Behavioral, Biochemical and Pathological Characterization of a new MDX Mouse Model of Duchenne Muscular Dystrophy

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Core tip:
Duchenne muscular dystrophy (DMD) is an X-linked inherited neuromuscular disorder due to mutations in the dystrophin gene. Animal models that accurately reflect pathological conditions and disease characteristics are key factors in the discovery and development of new anti-DMD drugs. Here, we described a novel DMD mouse model built up by the Nanjing Biomedical Research Institute of Nanjing University (NBRI). We found that the DMD mouse showed significant behavioral disorders and exhibited increased serum creatine kinase (CK) and lactate dehydrogenase. Western blotting and Immunofluorescence staining also showed significantly decreased expression level of dystrophin in the gastrocnemius (GAS) muscle. Besides, the mdx mouse of DMD developed fibrosis in both GAS and diaphragm (DIA). Taken together, our results indicated that the behavioral, biochemical and pathological characterization of the mdx mouse model is consistent with human DMD. This genetic mouse model may provide insights into the pathophysiology of DMD and the effects of anti-DMD drugs.

Abstract:
Background Duchenne muscular dystrophy (DMD) is an X-linked inherited neuromuscular disorder due to mutations in the dystrophin gene. Animal models that accurately reflect pathological conditions and disease characteristics are key factors in the discovery and development of new anti-DMD drugs.
Aim Here, we evaluated motor behavior, pathological and biochemical characters of a new DMD mouse model built up by the Nanjing Biomedical Research Institute of Nanjing University (NBRI).
Methods The pole test and open-field test were used to assess the movement disorders in DMD mouse model. The gastrocnemius (GAS), biceps, triceps, soleus, and tibialis anterior muscles of mice were subjected to
weight analysis to evaluate the skeletal muscle pseudohypertrophy. Meanwhile, immunofluorescence and Western blotting were used to detect the expression of dystrophin in the GAS. Serum levels of creatine kinase (CK) and lactate dehydrogenase (LDH) that accurately reflect muscle damage were detected. Masson staining was used to evaluate the fibrosis of GAS and diaphragm (DIA).

**Results** The novel DMD mouse showed significant behavioral disorders and exhibited high serum levels of CK and LDH. Western blotting and immunofluorescence staining showed decreased significantly with dystrophin level in the GAS. Besides, the mdx mouse of DMD developed fibrosis in both GAS and DIA.

**Conclusion** Taken together, our results indicated that the behavioral, biochemical and pathological characterization of the mdx mouse model is similar to human DMD. This mdx mouse model may provide insights into the pathophysiology of DMD and the effects of anti-DMD drugs.

**Key words** Duchenne muscular dystrophy; Behavioral disorder; Creatine kinase; Dystrophin; Fibrosis

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**INTRODUCTION**

Duchenne muscular dystrophy (DMD) is an X-linked recessive genetic disease. Most clinical patients are male, while women are mainly pathogen carriers with mild symptoms 1. DMD is primarily caused by mutations in the gene that encodes the dystrophin, resulting in loss of dystrophin function. Dystrophin functions as a cell scaffold to maintain muscle fiber integrity and muscle contraction. Usually, the clinical symptoms such as bradykinesia, abnormal gait, difficulty in standing, and the tendency toward falling appear at the aged of 3 to 5 in patients. Children with DMD older than 7 years old are usually unable to walk and require a wheelchair. After the age of 12, the disease progressively deteriorates 2 and most patients die around the age of 20 due to respiratory and circulatory failure 3. Fibrosis is a prominent pathological feature in patients with DMD, which directly leads to muscle dysfunction 4. Follow-up studies of 25 DMD patients with an average age over 10 showed that intramuscular fibrosis was uniquely associated with poor motor outcomes; the initial muscle biopsy showed pathological features such as muscle fiber atrophy, necrosis, and steatosis 5. Respiratory failure is one of the leading causes of death in DMD patients 6.

