

**Chronic Dietary Quercetin Enrichment and
Cardiac Pathology in Mdx and Mdx-Utrn^{+/-} Mice**

By

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Abstract

BACKGROUND: Duchenne Muscular Dystrophy causes declines in cardiac health resulting in mortality in up to 40% patients due to fibrosis and cardiopathology. Quercetin is a polyphenol possessing inherent anti-inflammatory and antioxidant effects and also potently activates SIRT1/PGC-1 α increasing mitochondrial biogenesis, antioxidant enzymes, and attenuates cardiopathology. **METHODS:** Our experiments tested whether life-long 0.2% dietary quercetin enrichment attenuates dystrophic cardiopathology in mdx and mdx-utrn ^{+/-} mice. Dystrophic animals were fed quercetin enriched or control diet for 14 or 10 months respectively. C57BL10 animals were fed a control diet for age-matched time. Cardiac function was assessed via 7T MRI. Post mortem, hearts were collected for histology and Western blotting. **RESULTS:** Findings reveal quercetin preserved overall cardiac function when compared to control mdx and mdx-utrn ^{+/-} mice. Histological analysis revealed increased expression of utrophin, α -sarcoglycan, utrophin/ α -sarcoglycan co-localization, decreased fibronectin, and decreased markers of cardiac damage versus control mdx and mdx-utrn ^{+/-} mice. Western blot analysis revealed quercetin increased cardiac PGC-1 α , cytochrome-c, ETC complexes I-V, citrate synthase, SOD2, and GPX versus control mdx and mdx-utrn ^{+/-} control mice. Western blot targets for inflammation revealed that quercetin decreased NF κ B, P- NF κ Bp65, P-IKBA α , while preserving IKBA α versus control mdx mice. Quercetin enrichment had a lower anti-inflammatory effect in mdx-utrn ^{+/-} mice. However, quercetin decreased TGF- β 1 and F4/80 versus control mdx and mdx-utrn ^{+/-} mice. **CONCLUSION:** Data suggest that long term quercetin enrichment attenuates cardiac dysfunction, increases mitochondrial biogenesis markers, antioxidant enzymes, decreases inflammation, and may partially reassemble the DGC in the dystrophic heart.

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Table of Contents

Abstract.....	i
Acknowledgments.....	ii
List of Abbreviations.....	iv
Chapter 1: Introduction.....	1
Chapter 2: Literature Review.....	4
Chapter 3: Pilot Data.....	18
Chapter 4: Methods and Experimentation.....	29
Chapter 5: Manuscript (MDX) for Submission/Publication.....	35
Chapter 6: Manuscript (MDX/UTRN ^{+/-}) for Submission/Publication.....	68
References	103

List of Abbreviations

DMD-Duchenne Muscular Dystrophy
ECG-Electrocardiogram
UTRN- Utrophin
SIRT1- Sirtuin 1
PGC-1 α - peroxisome proliferator-activated receptor gamma coactivator alpha
MMP9- matrix metalloprotease-9
KDa- Kilo Dalton
NF κ B65- nuclear factor kappa-light-chain-enhancer of activated B cells 65
P-NF κ B65- phospho-nuclear factor kappa-light-chain-enhancer of activated B cells 65
ROS- Reactive oxygen species
EF- Ejection Fraction
FS-Fractional Shortening
CO-Cardiac Output
SWT- Systolic wall thickness
EDV-End diastolic volume
ESV-End systolic volume
SV- Stroke volume
SOD2- Superoxide dismutase 2
GPX- Glutathione peroxidase
ETC-Electron transport chain
H&E- Hematoxylin and Eosin-y
IKB α - NF κ B light polypeptide gene enhancer in B-cells inhibitor, alpha
P-IKB α - phospho- NF κ B light polypeptide gene enhancer in inhibitor, alpha
COX2- Cyclooxygenase-2
iNOS- Inducible nitric oxide synthase
TGF- β 1- Transforming growth factor β -1
CD64- Cluster Differentiation 64
NIH-National Institute of Health
MRI-Magnetic Resonance Imaging

CHAPTER 1: INTRODUCTION

Duchenne muscular dystrophy (DMD) is an X-linked genetic disease that currently affects every 1 in 3,500 males [1]. Although multiple forms of muscular dystrophy exist, DMD is the most severe of the pathologies caused by a disturbance in the reading frame of the structural cytoskeleton protein dystrophin located on the Xp21 chromosome [2]. Physiologic consequences of DMD include fragile cell membranes, ionic disturbances, metabolic dysfunction, and cell death. Clinically, DMD is characterized by progressive muscle wasting and weakness with a wide range of heterogeneity. In a time-dependent disease progression, DMD leads to skeletal muscle degeneration and regeneration, respiratory complications, and also cardiac failure. Respiratory complications include problems breathing during sleep which result in headaches, fatigue, and poor appetite. Respiratory failure commonly causes death in many patients resulting in mortality in late teenage years [3]. However, due to recent improvements in secondary care and ventilation, cardiac health declines rapidly with age resulting in cardiac failure in many patients. Currently, up to 40% of all deaths resulting from DMD are due to complications resulting from cardiac pathology [4]. As disease severity increases with age, cardiac complications are evident including ECG abnormalities, cardiac remodeling, diastolic dysfunction, fibrotic tissue deposition, dilation of cardiac cavities, systolic dysfunction, and heart failure [5].

In absence of a cure, most treatments employed use corticosteroids and adjuvant therapies to decrease inevitable inflammation from the pathology. However, many treatments including the use of corticosteroids also cause other deleterious effects including excessive weight gain, immunosuppression, fatigue, increased blood glucose, and cardiac complications [6, 7]. While some treatments are successful in combating chronic inflammation characteristic of dystrophic pathology, numerous other physiologic and molecular aspects contribute to the severity of the phenotype of DMD. Thus, new and improved interventions need to be investigated in order to treat the collective pathology of DMD and both increase quality of life and life span of DMD patients.

To investigate and discover novel interventions that combat DMD, accurate and reliable models which recapitulate the disease are needed. Currently, most investigations for new

therapeutics are tested in animal models of DMD. Of those animal models, the mdx mouse, an inbred C57BL10 mouse strain developed through a spontaneous mutation of dystrophin, is one of the most popular and widely used. Furthermore, mdx/utrophin (utrⁿ) knockout mice are also useful to study DMD countermeasures because of accelerated pathology due to lack of the homologous structural protein utrophin which is thought to possess the ability to partially replace dystrophin [8]. Popularity of dystrophic mouse models is due to their relative cost effectiveness and approximate recapitulation of respiratory and cardiac distress characteristics in human DMD patients. Many interventions are effective in attenuating cardiac pathology in dystrophic mice including antioxidant therapy [9], inhibition of chronic inflammatory responses [5], exercise [10], and induction of adaptive gene transcripts including the Sirtuin 1 (SIRT1)/ peroxisome proliferator-activated receptor gamma coactivator alpha (PGC-1 α) axis which increases mitochondrial transcripts and expression of oxidative and membrane structural proteins such as utrophin [11]. In fact, previous work from our lab and others indicate that activation of PGC-1 α alleviates pathologies in dystrophin-deficient muscle [11, 12]. Consequently, there is currently no single therapeutic agent that successfully encompasses all of the subsequently mentioned successful therapeutic interventions in attenuating dystrophic cardiopathology.

However, a recent study has identified a class of molecules found in edible plants known as polyphenols that when consumed may attenuate the multi-faceted pathological mechanisms as a single therapeutic agent to attenuate dystrophic cardiopathology. Polyphenols are well known to combat oxidative stress [13], chronic inflammation [14], cardiac pathology [15], and induce adaptive gene expression through activation of SIRT/ PGC-1 α [16, 17]. Of the multiple polyphenols present in the human diet, quercetin makes up the majority of total polyphenol ingestion [18]. Quercetin is a flavonol with anti-inflammatory and antioxidant effects and is also a potent SIRT1/PGC-1 α activator capable of antioxidant up-regulation, mitochondrial biogenesis, and prevention of cardiac complications. Recent data from our lab and collaborators suggest that short-term moderate doses of 0.2% quercetin supplementation significantly elevate quercetin plasma levels and bioavailability, a finding that is closely associated with diminished pathology of dystrophin-deficient muscle [16]. However, it is currently less clear to which degree dietary quercetin enrichment can impact dystrophin-deficient hearts. Pilot data from our lab suggests that early short-term dietary quercetin enrichment in mdx mice increases mitochondrial biogenesis, antioxidant protein concentration, utrophin expression, and decreases

matrix metalloprotease-9 (MMP-9) in young mdx mouse hearts while also attenuating cardiac and fibrotic damage in older mdx mice [19]. While preliminary data are compelling, it is unknown whether long-term or chronic dietary quercetin enrichment affects physiologic variables similarly or could aid further in sustained attenuation of pathology. Also, more precise physiologic measurements such as cardiac function are needed to fully verify beneficial adaptations with quercetin enrichment. The overall aim of this research is to assess whether chronic dietary quercetin enrichment preserves cardiac function, decreases cardiac inflammation and damage, and induces adaptive gene expression in two dystrophic mouse models.

The underlying hypotheses of this proposal are:

Primary: Chronic 0.2% dietary quercetin enrichment *in vivo* will preserve cardiac function as assessed by MRI variables including: cardiac output, stroke volume, ejection fraction, end systolic volume, and end diastolic volume in hearts of mdx and mdx/utrn^{+/-} mice.

Secondary: Chronic 0.2% dietary quercetin enrichment *in vivo* will increase SIRT1/PGC-1 α expression causing biochemical signs of increased oxidative/mitochondrial protein expression, antioxidant expression, and adaptive gene expression in hearts of mdx and mdx/utrn^{+/-} mice.

Tertiary: Chronic 0.2% dietary quercetin enrichment *in vivo* will decrease histological signs of cardiac damage, fibrotic factors and proteins, and NF κ B mediated inflammatory markers in hearts of mdx and mdx/utrn^{+/-} mice.

CHAPTER 2: LITERATURE REVIEW

Duchenne Muscular Dystrophy

Duchenne Muscular Dystrophy (DMD) is an X-linked genetic disease caused by deletions or non-sense mutations of the dystrophin gene on chromosome Xp21 resulting in abnormal or absence of functional dystrophin in the sarcolemma or muscle fiber membrane [20]. DMD was termed after French physiologist Guillaume-Benjamin Duchenne who in the mid-19th century created the first published account of the pathology in infants with dystrophy [21]. Dystrophin, a 427 KDa rod-like protein, is expressed at the myotendinous junction and neuromuscular junction which create a mechanical link between the cytoskeleton of myofibrils and extracellular matrix in skeletal muscle and myocardium [22]. Since dystrophin has large elastic and flexible components in its rod-shaped domain, dystrophin protects myofibrillar membranes from contraction-induced stress [23]. However, deletions or mutations of other closely associated proteins with dystrophin such as sacroglycans and integrins are also implicated in DMD [4].

Independent of the precise molecular basis, the primary pathological consequence is rapid degeneration of muscle fibers causing a robust drive for regeneration. However, much of the futile regeneration for contractile tissue is overshadowed by infiltration of connective tissue (fibrosis) or fat deposition caused by inflammation. In time, this remodeling of dystrophic muscle leads to progressive muscle weakness. Clinical signs of weakness from DMD typically occur between 3-4 years of age with frequent falls and pseudohypertrophy of calf muscles. Disease progression is such that most patients cannot walk on their own or are wheel chair bound by 10 years of age [24]. Patients with DMD suffer from progressive weakening of respiratory accessory and diaphragm muscles leading to reliance on mechanical or medical intervention for ventilation in early adulthood to aid in prolonging survival [25]. Respiratory complications often occur including problems breathing during sleep which result in headaches, fatigue, and poor appetite. Complete respiratory failure commonly causes death in many patients resulting in mortality in late teenage years [3]. However, since the application of improved ventilation system interventions and secondary care prolongs years of survival with DMD, cardiac complications and progressive heart failure have emerged as pathology major form of morbidity

and mortality in those with DMD. Further prolongation of survival and attenuation of the pathology requires not only improving skeletal muscle function, but also interventions which can attenuate progression of heart disease.

Approximately 95% of the patients with DMD develop cardiac complications by 20 years of age, and for approximately 20% of these patients it is limiting for survival. Currently, up to 40% of all deaths resulting from DMD are due to complications resulting from cardiac pathology [4]. Patients with DMD exhibit cardiac complications in an age-dependent fashion as the pathology progresses. While phenotypic expression of DMD in skeletal and cardiac muscle are similar, cardiac-specific mechanisms responsible for dystrophic cardiopathy include oxidative stress, chronic inflammatory responses, cardiomyocyte death, and metabolic dysfunction. Indeed, declines in cardiac health seen with DMD are caused by a culmination of physiologic phenomena including mechano-induced membrane microruptures, ionic dyshomeostasis, intracellular Ca^{2+} signal amplification, oxidation of ionic transporters, mitochondrial dysfunction, and cardiomyocyte necrosis or apoptosis resulting in fibrotic tissue disposition [26]. As a result of physiological disturbances, numerous cardiac function outcomes also deteriorate as pathology progresses. ECG abnormalities and signs such as sinus tachycardia are typically identified in most DMD patients at early ages. As the pathology progresses, echocardiograms reveal contractile abnormalities of ventricular walls due to fibrotic areas. Dilated cardiomyopathy is evident by gradual enlargement and thinning of ventricular walls [26]. DMD patients develop consequent loss of cardiac contractility ultimately leading to heart failure. Arrhythmias also develop that may lead to sudden death [27]. Accordingly, additional research is warranted to further aid and help individuals affected with DMD. The following proposal introduces a new and exciting possible therapy for the primary molecular mechanisms underlying the fundamental exacerbation of DMD: oxidative stress, Ca^{2+} overload, mitochondrial dysfunction, and chronic inflammation.

Oxidative Stress and DMD

Underpinning the fundamental loss of functional dystrophin or dystrophin complex, other complications contribute to the overarching pathology of DMD. Oxidative stress is an underlying contributor to both the muscular and systemic pathology of DMD. Collectively, increases in oxidative stress cause free-radical mediated damage to almost all cellular constituents including proteins, lipids, and DNA [28]. Under healthy homeostatic conditions, endogenous defense

systems prevent oxidative damage and quench reactive oxygen species (ROS). However, recent evidence suggests that patients suffering from DMD are more vulnerable to damage from oxidative stress. Research with *in vitro* populations of mdx mouse myogenic cells reveal that oxidative injury contributes to the first pathologic change, namely cell death and muscle cell necrosis [29]. Redox imbalance is an underlying pathological factor in dystrophic muscle cells at early onset of life. Accordingly, many *in vivo* studies report that oxidative stress exacerbates and contributes significantly to the pathology of DMD [30, 31]. The diaphragm and respiratory muscles suffer from significant fibrosis and are more susceptible to free radical damage [32, 33]. Studies of diaphragm from mdx mice suggest that oxidant production is elevated and antioxidant enzyme activity is decreased contributing to fibrosis, weakness, and fatigue of respiratory muscles leading to a greater probability for respiratory failure [31, 34].

Oxidative damage is not limited to skeletal muscle, but also promotes tissue degeneration in the dystrophic myocardium. However unlike skeletal muscle, degeneration of myocardial tissue causes more robust deposition of connective tissue (fibrosis) due to the lack of regeneration of cardiomyocytes. Dystrophic ventricular cardiomyocytes exhibit specific characteristics when compared to healthy control animal counterparts: membrane fragility [35], ionic dyshomeostasis [36, 37], and importantly a shift in mitochondrial and intracellular redox status towards a more oxidative state [9, 26]. While subsequent characteristics can be viewed as individual contributors to the overarching cardiopathology, it should be mentioned that increased ROS production and oxidative stress exacerbates the overall phenotypical characteristic contributors. For example, increased free radical production causes lipid peroxidation of the phospholipid bilayer causing membrane damage and leakage [38]. Sarcolemmal damage abolishes both the ionic and osmotic gradient between intra- and extracellular compartments. Furthermore, oxidative stress causes oxidation and dysfunction of ion transport proteins such as ryanodine receptors (RyR) and sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) which contributes to 'Ca²⁺ overload' and Ca²⁺ signal amplification [36, 39]. Like dystrophic skeletal muscle, multiple *in vivo* studies reveal dystrophic hearts exhibit increased susceptibility to oxidative damage causing cardiomyocyte death, increased ventricular fibrosis, and decreased antioxidant enzyme activity [9, 40]. The culmination of subsequent damaging factors contributes to ventricular wall thinning, cardiac remodeling, and ventricular dilation. Moreover, ROS production causes direct consequences on myocardial contractile function by oxidizing

contractile proteins within the entire myocardium [41]. Oxidation and denaturation of contractile proteins leads to poor cardiac functional outcomes such as systolic and diastolic dysfunction, decreased stroke volume, and cardiac dilation. Thus, oxidative stress is a cornerstone contributor to the overarching disease of DMD and dystrophic cardiomyopathy. While direct production of ROS contributes significantly to cardiac dysfunction in DMD, other factors intensify and contribute simultaneously to a shift to an oxidative state including Ca^{2+} dysregulation and mitochondrial dysfunction.

Ca^{2+} and Mitochondrial Dysregulation and DMD

Endogenous Ca^{2+} levels within muscle tissue are tightly regulated and essential for proper muscle contraction and function [42]. Within the muscle, proteins and enzymes such as RyR, Dihydropyridine (DhP), SERCA etc. that control Ca^{2+} homeostasis and muscular contraction [43-45]. Dysregulation of Ca^{2+} or damage to Ca^{2+} handling proteins lead to ‘ Ca^{2+} overload’ which triggers pathologic cellular outcomes including necrosis, apoptosis, and mitochondrial damage. Furthermore, mitochondria become a secondary sink for elevated Ca^{2+} which leads to further metabolic dysfunction [46]. Studies reporting elevated Ca^{2+} accumulation in muscle biopsies of DMD patients lead to more investigation and an identity of another pathological mechanism that exacerbates DMD [47, 48]. Indeed, recent evidence indicates that increased influx of Ca^{2+} into muscle cells is observed with DMD [48]. *In vitro*, myotube mdx mouse muscle cells have an increase in a class of mechano-transducing Ca^{2+} channels in unique proportions to normal mice. In this same study, mdx skeletal myofibrillar cells had increased calcium entry through stretch activated ions channels [49]. Franco et al. supported this further *in vitro* showing that specific mechano-sensitive Ca^{2+} channels in mdx muscle exist in two gating modes and that mechanical stimuli cause an irreversible conversion between modes leading to an excess leakage of cytosolic Ca^{2+} [50]. In a comparable *in vitro* model, the presence of Ca^{2+} selective leak channels presented a 3-fold higher opening probability in mdx skeletal muscle preparation [51]. Yet, controversies still exist regarding the role of Ca^{2+} influx in these models [see selected review [52]]. However, it is widely accepted that the lack of dystrophin *in vivo* causes impairment in the trans-sarcolemmal connection [53] which results in increased frequencies of surface micro-tears of the sarcolemma during contraction causing transient dysregulation of cytosolic Ca^{2+} homeostasis [54-56]. Membrane ruptures can be re-sealed

relatively quickly, however increases in Ca^{2+} -leak channels are introduced into the dystrophic sarcolemma [57] exacerbating cytosolic Ca^{2+} levels to an even greater extent [58].

Currently, it is widely accepted that defects in Ca^{2+} handling, mitochondrial function, and excitation-contraction coupling are characteristic of failing hearts [59]. Pathological changes in Ca^{2+} handling in dystrophic hearts are due impart to changes in phosphorylation and abundance of Ca^{2+} handling proteins in addition to maladaptive modifications in pathways that control Ca^{2+} handling protein expression. Dystrophic murine hearts possess multiple defects in Ca^{2+} -handling proteins including sarcoplasmic reticulum (SR) Ca^{2+} binding proteins and SERCAs. Furthermore, dystrophic ventricular cardiomyocytes overexpress mechano-sensitive Ca^{2+} -leak channels similar to skeletal muscle which exacerbate cardiac dilation in mdx mice [60]. As a result, dystrophic cardiomyocytes suffer from abnormally elevated intracellular Ca^{2+} concentrations and increased Ca^{2+} transients [61]. The disruption in homeostatic Ca^{2+} conditions in dystrophic hearts causes contractile failure, arrhythmias, conduction abnormalities, and cardiomyocyte death [26]. As mentioned previously, alterations in Ca^{2+} regulation and oxidative stress work synergistically to damage dystrophic cardiac muscle. Increased oxidative stress and free radical damage causes increased Ca^{2+} influx into the myocardial cytoplasm, and consequently into both the mitochondria and the nucleus. Since the myocardium is such a bioenergetically demanding tissue, it requires tightly controlled functional mitochondria to synthesize high-energy phosphate molecules for proper metabolism and appropriate control of cell fate directly through signaling molecules. Under aerobic conditions, mitochondria can constitute up to 30% of the total myocyte volume [62]. With rising intracellular Ca^{2+} levels, mitochondria absorb the excess Ca^{2+} through pores and uncouple, become dysfunctional, and impair metabolism of the heart. Ca^{2+} further acts as a signal molecule to respond to oxidant stimuli or other components of the cell and activates calpains, proteases, repressors, and can affect gene transcription [63]. Change in redox status and Ca^{2+} overload cause cardiomyocyte necrosis, apoptosis, fibrosis, and metabolic dysfunction due to mitochondrial damage. In isolated mdx mice cardiomyocytes, ROS production is abnormally high and ROS scavengers prevent most of the acute Ca^{2+} signaling alterations, proposing an important role of the interplay of Ca^{2+} and oxidative stress [9, 36, 38]. Multiple studies demonstrate Ca^{2+} overload entry pathways such as membrane tears due to lipid peroxidation (i.e. microruptures) [37], RyR oxidative damage [39], and also DhP alterations [64]. Since alterations in Ca^{2+} regulatory proteins also allow

excess Na^+ to enter cardiomyocytes (e.g. SACCs, tears, TRP channels), simultaneous increases in intracellular Na^+ further exacerbate Ca^{2+} overload. In dystrophic cardiomyocytes, disruption in Na^+ homeostasis activates the reverse mode of $\text{Na}^+-\text{Ca}^{2+}$ exchanger (NCX) in an effort to restore homeostasis. While activation of the reverse mode of NCX relieves the cell of the elevated cytosolic Na^+ concentration, the activity is futile in that greater concentrations of Ca^{2+} are pumped intracellularly [26]. Collectively, Ca^{2+} dysregulation with the interplay of oxidative stress serves as a pathomechanism for typical cardiac pathology associated with DMD.

Inflammation and DMD

Among the multi-faceted aspects contributing to pathology, DMD is commonly characterized by chronic inflammation due to a robust drive of degeneration and regeneration of myocytes from the lack of functional dystrophin. Myocytes of patients with DMD commonly exhibit inflammatory changes and activation of multiple immune responses. Dystrophin mutations lead to membrane damage allowing for large amounts of immune cell infiltration leading to necrosis and cell death [65]. While healthy skeletal muscle possesses the ability to fully regenerate due to injurious signals, this ability is abolished in DMD possibly due to exhaustion of satellite cells during regeneration. Many different genome studies report data that provide evidence for coordinated activity of cytokine and chemokine signaling, leukocyte diapedesis, leukocyte adhesion molecules, and invasive cell type-specific markers [66]. Of particular interest, nuclear factor-kappa-B (NF κ B) is a major transcription factor responsible for modifying inflammatory and proliferative cellular responses [67]. Under normal conditions, NF κ B is constitutively present in an inactive form in the cytoplasm bound to an inhibitor I κ B α [68]. When activated, I κ B α is phosphorylated and degraded causing release and translocation of NF κ B (preferentially the NF- κ B-heterodimer p50/p65) into the nucleus [68]. In particular, nuclear translocation of phosphorylated NF- κ B is significantly higher in DMD patients than healthy normal individuals [69]. While NF- κ B is diverse and controls many pro-inflammatory responses from the cell, it particularly upregulates pro-inflammatory cytokines (i.e. TNF- α , IL-10, IL-6), fibrotic growth factors (i.e. TGF- β 1, CTGF), chemokines (i.e. IL-8, CCL20), and various inflammatory enzymes (i.e. iNOS, COX2) [68]. Genomic data on mdx mice reveal that elevation of these chronic pro-inflammatory processes subsequently mentioned dominate much of pathology in muscle deficient of dystrophin [70]. Immune response signals and overexpression of extracellular matrix genes such as matrix metalloprotease-9 (MMP-9) are also

evident in DMD muscle leading to extensive remodeling of cardiovascular and muscular tissue [71]. Many studies show that blunting inflammatory responses in DMD patients reduces muscle pathology and disease progression. Depletions of CD8+ and CD4+ T cells and macrophages *in vivo* attenuates overall pathology further cementing the role of immune cell response in progression of the disease [71]. Furthermore, specific pharmacological inhibition of the NF- κ B pathway results in improved outcome and preserves skeletal muscle function in mdx mice. Commonly implemented in the treatment of DMD, corticosteroids have strong anti-inflammatory effects and are one of the most widely used drugs to prevent chronic inflammation. DMD patients treated with prednisolone show significant attenuation of disease progression and significant prolongation of normal voluntary movement [72]. Consequently, long term use of corticosteroids causes many other deleterious effects including weight gain, decreased bone mineral density, fluid retention, and perturbations in blood glucose homeostasis [6, 7]. Despite adverse side-effects, corticosteroid administration has become a standard treatment and improves muscle pathology and decreases inflammation in DMD.

Myocarditis is widely accepted to contribute significantly to progressive heart failure in DMD [73]. Dystrophic cardiopathology is distinctly marked by increases in infiltration of immune cells, ventricular fibrosis, increases in inflammatory proteins, and necrotic cell death. Inflammatory signaling molecules are increased in dystrophic hearts, importantly activated NF κ B, leading to cascade signaling and upregulation of downstream factors contributing to the overarching pathology including TGF- β 1, MMP9, fibronectin, iNOS, and COX2. Like dystrophic skeletal muscle, pharmacological inhibition of NF κ B in dystrophic murine hearts is associated with preserved cardiac function and attenuation of cardiac damage likely due to attenuation of pathological signaling factors [74]. Of these gene transcripts, transforming growth factor β 1 (TGF- β 1) is markedly increased in dystrophic cardiac tissue and is a primary stimulating signal for fibrotic deposition and fibroblast proliferation. Evidence reveals TGF- β 1 is localized in atrophic, necrotic, and degenerating cardiomyocytes causing loss of contractile function. Blockade of TGF- β 1 signaling attenuates muscle degeneration in multiple models of myopathy [75]. Furthermore, TGF- β 1 significantly contributes to pathological left ventricular remodeling and dysfunction [76]. Other factors synergistically contributing to dystrophic cardiac remodeling are MMP9 and fibronectin. MMP9 is a secreted collagenase that causes basement membrane and extracellular matrix degradation. Metalloproteases such as MMP9 are elevated in

dystrophic myocardium and contributes to pathological remodeling [19]. Furthermore, evidence suggests inhibition of metalloproteases attenuate left ventricular remodeling, cardiomyopathy, and contractile dysfunction in models of heart failure [77]. Recent evidence also reveals that MMP9 contributes to other cardiac functional pathologies, including atrial fibrillation, further reinforcing cardiopathological consequences of elevated MMP9 expression [78]. Along with MMP9, elevated expression of the glycoprotein fibronectin is involved in dystrophic cardiac remodeling. Fibronectin, typically expressed by fibroblasts, is routinely involved in healthy cellular processes including cell adhesion, growth, and differentiation. However, altered expression and organization is associated with numerous pathologies. As cellular damage occurs, commonly seen with dystrophic cardiopathology, fibronectin is up-regulated by fibroblasts and contributes to fibrosis and collagen deposition synergistically with subsequently mentioned TGF- β 1. In failing hearts, fibronectin gene expression is over 3 fold higher when compared to normal healthy hearts [79]. Accompanying increases in fibronectin gene expression, similar 3 fold increases in collagen I and collagen III gene expression are expressed in the failing heart [79]. Pertaining to DMD, recent evidence from our lab revealed significant increases in fibronectin protein expression in aged mdx mice [16]. Thus, the aforementioned pro-fibrotic and tissue remodeling factors that contribute to inflammatory heart failure and could play a crucial role in decreased cardiac function and increased fibrosis in dystrophic cardiopathology.

