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ORIGINAL RESEARCH

Intraperitoneal Administration of Cardiolipin Enhances Mitochondrial Respiration in Skeletal Muscle of Male Mice

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Purpose: Cardiolipin is a phospholipid located in the inner mitochondrial membrane and is released following myocardial ischemia–reperfusion injury. While cardiolipin has documented effects in several tissues, its impact on skeletal muscle function and mitochondrial respiration remains unclear. The purpose of this study was to investigate the effects of exogenously elevated cardiolipin on aerobic capacity and mitochondrial respiratory function in skeletal muscle using a mouse model.

Methods: Male C57BL/6 mice were randomized into an experimental group (n = 11) receiving cardiolipin injections and a control group (n = 12) receiving a placebo solution. Mice were injected twice weekly for 6 weeks with 0.1 mL of cardiolipin (0.5 mg/mL) or placebo. Voluntary running distance was monitored throughout the intervention. Aerobic capacity was assessed at baseline, week 3, and week 6 by measuring time to exhaustion during treadmill running at a constant speed of 16 m min^{-1}. Following the intervention, mice were euthanized, the vastus lateralis muscle was excised, and mitochondrial respiratory capacity was evaluated using high-resolution respirometry. Mitochondrial density was assessed by immunoblotting.

Results: Mice receiving cardiolipin exhibited increased skeletal muscle oxygen consumption compared with controls. No differences in mitochondrial density were observed between groups, suggesting that the enhanced oxygen consumption was not associated with increased mitochondrial content but may instead reflect alterations in mitochondrial respiratory efficiency.

Conclusion: Exogenously elevated cardiolipin is associated with enhanced skeletal muscle mitochondrial respiratory function without altering mitochondrial density, which may indicate improved mitochondrial efficiency. These findings provide novel insight into the potential role of cardiolipin in skeletal muscle energy metabolism and aerobic performance. Future studies should explore the combined effects of cardiolipin administration and exercise training on skeletal muscle respiratory capacity and further investigate the underlying mechanisms.

Keywords: cardiolipin, OXPHOS, ATP production, aerobic capacity, respiratory efficiency

Introduction

Cardiolipin (CL) is a phospholipid that constitutes up to 20% of the total phospholipid content of the inner mitochondrial membrane.[1,2] Its structure is distinct as it contains four acyl chains and a negatively charged head group composed of two phosphate groups and a glycerol bridge, resulting in a conical shape.[3,4] In addition to its implication in cristae morphology, CL plays a multifaceted role in mitochondrial-dependent apoptosis by facilitating the recruitment of essential factors.[3,5,6]

CL plays a crucial role by interacting with key proteins in the electron transport chain (ETC), such as cytochrome C, as well as complex I, III and V.[7] These interactions are essential for efficient energy production within the mitochondria.

Mitochondria are critical for aerobic performance as the generation of adenosine triphosphate (ATP) is required for sustained exercise. Reduced mitochondrial respiration can impair oxidative phosphorylation (OXPHOS), resulting in decreased energy production. Conversely, individuals with higher rates of respiration typically demonstrate improved aerobic performance.[8]

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Changes in CL composition and abundance, impact mitochondrial efficiency and have been linked to several disorders. In conditions characterized by a loss of CL, such as Barth syndrome, a destabilization of mitochondrial respiratory complexes hinders energy production and results in reduced aerobic capacity.[9] Similarly, a reduction in CL through modulation of the very-long chain fatty acid (VLCFA) pathway results in decreased coupled respiration in skeletal muscle.[10]

Conversely, elevated levels of anti-CL antibodies have been found in young post-myocardial infarction patients, suggesting that CL is released into the blood upon cardiomyocyte necrosis.[11] Increased concentrations of CL mimicking post-infarction physiological conditions have been shown to negatively affect endothelial cell migration and proliferation as well as angiogenic sprouting, therefore impeding blood vessel formation.[12] Moreover, an acute increase in CL resulted in a decrease in mitochondrial respiration in vascular smooth muscle, impairing the tissue's ability to oxidize glucose and fatty acid substrates.[13]

Although CL has been shown to have various effects on body tissues, the impacts of an increase in CL on skeletal muscle have not yet been explored. The purpose of this study was to examine the effects of a chronic increase in CL levels on functional aerobic capacity and mitochondrial respiration in skeletal muscle. It was hypothesized that exogenously increased CL concentrations would negatively affect oxygen consumption and ATP production, consequently decreasing aerobic performance.

Material and Methods

Animals

Male C57Bl/6 mice aged 6–7 months were obtained from the Concordia University breeding colony. Females were excluded to eliminate the potential impact of estrous cycle variations on exercise performance.[14] The mice were screened and selected based on their ability and willingness to run on a rodent treadmill at a speed of 4m/min before random assignment to the control (CON) or cardiolipin (CL) group.[15] The animals were housed individually in cages equipped with a running wheel under a 12:12 light/dark photoperiod. Environmental conditions were maintained at 22°C with relative humidity between 40–50%, in accordance with Canadian Council on Animal Care guidelines. Food and water were available ad libitum. All procedures were approved by the Animal Ethics Committee of Concordia University (#30000259) and performed in compliance with guidelines set forth by the Canadian Council on Animal Care.

