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Chronotherapy with active vitamin D3 in aged stroke-prone spontaneously hypertensive rats, a model of osteoporosis

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Abstract

The chronotherapeutic effects of 1-α-(OH) vitamin D3, a pro-drug of 1,25(OH)2 vitamin D3 (1,25(OH)2D3), were evaluated by repeated dosing of the drug in aged stroke-prone spontaneously hypertensive male rats, a model of osteoporosis. Animals (7 months old) were kept in rooms with a 12-h light/dark cycle. Drug (0.5 μg/kg) or vehicle was given once daily at 2 or 14 h after lights on for 3 months. The severity of adverse effects such as body weight loss, hypercalcemia and hyperphosphatemia was significantly less when the drug was given at 14 h after lights on (14 HALO). Serum 1,25(OH)2 vitamin D3 concentrations of 2 h after lights on (2 HALO) group and 14 HALO group did not differ significantly after dosing. The decrease in parathyroid hormone (PTH) level 12 weeks after the start of the study was greater in the 14 HALO group than in the 2 HALO group. Urinary excretion of inorganic Ca and P in the 2 HALO group was greater than that in the 14 HALO group. Urinary excretion of deoxypyridiniline, an index of the bone resorption capacity of osteoclasts, was much suppressed in the 14 HALO group, suggesting that the efficacy of vitamin D3 for suppressing bone resorption might vary with the dosing time. The increase in bone density of both femurs, determined by dual-energy X-ray absorption at the end of the study, was greater in the 14 HALO group than in the 2 HALO group. This is the first study to show the dosing time-dependent efficacy and toxicity of active vitamin D3 in an animal model of osteoporosis. These results indicate that a chronopharmacological approach is beneficial for establishing a more effective and/or safer regimen of active vitamin D3 for the treatment of osteoporosis.

Keywords: Vitamin D3; Osteoporosis; Stroke-prone spontaneously hypertensive, rat; Chronotherapy

1. Introduction

Active vitamin D analogues are widely used for the treatment of osteoporosis and secondary hyperparathyroidism. Vitamin D increases serum inorganic Ca and P concentrations because it enhances (1) the absorption of Ca and P from intestine, (2) bone resorption by osteoclasts, and (3) renal Ca re-absorption (Parfitt, 1988). Patients treated with the drug sometimes have adverse effects such as hypercalcemia and hyperphosphatemia, which leads to a discontinuation of therapy. It is well known that serum inorganic Ca and P concentrations show diurnal changes in both humans and rats (Shinoda and Seto, 1985). We have recently reported that the hypercalcemic and hyperphosphatemic effects of 1,25(OH)2 vitaminD3 (1,25(OH)2D3) after single dosing varied with the dosing time in patients with secondary hyperparathyroidism and in normal rats as: hypercalcemia and hyperphosphatemia can be diminished by administering of the drug at night (Tsuruoka et al., 2000, 1999). However, it is uncertain whether the chronopharmacological profile of the drug is similar during repeated administration. Furthermore, it remains to be determined whether the efficacy of vitamin D3 also depends on the time of dosing. The 1-α-(OH) vitamin D3 (1-α-(OH)D3), a pro-drug of 1,25(OH)2D3, is one of the active vitamin D3 analogues and is prescribed for the treatment of osteoporosis as well as 1,25(OH)2D3. It is reported that this drug gives rise to a similar serum 1,25(OH)2D3 concentration as that after a single oral dose of 1,25(OH)2D3 in renal failure patients (Kimura et al., 1991). However, it was not determined whether

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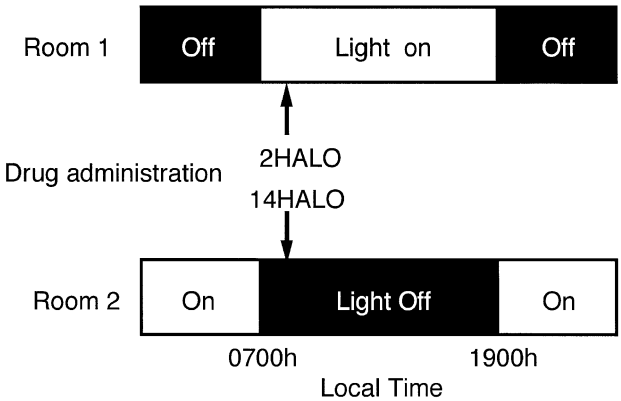

