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IMPAIRED RESPIRATORY FUNCTION IN *MDX* AND *MDX/UTRN+/−* MICE

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Abstract

Introduction—Muscle fibrosis is a prominent pathological feature that directly causes muscle dysfunction in Duchenne muscular dystrophy (DMD). The DMD mouse models, *mdx* mice and *mdx* mice with haploinsufficiency of the utrophin gene (*mdx/utrn*+/−), display progressive diaphragm fibrosis.

Materials and Methods—We performed unrestrained whole-body plethysmography (WBP) in *mdx* and *mdx/utrn*+/− mice, and compared them with wild-type controls.

Results—Respiratory function gauged by respiratory frequency, tidal volume, minute volume, peak inspiratory flow, and peak expiratory flow was significantly impaired in *mdx* mice. Consistent with more severe diaphragm fibrosis in *mdx/utrn*+/− mice, respiratory impairment was worse than in *mdx* mice at 6 months.

Discussion—WBP is useful in monitoring in vivo respiratory function of *mdx* and *mdx/utrn*+/− mice, and it may serve as an outcome measurement of therapies that target diaphragm fibrosis. *Mdx/utrn*+/− mice may be a better model than *mdx* mice for testing antifibrotic therapies, especially at a severe stage.

Keywords

Duchenne muscular dystrophy; *mdx* mice; diaphragm fibrosis; respiratory function; whole body non-invasive plethysmography

INTRODUCTION

DMD is the most common genetic muscle disease, affecting 1 in 3,500 live male births (1). Caused by a defective dystrophin gene on the X chromosome, DMD is characterized by progressive skeletal and cardiac muscle weakness with premature death (2). Muscle inflammation and fibrosis are prominent pathological features of DMD, which lead to muscle dysfunction and clinical weakness.

Mdx mice, which harbor a nonsense mutation in exon 23 of the dystrophin gene, have been widely used to study the pathophysiology and explore potential therapies for DMD. However, the muscular dystrophy phenotype in this mouse model is mild as compared to

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human DMD. Skeletal muscle inflammation starts at 3 weeks of age, peaks between 8 and 16 weeks, and then subsides spontaneously in limb muscles. Slowly progressive endomysial fibrosis only develops in the diaphragm (3–5) and causes impaired contractility (6, 7). To improve this model, mice deficient in both the dystrophin gene and its autosomal homolog utrophin gene (*dko* mice) were generated (8, 9). *Dko* mice display early onset of muscle dystrophy, severe muscle weakness, joint contracture, growth retardation, and premature death, which mimics the phenotype of human DMD (8). However, early death at around 10 weeks of age makes *dko* mice difficult to obtain or maintain for testing therapeutic interventions. To circumvent this difficulty, we previously characterized and quantified skeletal muscle fibrosis and inflammation in *mdx/utrn*+/− mice, and compared them with *mdx* mice (10). Diaphragm inflammation and fibrosis were worse in *mdx*/*utrn*+/− mice than in *mdx* mice at 3 and 6 months of age. At 6 months, diaphragm fibrosis was moderate in *mdx* mice but severe in *mdx/utrn*+/− mice. This may provide a better model for testing antifibrotic therapies (10).

There is no effective therapy for DMD at this point. *Mdx* mice have been widely used for testing potential therapies. In vivo functional improvement would be an important criterion to assess the effectiveness of potential therapies. Therefore, in this study we performed whole body plethysmography (WBP) in *mdx* and *mdx/utrn*+/− mice to address whether respiratory function was impaired in *mdx* mice and was more impaired in *mdx/utrn*+/− mice to correspond to more severe diaphragm fibrosis. We explored whether WBP could be used to serve as a functional measurement of diaphragm muscle dystrophy in *mdx* and *mdx/utrn*+/− mice.

