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Losartan Decreases Cardiac Muscle Fibrosis and Improves Cardiac Function in Dystrophin-Deficient Mdx Mice

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Abstract

Recent studies showed that chronic administration of losartan, an angiotensin II type I receptor antagonist, improved skeletal muscle function in dystrophin-deficient mdx mice. In this study, C57BL/10ScSn-Dmd^{mdx}/J female mice were either untreated or treated with losartan (n = 15) in the drinking water at a dose of 600 mg/L over a 6-month period. Cardiac function was assessed via in vivo high frequency echocardiography and skeletal muscle function was assessed using grip strength testing, Digiscan monitoring, Rotarod timing, and in vitro force testing. Fibrosis was assessed using picrosirius red staining and Image J analysis. Gene expression was evaluated using real-time polymerized chain reaction (RT-PCR). Percentage shortening fraction was significantly decreased in untreated (26.9% ± 3.5%) mice compared to losartan-treated (32.2% ± 4.2%; $P < .01$) mice. Systolic blood pressure was significantly reduced in losartan-treated mice (56 ± 6 vs 69 ± 7 mm Hg; $P < .0005$). Percentage cardiac fibrosis was significantly reduced in losartan-treated hearts ($P < .05$) along with diaphragm ($P < .01$), extensor digitorum longus ($P < .05$), and gastrocnemius ($P < .05$) muscles compared to untreated mdx mice. There were no significant differences in skeletal muscle function between treated and untreated groups. Chronic treatment with losartan decreases cardiac and skeletal muscle fibrosis and improves cardiac systolic function in dystrophin-deficient mdx mice.

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Keywords

Duchenne muscular dystrophy; dystrophin; mice; cardiomyopathy; angiotensin; echocardiography

Introduction

Duchenne muscular dystrophy (DMD) is an inherited X-linked disorder with an incidence of 1 in 3500 male births that is due to the absence of dystrophin, a large protein linking the intra-cellular cytoskeleton to the extracellular matrix.[1] Damage to the skeletal muscle is clinically evident first and later these patients develop respiratory and cardiac muscle damage. As the treatment of skeletal and respiratory systems improves, more patients with DMD are dying of cardiomyopathy due to cardiac muscle cell death. Cardiomyopathy now accounts for an estimated 10% to 20% of DMD deaths.[2]

Since the exact molecular etiology is unclear, DMD cardiomyopathy is treated similar to other forms of cardiomyopathy. There has been some success treating DMD cardiomyopathy with angiotensin converting enzyme inhibitors (ACEi). Ramaciotti et al did a retrospective analysis of clinical and echocardiographic data from 50 patients with DMD.[3] Twenty-seven (46%) of these patients developed systolic dysfunction and 10 (43%) of these patients exhibited normal function after treatment with the ACEi enalapril. Of interest, the type of DMD gene mutation did not predict response to enalapril. In another study, Jefferies et al showed that ACEi and/or β-blocker therapy improved cardiac function in 27 of 29 patients.[4] Duboc et al also showed that early treatment with the ACEi perindopril delayed the onset and progression of left ventricular (LV) dysfunction in children with DMD and led to decreased mortality during a 10-year follow-up period.[5,6]

Decreased cardiac function seen in cardiomyopathies stimulates the renin–angiotensin system and leads to the release of angiotensin II (ATII). Among its many actions, ATII is a potent stimulator of the profibrogenic cytokine transforming growth factor β (TGF-β), which stimulates fibrosis.[7] ACE's is modulate the production of ATII by preventing the conversion from angiotensin I to ATII and may benefit cardiac function by limiting the amount of fibrosis and scarring within the myocardium. These drugs are widely used and recommend by the American Heart Association for the prevention and treatment of heart failure.[8]

Losartan, an angiotensin II type 1 receptor blocker, was shown to be effective in preventing aortic aneurysms in mice heterozygous for the fibrillin I allele via modulation of TGF-β.[9] Cohn et al[10] recently reported the effects of losartan in mdx mice after a 6- to 9-month treatment. In this study, the diaphragm showed significantly decreased fibrosis and muscle fiber diameter. Losartan restored in vitro force-frequency in the mdx extensor digitorum longus (EDL) to wild type levels and also showed improved hindlimb grip strength in treated mice.[10] Matsuhisa et al[11] looked at the effects of losartan in BIO14.6 cardiomyopathic hamsters over 20 weeks. Losartan decreased ventricular dilation, myocardial fibrosis, and cardiac dysfunction by inhibiting oxidative stress and they also found upregulation of inducible nitric oxide synthase (iNos/Nos2) in the hamster heart. Losartan was postulated to disrupt NADPH generation of reactive oxygen species (ROS)

and may preserve the cardioprotective redox-signal transduction mediated by other ROS sources, such as activation of endothelial Nos (eNos/Nos3).[11]

