A Nitrate Ester of Sedative Alkyl Alcohol Improves Muscle Function and Structure in a Murine Model of Duchenne Muscular Dystrophy

Guqi Wang* and Qilong Lu

McColl-Lockwood Laboratory, Carolinas Medical Center, Charlotte, North Carolina 28232, United States

ABSTRACT: Nitric oxide (NO) has major physiological and cellular effects on muscle growth, repair, and function. In most muscle biopsies from humans with myopathies, sarcolemma-localized neuronal nitric oxide synthase (nNOS) is either reduced or not detected, particularly in dystrophin-deficient Duchenne muscular dystrophy (DMD). Abnormal NO signaling at the sarcolemmal level is integrally involved in the pathogenesis and accounts, at least in part, for the muscle weakness of DMD. Dystrophic muscle fibers exhibit an increased susceptibility to contraction-induced membrane damage. Muscle relaxants function to prevent muscle wasting by decreasing nerve impulses and reducing calcium influx that regulates tensing or tightening of muscle fibers. We have recently developed a new class of nitric esters that combines the pharmacological functions of NO and muscle relaxation. Here, we report the synthesis and properties of the nitric ester (MMPN) of 2-methyl-2-n-propyl-1,3-propanediol (MPP) and its effect in mdx dystrophic mice, a murine model of DMD. MMPN produced significant improvements in biochemical, pathological, and functional phenotypes in the mouse model. The endurance of exercise was extended by 47% in time to exhaustion and 54% in running distance. Serum CK level was decreased by 30%. Additionally, MMPN decreased intracellular free calcium concentration without causing skeletal muscle weakness. No hepatic or renal toxicities were observed during the study. Our investigations unveil a potential new treatment for muscular diseases.

KEYWORDS: nitric oxide, muscle relaxant, myostatin, myogenin, skeletal muscle, Duchenne muscular dystrophy.
excitation-contraction coupling of skeletal muscle by blocking the release of calcium from the sarcoplasmic reticulum into the cytoplasm, diminishes exercise-induced muscle damage, and normalizes serum CK in dystrophic animal models. These results suggest a potential therapeutic effect of sedatives or muscle relaxants for protecting muscles from degeneration. This notion was also supported by our previous results, namely, that the muscle relaxant, methocarbamol increased DNA synthesis in skeletal muscle, and was additive to the significant effects of NO on muscle regeneration in vivo. When muscles (as well as the body) become relaxed, so does the central nervous system responsible for circulation and immune functions. NO stimulates the immune system, promotes blood circulation, and increases delivery of nutrients and oxygen to damaged areas while removing waste, and improves cellular repair. If these functions together with relaxation could happen simultaneously, the body would get time for repair.

Thus, desirable options to alleviate dystrophic phenotype are to reduce contraction-related damage and improve repair process while maintaining essential muscle functions by a combined treatment of NO and muscle relaxant. To test this hypothesis, we have developed a new class of compounds having dual pharmacological functions of muscle relaxant and NO carrier. In this study, we report a nitrate ester of 2-methyl-2-n-propyl-1,3-propanediol that is structurally similar to the commonly used muscle relaxant, Meprobamate (structures are shown in Figure 1A), and its beneficial effects on skeletal muscle regeneration, repair, and functions in mdx mice.

MATERIALS AND METHODS

Synthesis of 2-Methyl-2-[(nitrooxy)methyl]pentyl Nitrate (MMPN). A schematic of the synthesis of MMPN is shown in Figure 1B. A sample of 42 mmol of concentrated nitric acid was added dropwise into 10 mL of acetic anhydride with continuous stirring and cooling in an ice bath. After the nitric acid had been added, 20 mmol of MPP was added in small amounts to the nitric acid−acetic anhydride mixture in the ice bath, and the mixture was left at room temperature for an additional 1 h, after which it was poured into 100 mL of ice-cold water. The product was extracted with 2 × 25 mL diethyl ether. The organic layer was collected and washed with 2 × 10 mL saturated sodium bicarbonate solution and then 3 × 10 mL water. The organic layer was dried over MgSO4 and concentrated by evaporation. The yield was over 90%. The identity and purity of the compound were established by NMR and high-performance liquid chromatography (Figures S1 and S2).

