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Quantification of histopathology in Haemotoxylin and Eosin stained muscle sections

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1 OBJECTIVE

This document describes a method and provides reference values for the histological characterisation of dystrophic muscle from *mdx* mice.

2 SCOPE AND APPLICABILITY

Dystrophic skeletal muscle pathology can be easily assessed on haematoxylin and eosin (H&E) stained transverse muscle sections. This SOP is written as a guide for those who wish to quantitate the amount of skeletal muscle necrosis (as an indication of the extent of dystropathology) and subsequent muscle regeneration in histological muscle sections from dystrophic *mdx* mice.

3 CAUTIONS

As with all histological staining, great care must be taken in preparation of the samples:

- Accurate removal of the muscle(s) from the mouse will ensure that the appropriate portion of the appropriate muscle is actually being consistently analysed.
- Fixation (or freezing) should be done carefully to minimize shrinkage of tissues (or ice-artifact). It is important to identify areas of ice-artifact in frozen sections and to distinguish this from actual areas of muscle necrosis.
- Accurate embedding is essential as all sections must be embedded the same way. Longitudinally embedded muscle samples cannot be quantitated and compared to transverse embedded muscle samples.
- Bad cutting (e.g. a blunt blade which leaves knife scores in the section) or folding of the muscle section can make it hard to analyse a section accurately.

4 MATERIALS

Animals: C57Bl/10$^{mdx/mdx}$, Animal Resource Centre Murdoch (Western Australia http://www.arc.wa.gov.au/) or a similar animal supplier such as Jackson Laboratories (http://jaxmice.jax.org) in the USA.

For all: Superfrost glass slides (Lomb SF41296), Harris Haematoxylin (BDH 35194.6T), Eosin yellowish (BDH 341973), 100% ethanol, 70% ethanol, Xylene, DPX mountant (BDH 36029.4H).

For frozen histology: Gum Tragacanth (Sigma G1128), Isopentane (BDH 103616V), Liquid Nitrogen, 1cm^3 cork squares.

For paffin histology: 4% Paraformaldehyde (Sigma P6148).

5 METHODS

Animal treatment:

Dystrophic mice do not necessarily need to be treated prior to assessing their histology. However, if treatments are involved it is important to have at least 2 groups in order to get interpretable information: for example, typically treated and untreated groups for drug testing, exercised and unexercised groups for exercise interventions, or combinations, depending on the experimental design.

Tissue preparation:

For paraffin histology, muscles (e.g. the tibialis anterior, TA) are removed from the mouse and immediately placed into 4% paraformaldehyde for fixation. If possible, placing an entire leg into paraformaldehyde will allow the muscles to hold their shape and minimise curling of the muscles. The muscles are left in paraformaldehyde for up to 48 hours (depending on the size of the muscle/leg) and once fixed moved into 70% ethanol. As an alternative to paraformaldehyde, various commercially available formalin solutions (Confix – Australian BioStain) can be used for basic muscle histology although these may not be suitable for antibody staining. After fixation, muscles are cut transversely, caged and processed overnight in an automatic processor e.g. Shandon, (ethanol through to paraffin wax) and embedded into paraffin blocks.

For frozen histology, the fresh muscles are bisected transversely and the 2 pieces mounted side-by-side on cork squares using tragacanth gum. The muscles are routinely frozen in a slurry of isopentane cooled in liquid nitrogen. Isopentane reduces surface tension and avoids trapping air around the muscle (which can slow the freezing process) thus producing a better frozen sample and excellent histology (with little or no freeze artefact).

Sectioning:

Skeletal muscle tissue sections are generally cut at a width of approximately 5µm for paraffin blocks, collected onto uncoated glass slides and stored in the dark at room temperature until stained. Frozen sections are cut at 8µm (on a cryostat) directly onto uncoated or silinated glass slides: ideally these are stained immediately but can be stored at -20ºC until stained.

