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Basic nutritional investigation

Combined exercise and calorie restriction therapies restore contractile and mitochondrial functions in skeletal muscle of obese–insulin resistant rats

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ABSTRACT

Objectives: A combined exercise training and calorie-restriction program is the mainstream treatment of obesity. However, the effect of the dual-action program on mitochondrial function in skeletal muscles has not yet been clarified. The aim of this study was to determine if the combined program, rather than a single program, restored both lost muscle activity and mitochondrial function in obesity.
Methods: The study included 30 female Wistar rats. Six rats fed a normal diet for 27 wk were used as the control group. The remaining 24 rats were fed a high-fat diet (HFD) for 27 wk. At week 20, the HFD rats were divided into the following four groups: sedentary lifestyle, endurance exercise five times per week, 60% of calorie restriction (CR) per day, and combined exercise training and CR. All conditions were maintained for 7 wk.
Results: We found that HFD-fed rats without therapy developed obese insulin resistance (IR) and impaired function of skeletal muscles. Skeletal muscles of the HFD-fed rats without therapy also exhibited early fatigability; impaired mitochondrial function, as indicated by increased reactive oxygen species production, membrane depolarization, and swelling; reduced mitochondrial dynamics as indicated by increased phosphorylation of *DRP1* and decreased *MFN2* expression; diminished mitochondrial biogenesis, as shown by decreased *PGC1α* and *CPT1* expression; and increased apoptosis. Both exercise and CR in HFD-fed rats equally attenuated the impairment of muscle functions. However, combined therapies in HFD-fed rats restored functions of skeletal muscles.
Conclusions: These findings reinforce the synergistic beneficial effects of combined exercise and CR on skeletal muscles of HFD-fed rats.

Introduction

As the prevalence of overweight and obesity, particularly in women, has increased up to 40.4% in the United States [1], the interest in the effect of obesity on skeletal muscles has been growing. Skeletal muscle takes in ≤80% of postprandial insulin-stimulated glucose [2]. Therefore, the roles of skeletal muscle in the cause and effect of obese–insulin resistant conditions need to be considered. In skeletal muscle, the insulin-resistant condition can be indicated by a disruption of protein phosphorylation in insulin signaling pathways. This disruption can include decreased tyrosine phosphorylation of insulin receptors and threonine phosphorylation protein kinase B (Akt), resulting in reduced glucose uptake and utilization [3,4]. An insulin resistant condition in skeletal muscles has been associated with mitochondrial dysfunction [5,6], imbalance of mitochondrial dynamics [7,8], and increased cell apoptosis [9]. These impairments can lead to abnormal skeletal muscle contractile function, indicated by increased muscle

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fatigability, the ability to withstand fatigue being one of the most important functions of skeletal muscle [10,11].

An exercise training (ET) program and calorie restriction (CR) are the conventional interventions for obesity. Previous results from both animal and clinical studies demonstrated that ET activated insulin signaling [12,13] and enhanced mitochondrial function of the skeletal muscle [5,6,12], leading to an increase in muscle contractile function [12,14]. In addition, a previous study reported that ET increased MFN1 protein expression [15], indicating the induction of mitochondrial fusion, and a decrease in DRP1 protein expression, indicating a reduction in mitochondrial fission [16] in the rat model. Although ET could ideally attenuate apoptosis in skeletal muscles, a previous study investigating the impact of ET on the skeletal muscle of rats failed to demonstrate any significant changes in the proapoptotic protein Bax and antiapoptotic protein Bcl2 [17]. Several previous studies showed the beneficial effects of CR in the rat model regarding an increase in insulin sensitivity and function of skeletal muscles [18], increased insulin signaling [19,20], and decreased apoptosis [21,22]. CR has been shown to have no effect on mitochondrial content and mitochondrial oxidative phosphorylation enzymes [23]. In addition, the effects of CR on mitochondrial dynamics in skeletal muscle of rats in the obese condition have not been investigated.

Several clinical studies found that the combination of ET with a CR program improved mitochondrial oxidative phosphorylation enzymes, oxidative capacity, insulin signaling, and contractile function of skeletal muscle in obese patients [23,24]. However, the combined effects of an ET with a CR program on mitochondrial dynamics and muscle fatigability in an obese–insulin resistant condition, in addition to the comparative effect of an ET, CR, and a combined ET/CR program on the function of skeletal muscles and mitochondria, have not yet been investigated to our knowledge. Therefore, the hypotheses of the present study were as follows:

1. In an obese condition, cell apoptosis increased and skeletal muscles developed insulin resistance (IR), contractile dysfunction, and impaired mitochondrial function, mitochondrial dynamics, and biogenesis.
2. A combination of ET and CR therapy in an obese–insulin resistant condition provides greater efficacy in improving the deleterious effects on skeletal muscles than ET or CR alone.

Methods

Study protocols

All experiments were conducted using a protocol approved by the Faculty of Medicine, Chiang Mai University Institutional Animal Care and Use Committee, in compliance with National Institutes of Health guidelines. Female Wistar rats (N = 30, body weight 200–220 g) were obtained from the National Laboratory Animal Center, Thailand, and were randomly assigned to be fed either a normal (ND) or high-fat diet (HFD). The ND group (n = 6) was given standard laboratory chow, which had an energy content of 4.02 kcal/g, with 19.77% of the total energy (%E) of the food being from fat (Mouse Feed Food No. 082, C.P. Company, Bangkok, Thailand). The HFD group (n = 24) was fed a high-fat diet with an energy content of 5.35 kcal/g and contained fat mostly from lard (59.28%E) [25]. Rats in both groups continued consuming their assigned diet for 27 wk. At week 21, ND-fed rats continued ingesting ND without any intervention until week 21. At week 21, HFD-fed rats were subdivided into four subgroups (n = 6 rats per subgroup). Each subgroup was designated as either sedentary living, exercise training, calorie restriction, or combined exercise and calorie restriction. The regimens continued for 7 wk. Blood samples were collected to determine metabolic parameters at the end of week 26. At the end of week 27, an oral glucose tolerance test (OGTT) was performed on each rat. The results of the test were calculated by collecting blood from the tail veins. The morning after the OGTT, the animals were deeply anesthetized with xylazine (0.15 mL/kg) and Zoletil (50 mg/kg). In situ muscle contraction studies were carried out using the gastrocnemius muscles measuring the time taken to fatigue of tetanic contraction. After this, insulin was injected intramuscularly

