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EFFECT OF 1α-HYDROXYVITAMIN D_3 ON CANCELLOUS BONE MATRIX

An Experimental Study on Adult Rats

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Two groups of adult male rats were treated perorally for 6 weeks with 0.1 μg and 1.0 μg of 1a-hydroxyvitamin D_3 (1a-OH-D_3), respectively. The effect of the treatment on cancellous bone matrix was studied by chemical analysis and morphometric measurements. The effect of the 1.0 μg dose on the inorganic composition, and on the calcification of the cancellous bone matrix, was significantly more pronounced, decreasing the amount of glycosaminoglycans. The lower dose level, 0.1 μg of 1a-OH-D_3, increased the collagen metabolism, whereas the higher dose level did not. The amount of cancellous bone determined morphometrically increased significantly during treatment with both dose levels. 1a-OH-D_3, converted in the organism to the hormonal form 1.25 $(OH)_2D_3$, induces new bone formation, probably by direct influence on the cancellous bone tissue itself.

Key words: 1a-hydroxyvitamin D_3; bone formation; dosage effect

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During the past few years vitamin D metabolites and their analogs have been available for use in experimental work and the treatment of metabolic bone diseases of various etiologies (Lindholm 1978, Lindholm & Sevastikoglou 1978, Lindholm et al. 1978, Lindholm 1979b). The analog 1a-hydroxyvitamin D_3 (1a-OH-D_3) is converted in the liver to the metabolite 1.25 $(OH)_2D_3$, which exerts its action directly through receptors in the actual bone tissue or via indirect mechanisms (Peacock et al. 1974, Kream et al. 1977a, b). As previously shown, 1a-OH-D_3 administered to adult male rats, reared on an ordinary laboratory diet, increased the bone mass and induced a change in the chemical composition of cortical bone (Lindholm et al. 1981). However, for the diagnosis and treatment of metabolic bone disease, a bone biopsy is usually taken from the iliac crest, where there is a preponderance of cancellous bone. Bone biopsy is supposed to be an excellent means of checking changes in the bone matrix brought about by treatment (Jowsey 1977).

We thus found it reasonable to study the changes in the different parameters of the cancellous bone matrix in adult rats after short-term treatment with 1a-OH-D_3.

MATERIAL

Adult male albino rats with a mean initial body weight of 492 g were used. The rats were divided into three randomized groups each containing 15 animals. One group of animals received 1 ml of propylenglycol perorally 5 days a week for 6 weeks. This group was used as a control; the animals in the other two groups received either 0.1 or 1.0 μg of 1a-OH-D_3, solubilized in 1 ml of propylenglycol, 5 days a week for 6 weeks.

All animals were kept on a diet consisting of 1.15 per cent calcium, 0.8 per cent phosphorus and 24.5 per cent protein per dry weight. The diet contained an average of 15 IU of vitamin D per 10 mg. The animals had free access to food and water.

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METHODS

At the end of the experimental period, the rats were sacrificed by exsanguination under ether anesthesia, and whole blood was collected. The *total serum calcium* was determined by atomic absorption flame photometry after precipitation of protein with trichloro-acetic acid. For biochemical analysis the hind leg bones were carefully cleaned of muscular and connective tissue. A bone cylinder measuring about 4 mm in length was excised with a saw from the proximal tibia. This piece of bone consisted mainly of cancellous matrix with a thin outside border of cortical bone. The bone pieces were carefully cleaned in distilled water to remove bone marrow. All the samples were frozen in liquid nitrogen and subsequently lyophilized. The dried spongeous bones were adapted for analyses of *calcium, phosphorus, magnesium, collagen, glycosaminoglycans* and *nucleic acids* as previously described (Lindholm et al. 1981).

The proximal parts of the femurs were cut off and embedded in methylmethacrylate. Sections were taken through the line between the major and minor trochanteric notches. The sections were measured and found to be about 80 μm thick. They were photographed on Kodak spectroscopic plates. The *microradiographs* were examined under a Zeiss microscope and microphotographs were obtained, for measurement of the bone area. The morphometric estimation of trabecular bone area was performed by the point-counting method (Weibel 1973). The *bone area* is given as the percentage of the area occupied by cancellous bone.

