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PRECLINICAL DRUG TRIALS IN THE *mdx* MOUSE: ASSESSMENT OF RELIABLE AND SENSITIVE OUTCOME MEASURES

CHRISTOPHER F. SPURNEY, MD, HEATHER GORDISH-DRESSMAN, PhD, ALFREDO D. GUERRON, MD, ARPANA SALI, MS, GOURI S. PANDEY, PhD, RASHMI RAWAT, PhD, JACK H. VAN DER MEULEN, PhD, HEE-JAE CHA, PhD, EMIDIO E. PISTILLI, PhD, TERENCE A. PARTRIDGE, PhD, ERIC P. HOFFMAN, PhD, and KANNEBOYINA NAGARAJU, DVM, PhD
Research Center for Genetic Medicine, Children's National Medical Center, 111 Michigan Avenue NW, Washington, DC 20010, USA

Abstract

The availability of animal models for Duchenne muscular dystrophy has led to extensive preclinical research on potential therapeutics. Few studies have focused on reliability and sensitivity of endpoints for *mdx* mouse drug trials. Therefore, we sought to compare a wide variety of reported and novel endpoint measures in exercised *mdx* and normal control mice at 10, 20, and 40 weeks of age. Statistical analysis as well as power calculations for expected effect sizes in *mdx* preclinical drug trials across different ages showed that body weight, normalized grip strength, horizontal activity, rest time, cardiac function measurements, blood pressure, total central/peripheral nuclei per fiber, and serum creatine kinase are the most effective measurements for detecting drug-induced changes. These data provide an experimental basis upon which standardization of preclinical drug testing can be developed.

Keywords

Duchenne muscular dystrophy; *mdx* mouse; preclinical trials; statistical analysis; phenotyping; muscle function tests; exercise; echocardiography

Duchenne muscular dystrophy (DMD), an inherited X-linked disorder with an incidence of 1 in 3500 male births, is due to the absence of dystrophin, a large protein that links the intracellular cytoskeleton to the extracellular matrix.^{24,38} DMD is typically diagnosed at about 3 years of age when patients present with weakening of skeletal muscles. Patients lose their ability to ambulate at around 10 years of age and develop multiple medical problems related to skeletal, respiratory, and cardiac muscle weakness.³² Currently, glucocorticoids are the main treatment for DMD, but there can be multiple side effects and variable therapeutic benefits. The identification of further treatment options is of great importance.

The *mdx* mouse is genetically and biochemically homologous to human DMD, and it has been used extensively as a preclinical model for DMD over the last 20 years. The clinical phenotype of the *mdx* mouse is commonly considered to be less severe than that of human DMD patients. An initial period of degeneration is seen at 3–4 weeks of age, followed by relatively successful regeneration with a subsequent mild phenotype and mild dystrophic changes on histopathology.^{1,28} The progressive deterioration among boys with DMD over 20 years, compared with the relative preservation of function over 1.5 years in the *mdx* mouse model, has led some to question the relevance of the *mdx* mouse as a preclinical drug-testing model for DMD.¹⁰ Because of this, many physiological challenges aimed to increase phenotypic severity of the *mdx* mouse are used. These include exercise, eccentric contractions, drugs, and double transgenics.

Despite extensive research and preclinical testing on the *mdx* mouse, there is no consensus regarding the most effective design of preclinical trials and most appropriate endpoints (clinical, histological, or biochemical). Different treatment protocols, age groups, and endpoints have been used to assess the efficacy of drugs in *mdx* mice^{2–4,6,8,9,11,14–16,20,21,31,33,37,39,42,45} (Table 1). Both trial design and endpoints should also consider data most attractive to the U.S. Food and Drug Administration in moving toward investigational new drug applications for initial human trials. These include appropriate powering of the design of preclinical trials and inclusion of endpoints that parallel human clinical trial endpoints focused on improvement of quality of life. For example, a commonly used human clinical trial endpoint for neuromuscular disease is the timed walk test (e.g., how far a patient can walk in 6 minutes, before and after drug treatment). For *mdx* mice, activity monitors or time to exhaustion on a treadmill might be relevant to the timed walk test, whereas physiological measures, such as eccentric contraction-induced strength loss, may be less relevant.

Preclinical testing in mouse models for both efficacy and toxicity are critical steps in translational medicine in the process of moving promising therapies from the bench to the patient bedside. The numbers of promising drug and biological treatments for DMD are increasing rapidly, thus there is a growing demand for preclinical testing in the *mdx* mouse. Moreover, scores of drugs have already been tested, but the lack of generally agreed upon endpoints makes it difficult to compare data or interpret many published trials. Although many of the recent investigations have not yet been published, there seems to be a trend toward extensive overlap in the testing of drugs by different groups. Therefore, a co-coordinated approach is needed to reduce unwarranted wasteful overlap of trials. In fact, recently, an international effort to standardize procedures was initiated by Washington, DC Wellstone Center (www.wellstone-dc.org) and discussed in two international workshops organized by the DC Wellstone Center and the TREAT-NMD Network (<http://www.treat-nmd.edu/activities/treatment/html>).

In this study, we present the methods and results obtained from a series of well-known and novel endpoints. Comparisons of normal and *mdx* mice are presented, as well as power calculations for expected effect sizes in *mdx* preclinical drug trials. The data presented provide an experimental basis upon which standardization of preclinical drug testing can be developed.

METHODS

Animal Care

All mice were handled according to the local institutional animal care and use committee guidelines. In general, 10–12-week-old female C57BL/10ScSn-Dmd^{mdx}/J (*mdx*) and C57BL/10ScSn (wild-type) mice weighing 20–30 g were purchased from the Jackson Laboratory (Bar Harbor, Maine). All mice were housed in an individually vented cage system with a 12-hour light–dark cycle and received standard mouse chow and purified water *ad libitum*. All animals were rested at least 10–14 days before starting acclimation and baseline readings.

