Progression of kyphosis in *mdx* mice

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Laws, Nicola, and Andrew Hoey. Progression of kyphosis in *mdx* mice. *J Appl Physiol* 97: 1970–1977, 2004. First published July 2, 2004; doi:10.1152/japplphysiol.01357.2003.—Spinal deformity in the form of kyphosis or kyphoscoliosis occurs in most patients with Duchenne muscular dystrophy (DMD), a fatal X-linked disorder caused by an absence of the subsarcolemmal protein dystrophin. *Mdx* mice, which also lack dystrophin, show thoracolumbar kyphosis that progresses with age. We hypothesize that paraspinal and respiratory muscle weakness and fibrosis are associated with the progression of spinal deformity in this mouse model, and similar to DMD patients there is evidence of altered thoracic conformation and area. We measured kyphosis in *mdx* and age-matched control mice by monthly radiographs and the application of a novel radiographic index, the kyphotic index, similar to that used in boys with DMD. Kyphotic index became significantly less in *mdx* at 9 mo of age (3.58 ± 0.12 compared with 4.27 ± 0.04 in the control strain; *P* ≤ 0.01), indicating more severe kyphosis, and remained less from 10 yr to 17 mo of age. Thoracic area in 17-mo-old *mdx* was reduced by 14% compared with control mice (*P* ≤ 0.05). Peak tetanic tension was significantly lower in *mdx* and fell 47% in old *mdx* latissimus dorsi muscles, 44% in intercostal strips, and 73% in diaphragm strips (*P* ≤ 0.05). Fibrosis of these muscles and the longissimus dorsi, measured by hydroxyproline analysis and histological grading of picrosirius red-stained sections, was greater in *mdx* (*P* < 0.05). We conclude that kyphotic index is a useful measure in *mdx* and other kyphotic mouse strains, and assessment of paralumbar and accessory respiratory muscles enhance understanding of spinal deformity in muscular dystrophy.

respiratory muscles; latissimus dorsi; intercostal muscles; kyphotic index; hydroxyproline

Duchenne Muscular Dystrophy (DMD) is a relentlessly progressive X-linked myopathy characterized by skeletal muscle necrosis and wasting as well as cardiomyopathy in affected males. Respiratory complications remain the main cause of death, with few patients surviving beyond 30 yr of age, despite continued improvements in nursing and physiotherapy care and the earlier implementation of ventilatory assistance (11). Chest deformity due to scoliosis, kyphosis, lordosis, or combined spinal curvatures contributes significantly to the morbidity associated with the disease and often leads to a restrictive respiratory pattern with diaphragm and inspiratory muscle weakness, ineffective cough mechanisms, mucus plugging of airways, and chronic alveolar hypoventilation (29, 30). Vital capacity remains normal in patients with neuromuscular disease if respiratory muscle strength is >50% of predicted; however, when strength is less than this, vital capacity becomes diminished to a greater extent than expected (9, 12). This decrease is thought to be associated with loss of compliance of the chest wall and lungs, with stiffening of ribcage tendons and ligaments and ankylosis of costosternal and thoracovertebral joints (2, 12). As well as exacerbating respiratory dysfunction in affected boys, kyphoscoliosis negatively impacts their quality of life in their wheelchair-dependent years, with most patients electing surgery for spinal fusion and stabilization and/or experiencing chronic pain due to poor posture and prolonged sitting (32).

The natural course of spinal deformity differs between patients, and a classification scheme has been established based on radiographic indexes, including the Cobb angle, pelvic obliquity, kyphotic index (KI), and sacral angle (26). Pulmonary function (as measured by plateau of vital capacity) correlates with the progression of spinal deformity and may be an indicator of the expected progression (35, 36). It has also been suggested that a particular clinical course may correlate with a specific molecular lesion (16, 17).