Experimental Protocol

Over a 6-week period, the mice received two intraperitoneal injections per week (0.1 mL) containing CL at a concentration of 0.5mg/mL (CL group) or a placebo solution containing physiological salt solution [135.5 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl, 11.6 mM glucose, 11.6 mM HEPES (pH 7.35)] (CON group). The placebo solution was identical in composition to that used for CL preparation, with matched ionic composition, pH, and osmolarity. This CL dose resembles physiological concentrations.[16] Specifically, the selected dose lies within a range previously shown to modulate mitochondrial function while avoiding cytotoxicity and non-specific effects.[12,13]

Voluntary Wheel Running

Running wheels were connected to a bike computer and gathered data on voluntary running activity. Prior to injection, total distance run was recorded. The data was then assessed on a meters per day basis.

Functional Aerobic Capacity Assessment

An adapted rodent treadmill was used to carry out maximal endurance tests at baseline, week 3 and week 6.[15] The mice were subjected to a 10-minute warm-up period consisting of three 2-min bouts of exercise at a speed of 4m/min, 8m/min and 12m/min, respectively, followed by a 4-min bout at 16m/min. After a 2-min rest with the treadmill off, the treadmill was turned on for a 2-min rest period with auditory acclimatization. Then, three 2-min bouts of exercise at a speed of 4m/min, 8m/min and 12m/min, respectively, were repeated. Thereafter, the speed was increased to 16m/min and time to

exhaustion was recorded to the nearest minute. Exhaustion was defined as the moment when the mouse remained in contact with the brush found at the bottom of the treadmill for 10 seconds or more despite tactile motivation.

Tissue Permeabilization

After the 6-week period, the mice were euthanized by isoflurane anesthesia, CO_2 asphyxiation and cervical dislocation. The vastus lateralis muscle was immediately extracted and permeabilized for mitochondrial respiratory assessment. Upon extraction, the vastus lateralis muscle was rid of connective tissue and the fibers were gently separated with forceps. The isolated tissue incubated for 30 minutes on ice in 50 µg/mL saponin and 2mL BIOPS buffer solution [2.77mM CaK_2 EGTA, 7.23mM K2EGTA, 5.77mM Na2ATP, 6.56mM MgCl2·6H2O, 20mM Taurine, 15mM Na2Phosphocreatine, 20mM Imidazole, 0.5mM Dithiothreitol, 50mM MES (pH 7.1).[17] Subsequently, the tissue underwent two consecutive 10-minute washes in MiR05 buffer [0.5mM EGTA, 3.0mM MgCl2·6H2O, 60mM K-lactobionate, 20mM Taurine, 10mM KH2PO4, 20mM HEPES, 110mM Sucrose, 1g/L BSA (pH 7.1)].[18]

Mitochondrial Respiratory Measurements

Mitochondrial oxygen consumption was measured via high-resolution respirometry (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria). Approximately 2–2.5 mg of muscle tissue were added to 2mL of MiR05 buffer in each chamber. The experiments were carried out at 37°C in a hyper-oxygenated environment to prevent oxygen limitation. Malate (2mM), glutamate (10 mM) and pyruvate (6 mM) were added in succession to stimulate LEAK respiration across complex I and to build the proton gradient across the inner mitochondrial membrane. ADP (5mM) was then incorporated to initiate complex I-dependent OXPHOS respiration. The outer mitochondrial membrane integrity was assessed upon the addition of cytochrome c (10µM). Subsequently, succinate (10mM) was added to measure maximal respiration, followed by oligomycin (2µg/mL) to inhibit ATP synthase and observe maximal LEAK respiration. Lastly, a carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (0.25µM) titration was carried out to assess maximal uncoupled respiration. The high-resolution respirometry values were obtained from stabilized O_2 flux per mass (pmol/s/mg) readings following the addition of each substrate. Data was analyzed by DatLab 7.0 software. The ADP stimulation ratio was determined by dividing ADP by pyruvate values while the respiratory control ratio (RCR) was calculated by dividing succinate by oligomycin values.

