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BRIEF REVIEW

DIAPHRAGM MUSCLE STRIP PREPARATION FOR EVALUATION OF GENE THERAPIES IN *mdx* MICE

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SUMMARY

1. Duchenne muscular dystrophy (DMD), a severe muscle wasting disease of young boys with an incidence of one in every 3000, 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 diaphagm strip preparation provided an ideal experimental model for investigations of skeletal muscle impairments in structure and function induced by interactions of disease- and age-related factors. Unlike the limb muscles of the *mdx* mouse, which 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, many 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 2 year life span of the *mdx* mouse, the impact of age-related effects can be studied in this model.

Key words: contractility, contraction-induced injury, Duchenne muscular dystrophy, force deficit, normalized power, specific force.

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 collagen-1 and collagen-3 in the extracellular matrix (ECM) is unknown. Lack of dystrophin expression in muscle fibres leads to a progressive weakness and wasting of skeletal muscles of boys with DMD that results in confinement to wheelchairs by approximately 12 years of age and most boys have died by their mid-20s owing to respiratory or cardiac failure.[3] The *mdx* mouse discovered by Bulfield *et al.* in 1984[4] 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 of the mouse.[5] The *mdx* mouse has also provided a model for the impact of the lack of dystrophin on the length of the life of the mouse,[6] on the structure and function of different skeletal muscles of the limbs,[7] on limb skeletal muscles throughout the life span,[5] on the susceptibility to contraction-induced injury[8–14] and on the efficacy of a variety of implantation[15] 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 caused by the absence of dystrophin expression. The major deficiency of the model is that adaptations occur in limb muscles of *mdx* mice and these adaptations result in hypertrophy of the skeletal muscles of the limbs.[5] The hypertrophy produces a substantial increase in muscle mass that maintains maximum isometric force but, owing to the presence of damaged fibres, results in an approximate 20% loss in maximum specific

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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 used the diaphragm strip preparation to investigate the progression of 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 *mdx*, wild-type (WT) and transgenic mice include the capacity for the development of maximum specific force, normalized power and the susceptibility to injury when subjected to protocols of lengthening contractions.

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.[25,26] In 1954, Ritchie[27] 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.[7,12,13,23,27] The rat was anaesthetized by an appropriate anaesthetic, administered as required to keep the rat unresponsive to tactile stimuli. The surgical procedure then consisted of excising the total diaphragm muscle from the anaesthetized 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 approximately 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.[23]

Such a diaphragm strip preparation obtained from *mdx* mice could 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.[16,18–20,28] Such studies with the diaphragm strip preparation have demonstrated the relative effectiveness of restoring various highly functional-length[16] or mini-dystrophin fusion genes for cell and gene therapy of DMD.[18–20,23]

DIAPHRAGM STRIP PREPARATION DURING AGEING OF *mdx* MICE

Dupont-Versteegen and McCarter[7] 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.[7] 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 approximately 205 and 245 kN/m^2 for *mdx* and WT mice, respectively.[7] 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 160–300 kN/m^2 compared with a normal range of 210–270 kN/m^2,[19,23] that age-related changes were not discernable even for WT mice. Despite the considerable variability among the specific forces of the diaphragm strips of both the *mdx* and WT mice in the Dupont-Versteegen and McCarter study,[7] the values for the specific forces

