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EFFECT OF 1, 25-DIHYDROXYVITAMIN D₃ ON THE FEMALE REPRODUCTIVE SYSTEM IN RATS

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Abstract.....Parenteral application of the active metabolite of vitamin D₃, 1,25-dihydroxyvitamin D₃, has been anticipated to have remarkable efficacy in secondary hyperparathyroidism. However, in a reproduction seg. I study in rats, poor reproductive performance was reflected in a decrease in the number of matings, implantations and live births. These changes were though reversible after treatment with the compound was discontinued.

In order to clarify the mechanism of these reversible toxicities, the following were examined in female rats treated with the D₃ metabolite : 1. effect on the estrous cycle (no treatment for 2 weeks, treatment for 3 weeks and recovery for 2 weeks), and 2. effect on the maintenance of pregnancy (treatment for 2 weeks before mating and during the gestation period). In both groups, the levels of calcium, calcitonin, PTH and progesterone in serum were measured, and histopathological examination of the thyroid, parathyroid, ovary and uterus was carried out.

The following results were observed : 1) disturbance of the estrous cycle, 2) hypofunctional changes in the corpus luteum in the ovary, and the epithelium, endometrium and uterine gland in the uterus with a decrease in the serum progesterone level and 3) hypercalcemia with a decrease in calcitonin or PTH levels in serum with morphological changes including atrophy and cyst-formation in the parathyroid. However, the above changes were reversible, and recovery was observed after administration of the compound was discontinued.

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These results indicate that the hypercalcemia caused by 1,25-dihydroxyvitamin D₃ disrupts endocrinological homeostasis which in turn temporarily disrupts the female reproductive system. Furthermore, it was suggested that 1,25-dihydroxyvitamin D₃ itself directly influences on endocrinological organs (hypothalamus, pituitary, parathyroid and thyroid) and reproductive organs (ovary and uterus).

Key words : Hypercalcemia, estrous cycle, reproduction, endocrinological parameter.

INTRODUCTION

The active form of vitamin D₃ (1,25-dihydroxyvitamin D₃), which is converted from vitamin D₃ through 25-hydroxyvitamin D₃ in the liver (Blunt et al., 1968 ; Poncheon and DeLuca, 1971) to 1,25-dihydroxyvitamin D₃ in the kidney (Fraser and Kodicek, 1970 ; Nowman et al., 1971), acts to promote the biosynthesis of calcium-binding protein in intestinal cells (Brehier and Thomasset, 1990 ; Liang et al., 1991), and induces the elevation of the circulating calcium level by accelerating intestinal calcium absorption (Haussler et al., 1976) and bone formation (Raisz et al., 1972).

In the past, oral clinical application of vitamin D₃ was extensively used in the treatment of osteoporosis and chronic renal failure for regulation of calcium metabolism. Parenteral clinical application of the active metabolite of vitamin D₃ has been anticipated to have remarkable efficacy in secondary hyperparathyroidism (Slatopolsky et al., 1984 ; Kurokawa, 1987 ; Delmez et al., 1989), since 1,25-dihydroxyvitamin D₃ directly inhibits the biosynthesis of parathyroid hormone (PTH) (Andress et al., 1989 ; Silver et al., 1986).

In our reproduction segment I study in rats treated intravenously with 1,25-dihydroxyvitamin D₃ (Takizawa et al., unpublished), poor reproductive performance was observed. There was a reduced number of matings, implantations and live births. However, these changes were reversible when treatment with the compound was discontinued. In order to clarify the mechanism of these reversible toxicities, the estrous cycle, biological and endocrinological parameters, and histopathological changes in reproductive and endocrinological organs were investigated in female rats (non-pregnant and pregnant) treated intravenously with 1,25-dihydroxyvitamin D₃.

MATERIALS AND METHODS

Animals : Female SD-slc rats were obtained at 7 weeks of age (for examination of the estrous cycle) and 10 weeks of age (for pregnancy experiments) from SLC Corp., Shizuoka, Japan. After 1 week acclimatization, healthy rats (body weight 200–250 g) were used in the experiments. They were housed in individual metal cages in a

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room with a temperature of $22 \pm 2^\circ\text{C}$ and a relative humidity of $55 \pm 5\%$, and a 12-hr light/dark cycle. Standard diet F-1 (Charles River Japan, Ltd.) and tap water were given *ad libitum*.