Protein Extraction, Immunoblotting and Immunofluorescence

Immunoblotting with an antibody specific for the voltage-dependent anion channel (VDAC) was used to determine mitochondrial density. Cell lysates were extracted in lysis buffer containing (in mmol/L) NaCl 250, HEPES 50, glycerol 10%, Triton X-100 1%, MgCl2 1.5, EGTA 1, Na4P2O7 10, NaF 1, Na3VO4 800 mol/L, pH 7.5 and centrifuged at 13 000g for 10 min. Supernatant was collected and equal volumes of lysates were separated on a 10% SDS–PAGE and transferred to a nitrocellulose membrane (0.02 µm, Biotrace NT Nitrocellulose) using 10 mmol/L sodium tetraborate buffer. Following protein transfer, membranes were stained with Ponceau S (0.1%) to verify transfer efficiency and, after imaging, were used for total protein-based normalization in accordance with Moritz (2020). The membranes were then blocked in 3% BSA in TBS-T buffer (10 mmol/L Tris–HCl, pH 7.5, 150 mmol/L NaCl, 0.05% Tween 20) for 1h at room temperature followed by overnight incubation at 4 °C with primary antibody VDAC (1:2000, ab14734 Abcam). The blots were washed 2×10 minutes in TBS-T, incubated with horseradish-peroxidase-conjugated secondary antibodies (anti-mouse, ab6728; Abcam), and visualized with a chemiluminescence system (Immun-Star Chemiluminescent; 1705070; Bio-Rad, Mississauga, Ontario, Canada). The bands were analyzed using the ImageJ software.

Statistical Analysis

Data are presented as mean with 95% confidence intervals (CI), whereas figures display mean ± standard error of the mean (SEM) where appropriate. Baseline refers to the period before the intervention, whereas Week 1 indicates the first week of injections. Normality was assessed using the Shapiro–Wilk test in conjunction with visual inspection of Q–Q plots. Given the small sample size, between-group comparisons were performed using two-tailed Welch's t-tests without assuming equal variances between groups to compare the CON and CL groups for mitochondrial respiratory capacity

measurements, ADP stimulation ratio, respiratory control ratio (RCR), and mitochondrial density. A linear mixed-effects model (mixed-model ANOVA) was used to assess the effects of group, time, and their interaction on voluntary running distance and functional aerobic capacity, with subject included as a random effect to account for repeated measures. This approach was used to accommodate repeated measurements and any missing data. Post hoc multiple comparisons were performed using Tukey-adjusted tests. To assess whether voluntary wheel running influenced treadmill endurance performance, correlation analyses were performed using pooled data across groups to examine the relationship between average daily running distance over weeks 1–3 and 1–6, and corresponding functional aerobic capacity. Given the small sample size and potential deviations from normality, nonparametric Spearman rank correlations were used with two-tailed tests.

A p-value of < 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism (version 10.4.0; GraphPad Software, San Diego, CA, USA).

Results

Voluntary Running Distance

There was no significant difference in daily voluntary running distance between the CON (n = 12) and CL (n = 11) groups at any time point (Figure 1), with no significant main effect of group ($F(1, 21) = 0.048$, $p = 0.828$). The mixed-model ANOVA revealed a significant main effect of time ($F(2.69, 55.90) = 35.34$, $p < 0.0001$), indicating that running distance increased over the course of the study. No significant group × time interaction was detected ($F(2.69, 55.90) = 0.600$, $p = 0.600$), suggesting that the pattern of change over time was similar between groups. Values increased from 39.74 ± 5.85 m/day and 46.85 ± 8.25 m/day (mean ± SEM) during week 1 to 86.53 ± 11.91 m/day and 85.11 ± 6.28 m/day during week 2 for the CON and CL mice, respectively. Thereafter, CON mice exhibited daily running distances of 93.10 ± 8.51 m/day, 98.12 ± 8.36 m/day, 96.86 ± 7.85 m/day, and 91.13 ± 7.15 m/day for weeks 3 to 6, respectively, while CL mice demonstrated values of 94.15 ± 6.97 m/day, 92.14 ± 5.93 m/day, 94.34 ± 7.52 m/day, and 87.81 ± 6.40 m/day. Post hoc analyses confirmed that both CON and CL mice exhibited significantly higher running distances from week 2 onward compared to week 1 (all adjusted $p \leq 0.021$), with no significant differences between subsequent weeks or between groups at any individual time point (all adjusted $p \geq 0.436$).

Functional Aerobic Capacity

No significant differences in running capacity were observed between CON (n = 12) and CL (n = 11) mice at any time point (Figure 2). The mixed-model ANOVA revealed a significant main effect of time ($F(1.60, 33.65) = 28.16$, $p <$

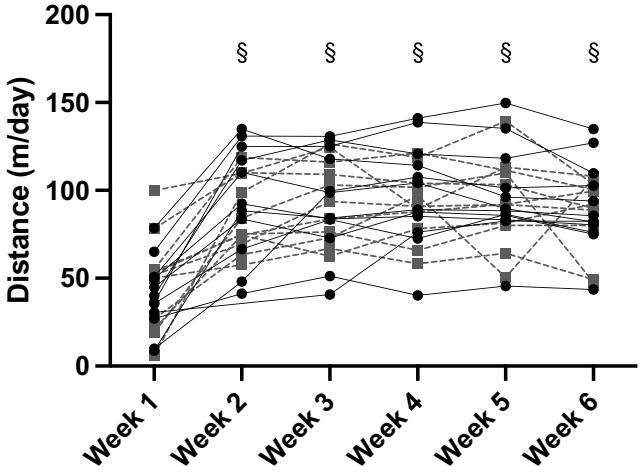

Figure 1 Voluntary running distance expressed in meters per day. No significance differences were found between CON (n=12) and CL (n=11). Black circles represent CON; grey squares represent CL. § denotes a statistically significant difference compared to week 1, where week 2 to week 6 mean values for both groups were found to be significantly increased.