Adding to the inflammatory pathology, NF κ B signaling also causes increases in various inflammatory proteins in dystrophic myocardium including inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX2). While nitric oxide stimulation is physiologically necessary for proper vascular and cardiac function under healthy conditions, the NF κ B-mediated activation of iNOS drives an inflammation-mediated overproduction of nitric oxide which leads to recruitment of proinflammatory signals such as cytokines and chemokines (i.e. TNF- α , TGF- β 1). In heart pathologies, iNOS expression is increased in dilated cardiomyopathy patients and patients with congestive heart failure when compared to healthy hearts [80]. Pertaining to DMD, upregulation of iNOS is reported to be increased several-fold in mdx hearts versus healthy controls by 12 months of age via increases in NF κ B activation. Furthermore, increases in iNOS are concomitantly accompanied by contractile dysfunction, decreased cardiac performance, and ECG abnormalities [81]. COX2 is distinctly involved in conversion of arachidonic acid to prostaglandin H₂. Functionally, prostaglandin H₂ is a precursor for many physiological

molecules. However, numerous COX2-derived prostaglandins cause recruitment of inflammatory cells, induce apoptotic cell death, and induce vasospasms if overexpressed. In patients with cardiomyopathy, a phenotype similarly seen with DMD, NFκB signaling and COX2 expression is highly increased and is accompanied by morphological abnormalities and fibrotic scarring [82]. Furthermore, treatment with natural polyphenolic compounds decrease NFκB signaling along with iNOS and COX2 expression revealing a novel therapeutic target [83]. While standard treatment with corticosteroids combats inflammation in dystrophic skeletal muscle, cardiac function is negatively affected [84, 85]. Thus, more research is warranted to elucidate more therapeutic approaches that safely deactivate or activate cellular pathways to decrease inflammation while also alleviating the multi-faceted consequences of DMD with minimal side effects from long term treatment.

Quercetin and SIRT1/PGC-1α Pathway

Compromised mitochondrial function is indicated in many diseases, including DMD, and causes metabolic and cardiovascular consequences [86]. Genetic basis for these consequences manifest themselves in decreased expression of genes controlling for aerobic capacity and mitochondrial biogenesis. One of the greatest factors controlling mitochondrial biogenesis and slow twitch muscle fiber shifts is the pleiotropic coactivator peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α) [87, 88]. Activity of PGC-1α is under control of cellular pathways that direct its methylation, phosphorylation, and acetylation by various molecules including Sirtuin 1 (SIRT1) [89]. Originally viewed for its role in the extension of lifespan, SIRT1 is a NAD⁺- dependent protein deacetylase that functions with PGC-1α to promote adaptation under caloric challenge and increases the gene expression of mitochondrial and gluconeogenic factors [90]. Activated PGC-1α stimulates mitochondrial biogenesis in concert with the increased expression of electron transport chain components (i.e. NADH dehydrogenase, succinate dehydrogenase) and mitochondrial metabolic enzymes (i.e. citrate synthase) which account largely for adaptive capability of skeletal and myocardial tissue [91]. PGC-1α activation selectively reduces mitochondrial Ca²⁺ overload by decreasing the number of Ca²⁺ uptake sites and pores while also increasing organelle volume [91]. Furthermore, PGC-1α activation upregulates antioxidant enzymes in the myocardium including manganese superoxide dismutase (SOD2) and glutathione peroxidase (GPX) which protect cellular components from free radical damage [92]. Recently, our group and collaborators found that induction of PGC-1α

gene expression in dystrophic muscle increases oxidative capacity and decreases dystrophic pathology [11]. Overall, therapeutic increases in PGC-1 α levels and induction positively affect the myocardium and upregulate energy production within the heart [93] and hold promise for DMD therapy. Conversely, much of the evidence of the physiological significance of PGC-1 α is revealed in studies that use PGC-1 α ^{-/-} mice or PGC-1 α down-regulation models. Various studies reveal decreases in activity of SIRT1 and PGC-1 α cause metabolic consequences and decrease lifespan presumably due to mitochondrial dysfunction much like the phenotype seen in DMD [94, 95]. PGC-1 α ^{-/-} mice exhibit decreases in myocardial antioxidant enzymes (i.e. SOD2), increases in markers of oxidative damage (i.e. 4-hydroxynoneal), and decreases of left ventricular fractional shortening and dP/dT_{max} [92]. Furthermore, PGC-1 α down-regulation causes decreased mitochondrial enzymes, metabolic dysfunction, and cardiac function abnormalities [96]. Thus, proper cardiac and mitochondrial function is dependent on PGC-1 α levels and activation. Since PGC-1 α is capable of inducing large beneficial adaptations in the myocardium, research in pathology is presently being conducted on how to induce increases in PGC-1 α levels and activity. In accordance with this research, dietary compounds that induce SIRT1/PGC-1 α activity are being researched to provide alternative avenues of stimulating adaptive gene expression.

Polyphenol compounds such as quercetin and resveratrol are well known for possessing antioxidant and anti-inflammatory properties [97, 98]. Perhaps most relevant to countermeasures against DMD pathology, a recent study reveals that polyphenols increase SIRT1 activity and induce mitochondrial activity through PGC-1 α [17, 99]. In mdx mice hearts, polyphenol administration prevents immune cell and macrophage infiltration while also inducing upregulation of adaptive genes such as SIRT1, PGC-1 α , and utrophin [97]. In particular, oral quercetin enrichment induces mitochondrial biogenesis in humans and mice through increased gene and protein expression of SIRT1 and PGC-1 α [99, 100]. Further research from our group indicates that short-term quercetin enrichment is associated with decreased muscle damage and injury in mdx mice through what appears to be activation of the SIRT1/PGC-1 α axis [16]. Regarding the heart, past studies reveal that chronic dietary quercetin enrichment prevents pathologic cardiac remodeling and Ca²⁺ overload in murine models, though the direct effects on dystrophic cardiopathology remain unknown [15, 101]. Thus, further research is warranted, as

dietary polyphenol and quercetin enrichment provides promising therapeutic effects for dystrophic cardiopathology.

Quercetin and metabolism

Polyphenols are organic compounds characterized by the presence of large phenol structural units and are present in various fruits, vegetables, grains, and leaves [102]. Flavonols comprise the largest groups of polyphenols in the humans diet, which consist of three-ring structures with two aromatic centers and an oxygenated heterocycle [103, 104]. Furthermore, flavonols are the most common dietary flavonoids of which quercetin (3,3',4',5,7-pentahydroxyflavone) is among the most abundant [103]. Among foods in the human diet, the largest sources of quercetin include capers, onions, kale, various peppers, blueberries, apples, and sweet potatoes [102, 104]. Average dietary flavonol consumption ranges from 13 to 64 mg/day of which quercetin comprises approximately 75% [18, 104]. In a dose-dependent fashion, quercetin is absorbed by humans in substantial amounts with an approximate half-life of 11-28 hours [103, 105, 106]. Concentrated supplements of quercetin are typically purified and isolated from various plants and are available over-the-counter at pharmacies and nutrition stores.

Quercetin is metabolized through the diet and conjugated to sugar derivatives or sulfates. Furthermore, these conjugated derivatives can also be variously modified including methylation. Once absorbed by the intestines, quercetin conjugates are present in the blood including quercetin-3'-sulfate and quercetin-3-glucuronide [107]. Following a single dose of quercetin, peak quercetin conjugates in human plasma can reach circulating levels of 200 $\mu\text{g/ml}$ or 0.6 μM with a reported half-life of 17 hours [108]. Absorption of quercetin and conjugated derivatives occurs rapidly possibly through sugar transporters in the intestine [109]. Elimination of quercetin and conjugated derivatives occurs slowly progressing further throughout the day. Furthermore, both aglycone (glycosylated) and conjugated quercetin significantly increase in porcine tissues following a single quercetin dose and 4-week quercetin enriched diet [110]. Conjugated quercetin is deconjugated to the aglycone form by β -glucuronidase within the liver, lungs, kidneys and muscles including the heart [110]. Thus, quercetin dietary enrichment significantly increases bioavailable quercetin derivatives to many different tissues within the body including the heart.

Chronic high-dose dietary quercetin enrichment does not cause any deleterious effects in both humans and rodents [111]. Multiple studies demonstrate that high dose (approximately 2,000 mg/kg/day) quercetin enrichment for 2 years does not cause toxicity in animals [112-114]. Furthermore, even higher doses (12,000 and 3,000 mg/kg/day) of dietary quercetin enrichment for 2 years in toxicological studies with animals did not produce deleterious outcomes including toxicological measures within the heart [115, 116]. Based off of these studies, the human equivalent doses would be ~1,621 mg/kg and ~243 mg/kg, respectively [117]. Currently, an upper limit or ideal dose for humans has not been established [111]. However, in multiple clinical studies no deleterious symptoms or perturbations in blood chemistries or cardiac abnormalities were found following 12 weeks of quercetin enrichment of 14 mg/kg/day [118, 119]. Further, higher quercetin doses ranging from 21-31 mg/kg safely decrease the severity of sarcoidosis, a chronic inflammatory disease resulting in increased oxidative stress, much like DMD [120]. In 2010, the Food and Drug Administration labeled quercetin as GRAS (Generally Recognized as Safe) and this orally available PGC-1 α activator is currently available at most supplement and vitamin stores. Given the well-established safety record of quercetin, even at high doses, success in animal studies could move quickly to clinical trial. Because of the efficacy, safety, and wide availability, quercetin is ideal for easy and effective therapeutic treatment of dystrophic cardiopathology.

Scientific Rationale of Quercetin-Mediated Rescue of Dystrophic Cardiopathology

DMD is a juvenile pathology caused by mutations in genes coding for dystrophin or dystrophin complex resulting in lack of functional dystrophin in the muscle membrane [20]. Primary consequences of DMD include rapid muscle fiber necrosis and deterioration. DMD is commonly marked by chronic inflammation, oxidative stress, and mitochondrial dysfunction which all contribute to dystrophic pathology as the disease progresses. Recently, improved secondary care prolongs survival rates in affected individuals such that cardiac complications and abnormalities now account for up to 40% of all DMD related deaths [4]. Lack of a cure drives a necessity for investigation of novel pragmatic therapies to attenuate the multifaceted pathology of DMD. Animal models including dystrophic murine modes recreate large facets of DMD disease etiology and allow for detailed mechanistic study to further therapeutic breakthroughs.

In murine models of DMD, cardiac pathology is exacerbated as a function of increasing age. Although 2 month old dystrophic mice have regular left ventricle function and ECGs [121], hearts of this age are vulnerable to overload stimuli [122]. By 9 months of age, dystrophic murine hearts become dilated, fibrotic, and exhibit contractile dysfunction [26]. Furthermore, cardiomyocytes from dystrophic murine models are prone to ionic dysregulation due to membrane fragility [36] as marked by elevated intracellular Na^+ and Ca^{2+} levels [38]. Decreased mitochondrial content, redox dyshomeostasis, and metabolic dysfunction also parallel ionic dysregulation exhibited in dystrophic hearts [9, 36].

Quercetin, a safe and naturally occurring flavonoid, is found in various types of plants and fruits present in the human diet [123]. Flavonoids, including quercetin, influence anti-oxidative gene expression [124, 125] and possess anti-inflammatory effects. Examples include inhibiting NF κ B signaling and inflammatory protein expression, associated with many disease conditions including the heart [83, 98, 126]. Quercetin also prevents pathological cardiac remodeling through inhibition of extracellular matrix breakdown, fibrotic scarring, and pathological morphological changes [127]. Along with being a potent stimulator of PGC-1 α which increases mitochondrial biogenesis, quercetin also possesses the ability to preserve electron transport chain function and prevent mitochondrial uncoupling thus preserving function [128, 129]. Enrichment of quercetin in the diet causes activation of post-translational modification and activation of the SIRT1/PGC-1 α pathway axis. Once activated, PGC-1 α activates various transcription factors of target genes in response to stress stimuli including ion dysregulation [130], oxidative stress [131], inflammation, and cytokines [132]. Furthermore, PGC-1 α promotes adaptive gene expression including mitochondrial transcript genes, slow oxidative muscle fiber genes, and structural accessory proteins like utrophin [87, 133]. Since utrophin and dystrophin are similar in size and structure and found at the neuromuscular junction (NMJ), utrophin can partially localize in the intracellular cytoskeleton and extracellular matrix linkage to the sarcolemma which may aid in membrane stabilization and partially alleviate DMD pathology [134, 135]. Thus, using quercetin to induce utrophin replacement or up-regulation in absence of dystrophin is a promising therapeutic strategy to compensate for the primary pathological mechanism of dystrophin deficiency. Quercetin is also inexpensive, safe, and widely available making it ideal for a potential therapeutic strategy to combat dystrophic cardiopathology. Since quercetin influences genes including anti-inflammatory genes,

antioxidant genes, and mitochondrial transcript genes, dietary quercetin enrichment provides further novel therapeutic potential to alleviate many secondary pathological mechanisms including chronic inflammation, oxidative stress, and mitochondrial dysfunction.

Research Paper

Histological and biochemical outcomes of cardiac pathology in *mdx* mice with dietary quercetin enrichment

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New Findings

- What is the central question of this study?
Does dietary quercetin enrichment improve biochemical and histological outcomes in hearts from *mdx* mice?
- What is the main finding and what is its importance?
Biochemical and histological findings suggest that chronic quercetin feeding of *mdx* mice may improve mitochondrial function and attenuate tissue pathology.

Patients with Duchenne muscular dystrophy suffer from cardiac pathology, which causes up to 40% of all deaths because of fibrosis and cardiac complications. Quercetin is a flavonol with anti-inflammatory and antioxidant effects and is also an activator of peroxisome proliferator-activated receptor γ coactivator 1 α capable of antioxidant upregulation, mitochondrial biogenesis and prevention of cardiac complications. We sought to determine the extent to which dietary quercetin enrichment prevents (experiment 1) and rescues cardiac pathology (experiment 2) in *mdx* mice. In experiment 1, 3-week-old *mdx* mice were fed control chow (C3w6m, $n = 10$) or chow containing 0.2% quercetin for 6 months (Q3w6m, $n = 10$). In experiment 2, 3-month-old *mdx* mice were fed control chow (C3m6m, $n = 10$) or 0.2% chow containing 0.2% quercetin for 6 months (Q3m6m, $n = 10$). Hearts were excised for histological and biochemical analyses. In experiment 1, Western blot targets for mitochondrial biogenesis (cytochrome c , $P = 0.007$) and antioxidant expression (superoxide dismutase 2, $P = 0.014$) increased in Q3w6m mice compared with C3w6m. Histology revealed increased utrophin ($P = 0.025$) and decreased matrix metalloproteinase 9 abundance ($P = 0.040$) in Q3w6m mice compared with C3w6m. In experiment 2, relative ($P = 0.023$) and absolute heart weights ($P = 0.020$) decreased in Q3m6m mice compared with C3m6m. Indications of damage (Haematoxylin- and Eosin-stained sections, $P = 0.007$) and Western blot analysis of transforming growth factor $\beta 1$ ($P = 0.009$) were decreased in Q3m6m mice. Six months of quercetin feeding increased a mitochondrial biomarker, antioxidant protein and utrophin and decreased matrix metalloproteinase 9 in young mice. Given that these adaptations are associated with attenuated cardiac pathology and damage, the present findings may indicate that dietary quercetin enrichment attenuates dystrophic cardiac pathology, but physiological confirmation is needed.

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Introduction

Duchenne muscular dystrophy (DMD) is a childhood disease caused by dystrophin gene mutations that result in the absence of functional dystrophin in the sarcolemma (Hoffman *et al.* 1987). The primary pathological consequence of DMD is muscle degeneration and muscle fibre necrosis. Duchenne muscular dystrophy is characterized by inflammation-induced fibrosis and fat deposition, leading to progressive muscle weakness. Improved secondary care in recent decades has prolonged survival rates in DMD patients such that cardiac complications and abnormalities now account for up to 40% of all DMD-related deaths (Costanza & Moggio, 2010). With the current lack of a cure, pragmatic therapies are needed to attenuate the multifaceted disease pathology of DMD. Animal models, including the *mdx* mouse, recapitulate many key facets of DMD disease aetiology and progression and are employed to further therapeutic breakthroughs.

The *mdx* mouse has a nonsense mutation on exon 23 of the dystrophin gene and is a widely used model of DMD (Sicinski *et al.* 1989). *Mdx* mice develop cardiac pathology as a function of increasing age, in that 2-month-old *mdx* mice have normal left ventricular function and ECGs (Quinlan *et al.* 2004), although hearts at this age are susceptible to mechanical overload (Danialou *et al.* 2001). Appearing at 9 months of age, *mdx* mouse hearts are fibrotic, hypertrophied and exhibit diminished contractility (Shirokova & Niggli, 2013). Due to sarcolemmal fragility, *mdx* cardiomyocytes are predisposed to ionic dysregulation (Jung *et al.* 2008) as marked by elevated intracellular Na^+ and Ca^{2+} levels (Prosser *et al.* 2011). Mitochondrial redox dyshomeostasis parallels Ca^{2+} accumulation in the matrix and results in metabolic dysfunction and deteriorating mitochondrial content in *mdx* hearts (Williams & Allen, 2007; Jung *et al.* 2008). Altogether, the phenotype of the *mdx* mouse exhibits many biochemical and pathological aspects of the DMD cardiac phenotype. Given these parallels to the human condition, the *mdx* mouse continues to serve as an experimental model for investigation of therapeutic interventions to mitigate DMD pathology.

Quercetin (3,3',4',5,7-pentahydroxyflavone) is a safe, naturally occurring and widely available flavonoid found in a variety of plants and fruits (Bhagwat *et al.* 2005). Dietary flavonoids, including quercetin, influence antioxidative genes (Dias *et al.* 2005; Ciz *et al.* 2008) and blunt inflammation associated with many disease conditions (Camuesco *et al.* 2006; Boots *et al.* 2008). Quercetin also prevents pathological cardiac remodelling (Erdman *et al.* 2005) and stimulates mitochondrial biogenesis (Davis *et al.* 2009; Nieman *et al.* 2010). When taken orally, quercetin upregulates post-translational modification of peroxisome proliferator-activated receptor gamma

coactivator 1 α (PGC-1 α); PGC-1 α activates numerous nuclear receptors and transcription factors of target genes in response to stress stimuli, including Ca^{2+} ion dysregulation (Szabadkai *et al.* 2006), oxidative stress (St-Pierre *et al.* 2006), inflammation and cytokines (Puigserver & Spiegelman, 2003). Activated PGC-1 α promotes slow oxidative muscle fibre gene expression, including beneficial mitochondrial transcripts (Wu *et al.* 1999) and structural accessory proteins such as utrophin (Lin *et al.* 2002). The upregulation of utrophin in the absence of dystrophin is promising therapeutic strategy to compensate for dystrophin deficiency. While more recent evidence shows notable differences in utrophin and its ability to duplicate dystrophin-like physiological functioning directly (Li *et al.* 2010; Belanto *et al.* 2014), both proteins are similar in size and structure and are found at the neuromuscular junction. Importantly, in the absence of dystrophin, utrophin can partly localize in the intracellular cytoskeleton and extracellular matrix linkage to the sarcolemma, which may aid in alleviating DMD pathology (Tinsley *et al.* 1998; Squire *et al.* 2002).

In the present study, we sought to determine the extent to which dietary quercetin supplementation alleviates dystrophic cardiac pathology by attenuating inflammation, fibrosis, oxidative stress and the decreased number of mitochondria, in addition to promoting increased utrophin abundance in *mdx* mice. In support of the present study rationale, exercise has previously been shown to improve cardiac function in *mdx* mice (Selsby *et al.* 2013). Researchers in our laboratory and others have revealed that activation of PGC-1 α ameliorates dysfunction by significantly blunting pathology (Handschin *et al.* 2007; Selsby *et al.* 2012; Hollinger *et al.* 2013). Moreover, we recently demonstrated that dietary quercetin enrichment mitigated biochemical and histological indices of pathology in diaphragms taken from *mdx* mice (Hollinger *et al.* 2014). For the present investigation, two independent experiments were conducted to evaluate the potential of quercetin dietary enrichment to prevent disease onset (experiment 1) or rescue already declining myocardium (experiment 2).

Methods

Study designs and animals

The experimental protocol was approved by the Iowa State University Animal Care and Use committee and guidelines established by the American Physiological Society for the use of animals in research. During experimentation, all animals from our colony at Iowa State University were housed on a 12 h–12 h light–dark cycle. Diets consisted of standard mouse chow or 0.2% quercetin-fortified rodent chow (Bioserv Inc., Frenchtown, NJ, USA). Quercetin

enrichment was founded on previous demonstration that 0.2% quercetin supplementation was adequate safely to elevate significantly the quercetin plasma levels and increase quercetin bioavailability (Zhang *et al.* 2010). In the prevention-directed experiment 1, 3-week-old *mdx* mice were randomly assigned to receive either 0.2% quercetin chow (Q3w6m, $n = 10$) or control rodent chow (C3w6m, $n = 10$) for 6 months. Likewise, in rescue-directed experiment 2, 3-month-old *mdx* mice were randomly assigned to control chow (C3m6m, $n = 10$) or 0.2% quercetin-enriched chow (Q3m6m, $n = 10$) for 6 months. All food and water were provided *ad libitum*. After each aim was completed, mice were weighed and anaesthetized intraperitoneally to a surgical plane with tribromoethanol prior to being killed by cervical dislocation.

Tissue harvesting and storage

Immediately after death, the mouse hearts were excised and rinsed in ice-cold 10 mM PBS. Hearts were blotted dry, placed into optimal cutting temperature (OCT) compound and flash frozen in liquid nitrogen-chilled 2-methyl-butane to facilitate rapid chilling without freeze fracture. Hearts were stored at -80°C until further study. The hearts harvested were obtained from the same cohort of mice from our collaborators that were used to assess the diaphragm pathology with quercetin enrichment (Hollinger *et al.* 2014)

Microscopy and immunofluorescence

In blinded conditions, 10- μm -thick heart cross-sections were cut on a Shandon Cryotome cryostat (-30°C) and placed on 1 mm microscope slides (Fischer Scientific, Pittsburgh, PA, USA). Selected slides were stained with Haematoxylin and Eosin Y (H&E) and imaged by light microscopy with Nikon software (Melville, NY, USA). A number of serial images (15–20) were taken to encompass the entire heart tissue for each cross-section in order to ensure consistent representation in the final analysis. Digital images were analysed using ImageJ software (National Institutes of Health, Bethesda, MD, USA) at $\times 40$ magnification as a ratio of total damaged area (in square micrometres) to healthy tissue area (in square micrometres).

Myocardial abundance and the localization of matrix metalloproteinase 9 (MMP9) and utrophin were determined using a histochemical fluorescence detection protocol. Heart cross-sections were fixed with a 10% formalin solution, washed, and permeabilized with Triton X-100 in 1% sodium citrate solution. Samples were blocked with 3% bovine serum albumin and 20% goat

serum. Tissues were incubated with rabbit antilaminin antibody (Sigma Aldrich, St Louis, MO, USA) and secondary antibody conjugated to Texas Red fluorescent tag (Vector Laboratories Inc., Burlingame, CA, USA). Tissues were incubated with primary antibodies for MMP9 (1:50 dilution, sc-6840; Santa Cruz Biotechnology, Dallas, TX, USA), or utrophin (1:50 dilution, sc-33700; Santa Cruz Biotechnology). Tissue sections were incubated with secondary antibody conjugated to fluorescent tags (1:100 dilution, sc-2356; Santa Cruz Biotechnology), sealed with a 4',6-diamidino-2-phenylindole mounting medium (Vector Laboratories Inc.) and imaged with a fluorescent microscope (Nikon, Melville, NY). Fluorescent images for 4',6-diamidino-2-phenylindole (nuclei), rhodamine (laminin) and FITC filters (MMP9 or utrophin) area combined and analysed for the mean FITC intensity area percentage (in square micrometres) using Nikon Software. Serial images (15–20) were acquired as described above for H&E analysis. All analysis and imaging were completed in blinded conditions by the same technician.

Western blotting

Briefly, 20 μg of myocardial protein per sample was obtained from frozen OCT samples previously used for histology and separated using standard SDS-PAGE techniques on 10–17% polyacrylamide gels. Following electrophoresis, proteins were transferred to polyvinylidene difluoride membranes and exposed to a rabbit polyclonal primary antibody for transforming growth factor $\beta 1$ (TGF- $\beta 1$; 1:500 dilution, sc-146; Santa Cruz Biotechnology), superoxide dismutase 2 (SOD2; 1:500 dilution, sc-30080; Santa Cruz Biotechnology), PGC-1 α (1:500 dilution, sc-13067; Santa Cruz Biotechnology) cytochrome *c* (1:500 dilution; Santa Cruz Biotechnology) and anti-rabbit IgG–HRP-conjugated secondary antibody (Cell Signaling, Danvers, MA, USA) for chemiluminescence detection. Membranes were reprobed for α -tubulin (normalizing protein; 1:1000 dilution; DSHB, Iowa City, IA, USA) and a monoclonal anti-mouse IgG–HRP-conjugated secondary antibody (Cell Signaling) for chemiluminescence detection. Western blots were normalized to α -tubulin and analysed using a UVP LLC digital imaging device (Upland, CA, USA). All experiments and analysis were completed in blinded conditions by the same technician.

Statistical analyses

Animal characteristic and key dependent variables from experiments 1 and 2 were examined with Student's unpaired *t* test using SPSS 21 statistical software (IBM,

Table 1. Heart weight and relative heart-to-body weight ratios for experiments 1 and 2

Characteristic	Experiment 1		Experiment 2	
	C3w6m	Q3w6m	C3m6m	Q3m6m
Heart weight (mg)	154.56 ± 11.01	150.57 ± 4.78	166.95 ± 9.73	138.57 ± 4.86*
Relative heart/body weight (mg g ⁻¹)	4.91 ± 0.20	4.72 ± 0.72	5.29 ± 0.14	4.80 ± 0.11

Mean absolute heart weight (in milligrams) and mean relative heart weight (in milligrams per gram) for experiment 1 (C3w6m and Q3w6m groups) and experiment 2 (C3m6m and Q3m6m groups) are presented. Data are means ± SEM. *Significant difference from respective experiment control value.

New York City, NY, USA). A significance threshold was determined at $P \leq 0.05$ *a priori*.

Results

Experiment 1 (prevention)

Animal characteristic data, including absolute heart weight and heart-to-body weight ratios, from experiment 1 are presented in Table 1. Heart weights were similar between Q3w6m and C3w6m mice ($P = 0.734$). Likewise, relative heart-to-body weight ratios were also similar between Q3w6m and C3w6m mice ($P = 0.415$).

Results and representative images of biochemical analyses from experiment 1 are presented in Fig. 1. Biochemical analyses of Western blots of cytochrome *c* revealed that hearts from Q3w6m mice exhibited on average 50% more relative protein content compared with C3w6m mice ($P = 0.007$; Fig. 1A). Likewise, SOD2 expression was 50% higher in Q3w6m *versus* C3w6m hearts ($P = 0.014$; Fig. 1B). However, no differences in TGF- β 1 ($P = 0.310$; Fig. 1C) or PGC-1 α ($P = 0.789$; Fig. 1D) were found between treatments in 3w6m mice.

Results and representative images of histological analyses from experiment 1 are presented in Fig. 2. Immunofluorescence analyses revealed that MMP9 abundance was attenuated by 50% in Q3w6m hearts compared with Cw6m hearts ($P = 0.040$; Fig. 2A). Also, a 40% increase in the expression of utrophin was observed in Q3w6m *versus* C3w6m hearts ($P = 0.025$; Fig. 2B). Cardiac damage, as determined by H&E staining, was not different between groups ($P = 0.763$; Fig. 2C).

Experiment 2 (rescue)

Animal characteristic data, including absolute heart weight and heart-to-body weight ratios, from experiment 2 are presented in Table 1. In Q3m6m mice, absolute heart weights were 17% lower than in C3m6m mice ($P = 0.023$). Likewise, relative heart-to-body weight ratios of Q3m6m mice were lower by 6% compared with C3m6m mice ($P = 0.020$).

Results and representative images of biochemical analyses from experiment 2 are presented in Fig. 3. Biochemical analysis of Western blots revealed no differences between treatment groups for cytochrome *c* ($P = 0.097$; Fig. 3A), SOD2 ($P = 0.421$; Fig. 3B) or PGC-1 α ($P = 0.325$; Fig. 3D). However, Western blot analysis of profibrotic TGF- β 1 was decreased by 40% ($P = 0.009$; Fig. 3C) in hearts from Q3m6m compared with C3m6m mice.