Test article and treatment : 1,25-dihydroxyvitamin D₃ was obtained from Hoffmann-La Roche Inc. (Nutley, NJ). Injectable test article solution, which had been prepared with vehicle (NaCl, 1.5 mg ; Monobasic sodium phosphate, 9.2 mg ; Edetate Disodium, 1.0 mg ; Isoascorbic acid, 10.0 mg ; Tween-20, 4.0 mg ; Sodium hydroxide q.s. pH 7.0 ; Dist. water q.s. ad 1.0 ml), was administered intravenously through the tail vein at doses of 0 (vehicle) and $0.45\ \mu\text{g/kg/day}$ with an administration volume of 0.2ml/100 g body weight.

Experimental schedule : The following two types of experiments using female rats treated with 1,25-dihydroxyvitamin D₃ were employed. (1) Effect on the estrous cycle. During the experimental period (no treatment for 2 weeks, treatment for 3 weeks, recovery for 2 weeks), the estrous cycle was checked daily by means of smear sampling. At the end of the 3-week treatment and 2-week recovery periods, the levels of calcium, calcitonin, parathyroid hormone (PTH) and progesterone in serum were measured, and histopathological specimens of thyroid, parathyroid, ovary and uterus were examined.

(2) Effect on maintenance of pregnancy. The biochemical, endocrinological and histopathological examinations referred to in (1) (treatment for 2 weeks before mating and during gestation) were performed on gestation days 3, 7, 10 and 14.

Estrous cycle estimation : The vaginal smear test was carried out once a day at a fixed time (9 : 00 a. m.) before administration, and the data on the estrous cycle (diestrus, pro- & metestrus, estrus) were quantitatively analyzed for each week by the modified dimension method (Mizuhara et al., 1958).

Measurement of calcium, progesterone, calcitonin and PTH : Calcium in serum was analyzed with an autoanalyzer, HITACHI 7150 (OCPC method), and hormones were measured by RIA methods (progesterone : progesterone kit from DPC (Los Angeles), calcitonin : calcitonin kit from EIKEN (Tokyo) and PTH : INS-PTH RIA kit from Nichols Institute Diagnostics (San Juan Capistrano)).

Pathology : At the time of necropsy, the pituitary, thyroids, parathyroids, ovaries and uterus were taken out, weighed and fixed in 15 % neutral buffered formalin, pH 7.4. After the specimen was embedded in paraffin and sectioned, it was stained with hematoxylin and eosin and evaluated using a light microscope.

RESULTS

Effect on the estrous cycle, biochemical and endocrinological parameters, and the histopathological findings index :

Alteration in the estrous cycle was represented as an area index by the modified dimension method (Mizuhara et al., 1958). The area index of the estrous cycle in normal rats is four to six per week. No disturbance was observed during the first

week of administration, but a marked decrease (area index; two to three) was observed during the second and third weeks of administration (Fig. 1). This indicates that most of the animals treated for 1 week or longer were in diestrus. This disturbance was still observed one week after administration was discontinued, but complete resolution occurred during week 2 of the recovery period. This rapid recovery after administration was discontinued shows that this disturbance is a reversible one.

Fig. 1. Estrous cycle in rats treated with 1, 25-dihydroxyvitamin D₃. Female rats were treated with 1,25-dihydroxyvitamin D₃ (0.45 μ g/kg, iv) for 3 weeks. Estrous cycle was determined after Wright-Giemsa staining through the experimental periods (for the 2-week no treatment, 3-week treatment, and 2-week recovery periods). The area index of the estrous cycle was calculated by a graph which indicated the area under the curve of the estrous cycle stage^a (vertically) for each day (horizontally). Values are the means $\pm$ SD.

