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Contents

Abbreviations

ANS	autonomic nervous system
AW/BW	ratio of left atrial + right atrial weight to body weight
BNP	brain natriuretic peptide
BW	body weight
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase type II
CPX	cardiopulmonary exercise testing
CR	cardiac rehabilitation
cDNA	complementary neoxyribonucleic acid
DNA	neoxyribonucleic acid
ECG	electrocardiography
FS	fractional shortening
HF	high frequency power
HRV	heart rate variability
HW/BW	ratio of heart weight to body weight
IgG	immunoglobulin G
LAD	left anterior descending artery
LF	low frequency power

LF/HF	ratio of low frequency power to high frequency power
LV	left ventricular
LVDd	left ventricular end-diastolic diameter
LVDs	left ventricular end-systolic diameter
LVEF	left ventricular ejection fraction
LVW/BW	ratio of left ventricle weight to body weight
LW/BW	ratio of lung weight to body weight
MI	myocardial infarction
miRNA	microRNA
mRNA	messenger ribonucleic acid
MI-Ex	myocardial infarction + exercise training
MI-Sed	myocardial infarction with no intervention
MI-βb	myocardial infarction + β-blocker treatment
Nppb	natriuretic peptide precursor B
PCR	polymerase chain reaction
Peak VO_2	oxygen consumption at peak exercise
PLN	phospholamban
PKA	protein kinase A
PP1	protein phosphatase 1
PP2A	protein phosphatase 2A

PVC	premature ventricular contraction
RNA	ribonucleic acid
RVW/BW	ratio of right ventricle weight to body weight
RyR2	ryanodine receptor type 2
SDNN	standard deviation of R-R intervals
SEM	standard error of the mean
SERCA2a	sarcoplasmic reticulum calcium ATPase
SMW/BW	ratio of the sartorius muscle weight to body weight
SR	sarcoplasmic reticulum
VT	ventricular tachycardia

Chapter 1 Introduction

1.1 Background

Myocardial infarction (MI) is caused by the occlusion of the coronary artery mainly due to atherosclerotic plaque rupture. Despite important advances in drug and device therapy, MI remains a leading cause of death and morbidity worldwide, especially because of heart failure and ventricular arrhythmia (Savard et al. 1997; Bahit et al. 2018). Acute ischemic injury leads to the activation of neurohormones and cytokines, subsequent myocardial remodeling, a further decline in cardiac function, and finally overt heart failure (Schober and Knollmann 2007; Zhang et al. 2010). During this pathophysiological progress, malignant arrhythmias such as ventricular tachyarrhythmias, associated with imbalance of the autonomic nervous system (ANS), are a major cause of death (Kalla et al. 2016; Shen et al. 2014).

β-adrenergic receptor blocker (β-blocker) has been established as a first-line medication for heart failure patients to reduce mortality. Long-term treatment with β-blockers can lessen the symptoms of heart failure and improve the patient's clinical status (Yancy et al. 2013).

There are increasing evidences that exercise training is an effective non-pharmacological treatment for cardiovascular diseases with a beneficial effect on disease progression and survival (Anderson et al. 2016; La Rovere et al. 2002). The American Heart Association guidelines for

heart failure published in 2005 recommend exercise training for stable outpatients with heart failure and a reduced left ventricular ejection fraction (LVEF) (Hunt et al. 2005). In post-MI patients, multiple previous studies reported that exercise could beneficially reduce post-MI remodeling and improve cardiac function (French et al. 2008; Guizoni et al. 2016; Kemi et al. 2007; Lu et al. 2002). Exercise training also causes a reverse remodeling of cardiac autonomic regulation such that parasympathetic activity is enhanced while sympathetic activity is reduced, resulting to improvement of heart rate variability (HRV) (Malfatto et al. 1996) and suppression of ventricular tachyarrhythmia (Billman 2009; Bonilla et al. 2012; Kukielka et al. 2011). Recently, intensive exercise training has been reported to have greater improvement than low intensive or moderate intensive training (Howden et al. 2018; Jaureguizar et al. 2016).

Cardiac rehabilitation (CR) programs are recognized as integral to comprehensive care of cardiac patients, with exercise therapy consistently identified as a central element, and have been given a Class I recommendation from the American Heart Association, the American College of Cardiology, and the European Society of Cardiology (Anderson et al. 2016). For acute MI patients, CR is known not only to improve cardiopulmonary function and the quality of life, but also allows for the more efficient management of risk factors (Zhang et al. 2018; Kim et al. 2012). During the process of CR, cardiopulmonary exercise testing (CPX) is the most useful clinical tool for evaluating the severity of disease and the limitations of physical activities in cardiac patients, which becomes the standard in making exercise prescription (Figure 1). Oxygen consumption at peak exercise (peak VO_2) reflects maximal cardiac output during exercise and is accordingly considered the standard for evaluating exercise

tolerance and cardiopulmonary function (Balady et al. 2010). Furthermore, it has also be accepted as the main indicator of predicting prognosis in cardiac patients and a gold standard in selecting patients for cardiac transplantation (Balady et al. 2010). Under the guidance of CPX, cardiac rehabilitation not only reduces the mortality rate of MI, but also lowers the recurrence rate of acute MI.

Although many cardiac disease patients may have benefited from the above-mentioned effects through cardiac rehabilitation, the actual cases of request for cardiac rehabilitation are not very common (Kim et al. 2012). At present, cardiac rehabilitation is still the short board in the overall treatment of coronary heart disease. The development of community-based cardiac rehabilitation in acute MI patients is still unsatisfactory, <25% of outpatients have been reported to enroll in CR, with <10% in elderly patients, within this small number of patients participating in CR, 30% to 40% of patients discontinued CR after 6 months, with up to 50% dropping out after 1 year (Wolkanin-Bartnik et al. 2011). While the need and the effects of cardiac rehabilitation are so well known as not to produce any disagreement, it is true that not only the patients who request cardiac rehabilitation but also the medical team who are involved in the cardiac rehabilitation have concerns regarding cardiovascular-related complications that may occur during monitoring exercises (Kim et al. 2012). Patients with heart failure were advised to avoid physical exertion at last century. There are controversial results about the effects of exercise, especially the high intensive exercise. Both experimental and clinical athlete studies demonstrated that intensive exercise was associated with enhanced myocardium fibrosis and dyssynchrony leading to electrical instability and arrhythmia (Guasch et al. 2013; Karjalainen et al. 1998). The risk of AMI

is also increased with approximately 5–10% of all infarcts being associated with intense physical activity (Thompson et al. 2003).

On the other hand, it remains to be fully elucidated what are the underlying mechanisms. Various researches attributed to different opinions (La Rovere et al. 2002; Kemi et al. 2007; Guizoni et al. 2016). Furthermore, it keeps unclear how does exercise training alter the cellular/molecular abnormalities to protect against malignant arrhythmias after MI and what are the underlying mechanisms.

Figure 1. Cardiac rehabilitation (CR) and cardiopulmonary exercise testing (CPX)

Cardiac rehabilitation (CR) programs are recognized as integral to comprehensive care of cardiac patients and have been given a Class I recommendation, with exercise therapy consistently identified as a central element. During the process of CR, cardiopulmonary exercise testing (CPX) is the most useful clinical tool for evaluating the severity of disease and the limitations of physical activities in cardiac patients, which becomes the standard in making exercise prescription.

1.2 Objective

In this study, we aim to explore the effects and mechanisms by intensive exercise training in post-MI mice, especially anti-arrhythmic potential, via comparing the effects of exercise versus an established first-line medicine β-blocker treatment.

Chapter 2 Methods

2.1 Ethics statement

All animal experimental procedures in this study were approved by the Institutional Animal Experiment Committee of the University of Tsukuba. Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and the Fundamental Guideline for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

2.2 Mouse model and study protocol

Total 96 wild-type male mice (C57Bl/6J) purchased from CLEA Japan, Inc. (Tokyo, Japan) were used in this study. After adapting the environment for 2-3 weeks and reaching 11-12 weeks old, MI operation was performed in 75 mice as described previously (Stujanna et al. 2017). Mice were anesthetized with ketamine and xylazine, followed by tracheal intubation and artificial ventilation (MiniVent 845; Harvard Apparatus, Holliston, MA, USA). Next, a thoracotomy was performed to expose the heart, and MI was induced by the permanent ligation of the left anterior descending coronary artery (LAD) with 7-0 polypropylene suture passed about 2 mm

from the inferior margin of the left atrial auricle. MI was confirmed by visual observation of myocardial color change and the following echocardiographic assessment at the 7th day after MI operation. 21 mice were subjected to a similar procedure without ligation of the LAD as the sham group. After closure of the thorax and recovery of spontaneous breathing, the mice were extubated and placed in a new box on the warm pad for recovery. In the first week after MI operation, 20 mice died (probably due to heart failure and/or arrhythmia). At the 7th day after MI/Sham operation 76 mice were performed echocardiographic assessment to confirm MI area and obtain initial parameters. Then, 55 MI mice were randomly divided into 3 groups: sedentary group (20 mice; MI-Sed), exercise training group (17 mice; MI-Ex) and β-blocker treatment group (18 mice; MI-βb). The study protocol is shown in Figure 2.

