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Original Article

MOLECULAR METABOLISM

Exercise mitigates sleep-loss-induced changes in glucose tolerance, mitochondrial function, sarcoplasmic protein synthesis, and diurnal rhythms

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ABSTRACT

Objective: Sleep loss has emerged as a risk factor for the development of impaired glucose tolerance. The mechanisms underpinning this observation are unknown; however, both mitochondrial dysfunction and circadian misalignment have been proposed. Because exercise improves glucose tolerance and mitochondrial function, and alters circadian rhythms, we investigated whether exercise may counteract the effects induced by inadequate sleep.

Methods: To minimize between-group differences of baseline characteristics, 24 healthy young males were allocated into one of the three experimental groups: a Normal Sleep (NS) group (8 h time in bed (TIB) per night, for five nights), a Sleep Restriction (SR) group (4 h TIB per night, for five nights), and a Sleep Restriction and Exercise group (SR+EX) (4 h TIB per night, for five nights and three high-intensity interval exercise (HIIE) sessions). Glucose tolerance, mitochondrial respiratory function, sarcoplasmic protein synthesis (SarcPS), and diurnal measures of peripheral skin temperature were assessed pre- and post-intervention.

Results: We report that the SR group had reduced glucose tolerance post-intervention (mean change ± SD, *P* value, SR glucose AUC: 149 ± 115 A.U., $P = 0.002$), which was also associated with reductions in mitochondrial respiratory function (SR: -15.9 ± 12.4 pmol $O_2.s^{-1}.mg^{-1}$, $P = 0.001$), a lower rate of SarcPS (FSR%/day SR: 1.11 ± 0.25%, $P < 0.001$), and reduced amplitude of diurnal rhythms. These effects were not observed when incorporating three sessions of HIIE during this period (SR+EX: glucose AUC 67 ± 57, $P = 0.239$, mitochondrial respiratory function: 0.6 ± 11.8 pmol $O_2.s^{-1}.mg^{-1}$, $P = 0.997$, and SarcPS (FSR%/day): 1.77 ± 0.22%, $P = 0.971$).

Conclusions: A five-night period of sleep restriction leads to reductions in mitochondrial respiratory function, SarcPS, and amplitude of skin temperature diurnal rhythms, with a concurrent reduction in glucose tolerance. We provide novel data demonstrating that these same detrimental effects are not observed when HIIE is performed during the period of sleep restriction. These data therefore provide evidence in support of the use of HIIE as an intervention to mitigate the detrimental physiological effects of sleep loss.

Keywords Sleep; Mitochondria; Glucose tolerance; Circadian rhythms; Exercise

1. INTRODUCTION

The detrimental effects of sleep loss on glucose tolerance are now well established, and insufficient sleep is a risk factor for the development of type 2 diabetes (T2D) [1]. In fact, sleep loss is comparable with other more traditional risk factors that are associated with the development of T2D, such as physical inactivity [1]. Several studies have shown that periods of sleep restriction, or reduced time in bed (TIB), typically with a sleep opportunity of 4–5 h per night, cause significant reductions in a range of indices related to glucose metabolism [2–5]. The severity of this effect can be seen with only one night of either sleep restriction (4 h TIB) or sleep deprivation (e.g., no sleep), which can reduce insulin

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sensitivity [6–9]. Despite these findings, there are limited data explaining the physiological and molecular changes that underpin these effects. As a large proportion of the population do not meet the current sleep recommendations (7–9 h per night) [10,11], and inadequate sleep is a consequence of many occupations [12,13], gaining a better understanding of these mechanisms may help to tailor specific interventions aimed at counteracting the detrimental effects of sleep loss. The physiological mechanisms that underpin the impairment of glucose tolerance following sleep restriction are likely multifactorial. While not previously investigated in the context of sleep loss, the development of insulin resistance has been associated with a reduction in mitochondrial content and impaired mitochondrial respiratory function [14,15]. Furthermore, reductions in citrate synthase activity (a surrogate marker for mitochondrial content [16]) and mitochondrial respiratory function has also been reported in T2D patients, compared to obese non-diabetics, suggesting a link between mitochondrial changes and the development of insulin resistance and T2D [14,15]. Therefore, sleep-loss-induced reductions in glucose tolerance may, in part, be a consequence of changes in mitochondrial content, function, or the processes that regulate these properties, including mitochondrial dynamics and mitochondrial protein synthesis [17]. In support of this, 120 h of sleep deprivation was associated with a 24% reduction in citrate synthase activity in human skeletal muscle [18]. However, how these results translate to the context of the sleep loss commonly experienced in society, such as repeated nights of partial sleep loss, has not been determined and remains a critical gap in the literature.

The detrimental effect of sleep loss on glucose metabolism may also be associated with the misalignment of circadian rhythms [7,8]. One night of sleep deprivation (commonly experienced by 20% of the world's population who perform shift-work) leads to a reduction in glucose tolerance and concomitant alterations in the expression of skeletal muscle clock genes (i.e., *Bmal1* and *Cry1* gene expression [7]) and the content of clock proteins (i.e., BMAL1) [8], which are known to regulate circadian rhythms at a molecular level [19]. The functional significance of disrupting the molecular clock has been shown in genetic mouse models (i.e., Clock mutant mice and the *Bmal1* KO mouse), which display reduced glucose tolerance, mitochondrial respiratory function, and skeletal muscle contractile function [20,21]. However, the effect of sleep restriction on markers of diurnal rhythms (e.g., skeletal muscle clock gene expression) and the potential implications of such changes have not previously been examined.

One approach to mitigate or ablate the impact of reduced sleep duration on glucose tolerance is via exercise [17]. Regular endurance exercise has been shown to exert beneficial effects on glycemic control via the activation of the contraction-mediated (insulin independent) glucose uptake signaling pathway [22]. High-intensity interval exercise (HIIE) is a time-efficient format of endurance exercise and also a potent stimulus for the induction of mitochondrial biogenesis [23], with increase in sarcoplasmic and mitochondrial protein synthesis and mitochondrial content and respiratory function, occurring concomitantly with improvements in glucose tolerance [24–27]. This raises the intriguing hypothesis that exercise may also be useful to combat sleep-loss-induced impairments to glucose tolerance, which are not necessarily reversed by a period of recovery sleep alone [28–30]. Furthermore, the same detrimental metabolic changes that occur in response to circadian misalignment and altered expression of clock genes may also be ameliorated by performing exercise [20,31,32]. Consequently, HIIE may mitigate the detrimental effects of sleep loss on glucose metabolism by increasing mitochondrial content and function and preventing changes in circadian rhythmicity [17].

Accordingly, the aim of this study was to investigate the effect of sleep restriction on glucose tolerance, and to examine the underlying physiological alterations that might contribute to these changes; specifically, we examined changes in mitochondrial content and function and diurnal rhythms. Furthermore, we examined the role of exercise as an intervention to mitigate the detrimental effects of sleep restriction. We hypothesized that sleep restriction would reduce mitochondrial content and respiratory function and disrupt diurnal rhythms, with a concomitant reduction in glucose tolerance, but that performance of HIIE would ameliorate these effects.

2. STUDY METHODOLOGY

2.1. Ethics approval

All procedures involved conform to the standards set by the latest revision of the Declaration of Helsinki (except for registration in a database) and were approved by the Victoria University Human Research Ethics Committee (HRE15-294).

2.2. Participants

Twenty-four healthy, recreationally active men, aged between 18 and 40 years of age, volunteered to participate. Eligible participants 1) were not taking any medications, 2) were not performing shift-work (within the previous three months), 3) had regular sleeping habits (6–9 h per night) and no previously diagnosed sleep disorders, 4) had not travelled overseas in the previous two months, and 5) had a body mass index (BMI) between 19 and 30.

2.3. Study overview

Eligible participants attended the exercise physiology laboratory for baseline anthropometric measurements (i.e., height and body mass), and aerobic fitness testing (peak oxygen uptake [$\acute{V}O_{2peak}$] and peak aerobic power [$\dot{W}_{Peak}$]) that was performed to volitional exhaustion on an electronically braked cycle ergometer (Excalibur, V2.0; Lode, Groningen, Netherlands) using an incremental ramp protocol (30 W/min). One week prior to the intervention, a resting skeletal muscle biopsy was obtained to determine baseline levels of deuterium oxide (D_2O) enrichment, and to assess basal mitochondrial respiratory function. Following baseline testing, the participants were allocated via minimization into one of the three experimental groups, in a counterbalanced order, so as to minimize differences in between-group baseline measures for age, BMI, habitual sleep duration, $\acute{V}O_{2peak}$, and mitochondrial respiratory function (Table 1).