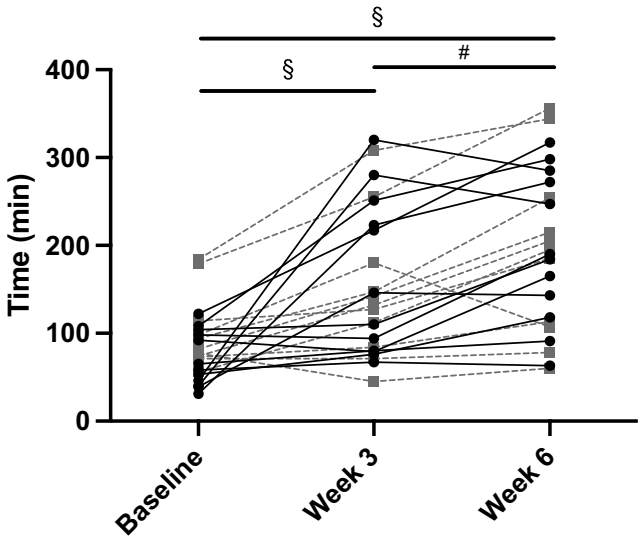

Figure 2 Time to exhaustion in minutes during the functional aerobic capacity assessment. No significant differences in running capacity were observed between CON (n=12) and CL (n=11). The increase in running capacity was significant over time. Values are expressed as mean. Black circles represent CON; grey squares represent CL. § denotes a significant change over time for both groups ($p< 0.05$). # denotes a significant change over time for the cardiolipin group only ($p<0.05$).

0.0001), indicating that running capacity increased over the course of the study. There was no significant main effect of group ($F(1, 21) = 0.008$, $p = 0.932$), nor a significant group × time interaction ($F(2, 42) = 1.20$, $p = 0.286$). Post hoc Tukey-adjusted comparisons demonstrated that both CON and CL mice exhibited significantly greater running times at week 3 (CON: 161.92 ± 26.28 min; CL: 145.73 ± 23.49 min) and week 6 (CON: 197.75 ± 24.63 min; CL: 192.00 ± 29.92 min) compared to baseline (CON: 71.33 ± 9.11 min; CL: 100.18 ± 12.99 min; all adjusted $p \leq 0.025$). No differences between groups were observed at baseline ($p = 0.0856$), week 3 ($p = 0.6507$), or week 6 ($p = 0.8835$). Within-group comparisons indicated a further increase in running capacity from week 3 to week 6 in the CL group ($p = 0.0345$), whereas this change did not reach statistical significance in CON mice ($p = 0.0616$). However, this occurred in the absence of a significant group × time interaction.

Average running distance was not correlated with treadmill endurance performance at either week 3 (weeks 1–3: Spearman $r = 0.11$, 95% CI: −0.34 to 0.52, $p = 0.619$) or week 6 (weeks 1–6: $r = -0.09$, 95% CI: −0.50 to 0.35, $p = 0.684$), indicating that functional aerobic capacity was not explained by voluntary running activity.

Mitochondrial Respiratory Assessment

High resolution respirometry showed a statistically significant increase in oxygen consumption for the animals that received exogenous CL (n=11) versus CON (n=12) (Figure 3. displays the results, Figure 4. is a representative graph). All datasets met normality assumptions based on Shapiro–Wilk testing, except for the CL ADP condition. However, visual inspection of Q–Q plots indicated only minor deviation from normality, and parametric analyses were therefore retained (Supplementary Table S1 and Supplementary Figure S1). During LEAK respiration, oxygen flux was significantly elevated in the CL group following addition of malate (3.04 vs 0.63 pmol s^{-1} mg^{-1}; $t(18.86) = 4.34$, $p = 0.0004$; mean difference = 2.42, 95% CI [1.25, 3.58]), glutamate (8.04 vs 4.11 pmol s^{-1} mg^{-1}; $t(12.76) = 3.92$, $p = 0.0018$; mean difference = 3.92, 95% CI [1.75, 6.09]), and pyruvate (12.67 vs 6.78 pmol s^{-1} mg^{-1}; $t(11.97) = 3.25$, $p = 0.0070$; mean difference = 5.89, 95% CI [1.94, 9.84]). ADP-stimulated Complex I-dependent respiration was also significantly higher in the CL group compared to CON (46.39 vs 30.68 pmol s^{-1} mg^{-1}; $t(19.23) = 2.31$, $p = 0.032$; mean difference = 15.71, 95% CI [1.49, 29.94]). Maximal respiratory capacity, assessed following succinate addition, remained significantly elevated in CL animals (68.70 vs 45.40 pmol s^{-1} mg^{-1}; $t(20.80) = 2.94$, $p = 0.0078$; mean difference = 23.30, 95% CI [6.83, 39.77]). Similarly, oligomycin-induced maximal LEAK respiration was higher in the CL group (38.55 vs 28.32 pmol s^{-1} mg^{-1}; $t(19.85) = 3.45$, $p = 0.0026$; mean difference = 10.23, 95% CI [4.04, 16.42]). Lastly, FCCP-induced maximal uncoupled respiration was also significantly greater in CL compared to CON (63.84 vs 48.92 pmol s^{-1} mg^{-1}; t

