Regenerative Capacity of the Dystrophic (mdx) Diaphragm After Induced Injury

Stefan Matecki *, Ghiabe H. Guibinga, and Basil J. Petrof

Meakins-Christie Laboratories and Respiratory Division, McGill University, Montreal, Quebec, Canada

RUNNING HEAD: mdx diaphragm regenerative capacity

Corresponding Author: Dr. Basil J. Petrof
Respiratory Division, Room L411
Royal Victoria Hospital
687 Pine Ave. West
Montreal, Quebec
CANADA H3A 1A1
TEL: (514) 934-1934, EXT. 35946
FAX: (514) 843-1695
E-mail: basil.petrof@mcgill.ca

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* The first two authors contributed equally to this work
ABSTRACT

Duchenne muscular dystrophy is characterized by myofiber necrosis, muscle replacement by connective tissue and crippling weakness. Although the mdx mouse also lacks dystrophin, most muscles show little myofiber loss or functional impairment. An exception is the mdx diaphragm, which is phenotypically similar to the human disease. Here we tested the hypothesis that the mdx diaphragm has a defective regenerative response to necrotic injury, which could account for its severe phenotype. Massive necrosis was induced in mdx and wild-type (C57BL10) mouse diaphragms in vivo by topical application of notexin, which destroys mature myofibers while leaving myogenic precursor satellite cells intact. At 4 hours after acute exposure to notexin, >90% of diaphragm myofibers in both wild-type and mdx mice demonstrated pathological sarcolemmal leakiness, and there was a complete loss of isometric force-generating capacity. Both groups of mice showed strong expression of embryonic myosin within the diaphragm at 5 days, which was largely extinguished by 20 days after injury. At 60 days post-injury, wild-type diaphragms exhibited a persistent loss (approximately 25%) of isometric force-generating capacity, associated with a trend toward increased connective tissue infiltration. In contrast, mdx diaphragms achieved complete functional recovery of force generation to non-injured values, and there was no increase in muscle connective tissue over baseline. These data argue against any loss of intrinsic regenerative capacity within the mdx diaphragm, despite characteristic features of major dystrophic pathology being present. Our findings support the concept that significant latent regenerative capacity resides within dystrophic muscles, which could potentially be exploited for therapeutic purposes.

Key Words: Duchenne muscular dystrophy, dystrophin, mdx mouse diaphragm, muscle regeneration, satellite cells
INTRODUCTION

Duchenne muscular dystrophy (DMD) is caused by defects in the gene encoding dystrophin, a subsarcolemmal protein which plays an important role in maintaining the physical integrity of the muscle cell surface membrane, or sarcolemma (18, 53). Muscles lacking dystrophin demonstrate an increased susceptibility to contraction-induced sarcolemmal injury (40) and necrosis. As the disease progresses, fiber loss and fibrosis occurs. A murine model of DMD, the mdx mouse, also lacks dystrophin due to a nonsense point mutation in exon 23 of the dystrophin gene (45). However, most skeletal muscles of mdx mice show little fibrosis or functional alteration until late in life (28, 37, 48). The relative sparing of muscle function in mdx mice, which is in striking contrast to the destructive muscle disease found in humans with the same genetic defect, has often been attributed to a superior regenerative capacity of skeletal muscle in the mouse model (35).

A notable exception to the mild muscle phenotype observed in mdx mice is the diaphragm, which exhibits significant fibrosis as well as greatly impaired contractile function from an early age (41, 48). In broad terms, the particularly severe dystrophic pathology found in the mdx mouse diaphragm can be considered to reflect an unfavorable imbalance between muscle damage and repair. In this regard, greater damage could be caused by the continuous nature of the respiratory muscle workload, thereby leading to a higher level of work-induced myofiber injury in the mdx diaphragm (48). On the other hand, it is also possible that the mdx diaphragm has an decreased capacity for self-repair following injury, which would imply a relative impairment of regenerative mechanisms.