Staining:

Dystrophic skeletal muscle pathology can be accurately assessed on sections stained with Haematoxylin and Eosin (H&E). Haematoxylin stains eosinophilic structures (e.g. muscle sarcoplasm) pink and Eosin stains basophilic structures (e.g. nuclei) dark purple, with high RNA producing paler purple staining in the cytoplasm (e.g. in young myotubes) (Fig. 1).
Basic H&E staining protocol for paraffin sections. **For frozen sections begin at 'f'**

a) Dewax paraffin sections – incubate slides at 60ºC for 20minutes.

b) Dewax sections – Xylene wash for 3 minutes (repeat x3).
c) Rehydrate sections – 100% ethanol for 3 minutes (repeat x2).
d) Rehydrate sections – 70% ethanol for 3 minutes.
e) Rinse – double distilled water for 1-3 minutes.
f) Stain - Haematoxylin for 30 seconds. **(Start frozen sections here)**
g) Stain - Remove excess stain in tap water (until nuclei turn blue).
h) Stain - 70% ethanol for 3 minutes.
i) Stain - Eosin for 15 seconds.
j) Dehydrate – 100% ethanol for 3 minutes (repeat x3).
k) Clear – Xylene wash for 3 minutes (repeat x3).
l) Mount - DPX mountant and coverslip.

Image analysis and quantitation:

The morphological features of the muscle are identified using digital images of the stained section. Non-overlapping images of a transverse muscle section can be tiled together to provide a single digital image of the entire muscle cross sectional area. Morphological features appear to be equally divided through-out the length of the muscle (e.g. from tendon to tendon) in unexercised *mdx* mice (Figure 2) so it is sufficient to take pictures at one location within the muscle i.e. the middle (van Putten et al 2010). For consistency it is necessary to keep the location constant in all muscles and to mention which part of the muscle is analyzed. Images are acquired using a bright-light microscope, digital camera and image capture software (e.g. Leica DM RBE microscope, a personal computer, a Hitachi HVC2OM digital camera, Image Pro Plus 4.5.1 software and Vexta stage movement software). More sophisticated digital slide scanners (e.g. Aperio Scanscope) can be used to collect images although not all researchers will have access to these machines.

Some researchers perform histological analysis on multiple single frame images taken from the same muscle section (fields of view) and do not analyse the entire cross sectional area. However, this method assumes that histological features are homogenous throughout the muscle cross section, which may not always be true, in particular when mice have been subjected to exercise.

Histological features are identified manually by the researcher and quantified using image analysis software (e.g. Image Pro Plus). To quantitate the abundance of a specific histological feature (e.g. myofibre necrosis) per muscle cross sectional area, first open the digital image in your image analysis software (e.g. Image Pro Plus) and measure (draw around) the entire cross-section area of the muscle. This can be done in pixels (sufficient when determining a percentage) or the software can be calibrated and features measured as an absolute value (e.g. μm^2). Second, identify and manually measure (draw around) all histological features of interest (e.g. all areas of infiltrating inflammatory cells and fragmented muscle sarcoplasm).

Once all the appropriate histological features have been measured in the muscle cross section, data can be exported directly into Microsoft Excel and a percentage (e.g. % myofibre necrosis) can be calculated (e.g. Total area of all histological features/ Total area of

muscle cross section x 100). The data are then subjected to the appropriate statistical analyses.

Analysis of all morphological features at once on whole cross sections can also be performed using the H&E colour deconvolution plugin of ImageJ (free software) which separates the Hematoxylin component and the Eosin component. Normal, undamaged myofibers are represented by the Eosin component and this area can be measured by thresholding. This can distinguish undamaged from damaged/regenerating myofibres and also identify areas of fibrosis. The total area can be determined on the original picture (van Putten et al. 2010).

6 EVALUATION AND INTERPRETATION OF RESULTS

Which muscle to analyse?

In all dystrophic *mdx* mice (both treated and untreated) it is vital to consider the age of the mouse when assessing skeletal muscle dystropathology and to compare age-matched mice (treated vs. untreated), since the dystrophic characteristics of muscle change with age. The tibialis anterior (TA) muscle of the lower hindlimb is widely used, since it is readily accessible, transverse sections are easily obtained and it contains mainly fast myofibres. The soleus is also of interest as a model of slow myofibres. There is also a wealth of information on other large hindlimb muscles such as the quadriceps and gastrocnemius, although these can be more complex to interpret since a true transverse section through all myofibres is not possible due to the architecture of some muscles. In addition, the plane of section and analysis is complicated since these muscles are composed of several groups of muscles. e.g. the gastrocnemius muscle has 2 heads that attach to the femur and the quadriceps muscle is composed of 4 individual muscles, the rectus femoris, vastus lateralis, vastus intermedius and vastus medialis.