Treadmill Exercise

The treadmill exercise uses a common commercially available setup (Columbus Instruments, Columbus, Ohio), which employs a moving belt. We subjected all experimental mice to a 30-minute run on a horizontal treadmill at 12 m/min, twice a week based on the protocol developed by Granchelli et al.^{8,9,17} All our animals were exercised in a room maintained at 70°F, 70% humidity, and <80 watts of light. The mice were exercised between 8:00 A.M. and 12:00 P.M., except on those days when functional data were obtained. Treadmill exercise was performed with constant monitoring by technicians to encourage mice to stay on the treadmill and was limited to morning hours.

Rotarod Test

Rotarod was performed as described previously.³⁶ Briefly, mice were trained on the rotarod (Ugo Basile, VA, Italy) for 2 days before collecting data. Each acclimatization session consisted of four training sessions, 2 per day, and each session lasted 120 seconds at a speed of 5 rpm. Each trial consisted of placing the mouse on the rod at 10 rpm for 60 seconds (stabilizing period) followed by acceleration from 10 rpm to 40 rpm within the first 25 seconds until the animal fell from the rod or until 180 seconds were reached. If animals fell during the stabilizing period, they were placed back on the rod to complete the session. The total testing time was 240 seconds (60-second stabilization time and 180-second test time). Each trial was done twice a day (2-hour interval between sessions) for 3 consecutive days. The latency to fall (seconds) was recorded, and all six scores per mouse were averaged and were recorded as latency to fall (in seconds) for each mouse.

Grip Strength Test

Grip strength was assessed using a grip strength meter (GSM) consisting of horizontal forelimb mesh and an angled hindlimb mesh (Columbus Instruments, Columbus, Ohio). The animals were acclimatized on fore- and hindlimb meshes for 3 consecutive days, at least 1 week before actual data collection. For forelimb strength assessment, the animal was allowed to hold the horizontal mesh with the forelimb paws and then was gently pulled back until its grip was broken. The force transducer retained the peak force reached when the animal's grip was broken and is shown on a digital display. This was repeated five times during each session within a 2-minute time-frame. For hindlimb strength, an angled mesh assembly was used. Mice were allowed to rest on the angled mesh assembly, facing away

from the meter and with hindlimbs at least halfway down the length of the mesh. The mouse's tail was pulled directly toward the meter and parallel to the mesh assembly. During this procedure the mice generally resisted by grasping the mesh with all four limbs. The pulling continued toward the meter until the hindlimbs released from the mesh assembly. Five successful hind- and forelimb strength measurements were recorded over a period of 2 minutes. The maximum values for each day over a 5-day period were used for subsequent analysis. The grip strength measurements were collected in the morning hours over a 5-day period, and data were normalized to body weight and expressed as KGF/kg.

Behavioral Activity Measurements

Open-field activity was measured using an open-field digiscan apparatus (Omnitech Electronics, Columbus, Ohio), as described previously.³² All mice were acclimatized for at least 1 week before actual data collection. The data were collected every 10 minutes over a 1-hour period each day for 4 consecutive days. Results were calculated as mean $\pm$ SE of all recordings. Over 20 different activity measures were recorded, but horizontal activity, vertical activity, total distance, and rest time were considered best to assess overall behavioral activity.

Echocardiography

Echocardiography was performed as detailed previously using 1–2% inhaled isoflurane anesthesia.⁴⁰ Qualitative and quantitative measurements were made offline using analytic software (VisualSonics, Toronto, Ontario, Canada). Electrocardiogram kilohertz-based visualization (EKV) software analysis produced offline reconstruction for simulated 250–1000-Hz static and cine-loop images. Echocardiographic measurements included vessel diameters, ventricular wall thickness, chamber size, and blood flow velocities across the atrioventricular and semilunar valves. Modified parasternal long-axis EKV loops were also used to measure ejection fraction (EF) via Simpson's method. M-mode images were used to measure left ventricular (LV) chamber sizes and wall thicknesses. Percent shortening fraction was calculated from M-mode measurements using the leading-edge to leading-edge method via the formula: percent shortening fraction (%SF) = left ventricular internal diameter (diastole) [LVID(d)] – left ventricular internal diameter (systole) [LVID(s)]/LVID(d).

Histological Evaluations

At the conclusion of the 28-week observation period (38–40 weeks of age), all animals were euthanized, and tissue samples were collected. Part of the dissected gastrocnemius muscle was kept in formalin for hematoxylin and eosin (H&E) staining. The remaining tissues were embedded in OCT compound and frozen in isopentane chilled in liquid nitrogen. Histological evaluations were performed by at least two individuals in a blinded manner using coded H&E-stained slides. Quantitative stereology (Olympus C.A.S.T. Stereology System; Olympus America, Inc., Center Valley, Pennsylvania) was used to evaluate the slides. Assessment criteria included: assessment of total fibers present; total fibers with central nuclei; total peripheral nuclei; total central nuclei; regenerating fibers (basophilic fibers); degenerating fibers; and inflammation (an interstitial group of >10 smaller inflammatory cell dark blue nuclei in a high-power field) in five high-power (40 $\times$) non-

overlapping fields in normal and *mdx* gastrocnemius muscle sections (Fig. 1 and Table 5). Fibers intersecting the left and top borders of the field were not counted, and nuclei more than one nuclear diameter from the fiber border were deemed “central.”

Quantitation of Fibrosis in the Diaphragm

Paraffin sections of diaphragm tissue were stained with Masson’s trichrome. The tissue was magnified under a light microscope at an objective of 4×, and a digital image was obtained using computer software (Olympus C.A.S.T. Stereology System). These digital images were processed using Image J (NIH) with additional threshold color plug-ins to process jpeg images. Pixels corresponding to the area stained in blue in the previously described technique were normalized to the total pixel area of the tissue image, and the results expressed as percent of collagen.