The muscle regeneration process involves the activation, proliferation and fusion of a population of resident myogenic precursor cells (satellite cells), which in their quiescent state are located between the basal lamina and plasma membrane of mature muscle fibers (30). Reductions in proliferative capacity have been reported in satellite cells obtained from limb muscle biopsies of DMD patients (6, 51) and with aging (43). Differences in satellite cell proliferative capacity have also been reported among different adult skeletal muscles (38). To our knowledge, only one study has examined the proliferative capacity of mdx diaphragm satellite cells, and reported no statistically significant defects of proliferation in vitro (3). However, the possibility that satellite cell...
function or other aspects of muscle repair are defective in the intact diaphragm of mdx mice in vivo cannot be excluded on this basis alone. Moreover, given the morphological (progressive fiber loss and fibrosis) and physiological (severe weakness) resemblance to human DMD, as well as the fact that DMD patients usually die from respiratory failure unless artificially ventilated, it is of considerable interest to gain a better understanding of the regenerative potential contained by the mdx diaphragm in its in vivo context.

In the present investigation, we reasoned that an impaired in vivo regenerative potential of the mdx diaphragm, if present, should be detectable when the regenerative capabilities of the muscle are greatly stressed by the imposition of a large injury stimulus. Therefore, the major objectives of the present study were three-fold. Firstly, we sought to establish a model of diaphragmatic injury/regeneration in vivo that would allow diaphragmatic myofiber damage to be imposed in a standardized manner, and which could then be verified as well as quantified using well-established morphological and functional criteria. Secondly, we wished to use this model to determine the extent to which the mdx mouse diaphragm is capable of functional regeneration following a bout of severe injury, with the latter being imposed at a point in the disease with established fibrosis and weakness of the muscle. Lastly, we ascertained whether mdx and wild-type mice differ in their abilities to achieve functional regeneration of the diaphragm after such injury.
MATERIALS AND METHODS

Animals

Dystrophin-deficient mdx and normal wild-type mice of the same genetic background (C57BL10) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and entered into the study at 6-7 months of age. This age group was selected because the mdx diaphragm shows clear and substantial dystrophic pathology, including fibrosis and weakness, at this point in the animal’s lifetime (48). Animals were anesthetized with intramuscular injection of ketamine (130 mg/kg) and xylazine (20 mg/kg) before the onset of the surgical procedure. The institutional animal ethics committee approved all the animal procedures described.

Experimental Model of Diaphragmatic Injury

To induce muscle regeneration in the diaphragm we employed notexin, a snake venom-derived compound which provokes transient necrosis of mature muscle cells followed by vigorous myofiber regeneration after injection into skeletal muscles (15). Notexin selectively destroys myofibers while leaving nerves, blood vessels, and satellite cells morphologically intact (15). A laparotomy was performed to expose the abdominal surface of the right hemidiaphragm. With the help of a dissecting microscope and forceps, the superficial fascial layer of the exposed hemidiaphragm was gently abraded. Immediately afterwards, a cotton swab immersed in notexin (10 ug/ml in 0.9% w/v NaCl saline) was applied to the entire hemidiaphragm for 30 seconds. The hemidiaphragm was subsequently rinsed with saline. The animals were maintained on a platform tilted at an angle of approximately 30° toward the targeted hemidiaphragm to avoid diffusion of notexin to the untreated side, which served as a within-animal control. The mice continued to breathe spontaneously throughout the procedure and recovered well from surgery with no apparent side effects.

