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The role of nitrite in muscle function, susceptibility to contraction injury, and fatigability in sickle cell mice

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Abstract

Sickle cell disease (SCD) patients can have limited exercise capacity and muscle dysfunction characterized by decreased force, atrophy, microvascular abnormalities, fiber distribution changes, and skeletal muscle energetics abnormalities. Growing evidence suggests that in SCD, there is alteration in nitric oxide (NO) availability/signaling and that nitrate/nitrite can serve as a NO reservoir and enhance muscle performance. Here, we examined effects of nitrite on muscle strength, exercise capacity, and on contractile properties of fast-(extensor digitorum longus, EDL) and slow-twitch (soleus) muscles in SCD mice. Compared to controls, homozygotes (sickling) had decreased grip strength, impaired wheel running performance, and decreased muscle mass of fast-twitch, but not slow-twitch muscle. Nitrite treatment yielded increases in plasma levels in controls, heterozygotes, and homozygotes but decreases in muscle nitrite levels in heterozygotes and

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Performed experiments, collected data: LW, LEFA, SK, JHM Analyzed and interpreted the data: PW, MQ, ZQ

Contributed reagents/materials/analysis tools: ZQ, JVM, KN

Wrote the paper: LW, LEFA, ZMNQ

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Declarations of interest

The authors have no conflict of interest to declare

homozygotes. Regardless of genotype, nitrite yielded increases in grip strength, which were coupled with increases in specific force in EDL, but not in soleus muscle. Further, nitrite increased EDL, but not soleus, fatigability in all genotypes. Conversely, in controls, nitrite decreased, whereas in homozygotes, it increased EDL susceptibility to contraction-induced injury. Interestingly, nitrite yielded no changes in distances ran on the running wheel. These differential effects of nitrite in fast- and slow-twitch muscles suggest that its ergogenic effects would be observed in high-intensity/short exercises as found with grip force increases but no changes on wheel running distances. Further, the differential effects of nitrite in homozygotes and control animals suggests that sickling mice, which have altered NO availability/signaling, handle nitrite differently than do control animals.

Keywords

muscle; strength; function; contractility; fatigue; pain

1. Introduction

As a result of a single point mutation on the beta subunit of hemoglobin, patients with sickle cell disease (SCD), develop hemolytic anemia, recurrent episodes of vaso-occlusion, and significant co-morbidities including cardiac, pulmonary, urogenital, central nervous system, and gastrointestinal dysfunctions, which result in decreased life expectancy[1; 2]. While less studied, skeletal muscle dysfunction, manifested by reduced muscle force and limitations in exercise capacity has also been reported in SCD patients [3; 4; 5; 6; 7]. While in part, these limitations could be explained by severe anemia and SCD-associated cardiovascular and pulmonary dysfunctions,[3; 5; 6; 7] recent studies show that SCD patients can also develop muscle atrophy, muscular microvascular abnormalities, changes in muscle fiber type distribution, and alterations in skeletal muscle energetics[8]. Therefore, while anemia and SCD-associated cardiopulmonary dysfunction can contribute to muscle dysfunction in SCD, other mechanisms possibly underlie skeletal muscle dysfunction in SCD [8].

The free radical nitric oxide (NO) is known to play an important role in regulating muscle perfusion and oxygen delivery, muscle mass, calcium handling, satellite cell activation, and glucose uptake both at rest and during muscle contraction [9; 10; 11; 12; 13; 14]. In fact, all NO synthases isoforms (NOS) are highly expressed and are believed to be a significant source of NO generation in skeletal muscle [14]. In SCD, researchers postulate that several factors associated with the disease process can lead to decreased NO availability [15; 16]. One such factor is hemolysis leading to release of free hemoglobin, which rapidly reacts with and scavenges NO [17; 18]. Hemolysis also leads to the release of arginase, which competes with NO synthases for their substrate, arginine, thus also limiting NO production in SCD [15]. Additionally, oxidation of the NOS co-factor tetrahydrobiopterin can contribute to NOS inactivation further adding to NO bioavailability deficits in SCD [15; 19; 20; 21; 22]. Therefore, given the critical role of NO in skeletal muscle metabolism, function, mass, and regeneration, it is reasonable to hypothesize that decreased NO bioavailability is likely to contribute to muscle dysfunction in SCD.

Nitrate and nitrite, known byproducts of NO metabolism, can serve as a NO reservoir as both can be converted back into NO particularly under ischemic and hypoxic conditions [23; 24]. In skeletal muscle, researchers showed that, both in animals and humans during basal conditions, nitrate levels in skeletal muscle are higher than in plasma levels [25; 26; 27] and in response to exercise, muscle nitrate levels decrease and nitrite levels increase [25; 26]. Others showed that nitrite supplementation increases nitrite levels in wild type and endothelial NOS knockout mice and that in models of ischemia/reperfusion injury in cardiac muscle it has salutary effects [28; 29]. Additionally, nitrite has also been shown to improve grip strength and decrease skeletal muscle inflammation in aging rodents [30]. Therefore, growing evidence suggests that in muscle, the nitrate/nitrite pathway can serve as a reservoir for NO production and may play a beneficial role during muscle contraction and on exercise-induced functional hyperemia. For these reasons, there is great interest on using the nitrate/nitrite pathway as a source of NO to improve muscle function and exercise performance in athletes and in patients with cardiovascular disease [31].

Humanized mouse models of SCD recapitulate the multi-organ dysfunction pattern observed in SCD patients. These animals have hemolysis, anemia, cardiopulmonary, hepatic and renal dysfunction, as well as somatosensory alterations (sensitization of sensory nerve fibers) and cognitive deficits similar to those observed in SCD patients [21; 32; 33; 34; 35; 36; 37; 38; 39; 40]. In concert with human findings of muscle dysfunction in SCD, we and others have shown that homozygous SCD mice have decreased muscle strength, decreased exercise capacity, impaired muscle force production capacity and higher muscle fatigability with intense exercise [40; 41; 42]. Using controls, heterozygotes, and homozygous (sickling) humanized Townes mice, we tested the hypothesis that nitrite would improve muscle function in SCD. We examined the effect of nitrite on muscle strength and exercise capacity in vivo, on contractile properties of fast- (extensor digitorum longus, EDL) and slow-twitch (soleus) muscles, susceptibility to contraction-induced injury, and on muscle fatigability ex vivo.

2. Material and Methods

2.1. Animals and preclinical trial

The NIH Clinical Center and Children's Research Institute Animal Care and Use Committees approved all experimental protocols. In this investigation, we used the humanized Townes model of SCD [32; 33; 34]. In these animals murine α and β globins were knocked-out and human hemoglobin genes knocked-in. All genotypes carry two alleles of the human α globin gene ($h\alpha$). Homozygotes ($h\alpha/h\alpha::\beta^S/\beta^S$) carry two alleles of the human sickle hemoglobin beta gene (β^S) and heterozygotes ($h\alpha/h\alpha::\beta^A/\beta^S$) carry one allele of the human β^S and one of the human wild-type hemoglobin beta gene (β^A). Control Townes mice ($h\alpha/h\alpha::\beta^A/\beta^A$) mice carry β^A genes. Animals' genotypes were determined prior to group assignment as described [40].

