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REVIEW PAPER

STEREOLOGICAL AND MORPHOMETRIC STUDIES OF MAMMALIAN MYOCARDIUM: A REVIEW

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ABSTRACT

This review article consists of two parts. The aim is to demonstrate the applicability of the stereological methods in cardiology. The first part briefly describes the histological and cytological structure of mammalian myocardium presented by a hierarchical model. The second part summarizes some sample articles dealing with cardiological problems and using stereological methods. Human research on autoptic and bioptic material is presented, as well as animal research on rats and other mammals, stressing the experimental investigations, which are of interest for theory and practice in human cardiology.

Key words: Cardiology, human, mammals, morphometry, myocardium, rats, stereology.

The aim of this review article is to demonstrate the applicability of the stereological methods in cardiology. Application of stereology requires technical knowledge described in textbooks (e.g. Weibel, 1979, 1980; Howard and Reed, 1998). Besides, certain knowledge of statistics is indispensable for a correct usage of stereological methods, as well as for interpretation of the data.

MAMMALIAN MYOCARDIUM

Mammalian myocardium consists of cardiac muscle tissue and interstitium, comprising connective tissue, blood and lymph vessels, and nerves. Cardiac tissue consists of cardiomyocytes. Each cardiomyocyte is surrounded by sarcolemma, has a nucleus and sarcoplasm, containing various organelles such as contracting myofibrils, energy producing mitochondria, regeneration enabling sarcoplasmic reticulum, impulse conducting transverse tubular system, desintegration enabling lysosomes and occasionally paraplasmatic inclusions (e.g. lipid droplets). Cardiomyocytes form muscle fibers and are separated by Z lines. Two functional types of cardiac tissue exist (conducting and working tissue) (Fig.1).

