Identification and Efficacy of Anti-Fibrotic Treatments for Duchenne Muscular Dystrophy Mouse Models

Honors Research Thesis

By

Neeraj S. Chimanji

The Ohio State University
June 2012

Project Advisor: Dr. Jill Rafael-Fortney, Department of Molecular and Cellular Biochemistry
Abstract

Duchenne Muscular Dystrophy (DMD) is an X-linked recessive disease caused by the absence of dystrophin at the muscle membrane. Duchenne Muscular Dystrophy patients suffer from progressive muscle deterioration, including respiratory muscles, and, until more recent advancements, died from skeletal muscle disease at a young age. Today, many patients live long enough to experience cardiomyopathy due to increased cardiac muscle damage and subsequent fibrosis. These patients often die in their late teens or twenties from complications due to heart failure rather than skeletal muscle disease. Through long-term treatment of 4 week and 8 week dystrophin-deficient, utrophin-haploinsufficient (het) mice with antifibrotic medications, we determined whether current standard-of-care drug treatment can be preventative of cardiac dysfunction if started prior to initial signs of damage. Spironolactone and lisinopril drug treatments were applied via water bottles three times per week until the mice reached the age of 20 weeks. Following treatment, cardiac magnetic resonance images (MRI) and both skeletal and cardiac muscle physiology data, along with histological data were obtained. The results of the project indicated that the combinatorial antifibrotic treatment significantly reduces cardiac damage and nearly restores cardiac function in the mouse models that begin treatment at 4 weeks of age. This project has direct implications for the treatment of Duchenne Muscular Dystrophy patients. After concluding the first project, a second project was started in which we tested the efficacy of spironolactone and lisinopril individually in comparison to the combination treatment.
**Introduction**

Duchenne Muscular Dystrophy is an X-linked disease that leads to severe muscle depletion. Duchenne Muscular Dystrophy affects one in every 3300 males, and is caused by mutations of the dystrophin gene (X chromosome P21) in humans, resulting in a lack of the dystrophin protein. Dystrophin localizes around the cytoplasmic face of the entire muscle membrane or sarcolemma and stabilizes the dystrophin glycoprotein complex, thereby protecting muscle membranes from damage (Lapidos, Kakkar et al. 2004).

As the regenerative capacity of skeletal muscle runs out, scar formation, known as fibrosis, becomes prominent. Fibrosis is typified by the deposition of extracellular matrix proteins such as collagen and fibronectin, which can diminish the functionality of muscle tissue. Increased sarcolemmal permeability due to a defective dystrophin-glycoprotein complex in Duchenne Muscular Dystrophy is evidenced by an influx of calcium into the sarcoplasm and the activation of proteases (Blake, Wier et al. 2001). These complications lead to myofiber necrosis and subsequent regeneration. Endomysial fibrosis directly contributes to progressive muscle dysfunction and the lethal phenotype of Duchenne Muscular Dystrophy. Patients are usually wheelchair-bound by the age of eleven and die by their early twenties.

Due to treatment objectives focused on maintaining limb and respiratory skeletal muscle functions such as assisted ventilation and corticosteroids, the longevity of Duchenne Muscular Dystrophy patients’ lives in general has increased significantly over the last five to ten years. As a result, cardiac complications are becoming more apparent since dystrophin has similar functions in both skeletal and
cardiac striated muscles. Approximately 25% of Duchenne Muscular Dystrophy patients exhibit preclinical cardiac involvement by the age of six (Ameen, Robson et al. 2010). Signs of complications involving the heart in Duchenne Muscular Dystrophy cases include dilated cardiomyopathy with reduced systolic function and cardiac arrhythmias such as bradycardic and tachycardic disturbances characterized by irregularities in left ventricular function and size. These cardiac complications typically display a later date of onset than skeletal muscle symptoms in Duchenne Muscular Dystrophy cases. Duchenne Muscular Dystrophy patients exhibit cardiac changes within the first ten to fifteen years of their lives.

