INFLUENCES OF LONG-TERM ADMINISTRATION OF 24R, 25-
DIHYDROXYVITAMIN D₃, A VITAMIN D₃ DERIVATIVE, IN RATS

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ABSTRACT — In order to examine the influences by long-term feeding of 24R, 25 dihydroxyvitamin D₃ [24R, 25(OH)₂D₃], an active form of vitamin D, Wistar rats (14-week-old, male, 20 rats/group) were fed a powder diet containing 0 or 5 ppm 24R, 25(OH)₂D₃ for 57 weeks. Final body weight and total food consumption were comparable between the groups. Urinary calcium levels were significantly (p<0.05 or 0.01) increased by the administration of 24R, 25(OH)₂D₃ at weeks 3, 22 and 56, although the levels of serum calcium did not differ between the groups at the termination of week 57. In the 24R, 25(OH)₂D₃ group, weights of the adrenals and femurs were significantly (p<0.01) increased. Histopathologically, this was found due to thickening of cortical bone in the femurs, and medullary hyperplasia and pheochromocytoma of the adrenals. Immunohistochemically, proliferating cell nuclear antigen (PCNA)-labeling indices for intact adrenal medulla, medullary hyperplasia and pheochromocytoma in the 24R, 25(OH)₂D₃ group were respectively 1.82±1.21, 5.88±4.13 and 16, all higher than that for the adrenal medulla in the control group (0.87±0.67). These results indicate that 24R, 25(OH)₂D₃ at a dose with which serum calcium is not chronically increased causes thickening of the cortex of the femur, and development of adrenal proliferative lesions, suggesting that rats may be too sensitive for results to be relevant to human risk assessment.

KEY WORDS: 24R, 25-dihydroxyvitamin D₃, Adrenal, Femur, Rat

INTRODUCTION

As a major active form of vitamin D, 1, 25(OH)₂D₃ has suppressive activities against colon (Belleli et al., 1992), skin (Chida et al., 1985), and mammary (Noguchi et al., 1989) carcinogenesis in rats. Because calcium supplementation has also been shown to inhibit colon carcinogenesis (Pence and Buddingh, 1988), it has been suggested that absorption of calcium may play a role in the chemopreventive effects. However, one of the toxicological influences of vitamin D derivatives in rats has been reported to be excessive hypercalcemia which gives rise to adrenal proliferative lesions such as medullary hyperplasia and pheochromocytoma (Tischler et al., 1996). It has been hypothesized that the mechanism underlying chromaffin cell proliferation may be due to stimulation by neurally derived signals that regulate catecholamine production and are released depending on serum calcium levels (Tischler et al., 1996).

Another active form of vitamin D, 24R, 25-dihydroxyvitamin D₃ [24R, 25(OH)₂D₃], has been reported to have physiological activities, and causes some histopathological as well as pharmacological changes in bone tissues (Birkenhager-Frenkel et al., 1995; Ornoy et al., 1978; Yamato et al., 1993; Nakamura et al., 1989). Recently, we found that 24R, 25(OH)₂D₃ inhibits glandular stomach carcinogenesis when given
in rats during the postinitiation phase (Ikezaki et al., 1996). Cancer chemopreventive effects of 24R, 25(OH)\(_2\)D\(_3\) have also been shown against colon carcinogenesis (Salim et al., 1997).

Although the toxicological profiles of 24R, 25(OH)\(_2\)D\(_3\) is little known, this vitamin D derivative may have low toxicity because some preliminary data suggest that it causes milder hypercalcemia than 1, 25(OH)\(_2\)D\(_3\) (Fenwick et al., 1984; Maeda et al., 1987). The present study was designed to investigate the influence, mainly on calcium homeostasis and the adrenal medulla of long-term administration of 24R, 25(OH)\(_2\)D\(_3\) in rats under conditions similar to those in our previous experiment concerned with stomach cancer prevention (Ikezaki et al., 1996).

**MATERIALS AND METHODS**

**Chemicals and animals**

24R, 25(OH)\(_2\)D\(_3\) was generously donated by Kureha Chemical Co. Ltd. (Tokyo, Japan). Male Wistar rats (Japan SLC Inc., Shizuoka, Japan) were housed five animals per polycarbonate cage and were maintained under standard laboratory conditions (room temperature, 23 ± 2°C; relative humidity, 60 ± 5%; 12 hr/12 hr light/dark cycle). For comparison with our previous experiment that inhibited glandular stomach carcinogenesis (Ikezaki et al., 1996), male 14-week-old Wistar rats and the dose level of 24R, 25(OH)\(_2\)D\(_3\) were selected in the present study. They were fed basal diet, CE-2 powder (Clea Japan, Inc., Tokyo), supplemented with or without the test chemical, and had free access to tap water throughout the study.