Results and representative images of histological analyses from experiment 2 are presented in Fig. 4. Immunofluorescence analyses revealed that MMP9 expression ($P = 0.608$; Fig. 4A) and utrophin expression ($P = 0.250$; Fig. 4B) were unaffected between Q3m6m and C3m6m treatment groups. Notably, consumption of a quercetin-enriched diet was associated with 50% mitigation of the pathological damage ($P = 0.007$; Fig. 4C) in Q3m6m hearts compared with C3m6m hearts using a rescue protocol.

Discussion

Duchenne muscular dystrophy patients have extended lifespans such that many sufferers die from cardiac-related complications (Shirokova & Niggli, 2013). Given the lack of an available cure, pragmatic countermeasures are needed to attenuate the cardiac pathology caused by dystrophin deficiency. The PGC-1 α pathway decreases dystrophic pathology and is implicated as a master regulator of adaptations in *mdx* mice (Selsby *et al.* 2012). Targeting of the PGC-1 α pathway represents a novel avenue for DMD therapy. Quercetin, an orally available isoflavone, activates PGC-1 α via sirtuin 1 (Boots *et al.* 2008). A recent companion paper from our collaborative group provides early evidence that quercetin may mediate protection of dystrophic skeletal muscle using the same cohort of *mdx* animals (Hollinger *et al.* 2014). The present experiments were derived from the hearts of the aforementioned study. As with our prior study, we treated *mdx* mice with a 0.2% quercetin-enriched diet for 6 months, beginning at weaning (experiment 1), to address the capacity for disease prevention and at 3 months of age (experiment 2) to address the capacity for rescue. In

experiment 1, histological markers of disease in treated and untreated groups were not observed, but indices of quercetin-mediated pathway activation were increased. In experiment 2, when mice were 9 months of age we detected histological evidence of significant myocardial injury, which appeared to be blunted in quercetin-treated mice. Hence, collective study findings suggest that dietary quercetin enrichment may attenuate some secondary pathological markers in the multifaceted DMD pathology in the heart.

Experiment 1 (prevention)

Quercetin has been well demonstrated to increase a variety of signalling pathways and gene products possibly related to PGC-1 α activation (Davis *et al.* 2009; Han *et al.* 2009; Nieman *et al.* 2010). Based on known responses to quercetin administration and efficacy, a strategic panel of outcome variables relevant to DMD cardiac pathology were chosen for the present study. These variables

included indirect biomarkers of mitochondrial density, cardiac remodelling, fibrotic desposition, endogenous antioxidant status, and possible alleviation of pathology by increased cardiac utrophin content. Hearts from young quercetin-fed mice exhibited more cytochrome *c*, which suggests elevated mitochondrial density in these hearts. The importance of this preliminary finding is underscored by prior understanding that untreated *mdx* mice exhibit decreased mitochondrial content and metabolic dysfunction due to Ca²⁺ overload (Vandebrouck *et al.* 2006). Moreover, functional dystrophin is required for proper mitochondrial localization and function (Percival *et al.* 2013). If correct, our interpretation of preserved mitochondrial content in hearts from quercetin-fed mice is a novel finding supported by prior understanding that quercetin upregulates mitochondrial activity and reverses mitochondrial defects (Karuppagounder *et al.* 2013). The present findings provide preliminary support for the increased mitochondrial density seen in hearts from quercetin-supplemented mice. While

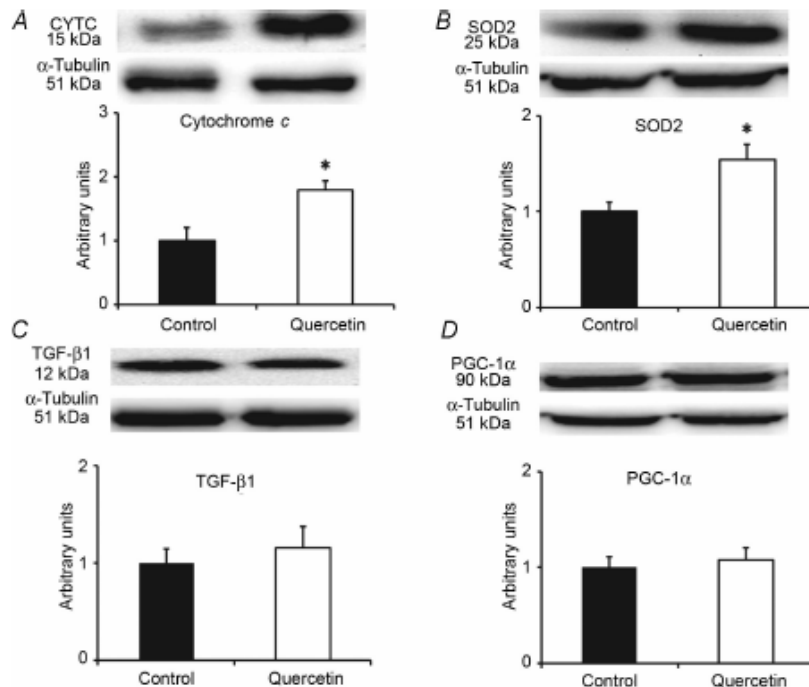


Figure 1. A quercetin-enriched diet increases cytochrome *c* and superoxide dismutase 2 (SOD2) protein abundance during prevention of pathology

Mean protein abundance and representative blots of cytochrome *c* (A), SOD2 (B), transforming growth factor β 1 (TGF- β 1; C) and peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α ; D) normalized to α -tubulin (in arbitrary units). Data are shown as means + SEM. *Significant difference from control value.

not conclusive, selected biomarker outcomes provide compelling early evidence within the context of an applied whole-animal feeding study that quercetin may be an efficacious therapeutic intervention against mitochondrial dysfunction in dystrophic hearts. However, future studies are warranted using gene expression, protein blotting of other mitochondrial biomarkers and/or enzyme kinetics to support these early findings fully.

While quercetin possesses direct antioxidant activity (Boots *et al.* 2008), the findings from experiment 1 reveal increased protein abundance of the endogenous antioxidant enzyme SOD2 in hearts from quercetin-fed mice. Primarily associated with the mitochondrial matrix of oxidative tissues, SOD2 serves to quench the pathological formation of reactive oxygen species in a way that benefits the heart (Van Remmen *et al.* 2001; Hamilton *et al.* 2004). The present findings are supported by the previous observation that quercetin-associated upregulation of SOD2 mitigates oxidative stress (Vásquez-Garzón *et al.* 2009). Moreover, increased cardiac SOD2 expression culminates in an innate protection for the mitochondria against oxidative damage associated with metabolic derangement in hearts from quercetin-fed mice. Additionally, the increase in SOD2 protein content in hearts from quercetin-fed mice bolsters

our preliminary finding and suggests that an increase in mitochondrial biogenesis may have occurred.

Cardiac utrophin protein abundance was elevated in quercetin-fed mice. Given that utrophin is similar in size and structure to dystrophin, it can partly replace absent dystrophin in the sarcolemma of DMD hearts and drastically decrease disease pathology (Squire *et al.* 2002). Prior investigation of dietary polyphenol treatment elicited an increase in utrophin gene transcript levels (Selsby *et al.* 2012; Gordon *et al.* 2013); however, the present study appears to be the first to demonstrate that polyphenol consumption increases utrophin protein expression in the *mdx* heart. The present findings of increased cardiac utrophin protein content are particularly significant in that prior evidence for increased utrophin protein expression due to dietary intervention is currently lacking in the published literature. However, caution is warranted with regard to this finding in that the immunofluorescence technique used to measure utrophin expression in the present study is semi-quantitative. As such, replicative findings that employ more definitive quantitative techniques are needed. Increased utrophin expression is marked further by the observation that cardiac MMP9 content was decreased in quercetin-fed mice. Matrix metalloproteinase 9 contributes to tissue

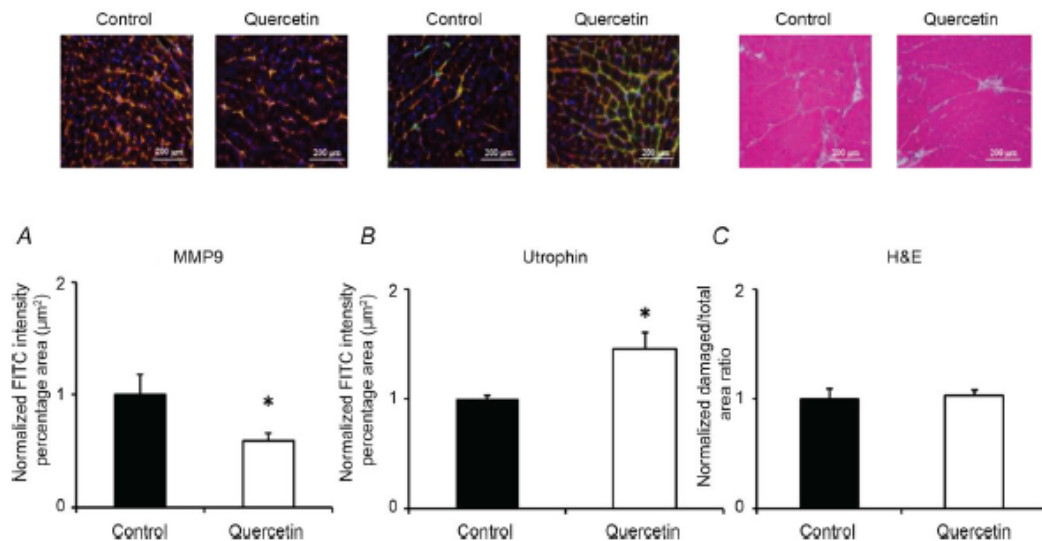


Figure 2. Dietary quercetin enrichment alters matrix metalloproteinase 9 (MMP9) and utrophin localization and abundance in prevention of pathology

A and B, mean normalized fluorescein isothiocyanate (FITC) intensity percentage area (in square micrometres) and representative images for MMP9 (A) and utrophin expression (B). C, mean normalized damaged-to-total area ratio of Hematoxylin- and Eosin Y (H&E)-stained sections. Data are shown as means \pm SEM. *Significant difference from control value.

remodelling and breakdown of the extracellular matrix, and previous evidence suggests that an increased MMP9 abundance is a telling biomarker of disease progression in DMD patients (Nadarajah *et al.* 2011). Matrix metalloproteinase 9 is directly involved in left ventricular remodelling and heart failure (Reinhardt *et al.* 2002), while inhibition or deletion of MMP9 attenuates left ventricular remodelling and preserves cardiac contractility (Moshal *et al.* 2008). Lower MMP9 expression in the hearts of quercetin-fed mice is supported by prior investigations, in which supplementation was cyto- and mitoprotective (Lee *et al.* 2011; Chakraborty *et al.* 2012). Saragusti *et al.* (2010) found that quercetin inhibits MMP9 activity, with structure–activity analysis confirming that flavonoid R3'–OH and R4'–OH substitutions contributed to MMP9 inhibitory properties. Once again, however, these findings are tempered to some degree because MMP9 expression was measured using a semi-quantitative immunofluorescence technique. Thus, attenuation of MMP9 may be another mechanism by which quercetin supplementation delays the onset of dystrophic cardiomyopathy.

Analysis of TGF- β 1, damage (H&E) and heart weights were unchanged in the mice of experiment 1 and reinforce earlier observations that cardiac remodelling in *mdx*

mice is not readily discernable prior to 9 months of age (Quinlan *et al.* 2004; Shirokova & Niggli, 2013). Cardiac PGC-1 α protein abundance was also unchanged in experiment 1 and refutes our working hypothesis for this protein. While quercetin and related polyphenols are potent sirtuin 1/PGC-1 α activators (Howitz *et al.* 2003), prior investigations that report PGC-1 α transcript levels following acute supplemental dosing do not include protein content (Davis *et al.* 2009; Nieman *et al.* 2010). As such, quercetin-induced PGC-1 α protein expression may be temporally unobserved. In support, the polyphenol-mediated regulation downstream of PGC-1 α targets can occur independent of corresponding changes in PGC-1 α protein (Selsby *et al.* 2012; Gordon *et al.* 2013).

Experiment 2 (rescue)

Mice supplemented with quercetin during the rescue protocol exhibited lower absolute and relative heart weights than control mice, which suggests that quercetin supplementation prevented pathological cardiac remodelling in comparison to untreated *mdx* mice. Previous evidence supports our findings that quercetin

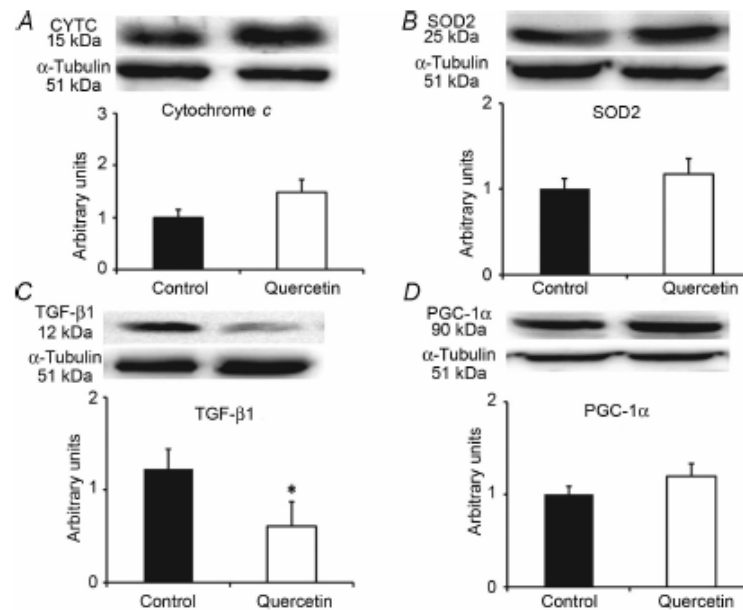


Figure 3. A quercetin-enriched diet decreases TGF- β 1 protein abundance during rescue from pathology. Mean protein abundance and representative blots of cytochrome c (A), SOD2 (B), TGF- β 1; (C) and PGC-1 α (D) normalized to α -tubulin (in arbitrary units). Data are shown as means + SEM. *Significant difference from control value.

supplementation prevents cardiac hypertrophy in other rodent disease models (Wang *et al.* 1999; Han *et al.* 2009). Consistent with the notion of cardioprotection, TGF- β 1 protein abundance was lower in quercetin-fed mice than in control animals. Transforming growth factor β 1 is a key regulator of fibrosis and collagen deposition (Chen *et al.* 2009), and increased TGF- β 1 expression is associated with cardiac hypertrophy (Villarreal & Dillmann, 1992). Quercetin blocks signalling of fibroblasts and TGF- β 1 (Phan *et al.* 2004), and may have clinical implications directly related to DMD (Costanza & Moggio, 2010). In accordance, H&E-stained histological sections revealed less ventricular damage in hearts of quercetin-fed mice *versus* control hearts. The mice in the present study were of an age at which cardiac pathology is readily identifiable (Shirokova & Niggli, 2013). While not confirmatory without physiological verification, the collective findings of lower TGF- β 1, lower heart weights and attenuated tissue damage in quercetin-fed mice suggests that rescue of cardiac function may also have occurred. We previously found that polyphenol administration was associated with decreased damage in skeletal muscle following H&E staining (Selsby *et al.* 2012; Hollinger *et al.* 2014). Thus, the present findings strongly suggest that quercetin supplementation attenuates the pathological phenotype in *mdx* hearts, a conclusion that requires physiological follow-up confirmation.

In contrast to experiment 1, the myocardial levels of cytochrome *c*, SOD2, MMP9 and utrophin and the protein abundance of PGC-1 α were unaltered by quercetin. Disparities between the prevention and rescue protocols are multifaceted. First, early elevations in the downstream targets observed in experiment 1 (prevention) may be less responsive to quercetin as a function of advancing age. Second, the mice in experiment 2 were 3 months old at the start of quercetin intervention, an age at which the mechanisms of disease aetiology may not be altered by quercetin to the extent observed in experiment 1. Indeed, we recently found only incomplete or slight PGC-1 α pathway activation in *mdx* mouse diaphragms following 6 months of feeding a quercetin-enriched diet (Hollinger *et al.* 2014).

Conclusion

We conducted this applied study in order to investigate a potential dietary countermeasure against cardiopathological symptoms associated with DMD. Given the limited tissue availability in mouse hearts, histological and biochemical outcomes were chosen strategically to gain preliminary understanding of whether chronic 0.2% quercetin feeding attenuates myocardial remodelling, redox control, mitochondrial content and

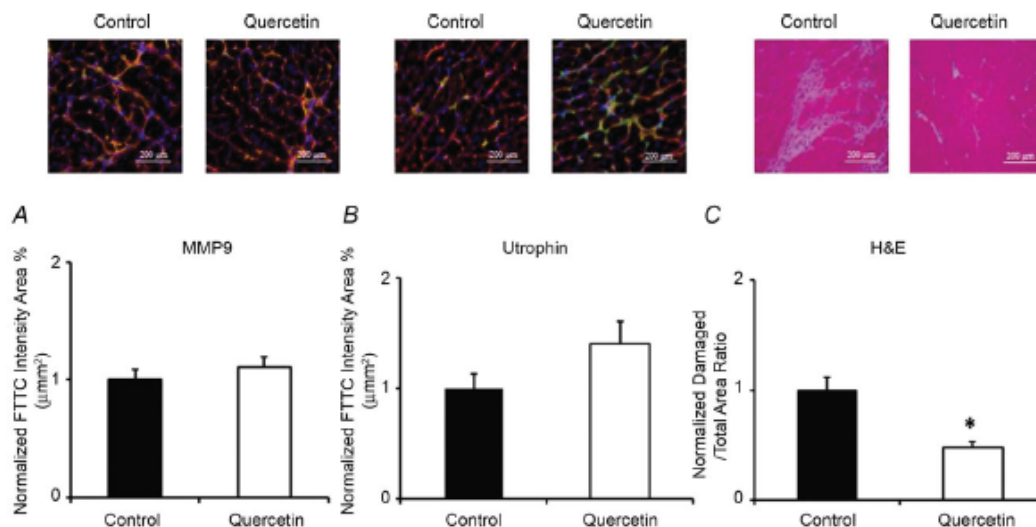


Figure 4. Dietary quercetin enrichment decreases fibrotic injury during rescue from pathology. A and B, mean normalized FITC intensity percentage area (in square micrometres) for MMP9 (A) and utrophin expression (B). C, mean normalized damaged-to-total area ratio of H&E-stained sections. Data are shown as means + SEM. *Significant difference from control value.

tissue damage in hearts of *mdx* mice. Two 6 month feeding experiments were used to quantify quercetin prevention (experiment 1) and rescue (experiment 2) in the *mdx* mouse heart. The findings of increased cytochrome *c* and SOD2 content implicate a possible scenario in which quercetin feeding may be associated with increased mitochondria and enzymatic antioxidant defenses. Moreover, the MMP9 content was lower in young quercetin-fed mice and may reflect attenuation in the pathological remodelling stimulus. Cardiac damage (H&E) and fibrotic growth factors (TGF- β 1) were attenuated in hearts in experiment 2. While it is tempting to speculate that the improved biochemical outcomes in experiment 1 pre-empted histological outcomes in experiment 2, follow-up studies with physiological confirmation are needed. Nonetheless, the findings of the present study provide exciting new evidence that dietary quercetin may be a pragmatic countermeasure against DMD cardiac pathology. Moreover, given the early findings in our companion study in skeletal muscle (Hollinger *et al.* 2014), follow-up investigation with physiological outcomes should be directed at understanding whether oral quercetin may prove to be a countermeasure against cardiac and skeletal muscle pathology.

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Additional information

Competing interests

None declared.

Author contributions

Conception and design of the experiments: C.G.B., J.T.S. and J.C.Q. Collection, analysis and interpretation of data: C.G.B., K.H., J.T.S., R.H.A. and J.C.Q. Drafting the article or revising it critically for important intellectual content: C.G.B., J.T.S., R.H.A. and J.C.Q. All the authors approved the final version of the manuscript for publication.

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CHAPTER 4: METHODS AND EXPERIMENTATION

DMD Models

The mdx mouse is a strain of mice developed through a spontaneous mutation (mdx) in inbred C57BL10 mice. Similar to DMD patients, mdx mice have complete loss of functional dystrophin in skeletal muscle and cardiac tissue. However, controversy exists whether the mdx mouse is an appropriate model to compare to humans in that the mdx mouse tends to have a less severe phenotype and can live to a near normal life span as compared to unaffected control mice. Much of the controversy has recently been resolved with the discovery that mdx mice do develop significant dystrophic cardiac pathology later in life and also have comparable diaphragm pathology and fibrosis. While young 2 month old mdx mice have normal ECGs [121], they are susceptible to mechanical overload damage when compared to healthy control mice [122]. ECG deviations gradually increase in the mdx mouse by the age of 6 months and severe abnormalities of the cardiac conduction system are evident [81, 136]. By 9 months of age, hearts from mdx mice are dilated, hypertrophied, fibrotic, and show poor function of contractility [121, 137]. Muscle tissues from mdx mice are highly susceptible to damage due to oxidative stress [34], increased inflammation through pathways including NFκB [34], Ca^{2+} dysregulation [138], decrease in force production with increased age [33], and degeneration and regeneration accompanied by fibrosis [29, 139]. Based off of these previous studies and more direct study, the mdx mouse exhibits many comparable characteristics to human DMD diaphragms and reproduces the changes and pathology in DMD [140].

In response to criticism of the mdx mouse, use of mdx/utrn^{+/-} mice has emerged as a more severe phenotypical murine model mimicking clinical manifestations of DMD. Utrophin is a cytoskeletal protein that is partially homologous to dystrophin and is widely distributed in muscle tissue similar to dystrophin [141]. Currently, it is accepted that utrophin can partially compensate for the lack of functional dystrophin and partially attenuate pathology associated with lack of functional dystrophin. Mdx/utrn^{+/-} mice show similar phenotypes as mdx mice but the pathology is accelerated and much more severe causing shorter life spans. Thus, the mdx/utrn^{+/-} mouse is thought to be a more clinically relevant murine model to mimic DMD. While there may be some limitations to the dystrophic mouse models as compared to a human

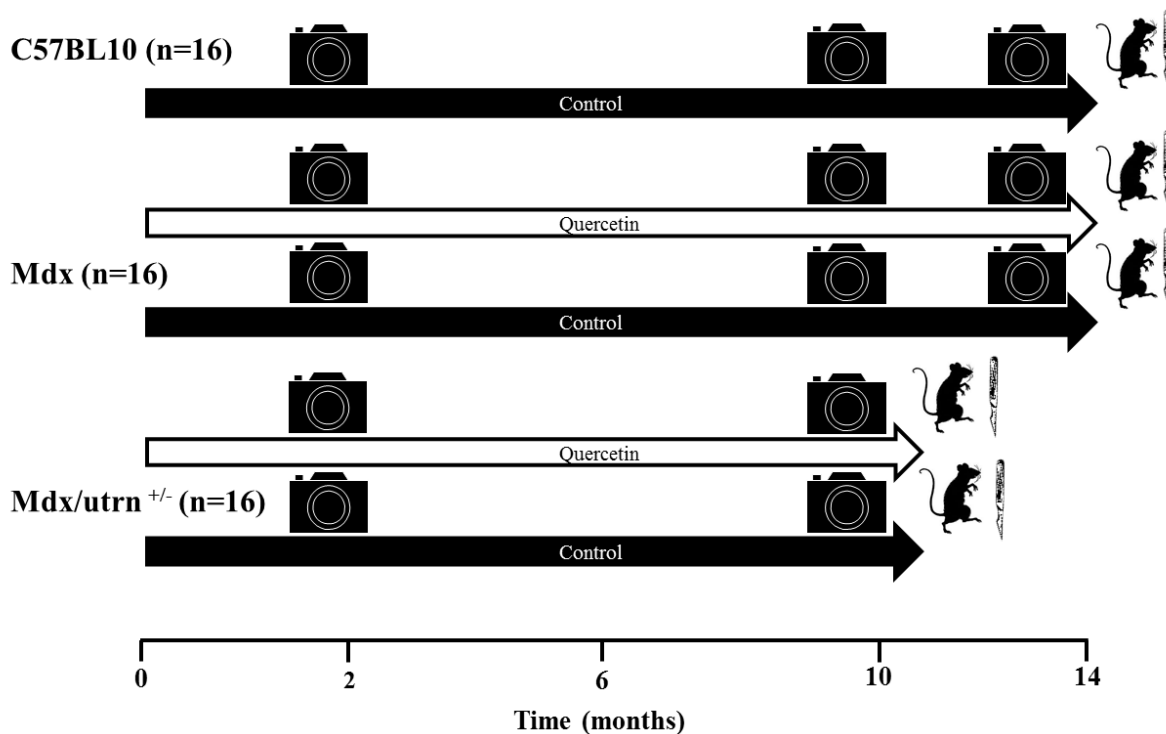
such as skeletal muscle phenotype severity, they are certainly useful models to both explore functional and physiological measures of consequences of the genetic defect of the lack of functional dystrophin. Thus, while every experimental model has its limitations, dystrophic mouse models provide adequate, cost-effective, longitudinal, and convenient ways to further study how to combat the pathology of DMD.

Study Design

Briefly, three different strains of mice (C57, mdx, and mdx utr^{n^{+/-}}) were split into two different treatment groups (n=8/group): 0.2% quercetin enriched or control diet. The concentration of quercetin enrichment was chosen based off of previous findings that 0.2% quercetin supplementation was adequate in significantly elevating quercetin plasma levels safely, increases quercetin bioavailability, and is near previously mentioned human consumption quantities [142]. At 2 months, 10 months, 14 months, 7-tesla magnetic resonance imaging (MRI) was used to image C57 and Mdx hearts with a small bore copper radio frequency (RF) coil. Mdx/utr^{n^{+/-}} were imaged identically at 2 months and 10 months due to decreased life span and accelerated pathology. Multiple variables were calculated including cardiac output, stroke volume, ejection fraction, end systolic volume, end diastolic volume, fractional shortening, and ventricular wall thickness. Furthermore, real time cinematic clips of each heart were obtained and used for further analysis. Before sacrifice, animals were assessed for amounts of physical activity using an animal activity scale.

Following collection of physical activity data, mice were weighed, put under a surgical plane of anesthesia with isoflourane and euthanized by cardiac excision. Hearts were weighed and placed in optimal cutting temperature (OCT) compound. Tissues were then frozen in 2-methylbutane chilled by liquid nitrogen, and stored at -80°C until subsequent biochemical and histological analysis.

Study Design



Study Design. C57, mdx, and mdx/utrn^{+/-} mice were randomly assigned into 2 groups: chronic 0.2% quercetin enriched (denoted by empty arrows) or control diet (denoted by black arrows). Using a 7T MRI, real time heart function was obtained and analyzed (denoted by heart). Before sacrifice, physical activity measures (denoted by mouse) and tissue collection (denoted by scalpel) were obtained.

Animal Characteristics and Physical Activity Assessment

To further characterize animals, body weight and amount of food eaten/day were recorded every month. Furthermore, absolute (mg) and relative heart weight (mg/g body weight) were calculated and recorded post-cardiac excision allowing for descriptive analysis of cardiac dilation and/or hypertrophy. At corresponding end time points, physical activity was obtained using a modified animal physical activity scale. Briefly, mice were observed for 10 minutes twice at corresponding morning and night times (ex. 6 am and 6 pm). During the 10 minutes of observation, mice were scored in 15 second intervals for the amount of times a certain physical act is performed. The following are the physical acts in increasing order of approximated metabolic equivalents per task (METS): sitting still, grooming, eating/drinking, socializing,

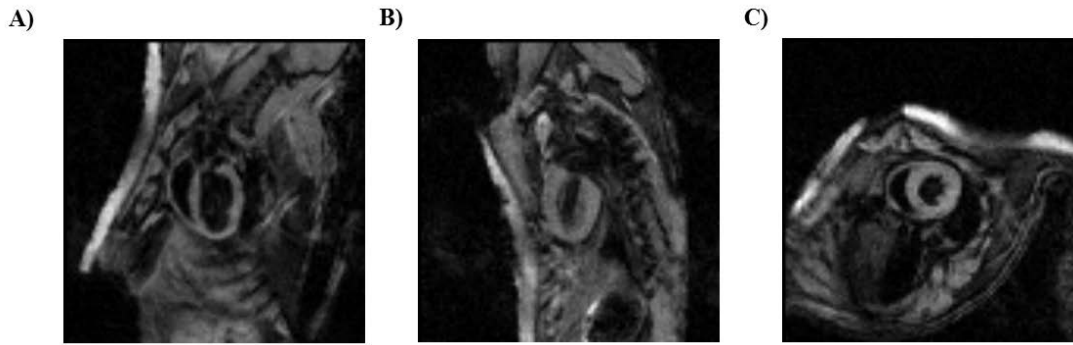
standing, walking, wall pacing, running, and jumping. Data are presented as occurrence of physical act/10 minutes at both morning and night time points.