Creatine Kinase Determination

The blood was collected by heart puncture immediately after euthanasia. Two hundred fifty microliters of blood was collected into Eppendorf tubes, allowed to clot, and kept at room temperature to allow clot contraction prior to centrifugation and serum collection. Creatine kinase (CK) determination was performed according to the manufacturer’s instructions using a standard spectrophotometric method with enzyme-coupled assay reagent from Fisher Scientific (CK10).⁴¹ Absorption at 340 nm was measured every minute for 2 minutes at 37°C to calculate enzyme activity. Duplicate measurements were done on each serum sample, and the data expressed as units per liter.

Statistical Analysis

Normality of each quantitative measurement (GSM, digiscan, echo) was confirmed prior to analysis. Measurements between wild-type and *mdx* mice were compared at each time-point using Student’s *t*-test for independent samples. Due to non-normality of the distribution, rest time was analyzed using Wilcoxon’s rank-sum test. Histology measurements were compared between wild-type and *mdx* mice using Poisson’s regression for count data. Calculations were done to determine the effect size necessary to detect a significant difference at the $P = 0.05$ level given an “*n*” of 10 *mdx* mice per comparison group and at a power of 80%. The analysis of measurements over time used repeated measures analysis of variance to determine both time and mouse strain effects. The percent variation attributable to weight was determined using linear regression models. Likelihood ratio tests were done comparing the r^2 -values from the full model (mouse strain and weight) with the constrained model (mouse strain only).

RESULTS

Body Weight

The C57BL/10ScSn-Dmd^{*mdx*}/J (*mdx*) females weighed significantly more than the wild-type females until 38–40 weeks of age. At baseline age of 10–12 weeks, wild-type females weighed 22.17 ± 0.96 g vs. 23.64 ± 1.33 g for *mdx* ($P = 0.002$). At 20–22 weeks, wild-type mice weighed 22.12 ± 1.05 g compared with 24.89 ± 0.88 g for *mdx* ($P < 0.0001$). At 38–40 weeks of age, a greater variation in size was found, and the differences were not significant.

Wild-type mice weighed 26.93 ± 2.06 g compared with 27.30 ± 1.50 g for *mdx*. Based on these values for body weight, an effect size of at least 7.5% at 10–12 weeks, 4.7% at 20–22 weeks, and 7.3% at 38–40 weeks is necessary to detect a significant difference when comparing groups of 10 *mdx* mice (Table 2).

Grip Strength

Analysis of grip strength found significant differences in fore- and hindlimb strength normalized for weight during the 28-week observation period. At baseline (10–12 weeks), wild-type normalized forelimb grip strength was 5.51 ± 0.27 KGF/kg vs. 4.41 ± 0.35 KGF/kg in *mdx* ($P < 0.0001$), 6.08 ± 0.46 vs. 4.09 ± 0.40 KGF/kg at 20–22 weeks ($P < 0.0001$), and 5.43 ± 0.72 vs. 4.05 ± 0.49 ($P < 0.0001$) KGF/kg at 38–40 weeks, respectively. Based on these values for *mdx*-normalized forelimb grip strength, an effect size difference of at least 10% at 10–12 weeks, 13% at 20–22 weeks, and 16% at 38–40 weeks is necessary to detect a significant difference when comparing groups of 10 *mdx* mice (Table 2).

Baseline normalized hindlimb grip strength in wild-type mice was 7.27 ± 0.38 KGF/kg vs. 6.24 ± 0.47 KGF/kg in *mdx* ($P < 0.0001$), 9.00 ± 0.59 vs. 7.49 ± 0.60 KGF/kg at 20–22 weeks ($P < 0.0001$), and 9.19 ± 1.21 vs. 8.46 ± 1.07 KGF/kg at 38–40 weeks of age (not significant), respectively. Based on these values for *mdx*-normalized hindlimb grip strength, an effect size difference of at least 10% at 10–12 weeks, 11% at 20–22 weeks, and 17% at 38–40 weeks is necessary to detect significant differences when comparing groups of 10 *mdx* mice (Table 2).

Rotarod

There were no significant differences in latency to fall on the rotarod apparatus between wild-type and *mdx* mice. Both groups showed decreases in time to fall over the trial period (Table 2). Due to significant variability in measurements, large effects sizes, from 13% to 31% depending on age, are required to determine significant effects of treatment on *mdx* mice.

Open-Field Behavioral Activity Monitoring

More than 20 different parameters can be measured by activity monitoring. We focused on four measurements that provide a general assessment of activity: vertical activity; horizontal activity; total distance traveled; and rest time. There were significant decreases in vertical activity, horizontal activity, and total distance, and an increase in rest time in *mdx* compared with control mice at baseline. At 20–22 weeks, significant decreases were seen in horizontal and vertical activity; however, there was a decreasing trend in total distance and an increasing trend in rest time. At ages 38 – 40 weeks, all activity parameters except rest time showed a decreasing trend in *mdx* mice in comparison to wild-type controls, but without reaching statistical significance. Rest time appears to have been the most sensitive indicator for *mdx* mice over the entire 28-week period. Significant differences in rest time could be detected with an effect size of 2.0% at 10 – 12 weeks, 1.4% at 20–22 weeks, and 3.3% at 38 – 40 weeks. Vertical activity requires a 35–103% change to obtain significance in this model (Table 3).

High-Frequency Echocardiography and Blood Pressure Monitoring

Using high-frequency echocardiography, M-mode imaging provided the best data for measuring heart size and calculating cardiac function. At baseline, *mdx* mice had normal cardiac function and a shortening fraction of $33.37 \pm 2.9\%$. Table 4 shows values for echocardiography and blood pressure measurements between wild-type and *mdx* mice at 38–40 weeks of age. A significant decrease in cardiac function, measured by percent shortening fraction, was seen in *mdx* mice ($P < 0.01$). A significant decrease was also seen in left ventricular internal diameter during diastole ($P < 0.01$). Heart rate was significantly increased in *mdx* mice ($P = 0.0001$). Diastolic and mean blood pressures were also significantly decreased in *mdx* mice ($P = 0.0001$). Using this technique, power analysis shows that, when groups of 10 *mdx* mice are compared, a difference of only 6.3% in shortening fraction is significant. Heart rate requires only an 8.5% difference, and measurement of left ventricular posterior wall thickness during diastole requires a 10.7% difference.

