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Late exercise training improves non-uniformity of transmural myocardial function in rats with ischemic heart failure.

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ABSTRACT

Aims. The exercise-induced beneficial mechanisms after long-term myocardial infarction (MI) are incompletely understood. The present study evaluated the effect of treadmill exercise training (5 weeks), started at a late stage of heart failure (13 weeks post-MI), on rat left ventricle (LV) remodeling and dysfunction of the regional global and cellular contractile functions.

Methods and Results. In vivo echocardiography confirmed that sub-endocardial (ENDO) layers contracts more (+86%) and faster (+50%) than the sub-epicardial (EPI) layers. This gradient was lost in MI rats due to a predominant reduction in the ENDO layer contractility. Exercise restored partially the amplitude and velocity of ENDO contraction resulting in a partial recovery of the pump function indexed by the aortic blood-flow velocity time integral. At the cellular level, MI impaired ENDO contractile properties, by reducing cell shortening (from 10% to 7%), calcium transient, and myofilament Ca$^{2+}$ sensitivity. These alterations were normalized by exercise. SERCA2a expression and MLC-2 phosphorylation in ENDO cells were significantly reduced after MI and were restored by exercise. The sub-epicardial layer was only slightly reduced in vivo without cellular alterations.

Conclusions. This study shows that exercise performed at a late stage after MI restored a transmural non-uniformity of myocardium lost during heart failure. Recoveries of Ca$^{2+}$ homeostasis and myofilament function of cardiomyocytes contribute to this beneficial effect.
LATE EXERCISE TRAINING IMPROVES NON-UNIFORMITY OF TRANSMURAL MYOCARDIAL FUNCTION IN RATS WITH ISCHEMIC HEART FAILURE.

Running title: LATE EXERCISE IN HEART FAILURE

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INTRODUCTION
After myocardial infarction (MI), the heart is characterized by reduced contractility and impaired filling resulting from changes in cardiac structure (hypertrophy, dilatation), cell death, maladaptive remodeling of the extracellular matrix, abnormal energy metabolism and cellular dysfunction. The underlying cellular mechanisms that have been suggested include altered calcium homeostasis, impaired myofilament calcium responsiveness, and reduced cross-bridge cycling rate. A hierarchy of cellular events during the development of heart failure (HF) has been proposed, starting with electrical remodeling and altered Ca\(^{2+}\) homeostasis during the early phase followed by myofilament dysfunction. Moreover, transmural myocardial contractile performance is non-uniform across the different layers of the LV wall. This non-uniformity of cardiac function plays a fundamental role in cardiac mechanical work. Its loss has been previously reported to be a sensitive index to discriminate physiological from pathological left ventricular (LV) remodeling. We previously reported that HF affects preferentially the contractile machinery of the sub-endocardial cells (ENDO), leading to loss of transmural heterogeneity.

In clinic, pharmacological treatments are used to maintain as much as possible the pump function of the failing heart. However non-pharmacological approaches may be attempted. For example, many clinical studies have shown that exercise training performed by patients with HF is beneficial for heart performance and quality of life. In the normal heart of small and large animals, exercise training increases cellular contractile function such as cardiomyocyte shortening, Ca\(^{2+}\) dynamics and myocyte power-generating capacity, and myocardial perfusion capacity. There are also clinical evidences that exercise after MI has a beneficial effect on disease progression and survival. A recent study has shown that early
exercise in MI mice had no effect on LV remodeling but attenuates global LV dysfunction, which can be essentially explained by the exercise-induced improvement of myofilament function.\textsuperscript{14} However after a small MI, exercise has either no effect or improved LV function independently of the starting point of exercise (early or late after MI).\textsuperscript{14} For large infarct, exercise can have detrimental effects when performed at an early stage while beneficial effects on LV remodeling and function appear when exercise is started late after MI.\textsuperscript{15,16} In animal models, most of the studies tested the effect of exercise on LV global and/or cellular functions, 4 weeks after MI, when LV remodeling is still ongoing.\textsuperscript{17,18} Moreover, it has been shown in rat models that 3-5 weeks after MI exercise capacity is still high and close to sham-operated animals while it decreased significantly from 10 weeks after MI.\textsuperscript{19} Thus the cellular adaptations between early and late exercise after MI may be different.

Moreover, part of the discrepancy on the effect of exercise on MI in the literature may be due to use of swimming training\textsuperscript{15}, which is known to have different responses from those to treadmill running, complicated by factors such as the diving reflex, mental stress, and episodes of hypoxia associated with diving.

The present study evaluated in a rat model the effect of exercise training, started at a late stage of HF (13 weeks post-MI), on LV remodeling and function of the global and cellular contractile properties in various regions of the LV free wall. The results indicated that \textit{in vivo} systolic function (Fractional Shortening and aortic Velocity Time Integral (VTI\textsubscript{Ao})) was reduced in MI rats in association with a reduction of the amplitude and speed of contraction of the ENDO layer and to a lesser extent of the sub-epicardial layer (EPI). These alterations led to a uniformity of the transmural myocardial function, which were partially reversed by exercise. Exercise improved
part of the systolic parameters and slightly reduced LV dilatation. The in vivo beneficial effect of exercise was associated with a restoration of the ENDO cellular properties, by reversing the MI-induced abnormalities in Ca$^{2+}$-handling function and proteins, phosphorylation status of contractile proteins, and myofilament function.

METHODS

For a detailed description, see expanded Materials and Methods in the online data supplement. Experiments complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publications No. 85-23, revised 1996) and with the approval of the French Ministry of Agriculture.

Animals and exercise training

Myocardial Infarction was produced by permanent ligation of the left coronary artery in male Wistar rats as previously described.$^3$ Thirteen weeks post-MI, rats were randomly assigned to the sedentary (MI) or 5 weeks treadmill exercised (MI-Ex, 40 min/day, 5 days/week, 16 m/min) groups. At the end of the training protocol, morphological and functional parameters were determined by echocardiography and compared to sham-operated animals. Animals were then sacrificed for cellular investigations.