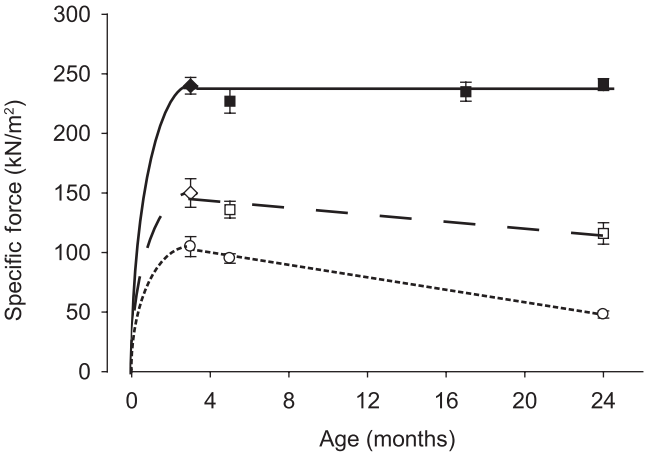

Fig. 1 Comparisons of the maximum specific forces of diaphragm strips obtained from *mdx* and wild-type (WT) mice at 3, 5, 17 (WT mice only) and 24 months of age. Maximum isometric forces were normalized to specific force by dividing the force developed by diaphragm strips of the WT mice by the total cross-sectional area (CSA) 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 CSA of the diaphragm strip including the damaged and 'ghost' fibres (dotted lines; see text for definition). (■), WT mice (data from Lynch *et al.*[23]); (□), *mdx* mice (data from Lynch *et al.*[23]); (◆), WT mice (data from Cox *et al.*[23]); (◇), *mdx* mice (data from Cox *et al.*[23]); (○), *mdx* mice (uncorrected for damaged fibres).

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.[5,7,16,22] The time point of 24 months of age is within a few months of the maximum life span (28 months) of *mdx* mice, compared with a life span of 36 months for WT mice.[6] For both young and old WT mice, histological sections of diaphragm strips show tightly packed viable fibres.[23] Consequently, the specific forces and normalized power are simply divided by the total cross-sectional area (CSA) of the diaphragm strip. Throughout the life span of the WT mice, values for the diaphragm strips range from 240 to 250 kN/m^2 for maximum specific forces and from 54 to 55 W/kg^2 for maximum normalized power; these values are in excellent agreement with each other and with control values for limb muscles of WT mice.[29,30] In contrast with WT mice with 100% of the CSA composed of viable muscle fibres, only 70% of the CSA of the diaphragm strips of 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%.[23] Even with corrections for the damaged and necrotic fibres, the diaphragm strip preparations of *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 values for WT mice (Fig. 1). Similarly, with the same corrections for volume, the normalized power of the diaphragm strips of young *mdx* mice was 45% that of the WT mice, whereas that of old *mdx* mice was only 30% (Fig. 2).

Although age-related changes in specific force have been investigated in limb muscles of *mdx* mice,[5,7] the limb muscles of *mdx* mice adapt to ongoing damage to fibres with a 20–30% hypertrophy.[5,7] 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

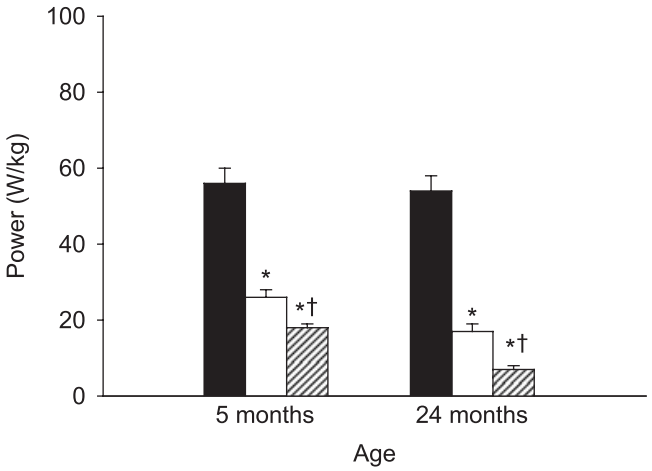

Fig. 2 Histograms showing maximum normalized power developed by diaphragm strips obtained from diaphragm muscles of wild-type (WT) and *mdx* mice. The maximum power developed by the diaphragm strips of *mdx* and WT mice was normalized by the mass of the total diaphragm strip for the WT mice, for the 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 *mdx* mice. $*P \leq 0.001$ compared with WT mice; $^{\dagger}P \leq 0.001$ compared with *mdx* (corrected) mice. (■), WT mice (data from Lynch *et al.*[23]); (□), *mdx* mice corrected for damaged fibres (data from Lynch *et al.*[23]); (▨), *mdx* mice uncorrected for damaged fibres.

