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Effects of $1,25(OH)_2D_3$ on Bone Tissue in the Rabbit: Studies on Fracture Healing, Disuse Osteoporosis, and Prednisone Osteoporosis

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Summary. A closed tibial fracture, which was controlled by an intramedullary stainless steel pin, was created in 16 rabbits. Eight rabbits were treated with 75 ng of $1,25(OH)_2D_3$ daily as subcutaneous (s.c.) injections. After three weeks, the fractured tibia resisted a force of 101,7 ± 21.0 Newtons in the control group and 57.3 ± 8.0 Newtons in animals given $1,25(OH)_2D_3$ (m ± SE, $P < 0.05$). In another group of eight rabbits, the left hindleg was immobilized in a plastic splint. Four rabbits were given 75 ng of $1,25(OH)_2D_3$/day s.c. and the effect of immobilization was studied on the calcaneus. Bone ash/cm^3 of the calcaneus on the immobilized side was decreased by 11 ± 2% in control rabbits and by 20 ± 2% in the group treated with $1,25(OH)_2D_3$ indicating a more advanced immobilization osteoporosis (m ± SE, $P < 0.05$), which was also demonstrated by studies of bone density. Eighteen rabbits were used in a study of the effects of $1,25(OH)_2D_3$ on the development of prednisolone osteoporosis. The dose of prednisolone was 2.5 mg per day, given by the oral route. After four months, the density of the femur was 1.53 ± 0.02 g/cm^2 in control rabbits and 1.42 ± 0.01 in prednisolone-treated animals ($P < 0.01$). In rabbits additionally given $1,25(OH)_2D_3$, the mean value for bone density was further lowered (n.s.). It appears that $1,25(OH)_2D_3$ exaggerates disuse osteoporosis and prednisolone osteoporosis and impairs fracture healing in rabbits. These results differ from what has been shown earlier with $1,25(OH)_2D_3$ treatment in the rat.

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The vitamin D endocrine system appears to serve as a regulator of mineral absorption in the intestine, thereby supporting the blood with adequate concentration of calcium and phosphate [1]. In the absence of such a system, there would be a risk for negative calcium balance and disturbed bone function.

Pharmacological doses of vitamin D compounds have been used in animal models of human bone disorders [2–5]. The rat is the most frequently used animal for such experiments because it is convenient and because it has a mineral metabolism that in many respects resembles the human. The rat differs from the human in that it appears to have a more active mineral absorption in the intestine, which prevents osteomalacia from developing in vitamin D deficiency, and osteoporosis from developing during prednisolone treatment [6, 7] if dietary calcium and phosphate are high. In addition, rats have a somewhat different bone structure in that Haversian systems are rarely seen. Rabbits on the other hand, have a microscopic bone structure that more closely resembles that of humans, although calcium metabolism is quite different. Rabbits readily absorb calcium after a meal, resulting in a marked postprandial hypercalcemia [8]. The excretion of calcium in the urine is very high with a normal laboratory diet [9].

These differences appeared to justify a study of the effect of $1,25(OH)_2D_3$ in rabbits under conditions where this substance has shown positive ffects in rats, that is, its influence on fracture healing,

disuse osteoporosis, and glucocorticoid-induced osteoporosis.

Materials and Methods

Seventeen male Dutch rabbits were used for the fracture study. The initial body weight was 2.0 ± 0.1 kg (mean ± SE). The rabbits were anesthetized with ketamine chloride and thiopental sodium and a 2 mm Kirschner wire was percutaneously inserted into the medullary cavity of the left tibia through the proximal metaphysis lateral to the patellar tendon. After this, a closed tibial fracture was created by bending. The fractures were always midtibial.

The rabbits were randomly divided into two treatment groups: one was given 75 ng of $1,25(OH)_2D_3$ dissolved in 0.1 ml propylene glycol, as daily s.c. injections, and the other group was given propylene glycol alone. The experimental period was three weeks at the end of which the rabbits were put under general anesthesia and killed by cardiac air injection. The tibiae and femurs were dissected free, and immediately put into plastic bags and frozen.

