Muscle damage and adaptation in response to plyometric jumping

by
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ABSTRACT

The aim of the study was to investigate skeletal muscle changes induced by an acute bout of plyometric exercise before and after plyometric training. The study consisted of an acute study and training intervention study. The acute study, investigated whether direct evidence of ultrastructural damage and identification of indirect factors were more evident in subjects presenting with rhabdomyolysis. Moreover the training intervention study investigated whether plyometric training would protect the muscle from ultrastructural damage and rhabdomyolysis.

During the acute intervention, twenty six healthy untrained individuals completed an acute bout of plyometric exercise (10 x 10 squat-jumps, 1 min rest). After, thirteen subjects continued with the training intervention. Eight of these subjects completed 8 weeks of plyometric jump training, while five subjects were instructed to rest from physical activity for 8 weeks. Seven days after the final training session the training and rest group repeated a second acute bout of plyometric exercise.

Acute Study: Creatine kinase (CK) activity increased significantly following the single bout of plyometric exercise in all subjects (baseline: 129 to day 4: 5348 U/l). This was accompanied by an increase in perceived pain, C-reactive protein (CRP) a marker of inflammation as well as white blood cells (WBCs). Electron micrographs of muscle biopsies taken 3 days post exercise showed evidence of ultrasructural damage and membrane damage was apparent by immunofluorescence by the loss of dystrophin staining. A stretch of the c-terminus of titin was observed by immunogold, and western blot analysis indicated an increase in calpain-3 autolysis. Based on individual CK responses (CK range: 153-71,024 U/L at 4days after exercise) the twenty six subjects were divided into two groups, namely the high (n=10) and low responders (n=16).

Training intervention: Following training the trained group did not experience: a rise of CK activity (110.0 U/l), perceived pain, CRP, WBCs, Z-line streaming, a stretch of titin or calpain-3 activation; while in the control group only two subjects presented with Z-line streaming.

The results indicate that high responders have a more pronounced inflammatory response compared to low responders after eccentric exercise, therefore more WBCs and more specifically neutrophils are recruited to damaged areas resulting in greater membrane damage by respiratory burst in high responders. This damage can be limited with training by remodelling sarcomeric proteins via calpain activation resulting in the stable assembly of proteins in the sarcomere preventing the release of proteins.

UITTREKSEL

Die doel van die studie was om skeletspier veranderinge wat teweeggebring is deur voor en na afloop van akute pleometriese oefening, te ondersoek. Die studie bestaan uit ‘n akute intervensie en ‘n oefeningsintervensie gedeelte. Die akute intervensie het ondersoek ingestel na die direkte bewyse van ultrastrukturele skade en identifikasie van indirekte faktore meer sigbaar is in proefpersone wat met rhabdomiolose presenteer. Meerso het die oefningsintervensie die moontlikheid dat pleometriese oefening die spier van ultrastrukturele skade en rhabdomiolose beskerm, ondersoek.

Tydens die akute intervensie is 26 gesonde ongeoefende individue die akute pleometriese oefeningsessie (10 x 10 hurkspronge, 1 min rus) voltooi. Hierna het 13 proefpersone voortgegaan met die oefeningsintervensie. Agt van hierdie proefpersone het agt weke pleometriese sprongsessie oefeninge voltooi, terwyl vyf proefpersone gevra is om vir 8 weke geen oefeninge te doen nie. Sewe dae na afloop van die finale oefeningssessie het die oefening en kontrole groep in ‘n tweede herhaalde akute pleometriese oefeningsessie deelgeneem.

Akute intervensie: kreatienkinase (KK) aktiwiteit het betekenisvol verhoog na die enkel pleometriese oefeningsessie in all proefpersone (basislyn: 129 tot op dag vier: 5348 U/l). Hierdie is vergesel met ‘n toename in die persepsie van pyn, c-reaktiewe proteïen (CRP) ‘n merker van inflammasie sowel as witbloedselle (WBS). Elektronmikrograwe van spierbiopsies wat geneem is drie dae na afloop van die oefeninge, het tekens van ultrastrukturele skade en membraanskade getoon wat ook deur immunofluoresensie duidelik warneembaar was deur die verlies van distrofienverkleuring. ‘n Verrekking van die c-terminus van titin is ook waargeneem deur middel van immunogold. Westernblot analyse het ‘n toename in calpain-3 outolise getoon. Gegrond op individuele KK

response (KK grense: 153-71,024 U/L na vier dae post oefening) is 26 proefpersone verdeel in twee groepe naamlik ‘n hoë (n=10) en lae responders (n=16).

Oefeningintervensie:: Na oefening het die geoefende groep nie ‘n toename in KK aktiwiteit getoon nie (KK aktiwiteit (110.0 U/l)), pynervaring, CRP, WBS, Z-lynstroming, ‘n strekking van titin of calpain-3 aktivering; terwyl in die kontrole groep daar slegs twee proefpersone met Z-lynstroming geïdentifiseer is.

