Metabolism Clinical and Experimental 94 (2019) 77–87

Contents lists available at ScienceDirect

Metabolism Clinical and Experimental

journal homepage: www.metabolismjournal.com

Combination of exercise and calorie restriction exerts greater efficacy on cardioprotection than monotherapy in obese-insulin resistant rats through the improvement of cardiac calcium regulation

Siripong Palee [a,b], Wanitchaya Minta [a,b,c], Duangkamol Mantor [a,b,c], Wissuta Sutham [a,b,c], Thidarat Jaiwongkam [a,b], Sasiwan Kerdphoo [a,b], Wasana Pratchayasakul [a,b,c], Siriporn C. Chattipakorn [a,b,d], Nipon Chattipakorn [a,b,c,*]

[a] Cardiac Electrophysiology Research and Training Center, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand
[b] Center of Excellence in Cardiac Electrophysiology Research, Chiang Mai University, Chiang Mai 50200, Thailand
[c] Cardiac Electrophysiology Unit, Department of Physiology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand
[d] Department of Oral Biology and Diagnostic Science, Faculty of Dentistry, Chiang Mai University, Chiang Mai, Thailand

ARTICLE INFO

Article history:
Received 18 September 2018
Accepted 11 February 2019

Keywords:
Heart
Insulin resistance
Mitochondrial dynamics
Obesity

ABSTRACT

Background: Long-term high-fat diet (HFD) consumption causes obese-insulin resistance which is known to be a major risk factor for cardiovascular diseases due to its impact on the impairment of left ventricular (LV) contractile function and cardiac mitochondrial function. Intracellular calcium [Ca^{2+}]i regulation plays an important role in the maintenance of LV function. Although either caloric restriction (CR) or exercise (Ex) are shown to strongly affect metabolic status and LV function, the combined effects of exercise and calorie restriction on cardiometabolic status, cardiac mitochondrial dynamics and cardiac [Ca^{2+}]i transient homeostasis under conditions of obese-insulin resistance have never been investigated.

Methods: Female rats were fed with either a high-fat diet (HFD: fat, 59.28%; protein, 26.45%; carbohydrate, 14.27%) or a normal diet (fat, 19.77%; protein, 28.24%; carbohydrate, 51.99%) for 13 weeks. HFD rats were then divided into 4 groups: 1) Vehicle (HFD + Veh); 2) Calorie restriction (HFD + CR); 3) Exercise (HFD + Ex) and 4) Combined therapy (HFD + CR + Ex). After 6-week intervention, the metabolic status, heart rate variability (HRV), LV function, cardiac mitochondrial dynamics, and [Ca^{2+}]i transients were determined.

Results: Insulin resistance developed in HFD rats as indicated by increased plasma insulin and HOMA index. Although HFD + Veh rats had markedly impaired LV function, indicated by reduced %LVFS and impaired cardiac mitochondrial dynamics and [Ca^{2+}]i transients, these impairments were attenuated in the HFD + CR, HFD + Ex and HFD + CR + Ex rats. However, the greatest improvement in cardiometabolic function was observed in HFD + CR + Ex rats.

Conclusions: Our findings indicated that a combination of calorie restriction and exercise exerted greater cardioprotection than a monotherapy through the improvement of cardiometabolic status, cardiac mitochondrial dynamics and cardiac [Ca^{2+}]i homeostasis in obese-insulin resistant rats.

1. Introduction

Long-term consumption of a high-fat diet (HFD) can lead to obese-insulin resistance [1], which is characterized by body weight gain, hyperinsulinemia with euglycemia and is also associated with metabolic dysregulation and dyslipidemia [1,2]. Obese-insulin resistance is one of the major risk factors for cardiovascular disease (CVD) due to its impact on the impairment of cardiac autonomic regulation, cardiac contractile function and cardiac mitochondrial function [3–6].

Abbreviations: Ca^{2+}, intracellular calcium; CR, caloric restriction; Cyt c, cytochrome c; DCFDA, dichlorohydro-fluorescein diacetate dye; Drp1, dynamin-related protein; EDP, end-diastolic pressure; ESP, end-systolic pressure; Ex, exercise; %FS, %fractional shortening; HF, high-frequency band of heart rate variability analysis; HFCB, high-fat diet fed rats treated with combined treatment; HFCR, high-fat diet fed rats treated with calorie restricted diet; HFEx, high-fat diet fed rats treated with exercise; HFV, high-fat-diet fed rats; HRV, heart rate variability; LF, low-frequency band of heart rate variability analysis; LV, left ventricular; LVEDP, left ventricular end diastolic pressure; LVESP, left ventricular end systolic pressure; Mfn2, mitofusin 2; OPA1, optic atrophy 1; ND, normal diet; P-V, Pressure-volume loop; ROS, reactive oxygen species; SEM, standard error of the mean; SV, stroke volume; TEM, transmission electron microscope.

* Corresponding author at: Cardiac Electrophysiology Research and Training Center, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand.
E-mail address: nchattip@gmail.com (N. Chattipakorn).

https://doi.org/10.1016/j.metabol.2019.02.003
0026-0495/

Cardiac contractile function is mainly regulated by intracellular calcium (Ca^{2+}) homeostasis which relies on cyclical changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]$i transient) [7]. Three major cardiac Ca^{2+} handling proteins, including sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA), ryanodine receptor Ca^{2+} release channel proteins (RYR) and Na^{+}-Ca^{2+} exchanger proteins (NCX), regulate $[Ca^{2+}]$i [8]. In addition, intracellular Ca^{2+} dysregulation can be facilitated through mechanisms associated with mitochondrial integrity, AMPK, JNK and IRS-1 serine phosphorylation [9,10]. Our previous study demonstrated that disturbances in cardiac $[Ca^{2+}]$i regulation were found in obese-insulin resistant rats as indicated by an increase in $[Ca^{2+}]$i diastolic level and a decrease in $[Ca^{2+}]$i transient amplitude and $[Ca^{2+}]$i transient decay rate [11]. In addition, the findings of the study also led to the proposal that cardiac mitochondrial dysfunction was a crucial mechanism in cardiac contractile dysfunction in that high-fat diet-induced obese-insulin resistant model [7]. Previous studies have also demonstrated that intracellular Ca^{2+} could modulate the activity of mitochondrial fission protein Drp1 leading to alteration of the balance of fusion/fission events and mitochondrial mobility, eventually resulting in morphological alterations [12–14]. Therefore, therapeutic approaches that can attenuate metabolic complications, cardiac $[Ca^{2+}]$i dyshomeostasis and cardiac mitochondrial dysfunction caused by obese-insulin resistance, may potentially lead to an improvement in previously impaired cardiac function.

Currently, lifestyle modifications including caloric restriction (CR) and exercise (Ex), are the first therapeutic approach for diabetic management. It has been shown that exercise can protect against obesity-induced mitochondrial dysfunction [15]. In addition, clinical studies have shown that CR combined with exercise attenuated metabolic disturbance, increased life span and reduced CVD risk factors in type 2 diabetes mellitus (T2DM) patients [16,17]. Despite these previous reports, the mechanisms responsible for the combined effects of exercise and calorie restriction on cardiometabolic status, cardiac mitochondrial dynamics and cardiac $[Ca^{2+}]$i transient homeostasis under conditions of obese-insulin resistance have never been investigated. In this study, we tested the hypothesis that the combination of exercise and calorie restriction attenuates cardiac dysfunction through the improvement of cardiac mitochondrial dynamics and $[Ca^{2+}]$i transient homeostasis in obese-insulin resistant rats.

2. Material and methods

2.1. Animal preparation and ethical approval

This study was approved by the Institutional Animal Care and Use Committee of the Faculty of Medicine, Chiang Mai University. Forty female Wistar rats (6 weeks of age, weighing 200–220 g) were obtained from the National Animal Center (Salaya campus, Mahidol University, Bangkok, Thailand). The rats were given time to acclimatize for 1 week and were individually housed in a cage in a temperature-controlled room (25 °C) with a 12-hour dark/light cycle setting.

2.2. Experimental protocol

Rats were randomly divided into two dietary groups: a normal diet (ND, a diet containing 19.77% energy from fat), and a high fat diet (HFD, a diet containing 59.28% energy from fat) [1,18,19]. Thirteen weeks after specific feeding, rats in the HFD group were divided into 4 subgroups to receive one of the following interventions for 6 weeks: 1) Calorie restriction (HFD + CR), 2) Exercise (HFD + Ex), 3) Combined Calorie restriction and Exercise (HFD + CR + Ex), and 4) HFD-control (no exercise and received HFD; HFD + Veh). Body weight and food intake of all rats were recorded throughout the experimental period. At the end of the treatment, blood samples were collected from the tail vein for determination of metabolic parameters. An oral glucose tolerance test (OGTT), heart rate variability (HRV) for cardiac autonomic balance, and echocardiography were carried out. At the end of the study protocol, rats were anesthetized with Xylazine (0.15 ml/kg) and Zolitil (50 mg/kg), and the left ventricular (LV) function was determined using a pressure-volume (P-V) loop recording system. Then, the heart was rapidly removed for the determination of cardiac mitochondrial function and $[Ca^{2+}]$i transients and for other biochemical studies (Fig. 1).

2.3. Calorie restriction

The CR was achieved in rats by switching from HFD to ND and also by decreasing the energy intake, after the 13-week average food intake, to 60% energy intake in ND form, this feeding protocol being continued for 6 weeks.

2.4. Exercise

The exercise regime was carried out using a motor-driven rodent treadmill (Columbus Instruments Ohio, USA) as previously described [20]. Exercise training was performed five days/week over a 6-week period. The treadmill was equipped with an aversive electrical stimulus (163 V of alternating current and 1.5 mA) in the back region of each lane to force the rats to run. Training sessions were held in the morning. During the running sessions sedentary rats were put in the same room as exercising rats. The intensity of exercise started at 10 min once a day at 22 m/min in the first week to accustom the rats to the equipment. Then, the intensity was increased to 30 min once a day at 25 m/min. This protocol was continued for 6 weeks. The intensity of exercise was 65% VO_2max and the intensity of exercise was classified as moderate intensity as previously described [21].