Thoracolumbar kyphosis also occurs in murine models of neuromuscular diseases, including the dystrophin-deficient (*mdx*) mouse (21), the dystrophin/utrophin-deficient (*mdx: utrn*) mouse (7, 15), and the kyphoscoliosis (*ky*) mouse (3, 10). Although *mdx* mouse diaphragm muscle has been shown to most closely mimic the pathological changes seen in DMD (33), there is also histological evidence of necrosis and fibrosis in postural and paraspinal muscles of *mdx* and *mdx: utrn* (*P* < 0.05). To date, limited respiratory studies have been performed in the *mdx* mouse model; however, there is recent evidence of significant attenuation of respiratory responses to hypercapnia (a potent ventilatory stimulant) in *mdx* mice compared with control mice, which was thought to be influenced by tumor necrosis factor-α (14).

There were three goals in this study. First, we applied a novel KI to determine the progression of spinal deformity in two groups of aging mice: *mdx* and their age-matched controls. We also assessed contractile function in paraspinal and respiratory muscles (latissimus dorsi muscles and intercostal strips) and compared them with diaphragm muscle. The latissimus, a flexor of the brachium, was chosen because of its origin from T8-T12 and the thoracolumbar fascia in the region kyphosis occurs and because of its well-documented contractile properties in other species (18). Functional parameters of intercostal muscles have been reported previously in larger species but not in mice. We report data using intercostal strips comprising four rib sections and adjoining intercostal muscles (external and internal intercostals). Finally, we measured fi...
brosis in the above muscles and longissimus dorsi muscles by means of hydroxyproline measurements and picrosirius red-stained sections and recorded histological changes indicating muscle degeneration and regeneration. We conclude that thoracolumbar kyphosis in mdx mice occurs before 1 yr of age then stabilized until 17 mo of age, and we further conclude that contractility of the intercostals and latissimus is correlated with the extent of fibrosis and histological evidence of regeneration. These findings extend our understanding of the mdx phenotype and are also commensurate with dystrophic changes contributing to thoracolumbar deformity in DMD patients.

METHODS

Animals. Male C57BL/10ScSn mice (control strain) were purchased from Animal Resource Centre, Nedlands, Perth, WA, at 7 wk of age. Male mdx mice were bred at the University of Southern Queensland Animal House, Toowoomba, Australia. The mice were housed in groups and given free access to laboratory chow and water, and all experiments were conducted in accordance with guidelines of the University of Southern Queensland Animal Ethics Committee. Four mice per strain were used for the radiographic study, with one mouse dying during the 17-mo duration of this study. An additional four mice per strain were also utilized for contractility experiments, hydroxyproline assays, and histology.

Radiographic studies and establishment of KI. Mice were sedated with 50 mg/kg ketamine HCl (Ketamil, Troy Laboratories) in combination with 10 mg/kg xylazine HCl (Ilium Xylazil-20, Troy Laboratories) administered by subcutaneous injection. At the end of the procedure, atipamezole (Antisedan, Novartis Animal Health) was given at a dose rate of 0.1 mg/kg to reverse α2-agonist effects of xylazine. Mice were lightly taped to the radiographic cassette with clear adhesive tape. Each animal was individually identified by tail markings, a radiodense (metal) number placed next to them, and a radiographic cassette label indicating date and animal grouping. Konica CM-H medical mammography film was exposed using a portable X-ray unit (either Showa X-ray, Tokyo, Japan, or Porta 1030 model, Job, Yokohama, Japan). Optimum exposure with our equipment was established at 48 kV, 1.8 MAS, with a film focal distance of 70 cm. Mice were radiographed once monthly from 4 mo until 17 mo of age.

Each whole body radiograph was photographed using a tripod-mounted Ricoh Caplio RR30 digital camera with images analyzed using Scion Image software Beta 4.0.2 (http://www.scioncorp.com). KI was calculated from a line drawn between the caudal margin of the last cervical vertebra to the caudal margin of the sixth lumbar vertebra (usually corresponding to the cranial border of the wing of the ilium) (line AB) divided by a line perpendicular to this from the dorsal edge of the vertebra at the point of greatest curvature (line CD). This correlates as closely as possible to those radiographic parameters used to assess KI in boys with DMD and is depicted in Fig. 1, A and B.

