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Effect of Vitamin D on Pregnant Rabbits and Their Offspring

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Summary

The morphologic and biochemical effects of vitamin D in pregnant rabbits and their offspring were studied. Four groups of four pregnant does were given 100,000 IU, 10,000 IU, and 1,000 IU vitamin D_2 or placebo im during pregnancy. Prestudy serum Ca, P, 25-hydroxy vitamin D (25-OHD), and cholesterol levels were not different between the control and vitamin D-supplemented does. At midgestation, the vitamin D-supplemented dose had significantly higher serum Ca levels than did controls (16.4 ± 0.6, mean ± SE, vs. 15.3 ± 0.4 mg/dl), higher serum P than control, (6.41 ± 0.06 vs. 4.50 ± 0.54 mg/dl), higher serum 25-OHD than controls (39 ± 6 vs. 21 ± 2 ng/ml), and higher serum cholesterol than controls (64.0 ± 17.2 vs. 27.5 ± 8.2 mg/dl, Wilcoxon rank, $P < 0.05$). At term, the vitamin D-supplemented does had significantly higher serum Ca than controls (15.2 ± 0.6 vs. 12.3 ± 0.10 mg/dl), higher serum P than controls (5.06 ± 0.48 vs. 2.96 ± 0.55 mg/dl), and higher serum 25-OHD levels than controls (50 ± 5 vs. 35 ± 5 ng/ml, Wilcoxon rank, $P < 0.05$). Two maternal does from the 100,000 D group that had serum 25-OHD levels greater than 100 ng/ml developed aortic medical calcifications. The 100,000 vitamin D does had a significantly higher number of abortions, 6 of 26 pregnancies, compared with control, 0 of 27 pregnancies (χ^2, $P < 0.01$). In the newborns, only the 10,000 D group had higher serum ionized Ca, 6.21 ± 0.28 vs. 5.26 ± 0.06 mg/dl in controls (Wilcoxon rank, $P < 0.001$). There were no significant differences in newborn 25-OHD and cholesterol levels among the four groups. Newborn aortic root showed supravalvular lesions in 2 of 11 newborns in the 10,000 D group (χ^2, $P < 0.05$) and 6 of 20 newborns in the 100,000 D group (χ^2, $P < 0.01$) compared with no lesions in the controls (0 of 27). No supravalvular lesions were found in the 1,000 D group. Thus, high doses of vitamin D during pregnancy affect fetal death, maternal calcium and cholesterol homeostasis, neonatal calcium homeostasis, and cause calcific aortic lesions in the mother and an apparent dose-related development of supravalvular aortic lesions in the newborn.

Speculation

Maternal vitamin D toxicity during pregnancy contributed to the development of supravalvular aortic lesions in the fetus which might lead to the infantile hypercalcemia syndrome. Vitamin D might have a direct toxic effect on maternal and fetal vascular areas of high turbulence flow.

Large doses of vitamin D may lead to deranged calcium (Ca) and fat metabolism (6, 7, 14). In offspring of pregnant rabbits that have been treated with large doses of vitamin D, there are lesions in the supravalvular aortic area thought to be similar to the lesions associated with infantile hypercalcemia (11). Clinically, the infantile hypercalcemia syndrome reached epidemic proportions in England following World War II when dietary vitamin D supplementation was increased (9, 25), and it has been suggested that vitamin D toxicity or sensitivity may be a factor in the pathogenesis of this condition (1, 2, 9–11, 25).

We undertook the present study to determine whether there was a dose relationship of vitamin D toxicity in Ca homeostasis and the development of supravalvular aortic lesions in pregnant rabbits and their offspring in association with alterations in Ca homeostasis.

MATERIALS AND METHODS

Sixteen adult New Zealand White rabbits were randomly divided into four experimental groups of four rabbits each. Each doe was mated twice with 2 of a pool of 10 healthy New Zealand White bucks. The initial mating pairs were determined randomly but in subsequent pairings, no buck was mated with more than one doe from an experimental group. Within 4 hr of her initial mating, each doe was given 1 mg pituitary luteinizing hormone (Armour-Baldwin Laboratories) iv to ensure ovulation.

The four treatment groups received placebo, or 1,000 U vitamin D_2 (Calciferol, vitamin D_2, 500,000 USP U/ml in sesame oil), or 10,000 U vitamin D_2, or 100,000 U of D_2. Vitamin doses were given im every other day for 14 doses. The total vitamin D doses given were 14,000, 140,000, and 1.4 million U during the course of pregnancy. Does were housed in an individual wire cage under identical environmental conditions during the gestation period. Each doe was fed a routine stock diet (Purina laboratory rabbit chow).