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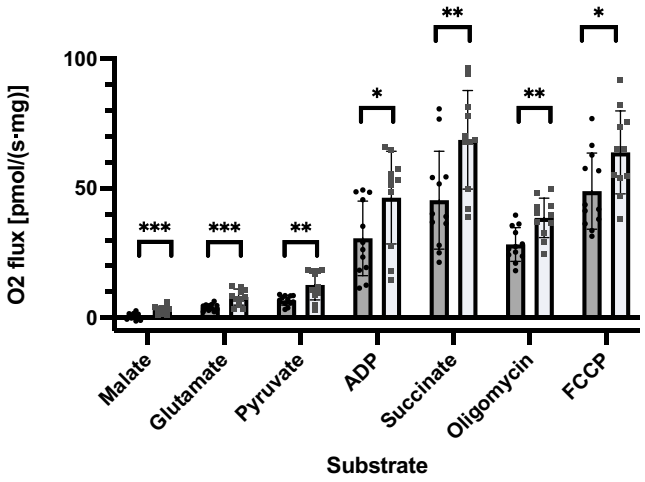

Figure 3 Mitochondrial respiratory capacity of permeabilized vastus lateralis muscle fibers measured by O_2 flux per milligram of tissue [pmol/(s·mg)]. The CL group (n=11) showed significantly increased rates of respiration for all substrates when compared to CON (n=12). For each substrate, independent t-tests were carried out to compare O_2 flux between groups. Values are expressed as mean ± SEM. Black circles represent CON; grey squares represent CL. *P<0.05, **P<0.01, ***P<0.001.

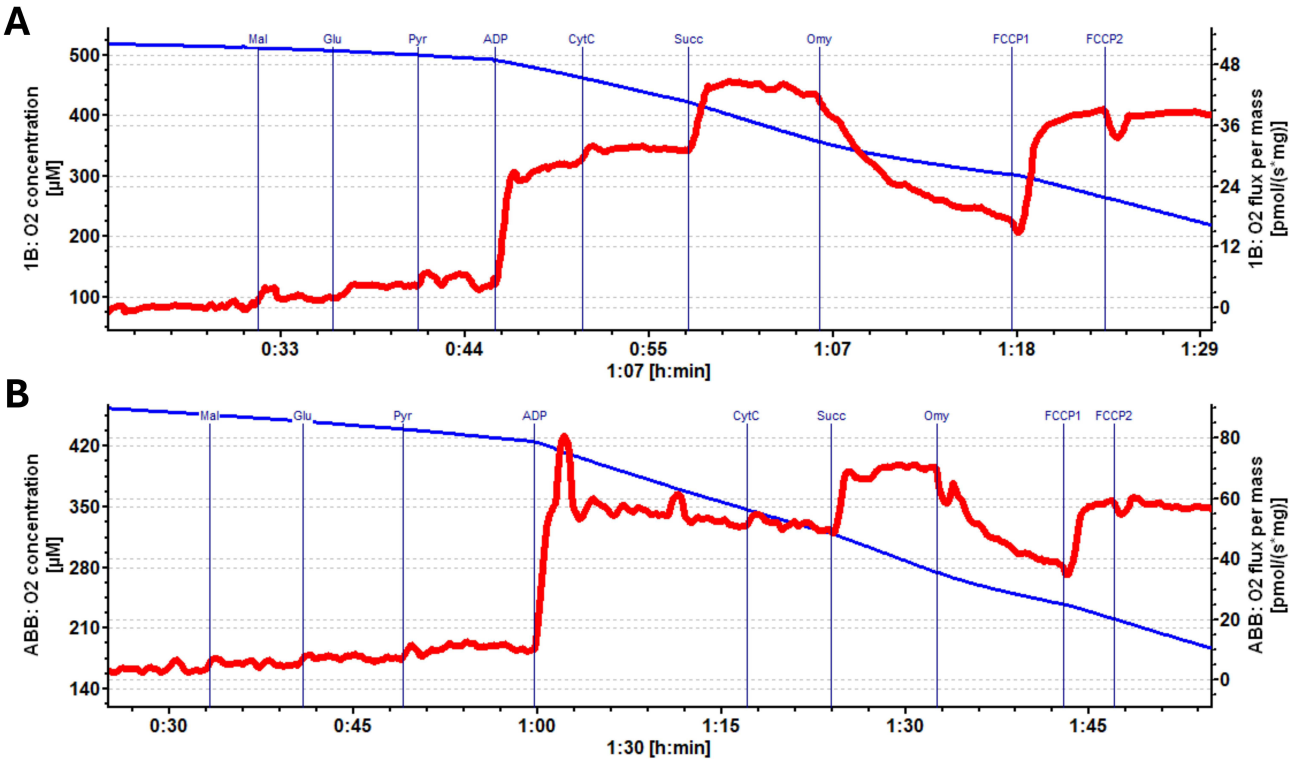

Figure 4 Representative trace of the respiratory flux from the mitochondrial respiratory assessment by high-resolution respirometry for CON (**A**) and CL (**B**). The blue line (y-axis, left) is oxygen concentration in the chamber. Red line (y-axis, right) is oxygen flux in the chamber. Addition of substrates and inhibitors as indicated in the figure. **Abbreviations**: Mal, malate; Glu, glutamate; ADP, adenosine diphosphate; CytC, cytochrome C; Succ, succinate; Omy, oligomycin; FCCP1 and FCCP2, represent an FCCP titration.

