Effects of Choline on Cardiovascular Lesions Induced by Feeding Large Doses of Vitamin D

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Twenty-six years ago, Kreitmair and Mall reported the production of vascular calcification in rats fed high doses of activated ergosterol, an observation that has since been confirmed repeatedly. Duguid gave detailed descriptions of this type of aortic sclerosis; Ham and Lewis provided excellent illustrations of the intimal sclerosis that rapidly develops in the coronary arteries of rats given massive single doses of vitamin D. These reports and others have been the subject of several reviews. In nearly all the experiments that have been reported, investigators employed commercially available rations for the basal diet. It has previously been reported from this laboratory that a significant number of young rats fed diets high in fat but low in choline regularly developed vascular lesions which were absent in pair-fed controls fed the same diet supplemented with choline. The gross appearance of the aortas in many of the choline-deficient rats resembled that of sclerotic aortas in rats subjected to hypervitaminosis D. Therefore, it was decided to determine whether or not dietary lipotropic supplements would alter significantly the incidence of cardiovascular lesions induced by excessive intakes of Vitamin D in rats fed a food mixture low in choline. In essence, our findings showed that lesions were absent in the coronary arteries of nearly all the animals receiving a dietary supplement of 0.85 per cent choline chloride in addition to 15,000 to 20,000 units of vitamin D daily, and all their aortas were normal. Lesions at comparable sites developed in 50 to 80 per cent of rats similarly treated but not provided with the lipotropic supplement.

Methods

One-hundred and sixteen Wistar rats (males; 120-150 Gm.) in four groups were used in the experiment. Twenty-five animals (Groups 1 and 7; Table I) were fed the basal low choline diet ad libitum. Twenty-five additional rats (Groups 2 and 8; Table I) were offered and consumed daily similar amounts of the basal diet supplemented with 0.85 per cent choline chloride. Thirty-three animals (Groups 3, 5, and 9; Table I) were pair-fed the basal diet supplemented with enough calciferol in corn oil so that each ingested daily 15,000 to 20,000 units of vitamin D. Animals in groups 4, 6, and 10 (Table I) received supplements of both choline and calciferol. The per cent composition of the basal diets was as follows: casein 10, alcohol-extracted peanut meal 30, alpha soya protein 5, LP salts 3, PDW* vitamins 1, cellulose flour 5, sucrose 10.5, lard 35.0 alpha tocopherol acetate 0.010, cod liver oil concentrate 0.015, cinnamon 0.03. Calciferol was dissolved in corn oil so that 1 ml. contained 10,000 international units of vitamin D. The mixture was added at the level of 20 per cent to the

* The ingredients of the salt mixture and the vitamin mixture have been published previously.
TABLE I

<table>
<thead>
<tr>
<th>Group No.</th>
<th>No. of rats</th>
<th>Dietary choline chloride (0.85 per cent)</th>
<th>Dietary calciferol (12,000-20,000 I.U./day)</th>
<th>No. of days on diet</th>
<th>No. of rats with cardiac muscle lesions</th>
<th>No. of rats with coronary arterial sclerosis</th>
<th>No. of rats with aortic sclerosis</th>
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basal diet at the expense of the lard. The cinnamon, which was added to all diets, served to stimulate the animals' appetites. Details of the methods of housing the cages, preparation of diets, etc., have been published previously.9

The experiment was carried out in three stages. In the first, 40 animals were used (Table I) in four groups (Nos. 1-4) of ten animals each. In the second stage, there were only two groups (Nos. 5 and 6) of eight rats in each; both received calciferol in their diets, but only one group (No. 6) was given the choline supplement (Table I). In the third stage of the experiment (Table I), again four groups (15 rats in each) were employed (Nos. 7, 8, 9, and 10). The various supplements fed to each group are indicated in the table. In the first two stages of the experiment the deficient animals were maintained for an average period of 35 days before sacrifice, and the choline-supplemented groups for 31 days. The discrepancy of four days developed because the groups were started at different times. In the third stage this time difference was avoided, and actually the choline-supplemented animals were observed for a slightly longer period than the deficient ones.

Animals were sacrificed under ether-inhalation anesthesia, and blocks of tissue from heart, aorta, liver, kidney, spleen, and pancreas were fixed by immersion in formol-calcium solution. Frozen sections were cut routinely of all hearts and aortas and stained by Wilson's modification of Lilly's supersaturated Isonpropynol Oil Red O Technique.10 In addition, a variety of special strains were carried out on these and other tissues (see below).

