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Reduced Myocardial Reserve in Young X-linked Muscular Dystrophy Mice Diagnosed by 2D Strain Analysis Combined with Stress Echocardiography

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Abstract

Background—Early, sensitive and reproducible evaluation of left ventricular (LV) function is imperative for diagnosis of cardiac dysfunction in Duchene muscular dystrophy (DMD) patients. We hypothesized that combining 2D-strain analysis with catecholamine stress could be a sensitive method for detecting early cardiac dysfunction.

Methods—*mdx* (C57BL/10ScSn-Dmd*mdx*/J, a mouse model of DMD) and control (C57BL/10ScSn) mice were studied with conventional M-mode and high frequency ultrasound based 2D speckle tracking echocardiography (STE) using long- and short-axis images of LV at baseline and after isoprenaline (2µg/g BW, i.p.).

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Results—Conventional M-mode analysis showed no differences in LV fractional shortening, wall thickness or internal diameter at diastole between *mdx* and control mice before the age of 6 months. Isoprenaline increased LV ejection fraction and fractional shortening to the same extent in *mdx* and control mice at young ages (3, 4 and 5 months). No differences in basal peak systolic strain (PSS) but increased standard deviations of times to PSS between young *mdx* and control mice were found. After isoprenaline, PSS and percentile changes of PSS were significantly diminished in *mdx* mice compared to control mice at young ages. Isoprenaline increased normalized maximum difference of times to PSS in young *mdx* mice but not in young control mice, suggesting isoprenaline reduces cardiac contractile synchrony in young *mdx* mice.

Conclusions—Our study suggests that catecholamine stress coupled with 2D strain analysis is a feasible and sensitive approach for detecting early onset of cardiac dysfunction, which is instrumental for early diagnosis of cardiac dysfunction and early treatment.

Keywords

Duchenne muscular dystrophy cardiomyopathy; Myocardial reserve; Stress echocardiography; Strain; *mdx* mouse

Introduction

Duchenne muscular dystrophy (DMD) is a hereditary X-linked recessive disease resulting from the mutation of the dystrophin gene [1], which affects 1 in 3500 male births. An early manifestation of DMD is weakness in the skeletal muscle [2]. A weakened diaphragm may cause death of DMD patients at young ages due to respiratory failure, but with the support of mechanical ventilators, DMD patients can now survive much longer than they did a decade ago [2]. Cardiomyopathy is becoming a more important cause of mortality and morbidity in DMD patients [3]. At rest, cardiac dysfunction can be detected during the early teenage years in DMD patients. With more advanced technologies, such as three dimensional (3-D) strain, distortion and dispersion analyses, cardiac dysfunction at rest can be detected as early as the age of 8 or 9 years [4, 5]. Reduced cardiac reserve is an early indication of cardiac dysfunction and is evaluated with a treadmill test or dobutamine test in the clinic. However, there were few reports regarding cardiac reserve in DMD patients.

Mouse models, such as x-linked muscular dystrophy (*mdx*) mice, have been used to investigate the pathophysiology of and to test new therapies for DMD [6, 7]. To develop a safe and effective therapy for cardiomyopathy in DMD, early, sensitive and reproducible measurements of left ventricular (LV) remodeling and dysfunction are needed. High frequency ultrasound has become a valuable tool to phenotype cardiac structure, hemodynamics, and global function in small animals [8–10]. Furthermore, high frequency ultrasound based 2D speckle tracking echocardiography (STE) has been used to quantitatively measure myocardial strains and strain rates as potentially sensitive markers for early regional myocardial dysfunction in *mdx* mice, showing that peak systolic strain (PSS) analysis is feasible and reproducible for detecting LV dysfunction in *mdx* mice [11].

The catecholamine (e.g., dobutamine in the clinic or isoprenaline in animal experiments) stress test has been used to evaluate cardiac reserve in many studies [12–14]. Previously,

using invasive *in vivo* intra-left ventricular hemodynamic measurement and *ex vivo* Langendorff system, we found that cardiac β -adrenergic response was reduced in 4-month old but not in 2-month old mdx mice. In a sixteen-year old DMD patient, it was found that there was reduced cardiac response to dobutamine stress although his left ventricular systolic function was normal at rest [15]. However, the use of the combination of the catecholamine stress test and high frequency ultrasound-based 2D STE to detect early cardiac dysfunction in DMD patients or animal models has not been reported. Therefore, we hypothesize that reduced abnormal β -adrenergic response of mdx at young ages could be detected by the combination of catecholamine stress with LV strain analysis but not by conventional echocardiography or 2D STE at rest alone.

