

## ORIGINAL PAPER

# Hibernating myocardium, morphological studies on intraoperative myocardial biopsies and on chronic ischemia experimental model

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### Abstract

Hibernating myocardium represent a prolonged but potentially reversible myocardial contractile dysfunction, an incomplete adaptation caused by chronic myocardial ischemia and persisting at least until blood flow restored. The purpose of this study was to investigate the morphological changes and weather relations exist among function, metabolism and structure in left ventricular hibernating myocardium. *Material and methods.* Experimental study is making on 12 dogs incomplete coronary obstruction during six weeks for morphologic studies of ischemic zones. On 48 patients with coronary stenosis myocardial biopsies was effectuated during aorto-coronarian bypass graft. On 60 patients with valvular disease associated with segmental coronary atherosclerotic obstructions during surgical interventions on a effectuated repeatedly biopsies from ischemic zones. Dyskinetic ischemic areas was identified by angiography, scintigraphy, low dose dobutamine echography to identify the cells viability. On myocardial biopsies various histological, histoenzymological, immunohistochemical and ultrastructural methods were performed. *Results.* The morphological cardiomyocytic changes can summarized: loss of myofilaments, accumulation of glycogen, small mitochondria with reversible lesions, decrease of smooth reticulum, absence of T tubules, depression of titin in puncted pattern, loss of cardiotonin, disorganization of cytoskeleton, dispersed nuclear heterochromatin, embryofetal dedifferentiation, and persistence of viability. Extracellular matrix is enlarged with early matrix protein such fibronectin, tenascin, fibroblasts. In experimental material the morphological changes present similarities with the human biopsies, but intermixed with postinfarction scar tissue. Redifferentiation of hibernating cells end remodeling of extracellular matrix is possible after quigle revascularization through aorto-coronary bypass grafts.

**Keywords:** hibernating myocardial biopsy, experiment, embryo-fetal dedifferentiation.

### Introduction

The concept of hibernating myocardium was introduced by Rahimtoola SH [1] when he hypothesized that chronic regional myocardium dysfunction could be viewed as a self protective adaptative process o down regulation of contractile function and metabolism in response to chronic hypofunction. This new study state of flow function match could eventually remain stable for a prolonged period of time and be reversed upon myocardial revascularization [2].

The definition of this state was preceding in the 1970 and early 1980 by several studies reporting on the correlation between regional contractile function and histology [3–5].

In more recent studies, peculiar mismatches have been described among regional wall motion metabolism and blood flow suggesting that the underlying myocardium is metabolic active, through functionally impaired [6–8].

Hence was suggested that more or less healthy myocardial structure underlies this by functional areas. Repeated stunned may be a cause of hibernating state. The contention that was gross structural abnormality are present in "hibernating" myocardium was also because

contractile function could resume rather quickly after restored of blood flow.

Rahimtoola SH [8] has challenged this original idea on functional recovery of myocardium after revascularization and classified the outcome of hibernating myocardium as either rapid/acute hibernating or show (subacute hibernation) to very slow (chronic hibernation).

Before Rahimtoola SH [9] introduced the term hibernating myocardium it was show by others [9, 10] that in fact, a group of patients exists that had significant coronary artery stenosis and impaired wall motion in the absence of myocardial infarction. It was show from intraoperative obtained biopsy material that these regions consisted of viable myocardium, which showed, or variable of structural abnormalities.

The material obtained from chronic dysfunction segments and analyzed in laboratory [1] came from patients who fulfilled the following criteria: severe stenosis of the coronary artery, decreased wall motion and regional ejection fraction; no sign of infarction on ECG; preoperative flow-metabolic mismatch as documented by positron emission tomography (PET) and postoperative (3 to 6 month) recovering of regional contractile function and ejection fraction.

## ☐ Material and methods

This study was made on a number of 60 repeated intraoperative myocardial biopsies (Candea V *et al.*) from patients with valvular dysfunctions associated with segmental coronary obstructive atherosclerosis and from 48 patients with coronary stenosis during aorto-coronary bypass (Mocanu I). The obstructive and diskinetic zone were preoperatory established with several investigations as: coronarography, echocardiography, dobutamide stress, regional ejection fraction estimation.

