Low load endurance activity and green tea extract represent potential therapies for Duchenne muscular dystrophy

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Abstract

Duchenne muscular dystrophy (DMD) is a progressive muscle wasting disease affecting 1 in every 3500 boys. The disease is characterized by the absence of the dystrophin protein from the sarcolemma of muscle cells. Muscle cells lacking dystrophin go through cycles of degeneration and regeneration and are considered susceptible to contraction-induced injury [144]. Eventually, the satellite cell proliferative capacity is exhausted and the muscle fibers are replaced by connective and adipose tissue that yields a progressive loss of force generating capability. DMD patients typically die by their early 20's, primarily due to respiratory or cardiac failure. The precise role of dystrophin is not presently known. However, its absence suggests that it may play a role in both cellular calcium regulation and oxidative stress [152]. Recent studies suggest increased reactive oxygen species (ROS) may precede the initial wave of wasting that marks disease onset [49]. Therefore, it is possible oxidative stress may contribute as a pathogenic mechanism of DMD. Strategies to reduce the deleterious effects of oxidative stress could be an effective therapeutic approach. Regular exercise is known to increase antioxidant capacity in humans and mice [146]. Green tea extract (GTE) is a powerful antioxidant that is easily supplemented in the diet [83].

The purpose of this study was to test the hypotheses that (1) voluntary endurance exercise alone, (2) a diet supplemented with 0.05% (wt/wt) GTE alone, or exercise and GTE combined will blunt the effects of ROS and improve muscle strength and endurance in young mdx mice. Male mdx mice at age 21-days were randomly divided into one of 4 treatment groups: Run Normal diet (RunNorm; n=8), Sedentary Normal diet (SedNorm; n=8), Run GTE diet (RunGTE; n=10), and Sedentary GTE diet (SedGTE; n=8). RunNorm and RunGTE mice were given free access to a running wheel for 3 weeks while SedNorm and SedGTE mice were restricted to normal cage movement. At the end

of 3 weeks, mice in each treatment group were sacrificed and assessed for daily and weekly running distances, content of actin and myosin proteins and fiber type distribution (tibialis anterior), contractile/mechanical and fatigue properties (extensor digitorum longus), creatine kinase levels and antioxidant capacity (serum), lipid peroxidation (gastrocnemius), and citrate synthase and beta-hydroxyacyl-CoA dehydrogenase activities (quadriceps and soleus).

The key findings of this study were: In normal diet running mice (RunNorm), average daily distance run was increased 300% (from 0.5 to 2.1 km/d, $P<0.05$) from week 1 to week 3. In GTE diet (RunGTE) compared to RunNorm mice, total distance over the 3 weeks was markedly improved 128% (61.2 vs. 26.8 km, $P<0.0001$). Running, independent of diet increased EDL muscle tetanic stress (18%), serum antioxidant capacity (22%), citrate synthase activity (35%), and beta-oxidation (37%; all $P<0.05$). GTE, independent of running decreased lipid peroxidation (gastrocnemius:-64%; heart: -29%) and serum creatine kinase (-36%), and increased citrate synthase activity (59%; all $P<0.05$).

These findings in dystrophic mice suggest that voluntary endurance exercise with or without GTE supplementation blunted the deleterious effects of ROS. If similar positive effects are evident in human DMD patients, then these approaches may be beneficial therapies either alone or in combination.

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List of Important Definitions

1. **DMD - Duchenne muscular dystrophy**
2. **ROS - Reactive oxygen species**
3. **DGC - Dystrophin glycoprotein complex**
4. **Ca^{2+} - Calcium**
5. **SDS - Sodium dodecyl sulfate**
6. **TBARS - Thiobarbituric acid reactive substances**
7. **BCA - Bicinchoninic acid**
8. **SedNorm - Sedentary, normal diet**
9. **RunNorm - Run, normal diet**
10. **SedGTE - Sedentary, green tea extract diet**
11. **RunGTE - Run, green tea extract diet**
12. **GTE - Green tea extract**

Chapter 1 Introduction

Introduction

Duchenne muscular dystrophy (DMD) is a progressive muscle wasting disease affecting 1 in every 3500 boys [56]. The disease is characterized by the absence of the dystrophin protein from inner surface of the muscle cell sarcolemma [184]. Muscle cells lacking dystrophin go through cycles of degeneration and regeneration and are considered susceptible to contraction-induced injury [167]. Eventually, the satellite cell proliferative capacity is exhausted and the muscle fibers are replaced by connective and adipose tissue that yields a progressive loss of force generating capability [167]. DMD patients typically die by their mid 20's, primarily due to respiratory or cardiac failure [114]. Recent studies suggest increased reactive oxygen species (ROS) may precede the initial wave of wasting that marks disease onset [49]. Therefore, it is possible oxidative stress may contribute to the pathogenic mechanism of DMD.

Oxidative stress is the accumulation of destructive reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, and the hydroxyl radical (i.e., $O_2\bullet^-$, H_2O_2, $OH\bullet$, respectively). When oxidative stress is severe and/or prolonged, antioxidants, the body's natural defense system, can be overwhelmed, leading to oxidative damage of lipids, proteins, and DNA. Studies involving the mdx mouse, a model of Duchenne muscular dystrophy (DMD), suggest oxidative stress is increased in muscles at age 10 days before disease onset [49].

Contributing factors to oxidative stress in mdx mice include increased motor movement associated with maturation, increased intracellular Ca^{2+} concentrations, and decreased antioxidant capacity.[152,148]. Once oxidative stress is initiated, it can cause irreversible damage to mitochondrial and cellular DNA which compromises the cell's ability to balance protein synthesis and degradation. Processes of oxidative stress can lead to the degradation of key muscle proteins (i.e., actin, myosin) that decrease contractile capability and initiate an inflammatory response. These processes may also oxidize lipids in the muscle fiber plasma membrane, compromising its integrity and leading to lesions through which normally regulated intracellular molecules diffuse out (e.g., creatine kinase) [152], and extracellular ions diffuse in (e.g., Ca^{2+}).

Increased antioxidant proteins (e.g., superoxide dismutase) in response to low intensity endurance exercise provide a potential treatment to alleviate oxidative stress [146]. Additionally, exercise training is known to increase fatigue resistance and reverse muscle atrophy in non-

diseased individuals [79]. For example, Fiatarone [61] observed improvements in muscle strength in elderly patients (age 72 – 98 years) after 10 weeks of progressive resistance exercise training. Assuming that similar positive adaptations in dystrophic muscle occur with endurance training, the quality of life and the longevity of DMD patients could be improved. However, it is not presently known if endurance exercise exacerbates the dystrophic condition in DMD patients, as few controlled human studies have been conducted [76]. Instead, studies have been carried out on the dystrophic mdx mouse. Unfortunately, the results of these studies are difficult to interpret because the type of training regimens (e.g., voluntary vs. involuntary), the age at which exercise training was initiated, and the training duration and frequency were so inconsistent [76]. Nonetheless, some voluntary wheel running studies provided evidence that low-intensity endurance training could promote beneficial adaptations in dystrophic mice. Carter [26] reported decreased plasma creatine kinase levels after voluntary wheel running for 4 weeks in young mdx mice (initiated at age 4 weeks). Creatine kinase is a ~87 kd molecule that can leak out of muscle fibers when their sarcolemmal membranes are compromised, and is a common serum marker of DMD. The data of Carter et al., therefore suggest that exercise has a protective effect against membrane leaks. Exercise training also increased mdx extensor digitorum longus (EDL) force output and decreased fatigability. Dupont-Versteegden [53] reported increased diaphragm force output after exercise training in mdx mice. For DMD patients, increased force output and fatigue resistance are both desired effects, especially in the diaphragm because of its critical role in breathing. For these reasons, exercise training may provide relief from oxidative stress and muscle wasting in DMD.

In addition to exercise training, another potential antioxidant therapy for DMD is green tea extract supplementation. Although there are few studies on the potential role of antioxidants as a DMD treatment, green tea extract is one antioxidant that has shown promise [22,51]. Buetler et al. reported chow supplemented with the natural polyphenol antioxidants found in green tea extract (GTE) delayed the initial wave of muscle wasting and elicited beneficial adaptations in dystrophic skeletal muscle (e.g., increased fatigue resistance). This study supports the idea that antioxidants have the potential to treat muscular dystrophy through mechanisms that attenuate oxidative stress.

For these reasons, a treatment regimen of exercise training and a powerful antioxidant (GTE) as a supplement in the diet may be beneficial. Other potential positive effects besides attenuation of oxidative stress could include decreased force loss and delayed disease onset and progression.

Statement of Problem

In summary, DMD is a fatal muscle wasting disease characterized by the absence of dystrophin. The mechanism of disease onset is unclear; however, oxidative stress is a likely contributor to its onset and/or progression. Because oxidative damage can destroy key components of the muscle cell including contractile proteins and the sarcolemmal membrane, increased antioxidant capacity associated with exercise training and GTE supplementation may provide relief. Furthermore, endurance exercise training may elicit desired adaptations such as increased strength and fatigue resistance. The purpose of this study was to determine if voluntary endurance exercise alone or in combination with GTE supplementation could attenuate dystrophic disease progression in maturing mdx male mice, a common mouse model of DMD.

Specific Aims

1. To determine if male mdx mice age 21 days can tolerate low load endurance exercise. A variety of methods will be used to assess beneficial/detrimental adaptations including: contractile/mechanical properties, contractile protein content, serum creatine kinase levels, lipid peroxidation, antioxidant capacity, and metabolic parameters.
2. To determine if GTE supplementation alone can benefit dystrophic muscle by attenuating oxidative stress. A variety of methods will be used to assess beneficial/detrimental adaptations including similar approaches to those listed above.
3. To determine if low load endurance exercise in combination with GTE supplementation will have a synergistic beneficial effect on dystrophic muscle.

Main Hypotheses:

1. Exercise training will have positive effects on running endurance, contractile/mechanical properties of the EDL, contractile protein content, serum

creatine kinase levels, lipid peroxidation, serum antioxidant capacity, and metabolic activities.

2. GTE supplementation will have positive effects on running endurance, contractile/mechanical properties of the EDL, contractile protein content, serum creatine kinase levels, lipid peroxidation, serum antioxidant capacity, and metabolic activities.
3. Both exercise training and GTE supplementation will be better than the no exercise training and no GTE supplementation.
4. Endurance exercise in combination with GTE supplementation will be more beneficial than endurance exercise or a diet of GTE alone.

Specific Hypotheses:

H_{01}: Low load endurance exercise (i.e., voluntary wheel running) will improve the overall running capacity of mdx mice. At least a 3-fold increase in daily running distance after 3 weeks of training is predicted.

H_{02}: Low load endurance exercise will improve dystrophic skeletal muscle function. Improvements in contractile properties (e.g., stress generation), in mechanical properties (e.g., stiffness), and decreased fatigability are predicted.

H_{03}: Low load endurance exercise will increase the expression of contractile proteins (i.e., actin and myosin) in dystrophic muscle.

H_{04}: Low load endurance exercise will decrease dystrophic gastrocnemius lipid peroxidation and serum creatine kinase levels.

H_{05}: Low load endurance exercise will increase serum antioxidant potential as assayed by an antioxidant capacity kit (Cayman Chemical).

H_{06}: A diet supplemented with the antioxidant green tea extract will improve running distance in the mdx mice after a 3 week training period.

H_{07}: A diet supplemented with the antioxidant green tea extract will improve dystrophic skeletal muscle function. Improvements in contractile properties (e.g., stress generation), no change in mechanical stiffness, and improvement in fatigability are predicted.

H_{08}: A diet supplemented with the antioxidant green tea extract will increase the content of contractile proteins (i.e., actin and myosin) in dystrophic muscle.

H_{09}: A diet supplemented with the antioxidant green tea extract will decrease dystrophic gastrocnemius lipid peroxidation and serum creatine kinase levels.

H_{10}**:** A diet supplemented with the antioxidant green tea extract will increase serum antioxidant potential as assayed by an antioxidant capacity kit (Cayman Chemical).

H_{11}: Combined low load endurance exercise and GTE in the diet will improve dystrophic skeletal muscle function (as described in Hypotheses 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10) better than either the exercise or diet treatment alone.

Chapter 2 Review of Literature

Review of Literature

Brief History of Duchenne Muscular Dystrophy

Duchenne muscular dystrophy was first observed in Italy circa 1836, but was not examined for its pathology until 1847. In 1836, two physicians, Conte and Gioja, reported two brothers aged 8-10 years exhibited progressive muscle weakness and associated hypertrophy of the calves, deltoids, and heart. They speculated the hypertrophy was the result of a change in the "nutritional process" of the muscle, but were unable to perform an autopsy to observe this and the pathology of the disease remained undefined [39].

Richard Partridge was the first to conduct a physical exam of a DMD patient in 1847; however, Edward Meryon is credited with the first pathological description of DMD in 1851 [178]. Partridge, reported a young boy's muscle weakness began in the proximal muscles of the legs and arms around age 2 years that was followed by muscle enlargement. The 14 year old male patient had a brother who was also affected with the disease but two sisters who were not. Meryon's autopsy report on the same patient noted that "all the voluntary muscles were wasted, retaining a fibrous appearance, but were flabby and of a yellowish colour." Meryon's full report submitted in 1851 contained the pathology of the disease as observed in three families with affected members. In all cases, women in the family were spared from the disease. The affected boys were noted as walking "heavy" and having impaired jumping ability as early as age 2 years. The ability to walk and stand in most cases was lost between age of 8-10 years and many patients died as young men due to a variety of respiratory illnesses. Through observations made during autopsies, Meryon noted abnormal spinal curvatures and rigid knee and hip-joints. He also described the replacement of muscle tissue with adipose tissue as the "breaking up of the sarcolemma." Partridge and Meryon were not initially given credit for their contributions to DMD. Partridge's brief description was not sufficient at the time to warrant a diagnosis and Meryon's observations were later challenged by Guillaume Duchenne as being cases of progressive muscle atrophy and not pseudohypertrophic muscular paralysis, which is why the disease ultimately became known as Duchenne's muscular dystrophy [178].

Duchenne, like Meryon, studied many cases of muscular dystrophy. Duchenne used a histologic harpoon on living patients to obtain muscle fibers and examine their pathological

condition. He observed increased volume of muscle fibers accompanied by increased "non-muscle tissue" that appeared to be connective or fibrotic. Duchenne later went on to publish an extensive account of the disease noting signs, symptoms, and possible intellectual impairment. Duchenne also noted the disease's prevalence in boys and occurrence in children within the same family [178].

Clinical Descriptions

Signs of DMD are evident by age 2 years and include delayed onset of walking, difficulty in performing a standing jump, difficulty in rising from the floor as demonstrated by use of the Gower's maneuver (i.e., maneuver used to rise from the floor to a standing position; utilizes the hands walking up the floor and legs to help extend the torso), and enlarged calf muscles. Symptoms of the disease, muscle weakness and wasting, first affect muscles of the hips, thighs, and shoulders, and in later stages, the heart and diaphragm. Most DMD patients are wheelchair bound by the age of 12 [167]. As DMD patients age, their respiratory muscles progressively deteriorate leading to an inability to generate proper inspiratory and expiratory forces, and death usually occurs due to respiratory or cardiac failure by the mid 20's [114,77].

A hallmark for diagnosing DMD is increased serum creatine kinase levels (~150 U/L Control vs. ~3000 U/L DMD). The method was developed by Okinaka [137] originally for assessing patients with neuromuscular diseases in general, but then was applied specifically to DMD by Emery [55].

In the past 20 years, researchers have identified the genetic defect responsible for DMD and localized it to band Xp21 on the human X chromosome [121,87]. Dystrophin was later identified as the protein product of the gene located at Xp21 and was shown to be greatly diminished (<3%) in muscle from DMD patients [87].

Dystrophin

Dystrophin is a 427 kd protein located on the inner surface of the muscle fiber sarcolemma and is thought to be involved in cell membrane stabilization via its interaction with the dystrophin glycoprotein complex [88,184]. Dystrophin is the largest gene identified in the human genome [3,108]. It's gene is 79 exons spanning 2.6 million base pairs and is transcribed to a

14 kb mRNA product [108]. Dystrophin's large size makes it very susceptible to mutations. These mutations are typically deletions (60%) of one or more exons that can prematurely abort dystrophin synthesis by disrupting the reading frame [100]. Mutations that do not completely disrupt the reading frame generate a phenotype with partially functional dystrophin known as Becker muscular dystrophy [100].

The dystrophin gene has 7 different tissue-specific promoters for 7 different protein isoforms (i.e., 2 brain, muscle, purkinje, retina, schwann, and general). The remainder of the Literature Review will report only on the effects of absent dystrophin in skeletal muscle. The cytoskeletal dystrophin protein consists of 3,685 amino acids [3,108]. It can be found in all 3 muscle types (skeletal, cardiac, and smooth), and in skeletal muscle can be found in all fiber types, making up approximately 5 percent of the surface membrane-associated cytoskeleton [106]. The dystrophin protein (Fig. 1) is made up of four domains: (1) the N-terminal is 240 amino acids in length and connects to F-actin of the cytoskeleton; (2) the central rod domain consists of 24 homologous spectrin-like repeats, each about 109 amino acids in length; (3) the cysteine rich region interacts with beta-dystroglycan and the sarcolemma; (4) the C-terminal is alpha-helical and interacts with alpha and beta syntrophins of the dystrophin-glycoprotein complex [99,58].

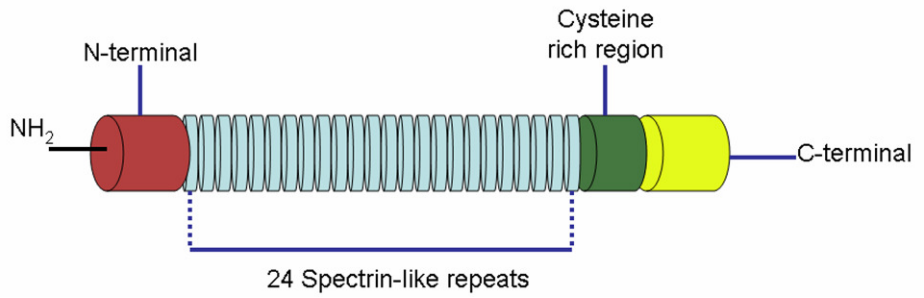

Fig. 1 - Dystrophin protein. The full length dystrophin is comprised of 4 different structural domains. The N-terminal (red), the central rod domain (blue), the cysteine rich region (green), and the C-terminal (yellow).

Dystrophin Glycoprotein Complex

Dystrophin is normally expressed in skeletal muscle in association with a multimolecular network of integral and subsarcolemmal proteins collectively known as the dystrophin glycoprotein complex (DGC) [57]. The DGC and additional proteins form costameres, which are rib-like lattices along the cytoplasmic face of the sarcolemma that facilitate even distribution of contractile forces laterally across the myofibril [45,57].

The DGC is comprised of a number of integral and subsarcolemmal proteins including: dystrobrevins, dystroglycans, sarcoglycans, and syntrophins [138]. Dystrobrevins are a family of proteins with C-terminal homology to dystrophin [12]. Only α-dystrobrevin is highly expressed in skeletal muscle where its C-terminal binds to the C-terminal of dystrophin in a coiled relationship [159]. Though α-dystrobrevin deficiency in humans does not cause disease, it does produce a mild muscular dystrophy in knockout mice [72]. Because of α-dystrobrevin's association with the sarcoglycans and syntrophins, its primary function is considered to be signal transduction.

The 2 dystroglycans (α and β) form an integral/transmembrane complex that create a link between the extracellular matrix and dystrophin, and dystrophin and the sarcoglycan complex [82,94]. The integral α-dystroglycan has a laminin-2 receptor and functions as a cellular proliferative and survival promoter via its signaling activation of the Ras/MAPK pathway [89]. The transmembrane β-dystroglycan binds to the cysteine-rich region of dystrophin and facilitates intracellular signaling cascades. In the absence of the dystroglycan complex in knockout mice there is a substantial loss in organization of the extracellular matrix around the DGC [191,188].

The sarcoglycan complex is comprised of 4 transmembrane proteins (α, β, γ, δ) that collectively link many integral and subsarcolemmal proteins of the DGC [57]. Both and β and δ-sarcoglycan make up the core of this complex and are thought to stabilize the DGC components at the sarcolemma [27]. In contrast, α and γ-sarcoglycan are considered to play an important role in cell adhesion signaling [193]. The absence of various components of the sarcoglycan complex yields various limb-girdle muscular dystrophies [139].

Two syntrophins (α1 and β1) are highly expressed in skeletal muscle and span the subsarcolemmal region [1,4]. One of the most important pathophysiological properties of the

syntrophins is their association with neuronal nitric oxide synthase to localize this important signaling molecule at the sarcolemma. Additionally, syntrophins associate with voltage-gated ion channels, suggesting interactions with additional signal pathways. Deficiencies of syntrophins at the sarcolemma lead to truncated signaling transduction [16,102].

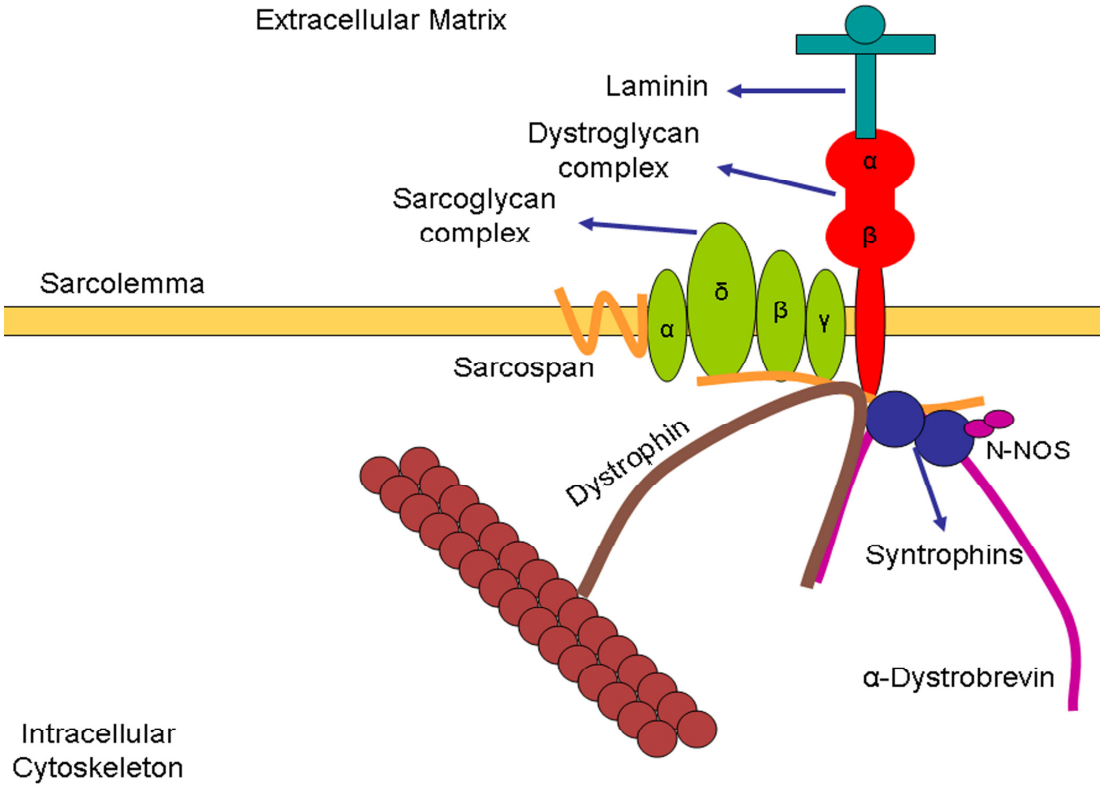

Fig. 2 Dystrophin glycoprotein complex with currently understood relationship of the components [150].