We examined the effects of nitrite treatment on grip strength, distance ran on the voluntary wheel in vivo, muscle contractile properties ex vivo, and biochemical parameters in control, heterozygous and homozygous Townes mice. We first examined the effect of four-week treatment with 0.05, or 1g/L of nitrite in drinking water (vehicle) on water and nitrite intake

and whole blood methemoglobin levels. As we had shown that nitrite diluted in drinking water is stable for at least one week and that double-distilled drinking water had undetectable nitrite levels[43], nitrite was administered in drinking water and prepared and changed twice every week. Given the results of the initial experiments (please see results), for the large cohort trial, we selected age-matched male and female mice from all genotypes to receive either double distilled drinking water (vehicle) or sodium nitrite (1g/L in vehicle, Sigma-Aldrich, St. Louis, MO) continuously for four weeks. All outcome measurements were obtained after four-week treatment was completed and while animals were still receiving nitrite or vehicle.

2.2. Behavior Studies

2.2.1. Grip Strength—We measured forelimb and total (forelimbs and hind limbs together) grip strength using the Grip Strength Meter (GSM, San Diego, Inc., San Diego, CA) as described [40; 41]. Briefly, prior to data collection, mice were trained on holding the instrument grids for grip strength measurement. Animals were held by the tail, directed and allowed to grasp a steel grip gauge with forelimbs only or with all four limbs. After gripping the steel grip gauge, mice were gently and steadily pulled away until the grip was released. The force measured at time of grip release (either forelimbs only or all four limbs) was recorded as grip strength. Measurements of forelimb and total grip strength were obtained daily for three consecutive days and daily forelimb and total grip strength were calculated as the average of five measurements [40; 41].

2.2.2. Voluntary wheel running—In order to evaluate overall physical activity performance, we measured distances ran daily over five days on the voluntary wheel in 16–23-week-old in homozygotes, heterozygotes, and control mice [40] as previously described. Mice were placed individually in transparent cages containing a stainless steel wheel (Mini-Mitter Company, Inc., Bend, OR). Prior to data collection, animals acclimated to the wheel for three days. Individual wheel revolutions and daily distances traveled by each animal were recorded. Animals remained in individual cages throughout the experiment.

2.3. Contractile properties of the extensor digitorum longus (EDL) and soleus muscles

We evaluated the contractile properties of the EDL and soleus muscles *ex-vivo* as previously described [44; 45]. Briefly, individual muscles were removed from the hind limb from anesthetized mice and immersed in an oxygenated bath (95% O₂, 5% CO₂) containing Ringer's solution (in mM: 137 NaCl, 24 NaHCO₃, 11 glucose, 5 KCL, 2 CaCl₂, 1 MgSO₄, 1 NaH₂PO₄ and 0.025 tubocurarine chloride; pH 7.4) at 25°C. The proximal tendon was tied securely to a dual-mode servomotor and the distal tendon was clamped to a fixed post (Aurora Scientific Inc., Aurora, Ontario, CA). Flanked by two stainless steel plate electrodes, the muscle was stimulated with twitches and adjusted to the optimal length for force generation. With the muscle held at optimal length, the EDL was stimulated for 300ms and the soleus muscle for 1000ms. The stimulation trains were separated by two-minute rest intervals. With each subsequent tetanus, the stimulation frequency was increased with 20 or 30Hz until the maximal force was reached. According to previous studies, the maximal force is found at 250Hz in EDL and 100Hz in soleus muscles. Once all studies were completed, the length of the muscle was measured and the muscle was taken from the bath and weighed.

Muscle specific force was calculated as the maximal isometric tetanic force normalized for the cross-sectional area of the muscle, which was estimated as the muscle mass divided by the product of fiber length and density of the tissue. The rationale for normalizing force to cross sectional area instead of muscle mass or volume is that by doing so, one takes into consideration the pennation angle of the fibers in the muscle. This is relevant as muscles with the same mass but with a different angle of pennation have different specific forces. This explains for example that the soleus muscle (smaller angle of pennation, endurance) has a lower specific force than the EDL muscle (larger angle of pennation, strength) [46; 47]. Fiber length was determined by multiplying the optimal muscle length by the ratio of fiber length to muscle length of 0.45 for the EDL muscle and 0.71 for the soleus muscle. We assumed muscle tissue density to be 1.056 kg/L as measured using the Archimedes' principle [46].

To study susceptibility to contraction-induced injury, the EDL muscles were subjected to a protocol of six lengthening contractions. Because the lengthening component adds high levels of strain on the muscle fibers, it is considered an eloquent method to show vulnerabilities in muscle function. To accomplish a lengthening contraction, the muscles were stimulated at 250Hz for 350ms at optimal length. Once a plateau of force generation was reached, the muscles were then lengthened from optimal length as they were stimulated. Once the muscle were lengthened over the desired length, the stimulation was stopped and the muscles were returned to the optimal length. During the first contraction, the muscles were lengthened over 2.5% of the fiber length. Before the protocol of lengthening contractions, a fiber to muscle length ratio of 0.45 was applied to the muscle length in order to calculate fiber length of the muscle. The second lengthening contraction was 5% of fiber length, followed by 10, 15, 20 and 25%. Each contraction was separated by one minute of rest. The deficit in force generation during lengthening contraction was determined by establishing the relationship between the force measured at each lengthening and that obtained before the experimental protocol started (expressed in percent of initial force).

Fatigability of EDL and soleus muscles were assessed by subjecting the muscles to 60 isometric contractions, once every five seconds. The duration of the isometric contractions was 400ms for the EDL muscles and 1000ms for the soleus muscles. The stimulation frequency during the isometric contractions for the EDL was 250Hz and 100Hz for the soleus muscle. After the fatigue protocol was completed at 5 min, the EDL muscles were allowed to rest for 10 min and the soleus for 5 min when a single isometric contraction was administered to assess the first phase of recovery. The deficit in force generation (fatigability) during the fatigue protocol was determined by establishing the relationship between the force measured at each time point and that obtained at baseline (expressed in percent of baseline force).

2.4. Methemoglobin

Methemoglobin was assayed as previously described [48]. Briefly, whole blood was diluted to 3% with double-distilled water and 200µl were loaded in duplicates on a clear bottom 96 well plate. An absorbance spectrum was obtained between 500 – 700nm (1nm interval). The average of each duplicate was obtained and the first derivative (D1) for each absorbance was

calculated by subtracting the absorbance at wavelength (x+1) minus absorbance at wavelength x. This method is based on the selective methemoglobin peak at 645nm observed at D1[48]. Total hemoglobin was calculated by obtaining a second spectrum of the same samples after addition of potassium ferricyanide III (10µl from a 100mM stock solution). The relative amount of methemoglobin was calculated by dividing the 645nm D1 peak recorded in the absence of potassium ferricyanide III by that recorded after its addition [48]. There is debate over the “best” method for methemoglobin assay and the method used here is known to yield higher methemoglobin levels than other methods [49], however we selected this method because it is not affected by the formation of nitrosyl-methemoglobin [50] and as animals were treated with nitrite for over 4 weeks, non-detection of nitrosyl-methemoglobin could generate abnormally low methemoglobin values.