The study consisted of an eight-night stay within a temperature-controlled sleep laboratory. All groups completed two initial nights of baseline sleep (8 h TIB from 23:00 h to 07:00 h), followed by a five-night intervention period, during which the Normal Sleep (NS) group spent 8 h TIB (23:00 h to 07:00 h), while both of the Sleep Restriction (SR) and Sleep Restriction and Exercise (SR+EX) groups spent 4 h TIB per night (03:00 h to 07:00 h). Between 23:00 h and 03:00 h, lighting was dimmed to below 15 lux to reduce the effect of lighting on circadian rhythms [33]. The SR+EX group also performed three exercise sessions during the intervention period on days 4, 5, and 6 at 10:00 h. Following the intervention period, all groups completed a final night of *ad libitum* recovery sleep. Participants were monitored throughout the protocol and provided with a standardized diet consisting of fixed proportions (relative to body mass) of carbohydrates (4.5 $g{\cdot}kg^{-1}{\cdot}d^{-1}$), protein (1.5 $g{\cdot}kg^{-1}{\cdot}d^{-1}$) and fat (1 $g{\cdot}kg^{-1}{\cdot}d^{-1}$). All mealtimes (six throughout the day) were kept constant. An overview of the study protocol is shown in Figure 1.

Table 1 — Baseline characteristics of participants.

	NS ($n = 8$)	SR ($n = 8$)	SR+EX ($n = 8$)	*P* Value
Age (y)	24 ± 4 (20, 30)	25 ± 5 (21, 32)	24 ± 4 (18, 29)	*P=0.754*
Height (cm)	177 ± 8 (169, 191)	179 ± 6 (173, 191)	179 ± 7 (172, 192)	*P=0.534*
Mass (kg)	78.7 ± 13.3 (62.8, 96.9)	74.5 ± 11.7 (63.8, 90.8)	80.2 ± 9.5 (61.4, 89.5)	*P=0.601*
BMI	25.2 ± 3.6 (20.3, 29.3)	23.3 ± 3.0 (20.0, 28.4)	24.6 ± 2.5 (20.8, 27.6)	*P=0.468*
$\dot{V}O_{2peak}$ (mL.kg^{-1}.min^{-1})	43.7 ± 9.7 (36.1, 64.8)	47.2 ± 6.7 (38.9, 56.9)	48.0 ± 5.0 (41.2, 56.6)	*P=0.486*
$\dot{W}_{peak}$ (W)	319 ± 59 (196, 399)	330 ± 44 (281, 415)	362 ± 48 (263, 425)	*P=0.242*
Habitual sleep duration (h:min)	7:37 ± 0:45 (6:36, 8:46)	7:08 ± 0:44 (6:15, 8:30)	7:17 ± 0:39 (6:18, 7:57)	*P=0.422*
Mitochondrial respiration (pmol.s^{-1}.mg tissue^{-1})	78.9 ± 17.7 (59.1, 113.7)	85.8 ± 25.3 (60.0, 133.3)	83.4 ± 19.7 (63.3, 122.0)	*P=0.802*

Values are mean ± SD, values in brackets are minimum and maximum values for each characteristic. There were no significant differences between the three groups for any of the baseline characteristics. NS — Normal Sleep, SR — Sleep Restriction, SR+EX — Sleep Restriction and Exercise, BMI — body mass index, $\dot{W}_{peak}$ — peak power (W), mitochondrial respiration — maximal ADP-stimulated oxidative phosphorylation with electron transfer via electron transfer flavoprotein, and complex 1 and 2, of the electron transport system (pre-study muscle biopsy).

2.4. Sleep and physical activity monitoring

Sleep was assessed for one week prior to and then throughout the study using wrist-watch activity devices (Actiwatch 2, Philips Respironics, Murrysville, PA, USA) [34]. Sleep architecture, duration, and quality were also determined via polysomnography (PSG) (Compumedics, AUS) on night 6 for a subset of participants from each condition ($n = 12$; 4 per group). Electrode placement for PSG monitoring was determined using the 10—20 electrode placement system and scored in accordance to standard criteria [35]. Habitual daily step counts were monitored using validated step-counting applications on the participants' personal mobile phone devices (i-Health app, Apple Inc., Cupertino, CA, USA; and Samsung Health, Samsung Electronics Co., Ltd., Suwon, South Korea) and these step counts were replicated throughout the study (Supplementary Table 1) (data modified from [36]).

2.5. High-intensity interval exercise (HIIE)

The HIIE protocol was adapted from previous studies, which demonstrated improvements in glucose tolerance [27,37] and consisted of 10 × 60-second intervals performed on a cycle ergometer (Velotron, Racer-Mate, Seattle, WA, USA) at 90% of each participant's $\dot{W}_{peak}$. Each interval was interspersed with 75 s of active recovery at 60 W. Each session started with a 3-minute warm up at 60 W. The mean power per interval was 318 ± 53 W, and the mean HR throughout the protocol was 156 ± 13 bpm.

2.6. Wrist skin temperature measurements and circadian statistical analysis

Wrist skin temperature was measured every 10 min at a sensitivity of +/− 0.0625 °C across two 48-h periods (pre-intervention - Days 2 and 3 (until 11 pm) and post-intervention - Days 6 and 7), using non-invasive temperature recording devices (iButtons, Thermochron iButton; Embedded Data Systems, Lawrenceburg, KY). This method has been shown to be reliable and valid for evaluating temperature circadian rhythmicity, with peripheral wrist skin temperature reported to have an inverse relationship to core body temperature [38,39]. As these measures were obtained under everyday life conditions, with

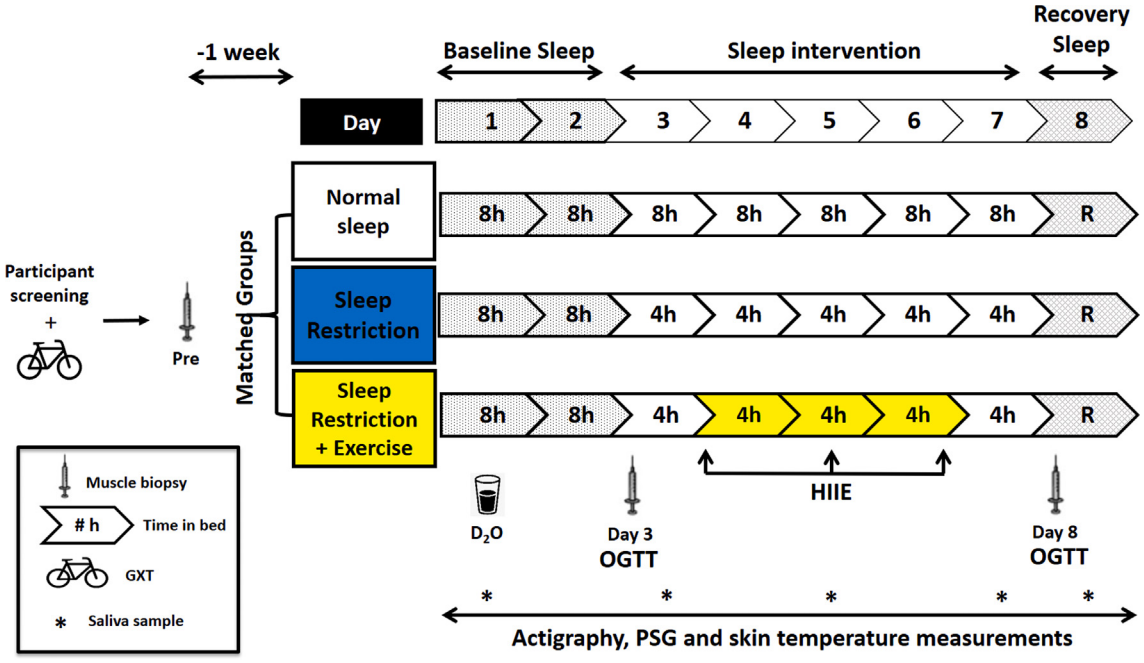

Figure 1: Schematic representation of the study protocol. OGTT — oral glucose tolerance test, GXT — graded exercise test, D_2O — deuterium oxide ingestion, HIIE — high-intensity interval exercise, R — *ad libitum* recovery sleep, PSG — polysomnography sleep monitoring, participant screening refers to medical questionnaires, exclusion criteria, and habitual sleep and physical activity monitoring.

regular meals and activity, they reflect diurnal rhythms, as opposed to circadian rhythms.

The diurnal analysis of the skin temperature data for each individual subject was performed with the 48 h of data for the pre- and post-intervention days. The data were fit with the following sine wave curve:

$$y_t = Amp \times \sin(F(t + Phase)) + Basal,$$

where t ($0 \leq t \leq 48$ [hours]) was the circadian time, y_t was the body temperature at time t, and the frequency F was fixed at 24-h period. *Amp*, *Phase*, and *Basal* were the amplitude, phase, and basal level of the sine wave curve, respectively. The amplitude is defined as the difference between the maximum (or minimum) value of the trace and the mesor across a 24-h period [42]; changes in amplitude have previously been proposed to provide an indication of the robustness of the rhythm [39,43]. The phase represents the time at which the temperature curve peaks, and basal for this dataset refers to the baseline skin temperature recordings over the 48 h. These measures represent the circadian parameters of the sinusoid fitting, which were estimated by a nonlinear least-square method using the R package *minpack.lm* [40]. We extracted the goodness-of-fit for the skin temperature data by the coefficient of determination R^2 ($0 \leq R^2 \leq 1$), with $R^2 = 1$ indicating perfect sinusoid fitting, and $R^2 = 0$ indicating no sinusoid fitting. The p-value of the sinusoid fitting was examined by F-test [41].