Maternal blood samples for ionized Ca (iCa), total Ca, P, Mg, 25-OHD, and cholesterol were drawn by cardiac puncture three times: prestudy, midgestation (14 days after mating), and term (before anesthesia for Cesarean section). Weights were also measured immediately prior to each blood collection.

At term, each doe was anesthetized with 20 mg sodium thiopental/kg and Cesarean sections were performed. Each newborn rabbit was immediately weighed and decapitated. Newborn blood samples were placed immediately in 5% CO_2-filled tubes for iCa, cholesterol, and 25-OHD determinations. Following delivery each doe was killed.

Postmortem cardiovascular examinations were performed on each doe and her offspring. Gross inspection of the hearts and ascending aortas of the mother rabbits was supplemented by a single longitudinal microscopic section that included myocardium, aortic valve ring, and aortic root. Gross inspection of the newborn rabbits' hearts was not performed because of their size. A longi-

tudinal apical-basilar slice that included ascending aorta was embedded and examined by a modified step section technique that provided histologic sections at various levels through the aortic root. All tissue taken for histologic examination was fixed in 10% buffered formalin, embedded in paraffin, and routinely stained with hematoxylin and eosin. Verhoff-Van Geison, Alcian blue, Von Kossa, and Masson trichrome stains were also used in selected cases.

STATISTICAL METHODS

Statistical analyses included χ^2 and regression (24). Nonparametric tests were used with data that were not normally distributed. Because of the small number of does in each experimental group, the data of the vitamin D-supplemented does were analyzed together and compared with the control does.

LABORATORY DETERMINATIONS

Ionized Calcium (iCa). Serum iCa levels were determined with a Ca selective flow-through electrode (Orion SS-20). Sample collecting and processing were performed as previously described (27), except that mercury and triethanolamine were omitted. Our 95% confidence limits for the true value in any sample is ±0.12 mg/dl. Standardization was accomplished through the use of serum and aqueous standards.

25-OHD. 25-OHD levels were determined according to the competitive binding radioassay of Belsey *et al.* (3). This assay used a specific binding protein isolated from rachitic rat serum, which has a high affinity for 25-OHD. Some investigators have proposed that other vitamin D metabolites might interfere with this assay without preparative chromatographic separation, and the 25-OHD values would be higher than the true concentrations, but the amount and degree of interference remain unclear (12, 17). The intra- and interassay coefficient of variation was 10%. The limit of sensitivity of this method was 5 ng/ml. Normal human values from our laboratory ranged from 20–80 ng/ml.

Cholesterol. Total serum cholesterol was determined by a gas-liquid chromatographic method, using 5-α-cholestane as the internal standard (15). The intraassay coefficient of variation was 1.2% and the interassay coefficient of variation was 2%. In 131 samples, cholesterol values obtained by the automatic colorimetric method and by gas-liquid chromatography were highly correlated (r = 0.99, $P < 0.001$) over a range in cholesterol values from 44–468 mg/100 ml (15). The limit of sensitivity of the gas-liquid chromatographic method was 10 mg/100 ml.

OTHER CHEMICAL DETERMINATIONS

Serum total Ca and Mg were determined by atomic absorption spectrophotometry (28) and serum P according to the method of Fiske and Subbarrow (18).

RESULTS

DOES

Mortality and Morbidity (Table 1). During the study, there were three maternal deaths consisting of one doe from each vitamin D group, except the control does. The doe from 1,000 and 10,000 D groups died after the blood drawing procedure at midgestation. The doe from the 100,000 D group was found dead near term and the death was unexplainable, despite autopsy examination.

Before the study, all maternal body weights were similar between controls and vitamin D groups. Mean maternal weight was

Table 1. *Mortality and morbidity of treatment groups*

Treatment	No.	Conceived	Maternal deaths	Abortions	Average maternal weight gain (kg)	Newborns
Control	4	3/4	0	0	0.36 ± 0.03[1]	27
1,000 D	4	3/4	1[2]	0	0.12 ± 0.02	13
10,000 D	4	3/4	1[2]	0	0.22 ± 0.12	11
100,000 D	4	4/4	1	6	0.04 ± 0.22	20

[1] Mean ± SE.
[2] Cardiac tamponade.