MDXQ-7	15	30	45	1	15	30	45	2	15	30	45	3	15	30	45	4	15	30	45	5	15	30	45	6	15	30	45	7	15	30	45	8	15	30	45	9	15	30	45	10		
Sit/Still	1	1				1							1	1	1	1	1		1		1	1																			10	Sit/Still
Groom									1	1	1																						1	1	1	1	1	1	1	1	14	Groom
Eat/Drink																									1	1	1	1													7	Eat/Drink
Socialize					1				1				1	1											1								1								6	Socialize
Stand					1																1	1			1	1	1	1	1												8	Stand
Walk		1	1		1	1	1	1	1	1	1						1	1	1	1	1	1	1	1					1	1	1		1	1							21	Walk
Wall Pace																													1	1							1	1			5	Wall Pace
Run																																					0	Run				
Jump					1								1																								2	Jump				

Example of a physical activity record. At 14 months, C57, mdx, and mdx/utrn^{+/-} mice were observed and physical activity was recorded using a modified animal activity scale. Data was reported as occurrence of physical act/ 10 minutes at corresponding morning and night time points.

Functional Cardiac Assessment (7T MRI)

To test for cardiac function, all animal hearts were imaged using a 7T MRI (Auburn University) with a small boar copper RF coil. Mice were put under anesthesia via isoflourane and ECG leads, respiration leads, and temperature probe were attached before being interested into scanner. C57 and Mdx animals were scanned at 2 months, 10 months, and 14 months. Mdx/utrn^{+/-} were scanned at 2 months and 10 months. Various serial section images were obtained including 2 chamber long axis views, 4 chamber long axis views, and 2 chamber short axis views to ensure complete depiction of the entire heart. Imaging was gated to ECG and respiration leads to ensure complete imaging of the entire cardiac cycle. Using image J software (NIH), the various serial sections were measured during systole and diastole for all axes. Furthermore, wall thickness of the left ventricle was calculated to assess for cardiomyopathy and used to calculate multiple variables such as cardiac output, stroke volume, ejection fraction, end systolic volume, and end diastolic volume by using a specialized ellipsoid equation. Furthermore, cinematic audio video interleaves (AVIs) were created to show real-time heart function.



Representative images. At corresponding time points, all mouse hearts were imaged using a 7T MRI. Various serial sections were obtained using different axes including a 4 chamber long axis (A), a 2 chamber long axis (B), and a two chamber short axis (C). All images above were imaged during diastole.

Histology and Biochemistry Measurements

Upon euthanasia, mouse hearts were excised and rinsed in ice cold 10mM PBS. Hearts were weighed, blotted dry, and placed into optimal cutting temperature (OCT) compound and flash frozen in liquid nitrogen chilled 2-methyl-butane to facilitate rapid chilling without freeze fracture. Hearts were stored at -80°C until further biochemical and histological analysis.

For histological analysis, hearts were cut into $10\ \mu\text{m}$ sections on a cryotome (-30°C) and placed on microscope slides. Myocardial abundance and localization of matrix metalloprotease-9 (MMP9), utrophin, and fibronectin were determined using a histochemical fluorescent detection protocol. Briefly, heart sections were fixed with a 10% formalin solution, washed, and permeabilized with a Triton X-100 in 1% sodium citrate solution. Samples were blocked with 3% Bovine Serum Albumin (BSA) and 20% goat serum. Tissues were then be incubated with a rabbit anti-mouse primary laminin antibody and secondary antibody conjugated to Texas red fluorescent tag to stain the basement membrane. Tissue was also incubated with primary antibodies for MMP9, utrophin, and fibronectin with corresponding secondary antibody conjugated to FITC. Slides were then sealed with a DAPI mounting medium, and imaged with a fluorescent microscope (Nikon, Melville, NY). Fluorescent images for DAPI (nuclei), TRITC (laminin), and FITC filters (MMP9, utrophin, or fibronectin) were combined and analyzed for mean FITC Positive Pixels (um^2) using Nikon Software. All analysis and imaging was completed under blinded conditions by the same technician.

For biochemical analysis, Western blotting was employed. Briefly, 20 μ g of myocardial protein was obtained from frozen OCT samples previously used for histologies and was separated using standard SDS-PAGE techniques on 10%-17% polyacrylamide gels. Following electrophoresis, proteins were transferred to PVDF membranes. To assess for fibrotic factors, membranes were exposed to anti-mouse primary antibodies for TGF- β 1 and fibronectin. To assess mitochondrial biogenesis and enzymes, membranes were exposed to anti-mouse primary antibodies for electron transport chain complexes (I-V), cytochrome- c, citrate synthase, and PGC-1 α . To measure myocardial antioxidant enzymes, membranes were exposed to anti-mouse primary antibodies for SOD-2 and GPX. For measuring inflammatory signaling, membranes were exposed to anti-mouse antibodies for IKB α , phospho-IKB α , NF- κ B, and phospho- NF- κ B p65, iNOS, COX2, F4/80, and CD64. All primary antibodies were incubated with corresponding HRP-conjugated secondary antibodies and imaged for chemiluminescence detection. Membranes were then stripped and re-probed for α -Tubulin for normalizing across blots. Western blots normalized to α -Tubulin were imaged using a chemiluminescent digital imaging device and analyzed using Image J (NIH). All analysis and imaging was completed under blinded conditions by the same technician.

CHAPTER 5: MANUSCRIPT (MDX) FOR SUBMISSION/PUBLICATION

Long-Term Dietary Quercetin Enrichment Attenuates Dystrophic Cardiac Pathology in Mdx Mice

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Key Words: Polyphenol, Heart Failure, Duchenne Muscular Dystrophy

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ABSTRACT

Duchenne Muscular Dystrophy (DMD) causes declines in cardiac health resulting in premature mortality in up to 40% patients due to fibrosis and cardiopathology. Quercetin is a polyphenol possessing inherent anti-inflammatory and antioxidant effects and also potently activates proliferator-activated γ coactivator 1 α (PGC-1 α) increasing mitochondrial biogenesis, antioxidant enzymes, and attenuates cardiopathology. Our experiment tested whether long-term 0.2% dietary quercetin enrichment attenuates dystrophic cardiopathology in mdx mice. Dystrophic animals were fed quercetin enriched or control diet for 14 months. Control C57BL10 animals were fed a control diet for 14 months. Cardiac function was assessed via 7T MRI at 2, 10, and 14 months. At 14 months, hearts were collected for histology and western blotting. Results reveal quercetin preserved ejection fraction (EF) ($p < 0.001$), fractional shortening (FS) ($p < 0.001$), systolic wall thickening (SWT) ($p < 0.001$), stroke volume (SV) ($p < 0.001$), and cardiac output (CO) ($p < 0.001$) when compared to control mdx mice. Histological analysis revealed increased expression of utrophin ($p < 0.001$), α -sarcoglycan ($p < 0.001$), utrophin/ α -sarcoglycan co-localization ($p < 0.001$), and decreased fibronectin ($p = 0.002$), and damage (Hematoxylin and Eosin-Y) ($p = 0.013$) versus control mdx mice. Western blot analysis revealed quercetin increased cardiac PGC-1 α ($p = 0.018$), cytochrome-c ($p = 0.027$), ETC complexes I-V ($p < 0.05$), citrate synthase ($p < 0.001$), SOD2 ($p = 0.040$), and GPX ($p = 0.039$) versus control mdx mice. Western blot targets for inflammation revealed that quercetin decreased NF κ B ($p < 0.001$), P-NF κ Bp65 ($p < 0.001$), P-IK β ($p = 0.001$), while preserving IK β ($p < 0.001$) versus control mdx mice. Furthermore, quercetin decreased TGF- β 1 ($p = 0.010$), COX2 ($p = 0.001$), and F4/80 ($p = 0.005$) versus control mdx mice. There was no difference in iNOS among groups ($p = 0.97$) or CD64 ($p = 0.64$). These data suggest that long-term quercetin enrichment attenuates cardiac dysfunction, increases mitochondrial biogenesis, antioxidant enzymes, decreases inflammation, and may partially reassemble the DGC in the dystrophic heart.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a childhood genetic disease primarily affecting males which causes premature mortality at early adulthood (1). Although multiple forms of muscular dystrophy exist, DMD is the most severe of the pathologies leading to a systemic dysfunction of the structural protein dystrophin (2). As dystrophin is key in mediating membrane stabilization during muscular contraction or mechanical stress, physiologic consequences of DMD include fragile cell membranes, ionic disturbances, metabolic dysfunction, and cell death. Clinically, DMD is characterized by progressive muscle wasting and weakness with a wide range of heterogeneity. In a time-dependent disease progression, DMD leads to robust muscle degeneration and regeneration, respiratory complications, and also cardiac failure. Respiratory failure commonly causes death in many patients resulting in mortality in late teenage years (3). However, due to recent improvements in secondary care and ventilation, life span of patients has extended such that the rapid decline in cardiac health with age is emerging as an impending life limiting factor. Currently, up to 40% of all deaths resulting from DMD are due to complications resulting from cardiac pathology (4). As pathology worsens with age, cardiac complications are evident including ECG abnormalities, cardiac remodeling, contractile dysfunction, fibrotic tissue deposition, dilation of cardiac cavities, and complete heart failure (5).

To investigate and discover novel interventions to combat DMD, accurate and reliable models which recapitulate the disease are necessary. Currently, most investigations for new therapeutics are tested in animal models of DMD. Of those animal models, the mdx mouse, an inbred C57BL10 mouse strain developed through a spontaneous mutation of dystrophin, is one of the most popular and widely used due to close recapitulation of respiratory and cardiac distress characteristic in human DMD patients. Many interventions are effective in attenuating cardiac pathology in dystrophic mice including antioxidant therapy (6), inhibition of chronic inflammatory responses (5), exercise (7), and induction of adaptive gene transcripts including the Sirtuin 1 (SIRT1)/ peroxisome proliferator-activated receptor gamma coactivator alpha (PGC-1 α) axis which increases mitochondrial transcripts and expression of oxidative and membrane structural proteins such as utrophin (8). In fact, previous work from our lab and others indicate that induction of PGC-1 α alleviates much pathology in dystrophin deficient muscle (8, 9).

However, currently there is no single therapeutic agent that successfully encompasses all of the previously mentioned therapeutic interventions in attenuating dystrophic cardiopathology.

Recent study has identified a class of molecules found in edible plants known as polyphenols which show promising evidence to attenuate the multi-faceted pathological mechanisms as a single therapeutic agent to attenuate dystrophic cardiopathology. Polyphenols are well known to combat oxidative stress (10), chronic inflammation (11), cardiac pathology (12), and induce adaptive gene expression through activation of SIRT/PGC-1 α (13, 14). Of the multiple polyphenols present in the human diet, quercetin (3,3',4',5,7-pentahydroxyflavone) makes up the majority of total polyphenol ingestion (15). Quercetin is a safe, naturally occurring flavonol with anti-inflammatory and antioxidant effects and is also a potent SIRT1/PGC-1 α activator capable of antioxidant up-regulation, mitochondrial biogenesis, and prevention of cardiac complications. Recent data from our lab and collaborators suggest that short-term moderate doses of 0.2% quercetin supplementation are adequate in significantly elevating quercetin plasma levels and bioavailability successfully alleviating pathology of dystrophin deficient muscle (13). However, it is currently less clear to what degree dietary quercetin enrichment can impact dystrophin deficient hearts. Preliminary data from our lab suggests that early short-term dietary quercetin enrichment in mdx mice increases mitochondrial biogenesis, antioxidant protein abundance, utrophin expression, and decreased matrix metalloprotease-9 (MMP-9) in young mdx mouse hearts while also attenuating cardiac and fibrotic damage in older mdx mice (16). While preliminary data are compelling, it is unknown whether long-term or chronic dietary quercetin enrichment affects physiologic variables similarly or possibly could aid further in pathology attenuation. Also, more precise physiologic measurements such as cardiac function are needed to further clarify beneficial adaptations with quercetin enrichment. The overall aim of this research was to assess whether chronic dietary quercetin enrichment preserves cardiac function as pathology progresses, decreases cardiac inflammation and damage, and induces mitochondrial and oxidative protein adaptation in dystrophic mouse hearts.

METHODS

Study Design and Animals

The experimental protocol was approved by the Auburn University Institutional Animal Care and Use Committee for the use of animals in research and in accordance with the American Physiological Society. All mdx mice were obtained from our colony at Iowa State University while control C57BL10 mice were purchased from Jackson Laboratories (Jackson Laboratory, Bar Harbor, Maine). During experimentation, all animals were housed on a 12h-12h light-dark cycle. Post-weaning, mdx mice were randomized and diets consisted of either standard rodent chow (n=8) or 0.2% quercetin-fortified rodent chow (n=8) (Bioserv Inc., Frenchtown, NJ) and control C57BL10 mice were fed standard rodent chow (n=8) for the total experimentation time of 14 months. All water and food were provided *ad libitum* throughout the experimentation period.

Functional Cardiac Assessment (7T MRI)

To assess cardiac function, all animal hearts were scanned using a 7 Telsa (7T) magnetic resonance imaging (MRI) system (Seimens Corp., USA) located at Auburn University. Prior to scanning, animals were put under anesthesia via isoflurane and ECG leads, respiration leads, and rectal temperature probes were attached. All animals were kept under anesthesia during the entire scanning process via vaporized isoflurane. Prior to being inserted into the 7T scanner for imaging, animals were placed into a custom made small bore copper radio frequency (RF) coil. All animals were scanned at 2 months, 10 months, and 14 months of age. Various serial section images were obtained including a 2 chamber (left atria and ventricle only) long-axis view, 4 chamber (all atria and ventricles) long-axis view, and 2 chamber (right and left ventricle only) short-axis view to ensure complete depiction of the entire heart. Imaging was gated to ECG and respiration to ensure quality and complete imaging of the entire cardiac cycle.

Cardiac performance was assessed using image J software (NIH). Specifically, MR images of the cardiac cross-sections were analyzed during systole and diastole for all axes. In addition, cardiac wall thickness of the left ventricle was calculated. (17). Cinematic audio video interleaves (AVIs) were created to show real-time heart function and aid in further analysis.

Tissue Harvesting and Storage

After the 14 month experimental period, all animals were weighed, and anaesthetized via vaporized isoflurane until a surgical plane was established. Animals were then sacrificed via

cardiac excision. Post cardiac excision, all hearts were rinsed in chilled 10 mM PBS. Hearts were weighed, placed in optimum cutting temperature compound (OCT) and flash frozen in liquid nitrogen chilled 2-methyl-butane to reduce risk for freeze fracture. After freezing, hearts were stored at -80°C until further analysis.

Histology and Immunofluorescence

Under blinded conditions, hearts were cut 10- μm thick on a Shandon Cryotome cryostat (-30°C) and set on 1 mm microscope slides (Fischer Scientific, Pittsburgh, PA, USA). To assess for damaged areas, selected slides were stained by a modified Hematoxylin and Eosin-Y (H&E) histological procedure. Stained slides were imaged via light microscopy with Nikon software (Melville, NY, USA). For each slide, 10-15 images were obtained at a magnification of 40x to characterize and ensure consistent representation of each heart cross section. Images were then analyzed using Image J software (NIH, Bethesda, MD, USA). Results of images were taken as a ratio of total damaged tissue to total healthy tissue (μm^2).

Tissue expression of utrophin, fibronectin, metalloprotease 9 (MMP9), α -sarcoglycan, and co-localization of α -Sarcoglycan and utrophin were determined using a histological immunofluorescence detection procedure. Briefly, slides with heart sections were fixed with a 10% formalin solution, rinsed, and permeabilized with Triton X-100 in 1% sodium citrate solution. Slides were rinsed in 10 mM PBS and blocked with a 3% bovine serum albumin and 20% goat serum solution. Selected slides were incubated with primary antibodies for proteins of interest: (Utrophin, 1:500 dilution; sc-33700; Santa Cruz Biotechnology), (Fibronectin, 1:500 dilution; F3648; Sigma-Aldrich), (MMP9, 1:500 dilution; sc-6840; Santa Cruz Biotechnology), (α -Sarcoglycan, 1:500 dilution; sc-28278; Santa Cruz Biotechnology). Tissues were then incubated with corresponding secondary antibodies for fluorescein isothiocyanate (FITC) (1:500, sc-2365; Santa Cruz Biotechnology)(1:500, sc-2010; Santa Crus Biotechnology) and/or Texas Red (TRITC, 1:500; T1-1000; Vector Laboratories Inc.) fluorescent tags. Slides were then sealed with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories Inc.) mounting medium and imaged with a fluorescent imaging microscope (Nikon, Melville, NY). Randomized images (10-15) were obtained as previously discussed with corresponding TRITC, FITC, and DAPI filters. Images were analyzed for mean fluorescent intensity area percentage (μmm^2) using Nikon Software. All imaging and analysis was conducted under blinded conditions by the same technician.

Western Blotting

Myocardial protein (60 µg/ sample) was obtained from hearts previously frozen in OCT from histology and separated using standard SDS-PAGE techniques on 6%-12% polyacrylamide gels through standard electrophoresis. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes and stained and imaged with Ponceau stain to ensure proper loading techniques. Membranes were then exposed to primary antibodies for the protein of interest: (Cytochrome-C, 1:500 dilution; sc-7159; Santa Cruz Biotechnology), (Citrate Synthase, 1:500 dilution; sc-390693; Santa Cruz Biotechnology), (CD64, 1:500 dilution, sc-15364p; Santa Cruz Biotechnology), (Cyclooxygenase 2, 1:1000; CS-12282; Cell Signaling Technology Inc.), (F4/80, 1:200 dilution; sc-25830; Santa Cruz Biotechnology) (Glutathione peroxidase, 1:500; sc-74498; Santa Cruz Biotechnology), (Inducible Nitric Oxide Synthase, 1:1000 dilution; CS-13120; Cell Signaling Technology Inc.), (NFKB, 1:1000 dilution; CS-8242; Cell Signaling Technology Inc.), (Phospho-NFKBp65, 1:1000 dilution; CS-3033; Cell Signaling Technology Inc.), (IKB α , 1:1000 dilution; CS-4814; Cell Signaling Technology Inc.), (Phospho-IKB α , 1:1000 dilution; CS-2859; Cell Signaling Technology Inc.), (OxPhos Complex Kit, 1:5000 dilution; 457999; Invitrogen), (PGC-1 α , 1:500 dilution; sc-13067; Santa Cruz Biotechnology),(SOD2, 1:500; sc-30080; Santa Cruz Biotechnology), (TGF β -1, 1:500 dilution; sc-146; Santa Cruz Biotechnology). Membranes were then exposed to corresponding IgG-HRP secondary antibodies from chemiluminescence detection: (anti-rabbit IgG HRP linked, 1:1000 dilution; CS-7074; Cell Signaling Technology Inc.), (anti-mouse IgG HRP linked, 1:1000 dilution; CS-7076; Cell Signaling Technology Inc.). Membranes were reprobed and exposed to corresponding normalizing protein primary antibodies: (α - Tubulin, 1:1000 dilution; DHSB),(β -Actin, 1:1000 dilution; CS-4970; Cell Signaling Technology Inc.), (GAPDH, 1:1000, GenTex). Corresponding secondary antibodies previously mentioned were used for chemiluminescence detection. Western blots were normalized to corresponding normalizing protein, Ponceau stain, and were analyzed using a UVP LLC digital imaging device (Upland, CA, USA). All experiments and analysis were conducted blinded and by the same technician.

STATISTICAL ANALYSES

For cardiac function variables, a 3x3 repeated measures ANOVA was used to test for differences of our primary dependent variables using SPSS 20 (IBM, New York City). For biochemical and histological variables, a one-way ANOVA was used to test differences for our secondary and tertiary dependent variables using SPSS 20 (IBM, New York City). Bonferroni post-hoc analyses were performed as needed. In the instances that a violation of homogeneity occurred (Levine's test), Welch's t-test were performed for all strain pair-wise comparisons to correct for unequal variances among groups (18). Significance was set at $p \leq 0.05$ *a priori*. Data are presented as mean \pm SEM.

RESULTS

Animal Characteristics

Animal characteristics and data are presented in [Table 1]. Results revealed no significant difference among groups in body weight (g) ($p = 0.322$). Absolute heart weights (mg) were also unaltered with no differences being detected among any of the groups ($p = 0.536$). Finally, relative heart weights (mg/g) were also unchanged among all groups ($p = 0.166$).

Cardiac Function

Results from cardiac function data are presented in [Table 2] and representative images (14 months) are presented in [Figure 1]. Ejection fraction (EF) was decreased in MDX mice versus control C57 and MDXQ by 10 months of age respectively ($p < 0.001$; $p < 0.001$). Furthermore, EF was decreased further at 14 months in MDX mice and was different than both C57 and MDXQ respectively ($p < 0.001$; $p < 0.001$) while also being significantly lower than previous 2 month value ($p < 0.0001$). End diastolic volumes (EDV) were significantly elevated in all strains at 14 months of age ($p < 0.001$). By 14 months, End systolic volume (ESV) was significantly increased in MDXQ mice when compared to respective 2 month value ($p < 0.001$). However, ESV at 14 months for MDX mice was increased from age matched control C57 and MDXQ mice respectively ($p < 0.001$; $p < 0.001$) and respective 2 month value ($p < 0.001$). Fractional shortening (FS) was significantly lower in MDX mice at 10 and 14 months when compared to C57 and MDXQ mice respectively ($p < 0.001$; $p < 0.001$) while also being significantly lower than previous 2 month value ($p < 0.0001$) at 14 months.. Systolic wall thickness (SWT) decreased in MDX mice by 10 months and further by 14 months. SWT was significantly decreased when compared to control C57 and MDXQ at both 10 and 14 months respectively ($p < 0.001$; $p < 0.001$). Stroke volume (SV) significantly decreased in MDX mice by 10 months and further by 14 months respectively ($p < 0.001$, $p < 0.001$). Furthermore, SV was significantly decreased in MDX from previous 2 month baseline values ($p < 0.001$). Finally, cardiac output (CO) was decreased in MDX mice at 10 and 14 months when compared to control C57 and MDXQ mice correspondingly ($p < 0.001$; $p < 0.001$). Furthermore, MDX mice CO had decreased significantly from respective 2 month values ($p < .001$).

Western Blotting

To assess key protein abundance of interest, various western blotting techniques were used and can be viewed in corresponding figures. PGC-1 α protein abundance was elevated in

MDXQ versus control C57 and MDX mice respectively ($p = 0.037$; $p = 0.018$) suggesting that quercetin in fact can inherently induce PGC-1 α protein abundance [Figure 2A]. As PGC-1 α is a fundamental transcription co-factor for mitochondrial biogenesis, strategic downstream mitochondrial proteins were also investigated. Cytochrome-C protein concentration was elevated above MDX mice in both control C57 and MDXQ mice respectively ($p = 0.047$; $p = 0.027$) [Figure 2B]. Citrate synthase, found exclusively in the mitochondria, was elevated in MDXQ mice when compared to control C57 and MDX mice correspondingly ($p < .0001$; $p < 0.001$) [Figure 2C]. To further characterize the full effect of quercetin enrichment on mitochondrial biogenesis, electron transport chain (ETC) complexes I-V protein abundance was probed and measured [Figure 3]. When compared to MDX mice, MDXQ mice had higher protein abundance of NADH Dehydrogenase (Complex I) ($p = 0.021$). MDXQ mice also possessed higher concentration of Succinate Dehydrogenase (Complex 2) when compared to MDX counterparts ($p = 0.017$). Complex III, Cytochrome bc1 complex, was also elevated in MDXQ mice compared to MDX mice ($p = 0.013$). Cytochrome-C Oxidase (Complex IV) protein abundance was lower in MDX mice when compared to MDXQ mice ($p = 0.019$). Finally, ETC Complex V (ATP Synthase) was elevated in MDXQ when compared to MDX counterparts ($p = 0.024$). There were no differences in ETC complexes I-V between MDXQ and control C57 mice. Furthermore, antioxidant enzymes associated with mitochondria and cardiac health were also measured. Superoxide Dismutase (SOD2) was increased in MDXQ animals when compared to MDX counterparts ($p = 0.040$) [Figure 4A]. Also, Glutathione Peroxidase (GPX) was elevated in MDXQ mice when compared to both control C57 and MDX mice respectively ($p = 0.039$; $p = 0.032$) [Figure 4B]. Collectively, long-term quercetin enrichment appears to stimulate myocardial mitochondrial biogenesis markers and enzymatic antioxidants.

Due to the chronic inflammation associated with DMD, strategic proteins and pathways were chosen to gain insight into changes due to quercetin enrichment. In particular, key protein markers in the NF κ B pathway were chosen to measure inflammation signaling. In MDXQ mice, I κ B α was approximately three to four fold higher than both control C57 and MDX groups respectively ($p < .0001$; $p < .0001$) [Figure 5A]. However, p-I κ B α was lower in MDXQ mice versus both control C57 and MDX mice respectively ($p = 0.001$; $p < 0.001$) [Figure 5B]. NF κ B expression was found to be lower in control C57 and MDXQ respectively ($p = 0.003$; $p < 0.001$) than MDX mice [Figure 5C]. Furthermore, phosphorylated (activated) p-NF κ Bp65 protein

abundance was lower in MDXQ versus both control C57 and MDX groups respectively ($p = 0.033$; $p < 0.001$) [Figure 5D]. Further downstream markers mediated by NF κ B were also measured. Myocardial iNOS was not significantly affected in any groups [Figure 6A]; however, COX2 was significantly lower in MDXQ mice versus both control C57 and MDX mice respectively ($p = 0.001$; $p = 0.001$) [Figure 6B]. The cytokine growth factor involved in fibrotic deposition, TGF β -1, was lower in MDXQ mice versus MDX mice ($p = 0.010$) [Figure 6C]. Cluster of Differentiation 64 (CD64) and F4/80 (a mouse homolog of EGF-like module-containing mucin-like hormone receptor-like 1) which are exclusively found in macrophages were assessed for immune cell infiltration. While there was an approximate 30% decrease in CD64 in MDXQ myocardium compared to MDX, there were no significant differences among the groups ($p = 0.64$) [Figure 6D]. However, there was approximate 40% lower protein abundance of F4/80 in MDXQ mice when compared to MDX mice ($p = 0.005$) [Figure 6E].

Histology

Results and representative images of histological data and analyses are presented in corresponding figures. Immunofluorescent analyses revealed a 2-fold increase in utrophin in MDXQ mice when compared to both MDX and C57 mice accordingly ($p < 0.001$; $p < 0.001$) [Figure 7A]. Analyses of α -Sarcoglycan, a central component of the DGC, revealed that compared to control C57 mice, MDX mice had an approximately 80% lower expression of α -Sarcoglycan ($p < 0.001$) while MDXQ had only an approximately 40% lower expression ($p < 0.001$). However, control C57 mice still maintained a higher expression of α -Sarcoglycan when compared MDXQ mice ($p < 0.001$) [Figure 7B]. Furthermore, in attempt to view if quercetin enrichment was associated with restoration of DGC with utrophin, further analysis of utrophin/ α -Sarcoglycan co-localization was performed. As was the case for α -Sarcoglycan, MDX mice displayed approximately 80% lower co-localization of utrophin and α -Sarcoglycan ($p < 0.001$) when compared to control C57. However, MDXQ mice had approximately 60% co-localization of utrophin and α -sarcoglycan when compared to control C57 mice, though this was still lower than C57 mice ($p < 0.001$) [Figure 7C]. To assess for cardiac remodeling, MMP9 was analyzed and found to be elevated in MDX mice when compared to C57 control animals ($p = 0.004$). While MDXQ mice had an approximately 30% lower MMP9 expression compared to MDX mice, this difference failed to reach significance ($p = 0.069$) [Figure 8A]. To measure fibrosis within the heart histological analysis of fibronectin, a key glycoprotein in fibrotic tissue, was

analyzed. Both C57 and MDXQ revealed lower levels of cardiac fibronectin content when compared to MDX mice correspondingly ($p < 0.0001$; $p = 0.002$). However, MDXQ mice did have higher fibronectin abundance than control C57 mice ($p < 0.001$) [**Figure 8B**]. Total cardiac damage was assessed by a modified H&E histological procedure and revealed that both control C57 and MDXQ mice displayed an almost 3-fold lower amount of total to damaged tissue ratio ($p = 0.005$; $p = 0.013$) [**Figure 8C**].