After obtaining the diurnal parameters for each subject, we performed statistical analysis of pre- vs. post-intervention values within each group (NS or SR or SR+EX). Pre-post *Amp*, *Basal*, and R^2 was compared at pre-visit (day 2—3) and at post-visit (day 6—7). The p-value for the pre-post comparison was obtained by a paired t-test, which captured the repeated measurement from the same subject.

To examine changes from pre-post in the diurnal parameters across intervention groups, linear mixed models were fit with subject specific random intercept by R package *lme4* [42], which accounts for the within subject correlation. Specifically, one of the parameters (*Amp*, *Basal* or R^2) was the outcome variable, intervention group, visit time (pre [day 2—3] or post [day 6—7]), and their interaction were the predictors. The p-value for the intervention interaction was used to benchmark the significance level for how pre-post changes in the circadian parameters differed by intervention group.

2.7. Oral glucose tolerance testing (OGTT)

To assess glucose tolerance, OGTT tests were performed on Day 3 (pre-intervention) and Day 8 (post-intervention) at 08:00 h, following an overnight fast. Participants consumed a 300-mL solution containing 75 g of glucose (Point of Care Diagnostics-Scientific, NSW, Australia), and blood samples were collected after 0, 10, 20, 30, 60, 90, and 120 min. Plasma glucose concentrations were measured on a glucose/lactate analyzer (YSI, 2300 STAT plus, Yellow Spring, OH, USA). Plasma insulin concentrations were assessed using an Insulin ELISA kit (ALPCO. 80-INSHU-E01.1, E10.1, Salem, NH, USA) and run according to the manufacturer's instructions.

2.8. Muscle biopsies

One week prior to commencing the study, and on Day 3 and Day 8 of the intervention, muscle biopsies were sampled from the *vastus lateralis* muscle using a suction-modified Bergström needle, and under local anesthesia of the skin and fascia (1% lidocaine). All samples were collected at 10:00 h. Samples on Day 3 and 8 were collected post OGTT. All samples were immediately frozen in liquid nitrogen and stored at −80 °C or set aside for mitochondrial respirometry.

2.9. High-resolution respirometry

Immediately following the muscle biopsies, muscle fibers were placed in ice-cold biopsy preserving solution (BioPS) and prepared as previously described [26]. Mitochondrial respiration was measured in triplicate (coefficient of variation [CV] = 12%) (from 2 to 4 mg wet weight of muscle fibers) in MiR05 at 37 °C using the high-resolution Oxygraph-2k (Oroboros, Innsbruck, Austria). Oxygen concentration (in nanomoles per milliliter) and flux (in picomoles per second per milligram) were recorded with DatLab software (Oroboros). The following substrate—uncoupler—inhibitor titration (SUIT) protocol was used to assess mitochondrial respiratory function; octanoyl-carnitine (0.2 mM) and malate (2 mM) for leak respiration (L) via electron transferring flavoprotein (ETF), (ETF)L, ADP (5 mM, saturating concentration) was added for the measurement of oxidative phosphorylation capacity (P), (ETF)P. Pyruvate (5 mM) was then added for measurement through Complex 1 (CI), (ETF+CI)P, followed by addition of succinate (10 mM) for the measurement of oxidative phosphorylation capacity through complex 1 and 2 combined (ETF+CI+II)P. Cytochrome C (10 mM) was then added to test for outer mitochondrial membrane integrity (an oxygen flux increase of <15% from (ETF+CI+II)P was considered acceptable). A series of stepwise carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) titrations (0.75—1.5 mM) for the measurement of ETS capacity (E) through (ETF+CI+II)E followed. Rotenone (0.5 mM), an inhibitor of CI, was then added to determine E through CII (ETF+CII)E, whereas the addition of antimycin A (2.5 mM), an inhibitor of CIII, allowed to measure and correct residual oxygen consumption (ROX), which is indicative of non-mitochondrial oxygen consumption. As there was no difference in mitochondrial respiration between 'Pre' and Day 3 resting biopsies (mean (ETF+CI+II)P pmol $O_2.s^{-1}.mg^{-1}$ ± SD; Pre — 81.4 ± 21.6 pmol $O_2.s^{-1}.mg^{-1}$, Day 3—80.6 ± 21.0 pmol $O_2.s^{-1}.mg^{-1}$, $P = 0.831$), 'Baseline' mitochondrial respiration was calculated as the mean from the 'Pre' study and Day 3 muscle biopsies.

2.10. Assessment of sarcoplasmic protein synthesis (SarcPS)

SarcPS was used as an indicator of mitochondrial protein synthesis (MitoPS), as performed previously [24], due to the large amount of muscle needed to assess MitoPS (e.g., 80—100 mg). In validating this approach, we measured CS activity (a validated biomarker of mitochondrial content [16]) in whole-muscle lysate, as well as the myofibrillar and sarcoplasmic fractions (Figure 5A). The whole-muscle CS activity was significantly higher than the myofibrillar fraction (mean difference CS activity (mol/h/kg protein) ± SD, 1.30 ± 0.48, CI [0.64, 1.97], $P < 0.001$). The sarcoplasmic fraction CS activity was also significantly higher than the myofibrillar fraction (2.05 ± 0.35, CI [-2.71, −1.39], $P < 0.001$) and whole-muscle fractions (0.74 ± 0.44, CI [-1.40, −0.07], $P = 0.027$) demonstrating that the sarcoplasmic fractions are enriched with mitochondria, compared to both the myofibrillar fraction and whole-muscle sample.

To determine SarcPS, on Day 1, each participant ingested 150 mL of deuterium oxide (D_2O) (70 atom %, Cambridge Isotope Laboratories) as previously described [43]. Saliva samples were collected prior to D_2O ingestion and then on Days 3, 5, 7, and 8 to determine body water enrichment via cavity ring-down spectroscopy (Picarro L2130-I analyzer, Picarro, Santa Clara, CA). Total body water ^2H enrichment was used as a surrogate for plasma alanine ^2H labelling, as previously described [43]. The mean body water enrichment (atom percent excess, APE) has been reported previously [36].

Frozen muscle samples (40—60 mg) were homogenized and prepared as previously described [44]. Cation exchange chromatography was then performed on the sarcoplasmic samples using columns containing Dowex resin (Dowex 50wx8-200 ion exchange resin, Sigma

Aldrich) to extract free amino acids from the sarcoplasmic fractions [44]. The amino acid samples were then derivatized as their N-acetyl-n-propyl-esters, as per previous protocols [45].

The $^{2}H/^{1}H$ ratio of the sarcoplasmic samples were determined using gas chromatography pyrolysis isotope ratio mass spectrometry (GC-P-IMS) (Metabolic Solutions, Nashua, NH, USA) to assess the incorporation of deuterium into protein-bound alanine. This was used to assess the fractional synthetic rate (FSR) of sarcoplasmic proteins with the use of the enrichment of body water, corrected for the mean number of deuterium moieties incorporated per alanine (i.e., 3.7) as previously described [43], as the surrogate precursor labelling between subsequent biopsies.

The following standard equation [43] was used to determine FSR:

$$FSR\ (\%/day) = ((E_{t1} - E_{t0})/(E_p \times time)) \times 100$$

where FSR = fractional synthetic rate, E_{t1} = APE Day 8, E_{t0} = APE Day 3, E_p = average saliva APE, time = time between biopsies, in days, and APE = atomic percentage excess.

2.11. Preparation of whole-muscle lysates for western blots and CS activity assay

Frozen muscle (10–20 mg) was homogenized as previously described [26] in an ice-cold lysis buffer (1:20 w/v) containing 50 mM of Tris, 150 mM of NaCl, 1 mM of EDTA, 1% IGEPAL, deionized water, and a protease/phosphatase inhibitor cocktail (Cell Signaling Technology (CST), Danvers, MA, USA), adjusted to pH 7.4. Protein concentration was determined in triplicate with a commercial colorimetric assay (Protein Assay kit-II; Bio-Rad, Gladesville, NSW, Australia) against bovine serum albumin standards (BSA, A9647; Sigma-Aldrich).

2.12. Citrate synthase activity assay

Citrate synthase (CS) activity was determined in triplicate on a 96-well microtiter plate by adding 7.5 μL of a 4 mg/mL muscle homogenate (freeze-thawed in liquid nitrogen twice), 40 μL of 3 mM acetyl CoA, 25 μL of 1 mM 5,59-dithiobis(2-nitrobenzoic acid) (DTNB), and 165 μL of 100 mM Tris buffer (pH 8.3, kept at 30 °C). After addition of 15 μL of 10 mM oxaloacetic acid, the plate was immediately placed in an xMark-Microplate spectrophotometer (Bio-Rad) at 30 °C, and after 30 s of linear agitation, absorbance at 412 nm was recorded every 15 s for 3 min. CS activity is reported as moles per hour per kilogram of protein.

2.13. Western blotting

Muscle homogenate was diluted in 4X Laemmli buffer, and equal amounts of total protein (15 or 20 μg) were loaded in different wells on Criterion™ 4–20% TGX Stain-Free™ precast gels (Bio-Rad, Australia). A stain-free system (Bio-Rad, Australia) was used as a loading control, with protein expression normalized to total protein loaded per lane (representative blot — Supplementary Figure 2). Each gel also contained four to six internal standards of varying dilutions, made from a mixed homogenate of every sample in equal concentrations. These standards were used to form a calibration curve, with density plotted against protein content. Protein abundance was then calculated from the measured band intensity for each sample on the gel, using the linear regression equation from the calibration curve.

