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1 **Endurance training and Hydroxyurea have synergistic effects**
2 **on muscle function and energetics in sickle cell disease mice**

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17
18
Abbreviations: SCD: Sickle cell disease, HU: Hydroxyurea, ³¹P-MRS: 31-Phosphorus Magnetic Resonance Spectroscopy, MRI: Magnetic Resonance Imaging, ATP: Adenosine triphosphate, PCr: Phosphocreatine, Pi: inorganic phosphate, TFP: Total force production, Pf: peak force, VPCr_{stim}: rate of PCr consumption, VPCr_{rec}: rate of PCr recovery, V_{max}: Maximal rate of oxidative ATP resynthesis

Abstract

19

20 Sickle cell disease (SCD) is an hemoglobinopathy resulting in the production of an abnormal Hb (HbS)
21 which can polymerize in deoxygenated conditions, leading to the sickling of red blood cells (RBC). These
22 alterations can decrease the oxygen-carrying capacity leading to impaired function and energetics of skeletal
23 muscle. Any strategy which could reverse the corresponding defects could be of interest. In SCD, endurance
24 training is known to improve multiples muscle properties which restores patient's exercise capacity but
25 present reduced effects in anemic patients. Hydroxyurea (HU) can increase fetal hemoglobin production
26 which can reduce anemia in patients. The present study was conducted to determine whether HU can
27 improve the effects of endurance training to improve muscle function and energetics. Twenty SCD Townes
28 mice have been trained for 8 weeks with (n = 11) or without (n = 9) HU. SCD mice muscle function and
29 energetics were analyzed during a standardized rest-exercise-recovery protocol, using Phosphorus-31
30 Magnetic resonance spectroscopy (³¹P-MRS) and transcutaneous stimulation. The combination of training
31 and HU specifically decreased fatigue index and PCr consumption while muscle oxidative capacity was
32 improved. These results illustrate the potential synergistic effects of endurance training and HU on muscle
33 function and energetics in sickle cell disease.

34 Keyword: Endurance training, Hydroxyurea, Sickle Cell Disease, Skeletal muscle, ³¹P-Magnetic
35 Resonance Spectroscopy

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Introduction

45

46 Sickle cell disease (SCD) is an inherited hemoglobinopathy resulting in the production of abnormal
47 hemoglobin (HbS). In deoxygenated conditions, HbS can polymerize eventually leading to the sickling of
48 the red blood cells (RBC) [1]. SCD patients suffer from severe chronic hemolytic anemia [2] and arterial
49 desaturation [3,4], resulting in deficient blood oxygen carriage and ultimately in tissue hypoxia [5,6]. This
50 decreased blood oxygen-carrying capacity may account, at least partly, for alterations in muscle structure,
51 function and energetics [7–14]. Such alterations have been reported in both patients [7–10] and mice with
52 SCD [11–14]. Because muscle structure, function and energetics are determinant for the quality of life of
53 patients [15], any strategy which could mitigate these muscular defects could be of interest.

54 Recent studies have suggested that regular physical activity could be considered as an interesting
55 complementary therapeutic strategy in SCD. In patients, moderate-intensity endurance-exercise has been
56 reported as safe and has been shown to improve patients' physical ability, capillary network and oxidative
57 enzymes activities [16–18]. Functional and metabolic improvements after endurance training have been also
58 observed in 7-month-old Townes SCD mice [19]. In addition, in another SCD mice model (SAD mice),
59 endurance training has been shown to mitigate systemic inflammation [20], a potential trigger of main SCD-
60 related complications [21], and severe organ damages [22]. Yet, in a context of anemia, the effects of
61 endurance training on muscle function and energetics were reported as limited compared to the
62 corresponding effects in healthy individuals [23] so that muscle function and energetics of trained anemic
63 individuals did not reach the level of non-anemic untrained subjects [23].

64 Hydroxyurea (HU) is a pharmacological treatment commonly used in SCD. HU can reactivate fetal
65 hemoglobin (HbF) production [24,25] thereby alleviating some SCD-related symptoms [26]. Indeed, HU
66 can lead to an increased total Hb content ultimately reducing the relative HbS content [26–31], enhancing
67 oxygen saturation [32] and hemodynamics [33] and increasing skeletal muscle perfusion [34]. In this
68 context, HU seems a perfect match for endurance training in SCD patients to maximize the metabolic
69 adaptations of a training intervention.

70 Additionally, HU treatment has also been related to an improved physical fitness in patients [35] and
71 has been shown to prevent muscle alterations over time in SCD mice [36]. Considering the HU-mediated
72 increase muscle perfusion and oxygen supply, one could expect some positive effects of HU on muscle
73 energetics. To the best of our knowledge, such an effect has never been assessed. Of interest, hyperoxia has
74 been shown to increase skeletal muscle oxygenation and improve oxidative capacity in trained but not in
75 sedentary subjects [37,38]. On that basis, it could be hypothesized that endurance training combined with
76 the HU-mediated muscle perfusion improvement could be translated into amplified positive effects.

77 Overall, endurance training is known to have considerable potential for the restoration of skeletal muscle
78 function and energetics although this is limited in SCD due to anemia. For this reason, its combination with
79 HU, which is known to also have its own positive effects, can be a perfect fit to achieve significant
80 improvement through an enhancement of their individual effectiveness. Indeed, on the one hand, HU can
81 reduce anemia thereby enhancing skeletal muscle adaptations to endurance training. On the other hand, HU
82 can increase oxygen supply which can be optimally used in endurance trained individuals. Overall, a
83 synergistic effect between endurance training and HU may be expected on skeletal muscle function and
84 energetics. Therefore, the purpose of the present study was to investigate the effects of endurance training
85 in association with HU treatment on skeletal muscle energetics and function in SCD mice.