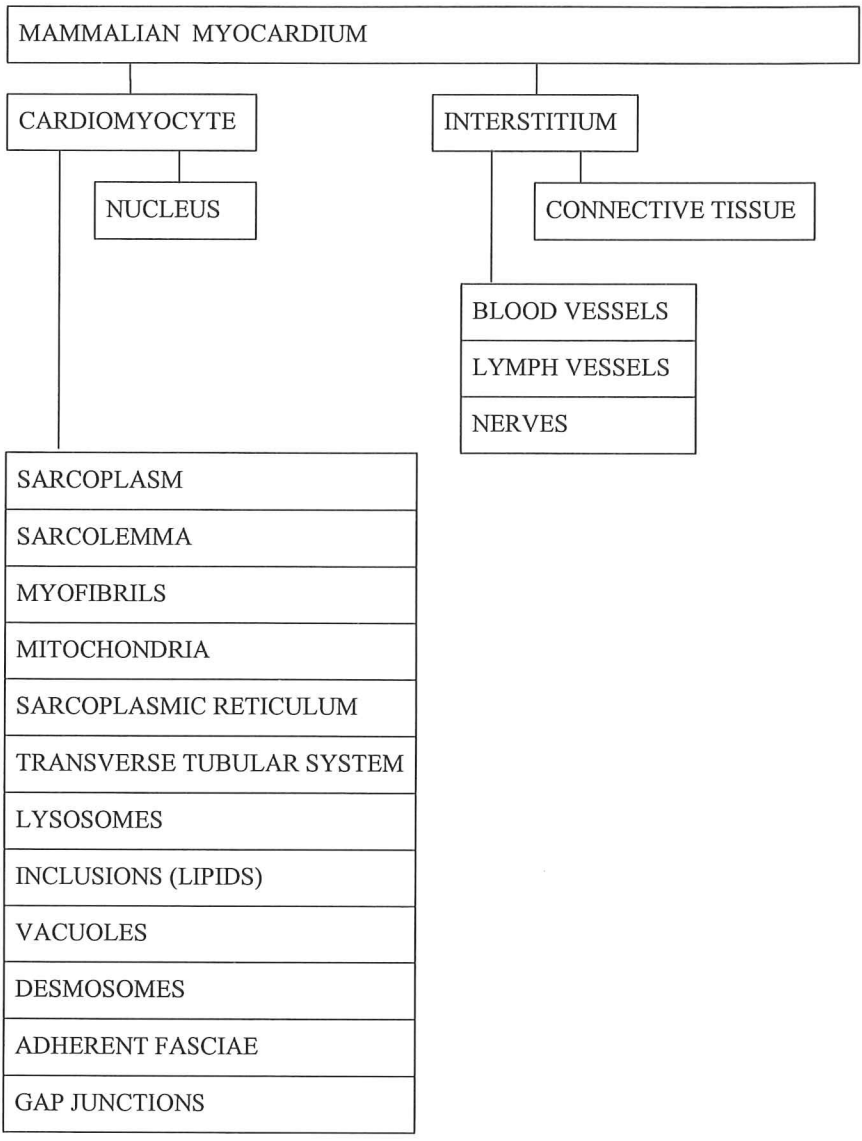

Fig 1. *Hierarchical model of mammalian myocardium.*

APPLICATION OF STEREOLOGY IN CARDIOLOGY

Since the foundation of the International Society for Stereology in 1961 stereological methods have been applied in several hundred papers concerning cardiology. A sample of these papers published mostly in the last two decades is presented here with short indicative abstracts or even annotations. "Qualitative microscopic studies, with expert interpretation and analysis, will continue to play a valid and useful role in the initial stages of many scientific problems. However, it is the use of quantitative methods that is the hallmark of modern scientific research" (Howard and Reed, 1998). The intention of this review article is to prove the validity of the above sentence in the field of cardiology.

Research work on human myocardium

Some problems of normal ontogeny, as well as some pathological problems have been studied on *autoptic material*. Two groups of authors have investigated prenatal and early postnatal development of human heart. The group in Rio de Janeiro (Mandarim de Lacerda et al., 1995, 1997) studied volume densities of myocytes and interstitium, numerical density of myocytes, total volume and mean volume of myocytes in embryonal and fetal period. The group in Nottingham (Austin et al., 1995, Mayhew et al. 1997) investigated the total number of myocytes and the mean volume of myocytes in fetuses and neonates. Both groups have found that proliferation of myocytes is linear toward birth but it abruptly or shortly ceases afterwards, though the mean volume of myocytes increases prenatally as well as postnatally.

Several groups of authors studied pathological problems of the conducting system and of myocardial hypertrophy. After serial sectioning of the interventricular septum in patients with chronic left anterior hemiblock Demoulin et al. (1975) have found that the density of fibrosis was significantly greater throughout the conducting system when compared with subjects of the corresponding age but with no conduction disturbances.

The group of authors in Heidelberg has found that in hypertrophic hearts the length and surface area of capillaries per unit tissue volume decrease with increasing heart size (Mattfeldt et al., 1989). The group in Poznan (Grajek et al., 1990a, 1990b) has studied myocytes in patients with cardiac muscle hypertrophy of various left ventricle weight. At the weight of 250 g, hyperplasia was absent, only hypertrophy was present. From 251 to 350 g, hypertrophy and signs of hyperplasia were present. At 350 g, marked signs of hyperplasia were present. In 1993 this group has confirmed the above mentioned findings (Grajek et al., 1993). Once a critical cardiac mass of 350 g has been reached, the hyperplasia of cardiomyocytes becomes the only way for further heart growth. The mean percentage of fibrosis also remained constant when the left ventricular mass increased over 250 g.

Rakusan et al. (1992) have demonstrated proportional capillary angiogenesis in response to congenital pressure overload hypertrophy in children, whereas in adult hypertrophic hearts it was not present.

Since the bioptome technique in obtaining fresh endomyocardial *biopsy samples* was introduced by Sakakibara and Konno (1962) a series of morphological and morphometrical biopsy studies were published concerning congestive cardiomyopathy. Kunkel et al. (1978) and Baandrup et al. (1981) have evaluated correlations between morphological and morphometrical alterations with ejection fraction and prognosis.

Mall et al. (1992) have measured fibre diameter, volume fraction of interstitial fibrous tissue and volume fraction of myofibrils in 3 groups of patients with dilated cardiomyopathy with low, medium or high (normal) ejection fractions. Nine of 21 patients with low ejection fraction (severe cardiomyopathy) died 2 years after biopsy. They had significantly higher fiber

diameters, higher volume fraction of fibrous tissue and lower volume fraction of myofibrils. In a study of patients with congestive cardiomyopathy Figulla et al. (1985) have found that reduced myofibril volume fraction (< 60%) has a prognostic significance for hemodynamic deterioration and death. From their point of view heart transplant should not be performed in patients with myofibril volume fractions of 60% or more, since prognosis is good for these patients.

Bosman et al. (1989) studied right ventricle affected with dilated cardiomyopathy. They have found increased mitochondria in number and size and dislocated myofibrils towards the sarcolemma.