(20.38) = 2.33, p = 0.0305; mean difference = 14.92, 95% CI [1.55, 28.29]). Cytochrome c addition resulted in minimal changes in respiration, with increases remaining below 10%, indicating preserved mitochondrial membrane integrity across samples.

No significant differences were observed in the ratio between oxidative phosphorylation and LEAK respiration across complex I. The ADP stimulation ratio was 4.79 ± 0.72 in the CON group and 4.31 ± 0.74 in the CL group (t(20.89) =

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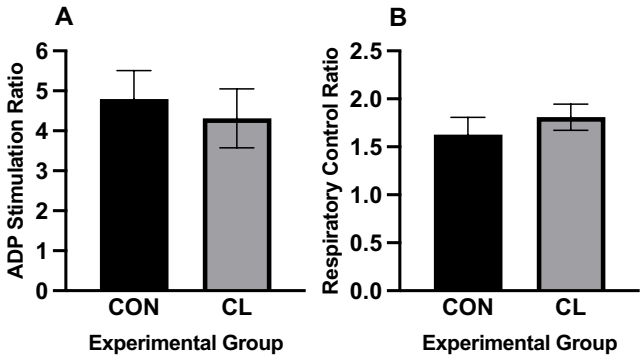

Figure 5 ADP stimulation ratio (**A**) and respiratory control ratio (RCR) (**B**). Both showed no difference between CON (n=12) and CL (n=11). An independent t-test was carried out to compare the ratios between groups. Values are expressed as mean ± SEM. Black represents CON; grey represents CL.

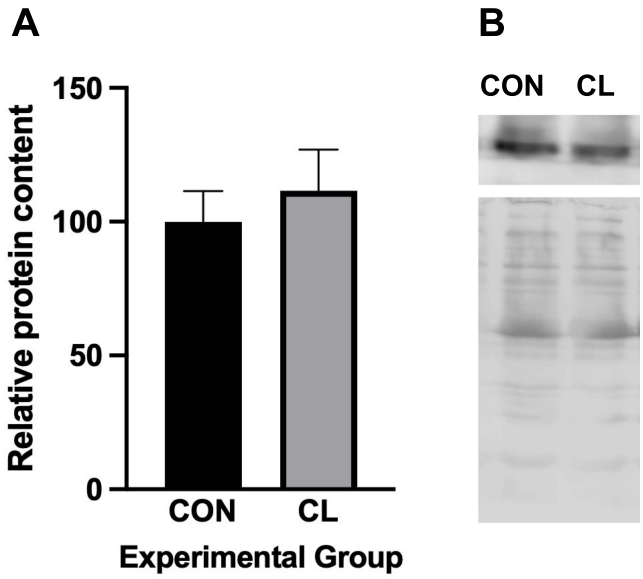

Figure 6 Determination of mitochondrial density using the voltage-dependent anion channel (VDAC) expression. No significant difference was observed between CON (n=11) and CL (n=11) groups (**A**). Representative blot and loading control (**B**). Values are expressed as mean ± SEM. Black represents CON; grey represents CL.

0.47, p = 0.646; mean difference = −0.48, 95% CI [−2.62, 1.66]; Figure 5A). Similarly, assessment of overall mitochondrial health via RCR showed no significant difference between groups, with a ratio of 1.63 ± 0.18 in CON mice and 1.81 ± 0.14 in CL mice (t(20.38) = 0.81, p = 0.428; mean difference = 0.18, 95% CI [−0.29, 0.65]; Figure 5B).

Mitochondrial Density

Immunoblotting using a VDAC-specific antibody revealed no significant difference in mitochondrial content between groups (CON: 100.0 ± 19.1 vs. CL: 111.5 ± 19.1, n = 11 per group; unpaired *t*-test, t = 0.60, df = 20, p = 0.55). The mean difference between groups was 11.55 (95% CI: −28.49 to 51.00), indicating no detectable effect of CL on mitochondrial density (Figure 6). One CL sample was excluded due to methodological error.

Discussion

This study set out to examine the effects of exogenously increased CL levels on aerobic capacity and skeletal muscle mitochondrial respiration in a male mouse model. Contrary to our hypothesis, the main finding of this research is that mice that received CL via intraperitoneal injection exhibited an increase in mitochondrial respiratory capacity in the vastus lateralis.

