IMPACT OF HEAT THERAPY ON SKELETAL MUSCLE FUNCTION IN A MODEL OF DUCHENNE MUSCULAR DYSTROPHY

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ABSTRACT

Duchenne muscular dystrophy (DMD) is an X-linked disease that affects about 1 in 3,500 male births worldwide. Mutations in the DMD gene result in unstable or even the absence of dystrophin, a key protein that stabilizes the skeletal muscle cell membrane by mechanically linking the cytoskeleton and the extracellular matrix. Although there is no cure for this disease, glucocorticoids or physiotherapy are commonly used to delay the progression of the disease and the loss of ambulation. However, long-term use of glucocorticoids is associated with several severe side effects, including weight gain, bone demineralization, and not all patients have access to physiotherapy. Contrary to the aforementioned option for DMD, heat therapy (HT) has no side effects, is practical, inexpensive, and amenable to be used in a home setting without medical supervision. Our recent work revealed that daily exposure to HT reduces fat accrual, promotes an increase in relative muscle mass, and enhances force development. Based upon these previous reports, the aim of this study was to: (1) examine the impact of treatment temperature on the skeletal muscle adaptations in DBA/2J mice; and (2) determine the impact of repeated HT for 3 consecutive weeks (5 days a week, 30 min per session) on the body composition and skeletal muscle function in D2.mdx, a model of DMD. Aiming to define the optimal temperature for HT application using a heat chamber, DBA/2J mice were randomly allocated (n = 6) to a control group or one of three different HT regimens (37, 39, 41℃) and underwent the treatment daily for 3 weeks. The second series of experiments was designed to contrast and compare the physiological adaptations to HT at 39℃ or control treatment (n = 12) for 3 weeks in D2.mdx mice. We hypothesized that the beneficial effects of HT would be dependent on the treatment temperature, being most evident at mild temperatures (37 and 39℃). In addition, we hypothesized that repeated exposure to HT would promote an increase in muscle mass and strength, and increase mitochondrial content. In study 1, we observed that relative muscle mass of both EDL and soleus muscle was significantly higher in animals exposed to HT at 39℃ (EDL treatment main effect, $p = 0.04$, soleus treatment main effect, $p = 0.02$). Moreover, when EDL muscle force was normalized to the estimated cross-sectional area, the 39℃ group showed significantly greater maximal force (treatment main effect, $p = 0.02$) compared to the 41℃ group. There were no significant differences between groups in body composition, forelimb grip strength, soleus muscle function, and mitochondrial content in DBA/2J mice. The results of

study 2 revealed that there were no significant differences between groups in relative muscle mass and contractile muscle function in D2.mdx mice. In conclusion, HT at 39°C for 3 weeks does not improve muscle function or increase muscle mass in a mouse model of DMD. Future studies are needed to define whether different HT treatment protocols elicit beneficial adaptations in the DMD.

CHAPTER 1. INTRODUCTION

1.1 Duchenne Muscular Dystrophy

Duchenne muscular dystrophy (DMD) is an X-linked recessive muscle disease that affects about 1 in 3,500 male births worldwide (McDonald et al., 2018). Mutations in the DMD gene result in unstable or even the absence of dystrophin, a key protein that stabilizes the skeletal muscle cell membrane by mechanically linking the cytoskeleton and the extracellular matrix (Hoffman et al., 1987; Pasternak et al., 1995; Rando, 2001). Dystrophic muscle exhibits a cycle of degeneration and regeneration accompanied by infiltration of inflammatory cells and progressive accumulation of fibrotic and adipose tissues. Of note, the degree of impact depends on specific skeletal muscle fiber type, with type II fibers being preferentially affected when compared to type I fibers (Talbot & Maves, 2016).

Patients with DMD manifest their first clinical symptoms at the age of 3–4 yr, including motor delay, abnormal gait, and Gower's sign (Manning & O'Malley, 2015). Progressive weakness of limb muscles, trunk muscles, and the diaphragm, leads to wasting, kyphoscoliosis, and severe respiratory problems. Individuals with DMD tend to walk on their toes, fall easily and are never able to jump (Manzur & Muntoni, 2009). Other clinical features include a waddling gait and pseudo-hypertrophy of the calf muscles. Affected boys lose independency by 13 years of age and become wheelchair bound (Yiu & Kornberg, 2015). Typically, patients usually die at the third decade of life due to respiratory or cardiac failure (van Putten et al., 2019; Hughes et al., 2019, 2020)

Glucocorticoids are the standard therapeutics used to delay the progression of the disease and the loss of ambulation (Hammers et al., 2020). However, long-term use of glucocorticoids is associated with several severe side effects, including weight gain, behavioral changes, cataracts, and skin changes (McDonald et al., 2018). Multidisciplinary rehabilitation, including physical therapy and postural correction, may also improve mobility and quality of life (QOL) (Birnkrant et al., 2018), but few patients with this condition have access to comprehensive care (Kinnett et al., 2015). Therefore, many studies are ongoing to identify potential therapeutic strategies by using animal model of DMD.

The C57BL/10ScSn-Dmdmdx/J (BL10-mdx) mouse is the most frequently used mouse model for DMD. However, several studies report that the disease phenotype in this model is much milder than that of patients with DMD. For example, mdx mice do not progressively lose muscle strength and therefore the ability to ambulate. Recent discoveries indicate that disease severity is exacerbated when muscular dystrophy mouse models are generated on a DBA2/J genetic background. Coley et al. reported that D2.mdx has more severe symptoms compared to BL 10-mdx (Coley et al., 2016). These animals display decrements in muscle mass, forelimb grip strength, ejection fraction, and a plethora of structural abnormalities, including a higher percentage of central nuclei and severe fibrosis (Hammers et al., 2020).

1.2 Heat Therapy for Skeletal Muscle Rehabilitation

Heat therapy (HT) modalities (e.g. shortwave diathermy, water-circulating garments, heat wraps, whirlpool therapy, etc.) are commonly employed in the management of chronic musculoskeletal conditions associated with pain, increased tissue stiffness, and reduced range of motion (Giombini et al., 2007). In this section, I review the literature on the effects of HT on muscle atrophy, regeneration, capillarization, mitochondrial content, and muscle strength.

1.2.1 Effects of Heat Therapy on Muscle Atrophy

Exposure to daily heat stress has been shown to mitigate immobilization-induced muscle atrophy. Naito and co-workers showed that muscle weight was significantly reduced after 8 days of the unweighted hind limb in 6-month-old adult female rats. However, exposure to heat in a form of a heat chamber for 60 min prior to 8 days of disuse reduced the loss of muscle mass by approximately 8% when compared to the control group (Naito et al., 2000). This result aligns closely with the report of Selsby and colleagues that exposure of male rats to whole body heat stress prior to 8 days of immobilization abrogated the loss in both absolute muscle mass and muscle mass-to-body mass ratio when compared to the control group. Of note, reloading in combination with heat stress augmented muscle growth (Selsby et al., 2007).

These positive effects of HT on atrophied skeletal muscle have also been documented in human studies. Hafen et al. have reported that daily heat stress with pulsed shortwave diathermy attenuates skeletal muscle atrophy during 10 days of immobilization in sedentary young

individuals. The subject's movement was limited by using a therapeutic knee brace and arm crutches for 10 days. After immobilization, the vastus lateralis cross-sectional area (CSA) and myofiber CSA decreased by 7.3% and 10.8%, respectively, in the sham-treatment group, while in the heat-treatment group the reductions were 4.5% and 5.8% (Hafen et al., 2019).

1.2.2 Effects of Heat Therapy on Muscle Regeneration Following Injury

Several studies have shown salutary effects of heat therapy on muscle regeneration after injury. Kojima and co-workers reported that exposure to heat stress for 60 min facilitated the regeneration of cardiotoxin-induced injured tibialis anterior muscle by activating Pax7+ satellite cells (Kojima et al., 2007). Similar findings were reported by Takeuchi and colleagues after crush injury to the extensor digitorum longus muscle (EDL) in rats (Takeuchi et al., 2014). Occasional heat therapy in the form of hot water immersion also stimulated Pax7+ satellite cells and overall soleus regeneration after bupivacaine injection in rats (Oishi et al., 2009). Shibaguchi and colleagues also showed that heat stress (42 °C, 30 min) applied every other day during 2-14 days after bupivacaine injection partially facilitated the recovery of muscle mass, protein content, and muscle fiber size of injured soleus. Indeed, the heat stress group exhibited a greater cross-sectional area of the soleus muscle and a higher number of Pax7+ satellite cells at day 28, compared to both icing and non-treated bupivacaine injected group (Shibaguchi et al., 2016).

1.2.3 Effects of Heat Therapy on Muscle Capillarization

Akasaki and colleagues first demonstrated the positive impact of heat therapy on endothelial nitric oxide synthase (eNOS) content and capillary density in a mouse model of hindlimb ischemia (Akasaki et al., 2006). Several studies have since confirmed these earlier findings in a wide range of models. Our group reported that long-term local HT increased eNOS content by 18% (4wk after) and 35% (8wk after) relative to the control leg (Kim et al., 2019). We have also demonstrated that a single session of HT increased the mRNA expression of vascular endothelial growth factor (VEGF) (Kuhlenhoelter et al., 2016). Conversely, long-term HT did not affect the expression of VEGF and angiopoietin 1 (ANGPT1) (Kim et al., 2020). Recently, Hesketh and colleagues investigated the effects of HT in a heat chamber for 6 wk (40-50 min at 40°C, 3×/wk) on skeletal muscle capillarization and eNOS content. When compared to

baseline levels, HT increased capillary density (21%), capillary-fiber perimeter exchange index (15%), and endothelial-specific eNOS content (8%) (Hesketh et al., 2019).