The forelimb muscles such as triceps and biceps are also sometimes measured and generally show similar changes with age as the hindlimb muscles.

The diaphragm is of special interest since it shows striking dystropathology, especially in older mice (Fig. 2). Due to the complex structure and varying width of the diaphragm muscle, extra care must be taken to cut sections at either equivalent locations for histological comparisons or at various intervals through the diaphragm.

Exercise will also influence the muscle analysed as some muscles are severely damaged by exercise (e.g. forced treadmill running, voluntary wheel running, swimming etc.) whereas other muscles may be relatively unaffected by a specific exercise regime. For example, voluntary wheel running almost doubles the amount of necrosis in the quadriceps, whereas the TA is almost unaffected (Radley et al. 2008); similarly, various muscles in the forelimbs and hindlimbs are differentially affected by treadmill running (Grounds et al, 2008).

The specific age of the *mdx* mice will greatly influence the histological features that are seen and analysed. For example a high level of muscle necrosis (e.g. 30%) in the TA muscle of a 23 day old *mdx* mouse is expected and the acute onset of muscle necrosis around 21 days of age is well documented (Grounds and Torrisi, 2004; Radley and Grounds, 2006). However, a high level of muscle necrosis in a sedentary adult (6 weeks^{+}) *mdx* mouse is not expected. Histological features in early and later stages of the disease can be very different. Reference data for 3 ages are shown in Tables 1 and 2 and Fig. 2.

Morphological features to be identified:

A) Normal / undamaged myofibres: Normal myofibres have peripheral nuclei, intact sarcolemma and non-fragmented sarcoplasm (Fig 1A). In young mice these myofibres represent areas of muscle that have not undergone the process of necrosis and regeneration. In adult mice these myofibres represent areas of muscle that have either never undergone necrosis (this is the usual interpretation), or have previously undergone necrosis and regeneration with long enough time for the myonuclei to have moved to the periphery.

B) Necrotic myofibres: Necrotic muscle is identified by the presence of infiltrating inflammatory cells (Fig 1B) (basophilic staining) and/or hypercontracted myofibres and degenerating myofibres with fragmented sarcoplasm (Fig 1C). Measurements of muscle necrosis in *mdx* mice are of much value in (i) young mice before and after the acute onset of myofibre necrosis, which occurs in limb muscles around 3 weeks of age and (ii) in older mice where necrosis is induced by exercise. The measurements of active muscle necrosis are generally of less value in sedentary adult *mdx* mice since the levels are very low in some limb muscles (e.g. around 5% of whole TA muscle affected and even lower after about 1 year of age) – thus it is difficult to detect a relative decrease in such already low values. The acute onset of dystropathology with high levels of necrosis provides a very sensitive assay to specifically evaluate therapeutic interventions designed to prevent or reduce myofibre necrosis.

C) Regenerating (recently necrotic): Regenerating muscle is identified by activated myoblasts and, 2 -3 days later, small basophilic myotubes (Fig 1D). These myotubes subsequently mature into plump myofibres with central nuclei (**regenerated myofibres**) (Fig 1E).

Cumulative skeletal muscle damage in young *mdx* mice consists of active myofibre necrosis plus the areas of subsequent regeneration (new myotubes/myofibres), see Table 2. It is noted that in *mdx* mice up to at least one year of age, there appears to be excellent capacity for new muscle formation in damaged limb muscles.

In older *mdx* mice, quantitation of **undamaged myofibres,** that look normal with peripheral nuclei (Fig 1A), is a useful measure of muscle that has NOT been subjected to necrosis (in the last 100 days)(McGeachie et al. 1993), since this indicates resistance of the myofibres to damage. This is a quicker parameter to measure (since it is smaller, often only 5-10% in untreated adult *mdx* mice) compared with measuring the remaining bulk of tissue

that contains myofibres with central nuclei (as an indicator of previously necrotic/regenerated tissue). If a treatment/intervention has significantly reduced the amount of necrosis then the area of unaffected myofibres will be greater. As indicated above, in older mice where the dystropathology has stabilised, measurements of active muscle necrosis and regeneration are often not very informative since the background level is usually very low (however, the specific question being addressed needs to be considered for such analyses).