2.5. Tail-cuff blood pressure measurement and echocardiography

Rats were placed in a restrainer to limit their mobility. Volume-pressure recording sensors (VPR) and occlusion cuffs (O-cuff) were attached to the tails. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were recorded and analyzed using a CODA 2 channel non-invasive blood pressure system (Kent Scientific Corporation, CT, USA) [22].

Non-invasive echocardiography was used to determine LV function. Animals were given light anesthesia, specifically 2% isoflurane, with oxygen (2 l/min). An echocardiography probe (S12, GE healthcare, CT, USA) was placed on the chest at the parasternal short axis, and connected to an echocardiography machine (GE vivid-i, GE healthcare, CT, USA). An M-mode echocardiogram at LV papillary muscle level was recorded, and %fractional shortening (%FS) was determined. A pulsed-wave Doppler spectrum of mitral flow was recorded from the apical four-chamber view with the guidance of the color Doppler. The mitral E/A ratio was measured.

2.6. Heart rate variability (HRV) measurement

HRV was performed by immobilizing the limbs of the rats in a prone position under 2.5% isoflurane inhalation anesthesia. A needle electrode was inserted subcutaneously into the position of lead II for the electrocardiogram (ECG). Rats were allowed to gain full consciousness prior to ECG recording. The ECG signals were recorded for 20 min through a signal transducer (PowerLab 4/25 T, ADInstruments, Sydney, Australia), and operated via a Chart 5.0 program (ADInstruments, Sydney, Australia). At least 300 consecutive RR intervals from the section of the tachogram were chosen for HRV analysis. LF and HF bands of HRV were analyzed using an analytical program. Increased LF/HF ratio indicates cardiac sympathovagal imbalance [22].

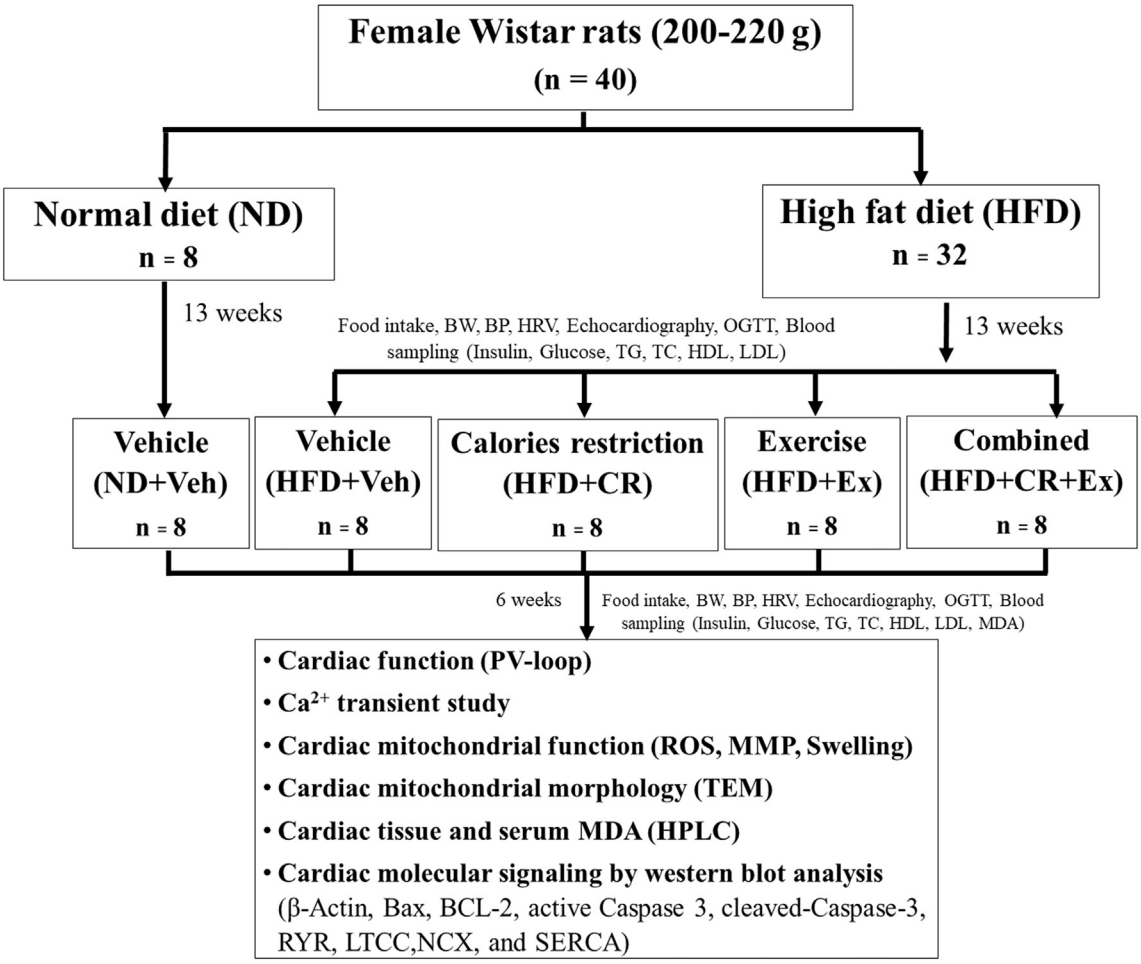

Fig. 1. The study protocol. Study protocol of the combined effects of exercise and calorie restriction on cardiometabolic status, cardiac mitochondrial dynamics and cardiac $[Ca^{2+}]i$ transient homeostasis under conditions of obese-insulin resistance.

2.7. Pressure-volume (P-V) loop study

Rats were anesthetized and ventilated with room air via a tracheostomy tube. The right carotid artery was identified and then a pressure-volume (P-V) loop catheter (Scisense, Ontario, Canada) was inserted. The catheter tip was directed into the LV chamber to record LV pressure and volume. The tip was left in place for a 10-minute period to ensure stable PV loop signals. After stable signals were obtained from the PV loop catheter all loops recorded during 10-minute period were used for data analysis. The investigated parameters obtained from the P-V loop study consisted of end-systolic pressure (ESP), end-diastolic pressure (EDP), maximum and minimum dP/dt (dP/dt_{max} and dP/dt_{min}), cardiac output (CO), % ejection fraction (%EF) and heart rate (HR). All P-V loop parameters were analyzed using Labscribe analytical software (Labscribe, Dover, NH, USA) [22,23].

2.8. Cardiac mitochondrial function study

To study cardiac mitochondrial function, the mitochondrial ROS production, mitochondrial membrane potential changes, and mitochondrial swelling were determined using the methods described previously [23]. Briefly, each rat heart was removed and cardiac tissues were homogenized and centrifuged to isolate cardiac mitochondria. Cardiac mitochondrial ROS production was measured by staining cardiac mitochondria with dichlorohydrofluorescein diacetate (DCFDA) dye for 25 min, after which a fluorescent microplate reader (Gen5 Microplate Reader, BioTek Instruments, VT, USA) was used to detect the ROS level using the excitation wavelength of 485 nm and emission wavelength of 530 nm [24]. For mitochondrial membrane potential change, the dye 5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolcarbocyanine iodide (JC-1) was used. The green fluorescence (JC-1 monomer) was excited at a wavelength of 485 nm and the emission detected at 590 nm while the red fluorescence (JC-1 aggregates) was excited at a wavelength of 485 nm and the emission detected at 530 nm. A decreased red/green fluorescence intensity ratio indicates depolarization of the mitochondrial membrane [25]. Mitochondrial swelling was determined using spectrophotometry at 25 °C as previously described [26–29]. Decreased light absorbance in a mitochondrial suspension at 540 nm indicates mitochondrial swelling [26]. Cardiac mitochondrial morphology was also studied using a transmission electron microscope (TEM; JEM-1200 EX II, JEOL Ltd., Japan).

2.9. Determination of cardiac mitochondrial dynamics

To determine cardiac mitochondrial dynamics, western blot analysis was used to determine protein expression of the mitochondrial fission protein dynamin-related protein 1 (Drp1) and the mitochondrial fusion protein mitofusin 2 (Mfn2) in the isolated crude mitochondrial fraction from the hearts. Briefly, the isolated cardiac mitochondrial fraction was mixed with a loading buffer consisting of 5% mercaptoethanol, 0.05% bromophenol blue, 75 nM Tris, 2% SDS, and 10% glycerol at pH 6.8. The mixture was then boiled for 5 min and loaded into 10% gradient SDS-polyacrylamide gels. Proteins were then transferred to a nitrocellulose membrane in the presence of a glycine/methanol transfer buffer containing 20 mM Tris, 0.15 M glycine, and 20% methanol (Bio-Rad). The

membranes were incubated in 5% skim milk in 1× TBST buffer containing 20 mM Tris at pH 7.6, 137 nM NaCl, and 0.05% Tween-20 for 1 h at room temperature then exposed to anti-Drp1, phospho-Drp1 (Ser616), MFN2 and VDAC (Cell Signaling Technology, Danvers, MA, USA) for 12 h. Bound antibody was detected by the conjugation of horseradish peroxidase with anti-rabbit IgG. Enhanced chemiluminescence (ECL) detection reagents were administered to visualize peroxidase reaction products.

2.10. Determination of oxidative stress

Malondialdehyde (MDA) concentrations in cardiac tissues were measured using a high-performance liquid chromatography (HPLC) system (Thermo Scientific, Bangkok, Thailand) as described previously [11]. Protein from cardiac tissues was mixed with 10% trichloroacetic acid (TCA) containing BHT then heated at 90 °C for 30 min and cooled to room temperature. The mixture was centrifuged, and the supernatant was mixed with 0.44 M H_3PO_4 and 0.6% thiobarbituric acid (TBA) solution to generate thiobarbituric acid reactive substances (TBARS). The solution was filtered through a syringe filter (polysulfone type membrane, pore size 0.45 μm, Whatman International, Maidstone, UK) and analyzed using the HPLC system. Data were analyzed using BDS software (BarSpec Ltd., Rehovot, Israel), and plasma TBARS concentration was determined directly from a standard curve generated from a standard reagent for MDA at different concentrations and reported as MDA equivalent concentration.