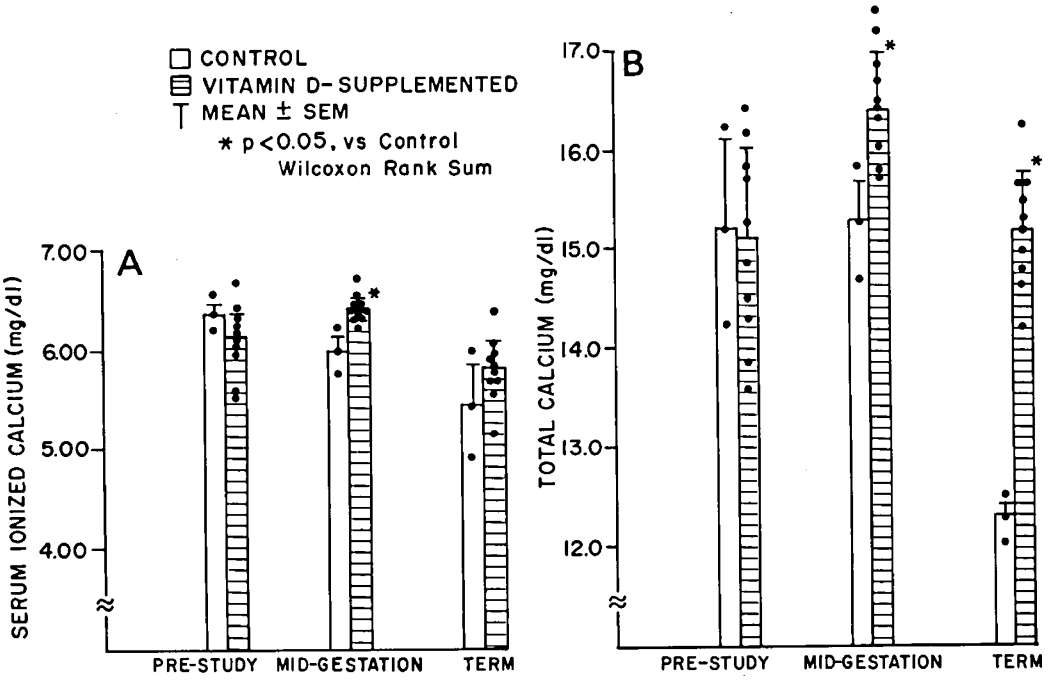

Fig. 1. *A:* Effects of vitamin D doses on iCa during pregnancy with rabbits given either placebo or vitamin D_2 every other day im. Prestudy values were taken 1 day postmating, midgestation at 14 days postmating, and term, 30 days postmating. Mean values and SEM are shown; P values are for Wilcoxon rank sum test. Prestudy iCa levels were not different between the two groups. At midgestation the vitamin D-supplemented groups had higher iCa levels than did controls. At term there were no differences. *B:* maternal total Ca during pregnancy. Prestudy total Ca levels were not different between the two groups. At midgestation and at term, the vitamin D-supplemented groups had significantly higher total Ca levels than did controls.

