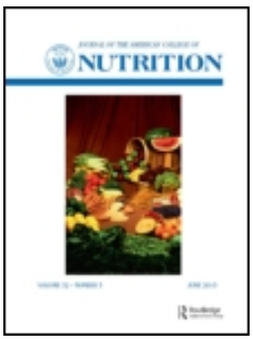

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Effects of a Dietary Magnesium Deficiency and Excess Vitamin D_3 on Swine Coronary Arteries

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Key words: magnesium deficiency, vitamin D_3, coronary artery, swine, ultrastructure

The effect of a moderate magnesium (Mg) deficiency on coronary arteries of 61 swine, fed various levels of vitamin D_3, was studied by light and electron microscopy. The effect of subnormal Mg intake on vitamin D_3-induced intimal lesions of the arteries showed a trend towards increased damage. The degree of cell degeneration and intimal thickening, which was induced by high vitamin D intakes, was as great in swine whose diet was low in Mg and moderately high in vitamin D as it was in those on twice as much vitamin D. Also, the degree of arterial calcification was intensified by inadequate Mg intake at the two higher vitamin D intakes. Present findings indicate that suboptimal dietary Mg, in combination with an excess of vitamin D, has an additive effect in the initiation of ultrastructural changes in the coronary arteries. Extension of the study is indicated to ascertain the extent to which further reduction of Mg intake can potentiate vitamin-D-induced coronary lesions.

INTRODUCTION

Magnesium (Mg) deficiency produces vascular diseases, such as ischemic heart disease, preeclampsia, and cerebrovascular attack [1–14]. Vasospasms appear to be a pathogenic feature of hypomagnesemia. Some investigators [1,2,6,15] have reported in vitro studies that indicate a link between Mg deficiency and vasospasms. Mg supplementation has been shown to exert a protective effect against the structural and functional damage of cardiac hypoxia in experimental animals. This protective effect of Mg suggests the inverse relationship between water hardness and the rate of sudden deaths from ischemic heart disease in epidemiological studies may have a physiological component [3,4]. There is evidence that Mg deficiency is widespread in the United States, possibly due to the modern technology of food processing and other factors that increase Mg needs [3,4,16–20]. Nutritional factors such as excessive dietary fat, salt, phosphate, calcium, and vitamin D increase Mg requirements and intensify Mg deficiency [4,5,21,22].

Though the optimum levels of dietary vitamin D_3 for humans and animals is still controversial, it appears that present day levels are excessive [19–24]. Numerous epidemiological and animal studies have implicated hypervitaminosis D as a risk factor in arteriosclerosis [20–25] and in infantile hypercalcemia and the supravalvular aortic stenosis syndrome [21]. Excessive doses of vitamin D in experimental swine fed a cholesterol-free diet quickly produce arterial lesions which contain many degenerated cells [26–28]. Since hypervitaminosis D and Mg deficiency are known to be related to risk of arteriosclerosis, the purpose of the present study was to evaluate by light and electron microscopy interactions between Mg deficiency and excessive doses of vitamin D_3.

MATERIALS AND METHODS

A total of 61 Yorkshire male piglets, 8 weeks of age and weighing 12–18 kg, were used in this study. The piglets were randomly divided into four groups, and subgroups A and B. The animals were housed in a facility with concrete slated floors in separate pens equipped with self-feeders and water. The animals were placed on experimental diets containing the following levels of vitamin D in IU per kg of ration (Table 1): group I, 300; group II, 2000; group III, 4000; and group IV, 24,000. The basal diet was composed of 87.25% ground yellow corn, 10% defatted soybean meal, and a 2.75% multiple minerals and vitamin premix (Table 2). Subgroup A was fed a ration which contained 517 mg of Mg per kg of

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Table 1. Summary of Dietary Supplements

Group	Subgroup	No. of swine	Vitamin D (IU/kg diet)	Magnesium (mg/kg diet)
I	A	12	300	518
	B	12	300	270
II	A	6	2000	518
	B	12	300	270
III	A	7	4000	518
	B	6	4000	270
IV	A	6	24,000	518
	B	6	24,000	270

Table 2. Composition of Diet[a]

Ingredients	%
Ground corn	87.25
Soybean meal, solvent-extracted	10.00
Mineral and vitamin mix	2.75