Animals and Drug Treatment. Mdx mice and C57BL6 mice were purchased from The Jackson Laboratory (Maine, USA) and bred in the vivarium of Carolinas Medical Center, which is accredited by Association for Assessment and Accreditation of Laboratory Animal Care International. All mice were maintained with 12 h light/dark cycles with food and water ad libitum and received humane care in compliance with Carolinas Medical Center Institutional Animal Care and Use Committee guidelines, which are in accordance with criteria set by the Office of Laboratory Animal Welfare.
MMPN was dissolved in corn oil freshly. In an initial acute toxicity study of MMPN, two month old C57BL6 mice were gavaged with a single large dose of MMPN (3.4 g/kg). Blood and tissue samples were collected 2 weeks after the treatment. In a chronic toxicity study, MMPN was gavaged into the mice daily in the dose of 60 mg/kg, Monday through Friday for 10 months. Blood and tissue samples were collected for serum chemistry and histological analysis.

Male mdx mice (4 weeks old) were used to test the therapeutic effect with 40 mg/kg (body weight) MMPN in 0.1 mL of corn oil Monday through Friday for 6 months by gastric gavage using 20 gauge bulb-tipped flexible feeding needle. Animals of the same age group were treated with the same amount of corn oil only as controls. The appearance and body weight of animals were checked and recorded daily until the end of the experiment.

**Assessment of Muscle Functions.** Overexercise may cause muscle damage in mdx mice and increase serum CK. To assess the effect of MMPN treatment on muscle function, grip strength and treadmill exercise tests were performed 10 days before ending the study. Considering muscle relaxant activity of the compound, all tests were done 12 h after the drug treatment.

A mouse grip strength meter (Columbus Instruments; Columbus, OH) was used to measure forelimb grip strength. The test was repeated five consecutive times within the same session, and the highest value from the five trials was recorded as the grip strength for that animal.

In treadmill exercise, mice were placed on a four-channel mouse treadmill system (Harvard Apparatus) equipped with a stimulus shock grid (setting at 0.6 mA). Mice were first trained for 5 min at 5 m/min and a 0° incline. After a 30 min break, the running exercise test was then started. The speed was set at 5 m/min at the beginning and then was increased 1 m/min every min up to 15 m/min. Mice that remained on the shock grid with stimulus for 10 s were considered to be exhausted.

ROTA-ROD for mice 7650 (Ugo Basile, Varese, Italy) was used in this study to evaluate muscle relaxation effect of the compounds in normal mice. C57BL6 mice were trained before testing. The rod was initially rotating at 4 rpm constant speed to allow the mice to become accustomed to walking forward and keeping their balance in their respective lanes. All mice had to stay on rod at 4 rpm for 60 s before moving on to the test. The apparatus was set to accelerating mode from 4 to 40 rpm in 300 s. The numbers of seconds before falling were used as the animals’ performance.

**Assessment of Experimental Samples.** Serum samples were sent to the Research Animal Diagnostic Laboratory (University of Missouri-Columbia, MO) for the assay of CK and other markers of liver and kidney function (ALT, ALP, BUN, bilirubin).

Tissues from the treated and control mice were dissected and immediately snap frozen in isopentane on dry ice. Sections of 6 μm were collected at −20 °C onto 3-aminopropyltriethoxysilane (Sigma-Aldrich, St. Louis, MO) coated glass slides using a Leica CM1850 cryostat and were subjected to H&E staining. Muscle fiber cross section area (CSA) was determined by measuring the shortest diameter of muscle fibers. Twenty fibers each from four corner and center areas in a field were measured; in total, 800 fibers from eight randomly selected fields were determined by using the NIH software ImageJ. Muscle fiber central nucleation was determined as an index of ongoing dystrophy as the ratio of number of the fibers with centered nuclei to the number of total fibers in a field. Eight randomly selected fields were counted.