Eight Dutch rabbits with a mean body weight of 2.2 ± 0.1 kg (mean ± SE) were used for the immobilization study. The left hindleg was immobilized in a plastic bandage (Hexcelite, Hexcel Medical Products, USA) under ketamine and thiopental anesthesia. The knee and the ankle joint were fixed at right angles. Usually the bandage had to be repaired or exchanged twice a week. The rabbits were killed after three weeks by cardiac air injection under general anesthesia. The calcanei were dissected free, put in plastic bags, and frozen.

Eight male New Zealand rabbits with a mean body weight of 3.2 ± 0.9 kg (mean ± SE) were used for a four month study on the effects of $1,25(OH)_2D_3$ as a prophylaxis for prednisolone osteopenia. The rabbits were randomly divided into three groups. They were given equal amounts of a commercial pelleted stock diet. The size of the portion was chosen so that in general all rabbits consumed the whole ration. The diet content of calcium and phosphate was 1.1 and 0.9%, respectively. Prednisolone dissolved in 95% ethanol was evenly spread on a thin layer of pellets using a nebulisator, and by the same method, $1,25(OH)_2D_3$ was also spread evenly on the diet and the ethanol was allowed to evaporate. By this method, three preparations of diet were made up: one was prepared with only ethanol, one with only prednisolone, and one with prednisolone and $1,25(OH)_2D_3$. The average daily consumption of prednisolone was 2.5 mg and the average daily consumption of $1,25(OH)_2D_3$ was 25 ng per rabbit. The food intake was carefully registered and the rations adjusted as necessary. Water was provided *ad libitum* but the consumption was measured. The urine was checked for glucose, and no glucosuria was detected. At the end of the observation period, the rabbits were put under general anesthesia with fentanyl-fluanison (Hypnorm®, Leo, Sweden) and killed by intracardiac air injection. The right hindlimb was dissected free from each rabbit and stored in a plastic bag at −20°C.

The concentration of the stock $1,25(OH)_2D_3$ was determined from the U.V. spectrum previous to use. The tibias and femurs from the fracture study were X-rayed. Immediately after thawing, the bones were subjected to a four point bending test and the breaking strength was recorded using a MTS equipment (Material Testing Systems, Minneapolis, Minn.) [2]. The bone mineral content (BMC) of the distal femur was measured using 125 I bone densitometry with the direct readout instrument developed by the medical physics group at the University of Wisconsin [10]. Measurement precision was typically 1–2% on the excised bones. The accuracy of this method, as assessed on ashed bone sections, was very high [11]. The long-term precision (four years) on standards was 1–2% [12]. Student's *t* test was used in the statistical analysis.

Table 1. Breaking strength by four-point bending 3 weeks after fracture (Newtons)

	n	Left tibia		Right tibia (fractured)
Untreated	7	234.8 ± 19.8	$P < 0.001$	101.7 ± 21.0
		n.s.		$P < 0.05$
$1,25(OH)_2D_3$ 75 ng/day s.c.	9	197.5 ± 17.1	$P < 0.001$	57.3 ± 8.0

Values are mean ± SE

Results

There were no significant changes in body weight between the rabbits in the experimental groups that sustained the fracture and those that were immobilized. Serum calcium and serum phosphate were not significantly influenced by administration of $1,25(OH)_2D_3$ in any of these groups. Serum calcium varied between 10–15 mg/100 ml whereas serum phosphate varied between 2.0 and 5.0 mg/100 ml. High levels of calcium and phosphate were apparently related to a recent intake of food.