2.11. Determination of metabolic parameters

Plasma insulin level was detected using a sandwich ELISA kit (Millipore, MI, USA). Plasma glucose and triglyceride levels were determined by colorimetric assay from a commercially available kit (Biotech, Bangkok, Thailand). Fasting plasma HDL and LDL were determined using commercially available kits (ERBA diagnostic, Mannheim, Germany) [4].

2.12. Cardiac expression of apoptotic proteins

For determination of cardiac apoptotic protein, Western blot analysis was used for measurement of expression of proteins Bax, Bcl-2, Caspase3, and Cleaved-Caspase3 as described previously [23]. Anti-Bax, Bcl-2, Caspase3, and Cleaved-Caspase3 (Cell Signaling Technology, Danvers, MA, USA), and anti-actin (Sigma-Aldrich, St. Louis, MO, USA) were used. Bound antibody was detected using horseradish peroxidase conjugated with anti-rabbit or anti-mouse IgG. Enhanced chemiluminescence (ECL) detection reagents were administered to visualize peroxidase reaction products [23].

2.13. Cardiomyocyte isolation and calcium transient measurement

Cardiomyocytes were isolated from the hearts of rats using a method described previously [30]. In brief, under deep anesthesia, the heart was removed immediately and placed into a Langendorff apparatus. The hearts were perfused with modified Krebs solution (130 mM NaCl, 4.5 mM KCl, 1.4 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 0.75 mM $CaCl_2$, 4.2 mM HEPES, 20 mM taurine, 10 mM creatine and 10 mM glucose), at pH 7.3 and 37 °C for 5 min, followed by a Ca^{2+}-free solution (100 μM EGTA) for 4 min, and a modified Krebs solution containing 100 μM $CaCl_2$ and 1 mg/ml type II collagenase for another 8 min. The ventricles were removed from the cannula, cut into small pieces and incubated in 10 ml of collagenase solution, 100% oxygen being bubbled through for 7 min at 37 °C, with regular trituration. The cardiomyocytes were separated from undigested ventricular tissues by filtering through a cell strainer, and were allowed to settle into a loose pellet. Then, the supernatant was removed and replaced with modified Krebs solution containing 1% BSA and 500 μM $CaCl_2$. This process was repeated with modified Krebs solution containing 1 mM $CaCl_2$. After this procedure, the cardiomyocytes were ready for the recording process.

The isolated cardiomyocytes were used for Ca^{2+} transient measurement using CELL^R imaging software (Olympus Soft Imaging Solutions GmbH, Germany). Isolated cardiomyocytes were loaded with Fura-2/AM and fluorescence intensity was recorded during electrical pacing (1 Hz, 10-ms duration, 15 V). The ratio of the emissions wavelengths is directly related to the amount of intracellular Ca^{2+}. When Ca^{2+} binds to a ratiometric indicator, it changes the optimum excitation or emission wavelength of the indicator. An elevation of Ca^{2+} concentration induces an increase in Fura-2 emission fluorescence when the indicator is excited at 340 nm, with a corresponding decrease in fluorescence at 380 nm excitation [31,32]. The experiments were performed in a temperature-controlled chamber system at 37 °C. Intracellular Ca^{2+} transient decay rate, which approximates the rate of Ca^{2+} uptake into the SR by sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), intracellular Ca^{2+} transient amplitude which approximates the intracellular Ca^{2+} level during the systolic period, and diastolic Ca^{2+} level which approximates to the intracellular Ca^{2+} level during the diastolic period were all calculated.

2.14. Determination of the expression of cardiac calcium regulatory proteins

To determine the levels of cardiac Ca^{2+} regulatory proteins, western blot analysis was used to examine expression of the SERCA, RYR, PLB and NCX proteins, using Anti-SERCA (#4388), RYR (#8153), NCX (#79350), and PLB (#8495) (Cell Signaling Technology, Danvers, MA, USA), LTCC (ab2864) (Abcam, Cambridge, MA, USA) and anti-actin (Sigma-Aldrich, St. Louis, MO, USA) for 12 h. Bound antibody was detected by the conjugation of horseradish peroxidase with anti-rabbit IgG. Enhanced chemiluminescence (ECL) detection reagents were administered to visualize peroxidase reaction products.

2.15. Statistical analysis

Data were expressed as mean ± SEM. Comparisons of variables were performed using a two-way ANOVA. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Calorie restriction, exercise and the combined therapy reduced metabolic disturbance in obese-insulin resistant rats

After 13 weeks of being fed on a HFD, HFD rats developed insulin resistance as indicated by markedly increased body weight and an increased HOMA index, when compared with ND + Veh rats (Table 1). However, the fasting blood glucose level was not significantly different between the HFD rats and ND rats, indicating a pre-diabetic condition in these HFD rats.

Six weeks after interventions there was an observable marked decrease in body weight and visceral fat deposition in all intervention groups (HFD + CR, HFD + Ex and HFD + CR + Ex), when compared with HFD + Veh rats (Table 1). Furthermore, plasma cholesterol levels in HFD + CR, HFD + Ex and HFD + CR + Ex rats were significantly decreased when compared with HFD + Veh rats (Table 1). Insulin sensitivity was also increased in HFD + CR, HFD + Ex and HFD + CR + Ex groups, in comparison to HFD + Veh rats (Table 1), as indicated by a decreased area under the curve (AUC) following an OGTT. Nevertheless, the combined therapy (HFD + CR + Ex rats) had a greater reduction in BW, visceral fat and the AUC, when compared with the HFD + CR and HFD + Ex groups (Table 1).

Table 1
Effect of calories restriction and exercise on metabolic parameters in obese-insulin resistant rats.

Parameters	Groups				
	ND + Veh	HFD + Veh	HFD + CR	HFD + Ex	HFD + CR + Ex
Body weight (g)	281.25 ± 3.80	353.33 ± 4.41*	300.00 ± 6.51*, †	322.50 ± 7.71*,†	266.43 ± 3.40*,†,‡,#
Visceral fat (g)	9.50 ± 1.01	28.69 ± 1.28*	15.19 ± 1.22*, †	21.23 ± 2.11*,†	10.18 ± 0.62*,†,‡,#
Glucose (mg/dl)	130.85 ± 2.15	139.92 ± 5.17	123.64 ± 12.50	135.36 ± 7.94	125.56 ± 4.94
Insulin (ng/ml)	1.36 ± 0.12	4.80 ± 0.40*	3.18 ± 0.58*,†	3.19 ± 0.15*,†	1.74 ± 0.18*,†,‡,#
HOMA index	10.52 ± 2.10	39.73 ± 3.10*	23.26 ± 7.54*,†	25.54 ± 3.54*,†	12.92 ± 2.35*,†,‡,#
Plasma glucose AUC (AUCg) (mg/dl × min × 10^4)	1.89 ± 0.07	2.62 ± 0.03*	2.31 ± 0.001*,†	2.32 ± 0.001*,†	1.97 ± 0.05*,†,‡,#
Cholesterol (mg/dl)	50.81 ± 7.97	99.46 ± 4.91*	62.36 ± 3.20*	67.94 ± 6.22*	59.29 ± 6.73*
HDL (mg/dl)	18.39 ± 1.41	22.48 ± 1.58	22.49 ± 0.94	21.62 ± 0.81	22.02 ± 0.86
LDL (mg/dl)	16.87 ± 4.61	59.02 ± 5.01*	20.90 ± 4.04*,†	30.07 ± 7.14*,†	21.25 ± 5.09*,†
Triglyceride (mg/dl)	62.61 7.97	69.60 ± 6.46	68.04 ± 9.99	67.35 ± 9.06	61.75 ± 5.17

Values are mean ± SEM (n = 8/group). *p < 0.05 vs ND + Veh, †p < 0.05 vs HFD + Veh, ‡p < 0.05 vs HFD + CR, and #p < 0.05 vs HFD + Ex. ND + Veh, normal-diet fed rats; HFD + Veh, high-fat-diet fed rats; HFD + CR, high-fat diet fed rats treated with calories restriction diet; HFD + Ex, high-fat diet fed rats treated with exercise; HFD + CR + Ex, high-fat diet fed rats treated with combine treatment; HOMA, Homeostasis model assessment.

3.2. The combined therapy improved LV function in obese-insulin resistant rats more extensively than calorie restriction and exercise alone

After ingestion of a HFD for 13 weeks, rats fed on a HFD (HFD + Veh, HFD + CR, HFD + Ex and HFD + CR + Ex) had a significantly increased SBP and DBP, compared to NDV rats (Fig. 1A and B). After six weeks of treatment HFD + CR, HFD + Ex and HFD + CR + Ex rats had decreased SBP and DBP, compared to HFD + Veh rats. In all treatment groups, HFD + Ex and HFD + CR + Ex rats showed equal improvement in BP (Fig. 2A and B). Echocardiograms demonstrated that HFD rats (HFD + Veh, HFD + CR, HFD + Ex and HFD + CR + Ex) also developed LV systolic dysfunction as indicated by decreased %FS and also LV diastolic dysfunction as indicated by a decreased mitral E/A ratio, when compared to ND + Veh rats (Fig. 2C and D). LV systolic dysfunction was attenuated in HFD + CR, HFD + Ex and HFD + CR + Ex rats as shown by an increased %FS, compared to HFD + Veh groups (Fig. 1C). However, HFD + Ex and HFD + CR + Ex rats had a more significant improvement of LV function than HFD + CR rats (Fig. 1C). Nevertheless, only HFD + CR + Ex rats had an increased mitral E/A ratio, compared to HFD + Veh groups indicating a greater improvement in diastolic function (Fig. 2D).

The P-V loop analysis was performed at the end of the experimental protocol to determine LV function invasively. Six weeks after treatment, the LV contractile function including LVESP, dP/dt_{max}, CO, and %EF were increased, whereas LV lusitropy function, including LVEDP and dP/dt_{min}, had decreased in the HFD + CR, HFD + Ex and HFD + CR + Ex groups, in comparison to HFD + Veh rats (Table 2). However, HFD + CR + Ex rats showed the greatest improvement in both LV contractile function and relaxation (Table 2).

