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Early Treatment With Lisinopril and Spironolactone Preserves Cardiac and Skeletal Muscle in Duchenne Muscular Dystrophy Mice

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Background—Nearly universal cardiomyopathy in Duchenne muscular dystrophy (DMD) contributes to heart failure and death. Because DMD patients show myocardial fibrosis well before functional impairment, we postulated that earlier treatment using drugs with antifibrotic effect may be beneficial.

Methods and Results—Three groups of 10 *utrn*^{+/-}; *mdx*, or “het” mice, deficient for dystrophin and haploinsufficient for utrophin with skeletal myopathy and cardiomyopathy that closely mimics clinical DMD were studied. One het group received spironolactone and lisinopril starting at 8 weeks of life (het-treated-8); a second received the same starting at 4 weeks of life (het-treated-4), and the third het group was untreated. At 20 weeks, all mice had normal ejection fractions though circumferential strain rate was abnormal (-0.21 ± 0.08) in untreated hets. This improved to -0.40 ± 0.07 in het-treated-8 mice ($P=0.003$) and further improved to -0.56 ± 0.10 in het-treated-4 mice ($P=0.014$ for het-treated-4 versus het-treated-8). Treated mice showed less cardiomyocyte damage, with a 44% reduction in intracardiomyocyte serum immunoglobulin G localization in het-treated-8 mice ($P<0.0001$) and a further 53% reduction in het-treated-4 mice ($P=0.0003$ versus het-treated-8); matrix metalloproteinases were similarly reduced. Cardiac, limb, and diaphragm function by ex vivo muscle testing remained at 80% of normal with early treatment compared to a decline to 40% of normal skeletal muscle function without treatment.

Conclusions—These findings offer clinically available medications with proven antifibrotic effect as a new therapeutic strategy in DMD. Early initiation greatly attenuated myocardial disease and, for the first time with these drugs, improved skeletal myopathy. Thus, early initiation of such agents warrants further clinical evaluation to maintain ambulatory, respiratory, and cardiac function for patients with DMD and related myopathies. (*Circulation*. 2011;124:582-588.)

Key Words: cardiomyopathy ■ muscles ■ aldosterone antagonists

Inherited myopathies produce progressive immobility due to limb muscle degeneration, respiratory failure due to diaphragm involvement, and cardiomyopathy due to myocardial disease.¹ The most common form is Duchenne muscular dystrophy (DMD), an X-linked disorder that leads to absence of the sarcolemmal protein dystrophin and impairs ambulation beginning in children age 3 to 7 years. Duchenne muscular dystrophy patients universally develop cardiomyopathy by the third decade of life, and present guidelines advocate periodic screening with echocardiography.^{2,3} Current guidelines dictate that drugs like angiotensin-converting enzyme (ACE) inhibitors may be started once there is evident left ventricular (LV) systolic dysfunction, particularly re-

duced LV ejection fraction (LVEF).⁴ Use of drugs like aldosterone antagonists that have antifibrotic effects are only advocated for treatment of patients with advanced heart failure.⁴ Recent evidence supports initiation earlier in the progression of cardiac disease albeit still only in symptomatic patients with evident LV dysfunction.⁵

Clinical Perspective on p 588

Our present study was motivated by increasing evidence suggesting that myocardial disease is developing in DMD patients well before LVEF becomes abnormal.⁶ Observing that the early histopathological changes that occur in both skeletal and cardiac muscle invariably leads to fibrosis, we

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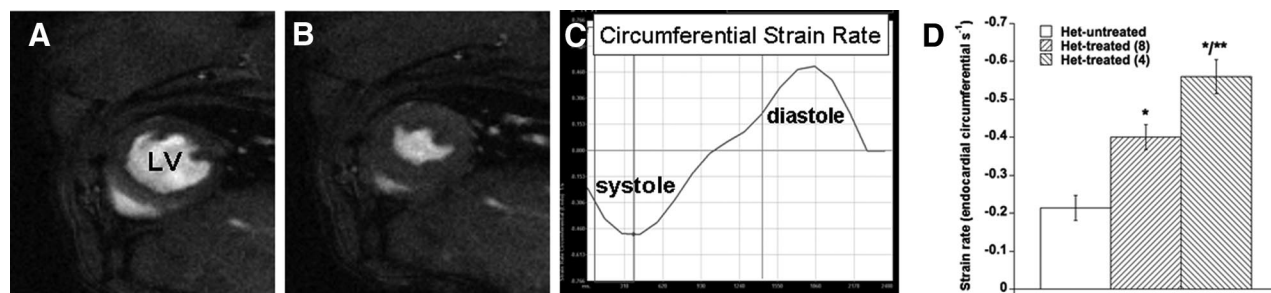


