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EFFECT OF AEROBIC INTERVAL TRAINING ON PATHOLOGICAL REMODELLING AND MITOCHONDRIAL DYSFUNCTION IN THE POST-INFARCTION FAILING RAT HEART

Doctoral Dissertation

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SVEUČILIŠTE U SPLITU
MEDICINSKI FAKULTET

Jasenka Kraljević

UČINAK AEROBNOG INTERVALNOG TRENINGA NA PATOLOŠKO REMODELIRANJE I MITOHONDRIJSKU DISFUNKCIJU U ŠTAKORA S POSLIJEINFARKTNIM ZATAJENJEM SRCA

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PREFACE

The studies included in this thesis have been carried out at the Department of Physiology, School of Medicine, at University of Split during the years 2010-2014 under supervision of my mentor Professor Jasna Marinović – Ljubković. The working hypothesis of the project was that aerobic interval training attenuates deterioration of mitochondrial function in post-myocardial infarction failing rat heart. The main part of the study included in this thesis was published in original paper below:

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FREQUENTLY USED ABBREVIATIONS

HF: heart failure

CHF: chronic heart failure

MI: myocardial infarction

ROS: reactive oxygen species

ETC: electron transfer chain

MPT: mitochondrial permeability transition

MPTP: MPT pore

NYHA: The New York Heart Association

ACC/AHA: American College of Cardiology/American Heart Association

LVSD: left ventricular systolic dysfunction

HFPEF: heart failure with preserved ejection fraction

HFNEF: heart failure with normal ejection fraction

HFPSF: heart failure with preserved systolic function

EF: ejection fraction

LV: left ventricle

CAD: coronary artery disease

cAMP: cyclic adenosine monophosphate

MMPs: matrix metalloproteinases

ACE: angiotensin converting enzyme

1. INTRODUCTION

1.1 Heart failure in general

Heart failure (HF) is a complex clinical syndrome defined as any structural or functional cardiac disorder that impairs the ability of the ventricle to fill with or eject blood. Heart failure is a leading cause of morbidity and mortality in the world as more and more patients survive myocardial infarctions due to advances in therapeutic strategies. The key feature of HF is the deficiency in the capability of the heart to adequately pump blood in response to systemic demands of the metabolizing tissues and is characterized by an altered cardiovascular, skeletal muscle, and neurohormonal function attempting to maintain circulatory homeostasis (1). The main manifestations of HF are dyspnea at rest or during exertion, fatigue, limited exercise tolerance, and fluid retention, which may lead to pulmonary congestion and peripheral edema. Both abnormalities can impair the functional capacity and quality of life of affected individuals, but they do not necessarily dominate the clinical picture at the same time. Because some patients present without signs or symptoms of volume overload at the time of initial or subsequent evaluation, the term “heart failure” is preferred over the older term “congestive heart failure.” It should be emphasized that HF is not equivalent to cardiomyopathy or left ventricular dysfunction. These terms describe possible structural or functional reasons for the development of HF (2). Nearly any form of heart disease may ultimately lead to the HF syndrome. Instead, HF is defined as a clinical syndrome that is characterized by specific symptoms in the medical history and signs on the physical examination (3).

Heart failure is a global problem with considerable morbidity and mortality despite improved understanding of the pathophysiology and better therapeutic options. The prevalence of heart failure can be estimated at 1–2% in the western world and the incidence approaches 5–10 per 1000 persons per year. The prevalence of heart failure in 70- to 80-year-old people is between 10 and 20% (4). However, the overall prevalence of heart failure is increasing due to ageing of the population, as well as increasingly successful therapy and prolonged survival of patients suffering from acute coronary events. However, despite the improved therapy, still 30–40% of HF patients die within a year of diagnosis and 60–70% die within 5 years (5).

1.2 Etiology

The causes of HF are very heterogeneous and range from disorders of pericardium, myocardium, endocardium, heart valves, great vessels or certain metabolic abnormalities, with the most common causes being acute or chronic myocardial ischemia, increased vascular resistance with hypertension, and abnormality in rhythm or conduction. In general, the etiology of HF may be divided in two categories: 1) *ischemic*, resulting from coronary artery disease and myocardial ischemia; and 2) *non-ischemic* (6). In developed world, heart failure most commonly develops as a sequel of myocardial infarction or cardiac pressure overload resulting from chronic arterial hypertension. Coronary heart disease is by far the most common cause of HF, being the initiating cause in 70% of patients with HF (7). Reduced coronary blood flow and oxygen delivery in CAD leads to myocardial hypoxia and impaired cardiac function. Myocardial infarction is the final and often fatal culmination of CAD. Degenerative valve disease is becoming more common and accounts for about 10% of HF. The exact etiology is unknown in 20-30% of cases of HF with depressed EF and therefore categorized in group of idiopathic cardiomyopathies (6).

1.3 Ischemic heart failure

Acute myocardial infarction is one of the most significant causes of HF in human population with the estimated incidence varying from 10% to 40%. Improvements in the management and therapy together with ageing population have contributed to a growing burden of heart failure. HF developed after myocardial infarction is associated with a markedly elevated risk of death, with an estimated median survival of about 4 years (8, 9). Progression to chronic heart failure after a myocardial infarction may or may not developed which depends on multifactorial causes involving the extent of initial myocardial damage, recurrent ischemia and the extent of myocardial remodelling and chronic neuroendocrine stimulation (10). Post-MI HF results from complex processes of pathological remodelling occurring in the

surviving myocardium after one or more MI(s) (11). Heart failure is the most common after anterior MI comparing to other sites (12, 13). Left ventricular systolic dysfunction is the single most common cause of post-MI heart failure. At the cellular level, the main substrate for the development of heart failure is a moderate amount of myocardial necrosis with consequent ventricular remodelling (14, 15). This adverse cardiac remodelling is a key feature of HF generally accepted as a major determinant of clinical course and outcome of the disease, generally preceding the development of symptoms and contributing substantially to worsening of symptoms despite treatment (16). The process of ventricular remodelling occurs rapidly in the early period after myocardial infarction and then more slowly thereafter. Therefore, early identification of adverse cardiac remodelling offers the potential to modify this process and reduce the risk of heart failure.

1.4 Cardiac remodelling after myocardial infarction

Pathological cardiac remodelling encompasses molecular, cellular and interstitial changes after cardiac injury resulting in changes of ventricular size, shape and function, ending in clinical manifestation of the HF syndrome. The process of pathological remodelling is regulated by mechanical (hemodynamic load), neurohormonal and genetic factors. Although remodelling in general is a normal physiological process of adaptation during growth and other physiological stimuli, it becomes pathological due to MI, hypertension or valvular heart disease. Therefore, cardiac remodelling has been described as both an adaptive and a maladaptive process, with the adaptive component enabling the heart to maintain function in response to pressure or volume overloading in the acute phase of cardiac injury (17). However, continued remodelling may not be necessary to maintain the integrity of the circulation after cardiac injury. Once established beyond a certain phase, it may be viewed as an adverse phenomenon that actually contributes to HF progression. However, there is no indication when the transition from possible adaptive to maladaptive remodelling occurs or how this might be identified in patients (18). Progressive remodelling is always associated with a poor prognosis and patients with major remodelling demonstrate progressive worsening of cardiac function. Cardiac

remodelling is now recognized as an important aspect of disease progression and is, therefore, emerging as a therapeutic target in HF of all etiologies (6).

In post-MI models, the process of LV remodelling begins within the first few hours of myocyte injury and is influenced by the combination of infarct expansion, and cardiac pressure and volume overload. Although the exact picture of all pathways involved in LV remodelling is still unclear, intense research efforts have identified several key factors responsible for the post-MI pathological remodelling in the remote non-infarcted myocardium (19, 20). The progression of remodelling and deterioration of heart failure probably occurs in two main ways. One is as a consequence of intervening cardiac events, and the other as a consequence of the systemic compensatory processes activated by the falling cardiac output (20).

1.4.1 Hemodynamic and cellular changes

In general, processes of ventricular remodelling encompass the entire LV in proportion to infarct size. As a result, ejection fraction will decrease in direct relation to the size of the infarction. The early post-MI remodelling involves infarct zone expansion and occurs within 72 hours of MI. Late remodelling involves global changes of LV including ventricular dilatation, distortion of shape and hypertrophy. These changes in LV arise from the profound hemodynamic changes that occur within the post-infarcted LV. The LV cavity enlargement is caused by the volume overload and reflects an increase in the length of the remaining contractile tissue. The increase LV volume augments the stroke volume by the Starling mechanism thus maintaining the relatively normal cardiac output at increased filling volumes. Initially, the compensatory hypertrophy in the remote myocardium makes up for the functional loss of the infarcted myocardium (19, 21, 22). However, over the time this adaptive hypertrophy becomes detrimental because the increased ventricular radius increases the wall stress by Laplace law and thus increases oxygen demand. Progressive left ventricular dilation and hypertrophy, infarct scar thinning, and alterations of the left ventricular geometry adversely affect cardiac function (23). Such long-term progressive remodelling of the LV with increases in the ventricular cavity size can occur up to 2 years post-infarction with the increased risk of cardiovascular death (21, 24).

At the cellular level, remodelling involves myocyte hypertrophy, necrosis, apoptosis, interstitial fibrosis with collagen deposition and fibroblast proliferation. Myocyte death within the infarcted and non-infarcted myocardium plays an important role in the expansion of infarction, thinning of the ventricle wall and the cell slippage in the remote myocardium (25). Apoptosis occurs in the infarcted, peri-infarcted area and the remote non-infarcted myocardium, while necrosis mostly occurs acutely in the infarction zone. Apoptosis is coordinated by multiple triggers, all of which occur in the myocardium after MI, including neurohormonal system activation, cytokines such as TNF alpha and interleukins, oxidative stress, mitochondrial damage, and other extra-cellular factors (26, 27). Both apoptosis and necrosis cause further deterioration in the composition and function of the ventricle (28).

Additionally, both the contractile apparatus and excitation-contraction coupling are significantly altered in failing cardiac myocytes (29). For example, there are changes in myosin heavy chain composition, down-regulation of alpha chains and up-regulation of beta chains (30, 31). Furthermore, disturbed sarcoplasmic reticulum function seems to play a central role for the altered systolic and diastolic performance. Under physiological conditions calcium released from the sarcoplasmic reticulum (SR) is the dominant source for systolic activation of contractile proteins. Diastolic relaxation depends on calcium removal from the cytosol by the sarcoplasmic reticulum and the sarcolemmal Na/Ca^{2+} - exchanger (32). In HF, there is a defect of excitation-contraction coupling resulting from decreased capacity of the SR to accumulate calcium and diastolic calcium accumulation in the cytosol causing the disturbed diastolic function. Such alterations of calcium handling by the SR significantly contribute to disturbed contractile function of individual myocytes as well as to the development of arrhythmias in patients with heart failure (32, 33).

Although the contribution of the interstitium to the remodelling process is still not completely clear, it is well established that alterations in the extra-cellular matrix are critical in the process of ventricular remodelling. The extra-cellular matrix supports the inter-cellular adhesion, coordinates cell signaling through integrins, matrix metalloproteinases and their tissue inhibitors. It is composed of many structural components including fibroblasts, collagen, elastin, and laminin (15, 34, 35). Between these structural components lay the zinc dependent enzymes, matrix metalloproteinases and their respective endogenous tissue inhibitors (36-38). The

balance of degradation and preservation of the structural components is controlled by the activity of matrix metalloproteinases and their tissue inhibitors. Matrix metalloproteinases are secreted from multiple cell types, including endothelial cells, fibroblasts, smooth muscle cells, and cardiomyocytes into the extra-cellular space. Over the past several years substantial evidence demonstrates that matrix metalloproteinases are actively involved in cardiac remodelling after MI (39, 40).

In summary, at the cellular level the final common pathway for the progression of cardiac remodelling and heart failure is the imbalance of hypertrophy and cell death over regeneration, which, combined with the significantly altered intracellular contractile apparatus, results in severe dysfunction at the level of whole left ventricle (41, 42).

1.4.2 Neurohormonal changes

There is substantial evidence that the neurohormonal activation plays a crucial role in cardiac remodelling and in the progression of HF. Neurohormonal activation in HF is major compensatory mechanism responding to falling cardiac output, but it is also a major component of disease progression and of the remodelling process. Sympathetic and renin-angiotensin-aldosterone system (RAAS) respond to decreased stroke volume and, at first, have compensatory role. They result in arterial and venous vasoconstriction, increased blood volume, and temporary improvement in systolic blood pressure and tissue perfusion (43). However, sympathetic and RAAS activation leads to salt and water retention and excessive vasoconstriction that may result in an elevation of cardiac preload and afterload, resulting in augmented cardiac strain, energy expenditure and further progression of HF. The increase in myocardial energy expenditure further decreases cardiac output and leads to myocardial cell death (44). Additional reduction in cardiac output further perpetuates a cycle through neurohormonal stimulation and adverse hemodynamic and myocardial responses. It was shown, that higher levels of circulating plasma norepinephrine correlate with a poorer long-term prognosis in HF patients. Moreover, neurohormonal activation also has direct cytotoxic effects on the myocytes and interstitium that together with stimulated myocardial fibrosis alter the structure and impair the cardiac performance in HF. The neurohumoral factors lead to myocyte hypertrophy and interstitial fibrosis,

resulting in increased myocardial volume, mass, and myocyte loss. As a result, the cardiac architecture changes, which, in turn, leads to further increase in myocardial volume and mass (18).

The role of renin angiotensin aldosterone system (RAAS) has been intensively investigated in heart failure development after a myocardial infarction. It is well established that chronic adrenergic system activation and resulting up-regulation of the RAAS plays a major role in post-MI cardiac remodelling. In the heart, angiotensin II has multiple direct cytotoxic effects on myocytes: inducing apoptosis, promoting cell hypertrophy, and stimulating myocardial fibrosis by angiotensin II type 1 receptor. For example, increased levels of both angiotensin II and aldosterone have been shown *in vitro* and *in vivo* to have cytotoxic effect and increase the rate of myocyte apoptosis (45). Also, locally produced angiotensin II leads to increased myocardial energy expenditure, thus having has similar actions as norepinephrine in heart failure. Finally, aldosterone itself plays a role in left ventricular remodelling, particularly myocardial fibrosis, by stimulating cardiac collagen synthesis, including collagen type I and type III (46-48).

1.4.3 Impaired energy metabolism

Energy depletion, evidenced by the loss of ATP, rise in ADP, and damaged energy transfer *via* creatine–phosphocreatine system, is implicated as a central factor in the development of cardiac contractile insufficiency cardiac contractile insufficiency (49, 50). The complex energetic state of the failing heart includes changes in substrate utilization from fatty acid to glucose, decreased oxidative capacity and energy production as a result of reduced mitochondrial biogenesis, reduced energy transfer by the phosphotransfer kinases, impaired energy utilization and efficiency of energy consumption (49, 50). Several lines of evidence support the concept of reversion to the fetal metabolic phenotype in HF that shifts away from fatty acids to carbohydrate utilization (51). However, decrease in fatty acids oxidation has not been observed in all HF studies and in human studies were not consistent to support the hypothesis of reversion to fetal pattern of metabolism (52). According to clinical studies data both fatty acids and glucose are required for optimal function of the failing myocardium and the observed different substrate selectivity is probably

dependent on severity of the disease. In the early phases of HF there is an increase in fatty acid utilization resulting from increased availability. This initially inhibits myocardial carbohydrate metabolism, but finally overloads the system by accumulation of fatty acids intermediates, and activates negative feedback of fatty acid oxidation (53). In the advanced phase of disease, there is reversion to fetal phenotype with significant reduction in fatty acid metabolism and increased glycolysis. This energetic remodelling may be explained as an adaptive mechanism of cardiomyocytes in response to stress (54-56).

1.5 Mitochondria in heart failure

Mitochondria are organelles, which, besides their obvious role in energy metabolism, have an essential role in cardiac calcium homeostasis, production of reactive oxygen species (ROS) and apoptosis (57). Mitochondria are at the center of cardiac energy metabolism, since they satisfy 90% of heart's daily energy requirements through oxidative phosphorylation (58). Recent studies suggest that alternations in regulatory processes and mitochondrial dysfunction may be a common participant in the major pathogenic pathways that lead to the progression of HF. Indeed, in the failing heart, mitochondria were shown to undergo pathological structural and functional remodelling (59). In myocardium after MI, a wide range of mitochondrial defects have been reported including: defective oxidative phosphorylation, inhibition of electron transfer chain respiratory complexes and adenine nucleotide translocase, increased proton leak in the inner membrane, oxidation of cardiolipin and membrane protein dysfunction, increased ROS production, opening of mitochondrial permeability transition pore (MPTP), activation of mitochondria-mediated apoptosis, nucleotide depletion and Ca^{2+} overload. These derangements could lead to deficit in cardiac energy production, increased oxidative stress-induced intracellular damage, increased rates of apoptotic cell death, which are all known to be adverse intra-cardiac events leading to progression of HF (60).