Morphological Evaluation of Diaphragmatic Injury and Repair

(i) Quantification of the Acute Injury Phase. At 4 hours after exposing the diaphragm to notexin, the level of acute sarcolemmal injury in diaphragm myofibers was evaluated by exposing muscles to a low-molecular weight (FW=631) fluorescent tracer
dye, Procion Orange (Sigma Chemical Co., St. Louis, MO). Because the sarcolemma of normal myofibers is impermeable to Procion Orange, fibers with sarcolemmal damage can be identified by their inability to exclude Procion Orange from the cytoplasm (26, 40). After removing a 2-mm wide muscle strip from each hemidiaphragm to evaluate isometric contractile properties (see below), the remainder of each hemidiaphragm was immersed for 60 min in Procion Orange solution at room temperature. The Procion Orange tracer dye (2% w/v in Ringers solution) was continuously oxygenated during this period. The muscles were subsequently rinsed, snap-frozen in isopentane precooled with liquid N₂, and stored at -80 °C. Frozen sections (7 um thick) from the mid-belly of the muscles were cut with a cryostat at -20 °C. The sections were mounted for viewing under epifluorescence microscopy (fluorescein filter settings, magnification 100x), and the images were captured to computer using a video camera. Using computer-assisted image analysis (ImagePro Plus, Media Cybernetics, Silver Springs, MD), the number of myofibers demonstrating clear cytoplasmic staining (i.e. fibers containing intracellular dye due to a loss of sarcolemmal integrity) was determined. Areas with sectioning artifacts (folds, tears, etc.) were avoided, and the edges of the sections were also excluded to avoid any fibers potentially damaged by the muscle dissection. A minimum of 400 myofibers from randomly selected microscopic fields were counted on each tissue section.

(ii) Immunostaining for Embryonic Myosin Heavy Chain (MyHC). Embryonic MyHC expression was examined as a marker of muscle regeneration (52), at 5 and 20 days after notexin exposure. Transverse cryostat sections of the diaphragm were reacted with a monoclonal antibody specific for the embryonic isoform of MyHC, diluted 1:75 in PBS as previously described (41). This was followed by incubation with a biotinylated goat anti-mouse secondary antibody at 1:150 dilution. Visualization of myofiber immunoreactivity was achieved using the peroxidase detection system (Elite Vectastain ABC kit, Vector Laboratories, Burlingame, CA).

(iii) Hematoxylin and Eosin (H&E) Staining. An increase in the muscle endomysial interstitium is found when connective tissue replaces muscle fibers within dystrophic muscles (34). Therefore, the area fraction of the diaphragm interstitial space
(excluding nerves and blood vessels) was measured to evaluate the degree of connective
tissue replacement within the diaphragm, both before and 60 days after (post-regeneration
phase) exposing the muscle to notexin. Randomly selected microscopic fields were
photographed using a video camera and the image was stored on computer. Computer-
assisted morphometric analysis of H&E-stained diaphragm sections to quantify the
interstitial space between myofibers was achieved by point counting, using a 100-point
grid projected onto the captured image as previously described (13).

**Physiological Evaluation of Diaphragmatic Injury and Repair**

Isometric contractile properties of the diaphragm were measured at 4 hours (acute
injury phase) and 60 days (post-regeneration phase) after applying notexin, as previously
described in detail (41). After euthanasia, the diaphragm was quickly removed and
transferred to chilled Ringers solution (composition 119 mM NaCl, 4.7 mM KCl, 2.5 mM
CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 20 mM NaHCO₃) perfused with 95% O₂:
5%CO₂ (pH 7.4). Muscle strips were dissected from each hemidiaphragm and mounted
in random order within a jacketed tissue bath chamber maintained at 25°C. One end of
each muscle was securely anchored to a platform near the base of the chamber, while the
opposite tendon was tied to the lever arm of a force transducer/length servomotor system
(model 300B, Cambridge Technology, Watertown, MA, USA). The latter was mounted
on a mobile micrometer stage (Newport Instrument, Toronto, Canada) to allow
incremental adjustment of muscle length. Electrical field stimulation was induced via
platinum plate electrodes placed into the bath on both sides of the muscle. Supramaximal
stimuli with a monophasic pulse duration of 2 ms were delivered using a
computer-controlled electrical stimulator (model S44, Grass Instruments, Quincy, MA,
USA) connected in series to a power amplifier (model 6824A, Hewlett Packard, Palo
Alto, CA, USA). Muscle force was displayed on a storage oscilloscope (Tektronix,
Beaverton, OR, USA), and the data were simultaneously acquired to computer
(Labdat/Anadat Software; RHT-Infodata, Montreal, Canada) via an analog-to-digital
converter at a sampling rate of 1000 Hz for later analysis.