DISCUSSION

Secondary care for DMD has improved to the point that cardiac dysfunction and ventricular pump failure have become a leading cause of morbidity and mortality (19). In the absence of cure or pragmatic long-term therapies, our lab group has sought to discover novel and practical means to mitigate DMD pathology. Preliminary data from our lab group suggests that PGC-1 α induction ameliorates many of the pathological processes in mdx mice (8, 9). Of note, our group has investigated quercetin as a potential avenue to activate the Sirtuin1/PGC-1 α metabolic axis in order to attenuate and preserve quality of life. Indeed, recently published data from our lab group show that short-term supplementation alleviates many factors of the multifaceted pathology of DMD in mdx mice (13, 16). Findings indicate that short-term dietary quercetin enrichment in young mdx mice is associated with increased cardiac levels of mitochondrial biogenesis, antioxidant protein concentration, utrophin expression, and decreased MMP-9. Moreover, these outcomes were associated with attenuated histological indices of fibrotic damage in hearts from 9 month old mdx mice (16). While these early findings are compelling, it remains unknown to what degree long-term dietary quercetin enrichment impacts dystrophin deficient hearts and whether improved biochemical and histological outcomes also translate to improved physiologic performance. Accordingly, the current research study was undertaken to determine whether long-term dietary quercetin enrichment affects physiologic variables similarly or possibly could aid further in pathology attenuation. Novel findings from the current study demonstrate for the first time that long-term quercetin enrichment preserves age-related declines in cardiac function. Post mortem tissue analyses from quercetin fed mice revealed corresponding improvements in mitochondrial biogenesis, endogenous antioxidant expression, and prevention of fibrosis, cardiac damage, and inflammation in dystrophin deficient hearts.

Cardiac Function

Previous reports suggest mdx mice develop cardiac dysfunction throughout their life-span (see selected review (19)). However, while 2 month mdx mice have normal heart function as compared to control strains, they are still subject to damage due to mechanical overload (20, 21). In a time-dependent manner, mdx mouse hearts become increasingly fibrotic and exhibit signs of contractile dysfunction by the age of 9 months (19). Findings of decreased EF, SV, FS, and CO in the current study are in agreement with previous reports that cardiac function declines with

age in mdx mice (20, 22). The novel discovery of the current study is that quercetin enrichment attenuated decreases in EF, FS, SV, and CO in mdx mice. Moreover, current measures of ESV confirm an age-dependent decline at 14 months of age (23), and finding that was mitigated in the hearts of mdx mice consuming quercetin. Not surprisingly, and in agreement with prior work (23), EDV was increased in all groups at 14 months of age, and apparently unaffected in quercetin fed mice as compared to mdx controls or C57 mice. While other studies show polyphenol consumption appears to promote beneficial adaptations in cardiac function (24, 25), the current study is the first to demonstrate that long-term quercetin alleviates cardiac dysfunction in dystrophic mice. A key methodological advantage in this regard is the novel application of 7T MRI to quantify cardiac function longitudinally. Of particular note, no single therapeutic agent alleviates declines in age-related cardiac dysfunction in dystrophic deficient hearts. While our early published histological and biochemical findings from a shorter duration feeding study in younger mice suggested that quercetin enrichment could be cardioprotective (16), current data link similar tissue-level outcomes to physiologic benefit in dystrophin deficient hearts from mice that consumed quercetin. Based on these collective findings, there is reason to believe that dietary quercetin may prove to be a pragmatic prospective therapy for those with DMD.

PGC-1 α , Mitochondrial, and Oxidative Adaptations

Quercetin and other polyphenols stimulate a variety of metabolic pathways including the SIRT1/PGC-1 α axis. Indeed, previous evidence indicates that polyphenols collectively stimulate the SIRT1/PGC-1 α axis and downstream transcripts (26-28). While previous studies in non-DMD models show increased gene expression of PGC-1 α following quercetin enrichment (26, 29), confirmation of protein expression outcomes are not well documented. Previously, our lab group found no change in PGC-1 α cardiac protein abundance following short-term quercetin enrichment (16). However, our current investigation provides novel findings in that long-term quercetin enrichment in mdx mice increased PGC-1 α protein abundance in the dystrophic myocardium. Disparate findings between our prior and current study may be due to animal age at sacrifice in that mitochondrial biogenesis and PGC-1 α abundance are characteristic of senescence (30, 31). In the current investigation, the animals were near the end-stage of life and nearly twice the age of our previous study (16). Thus, the current findings of increases in PGC-1 α with quercetin enrichment may manifest more potently in senescent mice. Second, PGC-1 α

expression is decreased in failing hearts, corresponding to decreased mitochondrial function. Furthermore, the decrease in PGC-1 α and mitochondrial function exacerbates cardiac pathology further (32). When comparing our current investigations on cardiac function and pathological heart failure in mdx mice, our findings of increased PGC-1 α expression mirror our preserved cardiac function in the mdx mice.

Further investigation for indices of altered mitochondrial biogenesis was performed in the current experiments. Findings revealed increases in citrate synthase and all electron transport complexes (NADH Dehydrogenase, Succinate Dehydrogenase, Cytochrome bc1 Complex, Cytochrome-C oxidase, and ATP synthase) with quercetin enrichment, which are all exclusively located within the mitochondrial matrix/membrane. To our knowledge the current data are the first to quantify elevated markers of bioenergetic capacity within the dystrophic heart with quercetin enrichment. Importance of these findings are emphasized by previous observation that mdx mice hearts are susceptible to mitochondrial damage and calcium overload (33). Collectively, these data support previous findings that increases in PGC-1 α enhance protein abundance of mitochondrial protein and enzymes in the myocardium (34) and support our previous data, which indicate that cytochrome-c was elevated in hearts from younger mice consuming an identical quercetin diet (16). Collectively, these data provide compelling evidence to suggest that dietary quercetin enrichment increased mitochondrial biogenesis within the myocardium of mdx mice and likely contributed to associated improvements in cardiac function.

Another aspect of mitochondrial adaptations is endogenous antioxidant enzyme up-regulation. Independent of quercetin's antioxidant properties (35), quercetin up-regulates endogenous antioxidant enzymes (10, 16). In agreement with our prior study, current results reveal that quercetin enrichment increased myocardial levels of SOD2. Furthermore, since SOD2 is primarily located within mitochondria, increased SOD2 levels further reinforce current evidence for increased mitochondrial biogenesis in hearts from quercetin fed mice. To further our inquiry of antioxidant enzyme status and quercetin enrichment within the dystrophic heart, we chose to investigate the influence of quercetin enrichment on glutathione peroxidase (GPX) abundance. GPX, an abundant antioxidant enzyme which catalyzes the reduction of hydrogen peroxide to water, is well established as a pivotal enzyme in protecting the heart against oxidative stress. Our data indicate that quercetin enrichment increased myocardial levels of GPX in dystrophic hearts. Our findings are in agreement with previous studies that show quercetin

possesses the possibility of inducing increases in GPX (36, 37). Furthermore, previous work shows that GPX overexpression attenuates cardiac remodeling and preserves cardiac function within pathological mouse hearts (38, 39). Thus, the increases in myocardial protective antioxidant enzymes may also contribute to the subsequently mentioned quercetin-mediated preservation in cardiac function.

Dystrophin Glycoprotein Complex (DGC) reassembly

In the absence of dystrophin, it is imperative to find a surrogate protein to replace or aid in stabilization of the membrane. Indeed, our data revealed that quercetin enrichment increased utrophin expression in the myocardium of mdx mice and further support our previous findings (16). Although utrophin exhibits some functional differences (40, 41), utrophin is considered a homolog protein of dystrophin and both are found at the neuromuscular junction. Of significant importance, utrophin can partially replace the function of dystrophin and alleviate much of DMD associated pathology if upregulated (42). In this investigation we additionally wanted to quantify whether increases in utrophin restored the costamere within the myocardium. To establish this, we probed for α -Sarcoglycan, an important glycoprotein complex protein. Findings revealed that quercetin enrichment was associated with increased membrane expression of α -sarcoglycan. Using microscopy software to assess co-staining on merged images, quercetin consumption was linked to co-localization of α -sarcoglycan and utrophin. While the results are semi-quantitative, these data suggest that reassembly of the DGC in the dystrophic heart may have occurred, though additional investigation is needed to verify this potential outcome.

Fibrosis and Damage

Assessment of fibrosis and damage to myocardial tissue was accomplished through microscopy techniques of staining for Hematoxylin and Eosin-Y (H&E) and also immunofluorescence for fibronectin and MMP9. Results from H&E reveal that long-term quercetin enrichment prevents overall cardiac damage and fibrosis in mdx hearts. Currently, it is well known that mdx mice which reach the age of approximately 9 months consistently exhibit cardiac damage and fibrosis histologically (19). Findings of MRI characterization of cardiac fibrosis in mdx mice of this age bolster these findings (17, 22). Current data suggest that quercetin can prevent cardiac damage and fibrosis and agree with our previous preliminary findings in hearts from younger mice (16). Current findings are further supported by previous investigation of other polyphenol applications as preventive against pathological cardiac

remodeling (12, 43). Fibronectin, a major glycoprotein in fibrotic tissue, was also attenuated in the dystrophic myocardium by quercetin enrichment in the current study. Diminished fibronectin content in quercetin fed hearts bolsters the notion that quercetin attenuates maladaptive remodeling in mdx mice and agrees with similar findings from our lab group in the mdx mouse diaphragm (13). While MMP9 levels were approximately 30% higher in mdx control mice versus quercetin enriched animals, MMP9 protein abundance was not significantly altered between quercetin and control fed mdx groups. While quercetin can inhibit MMP9 directly (44, 45), our current results are in agreement with our preliminary data that chronic quercetin enrichment did not influence MMP9 accumulation in the senescent mdx heart (16). Since increased MMP9 is a key factor to cardiac remodeling and cardiac dysfunction (46, 47), possible explanation for this phenomenon is that by 14 months, age-dependent cardiac remodeling was undetectable and had already occurred. Nonetheless, current data collectively indicate that quercetin enrichment prevents cardiac damage and protects the dystrophic heart against cardiac fibrosis.

Inflammation and Immune Cell Infiltration

Among the multi-faceted aspects contributing to pathology, DMD is commonly characterized by chronic inflammation due to a robust drive of degeneration and regeneration of myocytes from the lack of functional dystrophin. Commonly implemented in the treatment of DMD, corticosteroids have strong anti-inflammatory effects and are one of the most widely used drugs to prevent chronic inflammation. Disease progression in DMD patients treated with prednisolone is mitigated and normal voluntary movement is preserved (48). Consequently, long-term use of corticosteroids causes many other deleterious effects including weight gain, decreased bone mineral density, fluid retention, and more importantly causes cardiac complications (49, 50). Thus, we sought to determine whether quercetin enrichment could decrease indices of inflammation that contribute to heart failure in DMD (51). Since NF κ B signaling and protein is elevated in hearts from DMD patients (52, 53), we strategically sought to determine if quercetin enrichment affected the NF κ B inflammatory pathway in the dystrophic myocardium. Quercetin enrichment preserved levels of I κ B α and prevented increases in phosphorylation (P-I κ B α). Furthermore, quercetin enrichment lowered NF κ B protein abundance versus control mdx mice and indicates that the activated form, P-NF κ Bp65, was also lower in hearts from quercetin fed mice. Overall, the current data suggest that quercetin enrichment

inhibited the activation of the NF κ B inflammatory pathway in the dystrophic heart of mice. Examination of downstream markers of the NF κ B pathway revealed dramatically decreased COX2 protein expression in dystrophic mice. Supporting our findings are previous reports that polyphenols like quercetin suppress COX2 expression (54, 55). However, no difference in iNOS was observed currently between treatment groups and is difficult to explain currently. One possible explanation for this finding could be differences in iNOS expression may have been temporally unobserved. However, TGF- β 1, a key mediator in fibrotic deposition, was significantly lower in quercetin fed mdx mice which is in agreement with prior studies that polyphenols possess the ability to blunt TGF- β 1 (56). To assess for immune cell infiltration, CD64 and F4/80 were chosen to detect macrophages and immune cell infiltration signaling. Although quercetin enrichment was associated with lower levels of CD64 (30% lower than control fed mdx mice), numerical difference did not reach statistical significance. However, quercetin enrichment corresponded to lower F4/80 in dystrophic mouse hearts suggesting less immune cell infiltration occurred. Overall, these data reveal that quercetin enrichment appears to decrease markers of inflammation through suppression of the NF κ B inflammatory pathway while also preventing immune cell infiltration and signaling in dystrophic hearts.

CONCLUSION

Through this longitudinal study, our lab group sought novel insight into countermeasures against dystrophic cardiac pathology. Current results reveal important new information that chronic quercetin feeding at 0.2% of the total dietary intake provides robust cardioprotection against the development of ventricular dysfunction in the mdx mouse. Associative findings in cardiac tissue histological sections and biochemistry examination reveal that quercetin feeding was also associated with attenuation of pathological remodeling, improved mitochondrial biogenesis, and attenuated inflammation. These remarkable findings build on our previous investigations using a similar methodological approach of DMD. Our collective findings suggest that pleiotropic benefits of quercetin ingestion may be attributable to increased PGC-1 α expression which is well established to promote all of the tissue level benefits identified currently. Given the longitudinal, 14 month old endpoint approach used currently, verification of PGC-1 α modulation by quercetin is not possible. But given these dramatic outcomes, future investigations should be directed at understanding the specific time course for fully understanding the role of PGC-1 α as a trigger for beneficial outcomes due to quercetin feeding in the DMD heart. Moreover, whether quercetin works largely through PGC-1 α activation and/or off target effects remains to be determined. Ultimately, our results suggest that quercetin enrichment provides a novel and unique therapy for dystrophic cardiac pathology that may improve clinical outcomes in DMD patients.

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Table 1.

Strain ID	Body Weight (g)	Absolute Heart Weight (mg)	Relative Heart Weight (mg/g)
C57	40.9 ± 1.76	187 ± 11.45	4.6 ± 0.33
MDX	40.8 ± 1.79	182 ± 15.31	4.5 ± 0.38
MDXQ	36.1 ± 2.30	203 ± 13.6	5.2 ± 0.42

Animal Characteristics. Data are means ± SEM.

Table 2.

Time	Strain	EF (%)	EDV (μ L)	ESV (μ L)	FS	SWT (mm)	SV (μ L)	CO (μ L/min)
2 months	C57	59.3 \pm 1.4	67.2 \pm 3.1	27.4 \pm 1.6	46.6 \pm 2.3	0.42 \pm .04	39.8 \pm 2.0	20686 \pm 927
	MDX	53.2 \pm 1.4	64.5 \pm 1.7	30.2 \pm 1.2	38.8 \pm 2.3	0.37 \pm .05	34.3 \pm 1.2	17707 \pm 408
	MDXQ	59.0 \pm 1.3	58.2 \pm 3.8	23.7 \pm 1.4	45.3 \pm 1.8	0.35 \pm .03	34.5 \pm 2.6	18649 \pm 1340
10 months	C57	57.5 \pm 2.7	62.2 \pm 4.4	26.2 \pm 2.3	46.4 \pm 2.4	0.43 \pm .03	36.0 \pm 3.4	18998 \pm 1389
	MDX	46.7 \pm 2.0 * #	58.0 \pm 4.6	30.6 \pm 2.2	32.3 \pm 1.8 *#	0.29 \pm .02* #	27.3 \pm 2.8*	13344 \pm 1085*
	MDXQ	60.4 \pm 3.3	70.0 \pm 4.4	27.5 \pm 2.4	46.8 \pm 2.7	0.44 \pm .03	42.5 \pm 4.0	22471 \pm 2298
14 months	C57	56.6 \pm 1.9	74.7 \pm 3.1 †	32.4 \pm 2.1	44.9 \pm 1.6	0.54 \pm .04	42.2 \pm 2.1	18372 \pm 917
	MDX	32.6 \pm 2.1* # †	74.1 \pm 3.1 †	49.8 \pm 2.5 * # †	24.7 \pm 1.2*# †	0.28 \pm .02* #	24.2 \pm 2.0* #	10493 \pm 933*# †
	MDXQ	54.9 \pm 1.3	80.0 \pm 3.6 †	35.9 \pm 1.0 †	42.7 \pm 1.1	0.41 \pm .02	44.1 \pm 2.8	19962 \pm 1611

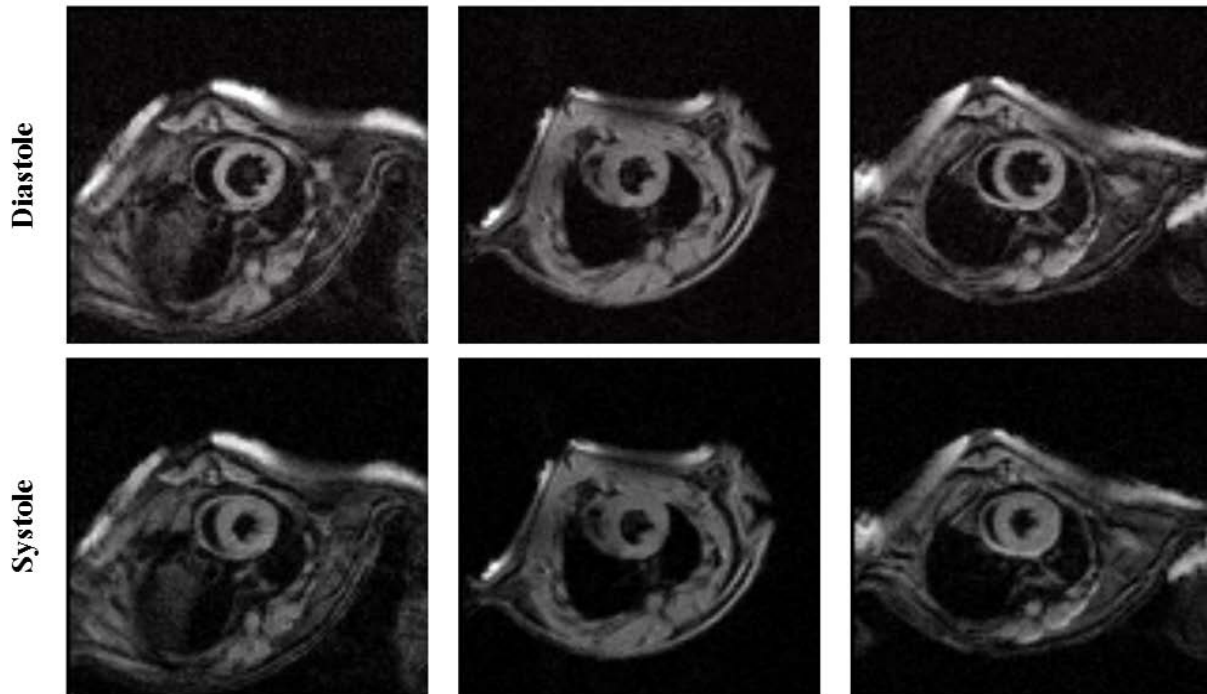
Cardiac Function. Data are means \pm SEM. * denotes significantly different from age matched MDXQ. # denotes significantly different from age matched C57. † denotes significantly different from strain 2 month value.

Figure 1.

C57

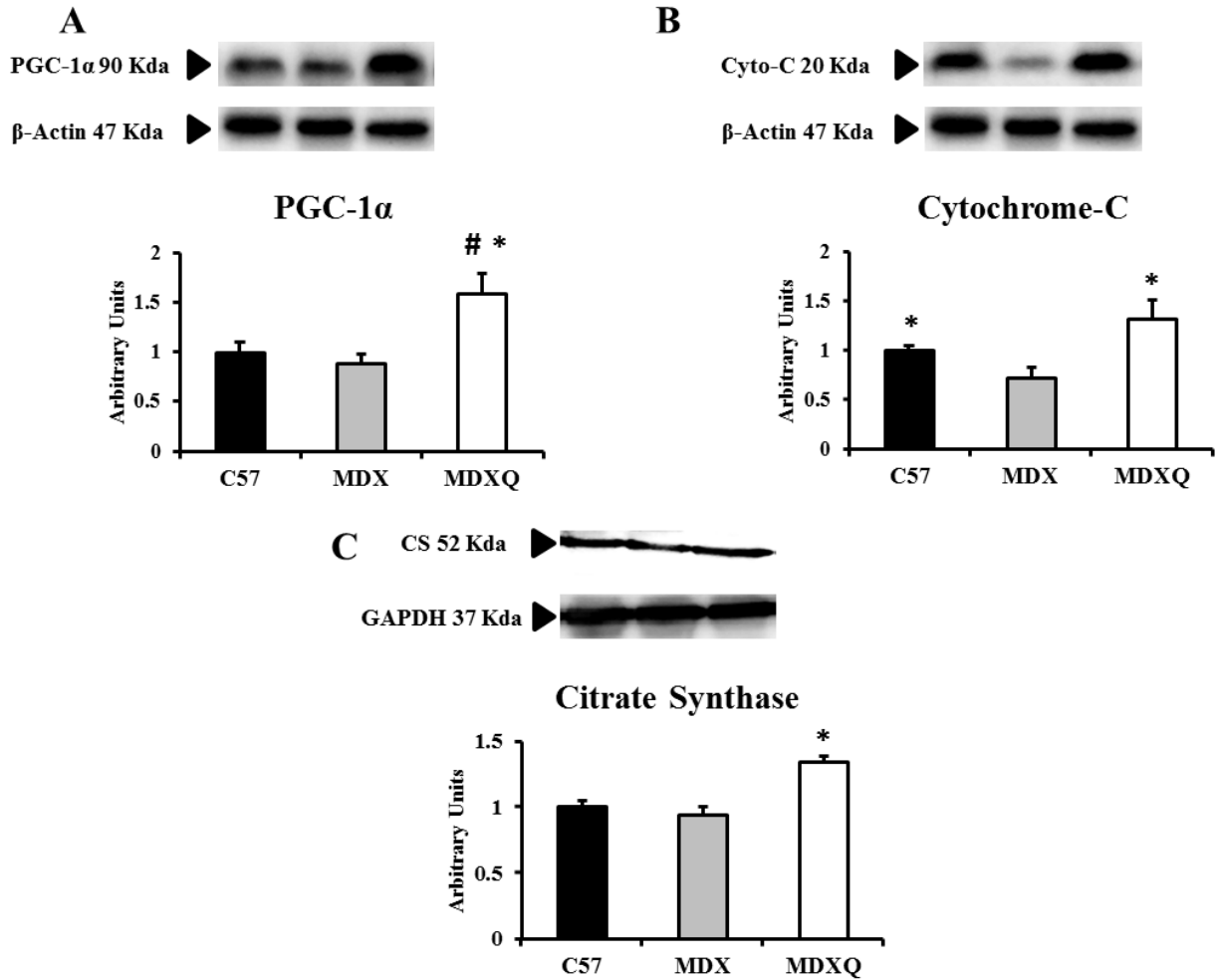
MDX

MDXQ



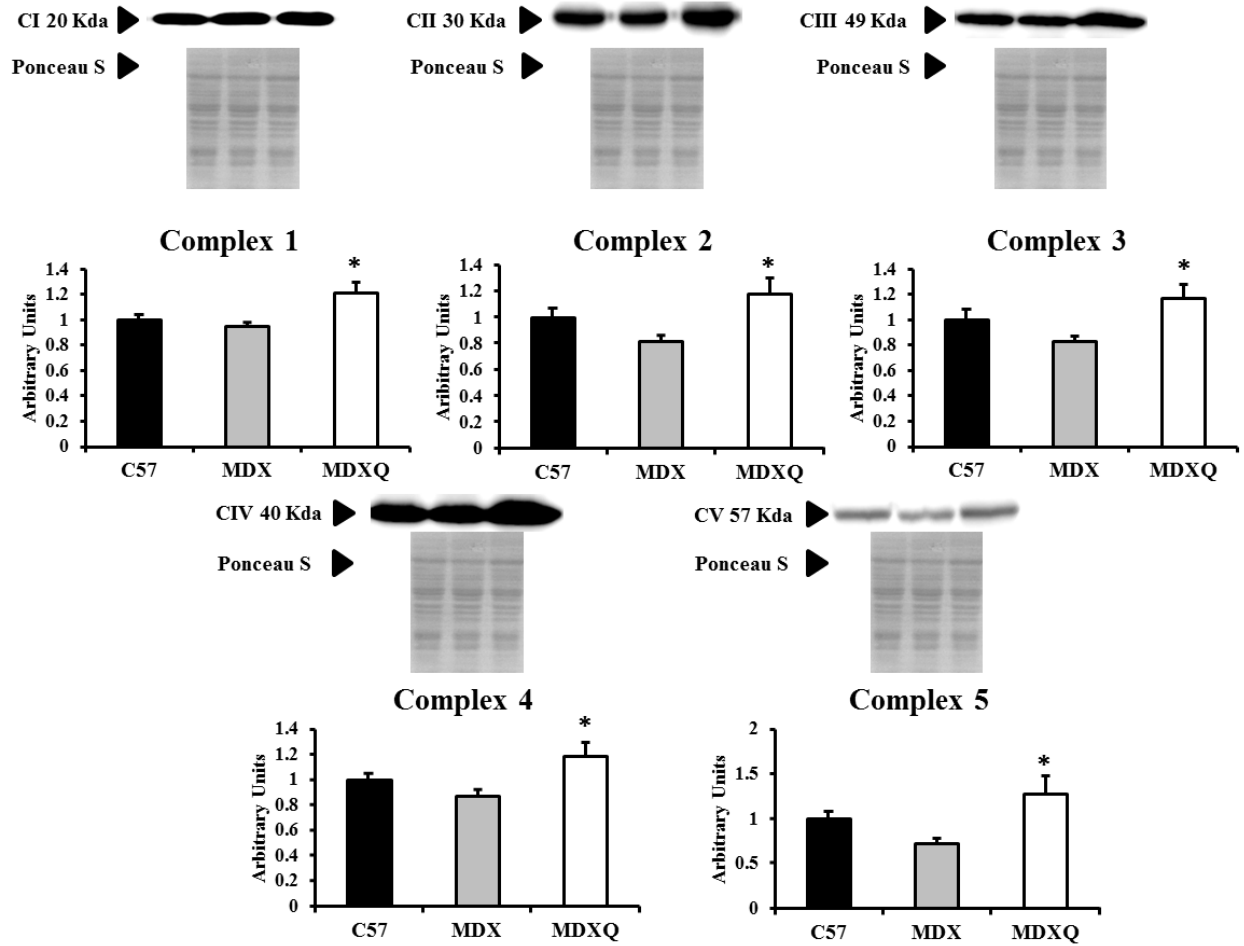
Representative images from 7T MRI. Top row represents beginning of diastole. Bottom row represents the end of systole.

Figure 2.