Figure 1. In vivo cine CMR in the het mouse showed normal global systolic function in all mice, treated and untreated (data not shown). **A** and **B**, Comparison of an end-diastolic frame (**A**) to an end-systolic frame (**B**) indicates systolic thickening in all regions, resulting in a normal LVEF. **C**, Measurement of myocardial deformation using vector velocity image segmentation allows measurement of LV circumferential strain rate, a sensitive measure of subtle abnormalities in both systolic and diastolic function. **D**, Despite normal LVEF, untreated het mice had abnormal circumferential strain rate that was significantly improved with treatment initiation at 8 weeks with lisinopril and spironolactone ($P=0.003$ compared with het-untreated mice); this improvement was even more marked if treatment was started at 4 weeks of life ($P=0.014$ comparing het-treated-8 to het-treated-4; $P<0.0001$ het-untreated versus het-treated-4). LV indicates left ventricle; Het-untreated, *utrn*^{+/-};mdx mice untreated with spironolactone and lisinopril; Het-treated (8), *utrn*^{+/-};mdx mice treated with spironolactone and lisinopril at 8 weeks; and Het-treated (4), *utrn*^{+/-};mdx mice treated with spironolactone and lisinopril at 5 weeks.

postulated that drugs with an antifibrotic effect may be most beneficial if started earlier in the disease course. Therefore, we implemented a prospective blinded study to test the hypothesis that early versus late treatment with clinically available ACE inhibitors and aldosterone antagonists provides significantly greater myocardial protection in a mouse model of DMD. In addition, because of (1) the reported antifibrotic properties of spironolactone and lisinopril,^{5,7-9} (2) the absence of any published investigations of these drugs on skeletal muscles in any muscular dystrophy model, and (3) the presence of large amounts of fibrosis in het mice, we decided to include analysis of skeletal muscle effects in addition to cardiac effects of the drug treatment in our studies.

Methods

All protocols were approved by the institutional laboratory animal care and use committee. For this study we used a mouse model deficient for dystrophin that is also haploinsufficient for its partially compensating homolog utrophin, *utrn*^{+/-};mdx “het” mice, because we have previously shown that their skeletal muscle fibrosis is more severe than that of *mdx* mice,¹⁰ making it a more accurate phenotypic model of DMD. Het mice were bred and genotyped as previously described.¹⁰ Groups of 10 of these mice, housed 2 to 3 to a cage, were given water bottles containing 66 mg/L lisinopril (Sigma L 6292) + 250 mg/L spironolactone (Sigma S3378) (dissolved in 0.1% ethanol) in reverse-osmosis water, starting at 4 or 8 weeks of age, or provided reverse-osmosis water only. Medicated water bottles were replaced 3 times per week. Mice were weighed and volume of water consumed was recorded to calculate the average drug dosages for the first several weeks of treatment, which were found to be reported effective dosages for a mouse of 10 mg · kg⁻¹ · d⁻¹ lisinopril and 37.5 mg · kg⁻¹ · d⁻¹ spironolactone.

Using medicated water bottles containing lisinopril and spironolactone (drug treatment), we treated 1 group of het mice starting at 4 weeks of age (het-treated-4, n=10), before onset of apparent cardiac damage and at an early stage of skeletal muscle damage, and a second group of het mice starting at 8 weeks of age (het-treated-8, n=10) when initial functional and histological signs of cardiomyopathy are apparent.^{11,12} We compared these groups to an untreated group (n=10) of het mice (het-untreated) at 20 weeks of age.