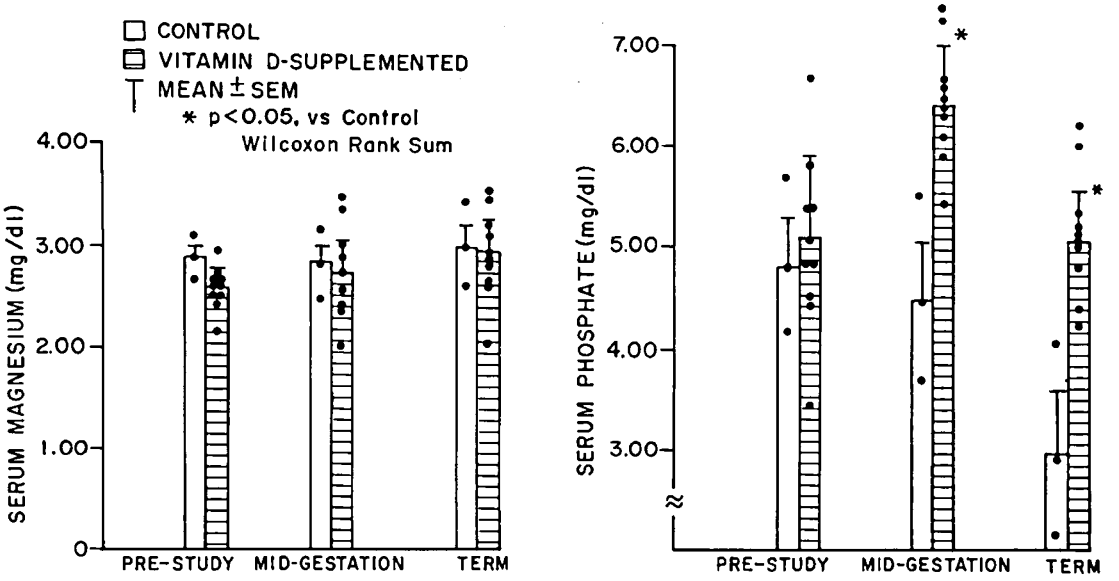

Fig. 2. Maternal serum Mg and P during pregnancy. There were no differences between the two groups in serum Mg at prestudy, midgestation, or term. Prestudy P levels were not different between the two groups. At midgestation and term the vitamin D-supplemented group had significantly higher serum P levels than controls (scheme as in Fig. 1*A*).

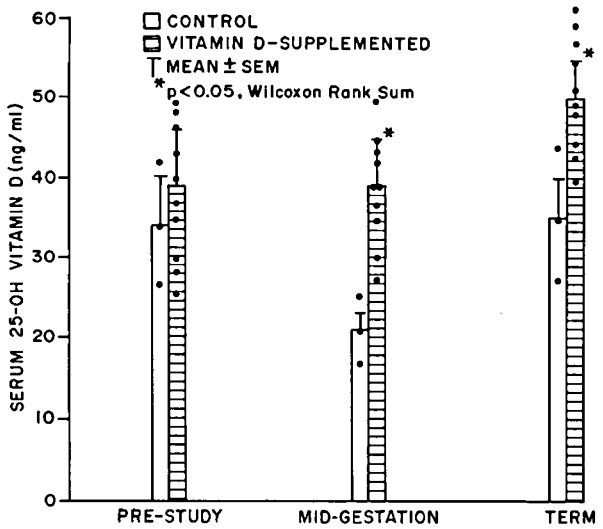

Fig. 3. Maternal serum 25-OHD during pregnancy. Prestudy serum 25-OHD levels were not different between the two groups. At midgestation and term the vitamin D-supplemented groups had significantly higher serum 25-OHD levels than did controls (scheme as in Fig. 1*A*).

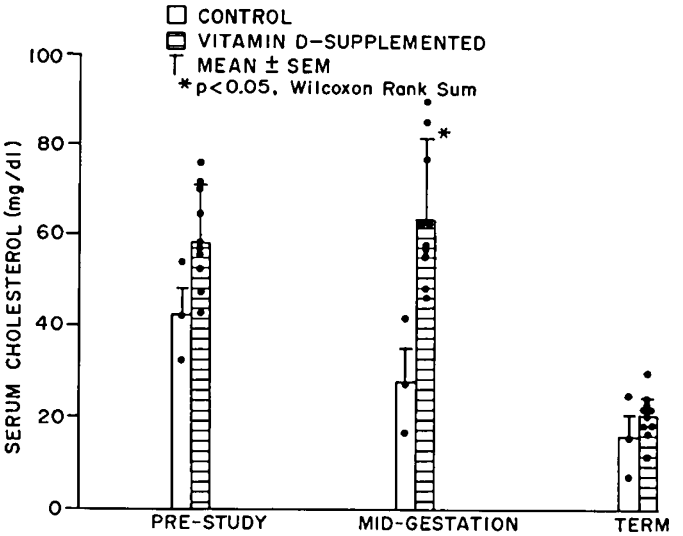

Fig. 4. Maternal serum cholesterol during pregnancy. Prestudy serum cholesterol levels were not different between the two treatment groups. At midgestation, the serum cholesterol level was significantly higher in the vitamin D-supplemented group compared with controls. At term there were no differences (scheme as in Fig. 1*A*).

4.03 kg (range 3.28–4.70 kg). By term the vitamin D-supplemented groups had gained less weight, 0.16 ± 0.09 kg (mean ± SE) compared with the control pregnant does, 0.37 ± 0.03 kg. (Wilcoxon rank sum, $P < 0.05$).

BIOCHEMICAL EFFECTS

Ionized Ca and Total Ca (Fig. 1). Prestudy ionized and total Ca were not different between the control and treatment groups. At midgestation, the vitamin D supplemented group had higher iCa (6.41 ± 0.09 mg/dl) than did controls (5.98 ± 0.11 mg/dl) (Wilcoxon rank sum, $P < 0.05$). At term, there were no differences in iCa between the control or vitamin D groups.

