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Original article

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

Bradley S. Gordon [a], Diana C. Delgado Díaz [a,b], Matthew C. Kostek [a,*]

[a] Department of Exercise Science, University of South Carolina, Columbia, SC, USA
[b] Universidad Industrial de Santander, Escuela de Fisioterapia, Bucaramanga, Colombia

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SUMMARY

Background & aims: Duchenne muscular dystrophy (DMD) is a lethal genetic disease with no cure. Reducing inflammation or increasing utrophin expression can alleviate DMD pathology. Resveratrol can reduce inflammation and activate the utrophin promoter. The aims of this study were to identify an active dose of resveratrol in mdx mice and examine if this dose decreased inflammation and increased utrophin expression.

Methods: 5-week old mdx mice were given 0, 10, 100, or 500 mg/kg of resveratrol everyday for 10 days. Sirt1 was measured by qRT-PCR and used to determine the most active dose. Muscle inflammation was measured by H&E staining, CD45 and F4/80 immunohistochemistry. IL-6, TNFα, PGC-1α, and utrophin gene expression were measured by qRT-PCR. Utrophin, Sirt1, and PGC-1α protein were quantified by western blot.

Results: The 100 mg/kg dose of resveratrol, the most active dose, increased Sirt1 mRNA 60 ± 10% ($p < 0.01$), reduced immune cell infiltration 21 ± 6% (H&E) and 42 ± 8% (CD45 immunohistochemistry ($p < 0.05$)), reduced macrophage infiltration 48 ± 10% (F4/80 immunohistochemistry ($p < 0.05$)), and increased IL-6, PGC-1α, and utrophin mRNA 247 ± 77%, 27 ± 17%, and 43 ± 23% respectively ($p \leq 0.05$). Utrophin, Sirt1, and PGC-1α protein expression did not change.

Conclusions: Resveratrol may be a therapy for DMD by reducing inflammation.

1. Introduction

Duchenne muscular dystrophy (DMD) is a lethal X-linked genetic disease caused by a mutation in the gene encoding dystrophin.[1] 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.[2] The loss of this connection leaves the muscle fibers susceptible to damage resulting in continuous rounds of muscle degeneration/regeneration.[3] The degeneration/regeneration process is coupled to and exacerbated by chronically elevated muscle inflammation. This inflammation is thought to contribute to the disease pathology.[4] The muscle fibers eventually lose the ability to regenerate, and they are replaced with fibrous and fatty tissue.[2] This results in a decrease in muscle function, loss of ambulation, and death in their mid to late 20's.[1] 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.[5,6] 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.[7] These mice have a premature stop codon in the dystrophin gene leading to the loss of a functional dystrophin protein product.[8] 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.[7] During this time, there is widespread muscle necrosis accompanied by infiltration of damaging

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* Corresponding author. University of South Carolina, Department of Exercise Science, Public Health Research Center, 3rd Floor, 921 Assembly Street, Columbia, SC 29208, USA. Tel.: +1 803 777 1462; fax: +1 803 777 0558.
E-mail address: mkostek@mailbox.sc.edu (M.C. Kostek).

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inflammatory cells, particularly macrophages.[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.[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.[4,10] Indeed, though the exact mechanism of corticosteroid treatment is not known, it is thought to be partially through reducing inflammation.[6] 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.[11,12] Studies have shown that increasing PGC-1α expression improves muscle function and reduces the disease pathology of mdx mice.[11,13,14] Conversely, a reduction in PGC-1α expression is associated with increased pathology.[15,16] Though PGC-1α has many transcriptional targets, of particular interest to DMD is the direct downstream target of PGC-1α, utrophin, a dystrophin homolog.[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.[17] This helps to restore the linkage between the intracellular cytoskeleton and the extracellular matrix, thereby drastically decreasing the disease pathology.[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.[18,19] In skeletal muscle, resveratrol can reduce inflammation, and improve muscle function in a variety of disease models.[18–20] Resveratrol's actions are thought to be primarily mediated through the NAD + dependent deaceytlase, Sirt1.[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.[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.[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. 2011[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. 2012[14] 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. 2012[14] used two resveratrol doses equal to 100 mg/kg and 400 mg/kg, it was given through the food allowing only a dose estimation which may be one reason for no increase in utrophin. Hori et al. 2011[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 reseratrol'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. 2011[23] and Selsby et al. 2012[14] 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/10ScSn$^{mdx/mdx}$) 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/10ScSn$^{mdx/mdx}$) 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/aceptromazine 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 frozen in liquid nitrogen cooled isopentane while tissues designated for molecular analysis were frozen in liquid nitrogen. After removal of tissue, mice were euthanized by cardiectomy. Gene expression of Sirt1 was used as a primary outcome to identify the most effective dose of resveratrol. We chose this as our primary outcome as the expression of Sirt1 has been routinely shown to increase with resveratrol treatment.[21,24–27] All further analysis was conducted with the dose that elicited a significant increase in Sirt1 gene expression.

2.2. Total RNA extraction and cDNA synthesis

RNA was extracted from the gastrocnemius using the TRIzol method (Invitrogen, Carlsbad, CA) as previously described.[28] RNA