3.3. The combined therapy attenuated cardiac sympathovagal imbalance in obese-insulin resistant rats more extensively than calorie restriction and exercise alone

The LF/HF ratio of the HRV was determined as an index of cardiac sympathovagal balance. Cardiac sympathovagal imbalance, as indicated by an increase in LF/HF ratio, was observed in all HFD rats (HFD + Veh, HFD + CR, HFD + Ex and HFD + CR + Ex), when compared with ND + Veh rats (Fig. 2E). Interestingly, six weeks after treatment, the LF/HF ratio in the HFD + CR, HFD + Ex and HFD + CR + Ex rats was significantly decreased when compared with HFD + Veh rats (Fig. 2E). However, HFD + CR + Ex rats had the greatest improvement with regard to cardiac sympathovagal balance. Furthermore, the cardiac MDA was markedly increased in all HFD rats with treatment (HFD + CR, HFD + Ex and HFD + CR + Ex), when compared with ND + Veh rats

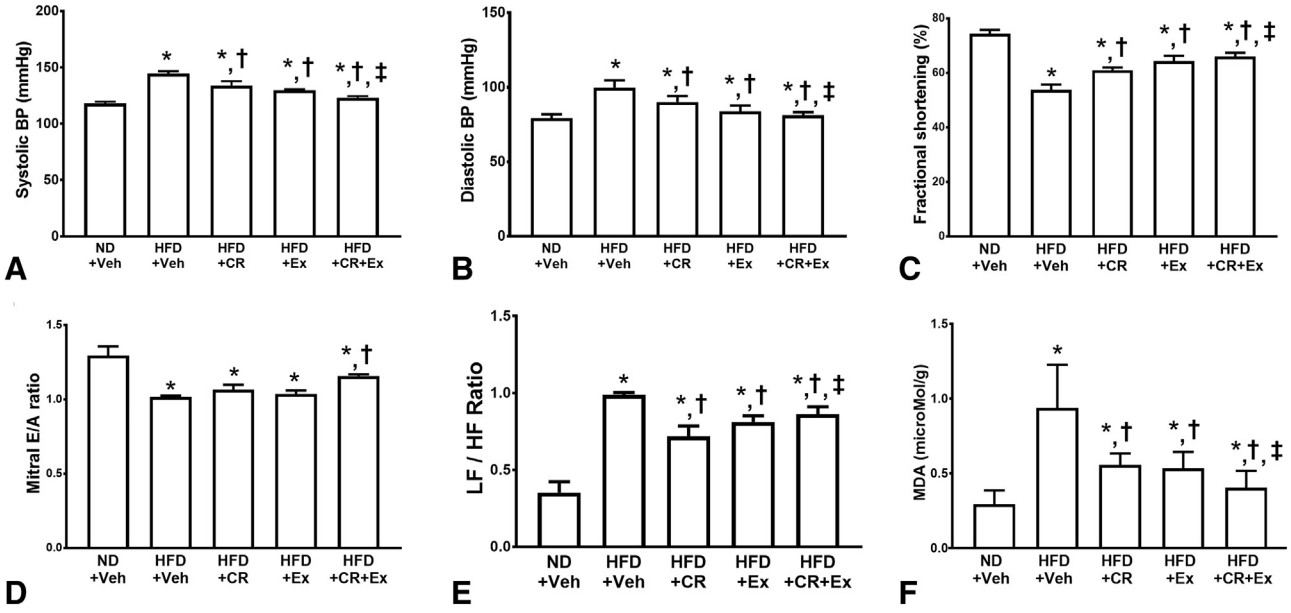

Fig. 2. Effects of calorie restriction and exercise on blood pressure, echocardiographic parameters, heart rate variability and oxidative stress in obese-insulin resistant rats. (A) Systolic blood pressure; (B) Diastolic blood pressure; (C) %Fractional shortening; (D) Mitral E/A ratio; (E) Heart rate variability; (F) MDA. *p < 0.05 vs ND + Veh; †p < 0.05 vs HFD + Veh; ‡p < 0.05 vs HFD + CR. ND + Veh, normal-diet fed rats; HFD + Veh, high-fat-diet fed rats; HFD + CR, high-fat diet fed rats treated with calorie restricted diet; HFD + Ex, high-fat diet fed rats treated with exercise; HFD + CR + Ex, high-fat diet fed rats treated with combined treatments; LF/HF ratio, low frequency/high frequency ratio; MDA, malondialdehyde.

Table 2
Effect of calories restriction and exercise on cardiac function in obese-insulin resistant rats.

Parameters	Groups				
	ND + Veh	HFD + Veh	HFD + CR	HFD + Ex	HFD + CR + Ex
Heart rate (bpm)	257 ± 13	342 ± 25*	283 ± 21*,†	287 ± 23*,†	278 ± 13*,†,‡,#
LVESP (mm Hg)	127 ± 11	104 ± 12*	115 ± 14*,†	117 ± 12*,†	123 ± 14*,†,‡,#
LVEDP (mm Hg)	12 ± 3	36 ± 5*	25 ± 3*,†	24 ± 7*,†	18 ± 6*,†,‡,#
dP/dtmax (mm Hg/s)	9959 ± 549	5584 ± 587*	7721 ± 614*,†	7684 ± 598*,†	8423 ± 612*,†,‡,#
−dP/dtmin (mm Hg/s)	−6938 ± 296	−3613 ± 472*	−5603 ± 625*,†	−5765 ± 62*,†	−6065 ± 626*,†,‡,#
CO/BW (ml/min/g)	0.38 ± 0.3	0.17 ± 0.2*	0.21 ± 0.4*	0.23 ± 0.2*,†	0.29 ± 0.4*,†,‡,#
LVEF (%)	72 ± 5	55 ± 4*	64 ± 2*,†	65 ± 4*,†	67 ± 3*,†,‡,#

Values are mean ± SEM (n = 8/group). *p < 0.05 vs ND + Veh, †p < 0.05 vs HFD + Veh, ‡p < 0.05 vs HFD + CR, and #p < 0.05 vs HFD + Ex. ND + Veh, normal-diet fed rats; HFD + Veh, high-fat-diet fed rats; HFD + CR, high-fat diet fed rats treated with calories restriction diet; HFD + Ex, high-fat diet fed rats treated with exercise; HFD + CR + Ex, high-fat diet fed rats treated with combine treatment; LVESP, left ventricular end systolic pressure; LVEDP, left ventricular end diastolic pressure; dP/dt_{max}, maximal slope of the systolic pressure increment; dP/dt_{min}, maximal slope of the diastolic pressure decrement; CO, cardiac output; LVEF, left ventricular ejection fraction.

(Fig. 2F). Six weeks after treatment, the cardiac MDA levels in the HFD + CR, HFD + Ex and HFD + CR + Ex rats were significantly decreased when compared with HFD + Veh rats (Fig. 2F). Similarly, HFD + Ex and HFD + CR rats had an equal reduction in cardiac oxidative stress.

3.4. The combined therapy attenuated intracellular calcium dyshomeostasis in obese-insulin resistant rats more extensively than calorie restriction and exercise alone

In this study, the intracellular Ca^{2+} transients were determined to assess intracellular Ca^{2+} homeostasis. After ingestion of a HFD for 19 weeks intracellular Ca^{2+} dyshomeostasis was found, the dyshomeostasis being indicated by a decreased intracellular Ca^{2+} transient amplitude and intracellular Ca^{2+} transient decay rate, compared to ND + Veh rats (Fig. 3A, B). Six weeks after treatment, intracellular Ca^{2+} transient amplitude and intracellular Ca^{2+} transient decay rate were markedly increased in HFD + CR, HFD + Ex and HFD + CR + Ex rats, compared to HFD + Veh rats (Fig. 3A, B).

Interestingly, HFD + CR + Ex rats had the greatest increase in intracellular Ca^{2+} transient amplitude, and intracellular Ca^{2+} transient decay (Fig. 3A, B). However, diastolic Ca^{2+} level was no different in all groups (Fig. 3C). The representative intracellular Ca^{2+} transient tracings are shown in Fig. 3D.

3.5. The combined therapy improved calcium regulation in obese-insulin resistant rats more extensively than calorie restriction and exercise alone

In this study, the Ca^{2+} handling proteins were investigated to assess intracellular Ca^{2+} regulation. Cardiac Ca^{2+} handling proteins (SERCA, NCX, RYR, LTCC) were significantly decreased and PLB was significantly increased in HFD rats (HFD + Veh, HFD + CR, HFD + Ex and HFD + CR + Ex), in comparison to ND + Veh rats (Fig. 4A–E). Six weeks after intervention, SERCA, NCX, RYR and LTCC protein expression was markedly increased and PLB protein expression was markedly decreased in HFD + CR, HFD + Ex and HFD + CR + Ex rats, compared to HFD + Veh rats (Fig. 4A–E). In all groups, HFD + CR + Ex rats had the greatest increase in SERCA, NCX, LTCC and decrease in PLB expression (Fig. 4A–F).