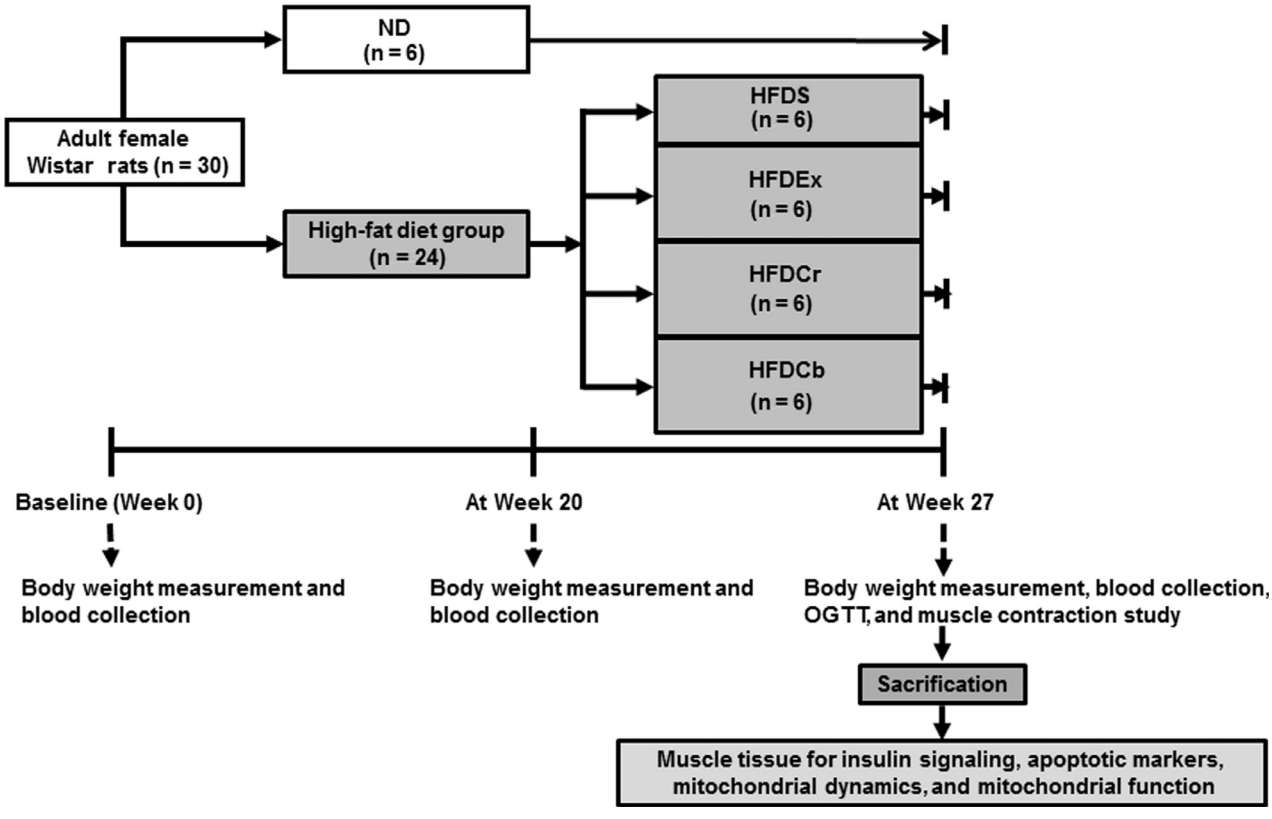

Fig. 1. Schematic diagram of the experimental design.

10 min before the rats were sacrificed and the vastus lateralis muscles were rapidly removed for the determination of insulin signaling, mitochondrial function, mitochondrial biogenesis, mitochondrial dynamics, oxidative stress, and apoptosis. A summary of the protocol is shown in Figure 1.

Calorie-restriction diet

CR provided 60% of the energy of the mean of basal freely available in the form of normal diet chow for 6 wk [26]. Body weight was monitored every week to prevent excessive body weight loss (≤3% per week).

Exercise training protocol

ET, in terms of endurance training, was performed using a motor-driven rodent treadmill 5 d/wk over a 6-wk period. The intensity was increased progressively from 10 min once a day at 22 m/min, with a 5% upgrade per week, up to 15 min twice a day at 25 m/min [27].

Plasma analysis

Plasma glucose, triacyliglyceride (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL), and total cholesterol (TC) concentrations were determined using colorimetric assay with a commercially available kit (Biotech, Bangkok, Thailand). Plasma insulin levels were measured using the Sandwich enzyme-linked immunosorbent assay protocol (LINCO Research, St. Charles, MO, USA).

Determination of IR: OGTT and HOMA

IR was assessed by the OGTT [28,29] and the homeostasis model assessment (HOMA) [30]. OGTT was performed after fasting overnight (12 h). Rats were given a bolus of glucose (2 g/kg body weight) via gavage feeding, and blood samples were collected from the tail veins at 0, 30, 60, 90, and 120 min after glucose administration. HOMA was calculated from fasting plasma insulin and fasting plasma glucose concentration.

Muscle homogenate preparation

Muscle strips were homogenized in an ice-cold lysis buffer: 50 mM HEPES (pH 7.4), 150 mM sodium chloride, 1 mM calcium dichloride, 1mM $MgCl_2$, 2 mM EDTA, 10 mM sodium fluoride, 20 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 10% glycerol, 1% Triton X-100, 2 mM Na_3VO_4, 10 μg/mL aprotinin and leupeptin, and 2 mM phenylmethylsulfonyl fluoride. After 20-min incubation on ice, the homogenates were centrifuged at 13 000*g* for 20 min at 4°C. Aliquots of the supernatant were frozen at −80°C, and a portion of these homogenates were used for the determination of total protein (bicinchoninic acid [BCA] method, Sigma Chemical, St. Louis, MO, USA) [31].

Immunoblotting

Level of expression of the proteins (antibody), including IR (SC-711, Santa Cruz Biotechnology, Santa Cruz, NM, USA); $Tyr^{1162/1163}$pIR (SC-25103, Santa Cruz Biotechnology); Akt (#9272, Cell Signaling Technology, Danvers, MA, USA); Thr^{308}pAkt (#9271, Cell Signaling Technology); Bax (ab 182733, Abcam, Cambridge, UK); Bcl2 (ab 196495, Abcam); *PPARδ* (PA5-29678, Thermo Fisher Scientific, Waltham, MA, USA); *PGC1α* (ab 154481, Abcam); *CPT1* (SC-393070, Santa Cruz Biotechnology); *MFN2* (#9482, Cell Signaling Technology); DRP1 (#5391, Cell Signaling Technology), and Ser^{616}p*DRP1* (#3455, Cell Signaling Technology), were determined using immunoblotting. The proteins were separated by electrophoresis on 10% polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA, USA) sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred into polyvinylidene fluoride membranes. Band intensity was quantified by Scion Image program, and the results are shown as average signal intensity (arbitrary units) [32].

Skeletal muscle mitochondrial isolation

We kept 68 to 150 mg of the extracted skeletal muscle in 1 mL isolation media. The muscle was cut into small pieces on ice. The skeletal muscle tissues were incubated in 1 mL of solution mixed in an isolation medium and 0.2 mg of Nagrase type XXVII for 2 min. The tissue was finely minced and homogenized for three sets. The final mitochondrial pellet was resuspended in 4 μL/g of muscle respiratory buffer to enable the measurement of mitochondrial reactive oxygen species (ROS), membrane potential, and mitochondrial swelling [33]. The volume of the added buffer was 4 μL/mg of skeletal muscle tissue. Concentrations of mitochondrial proteins were determined by the BCA assay [34].

ROS measurement in isolated skeletal muscle mitochondria

ROS in isolated skeletal muscle mitochondria was measured by fluorescent probe and using dichloro-hydro-fluorescein diacetate. Increase in fluorescent intensity represented an increase in skeletal muscle ROS [35].

Mitochondrial membrane potential measurement in isolated muscle mitochondria

Mitochondria membrane potential ($\Delta\Psi m$) change was measured using the dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl benzimidazole carbocyanine iodide (JC-1). JC-1 monomer (green) fluorescent was excited at the wavelength of 485 nm and detected at the emission wavelength of 590 nm, and the JC-1 aggregated form (red) fluorescent was excited at the wavelength of 485 nm and detected at the emission wavelength of 530 nm. The changes in mitochondrial membrane potential were calculated from the ratio of red to green fluorescence. Mitochondrial depolarization was represented as a decrease in the red-to-green ratio [36].

Isolated skeletal muscle mitochondrial swelling determination

Isolated skeletal mitochondria were examined to determine the changes in the absorbance of the suspension at 540 nm by using a microplate reader (Bio-Tek Instruments, Inc. Winooski, VT, USA). Mitochondria (0.4 mg/mL) were incubated in 2 mL respiration buffer. Mitochondrial swelling was represented as decrease in absorbance value [37].

In situ skeletal muscle contraction study

The left gastrocnemius muscle was surgically isolated, with the origin intact. The distal part of the sciatic nerve was inserted into a cuff lined with stainless steel stimulating wires. The Achilles tendon was attached to the force transducer. Tetanic contraction, using 50 Hz until 50% fatigue of the muscle, was performed. Time to fatigue of tetanic contraction was measured. At the end of the experiment, tendon-free muscle weight was determined, and force was normalized to muscle weight in grams [38,39].

Statistical analysis

Categorical variables were described using percentage of frequency. Normally distributed numerical variables were presented using arithmetic mean and SD. Differences of parameters among groups were compared using a two-way analysis of variance (ANOVA), followed by a post hoc Turkey test. Statistical analyses were performed using SPSS version 22 for Windows (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Results

HFD consumption-induced obese IR. ET and CR improved resultant metabolic function, and combined therapies restored metabolic parameters.