Muscle lysates were separated via gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were then blocked in 5% non-fat dry milk diluted in Tris-buffered saline with 0.1% Tween-20 (TBST) for 60 min. Membranes were then incubated overnight at 4 °C with the appropriate primary antibody and prepared at a 1:1,000 dilution in TBST with 5% BSA and 0.02% sodium azide (unless stated otherwise). The following primary antibodies were from Cell Signaling Technologies (CST) and include PGC-1α (CST2178), DRP1 (CST5341), MFN2 (CST9482), p53 (CST2527), GLUT4 (CST2213). The following antibodies were obtained from Abcam, BMAL1 (AB93806) and Total OXPHOS (AB110413). The membranes were then incubated at room temperature with the appropriate host species-specific secondary antibody for 60 min, before being exposed to a chemiluminescence solution (Clarity™ Western ECL substrate [Bio-Rad, Hercules, CA, USA] or SuperSignal™ West Femto Maximum Sensitivity substrate [ThermoFisher, ThermoFischer Scientific, Wilmington, DE, USA]). Images were taken with a ChemiDoc Imaging System fitted (Bio-Rad). Densitometry was performed with Image Lab 5.0 software (Bio-Rad). Images are typically displayed with at least five bandwidths above and below the band of interest.

2.14. Real-time quantitative polymerase chain reaction (qPCR)

RNA extraction with TRIzol (Life Technologies, 15596 026) from frozen muscle samples (10–20 mg), quantification, and reverse transcription (iScript™ Reverse transcription supermix, BioRad) were performed as previously described [46]. Relative mRNA expression was measured by qPCR (QuantStudio 7 Flex, Applied Biosystems, Foster City, CA) using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). Primers were designed using Primer-BLAST to include all splice variants and were purchased from Sigma-Aldrich (Supplementary Table 3). The expression of each target gene was normalized to the geometric mean of expression of the three most stably expressed reference genes (as previously described) [46] using the $2^{-\Delta\Delta Ct}$ method (where Ct is the quantification cycle). Because of insufficient muscle sample collections for one participant, only samples from seven participants in the NS group were prepared for RT-PCR.

2.15. Statistical analysis

Statistical analyses were conducted using the statistical software package GraphPad Prism (V7.03). Pre- to post-intervention changes in gene expression, protein content, mitochondrial respiratory function, glucose tolerance, citrate synthase activity, and plasma insulin concentrations were assessed for each group using a mixed analysis of variance (ANOVA) with one between-subjects measure (group) and a within-subjects measure (time). Significant effects of interaction (group × time), time (pre vs post), and group (NS vs SR vs SR+EX) are reported where effects are seen. Where significant interaction effects occurred, Bonferonni post-hoc testing was performed to locate the differences. Where appropriate, a Greenhouse-Geisser correction was used to adjust for sphericity. All statistical analyses of gene expression and protein content data were conducted using raw values. Gene expression data in-text are reported as percent fold-changes from pre-intervention values (mean % ± SD) with 95% confidence intervals (CI) from fold-change data (as a percentage), with the *P* value from the raw data reported. Data in figures represent fold-changes from pre-intervention values, with individual responses. A one-way ANOVA was used to assess baseline differences between groups, mean values for actigraphy and SarcPS throughout the intervention, and the mean differences of changes from pre- to post-intervention between groups. All data in text, figures and tables are presented as mean ± standard deviation (SD) and 95% confidence intervals, with P values ≤ 0.05 indicating statistical significance. Exact P values are presented unless $P < 0.001$ or >0.999.

3. RESULTS

3.1. Sleep data

To verify the efficacy of our sleep interventions, we measured the participants' total sleep time (TST) via actigraphy [34]. There was a significant difference between groups ($P < 0.001$), with mean nightly TST during the intervention significantly lower for the SR (mean difference for TST ± SD min, 95% CI, P value, −220 ± 8 min, CI [202, 237 min], $P < 0.001$) and SR+EX (−214 ± 8 min, CI [197, 232 min], $P < 0.001$) groups compared to the NS group (Supplementary Table 1). There was no difference in nightly TST between the SR and SR+EX group (−5 ± 6 min, CI [-23, 12 min], $P = 0.718$). Polysomnography (PSG), considered the gold standard assessment of sleep [47], was used to confirm actigraphy TST data and to also assess sleep architecture on night 6 of the study (n = 4 per group) (Supplementary Figure 1). Both the SR and SR+EX groups obtained significantly less time in rapid eye movement (REM) sleep, non-rapid eye movement (NREM) stage 1 sleep, and NREM stage 2 sleep, compared to the NS group. Despite differences in TST between NS and both the SR and SR+EX groups, there were no significant differences in the absolute amount of sleep in the NREM stage 3 (N3) sleep between any of the groups (N3 sleep ± SD, NS = 72 ± 17 min, SR = 75 ± 18 min, SR+EX = 71 ± 14 min, $P > 0.05$) (Supplementary Table 2). Thus, as reported previously [2], N3 sleep was preserved despite the reduced total sleep time.

3.2. Wrist skin temperature analysis

Next, we used peripheral wrist skin temperature, obtained over two 48-h periods, pre- and post-intervention, to assess whether the significant reduction in TST in the SR and SR+EX groups was associated with changes in aspects of diurnal skin temperature rhythms, as previously suggested [7,8]. To assess the robustness of the diurnal skin temperature rhythms, we extracted the amplitude of the rhythms and the coefficient of fit to a circadian wave pre-intervention and post-intervention. We obtained basal temperature levels also to check for any significant change in the range of temperatures among the groups. As shown in Figure 2A, there were no differences observed in basal skin temperature either pre- or post-intervention or between groups. For wrist skin temperature diurnal amplitude, we observed significantly diminished amplitude in both the SR ($P = 0.008$) and SR+EX ($P = 0.008$) post-intervention results when compared to their pre-intervention amplitude (Figure 2B). The difference between pre- and post-intervention amplitude values for the SR group compared to the NS group approached significance ($P = 0.055$), but the pre-post amplitude difference between the NS and the SR+EX group did not ($P = 0.100$). This finding suggests that the inclusion of exercise may serve to help maintain diurnal amplitude in skin temperature (mean amplitude change ± SD °C, 95% CI °C; NS: -0.13 ± 0.59 °C, CI [-0.37, 0.62 °C]), SR: -0.74 ± 0.57 °C, CI [0.25, 1.22 °C]) and SR+EX: -0.60 ± 0.47 °C, CI [0.21, 0.99 °C]) (Figure 2B). We also analyzed R^2 (the coefficient of fit to the circadian wave form) as an assessment of diurnal rhythm strength based on its similarity between pre- and post-intervention. Similar to diurnal amplitude, we did not see a change in R^2 for the NS group, but did observe significantly decreased R^2 when comparing pre- and post-intervention values for SR ($P = 0.004$) and SR+EX ($P = 0.009$). However, when assessing the change in R^2 between groups we did not detect any differences (mean R^2 change ± SD, 95% CI; NS: -0.057 ± 0.21, CI [-0.11, 0.23]; SR: -0.20 ± 0.13, CI [0.09, 0.31; SR+EX: -0.20 ± 0.16, CI [0.07, 0.34] (Figure 2C). Individual temperature traces for each subject both pre- and post-intervention can be found in the supplementary data (Supplementary Figure 3).

3.3. Glucose tolerance

As sleep loss has been associated with impaired glucose tolerance, we examined plasma glucose and insulin concentrations in response to an OGTT, performed before and after the sleep interventions. There were no significant differences between groups for pre-intervention glucose ($P = 0.771$) and insulin ($P = 0.137$) area under the curve (AUC) values. There was a significant change in total glucose AUC (interaction $P = 0.002$, day $P = 0.003$, group $P = 0.100$) from pre- to post-intervention. Post-hoc analysis indicated that there was a significant increase of total glucose AUC in the SR group (pre- and post-intervention glucose AUC ± SD, [95% CI of mean difference], P value; pre: 678 ± 92 vs post: 827 ± 56 A.U., [54, 243 A.U.], $P = 0.002$), but not the NS group (pre: 677 ± 188 vs post: 617 ± 136 A.U., [−36, 154 A.U.], $P = 0.356$) or the SR+EX group (pre: 638 ± 50 vs post: 705 ± 71 A.U., [−162 to 28 A.U.], $P = 0.239$). There was also a significant difference between groups for mean change in glucose AUC from pre- to post-intervention ($P = 0.002$), with post-hoc analysis indicating that the mean change for the SR group was significantly different from the mean change for the NS group (mean change difference ± SD for NS vs SR; 208 ± 168, [74, 342 A.U.], $P = 0.002$). However, there were no differences in the mean change for glucose AUC between NS and SR+EX (126 ± 135 A.U., [−8, 260], $P = 0.069$) or SR and SR+EX (−81 ± 129 A.U., [−216, 52], $P = 0.381$). There was no significant change (interaction $P = 0.085$, day $P = 0.225$, group $P = 0.130$) in insulin AUC within any group pre- to post-intervention, despite a 29% increase in mean insulin AUC following the SR intervention (NS pre: 5,845 ± 3,548 vs post: 5,264 ± 2,072 A.U., CI [−2,037, 874 A.U.]; SR pre: 4,454 ± 2,233 vs post: 5,729 ± 1,983 A.U., CI [−180, 2,731 A.U.]; and SR+EX pre: 3,095 ± 1,766 vs post: 3,614 ± 1,854 A.U., CI [−937, 1,975 A.U.]) (Figure 3 and Supplementary Tables 6 and 7). There were also no between-group differences for insulin AUC mean change ($P = 0.085$).

