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ScienceDirect

Neuromuscular Disorders 26 (2016) 865–872

www.elsevier.com/locate/nmd

Use of the six-minute walk test to characterize golden retriever muscular dystrophy

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Received 7 April 2016; received in revised form 27 July 2016; accepted 28 September 2016

Abstract

Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder in which loss of the dystrophin protein causes progressive skeletal/cardiac muscle degeneration and death within the third decade. For clinical trials and supportive animal studies, DMD disease progression and response to treatment must be established using outcome parameters (biomarkers). The 6-minute walk test (6MWT), defined as the distance an individual can walk in 6 minutes, is commonly used in DMD clinical trials and has been employed in dogs to characterize cardiac and respiratory disease severity. Building on methods established in DMD and canine clinical studies, we assessed the 6MWT in dogs with the DMD genetic homolog, golden retriever muscular dystrophy (GRMD). Twenty-one cross-bred golden retrievers were categorized as affected (*DMD* mutation and GRMD phenotype), carrier (female heterozygous for *DMD* mutation and no phenotype), and normal (wild type *DMD* gene and normal phenotype). When compared to grouped normal/carrier dogs, GRMD dogs walked shorter height-adjusted distances at 6 and 12 months of age and their distances walked declined with age. Percent change in creatine kinase after 6MWT was greater in GRMD versus normal/carrier dogs at 6 months, providing another potential biomarker. While these data generally support use of the 6MWT as a biomarker for preclinical GRMD treatment trials, there were certain limitations. Results of the 6MWT did not correlate with other outcome parameters for GRMD dogs when considered alone and an 80% increase in mean distance walked would be necessary to achieve satisfactory power.

Keywords: Six-minute walk test; Golden retriever muscular dystrophy; Duchenne muscular dystrophy; Creatine kinase; Biomarker

1. Introduction

Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder in which loss of the dystrophin protein causes progressive skeletal and cardiac muscle degeneration [1–3]. Mutations in the *DMD* gene can either be passed through families as a genetic trait or occur spontaneously [4,5]. Patients lose their ability to walk over a wide age range, from 6 to 15 years [2,6], suggesting that secondary mechanisms contribute to disease severity [7,8].

Dystrophin-deficient DMD animal models include the *mdx* mouse [9,10] and golden retriever muscular dystrophy (GRMD) dog [11–15]. Since GRMD clinical features more closely mirror those of DMD, preclinical studies with dystrophic dogs may better predict disease pathogenesis and treatment outcome [15]. Despite having the same *DMD* gene mutation, GRMD dogs demonstrate remarkable phenotypic variation [14–16], in keeping with the variable age at which DMD patients are confined to wheelchairs.

While various outcome parameters (biomarkers) are employed to track DMD disease progression [17,18], the six-minute walk test (6MWT) is now used as the primary outcome parameter in many clinical trials [19]. Defined as the distance an individual can walk in 6 minutes, the 6MWT has also been employed in dogs to define the severity of clinical respiratory and cardiac disease [20–22]. Building on methods established in DMD and canine clinical studies, we assessed the 6MWT in GRMD versus normal/carrier dogs.

The fact that the 6MWT correlates with other tests and predicts future disease progression has helped validate its use in

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http://dx.doi.org/10.1016/j.nmd.2016.09.024
0960-8966/

DMD clinical trials [23,24]. Thus, we correlated results of the 6MWT from our dogs with other biomarkers routinely used in our lab. In an attempt to establish an additional biomarker, the percent change in serum creatine kinase (CK) after completion of the 6MWT was compared between GRMD and normal/carrier dog groups. Finally, power analysis was done to determine the sensitivity of the 6MWT and percent CK value post-6MWT to detect efficacy in GRMD preclinical studies.

2. Materials and methods

2.1. Dogs

All dogs were used and cared for according to principles outlined in the National Research Council's Guide for the Care and Use of Laboratory Animals. Dogs were part of a colony maintained at Texas A&M University. GRMD dogs were identified at 1 day of age based on elevation of serum CK, and genotype was confirmed by PCR as previously described [25]. Characteristic clinical signs subsequently developed.

A total of 21 cross-bred golden retriever dogs (11 females and 10 males) from five litters were used. The dogs were categorized as affected (*DMD* mutation with GRMD phenotype), carrier (female heterozygous for *DMD* mutation and normal phenotype), and normal (wild type *DMD* gene and normal phenotype). Five females and four males were affected, six females were carriers, and six males were normal.