A thermoequilibration period of 10 min was observed before initiating contractile
measurements. After adjusting each muscle to optimal length (Lo, the length at which
maximal twitch force is achieved), five twitch stimulations were recorded and the mean value was used to determine the following: maximal isometric twitch force (Pt), twitch contraction time (CT), twitch half relaxation time (HRT). The tetanic force was evaluated at the following stimulation frequencies: 10, 30, 60, 90 and 120 Hz (train duration = 1s). The maximal isometric tetanic force (Po) was defined as the maximal force measured during generation of the force-frequency curve. The muscle was removed from the bath and Lo was directly measured under a dissecting microscope with calipers accurate to 0.1 mm. Total muscle strip cross-sectional area was determined by dividing muscle weight by its length and tissue density (1.056 g/cm³). This allowed diaphragmatic specific force to be calculated, which was expressed as N/cm².

**Statistical analysis**

All data are reported as means ± SE and were analyzed with a statistical analysis program (SigmaStat, SPSS, Inc., Chicago, IL). Differences between groups were tested by 2-way ANOVA or Student’s t test where appropriate. Statistical significance was defined as p<0.05.
RESULTS

Acute Injury in Normal and Dystrophic Diaphragms

Figure 1 shows the degree of intracellular Procion Orange dye uptake by diaphragmatic myofibers at 4 hours after acute treatment of the diaphragm with notexin. Note that in the control C57BL10 diaphragm, there was no intracellular uptake of Procion Orange at baseline prior to the application of notexin (Fig. 1A). On the other hand, there was a small degree of uptake by mdx diaphragm fibers under the same conditions (Fig. 1C), which reflects the underlying dystrophic process (40). However, both C57BL10 (Fig. 1B) and mdx (Fig. 1D) hemidiaphragms treated with notexin demonstrated widespread myofiber hyperpermeability to Procion Orange 4 hours later, thus indicating the induction of massive diaphragmatic sarcolemmal injury in both mouse strains. As shown by the group mean data presented in Fig. 2, there was no significant difference between C57BL10 and mdx mice in the level of diaphragmatic sarcolemmal injury produced by notexin at 4 hours post-treatment. In addition, at the same time point, the contralateral hemidiaphragms (i.e., not exposed to notexin) did not demonstrate any increases over baseline values of Procion Orange uptake in either C57BL10 or mdx.

To determine the physiological impact of notexin-induced diaphragmatic injury in C57BL10 and mdx mice, isometric contractile properties of the muscle were determined in vitro. The baseline force-generating capacity of the untreated mdx diaphragm was substantially reduced compared to the untreated C57BL10 diaphragm. This was reflected by a downward shift in the entire force-frequency relationship for the mdx diaphragm, as shown in Fig. 3. Notexin treatment had a dramatic adverse effect on force production by both mdx and C57BL10 diaphragms. Hence at 4 hours after being treated with notexin, there was no detectable force generation by either mdx or C57BL10 diaphragms at any of the applied frequencies of stimulation.

To further validate the model, immunohistochemical evidence of diaphragm muscle regeneration was sought at different time points after treatment of normal and dystrophic mouse diaphragms with notexin. Little or no embryonic MyHC expression was observed in the untreated diaphragm in either C57BL10 (Fig. 4A) or mdx (Fig. 4C) mice. At 5 days after notexin treatment, both the C57BL10 (Fig. 4B) and mdx (Fig. 4D)
diaphragms showed large numbers of small fibers expressing embryonic MyHC, thus indicating muscle regeneration as a response to the necrosis induced by notexin. Moreover, as shown in Figs. 4B and 4D, regenerating myofibers could be found throughout the entire thickness of the diaphragm muscle in both cases. Immunohistochemical detection of embryonic MyHC was largely extinguished by 20 days after notexin treatment in C57BL10 as well as mdx diaphragms (data not shown), suggesting that the myogenic regeneration process was subsiding at this point in time.