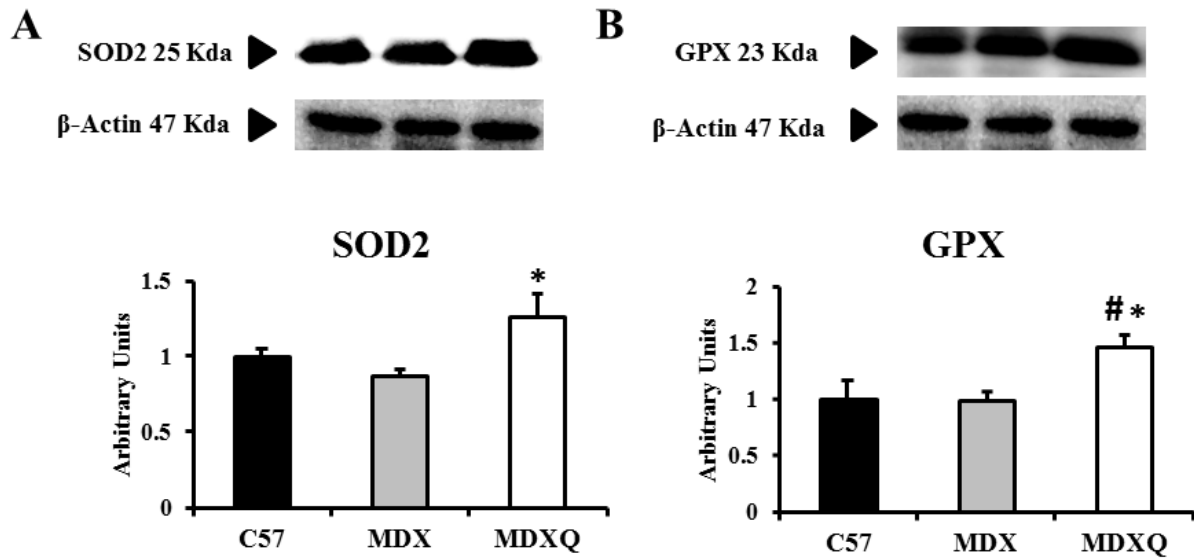
Quercetin increases PGC-1 α , Cytochrome-C, and Citrate Synthase expression in dystrophic hearts. Data are means \pm SEM. * denotes significantly different from MDX. # denotes significantly different from C57.

Figure 3.



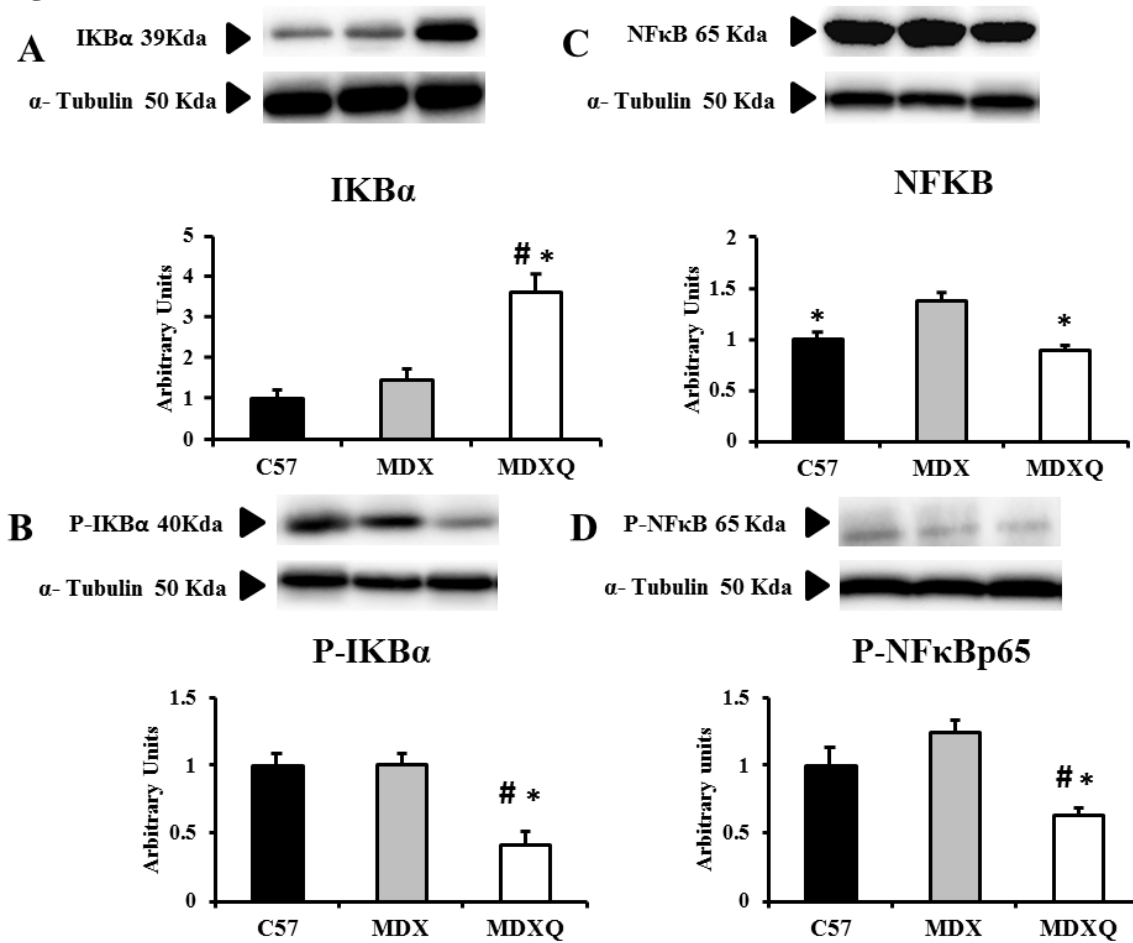
Quercetin increases mitochondrial ETC complexes I-V abundance in dystrophic hearts. Data are means \pm SEM. *denotes significantly different from MDX.

Figure 4.



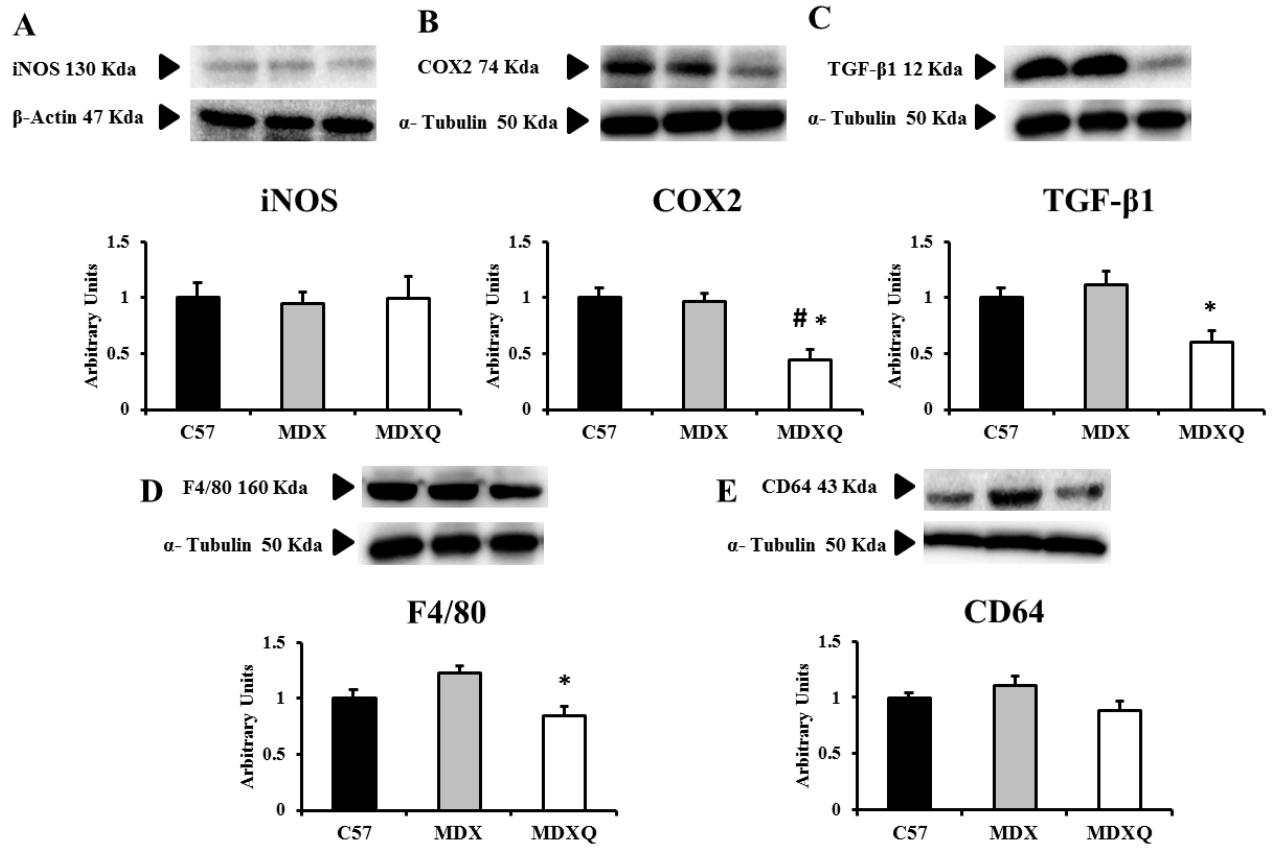
Quercetin increases antioxidant enzyme abundance in dystrophic hearts. Data are means \pm SEM. * denotes significantly different from MDX. # denotes significantly different from C57.

Figure 5.



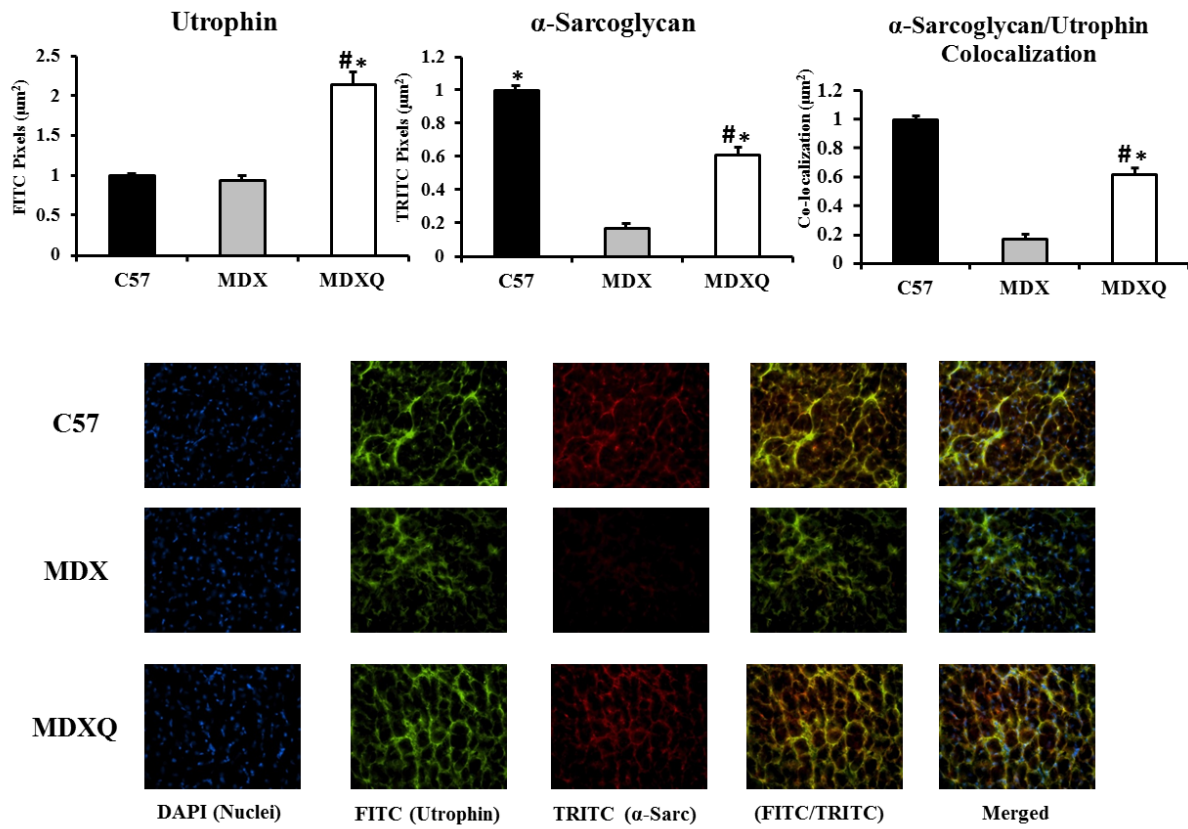
Quercetin decreases NFκB signaling protein abundance in dystrophic hearts. Data are means ± SEM. * denotes significantly different from MDX. # denotes significantly different from C57.

Figure 6.



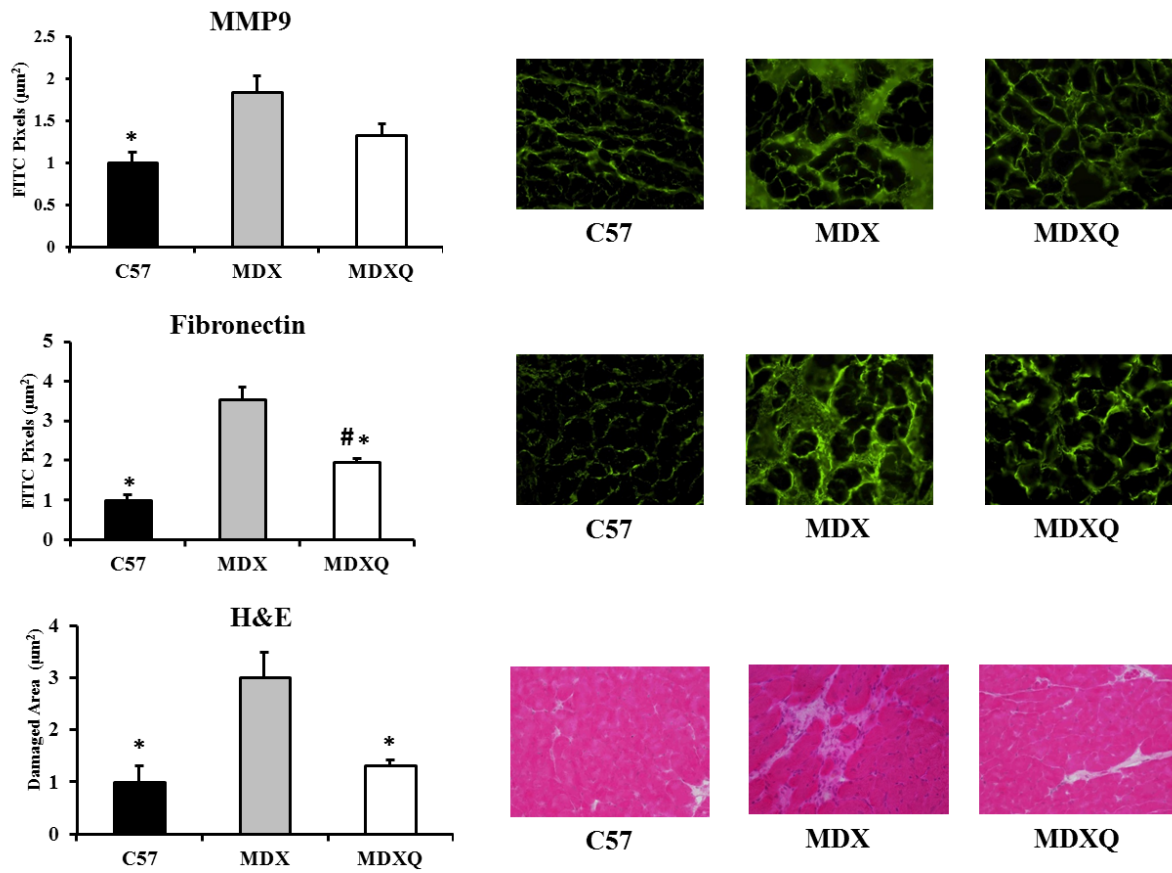
Quercetin decreases inflammation in dystrophic hearts. Data are means \pm SEM. * denotes significantly different from MDX. # denotes significantly different from C57.

Figure 7.



Quercetin increases utrophin expression, α - Sarcoglycan expression, and reassembly of costamere in dystrophic hearts. Data are \pm SEM. * denotes significantly different from MDX. # denotes significantly different from C57.

Figure 8.



Quercetin decreases fibrosis and damage in dystrophic hearts. Data are means \pm SEM. * denotes significantly different from MDX. # denotes significantly different from C57.

CHAPTER 6: MANUSCRIPT (MDX/UTRN^{+/-}) FOR SUBMISSION/PUBLICATION

Effect of Long-Term Quercetin Supplementation on Dystrophic Cardiac Pathology in Mdx/Utrn^{+/-} Mice

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Running Title: Quercetin Mdx/Utrn^{+/-} Mice Cardiac Pathology

Key Words: Polyphenol, Utrophin, Duchenne Muscular Dystrophy

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ABSTRACT

Duchenne Muscular Dystrophy (DMD) is commonly associated with progressive cardiac dysfunction and damage. Quercetin is a potent SIRT1/PGC1- α activator that may possess cardioprotective effects in the dystrophic heart. Our experiments *in vivo* tested whether long term 0.2% dietary quercetin enrichment attenuates dystrophic cardiopathology in mdx/utrn^{+/-} mice. Mdx/utrn^{+/-} mice were fed quercetin enriched (MUQ, n=8) or control diet (MU, n=8) for 10 months. Control C57BL10 (C57, n=8) animals were fed a control diet for 10 months. Using a 7T MRI, cardiac function was measured at 2 and 10 months. At 10 months, hearts were excised for histological and biochemical analysis. Data reveal quercetin preserved overall cardiac function including ejection fraction (EF) (p<0.05), systemic wall thickness (SWT) (p<0.05), % systolic wall thickness (%SWT) (p<0.05), and fractional shortening (FS) (p<0.05) when compared to control mdx/utrn^{+/-} mice. Histological analysis revealed higher expression of utrophin (p<0.001), α -sarcoglycan (p<0.001), and less utrophin - α -sarcoglycan co-localization (p<0.001). Furthermore, lower abundance of fibronectin (p<0.001), less cardiac damage (Hematoxylin Eosin-Y) (p<0.001), and less MMP9 (p=0.001) were observed in quercetin fed versus control mdx/utrn^{+/-} mice. Using Western blot techniques, data reveal quercetin higher cardiac PGC-1 α (p<0.001), cytochrome-c (p<0.001), ETC complexes I-V (p<0.05), citrate synthase (p<0.001), SOD2 (p=0.004), and GPX (p=0.003) versus control mdx/utrn^{+/-} mice. Western blot targets for inflammation revealed that quercetin lowered NF κ B (p=0.010), TGF- β 1 (p=0.007), and F4/80 (p=0.05) versus control mdx/utrn^{+/-} mice. However, quercetin-dependent changes in a panel of inflammatory markers (P-NF κ Bp65, P-IK β α , IK β α , CD64 and COX2) were not present. Collective findings suggest that long-term dietary quercetin enrichment preserves cardiac function in mdx/utrn^{+/-} mice. Secondary findings suggest that improved physiologic outcomes due to quercetin feeding are associated with increased mitochondrial biogenesis, increased antioxidant enzymes, and may partially reassemble the dystrophin glycoprotein complex (DGC). Histological analyses indicate a marked attenuation in pathological cardiac remodeling and further indicate that long-term quercetin consumption benefits the dystrophic heart.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a juvenile genetic disease typically affecting 1 in 3,500 males causing premature death as early as teenage years of age (1). Multiple forms of muscular dystrophy exist (i.e. Becker's, Limb Girdle); however, DMD is the most prevalent form of the disease. DMD is caused by a partial or complete loss of functional dystrophin due to a nonsense mutation (2). Dystrophin serves as a pivotal membrane stabilizing protein in the dystrophin glycoprotein complex (DGC). Membrane disruption occurs in the absence of adequate dystrophin content within the sarcolemma resulting in ionic disturbances, mitochondrial dysfunction, and cell necrosis. Physiologically, DMD is evident by muscle atrophy and weakness with a wide range of heterogeneity. Disease progression is marked by muscle degeneration and inadequate regeneration leading to respiratory and cardiac complications. Failure of the diaphragm and accessory respiratory muscles commonly cause death in many patients resulting in early mortality (3). Due to improvements in secondary care, the average life span of DMD patients has extended to the point that cardiac pathology accounts up to 40% of all deaths (4). As cardiac health deteriorates, cardiac complications manifest as ECG abnormalities, cardiac remodeling, contractile dysfunction, fibrotic tissue deposition, ventricular dilation, and heart failure (5). Accordingly, countermeasures directed against cardiac pathology in the DMD heart are needed.

Several mouse models exist as a cost effective means of vetting potential therapeutics for DMD. The dystrophin deficient mdx mouse is currently the most investigated animal model, though disease severity and lifespan in the mdx mouse imperfectly recapitulate the human condition (6, 7). In contrast, the mdx/utrn^{+/-} mouse is a comparatively less studied model of murine DMD that exhibits an exacerbated pathology and reduced life span. The mdx/utrn^{+/-} mouse lacks dystrophin and is a heterozygous knockout for utrophin, a homologous protein which appears to alleviate DMD pathology if upregulated and may partially replace dystrophin deficiency (8). Furthermore, aged mdx/utrn^{+/-} mice exhibit accelerated declines in cardiac health and dystrophic pathology.

Prior work clearly indicates that DMD is a multifaceted disease, whereby inflammation, mitochondrial dysfunction, and oxidative stress work in concert to promote cardiac pathology. Accordingly, countermeasures directed against individual disease facets including anti-inflammatory agents (5), and antioxidant administration (9) attenuate cardiac dysfunction. While

‘cocktail therapies’ are currently used in clinical settings to counter individual aspects of DMD, a single counter measure remains elusive. New findings reveal that a class of flavanones (primarily plant-derived polyphenols) may be effective as a single therapeutic agent in blunting the multifaceted pathological mechanisms associated with dystrophic cardiopathology.

Polyphenols can combat chronic inflammation (10), cardiac pathology (11), oxidative stress (12) and induce adaptive gene expression through activation of SIRT/ PGC-1 α (13, 14). This latter effect appears to be the fundamental mechanism underpinning the benefits of polyphenol use. Specifically, polyphenols promote induction of adaptive gene transcripts including the Sirtuin 1 (SIRT1)/ peroxisome proliferator-activated receptor gamma coactivator alpha (PGC-1 α) axis that increase mitochondrial transcripts and expression of oxidative and membrane structural proteins such as the aforementioned utrophin (15). Of the total ingestion of the multiple polyphenols in the human diet, quercetin (3,3',4',5,7-pentahydroxyflavone) makes up the majority of total polyphenol ingestion (16). Quercetin is a naturally occurring, safe flavonol with inherent anti-inflammatory and antioxidant properties. Moreover, quercetin is a potent SIRT1/PGC-1 α activator. Recent work from our lab group suggests that six months of moderate doses of 0.2% dietary quercetin enrichment is sufficient to elevate quercetin plasma levels and bioavailability successfully alleviating a major portion of the pathology of dystrophin deficient muscle (13). Prior study from our team and others suggest that induction of PGC-1 α alleviates considerable pathology in dystrophin deficient muscle (15, 17). Moreover, published data from our lab suggests that short term dietary quercetin enrichment promotes mitochondrial biogenesis, antioxidant protein abundance, and utrophin expression in young mdx mice. Histological analysis further indicated that quercetin consumption, was also associated with lower matrix metalloproteinase-9 (MMP-9) expression in young mdx mouse hearts and attenuated cardiac and fibrotic damage in older mdx mice (18). Currently, it is less clear to what degree dietary quercetin enrichment impacts dystrophin deficient hearts, especially in the mdx/utrn^{+/-} mouse. While these early findings are compelling, it is unknown whether long term or chronic dietary quercetin enrichment also translates to improved physiologic outcomes. More precise physiologic measurements such as cardiac function are needed to further clarify beneficial adaptations with quercetin enrichment. Further, more study is needed on the mdx/utrn^{+/-} mouse model and its suitability as a possibly superior model to study interventions for DMD. The aim of this study was to assess whether long term dietary quercetin enrichment preserves cardiac

function in mdx/utrn^{+/-} mouse hearts. Secondary to this, we sought to quantify histological and biochemical markers of cardiac adaptation to long term quercetin consumption.

METHODS

Study Design and Animals

The experimental protocol was approved by the Auburn University Institutional Animal Care and Use committee for the use of animals in research and in accordance with the American Physiological Society. To complete these experiments 2 month old mdx/utrn^{+/-} and C57BL10 mice were purchased from Jackson Laboratories (Jackson Laboratory, Bar Harbor, Maine). During experimentation, all animals were housed on a 12h-12h reverse light-dark cycle. Post-weaning, mdx/utrn^{+/-} mouse diets consisted of either normal rodent chow (MU, n=8) or 0.2% quercetin-fortified rodent chow (MUQ, n=8) (Bioserv Inc., Frenchtown, NJ) and control C57/BL10 mice were fed normal rodent chow (C57, n=8) for the total experimentation time of 10 months. All water and food were provided *ad libitum* throughout the experimentation period.

Functional Cardiac Assessment (7T MRI)

To assess cardiac function, mice were scanned at 2 months and 10 months of age using 7 Telsa (7T) magnetic resonance imaging (MRI) (Seimens Corp., USA) located at the Auburn University MRI Research Center. Prior to scanning, mice were anesthetized via isoflurane with ECG leads, respiration leads, and rectal temperature probes attached to monitor vital signs. All mice were maintained under anesthesia during the scanning processes via isoflurane. For the cardiac scan, anesthetized mice were placed in a custom made small bore copper radio frequency (RF) coil. Various cross-sectional images were obtained including a sagittal 2 chamber (left atrium and ventricle only) long-axis view, coronal 4 chamber (all atria and ventricles) long-axis view, and transverse 2 chamber (right and left ventricle only) short-axis view to ensure thorough depiction of the entire heart. Imaging was gated to ECG and respiration to ensure quality and complete imaging of the entire cardiac cycle.

Cardiac performance was assessed using image J software (NIH). Specifically, MR images of the cardiac cross-sections were analyzed during systole and diastole for all axes. In addition, cardiac wall thickness of the left ventricle was calculated. (19). Cinematic audio video interleaves (AVIs) were created to show real-time heart function and aid in further analysis.

Tissue Harvesting and Storage

After the 10 month longitudinal experiment, mice were weighed, and anaesthetized via isoflurane until a surgical plane was established. Mice were then sacrificed via cardiac excision.

Hearts were rinsed in chilled 10 mM PBS, weighed, placed in optimum cutting temperature compound (OCT), and flash frozen in liquid nitrogen chilled 2-methyl-butane to reduce the risk of freeze fracture. After freezing, hearts were stored at -80°C until further analysis.

Histology and Immunofluorescence

Hearts were cut 10 µm-15 µm thick on a Shandon Cryotome cryostat (-30°C) and set on 1 mm positively charged microscope slides (Fischer Scientific, Pittsburgh, PA, USA). To assess for total cardiac damage, slides were stained by a Hematoxylin and Eosin-Y histological procedure. Accordingly, stained slides were imaged via light microscopy with Nikon software (Melville, NY, USA). 10-15 images were obtained per slide at a magnification of 40x to characterize and confirm consistent representation of each cardiac cross section. Images were then analyzed using Image J software (NIH, Bethesda, MD, USA). Results of images were taken as a ratio of total damaged tissue to total healthy tissue (µm²). All imaging and analysis was conducted under blinded conditions by the same technician.

Cardiac expression of utrophin, fibronectin, metalloprotease 9 (MMP9), α-Sarcoglycan, and co-localization of α-Sarcoglycan and utrophin were determined using a histological immunofluorescence detection procedure. Briefly, slides were fixed with a 10% formalin solution, rinsed, and permeabilized with a Triton X-100 in 1% sodium citrate solution. Slides were rinsed in 10 mM PBS and blocked with a 3% bovine serum albumin and 20% goat or mouse serum solution. Slides were incubated with primary antibodies for proteins of interest: (Fibronectin, 1:500 dilution; F3648; Sigma-Aldrich), (Utrophin, 1:500 dilution; sc-33700; Santa Cruz Biotechnology), (α-Sarcoglycan, 1:500 dilution; sc-28278; Santa Cruz Biotechnology) (MMP9, 1:500 dilution; sc-6840; Santa Cruz Biotechnology). Heart sections were then incubated with corresponding secondary antibodies for Texas Red (TRITC, 1:500; T1-1000; Vector Laboratories Inc.) and/or fluorescein isothiocyanate (FITC) (1:500, sc-2365; Santa Cruz Biotechnology)(1:500, sc-2010; Santa Crus Biotechnology) fluorescent tags. Slides were sealed with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories Inc.) mounting medium and imaged with a fluorescent microscope (Nikon, Melville, NY). Random images (10-15) were obtained as previously discussed with corresponding DAPI, FITC, and TRITC filters. Images were analyzed for mean fluorescent positive pixels area percentage (µm²) using Nikon Software (Nikon, Melville, NY). All imaging and analysis was conducted under blinded conditions by the same technician.