1.2.4 Effects of Heat Therapy on Mitochondrial Content

Liu and Brooks first reported that, in C2C12 myotubes, 1 h of mild heat stress at 40°C upregulated the expression of key energy-sensing molecules regulating mitochondrial biogenesis (Liu & Brooks, 2012). Furthermore, repeated mild heat stress for 5 days increased the protein levels of several mitochondrial oxidative phosphorylation subunits. In mice, Tamura and colleagues showed that whole body heat stress produced by exposure to a hot environment (40°C, 30 min/day, 5 days/wk, 3 wk) induced mitochondrial adaptations such as increased mitochondrial enzyme activity (citrate synthase and 3-hydroxyacyl CoA dehydrogenase) and respiratory chain protein content (complexes I-V) in skeletal muscle in vivo (Tamura et al., 2014). In humans, Hafen and co-workers demonstrated that deep tissue heating of the vastus lateralis via pulsed shortwave diathermy (2 h daily for 6 consecutive days) augmented maximal coupled and uncoupled respiratory capacity, measured via high-resolution respirometry (Hafen et al., 2019). Conversely, our group did not observe changes in either maximal citrate synthase activity or the abundance of OXPHOS proteins following repeated local HT in humans (Kim et al., 2020).

1.2.5 Effects of Heat Therapy on Muscle Function

In addition to the aforementioned effects on muscle mass and morphology, HT has also been shown to alter muscle force development and fatiguability. Goto and colleagues first demonstrated that local HT applied to the quadriceps muscles for 8 h/day and 4 days/week by using a heat- and steam-generating sheet improved maximum isometric force during knee extension and enhanced the cross-sectional areas (CSAs) of vastus lateralis (VL, ~2.7%) and rectus femoris (~6.1%) muscles (Goto et al., 2011). Along the same lines, our group reported that daily local HT for 8 weeks augmented peak torque of the knee extensor muscles in sedentary individuals (Kim et al., 2020). Contrary to these findings, local HT for 6 weeks using heat pads (8 h/day, 5 days/week), had no effect on muscle cross-sectional area, peak twitch amplitude or

rate of torque development and maximal voluntary isometric or isokinetic torque (Labidi et al., 2021).

1.3 Summary

The use of corticosteroids or physiotherapy remains the mainstay of treatment options to alleviate the symptoms of DMD (Sussman, 2002; McDonald et al., 2018). However, prolonged glucocorticoid use has negative consequences, including bone demineralization and hastened cardiomyopathy (Hammers et al., 2020), and not all patients have access to physiotherapy (Kinnett et al., 2015). Meanwhile, HT has been shown to improve muscle function in both mice and human models of skeletal muscle injury (Kim et al., 2020). Contrary to other treatment options for DMD, HT has no side effects, is practical, inexpensive, and amenable to be used in a home setting without medical supervision.

1.4 Aims

The primary aim of this research project is to determine the effects of daily HT in the form of a heat chamber on the structure and function of limb (soleus and EDL) muscles in D2.mdx mice, a model of DMD.

1.5 Hypothesis

As reviewed above, repeated exposure to HT has been shown to: 1) prevent muscle atrophy during unloading, 2) accelerate recovery following muscle injury, 3) promote mitochondrial biogenesis, 4) increase muscle strength. Based upon these extensive data, we hypothesize that daily exposure to HT will enhance grip strength, muscle mass and maximal force, and muscle mitochondrial content compared to a control treatment. In addition, we anticipate that the beneficial effects of HT will be dependent on the treatment temperature, being most evident at mild temperatures (37 and 39°C).

CHAPTER 2. METHODS

2.1 Animals

Eight-week-old male wildtype (DBA/2J) and D2.mdx (D2.B10-DMD<mdx>/J+) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Both DBA/2J and D2.mdx mice were housed in groups of two to four in standard cages (18 × 29 cm and 12.5 cm height). The animals were kept in a temperature-controlled room (22℃, 60% humidity, 12:12h light-dark cycle) and they had ad libitum access to water and regular chow (Teklad global 18% protein rodent diet, Envigo Bioproducts Inc, Madison WI). All the protocols were approved by Institutional Animal Care and Use Committee at Purdue University (#2003002026).

2.2 Experimental Protocol

Aiming to define the optimal temperature for HT application, DBA/2J mice were randomly allocated (n = 6) to a control group or one of three different HT regimens (37, 39, 41℃). The second series of experiments was designed to contrast and compare the physiological adaptations to HT at 39℃ or control treatment in a model of DMD. Twenty-four male D2.mdx were randomly assigned to receive HT (n = 12) or a control treatment (n = 12). Upon arrival, both wildtype and D2.mdx mice were acclimated to the vivarium for at least 3 days prior to the treatment protocol. After acclimation, the animals were familiarized with heat stress in the incubator, forelimb grip strength testing, and the assessment of body composition. Both variables were also tested weekly and 24 h after the last treatment session. The treatments were applied for 30 min, 5 times/wk over 3 consecutive weeks. Forty-eight hours after the completion of the last treatment session, the animals were anesthetized using an isoflurane-oxygen mixture and the soleus and EDL muscles were harvested for the isolated muscle testing (Figure 1. Contains an overview of the experimental design).

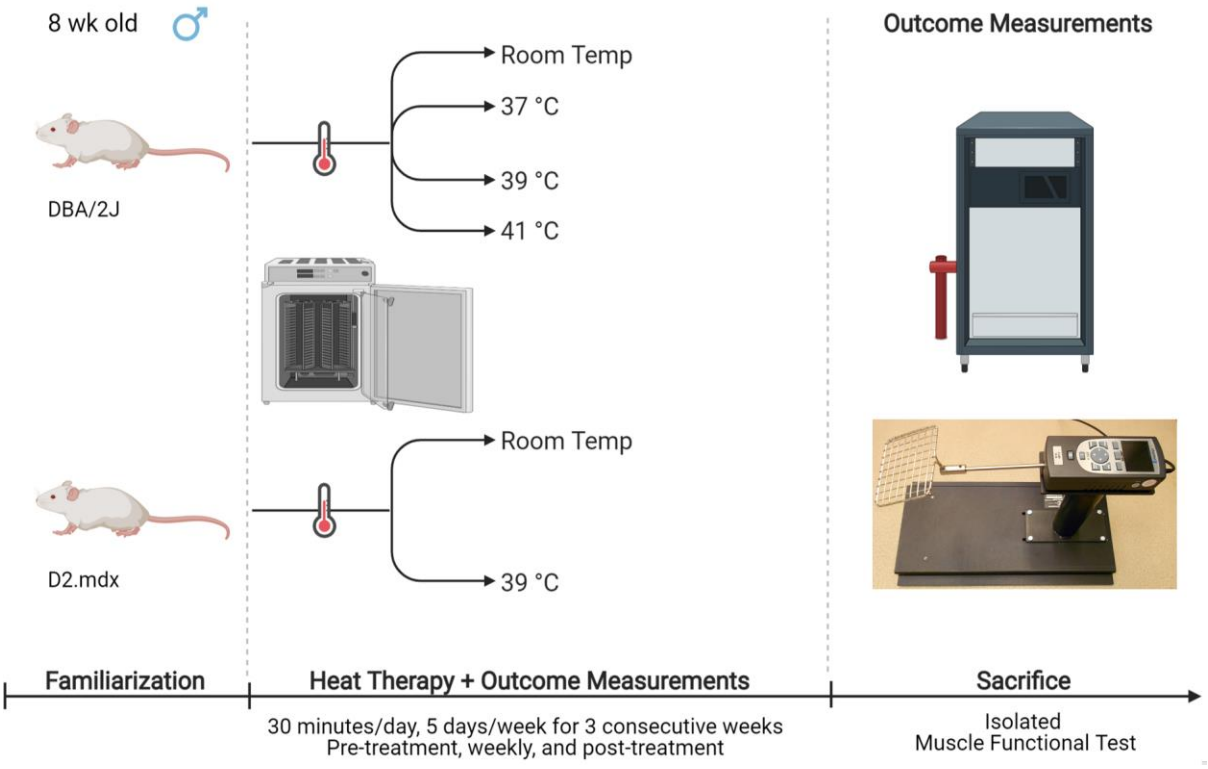

Figure 1: Overview of the experimental design

2.3 Heat Therapy

The animals were placed into a microbiological incubator (Heratherm advanced protocol incubators, Thermo Fisher scientific, Waltham, MA) with free access to food and water. Prior to the interventions, animals were habituated to the chamber for 3 days. On the first two days, animals were placed in the incubator set a room temperature for 15 min and 30 min, respectively. On the third day, animals were exposed to the assigned treatment for 30 min. The temperature of the chamber was continuously monitored using a digital thermometer (Fisher Scientific, 50-197-8021) and maintained within ± 0.5℃ of the desired targets. The duration and frequency of treatment were chosen based on the report by Tamura and coworkers that a similar regimen enhances mitochondrial adaptations in mouse skeletal muscle (Tamura et al., 2014).

2.4 Body Composition

Whole body composition was measured with magnetic resonance imaging (Echo-MRI 130 analyzer; Echo Medical System, Houston, TX) at baseline and once weekly throughout the

intervention. Unanesthetized mice were placed into a cylindrical holder, which was inserted into the chamber for approximately 2 min. Two successive scans were completed for each animal and the average of these two assessments is reported.

2.5 Forelimb Grip Strength

Forelimb grip strength was assessed using a digital grip-strength meter (Columbus Instruments, model:1027SM Grip Strength Meter with Single Sensor) at baseline and once weekly throughout the intervention. Mice were familiarized with the grip-strength meter daily for 3 consecutive days prior to baseline testing. Once mice grasped the metal pull bar attached to the digital transducer, animals were gently pulled backward by the tail. The peak force was recorded at the time when mice release the bar. There were at least 1 min gap between the trials and total 5 consecutive grip force were measured. The mean of all 5 attempts was used for further analysis. Body weight was also measured prior to the test. Grip strength testing was performed on the same day of the week, at the same time of day by the same investigator.