Experimental studies (with Constantinescu NM in “Carol Davila” University) was made in 1970 to 1979 on a number of 12 conscious dogs with incomplete obstruction on descending and only morphological examination on myocardial ischemic zones.

The following morphological studies was made:

- histological staining: Hematoxylin–Eosin, Van Gieson, PAS–Alcian blue, Lie, Scharlach, Gömöri;
- histoenzymological staining – histological cuttings at 20<sup>0</sup>C, in cryostat: SDH, cytochrome-oxidase, LDH, acid and alkaline phosphatase, cathepsin B, unspecified esterase, membrane ATP-ase.
- immunohistochemical stainings:  $\alpha$ -actin smooth muscle, vimentin;
- biological cellular stainings: TUNEL, propidium iodure;
- electronmicroscopical, after 4% glutaraldehyde fixation Epon 812 imbibitions and ultraphin sections examined with Philips ME 302.

Experimental studies made in 1970 on a number of 120 conscious dogs with Constantinescu NM, on “Victor Babeş” Institute and “Carol Davila” University: incomplete obstruction on left descending coronary artery during 60 days and only morphological examination of myocardial ischemic zones (the actually modern methods non-existed in 70<sup>th</sup> years).

## ☐ Results

The structural changes seen in chronic myocardial segments consist of a typical cellular remodeling which can be well appreciated white light microscopy, especial when tissue are stained with a polychrome stain or with PAS for glycogen. Two important qualitative changes were regularly observed in chronic hibernating myocardium [1].

First, myocardial cells become gradually depleted of contractile material (Figures 1 and 2) without however becoming less volume. The loss of contractile material in some cells was limited to the vicinity of the nucleus. Second, the area of myolysis becomes occupied by glycogen (Figure 3), which stained strongly with PAS.

At the ultrastructural level were a number of changes that are characteristic of poorly performing but viable myocardial segments. The electronmicroscopically changes were stereotypic and involve alterations of a non-degenerative nature, which may be interpreted as progressive structural adaptation with morphologic characteristics of “dedifferentiation”. The observed alterations in the structure were seen in varying degrees of intensity within the patient population and can be summarized:

- Depletion of contractile filaments without cellular volume loss;
- Accumulation of glycogen;
- Size and shape changes of mitochondria;
- Loss of T tubular invaginations.
- Absence of gross subcellular degeneration.

The most striking feature was the gradual depletion of contractile material (Figure 1 and 2) and his replacement by granules of glycogen. The depletion of sarcomeres was most obvious in the perinuclear area but often extend to the cell periphery. The peripherally located sarcomeres stands remained well organized, suggesting that a limited but orderly and unidirectional contractile ability might be preserved.

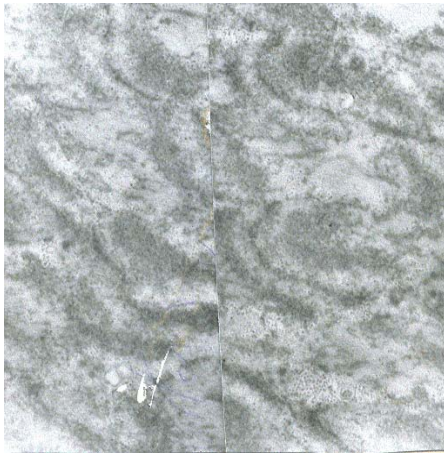
Another important feature (Figure 4) was the presence of many small mitochondria areas adjacent to the glycogen – rich perinuclear zones: most by them were found intermingled wit glycogen. A characteristic change involved the nuclei (Figure 1) in the majority of “hibernating” cells the nuclear heterochromatin was distributed evenly over the nucleoplasm.

The sarcoplasmic reticulum was in most cells “virtually” absent, whereas in few cells a network of unorganized profiles of reticular membranes remained present in the myolitic areas. Strands of endoplasmic reticulum were frequently seen in the center of the cell. The sarcolemma no longer projected protrusions (T tubules) in the cytosol. Very often, the sarcolemma presented numerous pinocytotic vesicles resembling those of endothelial cells. There was remarkable absence of degenerative changes. Most of these substructural alterations are reminiscent of structural characteristics seen in cardiomyocytes during embryonic and fetal development.