Trial radiographs of the same animals in three positions [1] hindlimbs and forelimbs placed in moderate extension, 2) overextension (stretching) of limbs, and 3) flexion of forelimbs and hindlimbs] showed there was some differences in measured KI, which was considered to be <10%. Care was then taken to avoid overextension or flexion of limbs and to ensure that limbs were only moderately extended. This could be confirmed when radiographs were analyzed because the femurs and humeri were close to parallel and perpendicular to the long axis of the spine. Several radiographs that did not meet these criteria or were under- or overexposed were excluded from analysis.

Measurement of thoracic area. With radiographs photographed using a fixed film-focal distance and utilizing the Scion Image program draw tool, a line was extended around the inside border of the thoracic cavity from T1 at the thoracic inlet following sternaebrae, diaphragm, and ventral edge of vertebras to allow an estimation of thoracic area at an age of 17 mo. This measurement was repeated three times, and results were averaged for each animal (n = 4 animals per group). Because thoracic area is related to body size, the calculated area was normalized for body weight to give a value of thoracic area/body weight (cm²/g).

Contractility studies. Seventeen-month-old mice were anesthetized with pentobarbitone sodium (Nembutal, Boehringer Ingelheim) at 70 mg/kg ip. Cessation of breathing occurred when the thorax was entered. The following three muscles were dissected and placed into ice-cold Krebs buffer solution bubbled with carbogen (95% O₂-5% CO₂). First was the diaphragm strip from left midcostal hemidia- phragm, with placement of silk suture material around the central tendon at one end and a small rib section at the other. Second was the latissimus dorsi muscle, which is a fan-shaped muscle with an aponeurosis originating from the spinal processes of T8–T12 and the thoracolumbar fascia, and a distinct tendon of insertion at the proximal humerus. A needle threaded with 6-0 surgical silk was passed through the aponeurosis and tied with a loop to attach to a force transducer. A short length of silk was also tied at the tendon to anchor to a fixed peg below the stimulating electrodes. Third, an intercostal section comprising four ribs and their attached intercostal muscles (internal and external), extending from T8–12, adjacent and parallel to the longissimus dorsi muscle were dissected. Silk sutures were passed with a needle around each rib at the top and bottom of the intercostal strip for mounting.

Muscles from the left side were collected and stored for histological analysis and hydroxyproline assays. Contralateral muscles were mounted in water-jacketed glass organ baths, maintained at 23°C, with 6-0 silk surgical suture thread attached to a fixed peg at one end and a force transducer at the other. Tissues were stimulated via a Grass S48 stimulator (W. Warwick, RI), and signals were amplified.

Fig. 1. Establishment of kyphotic index (KI) in mice. A: method of measurement of KI in boys with Duchenne muscular dystrophy (DMD) (diagram adapted from Ref. 32). With patient in sitting position, measurements are made from a lateral radiograph. AB is length of line drawn from anteroinferior edge of seventh cervical vertebra (C7) to the sacral promontory, and CD is the distance from the line to anterior border of the vertebral body that is furthest from that line. KI = AB/CD. B: KI in mice calculated from radiographs of anesthetised mice positioned in right lateral recumbency. Line AB is the length of a line drawn from posterior edge of C7 to the posterior edge of L6, usually where it contacts the wing of the ilium (which is more consistently identifiable than the sacral border). Line CD is the distance from line AB to the dorsal border of the vertebral body farthest from that line. KI = AB/CD.
with a preamplifier (EP500B, Audio Assemblies, Campbellfield, Victoria, Australia). Data was collected and analyzed with Chart 4.1.1 software. A square pulse of a duration of 0.2 ms was dispersed via two platinum electrodes positioned along the length of the muscle.