Fig. 1. Schematic representation of two reversed lighting schedules to provide different administration times. By reversing the light/dark 12:12 h lighting regimens in two different rooms, dosing at 0900 h provided treatment at two different circadian stages (2 and 14 HALO).

this drug has a similar chronopharmacological effect as 1,25(OH)2D3.

Stroke-prone spontaneously hypertensive rats (SHR-sp) were originally established to evaluate the mechanism of hypertension and its related cardiovascular events. Yamori et al. (1991) found that the calvaria of SHR-sp was more fragile than that of other rat strains. Thereafter, several studies reported that the aged SHR-sp was a suitable animal model of osteoporosis (Fukuda et al., 1992, 1995). The purpose of this study was to evaluate the chronopharmacological effect of active vitamin D3 following repeated administration in aged SHR-sp. We further evaluated its efficacy by examining changes in bone and serum variables.

2. Materials and methods

2.1. Animals

Male SHR-sp (SHR-sp/Izm, Funabashi-Noen, Chiba Japan), aged 7 months old, which are used as a model of osteoporosis (Yamori et al., 1991) (Fukuda et al., 1992, 1995), were used in this study ($n = 28$). To avoid the occurrence of stroke during the study, standard rat chow (CE-2 containing 1.18% Ca and 2.5 IU/g vit. D3, Japan Clea, Tokyo, Japan) and deionized water with 0.5% potassium chloride were used from 3 months of age (Sugimoto et al., 1992; Tobian, 1986). At 6 months, the rats were divided into four groups without any significant differences in body weight among the groups.

The animals were kept in two specific-pathogen-free rooms with a 12-h light/dark cycle and diverse lighting schedules (Tsuruoka et al., 2000; Yamauchi et al., 1998). In room 1, lights were on at 0700 h and off at 1900 h at local time. In room 2, lights were on at 1900 h and off at 0700 h (Fig. 1). Temperature and humidity in the rooms were maintained automatically. Two groups were kept in room 1 and two other groups were kept in room 2 until the end of the study. It is reported that most physiological parameters, such as neuronal, humoral, motor, and behavioral functions, are completely re-synchronized within 2 weeks after changing lighting schedules (Turek, 1985; Mrosovsky and Salmon, 1987; Takahashi and Zatz, 1982; Takamure et al., 1991; Yamauchi et al., 1998; Tsuruoka et al., 2000) and this maneuver is well accepted in the fields of chronobiology and chronopharmacology.

2.2. Experimental design

A pro-drug of 1,25(OH)2D3, 1-α-(OH)D3 (0.5 μg/kg, Alpharol®, generous gift from Chugai Pharmaceutical, Tokyo, Japan), which is quickly metabolized to 1,25 (OH)2D3, or its vehicle (olive oil) was given by gastric gavage at 2 or 14 h after light on (HALO), (e.g., 0900 h) once a day for 12 weeks. The animals were divided into four groups ($n = 7$ in each, Fig. 1):

Group 1: 2 HALO with 1-α-(OH)D3,
Group 2: 2 HALO with vehicle alone,
Group 3: 14 HALO with 1-α-(OH)D3 and
Group 4: 14 HALO with vehicle alone.