The animals used in DMD research are diverse, including mice, dogs, cats, fish, and *Caenorhabditis Elegans* 7. Mice are used in the primary pre-clinical DMD research for the well-known physiological and genetic characteristics. The most commonly used mouse model strain is the C57BL/10ScSn- Dmd<sup>mdx</sup> model mouse 8, which spontaneously mutated from normal British C57BL/10ScSn mice in 1977. A nonsense point mutation in exon 23 of dystrophin gene led to the early appearance of the stop codon, resulting in loss of expression of the full-length dystrophin 8, 9. Mdx mice were found increased creatine kinase (CK) and pronounced muscle fibrosis. Yet, it exhibited a mild non-progressive phenotype. Because large-scale muscle fiber necrosis generally occurred at 20 days of age. Then, the muscle regeneration process was well compensated after 60 days and muscle fiber destruction is much lower after 120 days 10.

Therefore, we commissioned Nanjing Biomedical Research Institute of Nanjing University (NBRI) to construct a novel DMD mice model through gene-edition in the *Dmd* exon 4 using the CRISPR/Cas9 and blastocyst injection technology. We assessed the new model from both the physical symptoms and the primary biomarkers (serum CK, LDH, muscle fibrosis, *et al.*) of DMD. This study may provide a new mouse model and methods for pre-clinical research of DMD therapy.

**MATERIALS AND METHODS**

**ANIMALS**

Seven-week-old male mouse (C57BL/10ScSnJNju- Dmd<sup>em3Cd4/Nju</sup>) and wild-type littermate controls were purchased from Nanjing Biomedical Research Institute of Nanjing University, China. The mice were housed at 23 ± 2 °C with a 12 h light/dark cycle and fed standard rodent chow and water *ad libitum*. The mice were acclimatized for one week before the experiments. All animal procedures were performed in accordance with the US NIH’s *Guide for the Care and
Use of Laboratory Animals” and approved by the Animal Ethics Committee of the Guangzhou University of Chinese Medicine, China (Approved number # 20181104002).

CRISPR/Cas9-MEDIATED MICE GENOME EDITION
KO mice were created using the CRISPR/Cas9 technique. gRNAs were designed based on the targeted sequences in the mouse mdx genes (Gene ID: 13405) and were generated by inserting gRNAs into pUC57 via BbsI restriction sites. The Cas9 gene (pSpCas9n(BB)-2A-Puro) was driven by a CMV promoter and the gRNA and its RNA scaffold by a T7 promoter. gRNA sequences are as follows: mdx exon 4 sgRNAs: 5’-TGCCCTTGTGCATTTTCAGgg-3’, 5’-ACCGGAACAGTCTGCACACCagg-3’, 5’-CTTGTAGATCCCTTTTCTTT tgg-3’, 5’-CAATGTCAACAAGGCACTGcgg-3’. Cas9 mRNA and sgRNAs were transcribed in vitro, and then were co-injected into the zygotes’ pronuclei at a concentration of 2.5 ng/μL. After the injection, zygotes were left for 4-6 h before being introduced into pseudopregnant host females and carried to term. Edited founders were identified by Sanger sequencing from digit biopsies. Mice carrying the frameshift mutation were crossed with C57BL/10ScNJ to ensure germline transmission and eliminate any possible mosaicism. Heterozygous animals with the same modification were then mated to generate homozygous offspring.

EXPERIMENTAL DESIGN
Experiments were procedure at 8 weeks. Behavioral tests were performed every 4 weeks. After 32 weeks, the mice were euthanized with 0.1% pentobarbital sodium (0.15 mL/10 g). Blood was drawn and the levels of ATP, CK, LDH, and superoxide dismutase (SOD) were measured. Immunofluorescence, Western blotting and Masson staining were also conducted in both DIA and GAS muscles.

