**Uniform low-level dystrophin expression in the heart partially preserved cardiac function
in an aged mouse model of Duchenne cardiomyopathy**

Nalinda B. Wasala¹, Yongping Yue¹, Jenna Vance¹, Dongsheng Duan^{1-4, *}

1, Department of Molecular Microbiology and Immunology, School of Medicine, The University of Missouri, Columbia, MO 65212, USA

2, Department of Neurology, School of Medicine, The University of Missouri, Columbia, MO 65212, USA

3, Department of Bioengineering, The University of Missouri, Columbia, MO 65212, USA

4, Department of Biomedical Sciences, College of Veterinary Medicine, The University of Missouri, Columbia, MO 65212, USA

*Corresponding Address: Dongsheng Duan Ph.D.
Professor
Department of Molecular Microbiology and Immunology
One Hospital Dr.
Columbia, MO 65212
Phone: 573-884-9584
Fax: 573-882-4287
Email: duand@missouri.edu

Abstract

Dystrophin deficiency results in Duchenne cardiomyopathy, a primary cause of death in Duchenne muscular dystrophy (DMD). Gene therapy has shown great promise in ameliorating the cardiac phenotype in mouse models of DMD. However, it is not completely clear how much dystrophin is required to treat dystrophic heart disease. We and others have shown that mosaic dystrophin expression at the wild-type level, depending on the percentage of dystrophin positive cardiomyocytes, can either delay the onset of or fully prevent cardiomyopathy in dystrophin-null mdx mice. Many gene therapy strategies will unlikely restore dystrophin to the wild-type level in a cardiomyocyte. To determine whether low-level dystrophin expression can reduce the cardiac manifestations in DMD, we examined heart histology, ECG and hemodynamics in 21-m-old normal BL6 and two strains of BL6-background dystrophin-deficient mice. Mdx3cv mice show uniform low-level expression of a near full-length dystrophin protein in every myofiber while mdx4cv mice have no dystrophin expression. Immunostaining and western blot confirmed marginal level dystrophin expression in the heart of mdx3cv mice. Although low-level expression did not reduce myocardial histopathology, it significantly ameliorated QRS prolongation and normalized diastolic hemodynamic deficiencies. Our study demonstrates for the first time that low-level dystrophin can partially preserve heart function.

Key words

Duchenne muscular dystrophy, DMD, Duchenne cardiomyopathy, dystrophin, gene therapy, heart, low-level expression, dilated cardiomyopathy, adeno-associated virus, AAV, ECG,

hemodynamics

Introduction

Deficiency of cytoskeletal protein dystrophin leads to Duchenne muscular dystrophy (DMD)[1, 2]. Skeletal muscle related symptoms (such as limited ambulation and respiratory restriction) are observed early on in young DMD patients[3]. While cardiac involvement appears at the later stage of the disease, all patients eventually develop cardiac dysfunction and heart failure causes up to 40% of death[4-6]. Currently, only palliative treatments are available for symptom management. Restoration of dystrophin expression using adeno-associated virus (AAV)-mediated micro/mini-dystrophin gene transfer, exon-skipping and genome editing are promising new approaches to treat DMD[7, 8]. However, these therapies may not restore dystrophin expression to the normal level in patients. An important issue is thus to determine whether low-level dystrophin expression is therapeutically relevant.

Numerous studies have investigated the amount of dystrophin required for treating skeletal muscle disease in mouse models of DMD and in human patients. These studies suggest that homogenous dystrophin expression at 20-30% of the wild-type level in every myofiber can significantly enhance muscle function and reduce muscle pathology [9-13]. Recent studies further suggest that uniform low-level dystrophin expression at even 5% of the normal level can still improve clinical outcome in dystrophic mice [14-17]. In the case of mosaic expression, approximately 50% myofibers have to express dystrophin in order to achieve a mild phenotype in skeletal muscle [18-20].