Vascular densities in cardiac and skeletal muscle were measured by endogenous endothelial alkaline phosphatase staining on frozen sections. Slides were postfixed in 4% paraformaldehyde and incubated with nitroblue tetrazolium (NBT)-5-bromo-4-chloro-3-indolyphosphate-p-toluidine salt for 1 h. The slides were then stained with eosin. Capillaries appear dark blue against a red background. Capillary density was measured by counting random 6 fields or a minimum of 200 fibers of each field from each muscle section.

**Western Blots.** Proteins were extracted from 15 μm thick muscle cryosections using a motorized hand-held homogenizer in the protein extraction buffer [125 mM Tris-HCl (pH 6.8), 4% SDS, 10% glycerol, 2 M urea, 5% mercaptoethanol, and protease inhibitor cocktail (Roche 04693159001)]. Protein (80 μg) was loaded onto 12% polyacrylamide gels for electrophoresis and blotting. The following antibodies were used for the Western blotting: rabbit antimonyosin polycional antibody (AB3239, Millipore, CA); mouse antimonogenon monoclonal antibody (the Developmental Studies Hybridoma Bank, IA); rabbit anti-TNF-α polyclonal antibody (ab6671, Abcam Inc., MA); rabbit antiutrophin polyclonal antibody (sc-15377, Santa Cruz Biotechnology Inc., CA). These primary antibody bindings were probed with the corresponding secondary antibodies conjugated with horseradish peroxidase and were visualized by using standard enhanced chemiluminescence. The results of Western blots were assessed visually by densitometric analysis of protein bands using NIH ImageJ (http://rsb.info.nih.gov/nih-image/).

**Intracellular Free Calcium Level Determination.** Human skeletal muscle cells (Cell Application, Inc. San Diego, CA) were grown in skeletal muscle growth medium (Cell Applications Inc., cat. nos. 151−500), and plated in a 96 well plate for 24 h until confluent. The cells were then treated with MMPN, MPP, and ISD (each at 200 μM) with MMPN, MPP, and ISD (each at 200 μM) for 5 min at 5 m/min and a 0° incline. After a 30 min break, the running exercise test was then started. The speed was set at 5 m/min at the beginning and then was increased 1 m/min every min up to 15 m/min. Mice that remained on the shock grid with stimulus for 10 s were considered to be exhausted.

ROTA-ROD for mice 7650 (Ugo Basile, Varese, Italy) was used in this study to evaluate muscle relaxation effect of the compounds in normal mice. C57BL6 mice were trained before testing. The rod was initially rotating at 4 rpm constant speed to allow the mice to become accustomed to walking forward and keeping their balance in their respective lanes. All mice had to stay on rod at 4 rpm for 60 s before moving on to the test. The apparatus was set to accelerating mode from 4 to 40 rpm in 300 s. The numbers of seconds before falling were used as the animals’ performance.

**Assessment of Experimental Samples.** Serum samples were sent to the Research Animal Diagnostic Laboratory (University of Missouri-Columbia, MO) for the assay of CK and other markers of liver and kidney function (ALT, ALP, BUN, bilirubin).

Tissues from the treated and control mice were dissected and immediately snap frozen in isopentane on dry ice. Sections of 6 μm were collected at −20 °C onto 3-aminopropyltriethoxysilane (Sigma-Aldrich, St. Louis, MO) coated glass slides using a Leica CM1850 cryostat and were subjected to H&E staining. Muscle fiber cross section area (CSA) was determined by measuring the shortest diameter of muscle fibers. Twenty fibers each from four corner and center areas in a field were measured; in total, 800 fibers from eight randomly selected fields were determined by using the NIH software ImageJ. Muscle fiber central nucleation was determined as an index of ongoing dystrophy as the ratio of number of the fibers with centered nuclei to the number of total fibers in a field. Eight randomly selected fields were counted.

Vascular densities in cardiac and skeletal muscle were measured by endogenous endothelial alkaline phosphatase staining on frozen sections. Slides were postfixed in 4% paraformaldehyde and incubated with nitroblue tetrazolium (NBT)-5-bromo-4-chloro-3-indolyphosphate-p-toluidine salt for 1 h. The slides were then stained with eosin. Capillaries appear dark blue against a red background. Capillary density was measured by counting random 6 fields or a minimum of 200 fibers of each field from each muscle section.

