High-Frequency Ultrasound to Grade Disease Progression in Murine Models of Duchenne Muscular Dystrophy

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Objective. This study used high-frequency ultrasound (HFU) imaging to assess muscle damage noninvasively in a longitudinal study of 2 transgenic murine models of Duchenne muscular dystrophy (DMD): mdx, which has mutated cytoskeletal protein dystrophin; and udx, which has mutated dystrophin and lacks another cytoskeleton protein, utrophin. The mdx group was further subdivided into exercised and nonexercised subgroups to assess exercise-induced damage. Methods. Muscle damage was assessed with HFU imaging (40 MHz) at biweekly intervals for 16 weeks. The assessment was based on the number of hyperechoic lesions, the lesion diameter, and muscle disorganization, giving a combined grade according to a 5-point scale. Results. High-frequency ultrasound discriminated the severity of muscle damage between wild-type and transgenic models of DMD and between mdx and udx models. Qualitative comparisons of 3-dimensional HFU images with serial histologic sections of the skeletal muscle showed the ability of ultrasound to accurately depict changes seen in the muscle architecture in vivo. Conclusions. High-frequency ultrasound images soft tissue in mice at high contrast and spatial resolution, thereby showing that this microimaging modality has the capability to assess architectural changes in muscle fibers due to myotonic dystrophy–related diseases such as DMD. Key words: calcification; disease progression; Duchenne muscular dystrophy; high-frequency ultrasound.

Duchenne muscular dystrophy (DMD) is a severe genetic neuromuscular disorder, which occurs in approximately 1 per 3500 live male births. Duchenne muscular dystrophy arises because of a loss of functional dystrophin, a cytoskeletal structural protein, through a point mutation in the dystrophin gene, thereby compromising sarcolemma integrity and leading to progressive muscle inflammation, membrane leakage, and myofiber degeneration. Although the diagnosis of DMD is usually made by family history and genotyping, analysis of disease progression typically relies on invasive measurements. To date, there is no effective means to reliably and noninvasively assess the progression of muscle wasting in DMD.

Traditional ultrasound analyses of dystrophic muscle in patients with DMD have shown that whereas a normal myofiber volume is preserved in early stages of muscle degeneration, there is an increase in the muscle echo intensity relative to healthy individuals. Similar find-
ings have also been reported in patients with less severe forms of muscle dystrophy such as Becker and limb girdle dystrophies; importantly, the observed changes in the muscle echo intensity were found to reflect the severity of the disease. Preclinical studies using ultrasound revealed abnormalities in the echo intensity associated with the initial stages of the clinical manifestation of muscle degeneration in many patients. Also, because the severity viewed on ultrasound images has been found to be related to age and clinical stages of disease progression, this imaging modality appears to provide an effective means of noninvasively visualizing structural changes in degenerative muscle.

High-frequency ultrasound (HFU; 40 MHz) holds the potential to track muscle deterioration serially in mouse models of DMD. It provides considerable backscatter in mice, which is necessary for the generation of detailed structural and anatomic images and enhanced soft tissue contrast not readily visible at clinical frequencies. We previously used HFU to discriminate hyper-echoic lesions in the dystrophic muscle of mdx mice, which have mutated dystrophin. The motivation for this study originated when differences were noted between the organized speckle patterns of healthy skeletal muscle contrasted with the speckled patterns seen in affected dystrophic muscle of mice. Healthy muscle structure is highlighted by the fibroadipose septa, resulting in a characteristic organized hyperchoic banding pattern, different from dystrophic muscle, which has disorganization and disruption of the fibroadipose septa possibly due to atrophy, macrophage infiltration, deterioration of myofibers, and calcification. It was hypothesized that the spatial frequencies observed within healthy and unhealthy skeletal muscle on ultrasound images would vary sufficiently to enable a semiquantitative grading method of discrimination between groups.

To the best of our knowledge, no established validated parameters using HFU exist in the literature regarding DMD. Thus, quantification of the structural appearance of HFU images of the musculature promises to be an excellent tool for diagnosis or measuring changes in muscle damage over time or in response to an intervention. We think that the advances afforded through the use of HFU imaging in preclinical murine models will directly translate to the enhancement of current clinical practices in the assessment of pathologic changes in myotonic diseases.