Minerals (mg/kg diet)		Vitamins (mg/kg diet)	
Calcium	6320.0	α-Tocopherol	11.3
Chlorine	2530.0	Biotin	0.032
Copper	8.58	Choline	384.9
Iodine	0.55	Folic acid	0.07
Iron	49.5	Niacin	42.2
Magnesium	517.0	Pantothenic acid	10.39
Manganese	40.2	Carotene	1.57
Phosphorus	5085.0	Pyridoxine	0.8
Potassium	1970.0	Riboflavin	3.65
Sodium	2840.0	Thiamine	3.8
Zinc	52.2	Vitamin B_{12}	17.6
L-Lysine	1520.0	Vitamin A (IU/kg)	3315.0
		Vitamin D_3 (IU/kg)	300.0

[a]The grain diet consists of crude protein (14.3%), fat (3.0%), carbohydrate (72.7%), and provides digestible energy (3483 kcal) and metabolizable energy (3246 kcal/kg diet).

diet; subgroup B was fed a ration which contained 270 mg of Mg per kg of diet. The water used in this experiment contained about 26 mg/L of Mg. All groups received this experimental dietary treatment continuously from 2 through 6 months of age.

For biochemical analysis, blood was collected at the time of sacrifice and stabilized with heparin. Plasma total cholesterol concentrations were determined by the method of Allain et al [29]. Plasma calcium (Ca) levels were determined by the method of Sarkar and Chauham [30] and magnesium (Mg) by means of Sigma diagnostic kit 595-A (Sigma, St. Louis). Serum 25-hydroxyvitamin D levels were determined by means of HPLC [31].

For pathological studies five specimens, measuring 3 cm, were excised from the proximal anterior descending coronary artery for transmission EM. The arterial tissues were immersed in a phosphate-buffered 3% glutaraldehyde solution (pH 7.4) immediately after their re-

Table 3. Weight Gain, Plasma Cholesterol, and Calcium Levels*

Group	Weight gain (kg)	Total cholesterol (mg/dl)	Calcium (mg/dl)
IA	117.9 ± 8.5^{a}	85.5 ± 16.6^{ab}	10.3 ± 0.4^{a}
IB	106.2 ± 14.1^{a}	70.0 ± 4.8^{a}	10.7 ± 0.5^{ab}
IIA	107.0 ± 11.5^{a}	86.4 ± 5.9^{ab}	11.0 ± 0.5^{b}
IIB	112.8 ± 17.7^{a}	79.0 ± 8.2^{ab}	11.9 ± 0.6^{c}
IIIA	118.4 ± 8.6^{a}	87.0 ± 8.8^{ab}	11.1 ± 0.8^{bc}
IIIB	118.2 ± 11.3^{a}	79.6 ± 15.0^{ab}	11.1 ± 0.9^{abc}
IVA	109.6 ± 8.4^{a}	95.0 ± 16.2^{b}	11.5 ± 0.4^{bc}
IVB	113.6 ± 18.8^{a}	ND	ND

*Data are expressed as mean ± SD. Means not sharing a common superscript letter in the same column are significantly different; $p < 0.05$; ND = not done.

Table 4. Intimal Thickening and Serum 25-OHD Level at 2 and 6 Months of Age

Level of vitamin D_3 in diet	Thickness (μm)	Serum 25-OHD level (ng/ml)
300 IU/kg for 2 months	5.6 ± 2.1	15.0 ± 7.6
300 IU/kg additional 4 months	19.2 ± 2.6	27.1 ± 4.9
2500 IU/kg additional 4 months	24.2 ± 2.6	55.6 ± 10.4
25,000 IU/kg additional 4 months	28.0 ± 3.8	438.7 ± 100.7

moval at the time of sacrifice. They were then cross-sectioned into lengths of 3 mm and fixed in a fresh glutaraldehyde solution for at least 24 hours. These specimens were then fixed in phosphate-buffered 1% osmium tetroxide, serially dehydrated in ethanol, and embedded in EM bed 812 epoxy resin. Thick sections were stained with alkaline toluidine blue and used for histological examination of coronary arterial lesions. The degree of intimal thickening was measured with an ocular micrometer. Ultrathin sections were cut with an ultramicrotome, stained with uranyl acetate and lead citrate, and observed with a Hitachi HV-12 electron microscope. Three epoxy resin-embedded tissue blocks from each animal were examined for comparison of the frequencies of degenerated cells, foam cells, and leukocytes. Cell counts were conducted routinely at a magnification of 6000. All data were analyzed by the difference between means and statistical significance as based on Fisher's least significant difference (LSD) multiple comparison test [32].