3.4. Mitochondrial content, function, and protein synthesis

As both insulin resistance and reduced glucose tolerance have been linked to changes in mitochondrial characteristics, we investigated whether skeletal muscle mitochondrial respiratory function, content, and protein synthesis (via sarcoplasmic protein synthesis) were influenced by the sleep loss and exercise interventions. There were no significant differences between groups for pre-intervention mitochondrial respiration ($P = 0.691$). There was a significant change for maximal coupled mitochondrial respiration $(ETF+CI+CII)_P$ (interaction $P = 0.032$, time $P = 0.001$, group $P = 0.960$), which revealed a reduction from pre- to post-intervention in the SR group (pre: vs post mean value ± SD pmol $O_2.s^{-1}.mg^{-1}$, [95% CI for change], P value) in the SR group (pre: vs post: 88.4 ± 24.6 vs post: 72.5 ± 22.6 pmol $O_2.s^{-1}.mg^{-1}$, CI [−25.6, −6.1 pmol $O_2.s^{-1}.mg^{-1}$], $P = 0.001$) (18% decrease and a coefficient of variation (CV) of 12%) (Figure 4A). This was not evident in the NS (pre: 80.8 ± 14.0 vs post: 72.7 ± 19.6 pmol $O_2.s^{-1}.mg^{-1}$, CI [−1.6 to 17.9 pmol $O_2.s^{-1}.mg^{-1}$], $P = 0.122$) or SR+EX groups (pre: 81.2 ± 18.7 vs post: 80.5 ± 24.0 pmol $O_2.s^{-1}.mg^{-1}$, CI [−9.1, 10.4 pmol $O_2.s^{-1}.mg^{-1}$], $P = 0.997$). There were also between-group differences for mean change in respiration between pre- and post-intervention ($P = 0.032$), with post hoc analysis indicating a difference between the mean change in respiration for SR, compared to SR+EX (SR vs SR+EX mean change difference ± SD, 15.2 ± 17.1 pmol $O_2.s^{-1}.mg^{-1}$, [1.3, 29.1 pmol

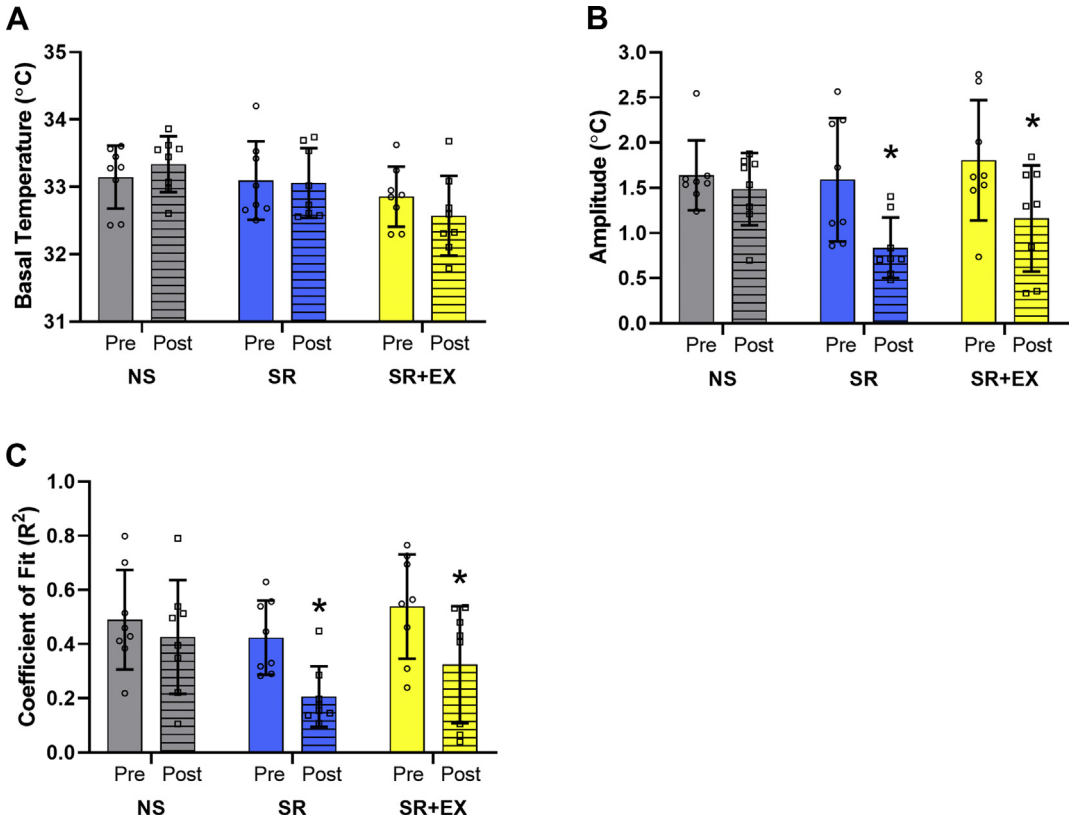

Figure 2: Measures of diurnal peripheral skin temperature A) basal skin temperature, B) amplitude, and C) coefficient of fit (R^2) pre- and post-intervention. Pre-intervention measurements are Day 2 and 3 (until 23:00 h) and the post-intervention measurements are Day 6 and Day 7. Normal Sleep (NS, $n = 8$), Sleep Restriction (SR, $n = 8$), and Sleep Restriction and Exercise (SR+EX, $n = 8$). * Denotes significant within-group differences from pre- to post-intervention ($P < 0.05$).

$O_2.s^{-1}.mg^{-1}$], $P = 0.029$), but not NS and SR (-7.7 ± 14.2 pmol $O_2.s^{-1}.mg^{-1}$, [-21.6, 6.2 pmol $O_2.s^{-1}.mg^{-1}$], P = 0.488) or NS and SR+EX (7.5 $\pm$ 13.7 pmol $O_2.s^{-1}.mg^{-1}$, [-6.4, 21.3 pmol $O_2.s^{-1}.mg^{-1}$], $P = 0.528$). These results show for the first time that sleep loss is associated with decreased mitochondrial respiratory function, which was not observed in the SR+EX group.

Mitochondrial content can be assessed by CS activity and the protein content of mitochondrial complex subunits [16]. There were no changes in whole-muscle CS activity from pre- to post-intervention for any of the groups (interaction, $P = 0.972$, time $P = 0.176$, group $P = 0.944$) (mean value $\pm$ SD, 95% CI, P value, NS pre: 2.81 $\pm$ 1.00 vs post: 2.71 $\pm$ 1.22 mol/h/kg protein, CI [-0.24, 0.44 mol/h/kg protein]); (SR pre: 2.82 $\pm$ 0.56 vs post: 2.69 $\pm$ 0.62 mol/h/kg protein, CI [-0.21, 0.47 mol/h/kg protein]) and (SR+EX pre: 2.69 $\pm$ 0.47 vs post: 2.60 $\pm$ 0.65 mol/h/kg protein, CI [-0.26, 0.43 mol/h/kg protein]) (Figure 4D). There were also no between-group differences for mean change of CS activity ($P = 0.917$). As a further validation of changes in mitochondrial content, the protein content for subunits of mitochondrial complexes were assessed via western blotting. No significant main effects were observed for the protein content of Complex 1, Complex 2, Complex 3, Complex 4, or Complex 5 (Figure 4E), collectively indicating that the sleep and exercise interventions did not affect mitochondrial content.

It has been argued that the best measure of mitochondrial biogenesis is mitochondrial protein synthesis (MitoPS) [48]. The sarcoplasm (skeletal muscle cytoplasm) is enriched with mitochondria (see Figure 5A), and protein synthesis within this fraction (sarcoplasmic protein synthesis - SarcPS) is likely reflective of MitoPS [24]. Therefore, for the first time, we assessed the effects of sleep restriction on SarcPS, which indicated that there was a significant difference between groups for fractional synthetic rate (FSR) ($P < 0.001$), with post-hoc testing indicating that FSR was significantly lower in the SR group compared to both the NS group (between groups difference FSR %/day $\pm$ SD, 95% CI, P value, -0.62 ± 0.11%, CI [-0.90, -0.33], $P < 0.001$) and the SR+EX group (-0.66 ± 0.12%, CI [-0.95, -0.37], $P < 0.001$); there was no difference in SarcPS between the NS and SR+EX groups (0.04 ± 0.10%, CI [-0.33, 0.24], $P > 0.999$) (Figure 5B). This new data suggest that participants in the SR+EX group maintained higher levels of SarcPS than those in the SR group.

