Resveratrol decreases inflammation and increases utrophin gene expression in the mdx mouse model of duchenne muscular dystrophy

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1 Introduction

Duchenne muscular dystrophy (DMD) is a lethal genetic disease caused by a mutation in the gene encoding dystrophin. This mutation leads to the loss of a functional dystrophin protein, the critical member of the dystrophin glycoprotein complex that creates a direct link between the intracellular cytoskeleton and the extracellular matrix of skeletal muscle. The loss of this connection leaves the muscle fibers susceptible to damage resulting in continuous rounds of muscle degeneration/regeneration. The degeneration/regeneration process is coupled to and exacerbated by chronically elevated muscle inflammation. This inflammation is thought to contribute to the disease pathology. The muscle fibers eventually lose the ability to regenerate, and they are replaced with fibrous and fatty tissue. Results in a decrease in muscle function, loss of ambulation, and death in their mid to late 20’s. Though reintroducing a functional dystrophin gene will alleviate or cure the disease, this technology has not currently been optimized. Corticosteroids are the only currently prescribed treatment, but they only show modest improvements in muscle function with many undesirable side effects such as bone loss, diabetes, hypertension, and behavioral changes. Therefore alternative therapies are needed to help alleviate the disease pathology.

The mdx mouse model of DMD is the most widely used animal for studying this disease. These mice have a premature stop codon in the dystrophin gene leading to the loss of a functional dystrophin protein product. Though the long term muscle pathology is not as severe as the human condition, allowing a relatively normal length life, mdx mice exhibit a similar muscle pathology to humans between 3 and 8 weeks of age. During this time, there is widespread muscle necrosis accompanied by infiltration of damaging...
inflammatory cells, particularly macrophages.\textsuperscript{7,9} By 12 weeks of age, the necrosis is replaced by regeneration of damaged muscle fibers, and the immune cell infiltration subsides with a corresponding change in macrophage phenotype which promotes the regeneration.\textsuperscript{9} Therefore, studying the effect of a therapy on muscle pathology should be done between 3 and 12 weeks of age when the pathology is most similar to that seen in humans.

Studies in the mdx mouse show that reducing inflammation through a variety of interventions improves muscle morphology and muscle function making inflammation a viable therapeutic target.\textsuperscript{4,10} Indeed, though the exact mechanism of corticosteroid treatment is not known, it is thought to be partially through reducing inflammation.\textsuperscript{9} In addition to inflammation, an emerging therapeutic target is activation of the protein Peroxisome proliferator-activated receptor Gamma Coactivator 1 alpha (PGC-1α). PGC-1α is a transcriptional coactivator that induces the expression of genes associated with slow oxidative muscle fibers as well as the neuromuscular junction.\textsuperscript{11,12} Studies have shown that increasing PGC-1α expression improves muscle function and reduces the disease pathology of mdx mice.\textsuperscript{11,13,14} Conversely, a reduction in PGC-1α expression is associated with increased pathology.\textsuperscript{12,16} Though PGC-1α has many transcriptional targets, of particular interest to DMD is the direct downstream target of PGC-1α, utrophin, a dystrophin homolog.\textsuperscript{11} Utrophin is similar in size and structure to dystrophin, and although it is primarily located at the neuromuscular junction in adult muscle, it can also functionally take the place of dystrophin throughout the muscle membrane.\textsuperscript{17} This helps to restore the linkage between the intracellular cytoskeleton and the extracellular matrix, thereby drastically decreasing the disease pathology.\textsuperscript{17} Therefore methods that increase the expression of utrophin are also good therapeutic targets.

Resveratrol is a compound found in foods like grapes and red wine, and it has recently gained popularity due to its anti-inflammatory and oxidative metabolic enhancing properties.\textsuperscript{18,19} In skeletal muscle, resveratrol can reduce inflammation, and improve muscle function in a variety of disease models.\textsuperscript{18–20} Resveratrol’s actions are thought to be primarily mediated through the NAD + dependent deacetylase, Sirt1.\textsuperscript{18} Resveratrol increases the expression and activity of Sirt1, and both Resveratrol and Sirt1 activate PGC-1α leading to increased expression of PGC-1α target genes as well as increased expression of itself.\textsuperscript{18,21} Recently, resveratrol has also been shown to activate the utrophin-A promoter in C2C12 cell culture, which may occur through the Sirt1-PGC-1α pathway.\textsuperscript{22} Therefore, besides resveratrol’s anti-inflammatory properties, resveratrol may also be a good therapy for DMD by increasing utrophin expression.