2.6 Assessment of Contractile Function

The contractile function of the slow-twitch soleus and the fast-twitch EDL was examined approximately 48 h after the completion of treatment. Under the isoflurane anesthesia, the left hindlimb was dissected and immediately placed into a dissecting dish filled with a bicarbonate-buffered solution (in mmol/l: 137 NaCl, 5KCl, 1 MgSO4, 1 NaH2PO4, 24 NaHCO3, and 2 CaCl2) equilibrated with 95% O2-5% CO2 (pH ~ 7.4). The right hindlimb muscles were also isolated, frozen in liquid nitrogen, and stored at -80℃ until Western blot analysis. Isometric contractile function of EDL muscle was assessed first, followed by the soleus muscle. Under a dissecting microscope, both the proximal and distal tendons were tied with a silk suture (18020-40, Fine Science Tools, Foster City, CA). The muscles were then incubated in a bicarbonate-buffered solution at room temperature with continued gasification (95% O2-5% CO2) and mounted in a force transducer (1200A Intact Muscle Test System, Aurora Scientific, ON, Canada). After establishing the optimal muscle length (Lo), the temperature of the bath was increased to 32℃, and muscle was allowed to equilibrate for 10 min. The force-frequency relationship was examined by using stimulation frequencies of 1, 15, 30, 50, 80, 120, 150, 250, and 300 Hz for EDL muscle

and 1, 15, 30, 50, 80, 120, 150, and 200 Hz for the soleus muscle. The muscles were then transferred to the dissecting dish, trimmed of connective tissue, blotted dry, and weighed. Muscle cross-sectional area (CSA) was estimated from the wet muscle mass, Lo and muscle-specific density (1.056 g/cm³). Specific force (N/cm²) was calculated by dividing the muscle force (N) by the CSA (cm²).

2.7 Protein Extraction and Western Blot Analysis

Muscle samples (~5 mg) were homogenized in ice-cold buffer containing 50 mM Tris · HCI, pH 7.4, 150 mM NaCl. 0.25% deoxycholic acid, 1 % NP-40, and 1 mM EDTA (RIPA Lysis Buffer; EMD Millipore), a protease inhibitor cocktail (P8340; Sigma-Aldrich) and phosphatase inhibitors (50 mM NaF and 0.2 mM Na3VO4) at a 1:20 dilution of wet muscle weight using a bead mill homogenizer (Bead Ruptor 12; Omni International). After centrifugation (13,500 g, 4℃, 20 min), the supernatant was collected and protein content was determined using a BCA protein assay kit (Thermo Scientific, IL). Samples were then subsequently mixed with homogenization buffer and reducing sample buffer (4× Laemmli sample buffer with 10% 2-mercaptoethanol), divided into small aliquots, and stored at -80℃.

An equal amount of protein (15 μg) was separated by SDS-PAGE on precast Stain Free 4-15% gels (Bio-Rad, CA) and transferred to PVDF membranes with the Trans-Blot Turbo transfer system (Bio-Rad, CA). After blocking with 5% nonfat milk in 1× Tris-buffered saline-Tween (TBST, 1% Tween 20) for 1 h, the membranes were incubated with a primary antibody (OXPHOS, ab110413; 1:1000, abcam) diluted in blocking buffer for 4 h at room temperature (~23℃). Membranes were then incubated with secondary antibody diluted in 1× TBST for 1 h at room temperature followed by exposure to an enhanced chemiluminescent solution (Clarity Western ECL; Bio-Rad) for 5 min. Membranes were washed with 1× TBST at least 3 times for 10 min each between incubation periods. The specific proteins were visualized with a densitometer (ChemiDoc Touch Imaging System; Bio-Rad) and quantified with image analysis software (Image Laboratory v.6.0.1; Bio-Rad). Target protein expression was normalized with total lane density from the stain-free blot after background signal adjustment (Taylor & Posch, 2014).

2.8 Statistical Analysis

All data were analyzed using SAS (version 9.4; SAS Institute, Cary, NC), with results expressed as means ± SE. Changes in body composition and grip strength throughout the intervention and contractile function data were analyzed by two-way repeated-measures ANOVA. A Turkey post hoc analysis was performed when appropriate. Muscle mass, peak twitch tension, and measurement of mitochondrial content were analyzed using one-way ANOVA. A value of $p < 0.05$ was considered statistically significant.

CHAPTER 3. RESULTS

3.1 Study 1 – Impact of HT on Body Composition and Muscle Function in Male DBA/2J Mice

3.1.1 Rectal Temperature

To characterize the magnitude and temporal profile of changes in core body temperature during exposure to HT in the incubator, rectal temperature was measured in a group of male DBA/2J mice (n = 4) prior to and during exposure to HT or control intervention. The measurements were performed daily over 5 consecutive days. The average changes in rectal temperature during exposure to HT and the control regimen are displayed in Figure 2. Rectal temperature was similar between groups before the intervention (Control: 35.7℃ ± 0.4℃; 37℃: 35.9℃ ± 0.5℃; 39℃: 35.5℃ ± 0.4℃; 41℃: 35.8℃± 0.4℃). While rectal temperature remained unaltered in the control group, a progressive increase was noted throughout the 30-min intervention in the groups exposed to HT. On average, rectal temperatures at the end of the treatment period were 38.2℃ ± 0.2℃, 39.5℃ ± 0.1℃, and 40.5℃ ± 0.2℃ in the groups exposed HT at 37℃, 39℃, and 41℃, respectively.

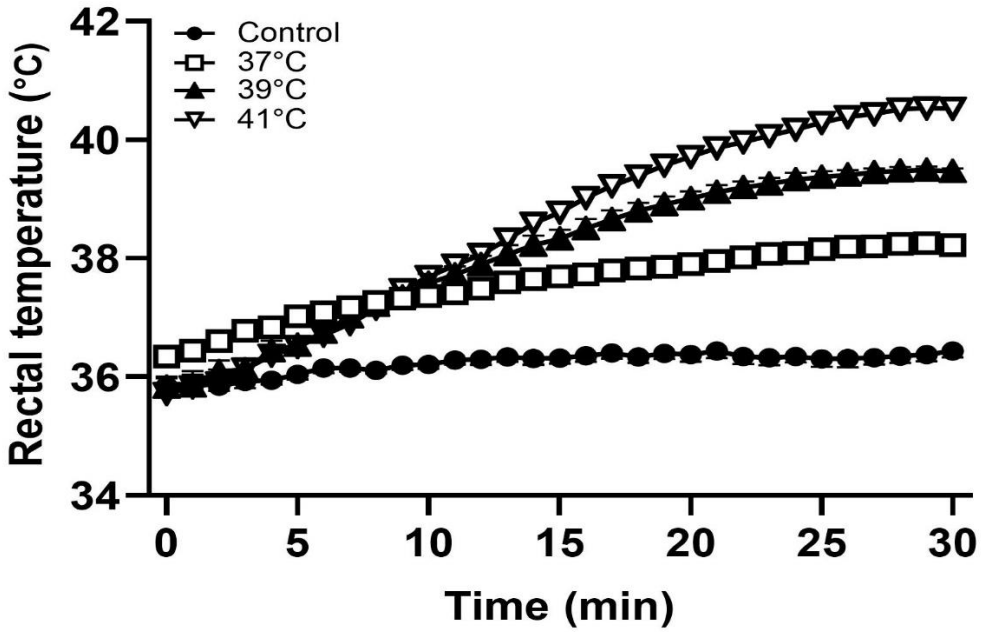

Figure 2. The rectal temperature during the single 30-min session of HT at 37℃ (□), 39℃ (▲), and 41℃ (▽), or the control treatment (●). Values are means ± SE. n = 1 of DBA/2J mice per group.

3.1.2 Body Composition

No significant group differences were found in body mass before and throughout the 3-wk intervention (treatment main effect, p = 0.27). One week after the onset of the intervention, the body mass of all HT groups showed a sharp increase (~7%) and remained stable in the subsequent weeks (Figure 3. panel A). Contrarily, the control group exhibited a significant increase of 6% after the first week of the treatment and 58% increase from week 2 to week 3 (Figure 3. panel A). There were no group differences in the changes in fat mass (treatment main effect, p = 0.87, Figure 3. panel B) and lean mass (treatment main effect, p = 0.10, Figure 3. panel C) throughout the 3-wk intervention. Of note, however, fat mass declined by ~31% from the second to the third week of treatment in the animals exposed to HT, while the control group displayed an increase of approximately 11%.

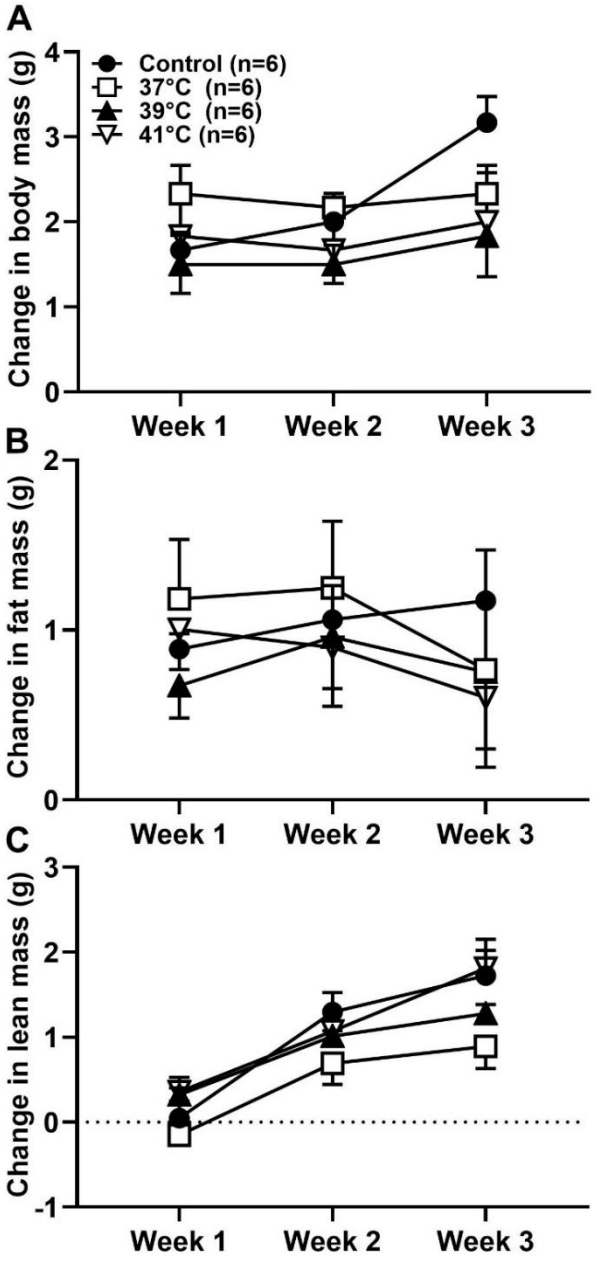

Figure 3. Changes in body mass (panel A), fat mass (panel B), and lean mass (panel C) throughout the 3-wk intervention of control treatment (●) and HT in 37°C (□), 39°C (▲), and 41(▽) in DBA/2J mice. Data were analyzed by using a 2-way ANOVA. Values are means ± SE. n = 6 DBA/2J in each group.