86

87

88

Animals and Methods

89 **Animal model, care & feeding**

90 Twenty HbSS Townes mice (Jackson Laboratories, Bar Harbor, USA) of 2-month-old were
91 investigated. Townes mice are a murine model of SCD which express the human hemoglobin gene and
92 display the typical clinical phenotype of SCD patients including anemia, sickling and organs dysfunction
93 [39]. The study was performed in conformity with the French guidelines for animal care and in conformity
94 with the European convention for the protection of vertebrate animals used for experimental purposes and
95 institutional guidelines n° 86/609/ CEE November 24, 1986. All animal experiments were approved by the
96 Institutional Animal Care Committee of Aix-Marseille University (permit number n°27800-
97 2020112517474997). Mice were housed collectively (4-5 mice per cage) in a controlled-environment
98 facility (12–12 h light–dark cycle, 22 °C) and received standard food *ad libitum*. Mice were also provided
99 with either water *ad libitum* or an HU treatment. For the latter and as previously described, HU was added
100 into drinking water at a concentration of 50 mg/kg/day [31,40] which was adjusted daily according to water
101 intake.

102

103 **Study design**

104 In order to assess the effectiveness of the addition of HU to endurance training and because the individual
105 effects of endurance training on the one hand [19] and HU on the other hand [36] have been already
106 documented, we presently analyzed the 2-month evolution of HU-supplemented trained and untrained SCD
107 mice. In that purpose, muscle function and energetics of the whole set of mice were initially assessed at 2-
108 month-old during a standardized rest-stimulation-recovery protocol. Mice were then randomly assigned to
109 two groups: SCD_{TR} (n = 9) and SCD_{TR-HU} (n = 11). Both groups were submitted to an 8-week endurance
110 training program. While SCD_{TR} did not receive any treatment, SCD_{TR-HU} received the HU-treatment during
111 the training period. Muscle function and energetics were then assessed a second time in response to the same
112 standardized protocol. Each assessment was identified with number 1 and 2 respectively, e.g. SCD_{TR1} and
113 SCD_{TR2}. One week after the second assessment, mice were anesthetized with vetflurane in an induction
114 chamber and then euthanized by cervical dislocation.

115 **Animal training**

116 SCD_{TR} and SCD_{TR-HU} mice were trained 5 days (D) a week for 8 weeks (W) on a treadmill (Columbus
117 Instruments, Columbus, OH, USA) as previously described [19]. Each training session started with a 5-min

118 warm-up period in order to progressively reach the target speed (*vide infra*). Mice were first submitted to an
119 acclimatization period over week 1 (W1). At D1, the target speed was 11 m/min for 10 minutes. Running
120 duration and target speed were then increased by 10 minutes and 1 m/min respectively on a daily basis until
121 D5. During W2, W3 and W4, mice ran 50 minutes at 15 m/min and the target speed was increased to 16
122 m/min at W5 and W6 and to 17 m/min at W7 and W8.

123 **Non-invasive investigation of posterior hindlimb muscle function and bioenergetics**

124 Investigations were conducted using an in-house experimental setup which has been designed to be
125 operational inside a PharmaScan® AVANCETM III HD 70/16 US equipped with a 90 mm BGA09S (760
126 mT/m) gradient insert (Bruker BioSpin MRI GmbH, Ettlinge, Germany). This setup is adapted from a
127 previously described model used on a 4.7 T MR scanner [41]. It allows (i) to get muscle volumetric
128 information using MR imaging (MRI), (ii) to assess its corresponding mechanical performance with a
129 dedicated ergometer consisting of a foot pedal coupled to a force transducer, and (iii) to monitor dynamically
130 its levels of high-energy phosphorylated compounds and acidosis using ³¹P-MRS. The posterior hindlimb
131 muscles, which form the belly of the calf, were studied because this muscle group i) is clearly distinct from
132 the other muscles of the leg, ii) is easily accessible for MR coils and large enough to give ³¹P-MR spectra in
133 a short time and with a good signal to noise ratio, and iii) has been shown to be preferentially activated using
134 a standardized experimental transcutaneous stimulation protocol [41].

135 **Animal preparation**

136 Mice were anesthetized in an induction chamber (Equipement vétérinaire Minerve, Esternay, France)
137 using an air flow (3 L/min) containing 4 % vetflurane. The left hindlimb was shaved and an electrode cream
138 was applied at the knee and heel regions so as to optimize electrical contacts. Each anesthetized animal was
139 then positioned supine within the experimental setup [41]. Corneas were protected from drying by applying
140 ophthalmic cream, and the animal's head was equipped with a facemask continuously supplied with 1-2 %
141 vetflurane mixed in 66 % room air (1 L/min) and 33 % O₂ (0.5 L/min). Breathing rate was monitored during
142 the protocol and maintained between 100 and 130 breaths/min thanks to vetflurane concentration
143 adjustments. The mouse's foot was positioned on the ergometer's pedal and the hindlimb was centered inside
144 a 16-mm diameter ¹H Helmholtz coil while the belly of the posterior hindlimb muscles was located above
145 an elliptic (8 x 6 mm²) ³¹P surface coil. Body temperature was controlled and maintained at a physiological
146 level throughout the experiment using a feedback loop including an electrical heating blanket, a temperature
147 control unit (ref. 507137, Harvard Apparatus, Holliston, MA, USA) and a homemade rectal temperature
148 probe as previously described [41].