Western Blotting

Heart protein (~50 µg/ sample) was obtained from hearts previously frozen in OCT from histology and separated using standard SDS-PAGE techniques on 6%-17% polyacrylamide gels through electrophoresis. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes and stained and imaged with Ponceau stain to ensure equal loading. Membranes were exposed to primary antibodies for each protein of interest: (PGC-1 α , 1:500 dilution; sc-13067; Santa Cruz Biotechnology), (SOD2, 1:500; sc-30080; Santa Cruz Biotechnology), (Citrate Synthase, 1:500 dilution; sc-390693; Santa Cruz Biotechnology), (Cytochrome-C, 1:500 dilution; sc-7159; Santa Cruz Biotechnology), (Glutathione peroxidase, 1:500; sc-74498; Santa Cruz Biotechnology), (CD64, 1:500 dilution, sc-15364p; Santa Cruz Biotechnology), (F4/80, 1:200 dilution; sc-25830; Santa Cruz Biotechnology), (Cyclooxygenase 2, 1:1000; CS-12282; Cell Signaling Technology Inc.), (NF κ B, 1:1000 dilution; CS-8242; Cell Signaling Technology Inc.), (Phospho-NF κ Bp65, 1:1000 dilution; CS-3033; Cell Signaling Technology Inc.), (I κ B α , 1:1000 dilution; CS-4814; Cell Signaling Technology Inc.), (Phospho-I κ B α , 1:1000 dilution; CS-2859; Cell Signaling Technology Inc.), (OxPhos Complex Kit, 1:5000 dilution; 457999; Invitrogen), (TGF β -1, 1:500 dilution; sc-146; Santa Cruz Biotechnology). Membranes were then exposed to corresponding HRP secondary antibodies for chemiluminescence detection: (anti-rabbit IgG HRP linked, 1:1000 dilution; CS-7074; Cell Signaling Technology Inc.), (anti-mouse IgG HRP linked, 1:1000 dilution; CS-7076; Cell Signaling Technology Inc.). Membranes were reprobed and exposed to corresponding normalizing protein primary antibodies: (GAPDH, 1:1000, GenTex) or (α - Tubulin, 1:1000 dilution; DHSB). Corresponding secondary antibodies previously mentioned were used for chemiluminescence detection. Western blots were normalized to corresponding normalizing protein, Ponceau stain, and were analyzed using a UVP LLC digital imaging device (Upland, CA, USA). All experiments and analysis were conducted blinded and by the same technician.

STATISTICAL ANALYSES

For cardiac function variables, a 3x3 repeated measures ANOVA was used to test for differences of our primary dependent variables using SPSS 20 (IBM, New York City). For biochemical and histological variables, a one-way ANOVA was used to test differences for our secondary and tertiary dependent variables using SPSS 20 (IBM, New York City). Bonfferoni post-hoc analyses were performed as needed. In the instances that a violation of homogeneity occurred (Levine's test), Welch's t-test were performed for all strain pair-wise comparisons to correct for unequal variances among groups (20). Significance was set at $p \leq 0.05$ *a priori*. Data are presented as mean \pm SEM.

RESULTS

Animal Characteristics

Animal characteristics and measurements are presented in [Table 1]. At the end of the study MU and MUQ mice were heavier than C57 controls ($p < 0.001$). C57 absolute heart weights were higher in MU mice compared to C57 ($p = 0.006$). Finally, relative heart weights (mg/g) of MUQ mice were significantly lower than C57 control mice ($p = 0.001$).

Cardiac Function

Measurements cardiac function MRI data are presented in [Table 2] and representative images (10 months) are presented in [Figure 1]. From 2 months of age, ejection fraction (EF) was lower in MU mice versus control C57 and MUQ by 10 months of age respectively ($p < 0.001$; $p < 0.001$). Furthermore, EF was lower than previous MU 2 month value ($p < 0.001$). At 2 months, end diastolic volumes (EDV) were lower in a strain dependent fashion in both MU and MUQ groups ($p < 0.001$). By 10 months, MU and MUQ groups EDV was elevated and were higher than strain respective 2 month values respectively ($p < 0.001$; $p < 0.001$). End systolic volume (ESV) was similar between all groups at 2 months. However, by 10 months, MU mice displayed higher ESV versus both C57 and MUQ mice respectively ($p < 0.001$; $p < 0.001$). Furthermore, ESV was higher in MU mice when compared to strain respective 2 month values ($p < 0.001$). MU mice revealed lower fractional shortening (FS) when compared to both C57 and MUQ groups at 2 months respectively ($p < 0.001$; $p < 0.001$). However, MU mice showed an even further decrease in FS by 10 months when compared to C57 and MUQ respectively ($p < 0.001$; $p < 0.001$). Stroke volume (SV) was lower in both MU and MUQ mice in a strain dependent manner at 2 months versus C57 mice ($p < 0.001$). However, by 10 months, both C57 and MUQ mice had significantly higher SV versus MU mice respectively ($p = 0.045$; $p = 0.045$) revealing that quercetin treatment improved SV. Like SV, cardiac output (CO) was lower in both MU and MUQ mice in a strain dependent manner at 2 months versus C57 mice ($p < 0.001$). However, quercetin feeding in the MUQ mice improved CO when compared to strain respective 2 month value ($p = 0.044$). Systolic wall thickness (SWT) was lowered in MU mice by 10 months when compared to C57 and MUQ mice respectively ($p < 0.001$; $p = 0.006$) revealing that quercetin enrichment preserved cardiac wall thickness in MUQ mice.

Western Blotting

To assess key protein abundance of interest for our proposed mechanism, various western blotting was performed for proteins of interest. PGC-1 α protein abundance was elevated in MUQ mice versus control C57 and MU mice respectively ($p = 0.002$; $p < 0.001$) [**Figure 2A**]. As PGC-1 α is considered the master regulator of mitochondrial biogenesis, strategic downstream mitochondrial proteins were also analyzed. As a primary mitochondrial biomarker, cytochrome-c protein concentration was assessed. Findings indicate cytochrome-c protein content was lower in MU mice when compared to C57 and MUQ mice respectively ($p = 0.033$; $p < 0.001$). Furthermore, cytochrome-c abundance was higher in hearts from MUQ mice as compared to C57 ($p = 0.005$) [**Figure 2B**]. Citrate synthase, an oxidative metabolic enzyme found in the mitochondria, protein abundance was lower in MU mouse hearts as compared to hearts from both C57 and MUQ mice correspondingly ($p = 0.037$; $p < 0.001$) [**Figure 2C**]. To further characterize the dietary quercetin enrichment on mitochondrial biogenesis, western blots for electron transport chain (ETC) complexes I-V protein abundance were performed [**Figure 3**]. MU mice displayed lower protein abundance of NADH Dehydrogenase (Complex I) than both C57 and MUQ mice respectively ($p = 0.045$; $p = 0.001$). MU mice also displayed lower abundance of Succinate Dehydrogenase (Complex 2) when compared to MUQ counterparts ($p = 0.001$). While hearts from MU possessed approximately 30% less Succinate Dehydrogenase than hearts from C57 mice, these values were not statistically different ($p = 0.059$). Complex III, Cytochrome bc1 complex, was also lower in MU mouse hearts as compared to C57 and MUQ hearts respectively ($p = 0.007$; $p < 0.001$). Further, Cytochrome-C Oxidase (Complex IV) protein abundance was lower in the hearts of MU mice when compared to C57 and MUQ mice correspondingly ($p = 0.012$; $p < 0.001$). Finally, ETC Complex V (ATP Synthase) content was lower in MU mice when compared MUQ counterparts ($p = 0.003$). While hearts from MU mice expressed approximately 35% less ATP Synthase when compared to C57, these numerical differences were not statically different ($p = 0.071$). Selected endogenous antioxidant enzymes associated with mitochondria and cardiac health were also measured in hearts from all three groups. Manganese Superoxide Dismutase (SOD2) protein abundance was significantly lower in MU mice when compared to C57 and MUQ counterparts correspondingly ($p = 0.026$; $p = 0.004$) [**Figure 4A**]. Furthermore, Glutathione Peroxidase (GPX) was protein content was higher in MUQ mice when compared to both control C57 and MU mice respectively ($p = 0.020$; $p = 0.003$) [**Figure 4B**].

Since robust inflammation is associated with DMD over time, strategic proteins and pathways related to tissue inflammation were assayed. In particular, key protein markers in the NF κ B pathway were chosen to measure inflammation signaling [Figure 5]. NF κ B65 protein abundance was higher in hearts from MU mice when compared to MUQ counterparts ($p=0.010$) [Figure 5A]. Further, while MU mice displayed approximately 20% more NF κ B65 protein content when compared to C57 mice, this numerical difference was not statistically different ($p=0.064$). Phosphorylated (activated) p-NF κ Bp65 protein abundance was not different among groups despite the fact that mean p-NF κ Bp65 abundance in MU mice was approximately 25% higher than the other two groups ($p=0.301$) [Figure 5B]. While mean I κ B α abundance was approximately 20% lower in MU mice compared to C57 and MUQ mice, statistical differences did not exist ($p=0.152$; $p=0.123$) [Figure 5C]. Furthermore, there was no statistical difference between groups of p-I κ B α abundance ($p=0.145$) [Figure 5D]. To further characterize the influence quercetin may have on inflammation, downstream markers that NF κ B mediate were also measured. While there was a strong COX2 trend in that mean COX2 protein abundance in MU mice was approximately 50% higher as compared to C57 and MUQ groups, significant differences were not present ($p=0.055$) [Figure 6A]. However, the cytokine and growth factor involved in fibrotic deposition, TGF β -1, was lower in MUQ mice versus MU mice ($p=0.007$) [Figure 6B]. To assess for macrophage infiltration, Cluster of Differentiation 64 (CD64) and F4/80 (a mouse homolog of EGF-like module-containing mucin-like hormone receptor-like 1) which are exclusively found in macrophages were analyzed. There was no difference among groups in CD64 protein expression ($p=0.090$) [Figure 6C]. Mean F4/80 expression was elevated approximately 35% and 65% in MU mice when compared to C57 and MUQ mice respectively, but these numerical differences were not statistically meaningful ($p=0.250$; $p=0.074$). However, hearts from MUQ mice displayed lower F4/80 expression when compared to hearts from C57 mice ($p=0.050$) [Figure 6D].

Histology

To further bolster our biochemical analysis, we employed strategic histological measurements. Immunofluorescent analyses revealed an approximately 90% lower expression of utrophin in MU mice when compared to C57 mice ($p < 0.001$). However, quercetin feeding in the MUQ group enhanced utrophin expression to 50% and was higher than MU mice ($p=0.011$) although this increase was lower than control C57 mice ($p=0.002$) [Figure 7A]. Analyses of α -

Sacroglycan, another scaffolding protein of the DGC, revealed that hearts from MU mice displayed an approximately 95% lower expression of α -Sacroglycan ($p < 0.001$) while MUQ displayed only an approximately 65% lower expression ($p < 0.001$). However, control C57 mice maintained a significantly higher expression of α -Sacroglycan when compared MUQ mice ($p < 0.001$) [Figure 7B]. Related to potential restoration of the DGC with utrophin, further measurements of utrophin/ α -Sacroglycan co-localization were performed. Compared to healthy control C57 mice, MU mice displayed an approximately 97% lower co-localization of utrophin and α -Sacroglycan ($p < 0.001$). However, MUQ mice had an approximately 65% co-localization of utrophin and α -Sacroglycan when compared to control C57s ($p < 0.001$) [Figure 7C]. To assess for a marker of cardiac remodeling, MMP9 was analyzed. Analyses revealed a 3-fold higher expression in MU mice when compared to C57 control animals ($p < 0.001$). MUQ mice displayed an approximate 2-fold lower MMP9 expression when compared to MU mice ($p < 0.001$), however MMP9 expression in MUQ mice was elevated when compared to control C57 mice ($p < 0.001$) [Figure 8A]. To assess fibrosis within the heart, fibronectin, a key glycoprotein in fibrotic tissue, was analyzed and measured. MU mice displayed an approximate 10-fold higher expression of fibronectin when compared to control C57 ($p < 0.001$). Quercetin feeding in the MUQ mice revealed an approximately 6-fold lower level of cardiac fibronectin content when compared to MU mice ($p < 0.001$). However, MUQ mice did display higher fibronectin abundance than control C57 mice ($p < 0.001$) [Figure 8B]. To assess cardiac damage, a modified Hematoxylin and Eosin-Y histological procedure was employed. Analyses revealed that MU mice displayed an approximately 4-fold lower total cardiac damage when compared to control MUQ mice ($p < 0.001$) and was 7-fold higher when compared control C57 mice ($p < 0.001$). While MUQ mice only displayed an approximate 3-fold lower amount of total to damaged tissue ratio this value was higher than control C57 mice ($p < 0.001$) [Figure 8C].

DISCUSSION

DMD patients experience time dependent cardiac abnormalities and myocardial pump failure leading to increases in morbidity and mortality from the pathology (7). Currently, no cure is available and current long term therapeutic approaches are limited. Based on this reality the purpose of this investigation was to pursue a novel and pragmatic therapy to alleviate DMD pathology. Fundamental to our approach, preliminary data from our lab group suggests that Sirtuin1/PGC-1 α induction via dietary quercetin enrichment ameliorates many of the pathological processes in mdx mice (13, 15, 17, 18). In the current study, a severe pathological murine model, the mdx/utrn^{+/-} mouse, was used to more closely recapitulate the human disease than the mdx mouse. Novel findings from the current study reveal that life-long quercetin enrichment attenuates age-related cardiac dysfunction characteristic of DMD. Interestingly, analyses from quercetin enriched mice post mortem revealed corresponding improvements in mitochondrial biogenesis, endogenous antioxidant expression, and prevention of fibrosis, and cardiac damage. However, quercetin enrichment showed only modest improvements in cardiac inflammation of mdx/utrn^{+/-} hearts further cementing the increasing of severity of pathology versus other DMD models.

Cardiac Function

Currently it is well established that dystrophic mice develop cardiac dysfunction in an age-dependent manner (see selected review (7)). While young dystrophic mice display relatively healthy cardiac function when compared to age matched controls, young dystrophic mice still experience damage due to mechanical overload (21, 22). Commonly, mdx mouse hearts become fibrotic and exhibit signs of contractile dysfunction by 9 months of age (7). However, in the mdx/utrn^{+/-} mouse, age-dependent changes in cardiac dysfunction and pathology have not been investigated. Current results indicate that EF, FS, and SWT are lower in mdx/utrn^{+/-} mice versus C57 controls. From the perspective of mouse models for DMD these current findings agree with previous reports that cardiac dysfunction increases with age in mdx mice as well (21, 23). The novel discovery of the current study is that quercetin enrichment attenuated decreases in EF, FS, and SWT in mdx/utrn^{+/-} mice. Furthermore, while it is currently documented that ESV increases in an age-dependent fashion at 14 months of age (24), novel findings in the current study reveal

that strain-dependent increases in ESV were attenuated in hearts of quercetin fed mdx/utrn^{+/-} mice.

Interestingly, results indicated some strain-dependent differences were present for mdx/utrn^{+/-} mice when compared to control C57 mice. EDV, SV, and CO were all significantly lower at 2 months when compared to control C57 mice. Even at this young age, the most likely cause of this finding is the increased severity of pathology due to the heterozygous knock-out of the dystrophin related protein utrophin. However, at 10 months of age EDV was similar between mdx/utrn^{+/-} and C57 mice. Age-dependent alterations in EDV have been observed previously (24), and were apparently unaffected by dietary quercetin enrichment. Similarly, SV was decreased in mdx/utrn^{+/-} mice at 2 months compared to control C57 mice. While SV in control mdx/utrn^{+/-} mice continued to decline, quercetin fed mice exhibited preserved SV that was similar to C57 mice at 10 months of age. Finally, CO was also decreased in mdx/utrn^{+/-} mice at 2 months compared to control C57 mice. Remarkably though, quercetin fed mice displayed age-dependent improvements in CO from 2 month values and CO was similar to that of healthy C57 mice at the 10 month examination period. Given that heart rates were not different between C57 and mdx/utrn^{+/-} treatment, quercetin-dependent improvements in CO are attributed to the corresponding preservation of SV.

While multiple published studies reveal flavanone or polyphenol consumption promotes beneficial alterations in cardiac function (25, 26), the current investigation is the first to demonstrate that long-term quercetin alleviates cardiac dysfunction in a pathologically severe model of DMD, the mdx/utrn^{+/-} mouse. A pivotal methodological benefit in the current study is the application of 7T MRI to quantify cardiac function over numerous time points. As previously mentioned, no single therapeutic agent exists to attenuate increases in age-related cardiac dysfunction in dystrophic hearts. While our preliminary published findings from a short duration feeding of quercetin in juvenile mdx mice suggested that quercetin feeding elicited beneficial cardiac adaptations (18), our current cardiac function results in a phenotypically more severe model provide physiologic evidence to support this notion. Collective findings further build upon a growing body of evidence to suggest that dietary quercetin consumption may be a novel and practical prospective therapy to improve cardiac health for those with DMD.

PGC-1 α , Mitochondrial, and Oxidative Adaptations

Flavanones and polyphenols such as quercetin robustly stimulate a variety of physiologic pathways including our particular pathway of interest in the SIRT1/PGC-1 α axis. A plethora of reports indicate that polyphenols are potent SIRT1/PGC-1 α activators and increase downstream transcripts and targets (27-29). Previously published studies report increases in mRNA and gene expression of the SIRT1/PGC-1 α axis with dietary quercetin feeding (27, 30); however, confirmation of protein abundance following quercetin enrichment is limited and relatively unknown. Preliminary data from our lab group found that short term dietary quercetin enrichment did not alter PGC-1 α cardiac protein abundance in dystrophic mice (18). Interestingly, the current investigation reveals that long term quercetin enrichment also increases cardiac PGC-1 α protein expression in a pathologically severe murine model of DMD. Even more intriguing, quercetin enrichment in the dystrophic mice increased PGC-1 α protein expression to a level comparatively higher than age matched C57 control mice. This latter finding may be of particular importance in that prior investigation reveals that PGC-1 α expression is decreased in failing hearts, and corresponds to decreased mitochondrial function and exacerbated cardiac pathology (31). Findings of increased cardiac levels of PGC-1 α and preserved cardiac function in the current study parallel outcomes in a nearly identical investigation using mdx mice and may hold important therapeutic implications for those with DMD.

Given the pleiotropic effects of PGC-1 α on mitochondrial expression and function, we examined a series of mitochondrial related biomarkers in cardiac tissues harvested at the end of the study. Results reveal that quercetin fed mice exhibited higher relative levels of citrate synthase protein abundance, cytochrome-c, and all electron transport complexes (NADH Dehydrogenase, Succinate Dehydrogenase, Cytochrome bc1 Complex, Cytochrome-C oxidase, and ATP synthase). While not confirmatory in the current study design, the current data appear to be the first to quantify elevated markers of bioenergetic capacity within the dystrophic heart with quercetin enrichment. Possibly indicative of pathology inherent to the DMD animal model, mdx/utrn^{+/-} mice expressed lower relative concentrations of the mitochondrial biomarkers as compared to C57 mice. The significance of these results are emphasized further by prior observation that dystrophic hearts are susceptible to mitochondrial damage (32). Thus, our results support previous findings that increases in PGC-1 α increase protein abundance of mitochondrial enzymes and proteins in the myocardium (33) and bolster our earlier data, which indicate that cytochrome-c levels were higher in the myocardia of young dystrophic mice

consuming an identical quercetin diet (18). Collectively, current findings deliver convincing indications to suggest that mitochondrial biogenesis is increased within the myocardium with long term dietary quercetin enrichment in mdx/utrn^{+/-} mice and likely contributed to attenuation of cardiac dysfunction characteristic of DMD.

We also sought to determine whether endogenous antioxidant enzyme abundance was increased in the myocardia of quercetin fed mice. Although quercetin possesses independent inherent antioxidant properties (34), polyphenols such as quercetin up-regulate endogenous antioxidant enzymes (12, 18). In agreement with our prior study, current findings indicate that quercetin enrichment increased myocardial abundance of SOD2. Since SOD2 is primarily located within mitochondria, increased SOD2 levels support our current interpretation that quercetin increased mitochondrial biogenesis in hearts from mdx/utrn^{+/-} mice. We also investigated the influence of quercetin enrichment on cardiac glutathione peroxidase (GPX) concentration. GPX, an abundant antioxidant enzyme which catalyzes the reduction of hydrogen peroxide to water, is well established as a pivotal enzyme in protecting the heart against oxidative stress. Our current findings show that quercetin enrichment increased myocardial abundance of GPX in mdx/utrn^{+/-} hearts. Current results coincide with previous reports that show quercetin possesses the possibility of inducing increases in GPX (35, 36). Furthermore, previous work shows that GPX overexpression attenuates cardiac remodeling and preserves cardiac function within pathological mouse hearts (37, 38). Thus, the increases in myocardial protective antioxidant enzymes may also contribute to the subsequently mentioned quercetin-mediated preservation in cardiac function.

DGC reassembly

Dystrophin is an important protein responsible for muscle membrane stabilization during contraction or mechanical stress. In the absence of sufficient dystrophin it is vital to find a substitute molecule or surrogate protein to prevent/attenuate membrane disruption. Currently, our findings reveal that quercetin enrichment is associated with increased utrophin expression. This finding is remarkable given the nature of the heterozygous knockout of the utrophin gene in the mdx/utrn^{+/-} mouse. Furthermore, these results bolster our previous finding that quercetin enrichment induces increased utrophin expression in dystrophic myocardium of younger mdx mice (18). Utrophin is a dystrophin homolog with some functional differences (39, 40). Published findings indicate that up-regulation or induction of utrophin in the absence of

dystrophin attenuates and alleviates DMD associated pathology (8, 41, 42). However the real benefit in utrophin up-regulation is dependent upon its role in restoration of the DGC. To investigate this currently, we probed for α -sarcoglycan, another protein found within the DGC. Findings revealed that membrane expression of α -sarcoglycan was associated with long term quercetin enrichment in dystrophic hearts. Furthermore, we also co-stained cardiac cross-sections to assess co-localization. Importantly long term quercetin enrichment was linked to co-localization of α -sarcoglycan and utrophin within the dystrophic heart. While current findings are semi-quantitative, they suggest that quercetin enrichment aids in DGC reassembly in the dystrophic heart. However, additional investigation is needed to confirm these observations.

Fibrosis and Damage

To assess for cardiac damage, fibrosis, and cardiac remodeling, a combination of histological assays were employed. Hematoxylin and Eosin-Y (H&E) staining along with immunofluorescent techniques to probe for fibronectin and MMP9 were performed in cardiac cross-sections. Findings from H&E analysis reveal that long term quercetin enrichment attenuated damage and fibrosis in mdx/utrn^{+/-} hearts. Mdx mice consistently exhibit histological indices of cardiac damage and fibrosis by 9 months of age (7). This finding supports our prior finding in aged mdx mice exposed to an identical quercetin feeding study (18). Moreover, prior investigations report similar anti-fibrotic effects with dietary application of polyphenols (11, 43). Prior published studies also support associative improvements in fibrosis and physiologic cardiac function as determined via MRI in similarly aged dystrophic mice (19, 23).

To refine our characterization of fibrotic disposition, we probed for fibronectin which is a glycoprotein highly expressed in connective and fibrous tissue. Quercetin enrichment severely diminished cardiac fibronectin expression further supporting our current findings of decreased cardiac damage and maladaptive remodeling while also agreeing with similar findings from our lab group in the mdx mouse diaphragm (13). To assess for pathological cardiac remodeling and extracellular matrix breakdown, we also strategically chose to probe for MMP9. Indeed, our results reveal that quercetin feeding diminished MMP9 expression and may have aided in attenuation of maladaptive cardiac remodeling. Our results agree with previous findings suggesting that quercetin inhibits MMP9 directly and may also be related to mito- and cyto-protection due to structure-activity analysis demonstrating that flavonoid R3-OH and R4-OH substitutions contribute to MMP9 inhibitory properties (44, 45). In support, prior findings from

mdx/utrn^{+/-} knockout mice indicate that cardiac pathology is largely due to matrix metalloprotease dysfunction (46). Furthermore, current findings reinforce our previous study in that quercetin enrichment decreases MMP9 expression in hearts from aged mdx mice (18). Current results demonstrate that quercetin enrichment attenuates cardiac damage, fibrotic tissue deposition, and potentially pathological cardiac remodeling. Collectively, these findings provide compelling evidence that quercetin-dependent prevention in pathological remodeling of the heart is directly related to preservation of cardiac function.

Inflammation and Immune Cell Infiltration

While DMD exhibits multi-faceted physiological consequences contributing to the overarching pathology, perhaps one of the largest maladaptation is chronic inflammation. Chronic inflammation results due to a robust drive of degeneration and regeneration of myocytes from the lack of functional dystrophin. Currently, corticosteroids are among the most common employed therapies for DMD patients to combat chronic inflammation. DMD patients treated with corticosteroids experience preserved mobility and attenuated respiratory complications (47). However, prolonged therapy of corticosteroids also produce maladaptive consequences such as glucose dyshomeostasis, increased BMI, decreased bone mineral density, and cardiac abnormalities (48, 49). Given this understanding, one of the secondary aims of the current study was to quantify whether quercetin consumption was associated with attenuated inflammation in cardiac tissues.

Previous findings suggest that NFκB protein, signaling, and downstream effectors are elevated in hearts from DMD patients (50, 51). Thus, we chose to determine if quercetin enrichment affected the NFκB inflammatory pathway in mdx/utrn^{+/-} hearts. Total NFκB protein abundance was lower with quercetin feeding, a finding in agreement with previous understanding that NFκB is decreased following quercetin enrichment (52). Interestingly, dietary quercetin enrichment did not alter other aspects of the pathway including cardiac levels of IKBα, P-IKBα, and P-NFκBp65.

We are currently unable to provide definitive answers for why quercetin was ineffective in this regard for our chosen markers of inflammation. Nonetheless, several explanations may exist for the largely negative anti-inflammatory results in quercetin fed mice. Firstly, the mdx/utrn^{+/-} mouse has a much more severe pathology compared to the mdx mouse. Next, mice in the current study were fed a modest dose of quercetin as compared to many other interventions to

investigate inflammation as an outcome. Lastly, samples were collected at a single time point so dynamic changes in these inflammatory markers, as would be evident in alternative time-course study designs could have been missed.

Previous studies report that polyphenols like quercetin suppress COX2 expression (53, 54). While COX 2 protein abundance was approximately 50% lower in quercetin fed mice as compared to control fed mdx/utrn^{+/-} mice this outcome was not statistically significant. In contrast, the cytokine and growth factor TGFβ-1, was attenuated with quercetin supplementation. TGFβ-1 is a major regulator in fibrosis and collagen deposition (55). Our current findings agree with previous reports that quercetin blunts fibroblast signaling and TGFβ-1 (56) and further supports our current findings of decreased fibronectin expression and also decreased cardiac damage. To assess for immune cell infiltration, CD64 and F4/80 were chosen to detect macrophages and immune cell infiltration and signaling. Quercetin enrichment did not alter CD64 expression. However, quercetin enrichment was associated with decreased F4/80 protein expression in mouse hearts suggesting the possibility that less immune cell infiltration occurred. Collectively, these results suggest that quercetin enrichment had only modest effects on the inflammatory response in mdx/utrn^{+/-} mouse hearts. Additional investigations that include additional considerations for tissue collection in order to account for dynamic cycles of inflammation are needed to better resolve a potential role of quercetin as an anti-inflammatory agent in mdx/utrn^{+/-} mice.