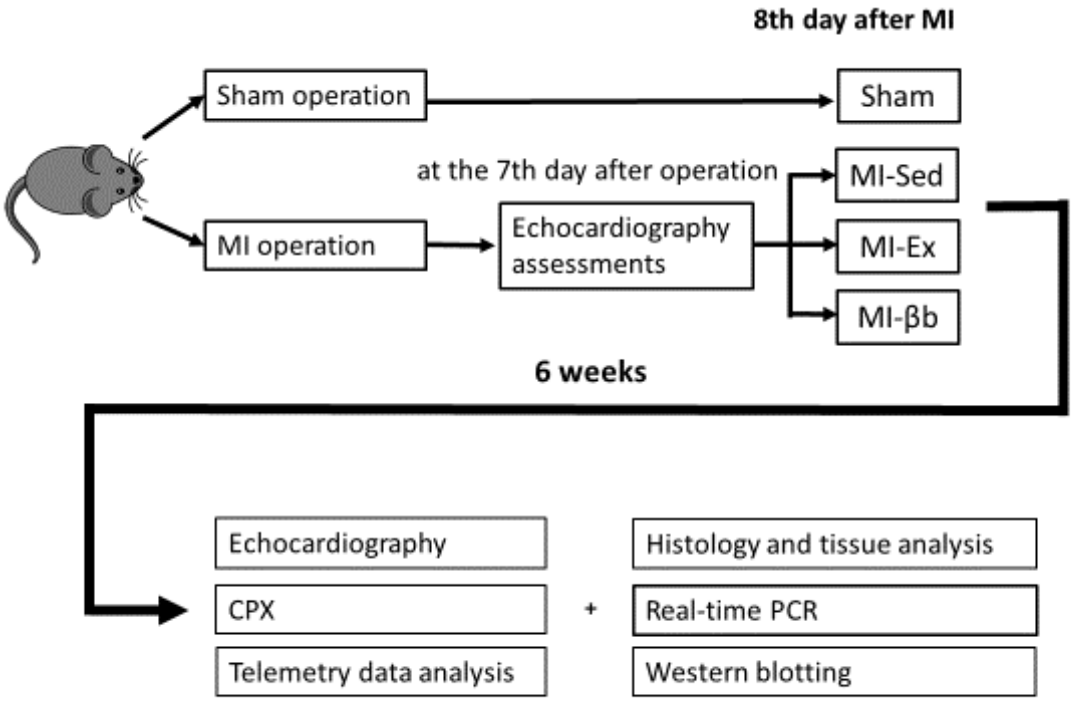

Figure 2. Study protocol

Wild-type male mice (C57Bl/6J) underwent MI/sham operation at the age of 11-12 weeks old. At the 7th day after operation mice were performed echocardiographic assessment. Then, mice were randomly divided into 4 groups: sham group (Sham), sedentary group of MI mice (MI-Sed), exercise training group (MI-Ex) and β-blocker treatment group (MI-βb). There was neither treatment nor exercise training in Sham and MI-Sed, while MI-Ex/MI-βb started treadmill training/Bisoprolol treatment from the 8th day after MI operation. After 6 weeks, mice were received assessments of echocardiography, exercise capacity and cardiopulmonary function. Telemetry devices were implanted into the body of mice to abtain data of electrocardiography (ECG) and heart rate variability (HRV) analysis. Then, mice were sacraficed and left ventricular (LV) tissues were excised for laboratory assessments.

2.3 Exercise training and β-blocker treatment protocol

Mice in MI-Ex started treadmill training from the 8th day after MI operation for 6 weeks. Training was performed with a ten-lane treadmill at 0% slope (MK-680, Muromachi Kikai Co., Ltd., Tokyo, Japan). The training intensity was determined as the maximum speed and endurance time when the animals were not able to run further. In the first week the training intensity started at 6 meters per minute (m/min) for 30 minutes and increased progressively in the following days, until 18 m/min for 45minutes/day when mice showed exhaustion. Then the maximal intensity (18m/min × 45minutes) was maintained for 5 weeks and consequent 5 days every week.

MI-βb mice were treated with Bisoprolol Hemifumarate (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) via water solution from the 8th day after MI operation for 6 weeks. The dosage was 1 mg/kg/day based on the previous report (Yamada et al. 2014). Firstly, we evaluated water intake per day of each mouse after operation (approximately 4 ml/day) and then the concentration was determined (0.0075 mg/mL).

2.4 Echocardiography

We performed echocardiographic assessments 3 times during the experiment (on 7th day after MI/sham operation, after 3 weeks of intervention and after 6 weeks of intervention). In brief, mice were anesthetized by isoflurane and echocardiographic images were obtained at

the papillary muscle level using a 40 MHz transducer connected to Doppler echocardiographic system (Vevo 2100; Visual Sonics, Toronto, Canada). Both the parasternal long-axis view and short-axis view were acquired. Left ventricular end-diastolic diameter (LVDd), LV end-systolic diameter (LVDs), LV ejection fraction (LVEF), and fractional shortening (FS) were measured or calculated in short-axis two-dimensional M-mode images (Tan et al. 2003). LVEF was calculated by the Teichholz method. FS was calculated as [(LVDd–LVDs)/LVDd] × 100%.

2.5 Assessment of cardiopulmonary function and exercise capacity

A metabolic running wheel chamber connected with a Mass Spectrometer for Respiratory Analysis (ARCO 2000; ARCO system Inc., Chiba, Japan) was used to assess exercise capacity and cardiopulmonary function. After 6 weeks of different interventions, 5-6 animals in each group were randomly chosen and weighed before the examination. Single mouse was put inside the sealed running wheel, forced to run at gradually increased speed, and maintained in the last velocity until exhaustion. Oxygen consumption at peak exercise (peak VO_2, Nml/min/kg) was determined and normalized by body weight in kilograms. Exercise capacity and cardiopulmonary function was accepted as peak VO_2 (Nml/min/kg) (Iwase et al. 2004) (Figure 3).

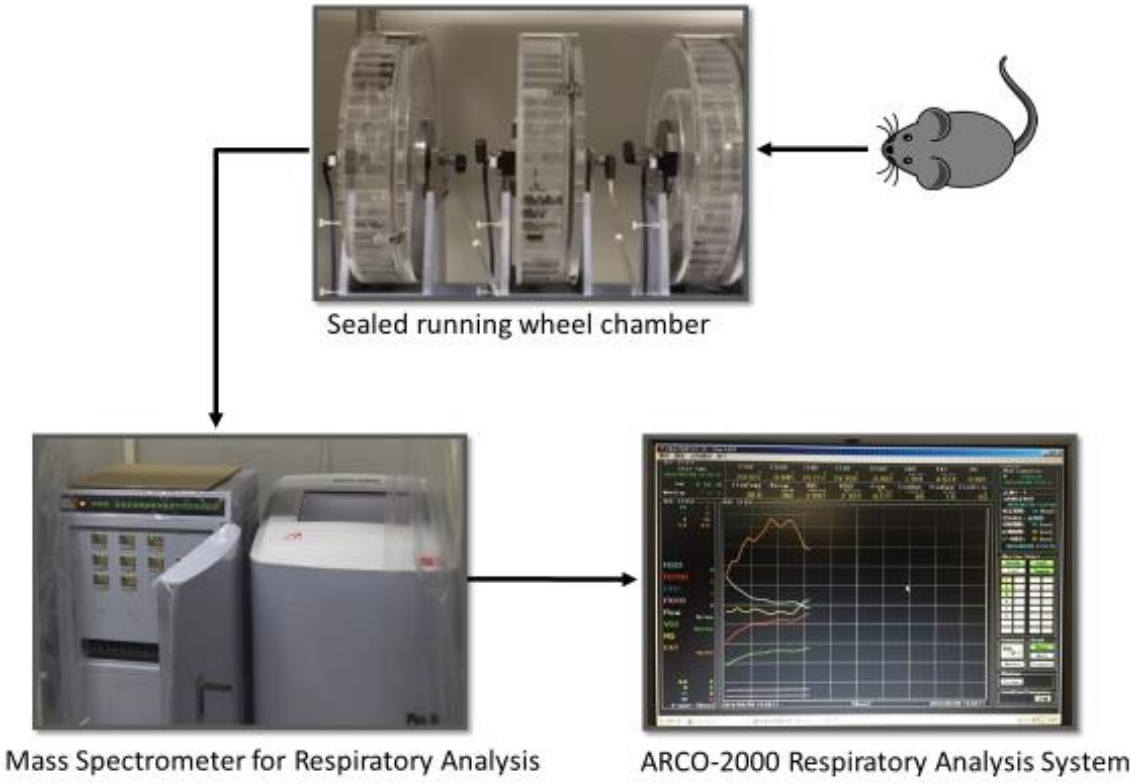

Figure 3. Assessment of cardiopulmonary function and exercise capacity

Single mouse was weighted and put inside the sealed running wheel chamber, forced to run at gradually increased speed, and maintained in the maximal velocity to keep running. The respiratory analysis was performed through the Mass Spectrometer connected with the running wheel chambers. Peak VO_2 (Nml/min/kg) was determined by the ARCO system software.

2.6 Electrocardiography telemetry recording and heart rate variability (HRV) analysis

Continuous 24 hours electrocardiography (ECG) recordings were obtained from implanted telemetry devices (Figure 4). After 6 weeks of intervention, randomly-selected 5-6 animals in each group underwent telemetry implantation. Mice were anesthetized with a mixture of ketamine and xylazine, and the tele-transmitters (ETA-F20, Data Sciences International DSI, USA) were subcutaneously implanted in the midline of their back (less invasive than intraperitoneal implantation, thus ensuring a higher survival rate in post-MI mice). After implantation, each mouse was housed in a single cage and given enough recovery time of several days before starting the measurement. An analog signal from the telemetric receiver was applied to ECG and ANS modulations. Data were collected and analyzed off-line by a commercially-available software (Dataquest A.R.T. Data Sciences International DSI). Premature ventricular contractions (PVC) and ventricular tachycardias (VT: more than 3 PVC beats in succession) were pick out and counted from ECG recordings. Heart rate variability (HRV) analysis is generally used to index ANS function. Mean R–R intervals (in ms) and standard deviation of R-R intervals (SDNN) in time-domain analysis are considered to reflect total autonomic variability (Shaffer and Ginsberg 2017). In frequency-domain analysis, the high frequency power (HF) band reflects parasympathetic activity, while the low frequency power (LF) band reflects the combined function of both sympathetic and parasympathetic activity. A high LF/HF (the ratio of low

frequency power to high frequency power) indicates sympathetic dominance (Thireau et al. 2008).