There are only two known reports of resveratrol in the mdx mouse model of DMD. Hori et al.\textsuperscript{2011}\textsuperscript{23} treated 9 week old mdx mice with resveratrol for 32 weeks and found that resveratrol reduced loss of muscle mass, reduced oxidative stress, and lastly reduced the accumulation of fibrotic tissue. Despite these very beneficial outcomes, resveratrol was not effective at reducing inflammation at this later (41 week) time point. Yet, inflammation is already reduced and non-cytotoxic at this later age. Recently, Selsby et al.\textsuperscript{2012}\textsuperscript{24} treated 4 week old mdx mice with resveratrol for 8 weeks reporting increased specific force and reduced fatigue in the soleus. Despite this improved function, they did not investigate resveratrol’s effect on disease pathology. Additionally, they also report that resveratrol did not increase the protein expression of utrophin. Though Selsby et al.\textsuperscript{2012}\textsuperscript{24} used two resveratrol doses equal to 100 mg/kg and 400 mg/kg, it was given through the food allowing only a dose estimate which may be one reason for no increase in utrophin. Hori et al.\textsuperscript{2011}\textsuperscript{23} also administered their treatment in the food and thus estimate a resveratrol dose of 500 mg/kg. Therefore, more precise administration may have different effects than previously reported necessitating the need for the direct comparison of different doses. Therefore, the aims of the current study are to 1) determine an active dose of resveratrol in mdx mouse muscle, 2) determine the effect of resveratrol on total immune cell and macrophage infiltration in young mdx mice, and 3) determine if resveratrol treatment induces the expression of utrophin. As Sirt1 is the most common activation pathway cited for reseratro’s effect we use Sirt1 gene expression as a marker of resveratrol’s effect in muscle tissue. We hypothesized that resveratrol would exhibit a dose response, resveratrol would reduce total inflammation, and resveratrol would increase the expression of utrophin. This study expands upon the very positive findings of Hori et al.\textsuperscript{2011}\textsuperscript{23} and Selsby et al.\textsuperscript{2012}\textsuperscript{24} to further investigate resveratrol’s therapeutic effect in the mdx mouse.

2. Materials and methods

2.1. Animal care and experimental design

The University of South Carolina IACUC approved all methods used in this study. Mice were housed and cared for in the animal facility at The University of South Carolina, kept on a 12:12 h light–dark cycle, and were given ad libitum access to water and Purina chow. Five-week old male mdx dystrophic mice (C57Bl/10ScSnmdx/mdx) were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were weighed upon arrival and evenly distributed into one of four treatment groups based upon body weight (n = 8 or 10/group). Experimental mice were given resveratrol (Sigma–Aldrich, St. Louis, MO; a kind gift from Narendra Singh, PhD at the USC Complementary and Alternative Medicine (CAM) center) at a dose of 10, 100, or 500 mg/kg of body weight suspended in 200 µl autoclaved tap water via oral gavage everyday for 10 days. Control mdx mice were given autoclaved tap water at a volume of 200 µl via oral gavage everyday for 10 days. A second cohort of five week old male mdx dystrophic mice (C57Bl/10ScSnmdx/mdx) were purchased from Jackson Laboratories (Bar Harbor, ME) and treated with the most active dose as determined from the first cohort in order to have additional tissue for analysis. Mice of the second cohort were housed and treated in the identical manner as the first cohort. All mice were sacrificed 24 h following their final resveratrol treatment. On the day of tissue collection, animals were anesthetized with a subcutaneous injection of ketamine (75 mg/kg)/xylazine (3 mg/kg)/acepromazine (5 mg/kg) at a final concentration of total cocktail equal to 1.4 ml/kg body weight. 100% Isoflurane was given periodically by a nose cone to maintain deep anesthesia if the ketamine/xylazine/acepromazine cocktail was not sufficient to maintain deep anesthesia as assessed by toe pinch during the surgical procedure. Gastrocnemius and tibialis anterior muscles were removed from the anesthetized mice, rinsed in PBS, blotted, weighed, frozen, and stored at –80°Celsius until analysis. Muscles designated for histology were fixed in 10% formalin in 10% phosphate buffered saline for 24 h. Paraffin sections were prepared and stained with hematoxylin and eosin (H&E) and imaged using a Leica DMI5000B microscope.