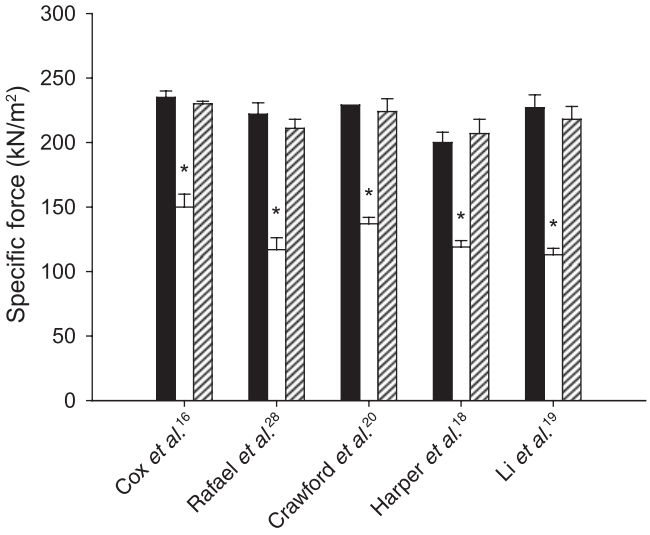

Fig. 3 Histograms showing the specific force developed by diaphragm strips obtained from diaphragm muscles of wild-type (WT; ■) and *mdx* (□) mice, as well as *mdx* mice treated with various genetic therapies (▨). In transgenic *mdx* mice that were 3 months of age, Cox *et al.*[16] overexpressed dystropin; in mice that were 4 months of age, Rafael *et al.*[28] expressed a truncated mini-gene; in mice that were 6 month of age, Crawford *et al.*[20] deleted the entire COOH-terminal domain of the dystrophin molecule; Harper *et al.*[18] deleted multiple regions of dystrophin in a variety of different combinations in mice that were 3 months of age; and, Li *et al.*[19] engineered a *miniDys-GFP* gene that removed much of the central rod domain of the dystrophin in mice that were 7–10 months of age. With each of these cell and gene therapy interventions, the specific forces of the diaphragm strips returned to values not different from those of the WT mice. $*P \leq 0.001$ compared with WT mice.

occur in skeletal muscles of *mdx* mice unimpeded by any adaptive changes.[5,7,22] Our data for the specific forces of the diaphragm strips for both *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 approximately 30% in maximum specific force and maximum normalized power by 28 months of age[29,30] 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.[3] 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 (cf. 27 and 36 months, respectively, for WT mice).[6] Consequently, with a linear decline in specific force and normalized power of the diaphragm muscle of *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%, respectively, of values for WT mice. These values place the *mdx* mice at considerable risk of respiratory failure. Consequently, the citing of respiratory failure and heart failure as the major causes of the shortened life-span of *mdx* mice is not surprising.[6]

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 above), the decision was made to restrict the assessments of cell and gene therapy to data obtained through collaborations between the Chamberlain laboratory performing interventions using cell and gene therapy techniques and the Faulkner laboratory performing assessments of muscle contractility.[16,18–20,28]