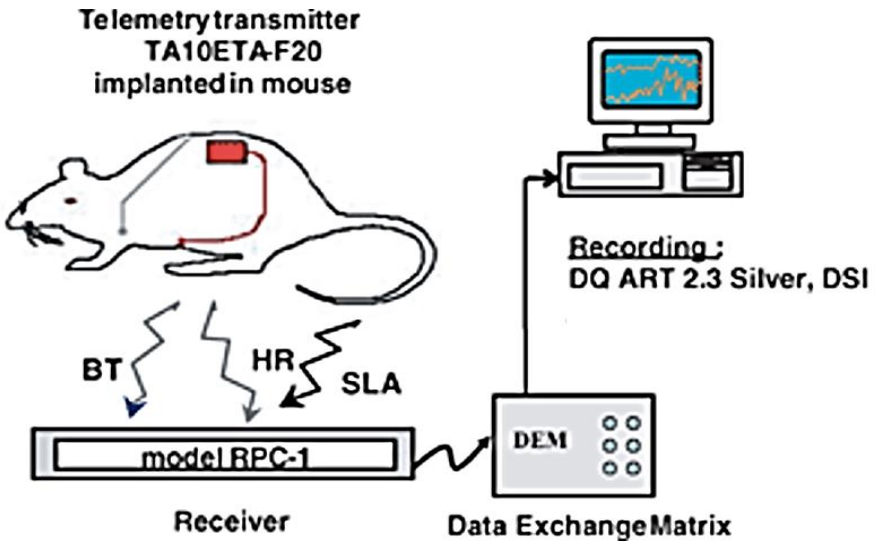

Figure 4. Telemetry implantation and electrocardiography recording

A tele-transmitter (ETA-F20, Data Sciences International DSI, USA) was implanted subcutaneously in the mouse's back, which sent radiofrequency signals to a receiver located below the mouse cage. A data exchange matrix converts the analog signal into a digital signal before storage in the microcomputer. Data were collected and analyzed off-line by Dataquest A.R.T. software.

2.7 Autonomic tone assessment

Autonomic tone components (reflecting parasympathetic and sympathetic tone) were assessed by mean of heart rate changes to sequential pharmacological blockade as described previously (Guasch et al. 2013). Propranolol is widely used to evaluate the inhibition of the sympathetic system, while atropine administration is used to evaluate the inhibition of parasympathetic nervous system (Garabedian et al. 2017). To estimate parasympathetic tone, propranolol (2 mg/kg) was administrated intraperitoneally; 15 to 20 minutes later, parasympathetic effects were blocked with atropine (1 mg/kg). The parasympathetic tone index was defined according to the difference between HR after propranolol alone versus propranolol + atropine. Sympathetic tone index was the heart rate difference between atropine alone and atropine + propranolol.

2.8 Histology and tissue analysis

After the 6 week-intervention, the hearts, lungs, and sartorius muscles were excised and weighed at postmortem. The LVs at the level of the papillary muscle were fixed with 4% paraformaldehyde, embedded in paraffin, sectioned into 4-μm thick slices, and stained with Masson's trichrome. Cross-section images of the LV were obtained by a digital microscope (Biozero BZ-X700; Keyence, Osaka, Japan), and MI area was calculated by dividing the collagen depositing area to the total area. In non-infarcted area, the fibrosis area was calculated by image analyzing software

(ImageJ analysis software ver.1.45; NIH; Bethesda, MD, USA). The anatomical ratios of tissue weights to body weight (BW) were calculated, including the ratios of heart weight to BW, LV weight to BW, right ventricular weight to BW, lung weight to BW, left atrial + right atrial weight to BW, and the sartorius muscle (on the femur) weight to BW.

2.9 Real-time PCR

We performed real-time PCR to assess gene expression levels in the LVs, as described previously (Stujanna et al. 2017; Xu et al. 2012). In brief, total RNA was extracted from LV tissues using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany). A 1 μg of RNA was then reverse transcribed to cDNA with a High-Capacity cDNA RT Kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The mRNA expression levels of the target genes were analyzed by an ABI Prism 7500 sequence detection system (Thermo Fisher Scientific) with gene-specific primers and probe sets (Integrated DNA Technologies, Coralville, IA, USA). The PCR mixture (10 μl total volume) consisted of the template, primer, and probe for each gene (250 nM), and the PrimeTime Gene Expression Master Mix (Integrated DNA Technologies, Skokie, IL, USA). PCR amplification was performed as follows: 1 cycle at 95°C for 10 min, and 40 cycles at 94°C for 15 s and 60°C for 1 min. The primers and probes used in the study were as follows: MHC-α(M00440359-a1), MHC-β (M01319005-g1) (Thermo Fisher Scientific); Nppb (BNP: brain natriuretic peptide) (Mm.PT.58.8584045.g), β1-AR (β1 adrenergic receptor; Mm.PT.58.41132658.g), Grk5 (Mm.PT. 58.11422186), β2-AR (β2

adrenergic receptor; Mm.PT.58.29310038.g), Chrm2 (Mm.PT.58.42964182.g), Col1a1 (Mm.PT.58.756.2513), Col3a1 (Mm.PT.58.13848686), TGF-β1 (Rn01475964), Atp2a2 (SERCA2a: sarcoplasmic reticulum calcium ATPase); Mm.PT.58.5303089), PLN (phospholamban; Mm.PT.58.43778023), and RyR2 (ryanodine receptor type 2; Mm.PT.58.45974879) (Integrated DNA Technologies). For analyzing microRNAs (miRNA), 500 μg of RNA was reverse transcribed to cDNA with a miRNA primer using the miRNeasy Mini Kit (Qiagen). The miRNA primer assays used were miR-1 (RT: 002064, PN4427975) and miR-133a (TM: 000458, PN4427975). The PCR mixture was made and amplified as described above. The quantitative values of target mRNA and miRNA were normalized to expression of 18S rRNA (4319413E; Thermo Fisher Scientific). Data were obtained from 3 independent measurements (n=8/group) performed in duplicate.

2.10 Western blotting analysis

We evaluated protein expression in the LVs by Western blotting, as described previously (Stujanna et al. 2017; Xu et al. 2012). In brief, isolated LVs were homogenized in PRO-PREP protein extraction solution (iNtRON Biotechnology, Inc., Kyungki-Do, Korea), and the supernatants were collected. A 10 μg of proteins were transferred by semidry electroblotting from gels (Bio-Rad Laboratory, Hercules, CA, USA) to polyvinylidene difluoride membranes. The blots were then blocked with the primary antibodies as follows: phospho-RyR2 (Ser 2814) (A010-31; Badrilla, Leeds, UK), phospho-RyR2 (Ser 2808) (A010-30, Badrilla),

RyR2 (PA5-36121; Thermo Fisher Scientific), SERCA2a (2A7-A1; Thermo Fisher Scientific), phospho-Phospholamban (p-PLN) (S16+T17; ab62170; Abcam, Cambridge, UK), total Phospholamban (PLN) (ab2865; Abcam), phospho-PKA (T197; ab75991; Abcam), PKA (ab187515; Abcam), phospho-CaMKII (T286) (ab32678; Abcam), Oxidized-CaMKII (Met281/282) (EMD Millipore, Darmstadt, Germany), total CaMKII (ab103840; Abcam), protein phosphatase 2A (PP2A)-B56-α (F-10; sc-271151; Santa Cruz, Dallas, TX, USA), phospho-Troponin-I (p-TNI) (Ser23/24) (#4004; Cell Signaling Technology, Danvers, MA, USA), total Troponin-I (TNI) (#4002; Cell Signaling Technology), PP1 (E-9; sc-7482; Santa Cruz), and β-actin (4967s; Cell Signaling Technology). The blots were incubated with an appropriate horseradish peroxidase-conjugated goat anti-rabbit IgG (ab6721; Abcam) or horseradish peroxidase-conjugated rabbit anti-mouse IgG (ab97046; Abcam) secondary antibody. Immunoreactions were visualized with an enhanced chemiluminescence method (ECL Prime Western Blotting Detection; GE Healthcare, Southeast, UK). Densitometric analysis was performed on scanned immunoblot images (ImageJ analysis software ver.1.45; NIH; Bethesda, MD, USA). Data were obtained from 3 independent measurements (n=6/group).

2.11 Statistical Analysis

All data are expressed as mean ± standard error of the mean (SEM). To compare the values between groups, continuous values were analyzed by one-way ANOVA followed by post hoc-testing with Bonferroni's test.

Significance was accepted when $P<0.05$. Statistical analysis was performed using IBM SPSS version 21.0 software (IBM Co. Ltd., Armonk, NY, USA).

Chapter 3 Results

3.1 Exercise training does not affect survival rate, MI size, and fibrosis in non-infarcted area, but enhances exercise capacity and cardiopulmonary function.

During the 6 weeks of training or treatment, 3 mice in MI-Sed (3/20: 15.0%) and 1 mouse in MI-Ex (1/17: 6.8%) died, while no mice died in Sham (0/21: 0.0%) and MI-βb (0/18: 0.0%) groups. There was no significant difference in the survival rate between the MI groups. As ventricular rupture was not observed in the 4 dead mice, we assumed death from cardiac arrhythmia or heart failure.

Masson's trichrome (MT) staining images of the LVs showed no differences in the infarct area between the three MI groups (Fig. 5A, B). We assessed the exercise capacity and cardiopulmonary function of mice via a metabolic running wheel chamber. Peak VO_2 was decreased in MI-Sed and MI-βb compared with Sham and was restored in MI-Ex (Fig. 5C).

In the non-infarcted area, real-time PCR for the fibrosis markers showed no significant differences in the gene expression levels of Col1a1, Col3a1 and TGF-β1 (Fig. 6A, B and C). MT staining images of the LVs showed there was no significant difference in the fibrosis area (Fig. 6D, E).

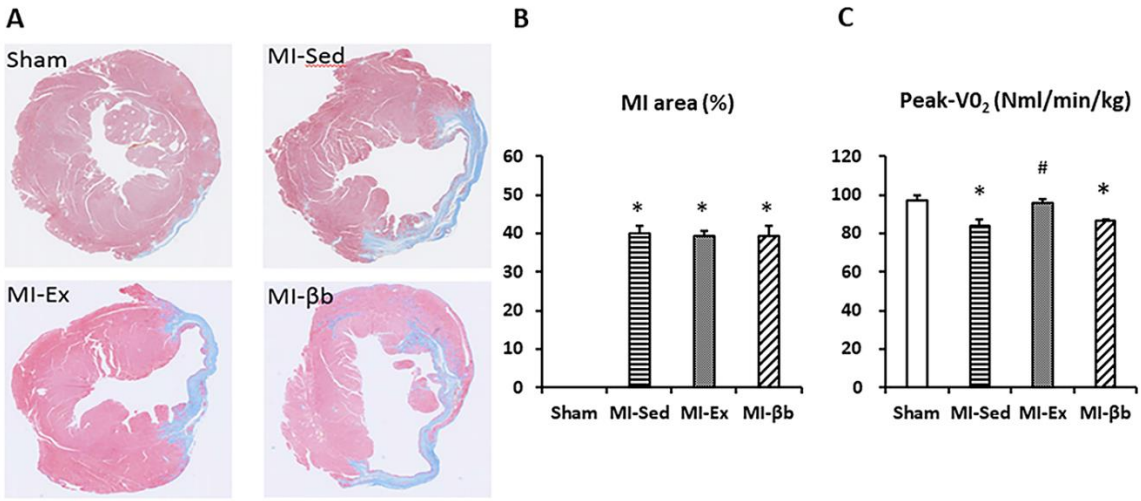

Figure 5. Cross-sectional findings of the left ventricles at the papillary muscle level and cardiopulmonary function.