Materials and Methods

Subjects

C57Bl6 (wild-type [wt]) and mdx mice, harboring a point mutation in the dystrophin gene, were purchased from Charles River Laboratories (Wilmington, MA) and The Jackson Laboratory (Bar Harbor, ME), respectively; mdx:utrn–/– (udx) animals, which have mutated dystrophin and also lack the cytoskeleton protein utrophin, were generated at Virginia Polytechnic Institute and State University through the breeding of mdx:utrn+/– pairs. Four groups of mice were used for imaging: (1) control wt (n = 9), which were exercised (Ex) involuntarily, as described below; (2) mdx (Ex) (n = 9); (3) mdx nonexercised (NE) (n = 9); and (4) udx (NE) (n = 11). The same 4 groups with 12 mice in each were used for histologic examinations at different times (see “Histologic Analysis”). All study procedures were approved by our institutional Animal Ethics Committee and conducted according to guidelines set by the Canadian Council on Animal Care.

Exercise and Nonexercise Protocols

The mdx and wt mice in the Ex groups began an involuntary running regimen after a baseline scan at 6 weeks of age for 30 minutes, 3 times weekly, for up to 16 weeks, at a speed of 15 m/min and a 7° positive incline on a motorized treadmill (AccuScan Instruments, Inc, Columbus, OH). To ensure that all animals in the study were handled similarly, mdx (NE) and udx (NE) mice walked on the treadmill 3 times weekly for 10 minutes at 5 to 7 m/min with no incline.

Three-Dimensional Ultrasound Image Acquisition

To obtain baseline scans, all mice were imaged at 6 weeks of age. Animals from each group were subsequently imaged every second week up to 16 weeks by an experienced sonographer. During each imaging session, mice were anesthetized under an induction level of 3% to 4% isoflurane/oxygen and then maintained with a 1.5% isoflurane/oxygen mixture; anesthetic-induced
hypothermia was avoided by using a heated imaging stage (THM-100; Indus Instruments, Houston, TX) to maintain the core temperature at 36°C. Depilatory hair removal cream (calcium thioglycolate based) was used on the lower leg and shank of the mice to enhance the contact of the ultrasound probe with the overlying skin above the muscles of interest. Ultrasound coupling gel (Aquasonic 100; Parker Laboratories, Inc, Fairfield, NJ) was applied to the depilated skin, and a 3-dimensional volume scan of the gastrocnemius was acquired by moving the ultrasound probe through a translation parallel to the long axis of the gastrocnemius medial head using multiple 2-dimensional images. The right leg of the animals was imaged in a standard supine position with the legs secured such that the medial head of the gastrocnemius was perpendicular to the probe. Ankle, knee, and thigh angles were kept consistently at 45° to standardize the procedure. Imaging was accomplished with a Vevo 770 HFU scanner (VisualSonics, Inc, Toronto, Ontario, Canada). Three-dimensional images were then reconstructed and visualized with VisualSonics software. The system uses single-element B-mode imaging with a 40-MHz center frequency probe that produces 40-µm axial and 80-µm lateral resolutions at a maximum focal depth of 6 mm. Three-dimensional images were reconstructed from the acquired 2-dimensional images using 3D-Quantify software, developed by one of the coauthors. The reconstructed 3-dimensional image was displayed in a dynamic cube view format. Sections of the “cube” in different orientations were displayed by moving a “cut” plane through the 3-dimensional ultrasound data interactively to view the different characteristics of the skeletal muscle. Two observers blinded to the source of the ultrasound images separately graded images according to a scoring system described below. Any differences between scores were resolved by a collaborating radiologist also blinded to the ultrasound images.