3.1.3 Forelimb Grip Strength

Figure 4 depicts the change in forelimb grip strength during the 3 weeks of treatment in DBA/2J mice. Data are expressed as absolute and relative forelimb grip strength (forelimb grip strength-to-body mass ratio). There were no differences in either absolute (treatment main effect, p = 0.23) or relative grip strength

(treatment main effect, p = 0.17). Absolute grip strength decreased in all groups one week after the onset of the intervention and increased progressively in subsequent weeks. Normalized grip strength was also lower in weeks 1 and 2 when compared to baseline. Of note, relative grip strength increased in all HT groups at the end of the last week of treatment, while no changes occurred in the control group.

3.1.4 Muscle Masses

There were no significant group differences in absolute masses of the EDL (treatment main effect, p = 0.83) and soleus (treatment main effect, p= 0.64) (Figure 5. panels A and C). In contrast, the group exposed to daily heat treatment at 39°C had significantly greater EDL (p = 0.04) and soleus (p = 0.02) relative muscle masses when compared to the control group.

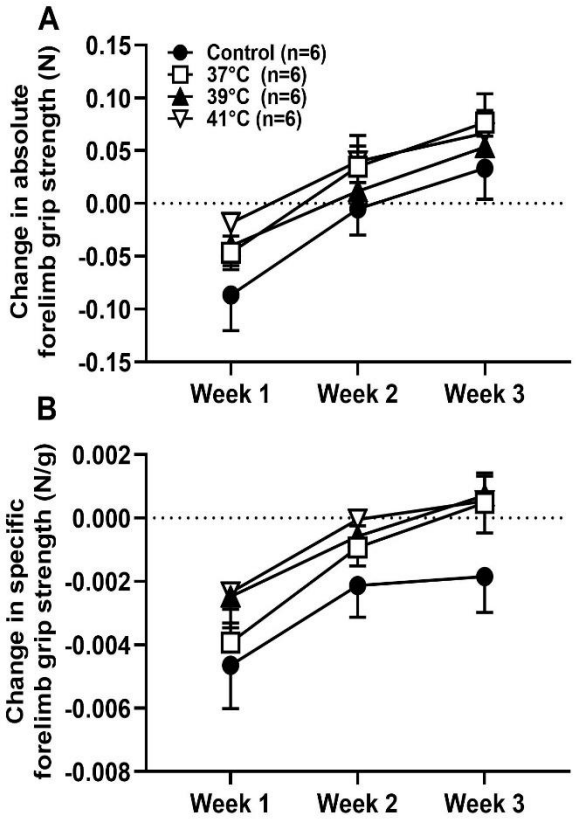

Figure 4. Changes in absolute (panel A) and relative (panel B) forelimb grip strength throughout the 3-week intervention of control treatment (●) and HT in 37°C (□), 39°C (▲), and 41°C (▽) in DBA/2J mice. Data were analyzed by using a 2-way ANOVA. Values are means ± SE. n = 6 DBA/2J in each group.

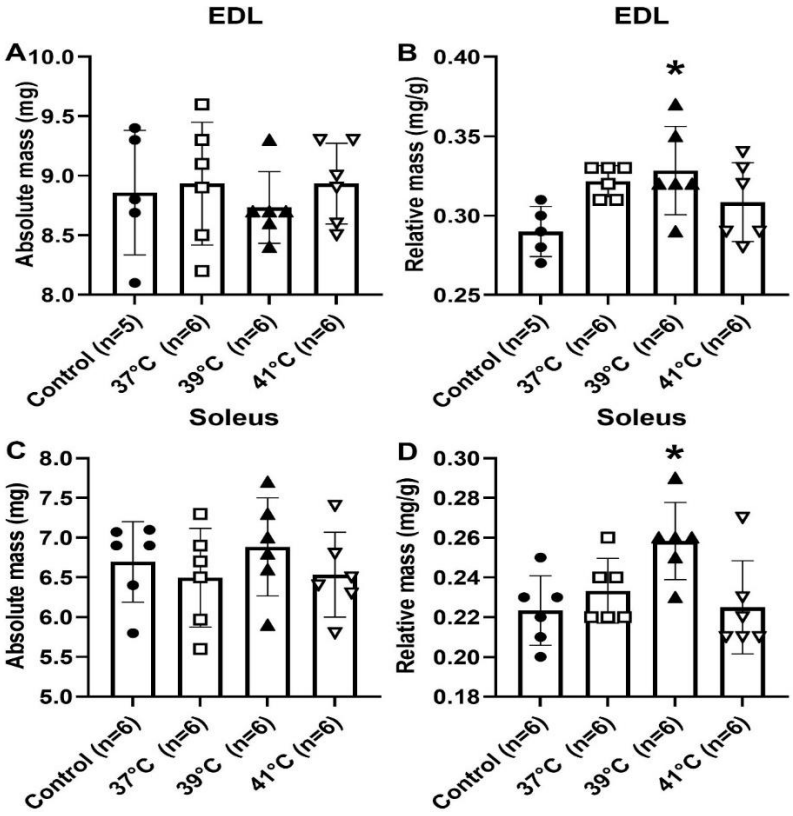

Figure 5**.** Absolute (panel A and C) and relative (panel B and D) muscle mass of extensor digitorum longus (EDL) and soleus of control treatment (●) and HT in 37°C (□), 39°C (▲), and 41°C (▽) in DBA/2J mice. Data were analyzed by using a 1-way ANOVA, with Turkey post hoc test for pairwise comparisons. Values are mean ± SE. n = 5 in control EDL, n = 6 in rest of the group DBA/2J. * Significantly different from control treatment.

3.1.5 Contractile Function

The twitch contraction parameters of isolated EDL and soleus muscles of DBA/2J mice exposed to control regimen and 3 different HT groups are given in Table 1. There was no significant difference between groups. In the EDL, peak twitch force was similar among the 4 experimental groups (treatment main effect, p = 0.46) (Figure 6. panel A). In the group exposed to HT at 39ºC, soleus peak twitch force was ~15% higher than in the control group, but this difference did not reach statistical significance (treatment main effect, p = 0.06) (Figure 6. panel B). Figures 7 and 8 show the force-frequency relationship and the maximal absolute and specific force of the EDL and soleus muscle, respectively. There was no significant effect of HT on the absolute force-frequency relationship (treatment main effect, p = 0.23) in the EDL muscle (Figure 7. panel A). However, when the values were normalized to the estimated cross-sectional area (Figure 7. panel

C), significant differences between the groups were observed. Between 120 and 300 Hz, the specific force of the 39℃ group was significantly higher than the 41℃ group (treatment × frequency interaction, $p < 0.001$). The maximal specific force (Figure 7. panel D) was also higher in the 39ºC group relative to the 41ºC group (treatment main effect, $p = 0.02$). There were no significant differences between groups in the force-frequency relationship and both maximal absolute and relative forces in the soleus muscle (Figure 8).

Table 1. The twitch contraction characteristics of isolated EDL and soleus muscles from DBA/2J mice. Values are listed as Mean ± SE. n = 6 (except for the control EDL: n =5)

	EDL					Soleus			
	Control	37℃	39℃	41℃		Control	37℃	39℃	41℃
Peak twitch tension, mN	46.90±2.0	57.89±5.3	54.38±3.6	56.95±7.0		28.06±1.8	32.02±1.6	34.17±1.4	29.55±4.6
Time to peak twitch tension, ms	14.80±0.7	15.83±0.2	15.17±0.5	16.17±0.4		26.17±0.8	26.83±0.8	26.67±0.7	26.83±0.8
Half-relaxation time, ms	15.08±0.8	15.09±0.7	14.66±0.6	16.32±0.4		30.77±1.9	31.29±3.9	29.00±1.6	34.09±1.5

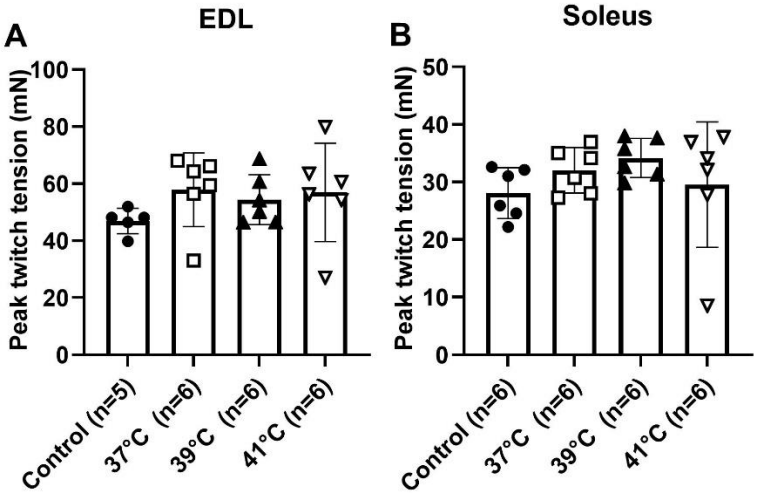

Figure 6. Peak twitch tension of EDL (panel A) and soleus (panel B) muscles of control treatment (●) and HT in 37℃ (□), 39℃ (▲), and 41℃ (▽) in DBA/2J mice. Data were analyzed by using a 1-way ANOVA. Values are means ± SE. n = 5 in control EDL, n = 6 in rest of the group DBA/2J.