The interest in studying in detail extracellular changes in the disfunctioning parts of the chronic hibernating left ventricle [12] is obviously related to the important contributory role the matrix may play in the degree and speed of functional recovery after revascularization. The collagen matrix, the structural component of the connective tissue plays an important role in maintaining [13] the functional integrity of the myocardium. It has been indicated that the composition and distribution of interstitial collagen determines the stiffness of the cardiac muscle [14].

An increase in interstitial collagen in experimentally induced overloaded cardiac hypertrophy has been described [15]. In addition, changes in the amount of interstitial collagen have been reported in non infarcted parts of human myocardium after infarction [16]. The increase in connective tissue was consistently in areas were structurally affected myocardial cells prevailed. Ultrastructural analysis showed aside from amount of collagen fibers (which appeared to be elevated in many of the samples) no striking abnormalities. Experimentally, the incomplete ligation of anterior descending coronary, are caused in ischemic myocardial areas various lesions. On are seen intermixed cardiomyocytes with morphological “hibernating” characteristics (described previous) with reversible and severe lesions also fibrotic post-necrotic scars. In some cases caused sudden cardiac death [17].

**Figure 1 – Human myocardium, mitral regurgitation, heart failure (cl. II–III NYHA), E.M. ×12 000: loss of myofilaments dispersed nuclear heterochromatin**



**Figure 2 – E.M., ×4500: modification of sarcomeres structures and disposition because of myofilaments loss**

**Figure 3 – E.M., ×8000: accumulation of glycogen granules**



**Figure 4 – E.M., ×34 000: small mitochondria, glycogen granules, depletion of myofilaments**

## ☐ Discussions

A very important problem was for recently performed studies [18] demonstrate the viable character of cardiomyocytes that showed myolysis and glycogen storage and underwent mitochondrial, nuclear and sarcoplasmic reticulum alterations.

In the first place, contrary to ischemic/ necrotic cardiomyocytes in acute myocardial infarction, fibronectin remained absent in the cytoplasm of hibernating cells [19]. Conversely transforming growth factor  $\beta$  (TGF- $\beta$ ) which is present in normal cardiomyocytes and absent in ischemic cells after infarction, remained present in hibernating cells. Secondly, there are observations that indicate that hibernating cells are actively metabolizing and possess a number of characteristics that classify them as healthy:

a) Cytochrome-oxidase activity should be demonstrated in the mitochondria despite the fact that they were morphologically altered [20];

b) The integrity of cellular calcium homeostasis was suggested by the absence of cytochemically demonstrable calcium overload.

The phospholipids bound fraction of calcium was preserved at the plasma membrane of hibernating cells and mitochondria were virtually devoid of calcium precipitate [21]. Hibernating cells suffered less from acute ischemia than their neighboring normally structured cells [16].

Examination of biopsies from hibernating myocardium taken at the end of CABG (coronary arterial bypass graft) revealed that "hibernating" cells were much susceptible to acute ischemia than were to adjacent cells, which had a normal ultrastructure [21]. After CABG, the mini-mitochondria of "hibernating" cells were not swollen, whereas in adjacent normally structural cells, mitochondria showed a more marked clarification of the matrix. In addition, the latter mitochondria possessed a lot more calcium deposits than these of the hibernating cells [21].

Moreover no difference in the structure of the sarcolemma-glycocalyx complex of hibernating cells seen between pre- and post-CABG biopsies. The intimate association of the external lamina of the glycocalyx with the sarcolemma was present and no discontinuities in the lipid bilayer were noticed. In contrast, clear abnormalities of the sarcolemma-glycocalyx complex were present in chromatin margination or chromatin clumping as did nuclei of normal cells in the post CABG biopsies.

Apoptotic cell changes were only rarely observed by electron microscopy. Most of the adaptive changes that hibernating cells undergo during chronic ischemia are changes that are renescent of dedifferentiation. Hence, the question can be asked whether hibernating cells are of an embryonic or fetal differentiation phenotype.

The corresponding to structural feature of embryonic or neonatal myocardial cells is:

- a) depletion of contractile filaments;
- b) accumulation of glycogen;
- c) presence of rough endoplasmic reticulum;

d) strong reduction in the amount of sarcoplasmic reticulum;

e) presence of rough endoplasmic reticulum;

f) virtual absence of T-tubules and

g) vesiculation of the sarcoplasm.

