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STEREOLOGICAL ANALYSIS OF PATHOLOGICAL CHANGES IN THE BLOOD-VESSEL WALL AFTER EXPERIMENTAL INDUCTION OF HYPERCHOLESTEROLEMIA

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ABSTRACT

The microarchitecture of blood-vessels of elastic type (aorta abdominalis, arteria iliaca) was observed at rabbits after a long time diet enriched by cholesterol. Using optical microscopy pathological changes along the blood-vessel were studied.

Using the Cavalieri principle the volume of atherosclerotic lesions is estimated and the precision of estimators investigated.

The permeability of blood-vessels is quantified by the ratio of lumen section area without and with plaques from transverse histological sections.

Key words: atherosclerotic plaques, blood vessel wall, Cavalieri principle, stereological methods

INTRODUCTION

The normal artery wall consists of three reasonably well-defined layers: the intima, the media and the adventitia. A single continuous layer of endothelial cells lines the lumen of all arteries. These cells are attached to one another by a series of junctional complexes and are also attached to an underlying meshwork of loose connective tissue, the basal lamina. These lining endothelial cells normally form a barrier that controls the entry of substances from the blood into the artery wall by specific transport systems. The intima is delimited on its own aspect by a membranose form of elastic tissue - the internal elastic lamina. The smooth-muscle cells in media form abundant collagen, elastic fibres, soluble and insoluble elastin, and glykosaminoglykans, cf. Salzmann et al.(1987). These cells metabolize glucose by both anaerobic and aerobic glykolysis. A variety of catabolic enzymes are present including fibrinolysis, mixed-function oxidases and lysosomal hydrolases. Arterial wall cells can synthetize fatty acids, cholesterol, phospholipids

and triglycerides from endogenous substrates which appear preferentially to utilize lipids from plasma lipoproteins transported into the wall. Circulating lipoproteins traverse endothelial cells in pinocytotic vesicles. Smooth-muscle cells possess specific high-affinity surface receptors for certain apoproteins on the surface of lipid-rich lipoproteins, thus facilitating the entry lipoproteins into the cell by adsorptive endocytosis. Free cholesterol entering the cell in this manner inhibits endogenous cholesterol synthesis, facilitates its own esterification and partially limits further entry of cholesterol by regulating number of lipoprotein receptors. However, lipoprotein cholesterol can gain entry into arterial smooth-muscle cells by receptor-independent pathways, potentially causing cholesterol ester accumulation.

The ability of the arterial wall to maintain the integrity of its endothelium, to prevent platelet aggregation, to prevent adherence of blood mononuclear cells, to prevent cholesterol accumulation and to ensure the nutrition of its middle portion may be critical determinants of the atherosclerotic process. Because of the prominence of lipids in atherosclerotic lesions, much attention has been directed to lipid metabolism in arteries, cf. Ross(1986).

Atherosclerosis represents an arterial dystrophy process affecting especially elastic arteries and involves primarily the intimal layer. Its extent is, however, variable and occurs most commonly in the abdominal aorta and its large renal and lower extremity branches. Atherosclerotic lesions are generally classified as fatty streaks, fibrous plaques and complicated lesions (Strong, 1992).

Fatty streaks may be the earliest lesions of atherosclerosis. They are characterized by an accumulation of lipid-filled smooth-muscle cells and macrophages (foam cells) and fibrous tissue in focal areas of the intima.

Fibrous atherosclerotic plaques are elevated areas of intimal thickening and represent the most characteristic lesion of advanced atherosclerosis. It consists of a central core of extracellular lipid and necrotic cell debris covered by fibromuscular layer or cap containing large number of smooth-muscle cells, foam cells and collagen. Thus the plaque is much thicker that in normal intima. The presence of multiple risk factor as hypercholesterolemia and hypertriglyceridemia, hypertension and cigarette smoking may be the most potent factors that further accelerates atherosclerosis.

The average cholesterol level is most closely related to the amount of fats in the diet . In experimental animals added dietary cholesterol and fat are essential for the production of atherosclerotic plaques. In general, vascular abnormalities cause clinical disease by progressively narrowing the lumina of vessels and producing ischemia of the tissue perfuse by that vessel, provoking intravascular thrombosis or weakening the walls, thereby leading to aneurysma or rupture (Ross, 1993).

In our experiment we tried to quantify the atherosclerotic thickening of vessel wall and determination of their areal densities in the lower part of abdominal aorta of rabbits after diet complemented by cholesterol. The percentages of changes of the arterial lumen after atherogenic processes is the fundamental knowledge for examination the possible relation between the biomechanical properties and structural wall alterations in atherosclerosis.

