Therapeutic interventions for Duchenne Muscular Dystrophy
– studies in the mdx mouse.

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Abstract

Duchenne Muscular Dystrophy (DMD) is a lethal X-linked muscle wasting disease resulting from defects in the myofibre subsarcolemmal protein dystrophin. The lack of functional dystrophin protein leads to myofibre membrane fragility, repeated cycles of myofibre necrosis and regeneration, and the eventual replacement of skeletal muscle by fatty and fibrous connective tissue. DMD is characterised by progressive muscle weakness and wasting with a limited life expectancy of approximately 20 years in humans. Myofibre necrosis is the fundamental destructive process in DMD although the exact mechanism by which the absence of dystrophin leads to myofibre necrosis is unknown; inflammation, oxidative stress, metabolic abnormality, mislocalisation of nNOS and increased intracellular calcium are all heavily implicated in the process. The mdx mouse is a very useful animal model for DMD to examine the potential mechanisms by which the absence of dystrophin leads to myofibre necrosis and to test potential therapeutic interventions to reduce the extent of dystropathology in vivo. While cell or gene therapy to replace the defective dystrophin is the definitive treatment option for DMD, there are many problems to overcome before clinical application. Corticosteroids remain the standard pharmacological treatment to maintain muscle mass and help prolong life, despite severe adverse side-effects including obesity, increased incidence of bone fractures, behavioural changes and cataracts.

The overall aim of this Thesis was to examine, using the mdx mouse, the potential benefits of 3 therapeutic interventions in vivo: 1) an anti-inflammatory drug, cV1q a mouse specific tumour necrosis factor (TNF) antibody, 2) an antioxidant drug, N-acetylcysteine (NAC) a scavenger of reactive oxygen species (ROS), and 3) dietary interventions, specifically a high fat (16%) and high protein (50%) diet. These interventions were tested in both sedentary and exercised dystrophic mdx mice of various ages, and assessed both histologically and biochemically using numerous techniques. All 3 therapeutic interventions showed some benefits in dystrophic mdx mice, confirming the involvement of inflammation, oxidative stress and metabolic abnormality in myofibre necrosis in dystrophic muscle.
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Comprehensive analyses of metabolism, including protein synthesis rate, energy expenditure and body composition, were also conducted using both young and adult mice to test the hypothesis that dystrophic mdx have an altered metabolism compared with control C57Bl/10 mice. Adult mdx mice show striking differences in response to a high fat diet (16%) in comparison to control C57Bl/10 mice, they are also very lean, with significantly increased muscle mass and increased protein synthesis rates. In addition dystrophic mdx mice exhibit continuous cycles of myofibre necrosis and regeneration and significant myofibre hypertrophy up to 24 weeks of age. These are all very energy expensive procedures and may explain why mdx mice (with the same energy intake compared to C57Bl/10 mice) do not exhibit the same negative side-effects of a high fat diet. However, at this stage, it is still unclear if the metabolic differences between strains are an innate feature of dystrophic muscle or a result of the continuous cycles of myofibre necrosis and regeneration, and the associated high energy demands of growing myofibres in dystrophic muscle.

Also contained in this Thesis is a standardised 30 minute treadmill protocol and time-course analysis of molecular and cellular changes after a single 30 minute treadmill exercise session. It was concluded that a single 30 minute treadmill session is a sufficient and conveniently fast screening test to evaluate some of the benefits of pre-clinical drugs in vivo, which has major applications to speed up pre-clinical drug screening in mdx mice.

A high level of variation in the extent of dystropathy in mdx mice is identified (and addressed) throughout this Thesis. This emphasises the need for establishing Standard Operating Procedures to enable comparison of data between laboratory groups worldwide when designing and conducting pre-clinical in vivo therapeutic trials in mdx mice.

These novel observations are of considerable interest to the field of preclinical DMD research because they demonstrate, for the first time, the potential in vivo benefits of 3 new therapeutic interventions, the major metabolic differences between dystrophic mdx and control C57Bl/10 mice, and the possible use of a 30 minute treadmill exercise session as a fast and reproducible in vivo screening test.
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Thank you.
Explanatory Note.

The regulations of The University of Western Australia allow candidates for the Doctor of Philosophy degree (PhD) to present their work as a series of manuscripts rather than as a conventional full text Thesis. These may be review manuscripts, or papers that have been published, submitted but not accepted, papers in preparation, or any combination of these. However, these papers must be related to the same central theme and be integrated by a general introduction and discussion.

This Thesis is comprised of 7 published papers, 2 submitted for publication and 2 papers in preparation for submission (see pages v-vi for a full list of publications). This series of papers is brought together in the Thesis by an Abstract of the Thesis, an Introduction which contains unpublished material and 3 published review papers (Publications #1-3), and the general Aims and Hypotheses of the Thesis. These 3 components form the introductory part of the Thesis (Chapter 1). Methodology is described in General material and methods (Chapter 2) and also within the methods section specific to each manuscript. A published methods manuscript (Publication #4) is also included. Thereafter the Thesis contains 5 results chapters (Chapters 3-7) each consisting of one research paper (Publications #5-9) that addresses the aims outlined in the introductory part, plus an additional Results chapter (Chapter 8) from other collaborative studies conducted during candidature (Publications #10 & 11). Finally a General Discussion integrates the body of knowledge derived from the Thesis and establishes the significance of the work.

For the 6 published manuscripts the re-print is included with the original published page numbering, figures and tables. The remaining manuscripts (submitted and/or in preparation) are formatted specifically for the intended journal, each manuscript has both an individual page numbering system and referencing system with references listed at the end of each manuscript. All other parts of the Thesis have a common page numbering system (bottom right-hand-side of the page) and a common referencing system with all references listed at the end of the Thesis (Chapter 10).
Hannah Crabb - Publications included in this PhD Thesis.

Publications are listed in order of appearance in the Thesis. The journal impact factor and citation count of each publication is also listed. On publications, my name has changed from Radley to Radley-Crabb (to reflect my married status).

General Introduction:


Methods:


Results:

5) Radley HG, Davies MD, Grounds MD. (2008). Reduced muscle necrosis and long-term benefits in dystrophic mdx mice after cV1q (blockade of TNF) treatment. Neuromuscular Disorders. 18: 227-238. (IF 2.67) (12)


8) **Radley-Crabb HG** and Grounds MD. The different impacts of a high fat diet on dystrophic mdx and control C57Bl/10 mice. *In preparation.*


Declaration

I hereby declare that the work contained within this Thesis is my own. Published work and/or work in preparation for publication which has been co-authored is clearly labelled and my individual contribution to the co-authored work is clearly stated.

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Hannah Crabb

September 2010


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Publication 7: Terrill J, Radley-Crabb HG, Shavlakadze T, Arthur PG, Grounds

**Chapter 6** – The different impacts of a high fat diet on dystrophic mdx and control C57Bl/10 mice.

**Publication 8:** Radley-Crabb HG, Grounds MD. The different impacts of a high fat diet on dystrophic mdx and control C57Bl/10 mice. In preparation.

**Chapter 7** – A comparison of metabolism and protein synthesis rates in young an adult dystrophic mdx and control C57Bl/10 mice.


**Chapter 8** – Additional work completed during candidature; not central to the Thesis.

**Publication 10:** Piers AT, Lavin T, Radley-Crabb HG, Bakker AT, Grounds MD, Pinniger G. Blockade of TNF (using cV1q antibody) in vivo reduces contractile dysfunction of skeletal muscle in response to eccentric exercise in dystrophic mdx and normal mice. Neuromuscular Disorders – Nov 3 [Epub ahead of print].


**Chapter 9** – General Discussion

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**Appendix 1** – Additional dietary intervention studies.
Introduction:

Overview: The following is a comprehensive review of background literature pertinent to the present study. The Introduction contains unpublished material and 3 published review manuscripts. Details of each manuscript, the impact factor of the journal and citation count (as of September 2010) along with the candidate’s individual contribution to each manuscript are listed below. Re-prints of the 3 review manuscripts are included at the end of the Introduction (Page 41-74).

Candidate completed approximately 85% of the work required for the manuscript.

Candidate completed approximately 35% of the work required for the manuscript.

Candidate completed approximately 40% of the work required for the manuscript.
1.1 Skeletal muscle structure and function:

Skeletal muscle is the most abundant tissue in the human body comprising approximately 40-50% of body mass; it is a highly specialised contractile tissue under voluntary nervous control. The primary function of skeletal musculature is movement; in addition to this muscles play a vital role in breathing, posture, heat regulation, metabolism and reflexes. There are over 600 individual skeletal muscles in the human body, individual skeletal muscles vary considerably in size, shape, and myofibre arrangement; ranging from the extremely tiny stapedium of the middle ear, to very large muscle masses such as the quadriceps of the thigh [Reviewed in (MacIntosh et al, 2006; Saladin, 2010)].

Despite extreme variation every skeletal muscle consists of both skeletal muscle cells and connective tissue, and requires both a nerve and blood supply. Individual skeletal muscles are enclosed by a tough connective tissue layer called the epimysium which acts to give each muscle its shape. Bundles (fascicles) of skeletal muscle myofibres are surrounded by perimysium, large blood vessels and nerves pass through perimysium. Inside the fascicles, individual myofibres are each enclosed by a layer of endomysium (Figure 1.1.1). The endomysium, approximately 60-120nm in diameter, is a dense network of extracellular proteins. Myofibres are long multi-nucleated muscle cells; the cytoplasm of a myofibre is referred to as sarcoplasm and the plasma membrane as sarcolemma. Located between the myofibre sarcolemma and the endomysium is the specialised basement membrane rich in laminins and other proteins which play many roles in signalling and scaffolding (Grounds et al, 2005c). Muscle satellite cells (undifferentiated mononuclear myogenic cells), are closely associated with the surface of the myofibres, and represent a reserve of muscle precursor cells to facilitate growth and muscle regeneration. Myofibres are peripherally nucleated and contain transverse tubules, sarcoplasmic reticulum, mitochondria and myofibrils. Myofibrils are specialised arrangements of contractile proteins organised into sarcomeres that are responsible for myofibre contraction (Figure 1.1.2) [Reviewed in (Charge and Rudnicki, 2004; Grounds et al, 2005c; MacIntosh et al, 2006; Saladin, 2010)].
Figure 1.1.1. Schematic gross structure of a skeletal muscle. Skeletal muscle is arranged in bundles. The bundles are encased within three sheaths of connective tissue; epimysium covers the whole muscle, perimysium covers the bundles of myofibres and the endomysium covers each individual myofibre. Figure scanned from Saladin (2010) Anatomy and Physiology 5th Edition. Figure 10.1 ‘Connective tissue of a Muscle’. Page 321.
Skeletal muscle is often referred to as striated muscle as the myofibres appear transversely striped or ‘striated’ when viewed longitudinally. This is due to the protein arrangement within the myofibrils. Thick protein filaments (myosin) produce a dark A band and thin protein filaments (actin, troponin and tropomyosin) produce a light I band (Figure 1.1.2). The entire array of thick and thin filaments between the Z lines is called a sarcomere. Shortening of the sarcomeres within a myofibril produces shortening of the myofibril, shortening of the myofibre and ultimately contraction of the skeletal muscle [Reviewed in (MacIntosh et al, 2006; Saladin, 2010)].
1.1.1 Plasticity of skeletal muscle (hypertrophy, atrophy and regeneration):

The plasticity of adult skeletal muscle is outstanding, muscles are able to respond and adapt to a wide variety of external stimuli including exercise, nutrition and temperature, however skeletal muscles are also susceptible to injury after direct trauma (e.g intense physical exercise, burns or laceration) or indirect causes such as disease or genetic defects. The maintenance of adult skeletal musculature is facilitated by the fine balance between protein synthesis and degradation and, the remarkable ability of skeletal muscle to regenerate [Reviewed in (Charge and Rudnicki, 2004; Karagounis and Hawley, 2010; Saini et al, 2009; Zierath and Hawley, 2004)].

Skeletal muscle hypertrophy

Skeletal muscle hypertrophy is an increase in the size and diameter (CSA - cross sectional area) of individual myofibres. All postnatal muscle growth, including hypertrophy, depends on the activation, proliferation and subsequent fusion of satellite cells with existing myofibres. Muscle hypertrophy also requires protein synthesis to exceed protein degradation. In adult skeletal muscle hypertrophy occurs in response to resistance exercise, increased physiological loading or enhanced nutritional uptake in vivo. Skeletal muscle adapts by increasing the size and amount of contractile proteins within the sarcomeres of each myofibre, however due to the fusion of satellite cells into hypertrophying myofibres the protein to DNA ratio of the myofibre stays the same. This increase in contractile proteins leads to an increase in the CSA of myofibres and associated force production, and also an alteration in protein synthesis and degradation rates. Numerous factors and signals can also trigger muscle hypertrophy, for example it is well known that Insulin-like growth factor-1 (IGF-1), hormones and anabolic steroids have a crucial role in inducing skeletal muscle hypertrophy in different situations [Reviewed in (Karagounis and Hawley, 2010; Saini et al, 2009; Shavlakadze et al, 2010; Shavlakadze and Grounds, 2006)].

Skeletal muscle atrophy

When protein degradation outweighs protein synthesis skeletal muscles shrink; atrophy is the reduction in CSA of individual myofibres, accompanied by a significant
decrease in myofibrillar protein content, decreased force production, and lower fatigue resistance. The biochemical and enzymatic responses to atrophy include the reduced capacity to synthesize new protein and the up-regulation of pathways leading to increased protein breakdown. Many factors (with different aetiologies) can lead to muscle atrophy including aging, disuse (immobilisation), denervation, inflammatory conditions associated with cancer (cachexia), burns and anorexia/starvation [Reviewed in (Lynch et al, 2007; Saini et al, 2009; Shavlakadze and Grounds, 2003; Shavlakadze and Grounds, 2006)].

**Skeletal muscle necrosis and regeneration**

Under normal conditions adult skeletal muscle is a stable tissue; however, when damage to skeletal myofibres results in necrosis this initiates regeneration and the formation of new muscle. In brief, the process of myofibre necrosis and regeneration is as follows: 1) injury/damage to the sarcolemmal leading to myofibre necrosis, 2) re-sealing of the damaged ends of the myofibre, 3) infiltration of inflammatory cells and phagocytosis of myofibre debris, 4) hyper-contraction of myofibrils, 5) revascularisation of injured myofibres, 6) activation and proliferation of muscle satellite cells, 7) fusion of myoblasts into myotubes and fusion of these with damaged myofibres (Figure 1.1.3), 8) re-innervation, growth and maturation of regenerated myofibres [Reviewed in (Charge and Rudnicki, 2004; Grounds, 1991)].

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**Figure 1.1.3. Regeneration of a damaged skeletal myofibre.** Damage to skeletal muscle initiates a reaction in which satellite cells are activated and become proliferating myoblasts. Myoblasts migrate (if necessary) to the damaged site where they fuse into myotubes, which then fuse with the existing end of damaged myofibres.
The term ‘myofibre necrosis’ refers to the irreversible breakdown of the sarcolemma and all sarcoplasmic structures, necrosis can occur through the whole length of a myofibre or only part of the myofibre can degenerate. Partial degeneration of myofibres (focal necrosis) is common in Duchenne Muscular Dystrophy (DMD). There are numerous causes of myofibre necrosis: disease (polymyositis, muscular dystrophies), eccentric exercise, ischemia, direct trauma (crush, cut, tear), transplantation (grafting), thermal injury or biological toxins (venom, streptococcus A) [Reviewed in (Engel and Franzini-Armstrong, 1994; MacIntosh et al, 2006; Wallace and McNally, 2009)].

A transient inflammatory response is the body’s natural reaction to acute tissue injury resulting from mechanical, physical or chemical damage. In skeletal muscle, the inflammatory response classically consists of early vascular changes producing oedema, the activation of resident leukocytes (mast cells and macrophages) and infiltration of additional leukocytes e.g. neutrophils (within the hour after muscle damage), release of pro-inflammatory cytokines and assistance with tissue phagocytosis and myofibre regeneration [Reviewed in (MacIntosh et al, 2006; Saini et al, 2009; Tidball, 1995)]. It is widely documented that excess (or dysregulated) inflammation may exacerbate muscle damage in both Duchenne Muscular Dystrophy (DMD) patients and the mdx mouse model of DMD; thus much pre-clinical research is conducted into anti-inflammatory drugs as potential therapeutic interventions for DMD, while waiting for gene/cell therapies to correct the fundamental gene defect (Evans et al, 2009a; Radley et al, 2008; Radley et al, 2007).

(For more detailed information on ‘The role of inflammation in Duchenne Muscular Dystrophy’, see sections 1.4 of this Introduction).

To minimise necrosis after injury, the damaged portion of the myofibre must be rapidly sealed off from the nearby undamaged sarcoplasm. Within 3 hours after damage, hyper-contraction of sarcomeric structures separates the undamaged viable parts of myofibre from the necrotic area. By 9-12 hours the necrotic tissue section is totally sealed off from the remaining viable parts of the damaged myofibre by formation of new plasmalemma (Grounds, 1991; Papadimitriou et al, 1990).
Myofibre necrosis can occur in the presence of a blood supply (e.g eccentric exercise) or in conditions of ischemia. Ischemic myofibre necrosis and regeneration occurs in transplanted whole or minced muscle grafts due to the loss of blood supply. In transplants (grafts) of whole muscles, some myofibres at the periphery do not degenerate due to diffusion of nutrients and gases from vessels in adjacent tissues (skin & muscle), new muscle formation in the centre of the graft is normally excellent suggesting a strong resistance to ischemia (Grounds, 1991; Grounds et al, 2005a; Radley et al, 2008; White et al, 2000). However, in very large grafts the central zone fails to re-vascularise, does not regenerate successfully and becomes fibrous, this may be due to prolonged ischemia which can favour the proliferation of fibroblasts. Revascularisation is an important event for ensuring successful new muscle formation [Reviewed in (Grounds, 1991; MacIntosh et al, 2006)].

Tissue resident adult stem cells play a crucial role in the maintenance of many body tissues and have been identified in bone marrow, brain, liver, intestines, heart and skeletal muscle [Reviewed in (Boonen and Post, 2008)]. Satellite cells, the resident stem cells of adult skeletal muscle, play a pivotal role in both postnatal growth and regeneration of skeletal muscle. Skeletal myofibres are terminally differentiated cells and cannot re-enter the mitotic cell cycle. Satellite cells are normally quiescent, however damage to skeletal muscle results in activation of satellite cells (then called myoblasts), they proliferate and migrate (if necessary) to the damaged site where they differentiate and fuse with existing or damaged myofibres (Figure 1.1.3) [Reviewed in (Boonen and Post, 2008; Buckingham and Montarras, 2008; Charge and Rudnicki, 2004; Grounds, 1991)].

The early events of muscle regeneration involving inflammation, re-vascularisation and activation and fusion of satellite cells into new myotubes, does not require innervation. However, reinnervation is a key factor in the restoration of muscle function and successful formation of synapses and motor endplates which connect muscles to the appropriate motorneurons completes the maturation of the newly regenerated muscle [Reviewed in (Grounds, 1991)].
When sarcolemmal damage is repaired and patched locally the myofibre avoids the response of necrosis (and subsequent regeneration is not required) (Bansal et al, 2003; Doherty and McNally, 2003). The process of skeletal muscle necrosis and regeneration is central to the lethal muscle disease DMD, which is the focus of this Thesis.

(For more detailed information on ‘Duchenne Muscular Dystrophy’, see section 1.2 of this Introduction).

1.1.2 Skeletal muscle and disease:

There are numerous disorders and diseases that can heavily affect skeletal muscle. The most common disorder is sarcopenia; the term used to describe the loss of skeletal muscle mass and strength that inevitably occurs with aging (Evans, 2004; Saini et al, 2009; Shavlakadze and Grounds, 2003). Neuromuscular diseases such as Parkinson’s or Multiple Sclerosis affect both skeletal muscle and/or their nervous control. Examples of other disease that affect muscle are the congenital myopathies (Central core disease, Nemaline myopathy and Minicore myopathy), mitochondrial myopathies, metabolic disorders (Pompe disease, McArdle disease and glycogen storage disease), inflammatory myopathies (dermatomyositis, polymyositis and inclusion body mitosis), and the muscular dystrophies (Duchenne, Becker, Emery-Dreifuss, limb-girdle and facioscapulohumeral) [Reviewed in (Hilton-Jones and Kissell, 2010)].

(For more detailed information of Duchenne Muscular Dystrophy, see section 1.2 of this Introduction).

1.2 Duchenne Muscular Dystrophy:

The muscular dystrophies are a set of inheritable muscle disorders, characterised by progressive muscle weakness and wasting, and elevated blood serum creatine kinase (CK) level. Based on the distribution of predominant muscle weakness seven major forms of dystrophy can be defined; Duchenne muscular dystrophy, Becker muscular dystrophy, Congenital muscular dystrophy, Emery-Dreifuss muscular dystrophy, Distal muscular dystrophy, Facioscapulohumeral muscular dystrophy, Occulopharyngeal muscular dystrophy and Limb-Girdle muscular dystrophy. Duchenne muscular dystrophy (DMD) is one of the most severe and the most common, affecting approximately 1/3500-6000 male births [Reviewed in (Bushby et al, 2010a; Emery, 2002; Manzur and Muntoni, 2009; Sinnreich, 2010)].
DMD is caused by a mutation in the X-linked gene that encodes for the sarcolemmal protein dystrophin, complete absence or impaired function of the dystrophin protein results in DMD. The dystrophin gene the largest in the human genome known to date, a feature which results in a very high spontaneous mutation rate. The gene is approximately 3 million base pairs in length, containing 79 exons; the full-length dystrophin protein is 427kDa in molecular mass [Reviewed in (Blake et al, 2002; Le Rumeur et al, 2010; Sinnreich, 2010)]. The gene is located on the short arm of the X chromosome (Xp21) thus the majority of DMD patients are male, however up to 10% of female carriers also manifest clinical symptoms due to the relative proportion of X-chromosomes that are inactivated (Matthews et al, 1995; Wenger et al, 1992). The majority of DMD patients inherit their dystrophin gene mutation from a carrier mother, however; approximately 30% of DMD cases occur in a child with no positive family history as the result of a de novo mutation (Manzur and Muntoni, 2009). Germ line mosaicism also complicates the rate of de novo mutations, with an 8.6% recurrence rate of a de novo mutation in the same healthy mother (Helderman-van den Enden et al, 2009).

Approximately 70% of DMD patients have a gene mutation located in the mutation ‘hot-spot’ in the central genomic region (exons 45–53) of the dystrophin gene [Reviewed in (Walmsley et al, 2010)]. The dystrophin deficient phenotype varies from the severe Duchenne (DMD) phenotype to the milder allelic form known as Becker Muscular Dystrophy (BMD). Mutations that result in the premature truncation of dystrophin typically result in the severe Duchenne phenotype because no functional dystrophin is produced; patients with the milder Becker phenotype have partial deficiencies of dystrophin that still enable translation of a semi-functional dystrophin protein [Reviewed in (Bushby et al, 2010a; Hoffman, 2001; Sinnreich, 2010)]. Most dystrophin gene mutations involve deletions or duplications of whole exons; less often, small point mutations are seen (Deburgrave et al, 2007).

Patients with DMD begin to show clinical symptoms (Gower’s manoeuvre and hypertrophied calf muscles) around 3 years of age. The absence of dystrophin in DMD patients is confirmed by immunohistochemical staining of a muscle biopsy, genetic
testing is also performed to determine the exact gene mutation(s). In young boys, walking is delayed and awkward and as the disease advances patients undergo a progressive loss of muscle mass and function; most boys lose the ability to ambulate and are wheelchair bound by 12 years of age. Loss of respiratory function usually begins early in the second decade and progresses to respiratory failure in the mid to late teens (Bushby et al, 2010a; Emery, 2002; Sinnreich, 2010). Many patients affected with the disease also have cognitive impairment (Giliberto et al, 2004; Polakoff et al, 1998). Without intervention the average survival age for an individual with DMD is approximately 19 years, with death occurring due to the overwhelming loss of muscle mass, respiratory and/or cardiac complications. However, corticosteroid, cardiac, respiratory and orthopaedic interventions have led to major improvements in quality of life, health and longevity; and children who are diagnosed with DMD today, have a potential life expectancy of up to 40 years [Reviewed in (Biggar, 2006; Bushby et al, 2010a; Toussaint et al, 2007)].

1.2.1 Current & potential therapies for Duchenne Muscular Dystrophy:

There is currently no cure or definitive treatment for DMD and the disease is fatal. The existing treatment for DMD patients is multidisciplinary including psychosocial management, palliative care and pharmacological treatment with corticosteroids (Bushby et al, 2010a; Bushby et al, 2010b). Corticosteroids offer the only available method for preserving some muscle function; however their exact mechanism of action in dystrophic muscle remains unknown and they are not effective in all patients. Two corticosteroids, prednisone and deflazacort have been used extensively in DMD patients and they appear to be equally effective in preserving skeletal muscle function. Unfortunately both drugs are associated with adverse side-effects although these, particularly weight gain, are less severe with deflazacort (Biggar et al, 2006; Campbell and Jacob, 2003). Recent critical review of corticosteroid use in DMD patients conclude that the use of steroids is limited due to detrimental side-effects but they are the best pharmacological treatment option currently available (Angelini, 2007; Manzur et al, 2008). Cell or gene therapy to replace the defective dystrophin is the ideal scenario and there have been many promising breakthroughs in the last 10 years, however interpretation of efficacy is sometimes complicated by the presence of dystrophin
positive revertant myofibres (Arechavala-Gomeza et al, 2010) and there are many problems to overcome before clinical application of these new potential interventions can become wide-spread (Bushby et al, 2009; Cossu and Sampaiolesi, 2007; Grounds and Davies, 2007; Guglieri and Bushby, 2010; Manzur and Muntoni, 2009; Nagaraju and Willmann, 2009; Odom et al, 2007; Partridge, 2010; Tremblay and Skuk, 2008; Wells, 2008).

Therapeutic approaches for DMD fall into three main strategies: (i) replacement of dystrophin by cellular, genetic or molecular interventions; (ii) enhancement of muscle regeneration and (iii) reduced muscle necrosis [Reviewed in (Bushby et al, 2009; Manzur and Muntoni, 2009; Radley et al, 2007; Tidball and Wehling-Henricks, 2004a)]. The latter approach is the main focus of this thesis which examines pharmacological treatment and dietary interventions as potential therapies to reduce muscle necrosis in dystrophic mdx mice.

1.2.2 Dystrophin & the Dystroglycan complex:

Through the use of different promoters, dystrophin is located in skeletal, smooth and cardiac muscle, neurons, Schwann cells and retinal synapses [Reviewed in (Blake et al, 2002; Hoffman, 2001; Sinnreich, 2010); however, skeletal muscle is by far the most severely affected in DMD and is the focus of this thesis. In normal skeletal muscle, dystrophin is located beneath the myofibre sarcolemma and forms part of the mechanical link between the internal cytoskeleton and the external extracellular matrix (ECM) of a myofibre. Dystrophin is part of the dystroglycan complex (DGC) which spans through the sarcolemma (Figure 1.2.1). Dystrophin links proteins within the cell sarcolemma to the cell cytoskeleton and plays a vital role in membrane stability and muscle strength (Grounds et al, 2005c; Lapidos et al, 2004; Roberts, 2001). The DGC also plays a vital role in cell signalling (Rando, 2001; Wehling et al, 2001). Dystrophin binds to cytoplasmic f–actin and, within the DGC, to sarcolemmal β-dystroglycan (βDAG), that binds to extracellular α-dystroglycan, that binds to laminin α2 in the basement membrane, ultimately joining the myofibre sarcolemma to the ECM (Grounds et al, 2005c).
Figure 1.2.1. The dystroglycan complex. Dystrophin (shown in red) is absent in DMD leaving dystrophic myofibre membranes fragile and susceptible to contraction induced damage. Adapted from Roberts et al (2001) (Roberts, 2001).

1.2.3 The absence of dystrophin:

It is well established that impaired function or absence of the skeletal muscle protein dystrophin, and numerous other dystrophin-associated glycoproteins, render dystrophic myofibres susceptible to sarcolemma damage during mechanical contraction (Gailly, 2002; Ohlendieck and Campbell, 1991; Petrof et al, 1993; Reed and Bloch, 2005; Watchko et al, 2002). Dystrophic myofibres are particularly vulnerable to damage after eccentric lengthening contractions (Brussee et al, 1997; Tegeler et al, 2010; Vilquin et al, 1998). The absence of functional dystrophin protein leads to myofibre membrane fragility, and myofibre necrosis. While regeneration is initially effective, repeat cycles of myofibre necrosis and subsequent regeneration (associated with inflammation and accumulating fibrosis) eventually leads to the replacement of myofibres by fatty and/or fibrotic connective tissue. The specific mechanism(s) leading to myofibre necrosis are still unclear, yet there are strong associations with excessive inflammation, increased intracellular calcium levels, elevated oxidative stress and metabolic abnormality (Davidson and Truby, 2009; Evans et al, 2009a; Even et al, 1994; Kuznetsov et al, 1998; Radley et al, 2008; Tidball and Wehling-Henricks, 2007; Whitehead et al, 2008).

(For more detailed information on Skeletal Muscle Necrosis, see section 1.4 of this Introduction).
The absence of dystrophin protein in the DGC also causes the incorrect anchoring of neuronal nitric oxide synthase (nNOS) at the myofibre sarcolemma. Nitric oxide is produced after increased muscle activity and plays an important role in regulating vasodilation and oxygenation. Disruptions in nNOS expression and signalling negatively impacts dystropathology [Reviewed in (Le Rumeur et al, 2010)]. Deregulation of nNOS may also contribute to high levels of reactive oxygen species (ROS) in dystrophic muscle (Grisotto et al, 2000; Thomas et al, 1998; Wehling et al, 2001).
(For more detailed information on The Role of ROS in Skeletal Muscle Necrosis, see section 1.4 of this Introduction).

1.2.4 Animal models for Duchenne Muscular Dystrophy:

Several animal research models for DMD have been identified and provide a wealth of information on the pathophysiology of dystrophin deficiency. Each animal model differs from both DMD patients and other animal models [Reviewed in (Allamand and Campbell, 2000; Banks and Chamberlain, 2008; Collins and Morgan, 2003; Grounds et al, 2008b; Partridge, 1997; Vainzof et al, 2008; Willmann et al, 2009)]. While dystrophin deficient worms (Caenorhabditis elegans) and zebra fish (Danio rerio) have been identified and described [Reviewed in (Collins and Morgan, 2003)], only the mammalian models are discussed here. The most widely used animal model is the mdx mouse (C57BL/10ScSnmdx/mdx) which was discovered during a screening for mutant glycolysis enzymes and occurred as a spontaneous mutation on the C57BL/10ScSn background (Bulfield et al, 1984). The mdx mouse (mdx/mdx homozygous females and mdx/Y heterozygous males) has total absence of the dystrophin protein and elevated serum CK levels. Mdx mice show some progressive disease symptoms, although they are not severe until later life [Reviewed in (Grounds et al, 2008b)].
(For more detailed information of the mdx mouse please see section 1.3 of this Introduction).

At least 2 dystrophin deficient cat models of muscular dystrophy (HFMD – Hypertrophic Feline Muscular Dystrophy) have been identified. Both show cycles of skeletal muscle necrosis and regeneration, marked myofibre hypertrophy and severe cardiac symptoms. Lingual and/or diaphragmatic muscle hypertrophy becomes so
severe that dystrophic cats succumb to de-hydration and/or starvation; the HFMD cats are not widely used in pre-clinical DMD research [Reviewed in (Carpenter et al, 1989; Collins and Morgan, 2003; Hoffman, 2001; Partridge, 1997)].

A sporadic mutation in golden retriever dogs (GRMD – Golden Retriever Muscular Dystrophy) discovered in 1983 was found to cause muscular dystrophy and follow X-linked recessive inheritance; the causative mutation was found to be a splicing mutation in exon 8 of the dystrophin gene. GRMD dogs most closely resemble DMD patients with a rapid course of muscle weakness, wasting and fibrosis. Severe onset of the disease is around 3 months of age with death occurring anywhere from the neonatal period to adulthood (Hoffman, 2001; Kornegay et al, 1988). GRMD colonies are challenging to work with due to their extremely severe phenotype, large size (25-30kg), high maintenance costs and high variability in muscle condition both between and within individual dogs (Ambrosio et al, 2009; Zucconi et al, 2010).

Recently, smaller dog animal models such as the Beagle, German Short Haired Pointer and Cavalier King Charles Spaniel have been established (Schatzberg et al, 1999; Walmsley et al, 2010). A dystrophic beagle colony, established and phenotyped in Japan (CXMDJ – Canine X-linked Muscular Dystrophy in Japan) has an obvious dystrophic phenotype, with a high neonatal death rate, elevated serum CK levels, diaphragmatic and limb muscle degeneration, and severe dysphagia and macroglossia in ‘old’ animals (Shimatsu et al, 2003; Shimatsu et al, 2005), although their phenotype is less severe and progresses more slowly in comparison to the Golden Retrievers. CXMDJ dogs have recently been the animal model of choice for pre-clinical cell and gene therapy studies due to their severe phenotype yet smaller size and thus greatly reduced pre-clinical research costs.

1.3. The Mdx mouse - overview:

The classic biochemical and genetic mouse model of DMD is the mdx mouse (C57BL/10ScSn<sup>mdx/mdx</sup>), a mutant with an inherited X-linked recessive trait, discovered in 1981 in a C57BL/10ScSn colony at the University of Leicester, UK (Bulfield et al, 1984; Hoffman et al, 1987). Dystrophic mdx mice have been widely used for pre-
clinical DMD research and intensively described in numerous papers (Coulton et al, 1988a; Coulton et al, 1988b; Grounds et al, 2008b; Willmann et al, 2009). The absence of dystrophin is due to a point mutation on the X chromosome, resulting in a premature stop codon in the mRNA of exon 23 of the dystrophin gene (Sicinski et al, 1989). The mdx mouse has no detectable dystrophin protein in skeletal muscles; however, sporadic revertant myofibres can express dystrophin which can complicate the interpretation of gene or cell replacement studies (Hoffman et al, 1990; Yokota et al, 2006).

The dystropathology of mdx mice begins early in embryonic development with the absence of dystrophin resulting in disrupted myogenesis, changes in skeletal and cardiac muscle patterning (Merrick et al, 2009) and disorganisation of Z-discs (Torres and Duchen, 1987). Some muscle necrosis is observed from 5 days of age in muscles of the head and shoulder and occasionally in the limb muscles from 14 days (Torres and Duchen, 1987). It must also be noted that a low level of apoptosis precedes any detectable necrotic changes in mdx muscle, and that this low level of apoptotic events continues in dystrophic muscle through the life cycle of the mdx mouse (Lim, 2004; Tidball et al, 1995). Dystrophic mdx mice experience severe dystropathology in both skeletal (Figure 1.3.1) and cardiac muscle (Grounds et al, 2008b; McNally and MacLeod, 2005; Zhang et al, 2008). Cardiac muscle falls outside the scope of this Thesis and only skeletal muscle is discussed here.

Mdx mice undergo an onset of severe skeletal myofibre necrosis and subsequent regeneration in limb and paraspinal muscles postnatally around 3 weeks of age (Figure 1.3.1) (Coulton et al, 1988b; Grounds et al, 2008b). The high level of muscle necrosis between 21 and 28 days provides an excellent model to study therapeutic interventions designed to prevent or reduce muscle necrosis, as a reduction in dystropathology is easily observed (Grounds and Torrisi, 2004; Radley et al, 2008; Radley and Grounds, 2006; Shavlakadze et al, 2004; Stupka et al, 2006). Skeletal muscle necrosis peaks around 4 weeks (Figure 1.3.2) and then decreases significantly and stabilises by 8-12 weeks of age to a relatively low level of damage where approximately 6% of each skeletal muscle is actively necrotic (McGeachie et al, 1993) (Radley-Crabb et al, submitted). Beyond 12 weeks of age, mdx mice show few clinical
dystrophic symptoms, they exhibit a low level of muscle necrosis and creatine kinase levels reduce dramatically, there is however a high level of cumulative damage/regeneration and a fibrosed diaphragm; the mdx diaphragm is the most severely affected muscle and its pathology most closely resembles that of DMD patients (Dupont-Versteegden and McCarter, 1992; Lynch et al, 1997; Stedman et al, 1991). Beyond 20 months of age, mdx mice exhibit a more severe dystrophic pathology; that is, the extensive replacement of skeletal muscle with fatty and/or fibrous connective tissue, reduced cardiac and respiratory function, reduced motor strength and a slightly shortened lifespan (Chamberlain et al, 2007; Lefaucheur et al, 1995; Willmann et al, 2009). The striking differences in disease severity between mdx mice and DMD patients, may largely reflect vast differences in the growth phase, final body size and life span of the two species [Reviewed in (Grounds, 2008; Grounds et al, 2008b; Willmann et al, 2009)].

Figure 1.3.1 Myofibre necrosis in Haematoxylin and Eosin stained dystrophic mdx muscle. (A) Pre-necrotic muscle in 20 day old mdx quadriiceps characterised by peripheral nuclei and a normal appearance. (B) Necrotic muscle in 23 day old mdx quadriiceps (outlined in yellow), characterised by inflammatory cells and fragmented sarcoplasm. Scale bar represents 100µm.

Some muscles of the head region, including extraocular muscles (Fisher et al, 2005), masseter (Muller et al, 2001) and the laryngeal muscles (Marques et al, 2007; Smythe, 2009), show a very mild dystrophopathy and are relatively spared from myonecrosis. The reasons for the mild dystrophopathy are not clear, although it is noted that these muscles may have an improved ability to regulate calcium homeostasis (Khurana et al, 1995; Marques et al, 2007) and selected mechanical properties that offer resistance to damage (Wiesen et al, 2007).
Figure 1.3.2 The relative level of myofibre necrosis across the first 10 weeks of life in a mdx mouse. There is an acute bout of myofibre necrosis around 3-4 weeks of age, beyond this myofibre necrosis remains at a low level.

The stimulus for the acute onset of myofibre necrosis around 21 days (Figure 1.3.2) is unknown. Studies suggest that it may be the result of an increase in motor activity (movement) at the time of weaning (Mokhtarian et al, 1995), onset is also associated with huge disruptions in intracellular signalling and excessive inflammation [Reviewed in (Evans et al, 2009a)]. Hind limb immobilization in young mdx mice (d14-28) results in reduced myofibre necrosis (and subsequent regeneration), suggesting that myofibre contraction and movement play an important role in the onset of myofibre necrosis (Mokhtarian et al, 1999).

The dystrophic mdx mouse strain (C57Bl/10ScSn\textsuperscript{mdx/mdx}) is an inbred strain, therefore unlike humans, both female (homozygous for the absence of dystrophin) and male mice (heterozygous) are used in pre-clinical research. Both sexes exhibit a similar pattern of myofibre necrosis and regeneration across their lifespan and have consistently elevated serum CK levels. However, specific differences have been documented between sexes – for example male mdx mice have a 2x fold higher blood serum CK level compared to age matched females at 12 weeks of age, yet they have a 4x fold lower serum CK level at 48 weeks of age (Salimena et al, 2004). It is recommended that when conducting pre-clinical research, experimental groups consistent of only one sex (Grounds et al, 2008b). A high level of variation in the extent of dystropathology (determined histologically) between individual mice is also seen in age, sex and muscle matched mdx mice (Radley and Grounds, 2006; Spurney et al, 2009)(Radley-Crabb et al, submitted).
1.3.1 Advantages / Limitations of the mdx mouse:

The suitability of the mdx mouse model for pre-clinical DMD research has been the subject of numerous reviews, conferences and recent discussions (Grounds et al, 2008b; Nagaraju and Willmann, 2009). The advantages and limitations of working with the mdx mouse strain are summarised in Table 1.3.1.

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small mammal – low husbandry costs in comparison to GRMD dogs</td>
<td>Mild phenotype in comparison to DMD patients and GRMD dogs</td>
</tr>
<tr>
<td>Large litters (4-8 pups)</td>
<td>High neonatal mortality rate</td>
</tr>
<tr>
<td>Short gestation period (19 – 21 days)</td>
<td>High variation in dystropathology (within and between both individual mice and whole litters)</td>
</tr>
<tr>
<td>Small mammal – low costs to pharmacologically treat mice in comparison to GRMD dogs</td>
<td>High number of mdx mice are required in pre-clinical studies due to high level of variation in dystropathology</td>
</tr>
<tr>
<td>Peak in dystropathology at 3-4 weeks of age</td>
<td>Mild phenotype in adult mdx mice - adult mdx mice require exercise in pre-clinical studies</td>
</tr>
<tr>
<td>Willingness to exercise</td>
<td>Marked muscle hypertrophy as adults (uncharacteristic of DMD patients and GRMD dogs)</td>
</tr>
</tbody>
</table>

Table 1.3.1 Summary of the main advantages and limitations of using the mdx mouse in pre-clinical DMD research.

1.3.2 The mdx mouse and exercise:

The low level of dystropathology in adult mdx mice can be made significantly worse by exercise that increases myofibre necrosis and reduces muscle strength (Brussee et al, 1997; Granchelli et al, 1996; Okano et al, 2005; Vilquin et al, 1998), thus enabling potential therapeutic interventions to be evaluated in adult mice (Archer et al, 2006; De Luca et al, 2005; Granchelli et al, 2000; Payne et al, 2006; Radley et al, 2008; Radley and Grounds, 2006). Exercise is a less invasive and a more clinically relevant option in comparison to previous methods (irradiation) designed to increase the extent of mdx dystropathology (Wakeford et al, 1991).
Our laboratory has used voluntary wheel exercise over 48 hours to increase myofibre necrosis in adult dystrophic mdx mice and to histologically assess the beneficial effects of various anti-inflammatory drugs on the muscles of adult mdx mice (Hodgetts et al, 2006; Radley et al, 2008; Radley and Grounds, 2006). Muscle necrosis is roughly doubled (increases from ~6 to 12%) in quadriceps muscle after 48 hours of voluntary exercise, although other muscles such as the tibialis anterior (TA) are barely affected (Archer et al, 2006; Radley et al, 2008). Histological analysis of muscle necrosis after 48 hours of voluntary exercise, allows induced necrosis to be readily measured without the ensuing complication of regeneration and new muscle formation, as new myotubes appear only after approximately 3 days after injury (McGeachie et al, 1993).

Voluntary wheel exercise is inexpensive and causes minimal stress to the animal since the mice (which are nocturnal) run voluntarily during the night (Hara et al, 2002). Mdx mice tend to run less total distance (km) and at a lesser speed (km/hr) than non-dystrophic control mice (Brussee et al, 1997; Grounds et al, 2008b; Hayes and Williams, 1996). The running patterns of mdx mice during voluntary exercise have previously been used as a non-invasive method to determine genotype, as mdx mice run a very distinct intermittent (stop-start) running pattern in comparison to a continuous running pattern seen in non-dystrophic mice (Hara et al, 2002; Radley and Grounds, 2006). However, long-term voluntary exercise has shown beneficial effects on muscle strength and fatigue resistance in the mdx mouse (Call et al, 2008; Dupont-Versteegden et al, 1994b; Hayes and Williams, 1996; Landisch et al, 2008) which may complicate the long-term analysis of pre-clinical drug interventions.

A widely used alternative to voluntary exercise is forced treadmill running. This occurs at a controlled speed and for a pre-determined length of time, thus potentially eliminating some of the variables experienced with voluntary exercise. Treadmill running is usually carried-out for convenience during the day, although normally mice are active at night and may benefit from being run at night as daytime exercise may be stressful for them [Reviewed in (Grounds et al, 2008b)]. A protocol of 30 minutes running on a horizontal treadmill at a speed of 12m/min, twice a week for at least 4 weeks, significantly increases the dystropathology of adult mdx mice (Burdi et al, 2009;
De Luca et al, 2005; De Luca et al, 2008; De Luca et al, 2003; Granchelli et al, 2000). The *in vivo* weakness produced by the above protocol is observed exclusively in mdx mice, with no effect in normal control C57Bl/10 mice (De Luca et al, 2003). There are concerns regarding such forced treadmill running because mdx mice can have problems coping and thus be reluctant to run, although a short warm-up period at a slower speed and training of the mdx mice to accustom them to this treadmill exercise appears to help with their running performance (Payne et al, 2006)(Radley-Crabb et al, submitted) in addition, the motivation for exercise may be influenced by various environmental and behavioural factors. The advantages and limitations of both voluntary and treadmill exercise are summarised in Table 1.3.2.

<table>
<thead>
<tr>
<th><strong>Voluntary wheel exercise</strong></th>
<th><strong>Controlled treadmill exercise</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Advantages:</strong></td>
<td></td>
</tr>
<tr>
<td>Mdx mice can run when they choose (usually at night)</td>
<td>Reduce variation between mice - pre-determined speed, distance &amp; protocol</td>
</tr>
<tr>
<td>Minimal supervision required by researcher</td>
<td>Sacrifice at a known time after exercise</td>
</tr>
<tr>
<td>Stop-start running pattern favoured by mdx mice</td>
<td>Up-hill / down-hill running if required</td>
</tr>
<tr>
<td>Non-invasive and non-stressful for the mice.</td>
<td>Cage mdx mice in groups when not running</td>
</tr>
<tr>
<td>Multiple inexpensive cage set-ups</td>
<td></td>
</tr>
<tr>
<td><strong>Limitations:</strong></td>
<td></td>
</tr>
<tr>
<td>Variation in speed, distance &amp; duration between mice</td>
<td>Time consuming for researcher</td>
</tr>
<tr>
<td>Heavily influenced by behaviour</td>
<td>Mdx mice forced to run during inactive phase</td>
</tr>
<tr>
<td>Wheels can ‘block-up’ overnight</td>
<td>Can be stressful for the mouse</td>
</tr>
<tr>
<td>Must cage mdx mice individually</td>
<td>Initially expensive</td>
</tr>
</tbody>
</table>

**Table 1.3.2** Summary of the main advantages and limitations of two different exercise models for the mdx mouse: voluntary wheel exercise and controlled treadmill exercise.

There are numerous mdx treadmill protocols documented in the literature with many differences in speed, duration, regime and surface incline/decline. Horizontal running is most common (Burdi et al, 2009; De Luca et al, 2005; De Luca et al, 2008; De Luca et al, 2003; Granchelli et al, 2000; Grounds et al, 2008b), however some researchers use up-hill (Ahmad et al, 2009; Okano et al, 2005), or down-hill (eccentric) treadmill running (Bizario et al, 2009; Brussee et al, 1997; Vilquin et al, 1998). Eccentric muscle contractions (muscle contraction while muscle is extended) are the most damaging for
dystrophic (and normal) muscle. Experimental lengthening contractions induced via nervous stimulation while the muscle is extended in vivo, avoids behavioural complications and produces reproducible contraction of the entire musculo-tendinous unit, allowing for more experimental control (Hamer et al, 2002; Piers et al, 2010; Ridgley et al, 2008; Weller et al, 1990), although these specialised exercise regimes using a dynanometer require expensive and specialised laboratory equipment (See Chapter 8 of this Thesis).

1.3.3 Development of Standard Operating Procedures:

Recently, the important issue of world-wide standard operating procedures for pre-clinical studies in the mdx mouse has been heavily discussed (Grounds et al, 2008b; Nagaraju and Willmann, 2009). Researchers have begun establishing a set of recommended standard operating procedures and endpoints for consistent and comparable assessment of pre-clinical interventions in the mdx mouse (Grounds et al, 2008b; Guerron et al, 2010; Spurney et al, 2010; Spurney et al, 2009) (Radley-Crabb et al, submitted). This was catalysed by ‘The first Brazilian International Workshop on preclinical tests for drug therapies for Muscular Dystrophy’ a 3 day workshop help in Brazil (Ribeirao Preto, Sao Paulo 2006) (Bizario and Costa, 2007) and an associated large review manuscript (Grounds et al, 2008b). There are also many standard operating procedures listed on the TREAT-NMD website http://www.treat-nmd.eu/research/preclinical/SOPs. The standard operating procedures listed on the TREAT-NMD website have been employed (chapters 5 & 6) and contributed to (chapter 2) in this thesis.

1.4. Myofibre Necrosis in dystrophic muscle:

Myofibre necrosis is the fundamental destructive process in several forms of inflammatory myopathies and muscular dystrophy—especially DMD. In contrast to healthy skeletal muscle, fragile dystrophic muscles undergo repeated cycles of sarcolemmal damage, myofibre necrosis and subsequent regeneration. The exact mechanism by which the absence of dystrophin leads to myofibre necrosis is unknown however inflammation, oxidative stress, metabolic abnormality, mislocalisation of
nNOS and increased intracellular calcium are all heavily implicated in the process [Reviewed in (Allen et al, 2010; Evans et al, 2009a; Evans et al, 2009b; Grounds et al, 2008a; Leighton, 2003; Radley et al, 2007; Tidball and Wehling-Henricks, 2004b; Tidball and Wehling-Henricks, 2005; Tidball and Wehling-Henricks, 2007; Wallace and McNally, 2009)].

The absence of dystrophin does not affect the activation or proliferation of satellite cells as dystrophin is not normally expressed in satellite cells or myoblasts. Satellite cells are hyperactivated in dystrophic muscle due to the need for myogenesis in response to necrosis. Initially satellite cells (myogenesis) can repair the damaged myofibres but eventually this process fails and dystrophic muscle is replaced with fatty and/or fibrous connective tissue [Reviewed in (Anderson and Wozniak, 2004; Karpati and Molnar, 2008)]. There are conflicting data surrounding satellite cell number and differentiation status between in vitro and in vivo studies and disease states; however, a recent study examining satellite cell numbers in muscle biopsies from DMD patients aged 2-7 years, reports an increased number of quiescent satellite cells (Pax-7 positive nuclei, located beneath the basement membrane on the surface of the myofibre) (Kottlors and Kirschner, 2010). It has also been proposed that growing dystrophic skeletal muscle is more susceptible to myofibre necrosis than adult muscle and that the cellular and molecular events contributing to acute myofibre necrosis (e.g. 3 week old young mice or exercised adult mdx mice) compared with chronic necrosis (e.g. low persistent necrosis in adult mdx mice) are quite different (Grounds, 2008).

1.4.1 Inflammation and dystrophic muscle:

A persistent inflammatory state is observed in dystrophic skeletal muscle, including (but not limited to) an increased presence of inflammatory (and immune) cells (e.g. neutrophils, macrophages, T-lymphocytes and mast cells), elevated levels of various pro-inflammatory cytokines (e.g. TNF and TGFβ) and increased oxidative stress (Figure 1.4.1) [Reviewed (Evans et al, 2009a; Evans et al, 2009b; Grounds et al, 2008a; Spencer and Tidball, 2001; Tidball and Wehling-Henricks, 2005; Tidball and Wehling-Henricks, 2007)]. It is hypothesised that activation of the inflammatory cascade, in response to sarcolemmal damage, exacerbates myofibre damage, leading to myofibre necrosis.
rather than repair of small sarcolemmal tears (Evans et al, 2009b; Grounds et al, 2008a; Grounds and Torrisi, 2004; Radley et al, 2008; Radley and Grounds, 2006; Spencer and Tidball, 2001).

Figure 1.4.1. Sequence of the inflammatory response in dystrophic skeletal muscle. Resident mast cells act early in the inflammatory cascade and rapidly degranulate in response to damage to release TNF and many other pro-inflammatory mediators. Figure taken from Radley et al (2006) (Radley and Grounds, 2006).

After muscle contractions which damage (tear) the dystrophic sarcolemma, there is an aggressive invasion of inflammatory (and immune) cells into dystrophic muscle, which can exacerbate myofibre damage, through the release of pro-inflammatory cytokines, increased oxidative stress and proteolysis (Figure 1.4.1) (Radley et al, 2008; Spencer et al, 2001; Tidball, 2005). Resident and rapidly infiltrating neutrophils can also damage dystrophic muscle via the production of hypochlorous acid (HOCl) and via the secretion of superoxide (SO•) which is then converted to the highly damaging hydroxyl radical (OH•) (Tidball, 2005).
Strong evidence that inflammatory cells can contribute to necrosis of healthy muscle cells comes from studies investigating the role of neutrophils, macrophages and oxidative damage in vitro (McLoughlin et al, 2003; Nguyen and Tidball, 2003b; Pizza et al, 2001; Toumi et al, 2006) and in vivo (Cheung and Tidball, 2003; Nguyen and Tidball, 2003a; Pizza et al, 2002). It has similarly been proposed that an excessive inflammatory response can directly damage myofibres in myopathic conditions such as dystrophies or myositis (Porter et al, 2002; Tidball and Wehling-Henricks, 2005) and recent data increasingly implicate inflammation and specifically tumour necrosis factor (TNF) in myofibre necrosis [Reviewed in (Evans et al, 2009b; Grounds et al, 2008a)].

TNF, previously referred to as TNFα (Clark, 2007), is a major pro-inflammatory cytokine that is expressed by a wide range of inflammatory cells and by myoblasts, myotubes and damaged skeletal muscle (Collins and Grounds, 2001; Kuru et al, 2003). TNF is also produced by adipose tissue (Fruhbeck, 2004; Weisberg et al, 2003) that is often pronounced within DMD patients as the disease progresses. In response to myofibre damage, TNF is rapidly released from resident mast cells and also by neutrophils that accumulate quickly at sites of tissue damage (Radley and Grounds, 2006; Tidball, 2005). TNF is a potent chemokine that attracts further inflammatory cells to the injured site. The chemotactic role of TNF was demonstrated in normal mouse muscle, where administration of TNF resulted in the accumulation of neutrophils and macrophages in the absence of any sarcolemma damage (Peterson et al, 2006).

Elevation of TNF protein locally in dystrophic muscles is supported by immunohistological studies that show increased staining for TNF associated with necrotic areas of dystrophic muscles of the mdx mouse (Collins, 1999) and biopsies from DMD patients (Kuru et al, 2003) and also with Western blotting analysis using anti-TNF antibodies in mdx muscle extracts (Messina et al, 2006). Few studies have accurately quantitated TNF in dystrophic muscles or blood; due mainly to problems with sensitivity thresholds of such immunodetection in muscle and blood. Numerous examples from the literature which demonstrate an increase in TNF in dystrophic muscle are summarised in Table 1.4.
Measurements of TNF in mdx mice and DMD patients.

1) TNF Protein

<table>
<thead>
<tr>
<th>Authors</th>
<th>Reference</th>
<th>Technique</th>
<th>TNF level</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chahbouni et al.</td>
<td>J. Pineal Res 2010; 48: 282-289.</td>
<td>Linco-Plex plate - blood plasma.</td>
<td>Plasma TNF 4.43ng/L (/ .0.61) in DMD patients vs 2.9ng/L (/ .0.29) in healthy controls.</td>
<td>2x increase.</td>
</tr>
<tr>
<td>Pan et al.</td>
<td>Mol Cells 2008; 25: 531-37.</td>
<td>ELISA – serum (JingMed Co PR China).</td>
<td>Serum TNF 120ng/L (+/ -40) in 28 day old mdx mice vs 10ng/L (+/ -10) in C57Bl/10 controls.</td>
<td>12x increase.</td>
</tr>
<tr>
<td>Kuru et al.</td>
<td>Acta neuropathol 2003; 105: 217-24.</td>
<td>Immunohistochemistry – muscle biopsy (Genzyme USA).</td>
<td>TNF positive muscle fibres 15.8% / 5.1% in DMD patient biopsys vs 0% TNF positive fibres in healthy controls.</td>
<td>Increased detection.</td>
</tr>
<tr>
<td>Messina et al.</td>
<td>Exp neurol 2006; 196: 234-41.</td>
<td>Western blot – mdx and C57Bl/10 quadriceps muscle (Chemicon USA).</td>
<td>TNF in mdx muscle approx 4x higher than in control muscle (arbitrary values).</td>
<td>4x increase.</td>
</tr>
<tr>
<td>Radley-Crabb et al.</td>
<td>2009 unpublished</td>
<td>Elisa – serum (Invitrogen Aus).</td>
<td>TNF in serum of unex/ex C57Bl/10 and mdx mice – undetectable (&lt;15.6ng/L).</td>
<td>-</td>
</tr>
</tbody>
</table>

2) TNF Gene expression

<table>
<thead>
<tr>
<th>Authors</th>
<th>Reference</th>
<th>Technique</th>
<th>TNF level</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Huang et al.</td>
<td>FASEB 2009; March epub.</td>
<td>Reverse transcription Polymerase Chain</td>
<td>TNF mRNA 2.5x higher in mdx diaphragm muscle in</td>
<td>2.5x increase.</td>
</tr>
</tbody>
</table>
Table 1.4. A comprehensive list of measurements of TNF in mdx mice and DMD patients. Both TNF protein and gene expression are listed.

<table>
<thead>
<tr>
<th>Author(s)</th>
<th>Year</th>
<th>Method</th>
<th>Description</th>
<th>Comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bizario et al.</td>
<td>2009</td>
<td>Reverse transcription Polymerase Chain Reaction – gastrocnemius muscle</td>
<td>TNF mRNA detected in chronic downhill exercised 10 wk old mdx mice.</td>
<td>No comparison to C57Bl/10 controls.</td>
</tr>
<tr>
<td>Radley-Crabb et al</td>
<td>2010 unpublished</td>
<td>Reverse transcription Polymerase chain reaction – gastrocnemius muscle samples.</td>
<td>TNF mRNA 4x higher in mdx gastrocnemius muscle in comparison to C57Bl/10 control muscle.</td>
<td>4x increase.</td>
</tr>
<tr>
<td>Radley-Crabb et al</td>
<td>2010 submitted</td>
<td>Reverse transcription Polymerase chain reaction – quadriceps muscle samples.</td>
<td>TNF mRNA 10 higher in mdx quadriceps muscle in comparison to C57Bl/10 control muscle.</td>
<td>5x increase.</td>
</tr>
<tr>
<td>Kuru et al.</td>
<td>2003</td>
<td>In-situ hybridisation – frozen muscle biopsys (Genzyme USA).</td>
<td>TNF mRNA detected in muscle fibres of DMD patient biopsys.</td>
<td>No comparison to controls.</td>
</tr>
</tbody>
</table>

Elevated TNF may exacerbate muscle damage through several pathways (Clark et al, 2004). One of the contributors to TNF induced muscle necrosis could be the inflammatory transcription factor nuclear factor-kappa beta (NFκB), since NFκB is activated in limb muscles (Acharyya et al, 2007) and the diaphragm (Acharyya et al, 2007; Kumar and Boriek, 2003) of mdx mice and also in muscle samples from DMD patients (Monici et al, 2003). Blockade of NFκB by pyrrolidine dithiocarbamate reduces skeletal muscle degeneration in mdx mice (Messina et al, 2006) and it has recently been shown that heterozygous deletion of the p65 subunit of NFκB is sufficient to decrease muscle necrosis in mdx mice (Acharyya et al, 2007). Dysregulation of signalling through activation of the NFκB pathway appears to be particularly important in many diseases (associated with inflammation) and chronically activated NFκB is a feature of DMD and mdx muscles (Peterson and Guttridge, 2008).

1.4.2 Anti-inflammatory therapies in dystrophic muscle:

A wide range of immunosuppressant, anti-inflammatory and specific anti-cytokine drugs have been tested in both mdx and DMD patients, with benefits for various indicators of muscle damage [Reviewed (Bogdanovich, 2004; Evans et al, 2009b; Manzur and Muntoni, 2009; Radley et al, 2007; Spurney et al, 2009)]. Treatments that
include (but are not limited to); blockade of TNF (Grounds and Torrisi, 2004; Hodgetts et al, 2006; Pierno et al, 2007; Radley et al, 2008), cromolyn prevention of mast cell degranulation (Granchelli et al, 1996; Radley and Grounds, 2006), cyclosporine treatment (De Luca et al, 2005), and depletion or reduction of CD4+ or CD8+ cells (T cells) (Spencer et al, 2001), neutrophils (Hodgetts et al, 2006) and macrophages (Wehling et al, 2001), reduce the severity of dystropathology in mdx mice. Other anti-inflammatory therapies, less specific and less commonly used, such as Imatinib and Melatonin have also demonstrated beneficial effects in young mdx mice (Bizario et al, 2009; Chahbouni et al, 2010; Huang et al, 2009).

Two drugs have been used to block TNF activity in the mdx mouse; Remicade® (an antibody to human TNF, also known as Infliximab) (Grounds and Torrisi, 2004); and Enbrel® (soluble receptor to TNF, also known as Etanercept) (Hodgetts et al, 2006; Pierno et al, 2007) are in wide clinical use to treat inflammatory disorders such as arthritis and Crohn’s disease. The high specificity of these anti-cytokine drugs, combined with their clinical success in other diseases and relatively few side effects suggests that they may be attractive alternatives to the existing use of corticosteroids to treat DMD. In the mdx mouse, long-term studies have further demonstrated that the mouse-specific antibody to TNF (cV1q) has equal efficacy to Remicade® and Enbrel® (Radley et al, 2008) (See chapter 3 of this thesis). It is noted that cV1q blockade of TNF has no effect on the low levels of chronic myofibre damage in un-exercised dystrophic muscle, in striking contrast to the marked protective effect on exercise-induced acute myofibre necrosis, raising the possibility of different roles for TNF (and other molecules) in these two different situations of myopathology (Radley et al, 2008). These results strongly support a key role for TNF in both inflammation and necrosis in dystrophic muscle.

While systemic depletion of inflammatory cells or cytokines is not clinically viable, studies in gene ‘knock-out’ mice provide a wealth of knowledge regarding inflammatory signalling in dystrophic muscle and the potential compensatory up-regulation of other cytokines (Rafael et al, 1997; Spencer et al, 2000). It was hypothesised that generation of dystrophin and TNF double knock-out mice (TNF-/mdx), would result in improved dystropathology. However, serum CK level and whole
body strength was unchanged in these mice, the extent of dystropathology was inconsistent across muscle groups and body weight of the mice was significantly reduced. These results suggest that the effects of complete TNF knock-out are complex, unpredictable, and may result in the up-regulation of numerous other inflammatory cytokines to compensate for the absence of TNF (Spencer et al, 2000). Phenotypic examination of these dystrophin and TNF gene ‘knock-out’ mice (TNF-/-mdx) was carried out on sedentary 8 week old mice, and different effects might have been seen in exercised mice.

1.4.3 Reactive oxygen species and oxidative stress:

The term reactive oxygen species (ROS) is used to describe oxygen radicals, including superoxide (O$_2^•^-$) and hydroxyl (OH•) and the non radical derivatives of oxygen, including hydrogen peroxide (H$_2$O$_2$) and hypochlorous acid (HOCl). ROS are generated by a wide range of biological functions in the human body. The most reactive of the ROS, the hydroxyl radical (OH•) is formed from hydrogen peroxide (H$_2$O$_2$) in the presence of metal ions (iron) via Fenton chemistry (Figure 1.4.2). Oxidative stress occurs when ROS generation is not counteracted by antioxidants; substances that delay prevent or remove oxidative damage to a target molecule Three of the main anti-oxidant systems are 1) superoxide dismutase enzymes (SODs) which remove superoxide O$_2^•^-$ by accelerating its conversion into hydrogen peroxide H$_2$O$_2$, 2) catalase enzymes which convert hydrogen peroxide H$_2$O$_2$ into water H$_2$O and oxygen O$_2$ and 3) the glutathione system which removes hydrogen peroxide H$_2$O$_2$ by using it to oxidize reduced glutathione GSH to oxidized glutathione GSSG. An excess production of ROS and/or an insufficient anti-oxidant defence within a cell can lead to a situation of oxidative stress [Reviewed in (Arthur et al, 2008; Halliwell and Gutteridge, 2007)]. ROS have a diverse range of actions in cells, ROS can cause irreversible oxidation (a gain of oxygen or loss of electrons) of cellular components, cross linking or fragmentation of protein, damage to DNA and permanent damage to membrane phospholipids (lipid peroxidation) (Figure 1.4.2) (Arthur et al, 2008; Berlett and Stadtman, 1997). These processes can lead to irreversible cell damage and death by both apoptosis and necrosis (Rando, 2002; Rando et al, 1998). ROS can also affect cell signalling pathways through the reversible modification of transcription factors and signal transduction
proteins, mediated by the reduction/oxidation (redox) state of protein thiols [Reviewed in (Arthur et al, 2008; Lui et al, 2010)].

<table>
<thead>
<tr>
<th></th>
<th>Superoxide</th>
<th>Hydrogen peroxide</th>
<th>Hydroxyl radical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negatively charged radical ion</td>
<td>O₂ + e⁻ → O₂⁻</td>
<td>H₂O₂ + e⁻ → ·OH + e⁻ → H₂O</td>
<td></td>
</tr>
<tr>
<td>Uncharged, nonradical, relatively stable</td>
<td>H₂O₂ + e⁻ → H₂O</td>
<td></td>
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</tbody>
</table>

**Figure 1.4.2. Formation and properties of some ROS molecules.** As shown, ROS are generated by the sequential addition of electrons. Figure taken from Arthur et al (2008) (Arthur et al, 2008).

### 1.4.4 Dystrophic muscle and oxidative stress:

After contraction (that damages vulnerable dystrophic myofibres) there is an aggressive invasion of inflammatory cells (Hodgetts et al, 2006; Radley et al, 2008; Radley and Grounds, 2006; Spencer et al, 2001; Spencer and Tidball, 2001), which can exacerbate myofibre damage, through cellular processes such as phagocytosis and proteolysis (Tidball, 2005; Tidball and Wehling-Henricks, 2005). Neutrophils can damage dystrophic muscle via the production of hypochlorous acid (HOCl) and the secretion of superoxide (SO•) which is then converted to hydroxyl radical (OH•) and this increase in ROS leads to further damage of the muscle tissue (Tidball, 2005; Tidball and Wehling-Henricks, 2005).

While it has been established that oxidative stress is increased in dystrophic muscle (shown by lipid peroxidation, glutathione status and Dihydroethidium staining - a fluorescent probe for superoxide) (Austin et al, 1992; Burdi et al, 2009; Disatnik et al, 2000; Dudley et al, 2006; Whitehead et al, 2008) and that dystrophic myotubes and myofibres are much more susceptible to oxidative damage (Ragusa et al, 1997; Rando et al, 1998), the exact role of oxidative stress in necrosis of dystrophic myofibres is uncertain. Some research suggests that ROS generation in dystrophic muscle is a primary occurrence, due to deregulation of nitric oxide (NO) (Grisotto et al, 2000; Thomas et al, 1998; Wehling et al, 2001), but others have suggested it is secondary to
excessive calcium influx and inflammation by contraction induced damage (Whitehead et al, 2008; Yeung et al, 2005).

In normal healthy muscle, neuronal nitric oxide synthase (nNOS) is directly bound to syntrophins in the dystroglycan complex (DGC) but, due to the absence of dystrophin, in dystrophic muscle nNOS is displaced from the sarcolemma into the cytoplasm. nNOS is an important member of the DGC complex as it synthesises nitric oxide (NO), a molecule that reacts with free radicals and regulates vascular tone. During muscle contraction, the production of NO allows blood vessels to vasodilate which prevents ischemia and subsequent damaging reperfusion. In the absence of dystrophin, NO production is significantly reduced and may lead to free radical mediated damage [Reviewed in (Brenman et al, 1995; Wallace and McNally, 2009)]. Lack of NO is proposed as a mechanism for damage in dystrophic muscle, and over-expression of nNOS in transgenic mdx mice decreases myofibre damage, CK levels and macrophage invasion (Wehling et al, 2001).