Based on these studies, we examined the effects of losartan on cardiac and skeletal muscle function in exercised dystrophin-deficient mdx mice. Mdx mice treated with losartan for 6 months significantly improved cardiac systolic function and significantly decreased cardiac and skeletal muscle fibrosis compared to untreated mdx mice. This study supports a potential role for losartan in the treatment of DMD cardiomyopathy and skeletal muscle fibrosis.

Materials and Methods

Animals

All mice were handled according to the local Institutional Animal Care and Use Committee guidelines under approved protocol DCVAMC #01079. Generally, 10- to 12-week-old female C57BL/10ScSn-Dmd^{mdx}/J (mdx) mice weighing 20 to 30 g were purchased from the Jackson Laboratory (Bar Harbor, Massachusetts). All mice were housed in an individually vented cage system with a 12-hour light-dark cycle and received standard mouse chow and purified water ad libitum. Experimental groups received losartan (Merck and Co, Inc, Whitehouse Station, New Jersey) at a concentration of 600 mg/L in the drinking water. Muscle tissue from age-matched untreated wild type controls (C57BL/10ScSn) was used for histology and real-time polymerized chain reaction (PCR) comparisons.

Treadmill Exercise

The treadmill exercise used a common commercially available apparatus (Columbus Instruments, Columbus, Ohio) that uses a moving belt. Mice were subjected to a 30-minute run on a horizontal treadmill at 12 m/min. The test was performed during the morning hours twice weekly over the course of the 6 months.

Rotarod Test

Rotarod tests were performed as described previously.[12] Each trial was done twice a day, with a 2-hour interval between sessions, for 3 consecutive days. The latency to fall was recorded, and all 6 scores were averaged for each mouse. The averaged data were expressed as latency to fall for each mouse.

Grip Strength Test

Grip strength tests were preformed as previously described.[13] Five successful hindlimb and forelimb strength measurements within 2 minutes were recorded. The maximum values for each day over a 5-day period were used for subsequent analysis. The grip strength measurements were collected in the morning hours over a 5-day period, and the data were normalized to body weight and expressed as kilogram force per kilogram (KGF/kg).

Behavioral Activity Measurement

Open-field activity was measured using an open field Digiscan apparatus (Omnitech Electronics, Columbus, Ohio) as described previously.[14] All mice were acclimated for 60

minutes before actual data collection. Data were collected every 10 minutes over a 1-hour period each day for 4 consecutive days.

In Vitro Skeletal Muscle Force Contraction

Experiments were conducted on the EDL muscle of the right hindlimb of mice. The muscle was isolated and 6-0 silk suture tied securely to the distal and proximal tendon. The muscle was carefully removed from the mouse and placed vertically in a bath containing buffered mammalian Ringer solution (composition in mmol/L: 137 NaCl, 24 $NaHCO_3$, 11 glucose, 5 KCL, 2 $CaCl_2$, 1 $MgSO_4$, 1 NaH_2PO_4 and 0.025 turbocurarine chloride) maintained at 25°C and bubbled with 95% O_2–5% CO_2 to stabilize pH at 7.4. One tendon of the muscle was tied securely to the lever arm of a servomotor/force transducer (model 305B, Aurora Scientific, Ontario, Canada) and the other tendon to a fixed bottom plate in the bath. The EDL muscle was stimulated between 2 stainless steel plate electrodes. The voltage of single 0.2-ms square stimulation pulses was increased until supra-maximal stimulation of the muscle was achieved and the muscle length was adjusted to the length that resulted in maximal twitch force (ie, optimal length for force generation). With the muscle held at optimal length, the force developed during trains of stimulation pulses was recorded, and stimulation frequency was increased until the maximal isometric tetanic force was achieved. The muscle length was measured with calipers and the optimal fiber length calculated by multiplying the optimal muscle length by 0.45, the established fiber length/muscle length ratio for EDL muscle.[15] The muscle mass was weighed after removal of the muscle from the bath. The muscle-specific force, a measure of intrinsic force generation of muscle, was calculated using the maximal isometric force, the muscle mass, and the fiber length according to the following equation:

$$\text{specific force} = \text{maximal isometric force} / (\text{muscle mass} \times [\text{density of muscle tissue} \times \text{fiber length}]^{-1}).$$

$$\text{Muscle tissue density used was } 1.056 \text{kg/L}$$

Echocardiography

Echocardiography was performed as detailed previously using 1% to 2% inhaled isoflurane anesthesia.[16] A blood pressure (BP) cuff was placed around the tail and the tail was then placed in a sensor assembly for noninvasive blood pressure monitoring (SC1000, Hatteras Instruments, Cary, North Carolina) during anesthesia. Qualitative and quantitative measurements were made offline using analytic software (VisualSonics, Toronto, Canada). Electrocardiogram kilohertz-based visualization (EKV) software analysis produced offline reconstruction for simulated 250 to 1000 Hz static and Cine loop images. Echocardiographic measurements included vessel diameters, ventricular wall thickness and chamber size, and blood flow velocities across the atrioventricular and semilunar valves. Modified parasternal long axis EKV loops were also used to measure ejection fraction (EF) via Simpson's method (Simp). M-mode images were used to measure LV chamber sizes and wall thicknesses. Percentage shortening fraction was calculated from M-mode measurements using the leading edge to leading edge method via the formula:

$$\%\,\text{Shortening Fraction}\,(\%\text{SF}) = \text{left ventricular internal diameter}\,(\text{diastole})\,[LVID(d)] - \text{left ventricular internal diameter}\,(\text{systole})\,[LVID(s)]/LVID(d)$$

Histological Evaluations

A portion of each of the dissected muscles (eg, gastrocnemius, EDL, diaphragm, and heart) was kept in formalin for hematoxylin and eosin (H&E) and Sirius Red staining. Paraffin sections and H&E staining were performed by Histoserv, Inc (Germantown, Maryland). Five nonoverlapping representative fields of the tissue were imaged under a light microscope at an objective of 20× and a digital image obtained using computer software (Olympus C.A.S.T. Stereology System, Olympus America Inc, Center Valley, Pennsylvania). The digital images were loaded into Image J (NIH) with additional plug-in to count cells. Two blinded investigators counted the total number of fibers, centralized nuclei, peripheral nuclei, and total number of cells with centralized nuclei per field. Fibers showing degeneration (loss of striations/homogenous appearance of fiber contents) or regeneration (basophilic cytoplasm, large peripheral or central nuclei with prominent nucleoli) and inflammatory foci per field were assessed as described previously.[13]

Myocyte Transverse Cross-Sectional Area

For cell membrane wheat germ agglutinin (WGA) staining, paraffin-embedded tissue sections at the ventricular equator were first deparaffinized and rehydrated by standard procedures. Following antigen retrieval in citric acid buffer (10 mmol/L, pH 6.0), the sections were incubated with Alexa Fluor 594-WGA (10 μg/mL [Invitrogen, Carlsbad, California]) and DAPI (4′-6-diamidino-2-phenylindole, dihydrochloride [Invitrogen, Carlsbad, California]) in blocking solution for 1 hour at room temperature. Seven to ten nonoverlapping randomly selected representative fields were imaged under Leica DM4000B fluorescence microscope at an objective of 40× and a digital image with the scale bar obtained using computer software (LeicaDM4000B, Leica Microsystems Inc, Exton, Pennsylvania). Approximately 65 to 85 cells were measured using Image J (NIH) to determine the myocyte cross-sectional area (CSA) for each mouse heart sample.

Quantification of Fibrosis

Paraffin sections of heart, EDL, gastrocnemius, and diaphragm tissue were stained with picrosirius red (Sigma-Aldrich, St. Louis, Missouri). The tissues were magnified under a light microscope at an objective of 4× and digital images obtained using computer software (Olympus C.A.S.T. Stereology System, Olympus America Inc). These digital images were processed using Image J (NIH), with additional threshold color plug-ins to process jpeg images. Pixels corresponding to the area stained in red were normalized to the total pixel area of the tissue image and the results were expressed as percentage of collagen.