Three mouse models for Duchenne Muscular Dystrophy are used in the laboratory. The first of these models, called \textit{mdx}, lacks dystrophin due to a mutation that results in a premature stop codon in exon 23 (Sickinci, Geng et al. 1989). \textit{Mdx} muscle appears histologically normal in the immediate postnatal period. Although these mice express the same genotype as human Duchenne Muscular Dystrophy patients, \textit{mdx} mice exhibit two-year life spans, comparable to the life span of wild type mice (Janssen, Hiranandani et al. 2005). This is due to compensation for the lack of dystrophin by proteins such as utrophin, which is structurally similar to dystrophin. Utrophin in humans does not compensate for a long period compared to the much longer human life span, leading to more severe phenotypes in humans. During the fetal development of mice, utrophin is present in high quantities at the sarcolemma of skeletal muscle fibers, before it is replaced by dystrophin. In the absence of dystrophin, utrophin is present at somewhat higher levels, accounting for the near-full life span of \textit{mdx} mice.
Double-knock-out mice deficient for both dystrophin and utrophin (DKO - mdx/utrn*+/) show many more clinical signs of Duchenne Muscular Dystrophy than mdx mice and reveal relative life spans more comparable to Duchenne Muscular Dystrophy patients. DKO mice exhibit slack posture, lack of mobility, abnormal breathing patterns and abnormal field behavior by four to six weeks of age (Deconinck, Rafael et al. 1997). They also undergo weight loss after weaning with the onset of joint contractures and kyphosis. This often results in premature death by 20 weeks of age. Taking into account the life span of mdx mice, the short life span of DKO mice suggests that utrophin and dystrophin play synergistic roles in the functional or developmental pathways in skeletal muscle. Furthermore, DKO mice exhibit severe cardiomyopathy and hallmarks of heart failure early in their relative lifespan (Janssen, Hiranandani et al. 2005).

The third mouse model is haploinsufficient for the utrophin gene and is deficient for the dystrophin gene (het = mdx/utrn*+/). Het mice were an advantageous model for this study because they live longer than DKO mice and develop more severe skeletal muscular and cardiac fibrosis than mdx mice, suggesting a mouse model analogous to humans with Duchenne Muscular Dystrophy (Zhou, Rafael-Fortney et al. 2008).

The purpose of this project was to test the prophylactic efficacy of current standard-of-care treatments including a combination of an angiotensin converting enzyme inhibitor and an aldosterone antagonist with reported antifibrotic properties on Duchenne Muscular Dystrophy mouse models exhibiting cardiomyopathy. We compared the effectiveness of treatment started prior to
cardiac pathology (4 weeks of age) with treatment started at the onset of cardiac pathology (8 weeks of age) using het (mdx/utrn+/−) mouse models.

Angiotensin converting enzyme inhibitors block the activity of angiotensin converting enzyme, which leads to the decrease of angiotensin II production. Angiotensin II is a chemical that causes constriction of the blood vessels and leads to increases in blood pressure. By reducing blood pressure, less strain is placed on the heart and overall heart function improves.

Spironolactone is an antifibrotic drug that has also been used in cases to treat high blood pressure. It is characterized as an aldosterone receptor antagonist, as it is also used to treat patients with hyperaldosteronism (patients exhibiting excess levels of aldosterone). Additionally, the Randomized Aldactone Evaluation Study has shown that spironolactone can have significant effects on patients who exhibit severe congestive heart failure (Pitt, Zannad et al. 1999). According to the study, congestive heart failure patients who were treated with spironolactone experienced a 30% relative-risk-of-death reduction.

**Figure 1:** This figure displays the similarities in left-ventricular cardiac scarring between DKO mice and human DMD patients. The light pink and white in figures A and B, respectively denote fibrosis The dark pink and black areas in fibures C and D denote healthy muscle, respectively. **A)** Major histology in DKO mouse **B)** Minor histology in DKO mouse **C)** Major histology in human DMD patient **D)** Minor histology in human DMD patient (Photo credited to Dawn Delfin, PhD and Dr. Subha Raman)
Following the first project, the second single-drug study tested the efficacy of spironolactone and lisinopril both individually and in combination. Het mice began treatment with either spironolactone only or lisinopril only at the age of 4 weeks and were treated until 20 weeks, at which point they underwent magnetic resonance imaging testing and then were sacrificed for physiological and histological data. Furthermore, biochemical testing was performed in order to analyze the mechanism of drug interaction. A small pilot study to determine whether earlier treatment prevented all skeletal muscle damage was performed initially with het mice. The first het group, both males and females, began treatment at 3 weeks of age until 8 weeks, at which point they were sacrificed and tissues were collected for histological analysis. The second het group, both males and females, began treatment as neonates until 8 weeks of age at which point they were sacrificed for histology. A second pilot study to determine whether spironolactone and lisinopril in combination could rescue the life span of DKO mice was performed, as DKO mice began treatment as neonates.