ROS production in dystrophic muscle could also be a secondary consequence of contraction induced injury, facilitated by excessive intracellular calcium and inflammatory cell cascade. Contraction of dystrophic muscle leads to excessive intracellular calcium via either increased sarcolemma tears or altered calcium channel function (Turner et al, 1988; Wallace and McNally, 2009; Yeung et al, 2005). This calcium imbalance can lead to excessive activation of proteases such as calpain (Whitehead et al, 2008; Whitehead et al, 2006a) which promote proteolysis (protein degradation). An overload of mitochondrial calcium stimulates the generation of ROS (Brookes et al, 2004).

Oxidative stress may also contribute to the pathology of dystrophies by disrupting cell signalling. Cellular protein thiols (sulfhydryl groups) respond to an increase in cellular ROS and oxidation leads to a reversible formation of disulphide bonds. These modifications can directly affect the activity of signal transduction proteins or transcription factors as well as cause indirect changes in signal transduction through modifications of ion transport proteins (Arthur et al, 2008; Kourie, 1998; Suzuki and Ford, 1999). Nuclear factor kappa B (NFκB) is an important redox regulated inducible
transcription factor activated by oxidative stress (Macaione et al, 2007). It regulates the expression of many genes, including those involved in the inflammatory and stress response. In particular, activation of the NFκB pathway increases pro-inflammatory cytokines such as TNF, and levels of both NFκB and TNF (Table 1.4) are elevated in mdx mice (Acharyya et al, 2007; Kumar and Boriek, 2003; Peterson and Guttridge, 2008).

1.4.5 Antioxidant therapies in dystrophic muscle:

An alternative (or perhaps complementary) approach to treating DMD is to decrease oxidative stress. N-acetylcysteine (NAC) is an anti-oxidant which decreases oxidative stress by scavenging ROS (hypochlorus acid, hydroxyl radicals and hydrogen peroxide) (Aruoma et al, 1989). NAC also has many effects on cell signalling and is a precursor to the cellular antioxidant glutathione which is preferentially oxidized by ROS thus protecting vital macromolecules such as DNA, proteins and lipids (Zafarullah et al, 2003). When administered to mdx mice, NAC reduced cellular ROS (shown by DHE staining), decreased nuclear protein expression of NFκB, and reduced muscle damage (histological quantitation of centrally nucleated myofibres) (Whitehead et al, 2008). The in vivo effects of NAC in exercised mdx mice were not investigated by Whitehead et al (2006); thus further analysis of myofibre necrosis and more specific ROS measurements were carried out in Chapter 5 of this Thesis.

Numerous in vivo studies have shown green tea (specifically the major polyphenol (-)epigallocatechin gallate –EGCG) to have anti-oxidant properties in vivo and beneficial effects on the extent of dystropathology in mdx mice (Buetler et al, 2002; Call et al, 2008; Dorchies et al, 2006; Evans et al, 2010; Nakae et al, 2008), possibly via down-regulation of NFκB (Evans et al, 2010). Call et al (2008) reported additional benefits of EGCG treatment when administered in combination with voluntary exercise in young mdx mice (21-42 days of age), possibly due to the beneficial effects of long-term voluntary exercise (Call et al, 2008).

NAC and EGCG function as antioxidants by scavenging radicals and reducing oxidative stress, but it is feasible that a more powerful way to prevent oxidative stress is to prevent ROS from forming. As mentioned previously, formation of the highly reactive
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hydroxyl radical requires an iron catalyst. Free iron, or Fe (II) is capable of catalysing the production of the hydroxyl radical. In this context, it is relevant to note that when skeletal muscle of rats is overloaded with iron, there is increased oxidative stress, decreased muscle strength and increased muscle atrophy (Reardon and Allen, 2009). In contrast, dystrophic mdx mice fed a low iron diet show a decrease in macrophage-invaded necrotic myofibres (Bornman et al, 1998).

In addition to excess (dysregulated) inflammation and oxidative stress, it is also proposed that the absence of dystrophin in skeletal muscle leads to many metabolic abnormalities, which may also be involved in the process of myofibre necrosis.

1.5 Skeletal muscle metabolism:

Metabolism refers to the sum of all chemical processes which occur in a living organism in order to maintain life. Metabolic processes enable living organisms to complete vital functions such as respiration, reproduction, growth, maintenance, and repair. Metabolism is divided into two types of processes – catabolic and anabolic. Catabolism is the set of metabolic processes that break down large molecules into components that can be used in anabolic processes e.g. digesting dietary protein into amino acids. Anabolism, on the other hand, is the set of constructive metabolic processes where the energy released by catabolism is used to synthesize complex molecules. Some of the main metabolic processes carried out by skeletal muscle are the synthesis and degradation of proteins, fatty acid catabolism (β-oxidation of fatty acids produces Acetyl-CoA which is used in the citric acid cycle), mobilization of glycogen stores for energy production and glycogen synthesis from glucose for energy storage. Hydrolysis of adenosine triphosphate (ATP) is the main source of energy for many enzymatic reactions that occur in skeletal myofibres, ATP is supplied by mitochondria located in the myofibre cytoplasm and since contraction is an energy demanding process myofibres are rich in mitochondria. Depending on the level of skeletal muscle activity, ATP is replenished from phosphocreatine stores, through the process of glycolysis or via oxidative metabolism [Reviewed in (Bettelheim et al, 2001; MacIntosh et al, 2006)].
1.5.1 Protein turnover in skeletal muscle:

Skeletal muscle protein mass is tightly regulated by (subtle) changes in the relative rates of protein synthesis and degradation. The whole-body protein synthesis rate represents the compilation of protein synthesis rates of all tissues and organs in the body and skeletal muscle represents the largest protein mass in the body. Growth, aging, nutrition and disease can all affect the protein synthesis rates of tissues and organs. Protein turnover is the net balance between protein synthesis and protein degradation. More synthesis than breakdown indicates an anabolic state that builds lean tissues, whereas more breakdown than synthesis indicates a catabolic state that burns lean tissues. Proteins are constantly synthesized and degraded in cells. Depending on the tertiary structure of the protein (stability), turnover rate can vary from minutes to weeks; rapid protein turnover allows cells and tissues to respond quickly to constantly changing conditions. Protein synthesis involves the process of translation of mRNA into an amino acid sequence to form a peptide, which occurs on ribosomes in the cytoplasm. Protein breakdown occurs generally in the lysosomes or proteasomes. The most commonly employed method for measuring actual protein synthesis is the use of isotopic tracers to measure the rate of incorporation of a labeled amino acid into tissue protein over-time [Reviewed in (Bettelheim et al, 2001; Davis et al, 1999a)].

Such issues of protein turnover are also influenced by nutrition, especially specific amino acids derived from protein ingestion, by available energy provided by different foods and influenced by the balance of energy demands within the body. It has been proposed (Leighton, 2003) that dystrophic boys have an altered metabolic rate and therefore this was investigated to explore the potential basis for meaningful dietary interventions or nutritional supplements for DMD boys.

1.5.2 Metabolic alteration in dystrophic muscle:

Boys with DMD can move between the spectra of over-nutrition to under-nutrition within their shortened lifespan. Young DMD patients are often over-weight or obese...
(due to a combination of inactivity, steroid therapies and proposed metabolism defects), yet patients are also often underweight and experience malnutrition in later life [Reviewed in (Davidson and Truby, 2009; Leighton, 2003)]. Despite these observations, there is limited literature on the nutritional and energy requirements for DMD patients. DMD is considered to be a hyper-metabolic condition [Reviewed in (Leighton, 2003)] with the basal metabolic rate (kcal/kg bw/24hr), dietary protein requirements (g protein/kg bw/24hr) and urinary excretion of 3-methylhistidine (an indicator of muscle protein breakdown) all higher in DMD patients (aged 11- 29 years) in comparison to healthy controls (Davidson and Truby, 2009; Leighton, 2003; Okada et al, 1992). Resting energy expenditure when adjusted to free fat mass (lean muscle tissue) is also higher in DMD patients (6-13 years) (Hankard et al, 1996; Zanardi et al, 2003).

Studies in mdx mice also indicate that dystrophin defects lead to altered skeletal muscle metabolism and an impaired energy status. Repeated cycles of myofibre necrosis and regeneration, increased demand on the sarcoplasmic reticulum to regulate intracellular calcium, defective mitochondrial function, increases in both protein synthesis and protein degradation rates, and disruption in nNOS signalling may all contribute to an altered metabolic state (Dupont-Versteegden et al, 1994a; Griggs and Rennie, 1983; Han et al, 2006; Kuznetsov et al, 1998; Landisch et al, 2008; MacLennan and Edwards, 1990; Passaquin et al, 2002; Whitehead et al, 2006b; Zanardi et al, 2003). In support of an altered metabolism in dystrophic muscle, 48 hours fasting significantly increased myofibre necrosis in muscles from the hind limb and lumbar region of 6 month old mdx mice (yet no change in muscle morphology of control C57BL/10 mice), suggesting a strong dependence on caloric intake to maintain dystrophic muscle structure (Helliwell et al, 1996).

Changes in protein metabolism have been reported in various mdx muscles both in vivo and ex vivo (Helliwell et al, 1996; MacLennan and Edwards, 1990; MacLennan et al, 1991a; MacLennan et al, 1991b; Turner et al, 1988). The rates of both protein synthesis and protein degradation are elevated in the gastrocnemius muscle of mdx
mice (various ages, sex unspecified) and it is suggested that this process is mediated by an increase in ribosome concentration in mdx muscles (MacLennan and Edwards, 1990). Interestingly, in mdx liver where there is no dystropathology, the rate of protein synthesis is similar to control C57BL/10 mice, indicating that altered protein metabolism in mdx mice is restricted to skeletal muscles (MacLennan and Edwards, 1990). The extent to which this reflects the increased protein demand of growing myotubes and myofibres compared with mature myofibres is unclear in dystrophic muscle.

Adult mdx mice maintain skeletal muscle mass and experience significant skeletal muscle hypertrophy (Anderson et al, 1987; Coulton et al, 1988a; Mokhtarian et al, 1996; Peter and Crosbie, 2006; Shavlakadze et al, 2010; Spurney et al, 2009), indicating that protein synthesis must outweigh protein degradation in adult mdx mice. Despite adult mdx mice experiencing significant muscle hypertrophy, dystrophic muscles are still ‘weaker’ than those from control C57Bl/10 mice due to the absence of dystrophin protein (Ridgley et al 2009). Skeletal muscle hypertrophy in adult mdx mice is also a feature of young DMD boys (~ 5 years of age) where hypertrophic calf muscles are a diagnostic feature (Bushby et al, 2010a; Hoffman, 2001). This is in striking contrast to older (6-18 years), wheel chair bound, DMD patients that lose lean muscle mass at an approximate rate of 4% per year (Griffiths and Edwards, 1988). Skeletal muscle hypertrophy in adult mdx mice may be linked to an increase in AKT signalling (Peter and Crosbie, 2006), since AKT is a key downstream molecule of IGF-1 signalling that results in increased protein synthesis and hypertrophy [Reviewed in (Shavlakadze and Grounds, 2006)]. There are also reports of increased circulating growth hormone in 8-10 month old female mdx (Anderson et al, 1994) and increased circulating IGF-1 levels in 8-10 week old (sex unspecified) mdx mice (De Luca et al, 1999).

It was hypothesised by Dupont-Vesteegden et al (1994) that continuous cycles of myofibre necrosis and subsequent regeneration, increased levels of intracellular calcium and elevated protein synthesis in dystrophic mdx muscles could lead to an overall increase in whole body metabolic rate; however they reported no change in the metabolic rate (kcal/kg bodyweight^0.75/24hr) of one year old adult mdx mice (mixed sex). In contrast, there was a decrease in the whole body metabolic rate (kcal/kg
bodyweight$^{0.75/24\text{hr}}$ of 4-6 week old (mixed sex) mdx mice, and it was proposed that this was a consequence of reduced physical activity and reduced food consumption (g food /g weight) (Dupont-Versteegden et al, 1994a). Mokhtarian et al (1996) also reported no difference in total energy expenditure, basal energy expenditure and spontaneous activity between control C57Bl/10 and mdx mice (sex unspecified) at 6-12 months of age (Mokhtarian et al, 1996). Clearly, mouse age, extent of dystrophopathy and the presence of regenerating (growing) muscle, may have a major impact on metabolic demand and energy usage.

1.5.3 Nutritional therapies for dystrophic muscle:

Dystrophin deficiency does not adversely affect muscle growth or function, in fact adult mdx mice experience muscle hypertrophy (Anderson et al, 1987; Coulton et al, 1988a; Mokhtarian et al, 1996; Peter and Crosbie, 2006; Shavlakadze et al, 2010; Spurney et al, 2009), however the absence of dystrophin does eventually lead to muscle wasting in both ‘old’ mdx mice and DMD patients (Bushby et al, 2010a; Emery, 2002; Sinnreich, 2010). Dietary supplementations have shown beneficial effects in both mdx mice and DMD patients [Reviewed in (Bogdanovich, 2004; Davidson and Truby, 2009; Leighton, 2003; Pearlman and Fielding, 2006; Radley et al, 2007)], in addition to this some recently completed clinical trials have examined the role of creatine (organic acid) and glutamine (amino acid) supplementation in DMD patients with some beneficial effects (Escolar et al, 2005; Mok et al, 2006; Tarnopolsky et al, 2004).

Creatine is a major intermediate of cellular energy transfer; and when fed to newborn mdx mice (10% w/w in chow) strongly reduced the onset of muscle necrosis in the fast-twitch EDL muscle and also improved mitochondrial respiration capacity (Passaquin et al, 2002). In addition, creatine, taurine (organic acid) and glutamine treatments have shown beneficial effects in treadmill exercised adult mdx mice (De Luca et al, 2003; Granchelli et al, 2000). A combined nutritional therapy (creatine monohydrate – organic acid, conjugated linoleic acid – essential fatty acid, α-lipoic acid – mitochondrial coenzyme and antioxidant, and β-hydroxy-β-methylbutyrate –leucine metabolite) administered, in addition to prednisolone, for 8 weeks increased muscle
strength and reduced the extent of dystropathology in 12 week old treadmill exercised mdx mice (Payne et al, 2006). A combination of taurine (1g/kg bw/day - orally) and prednisolone (1mg/kg bw/day - i.p. injection) treatment of treadmill exercised mdx mice (4-8 weeks of age) also markedly improved forelimb grip strength, compared to either taurine or prenisolone treatment alone (Cozzoli et al, 2010). In addition, a high protein diet (50%) improved muscle morphology in dystrophic laminin deficient (129ReJ dy/dy) mice and caused a shift to a more ‘normal’ protein metabolism (Zdanowicz et al, 1995).

A preliminary study to test the effects of a high fat diet on the dystrophic mdx heart (Personal communication - Andrew Hoey 2005, Queensland Australia, data unpublished), reported a striking difference in bodyweight of dystrophic mdx and control mice fed a high fat diet (~15%) for 9 weeks (from 6–15 weeks of age). As expected C57Bl/10 mice showed significantly increased body weight and % body fat, however; the bodyweight of mdx mice was unaffected by the change in diet. The dystropathology of skeletal muscles was not examined in this preliminary study, but these interesting results led us to propose that a high fat diet may reduce the extent of dystropathology, and may be metabolically beneficial to mdx mice due to their impaired energy status - see chapter 6 of this Thesis.

The information presented in this review of background literature summarises 3 main points 1) the suitability of the mdx mouse as an animal model for pre-clinical DMD research, 2) the dystropathology of mdx mice, in particular myofibre necrosis and 3) the identification of 3 potential therapeutic interventions that could be modulated in attempt to reduce dystropathology in mdx mice. The central hypothesis of the thesis is that inflammation, oxidative stress and metabolic abnormality all play a role in the extent of dystropathology and that modulation of these parameters via therapeutic intervention can ameliorate dystropathology. The experimental research presented in this thesis is an investigation of this central hypothesis.
1.6 Aims and Hypotheses of the Thesis

Overall Aim:
To identify potential therapeutic interventions for Duchenne Muscular Dystrophy using the mdx animal model.

Specific Aims:
1. Examine the potential therapeutic benefits of an anti-inflammatory drug (specifically cV1q a mouse specific TNF antibody) in both young and adult dystrophic mdx mice. (Chapter 3).
2. Develop a short (30 minute) and repeatable treadmill exercise protocol to efficiently test pre-clinical therapeutic drugs in adult mdx mice and identify key markers of muscle damage to rapidly monitor efficacy of pre-clinical drug treatments. (Chapter 4).
3. Examine the potential therapeutic benefits of an anti-oxidant drug (specifically N-acetylcysteine) in adult mdx mice and determine (if any) the mechanism(s) of protective action. (Chapter 5).
4. Examine the potential therapeutic benefits of a high fat and high protein diet in both sedentary and exercised dystrophic mdx mice, with respect to i) myofibre integrity; (ii) muscle growth and maintenance of muscle mass; (iii) the balance between muscle and fat formation. (Chapter 6).
5. Determine the metabolic differences (food intake, energy expenditure, activity level, body composition and protein synthesis rate) between young and adult mdx and C57Bl/10 mice. (Chapter 7).

Overall Hypothesis:
The central hypothesis of the thesis is that inflammation, oxidative stress and metabolic abnormality all play a role in the extent of myofibre necrosis in dystrophic muscle and that modulation of these parameters via therapeutic intervention can ameliorate dystropathology.
Specific Hypotheses:

1. TNF plays a key role in necrosis of dystrophic muscle and *in vivo* administration of cV1q a TNF antibody will ameliorate dystropathology in both young and adult mdx mice.

2. A single 30 minute treadmill exercise protocol will increase markers of muscle damage in adult mdx mice and thus provide a quick and efficient way to screen potential pre-clinical therapeutic drugs in adult mdx mice.

3. Oxidative stress plays a key role in myofibre necrosis of dystrophic muscle *in vivo* and administration of N-acetylcysteine (an antioxidant) will ameliorate dystropathology in both sedentary and treadmill exercised adult mdx mice.

4. Dystrophic muscle is subject to metabolic abnormalities and consumption of a high fat or high protein diet will ameliorate dystropathology in both sedentary and treadmill exercised adult mdx mice.

5. Due to the continuous cycles of muscle necrosis and regeneration in mdx mice there will be significant metabolic differences between mdx and C57Bl/10 mice. In addition, these differences will be amplified in young mdx mice where dystropathology is most severe.
**Materials and Methods.**

**Overview:** This methods chapter contains both an unpublished part and 1 published methods protocol (Publication #4). Details of the published protocol along with the candidate’s individual contribution to this protocol are listed below. A re-print of the published protocol is included in section 2.5 of the Methods chapter.


Candidate completed approximately 40% of the work required for the method.

Each paper (each Results chapter) within this thesis contains all specific information for the experimental methods specific to that paper. The following general text (Chapter 2) describes in detail the routine experimental methods employed throughout this Thesis.
2.1 In vivo animal protocols:

2.1.1 Animal usage and animal ethics: All experiments were carried out in non-dystrophic (normal) C57Bl/10ScSn and dystrophic mdx (C57Bl/10^mdx/mdx) mice. C57Bl/10ScSn mice are the parental strain for mdx. For experiments conducted at the University of Western Australia, all mice were obtained from the Animal Resource Centre, Murdoch, Western Australia. Mice were maintained at the UWA on a 12-h light/dark cycle, under standard conditions, with free access to food and drinking water. All animal experiments were conducted in strict accordance with the guidelines of the National Health and Medical Research Council Code of practice for the care and use of animals for scientific purposes (2004) and the Animal Welfare act of Western Australia (2002) and were approved by the Animal Ethics committee at the University of Western Australia.

(For details of animal experiments conducted in the USA see section 2.4 of this Materials and Methods chapter).

2.1.2 Voluntary exercise: The low level of muscle damage in dystrophic adult mdx mice can be elevated by voluntary exercise (Grounds et al, 2008b). One individual mouse is placed into a cage with a voluntary running wheel (Figure 2.1) and allowed free access to the wheel. Large perspex cages are used and the wheel is mounted to the roof of the cage; this allows for the height of the wheel (300mm) and prevents bedding on the cage floor from blocking up the exercise wheel.

![Figure 2.1. Voluntary exercise set-up. A metal exercise wheel (arrow) is attached to the cage lid with cable ties and the monitor from a bicycle speedometer (*) is taped to the inside of the mouse’s cage. The bicycle monitor records distance and speed run by the mouse.](image-url)
Exercise data were collected via a small magnet attached to the mouse wheel, and a sensor from a bicycle pedometer attached to the back of the cage. The pedometer records single wheel revolutions, allowing total distance (km) run by an individual mouse to be determined, as per (Hodgetts et al, 2006; Radley et al, 2008; Radley and Grounds, 2006). The mice run the most during the night since they are normally nocturnal (Hayes and Williams, 1996; Radley and Grounds, 2006). Mice are monitored daily throughout the experiment for food consumption, distance run and wheel function (See Results chapters 3 & 6).

### 2.1.3 Treadmill exercise:

Based on previous research (De Luca et al, 2003; Granchelli et al, 2000) and as per the TREAT-NMD recommended standard protocol “Use of treadmill and wheel exercise for impact on mdx mice phenotype M.2.1_001” [http://www.treat-nmd.eu/research/preclinical/SOPs/](http://www.treat-nmd.eu/research/preclinical/SOPs/) a treadmill exercise regime consisting of 30 minutes treadmill running at a speed of 12m/minute was used. The rodent treadmill used was an Exer 3/6 from Columbus Instruments (USA) (Figure 2.2).

**Treadmill Setup:** Individual running lanes were separated by clear Perspex dividers so that the mice could see each other while exercising. The treadmill was horizontal (0° incline) and mdx mice were run in groups of 3 or 4 as it is time consuming and inefficient to run mdx mice individually (Figure 2.2). For consistency, the treadmill running was routinely conducted between 8am – 11am.

![Figure 2.2. Treadmill exercise set-up. Lanes are separated by clear perspex dividers (arrow) and each mouse is given an individual lane to run in. Soft foam blocks (*) are placed at the end of each lane to prevent the mouse falling off the back of the treadmill.](image)
Exercise Protocol: Groups of 3 or 4 mdx mice were all (1) settled for 2 mins with the treadmill belt stationary, (2) then acclimatized with gentle walking for 2 mins at a speed of 4m/min, followed immediately by (3) a warm-up of 8 minutes at 8m/min and then (4) the main exercise session for 30 minutes at 12m/min. If during the 30 minutes exercise session a mouse fatigued and could no longer run, the procedure was as follows: turn the treadmill belt off and give all mice a 2 minute rest, turn the belt on at 4m/min for 2 minutes, increase the speed to 12m/minute and run for the remainder of the 30 minutes. Repeat this process if fatigue occurs again (up to 5 times for an individual mdx mouse) (Radley-Crabb et al, submitted) (See Results Chapters 4 & 5).

2.1.4 Forelimb Grip strength: Grip strength was measured using a Chatillon Digital Force Gauge (DFE-002) and a triangle metal bar, as per the TREAT-NMD recommended standard protocol “Use of grip strength meter to assess limb strength of mdx mice – M.2.2_001” [http://www.treat-nmd.eu/research/preclinical/SOPs/](http://www.treat-nmd.eu/research/preclinical/SOPs/). In brief, the mouse was placed on the front of the triangle bar (attached to a force transducer) and pulled gently until released. Each mouse underwent 5 consecutive grip-strength trials; the grip strength value for each mouse was recorded as the average of the 3 best efforts. Average grip strength was then normalized for body weight [Force (kg)/BW (g)]. Change in normalised grip strength was determined by subtracting normalised grip strength (8 weeks) from normalised grip strength (12 weeks) (See Results Chapter 4).

2.1.5 Dietary interventions: The three customised semi-pure diets; Control (7% fat, 19% protein - AIN93g), High Fat (16% fat, 19% protein - sf 06-040) and High Protein (7% fat, 50% protein - sf 00-252) were manufactured by and purchased from Specialty Feeds Glen Forest Western Australia ([www.specialtyfeeds.com.au](http://www.specialtyfeeds.com.au)). The three diets were designed and manufactured specifically for laboratory mice by Mr Warren Potts. Pilot studies were conducted with all custom diets for palatability, consumption and toxicity. All custom diets were administered as standard 12mm cubes. All mice were fed a cereal based standard rat and mouse chow (5% fat, 19% protein) prior to the commencement of any dietary interventions (this is the standard cereal diet fed to all
mice at UWA). Mice were monitored twice weekly for food consumption (weight g) and bodyweight (See Results Chapter 6).

2.1.6 Intra-peritoneal injections of cV1q antibody (Anti-inflammatory drug administration): Intra-peritoneal (IP) injections of cV1q and the isotype-matched, negative control antibody (cVaM) both provided by Centocor were given at a concentration of 20µg/g bodyweight /week. This procedure is simple and requires no anaesthesia; mice were picked up (scruffed) and held by a researcher while being injected. A 27.5 gauge insulin syringe (Sigma Aldrich Z192082) was used for the injection. Both cV1q and cVaM were diluted in PBS (See Results Chapter 3 & 8).

2.1.7 Drinking water administration of N-acetylcysteine (Anti-oxidant drug administration): N-acetylcysteine (Sigma Aldrich A7250) was added to standard acidified laboratory mouse drinking water (pH 2.5 – 3) at a concentration of either 1% or 4%. Mice were then given free access to the treated drinking water for 6 weeks (1%) or 1 week (4%). Drinking water was changed once a week as per standard animal husbandry practices. Mice were monitored every second day for drinking water consumption (ml) and bodyweight. It is estimated that laboratory mice drink approximately 3-5mls per day, this equates to 30-50µg N-acetylcysteine consumption per day, (e.g 1.6µg NAC/g bw/day [for a 30g mdx mouse]) (See Results Chapter 5).

2.1.8 Evans Blue Dye injection: A single Evans Blue Dye (Sigma Aldrich 46160) injection was given intraperitoneally (IP) 24 hours prior to animal sacrifice and sampling (Hamer et al, 2002). A 1% solution of EBD (diluted in filtered PBS) was injected at a dose of 1mg/g bodyweight (1% of mouse’ bodyweight). A 27.5 gauge insulin syringe (Sigma Aldrich Z192082) was used for the injection.

2.1.9 Whole muscle graft surgery: Whole muscle autografts were used as an in vivo bio-assay to assess the anti-inflammatory properties of cV1q in non-dystrophic
C57Bl/10 mice. Six weeks old female C57BL/10 mice were anaesthetised using 2% (v/v) isoflurane (Bomac Australia). The Extensor Digitorum Longus (EDL) muscle with both tendons was removed from the anatomical bed and transplanted onto the surface of the Tibialis Anterior (TA) muscle, the EDL tendons were sutured to the TA, the skin closed and wound left to heal, as described in (Grounds et al, 2005b; Roberts and McGeachie, 1992; Shavlakadze et al, 2009; White et al, 2000) (Figure 2.3). It is well documented that this procedure severs the nerve and blood supply to the EDL muscle and results in necrosis and subsequent inflammation and regeneration of the graft; grafts were examined histologically 5 or 7 days after surgery (See Results Chapter 3).

![Figure 2.3 Schematic diagram of whole muscle graft surgery.](image)

**Figure 2.3 Schematic diagram of whole muscle graft surgery.** The EDL is removed from the muscle bed and placed on top of the TA muscle. The EDL is sutured into place and collected after 5-7 days.

### 2.1.10 Animal sacrifice and tissue collection:
An individual mouse was placed into a small perspex box connected to an anaesthetic machine for initial anaesthesia with 2% v/v isoflurane (Bomac Australia) (see section 2.3.1). The anaesthetised mouse was then transferred into a small nose cone and remained under anaesthesia while 0.5ml of blood was collected from the mouse via direct cardiac puncture using a 27.5 gauge tuberculin syringe (Sigma Aldrich Z192082) - it is necessary to do this while the heart is still beating (see section 2.3.4). The anaesthetised mouse was then sacrificed by cervical dislocation. The tissues were collected in a strict sequence: firstly, tissues were rapidly collected for molecular or biochemical analysis by snap freezing immediately in liquid nitrogen. Secondly, tissues were collected for either frozen or paraffin histology. Lastly, tissues were collected for weight and morphometric measurements (e.g tibial length).
2.2 Histological protocols:

2.2.1 Sample preparation: For paraffin histology, muscles were removed from the mouse and immediately placed into 4% paraformaldehyde (Sigma Aldrich 158127) for fixation. Where possible, placing an entire leg into paraformaldehyde allows the muscles to hold their shape and length, which minimises curling of the muscles. The muscles were 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 were cut transversely, ‘caged’ and processed overnight in an automatic processor e.g. Shandon, (ethanol through to paraffin wax) and embedded into paraffin blocks. Skeletal muscle tissue sections were 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.

For frozen histology, the fresh muscles were bisected transversely and the 2 pieces mounted side-by-side on cork squares using tragacanth gum (Sigma Aldrich G1128). The muscles were 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). Frozen sections were cut at 8µm (on a cryostat) directly onto uncoated or silinated glass slides: ideally these were stained immediately but could be stored at -20°C until stained.

2.2.2 Haematoxylin and Eosin stain: Skeletal muscle pathology can be accurately assessed on frozen or paraffin sections stained with Haematoxylin and Eosin (H&E) (Publication #4 - section 2.5) (Grounds et al, 2008b). 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.
The basic H&E staining protocol was:

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

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 (Note – Can start staining frozen sections at “f”).

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.

2.2.3 Sirius red stain: Sirius red stain is used to assess fibrosis as it stains collagen red. Sirius red stain is also useful to measure myofibre cross sectional area as it stains myofibres yellow and collagenous myofibre membranes red. The Sirius red staining protocol was:

a) Bring sections to distilled water (heat for 20mins at 60ºC, dewax in 3x changes of xylene, dehydrate in 2x changes of 100% ethanol, 1x change of 70% ethanol and rinse in distilled water).

b) Stain nuclei with Haematoxylin for 1 minute.

c) Rinse well in tap water.

d) Stain in Solution A – Pico Sirius red for 70 minutes. This gives an equilibrium staining which does not increase with a longer time.

e) Wash in two changes of Solution B – Acidified water. (Separate the 500ml of acidified water into 2 glass staining containers, discard after use).

f) Remove most of the acidified water from the slides by shaking and blotting (do not rinse).

g) Dehydrate in 3x changes of 100% ethanol.

h) Clear in 3x changes of xylene and mount with DPX.

Solution A: Pico-sirius red: Sirius Red stain F3B (CI 35782) – 0.5g. Saturated aqueous solution of picric acid – 500ml (add a little solid picric acid to the solution to ensure saturation). Do not filter.

Solution B – Acidified water: Add 2.5ml of acetic acid to 500ml of distilled water. Make up fresh for each run and discard after use.

2.2.4 Image collection and analysis: Non-overlapping tiled images of transverse muscle sections provided a picture of the entire muscle cross section. Images were acquired using a Leica DM RBE microscope, a personal computer, a Hitachi HVC2OM
digital camera, Image Pro Plus 4.5.1 software and Vexta stage movement software. Histological analysis of muscle morphology was carried out on whole muscle cross sections. Muscle morphology was drawn manually by the researcher using Image Pro Plus 4.5.1 software and all section analysis was done ‘blind’. Muscle sections were routinely analysed for the following morphological features: normal /undamaged myofibres (myofibres with peripheral nuclei), myofibre necrosis (myofibres with fragmented sarcoplasm and/or areas of inflammatory cells), regeneration (myofibres with central nuclei), fibrosis and myofibre cross-section area (Grounds et al, 2008b). All Histological analysis was completed as per the TREAT-NMD recommended standard protocol “Hematoxylin and Eosin staining for histology of dystrophic skeletal muscle - M.1.2_007” <http://www.treat-nmd.eu/research/preclinical/SOPs/> (Publication #4 - section 2.5).

2.3 Molecular and Biochemical protocols:

2.3.1 Sample preparation: When using tissue samples for molecular or biochemical analysis is it important to remove the tissue from the mouse and snap freeze it immediately in liquid nitrogen to prevent degradation of various parameters. Snap frozen tissues were stored at -80ºC until analysed. Anaesthetic regimes should be standardised as changes may affect gene expression, protein synthesis and oxidative stress (Heys et al, 1989; Juhl, 2010). The standard terminal anaesthesia regime used throughout this thesis is: 400ml NO₂ (BOC Australia), 1.5L O₂ (BOC Australia) and 2% v/v isoflurane (Bomac Australia) (see section 2.1.10).

2.3.2 RNA extraction and reverse transcription: RNA was extracted from either the snap frozen gastrocnemius or quadriceps muscle using Tri-reagent (Sigma T9424) and quantitated using a Nano Drop Spectrophotometer (ND 1000) and ND 1000 software version 3.5.2. The RNA was DNase treated using Promega RQ1 RNase free DNase (M610A), RQ1 RNase free 10x buffer (M198A) and RQ1 DNase stop solution (M199A). RNA was reverse transcribed into cDNA using Promega M-MLV Reverse Transcriptase.
(M3682), random primers (C1181) and 10mM dNTPs (U1515) and the cDNA was purified using a MoBiol Clean up kit (12500-250).

2.3.3 Reverse Transcription Polymerase Chain Reaction (RT-PCR): RT-PCRs were run on a Corbett 3000 (Corbett Research) using QIAGEN quantifast SYBR green PCR mix (204054) and QIAGEN Quantitect Primer Assays for TNF (QT00104006), IL-1β (QT01048355), IL-6 (QT00098875), PPAR alpha (QT00137984), PPAR beta/delta (QT00166292), PPAR gamma (QT00095578), PGC-1alpha (QT00166292) and standardised to a house keeping gene L-19 (QT01779218). mRNA expression was calculated and standardised using Roto-gene 6.1 and Microsoft Excel software (See Results Chapters 4 & 6).

2.3.4 Creatine Kinase assay: While mice were under terminal anaesthesia, whole blood (approx 0.5ml) was collected via cardiac puncture using a 27.5 gauge tuberculin syringe (Sigma Z192082), into a 1.5ml tube (see section 2.1.10). Extensive experimentation revealed that storage of blood samples overnight at 4ºC to enable clotting leads to a false increase in serum CK levels and therefore blood samples were immediately spun down in a refrigerated centrifuge for 5 minutes (12000g), serum was removed and aliquoted (Radley-Crabb et al, submitted). Blood serum CK activity was determined in duplicate using the CK-NAC kit (Randox Laboratories) and analysed kinetically using a BioTek Powerwave XS Spectrophotometer using the KC4 (v 3.4) program. A minimum of 10µl serum is required to complete this assay. Prior to establishing this assay in our laboratory some CK assays were performed commercially at Murdoch Veterinary Hospital, WA (Radley et al, 2008).

2.3.5 TNF Elisa: A commercially available TNF Elisa (Invitrogen KMC3012 pre-coated 96 well plate) was used to measure TNF protein level in blood serum, according to manufacturer’s instructions. In brief the method consisted of: 1) Add standards, water blank, chromagen blank and TNF controls to first 12 wells. 2) Add 50µl of standard diluent buffer and 50µl of each serum sample to the remaining wells. 3) Add 50µl of
biotinylated anti-TNFα solution into each well (except chromagen blank) and tap plate to mix. 4) Cover plate and incubate at room temperature for 90 minutes. 5) Prepare working solution of Streptavidin-HRP (at approx 70 mins incubation). Warm streptavidin-HRP concentrate (100x concentrate in 50% glycerol) to room temperature and gently mix. Dilute 10ul of streptavidin-HRP concentrate in 1ml of streptavidin-HRP diluent (for each 8 well strip). 6) After 90 minutes empty wells and wash x4. 7) Add 100ul of Streptavidin-HRP working solution to each well (except chromagen blank). 8) Cover plate and incubate at room temperature for 30 minutes. 9) Empty well and wash x4. 10) Add 100ul of stabilized chromagen to each well (liquid in the wells will begin to turn blue). 11) Incubate at room temperature (and in the dark) for 30 minutes. Incubation time is determined by the microtiter plate reader used. Some plate readers have a maximum optical density (O.D) of 2.0. O.D values should be monitored and the reaction should be stopped before the positive wells exceed the instruments limit (2.0 O.D lies between std 2 and std 3). The O.D values can only be read at 450nm once the stop solution has been added. If using an instrument that has a maximum O.D of 2.0 an incubation time of 20 -25mins is suggested. 12) Add 100ul of stop solution to each well. 13) Blank the plate against the chromagen blank (well 10 – contains only chromagen and stop solution). 14) Read the absorbance of each well at 450nm (generate a standard curve for standards/wells 1-8). 15) Correct for dilutions of controls and samples in step 4,5,6 (i.e. added 50ul sample and 50ul of dilution buffer so need to multiply mouse TNF values by 2).

2.3.6 Novel ROS analysis (ratio of oxidised to reduced protein thiols): The following method was conducted by Miss Jessica Terrill (co-author on Publications #6&7). Oxidative stress was measured as a ratio of oxidised (di-sulphide) to reduced (sulfhydryl) protein thiols using a novel technique (USA PATENT #20080305495) developed by Dr Peter Arthur (Armstrong et al, submitted)(Lui et al, 2010). Muscle samples were collected very rapidly and immediately snap frozen in liquid nitrogen (section 2.1.10). Frozen quadriceps muscles were crushed under liquid nitrogen, before protein extraction with 20% trichloroacetic acid/acetone. Protein was solubilised in 0.5% sodium dodecyl sulphate 0.5 M tris (SDS buffer), pH 7.3 and thiols were labelled with the fluorescent dye BODIPY FL-N-(2-aminoethyl) maleimide (FLM,
Invitrogen). Following removal of the unbound dye using ethanol, protein was resolubilised in SDS buffer, pH 7 and oxidised thiols were reduced with tris(2-carboxyethyl) phosphine (TCEP, Sigma) before the subsequent unlabelled reduced thiols were labelled with a second fluorescent dye texas red maleimide (Invitrogen). The sample was washed in pure ethanol and resuspended in SDS buffer. Samples were read using a fluorescent plate reader (Fluostar Optima) with wavelengths set at excitation 485, emission 520 for FLM and excitation 595, emission 610 for texas red. A standard curve for each dye was created using ovalbumin and all results were expressed per mg of protein, quantified using Detergent Compatible protein assay (BioRad) (See Results Chapters 4 & 5).

2.4 Metabolism protocols: In May 2008, the candidate participated in a 5 week internship in the laboratory of Dr Marta Fiorotto, Baylor College of Medicine, Houston USA. The following protocols were all conducted during the internship and have been written-up as a collaborative manuscript (Publication #9 – Results Chapter 7).

2.4.1 Animal usage and animal ethics: All animal experiments and analysis were carried out at The Children’s Nutrition Research Centre (CNRC) at Baylor College of Medicine, Houtston USA. Experiments were conducted in strict accordance with the U.S. National Research Council’s Guide for the Care and Use of Laboratory Animals and all procedures were approved by the Baylor Medical College Animal Care and Use Committee. The young 3 - 4 week old male mice (mdx and C57Bl/10ScSn) were bred at the CNRC from breeders originally from Jackson Laboratories.

2.4.2 Whole Body Composition Scans. Total body lean muscle and fat mass were measured by dual X-ray absorptometry (DXA) using a PIXIImus (General Electric, USA). Whilst under isoflurane anaesthesia mice were aligned on a PIXIImus measuring tray and then inserted into the machine for measurements. All mice were measured in duplicate and then returned to their cage to recover from the anaesthetic, a minimum of 2 days elapsed before protein turnover measurements were performed. Correction factors derived specifically for the CNRC PIXIImus were applied to the fat and lean mass
data to adjust for the inherent errors in fat and lean mass obtained using all PIXImus instruments (http://www.bcm.edu/cnrc/intranet/MMRU/PIXImus%20corrections.xls).

2.4.2 Calorimetry. Mice were placed individually into calorimetry chambers for 72 hrs and energy expenditure was measured using a Columbus Instruments (Columbus OH) CLAMS Oxymax System. A known flow of air was passed through the chambers and the \( \text{O}_2 \) and \( \text{CO}_2 \) gas fractions were monitored at both inlet and outlet ports. The gas fraction and flow measurements were used to calculate \( \text{VO}_2 \) and \( \text{VCO}_2 \), from which the Respiratory Exchange Ratio and heat production (kcal/hr) are calculated. At the same time spontaneous activity level (both horizontal and vertical movements) was monitored from the interruption of infra-red beams projected across the cage (See Results Chapter 7).

2.4.3 Isotope infusion and tissue collection to measure rate of protein synthesis. Food was removed from all mice 7 hours prior to isotope infusion. A flooding dose of L-4-\(^{3}\text{H}\)-phenylalanine (American Radiolabeled Chemicals, Inc., St. Louis, MO) at 20mL/kg BW, 1.5mmol phenylalanine/kg and 250µCi/mouse was given through the tail vein fifteen minutes after injection mice were sacrificed (by decapitation) and trunk blood was immediately collected and acidified to 0.2M Perchloric acid (PCA). The supernatant was frozen and reserved for estimation of the specific radioactivity of blood free phenylalanine pool. The hindlimbs were immediately detached, wrapped in foil and chilled on ice. A range of muscles including; gastrocnemius and plantaris complex (referred to hereafter as the gastrocnemius), quadriceps, soleus, tibialis...
anterior, diaphragm and heart, were then dissected on ice and frozen in liquid nitrogen. The weights of all muscles were recorded and muscles were stored at -80°C.

**Laboratory method:** These are based on Fiorotto et al (2000) and Welle et al (1993) with some modifications (Fiorotto et al, 2000; Welle et al, 1993).

**Total Protein, and Myofibrillar Proteins isolation:** Frozen muscles from each mouse were powdered, weighed and 50mg was homogenized in a low salt homogenising buffer (50 mM potassium phosphate, 0.25 M sucrose, 1% Triton X-100 pH 7). For each muscle, an aliquot of the homogenate was retained for measurement of total protein; a second aliquot was acidified to 0.2M PCA and centrifuged; the supernatant containing the muscle free amino acid precursor pool was collected and neutralized as described below. The PCA-insoluble precipitate was washed, and after assaying for total RNA, were processed for the measurement of L-[4-3H] phenylalanine incorporated into all muscle proteins. The remainder of the low salt/sucrose buffer homogenate was left to incubate with agitation at 4°C for 60 minutes; the proteins that were insoluble after low speed centrifugation (1,500 g at 4°C for 10min) contained the myofibrillar proteins (Fiorotto et al, 2000; Welle et al, 1993). After several washes of the the pellet in the low salt/sucrose buffer followed by ice-cold water, the purified myofibrils were acidified to 0.2M PCA. The acid insoluble myofibrillar and total protein precipitates were separated from the supernatant by centrifugation at 10,000 g at 2°C for 30 min, and the pellets were washed three times in 0.2M PCA. The supernatant from the total protein fraction and the pellets from all protein fractions were processed for determination of L-[4-3H] phenylalanine specific radioactivity.

**Quantification of L-[4-3H] phenylalanine specific radioactivity in the protein bound and tissue free amino acid precursor pools:** The acid-insoluble pellets were first hydrolysed for 24 hrs in 6M HCL(ultrapure) at 110°C under N₂ gas. After evaporation of the HCl in a Savant Speedvac concentrator, the remaining precipitate was subject to several washes in water and finally resuspended in mQ water for HPLC analysis. The PCA-treated blood and muscle free pool supernatants samples were neutralized on ice with 4M KOH, evaporated to dryness and resuspended in 1M acetic acid. The amino acids were purified over a Dowex anion exchange column (AG-50w-x8, 100 - 200 mesh, Biorad). The amino acids were eluted in 3M NH₄OH evaporated to dryness in the Savant Speedvac concentrator and finally dissolve in MQ water. All samples were filtered using a 0.45µm syringe filter, supernatant dried down and each sample was re-
dissolved in 100ul of MQ water. Phenylalanine in the total and myofibrillar protein hydrolysates, muscle homogenate and blood supernatants were isolated by anion exchange HPLC (AminoPac1 Analytical column; Dionex, Sunnyvale, CA). Amino acids were post-column derivatized with o-phthalaldehyde reagent and detected with an online fluorimeter. The fraction of the eluant that included the phenylalanine peak was collected, and the associated radioactivity was measured in a liquid scintillation counter (Packard Tricarb, Perkin Elmer, Waltham, MA). The phenylalanine concentration was determined by comparing the peak areas of the samples with that of a known standard (Pierce Labs.)

**Calculations of in-vivo fractional protein synthesis rates:** The fractional rate of protein synthesis (FSR), i.e., the percentage of the protein mass synthesized in a day was calculated as follows: 

\[ \text{FSR} = \left( \frac{S_B}{S_A} \right) \times \left( \frac{1440}{t} \right) \times 100 \]

Where \( S_B \) is the specific radioactivity of the protein-bound labelled amino acid, \( S_A \) is the specific radioactivity of the precursor pool and \( t \) is the the labelling time in minutes (Davis et al, 1999a). It has been demonstrated that the specific radioactivity of the tissue free phenylalanine after a flooding dose of phenylalanine is in equilibrium with the aminoacyl-tRNA specific radioactivity; hence, the tissue free phenylalanine reflects the specific radioactivity of the tissue precursor pool (Davis et al, 1999b). Blood and tissue free phenylalanine specific radioactivity values were compared to verify equilibration.

**Protein quantification:** Total protein concentration of all muscle samples was determined using a standard BCA protein (Smith et al, 1985) assay after first solubilising the aliquot of total muscle homogenate in 0.1 M NaOH for 1 hr at 37°C.

**Total RNA measurement:** Because the RNA content of a tissue is dominated by ribosomal RNA, total RNA was measured quantitatively on all muscle samples and provides an estimate of ribosomal abundance. Total RNA was quantified in the total protein PCA-insoluble precipitate using a modified Schmidt-Thannhauser procedure as described by Munro and Fleck (1966) (Munro and Fleck, 1966).

**2.5 TREAT-NMD SOP published protocol:**

Re-print included in pages 90-100.
Chapter 3 (Results 1).

Reduced muscle necrosis and long-term benefits in dystrophic mice after cV1q (blockade of TNF) treatment.

Tumour necrosis factor (TNF) is a potent inflammatory cytokine that exacerbates damage of dystrophic muscle damage in vivo (see Introduction 1.4). This chapter examined the effects of cV1q, a monoclonal murine specific antibody that neutralises mouse TNF, in dystrophic mdx mice in vivo. This work was published in 2008 (Publication #5) in the Journal of Neuromuscular Disorders 18: 227-238 and has received 12 citations (as of September 2010). The candidate completed approximately 85% of the work required for this manuscript.

There is strong evidence to suggest that inflammatory cells and cytokines play a role in skeletal muscle damage [reviewed in (Evans et al, 2009a; Grounds et al, 2008a; Tidball, 2005)]. Antibody blockade of TNF with the human/mouse chimeric antibody infliximab (Remicade®) in young mdx mice results in a striking protective effect on dystrophic myofibres and suppresses the early acute phase of myofibre necrosis (Grounds and Torrisi, 2004). A similar protective effect in young dystrophic muscle was demonstrated with etanercept (Enbrel®) a soluble TNF receptor (Hodgetts et al, 2006; Pierno et al, 2007). These results strongly support a key role for TNF in both inflammation and necrosis in dystrophic muscle.

There is controversy regarding the cross-species binding of infliximab based on in vitro tests, yet at least 4 papers report anti-inflammatory effects in vivo in mice (Grounds and Torrisi, 2004), rats (Oruc et al, 2004; Woodruff et al, 2003) and pigs (Olmarker et al, 2003). As a result this controversy Centecor (USA) generally supplied us with the mouse specific anti-TNF antibody cV1q in 2007. The present paper tested the effectiveness of the cV1q (mouse-specific anti-TNF) antibody in comparison to infliximab in both dystrophic and non-dystrophic mice, in both short and long-term (up to 90 days) studies combined with voluntary exercise. Treatment with cV1q significantly delayed inflammation and thus muscle regeneration in whole muscle.
autografts in normal non-dystrophic C57Bl/10 mice and confirmed the bio-activity of cV1q. TNF antibody treatment (cV1q treatment) of dystrophic mdx mice reduced the extent of dystropathology in both young mdx litters and voluntarily exercised adult mdx mice. This paper highlights the central role of TNF in myofibre necrosis in dystrophic muscle and suggests the use of anti-inflammatory drugs as a potential clinical therapy for DMD patients.

Additional experimental work testing the protective effects of the cV1q antibody on in vivo functional parameters in adult mdx and normal mice using a dynamometer was conducted in collaboration with Mr Adam Piers, an Honours student, in 2009 and presented in Chapter 8 of this thesis (Publication #10).
Chapter 4 (Results 2).

The early consequences of two different treadmill exercise protocols on dystrophic mdx mice.

This chapter describes in detail two standardised protocols for the treadmill exercise of mdx mice and profiles changes in molecular and cellular events after a single 30 minute treadmill session or after 4 weeks of treadmill exercise. This work was re-submitted in November 2010 to the Journal of Neuromuscular Disorders (Publication #6). The candidate completed approximately 75% of the work required for this manuscript.

Bi-weekly treadmill exercise for 4 weeks is widely used in pre-clinical experiments to increase the extent of dystropathology in mdx mice, yet the cellular consequences of a single 30 minute treadmill exercise session have not been described. The present study standardised a protocol and conducted time-course analysis of molecular and cellular changes after a single 30 minute treadmill exercise session (Protocol A). These data were compared to data from age matched mdx mice subjected to 4 weeks of treadmill exercise (Protocol B) a protocol currently widely used for pre-clinical drug screening in mdx mice (Burdi et al, 2009; De Luca et al, 2003; Granchelli et al, 2000; Grounds et al, 2008b).

Both treadmill protocols increased multiple markers of muscle damage. It was concluded that a single 30 minute treadmill exercise session is a sufficient and conveniently fast screening test to evaluate the benefits of pre-clinical drugs in vivo. Myofibre necrosis, blood serum CK and oxidative stress (specifically the ratio of oxidised to reduced protein thiols) are reliable markers of muscle damage after exercise; however sampling time (after exercise) for these parameters is critical.

A more precise understanding of the changes in dystrophic muscle after exercise aims to identify the immediate molecular and cellular changes resulting from a single short treadmill exercise session; this novel information has the potential to identify new
reliable biomarkers and new therapeutic drug targets for Duchenne Muscular Dystrophy.

The detailed information gathered in this study (treadmill protocol, sampling time points and markers of muscle damage) was used to design an *in vivo* experiment which examined the potential benefits of N-acetylcysteine (NAC) treatment in dystrophic mdx mice (Chapter 5, Publication #6).
Chapter 5 (Results 3).

The benefits of N-acetylcysteine treatment in dystrophic mdx mice.

In addition to inflammation (discussed in chapters 4 & 8) and altered metabolism (discussed in chapters 6 & 7), oxidative stress is another proposed mechanism by which the absence of functional dystrophin protein leads to myofibre necrosis. Whether it is by direct macromolecule damage or via reversible modifications to protein function, oxidative stress has the potential to be a major contributor to the pathology associated with Duchenne Muscular Dystrophy [Reviewed in (Arthur et al, 2008; Tidball and Wehling-Henricks, 2007; Whitehead et al, 2006b)].

N-acetylcysteine (NAC) is an anti-oxidant which decreases oxidative stress by scavenging ROS; NAC is highly attractive as a clinical treatment since it is readily available, inexpensive and has few adverse side effects. This body of work initially began as a direct comparison between the potential therapeutic benefits of the anti-inflammatory drug cV1q (TNF antibody – see chapter 4) and the antioxidant drug NAC. It was hypothesised that conducting two parallel in vivo experiments testing the effects of both cV1q and NAC, by using identical experimental methods and analysis measures, would enable identification of the more ‘promising’ therapeutic intervention. Unfortunately, due to numerous problems with the cV1q experiments (e.g. concerns regarding efficacy of different antibody batches and conflicting results), the cV1q work was abandoned and only the NAC work was continued.

The final chapter (manuscript #7) examines the benefits of NAC treatment (at 2 different doses and treatment regimes) in both sedentary and treadmill exercised adult mdx mice in vivo and also attempts to determine the mechanism by which NAC works in dystrophic muscle. This chapter puts into practice the methods developed in chapter 4, including the exact 30 minute treadmill exercise protocol, mouse sampling time-points (after exercise) and biomarkers to measure muscle damage after treadmill exercise.

In sedentary mdx mice NAC treatment had no effect on myofibre necrosis or membrane damage (measured by serum CK level). NAC treatment had no effect on
malondialdehyde (MDA) level or protein carbonylation (two indicators of irreversible oxidative damage); however 4% NAC treatment did significantly reduce oxidized glutathione and oxidized protein thiols in sedentary mdx mice. Also, in treadmill exercised mdx mice, NAC treatment prevented exercise induced myofibre necrosis and membrane damage. These novel in vivo data show that NAC protects dystrophic muscle from exercise induced myofibre necrosis, the primary cause of severe muscle pathology seen in DMD. The exact mechanism by which NAC acts is unclear at this stage; however it appears to be multi-factorial.

This work was submitted to The International Journal of Biochemistry and Cell Biology in January 2011 a copy of the manuscript (manuscript #7) in included. The candidate completed approximately 40% of the work required for this manuscript.
Chapter 6 (Results 4).

The different impacts of a high fat diet on dystrophic mdx and control C57Bl/10 mice.

The absence of functional dystrophin protein in dystrophic mdx mice leads to fragile myofibre membranes and cycles of myofibre necrosis and regeneration. Studies in both DMD patients and mdx mice indicate that the absence of dystrophin may also (directly or indirectly) lead to alterations in skeletal muscle metabolism and an impaired energy status (Davidson and Truby, 2009; Dupont-Versteegden et al, 1994a; Landisch et al, 2008; MacLennan and Edwards, 1990; Passaquin et al, 2002; Zanardi et al, 2003). In addition to anti-inflammatory and antioxidant drug interventions such as cV1q and NAC, aimed to reduce the severity of dystropathology (see Chapters 3, 5 & 8), dietary interventions (e.g specific amino acid supplementation) have shown beneficial effects in both mdx mice and DMD patients [Reviewed in (Leighton, 2003; Pearlman and Fielding, 2006; Radley et al, 2007)].

This study (Chapter 6) directly compares the response of control C57Bl/10 and dystrophic mdx mice to a high protein (50%) and a high fat (16%) diet. Surprisingly, consumption of a high protein diet had minimal effects on the body composition or dystropathology of C57Bl/10 and mdx mice. In contrast, striking differences between the strains were seen in response to the high fat (16%) diet; this response also varied between mdx mice aged <24 weeks and up to weeks. C57Bl/10 mice demonstrated many negative side effects after consuming a high fat diet with significant weight gain, increased body fat and elevation of pro-inflammatory cytokines. In contrast, mdx mice (< 24 weeks) remained lean with minimal fat deposition and were resistant to changes in body composition after consuming a high fat diet. These results support the proposal that dystrophic mdx mice have an altered ‘energy status’ compared to normal C57Bl/10 mice since the mdx mice were more capable of efficiently processing and utilising increased dietary fat. However, older mdx mice (24-40 weeks old) exhibited some effects of a high fat diet but to a lesser extent than age matched C57Bl/10 mice. A high fat diet significantly increased the running ability (distance - km) of voluntarily exercised mdx mice and significantly reduced myofibre necrosis in 24
week old sedentary mdx mice, suggesting that increased dietary fat (and increased energy intake) can assist energy deficient mdx mice metabolically, providing them with additional energy to run further while maintaining muscle integrity. This research clearly identifies an ‘altered’ response to a high fat diet in dystrophic mdx mice compared to C57 controls. Energy deficient mdx mice appear to be utilising excess dietary fat to reduce the severity of dystropathology and increase voluntary exercise ability. Our new data highlight the potential benefits of a high fat diet on dystrophic muscle.

It must be noted that the custom diets used in this study, high fat and high protein, were initially designed to be isocaloric based on digestible energy content (HP – 18.2 MJ/Kg vs. HF – 18.1 MJ/Kg). Major differences in body composition were seen in response to the high fat and high protein diets and it became apparent that metabolisable energy content was a more appropriate way to assess energy intake of the mice. There are large differences in the metabolisable energy content of the high fat and high protein diet (HP – 14.09 MJ/Kg vs. HF – 16.96 MJ/Kg) which explains the major differences in energy intake and thus body composition in response to the two diets.

This manuscript has been prepared for submission in *Neurobiology of Disease* and has been formatted accordingly. The candidate completed approximately 90% of the work required for the manuscript.

In addition to the work presented in this chapter (Chapter 6), dietary interventions involving an Omega-3 enriched diet and dietary interventions in mdx litters and female mdx mice are briefly discussed in Appendix 1. These were major studies conducted over approximately 8 months that involved approximately 100 mice; however the work was fatally flawed due to errors in diet manufacture and experimental design. Because of these fatal flaws and the time and resources that would have been required to repeat this studies, the project and full analyses were terminated. This information is provided as an Appendix (Appendix 1 in this thesis) in case reference to the design of the study and the available data are of use for future work.
Chapter 7 (Results 5).

Comparison of metabolism and protein synthesis rates in young and adult dystrophic mdx and control C57Bl/10 mice.

In May 2008, the candidate participated in a 5 week internship in the laboratory of Dr Marta Fiorotto, at Baylor College of Medicine, Houston USA. The following work was conducted during the internship and has been written-up as a collaborative manuscript (Publication #9 – Chapter 7 that follows). The candidate completed approximately 50% of the work required for the manuscript. The manuscript has been prepared for submission to the Journal of Clinical Investigation and has been formatted accordingly.

Dystrophic skeletal muscle is subject to many metabolic abnormalities that can greatly impact on the maintenance of muscle mass and function. Repeated cycles of myofibre necrosis and regeneration, increased demand on the sarcoplasmic reticulum to regulate intracellular calcium, defective mitochondrial function, increases in both protein synthesis and protein degradation rates, and disruption in nNOS signalling may all contribute to an altered metabolic state (Dupont-Versteegden et al, 1994a; Griggs and Rennie, 1983; Han et al, 2006; Kuznetsov et al, 1998; Landisch et al, 2008; MacLennan and Edwards, 1990; Passaquin et al, 2002; Whitehead et al, 2006b; Zanardi et al, 2003).

Changes in protein metabolism have been reported in various mdx muscles both in vivo and ex vivo (Helliwell et al, 1996; MacLennan and Edwards, 1990; MacLennan et al, 1991a; MacLennan et al, 1991b; Turner et al, 1988) and, in contrast to older DMD patients, adult mdx mice maintain skeletal muscle mass and experience significant skeletal muscle hypertrophy (Mokhtarian et al, 1996; Peter and Crosbie, 2006; Shavlakadze et al, 2010; Spurney et al, 2009), indicating that protein synthesis must outweigh protein degradation in adult mdx mice. A previous study that examined the metabolic state of dystrophic mdx mice reported no change in the metabolic rate \([\text{kcal}/(\text{kg bodyweight}^{0.75} \cdot \text{d})]\) of one year old adult mdx mice, yet a decrease in the metabolic rate \([\text{kcal}/(\text{kg bodyweight}^{0.75} \cdot \text{d})]\) of 4-6 week old (mixed sex) mdx mice.
(Dupont-Versteegden et al, 1994a). Mokhtarian et al (1996) also reported no difference in total energy expenditure, basal energy expenditure and spontaneous activity between control C57Bl/10 and mdx mice (sex unspecified) at 6-12 months of age (Mokhtarian et al, 1996). Clearly, the mouse age, extent of dystropathology and the presence of regenerating (growing) muscle, may have a major impact on metabolic demand and energy usage.

The purpose of this longitudinal study (over approximately 10 days) was to measure food intake, energy expenditure, activity level, body composition and protein synthesis rate (of both whole muscles and of the myofibrillar fraction) of dystrophic mdx and control C57BL/10 mice at 2 different ages; 1) Young mice aged 3-4 weeks (shortly after the acute onset of muscle necrosis in mdx mice when muscle damage regeneration is at a peak); and 2) Adult mice aged 13-14 weeks (when adult mdx mice exhibit a lower level of muscle damage and dystropathology).

Young mdx mice had a ‘stunted’ growth; they were significantly lighter with reduced fat free mass (lean muscle mass). Young mdx mice also had a significantly increased protein synthesis rate (in both whole muscle samples and isolated myofibrillar fraction) and increased metabolic rate (Heat kcals/24hr/kg ffm). The acute onset of myofibre necrosis and regeneration at 3-4 weeks of age appears to have had a significant effect on many parameters related to muscle mass and function, and metabolic rate in young mdx mice. There was significant ‘catch-up’ growth in mdx mice between 4 and 14 weeks of age, with 14 week old adult mdx mice being heavier, with significantly increased muscle mass and bone length than C57Bl/10 mice. The adult mdx mice were very lean, with significantly increased fat free mass (lean muscle mass) and increased protein synthesis rates (in both whole muscle samples and isolated myofibrillar fraction) compared with non-dystrophic control mice.

These findings emphasise major metabolic differences, mainly due to protein synthesis rates, between control and dystrophic mdx mice and have many implications for understanding the basic pathology of DMD.
Chapter 8 (Results 6).

Additional work completed during candidature; not central to the Thesis.

In addition to the 5 main results chapters included in this thesis, the candidate made a significant contribution to 2 further published manuscripts. Re-prints of both manuscripts are included in this chapter (Publications #10 & 11).


Candidate completed approximately 20% of the work required for the manuscript. The candidate advised and trained on all aspects of in vivo work including animal treatment, cV1q dosages, animal sacrifice and tissue sampling, histological analysis and interpretation of histological results. This work is a direct continuation from Chapter 3 (Publication #5) in this thesis and was conducted in order to show the in vivo functional benefits of cV1q treatment in mdx mice in addition to the histological benefits already shown (Publication #5).

This study shows that blockade of the pro-inflammatory cytokine tumour necrosis factor (TNF) with the mouse specific antibody cV1q, protects dystrophic skeletal myofibres against initial contractile dysfunction and subsequent myofibre necrosis in adult mdx mice subject to eccentric exercise. Damaging eccentric exercise was conducted in vivo using a custom built mouse dynamometer (Hamer et al, 2002).

The candidate completed approximately 20% of the work required for the manuscript. The candidate advised and trained on all aspects of in vivo work including animal ethics, whole muscle autograft surgery, animal sacrifice and tissue sampling, histological analysis and interpretation of histological results. The collaborative work was conducted as part of an ARC grant investigating new ways of imaging whole muscles and development of new technologies as potential alternatives to animal sacrifice and subsequent histology.

This manuscript describes in detail the ability of Three-dimensional optical coherence tomography (3D-OCT) to evaluate the structure and pathology of whole muscle autografts ex vivo. 3D-OCT images, co-registered with histology, can readily distinguish necrotic and inflammatory tissue from intact healthy muscle fibres. These preliminary finding suggest that with further development 3D-OCT could be used as a tool for evaluation of small-animal (mouse) morphology in vivo.
Overall discussion and general conclusion.

Myofibre necrosis is the fundamental destructive process in Duchenne Muscular Dystrophy (DMD). The exact mechanism by which the absence of dystrophin leads to myofibre necrosis is unknown, however inflammation, oxidative stress, metabolic abnormality, mislocalisation of nNOS and increased intracellular calcium are all heavily implicated in the process (summarised in Figure 9.1) [Reviewed in (Evans et al, 2009a; Evans et al, 2009b; Grounds et al, 2008a; Leighton, 2003; Radley et al, 2007; Tidball and Wehling-Henricks, 2004b; Tidball and Wehling-Henricks, 2005; Tidball and Wehling-Henricks, 2007; Wallace and McNally, 2009)]. It seems likely that myofibre necrosis is due to a combination of many of these dysregulated mechanisms and not the result of one sole mechanism. The mdx mouse is a very useful animal model to examine the potential mechanisms by which the absence of dystrophin leads to myofibre necrosis and to test potential therapeutic interventions to reduce the extent of dystrophopathology in vivo. While replacement of the defective dystrophin by cell or gene therapy, or molecular interventions is the definitive treatment option for DMD, there are many problems to overcome before clinical application (Bushby et al, 2009; Cossu and Sampaolesi, 2007; Grounds and Davies, 2007; Guglieri and Bushby, 2010; Manzur and Muntoni, 2009; Nagaraju and Willmann, 2009; Odom et al, 2010; Odom et al, 2007; Partridge, 2010; Tremblay and Skuk, 2008; Wells, 2008).

The present body of work examined the potential benefits of 3 therapeutic interventions on dystrophic mdx and control mice in vivo: 1) an anti-inflammatory drug, cV1q a mouse specific TNF antibody, 2) an antioxidant drug, N-acetylcysteine a scavenger of ROS, and 3) dietary interventions, specifically a high fat (16%) and high protein (50%) diet, plus metabolic analyses of the 2 strains. All 3 therapeutic interventions showed some benefits in mdx mice, confirming the involvement of inflammation, oxidative stress and metabolic abnormality in myofibre necrosis in dystrophic muscle. A detailed discussion is given for each of these interventions in the appropriate manuscript/chapter and here an overall discussion is presented with a focus on potential clinical relevance.
Figure 9.1 Mechanisms for membrane degeneration in dystrophic muscle. The dystrophin glycoprotein complex (DGC) is a complex that links the extracellular matrix component to the actin cytoskeleton in muscle cells (Left). Absence of dystrophin causes DMD characterised by sarcolemmal disruptions (Right). In the absence of proper membrane-matrix attachment, tears may simply develop as a result of the mechanical stress. An increase in free radicals (green triangles) may be caused by the displacement of NOS from the plasma membrane. The high levels of free radicals in dystrophic muscle are thought to contribute to muscle degeneration via the oxidation of muscle membranes and recruitment of macrophages. Calcium-sensitive pathways also contribute to muscle degeneration. Calcium (pink spheres) may enter dystrophic muscle through membrane lesions or through calcium channels. Calcium dysregulation may also lead to abnormal mitochondrial function as well as the activation of the calcium-dependent protease calpain to degrade muscle membrane proteins. Adapted from Wallace and McNally (2009) (Wallace and McNally, 2009).

Anti-inflammatory therapy: The potential therapeutic benefits of cV1q, a monoclonal murine specific antibody that neutralises mouse TNF, in dystrophic mdx mice were examined (Chapter 3). Both short and long-term studies demonstrate that cV1q antibody treatment reduces the extent of dystrophopathy in both young and voluntarily exercised adult mdx mice (Radley et al, 2008) and supports the original hypothesis that TNF contributes to necrosis of dystrophic muscle. The protective benefits of cV1q were similar to those seen with the human/mouse chimeric TNF antibody infliximab (Remicade®) in young mdx mice (Grounds and Torrisi, 2004) and with etanercept (Enbrel®) a soluble TNF receptor (Hodgetts et al, 2006; Pierno et al, 2007). All these results strongly support a key role for TNF in both inflammation and necrosis in dystrophic muscle. Interestingly, long-term treatment with cV1q had no beneficial effects on sedentary adult mdx mice. This indicates that the already low
level of background dystropathology in adult mdx mice is not further reduced by cV1q blockade of TNF, whereas cV1q clearly protected against exercise induced acute myofibre damage (more representative of the severity of the human condition). The lack of effect in unexercised mice is in striking contrast to the benefits of cV1q in exercised mdx mice: whether this reflects differences in the cellular responses in these two situations is unknown (Radley et al, 2008). The study confirms that a drug intervention may not show benefits in unexercised mdx mice, thus exercise appears an essential part of the ‘standard operating procedure’ to effectively test the benefits of anti-inflammatory drugs in mdx mice in vivo [Reviewed in (Grounds et al, 2008b)].

Numerous studies report the beneficial effects of anti-inflammatory therapies in dystrophic mdx mice [Reviewed (Bogdanovich, 2004; Evans et al, 2009b; Manzur and Muntoni, 2009; Radley et al, 2007; Spurney et al, 2009)], but this is the first study to report the in vivo effects of a mouse specific antibody that targets TNF in dystrophic mdx mice. TNF blockade may be a relatively expensive clinical treatment option for DMD and the potential long-term side effects of TNF blockade in DMD patients is unknown, although infliximab (Remicade®) is widely used clinically to treat other human conditions in adults and children such as rheumatoid arthritis and Crohn’s disease with much success and relatively mild side-effects (Kageyama et al, 2006). The optimal regime and dosage, as well as the most appropriate humanised anti-TNF drug needs to be selected for possible trials in DMD boys. The development of new TNF drugs, such as TNF-TeAb (mini antibodies), further complicate the decision (Liu et al, 2007). We propose that TNF targeted therapies are more specific and may have fewer side effects than corticosteroid treatment and it is surprising that clinical trials of TNF based therapies have not yet been conducted.