The N-terminal of dystrophin is connected to the intracellular cytoskeleton and the cysteine rich region and C-terminal are bound to the DGC via β-dystroglycan and α-dystrobrevin, respectively. Thus, dystrophin via the DGC spans the sarcolemma to create a physical link between the intracellular cytoskeleton and the extracellular matrix. Studies have shown in the absence of dystrophin, the DGC fails to properly aggregate at the sarcolemma [57], and the link is compromised. It is postulated that this loss of the DGC decreases muscle fiber

integrity and muscle cells lacking the DGC are susceptible to contraction-induced injury leading to cycles of degeneration and regeneration resulting in cellular necrosis, muscle wasting and replacement by adipose tissue [140,167]. Two potential outcomes leading to disease onset and progression are (1) the sarcolemmal membrane is mechanically weaker and/or (2) signaling via the DGC is disrupted. At present, the precise mechanisms of muscle pathophysiology in DMD remain elusive.

Animal Models of Muscular Dystrophy

The discovery of the dystrophin gene in 1987 [87] made it possible to identify animal models with genetic similarities. Two of the most popular animal models for DMD are the Golden Retriever muscular dystrophy (GRMD) dog and the mdx mouse. The GRMD dog mimics the pathology of the disease better than any other animal model [40]. Onset of the disease is prenatal with lesions being identified *in utero* and muscle necrosis evident at birth [135]. Muscle pathology is most pronounced between ages 2-30 days, with initiation of fibrosis occurring during this period up to age 60 days [43]. The GRMD dog has elevated plasma creatine kinase levels and abnormal utrophin expression, experiences joint contractures, and typically dies young from cardiac or respiratory failure [135].

The mdx mouse is the most widely used animal model for DMD research. Discovered by Bulfield [23] the mdx mouse has a single point mutation of the dystrophin gene (i.e., exon 23) subsequently truncating the protein product. Though the mdx mouse is a genetic homologue of the human disease, it demonstrates a milder pathology and muscle function is less compromised. The skeletal muscles of mdx mice undergo a brief period of cycles of muscle degeneration and regeneration, yet the muscle degeneration stabilizes and the mice live a normal lifespan of ~2-2.5 years [38].

In the mdx mouse, the onset of the disease begins around age 2 weeks, with cycles of degeneration and regeneration peaking between age 3-4 weeks, and muscle pathology less pronounced after 8 weeks. During the time of evident muscle wasting (i.e., age 3-4 weeks), the concentration of creatine kinase in the blood is increased (i.e., ~300 U/L to ~3000 U/L) and myofiber regeneration is evident by centralized nuclei [112]. Muscle pathology is much milder for

the remainder of the animal's life though much older (i.e., 12 months) mdx mice do suffer from severe muscle wasting of the diaphragm [124].

The mdx/utrn$^{-/-}$, dystrophin and utrophin deficient mouse, presents an alternative model for DMD because of its severe phenotype. The mdx mouse has a partial replacement of the dystrophin protein by the homologous utrophin protein, which can ameliorate dystrophic pathology by partially restoring function [158]. The mdx/utrn$^{-/-}$ mouse experiences stunted growth, joint contractures, spinal curvature, reduced ability to produce force, and premature death [71]. The disadvantage of the mdx/utrn$^{-/-}$ mouse is the difficulties with breeding. Since mdx/utrn$^{-/-}$ mice are infertile they must be produced by parents that are mdx/utrn$^{+/-}$. This requires DNA screening for utrophin knockouts. There are typically only 1-2 mdx:utrn-/- pups per litter, so obtaining adequate numbers for studies can be difficult.

Potential Mechanisms that initiate Duchenne Muscular Dystrophy

Though the precise role of dystrophin is not presently known, it is hypothesized that dystrophin is involved in a number of mechanisms of muscle injury in DMD. The most consistent hypothesis is that dystrophin stabilizes the DGC and provides mechanical support to the cell membrane protecting against damage that can occur during muscle contractions, especially those that are eccentric [144]. Another possibility is that dystrophin could act as a mechano-transducer transmitting signals across the membrane in an outside to inside fashion, which would potentially activate muscle gene expression [20]. Dystrophin has also been linked to organizing other membrane-associated proteins such as nitric oxide synthase [15].

Mechanical

Several studies have shown the absence of the DGC compromises the integrity of the muscle membrane and reduces the muscle's ability to sustain repeated contractions [119,144]. This is believed to be the result of microlesions (small tears in the sarcolemma) that worsen with repeated contractions and inevitably lead to muscle cell death or necrosis via increased intracellular Ca^{2+} concentration and calpain activation (described below). Dystrophin was first thought to be involved in membrane stabilization when blood samples from DMD patients were found to have elevated concentrations of creatine kinase (~3000 U/L) [55]. It was thought that the

absence of dystrophin weakened the membrane of the muscle cell and allowed the intracellular molecule creatine kinase to pass into the blood stream. To test this possibility Petrof [144] subjected dystrophic mouse muscles to 4 protocols: (1) a series of eccentric stretches, (2) a series of maximal isometric contractions, (3) a series of passive lengthening contractions, and (4) a series of submaximal, isometric contractions. For all conditions, a contralateral muscle was kept at resting tension as a control, and both experimental and control muscles were maintained in an oxygenated bath with Ringer's solution and 0.2% procion orange, a fluorescent dye that passed through tears in the membrane, but was otherwise excluded. In the resting and all 4 experimental conditions, dystrophic muscles had greater dye uptake than muscles from wildtype mice. It was concluded that dystrophin is essential to stabilizing the sarcolemma and preventing tears. Therefore, leakage of creatine kinase into the blood stream may be attributed to microlesions in the sarcolemma. This supports the idea that dystrophin helps stabilize the sarcolemma and its absence possibly leads to tiny membrane tears.

The idea that the loss of dystrophin and the DGC renders the sarcolemma susceptible to mechanical injury may be age-dependent. Based on studies in vitro, muscles of older mdx mice are susceptible to contraction-induced injury (ages ~100 days) [144], but muscles of maturing mdx mice appear less susceptible (e.g., age 9-12 days; [75]). Grange [75] assessed the effects of a stretch protocol in vitro on the EDL muscles of pups aged 9-12 days from C57 control, mdx, and mdx/utrn$^{-/-}$ mice. Muscles subjected to a stretch had no increases in dye uptake within each genotype, and there was no change in stiffness throughout the stretch protocol, suggesting young dystrophic muscle may be able to handle mechanical stress better than old dystrophic muscle. Therefore, dystrophin's effect on the mechanical stability of the sarcolemma may be age-dependent. Wolff et al. [190] helped support this idea with the assessment of membrane stiffness values (**Δ**Force/**Δ**Length) of C57 control and mdx mice. Both passive (Wolff et al., 2006) and active (unpublished) stiffness values were similar between sedentary mdx and wild type mice at ages 14-35-days. Decreased membrane stability and increased susceptibility to mechanical induced injury may still play a role in disease progression, but this may not be the mechanism responsible for disease onset. An alternative pathogenic mechanism is aberrant signaling.

Signaling

In a normal muscle cell, dystrophin and the DGC are responsible for relaying external mechanical signals inside the cell, leading to signaling cascades that regulate gene and protein expression (mechanotransduction). De Bari [46] reported muscle from mdx mice injected with human synovial membrane-derived mesenchymal stem cells (hSM-MSCs) restored dystrophin at the sarcolemma, decreased numbers of centralized nuclei in the dystrophin positive myofibers, and mechanogrowth factor (MGF) was expressed. MGF is responsible for the replenishment of satellite cells in skeletal muscle and is absent in dystrophic mdx muscle [70]. The restoration of dystrophin with the injection of hSM-MSCs and the subsequent rescuing of MGF expression demonstrated the importance of dystrophin and the DGC in regulating cellular signaling.

Signaling molecules, such as nitric oxide (NO) and Ca^{2+}, are associated with the DGC and play an important role in cell survival. NO is produced by the DGC associated (via syntrophins) neuronal nitric oxide synthase (nNOS). The signaling potential of NO was first discovered when it was found to induce smooth muscle relaxation or vasodilation [90]. Additionally, NO serves as both a potential antioxidant and a potential free radical. In combination with oxidized lipids (lipid peroxyl radicals), NO can act as an antioxidant that prevents the chain reaction of lipid peroxidation; however, when combined with superoxide it can form the harmful free radical, peroxynitrite [154,189]. Therefore, the effects of the presence or absence of NO in the pathophysiology of DMD are interpretive.

Ca^{2+} is another potential signaling ion. Ca^{2+} is unique in that it mimics the size and coordination numbers of sodium and potassium, ions that readily pass through the cell membrane [44]. The charge-to-size ratio of Ca^{2+} and its flexible geometry allow it to selectively bind to multiple ligands at once [166]. The high kinetic rate of Ca^{2+} (i.e., water exchange rate 10^9/sec) ensure a fast response time, adding to Ca^{2+}'s signaling capabilities.

In dystrophic-deficient muscle the disruption of the DGC can lead to the upregulation of various signaling molecules (e.g., calcineurin and p38 MapK) [70]. This suggests that dystrophic-deficient muscle compensates for the loss of dystrophin and the DGC by upregulation of other proteins that play a part in mechanotransduction. The extent to which gene regulation plays a role in the dystrophic pathology has yet to be thoroughly explored; however, a recent studied

revealed that many genes responsible for muscle development and extracellular matrix remodeling were upregulated in DMD boys ages 1.5 – 24 months compared to age match controls [143].

Calcium

Ca^{2+} plays a very important role in skeletal muscle function acting as a primary and secondary messenger. The extracellular fluid surrounding the muscle cell has a concentration of Ca^{2+} of ~1 mM, which far exceeds the ~50 nM concentration in the cytosol of a resting muscle fiber [11]. This chemical gradient across the cell membrane is maintained by low permeability of Ca^{2+} and the expenditure of ATP to actively remove Ca^{2+} by Ca^{2+}ATPases located in the sarcoplasmic reticulum and sarcolemma [25]. During skeletal muscle stimulation, the cytosolic concentration of Ca^{2+} can increase 100-fold, but is short lived. Following stimulation cessation Ca^{2+} is rapidly sequestered by the sarcoplasmic reticulum. This reduces the binding of Ca^{2+} to troponin C, allowing tropomyosin to inhibit myosin head/actin binding, and facilitating muscle relaxation [68]. If Ca^{2+} is not properly scavenged from the cytosol it can activate mechanisms of muscle damage.

Calcium can be improperly scavenged from the cytosol three different ways: (1) improper function of the Ca^{2+}ATPases; (2) deficiency in Ca^{2+} binding proteins, and (3) increased permeability of the sarcolemma (e.g., damage to the membrane) [69]. One consequence of Ca^{2+} overload is the activation of Ca^{2+} activated proteases called calpains. Calpains are heterodimeric, calcium dependent cysteine proteases expressed ubiquitously [125]. Under physiological conditions calpains are not membrane bound but become so with increases in intracellular calcium concentration [120] resulting in the destruction of membrane constituents. Calcium concentrations for activating these calpains range from 0.5 μM to 400 μM. Calpain cleavage of various proteins is thought to contribute to changes in muscle function. Calpains have also been associated with the signaling of programmed cell death, or apoptosis [176].

Because creatine kinase can leak across the membrane to the extracellular fluid in dystrophin-deficient muscle, it is thought that Ca^{2+} can leak into the cytosol. For this reason, the role of calpains in DMD has been explored. In both DMD humans and mdx mice, muscle cells demonstrate increased cytosolic concentrations of Ca^{2+} at rest compared to non-diseased controls

[64]. In the mdx mouse specifically, calpain concentration is increased at times of cellular necrosis [171]. Calpains may play a role in the pathology of muscular dystrophy by cleaving proteins that subsequently change muscle function or by activating apoptotic pathways.

The Ca^{2+} hypothesis states that in the absence of dystrophin, either through cation-leak channels or stretch-activated channels, there is irregular Ca^{2+} influx [186,181]. Eventually the cell's capacity to maintain physiological levels of calcium is overridden and calpains are activated. As calpains cleave membrane constituents, membrane permeability is compromised and calcium influx worsens and leads to cell death. Although this theory has been demonstrated in older mdx mice, young mdx mice, age 14 days, do not appear to be affected, because cytosolic Ca^{2+} concentrations are similar to that of muscles from age-matched control mice [112].

As with the mechanical hypothesis, aberrant Ca^{2+} influx has been questioned as the primary event for disease onset because Ca^{2+} overload occurs too late (i.e., age 3-4 weeks) to initiate muscle fiber degeneration [155]. Once degeneration has been initiated, uptake of external calcium has been observed [112] suggesting Ca^{2+} mediated pathogenesis may be a secondary event. Still, the mechanism of disease onset in DMD in unclear, and discerning aberrant signaling and Ca^{2+} influx as primary or secondary events in the onset and progression of DMD is difficult.

Oxidative Stress

Key Players

Oxidative stress is the accumulation of destructive reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, and the hydroxyl radical (i.e., $O_2\bullet^-$, H_20_2, $OH\bullet$, respectively). When oxidative stress is severe and/or prolonged, antioxidants, the body's natural defense system, can be overwhelmed, leading to oxidative damage of lipids, proteins, and DNA. Oxidative stress can be initiated by the increased production of ROS by contracting skeletal muscles, increased Ca^{2+} concentration in the mitochondria, and infiltration of macrophages during inflammation. There are three important enzymes responsible for alleviating the effects of ROS: (1) superoxide dismutase, (2) glutathione peroxidase, and (3) catalase. The relationship between these enzymes, ROS, and NO are represented in Fig. 3.

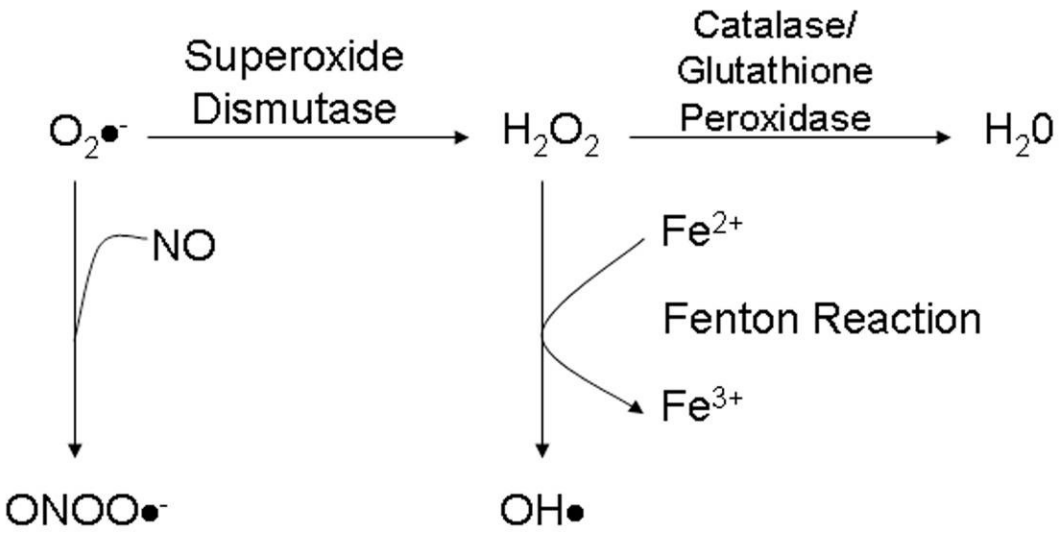

Fig. 3 Pathways of ROS

Mechanism of Cell Injury

ROS can readily oxidize proteins, lipids, nucleic acids, and carbohydrates. Polyunsaturated fatty acids (PUFAs), which help form plasmalemmas, are characterized by the presence of one or more CH_2 groups between double bonds (Fig. 4), and are preferentially attacked by ROS [129]. The hydrogens of the CH_2 group are double allylic-activated and can be easily removed by ROS, initiating a chain reaction that leads to the production of peroxydienyl radicals [172]. Peroxydienyl radicals can remove hydrogens from surrounding PUFAs, other phospholipids, sugars, and proteins. If this process is left unchecked it can lead to the degradation of biomolecules.

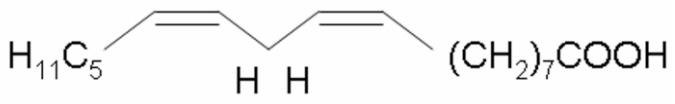

Fig. 4 Fisher projection of PUFA

Oxidative Stress and DMD Pathophysiology

The increased level of oxidative stress observed in mdx mice before disease onset may indicate that oxidative stress is a potential mechanism for DMD [49]. Disatnik et al. [50] cultured myotubes from different mouse models expressing various lengths of the dystrophin protein (0 kD to full-length 427 kD). Myotubes were then placed in a medium inducing lipid peroxidation (i.e., paraquat which is oxidized to generate $O_2\bullet^-$ and reacts with PUFAs). Disatnik et al. [50] observed that myotubes expressing no dystrophin had increased susceptibility to oxidative stress, while myotubes expressing the full length dystrophin had decreased cell death. This outcome suggested that in the complete absence of dystrophin, oxidative stress will be maximal. Oxidative stress can be perpetuated in dystrophic mice by, increased metabolism and motor movement associated with maturation (natal to pup), increased intracellular Ca^{2+} concentrations (~age 3 weeks), and decreased key protein antioxidants (e.g., glutathione peroxidase) [152,148].

Once muscle wasting is initiated, an inflammatory response follows. During this response, macrophages infiltrate the affected area and produce hydroxyl radicals ($OH\bullet$), which can initiate oxidative damage of proteins, lipids and DNA. The increased production of ROS is not the only contributing factor to increased oxidative stress. Rando [152] determined that mdx mice have a deficiency in the key antioxidant protein, cytosolic superoxide dismutase (~80% reduction in diaphragm of mdx compared to control). This protein is responsible for converting superoxide ($O_2\bullet^-$) into the less harmful H_2O_2. In superoxide dismutase's absence, $O_2\bullet^-$ readily combines with nitric oxide (NO) to form peroxynitrite ($ONOO\bullet^-$), one of the most reactive species in biological systems. Peroxynitrite, superoxide, and the hydroxyl radical are the primary ROS contributing to the oxidative stress in DMD.

A hallmark of DMD is the preferential destruction and sparring of different muscle fiber types. In both human and animal studies fast-glycolytic fibers appear to be more susceptible to muscle injury than slow-oxidative fibers [92,117]. This seems contradictive to the oxidative stress theory because it might by postulated that slow-oxidative fibers would be more affected due to their oxidative metabolism. This is not the case however, as it has been shown that slow-oxidative fibers compensate for oxidative stress with higher antioxidant capacities through the increased production of antioxidants (e.g., glutathione peroxidase) [160,9]. Because antioxidant

levels in normal and dystrophic fast-glycolytic fibers are depressed [10,152], they may have greater susceptibility to muscle injury.

Once oxidative stress is initiated, it can cause irreversible damage to mitochondrial and cellular DNA which compromises the cell's ability to balance protein synthesis and degradation. Processes of oxidative stress may lead to the degradation of key muscle proteins (i.e., actin, myosin) that initiate an inflammatory response. These processes may also oxidize lipids in the muscle fiber plasma membrane, compromising its integrity and leading to lesions through which normally regulated intracellular molecules diffuse (e.g., creatine kinase) [152].

In summary, the current evidence suggests that ROS contribute to the pathogenesis of DMD (Fig. 5). Though the mechanical hypothesis does not appear to cause disease onset, it still likely contributes to disease progression. The precise role of dystrophin in cell signaling is not presently known. However, its absence suggests that it may play a role in both cellular Ca^{2+} regulation and oxidative stress.

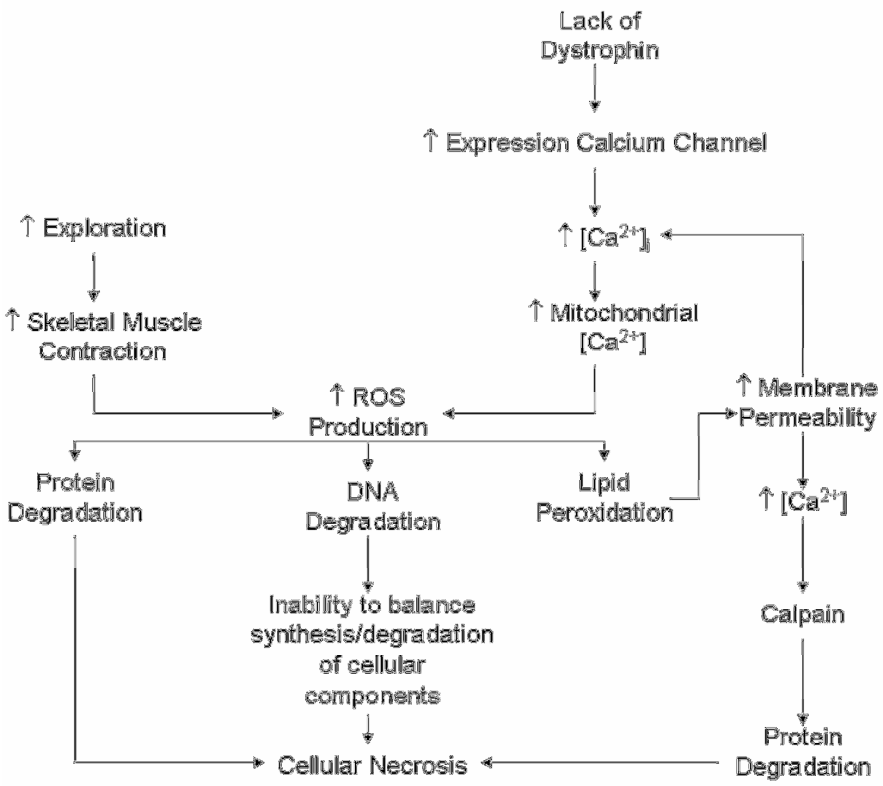

Fig. 5 Hypothesized mechanism of disease onset. ↑ = Increase; $[Ca^{2+}]_i$ = free intracellular calcium concentration; ROS = reactive oxygen species.

Ischemia

Ischemia refers to the condition of restricted blood flow to a certain area of the body. Skeletal muscle relies heavily on blood flow to deliver O_2 for proper function (i.e., oxidation of NADH by O_2 for sufficient ATP production). During extended periods of ischemia skeletal muscle can become hypoxic and if blood flow is cut off completely (anoxic), leading to cell death or necrosis. Sometimes the reintroduction of blood flow to an ischemic area, a condition known as reperfusion, can be just as damaging due to the increased production of reactive oxygen species (ROS) [116].

NO is an important signaling molecule produced by nitric oxide synthase (NOS), specifically endothelial NOS (eNOS) and neuronal NOS (nNOS). NO can effect skeletal muscle contraction [98], Ca^{2+} handling [59], and glucose metabolism [157]. Additionally, eNOS and nNOS are co-contributors to NO mediated vasodilation with eNOS generated NO regulating overall vasodilation and nNOS generated NO regulating vascular relaxation during skeletal muscle contraction [74]. nNOS is highly expressed in normal skeletal muscle [131] and is localized at the sarcolemma. Because of this it was suggested that nNOS was associated with the DGC [15]. Though nNOS does not directly bind to dystrophin (binds to α1 and β1-syntrophins), nNOS localization at the sarcolemma was depressed in dystrophin deficient muscle [28,15] leading to the hypothesis that dystrophic muscle may experience an ischemic effect during times of muscle contraction.

