Long-term quercetin dietary enrichment decreases muscle injury in mdx mice

Authors:
Katrin Hollinger, R. Andrew Shanely, John C. Quindry & Joshua T. Selsby

Abstract

Background & aims: Duchenne muscular dystrophy results from a mutation in the dystrophin gene, which leads to a dystrophin-deficiency. Dystrophic muscle is marked by progressive muscle injury and loss of muscle fibers. Activation of the PGC-1a pathway has been previously shown to decrease disease-related muscle damage. Oral administration of the flavonol, quercetin, appears to be an effective and safe method to activate the PGC-1a pathway. The aim of this investigation was to determine the extent to which long term dietary quercetin enrichment would decrease muscle injury in dystrophic skeletal muscle. We hypothesized that a quercetin enriched diet would rescue dystrophic muscle from further decline and increase utrophin abundance.

Methods: Beginning at three-months of age and continuing to nine-months of age mdx mice (n = 10/group) were assigned to either to mdx-control receiving standard chow or to mdx-quercetin receiving a 0.2% quercetin-enriched diet. At nine-months of age mice were sacrificed and costal diaphragms collected. One hemidiaphragm was used for histological analysis and the second hemidiaphragm was used to determine gene expression via RT-qPCR.

Results: The diaphragm from the mdx-quercetin group had 24% (p < 0.05) more muscle fibers/area and 34% (p < 0.05) fewer centrally nucleated fibers compared to the mdx-control group. Further, there were 44% (p < 0.05) fewer infiltrating immune cells/area, a corresponding 31% (p < 0.05) reduction in TNF gene expression, and a near 50% reduction in fibrosis. The quercetin-enriched diet increased expression of genes associated with oxidative metabolism but did not increase utrophin protein abundance.

Conclusions: Long-term quercetin supplementation decreased disease-related muscle injury in dystrophic skeletal muscle; however the role of PGC-1a pathway activation as a mediator of this response is unclear.
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Diaphragm

summary

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1. Introduction

Duchenne muscular dystrophy (DMD) is caused by dystrophin protein deficiency. Dystrophin serves as a link between the actin cytoskeleton and the extracellular matrix through the dystrophin-glycoprotein complex (DGO) [1]. DMD is marked by loss of muscle function resulting in wheelchair confinement and death due to respiratory or cardiac failure generally in the third decade of life [2].

This disease is modeled by the mdx mouse. The diaphragm of the mdx mouse most accurately recapitulates disease progression [3]. Transgenic overexpression and neonatal gene delivery of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (Ppargc1a; PGC-1α) prevented or delayed disease onset [4,5] and gene delivery also rescued actively declining skeletal muscle [6]. Nutraceutical-mediated activation of the PGC-1α pathway could make this approach translatable to DMD patients. Resveratrol, a polyphenol, is thought to be a PGC-1α activator via Sirtuin-1 (SirT1) [7] or AMPK [8]. Because of the equivocal results [4,7,9,10] and significant safety concerns [4] with resveratrol we sought out a compound that could drive the PGC-1α pathway, be rapidly administered to the DMD population, and had an excellent safety profile. Quercetin, a flavonol, met all of these requirements.
Table 1
Primer sequences used for qPCR.

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Quercetin is a potent Sirt1 activator [11] capable of entering muscle cells following oral delivery [12,13], which can lead to PGC-1α deacetylation and subsequent activation [14]. Importantly, oral administration of quercetin to mice [14] increased mRNA abundance of Sirt1 and PGC-1α and induced mitochondrial biogenesis in skeletal muscle. Quercetin may be beneficial to dystrophic skeletal muscle because of cellular pathway activation and/or through independent secondary effects including antioxidant and anti-inflammatory functions [15,16]. Also, quercetin is safe at high-doses (up to 12,000 mg/kg/day) for up to two years [17e21]. Therefore, the purpose of this investigation was to determine the extent to which long-term dietary quercetin enrichment would decrease injury in dystrophic skeletal muscle. We hypothesized that quercetin would rescue declining dystrophic skeletal muscle and increase utrophin abundance without causing obvious toxicities.