It is reported that 1-α-(OH)D3 is quickly metabolized to 1,25(OH)2D3, the active metabolite, by 25-hydroxylase in

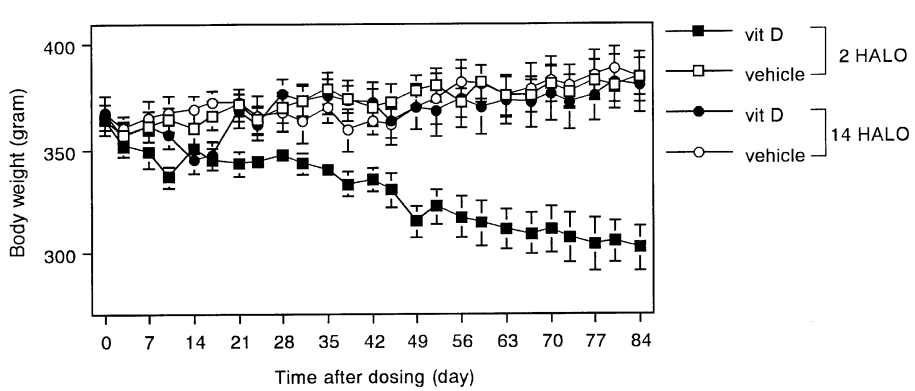

Fig. 2. Time course of body weight changes during repeated dosing of 1-α-(OH)D3 (vit. D) and vehicle at 2 and 14 HALO in aged SHR ($n = 7$ in each group). Means ± S.E..

the liver. The drug is also rapidly and directly converted to 1,25(OH)2D3 in osteoblasts (Koike et al., 1998).

Body weight was measured two times a week throughout the study. Blood samples were obtained at 0900 h (24 h after the last dose of the drug) just before and at 4, 8 and 12 weeks after the initiation of administration. Four-hour urine specimens were collected just before and 4, 8 and 12 weeks after the start of the study. For collection of urine, deionized water (3% of body weight) was given 30 min after dosing of 1-α-(OH)2D3 or vehicle and the animals were separately placed in metabolic cages for 4 h. Urine collection was performed 1 day before blood sampling. Both serum and urine were stored at −80 °C until the assay. Both femurs were obtained at the end of the study and frozen at −80 °C. All experiments were conducted in accordance with the Jichi Medical School Guide for Laboratory Animals.

2.3. Assay methods

Ca and P concentrations in serum and urine were determined by the orthocresolphthalein complexone method (Connerty and Briggs, 1966) and the ammonium molybdate method (Drewes, 1972) with an auto-analyzer, respectively. Creatinine concentration was measured by modified Jaffe's reaction with an auto-analyzer. Serum 1,25(OH)2D3 concentration was measured by radioreceptor assay (Fraser et al., 1997). Serum parathyroid hormone (PTH) concentration was measured by immunoradiometric assay (rat PTH IRMA kit, Immutopics, San Clemente, CA, USA). Its normal range is 10–40 pg/ml. Urine deoxy-pyridinoline (DPD), as an index of bone resorption, was measured by immunoassay (Seyedin et al., 1993).

Bone density of femurs was determined by dual-energy X-ray absorption (DEXA, DCS-600A, Aloka, Japan). The scan was performed every 2 mm along the axis of the bone from the proximal end. Usually, 14–17 scans were made for each bone. Averages of the first proximal were three scans, middle part four scans, and the last three scans are termed "proximal", "medial", and "distal", respectively. The average of all scans is termed "whole". "Medial" is exclusively cortical bone and "distal" is rich in cancellous bone (Shen et al., 1995).

2.4. Statistics

All data are presented as Mean ± S.E. Statistics was performed by analysis of variance or Student's *t*-test as appropriate. *P* values of less than 0.05 was regarded as significant.

3. Results

3.1. Change in body weight

The change in body weight is shown in Fig. 2. All animals completed the study without obvious symptoms of stroke. There was a small but significant ($P < 0.05$ vs. pre in each) increase in body weight during the study in the 2 HALO with vehicle and 14 HALO with vehicle and