D) Fibrosis and fatty connective tissue: Non-muscle tissue can be pronounced in older *mdx* mice (Fig 1F). Fibrosis and fatty tissue are readily observed in H&E stained sections but fibrosis can be emphasised by routine histochemical stains such as Van Gieson's or Masson's Trichrome, and lipids by specific stains such as Oil red O. Extensive fibrous connective tissue and some calcification are reported in limb muscles of *mdx* mice after about 16 months of age: in contrast the *mdx* diaphragm shows significant fibrosis much earlier and this increases in severity with age. Fibrosis, measured biochemically by hydroxyproline content, can also be quantitated in tissue extracts [see TREAT-NMD SOP: DMD_M.1.2.006].

E) Myofibre size: Muscle size is not widely measured for *mdx* mice although increased muscle size (hypertrophy) is an initial feature of this disease and decreased muscle size (atrophy) is seen. Muscle size can be measured as the cross sectional area of the entire muscle or of the individual myofibres but, while accurate for true transverse sections, values are distorted by myofibres cut obliquely (this is a major problem for clinical biopsies with variable myofibre orientation). This problem is avoided by instead measuring the minimal Feret's diameter of immunohistochemically stained frozen myofibres (See SOP DMD_M.1.2.001).

Interpretation:

The analysis of dystropathology on histological muscle sections is highly interpretive and thus can vary slightly between individuals and laboratories: a standard set of reference images to emphasise the precise features that are measured would help to reduce this variation globally (Fig 1A-F). Scientific analysis that involves any degree of interpretation should be carried out as 'blind' analysis using coded slides, to avoid any bias.

Potential advantages/disadvantages of methodology:

Muscles that are fixed (usually in freshly prepared 4% paraformaldehyde) and processed into paraffin blocks can simply be stored on the shelf indefinitely. Muscle sections from paraffin blocks are ideal for H&E analysis and for other routine histochemical stains, but can be limiting with respect to enzymatic or antibody staining, plus poor fixation can result in shrinkage of the muscle fibres (which is undesirable).

Frozen sections are routinely used as they avoid problems of shrinkage due to fixation (and can thus be used to measure myofibre size), can provide excellent histology and have the major advantage that the same tissue can be readily used for immunohistochemistry (since many antibodies do not work well on paraffin fixed muscle

sections). Disadvantages of frozen tissues are that skill is required to prepare (to avoid ice artefact damaging the muscle sample) and to cut sections, the tissues must be stored at -80°C and they can deteriorate over time.

7. REFERENCES

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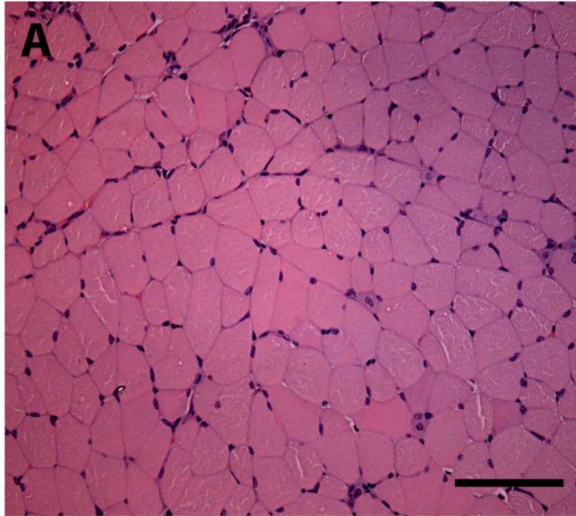
8. APPENDIX

Figure 1. Histological features in transverse sections of various mdx quadriceps muscles stained with Haematoxylin and Eosin.