**Differential Functional Recovery in Normal and Dystrophic Diaphragms**

Table 1 shows maximal force generation parameters along with twitch kinetics of the diaphragm in C57BL10 and mdx mice at 60 days after notexin treatment. There was a striking difference in the long-term response to notexin treatment in the two mouse strains. Hence in the C57BL10 group, there was a significant (approximately 25%) loss of maximal tetanic force production by the notexin-treated diaphragm when compared to the untreated contralateral side of the muscle in the same animal. On the other hand, under the same experimental conditions there were no differences in the levels of maximal force generated by the notexin-treated and untreated mdx diaphragm strips. Although there was a tendency for notexin treatment to result in faster twitch kinetics in both mouse strains (i.e., shorter values for CT and HRT), these trends did not achieve statistical significance.

Significant differences between normal and dystrophic mice in their respective abilities to achieve functional recovery from notexin-induced diaphragm myofiber necrosis were also observed at submaximal levels of stimulation. Hence Fig. 5A confirms the persistent net loss of force-generating capacity by the normal C57BL10 diaphragm across the entire spectrum of the force-frequency relationship at 60 days after notexin treatment. Under the same conditions, there was no significant alteration of diaphragmatic specific force at any stimulation frequency in the mdx group (Fig. 5B).

Finally, in order to assess whether there was any increase in endomysial fibrosis within the diaphragm at 60 days post-injury which could help to account for changes in force-generating capacity, the non-muscle interstitial space contribution to total muscle cross-sectional area was also determined (see Fig. 6). The baseline values for interstitial
space area fraction were significantly higher in dystrophic diaphragms than in the normal C57BL10 diaphragms as previously reported (34, 41). However, at 60 days after notexin-induced diaphragmatic injury, there was a trend toward greater interstitial space area fraction in the normal C57BL10 diaphragm (increased by approximately 42%; p=0.15), whereas no such trend was observed in the mdx diaphragms which had been treated by notexin in the same fashion. As a result, there were no longer significant differences in interstitial connective tissue space area fraction between mdx and wild-type diaphragms in the notexin-treated groups.
DISCUSSION

In the present investigation, we hypothesized that the mdx diaphragm would demonstrate incomplete functional recovery from experimentally-induced myofiber injury due to a reduction in the intrinsic regenerative potential of the muscle. Accordingly, we expected that in the aftermath of the massive diaphragmatic myofiber injury induced by notexin, defective muscle repair would be associated with a significant worsening of diaphragmatic contractile function. We instead found that despite the presence of advanced dystrophic histopathology and severe contractile impairment before being exposed to notexin, the mdx diaphragm was able to attain a complete restoration of force-generating capacity to non-injured values by 60 days after notexin treatment. In contrast, the wild-type mouse diaphragm demonstrated a relative impairment of functional regeneration under the same conditions, as evinced by the failure to achieve force recovery to baseline values despite being subjected to the same initial level of injury.

Comparison with Previous Studies

Considerable discrepancy exists among the small number of studies which have addressed the question of functional recovery from muscle injury in mdx skeletal muscles. Moens et al. (33) reported that 2 months after isotransplantation of the soleus muscle to induce a cycle of complete degeneration-regeneration, there were persistent but equivalent force deficits in both mdx and wild-type mice. Another study employed cryodamage in the soleus followed by implantation of immunologically incompatible myoblasts as a further method of producing injury, and reported that wild-type muscles were able to recover force to a greater extent than mdx (20). On the other hand, mdx extensor digitorum longus (EDL) muscles showed a return of absolute as well as weight-normalized force to baseline pre-injury levels 13 wks after denervation/devascularization-induced injury, whereas these parameters were persistently depressed in the non-dystrophic EDL at the same time point (32). Louboutin et al. (27) also reported better preservation of weight-normalized tetanic tension in mdx EDL muscles compared to wild-type after crush injury. Furthermore, at least in terms of histology alone, most studies have reported that the ability to reconstitute the muscle after massive injury is
either equivalent or superior in mdx as compared to non-dystrophic hindlimb muscles (2, 12, 21, 36, 54).