A: In Masson's trichrome staining images of the left ventricle (LV), the blue thinning region indicates the infarct area. B: There was no difference in the infarct area between the 3 MI groups (MI-Sed group: 40.1%±1.8% vs. MI-Ex group: 39.4%±1.4% vs. MI-βb: 39.3%±2.7 %; n=8/group). *P<0.05, vs. Sham. C: Oxygen consumption at peak exercise (Peak-VO_2) was decreased in MI-Sed and MI-βb groups, and was restored in MI-Ex, compared with Sham (Sham, 97.2 ± 2.3; MI-Sed, 83.8 ± 3.7; MI-Ex, 95.7 ± 2.4; MI-βb, 86.3 ± 1.0, Nml/min/kg) (n=5/group in Sham, MI-Sed, and MI-βb; n=6 in MI-Ex). *P<0.05, vs. Sham; #P<0.05, vs MI-Sed.

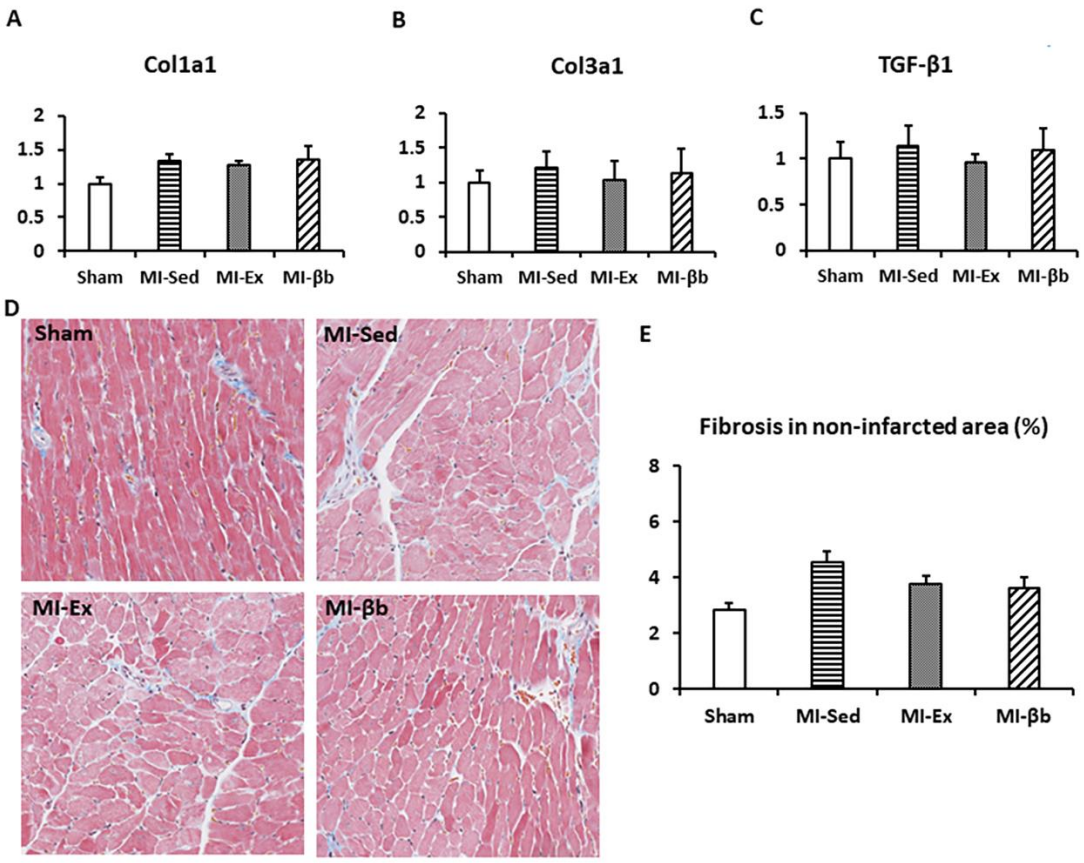

Figure 6. The fibrosis in non-infarcted area of the left ventricles.

A, B and C: There were no significant differences in the gene expression levels of Col1a1, Col3a1, and TGF-β1 between all groups. D: In Masson's trichrome staining images of non-infarcted area (at 20 × magnification), the regions stained blue indicate collagen deposition. E: There was no obvious difference in the collagen deposition area between groups in non-infarcted area (Sham, 2.86 ± 0.21 %; MI-Sed, 4.53 ± 0.40 %; MI-Ex, 3.75 ± 0.67 %; MI-βb, 3.62± 0.40 %; n=6/group).

3.2 Exercise training has no apparent effect on cardiac contractility or hypertrophy

3.2.1 Echocardiographic analysis

Before intervention, echocardiography at the 7th day after MI/sham operation demonstrated that LV was enlarged, and the LV systolic function was reduced in all MI groups compared with Sham, but there was no obvious difference between 3 MI groups (Figure 7). After 3-week intervention, LVDd and LVDs in all MI mice were progressively increased; however, there were no significant differences between MI groups. LVEF (%) and FS (%) were also not significantly different between MI groups. After 6-week intervention, the results showed the similar tendency as 3 weeks after MI. LVEF (%) was slightly improved by exercise and β-blocker treatment, while there were no significant differences in MI groups (Table 1).

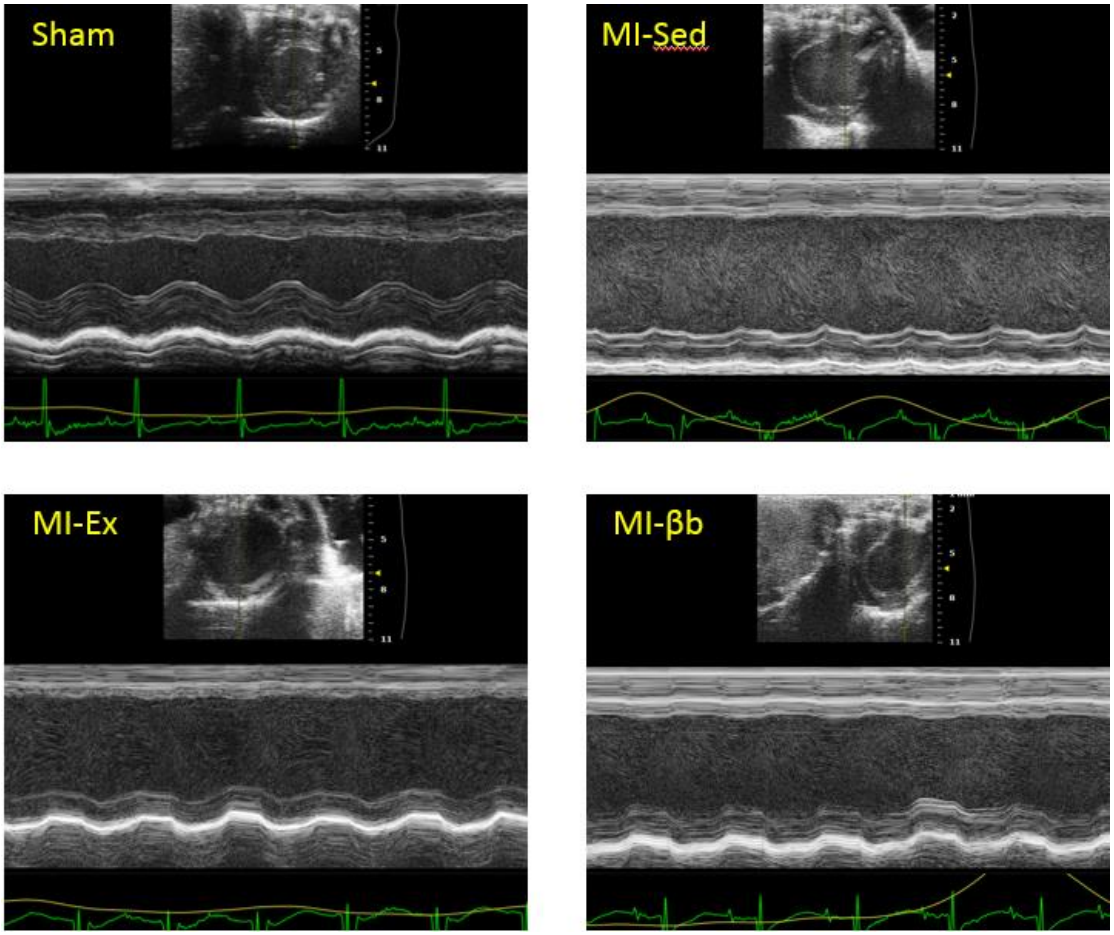

Figure 7. Representative images of echocardiographic analysis

The representative two-dimensional M-mode images of the left ventricular short-axis view show thinning of the anteroseptal wall, enlargement of the left ventricle, and reduced left ventricular systolic function in all MI groups compared with Sham.

Table 1. Echocardiographic analysis.