RESULTS

Plasma Cholesterol and Calcium

The plasma total cholesterol and calcium levels of each experimental group are listed in Table 3. The cholesterol levels were slightly lower in the low Mg subgroups although the reduction was not statistically significant ($p < 0.05$). Excess vitamin D_3 apparently did not affect plasma cholesterol levels. Relative to the control group, plasma Ca levels were significantly higher in groups with excess vitamin D_3 ($p < 0.05$), but there were no significant differences among the three excess vitamin D_3 groups. Although Mg deficiency caused a

Table 5. Incidence and Degree of Intimal Thickening of Coronary Artery*

Group	Incidence of intimal thickening (%)	Average of intimal thickening (μm)
IA	37.7[a]	19.2 ± 2.6[a]
IB	42.4[ab]	23.0 ± 3.1[ab]
IIA	43.3[ab]	23.4 ± 4.6[ab]
IIB	60.0[b]	30.1 ± 4.8[b]
IIIA	55.6[ab]	32.4 ± 4.7[b]
IIIB	57.1[ab]	33.8 ± 5.2[b]
IVA	53.3[ab]	33.3 ± 6.5[b]
IVB	54.8[ab]	33.5 ± 5.2[b]

*Average of intimal thickening is expressed as mean ± SE. Means not sharing a common superscript letter in the same column are significantly different; $p < 0.05$. Average intimal thickness at 2 months of age 5.6 ± 2.1 μm.

slight increase in plasma Ca levels in groups I and II, the overall effect of Mg deficiency on plasma Ca and Mg (data not shown) appeared inconclusive, possibly because of poor sensitivity of the Sigma method. The 25-hydroxyvitamin D serum levels increased in the animals fed the higher levels of vitamin D and increased intimal thickening (Table 4).

Light Microscopy

The incidence and magnitude of intimal thickening are shown in Table 5. Intimal thickening was most commonly observed at the bifurcation of the coronary arteries. Subgroup IIB and groups III and IV had significantly higher incidences of intimal thickening ($p < 0.05$) as compared to the control (subgroup IA). No significant difference in the number of intimal lesions was noted between group I and subgroup IIA.

Electron Microscopy

Coronary arteries from swine fed the basal diet with either deficient or sufficient levels of Mg (subgroups IA and IB) displayed only mild fibromuscular intimal thickening and nearly intact media. Swine fed varying levels of excess vitamin D_3 (groups II, III, and IV) developed frequent degeneration of smooth muscle cells (Fig. 1) consisting of both rarefaction and condensation types [33]. The rarefied smooth muscle cells contained electron lucent peripheral cytoplasm in which the myofilaments were indistinct. A diffuse increase in the density of both the nucleus and the cytoplasm occurred in the condensed smooth muscle cells. An increase in the number of smooth muscle cells, globular elastic fibers, collagen fibrils, and abundant glycosaminoglycans contributed mainly to the severe intimal thickening in these groups (Fig. 2).

The presence of foam cells in the intima is one of the most prominent features of atherosclerosis. A marked increase in the number of foam cells was observed in group IV irrespective of Mg level. Both macrophage and smooth muscle type foam cells were clearly present in the thickened intima in this group (Fig. 3). Counts and computations of degenerated smooth muscle cells, foam cells, and leukocytes are shown in Table 6. The frequencies of cell degeneration observed in the excess vitamin D_3 groups, ranging from 2000 to 24,000 IU/kg diet, were significantly higher than the control group, but there were no significant differences among the groups themselves. The number of foam cells observed in these groups appeared to increase as the level of vitamin D_3 increased. However, statistically, only group IV had a significantly higher frequency of foam cells as compared to the other excess vitamin D_3 groups. There was no specific trend in the appearance of leukocytes. Because of the low frequency of Ca deposition, quantitative estimates were not feasible. However, in the low Mg groups, especially subgroup IVB, Ca deposition was observed more frequently in low Mg groups than in the corresponding subgroups (Fig. 4).

