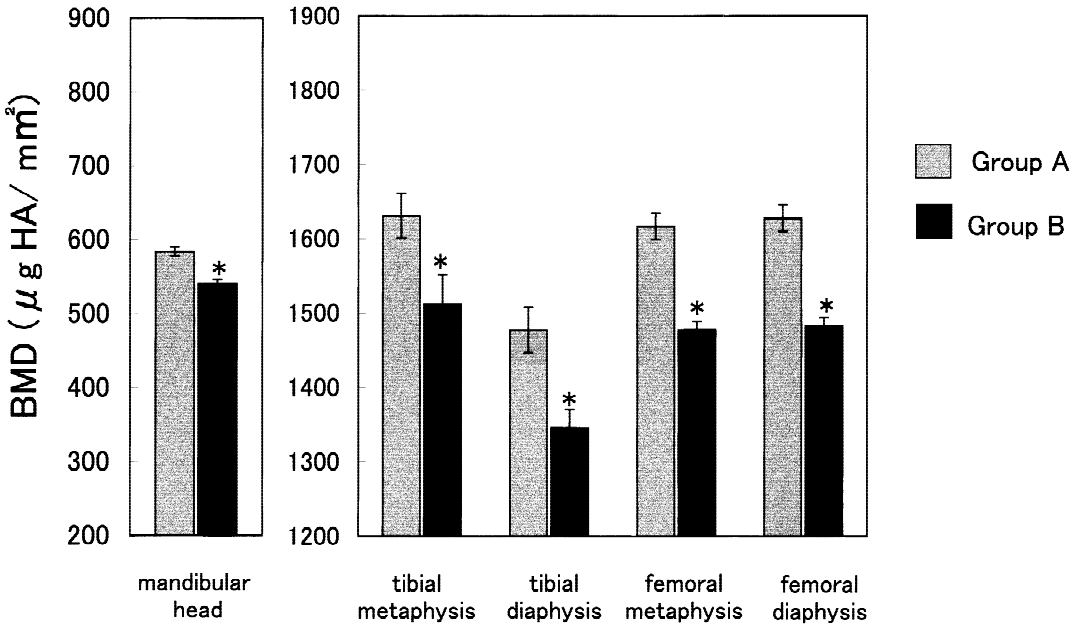

Fig. 4. Effects of phenytoin (20 mg/kg/day for 5 weeks) on BMD in rats. Each column and bar represents the mean ± SEM, respectively. *$P < 0.05$ between group A and group B.

mg/kg/day for 5 weeks decreased BMD in all regions of bones measured, but did not elicit any significant difference in the growth curves of rats. None of the body weights or bone sizes of phenytoin-treated rats showed any significant differences from the other groups. Thus, it is unlikely that adverse effects on growth caused the decreased BMD induced by phenytoin. Furthermore, it is notable that combined administration of either alfacalcidol, a derivative of vitamin D_3 [27] or calcitriol, an active form of vitamin D_3 [27] with phenytoin prevented the reduction in BMD induced by phenytoin. A lack of vitamin D_3 is well known to lead to rickets and/or osteomalacia in animals [3, 9, 11, 28, 29]. Previous clinical reports have shown that the use of high doses of phenytoin is frequently associated with development of hypocalcemia and osteomalacia [1, 2, 8, 11]. This mechanism was explained by the finding that phenytoin decreased intestinal transport of calcium [30, 31] and also induced decreases in calcium influx into osteoblasts [32]. Moreover, phenytoin is known to be an inducer of liver microsomal P-450-containing oxidases, which subsequently leads to an increased rate of catabolism of vitamin D and its derivatives to inactive metabolites [33, 34]. Accordingly, it has been proposed that the osteomalacia might be related to the reductions in circulating levels of biologically active vitamin D and its metabolites, which in turn might be responsible for the development of hypocalcemia

