Progression of kyphosis in *mdx* mice

NICOLA LAWS AND ANDREW HOEY

*Centre for Biomedical Research, Faculty of Sciences, University of Southern Queensland, Toowoomba, QLD, Australia 4352.*

Author for Correspondence

Andrew Hoey
Centre for Biomedical Research
Faculty of Sciences
University of Southern Queensland
TOOWOOMBA QLD 4350
Australia

Ph: 61 746 311 505
Fax: 61 746 311 530
Email: hoey@usq.edu.au
Abstract

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 sub-sarcolemmal protein dystrophin. *Mdx* mice, which also lack dystrophin, show thoracolumbar kyphosis which 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 (KI), similar to that used in boys with DMD. KI became significantly less in *mdx* at nine months of age (3.58 ± .12 compared to 4.27 ± .04 in the control strain, *P*<0.01), indicating more severe kyphosis, and remained less from 10-17 months of age. Thoracic area in 17 month old *mdx* was reduced by 14% compared to control mice (*P*<0.05). Peak tetanic tension (P0) was significantly lower in *mdx*; falling 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 KI 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.
DUCHENNE MUSCULAR DYSTROPHY (DMD) is a relentlessly progressive X-linked myopathy characterised 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 thirty years 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 contribute 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 (VC) remains normal in patients with neuromuscular disease if respiratory muscle strength is more than 50% of predicted, however when strength is less than this VC becomes diminished to a greater extent than expected (7,12). 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 (12), (2). As well as exacerbating respiratory dysfunction in affected boys, kyphoscoliosis negatively impacts on their quality of life in their wheelchair dependant years, with most patients electing surgery for spinal fusion and stabilisation 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 indices 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 (8,15) and the kyphoscoliosis (ky) mouse (3,10). Although mdx 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−/− (8,15,21). 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 to control mice, which was thought to be influenced by tumour necrosis factor-α (14).

There were three goals in this study. Firstly, we applied a novel Kyphotic Index (KI) to determine the progression of spinal deformity in two groups of aging mice, mdx and their aged matched controls. We also assessed contractile function in paraspinal and respiratory muscles (latissimus dorsi muscles and intercostal strips) and compared them to 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 due to 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 fibrosis 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 \textit{mdx} mice occurs before one year of age, then stabilised until 17 months 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 \textit{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 weeks of age. Male mdx mice were bred at the USQ Animal House, Toowoomba, Qld. 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 USQ Animal Ethics Committee. 4 mice per strain were used for the radiographic study, with one mouse dying during the 17 months duration of this study. An additional 4 mice per strain were also utilised for contractility experiments, hydroxyproline assays and histology.

Radiographic studies and establishment of Kyphotic Index. Mice were sedated with Ketamine HCl 50mg/kg (Ketamil, Troy Laboratories, Australia) in combination with Xylazine HCl 10mg/kg (Ilium Xylazil-20, Troy Laboratories, Australia) administered by subcutaneous injection. At the end of the procedure atipamezole (Antisedan, Novartis Animal Health, Australia) was given at a dose rate of 0.1mg/kg to reverse α-2 agonist effects of xylazine. Mice were lightly taped to the radiographic cassette using 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 Xray Co Ltd Tokyo, Japan or Porta 1030 model, Job Corporation, Yokohama, Japan). Optimum exposure with our equipment was established at 48KV, 1.8 MAS with a film focal distance of 70 cm. Mice were radiographed once monthly from 4 months until 17 months of age.

Each whole body radiograph was photographed using a tripod mounted Ricoh Caplio RR30 digital camera with images analysed 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 6th 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 Figure 1a and b.