**Experimental protocol**

Rats (14-week-old, 20 rats/group) were given the poweder diet containing 0 or 5 ppm 24R, 25(OH)\(_2\)D\(_3\) for 57 weeks. All rats were observed daily for symptoms of toxicity. Body weights were measured once a week for the first 13 weeks of the study and then once every 4 weeks. Urinary calcium and phosphorus levels were measured at three time points, 3, 22 and 56 weeks. After the final administration, animals were anesthetized with ether for the collection of blood for serum biochemical examination, and sacrificed for autopsy. At autopsy, the lungs, heart, liver, adrenals, kidneys, femur (right side only), thyroid, parathyroid, spleen, stomach and intestines of each animal were excised for histopathological examination, and the first six organs were weighed. Organs were fixed in 10% buffered formalin and paraffin-embedded sections were routinely prepared and stained with hematoxylin and eosin.

**PCNA immunohistochemistry**

The influence on cell proliferative activity in the adrenal medulla was evaluated in terms of proliferating cell nuclear antigen (PCNA) expression. For immunohistochemical detection of PCNA present in nuclei, the strept avidine-biotin-peroxidase complex (strept ABC) method was used (Shi et al., 1991) with an anti-PCNA monoclonal mouse antibody, PC-10, obtained from Dako Japan (Kyoto, Japan). The numbers of PCNA-positive nuclei (PCNA-labeling indices) in 100 cells from each lesion in the adrenal medulla were evaluated.

**Urinary excretion of calcium and phosphorous, and serum biochemistry**

Calcium and phosphorous levels in urine samples collected over 24-hr periods at 3, 22 and 56 weeks were analyzed using a Hitachi 736-60E autoanalyzer (Hitachi Ltd., Tokyo). Serum biochemical parameters such as total protein (TP), blood urea nitrogen (BUN), creatinine (CRN), magnesium (Mg), calcium (Ca), total cholesterol (TCho), triglyceride (TG) and alkaline phosphatase (ALP) were also determined using serum collected from the abdominal aorta immediately before autopsy.

**Statistical analysis**

The data for lesion incidences were analyzed using the Fisher's exact test (Fisher, 1950), and data for organ weights and biochemical quantitation were examined with the Student's t-test (Gad and Weil, 1982).

**RESULTS**

**Consumption of food and 24R, 25(OH)\(_2\)D\(_3\)**

Food consumption was comparable between groups at each measured time point (data not shown). The mean daily intake of 24R, 25(OH)\(_2\)D\(_3\) in the treatment group calculated from the food consumption and body weight data was 23.2 μg/100 g body weight per animal.

**Body and organ weights**

Growth curves were quite comparable between groups (Fig.1) and the final body weights did not significantly differ. In the 24R, 25(OH)\(_2\)D\(_3\) treatment group, the absolute and relative weights for the adrenal (bilateral) and femur (right) were significantly (p<0.05 or p<0.01) elevated. There were no statistical differences between groups in the other organ weights,
Influences of 24R, 25-dihydroxyvitamin D₃ in rats.

although the relative weight for the left but not right kidney was significantly \( (p<0.05) \) elevated as compared to the control group value (Table 1).

**Urinary excretion of calcium and phosphorus and serum biochemistry**

Fig. 2 and Table 2 respectively show data for the relationship between urinary excretion and serum levels of calcium and phosphorous, and the other serum biochemical data. Urinary calcium levels were significantly \((p<0.05 \text{ or } 0.01)\) increased by the administration of 24R, 25(OH)₂D₃ at all three investigated time points (Fig. 2). The urinary calcium level was highest at 22 weeks. Significant \((p<0.05)\) increase in urinary phosphorus levels was only noted at 22 weeks. The serum phosphorus level was significantly \((p<0.05)\) higher in the 24R, 25(OH)₂D₃ group than in the control group. There were no significant differences in the other serum parameters, including calcium.