Optimum preload ($L_o$) was defined as the length eliciting maximal single twitch force. Optimal voltage was also determined for each preparation, as was the frequency eliciting maximal tetanic force from a range of 50–180 Hz. A total of seven to eight mice per group was used for contractility studies. Reported data were the average of three individual single-twitch or tetanic stimulations per muscle strip after 25 min of equilibration and optimization of conditions. Muscles were measured at $L_o$ with a digital micrometer, blotted for 3 s, then weighed. Cross-sectional area (CSA) and normalization of force was calculated as described previously for diaphragm and latissimus weighed. Cross-sectional area (CSA) and normalization of force was pronounced. All animals were ambulatory and in fair to good health, showed a degree of thoracolumbar kyphosis that was not as pronounced. mdx mice at 17 mo of age. In addition, the control strain showed a more pronounced thoracolumbar kyphosis compared to mdx mice. The latissimus dorsi muscle showed the greatest variation between the two groups, with mdx muscles having considerably heavier ($P < 0.001$) muscles than C57, despite there being no significant differences between widths or $L_o$ for these muscle preparations. It is thought that this muscle (in addition to many mdx limb muscles) demonstrates considerable hypertrophy during the lifespan, with the presence of fibrosis contributing to increased weights. A difference between strains was also apparent during dissection; the diaphragm, intercostals, and latissimus muscles of C57 mice tended to be thinner and transparent, whereas in mdx mice they were thickened and opaque. There was a small difference in average diaphragm width selected for mounting, and, although this is unlikely to affect results, the resultant tissue strip was heavier in the mdx group.

In vitro isometric contractile properties of these muscles are shown in Table 3. All mdx muscles examined demonstrated reduced force production (twitch and tetanus) compared with control mice, with mdx diaphragm muscle showing the greatest reduction in tension generated (approximately two-thirds that of control mice, $P < 0.01$). The mdx latissimus dorsi and intercostal strip values were by contrast 50% of control levels for both twitch and tetanus tensions ($P < 0.05$ except for latissimus maximal tetanic force; $P < 0.01$). Rise times were similar, except for the attenuated diaphragm twitches of the mdx group.

### RESULTS

**Gross findings.** Kyphosis was palpable and clearly evident in all mdx mice at 17 mo of age. In addition, the control strain showed a degree of thoracolumbar kyphosis that was not as pronounced. All animals were ambulatory and in fair to good body condition; however, the mdx group showed a stiffer gait and moved less freely around their cages.

**Thoracic area.** There was no significant difference in body weights at 17 mo of age between mdx and C57 mice; however, there was a difference ($P < 0.05$) between thoracic area and normalized thoracic area in mdx and age-matched controls (Table 1), with mdx mice demonstrating lower values. It is thought that this muscle (in addition to many mdx limb muscles) demonstrates considerable hypertrophy during the lifespan, with the presence of fibrosis contributing to increased weights. A difference between strains was also apparent during dissection; the diaphragm, intercostals, and latissimus muscles of C57 mice tended to be thinner and transparent, whereas in mdx mice they were thickened and opaque. There was a small difference in average diaphragm width selected for mounting, and, although this is unlikely to affect results, the resultant tissue strip was heavier in the mdx group.

**Muscle contractility.** The characteristics ($L_o$, average width, and weight) of each isolated muscle strip is listed in Table 2. The latissimus dorsi muscle showed greatest variation between mouse strains, with mdx having considerably heavier ($P < 0.001$) muscles than C57, despite there being no significant differences between widths or $L_o$ for these muscle preparations. It is thought that this muscle (in addition to many mdx limb muscles) demonstrates considerable hypertrophy during the lifespan, with the presence of fibrosis contributing to increased weights. A difference between strains was also apparent during dissection; the diaphragm, intercostals, and latissimus muscles of C57 mice tended to be thin and transparent, whereas in mdx mice they were thickened and opaque. There was a small difference in average diaphragm width selected for mounting, and, although this is unlikely to affect results, the resultant tissue strip was heavier in the mdx group.