One day before they reached 20 weeks of age, injected mice were weighed and underwent cardiovascular magnetic resonance (CMR) on a vertical bore 11.7-Tesla 30-mm bore magnetic resonance imaging system (Bruker Biospin, Ettlingen, Germany) under gas anesthesia with electrocardiographic leads and temperature control maintaining body core temperature at 37°C. Fast low-angle shot

gradient-recalled echo cine imaging was acquired in contiguous short-axis planes covering the left ventricle. From short-axis cines, endocardial borders at end diastole and end systole were automatically delineated using dedicated software for CMR analysis (Segment, Medviso AB; Lund, Sweden), allowing computation of LVEF from end-diastolic volumes (EDV) and end-systolic volumes (ESV): LVEF=(EDV-ESV)/EDV, %. Myocardial strain and strain rate were computed from apical, mid, and basal short-axis cines using vector-based feature-tracking software (Vector Velocity Imaging, Siemens, Mountain View, CA).¹³

One day after in vivo CMR examination, ECGs were recorded and the mice were then euthanized. In vitro cardiac muscle function was assessed as previously described in detail for a related mouse model.¹² Briefly, small, linear, multicellular preparations were dissected and electrically stimulated to contract under near-physiological conditions. In addition to baseline function, the main 3 mechanisms (length-dependent activation, frequency-dependent activation, and β -adrenergic stimulation) that regulate in vivo myocardial force development were assessed. Isolated strips of diaphragm muscle were assessed on their force-generating ability whereas maximal tetanic strength of the *extensor digitorum longus* muscle as well as its susceptibility to repetitive eccentric stress were assessed as previously described.^{14,15}

The remainder of the heart tissue and skeletal muscles including diaphragm and quadriceps were embedded in optimal-cutting-temperature medium and frozen on liquid-nitrogen cooled isopentane for subsequent histological analyses. Eight μ m cryosections were stained by hematoxylin and eosin by standard methods or stained for intracellular immunoglobulin G (IgG). IgG immunostaining was performed using a CY3-conjugated antimouse IgG antibody (1:100) (Jackson Immunoresearch Laboratories 115-165-146) as previously described¹¹ with or without costaining with anti-Collagen I or anti-ERTR7 antibodies (Abcam ab7778 and ab51824, respectively). The percentage of IgG-stained pixels in composites of photomicrographs that represented the majority of heart or quadriceps sections from each mouse were quantified using Image J. For in situ zymography, heart cryosections were fixed and incubated for 9 hours at 37°C with a solution of gelatin conjugated to Oregon Green 488 (Invitrogen Molecular Probes) then washed with 10 mmol/L EDTA to stop activity. Summary values are presented as mean \pm SE. One-way ANOVA followed by posthoc test using Bonferroni adjustment, where applicable, was used to determine statistically significant differences.

Results

Cardiovascular magnetic resonance showed normal LVEF in all 3 groups. Strain analysis, however, showed measurable differences in both systolic and diastolic function (Figure 1);