2. Methods

2.1. Animal treatments

All animal procedures were approved by the IACUC at Iowa State University and were done in accordance with the guiding principles established by the American Physiological Society. At three-months of age 20 mdx mice were randomly divided in two groups receiving either standard chow (BioServe, Rodent diet) or standard diet enriched with 0.2% quercetin (BioServe, Rodent diet 0.2% quercetin) for six months. During the study period mice were weighed weekly and food consumption was recorded biweekly. At nine-months of age mice were anesthetized with a surgical plane with trichobromoanethol, sacrificed by cervical dislocation, and the costal diaphragm removed and divided into hemidiaphragms. One hemidiaphragm was snap frozen in liquid nitrogen for measures of gene expression and the other was coated in freezing media and frozen for histological analyses.

2.2. Histology

Ten-micron frozen sections were cut from the diaphragm and placed on slides. Hematoxylin and Eosin staining was performed according to standard techniques. Each diaphragm section was visualized using a Leica microscope at 100x and 264 images were taken of each diaphragm. Following a cursory inspection of slides from each group we hypothesized that diaphragms from quercetin treated animals had a greater abundance of muscle fibers compared to control fed mice. Using Image J [22] the total number of skeletal
muscle fibers and the number of skeletal muscle fibers with a centralized nucleus were counted in two images/section (77e330 fibers/image). Data are reported as the average (±SEM) of these two images.

To identify utrophin protein localization and abundance, utrophin antibody (1:10 dilution, Vector VP-U579) directly labeled with Zeon Alexa 568 (Invitrogen Z25006) was used as previously described [6]. To evaluate fibronectin abundance, an indicator of fibrosis, sections were first blocked with 5% BSA. Sections were incubated with anti-fibronectin antibody (1:100, Sigma F3648) at 4 °C overnight. The next day slides were washed three times with PBS. Following the wash, sections were incubated with fluorescein conjugated goat anti-mouse IgG at (1:100; Millipore) for 1 h in the dark. After the incubation period slides were washed three times in PBS and then cover slips were applied and sealed. To visualize fibronectin abundance for each Section 163 non-overlapping 200x images were taken under identical exposure conditions. To quantify protein abundance IHC images were converted into binary images using OpenLab (version 3.5.1, Perkin Elmer) as previously described [6].

Determination of fiber type differences by IHC was done in two serial sections. Sections were washed and blocked as described for fibronectin. One section was incubated with primary antibody for MhcI (A4,951, Hybridoma Bank University of Iowa developed by Blau, H.M.) and laminin (NeoMarkers, RB-092-A) the second section was incubated with primary antibody for MhcIIa (SC-71, Hybridoma Bank University of Iowa developed by Schiaffino, S.) and laminin. Primary myosin antibodies were detected using fluorescein conjugated goat anti-mouse IgG at 1:100 (Millipore, 126506) and laminin was detected with goat anti-rabbit rhodamine conjugated IgG (Millipore, 126510, respectively. Slides were imaged at 200x at the same area in both serial sections. For analyses, both the total number of cells/image and the number of positive staining cells/image were counted using Image J [22]. Cells that were negative for MhcI and MhcIIA were considered MhcII x/b and cells that were positive for both myosin antibodies were considered MhcI/IIA fibers. The cell counts from 2 to 3 images/section were pooled, resulting in approximately 100-400 cells/section.

2.3. Biochemistry

RNA was extracted from the remaining hemidiaphragm using Trizol according to manufacturer recommendations (Invitrogen, 15596-018). To eliminate organic carryover and DNA contamination RNA was purified using a Qiagen RNaseasy spin column (Qiagen RNeasy Mini Kit, 74106) in combination with an on-column DNase (Qiagen RNase free DNase set, 79254) treatment. RNA concentration was measured using a Nanodrop (Thermo Scientific). From 1 μg RNA, cDNA was synthesized following manufacturer’s instructions (QuantiTect Reverse Transcription Kit, Qiagen 205311) with the addition of 18S RT primers. To measure expression of genes of interest QuantiFast SYBR Green PCR kit (Qiagen, 204056), primers (Table 1), and 10 ng of cDNA template, were loaded into each well for a total 12.5 μL reaction volume. Gene expression was assayed with a Mastercycler EP Realplex (Eppendorf). All samples were measured in triplicate wells and normalized to 18S expression. Delta CT (dCT) values were calculated by subtracting the 18S CT value from the CT value for the gene of interest. ddCT was calculated by subtraction of the highest ddCT of a given gene from all the dCT for that gene.