Variable		Sham	MI-Sed	MI-Ex	MI-βb
LVDd (mm)	**pre**	3.36±0.13	4.54±0.26*	4.62±0.16*	4.81±0.12*
	mid		4.83±0.18	4.77±0.16	4.94±0.20
	post	3.49±0.08	5.01±0.10*	4.82±0.17*	5.00±0.13*
LVD s(mm)	**pre**	2.41±0.16	3.95±0.29*	4.02±0.16*	4.17±0.14*
	mid		4.16±0.20	4.14±0.17	4.27±0.18
	post	2.45±0.15	4.18±0.17*	4.13±0.18*	4.25±0.12*
LVEF (%)	**pre**	56.12±3.25	29.23±2.91*	28.06±1.32*	28.62±1.85*
	mid		29.78±2.40	28.45±2.16	29.20±1.68
	post	57.62±3.10	31.03±2.85*	30.91±2.09*	31.49±0.99*
FS (%)	**pre**	28.67±2.06	13.67±1.42*	13.05±0.64*	13.41±1.07*
	mid		14.03±1.21	13.33±1.11	13.73±0.85
	post	28.65±1.64	14.79±1.40*	14.61±1.08*	15.16±0.57*

Data are presented as mean ± standard error of the mean (SEM).

pre, at the 7th day after MI/sham operation;

mid, after 3 weeks of intervention;

post, after 6 weeks of intervention.

*P<0.05, vs. Sham.

3.2.2 Anatomical data

Anatomical data indicated that the ratios of LV weight to BW, right ventricular weight to BW, and lung weight to BW were significantly increased in all MI groups compared with Sham (Table 2). These values also showed a trend towards a slight increase after 6 weeks. However, only the ratio of the sartorius muscle weight to BW was significantly increased in MI-Ex compared with the others. These data suggest that exercise training strengthened the skeletal muscles but had no apparent effect on cardiac hypertrophy.

Table 2. Anatomical data

Variable	Sham	MI-Sed	MI-Ex	MI-βb
BW (g)	27.99±0.54	29.36±0.32	28.14±0.46	29.18±0.44
HW/BW (%)	4.01±0.06	4.78±0.12*	5.21±0.15*	4.95±0.13*
LVW/BW (%)	2.98±0.05	3.62±0.08*	3.85±0.11*	3.71±0.10*
RVW/BW (%)	0.78±0.03	0.86±0.03	1.01±0.05	0.94±0.03
LW/BW (%)	4.58±0.09	4.63±0.09	5.01±0.11	4.64±0.06
AW/BW (%)	0.24±0.01	0.28±0.01	0.34±0.02	0.31±0.02
SMW/BW (%)	8.16±0.19	7.30±0.26	9.10±0.28#	7.74±0.32

Data are presented as mean ± SEM.

BW, body weight; HW/BW (%), the ratio of whole heart weight to BW; LVW/BW (%), the ratio of left ventricular weight to BW; RVW/BW (%), the ratio of right ventricular weight to BW; LW/BW (%), the ratio of lung weight to BW; AW/BW (%), the ratio of left atrial + right atrial weight to BW; SMW/BW (%), the ratio of sartorius muscle (on the femur) weight to BW.

*P<0.05, vs. Sham; #P<0.05, vs. MI-Sed (n=9/group).

3.3 Exercise regulates the autonomic imbalance and reduces the occurrence of arrhythmias

3.3.1 The heart rate variability (HRV) analysis

HRV analysis in the time and frequency domains was used to evaluate cardiac autonomic function in conscious mice (Thireau et al. 2008). Mean heart rate (HR) was significantly lower in the MI-βb group compared with the other groups (Sham, 550 ± 17 beats per min (bpm); MI-Sed, 563 ± 51 bpm; MI-Ex, 554 ± 29 bpm; MI-βb, 476 ± 35 bpm) (Fig. 8A). In time-domain parameters, MI-βb mice demonstrated prolonged mean R–R intervals and standard deviation of R–R intervals (SDNN) (Fig. 8B, C). Frequency domain analysis indicated that the high frequency power (HF) and the low frequency power (LF) showed no significant differences. While LF/HF, an index of sympathetic tone activity, was decreased in MI-Ex and MI-βb compared with MI-Sed (Fig. 8D, E and F).

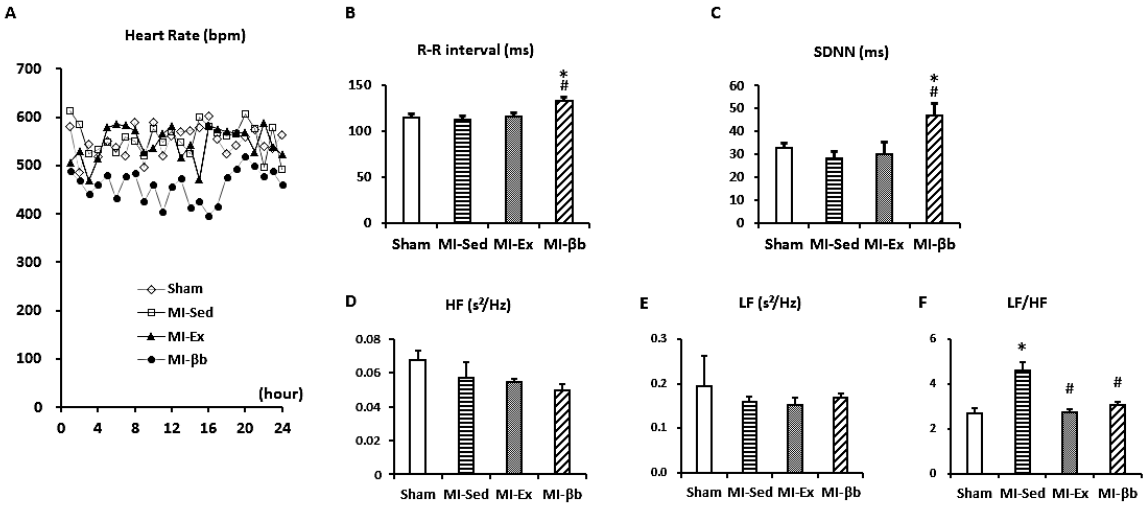

Figure 8. HRV analysis in the time and frequency domains

A: Mean heart rate (HR) was significantly lower in MI-βb compared with the other groups. B, C: In time-domain parameters of HRV analysis, MI-βb demonstrated prolonged mean R–R intervals (Sham, 114.1 ± 4.2 ms; MI-Sed, 112.2 ± 3.8 ms; MI-Ex, 115.4 ± 4.1 ms; MI-βb, 132.9 ± 3.6 ms) and standard deviation of R–R intervals (SDNN; Sham, 32.6 ± 2.0 ms; MI-Sed, 28.0 ± 3.0 ms; MI-Ex, 29.8 ± 5.4 ms; MI-βb, 46.7 ± 5.2 ms). D: In frequency domain analysis, HF showes a trend towards a decrease in MI groups (Sham, 0.068 ± 0.0059 s^2/Hz; MI-Sed, 0.057 ± 0.0096 s^2/Hz; MI-Ex, 0.057 ± 0.0021 s^2/Hz; MI-βb, 0.050 ± 0.0035 s^2/Hz); E: LF had no obvious difference (Sham, 0.19 ± 0.07 s^2/Hz; MI-Sed, 0.16 ± 0.01 s^2/Hz; MI-Ex, 0.15 ± 0.002 s^2/Hz; MI-βb, 0.17 ± 0.01 s^2/Hz). F: The LF/HF ratio, was significantly increased in MI-Sed compared with Sham, and it was restored in MI-Ex and MI-βb. (Sham, 2.60 ± 0.90 s^2/Hz; MI-Sed, 3.92 ± 0.38 s^2/Hz; MI-Ex, 2.83 ± 0.14 s^2/Hz; MI-βb, 3.68 ± 0.27 s^2/Hz), (n=4/group). *P<0.05, vs. Sham; #P<0.05, vs. MI-Sed.

3.3.2 Autonomic tone assessment

At baseline ECG there was no obvious difference in mean HR of Sham, MI-Sed and MI-Ex groups, except MI-βb. After injection of propranolol and following atropine (Figure 9), every group indicated an initial decrease and subsequent increase in HR. The HR difference between injection of propranolol and propranolol + atropine is considered as the parasympathetic tone index. Between them, MI-Ex displayed a significant difference, compared with others, which indicated an enhanced parasympathetic tone. But as to the sympathetic tone, the heart rates didn't show apparent change after initial injection of atropine and subsequent propranolol + atropine.

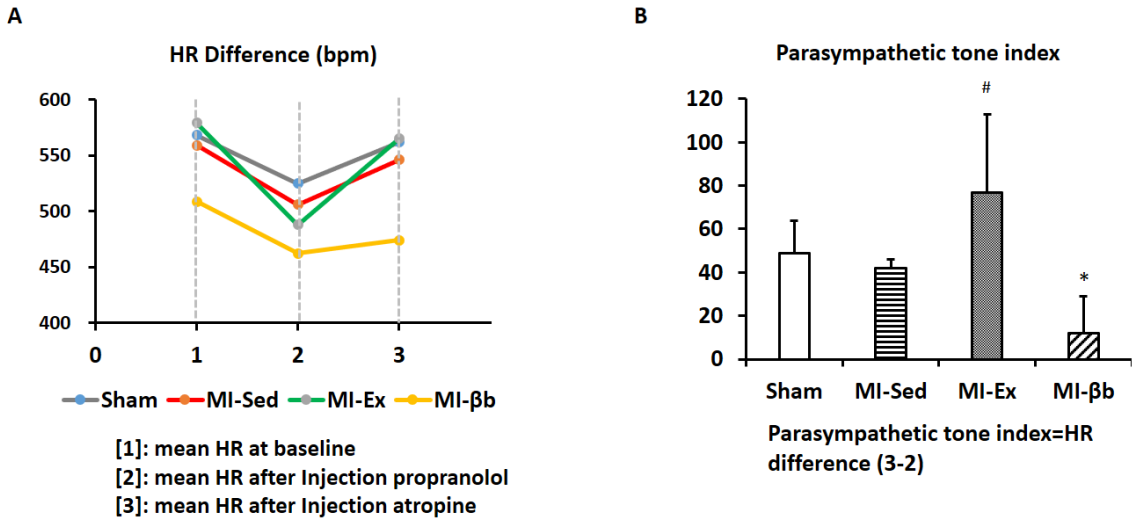

Figure 9. Autonomic tone assessment

(A)After injection of propranolol and following atropine, every group indicated an initial decrease [2] and subsequent increase [3] in HR. Between them, MI-Ex displayed a si*g*nificant difference, compared with others (B) (Sham, 49 ± 15; MI-Sed, 42 ± 7; MI-Ex, 77 ± 36; MI-βb, 12 ± 17, bpm), which indicated an enhanced parasympathetic tone. *P<0.05, vs. Sham; #P<0.05, vs. MI-Sed (n=5/group).

