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Original Article

Exercise improves muscle mitochondrial dysfunction-associated lipid profile under circadian rhythm disturbance

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ABSTRACT We investigated whether endurance exercise training (EXT) ameliorates circadian rhythm (CR)-induced risk factors by improving skeletal muscle (SKM) mitochondrial biogenesis, reducing oxidative stress, and modulating apoptotic protein expression. We distinguished between regular and shift workers using the National Health and Nutrition Examination Survey (NHANES) and investigated the health problems caused by shift work (CR disturbance) and the potential therapeutic effects of exercise. In our animal study, 36 rats underwent 12 weeks of CR disturbance, divided into regular and irregular CR groups. These groups were further split into EXT (n = 12) and sedentary (n = 12) for an additional 8 weeks. We analyzed SKM tissue to understand the molecular changes induced by CR and EXT. NHANES data were analyzed using SAS 9.4 and Prism 8 software, while experimental animal data were analyzed using Prism 8 software. The statistical procedures used in each experiment are indicated in the figure legends. Our studies showed that CR disturbance increases dyslipidemia, alters circadian clock proteins (BMAL1, PER2), raises apoptotic protein levels, and reduces mitochondrial biogenesis in SKM. EXT improved LDL-C and HDL-C levels without affecting muscle BMAL1 expression. It also enhanced mitochondrial biogenesis (AMPK, PGC-1α, Tfam, NADH-UO, COX-I), antioxidant levels (Catalase, SOD1, SOD2), and apoptotic protein (p53, Bax/Bcl2) expression or activity in SKM. We demonstrated that shift work-induced CR disturbance leads to dyslipidemia, diminished mitochondrial biogenesis, and reduced antioxidant capacity in SKM. However, EXT can counteract dyslipidemia under CR disturbance, potentially lowering the risk of cardiovascular disorders.

INTRODUCTION

Circadian rhythm (CR) is the internal biological clock governing various physiological and behavioral processes in organisms, including sleep-wake cycles, hormone secretion, metabolism, and gene expression [1]. The light-dark cycle synchronizes CR, regulating alertness and sleepiness in response to changing environmental light on a roughly 24-h cycle [2]. This mechanism plays a crucial role in regulating energy storage and expenditure that any disruption to this system can hinder internal physiological processes within the body.

Today, the necessity for natural light no longer limits working hours and so irregular working hours or shift work has become more normalized in various industries. However, working such

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hours accompanies inadequate sleep where disturbances in CR can lead to health issues like metabolic syndrome and cardiovascular diseases [3]. CR disorders are also significantly associated with skeletal muscle (SKM) dysfunction and apoptosis, potentially undermining metabolic function and overall well-being [1].

SKM is one of the major tissues in regulating energy expenditure, mainly using glucose and fatty acids for energy [4]. Thus, a break in this system in the context of muscle atrophy could contribute to the advancement of metabolic diseases, including cancer, aging, and obesity [5]. In fact, muscle atrophy, like sarcopenia, causes abnormal lipid metabolism known as dyslipidemia [6], characterized by increased serum total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), triglyceride (TG), and reduced levels of serum high density lipoprotein cholesterol (HDL-C) [7]. Considering dyslipidemia constitutes a risk factor for cardiovascular disease [8], muscle function and mass are crucial in preventing dyslipidemia-induced cardiovascular disease [9]. Targeting knockout (KO) clock protein, basic helix-loop-helix ARNT-like protein 1 (BMAL1), in SKM has shown to reduce mitochondrial function by affecting myogenic differentiation 1 (MyoD) [10]. Therefore, it appears that CR disturbance induced metabolic dysfunction in SKM may promote the development of metabolic diseases. To mitigate its progression, exercise training (EXT) was initiated in hopes to modify the CR clock mechanism in SKM [11-13] by influencing metabolic reprogramming through molecular alterations. Consequently, the physiological and biological effects triggered by exercise and CR intervention are closely intertwined. However, exercise effects during irregular sleep-wake cycles have not been fully elucidated. And so, we employed both the Korea National Health and Nutrition Examination Survey (KNHANES) and animal studies to gain a comprehensive understanding on how exercise affects CR disruption. Therefore, we will first verify through KNHANES data whether CR disruption increases dyslipidemia. Based on this, we aim to demonstrate whether CR disruption causes protein dysregulation in SKM and whether endurance EXT can improve protein dysregulation caused by CR disruption.

METHODS

Human study population and design

This study was based on data obtained by the KNHANES IV (2007–2009), V (2010–2012), VI (2013–2015), VII (2016–2018), and VIII (2019-2021). The details of the survey have been published elsewhere. KNHANES is a population-based cross-sectional survey that employs a continuous sampling design that incorporates a stratified, multistage, probability cluster survey to capture a representative sample of the Korean population [14]. This study included 41,798 individuals aged 19 years and older who took part in the survey between 2007 and 2021 as well as possessing all the necessary data to determine their shift worker status (Supplementary Fig. 1). After propensity score matching (PSM), a final dataset comprising 20,385 individuals was chosen based on their shift work status (Fig. 1).

Using this survey data in this manuscript was approved by the Institutional Review Board of Yonsei University Wonju Severance Christian Hospital (IRB No. CR323341).

Data collection

High TC was defined as TC ≥ 240 mg/dl or individuals taking cholesterol-lowering drugs. Low HDL-Ch referred to subjects with HDL-C levels below 40 mg/dl, while high TGs were defined as subjects with a TG level of 200 mg/dl or higher. The study centered on chronic diseases as defined by the health questionnaire, including the following conditions: dyslipidemia, obesity, stroke, myocardial infarction, angina, arthritis, osteoarthritis, rheumatoid arthritis, thyroid disease, renal failure, depression, hyperten-