**Western Blots.** Proteins were extracted from 15 μm thick muscle cryosections using a motorized hand-held homogenizer in the protein extraction buffer [125 mM Tris-HCl (pH 6.8), 4% SDS, 10% glycerol, 2 M urea, 5% mercaptoethanol, and protease inhibitor cocktail (Roche 04693159001)]. Protein (80 μg) was loaded onto 12% polyacrylamide gels for electrophoresis and blotting. The following antibodies were used for the Western blotting: rabbit antimonyosin polycional antibody (AB3239, Millipore, CA); mouse antimonogenon monoclonal antibody (the Developmental Studies Hybridoma Bank, IA); rabbit anti-TNF-α polyclonal antibody (ab6671, Abcam Inc., MA); rabbit antiutrophin polyclonal antibody (sc-15377, Santa Cruz Biotechnology Inc., CA). These primary antibody bindings were probed with the corresponding secondary antibodies conjugated with horseradish peroxidase and were visualized by using standard enhanced chemiluminescence. The results of Western blots were assessed visually by densitometric analysis of protein bands using NIH ImageJ (http://rsb.info.nih.gov/nih-image/).

**Intracellular Free Calcium Level Determination.** Human skeletal muscle cells (Cell Application, Inc. San Diego, CA) were grown in skeletal muscle growth medium (Cell Applications Inc., cat. nos. 151−500), and plated in a 96 well plate for 24 h until confluent. The cells were then treated with MMPN, MPP, and ISD (each at 200 μM) with MMPN, MPP, and ISD (each at 200 μM) for 5 min at 5 m/min and a 0° incline. After a 30 min break, the running exercise test was then started. The speed was set at 5 m/min at the beginning and then was increased 1 m/min every min up to 15 m/min. Mice that remained on the shock grid with stimulus for 10 s were considered to be exhausted.

ROTA-ROD for mice 7650 (Ugo Basile, Varese, Italy) was used in this study to evaluate muscle relaxation effect of the compounds in normal mice. C57BL6 mice were trained before testing. The rod was initially rotating at 4 rpm constant speed to allow the mice to become accustomed to walking forward and keeping their balance in their respective lanes. All mice had to stay on rod at 4 rpm for 60 s before moving on to the test. The apparatus was set to accelerating mode from 4 to 40 rpm in 300 s. The numbers of seconds before falling were used as the animals’ performance.
The reduction of CK after treatment may not be accompanied by a reduction of ALT and ALP in mdx mice. As shown in Table 2, both treated and untreated mdx mice had higher ALT and ALP levels relative to normal C57BL6 mice (Table 1). ALT and ALP are mainly used as biomarkers of hepato-biliary disorders. The dystrophic disease state in mdx mice also affects other nonmuscle organs, such as liver, which complicates interpretation of the abnormal levels of ALT and ALP.

Expression of Proteins Associated with Muscle Repair and Development. Myogenin plays an important role in muscle development. An increase of myogenin is a hallmark of myogenic differentiation. Myostatin is a growth and differentiation factor that regulates the size of muscles by inhibiting satellite-cell activation. As can be seen in Figure 2, mice treated with MMPN showed an increase in expression of myogenin and a decrease in myostatin levels. The results suggest that MMPN treatment enhanced skeletal muscle myogenesis.

Utrophin is the autosomal homologue of dystrophin. Up-regulation of utrophin has been shown to functionally compensate for the lack of dystrophin in mdx mice. NO donor, sodium nitroprusside, or l-arginine increased utrophin expression in wild type and mdx mice. We therefore examined the utrophin expression in the muscles of MMPN treated mdx mice. As shown in Figure 2, MMPN treatment increased the levels of utrophin expression, suggesting a possible mechanism for the observed improvement in muscle pathology.

Reduction of Muscle Inflammation and Degradation as well as Intracellular Free Calcium. H&E staining (Figure 3A) demonstrated significant reductions of tissue inflammatory infiltrates and fiber degradation in the biceps (upper panel) and tibialis anterior (TA, lower panel) muscles of mdx mice after MMPN treatment. Consistently, tumor necrosis factor (TNF)-α, a pro-inflammatory cytokine that is involved in the dystrophic pathology, was reduced (Figure 3B).