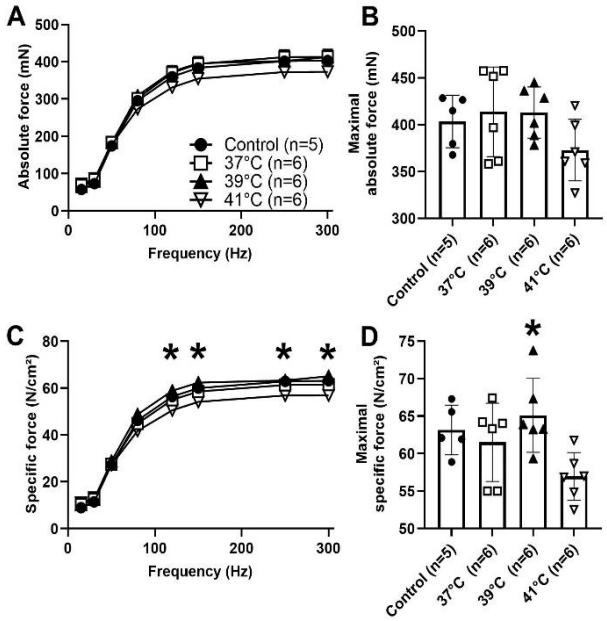

Figure 7**.** Absolute (panel A) and specific (panel C) force-frequency relationships in the extensor digitorum longus (EDL) muscle of control treatment (●) and HT in 37℃ (□), 39℃ (▲), and 41℃ (▽) in DBA/2J mice. The maximal absolute and relative forces are displayed on panels B and D, respectively. Data were analyzed by using a 2-way ANOVA, with Turkey post hoc test for pairwise comparisons. Values are means ± SE. n = 5 in control, n = 6 in HT groups DBA/2J. * Significantly different from 41℃ treatment.

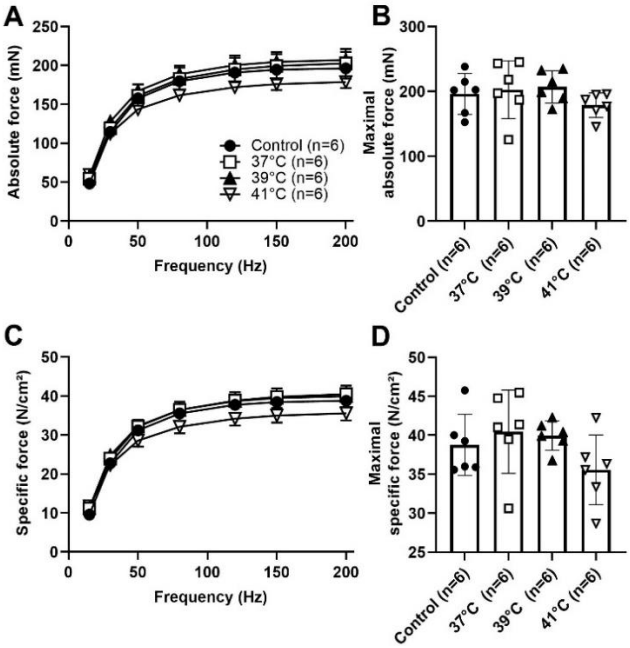

Figure 8**.** Absolute (panel A) and specific (panel C) force-frequency relationships in the soleus muscle of control treatment (●) and HT in 37℃ (□), 39℃ (▲), and 41℃ (▽) in DBA/2J mice. The maximal absolute and relative forces are displayed on panels B and D, respectively. Data were analyzed by using a 2-way ANOVA. Values are means ± SE. n = 6 in HT groups DBA/2J.

3.1.6 Mitochondrial Content

There were no group differences in the protein abundance of mitochondrial oxidative phosphorylation complexes in both the EDL (treatment main effect, complex I through V, p = 0.23, p = 0.83, p = 0.92, p = 0.48, p = 0.83, respectively) and the soleus (treatment main effect, complex I through V, p = 0.90, p = 0.96, p = 0.30, p = 0.98, p = 0.81) muscles (Figure 9).

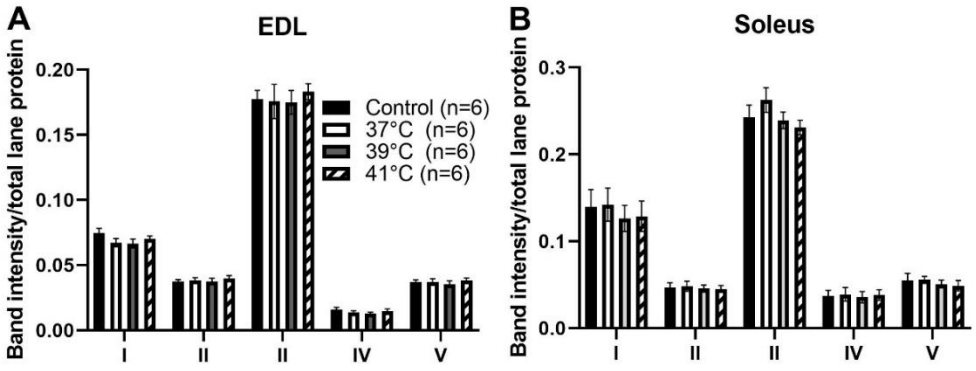

Figure 9. Protein abundance of mitochondrial oxidative phosphorylation complexes in the EDL (panel A) and soleus (panel B) muscles. Data were analyzed by using a 1-way ANOVA. Values are means ± SE. n = 6 DBA/2J.

3.2 Study 2 – Impact of HT on Body Composition and Muscle Function in Male D2.mdx Mice

The results of study 1 indicated that exposure to HT at 39ºC, but not at 37º or 41ºC, elicited an increase in relative muscle mass and tended to improve peak twitch force relative to the control group. Based upon these findings, D2.mdx mice were randomly allocated to receive HT at 39ºC or a control regimen.

3.2.1 Body Composition

Changes in body composition during exposure to the 3-week interventions are shown in Figure 10. There were no significant group differences in body mass (Figure 10. panel A, treatment main effect, p = 0.49), lean mass (Figure 10. panel B, treatment main effect, p = 0.40), or fat mass (Figure 10. panel C, treatment main effect, p = 0.27).

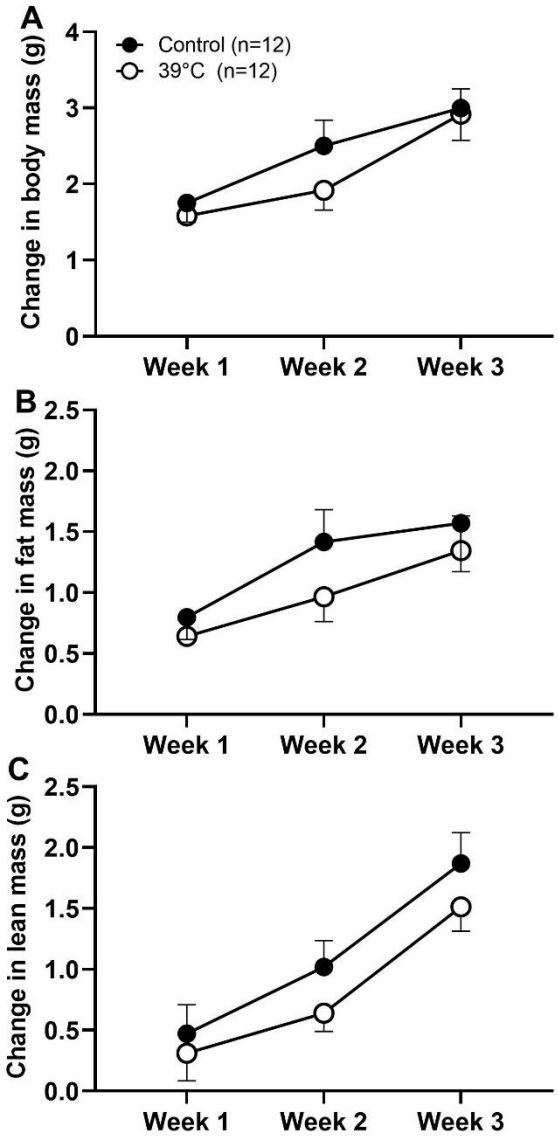

Figure 10. Changes in body mass (panel A), fat mass (panel B), and lean mass (panel C) throughout the 3-wk intervention of control treatment (●) and HT (○) in D2.mdx mice. Data were analyzed by using a 2-way ANOVA. Values are means ± SE. n = 12 D2.mdx in each group.

3.2.2 Forelimb Grip Strength

Figure 11 depicts the change in forelimb grip strength during the 3 weeks of treatment in the D2.mdx mice. Absolute forelimb grip strength increased gradually and similarly in both groups (treatment main effect, p = 0.64). When normalized to both mass, grip strength remained below the baseline levels in both the control and HT groups (Figure 11. panel B, treatment main effect, p = 0.89).

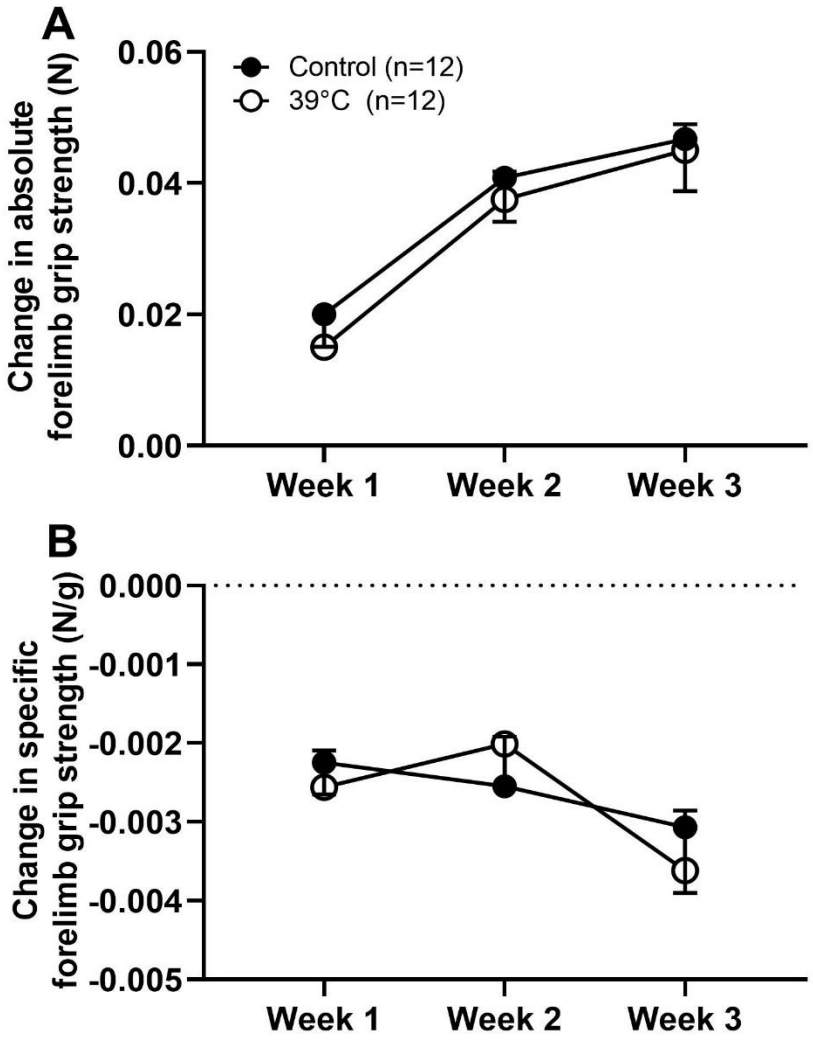

Figure 11. Change in absolute (panel A) and specific (panel B) forelimb grip strength in D2.mdx mice exposed to HT (○) or the control regimen (●). Data were analyzed by using a 2-way ANOVA. Values are means ± SE. n = 12 D2.mdx in each group.