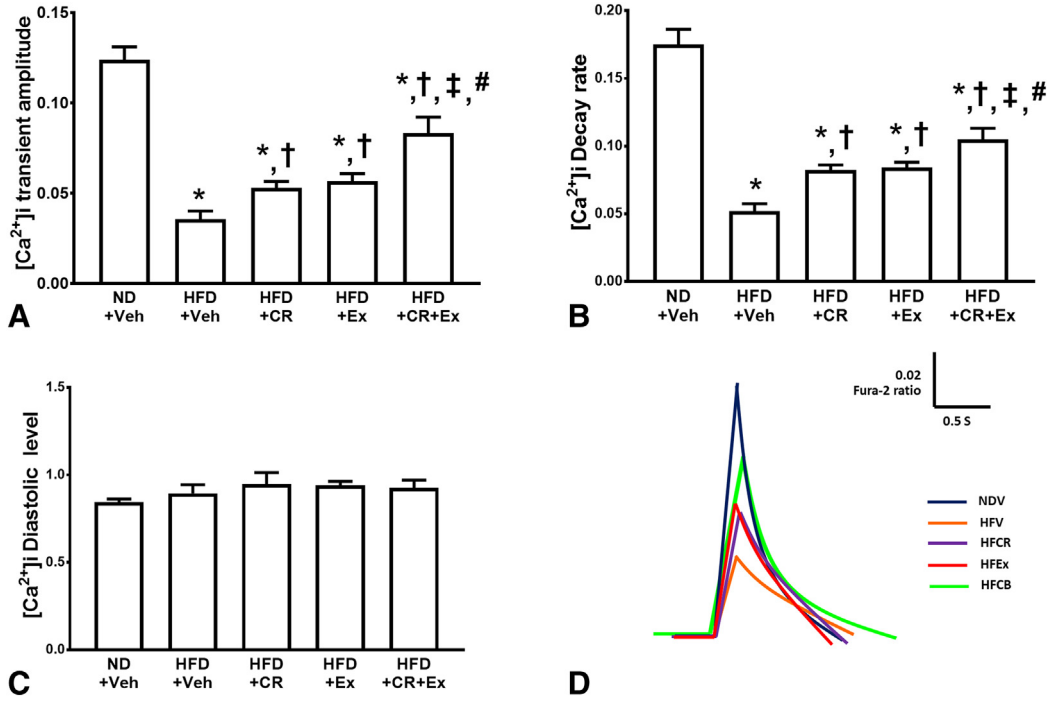

Fig. 3. Effects of calorie restriction and exercise on intracellular Ca^{2+} transients in cardiomyocytes of obese-insulin resistant rats. (A) intracellular Ca^{2+} transient amplitude; (B) increased intracellular Ca^{2+} transient decay rate; (C) increased intracellular diastolic Ca^{2+} levels; (D) Representative images of Ca^{2+} transient tracing. *p < 0.05 vs ND + Veh; †p < 0.05 vs HFD + Veh; ‡p < 0.05 vs HFD + CR; #p < 0.05 vs HFD + Ex ND + Veh, normal-diet fed rats; HFD + Veh, high-fat-diet fed rats; HFD + CR, high-fat diet fed rats treated with calorie restricted diet; HFD + Ex, high-fat diet fed rats treated with exercise; HFD + CR + Ex, high-fat diet fed rats treated with combined treatments.

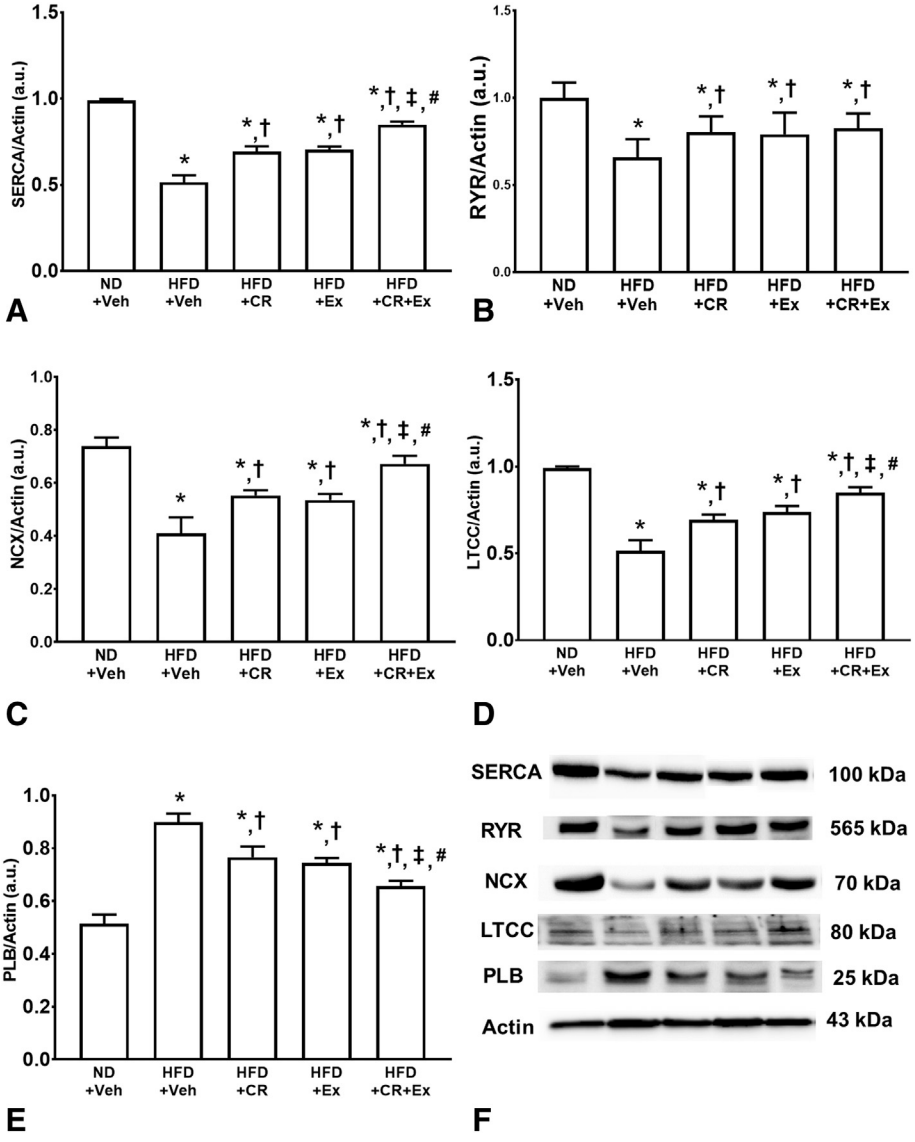

Fig. 4. Effects of calorie restriction and exercise on Ca^{2+} regulatory protein in obese-insulin resistant rats. (A) SERCA; (B) RYR; (C) NCX; (D) LTCC; (E) PLB expression; (F) Representative images of western blotting band. *$p < 0.05$ vs ND + Veh; †$p < 0.05$ vs HFD + Veh; ‡$p < 0.05$ vs HFD + CR; #$p < 0.05$ vs HFD + Ex. ND + Veh, normal-diet fed rats; HFD + Veh, high-fat-diet fed rats; HFD + CR, high-fat diet fed rats treated with calorie restriction diet; HFD + Ex, high-fat diet fed rats treated with exercise; HFD + CR + Ex, high-fat diet fed rats treated with combined treatments.

3.6. The combined therapy attenuated cardiac mitochondrial dysfunction in obese-insulin resistant rats more extensively than calorie restriction and exercise alone

In this study cardiac mitochondrial ROS production, cardiac mitochondrial depolarization, and cardiac mitochondrial swelling were determined to enable the assessment of cardiac mitochondrial function. After ingestion of a HFD for 19 weeks, cardiac mitochondrial dysfunction was evident as indicated by increased cardiac mitochondrial ROS levels, cardiac mitochondrial membrane depolarization, and mitochondrial swelling in comparison to ND + Veh rats (Fig. 5A–C). Six weeks after interventions, HFD + CR, HFD + Ex and HFD + CR + Ex rats had decreased cardiac mitochondrial ROS levels, cardiac mitochondrial membrane depolarization, and mitochondrial swelling when compared to HFD + Veh rats (Fig. 5A–C). However, the HFD + CR + Ex group had the greatest improvement in cardiac mitochondrial function as indicated by the lowest levels of ROS, membrane depolarization and mitochondrial swelling. Representative transmission electron micrographs of cardiac mitochondria morphology showed unfolding of cristae in the HFD rats compared to the ND + Veh rats (Fig. 5D). However, all interventions preserved cardiac mitochondria morphology as indicated by high cristae density and also correctly orientated cristae alignment, in comparison to the HFD + Veh group (Fig. 5D).

3.7. Calorie restriction, exercise and combined therapy improved cardiac mitochondrial dynamics in obese-insulin resistant rats

In this study, cardiac expression of mitochondrial fission proteins indicated by the phosphorylation of dynamin-related protein 1 (Drp1) at serine 616 and the mitochondrial fusion protein mitofusin 2 (Mfn2) were determined to assess cardiac mitochondrial dynamics. After ingestion of a HFD for 13 weeks, our results demonstrated that the phosphorylation of Drp1 at serine 616 was increased, whereas the Mfn2 expression was decreased in HFD + Veh rats, when compared to ND + Veh rats. However, 6 weeks after interventions, all treated rats had significantly decreased levels of phosphorylation of Drp1 at serine 616 in comparison to HFD + Veh rats (Fig. 5E), whereas cardiac mitochondrial expression of Mfn2 protein was increased, compared to HFD + Veh rats (Fig. 5F). Surprisingly, rats in the combined treatment group had the greatest reduction in the levels of phosphorylated Drp1 at serine 616, compared to HFD + CR and HFD + Ex rats.