The divergent findings among these studies may be related to the use of different methods for inducing injury, the different muscles examined, and in many cases a lack of quantitatively verifiable standardization of the initial injury. In addition, most studies have tended to focus upon histological outcome measures rather than muscle function parameters. However, it is important to recognize that when using a solely morphological approach, major alterations in muscle repair which are not manifested at the level of light microscopy can remain undetected. For example, in mice containing a null mutation of the MyoD gene (a key transcription factor in myogenesis), there is a significant downward shift in the diaphragmatic force-frequency relationship and a loss of maximal force-generating capacity despite the absence of apparent histological abnormalities (47). The present study is the first to assess functional regenerative responses in normal and dystrophic diaphragms using an appropriately controlled and standardized injury model.

Validation of Diaphragm Injury Model

We developed a novel and quantifiable model of acute diaphragmatic injury using notexin. Previous studies in which notexin was used to induce cycles of myofiber necrosis-regeneration in hindlimb and other non-respiratory skeletal muscles have entailed direct intramuscular injection of the compound. However, because the mouse diaphragm is extremely thin (10-15 muscle cell layers), we reasoned that it might be particularly well-suited to localized topical application of notexin. Indeed, based on our findings of Procion Orange dye uptake as well as embryonic MyHC staining in myofibers spanning the full thickness of the muscle, we conclude that notexin applied to the diaphragm in this manner is able to diffuse effectively across the entire myofiber layer which separates the peritoneal and pleural boundaries of the murine diaphragm.

In contrast to certain other established methods of inducing skeletal muscle injury (e.g., crushing, freezing), notexin administration elicits myofiber necrosis without causing morphological damage to the basal lamina, nerves or blood vessels (15, 25). In addition, satellite cells are spared from the necrotizing effects of notexin (15), which
allows one to test the ability of satellite cells (and possibly bone marrow-derived stem cells to a much lesser degree (11) to reconstitute a muscle after destruction of its mature, fully differentiated myofiber population. However, in order for any comparative study of the response to muscle injury to be valid, it is important to ensure that the initial level of induced damage is equivalent among groups. Indeed, it is notable that most studies comparing normal and mdx mouse skeletal muscle responses to injury have failed to do this in a quantitative fashion. Therefore, in order to specifically address this problem, we quantified the initial level of injury imposed on the diaphragms of wild-type and mdx animals at both the morphological and functional levels. Our data indicate that the initial levels of injury imposed on the diaphragms of normal and dystrophic mice were equivalent based upon: 1) over 90% of C57BL/10 as well as mdx diaphragm myofibers demonstrating sarcolemmal permeability defects at 4 hours after notexin treatment, and 2) a complete loss of diaphragmatic force-generating capacity in both groups of mice at the same time point.

It is also important to acknowledge the limitations of our model. In particular, it may not be entirely suitable for predicting the response to low-grade contraction-induced injury, since the latter involves a much more limited type of insult which does not necessarily entail necrotic death of the injured myofibers (31, 42, 55). However, our data are consistent with a recent study by Krupnick et al. (23), who increased myofiber injury in the mdx diaphragm by greatly augmenting the respiratory workload for several weeks. Despite the increased work-induced diaphragm myofiber injury produced under these conditions, the regenerative capacity of the diaphragm was sufficient to meet the demands of the increased damage, and there was no apparent acceleration of the dystrophic process within the mdx diaphragm (23).

Implications for Pathophysiology of DMD

Dystrophin and its associated protein complex form a link between the internal actin-based cytoskeleton and the extracellular matrix of muscle fibers (19). In the absence of dystrophin this linkage is disrupted, resulting in an increased vulnerability of muscle fibers to sarcolemmal injury and necrosis (40, 53). Muscle repair mechanisms are unable to match the pace of muscle injury in DMD, leading to a loss of muscle fibers
with attendant fibrosis. While the precise reasons for the failure of muscle repair mechanisms in DMD are not established, a leading hypothesis has been that multiple rounds of satellite cell division triggered by recurrent muscle fiber injury cause early replicative senescence of these cells and hence a decreased proliferation potential (6, 51). More recently, it has been reported that a subpopulation of radiation-resistant satellite cells are found at reduced levels in mdx limb muscles (16). However, the extent to which such changes in satellite cell function are actually linked to the failure of muscle regeneration in DMD is unclear. Muscle regeneration is an extremely complex process (17) involving many additional cell types (e.g., inflammatory cells) and soluble factors (cytokines, chemokines, growth factors, extracellular matrix elements) which could also be dysregulated in DMD.