1.5.1 Mitochondrial morphology

Observed by conventional transmission electron microscopy, mitochondria are elliptical organelles with the inner membrane organized in characteristic folds, termed cristae that protrude into the matrix and accommodate the respiratory chain complexes. It is well established that mitochondrial morphology and function respond to changes in homeostatic status of cardiomyocytes. Indeed, in various cardiac pathologies, diverse morphological alternations of mitochondria occur such as giant mitochondria, swelling, distortion of tree-dimensional structure, change in number, shape and orientation of cristae, decreased matrix density, and dense rods, vacuoles and crystalloids in both compartments (61, 62). Increased mitochondrial volume is linked to its increased permeability known as mitochondrial permeability transition (MPT) regulated by the MPT pore (MPTP), one of the key mediators of cardiomyocyte death (60). Giant mitochondria have been described in animal models of cardiac hypertrophy and in a number of human cardiomyopathies. In a HF models, besides changes in mitochondrial size, an increased number of smaller and fragmented mitochondria with loss of matrix density and disorganized cristae have been noted (62, 63).

Dynamic changes in mitochondrial morphology result from processes of mitochondrial fusion and fission, and disruption of these processes in the heart can be an important contributor to HF development. Mitochondria continuously join in the process of fusion that enables mixing of mitochondrial contents, protein complementation and repair of mitochondrial DNA (64). Mitochondrial fission, segregation into daughter organelles, requires synthesis of proteins and phospholipids controlled by mitochondrial and nuclear DNA. Fusion and fission are regulated by number of signaling enzymes, calcium homeostasis and the generation of ATP and ROS (65). Calcium overload, a common feature in HF, may increase mitochondrial fission and dysfunction by increased ROS generation. Recent studies demonstrate that inhibition of mitochondrial fission prevents ROS generation and mitochondrial permeability transition pore formation and subsequent cell death (65, 66). Therefore, mitochondrial fusion and fission may serve as potential therapeutic target for a variety of diseases associated with mitochondrial damage, including HF (67).

Mitochondrial biogenesis is a regulated process of mitochondrial growth and division relying on the coordinated synthesis of thousands of proteins encoded by both mitochondrial and nuclear DNA (64). The main regulator of mitochondrial biogenesis is considered to be peroxisome-proliferator-activated receptor γ coactivator 1α (PGC-1α), controlling the mitochondrial content optimal for normal cardiomyocyte function and stimulates mitochondrial biogenesis and respiration (67). The PGC-1α is protein that controls important metabolic functions and tissues with high oxidative activity, like heart, are enriched with PGC-1α. Mitochondrial biogenesis involves complicated interaction of transcription factors for target genes encoding enzymes for fatty acid transport and oxidation, oxidative phosphorylation and anti-oxidative defenses (65, 68). Accumulating data suggest that PGC-1α respond to metabolic challenges and external pathological stimuli and link them to the regulation of mitochondrial biogenesis and function. Recent studies report increased expression of PGC-1α in cardiac hypertrophy induced by exercise training. Moreover, it is demonstrated that PGC-1α down-regulation is linked to mitochondrial dysfunction in both cardiac and skeletal muscle in HF. This suggests that the decreased expression of PGC-1α probably play a significant role in the HF pathogenesis (67).

1.5.2 Mitochondria-mediated apoptosis and ROS-mediated damage

Oxidative stress has been suggested to play an important role in the pathogenesis of HF by inducing various aspects of mitochondrial damage. For example, oxidative stress was shown to induce mutations in mitochondrial DNA, which is particularly susceptible to mutations due to its limited repair mechanism. Indeed, in HF, ROS-induced damage of mitochondrial DNA was shown to lead to the mitochondrial dysfunction (69). On the other hand, mitochondria are the predominant source of ROS with mitochondrially-released ROS released playing an important role in mitochondrial bioenergetic dysfunction and triggering apoptosis. Studies suggest that increased ROS production in mitochondria is associated with signs of oxidative stress-related damage due to accumulation of lipid peroxidation by-products, mutations or deletions of mitochondrial DNA, mitochondrial membrane permeability and a consequent increase in release of cytochrome c and pro-caspases (70, 71). All of

these factors indicate that the mitochondria-induced apoptotic pathway is a requirement to cardiomyocyte death by apoptosis (28).

1.5.3 Mitochondrial energetics

Mitochondria are the major source of high energy compound adenosine triphosphate (ATP) which is synthesized mainly by oxidative phosphorylation in the inner mitochondrial membrane. Oxidative phosphorylation couples electron transfer and oxygen consumption with phosphorylation of ADP to ATP (72). The main energy substrates used by the heart are fatty acids and carbohydrates, which are catabolized into acetyl-CoA by β-oxidation and glycolysis, respectively. Molecules of acetyl-CoA enter the tricarboxylic (TCA) cycle and produce reduced intermediates NADH and $FADH_2$. These cofactors transfer electrons in the mitochondrial electron transport chain, ultimately generating ATP (**Figure 1**), (50, 72).

The metabolic flexibility of myocardium allows switching between carbohydrate and fat as fuel, in order to maintain constant rate of ATP production in diverse physiological conditions. However, FA catabolism provides almost 90% of ATP in normal conditions (55). Also, depending on energetic needs of the cardiomyocytes, mitochondrial function is regulated by various factors such as cAMP, Ca^{2+} and ROS (72).

In heart failure, and altered mitochondrial bioenergetics with a decreased mitochondrial capacity for substrate oxidation was shown. Experiments using isolated mitochondria, skinned fibers, isolated and *in vivo* hearts support the conclusion that mitochondria from the failing myocardium suffer from substantial reduction of oxygen consumption and energy production in all three components of cardiac energy metabolism: substrate utilization, oxidative phosphorylation, and high-energy phosphate metabolism (56). The structural support for oxidative phosphorylation is provided by four oxidoreductase complexes (I-IV) and the ATP synthase (complex V) (50). Three of these complexes generate inner membrane proton gradient that drives ATP synthesis by coupling electron transport with translocation of proton from mitochondrial matrix to the intermembrane space. Assembled in supercomplexes, the ETC complexes form a respiratory unit or respirasome with coenzyme Q and cytochrome c and transports electrons from NADH to reduce oxygen (72, 73). The ETC complexes are embedded in the phospholipid bilayer of the inner mitochondrial

membrane. Cardiolipin, a phospholipid exclusively present in mitochondrial inner membrane, plays a central role in organization of ETC in supercomplexes. Defective oxidative phosphorylation, defects in individual components of ETC and altered supra-molecular assembly of the electron transfer chain ETC complexes, as well as their decreased activities, have been described in CHF (74, 75). However, there is substantial heterogeneity of experimental data on activities of specific ETC complexes in CHF, with various studies pointing to different ETC components being primarily affected (73). Various studies on CHF report decreased enzymatic activity of complex I and in the combined activity of complexes I and III (76, 77). In contrast, other studies demonstrated normal activity of the exclusively nuclear-encoded complex II, or partially mitochondrially-encoded complexes III and IV (78, 79).

Therefore, understanding important aspects of mitochondrial dysfunction in CHF may result in new therapeutic approaches in order to prevent cardiac energetic failure, cardiomyocyte loss and attenuate pathological remodelling in heart failure (57, 80).

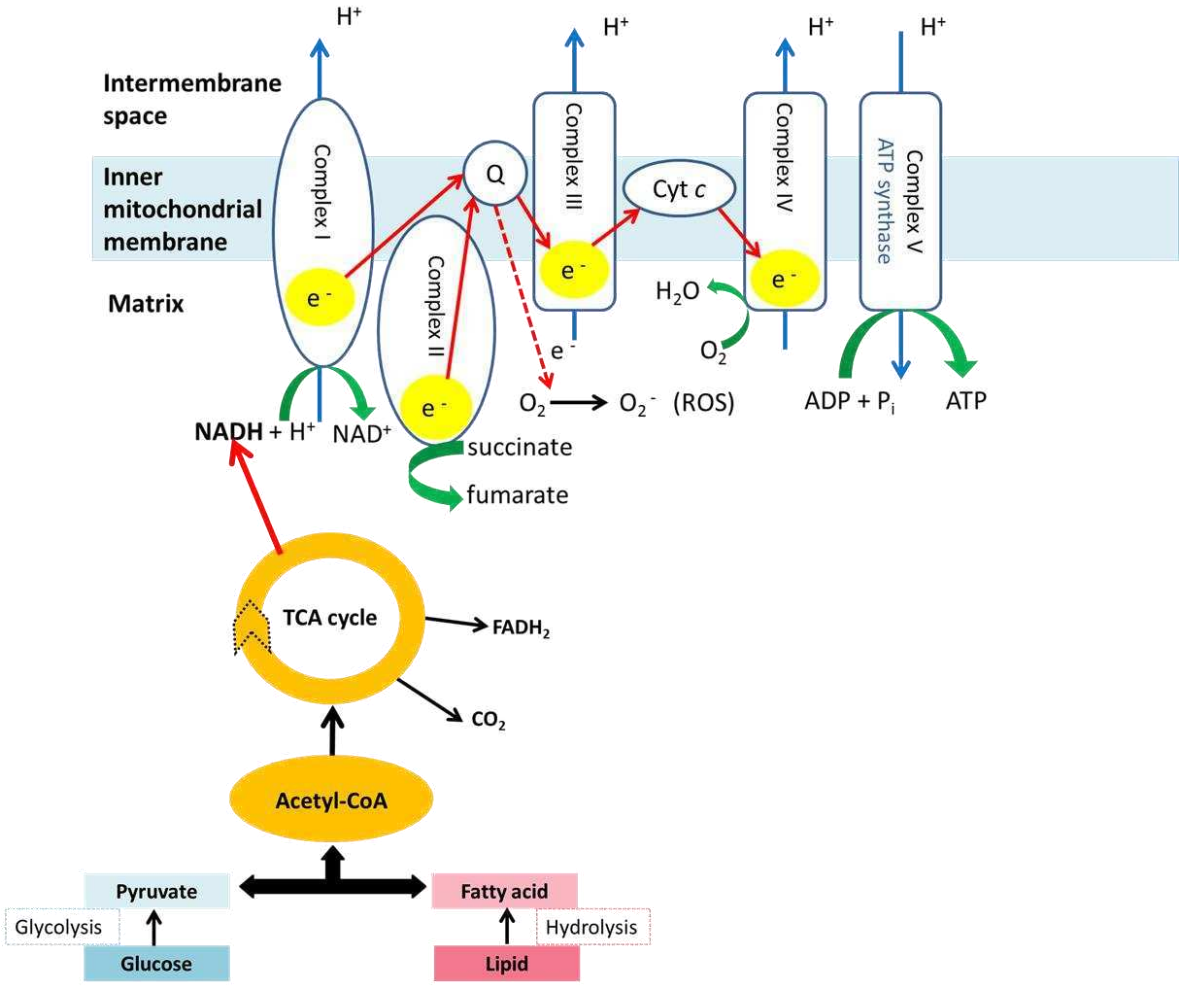


Figure 1. The basic mechanisms of the mitochondrial respiratory chain and oxidative phosphorylation system. The electron transport chain carries both protons and electrons, passing electrons from donors to acceptors (NADH to O_2), and transporting protons across a membrane.

1.6 Exercise training in chronic heart failure

1.6.1 Clinical aspect

Beneficial effects of exercise in chronic heart failure (CHF) are well established, with current treatment guidelines recommending exercise for patients with stable CHF in NYHA I–III groups (81). Exercise was shown to exert a number of beneficial effects in CHF regarding patients' quality of life, fatigue, and mortality. These changes, induced by exercise training, are associated with neurohormonal and metabolic changes, anti-inflammatory effects as well as cardiovascular, skeletal muscle, and pulmonary adaptations (82). Current evidence suggests that exercise yields beneficial adaptations in failing myocardium regarding cardiac remodelling and myocyte function. In patients with heart failure, exercise training was shown to improve exercise tolerance and cardiac performance by several mechanisms such as improved contractility, increased myocardial perfusion and angiogenesis, normalization of sympathetic-parasympathetic balance, improvement of cardiac energy metabolism, calcium handling, and peripheral arterial compliance. The data from animal models is consistent with aforementioned human studies demonstrating protective effects against pathological LV remodelling and deterioration of cardiac function (83).

Long-term aerobic interval training was demonstrated to improve left ventricular ejection fraction, cardiac output, and end-diastolic and end-systolic volumes even in elderly CHF patients. In meta-analysis that included 14 trials with measurement of cardiac performance the authors report an overall improvement in in maximal oxygen uptake (VO_{2max}) and ejection fraction as well as a decrease of end-diastolic and end-systolic volumes in endurance training studies (84). The HF-ACTION (Heart Failure–A randomized Controlled Trial Investigating Outcomes of exercise training) study demonstrated that exercise training is safe and offers clinical benefits in HF patients. Specifically, exercise training was associated with an 11% reduction in all-cause mortality or hospitalization, a 9% reduction in cardiovascular mortality or cardiovascular hospitalization, and a 15% reduction in cardiovascular mortality or heart failure hospitalization. For most of CHF patients, especially those in

advanced stages of functional impairment, the aerobic endurance training at 50–80% of VO_{2max} is the preferred training modality. Only one trial reported complications associated with training, but these complications were confined to the most severe patients with ejection fractions <30%. High-intensity interval training (HIIT) can be recommended in relatively low-risk HF patients to achieve higher training effectiveness (85). Other useful training programs may include inspiratory muscle training, strength training, and relaxation therapy. However, exercise training is only effective as long as it is maintained and continuation of regular exercise training needs to be encouraged after the initial cardiac rehabilitation phase (83).

1.6.2 Cellular and molecular effects of exercise in HF

The specific cellular and molecular mechanisms responsible for the beneficial effects of exercise training are not completely clear. Improvement in cardiac function mediated by exercise, observed in patients with HF and animal models, appears to be induced by amelioration of interstitial fibrosis, cardiomyocyte dysfunction and apoptosis associated with HF.

At the level of cardiomyocytes, chronic exercise training was shown to ameliorate pathological changes in Ca^{2+} regulation and improve the contractility of the failing myocardial cells (86, 87). In rats with myocardial infarction, aerobic endurance training attenuates ventricular and cellular hypertrophy and consistently restores contractile function, intracellular Ca^{2+} handling, and Ca^{2+}-sensitivity in cardiomyocytes (88). In animal models, studies showed that aerobic exercise training increases glycolysis and oxidative metabolism by selectively increasing the concentrations of regulatory enzymes of glycolysis and oxidative metabolism. Moreover, either increase or no change in fatty acid utilization capacity was reported (89, 90). In rabbit model of post MI-remodeled hearts, chronic exercise was shown to modulate autophagy and fatty acid utilization (91). Also, numerous factors have been proposed to contribute to exercise-induced improvement in cardiac function. The cellular adaptations include maintenance of a positive inotropic state, improved mitochondrial capacity, increased levels of mitochondrial antioxidant enzymes, decreased ROS production and inhibition of pro-apoptotic proteins (27, 92, 93). Exercise training decreases apoptotic processes, and protects mitochondrial function

from oxidative stress (94). Exercise training seems to improve cardiac energetic efficiency in heart failure. In studies performed on animal models of myocardial infarction, exercise training improves expression of cytochrome oxidase subunits, ventricular atrial natriuretic peptide, sarcoplasmic reticulum calcium ATPase and fatty-acid binding protein (95). Whether these results extend to human heart remains to be established. However, experimental data on the mitochondrial effects of exercise in failing cardiac muscle are still lacking (96).

2. AIMS OF THE STUDY

The main purpose of this dissertation was to gain insight into intracellular processes occurring in the post-ischemic failing myocardium after chronic exercise. The main hypothesis was that chronic exercise training significantly improves function of the failing myocardium by affecting one of the main contributors of pathological remodelling, the mitochondria. The hypothesis was tested by achieving the following specific aims:

1. a. To establish a reliable experimental animal model for post-myocardial infarction heart failure that shows adaptations characteristic for post-infarction cardiac remodelling.

b. To implement a valid and reproducible aerobic interval training protocol that will induce effective cardiovascular changes in post-myocardial infarction failing rat heart.

2. a. To determine the effect of aerobic interval training on cardiac morphology and functional parameters in post-infarction failing rat heart.

b. To examine the effect of aerobic interval training on mitochondrial function in post- infarction failing rat heart.

3. MATERIALS AND METHODS

Ethic statement

This study was conducted according to the Directive 2010/63/EU of the European Parliament and was approved by the Croatian Animal Care Committee and Ethical Committee of the University of Split, School of Medicine.