*Significantly different from the control (vehicle), $P < 0.05$

**Significantly different from the control (vehicle), $P < 0.01$

^a Grade of estrous cycle stage: Diestrus-0, Pro-& metestrus-1, Estrous-2

As shown in Table 1, the level of calcium in the serum was elevated at the end of the 3-week administration period, but returned to normal levels during the 2-week recovery period. In contrast to the elevation in calcium levels, progesterone and PTH levels in serum were decreased at the end of the 3-week administration period, but rose to normal levels during the 2-week recovery period. Calcitonin levels in

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Table 1. Biochemical & endocrinological parameters obtained from the serum of female rats treated with 1, 25-dihydroxyvitamin D₃.

Stage Dose ($\mu\text{g/kg}$) Number of animals	End of drug administration		End of recovery	
	Vehicle	0.45	Vehicle	0.45
	10	10	10	10
Calcium (mg/dl)	9.6 $\pm$ 0.7	12.2 $\pm$ 1.0*	10.1 $\pm$ 0.4	10.1 $\pm$ 0.3
Progesterone (ng/ml)	15.3 $\pm$ 11.4	5.2 $\pm$ 1.8**	8.9 $\pm$ 4.1	9.4 $\pm$ 9.0
Calcitonin (pg/ml)	175 $\pm$ 102	98 $\pm$ 21**	223 $\pm$ 130	102 $\pm$ 66**
PTH (pg/ml)	22.0 $\pm$ 3.1	15.9 $\pm$ 0.4**	21.8 $\pm$ 1.1	21.9 $\pm$ 3.0
Number of animals which showed each esrous stage on dissection				
Proestrus	0	0	0	1
Estrous	1	0	5	3
Metestrus	3	1	1	2
Diestrus	6	9	4	4

Female rats were treated with 1, 25-dihydroxyvitamin D₃ (0.45 $\mu\text{g/kg}$, iv) for 3 weeks followed by a 2-week recovery period. Serum samples were collected and calcium (H7150 autoanalyzer) and hormone (RIA) levels in serum were measured at the end of the 3-week administration period and at the end of the 2-week recovery period. Values are the means $\pm$ SD. In radioimmunoassay, all samples were tested in duplicate.

* Significantly different from the control (vehicle), $P < 0.05$

** Significantly different from the control (vehicle), $P < 0.01$

PTH : Parathyroid hormone

serum were decreased at the end of the 3-week administration period, and remained lower than the control level even after the 2-week recovery period.

In Table 2, the absolute weights of reproductive organs (ovary and uterus), especially the relative weight of the uterus, was appreciably reduced at the end of the 3-week administration period, but recovered somewhat to normal levels during 2-week recovery period. The relative weights of the endocrinological organs (pituitary and thyroid with parathyroid) were not changed during either the administration or recovery period, but their absolute weights were reduced in parallel with the reduction in body weight.

Table 2. Organ weights in female rats treated with 1, 25-dihydroxyvitamin D₃.

Stage	End of drug administration		End of recovery	
Dose ($\mu\text{g/kg}$)	Vehicle	0.45	Vehicle	0.45
Number of animals	10	10	10	10
Absolute organ weight (mg)				
Ovary (R+L)	65.7 $\pm$ 7.8	46.3 $\pm$ 10.2**	65.4 $\pm$ 8.6	55.4 $\pm$ 10.9*
Uterus	460 $\pm$ 119	263 $\pm$ 92**	539 $\pm$ 175	420 $\pm$ 153
Pituitary	12.3 $\pm$ 1.6	9.4 $\pm$ 1.4**	13.4 $\pm$ 2.6	11.5 $\pm$ 1.4
Thyroid (R+L)	12.0 $\pm$ 1.2	10.6 $\pm$ 1.6*	13.2 $\pm$ 2.8	11.5 $\pm$ 2.2
Relative organ weight (mg/100g body weight)				
Ovary (R+L)	29.3 $\pm$ 2.9	25.1 $\pm$ 4.9*	26.4 $\pm$ 3.3	25.2 $\pm$ 4.7
Uterus	204 $\pm$ 47	143 $\pm$ 41**	215 $\pm$ 55	183 $\pm$ 73
Pituitary	5.5 $\pm$ 0.7	5.2 $\pm$ 0.7	5.4 $\pm$ 1.1	5.0 $\pm$ 0.5
Thyroid (R+L)	5.4 $\pm$ 0.8	5.9 $\pm$ 1.2	4.6 $\pm$ 1.6	5.0 $\pm$ 0.9
Number of animals which showed each estrous stage on dissection				
Proestrus	0	0	0	1
Estrous	1	0	5	3
Metestrus	3	1	1	2
Diestrus	6	9	4	4