BEHAVIORAL TEST
The open-field test is an experimental test used to evaluate general locomotor activity levels. It was performed as previously described 11. Mice were placed in the center of the apparatus (50 × 50 × 50 cm) with a division of nine equal rectangles on the ground. The 5 minutes total distance of motion trace of each mouse were analyzed. As previously described 12, motor disorders in mice were also evaluated with a pole test using a homemade 50 cm stake with a diameter of 1 cm. The total time required for mouse to descend to the base of the pole is recorded for three trials.

IMMUNOFLUORESCENCE STAINING
Immunofluorescence staining was performed as previously described 11. Briefly, GAS muscle slides were incubated with the anti-dystrophin antibody (Abcam, ab15277) at 5 μg/mL overnight at 4 °C. The slides were then washed with TBST and incubated with the rabbit anti-mouse Alexa Fluor 488 conjugate secondary antibody (FD bio science, FDR007) in the dark at room temperature for 1 hour. Then, the slides were washed in TBST and counter stained with 4,6-diamino-2-phenyl indole (DAPI) for 5 minutes. Images of the slides were captured with an inverted fluorescence microscope (Olympus Corporation, Japan).

WESTERN BLOTTING ANALYSIS
Protein samples were loaded onto 10% or 12% polyacrylamide gels, and then electroblotted onto poly (vinylidene fluoride) (PVDF) membranes. After the membrane was blocked with 5% skim milk at room temperature for 1.5 hours, the membrane was covered with the specific primary antibody overnight at 4 °C. The primary antibodies used in the study included anti-β-actin (CST, 8457S), anti-dystrophin (Abcam, ab15277), and anti-TGF-β1 (Abcam, ab92486). After washed with TBST, membranes were incubated with the anti-mouse IgG-HP conjugated secondary antibody (1:2000) for further 1.5 hours. The ECL kit (Ford Biology, FD8020) was used to detect the proteins bound that were analyzed with a digital imaging system (Carestream...

VOL 10, ISSUE 06, YEAR 2020 121
The band intensities were quantified using the Carestream Molecular Imaging software.

**SERUM MEASUREMENT**
Changes of enzyme levels in muscle disease, which are directly related to muscle fiber necrosis or muscle damage and reflect the underlying disease processes. Serum CK and LDH are commonly used to help diagnose muscular dystrophy. The blood was clotted at room temperature for 30 minutes and then centrifuged at 1,500 r/min for 15 minutes to obtain the supernatants. The serum levels of CK, LDH, and SOD were measured using an automatic biochemical analyzer (Hitachi, Japan) according to the kit instructions (Xinyou Biotechnology, China). Values are reported as international units per liter (U/L).

**ATP CONTENT ASSAY**
The ATP level in GAS was detected using an ATP assay kit (Beyotime, S0026). Briefly, 30 mg GAS was fully lysed by adding 300 μL ATP lysis buffers for 30 minutes. The homogenate was centrifuged at 12,000 r/min for 15 minutes at 4 °C. According to the instructions, 50 μL of the sample homogenate was added to 50 μL ATP detection working solution and gently mixed. The OD value was measured at 570 nm using a multi-functional microplate reader (Bio Tek Corporation, USA). The ATP level in GAS was determined by a standard curve generated in the same assay.

**MASSON STAINING**
Paraffin-embedded muscle tissue slides (4 μm thick) in the DIA and GAS were deparaffinized in xylene, rehydrated in a graded alcohol series, and stained with Masson staining kit (Solarbio, G1340). The areas of fibrosis in DIA and GAS were measured using Materials Image Processing and Automated Reconstruction (MIPAR™) software (China).

**STATISTICAL ANALYSIS**
All results were expressed as means ± SEM. Data analyses were performed with GraphPad Prism software 6.0 (GraphPad, San Diego, CA, USA). An unpaired two-tailed Student’s t test or two-way ANOVA, were used for comparison between groups. A difference was considered statistically significant at \( P < 0.05 \).