In one study ischemia and reperfusion induced in mdx mice increased oxidation of proteins and lipids, increased damage to the sarcolemma, and decreased force output by the tibialis anterior [52]. Sander [161] reported that nNOS deficiency in human DMD patients also results in muscle ischemia. Thirteen healthy and ten DMD boys, age 7 – 15 years, were tested for forearm vascular conductance and tissue oxygen delivery during handgrip exercises while exposed to intermittent lower body negative pressure (LBNP) in the supine position. As expected, healthy boys performed better on the handgrip strength test, and forearm contractions lead to NO-induced alleviation of vasoconstriction. In contrast, there was no attenuation in LBNP induced, decreased muscle oxygenation with handgrip in DMD boys, therefore showing that NO mediated vasodilation is aberrant in dystrophin-deficient muscle from DMD boys, and as a result muscle ischemia persists.

Because NOS knockout mice have no pathology of muscle degeneration [29] and nNOS localization is not strictly dependent on DGC formation, NO's role in ischemia in DMD is interpretive. One interpretation is the two-hit hypothesis [151]. The two-hit hypothesis states that the deficiencies in NO lead to an ischemic condition (hit 1), which is exacerbated by the vulnerability of the sarcolemma to mechanical damage (hit 2) in dystrophin deficient mice. Whereas normal skeletal muscle would undergo recovery, dystrophic muscle undergoes necrosis.

In summary, the mechanism of disease onset is still not clearly defined. Mechanical damage, aberrant cell signaling, insufficient Ca^{2+} handling, oxidative stress, and improper NO regulation may all play a role in disease onset and progression. It is most likely that no one of these is exclusively responsible and that they probably interact with one another to some degree. Strategies to reduce the deleterious effects of these mechanisms could be effective therapeutic approaches.

Exercise

Prescribed exercise is defined physical activity to improve skeletal muscle and cardiovascular physiological function. The 3 principles for exercise prescription are duration, intensity, and frequency. Responses to exercise can be either acute or chronic. Acute responses refer to the response observed after a single bout of exercise. Chronic responses refer to the adaptations observed after extended periods of exercise training. Although desired health benefits result from regular exercise training, they are ultimately the result of micro-adaptations and overshoot transcription (RNA) after several single bouts of exercise [63].

Adaptations to Exercise

Skeletal muscle adaptations are specific to the type of exercise. Prolonged low-load, repetitive exercise can elicit a differentiation of fibers toward more fatigue-resistant phenotypes. Conversely, short bouts of high-loads can provoke a hypertrophic effect. In cycling tests for 30 minutes at aerobic threshold (when active muscle transitions from fatty acid to glycogen metabolism for ATP production), acute effects included increased mRNA levels of genes associated with beta-oxidation, fatty acid transport, and respiration [62]. Compared to pre-exercise levels, these effects increased ~4 fold at 8 hours post exercise. These acute adaptations in gene

expression after each exercise bout accumulate, eliciting structural-functional muscle adjustments, such as increased fatty acid metabolism for energy. Typical chronic adjustments in protein content are observed around 6 weeks of training [63].

It is generally accepted that responses during and after exercise lead to the activation of signaling pathways during exercise recovery that upregulate gene transcription [134]. Factors contributing to local and systemic activation of these signaling pathways in response to exercise include: (1) blood flow and substrate delivery; (2) muscle contraction; (3) energy depletion; and (4) pH and oxygen flux [79]. Activated signaling pathways include and are not limited to protein kinase pathways such as mitogen-activated protein kinase (MAPK) pathway and AMP-activated protein kinase (AMPK) pathway. These kinases are important intracellular messengers central to the upregulation of gene expression following exercise [84]. The post-exercise activation of these pathways lead to overshoot RNA transcription and subsequent mRNA translation [63]. Ultimately, the accumulation of these responses with regular bouts of exercise leads to adaptations in muscle metabolism (e.g., increased mitochondrial content) and contractile activity (e.g., increased MHC).

Exercise and Gender Differences in Mice

It has been suggested that gender may affect physical activity levels in mice [142]. Perrigo et al. [142] reported that female house mice exhibit more locomotor activity and consume more food than males. Koteja [103] compared selectively bred mice for high voluntary wheel running to random bred control lines. Koteja reported selective bred mice ran 70% greater distances than control mice, and that within all lines, females ran more than males (11.6 km vs. 4.4 km). Additionally, it was reported that the heavier male mice became the less they would run while the heavier female mice became the more they would run. Lightfoot [107] tested the genetic and gender variability in daily wheel running. Lightfoot [107] tested male and female A/J, AKR/J, BALB/cJ, C3H/HeJ, C57Bl/6J, C57L/J, C3He/FeJ, CBA/J, DBA/2J, SWR/J, MRL/MpJ, SPRET/Ei, and CAST/Ei mice for daily distance, daily running duration, and average running speed for 3 weeks. At the conclusion of the study C57L/J mice, independent of gender, averaged the greatest daily distance (7.96 km), for all 13 strains female mice averaged equal or greater daily distances (C57Bl/6: female 5.5 km/day vs. male 3.5 km/day), and for all 13 strains female

mice averaged equal or greater running velocities. It has been shown in female rats that voluntary running is at its peak during proestrus and at its lowest at metestrus, suggesting hormones may play a role in differences between genders [6].

Exercise and Duchenne Muscular Dystrophy

DMD patients suffer degrees of muscle wasting. One therapeutic suggestion has been exercise. Maintaining the ability to walk, participate in daily activities, and self-care are reasons exercise is considered beneficial for DMD patients. Since many patients die from respiratory failure, it would be helpful to find ways to strengthen the diaphragm. Therapies are desired to elicit adaptations in muscle to prevent muscle fatigue and maintain muscle function for longer durations. Therapies such as myoblast implantations and steroids have been used to increase muscle strength but because of their expense and adverse side effects, an alternative, less-expensive therapy such as exercise should be considered. However, exercise is in question because there is not a known amount to prescribe and overload must be avoided for an overall benefit [48].

Some of the current exercise practices include hydrotherapy, electronic stimulation, light aerobic training, light resistance training, respiratory training, stretching routines, and the use of orthoses such as ankle-foot-orthoses (AFOs) and knee-ankle-foot-orthoses (KAFOs) [54]. Exercises to be avoided are any that include eccentric contractions of the muscle such as walking down stairs, as these have been shown to accelerate the progression of the disease [145]. Many exercises focus on maintaining body symmetry because muscle imbalance is often observed in DMD. This is due to changes in muscle length, especially at weight bearing joints, which can lead to a loss of muscle force production [93]. These contractures, permanent tightening of the muscles (due to insufficient relaxation of the contractile apparatus) prevent movement especially in jointed areas, and are often a result of static posture such as sitting in a wheel chair for long periods of time with no change in position ultimately leading to a reduced range of motion [36]. Contractures can be lessened by the use of stretching routines and AFOs and KAFOs, which are splints applied at night to stretch the gastrocnemius-soleus complex and help maintain range of motion [170].

Few studies have directly assessed DMD patients, primarily due to lack of volunteers and ethical issues. However, there are some worth noting. In a 12 month home program where patients were asked to participate in high-resistance weight training 3-7 times a week, DMD boys experienced a non-significant decrease in strength while sedentary DMD boys experienced a significant decrease in strength [182]. In a separate 6 month study, where DMD boys were asked to perform 30 submaximal knee extensions (1 session) at a rate of 4-5 sessions a week, there was a non-significant strength improvement in all 4 DMD subjects [47]. Additional studies have shown that low frequency stimulation is not detrimental to dystrophic muscle [165] and that dystrophic muscle is capable of properly adapting to different exercise stressors (i.e., swimming: fiber type shift II to I; strength training: fiber type shift I to II) [65]. Overall, there is a lack of information available to determine the functional capabilities of dystrophic muscle making exercise prescription for DMD difficult [76].

Because the responses to exercise are not clearly defined for DMD, researchers conduct their studies first in dystrophic animal models (i.e., mdx mouse). The mdx mouse was first discovered by Bulfield et al. [23] and is used for experiments because it undergoes bouts of muscle degeneration and regeneration [17]. Exercise regimens include voluntary wheel running, involuntary treadmill running, and endurance swimming. Hayes [81] monitored voluntary wheel running started at age 4 weeks and continued to age 20 weeks. Average distance and speed were recorded for each week for both *mdx* and control mice. Following the training period, mice were sacrificed and the extensor digitorum longus (EDL) and soleus muscles were removed for isometric contractile tests. At the completion of these tests, the muscles were stained to identify fiber types (histochemical [18]). The mdx mouse ran shorter distances (29.8 km vs. 45.0 km, $P<0.05$) each week and had a slower average daily speed (1.62 km/h vs. 2.10 km/h). However, the mdx mouse spent a similar duration on the wheel as the control mouse suggesting that there was no difference in desire to run (18.1h vs. 21.3h). This suggests that the mdx mouse is a good dystrophy model to study for exercise because it has limited endurance capacity, yet does not appear to lack the motivation to run. Furthermore, histochemical results showed an increase in type I fibers in both the EDL and soleus muscle (+12.6% and +10.7%, $P<0.05$) of exercised mdx mice compared to sedentary mdx mice. This study demonstrated clear benefits of exercise on dystrophic muscle.

Dupont-Versteegden [53] also tested the effects of voluntary wheel running on mdx mice. Running was initiated at age 3 weeks and concluded at age 10 – 13 months. At sacrifice, the diaphragm and soleus muscles were removed and isometric contractile function was assessed. There was an observed increase in active tension (+30%), contraction time (+14%), and half relaxation time (+17%) in the diaphragm of exercised mdx mice compared to the sedentary mdx mice. The authors concluded that increased activity of the diaphragm, a highly affected muscle in DMD, was not deleterious and that endurance exercise might improve pulmonary function in DMD.

Carter [26] examined the effects of exercise on young (4 weeks) and old (6 months) mdx mice. Daily distance was recorded for 4 weeks on a running wheel and after that time the mice were sacrificed and EDL and soleus muscles were removed. Over the period of 4 weeks the young mdx mice increased their average daily distance (+150%) while the adult mdx did not. The tetanic tension in both the soleus and EDL muscle improved in the young mdx mice while the old mdx mice showed no improvements. The discrepancy between this study and the previous studies reporting exercised induced improvements in old mdx mice is the mouse age at which training was initiated (3-4 weeks [81,53] vs. 6 months [26]). These results suggest it is important to define a time point in which initiating exercise training will be most effective.

In summary, following voluntary wheel running there is evidence for increased resistance to fatigue based on: actual distance run by young mdx mice [26]; as well as in vitro stimulation of EDL and soleus muscle removed from exercised mdx mice [81,80]. Hayes reported shifts from type II fiber to type I fiber in exercised mdx mice. Dupont—Versteegden and Carter observed increases in active tension and tetanic tension, respectively. Overall, voluntary wheel running was beneficial because there was increased fatigue resistance in both fast and slow twitch muscles, as well as a shift to slow twitch muscle fibers. This would be considered beneficial because type II fibers have been shown to be more susceptible to injury then type I fibers [105].

Another method of exercise is involuntary treadmill running; however, in most instances involuntary treadmill running is used to exacerbate the dystrophic condition. This technique is often used to study the effects of eccentric contraction (muscle stretching while simultaneously being contracted) exercises, because the angle of the treadmill can be set to run downhill. Brussee [21] studied the effects of 2 month control and mdx aged mice running down hill (15°) at

10m/min for 10 minutes. After the third day of training the mice were sacrificed. Twenty-four hours prior to death, the mice were injected intravenously with Evans blue dye and after sacrifice, the limb muscles were removed, sectioned, and scanned for damage. There was a marked increase in damage in the extensor muscles in the exercised mdx mice. The authors concluded the eccentric running led to microlesions provoking muscle weakening and degeneration and regeneration of muscle fibers.

Vilquin [183] performed a similar study involving downhill running and mdx/beta-galactosidase transgenic mice. However, only the effect of one 5-minute exercise bout was examined. Creatine kinase and beta-galactosidase blood levels were measured before and after exercise and the results supported those of Brussee. There were increased levels of beta-galactosidase and creatine kinase in the blood following treadmill running suggesting that the exercise lead to microlesions in the membrane through which the intracellular molecules leaked out of. It was also noted that many mdx mice had trouble completing the 5-minute bout of exercise.

Fraysse [66] used treadmill running to observe membrane leakage after exercise. Four-week-old mdx and control mice were exercised on a horizontal treadmill for 30 minutes, twice a week, for 4-8 weeks. This non-eccentric exercise increased resting calcium concentration substantially in mdx mice compared to control mice. Membrane permeability also increased and the authors concluded that this was not a result of a damaged membrane but of leaky calcium channels. This led them to believe that dystrophin plays a substantial role in membrane channel regulation. They concluded that increases in calcium concentration leads to the progression of the DMD disease via calpains and that their study was the first to show that resting calcium concentration increased after exercise.

As expected, none of the studies in which involuntary treadmill running was used were there any benefits to the mdx mouse. Studies involving voluntary wheel running try to assess the technique as a possible therapy, whereas studies involving involuntary treadmill running purposefully try to exacerbate the dystrophic condition of the mdx mouse to study the elevated disease state. One of the major limitations of exercise research in DMD is the lack of homogeneity between the studies.

Green Tea

Green tea is the second most consumed beverage in the world [73]. Green tea has been intensely studied for its medicinal benefits which include antioxidant [130], anti-diabetic [111], anticarcinogenic [19], antibacterial [8], and antiatherogenic [118] effects. Green tea contains a large quantity of low molecular weight polyphenols, which have more antioxidant potential than vitamins C or E [156], and are thought to be responsible for these beneficial properties [83,179,41]. These polyphenols are powerful reducing agents whose structures promote oxidized state stabilization and therefore serve as antioxidants [179]. Unlike many other molecules, they can associate with ROS without being compromised. The four major polyphenols include gallocatechin (GC), epigallocatechin (EGC), epicatechin (EC), and epigallocatechin gallate (EGCG) (Fig. 6).

Green tea extract (GTE) refers to the hot water-soluble fraction of unfermented green tea leaves. Whereas polyphenols make up 13% of dry green tea, GTE's composition of polyphenols is 80% [177]. The antioxidant potential of polyphenols is related to their chemical structures, specifically the number of hydroxyl groups (-OH) attached to rings B and C [180]. These structures give green tea polyphenols an advantage over vitamins C and E in the ability to scavenge free radicals. In laboratory assays examining the effects of different antioxidants on the lipid peroxidation of rabbit erythrocytes, green tea polyphenols, especially EGCG, provided the most protection (85.8% reduction in lipid peroxidation compared to control) [149].

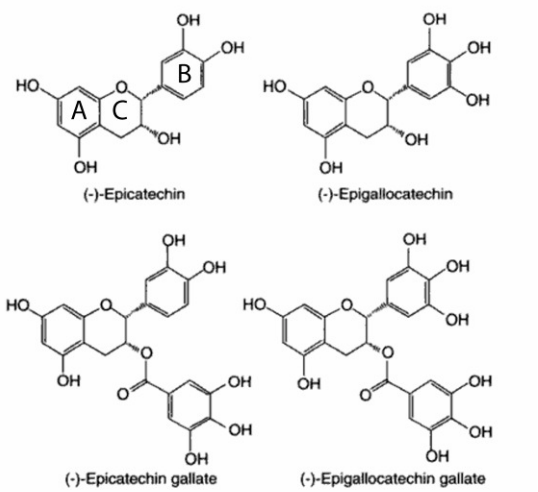

Fig. 6 Green tea polyphenols. From upper left clockwise: EC (with ring indentifications), EGC, EGCG, and ECG [153].

The mechanism of action for GTE involves the donation of a hydrogen atom or an electron to stabilize a ROS. A measure of a molecule's ability to donate an electron is measured by its reduction potential. A more negative reduction potential indicates a tendency to loss electrons to new species (reduce them). The reduction potentials of EC, EGC, ECG, and EGCG are listed in Table 1, along with their antioxidant activities.

Table 1. ***Reduction potentials and relative antioxidant activities*** [83]

Antioxidant	Reduction Potential (V)	Antioxidant Activity (mM)
(-)-Epicatechin	-0.57	2.4 ± 0.02
(-)-Epigallocatechin	-0.43	3.8 ± 0.06
(-)-Epicatechin gallate	-0.55	4.9 ± 0.02
(-)-Epigallocatechin gallate	-0.43	4.8 ± 0.06
Vitamin C	-0.28	1.0 ± 0.02
Vitamin E	-0.48	1.0 ± 0.03

Reduction potential at pH 7, 20ºC
Antioxidant activity (Trolox equivalent)

The effectiveness of an antioxidant is not only its ability to donate an electron but also its ability to sustain itself as an antioxidant radical. The C ring of EC and EGC, and the C ring and gallate attached to the B ring in ECG and EGCG, help stabilize unpaired electrons, making green tea

polyphenols very effective antioxidants [14]. Additionally, the gallate moiety at the 3 position of the C ring appears to increase the antioxidant activity of ECG and EGCG [132].

Understanding the bioavailability of green tea polyphenols is essential to understanding their overall significance in disease prevention and mechanisms of action. Green tea polyphenols are rapidly absorbed in the body [192] and there are a number of studies documenting its metabolism, distribution, and excretion in humans and animals [194,110,115,97,30]. Once digested, polyphenols are metabolized three different ways: (1) methylation (attachment of a methyl group); (2) glucuronidation (addition of glucuronic acid via glycosidic bond); and (3) sulfation (attachment of a sulfate groups) [60]. In rats, polyphenols transferred across the enterocytes of the jejunum to the serosal side being ~45% glucuronidated, ~30% methylated, ~20% O-methyl-glucuronidated, and the rest unmetabolized [104]. These percentages were different in isolated ileum sections, suggesting polyphenols are metabolized differently throughout the small and large intestine. The colon which contains large amounts of microorganisms, has a high catalytic activity for EGCG metabolizing it down to simple phenolic acids and glycine conjugates [101]. Additionally, the liver plays a role in polyphenol metabolism as many glucuronidated polyphenols are present [109].

Polyphenol metabolites pass through the small and large intestine where they are then carried via the portal vein to the liver where they are further metabolized. From the liver polyphenol metabolites can be distributed to other tissues or to the kidneys for excretion [60]. The cellular distribution of polyphenol metabolites is based on intracellular metabolism and membrane permeability [187]. For example, when A/J mice were given a dose of 0.3 mg/kg EGCG, the distribution of unmetabolized EGCG and metabolized 4'4"-Di-methyl-EGCG were found at their highest concentrations throughout the body on day 3 in the urine, feces, liver, kidney, and small intestine [115]. Also, SD rats given daily administrations of 0.6% EGCG (w/v) had maximal concentrations of EGCG on day 8 in the esophagus (0.61 nmol/g), prostate (0.13 nmol/g), spleen (0.09 nmol/g), bladder (0.44 nmol/g), lung (0.04 nmol/g), kidney (0.13 nmol/g), liver (0.05 nmol/g), heart (0.01 nmol/g), and thyroid (0.02 nmol/g) [97]. Additionally, humans given an oral dose of EGCG at a concentration of 200, 400, 600, or 800 mg had maximal plasma EGCG concentrations of 0.16, 0.24, 0.37, and 0.96 μM [31]. Finally, all major polyphenols present in GTE can pass through the placenta from dams to fetuses and be distributed in fetal organs as

well [33,34]. At present it is unknown if GTE can enter cells; although, these data suggest that polyphenols are rapidly absorbed, metabolized, and distributed to different organs in the body where they are involved in different mechanisms of disease prevention (e.g., antioxidant).

Though other molecules represent valid antioxidants (e.g., theaflavins in black tea, vitamins C) polyphenols in green tea are extensively studied for both their antioxidant capacity and possible signaling capabilities. Green tea polyphenols are rapidly absorbed by the body, can sustain unpaired electrons, and have been shown to be direct scavengers of NO and O_2^-, reducing the production of peroxynitrite [130]. Polyphenols also play an important role in signaling as they have been shown to interact with a number of signaling pathways [187]. According to Schroeter [164], polyphenols protected neurons from low density lipoprotein induced activation of JNK and c-jun, which when activated lead to mitochondrial dysfunction and apoptotic protease activation. Additionally, Afaq et al. [2] reported EGCG inhibited NF-kappa B pathways that can lead to inflammation and oxidative stress. Thus, the protective effects of the polyphenols in GTE not only come through their hydrogen donating antioxidant capacity, but also from their interaction with signaling cascades. Indeed, a receptor for EGCG was recently identified [174]. The 67 kd laminin receptor is found on cell surfaces and has a specific binding affinity for the gallate moiety of EGCG. This information, along with the report by Schroeter et al. [164] demonstrating that polyphenols had greater protective affects than 10-fold higher concentrations of Vitamin C, suggest that the amount of antioxidants available are not as important as the mechanisms through which those antioxidants act.

Exercise and Green Tea Extract in Mice

Green tea extract has been extensively studied for its effects to mediate cancer and diabetes. Recently, it was discovered that GTE prolonged time to exhaustion in swimming mice [127]. It was then reported that the same improved endurance was seen in running mice [128]. Green tea extract was provided to mice in their chow at a concentration of 0.2% and 0.5%. Mice were subjected to an initial treadmill running session until exhaustion. After the initial test, GTE feeding and an exercise prescription of 15m/min for 30 minutes a day was begun. Feeding and exercise training continued for 8 weeks at which point mice were sacrificed immediately after a final running to exhaustion test. Mice consuming a diet of 0.5% GTE had a 30% longer running

time than exercised mice on a standard chow ($P<0.05$). Additionally, mice consuming 0.5% GTE had decreased lactate concentration in the blood (-17%), an almost normal glycogen content in skeletal muscle (-7% compared to non-exercised, normal chow mice), decreased malonyl-CoA content in skeletal muscle (-210% compared to non-exercised, normal chow mice), and increased fatty acid beta-oxidation (+75% compared to non-exercised, normal chow mice). This study provided conclusive evidence that GTE supplementation increased running endurance by utilizing lipid metabolism, thus maintaining glycogen stores in skeletal muscle.

Green Tea and Duchenne Muscular Dystrophy

It is well established that oxidative stress is involved in either the onset or progression of DMD. Mdx mice experience increased levels of lipid peroxidation at ages of 12-16 days (+60% compared to age matched controls) [49]. Ragusa [148] reported lipid peroxidation levels of the extraocular, diaphragm, gastrocnemius, and soleus muscles from mdx mice were greater than control mice (+100%, +350%, +400%, +300% respectively). Additionally, a deficiency in the cytosolic antioxidant enzyme CuZnSOD was reported in mdx mice. These studies suggest that a level of oxidative stress is reached in dystrophic muscle that is deleterious. A possible therapy might be one that could offset the oxidative stress by the introduction of antioxidants.

GTE has been shown to be beneficial in the mdx mouse in two separate studies. Buetler [22] provided 0.01% and 0.05% GTE based diets to mdx mice at birth (via dams) and following weaning for 4 weeks. Cross sections from EDL muscles removed at the conclusion of the treatment period revealed a decrease in necrotic and regenerating surface area (-10% and -15% for 0.01% and 0.05% GTE respectively). Dorchies [51] provide 0.05% and 0.25% GTE based diets to mdx mice at age 21 days and continued treatment for a duration of 1 or 5 weeks. After 5 weeks (but not after 1 week) serum antioxidant levels were increased in the 0.05% and 0.025% GTE fed mice (+22% and +16% respectively). After 1 week (but not 5 weeks) necrotic and regenerating surface area was reduced in the 0.25% GTE fed mice (-17%). Additionally, tetanic stress was increased in 0.25% GTE fed mice after one week (+20.7%).