2.4. Statistics

For body weight and food consumption control and quercetin treated animals were compared with an ANOVA with repeated measures. All other data from control and quercetin-treated mdx mice were compared using a Student’s t-test. For gene expression statistics were performed on the dCT. Gene expression data are presented as fold change as calculated from ddCT. Significance was set at p < 0.05. All data are expressed as means ± SEM unless otherwise noted.

3. Results

Three-month old mdx mice were fed a control or 0.2% quercetin-enriched diet for six months. Food consumption fluctuated similarly throughout the study period in both groups with an average food consumption of 3.8 ± 0.03 g/day of control food and an average food consumption of 3.6 ± 0.02 g/day of quercetin enriched food (Fig. 1(A)). Body mass of the mice in both groups increased as they aged (p < 0.05) but did not differ between groups (Fig. 1(B)). Upon sacrifice at nine-months of age, mice on the control diet weighed 31.4 ± 1.3 g and mice on the quercetin-enriched diet weighed 29.5 ± 1.0 g. Gastrocnemius mass was similar between groups when considered as absolute mass (mdx-Con 172 ± 9 mg; mdx-Q 157 ± 8 mg) or normalized to body weight (mdx-Con 5.5 ± 0.1 mg/g; mdx-Q 5.3 ± 0.1 mg/g). In addition to normal growth, food consumption, and muscle mass, mice treated with the quercetin-enriched diet did not exhibit obvious signs of toxicity including failure to groom or lethargy. Importantly, and in contrast to previous experience with resveratrol, supplementation with quercetin did not result in unexpected deaths.

To determine the extent to which the quercetin-enriched diet protected dystrophic skeletal muscle we performed a histological

![Fig. 1. Body weight and food consumption during the six month study period in mdx mice. Data are means ± SEM. Food consumption did not differ between the control and quercetin diet treatment groups (A). The increase in body mass during the six month study period did not differ between the control and quercetin diet (B) treatment groups.](image-url)
evaluation of the diaphragms. Following H&E staining, the diaphragm sections from the quercetin-treated mdx mice exhibited less disease-related injury and appeared healthier than the diaphragm sections from the control-treated mice (Fig. 2A and B). To objectively quantify histological changes we measured the number of muscle fibers, percent of fibers with centralized nuclei, and fibrotic area. Diaphragms from quercetin-treated mice had 24% (p < 0.05) more muscle fibers/area than diaphragms from control-treated mice (Fig. 2C). Further, there was a 34% (p < 0.05) decrease in fibers with centralized nuclei (Fig. 2D). Because the quercetin-enriched diet led to a decrease in central nucleation, we measured transcript abundance of genes involved in muscle regeneration. Relative abundance of MyoC, MyoD, MyoZ, Myf5 and embryonic myosin heavy chain were similar between groups (Table 2).

Corresponding with a preservation of muscle fiber number was a 44% (p < 0.05) reduction in immune cell infiltration when normalized to section area or a 62% (p < 0.05) reduction when normalized to muscle fiber number (Fig. 2E and F). The reduction in immune cell infiltration was supported by a 31% (p < 0.05) reduction in Tnf transcript abundance in the diaphragms from the quercetin-enriched diet group compared to those in the control diet group (Fig. 2G). Fibrotic area was decreased by 47% (p < 0.05) in diaphragms taken from the quercetin-enriched diet group compared to mice in the control diet group (Fig. 3AeC).

To better understand the mechanism underlying quercetin-mediated interruption of the disease process we assessed changes indicative of increased oxidative metabolism as well as changes associated with the slow/neuromuscular junction gene

Fig. 2. Representative H&E stained diaphragm sections (200x). Dystrophin-deficient mice were fed a control diet (A) or a diet containing 0.2% quercetin (B) for 6 months starting at 3–6 mo of age. Following 6 months of quercetin treatment muscle fibers per area were preserved (C), central nucleation decreased (D), number of infiltrating immune cells was reduced per area (E) and per muscle fiber (F) compared to control fed mice. In support of decreased immune cell infiltration Tnf-a transcript abundance was also decreased (G). Data are means ± SEM. * indicates significantly different from control-fed animals (p < 0.05).
program. Both of these are likely contributors to PGC-1α mediated rescue of dystrophic muscle. Transcript abundance of mitochondrial transcription factors Tfam, Tfβ1m and Tfβ2m were measured in the diaphragms of both treatment groups. Tfam transcript abundance increased 46% (p < 0.05) and Tfβ1m abundance increased numerically by 28%, however, failed to reach significance (p = 0.08) (Fig. 4) and Tfβ2m did not differ between quercetin-treated and control groups. Abundance of the mitochondrial encoded transcript CoxII (Mtcox2) was increased 55% (p < 0.05), cytochrome C (Cycs) exhibited a non-significant 45% numerical increase (p = 0.07) with quercetin treatment, and Uqrc1, a component of the electron transport chain, was similar between groups. PGC-1α signals through Errα and Nrf1 resulting in mitochondrial biogenesis, however, transcript abundance of Errα and Nrf1 were similar between groups (Fig. 4).