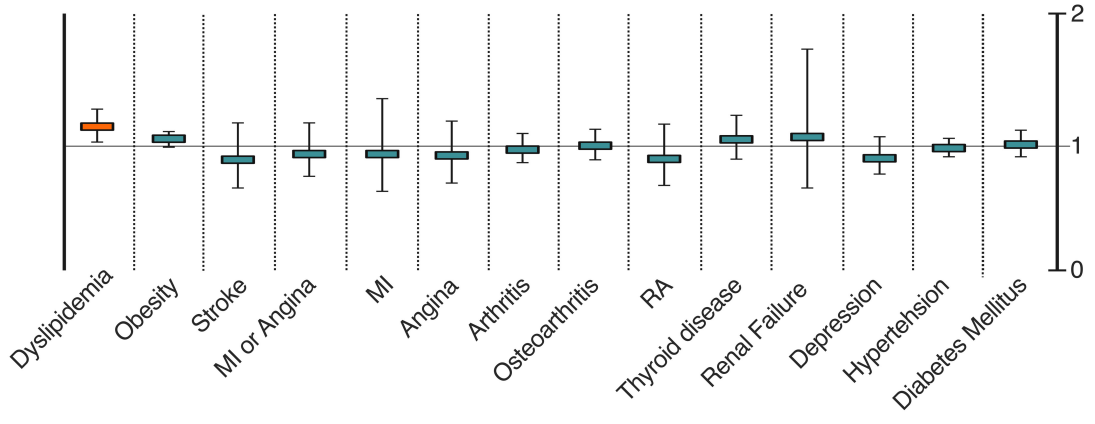

Fig. 1. Odds ratios and 95% confidence intervals illustrating the relationship between shift work and various chronic diseases. Adjustments were made for age, sex, TC, triglycerides, and HDL-C. The odds of having dyslipidemia were notably elevated among shift workers, as indicated by the highlighted red bar above. TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; MI, myocardial infarction; RA, rheumatoid arthritis.

sion, and diabetes mellitus. Obesity was identified as a body mass index of ≥ 25 kg/m^2 based on the criteria of the Asian-Pacific region [15]. Hypertension was recognized as a blood pressure ≥ 140/90 mmHg or use of current antihypertensive medication. Diabetes mellitus was defined based on [14] the use of insulin or oral hypoglycemic agents [15] or fasting plasma glucose ≥ 126 mg/dl.

Animal and experimental procedures

In this study, we used 36 male Sprague–Dawley (SD) rats obtained from DaeMul Science, each aged 12 weeks. The rats were housed in pairs per cage, maintaining humidity (40%–60%) and a consistent temperature (18°C–22°C). The feed (carbohydrates 58.9%, fat 12.4%, protein 28.7%) and water were provided ad libitum throughout the experimental period. A daily light-dark cycle was established, with the period from 8 AM to 8 PM designated as daytime.

Following a two-week acclimatization after obtaining the subjects, the SD rats were randomly divided into two group: the regular circadian rhythm group (RCR, n = 18) and the irregular circadian rhythm group (ICR, n = 18). While RCR rats were housed under a consistent light-dark cycle throughout the entirety of the experimental period, ICR rats experienced CR disruption by alternation of the light-dark cycle every three days. During the initial 12 weeks following group allocation, sleep cycles were manipulated to measure changes due to CR regulation, referencing the study by Sun *et al*. [16]. To achieve this, six rats from each group were sacrificed. The RCR (n = 12) and ICR (n = 12) groups were further categorized into sedentary (SED, n = 6) and EXT (n = 6) groups for an 8-week intervention period. After completing all interventions, the SD rats were sacrificed, and tissue samples were collected for analysis.

All procedures were conducted with the approval of the Institutional Animal Care and Use Committee of Jeonbuk National University (IACUC approval no. CBNU-2022-0067).

EXT

EXT was conducted for 8 weeks after 12 weeks of CR disturbance. During this period, CR conditions were administrated in the same manner as in the previous 12 weeks. EXT involved the use of a treadmill (TREADMILL, L.M.S. KOREA) at a fixed incline of 2%. The exercise intensity was set at approximately 60%–70% of maximum oxygen intake based on the study by Qin *et al*. [17]. The exercise regimen was structured accordingly: a warm-up phase (10–15 m/min, 20 min), the main exercise phase (15 m/min, 30 min), and a cool-down phase (10 m/min, 10 min) for a total of 60 min. For the ICR group, exercise sessions were conducted on the second and third days of the three-day interval sleep cycle, each session lasting for a duration of one hour (07:30–08:30 am). The treadmill exercise was modified and improved based on the method used in the study by Kim *et al.* [18].

Blood lipid parameters

After the 12-week CR disruption and the subsequent 8-week exercise intervention, blood samples were drawn from the inferior vena cava at each respective time point. The collected blood was subject to centrifugation to isolate the serum, which was subsequently stored at –80°C until analysis. Analysis of TGs (Triglycerides, Roche), TC (Cholesterol Gen.2, Roche), LDL-C (LDL-Cholesterol Gen.3, Roche), HDL-C (HDL-Cholesterol Gen.4, Roche), and free fatty acids (FFA, NEFA HR.II, Wako) was outsourced to Global Clinical Central Lab, a specialized clinical trial sample analysis company, prior to assessment.

Western blot

We analyzed protein expression using gastrocnemius muscle (GAS) samples collected at 12 and 20 weeks administration. SKM samples were collected 23 h after the final exercise session. The collected GAS samples were rapidly frozen in liquid nitrogen and stored at –80°C. Next, frozen tissues were powdered and homogenized using a cold buffer (50 mM Tris·HCl [pH 7.4], 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid [pH 7.4], 1 mM Pefabloc [Roche], 1 mM NaF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 0.1 mM bpV(phen), and 2 mg/ml β-glycerophosphate) with protease and phosphatase inhibitors. After homogenization, the supernatant was extracted and protein concentration in the supernatant was measured using the Bradford assay with bovine serum albumin as the standard. Each sample was dissolved in Laemmli sample buffer and subjected to gel electrophoresis to segregate proteins. These separated proteins were subsequently transferred onto a nitrocellulose membrane that had been blocked with 5% skim milk. The membranes were incubated with primary antibodies for two hours to overnight as follows: β-actin (Invitrogen, MA1-140); p-AMPK α1/2 (Millipore, #07-681); AMPK α1/2 (SCBT, sc-25792); peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC-1α; GenTex, GTX37356); mitochondrial transcription factor A (Tfam; SCBT, sc-166965); NADH:ubiquinone oxidoreductase subunit A9 (NADH; Abcam, ab110242); succinate dehydrogenase (SDH) subunits B (SDHB; ab14714, Abcam); cytochrome oxidase (COX) subunit I (COX-I; ab14705, Abcam); superoxide dismutase type 1 (SOD1; SCBT, sc-8637); superoxide dismutase type 2 (SOD2; SCBT, sc-18503); Catalase (SCBT, sc-271358); p-P53 (SCBT, sc-51690); P53 (SCBT, sc-55476); Bcl2 associated X (Bax; SCBT, sc-7480); B-cell lymphoma 2 (Bcl2; SCBT, sc-7382); brain and muscle ARNT-like 1 (BMAL1; SCBT, sc-365645); period circadian regulator 2 (PER2; Invitrogen, #PA5-100107). After incubation with primary antibodies, the membranes were incubated with appropriate secondary antibodies (mouse anti-goat [SCBT, sc-2354], mouse anti-rabbit [SCBT, sc-