Echocardiography and noninvasive hemodynamics

Doppler-echocardiography was performed with a MyLab 30 (ESAOTE, Italy). Wall Thicknesses and Left Ventricular End Diameters (LVED) were obtained at the
level of the papillary muscles. LV shortening fraction and End Systolic strain of the posterior wall, measured as deformation from end-diastole to end systole \[\frac{(PWTs – PWTd)}{PWTd}\times100\] were calculated. PWTs and PWTd are respectively end systolic and end diastolic Posterior Wall Thickness.\(^{20}\)

Ascending aortic blood flow was recorded, as previously described,\(^{21}\) via pulsed-wave Doppler permitting measurements of the aortic blood flow velocity time integrals (VTI\(_{Ao}\)). LV sub-endocardial (ENDO) and sub-epicardial (EPI) posterior wall displacements were measured offline at the level of the papillary muscles. Tissue Doppler imaging (TDI) was performed after each conventional echocardiography as previously described.\(^{22}\)

**Contractile properties in intact cardiomyocytes**

Single LV cardiomyocytes were isolated by enzymatic dissociation from the remaining inner free wall (ENDO) and from the outer free wall (EPI) as previously described (n=5 rats per group).\(^{23}\) Unloaded cell shortening and calcium concentration \([\text{Ca}^{2+}]\) (indo 1 dye) were studied using field stimulation (0.5Hz, 22°C, 1.8 mM external \(\text{Ca}^{2+}\)). Sarcomere length (SL) and fluorescence (405 nm and 480 nm) were simultaneously recorded (IonOptix system, Hilton, USA).

**Force measurements in permeabilized cardiomyocytes**

Isometric force was measured in single permeabilized cardiomyocytes at different \([\text{Ca}^{2+}]\), at 1.9 then 2.3 \(\mu\)m SL as described previously (n=5 rats per group).\(^{3,23}\) Force was normalized by the cross-sectional area measured from the imaged cross-section. Force-pCa relations were fitted to a Hill equation. To
prevent degradation, all solutions contained protease inhibitors (PMSF: 0.5 mmol/L; leupeptin: 0.04 mmol/L and E64: 0.01 mmol/L).

**Myosin-Heavy chain composition**

MyHC isoforms were separated on a 6% SDS-PAGE and silver stained as previously described. β-MyHC content was expressed relative to the total amount of MyHC protein.

**Western Immunoblotting**

SERCA2a and phospholamban (PLB) were separated on a gradient SDS-PAGE (2-20%) and then blotted onto nitrocellulose membrane. Proteins were revealed with specific antibodies and were expressed relative to Calsequestrin content.

Troponin I (TnI) was separated by 15% SDS-PAGE and the protein kinase A phosphorylated form of cardiac TnI was normalized by the total TnI form as previously described. Myosin light chain 2 (MLC-2) phosphorylated and non-phosphorylated forms were separated by a 10% urea gel and were specifically detected with a cardiac MLC-2 antibody. Immunodetection was revealed with ECL Plus system.

**Statistical analysis**

Data were analyzed using one-way or 2-way ANOVA between groups. When significant interactions were found, a Bonferroni t-test was applied with adjusted \( P < 0.05 \) (Sigmastat 3.5). Data are presented as mean±SEM.
Results

In vivo cardiac morphological and functional parameters

MI animals had large antero-lateral infarcts detected by visual inspection. In a preliminary longitudinal study (data not shown), we observed that in our model, most of the morphological and functional alterations occurred over 11-13 weeks. Minimal changes will occur after this period. Thirteen weeks after MI, LV diameters were increased in MI at the end-diastolic and end-systolic phases without hypertrophy of the posterior wall thickness (Figure 1A). The posterior wall end systolic strain during a cardiac cycle was largely reduced (-80%) in MI animals (Figure 1B). In sham animals, the LV sub-endocardial layer contracted faster (+50% of the section motion) than the sub-epicardial layers (Figure 1C). Heart failure significantly decreased contraction velocities of both EPI and ENDO layers by 15 and 32%, respectively, homogenizing the transmural velocities of contraction toward the slowest values. The global cardiac systolic function was also altered as shown by the reduction in fractional shortening (FS) (Figure 1D). Since the calculation of FS, measured at the level of papillary muscle, is highly affected by the akinetic infarct zone, we measured the aortic blood-flow velocity time integral (VTI\textsubscript{Ao}) as another index of systolic function. VTI\textsubscript{Ao} was also decreased after MI (Figure 1D). Additional weeks after MI corresponding to the training duration had little impact on these parameters (see below).

Effect of exercise on in vivo cardiac function

We then investigated the effect of exercise training while most of the remodeling and dysfunction of the heart had already occurred. Eighteen weeks post-
MI, hearts were hypertrophied as shown by the increase in the heart weight/body weight ratio between Sham and MI animals and further increased after exercise training (Table 1). Part of this exercise-induced hypertrophy may be explained by an increase of the posterior wall thickness although the difference did not reach significance (p= 0.069) (Table 1). The main effect of exercise on LV morphologies was a decrease of both end-systolic and end-diastolic left ventricular diameters (Table 1). Eighteen weeks after MI, we observed a large decrease in the end systolic strain (from 83±4% to 24±11% in sham and MI, respectively) and in the absolute ENDO posterior wall displacement between diastole and systole (from 2.7±0.2 mm to 1.3±0.2 mm in sham and MI, respectively). The values obtained were similar to the one measured 13 weeks post-MI confirming the stability of these indexes during this phase of the disease (Figure 2B). Exercise had a significant beneficial effect on the end systolic strain (63±7%) and on the ENDO posterior wall displacement (1.8±0.4 mm). Sm was similarly decreased between 13 and 18 weeks after MI in both regions. Exercise restored Sm completely in EPI and only partially in the ENDO layers (Figure 2C). The gradient of velocity across the LV defined as the difference of velocity between ENDO and EPI, observed in normal conditions (1.66±0.11 cm.s\(^{-1}\)), disappeared almost completely in MI animals (0.28±0.05 cm.s\(^{-1}\)) and was partially restored in MI-Ex (0.76±0.05 cm.s\(^{-1}\)) (Figure 2C). Finally, \(\text{VTI}_{Ao}\), used as an accurate index of LV stroke volume, decreased by 37% in MI animals and was partially restored by exercise in MI-EX remaining 14% lower than sham (Figure 2D). Exercise had no impact on the mortality of the animals (Figure 2E).