2.5. NOx and Nitrite levels

Total nitrate and nitrite (NOx) levels were measured with a fluorometric assay following the manufacturer instructions (Cayman Chemicals, Ann Arbor, MI). Plasma and muscle nitrite levels were measured using the acetic and ascorbic acids method as previously described[43; 51]. Briefly, nitrite in the sample is converted into nitric oxide (NO) in a reaction chamber, NO is then carried by an argon gas stream into a NO analyzer (Sievers 280i, GE Analytical Instruments, Boulder, CO) where it is measured in a chemiluminescence reaction with ozone generating an electrical signal. Given the small volume of EDL and soleus, muscle nitrite was assayed in a muscle bundle (EDL and soleus muscle combined) obtained from the hind limb not used for EDL and soleus removal for ex vivo studies. All plasma and muscle samples were processed in the presence of freshly prepared nitrite stabilization solution [52; 53] and processed for nitrite assay as described [43; 51].

2.6. Creatine kinase activity

Creatine kinase activity was measured in plasma using a standard enzymatic assay performed according to the manufacturer’s instructions (CK10, Fisher Scientific, Waltham, MA) as previously described[44; 45]. Creatine kinase enzymatic activity was determined by measuring light absorption in duplicates using a spectrometer set at 340 nm every minute for a total of 2 min at 37°C.

2.7. Histopathology

Soleus and EDL muscles were quickly embedded in OCT and snap frozen in liquid nitrogen/frozen isopentane. Cross sections (10 µm thick) were obtained from the center of the muscle and stained with hematoxylin-eosin for histological analysis.

2.8. Statistical analysis

For data recorded at one time point, i.e. for analyses that produced results shown in Figs. 1, 3, 4, 8, and supplemental, an Analysis of Variance (ANOVA) model with interaction terms (treatment by genotype, treatment by sex, sex by genotype) was used. For data with repeated measures, i.e. on three consecutive days (analyses that produced results in Fig. 2A, 2B and 2C), on five days (analysis for Fig. 2D), at several muscle lengthening (analyses for Fig. 5), or at multiple time points (analyses for Figs. 6 and 7), a repeated-measures mixed model

with interaction terms was used. Muscle lengthening was modeled as a continuous variable, and its squared value was included in the model. Day and time (fatigability study) were treated as categorical variables. For models with repeated measures, the best variance-covariance matrix structure was determined based on the Schwarz's Bayesian Information Criterion (BIC) [54; 55].

Model fit diagnostics were examined to check whether model assumptions were satisfied. In three cases, overall distance run on the voluntary wheel (Fig. 2D), plasma nitrite (Fig. 8A) and creatine kinase (Fig. 8D), they were not. The outcome measure was then transformed to the natural logarithmic scale to meet model assumptions. From each model, least-squares means with corresponding 95% confidence intervals were obtained. In cases where the outcome measure was log-transformed, least-squares means and corresponding variances were derived to convert results back to the original scale. Reported p-values for overall effects and interaction terms are based on Type 3 analyses.

Many statistical tests (comparisons) were performed, and many p-values reported. Because this is an exploratory study with its results meant to be hypothesis-generating rather than definitive, reported p-values are unadjusted for multiple comparisons [56; 57]. The reader is encouraged to keep that in mind when interpreting reported p-values, particularly the ones close to 0.05. Where appropriate, as an indication of effect size, we report the estimates of differences and 95% confidence intervals between experimental groups. All analyses were conducted using the statistical software SAS, version 9.4 (SAS Institute, NC, USA).

3. Results

Supplemental Table 1 lists number of animals in all experimental groups according to genotype, sex, and treatment for each experiment. Based on the number of males and females enrolled in the study (Supplemental Table 1), there was reasonable balance between the two sexes among experimental groups. Additionally, including treatment by sex and treatment by genotype interaction terms in the statistical model, allowed us to test whether the treatment effect was different for male and female mice and for controls, heterozygotes, and homozygotes. If an interaction term was statistically significant, the overall treatment effect could possibly be misleading, and one should focus instead on the treatment effect for each sex and each genotype as indicated in each subsection below.

3.1. Effect of nitrite treatment on methemoglobin levels

In keeping with previous reports [58], among vehicle-treated animals, homozygotes had higher water intake compared to control mice [186 (106, 266) ml/kg/day, difference estimate (95% CI), $p < 0.0001$, Fig. 1A]. Nitrite-treated animals had significantly lower water intake compared to vehicle-treated mice, regardless of genotype ($p < 0.0001$ for overall treatment effect, Fig. 1A). Regarding nitrite intake, with doses of 1g/L [265 (243, 288) g/kg/day, difference estimate (95% CI), $p < 0.0001$], but not with doses of 0.05 g/L [11 (-12, 35) g/kg/day, $p = 0.0337$] there were significant increases in the levels of nitrite intake compared with vehicle, Fig. 1B. Among vehicle-treated animals, homozygotes had greater percentages of methemoglobin compared with control mice [7.91 (4.14, 11.68) %, difference estimate (95% CI), $p < 0.0001$, Fig. 1C]. Interestingly, there was no overall effect of nitrite-treatment on

levels of methemoglobin ($p=0.412$ for overall effect of nitrite, Fig. 1C) compared with vehicle. Given that the dose of 1g/L was the only dose associated with significant increase in nitrite intake in homozygotes and this dose was not associated with changes in methemoglobin levels compared with vehicle, we chose the dose of nitrite at 1g/L in drinking water for the trial.

3.2. Effect of nitrite treatment on grip strength and voluntary wheel running

In concert with previous reports [40], vehicle-treated homozygotes had lower total (all limbs) and forelimbs grip strengths, compared with controls and heterozygotes (all $p<0.0001$, Fig. 2A, 2B). Nitrite treatment increased total grip strength as nitrite-treated controls [0.737 (0.050, 1.423), difference estimate (95%CI) g force/g body weight], heterozygotes [0.718 (0.069, 1.367) g force/g body weight], and homozygotes [1.114 (0.470, 1.758) g force/g body weight] had significantly greater grip strength compared to vehicle-treated animals [0.856 (0.473, 1.240) g force/g body weight, $p<0.0001$ for overall treatment effect, Fig. 2A).

Conversely, the patterns of nitrite treatment effects on mice forelimb grip strength significantly varied according to genotype ($p=0.028$, for treatment-by-genotype interaction, Fig. 2B). Compared to their respective vehicle-treated mice, nitrite-treated controls had similar [0.320 (-0.080, 0.720) g force/g body weight, difference estimate (95%CI), $p=0.116$], whereas nitrite-treated homozygotes [0.993 (0.608, 1.368) g force/g body weight, $p<0.0001$] and heterozygotes [0.395 (0.017, 0.773) g force/g body weight, $p=0.041$] had significantly greater forelimb grip strength. Interestingly, the nitrite effect pattern on grip strength also varied according to sex (Fig. 2C). Compared to respective vehicle-treated mice, nitrite-treated females had higher [0.945 (0.654, 1.241) g force/g body weight, $p<0.0001$], whereas, nitrite-treated males had similar [0.191 (-0.146, 0.527) g force/g body, $p=0.263$] forelimb grip strength and these patterns of nitrite treatment effects were significantly different ($p=0.001$ for treatment-by-sex interaction, Fig. 2C).