2.2. Total RNA extraction and cDNA synthesis

RNA was extracted from the gastrocnemius using the TRIzol method (Invitrogen, Carlsbad, CA) as previously described.\textsuperscript{25} RNA
quantity and purity was assessed spectrophotometrically (NanoDrop). RNA integrity was examined by visualization of the 28 s and 18 s ribosomal subunits after electrophoresis in a 1.1% agarose gel. Four μg of total RNA was added to DEPC H2O totaling 11 μl 1 μl of Oligo dT (Invitrogen) was added to all samples and incubated at 70˚Celsius for 10 min followed by incubation on wet ice for 2 min. Reverse transcription of RNA into cDNA was carried out using 4 μl buffer, 2 μl DTT, 1 μl deoxyNTP’s, and 1 μl Superscript III Reverse Transcriptase (Invitrogen) per sample. Reverse transcription was carried out for 1 h at 50˚Celsius followed by 5 min at 70˚Celsius to stop the reaction.

2.3. Gene expression

Gene expression for Sirt1, IL-6, PGC-1α, and utrophin were determined by quantitative Real Time Polymerase Chain Reaction using SybrGreen Master Mix (Applied Biosystems, Foster City, CA). Gene expression for TNFα was determined by quantitative Real Time Polymerase Chain Reaction using Taqman Master Mix (Applied Biosystems, Foster City, CA). Amplification using SybrGreen was carried out with 1 μl cDNA (20 ng), 12.5 μl SybrGreen Master Mix, 1 μl Forward Primer (200 nM), 1 μl Reverse Primer (200 nM), and 9.5 μl RNase free H2O in a final volume of 25 μl/well. Forward and Reverse primer sequences for all genes analyzed are outlined in Table 1. TNFα amplification was carried out in 12.5 μl Taqman Universal PCR Master Mix (AmpliTaq Gold DNA Polymerase, Passive Reference 1, buffer, dNTPs; AmpErase UNG), 1 μl cDNA, 9 μl RNase-free water, 1.25 μl 18 s primer (VIC) and 1.25 μl TNFα primers (FAM) in a final volume of 25 μl/well. Quantitative RT-PCR using Sybrgreen was performed in triplicate while quantitative RT-PCR using Taqman was performed in duplicate on an Applied Biosystems 7300 thermocycler. Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) was used as an internal control when using SybrGreen while 18 s was used as an internal control when using Taqman. A dissociation curve was performed to verify specific amplification with SybrGreen, and the PCR products were also run on a 2% agarose gel to verify specific amplification and proper amplicon size. Relative gene expression fold change was calculated using the delta delta Ct method. The delta Ct was determined for each sample by the equation (Ctexperimental gene – Ctinternal control). The delta Ct (ddCt) was calculated by the equation (delta Ct(reseratreated sample – mean delta Ct 0 mg/kg control)).

Fold change relative to the 0 mg/kg group was calculated by the equation (Fold change = 2ddCt). Sirt1, IL-6, TNFα, PGC-1α, and utrophin gene expression of the reseratreated groups are reported as fold change relative to the water treated control group. Statistical analysis was performed on the fold change.