RESULTS

The number of rats exhibiting lesions in heart muscle, coronary arteries, and aortas in the various groups are given in the table. In hearts and aortas of members of the basal choline-deficient groups (Nos. 1 and 7), lesions induced by choline deficiency per se developed in 25 to 60 per cent of time rats. The gross and microscopic appearances of this type of vascular injury have been described previously in reports from this laboratory.5,6 Choline supplements to the basal diets (without added calciferol) completely prevented lesions in the organs and vessels of control rats in both stages of the experiment (Groups Nos. 2 and 8). Addition of calciferol to the basal diet (Groups 3, 5, and 9) more than doubled the incidence of cardiovascular lesions (50 to 90 per cent) in 33 rats fed this ration. Aortas of all 33 control rats (Groups 4, 6, and 10) fed the basal diet supplemented with both choline and calciferol, were normal, and only minimal lesions were found in coronary arteries of five (15 per cent).

Not shown in the table are the effects on the kidneys of rats on the various dietary regimens. In almost every animal that did not receive the dietary supplement of choline chloride, the kidneys were moderately or severely damaged, particularly in those rats that had ingested calciferol. But neither
gI. oss nor microscopie evidence of renal damage could be found in any of the animals given the choline-supplemented diet with or without added calciferol. The type of renal injury induced by the low choline diets conformed to the descriptions previously published by Christensen\textsuperscript{11} and by one of us.\textsuperscript{12} The addition of calciferol to low choline diets intensified these lesions and increased the amount of calcium salt deposition in necrotic tubules.

\textbf{The Aortic Lesions}

The gross and microscopic appearances of the aortic lesions in the choline-deficient rats were not appreciably altered in those animals that also received added vitamin D (Figs. 1 and 2). In the advanced stages of the lesion the intima was frequently thickened and the lumen narrowed by plaques composed of hyperplastic endothelial cells. In the cytoplasm of these cells small droplets of stainable fat were frequently present. The underlying media appeared necrotic, calcified, and in some instances hyperplastic. In the example illustrated in Figure 1, a fracture of a calcified medial bar is in an early healing stage with callus formation ("collar-button lesion"). It is difficult to discern any consistent differences in the nature of the aortic lesions induced by choline deficiency per se and those by calciferol supplements to the low choline diet. Calcification frequently appeared to be as prominent a feature in the one case as in the other. In these experiments, aortic lesions were not produced by high intakes of calciferol unless the choline supplement was omitted from the diet consumed by the animals.

\textbf{Coronary Arterial Lesions}

In their most advanced form, coronary arterial lesions in members of the choline-deficient calciferol-supplemented groups could be readily distinguished from those seen in rats that had received the basal choline-deficient diet alone. Calciferol supplementation of the choline-deficient rats consumed by rats in Groups Nos. 3, 5, and 9 was associated with lesions in the coronary arteries that resembled those observed in the aortas, but on a smaller scale. The intima of the affected vessels was thickened and heaped up; stainable fat could be demonstrated in the proliferated endothelial cells; and hyperplasia and hypertrophy of the underlying media with some necrosis and calcification was frequently observed (Fig. 3).

In the coronary arteries of the choline-deficient animals that did not receive calciferol, lesions were neither as advanced nor as frequently encountered. Abnormalities were confined almost exclusively to simple deposition of stainable fat in intimal endothelial cells, media, and occasionally adventitia of affected vessels (Fig. 4). Aside from some thickening of the intima by hypertrophy of endothelial cells, there were no apparent alterations in the architecture of the arteries, although structural changes have been encountered in rats fed low choline diets for longer periods.\textsuperscript{8}

\textbf{Damage to Cardiac Parenchyma}

Abnormalities in the cardiac parenchyma of choline-deficient rats consisted of deposits of stainable fat within the cytoplasm of muscle cells, myocardial necrosis, lysis, and, in some instances, fibrosis. In those choline-deficient animals receiving calciferol supplements, necrotic muscle fibers were sometimes calcified, but otherwise no consistent differences could be detected in the type of lesion encountered in hearts of animals of the various groups. The microscopic appearance of the lesions in animals fed low choline, high fat diets has been reported;\textsuperscript{13} the addition of calciferol to similar diets appeared only to increase slightly the incidence of the lesions.