In embryonic cells, glycogen also accumulated around the nucleus and glucose is preferred over fatty acids as an energy source. The amount of glycogen in hibernating cells sometimes greatly exceeds the amount visualized in embryonic cells. The smooth sarcoplasmic reticulum is not extensively developed.

The morphologic resemblance between hibernating cells and embryonic or fetal cells is striking, it is necessary to investigate this relation in depth in order to make a firm case for dedifferentiation on hypothesis for hibernating myocardium. For this reason, the expression, assembly and (re)organization of markers of cardiac cell development were investigated. Was used monoclonal and polyclonal antibodies against contractile and cytoskeletal proteins to study the expression and organization of the muscle constitutions in biopsies of patients with chronically dysfunctional but viable myocardium;  $\alpha$ -smooth muscle actin were used as example. During cardiogenesis different structural proteins appear at different phases of myofibril formation and the organization of most of these muscle [specific] proteins changes, with differentiation as titin [22].

Marked changes in this organization and expression were present in hibernating cells. The most distinct mark – the double band cross – striation disappeared first. Titin become visible as single bonds in parts of sarcomeres. The distance between the cross-down regulation of titin appeared in the most severely affected cells in which titin appeared sparsely and only in a punctuate fashion [23].

In contrast to the titin disorganization, other sarcomeric protein such as actin, myosin, and tropomyosin were well organized in remaining sarcomere [23].

Cardiotonin is a recently described structural component of the myocardium in seems to be expressed only after birth. Cardiotonin in normal adult muscle is displayed as longitudinal filamentous structures between the myofibrils perpendicular to the desmin striation. In more severely affected cells, ones the cardiotonin filaments were sparse and ultimately their structure disappeared entirely;  $\alpha$ -smooth muscle actin as a protein found exclusively in cardiomyocytes during embryonic/fetal life. It is not present in cardiomyocytes of the normal myocardium and it was re-expressed in a large number of hibernating cells [25].

Small areas of hibernating myocardium uniformly presented a moderate expression of  $\alpha$ -smooth muscle actin in all cells. In cells with severe myolysis most of the  $\alpha$ -smooth muscle actin was located around the zone affected by myolysis.

In chronic hibernating myocardium, nuclear A-type lamins (lamin A and C) were shown to be down-regulated in majority of these cells. Vimentin protein is present during embryogenesis but absent from adult

cardiomyocytes. The differentiation of these cells has not reached the very development stage [20].

The remodeling of the extracellular matrix was also immunocytochemical investigated. On observed an increase in total collagen content and that this increase in both collagen subtypes I and III [20], but were seen throughout the enlarged interstitial space in chronic hibernating myocardial segment. Thus, protein (as fibronectin) was present in the interstitial space between the cardiomyocytes and at the sarcolemma of the myocardial cells.

In chronic hibernating myocardium fibronectin was found in high amounts throughout to increased interstitial space. The presence of an increased collagen content especially of type I collagen in the extracellular matrix may contribute to increased stiffness of the left ventricle. The collagen may impair contractile function on the cardiomyocytes. It was demonstrated that in the hibernating myocardium the number of cell of endothelial and fibroblast origin (vimentin positive) throughout the interstitial space is increased.

The early matrix proteins fibronectin and tenascin [19] should be demonstrated in the matrix mounding hibernating cells. Because these early matrix protein appear transiently during remodeling after infarction and are absent from mature myocardial scar tissue, it was throughout that these proteins provide an early and important matrix for the deposition and remodeling of other matrix protein such as collagen [23].

The sustained presence of tenascin in the matrix surrounding the hibernating myocardium might be indicative of ongoing interstitial remodeling. As tenascin is only transient present during myocardial scarring and disappeared when collagen deposition is present, it might also in indicative of the reversibility of the matrix increase and thus of the hibernating phenotype of the cardiomyocyte.

Next to mechanical factors such as stretch and circulating factors such as angiotensin II, locally produced cytokines such as TGF- $\beta$  have be considered in the regulation of early matrix protein synthesis [19]. The presence of TGF- $\beta$  in these hibernating cardiomyocytes may suggest that TGF- $\beta$  is involved in the modulation of hibernation. One hypothesis that can be put forward is that TGF- $\beta$  influences hibernating myocardium through modulation there are show product of specific matrix data available, which show an increased content of extracellular matrix in human myocardium.