Also in concert with previous results [40], among vehicle-treated animals, homozygotes [-1292 (-1944, -641) m, difference estimate (95%CI), $p=0.0002$] and heterozygotes [-964 (-1616, -312) m, $p=0.0043$] mice ran significantly shorter distances over five days compared to controls, Fig. 2D). Also in keeping with previous reports [40], females ran longer distances in the voluntary wheel compared to male mice [807 (417, 1197) m, difference estimate (95%CI), $p<0.0001$]. Unlike the effects observed on grip strength, nitrite treatment had no effect ($p=0.199$ for overall treatment effect) on distances ran on the voluntary wheel in any genotype (Fig. 2D).

3.3. Effect of nitrite on muscle mass and force production measured ex vivo

Controls, heterozygotes, and homozygotes had similar body mass ($p=0.126$, for overall genotype effect, Fig. 3A) and males had greater body mass than females [4.9 (3.8, 5.9) g, difference estimate (95%CI), $p<0.0001$ for overall effect of sex, Fig. 3B]. The effect of nitrite treatment on body mass varied according to mouse sex. Specifically, nitrite-treated females had significantly higher [2.2 (0.8, 3.7) g, $p=0.002$] whereas, nitrite-treated males [-0.03 (-1.54, 1.47) g, $p=0.969$] had similar body mass (weight) compared to their

respective vehicle-treated animals and these patterns of nitrite treatment effect in male vs. female were significantly different ($p=0.034$ for treatment-by-sex interaction, Fig. 3B).

We then examined mass and contractile properties of fast-twitch muscles. Homozygotes had significantly lower EDL muscle mass compared to controls [1.05 (0.56, 1.55) mg, difference estimate (95%CI), $p<0.0001$] and heterozygotes [0.89 (0.41, 1.35) mg, $p=0.0007$], $p<0.0001$ for overall genotype effect, Fig. 3C. While nitrite had no overall effect on muscle mass ($p=0.527$), the patterns of nitrite treatment effect in male vs. female were significantly different ($p=0.008$ for treatment-by-sex interaction, Fig. 3D). Specifically, compared with respective vehicle-treated animals, nitrite-treated males had significantly lower $[-0.68 (-1.26, -0.10)$ mg, difference estimate (95%CI), $p=0.021$], whereas nitrite-treated females $[0.42 (-0.13, 0.97)$ mg, $p=0.132$] had no significant changes in EDL muscle mass (Fig. 3D).

Overall, homozygotes had lower EDL maximal force compared to control $[-26.8 (-52.0, -1.5)$ mN, difference estimate (95%CI), $p=0.038$] and heterozygotes $[-30.6 (-54.4, -6.8)$ mN, $p=0.012$], ($p=0.029$, overall effect of genotype, Fig. 3E). Additionally, compared with vehicle, nitrite treatment was associated with an overall trend towards increases in maximal force irrespective of genotype $[10.3 (-0.27, 40.3)$ mN, difference estimate (95%CI), $p=0.056$ for overall treatment effect]. Further, nitrite-treated heterozygotes had significantly greater EDL maximal force compared with vehicle-treated mice $[34.4 (0.9, 67.9)$ mN, difference estimate (95%CI), $p=0.044$, Fig. 3E]. Homozygous and heterozygous mice had similar EDL specific force (maximal force/muscle cross sectional area) compared to control animals ($p=0.910$ for overall genotype effect, Fig. 3F) and independent of genotype, nitrite administration was associated with overall increases in EDL specific force compared with vehicle $[17.9 (3.4, 32.4)$ kN/m², difference estimate (95%CI), $p=0.017$ for overall treatment effect, Fig. 3F].

Regarding the *ex vivo* contractile properties of the soleus muscle (slow-twitch muscle), controls, heterozygotes, and homozygotes had similar soleus muscle mass, maximal force, and specific force (all $p>0.05$, Figs. 4A, 4B, and 4C). Additionally, nitrite treatment yielded no significant changes on soleus muscle mass or specific force irrespective of genotype or sex (all $p>0.303$, Figs. 4A and 4C). However, while there was no overall effect of nitrite treatment on soleus maximal force in all genotypes combined, nitrite-treated heterozygotes had greater soleus maximal force compared to vehicle-treated heterozygotes $[22.7 (0.3, 45.0)$ kN/m², difference estimate (95%CI)] $p=0.047$, Fig. 4B).

3.4. Effect of nitrite on muscle susceptibility to contraction-induced injury

Among vehicle treated mice, EDL muscle susceptibility to contraction-induced injury was predominantly similar among all genotypes except that EDL muscle from heterozygotes showed greater decreases in percent force following 10% $[-7.4 (-14.5, -0.4)$ %, difference estimate (95%CI), $p=0.039$] and 15% $[-7.8 (-15.3, -0.3)$ %, $p=0.042$] active stretches compared to homozygotes, Fig. 5, supplemental Tables 2 and 3.

Interestingly, the effect of nitrite on EDL susceptibility to contraction-induced injury in controls and heterozygous was significantly different from those observed in homozygous mice ($p=0.012$, for treatment-by-genotype interaction, Fig. 5, supplemental Tables 2 and 3).

In controls, nitrite ameliorated contraction-induced injury as EDL from nitrite-treated homozygotes had significantly less decreases in percent force following active stretches compared with EDL muscle from vehicle-treated mice ($p=0.015$ for 10% and $p=0.016$ for 15% active stretches, Fig. 5A, supplemental Tables 2 and 3). In heterozygotes, nitrite, compared with vehicle, was associated with similar decreases in percent force following active stretches thus yielding no changes in EDL muscle susceptibility to contractions-induced injury, Fig. 5B and supplemental Tables 2 and 3. Conversely, in homozygotes, nitrite exacerbated contraction-induced injury as EDL muscle from nitrite-treated homozygotes had significantly greater decreases in percent force following active stretches of 5 ($p=0.018$), 10 ($p=0.0009$), 15 ($p=0.0001$), 20 ($p<0.0001$) and 25% ($p<0.0001$) compared with EDL muscle from vehicle-treated mice, Fig. 5C and supplemental Tables 2 and 3.

Additionally, the overall effects of nitrite treatment compared with vehicle on EDL susceptibility to contraction-induced injury were similar among males and females (data not shown, $p=0.946$ for overall treatment-by-sex interaction).