Effect of exercise on myocyte shortening and Ca\(^{2+}\) transient
The effect of exercise on the excitation-contraction coupling of long-term MI was tested in intact LV unloaded cardiomyocytes. For this purpose, sarcomere length shortening and intracellular calcium content were simultaneously measured on field stimulated cardiomyocytes. Unloaded contraction decreased significantly with HF only in ENDO cells and was restored by exercise (Figure 3). The duration of unloaded shortening (data not shown) and relaxation (Figure 3B) were not different between the various groups. **However, the speed of contraction and relaxation were significantly reduced in ENDO MI cells and were restored by exercise (Figure 3C).** The amplitude of Ca\(^{2+}\) transient decreased significantly only in ENDO MI cells and was restored by exercise (Figure 4B). **In addition, the calcium transient decay in both EPI and ENDO MI myocytes was significantly slowed (increase in tau),** reflecting an altered calcium reuptake. All parameters were normal in MI-Ex. The calcium transient decay in MI is known to be altered due to changes in SERCA2a expression or in the inhibition of SERCA2a/PLB activity. We found that SERCA2a expression was significantly decreased in MI animals, both in ENDO and EPI layers. PLB expression was unchanged. Levels of protein expression in MI-Ex were similar to sham samples (Figure 4C).

**Force development in single permeabilized myocytes**

Force development of intact myocytes depends on the amount of calcium released by the sarcoplasmic reticulum and the myofilament Ca\(^{2+}\) sensitivity. Thus, we measured the myofilament Ca\(^{2+}\) sensitivity (pCa\(_{50}\)) at short length (1.9 \(\mu\)m SL) and at long length (2.3 \(\mu\)m SL). Passive force and maximal isometric tension measured at both SL were similar between Sham, MI, and MI-Ex (Table 2). Neither pathology nor exercise had any effect on myofilament Ca\(^{2+}\) sensitivity at short SL.
Stretching the cells to 2.3 µm SL induced a leftward shift of tension in all conditions reflecting an increase in myofilament Ca\(^{2+}\) sensitivity. However, pCa\(_{50}\) was significantly lower in the ENDO cells isolated from MI rats. Exercise restored Ca\(^{2+}\) sensitivity at 2.3 µm SL (Figure 5A). The difference between pCa\(_{50}\) at the long and short SL (ΔpCa\(_{50}\)), used an index of the length-dependent activation of contractile machinery, was significantly smaller in ENDO MI cells compared to the Sham and MI-Ex cells. EPI myocytes were affected neither by the pathology nor by the exercise at both lengths (Figure 5B).

We have previously shown that ΔpCa\(_{50}\) is closely related to passive tension rather than to SL.\(^3\) The differences in passive and active properties across the left free wall described a positive relationship in which ENDO cells develop more passive tension after a stretch to 2.3 µm SL associated with higher stretch-induced myofilament Ca\(^{2+}\) sensitivity than did EPI cells (Figure 5C). This correlation disappeared in MI rats, mostly due to a decrease in ΔpCa\(_{50}\) of ENDO cells. Interestingly, values obtained in MI-Ex myocytes were similar to those found in sham rats.

**Contractile protein isoforms and phosphorylation**

Rat heart expresses two isoforms of MyHC (α- or β-MyHC). Variation in α/β-MyHC expression influences cardiac function. In our conditions β-MyHC content increased after MI in both ENDO and EPI cells. Exercise had no effect on β-MyHC content in either ENDO or EPI cells of MI rats (Figure 6A).

TnI and MLC-2 phosphorylations are known to shift the tension-pCa curves in cardiac muscle. Western blot analysis was performed on non-stretched and stretched..
skinned muscle strips dissected from the sub-endocardial layer or the sub-epicardial layer. Phosphorylation of TnI on the PKA sites was similar between regions, before and after stretch, in sham and MI animals (Figure 6B). We have previously shown that the linear relationship between $\Delta pCa_{50}$ and passive tension was associated with changes in the phosphorylation level of MLC-2. As previously reported, stretch increased by ≈10% the amount of phosphorylated MLC-2 in the ENDO myocardium of sham animals and not in MI animals (Figure 6C). Exercise restored the stretch-induced increase in MLC-2 phosphorylation in MI animals. MLC-2 phosphorylation was not affected by pathology, stretch and exercise in EPI myocardium.

Discussion

The present study tested whether a remodeled end-stage failing heart following myocardial infarction could benefit from 5 weeks of endurance exercise training. To this end, we evaluated the effects of exercise on LV remodeling, regional in vivo function, cardiomyocyte contractility, Ca$^{2+}$ handling, and myofilament Ca$^{2+}$ sensitivity. The main findings were that: (1) normal myocardial function is non-uniform due to heterogeneous cellular properties across the wall (2) transmural non-uniformity of myocardial function was lost in MI rats due to ENDO cellular dysfunction (3) exercise recovered a transmural heterogeneity by improving LV function mostly in sub-endocardial layer, in relation with a normalization of MI-induced dysfunctions of both Ca$^{2+}$ handling and myofilaments Ca$^{2+}$ sensitivity in ENDO cardiomyocytes.
Regional pathophysiology of MI-induced LV dysfunction

It is now established that cardiac function is non-uniform across the wall due to larger and faster contraction of the sub-endocardial layer as compared with the sub-epicardial tissue one.\textsuperscript{7} A recent study using transmural bead markers under biplane cineradiography showed that the onset of myofiber shortening occurred earlier in endocardium than epicardium while the onset of myofiber relaxation occurred earlier in epicardium than endocardium.\textsuperscript{25} These differences in fiber strains may be explained by telediastolic and telesystolic transmural differences in wall stress, increasing toward the endocardium.\textsuperscript{26} Our data showed that the higher sub-endocardial fiber shortening during systole was likely due to higher basal cardiomyocyte contractility and a higher stretch-induced increase in myofilament Ca\textsuperscript{2+} sensitivity.

As previously reported,\textsuperscript{27,28} the remodeling of MI heart was characterized by LV dilatation, hypertrophy, and dysfunction. The cardiac pump dysfunction was characterized here by a reduction in LV fractional shortening and other indexes such as the aortic velocity time integral, and the amplitudes and velocities of contraction of the myocardial layers. We also observed that the pathology did not affect uniformly the heart. Following MI, wall contractility decreased mostly in the sub-endocardial layer with a higher decrease in the amplitude of displacement (-52\% vs -28\%, Endo vs Epi) and in the velocity of contraction (-36\% vs -13\%, Endo vs Epi). These changes resulted in a homogenization in regional myocardial function and the complete loss of transmural nonuniformity.