149 **Muscle contraction and force measurement**

150 Muscle contractions were induced by electrostimulation using two transcutaneous surface electrodes
151 connected to a constant-current stimulator (DS7A, Digitimer, Hertfordshire, United Kingdom). One
152 electrode was placed at the heel level and the other one was located just above the knee joint. Electrical
153 signal coming out from the force transducer of the ergometer was amplified (Two operational amplifiers:
154 AD620 and OP497, Analog Devices, Norwood, MA, USA, with a total dB gain: 70dB @ 0-5 kHz
155 bandwidth), continuously monitored and recorded on a personal computer using a dedicated data acquisition
156 system (Powerlab 16/36, AD Instruments, Sydney, Australia) and a dedicated software (LabChart 8, AD
157 Instruments, Sydney, Australia). The digital signal was converted to force (mN) according to a linear
158 calibration curve.

159 **Moderate exercise protocol**

160 Function and energetics of the posterior hindlimb muscles were evaluated throughout a 6-min
161 standardized moderate-intensity exercise consisting of maximal isometric contractions repeated at a
162 frequency of 1 Hz induced with square wave pulses (0.5 ms duration). Force production was acquired
163 continuously with the LabChart software (AD Instruments, Oxford, UK) and then processed by a custom-
164 written MATLAB routine computing mechanical performance parameters. Individual maximal stimulation
165 intensity was determined by a progressive increase of the stimulus intensity until no further force increase
166 was observed. The corresponding force was referred to as peak force (Pf, mN). Total force production (TFP,
167 N) was computed as the sum of the whole set of twitches. Pf and TFP were normalized to the posterior
168 hindlimb muscles volume (mm³), as these muscles are preferentially activated during our exercise protocol.
169 The corresponding volume was computed from anatomic hindlimb MR images and the normalized metrics
170 were referred to as specific Pf and specific TFP (sPf and sTFP, respectively). Force was analyzed at specific
171 time points i.e. initial and final (initial and final sPf, respectively) while the maximal recorded value was
172 referred to as maximal sPf. Additionally, the relative force increase, and the fatigue index were computed.
173 The relative force increase was measured as the ratio between the maximal and initial force measurements
174 and the fatigue index was computed as the ratio between the maximal and the final force measurements.
175 Both values were expressed as percentage (%).

176

177 **Multimodal MR data acquisition**

178 Ten consecutive non-contiguous axial slices (1 mm thickness, slice gap = 0.25 mm) were recorded at
179 rest in order to assess muscle volume. The corresponding region of interest covering the region from the

180 knee to the ankle was selected across the lower hindlimb. RARE images (Rare factor = 8, effective echo
181 time = 35.29 ms, actual echo time = 11.76 ms, repetition time = 5000 ms, one accumulation, 20 x 20 mm
182 field of view, 256 x 192 matrix size, acquisition time = 3 min 14 s). Posterior hindlimb muscles region was
183 manually delineated in each of the 6 largest slices. Then, muscle volume of each slice was extracted using
184 FSLeaves [42]. Total muscle volume (mm³) was calculated using the truncated cone volume formula
185 considering slice thickness and gap volumes. Multiecho T2-weighted images (16 echo times equally spaced
186 from 7.28 ms to 116.59 ms, 2000-ms repetition time, one accumulation, 20 x 30 mm field of view, 256 x
187 256 matrix size, total acquisition time = 8.32 min, slice thickness = 1 mm, slice gap = 1 mm) were recorded
188 at rest before the rest-exercise-recovery protocol. The T2-weighted images were processed to generate T2
189 maps on a pixel by pixel basis by fitting the corresponding data with a single exponential function. Mean
190 T2 values of posterior hindlimb muscles were measured on T2 maps and averaged on the 2 largest
191 consecutive slices from the outlined regions of interest. The corresponding values were quantified in the
192 gastrocnemius (GA) and the tibialis anterior (TA) muscles. ³¹P-MR spectra (8 kHz sweep width; 2048 data
193 points) from posterior hindlimb muscles were continuously acquired before (rest period; 4.7 min duration),
194 during and after (recovery period; 16 min duration) the 6-min moderate stimulation protocol. Spectra
195 acquisition was gated to muscle electrostimulation to reduce potential motion artifacts due to contraction. A
196 total of 800 saturated free induction decays (FIDs, 1.875 s repetition time) were acquired. The first 140 FIDs
197 were acquired in resting muscle and summed together. The next 450 FIDs were acquired during exercise
198 and were summed as blocks of 15, allowing a 30 s temporal resolution to assess the PCr time-dependent
199 changes. Due to reduced signal to noise ratio for the Pi signal, FIDs were also summed as block of 30
200 to assess pH_i changes with respect to time (1 min temporal resolution). The remaining 210 FIDs were recorded
201 during the recovery period and were summed as blocks of 30 FIDs.

202 **MRS data processing**

203 MRS data were processed using Python custom-written routines [43] integrating the AMARES Fortran
204 code [44] from jMRUI (<http://www.jmrui.eu/>). Relative concentrations of phosphocreatine (PCr), inorganic
205 phosphate (Pi) and ATP were obtained from ³¹P-MR spectra using the AMARES-MRUI-based time domain
206 fitting routine including appropriate prior knowledge for the ATP multiplets [43]. Absolute amounts of
207 phosphorylated compounds were expressed taking into account a 5-mM β-ATP basal concentration [45] and
208 were corrected taking into account T1 saturation effects for PCr and ATP. The PCr relative concentration
209 was expressed relative to the PCr content at rest which was set at 100%. Intracellular pH was calculated
210 from the chemical shift of the Pi signal relative to PCr according to the formula [46]: $pH_i = 6.75 + \log[(3.27 - \delta Pi) / (\delta Pi - 5.69)]$. Cytosolic ADP concentration was calculated, as previously described [47,48]: $[ADP] = ([Cr] \times [ATP]) / ([PCr] \times 10^{-pH} \times K_{CK})$, using the creatine kinase equilibrium constant ($K_{CK} = 1.66 \times 10^9 M^{-1}$