Rats fed an HFD for 27 wk demonstrated metabolic disturbance, as indicated by obesity (increased body weight and visceral fat weight), peripheral IR (as shown by hyperinsulinemia with euglycemia, increased HOMA index, and impaired OGTT), and dyslipidemia (increased plasma cholesterol and plasma LDL levels and decreased plasma HDL level; Table 1). A two-way ANOVA, determining the effects of two types of diet (CR or no CR) and physical activity (ET or no ET), revealed a significant effect of ET and CR on body weight ($F[3, 72] = 32.68$, $P < 0.001$; $F[1, 72] = 83.65$, $P < 0.001$), visceral fat ($F[3, 59] = 35.18$, $P < 0.001$; $F[1, 59] = 185.6$, $P < 0.001$), area under the curve of OGTT ($F[3, 70] = 3.222$, $P = 0.003$; $F[1, 70] = 44.51$, $P < 0.001$), plasma insulin ($F[3, 44] = 10.65$, $P < 0.001$; $F[1, 44] = 78.35$, $P < 0.001$), and HOMA index ($F[2, 44] = 34.65$, $P < 0.001$; $F[1, 44] = 72.34$, $P < 0.001$; ET and CR, respectively). The post hoc analyses revealed that ET and CR as individual regimens led to equally improved metabolic parameters, as indicated by decreased visceral fat, glucose intolerance from OGTT, plasma insulin, and HOMA index when compared with those of HFD-fed rats experiencing sedentary living (Table 1). A combination of ET with CR showed the greatest benefits in the improvement of metabolic function when compared with either monotherapy (Table 1). Focusing on plasma lipid profiles, a two-

Table 1
Effects of exercise, CT, and combined exercise and CR on metabolic parameters in HFD-fed rats

Metabolic parameters	Groups				
	ND	HFDS	HFSEx	HFDCr	HFSCb
Body weight (g)	281.15 ± 3.50	352.00 ± 4.16*	322.86 ± 6.53*,†	295.00 ± 8.16*,†	269.44 ± 3.27†,‡
Visceral fat (g)	9.31 ± 0.69	28.51 ± 1.12*	20.80 ± 1.83*,†	15.14 ± 1.06*,†	10.14 ± 0.54†,‡
Plasma glucose (mg/dL)	133.25 ± 3.03	139.66 ± 4.48	135.73 ± 7.12	125.41 ± 10.97	131.25 ± 7.06
Plasma insulin (ng/mL)	1.50 ± 0.17	4.76 ± 0.36*	3.34 ± 0.20*,†	3.01 ± 0.49*,†	1.75 ± 0.19†,‡
Plasma glucose AUC from OGTT (AUCg) (mg/dL × min × 10^4)	1.92 ± 0.07	2.57 ± 0.04*	2.31 ± 0.09*,†	2.26 ± 0.09*,†	1.97 ± 0.04†,‡
HOMA index	5 ± 2.4	16 ± 2.1*	11 ± 1.1*,†	9 ± 3.4*,†	6 ± 0.8†,‡
Plasma total cholesterol (mg/dL)	51.65 ± 6.79	98.55 ± 4.53*	70.33 ± 5.77*,†	63.46 ± 2.98*,†	56.61 ± 5.70†,‡
Plasma triacylglyceride (mg/dL)	61.30 ± 7.39	70.38 ± 5.65	66.21 ± 7.75	68.37 ± 8.45	63.07 ± 7.47
HDL cholesterol (mg/dL)	19.24 ± 1.49	22.64 ± 1.35	22.24 ± 0.94	22.64 ± 0.82	22.35 ± 0.81
LDL cholesterol (mg/dL)	19.29 ± 4.66	57.89 ± 4.66*	30.27 ± 6.18*,†	20.26 ± 3.56*,†	28.97 ± 8.89†,‡

AUC, area under the curve; CR, calorie restriction; ET, exercise training; HDL, high-density lipoprotein; HFDCr, high-fat diet with caloric restriction; HFDCb, high-fat diet with combined therapy; HFDEx, high-fat diet with exercise; HFDS, high-fat diet with sedentary living; HOMA, homeostatic model assessment; LDL, low-density lipoprotein; ND, normal diet; OGTT, oral glucose tolerance test.
*$P < 0.05$ compared with ND.
†$P < 0.05$ compared with HFDS.
‡$P < 0.05$ compared with HFDEx and HFDCr groups; n = 6 per group.

way ANOVA comparing the effects of the two types of diet (CR or no CR) and physical activity (ET or no ET) revealed a significant effect of ET and CR on TC (F[3, 48 = 7.05, $P < 0.001$; F[1, 48] = 70.57, $P < 0.001$) and LDL levels (F[3, 54] = 3.694, $P = 0.017$; F[1, 54] = 13.06, $P < 0.001$), but not TG (F[3, 70] = 0.051, $P = 0.985$; F[1, 70] = 0.340, $P = 0.562$) and HDL level (F[3, 50] = 0.012, $P = 0.994$; F[3, 50] = 0.329, $P = 0.804$; ET and CR, respectively). The post hoc analyses revealed that in the case of HFD-fed rats, all therapies led to significantly lower TC and LDL levels, but there was no difference in TG and HDL levels when compared with HFD-fed rats experiencing sedentary living (Table 1). These findings suggested the following:

1. HFD consumption caused an obese–insulin resistant condition.
2. ET and CR individually had similar effects in decreasing metabolic disturbance in obese–insulin resistant rats.
3. A combination of ET and CR demonstrated the greatest benefits on this improvement.

Enhanced early fatigability and decreased PPARδ protein expression in gastrocnemius muscles occurred in HFD-fed rats and were attenuated by ET and combined therapies

An in situ muscle contraction study of gastrocnemius muscles was used to determine muscle contractile dysfunction. HFD-fed rats in the sedentary-living group demonstrated a significant decrease in time-to-fatigue duration when compared with those of ND-fed rats. A two-way ANOVA comparing the effect of two types of diet (CR or no CR) and physical activity (ET or no ET) revealed a significant effect of ET (F[1, 14] = 44.88, $P < 0.001$), but not CR (F[1, 14] = 3.878, $P = 0.069$) on time-to-fatigue duration. The post hoc analyses also revealed that a fatigue-vulnerable property was absent in groups with ET and combined therapies. In addition, HFD-fed rats experiencing the combined therapies took a significantly longer time to 50% fatigue than those in the HFD-fed groups with ET (Fig. 2A, B). It is noticeable that the HFD-fed rats undergoing the combined therapies were the only group that had no difference in time to 50% fatigue when compared with ND-fed rats. This result suggested that only ET, not CR, attenuated skeletal muscle fatigability and combined therapies prevented fatigability in the obese–insulin resistant condition.

Muscle fatigability is mainly determined by the predominant type of muscle fiber in the muscle. *PPARδ* is part of the PPAR receptor family, which regulates muscle fiber type 1 construction. Corresponding with the results of the muscle contraction study, the results of the Western blot analysis also showed a significant decrease in *PPARδ* protein expression in sedentary-living, HFD-fed rats when compared with those of ND-fed rats (Fig. 2C). A two-way ANOVA, comparing two types of diet (CR or no CR) and physical activity (ET or no ET), revealed a significant effect of ET (F[1, 8] = 44.31, $P = 0.010$), but not CR (F[1, 8] = 1.087, $P = 0.109$) on *PPARδ* protein expression. The post hoc analyses also revealed that a decrease in PPARδ protein expression was attenuated in HFD-fed rats both with ET and with combined therapies. As expected, *PPARδ* protein expression of HFD-fed rats with combined therapies was significantly higher than those of HFD-fed rats with ET (Fig. 2C). It is significant that the combined therapies group was the only one that had no difference in *PPARδ* protein expression when compared with those of ND-fed rats.

HFD consumption induced IR in the skeletal muscle

Both ET and CR improved IR, and the combined ET and CR program restored insulin sensitivity in skeletal muscles.