The *statistical evaluation* of the results was performed using Student's *t*-test. The significance levels were: $P > 0.05$ = non significant (N.S.), $P < 0.05$ = almost significant (x), $P < 0.01$ = significant (xx) and $P < 0.001$ highly significant (xxx).

RESULTS

The rats withstood the treatment without developing complications and had a mean *final body weight* of 543 g. *Serum calcium* measured at sacrifice was significantly higher ($P < 0.001$) in the group treated with 1.0 μg of 1a-OH-D_3 than in the control group, whereas the lower dose level did not raise the serum calcium level significantly (Table 1).

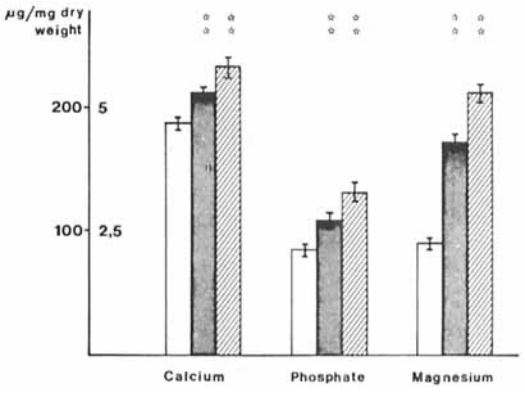


*Figure 1. Amounts of inorganic bone components. The values are given as μg/mg dry weight ± SEM. The left scale indicates amount of calcium or phosphorus while the right scale gives the amount of magnesium. Empty bars denote untreated rats, filled bars rats treated with 0.1 μg 1a-OH-D_3, hatched bars rats treated with 1.0 μg 1a-OH-D_3. The asterisks indicate significance level of the difference between untreated and 1a-OH-D_3 treated groups using Student's t-test, * denoting $P < 0.05$ and ** denoting $P < 0.01$. Each experimental group consists of 15 male adult rats.*

The content of *inorganic compounds* increased as a result of the treatment with 1a-OH-D_3 and was even more pronounced in the group given the higher dose (Figure 1). In all cases the increment was significant ($P < 0.01$) between the control group and the treated groups as well as between the treated groups reciprocally. The *calcium/phosphorus* ratio remained comparatively unchanged (Table 2) which indicates that the increment was caused by an increased amount of calciumhydroxyapatite.

The amounts of *hydroxyproline* and *nitrogen*

Table 1. Total serum calcium in the different groups at sacrifice

	Mean value mmol/l	S.D.	Significance (compared to untreated group)	Significance (between treated groups)
Untreated	2.63	0.08		
0.1 μg of 1a-OH-D_3	2.68	0.67	N.S.	$P < 0.05$
1.0 μg of 1a-OH-D_3	2.92	0.12	$P < 0.001$	

Table 2. Ratios between bone constituents in the 1a-OH-D_3 treated and untreated groups

		Mean value	S.D.	Significance (compared to untreated groups)	Significance (between treated groups)
Calcium/Phosphorus	Untreated	2.019	0.421		
	0.1 μg of 1a-OH-D_3	1.925	0.322	N.S.	
	1.0 μg of 1a-OH-D_3	1.753	0.354	N.S.	N.S.
Calcium/Hydroxyproline	Untreated	8.063	1.318		
	0.1 μg of 1a-OH-D_3	7.975	1.043	N.S.	
	1.0 μg of 1a-OH-D_3	10.061	1.991	$P<0.01$	$P<0.01$
Hydroxyproline/Nitrogen	Untreated	0.633	0.057		
	0.1 μg of 1a-OH-D_3	0.681	0.130	N.S.	
	1.0 μg of 1a-OH-D_3	0.783	0.156	$P<0.01$	$P<0.05$

increased ($P < 0.05$) with the lower dosage but remained quite unchanged with the higher dose level, which indicates a largely unchanged amount of matrix (Figure 2).