Trial radiographs of the same animals in 3 positions 1) hind limbs and forelimbs placed in moderate extension, 2) overextension (stretching) of limbs and 3) flexion of forelimbs and hind limbs showed there was some differences in measured KI, considered to be less than 10%. Care was then taken to avoid 2) or 3), and to ensure limbs were only moderately extended. This could be confirmed when radiographs were analysed 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 utilising 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 sternebrae, diaphragm and ventral edge of vertebrae to allow an estimation of thoracic area at age 17 months. This measure was repeated three times and results averaged for each animal (n=4 animals per group). Because thoracic area is related to body size the calculated area was normalised for body weight to give a value of thoracic area/body weight (cm²/gram)
Contractility studies. 17 month old mice were anaesthetised using pentobarbitone sodium (Nembutal, Boehringer Ingelheim, Australia) at 70 mg/kg IP. Cessation of breathing occurred when the thorax was entered. The following muscles were dissected and placed into ice-cold Krebs buffer solution bubbled with carbogen (95%O₂/5%CO₂); a) diaphragm strip from left midcostal hemi-diaphragm, with placement of silk suture material around the central tendon at one end and a small rib section at the other. b) 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 end to anchor to a fixed peg below the stimulating electrodes and c) intercostal section comprising 4 ribs and their attached intercostal muscles (internal and external), extending from T8-12, adjacent and parallel to the longissimus dorsi muscle. 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, using 6/0 silk surgical suture thread to 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, USA) and signals were amplified using a pre-amplifier (EP500B. Audio Assemblies, Campbellfield, Victoria, Aust). Data was collected and analysed using Chart 4.1.1 software. A square pulse of 0.2ms duration was dispersed via 2 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 7-8 mice per group were used for contractility studies. Reported data was the average of 3 individual single twitch or tetanic stimulations per muscle strip after 25 minutes of equilibration and optimisation of conditions. Muscles were measured at $L_o$ using a digital micrometer, blotted for 3 secs then weighed. Cross sectional area (CSA) and normalisation of force was calculated as described previously for diaphragm and latissimus muscles, where CSA equals tissue weight divided by length x 1.06 (density of mammalian muscle) (23). Intercostal muscle fibre CSA was shown to vary topographically (20) and in our preparations a strip comprises both muscle and rib cartilage and internal and external intercostal muscle layers consisting of differing fibre orientations and hence lengths. For these reasons intercostal forces were normalised to weight only. Time to peak force, 50% relaxation time and 90% relaxation time was calculated for each single twitch value.

**Hydroxyproline analysis.** We used hydroxyproline 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 hydrolysed in sealed tubes with 6M HCl overnight at 110°C. The samples were dried to entirety using filtered air under pressure and heat (50°C). The rest of the protocol has been described previously (34). Values are expressed as µgHP/mg tissue wet weight.

**Histology.** Each tissue (diaphragm, latissimus, longissimus and intercostal muscles) were pinned onto cork at optimal length and then fixed sequentially in Telly’s
fixative (formaldehyde, glacial acetic acid-ethanol fixative, 72 hours), Bouin’s solution (formaldehyde, glacial acetic acid-picric acid fixative, 24 hours) and 70% ethanol, prior to paraffin embedding, cutting and staining of 10 micron sections using 0.1% w/v picrosirius red solution (Sirius Red F3B, Chroma Dyes, Germany in saturated picric acid), a specific collagen stain. Additional 5 micron sections were stained with haematoxylin and eosin for determination of nuclear position and heterogeneity of muscle fibre size. Analysis was performed blinded to the strain of mouse, with sections viewed on a Nikon Eclipse E600 light microscope and captured with a Nikon FDX-35mm camera. Images were digitised and then analysed 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 (eg <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 an $n = 3$ animals for assessment of KI and $n=7$ animals for contractility experiments were adequate (28). Results are expressed as means ± S.E. Responses between mdx and control strain were analysed using Student’s unpaired $t$-tests, with the exception of differences in KI, where ANOVA was employed. $P$ values less than 0.05 were considered statistically significant.
Results

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

Kyphotic Index. KI as a measure of spinal deformity remained similar for mdx and control mice until approximately 9 months of age, after which a significant difference became apparent between mdx and normal mice (P<0.01 at 9 months). There was a plateau in both mouse strains after this age (Figure 2a). Figure 2b shows radiographs of young and aged mdx and control mice.

Thoracic Area. There was no significant difference in body weights at 17 months of age between mdx and C57 mice, however there was a difference (P<0.05) between thoracic area and normalised thoracic area in mdx and age-matched controls (Table 1) with mdx mice demonstrating lower values.

Muscle contractility. The characteristics (optimal muscle length (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 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 their 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 tended to be thin and transparent, while in \textit{mdx} 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 \textit{mdx} group.