Real-Time Polymerase Chain Reaction

Untreated mdx skeletal (gastrocnemius) and cardiac muscles (ventricular tissue) were compared to age-matched wild type (C57BL10ScSn/J) and losartan-treated mdx tissues. Tissues were frozen in isopentane cooled with liquid nitrogen immediately after the mice were euthanized. A tissue sample was placed into a tube with 1 mL of Trizol and homogenized. The total RNA was isolated and then cleaned using the Qiagen RNeasy Mini kit according to the manufacturer's instructions (Qiagen, Valencia, California). The resulting total RNA was checked on a gel for RNA integrity and quantified using the Nanodrop ND-100 Spectrophotometer (Nanodrop Technologies, Wilmington, Delaware). In all, 300 ng of total RNA was used with the ABI high capacity complementary DNA (cDNA) kit (Applied Biosystems, Foster City, California) to generate cDNA. The cDNA was added to TaqMan 2× Universal PCR mix and the desired TaqMan Gene Expression Assay primer (Applied Biosystems). HPRT1 was used as the control probe. Real-time PCR was performed using the 7900 HT Fast Real-Time PCR system (Applied Biosystems) in the relative quantification ΔΔct mode. Data was analyzed using manufacturer supplied SDS2.2 software.

Statistical Analysis

Normality of each quantitative measurement was confirmed prior to analysis and those not conforming to normality underwent data transformations (percentage fibrosis in gastrocnemius and heart underwent log transformations). Body and organ weights, grip strength, muscle strength, percentage fibrosis, echo, blood pressure, and behavioral measurements were compared between treated and untreated mice using a Student *t* test. The behavioral measurement of vertical activity was analyzed using quantile regression due to nonnormality. The histological parameters total fibers, fibers with centralized nuclei, peripheral nuclei, central nuclei, degenerating and regenerating fibers, and inflammation were analyzed using poisson or negative binomial regression where appropriate. Myocyte CSA was analyzed using the Wilcoxon rank sum nonparametric test. The differences were considered significant at a P value of <.05. Real-time PCR analysis compared $2^{-\Delta\Delta Ct} \pm$ SD (ΔCt) normalized to a HPRT1 control probe. All analyses were performed using Stata V10 (College Station, Texas).

Results

Body and Organ Weights

No significant change in body weights between untreated and losartan-treated mdx mice were noted at the completion of the trial. In losartan-treated mice, we did not see significant differences in the normalized weights of the EDL, gastrocnemius, soleus, and spleen weights, but the heart ($P < .04$) showed a lower normalized weight when compared to the untreated group (eTable 1).

Skeletal Muscle Functional Measurements

No significant differences in normalized forelimb or hindlimb grip strength were found between groups at the end of the trial. There were also no significant differences in RotaRod

latency to fall timing or Digiscan behavioral measurements including horizontal and vertical activity, total distance, and rest time between groups (eTable 2).

In Vitro Skeletal Muscle Force Contraction

There were no significant differences in the muscle force and specific muscle force between groups of mdx mice. Untreated mice had a maximal force of 418 ± 65 mN (milliNewtons) and losartan-treated mice had a value of 395 ± 34 mN ($P = .48$). For specific force, the maximal force normalized for the cross-section of the muscle, untreated mice measured 200 ± 32 mN/mm^2 and losartan-treated mice measured 178 ± 22 mN/mm^2 ($P = .28$).

Quantification of Muscle Fibrosis, Cardiac Myocyte CSA, and Muscle Histology

Significantly decreased fibrosis was found in the cardiac ($P < .05$; Figure 1, Panels A and B), diaphragm ($P < .01$), EDL ($P < .05$), and gastrocnemius ($P < .01$) muscles of losartan-treated mdx mice compared to untreated mdx controls (Table 1). Using independent samples of 3–month-old mdx and wild type mice, we found no significant difference in the amount of cardiac fibrosis (0.37 ± 0.21% vs 0.32 ± 0.24% [non-transformed data]; $P = .69$). We also found a small, but significant, decrease in the CSA of losartan-treated cardiomyocytes compared to untreated hearts (241 ± 5 μm^2 vs 269 ± 16 μm^2; $P < .04$; Figure 1, Panels C and D). There were no significant differences between groups in the histological analysis of skeletal muscle including total fibers per field, fibers with central nuclei per field, total peripheral and central nuclei per field, degenerating or regenerating fibers per field, and areas of inflammation per field (data not shown).