MATERIALS AND METHODS

Animals

Male *mdx* mice (C57BL/10J) were purchased from the Jackson laboratory or obtained from crossbreeding of *mdx* and *mdx/utrn*+/− mice. *Mdx/utrn*+/− mice were kindly provided by Dr. Jill Rafael-Fortney (Columbus, OH) and were crossed with *mdx* mice (C57BL/10J) from the Jackson lab for at least 9 generations. Wild-type C57BL/10J mice were also purchased from the Jackson laboratory. All three types of mice were in the C57BL/10J background. Mice at age 3 months (BL10: n=19, *mdx:* n=21, and *mdx/utrn*+/−: n=17) and 6 months (BL10: n=26, *mdx*: n=24, and *mdx/utrn*+/−: n=20) were used for the study. We followed the Cleveland Clinic guide for the care and use of laboratory animals.

Respiratory function analysis

Respiratory function in conscious and unrestrained mice was assessed by whole body non-invasive plethysmography (WBP) (Buxco Research Systems, Sharon, CT) and Biosytems XA software. Unrestrained Plethysmograph was calibrated according to the manufacturer's instructions (User manual for Whole Body Unrestrained Plethysmographs PLY3211). The Bias Flow Regulator was calibrated with room temperature and humidity and flow set at 1.2 liter per minute. Mice were placed in the "free moving" plethysmograph chamber and allowed to acclimate for 5 minutes. Afterwards, measurement of airway mechanics, including respiratory frequency (F), tidal volume (TV), minute volume (MV), peak inspiratory flow (PIF), and peak expiratory flow (PEF), were taken over a ten-minute interval. Values measured during the 10-minute sequence were then averaged.

Histopathological analysis

Muscle tissue was fresh-frozen in liquid nitrogen-cooled isopentane, sectioned at 8 micron (μm), stained with hematoxylin and eosin, and viewed under a bright field microscope.

Diaphragm collagen measurements

The diaphragm collagen content was measured by the Sircol collagen assay (Bioclor Ltd., Belfast, UK) following the manufacturer's instructions. Briefly, Sirius red reagent was added to each diaphragm homogenate and mixed for 30 minutes. The collagen-dye complex was precipitated by centrifugation at 16,000 g for 5 minutes, washed with ethanol, and dissolved in 0.5 M NaOH. The samples were then transferred to a microplate reader, and the absorbance was determined at 540 nm.

Statistical analysis

Data were expressed as mean ±SD. Differences of measurements among different groups were evaluated by the Kruskal-Wallis test. The association between the diaphragm collagen content and the individual respiratory parameters was evaluated by the Pearson correlation. A p-value of <0.05 was considered statistically significant.

RESULTS

Respiratory function was impaired in mdx and mdx/utrn+/− mice at 3 months of age

At 3 months of age, *mdx* and *mdx/utrn*+/− mice displayed multifocal endomysial inflammation in respiratory muscles, including diaphragm and intercostal muscles (Fig. 1) (10), and mild endomysial fibrosis in the diaphragm (10). To address whether respiratory function was impaired in *mdx* and *mdx/utrn*+/− mice as a result of respiratory muscle pathology, we performed whole body non-invasive plethysmography on these mice at 3 months of age, and compared them with age-controlled wild-type BL10 controls. Respiratory frequency, tidal volume, minute volume, peak inspiratory flow, and peak expiratory flow were all significantly reduced in *mdx* and *mdx/utrn*+/− mice (Table 1). Tidal volume adjusted by body weight (ml/kg) was also significantly reduced in *mdx* ($p<0.001$) and *mdx/utrn*+/− mice ($p<0.001$) as compared with controls. There was no significant difference in the respiratory function parameters between *mdx* and *mdx/utrn*+/− mice at this age.

