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Failure of Egg-Yolk Feeding to Accelerate Progress of Atherosclerosis in Older Female Swine*

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Misslingen, Atherosklerose bei alten weiblichen Schweinen durch Eigelbverfütterung zu beschleunigen

Summary. Ten older female swine (12—14 years) from the breeding herd that had been a consistent source of animals with more or less advanced atherosclerosis of aortas, extramural coronary, cerebral arteries and other sites were separated to individual pens. Five were continued on the customary garbage ration, and the remainder fed the garbage ration, supplemented by dried egg-yolk in amounts that would supply at least 35 persent of caloric intake. Egg-yolk feeding for 10 to 14 months was associated with significantly less aortic and extramural coronary atherosclerosis. Intramural coronary and cerebral atherosclerosis was not measurably different in the two groups. We suggest that earlier experiments that have found the progress of atherosclerosis in growing animals to be accelerated by egg-yolk feeding should be reexamined for other factors that could have contributed to this result.

Zusammenfassung. Bei alten Schweinen einer großen Zuchtherde wurden mehr oder weniger fortgeschrittene atherosklerotische Veränderungen an Aorta, Herzkranz-, Gehirn- und anderen Arterien festgestellt.

Zehn Mutterschweine im Alter von 12—14 Jahren aus dieser Zuchtherde wurden in Einzelbuchten abgesondert. Fünf Tiere wurden mit den üblichen Küchenabfällen gefüttert. Die anderen fünf erhielten zur Abfallnahrung zusätzlich Trockeneigelb in einer Menge von mindestens 35% der gesamten Kalorienaufnahme. Nach einer Versuchsdauer von 12 bis 14 Monaten war die Atherosklerose an Aorta und extramuralen Herzkranzarterien bei den mit Eigelb gefütterten Schweinen bedeutend weniger fortgeschritten als bei den Kontrolltieren. Die Veränderungen an den intramuralen Koronar- und an den Gehirnarterien erschienen bei beiden Gruppen ungefähr gleich.

Es wird vorgeschlagen, daß frühere Experimente, nach welchen Eigelbverfütterung Atherosklerose bei wachsenden Schweinen stark beschleunigt, nach anderen Faktoren, die möglicherweise zu diesen Ergebnissen beitrugen, neu überprüft werden.

Experiments designed to induce atherosclerosis in swine by manipulating diets usually have started with immature animals which matured during the study (Rowsell *et al.*, 1960, 1965). Therefore, lesions may reflect responses of growing animals to the inbalances of "atherogenic diets" and the arterial

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reorganization that accompanies growth. These variables have been avoided in the current study by using animals of relatively advanced ages (12 to 14 years). Further, these animals were obtained from the breeding herd that had been the source of other female swine of corresponding ages and in which advanced aortic, coronary, and intracranial atherosclerosis had been found (Luginbühl and Jones, 1965, a, b; Luginbühl, 1966, a, b). Thus, the present study also has had a high probability of testing the effects of egg-yolk feeding on preexisting lesions.

Materials and Methods

The herd that supplied these animals was crossbred mainly from Chester White, Hampshire, and Poland China breeds. It was maintained by an outdoor system of husbandry with five to eight female and one male in each enclosure. Cooked garbage from restaurants and military installations was the customary diet, supplemented seasonably by wastes from vegetables and fish. Samples of garbage collected at weekly intervals for analysis during a one-year period gave the following mean values: protein 6.62, fat 12.59, nitrogen-free extract 6.34, and moisture 71.63 percent (Vander Noot, 1963). The animals for this eyperiment were culled from the herd because of age and breeding failures, but were active, strong and only moderately undernourished. Weights were estimated to range from 130 to 200 kg.

Each animal was separated to an individual pen, walled solidly in wood and enclosing about 6 square meters of floor-space. The garbage ration was continued for all of them, but in addition each of the experimental group was fed 450 gm of dry egg-yolk per day for 11 to 14 months, determined by date of autopsy. Egg-yolk was added to, but not mixed with, the garbage ration. It was always eaten completely and was estimated to provide at least 35 percent of caloric intake. This level of egg-yolk in the diet had been found to be highly atherogenic when swine were started on the ration at about three months of age (Rowsell *et al.*, 1960).

Fasting blood samples were drawn from each animal at the start of the experiment and at intervals of about four weeks thereafter during a period of about 10 months for measurements of total cholesterol and lipoproteins. This phase was discontinued when values were found to range widely without a recognizable pattern. Blood cholesterol was measured in the laboratory of Dr. Arthur Whereat, University of Pennsylvania, and lipoprotein in the laboratory of Dr. David Kritchevsky, Wistar Institute of Anatomy and Biology.