3.5. Glucose, circadian, and mitochondrial-related gene expression and protein content

Given the changes observed for mitochondrial respiration, protein synthesis, circadian rhythm (in previous sleep studies [7,8]), and glucose tolerance, we investigated the expression of genes that regulate these processes (full list available in Table 2). With the remaining muscle biopsy tissue, we also assessed the content of proteins related to mitochondrial, circadian, and glucose-regulating processes. However, there were no significant differences within groups from pre- to post-intervention, or between groups ($P > 0.05$)

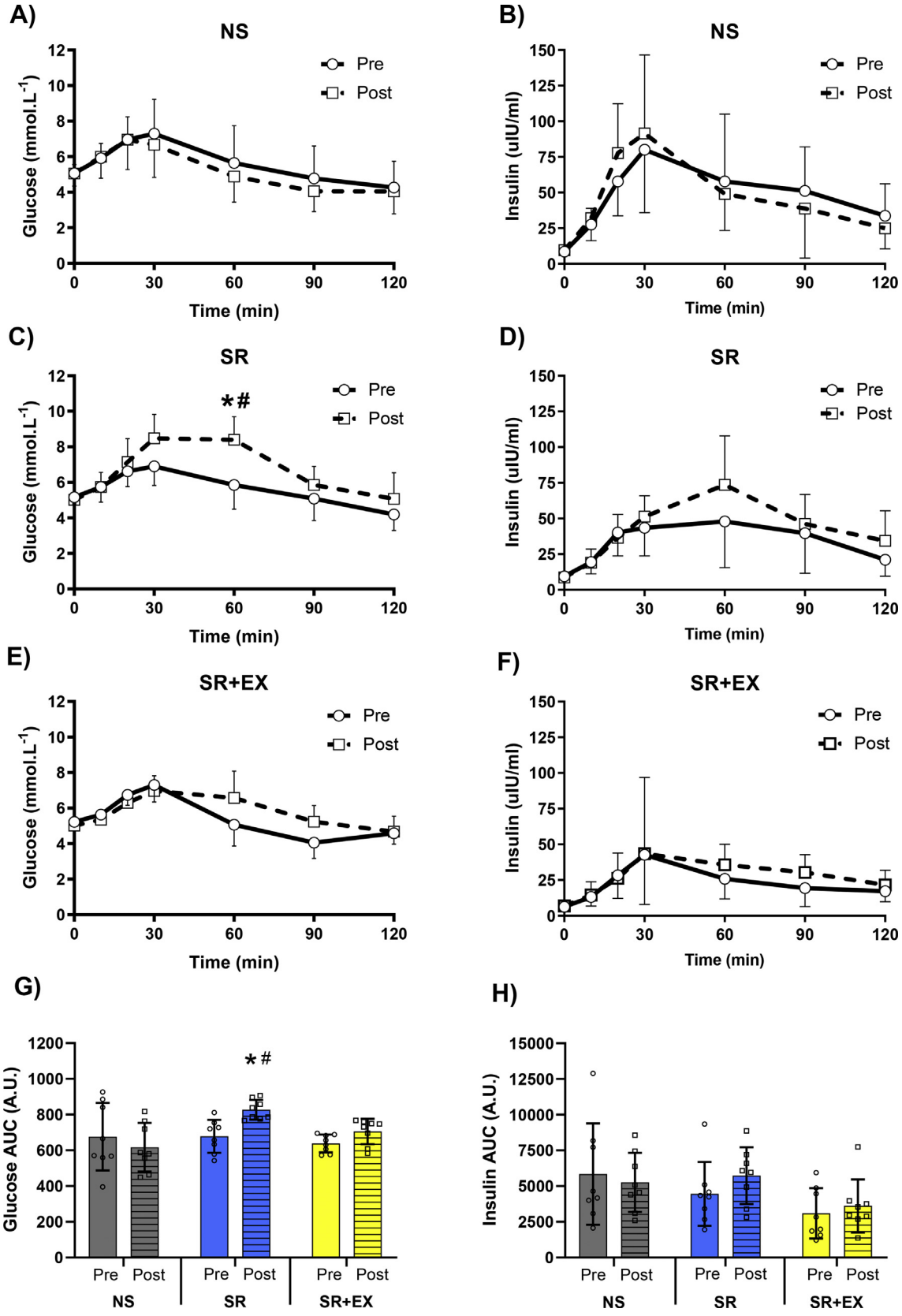

Figure 3: Plasma glucose and insulin concentrations for pre- and post-intervention oral glucose tolerance tests (OGTT). Plasma glucose and insulin concentrations throughout the 120-minute OGTT in the (A, B) Normal Sleep (NS), (C, D) Sleep Restriction (SR), and (E, F) Sleep Restriction and Exercise (SR+EX) groups. G) glucose and H) insulin total area under the curve (AUC) during the OGTT. Values are mean± SD, individual data points are shown, * Denotes significant within-group differences from pre- to post-intervention ($P < 0.05$). # Denotes significant between group difference for the change from pre- to post-intervention compared to the NS group ($P < 0.05$). $n = 8$ per group.

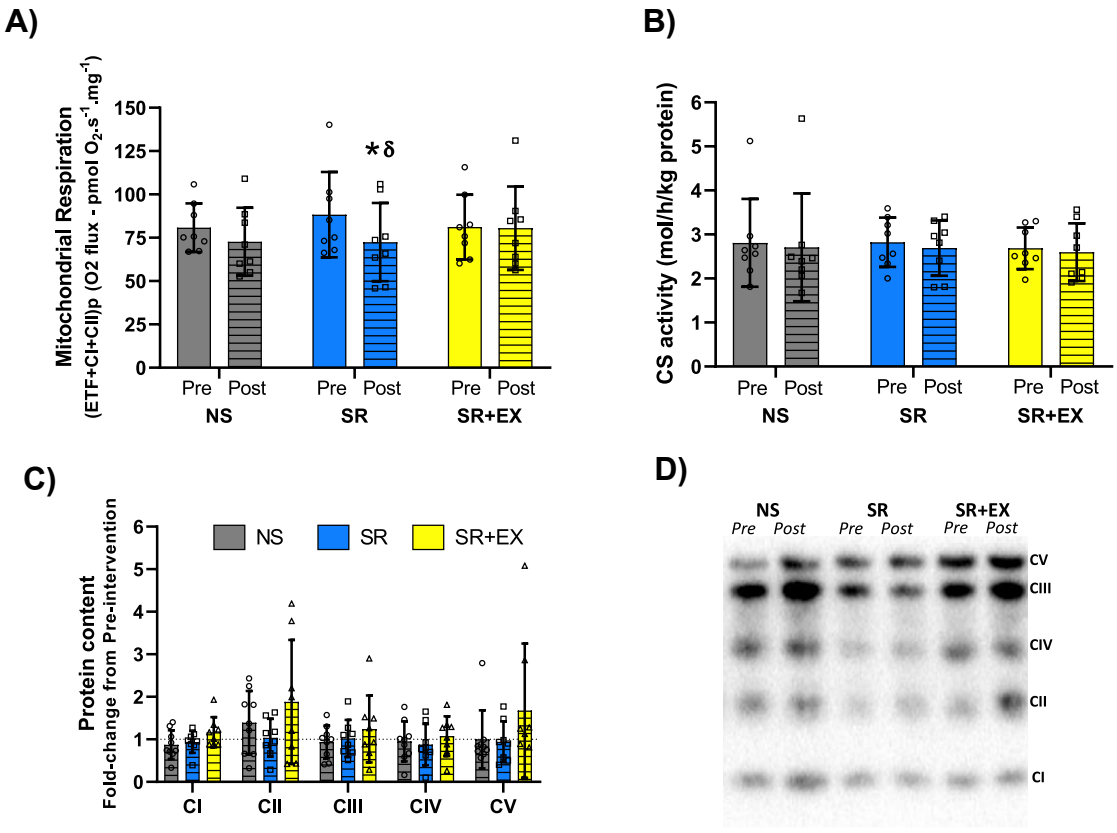

Figure 4: Mitochondrial respiratory function and markers of mitochondrial content from pre-intervention compared to post-intervention. A) Mitochondrial respiratory function in the Normal Sleep (NS), Sleep Restriction (SR), and Sleep Restriction and Exercise (SR+EX) groups. (B) Citrate synthase activity and (C) fold-change of protein content for subunits of mitochondrial complexes (I–V) from pre- to post-intervention. Mitochondrial complex 1 (CI) - NDUFB8, Complex 2 (CII) - SDHB, Complex 3 (CIII) - Core protein 2 (UQCCRC2), Complex 4 (CIV) – MTCO, and Complex 5 (CV) – ATP5A. (D) Representative image of protein content for mitochondrial complexes. $(ETF+CI+CII)_P$- maximal coupled mitochondrial respiration through ETF, CI and CII; Pre – pre-intervention, Post – post-intervention. $n = 8$ per group. * Denotes significant difference within group from pre- to post-intervention ($P < 0.05$). δ Denotes significant between group difference, for the change from pre- to post-intervention compared to the SR+EX group ($P < 0.05$).

for PGC-1α, DRP1, MFN2, p53, BMAL1, or GLUT4 protein content (Figure 6).

4. DISCUSSION

We discovered that in healthy young men, sleep restriction resulted in significantly impaired glucose tolerance, with concomitant changes in skeletal muscle mitochondrial respiratory function, sarcoplasmic protein synthesis (SarcPS) – a proxy for mitochondrial protein synthesis, and diurnal rhythms of peripheral skin temperature. However, in those performing three sessions of high-intensity interval exercise (HIIE) during the sleep restriction intervention, these same perturbations were not observed. Our study provides novel insights into the potential mechanisms underlying previously reported changes in glucose tolerance with sleep loss and suggests exercise may be used as a therapeutic intervention to attenuate such adverse effects.