Grading and Scoring System

The scoring system for muscle damage was based on the following characteristics on the HFU images of the gastrocnemius muscle in the right leg: (1) Number of hyperechoic lesions—Discernable regions that were more hyperechoic compared with the surrounding muscle tissue and were not related to the fibroadipose septa were counted (Figure 1). (2) Lesion diameter—

Figure 1. B-mode transverse HFU images of hind limbs from 3 dystrophic mice showing differences in the lesion number and size. A, Lesion measuring 375 mm within the gastrocnemius of a udx (NE) mouse at week 14 (lesion number score, 1; lesion diameter score, 4). B, Three visible lesions with the largest measuring 120 mm within the gastrocnemius of an mdx (Ex) mouse at week 14 (lesion number score, 3; lesion diameter score, 3). C, Five lesions with the largest measuring 55 mm within the gastrocnemius of an mdx (NE) mouse at week 14 (lesion number score, 5; lesion diameter score, 2). G indicates gastrocnemius; and S, soleus.
Long-axis diameters were measured by the embedded ultrasound software analysis tool in the Vevo 770 scanner. Visible lesions were given the following values: a value of 1 for lesions that ranged from 1 to 50 mm, a value of 2 for lesions that ranged from 51 to 100 µm, a value of 3 for lesions that ranged from 101 to 150 µm, and a value of 4 for lesions that were greater than 151 µm (Figure 1). (3) Myofiber disorganization—Normal muscle shows myofibers and fibroadipose septa in an organized regular repeating pattern that gives rise to the appearance of evenly distributed hyperechoic “streaks” and “bands” throughout the gastrocnemius (Figure 2A). Thus, analysis of myofiber disorganization was based on the disappearance of the regular banding pattern observed in healthy skeletal muscle. More specifically, a value of 0 was assigned for mice with anisotropic banding (Figure 2A), a value of 3 for mice with moderate skeletal muscle disorganization (Figure 2B), and a value of 6 for mice with marked disorganization (Figure 2C). Values for lesion number, lesion diameter, and myofiber disorganization were summed to give a final damage score, which was used to grade overall myofiber degeneration: 1, none (score 1–4); 2, minor (score 5–10); 3, mild (score 11–14); 4, moderate (score 15–20); and 5, marked (score 21+).

Histologic Analysis
Mice used for histologic analysis were treated and exercised in the same manner as the imaged mice; 12 extra mice were added to each group for euthanasia at specified time points: baseline (6 weeks of age), weeks 8 and 14, and end point (consisting of wt and mdx mice at 22 weeks of age and death of udx mice past 14 weeks of age) after the beginning of the exercise regimen. The gastrocnemius muscles were dissected from a minimum of 3 mice in each group at each of the time points. Muscles were fixed in 10% neutral-buffered formalin for a minimum of 48 hours. The fixed muscles were embedded in paraffin wax, cut transversely in 5-µm sections, and stained with hematoxylin-eosin (H&E). Comparisons of a minimum of 3 muscles with similar pathologic characteristics were made for each group and time point. Representative H&E slides were then compared with HFU scores as an indication of grades of degeneration. The presence of features seen on the HFU images, such as calcified lesions, macrophage infiltration, fat, degeneration of the endomysium, and changes in banding patterns from fibroadipose septa, were examined.

Figure 2. B-mode transverse HFU images of hind limbs from 3 mice showing different degrees of disorganization of fibroadipose septa. A, A score of 0 was assigned to mice with regular organized banding. B, A score of 3 was assigned to mice with moderate skeletal muscle disorganization. C, A score of 6 was assigned to mice with marked disorganization. Arrows indicate fibroadipose septa as depicted by HFU; and G, gastrocnemius.
Data Analysis
Statistical analyses were performed with the SPSS version 15.0 statistical software package for Windows (SPSS Inc, Chicago, IL). Repeated measures analysis of variance was used as an omnibus test to identify significant differences among groups and time points within groups for HFU scores. Tukey tests were used for the post hoc analyses of differences.