**Anti-oxidant therapy:** The second drug treatment examined in this Thesis was the antioxidant, N-acetylcysteine (NAC) (Chapter 5). The beneficial effects of NAC were examined in both sedentary and treadmill exercised mdx mice, using the exact experimental methods and analysis methods optimised in Chapter 4. NAC was chosen as the antioxidant therapy, as it contains a thiol which can directly scavenge some types of ROS directly (Zafarullah et al, 2003), it is also a precursor of L-cysteine which is required in the synthesis of the major intracellular antioxidant glutathione (Dilger and
NAC can be administered orally, it is readily available and it is widely used clinically. NAC has previously shown to improve muscle strength ex vivo and reduce some aspects of dystropathology in sedentary mdx mice (Whitehead et al, 2008), and sedentary Stra13 knock-out mice (which exhibit a dystrophy-like pathology) (Vercherat et al, 2009). To our knowledge this is the first study to extensively demonstrate the in vivo benefits of NAC treatment on muscle histopathology, serum CK level and oxidative state of both sedentary and exercised adult mdx mice.

NAC was administered via drinking water (1% or 4%) for a maximum duration of 6 weeks. NAC is reported to have an unpleasant bitter taste (Crouch et al, 2007; Pendyala and Creaven, 1995) and administration of 4% NAC to mice via drinking water resulted in a slight reduction in water intake and a slight reduction in body weight. For this reason, it is important to consider an alternative method of delivery when conducting long-term experiments with high concentrations of NAC. Miss Jessica Terrill, a PhD student in our laboratory, is currently investigating appropriate administration methods (e.g dietary supplement or gavage) and the best concentration of NAC for therapeutic in vivo studies in mdx mice. The ‘bitter’ taste of NAC is less of a factor when administering to human patients as it can be rationally tolerated or administered in an enclosed capsule; however a bitter taste may influence the compliance rate of treatment.

This work in Chapter 5 initially began as a comparison between the potential in vivo benefits of NAC treatment vs. cV1q treatment in exercised adult mdx mice. It was hypothesised that conducting two parallel in vivo experiments testing the effects of both cV1q and NAC, (using identical experimental methods and analysis measures) would enable identification of the more ‘promising’ therapeutic intervention. Unfortunately, due to numerous problems with the cV1q experiments (e.g. concerns regarding efficacy of different antibody batches and conflicting results), the cV1q work was abandoned and only the NAC work was continued; therefore a direct comparison between the in vivo effects of NAC vs. cV1q could not be made. However, when the relative improvements seen in the two separate studies (Chapter 3 vs. Chapter 5) are compared, both interventions appear to ameliorate dystropathology to a similar level. For example, both 1% and 4% NAC treatment, reduced myofibre necrosis.
approximately 3 fold (15% vs 6%) after 30 minutes of treadmill exercise and 4% NAC treatment reduced blood serum CK approximately 3 fold (12 000U/L vs 32 000U/L) after treadmill exercise. In comparison, cV1q treatment reduced myofibre damage (in young 24 day old mice) approximately 2 fold (15% vs. 30%), reduced myofibre necrosis after 48hrs voluntary exercise approximately 2 fold (7% vs 13%) and reduced serum CK in long-term voluntarily exercised mice approximately 4 fold (4000U/L vs. 18000U/L) (Radley et al, 2008).

Similar to the results seen with cV1q administration (Radley et al, 2008), differences in the extent of dystropathology (and thus the effectiveness of NAC treatment) varied between sedentary and exercised mdx mice, that is both 1% and 4% treatment showed limited beneficial effects in sedentary adult mdx mice (Chapter 5). This again demonstrates the importance of exercising adult mdx mice and confirms that exercise should be part of the ‘standard operating procedure’ to effectively test the effects of potential pre-clinical drugs in adult mdx mice in vivo [Reviewed in (Grounds et al, 2008b; Nagaraju and Willmann, 2009)]. NAC treatment showed many in vivo benefits in mdx mice and our novel in vivo data supports the proposed clinical trials of NAC in genetic myopathies. [http://www.treat-nmd.eu/about/TACT/previous-reviews---june-2010/](http://www.treat-nmd.eu/about/TACT/previous-reviews---june-2010/)

**Dietary interventions:** Previous studies in both DMD patients and mdx mice indicate that the absence of dystrophin may also (directly or indirectly) lead to alterations in skeletal muscle metabolism and an impaired energy status (Davidson and Truby, 2009; Dupont-Versteegden et al, 1994a; Landisch et al, 2008; MacLennan and Edwards, 1990; Passaquin et al, 2002; Zanardi et al, 2003). In addition to these published data, the striking differences in response to a high fat diet between mdx and C57Bl/10 mice (identified in chapter 6) strongly indicating that dystrophic mdx mice have an ‘altered’ metabolic state.

Chapter 6 directly compared the response of control C57Bl/10 and dystrophic mdx mice to a high protein (50%) and a high fat (16%) diet and identified striking differences between strains in response to the high fat (16%). C57Bl/10 mice demonstrated many negative side effects after consuming a high fat diet with
significant weight gain, increased body fat and elevation of pro-inflammatory cytokines. In contrast, mdx mice (< 24 weeks) remained lean with minimal fat deposition and were resistant to changes in body composition after consuming a high fat diet. These results support the proposal that dystrophic mdx mice have an altered ‘energy status’ compared to normal C57Bl/10 mice since the mdx mice were more capable of efficiently processing and utilising increased dietary fat. However, older mdx mice (24-40 weeks old), where energy intake (kJ/day and kJ/g bw /day) was significantly increased compared to mdx mice on the control diet, exhibited some negative effects of a high fat diet (increase in both bodyweight and standardised fat pad weight) but to a lesser extent than age matched C57Bl/10 mice. The negative effects of a high fat diet seen in older mdx mice (24-40 weeks) also corresponds to a very low level of myonecrosis and a cease in myofibre hypertrophy (indicative of diminished new muscle formation and thus reduced additional energy demands due to the status of the endogenous dystrophopathology). A high fat diet (and increased energy intake) significantly increased the running ability (distance - km) of voluntarily exercised mdx mice and significantly reduced myofibre necrosis in 24 week old sedentary mdx mice, suggesting that increased dietary fat (and increased energy intake) can assist energy deficient mdx mice metabolically, providing them with additional energy to run further while maintaining muscle integrity. This supports the notion of a high fat (or high energy) diet to reduce the severity of DMD, although this must be balanced against the potential for obesity.

It must be noted that the custom diets used in this study (Chapter 6), high fat and high protein, were initially designed to be isocaloric based on digestible energy content (HP – 18.2 MJ/Kg vs. HF – 18.1 MJ/Kg). Major differences in body composition were seen in response to the high fat and high protein diets and it became apparent that metabolisable energy content was a more appropriate way to assess energy intake of the mice. There are large differences in the metabolisable energy content of the high fat and high protein diet (HP – 14.09 MJ/Kg vs. HF – 16.96 MJ/Kg) which explains the major differences in energy intake and thus body composition in response to the two diets. Therefore it is important to determine if the lack of benefits on dystrophopathgy with a high protein diet is due to diet composition (i.e. an increase in dietary protein vs. Fat) or because consumption of the high protein diet did not result in an equivalent
increase in energy intake (kJ/day). This question could be addressed by administering a higher protein diet with a higher level of metabolisable energy and repeating the analysis methods employed in Chapter 6: this additional experiment is now planned. This is clearly of much interest since protein supplements are indeed used in an attempt to maintain muscle mass in DMD boys, yet there is little conclusive evidence for such nutritional supplements. It would seem that a high protein diet (with increased energy intake) may be more desirable than a high fat diet as a source of energy due to the potential adverse effects of a high fat diet.

**Altered metabolism in mdx mice:** The striking differences in response to a high fat diet between mdx and C57Bl/10 mice (chapter 6) raised the question ‘Why don’t mdx mice get fat after consuming a high fat diet?’ This led us to evaluate the differences in metabolism, body composition and protein turnover in muscle between dystrophic mdx and control C57Bl/10 mice, this research was conducted in collaboration with Dr Marta Fiorotto at Baylor Medical college, Houston USA (Chapter 7).

Young mdx mice (~4 weeks) have a ‘stunted’ growth, increased protein synthesis rates and increased metabolic rate and it appears that activity levels are reduced and energy intake is increased in order to help to compensate for this. It is highly likely that the acute onset of myofibre necrosis and initiation of new muscle formation during regeneration at 3-4 weeks of age has a significant effect on metabolic rate in young mdx mice and the onset of muscle hypertrophy, since growing new myofibres remain highly responsive to growth factors, whereas mature myofibres are less responsive to growth factors such as IGF-1 (Shavlakadze et al, 2010). There is significant ‘catch-up’ growth in mdx mice between 4 and 14 weeks of age, and 14 week old adult mdx mice are heavier than C57Bl/10 mice, with significantly increase muscle mass and bone length. Adult mdx mice are very lean, with significantly increased fat free mass (muscle mass) and increased protein synthesis rates. Similar to the results seen in Chapter 6, adult mdx mice appear to be focused on maintenance (and hypertrophy) of muscle mass and show very little fat deposition. In addition to increased protein synthesis rates and continuous cycles of myofibre necrosis and regeneration, dystrophic mdx mice exhibit significant myofibre hypertrophy up to 24 weeks of age (chapter 6), protein synthesis is a very energy expensive procedure and may explain why in mdx
mice (with the same increase in energy intake compared to C57Bl/10 mice) do not exhibit the same negative side-effects of a high fat diet.

Significant differences in body composition (e.g. muscle mass and fat mass) between young and adult mdx mice and between mdx and C57Bl/10 mice, may lead to changes in the metabolism and clearing of various therapeutic drugs and thus, where possible, dosage and half-life of different therapeutic interventions should be considered and optimised for mdx mice of various ages (Hanley et al, 2010).

At this stage, it is still unclear if the metabolic differences between strains are an innate feature of dystrophic muscle or a result of the continuous cycles of myofibre necrosis and regeneration, and the high energy demands of growing myofibres in dystrophic muscle. This question could be addressed by conducting similar metabolic studies on young ‘pre-necrotic’ mdx mice (<20 days old). However, it must be noted that conducting metabolic studies on young rapidly growing un-weaned mdx mice may be complicated by dystrophic mothers being energy deficient themselves and this impacting on milk availability for mdx pups, thus studies would need to be conducted on cross-fostered (non-dystrophic mother) mdx litters. Alternatively, metabolic analysis could be conducted \textit{in vitro} on mature myotubes in primary muscle cultures (Dystrophic vs. Control) since mdx myotubes do not manifest necrosis under routine tissue culture conditions.

In collaboration with Dr Matt McDonagh (Department of Primary Industries, Victoria), we are currently conducting a metabolomics screening study, using Nuclear Magnetic Resonance (NMR) spectroscopy (Griffin and Des Rosiers, 2009; Jones et al, 2005), of skeletal muscle metabolites in both pre-necrotic (15 day old litters) and adult (12 week old adult) mdx and C57Bl/10 mice. Information gathered from this collaboration will help to further understand possible subtle differences in metabolism between both pre-necrotic and adult dystrophic and control mice.

**Combined therapies:** It seems likely that excess inflammation, elevated oxidative stress and metabolic abnormality all contribute to myofibre necrosis via slightly different mechanisms and that targeting one mechanism alone may only partially
ameliorate dystropathology, and may not prevent it completely. Although not examined in this thesis, it is highly likely that combined therapeutic interventions (e.g. an anti-TNF or antioxidant drug treatment + a high fat/ high protein diet) may have cumulative beneficial effects in dystrophic muscle and should be pursued in pre-clinical mdx studies. Inflammation and oxidative stress are tightly linked [Reviewed in (Arthur et al, 2008; Tidball, 2005; Tidball and Wehling-Henricks, 2007)] and we have shown that the antioxidant NAC can act to reduce both oxidative stress and inflammation in dystrophic muscle (Chapter 5), therefore combined therapeutic intervention of both an anti-inflammatory and an antioxidant drug might not result in any additional benefit. However, combined therapy of a high fat diet (increased energy intake) with an anti-inflammatory or antioxidant drug may have a cumulative benefit as the two appear to be working through different mechanisms. Increasing numbers of recent studies in mdx mice are trialling such combined therapies and demonstrate that dietary interventions in combination with prednisolone have additive benefits to reduce the severity of dystropathology (Cozzoli et al, 2010; Payne et al, 2006). A deeper understanding of the precise mechanisms underlying the different aspects of dystropathology, specifically further clarification of the enigma of metabolic differences and benefits of different nutritional supplements, will greatly enhance the optimal design of such combined therapies.

**Development of a 30 minute treadmill protocol:** Chapter 4 in this Thesis, focused on the development of a short and repeatable *in vivo* treadmill exercise protocol. Bi-weekly treadmill exercise is widely used in pre-clinical experiments to increase the extent of dystropathology in mdx mice (Burdi et al, 2009; De Luca et al, 2003; Granchelli et al, 2000; Grounds et al, 2008b), yet the cellular consequences of a single 30 minute treadmill exercise session had not previously been described. The present study (Chapter 4) standardised a protocol and conducted time-course analysis of molecular and cellular changes after a single 30 minute treadmill exercise session. It was concluded that a single 30 minute treadmill exercise session is a sufficient and conveniently fast screening test to evaluate some of the benefits of pre-clinical drugs *in vivo*. This has major applications to speed up pre-clinical drug screening in mdx mice. This study also showed that myofibre necrosis, blood serum CK and oxidative stress (specifically the ratio of oxidised to reduced protein thiols) are reliable markers of
muscle damage after such treadmill exercise; however sampling time (after exercise) for these parameters is critical. This study further emphasises the need for standard operating procedures in order to compare experimental data between different laboratories. The methods developed in the study were employed when assessing the beneficial effects of NAC in vivo (Chapter 5).

While Chapter 4 identified 30 minutes of treadmill exercise to be an efficient screening method to assess potential therapeutic drugs in vivo, there are also many benefits of using voluntary exercise. For example, distance run (km) is a valuable indicator (albeit indirect) of exercise capacity and thus muscle function after therapeutic intervention (Call et al, 2008 11905; Radley et al, 2008), voluntary exercise also allows the mice to run at night during their normal ‘active’ hours and is thus less stressful (Grounds et al, 2008b; Radley and Grounds, 2006). The whole process does not require intensive researcher involvement and is easily conducted over many months. Further discussion of the advantages and limitations of both exercise methods is covered in section 1.3.2 of this Thesis.

The TNF antibody, cV1q (Chapter 3) (Radley et al, 2008) and the high fat and high protein dietary interventions (Chapter 6) were assessed in voluntarily exercised adult mdx mice. One reason for using voluntary exercise in these 2 studies is that they were conducted prior to the availability of a rodent treadmill in our laboratory group, plus the voluntary running is especially convenient for long term studies. It is striking that a single 30 minute bout of treadmill exercise resulted in a similar increase in myofibre necrosis to that seen with 48hrs voluntary exercise, endorsing a similar muscle susceptibility to both types of exercise.

<table>
<thead>
<tr>
<th>Specific exercise regime and gender</th>
<th>Myofibre necrosis in the quadriceps (% CSA)</th>
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<tr>
<td>48 hours voluntary exercise (F)</td>
<td>12.93% (\pm 3.08)</td>
</tr>
<tr>
<td>8 weeks voluntary exercise (F)</td>
<td>10.19% (\pm 1.96)</td>
</tr>
<tr>
<td>30 minutes treadmill exercise (24hrs post) (M)</td>
<td>15.06% (\pm 6.01)</td>
</tr>
<tr>
<td>4 weeks treadmill exercise (M)</td>
<td>11.83% (\pm 2.22)</td>
</tr>
</tbody>
</table>

**Table 9.1 Myofibre necrosis after exercise in mdx mice.** Myofibre necrosis in the quadriceps muscle is similar across the 4 exercise protocols used in this Thesis.
Indeed, it is very interesting to note that the amount of myofibre necrosis (% cross-sectional area) in the quadriceps muscle of exercised adult (6-12 weeks) mdx mice is similar after all 4 types of exercise used in this Thesis (Table 9.1)(Radley et al, 2008) and did not differ between male and female mice. This suggests that there is a certain number of myofibres at any one time that are ‘vulnerable’ to exercise induced damage and that increasing the duration of exercise (e.g increasing voluntary exercise from 48hrs up to 8 weeks) or increasing the number of exercise sessions a mouse is required to complete (e.g. a single 30 minute session vs. 8x 30 minute sessions) does not increase the percentage of myofibre necrosis. These data also demonstrates that dystrophic myofibres which undergo necrosis after contraction induced damage are fully capable of regenerating between exercise bouts (Chapter 4).

It is possible that if the ‘severity’ of the exercise protocol is increased (e.g. eccentric downhill running) that even more myofibre necrosis would be seen. While myofibre necrosis in the quadriceps muscle after eccentric exercise does not seem to be reported in the literature, higher levels of myofibre damage after eccentric downhill running for other muscles is described, for example: 3 downhill running sessions (15º decline) for 10 minutes at a speed of 10m/minute, caused 22.92% (+/- 1.08) damage in the triceps and 23.83% (+/- 1.18) damage in the gastrocnemius, as measured by Evans Blue Dye positive myofibres (Brussee et al, 1997). It is noted that Evans Blue Dye identifies ‘myofibre leakiness’ which does not always correspond to myofibre necrosis (Archer et al, 2006; Grounds et al, 2008b; Hamer et al, 2002; Straub et al, 1997) but these data do support higher levels of myofibre damage after more severe types of exercise.

**Variation in mdx mouse:** A high level of variation in the extent of dystropathology in mdx mice (within and between both individual mice and whole litters) is identified throughout this thesis (Chapter 3 & 4). This is an extremely important point to consider when conducting pre-clinical studies in mdx mice and treatment groups should always consist of age, sex and weight (where possible) matched mdx mice (Grounds et al, 2008b)(Willmann et al, in preparation). Analysis (histological or molecular) should also be conducted on the same muscle (or muscle group), since the extent of
A clear example of variation is documented in Chapter 4. Numerous experiments (control groups from 9 separate experiments) conducted in our laboratory revealed high levels of variation in the extent of dystropathology in various muscles of unexercised adult mdx mice. The variation in myofibre necrosis (% CSA) in the quadriceps muscle from 12 week old sedentary male mdx mice ranges from 1.04 – 23.1% for individual muscles (Chapter 4). When results from the 9 experiments were pooled together (n=60 quadriceps) the average amount of myofibre necrosis in the quadriceps muscle of a 12 week old unexercised male mdx mouse is 6.12% (Chapter 4). Similar variation was seen in the triceps and gastrocnemius muscles. These ‘pooled’ histological data were used in the assessment of myofibre necrosis after treadmill exercise (Chapter 4) and to assess the beneficial effects of NAC treatment (Chapter 5).

**Standard Operating Procedures:** Recently, the important issue of world-wide standard operating procedures for pre-clinical studies in the mdx mouse has been heavily discussed (Grounds et al, 2008b; Nagaraju and Willmann, 2009) and researchers have begun establishing a set of recommended standard operating procedures and endpoints for consistent and comparable assessment of pre-clinical interventions in the mdx mouse (Grounds et al, 2008b; Guerron et al, 2010; Spurney et al, 2010; Spurney et al, 2009)(Radley-Crabb et al, submitted; Willmann et al, in preparation). There are also many standard operating procedures listed on the TREAT-NMD website [http://www.treat-nmd.eu/research/preclinical/SOPs](http://www.treat-nmd.eu/research/preclinical/SOPs) and where possible these guidelines have been followed throughout this Thesis (Chapter 4 & 5). Included in this thesis (Methods – Chapter 2) is a standard operating procedure for ‘Haematoxylin and eosin staining for histological analysis of dystrophic muscle’. This method is published on the TREAT-NMD website and available worldwide for all researchers to follow and discuss.

**Conclusion:** The mdx mouse is a very useful animal model to examine the potential mechanisms by which the absence of dystrophin leads to myofibre necrosis and to test potential therapeutic interventions to reduce the extent of dystropathology *in vivo*. 
The exact mechanism by which the absence of dystrophin leads to myofibre necrosis is unknown, however the body of work presented in this thesis confirms the involvement of inflammation, oxidative stress and metabolic abnormality and that therapeutic interventions targeting these 3 processes can ameliorate dystropathology. It seems likely that myofibre necrosis is due to a combination of many dysregulated processes and combined pre-clinical therapeutic interventions should be pursued. The high level of variation in extent of dystropathology in mdx mice emphasises the need for establishing Standard Operating Procedures to enable comparison of data between laboratory groups world-wide when designing and conducting pre-clinical in vivo therapeutic trials in mdx mice.


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Medicine in focus

Duchenne muscular dystrophy: Focus on pharmaceutical and nutritional interventions

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Abstract

Duchenne muscular dystrophy is a lethal X-linked muscle disease resulting from a defect in the muscle membrane protein dystrophin. The absence of dystrophin leads to muscle membrane fragility, muscle death (necrosis) and eventual replacement of skeletal muscle by fat and fibrous connective tissue. Extensive muscle wasting and respiratory failure results in premature death often by the early 20s. This short review evaluates drug and nutritional interventions designed to reduce the severity of muscular dystrophy, while awaiting the outcome of research into therapies to correct the fundamental gene defect. Combinations of dietary supplementation with amino-acids such as creatine, specific anti-inflammatory drugs and perhaps drugs that target ion channels might have immediate realistic clinical benefits although rigorous research is required to determine optimal combinations of such interventions.

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Keywords: Duchenne muscular dystrophy; Mdx mouse; Pharmaceuticals; Nutritional supplements; Therapy

1. Duchenne muscular dystrophy and the mdx mouse model of DMD

There are many forms of muscular dystrophy but only Duchenne muscular dystrophy (DMD) is discussed here. DMD is an X-linked lethal muscle wasting disorder, affecting approximately 1/3500 male births. The disease is caused by a mutation in the gene that encodes for the sub-sarcolemmal protein dystrophin (Biggar, 2006). Dystrophin links the muscle cytoskeleton through a membrane complex to the extracellular matrix. Dystrophic myofibres are susceptible to damage during mechanical contraction, damage leads to myofibre necrosis and ultimately the replacement of myofibres by fibrous and fatty connective tissue (due to failed regeneration). While the genetic defect was identified in 1987, the specific mechanism of myofibre damage is still unclear (Whitehead, Yeung, & Allen, 2006) and there is still no effective treatment for DMD. Therapeutic approaches for DMD fall into three main strategies: (i) replacement of dystrophin by genetic, cell transplantation or molecular interventions; (ii) enhancement of muscle regeneration or reduction of fibrosis to combat failed regeneration; (iii) reduced muscle necrosis. This latter approach is the main focus of this review which outlines pharmacological interventions and nutritional supplementation as potential therapies to reduce myofibre necrosis in DMD. Most experimental studies use mdx mice and therefore data from this
model, along with clinical studies, form the basis of this review. DMD primarily affects skeletal and cardiac muscle and in addition other tissues (Biggar, 2006), but only the effects on skeletal muscle will be addressed.

The mdx mouse is the most widely used animal model for DMD. The absence of dystrophin results in a distinct disease progression with an acute onset of skeletal muscle necrosis around 3 weeks of age in young mdx mice (Fig. 1), necrosis then decreases significantly after 4–6 weeks to a relatively low level in adult mice (McGeachie, Grounds, Partridge, & Morgan, 1993): the pathology is far more benign than in DMD. The acute onset of myofibre necrosis provides an excellent model to study therapeutic interventions to prevent or reduce necrosis. In contrast, reduced necrosis can be difficult to detect in adult mice where there is little active myofibre breakdown but high cumulative muscle pathology. For this reason, exercise is often used to induce muscle damage enabling potential therapeutic interventions to be evaluated in adult mdx mice (Granchelli, Pollina, & Hudecki, 2000; Payne et al., 2006). The simplest form of exercise is voluntary wheel running, where muscle necrosis in the quadriceps is doubled (from ~6 to 12%) after 48 h (Hodgetts, Radley, Davies, & Grounds, 2006; Radley & Grounds, 2006). Forced exercise greatly increases muscle damage with the most severe injury resulting from forced downhill running (eccentric exercise), although such severe muscle damage caused by eccentric exercise is a poor model for pre-clinical drug screening. The symptoms of dystrophopathy are cumulative, with fibrosis becoming increasingly pronounced in older (>15 months) mdx mice. Symptoms are most severe in the mdx diaphragm that more closely resembles the severe pathology of DMD (Stedman et al., 1991).

Numerous parameters are measured to assess the in vivo effects of various interventions. In mdx mice, measurements on whole animals are combined with extensive tissue analysis. Physiological parameters such as Rotarod tests (to test motor co-ordination and fatigue resistance) and grip-strength tests (to measure the maximum amount of force an animal applies by grasping) assess changes in muscle endurance, muscle strength and overall functional capacity. Further physiological tests are conducted in vivo and on isolated muscles in situ or in vitro. Blood sampling and serum creatine kinase (CK) levels provide a qualitative indicator of muscle damage. Histological assessment of tissue sections quantifies cumulative muscle necrosis and regeneration, along with leaky myofibres and immunohistochemical staining identifies changes in location and levels of specific proteins. Other measurements include alterations in channel (Ca$^{2+}$ and Cl$^{-}$) function that contribute (or sensitise) to disrupted calcium homeostasis and to muscle necrosis (De Luca et al., 2003). A positive result with mdx mice can eventually lead to clinical studies in DMD patients. In humans, the main parameters measured are muscle strength, functional tests and CK levels. The Cooperative International Neuromuscular Research Group (CINRG) performs clinical trials on young DMD patients with various compounds, some of which showed positive results in an early screening program on dystrophic mice (Granchelli et al., 2000): while some of these trials have been published, ongoing results are available on http://www.cinrgresearch.org.

2. Steroids and anti-inflammatory drugs

Until a cure for DMD is found, treatment will involve the administration of corticosteroids combined with interventions to alleviate cardiac and respiratory prob-
lems. Corticosteroids have a catabolic effect on muscle (non-exercised muscle) and act to preserve existing muscle fibres and reduce inflammation, although their exact mechanism of action in dystrophic skeletal muscle is unknown. The two main corticosteroids used to treat DMD, Prednisone and Deflazacort, both seem equally effective in delaying the progression of muscle wasting. Unfortunately both are associated with adverse side-effects although these, particularly weight gain, are less severe with Deflazacort. Side-effects of Deflazacort include an increase in appetite requiring strict dietary control, retarded growth resulting in short stature and asymptomatic cataracts. It is recommended that daily calcium and Vitamin D supplementation is taken in conjunction with corticosteroids to maintain bone mineral density and reduce bone fractures (Biggar, 2006). The administration of steroids is not a cure for DMD but a therapy to improve quality of life and prolong lifespan.

DMD is characterized by aggressive inflammation and there is strong evidence that this contributes to myofibre necrosis both in vitro and in vivo (reviewed in Refs. (Tidball & Wehling-Henricks, 2005)). Other immunosuppressive drugs have demonstrated benefits in mdx mice, leading to increasing recognition for the damaging role of inflammation in DMD. Early clinical trials with the immunosuppressive drug cyclosporine in DMD returned promising results as 8 weeks of treatment (5 mg/kg/day) resulted in a significant increase in muscle force generation (Sharma, Mynhier, & Miller, 1993) and cyclosporine reduced the dystrophy in mdx mice (De Luca et al., 2005). However, cyclosporine exerts multiple dose-dependent effects and a correct dosage must be established especially when administered to young dystrophic patients during muscle development. These results are of clinical relevance, as immunosuppression may be required for enhancing efficiency of future gene/cell therapies.

Another potential anti-inflammatory drug that has attracted attention is pentoxifylline. Pentoxifylline has a wide range of anti-inflammatory and anti-coagulant effects; it reduces TNFα production in vitro (Vary et al., 1999), reduces fibrosis and may also play a role in normalising blood flow in dystrophic muscle. On the basis of increased muscle strength in exercised adult mdx mice (Granchelli et al., 2000), pentoxifylline is now the subject of two CINRG trials in DMD patients (one completed recently). However, a recent study in mdx mice involving pentoxifylline (16 mg/kg/day) administration for 4 weeks failed to reduce fibrosis or improve the contractile force of the diaphragm muscle (Gosselin & Williams, 2006), this study does not support the use of pentoxifylline as an anti-fibrotic drug in DMD patients. However, pentoxifylline counteracts, both in vitro and in vivo, the abnormal activity of calcium channels responsible for high sarcolemmal calcium permeability of dystrophic myofibres, suggesting a possible amelioration of dystrophic condition through alternative pathways (Rolland et al., 2006). Both cyclosporine and pentoxifylline (like corticosteroids) affect many cellular events and can have severe adverse side-effects, careful animal studies will be helpful in this regard.

Other anti-inflammatory drugs such as oxatomide (Granchelli et al., 2000) and cromolyn (Granchelli, Avosso, Hudecki, & Pollina, 1996; Radley & Grounds, 2006) that block mast cell degranulation are used widely for clinical treatment of allergies such as asthma and show benefits in mdx mice, indicating that mast cell products (including TNFα) have detrimental effects in dystrophic muscle. A recent CINRG trial with oxatomide (based on the study by (Granchelli et al., 2000)) in DMD showed some minor benefits.

3. Specific anti-cytokine drugs

Another promising approach involves targeting specific aspects of the inflammatory response (rather than the broadly acting anti-inflammatory drugs) in order to reduce muscle necrosis. While systemic depletion of specific inflammatory cells may not be clinically viable, modulation of specific cytokines has been very successful clinically in several severe inflammatory disorders. Tumour necrosis factor-alpha (TNFα) is a key pro-inflammatory cytokine that stimulates the inflammatory response and pharmacological blockade of TNFα activity with the neutralising antibody infliximab (Remicade) is highly effective clinically at reducing symptoms of inflammatory diseases such as rheumatoid arthritis and Crohn’s disease (Feldman & Maini, 2003). Similar successful clinical blockade of TNFα by the drug etanercept (Enbrel) results from the use of soluble receptors to TNFα. In mdx mice, infliximab delays and reduces the necrosis of dystrophic muscle in young mdx mice (Grounds & Torrisi, 2004). A protective effect of TNFα blockade is reinforced by two recent studies using etanercept that clearly reduces muscle necrosis in young mdx mice (Hodgetts et al., 2006; Pierno et al., 2006) and in exercised adult mdx mice (Hodgetts et al., 2006) with additional physiological benefits on muscle strength, chloride channel function and reduced CK levels being demonstrated in chronically treated exercised adult mdx mice (Pierno et al., 2006). Such emerging highly specific anti-inflammatory drugs designed for use in other clinical conditions, appear an attractive alternative (to steroids) for DMD, although their potential to reduce...
the severity of DMD remains to be determined. It may be possible to limit the use of these drugs to periods of intensive muscle growth in boys when muscle damage and deterioration can be especially pronounced. Patients undergoing long-term anti-cytokine treatment must be monitored carefully for serious infections, as is the case for any immunosuppressive drug.

4. Other pharmaceutical interventions

Antioxidants. It is widely recognized that high levels of reactive oxygen species can damage tissues, including skeletal muscle (Rando, 2002). Antioxidants that reduce oxidative damage in cells, such as Coenzyme Q10 (CoQ10) and green tea extract [(-)-epigallocatechin gallate] are the subject of recent research in mdx mice and DMD patients. Green tea extract supplemented diets fed to mdx mice (from birth), significantly reduced muscle damage (necrosis and regeneration) in the EDL muscle of 4-week-old mice and improved muscle function in 8-week-old mice after 5 weeks of treatment (Buetler, Renard, Offord, Schneider, & Ruegg, 2002; Dorchies et al., 2006). CoQ10 is essential for several enzymatic steps in the production of energy and functions as an antioxidant. CoQ10 was the subject of a CINRG pilot study in DMD patients to assess the effectiveness and safety in combination with steroid treatment. CoQ10 increased strength in some muscle groups and a larger follow-up study was recommended. This larger study that is currently being conducted by CINRG, is a 13-month, prospective, randomized study comparing daily Prednisone treatment (0.75 mg/kg/day), CoQ10 (>2.5 μg/mL) and a combined treatment (Prednisone and CoQ10) in older non-ambulatory patients.

4.1. Anabolic effects of β2-agonist drugs

Anabolic agents result in a net increase in protein content and muscle size and this is usually (but not always) associated with increased strength. Although commonly recognized as asthma drugs, high doses of some β2-agonists have anabolic effects on muscle and thus the potential to slow muscle degeneration. A 3-month pilot trial of the β2-agonist albuterol given to patients with fascioscapulohumeral disease improved maximum voluntary strength. This was followed by a year long trial where patients were treated with up to 16 mg of albuterol twice daily resulting in improved muscle mass and grip strength. Albuterol administered for 28 weeks (Fowler, Graves, Wetzel, & Spencer, 2004) to boys with DMD produced a modest increase in strength with no reported side-effects. It is noted that studies with β2-agonists in mdx mice have returned inconsistent results (Dupont-Versteegden, Katz, & McCarter, 1995; Lynch, Hinkle, & Faulkner, 2000) and that β2-agonists are associated with numerous undesirable side-effects including increased heart rate and tremors, which have limited their therapeutic potential. More recently synthesized β-agonists such as formoterol, have anabolic effects on skeletal muscle with minimal cardiac side-effects (Ryall, Silence, & Lynch, 2006) when administered at micro-molar doses in mice and thus may offer a greater potential for DMD (Harcourt, Schertzer, Ryall, & Lynch, 2006).

4.2. Disturbed ion channels and drugs to inhibit proteases

Damaged muscle membranes disturb the passage of calcium ions into the myofibre, and disrupted calcium homeostasis activates many enzymes, e.g. proteases, that cause additional damage and muscle necrosis. Ion channels that directly contribute to the pathological accumulation of calcium in dystrophic muscle are potential targets for drugs to treat DMD. There is evidence that some drugs, such as pentoxifylline, block exercise-sensitive calcium channels (Rolland et al., 2006) and antibiotics that block stretch activated channels reduce myofibre necrosis in mdx mice and CK levels in DMD boys (Whitehead et al., 2006). Calpains are calcium activated proteases that are increased in dystrophic muscle and may directly account for myofibre degeneration (Spencer, Croall, & Tidball, 1995). A new compound, BN 82270 (Ipsen) that has dual action as both a calpain inhibitor and an antioxidant (targeting both calpain and ROS induced muscle damage) increased muscle strength, decreased serum CK and reduced fibrosis of the mdx diaphragm, suggesting a potential therapeutic effect with this new compound (Burdi et al., 2006). A promising new compound of Leupeptin/Carnitine (Myodur) has recently been proposed for clinical trials in DMD patients.

Beyond the diverse approaches mentioned already, there has been much research into substances to help reduce the dystropathology via maintenance or improvement of myofibre size, strength and function. However, strategies such as increasing IGF-1 or other growth factors, inhibition of myostatin, normalising nitric oxide production and the use of poloxamer (P188), do not readily translate into the clinical situation at present. Although these interventions (and many others) have shown promising effects in mdx mice, adverse or unknown long-term systemic effects currently limit their therapeutic potential.
Chinese herbal medicine is becoming increasingly popular as an alternative approach to reduce the severity of symptoms associated with many diseases. For example, ginseng has a diverse range of effects in vivo and a study that showed a reduction in exercise-induced damage in normal muscle after ginseng supplementation (Hsu, Ho, Lin, Su, & Hsu, 2005) suggests possible benefits for DMD. A review of traditional Chinese medicines, such as massage, acupuncture and capsules (that contained herbs and other ingredients) that claimed to alleviate symptoms in DMD patients, was conducted in Beijing in 2003 (Urtizberea, Fan, Vroom, Recan, & Kaplan, 2003). Due to the small number of cases no definitive conclusions could be drawn from this study, although an overall mild frequency of contractures was noted and related possibly to the positive influence of acupuncture and massage. A follow-up study confirmed that high levels of glucocorticoids were present in the capsules and may account for the anecdotal improvement seen in patients (Courdier-Fruh, Barman, Wettstein, & Meier, 2003). Chinese herbal medicine was also found to improve locomotor activity in mdx mice (Chen, 2001). Traditional Chinese medicine is not formally regulated which potentially creates a large risk for incorrect dosing and dangerous content as medicines may unknowingly contain various heavy metals, herbicides, drugs, pesticides and micro-organisms. Furthermore, adverse drug interactions with cumulative effects may result when administered to DMD boys already receiving steroid treatment.

5. Nutritional interventions

Deficiencies of many substances (Selenium, Vitamin E or Vitamin D) can cause severe myopathies, suggesting the importance of nutritional supplementation to counteract these deficiencies: however it seems difficult to justify the application of such supplements to situations of adequate diet or DMD. Muscle wasting is associated with changes in the biochemistry of skeletal muscle that lead to reduced protein synthesis, increased protein breakdown (catabolism) and increased oxidative cell damage. The use of protein powders and specific amino acid supplements has been proposed for attenuating muscle protein loss and to provide a favourable environment for increasing protein synthesis and muscle mass and has received much attention in sports medicine and ageing. Dietary supplementation is a form of protective therapy potentially available for immediate use and broadly falls into three categories; antioxidants or Chinese herbs (both discussed above) and amino acid supplementation.

5.1. Amino acids

Amino acids such as creatine, taurine, glutamine and L-arginine have all been trialed in the mdx mouse with some benefits on muscle strength or dystrophopathy. Creatine is directly involved in the energy supply of muscle cells and supplementation into the diet (10%, w/w, in chow) of both exercised adult mdx mice and pregnant mdx mothers increased strength and improved dystrophopathy (De Luca et al., 2003; Passaquin et al., 2002). Similar benefits were seen after intraperitoneal injection of 10mg/kg in exercised adult mice (Granchelli et al., 2000). A double-blinded randomized creatine monohydrate (0.10 g/kg/day) trial in boys with DMD for 4 months showed increased hand grip strength and fat-free mass, independent of steroid usage (Tarnopolsky et al., 2004). A more recent clinical trial conducted by CINRG tested two amino acids (creatine 5g/day and glutamine 0.6 g/kg/day) in DMD boys aged 4–10 years (grouped as 4–7 years and 7–10 years). Although it did not significantly improve muscle strength, creatine was well tolerated by all patients and there was a trend towards less deterioration in other outcomes (Escolar et al., 2005). Creatine monohydrate supplementation in DMD is the subject of a recent review (Pearlman & Fielding, 2006) that concludes that it should be considered as a therapeutic agent for DMD, due to the potential for increased fat-free mass and increased muscle strength. However, additional long-term studies are required to elucidate the role of creatine in skeletal muscle growth and to accurately assess the degree to which creatine exerts protective musculoskeletal effects, without unwanted side-effects such as weight gain and kidney problems.

Taurine is a free amino acid abundant in skeletal muscle that exerts a wide spectrum of actions, ranging from osmolyte control, antioxidant action and anti-inflammatory effects. In skeletal muscle, taurine modulates ion channel function and calcium homeostasis (Conte Camerino et al., 2004). Supplementation of taurine (10%, w/w, in chow) is relatively safe and counteracts exercise-induced weakness after chronic exercise and ameliorates gCL (macroscopic chloride conductance, an index of degeneration-regeneration) in EDL muscles of mdx mice (De Luca et al., 2003). Clinically, taurine has been used with varying degrees of success in a wide variety of conditions (Birdsall, 1998) and is present in commercial food and beverages claimed to work as energizers.

Screening of numerous amino acids for potential efficacy in the mdx mouse (Granchelli et al., 2000) found that intraperitoneal injections of glutamine (10 mg/kg)
Table 1
Summary of potential pharmaceutical and nutritional interventions as therapeutic agents in (mdx mice and) DMD patients

<table>
<thead>
<tr>
<th>Substance</th>
<th>Action</th>
<th>Mdx mice</th>
<th>DMD patients (+/− benefit)</th>
<th>Immediate clinical potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclosporine</td>
<td>Immunosuppressant, anti-inflammatory</td>
<td>Maintained muscle strength, cellular parameters &amp; CK levels in exercised adult mouse (De Luca et al., 2005)</td>
<td>Eight-week trial—increased muscle force generation (Sharma et al., 1993)</td>
<td>May be (dosage to be carefully decided due to potential toxicity)</td>
</tr>
<tr>
<td>Pentoxifylline</td>
<td>Anti-inflammatory, anti-coagulant, anti-fibrotic</td>
<td>Increased muscle strength in exercised mice (Granchelli et al., 2000; Rolland et al., 2006). No improvement in mdx diaphragm fibrosis (Gosselin &amp; Williams, 2006) amelioration of calcium homeostasis (Rolland et al., 2006).</td>
<td>2×CINRG recent clinical trials (one trial on-going)</td>
<td>May be</td>
</tr>
<tr>
<td>Oxatomide</td>
<td>Anti-inflammatory histamine (H1) receptor antagonist (asthma therapy)</td>
<td>Increased muscle strength in exercised mice (Granchelli et al., 2000)</td>
<td>Recently completed CINRG clinical trial</td>
<td>Yes</td>
</tr>
<tr>
<td>Cromolyn</td>
<td>Anti-inflammatory mast cell stabilizer (asthma therapy)</td>
<td>Increased strength and reduce muscle necrosis in young and adult mice (Radley &amp; Grounds, 2006)</td>
<td>–</td>
<td>Yes</td>
</tr>
<tr>
<td>Infliximab (Remicade)</td>
<td>Anti-inflammatory TNFα antibody (arthritis, Crohn’s disease therapy)</td>
<td>Reduced muscle necrosis in young mice (Grounds &amp; Torrisi, 2004)</td>
<td>–</td>
<td>Yes (Monitor for possible infections)</td>
</tr>
<tr>
<td>Etanercept (Enbrel)</td>
<td>Anti-inflammatory TNFα antibody (arthritis, Crohn’s disease therapy)</td>
<td>Reduced muscle necrosis in young and exercised adult mice (Hodgetts et al., 2006; Pierro et al., 2006). Maintained muscle strength, cellular parameters &amp; CK levels in exercised adult mice (Pierro et al., 2006).</td>
<td>–</td>
<td>Yes (monitor for possible infections)</td>
</tr>
<tr>
<td>Coenzyme Q10</td>
<td>Antioxidant energy production</td>
<td>–</td>
<td>2× CINRG recent clinical trials (one trial on-going)</td>
<td>Yes</td>
</tr>
<tr>
<td>Green tea extract</td>
<td>Antioxidant</td>
<td>Reduced necrosis and improve muscle function in mice (Buetler et al., 2002; Dorchies et al., 2006)</td>
<td>–</td>
<td>Yes (necessary dosage still to be determined)</td>
</tr>
<tr>
<td>Chinese herbal medicine</td>
<td>Antioxidant</td>
<td>Improved locomotor activity in adult mdx mice (Chen, 2001)</td>
<td>Anecdotal improvement in patients (Urtizberea et al., 2003) Steroid content confirmed (Coudrier-Frhu et al., 2003)</td>
<td>No (exact composition of tablets unknown)</td>
</tr>
<tr>
<td>Old β-agonists: clenbuterol, albuterol</td>
<td>Anabolic effects on muscle. Possible anti-inflammatory (asthma therapy)</td>
<td>Inconsistent results in mdx mice (Dupont-Versteegden et al., 1995; Lynch et al., 2000)</td>
<td>Albuterol, 28 week trial resulted in strength increase (Fowler et al., 2004)</td>
<td>May be (potential for cardiac problems)</td>
</tr>
<tr>
<td>New β-agonists: formoterol</td>
<td>Anabolic effects on muscle. Possible anti-inflammatory</td>
<td>Improved muscle function in mdx mice (Harcourt et al., 2006)</td>
<td>–</td>
<td>May be/yes (minimal cardiac side-effects)</td>
</tr>
<tr>
<td>Substance</td>
<td>Action</td>
<td>Mdx mice</td>
<td>DMD patients (+/− benefit)</td>
<td>Immediate clinical potential</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>------------------------------------------------------------------------</td>
<td>--------------------------------------------------------------------------</td>
<td>------------------------------------------------------------------------------------------</td>
<td>---------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Calpain inhibitors: BN 82270, Myodur</td>
<td>Inhibit calcium dependent enzymes with additional pharmacodynamic or pharmacokinetic properties</td>
<td>BN 82270, increased muscle strength, decrease CK and muscle fibrosis in mice (Burdi et al., 2006)</td>
<td>Myodur proposed for clinical trials in 2006.</td>
<td>May be (pre-clinical toxicological studies are required)</td>
</tr>
<tr>
<td>Creatine</td>
<td>Amino acid (directly involved in muscle metabolism)</td>
<td>Increased strength and improved dystrophopathology in mice (De Luca et al., 2003; Granchelli et al., 2000; Passaquin et al., 2002)</td>
<td>Four-month trial—increased grip strength and decrease fat mass (Tarnopolsky et al., 2004) 6 month CINRG clinical trial—reduced deterioration of strength (Escolar et al., 2005)</td>
<td>Yes (upon monitoring of possible side-effects)</td>
</tr>
<tr>
<td>Taurine</td>
<td>Amino acid (control of calcium handling in vitro)</td>
<td>Maintained strength in exercised adult mice (De Luca et al., 2003)</td>
<td>–</td>
<td>Yes (upon monitoring of possible side-effects)</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Amino acid</td>
<td>Maintained strength in exercised adult mice (Granchelli et al., 2000)</td>
<td>CINRG clinical trial (Escolar et al., 2005) 10-day trial—reduced whole-body protein degradation (Mok et al., 2006)</td>
<td>Yes (upon monitoring of possible side-effects)</td>
</tr>
<tr>
<td>1-Arginine</td>
<td>Amino acid (substrate for NOS)</td>
<td>Upregulation of utrophin, improved dystrophopathology and reduced exercise induced muscle damage (Archer et al., 2006; Voisin et al., 2005)</td>
<td>–</td>
<td>Yes (upon monitoring of possible side-effects)</td>
</tr>
<tr>
<td>Chinese massage or acupuncture</td>
<td>Stimulation of skeletal muscles and joints</td>
<td>–</td>
<td>Anecdotal improvements in patients (Urtizberea et al., 2003)</td>
<td>Yes</td>
</tr>
</tbody>
</table>

*Note:* Even though some substances are shown as having the potential for immediate clinical intervention, the issue of adverse side-effects requires careful evaluation and, importantly, some of these substances when administered alone show only marginal benefits.
and a glutamine and alanine combination (10 mg/kg each) significantly improved whole body strength after 6 weeks of treadmill exercise. Clinical studies with glutamine show that oral supplementation (0.5 g/kg/day) over 10 days also inhibits whole-body protein degradation in DMD patients (Mok et al., 2006). Combinations of multiple dietary supplements (including creatine), both with and without prednisolone have recently shown improved muscle strength and reduced fatigue in exercised mdx mice (Payne et al., 2006). Such studies build a good case for promising benefits from combined interventions.

An additional possible treatment for DMD would be to compensate for the loss of dystrophin and nitric oxide (NO) with pharmacological agents. Administration of L-arginine (the substrate for nitric oxide synthase) increases NO production and up regulates utrophin expression in mdx mice. Six weeks of L-arginine treatment (200 mg/kg – intraperitoneal injection) improved muscle dystrophopathy and decreased serum CK in mdx mice (Voisin et al., 2005) and when given in combination with Deflazacort (Archer, Vargas, & Anderson, 2006) L-arginine (0.375% in drinking water) spared limb muscles from exercise induced damage and increased the distance (km) run voluntarily by an individual mouse. Since L-arginine can have adverse side effects, the drug isosorbide dinitrate that increases NO might be preferable for NO-based therapy in muscular dystrophy (Marques, Luz, Minatel, & Neto, 2005).

While amino acids have been proposed for many clinical conditions, possible benefits to DMD of supplementation with taurine, glutamine, alanine and arginine (alone or in combinations) remain to be formally evaluated.

6. Conclusion

The protective interventions briefly outlined in this review are those with some immediate possibility for clinical application in the near future; either drugs already in clinical use for other purposes or nutritional supplements as summarized in Table 1. When developing therapies for DMD, the goal is to maintain or promote skeletal muscle mass and function but at the same time reduce any deleterious side-effects, such as unwanted cardiovascular complications seen with powerful muscle anabolic agents. Early treatments administered before the pathology manifests are expected to be the most efficacious. Aggressive inflammation is a secondary characteristic of the disease and specific anti-inflammatory drugs seem to be a logical progression in the search for an alternative to steroids. Dietary supplementation in conjunction with some anti-inflammatory drug seems particularly promising and requires further rigorous investigation.

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References


IMPLICATIONS OF CROSS-TALK BETWEEN TUMOUR NECROSIS FACTOR AND INSULIN-LIKE GROWTH FACTOR-1 SIGNALLING IN SKELETAL MUSCLE

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SUMMARY

1. Inflammation, particularly the pro-inflammatory cytokine tumour necrosis factor (TNF), increases necrosis of skeletal muscle. Depletion of inflammatory cells, such as neutrophils, cromolyn blockade of mast cell degranulation or pharmacological blockade of TNF reduces necrosis of dystrophic myofibres in the mdx mouse model of the lethal childhood disease Duchenne muscular dystrophy (DMD).

2. Insulin-like growth factor-1 (IGF-1) is a very important cytokine for maintenance of skeletal muscle mass and the transgenic overexpression of IGF-1 within muscle cells reduces necrosis of dystrophic myofibres in mdx mice. Thus, IGF-1 usually has the opposite effect to TNF.

3. Activation of TNF signalling via the c-Jun N-terminal kinase (JNK) can inhibit IGF-1 signalling by phosphorylation and conformational changes in insulin receptor substrate (IRS)-1 downstream of the IGF-1 receptor. Such silencing of IGF-1 signalling in situations where inflammatory cytokines are elevated has many implications for skeletal muscle in vivo.

4. The basis for these interactions between TNF and IGF-1 is discussed with specific reference to clinical consequences for myofibre necrosis in DMD and also for the wasting (atrophy) of skeletal muscles that occurs in very old people and in cachexia associated with inflammatory disorders.

Key words: inflammation, insulin like growth factor 1, necrosis, skeletal muscle, tumour necrosis factor.

DUCHENNE MUSCULAR DYSTROPHY AND THERAPIES

Duchenne muscular dystrophy (DMD) is an inherited X-linked lethal childhood muscle disease caused by a defect in the gene for dystrophin, which affects young boys, causes extreme wasting and loss of function of skeletal muscles and leads to death usually by 20 years of age. Dystrophin is located beneath the sarcolemma and is part of a large dystrophin–dystroglycan complex that forms a critical link for force transmission between the contractile machinery of the muscle fibre and the extracellular matrix. Where dystrophin is defective or absent, the myofibre is fragile and the sarcolemma is readily damaged in response to exercise, leading to myofibre necrosis.1 Although it is widely considered that mechanical tears in the sarcolemma are the cause of the initial damage, other data indicate that changes in ion channels may be responsible for the initial influx of calcium that causes the damage;2 clearly, an accurate understanding of the basic mechanism will affect the targeting of potential therapeutic interventions. Although myofibre necrosis normally results in new muscle formation, in DMD (and, to a lesser extent, in the mdx mouse model of DMD) it appears that regeneration fails over time and the dystrophic muscle is progressively replaced by fatty and fibrous connective tissue.

Although the defective gene, dystrophin, was identified in 1987, there is still no effective treatment for DMD boys. Although cell or gene therapy to replace the defective dystrophin is the ideal scenario, the clinical application of such therapies is yet to become a reality.3,4 Meanwhile, many preclinical studies continue on the mdx mouse model of DMD.5

The existing treatment for DMD is administration of corticosteroids; these are broad-based anti-inflammatory drugs that decrease inflammatory cell populations in dystrophic muscle6 and increase myofibre mass, although the precise mechanism of action in DMD is not yet known and is under intense investigation.7,8 One disadvantage of steroids is that they are associated with severe adverse side-effects, such as weight gain and osteoporosis,9 and the response is variable between individual boys.10
Inflammatory response can directly damage myofibres in myopathic conditions, such as dystrophies or myositis, and recent data increasingly implicate inflammation, and specifically tumour necrosis factor (TNF), in myofibre necrosis.

Tumour necrosis factor is a major pro-inflammatory cytokine that is expressed by a wide range of inflammatory cells and by myoblasts, myotubes and damaged skeletal muscle. Tumour necrosis factor is also produced by adipose tissue, that is often pronounced within the wasted skeletal muscles in DMD. In response to even minor myofibre injury, TNF is rapidly released from resident mast cells and also by neutrophils, which accumulate quickly at sites of tissue damage, and TNF is a potent chemokine that attracts further inflammatory cells to the injured site. The chemotactic role of TNF was demonstrated in normal mouse muscle, where administration of TNF resulted in the accumulation of neutrophils and macrophages in the absence of any tissue damage.

In support of the proposal that TNF and neutrophils exacerbate initial sarcolemmal damage and provoke necrosis of dystrophic myofibres, in vivo blockade of TNF, cromolyn prevention of degranulation of mast cells (which normally release high levels of TNF) or depletion of host neutrophils protects dystrophic mdx mouse muscle from necrosis. Two drugs that were used to block TNF activity in the mdx mouse model of DMD, namely infliximab (an antibody to TNF) and etanercept (soluble receptor to TNF) are in wide clinical use already to treat inflammatory disorders such as arthritis and Crohn’s disease. The high specificity of these anticytokine drugs, combined with their clinical success in other diseases and relatively few side-effects, suggests that they may be attractive alternatives to the existing use of corticosteroids to treat DMD. In the mdx mouse, long-term studies have further demonstrated that the mouse-specific cVIq antibody to TNF has equal efficacy to Remicade and Enbrel. It is noted that this cVIq blockade of TNF has no effect on the low levels of chronic myofibre damage in unexercised dystrophic muscle, in striking contrast with the marked protective effect on exercise-induced acute myofibre necrosis, raising the possibility of different roles for TNF (and other molecules) in these two situations of myopathology.

The impact of exercise, as well as other factors such as age and gender that affect the severity of the pathology, should be taken into account when interpreting the expression profile of different molecules and the impact of drug interventions and other therapies in mdx mice.

That TNF protein is elevated locally in dystrophic muscles is supported by immunohistological studies showing increased staining for TNF associated with necrotic areas of dystrophic muscles of the mdx mouse (Fig. 1) and in biopsies from DMD patients, as well as Western blotting analysis using anti-TNF antibodies in mdx muscle extracts. It is noted that the issue of representative tissue from the small biopsy sample that can be taken from DMD muscles makes such measurements very difficult in humans. Few studies have quantified TNF in dystrophic muscles or blood. Although one study reported significantly higher plasma levels of TNF in dystrophic (DMD and Becker muscular dystrophy) patients than in age-matched control patients, another reported low levels of TNF levels in blood from DMD patients. It has proven difficult to detect elevation of TNF mRNA expression in skeletal muscles of adult non-exercised mdx mice, and in biopsies from DMD patients, as Western blotting analysis using anti-TNF antibodies in mdx muscle extracts have not been reported for dystrophic dogs.

Elevated TNF may exacerbate muscle damage through several pathways. One of the contributors to TNF induced muscle necrosis could be the inflammatory transcription factor nuclear factor...
(NF)-κB, because NF-κB is activated in limb muscles and the diaphragm of mdx mice, as well as in muscle samples from DMD patients. The blockade of NF-κB by pyrrolidine dithiocarbamate reduces skeletal muscle degeneration in mdx mice and it has recently been shown that heterozygous deletion of the p65 subunit of NF-κB is sufficient to decrease muscle necrosis in mdx mice.

Another possible mechanism for the damaging effects of TNF could be by activation of c-Jun N-terminal kinase (JNK). This is of special interest because activated JNK can inhibit the expression of insulin-like growth factor (IGF)-1 mRNA, as well as IGF-1 signalling, and such cross-talk between TNF and IGF-1 has many implications for muscular dystrophy and other conditions where inflammatory cytokines are elevated. A striking increase in phosphorylation of JNK1 has been reported in the diaphragm muscles of 7-week-old and 12-month-old mdx mice, whereas there was little increase in the limb muscles of 12-month-old mdx mice. Another study reported increased phosphorylation of JNK2, but not JNK1, in the limb muscle of 16-week-old non-exercised and exercised mdx mice. In marked contrast with the adverse effects of TNF on dystrophic muscle, increased levels of IGF-1 protect dystrophic muscle from necrosis and the roles of IGF-1 are discussed below.

COMPLEX ROLES OF IGF-1 AND IMPORTANCE IN SKELETAL MUSCLE

Insulin-like growth factor-1 plays a central role in myofibre hypertrophy and atrophy and this balance is of critical importance for muscle wasting in ageing (sarcopenia), in inflammatory disorders (cachexia), denervation, disuse atrophy and metabolic syndrome. An important finding was the demonstration that the reduced pathology in mdx mice that overexpress the Class 1 IGF-1Ea isoform is likely due to reduced myofibre necrosis and this protective effect may relate to increased protein synthesis and decreased protein degradation. The signalling pathways of IGF-1 are highly complex, with effects on not only atrophy/hypertrophy via promotion of protein synthesis and inhibition of protein degradation, but also on apoptosis, myoblast proliferation and muscle differentiation.

To further complicate the situation, there are at least six isoforms of IGF-1 and the specific biological function of different isoforms of IGF-1 are not defined. The recent development of transgenic mice that overexpress these different isoforms (N Winn (EMBL, Italy), unpubl. data, 2007) will hopefully help clarify their relative importance in skeletal muscle. It is noted that although the Class 1 IGF-1Ea isoform clearly reduced the dystrophopathy of mdx mice, transgenic mdx mice that overexpress the fully processed 70 amino acid human IGF-1 within myofibres (Rskα-actin/hIGF-1 transgene) and have elevated IGF-1 in muscles and blood showed no improvement in muscle pathology. Whether this lack of effect reflects the different form of IGF-1 overexpressed within the muscle or is due to increased IGF-1 levels seen only in the blood as well as skeletal muscles of these mdx/hIGF-1 transgenic mice is unclear, but these contrasting findings emphasize the complexity of interpreting transgenic data.

CROSS-TALK BETWEEN TNF AND IGF-1 SIGNALLING PATHWAYS VIA JNK

One of the main mechanisms by which TNF causes myofibre atrophy and myofibre necrosis may be through IGF-1, which is known to have both anti-apoptotic and growth-promoting effects.

We have undertaken intensive studies using transgenic mice that overexpress IGF-1 only within skeletal muscle. An important finding was the demonstration that the reduced pathology in mdx mice that overexpress the Class 1 IGF-1Ea isoform is likely due to reduced myofibre necrosis and this protective effect may relate to increased protein synthesis and decreased protein degradation. The signalling pathways of IGF-1 are highly complex, with effects on not only atrophy/hypertrophy via promotion of protein synthesis and inhibition of protein degradation, but also on apoptosis, myoblast proliferation and muscle differentiation.

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CROSS-TALK BETWEEN TNF AND IGF-1 SIGNALLING PATHWAYS VIA JNK

One of the main mechanisms by which TNF causes myofibre atrophy and myofibre necrosis may be through IGF-1, which is known to have both anti-apoptotic and growth-promoting effects.
Phosphorylation of the Ser307 residue leads to dissociation of IRS-1 from the IGF-1 receptor and inhibition of the tyrosine phosphorylation of IRS-1, which is required for the downstream signal transmission from the activated IGF-1 receptor. Use of the JNK inhibitors I-JNK and SP600125 has confirmed the effects of JNK as a negative regulator of IGF-1 signalling in C2C12 myoblasts, but these effects are yet to be tested in vivo. However, in cultured 3T3-L1 adipocytes, extracellular signal-regulated kinase (ERK) 1/2 rather than JNK seems to mediate IRS-1 phosphorylation at the Ser307 residue in response to TNF, because inhibition of ERK1/2 but not JNK1 was sufficient to abolish the Ser307 phosphorylation. In addition, the phosphorylation of IRS-1 on Ser307 takes place not only in response to TNF, but also following treatment with insulin and IGF-1, which represents a negative feedback loop responsible for insulin and IGF-1 resistance; this inhibition of IRS-1 by insulin and IGF-1 appears to be distinct from the signalling pathway activated by TNF.

The activation of a JNK1-mediated signal transduction cascade has been suggested to contribute to progression of the dystrophic mdx phenotype, independent of IRS-1 inhibition. Adenoviral expression of the JNK1 inhibitor JNK-interacting protein (JIP) 1 in skeletal myofibres of mdx mice that also lack MyoD protected them from degeneration and increased their cross-sectional area. That study suggested that the mechanism of JNK1 action in dystrophic muscle is due, at least in part, to serine phosphorylation and nuclear exclusion of the calcineurin-sensitive nuclear factor of activated T cells (NFAT) transcription factor. Data demonstrating the role of NFAT signalling in myofibre hypertrophy are controversial (for a review, see Shavlakadze and Grounds); however, in mdx muscle, upregulation of the calcineurin/NFAT pathway is protective against muscle degeneration. Furthermore, although deflazacort (a steroid used to treat DMD boys) did not alter JNK1 activity itself, it increased activity of the calcineurin phosphatase and upregulated NFAT-dependent gene expression, which, in turn, negates JNK1 inhibition. Taken together, these results suggest that further evaluation of JNK inhibitors, including JNK inhibitory peptides, and JNK ATP competitive inhibitors as new treatments for muscular dystrophy (with potential clinical application to DMD) should be considered.

Cross-talk between IGF-1 and TNF is further complicated by a report that IGF-1 can inhibit TNF signalling involved in protein catabolism, as shown in human colonic adenocarcinoma cells where pretreatment with IGF-1 reduced TNF-mediated nuclear localization of NF-κB. It was suggested that muscle-specific elevation of IGF-1 would also intercept TNF signalling and reduce the loss of muscle mass (cachexia) in inflammatory conditions; moreover, it has been demonstrated recently that inhibition of NF-κB signalling protects against denervation-induced muscle atrophy. Experiments are required to test the in vivo possibility that IGF-1 may play an inhibitory role in inflammatory mediated wasting of skeletal muscle.

BEYOND DYSTROPHY: CLINICAL IMPLICATIONS FOR AGEING AND OTHER MUSCLE CONDITIONS

Maintenance of skeletal muscle mass is governed by a complexity of signalling interactions and age-related muscle weakness and loss of muscle function (sarcopenia) presents many serious problems. Human studies show that, in the elderly, systemic low-grade inflammation associated with increased blood levels of TNF and interleukin-6 can contribute to loss of muscle mass and strength. Cytokines are responsible for muscle protein degradation in more severe cases of inflammation, such as cancer cachexia, sepsis and AIDS. Muscle wasting produced by TNF is associated with induction of oxidative stress, which is considered to be a major contributor to age-related sarcopenia. It has been suggested that the effects of TNF on muscle atrophy may also be mediated, in part, via interference with IGF-1 signalling and inhibition of the anabolic signalling cascade downstream of the IGF-1 receptor, which would lead to decreased protein synthesis and upregulation of atrophy related genes. Thus, attempts to minimize muscle wasting in various clinical conditions have focused on both anti-inflammatory drugs to block TNF action and the development of strategies to deliver IGF-1 to skeletal myofibre. Clarification of interactions between these two opposing pathways presents the possibility of new therapeutic targets and should provide valuable insight into molecular events determining the severity of muscular dystrophy and other muscle disorders.

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Review

Towards developing standard operating procedures for pre-clinical testing in the mdx mouse model of Duchenne muscular dystrophy

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ABSTRACT

This review discusses various issues to consider when developing standard operating procedures for pre-clinical studies in the mdx mouse model of Duchenne muscular dystrophy (DMD). The review describes and evaluates a wide range of techniques used to measure parameters of muscle pathology in mdx mice and identifies some basic techniques that might comprise standardised approaches for evaluation. While the central aim is to provide a basis for the development of standardised procedures to evaluate efficacy of a drug or a therapeutic strategy, a further aim is to gain insight into pathophysiological mechanisms in order to identify other therapeutic targets. The desired outcome is to enable easier and more rigorous comparison of pre-clinical data from different laboratories around the world, in order to accelerate identification of the best pre-clinical therapies in the mdx mouse that will fast-track translation into effective clinical treatments for DMD.

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Introduction
Therapeutic approaches to DMD

Duchenne muscular dystrophy (DMD) is a lethal X-linked muscle disease due to a defect in the sub-sarcolemmal protein dystrophin, that leads to membrane fragility, myofibre death (necrosis) and replacement of skeletal muscle by fibrous and fatty connective tissue (due to failed regeneration). This results in extensive wasting, weakness and loss of muscle function leading to death, often by the early 20s. DMD affects males although some carrier females can manifest and be severely affected (depending on the proportion of normal X-chromosomes that are inactivated during development) (Matthews et al., 1995; Wenger et al., 1992). While the genetic defect was identified in 1987, there is still no effective treatment for DMD. The therapeutic research approach that has received most attention to date is replacement of functional dystrophin by genetic, cell transplantation or molecular interventions, and there are many exciting developments in this field (Odom et al., 2007). In parallel, there is increasing interest in administration of exogenous factors (drugs or food supplements) to reduce the extent of myofibre necrosis, since promising protective effects have been reported for a variety of agents (Radley et al., 2007; Tidball and Wehling-Henricks, 2004) and combinations of such interventions present a daunting array of protocols to be tested. In addition it is feasible that pharmacotherapy may be necessary to increase the efficiency of genetic or molecular interventions, a factor that extends the importance of adequate pre-clinical tests for single or combined approaches.

During the last 20 years, various laboratories world-wide have focused on clarifying the pathogenic mechanisms and the eventual compensatory mechanisms, consequent to the primary defect in dystrophic muscles; this approach has led to the identification of new potential drug targets. Interestingly, different laboratories, using a variety of independent experimental approaches, generally obtain similar results in terms of factors aggravating the pathology and the potential efficacy of various drugs. However, comparing the relative efficacy of different drugs and interventions between laboratories is still difficult with consequent delay in data sharing and fragmentation of efforts. This observation pushes toward a concerted development of standard operating procedures (SOPs) for experiments in mdx mice that will simplify and hasten comparisons of data from different laboratories around the world and assist the research of scientists and pharmaceutical companies to optimise pre-clinical treatments for translation into clinical therapies.

Scope and limitations of the review

There are several animal models for DMD and all of these, like the human counterpart DMD, have defects in the sub-sarcolemmal protein dystrophin that make the muscle membrane fragile and result in necrosis of skeletal muscle fibres along with cardiac and other problems (Collins and Morgan, 2003; McNally and MacLeod, 2005). Only the skeletal muscle situation is addressed in this review. The mdx mouse, first identified in 1984, is the most widely used model due to ease of breeding, genetic uniformity, economy, and convenience for laboratory experiments. Similar pathology to mdx is seen in mice lacking alpha-sarcoglycan, another protein in the dystrophin dystroglycoprotein sarcocelomal complex (Duclos et al., 1998). Dystrophic dog models of DMD were first identified in 1988 (Kornegay et al., 1988) in the dystrophic golden retriever (Collins and Morgan, 2003) which has a much more severe pathology than the mdx mouse and...
more closely resembles the human condition: whereas the smaller dystrophic beagles exhibit a less severe pathology (Shimatsu et al., 1993; Yugeta et al., 2006). The highly variable phenotype of dystrophic dogs combined with expense of maintaining colonies has limited their use for pre-clinical testing. Beyond these mammalian models, complementary use is being made of invertebrate models that are readily manipulated genetically and are relatively easy and inexpensive to breed and maintain, such as the dystrophic worm Caenorhabditis elegans (Collins and Morgan, 2003) and dystrophic zebra fish (Bassett and Currie, 2004; den Hertog, 2005); although the usefulness of these models for drug screening for human conditions is debated.