Respiratory function was impaired in mdx and mdx/utrn+/− mice at 6 months of age, and it was worse in mdx/utrn+/− mice

Mdx and *mdx/utrn*+/− mice displayed remarkable diaphragm fibrosis at 6 months of age that was worse in *mdx/utrn*+/− mice (10). At this age, diaphragm fibrosis was moderate in *mdx* mice, and it was severe in *mdx/utrn*+/− mice (10). Intercostal muscles also showed scattered endomysial inflammation with mild fibrosis in *mdx* and *mdx/utrn*+/− mice (Fig. 1). To address whether respiratory function was impaired in *mdx* and *mdx/utrn*+/− mice as a result of prominent diaphragm fibrosis and intercostal muscle pathology, we performed whole body non-invasive plethysmography at 6 months of age, and compared the results with age-controlled wild-type BL10 controls. Respiratory frequency, tidal volume, minute volume, peak inspiratory flow, and peak expiratory flow were all significantly reduced in *mdx* and *mdx/utrn*+/− mice (Table 2). Tidal volume adjusted by body weight (ml/kg) was also significantly reduced in *mdx* ($p<0.001$) and *mdx/utrn*+/− mice ($p<0.001$) as compared with controls. Consistent with more severe diaphragm fibrosis in *mdx/utrn*+/− mice than in *mdx* mice (10) (Fig. 1), tidal volume ($p=0.001$), tidal volume adjusted by body weight ($p=0.002$), minute volume ($p=0.04$), and peak inspiratory flow ($p=0.04$) were more severely impaired in *mdx/utrn*+/− mice than in *mdx* mice. The degree of diaphragm fibrosis gauged by the diaphragm collagen content inversely correlated with tidal volume ($r=-0.59$, $p<0.01$), minute volume ($r=-0.64$, $p<0.001$), peak inspiratory flow ($r=-0.60$, $p<0.01$), and peak expiratory flow ($r=-0.41$, $p<0.05$), but not respiratory frequency (Fig. 2).

DISCUSSION

Skeletal muscle fibrosis is a prominent pathological feature of muscle in patients with DMD, and it directly causes muscle dysfunction and clinical weakness. Studies by our lab and others using the *mdx* mouse model showed that ameliorating skeletal muscle fibrosis may represent a useful therapeutic approach to slow down disease progression and improve the clinical phenotype (11, 12). The diaphragm is the major muscle in *mdx* mice that undergoes remarkable progressive fibrosis. It is thus useful for studying fibrogenesis associated with dystrophin deficiency and for testing potential antifibrotic therapies. Our previous study showed that diaphragm fibrosis was worse in *mdx/utrn*+/− mice than in *mdx* mice (10), which provides a better model for testing antifibrotic therapies, especially at a severe stage of fibrosis. Consistent with progressive diaphragm fibrosis, in vitro diaphragm contractility was greatly impaired in *mdx* mice (6, 7). However, in order to assess the effect of a potential antifibrotic therapy in the DMD mouse models, an easy and reliable in vivo function test is very much desired. Since the diaphragm is one of the respiratory muscles, which also include intercostal muscles, we assessed respiratory function by whole body non-invasive plethysmography to see if it was significantly impaired in *mdx* and *mdx/utrn*+/− mice and would correlate with diaphragm fibrosis. Our findings demonstrated that several respiratory function parameters gauged by WBP were significantly impaired in *mdx* and *mdx/utrn*+/− mice and were worse in *mdx/utrn*+/− mice. The diaphragm collagen content inversely correlated with the respiratory parameters, including tidal volume, minute volume, peak inspiratory flow, and peak expiratory flow. Therefore, WBP can serve as a valuable respiratory function test to assess the in vivo functional changes of the diaphragm and can be used as an outcome measurement of antifibrotic therapies.

At 3 months of age, both diaphragm and intercostal muscles show multifocal endomysial inflammation, and the diaphragm also shows mild endomysial fibrosis in *mdx* and *mdx/utrn* +/− mice (10). Impaired respiratory function at this age is likely the result of dysfunction of both types of respiratory muscles. At 6 months of age, inflammation remained in intercostal muscles of *mdx* and *mdx/utrn*+/− mice, and only mild morphological fibrosis was detected. However, diaphragm at this age shows remarkable endomysial fibrosis. It is moderate in *mdx* mice and severe in *mdx/utrn*+/− mice (10). Therefore, impaired respiratory function at this age is greatly affected by diaphragm dysfunction due to fibrosis. Consistent with more severe fibrosis in *mdx/utrn*+/− mice at this age, three respiratory function parameters, including tidal volume, minute volume, and peak inspiratory flow, are more severely reduced in *mdx/utrn*+/− mice than in *mdx* mice. Peak inspiratory flow mainly reflects the function of the diaphragm as it contracts and descends to create negative intrathoracic pressure for air inspiration. Peak expiratory flow mainly reflects the function of intercostal muscles. The significant reduction in PIF but not in PEF in *mdx/utrn*+/− mice as compared with *mdx* mice at 6 months of age is consistent with the finding that worsening of diaphragm fibrosis in *mdx/utrn*+/− mice is out of proportion to the worsening of intercostal muscle pathology in comparison with *mdx* mice.