Beginning with the original elimination of the dystrophic symptoms in the *mdx* mouse by the overexpression of dystrophin in transgenic *mdx* mice,[16] 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.[12,13,17–21,28] In 3-month-old *mdx* mice overexpressing dystrophin, the specific forces (Fig. 3) and normalized powers of the diaphragm strip were not different from those of WT mice of comparable age.[16] In 1994, Rafael *et al.*[28] 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 (Fig. 3) of the diaphragm strip. Dystrophin is a multidomain protein that links the actin cytoskeleton through the DGC to laminin in the ECM.[31] Crawford *et al.*[20] 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 (Fig. 3) in both limb muscles and diaphragm strip. Subsequently, Harper *et al.*[18] described detailed studies aimed at overcoming complications in attempts to develop gene therapy for DMD caused by the enormous size of the dystrophin gene. After a detailed functional analysis of the structural domains of the dystrophin protein, Harper *et al.* concluded that multiple regions of the protein could be deleted in various combinations that

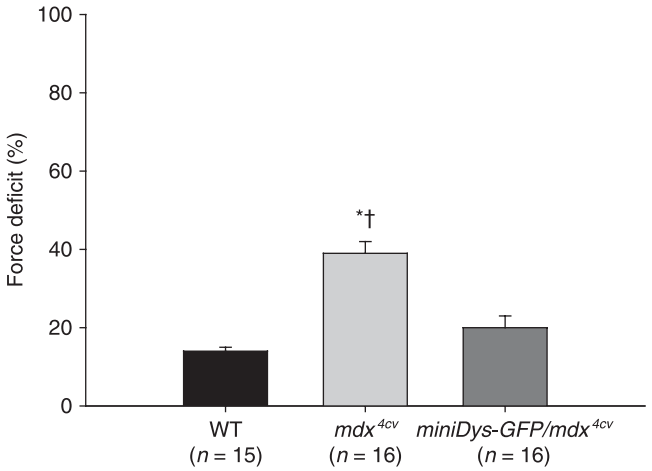

Fig. 4 The force deficit was assessed by expressing the decrease in maximum isometric force (Po; in mN) measured after two 30% lengthening contractions as a percentage of P_o before injury. Histograms show force deficits developed by diaphragm strips obtained from diaphragm muscles of wild-type (WT) and *mdx*^{4cv} mice, as well as *mdx* mice treated with an engineered *miniDys-GFP* gene that removed much of the central rod domain of the dystrophin.[19] With therapeutic intervention, the force deficit of the diaphragm strips returned to values not different from those of WT mice. *$P < 0.05$ compared with WT mice; †$P < 0.05$ comapred with transgenic mice.

generated highly functional mini- and microdystrophins. Diaphragm muscles that expressed even the smallest dystrophins had greater specific forces (Fig. 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.[19] 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 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.[19]

To avoid these difficulties, a 5.7 kb *miniDystrophin-GFP* fusion gene was engineered.[19] 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*^{4cv} mouse expressed the miniDys fusion protein under the control of a skeletal muscle-specific promoter. The green fusion protein was localized on the sarcolemma of muscle fibres and resulted in the assembly of the DGC. For the diaphragm strip, compared with age-matched WT mice, the *mdx*^{4cv} mice showed a 50% decrease in specific force.[19] In contrast, the specific forces for the diaphragm strip obtained from the transgenic *mdx*^{4cv} mice and those from WT mice were not different. Furthermore, after the lengthening contraction protocol, the force deficit of the diaphragm strip of the *mdx*^{4cv} mice was almost threefold greater than that of WT mice, whereas that of the transgenic *mdx*^{4cv} mice was not different (Fig. 4).

For WT mice, the phenomenon of contraction-induced injury to muscle fibres results only during 'lengthening contractions',[32] when muscles are stretched during a maximum, or near-maximum contraction. In contrast, muscles of *mdx* mice may also be injured by isometric contractions (DR Claflin and SV Brooks, unpubl. data, 2007). 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,[33] whereas those of old WT mice are much more easily injured.[34,35] The fibres in dystrophic muscles are extremely sensitive to contraction-induced injury (Fig. 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.[19] Consequently, the measurement of the sensitivity of skeletal muscles to contraction-induced injury produced by protocols of lengthening contractions provides 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*^{4cv} mice and that of WT mice (Fig. 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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