Beneficial effects of exercise training on MI cardiac function
In our MI model, most parameters of the LV remodeling and in vivo dysfunctions were achieved 13 weeks after MI and were stable 18 weeks after MI (Figures 1 and 2). We thus assessed whether exercise could improve myocardial function and restore the transmural heterogeneity in a remodeled MI heart. Studies in humans with large MI reported that exercise had either no\textsuperscript{29}, or a beneficial\textsuperscript{30} effect on ejection fraction and LV volumes. Previous studies in animal models reported that exercise had no effect on LV function parameters such as LV dP/dtmax or fractional shortening irrespective of whether exercise was started early or late after MI, despite an improvement in cell function.\textsuperscript{15,17} Neither was fractional shortening in MI improved by exercise in our study. However, other indexes of cardiac function such as aortic blood flow (VTIAo) and regional amplitude and velocities of contraction were improved after exercise. In several clinical studies, alterations in wall motion (including wall thickening) measured by echocardiography were shown to be good predictors of subsequent cardiac events of morbidity and mortality \textsuperscript{31, 32} and interventions that halt, slow or reverse these ventricular dysfunctions should markedly improve clinical outcomes.\textsuperscript{33} The higher exercise-induced improvement of amplitude and velocity of contraction in ENDO allowed to partially recovering a transmural nonuniformity. This may in turn contribute in the exercise-induced beneficial effect on cardiac function.

In view of the concern that late exercise may aggravate LV remodeling after a large MI and life expectancy, we investigated LV remodeling with or without exercise. Five weeks of exercise decreased LV end-diastolic and end-systolic diameters, and did not aggravate LV remodeling. Our exercise protocol was a moderate endurance protocol, confirmed by the fact that sham animals exercised at the same intensity did not show any change in any parameters investigated (data not shown). These
observations are in agreement with a recent study reporting that 8 weeks of moderate exercise in mice with MI cardiomyopathy\textsuperscript{14} and hypertrophic cardiomyopathy\textsuperscript{34} reversed collagen content with little effect on cardiac hypertrophy. In our study, exercise did not induce a higher mortality consistent with previous studies with human patients.\textsuperscript{16}

**Exercise-induced effects on contractile cellular properties**

The mechanisms for LV dysfunction after MI remain incompletely understood but have been proposed to be a consequence of cellular alterations and extracellular matrix remodeling.\textsuperscript{1} Recent studies have shown that early exercise in mice after MI had no effect on LV remodeling but slightly attenuates global LV dysfunction mostly by improving myofilament function without Ca\textsuperscript{2+} signaling.\textsuperscript{14} However, similar protocols performed in female Sprague-Dawley rat model showed a recovery of contraction function, Ca\textsuperscript{2+} handling and some indexes of improved myofilament Ca\textsuperscript{2+} sensitivity following early exercise after MI.\textsuperscript{17}

In our study, cell shortening decreased after MI exclusively in ENDO cells, most probably due to both reduced Ca\textsuperscript{2+} release and decreased myofilament function. Exercise fully restored the ENDO cellular properties without affecting the EPI cells. The recovery of calcium transient after exercise was associated with changes in SERCA2a\textsuperscript{17,present study} and Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger\textsuperscript{17} expression suggesting a recovery of the loading conditions of the sarcoplasmic reticulum. Further studies are needed to determine whether the exercise-induced improvement in systolic Ca\textsuperscript{2+} levels are due to changes in Ca\textsuperscript{2+} release properties through the ryanodine receptors.

**Myofilament Ca\textsuperscript{2+} sensitivity was depressed at end-stage HF in the present study.** Previous studies in single skinned myocytes have reported
either increases\textsuperscript{35} or decreases\textsuperscript{3,4} in myofilament Ca\textsuperscript{2+} sensitivity both in human and experimental HF. The reasons for these different findings are not entirely clear. One explanation for these different findings may relate to the different experimental preparations that were studied (multicellular vs. single myocyte skinned myocardium). Another explanation might be the level of neurohormonal stimulation present at the time of tissue preparation that will affect the balance between kinases and phosphatases. Indeed, most of the studies reported alterations of the level of phosphorylation of sarcomeric regulatory proteins that correlated with the changes in myofilament Ca\textsuperscript{2+} sensitivity. Finally the origin of heart failure (ischaemic, pressure overload) and the stage of HF may also affect the results.\textsuperscript{6}

Heart failure in small rodents is associated with a shift in isomyosin synthesis from predominantly α-myosin toward β-myosin.\textsuperscript{36} This shift observed during the LV remodeling has been proposed to improve myocardial work efficiency by generating cross-bridge force with a higher economy of energy consumption and thus maintaining contractility.\textsuperscript{6} Similar shift can be observed in exercised animals.\textsuperscript{24} In our study, β-MyHC content was increased by HF but was not affected by exercise. Thus the beneficial effect of exercise on MI myocardial function is independent on myosin expression.

The level of MLC-2 phosphorylation affects myofilament Ca\textsuperscript{2+} sensitivity.\textsuperscript{37} Increasing MLC-2 phosphorylation by incubating myofilaments with exogenous MLC Kinase (MLCK) increases myofilament Ca\textsuperscript{2+} sensitivity.\textsuperscript{38-40} A decrease in MLC-2 phosphorylation has been described in failing human\textsuperscript{41} and animal\textsuperscript{3} hearts and has been associated with the observed decrease in myofilament Ca\textsuperscript{2+} sensitivity; similar results were obtained in Endo MI
myocytes of the present study. Thus, the increase in MLC-2 phosphorylation in the sub-endocardial tissue exclusively, observed in the present study after exercise may by itself explain the improved myofilament Ca\textsuperscript{2+} sensitivity. The phosphorylation level of MLC-2 depends on the balance of activities between the MLCK and a protein phosphatase (PP)\textsuperscript{40}. MLCK activity is modulated by PKA and PKC phosphorylations, and Ca\textsuperscript{2+}-calmodulin interaction offering potential regulators for the exercise-induced effect on MLC-2 phosphorylation.\textsuperscript{40,42} The normalization of Ca\textsuperscript{2+} transient after exercise in MI may contribute to increasing MLCK activity and thus MLC-2 phosphorylation. MLC-2 dephosphorylation is classically attributed to PP1. Studies suggest that PP1 activity and expression is increased in end-stage CHF in dog\textsuperscript{43} but PP1 expression was found unchanged in rat with CHF.\textsuperscript{44} In our study, alteration in PP1 activity after MI and/or exercise, could account for the changes in MLC-2 phosphorylation and the lack of effect on TnI between MI and MI-Ex. However, more experiments are required to explore the kinase/phosphatase alteration after MI and exercise.