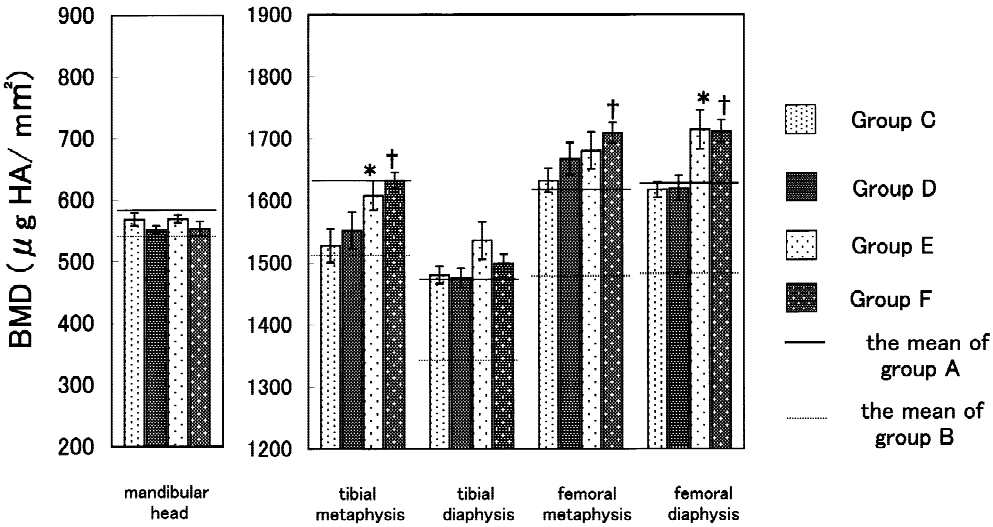

Fig. 5. Effects of combined administration of alfacalcidol or calcitriol with phenytoin (20 mg/kg/day) for 5 weeks on BMD in rats. Each column and bar represent the mean ± SEM, respectively. *$P < 0.05$ vs group C and †$P < 0.05$ vs group D.

and osteomalacia [5, 6]. In this study, we found that phenytoin produces effects on bone metabolism via different mechanism(s) from those proposed, i.e., induction of hypocalcemia and/or osteomalacia. Interestingly, hypocalcemia in phenytoin-treated rats was not observed in this experiment, although the treatment with phenytoin at 20 mg/kg/day for 5 weeks significantly decreased the BMD in all regions of bone measured. Consistent with this finding, Ohta et al. [20] reported that serum calcium levels were within the normal range for rats even after receiving daily injections of 150 mg/kg phenytoin for 47 days, although they also showed that phenytoin at a low dose (5 mg/kg) was osteogenic in rats. With regard to circulating levels of biologically active vitamin D_3 and its metabolite, Tomita et al. [35] suggested that phenytoin induces a decline in hepatic function, especially by inhibiting the hydroxylation step of alfacalcidol, as the reason for osteomalacia. This is because alfacalcidol, a derivative of vitamin D_3, is reported to be hydroxylated to calcitriol, an active form of vitamin D_3 in the liver [27, 36]. However, in this study, phenytoin did not affect the level of serum 25OHD. Combined administration of either alfacalcidol or calcitriol with phenytoin prevented the reduction in BMD induced by phenytoin with almost similar potencies, suggesting that inhibition of the hydroxylation step from alfacalcidol to calcitriol may not be significantly altered under our experimental conditions. Thus, these present data clearly showed that bone loss induced by phenytoin is not rachitic. In support of this finding, Marielle et al. [37] reported that daily phenytoin administration at a dose of 50 mg/kg for 22 days did not affect the plasma 25OHD levels in rats. In addition, it was reported that even when CCl_4 altered liver function, this hydroxylation step from alfacalcidol to calcitriol was normal [27]. In fact, clinical investigations of large numbers of epileptic patients with long-term courses of anti-epileptic drugs demonstrated that both osteomalacia and rickets were relatively uncommon [11, 12, 15]. To clarify the effects of phenytoin on the bone metabolism, we measured markers of bone metabolism. Our biochemical data indicated that the serum osteocalcin, a marker of bone formation, showed a slight but significant decrease in the phenytoin-treated group compared with the vehicle-treated group, suggesting that bone loss induced by phenytoin is caused by depression of bone formation. Hence, we could not detect any difference in pyridinoline between phenytoin- and vehicle-treated groups. Nevertheless, the bone histomorphometric measurements in secondary trabecular bone showed that there were increased numbers of osteoclasts, although it was not increased enough to be evident from measurements of circulating pyridinoline. Phenytoin decreased BV/TV, Tb.Th, and Tb.N, and increased Tb.Sp and Oc.N/BS. These findings suggest that the decrease in BMD by phenytoin was not due to an increase in the osteoid tissue in bone (osteomalacia), but rather to a decrease in bone mass, particularly in trabecular bone volume, by accelerating bone resorption in secondary trabecular bone. Combined administration of alfacalcidol was effective in preventing bone loss induced by phenytoin and did not cause a reduction of BV/TV. From these results it was indicated that the treatment with phenytoin at 20 mg/kg/day for 5 weeks caused osteopenia, which may be caused by both inhibition of bone formation and/or acceleration of bone resorption rather than osteoid accumulation. For this mechanism, there was no significant difference in the level of PTH between the vehicle-treated and phenytoin-treated group, suggesting that bone loss induced by phenytoin was not mediated by the secretion of PTH. Moreover, it is well recognized that the severity of drug-induced calcium and bone metabolism disorders depends on several factors including vitamin D intake, sunlight exposure, physical exercise, doses of antiepileptic