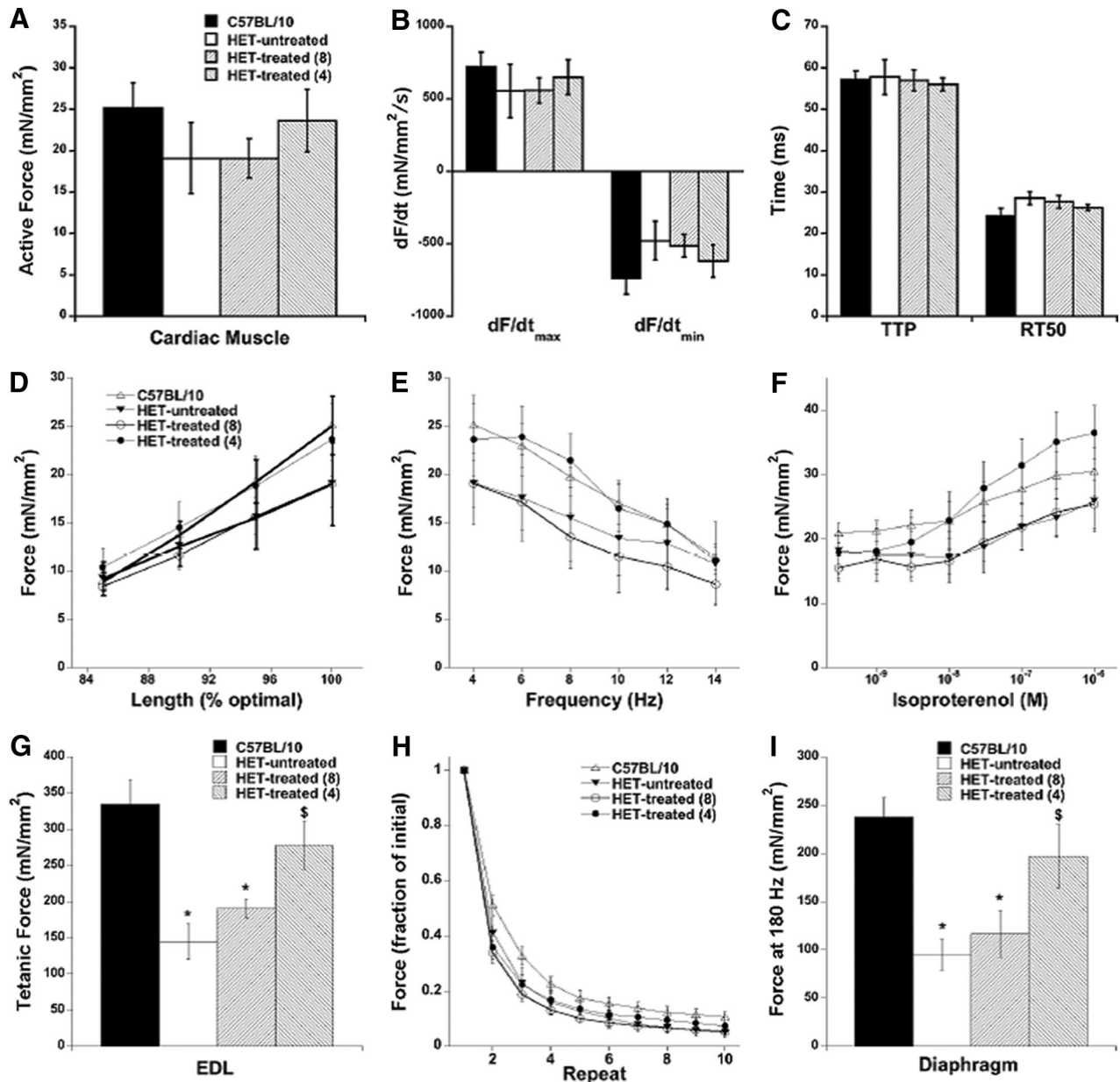


Figure 2. In vitro muscle physiology showed that compared to untreated het mice, trabeculae isolated from het-treated-4 mice trended to improve active developed force at 4 Hz, 37°C (A), and improve maximal rates of contraction and relaxation (B) and faster timing kinetics (C). Cardiac regulatory systems relative to length dependency (D), frequency dependency (E), and β -adrenergic responsiveness (F) were assessed similarly as previously reported¹² and trended to be normalized to levels in control mice. In *extensor digitorum longus* muscles, specific force (150 Hz tetanus for 500 ms at 30°C) was significantly improved on treatment (G), and resistance to eccentric contractions was also improved (H). In isolated diaphragm muscle (180 Hz tetanus for 500 ms at 37°C), specific force development was also significantly improved (I). n=6 to 9 per group, * P <0.05 versus C57BL/10 control, \$ P <0.05 versus het-untreated animals. Het-untreated indicates *utrn*^{+/-};*mdx* mice untreated with spironolactone and lisinopril; Het-treated (8), *utrn*^{+/-};*mdx* mice treated with spironolactone and lisinopril at 8 weeks; and Het-treated (4), *utrn*^{+/-};*mdx* mice treated with spironolactone and lisinopril at 4 weeks; dF/dt, maximal rate of contraction/relaxation; TTP, time to peak tension; RT50, time from peak tension to 50% relaxation; and EDL, *extensor digitorum longus*.