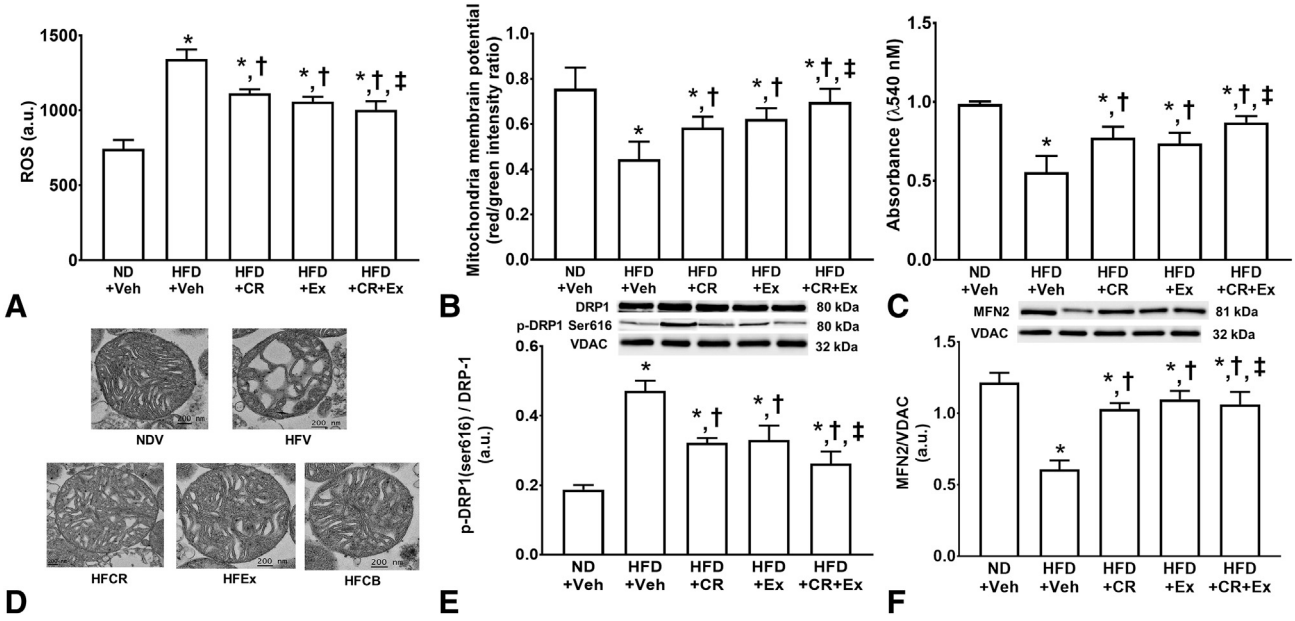

Fig. 5. Effects of calorie restriction and exercise on cardiac mitochondrial function and mitochondrial dynamics in obese-insulin resistant rats. (A) Cardiac mitochondrial ROS production; (B) Cardiac mitochondrial membrane potential; (C) Cardiac mitochondrial swelling; (D) TEM representative images of cardiac mitochondria; (E) Cardiac mitochondrial fission; (F) Cardiac mitochondrial fusion. *p < 0.05 vs ND + Veh; †p < 0.05 vs HFD + Veh; ‡p < 0.05 vs HFD + CR; #p < 0.05 vs HFD + Ex. NDV, normal-diet fed rats; HFD + Veh, high-fat-diet fed rats; HFD + CR, high-fat diet fed rats treated with calorie restricted diet; HFD + Ex, high-fat diet fed rats treated with exercise; HFD + CR + Ex, high-fat diet fed rats treated with combined treatments; ROS, reactive oxygen species; TEM, transmission electron microscopy; Drp1, dynamin-related protein 1; Mfn2, mitofusin 2; VDAC, voltage-dependent anion channels.

3.8. Calorie restriction, exercise and combined therapy all decreased cardiac apoptosis in obese-insulin resistant rats

Cardiac expression of apoptotic proteins Bax, Caspase 3, and Cleaved-Caspase3, and anti-apoptotic protein Bcl-2 were determined to assess cardiac apoptotic signaling. Six weeks after interventions HFD + CR, HFD + Ex and HFD + CR + Ex rats had decreased expression of apoptotic protein Bax, and Cleaved-Caspase3/Caspase 3, and increased anti-apoptotic protein Bcl-2 expression, compared to HFD + Veh rats (Fig. 6A–C). The representative western blot bands of Bax, Caspase 3, Cleaved-Caspase3, Bcl-2 and actin are shown in Fig. 6D.

4. Discussion

The major findings from this study demonstrate clearly that obese-insulin resistance causes metabolic disturbance, oxidative stress, cardiac autonomic imbalance, LV dysfunction, mitochondrial dysfunction, and increased mitochondrial fission. However, the severity of these

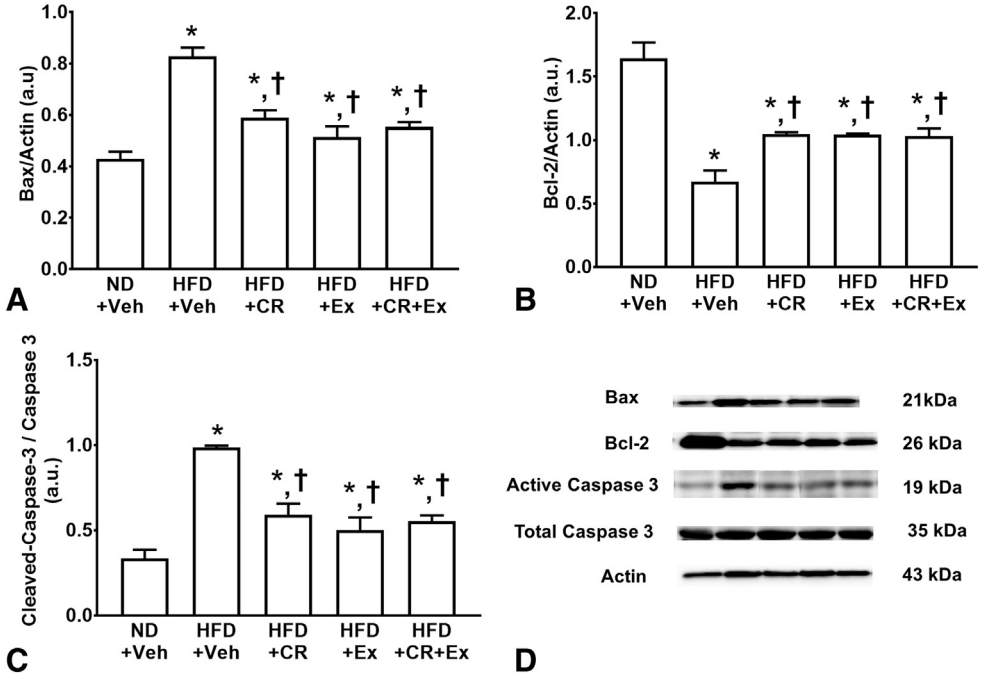

Fig. 6. Effects of calorie restriction and exercise on cardiac apoptosis in obese-insulin resistant rats. (A) Bax; (B) Bcl-2; (C) Cleaved-Caspase3; (D) Representative images of western blotting band. *p < 0.05 vs NDV; †p < 0.05 vs HFD + Veh; ‡p < 0.05 vs HFD + CR. ND + Veh, normal-diet fed rats; HFD + Veh, high-fat-diet fed rats; HFD + CR, high-fat diet fed rats treated with calorie restricted diet; HFD + Ex, high-fat diet fed rats treated with exercise; HFD + CR + Ex, high-fat diet fed rats treated with combined treatments.

metabolic and cardiac adverse effects was decreased after the interventions of calorie restriction, exercise and a combination of the two therapies. The combined therapy exerted the greatest cardioprotection in obese-insulin resistant rats. A summary of these findings is shown in Table 3.

Long-term consumption of a HFD is known to cause obese-insulin resistance, which is characterized by body weight gain, visceral fat accumulation, hyperinsulinemia with euglycemia, and dyslipidemia [1,2]. Our results showed that plasma glucose level was no different between ND and HFD rats, whereas plasma insulin level was significantly increased in HFD rats. This result confirmed that these rats developed an insulin resistance after HFD consumption. Previous studies have shown that either calorie restriction or exercise alone significantly reduced insulin resistance in both animal and clinical studies [16,33–35]. In this current study, plasma insulin levels were decreased in HFD rats treated with calorie restriction or exercise alone. However, we have demonstrated that the combination of calorie restriction and exercise provided the greatest improvement in insulin sensitivity caused by HFD consumption. In addition, although either calorie restriction or exercise alone improved the detriment caused to metabolic disturbance in obese-insulin resistant rats, a combination of calorie restriction and exercise treatments led to the greatest improvement with regard to metabolic function. These findings suggest that a combination of both calorie restriction and exercise had the greatest efficacy by attenuating insulin resistance.

As regards the heart, the obese-insulin resistant condition has been shown to increase cardiac oxidative stress [2,22]. Oxidative stress is known to have a strong influence on cardiac sympathetic hyperactivity [36,37], leading to cardiac autonomic imbalance and an increase in BP. Our study demonstrated that cardiac sympathovagal imbalance occurred in rats consuming a HFD as indicated by an increase in the LF/HF ratio and BP. Our results showed that although either calorie restriction or exercise alone attenuated cardiac autonomic imbalance, the combined therapy exerted the greatest efficacy in improving cardiac autonomic balance. This could be due to the higher rate of reduction in insulin resistance and oxidative stress found in the rats undergoing the combined treatment regime. In addition, the beneficial effects of caloric restriction and exercise found in this study could be as a result of both cardiac-selective and autonomic nerve system mechanisms. Previous studies reported that both exercise and calorie restriction exerted cardioprotection due to ROS detoxification [38–40]. Cardioprotection exerted by both exercise and calorie restriction could be moderated through the autonomic nerve system via an improvement in heart rate variability [41,42].

It has been shown that obese-insulin resistant rats have increased systemic, cardiac, and mitochondrial oxidative stress [4,43], resulting in impaired LV function. In this study, treatment with either calorie restriction or exercise alone effectively improved LV function in HFD-induced insulin resistant rats. This cardioprotective effect could be due to their increased level of protection of cardiac mitochondrial function. An impairment in cardiac mitochondrial function, including increased cardiac mitochondrial ROS production, mitochondrial membrane depolarization, and mitochondrial swelling, was observed in obese-insulin resistant rats, and these deleterious effects were alleviated in both the rats undergoing calorie restriction and exercise as single therapies. However, the combined therapy led to the lowest mitochondrial ROS level, mitochondrial depolarization, and mitochondrial swelling, which may explain why the combined therapy regime provided the greatest improvement in LV function, compared to a monotherapy.

Intracellular Ca^{2+} dyshomeostasis has been proposed as being a crucial determinant of cardiac contractile dysfunction [7]. Previous studies have demonstrated disturbances in cardiac $[Ca^{2+}]$i regulation in obese-insulin resistant rats as indicated by an increase in $[Ca^{2+}]$i diastolic level, and a decrease in $[Ca^{2+}]$i transient amplitude and $[Ca^{2+}]$i transient decay rate [11]. This aggravation could be due to impairment of the expression of cardiac Ca^{2+} handling proteins in obese-insulin resistant rats. It has been shown that the expression of SERCA protein [7,8] and Ca^{2+} uptake activities were decreased [8] in obese rats. Therefore, the therapeutic approaches that can attenuate metabolic complications, cardiac $[Ca^{2+}]$i dyshomeostasis and cardiac mitochondrial dysfunction caused by obese-insulin resistance may lead to improved LV function. In this study, although either calorie restriction or exercise alone improved intracellular Ca^{2+} homeostasis, the greatest improvement was observed in the rats receiving the combined therapies. These could be due to the highest reduction in oxidative stress found in the rats in the combined therapy group. In addition, our results also demonstrated that expression of the proteins SERCA, NCX, and LTCC were increased and decreased PLB in all intervention groups. However, the greatest increase in Ca^{2+} regulatory protein expression was observed in the rats receiving the combined therapy.