The precise mechanism of GTE's protective effect on dystrophic muscle is unknown. Oxidative stress is enhanced in the mdx mouse which could result in the damage of proteins and lipids in the muscle cell. GTE's antioxidant capability is probably able to alleviate oxidative

stress to a degree which slows the progression of the dystrophic disease in the mdx mouse. Additionally, it has been suggested that GTE: (1) optimizes muscle tension through regulation of myosin light chain thiol moieties, (2) promotes myotube formation from myoblasts, (3) enhances expression of muscle-specific proteins, and (4) interferes with mechanisms of Ca^{2+} dysregulation [7,51]. This makes GTE a potential therapy for DMD.

Summary

The role of dystrophin in the pathogenesis of DMD is still unknown. Currently, one of the leading hypotheses is damage results because of oxidative stress. Oxidative stress is initiated by muscle movement, increased Ca^{2+} load on the mitochondria, and inflammation. Because DMD is a muscle wasting disease with fast-fatigable fibers being damaged the most, a prescription of exercise may be beneficial in relieving muscle atrophy and promoting a shift toward more slow-fatigue resistant fibers. Additionally, GTE, a potent antioxidant with several signaling capabilities, is a possible therapy to attenuate the oxidative stress in DMD. For these reasons a treatment regimen of voluntary wheel running with GTE is proposed. To determine the efficacy of these treatments alone and in combination contractile/mechanical properties, antioxidant capacity, lipid peroxidation, serum creatine kinase, MHC content and distribution, and metabolic parameters will be assessed.

Chapter 3: Journal Article

Endurance capacity in maturing mdx mice is markedly enhanced by green tea extract

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Running Title:

Exercise and Green Tea Improve Mdx Endurance

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Abstract

Duchenne muscular dystrophy (DMD) is a progressive muscle wasting disease characterized by absence of dystrophin and the dystrophin glycoprotein complex from the sarcolemma membranes of muscle cells. Because dystrophic muscle cells are susceptible to damage from oxidative stress, strategies to reduce these deleterious effects could be an effective therapeutic approach. We tested the hypothesis that endurance exercise, which increases antioxidant capacity in humans and in mice, and green tea extract (GTE), a powerful antioxidant, would blunt oxidative stress and improve dystrophic muscle function as a result. Male mdx mice maintained normal cage activity or performed voluntary wheel running for 3 weeks starting at age 21 days. Mice were fed either normal chow (Norm) or chow supplemented with 0.5% GTE (wt/wt). In normal diet running mice (RunNorm), average daily distance run was increased 300% (from 0.5 to 2.1 km/d, $P<0.05$) from week 1 to week 3. In GTE diet (RunGTE) compared to RunNorm mice, total distance over the 3 weeks was markedly improved 128% (61.2 vs. 26.8 km, $P<0.0001$). Running, independent of diet increased EDL muscle tetanic stress (18%), serum antioxidant capacity (22%), citrate synthase activity (35%), and beta-oxidation (37%; all $P<0.05$). GTE, independent of running decreased lipid peroxidation (gastrocnemius:-64%; heart: -29%) and serum creatine kinase (-36%), and increased citrate synthase activity (59%; all $P<0.05$). Surprisingly, there were few additive adaptations with combined running and GTE, but the data suggest synergistic effects likely included improved aerobic metabolism, greater serum antioxidant capacity and decreased lipid peroxidation, so that endurance capacity was markedly increased and serum creatine kinase levels were dramatically reduced. These data suggest both approaches may be beneficial therapeutic strategies to treat DMD.

Key words: antioxidant capacity; muscular dystrophy; oxidative stress; voluntary wheel running.

Introduction

Duchenne muscular dystrophy (DMD) is a severe muscle wasting disease that affects 1 in every 3500 boys [56]. The pervasive and progressive skeletal muscle atrophy and weakness leaves patients wheelchair bound by age 12 years. Deterioration of respiratory muscles leads to an inability to generate proper inspiratory and expiratory forces, and death usually occurs due to respiratory or cardiac failure by the mid 20's [185]. DMD results from the absence of dystrophin, a 427 KD protein normally localized to the inner surface of the muscle fiber sarcolemma [184].

Dystrophin is expressed in control skeletal muscle and is associated with a multimolecular network of integral and subsarcolemmal proteins collectively known as the dystrophin glycoprotein complex (DGC) [57]. The DGC and additional proteins form costameres, which are rib-like lattices along the cytoplasmic face of the sarcolemma that facilitate even distribution of contractile forces laterally across the myofibril [45,57]. Thus, dystrophin via the DGC spans the sarcolemma to create a physical link between the intracellular cytoskeleton and the extracellular matrix. In the absence of dystrophin, the DGC fails to properly aggregate at the sarcolemma [57], and the link is compromised. Two potential outcomes leading to disease onset and progression are (1) the sarcolemmal membrane is mechanically weaker and/or (2) cell signaling via the DGC is disrupted. At present, the precise mechanisms of DMD pathophysiology remain elusive.

Because DMD results in severe muscle atrophy and progressive muscle weakness, appropriate exercise which is known to improve muscle strength and endurance may be a suitable therapy [76]. For example, exercise has been used to attenuate muscle atrophy and fatigue in non-diseased individuals because the parameters (duration, intensity, frequency) of exercise prescription are well defined [79]. However, it is not presently known if exercise will blunt or exacerbate disease progression in DMD and at present, exercise parameters have not yet been defined [76]. The greatest risk is that exercise may induce or accelerate muscle fiber damage. Because of this risk, we suggested that exercise training be first systematically tested in dystrophic mice (e.g., mdx) , and then in dystrophic dogs as a bridge to human studies [76].

Based on studies in vitro, muscles of older mdx mice are susceptible to contraction-induced injury (age ~100 days) [144], but muscles of maturing mdx mice appear less susceptible (e.g., age 9-12 days; [75]). Young mdx mice (e.g., age 4 weeks) [26] also appear to tolerate endurance training on a running wheel better than that of older mice (e.g., age 6 months) [26], perhaps suggesting that the muscles can better adapt to the stresses of physical activity when young. Thus, to potentially maximize the benefit of endurance training, we considered a reasonable age to begin was weaning age (21 days).

In addition to increased endurance capacity, another advantage of regular exercise for both humans and mice, is the upregulation of antioxidant proteins, enhancing the body's capability to balance increased reactive oxygen species (ROS) [133,146]. Oxidative stress appears to precede disease onset in the mdx mouse [49], thus increased antioxidant capacity associated with regular exercise may provide relief from oxidative stress in dystrophic muscle. To combat ROS, we considered that an antioxidant supplementation, such as green tea extract (GTE), may be a suitable adjunct to exercise training.

Green tea is the second most consumed beverage in the world [73], and has been intensely studied for its medicinal benefits including antioxidant [130], anti-diabetic [111], anti-carcinogenic [19], anti-bacterial [8], and anti-atherogenic [118] properties. The main constituent of GTE, epigallocatechin gallate (EGCG), is thought to be responsible for these beneficial properties [83,179,41]. Mdx mice supplemented with 0.05% and 0.25% GTE for periods of 1 to 5 weeks starting at weaning age showed delayed disease-onset and adaptations toward stronger and more fatigue resistant skeletal muscle fibers [22,51]. Although the precise mechanism by which GTE exerts its positive effects is not clear, these results suggest that the oxidative stress associated with DMD may be attenuated by GTE, and this may blunt disease progression.

In this study, we tested three hypotheses in male mdx mice; (1) 3 weeks of endurance exercise initiated immediately post-weaning (age 21 days) will improve skeletal muscle function and ANTIOXIDANT capacity, and decrease lipid peroxidation relative to non-running mice; (2) GTE without exercise will demonstrate similar beneficial effects as running; and, (3) GTE in combination with endurance exercise will be more beneficial than a diet of GTE or endurance exercise alone.

Methods

Mice

This study was performed on male mdx mice. Breeders were originally obtained from the Jackson Laboratory (Bar Harbor, ME), and a colony thereafter maintained at Virginia Tech. Mice were housed in plastic micro-isolator cages on a 12:12 hour light-dark cycle and were provided food and water ad libitum. All procedures performed in this study were approved by the Virginia Tech Institutional Animal Care and Use Committee.

Diet Groups

Sunphenon 90DCF, decaffeinated green tea extract (polyphenols > 80%, catechins > 80%, EGCG > 45%, caffeine < 1%) was a kind gift from Taiyo Kagaku (Minneapolis, Minnesota). Two commercial diets were supplemented with GTE at a 0.5% concentration by weight by Harlan Teklad (Madison, WI): #7004 for breeders, composed of 16.9% protein, 50.3% carbohydrate, and 11.4% fat; and #2018 for weaned pups, composed of 18.1% protein, 47.7% carbohydrate, and 5.8% fat. Male and female mdx breeder mice were paired at age ~35d, and randomly assigned and then maintained on normal diet (#7004) with or without 0.5% GTE. Pups were weaned at age 21 days and were provided normal diet (#2018) with or without 0.5% GTE. The normal and GTE diets were maintained for the duration of the study.

Body mass and food consumption were measured weekly. An initial body mass at age 21 days was obtained and subsequent measurements were made at ages 28, 35, and 42 days. Each mouse was provided with an initial food mass of 120 grams for the 3 week study. Food consumption was determined from the difference between the beginning and ending food mass each week (e.g., 120 g – 95 g = 25 g consumed). Weekly food consumption was then normalized by body mass to grams of food consumed per gram of body mass per week (g/g bm/wk).

Voluntary Wheel Running

Weaned male mdx mice (age 21 days) from both normal and GTE diet groups were randomly separated into sedentary and exercise groups and housed individually. There were four treatment groups in the study: (1) Sedentary, normal diet (SedNorm; n=8); (2) Sedentary GTE diet (SedGTE; n=8); (3) Run, normal diet (RunNorm; n=8); and, (4) Run GTE diet (RunGTE;

n=10). Sedentary mice were placed 2 to a cage (11"x7"). Run mice were placed in individual 11"x 9" plastic cages with a running wheel (0.16 m diameter; Silent Spinner) mounted to the side of the cage. Mice were allowed free access to the wheel 24 hours a day. A metal tab attached to the rear of the wheel was used to interrupt a light signal between a photo electric gate each revolution. Each signal interrupt was recorded on a laptop computer at a sampling rate of 1 Hz using a digital data acquisition card (National Instruments USB-6501, Part # 779205-01) and a custom Labview program. The running distance and average running speed were saved to a data file every 120 seconds.

Isometric Contractile and Mechanical Properties

At age 6 weeks, after 3 weeks of voluntary run training or normal cage activity, mice were deeply anesthetized (2 mg xylazine-20 mg ketamine per 100 g of body mass ip), and the fast-twitch extensor digitorum longus (EDL) muscles were excised. EDL muscles were incubated at 30ºC in an oxygenated (95% O_2-5% CO_2) physiological salt solution (PSS) as previously described [190]. Non-absorbable braided silk suture (4-0) was tied to the distal and proximal tendons at the myotendinous junctions. EDL muscles were then fixed between a clamp and arm of a dual-mode servomotor system (300B, Aurora Scientific) at a resting tension (L_0) of 1.0 g. EDL muscles were maintained at L_0 by a stepper motor (Wolff et al., 2006). The servomotor arm and stepper motor were controlled by Dynamic Muscle Control software (DMC Version 4.1.6, Aurora Scientific) to obtain the position and force output data.

The stimulated muscle protocol consisted of 5 steps: (1) a pre-twitch and tetanus; (2) a single passive stress relaxation; (3) a single active stretch; (4) a fatigue protocol; and (5) a post tetanus. The first and fifth steps were performed to determine the effects of steps 2 - 4 on contractile capability (Wolff et al., 2006). In step 1, the stimulated muscle was subjected to three isometric twitches and tetani (150 Hz) spaced 1 minute apart. In step 2, the muscle was stretched instantaneously to 1.05 L_0, held for 7.0 s, and then returned to 1.00 L_0 to determine passive parallel elastic stiffness (Wolff et al., 2006). After 5 minutes at L_0, the muscle was stimulated at 80 Hz for 700 ms (step 3). During the final 200 ms, the muscle was stretched at 0.5 L_0/s to a total strain of 0.1 L_0 (i.e., an eccentric contraction) to determine active stiffness. In step 4, the muscle was subjected to a 1 s submaximal tetanus (60 Hz) at a rate of 12 tetani/min for 5 min to assess fatigue. At 1 min intervals, beginning with the prefatigue measurement, the muscle force

output was recorded. After the fatigue protocol, the muscle was quiescent for 20 minutes at L_0, and was then subjected to a final tetanus (150 Hz, step 5). At the conclusion of the protocol, each muscle was weighed to the nearest 0.1 mg using an A-200D electronic analytical balance (Denver Instruments, Denver, Colorado) and snap frozen in liquid nitrogen. Additional muscles were also excised, snap frozen in liquid nitrogen, weighed to the nearest 0.1 mg using an A-200D electronic analytical balance (Denver Instruments, Denver, Colorado), and assayed as noted below.

Contractile and Mechanical Properties

Force and position output profiles were analyzed using Dynamic Muscle Analysis software (DMA Version 3.2, Aurora Scientific). For each twitch, the peak force, the time to peak stress (TPS), and half-relaxation time (HRT) were determined. Muscle cross-sectional area (CSA) was determined as previously described (Grange et al., 2002). Twitch and tetanic forces were normalized to muscle CSA to obtain twitch and tetanic stress. Passive parallel elastic stiffness (g/mm^2) for step 2 [190], and active stiffness for step 3 were determined as previously described [75].

Contractile Protein

Individual tibialis anterior (TA) were homogenized in 10 mM phosphate buffer (pH 7.0), assayed in triplicate using the BCA Protein Assay (Pierce), and then subjected to SDS-PAGE to determine sample myosin heavy chain and actin contents [122]. Briefly, homogenates were diluted with an equal volume of Laemmli sample buffer containing 5% beta-mercaptoethanol and boiled for 3 min. Ten µg total protein from each sample were separated on a 4% stacking, 7.5% separating Tris SDS-PAGE gel run at 150 V for 93 min. Four myosin heavy chain standards (2,3,4,5 µg) and four actin standards (1,2,3,4 µg) and 11 samples were run on each gel. Purified rabbit MHC was a kind gift of Dawn Lowe (University of Minnesota) and actin was purchased from Sigma (A2522). Gels were stained for 1 hour with 0.1% Coomassie Blue R-250, 30% methanol, 10% glacial acetic acid, and then destained overnight in 20% methanol and 10% glacial acetic acid. Stained gels were scanned using an EPSON (Expression 1680) Twain imaging densitometer and analyzed using SynGENE. Linear regressions of the optical density for MHC and actin protein standards were used to determine the contents of MHC and actin proteins for each sample

Myosin Heavy Chain Isoforms

The proportional content of MHC isoforms was determined using a modified method of Talmadge and Roy [175]. Briefly, frozen TA muscle obtained from SedNorm, RunNorm, SedGTE, and RunGTE mice aged 42 days were thawed on ice in microcentrifuge tubes, homogenized in 200 µL sample buffer using a micropestle, and heated to 60º C for 10 min. Each sample was subjected to SDS-PAGE using 4% stacking and 8% separating gels. Samples were run on a 20-cm vertical slab gel unit (CBS Scientific) for 40 h at a constant current of 4.2 mA. Gels were stained with Coomassie blue, destained, and scanned using an image analysis system (Alpha Imager 2000). Band densities for each MHC isoform were expressed as a percentage of total MHC band density.

Serum Creatine Kinase

Blood from cardiac puncture was collected (~1 mL) directly into Microtainer serum separator tubes (Becton Dickinson), and refrigerated (4°C) for 30 minutes to clot blood. The tubes were then centrifuged (Brinkmann Instruments, Inc., 5417 R) at 10,000 rpm for 10 minutes at 4ºC to separate serum. Serum was stored at -80ºC until analyzed for CK concentration by the Clinical Pathology Laboratory at Virginia Tech, using an Olympus AU400 chemistry analyzer (Olympus America, Center Valley, PA).

Serum Antioxidant Capacity

The ability of each serum sample to inhibit ABTS (2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) oxidation by metmyoglobin was determined with an antioxidant assay kit (Cayman Chemical). Briefly, each serum sample was diluted 1:20 with assay buffer (5 mM potassium phosphate, pH 7.4, containing 0.9% sodium chloride and 0.1% glucose). A Trolox standard range (0 - 0.660 mM) was obtained by diluting a 1 M Trolox stock solution with the same assay buffer. Trolox is a water-soluble tocopherol analogue. Reactions in duplicate were performed in a 90 well plate. To each well were added: 10 µl metmyoglobin, 150 µl chromogen, 10 µl of either standard or sample, and 40 ul of 441µM H_2O_2 to initiate the reaction. The plate was covered, placed on a shaker at low speed for 5 min, and then read at 750 nm (Bio-Tek Instruments, Inc., µQuant). Linear regressions of the absorption versus Trolox standard concentrations were used to determine the antioxidant capacity of the samples.

Lipid Peroxidation

Individual gastrocnemius and heart muscles were carefully excised, homogenized in 10 mM phosphate buffer (pH 7.0), and sonicated (550 Sonic Dismembrator, Fisher Sci.) at 40V over ice for 15 seconds. Homogenates were assayed in duplicate using the TBARS Assay Kit (Cayman Chemical) to determine the content of malondialdehyde (MDA). Homogenates were boiled for 1 hour, incubated on ice for 10 minutes, and then centrifuged at 1600 x g at 4ºC for 10 minutes. Samples were then loaded with standards onto a 90 well plate and read at 530 nm. Linear regressions of the absorption for MDA standards were used to determine the amount of MDA in each sample. Standards ranging from 0 to 50 uM MDA were prepared by diluting a 125 uM MDA stock solution with HPLC-grade water.

Citrate Synthase Activity

Citrate synthase catalyzes the formation of citrate and CoASH from acetyl-CoA and oxaloactetate. Because CoASH reduces DTNB, CS activity was determined for each sample in duplicate from the reduction of DTNB over time. Briefly, ten µl of a 1:5 diluted muscle homogenate (previously diluted 1:20 (1mg/µl in 10 mM phosphate buffer)) was added to 170 µl of a solution containing Tris buffer (0.1M, pH 8.3), DNTB (1mM, in 0.1M in Tris buffer) and oxaloacetate (0.01M, in 0.1M Tris buffer). Following a 2 min background reading, 30µl of 3 mM acetyl CoA was added to initiate the reaction. Absorbance was measured on a spectrophotometer (SPECTRAmax ME, Molecular Devices Corporation, Sunnyvale California) at 405 nm every 12 seconds for 7 minutes at 37℃. Maximum CS activity was calculated and reported as µmol/mg protein/min [169].

Beta-Hydroxy Acyl-CoA Dehydrogenase Activity

The third reaction of the Beta-oxidation cycle is the oxidation of the hydroxyl group at the Beta-position to produce a B-ketoacyl-CoA derivative. The second oxidation reaction is catalyzed by L-hydroxyacyl-CoA dehydrogenase, an enzyme that requires NAD^+ as a coenzyme. Each NADH produced in mitochondria by this reaction drives the synthesis of 2.5 molecules of ATP in the electron transport pathway. For the determination of β-hydroxyacyl-CoA dehydrogenase, oxidation of NADH to NAD was measured in triplicate. Briefly, 35 µl of whole muscle homogenate (1:20 wt/vol (mg/µl)) was added to 190µl of buffer containing 0.1M liquid

triethanolamine, 5mM EDTA tetrasodium salt dihydrate, 0.45mM NADH and 15 µl of 2mM acetoacetyl CoA to initiate the reaction. Absorbance was measured on a spectrophotometer (SPECTRAmax PLUS 384, Molecular Devices Corporation, Sunnyvale California) at 340 nm every 12 seconds for 6 minutes at 37ºC. Maximum BHAD activity was calculated and reported as µmol/mg protein/min [35].

Statistics

Results are expressed as means ± SE for figures and tables. Percentages (%) are expressed as either increases or decreases; not relative to initial values (e.g., a 100% increase means the original value has doubled). Daily wheel running data and weekly body mass and food intake data were analyzed by a two-way repeated measures ANOVA. All other data were analyzed by a two-way ANOVA (diet x activity) with Tukey's HSD post hoc analysis when necessary ($P<0.05$). In this report, when no interactions were detected, we describe a main effect of running as "independent of diet" (i.e., RunNorm and RunGTE data combined compared to SedNorm and SedGTE data combined), and a main effect of diet as "independent of running" (i.e., SedNorm and RunNorm data combined compared to SedGTE and RunGTE data combined).

Results

Voluntary Wheel Running

There was a significant interaction between Diets groups (RunNorm vs. RunGTE) and time ($P=0.0003$), indicating a variance of wheel running distance over time depending on diet. Within the RunNorm mice, average daily distance run each week was increased (i.e., week 1 < week 2 < week 3, $P<0.05$). Within the RunGTE mice average daily distance run was different between week 3 and weeks 1 and 2 ((week 1 = week 2) < week 3, $P<0.05$). During the second week of running, RunGTE had a 94% greater average daily distance than RunNorm mice (2.3 km/d vs. 1.2 km/d, $P<0.05$). During the third week of running, RunGTE mice had a 170% greater average daily distance compared to RunNorm mice, and had an average daily distance run statistically equivalent to C57Bl/10 (Fig. 7A). RunGTE mice ran a total distance 128% greater than RunNorm mice over the 3 week period (61.2km vs. 26.8 km, $P<0.0001$) (Fig. 7B).

Body mass and food consumption are reported in Fig. 8. There was not a significant interaction between treatment groups and time. Independent of diet, Run compared to Sedentary mice had greater body masses at weeks 1, 2, and 3 ($P<0.05$) of the treatment period. Additionally, Run mice gained more mass per week than Sedentary mice (4.6 g/wk vs. 3.6 g/wk, $P<0.05$). Independent of running, the GTE compared to Normal diet mice consumed more grams of chow per grams of body mass per week (1.51 g/g bm/wk vs. 1.34 g/g bm/wk, $P<0.05$).

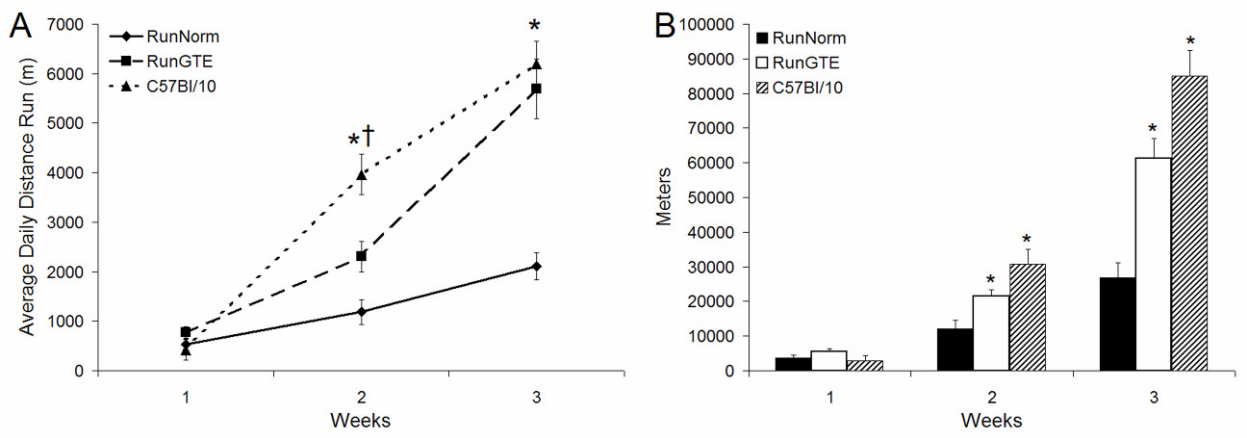

Fig. 7. Green tea extract increased running distance in mdx mice. A: Average daily distance run by week increased with GTE. B: After 2 and 3 weeks of training, RunGTE mice ran a greater total distance compared to RunNorm mice. Values are mean ± SE. * C57Bl/10 and RunGTE are greater than RunNorm ($P < 0.05$). † C57Bl/10 greater than RunGTE ($P<0.05$).