Results
Significant differences in muscle health were found between wt and dystrophic animals. All dystrophic mice had hyperechoic lesions on HFU images. Figures 1 and 2 depict differences in the lesion size and number and muscle disorganization within an HFU image of the gastrocnemius. On the basis of the overall muscle grade derived from the scores for the 3 parameters of muscle damage, we observed significant differences (P < .05) in damage of the gastrocnemius muscle between wt and dystrophic animals (Figure 3). The overall grade showed that udx (NE) mice were found to have greater degeneration than either mdx group over the course of the study (Figure 3). Muscle degeneration in mdx (Ex) mice peaked at 12 weeks of age at a grade of 3 (mean ± SD, 3 ± 0.32, mild), whereas that in mdx (NE) mice peaked at week 16 at a grade of 2 (2.28 ± 0.44, minor) over the course of the study, and thereafter there was a nonsignificant trend of improvement. In contrast, muscle degeneration in udx (NE) mice was progressive and stabilized at grade 4 (moderate) from week 12 to 18 after exercise before deterioration to marked degeneration (5 ± 0) at week 20.

Lesion numbers were greater (P < .05) in dystrophic animals compared with wt animals from week 8 onward (Figure 4). The udx (NE) mice showed the highest number of lesions over the course of the study, reaching a value of 11.3 ± 0.6 at 20 weeks, whereas no significant difference (P > .05) was found between mdx groups (Figure 4). The udx (NE) mice showed significantly higher numbers (P < .05) of lesions at weeks 12 (7.57 ± 1.6) and 18 (9.8 ± 0.4) compared with mdx (NE) (3.44 ± 1.1) and mdx (Ex) (7 ± 1.2) mice (Figure 4).

The lesion diameter of dystrophic mice increased significantly relative to wt animals (P < .05) from week 8 onward. Although a significant increase (P < .05) in the lesion diameter was observed in udx mice compared with mdx mice from week 14 onward, no significant differences (P > .05) were found between mdx (NE) and mdx (Ex) mice at any time points (Figure 5).

Fibroadipose septa in the muscles of wt animals appeared organized, resulting in multiple parallel linear echoes visible on HFU images of the gastrocnemius muscle (Figure 2A); these appear unorganized in dystrophic animals (Figure 2B). Significant differences (P < .05) between udx (NE) and mdx (Ex) and (NE) mice are represented by π and λ, respectively.

Figure 3. Grades of muscle degeneration derived from the sum (mean ± SD) of the lesion number, diameter, and muscle disorganization scores over 14 to 16 weeks of HFU imaging: 1, none (score 1–4); 2, minor (score 5–10); 3, mild (score 11–14); 4, moderate (score 15–20); and 5, marked (score 21+). The udx (NE) mice reached a moderate (4) to marked (5) grade of muscle damage, whereas both mdx (Ex) and (NE) mice only reached a minor (2) to mild (3) grade of muscle damage. Wild-type animals showed no muscle degeneration (<1) throughout the study. Significant differences (P < .05) between udx (NE) and mdx (Ex) and (NE) mice are represented by π and λ, respectively.

Figure 4. Mean number of lesions in gastrocnemius muscle over the time course of the study for each group: udx (NE), mdx (Ex), mdx (NE), and wt. Significant differences (P < .05) are represented by π between udx (NE) and mdx (NE) mice and λ between mdx (Ex) and udx (NE) mice.
normal muscle fibers had a disorganization score of 0 for all weeks (Figure 6). Both mdx groups showed some initial disorganization. The mdx (Ex) mice had a peak score of 2.7 ± 0.94 at 12 weeks of age, whereas the mdx (NE) mice showed a peak score of 2.6 ± 1 at 10 weeks (Figures 2B and 6) but in later weeks showed organization similar to that seen in wt animals (Figure 6). The udx mice showed disorganization from the baseline, which continually increased in severity until the end point of the study, reaching a value of 6 ± 0 (Figures 2C and 6). Both groups of mdx animals were found to have significantly lower ($P < .05$) disorganization scores than udx (NE) animals from week 6 onward (Figure 6).