**Limitations of the study**

From the present study, we cannot draw any conclusions as to the effect of this protocol on life expectancy since the animals were sacrificed at the end of the exercise protocol. In particular, future studies should determine how the improvement of the global and regional cardiac function and the normalization of the cellular dysfunctions post-MI at the end of the training could affect the occurrence of sudden cardiac death because of arrhythmic events. It would have been also interesting to obtain intraventricular pressure measurements
that would have given more in vivo functional index, allowing to further explore the effect of exercise training on diastolic (dys)function in our model.

The present study indicates that exercise training started late after a large MI improved regional LV function and molecular phenotype, without any adverse effects on LV remodeling and survival during the protocol. The beneficial effects of exercise on cellular function have been proposed to restore β1-adrenergic signaling. However, there is no sign here of such improvement that should affect the phosphorylation status of various proteins (TnI, PLB, titin-based passive tension). Future studies should be aimed at investigating whether this difference is due to the fact that exercise was performed in organisms in which the β-adrenergic signaling has been already extensively stimulated, suggesting other signaling pathways for exercise-induced beneficial effects. This is of particular interest since most patients with heart failure are treated with β-blockers. Another positive aspect of this study is that exercise preferentially restored the contractile properties of the tissue altered by the pathology (ENDO), allowing physiological cardiac contractile heterogeneity to be restored. Because of its relatively low cost, high availability and ease of use, exercise training is an intervention that could be accessible to most patients with heart failure.

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**Conflict of interest**  None
REFERENCES


Legends

Figure 1. Left ventricular morphological and functional characteristics 13 weeks post-MI. End-diastolic and end-systolic diameters and diastolic posterior wall thickness (PWTd) (A), end systolic strain (B), Systolic velocity of the LV Epicardial and Endocardial layers (C), global systolic function (D) as indexed by fractional
shortening (left), and aortic velocity time integral (VTI$_{Ao}$, right). * adjusted p<0.05 vs corresponding sham (n=11 hearts/group).

Figure 2. Left ventricular morphological and functional characteristics in MI and MI-Ex rats. A) Echocardiography images with an enlargement of the posterior wall. B) End systolic strain (left) and displacement of Epicardial and Endocardial layers during a cardiac cycle (right). C) Systolic velocity (left) and Gradient of velocity Endo–Epi (right). D) Aortic velocity time integral (VTI$_{Ao}$). E) Survival curve following the beginning of exercise program. * adjusted p<0.05 (n=12-17 hearts/group).

Figure 3. Sarcomere length shortening in isolated MI and MI-Ex cardiomyocytes. A) SL shortening examples of endocardial (left trace) and epicardial (right trace) sham, MI, and MI-Ex myocytes. B) Averaged data of SL shortening (in % on baseline) and time to 50% of relaxation (TR50). C) Speeds of contraction (left) and relaxation (right) (n=55-106 cells/5 hearts) * adjusted p<0.05

Figure 4. Calcium transient in isolated MI and MI-Ex cardiomyocytes. A) Ca$^{2+}$ transient illustrations of endocardial (left trace) and epicardial (right trace) sham, MI, and MI-Ex myocytes. B) Average data of calcium transient amplitude, and relaxation phase (tau). (n=55-106 cells/5 hearts) C) Immunoblot analysis of Ca$^{2+}$-handling proteins (SERCA and PLB) normalized with calsequestrin content (n=5 hearts). * adjusted p<0.05

Figure 5. Effect of MI and exercise on myofilament properties in skinned cardiomyocytes: Tension (normalized to maximal tension)-pCa curves of ENDO (A)
and EPI (B) myocytes, measured at 1.9 (solid line, solid symbols) and at 2.3 \( \mu \text{m} \) (dashed line, open symbols) sarcomere length in sham (square) MI (triangle) and MI-Ex (circle). (A and B, right) Myofilament Ca\(^{2+}\) sensitivity (pCa for half maximal activation) and the stretch-induced Ca\(^{2+}\) sensitization (\( \Delta \text{pCa}_{50} \) difference in pCa\(_{50}\) obtained at 2.3 and 1.9 \( \mu \text{m} \) SL). C) Correlation between \( \Delta \text{pCa}_{50} \) and passive tension across the ventricle. Linear regressions were calculated on a scatter plot, including all individual data in sham (solid line, square; y=0.15+0.005x, r=0.51 p=0.007); MI (dash-dot line, solid circle, y=0.18–0.005x, r=-0.34 p=0.16); and MI-Ex (dashed line, open circle, y=0.15+0.005x, r=0.34  p=0.15). * p<0.05

**Figure 6. Effect of MI and exercise on sarcomeric proteins:** SDS-PAGE for \( \alpha \) and \( \beta \)-MyHC isoforms; \( \beta \)-MyHC content was expressed in % of total MyHC in ENDO and EPI (A). Immunoblots with an anti-cardiac TnI and anti-cTnI phosphorylated by PKA (B), and with an anti-cardiac MLC\(_2\) (C), on ENDO (left) and EPI (right) strips, in relax state (R, open bar) and after stretch (S, gray bar). * adjusted p<0.05.
**Figure 2**

A. Sham, MI, MI-Ex

B. End systolic strain (%)

C. Section motion (cm.s$^{-1}$)

D. $V_{\text{ETI}_{\text{Ao}}}$ (mm)

E. Survival (%)
Figure 3

A

Endocardium

Epicardium

2 s

Sarcomere length (µm)

MI

MI-Ex

Sham

B

SL shortening (%)

* * *

endo EPI

TR50 (ms)

ENDO EPI

-2 -1 0

Contraction velocity (µm/s)

* *

Relaxation velocity (µm/s)

ENDO EPI

-2 -1 0

* *
**Figure 4**

A

![Graph showing F405/F480 ratio from Endocardium and Epicardium under Sham, MI, and MI-Ex conditions.]