In contrast to the abundant information on low-level dystrophin expression in skeletal muscle, little is known about the dystrophin level needed for correcting heart disease in DMD. A study in genetically modified mice suggests that expression in 3 to 5% of cardiomyocytes at the

wild-type level (in every dystrophin positive cell) may delay the onset of heart disease [21]. In a different study, Wu et al found that 5% dystrophin positive cells in the heart of adult mdx mice did not improve cardiac histology/baseline functional although mice tolerated dobutamine stress better [22]. We examined female carrier mice and found that normal level dystrophin expression in half of heart cells is sufficient to completely prevent dystrophic cardiomyopathy [23, 24]. While these results have provided critical insight on the percentage of dystrophin positive cells needed for treating cardiac manifestations, it should be noted that in all these studies dystrophin is expressed at the wild-type level in every positive cardiomyocyte. It remains unclear whether sub-physiological expression in a cardiomyocyte can benefit the heart.

We and others have previously shown that mdx3cv mice express marginal level dystrophin in skeletal muscle [14, 15, 25]. This residual level expression significantly enhanced skeletal muscle function although it did not improve histopathology [14, 26]. Mdx3cv mice were generated by Chapman et al using N-ethyl-N-nitrosourea mutagenesis [27]. A point mutation in intron 65 aborts full-length dystrophin expression. However, a slightly truncated $\Delta 65/66$ transcript is generated (**Supplementary Figure 1**). This results in the production of a near full-length dystrophin protein at ~ 5% of the wild-type level [15, 25].

To study the impact of low-level uniform dystrophin expression in the heart, we compared the cardiac phenotype among C57Bl/6 (BL6), mdx3cv and mdx4cv mice. All three strains are on the BL6 background. BL6 and mdx4cv mice are normal and dystrophin-null controls, respectively. The characteristic heart presentation in DMD is dilated cardiomyopathy. We have previously shown that dystrophin-deficient mice do not develop dilated cardiomyopathy until they reach 21 months of age [24, 28]. For this reason, we intentionally conducted our study in aged mice. We detected uniform dystrophin expression at ~3.3% of the

wild-type level in the heart of 21-m-old mdx3cv mice. Importantly, we observed significant improvement in some ECG and hemodynamic parameters suggesting low-level dystrophin expression can benefit the heart.

Results

The heart of aged mdx3cv mice expressed low-level dystrophin. We first performed dystrophin immunostaining in the heart (**Figure 1A**). We observed robust, no and very low expression in the heart of BL6, mdx4cv and mdx3cv mice, respectively. To quantify dystrophin expression, we performed whole heart lysate western blot (**Figure 1B**). Serially diluted BL6 heart lysate was used to show band intensity at 5, 25, 50 and 100% of the wild-type levels (**Figure 1B**). As expected, no dystrophin was detected in mdx4cv. Mdx3cv showed a faint band. On quantification, it reached approximately 3.3% of the wild-type level (**Figure 1B**).

Low dystrophin expression in the mdx3cv heart had minimal impact on the expression of utrophin and components of dystrophin-associated glycoprotein (DGC) complex. We have previously found that the hearts of 21-m-old normal BL10 mice and BL10-background dystrophin-null mdx mice had similar levels of utrophin expression on western blot [29]. Consistently, there was not much difference in the cardiac utrophin level among aged BL6, mdx3cv and mdx4cv mice (**Figure 1C**). We also compared the expression level of representative DGC components including β -dystroglycan, α -sarcoglycan, syntrophin and dystrobrevin. Compared to that of the BL6 mouse heart, there appeared a slight reduction of the DGC components in the heart of mdx3cv and mdx4cv mice (**Figure 1C**).

Low-level dystrophin expression did not improve cardiac histopathology. On H&E staining, BL6 mouse heart showed normal morphology (**Figure 2A**). Some myocardial distortion and mononuclear cell infiltration were noted in both mdx3cv and mdx4cv heart. But there was no

apparent difference between these two strains (**Figure 2A**). Cardiac fibrosis was examined using Masson trichrome staining(**Figure 2B**). The BL6 heart had no fibrosis. The hearts of mdx3cv and mdx4cv mice showed similar patchy myocardial fibrosis (**Figure 2B**). Cardiac inflammation was examined by immunohistochemical staining (**Figure 2C**). Abundant macrophages and neutrophils were detected in the heart of mdx3cv and mdx4cv mice but not BL6 mice (**Figure 2C**).