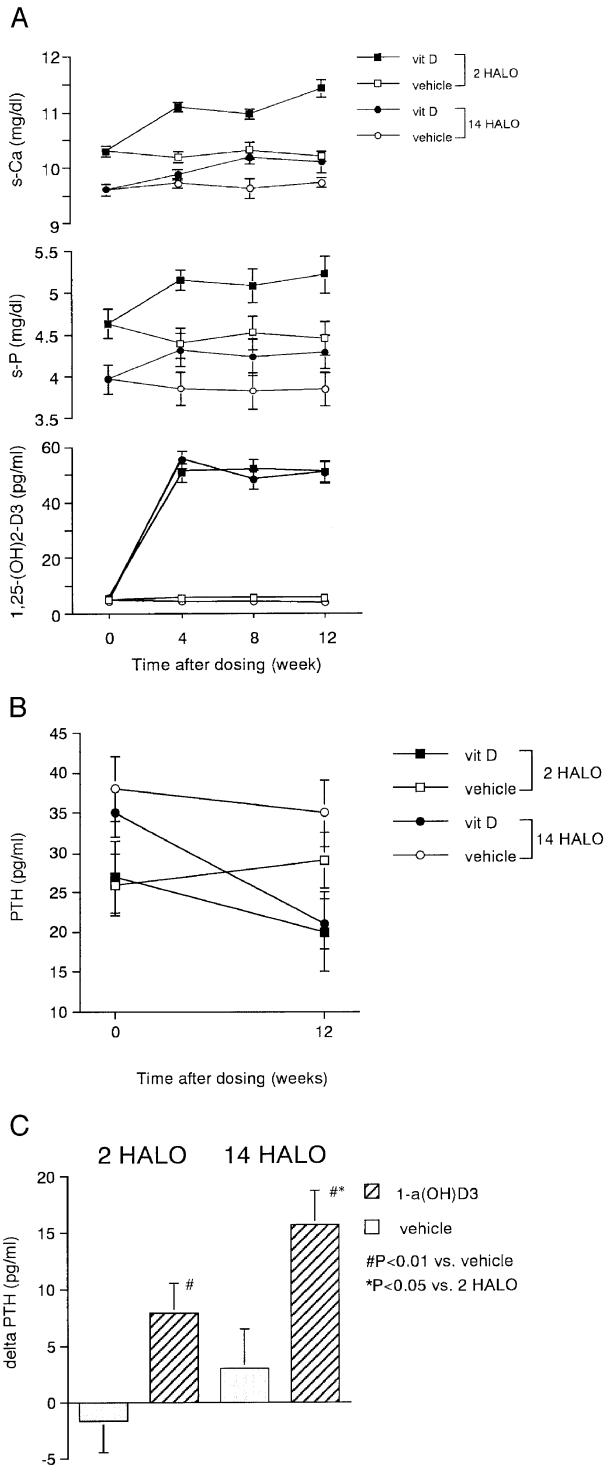

Fig. 3. (A) Changes in serum Ca (upper panel), P (middle panel) and 1,25(OH)2-D3 (lower panel) concentrations during repeated dosing of 1-α-(OH)D3 and vehicle at 2 and 14 HALO in aged SHR-sp. (B) Serum PTH concentration before and 12 weeks after the start of the study. (C) Change in serum PTH concentration 12 weeks after the study. ($n = 7$ in each group, Means ± S.E.).

1-α-(OH)D3 groups. However, body weight in the 2 HALO with 1-α-(OH)D3 group decreased significantly(mean reduction: 38 ± 9 g/3 months ($n = 7$)).

3.2. Serum inorganic Ca and P, PTH and 1,25(OH)2D3 concentrations

Serum inorganic Ca and P concentrations before the study were significantly different between the 2 HALO and 14 HALO groups (Fig. 3). These are compatible with our previous data (Tsuruoka et al., 2000) (Tsuruoka et al., 1999) and data in the literature (Shinoda and Seto, 1985; Calvo et al., 1991) for humans and normal rats. Treatment with 1-α-(OH)D3 significantly increased serum Ca concentration in both groups, and the increase was greater in the 2 HALO group than in the 14 HALO group (Fig. 3A, upper panel). Mean change in serum Ca concentration at the end of the study was 1.1 ± 0.2 mg/dl in the 2 HALO group and 0.5 ± 0.2 mg/dl in the 14 HALO group ($P < 0.05$). Similar findings were obtained for serum P concentration (Fig. 3B, middle panel). The mean change in serum P concentration at the end of the study was 0.5 ± 0.2 mg/dl in the 2 HALO group and 0.3 ± 0.2 mg/dl in the 14 HALO group. Serum P concentrations in the groups treated with vehicle were slightly and insignificantly ($0.05 < P < 0.1$) decreased, which is compatible with our previous results obtained in a single dosing study with humans and normal rats (Tsuruoka et al., 2000, 1999).