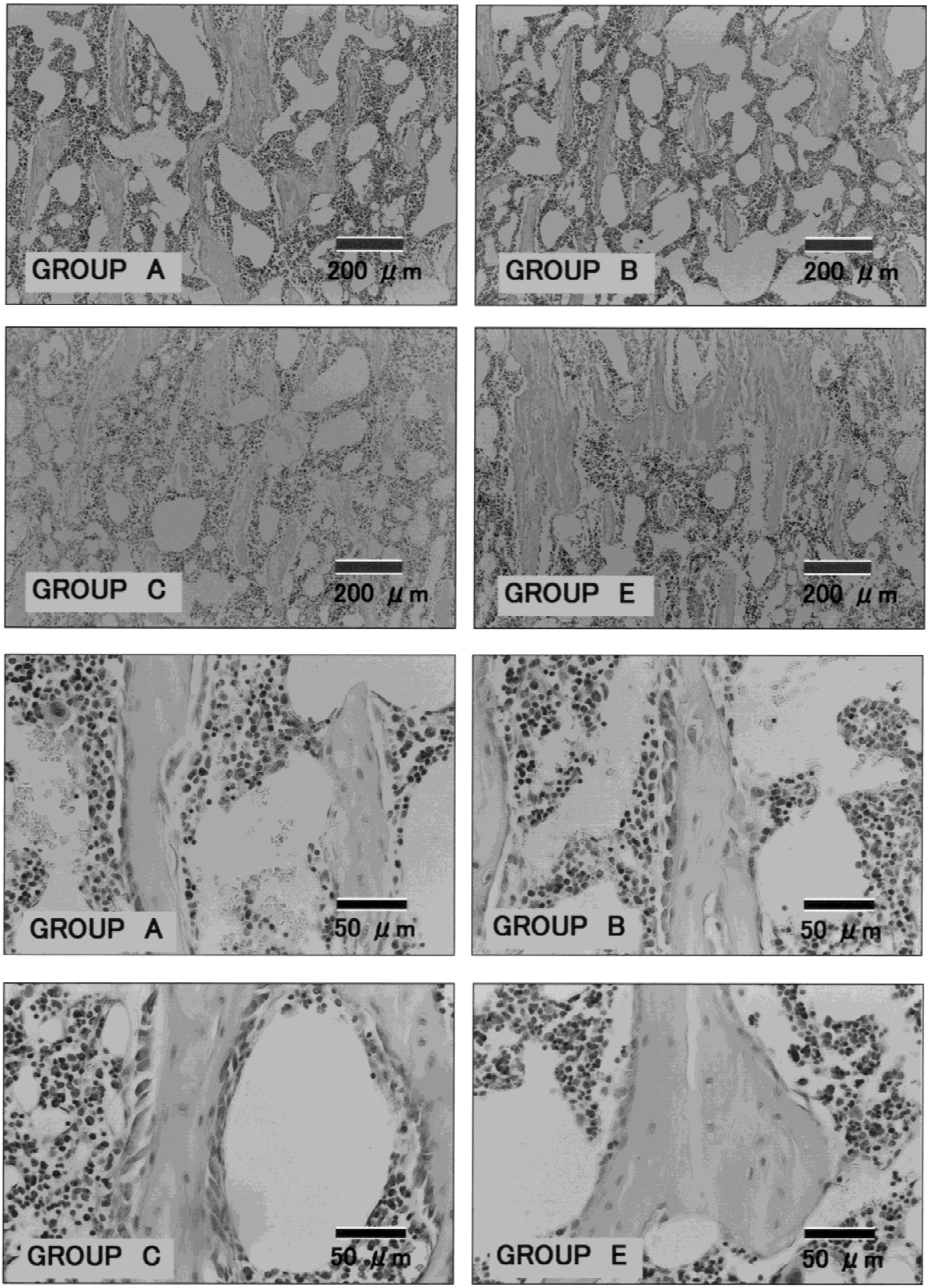

Fig. 6. Typical microphotomicrographs of the tibial metaphysis using hematoxylin-eosin (H-E) staining (upper 4 photos from groups A, B, C, and E ×100, lower 4 photos ×400). Lower 4 sections were treated with cyanuric chloride.

Table 1. Effect of phenytoin and/or alfacalcidol on serum bone makers

Group	Osteocalcin (nmol/l)	Pyridinoline (ng/ml)	25OHD (ng/ml)	PTH (pg/ml)
A	72.65 ± 3.48	2.87 ± 0.11	16.60 ± 1.25	11.83 ± 1.91
B	54.02 ± 3.26*[a]	3.17 ± 0.23	14.61 ± 1.51	10.40 ± 2.11
C	58.68 ± 2.24*[a]	3.00 ± 0.09	10.48 ± 1.28*[a,*b]	2.46 ± 0.71*[a,*b]
E	82.08 ± 3.06*[a,*b*c]	3.03 ± 0.22	11.97 ± 1.57*[a]	2.86 ± 0.61*[a*b]

Data represent the mean value ±SEM
* a, *b, and *c $P < 0.05$ vs group A, B, and C, respectively

Table 2. Effect of phenytoin and/or alfacalcidol on bone histomorphological parameters in sections from proximal tibia

Group	BV/TV (%)	Tb.Th (μm)	Tb.N (/mm)	Tb.Sp (μm)	O.Th (μm)	Oc.N/BS (mm)