We found that the $Tyr^{1162/1163}$pIR-to-IR ratio and Thr^{308}pAkt-to-Akt ratio, which represented the degree of insulin sensitivity, were significantly lower in HFD-fed rats experiencing sedentary living when compared with those of ND-fed rats. It has been proposed that ET and CR increase insulin sensitivity in skeletal muscles. A two-way ANOVA comparing two types of diet (CR or no CR) and physical activity (ET or no ET) revealed a significant effect of ET (F[1, 8] = 49.33, $P < 0.001$] and CR (F[1, 8] = 91.76, $P < 0.001$) on $Tyr^{1162/1163}$pIR-to-IR ratio. A two-way ANOVA also revealed a significant effect of ET (F [1, 8] = 8.735, $P = 0.018$) and CR (F [1, 8] = 8.444, $P = 0.020$) on the Thr^{308}pAkt-to-Akt ratio. The post hoc test also revealed that rats on either the ET or CR regimen had a significantly higher $Tyr^{1162/1163}$pIR-to-IR ratio and Thr^{308}pAkt-to-Akt ratio when compared with the sedentary-living, HFD-fed rat group. The combined therapy HFD-fed rat group had the highest $Tyr^{1162/1163}$pIR-to-IR ratio and Thr^{308}pAkt-to-Akt ratio out of all groups (Fig. 3A, B). However, the combined therapy group was the only group that had no difference in the $Tyr^{1162/1163}$pIR-tp-IR ratio and Thr^{308}pAkt-to-Akt ratio when compared with those of ND-fed rats. These findings suggest that both ET and CR in the obese–insulin resistant condition improved the insulin sensitivity in the skeletal

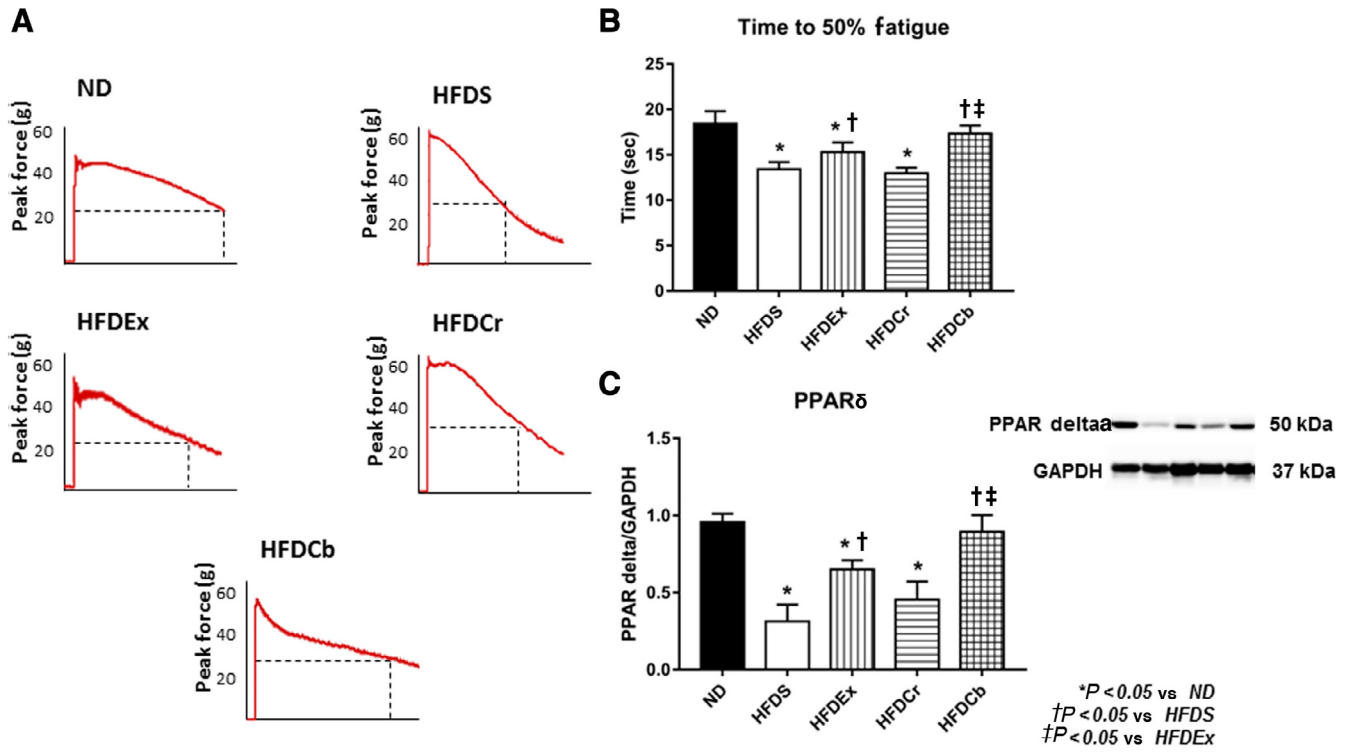

Fig. 2. Effects of high-fat diet consumption, exercise, CR, and combined exercise and CR on skeletal muscle fatigability and PPARδ protein expression. (**A**) Muscle contraction study tracing in each study group. (**B**) Comparison of time to fatigue parameter of muscle contraction studies between the study groups. (**C**) Comparison of PPARδ protein expression among the study groups. $^{*}P < 0.05$ compared with ND group. $^{\dagger}P < 0.05$ compared with HFDS group. $^{\ddagger}P < 0.05$ compared with HFDEx group; n = 6/group. CR, calorie restriction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HFD, high-fat diet; HFDCb, high-fat diet with combined therapy; HFDCr, high-fat diet with calorie restriction; HFDEx, high-fat diet with exercise; HFDS, high-fat diet with sedentary living; ND, normal diet; PPAR, peroxisome perforator-activated receptor.