At midgestation the vitamin D-supplemented groups had higher total Ca than did controls, 16.4 ± 0.6 vs. 15.3 ± 0.4 mg/dl (Wilcoxon rank sum, $P < 0.05$). By term, the vitamin D-supplemented groups had higher total Ca (15.2 ± 0.6 mg/dl) than did control does at term, 12.3 ± 0.10 mg/dl (Wilcoxon rank sum, $P < 0.05$).

Mg and P (Fig. 2). There were no differences between the control and vitamin D groups in serum Mg at either prestudy, midgestation, or term.

Prestudy P levels were not different between the control and vitamin D groups. At midgestation, the vitamin D groups had a significantly higher serum P than did controls, 6.41 ± 0.06 mg/dl vs. 4.50 ± 0.54 mg/dl (Wilcoxon rank sum, $P < 0.05$). At term the vitamin D groups had significantly higher serum P than did controls, 5.06 ± 0.48 vs. 2.96 ± 0.55 mg/dl (Wilcoxon rank sum, $P < 0.05$).

When serum Ca, iCa, and P levels were analyzed for each treatment group ($n = 3$ or 4 each) and compared with controls, no consistent significant differences were obtained.

25-OHD (Fig. 3). At prestudy there were no significant differences in serum 25-OHD levels between the treatment groups as compared with control. At midgestation and term, the vitamin D-supplemented groups had significantly higher serum 25-OHD levels as compared with controls, 39 ± 6 and 50 ± 5 ng/ml vs. 21 ± 2 and 36 ± 5 ng/ml, respectively (Wilcoxon rank sum, $P < 0.05$). There were two maternal does that had a term level of serum 25-OHD greater than 100 ng/ml in which aortic medial calcification also developed. All other serum 25-OHD levels were less than 60 ng/ml. Maternal vitamin D dose was not related to maternal Ca or 25-OHD levels at midgestation or term.

Cholesterol (Fig. 4). At prestudy and term, there were no significant differences in serum cholesterol levels between the vitamin D group as compared with the control group. At midgestation serum cholesterol in the vitamin D groups were significantly increased compared with controls, 64.0 ± 17.2 vs. 27.5 ± 8.2 mg/dl (Wilcoxon rank sum, $P < 0.05$). There was no correlation of maternal serum cholesterol and maternal vitamin D dose.

Morphologic Examination of Maternal Hearts and Ascending Aortas (Fig. 5). Gross and histologic examinations of heart and aortic roots were normal in the control, 1,000 D, and 10,000 D groups. One maternal doe from the 100,000 D group had numerous calcific plaques along the aorta on gross examination. Histologic sections revealed randomly scattered calcifications of the media of the ascending aorta in this and another rabbit from the 100,000 D group.

NEWBORN RABBITS

Abortions (Table 1). The 100,000 vitamin D dose group had a significantly higher number of abortions (6 of 26 pregnancies) compared with no abortions (0 of 51 pregnancies) in the other three treatment groups (χ^2, with Yate's correction compared with control 0 of 27, $P < 0.01$). Because of severe maceration, no histologic examinations were performed on any of the abortuses.

Litter Size and Newborn Weights (Table 1). There were 27 newborns that delivered in the control group of 3 does, 13 from the 1,000 D group with 1 doe failing to conceive, 11 newborns from the 10,000 D group with one doe failing to conceive, and 20 newborns were delivered from the 100,000 D group of 3 surviving does. There were no differences in newborn weights and placenta weights among the four groups. There were no correlations between newborn weights and maternal D dose. The number of fetal resorptions was not different in the four groups.

Ionized Ca (Fig. 6). Newborns from the 10,000 D group had significantly higher serum iCa, 6.21 ± 0.28 mg/dl vs. control

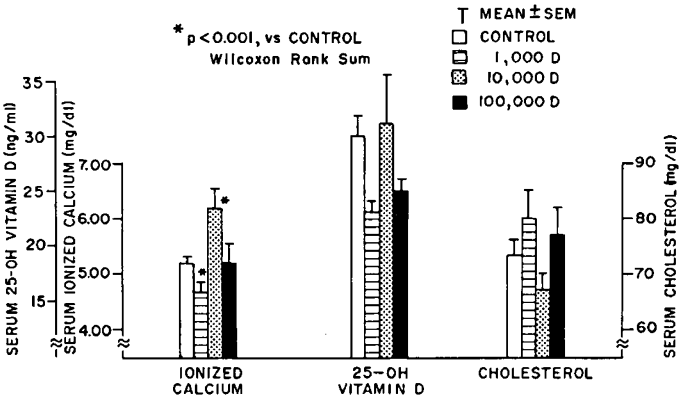

Fig. 6. Newborn serum ionized Ca, 25-OHD, and cholesterol levels. Serum iCa was significantly higher from newborns of mothers given 10,000 D compared with controls. Newborns from 1,000 D group had significantly lowered iCa compared with controls. Newborn serum 25-OHD and cholesterol levels were not significantly different among the four treatment groups (scheme as in Fig. 1*A*).