A	10.62 ± 0.35	29.24 ± 1.55	3.66 ± 0.11	245.54 ± 7.01	3.34 ± 0.13	0.51 ± 0.06
B	5.38 ± 0.28*[a]	21.07 ± 1.44*[a]	2.58 ± 0.13*[a]	371.62 ± 19.6*[a]	3.36 ± 0.09	1.04 ± 0.12*[a]
C	10.01 ± 0.31*[b]	20.10 ± 0.65*[a]	5.02 ± 0.28*[a,*b]	181.82 ± 8.89*[a,*b]	3.53 ± 0.21	0.37 ± 0.09*[a,*b]
E	11.30 ± 0.30*[b*c]	25.70 ± 1.30*[b*c]	4.46 ± 0.26*[a*b]	202.70 ± 11.52*[a*b]	3.44 ± 0.21	0.42 ± 0.07*[b]

Data represent the mean value ±SEM
* a, *b, and *c $P < 0.05$ vs group A, B, and C, respectively

drugs, duration of therapy, and individual susceptibility [15, 27, 40, 41]. Clinical work using photon-absorption measurements has demonstrated that most epileptic patients on long-term courses of antiepileptic drugs showed a moderate degree of bone loss [15]. Accordingly, bone loss induced by phenytoin in this study may be a convenient model for further research into the problem of a moderate degree of bone abnormality such as drug-induced osteopenia.

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