Abnormally high levels of cellular free calcium are associated with muscular dystrophy and are a key factor responsible for muscle damage and necrosis. Thus, decreasing intracellular calcium levels would be expected to provide beneficial effects on muscular dystrophy. We therefore examined the effect of MMPN and MPP on free calcium in human skeletal muscle cells.
cells. The results showed that, as expected, MMPN and MPP (a muscle relaxant) significantly reduced intracellular free calcium concentrations (Figure 4), as shown by decreases of calcium fluorescence intensity by 16% and 18%, respectively. This compares favorably with the effect of isosorbide dinitrate, which caused about 7% of reduction in cellular free calcium. This implicates an additional benefit of MMPN.

Effects of MMPN Treatment on Muscle Pathology of Mdx Mice. The central nucleation index is a marker of cumulative muscle regeneration and is commonly used as a pathological sign of muscular dystrophies. Reduction of NO-dependent vascular density and function were found in dystrophin-related myopathies. As shown in Table 3, the central nucleation was reduced mildly from 79% to 70%, which suggested a limited effect of this small molecule compound on histological improvement. However, the muscle fiber size and vascular density were increased significantly in the mdx mice after MMPN treatment. We also statistically analyzed the muscle fiber size of the treated and untreated groups. The treated group had a decrease of very small fibers (Figure 5). Taken together, these data indicate that MMPN treatment alleviates dystrophic pathology.

MMPN Treatment Improved Exercise Performance and Endurance in Mdx Mice. Muscle relaxants could reduce CK in mdx mice but cause muscle weakness, which is a major concern in the management of muscular dystrophy. In this study, we used a Rota-Rod treadmill to assess the effect of MMPN on fatigue resistance and motor coordination in C57BL6 mice. Mice were divided into three groups: (1) treated with muscle relaxant MPP; (2) treated with MMPN (nitrate ester of MPP); and (3) no drug treatment. As shown in Figure 6, MPP significantly weakened the mouse performance by 35%, while MMPN enhanced the performance by 26%. Although this enhancement was not statistically greater than that of the untreated group (perhaps due to the limited sample sizes), the

Figure 3. (a) H&E staining of a cross section of the skeletal muscles from MMPN treated mdx mice. Biceps (upper panel) and tibialis anterior (lower panel) muscles show significant reduction of tissue inflammation and degradation. Encircled areas indicate muscle damage and inflammation. (b) Western blotting analysis of TNF-α in the skeletal muscles of MMPN treated mdx mice. TNF-α levels were reduced by up to 60% in signal intensity of the TNF-α in the treated samples compared to the controls measured by NIH ImageJ. C and T represent the samples of control and treated mice, respectively. The images are representative of biological replicates from eight animals.
results are suggestive that MMPN may improve muscle function and not cause muscle weakness.

Muscle strength was assessed by treadmill and grip strength test at the end of 6 month course of treatment to mdx mice. As illustrated in Table 4, MMPN treatment extended the endurance of exercise by 47% in time to exhaustion and 84% in running distance ($p < 0.05$). Grip strength of the MMPN treated mice was also increased significantly compared to the untreated animals ($p < 0.01$).

## DISCUSSION

As a cell-signaling molecule responsible for many vascular and skeletal muscular functions, NO plays an important role in muscle growth and performance. However, compensating NO only may not be able to bring benefit to DMD and other muscular dystrophies. NO may prompt calcium leakage in dystrophic muscle, leading to elevated cellular calcium and consequently dystrophic pathogenesis. Supplement of NO can boost exercise performance. However, chronic uncontrolled exercise could exacerbate the impaired calcium homeostasis of dystrophic fibers and aggravate the disease. Therefore, it is desirable to prevent these potential side effects to dystrophic muscle when NO is used as a therapy. Alcohol is a well-known depressant, which inhibits calcium influx into the myocyte, leading to a decrease in muscle strength output and activity without causing structural muscle damage. Most alcohols (including diols) and their carbamates are known to be sedative-hypnotics and additionally have skeletal muscle relaxant properties. Branching of the alkyl chain increases depressant activity. In this study, we synthesized a nitrate ester of a branched diol, namely, 2-methyl-2-[(nitrooxy)-methyl]pentyl nitrate (MMPN), and tested the compound for its effect in dystrophic mouse model. We showed that the MMPN improves muscle pathology and functions in the mouse. The treatment considerably reduced serum levels of CK, a sensitive marker of cardiac diseases and muscular dystrophy, although the CK levels in treated animals were still higher than that in normal control mice. This decrease is likely more significant considering that the treated mice showed increase in voluntary exercise activity, which generally increases CK levels in dystrophic mice.