In our study, the mdx diaphragm was able to undergo effective regeneration following the massive necrotic insult induced by notexin despite the fact that injury was induced at a point in the animal’s life during which there is already advanced disease in this muscle. Therefore, our findings argue against a state of satellite cell exhaustion as the reason for more advanced pathology in the mdx mouse diaphragm in vivo. Accordingly, it is more plausible that myofiber loss and fibrosis in the mdx diaphragm are due to other microenvironmental factors which prevent effective myogenesis, rather than a problem of satellite cell function per se. This could consist of a loss of positive stimulatory signals and/or an increase in factors which actively inhibit regeneration or muscle growth (1, 4, 7). Indeed, cytokines that inhibit satellite cell proliferation or promote fibrosis, such as transforming growth factor (TGF)-β (5) and others, have been found to be upregulated in dystrophin-deficient muscles in vivo. Other characteristics associated with dystrophin deficiency such as dysregulation of skeletal muscle blood flow during muscle activation (49), a differential sensitivity to free radicals (9), and alterations in mechanotransduction cell signaling mechanisms (24) could also be involved in the accelerated dystrophic pathology of the mdx diaphragm. Exposure of the diaphragm to notexin, which in addition to destroying mature myofibers also has marked effects on proinflammatory mediators (17) and the extracellular matrix environment (22), may also transiently affect one or more of these factors.
Potential Therapeutic Implications

The results of the present investigation could have implications for a number of strategies that are currently being pursued for therapy of DMD patients. First, if a lack of appropriate stimulatory signals rather than satellite cell exhaustion underlies the progressive loss of muscle regeneration in dystrophic muscles, then supplying these signals through exogenous means might be beneficial. In support of this hypothesis, Barton et al. (4) have shown that overexpression of insulin-like growth factor 1 (IGF-1) in mdx mice augments muscle mass and reduces fibrosis within the diaphragm. Second, there is evidence that short-term strength or endurance training is useful for reducing the loss of diaphragmatic force-generating capacity and increased muscle fatigability associated with dystrophin deficiency (8, 10, 50). However, there has also been a fear that training could increase the level of contraction-induced injury and hence satellite cell turnover, thereby leading to an accelerated decline of regenerative potential (39). The present investigation suggests that the relatively low-grade injury induced by increased muscle activity (as compared with notexin-induced necrosis) is unlikely to be associated with any significant loss of regenerative potential within the dystrophic diaphragm. However, caution must nonetheless be exercised in extrapolating these results to humans with the disease. Finally, because experimentally-induced regeneration of the type performed in this study has been shown to improve the efficiency of therapeutic dystrophin gene transfer using viral vectors or cell therapy (14, 46), the results of this study may also have implications for the use of regenerative strategies as an adjunct to eventual gene-based therapies in DMD patients. Although notexin itself would be prohibitively toxic in humans, other methods for inducing regeneration (e.g., the application of electrical stimulation or ultrasound), could potentially be more clinically acceptable (29, 44). Further studies will be required to explore the full potential of these different approaches in treating the diaphragm as well as other muscles of DMD patients.
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Table 1. Isometric contractile properties 60 days after induction of muscle regeneration in the diaphragms of normal and dystrophic mice.
* P< 0.05, untreated C57BL10 versus notexin-treated C57BL10.
† P< 0.05, untreated C57BL10 versus untreated mdx.
‡ P< 0.05, notexin-treated C57BL10 versus notexin-treated mdx.

Figure 1. Acute sarcolemmal injury induced by notexin. Intracellular Procion Orange dye uptake by diaphragmatic myofibers indicates the presence of sarcolemmal damage. Under baseline conditions (i.e., no notexin exposure), there was little or no sarcolemmal injury in diaphragms of wild-type (A) and mdx (C) mice. Notexin produced widespread sarcolemmal injury in both wild-type (B) and mdx (D) diaphragms, as shown here at 4 hours post-exposure.