DISCUSSION

Magnesium plays a crucial role in the regulation of membrane stability. Altura et al [2] reported that an ar-

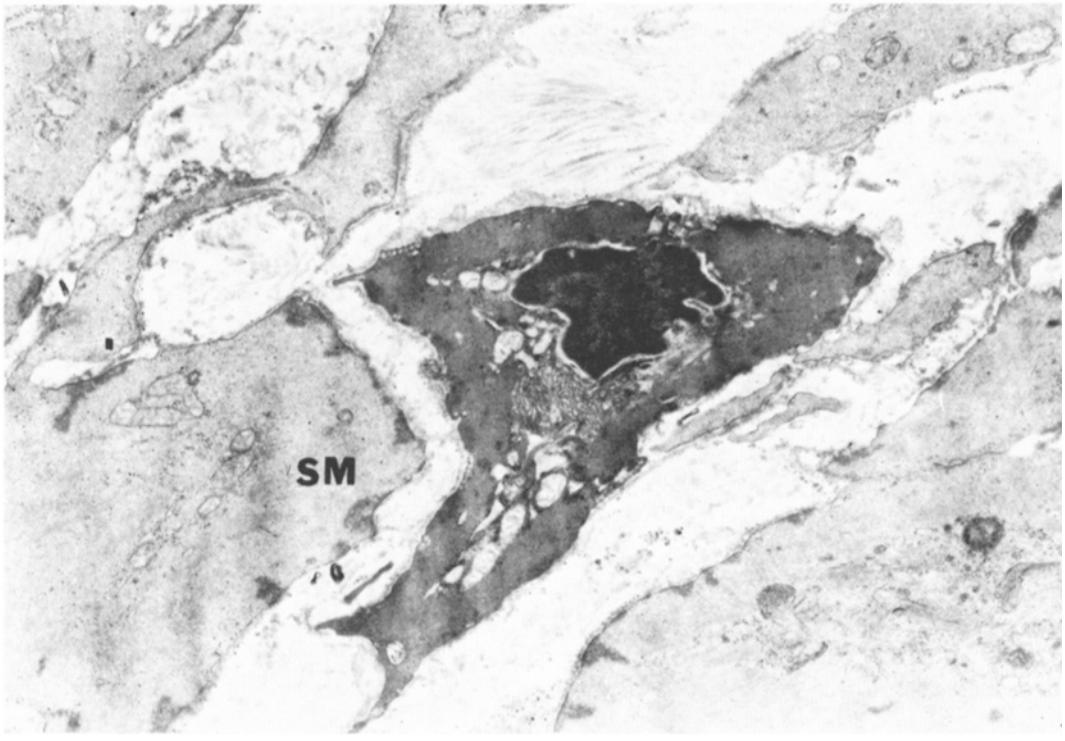

Fig. 1. Degenerated smooth muscle cell in an inner media from swine fed 2000 IU vitamin D_3/kg diet (group IIA). Note the diffuse increase in the density of both the nucleus and cytoplasm. SM = smooth muscle cell. ×12,000.