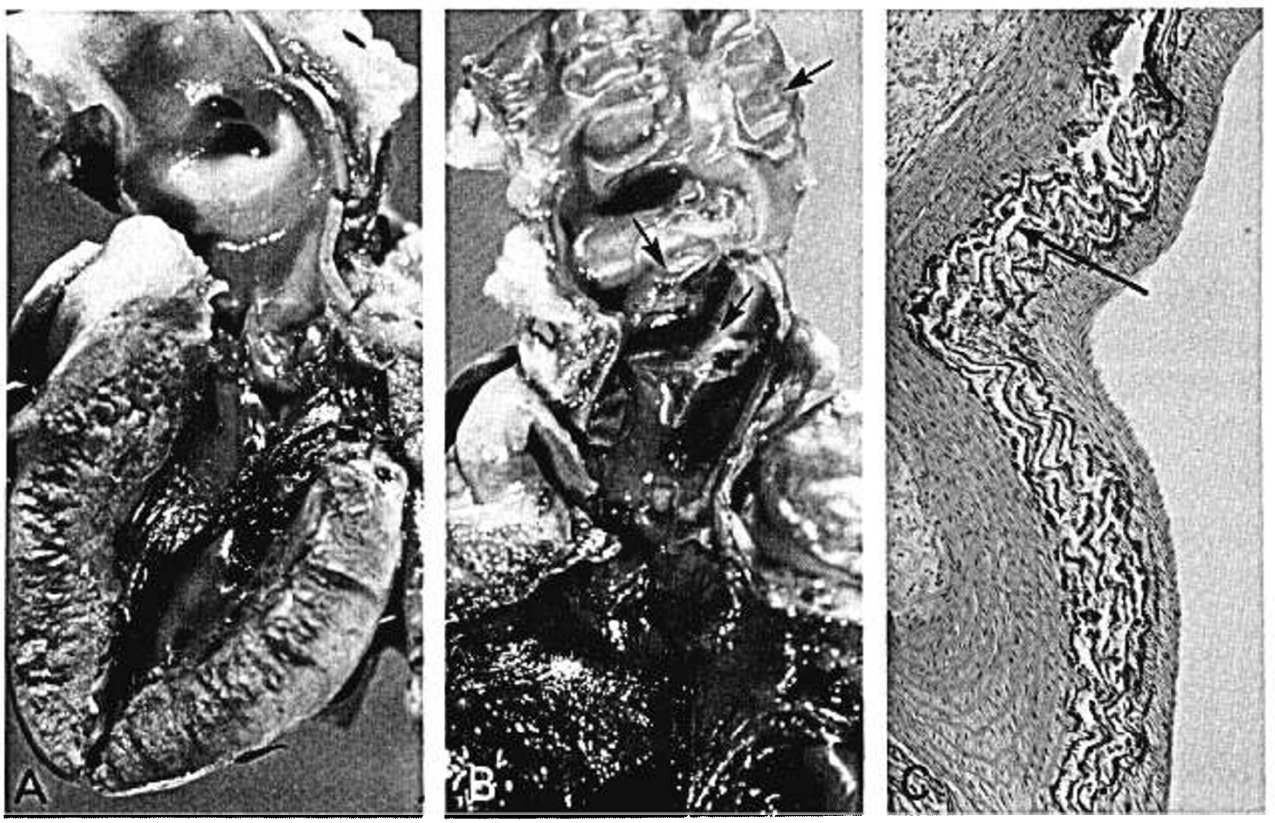

Fig. 5. Gross and microscopic appearance of aortic roots of maternal rabbits. *A:* Aorta of control maternal rabbit is grossly smooth and without lesions. *B:* Aorta of 100,000 D rabbit has numerous calcific plaques (*arrows*) on gross examination. *C:* randomly scattered calcifications of the media of the ascending aorta. Hematoxylin and eosin. ×65.