**Histopathology and immunohistochemistry**

As shown in Table 3, medullary hyperplasia and pheochromocytoma were respectively observed in 6 and 1 rats of the 24R, 25(OH)₂D₃ group, bilaterally in most cases (Photo 1). Immunohistochemically, the FCNA-labeling indices for medullary hyperplasia and pheochromocytoma were respectively 5.88±4.13 and 16, both being higher than the value for intact adrenal medulla \((1.82±1.21)\) of the 24R, 25(OH)₂D₃ group. However, this was also elevated \((p<0.05)\) as compared to the control group \((0.87±0.67)\). Thus, long-term feeding of 24R, 25(OH)₂D₃ induced hyperplasia and neoplasms of the adrenal medulla with increase in cell proliferative activity. Thickening of the cortical bone in the femur was also observed in some rats of the 24R, 25(OH)₂D₃ group (Photo 2). No apparent toxic changes including calcification were histopathologically recognized in the lungs, heart, liver, kidneys, thyroid, parathyroid, spleen, stomach or intestines.

**DISCUSSION**

The results of the present study indicate that 24R, 25(OH)₂D₃ induces adrenal medullary proliferative lesions associated with increased cell division, although only hypercalcuria but not hypercalcinemia was noted at the termination of the experiment. 24R, 25(OH)₂D₃ has been shown to increase calcium absorption from the small intestine (Wilhelm et al., 1986). This could account for the increased urinary calcium excretion. Notwithstanding the information on influence on bone cartilage (Birkenhager-Frenkel et al., 1995; Ornoy et al., 1978; Yamato et al., 1993; Nakamura et al., 1989),

![Fig. 1. Body weight curves.](image-url)

**Table 1. Absolute and relative organ weights.**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of rats</th>
<th>Lung (g) (R)</th>
<th>Lung (g) (L)</th>
<th>Heart (g)</th>
<th>Liver (g)</th>
<th>Adrenal (mg) (R)</th>
<th>Adrenal (mg) (L)</th>
<th>Kidney (g) (R)</th>
<th>Kidney (g) (L)</th>
<th>Femur (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute</td>
<td>20</td>
<td>0.92±0.48</td>
<td></td>
<td>1.16</td>
<td>10.2</td>
<td>24±0.09</td>
<td>24±0.09</td>
<td>1.33±0.36</td>
<td>1.36±0.36</td>
<td>1.60±0.08</td>
</tr>
<tr>
<td>Organ Weight</td>
<td>Control</td>
<td>0.92±0.10</td>
<td></td>
<td>1.14</td>
<td>10.4</td>
<td>20±0.09</td>
<td>21±0.09</td>
<td>1.31±0.33</td>
<td>1.33±0.33</td>
<td>1.37±0.09</td>
</tr>
<tr>
<td>Relative</td>
<td>20</td>
<td>0.22±0.12</td>
<td></td>
<td>0.28</td>
<td>2.41</td>
<td>5.7±0.09</td>
<td>5.7±0.09</td>
<td>0.315±0.32</td>
<td>0.324±0.38</td>
<td>0.38±0.10</td>
</tr>
<tr>
<td>Organ Weight</td>
<td>Control</td>
<td>0.22±0.02</td>
<td></td>
<td>0.27</td>
<td>2.40</td>
<td>4.8±0.09</td>
<td>4.9±0.09</td>
<td>0.304±0.31</td>
<td>0.311±0.31</td>
<td>0.32±0.04</td>
</tr>
</tbody>
</table>

Data are mean values±S.D.

*: \( p < 0.05 \), **: \( p < 0.01 \) versus control group.
the physiological effects of 24R, 25(OH)₂D₃, a metabolite of 25(OH)₂D₃ in the kidney, are not well known. It has been shown that the ratios of vitamin D₃ metabolites in the kidney depend on the serum calcium levels. Namely, 1, 25(OH)₂D₃ is predominantly produced when serum calcium levels are lower than 9 mg/dl, whereas production of 24R, 25(OH)₂D₃ is increased when serum calcium levels are higher than this value (Boyle et al., 1971). In relation to such data, 24R, 25(OH)₂D₃ is known to induce little, if any, hypercalcemia (Fenwick et al., 1984; Maeda et al., 1987). In fact, 24R, 25(OH)₂D₃ was not associated with hypercalcemia at the termination of week 57 in the present study, although serum calcium levels were not sequentially measured.