The review is comprised of two main parts. Part I is a description of the mdx mouse model and the high biological variation. In Part II the main parameters used to measure specific effects on the dystrophic muscles are discussed. Some basic protocols are proposed for both Parts I and II.

Part I. The mdx mouse (and biological variation)

Since the review is focused on the mdx mouse model of DMD, it is pertinent to first outline the variations of the mdx model that are available.

\[ Dmbmdx \]

The classical biochemical and genetic mouse model of DMD is the mdx mouse discovered in 1981 (Bulfield et al., 1984; Hoffman et al., 1987). Over the last 25 years, many elegant papers have been published on the mdx mouse and it is not our purpose to review this literature. In brief, there is an acute onset of pathology (increased myofibre necrosis and elevated blood CK) around 3 weeks of age, this reduces to a chronic level of damage by 8 weeks which persists throughout life but is further decreased by 1 year of age (McGeachie et al., 1993). The mdx mutation occurred spontaneously due to a premature stop codon resulting in a termination in exon 23 of the dystrophin gene. The mdx mouse has no detectable dystrophin protein, although sporadic revertant myofibres can express dystrophin: this can complicate interpretation of some studies especially those related to gene or cell replacement of dystrophin (Yokota et al., 2006).

Higher (3.5 fold) mortality in mdx litters is reported (Torres and Duchen, 1987) and recent studies show increased (45%) mortality before 7 days of age in mdx mice: this is not affected by litter size with some litters being unaffected and others totally lost [Radley and Grounds, 2006; De Luca et al., unpublished data 2007]. Mdx mice seem very susceptible to stress and this may be a contributing factor since avoiding all animal handling or routine cage cleaning reduces the neonatal mortality.

\[ Dma^{mdx-2Cv} \rightarrow Dmbmdx^{mdx-Scv} \]

Elevated plasma CK levels were used to screen the progeny of chemical mutagen-treated male mice to identify four new mutations of Dmd, called Dmbmdx-2Cv-3Cv-5Cv (Chapman et al., 1989). Preliminary data showed that mice with mdx2Cv and mdx3Cv mutations have muscular dystrophic phenotypes that do not grossly differ from the characterized mdx mutation, and spontaneous revertant fibres occur less frequently in the Dmbmdx^{mdx-4Cv} and Dmbmdx^{mdx-6Cv} mutants than in Dmbmdx. These additional mdx mutations have not been widely used.

Mdx52 mice

Since the point mutation in exon 23 of the dystrophin gene in mdx mice allows the expression of four other shorter isoforms of the dystrophin gene through differential promoter usage, exon 52 knockout mice (mdx52) were generated to simulate the DMD phenotype commonly seen in human patients (Araki et al., 1997). A major advantage of this mutation (especially for studies designed to replace the missing dystrophin gene) is the complete absence of dystrophin since there are no revertant myofibres. The skeletal muscle pathology of mdx52 is similar to that of the mdx mouse for limb and diaphragm muscles, although hypertrophy was reported in mdx52 limb muscles.

Response of different skeletal muscles to muscular dystrophy

Fast-twitch muscles are generally the most susceptible to muscular dystrophy (and also ageing) in all species (Lynch et al., 2001). The progress of the dystrophopathology has been extensively described in mdx mice in the 1980s (Coulton et al., 1988b; Torres and Duchen, 1987) (reviewed in Shavlakadze et al., 2004). The absence of dystrophin results in Z-line streaming of sarcomeres by 1 day post-natal, rare isolated necrotic myofibres are seen by 5 days, by 10 days necrosis is evident in rostral muscles such as the head (masseter) and shoulder girdle (parascapular) (Torres and Duchen, 1987) and other muscles may show some pre-necrotic changes (Coulton et al., 1988b). There is an abrupt onset of skeletal muscle necrosis around 21 days of age in hind limb and many other muscles (from 20 to 80% of the muscle can be affected) that stimulates muscle regeneration (Whitehead et al., 2006b) (Fig. 1). Most groups report that myonecrosis in limb muscles is rare before 21 days of age (Shavlakadze et al., 2004), although a slightly earlier onset at 16 to 17 days has been reported in quadriiceps muscles (Muntoni et al., 1993); these differences may reflect divergence between isolated mdx colonies in different countries. Necrosis peaks (30–60%, occasionally ~90%, of the TA muscle is affected during this acute damage phase) by 25–26 days (with many myotubules resulting from regeneration by day 28) and then decreases significantly to stabilize by 8 weeks of age to a relatively low level of active damage (~4–6%); the cyclic progression of necrosis (and regeneration) continues throughout life although reduces by 1 year (McGeachie et al., 1993). The acute onset of myofibre necrosis provides a good model to study therapeutic interventions designed to prevent or reduce necrosis, since a reduction in dystrophopathology is easily identified (Grounds and Torrisi, 2004; Radley and Grounds, 2006; Shavlakadze and Grounds, 2003; Stupka et al., 2001). However, drug interventions that may be toxic to the post-natal development of neuromuscular apparatus should be considered for such young mice. In contrast, reduced necrosis can be difficult to detect in adult mdx mice where there is little myofibre breakdown (~5%) and cumulative muscle pathology: for this reason exercise is often used to provoke myofibre damage in adult mdx mice.

While young mdx mice at the acute phase of dystrophopathology show muscle weakness and the mdx muscles appear more susceptible to fatigue in vivo than control mice, overall, adult mdx mice do not show in vivo functional muscle impairment up to 1 year of age (Coulton et al., 1988a; Muntoni et al., 1993; De Luca et al., 2003). The symptoms of dystrophopathy are cumulative, with fibrosis becoming increasingly pronounced in older (15 months old) mdx mice (Lefaucheur et al., 1995); there are several different stages in the severity of the dystrophopathy between growing and mature mdx mice (Keeling et al., 2007) and these are affected by gender (Salimena et al., 2004). The limb and diaphragm are the 2 main groups of muscle that have been studied in muscular dystrophy.

In addition, some muscles of the head and chest region, including extraocular muscles (Fisher et al., 2005), masseter (Muller et al., 2001) and the laryngeal muscles (Marques et al., 2007), show a very mild pathology and are relatively spared from myonecrosis. The reasons for the mild dystrophopathy are not clear, although it is noted that these muscles may have an improved ability to regulate calcium homeostasis (Khurana et al., 1995) and selected mechanical properties that offer resistance to damage (Wiesen et al., 2007).

Limb muscles

The hind limb muscles are the most widely studied and include the tibialis anterior (TA) and extensor digitorum longus (EDL), the gastrocnemius, quadriceps and the soleus muscles (Parry and Wilkinson, 1990;
Wang and Kernell, 2001). The TA typically first manifests muscle necrosis from 21 days after birth and this is more pronounced than in the quadriceps (Radley and Grounds, 2006; Shavlakadze et al., 2004). Another study observed more necrosis in soleus than EDL at 24 days with greater cumulative muscle damage in soleus (–86%) than EDL (–36%) muscle at 34 days (Passaquin et al., 2002). The precise reason for the acute onset of dystropathology at 3 weeks of age is unresolved: it may relate to adult-type locomotor activity or to striking developmental changes in expression of various genes, including down-regulation of utrophin (Khurana et al., 1991) and of key genes involved in creatine synthesis (McClure et al., 2007) as well as in proteins involved in excitation–contraction coupling mechanisms (Bertocchini et al., 1997; De Luca et al., 1990; Schiaffino and Reggiani, 1996).

Exercise has a different impact on various limb muscles (depending on which muscles are recruited for the specific exercise regimes) and this needs to be taken into account e.g. 48 h voluntary wheel running doubles necrosis in quadriceps muscles of adult mdx mice, but causes less damage to the TA and diaphragm (Archer et al., 2006; Hodgetts et al., 2006; Radley and Grounds, 2006) and the EDL is more affected than the plantaris and soleus muscles (Hayes and Williams, 1996). After 3 downhill running sessions (10 m/min for 10 min at a 15° decline) the muscles most affected are the diaphragm and triceps brachii, with little damage seen in either the TA or EDL (Brussee et al., 1997). A different exercise regimen, such as a protocol of chronic (at least 4 weeks) forced running on horizontal treadmill, increases muscle necrosis in the gastrocnemius muscle, with damage also being observed, although to a lesser extent, in TA and diaphragm (Burdì et al., 2006; Pierò et al., 2007) (Burdi and De Luca, data under review).

**Diaphragm**

Over time, the diaphragm shows a more severe pathology than the limb muscles with extensive replacement of muscle fibres with fibrous connective tissue in mdx mice, more closely resembling the severe pathology of DMD where loss of diaphragm function is a major problem (Lynch et al., 1997; Stedman et al., 1991). Normal diaphragm muscle is composed mainly of type 2X and type 2A myofibres as shown by histochemical staining or antibodies specific for type 2A and 2B myosins (Gregorevic et al., 2002; Shavlakadze et al., 2004). Yet in dystrophic mdx muscles, type 2B myofibres increase over time as a result of bouts of regeneration in response to necrosis and this is conspicuous by 12 weeks of age (Shavlakadze et al., 2004). However, at early stages the pathology of the diaphragm is very mild and quite different to the severe acute onset in limb muscles: isolated necrotic myofibres are evident earlier, by 15 days after birth, and the damage is mild at least up to 30 days (Shavlakadze et al., 2004), with increasing severity of myofibre degeneration and increasing fibrous connective tissue over time (Gosselin and Williams, 2006; Krupnick et al., 2003; Niebroj-Dobosz et al., 1997; Stedman et al., 1991). Intrinsic differences in collagen metabolism have been demonstrated between functionally different normal skeletal muscles (Gosselin et al., 2007).

**Impact of growth parameters**

The much less severe dystropathology in mdx mice compared with DMD boys may be due in large part to vast difference in growth parameters between mice and humans, specifically to the much shorter time-scale of growth and maturation of mice (about 3 months compared with 20 years), much smaller body size (about 30 g compared with 70 kg) and consequent greatly reduced load on the smaller muscles in mice. In addition, there is stress on different muscle groups due to the use of 4 legs in mice compared with the vertical bipedal posture of humans. A comparison of developmental milestones for mice and men (see Box 1) suggests that as a very rough indication: 2 weeks (for mouse) may be equivalent to 3 months (for human); 3 weeks to 6 months; 4 weeks to 10 years; 8 weeks to 20 years and 12 weeks to 25 years.

**Biological variation between and within mice**

**Factors influencing biological variation**

Great variation in the timing and severity of the dystropathology can be seen between and within different mdx mouse colonies,
When trying to draw parallels between the mouse and human for the study of muscle, the lines should be drawn on the basis of hormonal changes and physiological milestones beginning with muscle differentiation. It must be recognised that there is no linear scaling; the proportion of the life-span taken to sexual maturity is only about 5% for mice, whereas for man it is approximately 20%. (The long childhood is a unique characteristic of man and the apes; rodents and many other mammals do not have a "childhood" and they effectively go from baby to teenager.) There is also a rostro-caudal pattern of development so that the muscles in the upper part of the body are likely to be at a slightly more advanced stage than that described below which is derived mainly from the study of (male) mouse hind limb.

**Birth:** skeletal muscle is poly-innervated, the tubular systems are rudimentary, and neonatal myosin heavy chain predominates. At the same time activity of thyroid hormone and the HPA axis are suppressed. Human muscle is at a similar stage of differentiation at about 18–22 weeks of gestation.

**By 8–10 days post-natally:** the tubular system is more mature, fibres have become mono-innervated and enable the coordinated contraction of muscles to promote balance and increased locomotor activity. With respect to locomotor function, mice begin weight bearing from 1–2 weeks of age, this contributes to synapse elimination (Minatel et al., 2003) and maturation (Missias et al., 1996), whereas in humans this is completed by birth (Hesselmans et al., 1993). In mice, activity of the thyroid (McArdis et al., 1998) and hypothalamic pituitary adrenal (HPA) (Schmidt et al., 2003) hormonal axes are starting to increase, and replacement of immature MHC by the adult myosin heavy chains is occurring (Allen and Leinwand, 2001). Mice are suckled on milk which is high fat/low carbohydrate. This roughly corresponds with a newborn human (Bronson, 2001; Eilminger et al., 2001). In mdx mice, blood CK levels at 7 days are the same as normal mice, but by 10 days are elevated indicating early symptoms of the disease.

**14–16 days:** Milk is gradually becoming limiting and insulin levels decrease substantially. The GI tract is not fully capable of digesting complex carbohydrates, and there is evidence from the inflexion in the normal muscle growth curves, that growth capacity is limited. At this time myostatin is also increasing rapidly. Muscle IGF-II mRNA and muscle IGF 1 receptor levels are approaching a nadir. In the human this represents about 3 months of age.

**19–21 days:** Mice are fully weaned; muscle is mature (Allen and Leinwand, 2001). Diet is now largely chow, and there is very rapid growth of the muscle. Growth hormone starts to increase from 21 days and peaks around 28–30 days (Alba and Salvatori, 2004). This represents approximately 6 months in humans (Butler-Browne et al., 1990; Mehta et al., 2005). In mdx mice, acute muscle necrosis starts in hind limb muscles from 21 days: necrosis is seen in forelimb and other muscles at earlier ages.

**Developmental milestones in mice and men**

Composite data, compiled in close collaboration with Marta Fiorotto (Baylor College of Medicine, Houston, Texas)

<table>
<thead>
<tr>
<th>Developmental Milestone</th>
<th>Mouse</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>4–5 weeks</td>
<td>pre-adolescence in mouse; gender differences are just beginning to emerge: equivalent to about 10 years of human age.</td>
<td></td>
</tr>
<tr>
<td>5–7 weeks</td>
<td>puberty in mice (Jean-Faucher et al., 1978), they are fully fertile and growth rate decreases: about 14–18 years of age in humans (Rodriguez et al., 2007).</td>
<td></td>
</tr>
<tr>
<td>Around 10–15 weeks</td>
<td>muscle has reached a maximum size in mice and growth in general has reached a plateau (Balice-Gordon and Lichtman, 1993): &gt;20 years in the human. [In mdx mice, muscle necrosis stabilizes to a low persistence level of damage, &lt;10% of muscle affected, that is increased by exercise.]</td>
<td>12–18 months</td>
</tr>
<tr>
<td></td>
<td>in the mouse a gradual diminution in muscle mass becomes evident from 18 months, i.e. start of sarcopenia. In humans, this starts significantly after about 50 years of age (Shavlakadze and Grounds, 2003).</td>
<td>24+ months</td>
</tr>
</tbody>
</table>

### Summary of possible parallels for younger mdx mouse (during first 6 months)

<table>
<thead>
<tr>
<th>Mouse (weeks)</th>
<th>Human (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Newborn</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>16–18</td>
<td>20</td>
</tr>
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<td>25</td>
<td>35</td>
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<tr>
<td>30</td>
<td>30</td>
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between littermates and even between 2 legs of an individual mouse. Parameters affected include histological (e.g. the extent of necrosis and muscle damage varies from massive [60–80%] to moderate necrosis [20–40%] at 23 days with some mice showing almost no damage even at 26 days (Radley and Grounds, 2006); this occurs between littermates and between TA muscles in both legs of an individual mouse e.g. 0% compared to 24% for one mouse at 21 days [Radley and Grounds, unpublished data].) cellular (e.g. number of satellite cells (Schafer et al., 2005) and age-related increase in revertant myofibres (Yokota et al., 2006)); biochemical (serum creatine kinase (CK) levels in sedentary and exercised mice (Burdin et al., 2006; De Luca et al., 2005; Pierno et al., 2007; Vilquin et al., 1998) [see Blood measurements of CK and other proteins that leak out of damaged myofibres]; molecular [gene expression profiles (Turk et al., 2006)] and functional aspects (participation in voluntary wheel exercise (Radley and Grounds, 2006) or forced treadmill running (De Luca et al., 2003; Vilquin et al., 1998) [discussed in Voluntary wheel running and Forced treadmill running]. Such inherent variation necessitates constant conditions when grouping the animals, with large sample sizes (often at least 6–8 mice) to show statistically significant effects of treatments.

Epigenetic and genetic factors probably contribute to the wide range of phenotypic expression of muscular dystrophy (as discussed below). While it is clearly impractical to standardise many of these variables across laboratories globally, a deeper understanding of the many reasons for biological variation will help to initiate practises to minimise this problem.

### Developmental and in utero influences

It is now widely recognised that very early events before or at fertilisation, as well as in utero can have dramatic post-natal effects that contribute to large biological variation in anatomy, physiology, behaviour and the onset of disease (Gartner, 1990; Vandenbergh, 2004). Many of these are due to pre-natal hormone exposure;
however environmental epigenetic also plays an important role in disease susceptibility (Jirtle and Skinner, 2007; Vandenberghe, 2004). Some of the in utero factors to consider that may influence the severity of the dystrophopathy in individual mdx mice (within a litter and between litters) are the size of the litter, the position within the uterus, the number of male siblings and the proximity of female pups to male littersmates (this influences exposure to testosterone) along with a range of other factors (Vandenberghe, 2003). Some laboratories standardise litter size (e.g. 4–8 pups) for all experiments to minimise variation in litter size that can affect initial body weights and thus biological variation. However, this may be considered wasteful and is unlikely to become a standard, plus it does not take into account the consequence of early neonatal mortality of mdx litters. One simple solution that is strongly recommended to reduce effects of inter-litter variation is to always select pups from a single litter for both test and control mice.

Neonatal influences

The early post-natal environment is important with a wealth of data showing that maternal care mediates further variation in offspring phenotype and behaviour (Champagne et al., 2007) and catch-up post-natal growth by low birth weight humans and animals affects many metabolic and signalling events. Transmission of maternal behaviour (such as grooming and licking) and stress responses can occur from one generation to another by epigenetic modification of the chromatin around the glucocorticoid receptor gene (Francis et al., 1999; Weaver et al., 2004). The net result to the progeny is tighter regulation of stress hormone levels, an effect that is relayed to the next generation in subsequent maternal behaviour patterns.

These issues highlight some of the developmental variables that can influence the phenotype (severity of the dystrophopathy and potentially the propensity for voluntary exercise) of genetically equivalent inbred mice to contribute to the wide biological variation seem for mdx mice, both within and between litters.

Gender

Major sex differences have been noted in skeletal muscles in relation to energy metabolism, fibre type composition and contractile speed. Generally, muscles from males tend to be faster and have higher maximum power output than from females, whereas muscles from females are more fatigue resistant, recover faster from repeated contractions and show less mechanical damage after exercise (Glennmark et al., 2004). Striking differences have been noted in the dystrophopathy between male and female mdx mice at different ages (Salimena et al., 2004), with less susceptibility to muscle damage in young (6 weeks) female mice but greater fibrosis in old females (1 and 2 years) suggesting a role for female hormones in the pattern of myonecrosis and repair. Other studies show that levels of blood serum creatine kinase are higher in older male mdx mice (Yoshida et al., 2006). Sex differences in muscle membrane damage in response to intense exercise have been shown in animals and humans, with females being less susceptible than males: this appears to be due to a reduced inflammatory response in females, with many aspects being influenced by gender (Stupka et al., 2000). It is widely recognised that both the innate and adaptive immune responses of females are heightened and more robust than in males (Verthelyi, 2006) and recent evidence confirms that the innate and adaptive arms of the immune system are different in post-pubertal male and female mice (Lamason et al., 2006). These gender differences are largely attributed to oestrogen levels and the regulation of nitric oxide by oestrogen increasingly appears to play a key role (Verthelyi, 2006). There are also gender differences in response to drugs (Franconi et al., 2007) and to diets (see Food and water) and thus gender should be carefully considered when testing pharmaceutical or nutritional interventions. While such gender-related issues appear to be important in the mdx mouse, they have barely been considered. Interpretation and data comparison is complicated when the gender of the mice used in experiments is either not specified (Hall et al., 2007; Kaczor et al., 2007) or mixed males and females are used (Granchelli et al., 2000). Ideally pre-clinical tests should be performed on groups of mdx mice of the same sex.

Males. Testosterone has many effects; male mice of some strains are particularly aggressive and can be difficult to cage as placing males together may lead to fighting and thus additional muscle damage. This is generally not a problem if males are either caged together at weaning, many mice are caged together (since 2 males alone in one cage are more likely to fight), and the males are not near a stud male or females (due to pheromones). The male response to female odours can affect behaviour and is influenced by the major histocompatibility type of the foetus of pregnant females (Beauchamp et al., 2000). In addition, age-related changes in pheromones in urine appear to relate to altered immune function (Usada et al., 2003). The effects of such odours within an animal house can be minimised by using individually ventilated cage systems (see Cage design). In addition, inter-male aggression in grouped housing is influenced by different kinds of environmental enrichment (Van Loo et al., 2004). Beyond the sex determining region on the Y-chromosome (SRY) and testosterone, there are multiple pathways that control sexual differentiation; e.g. sexual differences in motoneurones may be affected by the testicular hormone Mullerian Inhibitory Substance that is present in the blood of pre-pubertal males, but not females (Wang et al., 2005). Since DMD affects boys, it might be considered that it is more appropriate to use male mice, although the overall significance of this issue has yet to be proven.

Females. Hormonal changes during the estrus cycle influence immune status, stress, activity levels and age-related changes in cognitive function (Kopp et al., 2006) and this may be a significant additional variable when using female mdx mice.

Genetics

Inbred mdx mice are homozygous and theoretically there is long-term genetic stability, but there will be a slow sub-line differentiation between colonies over many years (reviewed in Harris, 1997). Ideally, colonies should be replaced periodically from an international source. Furthermore, if inbred mice are not kept under specific pathogen free (SPF) conditions, their phenotype variability can be higher than outbred mice (reviewed in Biggers, 1958; Harris, 1997). The genetic background strain can greatly influence the severity of the dystrophic phenotype as demonstrated for sargoclycan deficient mice, although the gene loci that suppress the dystrophic phenotype remain to be identified (Heydemann et al., 2005).

While most mdx mice are maintained as homozygous/hemizygous colonies (where all X-chromosomes carry the gene mutation and thus all females and males are affected), maintenance as a heterozygous colony could more closely resemble the human situation and also provide appropriate negative littermate controls.

Stress

Numerous factors can stress animals and this has effects on the brain, behaviour, hormones, and the immune system. Some of the well known stressors that produce physiological effects are social stress related to housing and dominance/subordination (reviewed in Bartolomucci, 2007), transport, restraint and handling, in addition to blood sampling, surgery and anaesthesia. For example, even simply transferring mice to a different room can increase corticosterone levels and mice take about 4 days to acclimatize (Tuli et al., 1995). The rapid endocrine and metabolic response to the stress of handling is manifested by rapid changes in many blood components leading to the recommendation that any sampling be completed within 100 s of first touching an animal's cage (Gartner et al., 1980) since some
components are altered by 100% within 30 min. Considering that the in vivo procedures for drug treatment and evaluation cannot avoid a certain level of stress being experienced by the animals, it is essential to maintain standard handling procedures for all mice (test and controls) throughout the experimental procedure. Anecdotal evidence suggests that mdx mice are especially susceptible to stress.

Good husbandry practises to minimise biological variation

Environmental conditions such as housing and husbandry have a major impact on the laboratory animal throughout its life and will thereby influence the outcome of animal experiments. Much of the biological variation between and within mice (and in different laboratories) may reflect variation between aspects of animal husbandry (reviewed in Biggers, 1958; Harris, 1997; Reilly, 1998). Issues to consider for standard laboratory practise include: caging, housing systems, space recommendations and microenvironment—enrichment, bedding; temperature and humidity; ventilation: air quality, relative air pressure and individual cage ventilation; illumination: photoperiod, intensity; noises; food: types of diet, quality assurance; water; sanitation and cleaning; identification and record keeping. There is a wealth of literature on these topics and it is well recognised that they have a major impact on phenotypic variation (Gartner, 1990). Inbred mice such as mdx are far more susceptible than outbred animals to phenotypic variation induced by minimal environmental variability (such as pathogens), simply because the population lacks genetic diversity (reviewed in Harris, 1997). Some of these factors are discussed below.

Cage design

Cage design can modify the activity and behaviour of mice (Wurbel, 2001) with changes in the environments of animals, including environmental (supplemental) enrichment, having important effects on brain structure, physiology (including recovery from illness and injury), gene expression in various organs (Beneffel et al., 2005) and aggression between males (Van Loo et al., 2004). Individually ventilated cage systems generally help to maintain low ammonia and carbon dioxide concentrations; they support a low relative humidity, and reduce spread of allergens and infections. Additional benefits are that cages need to be cleaned less frequently (with reduced disturbance of the mice) and airborne pheromones (due to gender, stress, age) that can affect mouse hormonal responses are eliminated (Reeb-Whitaker et al., 2001).

Night and day (light and dark cycles)

Traditionally, mice are subjected to forced exercise and experiments are performed during the day (e.g. tissue samples collection and/or physiological experiments). Yet mice are nocturnal and normally active at night. Since the diurnal rhythms of mice and humans are out of phase, the nocturnal mouse more accurately corresponds to the daytime human (McLennan and Taylor-Jeffs, 2004). It is well documented that circadian rhythms profoundly influence many molecular, metabolic, endocrine, immunological and behavioural parameters (McCarthy et al., 2007; McLennan and Taylor-Jeffs, 2004) and thus it is important, within each laboratory, to maintain strictly the same time of day for exercising, treating, sampling or conducting physiological experiments on mice. One strategy to consider is the use of sodium lights to shift the day/night cycle in order and allow observations and interventions during the nocturnal phase with many scientific and welfare advantages (McLennan and Taylor-Jeffs, 2004).

Food and water

Diet can have a dramatic and rapid impact on cellular responses and gene expression, as illustrated by effects of soy or casein on cardiac hypertrophy (Stauffer et al., 2006) and how a diet enriched with omega-3 fatty acids can have potent post-natal effects on fetal programming (Wyrwoll et al., 2006); both of these studies emphasise the strong influence of gender. There is a huge literature on nutritional effects but these two examples illustrate the need for strictly standardised diets. Indeed nutritional interventions to ameliorate muscular dystrophy are being trialled in mdx mice and humans (Radley et al., 2007). It can be a challenge to rigorously control the quality of a well characterized diet (e.g. mouse chow) from a supplier, since even the standard ingredients may vary depending on the market source and the season. A further variable to consider is the impact of short-term (e.g. overnight) fasting on metabolic and cellular parameters. Awareness of such issues is important when evaluating variation between results from different laboratories and even within one laboratory over time.

Tap water that is traditionally used as drinking water in animal houses can vary very markedly in its mineral content and different additives such as chloride and fluoride, between different cities and may vary throughout the year (e.g. summer and winter) for the same location. Such differences may chemically interfere with some drugs. Whether such variations significantly influence the dystrophopathy is not known. To avoid such possible complications some laboratories, such as pre-clinical drug testing facility at CNMC in Washington (Nagaraju), now use purified water for all experimental mice.

Models of exercise-induced muscle damage to increase pathology in mdx mice

Endurance training produces many physiological, metabolic and vascular adaptations in skeletal muscle. This adaptive response may involve myokines, such as IL-6 and other cytokines, which are produced and released by contracting skeletal muscles and affect other organs of the body (Febrabio and Pedersen, 2005) combined with improvements in cardiac function. Beneficial effects of regular exercise on dystrophic mdx muscle are reported for free wheel running (Dupont-Versteegden et al., 1994; Hayes and Williams, 1996) and swimming that is a non-weight-bearing, low-intensity exercise (Hayes and Williams, 1998). The implications of such adaptation for strengthening dystrophic muscle, although still debated, are of much interest for physical therapy of DMD patients. However, here we will focus on exercise as an intervention to increase the severity of the phenotype in mdx mice, to enable more effective evaluation of drug treatment efficacy.

In adult mdx mice the muscle pathology is normally relatively mild and does not closely resemble the severity of DMD. The low level of damage in adult mdx mice can be elevated by exercise that increases myofibre necrosis and decreases muscle strength (Brussee et al., 1997; De Luca et al., 2003; Okano et al., 2005; Vilquin et al., 1998) enabling potential therapeutic interventions to be evaluated more rigorously throughout the in vivo treatment (Archer et al., 2006; De Luca et al., 2005; Granchelli et al., 2000; Payne et al., 2006; Radley et al., 2008). Muscles differ in their susceptibility to exercise-induced muscle damage, with fast fibres (Type 2) more likely to be damaged than slow (Type 1) fibres. Various models of exercise-induced muscle damage have been used to exacerbate the disease in mdx mice, including voluntary wheel running, treadmill running and swimming (also see Whole animals: in situ nerve stimulated contraction with dynamometer to measure function of muscle groups), but only two widely used in vivo running models are described here.

Voluntary wheel running

The simplest model is voluntary spontaneous exercise and this is generally well tolerated (Hayes and Williams, 1996). Voluntary wheel running allows the distance run by individual mice to be measured accurately and so relative activity can be related to the severity of the resultant muscle damage. Mice are voluntarily exercised using a metal mouse wheel placed (often suspended) inside the cage. Exercise data are collected via a small magnet attached to the mouse wheel and a
sensor from a bicycle pedometer attached to the back of the cage. The sensor records single wheel revolutions, allowing total distance (km) and speed run by an individual mouse to be determined (Archer et al., 2006; Dupont-Versteegden et al., 1994; Hayes and Williams, 1996; Radley and Grounds, 2006). The majority of voluntarily running is done at night (12 h dark cycle) (Hayes and Williams, 1996; Radley and Grounds, 2006). This exercise has the advantage that mice can be left with the exercise equipment continuously and their activity measured over many months. There can be wide variations in the amount of running between individual mice, one possible disadvantage is that mice are caged individually (even though the cages may have clear sides so they can see each other) and this lack of socialising may affect their behaviour. Another issue that is rarely considered is that dirt (e.g. urine and faeces) can accumulate and increase resistance of the wheel rotation. This can also be influenced by the design of the wheel, and thus the amount of effort required to turn the wheel will vary as will the impact on the muscles. Such variations in wheel resistance may contribute to different results between mice and between laboratories and should be considered: indeed some designs deliberately increase the wheel resistance to increase the workload (Konhilas et al., 2005). More sophisticated computerised monitoring systems can be used to collect precise data on the patterns of running and stopping (Hara et al., 2002; Radley and Grounds, 2006) and have revealed that mdx mice run more intermittently than wild type mice (Hara et al., 2002).

Muscle necrosis is roughly doubled (increases from ~6 to 12%) in quadriceps muscle after 48 h of voluntary exercise, although other muscles such as the TA are barely affected (Radley and Grounds, 2006) a finding reported by others even after a single night of running (Archer et al., 2006). Histological analysis after 48 h allows induced necrosis to be assessed without the ensuing complication of new muscle formation (myotubes appear by 3 days after injury). Adaptation to voluntary wheel running occurs over about 2 months in normal adult C57BL mice and varies between muscles (Konhilas et al., 2005). The capacity for voluntary wheel running over a long period (Brunelli et al., 2007; Dupont-Versteegden et al., 1994; Radley et al., 2008) probably reflects the relative health of mdx mice. Therefore, voluntary exercise can provide an additional measure of the protective benefits of interventions. (The use of voluntary or treadmill running to measure improvements in exercise capacity is also mentioned in in vivo measurements of whole body function and muscle strength in mice). When mdx mice voluntarily run greater distances, this puts the exercise equipment continuously and their activity measured over many months. There can be wide variations in the amount of running between individual mice, one possible disadvantage is that mice are caged individually (even though the cages may have clear sides so they can see each other) and this lack of socialising may affect their behaviour. Another issue that is rarely considered is that dirt (e.g. urine and faeces) can accumulate and increase resistance of the wheel rotation. This can also be influenced by the design of the wheel, and thus the amount of effort required to turn the wheel will vary as will the impact on the muscles. Such variations in wheel resistance may contribute to different results between mice and between laboratories and should be considered: indeed some designs deliberately increase the wheel resistance to increase the workload (Konhilas et al., 2005). More sophisticated computerised monitoring systems can be used to collect precise data on the patterns of running and stopping (Hara et al., 2002; Radley and Grounds, 2006) and have revealed that mdx mice run more intermittently than wild type mice (Hara et al., 2002).

Part I. Conclusions and recommendations

Part I has outlined the factors that influence biological variation and emphasised the need to be aware of these and to standardise conditions where possible (e.g. related to breeding, husbandry and gender). It seems that the 3 aspects that need to be addressed in order to help establish Standard Operating Procedures are:

1. Specify basic core experiments e.g. age of sampling, gender, exercise regime, onset of treatment (see Recommendation I in Box 2).
2. Define basic core methods of analysis (discussed in Part II).
3. Develop a scale to grade the efficacy of treatment (see Part II).

Part II. Parameters to measure muscle dystrophopathy and function in mdx mice

A range of histological analyses on muscle tissue sections, blood measurements and physiological parameters are used to assess the impact of various interventions on the pathology and function of
Box 2

**Recommendation I: Basic standard experimental regimes for pre-clinical testing**

Basic regimes to assist with global standardisation of studies in mdx mice are suggested. (Other issues of standardisation related to animal husbandry and developmental and neonatal influences can be more challenging to address). Key issues to consider are: age of onset of treatment and sampling (influenced by the experimental aim); sampling at standard ages to facilitate comparison of much data; gender (specify gender; males may be more suitable for testing some drugs); each litter should be divided into test and control mice to help reduce variation; exercise — either voluntary wheel (the total distance run by each mouse must be measured) or forced treadmill running. (Protocols for analysis are outlined in Part II).

**Regime A** To test the effects of interventions on the acute early stage of the disease treatments are started before the onset of necrosis (from about 14–17 days): two sampling regimes are outlined.

A1 Sample at 28 days. Treatment is started by day 17 with tissues sampled at 28 days (4 weeks) initially. This specifically targets the initial acute phase of myofibre necrosis.

A2 Sample at 12 weeks or later. Treatment can easily be extended to further sampling and analysis at 12 weeks (as for B1). Half of the mdx mice ideally should be exposed to exercise from 4 weeks of age (either voluntary wheel running or treadmill twice a week — or a combination) to increase the severity of the disease. Note: young mice before 4 weeks of age do not exercise well. (Longer term studies can build on this).

**Advantages:** treatment starts before the major initial bout of damage to mdx limb muscles. Interpretation is simplified due to absence of pre-existing background pathology. This young age relates to childhood events in DMD.

**Disadvantages:** this is an active period of growth and some drugs may have adverse effects or be difficult to administer to very young mice. Biological variation can be high due to differences in the time of onset and severity of pathology between young mice.

**Regime B** To test the effects of interventions on adult and older mice where there is a relatively low background level of pathology. These regimes all involve exercise

B1 Short term (2 day) studies to specifically test the impact on myonecrosis. This is a quick ‘proof-of-concept’ test. Treatment of mice exercised with a single bout of treadmill exercise (day 0), or overnight wheel running and sampled on day 2. Use of 12 week old mice corresponds to a standard sampling time.

B2 Short term (1 month) studies in young adult mice. Treatment of young adult mice (8 weeks) where active necrosis is low (but much pre-existing pathology exists). One month of treatment/exercise with sampling at 12 weeks is a simple protocol.

B3 Long term studies. Treatment/exercise starting at 4, 8 or 12 weeks and sampling at 12, 24 or 52 weeks.

**B4 Fibrosis and later aspects of the disease.** In some cases the treatment/exercise might start at 6 months. However, chronic exercise started at earlier stage can increase fibrosis, and thus some of these aspects can be investigated during B3 protocols.

**Advantages:** easy to obtain and work with older mice. Pathology has stabilized and is more standardised. Exercise exacerbates severity of the disease and can shorten the time required to show effects.

**Disadvantages:** the pre-existing background pathology can complicate interpretation of results.

Muscles of mdx mice. Issues associated with these different measurements are discussed. It is noted that some of the measurements e.g. whole body imaging and functional and physiological assessments (see Whole body imaging to measure histopathology over time, In vivo measurements of whole body function and muscle strength in mice and Whole animals: in situ nerve stimulated contraction with dynamometer to measure function of muscle groups) are done on intact animals, and often repeated throughout the study, prior to sacrifice.

**Measuring leakiness of myofibres**

Lack of functional dystrophin renders dystrophic myofibres susceptible to mechanical stresses, such as exercise-induced damage that result in small disruptions of the muscle sarcolemma. These membrane lesions may be rapidly resealed or lead to further myofibre breakdown and necrosis. The exact mechanisms determining whether initial lesions result in resealing of the damaged sarcolemma (Doherty and McNally, 2003; McNeil and Kirchhausen, 2005) or alternatively result in myofibre necrosis are of considerable interest and underpin much current therapeutic research. There is good in vitro and in vivo evidence to support the hypothesis that the initial sarcolemmal damage is exacerbated by inflammatory cells and cytokines that result in further damage leading to myofibre necrosis (Brunelli et al., 2007; Grounds and Torrisi, 2004; Hodgetts et al., 2006; Radley et al., 2008; Radley and Grounds, 2006; Spencer et al., 2001; Tidball and Wehling-Henriks, 2005). Two main approaches are used to measure the extent of damaged (leaky or necrotic) myofibres. One uses labels (such as dyes or proteins) that rapidly enter through damaged sarcolemma into myofibres and remain in the sarcoplasm, and the other measures blood levels of various proteins that diffuse out of damaged myofibres.

**Evans Blue Dye (EBD)**

Evans Blue Dye (EBD) is used to identify blood vessel and cell membrane permeability in vivo since it is non-toxic and can be injected systemically as an intravital dye (Hamer et al., 2002). EBD uses albumin as a transporter molecule and diffuses into cells through membrane discontinuities to readily identify myofibres with permeable (leaky or necrotic) sarcolemma. Damaged myofibres which stain positive for EBD can be viewed in two different ways. Macroscopic examination of the whole mouse once the skin is removed shows dark blue stained myofibres that demonstrate the extent and pattern of damage for all muscles in the body (Matsuda et al., 1995; Straub et al., 1997). Microscopic examination of frozen muscle sections under green fluorescent light (Hamer et al., 2002) identifies EBD positive myofibres by red auto-fluorescence and has been widely used to quantitate myofibre damage (as a proportion of total myofibres) in many studies using mdx mice (Archer et al., 2006; Brussee et al., 1997; Shavlakadze et al., 2004; Straub et al., 1997). Once EBD enters the myofibre it diffuses along the length of the myofibre and thus may be visible at
some distance (~150 μm) from the initial site of damage (Hamer et al., 2002; Straub et al., 1997): this is an important consideration when viewing transverse muscle sections. EBD persists in vivo for at least 4 days after intraperitoneal injection (Hamer et al., 2002).

Administration of EBD is by intraperitoneal (IP) or intravenous (IV) injection. IV is widely used as it ensures rapid availability to all tissues, although IV injections through the tail vein of (black) mdx mice can be difficult for the inexperienced: therefore the easier route of administration is IP injection. Recommended protocols for EBD administration are: an IP injection, 16–24 h prior to tissue sampling, of a 1% dye solution injected at 1% volume relative to body mass (Hamer et al., 2002) or IV injection (tail vein) of EBD that can be done within 3–6 h prior to sampling (Straub et al., 1997). The choice between these protocols is probably not critical for many experiments.

It is agreed that myofibres with histologically distinct necrosis always stain positive for EBD (Archer et al., 2006; Brussée et al., 1997; Matsuda et al., 1995; Straub et al., 1997) and that myofibres with an intact sarcolemma do not stain (Matsuda et al., 1995; Straub et al., 1997). Some studies report that hypercontracted myofibres stain EBD positive (Brussée et al., 1997; Matsuda et al., 1995) whereas others do not support this conclusion (Straub et al., 1997). EBD can be present within myofibres that appear morphologically normal in H&E stained sections (Brussée et al., 1997; Hamer et al., 2002) and EBD uptake (into leaky cells) does not always reflect severe myofibre damage (necrosis) that will provoke regeneration and require myogenesis (Archer et al., 2006). Thus, the number of EBD positive myofibres can exceed and not accurately reflect the number of necrotic myofibres (Shavlakadze et al., 2004; Straub et al., 1997). This must be considered when quantitating spectrophotometrically the total EBD (in a section or extracted from dystrophic muscle) as a measure of overall damage (Hamer et al., 2002).

Other markers (dyes and proteins) that enter damaged myofibres

Other techniques to measure sarcolemmal damage (reviewed in Hamer et al., 2002) include identification of proteins from the blood that have entered damaged myofibres, such as albumin (less sensitive than EBD bound to albumin but avoids the need for EBD administration) or fibronectin (Palacio et al., 2002); fluorescent labelled dextrans and Procion orange dye (POD). POD (FW 631 g mol⁻¹) is a smaller molecule than EBD and can be administered in vitro or in vivo (although there is some uncertainty about toxicity in the latter) (Pagel and Partridge, 1999; Palacio et al., 2002). POD is especially useful for post-sampling labelling of damaged myofibres by immersion of muscles in POD solution for 30–60 min (2% wt/volume in Ringer's solution), which conveniently avoids the need for in vivo administration (Wehling et al., 2001). However, in vitro immersion is limited by the possibility of additional myofibre injury during the procedure (Consolino and Brooks, 2004; Palacio et al., 2002).

Blood measurements of CK and other proteins that leak out of damaged myofibres

In blood samples (serum or plasma), a high level of activity of the enzyme creatine kinase (CK) (measured in a spectrophotometric assay) is another index of sarcolemmal fragility widely used as a diagnostic marker for muscular dystrophy (Zatz et al., 1991). It is assumed that the enzyme activity is a direct measure of the CK protein level in blood. The muscle (MM) isofrom of CK is produced by both skeletal and heart muscle and total CK measurement in serum can include isoenzymes of CK derived from other tissues. Normally this is not a major issue and can be addressed by measuring the specific CK isofroms if required. The relationship between muscle damage and serum CK is not always straightforward and can be influenced by many factors. Exercise generally increases serum CK, suggesting a direct correlation between mechanical stress and sarcolemmal damage especially in dystrophic muscles (De Luca et al., 2005) and lower serum CK levels are an indication of reduced pathology and drug efficacy. CK levels in mdx mice increase between 7 and 10 days post-natally indicating the onset of muscle leakiness, are higher in older males than females (Yoshida et al., 2006) and decrease by 1 year of age (Coulton et al., 1988b). CK measurements at rest range from about 1000–7000 U/l for females and up to 17,000 U/l for males and in exercised mdx mice range from 1500–30,000 U/l. Problems with CK measurements relate to high variability between individual mdx mice and changes related to age and the stage of disease. Humans studies show that after a single bout of strenuous exercise (that damages the sarcolemma), blood CK levels can increase immediately, peak by 24 hours and return to control values by 48 hours (McBride et al., 2008). However, many factors, including training and type and duration of exercise, affect the extent to which blood CK levels increase and persist (Branaccio et al., 2008) and the kinetics of blood CK levels might be affected by the dystrophic phenotype. There is also variability between assay runs and possible interference of chemicals used for plasma preparation with the diagnostic kits, so it is recommended that control normal mouse blood is included for all assays. Collection of a sufficient volume of blood (about 200–500 μl) blood is required to yield approximately 100–200 μl serum and at least 5–50 μl is required per assay) is invasive and stressful. Thus, rather than multipoint evaluation in the same animals, most studies collect blood from the heart under terminal anaesthesia. Due to the high variation, CK analysis requires quite large number of mice (n=6–8) for unequivocal statistical analysis. Increased blood CK levels may also reflect increased muscle mass or increased CK within myofibres; it is noted that up-regulation (about double) of the creatine synthetic pathway is reported in mature muscles of mdx mice (McClure et al., 2007). Despite these issues, dramatic changes in blood CK levels can be a very useful measure of the severity, or correction, of dystrophy and this blood marker is used widely. Other proteins that leak from damaged myofibres into the blood and have been used as a measure of muscle damage include the enzymes pyruvate kinase (Coulton et al., 1988b), aldolase, enolase, aspartate aminotransferase, and lactate dehydrogenase isoenzyme 5, as well as the muscle proteins myoglobin, troponin and alpha-actin (Martinez Amat et al., 2007) and some of these have been examined in mdx mice. In addition, leakage into serum of the soluble Ca(++)-binding protein parvalbumin that is very high in fast myofibres has been proposed as a useful diagnostic tool in mdx mice (Jockusch et al., 1990). To date, CK measurements remain the most widely used for monitoring muscle diseases but other more specific markers may emerge.

Whole body imaging to measure histopathology over time

Image capture technology aims to provide routine imaging of whole animals, body parts or whole muscles without the need for tissue biopsy or animal sacrifice, thus allowing repeat imaging from an individual mouse over time: this would be a great advantage. However, these techniques are still new and not yet established for routine laboratory use and they will face the same problems of standardisation to allow comparison of results between laboratory groups. A number of biomedical imaging modalities have been used to examine muscle tissue in vivo, including ultrasonography, magnetic resonance imaging (MRI) and confocal and multi-photon microscopy, with the potential of Optical Coherence Tomography (OCT) just starting to be investigated for mdx mice (Pasquesi et al., 2006) (reviewed in Klyen et al., 2008).

MRI can distinguish between healthy and damaged dystrophic muscles of mdx mice (McIntosh et al., 1998). The resolution is enhanced by combination with albumin-targeted contrast agents (taken into damaged cells) (Amthor et al., 2004), although another study in mice concluded that endogenous MR contrast was sufficient and did not require combination with a gadolinium based MRI contrast agent (Walter et al., 2005). MRI (without and with contrast...
agent) has recently been used in the dystrophic dog model where very high biological variation and the expense of dogs (Thibaud et al., 2007) makes 3-dimensional in vivo tissue analysis of an individual animal over time particularly attractive, especially for evaluating the effects of pre-clinical trials. The acquisition times for MRI are long and so motion due to cardiovascular-induced or breathing-induced artefacts can be an issue. The resolution of MRI is not very high (the resolution of 125 μm is insufficient to image the individual myofibres with an average diameter of 30–50 μm) but the development of MRI scanners specifically for mice, combined with enhanced image detail, may result in this technique becoming more widely used experimentally. MRI can also be used to monitor, non-invasively, transplanted stem cells pre-labelled by incubation with ferumoxide-polycation complexes that provide images with high spatial resolution (Cahill et al., 2004).

Confocal and multi-photon microscopy, with their superior resolution, can readily image individual myofibres in vivo under physiological and pathological conditions but depend on detection of fluorescent signals. For example, two-photon microscopy was used to characterize the topology and metabolic function of mitochondria within skeletal muscle of a living mouse (Rothstein et al., 2005). Whole animal imaging via fluorescent or luminescent labelling is a rapidly evolving and promising technique (reviewed in Ntziachristos, 2006). The power of this approach was elegantly demonstrated using transgenic α-sarcoglycan null mice that emit a fluorescent calpain-activated signal from damaged muscle (Bartoli et al., 2006), but this is not readily applied to conventional mdx mice (that lack the vital transgenic label).

Whether non-invasive imaging will become a routine procedure to repeatedly monitor the status of muscles in an individual animal over time in response to drug and other therapeutic treatments, remains to be demonstrated.

**Histological measurements to evaluate necrosis, regeneration and fibrosis**

Dystrophic skeletal muscle pathology in mdx mice is typically assessed on haematoxylin and eosin (H&E) stained transverse sections, with the tibialis anterior muscle of the lower hind limb being widely used, as it is readily accessible. When undertaking analysis it is important to consider the age of the mdx mice as different histological features change with age (discussed in Response of different skeletal muscles to muscular dystrophy).

**Young mdx mice (<4 weeks)**

The acute onset of myofibre necrosis occurs from around 21 days of age. 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. Normal (pre-necrotic) myofibres have peripheral nuclei, intact sarcolemma and non-fragmented sarcoplasm. Necrotic muscle is identified by the presence of infiltrating inflammatory cells (basophilic staining), hypercontracted myofibres and degenerating myofibres with fragmented sarcoplasm. Regenerating (recently necrotic) muscle is identified by activated myoblasts and, 2–3 days later, small basophilic myotubes. These myotubes subsequently mature into plump myofibres with central nuclei (regenerated myofibres). Cumulative skeletal muscle damage in young mdx mice consists of active myofibre necrosis plus the areas of subsequent regeneration (new myofibres) (Fig. 1). It is calculated that myonuclei of newly regenerated myofibres of mdx mice remain in a central location for about 50–100 days and thereafter 3–4% of myonuclei move to a peripheral sub-sarcolemmal position; i.e. numbers of central myonuclei may decline after 100 days of age (McGeachie et al., 1993). Myofibre size can be measured as the cross sectional area 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 myofibres (Briguet et al., 2004).

**Adult mdx mice (6 weeks +)**

After the acute onset of myofibre necrosis in young mice, skeletal muscle from adult mdx mice consists of a low level of necrotic and regenerating (recently necrotic) tissue, regenerated myofibres (with central nuclei) and some unaffected (intact) myofibres. As described previously, necrotic and regenerating and regenerated muscles have distinct histological features (Fig. 1). Unlike regenerated human myofibres, the nuclei of regenerated mouse myofibres stay central for many months. Therefore mdx myofibres with central nuclei are a reliable indicator of previously necrotic/regenerated tissue (although in other situations they might instead represent denervated myofibres). However, central nuclei do not indicate the ‘number’ of times that an individual myofibre has undergone necrosis and subsequent regeneration. In studies of older mdx mice, the area of muscle that has not succumbed to necrosis can be a useful measure, since this indicates resistance of the myofibres to damage: such unaffected intact myofibres look normal with peripheral nuclei (although it is noted that at a lower level the same myofibre might also contain central nuclei). Standardisation of key sampling times (e.g. 4 and 12 weeks and 6 months of age) greatly facilitates comparison of data between laboratories.

**Older mdx mice (6 months +) and fibrosis**

Fibrosis and fatty connective tissue and myofibre atrophy can be pronounced in older mdx mice. Fibrosis is readily observed in H&E stained sections but can be emphasised by routine histochemical stains such as Van Gieson’s or Masson’s Trichrome. The onset of progressive replacement of muscle by fibrous connective tissue (mild fibrosis) is reported in limb muscles from 10–13 weeks, with extensive fibrous connective tissue (fibrosis) and some calcification from 16 to 20 months of age (Keeling et al., 2007; Lefaucheur et al., 1995; Salimena et al., 2004). Intrinsic differences in collagen content (measured by amount of hydroxyproline) and metabolism have been demonstrated between functionally different normal and mdx skeletal muscles (Gosselin et al., 2007). It is well documented that the progression of dystropathology is different in the mdx diaphragm with significant fibrosis by 9 months (discussed in Diaphragm). With the diaphragm, extra care must be taken to cut sections at equivalent locations for histological comparisons, due to the complex structure and varying width of the diaphragm muscle (illustrated in Shavlakadze et al., 2004).

**Frozen vs. fixed/paraffin-embedded muscle tissue sections**

Frozen sections are routinely used as they avoid problems of shrinkage due to fixation, 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 fixed muscle sections). Muscles are routinely frozen in isopentane cooled in liquid nitrogen, since the isopentane reduces surface tension and avoids trapping air around the muscle that can slow the freezing process. Disadvantages of frozen tissues are that skill is required to prepare (to avoid ice artefact) and to cut sections, the tissues must be stored at −80°C and they can deteriorate over time. In contrast, muscles that are fixed (in 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.

Morphological features are usually identified manually by the researcher and quantified using various image analysis software. 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 (Fig. 1) would help to reduce this variation. Scientific analysis that involves any degree of interpretation should be carried out as ‘blind’ analysis.

**Immunohistochomical and molecular analyses**

**Immunological analysis**

Immunological determination can help to gain insight into tissue events accounting for the histological changes and a wealth of different antibodies can be used depending on the specific question being addressed (e.g. as a consequence of drug or other treatment); only two specific antibodies are discussed below. Determination of dystrophin levels may help to evaluate the percentage of spontaneously and/or therapy induced revertant myofibres (Yokota et al., 2006) and similar approaches apply for components of the dystrophin–glycoprotein complex. Other than for gene therapies, these approaches are useful when considering drugs able to force premature stop codon mutations, or to exert exon skipping, or to inhibit proteasome activity (Alter et al., 2006; Bonuccelli et al., 2007; Welch et al., 2007); ideally this results in uniform distribution of dystrophin in most myofibres. Similarly, immunological determination of utrophin expression is an important assay to evaluate compensatory mechanism in dystrophic muscle induced by experimental protocols and/or drugs (Moghadaszadeh et al., 2003; Nowak and Davies, 2004).

One cautionary note is that digital imaging of fluorescently labelled proteins or cells, in the hands of the inexperienced can sometimes result in false positives due to confusion with background fluorescence (that can be high in muscle tissue) and this can be exacerbated by image manipulation.

**Molecular analysis**

Treatment of animals with compounds can change gene expression profiles. Gene expression can be measured at the mRNA level, using real-time quantitative RT-PCR or microarray, or at the protein level by measuring changes in levels of specific proteins using Western blot, Elisa, proteomics (Ge et al., 2003) or phoshpo-protein profiling. Global gene expression profiling using microarrays is increasingly popular to monitor many mRNAs of target tissues before and after drug therapy. Microarray analysis of mdx mice at different stages of the disease and muscle biopsies from DMD patients, provides considerable new insights into muscular dystrophies (Chen et al., 2005; Haslett and Kunkel, 2002; Porter et al., 2003), have identified pathways that are amenable for therapeutic intervention early in the disease process and large muscle biopsy microarray data sets are now in the public domain (Bakay et al., 2002).

The powerful high-throughput tools of systems biology analysis (for RNA and protein) combined with biochemical techniques allow verification of the possible involvement of specific pathways in normal and diseased muscle (Hittel et al., 2007). However, gene expression patterns are not always easy to interpret, a change in gene expression does not always result in a change in protein expression and function, sub-threshold changes in both the gene and the protein may have a great impact for tissue function, and the relative (rather than absolute) amount of a protein may be the critical factor. Overall, the gene, protein and emerging non-protein-coding RNA (Pheasant and Mattick, 2007) array methods, which are difficult and expensive, require additional functional or biochemical analysis to detect real changes in gene product function.

In vivo measurements of whole body function and muscle strength in mice

Body weight is usually monitored (weekly or monthly) throughout chronic experiments as an index of general health, in addition to numerous tests on whole animals to evaluate overall functional capacity, muscle strength, muscle endurance and ability to fatigue and adapt. Apart from the measurements of activity (outlined in Whole animals: behavioural activity and response to exercise), there are several simple non-invasive methods to evaluate muscle function in intact whole animals (Whole animals: grip bar and rotator strength and coordination measurements). The techniques based on behavioural testing (open field, exercise, grip meters etc.) may be biased by effect of drugs on tissues other than skeletal muscle, modulating either animal motivation or animal metabolism that can modify strength and capacity to participate in the test, with the possibility of false positive or false negative results. Therefore detailed analysis of functional parameters is required for validation of a benefit. Muscles can be further tested in whole animals by invasive in situ procedures with some possibility of repeated measurements on an individual mouse (Whole animals: in situ nerve stimulated contraction with dynometer to measure function of muscle groups) and, finally, many detailed measurements are made in vitro on muscles removed from animals in terminal experiments (see Terminal physiological measurements of muscle function).

**Whole animals: behavioural activity and response to exercise**

Since activity (i.e. exercise) affects the amount of damage of dystrophic muscle, it is very important to determine whether a drug or treatment has any effect on mouse activity. The Digiscan open field apparatus measures exploratory locomotor activity of the animal, via a grid of invisible infrared light beams, with the position of the animal being determined when the beam is interrupted (Crawley, 1999; Hamann et al., 2003; Nagaraju et al., 2000). Such locomotion and behaviour is also clearly influenced by some drugs acting on cardiac and neurological systems. Behavioural tests are prone to variability and significant variation in absolute values can easily occur between different laboratories and between different experimenters within a same laboratory: therefore standard protocols must be used for these tests. Mdx mice at certain ages (e.g. between 10 and 28 weeks) show reduced locomotor activities in comparison to age and sex matched control normal mice [Nagaraju; unpublished data].

Other common ways to measure activity include monitoring voluntary wheel running or treadmill running (as outlined in Voluntary wheel running and Forced treadmill running (as outlined in Voluntary wheel running and Forced treadmill running)). The ability of mdx mice to run in either of these situations (measured as distance run/day or week, or the time taken to cover a particular distance) is an indication of their general well-being and muscle function (Brunelli et al., 2007; Dupont-Versteegden et al., 1994; Radley et al., 2008).

**Whole animals: grip bar and rotator strength and coordination measurements**

Functional strength in mice has been widely measured by exploiting the animals’ tendency to grasp a horizontal metal bar while suspended by its tail. The bar is attached to a force transducer and the force produced during the pull on the bar can be measured regularly (e.g. weekly). This is a relatively simple way of measuring body strength and repeated measurement can be made on the same individual throughout the life of the mdx mouse. This grip bar strength dynamometer is the most commonly used (for simplicity and economy) in vivo test for monitoring impaired limb strength caused by chronic exercise in mdx mice and whether a specific intervention can reduce muscle weakness (Anderson et al., 2000; Connolly et al., 2001; De Luca et al., 2005; De Luca et al., 2003; Granchelli et al., 2000; Payne et al., 2006; Smith et al., 1995). Some studies suggest that strength of mdx mice decreases after 3 months of age, but there is some controversy (Keeling et al., 2007).

Grip strength determination has to be performed under strict experimental condition as it may be affected by many variables (e.g. volition, cognition and fatigue) independent of muscle dysfunction. Therefore, as for the other behavioural approaches, the benefit of an intervention may not be through direct effects on muscle per se. It is
important that mouse strength is determined always by the same operator, using a constant protocol, i.e. a fixed number of determinations spaced by a fixed time, and possibly in a blind-fashion.

The Rotarod measures overall motor coordination; this is a motorised rotating treadmill which requires mice to maintain their grip and balance on a rotating drum, or simply lose their balance and fall off (Boglarianovich et al., 2002; Ozawa et al., 2006; Payne et al., 2006). Other tests for motor coordination involve coaxing an animal to walk along a narrow beam between two cages, to examine walking performance over successive weeks of treatment, or the wire hang-test where a mouse is placed on a wire cage lid, then held upside-down and latency to fall is recorded (Hamann et al., 2003).

Electromyographic studies are invasive but allow direct in vivo monitoring of electrical properties of muscles and disease progression in mdx mice (Han et al., 2006a). Other invasive procedures that directly measure muscle strength (without behavioural complications) are discussed below (Whole animals: in situ nerve stimulated contraction with dynamometer to measure function of muscle groups and Terminal physiological measurements of muscle function).

Whole animals: in situ nerve stimulated contraction with dynamometer to measure function of muscle groups

Measuring muscle function in situ in anaesthetized laboratory mice usually involves measuring the strength of an entire muscle group, such as the ankle plantarflexors or dorsiflexors using a dynamometer (Ashton-Miller et al., 1992; Brooks et al., 2001; Hamer et al., 2002; Miller et al., 1998). This is an invasive technique since the (peroneal or tibial) nerve innervating the muscle group of the leg is stimulated via surface, hook, or needle electrodes, with the foot of one leg secured to a force plate. When the muscle group is electrically stimulated, the apparatus measures the moment developed about the ankle during isometric, isovelocity shortening, or isovelocity lengthening contractions (Ashton-Miller et al., 1992). One advantage of this precise in vivo measurement is that changes in the functional properties, e.g. adaptation, of a muscle group can be repeatedly evaluated in an individual mouse over time [Ridgley, Grounds et al., paper under review]. This technique involves minimal surgery and there are no complicating factors such as muscle injury/repair that could affect force production. The major disadvantage is that such measurements require the use of elaborate custom-built hardware that is not widely available.

Terminal physiological measurements of muscle function

In many physiological studies, muscles are removed from the animal and various parameters measured in vitro: therefore these experiments are terminal, as are many in situ studies (below). Histological analysis can subsequently be carried out on whole muscles and these data reconciled with the physiological measurements.

In situ nerve stimulated contraction of individual whole muscles

For in situ analysis the distal tendon of (usually) a hind limb muscle such as EDL, soleus, medial gastrocnemius, or tibialis anterior is isolated and the tendon is sutured directly to the lever arm of a force/position controller while still attached to the muscle or muscle group (with the possibility of repeated experiments over time) (Brooks, 1998); alternatively the tendon is severed and then secured to the lever arm with suture (this is usually a terminal experiment). After the tendon is attached to the force-recording apparatus, the knee and body of the animal is secured, and the muscle in question stimulated by its nerve, e.g. the sciatic nerve (Brooks, 1998; Consolino and Brooks, 2004; Dellorusso et al., 2001; Schertzer et al., 2006; Stupka et al., 2006). The advantage of this technique (as for the in vitro evaluation in Whole animals: in situ nerve stimulated contraction with dynamometer to measure function of muscle groups) is that the muscle sarcolemma, basement membrane and extracellular matrix connections remain intact, and the nerve and blood supply are undamaged which permit evaluation of larger muscles (e.g. tibialis anterior, gastrocnemius) that cannot be evaluated in vitro due to difficulties with perfusion of such large muscles. A potential disadvantage of the in situ approach is that since the muscle is stimulated via the nerve, the accuracy of the measurements relies upon there being no interference with normal innervation. Some neuromuscular conditions can obviously affect peripheral nerves which may thus interfere with normal neurotransmission and invalidate the in situ approach.

Isolated whole muscles: in vitro measurements of function

Evaluation of muscle function in vitro requires careful surgical tendon-to-tendon excision of muscles from anaesthetized animals; these techniques take time to perfect. The slightest damage to muscle fibre integrity during surgery compromises muscle force-producing capacity. The isolated muscle (e.g. usually the whole EDL), is tied to a force recording apparatus which includes a fixed pin at one end and a lever arm of a dual-mode (force-length) servomotor or simply an isometric force transducer. The isolated muscle is stimulated by platinum plate electrodes that flank, but do not touch the preparation. The accurate measurement of maximum muscle force-producing capacity (and power output) in vitro is dependent upon many factors including surgical dissection, the use of accurate force recording equipment (to ensure that the muscle is stimulated adequately to recruit all motor units within the muscle) and adequate muscle perfusion to prevent any part of the muscle becoming anoxic (Lynch et al., 2001). Muscles can be stimulated to contract with muscle length either maintained (isometric contractions), or shortened (“concentric” contractions), or lengthened (eccentric or lengthening contractions). Such in vitro analysis also allows physiological parameters of diaphragm muscle to be measured (Lynch et al., 1997; Petrof et al., 1993; Steidman et al., 1991). In most cases, evaluation of muscle function capacity in vivo (Whole animals: in situ nerve stimulated contraction with dynamometer to measure function of muscle groups) or ex vivo (Terminal physiological measurements of muscle function) should produce very similar maximum forces. If there is a significant discrepancy between these values, there are deficiencies in the methodology and/or equipment. If any of these parameters are overlooked, the measurement of the muscle’s true functional capacity will be inaccurate and therefore evaluation of the efficacy of the pharmaceutical or nutritional intervention will be compromised. The relative advantages and disadvantages of the in vitro preparation for assessing muscle function, depend on whether an intact nerve and blood supply is desirable for a specific evaluation. The in vitro preparation can be advantageous for assessing whether a treatment affects the contractile apparatus directly, without contributions from the nerve and blood supply. Ideally, measuring muscle function in situ and in vitro (using a contralateral muscle) would provide the most comprehensive assessment of treatment efficacy. In practise, however, such an approach is beyond the scope of most studies both in terms of the time constraints for investigation and the technical expertise required.

Isolated individual myofibres in vitro

This technique uses isolated individual myofibres extracted from whole muscles, rather than assessment of isolated muscle fibre bundles or intact whole muscles. Experiments can be performed on membrane intact myofibres (Yeung et al., 2003) or on “skinned” myofibre preparations where the sarcolemma has been either mechanically peeled (Plant and Lynch, 2003) or chemically permeabilized (Lynch et al., 2000). This preparation allows for direct testing of the sensitivity of contractile filaments to activating Ca2+ (or other divalent cations, such as Sr2+) and an assessment of the effects of treatment on the contractile apparatus. Isolated myofibres can be attached directly between sensitive force transducers (at one end) and a length controller (at the other end) so that fibres can be activated (electrically or chemically) and fibre length either maintained,
shortened or lengthened, as per assessments on intact muscle preparations. These experiments can be informative for assessing the cellular mechanisms of action of novel treatments since they permit examination of the contractile apparatus and of excitation–contraction coupling. As for all studies using single myofibres, it is important to recognise that only a limited number of myofibres can be sampled and these may or may not be representative of the entire population of fibres within a muscle. In dystrophic muscles, the incidence of myofibres with atypical morphologies such as complex branching also needs to be considered for these physiological assessments (Williams et al., 1993). This helps to ensure that the myofibre sampling methods do not skew findings due to the inclusion of only less damaged myofibres. The single fibre assessment of muscle function provides a more physiological approach than examination of myotubes formed in culture (Han et al., 2006b).

Calcium regulation and ion channels measurements

Determination of calcium ion homeostasis

Altered Ca$^{2+}$ homeostasis, linked to altered activity of various ion channels, has been proposed as a key consequence of dystrophin-deficiency contributing to the dystrophic pathology and to calcium-dependent proteolytic events (Alderton and Steinhardt, 2000; De Luca et al., 2003; Whitehead et al., 2006b). However, it is still debated whether dysregulation of Ca$^{2+}$ is a primary consequence, or just another downstream player in the complex pathophysiological cascade. Changes in excitation–contraction coupling detected by electrophysiology are a functional integrated monitoring of altered calcium homeostasis (De Luca et al., 2001; Fraysse et al., 2004). A variety of methods including the use of radiolabelled calcium and Ca$^{2+}$-specific fluorescent dyes (like Fura-2 and FPP-18) in conjunction with microspectrofluorimetry or fluorescence digital imaging microscopy, have been used to measure alterations in resting intracellular free [Ca$^{2+}$]$_{i}$, sarcolemmal permeability and the events controlling Ca$^{2+}$ homeostasis. These techniques are usually applied ex vivo on collagenase dissociated individual myofibres, myotubes (often of the C2C12 cell line) or intact tendon-to-tendon bundles of muscle fibres, but similar methods can be used to visualize Ca$^{2+}$ events in peripherally located myofibres within intact muscles in vivo. The development of newer generation genetically encoded calcium biosensors offer the promise of improved Ca$^{2+}$ sensitivity both in vitro and in vivo and will allow for powerful two-photon Ca$^{2+}$ imaging of normal and dystrophic myofibres. A detailed discussion of these techniques is beyond the scope of this review although the determination of calcium homeostasis is an important end-point in pre-clinical testing. Despite different laboratories finding either dysregulation or no change in cytosolic Ca$^{2+}$ levels in dystrophic muscle fibres (De Backer et al., 2002), attributed primarily to differences in fibre preparations and the dyes employed, there is a general consensus that dystrophic myofibres exhibit an increased sarcolemmal permeability to Ca$^{2+}$, especially in response to osmotic and/or mechanical stress (Allen et al., 2005; Fraysse et al., 2004; Han et al., 2006b).

Channels

Altered Ca$^{2+}$ permeability and homeostasis in dystrophic muscle may result from a greater activity/expression of specific subset of voltage-independent ion channels, rather than from physical breaks in the sarcolemma (Fraysse et al., 2004; Whitehead et al., 2006b). Attention has been focused on various ion channels involved in sarcolemmal permeability including stretch-activated and transient-receptor-potential (TRP) channels, as this may lead to a better understanding of the pathogenetic events as well as identification of possible target for pharmacological intervention (Iwata et al., 2003; Rolland et al., 2006; Whitehead et al., 2006a). A variety of techniques are used for characterization of the candidate channels, such as biophysical (see below), immunofluorescent and biochemical approaches (RT-PCR, Western blotting etc.) in parallel with toxins, antibodies and drugs able to target specific channels (Rolland et al., 2006; Suchyna and Sachs, 2007; Yeung et al., 2005).