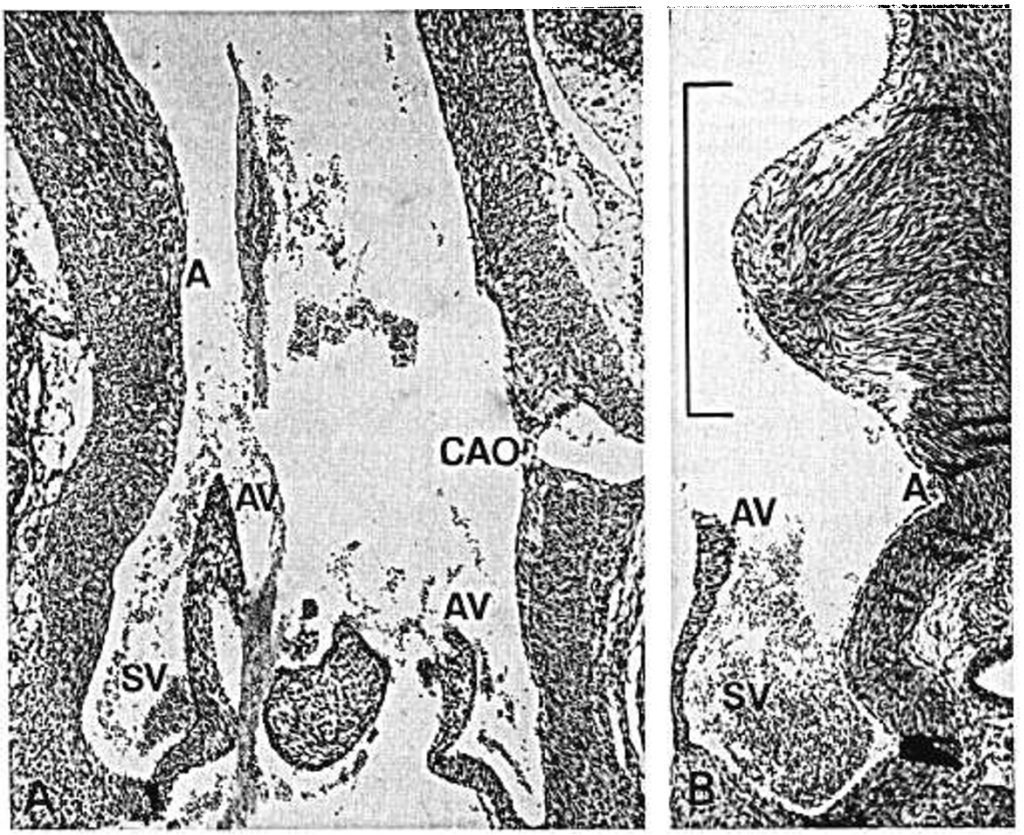

Fig. 7. *A:* aortic root of a control newborn with normal anatomical structures: aortic valves (AV), sinus of Valsalva (SV), coronary artery ostium (CAO), and ascending aorta (A). The aorta is smooth walled beyond the sinuses of Valsalva. Hematoxylin and eosin. ×65. *B:* Aorta of a newborn, of which the mother was given 100,000 IU vitamin D_2, shows a prominent protrusion impinging on the lumen in the region above the aortic valves (AV) and sinus of Valsalva (SV). The lesion (*bracketed*) consists of proliferation of intimal and medial components of the aorta. Hematoxylin and eosin. ×64.

Table 2. *Supravalvular aortic lesions in newborn rabbits of mothers given vitamin D*

Treatment	Supravalvular aortic lesions in the newborn	χ^2[1]
Control	0/27	
1000 D	0/13	
10,000 D	2/11	$P < 0.05$
100,000 D	6/20	$P < 0.01$

[1] χ^2 vs. control.

newborn 5.23 ± 0.06 mg/dl (Wilcoxon rank sum, $P < 0.001$). Newborns from the 1,000 D group had significantly lowered iCa, 4.66 ± 0.13 mg/dl compared with controls, 5.23 ± 0.06 mg/dl. There was no difference between the 100,000 D group newborn and controls, 5.24 ± 0.14 vs. 5.23 ± 0.06 mg/dl. There was no relation of newborn iCa and maternal dose of vitamin D.

Newborn 25-OHD and Cholesterol (Fig. 6). There were no significant differences in newborn 25-OHD levels among the four groups. Newborn serum cholesterol levels were not significantly different among the four groups.

Newborn Morphologic Examination of the Hearts and Ascending Aortas (Fig. 7 and Table 2). Histologic sections through the aortic root revealed supravalvular lesions in 6 of 20 newborns in the 100,000 D group (χ^2, $P < 0.01$) and 2 of 11 newborns in the 10,000 D group (χ^2, $P < 0.05$) compared with no supravalvular lesions in 27 controls. No lesions were found in the 1,000 D group.