Cardiac Echocardiographic Measurements and Calculations

Using noninvasive high frequency echocardiography images, untreated mdx mice had significantly decreased cardiac function measured via percentage shortening fraction ($P < .01$) and percentage ejection fraction ($P < .03$) compared to losartan-treated mdx mice (Figure 2; Table 2). There were no significant differences in cardiac chamber size, wall thickness, or Doppler velocity measurements.

Noninvasive Blood Pressure Measurements

Mdx mice treated with losartan showed significantly decreased blood pressure. Systolic blood pressure was 69 ± 7 mm Hg in untreated mice ($n = 10$) compared to 56 ± 6 mm Hg in treated mice ($n = 9$; $P = .0004$). Diastolic blood pressure was 43 ± 10 mm Hg in untreated mice compared to 27 ± 5 mm Hg in treated mice ($P = .0005$). Accordingly, mean blood pressure was also significantly reduced in treated mice (52 ± 8 mm Hg vs 36 ± 4 mm Hg; $P < .0001$).

Cardiac and Skeletal Muscle RT-PCR

Comparing untreated mdx cardiac ventricular tissue to wild type mice, there were significant increases in TGF-β ($P = .01$), periostin ($P < .0001$), thrombospondin 1 ($P < .0001$), and TGF-β receptor 1 ($P = .04$) messenger RNA (mRNA) expression (Table 3). When comparing untreated mdx cardiac tissue to losartan-treated mdx cardiac tissue, there were no significant differences for TGF-β, periostin, thrombospondin 1, or TGF-β receptor 1. Also,

no significant differences in the gene expression of NADPH oxidase 4 (Nox4); inducible nitric oxide synthase (Nos2); lysyl oxidase (Lox); TGF-β receptors 2 and 3; toll-like receptors (TLR) 2, 4, 7, and 8; connective tissue growth factor (CTGF); or osteopontin (Spp1) were found between untreated and losartan-treated mdx cardiac tissue (eTable 3).

Comparing untreated mdx gastrocnemius to wild type mice, there was a significant increase in TGF-β ($P < .001$), periostin ($P < .0001$), and thrombospondin 1 ($P = .04$) mRNA levels (Table 3). There was no significant difference found comparing TGF-β receptor 1 mRNA expression. When comparing untreated mdx gastrocnemius to losartan-treated mdx gastrocnemius, there were no significant differences for TGF-β, periostin, thrombospondin 1, or TGF-β receptor 1 mRNA levels.

Discussion

This is the first study to look at the effects of chronic losartan treatment in the heart of dystrophin-deficient mice. After treating mdx mice with losartan for 6 months, we found that it significantly reduced blood pressure levels, prevented the decrease in cardiac function found in untreated mdx mice, and significantly decreased the amount of fibrosis seen in cardiac and skeletal muscles compared to untreated mdx mice.

Using high frequency echocardiography, we showed that losartan-treated mice maintained a shortening fraction of 32% while untreated mdx mice decreased to 27% ($P = .006$). These measurements were validated by an evaluation of ejection fraction using endocardial border tracing in systole and diastole in a modified parasternal long axis ($P = .021$). This mild cardiomyopathy seen in mdx mice at 9 months of age was previously shown by our laboratory and is consistent with data from other groups.[16,17] Chronic treatment with losartan over 6 months prevented this decline in systolic cardiac function.

Noninvasive blood pressure measurements showed that afterload was significantly reduced ($P < .0006$) in losartan-treated mdx mice. The significant decrease in afterload produced via antagonism of ATII type I receptors is likely a main mechanism involved in the improvement of cardiac function. Due to the lack of dystrophin, the mdx myocardium is more sensitive to stress and increased afterload. The chronic after-load reduction provided by 6 months of losartan treatment indirectly minimizes myocardial injury and subsequent fibrosis. A previous study by Bauer et al showed that an 8-week treatment with the angiotensin-converting enzyme captopril improved cardiac contractility indirectly via decreased after-load using in vivo ventricular catheters.[18] Losartan treatment also significantly decreased cardiac fibrosis, heart weight, and myocyte CSA. Percentage fibrosis was 2.1% for losartan-treated mdx mice compared to 1.5% for untreated mice (log transformed data; $P = .04$). Cardiac fibrosis is known to increase in the mdx mouse over time and previous studies have documented fibrosis from age 9 months onward.[16,17,19–21] Losartan decreased cardiac fibrosis in other mouse models of dilated and hypertrophic cardiomyopathy.[22,23] Losartan also decreased myocyte CSA in previous cell culture, mouse and rat models.[24–26] Treatment in this study began at 3 months of age and there is no significant difference in cardiac fibrosis at this age between mdx and wild type mice. Therefore, this study does not delineate whether losartan modifies pathways that initiate

fibrosis, decreases pathways of ongoing fibrosis, or modulates other factors, such as afterload, that can lead to fibrosis.