All animals were brought to the laboratory to be killed and autopsied, not more than one per day at convenient intervals. Control animals were autopsied during a period of five months, and the egg-yolk group during a period of three months.

Tissues were fixed in 10 percent buffered neutral formalin, embedded in paraffin, sectioned at five to six microns and stained routinely by hematoxylin and eosin and the Van Gieson elastica method for collagen and elastic fibers. Movat's pentachrome stain and alcian blue-PAS were used for selected specimens. Frozen sections were stained by Oil-red "O" for lipids.

Aortic atherosclerosis was graded on an increasing scale from 0 through 5 without staining for lipids. The extent of intimal surface involved and developmental stages of the lesions were combined with changes found in longitudinal and transverse sections selected from representative parts of the thoracic and abdominal segments. Each section was graded individually for intimal and medial changes and all values combined to give the final grade.

For example, grades 1, 2, and 3 represented increasingly extensive intimal and medial involvement by progressively advanced or larger atheromata. Grade 4 however, combined the extensive intimal-medial lesions of grade 3 with aneurysmal dilatation of the abdominal segment. Grade 5, which was not encountered, would have combined the features of grade 4 with hemorrhages into atheroma, ulceration of atheroma and thrombosis.

Hearts were inspected for fibrosis, lesions of the valves and stenosis of the extramural coronary arteries. Extramural coronary stenosis was confirmed by microscopic sections of diseased segments and the grade of stenosis estimated from complete transverse sections and expressed numerically. For example, in grade 1 lesions, lumina had been reduced by

less than 25 percent, grade 2 by more than 25 but less than 50 percent and grade 3 by more than 50 but less than 75 percent. The numerical grade assigned to each animal (Table 2) for extramural coronary atherosclerosis was the maximum lesion found.

The severity of intramural coronary atherosclerosis was graded by the "heart-score" method, which has been described and its validity established by data contained in earlier publications (Ratcliffe and Snyder, 1965, 1967; Ratcliffe *et al.*, 1969). "Heart-score" values are estimates based upon two factors: (1) counts of cross-sections of arteries greater than 15—20 microns luminal diameter in sections of a complete standard transverse block of each heart cut through the walls of both ventricles at approximately the level of the mid portions of the left papillary muscles, and (2) estimated grades of stenosis in these arteries and the number of arteries affected by each grade. Values for stenosis represent estimated luminal reduction by cells, fibers, and intercellular substances between internal elastic membranes and endothelium whether concentric or not. In these estimates values of $1 =$ less than 25 percent luminal reduction; $2 <$ 50 percent, $3 <$ 75 percent, $4 >$ 75 percent and $5 =$ occlusion. Counts of arteries and estimates of stenosis are combined to derive heart-score values; e.g. $1 \times 23 + 2 \times 20 + 3 \times 21 + 4 \times 25 \div 164 = 1.38$, where 1, 2, 3 and 4 are grades of stenosis, 23, 20, 21 and 25 the number of arteries so classified and 164 the total arterial cross sections (75 without lesions) for a sample of myocardium. Values so derived are increased by 1.0 for convenience in statistical analysis, which, in this example would be "rounded" to 2.40. A heart-score of 1.0 would mean that no lesions were found. Values increase with the number of arteries found to be stenotic and the degrees of stenosis in each.

Brains were inspected macroscopically after fixation, for atherosclerotic stenosis of the extracerebral arteries; then cut transversely at intervals of about five millimeters and inspected for malacic foci. Transverse blocks that contained softened or liquified foci were taken for section. Sections were also prepared from corresponding blocks of brains in which these foci were not visible macroscopically.

Observations

Blood Cholesterol and Lipoproteins

Mean values for total blood cholesterol and lipoproteins, each representing six to ten individual measurements, are listed in Table I for the ten swine of this experiment. Swine numbered one through five were fed garbage plus 450 g of dry egg-yolk per day, while numbers six through ten were continued on the unsupplemented garbage ration.