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**Hydroxyproline analysis.** We used hydroxyproline (HP) content as a measure of collagen in diaphragm, intercostal muscles, latissimus dorsi, and longissimus dorsi muscles. Muscles were trimmed of fat, ribs, and tendons and stored at −80°C. Tissue was thawed and immediately weighed, then hydrolyzed in sealed tubes with 6 M HCl overnight at 110°C. The samples were dried to entirety using a filtered air under pressure and heat (50°C). The rest of the protocol has been described previously (34). Values are expressed as micrograms of HP per milligram of tissue wet weight.

**Histology.** Each tissue (diaphragm, latissimus, longissimus, and intercostal muscles) were pinned onto cork at $L_o$, and then fixed sequentially in Telly’s fixative (formaldehyde, glacial acetic acid/ethanol fixative, 72 h), Bouin’s solution (formaldehyde, glacial acetic acid-picric acid fixative, 24 h), and 70% ethanol before paraffin embedding, cutting, and staining of 10-µm sections using 0.1% wt/vol picrosirius red solution (Sirius Red F3B, Chroma Dyes, in saturated picric acid), a specific collagen stain. Additional 5-µm sections were stained with hematoxylin and eosin for determination of nuclear position and heterogeneity of muscle fiber size (see Fig. 7). Analysis was performed blinded to the strain of mouse, with sections viewed on a Nikon Eclipse E600 light microscope and captured with a Nikon FXD 35-mm camera. Images were digitized and then analyzed using Scion Image Beta 4.0.2 software. A visual grading scheme was applied to the picrosirius-stained sections, with grade 1 having minimum interstitial fibrosis (e.g., <10%), grade 2 with mild fibrosis (10–25%), grade 3 with moderate fibrosis (25–50%), and grade 4 with marked fibrosis (>50%).

**Statistics.** Pilot experiments on aged mice were performed to estimate standard deviations and suitable sample size. Post hoc tests of power confirmed that $n = 3$ animals for assessment of KI and $n = 7$ animals for contractility experiments were adequate (28). Results are expressed as means ± SE. Responses between mdx and control strain were analyzed by Student’s unpaired $t$-tests, with the exception of differences in KI, where ANOVA was employed. $P$ values of $<0.05$ were considered statistically significant.

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mdx group, which had significantly shorter rise times ($P < 0.001$). Relaxation was prolonged in mdx intercostal muscles compared with control levels, reflected by increased time to 50 and 90% relaxation ($P < 0.05$).

**Hydroxyproline analysis.** Four muscles were used for assays of hydroxyproline content, and the results are shown in Fig. 4. All mdx muscles had significantly more HP than control mice, with the diaphragm HP being 2.5 times higher than the other muscles examined. Interestingly, control mice also showed a higher level of fibrosis in this organ compared with other respiratory or paraspinal muscles, and when relative values were compared (control HP as a ratio of mdx HP) the diaphragm and latissimus displayed an equal tendency for fibrous tissue deposition (0.66), followed by intercostal muscles (0.58), and then longissimus dorsi (0.44). The magnitude of fibrosis may be a reflection of the workload of individual muscles.

**Histology.** The results of an applied histological grading scheme for muscle sections stained with the collagen-specific stain picrosirius red are listed in Fig. 5. Representative photomicrographs of all four muscles of mdx and control mice are shown in Fig. 5. The percentage of centronucleation of muscle fibers as visible on hematoxylin and eosin-stained sections indicates previous necrosis and degeneration and one or more cycles of regeneration and is displayed in Fig. 5B. It is apparent that mdx muscles demonstrate marked heterogeneity in cell size, a high incidence of centrally nucleated fibers, inflammatory cell infiltration, and fibrous tissue deposition compared with control muscles (Figs. 6 and 7).