The content of *hexosamines* was significantly lower in the treated groups than in the controls and this was also the case for *uronic acid* and *neutral sugars*, but their quantitative decrease was more pronounced for the higher dose of 1a-OH-D_3. The differences were all significant ($P < 0.01$) (Figure 3).

The *calcium/hydroxyproline* ratio considered to represent the degree of calcification was significantly ($P < 0.01$) elevated in the group treated with the higher dose of 1a-OH-D_3 while the lower dose gave a non significant difference compared with the controls (Table 2).

The *hydroxyproline/nitrogen* ratio also showed an increase for the vitamin treated groups, although it was significant ($P < 0.01$) only for the higher dose, while the *hexosamines/hydroxyproline* ratio showed a somewhat different pattern with a decrease ($P < 0.001$) in the 1a-OH-D_3 treated groups (Table 3), mainly accounted for by the decrease in *hexosamines* as shown in Figure 3. The *hexosamines/DNA* ratio also exhibited a significant ($P < 0.01$) decrease in the treated groups.

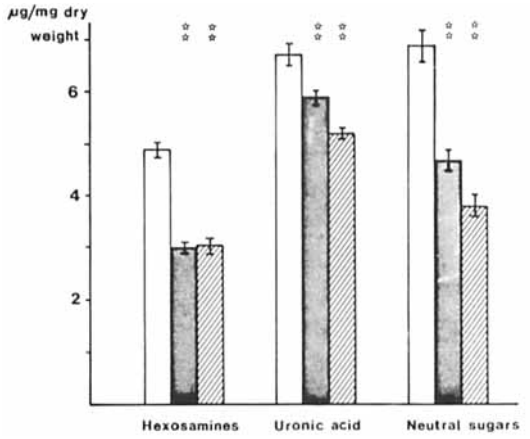


Figure 3. Amounts of hexosamines, uronic acid and neutral sugars. For symbols, see legend to Figure 1.

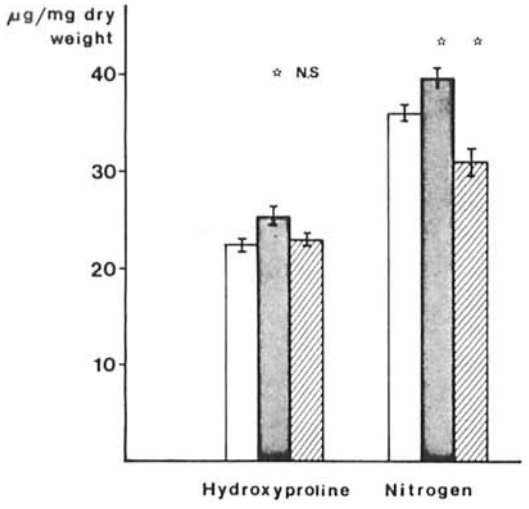


Figure 2. Amounts of hydroxyproline and nitrogen. For symbols, see legend to Figure 1.

Table 3. Ratios between bone constituents in the 1a-OH-D_3 treated and untreated groups

		Mean value	S.D.	Significance (compared to untreated group)	Significance (between treated groups)
Hexosamines/ Hydroxyproline	Untreated	0.204	0.040		
	0.1 μg of 1a-OH-D_3	0.115	0.016	$P<0.001$	
	1.0 μg of 1a-OH-D_3	0.132	0.025	$P<0.001$	$P<0.05$
Hexosamines/DNA	Untreated	3.086	1.172		
	0.1 μg of 1a-OH-D_3	1.581	0.461	$P<0.01$	
	1.0 μg of 1a-OH-D_3	1.115	0.345	$P<0.01$	$P<0.01$
RNA/DNA	Untreated	0.318	0.201		
	0.1 μg of 1a-OH-D_3	0.296	0.061	N.S.	
	1.0 μg of 1a-OH-D_3	0.150	0.044	$P<0.01$	$P<0.01$

Table 4. Morphometric estimation of trabecular bone area of untreated and 1a-OH-D_3 treated rats

		Mean value %	S.D.	Significance (compared to untreated groups)	Significance (between treated groups)
Trabecular bone area	Untreated	23.0	4.5		
	0.1 μg of 1a-OH-D_3	29.7	3.8	$P<0.001$	
	1.0 μg of 1a-OH-D_3	28.8	5.2	$P<0.01$	N.S.