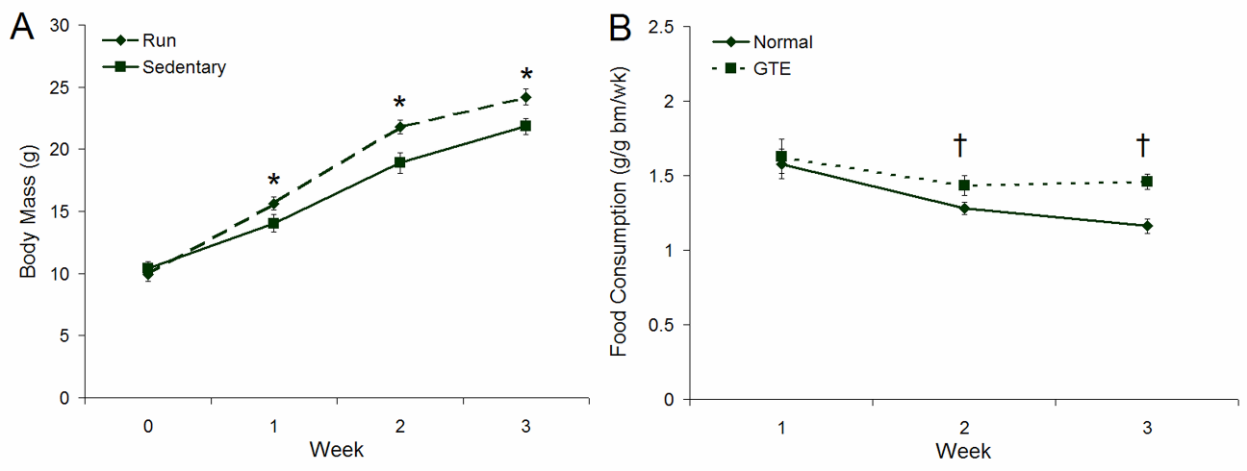

Fig. 8. Body mass and food consumption were increased with running and GTE, respectively. Values are mean ± SE. * Greater than Sedentary ($P < 0.05$). † Greater than Normal ($P < 0.05$).

Muscle Masses

Individual muscle masses are reported in Fig. 9. Running, independent of diet, increased gastrocnemius, soleus, and heart muscle mass (32%, 30%, and 27% respectively, $P<0.05$) compared to sedentary mice. RunGTE mice had greater TA masses (20%, $P<0.0342$) compared to SedGTE mice and RunGTE mice had greater heart masses compared to SedNorm, SedGTE, and RunNorm mice (30%, 46%, and 20%, respectively; $P<0.05$).

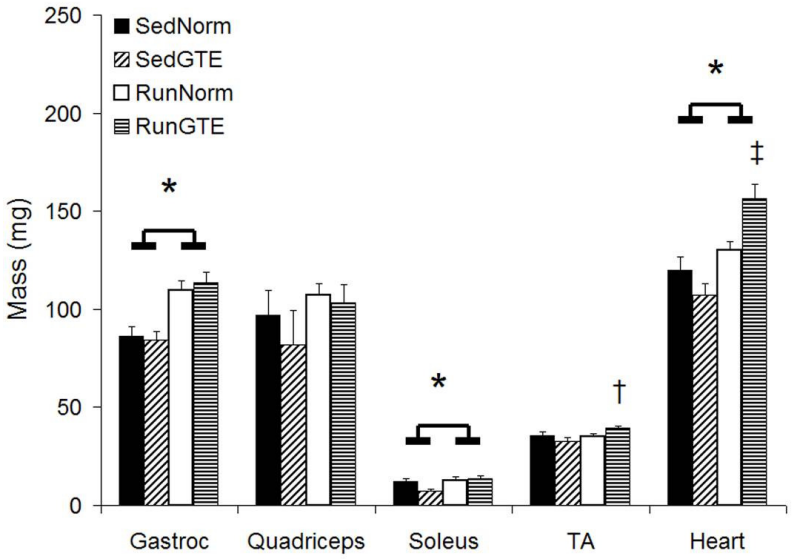

Fig. 9. Running elicited hypertrophy in both skeletal and cardiac muscle. A: Running increased muscle mass of the soleus, gastrocnemius, and heart. Values are means ± SE. * Runners greater than Sedentary mice (P<0.05). † Greater than SedGTE (P<0.05). ‡ Greater than SedNorm, SedGTE, and RunNorm (P<0.05).

Contractile and Mechanical Properties

Morphological, contractile, and mechanical data for the EDL muscles are shown in Table 2. Independent of diet, Sedentary mice had a slower time to peak stress than Run mice (13.1 ms vs. 11.4 ms, P<0.001). Independent of diet, Run mice produced a greater tetanic stress compared to Sedentary mice (24.2 g/mm^2 vs. 20.5 g/mm^2, P<0.0434) (Table 2). There were no main effects or interactions for fatigue resistance for the 4 treatment groups (data not shown).

Mechanical Properties

Passive and active stiffness values for EDL muscles from SedNorm, RunNorm, SedGTE, and RunGTE mice are shown in Table 2. Active stiffness was 16% greater in Run compared to Sedentary mice, independent of diet (1.80 MPa vs. 1.55 MPa, P<0.0121); and 42% greater in RunNorm mice compared to SedNorm mice (P<0.0171). Passive stiffness was increased 64% (P<0.0149) in SedGTE mice compared to RunGTE mice.

Contractile protein content and fiber type distribution

Independent of diet, Run compared to Sedentary mice demonstrated increased relative myosin, total protein, and total contractile protein contents (+6%, +1.2%, +2.5% respectively,

P<0.05) (Fig. 10A,B,C). We considered a shift in fiber type might be responsible for the increased endurance capacity and so we also assessed the fiber type distribution of the TA, but found no differences (Fig. 10D).

Antioxidant and membrane integrity markers

Antioxidant capacity was determined from the serum's ability to inhibit oxidation of ABTS® by metmyoglobin. Independent of diet, Run compared to Sedentary mice had a 22% greater antioxidant capacity (Fig. 11A; 156 mM vs. 128 mM Trolox, P<0.0017). Lipid peroxidation, a marker of oxidative stress, was determined from MDA content in gastrocnemius and heart homogenates. Independent of running, GTE compared to Normal diet mice demonstrated 64% less lipid peroxidation (Fig. 11B; 0.103 μmoles vs. 0.281 μmoles MDA/mg protein, P<0.0001) in the gastrocnemius and 29% less lipid peroxidation (0.311 μmoles vs. 0.22 μmoles MDA/mg protein, P<0.05) in the heart.

SedGTE mice had a CK levels 57% less than SedNorm mice (Fig. 11C; 3375 U/L vs. 5949 U/L, P<0.0308). Independent of running, GTE mice demonstrated lower serum CK (2981 U/L vs. 4659 U/L, P<0.0314). Independent of diet, RunNorm compared to SedNorm mice had insignificantly lower serum CK (3369 U/L vs. 5949 U/L, Fig. 11C).

Table 2. ***Morphological, contractile, and mechanical data following 3 weeks of diet and activity interventions***

		EDL Length	EDL Mass	CSA	TPS	HRT	P_t	P_o	PS	AS
	n	Mm	mg	mm^2	ms	ms	g/mm^2	g/mm^2	MPa	MPa
Diet										
Normal	16	10.0 (0.2)	8.1 (0.2)	0.78 (0.02)	11.9 (0.3)	25.7 (1.5)	4.4 (0.2)	21.3 (1.2)	1.23 (0.11)	1.64 (0.08)
GTE	18	10.0 (0.1)	8.3 (0.2)	0.78 (0.02)	12.6 (0.3)	25.1 (1.4)	4.1 (0.2)	23.5 (1.2)	1.39 (0.11)	1.70 (0.07)
Activity										
Sedentary	16	9.6 (0.2)	7.7 (0.2)	0.76 (0.02)	13.1.(0.3)	26.7 (1.5)	4.0 (0.2)	20.5 (1.2)	1.44 (0.11)	1.52 (0.08)
Run	18	10.4 (0.1)*	8.7 (0.2)*	0.80 (0.02)	11.5 (0.3)*	24.2 (1.4)	4.5 (0.2)	24.0 (1.2)*	1.18 (0.11)	1.82 (0.07)*
Treatment groups										
SedNorm	8	9.4 (0.2)	8.0 (0.3)[b]	0.80 (0.03)	12.2 (0.4)[b]	25.2 (2.1)	4.1 (0.3)	19.6 (1.7)	1.16 (0.16)	1.36 (0.12)[b]
RunNorm	8	10.5 (0.2)	8.3 (0.3)	0.75 (0.03)	11.7 (0.4)[b]	26.3 (2.1)	4.6 (0.3)	22.9 (1.7)	1.30 (0.16)	1.93 (0.11)[a]
SedGTE	8	9.7 (0.2)	7.4 (0.3)[b]	0.72 (0.03)[b]	14.0 (0.4)[a]	28.1 (2.1)	3.9 (0.3)	21.3 (1.7)	1.72 (0.16)[a]	1.69 (0.11)
RunGTE	10	10.3 (0.2)	9.1 (0.3)[a]	0.84 (0.03)[a]	11.3 (0.4)[b]	22.2 (1.9)	4.4 (0.2)	25.2 (1.5)	1.05 (0.14)[b]	1.71 (0.1)

Values are means (SE). CSA, cross sectional area; TPS, time to peak stress; HRT, half relaxation time; P_t, twitch stress; P_o, tetanic stress; PS, passive parallel elastic stiffness; AS, active stiffness determined from stretch protocol. * Different from Sedentary ($P<0.05$). Treatment means not followed by the same letter are significantly different ($P < 0.05$).

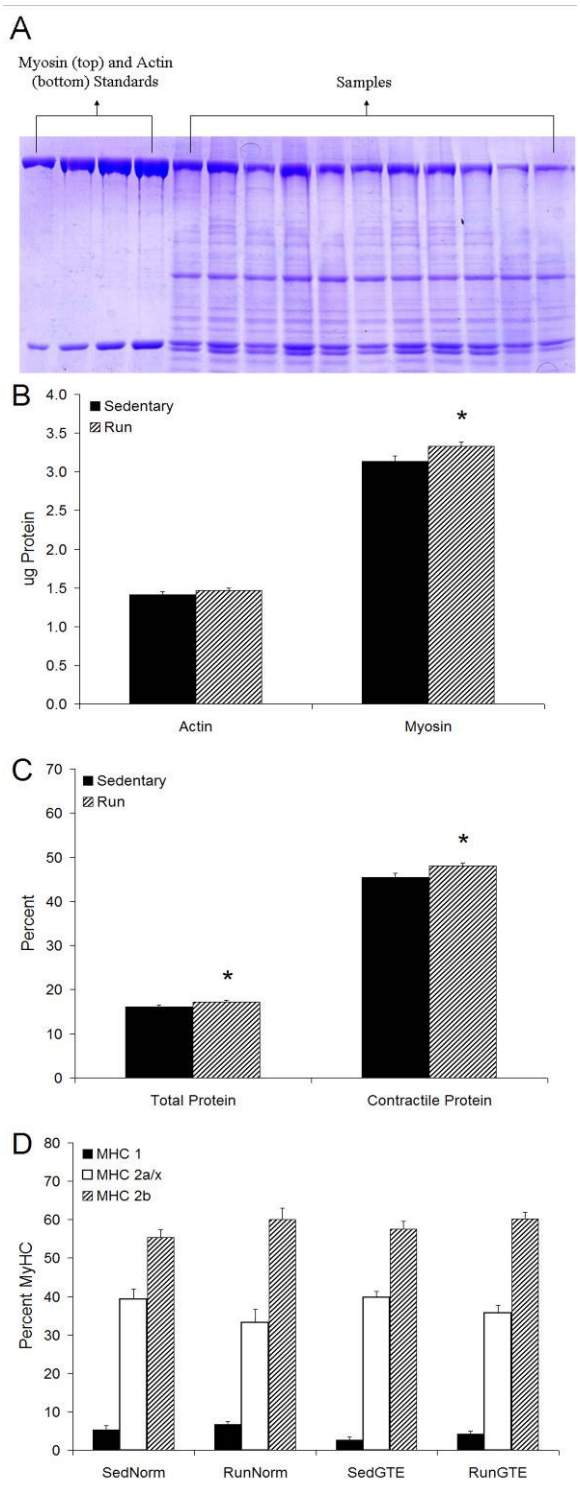

Fig. 10. Running resulted in greater contents of total protein, contractile protein, and myosin in mdx TA muscle relative to sedentary mdx TA muscle, but had no effect on fiber type distribution. A: Representative gel used to determine actin and myosin content. B: Actin and myosin contents expressed in μg per 10 μg total protein. C: Total protein is expressed as a percent of TA whole wet muscle mass and contractile protein is expressed as a percent of total protein. D: Fiber type distribution. Values are mean ± SE. * Greater than Sedentary ($P<0.05$).

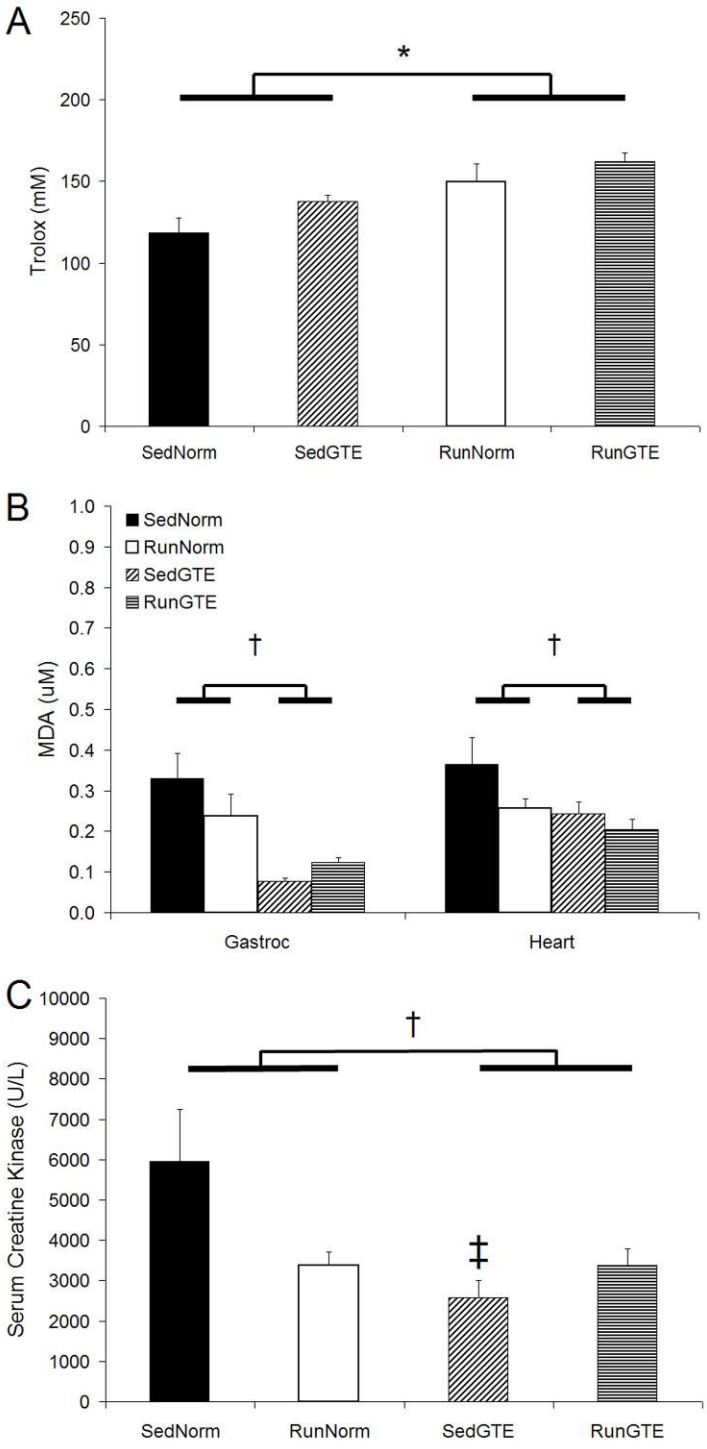

Fig. 11. Running and GTE both protect the sarcolemma from oxidative damage. A: Running, independent of diet increased serum antioxidant capacity. B: GTE, independent of running attenuated lipid peroxidation in the gastrocnemius and heart. C: GTE, independent of running reduced serum CK. Values are means ± SE. * Runners greater than Sedentary mice ($P<0.05$). † GTE diet mice less than Normal diet mice ($P<0.05$).

Markers of oxidative metabolism

Citrate synthase activity was determined in quadriceps, soleus, and heart muscles. Independent of running, GTE compared to Normal diet mice had 95% greater citrate synthase activity in quadriceps muscles (Fig. 12A; 448 vs. 285 µmol/mg prot/min, P<0.003). Additionally, citrate synthase activity was 35% greater in heart muscle in Run mice compared to Sedentary mice, independent of diet (Fig. 12A; 998 vs. 742 µmol/mg prot/min, P<0.0099).

Beta-hydroxyacyl-CoA dehydrogenase activity was determined in quadriceps and heart muscles. Independent of diet, Run compared to Sedentary mice had beta-oxidation activity that was 36% greater (242 vs. 178 µmol/mg protein/min, P<0.0028) in the quadriceps and 35% greater (200 vs. 148 µmol/mg prot/min, P<0.0099) in heart muscle (Fig. 12B).

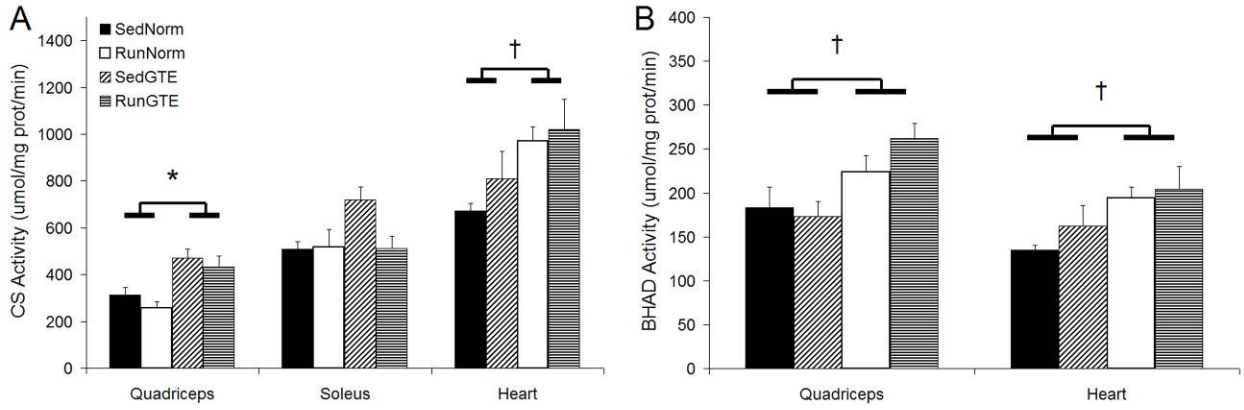

Fig. 12. GTE and Running increased metabolic activity differentially. A: Citrate synthase activity was increased with GTE in the quadriceps and with Running in the heart. B: Running increased beta-oxidation in the quadriceps and heart. Note, BHAD was not determined in the soleus because insufficient sample for the assay. Values are means ± SE. * GTE diet greater than Normal diet mice (P<0.05). † Runners greater than sedentary mice (P<0.05).

Discussion

The major findings of this study were 1) mdx males who began voluntary wheel running at age 21 days demonstrated a significant increase in weekly distance over the 3 week period, and 2) running endurance capacity was remarkably improved ~128% (comparison of total distance at conclusion of week 3) by 0.5% GTE in the diet. For each condition alone, there were beneficial, but no obvious deleterious, outcomes. Synergistic effects between running and GTE included improved skeletal muscle aerobic metabolism and serum antioxidant capacity, decreased lipid peroxidation, and cardiac hypertrophy. These data suggest combined endurance training and GTE can improve skeletal muscle function and membrane integrity in young mdx mice

Effects of Running

Running Alone. We began running male mdx mice at age 21 days because we have previously shown that young mdx mice do not experience the same membrane damage as older mdx mice [75,190] and because passive [190] and active (unpublished) stiffness of dystrophic EDL muscles are not different from WT. These data, along with reports that young (age 4 weeks) compared to older (age 6 months) mdx mice can better tolerate a physical stress such as running [26,81], provided a reasonable rationale to initiate training at weaning. Male mdx mice were tolerant of voluntary wheel running over 3 weeks, increasing the average daily distance run from the first to the last week ~300% (0.5 km/d vs. 2.1 km/d, $P<0.05$). Adaptations to support increased running capacity of mdx mice have been reported for long duration running (e.g., 16 weeks of voluntary wheel running; [81]). Nevertheless, our data indicated male mdx mice could positively adapt with minimal detrimental effects, in as little as 3 weeks. Taken together, these findings suggests that dystrophic muscle can positively respond to both short term and long term exercise training, particularly if it is initiated at a young age.

Surprisingly, running alone (i.e., significant interaction with RunNorm different than SedNorm) elicited only one significant effect in EDL muscles. Active stiffness was increased in RunNorm mice 42% compared to SedNorm mice. The increased active stiffness could reflect improved interaction between actin and myosin in the contractile

element [163] and improved transmission of force between the contractile element and the tendons. These data indicate male mdx mice can run voluntarily starting at age 21-d.

Running Independent of Diet. Body mass of runners was greater than that of sedentary mice (Fig. 8A), suggesting increased muscle mass (EDL Table 2, skeletal and heart muscle Fig. 9). Although wheel running would be considered endurance training, we did observe an ~18% increase in tetanic stress output from the EDL following running training (Table 2). We considered this increase could be due to at least three possible mechanisms: (1) increased expression and stability of contractile proteins (i.e., myosin and actin); (2) a shift in fiber type; and (3) improved membrane integrity. First, stress output of fast-twitch skeletal muscle is closely related to total myosin content ($R=0.81$, $P<0.05$; [24]) . We found modest increases in myosin content, total protein, and total contractile protein after 3 weeks of voluntary wheel running, independent of diet (Fig. 10B & C). In addition, our data also suggest decreased oxidative stress. Myosin heavy chain (MyHC) is highly susceptible to oxidative stress, which could lead to decreased contractile stress output [37]. Since decreased contractile stress output and increased oxidative stress are characteristic of DMD, an increased antioxidant capacity could alleviate oxidative stress detrimental to MyHC. In agreement with reports for humans that endurance activity increases antioxidant capacity, we observed a 22% increase in serum antioxidant capacity with running, independent of diet (Fig. 11C) and increased MyHC content in the TA. The active stiffness data from the EDL also supported the idea that serum antioxidant capacity may have helped protect MyHC from oxidative damage. We observed a 20% increase in active stiffness with voluntary wheel running, independent of diet (Table 2), suggesting an increased proportion of cross bridges in the strong binding state [163].