2357], or goat anti-mouse [SCBT, sc-2005]) for one hour at room temperature. Membranes incubated with secondary antibodies were then visualized using the ECL Western Blotting Detection Reagent (GE Healthcare) and quantified using the ChemiDoc XRS+ system (BIO-RAD). Each quantified protein was normalized to beta-actin, and phosphorylation was normalized to the corresponding relative protein levels.

Statistical analysis

To reduce the effect of selection bias and potential confounders, we adjusted for significant differences in the baseline characteristics of subjects using 1:2 PSM with caliper set to 0.25 [19]. The variables employed for PSM included age, sex, high TC, high TGs, and low HDL-C–all of which are directly associated with chronic diseases. After completing all PSM procedures, we conducted a comparison of baseline covariates between the groups. Considering the characteristics of the KNHANES data, a complex sample analysis was performed by examining the primary extraction unit (region), stratification variables, and weights. A complex sample design analysis and McNemar's analysis were conducted after PSM. To evaluate the relationship between shift work and chronic diseases, as well as the relationship between explanatory variables and the primary outcomes, logistic regression analyses were employed. All p-values less than 0.05 were considered statistically significant. Moreover, standardized differences of covariates used in PSM analysis less than 0.1 were considered significant and data were analyzed using SAS version 9.4 (SAS).

The statistical procedures employed in each experiment are indicated in the figure legends. Briefly, results are presented as the mean ± SD. Before any statistical analysis, the normality of the data was assessed using the Shapiro–Wilk test. According to the study design, either the unpaired Student's t-test or two-way ANOVA was performed. As for the Tukey's test, this was implemented for *post-hoc* analysis to determine significant differences between individual groups. ALL analyses were performed using the GraphPad Prism version 10.2.0 (GraphPad Software).

RESULTS

The baseline characteristics of the study subjects

The baseline characteristics of the study subjects are displayed in Table 1. The total number of subjects was 20,385, and the characteristics of the subjects were compared according to shift work. Shift workers were older and had a higher proportion of men. The rate of obesity was high in shift workers and was statistically significant.

Shift work is a potential risk factor for dyslipidemia

Fig. 1 and Supplementary Table 1 present the odds ratios (OR) and 95% confidence intervals (CI) depicting the relationship between shift work and each chronic disease, with adjustments made for age, sex, TC, TG, and HDL-C. Shift workers exhibited significantly increased odds for having dyslipidemia (OR = 1.14, 95% CI = 1.01–1.28). In a logistic regression analysis considering weights, shift workers were significantly more likely to be obesity (OR = 1.17, 95% CI = 1.08–1.26). The probability of developing

Table 1. Baseline characteristics of study population by shift work after propensity score matching

Characteristic	Total sample	Shift worker (+)	Shift worker (–)	p-value
Participants, n	20,385	6,795	13,590	
Age, y	40.23 (0.14)	40.47 (0.22)	40.10 (0.17)	0.148
Sex, male	10,178 (55.41)	3,419 (56.32)	6,759 (54.92)	0.101
High total cholesterol, yes	3,000 (13.08)	1,002 (13.11)	1,998 (13.07)	0.940
Low HDL cholesterol, yes	3,462 (16.29)	1,158 (16.35)	2,304 (16.26)	0.886
High triglycerides, yes	2,736 (13.51)	920 (13.78)	1,816 (13.38)	0.510
Chronic diseases, yes				
Dyslipidemia	2,082 (8.40)	729 (8.74)	1,353 (8.22)	0.247
Obesity	6,687 (38.61)	2,257 (40.98)	4,430 (37.36)	< 0.001
Stroke	215 (0.75)	65 (0.68)	150 (0.79)	0.376
MI or angina	326 (1.09)	103 (1.02)	223 (1.12)	0.511
MI	109 (0.39)	34 (0.36)	75 (0.41)	0.599
Angina	237 (0.77)	73 (0.70)	164 (0.81)	0.403
Arthritis	1,547 (5.54)	500 (5.51)	1,047 (5.57)	0.868
Osteoarthritis	1,355 (4.73)	445 (4.78)	910 (4.71)	0.815
RA	234 (0.96)	71 (0.90)	163 (0.99)	0.541
Thyroid disease	587 (2.36)	199 (2.53)	388 (2.27)	0.267
Renal failure	64 (0.29)	22 (0.41)	42 (0.23)	0.056
Depression	676 (3.15)	206 (2.97)	470 (3.24)	0.395
Hypertension	4,815 (19.91)	1,577 (20.04)	3,238 (19.85)	0.776
Diabetes mellitus	1,594 (6.57)	529 (6.66)	1,065 (6.52)	0.730

Data are presented by n (weighted %) or mean (SE). HDL, high density lipoprotein; MI, myocardial infarction; RA, rheumatoid arthritis.

dyslipidemia was not significant but showed a positive trend.

CR disturbance alters muscle BMAL1, PER2, and blood cholesterol levels

To validate the results obtained from the NHANES study, we investigated whether the same outcomes could be replicated in experimental animal models subject to a CR disturbance. To this end, we induced a CR disturbance in SD rats for 12 weeks (Fig. 2A) and found that CR disturbance decreases body weight (BW) (Fig. 2B) while increasing epididymal fat mass to body weight ratio (Epi/BW) (Fig. 2C), given epididymal fat mass is a form of visceral fat. We also observed that CR disturbance did not significantly change TG, FFA, and TC levels (Fig. 2D–F). Given that CR disturbance increased LDL-C levels (Fig. 2G), we expected a corresponding decrease in HDL-C levels. Nevertheless, while HDL-C exhibited a decrease due to CR disturbance, this reduction was not statistically significant (Fig. 2H).