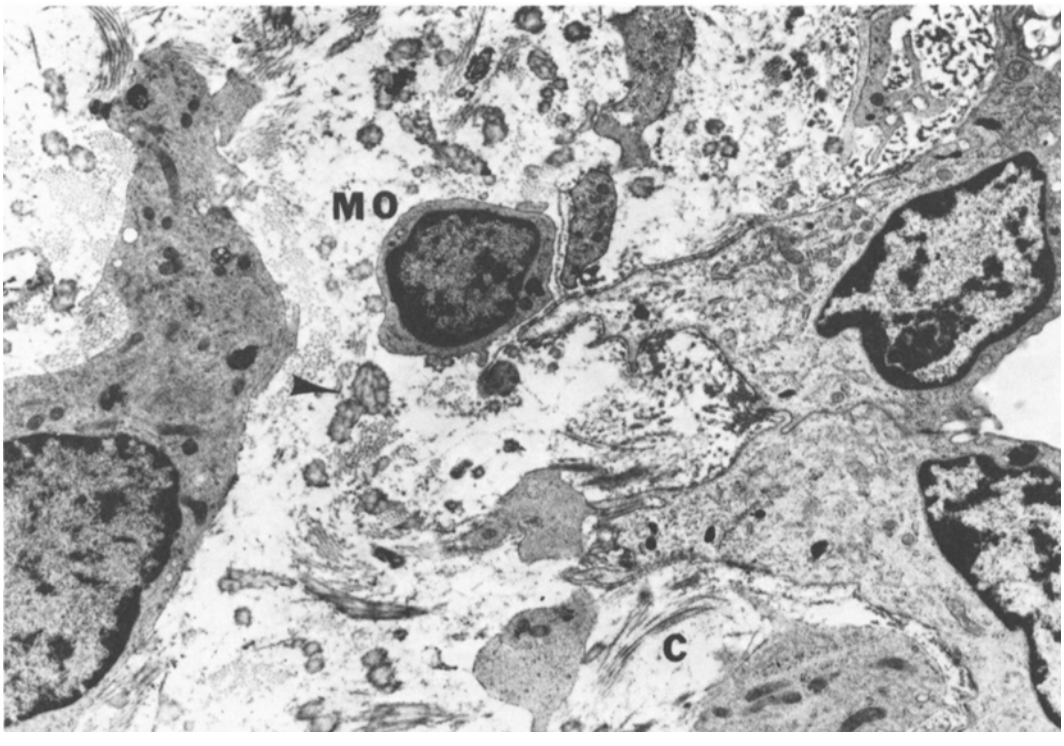

Fig. 2. Fibromuscular intimal thickening in a coronary artery from group IIB. The thickened intima is composed of globular elastic fibers (arrowheads), collagen fibrils (C), ground substance, and cells. The cell in the bottom of this micrograph is probably a modified smooth muscle cell. MO = monocyte; E = endothelial cell; L = lumen. ×12,000.

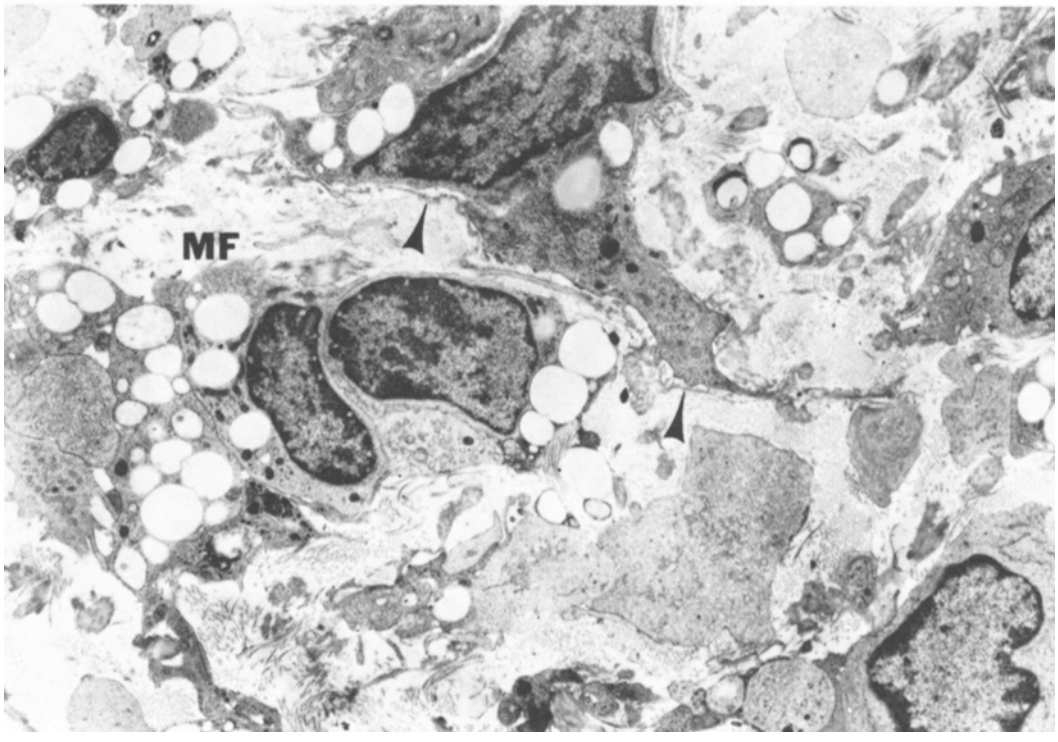

Fig. 3. Foam cells in a thickened intima from swine fed 24,000 IU vitamin D_3/kg diet in combination with adequate magnesium supplementation (group IVA). The smooth muscle cell type foam cell is identified by the presence of fusiform densities and basement membranes (arrowheads). MF = macrophage-type foam cell. ×9600.