**Flow chart for a typical pre-clinical efficacy drug trial**

<table>
<thead>
<tr>
<th>Procedures</th>
<th>Baseline</th>
<th>Mid point</th>
<th>End</th>
</tr>
</thead>
<tbody>
<tr>
<td>A) Bodyweight</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>B) Rotarod test</td>
<td>✓</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>B) Grip strength test</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>B) Open field or measurement of exercise</td>
<td>✓</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>B) Echocardiography</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B) MRI (in vivo)</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A) Blood collection</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A) Tissues for histology and immunohistochemistry</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B) In vitro force measure</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B) Electrophysiology or calcium homeostasis or other physiological end-points</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B) Gene microarray, ELISA, biochemical and molecular biology tests</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2. Example of a flowchart for a typical 3–6 months pre-clinical drug trial in mdx mice based on protocols used by the authors. The items shown in bold (indicated by A) are essential for all pre-clinical trials. Many of the remaining procedures (indicated by the letter B) are highly recommended, with additional analyses also included.
Other ion channels and biophysical recordings

Sarcosomal ion channels, gated by voltage/mechanical/chemical stimuli, are important in determining excitation patterns and a proper contractile response. In dystrophic myofibres alterations in ion channels can result either directly from dystrophin-related sarcosomal defects, or from downstream mechanisms modulating their function and/or expression. Electrophysiological techniques can measure ion channel function (voltage-clamp recordings) and membrane excitability characteristics (current clamp recordings), by electrode-mediated recordings in isolated muscles or myofibres. A detailed description of these specialised techniques is beyond the scope of this review. However, patch clamp recordings in freshly isolated myofibres and/or cultured myotubes are highly versatile allowing detection of a single channel current for any sort of ion channel. Although a large number of native freshly dissociated myofibres have to be sampled to be representative of all muscles, alteration in Ca²⁺ permeable channels have been clearly described in mdx myofibres by this approach (Rolland et al., 2006; Vandebrouck et al., 2002; Yeung et al., 2005).

Apart from over-activity of voltage-independent Ca²⁺ channels, the muscle chloride (Cl⁻) channels (CIC-1) are most affected by muscular dystrophy. These channels are responsible for the large conductance of Cl⁻(gCl), that provides electrical stability of the sarcosome, and are monitored by the intracellular microelectrode current clamp method in intact isolated muscle. A decrease in gCl is a functional cellular sign of spontaneous (as in diaphragm) or exercised-induced (as in EDL) damage in mdx muscles, and leads to an altered excitability pattern (De Luca et al., 1997, 2003; Pierno et al., 2007). This biophysical impairment account for an increased excitability-induced stress of dystrophic muscle as well as for the EMG abnormalities detected in both mdx mice and DMD patients (Han et al., 2006a).

Part II. Conclusions, recommendation and grading of data

The many techniques that can be used to measure specific aspects of the pathophysiology of muscular dystrophy (e.g. cellular changes and function), to assess the efficacy of an intervention, have been outlined. A suite of core methods for analysis of pre-clinical experiments in the mdx mouse model are outlined in Recommendation II (see Box 3). Additional techniques will be used depending on this initial analysis and the specific questions being addressed by various experiments. A flow chart outlining a broader range of analyses is shown in Fig. 2. One result of a recent Workshop on Pre-clinical testing for Duchenne dystrophy: End-points in the mdx mouse held in Washington, USA in October 2007, is that details of many methods for analysis will be collated and available in 2008 on the websites for TREAT-NMD (http://www.treat-nmd.eu/) and Wellstone-DC (http://www.wellstone-dc.org/) to help standardise the use of these procedures.

Scale to grade efficacy of treatment

To enable efficient comparison of data a universal grade to rank the relative efficacy of each treatment is needed. Since different interventions may affect one aspect (e.g. histology) more than another (e.g. physiology) it seems appropriate to grade the outcome for each parameter, rather than simply use one overall final score. A scale with 5 grades is proposed for each parameter ranging from 1 (best) to 5 (worst), with ‘1’ corresponding to the measurement in normal control C57Bl/10Scsn mice and ‘5’ to that in untreated mdx mice. (This grading might also be applied retrospectively to many published papers to try and facilitate some meaningful comparison of existing data).

Benchmarking

Benchmarking of reference data for mdx and normal C57Bl/10Scsn mice is required to help standardise measurements. While it is appreciated that measurements may vary slightly between different laboratories (and this may be more of an issue for some techniques), such reference data for both strains of mice would be very useful to help validate a technique and to establish the range of values for the grading scale. Much of this information is already published in research papers, but needs to be collated and readily available on websites (as outlined above). Reference data for all parameters for
both male and female mdx and control C57Bl/10ScSn mice at one standard age (e.g. 12 weeks), plus a range of other ages, including measurements after exercise, would be most helpful.

The global availability of such detailed background information on various parameters for analysis that will emerge from new collaborative networks, combined with agreement on basic core standard procedures for analysis, should rapidly advance the comparison of data between laboratories world-wide and accelerate research progress.

Summary

This review describes many factors that can influence the variation between similar experiments in different laboratories (Part I) and critically evaluates the technical and methodological approaches for analysis (Part II) to help coordinate pre-clinical tests in the mdx mouse. Although the mdx mouse is far from being the ideal model, its use allows us to gain more insight into the pathology and potential therapies for Duchenne muscular dystrophy. It is hoped that a more coordinated effort and the joining of different expertise may help to achieve greater and faster success in this important field. This review makes two major recommendations. The first relates to basic standardisation of experimental approaches (Recommendation I) and the second to minimal parameters for analysis with assistance between laboratories (Recommendation II). These guide-lines are not intended as a limitation to experimental approach, but rather as a suggestion to help provide more precise answers in a field with so many expectations. It is hoped that this review and its recommendations will serve as a basis for further discussion and refinements and as a starting point for the implementation of standard approaches for evaluating pre-clinical studies in mdx mice.

Acknowledgments

This review arose from discussions at The first Brazilian International Workshop on preclinical tests for drug therapies for muscular dystrophy held in Ribeirao Preto, Brazil in late 2006. MG thanks Ian McLennan (Dunedin, New Zealand) for stimulating discussions on animal husbandry. Current research support from various funding agencies is gratefully acknowledged with grants to: MG and HR, from the National Health & Medical Research Council (NH&MRC, Australia), L’Association Francaise contre les myopathies (France) and Parent Project, Germany (Aktion Benni & Co.); KN, the US Department of Defence, National Institute of Health, Foundation to Eradicate Dystrophy and the Jain Foundation; GL, the Muscular Dystrophy Association (USA), NH&MRC (Australia) and the Australian Research Council; and ADL, Telethon-Italy.

References


TREAT-NMD Activity A07: Accelerate preclinical phase of new therapeutic treatment development

Work package 7.4: Develop standardised protocols and procedures for harmonising and accelerating pre-clinical studies (including standardised data analysis)

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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<sup>mdx/mdx</sup>, Animal Resource Centre Murdoch (Western Australia [http://www.arc.wa.gov.au/](http://www.arc.wa.gov.au/)) or a similar animal supplier such as Jackson Laboratories ([http://jaxmice.jax.org](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<sup>3</sup> cork squares.

**For paraffin 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 throughout 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. µm²). 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 dystrophopathy 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 dystrophopathy, 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 dystrophopathy 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 dystrophopathy 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 dystroopathology 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


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.
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).

<table>
<thead>
<tr>
<th>Age/Muscle</th>
<th>Quadriceps</th>
<th>Gastrocnemius</th>
<th>Tibialis Anterior</th>
<th>Triceps</th>
<th>Diaphragm</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 months (12 wks)</td>
<td>6.12% (+/- 0.6)</td>
<td>2.5% (+/- 0.4)</td>
<td>6.89% (+/- 1.4)</td>
<td>8.5% (+/- 1.4)</td>
<td>2.32% (+/- 0.2)</td>
<td>Radley-Crabb 2010 Submitted</td>
</tr>
<tr>
<td>6 months (24 wks)</td>
<td>3.32% (+/- 0.4) (Not available)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>Radley-Crabb unpublished</td>
</tr>
<tr>
<td>10 months (40 wks)</td>
<td>2.23% (+/- 1.3)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>Radley-Crabb unpublished</td>
</tr>
</tbody>
</table>

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).

<table>
<thead>
<tr>
<th>Age/Muscle</th>
<th>Tibialis Anterior</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Necrosis</td>
<td>Regeneration</td>
</tr>
<tr>
<td>24 days</td>
<td>5.11 (+/- 1.45)</td>
<td>28.03 (+/- 7.7)</td>
</tr>
<tr>
<td>28 days</td>
<td>4.56 (+/- 1.1)</td>
<td>36.25 (+/- 9.7)</td>
</tr>
</tbody>
</table>
**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).
Reduced muscle necrosis and long-term benefits in dystrophic mdx mice after cV1q (blockade of TNF) treatment

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Abstract

Tumour necrosis factor (TNF) is a potent inflammatory cytokine that appears to exacerbate damage of dystrophic muscle in vivo. The monoclonal murine specific antibody cV1q that specifically neutralises murine TNF demonstrated significant anti-inflammatory effects in dystrophic mdx mice. cV1q administration protected dystrophic skeletal myofibres against necrosis in both young and adult mdx mice and in adult mdx mice subjected to 48 h voluntary wheel exercise. Long-term studies (up to 90 days) in voluntarily exercised mdx mice showed beneficial effects of cV1q treatment with reduced histological evidence of myofibre damage and a striking decrease in serum creatine kinase levels. However, in the absence of exercise long-term cV1q treatment did not reduce necrosis or background pathology in mdx mice. An additional measure of well-being in the cV1q treated mice was that they ran significantly more than control mdx mice.

Keywords: DMD; Mdx mouse; TNF; Myofibre necrosis; Voluntary exercise

1. Introduction

Duchenne muscular dystrophy (DMD) is a lethal muscle wasting disorder, affecting approximately 1/3500 male births [1,2]. Complete absence or impaired function of the skeletal muscle protein dystrophin leaves dystrophic myofibres susceptible to damage during mechanical contraction [3,4]. Consequently this initial damage progresses to myofibre necrosis. Repeated cycles of necrosis ultimately result in replacement of myofibres by fat and fibrotic connective tissue and loss of muscle function [2,5]. It is hypothesised that the initial myofibre damage is exacerbated by the endogenous inflammatory response [6–9] and that inflammatory cells and cytokines further damage the sarcolemma resulting in myofibre necrosis rather than the repair of minor membrane lesions. There is strong evidence to suggest that inflammatory cells and cytokines play a role in skeletal muscle damage (reviewed in [8]) and dystrophic muscle tissue has a considerably different gene expression pattern compared to non-dystrophic muscle with up-regulation of multiple genes involved in both the inflammatory response and muscle regeneration [10]. Blockade or depletion of resident T cells [11], neutrophils [9], macrophages [12,13] and mast cells [14,15] in mdx mice in vivo reduces the severity of dystropathology.

These cells all produce tumour necrosis factor (TNF) that is a potent pro-inflammatory cytokine that induces chemokine expression and upregulates adhesion protein expression on endothelial cells, resulting in cell infiltration to sites of inflammation [16,17]. TNF was previously referred to as TNFα, however as discussed in a recent review [18] the renaming of TNFβ as lymphotoxin (LTα and LTβ) leaves TNFα an orphan term and thus the appropriate term for use is now TNF. TNF is elevated in both DMD and mdx mouse muscles [19–21]. Antibody blockade of TNF with the human/mouse chimeric antibody infliximab (Remicade®) in young mdx mice, results in a striking protective effect on dystrophic myofibres and suppresses the early acute phase of myofibre necrosis [7]. A similar
protective effect in young dystrophic muscle was demonstrated with etanercept (Enbrel – a soluble TNF receptor) [9]. These results strongly support a key role for TNF in both inflammation and necrosis in dystrophic muscle. In addition, both infliximab and etanercept treatment prevented the inflammatory response normally seen at 5 days in whole muscle autografts in non-dystrophic C57BL/10 mice, confirming the efficacy of these drugs in mice [22].

The monoclonal antibody infliximab was generated to block human TNF [23], and is currently very effectively used in the treatment of Crohn’s disease and rheumatoid arthritis [24]. There is controversy regarding the cross-species binding of infliximab based on in vitro tests, yet at least four papers report anti-inflammatory effects in vivo in mice [7], rats [25,26] and pigs [27]. To increase the efficacy of TNF blockade in mice and to avoid potential problems of immune response to the human constant domain sequences of infliximab, a rat monoclonal antibody specific for mouse TNF [28] and chimerized using mouse kappa light chain and mouse IgG2a heavy chain constant domain sequences (cV1q) was identified [29]. The present paper tests the effectiveness of the cV1q (mouse-specific anti-TNF) antibody in both dystrophic and non-dystrophic mice, in both short and long-term (up to 90 days) studies combined with voluntary exercise. The use of the species specific antibody is considered important for long-term studies to minimise immune problems.

The mdx mouse, an animal model for DMD [30], undergoes a spontaneous onset of acute necrosis and subsequent regeneration in limb and paraspinal muscles around 3 weeks of age [7,31]. The high level of muscle necrosis between 21 and 28 days provides an excellent model to study therapeutic interventions designed to prevent or reduce muscle necrosis, as a reduction in dystrophopathy is easily observed [7,15,32,33]. Myofibre necrosis markedly decreases and stabilises by 6 weeks of age [34,35]. The low level of dystrophopathy in adult mice can be made significantly worse by exercise that increases myofibre necrosis [14,36,37] enabling potential therapeutic interventions to be evaluated in adult mdx mice [15,38–41].

2. Experimental overview

The cV1q antibody was tested in four in vivo models of inflammation. (1) Whole muscle autografts in adult non-dystrophic C57BL/10ScSn mice (the non-dystrophic parental strain for mdx), (2) young dystrophic mdx mice (male and female littermates), (3) adult dystrophic (mdx) mice subjected to 48 h voluntary exercise and (4) long-term (up to 90 days) cV1q treatment in both exercised and unexercised adult dystrophic (mdx) mice. Voluntary wheel running has several advantages compared to forced treadmill exercise; mice are able to run at night when they are normally inactive, also voluntary exercise is less stressful to the animal than forced high intensity exercise [44,45]. We also hypothesise that the amount of voluntary exercise undertaken by an individual mouse may reflect the overall health of the mouse and serve as an additional measure of well-being to test drug interventions. The effects of cV1q are compared with infliximab and etanercept for three of the experimental models [7,9,22].

3. Material and methods

Mice. Experiments were carried out using dystrophic mdx litters (male and female littermates), dystrophic female mdx (C57BL/10ScSn<sup>mdx/mdx</sup>) mice and non-dystrophic female C57BL/10ScSn mice (Table 1). All mice were obtained from the Animal Resources Centre (ARC) Murdoch, Western Australia, housed under a 12 h day–night cycle and allowed access to food and water ad libitum. Mice were treated in strict accordance with the Western Australian Prevention of Cruelties to Animals Act (1920), the National Health and Medical Research Council and the University of Western Australia Animal Ethics.

**cV1q treatment.** Intra-peritoneal (IP) injections of cV1q and the isotype-matched, negative control antibody (cVAM) both provided by Centocor were given at a concentration of 20 μg/g/mouse/week (Table 1). For the whole muscle autografts (experiment 1) cV1q was routinely injected 24 h prior to surgery (unless otherwise stated). In young mdx mice (experiments 2 and 4) injections began at 19 days of age. In the 48 h voluntarily exercised adult mice (experiment 3) a single injection was given 24 h prior to voluntary exercise.

**Whole muscle autograft surgery.** Whole muscle autografts were used as an in vivo bio-assay to assess the anti-inflammatory properties of cV1q in non-dystrophic mice. Six-weeks-old female C57BL/10 mice were anaesthetised using 2% (v/v) Rodia Halothane. The extensor digitorum longus (EDL) muscle with both tendons was removed form the anatomical bed and transplanted onto the surface of the tibialis anterior (TA) muscle, the EDL tendons were sutured to the TA, the skin closed and wound left to heal, as described in [22,46,47]. It is well-documented that this

<table>
<thead>
<tr>
<th>Table 1</th>
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<tbody>
<tr>
<td><strong>Animal treatment summary</strong></td>
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<tr>
<td><strong>Experiment</strong></td>
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<tr>
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<tr>
<td>(1) Whole muscle autografts</td>
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<tr>
<td>(2) Necrosis onset in young mdx litters</td>
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<tr>
<td>(3) 48 h voluntary exercise</td>
</tr>
<tr>
<td>(4) Long-term cV1q treatment</td>
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</table>

* Indicates that cV1q injections were usually given 1 day prior to whole muscle graft surgery; however some injections occurred at 1 week prior to surgery and some at 1 week prior to surgery plus again at 5 days after surgery.
procedure severs the nerve and blood supply to the EDL muscle and results in necrosis and subsequent inflammation and regeneration of the graft. Regeneration that results in new muscle formation begins with infiltration of inflammatory cells and revascularization [48–50]. This inflammatory zone is well-established by 5 days after surgery and provides a good model to observe anti-inflammatory interventions. To test the duration of biological efficacy, cV1q was administered at (A) 24 h prior to surgery (−1 day), (B) one week prior to surgery, or (C) as two injections, one 24 h prior to surgery and again on day 5. Whole muscle autografts were sampled at either 5 or 7 days after surgery and paraffin processed for histological analysis.

**Voluntary exercise.** Mice were voluntarily exercised using a metal mouse wheel (300 mm) placed inside the cage. Exercise data were collected via a small magnet attached to the mouse wheel with a sensor from a bicycle pedometer, attached to the back of the cage, that recorded single wheel revolutions, allowing total distance (km) run by an individual mouse to be determined, as per [9,15]. While an extended period of voluntary exercise might result in additional muscle necrosis, it also allows myofibres that became necrotic at the beginning of the exercise regime to commence regeneration resulting in new myofibre formation; however this makes interpretation difficult against the background pathology. We tested specifically for the effects of exercise on myofibre necrosis in adult mdx mice and therefore exercise was limited to only 48 h (experiment 3) to allow for analysis shortly after the onset of exercise-induced necrosis (and before new myotube formation—that normally starts about 2.5 days after necrosis) [51].

**Long-term voluntary exercise.** Young mdx mice were placed in cages containing exercise wheels from 28 days of age. Before 28 days of age mice are too small to run on the exercise wheels and mice did not run any considerable distance until 33 days of age; therefore, exercise measurements were recorded only from 33 days through to 90 days of age (8 weeks on the wheel—experiment 4). These mice were all injected weekly with cV1q, control cVaM or sterile water from 19 days of age.

**Tissue collection and image acquisition.** All mice were sacrificed by cervical dislocation while under terminal halothane (3%v/v) anaesthesia. The TA, grafted EDL or quadriceps muscles were sampled and prepared for paraffin processing. Transverse sections (5 μm) were cut through the mid-region of each muscle. Slides were stained with haematoxylin and eosin (H&E) for morphological analysis. Non-overlapping tiled images of transverse muscle sections provided a picture of the entire muscle cross-section. Images were acquired using a Leica DM RBE microscope, a personal computer, a Hitachi HVC2OM digital camera, Image Pro Plus 4.5.1 software and Vexta stage movement software. Tiled images were taken at 10x magnification.

**Histological image analysis.** Histological analysis was carried out on whole muscle cross-sections. Muscle morphology was drawn interactively by the researcher using Image Pro Plus 4.5.1 software and specific histological features measured as a percentage (area) of the whole muscle section. All section analysis was done ‘blind’. Different morphological features are quantitated in specific experiments and are therefore covered in the results section for each individual experiment. For each individual experiment, both the left and right leg from each mouse was analysed, the numeric value (e.g., % muscle necrosis) for each mouse (n) thus represents an average of both legs.

**Serum creatine kinase assay.** While under terminal anaesthesia blood from the mdx mice (experiments 2–4) was collected via cardiac puncture. Blood was refrigerated overnight, centrifuged for 3 min (1200 rpm) and serum removed. Blood serum creatine kinase (CK) analysis was completed at the Murdoch Veterinary Hospital, Murdoch, WA.

**Statistical analysis.** All statistical analysis was completed in SPSS (SPSS 15.0 for windows). Statistical analysis for direct comparison between two groups was performed by unpaired Student’s t-tests. Multiple comparisons between groups were made using a General Linear Model, univariate analysis of variance (ANOVA). A Least Significant Difference (LSD) posthoc t-test was used to identify differences between groups. For experiments 3 and 4 there was no difference (P > 0.7) between data from cVaM injected and untreated control exercised mdx mice, therefore both groups were pooled to form one single exercised ‘control’ group. Significance was set at P < 0.05 for all comparisons.

**4. Results**

**4.1. Experiment 1: whole muscle autografts**

Morphological analysis was carried out on H&E stained transverse muscle sections of the autografted EDL at either 5 or 7 days after transplantation (Fig. 1). The area within the centre of the graft (persisting necrotic tissue) was quantitated and expressed as a percentage (%) of the whole graft area. The remainder of the graft consists of inflammatory cell infiltration, surviving myofibres and regenerating muscle (myoblasts and myotubes) (Fig. 1). The extent of persisting necrotic muscle tissue is an inverse reflection of inflammatory cell activity and new muscle formation; with a larger area of persisting (central) necrotic tissue indicating less inflammatory cell infiltration (from the periphery).

TNF is a key pro-inflammatory cytokine involved in inflammation that is essential for the removal of necrotic tissue, stimulation of revascularization and activation of muscle precursor cells for regeneration [48,50]. Therefore it was hypothesised that blockade of TNF activity via the administration of cV1q would delay or prevent the inflammatory process and thus subsequent regeneration of the graft.

The average area of persisting necrotic tissue in autografts from cV1q treated mice (71.3%) sampled at 5 days after surgery, is significantly higher than in control grafts (35%) (Fig. 2, Table 2), therefore cV1q administration at
1 day ($P = 0.01$) prior to surgery significantly impaired inflammatory cell infiltration and subsequent muscle regeneration. cV1q intervention resulted in an approximate 2-fold increase in persisting necrotic autograft tissue sampled at 5 days after surgery. However after this time, inflammation does occur and at 7 days after surgery, autografts in cV1q treated have a similar appearance to untreated control mice with little necrotic tissue and extensive new muscle formation in all grafts (Fig. 2 and Table 2). To test how long the effects of cV1q persist, one single injection of cV1q was given one week prior to surgery, resulting in a slight improvement in day 5 grafts but this was not significant, in striking contrast with cV1q when given 24 h prior to surgery.

4.2. Experiment 2: young mdx mice (litters)

The onset of muscle necrosis in mdx mice shows high levels of biological variation between both individual mice...
and litters [15]. To help reduce biological variation, cV1q
injections were performed on half a litter of mdx pups with
the remaining half of the litter used as controls (cVaM
injected). This ensures that comparisons (cV1q vs. cVaM)
are made between age matched mice of one litter to reduce
inter-litter variation [15]. Mice were sampled on either day
24 or day 28: mice sampled on day 24 were injected once
(d19) and mice sampled on day 28 were injected twice
(d19 and d26).

Muscle necrosis was identified and measured on H&E
stained transverse sections of TA muscles by the presence
of infiltrating inflammatory cells (basophilic staining) and
degenerating myofibres with fragmented sarcoplasm.
Regeneration occurs in response to necrosis and results
(2–3 days later) in myotubes that, over time, mature into
myofibres with central nuclei. Cumulative skeletal muscle
damage in young mdx mice (up to 28 days of age) consists
of active myofibre necrosis (usually only necrosis present at
24 days) plus the areas of subsequent myotube formation
(present by 28 days).

cV1q treatment significantly reduced ($P = 0.03$) cumula-
tive muscle damage in the TA of 24 and 28-day-old mdx
mice (Fig. 3), with cV1q treatment, cumulative muscle
damage in 24-day-old mice was half that seen in control
(14.59%) vs. (33.16%) in an entire cross-sectional area
(CSA) of the TA. Further examination of cumulative muscle
damage at day 24 (Fig. 3) indicates a delayed onset of
necrosis after cV1q treatment, as reflected by a significantly
higher proportion of necrosis ($P = 0.01$) and a lower pro-
portion of regeneration ($P = 0.01$) in cV1q treated mice.
That is, given there are a more regenerating myofibres
(new myotubes) in the control mice and since myotubes
first form from about 2.5 days after necrosis [51]; the onset
of necrosis may have occurred earlier in the control mice.

While the process of muscle necrosis and hence regenera-
tion was delayed after cV1q administration, there were
no adverse effects on new muscle formation. Similarly no
adverse effects on muscle formation were seen after the
administration of infliximab [7] or etanercept [9].

Analysis of serum CK levels showed a clear trend for
reduced (roughly halved) serum CK levels in young
cV1q-treated mdx mice compared with controls (day 24
cV1q treated, 3330 U/L (±378); cVaM treated, 6300
(±3676). Day 28 cV1q treated, 1200 U/L (±608); cVaM
-treated, 2950 (±1484), although due to high variation
the effect of cV1q treatment on serum CK levels was not
statistically significant.

Due to the well-documented high variation in severity of
dystrophopathy between individual mdx mice, a direct
comparison of infliximab and cV1q data for mdx mice trea-
ted at the onset of myofibre necrosis (i.e., percentage mus-
cle necrosis) is inconclusive, that is absolute numbers
cannot be compared. A comparison of ‘relative change’ is
more appropriate with both infliximab [7] and cV1q pro-
ducing 2-fold reduction in myofibre necrosis in 24-day-
old mdx mice. cV1q treatment also demonstrated a strong
protective effect (2-fold) at 28 days of age (Fig. 3) whereas
infliximab treatment showed less benefit at this later time-
point. It is concluded that both treatments were highly
effective in reducing the extent of myofibre necrosis in
young mdx mice.

4.3. Experiment 3: 48 h voluntary exercise of adult mdx mice

After 48 h of voluntary wheel, the quadriceps muscle of
6-week-old female mdx mice were analysed histologically.
Measurements were calculated for: the area occupied by
necrotic myofibres, regenerating (small myotubes) and

![Fig. 3. Cumulative muscle damage in cV1q treated mdx mice aged 24 and 28-days-old compared with control (cVaM) littermates. cV1q treatment
significantly reduced ($P = 0.03$) cumulative muscle damage in the TA muscle of both 24 and 28-day-old dystrophic mice, as indicated by *.
! shows significantly more necrosis. # indicates significantly less regeneration in cV1q treated 24-day-old mice, this reflects the delay in muscle
damage and thus absence of new muscle formation (short-term up to day 28). Each time-point consists of 2 litters (cV1q treated and untreated litter
mate controls). Error bars represent standard error and $n = 7$, 6, 6 and 5 mice, respectively, across the groups.]
regenerated (myofibres with central nuclei) muscle and unaffected/intact myofibres as described previously [15]. 100% of the muscle cross-sectional area (CSA) is made up of necrotic tissue, regenerating tissue (recently necrotic), regenerated tissue (central nuclei), unaffected tissue (never been necrotic) and connective tissue. As reported previously, exercise-induced necrosis in the TA muscle of all mice was minimal and therefore only the quadriceps muscle was analysed [9,15,41]. No differences were found between control cVaM treated or untreated control exercised mdx mice, therefore for the purpose of statistical and graphical analysis both groups were pooled and are presented as one single exercised ‘control’ group (n = 6).

Voluntary exercise (48 h) resulted in a significant (P = 0.02) increase (approximately 2-fold) in muscle necrosis in the quadriceps muscle of control mdx mice (Fig. 4) as reported previously [9,15,41]. cV1q treatment significantly reduced (P = 0.04) the extent of exercise-induced myofibre necrosis from 12.93% (control) down to 7.58%; this low level of necrosis is similar (not significantly different) to the necrosis (6.22%) in unexercised quadriceps muscle of adult mdx mice. cV1q administration did not prevent an increase in serum CK levels after 48 h exposure to voluntary exercise (data not shown).

The mdx mice ran between 4.13 and 14.45 km over the 48 h; the average distance run by the control mice was 9.03 km and the average distance run by the cV1q treated mice was 8.27 km. It is important to note that the cV1q treatment had no significant affect on the distance run by the mice over 48 h, since reduced activity might result in reduced muscle damage (% necrosis). However, the distance run by an individual mouse does not directly correlate to either the percentage of myofibre necrosis in the quadriceps muscle or serum CK (data not shown) this is also documented in previous studies [9,15].

4.4. Experiment 4: long-term cV1q treatment in exercised and unexercised dystrophic (mdx) mice

The main objective of this study was to assess long-term treatment with an antibody to TNF in muscles of dystrophic mdx mice. cV1q treatment began at 19 days of age (before the onset of myofibre necrosis) and adult female mdx mice were sampled at 90 days of age (approximately 3 months). No differences were found between cVaM treated or untreated mice, therefore for the purpose of statistical analysis both groups were pooled and are presented as one single ‘control’ group (n = 6 unexercised mice or n = 8 exercised mice). Histological analysis was performed as per experiment 3.

cV1q treated mdx mice ran more than control mdx mice over the 8 week period (35–90 days of age) of voluntary exercise. cV1q treated mice ran significantly more (P = 0.03) in total distance (383.7 km n = 8) compared with control mdx mice (236.6 km n = 8) (Fig. 5, Table 3) and cV1q treated mice also ran more on average each week (Fig. 6) with the largest difference seen in weeks 2–5.

In unexercised mdx mice cV1q treatment had no effect on the amount of active muscle necrosis in the TA (data not shown) or in the quadriceps muscles (Fig. 7). Voluntary exercise over the 8 week period caused a significant (P = 0.017) increase in myofibre necrosis in the quadriceps muscle of mdx mice (Fig. 7) in comparison to unexercised mdx mice. cV1q treatment in long-term exercised mice
caused a reduction in the percentage of active myofibre necrosis in the quadriceps muscle, however this effect was not significant. It is interesting to note that the percentage of active muscle necrosis seen after 8 weeks of exercise is similar to that seen after 48 h voluntary exercise (10.2% vs. 12.9%) indicating no adaptation to the repeated exercise regime.

In unexercised adult mdx mice cV1q treatment had no effect on serum CK (Fig. 8). In striking contrast, voluntary exercise over the 8 week period (day 35–90) caused a dramatic (10-fold) and significant \( (P = 0.01) \) increase in the level of serum CK (Fig. 8, Table 2) whereas in cV1q treated mdx mice serum CK after exercise was significantly lower \( (P = 0.01) \) and, in fact, was the same as unexercised mdx mice. These data suggest that long-term voluntary exercise significantly increases myofibre ‘leakiness’ of control mdx muscle (although has no striking impact on the amount of myofibre necrosis) and this is prevented by cV1q treatment.

It was originally hypothesised that cV1q treatment would reduce the overall extent of dystropathology as

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**Table 3**

Summary – total distance run (km) over 8 weeks by cV1q treated and control exercised mice, combined with the data for % necrosis in the quadriceps muscle and serum creatine kinase (CK) levels

<table>
<thead>
<tr>
<th>Number</th>
<th>Treatment</th>
<th>Distance (Avg. km/wk)</th>
<th>Distance (total km/8wk)</th>
<th>Necrosis (quad% area)</th>
<th>CK (U/L)</th>
<th>Unaffected tissue (quad% area)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>cVaM</td>
<td>34.4</td>
<td>275.1</td>
<td>15.85</td>
<td>39900</td>
<td>2.1</td>
</tr>
<tr>
<td>2</td>
<td>cVaM</td>
<td>54.5</td>
<td>435.7</td>
<td>12.9</td>
<td>9850</td>
<td>1.7</td>
</tr>
<tr>
<td>3</td>
<td>cVaM</td>
<td>35.2</td>
<td>282.0</td>
<td>6.15</td>
<td>33600</td>
<td>2.6</td>
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<tr>
<td>4</td>
<td>cVaM</td>
<td>12.7</td>
<td>101.6</td>
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<td>168.8</td>
<td>11</td>
<td>6250</td>
<td>1.2</td>
</tr>
<tr>
<td>Average</td>
<td>Control</td>
<td>29.6 km/week</td>
<td>236.6 km total</td>
<td>10.19%</td>
<td>18850 U/L</td>
<td>1.95%</td>
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<tr>
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<td>3.8</td>
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<td>Average</td>
<td>cV1q</td>
<td>47.9*</td>
<td>383.7*</td>
<td>7.31</td>
<td>3718*</td>
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* Indicates a significant difference \( (P < 0.05) \) between control and cV1q treatment.
measured by quantitating the number of unaffected (normal) myofibres without central nuclei – an inverse representation of disease severity. Long-term voluntary exercise significantly \((P = 0.016)\) reduced the amount of unaffected myofibres indicating a more severe dystropathology in comparison with unexercised mdx muscle. In contrast, cV1q treatment significantly maintained \((P = 0.001)\) the area of unaffected myofibres in exercised adult mdx mice in comparison to control exercised mice (Fig. 9, Table 3). This suggests that cV1q treatment reduced the severity of dystropathology and protects against myofibre damage in long-term exercised mdx mice.

5. Discussion

These data from short and long-term studies demonstrate that cV1q antibody treatment, which neutralises murine TNF, reduces the extent of muscle necrosis in both young and exercised adult dystrophic mice and supports the original hypothesis that TNF contributes to the necrosis of dystrophic muscle. cV1q treatment also significantly increased the amount of voluntary exercise completed by dystrophic mice over 8 weeks, strongly indicating long-term functional benefits combined with reduced disease severity in the mdx mice.
The anti-inflammatory property of cV1q in mice was assessed using the whole muscle autograft model. Transplantation of whole muscle autografts produces a very large mass of avascular necrotic tissue and a situation of extreme pathology, which does not directly compare to the small foci of damage in dystrophic muscle, but the autograft model is useful to test the ability of cV1q to block TNF and the inflammatory response in vivo. The analysis of variance test for multiple comparisons between groups showed statistical difference ($P = 0.02$). Posthoc LSD $T$-tests showed a significant increase ($P = 0.012$) in CK levels after exercise (**) and no significant increase ($P = 0.013$) in CK level after exercise when treated with cV1q (*). Error bars indicate standard error, horizontal bar indicates exercised mice and $n = 6, 6, 8$ and $8$ mice, respectively.

The anti-inflammatory property of cV1q in mice was assessed using the whole muscle autograft model. Transplantation of whole muscle autografts produces a very large mass of avascular necrotic tissue and a situation of extreme pathology, which does not directly compare to the small foci of damage in dystrophic muscle, but the autograft model is useful to test the ability of cV1q to block TNF and the inflammatory response in vivo. The use of such an assay is also important to confirm the bioactivity of different batches of anti-inflammatory drugs in mice in vivo. The cV1q effect on autografts at 5 days (71.3% persistent necrosis) is similar to that seen with infliximab (64%) and etanercept (77.4%) [22]. The anti-inflammatory effects of infliximab and cV1q appear indistinguishable in this autograft model and TNF blockade by both drugs (and also etanercept) prevents this acute inflammatory cell response in vivo. cV1q treatment significantly inhibits the early stages of inflammatory cell infiltration (up to day 5).
but does not prevent the long-term response of inflammation that precedes regeneration (day 7 onwards): this is presumably due to some activation of a secondary pro-inflammatory pathway that is unaffected by TNF inhibition. Infliximab and etanercept treatment similarly showed no effect in day 7 grafts compared with controls [22]. This bio-assay (at day 5) confirms an inhibitory effect of cV1q on the inflammatory response in mice in vivo as anticipated. The inhibitory effect was reproducible across multiple grafts and it was specific – in that no effects were seen with control cVaM administration.

In dystrophic mdx mice, cV1q treatment significantly reduced the severity of skeletal muscle necrosis in both the TA of young mdx mice and the quadriceps muscle of exercised adult mdx mice. The voluntary wheel exercise significantly (P = 0.02) increases (approximately 2-fold) myofibre necrosis in the quadriceps of adult mdx mice and this was prevented by pre-treatment of the mice with cV1q. These short-term studies further validate the important principle that necrosis of dystrophic myofibres can be reduced by exogenous manipulation of the potent pro-inflammatory cytokine TNF.

A striking 10-fold increase in serum CK levels in mdx mice after 8 weeks of voluntary exercise was completely prevented by cV1q treatment, dramatically emphasizing the benefits of this long-term treatment. The serum CK levels in the long-term exercised mdx mice (18,850 ± 12,933 U/l) are very high; but do fall within the range reported by Granchelli (2000) (17,200 ± 11,600 U/l) after 2 x week 30 min exercise sessions on a horizontal treadmill for 4 weeks [38].

Long-term treatment with cV1q in the absence of exercise had no significant effect on myofibre necrosis or extent of undamaged myofibres in the quadriceps muscle, or on serum CK levels. This indicates that the already low level of background dystropathology in adult mdx mice is not further reduced by cV1q blockade of TNF, whereas cV1q clearly protected against exercise-induced acute myofibre damage. The lack of effect in the unexercised mice is in striking contrast to the cV1q effects seen in exercised mdx mice: whether this reflects differences in the cellular responses in these two situations is unknown. cV1q treatment in long-term exercised mdx mice showed (1) Improved muscle function – demonstrated by an increase capacity for exercise. (2) Reduced myofibre leakiness – demonstrated by a marked reduction in serum CK levels. (3) Reduced disease severity (myofibre necrosis and regeneration cycles) – demonstrated by a greater area of unaffected myofibres at 90 days of age.

It is calculated that myonuclei of newly regenerated myofibres remain in a central location for about 50–100 days in mdx mice and thereafter 3–4% of myonuclei move to a peripheral subsarcolemmal position every 100 days i.e., numbers of central myonuclei may decline after 100 days of age [34]. In this study, we presume that the central nucleus of a regenerated myofibre takes more than 70 days to move to the periphery (from day 21 with the acute onset of necrosis to day 90 when mice are sampled); thus it is considered that myofibres with only peripheral nuclei have never undergone myonecrosis and are deemed unaffected. Counting such non-centrally nucleated (unaffected) myofibres provides a converse indication of disease severity and supports the notion of reduced myofibre damage in long-term cV1q treated mdx mice. In contrast, myofibres with central nuclei must have regenerated at least once. If an individual myofibre undergoes multiple cycles of necrosis and regeneration the appearance is similar to a myofibre that has only regenerated once during the period of the experiment and thus this analysis does not give any indication of how many times an individual myofibre (with central nuclei) may have undergone cycles of necrosis and regeneration [51]. It seems likely that the cycles of necrosis and regeneration may have occurred more often in the control mice compared with cV1q treated mdx mice, although this cannot be determined from this basic histology.

In summary, the results achieved with cV1q administration in mdx mice are highly promising and similar to the results achieved by both infliximab and etanercept administration and are strongly supported by the long-term studies combined with exercise. The optimal regime and dosage, as well as the most appropriate humanised anti-TNF drug needs to be selected for possible trials in DMD boys. The development of new TNF drugs such as TNF-TeAb (miniantibodies) further complicate the decision [52]. The study confirms that a drug intervention may not show benefits in unexercised mdx mice, thus exercise appears an essential part of the ‘standard operating procedure’ to effectively test the effects of anti-inflammatory drugs in mdx mice in vivo.

Direct comparisons of cV1q treatment with other drugs such as HCT 1026; which appears to be more beneficial than prednisolone [53] is highly desirable as a prelude to selecting the most promising drug for potential clinical trials. There are many differences between the protocols used in our mdx study and the long-term study of HCT 1026 in mdx mice [53]: these include the duration of voluntary exercise; time (age) of animal sampling; and onset the of drug administration – with the crucial issue being that HCT 1026 treatment began after the acute onset of myofibre necrosis that starts at 21 days in mdx mice [7]. It appears that the gender of mdx mice also affects the severity of the dystropathology at different ages [54] although this has not been widely recognised and the impact on drug evaluations at different ages is unclear. We used female adult mdx mice in the present study; however other experiments show a similar level of background necrosis and the same doubling of exercise-induced myonecrosis in male mdx mice at 8 weeks of age, with similar high biological variation (Radley and Grounds unpublished data). It is noted that there are numerous studies where the gender of the mice is not stated [53,55,56] or a mix of both male and female mice are used [38]. Such fundamental differences emphasise the
need for standard operating protocols to facilitate important comparisons of promising therapies.

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References


A single 30 minute treadmill exercise session is suitable for ‘proof-of concept studies’ in adult mdx mice: a comparison of the early consequences of two different treadmill protocols.

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Abstract

The extent of dystropathology in sedentary adult mdx mice is very low and exercise is often used to increase myofibre necrosis. The early events in dystrophic muscle and blood in response to exercise (leading to myofibre necrosis) are unknown. This study describes in detail two standardised protocols for the treadmill exercise of mdx mice and profiles changes in molecular and cellular events after a single 30 minute treadmill session (Protocol A) or after 4 weeks of treadmill exercise (Protocol B). Both treadmill protocols increased multiple markers of muscle damage. We conclude that a single 30 minute treadmill exercise session is a sufficient and conveniently fast screening test and could be used in ‘proof-of-concept’ studies to evaluate the benefits of pre-clinical drugs in vivo. Myofibre necrosis, blood serum CK and oxidative stress (specifically the ratio of oxidised to reduced protein thiols) are reliable markers of muscle damage after exercise; many parameters demonstrated high biological variation including changes in mRNA levels for key inflammatory cytokines in muscle. The sampling (sacrifice and tissue collection) time after exercise for these parameters is critical. A more precise understanding of the changes in dystrophic muscle after exercise aims to identify biomarkers and new potential therapeutic drug targets for Duchenne Muscular Dystrophy.

Key Words

mdx mouse, treadmill exercise, skeletal muscle damage, myofibre necrosis, creatine kinase, inflammation, oxidative stress.
**Introduction**

Duchenne Muscular Dystrophy (DMD) is an X-linked, lethal muscle wasting disorder that affects mainly boys [1, 2]. Impaired function or absence of the sub-sarcolemmal protein dystrophin, renders dystrophic myofibres susceptible to sarcolemma damage in response to contraction [3-7]. This initial damage can progress to myofibre necrosis and subsequent regeneration; repeated cycles of necrosis ultimately result in the replacement of myofibres with fat and/or fibrotic connective tissue [8]. A progressive loss of muscle mass and function in DMD leads to premature death often due to respiratory or cardiac failure [9]. While the genetic defect was identified over 20 years ago the specific cause of myofibre necrosis is still unknown, although increased levels (or dysregulation) of inflammation, oxidative stress and intracellular calcium are all heavily implicated [7, 10-16].

Mdx mice (C57Bl/10ScSn\textsuperscript{mdx/mdx}) which also lack dystrophin, are an animal model for DMD and are widely used in pre-clinical research [11, 17]. In sedentary adult mdx mice, the extent of dystropathology is relatively mild, with usually <6% myofibre necrosis in the quadriceps muscle (expressed as % of cross sectional area [CSA]) and relatively low serum creatine kinase (CK) activity – a marker of myofibre leakiness. Exercise is routinely used to increase mdx myofibre damage and increase serum CK levels [18-21] thus enabling potential therapeutic interventions to be more rigorously evaluated *in vivo* [22-27].
In the past, our laboratory has used voluntary wheel exercise over 48 hours to increase myofibre necrosis and histology to demonstrate the benefits of anti-inflammatory drugs on dystrophic muscle in vivo [26-28]. Muscle necrosis is roughly doubled (~6 to 12% CSA) in quadriceps muscle after 48 hours of voluntary exercise, although other muscles such as the tibialis anterior (TA) are barely affected by voluntary exercise [11, 24, 26].

A widely used alternative to voluntary (usually nocturnal) exercise is controlled treadmill running (experiments usually conducted during the day). This occurs at a controlled speed for a pre-determined length of time, thus eliminating some of the behavioural variables experienced with voluntary exercise. A protocol of 30 minutes treadmill running on a horizontal treadmill at a speed of 12 m/min, twice a week for at least 4 weeks, causes a significant increase in the dystropathology of adult mdx mice and is widely used in pre-clinical research [20, 22, 25, 29, 30]. There is however some concerns regarding treadmill exercise because mdx mice can have problems coping and thus be reluctant to run, although a short warm-up period at a slower speed appears to help with treadmill running [23].

A single 30 minute treadmill exercise session represents a precise amount of controlled exercise that allows the time-course of early cellular and molecular events to be measured. It is of fundamental interest to determine the extent of the initial skeletal muscle damage and associated molecular changes in response to a single 30 minute exercise session (Protocol A) in unexercised mdx mice, compared with mice exercised for
4 weeks (Protocol B) which is a widely used exercise regime [20, 22, 25, 29, 30]. In the present study parameters measured included; histological quantification of myofibre necrosis, circulating blood CK activity, quadriceps muscle gene expression levels (mRNA) of the pro-inflammatory cytokines interleukin-1 β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor (TNF) and quantification of oxidative stress (protein thiol oxidation ratio and malondialdehyde (MDA) quantitation) in the quadriceps muscle.

The aims of the present study were to: 1) develop a short (30 minute) and repeatable in vivo treadmill protocol to increase myofibre necrosis in adult mdx mice; 2) profile the time course of multiple indicators of muscle damage immediately after a single exercise session; 3) compare these responses after a single treadmill exercise session to responses after 4 weeks of treadmill exercise; 4) establish if a single 30 minute exercise session is an appropriate protocol to increase muscle damage in adult mdx mice and thus be used in pre-clinical ‘proof-of-concept’ studies; 5) identify key parameters with potential as diagnostic biomarkers to rapidly monitor efficacy of pre-clinical drug treatments.

**Materials and Methods:**

1) **Animal procedures:**

All experiments were carried out on 8 - 12 week old (adult) male non-dystrophic control C57Bl/10 and dystrophic mdx mice; mice were obtained from the Animal Resource Centre, Murdoch, Western Australia. They were maintained at the University of Western Australia
on a 12-h light/dark cycle, under standard conditions, with free access to food and drinking water. Mice of each strain were caged in groups of 3-4. All animal experiments were conducted in strict accordance with the guidelines of the National Health and Medical Research Council Code of practice for the care and use of animals for scientific purposes (2004) and the Animal Welfare act of Western Australia (2002) and were approved by the Animal Ethics committee at the University of Western Australia.

1A) Establishing the 30 minute treadmill protocol:

Based on previous research [20, 22, 29] and as per the TREAT-NMD recommended standard protocol “Use of treadmill and wheel exercise for impact on mdx mice phenotype M.2.1_001” [http://www.treat-nmd.eu/research/preclinical/SOPs/] a treadmill exercise regime consisting of 30 minutes treadmill running at a speed of 12m/minute was used. The rodent treadmill was an Exer 3/6 from Columbus Instruments (USA). **Treadmill Setup:** Individual running lanes were separated by clear Perspex dividers so that the mice could see each other while exercising. The treadmill was horizontal (0° incline) and mdx mice were run in groups of 3 or 4 as it is time consuming and inefficient to run mdx mice individually. **Exercise Protocol:** Groups of 3 or 4 mdx mice were all (1) settled for 2 mins with the treadmill belt stationary, (2) then acclimatized with gentle walking for 2 mins at a speed of 4m/min, followed immediately by (3) a warm–up of 8 minutes at 8m/min and then (4) the main exercise session for 30 minutes at 12m/min. If during the 30 minutes exercise session a mouse fatigued and could no longer run, the procedure was as follows: turn the treadmill belt off and give all mice a 2 minute rest, turn the belt on at 4m/min for
2 minutes, increase the speed to 12m/minute and run for the remainder of the 30 minutes. Repeat this process if fatigue occurs again (up to 5 times for an individual mdx mouse).

1B) Treadmill regime and Animal Sample Groups:

All experiments (exercise and sampling) were started at 8am and completed by 11am each day.

Exercise protocol A (a single 30 minute treadmill exercise session): 12 week old (completely untrained) mdx and control C57Bl/10 mice were exercised for a single session on the rodent treadmill. All male mice were sampled at 12 weeks of age. The following 3 groups of male mdx mice were used for histological analysis: 1) unexercised, 2) mice subjected to 30 minutes treadmill exercise and sampled 24hrs or 3) 48hrs post exercise. Numerous treadmill exercise experiments have been conducted in our laboratory over the last year using 12 week old male mdx mice and the histological data from all experiments were pooled to provide large group numbers (see Table 1). The time course study was conducted on a total of 32 mdx and 16 C57Bl/10 12 week old mice representing 6 different groups with n=8 for each group. The following groups were used: 1) unexercised C57Bl/10, 2) exercised C57Bl/10 sampled immediately (0 mins) post exercise, 3) unexercised mdx, 4) exercised mdx sampled immediately (0 mins) or 5) 2hrs exercise or 6) 24hrs post exercise (see Table 1).
**Exercise protocol B (4 weeks of treadmill exercise):** mdx mice were exercised on the treadmill twice a week for 4 weeks, with a consistent 72 or 96hr break between each exercise session. The treadmill exercise started when mice were 8 weeks old and therefore all mice were 12 weeks old at time of sampling. For consistency all mice had a 72hr (3 day) break before the final (8th) exercise session and subsequent sampling. The 4 week treadmill exercise protocol was conducted on a total of 32 male mdx mice representing 4 different groups with n=8 for each group. The following groups were used: 1) unexercised mdx, 2) 4 week exercised mdx sampled immediately (0 mins) or 3) 24hrs or 4) 96hrs post exercise (see Table 1).

**1C) Forelimb grip strength:** Mice from Protocol B were also assessed throughout the study for forelimb grip strength (measured 24hrs prior to 1st, 5th and 8th treadmill exercise session). Grip strength was measured using a Chatillon Digital Force Gauge (DFE-002) and a triangle metal bar, as per the TREAT-NMD recommended standard protocol “Use of grip strength meter to assess limb strength of mdx mice – M.2.2_001” [http://www.treat-nmd.eu/research/preclinical/SOPs/](http://www.treat-nmd.eu/research/preclinical/SOPs/). In brief, the mouse was placed on the front of the triangle bar (attached to a force transducer) and pulled gently until release. Each mouse underwent 5 consecutive grip-strength trials; the grip strength value for each mouse was recorded as the average of the 3 best efforts. Average grip strength was then normalized for body weight [Force (kg)/BW (g)]. Change in normalised grip strength was determined by subtracting normalised grip strength (8 weeks) from normalised grip strength (12 weeks) [25].
2) Tissue collection and image acquisition:

All mice were sacrificed by cervical dislocation while under terminal anaesthesia (2% v/v Attane isoflurane Bomac Australia). Various muscles were collected, some were immediately snap frozen in liquid nitrogen for molecular analysis (quadriceps) and some were prepared for histology (quadriceps, triceps, gastrocnemius, diaphragm, tibialis anterior and extensor digitorum longus). Limb muscles were fixed immediately in 10% BFS (Confix Australian Biostain AB1020) and remained in solution for at least 72hrs. Tissues were placed into 70% ethanol, processed in a Shandon automatic tissue processor overnight, and paraffin embedded for sectioning. Transverse sections (5μm) were cut through the mid-region of each muscle. Slides were routinely stained with Haematoxylin and Eosin (H&E) for morphological analysis of the histology. Non-overlapping tiled images of transverse muscle sections provided a picture of the entire muscle cross section. Images were acquired using a Leica DM RBE microscope, a personal computer, a Hitachi HVC2OM digital camera, Image Pro Plus 4.5.1 software and Vexta stage movement software. Tiled images were taken at 10x magnification.

3) Histological image analysis:

Histological analysis of muscle necrosis was carried out on whole muscle cross sections. Muscle morphology was drawn manually by the researcher using Image Pro Plus 4.5.1 software. The area occupied by necrotic myofibres (i.e myofibres with fragmented sarcoplasm and/or areas of inflammatory cells) was measured as a percentage (area) of
the whole muscle section. All section analysis was done ‘blind’. Histological analysis was completed as per the TREAT-NMD recommended standard protocol “Histological measurements of dystrophic muscle - M.1.2_007” http://www.treat-nmd.eu/research/preclinical/SOPs/.

4) Blood collection and Serum Creatine Kinase (CK) assay:
While mice were under terminal anaesthesia, whole blood (approx 0.5ml) was collected via cardiac puncture using a 27.5 gauge tuberculin syringe (Sigma Z192082), into a 1.5ml tube. Extensive experimentation revealed that storage of blood samples overnight at 4ºC to enable clotting leads to a false increase in serum CK levels and therefore blood samples were immediately spun down in a refrigerated centrifuged for 5 minutes (12000g), serum was removed and aliquoted. Blood serum CK activity was determined in duplicate using the CK-NAC kit (Randox Laboratories) and analysed kinetically using a BioTek Powerwave XS Spectrophotometer using the KC4 (v 3.4) program. A minimum of 10µl serum is required to complete this assay.

5) Measuring cytokine gene expression by RNA extraction and RT-PCR:
Levels of mRNA for 3 inflammatory cytokines (IL-1β, IL-6 and TNF) was measured in the quadriceps muscle since this appeared to have the greatest amount of exercise-induced muscle damage, as indicated by the extent of myofibre necrosis (Figure 4 and 5). RNA was extracted from one half of a snap frozen quadriceps muscles using Tri-reagent (Sigma
T9424) and quantitated using a Nano Drop Spectrophotometer (ND 1000) and ND 1000 software version 3.5.2. The RNA was DNase treated using Promega RQ1 RNAse free DNase (M610A), RQ1 RNAse free 10x buffer (M198A) and RQ1 DNase stop solution (M199A). RNA was reverse transcribed into cDNA using Promega M-MLV Reverse Transcriptase (M3682), random primers (C1181) and 10mM dNTPs (U1515) and the cDNA was purified using a MoBiol Clean up kit (12500-250). RT-PCRs were run on a Corbett 3000 (Corbett Research) using QIAGEN quantifast SYBR green PCR mix (204054) and QIAGEN Quantitect Primer Assays for IL-1β (QT01048355), IL-6 (QT00098875) and TNF (QT00104006), and standardised to a house-keeping gene; ribosomal protein L-19 (QT01779218) as per [31]. mRNA expression levels were calculated and standardised using Rotor-gene 6.1 and Microsoft Excel software.

6) Quantitation of Oxidative stress:

Oxidative stress in the quadriceps muscle was measured in two different ways:

i) As a ratio of oxidised (di-sulphide) to reduced (sulphydryl) protein thiols – 2-Tag technique. Frozen quadriceps muscles were crushed under liquid nitrogen, before protein extraction with 20% trichloroacetic acid/acetone. Protein was solubilised in 0.5% sodium dodecyl sulphate 0.5 M tris (SDS buffer), pH 7.3 and thiols were labelled with the fluorescent dye BODIPY FL-N-(2-aminoethyl) maleimide (FLM, Invitrogen). Following removal of the unbound dye using ethanol, protein was resolubilised in SDS buffer, pH 7 and oxidised thiols were reduced with tris(2-carboxyethyl)phosphine (TCEP, Sigma) before the subsequent unlabelled reduced thiols were labelled with a second fluorescent dye
texas red maleimide (Invitrogen). The sample was washed in pure ethanol and resuspended in SDS buffer. Samples were read using a fluorescent plate reader (Fluostar Optima) with wavelengths set at excitation 485, emission 520 for FLM and excitation 595, emission 610 for texas red. A standard curve for each dye was created using ovalubumin and all results were expressed per mg of protein, quantified using Detergent Compatible protein assay (BioRad), as per (Armstrong et al 2010, submitted [32]).

ii) Malondialdehyde (MDA), a product formed via the decomposition of lipid peroxidation products [33] was quantitated using High Performance Liquid Chromatography (HPLC). Quadriceps muscles were ground under liquid nitrogen, homogenized in 10 x 5% perchloric acid and 150µl of supernatant mixed with 150µl of 40mM Thiobarbituric acid. Samples were then incubated at 50ºC for 90 minutes and cooled on ice for 15 minutes. Butanol (250µl) was added, before vortexing and centrifuging for 5 minutes. 20µl of the upper butanol layer was injected into a C18 HPLC column (5µl, 4.6 x 150 mm, Dionex) with an isocratic mobile phase of 60:40 50mM KH$_2$PO$_4$: methanol. Samples were run at a flow rate of 800µl/min for 7 minutes; the retention time was approximately 4.5 minutes. Fluorescent detection was achieved using the bandpass filters of 515 for excitation and 553 for emission. Tetraethoxypropane (Sigma) was used as a standard for absolute calculation. Approximately 30mg of tissue was required for this assay.
7) Statistics:
Statistical analysis was performed using Microsoft excel and SPSS 16.0. Data were checked for equal distribution and normality using Q-Q plots. Multiple variables were analysed by ANOVAs (one, two or three-way to account for exercise, sampling time and strain). All data are expressed as mean +/- S.E.M unless otherwise stated.

Results:
1) Ability of mdx mice to run on the treadmill

Protocol A: Preliminary studies revealed that approximately 45% of 12 week old untrained mdx mice could not complete a full 30 minutes of treadmill exercise at 12m/min (despite being rested 5 times during the 30 minute exercise session), some exhibited severe fatigue after 10 minutes exercise. This inability to exercise on a treadmill is similar to previous reports [23, 29, 34]. For this reason the additional ‘warm-up’ period (8 minutes at 8m/min) was included in the treadmill protocol to help the mdx mice to complete the treadmill exercise session, as per [23]. It is extremely important, especially when only conducting one single exercise session, to minimise biological variation in the experimental exercise protocol. Adding a ‘warm-up’ period significantly increased the ability of mdx mice (92%) to complete the treadmill exercise session. Out of the 24 mdx mice exercised in protocol A, 2 mice did not fully complete the 30 minutes treadmill exercise; these 2 mice each completed approximately 26 minutes exercise but, since neither produced any outlying results, the data were included in all analyses.
Protocol B: The average number of rests required to complete each exercise session reduced throughout the 4 week exercise period, particularly after the 3rd week, suggesting that mdx mice can improve their running ability with exercise training (Figure 1). Untrained 12 week old male mdx mice (Protocol A) required significantly (P<0.01) more rests to complete a 30 minute exercise session compared to 12 week old male mice that had been ‘trained’ for 4 weeks (Protocol B) (Figure 1). However, it must be noted that the running protocol used in this study involved resting all the mdx mice on the treadmill when only one was experiencing fatigue and this may have had an influence on mdx running ability overtime. There was no significant difference in the running ability of unexercised 8 week old (1st session of Protocol B) and unexercised 12 week old (Protocol A) mdx mice (Figure 1).

2) Forelimb grip strength (Protocol B only):

After 4 weeks of treadmill exercise (Protocol B) the forelimb grip strength of 12 week old mdx mice was significantly weaker than unexercised mice (Figure 2) as shown by a significant (P<0.01) decrease in both absolute forelimb strength (0.168 kg ++/ 0.007 unexercised vs 0.124 kg ++/ 0.005 exercised) and a significant (P<0.01) decrease in normalised change (normalised strength at 12 wks minus normalised strength at 8 wks) in forelimb strength (0.002 kg/g ++/ 0.0004 unexercised vs 0.0003 kg/g ++/ 0.00002 exercised) (Figure 2). This decrease in forelimb grip strength is similar to previous reports [29, 30] and falls within the expected general range discussed in the TREAT-NMD recommended
standard protocol “Use of grip strength meter to assess limb strength of mdx mice – M.2.2_001” http://www.treat-nmd.eu/research/preclinical/SOPs/.

3) Histological analysis of muscle necrosis.

Biological variation:

**Protocol A:** One striking feature of histological analysis was the high variation in the amount of myofibre necrosis (fragmented sarcoplasm and inflammatory cell infiltration) from both sedentary and exercised (age, sex and muscle matched) mdx mice. The variation in myofibre necrosis (% area) in quadriceps muscle from 12 week old sedentary male mdx mice is demonstrated in Figure 3 and ranges from 1.04 – 23.1% for each individual quadriceps muscle and 2.97 -17.15% average per experimental group. When results from 9 separate experiments were pooled together (n=60 quadriceps) the average amount (% CSA) of myofibre necrosis in the quadriceps muscle of an unexercised 12 week old male mice is 6.12%. Pooled histological data were also used for myofibre necrosis in unexercised triceps muscle - 8.5% (n=28) and unexercised gastrocnemius muscle - 6.89% (n=11).

**Exercise induced myofibre necrosis:**

**Protocol A:** High variation in myofibre necrosis was again seen in response to a single 30 minute treadmill exercise session and pooled histological data were also used for the various muscles sampled 24 hours after exercise from 4 experiments (n= 15-25) and at 48
hours after exercise from 6 experiments (n= 13-43) (Figure 4). The quadriceps muscle showed the highest level of myofibre necrosis (15.06 +/- 6.01%) after a single bout of treadmill exercise when sampled 24hrs, compared with triceps, gastrocnemius, (Figure 4) tibialis anterior (TA) and extensor digitorum longus (EDL) (data not shown). Necrosis was significantly increased in treadmill exercised (compared with unexercised) quadriceps muscles when sampled at either 24hrs (P<0.01) or 48hrs (P=0.04) post exercise. Both the TA and the EDL muscle from 12 week old mdx mice had a very low level of background myofibre necrosis (average <3%) and both appeared unaffected by the single treadmill exercise session with no consistent or significant increase in myofibre necrosis (data not shown), in accordance with previous reports [24, 26, 27].

**Protocol B:** Myofibre necrosis was significantly elevated in the quadriceps (P=0.04), triceps (P=0.05), diaphragm (P=0.04) and TA (P=0.05) muscles after 4 weeks of treadmill exercise training when mdx mice were sampled 24hrs after the last exercise session. Necrosis was also significantly elevated in the diaphragm muscle (P=0.02) when sampled immediately (0mins) after the last exercise session (Figure 5), suggesting prolonged myofibre necrosis after the penultimate exercise session or a particular sensitivity to exercise induced damage in the diaphragm.

Necrosis was most elevated in the quadriceps (2x fold) and diaphragm (3x fold) muscle at 24 hours after the last exercise session. The consistently elevated necrosis in the quadriceps is similar to that seen after a single exercise session (Protocol A). No significant
increase in myofibre necrosis was seen in the exercised gastrocnemius muscle possibly due to high level of variation in this parameter (Figure 5). Myofibre necrosis (fragmented sarcoplasm and inflammation) returned to unexercised levels (or below) in all muscles, within 96hrs after exercise (i.e. when the next exercise session would be due). This indicates that dystrophic myofibres can regenerate muscle to replace necrotic sarcoplasm and inflammation in between each treadmill exercise session. This also emphasises the importance of sampling time when quantitating myofibre necrosis after treadmill exercise.

4) Blood Serum CK levels as a measure of muscle leakiness.

Protocol A: No change in serum CK level was seen after 30 minutes treadmill exercise in control C57Bl/10 mice (unexercised 183.3U/L +/− 53.8 vs exercised 267.7U/L +/− 98.7). However, serum CK levels were rapidly elevated in response to treadmill exercise in mdx mice and were significantly higher (P=0.01) when blood was collected immediately (0mins) after exercise. The exercise induced increase in serum CK was transient and CK levels dropped rapidly down to unexercised level within 24hours (Figure 6).

Protocol B: In mdx mice subject to 4 weeks treadmill exercise, blood serum CK levels were significantly (P<0.01) elevated at 24hrs after the last exercise session and decreased to unexercised level within 96 hours (Figure 6). This is in marked contrast to the rapid elevation of CK in mdx mice subjected to a single exercise session (Protocol A).
5) **Inflammatory cytokine gene expression in the quadriceps muscle.**

**Protocol A:** IL-6 mRNA levels were significantly increased in the quadriceps muscle from C57Bl/10 mice after a single 30 minute treadmill session (Figure 7ii); although there was no significant change in IL-1β or TNF mRNA. Expression of all 3 inflammatory cytokines was significantly higher in unexercised mdx mice compared to unexercised C57Bl/10 mice (Figure 7i-iii). In mdx mice, mRNA levels for both IL-1β (2hrs post exercise P=0.03) and IL-6 (0mins post exercise, P=0.05 and 2hrs post exercise P=0.05) were significantly elevated after a single 30 minute exercise session compared to unexercised mice (Figure 7i & 7ii). This rapid increase in gene expression returned to the unexercised level for both genes within 24 hours. In contrast, levels of mRNA for TNF were significantly reduced after exercise (0mins post exercise P<0.01, 2hrs post exercise P=0.02 and 24hrs post exercise P<0.01) compared to unexercised mdx mice (Fig. 7iii).

**Protocol B:** In mdx mice there was no change in mRNA for IL-1β or IL-6 immediately after 4 weeks of treadmill exercise, although mRNA for both IL-1β and IL-6 was significantly (P=0.05) decreased for muscles sampled 96hrs post exercise, compared to unexercised mdx mice (Figure 8i and 8ii). TNF mRNA was significantly decreased in muscles sampled immediately (0mins) after exercise compared to unexercised mdx mice (Figure 8iii), but returned to unexercised level within 24 hours.
6) Oxidative stress measurement in the quadriceps muscle:

i) Ratio of oxidised (di-sulphide) to reduced (sulfhydryl) protein thiols.

**Protocol A:** Oxidative stress in the quadriceps muscle, specifically the ratio of oxidised to reduced protein thiols as measured by the novel 2-tag technique, was significantly (P=0.01) higher in unexercised mdx compared to unexercised C57Bl/10 12 week old male mice (Figure 9i). Protein thiol oxidation was also significantly elevated in mdx mice 0mins (P=0.015) and 2 hours (P=0.04) after a single treadmill session compared to unexercised mdx mice (Figure 9i). Protein thiol oxidation returned to unexercised level within 24hours.

**Protocol B:** Similarly, protein thiol oxidation was significantly increased (P=0.02) in mdx mice after 4 weeks of treadmill exercise when sampled immediately (0mins) after exercise (Figure 9ii) and returned to unexercised level within 24hours.

ii) Quantitation of Malondialdehyde (MDA).

**Protocol A:** Oxidative stress in the quadriceps muscle with respect to irreversible lipid peroxidation, measured as concentration of MDA, showed no significant difference between C57Bl/10 and mdx mice, and was unaffected by exercise even in mdx mice (Figure 10). MDA levels were not measured for Protocol B.

**Discussion**

Treadmill exercise is widely used in pre-clinical experiments to increase the extent of dystrophopathy in mdx mice, yet the cellular consequences of a single 30 minute treadmill exercise session (Protocol A) have not been described previously. The present
study analysed the time-course of molecular and cellular changes after a single standardised 30 minute treadmill exercise session (Protocol A). These data were compared to data from age matched mdx mice subjected to 4 weeks of treadmill exercise (Protocol B) a protocol currently widely used for pre-clinical drug screening in mdx mice [11, 20, 22, 25].

**Running ability of mdx mice:**

Adding a short warm-up period for 8 minutes at a slower speed (8m/min) produced much more consistent running by the mdx mice in both treadmill exercise protocols. With only a single exercise session it is important that all mice complete the exercise protocol to reduce variation. It is not recommended to remove the ‘non-running’ mice from the sample group as these may represent mice with the most severe dystropathology; in comparison to a full range of dystropathology being represented in unexercised control mice. Preliminary experiments showed that adding a warm-up period significantly increased the ability of mdx mice (from 45% up to 92%) to complete the 30 minute treadmill exercise session.

The intermittent running pattern of mdx mice and reduced capacity for exercise (compared to C57Bl/10 mice) on a voluntary exercise wheel is well documented [11, 35, 36]. With such voluntary running mdx mice can stop and start as they wish, yet they still manage to run a considerable total amount e.g. up to 14km over 48hrs and up to 435km over 8 weeks for untreated adult mdx mice [26-28]. In contrast, treadmill exercise requires
continuous running (for at least 30 minutes) and mdx mice can struggle with this type of exercise (indicating fatigue) [23, 29, 30, 34]. Thus, during long-term studies with repeat (often bi-weekly) treadmill sessions it is important to note the number of times that a mouse stops running during each treadmill exercise session as this provides some insight into running ability and thus muscle condition.