In a study about relations between quantitative morphologic findings and left ventricular contractile function in patients with idiopathic dilated cardiomyopathy Schwarz et al. (1983) have confirmed a positive correlation between volume fraction of myofibrils and ejection fraction, so as between reduction of volume fraction of myofibrils and increased fibrosis. Fiber diameter and fibrosis have shown a negative correlation.

Fleischer et al. (1980) have studied left ventricular biopsies obtained from patients with mitral insufficiency and from patients with aortic valve disease. The morphometrical results were compared with normal human left ventricle. The morphometrical differences between normal heart and volume loaded heart in mitral insufficiency were smaller than those between normal heart and pressure loaded left ventricle due to aortic valve disease.

Counting inflammatory cell profiles in myocardial tissue had improved the diagnosis of myocarditis established by endomyocardial biopsies. Edwards et al. (1982) using sections stained with hematoxylin-eosin have considered that a mean lymphocyte count of fewer than 5/high power field or 25-30/mm^2 of myocardium does not prove myocarditis. Schnitt et al. (1987) have shown excellent interobserver concordance for counting interstitial inflammatory cells, predominantly leucocytes, after immunoperoxidase staining using antibodies to leucocyte common antigen in endomyocardial biopsy samples of normal myocardium or myocardium with mild, nonspecific changes. However, the results must be interpreted with caution since this antibody stains other leucocytes in addition to lymphocytes too (e.g. mast cells). The morphometric study of Zorc et al. (1995) on endomyocardial biopsy samples from patients with dilatative cardiomyopathy and myocarditis revealed significantly higher numerical areal density of leucocytes and mast cells in patients with myocarditis.

Vassilev and Matchev (1986) have compared endomyocardial biopsies of patients with aortocoronary bypass to those with aortic valve replacement, before and after cardioplegic ischaemia. The mitochondria in cardiomyocytes in the first group were diminished in size and increased in number, though in the second group they were increased in size and diminished in number. The authors assume that in the second group a swelling or fusion of the mitochondria took place and so these patients were in a worse state of preservation after cardioplegic ischaemia and reperfusion. Similar results were obtained by Lindal et al. (1995).

Morphometry of different tissue components in aortocoronary autovenous bypass grafts from patients with occluded vessels have revealed a significant correlation between intimal proliferation and hypertension (Žemva et al. 1990).

Morphometrical methods were also used for evaluation of myocardial fibrosis in transplanted hearts. Pickering and Boughner (1990) have studied the effect of total ischemic duration of donor heart on myocardial fibrosis in cardiac transplant recipients. The total ischemic time was defined as the time from clamping of the donor aorta during harvesting to unclamping of the recipient aorta after aortic anastomosis. The time ranged from 70-363 minutes. The results showed that collagen volume fraction (degree of fibrosis) correlated with

the total ischemic time. Due to fibrosis which may contribute to the impared diastolic function, this relation should be taken into account when donor heart acceptability has to be evaluated.

Animal research

Research on rats

The majority of observations and experiments on animals has been performed on rats, some on other mammals also. The aim of these studies was to obtain additional information about the etiopathogenesis and therapy of various pathological entities in human.

Lindal et al. (1990) have compared two perfusion techniques performed on perfused rat hearts after prolonged global hypothermic cardioplegic ischemia: a gentle procedure with gradual rise in perfusate temperature and pressure to physiological levels in over 30 min, and an abrupt one. The degree of mitochondrial injury and myocyte edema was reduced by gentle reperfusion, corresponding to the results of Vassilev and Matchev (1986) as well as with the own results of Lindal et al. (1995) on human hearts.

Mandarim de Lacerda and Pessanha (1995) have found a high mitotic activity in the rat myocardium during prenatal life and after birth. Knaapen et al. (1996) have concluded alike that the increase of myocardial volume during embryonic life of rats is caused by cardiomyocytic hyperplasia and not by their hypertrophy. Mattfeldt and Mall (1987) have studied the normal growth process of myocardium cells and capillaries in the late postnatal period (after weaning) and have found that relative ventricular weight per body weight was decreasing continuously throughout the growth process and that the density of myocardial capillarization (length, surface area, and volume of capillaries per unit tissue volume) decreased with increasing heart size, though the total capillary length and left ventricular weight showed a high positive correlation. Schmucker and Sachs (1985) have examined quantitative submicroscopic changes in rats of various ages. They did not find any change in volume density of mitochondria, myofibrillar mass and lipids with the advanced age.