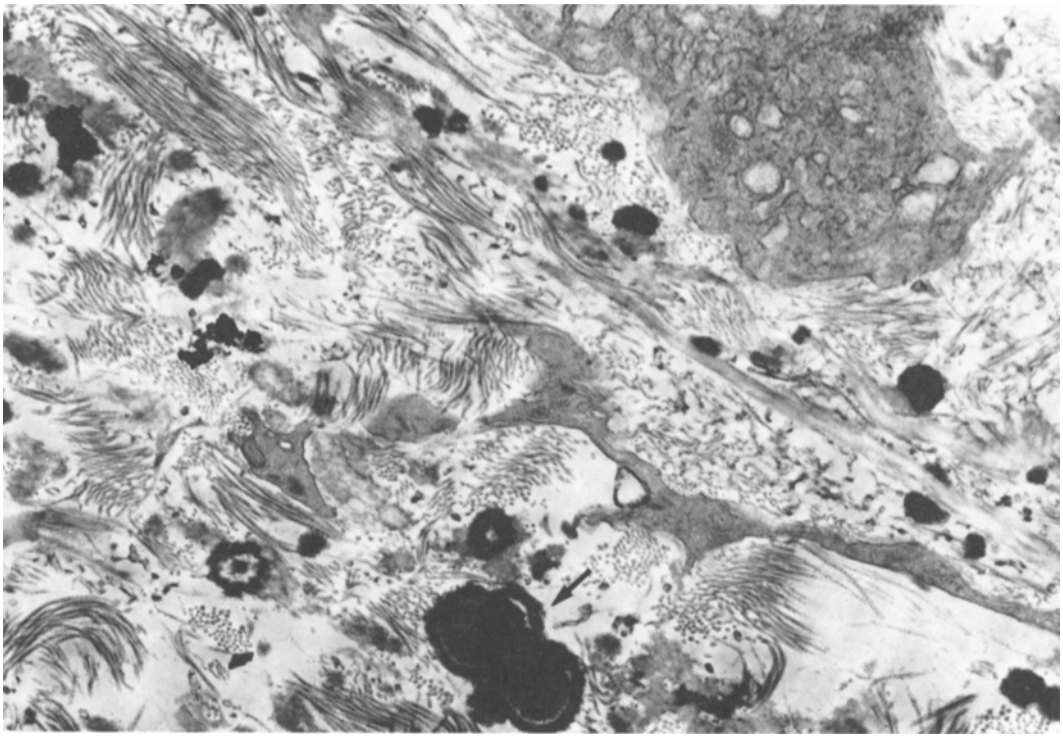

Fig. 4. Calcium deposits in an intimal lesion of a coronary artery from group IVB. Many calcified particles are observed as an electron dense granular structure adjacent to elastic fibers. Some of them show a characteristic feature with a corona of needle-shaped crystals (arrow). ×14,000.

Table 6. Frequency of Degenerated Cells, Foams Cells, Leukocytes, and Calcification*

Group	Degenerated cells (%)	Foam cells (%)	Leukocytes (%)	Calcification	Total cell counts
IA	4.3^{a}	1.9^{a}	1.3^{ac}	±	2532
IB	4.9^{a}	1.6^{ab}	1.3^{ac}	±	3186
IIA	7.7^{bc}	2.5^{a}	2.0^{c}	±	1641
IIB	9.9^{cd}	0.7^{b}	1.5^{ac}	±	1830
IIIA	10.9^{d}	2.6^{a}	1.0^{a}	±	1526
IIIB	9.3^{cd}	5.0^{c}	0.3^{b}	+	1617
IVA	6.8^{b}	6.1^{c}	1.0^{a}	+	1583
IVB	9.7^{cd}	5.0^{c}	0.8^{ab}	++	1488

*Calcification: ± = trace, + = less frequent, ++ = frequent. Means not sharing a common superscript letter in the same column are significantly different; $p < 0.01$.

tificial lowering of the Mg content of isolated vessels from rats, rabbits, piglets, and dogs induced a rapid potential contractile response. They also suggested that a lowering of the concentration of extracellular Mg increases the total exchangeable intracellular Ca fractions resulting in an enhanced influx and translocation of Ca into the vascular cells which causes probable blood vessel contraction. Enhanced membrane permeability to Ca is also considered a critical factor in the pathogenesis of arterial lesions induced by excess vitamin D_3 [22]. Siegel et al [6] emphasized the importance of a potassium (K) increase near the cell membrane in hypomagnesemia. It is well known that Mg deficiency induces vasospasms which may be involved in the initiation of atherosclerosis [34,35]. Vascular changes caused by Mg deficiency have been reported by previous investigators [36–38]. In those studies, intimal thickening, fibrosis, fragmentation of the internal elastic lamina, and calcification of the arteries were observed by light microscopy.