As the most abundant tissue in the body, SKM regulates circulating lipids levels for overall health [20]. Considering the role of muscle in adipogenesis regulation, we focused on changes in SKM that could potentially cause an increase in dyslipidemia [21]. BMAL1 and PER2 proteins were evaluated since these two vital components of the mammalian circadian clock regulate the timing of physiological and behavioral processes in response to the daily light-dark cycle [22]. And indeed, a Western blot analysis exhibited increased PER2 and decreased BMAL1 protein expression levels in both ICR group compared to the RCR group (Fig. 2I–K). In this regard, previous studies have demonstrated the existence of a negative feedback loop that governs gene expression between PER2 and BMAL1 [23,24]. Furthermore, the disruption of BMAL1 expression in SKM has been implicated in metabolic

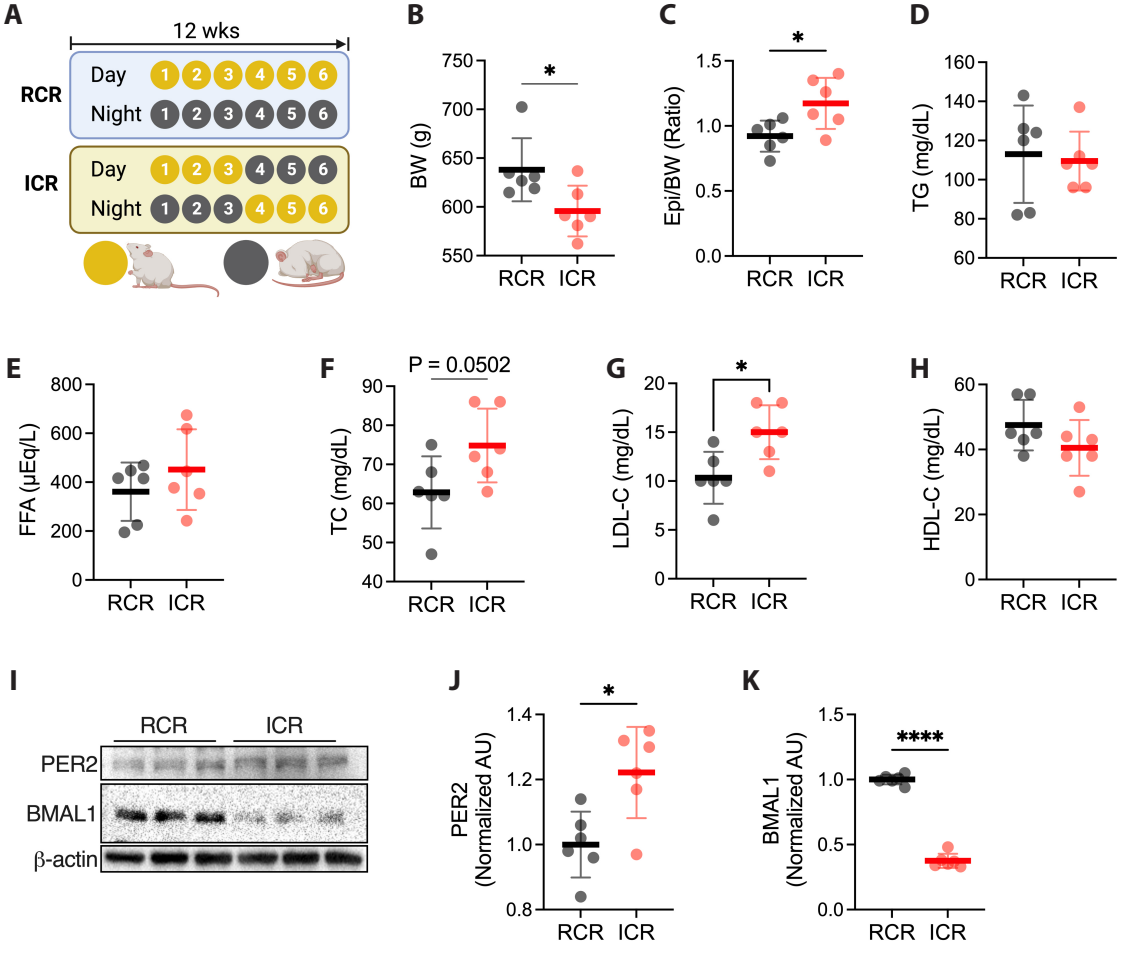

Fig. 2. CR disturbance altered blood profiles by increasing LDL-C levels despite decreasing body weight (BW). (A) Induction of 12 weeks CR disturbance on RCR (n = 6 rats per group) and ICR (n = 6 rats per group) groups. Significant changes include (B) BW decrease (C) epididymal fat mass to body weight ratio increase (Epi/BW). No meaningful change was observed in (D) TG, (E) FFA, (F) TC, (G) LDL-C, and (H) HDL-C levels. (I) A Western blot analysis of circadian clock protein levels reveal a significant difference in expression (I-K). All data are presented as mean ± SEM. CR, circadian rhythm; TC, triglyceride; FFA, free fatty acid; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; RCR, regular circadian rhythm; ICR, irregular circadian rhythm; PER, period circadian regulator 2; BMAL1, brain and muscle ARNT-like 1. *p < 0.05, ****p < 0.0001 as determined using Student's t-test (n = 6).

processes [25]. All things considered, alterations in BMAL1 and PER2 within SKM due to CR disruption appear to be associated with higher levels of LDL-C in the bloodstream, despite a decrease in weight, and are accompanied by adipogenesis.

