The diaphragm muscle strip preparation for evaluation of gene therapies in mdx mice

John A. Faulkner,** Rainer Ng,† Carol S. Davis,* Sheng Li‡§ and Jeffrey S. Chamberlain‡§

*Department of Molecular & Integrative Physiology, School of Medicine;  
†Department of Biomedical Engineering, College of Engineering, University of Michigan, Ann Arbor, Michigan 48109-2007, USA and  
‡Department of Neurology and †The Senator Paul D. Wellstone Muscular Dystrophy Cooperative Research Center, University of Washington, School of Medicine, Seattle, Washington 98195-7720, USA.

Summary

1. Duchenne muscular dystrophy (DMD), a severe muscle wasting disease of young boys with an incidence of one in every 3,000, results from a mutation in the gene that encodes dystrophin. The absence of dystrophin expression in skeletal muscles and heart results in the degeneration of muscle fibres and consequently severe muscle weakness and wasting. The mdx mouse discovered in 1984, with some adjustments for differences, has proven to be an invaluable model for scientific investigations of dystrophy.

2. The development of the diaphragm strip preparation by Ritchie in 195427 provided an ideal experimental model for investigations of the skeletal muscle impairments in structure and function, induced by the interactions of the disease-related and the age-related factors. Unlike the limb muscles of the mdx mouse that show adaptive changes in structure and function, the diaphragm strip preparation reflects accurately the deterioration in muscle structure and function observed in boys with DMD.

3. The advent of sophisticated servo motors and force transducers interfaced with state-of-the-art software packages to drive complex experimental designs during the 1990s greatly enhanced the capability of the mdx mouse and the diaphragm strip preparation to evaluate more accurately the impact of the disease on the structure-function relationships throughout the life span of the mouse.

4. Finally, during the 1990s and through the early years of the 21st century, a wide variety of promising, sophisticated genetic techniques have been designed to ameliorate the devastating impact of muscular dystrophy on the structure and function of skeletal muscles. During this period of rapid development of promising genetic therapies, the combination of the mdx mouse and the diaphragm strip preparation has provided an ideal model for the evaluation of the success, or failure, of these genetic techniques to improve dystrophic muscle structure, function, or both. With the two year life span of the mdx mouse, the impact of age-related effects can be studied in this model.

The mdx mouse as a model for Duchenne muscular dystrophy

In Duchenne muscular dystrophy (DMD), a mutation in the gene that encodes dystrophin results in the absence of dystrophin expression. Dystrophin and the dystrophin-glycoprotein complex (DGC) appear to link the costomeric structures of the cytoskeleton to the basement membrane of muscle fibres.1,2 The exact linkage of the basement membrane adjacent to the z-lines to the collagen-1 and collagen-3 in the extracellular matrix is unknown. The lack of dystrophin expression in muscle fibres leads to a progressive weakness and wasting of skeletal muscles of boys with DMD that leads to confinement to wheelchairs by ~12 years of age and most boys are deceased by their mid-twenties due to respiratory or cardiac failure.3 The mdx mouse discovered by Bulfield and his colleagues in 19844 lacks dystrophin expression in muscle and consequently provides an effective small animal model for studies of the effects of the lack of dystrophin on skeletal muscle structure and function throughout the life span.5 The mdx mouse has also provided a model for the impact of the lack of dystrophin on the length of life of the mouse,6 on the structure and function of different skeletal muscles of the limbs,7 on the limb skeletal muscles throughout the life span,5 on the susceptibility to contraction-induced injury8-14 and on the efficacy of a variety of implantation15 and genetic studies to reverse the dystrophic symptoms.16-19

The mdx mouse does have some short-comings as a model for investigations of the development of structural and functional deficiencies in skeletal muscles due to the absence of dystrophin expression. The major deficiency is that adaptations occur in limb muscles of mdx mice and these adaptations result in an hypertrophy of the skeletal muscles of the limbs.5 The hypertrophy produces a substantial increase in muscle mass that maintains maximum isometric force, but due to the presence of damaged fibres, results in an ∼20% loss in maximum specific force.5,7 Interestingly, the diaphragm muscle does not undergo such an adaptation. Consequently, the diaphragm muscle of the mdx mouse more accurately reflects the degenerative changes observed in both limb and diaphragm muscles of boys with DMD.7,12,13,19-24 Because of the more accurate representation of the degenerative changes, we have utilized the diaphragm strip preparation to investigate the progression of the muscle weakness and muscle wasting throughout the life span of the mdx mouse, as well as the effect of various genetic therapeutic interventions. The comparisons among the mdx, wild type (WT) and transgenic mice include the capacity for the development of maximum specific force, normalized power.
Diaphragm strips in gene therapy

and the susceptibility to injury when subjected to protocols of lengthening contractions.