The anatomic properties of the heart were similar between mdx3cv and mdx4cv mice. The absolute heart weight (HW) and ventricular weight (VW) were similar between mdx3cv and mdx4cv mice(**Table 1**). Both were significantly lower than those of BL6 mice. For the tibial length (TL) and anterior tibialis muscle weight (TW) normalized heart weight and ventricular weight (HW/TL, HW/TW, VW/TL and VW/TW), we did not see a difference between mdx3cv and mdx4cv mice. These ratios were all significantly lower than those of BL6 mice (**Table 1**). The body weight (BW) of BL6 and mdx3cv mice was comparable. However, the BW of mdx4cv mice was significantly reduced (**Table 1**). Hypertrophy of anterior tibialis muscle was obvious in mdx3cv and mdx4cv mice. Interestingly, the TW of mdx3cv mice was significantly higher than that of mdx4cv mice (**Table 1**).

Mdx3cv mice showed improved QRS duration. To study cardiac electrophysiology, we performed 12-lead ECG recordings using our published protocol [30, 31]. Compared with BL6, mdx4cv showed characteristic dystrophic ECG changes such as tachycardia, PR-interval reduction, QRS duration and QT interval prolongation, and a significant increase in the

cardiomyopathy index (**Figure 3**) [24, 28, 32, 33]. Surprisingly, we did not detect a significant change in the amplitude of Q wave among three strains (**Figure 3**). Compared to those of mdx4cv, several ECG parameters (the heart rate, QT interval and cardiomyopathy index) showed a trend of improvement in mdx3cv mice but did not reach statistical significance. The only ECG parameter that was significantly improved in mdx3cv mice was the QRS duration. It was significantly reduced compared to that of mdx4cv mice (**Figure 3**).

Low-level dystrophin in the heart normalized diastolic function in mdx3cv mice. We next examined the pump function of the heart using an ultra-miniature Millar ventricular catheter [30, 31]. Compared with BL6, mdx4cv showed the characteristic profile of dilated cardiomyopathy (**Figure 4**). Specifically, the end-systolic volume was significantly increased (**Figure 4A**). The end-diastolic volume also showed an apparent increase though not statistically significant (**Figure 4B**). Cardiac contractility (as reflexed by the maximum pressure, absolute values of dP/dt max and dP/dt min) was significantly reduced. The isovolumic relaxation time constant during diastole (tau) was prolonged (**Figure 4B**). As a result, the stroke volume, ejection fraction and cardiac output were all significantly decreased in mdx4cv mice (**Figure 4C**).

Low-level dystrophin expression in mdx3cv mice completely normalized diastolic parameters including the end diastolic volume, tau and dP/dt min (**Figure 4B**). The end systolic volume showed a trend of reduction (**Figure 4A**). However, overall heart performance (stroke volume, ejection fraction and cardiac output) was not significantly improved in mdx3cv mice.

Expression of sarcoplasmic/endoplasmic reticulum calcium ATPase 2a (SERCA2a), phospholamban and calsequesterin in aged BL6, mdx3cv and mdx4cv hearts. To gain molecular insight on how low-level dystrophin controls heart function, we performed quantitative western blot on the expression of three major calcium handling proteins in the heart including SERCA2a, phospholamban and calsequesterin. No statistically significant difference was detected among BL6, mdx3cv and mdx4cv mice (**Figure 5**). Unfortunately, our western blot for the phosphorylated form of phospholamban did not work.

Discussion

In this study, we tested the hypothesis that a uniform low-level dystrophin expression can benefit the heart in the mouse model of Duchenne cardiomyopathy. We found marginal level (approximately 3.3% of the wild-type level) homogenous dystrophin expression in the myocardium of aged mdx3cv mice (**Figure 1**). This residual level expression did not change the anatomic properties of the heart (**Table 1**). Neither did it reduce histological lesions in the heart (**Figure 2**). However, some aspects of heart function measures were significantly improved (**Figures 3 and 4**). Specifically, the abnormally elongated QRS duration was shortened and deficiencies in diastolic hemodynamics were completely prevented (**Figures 3 and 4**). Interestingly, there was no difference in the expression level of SERCA2a, phospholamban and calsequestrin (**Figure 5**). Our results suggest that low-level dystrophin is far from sufficient to cure Duchenne cardiomyopathy. However, it can still offer some protection to the heart.