Histological Evaluation

Results of histological evaluation (H&E staining) for wild-type and *mdx* mice showed significant increases in degenerating fibers, regenerating fibers, inflammation, central nucleated fibers, total central nuclei, and central/peripheral nuclei per fiber in *mdx* mice (Table 5). Examples of the measured pathology are shown in Figure 1. Although histological evaluation was very sensitive when comparing wild-type with *mdx* mice, it became much less so when comparing *mdx* mice with each other. For example, to detect significant treatment effects between groups of 10 *mdx* mice, an effect size of 22.6% for the number of central and 12.4% for the number of peripheral nuclei per fiber would be required. Analysis of other parameters showed yet further loss of sensitivity with groups of 10 mice to obtain $P < 0.05$. For degenerating and regenerating muscle fibers, 57.9% and 52.2% differences are required, whereas evaluation of inflammation is more subjective and still less sensitive, requiring a 71% difference.

Collagen, as assessed by Masson's trichrome staining, was significantly increased in *mdx* diaphragm muscle (Fig. 2 and Table 5). In wild-type mice, collagen occupied approximately 7% of the total muscle area compared with 23% in exercised *mdx* mice ($P < 0.002$). A 31% difference is required to obtain significance for a treatment effect on *mdx* mice.

Serum CK

Significant differences were seen in serum CK levels at 38 – 40 weeks of age. Wild-type mice ($n = 15$) had a serum level of 85 ± 75 , and *mdx* mice ($n = 9$) had a level of 6440 ± 2507 ($P < 0.001$) (Table 5). Due to large variation in CK values, an effect size of 37% is required to obtain significance for a treatment effect with *mdx* mice.

Variability in Measurements Attributable to Weight

An analysis of percent variability in measurements attributable to weight was performed by comparing the full model (mouse strain and weight) to the constrained model (mouse strain only). The only significant difference was found in maximum average forelimb grip strength

at 10–12 weeks of age. The likelihood ratio test between models showed a *P*-value of 0.011. All other measurements of grip strength and activity monitoring at all other time-points showed no significant variability attributable to mouse weight (Supplemental Table 1).

DISCUSSION

In this study we have presented evaluation methods for assessing efficacy in preclinical drug studies, including longitudinal non-invasive functional assessments of skeletal and cardiac muscle function. We have shown significant differences in body weight, normalized grip strength, activity monitoring, cardiac function, and histological measurements. Our analysis identifies parameters that can detect significant differences between wild-type and *mdx* mice and between treatment groups of *mdx* mice.

As we move toward comparison of results across labs to analyze therapeutic possibilities, it is important to minimize differences in the subject groups as much as possible. All experimental animals should be from a single genetic background and same sex. We have used female *mdx* mice based on the C57BL/10 background from Jackson Labs (Bar Harbor, Maine). Optimizing the use of the same breeding colony will help limit strain differences and outbreeding of mild phenotypes that can occur in less well-maintained breeding colonies. Unlike humans, female *mdx* mice are homozygous for the dystrophin mutation and therefore fully manifest the disease phenotype, similar to male *mdx* mice in the exercised model. This, coupled with the sometimes long delays in obtaining large numbers of male *mdx* mice, led us to perform all our experiments in female *mdx* mice. Our data clearly indicate that female *mdx* mice have a significant disease phenotype and are suitable for therapeutic interventions. We acknowledge that there are significant gender differences that influence disease progression, and it is important to consider gender differences in planning the experiments. Each gender has its own limitations, but using a single gender for a trial helps to minimize variation.

Other differences can also affect outcomes. Living conditions must be similar, including cage contents, light–dark cycles, and diet. Because *mdx* mice are generally stress-prone, both the route and frequency of drug administration significantly affect the disease progression. Therefore, our preferred method of administration for small molecule drugs is oral (via food or water). If parenteral routes (e.g., intravenous, intraperitoneal, or subcutaneous) are unavoidable, it is best to formulate drugs with relatively long half-lives to avoid frequent administration. Last, we have assessed all our baseline measurements at 10–12 weeks of age, after the initial phase of degeneration/regeneration and necrosis is complete, to reduce the variability associated with the initial disease onset. If an investigator's interest focuses more on early muscle necrosis, then a younger age may be more appropriate.

Because the muscle phenotype in *mdx* mice is clinically mild, several methods have been designed to accentuate the phenotype. Such methods include different exercise protocols. One type, eccentric downhill treadmill running, can have a severe effect on muscle pathology after short periods of exercise and can cause early death in an *mdx* mouse.^{5,43} In an effort to avoid premature death and maximize the development of muscle pathology over

a longer 6-month period, we have chosen a chronic exercise protocol.^{7,9,17} In protocols that utilize voluntary exercise, some beneficial effects on muscle strength in *mdx* mice have been found.^{13,22,44} Although these mice ran fewer kilometers per day than the control mice, they did not show the same age-associated loss in tension production found in sedentary *mdx* mice.⁴⁴ However, because changes in skeletal muscle strength are a primary endpoint in preclinical testing, we have selected a forced treadmill protocol to accentuate the phenotype and improve detection of beneficial effects of therapeutics over a 3–6 month trial period.