Wild-type mice showed no histologic changes among the 4 selected time points (weeks 6, 8, 14, and 20–22 from the start of the exercise regimen). All weeks showed features similar to those indicated by the representative image of a wt mouse at the end point (Figure 7A). The mdx (NE) mice showed initial damage/regeneration at the baseline with centrally located nuclei and the presence of macrophages (7B, i). Sections from week 8 (Figure 7B, ii) showed disruption of the endomysium and a moderate infiltrate, typically seen before the formation of calcified lesions. At week 14, myofibers were shown to remain in a state of regeneration, and a moderate influx of macrophages was noted, as well as the presence of calcified lesions (Figure 7B, iii). At the end point, there was little to no infiltration of macrophages, and some nuclei had reverted back to a peripheral position, as

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observed in wt mice, indicating repair, whereas other myofibers remained in a state of regeneration. The mdx (Ex) mice showed initial damage at the baseline (Figure 7C, i). Week 8 showed marked inflammation, centrally located nuclei suggesting ongoing regeneration, calcified lesions, and disruption of the endomysium (Figure 7C, ii). At week 14, calcified lesions and a mild infiltrate were present, but nuclei remained centrally located, suggesting that cells were in a state of regeneration/degeneration (Figure 7C, iii). Tissue sections from the end point showed calcified lesions, a mild macrophage infiltrate, and an intact endomysium, with some revertant myofibers showing peripherally located nuclei, as seen in wt mice. The udx (NE) mice showed very...
aggressive disease progression, with a marked infiltrate and regenerating/degenerating myofibers at the baseline (Figure 7D, i). Week 8 showed continued degeneration of myofibers, an infiltrate, and disruption of the endomysium, as well as early formation of calcified lesions (Figure 7D, ii). Week 14 showed the presence of macrophages and lesions (Figure 7D, iii). Tissue sections from the end point revealed all myofibers present, centrally located nuclei, leukocytic infiltrate remains, and extensive endomysium degeneration (Figure 7D, iv).

Discussion

The purpose of this study was to show the ability of using HFU imaging to noninvasively assess muscle degeneration in murine models of DMD.

Figure 7. (continued) C, iv, end point at the termination of the study (20–22 weeks of age). i, The mdx (Ex) mice showed initial damage at the baseline with centrally located nuclei (open arrowhead), and a small infiltrate of macrophages (filled arrowhead) was noted. ii, Week 8 showed marked inflammation (filled arrowheads), centrally located nuclei (open arrowhead), the initial stages of formation of a calcified lesion (thin arrow), and disruption of the endomysium (open arrow). iii, Week 14 showed calcified lesions (thin arrow) as well as a mild infiltrate (filled arrowhead), centrally located nuclei remained dominant (open arrowhead) despite a few peripherally located nuclei (open and filled arrows). iv, The end point showed calcified lesions (thin arrows), centrally located nuclei (open arrowhead), and an intact endomysium with a few revertant myofibers (filled arrows). D, Representative H&E-stained sections of gastrocnemius muscles isolated from udx (NE) mice (original magnification ×40; n = 3): i, baseline (6 weeks old); ii, week 8; iii, week 14; iv, end point at the termination of the study (14–22 weeks of age). i, The udx (NE) mice showed a marked infiltrate (filled arrowhead) and regenerating/degenerating myofibers at the baseline (open arrowhead). ii, Week 8 showed continued degeneration and centrally located nuclei (open arrowhead), an infiltrate (filled arrowhead), and disruption of the endomysium (open arrow), as well as early formation of calcified lesions (thin arrow). iii, Week 14 showed the presence of macrophages (filled arrowhead), centrally located nuclei (open arrowhead), and lesions (thin arrow). iv, The end point showed all myofibers in a state of regeneration/degeneration (open arrowhead), a leukocytic infiltrate (filled arrowhead), and extensive endomysium degeneration (open arrow).
To achieve this, a grading system was developed to classify and quantify the degree of muscle damage observed in HFU images. Two murine models of DMD were selected for comparison: the mdx and udx models. To exacerbate muscle damage in the mdx model, a less severe model of DMD, a subgroup of these mice were subjected to an involuntary treadmill running regimen; a udx (Ex) equivalent group was not created because of the severity of disease in this model. Our findings show that HFU can discriminate between animal models of DMD that have varying degrees of severity and, importantly, can accurately track degenerative changes occurring in muscle throughout the duration of the study.