The subcellular changes as formed in chronic hibernating myocardium are markedly different from these seen in acute stunned myocardium in animals [26] in acute severe leading to infarction [28–31], in transient ischemia [32–34] lead stage cardiomyopathies [34–40].

The subtle changes seen in stunned myocardium animals are frequently encountered in lumen biopsies prior to and after repeated short ischemic periods that occur during the aortic cross-changing procedure used for CABG [41].

Cells that border the fibrosis scar of infarction often of the “hibernating” type [40]. In cardiomyopathic

patients [41] “hibernating” cells are found intermingled with cells with which clearly show signs of degeneration with apoptosis [42].

Structural modification in cardiomyocytes strongly resemble those in hibernating myocardium [38] and including glycogen accumulation have been observed in atrial cells after sustained chronic atrial fibrillation [43].

Among the questions, which remain unanswered, the following deserve special attention [2].

1. Redifferentiation of hibernating cells and remodeling questions related to the reversibility of the “dedifferentiated” state of cardiomyocytes after restoration of blood flow [45]. The structural changes were reversible (redifferentiation) it would take the cells a prolonged period of time to remake a normal contractile machinery. This might explain the delay in recovery of ventricles function after revascularization that is seen in a number of patients with chronic hibernating myocardium [6–9].

Borisov AB [44] are described how cardiac muscle at early, intermediated and terminal stage of differentiation are capable of adaptative remodeling of their contractile system *in vivo* and *in vitro*. The hypo- or akinetic state of the myocardium during chronic hibernation may lead to a similar reduction in the amount of actin and other sarcomeric protein. There appears to be a direct relationship between the degree of cardiomyocyte alteration and amount of connective tissue in chronic hibernation [20].

The amounts of collagen and fibronectin increased considerably along with the number of mesenchymal cells, which produce this extracellular matrix protein. The fact that the matrix in contrast to scar tissue after infarction, still expressed early matrix proteins such as fibronectin [20] and tenascin favors the idea that chronic hibernating myocardium is a state of ongoing remodeling and probably amenable to reversal. In addition, it was shown that the mesenchymal cells were fibroblasts and myofibroblast by write of the absence of  $\alpha$ -smooth muscle actin [20]. This extracellular matrix was produced by these cells is different from that in matrix scar tissue. The presence of an extensive extracellular matrix compartment may be one major causes of the delay in recovery. Full recovery conceivably might only occur when the interstitial tissue is reduced to normal proportions and is of normal composition.

2. Relation between glycogen accumulation and clinical imaging of glucose uptake by positron emission tomography (PET): a point needing a more in dept discussion in the accumulation of glycogen in relation to the possible alteration or performance of metabolic substrate by hibernating cells. Glucose is preferred over fatty acids as an energy source in hibernating cells and the high amounts of glycogen observed may reflect such enhanced uptake of glucose (as in embryonic and fetal heart cells).

3. Experimental models remain a controversial issue. Lows flow ischemic models for acute hibernation have been described but a model for chronic hibernating myocardium without concomitant focal infarction is still lacking in animals.

## ☐ Conclusions

Hibernating myocardium represent a prolonged but reversible postischemic contractile dysfunction, a transient incomplete adaptation to reduced blood flow. The morphological myocardium cells changes can be summarized: loss of myofilaments, accumulation of glycogen, small mitochondria, decreased of smooth reticulum, absence of T tubules, depression of titin, in punctuated pattern, loss of cardiotonin, disorganization of cytoskeleton, dispersed nuclear heterocromatine, embryo-fetal dedifferentiation, maintain of viable character.

Extracellular matrix is enlarged with early matrix protein such as fibronectin, tenascin; fibroblasts. Redifferentiation of hibernating cells and remodeling of extracellular matrix is possible after quickly revascularization through aorto-coronary by-pass graft but the contractility delay in recovery because the presence of an extensive early matrix that is reduced slowly to normal.

In experimental material the morphological hibernating changes present similarity with that is in the human biopsies but intermixed with post infarction scar tissue, and some myocardial cells with degenerative lesions. This research is the first study that was effectuated on morphological in Romania.

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