2.4. Histology

Frozen gastrocnemius samples were mounted in Optimal Cutting Temperature compound (Tissue-Tek, Torrance, CA), sectioned (10 μm) and stained with H&E, anti-CD45, and anti-F4/80 for immunohistochemistry at two separate tissue depths separated by at least 200 μm in order to measure immune cell infiltration at two separate muscle depths. Muscle sections for CD45 and F4/80 staining were fixed in acetone for 10 min, air dried for 20 min, and washed with PBS (3 x 5 min). Exogenous peroxidase activity was quenched by incubation in 0.3% hydrogen peroxide in PBS for 10 min. Sections were washed with PBS (3 x 2 min) and blocked for 1 h at room temperature (CD45) or overnight at 4˚Celsius (F4/80) with 10% rabbit serum (Vector Laboratories, Burlingame, CA) in PBS. Slides were incubated for 1 h at room temperature with primary antibodies against CD45 (1:75); BD Biosciences, San Diego, CA) or 3 h at room temperature with primary antibodies against F4/80 (1:200; Biologic, San Diego, CA). After PBS washes (3 x 5 min), slides were incubated with anti-rat secondary antibody (1:400 CD45, 1:200 F4/80; Vector Laboratories) for 1 h at room temperature. Sections were washed with PBS (3 x 5 min) and incubated with the Vectastain ABC Reagent (Vector Laboratories) for 30 min. Sections were exposed to 3,3’-diaminobenzidine diaminobezidine tetrahydrochloride (DAB) with nickel (Vector Laboratories) for 3 min. The reaction was stopped with tap water. The sections were dehydrated in progressive ethanol washes, cleared in xylene, and mounted with permount. Secondary only control slides consisted of the same procedures except they remained in 10% rabbit serum instead of respective primary antibodies. Using an Olympus BX41 light microscope, 8–10 pictures (400 x) were randomly taken at each depth of the gastrocnemius for H&E, CD45, and F4/80 stained sections. Immune cell infiltration from H&E stained sections was quantified by a blinded investigator. The average number of immune cell infiltrate per picture analyzed was averaged for analysis. CD45 and F4/80 staining was quantified using Scion Image software. A pixel threshold from secondary only was determined by the point where no pixels were present, and this pixel threshold was used for analysis of CD45 and F4/80 stained Section 5 random pictures from 4 different secondary only samples were used for determining pixel threshold. The pixel area per picture analyzed was averaged and used for analysis.

2.5. Western blot

Total protein was extracted from the gastrocnemius and the tibalis anterior with Mueller extraction buffer consisting of HEPES (50 mM), Triton-X (0.1%), EGTA (4 mM pH 8), EDTA (10 mM pH 8), Sodium Pyrophosphate (15 mM), β-glycerophosphate (100 mM), Sodium Fluoride (25 mM), Sodium Orthovanadate (5 mM), and protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO). Total protein was quantified in triplicate using the Bradford method with BSA used as a standard.29 All samples and standards had a coefficient of variation less than 5 percent. 40–80 μg of total protein was fractionated on 8% polyacrylamide gels for Sirt1 and PGC-1α, and 4–12% Bis–Tris Gels (Invitrogen, Grand Island, NY) for utrophin. All gels were transferred overnight to PVDF membranes at 40 mA at 4˚Celsius. Membranes were then stained with Ponceau S (Sigma Aldrich, St. Louis, MO) to verify transfer and qualitatively assess equal loading. Membranes were blocked with 5% milk in phosphate buffered saline containing 0.1% Tween-20 (PBST) for 1 h at room temperature. Membranes for utrophin were incubated with primary antibodies against utrophin diluted in 5% milk in PBST for

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
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<tbody>
<tr>
<td>Sirt1</td>
<td>5'-AGA CCA GTA GCA CTA ATT CCA AGT TC -3'</td>
<td>5'-GAG GTG TTT GTG GTA CAA ACT CT -3'</td>
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<tr>
<td>PGC-1α</td>
<td>5'-AAG ACC GAT TGC CAT TT -3'</td>
<td>5'-AGT CCT AAG ACC CCT GCA TT -3'</td>
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<tr>
<td>Utrophin</td>
<td>5'-GCC CTC CCT GCA GAT TAT TTT G -3'</td>
<td>5'-CTG TCC AGT TGA CTT TTT AAT CTC TTC -3'</td>
</tr>
<tr>
<td>IL-6</td>
<td>5'-TGA TGC TGG TGA CCA CCA CG -3'</td>
<td>5'-CAG AAT TGC CAT TGC ACA ACT C -3'</td>
</tr>
<tr>
<td>Gapdh</td>
<td>5'-CGA GCC AAA CGG GTC ATC AT -3'</td>
<td>5'-TCA GGC AAC ATC TTG CCA GA -3'</td>
</tr>
</tbody>
</table>