**RESULTS**
**BEHAVIORAL DISORDERS IN DMD MODEL MICE**
In the open-field test, the movement distance of the model mice was significantly decreased from the 12th week compared with the WT mice (Fig. 1 A, B). Moreover, the model mice spent more time climbing down from the pole than the WT mice (Fig. 1 C), which indicated that the coordination of model mice was significantly worse than that of WT. More importantly, the movement disorders of model mice still appeared at the end of the experiment, which meant that the dyskinesia derived from the gene modification was stabilized.
MUSCLE WEIGHT OF MODEL MICE

It has been reported that hypertrophy in DMD patients is caused by deposits of fat and connective tissue. The body weight gradually decreases in late adolescence \cite{18, 19}. However, the increased muscle weight in the mdx mouse presented similar incomprehensible feature of muscle hypertrophy as DMD patients \cite{19, 20}. Such conditions lead to an unstable posture and joint contraction. As we observed, the weight of soleus, biceps brachii, and triceps brachii in model mice significantly increased than those in the WT group (Fig. 2 C, E, F), while there was no noticeable difference in the GAS and tibialis anterior (Fig. 2 B, D).

Figure 1 Behavioral disorders in model mice. (A) Total distance travelled in the open-field test; (B) Trajectories of WT and model mice in the open-field test. (C) Climbing time on pole test. Data are represented as mean ± SEM, #P < 0.05 and ###P < 0.001 relative to WT mice determined by two-way ANOVA.

Figure 2 Isolated muscle mass of model mice. (A) Isolated skeletal muscle; from left to right: gastrocnemius, soleus muscle, tibialis anterior muscle, biceps, and triceps; (B) - (F) Weight statistics of five isolated skeletal muscles. Data are represented as mean ± SEM, ###P < 0.001 by t-test.
DYSTROPHIN DEFECT IN DMD MODEL MICE

Dystrophin is localized on the cytoplasmic surface of the sarcolemma and has a cell scaffold function to maintain muscle fiber integrity and muscle contraction. Both immunofluorescence and Western blotting results of GAS showed similar significant reductions in the dystrophin expression in the model's skeletal muscles (Fig. 3). These results suggested that muscle fiber integrity and muscle contraction were somewhat disrupted.

Figure 3 Dystrophin is impaired in model mice. (A) Immunofluorescence staining of dystrophin in GAS (scale bar, 20μm). (B) Western blot analysis of dystrophin in GAS. (C) Quantification of the expression level of dystrophin in the mouse model and WT muscle. Data are represented as mean ± SEM. ##P < 0.01 by t-test. Four mice/group. DAPI= 4,6-diamino-2-phenyl indole.

SERUM CK AND LDH ELEVATED IN MODEL MICE

Serum CK is a hallmark of clinical DMD diagnosis for reflection of muscle damage. Serum CK level in DMD patients is usually high at a range of 5,000 to 150,000 U/L (while that in normal people is less than 200 U/L). Serum LDH levels also reflect tissue damage. The results showed that CK and LDH levels were significantly elevated in the model mice compared to the WT group (Fig. 4 A, B), indicating severe muscle and tissue damage in the model group.
MODEL MICE HAVE MITOCHONDRIAL DYSFUNCTION

We detected serum SOD and ATP levels in GAS, which are associated with mitochondrial dysfunction. The experimental results showed that the serum SOD level of model mice was significantly higher compared to the WT. On the contrary, the ATP level dramatically decreased in the model group (Fig. 4 C, D). These data demonstrated that there may be mitochondrial damage in the DMD model mice.

SEVERE MUSCLE FIBROSIS IN MODEL

Fibrosis is a pathological feature observed in DMD patients and mdx mice. To quantify the level of muscle fibrosis, we performed Masson staining on GAS and DIA, and then used MIPAR image analysis software to statistically analyze collagen. As illustrated in Fig. 5 A-D, masson staining showed that the fibrotic area of GAS and DIA in the model mice was much larger than that in the WT group, indicating severe fibrosis of GAS and DIA in model mice. The data showed in Fig. 5C and E demonstrated a significant up-regulation of TGF-β1 in GAS, which is in accordance with the published literature.