Chemicals

All chemicals used for this study, unless otherwise noted, were purchased from Sigma-Aldrich (Saint Louis, MO, USA).

Coronary artery ligation procedure

Adult female Sprague-Dawley rats weighing between 230 to 290 g were anesthetized with mixture of ketamine (Ketaminol 10, 90 mg/kg, Intervet International) and xylazine (Xylapan, 8 mg/kg, Vetoquintol) injected in the right hamstring muscles. After the rats were fully anesthetized (confirmed by the absence of corneal reflex), the trachea was intubated for artificial ventilation. A trans-abdominal trans-diaphragmal approach was used in order to avoid trauma to the myocardium (besides that related to the coronary artery ligation). Myocardial infarction (MI) was induced by permanent occlusion of the left coronary artery (LCA) with non-absorbable 8-0 suture. The immediate discoloration of the ventricular surface (pale appearance) was a sign of successful LCA ligation (**Figure 2**). Age-matched control rats (Sham animals) underwent the same surgical procedure without ligation of LCA.

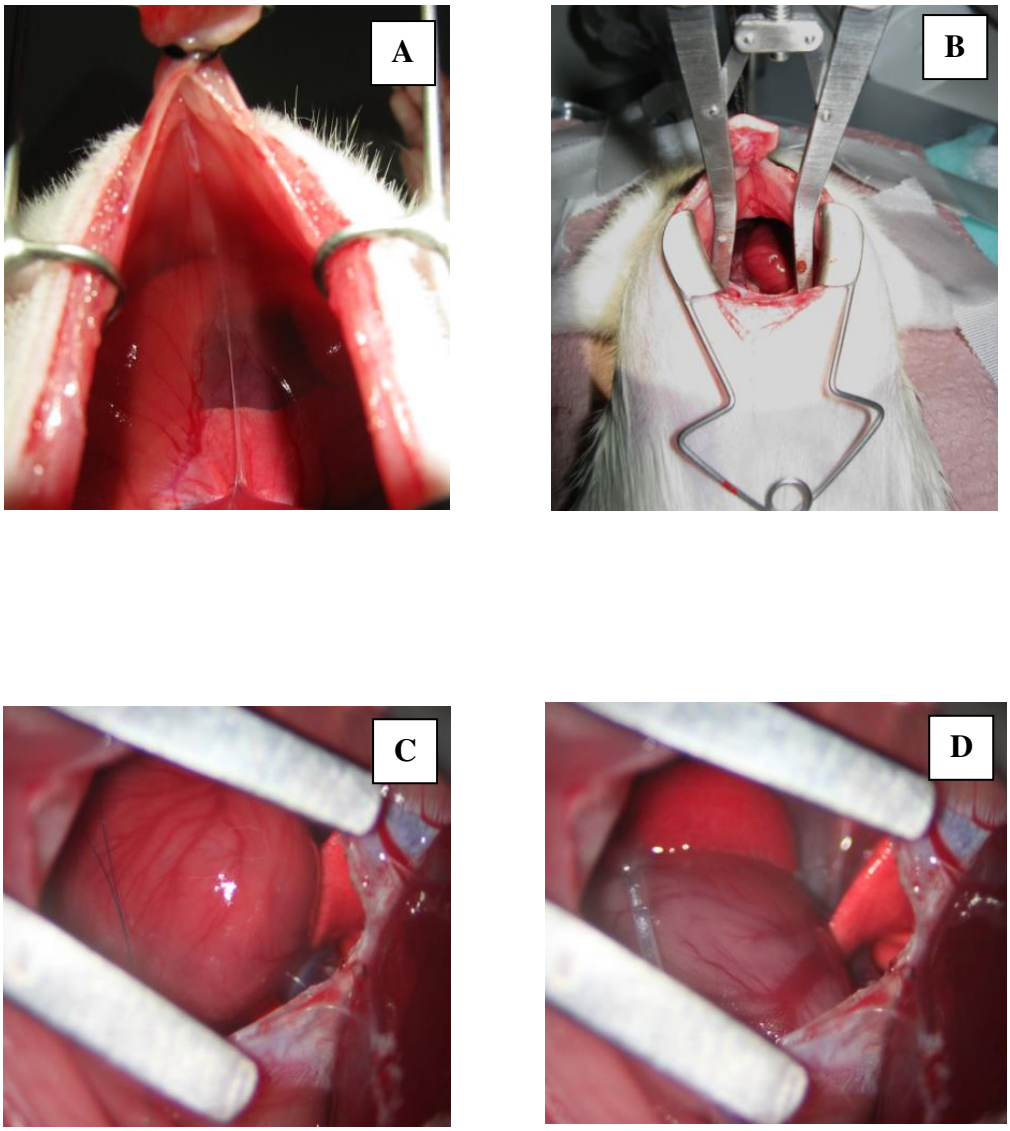


Figure 2. Photographs of the surgical procedure of the coronary artery ligation. **A.** Laparotomy and exposition of the diaphragm. **B.** Positioning retractor for trans-diaphragmal approach to the heart. **C.** Just before putting permanent occlusion of the left coronary artery (LCA) with non-absorbable 8-0 suture. **D.** Permanent occlusion of the left coronary artery and immediate pale appearance of the anterior surface of the heart.

Echocardiographic measurements

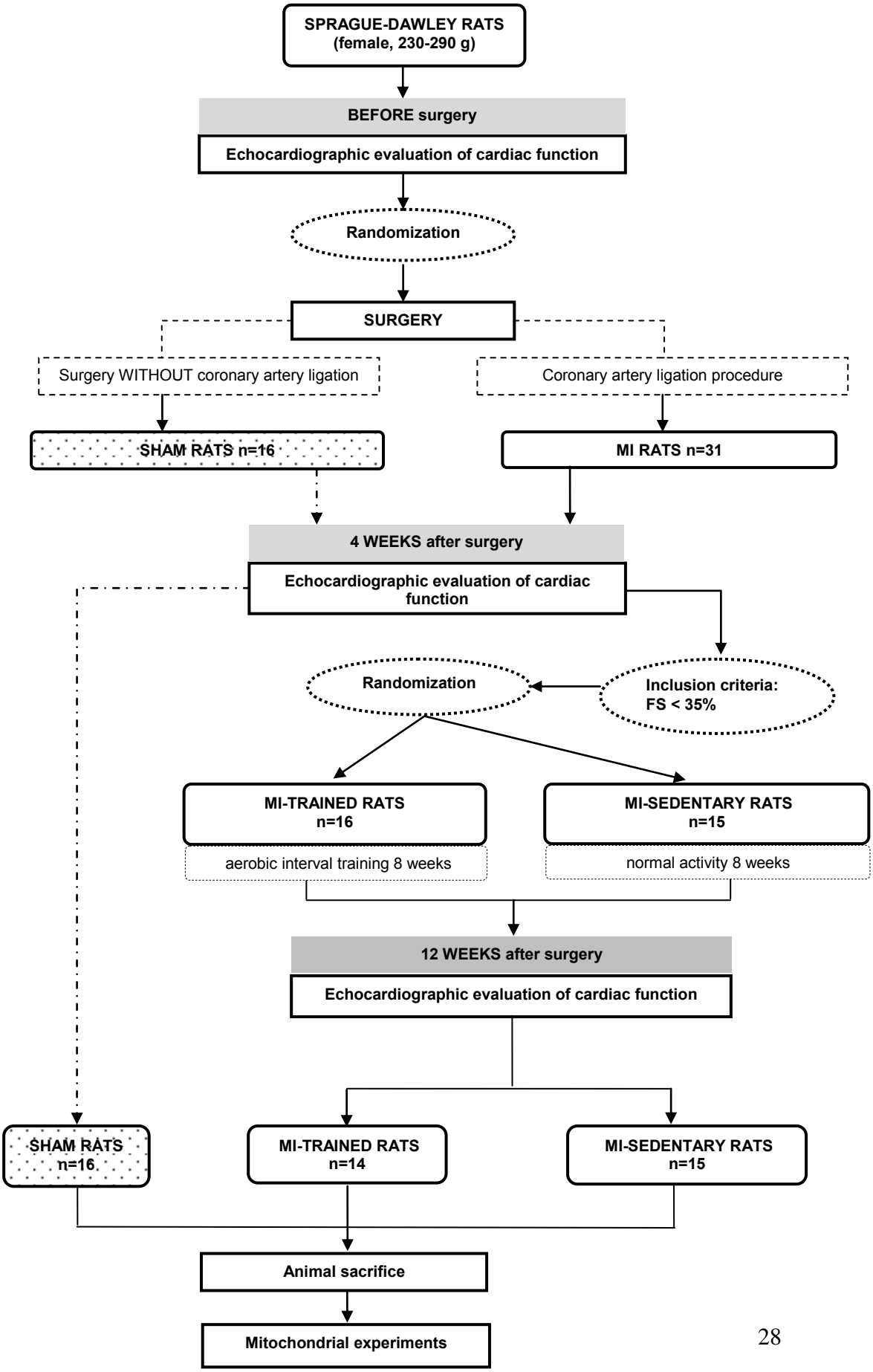
Transthoracic echocardiography was performed using a 12-MHz transducer connected to Vivid 3 Expert ultrasound system (General Electric, Milwaukee, WI, USA) under isoflurane anesthesia (1.5%) at 4 and 12 weeks after the surgery. Parasternal two dimensional short-axis view at the level of papillary muscles was used to measure the following parameters: left ventricular diameter in diastole and systole (LVDd and LVDs, respectively), anterior wall thickness in diastole and systole (AWTd and AWTs), and posterior wall thickness in diastole and systole (PWTd and PWTs). Left ventricular fractional shortening (FS, %), was calculated according to the following formula: FS = [(LVDd - LVDs) / LVDd] x 100. Echocardiographic assessment at 4 weeks after surgery was used to evaluate the extent of myocardial infarction (in MI-operated rats) and measure the cardiac contractile performance before commencement of further experimental procedures. The animals with high degree of myocardial damage and developed CHF, estimated at FS≤35%, were included in the the study. This selection criterion was based on previous reports that correlated echocardiographic FS values with invasive measurements of LV pressures for the assessment of heart failure in this animal model (97, 98) Out of 63 MI-operated animals that survived a 4-week postoperative period, 31 rats met the inclusion criteria and were subsequently randomized into exercised (MI-Trained) and sedentary (MI-Sedentary) groups. During the course of the next 8 weeks, 2 of the trained animals were excluded from the study due to insufficient compliance for exercise. At 12 weeks after surgery, echocardiography was again performed followed by animal sacrifice within 3 to 6 days. All echocardiographic evaluations were performed blinded to rats' group allocation. Finally, three groups of rats were assessed: MI-Trained (n=[14]), MI-Sedentary (n=15) and Sham sedentary (control) group (n=16).

Training protocol

Following echocardiographic evaluation at 4 weeks after surgery, MI-operated rats were randomly assigned to either MI-Sedentary or MI-Trained group. No statistical difference in any of the assessed echocardiographic parameters existed between the two groups at this time point. MI-Trained group started an eight-week aerobic interval training (AIT) protocol two days after the echocardiographic

evaluation was performed. Animals were running on a treadmill specially designed for small animals (Model Exer-3R, Columbus Instruments, Columbus, OH, USA), five days a week for 70 minutes, including 10 minutes of warm-up at 40–50% of estimated maximal oxygen consumption (VO_{2max}) and 60 minutes of interval running. Each interval consisted of 4 minutes of high-intensity running (estimated at approximately 85–90% of predicted VO_{2max}) and 2 minutes of active recovery (estimated at approximately 50–60% of predicted VO_{2max}). Running intensities for each week of training were based on the previous report studying the relationship between the running speed and VO_{2max} in the same post-MI rat model exposed to aerobic interval training for 8 weeks (99). Specifically, running speed was increased gradually for the first 6 weeks of training by 0.02 m/s per week, with last two weeks (7^{th} and 8^{th}) having the same intensity level as in the 6^{th} week. Treadmill inclination during training and testing was 25°.

STUDY PROTOCOL



Isolation of mitochondria

All animals were sacrificed for mitochondrial studies in the period of 3-6 days following second (12-week) echocardiographic evaluation and mitochondria were isolated from the viable part of left ventricle by differential centrifugation. After anesthetizing the animals with intramuscular injection containing the mixture of ketamine and xylazine (90 mg/kg and 8 mg/kg, respectively), the hearts were excised, atria and right ventricle removed, and viable part of left ventricle was placed into an ice-cold isolation buffer containing (in mmol/l): 50 sucrose, 200 mannitol, 5 KH_2PO_4, 1 EGTA, 5 MOPS, and 0.1% bovine serum albumin (BSA; pH 7.3) and minced into small pieces. The suspension was homogenized in the presence of 5 U/ml protease (from *Bacillus licheniformis*) by using Ultra Turrax T 25 homogenizer (IKA-Werke, Staufen, Germany). The homogenate was centrifuged at 8000 *g,* and the obtained pellet was resuspended in the isolation buffer using a Potter-Elvehjem homogenizer, and centrifuged at 900 *g*. The resulting supernatant was centrifuged at 8000 g, and the mitochondrial pellet was dissolved in the isolation buffer. [18] Portion of mitochondrial suspension was stored at -80°C for later measurements of enzyme activities, while the remaining was kept on ice and used immediately for measurements of mitochondrial respiratory parameters and ATP synthesis. Protein concentration was determined using a modified Lowry assay kit (DC protein assay kit, Bio-Rad, Hercules, CA, USA), using bovine serum albumin as a standard.

Citrate synthase activity

Citrate synthase activity was determined spectrophotometrically (at 412 nm, 25 ºC) in isolated mitochondrial preparations using the kit from Sigma-Aldrich (CS0720).

Measurement of mitochondrial oxygen consumption

Mitochondrial oxygen consumption was measured using an oxygen electrode (Oxygraph, Hansatech Instruments, Norfolk, UK). Experiments were conducted at 30°C in a respiration buffer containing 0.5 mg/ml mitochondrial protein and (in mmol/l): 130 KCl, 5 K_2HPO_4, 20 MOPS, 2.5 EGTA, 0.001 $Na_4P_2O_7$, 0.1% BSA (pH 7.4). State 2 respiration was stimulated with the combination of electron transfer chain complex I substrates pyruvate and malate (5 mmol/l each) or complex II substrate

succinate (5 mmol/l) in the presence of complex I inhibitor rotenone (1 µmol/l). Adenosine diphosphate (ADP) - stimulated (state 3) respiration was measured in the presence of ADP (250 µmol/l), and state 4 respiration after all ADP was consumed.

Measurement of mitochondrial ATP production rate

The rate of mitochondrial ATP production was determined with a chemiluminescence-based method utilizing firefly luciferase and luciferin (Molecular Probes, Invitrogen, Eugene, OR, USA). Reaction solution contained respiration buffer, dithiothreitol (0.91 mmol/l), luciferin (0.14 mg/ml), luciferase (1.14 mg/ml), mitochondria (0.01 mg/ml) and pyruvate/malate (5 mmol/l each) or succinate (5 mmol/L) as substrates. The reaction was initiated by the addition of ADP (30 µmol/l). Chemiluminescence was monitored in Glomax 20/20 luminometer (Promega, Madison, WI, USA) at room temperature for 120 s.The standard curve was obtained with defined ATP concentrations (0, 100, 1000 and 10000 nmol/l), from which the rate of mitochondrial ATP production measured in the presence of substrates and ADP was calculated.

Complex I (NADH: ubiquinone oxidoreductase) activity assay

Previously frozen mitochondria were thawed and solubilized on ice with 1% cholic acid in an MSM/EDTA buffer (220 mmol/l D-mannitol, 70 mmol/l sucrose, 5 mmol/l 3-(N-morpholino) propanesulfonic acid, 2 mmol/l EDTA, pH 7.4). Complex I enzymatic activity was determined by the rotenone-sensitive reduction of NADH absorbance using decylubiquinone as an acceptor. The reaction mixture, containing 20 µg/ml mitochondrial protein, 50 mmol/l KH_2PO_4, 0.1 mmol/l EDTA, 0.2% BSA, 0.15 mg/ml asolectin, 0.02 mmol/l antimycin A, and 0.2 mmol/l NADH, was warmed at 30°C, and transferred into a pre-warmed cuvette in a spectrophotometer (DU 800, Beckman Instruments, Fullerton, CA, USA). The reaction was initiated by adding decylubiquinone (0.075 mmol/l), and the change in NADH absorbance was measured at 30°C (regulated by Peltier temperature controller), at 340 nm (extinction coefficient = 6.22 $(mmol/l)^{-1}cm^{-1}$).