Recent progress in genetic engineering and molecular medicine is making gene therapy for DMD a reality [7, 8]. Large scale clinical trials have been conducted to test therapeutic benefits of exon-skipping [34, 35]. Systemic AAV micro-dystrophin therapy is slotted to start in next couple of years [7, 8]. Most recently, investigators have achieved remarkable proof-of-concept evidence in repairing the mutated dystrophin gene in mdx mice [36]. Despite these successes, it is still not completely clear whether sub-physiological level dystrophin expression can help mitigating dystrophic manifestations. A comprehensive understanding of the dystrophin expression level in striated muscle requires information on (a) the percentage of dystrophin positive myofibers and (b) the amount of dystrophin protein in these positive myofibers. The former is obtained by quantifying dystrophin immunostaining and the latter by

western blot. Accordingly, for DMD gene therapy we need to know what percentage of muscle cells should express dystrophin and what are the dystrophin levels in these cells.

Therapeutic relevance of mosaic dystrophin expression has been extensively examined [18-20, 23, 24]. These studies have documented remarkable disease amelioration and functionpreservation in both skeletal and cardiac muscles when half myofibers show positive dystrophin staining. Homogenous sub-physiological dystrophin expression has been shown to protect skeletal muscle by a number of laboratories [9-17]. However, it is not clear whether a low-level uniform dystrophin expression in the heart can reduce cardiomyopathy.

Townsend et al compared dystrophin expression in the heart of young adult (4-m-old) and aged (23-m-old) BL10 mice [37]. The authors observed a ubiquitous reduction of dystrophin content in every cardiomyocyte in aged mice. On average, the dystrophin level was reduced by 57% in theaged BL10 heart. Loss of dystrophin resulted in a decline of cardiac function in aged BL10 mice [37]. Our recent studies also suggest that removal of existing dystrophin from the myocardium can compromise the pump function of the heart [38]. These two studies suggest that sub-physiological level dystrophin is insufficient to maintain normal heart function. While this is an important conclusion, it does not tell us whether a heart with low-level dystrophin expression is structurally and/or functionally superior to a heart that has no dystrophin expression. Our study in aged mdx3cv mice is aimed to address this knowledge gap. Consistent with our previous studies on the mdx3cv mouse skeletal muscle[14, 15], we demonstrated a partial function preservation but not histopathology amelioration in the mdx3cv heart.

Little is known about the molecular mechanisms underlying electrophysiological defects and hemodynamic deficiencies in Duchenne cardiomyopathy. A number of hypotheses have been suggested such as myocardial necrosis and inflammation, cardiac fibrosis, vacuolar degeneration in the conduction system, perturbation of calcium homeostasis, oxidative stress, sarcolemma tearing, mitochondrial dysfunction and aberrant signaling [39-42]. We have previously demonstrated characteristic ECG changes in young adult (4-m-old) mdx mice in the absence of apparent histological lesions in the heart [43]. We have also shown significant ECG improvement but not histology amelioration in terminally aged (21 to 23-m-old) mdx mice by AAV micro-dystrophin gene therapy [32]. These data challenge a direct causal relationship between myocardial structural damage and ECG abnormality. In mdx3cv mice, residual level dystrophin expression did not reduce myocardial inflammation and fibrosis. Yet the extended QRS complex was significantly shortened. The QRS complex reflects depolarization of ventricular cells. In the absence of dystrophin, the time of ventricular depolarization was increased by 30% (**Figure 3**). With merely ~3.3% dystrophin, the speed of ventricular depolarization was significantly increased. As a result, the QRS duration was reduced in mdx3cv mice compared to that of dystrophin-null mdx4cv mice (**Figure 3**). Cardiomyocyte depolarization and repolarization is tightly controlled by various ion channels on the sarcolemma. Interestingly, dystrophin and some DGC components (such as syntrophin and nNOS) have been shown to regulate these ion channels [44-47]. Our results suggest that low-level dystrophin may partially restore dystrophin/DGC-mediated regulation in ion channels.