Figure 5 Severe fibrosis of GAS and DIA in model mice. (A) Masson staining of the GAS, the cytoplasm, and muscle fibers are stained red while the collagen fibers appear blue (scale bar, 200μm). (B) Masson staining of the DIA (scale bar, 200μm). (C) Quantitative analysis of GAS muscle fibrosis. (D) Quantitative analysis of DIA muscle fibrosis. (E) Western blot analysis of TGF-β1 in the GAS. (F) Quantitative analysis of TGF-β1 in the GAS. Data are represented as mean ± SEM. #P < 0.05 and ##P < 0.01 by t-test. Six mice/group. GAS= Gastrocnemius; DIA= Diaphragm.
DISCUSSION
DMD is a rare disease. Usually, patients behave normally at birth, and gradually lose motor ability after seven years. In one-third of patients, the lacking or insufficient of dystrophin which derived from the partial or points mutations in the stop codon of the dystrophin gene is the underlying mechanism of the disease. Dystrophin deficiency in DMD leads to the instability of myocyte membranes and an uncontrolled influx of calcium. This triggers a series of pathological processes that eventually lead to protein break down and cell damage in muscle cells. Subsequently, muscle tissue is replaced by fibroblasts and fat cells, resulting in collagen deposits (fibrosis). Later, the pseudo-hypertrophy of the calf muscles and the gradual weakness of the proximal limb muscles inevitably lead to wheelchair dependence.

A suitable animal model can reliably predict human responses to a given therapeutic intervention. Several DMD animal models, such as dogs, cats, mice, fish, and invertebrates have been constructed. Nevertheless, murine models are valuable for research because it is easy to breed and genetically engineer them compared to other large animal models. Though mdx mouse is the most widely used animal model for DMD, it shows a mild phenotype that differs from human. So, we are committed to designing a novel mouse model with a more severe phenotype.

CRISPR/Cas9 is a fast-growing genomic editing tool that is widely used in drug discovery and the development of animal models. NBRI targeted the gRNA of the mouse Dmd exon 4 using CRISPR/Cas9 and blastocyst injection technology. Then they screened for a mouse model that caused a frame shift mutation in the Dmd gene. And the model mouse is the background of C57BL/10, consistent with the mdx mouse model.

The mdx shows no obvious muscle weakness that differs from human while DMD mice have behavioral disorders in the 8th weeks and last for 24 weeks. The creatine kinase level in mdx mice is significantly higher than that in normal mice; however, it gradually decreases in the fifth week and eventually reaches 4000-6000 U/L at the 23rd week. Meanwhile, the creatine kinase levels in the model mice reach 6393-7973 U/L at 32 weeks, indicating that the muscle damage is more severe. Besides, the results showed that soleus, biceps, and triceps muscle in model mouse were obvious pseudo-hypertrophy and with increased mass. The immunofluorescence of dystrophin on the surface of the GAS membrane showed dimmer, which was consistent with the results of immunoblotting. And the model mice had lower ATP levels in the GAS. Besides, severe fibrosis was observed in the GAS and DIA muscles of model mice.

Of course, we need more experiments to determine the stability and reproducibility of the model and to perform further pathological analysis on other muscles, such as the myocardium. More importantly, it is necessary to determine the onset time and pathological process of model mice to better assist in drug development.

CONCLUSIONS
Currently, the establishment of an effective experimental model will help to screen a large number of drugs and make a significant contribution to drug advancement in the clinical arena. Our preliminary data on mouse model suggested that the model mice had a similar motor deficit and muscle pathology to human DMD. Thus, this mouse model may be a potential tool for exploring DMD drugs in the future.

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