Methods

Animal models

All mice were handled in compliance with the “Principles of Laboratory Animal Care” by the National Society for Medical Research and the “Guide for the Care and Use of Laboratory Animals” published by the National Institutes of Health (NIH publication 85-23, revised 1996). The research protocol was approved by the Institutional Animal Care Committee of the Temple University. *Mdx* (C57BL/10ScSn-Dmdmdx/J) and control mice (C57BL/10ScSn) (The Jackson Laboratory, ME) were used at the ages as indicated in the figures for studying cardiac morphology and function at rest. Both male and female animals were used. Three sets of animals were used for the study. We used the first set of animals (control: n=10; mdx: n=8) to detect cardiac function alterations for a year with conventional echocardiography. The 2nd set of animals (7 control and 7 mdx mice) were used for detecting the changes of cardiac strains at the ages of 6m, 9m and 12m with high frequency ultrasound 2D imaging followed by 2D STE analysis. The 3rd set of mice included a total of 21 mdx and 21 control mice; separate sets of 7 mdx and 7 control mice each were studied at three separate time points (3mos, 4mos, and 5mos) for combined high frequency ultrasound analysis and isoprenaline stress to avoid potential carryover effect of previous isoprenaline injection. Since the mdx mouse model is a model with mild disease progression [16], none of the mice died during the one-year study period.

Echocardiography

A high frequency ultrasound system (Vevo 770, VisualSonics, Toronto, Ontario) was used for acquiring ultrasound images. A single-crystal mechanical transducer (RMV 707B) with a central frequency of 30 MHz (15–45MHz) and focal length of 12.7 mm was used. The Vevo 770 machine with RMV 707B scanhead has a lateral resolution of 115 μ m, an axial resolution of 55 μ m and up to 240 frames per second, allowing us to acquire high resolution images for conventional echocardiography and speckle tracking analysis when combined with a proprietary software [17], Tomtec Image Arena 4.0.

Mice were anesthetized using 3% isoflurane initially and then maintained at 1.5%. The animals were laid in a supine position on a heated platform with all legs taped to ECG electrodes for ECG recording. Body temperature was monitored via a rectal thermometer

and maintained at 36–38°C. Hair was removed from the chest using chemical hair remover prior to imaging.

M-mode and 2D high frequency ultrasound

Eight *mdx* and ten control mice were followed for conventional M-mode analysis every two months until the age of 12 months to evaluate cardiac morphology and function. M-mode images were obtained using LV short-axis view at the mid papillary muscle level. M-mode gate was set in the middle between the two papillary muscles for ejection fraction and fractional shortening analysis.

For 2D STE studies, short-axis B mode cine loops were obtained at the mid LV level as described above and long-axis B mode cine loops were recorded with scanhead placed at the mid-LV level, i.e., along the left sternal border with clear view of both the apex and the outflow tract of LV [18]. At the ages of 3m, 4m, and 5m, 7 *mdx* and 7 control at each age were studied without reuse because they were also used for isoprenaline stress. For ages of 6m, 9m, and 12m, 7 *mdx* and 7 control mice were followed serially. The field of view was adjusted to maximize the display resolution of the region-of-interest (ROI) and the frame rates (up to 160fps for short-axis images and 120fps for long-axis images). Image gain and other parameters were adjusted carefully to delineate all myocardial segments. All images were acquired for multiple cardiac cycles and stored digitally in the hard drive for offline analysis.

LV ventricular remodeling and global functional measurements, including LV end-diastolic dimension (LVEDD), LV end-systolic dimension (LVESD), EF and FS were derived from M-mode using high frequency ultrasound by averaging 3 consecutive and stable cardiac cycles.

High frequency ultrasound with isoprenaline stress

High frequency ultrasound with isoprenaline stress was performed for *mdx* and control mice at the ages of 3, 4, and 5 months. At each age, 7 control and 7 *mdx* mice were used. Mice were not reused after isoprenaline stress to avoid potential complications. After long- and short-axis M-mode images and B-mode cine loops of LV were acquired at baseline, isoprenaline was injected intraperitoneally (2µg/g BW) [19]. The animals were fully anesthetized to avoid pain response during ISO injection, and had the heart rates between 400–450bpm. The relatively low heart rate also allowed us to get more images for each cardiac cycle and thus better strain analysis. Images were acquired every minute for up to 14 minutes after isoprenaline injection. Long-axis cine loops and M-mode images of the LV were acquired at 1min, 3min, 5min, 7min, 9min, 11min and 13min after injection. Short-axis cine loops and conventional M-mode images of the LV were acquired at 2min, 4min, 6min, 8min, 10min, 12min and 14min after injection. The images and cine loops showing maximum responses to isoprenaline were used for stress response analysis.