It is well known that neoplastic and neoplastic lesions in the adrenal medulla in rats are frequently induced by administration of agents such as retinol

![Fig. 2](image)

**Fig. 2.** Urinary excretion and serum levels of calcium and phosphorus.

* * * : Significantly different from the control group (\( *: p < 0.05, **: p < 0.01 \)).

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of rats</th>
<th>TP (g/dl)</th>
<th>BUN (mg/dl)</th>
<th>CRN (mg/dl)</th>
<th>Mg (mg/dl)</th>
<th>T. Cho (mg/dl)</th>
<th>TG (mg/kg)</th>
<th>ALP (IU/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24R, 25(OH)₂D₃</td>
<td>20</td>
<td>6.45 ± 0.17</td>
<td>19.8 ± 2.2</td>
<td>0.48 ± 0.05</td>
<td>1.83 ± 0.05</td>
<td>80.0 ± 6.16</td>
<td>97.5 ± 44.0</td>
<td>306 ± 154</td>
</tr>
<tr>
<td>Control</td>
<td>20</td>
<td>6.53 ± 0.05</td>
<td>18.3 ± 0.50</td>
<td>0.50 ± 0.00</td>
<td>1.85 ± 0.06</td>
<td>88.5 ± 9.68</td>
<td>144.7 ± 9.8</td>
<td>202 ± 33</td>
</tr>
</tbody>
</table>

Data are mean values ± S.D.
Influences of 24R, 25-dihydroxyvitamin D3 in rats.

Photo 1. Micrographs of adrenal medulla from 24R, 25(OH)2D3-treated rats, HE × 120. (a) Normal adrenal medulla, (b) Medullary hyperplasia. The cells are smaller and have more basophilic cytoplasm than normal medullary cells. (c) Pheochromocytoma. This neoplasm, composed of cells more hyperchromatic than normal medullary cells, compresses the adjacent gland.

Photo 2. Longitudinal sections of femur from control rat (a) or 24R, 25(OH)2D3-treated rat (b), HE × 24.
acetate (Kurokawa et al., 1985), vitamin D₃ (Tischler et al., 1996), lactose (Tischler et al., 1996) and xylitol (Tischler et al., 1996). These adrenal proliferative lesions occur spontaneously in aging rats and are also often observed in rat carcinogenicity studies of vitamin D derivatives, although this has not been reported in mice. Interestingly, although dietary carbohydrates such as lactose enhance calcium absorption and give rise to nephrocalcinosis in mice, there is no evidence of any carcinogenicity (Til et al., 1986). In humans, the tumors of chromaffin cells of the adrenal medulla are very rare. There is also no evidence suggesting that hypercalcemia might cause pheochromocytomas in man. In a review of 700 cases of hypercalcemia associated with primary hyperparathyroidism undergoing surgery at the University College Hospital, London, only one of these subjects was found to have a pheochromocytoma (Roe and Bar, 1985). Therefore, it is likely that the medulla is not a peculiar target for hypercalcemia in mice and humans. It can also be concluded that the rat adrenal medulla is too susceptible to be relevant to human risk assessment because adrenal medullary proliferation was induced even under the present experimental conditions in which prolonged hypercalcemia was not evident.

The present histopathological examination showed 24R, 25(OH)₂D₃ to induce cortical thickening of the rat femur correlated with increase of wet weight. It is known that 24R, 25(OH)₂D₃ increases bone density (Nakamura et al., 1989). Because it has been reported that 24R, 25(OH)₂D₃ exerts a specific inhibitory effect on the formation and function of osteoclastic cells stimulated by 1, 25(OH)₂D₃ or parathyroid hormone (Yamato et al., 1993), the cortical thickening observed in the present study might have been due to this mechanism. The results suggest that 24R, 25(OH)₂D₃ should be considered as a candidate for the purpose of curing osteoporosis and osteogenesis imperfecta. Because such adrenal lesions appear to be highly species-specific, the usefulness of 24R, 25(OH)₂D₃ in the treatment of bone diseases and chemoprevention against carcinogenesis warrants further attention.

In conclusion, our present findings show that 24R, 25(OH)₂D₃ induces hypercalciiurea and subsequently increases adrenal medullary proliferative lesions in rats.

REFERENCES


Influences of 24R, 25-dihydroxyvitamin D₃ in rats.