3.3.3 The occurrence of spontaneous arrhythmias

We calculated the occurrence of spontaneous ventricular arrhythmic events including PVCs and VTs (Fig. 10A to D) in continuous ECG recordings. Mice in MI-Sed showed an increased incidence of PVCs compared with Sham, while exercise training and β-blocker treatment effectively reduced the occurrence (Fig. 10E). Notably, VTs were frequently observed in 3 of 6 mice (50%) in MI-Sed and the longest duration of VT was 22 seconds in a MI mouse, while 1 of 6 mice (17%) in MI-Ex, and no VT was observed in Sham or MI-βb (Fig. 10F). These data suggest that chronic exercise training can reduce the episodes of spontaneous ventricular arrhythmias, similarly to β-blocker treatment.

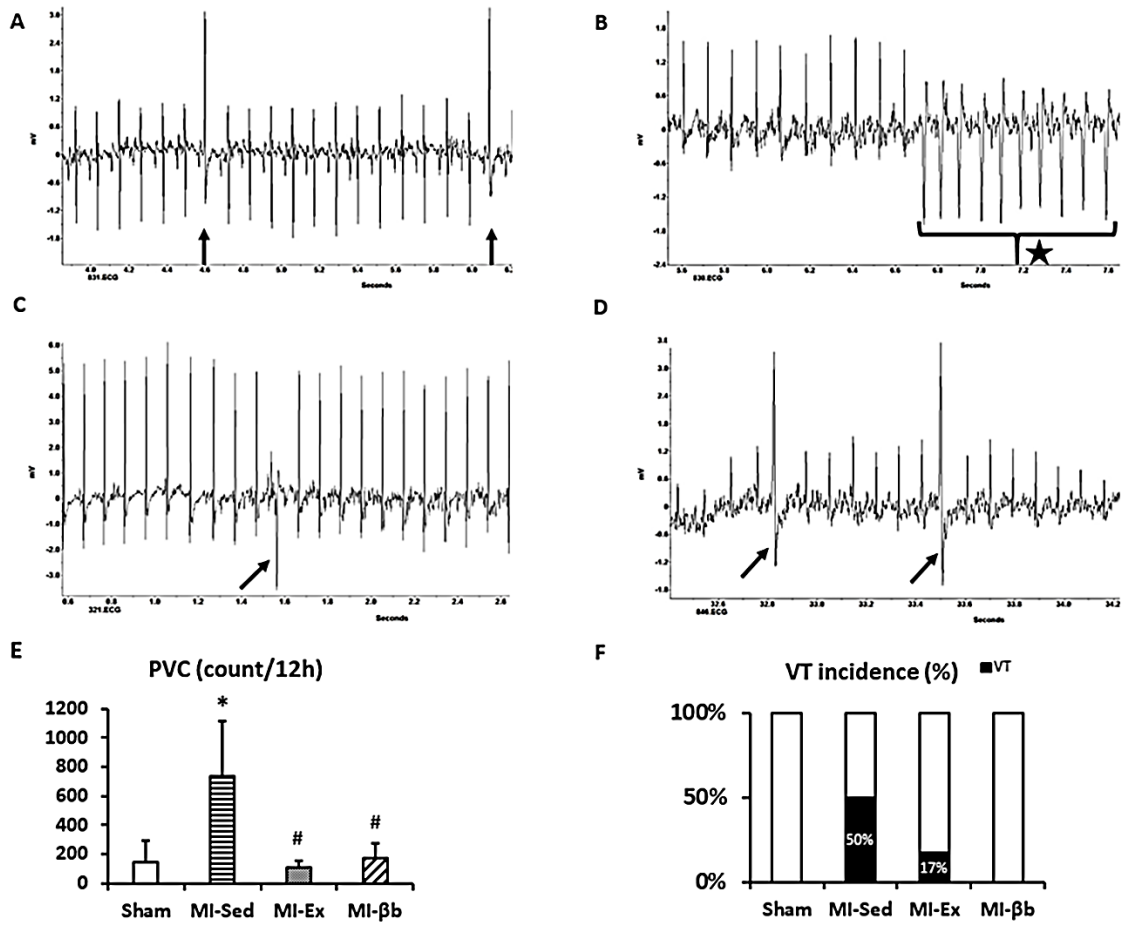

Figure 10. Representative images of the spontaneous ventricular arrhythmic events. PVC, premature ventricular contractions (indicated by the arrow); VT, ventricular tachycardias (indicated by the star).

A: in Sham; B: in MI-Sed,; C: in MI-Ex; D: in MI-βb. E: Mice in MI-Sed showed an increased incidence of PVCs compared with Sham, while exercise training and β-blocker treatment effectively reduced the occurrence (Sham, 148 ± 143 counts/12 h; MI-Sed, 739 ± 377 counts/12 h; MI-Ex, 109 ± 44 counts/12 h; MI-βb, 172± 102 counts/12 h, n=6/group). F: VTs were frequently observed in 3 of 6 mice (50%) in MI-Sed and 1 of 6 mice (17%) in MI-Ex. The longest duration of VT was 22 seconds in a MI-Sed mouse. However, no VT was observed in Sham and MI-βb (n=6/group). *P<0.05, vs. Sham; #P<0.05, vs. MI-Sed.

3.4 Exercise influences calcium handling-related molecules

3.4.1 Gene expressions of mRNAs

Next, we assessed the expression of relevant genes in LV tissues. The expression of Nppb, which encodes BNP, was significantly higher in MI-Sed versus Sham, but significantly lower in MI-Ex versus MI-Sed (Fig. 11A). The ratio of MHC α/β showed no obvious change (Fig. 11B). The expression level of β1-AR was slightly decreased, and the expression of Grk5 was slightly increased in MI-Sed compared to Sham. However, we couldn't find the significances between groups (Fig. 11C, D). The expressions of Chrm2 and β2-AR also showed no apparent changes (Fig. 11E, F).

The calcium handling pathway is highly involved in the incidence of cardiac arrhythmias and determines the force of myocardium contraction. RyR2 releases calcium from (SR) to the cytoplasm, while SERCA2a and PLN, an inhibitor of SERCA2a, coordinately mediate calcium reuptake into the SR (Lanner et al. 2010). Therefore, we investigated the changes in expressions of calcium handling related genes. The mRNA expressions of RyR2 and PLN were comparable in all groups, while the mRNA expression of Atp2a2 (SERCA2a) was significantly decreased in MI-Sed compared with Sham, and it was significantly increased in MI-Ex and MI-βb groups (Fig. 11G, H and I).

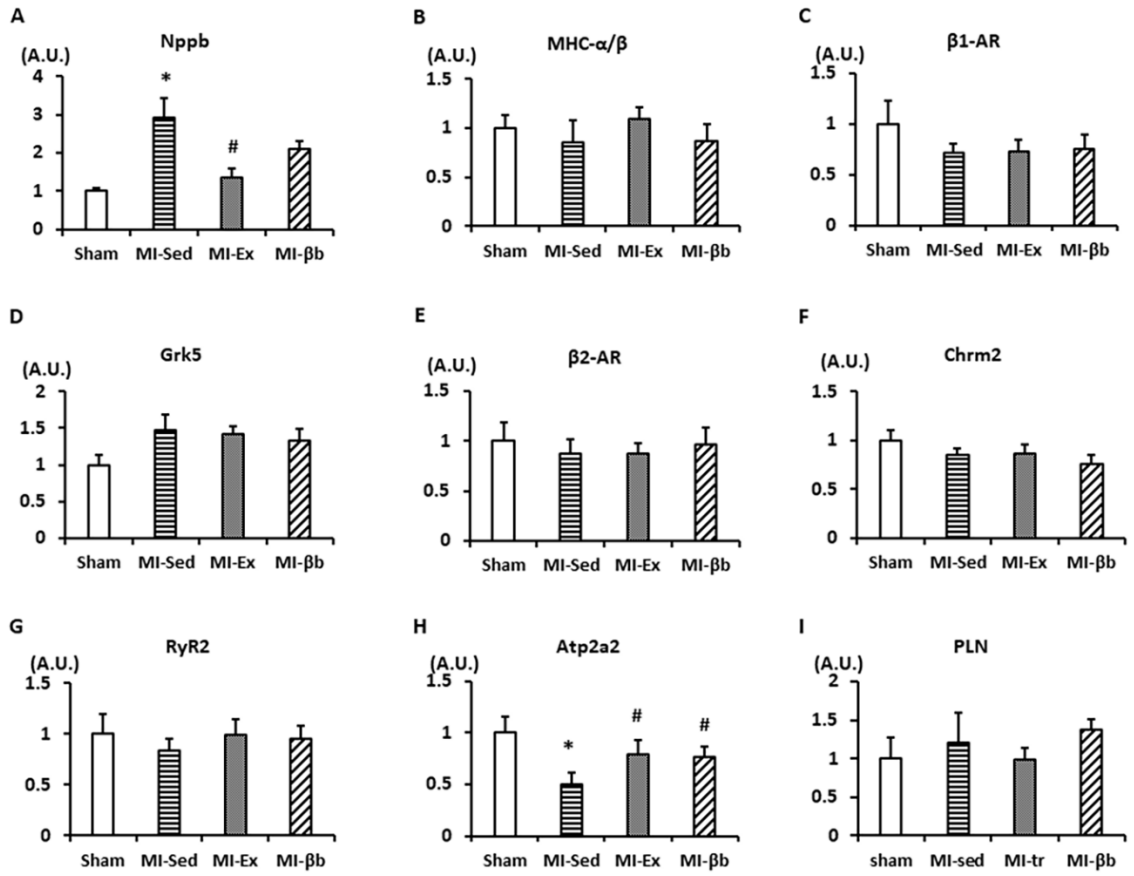

Figure 11. Gene expression levels analyzed by real-time PCR

A: The mRNA expression of Nppb (BNP) was significantly higher in MI-Sed than Sham and was significantly lower in MI-Ex and MI-βb than MI-Sed. B to F: The expression levels of MHC α/β, β1-AR, Grk5, β2-AR, and Chrm2 showed no obvious changes. G to I: In calcium handling related genes, there were no obvious differences in the expressions of RyR2 and PLN, while Atp2a2 (SERCA2a) was significantly lower in MI-Sed than Sham and was restored in MI-Ex and MI-βb. (n=8/group). *P<0.05, vs. Sham; #P<0.05, vs. MI-Sed.