these were more significant in basal LV segments compared to the apex, similar to DMD cardiomyopathy in humans that initially demonstrates abnormalities in the base versus the apex of the LV.¹⁶ Mean systolic circumferential strain rate measured in a short-axis cine view at the base of the heart was -0.21 ± 0.08 in untreated het mice, improved to -0.40 ± 0.07 in het mice whose treatment was initiated at 8 weeks of life, and improved even more to -0.56 ± 0.10 in het mice whose treatment was initiated at 4 weeks of life ($P=0.003$ for

het-untreated versus het-treated-8; $P<0.0001$ for het-untreated versus het-treated-4, and $P=0.014$ for het-treated-4 versus het-treated-8). Diastolic strain measurement showed similar improvements: 0.24 ± 0.09 in untreated hets, 0.42 ± 0.10 in hets whose treatment began at 8 weeks, and 0.59 ± 0.08 in hets whose treatment began at 4 weeks ($P=0.007$ for het-untreated versus het-treated-8, $P<0.0001$ for het-untreated versus het-treated-4, and $P=0.012$ for het-treated-4 versus het-treated-8).

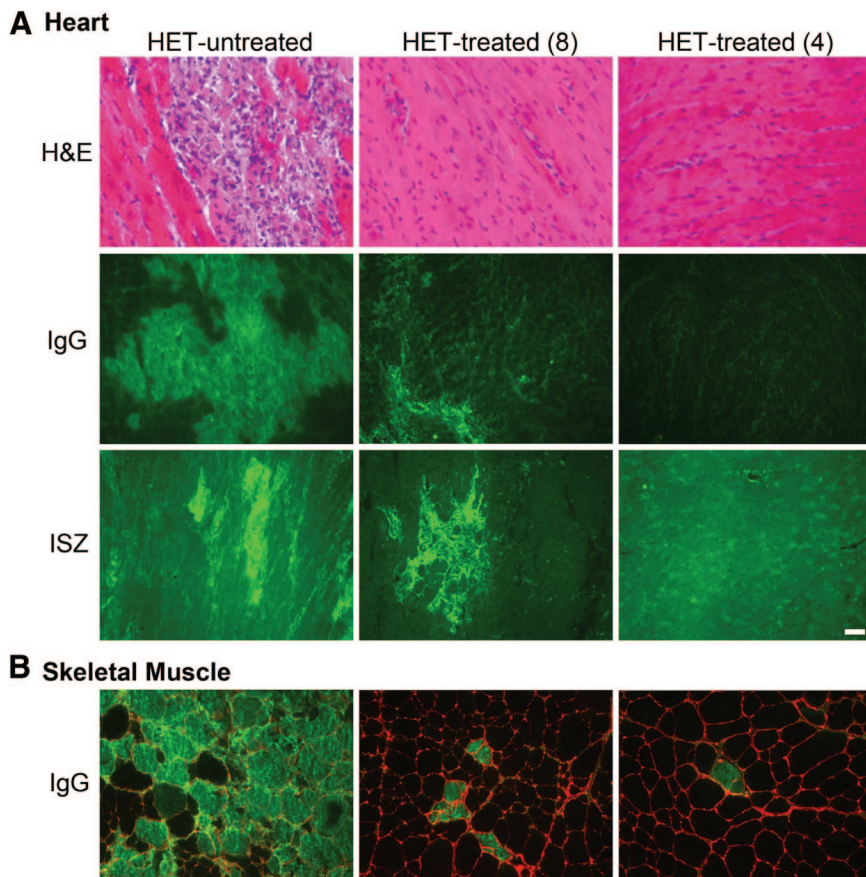


Figure 3. Drug treatment improves histological parameters of heart and skeletal muscles. **A**, Hematoxylin and eosin (H&E)-stained LV sections show the cardiac damage prevalent throughout het-untreated hearts that is almost completely prevented in both treatment groups. Intracellular localization of mouse IgG (green) indicates damaged myocardium that is significantly attenuated in het-treated-8 and even further improved in het-treated-4 hearts. Gelatinase in situ zymography (ISZ) shows the combined activity of matrix metalloproteinases 2 and 9 (bright green), indicative of ventricular remodeling, that is also attenuated in the het-treated-8 hearts and almost entirely prevented in the het-treated-4 hearts. **B**, Immunoglobulin G localization (green) in quadriceps skeletal muscle sections indicates a profound and significant reduction of ongoing myofiber damage in the het-treated-4 group, with intermediate effects in the het-treated-8 group compared to untreated hets. Localization of collagen I (red) in the matrix surrounding individual muscle fibers is shown to demonstrate the intracellular localization of the IgG staining. Bar=50 μ m. Het-untreated indicates *utm*^{+/-};*mdx* mice untreated with spironolactone and lisinopril; Het-treated (8), *utm*^{+/-};*mdx* mice treated with spironolactone and lisinopril at 8 weeks; and Het-treated (4), *utm*^{+/-};*mdx* mice treated with spironolactone and lisinopril at 4 weeks; H&E, hematoxylin and eosin; IgG, immunoglobulin G; and ISZ, in situ zymography.