Table 1: qRT-PCR primer sequences used with SybrGreen Master Mix.
Table 2

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Resveratrol</th>
<th>Resveratrol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(10 mg/kg)</td>
<td>(100 mg/kg)</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>20.5 ± 1.9</td>
<td>19.7 ± 0.7</td>
<td>19.2 ± 1.3</td>
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<tr>
<td>Gastrocnemius weight (mg)</td>
<td>82.4 ± 4.6</td>
<td>71 ± 3.9</td>
<td>71.1 ± 3.6</td>
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<tr>
<td>Tibialis anterior (mg)</td>
<td>32.9 ± 1.8</td>
<td>34 ± 2.5</td>
<td>31 ± 2.7</td>
</tr>
<tr>
<td>Gastroc/body weight</td>
<td>4.01 ± 0.1</td>
<td>3.6 ± 0.1</td>
<td>3.71 ± 0.1</td>
</tr>
<tr>
<td>TA/body weight</td>
<td>1.6 ± 0.1</td>
<td>1.8 ± 0.1</td>
<td>1.64 ± 0.1</td>
</tr>
</tbody>
</table>

Values are means ± SE.

1 h at room temperature (Tibialis Anterior- 1:500, BD Bioscience, San Jose, CA; Gastrocnemius- 1:1000, Mancho3, Developmental Studies Hybridoma Bank, The University of Iowa). Membranes for Sirt1 and PGC-1α were incubated with primary antibodies against Sirt1 (1:500, Cell Signaling, Danvers, MA) and PGC-1α (1:5000, Millipore [catalog # AB3242], Billerica, MA) overnight at 4 °C. Membranes were washed 4 × 5 min in PBST and incubated with anti mouse Ig HRP linked antibodies (GE Healthcare Life Sciences, Piscataway, NJ) for utrophin or anti rabbit Ig HRP linked antibodies (Cell Signaling) for Sirt1 and PGC-1α diluted 1:1000 in 5% milk in PBST for 1 h at room temperature. Membranes were washed 4 × 5 min with PBST. Enhanced chemiluminescence (GE Healthcare Life Sciences) was used to visualize the antibody–antigen interactions and developed by autoradiography. Digitally scanned blots were analyzed by measuring the optical density of each band using digital imaging software (Image J Software, Bethesda, MD). Sirt1, PGC-1α, and utrophin protein content is expressed as pixel density relative to the water treated control group.

2.6. Statistical analysis

Body weights, muscle weights, and Sirt1 gene expression were analyzed with independent student t-test’s to compare the 10, 100 and 500 mg/kg dose of resveratrol to the water treated control. This analysis was used because comparisons between resveratrol treated groups were not a primary outcome. Therefore, a Bonferroni correction was used to maintain the alpha level of 0.05. After the most active dose of resveratrol was determined, analysis was conducted between samples that received the active dose and the water treated control. Inflammation from H&E stained sections, CD45 stained sections, and F4/80 stained sections were analyzed using independent student’s t-test’s to compare the resveratrol treated samples to the water treated control. The remaining gene expression and protein data were analyzed using independent student’s t-test’s to examine the difference between the 100 mg/kg resveratrol treated mice to the water treated control mice. All data are presented as mean ± SEM, and significance was set at p ≤ 0.05 for all analyses.

3. Results

Three mice from each treatment group from the first cohort did not survive the initial gavage procedure due to fluid filling the lungs. Therefore, a modified gavage technique was employed after the second day of treatment. Briefly, the modified gavage consisted of slowly dripping the treatment into the throat of the mice allowing them to swallow the treatment. Using the modified gavage technique, no further complications or obvious signs of stress were noted of the mice and all mice received the full volume of all treatments. All mice of the second cohort survived the treatment period and received the full volume of all treatments. Table 2 illustrates body weights and muscle weights for both experimental and control mice from both cohorts. There was no significant difference between groups for body weights or muscle weights for either cohort.