**Materials and Methods**

The project consisted of 30 het mice divided into three groups of 10. The first group of 10 het mice started the combinatorial drug treatment at 4 weeks of age while the second group of 10 het mice started on the treatment at 8 weeks of age. An additional 10 het mice were left untreated and used as controls. The three groups were sex-matched and housed 2-3/cage. The mice were treated with the aldosterone antagonist spironolactone at a concentration of 250 mg/L and the angiotensin converting enzyme-inhibitor lisinopril at a concentration of 66 mg/L via water bottle solutions. Due to the low solubility of spironolactone (22 mg/L), the
drugs were first dissolved in ethanol prior to creating the water solutions. The overall water solutions contained 0.1% ethanol. The mice were provided continuous treatment through water bottles, which were replaced Mondays, Wednesdays and Fridays weekly until the mice reached the age of 20 weeks [Figure 2].

**Figure 2:** This diagram displays the treatment plan for the first project. The first group of mice (far left) are the control models; they do not receive treatment. The second group of mice (middle) receive prophylactic treatment beginning at 4 weeks of age, and the last group of mice (farthest right) receive treatment at the onset of cardiac fibrosis, beginning at 8 weeks of age.
One day prior to reaching 20 weeks of age, the mice were weighed and underwent cardiac magnetic resonance imaging at The Ohio State University Small Animal Imaging Core. They were then euthanized and tested for isolated muscle function. Cardiac trabeculae, diaphragm, and extensor digitorum longus were tested in parallel for contractile function. Additionally, heart, diaphragm, quadriceps, tibialis anterior, soleus, and extensor digitorum longus were tested for histological analysis in the laboratory of our collaborator, Dr. Paul Janssen. These tissues were frozen in liquid nitrogen-cooled isopentane, sectioned at a thickness of 8 micrometers, and stained with hematoxylin and eosin for microscope viewing to determine overall histology. Microscopic analysis was used to determine the degree of muscle fibrosis through immunofluorescence analysis of fibrosis markers such as collagen I. To perform this test, 8 micrometer heart tissue slides are blocked in potassium phosphate buffer solution + 0.2% gelatin (KPBSG) + 1% normal goat serum for primary binding with the collagen I antibody and secondary binding with CY3 anti-rabbit antibody. Furthermore, CY3-conjugated or Alexa 488 anti-mouse IgG was also used to stain heart sections with and without anti-Collagen I or anti-ERTR8 antibodies and analyzed to determine muscle degeneration. An experimenter blinded to the treatment groups visually scored all slides. This was done to determine qualitative differences between the three mouse groups in order to identify the most promising quantification experiments. Composites of the IgG-stained sections were created using Adobe Photoshop and the percentage of IgG-stained pixels were quantified relative to non-IgG-stained pixels using Image J [Figure 3].
Biochemical testing was performed on quadriceps, gastrocnemius, heart, and serum. Muscle samples were frozen in liquid nitrogen after dissection and homogenized in Newcastle buffer in preparation for protein quantification. Blood serum was contained and frozen directly. Homogenized tissues were used for immunoprobe of western blots, testing for tissue inhibitor of metalloproteinases -2 (TIMP2) activity. TIMP2 is a gene that inhibits matrix metalloproteinases, which break down the extracellular matrix in tissue remodeling. Western blots were placed in bovine serum albumin blocking solution for 2 hours, followed by incubation in 1% normal goat serum in tris-buffer saline + tween-20 (TBST) solution containing the primary antibody for 2 hours. The third incubation occurred in 1% normal goat serum in TBST solution containing the secondary antibody, followed by ECL incubation and 30 second, 1 minute, 5 minute, 20 minute, and overnight film exposures.

Furthermore, in situ zymography testing was performed by a postdoc in the laboratory, Dawn Delfin, to test the levels of matrix metalloproteinase activity. Heart
cryosections were fixed and incubated for 9 hours at 37°C. The in situ zymography was performed in a solution of gelatin conjugated to Orange Green 488 and washed with 10 mmol/L ethylenediaminetetraacetic acid (EDTA) to terminate the test (Fortney, Chimaji et al. 2011).
Results

In order to test the effectiveness of spironolactone and lisinopril administration to the mouse models, baseline data was established. Firstly, 26 week het (mdx/utrn+/−) and mdx baseline mouse models were sacrificed in order to harvest heart and quadriceps muscle tissue. The tissues were frozen in liquid nitrogen-cooled isopentane and sectioned at 8 micrometers for histochemical staining. These tissues were stained with hematoxylin and eosin for microscopy. Significant fibrosis was noted in the het heart muscle [Figure 4]. Additionally, het and mdx tissues from 26 week old mice were blocked in KPBSG + 1% normal goat serum for primary antibody binding with collagen I with CY3 anti-rabbit antibody for secondary binding. These tissues were compared to note qualitative differences in fibrosis between het and mdx mice. As evidenced by the data, there was substantial fibrosis in het models relative to mdx models of the same age [Figure 5].