The mdx diaphragm displays the highest scores on histological grading as expected, with marked fiber loss and replacement with interstitial collagen. The intercostals displayed an intensity of picrosirius staining and high score that is not perhaps reflected in measured hydroxyproline content of these tissues. The intercostal muscles are a complex mixed tissue when viewed microscopically, typically comprised of fibers in both cross and oblique section and containing fat and large blood vessels. The impression in mdx sections was for greater disarray in tissue structure, fiber loss, considerable variation in myocyte size, fibrosis, and inflammatory cell infiltration.

**DISCUSSION**

The mdx mouse is the most frequently used animal model for research into DMD, and much of our understanding of the pathophysiology of dystrophic muscle has been gained from studies in this model. These studies, however, show a milder phenotype than boys with DMD or the Golden Retriever muscular dystrophy dog.

**Table 2. Optimum fiber length, weight, and width of muscle strips from 17-mo-old control and mdx mice**

<table>
<thead>
<tr>
<th>Muscle</th>
<th>$L_o$, mm</th>
<th>Weight, mg</th>
<th>Width, mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Latissimus dorsi</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>31.14 ± 4.20</td>
<td>86.56 ± 13.55</td>
<td>2.84 ± 0.40</td>
</tr>
<tr>
<td>mdx</td>
<td>31.33 ± 1.01</td>
<td>158.09 ± 10.10†</td>
<td>3.68 ± 0.43</td>
</tr>
<tr>
<td>Intercostal strip</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8.28 ± 1.31</td>
<td>59.95 ± 9.04</td>
<td>5.53 ± 1.04</td>
</tr>
<tr>
<td>mdx</td>
<td>8.61 ± 0.39</td>
<td>55.65 ± 3.85</td>
<td>4.64 ± 0.37</td>
</tr>
<tr>
<td>Diaphragm strip</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>9.44 ± 1.34</td>
<td>8.15 ± 0.99</td>
<td>1.44 ± 0.21</td>
</tr>
<tr>
<td>mdx</td>
<td>8.56 ± 0.41</td>
<td>12.49 ± 1.75*</td>
<td>1.87 ± 0.18*</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n = 7$ (C57 mice) and 8 (mdx mice). Significant difference vs. control: *$P < 0.05$; †$P < 0.001$.
Although a reduction of vertebral support in humans with neuromuscular weakness can show as thoracolumbar deviation in a dorsal or ventral plane (kyphosis or lordosis, respectively) or a lateral deviation of the spine due to the effect of gravity (scoliosis), the quadrupedal gait of mice results in the development of kyphosis.

With careful positioning of animals, it is possible to accurately measure differences between animals not apparent by observation or palpation. We demonstrated that a significant decrease in KI occurred in a group of mdx mice at 9 mo of age or at approximately one-third of the mdx lifespan. This differs from mdx:utrn/H11002/H11002/H11002, which shows an earlier onset of spinal deformity (7, 15). This difference is probably attributable to the muscle hypertrophy demonstrable in most skeletal muscle of mdx, which for a time maintains whole muscle strength, although in vitro organ bath studies of the paraspinal muscles indicate that normalized forces (maximum isometric tension per unit CSA) are weaker. This is in agreement with experiments conducted on limb muscles (22).