213 ¹⁾ [49] and total creatine [Cr] calculated by assuming that PCr represents ~85% of the total Cr content [50].
214 The rate of PCr consumption ($V_{PCr_{stim}}$, in mM/min) at the onset of exercise was calculated as $V_{PCr_{stim}} =$
215 $\Delta PCr / \tau PCr_{stim}$, where ΔPCr is the extent of PCr depletion measured at the end of stimulation (relative to
216 basal value) and τPCr_{stim} is the time constant of PCr degradation. τPCr_{stim} was determined by fitting the time
217 course of PCr to a mono-exponential function using a least-means-squared algorithm. sPf-normalized non-
218 oxidative cost of contraction (PCr_{cost} in $\mu M/min/mN/cm^3$) was calculated as the ratio between $V_{PCr_{stim}}$ and
219 the amount of specific force produced at the beginning of exercise, considering that at that time PCr is the
220 only source of ATP production. Similarly, the PCr recovery parameter (τPCr_{rec}) was quantified during the
221 post-exercise period by fitting the time course of PCr resynthesis to a mono-exponential function. As
222 previously suggested, $V_{PCr_{rec}}$ (in mM/min) was considered as the rate of oxidative ATP production at the
223 end of exercise as $V_{PCr_{rec}} = \Delta PCr / \tau PCr_{rec}$ [51,52]. sPf-normalized oxidative cost of contraction (PCr_{rec} in
224 $\mu M/min/mN/cm^3$) was calculated as the ratio between $V_{PCr_{rec}}$ and the amount of specific force produced at
225 the end of exercise. The maximal rate of oxidative ATP resynthesis (V_{max} , mM/min), identified as the muscle
226 oxidative capacity, was calculated as previously described: $V_{max} = V_{PCr_{rec}} (1 + (K_m / [ADP_6]))$ [53], where
227 K_m is the affinity constant of the mitochondria for ADP (30 μM in gastrocnemius muscle of the mice), and
228 $[ADP_6]$ is the ADP concentration at the end of exercise.

229

230 **Statistics**

231 All values are presented as means \pm SD except on graphics for which SE are displayed for the sake of
232 clarity. Sample distribution was tested with the Shapiro-Wilk test. Considering that our main purpose was
233 to identify the improvements HU could achieve in SCD trained mice compared to training alone, the
234 statistical analyses were performed to identify the 8-week effects of each intervention i.e., SCD_{TR1} vs.
235 SCD_{TR2} and SCD_{TR-HU1} vs. SCD_{TR-HU2} . The additional or synergistic effect has been recognized when a given
236 significant change occurred in the SCD_{TR-HU} group but not in the SCD_{TR} group. Differences were assessed
237 using paired non-parametric Wilcoxon tests or parametric Student's t-tests according to the result of a
238 Shapiro-Wilk normality test. All statistics were performed using R (RStudio). The significance level was set
239 at $p < 0.05$. Exact p-values are reported, except for results applying to multiple groups where a combined p-
240 value was given for the sake of clarity (NS, $p > 0.05$ or $p < 0.05$).

241

242

243

Results

244

245 **Muscle volume and T2 relaxation times**

246 As summarized in table 1, body weight increased in each group over the 8-week period ($p < 0.05$).
247 Muscle volume also significantly increased over the 8-week period in SCD_{TR} mice ($p < 0.001$) but did not
248 change in SCD_{TR-HU} mice (NS, $p = 0.17$). The relative muscle volume calculated as the muscle volume scaled
249 to body weight did not change over the 8-week period in SCD_{TR} mice (NS, $p > 0.05$) but did decrease for
250 the combined SCD_{TR-HU} experimental condition ($p < 0.001$).

251 Over the same period, the average T2 value of tibialis anterior (TA) and gastrocnemius (GA) muscles
252 significantly decreased in each condition (table 1, $p < 0.05$).

253

254 **Muscle function**

255 The sPf time-course was classically biphasic with an initial increase followed by a progressive decrease
256 until the end of the stimulation period (figure 1A-B). sPf reached its maximum value at around 2 minutes of
257 stimulation.

258 The initial, maximal, and final force production sPf were significantly increased after the 8-week period
259 in each group ($p < 0.05$, table 1). The fatigue index was unchanged during the training intervention (SCD_{TR2},
260 $-2 \% \pm 14$, $p = 1.00$). On the contrary, the combined HU and endurance training intervention resulted in a
261 significant decrease of the fatigue index (SCD_{TR-HU2}, $-12 \% \pm 16$, $p = 0.02$, figure 1D).

262 As illustrated in figure 1C, sTFP was significantly higher after the 8-week period in both SCD_{TR2} ($+52$
263 $\% \pm 42$, $p = 0.0013$) and SCD_{TR-HU2} ($+34 \% \pm 26$, $p = 0.0007$) compared to their initial measurements
264 (SCD_{TR1} and SCD_{TR-HU1}, respectively).

265

266 **Posterior hindlimb muscle bioenergetics**

267 *PCr*

268 As illustrated in figure 2A-B, during the standardized rest-exercise protocol, the relative PCr time course
269 classically displayed 3 different phases i.e. first, an initial progressive decrease during the first 4 min of
270 exercise followed by a second slower one ending by a last period during which a progressive return towards
271 baseline values could be observed.

272 PCr consumption (Δ PCr) was unchanged by the training intervention only. On the contrary, the
273 corresponding parameter was significantly decreased in the SCD_{TR-HU} group over the 8-week TR and HU
274 co-intervention period (table 1).