MATERIAL AND METHODS

Ten seven-month old rabbits of both sexes (Nb breed, VUFB farm Konárovice, Czech Republic) were used. There were two groups of four experimental animals and the remaining

two animals were used as control. The first four animals were fed by a standard laboratory diet VELAZ complemented by 0.2% of cholesterol, the second four animals received the same diet complemented by 0.4% of cholesterol. The duration of the experiment was 65-75 days, in this time the body weight was 3200-3760 g (mean body weight 3450 g). The rabbits were killed by thiopental overdose. Immediately thereafter the distal parts of the abdominal aorta were dissected and immersed in Bouin fixative fluid for 48 hours. After fixation the samples of tissue measuring approximately 2cm were embedded in paraffin blocks and cut into 5μm thick slices. Twentyfour serial sections were stained for light microscopy with the haematoxylin - eosinealciane blue, or by Masson's green trichrome, respectively.

In order to confirm the occurence of a detailed cytology some tissue pieces were also prepared for transmission electron microscopy (TEM). Tissue blocks were fixed with 0.4% aquaeous osmium tetroxide solution (SPI-CHEM, Pa., USA) in 0.1 M phosphate buffer, pH 7.39 for 1 hour. After a through wash in the phosphate buffer (12 hours) the tissue blocks were dehydrated and embedded in mixture EPON 812 - DURCUPAN (SERVA). Semi-thin sections approximately 800-1000 nm were stained by alciane blue and examined. Thin sections were mounted on formwar-coated grids and double stained by uranyl acetate and lead citrate. Micrographs were obtained with TEM TESLA BS 500 operated at 60 kV, see Fig.1.

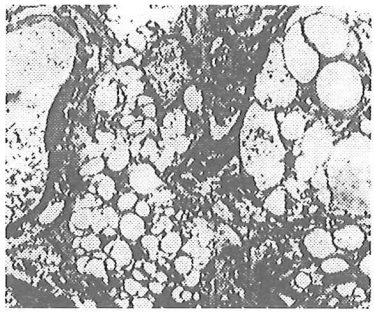

Fig.1: TEM image with details of foam cells, 23000×

The methods of quantitative analysis include the Cavalieri principle for volume estimation (Roberts et al. 1993) of atherosclerotic plaques from transverse sections of blood vessels. Let T be the distance between serial sections, m their number and A_i the area of plaque section in $i-$th plane, then the volume V of plaques in the block cut in m sections is estimated as

$$estV = T\sum_{i=1}^{m} A_i.$$

Using the point grid we estimate areas A_i as

$$estA_i = \frac{a}{pM^2}P_i \qquad (1)$$

and altogether

$$estV = T\frac{a}{pM^2}\sum_{i=1}^{m} P_i$$

where M is the magnification, a/p the test area per grid point and P_i are the data - number of grid points in measured object. It is useful to define a parameter V_L - mean volume per unit length of the blood vessel. Its estimator is obtained as

$$estV_L = estV/mT. \tag{2}$$

The permeability decrease of the blood vessel at a given location is quantified by the parameter $\mathcal{R}$ derived from lumen area $\mathcal{L}$ and plaque area $\mathcal{P}$, cf. Fig. 2b,

$$\mathcal{R} = \frac{\mathcal{L}}{\mathcal{L}+\mathcal{P}}. \tag{3}$$

Both $\mathcal{L}$ and $\mathcal{R}$ are estimated using a point grid.

The coefficient of error $CE(estV)$ was previously quantified by

$$CE(estV) = (\sum_{i=1}^{m} P_i)^{-1}[\frac{1}{12}(3\sum_{i=1}^{m} P_i^2 + \sum_{i=1}^{m-2} P_iP_{i+2} - 4\sum_{i=1}^{m-1} P_iP_{i+1})]^{1/2}. \tag{4}$$

This formula does not include the nugget effect caused by point grid estimation. Recently the theory was developed by Kieu(1997), where different formulas for $CE(estV)$ were derived (both with and without nugget effect) depending on the smoothness of area section function, cf. Fig. 2a. For irregular functions (with nugget effect) the approximation(the standard formula for independent and identically distributed data)

$$CE(estV) = \frac{SD}{\sqrt{n}Mean} \tag{5}$$

takes place as an extremal worst case. Here standard deviation SD and $Mean$ are evaluated from the sample $\{P_i\}$.

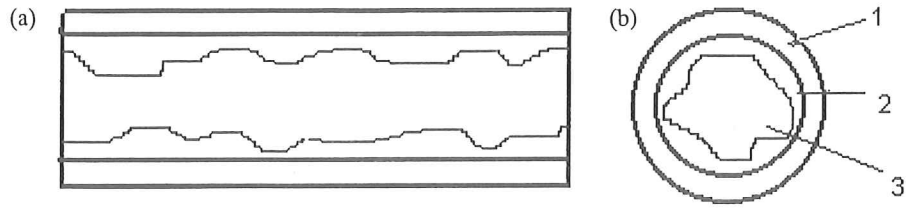

Fig.2: Scheme of a longitudinal (a) and transverse (b) section; 1-vessel wall, 2-plaques, 3-lumen.