An unexpected finding of our study is the full normalization of diastolic hemodynamic parameters in mdx3cv mice. This suggests that low-level dystrophin may meet the need of myocardial relaxation during the cardiac cycle. However, a much higher level of dystrophin

expression is needed to enhance ventricular muscle contraction in order to improve the blood pumping function of the heart. We would like to point out that it is not unusual that different levels of dystrophin expression are needed for the correction of different aspects of disease. For example, a recent study in skeletal muscle by Godfrey et al suggests that protection against eccentric contraction-induced injury requires homogenous dystrophin expression at the 15% of wild-type level. However, reduction of skeletal muscle histopathology requires much more dystrophin[11].

While the ultimate goal of the study is to translate our findings into human patients, it is important to remember that scaling up to a large dystrophic mammal is much more complex than we can model in mice. Whether marginal level expression can result in clinically appreciable improvement in human patients will depend on a number of factors, such as the configuration of the therapeutic dystrophin protein (full-length, moderately truncated mini-dystrophin, or highly abbreviated micro-dystrophin), treatment regime (the age at the start of the therapy, the duration of the study and the gene therapy vector dose etc.), and the abundance of dystrophin (percentage of dystrophin expressing cells and dystrophin level in these cells). Our data suggest that uniform low-level dystrophin expression may have therapeutic implications for treating Duchenne cardiomyopathy. Future studies in large animal models of DMD (such as dystrophic dogs) may testify whether this observation can be translated to large mammals.

Materials and Methods

Experimental Animals. All animal experiments were approved by the institutional animal care and use committee and were in accordance with NIH guidelines. Experimental mice were generated in a barrier facility using founders from The Jackson Laboratory (Bar Harbor, ME). Female mice were used in the study because we have previously shown that female mice are better than male mice in modeling Duchenne cardiomyopathy seen in human patients [28]. All mice were maintained in a specific-pathogen free animal care facility on a 12-hour light (25 lux):12-hour dark cycle with access to food and water *ad libitum*. Mice were euthanized following functional assays for tissue collection.

Morphological studies. Dystrophin expression was evaluated by immunofluorescence staining using the Dys2 monoclonal antibody (1:30; Vector Laboratories, Burlingame, CA). General histology was examined by hematoxylin and eosin (HE) staining. Fibrosis was examined by Masson trichrome staining. Inflammation was studied with immunohistochemistry staining using antibodies specific to mouse neutrophils (Ly-6G, 1:8,00, BD Pharmingen, San Diego, CA) and macrophages (F4/80, 1:200, Caltag Laboratories, Burlingame, CA) according to our published protocol [29]. Slides were viewed at the identical exposure setting using a Nikon E800 fluorescence microscope. Photomicrographs were taken with a QImage Retiga 1300 camera [31].

Western blot. Whole heart lysate was prepared as we described before [48]. Briefly, the freshly isolated heart was snap frozen in liquid nitrogen. The frozen heart sample was ground to fine powder in liquid nitrogen followed by homogenization in a buffer containing 10% sodium

dodecyl sulfate, 5mM ethylenediaminetetraacetic acid, 62.5mM Tris at pH6.8 and the protease inhibitor cocktail (Roche, Indianapolis, IN). The crude lysate was heated at 95°C for 3 min, chilled on ice for 2 min and then centrifuged at 14,000 rpm for 2 min (Eppendorf 5418, Hauppauge, NY). Supernatant was collected as the whole muscle lysate. Protein concentration was measured using the DC protein assay kit (Bio-Rad, Hercules, CA). For western blot, we loaded 5 to 100µg of protein per lane as indicated in the figures. Dystrophin was detected with Dys2 (1:100 Vector Laboratories, Burlingame, CA) and DysB (1:100, clone 34C5, IgG1; Novocastra, Newcastle, United Kingdom) antibodies. Utrophin was detected with a mouse monoclonal antibody against utrophin amino acid residues 768-874 (1:200; clone 55, IgG1; BD Biosciences, San Diego, CA). β -Dystroglycan was detected with a mouse monoclonal antibody against the β -dystroglycan C-terminus (NCL-b-DG, 1:100; clone 43DAG1/8D5, IgG2a; Novocastra, Newcastle, United Kingdom). α -Sarcoglycan was detected with a mouse monoclonal antibody against α -sarcoglycan amino acid residues 217-289 (VP-A105; 1:1,000; clone Ad1/20A6, IgG1; Vector Laboratories, Burlingame, CA). Syntrophin was detected with a pan-syntrophin mouse monoclonal antibody that recognizes the syntrophin PDZ domain (ab11425, 1:2,000; clone 1351, IgG1; Abcam, Cambridge, MA). Dystrobrevin was detected with a mouse monoclonal antibody against dystrobrevin amino acid residues 249 to 403(#610766, 1:1,000; clone 23, IgG1; BD Biosciences, San Diego, CA). The calcium handling proteins were detected using sarcoplasmic/endoplasmic reticulum calcium ATPase 2a (SERCA2a, 1:2500 Badrilla, Leeds UK), calsequestrin (1:2500, Thermo Scientific, Grand Island NY) and phospholamban (1:2500 Badrilla, Leeds UK) antibodies. For the loading control, we used an antibody against glyceraldehyde 3-phosphate dehydrogenase (1:3000; Millipore, Billerica, MA) and the α -tubulin antibody (1:3,000; clone B-5-1-2; Sigma, St Louis, MO). Western blot