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In accordance with previous findings that CL supplementation can rescue respiratory coupling efficiency in isolated mitochondria with CL deficiency, our study highlights that CL content plays a prominent role in mitochondrial function in skeletal muscle.[10] The overall increase in OXPHOS (Figure 3) further supports that CL has a positive effect on bioenergetics.

Exogenous CL administration resulted in a robust enhancement of mitochondrial respiratory function, as evidenced by consistently higher oxygen consumption rates across multiple respiratory states. Specifically, CL-treated animals exhibited significantly elevated LEAK respiration following the addition of malate, glutamate, and pyruvate, suggesting an increased basal electron flux through Complex I-supported pathways. This effect extended to ADP-stimulated respiration, indicating that CL supplementation augments oxidative phosphorylation capacity under phosphorylating conditions. In addition to Complex I-supported respiration, maximal respiratory capacity was also enhanced in the CL group following succinate addition, reflecting improved convergent electron flow through both Complex I and II. Similarly, elevations in oligomycin-induced LEAK respiration and FCCP-induced uncoupled respiration further support the notion that CL enhances the overall capacity of the electron transport system. In addition, the large effect sizes observed across these comparisons underscore the substantial impact of CL on mitochondrial bioenergetics.

Our data demonstrate an increase in oxygen flux across substrates, however, the mechanisms underlying this observation were not directly assessed in the present study. Though, it can be hypothesized that CL contributes to the generation of the proton gradient, as reflected by the significant increase in oxygen consumption observed in response to malate, glutamate and pyruvate. One proposed mechanism is that CL acts as a proton trap within the mitochondrial membrane.[19] As such, a sequestration of hydrogen ions would contribute to building the proton gradient, which may also explain the significant increase in LEAK respiration seen upon the addition of oligomycin, an inhibitor of ATP synthase. Similarly, a greater concentration of hydrogen ions within the intermembrane space would result in a greater amount of uncoupled respiration.[20] This could explain the significantly increased rates of oxygen consumption observed in the CL group upon the addition of FCCP. Collectively, these findings suggest that exogenous CL enhances mitochondrial respiratory capacity in a physiologically meaningful manner, without adversely affecting mitochondrial quality or efficiency. In addition, CL plays a central role in the formation and stability of mitochondrial supercomplexes, which are structures that increase the efficiency of the ETC and reduce the rate of reactive oxygen species production.[21,22] The presence of exogenous CL may enhance electron flow, which could explain the significant increases in ADP-stimulated complex I respiration and succinate-induced maximal respiration observed throughout this experiment.

The ADP stimulation ratio and RCR, which compare oxidative phosphorylation to LEAK respiration across complex I and complex I+II, respectively, indicated no change in overall mitochondrial health between the two groups (Figure 5A and B). This indicates that while CL enhances mitochondrial capacity, it does not compromise coupling efficiency or induce mitochondrial dysfunction. It can be proposed that exogenous CL affects both active and resting respiration proportionally, resulting in similar ratios as those seen in the control group.

Higher mitochondrial respiration rates are a result of increases in mitochondrial density and/or efficiency.[23] Given that the immunoblotting indicated that there was no significant difference in expression between the groups (Figure 6), our data suggest that the elevated rates of respiration are unlikely to be driven by changes in mitochondrial content. Consequently, it can be proposed that added CL may enhance the generation of the proton gradient and improve mitochondrial membrane integrity, ultimately leading to higher respiratory rates.[7,19]

The relatively large increase in running distance from Week 1 to Week 2 is consistent with this well-described phenomenon in voluntary wheel-running paradigms. As noted by Manzanares et al (2018), early variability in wheel-running behavior is frequently observed and likely reflects both behavioral acclimatization and the progressive establishment of stable running patterns. Although running wheels do not require a formal familiarization period per se, total daily running distance has been shown to increase over the first 2–5 weeks before reaching a plateau, with notable day-to-day variability reported in the literature.[24] In the present study, these known patterns align with the observed increase in voluntary running following Week 1, after which activity levels stabilized for the remainder of the intervention in both groups (Figure 1). All wheel systems were calibrated prior to study initiation and routinely checked to ensure accurate data acquisition; however, we cannot fully exclude the possibility that early recordings slightly underestimated true running activity. Accordingly, greater interpretive weight was placed on data collected following the initial adaptation

period. Despite the absence of a significant difference in functional aerobic capacity between the two groups at any of the timepoints, the opportunity to voluntarily exercise likely contributed to the significant increase in time-to-exhaustion during the treadmill tests at week 3 and week 6 (Figure 2). Voluntary running has been shown to result in endurance-like adaptations in skeletal and cardiac muscle after 4 weeks.[24] In addition, increases in endurance capacity of approximately 90% on treadmill tests have been seen in mice subjected to voluntary wheel running.[24]

An important limitation of the present study relates to the interpretation of the exercise performance data in the context of whole-body oxygen transport and utilization. The treadmill protocol employed was a constant-speed, time-to-exhaustion test performed at a submaximal workload, and therefore does not provide a direct measure of maximal oxygen uptake (VO_2max). As such, while the observed improvements in endurance performance may be consistent with enhanced mitochondrial function, the current design does not allow us to determine whether these adaptations translate to changes in systemic oxygen delivery or utilization. Consequently, caution is warranted when extrapolating the present findings to integrative respiratory capacity. Future studies incorporating graded exercise testing, alongside measures of VO_2max and central hemodynamics (eg., cardiac output), will be necessary to more definitively establish the relationship between mitochondrial adaptations and whole-body physiological performance.