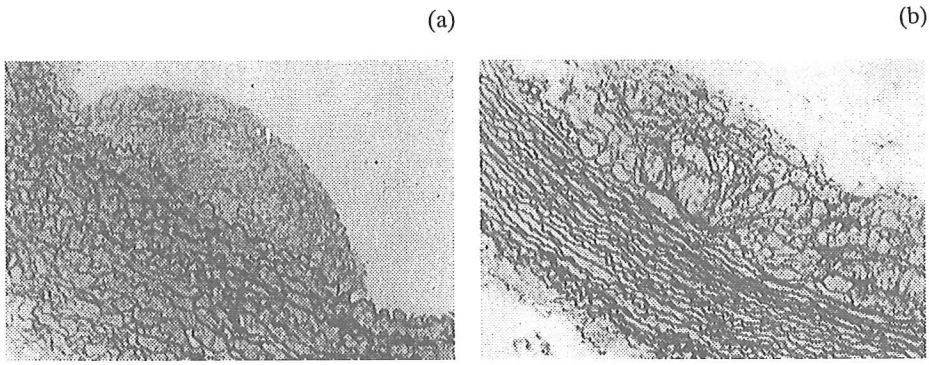

Fig.3: Atherosclerotic plaques in experimental animals, (a) diet 0.2%, 60× (b) diet 0.4%, 160×

RESULTS

In Fig.3 the details of measured atherosclerotic plaques are presented.

Finally, complete results of Cavalieri estimation were obtained for two animals of each group (corresponding to 0.2% and 0.4% of cholesterol) as presented in Table 1. The ratio $\mathcal{R}$ is according to formula 3 (maximum of $m = 24$ sections), the corresponding plaque area $\mathcal{L}$ from formula 1. The V_L parameter is obtained from formula 2.

no. of animal	cholesterol	$\mathcal{R}$	$\mathcal{L}\ [mm^2]$	$V_L\ [mm^2]$
1196	0.4	0.58	2.11	1.54
1200	0.4	0.68	2.47	1.16
1198	0.2	0.70	2.54	1.09
1199	0.2	0.86	3.12	0.59

Table 1: Results of quantitative analysis.

Finally the graphs of $CE(estV)$ with respect to number of sections were presented using the analysis of subsamples, cf. Roberts et al.(1993). In Fig.4 the classical formula (4) was used, while in Fig.5 $CE(estV)$ is evaluated from formula (5). These theoretical values are compared empirical CE's obtained from subsamples.

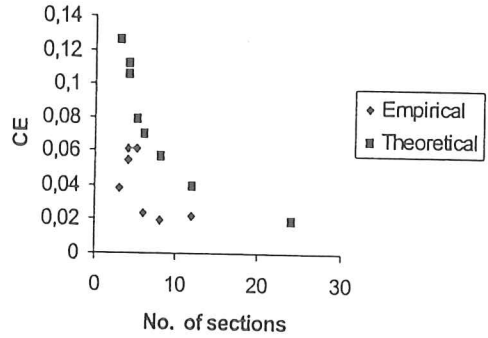

Fig.4: Graphs of $CE(estV)$, animal 1196, cholesterol 0.4%, theoretical values from formula (4).

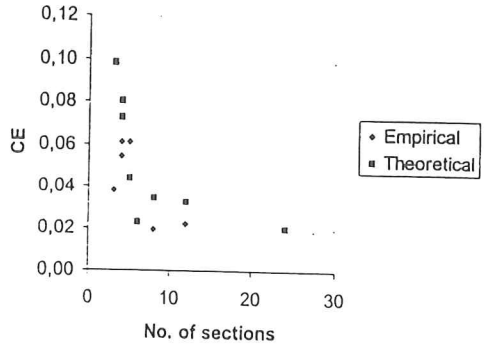

Fig.5: Graphs of $CE(estV)$, animal 1196, cholesterol 0.4%, theoretical values from formula (5).

DISCUSSION

In summary, exposure of experimental rabbits fed with cholesterol addition was shown to induce distinctive atherosclerotic lesions. We observed larger plaques at richer diet, even if limited results do not serve a base for significant statistical test. The detailed morphometric study related to sex difference and fine ultrastructural details involved with athero sclerosis are not yet finished.

The material is extremely interesting for the study of error structure of Cavalieri volume estimator. Along short vessel pieces studied the area function of atherosclerotic plaques is rather flat and its estimator irregular (pure nugget effect). Therefore from variety of formulas proposed by Kieu(1997) for different smoothness of area functions the smallest differences between theoretical and empirical $CE(estV)$ are in Fig.5. This behaviour was already explained in Roberts et al.(1993) using a different biomaterial.

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