3.2.3 Muscle Masses

There were no significant group differences in absolute masses of EDL (treatment main effect, p = 0.39) and soleus (treatment main effect, p = 0.30) muscles. The EDL relative muscle mass tended to be higher in the group exposed to HT compared to the control group (Figure 12. panel B, treatment main effect, p = 0.07). Conversely, relative soleus muscle mass was similar between groups (Figure12. panel D).

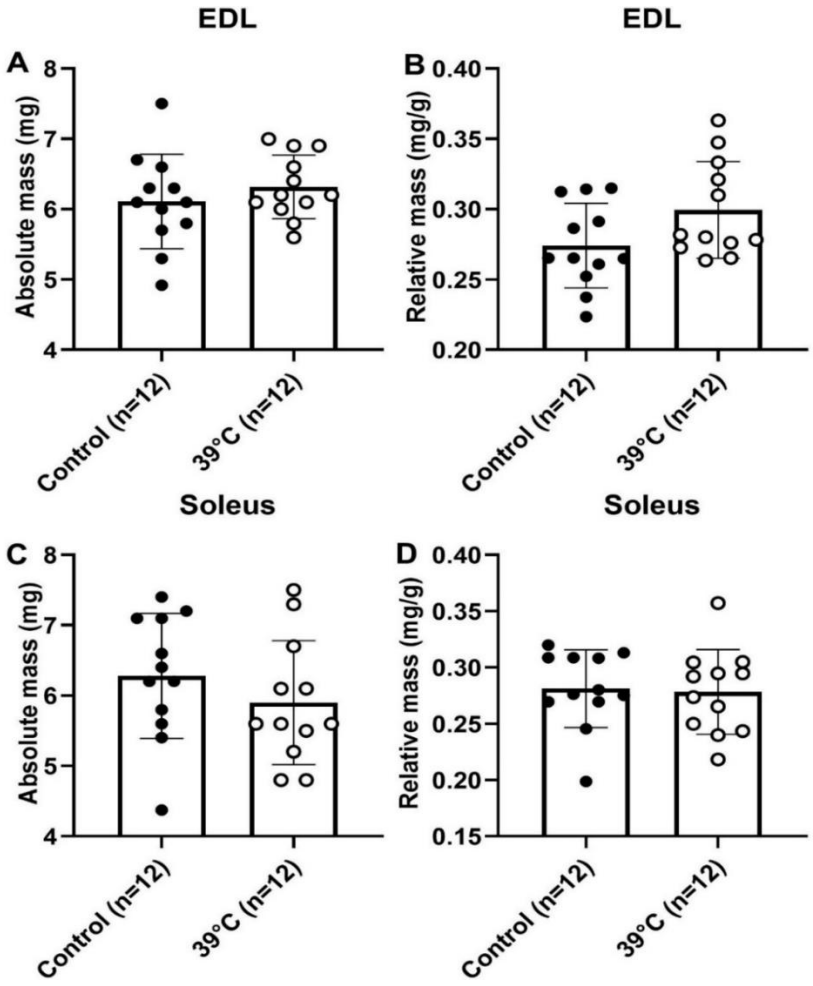

Figure 12. Absolute (panel A and C) and relative (panel B and D) muscle mass of extensor digitorum longus (EDL) and soleus of control treatment (●) and HT (○) in D2.mdx mice. Data were analyzed by using a 1-way ANOVA. Values are means ± SE. n = 12 D2.mdx in each group.

3.2.4 Contractile Function

The twitch contraction parameters of isolated EDL and soleus muscles of D2.mdx mice exposed to control regimen and HT group are provided in Table 2. No group difference was

found in the twitch contraction characteristic. Peak twitch tension was similar between groups in both the EDL (treatment main effect, p = 0.75) and the soleus (treatment main effect, p = 0.96) (Figure 13). The force-frequency relationship and maximal forces were also comparable between the HT and control groups for both the EDL (Figure 14) and the soleus (Figure 15).

Table 2. The twitch contraction characteristics of isolated EDL and soleus muscles from D2.mdx mice. Values are listed as Mean ± SE. n = 12

	EDL			Soleus	
	Control	39℃		Control	39℃
Peak twitch tension, mN	26.37±2.6	29.14±1.5		18.68±1.1	18.77±1.4
Time to peak twitch tension, ms	13.92±0.4	14.25±0.5		24.00±0.7	24.58±0.6
Half-relaxation time, ms	15.84±0.3	16.40±0.4		31.78±1.4	32.26±1.4

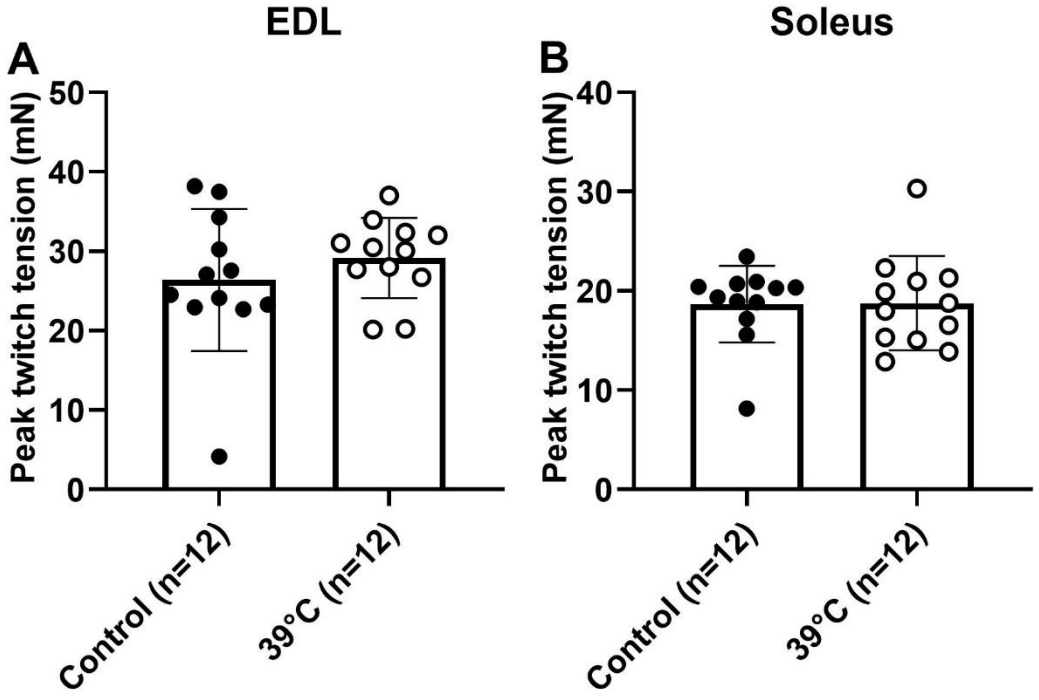

Figure 13. Peak twitch tension of EDL (panel A) and soleus (panel B) muscle of control treatment (●) and HT (○) from mdx mice are displayed. Both EDL and soleus, did not showed significant difference between the group. Data were analyzed by using a 1-way ANOVA. Values are means ± SE. n = 12 D2.mdx in each group.

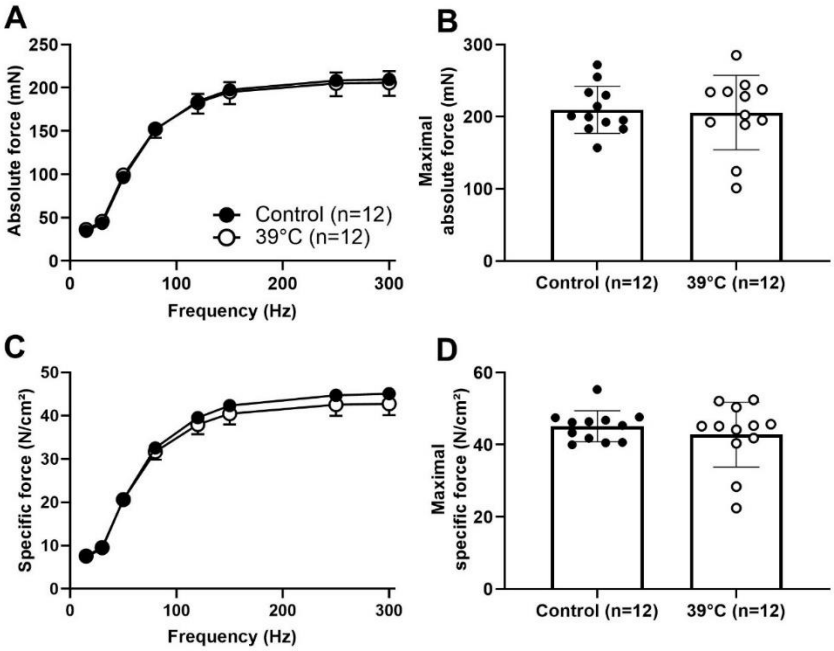

Figure 14. Absolute (panel A) and specific (panel C) force-frequency relationships in the extensor digitorum longus (EDL) muscle of control treatment (●) and HT (○) in D2.mdx. The maximal absolute and relative forces are displayed on panels B and D, respectively. Data were analyzed by using a 2-way ANOVA. Values are means ± SE. n = 12 D2.mdx in each group.