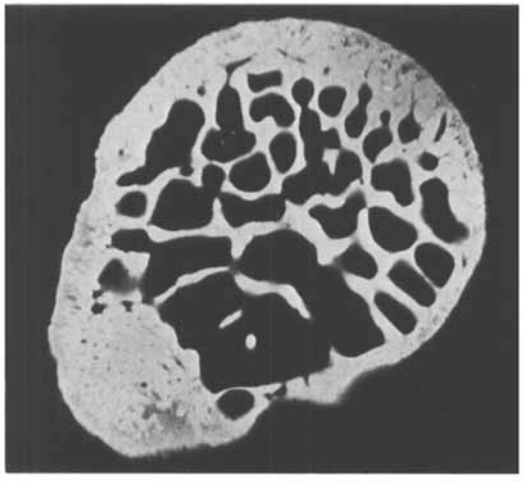
The ratio between the two types of *nucleic acids* showed a tendency towards a more pronounced difference which was significant ($P < 0.01$) for the group treated with the 1.0 μg dose. There was a slight quantitative increase in *DNA*, while *RNA* remained largely unchanged (not shown in table).

The morphometric measurements using the point-counting method for estimating the area of trabecular bone showed a clearly significant ($P < 0.01$) increase in the 1a-OH-D_3 treated groups compared with controls, whereas the difference between the treated groups was not significant (Table 4).

DISCUSSION

Vitamin D may directly influence the production of bone matrix, and bone resorption and calcification. The direct effect on the production of bone matrix (Canas et al. 1969, Corvol et al. 1978, Raisz et al. 1978) and the effect on bone resorption (Raisz et al. 1972, Reynolds et al. 1974, Hermann-Erle & Gaillard 1978) has been previously demonstrated. Even an effect on bone ossification in animals (Henry & Norman 1978) has been shown and in the treatment of patients suffering from uremic bone disease vitamin D has been reported to increase the formation of mineralized bone measured both at the cellular level and as bone formation rates (Melsen & Mosekilde 1978, Mosekilde & Melsen 1978).

In this experiment we demonstrated the effect of two dose levels, i.e. 1.0 μg and 0.1 μg, of 1a-OH-D_3 on the formation of cancellous bone matrix. The doses were administered for 6 weeks to adult male rats reared on a standard laboratory diet. The daily oral dose of 1.0 μg of 1a-OH-D_3 significantly increased the absolute amount of inorganic compounds, as did the lower dose level of 0.1 μg per day, but at the same time significantly decreased the amount of glycosaminoglycans. However, the low dose of 0.1 μg also increased



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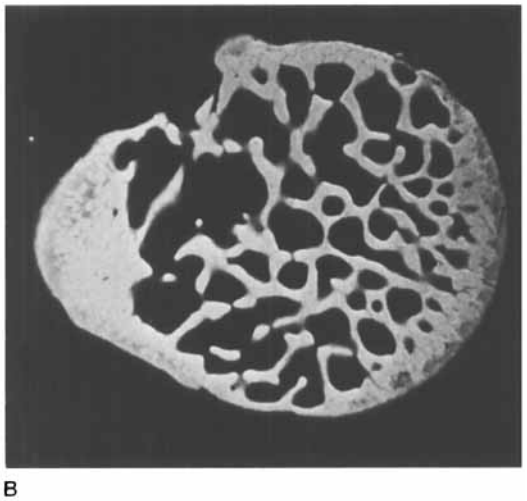