Second, a modest increase in stress output coupled with improved endurance capacity could reflect increased fast glycolytic-oxidative fibers (e.g., Type 2a). There is a lack of consistency and contradicting evidence on the amount of voluntary wheel running required to elicit fiber type shifts in skeletal muscle [5,141]. Allen et al. reported a transition toward more fatigue resistant MyHC 2a fibers in TA of male C57Bl/6 mice after 1 week of voluntary wheel running; whereas, Pellegrino et al. reported no changes in TA of male C57Bl/6 mice after 8 weeks of voluntary wheel running. We observed no differences in

fiber type distribution (Fig. 4D) in the mdx mice after 3 weeks of voluntary wheel running possibly because of (1) the volume of running as a stimulus was insufficient to cause a fiber type shift or (2) MyHC in dystrophic muscle does not adapt to exercise in the same way that non-diseased muscle does. Although, with regard to the latter, 16 weeks of voluntary wheel running did elicit similar fiber type shifts in EDL and soleus muscles of C57Bl/10 and mdx mice [81]; however, in the current study we only looked at fiber type shift in the TA.

Third, the dystrophic process is thought to render the sarcolemma susceptible to mechanical-induced injury, which contributes to depressed whole muscle stress output [144]. It is possible that this occurs more readily in old compared to young mdx muscles [75,190]. In support of this possibility, we observed an ~20% increase in muscle tetanic stress output and modest but not significant decreases in serum creatine kinase and lipid peroxidation levels with running, both of which could be considered indexes of muscle fiber integrity. Thus, our data suggests voluntary wheel running, independent of diet, improves contractile stress and does not exacerbate sarcolemmal membrane leakiness, but may actually reduce it.

Metabolic Properties. To account for the improved endurance capacity, we considered metabolic adaptations in (1) citrate synthase (CS) and (2) beta-hydroxyacyl-CoA dehydrogenase (BHAD) activities. Running on a Normal or on a GTE diet alone did not increase either CS or BHAD activities above those of the respective sedentary diet groups (SedNorm and SedGTE) (Fig. 12A-B). Runners, independent of diet did demonstrate increased CS activity, an index of mitochondrial content, in cardiac muscle but not in the skeletal gastrocnemius muscle. It is possible that capillary density was increased to enhance oxygen diffusion into muscle fibers and/or efficiency of the electron transport chain was enhanced to increase ATP generation, and these changes accounted for improved running endurance capacity. However, we did find BHAD activity was increased 37% in Run compared to Sedentary mice independent of diet in the gastrocnemius, as well as an increase of 35% in cardiac muscle. These results were similar to the 20% increase reported for 8-wk treadmill trained wildtype FVB mice [32]. This outcome suggests dystrophic muscle can adapt to exercise by increasing fatty acid

metabolism to meet energy demands, and this likely contributed in part to the improved running capacity.

Effects of GTE

GTE Alone. GTE had a beneficial effect on running male mdx mice as endurance capacity was markedly enhanced so that the average daily distances covered per day during the 3rd week of training were approaching those of C57BL/10 male runners (herein; [26,81]). GTE also had several beneficial outcomes on sedentary dystrophic muscle. Previous studies have reported that GTE reduces necrosis and regenerating surface area in dystrophic muscle along with increases in tetanic stress and fatigue resistance through undetermined mechanisms [22,51]. Our data suggest that one of GTE's primary beneficial effects is to enhance membrane integrity, potentially mediated through cytoskeletal remodeling and/or antioxidant effects. We observed increases in both passive (+48%) and active (+24%) stiffness (Table 2), coincident with decreased serum creatine kinase (-52%, Fig. 11C). Recently it was reported that a 67 kd laminin receptor can bind EGCG, the main constituent of GTE [174], to modulate actin remodeling and inhibit degranulation of human basophilic KU812 cells [67]. If a similar effect was evident in mdx skeletal muscle cells, cytoskeletal remodeling could improve sarcolemmal integrity. The increased passive parallel elastic stiffness we observed with GTE alone may be indicative of similar cytoskeleton remodeling, possibly through GTE binding to α7β1 integrin. The α7β1 integrin is a laminin receptor upregulated as a compensatory mechanism to possibly stabilize the costameric lattice in human DMD patients and mdx mice [86]. However, at present, the precise mechanisms of GTE action remain undefined, but could operate through various signaling cascades to promote cytoskeletal remodeling and/or through direct interactions with ROS to attenuate oxidative stress. Because membrane fragility and increased permeability are thought to contribute to dystrophic fiber degeneration, our data support the idea that necrosis and regenerating surface area were reduced [51,22].

GTE Independent of Running. The most intriguing findings were the significant decreases in lipid peroxidation, an index of membrane degradation, and subsequent decreases in serum CK, a benchmark index of muscle fiber damage Fig. 11C & B). Polyunsaturated fatty acids comprise the plasmalemma (i.e., the lipid bilayer) and are highly susceptible to oxidative damage [129]. GTE protected C2C12 myotubes from

induced oxidative stress [22]. Our results suggest that similar protection may be elicited in vivo, as we observed a strong correlation (R=0.82; data not shown) between lipid peroxidation and serum CK levels. At least part of the protection may be the direct ROS scavenging properties of GTE [130]. Additionally, oxidative stress is a potential mechanism of cardiac failure [147]. If GTE could elicit similar decreases in lipid peroxidation of the heart in DMD patients, whom ~40% suffer fatal myocardial dysfunction [123,136], it would be a beneficial outcome.

Metabolic Properties. Citrate Synthase (CS) activity is a common index of mitochondrial content. When GTE was considered independent of running, we observed increased CS activity in quadriceps (+59%) but not in soleus. In soleus, running blunted the increased CS activity induced by GTE. However, in both muscles the data suggest that mitochondrial capacity could be greatly improved with GTE supplementation, even when physical exercise may not be possible. This may be especially important for DMD patients who could benefit from improved fatigue resistance. GTE alone has been shown to reduce obesity by suppressing fatty acid synthesis [162] and increasing liver beta oxidation[126], but we observed no increase in skeletal muscle beta-oxidation either in SedGTE vs. SedNorm, nor in RunGTE vs. RunNorm mice.

Effects of Running and GTE combined

Though running and GTE both independently appear to elicit beneficial outcomes in dystrophic muscle, the major synergistic effects of combined running and GTE was an ~128% increase in endurance running capacity and an ~30% increase in heart mass. Surprisingly, rather than additive positive adaptations when running and GTE were combined, several of the beneficial effects of GTE alone were attenuated by running. These included: passive parallel elastic stiffness, active stiffness, serum creatine kinase, lipid peroxidation, and CS activity. However, although these effects were attenuated, values for each did not return to levels seen in Sedentary normal fed mice (e.g., serum creatine kinase). Our data suggest that RunGTE mice had dramatically improved endurance capacity, likely because GTE improved CS activity and running improved BHAD activity, and other deleterious effects such as lipid peroxidation were depressed. Although these changes likely accounted in part for the improved running capability of the RunGTE mice, it also likely that positive adaptations occurred in the cardio-

respiratory system, as both the diaphragm and heart are affected in DMD [173,96]. The diaphragm experiences degeneration without the regenerative capacity observed in limb muscles of mdx mice [173], while mdx cardiac tissue is susceptible to decreased cellular integrity, aberrant energy production, and decreased aortic flow [96]. Because DMD patients often die due to cardiac and/or respiratory failure, attenuating these deleterious effects would be beneficial. Indeed, exercise (running) and GTE each alone have promise as therapies [53,85]. For example, increased active tension was evident in dystrophic diaphragm muscles following long term voluntary wheel running, while GTE supplementation rescued post-ischemic guinea pig hearts by decreasing oxidative stress, increasing high energy phosphates, and increasing coronary flow rate [85]. Similar positive effects of GTE may also be evident in the dystrophic mouse heart.

Conclusion

In summary, male mdx mice on a Normal diet tolerated voluntary wheel running starting at age 21 days and improved their daily distance over a 3 week period. In none of the parameters assessed (e.g., tetanic stress, serum creatine kinase) was running detrimental. GTE supplementation appeared to be a beneficial supplement with or without exercise, but markedly increased endurance running distance in those mice provided running wheels. Our findings suggest the beneficial effects of endurance exercise and GTE result from adaptations in both skeletal and cardiac muscle.

Chapter 4: Discussion

Motivation

Increased oxidative stress has been observed in dystrophic muscle before disease onset [49]. Additionally, there are reports of increased lipid peroxidation and decreased antioxidant capacity in DMD boys and mdx mice [91,95,113]. Prolonged oxidative stress can lead to the destruction of biomolecules such as MHC, which are highly susceptible to oxidative damage [37]. Though the precise role of dystrophin in the dystrophic process is not presently known, its absence suggests that it may play a role in both cellular calcium regulation and oxidative stress [152]. Because increased oxidative stress and decreased force output are characteristic of DMD attenuation of oxidative damage should be considered when assessing different therapeutic approaches. Endurance exercise increases antioxidant capacity in both humans and mice, and for this reason it is considered a potential therapy for DMD. Additionally, supplementation with antioxidants such as GTE should also be considered because of their ability to blunt oxidative stress. The purpose of the present study was to evaluate the initiation of low load endurance exercise at weaning (i.e., age 21 days) in male mdx mice on dystrophic muscle, as well as to evaluate the powerful antioxidant GTE, as a therapy to attenuate oxidative stress. A few of the parameters explored to determine the efficacy of these approaches include: contractile/mechanical properties, antioxidant capacity, serum creatine kinase, lipid peroxidation, MHC content, fiber type distribution, and metabolic activity.

Major Findings

The major findings of this study included: (1) increased daily distance by RunNorm mice over a 3 week period; (2) a substantial increase in daily distance and total distance by RunGTE mice compared to RunNorm mice; (3) increased EDL stress output of mdx mice subjected to voluntary wheel running; (4) increased antioxidant capacity of mdx mice subjected to voluntary wheel running; (5) decreased serum creatine kinase levels and lipid peroxidation in mice consuming 0.5% GTE; and (6) increased CS activity in mice consuming GTE.

Additionally, one unexpected outcome was the emergence of two different running groups from the male mdx mice that were initiated to voluntary wheel running at

age 21 days: (1) a low runner group that gradually increased running distance over three weeks of training; and (2) a high runner group which ran between 2 – 4 times as far as the low runners, then became extremely lethargic and typically died by the 4th day of running. Low runners typically remained under ~750 meters of daily running within the 1st week of training while high runners typically exceeded ~750 meters of daily running within the first few nights before they died (Fig. 13A, Appendix B).

Mortality rates observed for each treatment group were 27% for SedNorm (3/11), 47% for RunNorm (7/15), 27% for SedGTE (3/11), and 23% for RunGTE (3/13) (Fig. 13B, Appendix B). Initially, the increased mortality rate of the RunNorm mice was speculated as being related to either cardiac myopathy or external environmental stressors. All mice were kept in an animal facility with a 12-12 hour light-dark cycle, weekly changing of cages, and available food and water ad libitum. However, at the time at which mice for the current study were being tested, an extensive amount of construction was being done on the exterior of the animal house facility. To understand the normal mortality rate for mdx mice, the Jackson Laboratory, our supplier of the original mdx breeders, was contacted (E-mail communication, Appendix B). A Technical Information Scientists (Peter Kelmenson) reported that the mdx colony is classified as fragile at the Jackson Laboratory, approximately 10-20% of mdx pups are considered runts and are culled immediately from the litter, 90% of the remaining pups survive until weaning (age 28 days at Jackson), and mdx mice are particularly sensitive to noise. Other investigators whom have previously studied voluntary wheel running in mdx mice were contacted and they did not observe any mortality rates as high as in the current study (E-mail communication with Dupont-Versteegden and Carter, Appendix B). Additionally, current ongoing voluntary wheel running studies from the current animal housing facility have not observed similar mortality rates (e.g., Kevin Voelker, Leucine Study) Overall in the mdx litters since the conclusion of this study, the mortality rate has been minimal (less than 10%, based on retrospective analysis). One potential confounding factor in our study was the work conducted on Litton Reaves Hall during the study period. Because the mdx mice are sensitive to noise, this may have exacerbated the mortality rate beyond the typical 10% observed by Jackson (after the runts are culled). If it assumed that the runts in our litters were sensitive to the noise in the sedentary and

running groups and died, this may have contributed to the higher mortality rate. Taken together, and without additional substantive data to support the increased mortality rate since the study was conducted (the building work has greatly diminished), we believe the rates we observed for the Sedentary groups were higher than observed by Jackson (possibly by 2-3 fold). It is therefore unclear if the increased mortality we observed in the RunNorm group was due to running or noise. For this reason it was decided not to report the mortality rates in the manuscript. Dr. Grange will continue to document the colony mortality rates.

Summary

There are several limitations to prescribing exercise for DMD patients because of insufficient data [76]. A few of these limitations include: (1) exercise may exacerbate the dystrophic condition; (2) dystrophic muscle may not adapt to exercise the same way non-diseased muscle does; and (3) benefits may not be as pronounced in dystrophic muscle [76].

With regard to the first limitation, it has previously been demonstrated that voluntary wheel running when initiated at an early age (i.e., age 3-4 weeks) does not exacerbate the dystrophic process [81,26,53]. There are involuntary treadmill exercise studies where the dystrophic condition has been worsened (e.g., increased serum CK and centralized nuclei) [183,66]. However, it should be noted that mdx mice tend to run in an intermittent "stop and go" pattern [78]. This suggests a possible internal mechanism that keeps mdx mice from over exerting, which cannot be controlled on a treadmill. The current study used voluntary wheel running and confirms that a low load, voluntary endurance exercise does not exacerbate the dystrophic condition. Because voluntary running for a DMD patient would demand an increased load to be placed on the joints, a low load endurance exercise such as swimming is recommended [54].

With regard to the second limitation, in non-diseased skeletal muscle an increase in physiological stressors, such as increased contractions associated with exercise, can lead to the stimulation of various pathways that promote upregulation and expression of many genes and proteins [168]. Examples of these proteins may be those contributing to substrate delivery (GLUT4), mitochondrial function (cytochrome c), and muscle contraction (MyHC) [13]. In the current study antioxidant capacity, MyHC, and BHAD

activity were each increased. The increase in antioxidant capacity may be the result of enhanced expression of antioxidant proteins (i.e., superoxide dismutase). The increase in MyHC was moderate and the data support two possible explanations: (1) an actual increase in MyHC expression or (2) protection of MyHC by the increased antioxidant capacity from oxidative damage. The increase in BHAD activity suggests increased fatty acid metabolism, perhaps promoting an efficient adaptation to meet the energy demands of the muscle. If similar responses were to occur in DMD patients all three of these responses to exercise in dystrophic muscle would be beneficial by: (1) alleviating oxidative stress; (2) attenuating muscle force loss; and (3) increasing the production energy for the muscle.

Therefore, it is reasonable to believe mdx mice that begin voluntary wheel running at weaning can experience moderate benefits (e.g., increased tetanic stress) from voluntary wheel running [81,26,53]. Interestingly, similar responses are not seen when much older mdx mice (i.e., 6 months) perform voluntary running. However, when younger mice are initiated to voluntary wheel running and continue running well past age 6 months, these benefits persist [26]. This suggests an initiation time frame which will determine if exercise will produce beneficial outcomes in dystrophic muscle. This would be an important factor to consider when considering exercise prescription for DMD patients. Because childhood onset is approximately age 2 years and most DMD boys are wheelchair bound by age 12, it would be beneficial to start exercise training at an early age.

Finally, the third limitation is the possibility that if dystrophic muscle can adapt to exercise it may lack the capacity to do so to the same extent as non-diseased muscle. The diaphragms of age 45 week mdx mice initiated to voluntary wheel running at age 3 weeks had a greater increase in active tension; however, even the increased active tension was still less than that of the age matched, trained wildtype mice[53]. In a separate study, EDL and soleus muscles from age 20 week mdx mice initiated to voluntary wheel running at age 4 weeks, had similar increases in tetanic stress and fiber type shifts from IIb to IIa as age matched, trained wildtype mice; however, they still ran significantly less distance each week [81]. This current study confirms that while mdx mice initiated to voluntary

wheel running may be able to benefit from the exercise, they are not able to reach the functional (i.e., distance run) limits as the wildtype mice (Fig. 7A).

Because mdx mice do experience modest benefits from voluntary wheel running, but do not reach the functional capacity of wildtype mice, it suggests exercise alone is not adequate to attenuate the dystrophic process. GTE is a known antioxidant that is extensively studied for its many medicinal uses [83,42]. Although GTE did not fully restore functional capacity of the mdx mouse to that of the C57Bl/10 mouse (Fig. 7A) it did elicit daily distances in the mdx mice that approached those of the C57Bl/10 mice. Murase previously reported increased running endurance in BALB/c mice consuming 0.5% GTE. The increased endurance was mediated by increased use of fat for energy and the sparing of intramuscular glycogen. Additionally, Murase reported that GTE acted post-transcriptionally, as it elicited no increases in PPAR-alpha or gamma expression. Though it has not been determined for all characteristics of muscle, at least with regard to metabolism, dystrophic muscle responds in the same fashion as non-diseased muscle to GTE supplementation. Since fat metabolism is utilized mainly by slow-oxidative fibers, GTE may contribute to a fiber type shift.

Previous studies have demonstrated that GTE delays disease onset in the mdx mouse [22,51].

Buetler reported a dose-dependent protection of C2C12 myotubes from oxidation by GTE; however, this may not be the case in vivo as Dorchies reported no difference in serum antioxidant capacity in mice consuming 0.05% and 0.25% GTE. Dorchies also reported that GTE exerts its beneficial properties by influencing myogenesis and enhancing expression of muscle specific proteins in normal mice. It was yet to be determined whether dystrophic muscle responded in this same manner. This study has shown that this is not the case, as we found no increase in myosin or actin content in mice consuming a GTE based diet. One explanation for this is the short duration of this current study experiment.

The current study demonstrates that GTE decreases lipid peroxidation in the mdx mouse and may elicit functional changes in muscle by altering its metabolism (i.e., increased CS activity). The precise mechanism through which GTE exerts its benefits is unknown; however, it has recently been shown that EGCG binds to laminin receptors [174].

Though not fully tested, a few of the possible beneficial outcomes include: (1) initiation of signaling cascades through laminin receptors such as alpha 7 integrin; (2) increased stabilization of membrane to the ECM via laminin receptor-EGCG-laminin connection; and (3) increased protection of alpha 7 integrin from oxidative damage. The fact that decreased lipid peroxidation was strongly correlated with decreased serum creatine kinase levels (an index of muscle fiber damage) in this current study, supports the possible protection of the sarcolemma from oxidative damage by GTE.

In summary, GTE may be a beneficial approached to attenuating oxidative stress in DMD with or without exercise. Though there are a few studies explore GTE and dystrophic muscle with animal models there is insufficient data on GTE and dystrophic muscle with DMD boys.

Research Hypothesis Conclusion

Main Hypothesis 1: Exercise training did have a beneficial effect on dystrophic muscle. There was a substantial training effect observed during the 3 week training period. Independent of diet, voluntary wheel running improved EDL stress output, active stiffness, MHC content, antioxidant capacity, and fatty acid metabolism. In conclusion, voluntary wheel running when initiated at an early age in mdx male mice is beneficial.

Main Hypothesis 2: GTE had positive effects on running endurance, lipid peroxidation, serum creatine kinase levels, and citrate synthase activity. GTE did not improve contractile/mechanical properties of the EDL, contractile protein content, or serum antioxidant capacity, but was not detrimental. In conclusion, GTE is still a formidable supplement to alleviate a degree of oxidative stress in DMD and may promote cytoskeletal remodeling in dystrophic muscle.

Main Hypothesis 3: None of the data suggested that the SedNorm mice were any better than RunNorm, SedGTE, or RunGTE mice with respect to contractile/mechanical properties, contractile protein content, lipid peroxidation, serum creatine kinase levels, or serum antioxidant capacity.

Main Hypothesis 4: When voluntary wheel running and GTE supplementation were combined there was a marked increase in endurance capacity and significant increases in antioxidant capacity and fatty acid metabolism.

Future Directions

Endurance Exercise: This study investigated initiating voluntary wheel running at age 21 days in mdx mice. In keeping with the theme of exercise prescription, it would be beneficial to control the amount of running to better explore the differences in adaptations to exercise experienced by mdx mice and wildtype mice. Additionally, it would be beneficial to explore placing load on the wheel to elicit muscle hypertrophy.

Green Tea Extract: Because this study revealed that 0.5% GTE attenuated lipid peroxidation and increased CS activity, it would be beneficial to explore the mechanisms behind these findings. Because EGCG can bind to laminin receptors, GTE supplementation on mdx/alpha-7 integrin knockout mice might help determine a particular mechanism through which GTE protects membranes from oxidative damage.

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Appendix A: Methods

Appendix A

Isometric Contractile and Mechanical Properties

At age 6 weeks, after 3 weeks of voluntary run training, mice were deeply anesthetized (2 mg xylazine-20 mg ketamine per 100 g of body mass ip), and the fast-twitch extensor digitorum longus (EDL) muscles were excised. Additional muscles were also excised and assayed as noted below. EDL muscles were incubated at 30ºC in an oxygenated (95% O_2-5% CO_2) physiological salt solution (PSS) as previously described [190]. Non-absorbable braided silk suture (4-0) was tied to the distal and proximal tendons at the myotendinous junctions. EDL muscles were then fixed between a clamp and arm of a dual-mode servomotor system (300B, Aurora Scientific) at a resting tension (L_0) of 1.0 g. EDL muscles were randomly selected as stimulated or control muscles. The stimulated muscle underwent all experimental steps as described below; the control muscle was hung in the bath at 1.0 g resting tension but was not subjected to any of the experimental steps. Both muscles were maintained at L_0 by a stepper motor (Wolff et al., 2006). The servomotor arm and stepper motor were controlled by Dynamic Muscle Control software (DMC Version 4.1.6, Aurora Scientific) to obtain the position and force output data.

The stimulated muscle protocol consisted of 5 steps: (1) a pre-twitch and tetanus; (2) a single passive stress relaxation; (3) a single active stretch; (4) a fatigue protocol; and (5) a post tetanus. The first and fifth steps were performed to determine the effects of steps 2 - 4 on contractile capability (Wolff et al., 2006). In step 1, the stimulated muscle was subjected to three isometric twitches and tetani (150 Hz) spaced 1 minute apart. In step 2, the muscle was stretched instantaneously to 1.05 L_0, held for 7.0 s, and then returned to 1.00 L_0 to determine passive parallel elastic stiffness (Wolff et al., 2006). After 5 minutes at L_0, the muscle was stimulated at 80 Hz for 700 ms (step 3). During the final 200 ms, the muscle was stretched at 0.5 L_0/s to a total strain of 0.1 L_0 (i.e., an eccentric contraction) to determine active stiffness. In step 4, the muscle was subjected to a 1 s submaximal tetanus (60 Hz) at a rate of 12 tetani/min for 5 min to assess fatigue.

At 1 min intervals, beginning with the prefatigue measurement, the muscle force output was recorded. After the fatigue protocol, the muscle was quiescent for 20 minutes at L_0, and was then subjected to a final tetanus (150 Hz, step 5). At the conclusion of the protocol, each muscle was weighed to the nearest 0.1 mg using an A-200D electronic analytical balance (Denver Instruments, Denver, Colorado) and snap frozen in liquid nitrogen.

Contractile and Mechanical Properties

Force and position output profiles were analyzed using Dynamic Muscle Analysis software (DMA Version 3.2, Aurora Scientific). For each twitch, the peak force, the time to peak stress (TPS), and half-relaxation time (HRT) were determined. Muscle cross-sectional area (CSA) was determined as previously described (Grange et al., 2002). Twitch and tetanic forces were normalized to muscle CSA to obtain twitch and tetanic stress. Passive parallel elastic stiffness (g/mm^2) for step 2 [190], and active stiffness for step 3 were determined as previously described [75].