Western Blotting

Following excision, rat hearts were placed in an ice-cold PBS buffer, weighed, and atria, right ventricle and scar tissue were removed. Left ventricles were then snap frozen in liquid nitrogen and stored at -80°C. Left ventricles were homogenized using Ultra-Turrax T25 in lysis buffer (1 ml of buffer per 100 mg of tissue) containing (in mmol/l): 20 Tris HCl (pH 7.5), 150 NaCl, 1 Na_2EDTA, 1 EGTA, 1% Triton X, 1% Na-deoxycholate, 1 β-glycerophosphate, 0.2 phenylmethylsulfonyl fluoride, 2.5 NaPPi, 1 Na_3VO_4, 1 dithiothreitol, 5 NaF and a protease inhibitor cocktail tablet (Roche Diagnostics, Basel, Switzerland). Protein concentration in cardiac homogenates was determined using DC protein assay kit. Cardiac homogenate proteins were then separated by SDS-PAGE on 12% gel with 30 μg of protein loaded in each lane. In order to allow for gel-to-gel comparison, a standard sample was loaded on each gel and all tested protein bands were normalized to this standard control band. After electrophoresis, proteins were transferred to a nitrocellulose membrane, blocked in 5% milk and then incubated with Mitoprofile Total OXPHOS antibody cocktail (MS601, MitoSciences, Eugene, OR, USA) containing mouse monoclonal antibodies against structural components of four of the five mitochondrial respiratory complexes (the 22-kDa NDUFB8 subunit of complex I, the 30-kDa Ip subunit of complex II, the 47-kDa core 2 subunit of complex III, and the α-subunit of the F_1F_0 ATP synthase of complex V). After incubation with secondary HRP-conjugated antibody and incubation with chemiluminescent substrate (Supersignal West Pico, Pierce Biotechnology, Rockford, IL, USA), the blots were imaged using Chemidoc imaging system (Bio-Rad). After stripping with 0.4 mol/l NaOH, the blots were re-probed with anti-β-actin antibody (A5441, Sigma) that served as loading control. The densities of bands from MI-Sedentary and MI-Trained groups (normalized to the standard sample and loading control) were analyzed using Image Lab 3.0 software and expressed relative to the Sham group.

Detection of protein oxidation

Protein carbonylation was measured using OxyBlot protein oxidation detection kit (S7150, Merck Millipore). Briefly, left ventricle homogenates were supplemented with dithiothreitol to a final concentration of 50 mM, followed by treatment with 2,4-dinitrophenylhydrazine (DNPH) according to manufacturer's

instructions. The DNP-derivatized protein samples were separated on 10 % polyacrylamide gel followed by transfer to the nitrocellulose membrane. DNP groups were immunodetected with a rabbit anti-DNP primary antibody (1:150) followed by secondary anti-rabbit HRP antibody and chemiluminescence detection using Chemidoc imaging system (Bio-Rad) as described earlier. Ponceau S staining (after protein transfer and before blocking) was used as a loading control.

Electron microscopy

The pieces of left ventricle were fixed in 3.5 % paraformaldehyde in 0.1 M phosphate buffer solution (pH 7.3) during 24 h at 4 °C and then in 3% glutaraldehyde in 0.1 M phosphate buffer solution (pH 7.2) during 2 h. The post-fixation was done in 2 % osmium tetroxide in the same buffer solution. The tissue was embedded in Epoxy resin and cut in ultrathin sections (0.05 μm) followed by staining with uranyl-acetate and lead citrate. The electron microscope Zeiss EM 10A was used for visualization. The mitochondrial surface area was analyzed with Image J.

Statistical analysis

Data are presented as means ± standard deviation. Differences between MI-Trained, MI-Sedentary and Sham sedentary rats were tested using Kruskal-Wallis test followed by *a posteriori* Mann-Whitney comparisons. Differences in echocardiographic parameters at 4 weeks post-surgery between sham-operated and MI-operated animals were tested with Mann-Whitney test for independent samples. Differences between 4 weeks and 12 weeks post-operative echocardiographic values within the same group of rats were probed with Wilcoxon test for paired samples. Statistical analysis was performed by employing commercially available software (MedCalc, Mariakerke, Belgium), and significance was accepted at $P<0.05$.

4. RESULTS

Echocardiography data are presented in **Table 1.** Four weeks after surgery, animals that underwent coronary artery ligation and exhibited LV fractional shortening below 35% were selected for the study (MI-operated group). As seen in the table, and also illustrated in **Figure 3A**, these animals had enlarged ventricular cavity and thinned myocardium comparing to the sham-operated animals. Twelve weeks after surgery, the contractile function of the left ventricle deteriorated even further in MI-Sedentary group (**Figure 3B**), while FS in MI-Trained group remained at the 4-week level.

Twelve weeks after surgery, the expression of atrial natriuretic peptide (ANP) in LV, which is often found enhanced in pathological cardiac hypertrophy, was increased to the similar extent in MI-Trained and MI-Sedentary groups, as compared to Sham (**Figure 4**). The expression of citrate synthase, a mitochondrial marker, was not changed in any experimental group (**Figure 4**), nor was its specific enzymatic activity different in isolated mitochondria (1793±191 mU/mg protein, 1925±205 and 1853±212 for Sham, MI-Sedentary and MI-Trained, respectively (data not shown)). Furthermore, no difference was found in LV expression of PGC-1α, a marker of mitochondrial biogenesis (**Figure 4**).

Respiratory function of LV mitochondria was also analyzed at 12 weeks after surgery. As displayed in **Figure 5A**, in the presence of complex I ETC substrates, ADP-supported respiration (state 3) was significantly reduced in mitochondria from MI rats, as compared to Sham. However, it was better preserved in animals exposed to 8 weeks of exercise training (211.5 ± 38.1 nmol O_2/min/mg protein vs. 160.2 ± 45.4 in MI-Sedentary rats and 254.1 ± 38.8 in Sham). The respiratory control ratio (RCR), calculated as the ratio of state 3 and state 4 respiration and used as an indicator of coupling of O_2 consumption and phosphorylation, was reduced only in MI-Sedentary rats, while in the trained animals it remained at the same level as in Sham (**Figure 5B**). When mitochondria were fuelled with substrate for complex II, no difference in ADP-supported respiration or the RCR between the Sham and any of the MI-operated animal groups was observed (**Figure 6 A, B**). Measurements of mitochondrial ATP production were conducted in parallel with oxygen consumption experiments. In the presence of pyruvate and malate, the rate of ATP generation was

significantly decreased only in mitochondria from MI-Sedentary rats, as compared to Sham (**Figure 7A**). When succinate was used as an electron donor, no change in ATP production was observed in any of the tested groups.

Since data obtained from respiratory and ATP production measurements suggested ETC damage primarily at the level of complex I, we next measured the specific enzymatic activity of NADH: ubiquinone oxidoreductase in isolated mitochondria. Recording the rate of complex I enzymatic turnover revealed a similar pattern as detected in respirometry experiments. As can be inferred from **Figure 7B**, relatively high rate of NADH oxidation observed in Sham mitochondria (641.2 ± 68.3 nmol NADH/min/mg mitochondria) was substantially impaired in MI-Sedentary hearts (486.8 ± 35.3 nmol NADH/min/mg). In mitochondria isolated from MI-Trained rats, although still depressed comparing to Sham, the complex I activity was significantly better preserved than in MI-Sedentary group (552.3 ± 39.4 nmol NADH/min/mg for MI-Trained).

Probing the expression of the representative subunits for mitochondrial ETC respiratory complexes I, II, III and V revealed no differences between the three experimental groups (**Figure 8A, B**). Whether a reduction in complex I activity is accompanied with altered degree of oxidative stress was also investigated. Protein carbonylation, an indicator of oxidative stress, was significantly increased in MI-Sedentary hearts with respect to Sham (**Figure 8C, D**), while MI-Trained hearts showed no significant difference compared either to Sham or MI-Sedentary rats.

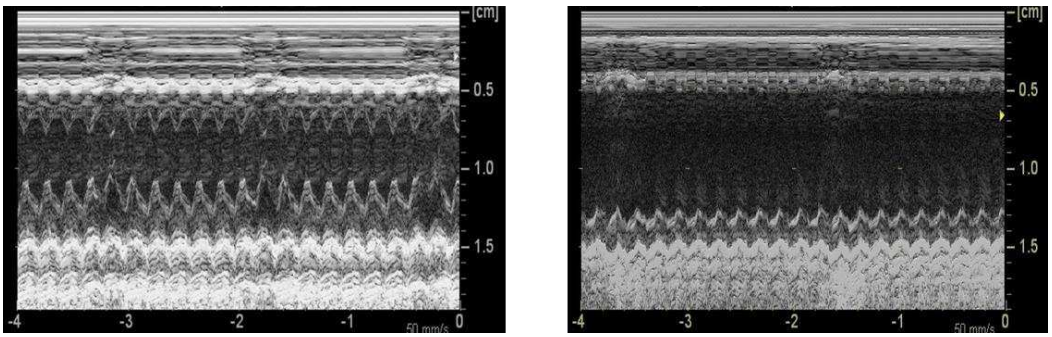
Electron microscopy images of cardiac tissue sections revealed significantly smaller mitochondria in hearts of sedentary MI-rats as compared to Sham and trained MI- hearts (**Figure 9** and **Figure 10**).

4-week post-surgery	Sham	MI-operated	
FS (%)	53.1 ± 5.5	28.28 ± 5.7 *	
LVDd (mm)	5.82 ± 0.26	7.92 ± 0.59 *	
LVDs (mm)	2.73 ± 0.39	5.81 ± 0.91 *	
AWTd (mm)	1.76 ± 0.05	1.4 ± 0.39	
AWTs (mm)	2.78 ± 0.13	2.00 ± 0.48 *	
PWTd (mm)	1.70 ± 0.18	1.59 ± 0.19	
PWTs (mm)	2.88 ± 0.23	2.47 ± 0.35 *	
W_B (g)	257.4 ± 11.5	259.92 ± 13.10	
Number of animals	16	31[a]	
12-week post-surgery	**Sham**	**MI-Sedentary**	**MI-Trained**
FS (%)	58.3 ± 8.9	21.7 ± 5.5 *[†]	30.4 ± 8.5 [#]
LVDd (mm)	5.86 ± 0.31	9.10 ± 0.98 *	7.84 ± 1.09 *
LVDs (mm)	2.46 ± 0.65	7.16 ± 1.17 *	5.50 ± 1.30 [#]
AWTd (mm)	1.52 ± 0.19	1.22 ± 0.61	1.49 ± 0.33
AWTs (mm)	2.78 ± 0.31	1.76 ± 0.83	2.36 ± 0.74
PWTd (mm)	1.78 ± 0.16	1.48 ± 0.40	1.81 ± 0.20
PWTs (mm)	2.84 ± 0.40	2.34 ± 0.50	2.63 ± 0.48
W_B (g)	277.4 ± 18.2	278.9 ± 17.5	277.6 ± 15.56
W_H (mg)	779.4 ± 57.9	1031.1 ± 139.2 *	1070.44 ± 126.1 *
W_H/W_B (mg/g)	2.87 ± 0.29	3.70 ± 0.46 *	3.86 ± 0.42 *
Number of animals	16	15	14

Table 1. Cardiac morphological and functional parameters after surgery and exercise training. Values presented are mean + SD. FS, left ventricular fractional shortening; LVDd and LVDs, left ventricular diameter in diastole and systole, respectively; AWTd and AWTs, anterior wall thickness in diastole and systole, respectively; PWTd and PWTs, posterior wall thickness in diastole and systole, respectively; WB, body weight; WH, heart weight. [a]Two animals randomized into the MI-Trained group were excluded from the study during the course of training.

**P <0.05 vs. Sham. [#]P <0.05 vs. Sham and MI-Sedentary. [†]P <0.05 vs. 4-week value.*

A



Sham-operated MI-operated

B

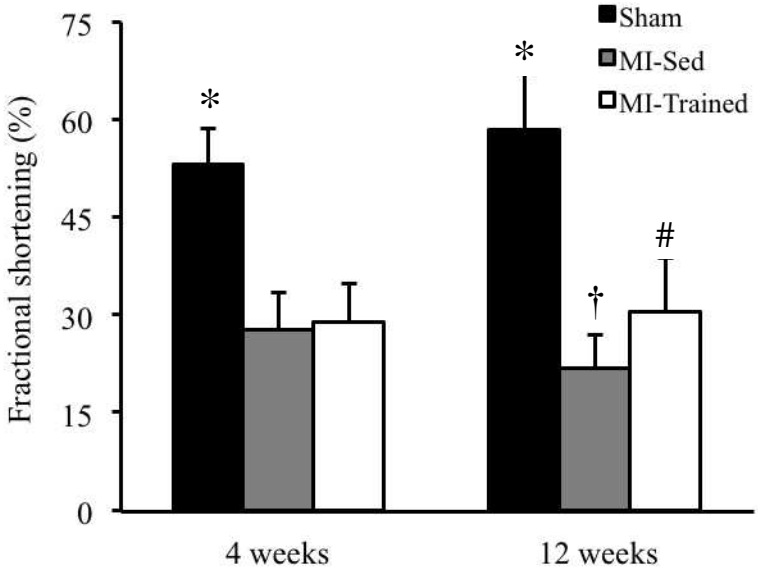


Figure 3. Echocardiographic evaluation of operated animals. **A**, Examples of M-mode of two-dimensional echocardiograms taken at 4 weeks after surgery in sham-operated and animal with coronary artery ligation (MI-operated). In animal with induced myocardial infarction, increased left ventricular cavity dimensions and reduced contractility and thickness of the ventricular muscle can be seen. **B**, Left ventricular fractional shortening (FS) at 4 and 12 weeks after surgery. Sham, sham operated sedentary animals (n=16); MI-Sed, sedentary animals with induced myocardial infarction (n=15); MI-Trained, animals with myocardial infarction exposed to 8 weeks of exercise training (n=14). **P<0.05 vs. MI-Sed and MI-Trained, #P<0.05 vs. MI-Sed, †P<0.05 vs. 4-weeks value in the same group.*

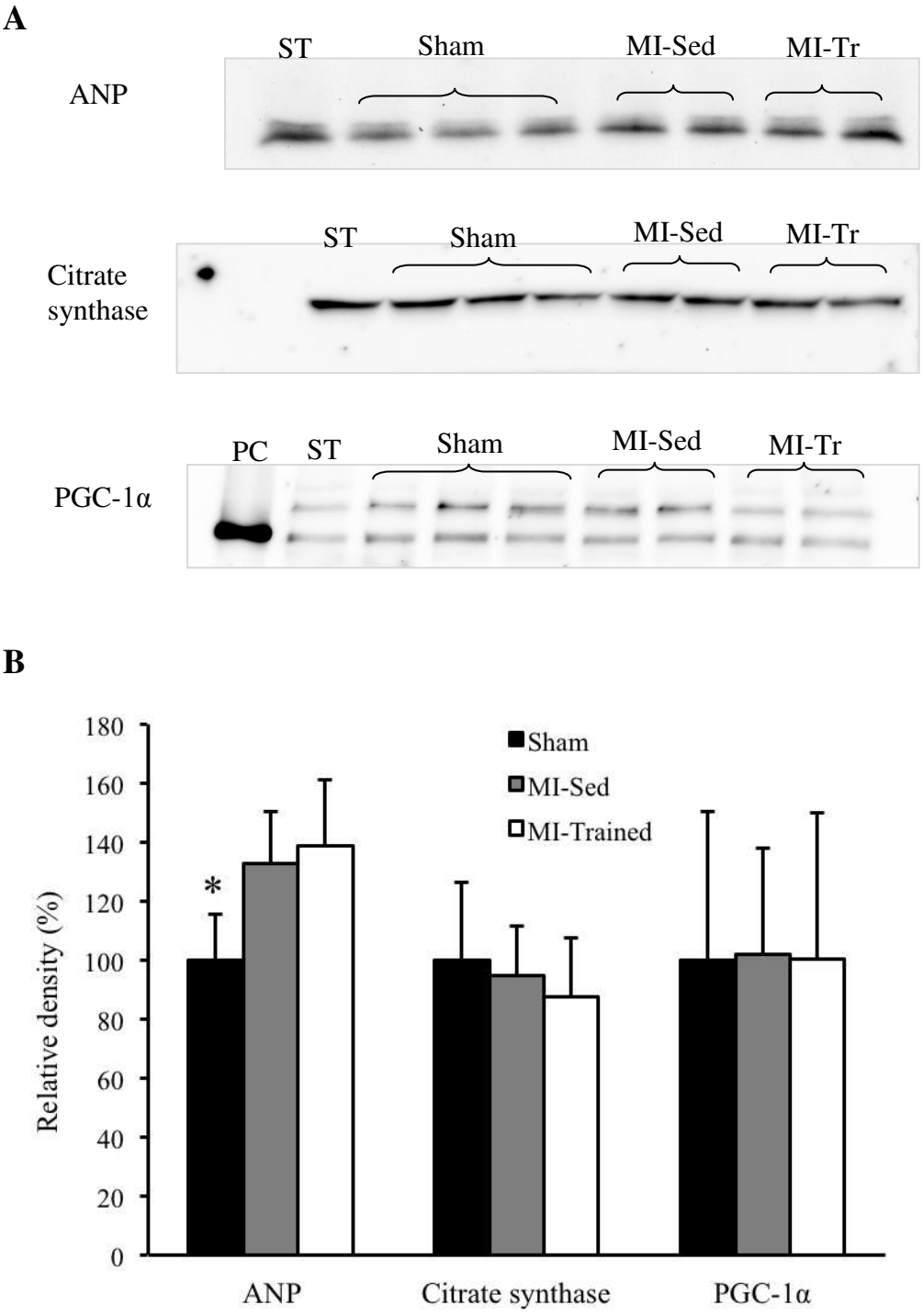


Figure 4. Expression of ANP, citrate synthase and PGC-1α. **A**, Image of representative Western blots probed with anti-ANP, anti-citrate synthase and anti-PGC-1α primary antibodies. **B**, Average densities normalized to β-actin expressed relative to the Sham group. **P<0.05 vs. MI-Sedentary (MI-Sed) and MI-Trained (MI-Tr), n=6 animals per group. PC = PGC-1α positive control.*