An additional major limitation of the present study is the absence of direct quantification of CL levels in plasma, skeletal muscle, or mitochondrial fractions. As a result, it cannot be confirmed whether intraperitoneal administration of CL led to increased intracellular or mitochondrial CL content within the vastus lateralis. This represents an important mechanistic gap, as the observed increases in mitochondrial respiration cannot be definitively attributed to alterations in CL availability at the tissue or organelle level. Consequently, the findings of this study should be interpreted as associative rather than causal. An important consideration emerging from both the existing literature and the present findings is the context-dependent, potentially dual role of CL in skeletal muscle. CL is essential for maintaining mitochondrial integrity and function, notably through its role in stabilizing ETC complexes and supporting efficient oxidative phosphorylation. However, alterations in CL content, fatty acid composition, or remodeling processes have been associated with impaired mitochondrial function, particularly under conditions such as oxidative stress, aging, or metabolic dysfunction.

These seemingly opposing effects underscore the importance of carefully interpreting the role of CL within specific physiological and pathological contexts. In the present study, our findings should therefore be considered in light of this balance between beneficial and potentially detrimental effects, depending on the underlying cellular environment.

From an applied perspective, interventions targeting CL must account for this complexity. Modulating CL levels or remodeling pathways may yield beneficial outcomes when restoring deficient or dysfunctional mitochondrial systems, yet inappropriate or excessive manipulation could disrupt mitochondrial bioenergetics. Accordingly, future work should aim to better delineate the conditions under which CL modulation is advantageous, with particular attention to tissue state, redox environment, and the dynamics of CL turnover.

Consequently, forthcoming studies should aim to directly investigate the mechanisms underlying the observed increases in mitochondrial respiration. Lipidomic quantification of CL in plasma, skeletal muscle, and mitochondrial fractions would be essential to confirm uptake and tissue distribution. Additional approaches could include measurements of mitochondrial membrane potential, reactive oxygen species production, and CL species composition, as well as structural analyses of mitochondrial cristae and supercomplex assembly. Such studies would be necessary to determine whether the proposed mechanisms contribute to the functional effects observed in the present work.

The use of a male-only design represents another significant limitation of the present study, as it restricts the generalizability of our findings and does not account for potential sex-specific differences in mitochondrial function and exercise capacity. Estrogen has been shown to influence mitochondrial bioenergetics, including modulation of substrate utilization, mitochondrial respiration, and the expression of genes involved in energy metabolism.[25,26] Fluctuations in ovarian hormones across the estrous cycle have been demonstrated to alter skeletal muscle function and endurance-related outcomes in rodents, with lower estrogen phases associated with reduced muscle performance and changes in metabolic capacity.[14,27] Consequently, inclusion of female mice without controlling for estrous cycle phase could introduce biologically meaningful variability in mitochondrial respiration and aerobic capacity measures, thereby

confounding interpretation of the results. Future studies should incorporate female mice with appropriate estrous cycle phase control to better elucidate the role of sex hormones in these outcomes.

Physiological adaptations associated with endurance exercise can take up to 12 weeks or more to manifest.[28] Therefore, a possible limitation of this study is the duration of the experimental period, whereby the longer-term impacts of CL on functional capacity might not have been fully captured within the study's six-week timeframe. Additionally, the absence of a direct measure of cardiac output may have restricted our ability to comprehensively evaluate the effects of CL supplementation. Furthermore, muscle wet weight was not collected, which lessens the ability to contextualize mitochondrial respiration findings at the whole-tissue level, despite normalization of respiration to tissue mass. Finally, the absence of direct VO_2max assessment may have limited the ability to fully characterize functional aerobic capacity, as the skeletal muscle may not have been sufficiently challenged to reveal performance-level adaptations.

Conclusion

In conclusion, the present results indicate that exogenously elevated levels of CL are associated with significantly increased skeletal muscle mitochondrial respiratory capacity in male mice. No differences in daily voluntary running distance or functional aerobic capacity were observed. Exploring the effects of exogenous CL on skeletal muscle function provides valuable insights into its role beyond the cardiovascular system.

These findings highlight a potential relationship between CL exposure and mitochondrial function, which can have implications for exercise physiology and supplementation. Future considerations include exogenous CL administration paired with imposed exercise training, such as high-intensity interval training (HIIT), to further investigate changes in mitochondrial capacity and performance. Additionally, lipidomic analyses should be used to confirm CL uptake and better define its mechanistic role in skeletal muscle bioenergetics.

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Disclosure

The authors report no conflicts of interest in this work.

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