With activation of the PGC-1α pathway a fast-to-slow myosin heavy chain shift is expected. In the current study frequency of type I (slow), Ia, and IIb/x (fast) skeletal muscle fiber types did not differ between groups (mdx/control: 1:20.9 ± 4.5%, I/IIa:2.2 ± 0.6%, IIa:

Fig. 3. Representative diaphragm images of thionin IHC. mdx-control (A) and mdx-quercetin (B) mice were either fed a control diet or a quercetin-enriched diet, respectively. Fibrosis was significantly reduced in mdx-quercetin diaphragms compared to mdx-control (C). Data are means ± SEM. * indicates significantly different from control-diet animals (p < 0.05).

Fig. 4. Oxidative gene expression. The relative difference between mdx-quercetin and mdx-control mRNA abundance was measured. Data are means ± SEM. * indicates significantly different from control-fed animals (p < 0.05) z indicates (p < 0.1).

74 ± 5.0%, and IIb/x:2.7 ± 0.9%; mdx-quercetin: I-16.4 ± 3.2%, I/IIa-3.3 ± 0.9%, IIa-75.9 ± 3.5%, and IIb/x-4.4 ± 1.3%). Further, transcript abundance of Mbc1 (Myh7), Mbc1la (Myh2), Mbc1lx (Myh1), and Mbc1lb (Myh4) did not differ between groups (Table 2). Transcript abundance of Nrf2, a transcriptional activator of the slow gene program, did not differ between groups (Table 2). Associated with the slow/neuromuscular gene program is increased utrophin abundance. We and others have previously measured increased utrophin gene and protein abundance following PGC-1α pathway activation [4,6]. In this investigation we found that utrophin transcript (Table 2) and protein (Fig. 5(AeC)) abundance were similar between groups.

4. Discussion

While the cause of DMD is well known, effective therapies to treat this disease have been slow in reaching the patient
population. We have previously shown that up regulation of PGC-1α protein expression via viral gene transfer prevents disease onset and rescues dystrophic skeletal muscle from typical disease progression [4,6]. In order to translate this experimental approach to an immediately available therapy we sought nutraceuticals that increase PGC-1α pathway activation and have a demonstrated safety profile. In the current longterm feeding study we demonstrate that oral delivery of quercetin decreased indices of disease severity in diaphragms from mdx mice without obvious signs of toxicity.

The current study demonstrates mdx mice fed a 0.2% quercetin-enriched diet had reduced muscle histopathology including preservation of muscle fiber number and decreased fibrosis. Further, we also demonstrate that remaining fibers from mdx-quercetin diaphragms were in generally better health than those from mdx-control as central nucleation was decreased following consumption of the quercetin-enriched diet. Consistent with these observations, consumption of the quercetin-enriched diet was also associated with decreased inflammation. It was beyond the scope of this investigation to determine the extent to which quercetin treatment decreased fibrosis and inflammation directly or indirectly by interfering with other aspects of disease progression and skeletal muscle injury.