\textit{In vitro} isometric contractile properties of these muscles are shown in Table 3. All \textit{mdx} muscles examined demonstrated reduced force production (twitch and tetanus) compared to control mice, with \textit{mdx} diaphragm muscle showing the greatest reduction in tension generated (approximately two-thirds that of control mice, \textit{P}<0.01). \textit{Mdx} latissimus dorsi and intercostal strip values were by contrast 50\% of control levels for both twitch and tetanus tensions (\textit{P}<0.05 except for latissimus Po (\textit{P}<0.01)). Rise times (TPT) were similar, except for the attenuated diaphragm twitches of the \textit{mdx}, which had significantly shorter TPT (\textit{P}<0.001). Relaxation was prolonged in \textit{mdx} intercostal muscles compared to control levels, reflected by increased TR_{50} and 90 (\textit{P}<0.05).

\textit{Hydroxyproline analysis.} Four muscles were used for assays of hydroxyproline content, and results are shown in Figure 4. All \textit{mdx} muscles had significantly more HP than control mice, with the diaphragm HP 2.5 times higher than the other muscles examined. Interestingly control mice also showed a higher level of fibrosis in this organ compared to other respiratory or paraspinal muscles, and when relative values were compared (control HP as a ratio of \textit{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 Figure 5a. Representative photomicrographs of all four muscles of *mdx* and control mice are shown in Figure 5. The percentage of centronucleation of muscle fibres as visible on H&E stained sections indicates previous necrosis and degeneration and one or more cycles of regeneration, and is displayed in Figure 5b. It is apparent that *mdx* muscles demonstrate marked heterogeneity in cell size, a high incidence of centrally nucleated fibres, inflammatory cell infiltration and fibrous tissue deposition compared to control muscles. *Mdx* diaphragm displays the highest scores on histological grading as expected, with marked fibre 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 fibres in both cross and oblique section and containing fat and a large blood vessel. The impression in *mdx* sections was for greater disarray in tissue structure, fibre 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. They however, show a milder phenotype than boys with DMD or the Golden Retriever Muscular Dystrophy dog (GRMD).

While 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 Kyphotic Index occurred in a group of *mdx* mice at 9 months of age, or at approximately one third of the *mdx* lifespan. This differs from *mdx:utrn* which shows an earlier onset of spinal deformity (8, 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 indicates that normalised forces (maximum isometric tension per unit cross-sectional area) are weaker. This is in agreement with experiments conducted on limb muscles (23).

It is likely that the level of anaesthesia of mice will affect KI, and early trials utilizing only light sedation resulted in animals struggling against the tape restraint causing movement blur. Ketamine/xylazine combination provided muscle relaxation as well as immobilisation, 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 6 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 hind limb musculature of young mdx (3-12 months 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⁻/⁻ (8)). Certainly in DMD patients diaphragmatic and respiratory muscle weakness coupled with severe thoracic deformity leads to hypoventilation and hypoxaemia, 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 to 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-characterised muscles. Mdx diaphragm strips generate significantly lower maximum tensions compared to age-matched control mice as reported previously (22,33) 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 as they have an augmentative, though lesser, role 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 TR_{50} and TR_{90} compared to control mice. We did not separate internal and external intercostal muscle layers due to the risk of damage to individual fibres. We chose the direction of dissection as parallel to external intercostal fibres, as external intercostal muscle fibres contribute most to force generation during normal respiratory movements. Previous intercostal studies on larger species including rabbits (5), dogs (9,13) and hamsters (19,20) utilised separated muscles. Our preparations spanning 4 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 (24) (4).

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 P_t and P_o 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 the latissimus dorsi is also a fast twitch muscle in older mice, although this needs to be verified by fibre 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 to 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 to control mice. The measurement of kyphosis by radiographic indices 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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Address for reprint requests and other correspondence: A. Hoey, Centre for Biomedical Research, Faculty of Sciences, University of Southern Queensland, Toowoomba, QLD, Australia 4352. (email; hoey@usq.edu.au)
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**TABLE 1** Mean (± SE) of thoracic area, body weight and normalised thoracic area in 17-month-old mice. \( n=3(C57), \ n=4(mdx). \) \(*P<0.05\)

<table>
<thead>
<tr>
<th></th>
<th>Thoracic area (cm²)</th>
<th>Body weight (g)</th>
<th>Thoracic area/bw (cm²/g)</th>
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<tbody>
<tr>
<td><strong>Control</strong></td>
<td>4.49 ± 0.12</td>
<td>32.2 ± 0.86</td>
<td>0.14 ± 0.007</td>
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<tr>
<td><strong>Mdx</strong></td>
<td>3.71 ± 0.25 *</td>
<td>31.65 ± 1.2</td>
<td>0.12 ± 0.004 *</td>
</tr>
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**FIGURE 1** Establishment of Kyphotic Index in mice