Many therapeutic attempts have been proposed to modify the expression profiles of patients with a variety of inherited and acquired forms of neuromuscular diseases. Recent observations in myopathic biopsies have revealed that sarcolemma-localized nNOS is either reduced or undetectable,
contributing to the disease progression. NO-nNOS signaling might be a relative nonspecific, downstream target, and NO therapy would be a more likely effective treatment. In mdx mice, deficiency of dystrophin leads to nNOS mislocalization and reduction. Unlike DMD, the clinical manifestation of the disease is substantially milder in mdx mice. Nevertheless, the animals share similar defects in nNOS and NO production, which impair skeletal muscle contraction, vascular dilation, and low arteriolar density. Supplements of NO as a complementary treatment for DMD have therefore been considered as a potential therapy. Isosorbide dinitrate (ISD), a NO donor, has been reported to improve bupivacaine-induced muscle regeneration significantly in mdx mice but did not affect spontaneous muscle regeneration. NO donors also improve effects of prednisone on dystrophic phenotype of the diaphragm in mdx mouse. Brunelli et al. reported that combination of NO with nonsteroidal anti-inflammatory agent alleviates pathology and enhances effect of stem cell therapy in dystrophic animals. Targeting NO-cGMP signaling pathways by a phosphodiesterase 5 (PDE-5) inhibitor, sildenafil, has been reported to compensate for the loss of NO at the muscle membrane and reverse cardiomyopathy in mdx mice. However, it is unclear whether the drug also has beneficial effects to other muscles in the model. Currently, the PDE-5 inhibitor as a drug to provide protection to skeletal and cardiac muscle of DMD patient is under clinical evaluation. Attempts to increase NO production by dietary supplement of NO substrate l-arginine have been reported to produce beneficial effects in mdx mice; however, long-term supplementation exacerbates fibrosis of the dystrophic heart and muscles and has a potential risk for increasing the pathology.

Our results indicate that MMPN alleviates muscular dystrophy through several different pathways. MMPN treatment increases the levels of myogenin but reduces the levels of myostatin modestly, suggesting that the treatment improves satellite cell activation, proliferation, and myoblast differentiation. These effects are consistent with the increase in muscle fiber size and decrease in population of regenerating small fibers and very large fibers. Dramatically eliminating myostatin in dystrophic animal may have undesirable effects; therefore, we consider the modest alteration of myogenin and myostatin with MMPN would be beneficial. In dystrophic muscles, insufficient vascular density, a possible outcome of reduced NO production, results in impairment in muscle contraction. The treatment of MMPN increases vascular density (Table 3) in heart and skeletal muscles. This effect is likely beneficial to the function of the dystrophic muscles and explains the significant improvement of exercise performance in the treated mdx mice. Our study also shows that MMPN decreased intracellular free calcium in human skeletal muscle cells. This effect is similar to that achieved by muscle relaxant, MPP. Although muscle relaxants (e.g., dantrolene) can relieve muscle stiffness of muscular dystrophy and was reported to benefit DMD and mdx mice, they may worsen muscle weakness in patients. We had the same concerns in light of the data we obtained for MPP (Figure 6). Interestingly, when MPP was conjugated with NO, it did not impair muscle strength and conversely enhanced exercise endurance to some extent. NO here seems to work as an antagonist of the muscle relaxant. It is well-documented that NO enhances muscle strength. However, the mechanism that NO prevents muscle weakness caused by muscle relaxant remains to be investigated.