Mayo et al [39] postulated that the Mg requirement for swine weaned at 9 weeks of age is 505 mg/kg total ration and reported that swine fed 417 mg/kg diet or less suffer from deficiency symptoms. These levels were determined from a 42-day feeding trial involving swine. We selected Mg levels of 517 and 270 mg per kg of diet as sufficient and deficient levels, respectively. The swine used in this study were fed these levels of Mg continuously for 4 months. Plasma Mg concentrations did not reach statistical significance in this study, but other studies have shown that, although they may be significantly correlated to dietary Mg levels [6,39], other tissue levels are better indices [40]. Analysis of variance revealed that dietary Mg levels of 270 and 517 mg/kg diet had no significant effect on weight gain (Table 2); this finding agrees with the results of others [39]. Furthermore, during a 4-month feeding period, no deaths occurred in animals fed the low-Mg diets, but hyperirritability and a slight pastern weakness were noted. We repeated the feeding study four times (data not shown) but were not able to find significant differences in serum Mg levels in swine fed 517 and 270 mg per kg of diet, probably due to the limits of error in methodology [41–43].

It has been shown that atherosclerosis is linked with the possible role of hypervitaminosis D and with its accentuation by Mg deficiency in atherosclerosis [25,44,45]. The degree of Mg deficiency induced in the present study did not cause significant arterial lesions in the coronary arteries of piglets not given excessive vitamin D_3 supplementation. This observation differs somewhat from those obtained with Mg deficiency in rat and dog studies, which caused coronary arterial lesions [37,38]. Whether this discrepancy is due to species differences or lack of severity of Mg deficiency is not clear. However, the effects of various degrees of pure Mg deficiency alone on experimental atherosclerosis in swine deserves further investigation. A definite effect of Mg deficiency was obtained in the animals in subgroup IIB, which received 2000 IU vitamin D/kg diet. They exhibited a significant degree and incidence of arterial lesions, while subgroup IIA, on the same high vitamin D intake, but adequate in Mg, sustained only a high frequency of cell degeneration without intimal thickening. In groups III and IV, obvious Mg deficiency effects were not observed except for enhancement of calcification. As shown in our previous studies, the vitamin D_3 levels used in groups III and IV have proved to be critical levels for the induction of atherosclerosis [46]. These levels cause a great magnitude of fibromuscular thickening and a

high frequency of smooth muscle cell degeneration in coronary arteries. We speculate that these excessive levels of vitamin D_3 induce more damage than that caused by the moderate Mg deficiency we induced; the effects of excess vitamin D might mask the effects of a moderate Mg deficiency in terms of promoting intimal thickening. But in these groups enhancement of arterial calcification occurred, an important feature in advanced atherosclerosis. Smooth muscle cell degeneration in the coronary arteries that occurred when vitamin D_3 was supplemented at levels of 2000 IU/kg diet or greater indicates that even a mild excess level of vitamin D promotes cell degeneration. The high incidence of cell degeneration probably resulted from the cytotoxic effect of hypervitaminosis D [28]. Smooth muscle cell death is postulated to be an important morphological event in the development of atherosclerosis [47]. In this study there was no evidence that moderate Mg deficiency exacerbated cell degeneration, but the presence of smooth muscle cell degeneration is considered important for the induction of Mg deficiency effects.

CONCLUSION

An examination of dietary ingredients indicated that a typical commercial swine ration contains approximately 14 times more vitamin D_3 than the amount recommended by the National Research Council [48]. Furthermore, the 25-hydroxyvitamin D_3 levels in plasma of swine from group II were similar to those of the American population [49], suggesting that the humans sampled had ingested much more than their daily requirements. Based on the incidence of infantile hypercalcemia, the supravalvular aortic stenosis syndrome, and renal acidosis, Seelig has suggested that there is a variation in the range of individual reactivity to vitamin D_3 [19,21]. Those who are hyperreactive to vitamin D_3 could be highly susceptible to its toxicity even with moderately high intakes of vitamin D_3, especially in combination with Mg inadequacy; in infancy it may play a role in the early roots of cardiovascular disease [3,25]. In this study, levels of vitamin D_3 much lower than those of the average American population [47] were able, in combination with a moderate Mg deficiency, to initiate coronary atherosclerosis. This suggests that even a moderate Mg deficiency could be a potential or additive risk factor for the norm of the American population in terms of atherosclerosis and heart disease.

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