![Diagram of Kyphotic Index measurement in mice](image)

**Figure 1a.** Method of measurement of KI in boys with DMD (diagram adapted from Smith AD et al 1989). 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

**Figure 2b.** KI in mice, calculated from radiographs of anaesthetised 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
Kyphosis Index of aging mdx and control mice. Mice were radiographed monthly from 4 months to 17 months of age and measurements were made every 2 months. \( n=4 \text{(mdx)}, n=3 \text{(C57)} \) *\( P<0.05 \), **\( P<0.01 \).
Figure 3. Examples of mouse whole body radiographs used for calculation of kyphotic index. A and C = young and aged (5 month old and 17 month old) control mouse, B and D=young and aged *mdx* mouse. The progression of spinal deformity results in a decrease in KI, and alteration of thoracic shape and size.
Table 2. Mean (± SE) optimum fibre length, weight and width of muscle strips from 17-month-old control and *mdx* mice. *n=7(C57), n=8(mdx)*** *P*<0.001 *P*<0.05

<table>
<thead>
<tr>
<th>Muscle</th>
<th>Lo (mm)</th>
<th>Weight (mg)</th>
<th>Width (mm)</th>
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<tr>
<td><strong>Latissimus dorsi</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>31.14± 4.2</td>
<td>86.56±13.55</td>
<td>2.84± 0.4</td>
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<tr>
<td><em>mdx</em></td>
<td>31.33± 1.01</td>
<td>158.09± 10.1</td>
<td>3.68± 0.43</td>
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<td><strong>Intercostal strip</strong></td>
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</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><em>mdx</em></td>
<td>8.61± 0.39</td>
<td>55.65± 3.85</td>
<td>4.64± 0.37</td>
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<tr>
<td><strong>Diaphragm strip</strong></td>
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<td></td>
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<tr>
<td>Control</td>
<td>9.44± 1.34</td>
<td>8.15± 0.99</td>
<td>1.44± 0.21</td>
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<tr>
<td><em>mdx</em></td>
<td>8.56± 0.41</td>
<td>12.49± 1.75*</td>
<td>1.87± 0.18*</td>
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Table 3: Contractile properties of latissimus dorsi muscles, intercostal strips and diaphragm strips from 17-month-old mdx and control mice: Time to peak tension (TPT), Time to 50% relaxation (TR50), Time to 90% relaxation (TR90), peak twitch force (Pt) and maximal isometric tetanic force (Po). n=7 (C57), n=8 (mdx). *P<0.05, **P<0.01, ***P<0.001. Pt and Po normalised using muscle CSA for diaphragm and latissimus muscles, and wet weight only for intercostal preparations.
Figure 4 Hydroxyproline content of paraspinal and respiratory muscles in mdx and control mice, as a measure of tissue fibrosis. *P<0.05, **P<0.01, ***P<0.001
Figure 5a. Histological score based on interstitial fibrosis, calculated on picrosirius red stained muscle samples. Scoring scheme was determined on viewing 10 fields per tissue (20X magnification) with a score of 4 indicating greater than 50% fibrosis. \( n = 7(\text{C57}), n = 8(\text{mdx}) \). ** \( P < 0.01 \) *** \( P < 0.001 \)

Figure 5b Frequency distribution of fibres with internal nuclei. Figures are based on counting 100 fibres per muscle on H&E stained sections. \( n = 7(\text{C57}), n = 8(\text{mdx}) \) \( P < 0.0001 \) for all muscles.
Figure 6. Picrosirius red stained muscle sections. The dark red stained areas represent collagen. The relative amounts of staining were used to grade samples from 1-4, corresponding with the level of interstitial fibrosis. A,C,E,G: mdx. B,C,F,H: C57 A,C =Grade 2, E=Grade 3, G=Grade 4. All control mice were considered Grade 1. Sections C,D and F shown at 10X magnification, other muscles at 20X.
Figure 7 Photomicrographs of haematoxylin and eosin stained sections. A; mdx longissimus, B; mdx intercostals, C; mdx latissimus, D; control diaphragm. 20X magnification. The mdx cells show dystrophic features including centronucleation, variation in fibre size, inflammatory cell infiltration and interstitial fibrosis, indicating cycles of degeneration and regeneration. In contrast the control diaphragm shows greater uniformity of fibre size and little evidence of fibrosis.