The sleep restriction protocol used in this study (consisting of 4 h TIB per night, for five consecutive nights) resulted in a significant impairment in glucose tolerance. Furthermore, the plasma insulin response during the OGTT for the SR group increased by 29% from pre- to post-intervention (although not statistically significant). These results are consistent with previous studies [3–5], including Rao et al. [2] who reported a 25% decrease in whole-body insulin sensitivity (measured with a hyperinsulinemic-euglycemic clamp) following a similar sleep restriction protocol [2]. Therefore, the 22% increase in plasma glucose AUC following sleep restriction observed in young, healthy men in this study solidifies the evidence supporting the detrimental effect of even short periods of sleep restriction on glucose tolerance.

A novel aspect of this study was to examine the effect of three sessions of HIIE during the period of sleep restriction as a means of mitigating sleep restriction-induced reductions in glucose tolerance. In contrast to the SR group, adverse changes in glucose tolerance were not observed in the SR+EX group. While others have shown acute positive [49] and protective [50] effects of exercise on sleep-loss-induced changes in glucose tolerance, these studies assessed glucose tolerance either immediately after or within 24 h of exercise. This raises the possibility that these findings were confounded by the acute effects of exercise on glycemic control, which are known to persist for up to 48 h [22]. In contrast, we performed the OGTT 48 h post-exercise, thus demonstrating for the first time that the negative effects of sleep loss on glucose tolerance were not observed when performing HIIE during a period of sleep restriction.

At the molecular level, we reported a 33% change in *Glut4* mRNA (that was not statistically significant) in the SR group, with no change in GLUT4 protein. Our findings mirror those from Cedernaes et al. [8],

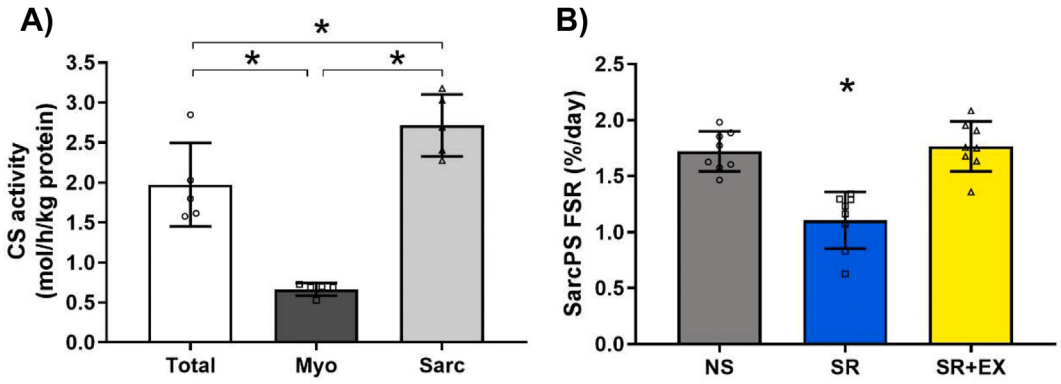

Figure 5: Citrate synthase (CS) activity from fractionated skeletal muscle samples and sarcoplasmic protein synthesis (SarcPS) – A) CS activity of whole-muscle lysate (Total), myofibrillar (Myo), and sarcoplasmic (Sarc) fractions were assessed from the same muscle samples ($n = 5$). B) Fractional synthetic rate (FSR) of SarcPS during the sleep intervention. Data are mean ± SD. Normal Sleep (NS), Sleep Restriction (SR), and Sleep Restriction + Exercise (SR+EX), $n = 8$ per group. * Denotes significantly different from other groups ($P < 0.05$).

who reported no significant change in *Glut4* mRNA or GLUT4 protein following one night of total sleep deprivation. While exercise is known to acutely increase skeletal muscle *Glut4* mRNA [22], we did not observe an increase in the SR+EX group. This may be explained by the timing of our muscle sample, which was collected 48 h post the final exercise session, by which time *Glut4* mRNA expression may have returned to baseline levels [22,51]. Therefore, while no changes to *Glut4* mRNA or protein content were observed in tandem with changes in glucose tolerance, it is plausible that GLUT4 translocation to the cell membrane may be impaired, which would support previous research that has indicated impairments within the insulin signaling pathway following sleep restriction [52,53].

Insulin resistance and reductions in glucose tolerance have previously been linked to altered mitochondrial characteristics [14,15,54]; therefore, we also assessed changes in skeletal muscle mitochondrial respiratory function. To our knowledge, our study is the first report of an SR-induced reductions in skeletal muscle mitochondrial respiratory function in humans. Previously, a study in humans investigating a single night of 4 h TIB reported a decrease in insulin sensitivity with a concomitant increase in plasma acylcarnitines [9], which was suggested to be indicative of both reduced fatty acid oxidation and impaired mitochondrial function. The maintenance of mitochondrial respiratory function in the SR+EX group is consistent with previous reports of the potency of HIIE for improving mitochondrial respiratory function [26,55]; however, we do not have a Normal Sleep and HIIE group that would allow us to ascertain whether HIIE enhanced mitochondrial function. Nonetheless, our results do provide evidence supportive of a link between reduced mitochondrial respiratory function and impaired glucose tolerance, which was not observed in the SR+EX group.

Table 2 – Glucose, circadian, and mitochondrial-related skeletal muscle mRNA responses.

Name	NS	SR	SR+EX	Interaction effect	Time effect	Group effect
Glucose metabolism-related mRNA						
Glut4	−14 ± 18	−38 ± 33	16 ± 83	P = 0.192	**P = 0.022**	P=0.988
Ampk1α	−17 ± 30	−1 ± 52	−15 ± 24	P = 0.952	P = 0.060	P=0.334
Pdk4	0 ± 105	124 ± 286	60 ± 111	P = 0.862	P = 0.850	P=0.995
β-Had	−30 ± 42	−25 ± 39	−15 ± 42	P = 0.872	**P = 0.011**	P=0.547
Circadian-related mRNA						
Bmal1	2 ± 22	−29 ± 33	7 ± 51	P = 0.154	**P = 0.041**	P=0.319
Clock	6 ± 75	−7 ± 45	−14 ± 37	P = 0.775	P = 0.407	P=0.189
Per1	−7 ± 44	−28 ± 48	2 ± 71	P = 0.527	P = 0.123	P=0.853
Per2	−4 ± 32	−7 ± 51	−6 ±48	P = 0.719	P = 0.410	P=0.653
Cry1	7 ± 45	12 ± 34	−10 ± 21	*P* = 0.466	*P* = 0.982	*P*=0.466
Rev-Erb α	−9 ± 38	−9 ± 47	−20 ± 42	*P* = 0.583	*P* = 0.130	*P*=0.231
Mitochondrial-related mRNA						
Pgc1α	−33 ± 51	−23 ± 49	14 ± 61	*P* = 0.552	*P* = 0.076	*P*=0.162
p53	48 ± 58	89 ± 100	6 ± 41	*P* = 0.523	*P* = 0.067	*P*=0.552
Tfam	−20 ± 33	−22 ± 41	−7 ± 59	*P* = 0.816	***P* = 0.035**	*P*=0.806
Nrf2	−1 ± 34	−11 ± 33	−4 ± 43	*P* = 0.777	*P* = 0.179	*P*=0.309
Dnm1l	−14 ± 45	−3 ± 65	−4 ± 72	*P* = 0.937	*P* = 0.170	*P*=0.625
Mfn1	6 ± 39	−12 ± 47	−5 ± 53	*P* = 0.414	*P* = 0.524	*P*=0.791
Mfn2	18 ± 28	−35 ± 28	2 ± 68	*P* = 0.562	***P* = 0.002**	*P*=0.901
Lc3b	4 ± 50	−16 ± 40	−11 ± 55	*P* = 0.486	*P* = 0.177	*P*=0.244
p62	39 ± 75	−1 ± 39	−8 ± 69	*P* = 0.088	*P* = 0.628	*P*=0.096
Pink1	−21 ± 34	−32 ± 45	2 ± 74	*P* = 0.895	*P* = 0.204	*P*=0.784
Cox4	−11 ± 22	−23 ± 28	−3 ± 65	*P* = 0.537	*P* = 0.088	*P*=0.609

Values are mean ± SD percent (%) changes from pre-intervention mRNA values. Normal Sleep (NS, $n = 7$), Sleep Restriction (SR, $n = 8$), and Sleep Restriction and Exercise (SR+EX, $n = 8$). Significant main effects ($P < 0.05$) are highlighted in bold. * Denotes significantly different from pre-intervention value ($P < 0.05$).