Using plethysmography to compare respiratory function in *mdx* and BL10 mice has been previously reported by a few groups (6, 13, 14). Gosselin et al reported that tidal volume was not significantly different in wild-type and *mdx* mice at 7 months of age, but it was lower in *mdx* mice than in wild-type mice in response to hypercapnia (6). Gayraud et al reported no difference in respiratory rate, tidal volume, or minute ventilation during air breathing or hypercapnia in *mdx* and wild-type mice at 5 months of age, but respiratory rate and minute ventilation were significantly lower in *mdx* mice than in wild-type controls at 16 months of age in response to hypercapnia (13). We used more recent and better version of plethysmography than these two groups did, and our results were different. Our study showed that respiratory rate, tidal volume, and minute volume during air breathing were all

significantly reduced in *mdx* and *mdx/utrn*+/− mice at both 3 and 6 months of age. Ishizaki et al (14) appeared to use a similar version of WBP from Buxco Electronics as we did. They showed that respiratory rate was reduced in *mdx* mice at 2 and 4 months of age, but it increased at 7 months of age as compared with wild-type controls. Our results showed reduced respiratory frequency at both 3 and 6 months of age. Their study showed no significant change in tidal volume in *mdx* mice as compared with wild-type mice at 2 and 4 months of age, but it was reduced at 7 months of age. Our study showed unequivocal reduction of tidal volume and minute volume (even adjusted by body weight) in *mdx* and *mdx/utrn*+/− mice at both 3 and 6 months of age, which was worse in *mdx/utrn*+/− mice at 6 months of age. In addition, they did not show PIF and PEF results, and our results showed that both PIF and PEF were significantly reduced in *mdx* and *mdx/utrn*+/− mice at 3 and 6 months of age, with PIF being worse in *mdx/utrn*+/− mice than in *mdx* mice at 6 months of age. The discrepancies may be in part due to differences in plethysmography calibration which is critical, and their method of calibration was not described (14). Since we noticed individual variations in respiratory parameters, we used a large number of mice (around 20 per group) for our study. Ishizaki et al (14) used only 4–8 mice per group for their study, which was probably not powerful enough to detect significant differences in respiratory parameters between *mdx* and control mice.

We chose to study respiratory function in mice at 3 and 6 months of age, because our previous diaphragm muscle pathology quantification and comparisons were done in *mdx* and *mdx/utrn*+/− mice at these two ages. Our previous studies showed that diaphragm fibrosis was mild in *mdx* and *mdx/utrn*+/− mice at 3 months of age, moderate in *mdx* mice at 6 months of age, and severe in *mdx/utrn*+/− mice at 6 months of age. Consistent with the pathology findings, this study showed that respiratory function was impaired in *mdx* and *mdx/utrn*+/− mice at both ages, worse in *mdx/utrn*+/− mice at 6 months of age. Our findings suggest that the respiratory parameters, including TV, MV, PIF and PEF, can be used to assess in vivo respiratory muscle function in DMD mouse models and can serve as a useful functional outcome measurement for antifibrotic therapies. The findings further support the notion that with more severe diaphragm fibrosis and greater impairment of respiratory function, *mdx/utrn*+/− mice may serve as a better model than *mdx* mice for testing antifibrotic therapies, especially at a severe stage of muscle fibrosis.