This study has described the effectiveness of non-invasive longitudinal measurements for the assessment of potential therapies in the *mdx* mouse. In many of the outcome measures reported herein, we found marked differences between normal controls and *mdx* mice with very high statistical significance, suggesting that such parameters would be sensitive and reliable outcome measures for drug trials in *mdx* mice. However, some of these measures also showed poor sensitivity when calculating statistical power in comparing groups of 10 *mdx* mice. This apparent discrepancy is explained by the variability seen in *mdx* mice. Variability was recently reviewed by Grounds et al., and numerous factors were identified, including gender, diet, cage design, neonatal influences, and stress.¹⁸ Wide variability for an outcome measure between individuals in an *mdx* group generates high standard deviations and a reduction of the calculated power, leading in turn to high effect sizes required for statistical significance in groups of 10 animals (Tables 2–5). Some of the criteria with high variance are well known, such as quantitative histological indices of degeneration and regeneration, where groups of 10 *mdx* mice showed low sensitivity, requiring some 57.9% to 52.2% change to achieve 5% statistical significance (Table 5). On the other hand, some were unexpected and novel to this report. These parameters include body weight, normalized grip strength, rest time, cardiac function measurements (% fractional shortening, % ejection fraction), blood pressure, total nuclei per fiber, and serum CK.

Our data show significant weight increases in exercised *mdx* mice at 10–12 and 20–22 weeks of age. These 10–12-week data are similar to weights obtained by De Luca et al. at 8–9 weeks of age in exercised *mdx* mice.⁹ The *mdx* mice continued to weigh more than wild-type mice at 38–40 weeks of age, but the difference was not significant. Keeling et al.²⁷ reported a 15% increase in *mdx* body weights compared with wild-type from 10–12 weeks until 48–52 weeks. However, this was found in sedentary *mdx* mice, and our weights were a few grams less, possibly related to our exercise protocol.²⁷ The exact etiology of increased body weight in *mdx* mice compared with wild-type mice is unknown, but inflammatory changes with muscle edema, compensatory muscle hypertrophy, and decreased activity (discussed later) are possibilities for the significant differences at earlier ages.

As mentioned previously, skeletal muscle strength is an optimal endpoint, because there is clinical correlation with DMD patients. We have chosen to measure fore- and hindlimb grip strength as the primary endpoint for the evaluation of skeletal muscle strength. De Luca et al. also measured fore-limb grip strength in 4–5- and 8–9-week-old *mdx* mice.⁹ They found significantly decreased forelimb grip strength at 8–9 weeks compared with controls, but the values were higher than those in our study (approximately 0.125 kg vs. 0.104 kg). They also showed a significant decrease in the normalized strength of exercised *mdx* mice to approximately 60% lower than in sedentary *mdx* mice. We showed a 20% decrease in

normalized grip strength in exercised *mdx* mice compared with exercised controls. Payne et al. did not find any significant differences in grip strength between exercised wild-type and placebo-treated *mdx* mice at 12 weeks of age.³³ The exact reasons for these differences are currently unclear, but they warrant future pilot trials between different laboratories using standard operating procedures, which are currently being developed by an international working group.

As is also evident in the data from De Luca et al., we consider it very important to normalize grip strength measurements. We have already confirmed the increased weights in *mdx* mice, and there is concern that the methodology of grip strength measurement is not independent of body weight. In fact, we analyzed all of our grip strength and activity measurements to look for a significant effect due to body weight. The only measurement with significant variability attributable to body weight was forelimb grip strength ($P = 0.011$) (Supplemental Table 1). Hindlimb grip strength measurements also showed increased variability, but this did not reach statistical significance. Thus, in order to minimize differences within a lab and between laboratories, especially for drugs that affect body weight, normalization is paramount for data analysis to completely assess whether results are related to a drug effect.

To our knowledge, this is the first study to use behavioral monitoring in the *mdx* mouse. Hudecki et al. used small animal activity chambers that recorded spontaneous physical movements as “events” in *mdx* mice.²⁵ They found decreased activity in *mdx* mice over time, but this was no different from findings in age-matched controls. They did see a significant elevation in activity in *mdx* mice treated with dexamethasone and imipramine. The monitors used in this study were more advanced and able to quantify 24 different movements. We found significant decreases in four parameters in 10–12-week-old and two parameters in 20–22-week-old *mdx* mice. Activity monitors have many limitations, especially regarding ambient activity in the rooms and time of monitoring. Although we made every effort to minimize these differences, small environmental changes could have significant effects on measured activity. All activity monitors need to be placed in a separate room to minimize effects of human activity or undue noise during testing. Also, behavioral adaptation to the open-field digiscan apparatus and its environment likely affects the activity levels.

To optimize imaging and evaluation of cardiac function, we utilized high-frequency echocardiography. We showed a significant decrease in cardiac function as measured by percent shortening fraction in 38 – 40-week-old *mdx* mice, as compared with wild-type. Although we have presented only four echo-derived parameters, many other functional assessments are possible to evaluate both systolic and diastolic function.⁴⁰ Non-invasive echocardiography used by Quinlan et al. showed decreased cardiac function in 42-week-old *mdx* mice.³⁵ We found no significant differences at 10–12 and 20–22 weeks of age, consistent with findings by Quinlan et al.³⁵ We also found significantly decreased left ventricular internal diameter at the end of diastole. These measurements differ from those of Quinlan et al., who showed a dilated left ventricle at the end of diastole in conscious *mdx* mice. It is not clear why our measurements were smaller, but potential side effects of anesthesia must be considered.

The *mdx* mice were also found to have significantly increased heart rates compared with controls, and this may represent compensation secondary to decreased cardiac function. Non-invasive tail cuff blood pressure monitoring also showed significantly decreased diastolic and mean blood pressures in *mdx* mice. Although the etiology of this effect is unknown, possible dysregulation of nitric oxide may adversely affect endothelial tone.^{29,30} Significantly increased cardiac fibrosis in 9-month-old, unexercised *mdx* mice was previously detected by our lab and others.^{35,40} Cardiac fibrosis is a significant endpoint and is a more readily available technique that can be performed consistently between different labs. Because there is only mildly decreased cardiac function in situ at age 9–12 months, adequate measurement of this parameter is significantly improved using the expertise of a cardiologist and high-frequency ultrasound. These resources may not be available to all laboratories and can limit functional cardiac endpoints for many investigators.