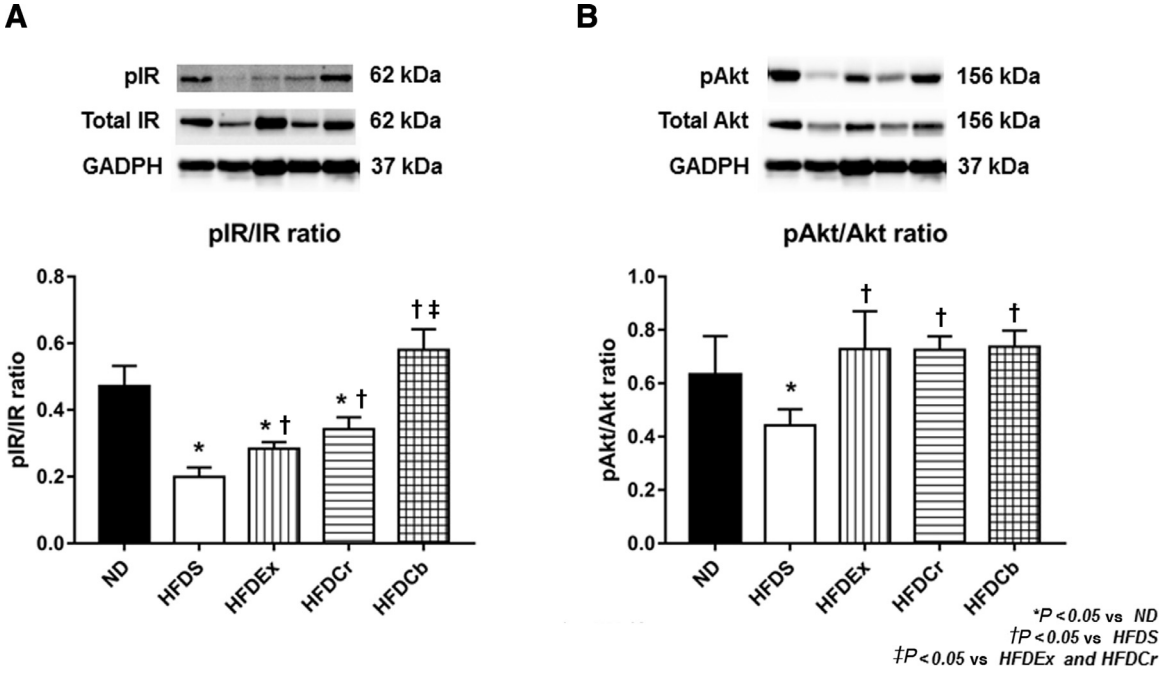

Fig. 3. Effects of high-fat diet consumption, exercise, CR, and combined exericise and CR program on skeletal insulin signaling. (**A**) Comparison of ratios of $Tyr^{1162/1163}$pIR to total insulin receptor among the study groups. (**B**) Comparison of ratios of Thr^{308}pAkt to total Akt among the study groups. $^{*}P < 0.05$ compared with ND group. $^{\dagger}P < 0.05$ compared with HFDS group. $^{\ddagger}P < 0.05$ compared with HFDEx and HFDCr group; n = 6 per group. Akt, protein kinase B; CR, calorie restriction; HFDCb, high-fat diet with combined therapy; HFDCr, high-fat diet with calorie restriction; HFDEx, high-fat diet with exercise; HFDS, high-fat diet with sedentary living; ND, normal diet; Thr^{308} pAkt, phosphorylated protein kinase B; $Tyr^{1162/1163}$pIR, tyrosine phosphorylated insulin receptor.

muscles and the combined ET and CR program in HFD-fed rats restored it.

Impaired skeletal muscle mitochondrial function was found in obese–insulin resistant rats and could be attenuated by the combined ET and CR program

We found that skeletal muscle was one of the end organs affected by an insulin-resistant condition, and the resulting effect is an impairment of mitochondrial function in the muscle. Mitochondrial ROS production was significantly higher in HFD-fed rats experiencing sedentary living when compared with those of ND-fed rats (Fig. 4A). This result was compatible with an increase in mitochondrial membrane potential change (Fig. 4B) and mitochondrial swelling (Fig. 4C). A two-way ANOVA comparing two types of diet (CR or no CR) and physical activity (ET or no ET) revealed a significant effect of ET ($F [1, 10] = 7.898$, $P < 0.020$) and CR ($F [1, 14] = 14.6$, $P = 0.003$) on mitochondrial ROS. It also revealed a significant effect of ET and CR on mitochondrial membrane potential change and mitochondrial swelling ($F [2, 14] = 76.5$, $P < 0.001$; $F [1, 14] = 10.24$, $P = 0.006$ and $F [1, 9] = 11.16$, $P < 0.009$; $F [1, 9] = 10.64$, $P < 0.010$ respectively). However, the post hoc analyses demonstrated that neither the ET or CR program alone attenuated any parameters pertinent to mitochondrial dysfunction, but the combined ET and CR program led to restoration of mitochondrial function, represented by a decrease in mitochondrial ROS production, membrane potential change, and mitochondrial swelling (Fig. 4A–C).

An imbalance in mitochondrial dynamics was observed in skeletal muscles of obese–insulin resistant rats, and this imbalance was restored by the combined ET and CR therapies.

Mitochondrial dynamics involve a reciprocal change in the morphology between a fission and fusion stage of mitochondria. The present study demonstrated an increase in the ratio of Ser^{616}p*DRP1* to total *DRP1* and a decrease in the *MFN2* protein expression of HFD-fed rats experiencing sedentary living when compared with that of ND-fed rats (Fig. 5A, B). A two-way ANOVA comparing two types of diet (CR or no CR) and physical activity (ET or no ET) revealed a significant effect of ET ($F [1, 8] = 64.73$, $P < 0.001$) and CR ($F [1, 8] = 36.1$, $P < 0.001$) on the ratio of Ser^{616}p*DRP1* to total *DRP1*. A two-way ANOVA also revealed a significant effect of ET ($F [1, 8] = 120$, $P < 0.001$) and CR ($F [1, 8] = 109.4$, $P < 0.001$) on *MFN2* protein expression. The post hoc analyses demonstrated that all interventions, that is, ET, CR, and the combined therapies, led to a decreased ratio of Ser^{616}p*DRP1* to total *DRP1* and increased MFN2 protein expression in HFD-fed rats. In addition, the ratio of Ser^{616}p*DRP1* to total *DRP1* and *MFN2* expression of HFD-fed rats undergoing the combined therapies did not differ significantly from those of ND-fed rats (Fig. 5A, B). These results indicated that ET and CR in rats with the obese–insulin resistant condition improved mitochondrial dynamics and the combined therapies restored it.

HFD-induced IR decreased mitochondrial biogenesis and fatty acid oxidation of skeletal muscles, which were attenuated by either ET or CR, and the two therapies combined restored them

Compared with ND-fed rats, sedentary-living, HFD-fed rats had significantly lower PGC1α and CPT1 protein expression, indicating a reduction in fatty acid oxidation metabolism in skeletal muscles (Fig. 6A, B). A two-way ANOVA, comparing two types of diet (CR or no CR) and physical activity (ET or no ET), revealed a significant effect of ET and CR on *PGC1α* and *CPT1* protein expression ($F [1, 8] = 33.18$, $P < 0.001$; $F [1, 8] = 23.8$, $P = 0.001$ and $F [1, 8] = 16.89$, $P = 0.003$; $F [1, 8] = 14.2$, $P < 0.006$, respectively). The post hoc analyses demonstrated that HFD-fed rats undergoing ET, CR, or combined therapies had significantly higher *PGC1α* and *CPT1* protein expression compared with sedentary-living, HFD-fed rats. In addition, the expression of *PGC1α* and *CPT1* in the combined therapies group was not significantly different from those of ND-fed rats (Fig. 6A, B). These results indicated that ET and CR in rats with the

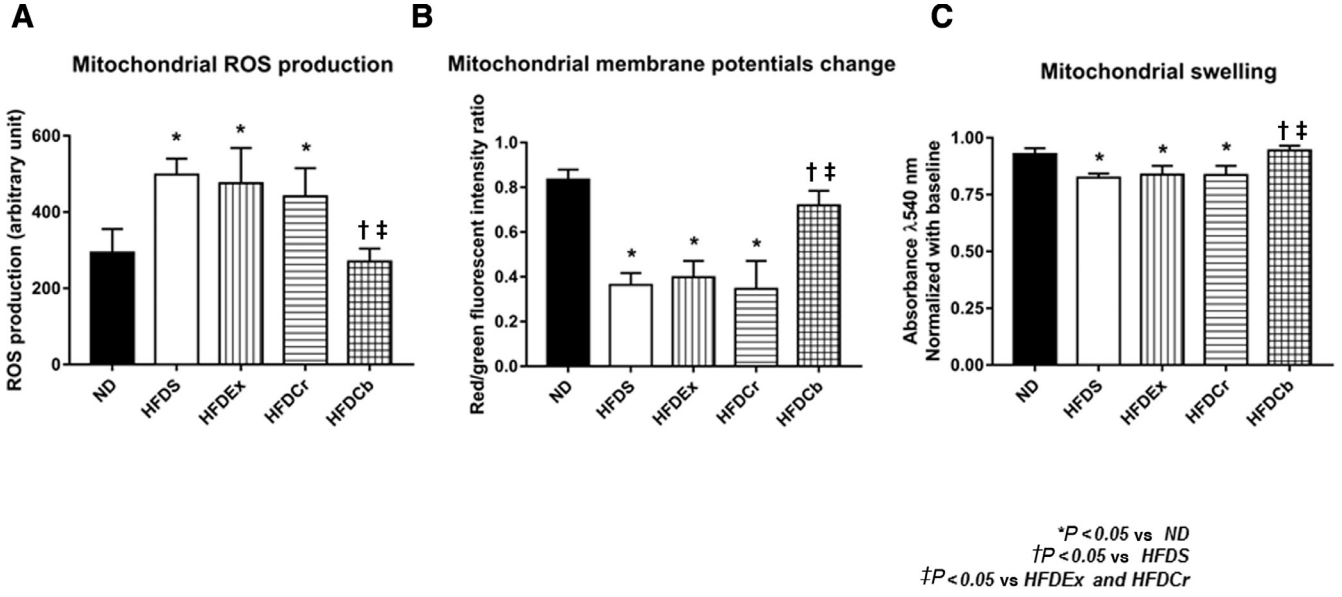

Fig. 4. Effects of high-fat diet consumption, exericise, CR, and combined exericise and CR program on skeletal muscle mitochondrial function. (**A**) Comparison of mitochondrial ROS among the study groups. (**B**) Comparison of mitochondrial membrane potential change between the study groups. (**C**) Comparison of mitochondrial swelling between the study groups. *$P < 0.05$ compared with ND group. †$P < 0.05$ compared with HFDS group. ‡$P < 0.05$ compared with HFDEx and HFDCr groups.; n = 6 per group. CR, calorie restriction; HFDCb, high-fat diet with combined therapy; HFDCr, high-fat diet with calorie restriction; HFDEx, high-fat diet with exercise; HFDS, high-fat diet with sedentary living; ND, normal diet; ROS, reactive oxygen species.