\textbf{Livers}

In all the choline-deficient animals, whether receiving calciferol supplements or not, the livers contained abnormal deposits of stainable fat; choline prevented the accumulation of lipid in the livers of the control rats. Hepatic fibrosis or cirrhosis did not develop in any of the choline-deficient rats, presumably because of the short duration of the experimental period.
Fig. 1. Advanced aortic sclerosis in a choline-deficient rat (that did not receive added vitamin D). Note the thickened intima and the healing fracture of the underlying calcified media; × 100. The inset demonstrates the presence of stainable fat droplets (black) in the intima; × 800. Frozen section stained with Oil red O.

Fig. 2. Stain and magnifications as for Fig. 1. An early stage of aortic sclerosis is seen in this aorta from a choline-deficient rat that received the excessively high supplement of vitamin D. Calcium has dropped out of the hole in the necrotic media which underlies the slightly thickened intima. Little fat is present in the intima (inset). In many rats of this group, the aortic lesions advanced to a stage indistinguishable from that shown in Fig. 1.
Fig. 3. Coronary artery from a choline-deficient rat that did not receive the supplement of vitamin D. Fat (black) is deposited in media and adventitia. Frozen section stained with Oil red O; × 500.

Fig. 4. Stain and magnification as for Fig. 3. The tremendously thickened subintima and media are shown in this coronary artery which is considerably narrowed. Only small amounts of stainable fat are present in the lesion at this stage. The vessel is from a choline-deficient rat that received a high dose of vitamin D.
DISCUSSION

Despite excessively high doses of vitamin D, those animals that also received the dietary supplement of choline chloride (0.85 percent) were clearly afforded a large measure of protection against the type of cardiovascular damage observed in many of the rats consuming the basal diet containing calciferol without added choline. Calculations based on the food intake of the animals (which averaged 8 to 10 Gm. per day per rat) indicated that rats consuming the calciferol-supplemented diet obtained between 15,000 and 20,000 international units of vitamin D per day. Our data provide no information on the protective effect of a choline supplement under conditions where rats may receive appreciably more than this amount of vitamin D. But it seems likely that with higher doses of the vitamin the protective effect of choline would be lost. It is also possible that the protective effect of choline would not have been demonstrated had the same daily intake of vitamin D used in the present experiment been continued for longer periods.

Comparison of Calciferol-induced and Choline-deficient Cardiovascular Damage

Neither gross nor microscopic differences that could be related to the amount of vitamin D in the diet were detected in the appearance of the aortic lesions. But by their lesions the coronary arteries of choline-deficient rats that were given calciferol could be easily distinguished from arteries of rats that did not receive extra vitamin D. In the coronary arteries of the choline-deficient animals, abnormal deposits of lipid were present with hypertrophy but not hyperplasia of intimal endothelial cells, whereas the lesions in the choline-deficient, calciferol-supplemented rats exhibited more profound structural abnormalities (See Fig. 4). The difference in the lesions, however, may be more apparent than real, because it may only be associated with the stage and severity of the injury. In previously reported experiments, the role of renal damage in the production of vascular lesions in choline-deficient rats has already been considered. It is possible that choline deficiency per se induces vascular lesions in rats as a result of initial renal damage. Conclusive evidence concerning this point is not yet available, but the investigations of Lehr and...
Chung,14 Wissler,15 and Holman16 indicate that kidney damage induced by a variety of methods in susceptible animals may be regularly associated with lesions of aortas and coronary arteries. If cardiovascular damage in choline-deficient rats should prove to be of renal origin, the prevention of damage to the vessels of calciferol-fed rats by choline supplements may be simply a manifestation of the protective action of choline on the kidney. Whichever mechanism proves to be true, however, there is now considerable evidence to indicate that, directly or indirectly, lipotropic supplements aid in the maintenance of the heart and blood vessels of experimental animals.