2.2. Six-minute walk test (6MWT)

Prior to assessing the 6MWT, tibiotarsal joint (TTJ) extension and flexion force, eccentric contraction decrement (ECD), and pelvic limb joint angles were assessed using established methods [13,15,26,27].

Dogs were acclimated to the course and method for the 6MWT beginning at 2 months of age. Results were recorded monthly from 3 months to a year. The protocol was adapted from one used for both dogs [20–22] and humans [19,23]. A 6MWT course was laid out in the hallway of a building used for veterinary care. Testing was done at a consistent time during the day when there was minimal other activity that could be a distraction. Cones were placed 8.8 meters apart, such that the dog would repeatedly circle the cones over a period of 6 minutes. Body weight, height at the withers, length from the occiput to the rump, and heart and respiratory rates were recorded before and/or after each test (Supplementary Table S1**)**. Blood was drawn immediately before and after the 6MWT for serum CK measurement in some, but not all, dogs; thus, fewer dogs were included in the CK portion of the study.

Two leashes of equal length were crossed around the neck and one each per thoracic limb to create a body harness. Dogs were leash walked by the same technician, while another technician sat at the far end of the course. A timer was set to six minutes and the number of laps completed was recorded. Dogs were encouraged to walk by verbal prompts and given food treats. A slight leash tug was applied if the dog stopped. The number of times a dog rested was recorded, while time continued to elapse on the timer. If the dog stopped to urinate or defecate, the timer was stopped and resumed when the dog continued to walk.

2.3. Statistical analysis

Data were imported into a commercial statistical software program (SAS, version 9.4; SAS Institute Inc., Cary, NC) for analysis. Normal and carrier dogs were combined as a single group, as their 6MWT distances did not differ significantly. Median absolute (m) and height-adjusted (m/cm) 6MWT distances were compared between GRMD versus grouped normal/carrier dogs at 3, 6, and 12 months using the Wilcoxon rank-sum test. Height-adjustment was intended to correct for the effects of stunting with associated reduced stride length in GRMD versus normal/carrier dogs. Absolute and height-adjusted distances walked were also compared among the different ages within the GRMD and normal/carrier study groups using Friedman's test. Spearman correlation coefficients were used to assess correlation between height-adjusted distances walked and other functional tests at 6 and 12 months. Median percent change in CK after the 6MWT at 6 and 12 months was compared in GRMD versus grouped normal/carrier dogs using the Wilcoxon rank-sum test. For all analyses, p-values < 0.05 were considered significant. We also performed power analysis on the mean-height-adjusted 6MWT distance and mean post-exercise CK increase data at 6 months, both assuming the use of a 2-tailed test, a group size of 6 GRMD dogs, alpha of 0.05, power of 80%, and a standard deviation derived from our data.

3. Results

3.1. Median absolute [m] and height-adjusted [m/cm] 6MWT in GRMD versus normal/carrier dogs

At 3 months, the median absolute distance walked during the 6MWT was significantly greater ($p = 0.048$) in the normal/carrier (283.0 m) versus GRMD dogs (247.5 m) (Table 1**)**. The difference between normal/carrier and GRMD dogs became increasingly more pronounced at 6 (389.1 vs. 193.3 m; $p = 0.001$) and 12 (350.0 vs. 79.6 m; $p = 0.0006$) months (Fig. 1A).

The median height-adjusted distance walked during the 6MWT at 3 months did not significantly differ ($p = 0.2$) in normal/carrier (8.0 m/cm) versus GRMD dogs (7.3 m/cm). In contrast, there were significant differences at 6 (8.6 m/cm vs. 4.5 m/cm; $p = 0.001$) and 12 (6.8 m/cm vs. 1.6 m/cm; $p = 0.0006$) months (Fig. 1B).

3.2. Median absolute [m] and height-adjusted [m/cm] 6MWT at different ages for GRMD and normal/carrier dogs

Absolute distances walked at 3, 6, and 12 months were significantly different for the GRMD dogs ($p = 0.0006$) but only marginally significant for the normal/carrier dogs ($p = 0.08$) (Fig. 2A). Height-adjusted distances walked at 3, 6, and 12 months were significantly different for both the GRMD ($p = 0.0001$) and normal/carrier ($p = 0.01$) dogs (Fig. 2B).

3.3. Correlation of distance walked in the 6MWT with other phenotypic biomarkers

We have previously shown that results from biomarkers traditionally used in our laboratory to assess GRMD dogs tend