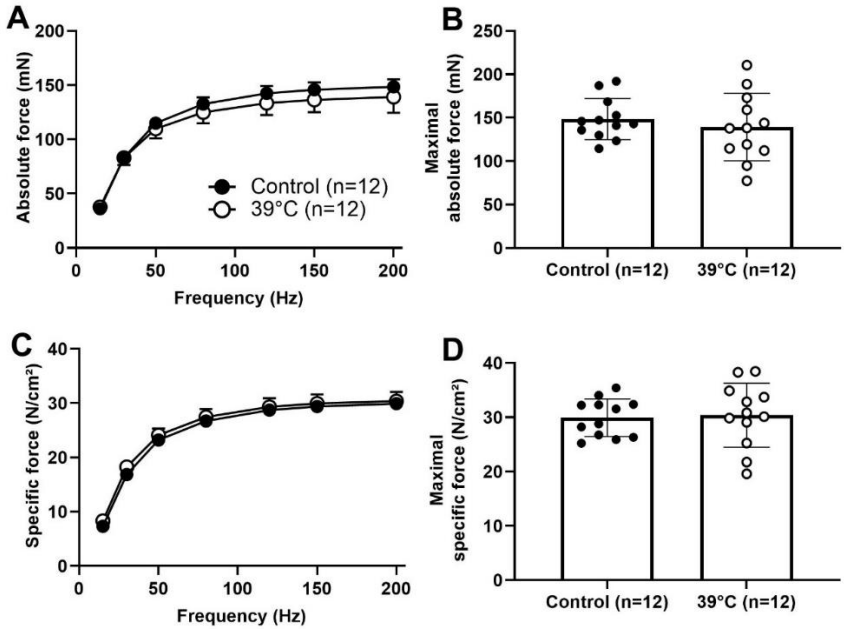

Figure 15. Absolute (panel A) and specific (panel C) force-frequency relationships in the soleus muscle of control treatment (●) and HT (○) in D2.mdx. The maximal absolute and relative forces are displayed on panels B and D, respectively. Data were analyzed by using a 2-way ANOVA. Values are means ± SE. n = 12 D2.mdx in each group.

3.2.5 Mitochondrial Content

Exposure to HT had no significant effect on the abundance of the mitochondrial OXPHOS proteins in both EDL (treatment main effect, complex I through V, p = 0.90, p = 0.95, p = 0.89, p = 0.92, p = 0.80, respectively) and soleus muscle (treatment main effect, complex I through V, p = 0.49, p = 0.78, p = 0.59, p = 0.78, p = 0.54) (Figure 16).

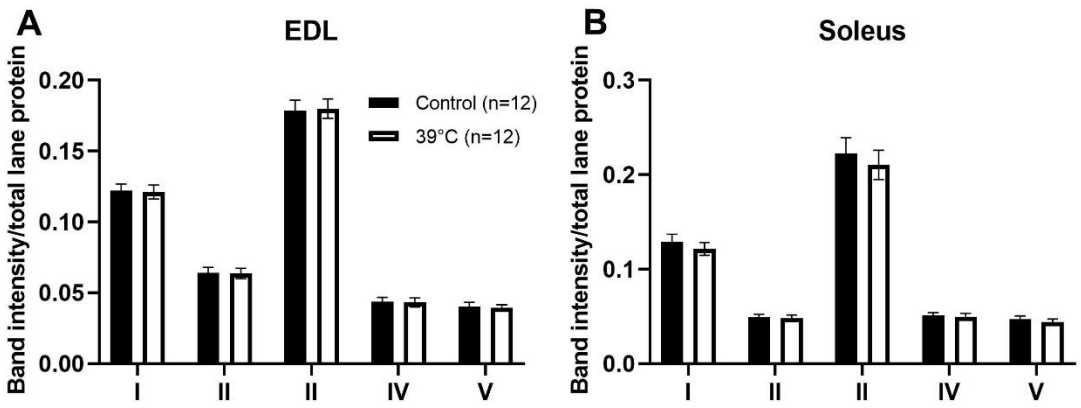

Figure 16. Protein abundance of mitochondrial oxidative phosphorylation complexes in the EDL (panel A) and soleus (panel B) muscles. Data were analyzed by using a 1-way ANOVA. Values are means ± SE. n = 12 D2.mdx in each group.

CHAPTER 4. DISCUSSION

4.1 Main Findings

The primary aims of this study were twofold: first, we conducted a dose-response experiment in male DBA/2J mice to define the optimal HT temperature in an incubator; second, we examined the effects of repeated exposure to HT on body composition and skeletal muscle function in the D2.mdx model of DMD. In agreement with our previous findings in a model of ischemia-induced muscle damage, we report that the skeletal muscle adaptations to HT depend on the treatment temperature. Male DBA/2J mice exposed to HT at 39℃, but not at 37º or 41℃, displayed a significant increase in the relative mass of the EDL and soleus muscles when compared to the sham-treated group. Noteworthy, the maximal specific force of the isolated EDL muscle was significantly higher in the 39℃ group when compared to the 41℃ group. Building upon these findings, we chose to treat the D2.mdx mice with HT at 39ºC for 3 consecutive weeks. Contrary to our hypothesis and our previous findings in a model of ischemia-induced muscle damage, repeated HT had no impact on body composition, muscle mass and isolated muscle function.

4.2 Study 1 – Impact of HT on Body Composition and Muscle Function in Male DBA/2J Mice

We have previously reported that the effects of heat therapy, in the form of a heated water bath, depend on treatment temperature. In a model of ischemia-induced muscle damage, we observed that daily hindlimb immersion in water at 39ºC, but not at 37ºC or 41ºC, increased relative soleus muscle mass and force development (Kim et al.,2019). Herein, the animals were exposed to whole-body HT in an incubator rather than water-immersion. Although this HT modality has been used previously by other groups, the optimal incubator temperature necessary to elicit beneficial skeletal muscle adaptations has not been determined. Therefore, in our first experiment, male wild-type animals were randomly allocated to receive HT in an incubator set at 37ºC (n = 6), 39ºC (n = 6), and 41ºC (n = 6), while the control group was placed in the incubator set at room temperature (n = 6). Below we discuss in detail the effects of treatment temperature

on body composition, forelimb grip strength, muscle mass and isolated contractile function, and mitochondrial content.

4.2.1 Body Composition

We recently reported that repeated exposure to HT at 39ºC prevented an increase in body mass due to reduced fat accrual in a model of combined obesity and ischemia-induced muscle damage. These previous observations prompted us to examine the impact of HT at different temperatures on body composition in healthy animals. Contrary to our observations in obese mice, there were no statistically significant differences between groups in body mass, fat mass, and lean mass. These findings are congruent with the observations of Tamura and colleagues in ICR mice subjected to HT (40°C, 30 min/day, 5 days/wk, 3 wk) (Tamura et al., 2015). However, it is worth noting that from the second to the third week of treatment, the behavior of changes in body mass and fat mass was quite contrasting between the animals exposed to HT and the control group. Indeed, while an increment in both body and fat masses was noted in the control group, the animals exposed to HT displayed a reduction in fat mass, thereby preventing an increase in body mass. It is thus tempting to speculate that a longer treatment duration (i.e., 4 weeks or more) may be necessary to elicit significant changes in body composition in healthy animals. Additional studies are needed to define the impact of treatment duration and temperature on body composition in non-obese animals.

4.2.2 Muscle Masses

Treatment with HT at 39°C has been shown to increase the relative mass of the soleus, but not the EDL muscle, in a model of ischemia-induced muscle damage (Kim et al., 2019). In this previous study, C57BL/6 mice were subjected to bilateral ligation of the femoral artery and received HT (immersion in a water bath) 6 days/week for 3 weeks. We extend these previous findings in the present study by showing that HT at 39ºC, but not at 37 or 41ºC, increases the relative mass of both the soleus (16%) and the EDL (12%) muscles. The close agreement between the findings of these two studies indicates that, among the three temperatures tested, 39ºC appears to be the temperature that elicits the greatest increase in relative muscle mass. The causes of these marked changes in relative muscle mass following HT at 39ºC are undefined.

Given the lack of changes in absolute mass between groups, one possibility is that the aforementioned changes in body composition following HT, most notably the decline in fat mass and the absence of weight gain from the second to the third week of treatment, are responsible for the change in relative muscle mass. However, it is important to note that while body mass remained unaltered from the second to the third week of the intervention in all three groups exposed to HT, the increase in relative mass was most evident in the 39ºC group. These observations indicate that factors beyond body mass may also contribute to the changes in relative muscle mass.

4.2.3 Isolated Muscle Contractile Function

In our previous study in a model of ischemia-induced muscle damage, soleus absolute isometric tetanic force in animals treated with HT at 37°C and 39°C was significantly higher than the control group (Kim et al., 2019). In contrast, no treatment effects were observed in DBA/2J mice in the current study. It is important to highlight, nonetheless, that peak twitch tension in the soleus muscle tended to be higher in the 39℃ group when compared to the control group. As mentioned above, it is conceivable that a longer treatment duration is needed in order to elicit palpable changes in muscle strength in healthy, uninjured animals.

One important observation in our dose-response study was that mice treated with HT at 39℃ group had significantly higher force development in the EDL muscle when compared to the 41℃ group. This finding adds further support to the notion that the adaptations to HT depend on treatment temperature. There appears to be a temperature threshold beyond which the effects of HT are negated. Additional studies are warranted to define the mechanisms underpinning the temperature-dependent effects of HT.