Atrial natriuretic peptide (ANP) in rat hearts has been the object of investigation of three research groups. Skepper et al. (1989) have seen a reduction in the number of ANP containing granules in the right atrium after expansion of blood volume, what was additionally supported by bilateral cervical vagotomy. Todd et al. (1990) have observed decreased cardiomyocyte granularity in rats with diabetes induced by streptozocin. Cavallini et al. (1992) have found that application of triiodothyronine in rats has provoked an initial elevation followed by a fall and again an elevation in plasma ANP. It was accompanied by corresponding changes in numerical density of specific granules in atrial myoendocrine cells.

Several research groups have studied various aspects of hypertension in rats.

Loud et al. (1978) have induced hypertension in rats by constriction of the left renal artery. After 1-4 weeks of hypertension endocardial myocytes were enlarged for 21 %, while epicardial myocytes showed 37 % hypertrophy. Epicardial cells nuclei were significantly longer than the nuclei of endocardial cells. (Similar results were obtained by the scientists from Leiden (Vliegen et al., 1987) who found that in hypertrophic human heart myocytes the volume in subepicardial area had increased by 78 % and in subendocardial left ventricular area by 43 % respectively, indicating transmural differences in the extent of myocyte hypertrophy.)

Olivetti et al. (1987) studied hyperplasia of myocytes in rats after experimental cardiac hypertrophy. Six months after constriction of pulmonary artery there were 76 % increase in right ventricular weight, 41 % increase of total number of myocytes nuclei and 28 % increase

of average myocyte cell volume per nucleus. No morphometrical changes were observed in the left ventricle.

Tomanek (1989) has shown that sympathetic nerves inhibit cardiocyte mitochondrial growth and capillary proliferation during normal and pressure overload induced cardiac enlargement in spontaneously hypertensive rats. Pereira and Mandarim de Lacerda (1997) have induced arterial hypertension in rats by chronic inhibition of nitric oxide synthesis, resulting in hypertrophy of the myocytes, fibrosis and decrease in both length and surface densities of the blood vessels. The group from Heidelberg has studied the length and surface density of myocardial cells and capillaries in rats exposed to a training program as well as with a surgical stenosis of the left renal artery. At hypertrophy induced by running exercise the volume, surface and length density of capillaries per tissue volume remained unchanged, whereas all these estimates were significantly decreased at hypertrophy induced by pressure overload. The ratio of the length density of capillaries to length density of fibers was significantly increased at hypertrophy induced by exercise only (Mattfeldt et al., 1985, 1987).

Mall and al. (1990) studying capillary length density in hypertrophy and physiologic growth in rat heart showed that in hypertrophy induced by physical exercise or by chronic application of thyroxin capillary neoformation in parallel connection counterbalances the increase of oxygen diffusion distance due to myofiber enlargement. In renovascular hypertension, capillary neoformation does not occur in parallel connection.

Amann et al. (1992) studied the therapeutical effects of nifedipin and moxonidine on spontaneously hypertensive rats. They have found that both drugs efficiently prevented myocardial fibrosis, reduced capillarization and regressive changes of myocytes, which were the results of hypertension. Nifedipin additionally enhanced capillary supply beyond the normal level by induction of capillary neoformation (Amann et al., 1992). The same group has failed to substantiate noxious synergistic effects of hypertension and diabetes on rat heart by qualitative morphology. Vascular abnormalities were not observed. However, by stereological methods, they have revealed microstructural reactions which were observed exclusively in hypertensive diabetic groups (and neither in non diabetic hypertensives nor in diabetics): the volume ratio of mitochondria to myofibrils was decreased, the surface to volume ratio of mitochondria was increased (reduction of mitochondrial size) and the mean cross sectional area of capillaries was decreased (Mall et al., 1987; Fischer et al., 1992).

Mattfeldt et al. (1987, 1989) have studied the hypertrophic hearts of rats after thyroxin application. They have found significant increase of ventricular weight and significant increase of mean cross-sectional area of myocardial cells, though the volume, surface and length densities of capillaries pro volume tissue unit remained constant. Besides, the ratio of length density of capillaries to length density of fibers increased significantly; the volume density of mitochondria increased while that of myofibrils decreased significantly.

Two other studies have dealt with experimental diabetes induced by streptozotocin, indicating that the metabolic disturbances of myocardial cells in diabetes might be an important cause of the occurrence of diabetic cardiomyopathy. In diabetic rats the volume densities of mitochondria, sarcoplasmic reticulum, lipid droplets and lysosomes were markedly increased and volume density of myofibrils was significantly decreased (Li, 1990). Moreover, the volume fraction of extracellular components was three times increased and the volume fraction of capillaries was lowered. The surface density and total surface area of capillaries were reduced, and oxygen diffusion distance to myocyte mitochondria increased (Warley et al., 1995).