Female rats were treated with 1, 25-dihydroxyvitamin D₃ 0.45 $\mu\text{g/kg}$, iv) for 3 weeks followed by a 2-week recovery period. Dissection was carried out at the end of the drug administration period and at the end of the recovery period, and reproductive and endocrinological organs were weighed. Values are the means $\pm$ SD.

* Significantly different from the control (vehicle), $P < 0.05$

** Significantly different from the control (vehicle), $P < 0.01$

Histopathological findings showed atrophic changes in the parathyroid and hypofunctional changes in the ovaries and uterus in the animals receiving the compound. Detailed microscopic findings were as follows (Table 3, Photo. 1): Slight to moderate atrophy of parathyroid cells and cyst-formation in the parathyroids were observed at the end of the 3-week administration period, and these changes had a tendency to revert to normality during the recovery period. In the reproductive organs, a decrease in size of the corpus luteum in the ovary and hypofunctional changes in the epithelium, endometrium and uterine gland in the uterus were observed at the end of the 3-week administration period, and these changes reverted to normality during the recovery period.

The animals possessed the above histopathological changes corresponded to that showed diestrus of estrous cycle for a long time.

Effect on maintenance of pregnancy :

Only one or two pregnant animals at each 3, 7, 10 and 14 days of gestation were obtained because mating was difficult in female rats receiving the compound during

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Table 3. Histopathological findings in female rats treated with 1, 25-dihydroxyvitamin D₃.

Stage Dose ($\mu\text{g/kg}$)	End of drug administration		End of recovery	
	Vehicle	0.45	Vehicle	0.45
Number of animals	10	10	10	10
Parathyroid :				
Atrophy of parathyroid cells				
grade 0	10	0	10	3
1	0	8	0	7
2	0	2	0	0
Cystic changes				
grade 0	10	7	10	9
1	0	1	0	1
2	0	2	0	0
Ovary :				
Decrease in size corpus luteum				
grade 0	10	5	10	10
1	0	3	0	0
2	0	2	0	0
Uterus :				
Hypofunctional changes in the epithelium, endometrium and uterine gland				
grade 0	10	5	10	10
1	0	4	0	0
2	0	1	0	0

Female rats were treated with 1,25-dihydroxyvitamin D₃ (0.45 $\mu\text{g/kg}$, iv) for 3 weeks followed by a 2-week recovery period. Dissection and histopathological examination were carried out at the end of the drug administration period and at the end of the recovery period.

grade 0 : no change, grade 1 : slight, grade 2 : moderate

pre-mating period. For the few pregnant animals that were used for the measurement of the biochemical and endocrinological parameters, the calcium levels were elevated throughout the pregnancy period, but progesterone, PTH and calcitonin levels decreased conversely (Fig. 2).

In parallel with the changes in the endocrinological parameters, the weight of the reproductive organs (ovary and uterus) in pregnant rats receiving the compound were reduced, and incomplete changes in pregnancy status such as decidual reaction of endometrium and decreased number of corpus luteum gravidilatis were also found in the treated animals. Other pathological changes with compound treatment (hypofunctional changes in ovary, uterus and vagina) were observed in the pregnant rats on gestation day 7 to 14 (Data not shown). These histopathological changes indicate that implantation and the maintenance of pregnancy were difficult during treatment with the compound.