Electrocardiographic recordings obtained in conscious unrestrained mice showed no significant difference in heart rate among C57BL/10 controls, het-untreated, het-treated-8 and het-treated-4 mice ($P=0.19$), nor were there significant differences across groups in QT interval ($P=0.31$).

In vitro cardiac muscle force and response to isoproterenol also showed a clear trend toward improvement in the het-treated-4 group (Figure 2A through F) at 20 weeks of age compared to untreated het mice. The profound cardiac dam-

age present in untreated het mice was almost completely prevented by the drug treatment in both groups of treated mice (Figure 3A). Degenerating cardiomyocytes, as detected by intracellular serum IgG localization were prevalent throughout the left ventricle of untreated het mice (Figure 3A). These were reduced 44% in het-treated-8 mice ($P<0.0001$ versus het-untreated mice) and showed a further 53% reduction from these levels in the het-treated-4 group ($P<0.0001$ versus het-untreated mice; $P=0.0003$ versus het-

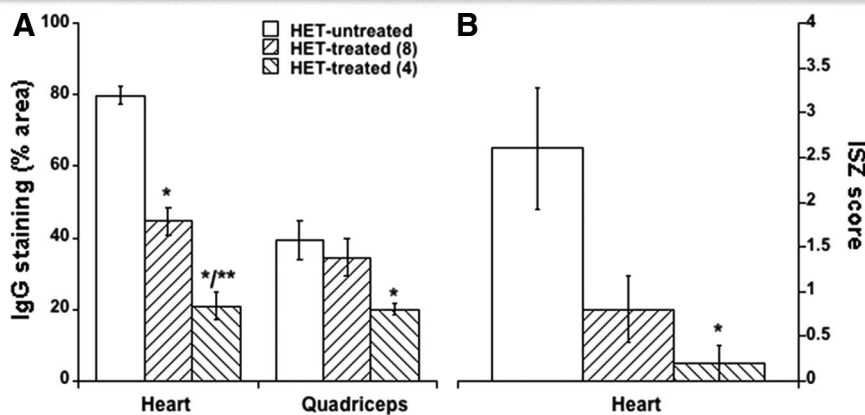


Figure 4. A, The average percentage (\pm SE) of section area stained for mouse IgG for heart (left) and quadriceps skeletal muscles from het-untreated and treated groups shows significant reductions in ongoing muscle damage. **B**, Blinded visual scoring of gelatinase activity from in situ zymography supports reductions of matrix metalloproteinase remodeling in treated groups. * $P<0.03$ versus untreated, ** $P<0.05$ versus 8-week. Panel **A**, $n=7$ to 10 per group. Panel **B**, $n=5$ per group. IgG indicates immunoglobulin G; Het-untreated, *utm*^{+/-};*mdx* mice untreated with spironolactone and lisinopril; Het-treated (8), *utm*^{+/-};*mdx* mice treated with spironolactone and lisinopril at 8 weeks; and Het-treated (4), *utm*^{+/-};*mdx* mice treated with spironolactone and lisinopril at 8 weeks; and ISZ, in situ zymography.