Rat myocardium has also been studied stereologically in experimental uraemia (Amann et al., 1995), in chronic anaemia (Datta and Silver, 1975), and in chronic thiamine deficiency

(Mall et al., 1986). A Russian group has reported about the results of stereologically analysed changes in rat myocardium during hypokinesia (Nepomniashchikh et al., 1985), during starvation (Nepomniashchikh et al., 1989), at high altitude (Lushnikova et al., 1993), during cooling (Nepomniashchikh et al., 1987), and in whole body overheating (Lushnikova et al., 1993). Some stereological studies have been performed on cardiotoxic effects of alcohol (Mall et al., 1980; Nepomniashchikh et al., 1989), ethyl ether of 3,3-dimethyl-4,4,4-trichloro-5-hexanoic acid (Sarbaeva et al., 1993), of enzymatically generated oxygen radicals (Ytrehus et al., 1987) and anthracycline antibiotic adriablastin (Nurmukhambetov and Ivanov, 1986).

Gevondian (1989) has proposed an equation for estimation of contraction force generated by 1 mm^3 of myocardial cell mass. The evaluation was based on some stereological ultrastructure parameters of rat myocardium. The author claims to obtain contractility data by direct mechanocardiography being quantitatively parallel to those calculated by his equation. His data have not been confirmed yet. The idea that stereology can build a bridge between the morphology and physiology or between structure and function has been expressed before (Weibel, 1982).

Research on other mammals

Marino et al. (1991) have induced progressive hypertrophy in cat hearts by norepinephrine administration. This type of changes was similar to that seen in response to volume overload rather than that seen in response to pressure overload. Jordalen et al. (1985) have reported about lipid accumulation in borderline tissue between ischaemic and normal cat myocardium tissue. According to Grong et al. (1986) non selective beta adrenergic blockade with timolol reduces the appreciable lipid accumulation in borderline tissue of cat myocardium with experimentally produced infarctions.

Lichtig and Brooks (1975) have found that morphological changes after experimental occlusion of the anterior descending coronary artery in pig hearts are not always correlated with functional changes. They believed, that refined stereological measurements will help to overcome the present gap between function and morphology in early myocardial ischaemia.

Quantitative study of normal myocardial tissue in Yucatan swine revealed ultrastructural regional differences in the contents of some subcellular components, consistent with metabolic activity of different cardiac zones (Singh et al., 1981).

Kassab et al. (1993) have published some topological and morphometric data about coronary arterioles, capillaries, and venules in the normal pig heart.

Lukacikova et al. (1989) have produced chronic hemodynamic overload of rabbit heart by perforation of aortic valves. Following heart failure was characterised by an increased volume of mitochondria and decreased volume of myofibrils.

Some descriptive morphological research using stereological methods has been done also on other mammals, e.g. primates baboon (Mandarim de Lacerta and Costa, 1993), marmoset (Burity et al., 1996), dog (leGrice et al., 1995), mice (Forbes et al., 1985, Cluzeaut et al., 1986), hibernating rodent red cheeked suslik (Nepomniashchikh and Kolesnikova, 1989) and bat (Navarathnam et al., 1986).

CONCLUSION

Stereological methods have been presented as useful and valid procedures in quantitative estimation of the three dimensional inner structure of bodies, applicable to the

field of cardiology. These methods are especially recommendable in cases where a qualitative analysis does not give answers or solutions of the problems, e.g. where differences between experimental or observed and control groups seem to be small and inconspicuous, or where the variability of the results blurs these differences. The results of the research in this field so far seem to be important not only for theoretical knowledge in cardiology, but also for practical cardiology, concerning etiopathogenesis, diagnostics, therapy and prognosis of human heart diseases. Let us quote here the words of Shanes et al. (1987): "When the same slides of myocardial biopsy were independently reviewed by seven pathologists they assessed fibrosis in range from 25-69 %, hypertrophy from 19-88 %, nuclear changes from 31-94 % and abnormal lymphocyte count from 0-38 %. Interobserver variability in interpreting biopsy specimens is high so that introducing of a quantitative and standardised method is indubitably important to increase diagnostic consistency.«

More quantitative morphological data obtained by unbiased stereological methods are promising to correlate with functional data, making the research of the inner structure useful for better understanding of human heart in health and disease.

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