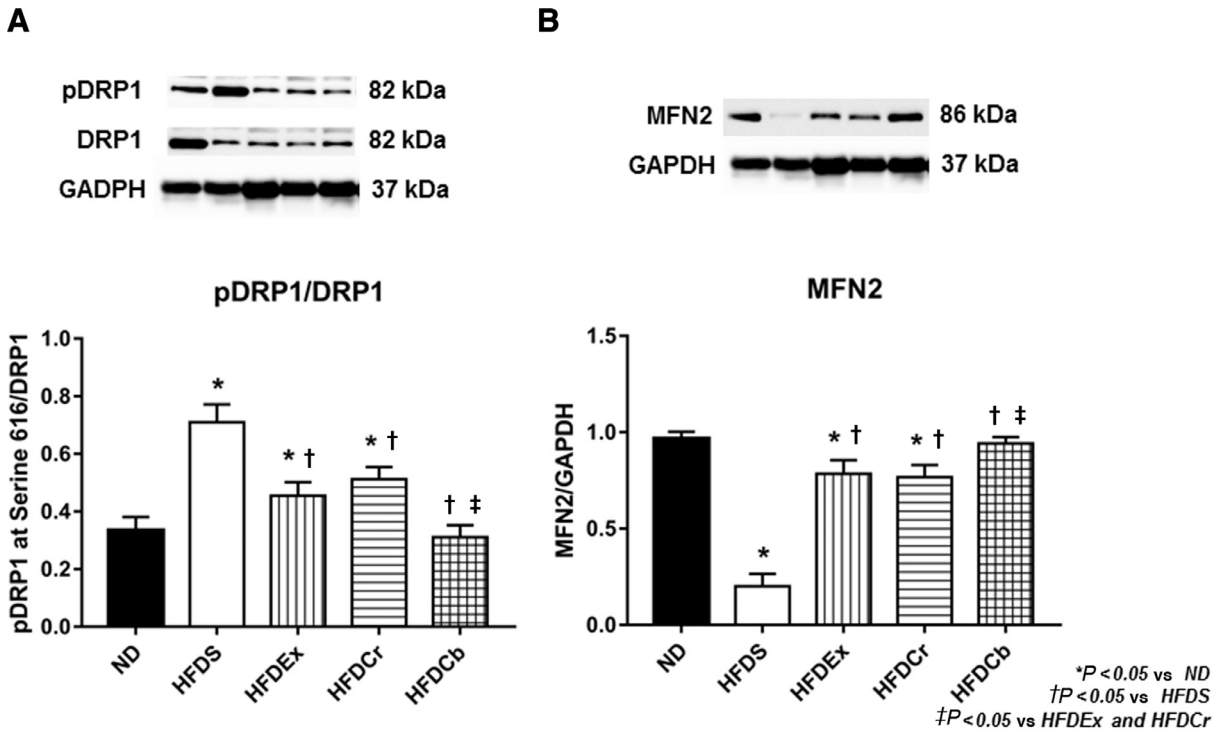

Fig. 5. Effects of high-fat diet consumption, exericise, CR, and combined exericse and CR program on skeletal muscle mitochondrial dynamics. (**A**) Comparison of ratios of Ser^{616}pDRP1 to total DRP1 among the study groups. (**B**) Comparison of MFN2 protein expression between the study groups. $^{*}P < 0.05$ compared with ND group. $^{\dagger}P < 0.05$ compared with HFDS group. $^{\ddagger}P < 0.05$ compared with HFDEx and HFDCr groups; n = 6 per group. CR, calorie restriction; DRP1, dynamin-1-like protein 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HFDCb, high-fat diet with combined therapy; HFDCr, high-fat diet with calorie restriction; HFDEx, high-fat diet with exercise; HFDS, high-fat diet with sedentary living; MFN2, mitofusin-2; ND, normal diet; pDRP1; phosphorylated dynamin-1-like protein 1.

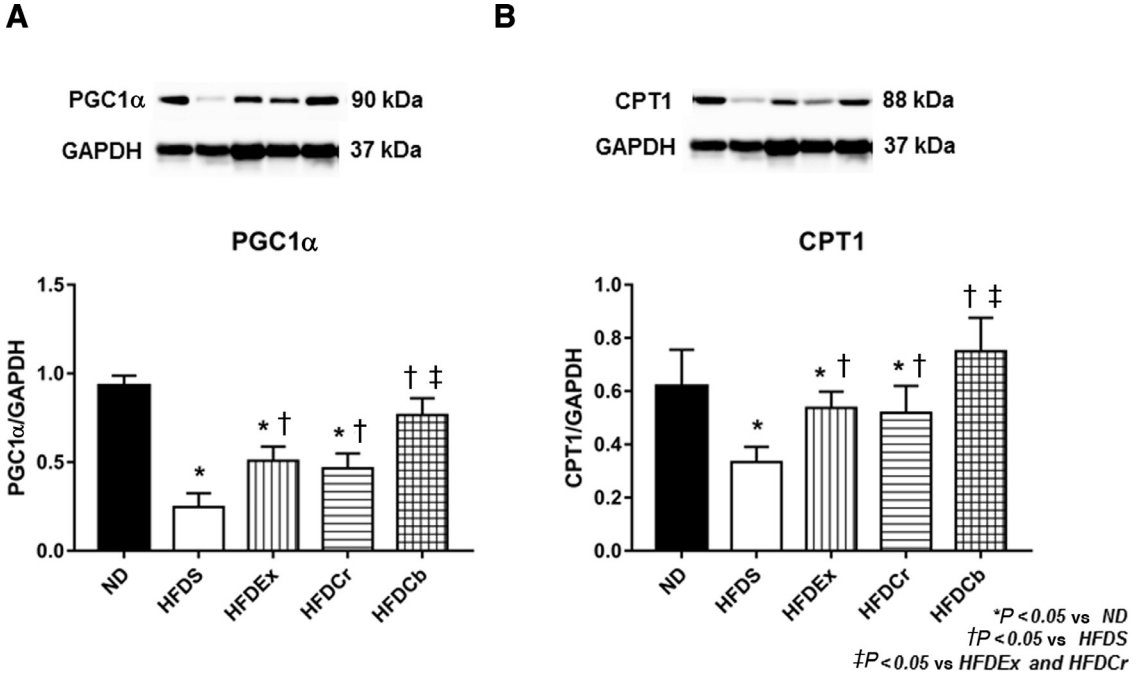

Fig. 6. Effects of high-fat diet consumption, exercise, CR, and combined exercise and CR program on skeletal muscle mitochondrial biogenesis. (**A**) Comparison of *PGC1α* protein expression between the study groups. (**B**) Comparison of *CPT1* protein expression between the study groups. $^{*}P < 0.05$ compared with ND group. $^{\dagger}P < 0.05$ compared with HFDS group. $^{\ddagger}P < 0.05$ compared with HFDEx and HFDCr groups; n = 6 per group. *CPT1*, carnitine palmitolytransferase-1; CR, calorie restriction; ET, exercise training; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HFDCb, high-fat diet with combined therapy; HFDCr, high-fat diet with calorie restriction; HFDEx, high-fat diet with exercise; HFDS, high-fat diet with sedentary living; ND, normal diet; *PGC1α*, peroxisome perforator-activated receptor coactivator-1 alpha.

obese–insulin resistant condition improved mitochondrial biogenesis as well as the fatty acid oxidation capacity of skeletal muscles, and a combined ET and CR program restored them.