3.5. Effect of nitrite on muscle fatigability

Among vehicle-treated mice, fatigability and the initial phase of recovery of force producing capacity in EDL muscles from controls, heterozygotes, and homozygotes were similar (all $p>0.05$, Fig. 6, supplemental Table 4). Nitrite treatment increased EDL muscle fatigability in all genotypes as EDL from nitrite-treated controls, heterozygotes, and homozygotes had greater decreases in force producing capacity from baseline compared with EDL from respective vehicle-treated mice, Fig. 6, supplemental Tables 4 and 5. Interestingly, from the last time point in the fatigue protocol (5 min) to the initial phase of recovery (at 15 min), EDL muscles from nitrite-treated controls and heterozygotes, but not homozygotes, exhibited greater increases in force producing capacity compared with EDL muscle from respective vehicle-treated mice ($p=0.0001$, $p=0.012$, and $p=0.115$ for controls, heterozygotes, and homozygotes, respectively, Fig. 6 and supplemental Tables 4 and 5).

Among vehicle-treated mice, fatigability or recovery of force producing capacity in soleus muscles from controls, heterozygotes, and homozygotes were similar (all $p>0.05$, Fig. 7). Additionally nitrite-treatment had no effect on fatigability of soleus muscle from animals of any genotype. However, during recovery, 5 min after the fatigue protocol, soleus muscle from nitrite-treated heterozygotes and homozygotes generated less force compared to respective vehicle-treated animals [-10.293 (-17.767 , -2.818) %, difference estimate (95%CI), $p=0.007$, Fig. 7B for heterozygotes, and -9.103 (-16.761 , -1.445) %, $p=0.020$, Fig. 7C, for homozygotes].

3.6. Effect of nitrite treatment on plasma and muscle nitrite levels and plasma creatine kinase activity

Among vehicle-treated mice, controls, heterozygotes, and homozygotes had similar plasma nitrite levels ($p=0.123$ for all comparison, Fig. 8A). Additionally, contrary to plasma nitrite levels, among vehicle-treated mice, homozygous and heterozygous mice had significantly higher muscle nitrite levels compared to controls [1.450 (0.376 , 2.525) nmol/g, difference

estimate (95%CI), $p=0.008$] for homozygotes and [1.368 (0.306, 2.429) nmol/g, $p=0.012$] for heterozygotes vs. controls respectively, Fig. 8B]. Notably, in all genotypes, muscle nitrite levels were approximately three to four-fold higher than plasma nitrite (Fig. 8A and 8B). Nitrite treatment increased plasma nitrite levels independently of genotype, as nitrite-treated mice had significantly higher plasma nitrite levels compared to vehicle-treated animals ($p<0.0001$ for overall treatment effect, Fig. 8A). Interestingly, among nitrite-treated animals, nitrite plasma levels were significantly higher in controls compared to heterozygotes ($p=0.006$) and homozygotes ($p=0.002$), Fig. 8A. Conversely, the effect of treatment on muscle nitrite levels varied according to genotype and sex. Specifically, compared to respective vehicle-treated animals, nitrite-treated controls had significantly higher [1.205 (0.220, 2.190) nmol/g, difference estimate (95%CI), $p=0.017$], whereas homozygotes [-1.312 (-2.229, -0.396) nmol/g, $p=0.005$] and heterozygotes [-0.988 (-1.861, -0.116) nmol/g, $p=0.026$] had significantly lower muscle nitrite levels. These differential nitrite-treatment effect patterns were significantly different ($p=0.0007$, for treatment-by-genotype interaction, Fig. 8B). Additionally, compared to vehicle-treated animals, nitrite-treated females had significantly higher [0.776 (0.001, 1.552) nmol/g, difference estimate (95%CI), $p=0.049$], whereas nitrite-treated males had significantly lower [-1.507 (-2.242, -0.772) nmol/g, $p<0.0001$] muscle nitrite levels and these patterns of effect were significantly different ($p<0.0001$ for treatment-by-sex interaction, Fig. 8C).

Regarding plasma creatine kinase activity, among vehicle-treated mice, homozygotes had significantly higher creatine kinase activity levels compared to control mice [1.133 (0.260, 2.001) Unit/L, difference estimate (95%CI), $p=0.011$, Fig. 8D]. Interestingly, the effect of nitrite treatment on creatine kinase activity levels varied according to genotype ($p=0.028$ for overall treatment-by-genotype interaction, Fig. 8D). Specifically, compared to respective vehicle-treated mice, nitrite-treated homozygotes had similar [0.390 (-0.333, 1.112) Unit/L, difference estimate (95%CI), $p=0.291$], whereas heterozygotes [0.978 (0.277, 1.679) Unit/L, $p=0.006$] and control animals [1.954 (1.093, 2.815) Unit/L, $p<0.0001$] had significantly higher plasma creatine kinase activity, Fig. 8D.

We also measured muscle NOx levels (nitrate and nitrite combined, supplemental Figure) and found that controls, heterozygotes, and homozygotes had similar muscle NOx levels ($p=0.165$) and that nitrite administration had no effect on muscle NOx levels ($p=0.669$, supplemental Figure).

3.7. Effect of sickle cell genotype on muscle histopathology

A pathologist (MQ) blinded to genotype and sex examined H&E-stained transverse EDL and soleus muscle sections from vehicle treated mice. The degree of fiber size variation, fiber shape, subsarcolemmal collection (mitochondria), and nuclei internalization were similar comparing control, heterozygous, and homozygous mice (data not shown).

4. Discussion

Here we showed that that homozygotes (sickling mice), despite having lower grip strength and decreased exercise capacity, in vivo, on ex vivo studies had only mild skeletal muscle weakness compared to controls. In homozygotes, muscle mass and maximal force of

isolated fast-twitch (EDL), but not slow-twitch muscle (soleus), were lower and plasma creatine kinase activity were higher compared to controls. We then found that chronic nitrite treatment yielded increases in grip strength *in vivo*, but did not alter distances ran on the voluntary wheel, regardless of genotype (control, heterozygotes, and homozygotes). Further, the increases in grip strength associated with nitrite treatment *in vivo* were coupled with increases in specific force in EDL, but not in soleus muscle. Surprisingly, nitrite treatment increased EDL muscle susceptibility to lengthening contraction injury in homozygotes. In contrast, nitrite treatment decreased EDL muscle susceptibility to lengthening contraction in control mice. Interestingly, nitrite treatment increased EDL fatigability in all genotypes. However, during the initial phase of recovery after the fatigue protocol, EDL muscle from nitrite-treated controls and heterozygotes, but not homozygotes, showed increased force generating capacity, an effect not observed in soleus muscle. Notably, the effect of nitrite treatment in some of the outcomes examined varied according to animal sex. Taken together these findings suggest that the effects of nitrite on muscle function can vary according to the type of muscle studied (fast- or slow-twitch muscle), the mechanism of muscle injury (lengthening contraction or fatigability), and mouse sex and genotype (type of hemoglobin expressed).