CR disturbance increases apoptotic protein and decreases mitochondrial biogenesis in SKM

BMAL1 gene is involved in the maintenance of muscle phenotype and function [10], which are both affected by reactive oxygen

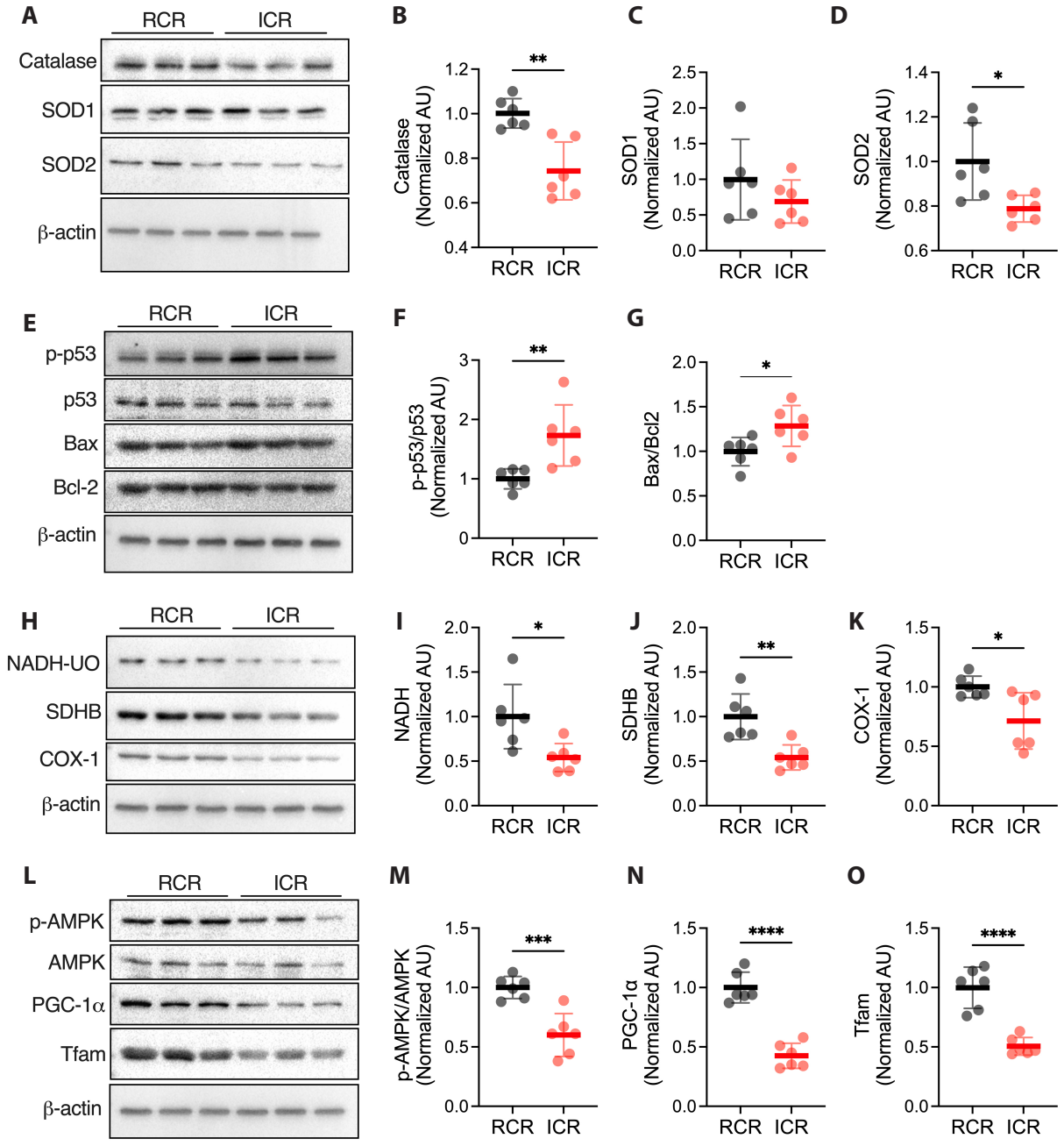

Fig. 3. Circadian rhythm disturbance decreases antioxidant expression and mitochondrial biogenesis, but increases apoptotic protein expression. Representative Western blot analyses reveal (A-D) decreased catalase and superoxide dismutase type 2 (SOD2) levels with no significant change in superoxide dismutase type 1 (SOD1) levels. (E-G) Increased apoptotic protein expression was observed. (H-K) Decreased expression of major proteins in the electron transport chain and (L-O) proteins governing mitochondrial biogenesis was presented. All data are presented as mean ± SEM. RCR, regular circadian rhythm; ICR, irregular circadian rhythm; Bcl2, B-cell lymphoma 2; Bax, Bcl2 associated X; NADH, reduced nicotinamide adenine dinucleotide; SDHB, succinate dehydrogenase subunit B; COX-I, cytochrome oxidase (COX) subunit I; AMPK, AMP-activated protein kinase; PGC-1α, peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1alpha; Tfam, mitochondrial transcription factor A. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ as determined using two-way ANOVA and Tukey's *post-hoc* analyses (n = 6).

species (ROS) levels and the degree of apoptosis. It is well known that high levels of ROS in muscle tissue can lead to apoptosis, contributing to a decline in SKM mass and function [26]. Hence, catalase and superoxide dismutase were incorporated into this study to assess their potential in counterbalancing such elevation in ROS and protect SKM function [26]. Western blot analyses were performed to investigate the impact of CR disturbance on skeletal function by examining changes in the levels of antioxidants, apoptotic proteins, and proteins associated with mitochondrial biogenesis. Our results revealed a decrease in the protein expression levels of catalase and SOD2 in the ICR group compared to the control RCR group (Fig. 3A, B, D). However, there was no significant change observed in SOD1 levels (Fig. 3C).

CR disturbance on expression levels of apoptotic proteins, p53 and Bax/Bcl2, which regulate programmed cell death, were also assessed [27]. p53 activates the expression of pro-apoptotic genes, including Bax, while suppressing anti-apoptotic genes, such as Bcl-2 [28]. In response to stress, p53 increases the ratio of Bax to Bcl-2, stimulating damaged cells to undergo apoptosis [29]. Therefore, the ratio of Bax to Bcl-2 is crucial in determining whether cells survive or undergo apoptosis. In this regard, we observed that phosphorylation of p53 and ratio of Bax to Bcl2 increased in the ICR group than that in the RCR group (Fig. 3E–G). Considering both antioxidant and apoptotic protein expression levels, the significant decrease in antioxidant expression by CR disturbance appears to have triggered the increase in apoptotic protein expression.