Offline strain analysis

Since cardiac muscle degeneration and cardiac dysfunction are heterogeneous in different regions of the heart in DMD patients [20] and there has been thus far no report about

epicardial and endocardial strains in mdx mice, we analyzed endocardial and epicardial strains of different regions of the heart including 6 segments of short-axis cine loops and 6 segments of long-axis cine loops. B-mode cine loops of short-axis and long-axis recorded with a 707B scanhead (15–45MHz, 100–240 frames per second) of a Vevo 770 System (FUJIFILM Visual Sonics, Inc., Toronto, Canada) were converted to DICOM format without compression and imported into Tomtec Image Arena 4.0 (Tomtec, Munich, Germany) for offline strain analysis. Tomtec Image Arena has been widely used as a vendor-independent cardiac strain and time to peak analysis. All analyses were based on at least three successive and stable cardiac cycles. Longitudinal, circumferential and radial global peak systolic strains (PSS) of the left-ventricular (LV) endocardium and epicardium were obtained by manual tracing, editing and semi-automated tracking of LV myocardial deformation. The software automatically divides the long-axis LV images into 6 segments: basal anterior (bAnt), mid anterior (mAnt), apical anterior (aAnt), apical posterior (aPst), mid posterior (mPst) and basal posterior (bPst) (Supplemental Figure 1A). The software also divides the short-axis images into 6 segments: anterior (A), anterior septum (AS), posterior septum (PS), inferior (I), posterior (P) and lateral (L) (Supplemental Figure 1B). Strain measurements in each segment were averaged and the peak systolic strains of all segments were further averaged to obtain the peak systolic strain (PSS). Five PSS measurements were determined: longitudinal PSS of the endocardium (L-Endo), longitudinal PSS of the epicardium (L-Epi), circumferential PSS of the endocardium (C-Endo), circumferential PSS of the epicardium (C-Epi), radial PSS (R) (Supplemental Figure 2 and 3). L-Endo and L-Epi PSS were analyzed with long-axis cine loops and C-Endo, C-Epi and R PSS were obtained from short-axis cine loops obtained at the mid-LV level. EF, FS and PSS measurements were made under baseline and isoprenaline stress conditions. Maximum values for EF, FS and PSS after ISO injection were selected for statistics. Percentage increase of PSS measurements were calculated as: (difference between the measurements after and before isoprenaline injection/measurement before isoprenaline injection) $\times$ 100 %. To determine dyssynchrony of left ventricle segments, the maximum time difference between times to peak systolic strain of segments (maximum Tpeak) was determined. The standard deviation of times to peaks of six segments of each heart were normalized to the cardiac cycle (i.e., RR interval) to further characterize the dyssynchrony of contractions. The person performing the echo analysis was blinded to the mice population.

Intra-observer and Inter-observer Variability

A total of 20 data sets at rest and 20 data sets during stress were selected, and measurements were made by two blinded, experienced observers to determine intra- and inter-observer variability. To determine the intra-observer variability, one observer analyzed and measured the strain data (PSS) from rest and stress echocardiography results twice at a 4-week interval, while blinded to the results of the first measurements. A second observer, blinded to the results of the first observer, also analyzed and measured the strain data independently to determine the inter-observer variability. The intra- and inter-observer variabilities were expressed as percentile differences: (absolute difference between the measurements/mean of the measurements) $\times$ 100 %.

Statistical Analysis

Continuous variables were presented as mean $\pm$ standard error of the mean (SEM). The difference between 2 continuous variables was tested using a *t*-test with a 2-tailed distribution or one-way or two-way ANOVA with or without repeated measurements. Bonferroni adjustment was done for comparison between pairs for ANOVA analyses. Statistical significance was defined as $P < 0.05$. All statistical analyses were done using SPSS statistical software package (version 17.0; SPSS Inc, Chicago, IL).

Results

Conventional M-mode high frequency ultrasound analysis in combination with isoprenaline stress test cannot detect cardiac dysfunction in young *mdx* mice

We first used conventional M-mode analysis to determine cardiac function in *mdx* and control mice every two months from the age of two months until the age of 12 months (Figure 1). This technique only detected reduced cardiac dysfunction in *mdx* mice at rest after the age of 8 months (Figure 1B) while diastolic LV posterior and septal wall thickness increased (Figure 1C and D). The diastolic LV internal diameter decreased slightly due to the increases in LV wall thickness in *mdx* mice seen at the age of 8 months (Figure 1). These results agree with those from previous studies [14, 21].

Since altered β -adrenergic reserve is an early indication of cardiac dysfunction [22], we tested if young *mdx* mice (ages 3, 4, and 5 months) had reduced cardiac reserve with a catecholamine (isoprenaline, 2 μ g/g BW, i.p.) stress test assessed by conventional M-mode echocardiography. During stress, LVEF and LVFS increased gradually after injection and reached peak value within 3–7mins (4.25 $\pm$ 1.16mins) for all *mdx* and control mice. ISO significantly increased heart rate (HR), LVEF and LVFS in both control and *mdx* mice at the ages of 3, 4 and 5 months. After ISO, there was no difference in HR, EF and FS between control and *mdx* mice of the same age (Figure 2).