We also evaluated the trough concentration of serum 1,25(OH)2D3 (Fig. 3C, lower panel). Mean concentration before the study was 7.9 ± 0.9 pg/ml in the 2 HALO group and 8.7 ± 1.1 pg/ml in the 14 HALO group ($n = 14$). The serum concentration at a trough level increased after treatment with 1-α-(OH)D3 in both groups. No significant differences were observed at any observation points. Serum PTH concentration was measured before and 12 weeks after the start of the study (Fig. 3B and C). Its mean value before the study was relatively but not significantly higher ($P = 0.07$) in the 14 HALO group than in the 2 HALO group (26 ± 4 and 36 ± 4 pg/ml, 2 HALO and 14 HALO, respectively), although these levels were within the normal range. Drug treatment significantly reduced the PTH concentration at both times of administration, however, the decrease was significantly greater in the 14 HALO group (Fig. 3C).

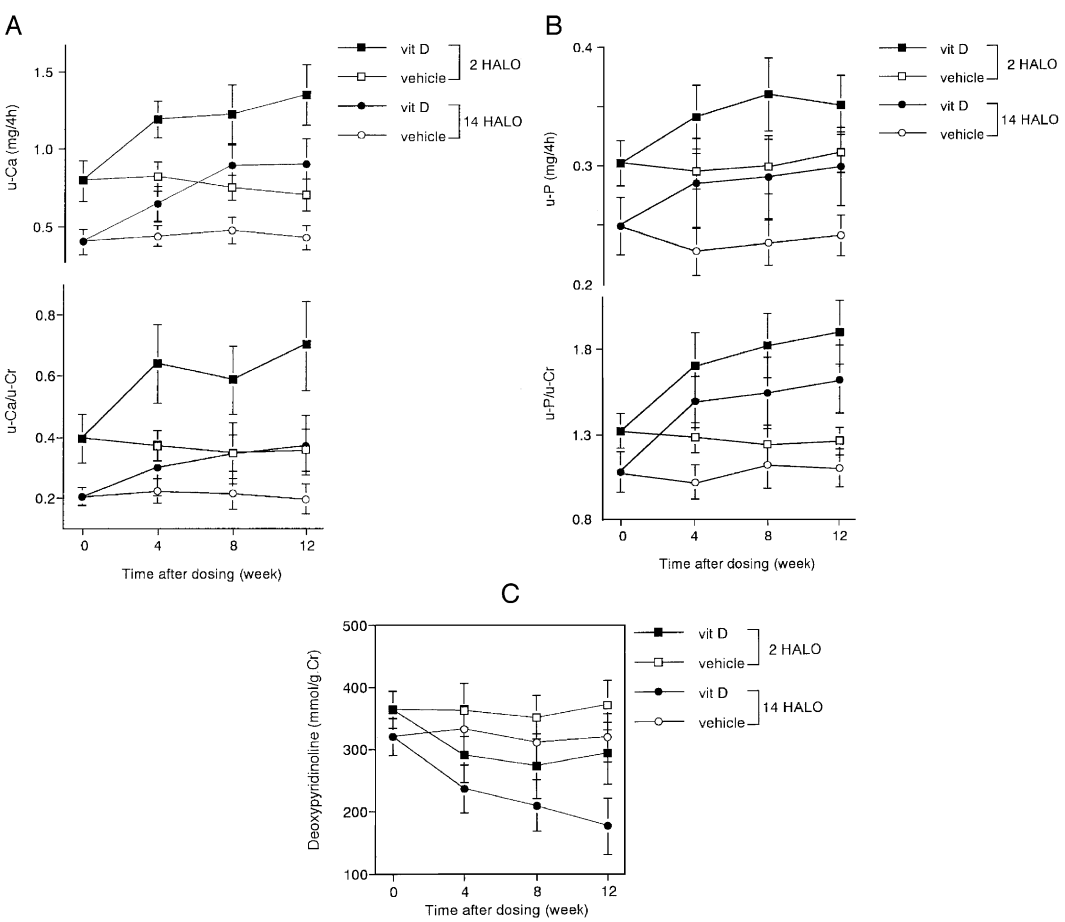

Fig. 4. Changes in urinary excretion of Ca ((A), top), ratio of u-Ca/u-creatinine ((A), bottom), phosphate excretion ((B), top), u-P/u-creatinine ((B), bottom) and deoxypyridinoline (C) during repeated dosing of 1-α-(OH)D3 and vehicle at 2 and 14 HALO in aged SHR-sp ($n = 7$ in each group). Means ± S.E.

3.3. Urinary Ca, P, creatinine and DPD excretions