The mean values for cholesterol may be interpreted as indicating that egg-yolk feeding was accompanied by increases in the blood level of this substance. However, values for both cholesterol and lipoproteins for each sample ranged widely without recognizable relations to individual animals or the seasons of the year. Standard deviations for each series of values were therefore large and

Table 1. *Mean values for total blood cholesterol and for lipoprotein in five swine on dried egg-yolk and garbage (Nos. 1—5) and five on the unsupplemented garbage ration (Nos. 6—10). Means represent 6 to 10 determinations*

Swine number	Total cholesterol	Lipoprotein		Swine number	Total cholesterol	Lipoprotein	
		α	β			α	β
1	135.10	13.7	46.3	6	112.10	18.8	31.8
2	143.70	20.3	64.9	7	142.00	18.7	73.2
3	110.00	10.6	51.6	8	109.33	14.6	41.4
4	126.77	15.4	41.3	9	84.40	10.6	68.1
5	174.10	14.6	77.3	10	99.70	21.9	56.7

Table 2. *Aortic, coronary and intracranial atherosclerosis and associated lesions of older female swine after egg-yolk feeding for 10 to 14 months (1 through 5) and without egg-yolk*

Swine number	Aortic score	Heart			Brain		
		stenosis EMC	heart score	fibrosis	Arterial stenosis		malacia
					ECA	MA	
Egg-yolk							
1	1	0	2.55	1	2	2	++
2	4	1	2.80	2	2	3	++
3	2	0	3.25	2	1	1	0
4	1	0	2.45	0	2	2	+
5	1	0	2.65	1	1	4	++
Control							
6	4	4	2.45	4	0	1	0
7	4	2	2.55	0	2	2	+
8	3	2	2.55	2	0	2	+
9	3	2	2.60	2	1	4	++
10	3	0	2.65	1	2	4	++

EMC = Extramural coronary arteries, ECA = Extracerebral arteries, MA = Meningeal arteries.

indicated that differences in mean values did not measure significant changes. Certainly these values did not correspond in any way to the grades of atherosclerosis found at autopsy (compare Tables 1 and 2).

Atherosclerosis and Associated Lesions

Grades of aortic atherosclerosis and atherosclerosis of the coronary and intracranial (extracerebral and meningeal) arteries, myocardial fibrosis and cerebromalacia found in the experimental and control animals are listed in Table 2. Grades of aortic and extramural coronary atherosclerosis were derived from examination of complete specimens. Similarly, heart-scores represent adequate samples. Therefore, differences in the values for these three lesions may be tested statistically. Other values given in Table 2 are less exact, and differences may not be examined statistically.

The aortic score of only one experimental animal equalled or exceeded aortic scores of controls. This was animal number 2 for which the aorta was graded 4 (Fig. 1). Differences in lesions of the extramural coronary arteries were more pronounced. Only one of the animals on egg-yolk feeding had an extramural coronary lesion that caused an appreciable reduction ($<25\%$) in luminal diameter. In contrast, luminal diameters were reduced to a greater degree in four of the controls. In three of these animals, the reduction amounted to more than 25 percent but less than 50 percent. In the fourth animal, the grade of luminal reduction was more than 75 percent.

This fourth animal was exceptional for the series as a whole which has included about 50 older females from this herd. It had also developed grade 3 atherosclerosis of the proximal right coronary artery. At the same time,

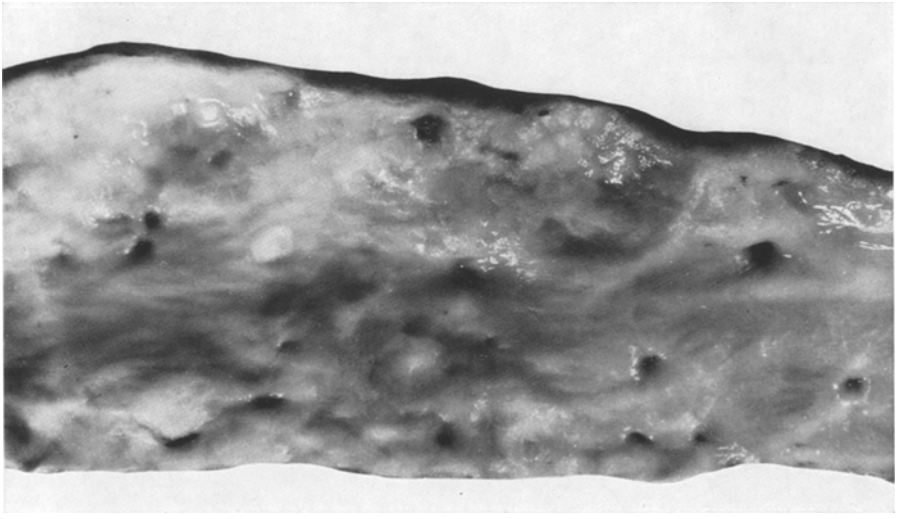

Fig. 1. Swine, aged approximately 13 years. Segment of abdominal aorta with atherosclerotic changes involving much of intimal surface

atheroma formation had reduced the lumen of the left anterior coronary artery by more than 75 percent (Fig. 2), but neither complete occlusion nor thrombosis was found.