Photo 1. Histopathological effect of 1, 25-dihydroxyvitamin D₃ on endocrinological and reproductive organs in female rats.
 Female rats were treated with 1, 25-dihydroxyvitamin D₃ (0.45 µg/kg) or formulated buffer intravenously for 3 weeks. Histopathological specimens of endocrinological and reproductive organs were prepared with hematoxylin and eosin staining. The parathyroid (1), ovary (3) and uterus (5) in the control groups showed no abnormal changes, but the animals treated with 0.45 µg/kg of 1, 25-dihydroxyvitamin D₃ showed cystic changes in the parathyroid (2), hypofunctional changes in the corpora lutea in the ovary (4) and hypofunctional changes in the endometrium and uterine gland in the uterus (6). An arrow mark indicates the position where the alterations were observed.

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Fig. 2. Biochemical and endocrinological parameters in serum of pregnant rats treated with 1, 25-dihydroxyvitamin D₃. After pre-treatment with 1,25-dihydroxyvitamin D₃ (0.45 μ g/kg, iv) for 2 weeks, female rats were mated with non-treated male rats. The day when sperm was observed in the vaginal smear was designated gestation day 0. Pregnant rats were dissected on gestation day 3, 7, 10 and 14. Serum samples were collected, and calcium (H7150 autoanalyzer) and hormone (RIA) levels in serum were measured. Values are the means $\pm$ SD. In radioimmunoassay, all samples were tested in duplicate. Statistical analysis was not performed because of small sample size resulting from the decrease in mating ability in treated female rats.

DISCUSSION

For regulation of the normal estrous cycle, a relevant amount of hormones such as FSH and LH from the pituitary, and estrogen and progesterone from the ovary should be secreted at an appropriate time during the estrous cycle (Rivier et al., 1989; Piquette et al., 1991; DePaolo, 1991; Blake, 1978; Fallest and Schwartz, 1991; Peluso et al., 1991). In the present experiment, reduced levels of progesterone in the serum indicate a disturbance of the secretion or production of related hormones (FSH and LH). This disturbance might induce the continuation of a diestrus status, and lead to the decrease in mating ability. Namely, after the 3-week administration period, two thirds of animals were in diestrus and one third had normal estrous cycles.

Progesterone, which is produced in the corpus luteum in the early stage of pregnancy and secreted from the placenta in the late stage of pregnancy, is necessary for the maintenance of pregnancy (Gibori and Richards, 1978; Peluso et al., 1991; Woodruff et al., 1991). If a substantial decrease in serum progesterone is detected at the early stage of treatment, successful implantation and the maintenance of pregnancy would be difficult. These fewer implantations and live births can be thought of a secondary effect. It is also probable that the levels of LH, FSH and estradiol, which are involved in ovulation and the formation of corpus luteum, will decrease as a result of treatment with 1,25-dihydroxyvitamin D₃. Since half of the animals that had mated in the reproduction segment I study had traces of an implantation site and the other half of the mated animals did not have traces of an implantation site, it seemed that implantation was affected in half of the mated animals treated with 1,25-dihydroxyvitamin D₃ and that pregnancy was affected in the other half of the mated animals. This indicates that these effects on reproductive performance in female rats varied with respect to the timing of manifestation. In one study (Nebel and Ornstein, 1966), vitamin D₂ was administered before mating or from day five of pregnancy. Treatment before mating affected the mating and the maintenance of pregnancy, but treatment from day five of pregnancy did not affect the maintenance of pregnancy and caused no complications in delivery and no observable physical changes in the neonates. Based on these results, we assumed that vitamin D₂ would affect the progesterone secretion from the corpora lutea but would not affect the progesterone secretion from placenta, since the maintenance of pregnancy by progesterone is regulated by the corpora lutea in the early stage of pregnancy and placenta in the late stage of pregnancy. In the present study, it was supposed that 1,25-dihydroxyvitamin D₃ also had an effect on the progesterone secretion from the corpora lutea.