The 4-h urinary Ca excretion is shown in Fig. 4A. Under basal conditions, the urinary Ca excretion in the 2 HALO group was significantly greater than that in the 14 HALO group. Treatment with 1-α-(OH)D3 increased the urinary Ca excretions, which was higher in the 2 HALO group than in the 14 HALO group. The ratio of urinary excretion of Ca and creatinine (u-Ca/u-Cr) showed a similar tendency. The basal value was higher in the 2 HALO group than in the 14 HALO group. The drug increased the ratio more in the 2 HALO group than in the 14 HALO group (Fig. 4A).

Regarding P excretion, the 4-h basal urinary phosphate excretion and u-P/u-Cr were higher in the 2 HALO group than in the 14 HALO (Fig. 4B). Both values were increased by dosing with the drug. However, there were no significant differences in the changes in these values between the 2 HALO and the 14 HALO groups ($P = 0.08$ for u-P and 0.09 for u-P/u-Cr). Urinary excretion of deoxypyridinoline (DPD) was also evaluated. It was slightly higher in the 2 HALO group at baseline; however, the difference was not statistically significant. Administration of 1-α-(OH)D3 reduced its excretion in both groups and this change was more prominent in the 14 HALO group (Fig. 4C).

3.4. Bone density

Bone density of the femur was determined by DEXA at the end of treatment with the drug for 3 months. There was no significant difference between the 2 HALO- and 14 HALO-vehicle groups (Fig. 5A–D). Bone density increased during repeated dosing with 1-α-(OH)D3 in the 2 HALO and 14 HALO groups. However, the increase in the 14 HALO group was significantly greater than that in the 2 HALO group (Fig. 5A–D).