The primary dependent variable to determine resveratrol activity at the muscle level was Sirt1 gene expression. Figure 1 illustrates the Sirt1 fold change for each resveratrol treated group relative to the mdx water treated control. The 100 mg/kg dose of resveratrol increased Sirt1 gene expression 60 ± 10% (p < 0.01), and this dose was the only one that reached statistical significance (Fig. 1A). However, the 100 mg/kg dose of resveratrol did not increase the protein expression of Sirt1 after 10 days of treatment (Fig. 1B). Due to this data, all further analysis was performed on mdx mice treated with the 100 mg/kg dose of resveratrol and compared to the water treated control.

Total immune cell infiltration of the gastrocnemius was quantified through H&E stained sections and CD45 immunostaining (Fig. 2). Figure 2A demonstrates that total immune cell infiltration as assessed by H&E stained sections was significantly reduced.
21 ± 6% with resveratrol treatment \((p < 0.05)\). Figure 2B verifies these results using CD45 as a more specific marker of inflammatory cell infiltration, illustrating a 42 ± 8% \((p < 0.05)\) decrease with resveratrol treatment. Since macrophages are a significant source of muscle damage in mdx mice,\(^9\) we performed F4/80 immunohistochemistry to determine if macrophages were affected by resveratrol treatment. 10 days of resveratrol treatment significantly decreased the infiltration of macrophages 48 ± 10% \((p < 0.05)\) relative to the water treated control group (Fig. 2C). To determine if the gene expression of pro-inflammatory cytokines accompanied the reduction in immune cell infiltration, we quantified the gene expression of IL-6 and TNF\(_\alpha\) in the gastrocnemius (Fig. 3). Resveratrol treatment induced IL-6 gene expression 247 ± 77% \((p < 0.05)\) relative to the water treated control group while there was no change in TNF\(_\alpha\) gene expression. We also measured the gene expression of these cytokines in the 10 mg/kg and 500 mg/kg resveratrol treated groups with no significant difference observed (data not shown).

Because resveratrol is known to activate and induce the expression of the transcriptional coactivator PGC-1\(_\alpha\),\(^{18}\) we examined the gene and protein expression of PGC-1\(_\alpha\) in the gastrocnemius (Fig. 4). Resveratrol treatment induced PGC-1\(_\alpha\) gene expression 27 ± 17% \((p = 0.05)\) relative to control treated mice. Resveratrol treatment did not affect the protein content of PGC-1\(_\alpha\) after 10 days of treatment relative to the water treated control group (Fig. 4B). We next quantified the gene and protein expression of utrophin in the gastrocnemius and protein in the tibialis anterior after 10 days of resveratrol treatment (Fig. 5). 10 days of resveratrol treatment significantly increased utrophin gene expression 43 ± 23% \((p < 0.05)\) relative to water treated control group (Fig. 5A). Resveratrol did not increase utrophin protein content in the gastrocnemius (Fig. 5B) or in the tibialis anterior (Fig. 5C) when compared to the water treated control group.

### 4. Discussion

The aims of this study were to 1) determine an active dose of resveratrol in mdx mice, 2) determine the effect of resveratrol on skeletal muscle inflammation, and 3) determine the effect of resveratrol on the expression of PGC-1\(_\alpha\) and the PGC-1\(_\alpha\) target gene, utrophin. The major findings from this study illustrate that 1) only the 100 mg/kg dose of resveratrol significantly increased Sirt1 gene expression in the gastrocnemius after 10 days of treatment, 2) the 100 mg/kg dose of resveratrol significantly reduced total immune cell infiltration and macrophage infiltration in the gastrocnemius after 10 days of treatment, and 3) the 100 mg/kg dose of resveratrol significantly increased the gene expression of PGC-1\(_\alpha\) and utrophin in the gastrocnemius muscle of mdx mice after 10 days of treatment. These findings suggest that resveratrol...
may be an effective treatment for patients with DMD as inflammation is reduced with resveratrol treatment.