A) Normal myofibres (undamaged/pre-necrotic), characterised by peripheral nuclei, intact sarcoplasm and regular shape are mainly visible. A few very small basophilic myofibres (myotubes) with a central nucleus are also present.
B) Necrotic myofibres (inflammation) characterised by many inflammatory cells which have infiltrated dystrophic myofibres (sarcoplasm is barely visible).
C) Necrotic myofibres (degeneration) characterised by fragmented sarcoplasm of dystrophic myofibres with irregular shape and few myonuclei; inflammatory cells are not conspicuous.
D) Recent regeneration, shown by many small dystrophic myofibres (sometimes seen as smaller myotubes) with central nuclei: most of these are basophilic (stain slightly purple) due to large amounts of RNA in these actively differentiating and growing cells.
E) Regenerated myofibres, indicated by large plump mature dystrophic myofibres with central nuclei.
F) Fat deposition in dystrophic muscle, between myofibres in the interstitial space.

Pictures A - C were all taken from a 22 day old male *mdx* mouse. Picture D was taken from a 28 day old male *mdx* mouse. Pictures E & F were taken from a 12 week old treadmill exercised male *mdx* mouse. Scale bar represents 100µm.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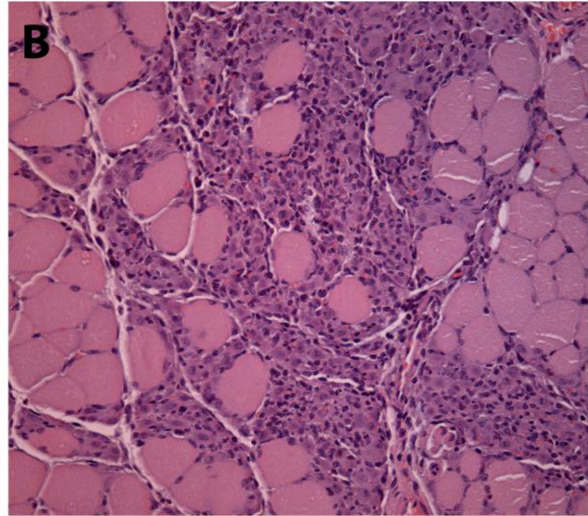
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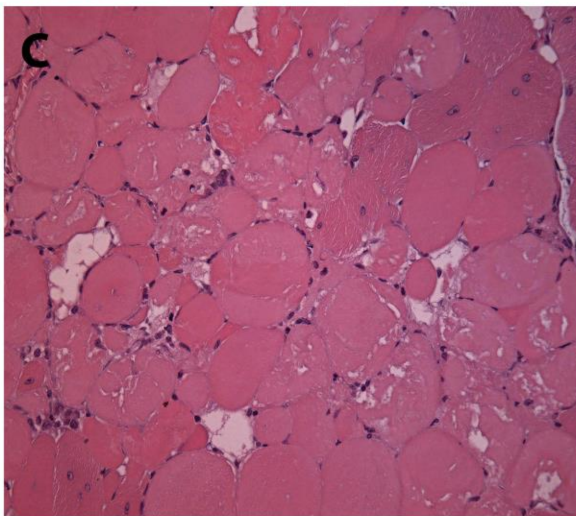
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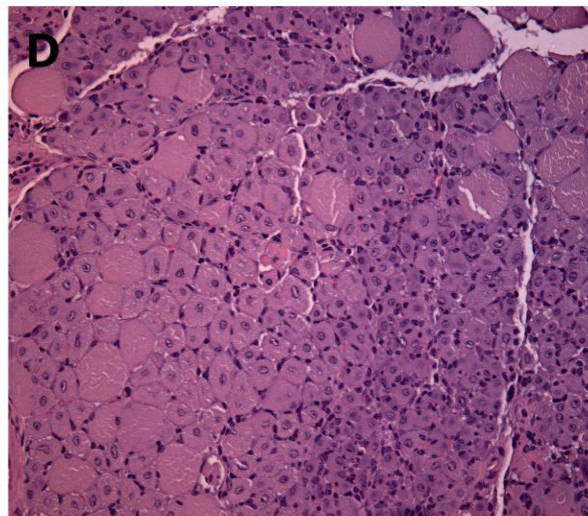
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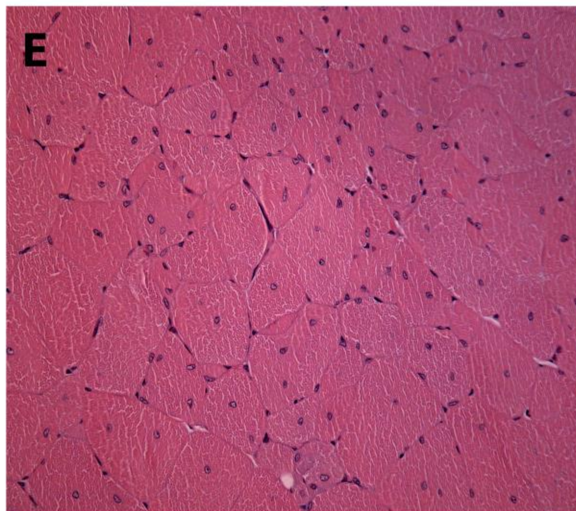
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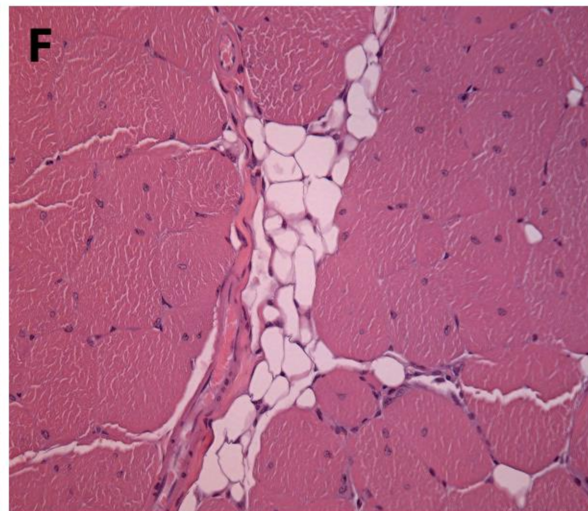
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Table 1. Myofibre necrosis in male *mdx* mice. Values shown are average percentage (%) of whole cross sectional area $^{+}/_{-}$ s.e.m. Myofibre necrosis is identified as the presence of infiltrating inflammatory cells (Fig 1B) (basophilic staining) and/or hypercontracted myofibres and degenerating myofibres with fragmented sarcoplasm (Fig 1C).