At the completion of the trial, skeletal muscle tissue is obtained for histological evaluation. The timing for tissue collection should be standardized across trials to improve comparison. Currently, different investigators use different muscles for histological analysis, and there is no consensus on which muscle best represents the histological phenotype (increase in fibrosis, central nucleation, and degeneration) seen in skeletal muscle of DMD patients. We used the predominantly fast-twitch fiber gastrocnemius, but it is also acceptable to use the extensor digitorum longus for a mixed muscle fiber type composition. Histological measurements have been a mainstay of preclinical studies to date, although methods and measurements have varied across studies. We found significant changes in all four parameters measured in skeletal muscle: degenerating fibers; regenerating fibers; inflammatory infiltrates; and total central nuclei. The *mdx* skeletal muscle also showed a significant increase in total, central, and peripheral nuclei per fiber. The latter incorporated a minor contribution of satellite cells as well as genuine peripheral myonuclei. These data suggest that the exercise regime augments accumulation of myofiber nuclei in 40-week-old exercised *mdx* mice, probably through enhanced satellite cell proliferation and fusion. This is consistent with exercise data, which showed exercised-induced increases in both myofiber nuclei and satellite cell numbers in human volunteers.^{12,34}

Our results concur with previous studies that used histological parameters.^{2,3,9,25,39} These parameters are also used to successfully evaluate drug interventions, and can validate results from longitudinal non-invasive muscle function testing at completion of the trial.^{2,4,8,9,11,15,20,21,26,33,39,45} Collagen-sensitive stains can be used to quantitate the amount of fibrosis in cardiac and skeletal muscles, as shown in Figure 2, and Evans blue dye uptake can also be used to assess muscle cell membrane integrity.^{3,19,39,40,42}

Along with histology, quantification of creatine kinase levels is a validated method to quantify muscle damage. Similar to DMD patients, *mdx* mice have increased levels of serum CK.^{2,3,9,17,25} However, because *mdx* mice undergo an initial period of degeneration/regeneration and maintain a mild phenotype, correlation of CK levels with function and histological parameters is difficult. Some studies have shown improved functional/histological parameters with no changes in increased CK levels.^{2,17} CK levels were shown to increase with age, exercise, and male gender.^{9,25,45} CK values tended to have large variations within studies and could vary dramatically across studies. It is critical in the

methodology to avoid artificial increases. For example, squeezing the tail to obtain more blood or manipulating muscles under anesthesia prior to euthanasia can increase CK levels. Due to these factors, the overall effectiveness of this evaluation is limited to a comparison of measured levels and not the actual values obtained.

Our statistical analysis also identified parameters that are not as useful. These measurements showed little consistent differences between *mdx* and wild-type mice and thus required a large effect size to achieve significance between groups of *mdx* mice ($n = 10$). These parameters included latency to fall on rotarod apparatus, cardiac chamber size and wall thickness measurements, and H&E analysis of degenerating/regenerating fibers and inflammation. H&E is a relatively insensitive method to clearly detect regeneration/degeneration as well as specific inflammatory cells in muscle tissue. Incorporation of immunohistochemical evaluation that uses specific markers of regeneration and inflammation should greatly enhance the usefulness of histology in pre-clinical trials.

Rotarod analysis did not show any significant differences in *mdx* mice. Hockly et al. discussed limitations of rotarod testing, which we also faced, including control mice that reached the time ceiling and prolonged periods of clinging on the rotating rod by both control and *mdx* mice.²³ Although we used a protocol similar to Hockly et al., which minimizes these limitations, we still did not see any significant differences in exercised *mdx* mice, and this test produced no significant results. Because we also recorded a decline in timing for normal mice, we believe there may be learning or training effects that apply equally to both strains of mouse. Payne et al. found significantly decreased rotarod timing in 4–12-week-old *mdx* mice.³³ Their protocol utilized a maximum speed of 20 rpm after 15 seconds. These results may be related to the age of the mice and the active muscle degeneration/regeneration that occurs at this time. Our trial intentionally avoided this period to assess more chronic muscle changes. However, Payne et al.³³ demonstrated that rotarod timing has a role in drug trial evaluations. The current protocol seems to work well at a younger age, but, as both control and *mdx* mice get older, the differences diminish. Because of this, we may need an age-specific protocol to expose pathology during aging.

Postmortem, there are many other parameters that can be studied. It will always be advantageous to further analyze specific aspects of muscle function, but this is often limited to specific laboratories with expertise in these techniques. For example, De Luca et al.^{7,8} expanded the analysis of exercised muscle function in *mdx* mice using muscle chloride channel function.^{7,8} Many researchers have evaluated isometric force contraction parameters in leg muscles and diaphragm.^{3,11,15,20,39} Such functional cellular parameters can validate therapeutic interventions in exercised models and add valuable insight to muscle function and response to drugs. These tests are not available to all labs, so consistent non-invasive functional parameters are paramount for correlation of these more specific assessments. The benefits of our described non-invasive assessments are the ability to monitor and quantify disease progression and treatment effects without sacrificing the mice.

As we move toward standardization of preclinical studies, we come to understand the limitations involved. These non-invasive longitudinal assessments are very sensitive to environmental conditions (light, sound, and time of day) and stress (timing of injections,

animal handling, and repeated measurements). We hope that our methods and results encourage the process of standardization to limit experimental variability. As a community, we can build on these results and continue to develop standard operating procedures for preclinical drug testing in *mdx* mice. Once this is accomplished, potential therapeutics can be evaluated by multiple labs and results compiled to provide significant data for the initiation of human trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CK	creatinase kinase
DMD	Duchenne muscular dystrophy
d	diastolic
EF	ejection fraction
EKV	electrocardiogram kilohertz-based visualization
H&E	hematoxylin and eosin
KGF	kilogram-force
LV	left ventricular
LVID	left ventricular inner diameter
SF	shortening fraction

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FIGURE 1.