Since mitochondria play a major role in regulating apoptosis, we analyzed mitochondrial biogenesis in SKM and found that major proteins in the electron transport chain, including NADH-UO, SDHB, and COX-I, decreased in the ICR group compared to the RCR (Fig. 3H–K). In addition, AMPK phosphorylation, PGC-1α, and Tfam protein expression, all of which regulate mitochondrial biogenesis, decreased in the ICR group compared to the RCR (Fig. 3L–O). Taking all these data into consideration, CR disturbance leads to decreased mitochondrial biogenesis and antioxidant protein expression, which in turn appears to elevate the occurrence of apoptosis in SKM.

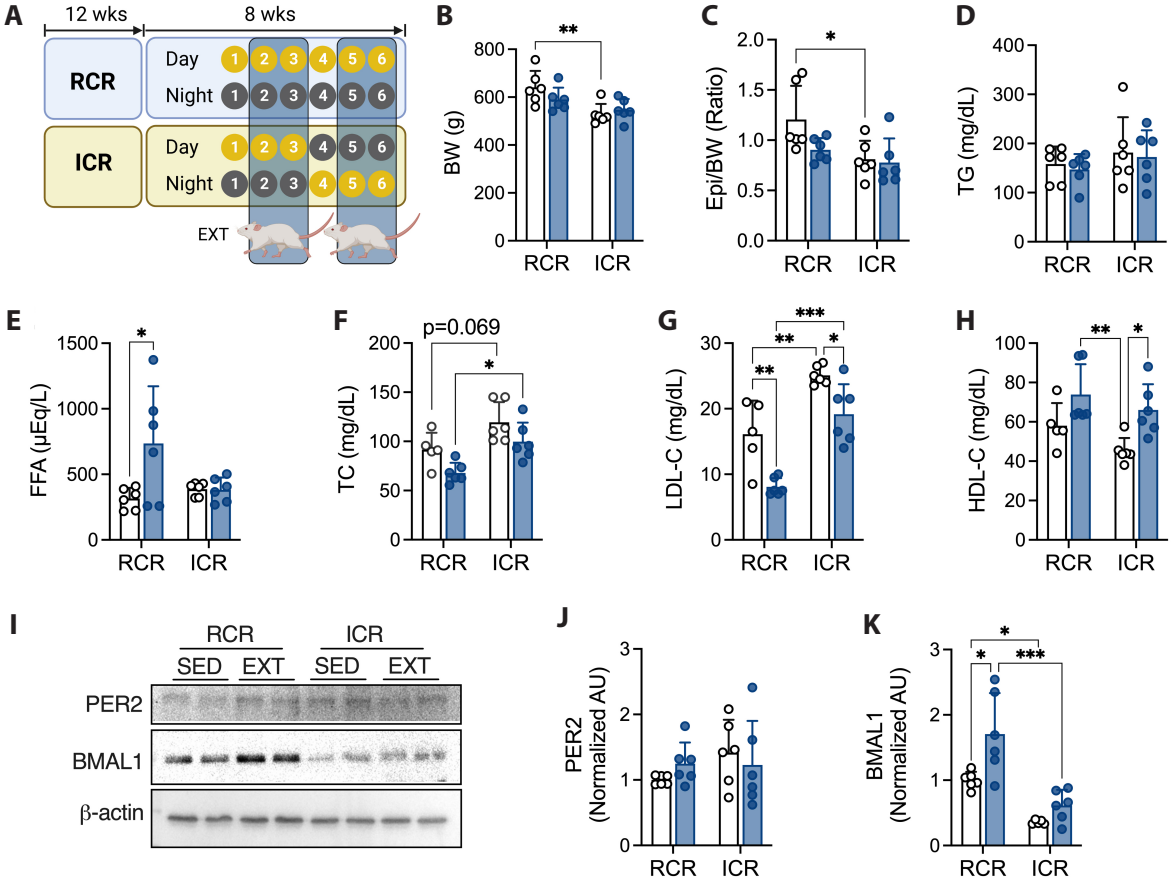

Fig. 4. EXT improves LDL-C and HDL-C levels without altering body composition and BMAL1 muscle gene expression. (A) Induction of 8 weeks endurance training followed by 12 weeks CR disturbance. (B, C) Body composition and (D-F) blood profiles did not show improvement. (G) LDL-C levels (RCR n = 5) decreased while (H) HDL-C (RCR n = 5) expression increased. (I-K) A Western blot analysis presents no significant change in circadian clock protein levels. All data are presented as mean ± SEM. EXT, endurance exercise training; LDL-C, low-density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; CR, circadian rhythm; RCR, regular circadian rhythm (n = 6); ICR, irregular circadian rhythm (n = 6); BW, body weight; Epi/BW, epididymal fat to body weight ratio; TG, triglyceride; FFA, free fatty acid; TC, total cholesterol; PER2, period circadian regulator 2; BMAL1, brain and muscle ARNT-like 1; SED, sedentary. *p < 0.05, **p < 0.01, ***p < 0.001 as determined using two-way ANOVA and Tukey's *post-hoc* analyses (n = 6).