In the present study, 4 weeks of treadmill exercise training (Protocol B) significantly improved the running ability of mdx mice (Figure 1). Despite exhibiting a significant reduction in both absolute forelimb strength (kg) and change in normalised forelimb strength (kg/g bw) compared to unexercised mdx mice (Figure 2), exercised mdx mice show a small increase in normalised forelimb strength after 4 weeks of treadmill exercise (Protocol B). This increase in normalised forelimb strength along with behavioural adaptation to exercise training may account for the improvement in treadmill running ability. However, it must be noted that during the treadmill exercise protocol mice were run in groups of 3 or 4 and if one mouse fatigued during the 30 minute protocol all mice on the treadmill were rested and this may have impacted the results. Some studies document an improvement in the voluntary wheel exercise ability (distance run) of mdx mice over time, especially when exercise is started at a young age [Reviewed in [37]]. However, an improvement in running capacity on a treadmill over-time has not been previously reported for mdx mice.
Myofibre necrosis:

Increased myofibre necrosis after both treadmill exercise protocols is transient (Figure 4 and 5) and muscles must be sampled between 24 - 48 hours after exercise to visualise the increase in this histological parameter. While dystrophic skeletal muscles appear fully capable of regenerating between repeat exercise sessions (Figure 5) it must be noted that grip strength is significantly reduced in mdx mice subjected to repeated bouts of treadmill exercise compared with age-matched unexercised mdx mice. Skeletal muscle fibrosis was not measured in this study although it is likely that, due to exercise induced cycles of myofibre necrosis associated with inflammation (and regeneration), fibrosis is indeed progressively increased after 4 weeks of treadmill exercise and may impact negatively on forelimb grip strength.

Similar to histological results seen after voluntary wheel exercise, treadmill exercise induces a large amount of damage in the quadriceps muscle [24, 26, 27]. Large variation in the extent of myofibre necrosis is seen for most muscles; with the quadriceps muscle showing the highest increase in necrosis after a single 30 minute exercise session (Protocol A - Figure 4) and the quadriceps, triceps and diaphragm muscles all showing increased necrosis after 4 weeks of treadmill exercise (Protocol B - Figure 5).

4 weeks of treadmill exercise (Protocol B) induced a more consistent increase in myofibre necrosis in many muscles (excluding the gastrocnemius) compared to a single 30 minute exercise session (Protocol A). These data emphasise that large groups of mdx mice (at
least 8 mice) are required for histological analysis, due to the notorious variation in mdx mice [Reviewed in detail [11, 17]] and that the choice of muscle is critical to assess the impact of exercise induced damage.

**Blood serum creatine kinase level:**

Serum CK activity is widely used as an indirect measure of muscle damage (sarcolemma leakiness) and levels are consistently increased in mdx mice after exercise [22, 26, 28, 29, 38]. There is no absolute correlation between the extent of dystopathology in an individual mdx mouse and CK activity [28], with many factors including stress and muscle mass influencing CK activity [Reviewed in [11]]. CK is an enzyme and has a short circulating half life of approximately 12 hours [39], thus there is considerable interest in understanding the kinetics of CK release from dystrophic muscle after treadmill exercise.

Serum CK was strikingly increased immediately (0 mins) after a single 30 minute exercise session (Protocol A) and returned to baseline within 24 hours post exercise (Figure 6). This elevated level is similar to the transient elevation in mdx serum CK reported at 1 hour after eccentric exercise (16º downhill, 10m/min for 5 min) [19] and to the increased (x10 fold) serum CK after 8 weeks of voluntary wheel exercise [26]. This immediate increase in serum CK after exercise indicates that Protocol A was sufficiently strenuous to render myofibres ‘leaky’ and allow the release of CK into circulation; however it was not damaging enough to induce widespread myofibre necrosis (Figure 4) in many muscles with the exception of the quadriceps. Serum CK activity is a measure of muscle damage
through-out the whole body, unlike histological analysis of myofibre necrosis which exclusively examines sections of an individual muscle. In addition to the results presented, some mice received Evans Blue Dye injections 24 hours prior to the single 30 minutes exercise session to enable histological quantitation of ‘leaky’ myofibres; however this did not produce any consistent results (data not shown).

In contrast to a single exercise session (Protocol A), CK levels in mdx mice subjected to 4 weeks treadmill training (Protocol B) were significantly increased at 24hrs after exercise, but not immediately after exercise (Figure 6). This suggests either a delayed or sustained release of CK from leaky myofibres to account for the prolonged elevation of blood CK activity. Many factors including training and type of exercise can affect the level and duration of CK increase; for example in humans a single session of high intensity resistance exercise immediately increases blood serum CK which peaks at 24 hours and begins to decline within 48 hours [40]. Our result emphasises the importance of documenting the timing of such events after different exercise regimes in order to determine the optimal time for sampling (sacrifice and tissue collection) after exercise when measuring specific parameters.

**Gene expression of inflammatory cytokines:**

IL-1β, IL-6 and TNF are 3 major pro-inflammatory cytokines; however it is also recognised that IL-6 is a myokine, with many important anti-inflammatory and metabolic effects, produced by and released from contracting myofibres *in vivo* [Reviewed in [41, 42]].
Accordingly, a significant increase in IL-6 mRNA was seen in the quadriceps muscle from both C57Bl/10 and mdx mice after a single 30 minute treadmill session (Figure 7ii). The increased IL-6 mRNA in C57Bl/10 mice after treadmill exercise does not coincide with any inflammation (no change in the expression level of IL1β or TNF – Figure 7i & 7iii) or with muscle damage (no increase in blood serum CK level or muscle necrosis) and further demonstrates the capacity of normal muscle contraction to increase IL-6 production. The pronounced increase in IL-6 mRNA in mdx mice after a single 30 minute exercise session (Protocol A) is presumably caused by both a response to myofibre contraction and exercise induced muscle damage. There was no significant increase in IL-6 mRNA in mdx mice after 4 weeks of treadmill exercise (Protocol B); this may reflect an adaptation (training) in response to treadmill exercise over time.

The level of TNF mRNA was significantly reduced after both treadmill exercise protocols in mdx mice. While there is strong evidence that TNF plays a major role in the dystropathology of mdx mice and blockade of TNF can reduce myofibre necrosis [26, 28, 38, 43], increased IL-6 after exercise can inhibit TNF [44-46] and this may explain the transient reduction in TNF mRNA seen in response to exercise in the present study.

While there were transient changes in TNF, IL1β and IL-6 mRNA expression after exercise, changes and bioavailability at the protein level were not measured. This is in part because of issues with sensitivity of quantification [47]. Indeed, attempts were made to measure TNF protein levels in blood serum using a standard ELISA (Invitrogen, USA), however
serum TNF levels for all mice were below the lowest standard (15.6pg/ml) and therefore results were uninformative (data not shown). Another important factor to consider is that high levels of TNF (and other cytokine) protein are already present, yet sequestered, within resident and invading inflammatory cells (e.g. mast cells, neutrophils and macrophages) in dystrophic muscle [13, 27, 28, 48-51]. Protein quantification makes no comment on the bioavailability or the re-distribution of these cytokines: for example these proteins are rapidly released from activated mast cells and other inflammatory cells (e.g. neutrophils and macrophages) that accumulate after myofibre damage. Thus it is likely that, despite a reduction in TNF mRNA after treadmill exercise, localised bioavailable TNF protein increases rapidly independent of gene transcription [52].

**Oxidative stress:**

A significant increase in oxidative stress in unexercised mdx compared to unexercised C57Bl/10 quadriceps muscle was demonstrated by the increased ratio of oxidised to reduced protein thiols (reversible oxidative modification) as measured by the novel 2-tag technique (Armstrong et al, 2010 submitted [32]. This elevated oxidative stress in dystrophic muscle supports earlier reports that measured oxidative stress in dystrophic muscle (from both DMD patients and mdx mice) [16, 25, 53-56].

Our second measurement of oxidative stress, using a HPLC method to quantitate MDA and irreversible peroxidation of membrane lipids, showed no significant difference in oxidative stress between C57Bl/10 and mdx quadriceps muscle and MDA levels were not
increased in mdx muscle after 30 minutes treadmill exercise (Protocol A). While previous studies have reported increased MDA in the hind limbs of young (<20days) mdx mice [56] and the gastrocnemius muscle of 90 day old mdx mice [53], compared to age-matched C57Bl/10 mice, there are numerous reasons for this discrepancy in MDA results, including animal age and specific muscle examined. It is also important to note that many commonly used methods for measuring MDA have specificity issues and only HPLC based methods are recommended [Reviewed in [57]].

Despite no evidence of increased lipid peroxidation in adult mdx quadriceps muscle, reversible changes in oxidative state were evident by the ratio of oxidised to reduced protein thiols in mdx muscle. Importantly this 2-tag method identified rapid and significant increases in protein thiol oxidation immediately (0 mins) and 2 hours after a single 30 minute treadmill exercise session, emphasising the speed of such changes in vivo and the sensitivity of this specific assay for oxidative stress.

There is an increasing need for standardised experiments involving mdx mice in order to readily compare data between laboratory groups globally; this is the subject of recent reviews [11, 17] that aim to establish a set of recommendations for pre-clinical mdx drug trials. The present study provides strong support for a single 30 minute exercise session in adult mdx mice as an appropriate fast protocol to conduct preliminary ‘proof-of-concept’ testing of potential therapeutic drugs to reduce the severity of myofibre necrosis associated with muscular dystrophy. We have successfully conducted in vivo studies in
adult mdx mice examining the potential benefits of on N-acetylcysteine (NAC) using the 30 minute treadmill exercise protocol established in this manuscript (Terrill et al, submitted). A single 30 minute exercise session (Protocol A) results in a similar level of muscle damage (muscle necrosis, serum CK, oxidative stress) as 4 weeks of treadmill exercise (Protocol B) and thus the single exercise session appears suitable as a high through-put screening test. However a short term protocol does not allow for monitoring of running pattern or changes in normalised grip strength over time. It is noted that potential therapeutic drugs for DMD (identified in pre-clinical proof-of-concept short studies) should be tested chronically to examine efficiency, toxicity and also possible negative side effects e.g on heart function.

We conclude that a single 30minute treadmill exercise session is a suitable screening protocol for assessing therapeutic interventions in adult mdx mice (proof-of-concept studies), that serum CK level and myofibre necrosis and oxidative stress in the quadriceps muscle are key endpoints which should be monitored when assessing the efficacy of drug treatments in combination with treadmill exercise (summarised in Table 2), and emphasise the importance of specific muscle and sampling time (sacrifice and tissue collection). It is hoped that this simplified single exercise protocol will help accelerate pre-clinical drug trials in mdx mice and that further insight into the very early events that lead to myofibre necrosis will identify more precise and better targets for drug interventions to reduce the severity of the dystrophopathy.
Acknowledgements

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Table 1. Summary of the animal groups and numbers used for both Protocol A (1x single 30 minute exercise session) and Protocol B (4 weeks of treadmill exercise).

Table 2) Summary of reliable indicators of muscle damage after treadmill exercise in dystrophic mdx mice. * indicates an extremely high level of high biological variation (large standard error) thus making the parameter unreliable as an indicator of muscle damage.

Figure 1. Running pattern of mdx mice over 4 weeks of treadmill exercise (8 sessions) compared to mice subjected to a single exercise session. The average number of rests required to complete each 30 minute exercise sessions is shown. Mice were 8 weeks old at the start of the Protocol B and all mice were sampled when 12 weeks old (Protocol A & B). The number of rests is significantly reduced (*) in 12 week old mdx mice after 4 weeks of treadmill exercise (Protocol B) in comparison to both untrained 12 week old (Protocol A) and untrained 8 week old mdx mice. P<0.05, N=24 mice in each session. Bars represent standard error.

Figure 2. Forelimb grip strength (kg) and normalised strength change (kg/g bw); a comparison of unexercised mdx mice with mice subjected to 4 weeks of treadmill exercise (Protocol B). Absolute forelimb strength is significantly decreased (*) in 12 week old treadmill exercised mice and normalised change in forelimb strength is also decreased after 4 weeks of treadmill exercise. Normalised change in forelimb strength was calculated by normalising absolute force to bodyweight (kg force / g body weight) and by subtracting normalised forelimb strength of 12 week old mice from normalised forelimb strength of 8 week old mice. * denotes significant difference (P<0.05), in exercised compared to unexercised mdx mice. N= 32, 8, 24, 8, 24 mice respectively. Normalised change in forelimb strength is increased by a scale factor of 100 for graphical purposes only.

Figure 3. Biological variation in myofiber necrosis in the quadriceps muscle of 12 week old unexercised male mdx mice. Each bar represents the unexercised control group from a separate experiment conducted in our laboratory over a period of 12 months. When pooled together (n=60 muscles) the average amount of myofibre necrosis in the quadriceps muscle of unexercised 12 week old male mice is 6.12%. Bars represent standard deviation (to highlight range) and n = 4-8 muscles per group. A,B denotes significant differences, groups with different letters are significantly different from each other (P<0.05).

Figure 4. Myofibre necrosis (% CSA) in the quadriceps, triceps and gastrocnemius muscles of 12 week old male mdx mice: a comparison of unexercised mice with mice subjected to a single 30 minute exercise session (Protocol A). Myofibre necrosis in the quadriceps muscle is significantly increased in treadmill exercised mice when sampled both 24hrs (n=25) and 48hrs (n=43) after exercise in comparison to unexercised mice (n=60). Bars represent standard error. N= 28, 19, 12 respectively for triceps and 11, 15, 20 respectively for gastrocnemius muscle. * denotes significant difference (P<0.05), in exercised quadriceps compared to unexercised quadriceps.

Figure 5. Myofibre necrosis (% CSA) in the quadriceps, triceps, gastrocnemius, diaphragm and tibialis anterior muscles of 12 week old male mdx mice: a comparison of unexercised mdx mice with mdx mice sampled after 4 weeks of treadmill exercise (Protocol B). * denotes significant increase in necrosis in exercised muscle in comparison to unexercised muscle (same muscle only). # denotes significant decrease in necrosis in exercised muscle in comparison to unexercised
muscle (same muscle only). For unexercised mice n= 60, 28, 11, 8, 8 muscles respectively. N=8 for all other groups. Bars represent standard error and significant differences were determined by P <0.05.

Figure 6. Blood serum CK in 12 week old male mdx mice, comparing unexercised mice with mice subjected to a single 30 minute treadmill session (Protocol A) and 4 weeks of treadmill exercise (Protocol B). Serum CK is significantly increased after a single 30 minute exercise session (Protocol A) when mdx mice are sampled 0mins and 2hrs post exercise in comparison to unexercised mdx mice. Serum CK is significantly elevated in mdx mice subject to 4 weeks of treadmill exercise (Protocol B) when sampled 24hrs post exercise in comparison to unexercised mdx mice. N=8 mice per group and bars represent standard error. A,B,C denotes significant differences, groups with different letters are significantly different from each other.

Figure 7. Gene expression (mRNA) changes in the quadriceps muscle of non-dystrophic C57Bl/10 mice and dystrophic mdx mice in response to a single 30 minute exercise session (Protocol A) for (i) IL-1β, (ii) IL-6 and (iii) TNF. IL-1β mRNA is significantly increased in mdx mice at 2hrs post exercise compared to unexercised mdx mice. IL-6 is significantly increased in both C57Bl/10 and mdx mice immediately after treadmill exercise. TNF is significantly decreased after treadmill exercise at all times in mdx mice. Bars represent standard error. N= 7-8 mice per group. A,B,C denotes significant differences, groups with different letters are significantly different from each other (P<0.05).

Figure 8. Gene expression (mRNA) changes in the quadriceps muscle of mdx mice in response to 4 weeks treadmill exercise (Protocol B) for (i) IL-1β, (ii) IL-6 and (iii) TNF. Both IL-1β and IL-6 mRNA are significantly decreased after 4 weeks of treadmill exercise when mdx mice are sampled 96hrs post exercise. TNF mRNA is significantly decreased at 0mins after exercise compared to unexercised mdx mice. Bars represent standard error. N= 8 mice per group. A,B,C denotes significant differences, groups with different letters are significantly different from each other (P<0.05).

Figure 9. Ratio of oxidised to reduced protein thiols in the quadriceps muscle of non-dystrophic C57Bl/10 and dystrophic mdx mice in response (i) a single 30 minute treadmill exercise session (Protocol A) and (ii) 4 weeks of treadmill exercise (Protocol B). Oxidative stress is significantly higher in 12 week old male mdx mice compared to C57Bl/10 mice. It is further elevated in mdx mice at both 0mins and 2 hours after a single treadmill exercise session (Protocol A) and at 0 mins after 4 weeks of treadmill exercise (Protocol B). A,B,C denotes significant differences, groups with different letters are significantly different from each other (P<0.05).

Figure 10. Malondialdehyde (MDA) concentration in the quadriceps muscle of C57Bl/10 and mdx mice in response to a single 30 minute treadmill exercise session (Protocol A). There is no difference in the MDA level between non dystrophic C57Bl/10 and mdx muscle from both unexercised mice and mice sampled immediately after exercise (0 mins). A,B denotes significant differences, groups with different letters are significantly different from each other (P<0.05).
Figure 1.

Figure 2.
Figure 3.

Figure 4.
Figure 5.

Figure 6.
Figure 7.
Figure 8.
Figure 9.
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N-acetylcysteine treatment of dystrophic mdx mice prevents exercise induced myofibre necrosis

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Abstract.

Duchenne Muscular Dystrophy (DMD) is a lethal muscle wasting disorder caused by the absence of functional dystrophin protein in skeletal muscles. Myofibre necrosis is the primary pathological consequence in DMD and oxidative stress is implicated in dystropathology. Consequently, strategies to minimize oxidative stress have the potential to be useful treatment options for DMD. In this study we examined the potential beneficial effects of N-acetylcysteine (NAC) treatment (1% and 4% in drinking water) on dystrophic mdx mice. NAC prevented treadmill exercise induced myofibre necrosis and membrane damage. While NAC had no effect on malondialdehyde level or protein carbonylation (two indicators of irreversible oxidative damage), treatment with 4% NAC significantly decreased the oxidation of glutathione and protein thiols in sedentary mdx mice. Our data provide evidence that the protective effects of NAC are multi-factorial, as NAC caused a decrease in membrane permeability and enhanced protein thiol content. NAC is highly attractive as a clinical treatment since it is readily available, inexpensive and a clinically proven drug with few adverse side effects.

Key words.

Duchenne Muscular Dystrophy; mdx mouse; N-acetylcysteine; treadmill exercise; oxidative stress; protein thiol oxidation.
1. Introduction

The muscular dystrophies are inherited muscle disorders characterised by progressive muscle wasting and weakness. The most common is Duchenne Muscular Dystrophy (DMD) a lethal, X-chromosome linked disease affecting about 1 in 3500-6000 boys worldwide (Reviewed in [1, 2]). DMD and the milder Becker Muscular Dystrophy are caused by mutations in the dystrophin gene resulting in the absence or decreased functional dystrophin protein. Skeletal and cardiac myofibres lacking functional dystrophin have an increased susceptibility to sarcolemma damage after muscle contraction which ultimately leads to myofibre necrosis and regeneration [3, 4]. Repeat cycles of widespread myofibre necrosis lead to the progressive loss of muscle mass and function in DMD, eventuating in premature death often due to respiratory or cardiac failure (Reviewed in [2, 5]).

There is no definitive treatment for DMD and, despite many negative side-effects, corticosteroids remain the standard pharmacological treatment to maintain muscle mass and strength and help prolong life [2]. Numerous approaches to replace the defective dystrophin gene have been investigated, however they are not yet clinically

Abbreviations

APF, 2-[6-(4-aminophenoxy)-3-oxo-3H-xanthen-9-yl]benzoic acid; CTAB, hexadecyltrimethylammonium bromide; GSH, reduced glutathione; GSSG, oxidised glutathione; HPLC, high performance liquid chromatography; MDA, malondialdehyde; NAC, N-acetylcysteine; SDS, sodium dodecyl sulphate; TBA, thiobarbituric acid; TCA, trichloroacetic acid; TCEP, tris(2-carboxyethyl)phosphine.
established [6]. Until genetic or molecular therapies are available, pharmacological interventions may be useful as interim measures. Particularly attractive are pharmacological agents where the toxicological information is already documented for humans, as this should accelerate clinical adoption for DMD.

Myofibre necrosis is the primary pathological consequence in DMD, thus pharmacological interventions to prevent necrosis are appealing. The mechanism by which the absence of dystrophin leads to myofibre necrosis is not certain; but there is evidence that a lack of dystrophin causes myofibre membrane fragility, which leads to susceptibility to contraction induced injury and excess reactive oxygen species production, possibly mediated by increased calcium and inflammation [7, 8]. Reactive oxygen species (ROS), such as hydroxyl radicals, can cause cellular damage by directly damaging macromolecules such as proteins, membrane lipids and DNA [9]. ROS, such as hydrogen peroxide, can also cause the reversible oxidation of protein thiols which can affect the function of many intracellular proteins including signal transduction proteins and transcription factors [10, 11]. For example, ROS can mediate activation of the transcription factor nuclear factor kappa B (NFκB) [12] which itself regulates the expression of many genes, including those involved in the inflammatory and stress response [13]. Whether it is by direct damage or via reversible modification of protein thiols, ROS have the potential to be major contributors to the pathology associated with muscular dystrophies. Consequently, strategies to minimize the generation or actions of ROS have the potential to be useful treatment options for DMD.
A pharmacological agent that targets ROS is the orally available N-acetylcysteine (NAC); NAC has antioxidant properties [14] and it has been used clinically to treat various conditions, including acetaminophen overdose (Reviewed in [15]). NAC can exert its antioxidant properties directly, as it contains a thiol which can directly scavenge some types of ROS [14-16] and NAC is also a precursor of L-cysteine which is required in the synthesis of the major intracellular antioxidant glutathione [17, 18].

NAC has shown promise as an antioxidant to decrease contraction induced injury in the mdx mouse model of DMD. Treatment of EDL muscles isolated from mdx mice with 20 mM NAC improved force production and significantly decreased the impact of eccentric contractions on myofibre membrane integrity [19]. Our objective was to test whether NAC could prevent contraction induced injury in vivo. We establish that NAC, when taken orally, decreased myofibre membrane damage and necrosis in exercised mdx mice. Consistent with the actions of NAC as an antioxidant, there is evidence of decreased oxidative stress in dystrophic muscle of mdx mice treated with NAC.


2.1 Animal procedures:

All experiments were carried out on 6 - 12 week old male dystrophic mdx (C57Bl/10ScSn<sup>mdx/mdx</sup>) and non-dystrophic control C57Bl/10ScSn (C57) mice (the parental strain for mdx). All mice were obtained from the Animal Resource Centre, Murdoch, Western Australia. They were maintained at the University of Western Australia on a 12-h light/dark cycle, under standard conditions, with free access to
food and drinking water. All animal experiments were conducted in strict accordance with the guidelines of the National Health and Medical Research Council Code of practice for the care and use of animals for scientific purposes (2004), and the Animal Welfare act of Western Australia (2002), and were approved by the Animal Ethics committee at the University of Western Australia. For consistency, all experiments (exercise and sampling) were started at 8am and completed by 11am each day.

2.2 NAC treatment:

NAC (Sigma Aldrich) treatment was administered as either a 1% drinking water solution for 6 weeks as previously described [19], or as a short term high dosage regime of 4% drinking water solution for one week, where mice consumed about 2g of NAC/kg per day) [20].

2.3 Exercise protocol:

In order to initiate contraction induced myofibre necrosis and myofibre membrane damage, mdx mice were exercised for one single 30 minute exercise session on a horizontal rodent treadmill (Columbus Instruments USA), according to a protocol established by our laboratory group [21]. In brief, groups of 3 or 4 mdx mice were (1) settled for 2 minutes on the stationary treadmill belt, (2) acclimatized with gentle walking for 2 minutes at a speed of 4 meters/minute, followed immediately by (3) a warm–up of 8 minutes at 8 meters/minute and then (4) the main exercise session for 30 minutes at 12 meters/minute. Mice were sampled either immediately after exercise (0 minutes) or 24 hours after treadmill exercise.
2.4 Tissue collection and image acquisition:

All mice were sacrificed at 12 weeks of age by cervical dislocation while under terminal anesthesia (2%v/v Attane isoflurane Bomac Australia). Various muscles were collected (while mice were under terminal anesthesia) and immediately snap frozen in liquid nitrogen for biochemical analysis, or prepared for histology. For histology, quadriceps muscles were cut in half and mounted on cork board using tragacanth gum (Sigma Aldrich G1128). Muscles were quenched in isopentane cooled in liquid nitrogen and stored at -80°C until used for sectioning. Transverse sections (5-8 μm) were cut through the mid-region of each muscle on a Leica CM3050S cryostat, as per [21]. Slides were routinely stained with Haematoxylin and Eosin (H&E). For morphological analysis, non-overlapping tiled images of transverse muscle sections provided a picture of the entire muscle cross section. Images were acquired using a Leica DM RBE microscope, a personal computer, a Hitachi HVC2OM digital camera, Image Pro Plus 4.5.1 software and Vexta stage movement software. Tiled images were taken at 10x magnification.

2.5 Histological image analysis:

Histological analysis of muscle necrosis was carried out on whole muscle cross sections of the quadriceps muscle. Muscle morphology was drawn manually by the researcher using Image Pro Plus 4.5.1 software. The area occupied by necrotic myofibres (i.e myofibres with fragmented sarcoplasm and/or areas of inflammatory cells) was measured as a percentage (area) of the whole muscle section. All section analysis was done ‘blind’. Histological analysis was completed as per the TREAT-NMD recommended standard protocol “Histological measurements of dystrophic
Due to the level of variation in myofibre necrosis in 12 week old mdx mice, histological data for untreated mdx mice consists of pooled data from previous experiments (n=60 for unexercised mice and n= 25 for exercised mice).

2.6 **Blood collection and Serum Creatine Kinase (CK) assay:**

While mice were under terminal anaesthesia, whole blood (about 0.5 ml) was collected via cardiac puncture using a 27.5 gauge tuberculin syringe (Sigma Aldrich), into a 1.5 ml tube. Blood samples were immediately spun down in a refrigerated centrifuged for 5 minutes (12000g), serum was removed and aliquoted. Blood serum CK activity was determined in duplicate using the CK-NAC kit (Randox Laboratories) and analysed kinetically using a BioTek Powerwave XS Spectrophotometer using the KC4 (v 3.4) program. In order to confirm the sampling method and analytical method, levels of CK were also assayed in the control (non-dystrophic) C57 mice. These levels were very low (approximately 400 units/L) and comparable to previous reports [22].

2.7 **Carbonylated protein:**

Oxidative damage to proteins in mdx gastrocnemius muscles was determined by measuring the carbonyl content with 2,4-dinitrophenylhydrazine as previously described [23, 24]. Frozen muscles were crushed under liquid nitrogen, before protein was extracted with 20% TCA/acetone. The protein pellets were washed in acetone and ethanol, precipitated, dried, re-suspended in 10 mM DNPH in 2 M HCl and incubated for 30 min at room temperature in the dark. Proteins were washed with ethyl acetate/ethanol (1:1) and dissolved in 6 M guanidine, and absorbance was
measured at 370 nm. Protein concentration (mg/ml) was determined using the Bio-Rad Bradford protein assay. Carbonyl concentrations are expressed as nmol of carbonyl per mg protein.

2.8 Malondialdehyde levels:
Malondialdehyde (MDA) was measured by High performance liquid chromatography (HPLC) with slight modifications to the method of Seljeskog (2006) [25]. Quadriceps muscle was ground under liquid nitrogen before the addition of 100 µl per mg of 5% perchloric acid. This was vortexed and incubated for 1 hour at 4°C. After centrifugation, 150 µl of supernatant was removed and mixed with 150 µl of 40 mM TBA. Samples and MDA standards were then incubated at 50°C for 60 minutes, 250 µl butanol was added and separation of the upper butanol layer was achieved with a C18 column (5 µl, 4.6 x 150 mm, Dionex) using a Dionex Ultimate 3000 HPLC system. MDA concentrations are expressed as nmol of MDA per mg tissue.

2.9 Glutathione assay:
The levels of reduced and oxidized glutathione were assayed using HPLC with dansyl derivatives as fluorescent markers, based on Jones (1998) with slight modifications [26]. Protein was extracted from ground quadriceps muscles by the addition (1 ml solution per 100 mg tissue) of 10% perchloric acid containing 0.2 M boric acid (Sigma Aldrich) and 20 µM γ-glutamylglutamate (internal standard). Separation was achieved using a 3-Aminopropyl column (5µm, 100x2mm, Phenomenex) using a Dionex Ultimate 3000 HPLC system. Concentrations of reduced and oxidized glutathione were expressed as nmoles per mg tissue.
2.10 Protein thiols:

Reduced and oxidized protein thiols were measured as described by Lui et al (2010) with modifications. Frozen quadriceps muscle was crushed under liquid nitrogen, before protein was extracted with 20% TCA/acetone. Protein was solubilized in 0.5% sodium dodecyl sulphate, 0.5 M tris at pH 7.3 (SDS buffer) and protein thiols were labeled with the fluorescent dye BODIPY FL-N-(2-aminoethyl) maleimide (FLM, Invitrogen). Following removal of the unbound dye using ethanol, protein was re-solubilized in SDS buffer, pH 7 and oxidized thiols were reduced with tris(2-carboxyethyl)phosphine (TCEP, Sigma Aldrich) before the subsequent unlabeled reduced thiols were labeled with a second fluorescent dye Texas Red C2-maleimide (Invitrogen). The sample was washed in ethanol and resuspended in SDS buffer. Samples were read using a fluorescent plate reader (Fluostar Optima) with wavelengths set at excitation 485, emission 520 for FLM and excitation 595, emission 610 for texas red. A standard curve for each dye was created using ovalbumin and results were expressed per mg of protein, quantified using Detergent Compatible protein assay (BioRad).

2.11 Statistics:

Significant differences between groups were determined using one way ANOVA, and all data are presented as mean ± standard error of the mean. Significance was set at p < 0.05.
3. Results.

3.1 Muscle damage (exercised mice):

We tested whether oral dosing with 1% NAC for 6 weeks could protect dystrophic muscle from exercise induced myofibre membrane damage and necrosis in vivo. In untreated mdx mice, a single 30 minute treadmill exercise session caused a significant increase in necrosis in the quadriceps muscle of mdx mice (Fig. 1A). NAC completely prevented this increase in exercise induced myofibre necrosis (Fig. 1A). The levels of necrosis were comparable to the levels of necrosis measured in NAC treated and untreated mice that had not been exercised (Fig. 1A).

We hypothesized that NAC would prevent myofibre necrosis by stabilizing dystrophic sarcolemma membranes (as measured by serum creatine kinase level). NAC treatment did not affect the levels of CK measured in unexercised mice (Fig. 1B). Following exercise, there was a rapid elevation in serum CK in untreated mdx mice, indicative of membrane damage (Fig. 1B). However, while there was a trend for decreased CK level, treatment with 1% NAC did not significantly attenuate the increase in CK release after exercise (Fig. 1B).

We tested whether an increase in NAC dosage to 4% would be more successful in preventing the exercise induced increase in plasma CK. After 1 week of treatment with 4% NAC, the level of CK was not affected in unexercised mdx mice. However, in mice treated with 4% NAC, there was about a 2.5 fold smaller increase in CK following exercise compared to untreated exercised mdx mice (Fig. 2B). Similar to the data for 1% NAC (Fig. 1A), the exercise-induced increase in myofibre necrosis in the quadriceps muscle was completely prevented by 4% NAC treatment (Fig. 2A).
3.2 Oxidative damage:

Since NAC has antioxidant properties, we examined if the protective effects of NAC could be mediated by a decrease in oxidative stress. The protein carbonyl assay is commonly used as an indicator of irreversible oxidative damage to proteins [27]. Treatment with either 1% or 4% NAC did not change the levels of protein carbonylation in unexercised mdx gastrocnemius muscles (Fig. 3A). However, it was unlikely that NAC protected mdx muscle via the prevention of irreversible oxidative damage to proteins as protein carbonylation was not increased from pre-exercise levels (4±0.4 nmol/mg protein, n=6) immediately after exercise (6.5±0.5 nmol/mg protein, n=5) or 24 hours post-exercise (6.5±0.8 nmol/mg protein, n=7). MDA widely used as a measure of irreversible oxidative damage to lipids [27]. Treatment with either 1% or 4% NAC did not change the levels of MDA in unexercised mdx quadriceps (Fig. 3B). However, it was unlikely that NAC protected mdx muscle via the prevention of irreversible oxidative damage to lipids as MDA was not increased from pre-exercise levels (0.38±0.06 nmol/mg tissue, n=9) immediately after exercise (0.37±0.06 nmol/mg tissue, n=8) or 24 hours post-exercise (0.28±0.04 nmol/mg tissue, n=9).

An increase in protein carbonyls or MDA following exercise would be indicative of oxidative damage to proteins or membrane lipids leading to membrane destabilization or necrosis in dystrophic muscle. However, protein carbonylation was not increased immediately after exercise (6.5±0.5 nmol/mg protein, n=5) or 24 hours post-exercise (6.5±0.8 nmol/mg protein, n=7) from pre-exercise levels (4±0.4 nmol/mg protein, n=6). Furthermore, MDA was not increased immediately after
exercise (0.37±0.06 nmol/mg tissue, n=8) or 24 hours post-exercise (0.28±0.04 nmol/mg tissue, n=8) from pre-exercise levels (0.38±0.06 nmol/mg tissue, n=9). These data indicate that oxidative damage to proteins or membrane lipids was not a likely cause of membrane destabilization or necrosis in dystrophic muscle following exercise.

3.3 Thiol oxidation:

NAC can scavenge reactive oxygen species indirectly by enhancing glutathione content [17]. However, the protective effects of NAC were not likely mediated by an increase in glutathione content as total glutathione content was not significantly altered in the quadriceps muscle by 1% or 4% NAC treatment (Fig. 4A). The percentage of oxidized glutathione can also be used as an indicator of oxidative stress [28]. Treating mdx mice with 1% NAC did not significantly decrease the percentage of glutathione in the oxidized form (Fig. 4B). However, 4% NAC significantly decreased the percentage of oxidized glutathione (Fig. 4B). These data are consistent with NAC acting as a thiol antioxidant in dystrophic muscle.

As NAC is a thiol containing antioxidant we assessed whether NAC protected protein thiol groups from oxidation. Treating mdx mice with 1% NAC did not change the total protein thiol content of quadriceps mdx muscle, nor the percentage of protein thiols oxidized (Fig. 4C and 4D). However, increasing NAC to 4% caused a significant decrease in the extent of protein thiol oxidation (19 % untreated vs. 12% NAC treated) (Fig. 4D). The decrease in the extent of protein thiol oxidation was not a consequence of decreased oxidized protein thiol content; instead, it was a
consequence of over a two fold increase in reduced protein thiol content from 40 to 86 nmol/mg protein.

An increase in oxidized glutathione or protein thiol oxidation following exercise would implicate thiol oxidation as a potential cause of membrane destabilization or necrosis in dystrophic muscle. For glutathione, the percentage of oxidized glutathione was not increased immediately after exercise (13±3%, n=7) or 24 hours post-exercise (10±2.8%, n=9) from pre-exercise levels (11.4±1.4%, n=9). However, for protein thiols, there was a 30% increase in protein thiol oxidation immediately after exercise (Fig 5B). As protein thiol state was affected by exercise, we examined the effect of 4% NAC treatment on protein thiol state following exercise in mdx quadriceps muscle. Consistent with the observation in unexercised mice, total protein thiols were increased in exercised mdx mice treated with 4% NAC. (Fig. 5A) In addition, significantly more protein thiols were oxidized in muscles from 4% NAC treated mice (20 nmol/mg protein) than untreated mice (13 nmol/mg protein) (Fig. 5B). Treatment with 1% NAC had no effect on protein thiol groups after treadmill exercise (data not shown). Together these data indicate that the protective properties were a consequence of NAC acting as a thiol antioxidant.

4. Discussion.
The effectiveness of NAC in preventing myofibre necrosis in dystrophic muscle has not been previously reported in vivo. In this study we show that NAC prevented exercise induced myofibre necrosis, using two different dosing regimens of 1% NAC for 6 weeks and 4% NAC for 1 week. Prevention of exercised induced myofibre necrosis is an important outcome, because repeated bouts of myofibre necrosis
eventually lead to the replacement of myofibres with fatty and fibrous connective tissue and thus a loss of function of dystrophic muscle [5, 29].

One possible mechanism by which NAC protects dystrophic muscle from exercise induced myofibre necrosis is by stabilizing fragile dystrophic sarcolemma membranes. Previous *in vitro* work supports this hypothesis, as uptake of Evans Blue Dye into isolated myofibres following stretched induced contractions decreased with exposure to NAC [19]. For the current *in vivo* experiments we used CK release to assess myofibre membrane damage; mdx mice have elevated CK levels [30] which are greatly exacerbated after treadmill exercise [22]. While there was a trend for attenuation in CK level following exercise with 1% NAC treatment, this was not statistically significant. This may be due to the high variation in CK levels following exercise in mdx mice [31], resulting in poor sensitivity to the protective effects of NAC (Type II error). However, when the dose of NAC was increased to 4%, there was a significant attenuation of increased blood CK after treadmill exercise. These findings are important as they indicate that NAC can rapidly (within one week) protect dystrophic muscle from damage *in vivo*.

These data also imply that doses of NAC higher than 1% are more protective. This will need to be further tested with consistent treatment protocols to determine the optimal dose for *in vivo* treatment. Overall, our data and evidence from a previous *in vitro* study [19] are consistent with the concept that NAC stabilizes fragile dystrophic membranes, although how this occurs is not clear.
The beneficial actions of NAC may primarily reflect its antioxidant properties since oxidative stress has been identified as a possible cause of myofibre necrosis and muscle wasting of dystrophic muscle [11, 32]. ROS can cause irreversible damage to macromolecules such as membrane proteins and lipids, with extensive damage potentially leading to membrane permeability. Such irreversible oxidation of proteins can be assessed by measuring the protein carbonyl content of muscles. Increased protein carbonyl content has been reported for both DMD biopsies and mdx muscles [33, 34]. Oxidative damage to proteins can be caused by hydroxyl radicals, which can be prevented by NAC [15, 16]. However, in the current study NAC treatment did not decrease protein carbonyl content in mdx muscle. Oxidative damage to lipids (lipid peroxidation) has also been reported in young mdx muscle [34, 35] and irreversible damage to membrane lipids can be assessed by measuring malondialdehyde (MDA), generated as a consequence of lipid peroxidation. However, we did not observe a decrease in MDA level in mdx mice after either NAC treatment. Overall, our data indicate that the protective effects of NAC in dystrophic muscle do not seem to be a consequence of a decrease in irreversible oxidative damage to protein and lipids.

Another mechanism proposed for the antioxidant properties of NAC is up-regulation of the cellular antioxidant glutathione [17], since NAC is a precursor, via cysteine, for the synthesis of glutathione [14, 36]. Glutathione, a non-protein containing thiol, is a naturally occurring antioxidant present at high concentrations (mM) in tissues that can react directly with ROS. Of particular significance, glutathione is also an essential co-factor involved in the removal of hydrogen peroxide by glutathione peroxidase [9, 37, 38]. In this study, both 1% and 4% NAC treatment produced no
increase in total glutathione in dystrophic myofibres. These observations imply that decreased myofibre necrosis caused by NAC was not a consequence of increased glutathione synthesis.

An effect of NAC treatment which has not been previously described was the increase in reduced protein thiols in mdx muscle. We propose the protective properties of NAC are a consequence of this increase in reduced protein thiol content. As reduced protein thiols can become preferentially oxidized as a result of oxidative stress, protein thiols can be considered to be acting as antioxidants, somewhat analogous to glutathione [36]. In NAC treated mice, the increase in reduced protein thiol content in tissue would have enhanced the antioxidant capacity of muscle protecting the muscle from increased oxidative stress. Consistent with concept, there was considerably more oxidized protein thiols following exercise in NAC treated mice than in untreated mice, suggesting the oxidation of the increased protein thiol pool is preventing oxidation of other constituents of the dystrophic muscle that leads to necrosis of the myofibres [36].

In summary, our novel in vivo data show that NAC protects dystrophic muscle from exercise induced myofibre necrosis, the primary cause of severe muscle pathology seen in DMD. The exact mechanism responsible for these benefits of NAC is unclear at this stage, mainly because there is still uncertainty regarding the precise pathways leading to necrosis of dystrophic myofibres. Membrane permeability and oxidative stress have been implicated in causing necrosis. Our in vivo data provide evidence that the protective effect of NAC could be multifactorial, as NAC caused a decrease in membrane damage and permeability and enhanced protein thiol
content. NAC is highly attractive as a readily available, inexpensive and clinically proven drug with few adverse side effects. Our data strongly support further preclinical investigation into the potential dose of NAC to mitigate aspects of dystrophic pathology.

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**Author Disclosure Statement.**

The authors declare they have no conflicts of interest.
References.


List of Figure Captions:

Figure 1. Effect of exercise on mdx mice treated with 1% NAC. (A) Myofibre necrosis (% CSA) was assessed in the quadriceps muscles of untreated and 1% NAC treated mdx mice prior to exercise (Unex mdx) and 24 hours after exercise (24hrs after ex.). n= 60, 8, 25 and 6 respectively. (B) CK was measured in blood serum from untreated and 1% NAC treated mdx mice prior to exercise (Unex mdx) and 0 minutes after exercise (0mins after ex.). n= 13, 8, 8 and 8 respectively.*Significantly different between untreated and 1% NAC treated. #Significantly different between unexercised and exercised mice.

Figure 2. Effect of exercise on mdx mice treated with 4% NAC. (A) Myofibre necrosis (% CSA) was assessed in the quadriceps muscles of untreated and 4% NAC treated mdx mice prior to exercise (Unex mdx) and 24 hours after exercise (24hrs after ex.). n= 60, 8, 25 and 6 respectively. (B) CK was measured in blood serum from untreated and 4% NAC treated mdx mice prior to exercise (Unex mdx) and 0 minutes after exercise (0mins after ex.). n= 13, 8, 8 and 8 respectively. *Significantly different between untreated and 1% NAC treated. #Significantly different between unexercised and exercised mice.

Figure 3. Oxidative damage in unexercised mdx mice treated with 1% and 4% NAC. (A) Carbonyl content in gastrocnemius muscle of untreated and NAC treated mdx mice. n= 6, 7 and 7 respectively. (B) Malondiadehyde content in quadriceps muscle of untreated and NAC treated mdx mice. n= 9, 8 and 6 respectively.
**Figure 4. Thiols in unexercised mdx mice treated with 1% and 4% NAC.** (A) Total glutathione content and (B) percentage of oxidised glutathione in quadriceps muscle of untreated and NAC treated mdx. n= 9, 7 and 7 respectively. (C) Total protein thiol content and (D) percentage of oxidised protein thiol content quadriceps muscle of untreated and NAC treated mdx mice. n= 9, 8 and 4 respectively. *Significantly different from untreated.

**Figure 5. Effect of exercise on protein thiols for mice treated with 4% NAC.** (A) Total protein thiol content and (B) percentage of oxidised protein thiol content of untreated and NAC treated mdx quadriceps muscle prior to exercise (Unex mdx) and 0 minutes after exercise (0mins after ex.). *Significantly different between untreated and NAC treated. #Significantly different between unexercised and exercised mice. n= 9, 6, 4 and 7 respectively.
Fig. 1
Fig. 2

A

Muscle Necrosis (% CSA)

- Untreated
- 4% NAC treated

Unex mdx | 24hrs after ex

B

Units CK per litre serum

- Untreated
- 4% NAC treated

Unex mdx | 0min after ex

# *
Fig. 3
Fig. 4
Fig. 5
The different impacts of a high fat diet on dystrophic mdx and control C57Bl/10 mice.

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Key words:
Duchenne muscular dystrophy, mdx mouse, skeletal muscle, high fat diet, high protein diet, body composition, dystropathology.
Abstract:
The absence of functional dystrophin protein in patients with Duchenne muscular dystrophy (DMD) and dystrophic mdx mice leads to fragile myofibre membranes and cycles of myofibre necrosis and regeneration. Studies in both DMD patients and mdx mice indicate that dystrophic muscles have altered metabolism and impaired energy status and has led to the proposal that nutritional supplementation may reduce the severity of dystropathology. This research compares the in vivo responses of dystrophic mdx and normal control C57Bl/10 mice to a high protein (50%) or a high fat (16%) diet. Consumption of a high protein diet had minimal effects on the body composition or muscle morphology in both strains of mice. In contrast, striking differences between the strains were seen in response to the high fat diet; which also varied between mdx mice aged <24 weeks, and mdx mice aged 24 - 40 weeks. C57Bl/10 mice demonstrated many negative side effects after consuming a high fat diet including weight gain, significantly increased body fat and elevated inflammatory cytokines. In contrast, mdx mice (≤ 24 weeks) remained lean with minimal fat deposition and were resistant to changes in body composition after consuming the high fat diet. These results support the proposal that dystrophic mdx mice have an altered ‘energy status’ compared to normal C57Bl/10 mice because the mdx mice appeared more capable of metabolising the high fat diet and avoiding fat deposition. However, older mdx mice (24 - 40 weeks old), with increased energy intake, exhibited some mild adverse effects of a high fat diet but to a far lesser extent than age-matched C57Bl/10 mice. Benefits of the high fat diet on dystrophic muscles of young mice were demonstrated by the significantly increased running ability (km) of voluntarily exercised mdx mice and significantly reduced myofibre necrosis in 24 week old sedentary mdx mice. These novel data clearly identify an ‘altered’ response to a high fat diet in dystrophic mdx compared to normal C57Bl/10 mice. The high fat diet was beneficial to young mdx mice and our data indicate that energy deficient mdx mice may utilise excess dietary fat to improve muscle function and reduce muscle damage.
Introduction:

Duchenne Muscular Dystrophy (DMD) is a lethal X-linked muscle wasting disease resulting from defects in the myofibre subsarcolemmal protein dystrophin. DMD is characterised by progressive muscle weakness and wasting with a limited life expectancy of approximately 20 years in humans (Biggar, 2006; Bushby et al, 2010; Emery, 2002; Sinnreich, 2010). The lack of functional dystrophin leads to myofibre membrane fragility, repeated cycles of myofibre necrosis and regeneration, and the eventual replacement of skeletal muscle by fatty and fibrous connective tissue. Corticosteroids remain the standard pharmacological treatment to maintain muscle mass and help prolong life, despite severe adverse side-effects including obesity, Cushingoid symptoms, immune suppression, hypertension, behavioural changes and cataracts (Angelini, 2007; Biggar et al, 2006; Bushby et al, 2010; Manzur et al, 2008). Promising approaches to replace the defective dystrophin gene are not yet established for clinical use although numerous potential treatment options have been thoroughly investigated over the last 10 years (Cossu and Sampaolesi, 2007; Guglieri and Bushby, 2010; Manzur and Muntoni, 2009; Nagaraju and Willmann, 2009; Radley et al, 2007; Smythe et al, 2001; Wells, 2008). The specific mechanism(s) leading to myofibre necrosis are still unclear, yet there are strong associations with excessive inflammation, increased intracellular calcium levels, elevated oxidative stress and metabolic abnormality (Davidson and Truby, 2009; Evans et al, 2009; Even et al, 1994; Kuznetsov et al, 1998; Radley et al, 2008; Tidball and Wehling-Henricks, 2007; Whitehead et al, 2008).

Despite there being fundamental differences in growth parameters, body size and muscle loading, that lead to very different disease severity between dystrophic mdx mice (C57Bl/10ScSn<sup>mdx/mdx</sup>) and DMD patients; the mdx mouse is a widely used animal model for pre-clinical DMD research [Reviewed in (Grounds, 2008; Grounds et al, 2008b; Vainzof et al, 2008; Willmann et al, 2009)]. Studies in both DMD patients and mdx mice indicate that dystrophin defects may also lead to altered skeletal muscle metabolism and an impaired energy status. Repeated cycles of myofibre necrosis and regeneration, increased demand on the sarcoplasmic reticulum to regulate
intracellular calcium, defective mitochondrial function, increases in both protein synthesis and protein degradation rates, and disruption in nNOS signalling may all contribute to an altered metabolic state (Dupont-Versteegden et al., 1994; Griggs and Rennie, 1983; Han et al., 2006; Kuznetsov et al., 1998; Landisch et al., 2008; MacLennan and Edwards, 1990; Passaquin et al., 2002; Whitehead et al., 2006; Zanardi et al., 2003). In support of an altered metabolism in dystrophic muscle, 48 hours of fasting significantly increased myofibre necrosis in muscles from the hind limb and lumbar region of 6 month old mdx mice (yet no change in muscle morphology of control C57BL/10 mice), suggesting a strong dependence on an adequate energy intake to maintain dystrophic muscle structure (Helliwell et al., 1996).

Dietary interventions in the form of various amino acids (with different biochemical effects) or other nutritional supplements have shown variable beneficial effects in both mdx mice and DMD patients [Reviewed in (Bogdanovich, 2004; Davidson and Truby, 2009; Leighton, 2003; Pearlman and Fielding, 2006; Radley et al., 2007)]. Recently completed clinical trials have examined the role of specific amino acid supplementation (creatine and glutamine) in DMD patients and demonstrated some beneficial effects (Escolar et al., 2005; Tarnopolsky et al., 2004). In mdx mice, a creatine enriched diet (10% w/w in chow) fed to new-borns strongly reduced the onset of muscle necrosis in the fast-twitch EDL muscle and also improved mitochondrial respiration capacity (Passaquin et al., 2002). In addition, creatine, taurine and glutamine treatments have shown beneficial effects in treadmill exercised adult mdx mice (De Luca et al., 2003; Granchelli et al., 2000). A combined nutritional therapy (creatine monohydrate, conjugated linoleic acid, α-lipoic acid, and β-hydroxy-β-methylbutyrate) administered, in addition to prednisolone, for 8 weeks increased muscle strength and reduced the extent of dystropathology in 12 week old treadmill exercised mdx mice (Payne et al., 2006). A combination of taurine (1g/(kg bw.day) - orally) and prednisolone (1mg/(kg bw.day) - i.p. injection) treatment of treadmill exercised mdx mice (4-8 weeks of age) also markedly improved forelimb grip strength, compared to either taurine or prenisolone treatment alone (Cozzoli et al., 2010). In addition, a high protein diet (50%) improved muscle morphology in dystrophic laminin
deficient (129ReJ dy/dy) mice and caused a shift to a more ‘normal’ protein metabolism (Zdanowicz et al., 1995). In many cases, the molecular/metabolic basis for the variable benefits reported for the different interventions in dystrophic skeletal and heart muscle remains to be determined. Additional work is required to determine the optimal dietary intervention to reduce the severity of dystrophopathy for potential application to DMD.

We were impressed by a preliminary study to test the effects of a high fat diet on the dystrophic mdx heart (Andrew Hoey 2005, Queensland, data unpublished) that reported a striking difference in bodyweight of dystrophic mdx and control mice fed a high fat diet (~15% w/w) for 9 weeks (from 6–15 weeks of age). As expected, C57Bl/10 mice showed significantly greater body weight and % body fat; however, the body weight of mdx mice was unaffected by the change in diet. The dystropathology of skeletal muscles was not examined in this study, but these interesting results led us to propose that a high fat diet may reduce the extent of dystrophopathy, and may be metabolically beneficial to mdx mice due to their altered energy status.

The aims of the present study were to compare the effects of a high protein and high fat diet on the body composition, muscle morphology and gene expression levels in sedentary adult and voluntarily exercised C57Bl/10 and mdx mice. Body composition was assessed for both strains by measuring total body weight, gastrocnemius muscle weight, myofibre cross-sectional area and epididymal fat pad weight. Muscle morphology and dystropathology (for mdx mice only) was assessed by histological quantification of myofibre necrosis and adipocyte infiltration into the quadriceps muscle, blood serum creatine kinase (CK) level and the distance run during voluntarily exercise. Gene expression levels of 3 major inflammatory cytokines; Tumor necrosis factor (TNF), Interleukin 1β (IL-1β) and Interleukin 6 (IL-6) were measured because of their role in exacerbating myofibre necrosis in dystrophic muscle (Evans et al., 2009; Grounds et al., 2008a; Radley et al., 2008; Tidball and Wehling-Henricks, 2005) and because long-term consumption of a high fat diet and subsequent increase in visceral
obesity is associated with low-grade chronic inflammation [Reviewed in (Pedersen, 2007; Stienstra et al, 2007; Wisse, 2004)]. Gene expression of the peroxisome proliferator-activated receptors (PPARs), a group of nuclear receptor proteins (transcription factors); PPAR alpha, PPAR delta/beta, PPAR gamma and PPAR gamma coactivator 1 alpha (PGC-1α), were measured due to their pivotal role in metabolism, inflammation, adiposity, and remodelling of skeletal myofibre type composition [Reviewed in (Liang and Ward, 2006; Stienstra et al, 2007; Zierath and Hawley, 2004)].

Methods:
Animals. Experiments were carried out on male non-dystrophic (control) C57Bl/10ScSn (hereafter referred to as C57) and dystrophic mdx (C57Bl/10ScSn^{mdx/mdx}) mice; all mice were obtained from the Animal Resource Centre, Murdoch, Western Australia. They were maintained at the University of Western Australia on a 12-h light/dark cycle, under standard conditions, with free access to food and drinking water. All animal experiments were conducted in strict accordance with the guidelines of the National Health and Medical Research Council Code of practice for the care and use of animals for scientific purposes (2004), and the Animal Welfare act of Western Australia (2002), and were approved by the Animal Ethics committee at the University of Western Australia.

Experimental groups and custom diets. This study was conducted over three separate experiments using the following groups of mice: Group 1) sedentary 8-24 week old C57 and mdx mice, Group 2) sedentary 24-40 week old C57 and mdx mice and Group 3) voluntarily exercised 8-12 week old mdx mice. All mice were fed a standard (cereal based) mouse chow (meat free, 5% fat, 19% protein) prior to the study. Sedentary mice (groups 1 and 2) were fed a customised semi-purified diet (control, high fat or high protein) for 16 weeks and voluntarily exercised mice were fed a customised semi-purified diet for 4 weeks. N=8 mice for all groups. The three customised semi-purified diets; Control (7% fat, 19% protein - AIN93G), High Fat (16% fat, 19% protein - SF 06-040) and High Protein (7% fat, 50% protein – SF 00-252) were manufactured (all in
pellet) form by Specialty Feeds Glen Forest Western Australia www.specialtyfeeds.com.au (Table 1). Pilot trials were conducted prior to commencement of the study to ensure that all diets were palatable. Throughout the study food intake and body weight were continuously monitored. Food intake was measured by removing and weighing all food from both the food compartment on the cage lid and from inside the cage (e.g separated from the cage bedding). Energy intake was calculated by adjusting food consumption (g) to metabolisable energy content of each diet (Table 1): metabolisable energy content was calculated according to guidelines of Food and Agriculture Association of the United Nations, http://www.researchgate.net/journal/0254-4725_FAO_food_and_nutrition_paper.

Voluntary exercise (8-12 week old mdx mice only). The low level of muscle damage in dystrophic adult mdx mice can be elevated by voluntary exercise (Grounds et al, 2008b). Mice were caged individually with free access to a voluntary running wheel for 4 weeks (8-12 weeks of age). Exercise data were collected via a small magnet attached to the mouse wheel, and a sensor from a bicycle pedometer attached to the back of the cage. The pedometer records single wheel revolutions, allowing total distance (km) run by an individual mouse to be determined, as per (Hodgetts et al, 2006; Radley et al, 2008; Radley and Grounds, 2006). The mice run the most during the night since they are normally nocturnal (Hara et al, 2002; Radley and Grounds, 2006). Mice were monitored daily throughout the experiment for food consumption, distance run and wheel function.

Tissue collection. Mice were sacrificed at either 12, 24 or 40 weeks of age by cervical dislocation, while under terminal isoflurane (Bomac Australia) anaesthesia (2%v/v). Total body weight, tibial length, epididymal fat pad weight and gastocnemius muscle weight were recorded immediately. Blood serum was collected via cardiac puncture, the gastrocnemius muscle was snap frozen in liquid nitrogen for molecular analysis and the quadriceps and tibialis anterior (TA) muscles were collected for histology (paraffin and frozen section preparation, respectively).
Skeletal muscle histology. The quadriceps muscles were collected and immediately fixed in 4% paraformaldehyde (Sigma P6148) for 48 hours. Muscles were then placed into 70% ethanol, processed in a Shandon automatic tissue processor overnight, and finally paraffin embedded for sectioning. Transverse sections (5μm) were cut through the mid-region of each muscle on a Leica retractable microtome. Slides were stained with Haematoxylin and Eosin (H&E) for morphological analysis of dystropathology (e.g. myofibre necrosis and adipocyte content) as per the TREAT-NMD recommended standard protocol “Histological measurements of dystrophic muscle - M.1.2.007” http://www.treat-nmd.eu/research/preclinical/SOPs/. The frozen TA muscles were collected, mounted on a small cork block and stabilised (embedded) in tragacanth gum (Sigma Aldrich G1128), quenched in isopentane cooled in liquid nitrogen and stored at -80°C until cut on a CM3050S Leica cryostat. TA sections were stained with Sirius red, which readily distinguishes myofibres (yellow) from collagenous myofibre membranes (red), to allow for easy and accurate quantification of myofibre cross-sectional area (CSA).

Image capture and Histological image analysis. Non-overlapping tiled images of transverse muscle sections provided a picture of the entire muscle cross section. Images were acquired using a Leica DM RBE microscope, a personal computer, a Hitachi HVC2OM digital camera, Image Pro Plus 4.5.1 software and Vexta stage movement software. Tiled images were taken at 10x magnification. Histological analysis was carried out on whole cross sections of the quadriceps muscle (dystropathology and adipocyte content) and the frozen TA muscle (myofibre CSA). Muscle morphology was drawn interactively by the researcher using Image Pro Plus 4.5.1 software.

Serum Creatine Kinase Assay. While under terminal anaesthesia blood from the mdx mice was collected via cardiac puncture. Blood was refrigerated overnight, centrifuged
for 3 min (1200 rpm) and serum removed. Blood serum creatine kinase (CK) analysis was completed at the Murdoch Veterinary Hospital, Murdoch, WA.

**Quantitation of Gene expression (relative to L-19).** Consumption of a high fat diet induced many changes in the body composition of both C57 and mdx mice, whereas a high protein diet did not. Therefore gene expression was quantified only in mice which consumed the control or high fat diet. RNA was extracted from snap frozen gastrocnemius muscles using Tri-reagent (Sigma T9424) and quantified using a Nano Drop Spectrophotometer (ND 1000) and ND 1000 software version 3.5.2. The RNA was DNase treated using Promega RQ1 RNAse free DNAse (M610A), RQ1 RNAse free 10x buffer (M198A) and RQ1 DNAse stop solution (M199A). RNA was reverse transcribed into cDNA using Promega M-MLV Reverse Transcriptase (M3682), random primers (C1181) and 10mM dNTPs (U1515) and the cDNA was purified using a Mo Biol Clean up kit (12500-250). RT-PCRs were run on a Corbett 3000 (Corbett Research) using QIAGEN quantifast SYBR green PCR mix (204054) and QIAGEN Quantitect Primer Assays for TNF (QT00104006), IL-1β (QT01048355), IL-6 (QT00098875), PPAR alpha (QT00137984), PPAR delta/beta (QT00166292), PPAR gamma (QT00100296), PGC-1α (QT00095578) and standardised to an appropriate house-keeping gene; ribosomal protein L-19 (QT01779218) as per (Shavlakadze et al, 2010). mRNA expression was calculated and standardised using Roto-gene 6.1 and Microsoft Excel software.

**Statistical analysis.** Analysis was completed using Microsoft excel and SPSS 16.0. All variables were analysed by ANOVAs (to account for diet, strain, age and exercise) and Least Significant Difference (LSD) post-hoc tests. All data are expressed as mean +/- S.E.M throughout the manuscript.

**Results:**

**Food consumption.**

There were no significant differences in average daily food consumption (g/g bw/day) across the 3 custom diets in both strains of 8-24 week old mice (Figure 1A). However
there was a significant decrease in food consumption (g/g bw/day) with age in both strains of mice and a significant decrease in food consumption in 24-40wk old C57 mice on the high fat diet compared to both the control and high protein diet (Figure 1A). Absolute energy intake (kJ/day) was significantly decreased in both strains of 8-24 week old mice on the high protein diet (Figure 1B). There was a slight increase in energy intake (kJ/day) in both strains of mice on the high fat diet at 8-24 weeks of age compared to mice on the control diet; however this difference was not significant. In the older mice (24-40 wks) on the high fat diet there was a significant increase in energy intake (kJ/day) in both strains compared to mice on both the control and high protein diet (Figure 1B). There were no differences in standardised energy intake (kJ/g bw/day) between 8-24 week old C57 or mdx mice on the control diet, however both C57 and mdx mice on the high protein diet consumed significantly less standardised energy compared to mice on both the control and high fat diet (Figure 1C). There was a significant decrease in energy intake in both strains of mice with age, and 24-40 week old mdx mice on the high fat diet consumed significantly (P<0.02) more energy (kJ/g bw/day) than all other groups of mice (Figure 1C).

**Body Composition.**

The linear growth of both adult C57 and mdx mice, as reflected by tibial bone length, was not affected by diet (data not shown).

**Group 1) 8 - 24 week old sedentary mice subjected to 3 diets.** 8 week old dystrophic mdx mice (weight at beginning of the study) fed a standard (cereal based) mouse chow were significantly heavier (P=0.02) than age matched C57 mice (C57 21.6g+/−0.23 vs. mdx 23.4g+/−0.72). This significant difference in body weight was maintained throughout the study until 24 weeks of age (C57 30.6g+/−0.74 vs. mdx 35.5g+/−0.98) (Figure 2A). The high protein diet had no significant effect on the bodyweight of either strain of mice (Figure 2A). After consuming the high fat (HF) diet for 16 weeks there was a significant (P=0.04) increase in the bodyweight of 24 week old C57 mice compared to C57 mice on the control (C) diet (HF 34.54g+/−1.2 vs. C 30.63g+/−0.73). In contrast, consumption of the high fat diet caused no significant change in the bodyweight of mdx mice (HF 34.97g+/−0.53 vs. C 35.54g+/−0.98) (Figure 2A). The largest gain in body weight (body weight at 24wks minus body weight at 8wks) was
seen in C57 mice after consuming the high fat diet. The average group percentage changes in body weight for C57 mice were 130% after control diet, 140% after high protein diet and 159% after high fat diet. The average group percentage changes in body weight for mdx mice were 130% after control diet, 132% after high protein diet and 137% after high fat diet.

Obesity is the direct result of an imbalance between energy intake and energy expenditure, with excess energy being primarily stored in adipose tissue in the form of triglycerides [Reviewed in (Stienstra et al, 2007)]. Epididymal fat pad weight increases in male C57Bl/6J mice after consumption of a high fat diet (Turpin et al, 2009) and was measured in this study as an indicator of obesity. After consuming the control diet, the standardised epididymal fat pad weight (g fat /g bw) was significantly (P<0.001) heavier in C57 compared to mdx mice at 24 weeks of age (C57 0.019g/g bw †/. 0.009 vs. mdx 0.0066g/g bw †/. 0.002) (Figure 2B). The high protein diet had no significant impact on epididymal fat pad weight in either strain of mice, in contrast with the striking effects of the high fat diet. After consuming the high fat diet (from 8-24 weeks), the standardised epididymal fat pad was significantly (P=0.015) increased in C57 mice (HF 0.028g/g bw †/. 0.002 vs. C 0.019g/g bw †/. 0.009) (Figure 2B) and, at sacrifice, large amounts of adipose tissue were conspicuous around the sternum, kidneys and hip joints. In striking contrast, 24 week old mdx mice were very lean and had very small epididymal fat pads (Figure 2B); upon opening the abdominal cavity the epididymal fat pads often could not be seen until the testes were pulled up into the abdominal cavity and consumption of a high fat did not increase epididymal fat pad weight in 24 week old mdx mice.

Standardised gastrocnemius muscle weight (g muscle /g bw) was significantly (P=0.04) heavier in mdx mice fed a control diet compared to C57 mice at 24 weeks of age (C57 0.0050g/g bw †/. 0.0002 vs. mdx 0.0056g/g bw †/. 0.0001) (Figure 2C). Absolute gastrocnemius muscle weight was also significantly (P<0.05) heavier in mdx mice at this age (C57 0.152g †/. 0.002 vs. mdx 0.190g †/. 0.004) (data not shown). Neither the high protein nor high fat diet caused any changes in standardised or absolute gastrocnemius muscle weight in either strain of mice.
Group 2) 24 - 40 week old sedentary mice subjected to 3 diets. There was no significant difference in the body weight of 40 week old C57 mice on the control diet compared to mdx mice (C57 37.3g+/−. 2.2 vs. mdx 34.2g+/−. 0.76) (Figure 2A), nor did the high protein diet have any significant effect of the body weight of either strain of mice (Figure 2A). However, after consuming the high fat diet for 16 weeks the body weight of 40 week old C57 mice was significantly (P<0.01) increased (approximately 10g) compared to C57 mice on the control diet (HF 47.3g+/−. 1.5 vs. C 37.3+/−. 2.2g). Consumption of the high fat diet also significantly (P=0.04) increased the body weight of the older mdx mice (HF 37.2g+/−. 1.1 vs. C 34.2g+/−. 0.76) (Figure 2A). The largest increase in body weight (body weight at 40wks minus body weight at 24wks) was seen in C57 mice on the high fat diet. The (average group percentage) change in bodyweight for C57 mice was 116% after control diet, 102% after high protein diet and 141% after high fat diet. The change in body weights for mdx mice were 103% after control diet, 94% after high protein diet and 110% after high fat diet.

On the control diet the standardised epididymal fat pad weight (g/g bw) was significantly (P<0.01) increased in 40 week old C57 mice compared to mdx mice (C57 0.027g/g bw+/−. 0.005 vs. mdx 0.007g/g bw+/−. 0.0003) (Figure 2B). The high protein diet had no impact on the epididymal fat pad weight in either strain of the older mice. On the high fat diet, there was a slight increase (but not significant – presumably due to the large increase in total bodyweight) in standardised epididymal fat pad weight (g fat/ g bw) of C57 mice (HF 0.032g/g bw+/−. 0.002 vs. C 0.027g/g bw+/−. 0.005) (Figure 2B). The absolute epididymal fat pad weight (g fat) was significantly (P=0.03) increased (HF 1.42g+/−. 0.06 vs. C 1.16g+/−. 0.2) (data not shown) and observations made during tissue collection showed very large fat pads and pronounced adipose tissue around the sternum, kidneys, intestines and hip joints. 40 week old mdx mice were still very lean with no change in the standardised epididymal fat pad weight between 24 and 40 weeks for mdx mice fed a control diet (Figure 2B). The high fat diet significantly (P=0.035) increased epididymal fat pad weight (HF 0.012g/g bw+/−. 0.002 vs. C 0.007g/g bw+/−. 0.0003) in 40 week old mdx mice (Figure 2B), to a much lesser extent than C57 mice, whereas this effect was not seen in the younger 8-24 week old mdx mice.
Standardised gastrocnemius muscle weight was significantly (P=0.045) heavier in mdx mice fed a control diet compared to C57 at 40 weeks of age (C57 0.0048g/g bw +/ 0.0002 vs. mdx 0.0059g/g bw +/ 0.0001) (Figure 2C). Absolute gastrocnemius muscle weight was also significantly (P<0.05) heavier in mdx mice at this age (C57 0.178g +/ 0.003 vs. mdx 0.198g +/ 0.005) (data not shown). There was no change in standardised gastrocnemius muscle weight in mdx mice with age (e.g 24wk old mdx vs 40wk old mdx), however there was a significant (P<0.05) increase in absolute gastrocnemius muscle weight with age in C57 mice (24wk 0.152g/ +/ 0.002 vs. 40wk 0.178g +/ 0.003) (data not shown). The high protein diet had no effect on the muscle weights of either strain. The high fat diet caused no change in absolute gastrocnemius muscle weight in either strain of mice (data not shown), thus due to the large increase in body weight in C57 mice after consuming a high fat diet there was a strikingly significant (P=0.01) reduction in standardised gastrocnemius muscle weight in C57 mice (HF 0.003g/g bw +/ 0.0006 vs. C 0.0048g/g bw +/ 0.0002) (Figure 2C), yet had no change on the standardised gastrocnemius muscle weight in mdx mice.