The Role of Lipotropic Substances in the Treatment of Arterial Disease in Man

Katz and Stamler17 have recently reviewed the role of lipotropic factors in the prevention and treatment of both experimental and clinical vascular disease. They quote their previous conclusion:18 "Neither on theoretical nor experimental nor clinical grounds is there today a firm scientific basis for the widespread clinical use of costly lipotropic preparations in the prophylaxis and/or therapy of human atherosclerosis." This conclusion is based on the apparently negative result of clinical trials of the therapeutic use of lipotropic factors in the treatment of human atherosclerosis, despite initial encouraging reports by Steiner19 and others. A number of investigators20,21 have reported that choline supplements failed to protect rabbits against cholesterol-induced atheroma. In these experiments, the basal diets employed already contained adequate amounts of lipotropic factors and their cholesterol content was very high. Under these conditions, the published data clearly indicate that dietary choline does not afford any protection to animals against vascular injury so induced. In our experiments, we have been able to show a protective effect of choline supplements against calciferol-induced injury to vessels only when the basal diet was low in lipotropic factors. Whether or not choline deficiency is ever responsible for directly inducing disease in vessels of man or for facilitating their injury by other agents remains to be demonstrated. We are in agreement with the conclusions of Katz and others that at the present time clinical use of lipotropic factors in human arterial disease is entirely unwarranted. Nevertheless, we believe that the available results do justify further exploration of any relations that may exist between dietary choline deficiency and cardiovascular disease in experimental animals and possibly also in man. Heart disease is high in the list of causes of death in most countries. Every experimental approach that may add to our knowledge of factors responsible for the health of the heart and vessels should be pursued vigorously.

SUMMARY AND CONCLUSIONS

Nine of 25 rats fed a basal choline-deficient, high fat diet developed varying degrees of aortic sclerosis. Nineteen of 33 choline-deficient rats fed the same basal diet but supplemented with high doses (15,000–20,000 international units per rat per day) of vitamin D (calciferol) developed essentially the same type of aortic lesions. Choline supplements added to the basal diet completely prevented the development of aortic sclerosis in 25 rats and was also successful in preventing lesions in an additional 33 animals fed the basal diet supplemented with large amounts of vitamin D. A similar but not completely protective effect of the choline supplement on their coronary arteries was demonstrated.

Choline supplements in the basal diets (with or without added calciferol) consumed by the rats prevented the development of renal damage.

The mechanism of the protective action of choline supplements on the cardiovascular system of rats given excessive amounts of vitamin D and the possible role of the kidney in this regard is discussed.

Clinical applications of these findings to either the prevention or treatment of arterial disease in man are not apparent at the present time. However, in view of the data presented, it would seem that therapeutic use
of large doses of vitamin D, for example, in the treatment of scleroderma or arthritis, should be accompanied by administration of abundant amounts of lipotropic factors.

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Mr. William D. Wilson was responsible for the preparation of histological material, and Mrs. M. E. Lindsay aided in the preparation of the manuscript. Their expert and willing assistance is gratefully acknowledged.

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RESUMEN

Efectos de la colina sobre las lesiones cardiovasculares inducidas por la suministración de grandes dosis de vitamina D

De 25 ratas alimentadas con una dieta basal deficiente en colina, 9 mostraron varios grados de esclerosis aórtica. Diez y nueve de 33 ratas deficientes en colina alimentadas con la misma dieta basal pero suplementada con grandes dosis (15.000–20.000 u.i./rata/día) de vitamina D (calciferol) mostraron lesiones aórticas esencialmente del mismo tipo. La adición a la dieta basal de suplementos de colina impidió completamente el desarrollo de
la esclerosis ártica en 25 ratas y logró también prevenir la aparición de lesiones en otros 33 animales alimentados con la dieta basal suplementada con grandes cantidades de vitamina D. El suplemento de colina tuvo un efecto similar, pero no completamente protector, sobre las arterias coronarias.

La suplementación con colina de las dietas basales (con o sin adición de calciferol) ingeridas por las ratas impidió el desarrollo del daño renal.

Se discuten el mecanismo de la acción protectora de los suplementos de colina sobre el sistema cardiovascular de ratas que han recibido cantidades excesivas de vitamina D y el posible papel del riñón en este respecto.

Las aplicaciones clínicas de estos hallazgos, sea a la profilaxis sea al tratamiento de las enfermedades arteriales en el hombre, no son aparentes en la actualidad. Sin embargo, a la luz de los hechos presentados, pareciera que el uso terapéutico de grandes dosis de vitamina D—en el tratamiento de la esclerosis o de la artritis, por ejemplo—debe acompañarse de la administración de cantidades abundantes de los factores lipotrópicos.