High frequency ultrasound speckle tracking echocardiography (STE) does not detect cardiac dysfunction in young *mdx* mice at baseline

It has been reported that cardiac strain analysis is a more sensitive method for detecting local and global cardiac dysfunction [23]. We used this method to determine if it could reveal a difference between young *mdx* and control mice. At the ages of 3 and 4 months, there was no difference in PSS between *mdx* and control mice. PSS started to decrease in *mdx* mice at the age of 5 months, and the differences in L-endo PSS and Radial PSS reached statistical significance at the age of 6 months, progressing with age. Differences in L-epi and C-endo PSS between *mdx* and control mice were significant at the ages of 9 months and 12 months (Figure 3). The impairment of strain in *mdx* mice appeared earlier and more severe in the endocardium than in the epicardium, indicating endocardial PSS were more sensitive measurements of regional cardiac dysfunction in *mdx* mice. These data show that strain analysis is only able to detect cardiac contraction differences between control and *mdx* mice after the age of 6 months.

Peak systolic strains in young *mdx* mice had reduced response to isoprenaline

During stress, LVEF, LVFS and PSS increased gradually after injection and all reached peak value between 3–7mins (4.25 ± 1.16 mins) for all *mdx* and control mice. Endocardial and epicardial PSS in longitudinal, circumferential and radial directions increased significantly in control and *mdx* mice with isoprenaline stimulation (Figure 4). In control mice of 3–5 months, age did not significantly change PSS at baseline or after isoprenaline. Isoprenaline also significantly increased PSS in young *mdx* mice, but the increases in most PSS were less in *mdx* mice than in control mice except in L-Epi and C-Epi PSS at the age of 3 months. From the age of 3 months to 5 months, PSS responses to isoprenaline in *mdx* mice tended to decrease with age. For L-Endo and R PSS during stress, significant differences between *mdx* and control mice appeared in all age groups from 3 to 5 months (Figure 4). However, for L-epi and C-endo PSS, the differences between *mdx* and control mice after isoprenaline were not significant at the age of 3 months but reached significance at the age of 5 months. For C-Epi PSS, differences between *mdx* and control mice could not be detected in the 3, 4, and 5 month groups.

Percentage increases of LV PSS were less in young *mdx* mice as compared to young control mice

Since there were subtle differences in baseline PSS between *mdx* and control mice, we determined the percentage increase of LV PSS to better compare the effects of isoprenaline on (Figure 5). There were significantly lower percentage increases of L-Endo, C-Endo and R PSS in *mdx* mice than in control mice of 3, 4, and 5 months (Figure 5A, C and E). The percentage increases of L-Epi PSS and C-Epi PSS were not significantly different between *mdx* and control mice at the ages of 3 and 4 months. At the age of 5 months, the increases of these two PSS reached statistical significance between *mdx* and control mice (Figure 5B and D). These data suggest that the combination of 2D strain analysis and isoprenaline stress can detect reduced cardiac functional reserve in *mdx* mice starting at a very young age (3 months). L-endo PSS, C-endo PSS and R PSS were more sensitive than L-epi PSS and C-epi PSS for distinguishing between control and *mdx* mice.

β -adrenergic stimulation decreases myocardial contraction synchrony in young *mdx* mice

To determine if there is dyssynchrony of myocardial contractions at baseline or after β -adrenergic stimulation in young *mdx* mice, standard deviations and maximum differences of times to peak of strains ($T_{\text{peak,max}}$) were analyzed for epicardium and endocardium in the longitudinal, circumferential and radial directions. At baseline, no difference in $T_{\text{peak,max}}$ in all directions was observed between 3-, 4-, and 5-month old control and *mdx* mice. ISO stimulation did not significantly alter $T_{\text{peak,max}}$ in control mice of all three age groups or in 3-month *mdx* mice. However, ISO significantly increased L-endo and C-endo $T_{\text{peak,max}}$ in 5-month old *mdx* mice, suggesting that ISO increases dyssynchrony in 5-month old *mdx* mice (Supplemental Figure 5). Since ISO increased the heart rate and reduced cardiac cycle duration, we normalized $T_{\text{peak,max}}$ to cardiac cycle duration. Again, ISO did not significantly change $T_{\text{peak,max}}/\text{CC}$ in control mice but significantly increased L-endo, C-endo and radial $T_{\text{peak,max}}/\text{CC}$ in 4-month *mdx* mice and $T_{\text{peak,max}}/\text{CC}$ in all directions (L-endo, L-epi, C-endo, C-epi and radial) in 5-month old *mdx* mice (Figure 6). It seems that the

normalization of $T_{\text{peak,max}}$ to CC is a more sensitive parameter for evaluating myocardium dyssynchrony, and ISO increases myocardium dyssynchrony in *mdx* mice. The early contraction or delayed contraction was not restricted to a specific region in the mid-level short axis and long axis videos (**Supplemental Tables 1–3**).