The mechanisms of the reciprocal action between reproductive/endocrinological and morphological changes induced by the excessive administration of active vitamin D₃ is still unclear. Based upon the results obtained in the present experiment, we propose the following (Fig. 3): (1) an indirect action with vitamin D₃-induced

hypercalcemia, (2) a secondary effect by the decreased blood levels of calcitonin and PTH related to calcium metabolism, and (3) a direct action on target organs, such as the hypothalamus, pituitary, thyroid, parathyroid, ovary and uterus, by active vitamin D₃. It is well known that excessive dosage and repeated administration of active vitamin D₃ causes hypercalcemia which in turn generates a rise in calcitonin and decrease in PTH levels (Mayer and Hurst, 1978 ; Brown, 1983 ; Care et al., 1968). However, it is said in another report (Raue et al., 1984) that basal serum calcitonin levels do not vary in chronic hypercalcemia induced by 1,25-dihydroxyvitamin D₃-administration in female rats. In this experiment even though there was hypercalcemia, the calcitonin level fell during parenteral treatment with 1,25-dihydroxyvitamin D₃. The secretion of calcitonin should increase when calcium levels in serum are elevated, but no changes in the mRNA level, an index of calcitonin biosynthesis, were observed in rats in a similar experiment (Naveh-Many et al, 1989). Recently, another report suggested that the active vitamin D₃ metabolite inhibits the mRNA -directed biosynthesis of calcitonin (Naveh-Many and Silver, 1988). These two reports suggested that pooled calcitonin in thyroid C cells would be released at the early stage of hypercalcemia caused by repeated-administration, and thereafter biosynthesis of calcitonin would be constantly inhibited as to maintain continued secretion.

The PTH level was lower than usual during parenteral 1,25-dihydroxyvitamin D₃ treatment, but normal levels were again observed after treatment was stopped. Morphological changes such as atrophy and cyst-formation in the parathyroid cells reflected lower PTH levels in serum. This lower PTH level in serum and atrophy/cyst-formation in the parathyroid have been reported in rats treated with active vitamin D₃ (Koyama et al., 1984). In general, hypercalcemia causes the lowering of PTH in the parathyroid as a feed-back mechanism. However, it was reported that PTH mRNA levels would be directly decreased by 1,25-dihydroxyvitamin D₃ by suppression of transcription of the pre-proparathyroid hormone gene (Russell et al., 1986).

Reversibility of the changes in the reproductive and endocrinological system by 1,25-dihydroxyvitamin D₃ was confirmed with the 2-week recovery test. Rapid recovery was observed in the reproductive system. Where there were hypofunctional changes in the ovary and uterus, and a decrease in progesterone levels in serum, normal levels were observed soon after treatment was discontinued.

In the endocrinological system, atrophic and cystic changes were observed in the parathyroid cells, but almost normal physical states were observed after the 2-week recovery period. The PTH level in serum was completely restored to the normal range, but the calcitonin level was not.

These results indicate that the changes in the reproductive system of the female recovered more rapidly and completely than those in the endocrinological system.

On the whole, it was concluded that 1,25-dihydroxyvitamin D₃ affected the female reproductive system functionally and morphologically, and these effects might

Fig. 3. Speculative schema of the endocrinological effect of 1, 25-dihydroxyvitamin D₃ (D₃ metabolite) on the female reproductive system.

Excess administration of D₃ metabolite induces morphological/functional endocrinological changes in reproductive organs by direct action on target organs, indirect action with hypercalcemia and/or a secondary hormonal effect of calcitonin/PTH. Hypercalcemia induced by D₃ metabolite would have an influence on ovary/uterus and thyroid/parathyroid whose hormones (progesterone, calcitonin, PTH) might regulate the function of the calcium metabolism in the living body. Direct effect of D₃ metabolite on reproductive and endocrinological organs would also have a role of regulating the feedback mechanism. These hormonal disturbances might cause disturbance of estrous cycle, which would lead to poor mating ability, and hypofunctional changes in the ovary and uterus, which would induce a decrease in implantation or live fetuses.

GnRH : Gonadotropin releasing hormone

FSH : Follicle stimulating hormone

LH : Luteinizing hormone

PTH : Parathyroid hormone

lead to a decrease in mating ability, as well as the number of implantations and live births. All these alterations were reversible after treatment with the compound was discontinued.

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