The diaphragm strip preparation of the mdx mouse

Our laboratory has had a long and sustained interest in the structure and function of the diaphragm muscle. In 1954, Ritchie first described the diaphragm strip preparation of the rat. The diaphragm strip provided an immediate, novel and innovative approach to the function of the diaphragm. The rat was anesthetized by an appropriate anesthetic administered as required to keep the rat unresponsive to tactile stimuli. The surgical procedure then consisted of excising the total diaphragm muscle from the anesthetized rat, and immersing the diaphragm muscle with rib attachments and central tendon intact in an oxygenated bath of physiological saline. A small strip of intact muscle fibres ~5 mm wide was then dissected carefully from the central tendon to the attachment of the fibres on a single rib. The diaphragm strip was then attached to a servo motor and a force transducer and measurements of force were made during shortening, isometric and lengthening contractions.

Such a diaphragm strip preparation obtained from mdx mice could then be used for investigations of the effects of the lack of dystrophin on the mechanical properties, maximum specific force, maximum normalized power and susceptibility to injury during lengthening contractions, or of the effectiveness of various gene therapies in reversing the dystrophic symptoms. Such studies with the diaphragm strip preparation have demonstrated the relative effectiveness of restoring various highly functional full length or mini-dystrophin fusion genes for cell and gene therapy of DMD.

The diaphragm strip preparation during aging of mdx mice

Dupont-Versteegen & McCarter provided an early, insightful comparison of the age-related changes up to 20 months of age for the maximum specific forces of diaphragm strips, soleus muscles and extensor digitorum longus (EDL) muscles of mdx and WT mice. The soleus and EDL muscles of mdx and WT mice showed rapid increases in specific force up to 4 months of age with a plateau at ~205 kN/m² for mdx and ~245 kN/m² for WT mice. For both mdx and WT mice, the plateau in force extended to 20 months of age. Unfortunately, the values for specific forces for diaphragm strips of WT mice varied so greatly with a range of 160 to 300 kN/m², compared with a normal range of 210 to 270 kN/m², that age-related changes were not discernable even for WT mice. Despite the great variability among the specific forces of the diaphragm strips of both the mdx and WT mice in the Dupont-Versteegen & McCarter study, the values for the specific forces of the two groups showed no overlap at any age providing strong evidence of a deficit for mdx mice.

Our data on diaphragm strips now extend from sampling points between 14 days and 24 months of age. The 24 months of age is within a few months of the maximum life span of 28 months for mdx mice, compared with the 36 months of age life span for WT mice. For both young and old WT mice, the histological sections of the diaphragm strips showed tightly packed viable fibres. Consequently, the specific forces and normalized power were simply divided by the total CSA of the diaphragm strip. Throughout the life span, the values for the diaphragm strips of the WT mice from 240 to 250 kN/m² for the maximum specific forces, and from 54 to 55 W/Kg. for maximum normalized powers are in excellent agreement with each other and with control values for limb muscles of WT mice. In contrast to the WT mice with 100% of the CSA composed of viable muscle fibres, only 70% of the CSA of the diaphragm strips of the young mdx mice was composed of viable fibres and for the old mdx mice the percentage of the CSA composed of viable fibres had decreased to 41%. Even with corrections for the damaged and necrotic fibres, the diaphragm strip preparations of the mdx mice at 3 months of age had specific forces only 62% of the value for WT mice and at 24 months only 48% of the WT value (Figure 1). Similarly, with the same corrections for volume, the normalized power of the diaphragm strips of the young mdx mice were 45% that of the WT mice and for the old mdx mice 30% (Figure 2).

Figure 1. Comparisons of the maximum specific forces of the diaphragm strips obtained from mdx and wild type (WT) mice at 3, 5, 17 (WT mice only) and 24 months of age. The maximum isometric forces were normalized to specific force by dividing the force developed by the diaphragm strips of the WT mice by the total cross-sectional area of the diaphragm strips and for the mdx mice by dividing the maximum force by the total area of viable fibres in the diaphragm strip (dashed lines), or by the total cross-sectional area of the diaphragm strip including the damaged and ‘ghost’ fibres (see text for definition) (dotted lines).
mice throughout their life span, the specific force and normalized power of the diaphragm muscle of the WT mice.