HFD-induced IR increased skeletal muscle apoptosis, which was attenuated by ET, CR, and combined therapies

There is evidence to demonstrate that HFD-induced IR could enhance apoptosis in skeletal muscle tissue by increasing levels of proapoptotic protein-Bax and decreasing antiapoptotic protein-Bcl2 [9]. Compared with ND-fed rats, sedentary-living, HFD-fed rats had significantly higher Bax protein expression (Fig. 7A), lower Bcl2 protein expression (Fig. 7B), and a higher Bax-to-Bcl2 ratio (Fig. 7C) in skeletal muscles.

The effects of exercise and calorie restriction were also demonstrated in this study. A two-way ANOVA comparing two types of diet (CR or no CR) and physical activity (ET or no ET) revealed a significant effect of ET and CR on Bax ($F [1, 8] = 36.28$, $P < 0.001$; $F [1, 8] = 34.16$, $P = 0.001$, respectively) but not Bcl2 protein expression ($F [1, 8] = 3.308$, $P < 0.106$; $F [1, 8] = 3.739$, $P = 0.089$, respectively). It also revealed a significant effect of ET ($F [1, 8] = 12.47$, $P = 0.008$) and CR ($F [1, 8] = 13.65$, $P = 0.006$) on the Bax-to-Bcl2 ratio. The post hoc analyses demonstrated that HFD-fed rats in the ET or CR groups had significantly lower Bax protein expression and significantly higher Bcl2 protein expression than those of sedentary-living, HFD-fed rats. A significant decrease in the Bax-to-Bcl2 ratio in combined-therapy, HFD-fed rats was observed when compared with that of the monotherapy groups, but the Bax-to-Bcl2 ratio of combined-therapy group was no different from that of ND-fed rats (Fig. 7A–C). These results indicated that an ET and CR attenuated apoptosis in skeletal muscles of HFD-fed rats and combined therapies restored it.

Discussion

The major findings of the study are as follows:

1. Long-term HFD consumption led to obese IR and pathologic changes in skeletal muscles, including IR, enhanced early fatigability, increased apoptosis, and impaired mitochondrial function, as well as imbalanced mitochondrial dynamics and decreased biogenesis.
2. ET in the induced obese–insulin resistant condition improved metabolic function, insulin signaling, fatigability, apoptosis, mitochondrial biogenesis, and mitochondrial dynamics of skeletal muscles.
3. CR in the induced obese–insulin resistant condition also improved metabolic function, insulin signaling, apoptosis, and mitochondrial dynamics of skeletal muscles.
4. Combined ET and CR therapy in the induced obese–insulin resistant condition reversed those pathologic conditions in skeletal muscles.

Contractile function is one of the most important functions of skeletal muscle. Although it is dependent on several parameters, the parameter that has the most significant effect on human mobility is fatigability of tetanic contraction [40]. Fatigability is determined by many factors, including the type of muscle fiber. Skeletal muscle fibers can be roughly divided into type 1 (slow-twitch) and type 2 (fast-twitch) muscle fibers. Type 1 fibers have a lower

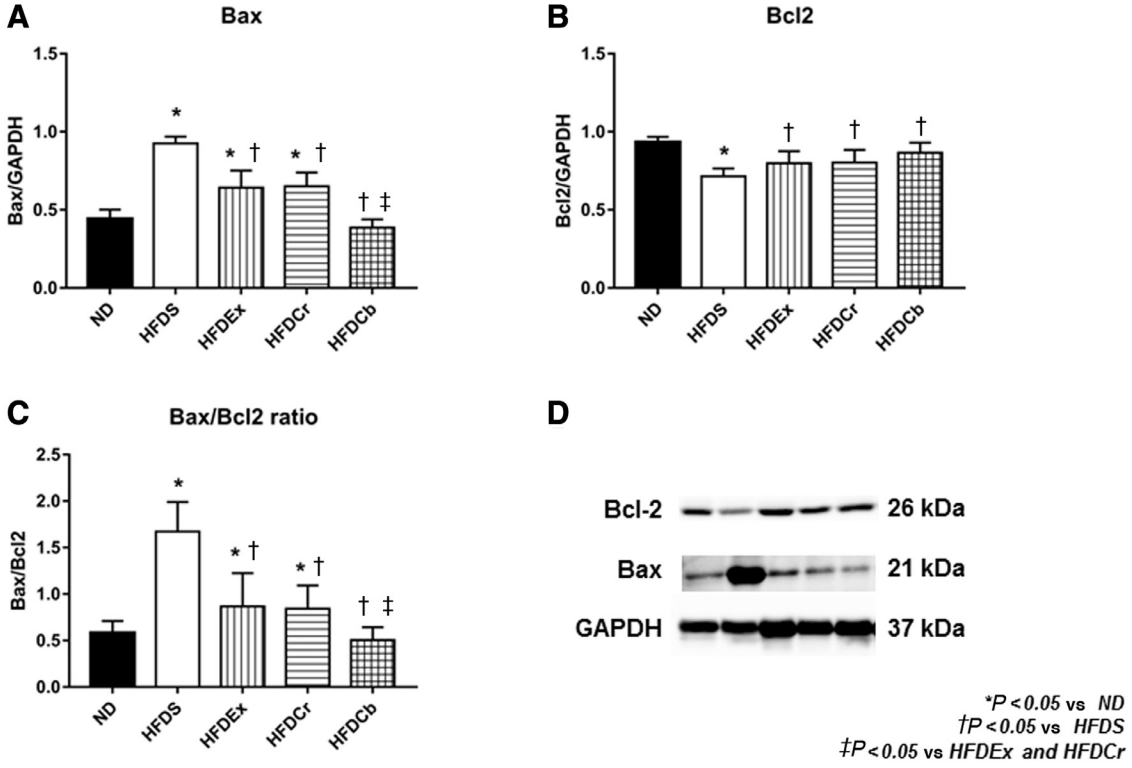

Fig. 7. Effects of high-fat diet consumption, exercise, CR, and combined exercise and CR program on skeletal muscle apoptosis. (**A**) Comparison of Bax protein expression among the study groups. (**B**) Comparison of Bcl2 protein expression among the study groups. (**C**) Comparison of Bax-to-Bcl2 ratio among the study groups. (**D**) Representative blots from all groups. $^{*}P < 0.05$ compared with ND group. $^{\dagger}P < 0.05$ compared with HFDS group. $^{\ddagger}P < 0.05$ compared with HFDEx and HFDCr groups; n = 6 per group. Bax, Bcl2 associated X protein; Bcl2, B-cell lymphoma 2 protein;CR, calorie restriction; ET, exercise training; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HFDCb, high-fat diet with combined therapy; HFDCr, high-fat diet with calorie restriction; HFDEx, high-fat diet with exercise; HFDS, high-fat diet with sedentary living; ND, normal diet.

fatigability than type 2 fibers [41]. There is evidence to demonstrate that the fatigability of each skeletal muscle is correlated with the percentage of type 1 fiber in the muscle [41]. One of the factors determining the production of type 1 fiber is *PPARδ* [42,43]. The percentage of type 1 fiber in each muscle has been associated with the amount of *PPAR*δ protein expression in the muscle [42,43]. Therefore, the enhancement of early fatigability of sedentary-living, HFD-fed rats might be as a result of a decrease in the percentage of type 1 muscle fiber, which shows a correlation with a decrease in *PPARδ* protein expression of the sedentary-living, HFD-fed rats when compared with those of ND-fed rats.

We also demonstrated that sedentary-living, HFD-fed rats had impaired mitochondrial function and imbalance of mitochondrial dynamics and increased cell apoptosis, all of which showed a correlation with the degree of IR. Several previous studies have demonstrated that mitochondrial dysfunction [5,6], imbalance of mitochondrial dynamics [7,8], and increased cell apoptosis [9] are associated with an insulin-resistant condition in skeletal muscles.