In order to better define the location of the lesion, two hearts and ascending aortas from the 100,000 D group were serially sectioned at 6-μm intervals in cross-section. One of these hearts was found to have a lesion that was located just above the aortic valve insertion and sinuses of Valsalva and consisted of a proliferation of the intimal and medial layers of the aorta and protruded into the lumen. Under higher magnification, the lesion was characterized by cellular disarray of smooth muscle cells and fibroblasts with occasional mitotic figures. Special stains for Ca and mucopolysacharide were negative. Stains for elastic tissue were also normal.

DISCUSSION

In the present study, large doses of vitamin D during pregnancy affected fetal deaths. There was a significant number of abortions (6 of 26 pregnancies) in the high-dose vitamin D-treated group, compared with no abortions in 51 pregnancies in the other 3 groups (2 lower dose vitamin D, one control). In rats a dose of 20,000 IU D_2 given to the mother had been shown to damage follicle maturation and impede fertilization and implantation (19, 20). Hypercalcemia in the rat produced by hypervitaminosis D has been thought to interfere with fertilization by producing fallopian tube spasm (16).

Vitamin D increases extracellular Ca and P by increasing intestinal absorption of Ca and P through mobilization of these minerals from bone. At midgestation on day 15 (3) and at term, the vitamin D-supplemented does had significantly higher serum Ca, serum P, and 25-OHD levels than did the controls. In the present study, there were two maternal rabbits that developed calcified lesions in the media of their ascending aorta. Both of these rabbits were from the 100,000 D group. Although their serum Ca and P were normal, the serum 25-OHD levels in both affected rabbits were in a toxic range at term (>100 ng/ml). It is unclear whether Ca or vitamin D, or both, are involved in the pathogenesis of the medial lesions.

During pregnancy, there appears to be a protective resistance to hypervitaminosis D. Increased production of estrogen, progester-

one, and cortisone during pregnancy might be protective because of their effects in lowering serum Ca (4, 21–23). The failure to detect very high levels of 25-OHD in the vitamin D group may be due to the inhibition of hepatic 25-hydroxylation by massive doses of vitamin D (5). In human pregnancy, hepatic microsomal enzymes might be induced which may further increase the metabolism of 25-OHD (8).

In newborns of the present study, only the 10,000 D group had higher serum Ca levels than did controls. Neonatal serum 25-OHD levels were not different in the four groups. The lack of correlation between vitamin D dose and the newborn 25-OHD levels may be due to the interference of vitamin D metabolites and the possibility that vitamin D serum levels might not accurately reflect tissue levels. Also im injections of oily solutions of vitamin D may be absorbed erratically in the does and this may account for apparent lack of correlation between maternal and newborn 25-OHD levels. However, there was an apparent dose-related risk in the development of supravalvular aortic lesions in the newborn rabbits; only the newborns of mothers receiving 10,000 and 100,000 D during pregnancy developed the supravalvular lesions. These lesions were similar to the ones described by Friedman and Mills (10) and Friedman and Roberts (11) which theoretically might lead to infantile supravalvular aortic stenosis. Since these lesions occurred in an area of high turbulence it appeared that these lesions were flow induced. Vitamin D increases collagen and decreases elastin of the aorta in the rat (29), and it is speculated that vascular lesions may develop in an area of high turbulence flow from a direct toxic effect of vitamin D. We found no clear correlation with the development of the supravalvular lesion and newborn Ca, 25-OHD, or cholesterol levels.

In the present study, maternal serum cholesterol levels in the vitamin D-supplemented groups were significantly increased, compared with controls at midgestation. However, newborn cholesterol levels were not different in the four groups. We found no relationship between cholesterol levels and maternal vitamin D doses. In the rat high doses of vitamin D increase plasma-free fatty acids and triglyceride levels (22). Vitamin D also stimulates ^{32}P incorporation into phospholipid (26) and increases hepatic cholesterol total fat and fatty acid content (7).

In this study, no effects of vitamin D on maternal and neonatal serum Mg levels were seen. In rats, vitamin D has been reported to decrease serum Mg levels (13).

CONCLUSION

High doses of vitamin D during pregnancy affect fetal death, maternal calcium, phosphate and cholesterol homeostasis, and neonatal calcium homeostasis. Although there are no clear relations between biochemical derangements and the cardiovascular lesions in the mother or newborn, vitamin D toxicity can produce calcific aortic lesions in the mother and an apparent dose-related development of supravalvular aortic lesions in their newborn.

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