Guided by our previous findings in studies using gene transfer of PGC-1α we tested several hypotheses to better understand the mechanisms leading to quercetin-mediated interruption of the disease process. Consistent with previous work, we found that quercetin treatment led to increased expression of oxidative genes. This outcome is important as metabolic dysregulation [23,24] and depleted ATP [24] content are characteristic of dystrophic muscle. We did not detect evidence suggesting enhanced muscle regeneration, which we found in a previous study where PGC-1α gene transfer was used to rescue dystrophic muscle from disease progression [6]. Contrary to our hypothesis, we did not increase relative utrophin transcript or protein abundance. Utrophin has been shown to substitute for the missing dystrophin protein in dystrophic skeletal muscle and restore resistance to contraction-induced injury [25,26]. Additionally, utrophin has been repeatedly increased by PGC-1α gene transfer studies, however, supplementation with resveratrol, another dietary flavonoid, has, like quercetin, also failed to increase utrophin protein abundance [4,9]. A recent report supports our hypothesis that PGC-1α pathway activation functions through multiple therapeutic avenues and is not solely utrophin-dependent [27]. Importantly, it seems likely that PGC-1α can lead to increased mitochondrial biogenesis independent of inducing gene expression associated with the neuromuscular junction and slow skeletal muscle as the former is driven through an Nrf1 dependent pathway [28] while the latter is driven through an Nrf2 dependent pathway [5]. Muscle and body mass were also similar between groups, which is counter to expectations given that virally-mediated PGC-1α overexpression led to reduced muscle mass. These findings and inconsistencies with viral gene transfer of PGC-1α suggest that, while dietary quercetin consumption did successfully decrease skeletal muscle injury, quercetin led to only partial PGC-1α pathway activation.

Careful scrutiny of several previous reports of oral quercetin administration indicates dose, method, and frequency of delivery may be key to more robustly activating the PGC-1α pathway. In the current investigation quercetin was delivered in the food likely resulting in a consistent, though relatively small, increase in quercetin concentration for the duration of the project. This dose was based on previous work demonstrating a 0.2% quercetin diet increased plasma quercetin concentration [29]. In contrast, studies using the gavage technique to deliver quercetin [14,30] found increased PGC-1α pathway activation after one and six weeks, respectively, of a similar daily quercetin dose, likely causing a transient spike in bioavailable quercetin. In order to robustly activate the PGC-1α pathway in skeletal muscle pulsatile spikes in plasma quercetin concentration may be needed rather than a prolonged low-level plasma quercetin concentration. Another consideration is that the current investigation was carried out for six months where the former were considerably shorter in duration (days to weeks). How the prolonged dosing regimen may affect efficacy is unclear, however, this remains an important

Fig. 5. Representative images for utrophin IHC. 100x images of diaphragms from mdx-control (A) and mdx-quercetin (B) treated mice. Utrophin abundance did not differ between mdx-control and mdx-quercetin treatments (C). Data are means ± SEM.
consideration as DMD patients would likely be treated with quercetin for an extended period. Another possibility as to why the present results do not more closely match those of PGC-1α gene transfer studies is that PGC-1α protein content is decreased in dystrophic skeletal muscle compared with healthy skeletal muscle [31], thereby limiting the capacity for activation through deacetylation via Sirt1. This biological limitation is overcome in gene transfer studies; however magnitude of change may also be important as it is unlikely that supplementation studies will increase PGC-1α content to that achieved in transgenic or gene transfer studies.

While data presented in this investigation suggest at least partial activation of the PGC-1α pathway an important consideration is that quercetin may be acting through PGC-1α-independent mechanisms. Dystrophin deficiency leads to a host of maladaptations including oxidative stress and inflammation. Targeting of these contributors to muscle pathology has been previously shown to decrease disease severity [32-34]. Importantly, quercetin functions as an antioxidant and/or anti-inflammatory in a variety of cell types [15,55,56,37] and skeletal muscle taken from obese ob/ob mice [16]. Further, quercetin has recently been tested as an ameliorative anti-inflammatory and antioxidant therapy for sarcoidosis patients, an inflammatory disease with components of oxidative stress much like DMD. In this investigation quercetin decreased lipid peroxida

dation and inflammatory signaling [38].

In conclusion, we demonstrate that dietary quercetin enrichment decreased disease indices in dystrophic skeletal muscle and appears to be a promising therapeutic avenue. The mechanism responsible for the observed improvements remains to be elucidated. Increased expression of oxidative genes points toward activation of the PGC-1α pathway, however, a failure to increase utrophin transcript or protein abundance indicates this pathway was only partially activated. We postulate that PGC-1α pathway activation can be maximized following careful study of dosing schemes. Importantly, and consistent with the established safety profile of quercetin, animals receiving the quercetin-enriched diet did not exhibit signs consistent with toxicity.

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Statement of authorship

Contributions of authors to the manuscript: KH, RAS, JCQ, and JTS designed study: KH and JTS conducted research: RAS provided essential reagent: KH and JTS analyzed data: KH, RAS, JCQ, and JTS wrote the paper: JTS had primary responsibility for final content. All authors read and approved the final manuscript.

Conflict of interest

The authors have no conflict of interest.

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