It is known that obese-insulin resistance is associated with mitochondrial dynamic imbalance, including increased mitochondrial fission and reduced mitochondrial fusion, and cardiac cell apoptosis [44]. In this study, HFD rats showed increased levels of mitochondrial fission indicated by increased phosphorylated Drp1 at serine 616 and decreased cardiac mitochondrial fusion. All interventions effectively decreased mitochondrial fission and increased mitochondrial fusion, thus improving the mitochondrial dynamic balance which had been disturbed by the consumption of a HFD. However, the greatest decrease in mitochondrial fission was observed in the rats receiving the combined therapy. Mitochondria undergoing the fission process, concomitantly with Bax activation, could lead to increased Bax-induced Cyt *c* release from mitochondria, thus promoting caspase-3 activation and cardiomyocyte apoptosis [45,46]. Our results demonstrated that all intervention groups had decreased cardiac apoptosis when compared to the control group, indicated by decreased Bax and caspase-3 activation. Moreover, all intervention groups also showed increased anti-apoptotic Bcl-2 expression. A reduction in apoptotic associated proteins and mitochondrial fission in all intervention groups could be responsible for the improvement in cardiac function found in this study. In addition, the greatest decrease in mitochondrial fission observed in the rats receiving the combined therapies could also be responsible for the highest improvement in cardiac function. All of these findings could be responsible for the improvement in cardiac function. These are summarized in Fig. 7.

In conclusion, our findings indicated that the combination of calorie restriction and exercise exerted greater cardioprotection than either of the monotherapies through the improvement in cardiometabolic status, cardiac mitochondrial dynamics and cardiac $[Ca^{2+}]$i homeostasis in obese-insulin resistant rats. The clinical implications of our findings are that a combination of calorie restriction and exercise will exert greater cardioprotection than exercise alone or calorie restriction alone in obese patients.

Table 3
A summary of cardiometabolic impairment in the experimental groups.

Impairment	Groups				
	ND + Veh	HFD + Veh	HFD + CR	HFD + Ex	HFD + CR + Ex
Metabolic disturbance	↔	↑↑↑	↑↑	↑↑	↑
LV contractile dysfunction	↔	↑↑↑	↑↑	↑↑	↑
Cardiac autonomic imbalance	↔	↑↑↑	↑↑	↑↑	↑
Oxidative stress	↔	↑↑↑	↑↑	↑↑	↑
Cardiac calcium dysregulation	↔	↑↑↑	↑↑	↑↑	↑
Cardiac mitochondrial dysfunction	↔	↑↑↑	↑↑	↑↑	↑
Cardiac mitochondrial fission	↔	↑↑↑	↑↑	↑↑	↑

ND + Veh, normal-diet fed rats; HFD + Veh, high-fat-diet fed rats; HFD + CR, high-fat diet fed rats treated with calories restriction diet; HFD + Ex, high-fat diet fed rats treated with exercise; HFD + CR + Ex, high-fat diet fed rats treated with combine treatment; LV, left ventricular.

4.1. Limitations

We did not measure the contractile function in the cardiomyocytes in this study. However, previous studies reported that obese rats had

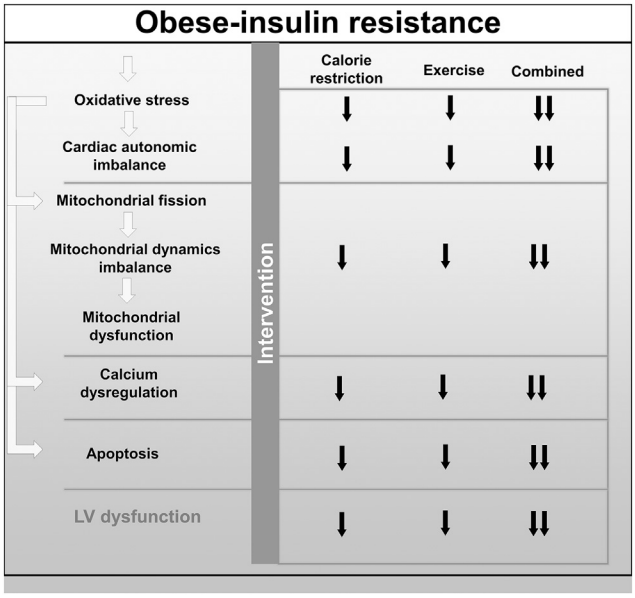

Fig. 7. Diagram summarizing the effects of calorie restriction, exercise and combined therapy on cardiometabolic function in obese-insulin resistant rats.

impaired contractile function [47,48]. In addition, we determined the adiposity in our rats by weighing the abdominal fat pads. We did not measure the total body fat. However, the abdominal fat pads made up the majority of fat tissue in the rats, therefore, could be assumed as equivalent and comparative total body fat accumulation.

Author contributions

SCC and NC designed the experiments. SP, SCC and NC wrote the paper. SP, WM, DM, WS, TJ, SK and WP performed the experiments. SP analyzed the data. All authors approved the final version of the paper.

Funding

This work was supported by the Thailand Research Fund grants RSA6180056 (SP), RSA6180071 (WP), and RTA6080003 (SCC); the NSTDA Research Chair grant from the National Science and Technology Development Agency Thailand (NC), and the Chiang Mai University Center of Excellence Award (NC).

Declaration of interest

None.

References

[1] Pratchayasakul W, Kerdphoo S, Petsophonsakul P, Pongchaidecha A, Chattipakorn N, Chattipakorn SC. Effects of high-fat diet on insulin receptor function in rat hippocampus and the level of neuronal corticosterone. Life Sci 2011;88:619–27. https://doi.org/10.1016/j.lfs.2011.02.003.

[2] Apaijai N, Pintana H, Chattipakorn SC, Chattipakorn N. Cardioprotective effects of metformin and vildagliptin in adult rats with insulin resistance induced by a high-fat diet. Endocrinology 2012;153:3878–85. https://doi.org/10.1210/en.2012-1262.

[3] Apaijai N, Pintana H, Chattipakorn SC, Chattipakorn N. Effects of vildagliptin versus sitagliptin, on cardiac function, heart rate variability and mitochondrial function in obese insulin-resistant rats. Br J Pharmacol 2013;169:1048–57. https://doi.org/10.1111/bph.12176.

[4] Sivasinprasasn S, Sa-Nguanmoo P, Pratchayasakul W, Kumfu S, Chattipakorn SC, Chattipakorn N. Obese-insulin resistance accelerates and aggravates cardiometabolic disorders and cardiac mitochondrial dysfunction in estrogen-deprived female rats. Age (Dordr) 2015;37(28). https://doi.org/10.1007/s11357-015-9766-0.

[5] Ginsberg HN. Insulin resistance and cardiovascular disease. J Clin Invest 2000;106:453–8. https://doi.org/10.1172/JCI10762.

[6] Maas AH, van der Schouw YT, Regitz-Zagrosek V, Swahn E, Appelman YE, Pasterkamp G, et al. Red alert for women's heart: the urgent need for more research and knowledge on cardiovascular disease in women: proceedings of the workshop held in Brussels on gender differences in cardiovascular disease, 29 September 2010. Eur Heart J 2011;32:1362–8. https://doi.org/10.1093/eurheartj/ehr048.

[7] Dunay GA, Paragi P, Sara L, Acs N, Balazs B, Agoston V, et al. Depressed calcium cycling contributes to lower ischemia tolerance in hearts of estrogen-deficient rats. Menopause 2015;22:773–82. https://doi.org/10.1097/GME.0000000000000377.

[8] Bupha-Intr T, Wattanapermpool J. Regulatory role of ovarian sex hormones in calcium uptake activity of cardiac sarcoplasmic reticulum. Am J Physiol Heart Circ Physiol 2006;291:H1101–8. https://doi.org/10.1152/ajpheart.00660.2005.

[9] Turdi S, Hu N, Ren J. Tauroursodeoxycholic acid mitigates high fat diet-induced cardiomyocyte contractile and intracellular Ca2+ anomalies. PLoS One 2013;8:e63615. https://doi.org/10.1371/journal.pone.0063615.

[10] Ceylan-Isik AF, Sreejayan N, Ren J. Endoplasmic reticulum chaperon tauroursodeoxycholic acid alleviates obesity-induced myocardial contractile dysfunction. J Mol Cell Cardiol 2011;50:107–16. https://doi.org/10.1016/j.yjmcc.2010.10.023.

[11] Apaijai N, Chinda K, Palee S, Chattipakorn S, Chattipakorn N. Combined vildagliptin and metformin exert better cardioprotection than monotherapy against ischemia-reperfusion injury in obese-insulin resistant rats. PLoS One 2014;9:e102374. https://doi.org/10.1371/journal.pone.0102374.

[12] Szabadkai G, Simoni AM, Bianchi K, De Stefani D, Leo S, Wieckowski MR, et al. Mitochondrial dynamics and Ca2+ signaling. Biochim Biophys Acta, Mol Cell Res 2006;1763:442–9. https://doi.org/10.1016/j.bbamcr.2006.04.002.

[13] Nicholls DG. Mitochondria and calcium signaling. Cell Calcium 2005;38:311–7. https://doi.org/10.1016/j.ceca.2005.06.011.

[14] Szabadkai G, Simoni AM, Chami M, Wieckowski MR, Youle RJ, Rizzuto R. Drp-1-dependent division of the mitochondrial network blocks intraorganellar Ca2+ waves and protects against Ca2+−mediated apoptosis. Mol Cell 2004;16:59–68. https://doi.org/10.1016/j.molcel.2004.09.026.

[15] Heo JW, No MH, Park DH, Kang JH, Seo DY, Han J, et al. Effects of exercise on obesity-induced mitochondrial dysfunction in skeletal muscle. Korean J Physiol Pharmacol 2017;21:567–77. https://doi.org/10.4196/kjpp.2017.21.6.567.