B

![Bar graph showing Amplitude of Ca^2+ transient in Endocardium (ENDO) and Epicardium (EPI) for Sham, MI, and MI-Ex.]

C

![Western blots showing SERCA, PLB, and CSQ levels in Endocardium and Epicardium for sham, MI, and MI-Ex. Bar graph showing SERCA/CSQ (A.U) in Endocardium (ENDO) and Epicardium (EPI) with statistical significance indicated.]
Figure 5

A. Endocardium

B. Epicardium

C. 

\[ \Delta \text{pCa}_{50} \]

vs.

Passive tension (mN/mm²)

EPI

ENDO
**Figure 6**

(A) Bar graph showing the percentage of \( \beta \)-Myosin Heavy Chain (MHC) in Endocardium (ENDO) and Epicardium (EPI) for Sham MI and MI-Ex groups. The graph includes error bars.

(B) Western blot analysis of TnI and P-TnI in Endocardium and Epicardium for Sham, MI, and MI-Ex groups. The graphs show the expression levels of P-TnI/TnI in arbitrary units (A.U.).

(C) Western blot analysis of MLC-2 and P-MLC-2 in Endocardium and Epicardium for Sham, MI, and MI-Ex groups. The graphs show the expression levels of P-MLC-2 as a percentage of MLC total.
### Table 1: Cardiac morphological and functional parameters:

<table>
<thead>
<tr>
<th></th>
<th>Sham (n=12)</th>
<th>MI (n=17)</th>
<th>MI-Ex (n=14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>582.9 ± 22.3</td>
<td>605.6 ± 19.2</td>
<td>559.3 ± 16.0</td>
</tr>
<tr>
<td>Heart mass (g)</td>
<td>2.11 ± 0.08</td>
<td>2.56 ± 0.11*</td>
<td>2.73 ± 0.11*</td>
</tr>
<tr>
<td>Heart mass/body weight (mg/g)</td>
<td>3.65 ± 0.12</td>
<td>4.28 ± 0.22*</td>
<td>4.92 ± 0.24*†</td>
</tr>
<tr>
<td>LVEDs (mm)</td>
<td>5.4 ± 0.1</td>
<td>11.6 ± 0.3*</td>
<td>10.6 ± 0.3*†</td>
</tr>
<tr>
<td>LVEDd (mm)</td>
<td>9.5 ± 0.1</td>
<td>13.3 ± 0.3*</td>
<td>12.3 ± 0.2*†</td>
</tr>
<tr>
<td>AWTd (mm)</td>
<td>1.2 ± 0.1</td>
<td>0.4 ± 0.1*</td>
<td>0.4 ± 0.1*</td>
</tr>
<tr>
<td>PWTd (mm)</td>
<td>1.2 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>FS (%)</td>
<td>43.1 ± 0.6</td>
<td>12.2 ± 0.9*</td>
<td>13.8 ± 0.9*</td>
</tr>
<tr>
<td>VTI_Ao (mm)</td>
<td>51 ± 1</td>
<td>32 ± 1*</td>
<td>44 ± 2*†</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>280 ± 6</td>
<td>282 ± 5</td>
<td>283 ± 12</td>
</tr>
</tbody>
</table>

LVEDs, left ventricular end-systolic diameter; LVEDd, left ventricular end-diastolic diameter; AWTd, end-diastolic anterior wall thickness; PWTd, end-diastolic posterior wall thickness; FS, Fractional shortening. VTI_Ao, aortic Velocity Time Integral. HR, heart rate. Values are means ± SEM (n= number of cells).

Sham vs MI. † MI vs MI-Ex; p<0.05.
Table 2: Regional mechanical properties of cardiomyocytes isolated from sham, myocardial infarcted (MI), and MI exercised (MI-Ex) rats:

<table>
<thead>
<tr>
<th></th>
<th>Sarcomere length, 1.9 µm</th>
<th>Sarcomere length, 2.3 µm</th>
<th>( \Delta p_{Ca_{50}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( n )</td>
<td>T(_{pass} )</td>
<td>Tmax</td>
</tr>
<tr>
<td><strong>Endocardium</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>15</td>
<td>0.9±0.2</td>
<td>47±4</td>
</tr>
<tr>
<td>MI</td>
<td>10</td>
<td>0.9±0.3</td>
<td>40±3</td>
</tr>
<tr>
<td>MI-Ex</td>
<td>10</td>
<td>1.0±0.2</td>
<td>45±5</td>
</tr>
<tr>
<td><strong>Epicardium</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>12</td>
<td>0.8±0.1</td>
<td>36±4</td>
</tr>
<tr>
<td>MI</td>
<td>8</td>
<td>0.8±0.3</td>
<td>35±4</td>
</tr>
<tr>
<td>MI-Ex</td>
<td>13</td>
<td>0.9±0.3</td>
<td>38±3</td>
</tr>
</tbody>
</table>

Passive (T\(_{pass}\)) and maximal active (T\(_{max}\)) tensions in mN/mm\(^2\) were measured at \( p_{Ca} 9 \) and 4.5 and at 1.9 and 2.3 mm sarcomere length, respectively, on sub-epicardial (EPI) and sub-endocardial (ENDO) cells isolated from sham, MI, and MI-Ex hearts. \( p_{Ca_{50}} \) (pCa for half-maximal activation) and \( n_H \) (Hill coefficient), were calculated by fitting the force-pCa relation (see materials and methods). Values are means ± SEM (n= number of cells; 5 rats per group). * EPI vs ENDO, † sham vs MI, ‡ MI vs MI-Ex ; p<0.05, two-way ANOVA.
Expanded methods

Animals and exercise training:

Male Wistar rats (5 weeks old) were subjected to coronary artery ligature to produce MI (Myocardial Infarcted) rats as previously described.\textsuperscript{1-3} Thirteen weeks after myocardial infarction, rats were subdivided into two groups: sedentary (MI) and exercised (MI-Ex) animals. The exercise program consisted of 5 weeks exercise on a treadmill (40 min/day, 5 days/week). The speed of running was gradually increased from 10 m/min to 16 m/min. At the end of the training protocol, morphological and functional parameters were determined by echocardiography and compared to sham-operated animals that were subjected to the same surgical operation without tightening the coronary ligature.