Following the tibial fracture, the rabbits avoided weight-bearing on their fractured limb and usually kept the foot in moderate outward rotation. After three weeks, the fracture appeared to be stable and the rabbits seemed to function normally. The tibial fracture was in all cases mid-diaphyseal and transverse, occasionally with a small posterior fragment. One tibia could not be examined due to a technical error in the procedure. As seen in Table 1, the breaking strength at the fracture was significantly lower in the group given $1,25(OH)_2D_3$. Densitometry of the distal femur did not show any bone mineral content (BMC) differences between the two groups (Table 2). On the fractured side, the BMC was decreased between 5 and 10% compared with the contralateral side. The rabbits immobilized in the Hexcelite cast occasionally had pressure sores. The ankle joint was usually quite stiff, whereas the knee joint did not seem to be affected. This treatment caused a decrease exceeding 40% in bone density of the calcaneus (Table 3). The density was significantly lower in immobilized calcanei of the $1,25(OH)_2D_3$ group as indicated by the percentage difference between the nonimmobilized and the mobilized side compared with the untreated group.

Table 2. Bone mineral content of distal femur 3 weeks after right tibial fracture (g/cm)

	n	Left femur		Right femur	Right as % of left
Untreated	8	0.3172 ± 0.053	$P < 0.001$	0.2985 ± 0.003	93.9 ± 0.8
		n.s.		n.s.	n.s.
$1,25(OH)_2D_3$ 75 ng/day s.c.	9	0.3018 ± 0.0090	$P < 0.05$	0.2831 ± 0.0105	93.9 ± 2.5

Values are mean ± SE

Table 3. Effects on calcaneus of immobilization of the right lower extremity

	n	Whole bone density (g/cm^3) Left calcaneus	Right calcaneus	Ash/volume (g/cm^3) Left calcaneus	Right calcaneus	Difference between immobilized and nonimmobilized side (%) Density	Ash/volume
Untreated	4	1.4375 ± 0.0517	1.2739 ± 0.0409	0.5897 ± 0.0508	0.3652 ± 0.0406	38 ± 4	11 ± 2
		$P < 0.001$		$P < 0.01$		$P < 0.05$	$P < 0.05$
		n.s.	n.s.	n.s.	n.s.		
$1,25(OH)_2D_3$ 75 ng/day s.c.	4	1.4566 ± 0.0263	1.1694 ± 0.0147	0.5652 ± 0.0515	0.2622 ± 0.0325	53 ± 4	20 ± 2
		$P < 0.001$		$P < 0.01$			

Values are m ± SE

This demonstrates that the immobilization osteopenia was exaggerated due to $1,25(OH)_2D_3$.

During the prednisolone experiment, one control rabbit died for reasons unrelated to the experiment. The prednisolone-treated rabbits showed a lesser increase in body weight than the controls (Table 4). Prednisolone treatment led to lower values of femur ash; the group given $1,25(OH)_2D_3$ in addition to prednisolone showed the lowest mean values of femur ash. The density of the femur demonstrated similar results. The density findings suggest that the prednisolone diet caused osteoporosis and that osteoporosis was not prevented by $1,25(OH)_2D_3$.

Discussion

Rabbits have previously been used in a number of experiments on bone and mineral metabolism. However, the calcium metabolism is different in rabbits than in most other species. Rabbits normally have extensive fluctuations in the serum calcium concentration which invalidates its use as an indicator in disorders where a measurable hypercalcemia would be found in other species. On a normal laboratory diet, there is also a very high concentration of calcium in the urine. This makes it also impractical to use the urinary calcium excretion as a measure of changes involving the calcium metabolism. It has been proposed that in the rabbit the kidneys are more important in the regulation of the calcium homeostasis than is the intestive [9], and therefore, the rabbit might be unsuitable for vitamin D experiments with relevance to overall calcium metabolism in the human. However, in studies of bone tissue, the rabbit might be preferable at least to the rat, because of the greater resemblance in microscopic bone structure between rabbits and humans.

The big variations in serum calcium of rabbits make it difficult to adjust the dose of $1,25(OH)_2D_3$. The doses used in the fracture experiments were less than half of what has been used in rats where the fracture healing was augmented [2], and considerably lower than doses tolerated by rats before bone resorption occurs. Still, the negative findings from $1,25(OH)_2D_3$ treatment of disuse osteoporosis and fracture healing may be dose-dependent. In the prednisolone study, however, the doses used were as low as the therapeutic doses recommended in human medicine.