The functional tests of this study demonstrate a significant improvement of exercise performance in mdx mice that indicate the restoration of muscle strength after MMPN treatment. Yet this is associated with mild reduction in central nucleation of skeletal muscles. Collectively, these results suggest that pharmacological treatment with MMPN could be clinically useful for improving the symptoms of DMD, even though it is less likely to repair the primary defect of DMD. Nevertheless if it will delay the progression of the disease, it will still be very significant. It has been widely conceived that effective treatment of DMD will likely involve a multitarget, cocktail approach rather than a single drug. Therefore it is possible that our compound could be more valuable when used in combination with other types of approaches such as vascular gene transfer, exon skipping, myostatin inhibition, and prednisone.

It is worth noting that the beneficial effects of NO to dystrophic muscles may also depend on the host environment and quantity of NO released locally. Organic nitrates may cause hypotension in some patients. High concentrations of NO can also cause cellular nitrosative (nitrogen involved peroxidation) stress, inflammation, and tissue damage. Development of tolerance to the pharmacologic and therapeutic effects of MMPN may occur as reported with many organic nitrate therapies. This is the rationale behind our treatment regime wherein the drug was given only for 5 days every week during this study. Therefore optimization of dosing, formulation, and regime could be critical for achieving therapeutic effect in clinical application. Furthermore, many other factors related to pharmacokinetics and pharmacodynamics of MMPN as a drug, its absorption, protein binding, biotransformation, half-life, onset of action, side effects, and duration of action need to be established in future studies.

We have developed a new concept that combines the pharmacological functions of NO and muscle relaxant for the treatment of muscular diseases. The compound we report in this study provided significant benefits to muscle functions and structure in mdx dystrophic mice. To develop new compounds and demonstrate their positive effects in improving the outcome of muscular dystrophy in animal models will advance

Table 4. Treadmill and Grip Strength Test Results

<table>
<thead>
<tr>
<th></th>
<th>running time (min)</th>
<th>running distance (meter)</th>
<th>grip strength (LBF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>treated control</td>
<td>treated control</td>
<td>treated control</td>
<td>treated control</td>
</tr>
<tr>
<td>19.9 ± 2.2°</td>
<td>13.5 ± 1.0</td>
<td>248 ± 39°</td>
<td>0.24 ± 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>135 ± 15</td>
<td>0.17 ± 0.01</td>
</tr>
</tbody>
</table>

“Male mdx mice (one month old) were gavaged with MMPN daily for 6 months. Mice were placed on a four-channel mouse treadmill system (Harvard Apparatus) equipped with a shock plate (setting at 0.6 mA). Mice were first trained for 5 min at 5 m/min and 0° incline. After a 30 min break, running exhaustion test then started. The speed was set at 5 m/min at beginning and then was increased 1 m/min every min up to 15 m/min. The treatment significantly increased the animal’s endurance of exercise; a mouse grip strength meter (Columbus Instruments; Columbus, OH) was used to measure forelimb grip strength. The test was repeated five consecutive times within the same session, and the highest value from the five trials was recorded as the grip strength for that animal. Data are presented as mean ± SE, n = 8, *p < 0.05, **p < 0.01.”
our current understanding of NO-based treatment in muscle diseases and will lead to the development of new therapies for common neuromuscular diseases and muscle wasting conditions.

ACKNOWLEDGMENTS

This work was supported by the Carolinas Muscular Dystrophy Research Endowment at the Carolinas HealthCare Foundation. Authors wish to acknowledge technical assistance from Ehsan Benrashid and Olga Dimov. The authors would like to thank Dr. Bonkovsky for his helpful discussion and advice in the manuscript preparation.

Abbreviations

CSA, cross section area; EDL, extensor digitorum longus; TA, tibialis anterior; CK, creatine kinase; MMPN, 2-methyl-2-[(nitrooxy)methyl]pentyl nitrate; MPP, 2-methyl-2-n-propyl-1,3-propanediol; NO, nitric oxide; nNOS, neuronal nitric oxide synthase

REFERENCES