Echocardiography:

Doppler-echocardiography was performed in anesthetized animals (50 to 75 mg/kg ketamine and 10 to 15 mg/kg xylazine, IP) with the use of a MyLab 30 (ESAOTE, Italy) equipped with a high frequency transducer (10-12 MHz). Wall thicknesses and left ventricular end dimensions (LVED) were obtained from a short axis view, at the level of the papillary muscles. LV shortening fraction was calculated as \( \frac{(\text{LVEDd} - \text{LVEDs})}{\text{LVEDd}} \times 100 \), where LVEDd and LVEDs are respectively end diastolic and end systolic LVED. Posterior Wall thickening was calculated as \( \frac{\text{PWTs} - \text{PWTd}}{\text{PWTd}} \), where PWTs and PWTd are respectively end systolic and end diastolic Posterior wall thickness. End Systolic strain of the posterior wall was measured as deformation from end-diastole to end systole and was therefore calculated as \( \frac{((\text{PWTs} - \text{PWTd})}{\text{PWTd}} \times 100 \).

Noninvasive hemodynamics

Ascending aortic blood flow was recorded, as previously described\textsuperscript{5} via pulsed-wave Doppler from a suprasternal view permitting measurements of the aortic velocity time integrals (VTI\textsubscript{Ao}, VTI\textsubscript{ao})
was preferentially used in this study as an accurate index of LV stroke volume and therefore of LV global function because this index is less sensitive to error measurement from the aortic annulus than the stroke volume.\(^6\) LV sub-endocardial (ENDO) and sub-epicardial (EPI) posterior wall displacement was measured offline on the short axis view at the level of the papillary muscles. Tissue Doppler imaging (TDI) was performed after each conventional echocardiography as previously described.\(^7\)

Measurement of myocardial velocities resulting from the LV radial contraction was performed on the short axis view at the level of the papillary muscles. TDI analysis was performed offline with the MyLabDesk software (ESAOTE, Italy). The angle of interrogation of the myocardial velocities was carefully aligned to be perpendicular to LV walls. The TDI sample was manually positioned along the posterior wall within the Endo and Epi layers to obtain the ENDO and EPI velocities. Intra- and inter-observer Doppler echocardiography and TDI variabilities are evaluated during a previous study in Wistar rats (n=6) by means of variation coefficients. Coefficients were all equal to or lower than 6.5 % (aortic VTI) with a minimal value for LVEDd (1.5 %).

**Contractile properties in intact cardiomyocytes**

Eighteen weeks post-infarctus, single ventricular myocytes were isolated by enzymatic digestion as previously described (n=5 rats par group).\(^8\) Briefly, hearts were quickly removed and mounted on a Langendorff apparatus. The heart was perfused 10 min with a Ca\(^{2+}\)-free Hanks-HEPES solution followed by perfusion with an enzymatic solution for 10-20 min at 37\(^\circ\)C (see below for solution compositions). Small pieces of ventricular tissues from the inner free wall Endocardium and from the outer free wall Epicardium were dissected and gently dissociated with a pipette in Ca\(^{2+}\) free solution. Calcium concentration was gradually increased to 1 mmol/L Ca\(^{2+}\). To prevent protein degradation after collagenase treatment, all solutions contained protease inhibitors (PMSF: 0.5 mmol/L and E64d [trans-epoxysuccinyl-1-leucine-guanidobutylamide]: 0.01 mmol/L).

Cells were loaded for 30 min at room temperature with Indo-1 AM (10\(\mu\)mol/L Invitrogen inc., France). Experiments were performed in 1.8 mM Ca\(^{2+}\) containing solution. Cells were electrically stimulated at a frequency of 0.5 Hz (20V, 1 ms), and simultaneously illuminated at 305 nm using a xenon arc bulb light. Sarcomere length (SL) and fluorescences emitted at 405 nm and 480 nm were simultaneously recorded using IonOptix acquisition software (IonOptix system, Hilton, USA).
Force measurements in permeabilized cardiomyocytes

Ventricular myocytes were isolated by mechanical dissociation as described previously. The heart was pre-skinned by perfusing relaxing solution (for composition see below) containing 1% Triton X-100 and protease inhibitors (see above) for 10 min. Right ventricle and PMI fibrotic tissue were discarded. Several left sub-epicardial and sub-endocardial skinned strips (8-mm long, 2-mm width, 1-mm thick) were dissected under a microscope following the orientation of the fibers in the skinning solution, and were further skinned 10 min at 4°C in relaxing solution containing 1% Triton X-100. Strips were frozen in liquid nitrogen at slack length or after stretching by ~20-30% of initial slack length to 2.30±0.05 µm SL in relaxing solution for biochemistry or prepared for mechanical experiments as follow. The strips were mechanically disrupted at 11,000 rpm for 2-3 sec (Polytron PT45-80 with a PTA 10TS shaft, KINEMATICA AG, Switzerland) in fresh ice-cold relaxing solution, resulting in a suspension of small clumps of myocyte-sized preparations. The homogenized tissue was filtered and centrifuged at 1,000 rpm for 1 min at 4°C. The pellet containing the myocytes was further skinned in 0.3% Triton X-100 solution for 6 min to remove remaining sarcolemma membranes, and then extensively washed in relaxing solution. To prevent degradation, all solutions contained protease inhibitors (PMSF: 0.5 mmol/L; leupeptin: 0.04 mmol/L and E64: 0.01 mmol/L). Cells were kept on ice and used within the day. Cells were used immediately after cell isolation within one day for mechanical experiments.