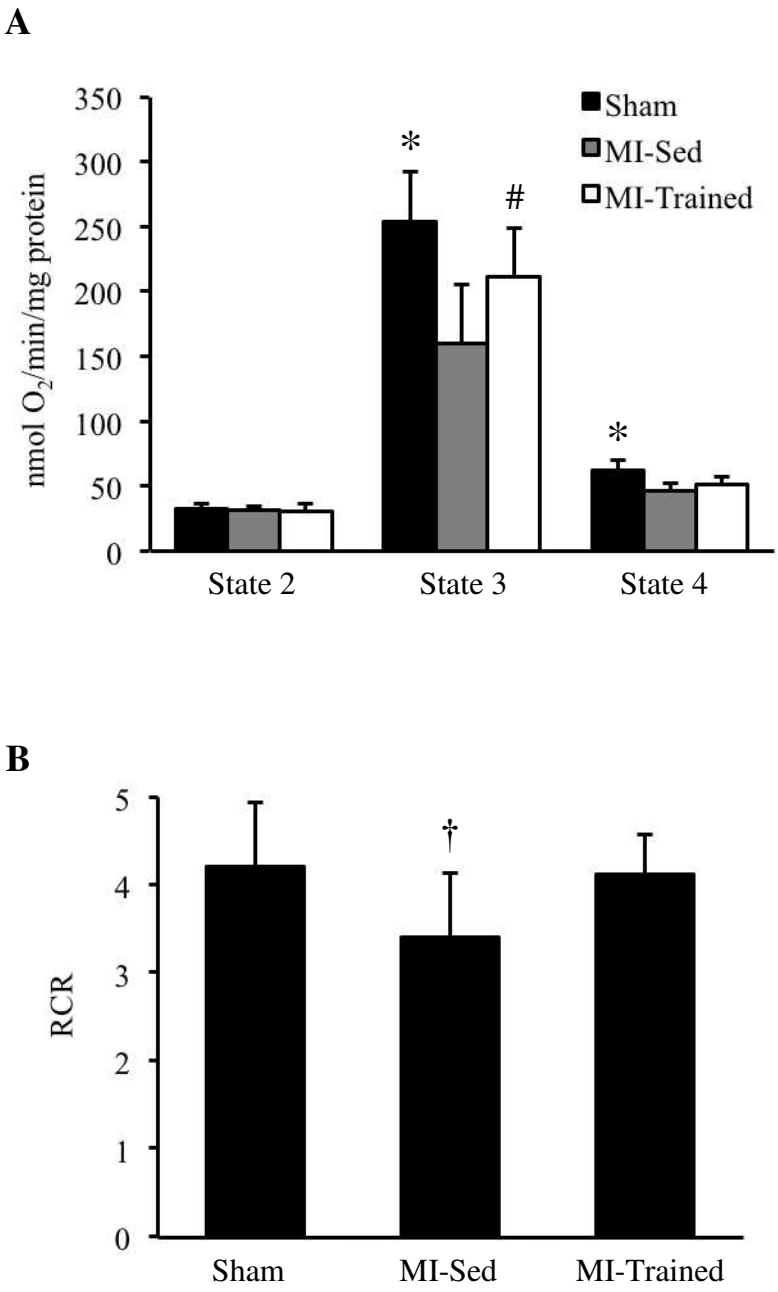


Figure 5. Mitochondrial respiratory function assessed with substrates for complex I. **A**, Oxygen consumption was recorded in isolated mitochondria in the presence of pyruvate and malate (State 2), upon addition of ADP (State 3) and after all ADP was consumed (State 4). **B**, Respiratory control ratio (RCR), an indicator of mitochondrial coupling, was calculated as State 3/State 4 for each of the experimental groups. **P<0.05 vs. MI-Sedentary (MI-Sed) and MI-Trained (MI-Tr), #P<0.05 vs. MI-Sed, †P<0.05 vs. Sham and MI-Tr, n=8 animals for each experimental group.*

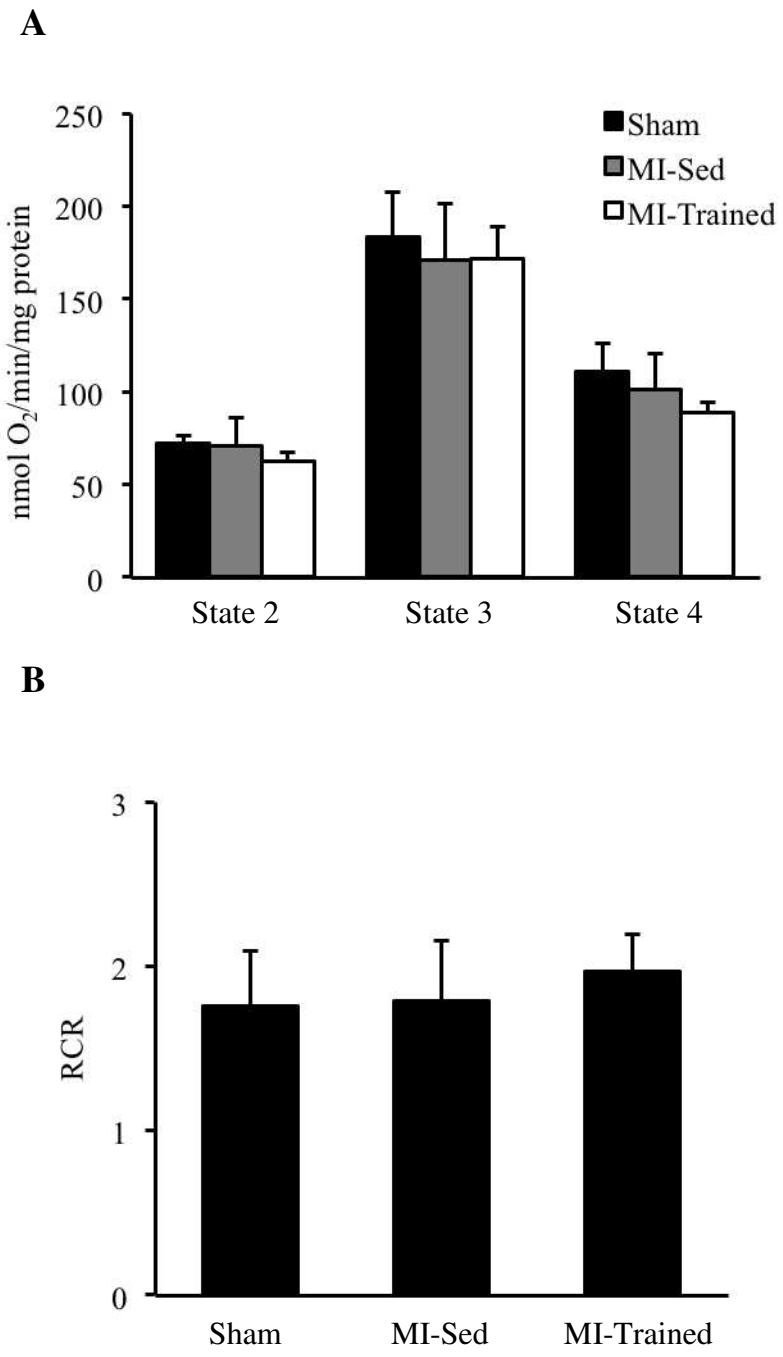


Figure 6. Mitochondrial respiratory function evaluated with substrate for complex II. **A**, Mitochondrial oxygen consumption was monitored in the presence of succinate and rotenone (State 2), after addition of ADP (State 3) and when the entire ADP was converted into ATP (State 4). **B**, Calculated respiratory control ratio for the three groups of rats. *n=8 animals for each group.*

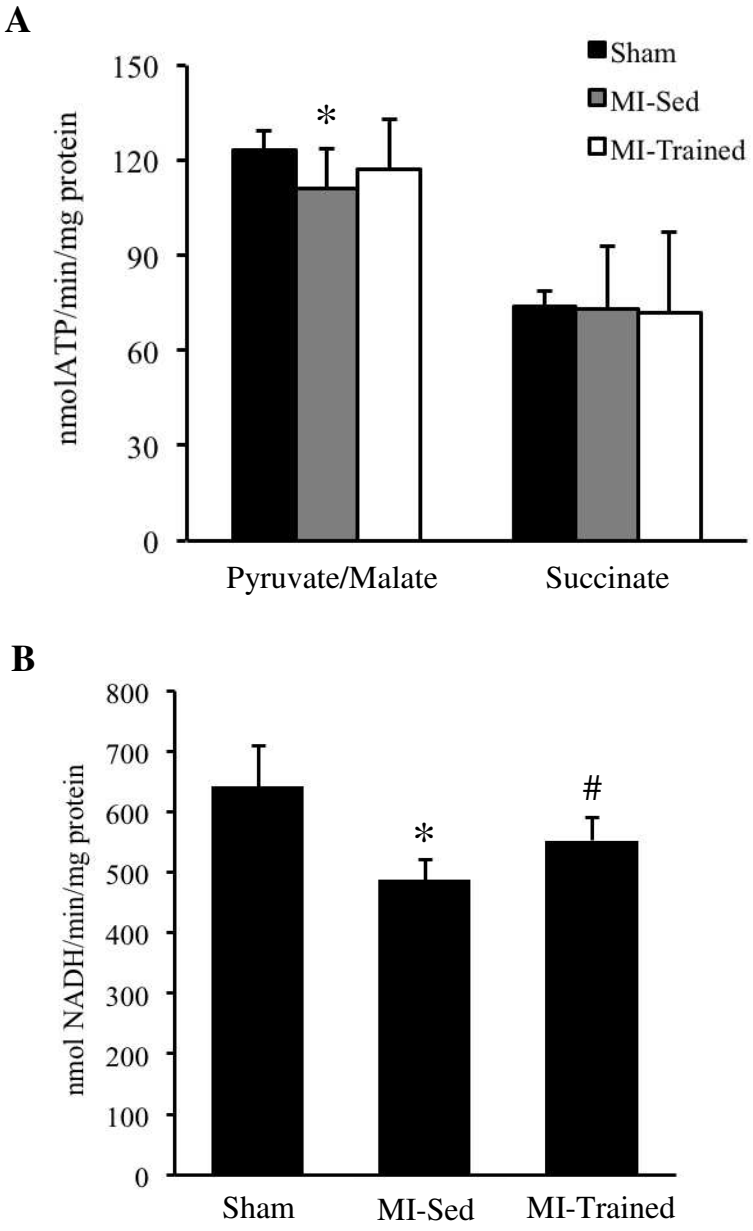


Figure 7. Analysis of ATP production rate and the activity of complex I of the electron transfer chain. **A**, The rate of ATP generation was assessed in isolated mitochondria in the presence of respiratory chain substrates, pyruvate and malate (complex I) or succinate (complex II), n=8 animals for MI-Sedentary (MI-Sed) and MI-Trained and 7 animals for Sham group. **B**, The enzymatic turnover of NADH: ubiquinone oxidoreductase (complex I) was assessed in solubilized mitochondria by recording the change of NADH absorbance in a reaction stimulated with decylubiquinone. **P<0.05 vs. Sham, #P<0.05 vs. MI-Sed and Sham, n=8 animals for each group.*

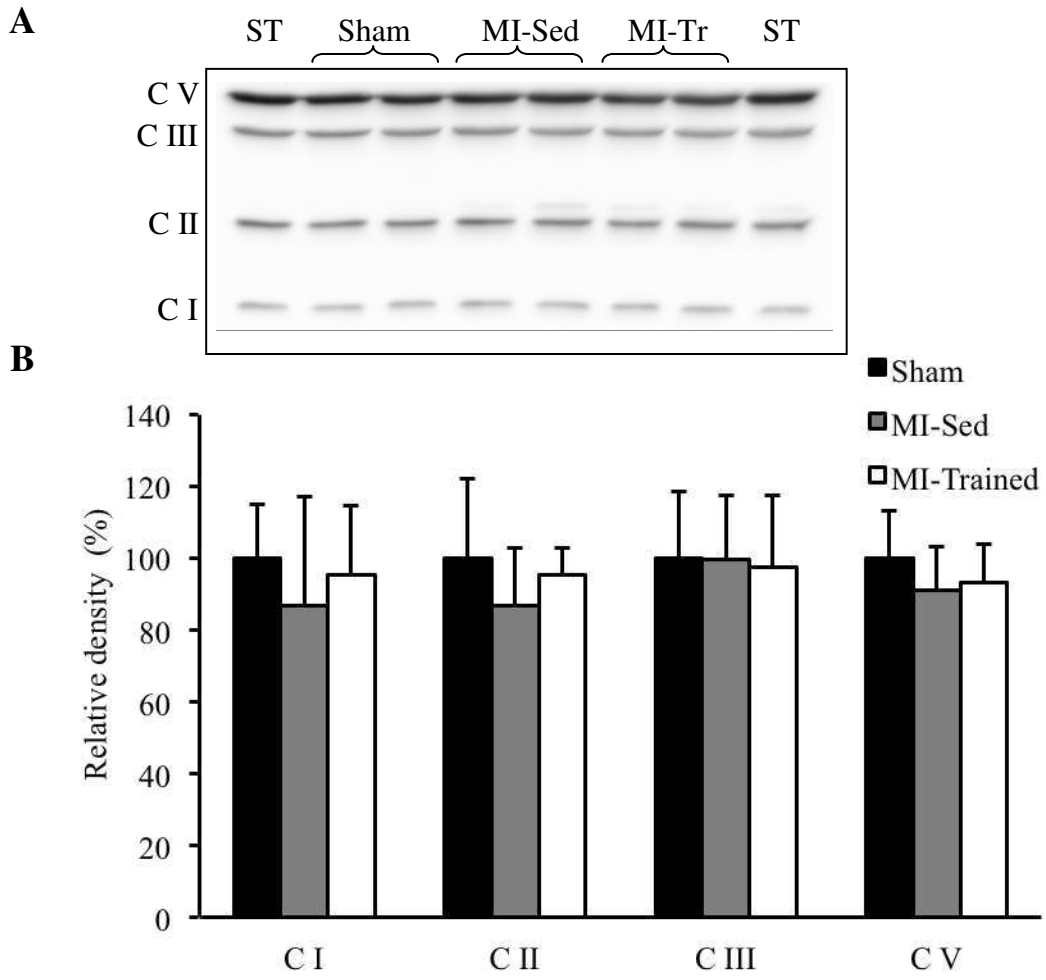

A
ST
Sham
MI-Sed
MI-Tr
ST
C V
C III
C II
C I
B
Sham
MI-Sed
MI-Trained
Relative density (%)
140
120
100
80
60
40
20
0
C I
C II
C III
C V
C
Sham
MI-Sed
MI-Trained
D
Relative carbonylation (%)
200
150
100
50
0
*
Sham
MI-Sed
MI-Trained

Figure 8. Expression of the electron transfer chain respiratory complexes and protein carbonylation. **A**, Image of the representative Western blot showing bands corresponding to mitochondrial respiratory complexes I, II, III and V. **B**, Average densities normalized to β-actin expressed relative to the Sham group. CI corresponds to complex I; CII, complex II; CIII, complex III and CV is complex V, n=6 animals for each group. **C**, Image of representative Western blot showing carbonylated proteins after derivatization with dinitrophenyl hydrazine. **D**, Average protein carbonylation normalized to Ponceau staining and expressed relative to the Sham group. **P<0.05 vs. Sham, n=6 animals per group.*