Coronary atherosclerosis in this animal was associated with circumferential fibrosis extending irregularly over a relatively large segment of the left ventricular myocardium including the septum. Transverse sections of this heart (perpendicular to the longitudinal axis) demonstrated that the fibrotic zone extended from about the level of the mid portion of the left papillary muscles into the apical region. About three centimeters from the apex, fibrosis was localized more or less to the anterior left ventricular wall where thickness was reduced and the muscle more completely replaced by scars. The appearances of the scars in this region indicated at least three episodes of ischemic necrosis and a focus of intensely eosinophilic fibers from which nuclei had disappeared was taken to indicate a fourth ischemic episode within about 48 hours before the animal was killed.

Atherosclerosis of the intramural coronary arteries as measured by the heart-score method apparently was not influenced significantly by egg-yolk feeding. Nevertheless, values for the animals fed egg-yolk ranged more widely and the values for animal number 3 of this group recalls the heart-scores for separated females in another experiment (Ratcliffe *et al.*, 1969). Mean values for animals with and without egg-yolk feeding differed by a relatively small fraction (0.20), however, and with the exception of swine number 3, heart-scores for this series were at the lower limits of values which in other experiments have been found to be associated with myocardial necrosis and fibrosis (Ratcliffe and Snyder, 1967; Ratcliffe *et al.*, 1969). Further, intramural coronary stenosis probably developed more slowly in this series than in the animals of these other experiments, which would have allowed opportunity for collateral channels to develop.

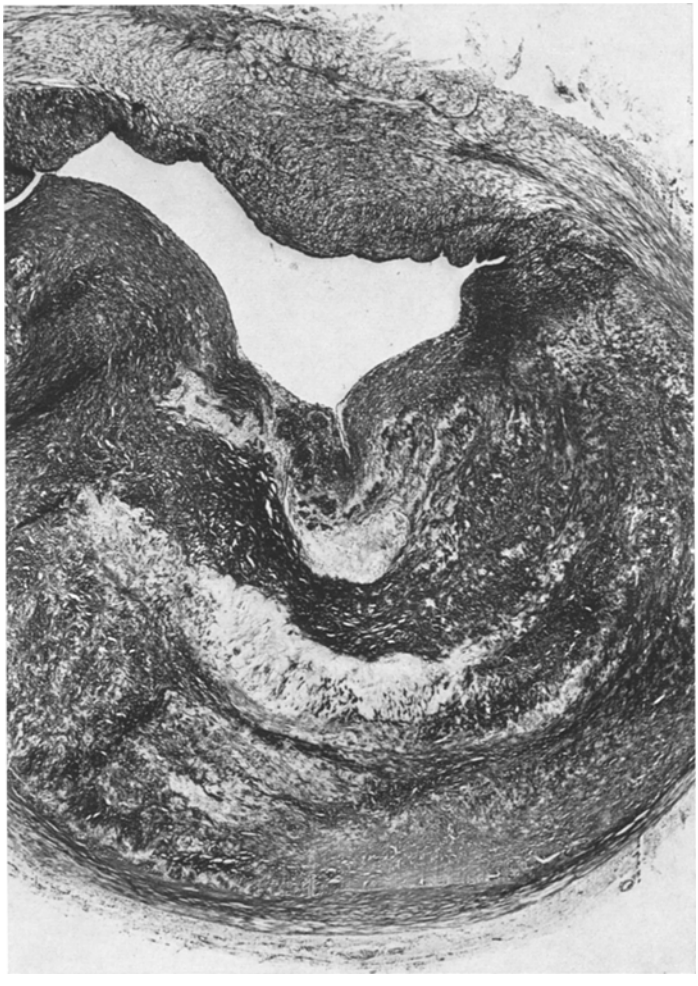

Fig. 2. Swine, female, aged approximately 13 years. Left coronary artery, transverse section; advanced stenosis by atheroma formation, media reduced in thickness to a fraction of the atherosclerotic intima; Movat pentachrome stain, low power

We have assumed that grades of aortic and extramural coronary atherosclerosis (Table 2) of controls represented expected values. This assumption is based upon study of about 40 other females from this herd and younger by 2 to 3 years than the females of this experiment. Accordingly, these values for controls have been compared with values for the experimental animals by means of the chi-square test. This indicates that egg-yolk feeding was associated with significantly less aortic and extramural coronary atherosclerosis ($P<0.05$).