quantification was performed using the ImageJ software (<http://rsbweb.nih.gov/ij/>) and LI-COR Image Studio Version 5.0.21 (<https://www.licor.com>) software. The intensity of the respective protein band was normalized to the corresponding loading control in the same blot. The relative band intensity in mdx3cv and mdx4cv mice was normalized to that of BL6 mice.

ECG and hemodynamic assay. A 12-lead ECG assay was performed using a commercial system from AD Instruments (Colorado Springs, CO) according to our previously published protocol [30, 31]. The Q wave amplitude was determined using the lead I tracing. Other ECG parameters were analyzed using the lead II tracing. The QTc interval was determined by correcting the QT interval with the heart rate as described by Mitchell et al [49]. The cardiomyopathy index was calculated by dividing the QT interval by the PQ segment [50]. Left ventricular hemodynamics was evaluated using a Millar ultra-miniature pressure–volume (PV) catheter SPR 8329. The catheter was placed in the left ventricle using a closed chest approach as we have previously described [30, 31]. The resulting PV loops were analyzed with the PVAN software (Millar Instruments, Houston, TX). Detailed protocols for ECG and hemodynamic assays are available at the Parent Project Muscular Dystrophy standard operating protocol web site

(http://www.parentprojectmd.org/site/PageServer?pagename=Advance_researchers_sops) [51].

Statistical analysis. Data are presented as mean $\pm$ stand error of mean. One-way ANOVA with Bonferroni's multiple comparison analysis was performed using the GraphPad PRISM software version 6.0 for Mac OSX (GraphPad Software, La Jolla, CA, www.graphpad.com). A $P < 0.05$ was considered statistically significant.

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Figure Legends

Figure 1. Mdx3cv mouse heart expressed low-level dystrophin. **A**, Representative photomicrographs of dystrophin immunofluorescence staining in BL6, mdx3cv and mdx4cv heart. Upper panel shows the whole heart view and the lower panel shows a higher magnification of the corresponding boxed region in the whole heart view. **B**, Top panel, Representative heart western blot from BL6, mdx3cv and mdx4cv mice. The BL6 heart lysate was loaded at 100%, 50%, 25% and 5%. The mdx3cv and mdx4cv heart lysate was loaded at 100%; Bottom panel, Densitometry quantification of cardiac dystrophin expression (N=3 for each group). Dys-2, a monoclonal antibody against the dystrophin C-terminal domain. The heart of mdx3cv mice showed uniform dystrophin expression at approximately 3.3% of the wild-type level. **C**, Representative cardiac western blots for utrophin and selected components of dystrophin-associated glycoprotein complex (β -dystroglycan, α -sarcoglycan, syntrophin and dystrobrevin). DysB, a monoclonal antibody against the dystrophin exons 10-12; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Figure 2. Low-level dystrophin expression did not ameliorate myocardial inflammation and fibrosis in mdx3cv mice. **A**, Representative heart HE staining photomicrographs from BL6, mdx3cv and mdx4cv mice. Left panel, whole heart cross-sectional images; right panel, high-power images of the respective boxed areas in the whole heart view. **B**, Representative Masson trichrome staining photomicrographs of the BL6, mdx3cv and mdx4cv heart. Left panel, whole heart cross-sectional images; right panel, high-power images of the respective boxed areas in the whole heart view. The blue color in Masson trichrome staining marks myocardial fibrosis. **C**,

Representative macrophage and neutrophil immunohistochemical staining photomicrographs of the BL6, mdx3cv and mdx4cv heart. Arrow, dark brown stained macrophages and neutrophils.