Contractile Protein

Individual tibialis anterior (TA) were homogenized in 10 mM phosphate buffer (pH 7.0), assayed in triplicate using the BCA Protein Assay (Pierce), and then subjected to SDS-PAGE to determine sample myosin heavy chain and actin contents [122]. Briefly, homogenates were diluted with an equal volume of Laemmli sample buffer containing 5% beta-mercaptoethanol and boiled for 3 min. Ten µg total protein from each sample were separated on a 4% stacking, 7.5% separating Tris SDS-PAGE gel run at 150 V for 93 min. Four myosin heavy chain standards (2,3,4,5 ug) and four actin standards (1,2,3,4 ug) and 11 samples were run on each gel. Purified rabbit MHC was a kind gift of Dawn Lowe (University of Minnesota) and actin was purchased from Sigma (A2522). TA and diaphragm muscles were run on separate gels. Gels were stained for 1 hour with 0.1% Coomassie Blue R-250, 30% methanol, 10% glacial acetic acid, and then destained overnight in 20% methanol and 10% glacial acetic acid. Stained gels were scanned using an EPSON (Expression 1680) Twain imaging densitometer and analyzed using SynGENE. Linear regressions of the optical density for MHC and actin protein standards were used to determine the contents of MHC and actin proteins for each sample

Myosin Heavy Chain Isoforms

The proportional content of MHC isoforms was determined using a modified method of Talmadge and Roy [175]. Briefly, frozen TA muscle obtained from SedNorm, RunNorm, SedG, and RunGTE mice aged 42 days were thawed on ice in microcentrifuge tubes, homogenized in 200 µL sample buffer using a micropestle, and heated to 60º C for 10 min. Each sample was subjected to SDS-PAGE using 4% stacking and 8% separating gels. Samples were run on a 20-cm vertical slab gel unit (CBS Scientific) for 40 h at a constant current of 4.2 mA. Gels were stained with Coomassie blue, destained, and scanned using an image analysis system (Alpha Imager 2000). Band densities for each MHC isoform were expressed as a percentage of total MHC band density.

Serum Creatine Kinase

Blood from cardiac puncture was collected (~1 mL) directly into Microtainer serum separator tubes (Becton Dickinson), and refrigerated (4°C) for 30 minutes to clot blood. The tubes were then centrifuged (Brinkmann Instruments, Inc., 5417 R) at 10,000 rpm for 10 minutes at 4ºC to separate serum. Serum was stored at -80ºC until analyzed for CK concentration by the Clinical Pathology Laboratory at Virginia Tech, using an Olympus AU400 chemistry analyzer (Olympus America, Center Valley, PA).

Serum Antioxidant Capacity

The ability of each serum sample to inhibit ABTS (2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) oxidation by metmyoglobin was determined with an antioxidant assay kit (Cayman Chemical). Briefly, each serum sample was diluted 1:20 with assay buffer (5 mM potassium phosphate, pH 7.4, containing 0.9% sodium chloride and 0.1% glucose). A Trolox standard range (0 - 0.660 mM) was obtained by diluting a 1 M Trolox stock solution with the same assay buffer. Trolox is a water-soluble tocopherol analogue. Reactions in duplicate were performed in a 90 well plate. To each well were added: 10 µl metmyoglobin, 150 µl chromogen, 10 µl of either standard or sample , and 40 ul of 441µM H_2O_2 to initiate the reaction. The plate was covered, placed on a shaker at low speed for 5 min, and then read at 750 nm (Bio-Tek Instruments, Inc., µQuant). Linear regressions of the absorption versus Trolox standard concentrations were used to determine the antioxidant capacity of the samples.

Lipid Peroxidation

Individual gastrocnemius muscles were carefully excised and 25 mg placed in a 1.5mL sample tube. Tissues were homogenized in 250 uL RIPA buffer and sonicated (550 Sonic Dismembrator, Fisher Sci.) at 40V over ice for 15 seconds. Homogenates were assayed in duplicate using the TBARS Assay Kit (Cayman Chemical) to determine the content of malondialdehyde (MDA). Homogenates were boiled for 1 hour, incubated on ice for 10 minutes, and then centrifuged at 1600 x g at 4ºC for 10 minutes. Samples were then loaded with standards onto a 90 well plate and read at 530 nm. Linear regressions of the absorption for MDA standards were used to determine the amount of MDA in each sample. Standards ranging from 0 to 50 uM MDA were prepared by diluting a 125 uM MDA stock solution with HPLC-grade water.

Citrate Synthase Activity

Citrate synthase catalyzes the formation of citrate and CoASH from acetyl-CoA and oxaloactetate. Because CoASH reduces DTNB, CS activity was determined for each sample in duplicate from the reduction of DTNB over time. Briefly, ten µl of a 1:5 diluted muscle homogenate (previously diluted 1:20 (1mg/µl in 10 mM phosphate buffer)) was added to 170 µl of a solution containing Tris buffer (0.1M, pH 8.3), DNTB (1mM, in 0.1M in Tris buffer) and oxaloacetate (0.01M, in 0.1M Tris buffer). Following a 2 min background reading, 30µl of 3 mM acetyl CoA was added to initiate the reaction. Absorbance was measured on a spectrophotometer (SPECTRAmax ME, Molecular Devices Corporation, Sunnyvale California) at 405 nm every 12 seconds for 7 minutes at 37℃. Maximum CS activity was calculated and reported as µmol/mg protein/min [169].

Beta-Hydroxy Acyl-CoA Dehydrogenase Activity

The third reaction of the Beta-oxidation cycle is the oxidation of the hydroxyl group at the Beta-position to produce a B-ketoacyl-CoA derivative. The second oxidation reaction is catalyzed by L-hydroxyacyl-CoA dehydrogenase, an enzyme that requires NAD^+ as a coenzyme. Each NADH produced in mitochondria by this reaction drives the synthesis of 2.5 molecules of ATP in the electron transport pathway. For the determination of β-hydroxyacyl-CoA dehydrogenase, oxidation of NADH to NAD was measured in triplicate. Briefly, 35 µl of whole muscle homogenate (1:20 wt/vol (mg/µl)) was added to 190µl of buffer containing 0.1M liquid triethanolamine, 5mM EDTA tetrasodium salt dihydrate, 0.45mM NADH and 15 µl of 2mM acetoacetyl CoA to

initiate the reaction. Absorbance was measured on a spectrophotometer (SPECTRAmax PLUS 384, Molecular Devices Corporation, Sunnyvale California) at 340 nm every 12 seconds for 6 minutes at 37ºC. Maximum BHAD activity was calculated and reported as μmol/mg protein/min [35].

Statistics

Results are expressed as means ± SE for figures and means (SD) for tables. Data were analyzed by a two-way ANOVA with two between factors, diet and running. Significant main effects and/or interactions were assessed by one-way ANOVA, and the differences between means determined by Tukey's HSD post hoc analysis ($P<0.05$). Daily voluntary wheel running data for the RunNorm and RunGTE groups were analyzed by a repeated measures AVOVA ($P<0.05$). In this report, we described a main effect of running as "independent of diet" (i.e., RunNorm and RunGTE data combined compared to SedNorm and SedGTE data combined), and reported a main effect of diet as "independent of running" (i.e., SedNorm and RunNorm data combined compared to SedGTE and RunGTE data combined). Relevant means in figures are plotted as follows: (1) treatment means were plotted if an interaction was significant; (2) if there were no interaction, but both main effects were significant, then treatment means were plotted; and (3) if there was no interaction and only one main effect was significant, then factor means were plotted.

Appendix B: Raw Data

Appendix B

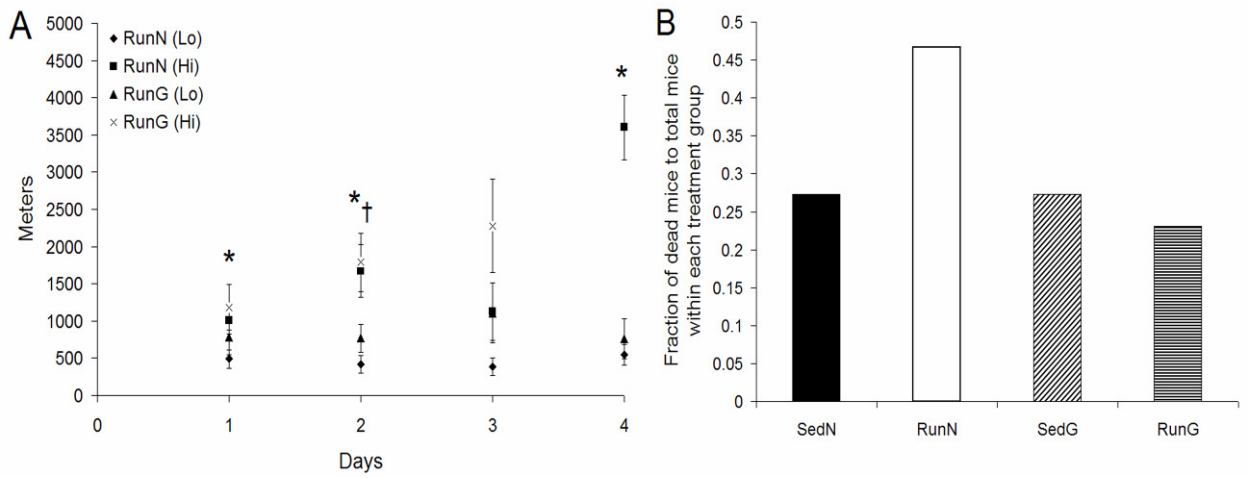

Fig. 13 – Mdx mice that voluntarily exceed a distance threshold the first night (e.g., Hi runners) die within 4 days; those runners in the Lo group all successfully completed the 3 weeks of training. A: RunNorm and RunGTE mice divided into Hi and Lo running groups. B: The fraction of mice within each treatment group that died with in the first days of training: (SedNorm: 3/11) (RunNorm: 7/15) (SedGTE: 3/11) (RunGTE 3/13). * RunNorm (Hi) is greater than RunNorm (Lo). † RunGTE (Hi) is greater than RunGTE (Lo).

Jackson Laboratory Communications

Dear Jarrod,

Thank you for your inquiry. Our colony is maintained using homozygous female (mdx/mdx) x hemizygous male (mdx/Y) matings.
C57BL/10ScSn-Dmd<mdx>/J mice (Stock #001801) are characterized as "fragile"
by the colony manager who is responsible for their care. They are particularly sensitive to noise. Handling them slowly and gently, and maintaining them in a quiet environment is recommended. Although adult Dmd<mdx> homozygous mice often breed well, multiple customers have reported problems maintaining this line, and we occasionally have had problems with high mortality among pups in our colony as well.

In our facility, females in our 001801 colony average 6 pups per litter and average 5 litters during their ~ 6 month breeding rotation. First litters typically are born ~ 3 weeks after pairing. Considering their neuromuscular phenotype, our colony manager describes these mice as "great" breeders, provided the mice are handled carefully, with only 1% becoming
non-productive prior to retirement age. Runts are frequently produced in
our colony (incidence ~10-20%). These mice are culled, but about 90% of the remaining pups survive to weaning. Mice are weaned at 4 weeks of age.
There can be some variation in the severity of the phenotype, so it is advisable to avoid the most severely effected mice when selecting mice as breeders.

I hope that this information is helpful to you.

Sincerely,

Peter

Peter Kelmenson
Technical Information Scientist
The Jackson Laboratory
610 Main St. _ Bar Harbor, ME 04609
Ph: 1-800-422-MICE (6423) _ Fax: 1-207-288-6629
email: micetech@jax.org
Technical Information Express Email Form:
http://jaxmice.jax.org/micetech/ In Vivo Research Systems--offering state-of-the-art phenotyping and compound dosing studies for efficacy and adverse effects testing.
Learn more now at http://jaxmice.jax.org/services/invivo.html

Dupont-Versteegden Communication
From: Jarrod Call [mailto:trijc@vt.edu]
Sent: Monday, August 06, 2007 5:03 PM
To: Dupont-Versteegden, Esther E
Subject: RE: Voluntary wheel running in mdx mice

Hi Dr. Dupont-Versteegden,

My name is Jarrod Call and I contacted you in May about your 1994 article on the effects of voluntary wheel running on mdx mice diaphragms. In my current study, I am using male mdx mice that began running at age 21 days. I observe 2 different groups of runners: one that runs < ~750 meters the first few nights and gradually increases daily distance over a three week period; and a second group that runs 2 – 4 times the distance of the low runners the first few nights, but then the mice in the latter group become lethargic and die within the first 3-4 days of training. I was curious as to whether or not you happened to observe similar behavior and mortality in your 1994 study, or have you heard of this occurring in any other studies? Your help would be much appreciated.

Sincerely,
Jarrod Call
trijc@vt.edu

Dear Jarrod,

No, I did not observe the death after running. I can not recall any of the animals dying after they started running. I have not heard this before. Sorry.

Esther

Esther E. Dupont-Versteegden, Ph.D.
Associate Professor
Division of Physical Therapy, Dept. Rehabilitation Sciences
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Carter Communications

----- Original Message -----
From: Jarrod Call
To: gtcarter@u.washington.edu
Sent: Thursday, May 17, 2007 1:50 PM
Subject: voluntary wheel running in mdx mice

Hi, Dr. Carter.

My name is Jarrod Call and I am currently conducting a mdx mouse running study at Virginia Tech. I was doing a literature review on voluntary wheel running in mdx mice and came across your paper Effect of voluntary wheel-running exercise on muscles of the mdx mouse. I was just writing to confirm whether or not you used both male and female mdx mice for runners in this study? Thank you for your help.

Sincerely,

Jarrod Call

trijc@vt.edu

From: Greg Carter [mailto:gtcarter@comcast.net]
Sent: Wednesday, May 23, 2007 2:36 PM
To: Jarrod Call
Subject: Re: voluntary wheel running in mdx mice

wow, what a blast from the past...the mice were all male to control for the hormone effect and the fact that we were comparing to the mdx mouse, which is an x-linked disease affecting only male mice

Feel free to ask any other questions...I have stopped doing wheel running studies but am still active in this area of research

Greg C

----- Original Message -----
From: Jarrod Call
To: 'Greg Carter'
Sent: Monday, August 06, 2007 1:59 PM
Subject: RE: voluntary wheel running in mdx mice

Hi Dr. Carter,

My name is Jarrod Call and I contacted you in May about your 1995 article on the effects of voluntary wheel running on mdx mice. In my current study, I am using male mdx mice as you did, but I initiated running at age 21 vs 28 days. I observe 2 different groups of runners: one that runs < ~750 meters the first few nights and gradually increases daily distance over a three week period; and a second group that runs 2 – 4 times the distance of the low runners the first few nights, but then the mice in the latter group become lethargic and die within the first 3-4 days of training. I know you mentioned you are no longer involved in running studies, but since you are still active in the field, did you happen to observe similar behavior and mortality in your 1995 study, or have you heard of this occurring in any other studies? Your help would be much appreciated.

Sincerely,
Jarrod Call
trijc@vt.edu

From: Greg Carter [mailto:gtcarter@comcast.net]
Sent: Saturday, August 11, 2007 5:34 PM
To: Jarrod Call
Cc: Jay Han; Ted Abresch
Subject: Re: voluntary wheel running in mdx mice

Jarrod:

Yes, actually that is similar to what I (and others have observed). What we observed, as best I can recall now, is that a) mdx mice, when left in their cages and allowed to roam or run "ad libitum", will exercise less and overall have less movement in general; b) there is some evidence that strenuous exercise in mdx mice is associated with increased mortality...probably due to cardiac issues, but to my knowledge, no one has looked at this in detail.

I am copying this to Dr. Jay Han, who was a research fellow here at the University of Washington (where I am now) and is now faculty at the University of California, Davis (where I was when this work was done). I am also copying Ted Abresch, who is the director of research at the University of California, Davis and probably knows as much about exercise in mdx mice as anyone in the entire world. Perhaps these guys could collaborate with you. I think the mouse gym is still around, although ther! ! e may be a heavy layer of dust on it (along with mice feces...) I would also be interested in being involved, primarily from an intellectual curiosity standpoint as I still think there are many, many ***unanswered, important questions*** to be looked at regarding exercise in dystrophinopathy and the mdx mouse is a great model to use given its size and homologous genotype

Greg C~

---------- Original message ----------
From: "Ted Abresch" <rtabresch@ucdavis.edu>
Hi Jarrod,

I don't recall that we had any increased mortality in the mice that were allowed to run ad libitum on the exercise wheels. I do recall that we had some major differences in the amount that each of the mice ran, but we attributed to just differences in the individual mice. However, I recall that there was almost no overlap between the amount that the mdx and control mice ran. It seems to me that you may need to run many more mice to chase down the cause of this response. I don't know if your campus vet could help discern changes in cardiac function. Perhaps Jackson labs could shed some light on the matter. You might want to find out their mortality data with their mdx mice.

R. Ted Abresch
Director of Research, Center for Neuromuscular Disease
Med: PM&R, TB 191, UC Davis
One Shields Avenue, Davis CA 95616
(530) 752-9085 (p)
(530) 752-3468 (f)

Thanks Ted...those are great ideas. Jarrod, I would definitely call Jackson labs. They breed the mice and care for them so they are going to have the best mortality info.

Ted, I seem to recall that we did lose some mdx mice in the exhaustive wheel running protocol, no?

I will try and dig out my old coffee-stained lab book. That work was done when I was a fellow, hard to believe...that was 15+ years ago

I am glad you guys are looking at this. Where are you submitting the work? I would suggest Muscle & Nerve. I am on the editorial board. Request me as a reviewer (that is all allowed for this journal, which is not blinded, remarkably). Of course I can't guarantee acceptance but I will give you a fair review and advocate for you, if appropriate.

Let me know what you find out from Jackson labs...

Greg

ID	Genotype	Diet	Activity	Initial Body Mass, g	Week 1 Body Mass, g	Week 2 Body Mass, g	Week 3 Body Mass, g
914	mdx	GTE	Run	10.3	12.4	19.1	21.8
907	mdx	GTE	Run	11.2	15.9	22.7	26.4
555	mdx	GTE	Run	13.3	17.2	23.2	27.2
1111	mdx	GTE	Run	12.3	15.6	20.9	24.3
1212	mdx	GTE	Run	14.3	18.0	26.3	29.0
919	mdx	GTE	Run	13.4	20.5	21.0	25.7
910	mdx	GTE	Run	11.7	17.2	26.3	28.9
968	mdx	GTE	Run	10.7	17.3	19.5	21.0
333	mdx	GTE	Run	10.1	16.4	22.7	26.4
1313	mdx	GTE	Run	9.5	15.5	21.9	25.0
915	mdx	GTE	Sedentary	9.4	15.3	20.1	19.7
916	mdx	GTE	Sedentary	11.8	19.0	25.3	25.3
970	mdx	GTE	Sedentary	11.7	17.5	21.7	23.8
971	mdx	GTE	Sedentary	11.4	15.3	20.8	24.2
1	mdx	GTE	Sedentary	5.5	15.7	17.8	20.3
2	mdx	GTE	Sedentary	8.8	13.3	16.5	19.1
1024	mdx	GTE	Sedentary	11.5	15.3	17.5	22.3
1025	mdx	GTE	Sedentary	9.6	14.6	16.8	20.0
663	mdx	Normal	Run	10.3	15.1	22.3	24.6
665	mdx	Normal	Run	11.2	17.2	23.9	25.8
668	mdx	Normal	Run	9.3	15.9	23.3	23.1
669	mdx	Normal	Run	7.0	14.7	20.0	25.2
696	mdx	Normal	Run	6.4	12.2	19.6	21.4
700	mdx	Normal	Run	8.9	15.4	22.0	24.2
697	mdx	Normal	Run	9.0	14.1	20.0	21.7
903	mdx	Normal	Run	9.4	15.2	23.7	24.9
661	mdx	Normal	Sedentary	8.9	12.3	20.0	23.5
664	mdx	Normal	Sedentary	9.8	12.3	18.5	21.6
676	mdx	Normal	Sedentary	12.8	13.9	17.5	21.4
677	mdx	Normal	Sedentary	12.3	14.1	17.3	22.3
673	mdx	Normal	Sedentary	10.7	10.7	13.7	18.2
672	mdx	Normal	Sedentary	12.3	12.6	15.4	20.0
901	mdx	Normal	Sedentary	10.1	10.4	21.1	24.7
1023	mdx	Normal	Sedentary	8.6	10.3	15.6	21.2

ID	Genotype	Diet	Activity	Wk 1 Food, g/g bm/wk	Wk 2 Food, g/g bm/wk	Wk 3 Food, g/g bm/wk	Total Dist. Wk 1, meters	Total Dist. Wk 2, meters	Total Dist. Wk 3, meters
914	mdx	GTE	Run	1.27	1.35	1.39	7928	16645	62814
907	mdx	GTE	Run	1.46	1.19	1.26	6437	14176	35220
555	mdx	GTE	Run	1.38	1.25	1.47	2594	34683	97014
1111	mdx	GTE	Run	1.70	1.29	1.60	2911	19580	53593
1212	mdx	GTE	Run	1.72	1.42	1.08	2320	21572	72233
919	mdx	GTE	Run	1.66	1.38	1.45	4192	19946	42655
910	mdx	GTE	Run	1.26	1.40	1.59	7219	23141	62653
968	mdx	GTE	Run	2.40	1.62	1.85	8271	19740	50543
333	mdx	GTE	Run	1.54	1.35	1.40	3406	20047	57825
1313	mdx	GTE	Run	1.50	1.58	1.85	9068	26295	78564
915	mdx	GTE	Sedentary	1.33	1.24	1.39			
916	mdx	GTE	Sedentary	1.44	1.25	1.43			
970	mdx	GTE	Sedentary	1.20	1.38	1.45			
971	mdx	GTE	Sedentary	2.29	1.83	1.37			
1	mdx	GTE	Sedentary	2.17	1.96	1.63			
2	mdx	GTE	Sedentary	1.53	1.52	1.43			
1024	mdx	GTE	Sedentary	2.14	1.78	1.55			
1025	mdx	GTE	Sedentary	2.09	1.75	1.54			
663	mdx	Normal	Run	1.60	1.41	1.22	3762	12599	25041
665	mdx	Normal	Run	1.63	1.31	1.11	2816	10560	25382
668	mdx	Normal	Run	1.62	1.36	1.42	2555	9729	27060
669	mdx	Normal	Run	1.98	1.25	1.35	6424	24622	47559
696	mdx	Normal	Run	2.16	1.39	1.41	775	3918	12157
700	mdx	Normal	Run	2.06	1.32	1.20	1307	3945	10938
697	mdx	Normal	Run	2.15	1.46	1.29	6673	18819	37471
903	mdx	Normal	Run	1.78	1.35	0.93	5254	11891	28647
661	mdx	Normal	Sedentary	1.42	1.31	1.26			
664	mdx	Normal	Sedentary	1.42	1.42	1.37			
676	mdx	Normal	Sedentary	1.03	0.97	0.95			
677	mdx	Normal	Sedentary	1.01	0.98	0.91			
673	mdx	Normal	Sedentary	1.33	1.24	1.12			
672	mdx	Normal	Sedentary	1.13	1.10	1.02			
901	mdx	Normal	Sedentary	1.35	1.36	0.89			
1023	mdx	Normal	Sedentary	1.23	1.10	1.00			