275 After the 8-week period, the ADP concentration at the end of exercise was significantly increased in
276 both the SCD_{TR} and SCD_{TR-HU} groups ($+50 \pm 72$ %, $p = 0.033$ and $+40 \pm 54$ %, $p = 0.048$, respectively).

277 The rate of PCr consumption ($V_{PCr_{stim}}$) remained unchanged after the 8-week period in both groups
278 (with an average of $+0.05 \pm 1.73$ mM/min, table 1). On the contrary, the rate of PCr recovery ($V_{PCr_{rec}}$) was
279 significantly accelerated after TR ($+1.17 \pm 1.25$ mM/min, $p = 0.0078$) and TR-HU intervention ($+1.47 \pm$
280 1.32 mM/min, $p = 0.0049$, table 1).

281 The maximal rate of PCr recovery, V_{max} , remained unchanged over the 8-week period in SCD_{TR} mice
282 ($+1.18 \pm 2.29$ mM/min, $p > 0.05$) but increased significantly in SCD_{TR-HU} over the same period ($+2.11 \pm$
283 2.38 mM/min, $p = 0.027$).

284 pH_i

285 pH_i at rest was similar before and after the intervention for both conditions ($p > 0.05$, table 1).

286 As illustrated in figure 2C-D, during the stimulation period, a transient alkalinization was observed,
287 followed by a pH_i drop. The acidotic phase was initially fast and then slower. When the stimulation ceased,
288 pH_i progressively increased toward resting values.

289 The extent of intracellular acidosis (ΔpH_i) in response to the stimulation period was not significantly
290 different between the groups (table 1). The pH_i at end of the recovery period, $pH_{i_{end}}$, was not significantly
291 different over the 8-week period in SCD_{TR} group ($+1.32 \pm 2.76$, $p = 0.88$). On the contrary, $pH_{i_{end}}$ was
292 significantly higher after the combined training-HU intervention ($+2.01 \pm 2.20$ %, $p = 0.025$).

293

294

Discussion

295

296 In the present study, we aimed at determining whether HU can improve endurance training effects by
297 addition or synergia on skeletal muscle function and energetics in a sickle cell disease murine model.
298 According to our initial hypotheses, a synergistic effect has been identified in SCD_{TR-HU} for multiple
299 parameters. During the standardized exercise part, both the fatigue index and PCr breakdown were reduced.
300 During the post-exercise recovery period, the maximal aerobic capacity (V_{max}) was improved. Of note, mice
301 submitted to the combined intervention did not show any increase in muscle volume over the 8-week period
302 whereas the trained mice did.

303 The present results are supportive of the expected effects of endurance training. Indeed, oxidative ATP
304 production was improved in SCD_{TR} mice as illustrated by the increased $VPCr_{rec}$ and so in agreement with
305 the previously reported improved oxidative capacity [54,55] and faster PCr resynthesis [56–59]. At the same
306 time, an increased force production was measured. This increased performance is in agreement with previous
307 results showing an increased force production in SCD_{mice} after an 8-week endurance training program [19].
308 One could have also expected an increased V_{max} , a recognized index of oxidative function and maximal
309 aerobic capacity [53] as previously reported [54,55]. This was not observed in the present study. Overall,
310 these results further support the positive effects of training on oxidative muscle function of SCD mice
311 [54,55].

312 The rationale of this study was based on the fact that endurance training is known to improve muscle
313 function and energetics in SCD mice [19] with limited effects in anemic individuals. We hypothesized that
314 the positive effects of HU could overcome this issue [36] given that previous results were supporting this
315 idea [26–30,34]. We aimed at identifying whether synergistic effects could be identified when using both
316 interventions at the same time. This synergy was confirmed when changes occurred as a result of the
317 combined intervention whereas no change was identified for a single condition and when literature does not
318 support any effects of HU on the considered parameters.

319 The combined interventions led to changes during the standardized exercise i.e. a reduced fatigue index
320 and a reduced PCr consumption and the following recovery period i.e. an increased V_{max} . Different
321 interactions between endurance training and HU could account for these synergetic effects. HU has been
322 recognized to alleviate anemia and improve muscle perfusion and oxygen supply [26–30,34], both of which
323 contributing to improve the condition of SCD patients. On the one hand, training has been reported as less
324 effective in anemic rats than in control rats [23]. The recognized HU-mediated effects on anemia would be
325 expected to enhance the beneficial effects of training. On the other hand, it has been previously evidenced

326 that endurance-trained individuals were more responsive to an increased oxygen supply [60]. More
327 specifically, the beneficial effect of hyperoxia on mitochondrial function has been identified in trained
328 subjects only and not in sedentary counterparts [37,38]. Therefore, the HU-mediated increased oxygen
329 supply could then be optimally used in trained individuals. Overall, these synergetic effects would strongly
330 support the hypothesis that hydroxyurea (HU) supplementation enhances training-induced adaptations in
331 skeletal muscle and can most likely be explained through the two mentioned effects.

332 More particularly, our study indicated a fatigue index reduction which has been previously related to
333 endurance training [61,62]. Endurance training could partly restore muscle oxygenation through capillary
334 supply [63,64] thereby contributing to lowering muscle fatigue [65]. The effects of HU on fatigue are still a
335 matter of discussion [36,66]. In a protocol similar to the one presented here, no effect of HU has been
336 reported on fatigue in SCD mice [36] whereas in SCD patients, HU has been reported to increase the time
337 before exhaustion and so likely as a result of the higher Hb level [66]. In the present study, endurance training
338 alone was not sufficient to reduce fatigue. However, as the combination with HU did, one can hypothesize
339 that the combination of HU with training may have potentiated the training effects and led to a reduced
340 fatigue through an anemia reduction and an increased oxygen supply [26–30,34].