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Although age-related changes in specific force had been investigated in limb muscles of mdx mice, the limb muscles of mdx mice adapt to ongoing damage to fibres with a 20% to 30% hypertrophy. In contrast, the diaphragm muscle of the mdx mouse does not appear to undergo such an adaptation and consequently the diaphragm strip has been extremely useful in following the age-related changes that occur in skeletal muscles of mdx mice. The age-related changes that occur in skeletal muscles of mdx mice unimpeded by any adaptive changes. Our data for the specific forces of the diaphragm strips for both the mdx and WT mice indicate a clear age-related decline in specific force for diaphragm strips normalized for viable fibre CSA and even more steeply for specific forces normalized for total CSA of the diaphragm strips. For WT mice, the EDL and soleus muscles display a loss of ~30% in maximum specific force and maximum normalized power by 28 months of age and presumably the diaphragm strips from diaphragm muscles of WT mice would show a similar deficit. Certainly, respiratory function of elderly humans is decreased considerably. For mdx mice similar deficits in structure and function of the diaphragm muscles undoubtedly occur, but at an even earlier age than for the WT mice. For mdx mice, the average life span (50% of the cohort deceased) is 22 months and the maximum life span is 28 months, compared with 27 months and 36 months for WT mice. Consequently, with a linear decline in specific force and normalized power of the diaphragm muscle of the mdx mice throughout their life span, the specific force and normalized power of the total diaphragm strips, not corrected for damaged fibres are 19% and 10% of the values for WT mice. These values place the mdx mice at grave risk of respiratory failure. Consequently, the citing of respiratory failure, along with heart failure, as the major causes of the shortened life-span of the mdx mice, is not surprising.

The diaphragm strip preparation of the mdx mouse in assessments of cell and gene therapy

Based on the difficulties that arose through our comparisons of data on the specific forces measured on diaphragm strips of mdx and WT mice by different laboratories (see Section on Age-related Changes), the decision was made to restrict the assessments of cell and gene therapy to data obtained through collaborations between the Chamberlain laboratory performing interventions utilizing cell and gene therapy techniques and the Faulkner laboratory performing the assessments of muscle contractility.

**Figure 2.** Bar-graphs of the maximum normalized power developed by the diaphragm strips obtained from the diaphragm muscles of the WT and mdx mice. The maximum power developed by the diaphragm strips of mdx and WT mice were normalized for the WT mice by the mass of the total diaphragm strip, for the mass of the viable fibres in the diaphragm strips of the mdx mice and by the total mass of the diaphragm strip including the mass of the damaged and even ‘ghost’ (see text for definition) fibres for the mdx mice. Asterisk indicates a difference (P ≤ 0.001) from WT; double asterisk indicates a difference from mdx (corrected) mice.

**Figure 3.** Bar-graphs for the specific force developed by the diaphragm strips obtained from the diaphragm muscles of the WT and mdx mice and mdx mice treated with various genetic therapies. In transgenic mdx mice, 3 months of age, Cox et al., expressed dystrophin; 4 months of age, Rafael et al., expressed a truncated mini-gene; 6 months of age, Crawford et al., deleted the entire COOH-terminal domain of the dystrophin molecule; 3 months of age, Harper et al., deleted multiple regions of the dystrophin in a variety of different combinations; and 7-10 months of age, Li et al., engineered a miniDys-GFP gene that removed much of the central rod domain of the dystrophin. With each of these cell and gene therapy interventions the specific forces of the diaphragm strips were returned to values not different from those of the WT mice. Asterisk indicates a difference (P ≤ 0.001) from WT.
Diaphragm strips in gene therapy

Beginning with the original elimination of the dystrophic symptoms in the mdx mouse by the overexpression of dystrophin in transgenic mdx mice, the diaphragm strip preparation has provided a highly valuable muscle preparation for the evaluation of a wide variety of gene therapies for the treatment of muscular dystrophy in the mdx mouse. In three month old mdx mice overexpressing dystrophin, the specific forces (Figure 3) and normalized powers of the diaphragm strip were not different from those of WT mice of comparable age. In 1994, Rafael et al. demonstrated that expression of a truncated dystrophin mini-gene that was missing exons 71-74 similarly eliminated the dystrophic pathology in both the structure and function (Figure 3) of the diaphragm strip. Dystrophin is a multidomain protein that links the actin cytoskeleton through the DGC to laminin in the extracellular matrix (ECM). Crawford et al. demonstrated that transgenic mouse lines with deletions throughout the entire COOH-terminal domain assembled the DGC successfully and had WT values for muscle structure and function (Figure 3) in both limb muscles and diaphragm strip. Subsequently, Harper et al. described detailed studies aimed at overcoming complications in attempts to develop gene therapy for DMD due to the enormous size of the dystrophin gene. After a detailed functional analysis of the structural domains of the dystrophin protein, Harper and his colleagues concluded that multiple regions of the protein could be deleted in various combinations that generated highly functional mini- and micro-dystrophins. Diaphragm muscles that expressed even the smallest dystrophins had greater specific forces (Figure 3) than dystrophic mdx muscles. Power was not measured, but presumably would have been similarly improved.