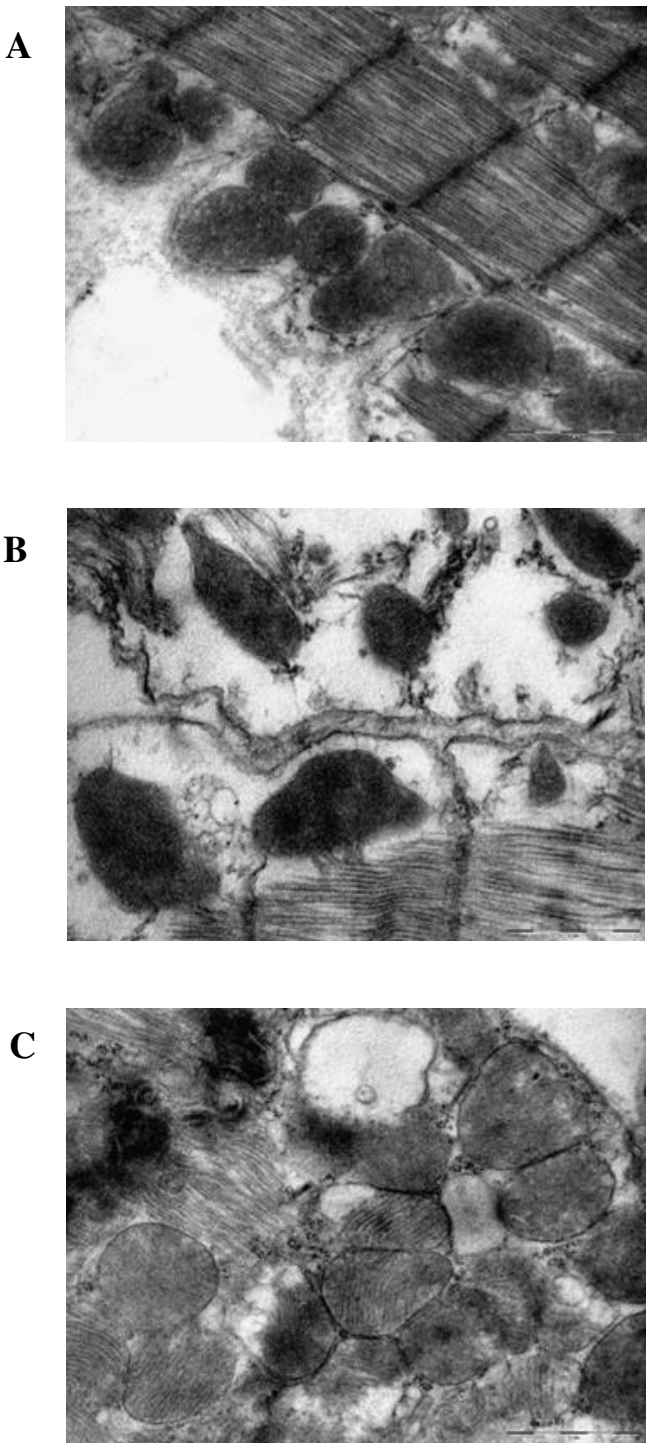


Figure 9. Electron micrographs of mitochondria. **A**, Sham, **B,** MI-Sedentary and **C,** MI-Trained rats.

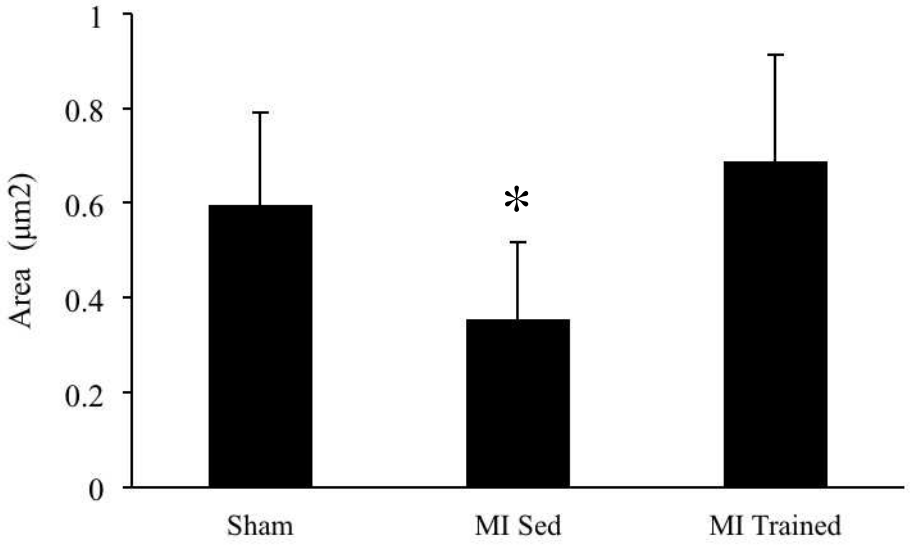


Figure 10. Average mitochondrial surface area measured in electron microscopy images using Image J. *$^{*}P<0.05$ vs. Sham and MI-Tr*

5. DISCUSSION

The main finding of the present study is that aerobic interval training attenuates deterioration of mitochondrial function in post-infarction heart failure in rats. This is evidenced by better preservation of mitochondrial respiratory capacity and activity of the complex I of the electron transfer chain in animals that underwent 8 weeks of aerobic interval training, as compared to their sedentary counterparts.

In our study, we observed impaired mitochondrial function in LV of the failing hearts, which is primarily attributable to the reduced activity of complex I of the electron transfer chain. This is evidenced by significantly decreased respiratory rates observed in mitochondria energized with specific complex I substrates. Moreover, measurement of complex I enzymatic activity revealed a diminished rate of NADH oxidation in mitochondria from infarcted hearts of sedentary animals, as compared to sham. Decreased complex I-dependent respiratory capacity was reported in saponin-permeabilized cardiac fibers in a similar animal model, (100) but also in dog (101) and human (73, 74) ischemic dilated cardiomyopathy. Furthermore, a specific decrease in complex I enzymatic function, with preserved activity of other ETC complexes, was found in explanted failing human hearts, as compared to healthy donor hearts (77). Reduced complex I function may result from its diminished expression, as reported previously in a mouse model of post-infarction heart failure, where it was linked to LV mitochondrial DNA damage (69). In our model, this possibility is less likely, since the expression level of complex I representative subunit was not altered in MI-Sedentary rats. This is also in line with the study of Scheubel et al. (77) where the mRNA levels of complex I subunits were not differentially expressed in human failing LV, despite the enzymatic activity of the entire complex being reduced. Alternatively, complex I activity may be depressed due to its specific inhibition. For example, a state of prolonged inflammation that persists in chronically failing heart was linked to enhanced activity of inducible nitric oxide synthase (NOS2), (102). NO may act directly (S-nitrosylation) or via peroxynitrite formation (generated through interaction with reactive oxygen species) on complex I, selectively reducing its activity (103).

Reduced mitochondrial capacity for substrate metabolization, as observed here, may result in decline of ATP production, which would cause substantial energy deficits in the muscle required to daily provide up to 30 kg of this energy-rich molecule (104, 105). Indeed, in cardiac mitochondria of post-MI sedentary animals, the rate of ATP production with complex I substrates were significantly reduced compared to sham-operated animals. This decreased ATP-producing potential, which may be coupled with the reported reduction in creatine kinase activity and creatine transporter function, (106, 107) is likely to contribute to cardiac contractile dysfunction, due to deficit in production and intracellular transfer of high-energy phosphates. Furthermore, deficiencies in complex I of the ETC were linked to excessive production of reactive oxygen species, (108) inflicting damage to other cellular and mitochondrial elements (109). Indeed, we detected increased protein carbonylation in post-infarcted myocardium, indicating increased levels of oxidative stress. Therefore, due to mitochondrial involvement in a number of cellular processes and a central role in myocardial energy production, their dysfunction likely causes further damage to the cardiac muscle and elicits progression of the disease (110). In our animal model, echocardiographic evaluation also revealed progressive deterioration of LV contractile function in post-MI sedentary animals from 4 weeks to 12 weeks after surgery, indicating further aggravation of the disease.

In contrast to the post-MI sedentary animals, rats with high degree of myocardial damage that underwent 8 weeks of exercise training exhibited better preservation of LV contractile function and reduced cavity dilatation. This is in agreement with data, both from patients and animal models, showing that physical activity attenuates or even reverses pathological ventricular remodelling in CHF of various etiology (111, 112). Therefore, exercise training, which was previously considered a risk for patients with post-infarction chronic heart disease, has recently been attributed a strong therapeutic potential for this condition. Although the beneficial effects of physical activity in a diseased heart have been associated with exercise training of different mode, intensity and duration, (113) aerobic interval training, with alternating high and low intensity exercise periods, was reported superior to other types of exercise, such as strength, endurance or moderate continuous training (112, 114). Its protective potential has been demonstrated in

animal and human studies, through the significant reduction of pathological left ventricular remodelling (112, 115).

There are multiple mediators and pathways suggested to underlie the beneficial exercise-induced effects in pathologically remodeled hearts. Some of them include enhanced endothelium-dependent vasodilatation and improved coronary blood flow, which may improve oxygen and substrate supply to the myocardium (116, 117). In cardiac myocytes, chronic exercise has been reported to restore Ca^{2+} sensitivity and handling, thus improving their contractility (88, 118). In the model of post-MI failing heart used here, the existence of energetic imbalance in the myocardium is very likely, due to additive effect of at least two factors: the observed reduction in mitochondrial function, as well as greater energy demand in a dilated heart (119). Therefore, we sought to investigate whether exercise improves the function of pathologically remodeled mitochondria in the failing myocardium. We demonstrated that mitochondrial respiratory capacity, which was significantly compromised by post-MI remodelling in sedentary animals, was better preserved in rats subjected to the training protocol. Higher respiratory rates observed in the trained animals were likely due to better preservation of complex I function. Measurements of complex I enzymatic activity further support this observation, as the NADH oxidation was maintained at the higher level in cardiac mitochondria of post-MI trained than in MI-sedentary rats. The extent of protein carbonylation and oxidative stress in MI-Trained animals was found to be somewhere between Sham and MI-Sedentary group with no statistically significant difference with either of them. Exercise-induced reduction in myocardial remodelling and mitochondrial protection was also documented recently in the swine model of pressure-overload heart failure; although the specific contribution of complex I was not investigated, cardiac mitochondria from trained animals exhibited lesser increase in sensitivity to the permeability transition (120).

Mechanism of the protective effect of exercise on complex I activity and on the overall mitochondrial function in the failing heart is still unknown. High levels of pro-inflammatory cytokines associated with this disease can elicit a number of cellular events (such as increased expression of NOS2 (121)), thus potentially contributing to the progression of ventricular remodelling and mitochondrial dysfunction (122). Exercise training was reported to oppose the inflammatory

cytokine effects in experimental models of chronic heart disease, which might counterbalance their detrimental actions, including those affecting mitochondria (123, 124). Increased myocardial expression of antioxidant enzymes, also related to exercise training, may also confer mitochondrial protection against the proposed ETC inhibition by reactive oxygen and nitrogen species (125). Furthermore, exercise was shown to reduce the excessive sympathetic activity that is usually found in CHF (124) and may act detrimentally through multiple pathways, for example by reducing the expression of PGC-1α,(126) a master regulator of mitochondrial metabolic function and biogenesis. However, the PGC-1α down regulation is not uniformly present in the failing myocardium,(127) and in our model we did not observe altered PGC-1α levels in any post-MI animal group.

In conclusion, we demonstrated that aerobic interval training in rat post-infarction chronic heart failure model mitigated contractile deterioration of the left ventricle. This was paralleled with better preservation of mitochondrial functional parameters, as evidenced by preserved activity of complex I of the electron transfer chain. Since damaged mitochondria contribute to the myocardial functional decline through multiple pathways, their protection conferred by exercise has a significant potential to repress this vicious cycle and alleviate the progression of contractile dysfunction in post-infarction failing heart.

6. MAIN CONCLUSIONS

1. Our experimental rat model for post-infarction heart failure shows adaptations characteristic for post-infarction left ventricular remodelling. After myocardial infarction animals had enlarged ventricular cavity, thinned myocardium and deteriorated contractile function of the left ventricle comparing to the sham-operated animals.

2. Aerobic interval training induces effective cardiovascular changes in rat heart after myocardial infarction.

3. Aerobic interval training attenuates deterioration of mitochondrial function in post-infarction heart failure in rats that underwent 8 weeks of aerobic interval training:
    a. better preservation of mitochondrial respiratory capacity
    b. better activity of the complex I of the electron transfer chain

SUMMARY

Effect of aerobic interval training on pathological remodelling and mitochondrial dysfunction in the post-infarction failing rat heart

Aims: Following a large myocardial infarction (MI), remaining viable muscle often undergoes pathological remodelling and progresses towards chronic heart failure (CHF). Mitochondria may also be affected by this process and, due to their functional importance, likely contribute to the progression of the disease. Aerobic interval training (AIT) has been shown effective in diminishing pathological myocardial transformation, but the effects of AIT on mitochondrial function in hearts undergoing remodelling are not known.

Methods and Results: Adult female Sprague-Dawley rats were randomized to either 8 weeks of aerobic interval treadmill running (5 days/week), which started 4 weeks after left coronary artery ligation (MI-Trained), or a sedentary group (MI-Sedentary). Echocardiography was performed before and after the 8-week period, at which point the left ventricles (LV) were also harvested. Twelve weeks after surgery, MI-Sedentary rats had significantly lower LV fractional shortening compared to MI-Trained. Complex I – dependent respiration assessed in isolated LV mitochondria was decreased by ~37% in MI-Sedentary and 17% in MI-Trained (group differences $p<0.05$), compared to sham-operated animals. This was paralleled with diminished ATP-production and increased degree of protein oxidation in MI-Sedentary rats. The enzymatic activity of complex I was also decreased to the greater extent in MI-Sedentary than in MI-Trained animals, with no evidence of its reduced expression. When complex II substrate was used, no differences among the three groups were observed.

Conclusions: Exercise reduces the left ventricle contractile deterioration in post-infarction heart failure and alleviates the extent of mitochondrial dysfunction, which is paralleled with preserved complex I activity.

Keywords: myocardial infarction; heart failure; aerobic interval training; animal model; mitochondria

SAŽETAK

Učinak aerobnog intervalnog treninga na patološko remodeliranje i mitohondrijsku disfunkciju u štakora s poslijeinfarktnim zatajenjem srca

Uvod: Nakon opsežnog infarkta miokarda (IM) u dijelu srčanog mišića izvan zahvaćenog područja dolazi do patološkog remodeliranja koje ako napreduje dovodi do razvoja kroničnog srčanog zatajenja. Patološko remodeliranje obuhvaća makroskopske i mikroskopske promjene na razini srčanog mišića. Remodeliranje na razini mitohondrija vjerojatno je važni čimbenik napredovanja bolesti zbog njihove ključne uloge u energijskom metabolizmu stanice. Aerobna intervalna tjelovježba učinkovito ublažava patološku transformaciju miokarda iako još uvijek nisu dovoljno istraženi mehanizmi preko kojih se to događa.

Materijali i metode: Ženke štakora Sprague-Dawley randomizirane su u dvije skupine: jedna je skupina 4 tjedna nakon podvezivanja lijeve koronarne arterije bila podvrgnuta aerobnoj intervalnoj tjelovježbi koja se sastojala od trčanja na pokretnoj traci (5 dana/tjedan) dok je druga tijekom istog razdoblja živjela sedentarnim životom. Prije i poslije perioda tjelovježbe od 8 tjedana učinjeno je ehokardiografsko testiranje. Nakon provedenih ispitivanja životinje su bile žrtvovane, a iz srca izolirani dijelovi lijevog ventrikula izvan područja zahvaćenog infarktom.

Rezultati: 12 tjedana nakon kirurškog zahvata sedentarne su životinje imale značajno nižu frakciju skraćenja lijevog ventrikula u usporedbi s treniranim životinjama. U mitohondrijima izoliranim iz lijevog ventrikula respiracija preko kompleksa I bila je 37% manja u sedentarnih i 17% manja u treniranih životinja (statistička razlika $p<0.05$) u usporedbi s kontrolnom skupinom (Sham štakori). Uz ove promjene u sedentarnih životinja postoji i smanjena proizvodnja ATP-a i povećana oksidacija proteina. Enzimska aktivnost kompleksa I respiracijskog lanca elektrona značajno je smanjena u sedentarnih u usporedbi s treniranim životinjama bez razlike u ekspresiji proteina kompleksa I. Nema statistički značajne razlike među grupama kada se respiracija odvijala uz supstrate za respiraciju preko kompleksa II.

Zaključci: Tjelovježba ublažava propadanje kontraktilne funkcije lijevog ventrikula i ublažava mitohondrijsku disfunkciju uz očuvanu aktivnost kompleksa I u srčanom mišiću nakon infarkta miokarda

Ključne riječi: infarkt miokarda; kronično zatajenje srca; aerobni intervalni trening; životinjski model; mitohondriji

REFERENCES

1. Yancy CW, Jessup M, Bozkurt B, Butler J, Casey DE, Jr., Drazner MH, et al. 2013 ACCF/AHA guideline for the management of heart failure: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. Journal of the American College of Cardiology. 2013;62(16):e147-239.
2. McMurray JJ, Adamopoulos S, Anker SD, Auricchio A, Bohm M, Dickstein K, et al. ESC guidelines for the diagnosis and treatment of acute and chronic heart failure 2012: The Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2012 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association (HFA) of the ESC. European journal of heart failure. 2012;14(8):803-69.
3. Fonseca C. Diagnosis of heart failure in primary care. Heart failure reviews. 2006;11(2):95-107.
4. Mosterd A, Hoes AW. Clinical epidemiology of heart failure. Heart. 2007;93(9):1137-46.
5. Morgan S, Smith H, Simpson I, Liddiard GS, Raphael H, Pickering RM, et al. Prevalence and clinical characteristics of left ventricular dysfunction among elderly patients in general practice setting: cross sectional survey. Bmj. 1999;318(7180):368-72.
6. Johnson FL. Pathophysiology and etiology of heart failure. Cardiology clinics. 2014;32(1):9-19, vii.
7. Wood DA, Coats AJ, Thompson SG, Poole-Wilson PA, Suresh V, Sutton GC. Incidence and aetiology of heart failure; a population-based study. European heart journal. 1999;20(6):421-8.
8. Jhund PS, McMurray JJ. Heart failure after acute myocardial infarction: a lost battle in the war on heart failure? Circulation. 2008;118(20):2019-21.
9. Hellermann JP, Jacobsen SJ, Redfield MM, Reeder GS, Weston SA, Roger VL. Heart failure after myocardial infarction: clinical presentation and survival. European journal of heart failure. 2005;7(1):119-25.

10. Latini R, Masson S, Staszewsky L, Barlera S. Neurohormonal modulation in heart failure of ischemic etiology: correlates with left ventricular remodeling. Current heart failure reports. 2006;3(4):157-63.
11. Francis GS. Pathophysiology of chronic heart failure. The American journal of medicine. 2001;110 Suppl 7A:37S-46S.
12. Lamblin N, Fertin M, de Groote P, Bauters C. Cardiac remodeling and heart failure after a first anterior myocardial infarction in patients with diabetes mellitus. Journal of cardiovascular medicine. 2012;13(6):353-9.
13. Meimoun P, M'Barek D, Dragomir C, Luycx-Bore A, Elmkies F, Boulanger J, et al. [Incidence, associated factors, and follow-up of hospital heart failure complicating acute anterior myocardial infarction successfully treated by primary angioplasty]. Annales de cardiologie et d'angeiologie. 2013;62(5):293-300.
14. Ikeda Y, Yutani C, Huang Y, Masuda K, Yuasa T, Kawaguchi O, et al. Histological remodeling in an ovine heart failure model resembles human ischemic cardiomyopathy. Cardiovascular pathology : the official journal of the Society for Cardiovascular Pathology. 2001;10(1):19-27.
15. Gallagher GL, Jackson CJ, Hunyor SN. Myocardial extracellular matrix remodeling in ischemic heart failure. Frontiers in bioscience : a journal and virtual library. 2007;12:1410-9.
16. Konstam MA. Patterns of ventricular remodeling after myocardial infarction: clues toward linkage between mechanism and morbidity. JACC Cardiovascular imaging. 2008;1(5):592-4.
17. Konstam MA, Udelson JE, Anand IS, Cohn JN. Ventricular remodeling in heart failure: a credible surrogate endpoint. Journal of cardiac failure. 2003;9(5):350-3.
18. Hein S, Arnon E, Kostin S, Schonburg M, Elsasser A, Polyakova V, et al. Progression from compensated hypertrophy to failure in the pressure-overloaded human heart: structural deterioration and compensatory mechanisms. Circulation. 2003;107(7):984-91.
19. Pfeffer MA, Braunwald E. Ventricular remodeling after myocardial infarction. Experimental observations and clinical implications. Circulation. 1990;81(4):1161-72.

20. Schaper J, Kostin S, Hein S, Elsasser A, Arnon E, Zimmermann R. Structural remodelling in heart failure. Experimental and clinical cardiology. 2002;7(2-3):64-8.
21. Mitchell GF, Lamas GA, Pfeffer MA. Ventricular remodeling after myocardial infarction. Advances in experimental medicine and biology. 1993;346:265-76.
22. Mitchell GF, Lamas GA, Vaughan DE, Pfeffer MA. Left ventricular remodeling in the year after first anterior myocardial infarction: a quantitative analysis of contractile segment lengths and ventricular shape. Journal of the American College of Cardiology. 1992;19(6):1136-44.
23. Erlebacher JA, Richter RC, Alonso DR, Devereux RB, Gay WA, Jr. Early infarct expansion: structural or functional? Journal of the American College of Cardiology. 1985;6(4):839-44.
24. Erlebacher JA, Weiss JL, Weisfeldt ML, Bulkley BH. Early dilation of the infarcted segment in acute transmural myocardial infarction: role of infarct expansion in acute left ventricular enlargement. Journal of the American College of Cardiology. 1984;4(2):201-8.
25. Hasenfuss G, Just H. Myocardial phenotype changes in heart failure: cellular and subcellular adaptations and their functional significance. British heart journal. 1994;72(2 Suppl):S10-7.
26. Yu ZB. [The role of cardiomyocyte apoptosis on mechanism of heart failure]. Sheng li ke xue jin zhan [Progress in physiology]. 2000;31(3):265-8.
27. Kang PM, Izumo S. Apoptosis and heart failure: A critical review of the literature. Circulation research. 2000;86(11):1107-13.
28. Nadal-Ginard B, Kajstura J, Leri A, Anversa P. Myocyte death, growth, and regeneration in cardiac hypertrophy and failure. Circulation research. 2003;92(2):139-50.
29. Brixius K, Frank KF, Bolck B, Hoyer F, Schwinger RH. [Reverse remodeling of the intracellular Ca(2+)-homeostasis: new concepts of pathophysiology and therapy of heart failure]. Wiener medizinische Wochenschrift. 2006;156(7-8):209-15.
30. Vescovo G, Dalla Libera L, Serafini F, Leprotti C, Facchin L, Volterrani M, et al. Improved exercise tolerance after losartan and enalapril in heart failure: correlation with changes in skeletal muscle myosin heavy chain composition. Circulation. 1998;98(17):1742-9.

31. Reiser PJ, Portman MA, Ning XH, Schomisch Moravec C. Human cardiac myosin heavy chain isoforms in fetal and failing adult atria and ventricles. American journal of physiology Heart and circulatory physiology. 2001;280(4):H1814-20.

32. Pott C, Eckardt L, Goldhaber JI. Triple threat: the Na+/Ca2+ exchanger in the pathophysiology of cardiac arrhythmia, ischemia and heart failure. Current drug targets. 2011;12(5):737-47.

33. Baartscheer A, Schumacher CA, Belterman CN, Coronel R, Fiolet JW. SR calcium handling and calcium after-transients in a rabbit model of heart failure. Cardiovascular research. 2003;58(1):99-108.

34. Chen YW, Pat B, Gladden JD, Zheng J, Powell P, Wei CC, et al. Dynamic molecular and histopathological changes in the extracellular matrix and inflammation in the transition to heart failure in isolated volume overload. American journal of physiology Heart and circulatory physiology. 2011;300(6):H2251-60.

35. Jane-Lise S, Corda S, Chassagne C, Rappaport L. The extracellular matrix and the cytoskeleton in heart hypertrophy and failure. Heart failure reviews. 2000;5(3):239-50.

36. Schaper J, Mollnau H, Hein S, Scholz D, Munkel B, Devaux B. [Interactions between cardiomyocytes and extracellular matrix in the failing human heart]. Z Kardiol. 1995;84 Suppl 4:33-8.

37. Vanhoutte D, Heymans S. TIMPs and cardiac remodeling: 'Embracing the MMP-independent-side of the family'. Journal of molecular and cellular cardiology. 2010;48(3):445-53.

38. Vanhoutte D, Schellings M, Pinto Y, Heymans S. Relevance of matrix metalloproteinases and their inhibitors after myocardial infarction: a temporal and spatial window. Cardiovascular research. 2006;69(3):604-13.

39. Feldman AM, Li YY, McTiernan CF. Matrix metalloproteinases in pathophysiology and treatment of heart failure. Lancet. 2001;357(9257):654-5.

40. Polyakova V, Hein S, Kostin S, Ziegelhoeffer T, Schaper J. Matrix metalloproteinases and their tissue inhibitors in pressure-overloaded human myocardium during heart failure progression. Journal of the American College of Cardiology. 2004;44(8):1609-18.

41. Figueredo VM, Camacho SA. Basic mechanisms of myocardial dysfunction: cellular pathophysiology of heart failure. Current opinion in cardiology. 1995;10(3):246-52.

42. Minamino T, Kitakaze M. Cellular mechanisms for the treatment of chronic heart failure: the nitric oxide- and adenosine-dependent pathways. Expert opinion on emerging drugs. 2002;7(1):99-110.

43. Ikram H. The renin-angiotensin system and cardiac remodelling after acute myocardial infarction. Heart. 1996;76(3 Suppl 3):68-72.

44. Orn S, Aukrust P. The prediction of adverse cardiac remodelling following myocardial infarction: defining the need for a dynamic multimarker approach. Heart. 2012;98(15):1112-3.

45. Isidoro Tavares N, Philip-Couderc P, Baertschi AJ, Lerch R, Montessuit C. Angiotensin II and tumour necrosis factor alpha as mediators of ATP-dependent potassium channel remodelling in post-infarction heart failure. Cardiovascular research. 2009;83(4):726-36.

46. Lang CC, Struthers AD. Targeting the renin-angiotensin-aldosterone system in heart failure. Nature reviews Cardiology. 2013;10(3):125-34.

47. Sayer G, Bhat G. The renin-angiotensin-aldosterone system and heart failure. Cardiology clinics. 2014;32(1):21-32, vii.

48. Yu Y, Wei SG, Zhang ZH, Gomez-Sanchez E, Weiss RM, Felder RB. Does aldosterone upregulate the brain renin-angiotensin system in rats with heart failure? Hypertension. 2008;51(3):727-33.

49. Ventura-Clapier R, Garnier A, Veksler V, Joubert F. Bioenergetics of the failing heart. Biochimica et biophysica acta. 2011;1813(7):1360-72.

50. Liu J, Wang C, Murakami Y, Gong G, Ishibashi Y, Prody C, et al. Mitochondrial ATPase and high-energy phosphates in failing hearts. American journal of physiology Heart and circulatory physiology. 2001;281(3):H1319-26.

51. Ardehali H, Sabbah HN, Burke MA, Sarma S, Liu PP, Cleland JG, et al. Targeting myocardial substrate metabolism in heart failure: potential for new therapies. European journal of heart failure. 2012;14(2):120-9.

52. Jaswal JS, Keung W, Wang W, Ussher JR, Lopaschuk GD. Targeting fatty acid and carbohydrate oxidation--a novel therapeutic intervention in the ischemic and failing heart. Biochimica et biophysica acta. 2011;1813(7):1333-50.

53. Abdurrachim D, Luiken JJ, Nicolay K, Glatz JF, Prompers JJ, Nabben M. Good and bad consequences of altered fatty acid metabolism in heart failure: evidence from mouse models. Cardiovascular research. 2015.

54. Pratt NG. Pathophysiology of heart failure: neuroendocrine response. Critical care nursing quarterly. 1995;18(1):22-31.

55. Azevedo PS, Minicucci MF, Santos PP, Paiva SA, Zornoff LA. Energy metabolism in cardiac remodeling and heart failure. Cardiology in review. 2013;21(3):135-40.

56. Neubauer S. The failing heart--an engine out of fuel. The New England journal of medicine. 2007;356(11):1140-51.

57. Marin-Garcia J, Akhmedov AT, Moe GW. Mitochondria in heart failure: the emerging role of mitochondrial dynamics. Heart failure reviews. 2013;18(4):439-56.

58. Chen L, Knowlton AA. Mitochondria and heart failure: new insights into an energetic problem. Minerva cardioangiologica. 2010;58(2):213-29.

59. Rosca MG, Hoppel CL. Mitochondria in heart failure. Cardiovascular research. 2010;88(1):40-50.

60. Baumgartner HK, Gerasimenko JV, Thorne C, Ferdek P, Pozzan T, Tepikin AV, et al. Calcium elevation in mitochondria is the main Ca2+ requirement for mitochondrial permeability transition pore (mPTP) opening. The Journal of biological chemistry. 2009;284(31):20796-803.

61. Palmer JW, Tandler B, Hoppel CL. Biochemical differences between subsarcolemmal and interfibrillar mitochondria from rat cardiac muscle: effects of procedural manipulations. Archives of biochemistry and biophysics. 1985;236(2):691-702.

62. Ong SB, Hausenloy DJ. Mitochondrial morphology and cardiovascular disease. Cardiovascular research. 2010;88(1):16-29.

63. Rosca MG, Tandler B, Hoppel CL. Mitochondria in cardiac hypertrophy and heart failure. Journal of molecular and cellular cardiology. 2013;55:31-41.

64. Tsutsui H, Kinugawa S, Matsushima S. Oxidative stress and mitochondrial DNA damage in heart failure. Circulation journal : official journal of the Japanese Circulation Society. 2008;72 Suppl A:A31-7.

65. Cooper MP. Interplay of mitochondrial biogenesis and oxidative stress in heart failure. Circulation. 2013;127(19):1932-4.

66. Lu FH, Fu SB, Leng X, Zhang X, Dong S, Zhao YJ, et al. Role of the calcium-sensing receptor in cardiomyocyte apoptosis via the sarcoplasmic reticulum and mitochondrial death pathway in cardiac hypertrophy and heart failure. Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology. 2013;31(4-5):728-43.

67. Ventura-Clapier R, Garnier A, Veksler V. Transcriptional control of mitochondrial biogenesis: the central role of PGC-1alpha. Cardiovascular research. 2008;79(2):208-17.

68. Rimbaud S, Garnier A, Ventura-Clapier R. Mitochondrial biogenesis in cardiac pathophysiology. Pharmacological reports : PR. 2009;61(1):131-8.

69. Ide T, Tsutsui H, Hayashidani S, Kang D, Suematsu N, Nakamura K, et al. Mitochondrial DNA damage and dysfunction associated with oxidative stress in failing hearts after myocardial infarction. Circulation research. 2001;88(5):529-35.

70. Merkle S, Frantz S, Schon MP, Bauersachs J, Buitrago M, Frost RJ, et al. A role for caspase-1 in heart failure. Circulation research. 2007;100(5):645-53.

71. Narula J, Pandey P, Arbustini E, Haider N, Narula N, Kolodgie FD, et al. Apoptosis in heart failure: release of cytochrome c from mitochondria and activation of caspase-3 in human cardiomyopathy. Proceedings of the National Academy of Sciences of the United States of America. 1999;96(14):8144-9.

72. Rosca MG, Vazquez EJ, Kerner J, Parland W, Chandler MP, Stanley W, et al. Cardiac mitochondria in heart failure: decrease in respirasomes and oxidative phosphorylation. Cardiovascular research. 2008;80(1):30-9.

73. Lemieux H, Semsroth S, Antretter H, Hofer D, Gnaiger E. Mitochondrial respiratory control and early defects of oxidative phosphorylation in the failing human heart. The international journal of biochemistry & cell biology. 2011;43(12):1729-38.

74. Sharov VG, Todor AV, Silverman N, Goldstein S, Sabbah HN. Abnormal mitochondrial respiration in failed human myocardium. Journal of molecular and cellular cardiology. 2000;32(12):2361-7.

75. Nojiri H, Shimizu T, Funakoshi M, Yamaguchi O, Zhou H, Kawakami S, et al. Oxidative stress causes heart failure with impaired mitochondrial respiration. The Journal of biological chemistry. 2006;281(44):33789-801.

76. Ide T, Tsutsui H, Kinugawa S, Utsumi H, Kang D, Hattori N, et al. Mitochondrial electron transport complex I is a potential source of oxygen free radicals in the failing myocardium. Circulation research. 1999;85(4):357-63.

77. Scheubel RJ, Tostlebe M, Simm A, Rohrbach S, Prondzinsky R, Gellerich FN, et al. Dysfunction of mitochondrial respiratory chain complex I in human failing myocardium is not due to disturbed mitochondrial gene expression. Journal of the American College of Cardiology. 2002;40(12):2174-81.