We also studied the dyssynchrony of PSS by analyzing the standard deviation of time to peak ($SD_{T_{\text{peak}}}$) of PSS. Baseline $SD_{T_{\text{peak}}}$ was not significantly changed in control mice but increased in *mdx* mice during the growth from 3m to 5m, reaching statistical significance between these two groups of mice at the age of 5 months for all PSS and at the age of 4m for L-endo PSS. ISO decreased $SD_{T_{\text{peak}}}$ in all mice but did not further enhance the difference in $SD_{T_{\text{peak}}}$ between control and *mdx* mice (Supplemental Figure 6). When $SD_{T_{\text{peak}}}$ was normalized to cardiac cycle ($SD_{T_{\text{peak}}}/CC$), there were significant differences in the $SD_{T_{\text{peak}}}/cc$ of L-endo and L-epi PSS at the age of 4 months and of all PSS at the age of 5 months between control and *mdx* mice at baseline or after ISO (Figure 7).

Intra-observer and Inter-Observer Variability in STE measurements

Intra-observer and inter-observer variabilities for specific PSS were less than 10% as shown in Table 1 and the Bland-Altman plots in supplemental Figures 7–8. The intraclass correlation of intra-observer and inter-observer observations were all greater than 0.90.

Discussion

In this study, we evaluated the cardiac function of *mdx* mice and control mice serially with conventional echocardiography and 2D STE strain analysis both with and without a concurrent catecholamine stress test. We made the following observations that: (1) At baseline, neither conventional echo nor 2D STE strain analysis is sensitive enough to detect cardiac dysfunction of *mdx* hearts younger than 5 months; however, 2D STE strain analysis combined with a catecholamine stress test clearly showed reduced β -adrenergic responsiveness in 3-month old *mdx* mice. (2) ISO stimulation revealed that there was regional contraction dyssynchrony within seemingly normal young *mdx* hearts at baseline. These results suggest that the combination of catecholamine stress and echocardiographic 2D strain analysis can be a sensitive approach to detect reduced cardiac reserve in young *mdx* mice.

Detection of cardiac dysfunction with conventional echo, 2D-STE in combination with catecholamine stress in *mdx* mice

Conventional M-mode analysis has been used to evaluate global cardiac functions and morphology such as EF, FS, wall thickness and chamber diameter in *mdx* mice and DMD patients. It detects cardiac dysfunction during the teenage years in DMD patients [24] and after the age of 6–9 months in *mdx* mice [25–27]. The echocardiography technology has now advanced from global to local (e.g., motility, torsion, and strain) analysis [8, 23], which has been used in DMD patients [4, 20, 28, 29] and *mdx* mice [11] to diagnose cardiac dysfunction and is more sensitive than conventional echo in detecting cardiac function abnormalities. In our study, it seems that PSS is more sensitive for detecting the decrease in cardiac function than conventional M-mode.

Using strain analysis to measure cardiac dysfunction in young muscular dystrophy patients has been just considered as feasible and reproducible in 2015 [5] and a clinical trial to prove this has just begun in 2015 and is enrolling patients (ClinicalTrials.gov Identifier: NCT02418338).

The catecholamine stress test has been widely used in clinical diagnoses of patients [30–32] and animal models to detect altered β -adrenergic response [33, 34]. Dobutamine, a β 1-adrenergic receptor selective agonist, is often used for patients while isoprenaline, an adrenergic agonist activating both β 1- and β 2-adrenergic receptors, is often used for animals [30–32]. Combining dobutamine stress with STE has been used in the clinic to determine ischemic region [35] and predict myocardial recovery after revascularization [36]. In this study, we combined isoprenaline stress with echocardiography to determine if β -adrenergic responses of 3–5 month old *mdx* hearts were altered. Two dimensional-strain analysis in combination with catecholamine stress showed significant decreases in the responses of L-endo and radial PSS to isoprenaline at the age of 3 months. These results suggest that the combination of 2D-STE strain analysis and catecholamine stress is more sensitive than both 2D-STE strain analysis alone and the combination of conventional M-mode and catecholamine stress, which is a novel finding in this study. It seems that at rest and after isoprenaline, endocardial PSS are more sensitive than epicardial PSS to detect cardiac dysfunction in *mdx* mice. This could reflect the true sensitivity but also could be due to greater variation of epicardial strains. As Tomtec Image Arena 4.0 could not analyze average PSS of the full thickness of the left ventricular wall, it warrants further study to determine if the average PSS of the full thickness of the left ventricular wall could have the same sensitivity as endocardial PSS with other ultrasound imaging systems.

It is evident that different PSS measurements have varying sensitivities in distinguishing *mdx* and control mice. L-Endo and R PSS are the most sensitive measurements for detecting the difference between control and *mdx* mice. Endocardial PSS were more sensitive than epicardial PSS to distinguish between *mdx* and control mice. This may be caused by the structural and functional differences between the endocardium and epicardium. The endocardium contributes more to systolic movement of the heart than the epicardium [37–39]. Therefore, the sub-endocardial strain analysis may provide more information for early diagnosis of abnormal cardiac function/reserve.