We studied mRNA expression levels to determine whether any specific pathways of fibrosis were activated in the mdx heart. We found significantly increased TGF-β, CTGF, TSP1, and periostin mRNA expression in mdx cardiac tissue compared to wild type, but found no differences between losartan and untreated mdx mice. We also interrogated other pathways that may have been modified by losartan treatment. We found no differences in the expression levels of TLR2, 4, 7, 8 or Spp1.[27–30] We showed no significant differences in mRNA expression of Nox4 or Nos2 between treated and untreated mdx mice and no significant differences in protein levels of Nos2 or Nos3 (data not shown). There are also other pathways involved in TGF-β signaling that we did not interrogate. These include TAK1, mitogen-activated protein (MAP) kinase, Rho-like GTPase and PI3K/AKT pathways.[31] Further studies are needed to fully understand cardiac TGF-β genetic pathways and post-translational modifications occurring in dystrophin-deficient cardiac tissue.

In this study, mdx were exercised to increase muscle pathology over the 6-month period. Exercise was shown to increase the heart–to-body weight ratio and histopathology markers including interstitial fibrosis, adipose tissue, and inflammatory cells.[32] Our study shows that losartan decreased the normalized heart weight and cardiac fibrosis of exercised mice, but we did not see any significant differences in inflammatory cell infiltrates between treated and untreated mdx mice (eTable 1). Drug effects on exercised muscle are quite important. Townsend et al showed that mdx mice with a mini-dystrophin transgene increased their exercise compared to nontransgene mdx mice and developed cardiomyopathy at a younger age.[33] The authors postulated that the increased exercise may lead to increased stress on the myocardium and worsening cardiomyopathy. This is an important consideration as researchers focus on skeletal muscle treatment without fully understanding the effects of improved skeletal muscle function on cardiac muscle in dystrophy patients.

Losartan was previously shown to improve mdx skeletal muscle function.[10] Our results showed no significant differences. This trial included forced exercise and this may have lead to skeletal muscle functional changes that were unable to be ameliorated by this losartan dosing. Although losartan dose and length of treatment were similar in the 2 studies, Cohn et al reported greater decreases in skeletal muscle and diaphragm fibrosis in losartan-treated mice.[10] This study also found significant decreases in the gastrocnemius and EDL muscles. These decreases were smaller than Cohn et al found and may have been unable to significantly improve skeletal muscle strength and force measurements.

This study is limited by the small numbers of samples in some analyses. Although we used losartan dosing and treatment duration consistent with a previous study, the mice in this trial did not show a similar degree of significance in fibrosis results and we did not find any significant differences in skeletal muscle function. Perhaps, the actual drug amounts ingested by these mice was less than that of the previous trial or delivery was suboptimal, even though a pretest evaluation showed equal amounts of losartan(+) and losartan(−) water were ingested by mice. Further investigations are needed to better understand the effects of exercise and fibrosis on skeletal muscle function assessments. Although this study confirms

the ability of losartan to decrease fibrosis, we could not confirm molecular changes at the mRNA level in fibrotic pathways.

Conclusion

This study shows that losartan treatment decreased afterload, muscle fibrosis, and prevented cardiomyopathy in the dystrophin-deficient mouse. Further testing is needed to better understand the mechanisms involved in losartan therapy, but this preclinical trial supports further investigation of losartan therapy in patients with DMD.