4.2.4 Forelimb Grip Strength

In alignment with the findings of unaltered maximal force development in isolated muscles, there were no group differences in either absolute or specific forelimb grip strength in DBA/2J mice. Noteworthy, a distinct profile is evident between HT-treated and control animals for specific strength, which accounts for body mass, between the second and third week of treatment. While the animals exposed to HT displayed an increase in specific strength from week

2 to week 3, no changes were seen in the control group. This response is likely the result of the aforementioned differences in body mass. Between the second and third week, the HT-treated animals displayed an increase in absolute grip strength concomitant with minimal or no changes in body mass, thus resulting in an increase in relative strength. Conversely, the increase in body mass in control animals in the third week prevented the increase in relative grip strength. These results highlight that the changes in body composition are a central adaptation to repeated HT in rodents.

4.2.5 Mitochondrial Content

Tamura and colleagues reported that daily whole body HT (40ºC, 30 min/day, 5days/week) for 3 consecutive weeks increased the abundance of mitochondrial respiratory chain proteins (complexes I-V) in the plantaris and soleus muscle of six-week-old male ICR mice (Tamura et al., 2014). Based upon these findings, we hypothesized that HT would increase mitochondrial content in the muscles from DBA/2J mice. However, we did not detect any changes in the abundance of mitochondrial respiratory chain proteins in either the soleus or the EDL muscles. The reasons for this discrepancy are unclear, especially when considering that our HT protocol was similar to the one used by Tamura and colleagues. As our study was performed in DBA/2J mice while Tamura and colleagues used ICR mice, it is fair to speculate that strain differences exist in the mitochondrial adaptations to HT.

4.3 Study 2 – Impact of HT on Body Composition and Muscle Function in Male D2.mdx Mice

Guided by the findings of our first experiment in DBA/2J mice, the goal of the second experiment was to assess the effects of repeated HT at 39ºC on body composition and muscle function in D2.mdx mice, a model of Duchenne muscular dystrophy. We chose the D2.mdx model, as opposed to the more commonly used C57BL/10-mdx (BL 10-mdx) strain, because D2.mdx mice have a more severe dystrophic phenotype, including lower muscle weight, impaired muscle function, and a greater level of fibrotic tissue (van Putten et al., 2019; Verhaart et al., 2020; Hammers et al., 2020; Spaulding et al., 2020).

4.3.1 Body Composition

Patients with DMD display an increase in fat mass that is positively correlated with age and a progressive loss of lean mass that results from the dystrophic process. Further complicating this scenario, DMD patients often receive steroids, which significantly alter the metabolic function and lead to an increase in obesity (Davidson et al., 2014). There are currently no therapies that are capable of combating the increase in adiposity while preserving or increasing lean mass in the patients. Our findings that repeated HT attenuates fat accrual in a model of diet-induced obesity led us to hypothesize that this novel treatment would alter body composition in the D2.mdx mice. Contrary to this hypothesis, HT at 39ºC HT had no impact on fat mass, lean mass, and body mass in D2.mdx. Indeed, as opposed to the findings in the DBA/2J mice, in which a clear reduction in fat mass was observed in the last of the three weeks of treatment, the fat mass increased similarly throughout the intervention in D2.mdx mice treated with HT and the sham-treated animals. Based on this comparison, one interpretation is that D2.mdx mice are resistant to the effects of HT on fat metabolism. Another possibility is that the treatment dose, including treatment temperature and the frequency and duration of sessions, was not optimal to elicit metabolic adaptations in the D2.mdx mice.

4.3.2 Muscle Masses

The progressive loss of skeletal muscle, which is replaced with fatty fibrotic tissue, is a central cause of functional impairments in DMD (Sussman., 2002). Treatment with HT has also been shown to attenuate skeletal muscle atrophy during immobilization and unloading and rescue denervation-induced atrophy (Naito et al., 2000; Selsby et al., 2007; Tamura et al., 2015). These findings in other models of muscle atrophy prompted the hypothesis that HT would increase muscle mass in the D2.mdx mice. Conversely, we report that there were no significant differences in either absolute and relative muscle mass in EDL and soleus from D2.mdx mice. Of note, however, the relative mass of the fast-twitch EDL muscle of animals exposed to HT tended to be greater when compared to the muscles from the control animals, despite no differences in body mass between groups. This finding needs to be further explored in part because type 2 fibers are preferentially affected in DMD (Webster et al., 1988; Talbot & Maves, 2016).

Additional studies are also needed to define why, contrary to the observations in DBA/2J mice, the relative mass of the soleus muscle was not affected by HT.

4.3.3 Muscle Strength

Loss of muscle strength and skeletal functional mass in DMD negatively affects the health outcomes leading to an increased risk for disability and morbidity as well as decreased quality of life (Yiu & Kornberg., 2015). Hammers and co-worker reported that maximum absolute force production of isolated EDL was reduced by 40-50% in D2.mdx compared to age-matched wildtype value. In addition, when the isolated EDL maximum force production was normalized to muscle cross-sectional area, it showed approximately 30% decrement from age-matched wildtype (Hammers et al., 2020). In agreement with previous reports, our findings reveal that the maximal absolute strength of the isolated EDL and soleus muscles is severely depressed in D2.mdx mice when compared to healthy controls. The magnitude of impairment of maximum absolute force production of isolated EDL relative to DBA/2J mice was 48% and 50% in the control and 39 ºC HT group, respectively. The same trend was observed in isolated soleus muscle, although impairment was not severe as EDL muscle. Maximal force was, on average, 29% and 34% lower in the control and 39ºC HT groups, respectively, compared to wildtype mice. We also show that exposure to HT at 39ºC had no impact on either absolute and specific force development in the EDL and soleus. These findings contrast with our previous report of increased maximal strength of the soleus muscle in a model of ischemia-induced muscle damage (Kim et al., 2019). As detailed above, the treatment protocol employed herein was based on experiments performed in healthy, control animals and may not be optimal to reverse the severe contractile dysfunction observed in D2.mdx mice. Future studies are needed to define whether different HT treatment protocols can improve muscle strength in D2.mdx animals.

4.3.4 Forelimb Grip Strength

In line with the findings of unaltered isolated muscle force development following HT, there was no treatment effect on forelimb grip strength in D2.mdx mice. The Control group showed a higher but not significant increase in lean mass throughout the intervention, but normalized forelimb grip strength did not differ from the HT group. The difference between

muscle mass and muscle function can be possibly speculated muscle integrity. Muscle integrity and stability are the important roles of the myofiber membrane protein, called Dystrophin (Campbell & Kahl, 1989; Ervasti & Campbell, 1993; Yue et al., 2020). Therefore, future study to define the gap of the lean mass and muscle function is needed.

It was striking that mdx mice showed a decrease in specific forelimb grip strength in all 3 weeks of the intervention compared to baseline. During 3 weeks of the intervention, the control and HT groups showed an average of decrease around 0.00238 (N/g) and 0.00265 (N/g), respectively. Van Putten et al. reported a similar trend of normalized forelimb grip strength in D2- wildtype and D2.mdx. In that study, mdx mice showed a substantial reduction in the normalized forelimb grip strength after 8 weeks of age, while D2-wildtype increased between the age of 8 and 12 weeks (van Putten et al., 2019). These results indicate that the animal's age may impact the treatment outcomes. Rafael-Fortney et al. reported that the start of the treatment with combination of lisinopril and spironolactone at 4 weeks of age did improved skeletal muscle function, while there was no improvement when the treatment was initiated at 8 weeks of age (Rafael-Fortney et al., 2011). This finding may be explained due to the development of pathological features across the lifespan. Hammers and colleagues documented that degeneration and inflammation peak at 8 weeks of age and decrease along with time in D2.mdx mice, while muscle fibrosis starts to increase rapidly after 8 weeks. (Hammers et al., 2020).

4.3.5 Mitochondrial Content

Several studies reported that dystrophin deficiency may be associated with an impairment in mitochondrial function in mice and human skeletal muscle (Kuznetsov et al., 1998; Rybalka et al., 2014; Ramos et al., 2020). For example, Hughes et al. demonstrated that mdx mice have lower content of mitochondrial complexes I through V in the quadriceps muscle. However, no significant difference was founded between wildtype and mdx in white gastrocnemius muscle (Hughes et al., 2019). In accordance, Ramos et al. reported that no change was observed between wildtype and mdx in all five complexes in the EDL muscle (Ramos et al., 2020). To our knowledge, this is the first study to examine the impact of HT on mitochondrial biogenesis in D2.mdx mice. We did not observe a decrease of subunit content in all five complexes in D2.mdx mice relative to wildtype animals. In addition, HT did not affect the abundance of respiratory

chain proteins in D2.mdx skeletal muscle. This finding is consistent with previous reports from our laboratory (Kim et al., 2020; Kim et al., 2020).

4.4 Limitations

First, in the present study, we have used whole-body HT not a local HT. So, the effect of HT we have examined could be not only due to the muscle adaptation but also due to the combination of neural or immune system adaptation. Also, one of the limitations of the current study is that the sample size was too small for study 1. Only 6 of DBA/2J mice were randomly distributed in the control regime and HT group. Even in the EDL muscle contractile test, one of the muscles could not assess so there were only 5 samples in the control group. Secondly, while measuring the rectal temperature during the HT, mice were inside the restrainer. In normal treatment session, mice were actively moving in the cage. Also, food and drink were placed in the incubator. However, during the rectal temperature measurement, since mice were in the restrainer the circumstances were slightly different from the actual treatment setting. In the second set of the study, we only examined the adaptations to HT at 39℃ by using D2.mdx mice. Therefore, future studies are needed to identify the proper treatment dose, including treatment temperature, frequency, and duration. In addition, during both sets of studies, we only focused on the same age of mice, but different ages of treatment initiation could differentiate the results. Thus, additional studies are warranted to clarify the impact of animal age on the outcomes of HT in a DMD model.

4.5 Conclusion

In summary, the current study demonstrates that the skeletal muscle adaptations to daily whole-body HT depends on the treatment temperature in DBA/2J mice. Treatment with HT at 39℃, but not 41℃, promotes an increase in relative muscle mass in both EDL and soleus muscle. Additionally, daily HT for 3 weeks had no effects on body composition and muscle mass and function in D2.mdx mice. Additional studies are warranted to define the importance of the treatment protocol characteristics as well as the animal's age on the outcomes of HT in DMD.

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