**Myofibre Size (control diet only).**

There was no difference in myofibre size (CSA) in the tibialis anterior (TA) muscle from 12 week old C57 and mdx mice on the control diet (Figure 3). Between 12 -24 weeks of age, dystrophic myofibres in the mdx TA continue to grow (hypertrophy) and were significantly larger than C57 myofibres at 24 weeks (P=0.02) (Figure 3). Dystrophic myofibres do not continue to infinitely hypertrophy and myofibre size in 40 week old mdx mice was significantly reduced (P=0.025) compared to 24 weeks and there was no difference in myofibre size between 12 and 40 week old mdx mice. There was no change in myofibre size between 12, 24 and 40 week old C57 mice.

**Muscle morphology and dystropathology.**

**8 – 24 week old sedentary mice.** Myofibre necrosis (% cross sectional area) was significantly (P=0.04) decreased in the quadriceps muscle of 24 week old mdx mice fed the high fat diet, compared to the control diet (HF 1.49% +/1.7 vs. C 3.3% +/0.4)
(Figure 4A); however the high protein diet had no effect on myofibre necrosis (compared to control diet). Myofibre necrosis is not a feature of control C57 mice and therefore it was not measured. There were no significant differences in adipocyte content of the quadriceps muscle in 24 week old C57 and mdx mice after consuming any of the 3 diets.

**24 – 40 week old sedentary mice.** There was no change in the level of myofibre necrosis in the quadriceps muscle between 24 and 40 week old mdx mice (Figure 4A) and no effect of the high protein nor high fat diet on myofibre necrosis in 40 week old mdx mice (Figure 4A). The high protein diet had no effect on the adipocyte content of the quadriceps muscle in either strain of 40 week old mice. Adipocyte content of the quadriceps was significantly (P=0.03) increased with age in C57 mice (24wk 0.06% +/- 0.014 vs. 40wk 1.16% +/- 0.035) fed a control diet (Figure 4B) and was significantly (P<0.001) increased further in C57 mice fed a high fat diet (HF 2.94% +/- 0.72 vs. C 1.16% +/- 0.35) (Figure 4B). The high fat diet also significantly increased adipocyte content between 24 and 40 week old mdx mice (Figure 4B).

Blood serum CK levels are always lower (approximately 10 fold) in C57 mice compared to mdx mice (Grounds et al, 2008b; Radley-Crabb et al, 2010; Spurney et al, 2009) and were not measured for C57 mice in this study. For the mdx mice there was no change in serum CK level between 24 and 40 weeks (24wk 3171U/L +/- 460 vs. 40wk 2791U/L +/- 422) and no change in CK level after consuming either a high fat or high protein diet for both 24 and 40 week old mdx mice (data not shown).

**Gene expression.**

**8 – 24 week old sedentary mice.** mRNA levels of TNF and IL1β were significantly elevated (approximately 2 fold) in the gastrocnemius muscle from 24 week old mdx compared to C57 mice (Figure 5A&B). Consumption of the high fat diet significantly increased the mRNA levels of all 3 inflammatory cytokines (TNF, IL1β and IL-6) in C57 mice (Figure 5A-C), however there was no change in 24 week old mdx mice.
Levels of mRNA for PPAR alpha, PPAR delta/beta and PGC-1α alpha (but not PPAR gamma) were significantly decreased (approximately 2 fold) in the gastrocnemius muscle from 24 week old mdx compared to C57 mice (Figure 6A,C,D). Expression of the 3 PPARs was unchanged in either strain by consumption of the high fat diet. However, PGC-1α was significantly reduced (approximately 2 fold) in 24 week old C57 mice fed the high fat compared to the control diet.

**24 – 40 week old sedentary mice.** mRNA levels of TNF were significantly elevated (approximately 2 fold) with age (24wk C57 vs. 40wk C57) in C57 mice (Figure 5A). The high fat diet significantly increased (approximately 2x fold) TNF and IL-6 mRNA in 40 week old C57 mice and IL-6 mRNA (approximately 3x fold) in mdx mice (Figure 5A & C). mRNA levels of PGC-1α were significantly decreased (approximately 2x fold) with age (24wk C57 vs. 40wk C57) in C57 mice (Figure 5D) and PPAR alpha and PPAR delta/beta mRNAs were significantly decreased (approximately 2x fold) in the gastrocnemius muscle of 40 week old mdx mice compared to C57 mice (Figure 5A & C). The high fat diet caused no change in the mRNA level of the 3 PPARs or PGC-1α in either strain.

**Group 3) 8-12 week old voluntarily exercised mice.** Dystrophic mdx mice were voluntarily exercised for 4 weeks to increase the low level of dystropathology in skeletal muscles (Radley et al, 2008; Radley and Grounds, 2006). Increasing the extent of dystropathology allowed for further evaluation of the beneficial effects of a high protein or high fat diet in mdx mice.

**Food consumption.**
There was no significant difference in average food consumption (g/ g bw /day) across the 3 custom diets in both sedentary and exercised mdx mice (Figure 7A); however, food consumption of all 3 custom diets (g /g bw /day) was significantly (P<0.04) increased (approximately 30%) by voluntary exercise (Figure 7A). Standardised energy intake (kJ /g bw/day) was unchanged in sedentary 8-12 week old mdx mice across the 3 diets (Figure 7B); however energy intake was significantly increased in exercised mdx mice for all three diets. Absolute energy intake (kJ/day) showed the same pattern as standardised energy intake (data not shown). Voluntarily exercised mdx mice on the
high fat diet had a higher standardised energy intake (kj/g bw/day) than exercised mdx mice on both the control and high protein diet (C Ex 1.91 kj/g bw/day +/- 0.08 vs. HF Ex 2.28 kj/g bw/day +/- 0.08) (Figure 7B). Absolute energy intake (kj/day) was also significantly increased in exercised mice on the high fat diet (C Ex 50.4 kj +/- 4.2 vs. HF Ex 63.7 kj +/- 7.6) (data not shown).

Body composition.
Voluntary wheel exercise for 4 weeks caused no change in the body weight of 12 week old mdx mice (sed 29.1g +/- 2.8 vs. ex 27.5g +/- 1.9), neither did consumption of a high protein or high fat diet (Figure 8A). Exercising 8-12 week old mdx mice significantly (P=0.03) reduced the standardised epididymal fat pad weight (g fat/g bw), compared to sedentary mdx mice on a control diet (sed 0.0081g +/- 0.0005 vs. ex 0.0051g +/- 0.0002) (Figure 8B). Neither the high protein nor the high fat diet had any significant effect on the epididymal fat pad weight in sedentary 12wk old mdx mice; however the high fat diet significantly (P=0.045) increased epididymal fat pad weight in exercised mdx mice (HF 0.0064g +/- 0.0006 vs. C 0.0051 +/- 0.0002) (Figure 7B). No significant change in standardised or absolute gastrocnemius muscle weight was seen after voluntary exercise or diet change (Figure 8C).

Dystropathology.
Exercise was completely voluntary and distance run is an indirect indicator of muscle function and exercise capacity. Mdx mice fed the high fat diet ran significantly (P=0.003) further during 4 weeks of exercise (approximately 50% further) compared to mdx mice on the control diet (HF 221.8km +/- 13.2 vs. C 152.4km +/- 12.1) (Figure 9A) and there was no effect of a high protein diet compared with control diet.

Voluntary exercise significantly (P=0.02) increased (approximately 2x fold) myofibre necrosis (% CSA) in the quadriceps muscle from 12 week old mdx mice (sed mdx 3.68% +/- 0.49 vs. ex mdx 8.75% +/- 0.91). The high fat diet for 4 weeks had no effect on myofibre necrosis in either voluntarily exercised or sedentary mdx mice. The high protein diet had no effect on sedentary mice, but significantly (P=0.04) increased
myofibre necrosis in exercised mdx mice (HP 12.51% +/− 2.00 vs. C 8.75 +/− 0.91) (Figure 9B).

Blood serum CK level was significantly (P=0.04) higher (approximately 3x fold) in voluntarily exercised compared to sedentary 12 week old mdx mice (sed mdx 4697U/L +/− 1898 vs. ex mdx 12913U/L +/− 4537). The CK levels in mdx mice were not affected by a high protein or high fat diet (data not shown).

Exercise did not change the adipocyte content of the quadriceps muscle from 12 week old mdx mice (sed mdx 0.54% +/− 0.07 vs. ex mdx 0.43% +/− 0.07), nor was there any change in adipocyte content after consuming a high protein or high fat diet in sedentary or exercised mice (data not shown).

Gene expression.
Voluntary exercise (for 4 weeks) and/or a high fat diet caused no significant change in the mRNA levels of TNF, IL1β or IL-6 in 12 week old mdx mice (data not shown). Consumption of the high fat diet had no significant affect on mRNA levels of the 3 PPARs or PGC-1α in sedentary 12 week old mdx mice; however the combination of voluntary exercise and a high fat diet significantly increased (approximately 3x fold) the mRNA levels of PPAR alpha, PPAR gamma, PPAR delta/beta and PGC-1α (Figure 10A-D).

Discussion:
Sedentary mdx and C57 mice.
To our knowledge, the response of adult mdx mice to a high fat diet has not been previously described; furthermore, while it is widely documented that C57Bl/6J mice are highly susceptible to diet induced obesity (obesity-prone) (Alexander et al, 2006), the effect of a high fat diet on C57Bl/10 mice does not seem to have been reported. It is commonly accepted that obesity is the direct result of an imbalance between energy intake and energy expenditure, that long-term consumption of a high fat diet increases deposition of adipose tissue and eventually leads to obesity in both sedentary laboratory rodents and humans and that obesity is associated with numerous changes
in cell signalling that are believed to underlie much of the morbidity associated with the condition [Reviewed in (Buettner et al, 2007; Pedersen, 2010; Stienstra et al, 2007)].

Dystrophic mdx mice were significantly heavier than control C57 mice at both 8 and 24 weeks of age (Figure 2A), primarily due to a well described increase in lean muscle mass (Anderson et al, 1987; Connolly et al, 2001; Coulton et al, 1988; Mokhtarian et al, 1996; Peter and Crosbie, 2006; Shavlakadze et al, 2010; Spurney et al, 2009). In contrast, 40 week old mdx mice were the same weight as C57 mice; this equalising in bodyweight occurred at approximately 32 weeks of age (data not shown) and was most likely due to an increase in body fat in C57 mice (Figure 2B). The body weight of mdx mice did not increase between 24 and 40 weeks of age, nor was there a change in standardised epididymal fat pad nor standardised gastrocnemius muscle weights (Figure 2A), supporting reports of stabilisation of the dystrophic phenotype in adult mdx mice (Grounds et al, 2008b; Shavlakadze et al, 2010). Both 24 and 40 week old mdx were very lean, with significantly smaller epididymal fat pads, larger gastrocnemius muscles and larger myofibres CSA compared to C57 mice (Figures 2B, 2C, 3).

The muscular body composition of mdx mice (<40 weeks) is in stark contrast to DMD patients who can move between the spectra of over-nutrition (and obesity) to under-nutrition within their shortened lifespan, and lose muscle mass at a rate of 4% per year as adults (Davidson and Truby, 2009; Griffiths and Edwards, 1988; Leighton, 2003). It is important to consider that the striking loss of muscle mass in DMD patients occurs during the growth phase of the boys (approximately 20 years). In mice however, the damaging main growth phase is exceedingly short by comparison (approximately 6 weeks) (Grounds, 2008; Grounds et al, 2008b) with a reduction in myofibre necrosis occurring after growth has ceased in mdx mice.

There was significant myofibre hypertrophy in 24 week old mdx mice (compared to all C57 and 12 week old mdx mice); however, dystrophic myofibres do not continue to hypertrophy indefinitely, and by 40 weeks of age myofibre size returned to the same
size as 12 week old mdx mice (Figure 3). We propose that the reduced myofibre size in 40 week old mdx mice was due to myofibre splitting or branching. Shavlakadze et al (2010) reported no difference in myofibre size in the quadriceps muscle between 12 and 52 weeks due to significant myofibre splitting in 52 week old mdx mice (Shavlakadze et al, 2010). Our study examined myofibre size at 24 weeks and identified significant myofibre hypertrophy in mdx mice at this age, so it seems that 24 weeks is as a suitable time point to visualise myofibre hypertrophy in the TA muscle before myofibre splitting occurs (by 40 weeks onwards).

The body composition of dystrophic mdx mice changed between 12 and 24 weeks of age, with significant increases in body weight, absolute and standardised gastrocnemius muscle weight and individual myofibre CSA (µm²) (Figures 2A, 3 & 8A).

The 8-24 week old mdx mice had similar food consumption and energy intake levels across the 3 diets compared to age matched C57 mice (Figure 1A-C) with both strains on the high fat diet showing a slight increase in energy intake (kJ/day) compared to the control diet. Yet, there was a large difference in the way the two strains of mice responded to the high fat diet. The resistance of dystrophic mice (≤24 weeks of age) to the high fat diet (i.e. they did not deposit significant body fat) may be due to excess dietary fat (or increased energy intake) assisting with muscle growth and hypertrophy of individual myofibres (Figure 3). Between 24 and 40 weeks of age, there was no change in bodyweight (Figure 2A) or gastrocnemius muscle weight (absolute or standardised) and myofibre CSA was significantly reduced (Figure 3) for mdx mice on a control diet. The older mdx mice (24-40 weeks) were susceptible to the negative effects of a high fat diet, showing significant increases in total bodyweight and standardised fat pad weight (Figures 2A&B). This altered trend is presumably due to the large increase in energy intake (Figure 1B&C) without the corresponding high energy expenditure associated with ongoing myogenesis/regeneration, due to a progressive reduction of myofibre necrosis in old mdx mice; e.g. 6% myofibre necrosis in 12 week old mdx mice (Radley-Crabb et al, submitted) reducing to approximately 2% by 40 weeks of age.
In contrast to mdx mice, after consumption of a high fat diet (for 16 weeks) C57 male mice at both 24 and 40 weeks exhibit significantly increased bodyweight primarily due to a gain in body fat (Figure 2A&B) and significant deposition of adipocytes in the quadriceps muscle of 40 week old mice (Figure 4B). The increase in daily energy intake (kj/day) in 8-24 week old C57 mice on the high fat diet is only minor compared to mice on the control diet (Figure 1B), but when accumulated chronically it was enough to cause large changes in body composition; whereas the increase in energy intake (kj/day) in 24-40 week old C57 mice on the high fat diet is significantly increased compared to mice on the control diet (Figure 1B).

In contrast to the high fat diet (16% fat, 19.4% protein), a high protein diet (50% protein, 7% fat) had little impact on the body composition of 8 – 40 week old C57 and mdx mice. Initially these two diets were designed to be isocaloric based on digestible energy content (HP – 18.2 MJ/Kg vs. HF – 18.1 MJ/Kg) (Table 1). Major differences in body composition were seen in response to the high fat and high protein diets and it became apparent that metabolisable energy content was a more appropriate way to assess energy intake of the mice. There are large differences in the metabolisable energy content of the high fat and high protein diet (HP – 14.09 MJ/Kg vs. HF – 16.96 MJ/Kg) due to the incomplete in vivo oxidation of dietary proteins which may explain the major differences in energy intake and thus body composition in response to the two diets. A high protein diet or supplementation with certain amino acids (such as leucine) can stimulate skeletal muscle protein synthesis in both young and adult animals, but there is a threshold of protein requirement for maximum growth and once reached, additional protein intake does not further stimulate protein synthesis in skeletal muscle [Reviewed in (Shavlakadze and Grounds, 2006)]. Therefore, it is possible that in well nourished sedentary mice (with adequate protein intake), the high protein diet had no effect on muscle mass. While it is possible that a beneficial response to high protein consumption might be seen in combination with resistance exercise, such demanding exercise is usually not recommended for dystrophic boys or animal models of DMD due to likely additional damage to dystrophic muscles.
Our initial hypothesis was that a high protein and/or high fat diet would help to maintain myofibre integrity and thus reduce dystropathology in mdx mice. Consumption of either diet for 16 weeks did not reduce levels of serum CK (an indicator of myofibre leakiness), however, the high fat diet did significantly reduce (approximately 2.5 fold) myofibre necrosis in the quadriceps muscle of 24 week old mdx mice. It is possible that a high fat diet (and increased energy intake) assists mdx mice metabolically and thus helps them to maintain skeletal muscle structure and mass, or that increased lipid coming from the high fat diet stabilises fragile membrane lipids and prevents myofibre necrosis.

The lack of any benefits with the high protein diet contrasts with studies in mdx mice that show mildly reduced dystropathology after dietary supplementation of creatine, taurine and glutamine (both alone and in combination with prednisolone) (Cozzoli et al, 2010; De Luca et al, 2003; Granchelli et al, 2000; Passaquin et al, 2002; Radley et al, 2007). Taurine and glutamine are not substrates for protein synthesis, but may improve dystropathology via regulation of calcium homeostasis (Conte Camerino et al, 2004) or by inhibiting whole body protein degradation (Mok et al, 2006). Amino acid supplementation is also widely used amongst DMD patients despite there being no conclusive supporting evidence in the literature (Bushby et al, 2010; Davidson and Truby, 2009; Radley et al, 2007). It is important to consider possible counteracting effects when combined interventions are administered. Our data from mdx mice on the high protein diet (which contains increased concentrations of the essential amino acids such as leucine and phenylalanine which are used in protein synthesis, but no increase in metabolisable energy - Table 1) do not support such an intervention and elevated myofibre necrosis was observed in voluntarily exercised mdx mice.

A prolonged positive imbalance between energy intake and expenditure resulting in obesity, is associated with chronic inflammation and may be a potential mechanism by which obesity leads to insulin resistance (in both humans and mice) [Reviewed in (Arkan et al, 2005; Stienstra et al, 2007; Wisse, 2004)]. Expression of 3 major pro-inflammatory cytokines, TNF, IL1β and IL6 was up-regulated in the gastrocnemius
muscle of both 24 and 40 week old C57 mice after consumption of the high fat diet (Figure 5A-C). These increases in gene expression were not seen in mdx mice (Figures 5A-C) which demonstrates, in addition to the lack of changes in body composition, the resistance of mdx mice (particularly ≤24 weeks) to a high fat diet. Inflammation plays a major role in myofibre necrosis and regeneration in skeletal muscle, thus the inflammatory state can be an indirect indicator of the extent of dystrophopathy (Grounds et al, 2008a; Radley et al, 2008). Consistent with the lack of significant adiposity, there were no major changes in cytokine gene expression in mdx mice after consuming a high fat diet.

The high fat diet significantly reduced PGC-1α gene expression in 24 week old C57 mice but, apart from this, there were no other significant changes in PPAR expression caused by a high fat diet in either strain of mice. Compared with C57 mice, the levels of PGC-1α, PPAR alpha and PPAR delta/beta were all much lower (approximately half) in mdx mice on a control diet; again endorsing altered metabolic processes in dystrophic mdx mice.

Due to the multiple systemic effects that the 3 PPARs and PGC-1α have on whole body metabolism (e.g. lipid metabolism) (Ehrenborg and Krook, 2009; Liang and Ward, 2006; Stienstra et al, 2007) we assume that decreased gene expression of the 3 PPARs and PGC-1α in dystrophic skeletal muscle would have a wide range of effects. However the precise consequence(s) in dystrophic skeletal muscle, to our knowledge, are not documented. Decreased PGC-1α expression in skeletal muscles of C57Bl/6J mice decreases exercise capacity and fatigue resistance [Reviewed in (Liang and Ward, 2006)] and low levels of PGC-1α mRNA in mdx mice on a control diet, correspond with decreased exercise capacity and increased fatigue that are common features of dystrophic muscle (Grounds et al, 2008b; Hara et al, 2002; Piers et al, 2010). It has also been shown that several gene programmes linked to PGC-1α are dysregulated in dystrophic muscle (e.g mitochondrial function, calcium handling and ROS) and introduction of a muscle specific PGC-1α transgene into mdx mice improved muscle function and structure, possibly via up-regulation of utrophin (Davies and Khurana, 2007; Handschin et al, 2007). Overall there seems to be an association with decreased
PGC-1α expression having detrimental effects in both dystrophic and normal muscle. Although the combined consequences of generally PGC-1α, PPAR alpha and PPAR delta/beta in dystrophic (compared with normal) muscle are not clear, they are all elevated in mdx muscles in response to voluntary exercise combined with a high fat diet (see below) with beneficial effects.

**Voluntarily exercised mice.**

In order to further examine the potential benefits of a high fat or high protein diet on dystrophic muscle, 8-12 week old mdx mice were voluntarily exercised, to exacerbate the dystrophopathy (Hodgetts et al, 2006; Radley et al, 2008; Radley and Grounds, 2006). The 12 week old mdx mice were very lean, yet there was a significant further reduction in epididymal fat pad weight after 4 weeks of voluntary exercise (Figure 8B). It appears that mdx mice were able to maintain body weight and muscle mass (Figures 8A & C) over 4 weeks of voluntary exercise by increasing food consumption and energy intake (Figure 7A&B).

Despite a high fat diet reducing myofibre necrosis in sedentary 24 week old mdx mice (Figure 4), this diet had no significant benefit on myofibre necrosis or serum CK levels in voluntarily exercised mdx mice. Energy intake was significantly increased in voluntarily exercised mdx on the high fat diet compared to mdx mice on the control diet (Figure 7B) and this may have enabled mdx mice to run significantly more (approximately 50%); and yet this increased exercise did not result in increased muscle damage (e.g. myofibre necrosis or serum CK level). It appears that a high fat diet is assisting energy deficient mdx mice metabolically, providing them with additional energy to run further while also maintaining myofibre integrity.

A striking increase in the mRNA levels of PPAR alpha, PPAR gamma, PPAR delta/beta and PGC-1α was seen in the gastrocnemius muscle of exercised mdx mice on the high fat diet. Exercise is known to increase PGC-1α expression (due to increased neuromuscular input and elevated levels of MEF2 and CREB expression with exercise) (Liang and Ward, 2006) and increases in PGC-1α and PPAR delta/beta are both beneficial to mdx dystrophopathlogy in vivo (Handschin et al, 2007; Miura et al, 2009).
Therefore it is proposed that the increased capacity for exercise in dystrophic mdx mice fed a high fat diet may be modulated, in part, via up regulation of the 3 PPARs and PGC-1α.

**Conclusion:**
This research clearly identifies an ‘altered’ response to a high fat diet in dystrophic mdx mice compared to C57 controls; this response was pronounced in young mdx mice <24 weeks old, but diminished with age (by 40 weeks). Energy deficient mdx mice appear to be utilising excess dietary fat to reduce the severity of dystropathology and increase voluntary exercise ability. The high protein diet had no significant effects on the body composition of either strain of mice and no benefit on mdx dystrophopathy (this may be due in part to the fact that while it was isocaloric for digestible energy it was lower in metbolisable energy content compared with the high fat diet). While it is appreciated that in corticosteroid treated inactive DMD patients, a high fat diet may negatively contribute to obesity and thus impact on disease severity (especially since corticosteroid usage is known to contribute to obesity and Cushingoid symptoms), our new data highlight the potential benefits of a high fat diet on dystrophic muscle. It is not yet clear if the extent to which this benefit of a high fat diet is due largely to increased energy (kJ) per se, relative to specific benefits of increased dietary fat on dystrophic muscles. Clearly careful management is required to achieve a fine balance between the increased metabolic demands of dystrophic muscle, disease treatment and the potential negative effects of a high fat diet.

**Acknowledgements:**
The authors gratefully acknowledge the expert assistance of Dr Marta Fiorotto of Baylor College, Houston, USA and her valuable comments regarding the manuscript. The authors also thank Mr Greg Cozens (School of Anatomy & Human Biology, UWA) for excellent technical assistance, Dr Andrew Hoey (University of Southern Queensland) for consultation regarding the preliminary studies and Mr Warren Potts (Specialty Feeds, WA) for design and manufacture of the 3 semi-pure diets. Research funding from the Australian National Health and Medical Research Council (MG) and Australian Postgraduate Award Scholarships (HR-C) are gratefully acknowledged.
Table captions:

Table 1. Diet specifications for the 3 semi-pure diets used in this study: control (AIN93g), high protein (SF00-252) and high fat (SF06-40). Energy intake was calculated based on the metabolisable energy content of each diet. All diets were manufactured by Specialty Feeds, Glen Forrest, WA.

Figure captions:

Figure 1. Average daily food consumption (g/ g bw/ day) (A), absolute energy intake (kJ/ day) (B) and standardised energy intake (kJ /g bw /day) (C) for C57 and mdx mice; a comparison of sedentary mice on a control diet, high fat diet or high protein diet between 8-24 weeks of age and 24-40 weeks of age. Bars represent standard error. N= 8 for all groups. A,B,C denotes significant differences, groups with different letters are significantly different from each other (P<0.05).

Figure 2. Body composition of sedentary 24 week and 40 week old C57 and mdx mice; a comparison of sedentary mice on a control diet, high fat diet or high protein diet. A) Total body weight. B) Standardised epidyiymal fat pad weight (g fat/g bw). C) Standardised gastrocnemius muscle weight (g muscle /g bw). Bars represent standard error. N= 8 for all groups. A,B,C,D denotes significant differences, groups with different letters are significantly different from each other (P<0.05).

Figure 3. Myofibre cross-sectional area in 12, 24 and 40 week old sedentary C57 and mdx mice. Bars represent standard error. N= 6 mice for all groups (at least 500 myofibres measured per mouse). A,B denotes significant differences, groups with different letters are significantly different from each other (P<0.05).

Figure 4. Myofibre necrosis in sedentary mdx mice and adipocyte content of both C57 and mdx mice aged 24 and 40 weeks; a comparison of sedentary mice on a control diet with mice on either a high fat or high protein diet. A) Myofibre Necrosis in
the quadriceps muscle (mdx only). B) Adipocyte content in the quadriceps muscle of both strains. Bars represent standard error. N= 8 for all groups. A,B,C denotes significant differences, groups with different letters are significantly different from each other (P<0.05).

Figure 5. Gene expression (mRNA) changes in the gastrocnemius muscle of 24 and 40 week old C57 and mdx mice; a comparison of sedentary mice on a control diet with mice on a high fat diet. A) Tumour Necrosis Factor (TNF). B) Interleukin 1β (IL-1β). C) Interleukin 6 (IL-6). N= 8 mice per group. A,B,C denotes significant differences, groups with different letters are significantly different from each other (P<0.05).

Figure 6. Gene expression (mRNA) changes in the gastrocnemius muscle of 24 and 40 week old C57 and mdx mice; a comparison of sedentary mice on a control diet with mice on a high fat diet. A) Peroxisome proliferator-activated receptor (PPAR) alpha. B) PPAR gamma. C) PPAR delta/beta. D) Peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1-α). N= 8 mice per group. A,B denotes significant differences, groups with different letters are significantly different from each other (P<0.05).

Figure 7. Average daily food consumption (g/ g bw/ day) (A) and energy intake (kj /g bw /day) (B) for mdx mice. Data for dietary consumption between 8-12 weeks of age is shown for both sedentary and voluntarily exercise mdx mice on either a control diet, high fat diet or a high protein diet. Bars represent standard error. N= 8 for all groups. A,B,C denotes significant differences, groups with different letters are significantly different from each other (P<0.05).

Figure 8. Body composition of 12 week old sedentary and voluntarily exercised mdx mice; a comparison of mice on a control diet with a high fat diet or high protein diet. A) Total body weight. B) Standardised epidyiymal fat pad weight (g fat /g bw). C) Standardised gastrocnemius muscle weight (g muscle /g bw). Bars represent standard
error. N = 8 for all groups. A, B denotes significant differences, groups with different letters are significantly different from each other (P<0.05).

Figure 9. Total distance run (km) over 4 weeks of voluntary exercise and myofibre necrosis in sedentary and exercised mdx 12 week old mice; a comparison of mice on a control diet, high fat diet or high protein diet. A) Total distance run (km) over 4 weeks of voluntary exercise (mdx only). B) Myofibre necrosis in the quadriceps muscle (mdx only). Bars represent standard error. N = 8 for all groups. A, B, C denotes significant differences, groups with different letters are significantly different from each other (P<0.05).

Figure 10. Gene expression (mRNA) changes in the gastrocnemius muscle of 12 week old sedentary and voluntarily exercised mdx mice; a comparison of sedentary mice on a control diet with mice on a high fat diet. A) PPAR alpha. B) PPAR gamma. C) PPAR delta/beta. D) PGC1-α. N = 8 mice per group. A, B denotes significant differences, groups with different letters are significantly different from each other (P<0.05).
### Table 1.

#### DIET SPECIFICATIONS

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Figure 1.
Figure 2.
Figure 3.
Figure 4.

(A) Necrosis in quadriceps (% CSA)

- Control
- High Fat
- High Protein

(B) Adipocytes in quadriceps (% CSA)

- Control
- High Fat
- High Protein

Radley-Crabb and Grounds, 2010. 32
Figure 5.
Figure 6.
Figure 7.

A

Food consumption (g/g bw/day)

Sedentary mdx

Exercised mdx

Control

High Fat

High Protein

B

Energy intake (kJ/g bw/day)

Sedentary mdx

Exercised mdx

Control

High Fat

High Protein
Figure 8.
Figure 9.

A

Total distance run (km) over 4 weeks.

8-12 wks old mdx

Control
High Fat
High Protein

B

Necrosis in quadriceps (% CSA)

Sedentary mdx
Exercised mdx

Control
High Fat
High Protein

Radley-Crabb and Grounds, 2010. 37
Figure 10.
References


Hodgetts S, et al, 2006. Reduced necrosis of dystrophic muscle by depletion of host neutrophils, or blocking TNFalpha function with Etanercept in mdx mice. Neuromuscul Disord. 16, 591-602.


A comparison of metabolism and protein synthesis rates in young and adult dystrophic mdx and control C57Bl/10 mice.

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Key words:
Mdx mice, body composition, energy expenditure, protein turnover.
Abstract:
The mdx mouse is a widely used animal model for pre-clinical Duchenne Muscular Dystrophy (DMD) research. Impaired function or absence of the skeletal muscle subsarcolemmal protein dystrophin leaves dystrophic myofibres susceptible to damage during contraction, yet the specific mechanism by which the absence of dystrophin leads to myofibre necrosis is still unclear. Dystrophic skeletal muscle is also subject to many metabolic abnormalities that can greatly impact on the maintenance of muscle mass and function. This research thoroughly investigated the metabolic differences between young (4 week old) and adult (14 week old) dystrophic mdx and control (normal) C57Bl/10 mice specifically measuring; energy expenditure, activity level, body composition and protein synthesis rates. Striking differences were observed in the metabolic processes between both young and adult dystrophic and control mice. Young mdx mice had a ‘stunted’ growth; they were significantly lighter with reduced fat free mass (lean muscle mass). Young mdx mice also had a significantly increased protein synthesis rate (in both whole muscle samples and isolated myofibrillar fraction) and increased metabolic rate (Heat kcals/24hr/kg ffm). The acute onset of myofibre necrosis and regeneration at 3-4 weeks of age appears to have a significant effect on many parameters related to muscle mass and function, and metabolic rate in young mdx mice. There is significant ‘catch-up’ growth in mdx mice between 4 and 14 weeks of age, with 14 week old adult mdx mice being heavier, with significantly increase muscle mass and bone length than C57Bl/10 mice. The adult mdx mice were very lean, with significantly increased fat free mass (lean muscle mass) and increased protein synthesis rates (in both whole muscle samples and isolated myofibrillar fraction). These findings have many implications for understanding the basic pathology of DMD especially with respect to susceptibility to myofibre damage of both growing and mature muscle and impact on potential therapeutic interventions to protect dystrophic muscle from damage.
**Introduction:**
Duchenne Muscular Dystrophy (DMD) is an X-linked lethal muscle wasting disorder affecting approximately 1/3500-6000 male births (1, 2). Patients with DMD exhibit a progressive loss of muscle mass and function with premature death frequently occurring due to respiratory and/or cardiac complications (1, 3). There is currently no cure for DMD and corticosteroids are still the most widely used pharmacological treatment for patients with the disease (4, 5), however numerous interventions to correct the gene defect (and replace dystrophin), or other drugs to ameliorate the severity of dystrophopathy have been identified over the last 10 years [Reviewed in (6-9, 10)].

Impaired function or absence of the skeletal muscle subsarcolemmal protein dystrophin leaves dystrophic myofibres susceptible to damage during mechanical contraction (11, 12) and this initial membrane damage progresses to myofibre necrosis. While successful muscle regeneration occurs initially, over time regeneration is impaired and myofibres are permanently replaced by fatty/fibrotic connective tissue (2, 13). The primary defect in the dystrophin gene was identified in 1987, although the specific mechanism(s) leading to myofibre necrosis are still unclear. There are strong associations with excessive inflammation, increased intracellular calcium levels, oxidative stress and metabolic abnormality, yet the precise importance of these aspects requires *in vivo* elucidation (14-20).

Boys with DMD can move between the spectra of over-nutrition to under-nutrition within their shortened lifespan. Young DMD patients are often over-weight or obese (due to a combination of inactivity, steroid therapies and metabolic abnormalities), yet patients are also often underweight and experience malnutrition in later life [Reviewed in (20, 21)]. Despite these observations, there is limited literature available on the precise nutritional, metabolic and energy requirements for DMD patients.
Skeletal muscle is a metabolically active tissue and therefore loss of muscle mass in DMD patients should lead to a decline in resting energy expenditure. However, DMD is considered to be a hyper-metabolic condition [Reviewed in (20, 21)] and resting energy expenditure, when adjusted to muscle mass (fat free mass), is increased in DMD patients aged 6-13 years (kJ/kg FFM) compared to healthy controls (22, 23). Similarly, Okada et al (1992) reported an increase in basal metabolic rate (kcal/kg/day) in DMD patients aged 11-29 years (24).

The confounding effects of different proportions of undamaged mature myofibres, actively growing myotubes and myofibres (regenerating muscle), and fatty/fibrotic connective tissue throughout all muscles of individual boys at different ages and stages of the disease, makes fundamental differences in the metabolism of dystrophic muscle difficult to evaluate. The problem is further complicated by gross changes in body composition (obesity vs. malnutrition) and corticosteroid therapies. Thus, an experimental model, such as the mdx mouse is useful to address the issue of metabolic status of dystrophic muscle because it enables more invasive measurements to be performed that can isolate the numerous variables that contribute to the alterations in whole body measurements.

The mdx mouse (C57BL/10ScSn\textsuperscript{mdx/mdx}) is an animal model for DMD, and has been extensively described and widely used in pre-clinical experiments to investigate DMD [Reviewed in (25-28)]. The severity of dystropathology varies throughout the life of mdx mice. There is a spontaneous onset of acute myofibre necrosis and subsequent regeneration in limb and paraspinal muscles around 3 weeks of age (29, 30), and skeletal muscle damage (necrosis and regeneration) peaks around 4 weeks. Muscle damage then decreases significantly to stabilise by 8-12 weeks of age to a relatively low level of damage where approximately 6% of the quadriceps muscle is actively necrotic (14, 26, 31, 32).
The extent and progression of dystropathology is far more pronounced in humans compared to the relatively small and short-lived mdx mouse (33). The striking difference in the severity of pathology between mdx mice and DMD patients is widely acknowledged and may reflect the size, lifespan and especially the duration of the postnatal growth phase (e.g. approximately 6 weeks in mice vs 20 years in humans) when muscle may be especially susceptible to damage (33). Continuous cycles of myofibre necrosis and regeneration, regenerating myofibres in various stages of growth, increased demand on the sarcoplasmic reticulum to regulate intracellular calcium, defective mitochondrial function and disruption in nNOS signalling may all contribute to an altered metabolic state in dystrophic muscle (17, 23, 34-39).

Changes in protein metabolism have been reported in various mdx muscles both in vivo and ex vivo (40-44). The rates of protein synthesis and protein degradation are elevated in the gastrocnemius muscle of mdx mice (various ages, sex unspecified) and it is suggested that this process is mediated by an increase in ribosome concentration in mdx muscles (42). Interestingly, in mdx liver where there is no dystropathology, the rate of protein synthesis is similar to control C57BL/10 mice, indicating that altered protein metabolism in mdx mice is restricted to skeletal muscles (42). The extent to which this reflects the increased protein demand of growing myotubes and myofibres compared with mature myofibres is unclear in dystrophic muscle.

Adult mdx mice maintain skeletal muscle mass and experience significant skeletal muscle hypertrophy (27, 45-49), indicating that protein synthesis must outweigh protein degradation in adult mdx mice. Skeletal muscle hypertrophy is also pronounced in dystrophic cat models of muscular dystrophy (HFMD) (50, 51). Skeletal muscle hypertrophy is also a feature of young DMD boys (~ 5 years of age) where hypertrophic calf muscles are a diagnostic feature (1, 51). This is in striking contrast to older (6-18 years), wheel chair bound, DMD patients that lose lean muscle mass at an approximate rate of 4% per year (52). Skeletal muscle hypertrophy in adult mdx mice may be linked to an increase in AKT signalling (45), since AKT is a key downstream
molecule of IGF-1 signalling that results in increased protein synthesis and hypertrophy of growing muscles such as that found during the regenerative process in mdx mice (48, 53). There are also reports of increased circulating growth hormone in 8-10 month old female mdx (54) and increased circulating IGF-1 levels in 8-10 week old (sex unspecified) mdx mice (55).

It was hypothesised by Dupont-Vesteegden et al (1994) that continuous cycles of myofibre necrosis and subsequent regeneration, increased levels of intracellular calcium and elevated protein synthesis in dystrophic mdx muscles could lead to an overall increase in whole body metabolic rate. However, they reported no change in the metabolic rate $[\text{kcal}/(\text{kg bodyweight}^{0.75} \cdot \text{d})]$ of one year old adult mdx mice (mixed sex). In contrast, there was a decrease in the whole body metabolic rate $[\text{kcal}/(\text{kg bodyweight}^{0.75} \cdot \text{d})]$ of 4-6 week old (mixed sex) mdx mice, and it was proposed that this was a consequence of reduced physical activity and reduced food consumption (g food/g body weight) (34). Mokhtarian et al (1996) also reported no difference in total energy expenditure, basal energy expenditure and spontaneous activity between mdx and control C57Bl/10 mice (sex unspecified) at 6-12 months of age (49). Clearly, mouse age, extent of dystropathology and the presence of regenerating (growing) muscle, may have a major impact on metabolic demand and energy usage.

The purpose of this longitudinal study (over approximately 10 days) was to measure food intake, energy expenditure, activity level, body composition and protein synthesis rate (for both whole muscles and the myofibrillar fraction) of dystrophic mdx and control C57BL/10 mice at 2 different ages; 1) Young mice aged 3-4 weeks (shortly after the acute onset of muscle necrosis in mdx mice when muscle damage regeneration is at a peak). 2) Adult mice aged 13-14 weeks (when adult mdx mice exhibit a lower level of muscle damage and dystropathology).
It was hypothesised that:

A) There would be no difference in absolute daily food intake (g) between strains. However, due to their higher fat free mass (lean muscle), mdx mice will consume less food (g), when adjusted to fat free mass. B) Continuous cycles of muscle necrosis and regeneration in both young and adult mdx mice will promote an increase in whole body metabolic rate (relative to control mice) and that this difference will be amplified in young mdx mice when muscle necrosis and regeneration are most pronounced. C) As hypertrophy is a feature of adult mdx muscle, PIXImus body composition scans will show a higher total % lean muscle mass and a lower total % fat mass in adult mdx mice compared to age-matched control mice. However, there will be no marked differences in body composition between young strains of mice.

Materials and Methods:

Animals

All animal experiments and analysis were carried out at The Children’s Nutrition Research Center (CNRC) at Baylor College of Medicine, Houston USA. Experiments were conducted in strict accordance with the U.S. National Research Council’s Guide for the Care and Use of Laboratory Animals and all procedures were approved by the Baylor Medical College Animal Care and Use Committee. The young 3 - 4 week old male mice (mdx and C57Bl/10) were bred at the CNRC from breeders originally from Jackson Laboratories. Litters were standardised to 7 pups per dam on the day of birth and mice were changed to a wire bottom cage on the day after weaning (22 days of age). Only male mice were studied. Adult 13-14 week old male mice (mdx and C57BL/10) were ordered from Jackson’s laboratory (USA) and changed to a wire bottom cage on the day after arrival. Mice were left to stabilize for a minimum of 6 days at CNRC prior to experimentation (3 days stabilization was allowed before food consumption was measured). A typical experimental timeline for both young (4wks) and adult (14wks) mdx and C57Bl/10 mice is outlined in Table 1. An additional set of 8 (4 C57 and 4 mdx) 14-wk-old mice were used only for calorimetry and activity
measurement, as the complete set of the activity measurements was not obtained on the first set of mice.

**Food consumption.** All mice were maintained on a standard diet based on AIN93G containing 19% protein (Research Diets, New Brunswick, NJ). Daily food intake (g) was measured before and during measurements of energy expenditure. Food intakes determined before the energy expenditure measurements were estimated from the change in the weight of food in the food hoppers corrected for losses. Measurements made during the energy expenditure determinations utilized the automated in cage feeding assembly of the CLAMS system (Columbus Instruments)

**Calorimetry.** Mice were placed individually into calorimetry chambers for 72 hrs and energy expenditure was measured using a Columbus Instruments (Columbus OH) CLAMS Oxymax System. A known flow of air was passed through the chambers and the O₂ and CO₂ gas fractions were monitored at both inlet and outlet ports. The gas fraction and flow measurements were used to calculate VO₂ and VCO₂, from which the Respiratory Exchange Ratio and heat production (kcal/hr) were calculated. At the same time spontaneous activity level (both horizontal and vertical movements) was monitored from the interruption of infra-red beams projected across the cage.

**Whole Body Composition Scans.** Total body lean muscle and fat mass were measured by dual energy X-ray absorptiometry (DXA) using a PIXIImus (General Electric, USA). Whilst under isoflurane anaesthesia mice were aligned on a PIXIImus measuring tray and then inserted into the machine for measurements. All mice were measured in duplicate and then returned to their cage to recover from the anaesthetic, a minimum of 2 days elapsed before protein turnover measurements were performed. Correction factors derived specifically for the CNRC PIXIImus were applied to the fat and lean mass data to adjust for the inherent errors in fat and lean mass obtained using all PIXIImus instruments ([http://www.bcm.edu/cnrc/intranet/MMRU/PIXImus%20corrections.xls](http://www.bcm.edu/cnrc/intranet/MMRU/PIXImus%20corrections.xls)).
Isotope infusion and tissue collection to measure rate of protein synthesis. Food was removed from all mice 7 hours prior to isotope infusion. A flooding dose of L-4-[\(^3\)H]-phenylalanine (American Radiolabeled Chemicals, Inc., St. Louis, MO) at 20mL/kg BW, 1.5mmol phenylalanine/kg and 250µCi/mouse was given through the tail vein, 15 minutes after injection mice were sacrificed (by decapitation) and trunk blood was immediately collected and acidified to 0.2M Perchloric acid (PCA). The supernatant was frozen and reserved for estimation of the specific radioactivity of blood free phenylalanine pool. The hindlimbs were immediately detached, wrapped in foil and chilled on ice. A range of muscles including; gastrocnemius and plantaris complex (referred to hereafter as the gastrocnemius), quadriceps, soleus, tibialis anterior, diaphragm and heart, were then dissected on ice and frozen in liquid nitrogen. The weights of all muscles were recorded and muscles were stored at -80°C.

Laboratory methods: These are based on Fiorotto et al (2000) and Welle et al (1993) with some modifications (56, 57).

Total Protein, and Myofibrillar Proteins isolation: Frozen muscles from each mouse were powdered, weighed and 50mg was homogenized in a low salt homogenising buffer (50 mM potassium phosphate, 0.25 M sucrose, 1% Triton X-100 pH 7). For each muscle, an aliquot of the homogenate was retained for measurement of total protein; a second aliquot was acidified to 0.2M PCA and centrifuged; the supernatant containing the muscle free amino acid precursor pool was collected and neutralized as described below. The PCA-insoluble precipitate was washed, and after assaying for total RNA, was processed for the measurement of L-[4-\(^3\)H] phenylalanine incorporated into all muscle proteins. The remainder of the low salt/sucrose buffer homogenate was left to incubate with agitation at 4°C for 60 minutes; the proteins that were insoluble after low speed centrifugation (1,500 g at 4°C for 10min) contained the myofibrillar proteins (56, 57). After several washes of the pellet in the low salt/sucrose buffer followed by ice-cold water, the purified myofibrils were acidified to 0.2M PCA. The acid insoluble myofibrillar and total protein precipitates were separated from the supernatant by centrifugation at 10,000 g at 2°C for 30 min, and the pellets were
washed three times in 0.2M PCA. The supernatant from the total protein fraction and the pellets from all protein fractions were processed for determination of L-[4-³H] phenylalanine specific radioactivity.

**Quantification of L-[4-³H] phenylalanine specific radioactivity in the protein bound and tissue free amino acid precursor pools:** The acid-insoluble pellets were first hydrolysed for 24 hrs in 6M HCL (ultrapure) at 110°C under N₂ gas. After evaporation of the HCl in a Savant Speedvac concentrator, the remaining precipitate was subject to several washes in water and finally resuspended in mQ water for HPLC analysis. The PCA-treated blood and muscle free pool supernatants samples were neutralized on ice with 4M KOH, evaporated to dryness and resuspended in 1M acetic acid. The amino acids were purified over a Dowex anion exchange column (AG-50w-x8, 100 - 200 mesh, Biorad). The amino acids were eluted in 3M NH₄OH evaporated to dryness in the Savant Speedvac concentrator and finally dissolve in mQ water. All samples were filtered using a 0.45µm syringe filter, supernatant dried down and each sample was re-dissolved in 100ul of mQ water. Phenylalanine in the total and myofibrillar protein hydrolysates, muscle homogenate and blood supernatants were isolated by anion exchange HPLC (AminoPac1 Analytical column; Dionex, Sunnyvale, CA). Amino acids were post-column derivatized with o-phthalaldehyde reagent and detected with an online fluorimeter. The fraction of the eluant that included the phenylalanine peak was collected, and the associated radioactivity was measured in a liquid scintillation counter (Packard Tricarb, Perkin Elmer, Waltham, MA). The phenylalanine concentration was determined by comparing the peak areas of the samples with that of a known standard (Pierce Labs.)

**Calculations of in vivo fractional protein synthesis rates:** The fractional rate of protein synthesis (FSR), i.e., the percentage of the protein mass synthesized in a day was calculated as follows: FSR = \( \frac{S_B}{S_A} \) \( H (1440/t) \) H 100

Where \( S_B \) is the specific radioactivity of the protein-bound labelled amino acid, \( S_A \) is the specific radioactivity of the precursor pool and \( t \) is the the labelling time in minutes (58). It has been demonstrated that the specific radioactivity of the tissue free phenylalanine after a flooding dose of phenylalanine is in equilibrium with the
aminoacyl-tRNA specific radioactivity; hence, the tissue free phenylalanine reflects the specific radioactivity of the tissue precursor pool (59). Blood and tissue free phenylalanine specific radioactivity values were compared to verify equilibration.

**Protein quantification:** Total protein concentration of all muscle samples was determined using a standard BCA protein assay (60) after first solubilising the aliquot of total muscle homogenate in 0.1 M NaOH for 1 hr at 37°C.

**Total RNA measurement:** Because the RNA content of a tissue is dominated by ribosomal RNA, total RNA was measured quantitatively on all muscle samples and provides an estimate of ribosomal abundance. Total RNA was quantified in the total protein PCA-insoluble precipitate using a modified Schmidt-Thannhauser procedure as described by Munro and Fleck (1966) (61).

**Statistical analysis:**

Statistical analysis was completed by using Microsoft excel and SPSS software. Values throughout the manuscript are expressed as mean ± standard error of measurement (SEM). Significant difference between 2 groups was determined by conducting student’s T-test. To establish the extent to which differences in the variables related to energy balance might be accounted for by differences in body weight or fat free mass (FFM), ANOVA was performed, with strain as the main independent effect and body weight or FFM as covariates. Results are presented as least square means ± the standard error.

**Results - Young mice:**

**Body composition:** The young dystrophic mdx mice weighed significantly less (approximately 25%) than age-matched C57Bl/10 mice (16.5 g ± 0.3 vs. 20.7 g ± 0.4). This difference in body weight is due to a significant and proportional reduction in both fat free mass (FFM) (e.g., lean muscle tissue) and fat mass as measured by PIXImus (Table 2). Bone mineral content (BMC) was also significantly reduced in dystrophic mdx mice. The reduction in BMC was attributable in part to a reduction in total bone area (5.56 ± 0.10 cm² vs. 6.55 ± 0.06 cm², P< 0.001), but also to a reduction in bone
mineralization as indicated by the bone mineral density (Table 2). In addition to the PIXImus body scans, individual muscle weights (g) and bone lengths (mm) recorded at time of animal sacrifice were significantly lower for the gastrocnemius and tibialis anterior muscles and there was also a significantly shorter length of femur and tibia bone in young mdx mice (Table 2). After adjusting for these differences in bone length, only the gastrocnemius muscle remained significantly smaller in the mdx mice (P<0.05). Absolute heart and diaphragm weights were similar between young mdx and C57Bl/10 mice, however when adjusted to FFM (an indicator of the body’s active metabolic mass) there was a significant increase in both heart and diaphragm weights of young mdx mice (Table 2).

**Calorimetry and activity level:**

As the same diet was consumed by both strains at all ages, differences in nutrient intakes are entirely explained by differences in daily food intake. Energy intake (kcal/d) was significantly lower in young mdx mice compared to C57Bl/10 mice (10.3 +/- 0.5 vs 11.3 +/- 0.3) (Table 3). However, when adjusted to individual bodyweight and/or fat free mass (FFM), young mdx mice consume significantly more energy than C57Bl/10 mice (Table 3). Respiratory exchange ratio (RER – ratio of carbon dioxide production / oxygen production) was similar between strains suggesting both strains are using the same type of fuel over a 24hr period. Total energy expenditure (heat production - kcal/d) was unchanged between strains; however, when adjusted for difference in FFM, energy expenditure was significantly increased in young mdx mice (Table 3). Daily energy balance, calculated as the difference between energy intake and energy expenditure was significantly less positive in mdx mice. After adjusting for differences in body weight or FFM, the values remained numerically smaller but were no longer significant. Total daily activity was reduced by approximately 35% in young mdx mice (Table 3), especially at night (during their active phase). The young mdx mice performed significantly less vertical movements (standing on back legs) during the day (819 +/- 71 vs 1683 +/- 294) and during night (3812 +/- 625 vs 17093 +/- 2389) compared
to young C57Bl/10 mice. The mdx mice also performed significantly less horizontal movement during the night (33486 ± 1887 vs 46797 ± 3490) (Table 3).

**Protein synthesis:** We initially compared fractional synthesis rates (FSR) for total and myofibrillar proteins in the quadriceps, gastrocnemius and diaphragm muscles in a subset of mice. The values were quite similar for the gastrocnemius and quadriceps, and thus, for the full set of mice we performed measurements only on the gastrocnemius. Additionally, we evaluated the response of the diaphragm to establish if the differences in the pathophysiology of this muscle in the mdx mouse are manifested in protein synthesis rates. FSR of both total and myofibrillar proteins were greatly increased in both gastrocnemius and diaphragm muscles of young mdx mice (Table 4). Total proteins FSRs were more than 2 fold higher in young mdx mice than in controls, and there was no difference between muscles. The myofibrillar protein FSR was approximately 2.5 fold higher in the gastrocnemius muscle of the young mdx mice, whereas for the diaphragm the increase was only approximately 2 fold; there was no difference between the FSR of myofibrillar proteins in diaphragm and gastrocnemius muscles of the control mice (strain X muscle, P=0.02) (Table 4). Absolute rates of synthesis for total proteins (mg/d) reflected the differences between strains in FSR (mdx>C57, P<0.001). Absolute rates of myofibrillar protein synthesis could not be calculated as the isolation of myofibrillar proteins was not quantitative.

Although the FSR values were similar for the two muscles in both strains of mice, this was achieved by slightly different mechanisms. The increase in gastrocnemius FSR in the young mdx mice was due, in part, to an 80% increase in ribosome abundance (RNA/TP – mg/g) together with a 40% increase in their translational efficiency (KRNA – mg/g) (Table 4). The ribosomal abundance in the diaphragm was higher overall than in gastrocnemius muscle for both strains of mice, and the difference in ribosomal abundance between mdx and C57Bl/10 in the diaphragm was approximately 30%. Yet, the overall translational efficiency of the diaphragm ribosomes was significantly lower than for the gastrocnemius ribosomes in mdx mice and the difference between
C57Bl/10 and mdx diaphragm ribosomes was significantly greater (approximately 60%) than for the gastrocnemius muscle.

Despite the 80% increase in total gastrocnemius muscle protein synthesis rate (mg/d) of the mdx mice, total protein content (mg) of the gastrocnemius muscle was decreased by approximately 4mg or 40% in young mdx mice (Table 4), and this can only be explained by a substantial increase in protein degradation. Myofibrillar FSR is increased in both the gastrocnemius and diaphragm muscle in mdx mice; however the difference between strains is much greater in the gastrocnemius.

The strain difference in total muscle protein synthesis rate (mg/d) was greater in the diaphragm (increased in mdx approximately 100%) than the gastrocnemius muscle (increased in mdx approximately 85%), yet there was no change in total muscle protein (mg) in the diaphragm muscle between strains (Table 4). Thus, protein degradation rate in the diaphragm must be substantially higher in the mdx mice compared to C57Bl/10, but probably not to the same extent as in the gastrocnemius muscle, as the mdx diaphragm is able to maintain protein mass (mg). As the strain difference in myofibrillar FSR in the diaphragm was not as great as in the gastrocnemius muscle, one interpretation is that the replacement of nonmyofibrillar proteins makes a greater contribution in the diaphragm of mdx mice either because the protein composition is different or because the contractile proteins are less impacted by dystrophy than in the gastrocnemius muscle.

Results - Adult mice:

Body composition: There was no difference in body weight of adult mdx mice and age-matched C57BL/10 mice (Table 5). PIXImus body composition scans revealed that mdx mice had significantly more FFM (approximately 15%); this increase largely reflects increased lean skeletal muscle but also includes some other tissues such as gut and liver. Mdx mice also had a huge reduction (approximately 50%) in fat mass which, together with the increase in FFM, was manifested as a 45% reduction in percentage
body fat. In addition to the PIXImus body scans, individual muscle weights and bone lengths (recorded at time of animal sacrifice) showed significantly heavier gastrocnemius, quadriceps, soleus, tibialis anterior, and diaphragm muscles in mdx mice (Table 5) and also significantly longer femur and tibia bone length (Table 5). Although there was no difference in total bone mineral content (g), given the larger bone area, bone mineral density (g/cm²) was significantly lower in the mdx mice. Absolute heart weight (g) was similar between adult mdx and C57Bl/10 mice; however when adjusted for FFM, there was a significant decrease in the heart weight of mdx mice (g/kg FFM) (Table 5).

Calorimetry and activity level:
There were no differences in energy intake (kcal/d), energy expenditure (heat production – kcal/d) or energy balance (both absolute or when adjusted for body weight or FFM) between strains (Table 6). RER was similar between strains suggesting both were using the same fuel over a 24hr period. Total activity level was significantly lower in the adult mdx mice, and was attributable primarily to a reduction in vertical movements (stand on back legs) at night (during their active phase) compared to adult C57Bl/10 mice (Table 6).

Protein synthesis:
Fractional synthesis rates (FSR) of both total and myofibrillar proteins in both the gastrocnemius and diaphragm muscles exhibited the normal developmental decline in both strains of mice. FSRs of the gastrocnemius muscle in adult mdx mice were significantly higher than age-matched C57Bl/10 mice; total protein FSR was approximately 2.5x fold higher and myofibrillar protein FSR was approximately 2x fold higher in adult mdx mice (Table 7); the age-related decline in myofibrillar FSR was significantly greater for the mdx mice than controls. Total protein synthesis (mg/day) and total muscle protein content (mg) in the gastrocnemius muscle were also significantly increased in adult mdx mice: total protein synthesis rate was approximately 3x fold higher in adult mdx and total protein content of the
gastrocnemius muscle was increased by approximately 4 mg or 20% (Table 7). These increases in FSR were due to the combination of an increase in ribosome abundance (40% greater) and ribosomal translational efficiency (70% greater) in adult mdx mice (Tables 7).

A quantitative dissection of the diaphragm in the adult mice was achieved for only 4 mice of each strain. All other parameters evaluated did not differ between this sub group of mice and the total group. Thus, the average trends over time and differences between strains and muscles are likely to be valid reflections for the diaphragm muscle. The developmental decline in FSR for the diaphragm was less marked than for the gastrocnemius muscle proteins, and was more pronounced in the mdx. Total protein FSR was approximately 60% higher and myofibrillar protein FSR was approximately 50% higher in the diaphragm of adult mdx compared to C57Bl/10 mice (Table 7). Total daily protein synthesis rates (mg/d) and total diaphragm protein mass (mg) were significantly greater in mdx than C57Bl/10 mice; this difference in protein mass was more pronounced in the diaphragm than the gastrocnemius muscle. In contrast to the gastrocnemius muscle, the difference in ribosomal abundance between strains (45% greater in mdx) made a greater contribution than differences in ribosomal efficiency (20% greater in mdx).

**Percentage change from 4-14 weeks of age:**
Despite being significantly (approximately 4g) lighter at 4 weeks of age, mdx mice were similar in weight to the C57Bl/10 mice at 14 weeks of age, representing a 176% increase in bodyweight over 10 weeks, compared to a 142% increase for C57Bl/10 mice. Over this 10 week period, mdx mice showed a large increase in FFM (approximately 12.5 g) and deposited very little fat mass (approximately 0.2 g); this was in stark contrast to C57Bl/10 mice that gained approximately 5.9 g of FFM and deposit 2.8 g of fat over the 10 weeks (Table 8). The percentage growth of all individual muscles (except heart) over this time was also much higher in mdx compared to C57Bl/10 mice; for example, there was a 240% increase in weight in the
mdx gastrocnemius compared to only a 152% increase in the C57Bl/10 gastrocnemius (Table 8). The lack of fat deposition suggests that mdx mice, in contrast to the C57Bl/10 controls, were not consuming energy in excess of their needs during this time, and that a greater portion of dietary protein was partitioned toward protein deposition rather than being oxidized. In addition to rapid muscle growth, bone length also caught up and overtook the controls during these 10 weeks.

Discussion:

Young mice:

Young mdx mice (aged 3-4 weeks) were significantly smaller (by approximately 25%) than age-matched C57Bl/10 mice due to reductions in both fat free mass (lean muscle tissue), fat mass, and individual muscle weights, with bone length and bone mineral content also significantly reduced in young mdx mice (Table 2). Since bone strength (and thus mineral content) is determined by the dynamic load from skeletal muscles this smaller bone size may directly result from the reduced muscle mass. In addition, 15 day old dystrophic mdx mice are significantly lighter than aged matched C57Bl/10 mice (Mdx 7.53g +/- 0.18 vs. C57 8.24g +/- 0.16) (Radley-Crabb 2010 unpublished) and it is also known that the dystropathology of mdx mice begins in early development (62). These combined data indicate that growth of young mdx mice is decreased (stunted) during development, and this occurs well before the onset of severe pathology (around 3 weeks of age).

Multiple factors could contribute to the reduced growth of young mdx mice. The rate of protein synthesis (both total protein and myofibrillar proteins) is more than doubled (2.5 fold higher) in young (growing) mdx mice compared to C57Bl/10 control mice (Table 4) and protein synthesis is a very energetically expensive process [Reviewed in (63)] that, if not supported by adequate increases in food intake, may occur at the expense of overall growth. After accounting for the differences in size, young mdx mice (26-28 days) have an increased metabolic rate which supports the increased energy demands of a high protein synthesis rate and the increased energy demands of
myofibre regeneration and myofibre growth due to the acute onset of dystrophy at this time. However, this result disagrees with a previous study that measured metabolic rate in 4-6 week old mdx mice (34). Nonetheless, it should be noted, that our data indicating greater energy expenditure are supported by the significantly increased heart weight, even after adjusting for differences in FFM, and cardiac hypertrophy is a classic functional response to an increase in the oxygen demand of body tissues.

It appears that the increased energy needs of young mdx mice were met partly by a relative increase in food intake (after taking into account differences in body size) and partly by expending less energy by being less active, especially at night (Table 3). This possibly reflects the acute onset of pathology around this age; perhaps this may be an attempt to conserve energy at this time of high muscle damage or the mdx mice may feel ‘unwell’. It must also be considered that mdx mothers (who are energy deficient themselves) may not produce sufficient milk for their pups.

It seems likely that the cycles of myofibre necrosis and regeneration (which have an acute onset around 3 weeks of age and peak around 28 days in mdx mice) are responsible (entirely or in part) for the increase in protein fractional synthesis rate in young mdx mice (e.g. due to the acute inflammation, proliferation of myoblasts and rapidly growing myotubes in the regenerating muscle). The large difference between protein synthesis rates in whole mdx muscle (Total protein FSR) and the isolated sarcomeric myofibrillar fraction (myofibrillar protein FSR) is probably due to the many inflammatory cells present in necrotic/regenerating dystrophic muscles at this young age (14, 26), since the inflammatory cells inherently have higher protein synthesis rates than myofibrillar proteins.

It remains unknown if ‘intact’ dystrophic skeletal myofibres have an inherent increase in protein synthesis rate or if the elevated protein synthesis is a result of continuous cycles of myofibre necrosis and regeneration. However, the delayed growth of young
mdx mice, that must occur in the pre-necrotic neonatal phase strongly supports the proposal that there are inherent differences in the metabolic rate of ‘intact’ dystrophic myofibres. This issue might be addressed, in part, by similar studies conducted in pre-necrotic mdx mice, i.e. ≤20 day old, that have either been cross-fostered onto normal non-dystrophic mothers, to avoid complications of reduced milk quality or quantity by mdx mothers, or born to heterozygous mdx mothers to mimic the human situation.

**Adult mice:**

Adult male mdx mice (14 wks) attained the same weight as C57Bl/10 mice due to a significant increase in fat free mass (lean muscle tissue) and significant hypertrophy of many individual muscles (Table 5). Adult mdx mice are very lean and have very little fat mass (approximately 50% less than C57Bl/10 mice) and a significantly reduced percentage body fat (Table 5). The same differences in body composition are seen in 40 week old mdx and C57Bl/10 mice (Radley-Crabb et al, 2010 unpublished). The body composition of adult mdx mice is in stark contrast to DMD patients who may experience obesity (due to a combination of inactivity and steroid therapies) and extreme loss of muscle mass and function as the disease progresses during the growth phase.

The increased protein synthesis rate and increased ribosomal efficiency in adult mdx mice (Table 7) facilitates skeletal muscle hypertrophy. Previous studies also report an increased protein synthesis rate (approximately 2x fold) in the gastrocnemius, soleus and EDL muscles from adult mdx mice (40, 42, 44). The present data show that the myofibrillar protein synthesis rate of mdx mice is about twice as high as C57Bl/10 mice in both adult and young mice. This suggests that the increased myofibrillar protein synthesis rate in mdx mice may reflect inherent differences in dystrophic myofibres (at all ages) rather than merely the consequence of necrosis (with associated inflammation) and regeneration since myofibre necrosis is far less pronounce in adult compared with young mdx mice. It should be noted that all the measurements in this study were performed with the mice in the fasted condition, and it is difficult to infer if
these differences are maintained, or are greater or smaller in the postprandial condition. Because ribosomal abundance is increased, it is likely that in the postprandial condition FSR values would remain higher in the mdx muscle than in the controls, even if translational efficiency were to increase more in the control muscles. The ability of the mdx muscle to sustain higher translational efficiency even in the fasted state is unusual, as translational efficiency is highly dependent on the activation of the insulin and mTOR signalling pathways (64), and these are usually inactive in the fasted state. However, these pathways can be activated by stimulation of the type IGF1-R, and both IGF-I and IGF-II expression are relatively high in the early stages of muscle regeneration in young mdx mouse muscles (65, 66).

Overall energy expenditure was not significantly different between adult mdx and C57 mice even after adjusting for the differences in FFM. There also was no difference in energy intake, so that overall there was no difference in energy balance at this age. The adult mdx mice, however, were significantly less active than controls (Table 6) primarily because they reduced vertical movements (rear-up on back legs) at night. This result agrees with previous studies which document significantly reduced general activity levels in mdx mice (27, 67). The reduction in energy expenditure for activity likely compensated for the greater energy demands of maintaining their higher metabolic activity. This could be demonstrated by performing measurements of resting energy expenditure when there would be no contribution from activity and the thermal effect of food on total energy expenditure.

It appears, therefore, that adult mdx mice focus their energy on the maintenance of skeletal muscle mass and growth of new myofibres (that leads to hypertrophy) and because they do not increase food intake, they do not have any excess energy to deposit as adipose tissue. In support of the importance of this vulnerable metabolic/energy balance, 48 hours fasting causes a significant increase in myofibre necrosis in muscles from the hind limb and lumbar region of 6 month old mdx mice, yet no change
in the muscle morphology of control C57BL/10 mice, suggesting a strong dependence on energy intake to maintain dystrophic muscle structure (44).

These findings have many implications for understanding the basic pathology of DMD especially with respect to susceptibility to myofibre damage of both growing and mature muscle and the design and impact of potential nutritional therapeutic interventions to protect dystrophic muscle from damage.

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References:


Table Captions:

Table 1) Example experimental Timeline for both young (4 wks) and adult (14 wks) mdx and C57Bl/10 mice. *Actual sacrifice age of young mice was between 31-32 days of age and for adult mice was between 90 – 100 days of age.

Table 2) Body composition parameters, individual muscle weights and bone lengths in young (4 wks) male mice. n=12 for mdx and n=11 for C57Bl/10 mice for body composition parameters and n=8 for individual muscle weights and bone lengths. Significant differences are shaded in grey.

Table 3) Body composition parameters, individual muscle weights and bone lengths in young (4 wks) male mice. n=8 for mdx and n=11 for C57Bl/10 mice for body composition parameters and n=8 for individual muscle weights and bone lengths. Significant differences are shaded in grey.

Table 4) Body composition parameters, individual muscle weights and bone lengths in adult (14 wks) male mice. n=16-20 for mdx and n=15 for C57Bl/10 mice for body composition parameters and n=8 for individual muscle weights and bone lengths; *n=4 per strain for diaphragm. Significant differences are shaded in grey.

Table 5) Body composition parameters, individual muscle weights and bone lengths in adult (14 wks) male mice. n=16-20 for mdx and n=15 for C57Bl/10 mice for body composition parameters and n=8 for individual muscle weights and bone lengths; *n=4 per strain for diaphragm. Significant differences are shaded in grey.

Table 6) Body composition parameters, individual muscle weights and bone lengths in adult (14 wks) male mice. n=16-20 for mdx and n=15 for C57Bl/10 mice for body composition parameters and n=8 for individual muscle weights and bone lengths; *n=4 per strain for diaphragm. Significant differences are shaded in grey.