ID	Genotype	Diet	Activity	EDL Length, mm	EDL Mass, mg	EDL CSA, mm^2	Time to Peak Tension, s	Half-Relaxation, s
914	mdx	GTE	Run	10.1	6.6	0.62	0.012	0.039
907	mdx	GTE	Run	10.1	8.5	0.80	0.012	0.020
555	mdx	GTE	Run	10.0	8.5	0.80	0.011	0.024
1111	mdx	GTE	Run	10.5	9.0	0.81	0.012	0.021
1212	mdx	GTE	Run	11.0	11.0	0.95	0.011	0.022
919	mdx	GTE	Run	9.4	9.0	0.91	0.013	0.020
910	mdx	GTE	Run	10.0	9.0	0.85	0.011	0.026
968	mdx	GTE	Run	11.0	9.2	0.79	0.011	0.015
333	mdx	GTE	Run	10.0	10.0	0.95	0.010	0.017
1313	mdx	GTE	Run	10.5	10.5	0.95	0.010	0.019
915	mdx	GTE	Sedentary	9.8	7.3	0.71	0.015	0.031
916	mdx	GTE	Sedentary	9.8	6.7	0.65	0.015	0.031
970	mdx	GTE	Sedentary	10.0	6.3	0.60	0.015	0.038
971	mdx	GTE	Sedentary	10.6	7.5	0.67	0.015	0.034
1	mdx	GTE	Sedentary	10.5	7.5	0.68	0.012	0.024
2	mdx	GTE	Sedentary	10.0	8.5	0.80	0.013	0.030
1024	mdx	GTE	Sedentary	8.5	6.5	0.72	0.016	0.022
1025	mdx	GTE	Sedentary	8.5	8.7	0.97	0.011	0.016
663	mdx	Normal	Run	9.7	8.3	0.81	0.010	0.018
665	mdx	Normal	Run	10.8	9.0	0.79	0.012	0.023
668	mdx	Normal	Run	9.6	8.0	0.79	0.011	0.023
669	mdx	Normal	Run	11.2	8.4	0.71	0.012	0.026
696	mdx	Normal	Run	10.1	8.0	0.75	0.012	0.031
700	mdx	Normal	Run	10.8	7.9	0.69	0.015	0.038
697	mdx	Normal	Run	11.0	8.0	0.69	0.011	0.026
903	mdx	Normal	Run	11.0	9.0	0.77	0.011	0.026
661	mdx	Normal	Sedentary	9.7	8.5	0.83	0.011	0.024
664	mdx	Normal	Sedentary	9.8	7.3	0.71	0.012	0.031
676	mdx	Normal	Sedentary	9.1	8.5	0.88	0.012	0.020
677	mdx	Normal	Sedentary	9.7	8.2	0.80	0.012	0.030
673	mdx	Normal	Sedentary	8.5	7.1	0.79	0.013	0.029
672	mdx	Normal	Sedentary	9.0	7.3	0.77	0.013	0.021
901	mdx	Normal	Sedentary	9.8	8.3	0.80	0.010	0.023
1023	mdx	Normal	Sedentary	9.8	8.5	0.82	0.013	0.025

ID	Genotype	Diet	Activity	Twitch Stress, g/mm^2	Tetanic Stress, g/mm^2	Passive Stiffness, MPa	Active Stiffness, MPa
914	mdx	GTE	Run	4.3	21.1	1.7	2.3
907	mdx	GTE	Run	4.5	21.9	0.9	1.7
555	mdx	GTE	Run	4.5	24.2	0.9	1.5
1111	mdx	GTE	Run	5.2	28.0	1.1	1.8
1212	mdx	GTE	Run	3.9	23.7	0.9	1.5
919	mdx	GTE	Run	4.2	24.5	1.4	1.8
910	mdx	GTE	Run	4.9	30.1	1.7	2.1
968	mdx	GTE	Run	3.0	21.4	0.6	1.4
333	mdx	GTE	Run	4.7	24.9	0.7	1.5
1313	mdx	GTE	Run	4.6	32.2	0.8	1.5
915	mdx	GTE	Sedentary	3.9	17.2	1.9	1.5
916	mdx	GTE	Sedentary	4.0	21.4	2.1	2.2
970	mdx	GTE	Sedentary	4.6	28.5	1.5	1.9
971	mdx	GTE	Sedentary	4.1	22.4	0.9	1.6
1	mdx	GTE	Sedentary	3.5	22.3	1.5	1.7
2	mdx	GTE	Sedentary	4.3	18.7	2.5	2.0
1024	mdx	GTE	Sedentary	3.9	19.8	1.0	1.5
1025	mdx	GTE	Sedentary	2.9	20.5	2.4	1.1
663	mdx	Normal	Run	5.1	25.8	0.8	1.3
665	mdx	Normal	Run	5.5	32.3	0.9	2.0
668	mdx	Normal	Run	1.7	7.0	1.3	
669	mdx	Normal	Run	5.0	27.3	2.0	2.4
696	mdx	Normal	Run	4.2	22.8	1.5	1.6
700	mdx	Normal	Run	5.3	22.5	1.2	2.0
697	mdx	Normal	Run	5.3	23.5	1.7	2.4
903	mdx	Normal	Run	4.4	22.1	1.0	1.7
661	mdx	Normal	Sedentary	4.3	25.2	1.1	
664	mdx	Normal	Sedentary	3.7	18.6	1.9	
676	mdx	Normal	Sedentary	4.9	20.5	1.0	1.2
677	mdx	Normal	Sedentary	4.2	17.4	1.4	1.4
673	mdx	Normal	Sedentary	4.6	20.2	1.2	1.4
672	mdx	Normal	Sedentary	4.0	21.2	1.3	1.5
901	mdx	Normal	Sedentary	3.0	10.5	0.6	1.4
1023	mdx	Normal	Sedentary	4.2	23.7	0.9	1.4

ID	Genotype	Diet	Activity	Lipid Peroxidation, uM MDA	Creatine Kinase, U/L	ANTIOXIDANT Capacity, mM Trolox
914	mdx	GTE	Run	0.132	3276	163
907	mdx	GTE	Run	0.121	3557	167
555	mdx	GTE	Run	0.169		166
1111	mdx	GTE	Run	0.092	2349	161
1212	mdx	GTE	Run	0.144	3221	
919	mdx	GTE	Run	0.121	3085	133
910	mdx	GTE	Run	0.185	5646	171
968	mdx	GTE	Run	0.094	2493	175
333	mdx	GTE	Run	0.093		
1313	mdx	GTE	Run	0.085		
915	mdx	GTE	Sedentary	0.064	1376	122
916	mdx	GTE	Sedentary	0.074	1453	135
970	mdx	GTE	Sedentary	0.070	1975	153
971	mdx	GTE	Sedentary	0.065	2537	141
1	mdx	GTE	Sedentary	0.069	2966	136
2	mdx	GTE	Sedentary	0.075	3259	134
1024	mdx	GTE	Sedentary	0.126	4544	146
1025	mdx	GTE	Sedentary	0.076		
663	mdx	Normal	Run	0.498	4880	163
665	mdx	Normal	Run	0.097	2839	169
668	mdx	Normal	Run	0.394	3439	152
669	mdx	Normal	Run	0.244	3917	155
696	mdx	Normal	Run	0.207	2770	164
700	mdx	Normal	Run	0.125	3603	162
697	mdx	Normal	Run	0.125	2137	85
903	mdx	Normal	Run	0.210		
661	mdx	Normal	Sedentary		10000	100
664	mdx	Normal	Sedentary	0.449	4099	113
676	mdx	Normal	Sedentary	0.259	6589	131
677	mdx	Normal	Sedentary	0.552	9500	135
673	mdx	Normal	Sedentary	0.167	7863	132
672	mdx	Normal	Sedentary	0.439	1963	75
901	mdx	Normal	Sedentary	0.100	1631	144
1023	mdx	Normal	Sedentary	0.345		

ID	Genotype	Diet	Activity	Actin, ug	Myosin, ug	Total Prot, %	Cont. Prot., %
914	mdx	GTE	Run	1.3	3.2	20.1	45.4
907	mdx	GTE	Run	1.6	3.6	17.1	52.3
555	mdx	GTE	Run	1.5	3.1	15.4	45.9
1111	mdx	GTE	Run	1.5	3.6	19.1	50.7
1212	mdx	GTE	Run	1.4	3.1	15.5	45.1
919	mdx	GTE	Run	1.4	3.2	17.8	46.2
910	mdx	GTE	Run	1.3	3.3	15.0	46.9
968	mdx	GTE	Run	1.5	3.7	19.2	51.4
333	mdx	GTE	Run	1.5	3.7	14.8	52.0
1313	mdx	GTE	Run	1.6	3.6	14.6	52.2
915	mdx	GTE	Sedentary	1.2	2.7	14.1	38.5
916	mdx	GTE	Sedentary	1.4	3.2	16.0	45.7
970	mdx	GTE	Sedentary	1.4	3.0	14.9	43.9
971	mdx	GTE	Sedentary	1.4	3.0	15.1	44.1
1	mdx	GTE	Sedentary	1.5	3.5	17.0	49.7
2	mdx	GTE	Sedentary	1.4	3.4	17.2	48.4
1024	mdx	GTE	Sedentary	1.7	3.6	15.6	53.0
1025	mdx	GTE	Sedentary	1.4	3.0	15.2	43.6
663	mdx	Normal	Run	1.5	3.3	18.5	48.2
665	mdx	Normal	Run	1.4	3.2	19.1	46.1
668	mdx	Normal	Run	1.5	3.4	18.7	49.0
669	mdx	Normal	Run	1.8	3.1	16.7	49.1
696	mdx	Normal	Run	1.3	2.9	16.1	42.2
700	mdx	Normal	Run	1.5	3.0	17.9	45.0
697	mdx	Normal	Run	1.2	3.4	16.2	46.9
903	mdx	Normal	Run	1.5	3.5	17.6	49.1
661	mdx	Normal	Sedentary	1.4	3.3	17.9	46.4
664	mdx	Normal	Sedentary	1.3	3.1	17.8	44.4
676	mdx	Normal	Sedentary	1.3	3.0	18.5	43.5
677	mdx	Normal	Sedentary	1.7	3.0	16.3	47.1
673	mdx	Normal	Sedentary	1.4	3.5	16.3	49.0
672	mdx	Normal	Sedentary	1.5	2.6	15.8	41.6
901	mdx	Normal	Sedentary	1.1	2.9	14.8	40.2
1023	mdx	Normal	Sedentary	1.5	3.3	16.0	48.2

ID	Genotype	Diet	Activity	Quad CS, umol/mg prot/min	Soleus CS, umol/mg prot/min	BHAD, umol/mg prot/ min	MHC 1, %	MHC 2ax, %	MHC 2b, %
914	mdx	GTE	Run	211	357	260	3.7	38.4	58.0
907	mdx	GTE	Run	305	543	237	8.2	29.1	62.7
555	mdx	GTE	Run	429	608	215			
1111	mdx	GTE	Run	594	605	188			
1212	mdx	GTE	Run	553	411	317	2.2	40.1	57.7
919	mdx	GTE	Run	664	728	231	3.4	41.0	55.6
910	mdx	GTE	Run	440	695	261	4.7	34.9	60.4
968	mdx	GTE	Run	528	279	364	2.8	30.5	66.8
333	mdx	GTE	Run	240	377	223			
1313	mdx	GTE	Run	361		321			
915	mdx	GTE	Sedentary	498	760	211	4.1	44.4	51.6
916	mdx	GTE	Sedentary	477	423	216	2.9	42.0	55.1
970	mdx	GTE	Sedentary	663	670	225	2.6	35.7	61.7
971	mdx	GTE	Sedentary	421	870				
1	mdx	GTE	Sedentary	388	825	131	3.8	38.9	57.4
2	mdx	GTE	Sedentary	478	794	133	0.0	38.1	61.9
1024	mdx	GTE	Sedentary	377	844	176			
1025	mdx	GTE	Sedentary		566	119			
663	mdx	Normal	Run	285	825	209			
665	mdx	Normal	Run	373	807	267			
668	mdx	Normal	Run	206	521		7.8	28.3	63.9
669	mdx	Normal	Run	314	425	314	7.6	31.6	60.8
696	mdx	Normal	Run	225	320	194	8.9	26.7	64.3
700	mdx	Normal	Run	177	343	217	6.7	24.9	68.4
697	mdx	Normal	Run	216	293	175	5.9	43.1	51.0
903	mdx	Normal	Run		617	190	3.5	44.3	52.3
661	mdx	Normal	Sedentary	394	452	139			
664	mdx	Normal	Sedentary	259	447	136	6.7	35.1	58.2
676	mdx	Normal	Sedentary	456	611	241	6.5	46.6	46.9
677	mdx	Normal	Sedentary	293	644	244	6.5	35.7	57.9
673	mdx	Normal	Sedentary	411	496	123	5.8	35.4	58.8
672	mdx	Normal	Sedentary	271	481	133	6.1	35.3	58.7
901	mdx	Normal	Sedentary	235	440	294	0.0	48.0	52.0
1023	mdx	Normal	Sedentary	175		155			

Appendix C: Statistical Analysis

Two-Way Analysis of Variance for Initial Body Mass

Source of Variation	DF	SS	MS	F	P
Diet	1	8.57	8.57	2.8	0.1042
Activity	1	0.00	0.00	0.00	0.9986
Diet x Activity	1	25.31	25.31	8.29	0.0073
Error	30	91.58	3.05		
Total	33	127.27			

Two-Way Analysis of Variance for Week 1 Body Mass

Source of Variation	DF	SS	MS	F	P
Diet	1	59.14	59.14	19.59	0.0001
Activity	1	29.61	29.61	9.81	0.0039
Diet x Activity	1	8.85	8.85	2.93	0.0972
Error	30	90.57	3.02		
Total	33	189.04			

Two-Way Analysis of Variance for Week 2 Body Mass

Source of Variation	DF	SS	MS	F	P
Diet	1	15.18	15.18	2.49	0.1253
Activity	1	110.96	110.96	18.18	0.0002
Diet x Activity	1	5.84	5.84	0.95	0.3360
Error	30	183.13	6.10		
Total	33	316.57			

Two-Way Analysis of Variance for Week 3 Body Mass

Source of Variation	DF	SS	MS	F	P
Diet	1	7.86	7.86	1.57	0.2195
Activity	1	75.35	75.35	15.07	0.0005
Diet x Activity	1	4.63	4.63	0.93	0.3438
Error	30	150.01	5.00		
Total	33	243.84			

Two-Way Analysis of Variance for Week 1 Food Consumption

Source of Variation	DF	SS	MS	F	P
Diet	1	0.13	0.13	1.35	0.2543
Activity	1	0.42	0.42	4.28	0.0473
Diet x Activity	1	1.40	1.40	14.27	0.0007
Error	30	2.94	0.10		
Total	33	4.80			

Two-Way Analysis of Variance for Week 2 Food Consumption

Source of Variation	DF	SS	MS	F	P
Diet	1	0.40	0.40	12.74	0.0012
Activity	1	0.00	0.00	0.08	0.7749
Diet x Activity	1	0.30	0.30	9.59	0.0042
Error	30	0.93	0.03		
Total	33	1.60			

Two-Way Analysis of Variance for Week 3 Food Consumption

Source of Variation	DF	SS	MS	F	P
Diet	1	0.93	0.93	28.57	0.0001
Activity	1	0.08	0.08	2.51	0.1235
Diet x Activity	1	0.05	0.05	1.54	0.2239
Error	30	0.98	0.03		
Total	33	2.05			

Two-Way Analysis of Variance for Week 1 Total Distance

Source of Variation	DF	SS	MS	F	P
Diet	1	13438701	13438701	2.23	0.1550
Activity	0	0	0		
Diet x Activity	0	0	0		
Error	16	96494839	6030927		
Total	17	109933540			

Two-Way Analysis of Variance for Week 2 Total Distance

Source of Variation	DF	SS	MS	F	P
Diet	1	407242423	407242423	10.32	0.0054
Activity	0	0	0		
Diet x Activity	0	0	0		
Error	16	631542226	39471389		
Total	17	1038784649			

Two-Way Analysis of Variance for Week 3 Total Distance

Source of Variation	DF	SS	MS	F	P
Diet	1	5299084588	5299084588	21.57	0.0003
Activity	0	0	0		
Diet x Activity	0	0	0		
Error	16	3931577354	245723585		
Total	17	9230661943			

Two-Way Analysis of Variance for EDL Length

Source of Variation	DF	SS	MS	F	P
Diet	1	0.08	0.08	0.24	0.6306
Activity	1	4.41	4.41	13.40	0.0010
Diet x Activity	1	1.07	1.07	3.25	0.0819
Error	29	9.54	0.33		
Total	32	15.10			

Two-Way Analysis of Variance for EDL Mass

Source of Variation	DF	SS	MS	F	P
Diet	1	0.00	0.00	0.00	0.9626
Activity	1	10.80	10.80	15.00	0.0006
Diet x Activity	1	5.08	5.08	7.05	0.0127
Error	29	20.88	0.72		
Total	32	37.26			

Two-Way Analysis of Variance for EDL CSA

Source of Variation	DF	SS	MS	F	P
Diet	1	0.00	0.00	0.14	0.7092
Activity	1	0.02	0.02	4.08	0.0526
Diet x Activity	1	0.08	0.08	15.70	0.0004
Error	29	0.15	0.01		
Total	32	0.26			

Two-Way Analysis of Variance for TPT

Source of Variation	DF	SS	MS	F	P
Diet	1	0.00	0.00	4.45	0.0437
Activity	1	0.00	0.00	18.33	0.0002
Diet x Activity	1	0.00	0.00	9.93	0.0038
Error	29	0.00	1.43e-6		
Total	32	0.00			

Two-Way Analysis of Variance for Half-Relaxation Time

Source of Variation	DF	SS	MS	F	P
Diet	1	0.00	0.00	0.02	0.89
Activity	1	0.00	0.00	2.68	0.1124
Diet x Activity	1	0.00	0.00	4.67	0.0381
Error	29	0.00	0.00		
Total	32	0.00			

Two-Way Analysis of Variance for Twitch Stress

Source of Variation	DF	SS	MS	F	P
Diet	1	0.36	0.36	0.60	0.4429
Activity	1	1.83	1.83	3.06	0.0904
Diet x Activity	1	0.00	0.00	0.00	0.9878
Error	30	17.97	0.60		
Total	33	20.09			

Two-Way Analysis of Variance for Tetanic Stress

Source of Variation	DF	SS	MS	F	P
Diet	1	33.31	33.31	1.39	0.2471
Activity	1	106.38	106.38	4.45	0.0434
Diet x Activity	1	0.67	0.67	0.03	0.8679
Error	30	717.31	23.91		
Total	33	866.64			

Two-Way Analysis of Variance for Passive Stiffness

Source of Variation	DF	SS	MS	F	P
Diet	1	0.21	0.21	1.03	0.3165
Activity	1	0.60	0.60	2.90	0.0988
Diet x Activity	1	1.38	1.38	6.67	0.0149
Error	30	6.21	0.21		
Total	33	8.41			

Two-Way Analysis of Variance for Active Stiffness

Source of Variation	DF	SS	MS	F	P
Diet	1	0.02	0.02	0.26	0.6162
Activity	1	0.65	0.65	7.24	0.0121
Diet x Activity	1	0.58	0.58	6.46	0.0171
Error	27	2.44	0.09		
Total	30	3.52			

Two-Way Analysis of Variance for Lipid Peroxidation

Source of Variation	DF	SS	MS	F	P
Diet	1	0.27	0.27	25.28	0.0001
Activity	1	0.00	0.00	0.40	0.5299
Diet x Activity	1	0.04	0.04	3.63	0.0667
Error	29	0.31	0.01		
Total	32	0.61			

Two-Way Analysis of Variance for Creatine Kinase

Source of Variation	DF	SS	MS	F	P
Diet	1	19711466	19711466	5.22	0.0314
Activity	1	5618816	5618816	1.49	0.2342
Diet x Activity	1	19852676	19852676	5.26	0.0308
Error	24	90542999	3772625		
Total	27	135725957			

Two-Way Analysis of Variance for Actin Content

Source of Variation	DF	SS	MS	F	P
Diet	1	0.00	0.00	0.11	0.7469
Activity	1	0.02	0.02	1.07	0.3080
Diet x Activity	1	0.00	0.00	0.04	0.8400
Error	30	0.68	0.02		
Total	33	0.71			

Two-Way Analysis of Variance for Myosin Content

Source of Variation	DF	SS	MS	F	P
Diet	1	0.12	0.12	1.79	0.1913
Activity	1	0.31	0.31	4.70	0.0382
Diet x Activity	1	0.03	0.03	0.50	0.4851
Error	30	1.95	0.06		
Total	33	2.44			

Two-Way Analysis of Variance for TA Total Protein Percentage

Source of Variation	DF	SS	MS	F	P
Diet	1	6.46	6.46	2.91	0.0985
Activity	1	9.52	9.52	4.29	0.0471
Diet x Activity	1	0.18	0.18	0.08	0.7778
Error	30	66.67	2.22		
Total	33	82.08			

Two-Way Analysis of Variance for TA Contractile Protein Percentage of Total Protein

Source of Variation	DF	SS	MS	F	P
Diet	1	15.18	15.18	1.35	0.2551
Activity	1	50.16	50.16	4.45	0.0434
Diet x Activity	1	2.23	2.23	0.20	0.6593
Error	30	338.31	11.28		
Total	33	411.20			

Two-Way Analysis of Variance for Serum ANTIOXIDANT Capacity

Source of Variation	DF	SS	MS	F	P
Diet	1	1776.04	1776.04	4.10	0.0543
Activity	1	5432.14	5432.14	12.53	0.0017
Diet x Activity	1	82.29	82.29	0.19	0..6670
Error	24	10406	433.60		
Total	27	17697			

Two-Way Analysis of Variance for Quad CS Activity

Source of Variation	DF	SS	MS	F	P
Diet	1	220588	220588	17.02	0.0003
Activity	1	17356	17356	1.34	0.2569
Diet x Activity	1	487	487	0.04	0.847
Error	28	362855	12959		
Total	31	590966			

Two-Way Analysis of Variance for Soleus CS Activity

Source of Variation	DF	SS	MS	F	P
Diet	1	80666	80666	3.08	0.0904
Activity	1	78280	78280	2.98	0.0951
Diet x Activity	1	92801	92801		
Error	28	734518	26233		
Total	31	988101			

Two-Way Analysis of Variance for BHAD Activity

Source of Variation	DF	SS	MS	F	P
Diet	1	1504	1504	0.49	0.487
Activity	1	32602	32602	10.73	0.0028
Diet x Activity	1	4475	4475	1.47	0.2351
Error	28	85082	14191		
Total	31	127655			

Two-Way Analysis of Variance for MHC 1

Source of Variation	DF	SS	MS	F	P
Diet	1	37.9	37.9	8.47	0.0900
Activity	1	12.5	12.5	2.78	0.1118
Diet x Activity	1	0.001	0.001	0.0001	0.9911
Error	19	85.1	4.48		
Total	22	133.8			

Two-Way Analysis of Variance for MHC 2a/x

Source of Variation	DF	SS	MS	F	P
Diet	1	12.7	12.7	0.33	0.5694
Activity	1	153	153	4.02	0.0592
Diet x Activity	1	5.98	5.98	0.16	0.6959
Error	19	172	57.4		
Total	22	894.3			

Two-Way Analysis of Variance for MHC 2b

Source of Variation	DF	SS	MS	F	P
Diet	1	6.96	6.96	0.25	0.6217
Activity	1	77.38	77.38	2.80	0.1107
Diet x Activity	1	5.95	5.95	0.22	0.6481
Error	19	525	27.6		
Total	22	619.3			

Vita

Jarrod Alan Call, the son of Jerry and Dianna Call, was born on June 11, 1983 in Marion, Ohio.
He graduated from Marion River Valley High School in May 2002. He attended Wittenberg University where he received his Bachelor of Science in Biology in December 2005. Jarrod will graduate with a Master of Muscle Physiology and Biochemistry from Virginia Polytechnic Institute and State University in August 2007.