Figure 4: Hematoxylin and Eosin Staining of 26 Week het (mdx/utrn+/−) murine model heart. Light pink areas denote fibrosis relative to the healthy, dark pink cardiac muscle.
26 Week Heart

Figure 5: Significant differences in fibrosis are noted through Collagen I immunofluorescence as seen by the bright orange collagen in the 26-week het model hearts (above) versus the 26-week mdx model hearts (below). These images typify maximum instances of fibrosis for both het and mdx tissues.
Magnetic resonance data collected from The Ohio State University MRI Core exhibited normal left ventricular ejection volume (LVEF) for all three mouse groups (4 weeks, 8 weeks, untreated); however, there were notable differences in systolic and diastolic function. The mean systolic circumferential strain rate improved significantly between the untreated het mice (-0.21 ± 0.08 s⁻¹), the het mice that began treatment at 8 weeks of age (-0.40 ± 0.07 s⁻¹), and furthermore the het mice that began treatment at 4 weeks of age (-0.56 ± 0.10 s⁻¹) [Figure 6]. Statistically, between the het-untreated and the het-treated-8 groups P = 0.003; between the het-untreated and the het-treated-4 groups P<0.0001; between the het treated-4 and het-treated-8 groups P = 0.014. Analogous improvement was noted for diastolic strain measurement (Fortney, Chimamji et al. 2011).

**Figure 6**: Endocardial circumferential strain rate is significantly improved between the three het treatment groups.
Moreover, physiology testing in Dr. Paul Janssen’s lab indicated that cardiac muscle force and response to isoproterenol exhibited an improving trend amongst the three mouse groups, with the het-treated-4 group displaying cardiac muscle force near the same levels as C57 wild-type mice (Fortney, Chimanji et al. 2011).

IgG localization, which indicates cardiomyocytic degeneration, indicated significant improvements between the three treatment groups when quantified – between the het-untreated mice and the het-treated-8 mice, there was a 44% decrease in degenerating cardiomyocytes (P<0.0001) [Figure 7]. Furthermore, there was a further reduction of 53% in degenerating cardiomyocytes between the het-treated-8 mice and the het-treated-4 mice (P<0.0001 vs. het-untreated mice; P = 0.0003 vs. het-treated-8).

Statistical analysis for MRI, histology and physiology data was performed by Dr. Paul Janssen. P-value tests were performed with a significance level of P = 0.05.

**Figure 7:** A) The average percentage (± SE) of section area stained for mouse IgG for heart (left) and quadriceps skeletal muscles from het-untreated and treated groups shows significant reductions in ongoing muscle damage. B) Blinded visual scoring of gelatinase activity from *in situ* zymography supports reductions of MMP remodeling in treated groups. * P<0.05 versus untreated, ** P<0.05 vs. 8-week. Panel A, n=7-10/group. Panel B, n=5/group.
Hematoxylin and eosin staining of the left ventricle shows dramatic improvement between the three mouse groups as well. While fibrosis is readily apparent in the het-untreated group, it is drastically reduced in the het-treated-8 group and non-evident in the het-treated-4 group. [Figure 8]

**Figure 8: A)** Hematoxylin and Eosin (H&E)-stained left ventricular sections show the cardiac damage prevalent throughout het-untreated hearts that is almost completely prevented in both treatment groups. **B)** Intracellular localization of mouse IgG (green) indicates damaged myocardium that is significantly attenuated in het-treated-8 and even further improved in het-treated-4 hearts. **C)** Gelatinase in situ zymography (ISZ) shows the combined activity of matrix metalloproteinases 2 and 9 (bright green), indicative of ventricular remodeling, that is also attenuated in the het-treated-8 hearts, and almost entirely prevented in the het-treated-4 hearts.
The TIMP2 immunoprobe western blots indicated upregulation of TIMP2 in het-treated-4 mice compared to the control mice [Figure 9]. This indicates that TIMP2 may play a role in inhibiting protease activity in tissues that typically undergo remodeling.