It is likely that the level of anesthesia of mice will affect KI, and early trials utilizing only light sedation resulted in animals

Table 3. Contractile properties of latissimus dorsi muscles, intercostal strips, and diaphragm strips from 17-mo-old mdx and control mice

<table>
<thead>
<tr>
<th></th>
<th>TPT, ms</th>
<th>TR50, ms</th>
<th>TR90, ms</th>
<th>Pt, mN/mm²</th>
<th>Po, mN/mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Latissimus dorsi</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>26.5±1.2</td>
<td>20.6±0.9</td>
<td>43.3±1.5</td>
<td>18.76±3.90</td>
<td>86.8±13.65</td>
</tr>
<tr>
<td>mdx</td>
<td>25.0±1.0</td>
<td>20.2±1.0</td>
<td>43.8±3.0</td>
<td>10.62±1.19</td>
<td>45.9±5.60</td>
</tr>
<tr>
<td>Intercostal strip</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>24.2±0.8</td>
<td>17.4±0.8</td>
<td>36.8±1.7</td>
<td>0.23±0.05</td>
<td>1.04±0.20</td>
</tr>
<tr>
<td>mdx</td>
<td>25.8±1.0</td>
<td>20.1±1.0*</td>
<td>43.0±2.0*</td>
<td>0.11±0.02*</td>
<td>0.58±0.09*</td>
</tr>
<tr>
<td>Diaphragm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>41.5±1.4</td>
<td>30.3±0.9</td>
<td>62.0±2.3</td>
<td>31.93±6.67</td>
<td>117.8±23.1</td>
</tr>
<tr>
<td>mdx</td>
<td>33.1±1.0</td>
<td>31.8±1.0</td>
<td>62.6±3.0</td>
<td>5.83±0.61†</td>
<td>31.86±3.2†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 7 (C57 mice) and 8 (mdx mice). TPT, time to peak tension; TR50, time to 50% relaxation; TR90, time to 90% relaxation; Pt, peak twitch force; Po, maximal isometric tetanic force. Pt and Po were normalized using muscle cross-sectional area for diaphragm and latissimus muscles and wet weight only for intercostal preparations. Significant difference vs. control: * P < 0.05; † P < 0.01; ‡ P < 0.001.

Fig. 4. Hydroxyproline content of paraspinal and respiratory muscles in mdx and control mice, as a measure of tissue fibrosis. Values are means and SE; n = 7 (C57), n = 8 (mdx). Lat, latissimus; Long, longissimus. Significant difference vs. control mice: * P < 0.05; ** P < 0.01; *** P < 0.001.

Fig. 5. A: histological score based on interstitial fibrosis, calculated on microsirius red-stained muscle samples. Scoring scheme was determined on viewing 10 fields per tissue (>20 magnification) with a score of 4 indicating >50% fibrosis. Values are means and SE; n = 7 (C57), n = 8 (mdx). B: frequency distribution of internal nuclei in paraspinal and respiratory muscles. Figures are based on counting 100 fibers per muscle on hematoxylin and eosin-stained sections. Values are means and SE; n = 7 (C57), n = 8 (mdx). P < 0.0001 for all muscles. Significant difference vs. control mice: ** P < 0.01; *** P < 0.001.

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struggling against the tape restraint and causing movement blur. Ketamine/xylazine combination provided muscle relaxation as well as immobilization and offered the benefit of allowing xylazine reversal by atipamezole, which may enhance recovery in aged mdx with cardiac impairment. Right lateral recumbent views were chosen for ease and consistency, allowing up to six mice per cassette to be radiographed. It is also possible to choose prone positioning with cross-table lateral views; however, this would allow only two mice to be radiographed at one time. There was also concern that the heavy shoulder and hindlimb musculature of young mdx (3–12 mo old, corresponding to muscle hypertrophy stage) would hinder correct positioning when prone.

We also showed that thoracic area measurements of mdx was less than those of control mice, and it is likely that such thoracic deformity in aged mdx will affect pulmonary function [or contribute to the reduced lifespan of more severely kyphotic mdx:utrn<sup>+/−</sup> (7)]. Certainly in DMD patients, diaphragmatic and respiratory muscle weakness coupled with severe thoracic deformity leads to hypoventilation and hypoxemia, sleep abnormalities, and susceptibility to respiratory tract infections (30, 31).