Acknowledgments

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Abbreviations

DMD	Duchenne muscular dystrophy
WBP	whole-body plethysmography
F	respiratory frequency
TV	tidal volume
MV	minute volume
PEF	peak expiratory flow
PIF	peak inspiratory flow

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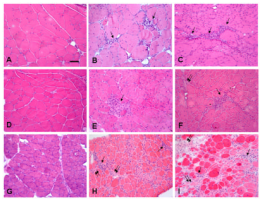

Figure 1.

Inflammation and fibrosis in respiratory muscles of *mdx* and *mdx/utrn*+/− mice.

H&E staining showed multifocal small foci of endomysial inflammatory cells (arrows) in intercostal muscles of *mdx* (B, E) and *mdx/utrn*+/− (C, F) mice at 3 (B, C) and 6 (E, F) months of age. Inflammatory cells (arrows) were more widespread in the endomysium of diaphragm muscles of *mdx* (H) and *mdx/utrn*+/− mice (I) at 6 months of age. Focal areas of increased endomysial collagen deposition were detected at 6 months of age (double arrows). They were mild in intercostal muscles of *mdx/utrn*+/− (F) mice, moderate in the diaphragm of *mdx* mice (H), and severe in the diaphragm of *mdx/utrn*+/− mice (I). Wild-type BL10 mice showed no inflammation or fibrosis in intercostal muscles at 3 (A) or 6 (D) months of age or diaphragm at 6 months of age (G). Bar=50μm.

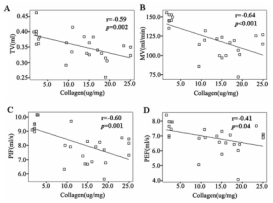

Figure 2.

Diaphragm collagen content inversely correlated with respiratory parameters.

The scatter plots show that the diaphragm collagen content inversely correlated with tidal volume (A), minute volume (B), peak inspiratory flow (C), and peak expiratory flow (D).

Table 1

Respiratory Parameters in BL10, *Mdx*, and *Mdx/utrn*+/− Mice at 3 Months

	BL10	***Mdx***	***Mdx/utrn*+/−**	***p1**	***p2**	***p3**
F (breath/min)	426.5±24.1	347.6±50.2	344.9±31.6	<0.001	<0.001	0.97
TV (ml)	0.37±0.05	0.29±0.04	0.28±0.05	<0.001	<0.001	0.55
TV/BW (ml/kg)	12.5±1.9	9.3±1.3	8.9±1.5	<0.001	<0.001	0.40
MV (ml)	154.4±19.4	100.5±20.9	95.5±19.4	<0.001	<0.001	0.48
PIF (ml/s)	10.7±1.6	7.6±1.5	7.3±1.1	<0.001	<0.001	0.51
PEF (ml/s)	8.1±1.1	6.4±1.6	6.4±1.2	<0.001	<0.001	0.90

* p1: comparisons between BL10 and *mdx* mice;

p2: comparisons between BL10 and *mdx/utrn*+/− mice;

p3: comparisons between *mdx* and *mdx/utrn*+/− mice.

Table 2

Respiratory Parameters in BL10, *Mdx*, and *Mdx/utrn*+/− Mice at 6 Months

	BL10	***Mdx***	***Mdx/utrn*+/−**	***p1**	***p2**	***p3**
F (breath/min)	400.9±26.9	352.16±49.7	349.3±23.8	<0.01	<0.001	0.80
TV (ml)	0.42±0.06	0.37±0.05	0.32±0.03	0.002	<0.001	0.001
TV/BW (ml/kg)	12.1±1.7	10.2±1.5	9.1±0.7	<0.001	<0.001	0.002
MV (ml)	166.0±23.8	126.8±27.5	111.7±15.6	<0.001	<0.001	0.04
PIF (ml/s)	11.1±1.5	8.9±2.0	7.7±0.9	0.001	<0.001	0.04
PEF (ml/s)	9.1±1.9	7.8±2.0	6.9±1.0	0.03	<0.001	0.10

* p1: comparisons between BL10 and *mdx* mice;

p2: comparisons between BL10 and *mdx/utrn*+/− mice;

p3: comparisons between *mdx* and *mdx/utrn*+/−mice.