When the increase of PSS is expressed as the percentage of increase, it is more sensitive than absolute PSS increase in differentiating *mdx* mice from control mice. For example, the C-Endo PSS could only detect altered cardiac β -adrenergic response in 5-month old *mdx* mice, but the percentage of increase in C-Endo PSS could differentiate between *mdx* mice and control mice of 3, 4, and 5 months. In normal human subjects, the strain responses to dobutamine depends on the age and the dose of dobutamine. Strains of young and healthy hearts have positive responses to low doses of dobutamine but may have plateau or slight decreases at peak doses of Isoprenaline such as 40 μ g/kg BW when there are significant increase of heart rates and decrease of stroke volume [40]. Even at high dobutamine or isoprenaline doses, the strain rate may still have a positive response [40] and thus could be more sensitive than strain for assessing cardiac β -adrenergic reserve.

Potential mechanisms of loss of β -adrenergic response in young *mdx* mice

Reduced β -adrenergic reserve is often an early alteration in the diseased heart [41]. The loss of dystrophin in DMD patients and *mdx* mice makes cardiomyocytes prone to death, causing injury of the heart even in young patients and *mdx* mice [42]. We detected significant myocyte dropout and replacement fibrosis in 2-month old *mdx* mouse hearts, but normal baseline cardiac function with ultrasound analysis [14]. The loss of cardiomyocytes in *mdx* mice may cause reactive β -adrenergic/sympathetic system activation, which has been indicated by the high basal heart rate in *mdx* mice and DMD patients [43] and high basal PKA activity in *mdx* mice [14]. Upon persistent activation of the sympathetic/adrenergic system (SAS), the myocytes may develop desensitization, e.g., the decrease of β 1-AR adrenergic receptors in ventricular myocytes, as we reported previously [14]. We also showed that young *mdx* mouse myocytes functionally remodel to enhance myocyte contractility to compensate for the loss of myocytes. This remodeling leaves less room for β -adrenergic agonists to stimulate myocytes [14]. In a single patient case report, it was shown that a 16-year old DMD patient had reduced cardiac contractile reserve upon dobutamine stimulation [15], supporting that dobutamine stress combined with echocardiography as an effective tool to diagnose cardiac dysfunction in DMD patients. In fact, 3-month old *mdx* mice is considered at very early stage of muscular dystrophy because of the slow progression of the disease in this model [16], but our approach was able to detect significant cardiac dysfunction.

Interestingly, we found no change in HR responses to isoprenaline between *mdx* and control mice at the ages of 3, 4 and 5 months, suggesting that the alterations of β -adrenergic responses of different cardiac regions, i.e., the sinoatrial node versus ventricular myocardium, could be different. Such dissociation of chronotropic and inotropic response to β -adrenergic stimulation has been reported in normal aging human heart [44].

The increases in myocardial dyssynchrony in young *mdx* mice after ISO stimulation

The normal function of the left ventricle relies on synchronous contraction of all portions of the ventricular wall to effectively pump the blood out of the heart [45]. The synchrony of contractions of different myocardial regions can be assessed by maximum difference in times to peak strains ($T_{\text{peak, max}}$) and standard deviation of T_{peak} . Dyssynchronized contraction of different portions of the left ventricular wall is often a subtle alteration of cardiac function before the appearance of overt cardiac dysfunction. In DMD patients, it was reported that posterolateral myocardial strain was delayed [20] corresponding to worse myocardial atrophy and fibrosis in this region. However, in young *mdx* mice, the fibrosis displays a random and scattered pattern [46] and we found no delay in a specific region (Supplemental Tables 1–3) at rest. Accordingly, we found that at baseline, all portions of the left ventricle in young *mdx* and control mice contract synchronically with the $T_{\text{peak, max}}$ less than 30 milliseconds at the heart rate of 400–450 beats per minute. Isoprenaline stimulation caused insignificant decreases in $T_{\text{Peak, max}}$ and T_{peak} SD/CC in control mice. In contrast, isoprenaline significantly increased the dyssynchrony in 4- and 5-month *mdx* mice though not at a specific region (Supplementary Table 1–3), which has not been reported previously. It has been reported that β -adrenergic stimulation can cause cardiac dyssynchrony if different portions of the myocardium have different sensitivities to β -

adrenergic agonists [47]. This could also be the cause of increased dyssynchrony induced by isoprenaline in *mdx* mice but needs to be further studied.

Significance of this study

Our study shows that catecholamine stress combined with high frequency ultrasound 2D STE is a sensitive noninvasive approach to detect early cardiac dysfunction in *mdx* mice as young as 3-month old. The reduced response of strains and induced regional contraction dyssynchrony by β -adrenergic stimulation may account for reduced exercise capability of young DMD patients other than degenerated skeletal muscles. So far this is the most sensitive method for studying cardiac abnormalities in this DMD mouse model. It has the potential to diagnose early cardiac dysfunction in very young (e.g., several years old or early teens) DMD patients and help decide when to start the treatment for cardiac dysfunction. This approach is instrumental for evaluating responses to treatment or the effect of different genetic variations in DMD patients and muscular dystrophy animal models. It can also be used to tackle other issues that might be difficult to study in patients with muscular dystrophy.