An eventual cure for DMD will be complex for many reasons, some of which include the size of the dystrophin gene, the multitude of mutations that cause the disease, and the complexity of a successful and specific gene delivery to the affected tissues. This necessitates the need for alternative therapies to reduce the severity of the disease until a cure can be found. Currently, the only prescribed treatment for boys with DMD is corticosteroids, which provide only a small improvement in muscle function with many undesirable side effects.\(^6\) Though the exact mechanism by which corticosteroids are effective is not known, their ability to reduce inflammation probably plays a role.\(^6\) The results of this study show that total immune cell infiltration, specifically macrophage infiltration, can be significantly reduced with 10 days of resveratrol treatment. These findings have important implications as previous reports show that inflammation, particularly macrophages, in young mdx mice exacerbates muscle damage decreasing muscle function.\(^9,30\) Additionally, many of the negative side effects such as bone loss, heart problems, and diabetes seen with corticosteroid treatment\(^6\) have not been reported with resveratrol treatment. Indeed, resveratrol even has many healthy side effects.\(^18,19\)

Our findings are in contrast with Hori et al. 2011\(^{23}\) who found that daily treatment of resveratrol estimated at 500 mg/kg/day did not reduce the infiltration of inflammatory cells for which there are several explanations. The first and most likely is that Hori et al. 2011\(^{23}\) measured total immune cell infiltration at 41 weeks of age compared to our study at 6 weeks of age. This younger age (3–12 weeks of age) corresponds to the time point of peak inflammation in this animal model.\(^{31}\) By 12 weeks of age, a reduction in the amount of inflammation and a shift in macrophage phenotype from the cytotoxic/proinflammatory M1 phenotype to the pro growth/repair M2 phenotype occurs.\(^{31,32}\) Our current results coupled with those of Hori et al. 2011 indicates that resveratrol reduces immune cell infiltration, particularly macrophages, during periods of cytoxic inflammation but has no effect during periods of low inflammatory stress.

Another potential explanation for the differing results between these studies is that an estimated higher dose of resveratrol (i.e. 500 mg/kg) may not affect inflammation the same as a lower dose. Although Hori et al. 2011\(^{23}\) found a significant reduction in oxidative stress and fibrosis, which would most likely benefit a person with DMD, further studies are needed to compare the effects of our lower dose of resveratrol to the one used by Hori et al. 2011\(^{23}\) at a later age in the mdx mouse. Additionally, higher and lower resveratrol doses should be examined for their effect on inflammation at this younger time point as the dose examined here was the most active at increasing Sirt1 mRNA but may not be the most active at reducing inflammation.

Though the reduced inflammation in our study is one potential therapeutic target for DMD by possibly decreasing muscle necrosis, reducing inflammation would have no effect on the underlying

Fig. 3. IL-6 and TNF\(_\alpha\) gene expression in the gastrocnemius of mdx mice. 10 days of resveratrol treatment increased IL-6 gene expression 247 ± 77% relative to water treated control as measured by qRT-PCR (3A). There was no difference in TNF\(_\alpha\) gene expression between treatments (3B). Gapdh was used as an internal control for IL-6 while 18 s was used as an internal control for TNF\(_\alpha\). N = 7 per group. * indicates \(p < 0.05\).