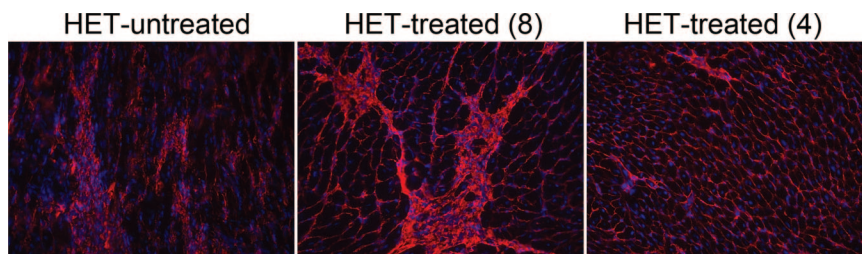


Figure 5. Immunolocalization (red) with an antibody against the fibroblast marker ER-TR7 in heart sections shows that the mononuclear cell infiltrate (nuclei stained blue) in damaged regions of het-untreated cardiac muscle is mostly composed of fibroblasts. The less frequent areas of damage in the het-treated-8 group still contain fibroblasts, suggesting that the drug treatment is not acting directly on fibroblast proliferation or migration. Because very few areas of cardiac damage are present in het-treated-4 hearts, clusters of activated fibroblasts are not typically present. Het-untreated indicates *utrn*^{+/-};*mdx* mice untreated with spironolactone and lisinopril; Het-treated (8), *utrn*^{+/-};*mdx* mice treated with spironolactone and lisinopril at 8 weeks; and Het-treated (4), *utrn*^{+/-};*mdx* mice treated with spironolactone and lisinopril at 4 weeks.

treated-8) (Figure 4). Drug treatment also prevented matrix metalloproteinase gelatinase activity, a key indicator of ventricular remodeling (Figure 3) and the infiltration of activated fibroblasts that secrete both matrix metalloproteinases and collagen (Figure 5).

Surprisingly, het mice started on drug treatment at 4 weeks of age showed a dramatic improvement in both diaphragm and *extensor digitorum longus* muscle function to 80% of isogenic normal (C57BL/10) control muscle force compared to 40% of normal force in untreated het mice, (Figure 2G, *extensor digitorum longus*: $P=0.0006$ C57BL/10 versus het-untreated mice; $P=0.0067$ C57BL/10 versus het-treated-8; $P=0.013$ het-untreated versus het-treated-4; Figure 2I, diaphragm: $P=0.0017$ C57BL/10 versus het-untreated; $P=0.0086$ C57BL/10 versus het-treated-8; $P=0.044$ het-untreated versus het-treated-4).

Limb muscles demonstrated a similar reduction of ongoing muscle degeneration as shown in the heart (Figure 3B), with a 2-fold reduction in the het-treated-4 group ($P=0.025$ het-untreated versus het-treated-4) (Figure 4). However, gross limb muscle histopathology was similar in all groups. Despite the profound functional improvement in diaphragm conferred by early drug treatment initiation in het-treated-4 muscles, the diaphragm showed no obvious improvement in any histopathological parameter assessed (not shown).

Discussion

We show for the first time in a mouse model of DMD a remarkable protective effect on both cardiac and skeletal muscle of very early initiation of treatment with an aldosterone antagonist plus an ACE inhibitor. Treatment was initiated well before evident cardiac dysfunction, and even untreated animals at 20 weeks of life still showed preserved EF despite extensive myocardial fibrosis and injury. Despite genetics that would otherwise dictate inevitable cardiomyopathy and skeletal muscle disease, early treatment with spironolactone in combination with lisinopril provided near normalization of muscle function and considerable prevention of tissue changes.

Gross histopathology of limb muscle was similar in all groups despite less muscle degeneration by IgG staining and matrix metalloproteinase activity; this may be explained by ongoing damage in dystrophic skeletal muscle at 4 weeks of age when such damage is not yet evident in cardiac muscle. Also, despite dramatic improvement in diaphragm function,

there was no appreciable histopathological improvement in diaphragm. This result is likely due to the well documented crisis period of damage that occurs in dystrophin-deficient mouse diaphragm between 3.5 and 4 weeks of age,¹⁷ before the initiation of treatment in either group, and supports that treatment preceding damage likely results in the most improvement in all parameters.