Age/Muscle	Quadriceps	Gastrocnemius	Tibialis Anterior	Triceps	Diaphragm	Reference
3 months (12 wks)	6.12% ($^{+}/_{-}$ 0.6)	2.5% ($^{+}/_{-}$ 0.4)	6.89% ($^{+}/_{-}$ 1.4)	8.5% ($^{+}/_{-}$ 1.4)	2.32% ($^{+}/_{-}$ 0.2)	Radley-Crabb 2010 Submitted
6 months (24 wks)	3.32% ($^{+}/_{-}$ 0.4)	N/A (Not available)	N/A	N/A	N/A	Radley-Crabb unpublished
10 months (40 wks)	2.23% ($^{+}/_{-}$ 1.3)	N/A	N/A	N/A	N/A	Radley-Crabb unpublished

Table 2. Cumulative muscle damage in young mdx litters. Values shown are average percentage (%) of whole cross sectional area $^{+}/_{-}$ s.e.m. Cumulative skeletal muscle damage in young *mdx* mice consists of active myofibre necrosis plus the areas of subsequent regeneration (new myofibres).

Age/Muscle	Tibialis Anterior			Reference
	Necrosis	Regeneration	Cumulative damage	
24 days	5.11 ($^{+}/_{-}$ 1.45)	28.03 ($^{+}/_{-}$ 7.7)	33.16 ($^{+}/_{-}$ 6.7)	Radley et al 2008 Neuromus Dis 18; 227-238
28 days	4.56 ($^{+}/_{-}$ 1.1)	36.25 ($^{+}/_{-}$ 9.7)	40.84 ($^{+}/_{-}$ 9.4)	Radley et al 2008 Neuromus Dis 18; 227-238

Figure 2. Quantification of histopathology between different areas within a muscle in 16 week old *mdx* males. In 4 different skeletal muscles and the diaphragm, the percentage of damage (necrosis and fibrosis) was determined at five locations (A-E) from tendon to tendon. No significant difference was found between different areas within one muscle. Significant differences in histopathological severity were found between different muscles; the tibialis anterior and the diaphragm were the least and most severely affected muscles respectively. Based on (van Putten et al. 2010).