Examples of parameters used for skeletal muscle measurements in H&E staining of the gastrocnemius of *mdx* mice. **(A)** Myofiber degeneration. The arrow points to an area of degeneration noted by light pink staining of muscle fibers, irregular shapes, and few myonuclei. **(B)** Myofiber regeneration. The arrow points to small myofibers with increased basophilic staining. **(C)** Myofiber inflammation. The arrow points to increased cellular infiltrate with small, darkened nuclei surrounding a myofiber. **(D)** Central nucleation. The arrow points to large, mature, regenerated myofibers containing multiple central nuclei.

FIGURE 2.

Significantly increased fibrosis is seen in diaphragm muscle of *mdx* mice (**B**) as compared with wild-type mice (**A**), using Masson's trichrome staining. The areas of blue staining indicate collagen, which comprises 23% of the total muscle area in *mdx* mice compared with 7% in wild-type mice ($P < 0.002$).

Table 1

Different treatment durations, age groups, and methods used to evaluate efficacy of different drugs in *mdx* mice.

Drug	Duration	Age	Tissue type	Methods used	Reference
IL-15	4 wk	8 wk	Diaphragm	Isometric force contraction, histology	Harcourt et al. (2005) ²⁰
Prednisone	8 wk	4 wk	Diaphragm	TGF- β hydroxyproline	Hartel et al. (2001) ²¹
Deflazacort	4 wk	3 wk	TA, diaphragm	Histology, serum CK	Anderson et al. (1996) ²
Pentoxifylline	4 wk	4 wk	Diaphragm	Isometric force contraction, hydroxyproline	Gosselin et al. (2006) ¹⁵
L-arginine	4 wk	8 wk	Diaphragm, TA, EDL, Quad	Isometric force contraction, EBD uptake, serum CK and nitrate, histology	Barton et al. (2005) ³
Cromolyn	3–4 wk	3–6 wk	TA, Quad	Histology	Radley et al. (2006) ³⁷
Cromolyn	2 wk	2 wk	TA, diaphragm, sternomastoid	Histology, EBD uptake	Marques et al. (2008) ³¹
Cromolyn, prednisone, 48/80	8 wk	2 wk		Treadmill, muscle strength, serum CK, mast cell tryptase	Granchelli et al. (1996) ¹⁶
Pirfenidone	~28 wk	~32 wk	Heart	Langendorff experiments, histology, hydroxyproline	Van Erp et al. (2006) ⁴²
Curcumin	2 wk	3 wk	Diaphragm, soleus	Isometric force, NF- κ B activity	Durham et al. (2006) ¹⁴
Creatine, taurine, IGF-1, methylprednisone	4–8 wk	4 wk	Diaphragm, EDL, TA	Treadmill running, grip strength, chloride conductance, histology	De Luca et al. (2003) ⁸
IGF-1	8 wk	20–23 wk	TA	Isometric force contraction, histology, EBD	Schertzer et al. (2006) ³⁹
Calpain inhibitor	4–6 wk		EDL, diaphragm	Chloride channel function, histology	Burdi et al. (2006) ⁶
Cyclosporine	4–8 wk	4–5 wk	Diaphragm, EDL, gastrocnemius	Histology, chloride conductance, mechanical threshold, grip strength, serum CK	De Luca et al. (2005) ⁹
HCT 1026	24 wk	4 wk	TA, diaphragm	Histology, serum CK, EBD treadmill/running wheel	Brunelli et al. (2007) ⁴
EGCG	1 wk, 5 wk	3 wk	EDL, soleus, triceps surae	Histology, isometric force contraction	Dorchies et al. (2006) ¹¹
CrM, CLA, MB, α -LA, methylprednisone	8 wk	4 wk	Gluteus medius, diaphragm	Grip strength, rotarod, serum CK, histology, muscle biochemistry	Payne et al. (2006) ³³
Sodium chloride	1 wk, 3 wk, 9 wk, 12 wk	1 wk	TA	Histology, serum CK, electrolytes	Yoshida et al. (2006) ⁴⁵

Histology: H&E evaluation. IGF-1, insulin-like growth factor-1; TA, tibialis anterior; EDL, extensor digitorum longus; Quad, quadriceps; EBD, Evans blue dye; EGCG, (–)-epigallocatechin gallate; CK, creatine kinase; CrM, creatine monohydrate; CLA, conjugated linoleic acid; HMB, β -hydroxy- β -methylbutyrate; α -LA, α -lipoic acid.

Table 2

Comparison of body weight, normalized grip strength, and rotarod timing for C57BL/10 and *mdx* mice.

Measurement	Age (wk)	C57BL/10* (N = 15) [†]	<i>mdx</i> * (N = 13) [†]	P-value	Effect size with (N = 10) [‡]
Weight (g)	10–12	22.17 ± 0.96	23.64 ± 1.33	0.0023	7.5%
Norm max FL (KGF/kg)		5.506 ± 0.274	4.409 ± 0.346	<0.0001	10.4%
Norm max HL (KGF/kg)		7.273 ± 0.380	6.244 ± 0.466	<0.0001	9.9%
Rotarod (s) [§]		14.329 ± 1.478	14.714 ± 1.387	NS	12.5%
Weight (g)	20–22	22.12 ± 1.05	24.89 ± 0.88	<0.0001	4.7%
Norm max FL (KGF/kg)		6.077 ± 0.457	4.090 ± 0.400	<0.0001	13.0%
Norm max HL (KGF/kg)		9.004 ± 0.586	7.494 ± 0.598	<0.0001	10.6%
Rotarod (s) [§]		12.931 ± 1.788	12.747 ± 2.199	NS	22.9%
Weight (g)	38–40	26.93 ± 2.06	27.30 ± 1.50	NS	7.3%
Norm max FL (KGF/kg)		5.430 ± 0.722	4.054 ± 0.485	<0.0001	15.9%
Norm max HL (KGF/kg)		9.189 ± 1.207	8.455 ± 1.067	NS	16.7%
Rotarod (s) [§]		12.544 ± 1.437	12.352 ± 2.859	NS	30.7%

Norm, normalized; max, maximum; FL, forelimb; HL, hindlimb; NS, not statistically significant.