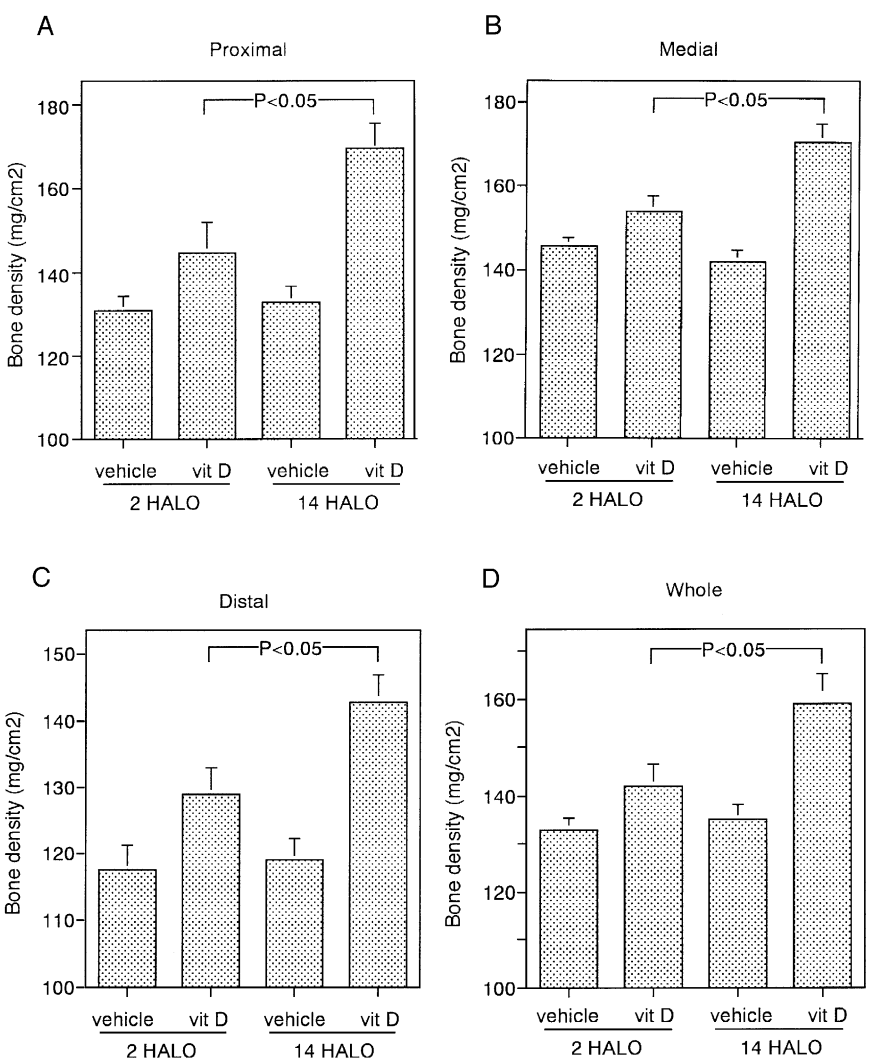

Fig. 5. Bone density of femur at the end of the study ($n = 7$ in each group). (A) Proximal, (B) medial, (C) distal, (D) whole. Means ± S.E.

4. Discussion

In this study, we found that the severity of adverse effects, such as body weight loss, hypercalcemia and hyperphosphatemia, was less when 1-α-(OH)D3 was repeatedly administered at 14 HALO. Thus, this study extended our and previous chronopharmacological data obtained for single dosing of vitamin D3 (Shinoda and Stern, 1992; Tsuruoka et al., 2000, 1999). This is the first study to show the importance of the time of dosing for treatment with active vitamin D3 in a model of osteoporosis. Serum concentrations of Ca and P increased in a dose time-dependent manner and the difference lasted until the end of the study. In addition, the change in body weight varied with dosing time. We do not have a definite explanation for this finding. Serum 1,25(OH)2D3 concentration was not significantly different between the 2 HALO and 14 HALO groups, which is good agreement with our previous finding for single dosing of 1,25(OH)2D3 in patients with secondary hyperparathyroidism (Tsuruoka et al., 1999). Therefore, a pharmacokinetics-related mechanism might not be involved. Hypercalcemia and hyperphosphatemia, which were pronounced in the 2 HALO group, might affect the activity of central nervous and gastrointestinal systems, which, in turn, might alter eating behavior and cause the reduction in body weight. Thus, we speculate that anorexia due to hypercalcemia is the major reason for the reduction in body weight of animals in the 2 HALO dosing group; however we did not measure food intake during the study, which is a limitation of the study.

Urinary excretion of Ca and P also varied with dosing time in this study. Basal excretion was higher in the 2 HALO group than in the 14 HALO group, which is consistent with our previous finding (Tsuruoka et al., 2000). Both the total excretion of urinary Ca and P and the ratio to creatinine were higher in the 2 HALO group than in the 14 HALO group. This finding suggests that the contribution of renal re-absorption to the dosing time-dependent changes in serum Ca and P induced by active vitamin D3 is small, if any.