We have previously shown that homozygous SCD mice have decreased grip strength and decreased performance on the voluntary wheel running and that those deficits correlate with aging and worsening anemia [40 {Wang, 2016 #2738; 41}. Here we asked the question, is the decreased grip strength and impaired performance on the voluntary wheel associated with skeletal muscle contractile dysfunction? We found that during baseline conditions, homozygotes SCD mice had only mild decreases in EDL muscle mass and maximal force, as well as increases in plasma creatine kinase activity. Notably, the decreases in muscle mass and maximal force, while observed in EDL, a predominantly fast-twitch, were not shown on soleus muscle (a slow-twitch muscle) and were not associated with changes in muscle histopathology. Others have also shown that *in vivo*, muscle force production capacity is impaired in SCD and those deficits can be exacerbated by intense exercise [42; 59]. However, researchers have also shown that decreases in grip strength, at least in part, can be explained by muscle hyperalgesia/pain given that the administration of analgesics (opioids, cannabinoid, or α_2 -adrenoreceptor agonists) increases grip force [38; 41]. Thus, taken together, these findings support the notion that several factors including muscle hyperalgesia, cardiopulmonary dysfunction, anemia, and intrinsic muscle alterations affecting predominantly fast-twitch muscles, contribute to decreased muscle function and exercise capacity in SCD.

A great deal of research indicates that NO has complex and multifocal effect on skeletal muscle excitation/contraction coupling, regulation of blood flow, mitochondrial respiration, glucose homeostasis, and myocyte differentiation [9; 10; 11; 12; 13; 14]. Recently, supplementation of nitrate/nitrite to boost the bioavailability/signaling of NO has been explored as a strategy to improve muscle function and performance. Studies in humans and animals suggest that oral supplementation of nitrate increases circulating nitrite, improves skeletal muscle mitochondrial efficiency, reduces oxygen consumption, improves contractility and increases voluntary running [25; 31; 60; 61; 62; 63; 64; 65]. We found that in control mice, nitrite treatment increased plasma and muscle nitrite levels, increased grip

strength and EDL, but not soleus, specific force, and was protective of EDL contraction-induced injury. However, in controls nitrite also increased plasma creatine kinase activity and EDL, but not soleus, fatigability. These differential effects of nitrite in fast- (EDL) and slow-twitch (soleus) are in concert with findings reported by others [63]. In fact, researchers showed that nitrate (a source of nitrite) supplementation in mice, increased contractile force in fast-, but not in slow-twitch muscle, and these changes in nitrate-treated animals were associated with increased expression of calcium handling proteins and the dihydropyridine receptor [63]. Therefore, these preferential beneficial effects of nitrate/nitrite supplementation on fast twitch muscle may suggest that its ergogenic effects would be observed in high intensity short exercises as we found here with increases in grip force but no changes on the distances ran on the voluntary wheel. Further, while it might be tempting to speculate that the nitrite effects observed in this study are related to increased NO availability/signaling, the determination of whether the effects of nitrite in muscle contractility and in vivo exercise capacity observed here are mediated by NO will require additional studies.

A significant body of literature including a few clinical studies support the hypothesis that altered NO bioavailability/signaling plays a significant role in the pathobiology of sickle cell disease [66]. However, this hypothesis has been disputed [67] and therapies that enhance NO availability have been proven ineffective and/or harmful [68; 69]. Researchers have shown that nitrite can be reduced into NO by a number of enzymes including deoxyhemoglobin, xanthine oxidoreductase (XOR), and nitric oxide synthase (NOS) among others [70; 71; 72; 73]. We found that plasma nitrite levels in homozygous mice are similar to those found in heterozygous and control animals. Surprisingly in vehicle-treated animals, muscle nitrite levels were actually higher in homozygotes and heterozygotes than in control animals. These results of increased muscle nitrite levels in SCD mice may thus suggest that in SCD there may actually be an increase in the NO reservoir nitrite [74], at least in some tissues. Notably, with nitrite treatment, compared with vehicle, while plasma nitrite levels increased in all genotypes, nitrite muscle levels actually decreased in heterozygotes and homozygotes, and increased in controls. These findings are intriguing and may not support the hypothesis of decreased nitrite/NO bioavailability in SCD. Moreover, these findings suggest that, by mechanisms incompletely understood, animals expressing sickle hemoglobin (heterozygotes and homozygotes) handle nitrite in a significantly different manner than do animals expressing hemoglobin A (control mice).

Notably, some of the effects of nitrite on the outcomes studied were different in sickling mice compared to controls. In homozygotes, nitrite treatment, increased susceptibility to contraction-induced injury in EDL muscle as evidenced by significantly greater decreases in force following active stretches, an effect significantly different than that observed in controls or heterozygotes. Further, this nitrite-induced increased susceptibility to EDL stretch injury in homozygotes was associated with decreases in muscle nitrite levels and no changes in plasma creatine kinase activity. Given the findings reported by others that chronic nitrate increases the expression of sarcoplasmic reticulum calcium handling proteins [63], it is conceivable that the nitrite treatment regimen used in this study resulted in changes in muscle structure and alterations in protein expression, thus rendering the EDL muscle membrane more susceptible to stretch injury in mice expressing sickle hemoglobin

(compared to mice expressing hemoglobin A). This hypothesis is worthy of testing in future studies.

While a matter of debate, some reports suggest that compared to the general population, sickle cell trait carriers (individuals expressing less than 50% of sickle hemoglobin) might have increased risk of exertional rhabdomyolysis, muscle weakness, and sudden death as a result of exercise [75]. Additionally, sickle cell trait carriers have decreased exercise performance when it involves short bouts of anaerobic exercises with limited recovery between bouts and lower muscle cytochrome C oxidase activity, which could explain the lower aerobic capacity observed in individuals with sickle cell trait [76; 77]. Further, in an analysis of muscle pathology in patients with SCD and individuals with sickle cell trait, researchers showed that the degree of microvascular remodeling was associated with the degree of sickle hemoglobin expression [8]. Specifically, individuals with sickle cell trait (heterozygotes) have degrees of microvasculature remodeling that are intermediary to those exhibited by control subjects and SCD patients (homozygotes) [8]. Our findings that heterozygotes have impaired performance on the voluntary wheel running compared to controls, as well as increased susceptibility to contraction injury of EDL muscle, showing decreases in percent force following 10 and 15% active stretches compared to homozygotes, support the notion that heterozygous mice can have muscle dysfunction and increased susceptibility to muscle injury. Using a different paradigm than that used in this investigation, others have also shown that muscle force parameters measured in heterozygous Townes mice are also impaired compared to controls [42]. Thus, taken together our findings and those of others support the notion that heterozygous SCD Townes mice might provide a valuable model for studies aimed at characterizing the mechanisms of altered exercise performance associated with sickle cell trait.

We found that for a number of outcomes including grip strength, muscle mass, and muscle nitrite levels the effects of nitrite varied according to mouse sex. As most human and animal studies evaluating the effect of nitrate/nitrite on muscle performance have been performed in males, it remains unknown whether there are differential effects of the nitrate/nitrite pathway between sexes. However, our findings do suggest that the handling of nitrite in muscle and its effects on body and muscle mass and muscle strength in males is different than in female mice and these hypotheses should be tested in clinical studies. These findings also lend support to the notion that the inclusion of male and females in animal and human studies of muscle function are of utmost importance.