3.4.2 The protein expressions of calcium handling related molecules

Next, we assessed the protein expressions of calcium handling related molecules. The ratio of phosphorylated RyR2 at Serine 2814 to total RyR2 was significantly increased in MI-Sed compared with Sham and decreased in MI-Ex and MI-βb groups (Fig. 12A). By contrast, there were no differences in the ratio of phosphorylated RyR2 at Serine 2808 to total RyR2 between the MI groups. The expression of SERCA2a was significantly decreased in MI-Sed compared with Sham, and restored in MI-Ex and MI-βb groups (Fig. 12B). The expression of p-PLN/PLN showed slightly increased in MI- Ex, but the difference was not significant between all groups (Fig. 12C). It has been reported that reduced p-TNI could be an indicator of increased Ca2+ sensitivity in post-MI (van der Velden et al. 2004). In our study, although both the expressions of TNI and p-TNI were decreased in MI groups compared with Sham, the ratio of p-TNI to TNI showed no obvious difference (Fig. 12D).

We further determined the protein activity of the upstream kinases that regulate the amplitude and kinetics of calcium cycling. Exercise and β-blocker treatment suppressed CaMKII hyperphosphorylation without alteration of expression levels of oxidized-CaMKII and total CaMKII (Fig. 12E). By contrast, there were no significant differences in PKA and phosphorylated PKA (T197) expressions (Fig. 12F).

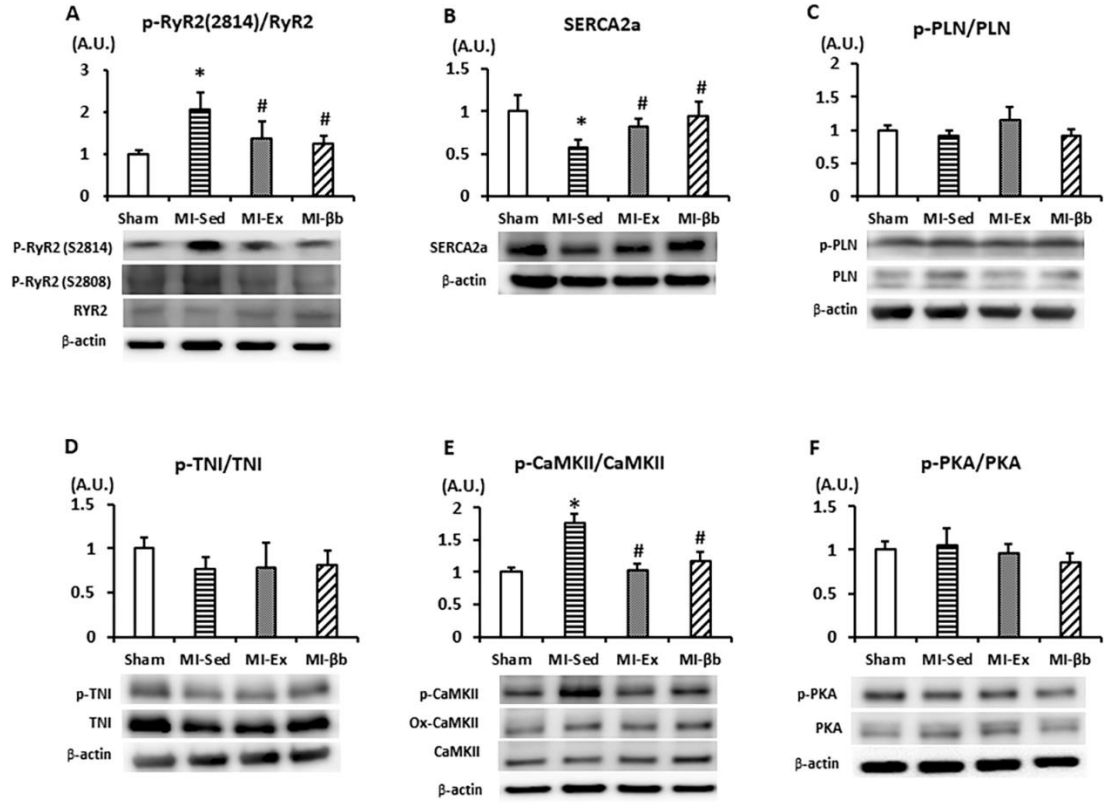

Figure 12. Western blotting analysis.

A: The phosphorylated RyR2 at Serine 2814 site was significantly increased in MI-Sed compared with Sham and was decreased in MI-Ex and MI-βb. There was no change in the expression of phosphorylated RyR2 at Serine 2808 site or total RyR2. B: The protein expression of SERCA2a in MI-Sed was significantly decreased compared with Sham and was recovered in MI-Ex and MI-βb. C: The expression of p-PLN/PLN showed slightly increased in MI- Ex, but the differences were not significant between all groups. D: Although both the expressions of TNI and p-TNI were decreased in MI groups compared with Sham, the ratio of p-TNI to TNI showed no obvious difference. E: The expression level of p-CaMKII was obviously increased in MI-Sed, and it was decreased in MI-Ex and MI-βb without significant alterations of oxidized-CaMKII and total CaMKII

expressions. F: There were no significant differences in PKA and phosphorylated PKA (T197) expressions between all groups. (n=6/group). *P<0.05, vs. Sham; #P<0.05, vs. MI-Sed.

3.5 Exercise decreases miR-1 expression and improves PP2A expression.

The miRNAs are a class of small single-stranded and highly conserved non-coding RNAs, which post-transcriptionally regulate gene expression (Bartel 2009). In the miRNA family, miR-1and miR-133a were the first described striated muscle-specific miRNAs and are closely linked to cardiac disorders such as MI, heart failure, and arrhythmia (Belevych et al. 2011; Matkovich et al. 2009). Therefore, we evaluated the expression of miR-1 and miR-133a. We found that miR-1 expression was significantly elevated in MI-Sed, but restored by exercise and β-blocker treatment (Fig. 13A), while there was no significant difference in miR-133a expression (Fig. 13B).

Protein phosphorylation is regulated by the balance of kinase and phosphatase activities. PP1 and PP2A are major serine–threonine phosphatases involved in the dephosphorylation of specific substrates (Terentyev et al. 2009). The expression of the PP2A regulatory subunit B56α (F-10) was significantly decreased in MI-Sed, and restored in MI-Ex and MI-βb groups (Fig. 13C). However, there were no differences in PP1 protein expression between the groups (Fig. 13D).

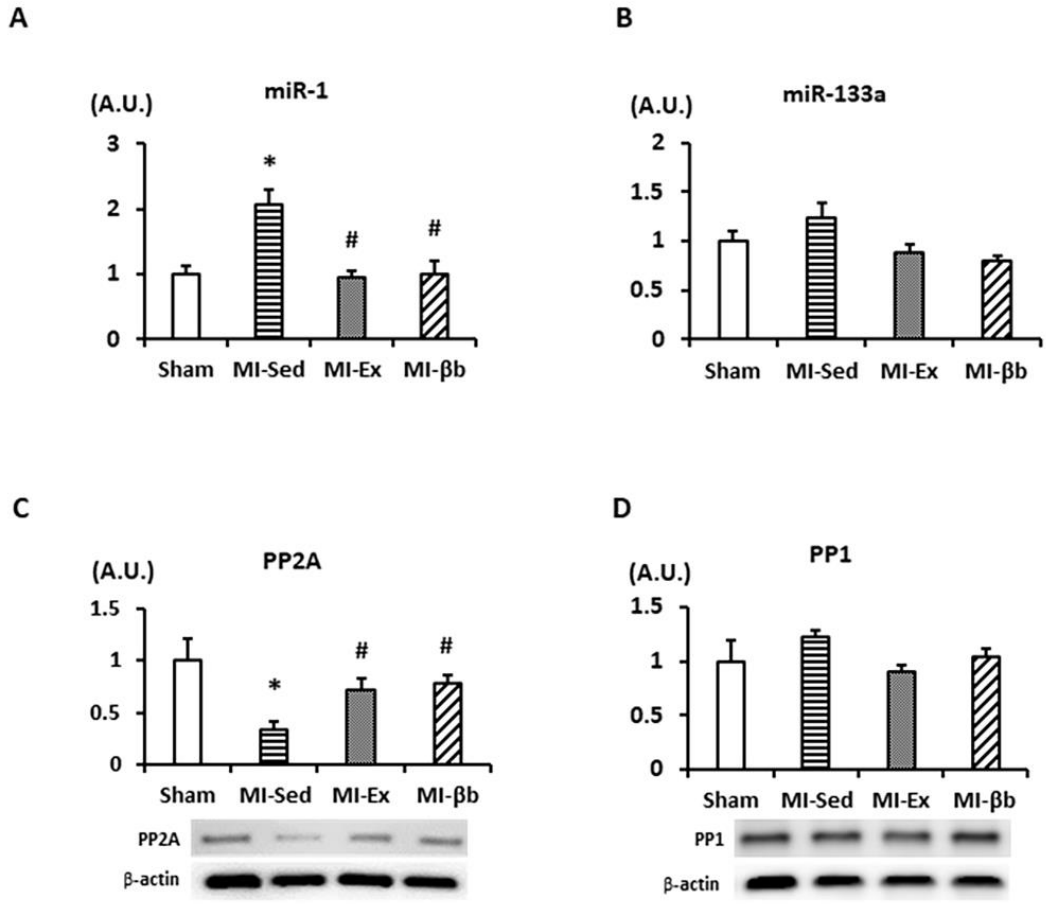

Figure 13. Exercise decreases miR-1 expression and improves PP2A expression.

A: The expression of microRNA-1 (miR-1) in the LV was elevated in MI-Sed and reduced by exercise and β-blocker treatment. B: There was no difference in the expression of miR-133a between the groups. (n=5/group). C: There was no difference in expression of protein phosphatase 1 (PP1), while PP2A-B56-α (F-10) expression was significantly decreased in MI-Sed compared to that in Sham and reversed in MI-Ex and MI-βb (D). (n=6/group) *P<0.05, vs. Sham; #P<0.05, vs. MI-Sed.