Fig. 4. PGC-1\(\alpha\) gene and protein expression in the gastrocnemius of mdx mice. 10 days of resveratrol treatment increased PGC-1\(\alpha\) gene expression 27 ± 17% (4A) relative to water treated control as measured by qRT-PCR. 10 days of resveratrol treatment did not increase PGC-1\(\alpha\) protein content in the gastrocnemius relative to the water treated control group (4B). Gapdh was used as an internal control. N = 7 per group. * indicates \(p = 0.05\).
cause of the pathology, i.e. lack of dystrophin protein. Utrophin is a known downstream target of PGC-1α,11,14 the utrophin promoter is activated with resveratrol treatment,22 and previous reports show that utrophin can functionally take the place of dystrophin helping to restore the dystrophin glycoprotein complex, stabilize the sarcolemma, and alleviate the disease pathology.17,33 Here we show that both PGC-1α and utrophin gene expression are significantly increased after 10 days of resveratrol treatment indicating that PGC-1α may have been activated with resveratrol treatment. Despite the increases in both Sirt1 and PGC-1α mRNA expression with resveratrol treatment, the corresponding protein product for each gene was not different between treatment groups. Yet, an increase in the activity of both Sirt1 and PGC-1α prior to the increase in protein amount, has been shown to occur.34 Additionally, the corresponding protein content of utrophin in both the gastrocnemius and tibialis anterior were not affected with resveratrol treatment. This finding indicates that either the time point analyzed did not allow sufficient time for translation of the utrophin mRNA transcript, or that there is a disconnect between transcription and translation of utrophin. Miura et al. 200535 show that the 5' untranslated region (UTR) of the utrophin mRNA transcript exhibits a regulatory role in the translation of the transcript. In undamaged muscle, the 5’ UTR inhibits translation of the protein while this region allows translation during development and regeneration of muscle.35 Therefore, the results of the current study may indicate that though resveratrol increased the mRNA expression of utrophin, the translation of the mRNA transcript may be inhibited in non regenerating muscle fibers. Therefore, our results would agree with a previous report showing resveratrol activates the utrophin promoter,22 and Selsby et al. 201214 who showed that utrophin protein was not increased with resveratrol treatment.

Despite the possibility that resveratrol is enhancing utrophin mRNA expression via PGC-1α, another possible explanation for the increased PGC-1α and utrophin mRNA expression could be that resveratrol enhanced the muscle regenerative process. Utrophin, PGC-1α, and IL-6 increase expression during muscle differentiation.36,37 This could be a direct effect of resveratrol or might only be possible because of the decreased inflammation, or even a currently unknown effect of resveratrol in mdx muscle. In regards to the changes in inflammation, IL-6 injections in young mdx mice can increase the expression of utrophin,38 and IL-6 is also known to increase muscle differentiation in-vitro.39 Therefore, the significant increase in PGC-1α, utrophin, and IL-6 gene expression noted in this study would support this alternative conclusion of increased muscle regeneration but it is likely that the regeneration was allowed due to the decreased inflammation as opposed to direct growth effects of resveratrol.

While there are very promising cellular and molecular findings in the current study, there is now a need for studies of muscle function, in-depth analysis of inflammation, and consideration of translation to humans. The current study, however, was needed to enhance the quality (i.e. precise dose) and impact of longer resveratrol treatment studies, which are currently underway in our laboratory or recently published.14 Now that the overall decrease in inflammation is noted, future studies should further examine the type of inflammation affected, as well as whether inflammation is reduced in other tissues and systemically. As with any animal study there is a limitation of crossover from a mouse model to humans. Resveratrol, or this particular dose of resveratrol, may not have the same effect in a human as observed in the mdx mouse. Despite this potential limitation, resveratrol is currently being examined in several ongoing human clinical trials for other conditions with few if any side effects, and is thus a logical candidate therapeutic agent to at least decrease inflammation in humans with DMD.

In summary, we found that resveratrol significantly reduced the infiltration of immune cells, particularly macrophages, and increased the mRNA expression of utrophin in the mdx mouse after 10 days of treatment. Although a multitude of studies have found

**Fig. 5.** Utrophin gene expression and protein content in the gastrocnemius and tibialis anterior of mdx mice. 10 days of resveratrol treatment increased utrophin gene expression 43 ± 23% relative to the water treated control group (5A). 10 days of resveratrol treatment did not affect the protein expression of utrophin in either the gastrocnemius (5B) or the tibialis anterior (5C) relative to the water treated control. * indicates p < 0.05.
resveratrol to be beneficial in other disease models, its effect on DMD is only beginning to emerge. Our results suggest resveratrol may be a novel therapeutic compound to alleviate the DMD pathology, through more than one molecular pathway.

**Statement of authorship**

BG performed resveratrol treatments, gene expression, protein expression, data analysis, drafted manuscript. DD assisted with surgery, assisted with data collection and analysis. MK designed and supervised the study, assisted with manuscript drafting. All authors read and approve the final manuscript.

**Conflict of interest**

The authors report no conflict of interest.

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