Limitations

Though our study suggests that the combination of echocardiography and catecholamine or exercise stress test could be a novel way to detect early cardiac dysfunction in young DMD patients, this type of test can only be done in those patients capable of exercise or tolerant of catecholamine/dobutamine stress. Furthermore, our study was performed with a DMD animal model (*mdx* mouse model) that does not have exactly the same disease process as DMD human patients [21]. Thus, our study needs to be verified with human patients. For example, in human patients, high doses of dobutamine may slightly decrease PSS while low doses of dobutamine may increase PSS. Therefore, we may need to use low dose of dobutamine in DMD patients and normal reference values need to be established in healthy age-matched volunteers. As our study was done under anesthesia causing the reduction of heart rate and cardiac muscle contractility, the ejection fraction and strain values are lower than those obtained at heart rates closer to physiological conditions [18]. Technically, the scanhead (RMV 707B) frame rate used in the study (up to 160fps) may not be ideal for strain analysis at high heart rates (e.g., after ISO) and the algorithm of strain analysis by Tomtec Image Arena may need to be validated with other software more specifically designed for small animals such as the proprietary software used by VisualSonics. However, when the heart rate was comparable (e.g., 500–550 bpm), the strain values obtained with our approach were comparable to those reported with the Vevo 2100 machine [18]. Nonetheless, the low frame rates of Vevo 770 may decrease the absolute PSS values measured. In addition, *mdx* mice younger than 3 months have not been studied.

Conclusions

Our study suggests that 2D-STE could detect the systolic dysfunction of *mdx* mouse hearts as early as 5 months of age and at even younger ages (3–4 months) when combined with catecholamine stress. This method is feasible, reproducible and sensitive for detecting LV myocardial dysfunction in young *mdx* mice. The approach that we have used may allow us

to evaluate responses to treatments, the effect of different genetic variations, and early cardiac dysfunction in patients with muscular dystrophy. Further investigation is warranted to confirm the utility of this new, sensitive and quantitative technique for detecting early LV dysfunction, and for assessing novel, safe, and efficacious preventive therapies for DMD patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Cardiac dysfunction becomes an important contributing factor for mortality and morbidity of muscular dystrophy patients
- There is still a lack of reliable approach to diagnose early cardiac dysfunction in DMD patients
- The combination of 2D strain analysis with isoprenaline stress showed reduced β -adrenergic reserve and increased contractile dyssynchrony in mdx mice at a very early stage
- 2D strain analysis with stress test is a feasible and sensitive approach to diagnose early cardiac function and to evaluate treatments in DMD patients
- The treatment of cardiac dysfunction should be started at young age for DMD patients

Conventional 2D ECHO

Figure 1.

Cardiac morphology and function evaluation by conventional M-mode echocardiography in control and *mdx* mice at the ages of 2, 4, 6, 8, 10 and 12 months. When the animals were imaged, the heart rate was kept in the range of 400–450 beats per minute (bpm) and there was no difference in heart rates between groups (A). Fractional shortening (B), diastolic left ventricular posterior wall (LVPD;d) thickness (C), diastolic interventricular septum (IVS;d) thickness (D), diastolic left ventricular internal diameter (LVID;d) (E) and corrected LV mass (F) were compared between control and *mdx* mice at different ages. ** or *: $p < 0.01$ or $p < 0.05$ *mdx* vs. control at the same age; ## or #: $p < 0.01$ or $p < 0.05$ vs. 2m-old *mdx* mice. \$\$ or \$: $p < 0.01$ or $p < 0.05$ vs. 2m-old control mice. The black numbers are the numbers of control mice studied and the gray numbers are the numbers of *mdx* mice studied.

Conventional 2D ECHO + Isoprenaline stress

Figure 2.

Effects of isoprenaline (ISO) on cardiac function evaluated by conventional echocardiography. **A & B.** M-mode images of control (A) and *mdx* (B) mice at baseline and after ISO. **C.** Heart rates of control (3-, 4- or 5- month old, 5 mice per age group) and *mdx* mice (3-, 4- or 5- month old, 7 mice per age group) before and after ISO. **D.** Left ventricular fractional shortening of control and *mdx* hearts before and after ISO. ** or *: $p < 0.01$ or $p < 0.05$ ISO vs. rest at the same age of control mice; ## or #560: $p < 0.01$ or $p < 0.05$ ISO vs. rest at the same age of *mdx* mice.

2D strain analysis-peak strain

Figure 3.

Peak systolic strain (PSS) of control and *mdx* mice at the ages of 3, 4, 5, 6, 9 and 12 months. Longitudinal endomyocardial (L-endo) PSS (A), longitudinal epimyocardial (L-epi) PSS (B), circumferential endomyocardial (C-endo) PSS (C), circumferential epimyocardial (C-epi) PSS (D), and radial PSS (E) were measured at rest. *: $p < 0.05$ *mdx* vs. control mice at the same age.