* Data expressed as mean ± SD.

[†] For 20–22 weeks: *mdx* N = 9; for 38–40 weeks: BL/10 N = 14 and *mdx* N = 10.

[‡] Effect size detectable in *mdx* mice at the P = 0.05 level with an N = 10 (power = 80%).

[§] Rotarod shown as square root transformed data.

Table 3

Comparison of behavioral activity monitoring measurements for C57BL/10 and *mdx* mice.

Measurement	Age (wk)	C57BL/10* (N = 15) [†]	<i>mdx</i> * (N = 13) [†]	P-value	Effect size with N = 10 [‡]
Horizontal activity	10–12	1650.32 ± 270.75	1014.10 ± 230.03	<0.0001	21.3%
Total distance (cm)		343.94 ± 72.16	196.26 ± 96.90	0.0001	46.5%
Rest time (s)		562.22 ± 8.45	576.50 ± 12.15	0.0012	2.0%
Vertical activity		26.87 ± 10.66	12.01 ± 13.11	0.0028	103%
Horizontal activity	20–22	1302.40 ± 224.36	968.25 ± 154.54	0.0007	15.0%
Total distance (cm)		252.08 ± 77.85	194.39 ± 68.48	NS	33.1%
Rest time (s)		571.54 ± 8.45	576.95 ± 8.59	NS	1.4%
Vertical activity		15.62 ± 5.39	9.96 ± 3.70	0.0112	35.0%
Horizontal activity	38–40	1394.91 ± 344.50	1326.62 ± 416.72	NS	29.5%
Total distance (cm)		282.96 ± 114.67	275.15 ± 176.11	NS	57.7%
Rest time (s)		569.05 ± 13.17	569.63 ± 20.00	NS	3.3%
Vertical activity		19.39 ± 11.23	22.75 ± 18.54	NS	76.6%

* Data expressed as mean ± SD. NS, not statistically significant.

[†] For 20–22 weeks: *mdx* N = 9; for 38–40 weeks: BL/10 N = 14 and *mdx* N = 10.

[‡] Effect size detectable in *mdx* mice at the P = 0.05 level with N = 10 (power = 80%).

Table 4

Comparison of echocardiography and blood pressure measurements for C57BL/10 and *mdx* mice at 38 – 40 weeks of age.

Measurement	C57BL/10* (N = 14)	<i>mdx</i> * (N = 10)	P-value	Effect size with N = 10 [†]
LVPW (d)	0.69 ± 0.08	0.70 ± 0.08	NS	10.7%
LVID (d)	3.90 ± 0.14	3.70 ± 0.19	0.0077	48.3%
LV %FS	30.63 ± 2.58	27.89 ± 1.86	0.0091	6.3%
Simpson %EF	46.59 ± 7.90	42.95 ± 5.48	NS	12.0%
Heart rate	408.36 ± 31.24	481.40 ± 43.50	0.0001	8.5%
Systolic BP [‡]	79.15 ± 3.08	74.10 ± 8.62	NS	10.9%
Diastolic BP [‡]	68.54 ± 6.50	50.60 ± 8.36	<0.0001	15.5%
Mean BP [‡]	71.85 ± 4.83	58.20 ± 8.08	0.0001	13.1%

LVPW(d), left ventricular posterior wall thickness in diastole; LVID(d), left ventricular internal diameter in diastole; LV %FS, left ventricular percent shortening fraction; Simpson %EF, percent ejection fraction measured via Simpson's method; BP, blood pressure.

* Data expressed as mean ± SD.

[†] Effect size detectable in *mdx* mice at the P = 0.05 level with an N = 10 (power = 80%).

[‡] Blood pressure measurements done on 13 C57BL/10 mice and 10 *mdx* mice.

Table 5

Comparison of histological parameters in the gastrocnemius muscle, percent fibrosis in the diaphragm, and serum CK levels in C57BL/10 and *mdx* mice at 38 – 40 weeks of age

Measurement	C57BL/10 (N) [*]	<i>mdx</i> (N) [*]	P-value	Effect size with N = 10 [†]
Total number of fibers/field	76.89 ± 15.35 (7)	96.32 ± 22.52 (7)	0.083	22.0%
Degenerating fibers/field	1.42 ± 1.15 (13)	5.42 ± 3.34 (7)	<0.001	57.9%
Regenerating fibers/field	1.23 ± 1.09 (13)	12.07 ± 6.69 (7)	<0.001	52.2%
Fibers with central nuclei/field	0.74 ± 0.56 (7)	43.05 ± 10.97 (7)	<0.001	24.0%
Peripheral nuclei/field	101.68 ± 18.17 (7)	108.94 ± 26.17 (7)	0.558	22.6%
Central nuclei/field	0.74 ± 0.56 (7)	59.74 ± 15.77 (7)	<0.001	24.8%
Peripheral nuclei/fiber	1.33 ± 0.11 (7)	1.142 ± 0.151 (7)	0.0211	12.4%
Central nuclei/fiber	0.010 ± 0.007 (7)	0.633 ± 0.152 (7)	<0.001	22.6%
Total nuclei/fiber	1.34 ± 0.10 (7)	1.77 ± 0.28 (7)	0.0029	15.3%
Inflammation/field	0.53 ± 1.23 (13)	1.92 ± 1.45 (7)	0.006	71.0%
Serum CK level (U/L)	85.20 ± 74.66 (15)	6439.66 ± 2506.59 (9)	<0.001	36.6%
Percent fibrosis (diaphragm) [‡]	7.39 ± 0.80% (5)	22.90 ± 7.52% (5)	0.0018	30.8%

* Data expressed as mean ± SD. CK, creatine kinase.

[†] Effect size detectable in *mdx* mice at the P = 0.05 level with N = 10 (power = 80%).

[‡] Percent fibrosis as estimated using Masson's trichrome staining was determined in the diaphragm muscle.