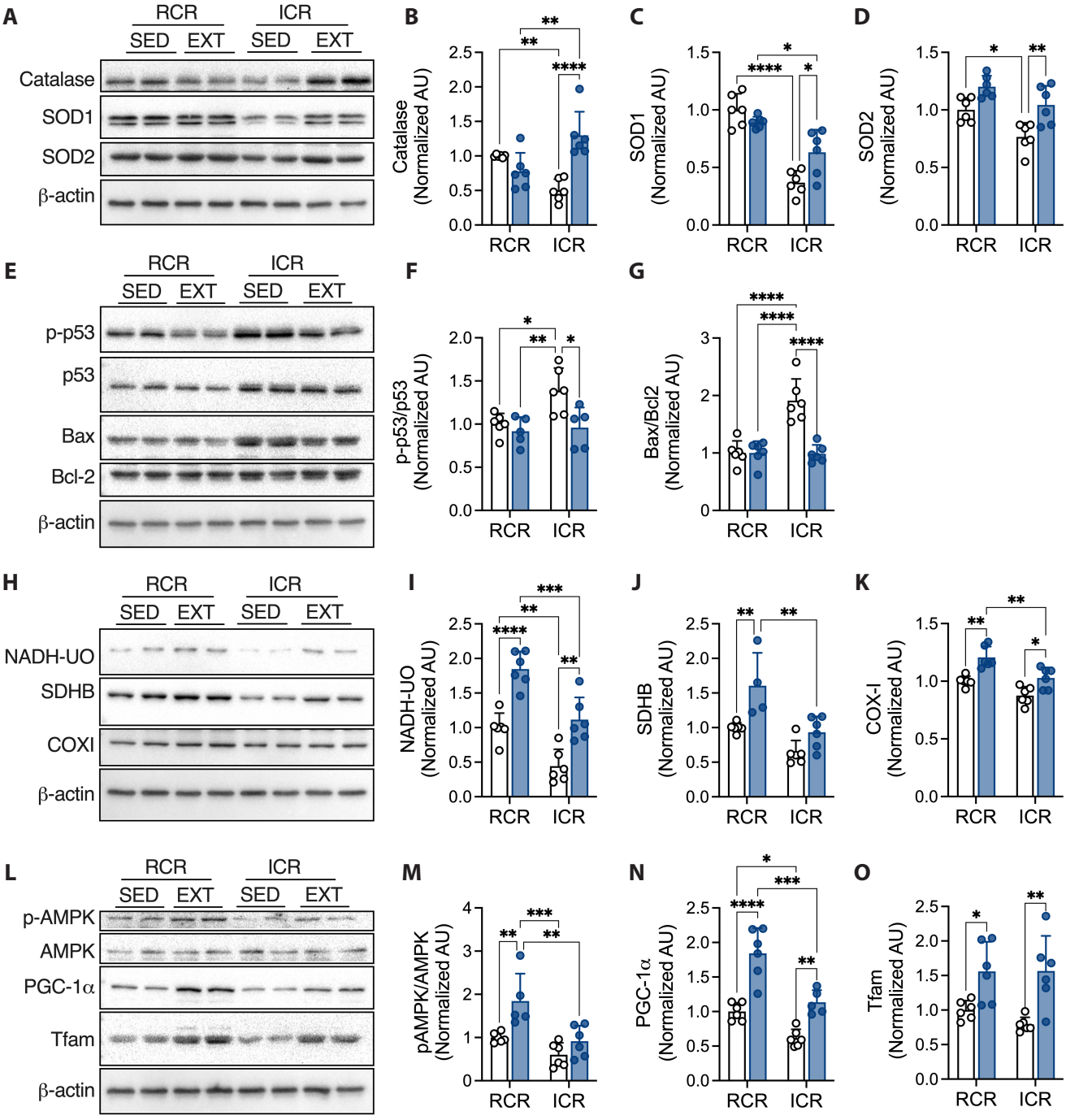

Fig. 5. EXT increased antioxidant expression, decreased apoptotic protein activity, and enhanced expression of proteins involved in mitochondrial biogenesis. Western blot analyses reveal (A-D) increased antioxidant levels stimulated a (E-G) decrease in apoptotic protein expression. (H-K) Results exhibit heightened expression of proteins involved in the electron transport chain and (L-O) proteins governing mitochondrial biogenesis. All data are presented as mean ± SEM. EXT, endurance exercise training; SED, sedentary; RCR, regular circadian rhythm; ICR, irregular circadian rhythm; SOD1 & 2, superoxide dismutase type 1 & 2; Bcl2, B-cell lymphoma 2; Bax, Bcl2 associated X; NADH, reduced nicotinamide adenine dinucleotide; SDHB, succinate dehydrogenase subunit B; COX-I, cytochrome oxidase (COX) subunit I; AMPK, AMP-activated protein kinase; PGC-1α, peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1alpha; Tfam, mitochondrial transcription factor A. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 as determined using two-way ANOVA and Tukey's *post-hoc* analyses (n = 6).

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EXT improves LDL-C and HDL-C without a change of the BMAL1

Based on the cholesterol data collected from human and animal studies, we are curious whether EXT improves circulating cholesterol levels and circadian clock proteins expression. Hence, EXT intervention was administered to SD rats under continuous RCR or ICR treatment (Fig. 4A). We found that the eight weeks of EXT did not improve BW (Fig. 4B). We expected that extending the CR disturbance by an additional 8 weeks, following the initial 12-week intervention, would further increase Epi/BW (Fig. 4C). Interestingly, the extended 8-week CR disturbance resulted in a decrease in Epi/BW, indicating no improvement in Epi/BW (Fig. 4C). TG levels were unchanged by an additional 8 week-CR disturbance or EXT intervention (Fig. 4D). Interestingly, FFA levels increased in EXT compared to SED in the RCR group, but no significant change in FFA levels in EXT was found in the ICR group (Fig. 4E). Contrary to increased TC levels during the initial 12-week intervention, EXT tended to decrease TC levels in both RCR and ICR groups, despite the lack of a significant difference (Fig. 4F). While the initial 12-week CR increased LDL-C levels, EXT significantly improved LDL-C levels in the ICR group, even though these levels were significantly higher compared to EXT levels in the RCR group (Fig. 4G). CR disturbance tended to decrease HDL-C, but EXT significantly increased HDL-C levels to baseline (Fig. 4H). Note, a t-test shows that inducing a further 8 weeks CR disturbance following a 12-week CR intervention resulted in significantly increased LDL-C levels and decreased HDL-C levels (Supplementary Fig. 2).

Dysregulation of BMAL1 gene in muscle is related to dyslipidemia [30]. Since EXT improved LDL-C and HDL-C levels in both ICR groups, we tested whether EXT improved circadian clock protein expression. A Western blot analysis indicates that neither an additional 8-week CR disturbance nor EXT increases PER2 expression with similar levels of PER2 being presented in both ICR and RCR groups (Fig. 4I, J). In contrast to PER2 expression, we observed that while EXT increases BMAL1 protein expression, administering EXT under CR disturbance did not clearly improve BMAL1 levels. Instead, BMAL1 levels seemed to significantly increase in the RCR group due to EXT (Fig. 4K). Taken together, EXT under CR disturbance improves dyslipidemia, as evident in changing LCL-C and HDL-C expression, without altering body composition and BMAL1 muscle gene expression. In this regard, our human study also showed that EXT participants in shift workers tend to reduce developing dyslipidemia (Supplementary Table 2).