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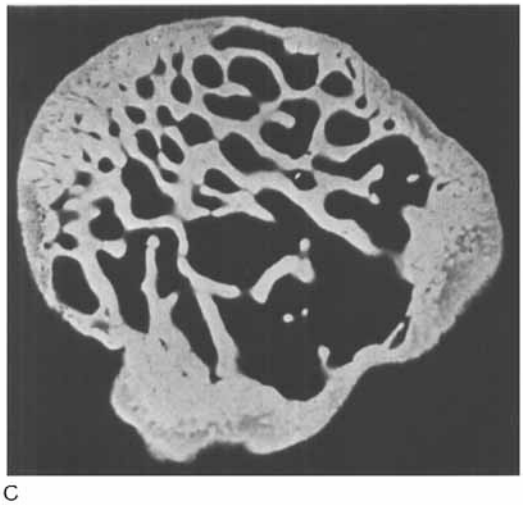

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almost significantly the amount of hydroxyproline and nitrogen of cancellous bone which was contrary to the effect of the high dose of 1.0 μg. From other studies moreover we know that vitamin D influences the biosynthesis of collagen and the nature of cross-linking (Barnes & Lawson 1978).

The dose level of 1.0 μg caused a significant increase in calcification of cancellous bone matrix (Hydroxyproline/Nitrogen, Calcium/Nitrogen) which was not the case with the lower dosage, although the effect on the area of trabecular bone was somewhat more pronounced with the lower dose level. The same correlation can be observed for the RNA/DNA ratio. The ratios of Hexosamines/Hydroxyproline and Hexosamines/DNA diminished to the same extent for both dose levels of 1a-OH-D_3. However, in a previous experiment it was shown that a daily dose of 0.09 μg increased the ash content, the bone volume and the thickness of cortical bone more definitely than did a dose of 0.9 μg, and, furthermore, that the content of hydroxyproline increased with the lower dose level, whereas that of nitrogen remained unchanged. The amount of calcium and the calcium/nitrogen ratio were, however, affected to a greater extent by the dose of 0.9 μg, while the effect on glycosaminoglycans was identical with that in the cancellous bone matrix. Thus, small amounts of 1a-OH-D_3 may increase measured parameters of cortical bone mass and mineral to a greater extent, while higher dose levels may exert a more pronounced effect on the chemical composition of the cancellous bone matrix, although the differences are rather small and may be due to variations in experimental conditions (Lindholm et al. 1981). In other experiments, too, 1.25 $(OH)_2D_3$ was observed to induce hypertrophy and hyperplasia of osteoblasts without increasing the amount of osteoclasts in adult male rats reared on an optimal calcium diet. Thus, 1.25 $(OH)_2D_3$ may be able to selectively stimulate osteoblastic activity without increasing bone resorption (Weisbrode et al.

Figure 4. Microradiographs of bone sections used for the estimation of trabecular bone area: (A) Untreated (control); (B) 0.1 μg 1a-OH-D_3; (C) 1.0 μg 1a-OH-D_3.

1979). 1a-OH-D_3 which is converted to 1.25 $(OH)_2D_3$ in the liver may be useful in treating certain osteopenic conditions such as osteoporosis (Lindholm 1979a, b).

There is some evidence that the production of other metabolites, e.g. 24,25$(OH)_2D_3$ may be stimulated by 1.25$(OH)_2D_3$, and therefore some of the effects of 1.25$(OH)_2D_3$ may be mediated by endogenous synthesis of 24.25$(OH)_2D_3$ or other metabolites of vitamin D (Taylor 1979).

Recently, 1.25$(OH)_2D_3$ binding proteins have been identified in human and avian parathyroid glands (Haddad et al. 1976, Hughes & Haussler 1978) as well as in rat intestine (Kream et al. 1977a) and bone (Kream et al. 1977b). The cytosol binding protein for 1.25$(OH)_2D_3$ apparently performs a receptor function, reminiscent of those characterized for steroid binding proteins in their target tissue (Haddad 1979).

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