Numerous pharmacological treatment approaches have been investigated to attenuate the devastating sequelae of cardiomyopathy and skeletal myopathy in DMD, though none before this work have shown such remarkable efficacy for both myocardial and skeletal muscle disease. A recent critical review of the literature listed therapeutics with sufficient evidence for clinical use, and endorsed the use of glucocorticoids that limit decline in muscle strength and function.¹⁸ However, long-term steroid therapy brings a wide range of attendant complications.¹⁹ Further, glucocorticoids have shown inconsistent effects on myocardial disease, ranging from modest protection²⁰ and reduced fibrosis²¹ to accelerated progression²² and even increased fibrosis.²³ Standard heart failure medications such as ACE inhibitors and β -blockers are advocated once LVEF is abnormal by echocardiography, though evidence suggests that changes in myocardial structure and function are well underway even with normal LVEF using more sensitive CMR-based strain and fibrosis imaging techniques.⁶

Mechanistic insights from this work include evidence supporting that the drugs' benefit is likely not mediated via inhibition of fibroblast proliferation and migration. As precise molecular pathways are better defined through ongoing preclinical studies, these results behoove initiation of clinical trials to evaluate the potentially significant therapeutic benefits across a broad range of genetic and acquired cardiomyopathies. Any myocardial disease marked by fibrosis, from hypertensive heart disease to diabetic cardiomyopathy, warrants evaluation of earlier use of aldosterone antagonism than what is presently advocated. Similarly, our findings of these drugs' effect on attenuating damage and maintaining strength of skeletal muscle suggest potential benefit for conditions such as sarcopenia and cachexia where therapeutic options are presently limited.

The threshold doses for lisinopril and spironolactone were not established in this study but were based on therapeutic doses commonly used in other mouse studies and relate to the allometric scaling needed to account for different pharmacoki-

netics between rodents and humans. Clinical trials testing the efficacy of these drugs in DMD patients will likely begin at the comparable per-kilogram body weight doses typically used clinically. Because both lisinopril and spironolactone were administered, we cannot determine from these data if the benefits demonstrated were due to the combination of drugs or spironolactone alone. Ongoing studies with administration of each drug individually should address this question.

In conclusion, combining aldosterone antagonism with ACE inhibition at an extremely early stage of genetically determined myocardial and skeletal muscle disease potentially offers maximal inhibition of tissue injury and fibrosis with superior outcomes. Our work indicates that such a combined approach yields (1) improvements in both skeletal and cardiac muscle histology, unlike prior investigations that showed improvements in one tissue but not in another, and (2) preservation of function to an extent that far exceeds that of any prior pharmacological therapy regimen studied in mouse models of DMD.^{24–26}

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Disclosures

None.

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CLINICAL PERSPECTIVE

Duchenne muscular dystrophy (DMD) is a universally fatal disorder, with cardiomyopathy and skeletal myopathy leading to progressive immobility, respiratory impairment, heart failure, and death. Current treatment options are limited to glucocorticoids that produce inconsistent effects on myocardial disease. Guidelines for management of cardiomyopathy advocate medications such as angiotensin-converting enzyme inhibitors and β -blockers only when left ventricular ejection fraction drops below normal. We tested the hypothesis that early treatment using the angiotensin-converting enzyme inhibitor lisinopril in combination with the aldosterone antagonist spironolactone with proven antifibrotic effect might prevent muscle damage and preserve muscle function. We found that these drugs dramatically improved function of cardiac muscle, leg muscle, and diaphragm in *utrn*^{+/-};mdx or “het” mice whose skeletal myopathy and cardiomyopathy closely mimics clinical DMD. Furthermore, histology demonstrated striking reduction in cardiomyocyte damage. These results offer clinically available medications as a new therapeutic strategy in DMD. For the first time, these historically cardioprotective drugs were shown to protect skeletal muscle as well. These preclinical results suggest that angiotensin-converting enzyme inhibition plus aldosterone antagonism may deliver improved ambulation, respiratory function, and cardiac status for patients with DMD. Further investigations are also warranted to test these drugs’ efficacy in attenuating the skeletal myopathy that accompanies a variety of myocardial disorders.



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