The ET in this study was an endurance exercise program. In this study, HFD-fed rats with ET had significantly lower peripheral and skeletal muscle IR when compared with sedentary living rats. HFD-fed rats with ET also showed an improvement in metabolic function, insulin sensitivity, muscle fatigability, mitochondrial biogenesis, and balance in mitochondrial dynamics and a decreased level in apoptosis of skeletal muscles. These findings might be as a consequence of an activation of adenosine monophosphate kinase (AMPK) during exercise training. AMPK is the main signaling protein responsible for energy control of the body cells [44]. There is evidence to demonstrate that endurance exercise activates AMPK by causing energy deprivation, and the beneficial effects of exercise are dependent on its activation [45]. AMPK is an upstream signaler of *PGC1α*, which is a coactivator of several proteins involved in many important metabolism reactions. These proteins include *CPT1* for fatty acid oxidation [46] and PPARδ for type 1 skeletal muscle fiber transformation [44].

Another beneficial effect of ET in this study was an improvement in the balance of mitochondrial biogenesis, as indicated by a decrease in mitochondrial fission markers, which occurred simultaneously with an increase in mitochondrial fusion markers. These results were compatible with the results from previous studies, which also demonstrated a decrease in mitochondrial fission markers [16] and an increase in mitochondrial fusion markers [15] after ET. However, the mechanism involved in the effect of exercise on improving the balance of mitochondrial dynamics is still elusive. Although this beneficial effect of ET was compatible with results from previous studies, this study is, to our knowledge, the first to demonstrate the reciprocal change between mitochondrial fission and fusion makers in the same study model.

Apoptosis is programmed cell death that mostly results from mitochondrial dysfunction. In the present study, a decrease in proapoptotic proteins and an increase in antiapoptotic proteins were demonstrated in HFD-fed rats with ET compared with those undergoing sedentary living. The mechanism of an exercise-induced reduction of apoptosis might be associated with an improvement in the insulin-resistant condition after ET. This result was different to the results of a previous study [17]. That study, with obese- Zucker rats, demonstrated that after 9 wk of endurance exercise (treadmill running), there was no change in apoptosis-related markers, specifically Bax, Bcl2, and the Bax-to-Bcl2 ratio, in the skeletal muscle of obese rats when compared with lean controls [17]. These different findings might be due to the difference in obese modes. Zucker rats developed the insulin-resistant condition from a genetic defect, resulting in prolonged hyperinsulinemia, whereas the HFD-induced insulin resistant condition has a shorter period of onset of the hyperinsulinemic condition.

The CR in this study provided 60% of the basal calorie requirement. Earlier evidence has demonstrated that CR could attenuate peripheral IR and increase insulin signaling in skeletal muscle tissue [19,20]. Some of our findings were compatible with the previous studies in that HFD-fed rats with CR demonstrated a significantly lower peripheral and skeletal muscle IR. HFD-fed rats with CR also exhibited increased skeletal muscle insulin sensitivity, reduced apoptosis, improved mitochondrial dynamics, and mitochondrial biogenesis. The mechanism behind these positive results is not clear but might be associated with an improvement in insulin sensitivity. It has been proposed that the underlying mechanism of CR behind improvement in insulin sensitivity is related to the activation of AMPK, which might result from an energy-deprivation stage during caloric restriction [47]. Although we found an increase in *CPT1*, which is a part of the downstream signaling mechanism of AMPK via *PGC1α*, we did not find any difference in *PPARδ* protein expression or muscle fatigability in HFD rats with CR compared with those undergoing sedentary living. These findings were consistent with the results from a previous study, demonstrating no change in percentage of type 1 muscle fiber of the subjects with CR compared with those without CR [48]. *PGC1α* is induced by several mechanisms, such as an activation of AMPK from energy deprivation and an induction of Calcium-dependent signaling (CaMKIV, calcineurin) from repetitive muscle contraction [40]. The findings differ in the two groups. ET induced not only energy deprivation but also repetitive muscle contraction. CR induced only energy deprivation. This might be the reason for the inconsistent findings between the effect of ET and CR on the induction of *PPARδ* protein expression and fatigability. We also found that HFD-fed rats with CR had a lower apoptotic process compared with those in the sedentary living group. This result corresponded with the results from a previous study, which demonstrated CR preventing apoptosis in rat skeletal muscle [20]. The possible mechanism of decreased apoptosis of CR might be associated with an improvement in insulin sensitivity [22,49].

The combined CR and ET therapy in this study included both endurance exercise and a 60% CR program. The study demonstrated that a combination of the therapies resulted in the greatest benefit for almost all of the parameters pertaining to the skeletal muscles. The beneficial results on skeletal muscles from the three interventions in the obese–insulin resistant condition could be summarized into three main findings.

1. There was an equal incidence of positive responses observed with ET and combined therapies, but not with CR.
2. The combined therapies had the synergistic effects from ET and CR.
3. Some favorable responses were found only in the combined therapy groups.

These different responses from interventions could reflect the underlying mechanisms of each intervention.

The parameters, which showed positive responses to ET and the combined therapies, but not CR, were skeletal muscle fatigability and *PPARδ* protein expression. The possible mechanisms causing these differences could be associated with the activation of the Calcium-dependent signaling pathway. The next parameters, from which the combined therapies had the synergistic effects from ET and CR, were increased peripheral and skeletal muscle insulin sensitivity, mitochondrial biogenesis, and mitochondrial dynamics, and decreased apoptosis. We propose that a combination of ET and CR therapy has a greater beneficial effect than either ET or CR alone

in terms of enhancing the balance of mitochondrial dynamics, improving mitochondrial biogenesis, and attenuating the rate of apoptosis in skeletal muscles. These improvements led to the restoration of peripheral and skeletal muscle insulin sensitivity. The parameters in the third group, specifically the beneficial effects only seen in the combined-therapy group, were those relating to mitochondrial function, including attenuation of mitochondrial ROS, mitochondrial membrane potential, and mitochondrial swelling. These exclusive effects might be associated with the synergistic activation of AMPK signaling from both the ET and CR program [45,47]. We proposed that the combined therapies could induce activation of the AMPK pathway at an adequate rate that the ET or CR therapy alone could not adequately maintain.

This study had some limitations. First, the function of glucose uptake in skeletal muscles, for example 2-deoxyglucose uptake study, was not directly determined. Next, although the OGTT, which is one of the recognized assessments of insulin sensitivity [50], was performed in this study, an insulin tolerance test should be performed to confirm insulin sensitivity in a future study. Finally, it has been demonstrated that the effects on IR and skeletal muscle functions between males and females are different [51]. To limit variables, we decided to use animals in only the female sex for the present study. This limits the generalization possibilities of the study since the period of menopause comes earlier than andropause, exposing women to a lack of the sex hormone longer. There is also a prevalence of the overweight and obese condition in women, the increase being up to 40.4%. To enable the generalization of the results of this study, a further study will aim to investigate the effects of exercise training and caloric restriction on skeletal muscle function in obese–insulin resistant male rats.

Conclusion

Long-term consumption of an HFD induced peripheral IR and decreased cellular insulin signaling. In addition, it led to increasing fatigability, apoptosis, and mitochondrial dysfunction in skeletal muscles. ET alone improved cellular insulin signaling, fatigability, apoptosis, mitochondrial biogenesis, and mitochondrial dynamics in skeletal muscles. CR alone improved cellular insulin signaling, mitochondrial biogenesis, apoptosis, and mitochondrial dynamics in skeletal muscles. A combination of ET and CR improved skeletal muscle fatigability in comparison with ET alone. In addition, the combined therapy had additive effects on improving insulin signaling, apoptosis, mitochondrial biogenesis, and mitochondrial dynamics in skeletal muscles. The combination of ET and CR caused an exclusive positive effect on mitochondrial function, which ET or CR alone could not instigate. These results demonstrate the beneficial synergistic effects of the combined ET and CR in an obese–insulin resistant condition on improving the function of skeletal muscle and associated mitochondria.

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