341 Regarding the reduced PCr consumption, one must keep in mind that PCr changes illustrate the balance
342 between the ongoing PCr consumption and resynthesis. On that basis, the corresponding reduction can be
343 related to a reduction of the catabolic process i.e. PCr demand or as an increase of PCr resynthesis. A reduced
344 PCr consumption has been previously reported in SCD mice submitted to an endurance training program
345 [19,67] further supporting an enhanced contractile efficiency although the exact mechanisms have not been
346 identified. An improved PCr resynthesis rate illustrates a higher oxidative contribution to ATP production
347 as previously described as a result of training [56–59,68,69]. In the present study, $VPCr_{rec}$ was increased in
348 both SCD_{TR} and SCD_{TR-HU} thereby indicating no synergistic effect. On the contrary, V_{max} , an index of the
349 maximal aerobic capacity [53], was only increased in the SCD_{TR-HU} group. V_{max} can be understood as the rate
350 of aerobic ATP production in a given condition of end-of-exercise ADP, a known activator of aerobic ATP
351 production. Overall, the combination of HU with endurance training maximized the metabolic adaptations
352 contributing to a significant improvement of the maximal aerobic capacity, V_{max} . Indeed, V_{max} is related to
353 both $VPCr_{rec}$ and $[ADP]_{end}$. Although $VPCr_{rec}$ and $[ADP]_{end}$ increased significantly in both SCD_{TR} and
354 SCD_{TR-HU}, the improvement of V_{max} only in SCD_{TR-HU} highlights the fact that the combination of HU and
355 endurance training allowed different modifications and ultimately improved the maximal aerobic capacity.

356 Over the 8-week period, muscle volume increased as a result of training (SCD_{TR}) but not when training
357 and HU supplementation were combined (SCD_{TR-HU}) whereas body weight increased in both groups. In a

358 recent study, Hill *et al* (2020) reported that muscle cross sectional area and muscle mass increased up to 52
359 weeks of age in control mice [70]. Our results might indicate that, in SCD mice, the combination of
360 endurance training and HU would interfere with growth whereas training only would not. However, it has
361 been shown that repeated high dose (205 mg/kg) of HU did not prevent muscle growth *in vitro* [71] and that
362 patients with and without HU displayed the same lean mass over 12 months [35]. Overall, the absence of
363 muscle growth in SCD_{TR-HU} mice can hardly be related to a negative effect of HU. Taking into account that
364 both groups reached comparable muscle volume after the 8-week period, one can think that the reported
365 differences in muscle volume evolution might originate from an earlier muscle growth in SCD_{TR-HU} mice.
366 Another unknown synergistic effect might also have occurred.

367 ***Limitations***

368 We have to acknowledge the absence of Hb measurement and of an untrained group as a limitation of
369 the present study. First, Townes mice are largely acknowledged as anemic with a low hemoglobin
370 concentration (7.1 ± 0.9 g/dL) [39]. A large range of Hb concentrations has been reported in this model
371 (from $\sim 6 \pm 1$ g/dL to 10.06 ± 0.7 g/dL) [31,72]. Nevertheless, thanks to our longitudinal assessment where
372 each mouse is his own control, we can consider any severity of anemia and focus on the analysis of the
373 individual evolution of a mouse in response to endurance training and an additional HU supplementation.
374 We considered that such a group would not have been informative regarding the synergistic effects of
375 training and HU supplementation. For obvious ethical reasons, we have decided to keep the number of
376 animals investigated as small as possible. We did not assess the time effects which could also partly account
377 for the results reported as a result of endurance training.

378

379

Conclusion

380 Synergistic effects of endurance training and hydroxyurea treatment have been observed for skeletal
381 muscle function and energetics in a sickle cell disease mice model. The co-intervention minimized fatigue,
382 reduced the PCr consumption and improved the muscle maximal aerobic capacity. Such an enhanced aerobic
383 capacity in patients would be expected to improve daily life and quality of life. The reduced fatigue should
384 also be a key factor for patients given that fatigue has been described as a major symptom of the disease
385 affecting daily life.

386

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392 **Data availability**

393 Data will be made available upon reasonable request.

394

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611
612

613 Figures

614 **Figure 1:** Muscle function parameters with: time-dependent changes in specific peak force throughout
615 the 6-min moderate stimulation protocol for SCD_{TR} (A) and SCD_{TR-HU} mice (B), presented as mean ± SEM,
616 and specific total force production quantified throughout the 6-minute moderate stimulation protocol of each
617 condition before and after intervention (C) and fatigue index (%) in each condition (D), presented as
618 boxplots. SCD_{TR}: mice before (1) and after (2) an 8-week endurance training period. SCD_{TR-HU}: mice before
619 (1) and after (2) an 8-week endurance training combined to an HU treatment period.

620 **Figure 2:** Muscle metabolic parameters with time-dependent changes in phosphocreatine (PCr) during
621 the 6-min moderate stimulation protocol for endurance training (A) and combined intervention (B) and the
622 time-dependent changes in intracellular pH (pHi) for SCD_{TR} (C) and SCD_{TR-HU} mice (D) presented as means
623 ± SEM. SCD_{TR}: mice before (1) and after (2) an 8-week endurance training period. SCD_{TR-HU}: mice before
624 (1) and after (2) an 8-week endurance training combined to an HU treatment period.