In the three week experiment, parenteral dosing with $1,25(OH)_2D_3$ was used. This could have led to high peak concentrations which are known to cause bone resorption. On the other hand, in the rat and

Table 4. Results from daily oral treatment with 2.5 mg of prednisolone alone or in combination with 25 ng of $1,25(OH)_2D_3$ in rabbits (experimental period = 4 months)

	n	Final body weight (kg)	Total ash (g)		Density (g/cm^3)	
			Femur	Tibia	Femur	Tibia
Control	5	4.5 ± 0.3	66.2 ± 0.4	66.9 ± 0.7	1.53 ± 0.02	1.63 ± 0.03
Prednisolone	6	3.4 ± 0.2[b]	63.6 ± 0.9[a]	65.8 ± 0.4	1.42 ± 0.01[b]	1.54 ± 0.02[a]
Prednisolone + $1,25(OH)_2D_3$	6	3.4 ± 0.1[b]	62.8 ± 0.7[b]	65.1 ± 0.4[a]	1.40 ± 0.01[c]	1.50 ± 0.03[b]

Values are mean ± SE. The statistical significance is determined vs control
[a] $P < 0.05 > 0.01$
[b] $P < 0.01 > 0.001$
[c] $P < 0.001$

dog experiments cited above, resorption was not dominant. In the four month experiment described here, oral dosing was used which should account for a more even concentration of $1,25(OH)_2D_3$ in serum.

The findings that the fracture healing was impaired whereas disuse osteoporosis was increased are somewhat contradictory. It is likely that the high dose of $1,25(OH)_2D_3$ caused an increase in bone turnover. The rate of bone loss due to trauma and immobilization could be increased under such conditions [13]. If, indeed, the skeletal reactions were accelerated by this treatment, the rate of fracture healing might also be expected to increase. The reason for our results might be that fracture healing primarily involves tissues other than bone, and also under these conditions, a selective increase in bone resorption might have occurred.

In order to use the rabbit as a model for the prednisolone-induced osteoporosis in humans, the doses have to be kept quantitatively comparable to human doses. Higher doses have frequently been given to rabbits and can cause serious disease such as diabetes, decreased body weight, and death due to various complications. These higher doses, however, cause a more rapid development of osteoporosis, whereas the low doses used in this experiment require several months to give a measurable loss of compact bone (own unpublished data).

After four months of prednisolone administration, the body weight was significantly lower than in controls and there was a low trabecular bone density indicative of osteoporosis. The total bone and ash density showed the lack of positive effect from $1,25(OH)_2D_3$ on this type of osteoporosis. The lack of prophylactic effect might be due to the different mineral metabolism in rabbits compared to rats and humans. It is possible that the calcium absorption is not a limiting factor during prednisolone treatment in the rabbit and therefore prednisolone may only affect the bone directly. Both from the theoretical aspect and from practical experience it seems that $1,25(OH)_2D_3$ counteracts the prednisolone effects, especially at the intestinal level [5, 14]. In this species, as might be anticipated from the earlier discussion, no such effect was seen from $1,25(OH)_2D_3$. This also indicates that no antagonism between $1,25(OH)_2D_3$ and prednisone exists at the bone level. Although there is reason to believe that the present results may not be applicable to human conditions, it must be remembered that other animal studies are similarly limited. For example, in the case of the rat, where bone is less prone to be resorbed from high doses of vitamin D, the risk for bone resorption is obscured.

In conclusion, $1,25(OH)_2D_3$ had undesirable effects on fracture healing, immobilization osteoporosis, and prednisolone osteoporosis in the rabbit. The results may be due to a higher sensitivity of rabbit bone to vitamin D and to a different mineral metabolism in the rabbit compared to rats and humans. The results emphasize that species difference must be taken into account in interpreting experimental studies.

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