Figure 2. Quantification of acute notexin-induced sarcolemmal injury. At 4 hours after acute exposure to notexin, over 90% of diaphragmatic myofibers in both wild-type (C57BL10) and mdx mice demonstrated intracellular Procion Orange dye uptake. Values represent means ± SE (n=6 per group).
* P<0.05, untreated versus notexin-treated hemidiaphragm

Figure 3. Acute effects of notexin-induced injury on the diaphragmatic force-frequency relationship. At 4 hours after notexin exposure, neither wild-type (C57BL10) nor mdx diaphragms were able to generate any detectable level of force production across the entire spectrum of the force-frequency relationship. Values represent means ± SE (n=6 per group).
* P<0.05, untreated versus notexin-treated hemidiaphragm

Figure 4. Widespread muscle regeneration in wild-type and mdx diaphragms at 5 days after notexin-induced injury. Positive immunoreactivity to embryonic MyHC is indicated by dark brown staining of the myofiber cytoplasm on cross-sections of the diaphragm. (A) Wild-type (C57BL10) diaphragm under baseline (i.e., no notexin
exposure) conditions; (B) mdx diaphragm under baseline conditions; (C) Wild-type diaphragm at 5 days after notexin exposure; (D) mdx diaphragm at 5 days after notexin exposure. See text for further explanation.

**Figure 5. Functional recovery from notexin-induced diaphragmatic injury during the post-regeneration phase.** At 60 days after notexin exposure, wild-type diaphragms exhibited a persistent defect in diaphragmatic force production across entire the force-frequency relationship as compared to untreated hemidiaphragms (A). In contrast, the mdx diaphragms treated with notexin 60 days earlier achieved the same levels of force production as their untreated counterparts (B). Values represent means ± SE (n=5 per group).

* P<0.05, untreated versus notexin-treated hemidiaphragm

**Figure 6. Post-regeneration phase contribution of the connective tissue interstitium to the diaphragm.** As expected, the percent contribution (area fraction) of connective tissue interstitial space elements was significantly higher in mdx than in wild-type diaphragms under baseline conditions. However, only the wild-type diaphragms showed a trend toward increased connective tissue accumulation at 60 days after notexin exposure. Values represent means ± SE (n=5 per group).

* P<0.05, C57BL10 versus mdx under the same conditions
Table 1. Isometric contractile properties 60 days after induction of muscle regeneration in the diaphragm of normal and dystrophic mice

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<td>2.1±0.6‡</td>
<td>10.1±0.3†</td>
<td>0.19±0.04 †</td>
<td>48.0±7.4</td>
<td>53.0±4.5</td>
</tr>
<tr>
<td>Notexin</td>
<td></td>
<td>2.4±0.6‡</td>
<td>9.4±1.4‡</td>
<td>0.22±0.04</td>
<td>42.5±4.5</td>
<td>42.5±3.5</td>
</tr>
</tbody>
</table>

Definition of abbreviations: n, number of animals; $P_t$ = maximal twitch force; $P_o$ = maximal tetanic force; CT = contraction time; HRT = half relaxation time. All values are means ± SE.

* p< 0.05, untreated C57BL10 versus notexin-treated C57BL10.
† p< 0.05, untreated C57BL10 versus untreated MDX.
‡ p< 0.05, notexin-treated C57BL10 versus notexin-treated MDX.
Figure 1
Figure 2

Fibers with Sarcolemmal Damage (%)

- Untreated
- Notexin

C57BL10

MDX

* *
**A**

C57BL10

- UNTREATED
- NOTEXIN

**B**

MDX

- UNTREATED
- NOTEXIN

**Figure 3**

Specific Force (N/cm²) vs. Frequency
Figure 4
Figure 5

A

C57BL10

- Untreated
- Notexin

Specific Force (N/cm²)

0 20 40 60 80 100 120 140

Frequency

B

MDX

- Untreated
- Notexin

Specific Force (N/cm²)

0 20 40 60 80 100 120 140

Frequency
Figure 6

![Graph showing interstitial space (% of area) for C57BL10 and MDX untreated and treated with Notexin.](image)