EXT improves CR disturbance-induced mitochondrial biogenesis, antioxidants, and apoptosis-regulating proteins in SKM

Altering mitochondrial functions is associated with dyslipidemia [31]. Since EXT improved LDL-C and HDL-C levels under CR disturbance, we conducted Western blot analyses to determine if EXT could enhance the expression of protein-related mitochondrial biogenesis and function, which were impacted by CR disturbance.

We found that EXT clearly improved CR disturbance-induced Catalase, SOD1 and SOD2 levels (Fig. 5A–D). Not to mention, EXT recovered CR disturbance-induced phosphorylation of P53 and BAX/BCL2 levels (Fig. 5E–G). We also discovered that CR disturbance induced a reduction in electron transport chain protein expression, including NADH-UO, SDHB, and COX-I; however, EXT reversed these proteins to SED levels in the RCR group (Fig. 5H–K). While mitochondrial biogenesis regulating proteins, PGC-1α (Fig. 5N) and Tfam (Fig. 5O), recovered to normal levels due to EXT, AMPK phosphorylation (Fig. 5M) only showed a significant increase by EXT in the RCR group. All things considered, EXT enhances the expression of mitochondrial biogenesis, antioxidant, and apoptotic proteins affected by CR disturbance in SKM, except for the BMAL1 gene.

DISCUSSION

The physiology and biology induced by exercise and CR intervention are closely intertwined; however, the contribution of exercise in countering the effects of CR disturbance remains controversial. In this study, we have illustrated that CR disturbance leads to elevated dyslipidemia in both humans and rats. CR-induced decline in mitochondrial biogenesis and antioxidant capacity in SKM is counteracted by EXT, normalizing dyslipidemia without improving BMAL1 protein expression. Our human research also shows that implementing EXT tends to lower the risk of dyslipidemia development.

SKM of BMAL1 KO mice have been shown to exhibit functional deficits in contractile force, disrupted myofilament structure, and altered MyoD target gene expression [10]. MyoD directly binds to various metabolic genes involved in mitochondrial biogenesis, fatty acid oxidation, and the electron transport chain. Previous study demonstrates how MyoD regulates oxidative metabolism in SKM [32]. Additionally, our findings highlight that CR disturbance decreases mitochondrial biogenesis and antioxidants, potentially increasing apoptosis-related factors in muscle cells. While SKM is the main site of fat oxidation, during resting and fasting, fatty acids are the predominant fuel source in SKM [33]. Since SKM has a comparatively high capacity for fatty acid oxidation [34], metabolic dysfunction in SKM stands as a risk factor for dyslipidemia. We discovered that CR disturbance can trigger dyslipidemia in both humans and animals; notably, this dyslipidemia can manifest independently of obesity. However, we did not provide evidence that CR disruption decreases the capacity for fatty acid oxidation in skeletal SKM, and that these effects can directly lead to dyslipidemia. Further studies are necessary

to understand how CR disruption-induced dysfunction of SKM triggers whole-body dyslipidemia. Although we did not provide direct evidence, our findings partially indicate that CR disruptions can lead to dyslipidemia through distinct mechanisms separate from those associated with a high-fat diet. In this context, we propose that CR disturbance-induced mitochondrial dysfunction in SKM may contribute to the development of dyslipidemia.

EXT has been demonstrated to increase blood HDL-C levels through elevated lipoprotein lipase concentration and human SKM activity [35], which decreases LDL-C levels in blood. We showed that EXT under CR disturbance increases HDL-C levels and improves LDL-C levels to basal levels without improving PER2 and BMAL1 protein expression in SKM. Despite not fully restoring BMAL1 protein levels in SKM, EXT partially improved mitochondrial and antioxidant protein expression, as well as apoptosis-related proteins, alongside PGC-1α and Tfam. These data indicate that BMAL1 is necessary in preserving mitochondrial biogenesis at rest and during exercise. However, other mechanisms can be involved in exercise-induced mitochondrial biogenesis. Interestingly, Apolipoprotein A-I KO mice and transgenic mice studies have shown that HDL-C modulates muscle function *via* mitochondrial respiration [36]. This and our data suggest that exercise-induced HDL-C elevation may enhance SKM function and mitochondrial biogenesis through autocrine and endocrine pathways.

Mitochondrial functions, linked to ROS generation and apoptosis [37], are influenced by BMAL1, which can affect ROS levels tied to mitochondrial respiration [38]. P53 regulates cell growth and apoptosis [39], while Bax and Bcl2 control mitochondrial membrane permeability and apoptosis [27,40]. Our study reveals that reduced BMAL1 expression in muscles leads to lower antioxidants and elevated apoptotic proteins, potentially increasing apoptosis due to CR disturbance. However, as anticipated, EXT lowered apoptosis-related proteins to baseline levels despite not restoring BMAL1 protein levels. We posit that this is caused by improved mitochondrial antioxidant enzyme levels, such as catalase and SOD2, in SKM. These results are supported by research that exercise reduces the ratio of Bax to Bcl2, thereby regulating apoptosis and cell survival [22,41]. Furthermore, exercise is known to elevate the expression of SKM homeostatic enzymes, including antioxidant enzymes, while reducing oxidative stress levels [42,43]. Although the previous study demonstrated that BMAL1 KO in the skin increases ROS levels in those cells [38], this study has the limitation of not analyzing ROS levels and their induced apoptosis in the muscle. Therefore, further study is necessary to determine whether reduced BMAL1 in SKM directly increases ROS levels and apoptosis in those cells.

Our human and animal model (rats) studies demonstrated that EXT under CR disturbance improves mitochondrial biogenesis, antioxidant enzyme expression, and apoptosis in SKM, which in turn improves dyslipidemia. Indeed, CR-induced dyslipidemia can typically be reversed by altering sleep patterns. Despite sleep disruptions, consistent exercise can be a strategy to reduce the risk factors associated with dyslipidemia, potentially mitigating conditions like cardiovascular diseases, hypertension, diabetes, and vascular dementia.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary data including two tables and two figures can be found with this article online at https://doi.org/10.4196/kjpp.2024.28.6.515

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