Atherosclerosis of the extracerebral arteries of these swine is shown in Table 2 as having been more advanced in the egg-yolk group. It will be recognized, of course, that grades of stenosis shown in the table represent impressions at best. Size of these arteries limited macroscopic observations, and sections did not include all foci of apparent stenosis.

Lesions of meningeal arteries were estimated from microscopic sections which have indicated that lesions of these smaller arteries were not uniformly distributed. Accordingly, grades of stenosis given in the table for meningeal arteries may have been influenced by chance distribution of lesions within the sections.

Presumably, cerebromalacic foci found in these animals were a result of ischemia following atherosclerotic stenosis of intracranial arteries. The number and size of these cerebral foci did not differ appreciably between the two groups of animals. The records of Table 2 also indicate that atherosclerosis of the aorta, coronary arteries and intracranial arteries are independent lesions.

Discussion

The swine for this experiment were supplied by the breeding herd that had been a rich and consistent source of animals with advanced atherosclerosis of the aorta, the extramural coronary arteries and the cerebral arteries (Luginbühl, 1966, a, b; Luginbühl and Jones, 1965, a, b). Further, these animals were older by two to three years than others examined earlier from this herd. It seems reasonable therefore, to assume that atherosclerosis of aortas, extramural coronary arteries and cerebral arteries was not less advanced in them than in animals studied earlier, and that at the start of this experiment the range of development of the lesions was approximately equal in the two groups. When the experiment was terminated however, lesions of aortas and extramural coronary arteries were measurable smaller in the animals that had been fed egg-yolk. Therefore, we suggest that under the conditions of this experiment spontaneous atheromata of aortas and extramural coronary arteries were reversable.

Thus, the results of this experiment do not support the view that high levels of certain dietary fats alone accelerate atherosclerosis in susceptible species. Rather, these results encourage further reappraisal of the evidence that supports the concept that high levels of certain dietary fats are important in the etiology of naturally occurring atherosclerosis. The cause or causes of spontaneous atherosclerosis have not been identified but certainly dietary fats do not appear to be a primary factor within the customary levels of intake (Rowsell *et al.*, 1960, 1965; Ratcliffe, 1966).

However, experiments designed to induce atherosclerosis apparently have been based on the assumption that high dietary fats merely accelerate a disease process that has been underway since birth. Thus, customary diets are said to be "enriched" by fats or fats and cholesterol and usually are fed to growing animals. Lesions that develop under the influence of such dietary alterations may not have a direct relation to those that develop with increasing age in species that are susceptible to spontaneous atherosclerosis.

The present study also shows that levels of blood cholesterol and lipids need not be elevated for the development of advanced aortic and extramural coronary atherosclerosis.

Still, there is evidence for attributing aortic atherosclerosis at least, to multiple nutritional deficiencies (Rinehart and Greenberg, 1951). This has been amplified by experiences of the Philadelphia Zoological Garden (Ratcliffe and Cronin, 1958; Ratcliffe *et al.*, 1960).

Comparisons between the distribution and severity of atherosclerosis in these garbage-fed swine and younger swine (6—9 years) on controlled rations of high quality suggest that diet (cooked garbage) may have contributed to the disease process (Ratcliffe *et al.*, 1969). The improved physical status of the garbage-fed animals with egg-yolk feeding suggests that protein deficiency (ca 7%), combined with the relatively high content of fat (ca 13%) may have been a major factor in the development of atherosclerosis. Possibly, this factor also contributed to atherosclerotic stenosis of the extracerebral arteries, but apparently not of the smaller meningeal arteries. Stenosis of the meningeal arteries has been more advanced in younger animals on diets of high quality.

The results of this experiment also illuminate a fallacy inherent in experiments that depend upon "enriched" diets and assume that lesions in the aorta, for example, are equivalent to lesions in other arteries. This study demonstrates that atherosclerosis of the aorta and coronary arteries develops independently, which corresponds to the results of other studies (Baker *et al.*, 1961; Ratcliffe, 1965, Ratcliffe and Snyder, 1965, 1967; Ratcliffe *et al.*, 1969; Solberg *et al.*, 1968).

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