**Figure 9:** The TIMP western indicates upregulation of TIMP2 in 4 week mice relative to the untreated control mice. Additionally, TIMP2 levels can be compared to C57 wild-type mice.

In situ zymography of the three mouse groups indicated higher levels of matrix matelloproteinases 2 and 9 (MMP2, MMP9) in the untreated groups than the treated groups. Blinded visual scoring of in situ zymography by an experimenter in the lab supported a reduction of matrix metalloproteinases in the treated groups [Figure 7].

Regarding the pilot studies, it appeared that earlier treatment of the het mice did not lead to improved skeletal muscle function relative to the het-treated-4 mice in the first study. Furthermore, neonatal treatment of DKO mice did not restore cardiac or skeletal muscle function in the DKO mice.

Due to increased heat in the vivarium (>85°F temperatures for 3+ weeks) during treatment of the mice in the second het study (single treatment), the data
were thrown off as many of the mice experienced abnormal physical stress. Additionally, the drugs may have been unstable in water at this temperature. The treatments have since been restarted as the vivarium conditions have now stabilized.

**Discussion**

The results of this experiment show clear evidence that prophylactic combinatorial aldosterone analog and angiotensin converting enzyme inhibitor drug treatment is an effective form of reducing scarring and improving cardiac function in a Duchenne Muscular Dystrophy mouse model. Rather than treating cardiomyopathy as it arises, the early treatment model works in a preventative manner to protect against cardiac dysfunction before it even arises. Since cardiac complications are virtually guaranteed in all Duchenne Muscular Dystrophy patients that live long enough to exhibit it, we are in a unique position in which we can use standard-of-care treatments against cardiac complications in a preventative manner. While the het-treated-8 mice still exhibit some cardiac dysfunction, the prophylactic het-treated-4 mouse group’s overall improvement surpasses standard-of-care treatments and practically restores heart function.

While we have shown that the combinatorial drug-treatment restores heart function in the prophylactic het-treated-4 mice, we then focused on the mechanism by which this occurred. Data obtained from the TIMP2 western blot indicates that TIMP2 may be upregulated in the treated mouse groups, suggesting that TIMP2 is preventing matrix metalloproteinases from degrading the cardiac extracellular matrix. This is supported by the in situ zymography data, as there is a reduction in
matrix metalloproteinase remodeling in treated groups – it is likely the mechanism is acting upstream of matrix metalloproteinase remodeling.

It is likely that the early-treatment pilot studies were not effective because the drugs cannot act on the mice after they reach certain physical developments. Although we now have preliminary data demonstrating the presence of mineralocorticoid receptors in adult skeletal muscle, we still need to investigate whether they are present prior to 4 weeks-of-age. Mineralocorticoid receptors are the receptors that bind spironolactone, allowing it to exert its effects.

The results of these studies led to clinical trials employing aldosterone antagonists added to angiotensin converting enzyme inhibitors in young Duchenne Muscular Dystrophy patients. These trials have already begun under the investigator Dr. Subha V. Raman at The Ohio State University Wexner Medical Center and the University of Cincinnati Medical Center. This trial will use the magnetic resonance-specific antagonist eplerenone (analogous to spironolactone in this study) in conjunction with lisinopril, the cardiac standard-of-care target treatment for Duchenne Muscular Dystrophy patients, and the glucocorticoid prednisone, the neuromuscular standard-of-care treatment for Duchenne Muscular Dystrophy patients. This study will be conducted on Duchenne Muscular Dystrophy males at least 7 years of age. The checkpoint for this study will focus on myocardial strain, which exhibited significant improvement in the first drug study. The second clinical study, occurring in coordination with Muscular Dystrophy Association clinical centers across the country led by Dr. Kevin Flanigan at Nationwide Children’s Hospital in Columbus, OH will employ spironolactone in 5-6 year old Duchenne Muscular Dystrophy boys who have not been exposed to prednisone and
have not yet exhibited any signs of cardiomyopathy. This study will have an outcome measure of improved skeletal muscle function.
References


Lapidos, K., R. Kakkar, et al. (2004). "The dystrophin glycoprotein complex: Signaling strength and integrity for the sarcolemma." Circ. 94, 1023-1031