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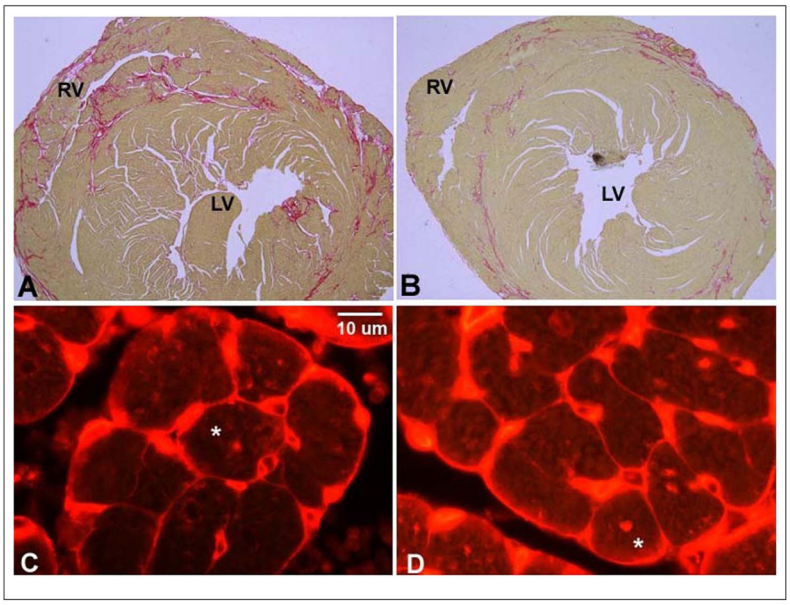

Figure 1.

Significantly decreased fibrosis and myocyte cross-sectional area in the hearts of losartan-treated mdx mice compared to untreated mdx mice. Panel A shows a cross-section of an untreated mdx heart with the left ventricle (LV) and right ventricle (RV) labeled. The section is stained with picrosirus red, which colors collagen red. The image corresponds to 12.5% fibrosis (not log transformed data). Panel B shows a cross-section of a losartan-treated mdx heart at a similar level to Panel A. The image corresponds to 5.6% fibrosis (not log transformed data). Panel C is a representative image of untreated mdx left ventricular tissue with WGA staining. Measurement of the myocyte cross-sectional area (representative cells marked with *) shows increased area compared to losartan-treated mdx left ventricular tissue (Panel D).

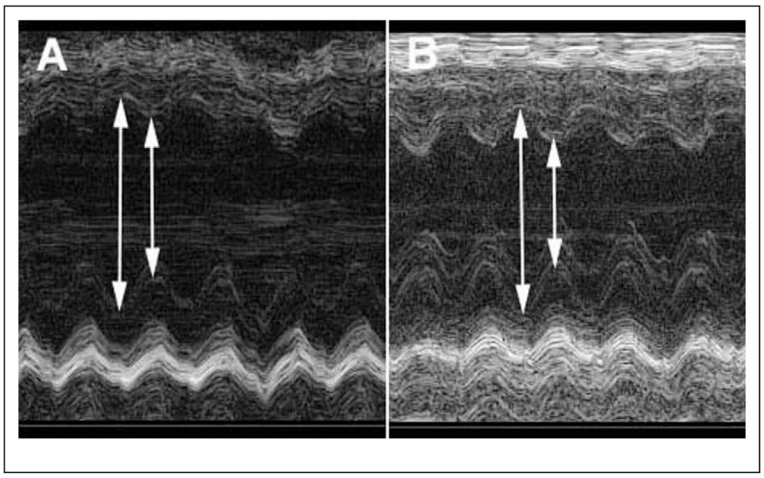

Figure 2.

High frequency echocardiography M-mode images of untreated (A) and losartan-treated (B) hearts. The arrows on the left in each panel show the left ventricular internal diameter (LVID) in diastole and the arrow on the right shows the LVID in systole. Panel A corresponds to a percentage shortening fraction (%SF) of 24% and panel B corresponds to a %SF of 31% (%SF = LVID diastole – LVID systole/LVID diastole).

Table 1

Significant Decreases in Percentage Fibrosis Are Seen in Cardiac and Skeletal Muscle of Losartan-Treated mdx Mice Compared to Untreated mdx Mice at 9 Months of Age

Percentage Fibrosis	Untreated (N = 8) Mean ± SD	Losartan (N = 8) Mean ± SD	*P* Value
Heart[a,b]	2.12 ± 0.20	1.51 ± 0.42	.044
Diaphragm	38.07 ± 7.88	26.91 ± 6.31	.007
EDL	10.47 ± 1.70	8.03 ± 2.62	.041
Gastrocnemius[a]	2.45 ± 0.26	1.98 ± 0.29	.003

NOTE: EDL = extensor digitorum longus.

[a] Log transformation of data performed due to nonnormality of measurements.

[b] Untreated N = 5.