[16] Wycherley TP, Brinkworth GD, Noakes M, Buckley JD, Clifton PM. Effect of caloric restriction with and without exercise training on oxidative stress and endothelial function in obese subjects with type 2 diabetes. Diabetes Obes Metab 2008;10:1062–73. https://doi.org/10.1111/j.1463-1326.2008.00863.x.

[17] Foster-Schubert KE, Alfano CM, Duggan CR, Xiao L, Campbell KL, Kong A, et al. Effect of diet and exercise, alone or combined, on weight and body composition in overweight-to-obese post-menopausal women. Obesity (Silver Spring) 2012;20:1628–38. https://doi.org/10.1038/oby.2011.76.

[18] Pratchayasakul W, Chattipakorn N, Chattipakorn SC. Effects of estrogen in preventing neuronal insulin resistance in hippocampus of obese rats are different between genders. Life Sci 2011;89:702–7. https://doi.org/10.1016/j.lfs.2011.08.011.

[19] Pipatpiboon N, Pintana H, Pratchayasakul W, Chattipakorn N, Chattipakorn SC. DPP4-inhibitor improves neuronal insulin receptor function, brain mitochondrial function and cognitive function in rats with insulin resistance induced by high-fat diet consumption. Eur J Neurosci 2013;37:839–49. https://doi.org/10.1111/ejn.12088.

[20] Zoth N, Weigt C, Laudenbach-Leschowski U, Diel P. Physical activity and estrogen treatment reduce visceral body fat and serum levels of leptin in an additive manner in a diet induced animal model of obesity. J Steroid Biochem Mol Biol 2010;122:100–5. https://doi.org/10.1016/j.jsbmb.2010.03.029.

[21] Posa A, Szabo R, Kupai K, Csonka A, Szalai Z, Veszelka M, et al. Exercise training and calorie restriction influence the metabolic parameters in ovariectomized female rats. Oxidative Med Cell Longev 2015;2015:787063. https://doi.org/10.1155/2015/787063.

[22] Tunapong W, Apaijai N, Yasom S, Tanajak P, Wanchai K, Chunchai T, et al. Chronic treatment with prebiotics, probiotics and synbiotics attenuated cardiac dysfunction by improving cardiac mitochondrial dysfunction in male obese insulin-resistant rats. Eur J Nutr 2017. https://doi.org/10.1007/s00394-017-1482-3.

[23] Palee S, Weerateerangkul P, Chinda K, Chattipakorn SC, Chattipakorn N. Mechanisms responsible for beneficial and adverse effects of rosiglitazone in a rat model of acute cardiac ischaemia-reperfusion. Exp Physiol 2013;98:1028–37. https://doi.org/10.1113/expphysiol.2012.070433.

[24] Ding M, Dong Q, Liu Z, Liu Z, Qu Y, Li X, et al. Inhibition of dynamin-related protein 1 protects against myocardial ischemia–reperfusion injury in diabetic mice. Cardiovasc Diabetol 2017;16. https://doi.org/10.1186/s12933-017-0501-2.

[25] Jheng HF, Tsai PJ, Guo SM, Kuo LH, Chang CS, Su IJ, et al. Mitochondrial fission contributes to mitochondrial dysfunction and insulin resistance in skeletal muscle. Mol Cell Biol 2012;32:309–19. https://doi.org/10.1128/mcb.05603-11.

[26] Ruiz-Meana M, Garcia-Dorado D, Miro-Casas E, Abellan A, Soler-Soler J. Mitochondrial Ca2+ uptake during simulated ischemia does not affect permeability transition pore opening upon simulated reperfusion. Cardiovasc Res 2006;71:715–24. https://doi.org/10.1016/j.cardiores.2006.06.019.

[27] Morikawa D, Kanematsu K, Shibata T, Haseda K, Umeda N, Ohta Y. Detection of swelling of single isolated mitochondrion with optical microscopy. Biomed Opt Express 2014;5:848–57. https://doi.org/10.1364/BOE.5.000848.

[28] Di Lisa F, Menabo R, Canton M, Barile M, Bernardi P. Opening of the mitochondrial permeability transition pore causes depletion of mitochondrial and cytosolic NAD + and is a causative event in the death of myocytes in postischemic reperfusion of the heart. J Biol Chem 2001;276:2571–5. https://doi.org/10.1074/jbc.M006825200.

[29] Scorrano L, Petronilli V, Di Lisa F, Bernardi P. Commitment to apoptosis by GD3 ganglioside depends on opening of the mitochondrial permeability transition pore. J Biol Chem 1999;274:22581–5.

[30] Palee S, Apaijai N, Shinlapawittayatorn K, Chattipakorn SC, Chattipakorn N. Acetylcholine attenuates hydrogen peroxide-induced intracellular calcium dyshomeostasis through both muscarinic and nicotinic receptors in cardiomyocytes. Cell Physiol Biochem 2016;39:341–9. https://doi.org/10.1159/000445628.
[31] Waldeck-Weiermair M, Bischof H, Blass S, Deak AT, Klec C, Graier T, et al. Generation of red-shifted Cameleons for imaging Ca(2)(+) dynamics of the endoplasmic reticulum. Sensors (Basel) 2015;15:13052–68. https://doi.org/10.3390/s150613052.
[32] Bootman MD, Rietdorf K, Collins T, Walker S, Sanderson M. Ca2+-sensitive fluorescent dyes and intracellular Ca2+ imaging. Cold Spring Harb Protoc 2013;2013:83–99. https://doi.org/10.1101/pdb.top066050.
[33] Park S, Komatsu T, Hayashi H, Yamaza H, Chiba T, Higami Y, et al. Calorie restriction initiated at middle age improved glucose tolerance without affecting age-related impairments of insulin signaling in rat skeletal muscle. Exp Gerontol 2006;41:837–45. https://doi.org/10.1016/j.exger.2006.06.055.
[34] Sharma N, Bhat AD, Kassa AD, Xiao Y, Arias EB, Cartee GD. Improved insulin sensitivity with calorie restriction does not require reduced JNK1/2, p38, or ERK1/2 phosphorylation in skeletal muscle of 9-month-old rats. Am J Phys Regul Integr Comp Phys 2012;302:R126–36. https://doi.org/10.1152/ajpregu.00372.2011.
[35] Yu J, Zheng J, Liu XF, Feng ZL, Zhang XP, Cao LL, et al. Exercise improved lipid metabolism and insulin sensitivity in rats fed a high-fat diet by regulating glucose transporter 4 (GLUT4) and musclin expression. Braz J Med Biol Res 2016;49:e5129. https://doi.org/10.1590/1414-431x20165129.
[36] Campese VM, Shaohua Y, Huiquin Z. Oxidative stress mediates angiotensin II-dependent stimulation of sympathetic nerve activity. Hypertension 2005;46:533–9. https://doi.org/10.1161/01.hyp.0000179088.57586.26.
[37] Ye S, Zhong H, Yanamadala S, Campese VM. Oxidative stress mediates the stimulation of sympathetic nerve activity in the phenol renal injury model of hypertension. Hypertension 2006;48:309–15. https://doi.org/10.1161/01.HYP.0000231307.69761.2e.
[38] Ungvari Z, Parrado-Fernandez C, Csiszar A, de Cabo R. Mechanisms underlying caloric restriction and lifespan regulation: implications for vascular aging. Circ Res 2008;102:519–28. https://doi.org/10.1161/circresaha.107.168369.
[39] Cruzen C, Colman RJ. Effects of caloric restriction on cardiovascular aging in non-human primates and humans. Clin Geriatr Med 2009;25:733–43. https://doi.org/10.1016/j.cger.2009.07.001.
[40] Ahmet I, Tae HJ, de Cabo R, Lakatta EG, Talan MI. Effects of calorie restriction on cardioprotection and cardiovascular health. J Mol Cell Cardiol 2011;51:263–71. https://doi.org/10.1016/j.yjmcc.2011.04.015.
[41] Ito H, Ohshima A, Tsuzuki M, Ohto N, Yanagawa M, Maruyama T, et al. Effects of increased physical activity and mild calorie restriction on heart rate variability in obese women. Jpn Heart J 2001;42:459–69.
[42] Nicoll R, Henein MY. Caloric restriction and its effect on blood pressure, heart rate variability and arterial stiffness and dilatation: a review of the evidence. Int J Mol Sci 2018;19. https://doi.org/10.3390/ijms19030751.
[43] Sivasinprasasn S, Tanajak P, Pongkan W, Pratchayasakul W, Chattipakorn SC, Chattipakorn N. DPP-4 inhibitor and estrogen share similar efficacy against cardiac ischemic-reperfusion injury in obese-insulin resistant and estrogen-deprived female rats. Sci Rep 2017;7(44306). https://doi.org/10.1038/srep44306.
[44] Ilkun O, Boudina S. Cardiac dysfunction and oxidative stress in the metabolic syndrome: an update on antioxidant therapies. Curr Pharm Des 2013;19:4806–17.
[45] Martinou JC, Youle RJ. Which came first, the cytochrome c release or the mitochondrial fission? Cell Death Differ 2006;13:1291–5. https://doi.org/10.1038/sj.cdd.4401985.
[46] Renault TT, Floros KV, Chipuk JE. BAK/BAX activation and cytochrome c release assays using isolated mitochondria. Methods 2013;61:146–55. https://doi.org/10.1016/j.ymeth.2013.03.030.
[47] Dong F, Zhang X, Yang X, Esberg LB, Yang H, Zhang Z, et al. Impaired cardiac contractile function in ventricular myocytes from leptin-deficient ob/ob obese mice. J Endocrinol 2006;188:25–36. https://doi.org/10.1677/joe.1.06241.
[48] Essop MF, Anna Chan WY, Valle A, Garcia-Palmer FJ, Du Toit EF. Impaired contractile function and mitochondrial respiratory capacity in response to oxygen deprivation in a rat model of pre-diabetes. Acta Physiol (Oxford) 2009;197:289–96. https://doi.org/10.1111/j.1748-1716.2009.02024.x.