Table 7) Protein synthesis measures in gastrocnemius and diaphragm muscles of adult (14 wks) male mice. n=8 for both mdx and C57Bl/10 mice. Significant differences are shaded in grey.

Table 8) Percentage increase in body composition parameters (comparison of group averages shown in Table 2 & 5), individual muscle weights and bone lengths in mdx and C57Bl/10 mice over 10 weeks (4-14 weeks of age). Calculated as: (14wks/4wks)x100. n=16-20 for mdx and n=15 for C57Bl/10 mice for body composition parameters and n=8 for individual muscle weights and bone lengths. Important differences are highlighted in grey.
**Table 1.**

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**Table 2.**

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<td>Bone Mineral Density (g/cm²)</td>
<td>0.032 0.001</td>
<td>0.034 0.0004</td>
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<td>18.39 0.31</td>
<td>P&lt;0.001</td>
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<td>Fat tissue (g)</td>
<td>1.96 0.16</td>
<td>2.30 0.09</td>
<td>P&lt;0.05</td>
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<tr>
<td>Body fat (%)</td>
<td>11.8 0.9</td>
<td>11.1 0.3</td>
<td>NS (P=0.63)</td>
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</tbody>
</table>

**Muscle weight**

| Gastrocnemius (g) | 0.085 0.003 | 0.10 0.002 | P<0.001 |
| Quadriceps (g)   | 0.122 0.003 | 0.127 0.002 | NS (P=0.1) |
| Soleus (g)       | 0.007 0.0002 | 0.008 0.0001 | NS (P=0.07) |
| Tibialis anterior (g) | 0.030 0.0006 | 0.032 0.001 | P=0.03 |
| Diaphragm (g)    | 0.054 0.003 | 0.052 0.002 | NS (P=0.7) |
| Diaphragm (g/kg ffm) | 3.71 0.19 | 2.87 0.11 | P=0.002 |
| Heart (g)        | 0.089 0.005 | 0.086 0.003 | NS (P=0.7) |
| Heart (g / kg ffm) | 6.08 0.325 | 4.71 0.16 | P=0.002 |

**Bone length**

<p>| Femur (mm)       | 12.66 0.057 | 13.03 0.079 | P&lt;0.001 |
| Tibia (mm)       | 15.54 0.029 | 15.99 0.049 | P&lt;0.001 |</p>
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<th>+/- SE</th>
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<td>Energy intake (kcal/d)</td>
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<td>80167</td>
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<td>14596</td>
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<td>1887</td>
<td>46797</td>
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<td>Vertical Activity - Night (cts/12hr)</td>
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<td>625</td>
<td>17093</td>
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<td>Total Activity – Night (cts/12hr)</td>
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<td>2416</td>
<td>63889</td>
<td>5306</td>
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Table 3.

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<th>sterr +/-</th>
<th>C57Bl/10</th>
<th>sterr +/-</th>
<th>P for Strain effects</th>
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<tr>
<td>Gastrocnemius</td>
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<tr>
<td>Total Protein FSR (% / d)</td>
<td>31.13</td>
<td>2.22</td>
<td>12.31</td>
<td>0.57</td>
<td>P&lt;0.001</td>
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<td>Myofibrillar proteins FSR (% / d)</td>
<td>24.67</td>
<td>1.54</td>
<td>10.27</td>
<td>0.59</td>
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<td>Total protein synthesis (mg/d)</td>
<td>3.46</td>
<td>0.20</td>
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<td>Ribosome abundance (RNA/TP - mg/g)</td>
<td>18.28</td>
<td>0.95</td>
<td>10.24</td>
<td>0.46</td>
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<tr>
<td>Ribosome efficiency (K&lt;sub&gt;RNA&lt;/sub&gt; - mg/g)</td>
<td>17.16</td>
<td>1.23</td>
<td>12.16</td>
<td>0.70</td>
<td>P&lt;0.001</td>
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<tr>
<td>Total muscle protein (mg)</td>
<td>10.93</td>
<td>0.42</td>
<td>15.07</td>
<td>0.33</td>
<td>P&lt;0.001</td>
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<tr>
<td>Diaphragm</td>
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<tr>
<td>Total Protein FSR (% / d)</td>
<td>28.23</td>
<td>1.81</td>
<td>13.91</td>
<td>0.90</td>
<td>P&lt;0.001</td>
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<tr>
<td>Myofibrillar proteins FSR (% / d)</td>
<td>20.16</td>
<td>1.44</td>
<td>11.55</td>
<td>0.82</td>
<td>P&lt;0.001</td>
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<td>Total protein synthesis (mg/d)</td>
<td>2.12</td>
<td>0.24</td>
<td>1.05</td>
<td>0.09</td>
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<tr>
<td>Ribosome abundance – RNA/TP - mg/g</td>
<td>25.01</td>
<td>1.30</td>
<td>19.62</td>
<td>1.19</td>
<td>P&lt;0.01</td>
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<td>Ribosome efficiency – K&lt;sub&gt;RNA&lt;/sub&gt; - mg/g</td>
<td>11.35</td>
<td>0.58</td>
<td>7.13</td>
<td>0.31</td>
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<td>Total muscle protein (mg)</td>
<td>7.35</td>
<td>0.37</td>
<td>7.49</td>
<td>0.39</td>
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Table 4.
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<th>Strain/Parameter</th>
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<th>C57Bl/10 sterr +/-</th>
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<tr>
<td>Weight (g)</td>
<td>29.18 0.39</td>
<td>29.37 0.80</td>
<td>NS (P=0.81)</td>
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<tr>
<td>Bone Mineral Content (g)</td>
<td>0.47 0.006</td>
<td>0.47 0.01</td>
<td>NS (P=0.56)</td>
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<tr>
<td>Bone Mineral Density (g/cm²)</td>
<td>0.049 0.0003</td>
<td>0.051 0.001</td>
<td>P&lt;0.01</td>
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<td>Fat free mass (g)</td>
<td>27.01 0.33</td>
<td>24.28 0.43</td>
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<tr>
<td>Fat tissue (g)</td>
<td>2.17 0.13</td>
<td>5.10 0.56</td>
<td>P&lt;0.001</td>
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<tr>
<td>Body fat (%)</td>
<td>7.39 0.38</td>
<td>16.89 1.49</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Strain/Parameter</td>
<td>mdx sterr +/-</td>
<td>C57Bl/10 sterr +/-</td>
<td>P for Strain effects</td>
</tr>
<tr>
<td>Gastrocnemius (g)</td>
<td>0.203 0.007</td>
<td>0.152 0.002</td>
<td>P&lt;0.001</td>
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<td>Quadriceps (g)</td>
<td>0.317 0.016</td>
<td>0.212 0.005</td>
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<td>Soleus (g)</td>
<td>0.0127 0.001</td>
<td>0.010 0.0003</td>
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<td>Tibialis anterior (g)</td>
<td>0.071 0.004</td>
<td>0.044 0.0004</td>
<td>P&lt;0.001</td>
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<tr>
<td>Diaphragm (g)*</td>
<td>0.113 0.005</td>
<td>0.077 0.001</td>
<td>P&lt;0.001</td>
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<tr>
<td>Diaphragm (g/kg FFM)*</td>
<td>3.96 0.20</td>
<td>3.41 0.05</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>Heart (g)</td>
<td>0.119 0.005</td>
<td>0.113 0.002</td>
<td>NS (P=0.26)</td>
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<tr>
<td>Heart (g / kg ffm)</td>
<td>4.32 0.17</td>
<td>4.85 0.04</td>
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</tr>
<tr>
<td>Strain/Parameter</td>
<td>mdx sterr +/-</td>
<td>C57Bl/10 sterr +/-</td>
<td>P for Strain effects</td>
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<tr>
<td>Femur (mm)</td>
<td>16.06 0.06</td>
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<td>Tibia (mm)</td>
<td>18.15 0.06</td>
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<td>Table 5.</td>
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<tr>
<td>Energy intake (kcal/d)</td>
<td>11.08 0.49</td>
<td>10.74 0.3</td>
<td>NS (P=0.241)</td>
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<tr>
<td>Energy intake (kcal/ d) §</td>
<td>12.8 0.5</td>
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</tr>
<tr>
<td>Energy intake (kcal/d) ¶</td>
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<td>NS</td>
</tr>
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<td>Heat (kcal/d)</td>
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<td>Energy balance (kcal/d) §</td>
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<td>Vertical Activity (cts/d)</td>
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<td>24761 4869</td>
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<td>Total Activity (total cts/d)</td>
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<td>14611 846</td>
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<td>Total Activity – Day (cts/12hr)</td>
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<td>Vertical Activity – Night (cts/12hr)</td>
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<td>Strain/Parameter</td>
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<tr>
<td><strong>Gastrocnemius</strong></td>
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<td>Total protein FSR (% / d)</td>
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<td>Total muscle protein (mg)</td>
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<td>Total muscle protein (mg)*</td>
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Table 7.

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<td>Fat free mass (g)</td>
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<td>Fat mass (g)</td>
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<td>% body fat</td>
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<td>Gastrocnemius (g)</td>
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<td>Quadriceps (g)</td>
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<td>Soleus (g)</td>
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<td>Tibialis anterior (g)</td>
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<td>137</td>
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<td>Diaphragm (g)</td>
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<td>148</td>
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<td>Heart (g)</td>
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<td>Femur (mm)</td>
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<td>Tibia (mm)</td>
<td>117</td>
<td>112</td>
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Table 8.
Blockade of TNF in vivo using cV1q antibody reduces contractile dysfunction of skeletal muscle in response to eccentric exercise in dystrophic mdx and normal mice

A.T. Piers a,b, T. Lavin a, H.G. Radley-Crabb b, A.J. Bakker a, M.D. Grounds b, G.J. Pinniger a,*

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b School of Anatomy and Human Biology, The University of Western Australia, Crawley, WA 6009, Australia

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Abstract

This study evaluated the contribution of the pro-inflammatory cytokine, tumour necrosis factor (TNF) to the severity of exercise-induced muscle damage and subsequent myofibre necrosis in mdx mice. Adult mdx and non-dystrophic C57 mice were treated with the mouse-specific TNF antibody cV1q before undergoing a damaging eccentric contraction protocol performed in vivo on a custom built mouse dynamometer. Muscle damage was quantified by (i) contractile dysfunction (initial torque deficit) immediately after the protocol, (ii) subsequent myofibre necrosis 48 h later. Blockade of TNF using cV1q significantly reduced contractile dysfunction in mdx and C57 mice compared with mice injected with the negative control antibody (cVaM) and un-treated mice. Furthermore, cV1q treatment significantly reduced myofibre necrosis in mdx mice. This in vivo evidence that cV1q reduces the TNF-mediated adverse response to exercise-induced muscle damage supports the use of targeted anti-TNF treatments to reduce the severity of the functional deficit and dystrophy in DMD.

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1. Introduction

Dystrophin is part of the dystrophin–glycoprotein complex that links intra-cellular F-actin to the extra-cellular matrix [1] and provides mechanical protection for the sarcolemma during muscular contraction [2,3]. In patients with Duchenne muscular dystrophy (DMD) and in the mdx mouse and golden retriever (GRMD) dog models of DMD, the absence of dystrophin disrupts normal force transmission and reduces the stability of the sarcolemma [4]. As a result, dystrophic skeletal muscles are more susceptible to exercise-induced muscle damage (EIMD), particularly during lengthening (eccentric) muscle actions [5,6]. The high susceptibility to muscle damage and repeated cycles of myofibre necrosis, especially throughout the growth phase [7], ultimately results in the failure of myofibres to regenerate and the replacement of myofibres with fat or fibrous connective tissue. It is likely that an elevated inflammatory response contributes to the progressive loss of skeletal muscle mass and function seen in the dystrophic condition [8]. While the pro-inflammatory cytokine tumour necrosis factor (TNF) has been associated with chronic muscle wasting conditions such as cachexia [9] and sarcopenia [10], it has also been implicated in the acute inflammatory response that exacerbates muscle damage and contractile dysfunction in mdx mice [11–14].

EIMD is characterized by an initial decrease in the force producing capacity of the muscle (contractile dysfunction)
immediately after the exercise and a subsequent secondary decline in force producing capacity that develops over the following days. Although the initial force deficit has been attributed to the impairment of excitation–contraction coupling [15] and/or disruption of the contractile filaments [16], this muscle damage also coincides with an inflammatory response including the rapid release of TNF from the damaged muscle and resident mast cells [17,18]. TNF attracts neutrophils and further inflammatory cells (that also produce TNF) to the injured site thereby increasing the inflammatory cascade. When myofibre injury occurs, inflammation and associated cytokines are essential for coordinating the removal of damaged tissue and for formation of new skeletal muscle [19]. However, in situ experiments examining EIMD in mice have shown that the accumulation of neutrophils and macrophages after injury contributes to further muscle damage [20,21] and, along with ex vivo studies on cultured myotubes [22], it has been suggested that this effect may be mediated by increased production of reactive oxygen species (ROS). In the mdx mouse, an excessive state of inflammation can exacerbate myofibre necrosis [19,23]. In normal (non-dystrophic) muscle, experimentally elevated TNF (in vivo for 7 days) results in the accumulation of inflammatory cells without necrosis [24]; however, elevated TNF (ex vivo) has rapid adverse effects on contractile function of normal myofibres with early loss of force due (at least in part) to associated oxidative stress [25]. Therefore, the immediate acute increases in TNF may contribute to the initial muscle weakness following EIMD, whereas a prolonged inflammatory response may lead to the myofibre necrosis and progressive muscle wasting that is characteristic of the dystrophic pathology.

Despite some equivocal reports [26], numerous studies indicate that TNF levels are elevated in mdx mice and human DMD patients [27–30]. Elevated local TNF, when combined with the increased susceptibility to EIMD, contributes to myofibre necrosis and, ultimately, the fibrosis inherent in DMD, GRMD and mdx muscle [23]. In vivo experiments on mdx mice have demonstrated that blockade of TNF [11,12,14], or depletion of host neutrophils [13], reduces the severity of the dystrophic pathology. The two drugs used to block TNF activity, Remicade® (antibody to human TNF, also known as Infliximab) and Enbrel® (soluble receptor to TNF, also known as Etanercept), are widely used clinically to treat inflammatory disorders such as arthritis and Crohn’s disease [31]. To increase the efficacy of TNF blockade in mice and to avoid potential problems of immune response to the human constant domain sequences of infliximab, a rat monoclonal antibody specific for mouse TNF [32] was modified to generate a chimerical sequences of infliximab, a rat monoclonal antibody with rat variable and mouse IgG2a, kappa constant domains (cV1q; [11,33]).

This study used a custom built mouse dynamometer [34] to quantify precisely the initial damage of dystrophic muscle and the impact of cV1q mediated TNF blockade on the initial contractile dysfunction and subsequent myofibre necrosis in response to a bout of EIMD performed in situ.

2. Materials and methods

2.1. Animals

All experiments were performed on 10–13 week old adult male dystrophic C57BL/10ScSnmdx/mdx and control C57BL/10ScSn mice, hereafter referred to as mdx and C57, respectively. Adult mdx mice were used in these experiments since the level of myofibre necrosis and regeneration has stabilised to a relatively low level at this age [35,36]. The mice, obtained from a specific pathogen free colony at the Animal Resource Centre, (Murdoch, Western Australia), were housed in cages, supplied with food and water without restriction, and maintained in a 12 h light/dark air-conditioned (20–25 °C) environment. All animal procedures were approved by the Animal Ethics and Experimentation Committee of the University of Western Australia in accordance with the guidelines of the National Health and Medical Research Council of Australia.

2.2. Experimental outline

Experiments were performed using a custom built mouse dynamometer [34] to induce controlled and consistent eccentric EIMD in vivo and to assess the contractile parameters of muscles from mdx and C57 mice. Initial contractile dysfunction was quantified by comparing the isometric torque production before and after the EIMD protocol (initial damage), while histological techniques were used to quantify subsequent (secondary) myofibre necrosis. The effect of cV1q mediated blockade of TNF on initial and secondary muscle damage was compared with un-treated and sham injected (cVaM) control mice.

2.3. Experimental procedures

Mice were anaesthetised by inhalation of a gaseous mixture of isoflurane (isoflurane, 0.4 L/min N 2O, 0.4 L/min O 2) which was maintained for the duration of the experiment (approximately 1 h) via a flow-through facemask over the mouse’s head. Anaesthetised mice were connected to the dynamometer for the measurement of contractile function and to induce EIMD. The dynamometer protocol performed in this experiment is described in detail by Hamer et al. [37] and explained briefly below.

2.4. Dynamometer protocol for eccentric exercise-induced muscle damage

Before performing the EIMD protocol, optimal stimulation parameters were determined based on the torque–volt and torque–frequency relationships for isometric contractions (200 ms train) recorded at a neutral ankle angle (i.e. the foot positioned perpendicular to the tibia). The optimal stimulation voltage from all experiments ranged from 1 to 10 V and the optimal stimulation frequency ranged from 125 to 300 Hz. There were no significant differences in these...
parameters between mouse strain or treatment group, nor did the EIMD protocol significantly affect these parameters. These stimulation parameters were then used when recording the torque–angle relationship and when performing the EIMD protocol consisting of 20 eccentric contractions. After a 10 min recovery period following EIMD, the initial procedures of torque–volt, torque–frequency and torque–angle were repeated for assessment of initial contractile dysfunction. Specific details of these procedures are as follows.

2.5. Torque–angle relationship

A torque–angle relationship for the anterior crural muscles was determined from isometric contractions elicited at 5° increments between 15° dorsiflexion and 55° plantar flexion. The foot was passively rotated to 15° dorsiflexion and a 200 ms train of pulses delivered to the common peroneal nerve to elicit an isometric contraction. Immediately after stimulation had ceased the ankle was rotated 5° towards plantar flexion and rested at this angle for 30 s prior to recording of isometric torque at the new joint angle. The procedure was repeated at 5° increments up to 55° plantar flexion, with the isometric torque being recorded at each position.

Each isometric contraction was analysed for the mean peak torque during the final 80 ms of activation. The peak torque was plotted against joint angle and a Gaussian fit was applied using Curve Expert v1.36. The Gaussian model fitted was of the form:

\[ y = a \exp\left(-\frac{(x-b)^2}{2c^2}\right) \]

The coefficients of this fit are: \( a \) = the amplitude of the Gaussian fitted curve, equivalent to the peak joint torque; \( b \) = the angle at which peak torque was generated and \( c \) is the width at half the peak.

2.6. Exercise-induced muscle damage

After the torque–angle relationship was established, an exercise protocol of 20 eccentric contractions was performed between ankle angles of 15° and 55° plantar flexion with a 30 s rest interval between each trial. The anterior crural muscles were stimulated initially at an ankle angle of 15° plantar flexion to produce an isometric contraction (Fig. 1A). After 100 ms of stimulation the ankle was rapidly rotated to 55° plantar flexion (Fig. 1B) at an angular velocity of 1000 °s\(^{-1}\), thus producing an eccentric contraction in the anterior crural muscles. Immediately following the eccentric contraction, the position of the foot was passively returned to the starting position at 10 °s\(^{-1}\) under servomotor control. After a 10 min rest period, the torque–volt, torque–frequency and torque–angle procedures were repeated to determine the decrease in the peak joint torque resulting from the EIMD protocol (Fig. 2). At completion of the final procedure the incision was sutured and the mouse was allowed to recover. All mice recovered quickly from anaesthesia, were fully mobile within minutes of regaining consciousness and displayed no signs of abnormal limb movement.

2.7. Evans Blue Dye injections

Intra-peritoneal (IP) injections of a 1% sterile solution (w/v) of Evans Blued Dye (EBD) in phosphate–buffered saline (PBS, pH 7.5) at a volume of 1% of body weight were administered 24 h before the EIMD protocol. Prior to injection, the EBD solution was sterilised by passage through a Milli\(^{\circ}\)-GP 0.22 mm filter and stored at 4 °C. After the injection, animals were returned to their cage and allowed food and water ad libitum.

2.8. Histological assessment of myofibre necrosis and sarcolemmal damage

Mice were sacrificed by cervical dislocation while under terminal anaesthesia at 48 h after the EIMD protocol. Body weight was recorded prior to sacrifice. The TA muscles of the test limb (subjected to EIMD) and the non-tested (contralateral) limb were excised, sliced transversely through the mid-belly of the muscle, mounted vertically in

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Fig. 1. Mouse dynamometer apparatus with the ankle of the right hind limb at (A) 15° plantar flexion and (B) 55° plantar flexion during the EIMD protocol. The knee was clamped and the foot attached to a foot plate with the ankle aligned with the axis of the torque transducer. The anterior crural muscles were stimulated to contract via hook electrodes connected to the common peroneal nerve via a small incision in the skin and muscle fascia near the head of the fibular. During the EIMD protocol the anterior crural muscles were stimulated to contract at an initial ankle angle of 15° PF and rapidly rotated at 1000 °s\(^{-1}\) to 55° PF during the contraction.

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tragacanth gum on cork blocks and snap frozen in isopentane, cooled by liquid nitrogen, and stored at −80°C. Alternate frozen sections (8 μm) were cut for EBD and Haemotoxylin and Eosin (H&E) slides on a Leica (CM3050) cryostat at −21°C.

The H&E stained muscle sections were observed with bright-field light microscopy, non-overlapping tiled images of transverse muscle sections were taken at 10× magnification to produce an overall image of the entire muscle cross-section. Images were acquired using a Leica DM RBE microscope, Stage Pro movement software and a Q Imaging Micropublisher 3.3 RTV digital camera. Muscle morphology was analysed from the tiled images using Image Pro Plus 6.2 software. Areas of myofibre necrosis were identified by the presence of fragmented sarcoplasm and the infiltration of inflammatory cells. Myofibre necrosis was calculated as a percentage of the whole muscle cross-sectional area.

The unstained EBD sections were viewed by red auto-fluorescence microscopy (Fluoro filter N.2.1: Green) at 10× magnification and tiled the same as for H&E. Sections with less than 20 EBD positive fibres (less than 1% of total area) were not tiled. EBD positive fibres were measured using Image Pro Plus 6.2 and expressed as a percentage of total muscle cross-sectional area.

2.9. Use of cV1q antibody to block TNF

This study investigated the effectiveness of blocking TNF using the mouse specific anti-TNF antibody cV1q (Centocor USA), which is a rat/mouse chimeric, specific for murine TNF [11]. A negative control solution of cVaM was also administered, which is an isotope matched control antibody for cV1q. These reagents were generously provided to us by Centocor (USA). In the initial experiments (cV1q-1w) both mdx and C57 mice received two intra-peritoneal injections of either cV1q or cVaM made up in PBS at 1 week and 1 h prior to the EIMD at a concentration of 20 μg/g body weight. In additional experiments (cV1q-4 h), mdx mice received a single injection of cV1q (20 μg/g body weight) at 4 h prior to EIMD (dosages based on [11]).

2.10. Data analysis

All data are presented as mean ± SE, unless stated otherwise. Unpaired student t-tests were performed for direct comparison of untreated C57 and mdx mice. Multiple comparisons between all groups and between tested and un-tested (contralateral) limb muscles were made using univariate analysis of variance (ANOVA). A least significant difference (LSD) post-hoc t-test was used to identify differences between groups. Significance was set at P ≤ 0.05 for all groups.

As a quantitative analysis of the benefit of cV1q treatment, a ‘recovery score’ for the correction of the mdx defect was calculated as ([cV1q-untreated mdx]/[C57-untreated mdx]) × 100 where a score of 100% indicates that the parameter in treated mdx mice was equal to that of control C57 mice, and 0% indicates that no gain was

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Fig. 2. The joint torque–angle relationships before (solid symbols) and after (open symbols) the dynamometer EIMD protocol in mdx and C57 mice. Data are presented as the mean (±SE) normalised joint torque at ankle joint angles from −15° (dorsiflexion) to 55° (plantar flexion) for (A) untreated C57 mice, (B) C57 mice treated with cV1q, (C) untreated mdx mice, and (D) mdx mice treated with cV1q. Vertical dashed line indicates the neutral ankle angle (0°, when the foot is perpendicular to the leg).

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obtained compared with untreated mdx mice [38]. Recovery scores were generated for the initial torque deficit and subsequent myofibre necrosis for mdx mice from the cV1q-1w and cV1q-4h protocols.

3. Results

The number of animals in each group, along with mean age and body weight, are presented in Table 1. When the body weights of mice in each treatment were grouped by mouse strain, the mean body weight for mdx mice (29.3 ± 0.53 g) was significantly higher than for C57 mice (24.9 ± 0.38 g, $P<0.05$). Due to the significant difference in body weights between mdx and C57 mice, peak isometric torque was normalised to body weight and presented in units Nmm/kg.

3.1. Muscle contractile properties of untreated mdx and C57 mice

3.1.1. Peak torque production and optimal joint angle

Peak isometric torque normalised to body weight (Fig. 3A) was significantly lower in mdx mice compared to C57 mice ($P=0.029$). The muscles of mdx mice were ~25% weaker than the non-dystrophic C57 mice, which is consistent with the significantly lower specific force in TA recorded by Dellorusso et al. [6], from in situ experiments in mdx and C57 mice. Furthermore, the optimal angle at which the peak torque was recorded was significantly greater for mdx mice compared to C57 mice (Fig. 3B). This indicates a shift in maximal force production to longer muscle lengths which may reflect an increase in series compliance within the muscle [39] due to the underlying dystrophopathy in mdx mice.

3.1.2. Susceptibility to EIMD

The effects of the EIMD protocol on torque–angle relationships for C57 and mdx mice are presented in Fig. 2A and C, respectively. Initial contractile dysfunction was quantified physiologically as the percentage decrease in peak isometric torque after the EIMD protocol (Fig. 3C). Both mdx and C57 mice experienced a marked decrease in mean peak joint torque after the 20 eccentric contractions. However, the deficit in mean peak joint torque resulting from the EIMD protocol was twofold greater in mdx compared to C57 mice ($P=0.02$). The significantly greater torque deficit in mdx compared to C57 mice confirms that dystrophic muscles are more susceptible to EIMD resulting from controlled eccentric contractions, as performed here in vivo with the mouse dynamometer. This is consistent with previous EIMD studies in mdx mice using isolated EDL muscles [40] and also for in vivo dynamometer studies in GRMD dogs [5].

3.1.3. Histological assessment of myofibre necrosis and sarcolemmal damage

Myofibre necrosis was not evident in any C57 muscles, in accordance with previous observations in our laboratory following voluntary wheel running or horizontal treadmill running (Radley-Crabb, unpublished data). Therefore, to obtain a reliable measure of the extent of myofibre necrosis resulting from EIMD in mdx mice, comparisons were made between the TA muscles of the test limb and the un-tested (contralateral) limb. The mean area of necrosis increased about fourfold in tested mdx muscle compared to un-tested (contralateral) muscle ($P=0.002$) (Fig. 3D). Evidence of necrosis in the un-tested control leg gives an
indication of the severity of the background dystrophology in both legs prior to the EIMD protocol, although it is recognised that there can be biological variation even between both legs of an individual mdx mouse [35].

As for the analysis of myofibre necrosis, there was no evidence of EBD positive fibres in any of the tested muscle from C57 mice. For the TA muscles of mdx mice, however, EBD positive fibres accounted for 5.6 ± 1.3% of total muscle cross-sectional area (Fig. 8A). These results indicate that the muscles of the mdx mice are weaker (lower peak torque) and more susceptible to EIMD than non-dystrophic C57 mice. The absence of myofibre necrosis and EBD positively stained fibres in any muscles from the C57 mice reflects the capacity for normal (non-dystrophic) mice to tolerate EIMD.

3.2. The effect of cV1q treatment on mdx and C57 muscles after EIMD

3.2.1. The effect of cV1q on peak torque and optimal joint angle

Peak isometric torque prior to EIMD normalised to body weight for all mdx and C57 groups is presented in Fig. 4A. There was a significant main effect of mouse strain on peak torque ($F_{1,28} = 10.6, P < 0.001$) (mdx versus C57) and post-hoc analysis revealed that the mdx mice were significantly weaker than the non-dystrophic C57 mice ($P < 0.01$). There was no significant main effect of treatment ($F_{2,28} = 8.6, P = 0.08$) on the peak torque. The joint angle at which the peak torque was recorded (optimal angle) for all mdx and C57 groups is presented in Fig. 4B. The optimal angle for untreated mdx and C57 mice that was reported in Fig. 3B is also included here for comparison with the treatment groups. There was no significant main effect of treatment ($F_{2,28} = 1.09, P = 0.35$) on the optimal joint angle.

3.2.2. Effect of cV1q treatment on susceptibility to EIMD

The torque-angle relationships recorded before and after the EIMD protocol in cV1q-treated C57 and mdx mice are presented in Fig. 2B and D, respectively. The initial contractile dysfunction as determined by torque deficit after EIMD is presented in Fig. 5 for all mdx and C57 groups. There was a significant main effect of mouse strain ($F_{1,28} = 43.3, P < 0.001$) (mdx versus C57) on the torque deficit after EIMD. Post-hoc analysis revealed that the C57 mice experienced significantly less torque deficit than the mdx mice ($P < 0.001$). Furthermore, there was a significant main effect of treatment ($F_{2,28} = 8.6, P = 0.002$) with cV1q-1w significantly reducing the torque deficit in both mdx and C57 mice ($P < 0.001$). The acute cV1q-4h treatment, examined in mdx mice, also significantly reduced the torque deficit following EIMD ($P < 0.05$).

3.2.3. Assessment of cV1q treatment on necrosis and sarcolemmal damage after EIMD

Necrosis was quantitated on H&E stained sections (Fig. 6): since necrosis was not observed in any C57 mice, only data from mdx mice are presented (Fig. 7). The % area of necrosis in the TA muscle of tested limbs was compared to the non-tested contralateral limb. Significant main effects of leg ($F_{1,28} = 19.0, P < 0.001$) (whether dynamometer tested or contralateral un-tested limb) and treatment ($F_{2,28} = 4.0, P = 0.029$) were observed on muscle necrosis in mdx mice. Post-hoc analysis revealed that for all treatment groups the level of necrosis was significantly greater in the TA muscle of the tested limb compared to the un-tested limb ($P < 0.05$). Furthermore, exposure to cV1q for 1 week (cV1q-1w) significantly reduced the level of myofibre necrosis in the TA muscles of both the tested limb (Fig. 6) and the un-tested limb, compared to untreated mdx...
mice ($P < 0.05$). However, the level of necrosis in the acute cV1q-4h treated mdx mice was not significantly different from the untreated mdx mice (in either tested or un-tested limbs). These results show that 1 week exposure to cV1q in mdx mice significantly reduced necrosis in both the tested and un-tested (control) limbs compared to untreated mdx mice. The effects of cV1q-1w and cVaM treatments on sarcolemmal damage were evaluated by the analysis of EBD positively stained myofibres (Fig. 8). Neither cV1q-1w nor cVaM treatments had any significant effect on the number of EBD positive fibres in mdx mice ($F_{2,15} = 0.52$, $P = 0.60$). EBD staining was not performed in cV1q-4h treated mice.

![Typical examples of myofibre necrosis in H&E stained transverse sections of TA muscles subjected to the dynamometer EIMD protocol. (A) Untreated mdx muscle showing myofibre necrosis, fragmented sarcomeres and inflammatory cell accumulation. Similar necrosis is observed in (B) cVaM (control IgG) treated mdx muscle. (C) mdx muscle treated with cV1q (anti-TNF IgG) for 1 week prior to EIMD protocol showing areas of centrally nucleated myofibres but far less myofibre necrosis. Scale bars represent 0.1 mm.](image)

![Mean (±SE) area of myofibre necrosis in dynamometer tested and un-tested (control) TA muscles of mdx mice sampled 48 h after the dynamometer EIMD protocol. *significantly different from corresponding test leg; # significantly different from untreated group; $P < 0.05$.](image)

![TA cross sections showing positive staining for Evans Blue Dye (EBD) in myofibres 48 h after the EIMD protocol for (A) untreated mdx, (B) mdx mice treated with cV1q for 1 week, and (C) mdx mice treated with cVaM for 1 week. Scale bars represent 500 μm. (D) Mean (±SE) area of EBD positively stained fibres in TA muscles of mdx mice sampled 48 h after the dynamometer EIMD protocol. Note: There were no (<1%) EBD positive fibres observed in C57 mice.](image)
Calculation of the ‘recovery score’ shows a correction by cV1q-1w treatment of the mdx values (control untreated value as 0%) towards those for normal C57 mice (considered as 100%) of 78% for torque deficit and 56% for myofibre necrosis. The recovery score for the torque deficit in this study is similar to the 81% recovery reported by Gillis [38] for isolated EDL muscles of transgenic mdx mice over-expressing a truncated form of utrophin. The recovery score for mdx mice exposed to a single cV1q-4h injection was 56% for torque deficit but only 5% for necrosis.

4. Discussion

The contractile dysfunction of dystrophic mdx muscles (compared with normal controls) measured by the dynamometer in this study is similar to that for the GRMD dog model of DMD [5] and for transgenic mdx mice that over-express insulin-like growth factor-1 [34]. The main findings of this study support our hypothesis that blockade of TNF (using cV1q antibody) would significantly reduce the extent of contractile dysfunction and (in some cases) subsequent myofibre necrosis in mdx mice following a bout of damaging eccentric exercise. These data further support the use of cV1q administration to protect dystrophic muscles from initial and longer-term damage.

Exposure to cV1q for 1 week before EIMD significantly reduced the initial contractile dysfunction (recovery score = 78%) and the subsequent myofibre necrosis (recovery score = 56%) in response to EIMD. The beneficial effects of cV1q in reducing myofibre necrosis has also been demonstrated in mdx mice exposed to voluntary wheel running [11] with similar effects observed for other anti-TNF treatments (e.g. Remicade and Enbrel [12–14]). Interestingly, TNF blockade was also beneficial and reduced the initial contractile dysfunction in C57 mice, although to a lesser extent than for mdx mice. This observation suggests that, in addition to its contribution to myofibre necrosis, TNF also plays a crucial role in mediating the initial functional weakness following EIMD in both normal and dystrophic muscles: this accords with ex vivo studies on non-dystrophic muscles [25].

It is worth noting that the exposure to cV1q for 1 week reduces not only necrosis after EIMD (in the test leg) but also the background pathology in the mdx mice as indicated by a significant (albeit small) reduction in necrosis in the un-tested limb. To distinguish between possible longer-term beneficial effects of cV1q on the muscle environment compared with more short-term effects of blocking TNF, we conducted experiments with a single cV1q injection administered 4 h before EIMD. We chose a 4 h time-point to ensure that the cV1q had sufficient time to take effect following the IP injection. This short-term exposure to cV1q in mdx mice also reduced the contractile dysfunction following EIMD (recovery score = 56%). However, this protective effect on force production did not extend to myofibre necrosis, which was still elevated in the TA of the tested mdx limb (recovery score = 5%).

Furthermore, the level of necrosis in the un-tested limb was not significantly different from that of untreated mdx mice which would indicate that the background level of pathology was unaffected. Therefore, at least the protective effect of cV1q on contractile dysfunction must be mediated by the short-term blockade of TNF rather than some major adaptation of the mdx environment. This is consistent with the beneficial effects of cV1q in normal C57 mice in reducing the contractile dysfunction after EIMD. Therefore, we propose that elevated levels of TNF in response to EIMD have direct effects on contractile function.

TNF has been widely implicated as a possible mediator of the muscle weakness observed in conditions of chronic and acute inflammation (e.g. muscle damage/trauma, chronic obstructive pulmonary disorder, AIDS, congestive heart failure etc.). Elevated systemic TNF in mice results in loss of body weight (by 3 weeks) and decreased muscle fibre cross-sectional area (by 5 weeks) [9] and, in tissue cultured myotubes, chronic TNF exposure (up to 3 days) results in muscle protein catabolism [41]. Acute exposure of isolated muscle preparations to TNF (for 4 h), however, causes contractile dysfunction and weakness, without evidence of catabolism [25]. Our dynamometer results are in accord with the experiments of Reid et al. [25] who demonstrated that TNF administration to isolated mouse muscles in vitro depressed tetanic force without altering the intracellular Ca2+ concentration ([Ca2+]i) in diaphragm and limb muscles of non-dystrophic mice. Other in vivo experiments by Hardin et al. [42] show that IP administration of TNF acts quickly (within 1 h) via the TNF-1 receptor to increase ROS production and decrease specific force in non-dystrophic mouse diaphragm. These effects were abolished by treatment with the antioxidant Trolox leading to the suggestion that muscle-derived oxidants are essential post-receptor mediators of TNF-induced force loss [42].

Altered cytosolic Ca2+ homeostasis has also been implicated as a putative pathway in development of the dystrophopathy [43]. Elevated [Ca2+]i may arise from transient tears in the fragile sarcolemma of dystrophic myofibres, and/or through mechanosensitive (stretch-activated) calcium channels. The uptake of EBD following the in vivo eccentric exercise protocol was limited to ~5% of myofibres in mdx mice, which is slightly less than that reported by Whitehead et al. (8.6%) from in vitro experiments on EDL muscles from mdx mice [44]. Interestingly, the level of EBD staining was unaffected by cV1q treatment suggesting that the beneficial effects of cV1q reported in this study are not mediated by reducing the sarcolemmal tearing in dystrophic myofibres. This does not preclude the possibility that the contractile dysfunction is mediated by altered calcium homeostasis as numerous recent studies have implicated the contribution of transient receptor potential channels (TRPC) to the dystrophopathy [45,46] and elevated expression of TRPC1 has been reported in mdx muscle [47,48]. Regardless of the source of entry, the influx of Ca2+ into cells has been linked to ROS production and activation of the nuclear factor-kappa B (NF-κB) pathway.

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[43,49] that regulates pro-inflammatory cytokine expression, in particular TNF [43]. Thus, a putative mechanism involved in the exercise-induced contractile dysfunction in mdx mice may involve a TNF mediated positive feedback relationship with NF-κB [30,50] and ROS [51]. The protective effect of TNF blockade using cV1q might be mediated (at least in part) by altering this positive feedback cycle, although further quantitative studies are required to clarify this hypothesis.

5. Summary

We have shown that cV1q administration (1 week before EIMD) protects dystrophic skeletal myofibres against initial contractile dysfunction and subsequent myofibre necrosis in adult mdx mice subjected to a damaging eccentric contraction protocol in vivo using a custom built mouse dynamometer. This technique enables the precise in situ quantitation of the initial physiological damage of muscles and evaluation of the impact of cV1q mediated TNF blockade on both contractile dysfunction and myofibre necrosis. The results from this study indicate that TNF acts quickly (within 30 min) to cause muscle weakness. Short term (4 h) administration of cV1q protects against TNF-mediated contractile dysfunction but not necrosis of mdx muscles, indicating that different mechanisms contribute to these events. The controlled EIMD produced by the dynamometer, with precise timing of damage combined with the ability to repeatedly measure functional parameters in vivo (including force production, muscle fatigue and adaptation [34]) makes this a powerful tool to test the efficacy of various therapeutic interventions and to more precisely define the molecular mechanisms responsible for initial and longer-term damage of dystrophic muscles.

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Three-dimensional optical coherence tomography of whole-muscle autografts as a precursor to morphological assessment of muscular dystrophy in mice

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Abstract. Three-dimensional optical coherence tomography (3D-OCT) is used to evaluate the structure and pathology of regenerating mouse skeletal muscle autografts for the first time. The death of myofibers with associated inflammation and subsequent new muscle formation in this graft model represents key features of necrosis and inflammation in the human disease Duchenne muscular dystrophy. We perform 3D-OCT imaging of excised autografts and compare OCT images with coregistered histology. The OCT images readily distinguish the necrotic and inflammatory tissue of the graft from the intact healthy muscle fibers in the underlying host tissue. These preliminary findings suggest that, with further development, 3D-OCT could be used as a tool for the evaluation of small-animal muscle morphology and pathology, in particular, for analysis of mouse models of muscular dystrophy. © 2008 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.2870170]

Keywords: optical coherence tomography; three-dimensional imaging; muscle; muscular dystrophy; small-animal imaging.

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1 Introduction

Muscle tissue from experimental animal models is routinely studied to understand the cause, disease progression, and efficacy of treatment of human Duchenne muscular dystrophy. This common form of muscular dystrophy, occurring in approximately 1 in 3500 male births worldwide, is characterized by the progressive degeneration and loss of skeletal muscle, leading to death from respiratory or cardiac failure. Myofiber (muscle fiber) necrosis (cell death) normally results in new muscle formation to repair the damaged tissue, but in Duchenne muscular dystrophy repeated cycles of necrosis over time result in failed regeneration with the progressive loss of myofibers and replacement with fibrous and fatty connective tissue.

The mouse whole-muscle autograft provides a large mass of tissue with distinctive histological features of muscle necrosis, inflammation, and regeneration (representing events in human disease): this model is well characterized, highly predictable, and reproducible between animals. The model also provides a convenient in vivo bioassay for assessing pharmacological interventions designed to reduce inflammation associated with necrosis (as also occurs in muscular dystrophy). Such drug interventions have been shown to ameliorate skeletal muscle necrosis and pathology in the X-chromosome-linked muscular dystrophy (mdx) mouse experimental animal model for human Duchenne muscular dystrophy, and in patients with the disease.

Histology is conventionally used to study the morphology of skeletal muscle. Typically (e.g., for whole-muscle grafts), the muscle tissue is removed from the animal, processed, and cut into transverse sections that are stained to identify and measure the amount of healthy and necrotic muscle tissue, the extent of inflammation, and the formation (density and size) of myotubes (multinucleated new muscle fibers) within the regenerating region. However, the full histological process is laborious and time consuming and not amenable to examining large tissue volumes. It requires sacrifice of the animal for tissue harvesting, tissue processing, and preservation; sectioning and staining; and microscopic examination of many images. It would be preferable to be able to perform detailed 3D analysis on large volumes of tissue in situ without the need for muscle biopsy or animal sacrifice, and subsequent sectioning.
staining, and imaging. Furthermore, longitudinal studies require the sacrifice of multiple animals for each time point, preventing the observation of sequential development of the same tissue within an individual animal. In vivo imaging, without the need for sacrifice, would enable repeated observation on an individual animal (optimal experimental scenario) and decrease the number of animals necessary per experiment (this is ethically desirable and has the added advantage of reduced cost). The opportunity to track the same tissue over time would greatly reduce the complication of biological variation between animals (at each time point) that is a considerable problem in conventional sampling. Such an increase in the efficiency and throughput of tissue characterization would signify a major shift in current experimental technique with wide application to animal studies and potential human tissue analysis.

Biomedical imaging modalities that could potentially be used to characterize skeletal muscle pathology, ex vivo and in vivo, include ultrasonography, magnetic resonance imaging (MRI), confocal and multiphoton microscopy, and optical coherence tomography. Ultrasonography has been used clinically to probe the gross structure of human muscle tissue with large penetration depth, but is unable to resolve individual myofibers with an average diameter of 30 to 50 μm. MRI has been used for in vivo studies of muscle in whole live animals, but requires an exogenous contrast agent for localized detection of muscle pathology, and its resolution of 125 μm is insufficient to image the individual myofibers. Confocal and multiphoton microscopy, with their superior resolution, can resolve individual myofibers in vivo but are limited in penetration depths to a few hundred micrometers, and routinely depend on the detection of fluorescent signals from exogenous contrast agents.

Optical coherence tomography (OCT) is an emerging imaging modality capable of real-time, noninvasive, cross-sectional imaging of thick biological tissue approaching micrometer resolution. OCT uses near-IR light to perform optical ranging analogous to ultrasound B-mode imaging, but with resolution of two orders of magnitude greater. Typically, cross-sectional images are generated, which represent the spatially localized intensity of backscattered light with a penetration depth of up to 2 to 3 mm in tissue. A third scan dimension can be added to enable 3D imaging of samples.

OCT has been used to investigate a wide range of biological tissues and structures, but its use in the study of muscle tissue has been limited. Recently, Pasquesi et al. reported the first study directed specifically toward skeletal muscle tissue and muscular dystrophy. This study used polarization-sensitive OCT to measure changes in the birefringence of skeletal muscle of the mdx mouse model for Duchenne muscular dystrophy, and correlated these changes with myofiber damage due to dystrophy. Other previous studies with OCT have involved muscle tissue only incidentally; e.g., measuring the refractive index of cardiac muscle in humans, and determining the birefringence of animal skeletal and cardiac muscle using polarization-sensitive OCT.

The purpose of this study is to determine if 3D-OCT is capable of imaging large volumes of ex vivo unstained mouse skeletal muscle in sufficient detail to be able to differentiate healthy and pathological tissue. Such capability is a precursor to eventual noninvasive in vivo assessment of muscular dystrophy in mouse models. We compare, for the first time, 3D-OCT images of whole-muscle autografts with conventional histology, to identify damaged and regenerating muscle adjacent to healthy undamaged myofibers. In the remainder of this paper, we describe the imaging method, the anatomy of the whole-muscle autograft, and the surgical procedure. We present coregistered OCT images and conventional histology of the autografted muscle tissue, as well as 3D-OCT volume reconstructions, and discuss the results and prospects for in vivo imaging.

2 Methods
2.1 OCT

We performed 3D-OCT imaging using a 3D-scanning optical fiber interferometer, as shown in Fig. 1(a). The input light is split by a 50/50 fiber optic coupler to reference and sample arms, and a circulator is used to enable dual-balanced detection to reduce optical intensity noise. The broadband light source is based on a semiconductor optical amplifier (JDS Uniphase, Milpitas, California) with a center wavelength of 1330 nm and a full-width-at-half-maximum bandwidth of 45 nm.

Measurement of the sample in the axial direction (along the beam) was performed by scanning the path length of the reference arm, using a frequency-domain optical delay line, with a measured resolution in air of 19.4 μm. Transverse scanning in a single dimension was performed by a galvanometer mirror and objective lens setup with a beam numerical aperture of 0.07, measured in-focus transverse resolution (1/e² intensity beam diameter) of ~11 μm and calculated axial Rayleigh range of ~72 μm. The dual-balanced photodetected difference signal was bandpass filtered and logarithmically demodulated before analog-to-digital conversion (PCI-6111E, National Instruments, Austin, Texas). The OCT system had a measured detection sensitivity of ~95 dB with 2 mW incident on the sample.
placed in phosphate-buffered saline (PBS) and allowed to recover. Five days after the surgery, the mice were anaesthetized and sacrificed via cervical dislocation, and the entire TA muscle with attached EDL muscle was excised and stained with hematoxylin and eosin (H&E) for light microscopy (DM RBE, Leica Microsystems GmbH, Wetzlar, Germany). The mice were housed and treated according to the guidelines of the Animal Resources Centre, Murdoch, Western Australia. The mice were housed and treated according to the Western Australian Prevention of Cruelties to Animals Act 1920 and the Australian National Health and Medical Research Council guidelines, under protocols approved by the University of Western Australia Animal Ethics Committee.

Two-dimensional (2D) OCT images (512 × 512 pixels) were acquired in 1 s, and the third dimension was obtained by sequential cross-sectional (y-z) scans at 10-μm intervals along the x axis obtained by linear translation of the objective lens. The 3D data sets consisting of ~400 images were acquired in 5 min and measured 4 × 1.8 × 1 mm (x × y × z). En face (x-y) OCT images at various sample depths were reconstructed from the 3D data set.

2.2 Animal Model and Whole-Muscle Autograft Surgery

Three female C57BL/10ScSn 6-week-old mice were obtained from the Animal Resources Centre, Murdoch, Western Australia. The mice were housed and treated according to the Western Australian Prevention of Cruelties to Animals Act (1920) and the Australian National Health and Medical Research Council guidelines, under protocols approved by the University of Western Australia Animal Ethics Committee.

For whole-muscle autograft surgery, the mice were anaesthetized with 1.5% (v/v) Rodia halothane (Merial, Parramatta, Australia), N₂O, and O₂ and transplantation of the whole intact extensor digitorum longus (EDL) muscle was performed on one or both legs, described in detail elsewhere. Briefly, the EDL muscle with both tendons attached was removed from the anatomical bed of the hindlimb of the mouse and transplanted onto the anterior surface of the tibialis anterior (TA) muscle of the same leg. The tendons of the EDL were sutured onto the host TA muscle, the skin closed, and the mice allowed to recover. Five days after the surgery, the mice were anaesthetized and sacrificed via cervical dislocation, and the entire TA muscle with attached EDL muscle was excised and placed in phosphate-buffered saline (PBS) on ice for transportation for ex vivo 3D-OCT imaging.

2.3 OCT Imaging Protocol

All muscle samples were imaged within 2 h of animal sacrifice. A number of imaging geometries were investigated on the first two samples, and the optimum protocol, used on subsequent samples, is described in the following. The sample was bisected along the midregion to expose the transverse face of both the host TA and graft EDL, and then placed on an imaging window and bathed in PBS to prevent drying, and to reduce the refractive-index mismatch by displacing any air gaps between the glass plate and sample. The muscle sample was oriented with its myofibers parallel to the optical axis and its transverse face parallel to the en face imaging plane, as shown in Fig. 1(b), and full-volume 3D-OCT scans were obtained. The image axial depth was scaled by 1.4, the approximate refractive index of muscle tissue. 30 This sample alignment was preferred as it enabled the gross muscle tissue morphology and individual myofibers of the transverse plane of the EDL and TA to be displayed over the large area (4 × 1.8 mm²) of the en face OCT image plane, for comparison with conventional transverse histology.

2.4 Histological Processing and Image Coregistration

Immediately following OCT imaging, samples were fixed in 4% (w/v) paraformaldehyde (pH 7.6) for 30 min, transferred to 70% (v/v) ethanol followed by automated standard paraffin preparation (Shandon Citadel 1000, Thermo Fisher Scientific, Inc., Waltham, Massachusetts). Transverse 5-μm-thick sections were cut on a microtome and collected on glass slides, and stained with hematoxylin and eosin (H&E) for light microscopy (DM RBE, Leica Microsystems GmbH, Wetzlar, Germany). Histological sections were imaged with a DM RE microscope equipped with an epifluorescence module (Leica Microsystems GmbH, Wetzlar, Germany).

Fig. 2 (a) En face OCT image and (b) corresponding histology of a transverse section of the autografted muscle sample showing the grafted EDL overlying the host TA muscle, separated by a dashed line added for clarity. A 3× magnified view (right) shows the graft boundary, with healthy myofibers (H); surviving myofibers (S); and inflammatory cells (I).
graft boundary

agocytosed, although some myofibers survive at the periphery

the peripheral zone of inflammation, the necrotic tissue is ph-

the revascularization of the graft in part from the host TA. In

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Fig. 3 (a), (c), and (e) En face OCT images, and (b), (d), and (f) the corresponding histology of transverse sections of the autografted muscle sample at depths of (a) and (b) 100, (c) and (d) 250, and (e) and (f) 500 μm. Surviving myofibers (S). The 2× magnified view (insets) show the graft boundary.

Germany) and digital image capture (DXM 1200F, Nikon Corporation, Ontario, Canada). Nonoverlapping images of the grafts were obtained with an automated microscope translation stage, and images were tiled and stitched together using commercial software (ImagePro Plus 4.5.1, Media Cybernetics Inc., Silver Spring, Maryland) on a personal computer. Coregistration of OCT and light microscopy images was performed by matching for depth and visually correlating structural features of interest.

3 Results

All en face images taken from 3D-OCT data sets obtained using the protocol described in Sec. 2.3 were found to be consistent in appearance. The typical results presented in this section were taken from one mouse. Figure 2 shows a representative en face OCT image and the corresponding H&E-stained histological image of the transverse face of the EDL and TA tissues through the midregion of the muscle sample at a depth of 100 μm. A 3× magnified image (right) shows the graft boundary (dashed line) and nearby healthy myofibers (H) of the healthy host TA tissue. A mean filter (1×3 pixels) was applied to this image to reduce speckle noise. The kernel size was chosen to roughly match the OCT in-focus transverse resolution. As a result of surgery, the blood and nerve supply to the EDL muscle is severed, causing the grafted tissue to undergo necrosis, subsequent inflammation, and regeneration. The histology in Fig. 2(b) shows inflammatory cells (I) infiltrating the grafted EDL; this is associated with the revascularization of the graft in part from the host TA. In the peripheral zone of inflammation, the necrotic tissue is phagocytosed, although some myofibers survive at the periphery (S), and myoblasts (precursor muscle cells) are activated and fuse to form myotubes. The centrally located area of persisting necrotic tissue (N) remaining within the EDL is termed the necrotic core, and constitutes approximately 50% of the entire area of the graft in these samples (at 5 days).

In the OCT image in Fig. 2(a), the grafted EDL tissue is readily distinguished from the host TA tissue, meeting at a clearly defined boundary (dashed line). The TA tissue displays a regular pattern of circular features, in marked contrast to the EDL graft, which lacks any such prominent features, instead exhibiting a more uniform texture corrupted by the speckle noise typical of OCT images. The 3× magnified image (right) highlights the distinction between the two muscle types, and the circular features of the OCT image in Fig. 2(a, right) are of a size and shape that correlate well with the healthy myofibers (H) of the corresponding histology [Fig. 2(b, right)]. In the entire 3D-OCT data set, we observed the grafted EDL tissue to be clearly distinguished from the host TA tissue with a distinct boundary between the two.

Three representative en face OCT images at different depths within the whole-muscle autograft are presented in Fig. 3, along with coregistered histology. At all depths, the OCT images [Figs. 3(a), 3(c), and 3(e)] correspond well in overall structure with the histology [Figs. 3(b), 3(d), and 3(f)]. The pattern of circular structures in the TA tissue persists throughout the 3D-OCT data set, with a shape and size similar to that expected for healthy myofibers. In the grafted EDL tissue, however, a similar pattern is lacking throughout. The tissue generally appears more uniform in texture, although there are sparsely distributed small regions of high intensity, which resemble the circular features found in the TA tissue.
The contrast between the two muscle tissue types is more apparent, particularly at the graft boundary (see Fig. 3 insets), as the image depth increases. Another difference between the grafted EDL and host TA tissue is that the average signal intensity, particularly at the graft boundary as seen in Fig. 3(a), (c), and (d) (insets), is attenuated to a greater extent in the grafted EDL tissue than in the neighboring region of TA tissue at the same depth. In the histological sections, the necrotic core within the EDL graft is obvious in all sections, and constitutes approximately 50% of the entire area of the graft. However, this feature is not evident in the corresponding OCT images. The average myofiber diameter of this region is smaller than those of the host TA muscle.

The 3D-OCT cutaway reconstructions of the autografted muscle sample reveal the transverse face of the graft EDL tissue and host TA tissue at three depths into the sample in Fig. 4. The reconstructed *en face* plane of the 3D-OCT data set is by far the most informative section for revealing the muscle morphology. The *x-z* and *y-z* image views in Fig. 4 show far fewer readily identifiable features.

4 Discussion

This preliminary investigation has demonstrated that 3D-OCT is a promising tool for examining the morphology of muscle pathology and identifying individual muscle fibers. Previous studies involving muscle tissue using conventional B-scan oriented OCT have presented images that do not show individual muscle fibers or reveal significant morphological detail of the muscle tissue. A possible exception is the study by Hsiung et al. of the hamster cheek pouch at ultra-high resolution (1.5 μm at 800 nm), wherein, within the superficial (250 μm) and deep muscle layers (750 μm), structures resembling muscle fibers are observed, although the origin of these structures was not discussed. Similarly, Guo et al. presented a B-scan OCT image of rabbit muscle in which structure is evident, but the nature of this structure was not discussed, nor was there comparison with histology.

In working with excised muscle, we were able to optimally orientate the sample to match the largest scan area (*en face*) to the transverse face of the whole-muscle autograft, which is the orientation of most interest to experimental biologists and conventionally examined in histological sections. We have found the availability of 3D data indispensable in identifying gross and fine features in the autografted muscle sample. The availability of alternative 2D sectional views provided by the 3D data set, as well as the ability to visualize the muscle sample in cut-away 3D movies (not presented here), proved invaluable in orientating the samples and presenting the *en face* images shown here. By contrast, our early work (not reported) recording only 2D sections was less successful. *En face* OCT images at all depths were found to be significant for visualizing the transverse-face whole-muscle autograft structure and features of interest; in contrast, images from the *x-z* or *y-z* planes were less informative.

The *en face* OCT images generated from the 3D-OCT data set reveal a distinct difference between the muscle structure of the grafted EDL and the host TA, which correlates well with the corresponding histology. We observed that the muscle tissue features were smaller in the histology than in the OCT images. This shrinkage is due to dehydration of the tissue during the histological process, as previously reported. The most noticeable feature of the OCT images, confirmed by histology, is the observation of healthy myofibers (H) in the TA muscle, and their general absence in the graft EDL [Figs. 2(a), 3(a), 3(c), and 3(e)] with a few exceptions. Surviving myofibers (S) were identified in OCT images of the EDL [Figs. 2(a), 3(a), and 3(c)] (confirmed in the corresponding histological sections) by their distinctive high-intensity circular shape comparable with the appearance of the healthy myofibers of the TA, which differentiated them from the generally featureless and lower intensity surrounding regions containing necrotic tissue and inflammatory cells. The 3D-OCT volume enhanced identification of surviving myofibers, as they could be tracked in depth and seen in successive *en face* OCT images reconstructed along the *z* axis [Figs. 3(a) and 3(b)].

Fig. 4 Three 3D-OCT reconstructions of the autografted muscle sample, with cut-away sections revealing the transverse face at depths of (a) 100, (b) 250, and (c) 500 μm into the tissue. A dashed line added for clarity separates the EDL and TA tissues.
By 5 days after transplantation, revascularization of the EDL muscle and infiltration of inflammatory cells into the grafted EDL tissue is well advanced. The inflammatory tissue in the en face OCT images of the EDL has an appearance similar to those features observed in the study by Cobb et al. of wound healing in mouse skin, particularly the granulation tissue in the dermis. The presence of the inflammatory cells in the EDL with the breakdown of necrotic muscle tissue significantly increases the density of cell nuclei is this region, staining dark blue in the histology of Fig. 2(b). These nuclei (~1 to 2 µm in diameter) are around an order of magnitude smaller than the resolution of the OCT system and therefore cannot be visualized. This is a plausible explanation for the more uniform appearance of the EDL muscle graft, which is especially obvious at the boundary of the two tissues, as seen in Fig. 3 (insets). The presence of inflammatory cells and subsequent increase in the density of nuclei in the EDL tissue, particularly at the graft boundary, should also affect the attenuation of the OCT signal with depth (A-scan profile). The expected relatively stronger scattering in the EDL is confirmed in Fig. 3 (insets), which shows that the average signal strength in the EDL tissue is lower than in the neighboring TA tissue, increasingly so at greater depth [Fig. 3(c)].

While the morphology evident in the en face OCT images presented here correlates well with the corresponding histology, several issues must be resolved for the technique to be a useful replacement for conventional histology. New regenerating myofibers, which are of particular interest in the study of inflammation and regeneration in muscle tissue, could not be readily identified in the OCT images. Typically, activated myoblasts have fused to form myotubes and new myofibers are visible at the edge of the graft by day 5, becoming evenly distributed throughout the EDL around days 7 to 8. Discrimination in histology relies on resolving the nucleus and its position with respect to the myofiber, as myotubes and regenerated myofibers have centrally located nuclei, while original myofibers have peripherally located nuclei. Such resolution is beyond the ability of the OCT setup used in this experiment but may be attainable with ultrahigh-resolution OCT (Ref. 32) and by utilizing recently published resolution enhancement methods. The distinction between myofiber types may also be possible through examination of the variation in the A-scans, but more work is required to establish this. En face OCT images could not be used to readily identify the necrotic core within the grafted EDL tissue and, therefore, could not be used to assess the percentage of this necrotic tissue relative to the total graft area, an important measure that is used to assess the effectiveness of drugs aimed at reducing inflammation.

Future in vivo 3D-OCT imaging of mouse skeletal muscle tissue must contend with the anatomical orientation of muscle tissue and the presence of overlying skin tissue. Beyond improvements to the resolution afforded by ultrahigh resolution and high speed, there are other potential strategies to facilitate in vivo imaging. Optical clearing agents could potentially be used to increase the imaging depth of OCT through the skin and cutis to the underlying muscle tissue of interest. Less desirably, direct access to muscle could be made via surgical incision or needle incision.

5 Conclusion

The findings here demonstrate the promise of 3D-OCT as a tool for the study of the morphology of mouse skeletal muscle. In the whole-muscle autograft model of damage and inflammation, OCT clearly shows the boundary between the tissues of the grafted and host muscles. Further improvements to enable the distinction between muscle fiber types (original undamaged versus regenerated) and the detection of the necrotic core, however, are required. Our preliminary results suggest that OCT may have a future role in the morphological assessment of the pathology of mouse skeletal muscle with applications in the study of muscular dystrophy.

Acknowledgments

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References


Appendix 1 – Additional dietary intervention studies.

In addition to the work presented in Chapter 6, dietary interventions involving female mice, mdx litters, and an Omega-3 enriched diet were also conducted. Due to fatal flaws in experimental design and diet manufacture each of these studies was terminated and data were not analysed in full. Each experiment is discussed briefly below.

Custom diets: All custom diets were manufactured by Mr Warren Potts at Specialty Feeds Glen Forrest WA.

A) Standard meat free rat and mouse chow – cereal based – 19% protein, 5% fat.
B) AIN93g – semi pure control – 19% protein, 7% fat.
C) High fat – semi pure – 19% protein, 16% fat.
D) High protein – semi pure – 50% protein, 7% fat.
E) Low protein – semi pure – 8% protein, 7% fat.
F) Omega 3 enriched – semi pure – increase Omega -3 content of AIN93G diet up to 7% (replace canola oil with fish oil).

1) Dietary interventions in female mice:
Dietary intervention studies were initially conducted on female control C57 and dystrophic mdx mice. Customised diets were fed to female mice from 8-24 weeks of age: before starting the custom diet at 8 weeks of age all mice were fed a standard mouse chow diet. There was no significant change in body weight of C57 or mdx female mice after consumption of the 4 custom diets for 16 weeks. At the time of sacrifice (24 weeks of age) there were no significant differences in the absolute body weight (Figure A1) or retroperitoneal fat pad weight between any groups of mice (Figure A2), however dystrophic mdx mice were significantly heavier than control C57 mice (Figure A1). Similar parallel studies that were being conducted on male mice showed numerous significant differences in body weight and body composition, and were therefore pursued in detail (Chapter 6). Due to time constraints detailed analysis of dietary interventions was only conducted on male mice and the female dietary intervention studies were not continued (e.g. extent of dystropathology was not analysed), nor was the difference in gender response examined in detail. The major differences in response to dietary interventions may be due to sex or less likely, to differences in the batches of custom diets used for the 2 studies.
2) Dietary interventions in mdx litters:
Dietary interventions were conducted in young mdx mice in attempt to reduce the acute onset of dystropathology around 3-4 weeks of age. Dietary interventions were administered in two different ways A) in utero – postnatal day 28, B) postnatal day 15 – 28.

In utero dietary interventions consisted of feeding a custom diet to a female mdx mouse 2 weeks before mating/conception. The major problem with this experiment was that litter size was not standardised. Dystrophic mdx mice have a high neo-natal death rate and litters are quite variable in size. When studying growth and development of neo-natal mice it is important to standardise litter size as all neo-natal mice depend on milk provided by the mother for survival and growth (e.g. smaller litter size = larger pups). The number of mice used in this study (based on 2 litters per sample group) is summarised in Table 1. Another major problem with this early study was that all work was conducted using dystrophic mdx mice as mothers: as our knowledge of the metabolic abnormalities in dystrophic mdx mice increased (Chapter 6 & 7) it became apparent that this work should have been conducted using neo-natal mdx mice cross-fostered onto non-dystrophic mothers at birth. This would have eliminated any complications resulting from dystrophic mdx mice being energy deficient themselves and thus having limited milk producing capacity.
<table>
<thead>
<tr>
<th>Group / Diet</th>
<th>High fat</th>
<th>High protein</th>
<th>Omega-3</th>
<th>AIN93g</th>
<th>Chow</th>
</tr>
</thead>
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<td>N=6 (3, 3)</td>
<td>N=9 (4, 5)</td>
<td>N=13 (7, 6)</td>
<td>N=9 (5, 4)</td>
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<td>d15 – d28</td>
<td>N=11 (5, 6)</td>
<td>N=8 (6, 2)</td>
<td>N=8 (5, 3)</td>
<td>N=11 (5, 6)</td>
<td>N=8 (4, 4)</td>
</tr>
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Table 1. Sample group size for dietary interventions in young mdx mice (size of individual litters is shown in brackets).

There were no significant differences in body weight after dietary interventions in 28 day old mdx mice (Figure A3). This result is heavily influenced by litter size and different results may have been seen if litters were standardised to a specific number.

The extent of dystrophathy was assessed histologically on H&E stained muscle sections. There were no significant differences in myofibre necrosis (Figure A4 - A) or myofibre regeneration (Figure A4 – B) in the quadriceps muscle of 28 day old mdx mice after dietary interventions from 15 – 28 days of age. Similar results were seen in the TA muscle and after consuming the dietary interventions from In Utero – d28. In summary this was a poorly designed experiment and would need to be completely re-designed in order to achieve sound scientific results.
Figure A4). Myofibre necrosis and regeneration (quadriceps) in 28 day old mdx mice, after consumption of a dietary intervention from 15 – 28 days of age. A) Necrosis, B) Regeneration. For N, see table 1. Bars represent s.e.m.

1) **Omega-3 enriched diet**: An omega-3 enriched diet was fed to dystrophic mdx mice in attempt to reduce inflammation and thus reduce the extent of dystropathology. The omega-3 enriched diet was fed to male mdx mice (8-12 weeks +/− voluntary exercise, 8-24 weeks and 24-40 weeks). Half way through conducting this study on sedentary adult mice we were notified by the manufacturer that there was a major problem with the diet composition (failure to add vitamin B), which would result in significant weight loss in the mice, and that all experiments should cease. This resulted in the loss of 12 weeks of experimental work.

This study was repeated and upon sampling mice that had consumed the omega-3 enriched diet for 16 weeks a large number of mice showed significant weight loss (or failure to gain weight) and had inflamed and gaseous stomach/bowels. No detailed analysis of dystropathology was conducted on these mice. Attempts to again repeat this study encountered major problems with finding a reliable and consistent source of omega-3 rich fish oil, the oxidation of omega-3 fatty acids in the manufacturing process, and further oxidation of omega-3 fatty acids in the autoclaving process. Thus this dietary intervention was subsequently abandoned at the time.

**Additional points of interest**: There are a few additional points of interest to come out of these failed dietary intervention studies.
1) The neo-natal death rate of dystrophic mdx mice is very high (approximately 35%). If mdx mothers and their new pups are left untouched (e.g. cage unchanged and pups not handled) for at least 10 days after birth, the neo-natal death rate is significantly reduced to approximately 10%. Based on observations of approximately 50 pups.

2) The litter size of mdx pups is increased on the AIN93G semi-pure diet (19% protein, 7% fat) in comparison to mice fed the standard (cereal based) rat and mouse chow (19% protein, 5% fat). The average litter size for AIN93G diet is 6.7 pups (n=4 litters) and the average litter size for standard chow diet is 4.1 (n=4 litters).

3) Both female and male dystrophic mdx mice are significantly heavier than control C57Bl/10 mice at 8 weeks of age (Figure A5) (that is mdx mice undergo significant muscle hypertrophy before 8 weeks of age).

Figure A5. Absolute body weight (g) of 8 week old mdx and C57Bl/10 mice. A) Female mice, B) Male mice. Bars represent s.e.m. * indicates significant difference between strains (P<0.05). N indicated on each graph.