Table 2

Losartan Treatment Significantly Prevents the Decrease in Percentage Shortening Fraction and Ejection Fraction Using High-Frequency Echocardiography in 9-Month-Old mdx Mice

Parameter	Untreated (N = 11) Mean ± SD	Losartan (N = 9) Mean ± SD	*P* Value
%Fractional shortening	26.86 ± 3.49	32.23 ± 4.22	.006
%Ejection fraction	52.25 ± 5.35	59.46 ± 7.42	.021
LVID (d; mm)	3.72 ± 0.23	3.68 ± 0.32	.705
LVPW (d; mm)	0.76 ± 0.10	0.72 ± 0.20	.551
MPI	0.77 ± 0.24	0.67 ± 0.15	.285
Heart rate (bpm)	413.4 ± 39.4	389.3 ± 39.6	.190
Ao Vmax (mm/s)	888.7 ± 79.4	813.4 ± 139.4	.155
PA Vmax (mm/s)	676.5 ± 63.7	674.4 ± 33.8	.930
MV Vmax (mm/s)	628.5 ± 55.6	588.9 ± 89.0	.240
TV Vmax (mm/s)	373.4 ± 37.1	388.4 ± 56.4	.483

NOTE: Ao = aorta; bpm = beats per minute; LVID(d) = left ventricle internal diameter in diastole; LVPW(d) = left ventricular posterior wall thickness in diastole; MPI = myocardial performance index; MV = mitral valve; PA = pulmonary artery; TV = tricuspid valve.

Table 3

RT-PCR Results Showing Significant Up-Regulation of TGF-β, Periostin and Thrombospondin-1 mRNA in the Heart and Gastrocnemius of Untreated and Losartan Treated mdx Mice Compared to Untreated Wild Type Controls[a]

	Group	Mean ± SD	*P* Value	Group	Mean ± SD	*P* Value	Group	Mean ± SD	*P* Value
Heart									
TGF-β	wt	1.0 ± 0.52	.01	wt	1.0 ± 0.52	.005	Mdx untx	1.0 ± 0.78	.98
	Mdx untx	2.74 ± 0.78		Mdx los	2.72 ± 0.60		Mdx los	0.99 ± 0.60	
Periostin	wt	1.0 ± 0.82	<.0001	wt	1.0 ± 0.82	.04	Mdx untx	1.0 ± 0.87	.74
	Mdx untx	7.83 ± 0.87		Mdx los	2.43 ± 0.76		Mdx los	0.80 ± 0.76	
Thrombospondin-1	wt	1.0 ± 0.88	<.0001	wt	1.0 ± 0.88	<.0001	Mdx untx	1.0 ± 1.09	.18
	Mdx untx	16.3 ± 1.10		Mdx los	46.3 ± 2.15		Mdx los	2.84 ± 2.15	
TGF-β receptor -1	wt	1.0 ± 0.16	.04	wt	1.0 ± 0.16	.2	Mdx untx	1.0 ± 0.34	.69
	Mdx untx	1.49 ± 0.34		Mdx los	1.9 ± 1.3		Mdx los	1.28 ± 1.32	
Gastrocnemius									
TGF-β	wt	1.0 ± 1.39	.0004	wt	1.0 ± 1.39	.0004	Mdx untx	1.0 ± 0.28	1.0
	Mdx untx	5.91 ± 0.28		Mdx los	6.09 ± 0.44		Mdx los	1.03 ± 0.44	
Periostin	wt	1.0 ± 1.83	<.0001	wt	1.0 ± 1.83	<.0001	Mdx untx	1.0 ± 0.39	.3
	Mdx untx	10.9 ± 0.39		Mdx los	16.6 ± 0.89		Mdx los	1.52 ± 0.89	
Thrombospondin-1	wt	1.0 ± 1.25	.04	wt	1.0 ± 1.25	.05	Mdx untx	1.0 ± 0.66	.8
	Mdx untx	2.88 ± 0.66		Mdx los	2.62 ± 0.40		Mdx los	0.91 ± 0.40	
TGF-β receptor-1	wt	1.0 ± 2.79	.7	wt	1.0 ± 2.79	.9	Mdx untx	1.0 ± 1.89	.17
	Mdx untx	0.37 ± 1.89		Mdx los	0.97 ± 0.73		Mdx los	2.6 ± 0.73	

NOTE: Los = losartan; RT-PCR = real-time polymerized chain reaction; untx = untreated; wt = wild type (C57BL10/ScSnJ).

[a]No significant differences were seen between losartan-treated and untreated mdx mice. Mean equals $2^{-\Delta\Delta Ct}$.