Chapter 4 Discussion

In the present study, we found that chronic intensive exercise training improved the imbalance of sympathetic and parasympathetic activities, reduced the incidence of PVC and VT, and restored calcium handling in MI mice, despite no obvious alterations in cardiac structure or function. Thus, exercise training in subacute to chronic phase of MI did not increase the risk of malignant arrhythmias, but rather restores autonomic function and cardiac electrical stability.

Although both exercise and treatment with β-blockers did not alter MI area and cardiac systolic function, we found that exercise training significantly improved peak VO_2. This finding is consistent with the previous observation that exercise has a better effect on cardiopulmonary function and exercise capacity (Jaureguizar et al. 2016). We also found that 6-week exercise restored autonomic imbalance showed by LF/HF and decreased spontaneous ventricular arrhythmias. Autonomic nervous remodeling occurs after MI, which is critical for the incidence of tachyarrhythmias and sudden cardiac death (Shen et al. 2014). Billman GE and colleagues reported that β2-adrenergic stimulation was relatively activated, and its receptor antagonist could suppress the incidence of VF in canine MI model through restoring intracellular Ca^{2+} transients (Billman et al. 1997). They also reported that endurance exercise training normalized repolarization and calcium-handling abnormalities, prevented ventricular fibrillation (VF) in ischemia-induced canine model of sudden cardiac death (Billman 2009; Bonilla et al. 2012). Therefore, autonomic imbalance and calcium handling abnormality play critical roles in the occurrence of

ventricular tachyarrhythmias after MI, and exercise training could ameliorate such pathological conditions. In the failing heart, RyR2 hyperphosphorylation at serine 2814 site by CaMKII increases diastolic calcium leak, leading to delayed after-depolarization and triggering of VT (Gonano et al. 2011; Lanner et al. 2010). We showed that phosphorylated CaMKII and Ser2814-phosphorylated RyR2 were significantly increased in MI-Sed compared with Sham, which were suppressed by exercise. Exercise training was previously reported to reduce RyR2-induced calcium release from the SR and reduce VT in diabetic mice after MI (Rolim et al. 2015). Thus, inhibition of CaMKII dependent-RyR2 hyperphosphorylation by chronic exercise training may be a key mechanism underlying the suppression of ventricular arrhythmias in heart failure after MI.

In the present study, bisoprolol treatment also restored the hyperphosphorylation of CaMKII and RyR2 at Ser2814, as well as the expression of SERCA2a, suggesting an improvement in calcium handling, as for exercise training. Although β-blocker treatment did not improve cardiopulmonary function and exercise capacity, it showed a more powerful effect on improving HRV (prolonging R-R interval and increasing SDNN) and reducing ventricular arrhythmias. Thus, the combination of these two therapies may lead to a better integrative outcome. Vanzelli et al. reported that combined aerobic exercise training and carvedilol treatment had a positive impact on heart failure using a genetic model of sympathetic hyperactivity-induced heart failure, albeit via a different mechanism (Vanzelli et al. 2013). The beneficial effects of combined therapies, including improved cardiac calcium handling, are mainly related to the effects of exercise training and the reduced myocardial oxidative stress and reversed ventricular remodeling associated

with carvedilol therapy (Vanzelli et al. 2013). Our contrasting findings may be attributed to differences in the model, pharmacological agent, and study protocol.

There is now strong evidence for an important role of miRNAs in regulating cardiac function and structural changes during normal cardiac development, as well as in pathological conditions such as heart failure (Bartel 2009; Olson 2014; Pinti et al. 2017). Of more than 1000 miRNAs, miR-1 and miR-133 are recognized as muscle-specific miRNAs and play an important role in the pathogenesis of heart failure and arrhythmia (Li et al. 2015). However, the behavior of miRNAs in heart failure remains controversial. While some studies have reported a reduction in miR-1 expression in hypertrophy and heart failure (Diniz et al. 2017; Ikeda et al. 2009; Li et al. 2015), others have reported an increase (Long et al. 2012; Matkovich et al. 2009). In the present study, miR-1 expression was upregulated, while miR-133 was unchanged, in MI groups compared with Sham. The differences in cardiac miR-1 and miR-133 expression may depend on the stages and etiology of heart failure. We showed that elevated miR-1 expression in MI was significantly restored by chronic exercise training and β-blocker treatment, which is consistent with a previous study showing that β-adrenergic antagonists can downregulate miR-1 (Lu et al. 2009). Moreover, some studies reported that exercise training restored cardiac miR-1 and regulating calcium handling (Melo et al. 2015; Silveira et al. 2017). Terentyev et al. also reported that miR-1-overexpressing cardiomyocytes exhibited spontaneous arrhythmogenic oscillations of intracellular calcium, via a selective increase in phosphorylation of the L-type calcium channel and RyR2 at Serine 2814 (CaMKII-dependent

phosphorylation site), and downstream regulation of PP2A activity (Terentyev et al. 2009).

PP2A is the target molecule of miR-1(Terentyev et al. 2009), and the authors reported that expression of miR-1 and miR-133 were significantly increased in heart failure, while expression of PP2A catalytic and regulatory subunits (putative targets of miR-133 and miR-1) were decreased (Belevych et al. 2011). Moreover, the decreased PP2A activity observed in heart failure was accompanied by enhanced CaMKII-mediated phosphorylation of RyR2 at Ser-2814, and increased frequency of diastolic calcium waves and after-depolarizations, in heart failure rats compared with controls (Terentyev et al. 2009). Taken together, these findings suggest that the antiarrhythmic effects of chronic exercise or β-blocker treatment are, at least in part, related to regulation of miR-1 expression, and downstream phosphatase activity of calcium handling-related molecules, in cardiomyocytes.

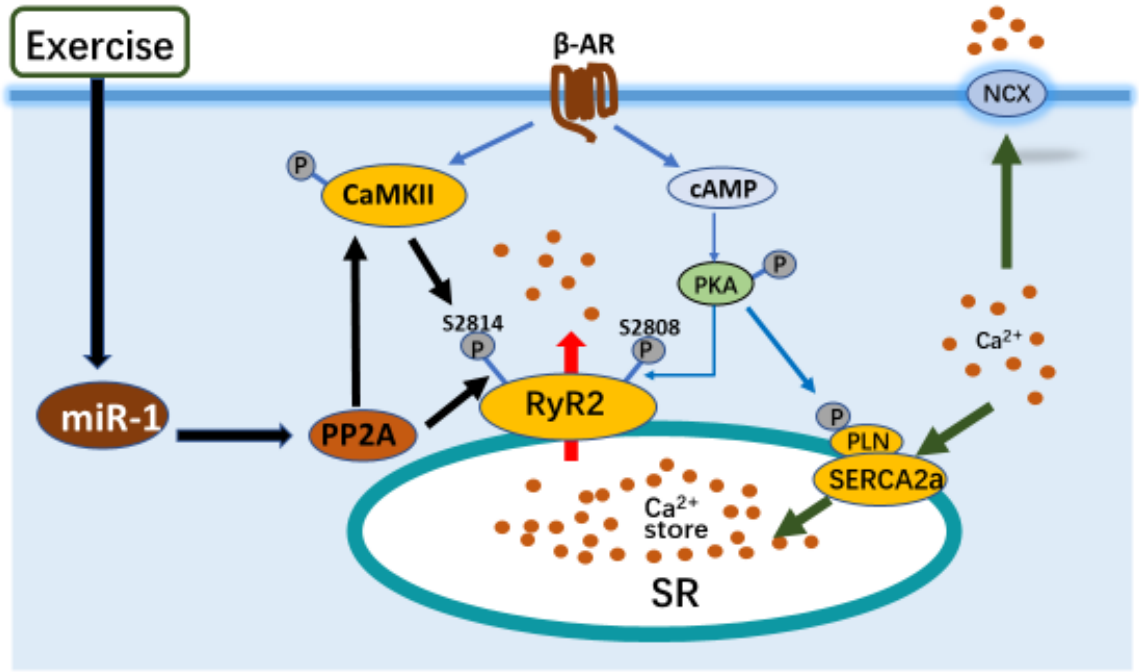

Figure 14. The effect of exercise in calcium modulating.

In failing heart, RyR2 hyperphosphorylation at serine 2814 site by CaMKII increases diastolic Ca^{2+} leak, leading to delayed after depolarization and triggering ventricular tachyarrhythmias. Exercise training restored the elevated expression of miR-1 in MI. As the targeting subunit of miR-1, PP2A, which committed to dephosphorylation of CaMKII and RyR2, was recovered by exercise. Therefore, it is suggested that inhibition of CaMKII dependent-RyR2 hyperphosphorylation by chronic exercise training reduced the abnormal Ca^{2+} leak and is a main mechanism of suppression of ventricular arrhythmias in heart failure after MI.

Chapter 5 Limitations

There are several limitations in this study. Although we analyzed the protein expression levels of calcium handling molecules, we didn't measure intracellular calcium content. This is recognized as a major limitation in our study. In addition, the valid sample number in HRV analysis was only 4, and although we showed SDNN value as a major parameter of HRV, the others such as PNN50 were not obtained. We decided to keep 6-week intervention to obtain enough effectiveness according to the previous researches (Rolim et al. 2015; Speaker et al. 2014). Because structural remodeling process might be almost completed within 1 month after MI or even shorter, we supposed longer intervention would not have yield different results. However, we cannot exclude the possibilities that longer or shorter period of intervention or start period of intervention would yield different results in this study, and further research will be recommended.

Chapter 6 Conclusion

In summary, we conclude that continuous intensive exercise training can suppress ventricular arrhythmias in subacute to chronic phase of MI through restoring autonomic imbalance and impaired calcium handling, similarly to that for β-blockers. It is suggested that the antiarrhythmic effects of chronic intensive exercise or β-blocker treatment are, at least in part, attributed to regulations of miR-1-mediated PP2A activity and its downstream targets, hyperphosphorylated CaMKII-RyR2, in MI. Thus, exercise may be a safe and effective therapy for improving outcome in heart failure patients after MI.

Chapter 7　Acknowdegement

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Chapter 8 The Source

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