Active and passive forces were measured as described previously. Myocytes were attached to a piezoresistive strain gauge (AE801 sensor, Memscap, Crolle, France) and to a stepper motor driven micromanipulator (MP-285, Sutter instrument company, Novato CA, USA) with thin needles and optical glue (NOA 63, Norland products Inc, North Brunswick, NJ) that polymerized by 2 min UV illumination. SL was determined online throughout the experiment at 50 Hz by using a Fast Fourier Transform algorithm on the video images of the cell. Force was normalized by the cross-sectional area measured from the imaged cross-section. After a test-activation at pCa 4.5, the cell was stretched to various SL in relaxing solution using a stepper motor driven micromanipulator at a speed of 0.1 length/sec to evaluate passive tension. Steady state passive tension (after the rapid phase of stress relaxation) was sequentially measured at 1.9, and 2.3 µm SL. Then pCa-force relationships were established at two SL, 1.9 then 2.3 µm at 22°C. The cell was kept five minutes at slack length in relaxing solution between each phase of the protocol for complete refolding of titin. Active tension at
each pCa was the difference between total tension and relaxed tension. Cells that did not maintain 80% of the first maximal tension or a visible striation pattern were discarded. The SL change varied somewhat from cell to cell, and we continued with cells that were well attached with minimal SL changes (< 0.1 µm). When required, cell length was varied during contraction in order to keep SL constant. Active tensions at submaximal activations were normalized to maximal isometric tension (classically obtained at pCa 5) at the same SL. The relation between force and pCa was fitted to the following equation: 

$$force = \frac{[Ca^{2+}]^n}{K + [Ca^{2+}]^n},$$

where \( n_H \) is the Hill coefficient and pCa\(_{50} \), pCa for half-maximal activation equals –(log K)/\( n_H \).

**Experimental solutions**

For isolating intact cells, three different solutions were used. (i) Hanks-HEPES solution contained (mmol/L): NaCl 117, KCl 5.7, NaHCO\(_3\) 4.4, KH\(_2\)PO\(_4\) 1.5, MgCl\(_2\) 1.7, HEPES 21, glucose 11, taurine 20, PMSF 0.5, pH 7.2 adjusted with NaOH. (ii) The enzymatic solution contained in addition 1.25 mg.ml\(^{-1}\) collagenase type IV (Worthington Freehold, NJ, U.S.A). (iii) Washing solutions contained 0.1% bovine serum albumin and 15 mmol/L 2.3-butanedione monoxime (BDM) with increasing Ca\(^{2+}\) concentrations. Cells were kept on a 1 mmol/L Ca\(^{2+}\) containing solution did not contain BDM and BSA.

Ca\(^{2+}\)-activating solutions were prepared daily by mixing relaxing (pCa 9.0) and maximal activating (pCa 4.5) solutions.\(^8\) The relaxing and activating buffers contained (in mmol/L): phosphocreatine 12, imidazole 30, free Mg\(^{2+}\) 1, EGTA 10, Na\(_2\)ATP 3.3, and dithiothreitol 0.3 with pCa 9.0 (relaxing solution) and pCa 4.5 (maximal activating solution), protease inhibitors (PMSF 0.5 mmol/L, leupeptin 0.04 mmol/L, and E64 0.01 mmol/L) pH 7.1 adjusted with acetic acid. Sufficient potassium acetate was added to adjust ionic strength to 180 mmol/L.

**Protein analysis:**

MyHC isoforms were separated on a 6% SDS-PAGE as previously described \(^9\). Intact myocytes were dissolved in an SDS lysis buffer (Tris-HCl 50 mM, 2% (w/v) SDS, urea 8 mol/L, EGTA 1 mmol/L, EDTA 1 mmol/L, DTT 80 mmol/L, 10% (v/v) glycerol, pH 6.8, protease inhibitors) and heated 6 min at 50°C. Gels were silver stained and analyzed with an imaging system (Kodak Image Station 2000R). \( \beta \)-MyHC content was expressed relative to total amount of the MyHC protein.
CVR-2008-687R1

For the SERCA and phospholamban (PLB) intact myocytes were dissolved 15 min at 37°C in a lysis buffer containing in mmol/L : Tris-HCl 50, NaCl 200, β-glycerophosphate 20; NaF 50, EDTA 2, benzamidin 10, Na₃VO₄ 0.2, DTT 5, Triton 1% and proteases inhibitors. Proteins were separated on a 2-20% SDS-PAGE and blotted onto nitrocellulose membrane (Protran, Schleichen and Schuele, Dassel, Germany). Membranes were blocked 30 min in 3% BSA TBS-Tween. SERCA was specifically detected with a SERCA2a antibody (A010-20, Badrilla, UK) and PLB with was detected with a PLB antibody (A010-14, Badrilla, UK). Both SERCA and PLB were expressed relative to Calsequestrin content (Cat # PA1-913, ABR, USA) on the same membrane.

The phosphorylation levels of troponin I (TnI) and myosin light chain 2 (MLC-2) were performed as previously described on skinned muscle strips dissected from the ENDO and EPI layers (see above). Samples were treated with ice cold trichloroacetate (TCA 10%) to maintain contractile protein phosphorylation status, washed three times in acetone/10mmol/L DTT, and then pellets were dried and mixed 1 hour at room temperature with urea buffer (containing in mol/L : urea 8, Tris 0.2, glycine 0.25, dithiothreitol (DTT) 0.5, sucrose saturated, ethylenediaminetetra acetic acid (EDTA) 0.4, proteases inhibitors). Protein concentration was determined with RCDC kit (Bio-Rad). Proteins (20 µg) were separated either on 15% SDS-PAGE (for TnI) or 10% urea gel (for MLC-2). For both studies, all samples from one animal in the various conditions (ENDO/EPI, relax/stretch) were always loaded on the same gel. MLC-2 phosphorylated and non-phosphorylated forms were separated by a 10% urea gel and were specifically detected with a cardiac MLC-2 antibody (Coger SA, Paris, France). Total TnI content was determined on the first wells of the membrane with a total cardiac TnI antibody (Cat#4T21, Hytest, Turku, Finland) and the protein kinase A phosphorylated form of cardiac TnI antibody on the other wells (Cat#4T45, Hytest). Immunodetection was revealed with ECL Plus system (Amersham Pharmacia, Little Chalfont Buckinghamshire, England).

Statistical analysis
One-way or 2-way ANOVA was applied for comparison between groups. When significant interactions were found, a Bonferroni t-test was applied with P < 0.05 (Sigmastat 3.5). Data are presented as mean±SEM.
References:


