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Abstract

Citrulline malate (CM) has been shown to improve muscle performance in healthy participants during a single exercise session. Yet, within the framework of exercises repeated at close time interval, the consequences of CM ingestion on mechanical performance are controversial and the bioenergetics side remains undocumented. The aim of this double-blind placebo-controlled study was to evaluate in vivo the effect of short-term (7 doses in 48h) oral administration of CM upon gastrocnemius muscle function and bioenergetics using noninvasive multimodal NMR techniques in healthy rats. The experimental protocol consisted of two 6-min bouts of fatiguing exercise spaced by an 8-min recovery period. CM treatment did not affect the basal bioenergetics status and increased the half-fatigue time during the first exercise bout. With exercise repetition, it prevented PCr cost alteration and decreased both the glycolytic ATP production and the contractile ATP cost in working muscle, but these changes were not associated to any improvement in mechanical performance. Besides, CM did not influence the replenishment of high-energy phosphorylated compounds during the post-exercise recovery periods. Therefore, short-term CM administration enhances muscle bioenergetics throughout fatiguing bouts of exercise repeated at close time interval but this enhancement does not benefit to mechanical performance.

Keywords: Stimol®; fatigue; energy metabolism; training; sport performance; exercise recovery.

1. Introduction

Athletes are often required to perform training sessions consisting of repeated exercises at close interval. However, a reduced recovery time induces fatigue and ultimately decreases muscle performance (Cook et al., 2007; Komi and Viitasalo, 1977). Considering the crucial role of energy metabolism in contractile activity (Chin and Allen, 1997; Fitts, 1994; Sahlin et al., 1998), any strategy that allows acceleration of intramuscular energy pool reconstitution between exercise bouts should be efficient for increasing mechanical performance. Since glycogenogenesis is a relatively slow phenomenon, with several tens of hours to restore the initial reserves after intense exercise in the trained subject (Murray and Rosenbloom, 2018), bioenergetics recovery over short interval periods is exclusively related to high-energy phosphorylated compounds, i.e., phosphocreatine (PCr) and ATP. Therefore, any exogenous substance able to accelerate this regeneration warrants consideration within the framework of physical training.

In this regard, citrulline malate (CM) has been shown to markedly improve muscle mechanical performance in weakened and healthy subjects. In asthenic rats, ingestion of doses of CM at 1 to 2.4 g/kg three times a day during 1 to 2 days extends running endurance during treadmill test (Verleye et al., 1995) and increases lower hindlimb muscle force in response to electrical stimulation (Giannesini et al., 2009; Goubel et al., 1997). Furthermore, chronic (15 days) oral supplementation with 6 g/day CM reduces the sensation of fatigue following repeated finger flexions in sedentary men suffering from asthenia after acute disease (Bendahan et al., 2002). In healthy rat, CM supplementation (3 g/kg/day during 48h) produces an ergogenic effect in electrically-stimulated gastrocnemius muscle in vivo (Giannesini et al., 2011). These beneficial effects would be mediated by changes in muscle bioenergetics. Because it is a precursor of nitric oxide, a compound known for its vasodilator properties, citrulline has been proposed to increase blood flow during and after exercise (Goubel et al., 1997; Verleye et al., 1995). This increased flow may in return improve oxygen and nutrient delivery to muscle, thereby enhancing the mitochondrial respiration (Brevetti et

al., 2003; Tousoulis et al., 2012; Young et al., 1997). Citrulline is also involved in the urea cycle and could facilitate the elimination of by-products of energy metabolism generated during exercise, especially ammonium ions whose intramyoplasmic accumulation would slow down the oxidative ATP production by disrupting the NAD^+/NADH ratio (Newsholme and Leech, 1983). In addition, malate is an intermediate of the Krebs cycle and its supplementation could accelerate the mitochondrial synthesis of ATP (Wagenmakers, 1998). Accordingly, an increased oxidative ATP production (Bendahan et al., 2002) and a reduced oxidative cost of contraction (Giannesini et al., 2011) have been previously reported during a single session of fatiguing exercise in healthy subjects supplemented with CM. Moreover, it is important to emphasize that the resynthesis of PCr during the post-exercise recovery period relies exclusively on oxidative ATP production (Arnold et al., 1984; Giannesini et al., 2002; Kemp et al., 1993). Collectively, these data suggest that CM supplementation accelerates the reconstitution of high-energy phosphorylated compounds reserves during the post-exercise recovery period, hence improving muscle function during any subsequent exercise.

Nevertheless, very few studies have examined so far the effects of CM on mechanical performance during exercises repeated at close interval and the corresponding results are controversial. It has been reported in resistance-trained men and women that a single dose (8 g) of CM ingested one hour prior to exercise improves fatigue resistance during body weight (chin-ups and push-ups) sessions (Wax et al., 2016) and multi-bout upper- and lower-body submaximal weightlifting activities (Glenn et al., 2017; Perez-Guisado and Jakeman, 2010; Wax et al., 2015), in association with a significant decrease in postexercise muscle soreness (Perez-Guisado and Jakeman, 2010). By contrast, other studies using similar dosages (8-12 g) and dose timing administration (40 to 60 min prior to exercise) in trained participants have shown that acute CM supplementation does not improve muscle performance during repeated sessions of high-intensity dynamic body movements (Farney et al., 2019), maximal cycle sprints (Cunniffe et al., 2016) and upper-body submaximal weightlifting exercises (Gonzalez et al., 2018) in trained men. These

discrepancies among the literature might be linked to methodological differences related to exercise protocols, subjects training status and heterogeneity. In addition, the corresponding effects on muscle bioenergetics have never been investigated.

The aim of this double-blind placebo-controlled study was to characterize *in vivo* the effect of CM supplementation on muscle function and bioenergetics in healthy rats submitted to two fatiguing bouts of exercise spaced by a short recovery period. Specifically, we have tested whether short-term (7 doses in 48h) supplementation of CM at 1 g/kg (i) improves oxidative metabolism during exercises and (ii) accelerates the replenishment of high-energy phosphorylated compounds during recovery periods. For this purpose, we have assessed noninvasively gastrocnemius muscle force-generating capacity, bioenergetics and anatomy using magnetic resonance (MR) imaging and 31-phosphorus (31P) MR spectroscopy. ^{31}P-MR spectroscopy allows to dynamically monitor pH and high-energy phosphorylated compounds changes in exercising conditions and to characterize muscle oxidative capacity and ATP turnover (Cea et al., 2002; Kemp and Radda, 1994).

2. Materials and methods

2.1. Animal care and feeding

Forty 3-month-old Wistar male rats (Charles River Laboratories, L'Arbresle, France) were used for these experiments conducted in strict accordance with the guidelines of the European Communities Council Directive 86/609/EEC for Care and Use of Laboratory Animals. All animal procedures were performed with the approval of the animal experiment committee of Aix-Marseille University and under the supervision of BG (license number 13.164 2008/11/25). Every attempt was made to minimize the number and the suffering of animals. Rats were socially housed as two per cage in an enriched and controlled environment (12-12 h light-dark cycle, 22°C) with free access to commercial standard food and water until the time of the experiment.

134

135 *2.2. Study design*

136 This study utilized a randomized, double-blind placebo-controlled design. Animals
137 were randomly distributed to one of the two groups: CM (Biocodex, Gentilly, France)
138 supplemented group (n = 20) and vehicle group (n = 20). This process was conducted in a
139 blind fashion at the receipt of the animals from the breeding center by a person not involved
140 in this study. In each group, the noninvasive MR investigation of the right gastrocnemius
141 muscle function and bioenergetics was performed after the treatment in half of the animals (n
142 = 10 per group). For the other half, after the treatment period (vehicle or CM), gastrocnemius
143 muscles were quickly removed, freeze-clamped with liquid nitrogen-chilled metal tongs, and
144 stored at -80°C for subsequent *in vitro* analytical measurements. Sample size was
145 determined on the basis of previous works performed in our laboratory in order to evaluate
146 the effect of CM ingestion upon skeletal muscle function in vivo in rat (Giannesini et al.,
147 2009; Giannesini et al., 2011). Both CM and vehicle treatments were administrated by a
148 person not involved in data collection and analysis. It consisted of seven administrations of
149 CM (1 g/kg) or vehicle using an esophageal cannula at 48 h, 44 h, 40 h, 24 h, 20 h, 16 h and
150 1 h before the MR experiment or the sacrifice. These dosage and timings were established
151 based on prior studies reporting beneficial effect of oral CM supplementation on muscle
152 function throughout a single exercise session in asthenic (Giannesini et al., 2009; Goubel et
153 al., 1997; Verleye et al., 1995) and healthy (Giannesini et al., 2011) rat.

154

155 *2.3. Noninvasive investigation of gastrocnemius muscle function and bioenergetics*

156 Investigations were performed using a home-built experimental device operating in
157 the 4.7 Tesla horizontal magnet of a MR system (47/30 Biospec Avance, Bruker, Karlsruhe,
158 Germany) as described previously (Giannesini et al., 2005). This device allows to induce
159 gastrocnemius muscle contraction by transcutaneous electrostimulation and to obtain
160 information about mechanical performance (using an ergometer composed of a foot pedal

coupled to a force transducer), muscle volume (using proton MR imaging) and bioenergetics (using ^{31}P-MR spectroscopy). The gastrocnemius muscle was chosen because it is easily accessible for MR measurements and preferentially activated with our methods (Giannesini et al., 2005).

2.3.1. Animal preparation

Each animal was anesthetized in an induction chamber with 4% isoflurane (Forene; Abbott France, Rungis, France) mixed with air (3 L/min). Once the right hindlimb was shaved for optimizing transcutaneous electrostimulation, the anesthetized animal was placed supine in the home-built experimental device. Its right foot was placed on the pedal of the ergometer and the hindlimb was centered inside a 30 mm-diameter Helmholtz MR imaging coil while the belly of the gastrocnemius muscle was positioned above an elliptic (10 x 16 mm^2) ^{31}P-MR spectroscopy surface coil. Ophthalmic cream was applied to protect corneas from drying and animal's head was inserted in a facemask continuously supplied with 2.5% isoflurane in 33% O_2 (0.2 L/min) and 66% N_2O (0.4 L/min). Throughout the experiment, animal body temperature was controlled and maintained at a physiological level using a feedback loop including an electrical heating blanket (Prang+Partner AG, Pfungen, Switzerland), a temperature control unit (ref. 507137; Harvard Apparatus France, Les Ulis, France) and a rectal probe (ref. 507145, Harvard Apparatus France).

2.3.2. Muscle electrostimulation procedure

Muscle contractions were elicited using two transcutaneous electrodes integrated in the experimental device and connected to a constant-current stimulator (DS7A, Digitimer, Welwyn Garden City, UK). One electrode was located at the heel level and the other one just above the knee joint. Electrical signal coming out from the ergometer's force transducer was amplified with a home-built amplifier (based on an operational amplifier AD620; Analog Devices, Norwood, MA, USA) and converted to a digital signal, which was monitored and

recorded on a personal computer using the Powerlab 35/series system (AD Instruments, Oxford, UK). Before the MR acquisition, a progressive increase of the electrostimulation intensity (square wave electrical pulses of 1-ms duration) until there was no further peak twitch force increase.

2.3.3. Repeated exercises protocol

The repeated exercises protocol consisted of two 6-min bouts of moderate-intensity exercise spaced by an 8-min recovery period. For each bout, maximal isometric contractions were induced electrically with 1-ms duration square-wave pulses at a frequency of 2 Hz. In addition, two tetanic contractions (150 Hz; 0.5-s duration) were performed: the first one before the repeated exercises protocol, and the second one immediately after the 20-min post-exercise recovery period consecutive to the second bout of exercise.

2.3.4. Force output measurement

Force output was scaled to gastrocnemius muscle volume, which was measured from MR images (see below). For each bout of exercise, force-generating capacity was calculated as percent of the maximal force reached during exercise, and the half-fatigue time was defined as the time required for the force to halve compared to its start exercise value.

2.3.5. MR data acquisition and processing

Sixteen consecutive non-contiguous axial slices (1-mm thickness; 0.5-mm spaced), covering the region from the knee to the ankle, were positioned across the hindlimb. Rapid acquisition relaxation-enhanced (RARE) images of these slices (6 echoes; 49.3-ms echo time; 2000-ms repetition time; 32 x 32 mm^2 field of view; 256 x 256 matrix size) were recorded at rest. ^{31}P-MR spectroscopy measurements (4 kHz spectral width; 1024 data points) from the gastrocnemius muscle region were continuously acquired throughout a 48-min period consisting in 8 min of rest, 6 min of electrostimulation (exercise #1), 8 min of post-

exercise recovery (recovery #1), 6 min of electrostimulation (exercise #2), and 20 min of post-exercise recovery (recovery #2). A fully relaxed ^{31}P-MR spectrum (12 scans; 20-s repetition time; 4-min duration) was acquired at rest and was followed by a total of 1320 partially saturated free induction decays (FID; 2-s duration). The first 120 FID were acquired at rest and summed together. The next 810 FIDs (exercise #1, recovery #1, exercise #2 and the first 7 min of recovery #2) were summed by packets of 15, allowing a temporal resolution of 30 s. The remaining 390 FIDs (corresponding to the last 13 minutes of recovery #2) were summed as 13 packets of 30 FIDs (60-s resolution time). During both exercise periods, FIDs acquisition was gated to muscle electrostimulation in order to reduce potential motion artifacts due to contraction.

MR data were processed using a custom-written image analysis program developed with the IDL software (Interactive Data Language, Research System, Inc., Boulder, CO, USA). For each MR image, gastrocnemius muscle region was manually outlined so that the corresponding cross-sectional areas was measured. Gastrocnemius muscle volume was then calculated as the sum of the volumes included between consecutive slices. For MR spectra, relative concentrations of PCr, inorganic phosphate (P_i) and β-ATP were obtained by a time-domain fitting routine using the AMARES-MRUI Fortran code and appropriate prior knowledge of the ATP multiplets (Vanhamme et al., 1997). Absolute concentrations of these compounds were expressed relative to a resting β-ATP concentration determined by bioluminescence in muscle extracts as detailed below. Intracellular pH (pH_i) was calculated from the chemical shift of the P_i relative to the PCr signal (Arnold et al., 1984). [ADP] was calculated from [PCr], [ATP] and pH_i using the creatine kinase (CK) equilibrium constant (K = 1.67 10^9 M^{-1}) (Roth and Weiner, 1991).

2.3.6. Oxidative capacity and metabolic fluxes calculations

ATP productions from CK reaction, oxidative phosphorylation and glycolysis were calculated *in vivo* in exercising muscle according to the quantitative interpretation of ^{31}P-MR spectroscopy bioenergetics data (Kemp et al., 2015; Kemp and Radda, 1994).

ATP production rate from PCr degradation throughout the CK reaction (D) was directly calculated using the [PCr] time-course: $D = -dPCr/dt$.

Oxidative ATP production rate (Q) was calculated considering that oxidative ATP synthesis is stimulated by [ADP] through a hyperbolic relationship: $Q = Q_{max}/(1 + K_m/[ADP])$, in which K_m (the ADP concentration at half-maximal oxidation rate) is 50 µM and Q_{max} is the maximal oxidative rate. Q_{max} was calculated using the rate of PCr resynthesis at the start of the post-exercise recovery period (VPCr$_{rec}$) and the concentration of free cytosolic ADP measured at the end of exercise ([ADP]$_{end}$): $Q_{max} = V\text{PCr}_{rec}\ (1 + K_m/[ADP]_{end})$. VPCr$_{rec}$ was the product of k (the pseudo-first-order rate-constant of PCr recovery) and [PCr]$_{cons}$ (the amount of PCr consumed at the end of exercise). In order to determine k, the PCr time-course during the post-exercise recovery period was fitted to a first-order exponential curve with a least means-squared algorithm: $[PCr]_t = [PCr]_{rest} - [PCr]_{cons}\ e^{-kt}$.

Glycolytic ATP production rate (L) was determined considering that it is related to glycolytic proton generation (H_{Gly}) with a stoichiometry of 1.5 moles of ATP per mole of proton: $L = 1.5H_{Gly}$. Indeed, the degradation of a mole of glycosyl unit generates 3 moles of ATP while the hydrolysis of 3 moles of ATP is coupled to the production of 2 moles of protons (Hochachka and Mommsen, 1983). Proton generation can be inferred from the observed changes in pH_i and taking into account (i) protons consumed by PCr degradation throughout the CK reaction (H_{CK}), (ii) protons passively buffered in the cytosol (H_β), (iii) protons leaving the cell (rate of net proton efflux, H_{Efflux}) and (iv) protons produced by oxidative phosphorylation (H_{Ox}): $H_{Gly} = H_{Ox} - H_{CK} - H_\beta - H_{Efflux}$. The rate of aerobic proton production coupled to oxidative ATP synthesis was quantified as follows: $H_{Ox} = mV\text{PCr}_{rec}$, with $m = 0{,}16/(1+10^{(6{,}1-pH)})$. Calculation of H_{CK} was done from the time-dependent changes in

[PCr] and with the stoichiometric coefficient φ = 1/(1+10$^{(pHi-6.75)}$), which represents the number of protons generated per mole of PCr degraded: $H_{CK} = \varphi$ dPCr/dt. Besides, H_{β} was the product of β_{total} (in Slykes, millimoles acid added per unit change in pH_i) and pH_i changes ($\Delta pH_i = pH_{observed} - pH_{rest}$): $H_{\beta} = (-\beta_{total}\Delta pH_i)$. The apparent buffering capacity (β_{total}) takes into account the buffering capacity of P_i (β_{Pi}) and the buffering capacity of muscle tissue (β_{tissue}): $\beta_{total} = \beta_{Pi} + \beta_{tissue}$, where $\beta_{Pi} = 2.3[P_i]/((1+10^{(pHi-6.75)})(1+10^{(6.75-pHi)}))$. It has been demonstrated that β_{tissue} varies linearly between pH 7 (16 Slykes) and pH 6 (37 Slykes) in murine gastrocnemius muscle. Accordingly, β_{tissue} was calculated as follows: $\beta_{tissue} = -21pH_i + 163$. During exercise, H_{efflux} was calculated using the proportionality constant λ (in µmol/s/pH unit) referring to the ratio between the rate of proton efflux and pH_i: $H_{efflux} = -\lambda\Delta pH_i$. This constant was determined at the start of the post-exercise recovery period as $\lambda = -V_{eff}/\Delta pH_i$. At that time, although protons are generated throughout the aerobic PCr resynthesis, pH_i recovers back to basal because of net proton efflux from the cell: H_{efflux} can then be calculated taking into account proton loads associated with CK reaction and mitochondrial ATP synthesis, and the rate of pH changes, $H_{efflux} = H_{CK} + H_{Ox} + \beta_{total}dpH_i/dt$.

2.4. In vitro analytical procedures

Intramuscular contents for ATP, glycogen and glucose were determined in 40-60 mg of freeze-clamped muscles homogenized in 1.2 mL of ice-cold 0.6 M perchloric acid using a Polytron PT2100 (Kinematica AG, Luzern, Switzerland). After incubation for 15 min at 4°C, the homogenates were centrifuged (15 min, 2000 x g, 4°C). The supernatants were neutralized with K_2CO_3, placed for 30 min at 4°C and centrifuged (15 min, 2000 x g, 4°C) to remove precipitates. ATP concentration was determined using the bioluminescence ATP Determination Kit (ref. A22066; Invitrogen, Eugene, OR, USA). Glycogen and glucose contents were assessed by colorimetric procedure using the Glycogen Assay Kit (ref. E2GN-100; EnzyChrome, Hayward, CA, USA).

Citrate synthase activity was assessed in another part (20-30 mg) of the freeze-clamped muscle, which was homogenized with a lysis reagent (ref. C3228; Sigma-Aldrich) and a protease inhibitor cocktail (P8340, Sigma-Aldrich). Citrate synthase activity was measured using the colorimetric Citrate Synthase Assay Kit (ref. CS0720; Sigma-Aldrich) and was normalized by the protein content measured using the colorimetric Pierce BCA Protein Assay Kit (ref. 23225; Thermo Fisher Scientific, Waltham, MA, USA). All *in vitro* measurements were done on a microplate reader (Victor X3; PerkinElmer, Waltham, MA, USA).

2.5. Statistical analysis

Data are expressed as mean ± SEM. Statistical analyses were performed using the Statistica 10 software (StatSoft France, Maisons-Alfort, France) and the significance level was set at $P < 0.05$. Sample distribution was tested with the Shapiro-Wilk test. Differences were tested with nonparametric Mann-Whitney tests (citrate synthase activity and basal levels of glycogen and Pi,) or parametric two-tailed Student's *t*-tests (body weight, gastrocnemius muscle volume, oxidative capacity, and [PCr]/[ATP] ratio, pH, and levels of PCr, ADP, glucose, ATP in resting muscle). Otherwise, differences were tested with two-factor (group × time) ANOVAs with repeated measures on time followed when appropriate by post-hoc LSD Fisher tests (for all other parameters).

3. Results

3.1. Animal morphology

After treatment with vehicle or CM, there were no differences between both groups for body weight (vehicle: 323 ± 3 g; CM: 316 ± 4 g) and gastrocnemius muscle volume (vehicle: 1483 ± 28 mm^3; CM: 1479 ± 20 mm^3).

3.2. Mechanical performance

All force data reported here refers to specific force, i.e., absolute force normalized by gastrocnemius muscle volume measured from MR images. There were no differences between both groups for tetanic force measured before (vehicle: 4.1 ± 0.2 N/cm^3; CM: 3.9 ± 0.2 N/cm^3) and after (vehicle: 3.2 ± 0.2 N/cm^3; CM: 3.3 ± 0.2 N/cm^3) the repeated exercise protocol. Importantly, exercise repetition reduced the tetanic force in both groups (vehicle: -22%, $P < 0.001$; CM: -15%, $P = 0.023$). The time courses of force production and force-generating capacity are shown in Fig. 1A and 1B, respectively. In both groups, exercise repetition reduced ($P < 0.001$ for each test) the start-exercise force (vehicle: -32%; CM: -29%), the maximal force (vehicle: -33%; CM: -32%), the end-exercise force (vehicle: -22%; CM: -25%), the total force production (vehicle: -36%; CM: -38%) and the half-fatigue time (vehicle: -44%; CM: -51%), whereas it increased (vehicle: +14%, $P = 0.038$; CM: +11%, $P = 0.010$) the force-generating capacity (Table 1). There were no differences between both groups for most of these variables, except the half-fatigue time, which was longer (+19%; $P = 0.022$) in the CM-supplemented group during the first bout of exercise (Table 1), thereby indicating that CM slows down the development of muscle fatigue.

3.3. Energy metabolism

Muscle bioenergetics was assessed at rest, and during exercise and post-exercise recovery periods. In resting muscle, there were no differences between both groups for [PCr]/[ATP] ratio, pH, levels of PCr, P_i, ATP, ADP, glucose and glycogen, citrate synthase activity and oxidative capacity (Table 2).

For each group, both electrostimulation protocols caused PCr (Fig. 2A) and ATP (Fig. 2B) degradation, ADP accumulation (Fig. 2C) and acidosis (Fig. 2D). PCr was rapidly degraded at the start of exercise (Fig. 2A). The initial rate of this degradation ($V\text{PCr}_{cons}$) did not differ between both groups during the first bout of exercise but was lower (-26%; $P = 0.034$) in CM-treated animals during the second bout (Table 3). After 2 min of exercise, PCr

346 level reached a plateau that was maintained until the exercise end (Fig. 2A), which means
347 the establishment of a metabolic steady state. At the end of the first exercise bout, PCr
348 consumption was lower (-11%; P = 0.048) in the CM group (Table 3). ATP level decreased
349 slightly during exercise (Fig. 3B), and neither the treatments nor the exercise repetition did
350 affect the extent of ATP reduction (∆ATP) measured at exercise end (Table 3). Moreover,
351 ADP – the main stimulator of oxidative ATP synthesis in vivo (Gyulai et al., 1985; Kemp and
352 Radda, 1994) – continuously accumulated during exercises (Fig. 2C). In both groups,
353 exercise repetition increased (vehicle: +59%, P = 0.019; CM: +68%, P < 0.001) the extent of
354 ADP accumulation (∆ADP) and reduced (vehicle: -0.29 pH unit; CM: -0.34 pH unit; P < 0.001
355 for each condition) the acidosis measured at exercise end (Table 3).
356 During the initial phase of the post-exercise recovery periods (Table 4), neither the
357 treatment nor the exercise repetition did affect the pH (i.e., the intracellular proton load) and
358 the rate of PCr resynthesis. However, for CM-treated animals, the initial rate of acidosis was
359 slower (-59%; P = 0.005) during the first recovery period, whereas the proton efflux at this
360 time was faster (+51%; P = 0.046). In addition, the initial rate of acidosis was increased
361 (+160%, P = 0.007) with exercise repetition in the CM group (Table 4). In both groups,
362 exercise repetition also caused reduction of the extent of PCr consumption (∆PCr; vehicle: -
363 56%; CM: -51%; P < 0.001 for each condition) and acidosis (∆pH; vehicle: -0.15 pH unit; CM:
364 -0.13 pH unit; P < 0.001 for each condition) measured at the end of the recovery periods
365 (Table 4).
366
367 *3.4. Metabolic fluxes and contractile cost*
368 For each exercise, there were no differences between both groups for the PCr cost of
369 contraction, the oxidative and anaerobic ATP productions, the relative contribution of the
370 oxidative process to the total ATP production, and the contractile ATP cost (Table 3).
371 However, in each group, the relative contribution of the oxidative process to total ATP
372 production was larger (vehicle: +40%, P = 0.005; CM: +43%, P = 0.003) during the second

373 bout of exercise as compared to the first one. Furthermore, exercise repetition increased the
374 PCr cost of contraction (+30%; *P* = 0.003) in the vehicle group and reduced both the
375 anaerobic ATP production (-43%; *P* = 0.003) and the contractile ATP cost (-26%; *P* = 0.015)
376 in the CM group.

4. Discussion

To our knowledge, this is the first study to investigate the effect of CM supplementation on muscle bioenergetics during repeated bouts of fatiguing exercise spaced by a short recovery period. The main findings from these in vivo investigations in rat gastrocnemius muscle are that short-term CM treatment (i) reduces the PCr consumption and lengthens the half-fatigue time during the initial bout of exercise, (ii) decreases the anaerobic ATP production and the contractile ATP cost during the second bout, and (iii) softens the intramuscular acidosis at the onset of the recovery period consecutive to the first exercise. On the other hand, exercise repetition suppresses the beneficial effect of CM on half-fatigue time, and CM does affect neither the rate of PCr resynthesis nor the replenishment extent of both PCr and ATP levels in recovering muscle.

Our data did not show any difference in resting muscle between CM and vehicle groups for the pre-exercise tetanic force, the intracellular pH, the citrate synthase activity, the levels of high-energy phosphorylated compounds and the glucidic content. These findings demonstrate that the initial force-generating capacity and the basal bioenergetics status were not altered by short-term CM treatment, which is in line with previous studies in asthenic men (Bendahan et al., 2002) and healthy rat (Giannesini et al., 2011). Likewise, we found that neither the treatments nor the exercise repetition did affect the extent of ATP reduction measured at exercise end, thereby indicating that the metabolic stress induced by muscular activity was similar in both groups.

The primary goal of this work was to test for the first time whether short-term CM supplementation improves oxidative metabolism throughout repeated bouts of fatiguing exercises. According to the quantitative interpretation of bioenergetics data (Kemp et al., 2015; Kemp and Radda, 1994), we have estimated the ATP production from each energy pathway in exercising muscle. We found that exercise repetition reduced the ATP production from anaerobic process in CM-treated animals but did not affect that from oxidative pathway, which leads at first glance to assumption that CM treatment does not affect the oxidative

metabolism. Nevertheless, the analysis of PCr level changes provides interesting information. The intracellular PCr level is under the control of the creatine kinase (CK), an enzyme that reversibly transfers high energy phosphate from PCr to ADP to form ATP. The PCr-CK system acts to maintain ATP pool highly charged in exercising muscle, functioning as an energy buffer at the transition from rest to exercise when PCr breakdown is the only pathway for regenerating ATP (Meyer et al., 1984; Wallimann et al., 1992). With exercise prolongation, the PCr-CK system works as an energy carrier directly involved in the transport of high-energy phosphate between the sites of production (mitochondria) and utilization (myofilaments) of ATP, which leads the PCr level to reach a steady state (Bessman and Geiger, 1981; Meyer et al., 1984; Wallimann et al., 1992). Here, the PCr cost of contraction, i.e., the rate of PCr degradation normalized to force output, was comparable in both groups in the early stage of each exercise session. However, exercise repetition increased this cost in the vehicle group whereas it was kept constant in the animals supplemented with CM. Since an increased PCr cost of contraction is associated with a reduced oxidative ATP synthesis (Korzeniewski and Zoladz, 2004; Willcocks et al., 2010), it can be extrapolated from our data that the oxidative capacity was preserved in CM animals but reduced in the vehicle group. Noteworthy, PCr consumption measured at the end of the first exercise bout was reduced in the CM group. This reduction indicates that CM might improve the energy carrier function of the PCr-CK system, hence allowing to optimize the transport of ATP produced by oxidative phosphorylation. Overall, it can be assumed from our findings that short-term CM administration is beneficial for oxidative metabolism throughout repeated moderate-intensity exercises. Our assumption is in line with previous works in our laboratory showing that the same treatment as used here improves the oxidative capacity efficiency during a single high-intensity exercise in healthy rat (Giannesini et al., 2011) and 15 days of oral supplementation with 6 g/day CM increases the rate of oxidative ATP production during a session of forearm low-intensity weightlifting in sedentary asthenic men (Bendahan et al., 2002).

Concerning mechanical performance, it might be useful to report that, within each group, exercise repetition reduced the initial-, maximal- and end-exercise force values, the total force production and the half-fatigue time, but increased the end-exercise force-generating capacity. There were no differences between both groups for all these variables, except the half-fatigue time that was larger in animals treated with CM during the first exercise bout, which is in agreement with a previous study in rat showing that short-term CM treatment enhances running endurance during a single treadmill test (Verleye et al., 1995). Nevertheless, we observed that exercise repetition suppressed the difference between both groups for this variable. Therefore, our data evidence that CM treatment did not improve muscle function within the framework of exercises repeated at close time interval. This finding is in accordance with other studies reporting that acute supplementation of CM (8-12 g) in trained participants has no effect on muscle performance during repeated sessions of high-intensity dynamic body movements (Farney et al., 2019), maximal cycle sprints (Cunniffe et al., 2016) and upper-body submaximal weightlifting exercises (Gonzalez et al., 2018).

In addition, it is worth mentioning that exercise repetition reduced the contractile ATP cost in CM-treated rats. The energy cost of contraction is considered as a key factor in muscle performance and physical ability, and any reduction is expected to increase muscle endurance (Lauretani et al., 2003; Tevald et al., 2010). Surprisingly, we did not measure in this group any concomitant increase in the half-fatigue time. However, it must be kept in mind that fatigue is a complex multifactorial phenomenon (Fitts, 1994) and it is possible that other factors such as failure of action potential propagation along the sarcolemma or intramyoplasmic accumulation of energy metabolism by-products would counterbalance the potent beneficial effect of the reduction in the contractile ATP cost. Moreover, the protocol used in the present study involves two repeated exercise bouts lasting 6 minutes, and it cannot be ruled out that the reduced ATP cost of contraction translates into enhanced fatigue resistance during longer bout.

458 The second goal of our investigation was to test whether CM supplementation
459 accelerates the replenishment of high-energy phosphorylated compounds in recovering
460 muscle. Because we did not observe any difference between both groups for the rate of PCr
461 resynthesis and for the levels of PCr and ATP reached at the end of each recovery period, a
462 potential effect on the post-exercise recovery process can be ruled out. However, we found
463 that CM administration reduced the rate of acidosis at the onset of the first recovery period.
464 Importantly, intracellular pH value results from the balance between mechanisms producing
465 and consuming protons (Fitts, 1994; Juel, 1998). In the early stage of post-exercise recovery
466 period, intramuscular acidosis occurs when proton production associated with PCr
467 resynthesis exceeds the ability of removing protons out of the cell (Kemp et al., 1994;
468 Mainwood and Renaud, 1985). Here, the fact that CM did not affect the rate of PCr
469 resynthesis means that the intracellular production of proton was similar between both
470 groups at the onset of the first recovery period. Thus, the larger proton efflux we quantified in
471 CM-treated animals could fully explain the reduced rate of acidosis in this group. Our
472 hypothesis is in agreement with previous studies suggesting that citrulline improves muscle
473 function given its involvement in the elimination of muscle metabolism by-products (Briand et
474 al., 1992; Verleye et al., 1995). Furthermore, the repetition of exercise increased the rate of
475 acidosis during recovery in the CM group and the value of this increased rate did not differ
476 from that measured in the vehicle group. On that basis, it can be concluded that the potential
477 effect of CM on pH homeostasis no longer exists when exercise is repeated. Consequently,
478 CM supplementation would not be beneficial for high-energy phosphorylated compounds
479 recovery when multiple exercise bouts are repeated.
480 A few limitations of the current study can be mentioned. The first one could lie in the
481 specific metabolism of the studied muscle. Indeed, our findings were obtained in the
482 gastrocnemius muscle which is predominantly glycolytic so that they might not be
483 extrapolated to type I oxidative muscle such as soleus muscle. Second, we have limited the
484 protocol to two bouts of moderate-intensity exercise lasting six minutes, and the use of
485 additional, longer and/or more intensive bout(s) of exercise could bring additional interesting

information. Finally, this work has been conducted in sedentary animal and cannot generalized to human recreational practitioners and/or athletes.

5. Conclusions

This study evidences that the effects of short-term CM administration upon muscle function and bioenergetics during repeated bouts of fatiguing exercises are contrasted. On the one hand, our findings demonstrate that CM treatment increases fatigue resistance during the initial exercise bout and increases the oxidative capacity and the contractile efficiency with exercise repetition, which supports the notion that CM improves muscle function. Nevertheless, the beneficial effect of CM on mechanical performance is suppressed by exercise repetition and CM does not favor the replenishment of high-energy phosphorylated compounds in post-exercise recovering muscle. In light of these preclinical data specific to rat, it is still premature to recommend CM administration to practitioners seeking nutritional aids for improving performance and recovery from training sessions consisting of repeated exercises at close interval. Further researches should use different exercise and CM supplementation paradigms, as well as investigations on various populations including individuals with oxidative deficit such as the eldery or patients suffering from muscle wasting.

Declaration of competing interest

The authors declare they have no conflicts of interest, financial or otherwise that could have influence the work reported in this paper.

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638

Table 1. Mechanical performance.

	Vehicle (n = 10)		CM (n = 10)	
	Exercise 1	Exercise 2	Exercise 1	Exercise 2
Start-exercise force (N/twitch/cm^3)	1.83 ± 0.07	1.25 ± 0.09*	1.67 ± 0.14	1.18 ± 0.07*
Maximal force (N/twitch/cm^3)	2.00 ± 0.07	1.35 ± 0.08*	1.89 ± 0.12	1.27 ± 0.08*
End-exercise force (N/twitch/cm^3)	0.94 ± 0.07	0.73 ± 0.06*	0.92 ± 0.05	0.69 ± 0.04*
Total force production (N/cm^3)[a]	1028 ± 42	660 ± 44*	998 ± 43	618 ± 39*
End-exercise force generating capacity (%)[b]	47 ± 3	54 ± 3*	49 ± 2	55 ± 2*
Half-fatigue time (min)[c]	3.0 ± 0.1^{CM1}	1.7 ± 0.1*	3.6 ± 0.3^{Veh1}	1.8 ± 0.1*

Data are means ± SEM. Start- and end-exercise force were averaged over the first and last 15-s intervals of the 6-min exercise, respectively. [a] Total force production produced during the whole exercise. [b] Expressed as % of the maximal force reached during the exercise. [c] Time required for the force to halve compared to the start-exercise value within the same bout.

* Significantly different from exercise 1 within the same group.

^{Veh1} Significantly different from vehicle for exercise 1.

^{CM1} Significantly different from CM for exercise 1.

652 **Table 2.** Basal energy metabolism.

653

	Vehicle (n = 10)	CM (n = 10)
PCr/ATP	2.8 ± 0.1	2.7 ± 0.1
PCr (mM)	18.0 ± 0.5	17.0 ± 0.7
P_i (mM)	1.9 ± 0.1	1.6 ± 0.2
ATP (mM)	6.5 ± 0.1	6.4 ± 0.1
ADP (μM)	10.4 ± 0.5	9.3 ± 0.4
pH	7.17 ± 0.02	7.14 ± 0.02
Glucose (mM)	1.0 ± 0.1	0.9 ± 0.1
Glycogen (mM)	19.0 ± 0.5	18.5 ± 0.5
Citrate synthase (nmol/mg prot/min)	74 ± 3	76 ± 5
Oxidative capacity (mM/min)	10.3 ± 1.4	11.7 ± 1.3

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655 Data are means ± SEM. No significant difference exists.

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Table 3. Energy metabolism during the fatiguing bouts of exercise.

	Vehicle (n = 10)		CM (n = 10)	
	Exercise 1	Exercise 2	Exercise 1	Exercise 2
Start of exercise				
VPCr$_{cons}$ (mM/min)[a]	15.5 ± 1.3	16.8 ± 1.4^{CM2}	12.8 ± 0.8	12.4 ± 2.0^{Veh2}
PCr cost of contraction (μmole/N)	0.28 ± 0.02	0.45 ± 0.03*	0.26 ± 0.03	0.37 ± 0.08
End of exercise				
ΔPCr (mM)[b]	13.8 ± 0.5^{CM1}	12.9 ± 0.4*	12.3 ± 0.6^{Veh1}	11.9 ± 0.6
ΔpH (pH unit)[b]	0.69 ± 0.04	0.39 ± 0.06*	0.67 ± 0.03	0.33 ± 0.04*
ΔATP (mM)[b]	2.1 ± 0.2	2.5 ± 0.3	2.4 ± 0.3	2.2 ± 0.4
ΔADP (mM)[b]	14 ± 2	38 ± 5	24 ± 2	38 ± 6
Whole exercise				
Anaerobic ATP production (μmol/N)	0.13 ± 0.01	0.10 ± 0.01	0.13 ± 0.01	0.08 ± 0.01*
Oxidative ATP production (μmol/N)	0.07 ± 0.01	0.09 ± 0.01	0.08 ± 0.01	0.08 ± 0.01
Oxidative contribution (% of total ATP production)	34 ± 3	48 ± 3*	36 ± 2	51 ± 3*
Contractile ATP cost (μmol/N)	0.20 ± 0.01	0.19 ± 0.02	0.21 ± 0.02	0.15 ± 0.01*

Data are means ± SEM. [a]VPCr$_{cons}$, initial rate of PCr degradation at the exercise start. [b] Relative to basal value.

* Significantly different from exercise 1 within the same group.

^{Veh1} Significantly different from vehicle for exercise 1.

^{Veh2} Significantly different from vehicle for exercise 2.

^{CM1} Significantly different from CM for exercise 1.

^{CM2} Significantly different from CM for exercise 2.

Table 4. Energy metabolism in recovering muscle.

	Vehicle (n = 10)		CM (n = 10)	
	Recovery 1	Recovery 2	Recovery 1	Recovery 2
Initial phase of recovery period				
VPCr$_{rec}$ (mM/min)[a]	5.4 ± 0.7	5.5 ± 0.5	5.0 ± 0.6	4.8 ± 0.5
pH	6.45 ± 0.04	6.75 ± 0.07*	6.46 ± 0.05	6.77 ± 0.05*
VpH (pH unit/min x 1000)[a]	-69 ± 14^{CM1}	-55 ± 8	-28 ± 9^{Veh1}	-73 ± 9*
Proton efflux (mM/min)	1.4 ± 0.8 ^{CM1}	2.0 ± 0.4	2.7 ± 0.5 $_{Veh1}$	1.4 ± 0.3
End of recovery period				
∆PCr (mM)[b]	3.8 ± 0.5	1.7 ± 0.6*	3.3 ± 0.6	1.7 ± 0.4*
∆pH (pH unit)[b]	0.52 ± 0.07	0.15 ± 0.05*	0.47 ± 0.05	0.13 ± 0.02*
∆ATP (mM)[b]	2.2 ± 0.2	1.8 ± 0.3	2.2 ± 0.2	1.8 ± 0.2

Data are means ± SEM. [a] VPCr$_{rec}$ and VpH are the initial rates of PCr resynthesis and acidosis at the start of the recovery period, respectively. [b] Relative to basal value.

* Significantly different from recovery 1 within the same group.

^{Veh1} Significantly different from vehicle for recovery 1.

^{CM1} Significantly different from CM for recovery 1.

Figure legends

Figure 1. Gastrocnemius muscle mechanical performance. Time courses of force production (A) and force-generating capacity (B) throughout the 6-min fatiguing bouts of exercise performed simultaneously to the dynamic ^{31}P-MR spectroscopy acquisition after treatment with vehicle or CM (*n* = 10 animals for each condition). Data are expressed as means ± SEM.

Figure 2. Noninvasive investigation of gastrocnemius muscle bioenergetics using dynamic ^{31}P-MR spectroscopy. Time courses of [PCr] (A), [ATP] (B), [ADP] (C) and pH (D) during exercise and post-exercise recovery periods after treatment with vehicle or CM (*n* = 10 animals for each condition). For each panel, the first point (t = 0) indicates the resting value. Data are expressed as means ± SEM.

Figure 1

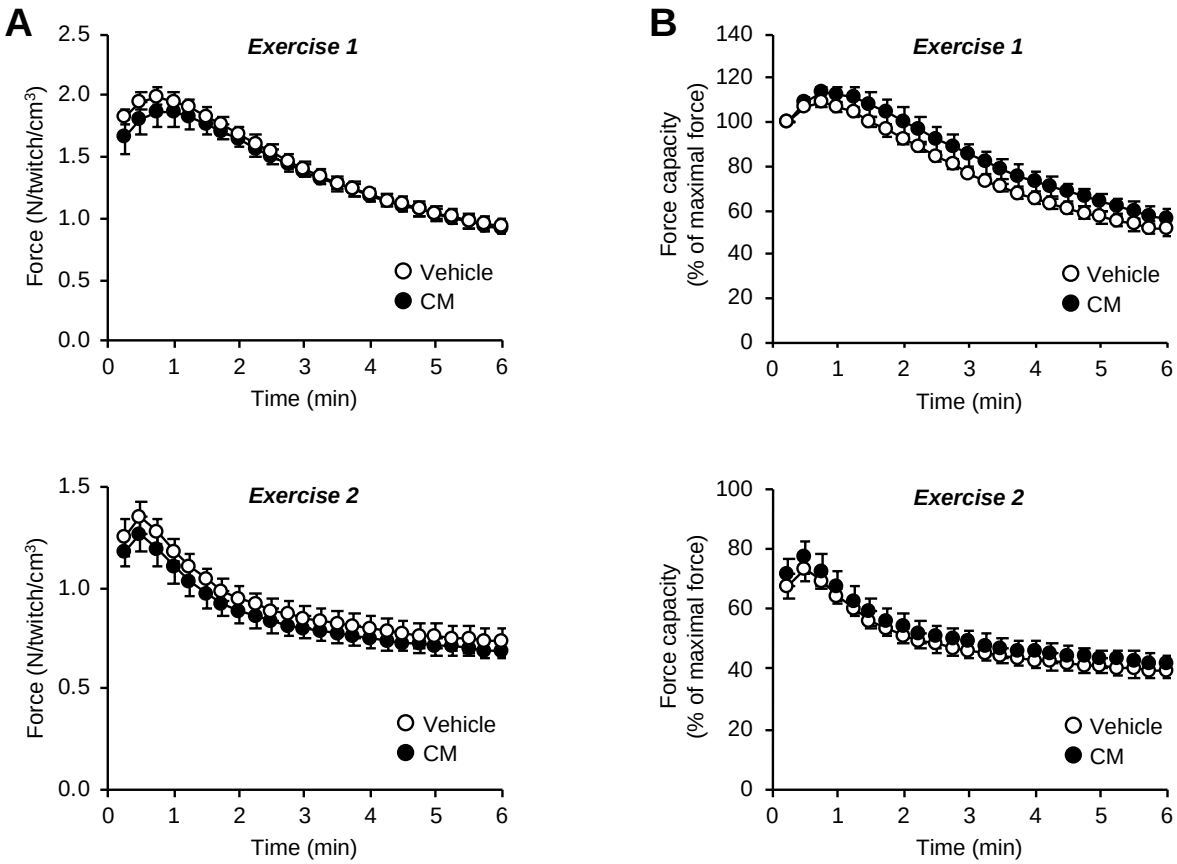
A
Exercise 1
Force (N/twitch/cm³)
Time (min)
Vehicle
CM
B
Exercise 1
Force capacity (% of maximal force)
Time (min)
Vehicle
CM
Exercise 2
Force (N/twitch/cm³)
Time (min)
Vehicle
CM
Exercise 2
Force capacity (% of maximal force)
Time (min)
Vehicle
CM

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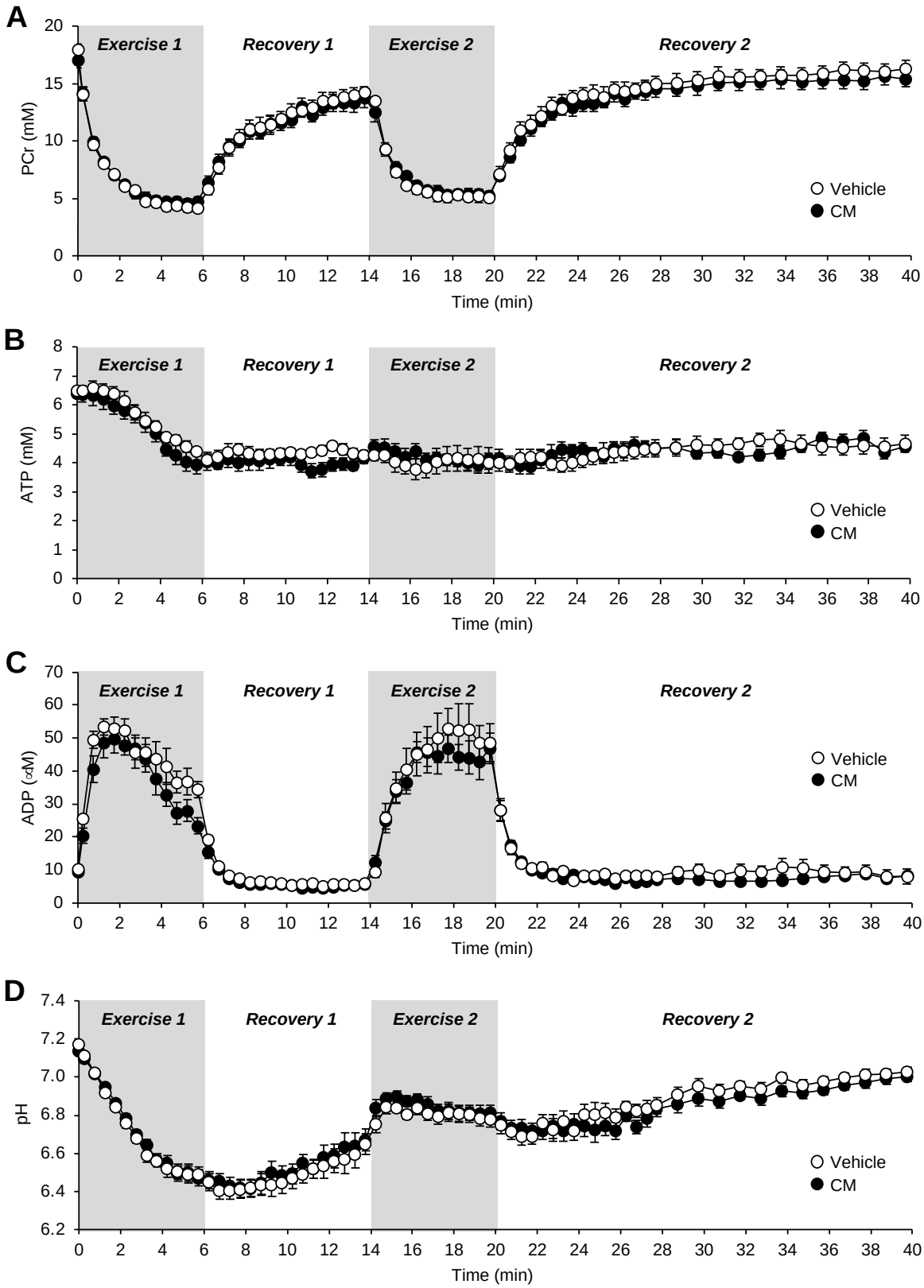
Figure 2



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