625

626 Tables

627 Table 1: Anatomical, functional and metabolic characteristics in SCD_{TR} and SCD_{TR-HU} groups.

	SCD _{TR1}	SCD _{TR2}	SCD _{TR-HU1}	SCD _{TR-HU2}
<i>Anatomical characteristics</i>				
Body weight (g)	30.70 ± 1.42	33.77 ± 1.89***	29.68 ± 1.49	34.07 ± 2.19***
Hindlimb muscle volume (mm ³)	124.08 ± 6.54	140.22 ± 6.49***	133.40 ± 9.00	138.45 ± 11.54

Relative hindlimb muscle volume (mm ³ /g)	4.04 ± 0.18	4.16 ± 0.28	4.50 ± 0.25	4.06 ± 0.16***
<i>T2 muscles values</i>				
Tibialis Anterior (ms)	26.98 ± 0.88	26.28 ± 0.44*	28.52 ± 1.75	25.36 ± 3.69*
Gastrocnemius (ms)	32.30 ± 1.41	31.08 ± 1.12*	35.14 ± 3.43	32.17 ± 0.96*
<i>Muscle function characteristic</i>				
Initial force (mN/cm ³)	524.98 ± 113.52	787.47 ± 235.02*	488.98 ± 111.07	612.51 ± 138.75**
Force increase (%)	17.22 ± 8.47	18.66 ± 14.86	18.94 ± 8.76	18.02 ± 15.73
Maximal force (mN/cm ³)	630.50 ± 131.67	907.92 ± 172.44**	658.72 ± 129.98	708.76 ± 121.71*
Final force (mN/cm ³)	501.27 ± 156.35	725.26 ± 117.32**	414.08 ± 98.57	590.74 ± 108.59**
Fatigue index (%)	-21.62 ± 12.43	-19.47 ± 7.69	-28.39 ± 8.71	-16.46 ± 7.74*
sTFP (N/cm ³)	205.03 ± 47.53	297.25 ± 56.15**	180.33 ± 39.92	232.06 ± 42.20*
<i>Muscle metabolic indices</i>				
pH _{i rest}	7.17 ± 0.18	7.22 ± 0.11	7.19 ± 0.14	7.17 ± 0.17
Δ pH _i	-0.24 ± 0.11	-0.26 ± 0.12	-0.30 ± 0.11	-0.23 ± 0.15
Δ pH _i / sTFP	-0.0012 ± 0.0007	-0.0009 ± 0.0005	-0.0020 ± 0.0007	-0.0010 ± 0.0006*
pH _{i end}	7.17 ± 0.18	7.26 ± 0.04	7.13 ± 0.15	7.27 ± 0.10*
Δ PCr (%)	-68.27 ± 6.22	-68.91 ± 4.91	-72.46 ± 5.04	-67.84 ± 5.71**
Δ PCr / sTFP	-0.36 ± 0.12	-0.24 ± 0.04*	-0.43 ± 0.12	-0.30 ± 0.05*

ADP ₆ (μM)	41.03 ± 16.69	52.25 ± 10.56*	42.97 ± 10.20	59.44 ± 23.20*
VPCr _{stim} (mM/min)	4.59 ± 1.66	4.56 ± 1.22	4.91 ± 1.57	5.41 ± 1.58
VPCr _{rec} (mM/min)	2.01 ± 0.60	3.18 ± 1.03**	2.16 ± 0.59	3.63 ± 1.05*
V _{max} (mM/min)	3.81 ± 1.62	4.99 ± 1.33	3.70 ± 0.88	5.81 ± 2.14*

628 *Data are presented as means ± SD, * Significantly different from the pre-intervention condition *(p < 0.05), ***
629 *(p < 0.005), ***(p < 0.0005). SCD_{TR}: mice before (1) and after (2) an 8-week endurance training period. SCD_{TR-HU}:*
630 *mice before (1) and after (2) an 8-week endurance training combined to an HU treatment period. sTFP, specific total*
631 *force production; VPCr_{stim}, rate of PCr consumption; VPCr_{rec}, rate of PCr resynthesis; pH_{iend} : intracellular pH at*
632 *the end of the recovery period; ADP₆: ADP quantity at the end of the exercise i.e. 6 minutes of stimulation.*

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Table 1: Anatomical, functional and metabolic characteristics in SCD_{CON}, SCD_{TR-HU} and SCD_{TR} groups.

	SCD _{CON1}	SCD _{CON2}	SCD _{TR1}	SCD _{TR2}	SCD _{TR-HU1}	SCD _{TR-HU2}
Anatomical characteristics						
Body weight (g)	27.48 SD 2.08	33.08 SD 1.98*	30.70 SD 1.42	33.77 SD 1.89***	29.68 SD 1.49	34.07 SD 2.19***
Hindlimb muscle volume (mm ³)	111.83 SD 15.82	134.17 SD 13.98*	124.08 SD 6.54	140.22 SD 6.49***	133.40 SD 9.00	138.45 SD 11.54
Relative hindlimb muscle volume (mm ³ /g)	4.05 SD 0.31	4.05 SD 0.30	4.04 SD 0.18	4.16 SD 0.28	4.50 SD 0.25	4.06 SD 0.16***
T2 muscles values						
Tibialis Anterior (ms)	26.69 SD 0.44	25.93 SD 0.69*	26.98 SD 0.88	26.28 SD 0.44*	28.52 SD 1.75	25.36 SD 3.69*
Gastrocnemius (ms)	32.27 SD 0.80	30.81 SD 1.36*	32.30 SD 1.41	31.08 SD 1.12*	35.14 SD 3.43	32.17 SD 0.96*
Muscle function characteristic						
Initial force (mN/cm ³)	484.20 SD 141.49	765.61 SD 189.94**	524.98 SD 113.52	787.47 SD 235.02*	488.98 SD 111.07	612.51 SD 138.75**
Force increase (%)	41.21 SD 37.54	16.44 SD 12.56	17.22 SD 8.47	18.66 SD 14.86	18.94 SD 8.76	18.02 SD 15.73
Maximal force (mN/cm ³)	655.41 SD 161.42	882.98 SD 189.10**	630.50 SD 131.67	907.92 SD 172.44**	658.72 SD 129.98	708.76 SD 121.71*
Final force (mN/cm ³)	535.15 SD 144.14	653.58 SD 167.53	501.27 SD 156.35	725.26 SD 117.32**	414.08 SD 98.57	590.74 SD 108.59**
Fatigue index (%)	-18.12 SD 12.05	-26.65 SD 4.38	-21.62 SD 12.43	-21.62 SD 12.43	-28.39 SD 8.71	-16.46 SD 7.74*
sTFP (N/cm ³)	210.67 SD 48.60	276.40 SD 62.12*	205.03 SD 47.53	297.25 SD 56.15**	180.33 SD 39.92	232.06 SD 42.20*
Muscle metabolic indices						
pH _{i rest}	7.16 SD 0.12	7.12 SD 0.19	7.17 SD 0.18	7.22 SD 0.11	7.19 SD 0.14	7.17 SD 0.17
Δ pH _i	-0.27 SD 0.10	-0.27 SD 0.09	-0.24 SD 0.11	-0.26 SD 0.12	-0.30 SD 0.11	-0.23 SD 0.15