78. Rosca M, Minkler P, Hoppel CL. Cardiac mitochondria in heart failure: normal cardiolipin profile and increased threonine phosphorylation of complex IV. Biochimica et biophysica acta. 2011;1807(11):1373-82.

79. Drose S. Differential effects of complex II on mitochondrial ROS production and their relation to cardioprotective pre- and postconditioning. Biochimica et biophysica acta. 2013;1827(5):578-87.

80. Bayeva M, Gheorghiade M, Ardehali H. Mitochondria as a therapeutic target in heart failure. Journal of the American College of Cardiology. 2013;61(6):599-610.

81. Dickstein K, Cohen-Solal A, Filippatos G, McMurray JJ, Ponikowski P, Poole-Wilson PA, et al. ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure 2008: the Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2008 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association of the ESC (HFA) and endorsed by the European Society of Intensive Care Medicine (ESICM). European heart journal. 2008;29(19):2388-442.

82. Belardinelli R, Georgiou D, Cianci G, Purcaro A. Randomized, controlled trial of long-term moderate exercise training in chronic heart failure: effects on functional capacity, quality of life, and clinical outcome. Circulation. 1999;99(9):1173-82.

83. Lachance D, Plante E, Bouchard-Thomassin AA, Champetier S, Roussel E, Drolet MC, et al. Moderate exercise training improves survival and ventricular remodeling in an animal model of left ventricular volume overload. Circulation Heart failure. 2009;2(5):437-45.

84. Experience from controlled trials of physical training in chronic heart failure. Protocol and patient factors in effectiveness in the improvement in exercise tolerance. European Heart Failure Training Group. European heart journal. 1998;19(3):466-75.

85. Mentz RJ, Schulte PJ, Fleg JL, Fiuzat M, Kraus WE, Pina IL, et al. Clinical characteristics, response to exercise training, and outcomes in patients with heart failure and chronic obstructive pulmonary disease: findings from Heart Failure and A Controlled Trial Investigating Outcomes of Exercise TraiNing (HF-ACTION). American heart journal. 2013;165(2):193-9.

86. Lou Q, Janardhan A, Efimov IR. Remodeling of calcium handling in human heart failure. Advances in experimental medicine and biology. 2012;740:1145-74.

87. Marban E. Calcium and heart failure. Cardiovascular research. 1998;37(2):277-8.

88. Wisloff U, Loennechen JP, Currie S, Smith GL, Ellingsen O. Aerobic exercise reduces cardiomyocyte hypertrophy and increases contractility, Ca2+ sensitivity and SERCA-2 in rat after myocardial infarction. Cardiovascular research. 2002;54(1):162-74.

89. Terblanche SE, Gohil K, Packer L, Henderson S, Brooks GA. The effects of endurance training and exhaustive exercise on mitochondrial enzymes in tissues of the rat (Rattus norvegicus). Comparative biochemistry and physiology Part A, Molecular & integrative physiology. 2001;128(4):889-96.

90. Iemitsu M, Miyauchi T, Maeda S, Sakai S, Fujii N, Miyazaki H, et al. Cardiac hypertrophy by hypertension and exercise training exhibits different gene expression of enzymes in energy metabolism. Hypertension research : official journal of the Japanese Society of Hypertension. 2003;26(10):829-37.

91. Chen CY, Hsu HC, Lee BC, Lin HJ, Chen YH, Huang HC, et al. Exercise training improves cardiac function in infarcted rabbits: involvement of autophagic function and fatty acid utilization. European journal of heart failure. 2010;12(4):323-30.

92. Nediani C, Raimondi L, Borchi E, Cerbai E. Nitric oxide/reactive oxygen species generation and nitroso/redox imbalance in heart failure: from molecular mechanisms to therapeutic implications. Antioxidants & redox signaling. 2011;14(2):289-331.

93. Tsutsui H. Oxidative stress in heart failure: the role of mitochondria. Internal medicine. 2001;40(12):1177-82.

94. Ascensao A, Ferreira R, Magalhaes J. Exercise-induced cardioprotection--biochemical, morphological and functional evidence in whole tissue and isolated mitochondria. International journal of cardiology. 2007;117(1):16-30.

95. Freimann S, Scheinowitz M, Yekutieli D, Feinberg MS, Eldar M, Kessler-Icekson G. Prior exercise training improves the outcome of acute myocardial infarction in the rat. Heart structure, function, and gene expression. Journal of the American College of Cardiology. 2005;45(6):931-8.

96. Ventura-Clapier R. Exercise training, energy metabolism, and heart failure. Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme. 2009;34(3):336-9.

97. Li J, Sinoway AN, Gao Z, Maile MD, Pu M, Sinoway LI. Muscle mechanoreflex and metaboreflex responses after myocardial infarction in rats. Circulation. 2004;110(19):3049-54.

98. Migrino RQ, Zhu X, Morker M, Brahmbhatt T, Bright M, Zhao M. Myocardial dysfunction in the periinfarct and remote regions following anterior infarction in rats quantified by 2D radial strain echocardiography: an observational cohort study. Cardiovasc Ultrasound. 2008;6:17.

99. Hoydal MA, Wisloff U, Kemi OJ, Ellingsen O. Running speed and maximal oxygen uptake in rats and mice: practical implications for exercise training. Eur J Cardiovasc Prev Rehabil. 2007;14(6):753-60.

100. Sanbe A, Tanonaka K, Hanaoka Y, Katoh T, Takeo S. Regional energy metabolism of failing hearts following myocardial infarction. Journal of molecular and cellular cardiology. 1993;25(9):995-1013.

101. Sharov VG, Goussev A, Lesch M, Goldstein S, Sabbah HN. Abnormal mitochondrial function in myocardium of dogs with chronic heart failure. J Mol Cell Cardiol. 1998;30(9):1757-62.

102. Drexler H, Kastner S, Strobel A, Studer R, Brodde OE, Hasenfuss G. Expression, activity and functional significance of inducible nitric oxide synthase in the failing human heart. J Am Coll Cardiol. 1998;32(4):955-63.

103. Clementi E, Brown GC, Feelisch M, Moncada S. Persistent inhibition of cell respiration by nitric oxide: crucial role of S-nitrosylation of mitochondrial complex I and protective action of glutathione. Proc Natl Acad Sci U S A. 1998;95(13):7631-6.

104. Neubauer S, Horn M, Cramer M, Harre K, Newell JB, Peters W, et al. Myocardial phosphocreatine-to-ATP ratio is a predictor of mortality in patients with dilated cardiomyopathy. Circulation. 1997;96(7):2190-6.

105. Ashrafian H, Frenneaux MP. Metabolic modulation in heart failure: the coming of age. Cardiovasc Drugs Ther. 2007;21(1):5-7.

106. Weiss RG, Gerstenblith G, Bottomley PA. ATP flux through creatine kinase in the normal, stressed, and failing human heart. Proc Natl Acad Sci U S A. 2005;102(3):808-13.

107. Nascimben L, Ingwall JS, Pauletto P, Friedrich J, Gwathmey JK, Saks V, et al. Creatine kinase system in failing and nonfailing human myocardium. Circulation. 1996;94(8):1894-901.

108. Ide T, Tsutsui H, Kinugawa S, Suematsu N, Hayashidani S, Ichikawa K, et al. Direct evidence for increased hydroxyl radicals originating from superoxide in the failing myocardium. Circ Res. 2000;86(2):152-7.

109. Suematsu N, Tsutsui H, Wen J, Kang D, Ikeuchi M, Ide T, et al. Oxidative stress mediates tumor necrosis factor-alpha-induced mitochondrial DNA damage and dysfunction in cardiac myocytes. Circulation. 2003;107(10):1418-23.

110. Rosca MG, Hoppel CL. New aspects of impaired mitochondrial function in heart failure. Journal of bioenergetics and biomembranes. 2009;41(2):107-12.

111. Giannuzzi P, Temporelli PL, Corra U, Tavazzi L. Antiremodeling effect of long-term exercise training in patients with stable chronic heart failure: results of the Exercise in Left Ventricular Dysfunction and Chronic Heart Failure (ELVD-CHF) Trial. Circulation. 2003;108(5):554-9.

112. Wisloff U, Stoylen A, Loennechen JP, Bruvold M, Rognmo O, Haram PM, et al. Superior cardiovascular effect of aerobic interval training versus moderate

continuous training in heart failure patients: a randomized study. Circulation. 2007;115(24):3086-94.

113. Hambrecht R, Gielen S, Linke A, Fiehn E, Yu J, Walther C, et al. Effects of exercise training on left ventricular function and peripheral resistance in patients with chronic heart failure: A randomized trial. JAMA. 2000;283(23):3095-101.

114. Haykowsky MJ, Liang Y, Pechter D, Jones LW, McAlister FA, Clark AM. A meta-analysis of the effect of exercise training on left ventricular remodeling in heart failure patients: the benefit depends on the type of training performed. J Am Coll Cardiol. 2007;49(24):2329-36.

115. Kemi OJ, Hoydal MA, Macquaide N, Haram PM, Koch LG, Britton SL, et al. The effect of exercise training on transverse tubules in normal, remodeled, and reverse remodeled hearts. J Cell Physiol. 2011;226(9):2235-43.

116. Crimi E, Ignarro LJ, Cacciatore F, Napoli C. Mechanisms by which exercise training benefits patients with heart failure. Nature reviews Cardiology. 2009;6(4):292-300.

117. Gielen S, Schuler G, Hambrecht R. Exercise training in coronary artery disease and coronary vasomotion. Circulation. 2001;103(1):E1-6.

118. de Waard MC, van der Velden J, Bito V, Ozdemir S, Biesmans L, Boontje NM, et al. Early exercise training normalizes myofilament function and attenuates left ventricular pump dysfunction in mice with a large myocardial infarction. Circ Res. 2007;100(7):1079-88.

119. Pfeffer JM, Pfeffer MA, Fletcher PJ, Braunwald E. Progressive ventricular remodeling in rat with myocardial infarction. Am J Physiol. 1991;260(5 Pt 2):H1406-14.

120. Emter CA, Baines CP. Low-intensity aerobic interval training attenuates pathological left ventricular remodeling and mitochondrial dysfunction in aortic-banded miniature swine. Am J Physiol Heart Circ Physiol. 2010;299(5):H1348-56.

121. Singh K, Balligand JL, Fischer TA, Smith TW, Kelly RA. Regulation of cytokine-inducible nitric oxide synthase in cardiac myocytes and microvascular endothelial cells. Role of extracellular signal-regulated kinases 1 and 2 (ERK1/ERK2) and STAT1 alpha. J Biol Chem. 1996;271(2):1111-7.

122. Lewis NP, Tsao PS, Rickenbacher PR, Xue C, Johns RA, Haywood GA, et al. Induction of nitric oxide synthase in the human cardiac allograft is associated with contractile dysfunction of the left ventricle. Circulation. 1996;93(4):720-9.

123. Conraads VM, Beckers P, Bosmans J, De Clerck LS, Stevens WJ, Vrints CJ, et al. Combined endurance/resistance training reduces plasma TNF-alpha receptor levels in patients with chronic heart failure and coronary artery disease. Eur Heart J. 2002;23(23):1854-60.

124. Roveda F, Middlekauff HR, Rondon MU, Reis SF, Souza M, Nastari L, et al. The effects of exercise training on sympathetic neural activation in advanced heart failure: a randomized controlled trial. J Am Coll Cardiol. 2003;42(5):854-60.

125. Atalay M, Sen CK. Physical exercise and antioxidant defenses in the heart. Ann N Y Acad Sci. 1999;874:169-77.

126. Arany Z, Novikov M, Chin S, Ma Y, Rosenzweig A, Spiegelman BM. Transverse aortic constriction leads to accelerated heart failure in mice lacking PPAR-gamma coactivator 1alpha. Proc Natl Acad Sci U S A. 2006;103(26):10086-91.

127. Karamanlidis G, Nascimben L, Couper GS, Shekar PS, del Monte F, Tian R. Defective DNA replication impairs mitochondrial biogenesis in human failing hearts. Circ Res. 2010;106(9):1541-8.

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Working experience

- 2013 – current: Residency in Abdominal Surgery, University Hospital Split
- 2010 – 2013: PhD student, Department of Integrative Physiology, School of Medicine Split
- 2009 – 2010: Internship at the University Hospital Split

Education

- 2010 – 2013: Postgraduate School „Evidence Based Medicine"
- 2008: M.D. (Doctor of Medicine) - University of Split, School of Medicine *Dissertation: Natural Orifice Transluminal Endoscopic Surgery (N.O.T.E.S.): Experimental Training Models and Possibilities for Clinical Application*

Awards

- 2014: The Best Research and Scientific Publication in Basic Medical Sciences Annual Award 2012/2013, School of Medicine Split
- 2008: Dean´s Award for the best graduate student of the School of Medicine Split

Research activities

- Project participation
  - 2013
    - *Myocardial energetics as a target for treatment of ischemic heart disease: A translational approach from patient to mitochondria, Croatian Science Foundation Guest researcher at The Department of Circulation and Medical Imaging, Norwegian University of Science and Technology, Trondheim, Norway*

- 2010
  - *Exercise-induced improvement of chronic heart failure: the role of KATP channels and mitochondria, scientific project of Unity Through Knowledge Fund, reintegration grant (3B)*
  - *Physiology of SCUBA diving, scientific project of Unity Through Knowledge Fund, crossing borders grant (1B)*
- Project participation
  - 2009
    - *Natural Orifice Transluminal Endoscopic Surgery (N.O.T.E.S.), Endoscopic Surgery, Experimental Training Models and Possibilities for Clinical Application, The Experimental Surgery Laboratory, Department of Surgery*
- Research grants
  - *Diving with compressed air and cardiovascular system, scientific project (216-2160133-0130) – co-investigator*

Publications

- Aerobic interval training attenuates remodelling and mitochondrial dysfunction in the post-infarction failing rat heart. **Kraljevic J**, Marinovic J, Pravdic D, Zubin P, Dujic Z, Wisloff U, Ljubkovic M. Cardiovasc Res. 2013 May 2.
- The influence of varying inspired fractions of O_2 and CO_2 on the development of involuntary breathing movements during maximal apnoea. Breskovic T, Lojpur M, Maslov PZ, Cross TJ, **Kraljevic J**, Ljubkovic M, Marinovic J, Ivancev V, Johnson BD, Dujic Z. Respir Physiol Neurobiol. 2012 Apr 30; 181(2):228-33.
- First Croatian transvaginal laparoscopically assisted cholecystectomies. Perko Z, Cala Z, Mimica Z, Stipić R, Bakotin T, **Kraljević J**, Radonić V, Strinić T, Jakus IA, Simunić M. Hepatogastroenterology. 2012 Mar-Apr; 59(114):351-2.
- Cardiovascular changes during underwater static and dynamic breath-hold dives in trained divers. Breskovic T, Uglesic L, Zubin P, Kuch B, **Kraljevic J**, Zanchi J, Ljubkovic M, Sieber A, Dujic Z. J Appl Physiol. 2011 Sep; 111(3):673-8.
- Laparoscopic transabdominal preperitoneal approach for inguinal hernia repair: a five-year experience at a single center. Perko Z, Rakić M, Pogorelić Z, Družijanić N, **Kraljević J**. Surg Today. 2011 Feb; 41(2):216-21.

- Laparoscopic cholecystectomy in Cantonal Hospital Livno, Bosnia and Herzegovina and University Hospital Center Split, Croatia. Perić B, Perko Z, Pogorelić Z, **Kraljević J**. Coll Antropol. 2010 Mar; 34 Suppl 1:125-8.
- Transvaginal laparoscopically assisted cholecystectomy: a first Croatian experience. Perko Z, Mimica Z, Stipić R, Radonić V, Cala Z, Bakotin T, **Kraljević J**, Strinić T, Jakus IA, Simunić M. Lijec Vjesn. 2009 Mar-Apr; 131(3-4):100-1.
- N. O. T. E. S.--natural orifice transluminal endoscopic surgery. Perko Z, **Kraljević J**. Lijec Vjesn. 2007 Oct-Nov; 129(10-11):371-2.

Book Chapters

- Knjiga: Bol - uzroci i liječenje**.** Poglavlje: "Visceralna bol u trbuhu"; Perko Z, **Kraljevic J**. Urednici: Jukić M; Majerić Kogler V; Fingler M; Izdavač: Medicinska naklada, Zagreb, 2011. ISBN: 978-953-176-489-6

Teaching activities

- 2010 to 2013 - Teaching assistant, University of Split, School of Medicine Courses:
  - Physiology at Graduate Degree program in Medicine
  - Physiology at Graduate Degree program in Dental Medicine
  - Physiology at Undergraduate program in Physiotherapy
  - Physiology at Undergraduate program in Radiology
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Affiliations

- Croatian Medical Chamber
- Croatian Physiological Society
- Croatian Society for Endoscopic Surgery
- The European Association for Endoscopic Surgery