The basal urinary excretion of DPD, which reflects bone resorption by osteoclasts (Seyedin et al., 1993), was higher in the morning (2 HALO) than in the evening (14 HALO) in this study. This is compatible with previous results in postmenopausal women (Schlemmer et al., 1992). Treatment with 1-α-(OH)D3 reduced the excretion of DPD in the 2 HALO and 14 HALO groups, and this change was greater in the 14 HALO group in spite of similar serum 1,25(OH)2D3 concentration. This finding indicates that the sensitivity of bone resorption to active vitamin D3 varies with the time of dosing. This is one of the important observations of this study. The mechanisms of this phenomenon are uncertain. There are no reports about a diurnal rhythm in the expression of molecules that are bound to 1,25(OH)2D3 (such as vitamin D receptor and vitamin D-binding proteins). The suppression of bone resorption by vitamin D is believed to be indirect effect of 1,25(OH)2D3 via the suppression of PTH. Although serum PTH concentration shows a diurnal rhythm in both human and rats, it is reported that the rhythm of PTH secretion is endogenous and not affected by lighting schedules in humans (el-Hajj Fuleihan et al., 1997). A recent study showed that basal serum PTH concentrations tended to be higher in 14 HALO than 2 HALO normal rats (Tsuruoka et al., 2000), as found in this study. In addition, although PTH concentrations were not significantly decreased at 4 h after dosing of 1,25(OH)2D3 in the two groups, PTH concentrations value tended to be lower in the 14 HALO group (Tsuruoka et al., 2000). In this study, we proved that the decrease in PTH concentration was significantly greater in the 14 HALO group after repeated dosing. Thus, the dosing time-dependent change in the PTH-suppressive effect of the drug is involved in this phenomenon. Furthermore, calbindin 28 K expression in the suprachiasmatic nuclei is altered by light(Silver et al., 1996). This might also contribute to the phenomenon because the expression of calbindin affects that of vitamin D receptors, and vice versa, in some cells (Li et al., 1998). Further study is needed to address this issue. We have previously reported that the intestinal absorption of Ca is one of cause of the 1,25(OH)2D3-induced serum Ca increase in normal rats (Tsuruoka et al., 2000). Although we think that a time-dependent change in the sensitivity of the intestine to 1,25(OH)2D3 might contribute to this phenomenon, other mechanisms via bone resorption must also be involved.

We also found that the effect of 1-α-(OH)D3 on bone density varied with the dosing time. Dosing-time-dependent changes in the effect of 1-α-(OH)D3 on bone density and urinary DPD excretion are compatible with the hypothesis that the sensitivity of osteoclasts to active vitamin D3 varies with the dosing time. However, systemic toxicity by 1-α-(OH)2D3 was detected in the 2 HALO group, and therefore other mechanisms, such as anorexia, might affect the bone density. Indeed, hypercalcemia, which was observed in the 2 HALO group, sometimes induces anorexia and disturbances of consciousness in clinical situation. Because serum 1,25(OH)2D3 concentration did not vary with the dosing schedule, drug-related hypercalcemia per se might be a cause of the weight loss in the 2 HALO group. Although the dosing time-dependent excretion of DPD strongly indicated that the efficacy of the drug to cause bone mineralization was higher in the 14 HALO group than in the 2 HALO group, the bone density in this study reflect a combination of both dosing-time-dependent efficacy and toxicity. Further study involving patients with osteoporosis is needed to determine the efficacy of 1-α-(OH)2D3.

It is generally accepted that rats tend to forage at night (dark phase) and sleep during the day (light phase), while humans do the opposite. However, it is also accepted that the serum profiles of calcium and phosphate concentrations show similar fluctuations in the two species (i.e.,

higher in light phase and lower in dark phase). When we apply the present findings to the treatment of patients with osteoporosis, we need to consider these differences.

In conclusion, we report the first study to show the chronotherapeutic effects of active repeated dosing of vitamin D3 using an animal model of osteoporosis. The severity of adverse effects such as hypercalcemia and hyperphosphatemia was less and bone density was increased by dosing of the drug in the dark phase. These effects obtained by repeated dosing of 1-α-(OH)D3 were compatible with those obtained by single dosing of 1,25(OH)2D3 in our previous reports. Suppression of bone resorption by the drug might contribute to the mechanisms, in addition to the suppression of intestinal Ca absorption. Such a chronopharmacological approach is useful for establishing more effective and/or safer treatment regimens for osteoporosis.

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