High-frequency ultrasound, at 40 MHz as used in this study, can theoretically resolve groups of hypertrophic myofibers, consisting of small functional groups of muscle cells having undergone substantial dystrophic changes and potential calcification. Additional changes in the normal echoic speckle pattern of myofiber organization can be caused by an increase or decrease in reflectance due to a biomechanical strain or an increase in cellularity. In our study, these changes were assessed to define a scoring system for muscle damage.

Discrete lesions seen on HFU images of dystrophic mice were either aggregations of infiltrating leukocytes or calcified necrosis of myofibers. The mdx mice revealed early stages of degeneration with macrophage infiltration in histologic sections of the gastrocnemius muscle. The absence of functional dystrophin promotes muscle instability by increasing contraction-induced damage to the sarcolemma, allowing cytosolic calcium ion levels to increase. This initiates a cascade of intracellular events that lead to necrosis. In our histologic study, there were dense regions of macrophage accumulation and calcification around degenerative myofibers.

Elevated calcium ion concentrations in dystrophic muscle have previously been noted and have been attributed to persistently activated calcium channels, which are incorporated into the sarcolemma membrane during repair. It has been suggested that with continual activation of calcium channels, calcium loading within the myocyte results in myonecrosis and tissue calcification. We think that the accretion of macrophages around calcified lesions in damaged muscle fibers, followed by macrophage withdrawal, may explain changes in the size of lesions seen in mdx mice on HFU images. With increasing macrophage infiltration and calcification, a lesion observed on HFU images may be perceived as having a larger diameter compared with lesions having only macrophages present or calcified scar tissue. The proposed explanation is supported by our histologic data on mdx mice. We observed early macrophage infiltration from initial damage, which was followed by up-regulation of utrophin, leading to an increase in muscle stability, a reduction in myofiber necrosis, and macrophage infiltration. In udx mice, because the utrophin gene is knocked out, the stabilization effect from utrophin up-regulation is lost. Thus, persistent muscle damage and macrophage infiltration are present after birth in this model, contributing to a continual increase in the number and size of lesions.

Muscle disorganization was scored according to the visibility of the linear echo banding pattern produced by the normal organization of fibroadipose septa in gastrocnemius muscle. Studies have shown that ultrasound has the capability to assess damage in muscle based on fibroadipose septa organization. The mdx mice in comparison with the wt mice showed more muscle disorganization in the initial weeks due to damage. However, with healing/restoration from up-regulation of utrophin, the mdx mice showed patterns more similar to those of the wt mice in later weeks of the study. The udx mice showed very little organization in the hyperechoic banding pattern from the baseline, and with time, more progressive degeneration was evident. Our histologic data as well as others’ suggest that groups of degenerative myofibers, endomysium degeneration, fat/collagen deposits, and macrophage infiltration may all contribute to the extreme disorganized hyperechogenicity seen in the udx and to a lesser extent the mdx mice.

In conclusion, HFU images soft tissue in mice at high contrast and spatial resolution, thereby showing that this microimaging modality has the capability to assess architectural changes in muscle fibers in murine models of DMD. Using our scoring and grading system on HFU images, we were able to assess a higher degree of muscle

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damage/degeneration in udx mice rather than mdx mice, which was corroborated by our histologic data. To the best of our knowledge, no previous study has longitudinally tracked muscle degeneration using HFU in 2 murine models of DMD and shown the ability of HFU to differentiate not only between wt and disease models of DMD but also within disease models. The grading scheme also holds promise for application in preclinical therapeutic trials to monitor treatment effects. Because our techniques for studying mice were adapted from clinical studies, the same methods, including the scoring and grading schemes, can be applied, in principle, to monitor the progression of muscle damage in patients with DMD and other muscular dystrophies. However, a compromise between the depth of penetration and spatial resolution has to be made such that a lower frequency can be used in patients. As such, a preliminary study on patients to show the feasibility of our preclinical technique in the clinical environment is warranted.

References