We note some limitations of this study. For the large trial, we used only one dose of nitrite, which is higher than the doses used by others [30] showing that nitrite improves motor function in old mice. Additionally, as we administered nitrite in drinking water, we could not control the amount of water intake. However, while homozygotes, compared with controls, had higher water and nitrite intakes, among nitrite-treated animals, the levels of plasma nitrite were significantly lower in homozygotes than in control mice. Additionally, the dose of nitrite studied was well tolerated by all genotypes and was not associated with increases in methemoglobinemia, findings also reported by others [78]. Another consideration when interpreting the data is that we examined fatigability *ex vivo*, which exclude motor neuron input and blood supply, amongst other factors, that are known to play important roles in

muscle fatigue resistance [79]. Therefore, it is conceivable that different results could be observed during in vivo examination of fatigability. Nevertheless, despite these limitations, with the dose studied here we were able to identify beneficial effects of nitrite in muscle performance as well as to elicit increased fatigability and susceptibilities to stretch-induced injury in control and SCD mice. Further, we showed that nitrite has differential effects in fast and slow-twitch muscle types and in male and female mice. Lastly, we showed that handling of nitrite in heterozygous and homozygous SCD mice is different from that in control animals. Therefore, given the growing evidence that nitrate/nitrite can serve as a reservoir for NO and that this pathway is being explored for their ergogenic effect, the demonstration that sex and altered NO availability impact on the effects of nitrate/nitrite are relevant for the design of future studies evaluating this therapeutic strategy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. Sick cell patients have muscle dysfunction and decreased exercise capacity
2. Sick cell mice also have decreased muscle strength and decreased exercise capacity
3. Nitrite improved grip strength but not distance ran on the voluntary wheel
4. Nitrite improved specific force of fast but not slow-twitch muscles in all genotypes
5. Nitrite increased susceptibility to contraction injury in homozygous sickling mice

Figure 1. Effect of nitrite treatment on water and nitrite intake and on blood methemoglobin levels.

The data were analyzed using an analysis of variance model with a treatment by genotype interaction term included. Results are shown as least-square mean estimates and their corresponding 95% confidence interval. A. Among vehicle-treated mice, homozygotes had higher water volume intake compared to control mice ($p < 0.0001$). Regardless of genotype, nitrite-treated animals had significantly lower water volume intake compared to vehicle-treated mice, ($p < 0.0001$ for overall treatment effect). B. With doses of 1g/L of nitrite, but not with doses of 0.05 g/L, there were significant increases in the levels of nitrite intake

compared with vehicle ($p<0.0001$ and $p=0.171$ for 1g/L and 0.05g/L dose overall compared with vehicle). C. In vehicle-treated animals, homozygotes had greater percentages of methemoglobin compared with control mice ($p<0.0001$). Interestingly, compared with vehicle, there was no overall effect of nitrite-treatment on levels of methemoglobin ($p=0.412$ for overall effect of nitrite). * reflects $p<0.05$, † reflects $p=0.01$, ‡ reflects $p=0.001$, and § reflects $p=0.0001$. Forty-five mice were included in this experiment, N=6 to 9 per each of the six experimental groups.

Figure 2. Effect of nitrite treatment on grip strength and voluntary wheel running in SCD mice. The data were analyzed using a repeated-measures mixed model with interaction terms (treatment by genotype, treatment by sex, sex by genotype). Results are shown as least-square mean estimates and their corresponding 95% confidence interval. Hetero indicates heterozygotes and homo homozygotes. A. Homozygotes (homo) had lower grip strength, compared to controls and heterozygotes (hetero, $p < 0.0001$) and nitrite treatment increased grip strength as nitrite-treated controls, heterozygotes, and homozygotes had significantly greater grip strength compared to vehicle-treated animals ($p < 0.0001$ for overall treatment effect). B. Compared to respective vehicle-treated mice, nitrite-treated homozygotes ($p < 0.0001$) and heterozygotes ($p = 0.041$) had significantly higher, whereas control mice had no significant changes ($p = 0.116$) on forelimb grip strength and these nitrite-treatment effect patterns were significantly different ($p = 0.028$, for genotype-by-treatment interaction). C. Mouse sex affected the effect of nitrite on grip strength as compared to vehicle-treated mice, nitrite-treated females had higher ($p < 0.0001$), whereas, nitrite-treated males had similar ($p = 0.263$) forelimb grip strength and these patterns of treatment effect were significantly different ($p = 0.001$ for sex-by-treatment interaction). D. Homozygous and heterozygous mice run shorter distances over five days compared to control mice ($p = 0.0002$ and $p = 0.0043$ for vehicle-treated homozygotes and heterozygotes respectively compared to vehicle-treated controls). Nitrite treatment had no effect ($p = 0.199$ for overall treatment effect) on distance run on the voluntary running wheel in any of the genotypes studied. Symbols above nitrite-

treated bar reflects post-hoc comparisons with respective vehicle-treated group within a given genotype and those above vehicle-treated bars reflect comparison with the vehicle-treated control genotype. * reflects $p < 0.05$, † reflects $p \leq 0.01$, ‡ reflects $p \leq 0.001$, and § reflects $p \leq 0.0001$. One hundred and one mice were included in this experiment, $N=13-21$ underwent grip strength measurements and $N=9-18$ voluntary wheel running per each experimental group including males and females.

Figure 3. Effect of nitrite on body mass and on fast-twitch muscle (extensor digitorum longus, EDL) mass and force production.

The data were analyzed using an analysis of variance model with interaction terms (treatment by genotype, treatment by sex, sex by genotype). Results are shown as least-square mean estimates and their corresponding 95% confidence interval. Hetero indicates heterozygotes and homo homozygotes. A. Controls, heterozygotes, and homozygotes had similar body mass ($p=0.126$, for overall genotype effect). B. The effect of nitrite varied according to mouse sex but not according to genotype. Specifically, nitrite-treated females had significantly higher ($p=0.002$) whereas, nitrite-treated males had similar ($p=0.969$) body

mass compared to respective vehicle-treated animals and these patterns of nitrite treatment effect in male vs. female were significantly different ($p=0.034$ for treatment-by-sex interaction). C. Compared to control and heterozygotes, homozygotes had significantly lower EDL muscle mass ($p<0.0001$, overall effect of genotype) and administration of nitrite had no effect on EDL muscle mass ($p=0.527$, overall treatment effect) in any genotype. D. The effects of nitrite on EDL muscle mass had significantly different patterns in males compared to females ($p=0.0077$ for treatment-by-sex interaction). Nitrite-treated males had significantly lower EDL muscle mass compared with vehicle-treated animals ($p=0.021$), whereas nitrite-treated females, had no significant changes ($p=0.132$) in EDL muscle mass. E. Homozygotes had lower EDL maximal force compared with controls and heterozygotes ($p=0.029$, overall effect of genotype) and nitrite administration was associated with a trend towards increases in maximal force irrespective of genotype ($p=0.056$ for overall treatment effect). Nitrite-treated heterozygotes had significantly greater EDL maximal force compared with vehicle-treated mice ($p=0.044$). F. Homozygous and heterozygous mice had similar EDL specific force (maximal force/muscle cross sectional area) compared to control animals ($p=0.910$ for genotype effect). Additionally, independent of genotype, nitrite administration was associated with overall increases in EDL specific force compared with vehicle ($p=0.017$ for overall treatment effect). Symbols above nitrite-treated bars reflects post-hoc comparisons with respective vehicle-treated group within a given genotype and those above vehicle-treated bars reflect comparison with the vehicle control genotype. * reflects $p<0.05$, † reflects $p \leq 0.01$. Ninety-one mice were included in this experiment, $N=13-20$ per each experimental group including males and females.