CONCLUSION

The current study is the first to perform a longitudinal examination of dietary quercetin ingestion as a counter therapy in the mdx/utrn^{+/-} mouse. In addition to the longitudinal approach, the current experimental model is a comprehensive investigation with concurrent examination of physiological, histological, and biochemical outcomes. Findings reveal that long term 0.2% dietary quercetin feeding provides robust protection against the development of cardiac dysfunction in the mdx/utrn^{+/-} mouse as determined by 7T MRI diagnostics. Further, results in post mortem cardiac tissue with histological and biochemical analysis revealed that quercetin enrichment was also accompanied with attenuation of cardiac damage, fibrosis, cardiac remodeling, and improved mitochondrial biogenesis. These noteworthy results bolster our previous investigations using similar methodological approaches in the mdx mouse model of DMD. In contrast to prior work in mdx mice, use of the more pathologically severe mdx/utrn^{+/-} mouse model further advocates the benefits of quercetin consumption as a potential therapy against DMD. Collectively, our findings support the pleiotropic benefits of dietary quercetin consumption. We present compelling, albeit indirect, evidence to suggest that increased PGC-1 α expression may be the cellular mechanism underpinning the benefits observed in hearts from quercetin fed animals. However, given the nature of the longitudinal, 10 month old endpoint employed in the current study we cannot confirm whether quercetin alone modulated PGC-1 α . Future investigations should be directed at understanding the specific time course for fully understanding the role of PGC-1 α as a trigger for beneficial outcomes due to quercetin feeding in the DMD heart. Furthermore, whether quercetin works largely through increased PGC-1 α protein abundance, activity, and/or off target effects remains to be determined. Current results suggest that quercetin enrichment may be a novel and unique therapy for dystrophic cardiac pathology that may improve clinical outcomes in DMD patients.

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Table 1.

Strain ID	Body Weight (g)	Absolute Heart Weight (mg)	Relative Heart Weight (mg/g)
C57	30.8 ± 2.50	162 ± 11.45 *	5.2 ± 0.33
MU	41.2 ± 2.73 #	194 ± 15.31	4.8 ± 0.38
MUQ	42.0 ± 2.34 #	183 ± 13.6	4.4 ± 0.42 #

Animal Characteristics. Data are means ± SEM.* denotes different from MU. # denotes different from C57

Table 2.

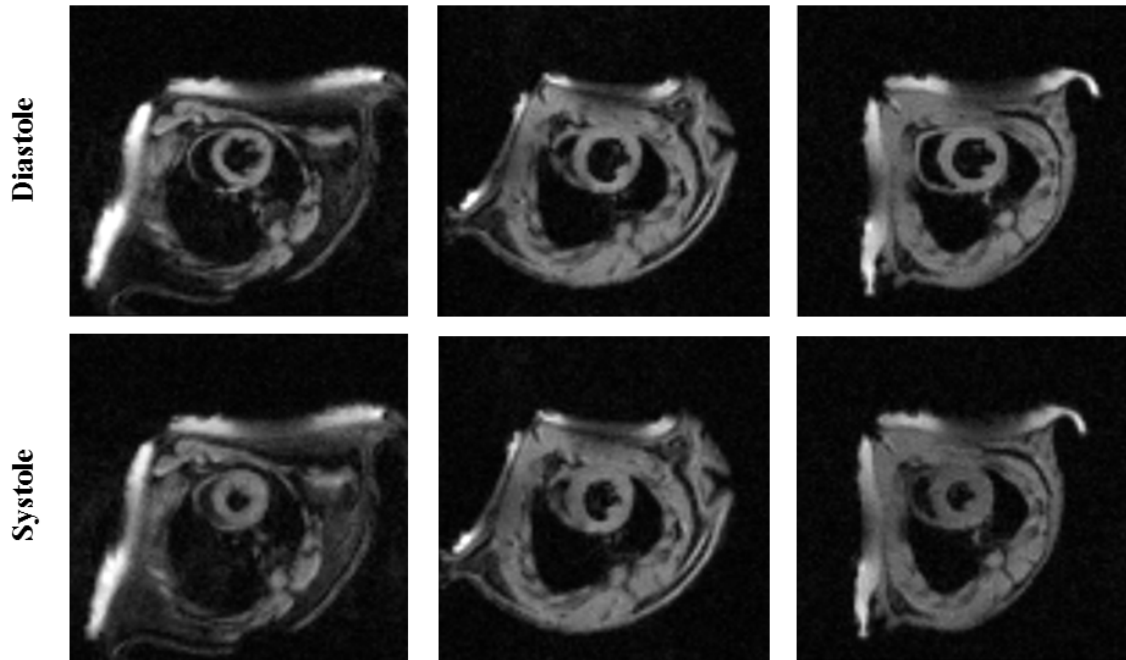
Time	Strain	EF (%)	EDV (μL)	ESV (μL)	FS	SWT (mm)	SV (μL)	CO ($\mu\text{L}/\text{min}$)
2 months	C57	59.3 \pm 1.4	67.2 \pm 3.1	27.4 \pm 1.6	46.6 \pm 2.3	0.42 \pm .04	39.8 \pm 2.0	20686 \pm 927
	MU	52.3 \pm 2.3	46.6 \pm 2.6 #	22.4 \pm 2.1	35.5 \pm 2.0 *	0.34 \pm .02	24.2 \pm 1.6 #	9110 \pm 617 #
	MUQ	53.2 \pm 2.1	47.4 \pm 3.8 #	23.7 \pm 1.4	45.5 \pm 2.4	0.39 \pm .01	24.8 \pm 2.4 #	9602 \pm 862 #
10 months	C57	57.5 \pm 2.7	62.2 \pm 4.4	26.2 \pm 2.3	46.4 \pm 2.6	0.43 \pm .03	36.0 \pm 3.4	18998 \pm 1389
	MU	39.3 \pm 1.3 * # †	73.8 \pm 1.9 †	44.2 \pm 0.9 * # †	29.1 \pm 0.9 * #	0.26 \pm .03* #	28.8 \pm 1.6 * #	10371 \pm 678
	MUQ	53.7 \pm 1.9	67.3 \pm 4.3 †	30.8 \pm 1.7	46.2 \pm 2.1	0.34 \pm .03	36.4 \pm 2.9	13125 \pm 1056 †

Cardiac Function. Data are means \pm SEM. Representative Images are from 10 month scans. * denotes significantly different from age matched MU. # denotes significantly different from age matched C57. † denotes significantly different from strain 2 month value.

Figure 1. C57

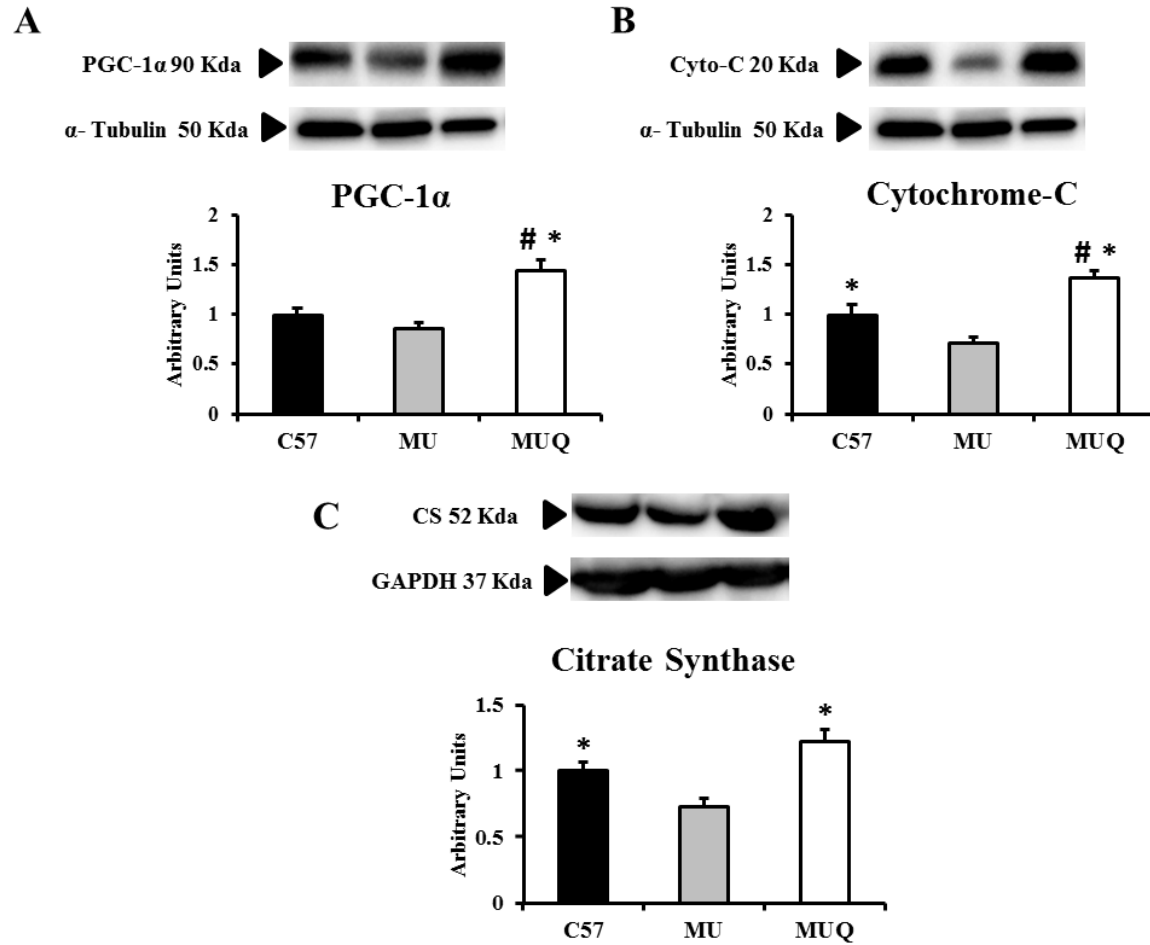
MU

MUQ



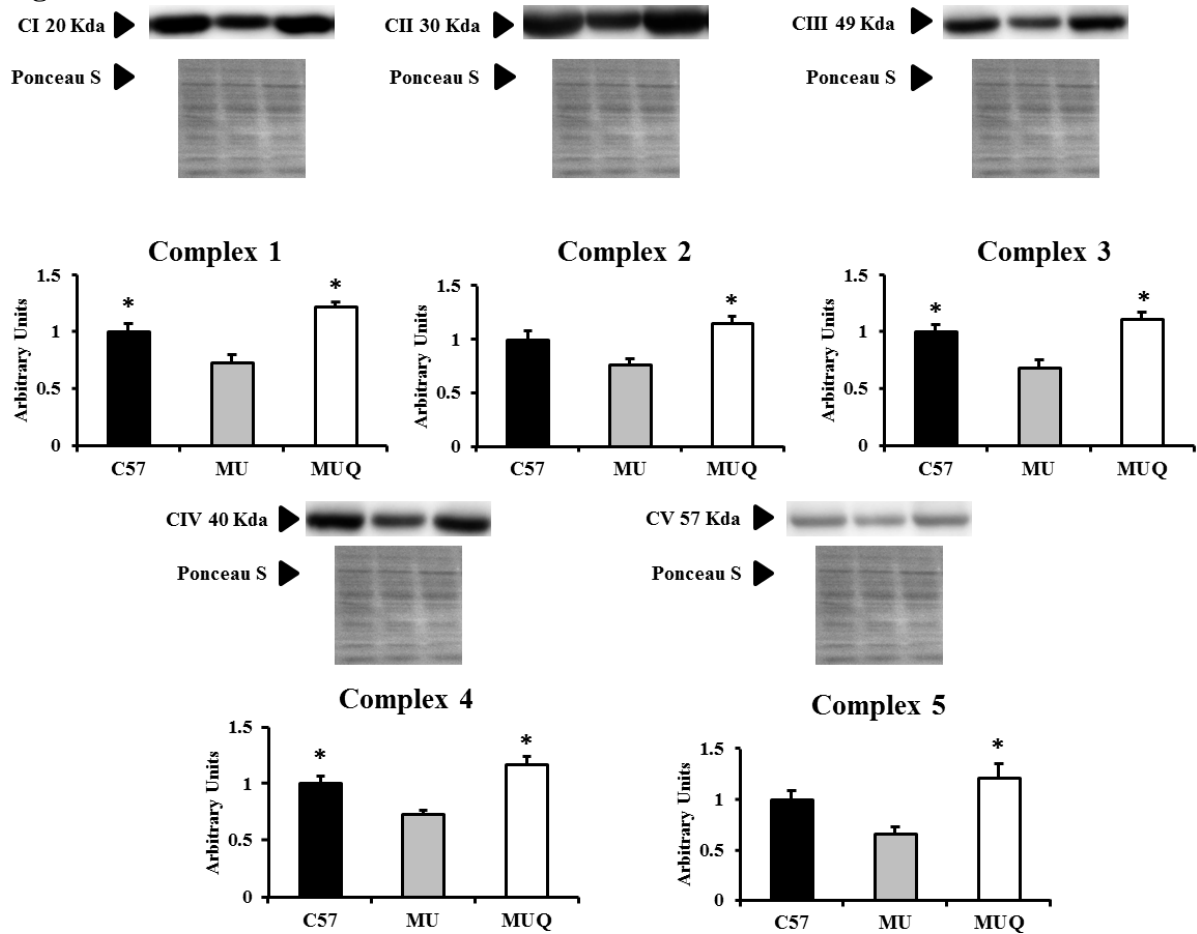
Representative images from 7T MRI. Top row represents beginning of diastole. Bottom row represents the end of systole.

Figure 2.



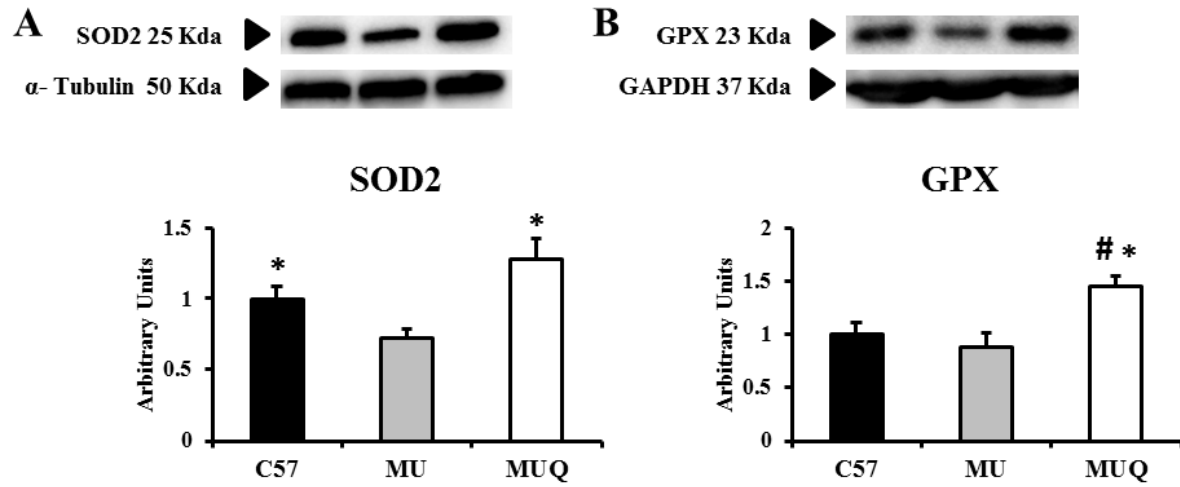
Quercetin increases PGC-1α, Cytochrome-C, and Citrate Synthase expression in mdx/utrn^{+/-} hearts. Data are means ± SEM. * denotes significantly different from MU. # denotes significantly different from C57.

Figure 3.



Quercetin increases mitochondrial ETC complexes I-V abundance in mdx/utrn^{+/-} hearts. Data are means \pm SEM. *denotes significantly different from MU.

Figure 4.

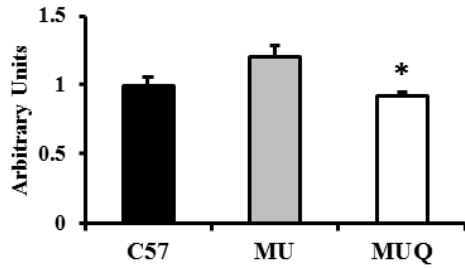


Quercetin increases antioxidant enzyme abundance in mdx/utrn^{+/-} hearts. Data are means \pm SEM. * denotes significantly different from MU. # denotes significantly different from C57.

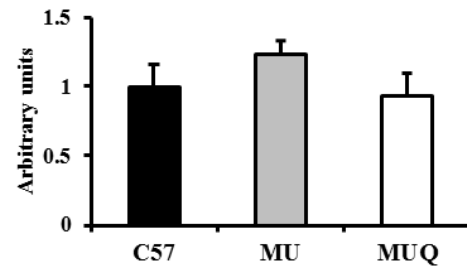
Figure 5.



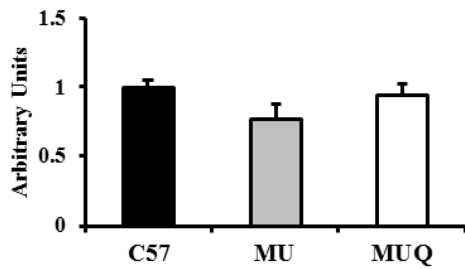
NFκB



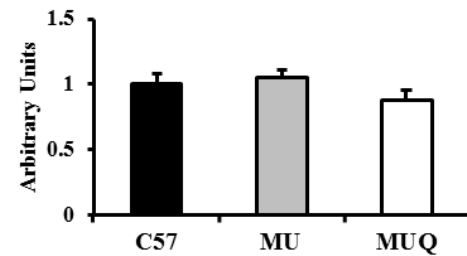
P-NFκBp65



IκBα

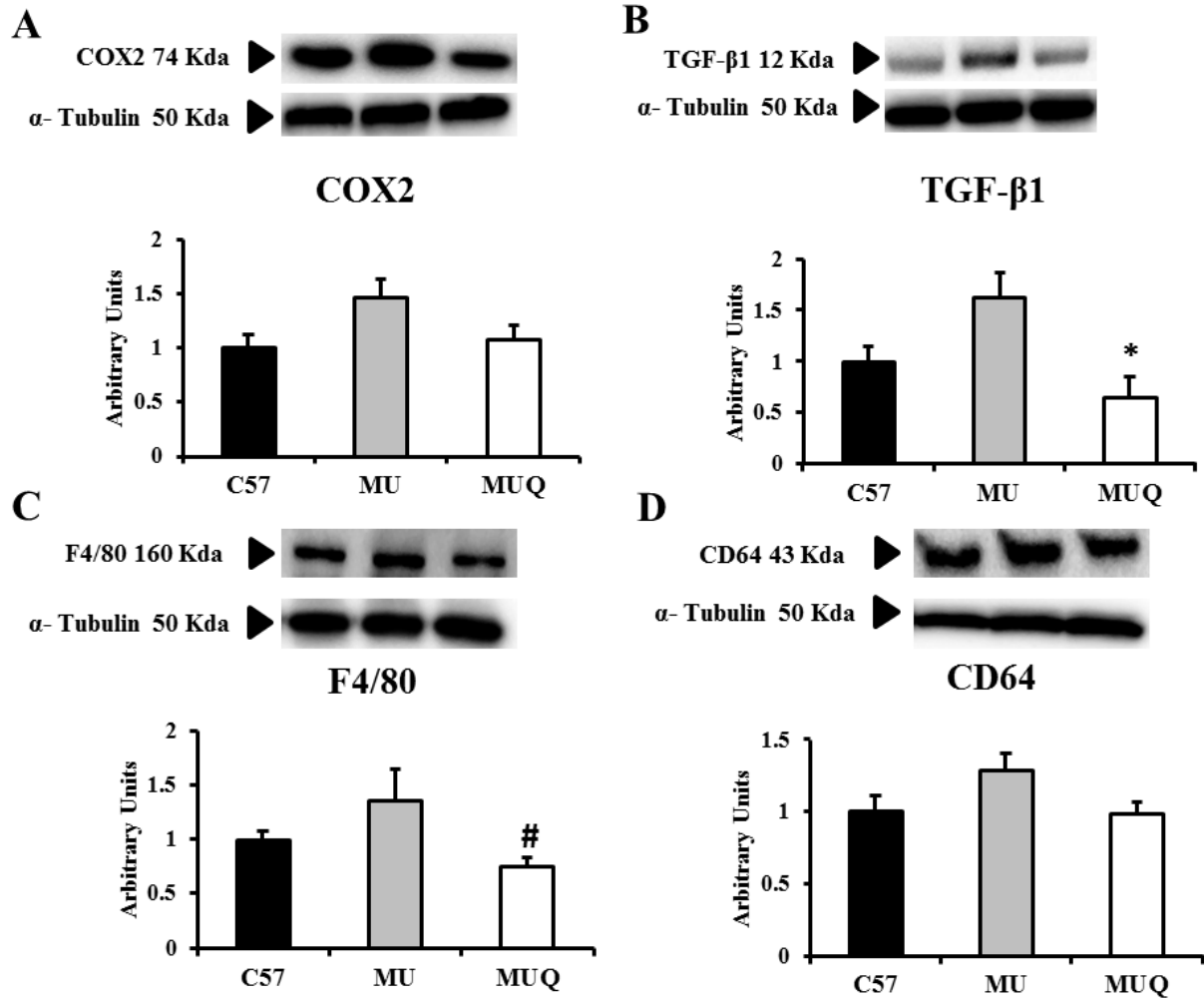


P-IκBα



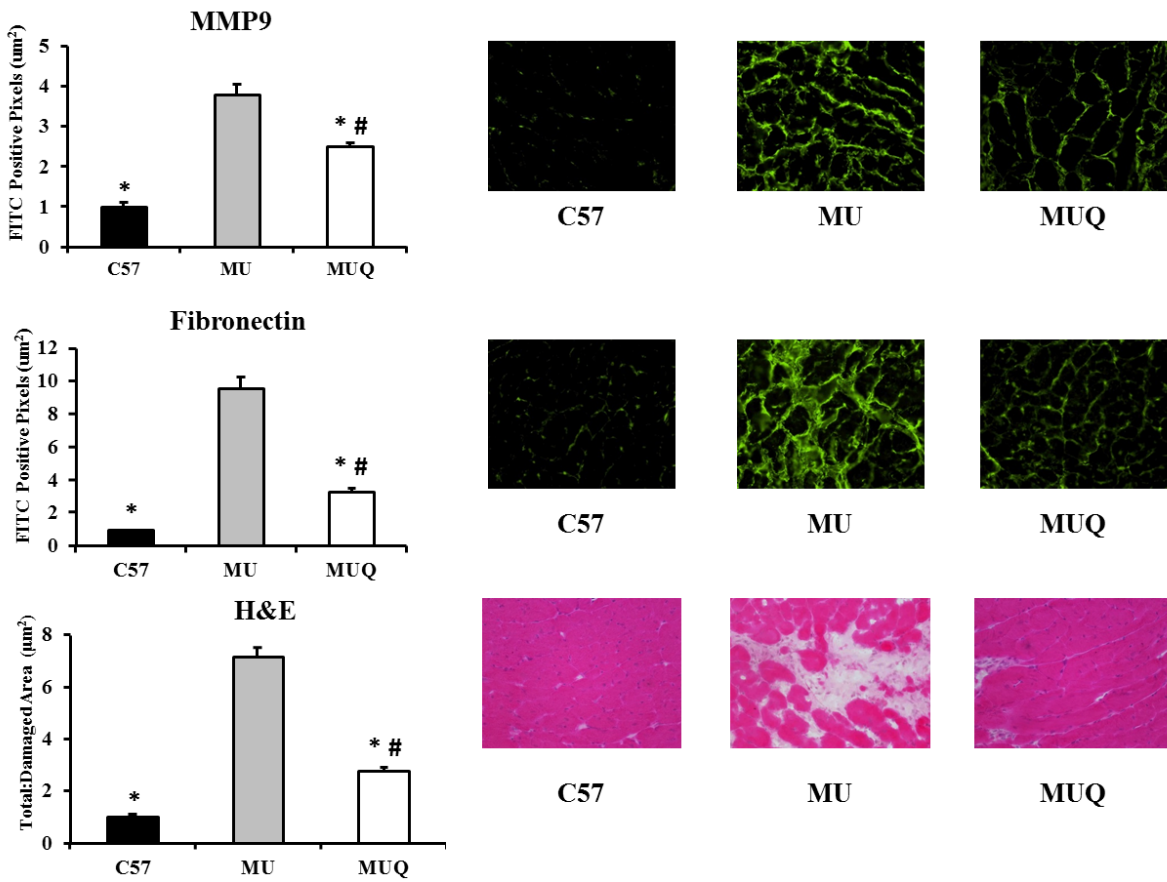
Quercetin decreases NFκB abundance in mdx/utrn^{+/-} hearts. Data are means ± SEM. * denotes significantly different from MU.

Figure 6.



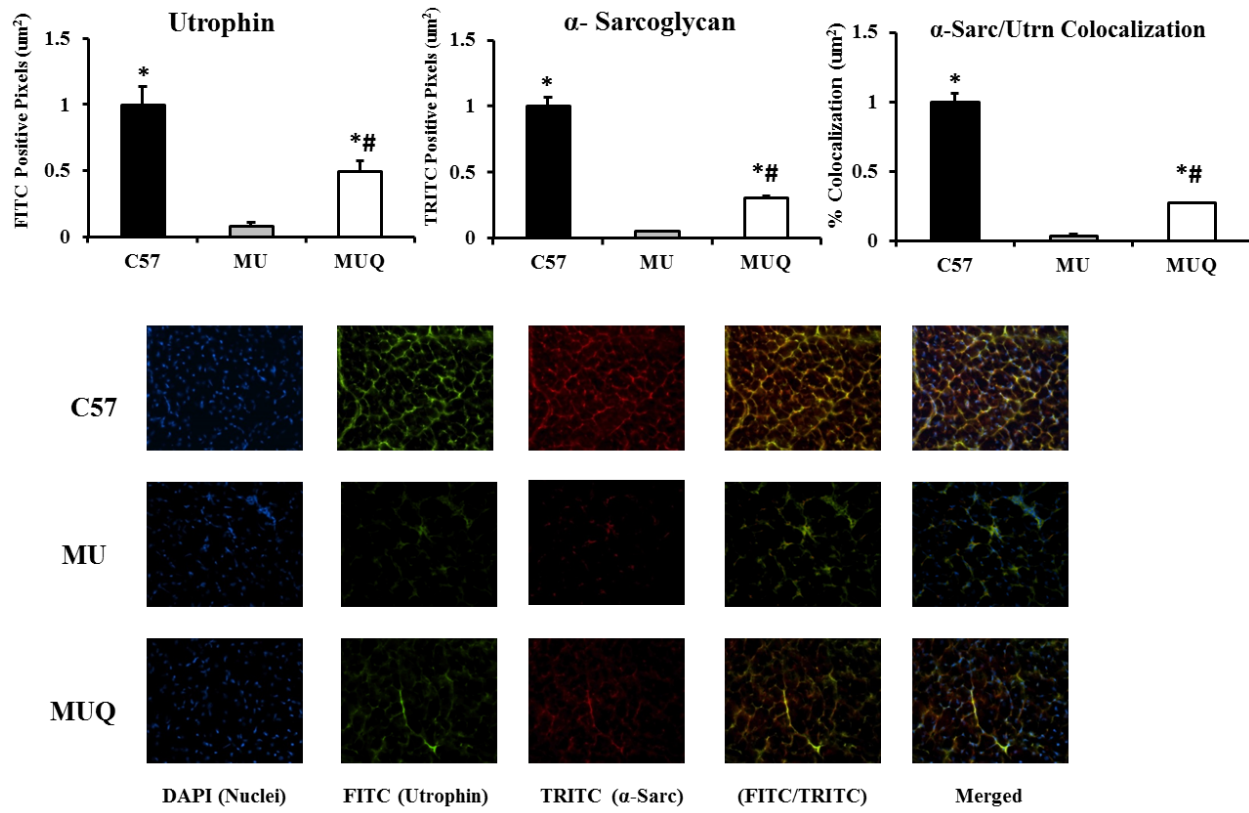
Quercetin decreases TGF- β 1 and F4/80 abundance in mdx/utrn^{+/-} hearts. Data are means \pm SEM. * denotes significantly different from MU. # denotes significantly different from C57.

Figure 7.



Quercetin decreases MMP9, Fibronectin, and cardiac damage in mdx/utrn^{+/-} hearts. Data are means ± SEM. * denotes significantly different from MU. # denotes significantly different from C57.

Figure 8.



Quercetin increases utrophin expression, α - Sarcoglycan expression, and reassembly of DCG in *mdx/utrn*^{+/-} hearts. Data are \pm SEM. * denotes significantly different from MU. # denotes significantly different from C57.

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