Figure 3. Low-level dystrophin expression improved QRS duration but not other ECG parameters in mdx3cv mice. Quantitative evaluation of the heart rate, PR interval, QRS duration, Mitchell corrected QT interval (QTc), cardiomyopathy index and the Q wave amplitude (Q Amp). The QTc interval was determined by correcting the QT interval with the heart rate as described by Mitchell et al [49]. Asterisk, statistically significant ($p < 0.05$).

Figure 4. Low-level dystrophin expression partially improved hemodynamics in mdx3cv mice. **A**, Quantitative evaluation of systolic hemodynamic parameters. **B**, Quantitative evaluation of diastolic hemodynamic parameters. End-diastolic volume, tau and dP/dt min were all normalized in mdx3cv mice. **C**, Quantitative evaluation of overall heart function. Asterisk, statistically significant ($p < 0.05$). The heart rate at the hemodynamic assay was 616.9 ± 9.10 bpm, 619.9 ± 25.5 bpm and 629.3 ± 8.3 bpm for BL6, mdx3cv and mdx4cv, respectively. There is no statistically significance difference.

Figure 5. Western blot evaluation of SERCA2a, calsequestrin and phospholamban in the heart. **A**, Representative western blot results from BL6, mdx3cv and mdx4cv mice. **B**, Densitometry quantification of calcium handling proteins shown in panel A ($N=3$ for each group).

Tables

Table 1. Anatomical measurements and ratios

	BL6	mdx3cv	mdx4cv
Sample Size (N)	11	28	19
Age (m)	21.67 ± 0.54	21.85 ± 0.33	20.76 ± 0.19
BW (g)	26.78 ± 0.79	27.60 ± 0.37	23.59 ± 0.91 ^a
HW (mg)	117.80 ± 3.42	101.99 ± 2.73 ^b	101.58 ± 3.22 ^b
VW (mg)	111.49 ± 3.25	89.91 ± 2.52 ^b	94.32 ± 3.10 ^b
TL (mm)	18.51 ± 0.09	18.42 ± 0.08	18.94 ± 0.09 ^a
TW (mg)	36.75 ± 1.14 ^a	63.14 ± 2.02 ^a	55.42 ± 2.32 ^a
HW/BW (mg/g)	4.41 ± 0.11	3.71 ± 0.11 ^a	4.38 ± 0.16
HW/TL (mg/mm)	6.36 ± 0.17	5.21 ± 0.10 ^b	5.17 ± 0.16 ^b
HW/TW (mg/g)	3.25 ± 0.16	1.81 ± 0.25 ^b	1.89 ± 0.10 ^b
VW/TL (mg/mm)	6.02 ± 0.16	4.81 ± 0.11 ^b	4.78 ± 0.15 ^b
VW/TW (mg/g)	3.08 ± 0.15	1.60 ± 0.23 ^b	1.76 ± 0.09 ^b

Abbreviations: BW, body weight; HW, heart weight; VW, ventricle weight; TL, tibia length; TW, anterior tibialis muscle weight.

a- Significantly different from other two groups

b- Significantly different from BL6 mice

A

BL6

mdx3cv

mdx4cv

B

Heart

C

A

BL6

mdx3cv

mdx4cv

B

BL6

mdx3cv

mdx4cv

C

Neutrophil

Macrophage

BL6

mdx3cv

mdx4cv

■ BL6 (n=21)

■ mdx3cv (n=27)

□ mdx4cv (n=20)

■ BL6 (n=35)

■ mdx3cv (n=25)

□ mdx4cv (n=12)

A

B

C

A **B**