Figure 4. Effect of nitrite on slow-twitch muscle (soleus) mass and force production.

The data were analyzed using an analysis of variance model with interaction terms (treatment by genotype, treatment by sex, sex by genotype). Results are shown as least-square mean estimates and their corresponding 95% confidence interval. A. Regarding the *ex vivo* contractile properties of the soleus muscle (slow twitch muscle), there were no significant effects of genotype or nitrite treatment on soleus muscle mass. B. Soleus muscle maximal force was similar among all genotypes $p > 0.05$. While there was no overall effect of nitrite treatment on soleus maximal force in all genotypes combined, nitrite-treated

heterozygotes had greater soleus maximal force compared to respective vehicle-treated mice ($p=0.047$). C. There were no significant effects of genotype or nitrite treatment on soleus muscle specific force. Symbols above nitrite-treated bars reflects post-hoc comparisons with respective vehicle-treated group within a given genotype and those above vehicle-treated bars reflect comparison with the vehicle control genotype. * reflects $p<0.05$. Eighty-three mice were included in this experiment, $N=11-19$ per each experimental group including males and females.

Figure 5. Effect of nitrite on extensor digitorum longus (EDL) susceptibility to contraction-induced injury.

The data were analyzed using a repeated-measures mixed model with interaction terms (treatment by genotype, treatment by sex, sex by genotype, treatment by genotype by length). Results are shown as least-square mean estimates and their corresponding 95% confidence interval. A. In EDL muscle from control mice nitrite treatment ameliorated the decreases in force generation in EDL muscle following active stretches compared with vehicle, ($p=0.015$ for 10% and $p=0.016$ for 15% active stretches). B. In heterozygotes Townes, compared with vehicle, nitrite treatment had no significant effect on the decreases in force generation in EDL muscle following active stretches. C. In homozygotes, nitrite treatment exacerbated the decreases in force following active stretches of EDL muscle compared with vehicle as EDL muscle from nitrite-treated homozygotes had significantly greater decreases in percent force following active stretches of 5, 10, 15, 20 and 25% compared with EDL muscle from vehicle-treated mice. Importantly, the effect of nitrite on EDL susceptibility to contraction-induced injury in homozygous mice were significantly different from those observed in controls and heterozygotes ($p=0.012$, for treatment-by-genotype interaction). Symbols reflect post-hoc comparison between nitrite- and vehicle-treated groups at the level of muscle stretch indicated. * reflects $p<0.05$, † reflects $p<0.01$, ‡ reflects $p<0.001$, and § reflects $p<0.0001$. Eighty-three mice were included in this experiment, $N=13-18$ per each experimental group including males and females.

Figure 6. Effect of nitrite on EDL fatigability.

The data were analyzed using a repeated-measures mixed model with interaction terms (treatment by genotype, treatment by sex, sex by genotype, treatment by genotype by time). Results are shown as least-square mean estimates and their corresponding 95% confidence interval. Nitrite treatment increased fatigability in EDL muscle from controls (A), heterozygotes (B), and homozygotes (C). Interestingly, from the last time point in the fatigue protocol (5 min) to the initial phase of recovery (15), EDL muscles from nitrite-treated controls (A) and heterozygotes (B), but not homozygotes (C), exhibited greater increases in

force producing capacity compared with muscles from respective vehicle-treated mice ($p=0.0001$, $p=0.012$, and $p=0.115$ for controls, heterozygotes, and homozygotes, respectively). Symbols reflect post-hoc comparison between nitrite- and vehicle-treated groups at the time indicated. * reflects $p<0.05$, † reflects $p \leq 0.01$, ‡ reflects $p \leq 0.001$, and § reflects $p \leq 0.0001$. Eighty-three mice were included in this experiment, $N=11-20$ per each experimental group including males and females.

Figure 7. Effect of nitrite soleus fatigability.

The data were analyzed using a repeated-measures mixed model with interaction terms (treatment by genotype, treatment by sex, sex by genotype, treatment by genotype by time). Results are shown as least-square mean estimates and their corresponding 95% confidence interval. Nitrite-treatment had no impact on fatigability of the soleus muscle from controls (A), heterozygotes (B), or homozygotes (C). However, during recovery (10 min), nitrite-treated heterozygotes (B) and homozygotes (C) had less force producing capacity compared to respective vehicle-treated mice. Symbols reflect post-hoc comparison between nitrite- and

vehicle-treated groups at the time indicated. * reflects $p < 0.05$, † reflects $p \leq 0.01$. Seventy-six mice were included in this experiment, $N = 9-17$ per each experimental group including males and females.

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Figure 8. Effect of nitrite treatment on plasma and muscle nitrite levels and plasma creatine kinase activity.

The data were analyzed using an analysis of variance model with interaction terms (treatment by genotype, treatment by sex, sex by genotype). Results are shown as least-squares mean estimates and their corresponding 95% confidence interval. A. Among vehicle-treated mice, controls, heterozygotes, and homozygotes had similar plasma nitrite levels. Nitrite administration increased plasma nitrite levels in controls, heterozygotes and homozygotes ($p < 0.0001$ for overall treatment effect). B. Muscle nitrite levels were approximately three to four-fold higher than plasma nitrite in all genotypes. Contrary to

plasma nitrite levels, among vehicle-treated mice, homozygous and heterozygous Townes had significantly higher muscle nitrite levels compared to controls. The effect of nitrite treatment on muscle nitrite levels varied according to genotype. Specifically, compared to vehicle-treated animals, nitrite-treated homozygotes and heterozygotes had significantly lower, whereas control mice had significantly higher levels of muscle nitrite levels and these differential nitrite-treatment effect patterns were significantly different ($p=0.0007$, for genotype*treatment interaction). C. Compared to vehicle-treated animals, nitrite-treated females had higher ($p=0.0498$), whereas, whereas nitrite-treated males had significantly lower muscle nitrite levels ($p<0.0001$) and these patterns of effect were significantly different ($p<0.0001$ for sex*treatment interaction). D. Among vehicle-treated mice, homozygotes had significantly higher creatine kinase activity levels compared to control mice. Compared to vehicle-treated mice, nitrite-treated homozygotes had similar, whereas heterozygotes and control animals had significantly higher plasma creatine kinase activity, and these differential nitrite-treatment effect patterns were significantly different ($p=0.028$ for treatment-by-genotype interaction). Symbols above nitrite-treated bars reflects post-hoc comparison with respective vehicle-treated group within a given genotype and those above vehicle-treated bars reflect comparison with the vehicle control genotype. * reflects $p<0.05$, † reflects $p \leq 0.01$, ‡ reflects $p \leq 0.001$, and § reflects $p \leq 0.0001$. Measurements were obtained from 110 mice, N=11–29 had plasma nitrite, N=10–26 muscle nitrite, and N=8–20 creatine kinase measurements per experimental group including males and females.