Certain mdx skeletal muscles show profound dystrophic changes, particularly the diaphragm, slow-twitch limb muscles, and postural muscles (21, 27, 33). These are muscles with either an obligatory constant workload or a role in resisting gravitational forces compared with fast-twitch, intermittently active muscles such as the extensor digitorum longus. We utilized the diaphragm in our experiments because its contractile, morphometric, and histopathologic properties are well documented (1, 6, 33) and it serves as a useful benchmark for the severity of dystrophic changes in other less well-characterized muscles. Mdx diaphragm strips generate significantly lower maximum tensions compared with age-matched control mice as reported previously (23, 33), and there was an inverse relationship to hydroxyproline content. Histological changes showed severe interstitial fibrosis and myocyte disarray typical of this organ in mdx mice.

It is perhaps not surprising that intercostal muscles are also subject to a high degree of dystrophic changes because they have an augmentative role, although lesser than the diaphragm, in respiration. During inspiration, contraction of the parasternal intercostals causes elevation of the ribs and flaring of the sternum synergistic to diaphragmatic contractions (13). Several
of the *mdx* intercostal sections examined scored equally to diaphragm strips (grade 4, equivalent to >50% fibrosis). The intercostal muscles showed prolonged relaxation properties, with significant increases in time to 50 and 90% relaxation compared with control mice. We did not separate internal and external intercostal muscle layers due to the risk of damage to individual fibers. We chose the direction of dissection as parallel to external intercostal fibers, because external intercostal muscle fibers contribute most to force generation during normal respiratory movements. Previous intercostal studies on larger species, including rabbits (5), dogs (8, 13), and hamsters (19, 20), utilized separated muscles. Our preparations spanning four ribs were very similar in dimensions to these hamster preparations, and we also extrapolated from studies in guinea pigs where tracheal segments comprising a series of tracheal rings are mounted via silk suture around cartilages in organ bath experiments (4, 24).

The latissimus dorsi is a fan-shaped muscle, which because of its superficial position on the trunk is easily dissected. This muscle also showed centronucleation and fibrosis, with reduced peak twitch force and maximal tetanic force seen in other skeletal muscles of older *mdx*. Previous contractility studies using rabbit latissimus dorsi confirm its fast-twitch properties (18). Twitch kinetics from our experiments suggest that the latissimus dorsi is also a fast-twitch muscle in older mice, although this needs to be verified by fiber typing.

The longissimus dorsi is an important member of the erector spinae group involved in spinal rotation and extension. It is not amenable to organ bath studies because of multiple branching and insertions on many vertebral processes; however, there was histological and biochemical evidence of dystrophy similar to the other *mdx* muscles examined.

In humans, comparisons have been made between limb muscle and joint contracture seen in neuromuscular diseases and the fibrosis and contracture of respiratory muscles, stiffening of tendons and ligaments of the rib cage, and ankylosis of costovertebral and costosternal articulations (2). Failure to fully expand the lungs causes increases in lung tissue and chest wall elastance and decreases in compliance (25), alterations that contribute markedly to the total mechanical work of breathing.

Although the spinal deformity of dystrophin-deficient mice is not as extreme as that seen in patients with DMD, we suggest that the hallmarks of dystrophy, muscle weakness, and fibrosis, and not just aging per se, are implicated in the progression of kyphosis and thoracic deformity in this model. It is likely also that the relative inactivity of aged *mdx* mice compared with their control strain, noted by ourselves and others (27), is due in large part to the presence of these skeletal malformations and associated muscle contracture and increase in stiffness. It is only conjecture at this stage that significant respiratory insufficiency may also occur in *mdx* mice and could be implicated in this failure to move freely.

In addition, we demonstrated the application of a radiographic index for the measurement of kyphosis in mice and showed how this index changed in *mdx* mice compared with control mice. The measurement of kyphosis by radiographic indexes presented here is a method of quantitative comparison between mouse strains and may also have application for long-term therapeutic studies or gene-therapy trials in the *mdx* or other kyphotic mouse strains.

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