Figure 4.

Peak systolic strain (PSS) of young control and *mdx* mice at the ages of 3, 4, and 5 months before and after maximum isoprenaline effect. Animals were anesthetized with isoflurane to keep the heart rate within the 400–450 beats per minute (bpm) range. Baseline short-axis and long-axis B-mode images were recorded at rest and then isoprenaline (ISO, 2 μ g/g BW) was injected intraperitoneally. Short-axis and long-axis B-mode images were recorded every minute until 15 minutes after ISO injection. Maximum PSS values after ISO were used to reflect maximum effect of ISO. Longitudinal endomyocardial (L-endo) PSS (A), longitudinal epimyocardial (L-epi) PSS, circumferential endomyocardial (C-endo) PSS (C), circumferential epimyocardial (C-epi) PSS (D), and radial PSS (E) were measured before and after ISO. *, **, ***: $p < 0.05$, $p < 0.01$, $p < 0.001$ ISO vs. baseline in control mice at the same age; #: $p < 0.05$, ISO vs. baseline in *mdx* mice at the same age; \$574, \$\$, \$\$\$575: $p < 0.05$, $p < 0.01$, $p < 0.001$ control vs. *mdx* after ISO at the same age. Two-way repeated ANOVA with post-hoc tests with Bonferroni adjustment was done for comparing pairs.

Figure 5.

Percentage of increase in each PSS by isoprenaline (ISO, 2 μ g/g BW, i.p.) in control and *mdx* mice at the ages of 3, 4 and 5 months. The percentage of increase of L-endo (A), L-epi (B), C-endo (C), C-epi (D) and radial (E) PSS by ISO was compared between young *mdx* and control mice. *, **, ***: $p < 0.05$, $p < 0.01$, and $p < 0.001$ *mdx* vs. control at the same age; statistical analysis was done using one-way ANOVA with post-hoc test with Bonferroni adjustment.

2D strain analysis + isoprenaline stress
 $-\Delta t_{\text{peak, max}}$ normalized to cardiac cycle duration

Figure 6.

Maximum differences in time to peak ($T_{\text{peak, max}}$) of systolic strains normalized to the duration of cardiac cycle (CC) of young control and *mdx* mice at the ages of 3, 4, and 5 months before and after maximum isoprenaline effect. $T_{\text{peak, max}}$ was measured when a maximum effect of ISO ($2\mu\text{g/g BW}$) was observed. $T_{\text{peak, max}}/\text{CC}$ ratios of longitudinal endomyocardial (L-endo) (A), longitudinal epimyocardial (L-epi) (B), circumferential endomyocardial (C-endo) (C), circumferential epimyocardial (C-epi) (D), and radial (E) strains were analyzed before and after ISO. #, ##587: $p<0.05$, $p<0.01$, ISO vs. baseline in *mdx* mice at the same age; \$, \$\$588: $p<0.05$, $p<0.01$, control vs. *mdx* after ISO at the same age. Two-way repeated ANOVA with post-hoc tests with Bonferroni adjustment was done to compare pairs.

2D strain analysis + isoprenaline stress
-Standard deviation of Tpeak normalized to cardiac cycle

Figure 7.

Standard deviations (SD) of T_{peak} of systolic strains normalized to cardiac cycle (CC, i.e., R-R interval) of control and mdx mice at baseline and after maximum isoprenaline effect. SD of T_{peak}/CC of L-endo (A), L-epi (B), C-endo (C), C-epi (D) and radial (E) strains. \$594: $p < 0.05$, mdx vs. control at baseline at the same ages; &595: $p < 0.05$, $p < 0.01$, control vs. mdx after ISO at the same age. Two-way repeated ANOVA with post-hoc tests with Bonferroni adjustment was done for comparing pairs.

Intra-observer and inter-observer variation and intraclass correlation of L-Endo, L-Epi, C-Endo, C-Epi, and R PSS at rest and after ISO stress (mean±SD)

Table 1

Strains	Rest (n=20)				Stress (n=20)			
	Intra-observer		Inter-observer		Intra-observer		Inter-observer	
	Variation (%)	ICC	Variation (%)	ICC	Variation (%)	ICC	Variation (%)	ICC
L-Endo	5.30±3.16	0.965	6.58±4.43	0.947	5.57±3.95	0.958	6.79±4.73	0.921
L-Epi	6.09±4.41	0.932	7.08±3.85	0.970	6.21±4.62	0.927	7.52±4.74	0.960
C-Endo	5.74±3.26	0.936	6.64±3.33	0.958	6.13±4.12	0.931	6.91±4.02	0.951
C-Epi	6.82±4.07	0.900	7.06±3.91	0.960	7.12±4.62	0.920	8.16±4.59	0.934
R	5.73±3.84	0.929	6.54±3.71	0.956	5.94±3.80	0.932	7.88±4.68	0.917

ICC: intraclass correlation.