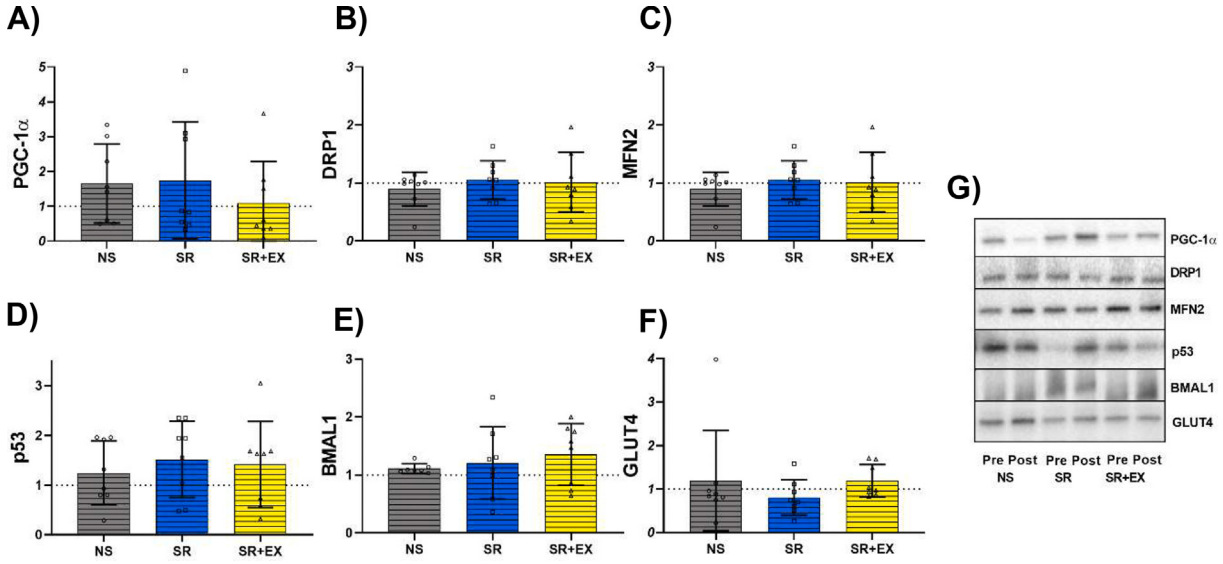

Figure 6: Skeletal muscle mitochondrial-, circadian-, and glucose-related protein content A) PGC-1α, B) DRP1, C) MFN2, D) p53, E) BMAL1, F) GLUT4, and G) representative western blot images. Data are mean values ± SD, normalized to pre-intervention values. Normal Sleep (NS), Sleep Restriction (SR) and Sleep Restriction and Exercise (SR+EX), $n = 8$ per group. * Denotes significant change from pre-intervention ($P < 0.05$).

Mitochondria are dynamic organelles, and the production of new mitochondrial proteins is an important determinant of their overall function [48]. Mitochondrial protein synthesis (MitoPS) is the measure that best reflects the process of mitochondrial biogenesis [23,48]. However, tissue availability for this project necessitated the use of sarcoplasmic protein synthetic rate (e.g., SarcPS — Figure 5A) as a proxy for MitoPS. For the first time, we report a lower rate of SarcPS in the SR group compared to both the NS and SR+EX groups (Figure 5B). The significance of the lower rate of SarcPS in the SR group is difficult to ascertain; however, we hypothesizes that it may underpin a reduced turnover of mitochondrial proteins, with a subsequent impact on mitochondrial respiratory function [48]. Support for this notion comes from the observation that changes in MitoPS and mitochondrial respiratory function have been shown to occur concomitantly [23,56]; nonetheless, a direct link is yet to be established. Moreover, HIIE has consistently been reported to increase both SarcPS and MitoPS [24,57], and this likely accounts for the higher rate of SarcPS in the SR+EX group compared to the SR group. Indeed, previous reports indicate that SarcPS remains elevated for 48 h following endurance exercise [58], and these increases are likely reflected in the integrative measure of protein synthesis used in this study, which represents a summation of SarcPS throughout the entire intervention.

Considering the changes observed to SarcPS, the effect of sleep restriction on markers of mitochondrial content was also assessed. There were no changes to the protein content of mitochondrial complex subunits or CS activity, both of which are valid markers of mitochondrial content [16]. While reduced CS activity has previously been reported following sleep interventions, this was in response to extreme sleep deprivation (i.e., 120 h of continuous wakefulness) [18]; thus, a direct comparison with our findings is difficult. Despite no change in CS activity or protein content of mitochondrial complex subunits in the SR+EX group, endurance exercise is well known to increase mitochondrial content [25]. Our results suggest changes in mitochondrial respiratory function and SarcPS occurred independently of detectable changes in mitochondrial content. This dissociation between changes in mitochondrial content and respiratory function has previously been reported, and it has been suggested that these properties may be differentially regulated [26,56]. One explanation for our observations may involve the processes regulating mitochondrial dynamics/remodeling (i.e., fission and fusion), which can alter the efficiency and oxidative capacity of the mitochondria, without necessarily altering mitochondrial content [23]. Therefore, we assessed genes and proteins known to be central to the regulation of mitochondrial remodeling (e.g., fission - Drp1, and fusion — Mfn1/2) [59]. However, despite a non-significant 35 ± 28% change in *Mfn2* mRNA in the SR group, we observed no changes in the molecular regulators of mitochondrial dynamics. Considering that exercise can also acutely influence markers of mitochondrial dynamics [59], this suggests the chosen muscle sampling time points may not have been appropriate to detect these potential changes [51].

As many of the molecular processes that regulate mitochondrial content, function, and dynamics are regulated in a circadian manner [60,61] and considering that molecular markers of circadian rhythm are altered by a night of sleep deprivation [7,8], we also examined aspects of diurnal rhythmicity using the measures of peripheral skin temperature (obtained over 48 h, pre- and post-intervention) and analysis of molecular clock gene expression. Peripheral skin temperature measures oscillate inversely to the rhythms of core body temperature, and therefore provides a physiological index of circadian output. We show that the amplitude of the temperature rhythms decreased significantly in both SR and SR+EX when compared to pre-intervention values. This is consistent with previous human studies demonstrating diminished temperature amplitude in other populations with disrupted sleep including shift work, metabolic syndrome, and sleep-disordered breathing [62—64]. In fact, similar to our study, Martinez-Nicolas et al. [63] demonstrated improved circadian temperature amplitude in response to a continuous positive airway pressure (CPAP) intervention, which suggests that physiological interventions can mitigate some circadian aspects of sleep disruption. Previously, Moller-Levet et al. [65] reported reduced amplitude of circadian expression of core clock genes following seven nights of sleep restriction (6 h TIB each night), in human white blood cells. However, at the skeletal muscle level, we did not see

any changes in circadian clock genes (despite a 29 ± 33%, reduction in *Bmal1* mRNA in the SR group). This is in contrast to previous findings from Cedernaes et al. [7,8] who reported a decrease in *Bmal1* mRNA expression in 15 participants following 24 h of sleep deprivation. This is potentially explained by the differences in length and severity of the sleep loss interventions and/or the difference in participant numbers and statistical power. We also report no alteration in skeletal muscle *Bmal1* mRNA in the SR+EX group. While others have reported exercise-induced changes in skeletal muscle clock gene expression [32,66], this is thought to be a time-of-day dependent effect [66]. Whether performing exercise at alternate times of the day might influence clock gene expression in human skeletal muscle differently remains to be elucidated. Considering the negative metabolic consequences, such as reduction in insulin sensitivity and mitochondrial function, attributed to both circadian misalignment and sleep loss [21,67] and the potential benefits of exercise in this context [17,68], further research is warranted.

This study has some limitations. Because of the extremely challenging and invasive nature of the protocol, a parallel group design was chosen. To offset the challenges associated with such designs for comparing results between groups, we adopted an approach that allowed us to closely match the groups for baseline characteristics (Table 1). Further, it is also important to note that muscle samples were collected following an OGTT to minimize any potential effect caused by the biopsy procedure on the participant's glycemic response. This may have altered molecular signaling pathways, although we did not observe any differences between the groups. Finally, the inclusion of a normal sleep and exercise group (NS+EX), would have helped to elucidate the contribution of HIIE to prevent the negative metabolic consequences observed in the SR group.

In summary, we have provided the first direct evidence of a concomitant decrease in mitochondrial respiratory function, SarcPS (of which MitoPS is a contributor), diurnal rhythms, and glucose tolerance, following sleep restriction in otherwise healthy young men. Despite this, we did not observe any alterations in mRNA or protein content associated with the regulation of these processes. Collectively, these results highlight several potential mechanisms by which sleep restriction may lead to reduction in glucose tolerance. Importantly, these same detrimental effects to mitochondrial function and SarcPS were not observed when HIIE was performed during the period of sleep restriction. While further research is still required, these data provide a basis for the development of evidence-based health guidelines and recommendations for those experiencing inadequate sleep by highlighting some of the underlying biological mechanisms that can be targeted by therapeutic interventions such as exercise.

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AUTHOR CONTRIBUTIONS

NS, GR, SP, DB, and JB were involved in the conception and design of the work, NS, ML, NP, AG, TS, JK, KE, ZH, ES, SP, DB, and JB were involved in the acquisition, analysis or interpretation of the data for the work. NS, ML, NP, AG, GR, TS, JK, KE, ZH, ES, SP, DB, and JB were involved in drafting the work and revising it critically for important intellectual content. All authors approved the final version of the manuscript. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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CONFLICT OF INTEREST

The authors have no competing interests to declare.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2020.101110.

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