$\Delta \text{pH}_i / \text{sTFP}$	-0.0013 SD 0.0003	-0.0010 SD 0.0002	-0.0012 SD 0.0007	-0.0009 SD 0.0005	-0.0020 SD 0.0007	-0.0010 SD 0.0006*
$\text{pH}_{i \text{ end}}$	7.19 SD 0.18	7.34 SD 0.08	7.17 SD 0.18	7.26 SD 0.04	7.13 SD 0.15	7.27 SD 0.10*
$\Delta \text{PCr} (\%)$	-71.76 SD 8.65	-79.79 SD 5.60	-68.27 SD 6.22	-68.91 SD 4.91	-72.46 SD 5.04	-67.84 SD 5.71**
$\Delta \text{PCr} / \text{sTFP}$	-0.36 SD 0.12	-0.31 SD 0.11	-0.36 SD 0.12	-0.24 SD 0.04*	-0.43 SD 0.12	-0.30 SD 0.05*
$\text{ADP}_6 (\mu\text{M})$	52.75 SD 24.02	81.66 SD 36.34	41.03 SD 16.69	52.25 SD 10.56*	42.97 SD 10.20	59.44 SD 23.20*
$\text{VPCr}_{\text{stim}} (\text{mM}/\text{min})$	5.13 SD 1.43	4.63 SD 0.54	4.59 SD 1.66	4.56 SD 1.22	4.91 SD 1.57	5.41 SD 1.58
$\text{VPCr}_{\text{rec}} (\text{mM}/\text{min})$	2.34 SD 0.80	3.17 SD 0.50	2.01 SD 0.60	3.18 SD 1.03**	2.16 SD 0.59	3.63 SD 1.05*
$V_{\text{max}} (\text{mM}/\text{min})$	3.80 SD 1.01	4.57 SD 1.14	3.81 SD 1.62	4.99 SD 1.33	3.70 SD 0.88	5.81 SD 2.14*

*Data are presented as means SD, * Significantly different from the pre-intervention condition *($p < 0.05$), ** ($p < 0.005$), ***($p < 0.0005$). SCD_{CON} : mice before (1) and after (2) an 8-week control period. SCD_{TR} : mice before (1) and after (2) an 8-week endurance training period. $\text{SCD}_{\text{TR-HU}}$: mice before (1) and after (2) an 8-week endurance training combined to an HU treatment period. sTFP , specific total force production; $\text{VPCr}_{\text{stim}}$, rate of PCr consumption; VPCr_{rec} , rate of PCr resynthesis; $\text{pH}_{i \text{ end}}$: intracellular pH at the end of the recovery period; ADP_6 : ADP quantity at the end of the exercise i.e. 6 minutes of stimulation.*

Table 2: Relative indexes variations over the 8-week period in each group.

	SCD _{CON}	SCD _{TR}	SCD _{TR-HU}
Body weight (%)	+ 20.75 SD 10.23	+ 10.07 SD 5.63	+ 14.74 SD 3.21
T2 values in TA (%)	- 2.79 SD 3.77	- 2.53 SD 2.79	- 10.35 SD 18.03
T2 values in GA (%)	- 4.40 SD 6.24	- 3.65 SD 4.39	- 7.85 SD 7.45
Muscle volume (%)	+ 20.93 SD 10.40	+ 13.07 SD 3.08	
Initial force (%)	+ 68.30 SD 59.62	+ 53.45 SD 41.11	26.74 SD 20.90
Maximal force (%)	+ 36.90 SD 20.61	49.62 SD 40.48	25.74 SD 24.88
Final force (%)		58.49 SD 56.73	49.14 SD 38.65
sTFP (%)	+ 32.30 SD 18.51	51.63 SD 42.34	32.45 SD 25.79
VPC _{rec} (mM/min)		+1.17 SD 1.25	+1.47 + 1.32
Δ PCr / sTFP		+ 0.12 SD 0.12	+ 0.13 SD 0.09

Results are presented as means SD. No significant variation was found. TFP, total force production; TA: tibialis anterior muscle; GA: gastrocnemius muscle; SCD_{CON}: mice submitted to a control period; SCD_{TR}: mice submitted to a training period; SCD_{TR-HU}: mice submitted to a combined training and HU-treatment period.