The autologous transplantation of myogenic stem cells transduced with a therapeutic expression cassette also offers a highly promising technique for the treatment of DMD. Despite the promise of this technique, the development of this method has been hampered by a number of critical difficulties that include: (i) the very low frequency rates for the engraftment of cells, (ii) the tracing of the specific cells that have been transplanted, (iii) the inability to halt muscle necrosis due to the rapid loss of autologous cells carrying marker genes, and (iv) the inefficient transfer of a large dystrophin gene into myogenic stem cells.

To avoid these difficulties, a 5.7 kb miniDystrophin-GFP fusion gene was engineered. The miniDystrophin-GFP fusion gene replaced the dystrophin COOH-terminal domain with an enhanced green fluorescent protein (eGFP) coding sequence after the removal of much of the dystrophin central rod domain. A transgenic mdx mice expressed the miniDys fusion protein under the control of a skeletal muscle-specific promoter. The green fusion protein localized on the sarcolemma of muscle fibre and resulted in the assembly of the DGC. For the diaphragm strip, compared with age-matched WT mice, the mdx mice showed a 50% decrease in specific force. In contrast, the specific forces for the diaphragm strip obtained from the transgenic mdx mice and those from the WT mice were not different. Furthermore, after the lengthening contraction protocol, the force deficit of the diaphragm strip of the mdx mice was almost three-fold greater than that of the WT mice, whereas that of the transgenic mdx mice were not different (Figure 4).

![Figure 4](image-url)

**Figure 4.** The force deficit was assessed by expressing the decrease in $P_o$ (mN) measured after two 30% lengthening contractions as a percentage of the $P_o$ (mN) before injury. Asterisk indicates a difference ($P < 0.05$) from WT; double asterisk indicates a difference from transgenic mice. Bar graphs for the force deficits developed by the diaphragm strips were obtained from the diaphragm muscles of the WT and mdx mice and mdx mice treated with engineered a miniDys-GFP gene that removed much of the central rod domain of the dystrophin. With therapeutic intervention the force deficit of the diaphragm strips were returned to values not different from those of the WT mice.

For WT mice, the phenomenon of contraction-induced injury to muscle fibres results only during ‘lengthening contractions’, when muscles are stretched during a maximum, or near-maximum contraction. In contrast, muscles of mdx mice may also be injured by isometric contractions (unpublished data). The most useful measure of the susceptibility to injury of fibres, or whole muscles, is the force deficit (the decrease in isometric force developed after an injury producing protocol of lengthening contractions expressed as a percentage of the initial isometric force developed by the uninjured muscle prior to the procedure). The fibres in skeletal muscles of young WT mice are highly resistant to injury, whereas those of old WT mice are much more easily injured. The fibres in dystrophic muscles are extremely sensitive to contraction-induced injury (Figure 4). Protocols of lengthening contractions that cause only slight injuries, with force deficits of 10% for EDL muscles and 14% for diaphragm strips of young WT mice cause severe injury to dystrophic muscles with force deficits of 75% for EDL muscles and 40% for diaphragm strips. Consequently, the measurement of the sensitivity of skeletal muscles to...
contraction-induced injury produced by protocols of lengthening contractions provide a powerful tool to assess the efficacy of gene therapy, as evidenced by no difference between the force deficits for the diaphragm strip obtained from the transgenic mdx<sup>cv</sup> mice and that of WT mice (Figure 4).

We conclude that diaphragm strips dissected from the diaphragm muscles of young and old WT, mdx, and transgenic mdx mice provide an extremely useful preparation for the evaluation of age-related changes in skeletal muscle structure and function and on the efficacy of a wide range of genetic manipulations and interventions. Compared with limb muscles, the diaphragm strip has the advantage of assessing a comparable deficit in specific force and normalized power and a magnitude of contraction-induced injury comparable to the magnitude of the disturbances observed in the human expression of the disease. As such, the interpretation of the success or failure of an intervention is much more clearly defined.

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