Die resultate wyse daarop dat hoë responders ‘n meer uitgesproke inflammatoriese reaksie toon vergeleke met die lae responders na afloop van essentriese oefening. Daar word dus meer WBS en spesifiek meer neutrofiele na beskadigde areas gelokaliseer wat in grootter membraanskade deur respiratoriese inspanning in die hoë responders. Hierdie skade kan beperk word deur oefening waardeur hermodulering van sarkomeriese proteïene via calpain aktivering tot stabiele rangskiking van proteïene in die sarcomere lei en daardeur proteïen vrystelling verhinder.

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ABBREVIATIONS

CK	creatine kinase
CSA	cross-sectional area
VO2 max	maximal oxygen consumption
SSC	stretch-shortening cycle
DOMS	delayed-onset muscle soreness
Mb	myoglobin
LDH	lactate dehydrogenase
CRP	C-reactive protein
LGMD2A	limb-girdle muscular dystrophy type 2A
MHC	myosin heavy chain
SNPs	single nucleotide polymorphisms
MYLK	myosin light chain kinase gene
MLCK	myosin light chain kinase
ACTN3	α-actinin 3 gene
IGF2	insulin-like growth factor II gene
ACE	angiotensin-converting enzyme gene
IL6 (-174)	interleukin-6 gene
IL-6	interleukin-6
TNFA (-308)	tumor necrosis factor-alpha gene
RLC	regulatory light chain
ACTN3	α-actinin 3 gene
CK-MM	muscle specific CK
β-MEtOH	beta-mercaptoethanol
SDS	sodium dodecyl sulfate
TRIS	tris(hydroxymethyl)aminomethane

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PUBLICATIONS AND CONFERENCE PROCEEDINGS

Peer-reviewed Paper

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CHAPTER 1: Literature Review

1.1 Introduction

Many human exercise models have been developed to describe and study skeletal muscle damage and remodeling; the models used most are eccentric exercise interventions or post-competition monitoring. Studies on eccentric exercise models are more commonly found in the literature, but a limitation with these models is that they are highly standardized and do not replicate normal daily life movement; whereas with competitive event studies participants are followed during and after sport competition making this type of model more difficult to control. Therefore a plyometric jump model was adopted in this study, which incorporates eccentric contraction of the lower muscle groups.

Structural muscle damage that results from eccentric exercise occurs when the sarcomere maximally contracts to resist an applied external load and once the external load is too high, a 'damage threshold' will be reached resulting in sarcomere disruption. A muscle proteinase, calpain-3, is specifically activated following eccentric exercise and is responsible for initiating muscle breakdown (Cagan et al. 1991; Huang and Forsberg 1998); however controversy in the literature exists as to how this protein is regulated. Researchers are divided on whether calpain-3 is activated by mechanical stretch of titin or by small increases in resting cytoplasmic Ca^{2+} concentration (Murphy et al. 2007; Murphy and Lamb 2009).

It has previously been determined using morphological methods that eccentric muscle damage results in preferential type II fibre disruption in human subjects (Friden et al. 1988; Friden et al. 1983). Currently the literature suggests that there is preferential type II muscle fibre damage in response to plyometric exercise and this is based on indirect methods by

using changes in peak power output at different velocities and fatigue (Twist and Eston 2005).

Certain markers of muscle damage can be detected in blood samples since these proteins are released into circulation following sarcolemmal disruption (Feasson et al. 2002). Creatine kinase (CK) is the most used indirect marker of muscle damage and is used as a diagnostic tool in the detection of a condition known as rhabdomyolysis (Chimera et al. 2004). Exertional rhabdomyolysis is clinically diagnosed when increases in CK activity exceed 1,000 U/L (Thoenes 2010). A biological phenomenon that remains unexplained (Sayers and Clarkson 2002), is the way in which CK release in response to unaccustomed exercise varies amongst individuals with similar characteristics (Clarkson et al. 2005b); some may experience a greater increase in CK activity (>60,000 U/L) than others. Several single-nucleotide polymorphisms have been identified and been implicated with this varied response (Clarkson et al. 2005b; Devaney et al. 2007; Yamin et al. 2007; Yamin et al. 2008); however this does not explain why high responders experience a blunted CK response after a second bout of eccentric exercise.

Due to skeletal muscles plasticity, the tissue is highly adaptable and responds to the above-mentioned stressors through remodeling (Proske and Morgan 2001). Plyometric training has been shown to increase muscle power and improve both vertical and horizontal jump performance (Markovic 2007; Markovic and Mikulic 2010; Vissing et al. 2008). Ultrastructural adaptations occur in response to plyometric training, which can be observed by the absence of z-disk streaming following training. Studies investigating the effect of plyometric training on muscle fibre type transitions and cross-sectional area (CSA) in human subjects are few. Results are contradictory regarding the effects of plyometric training on human muscle fibre type transitions, although evidence points to increases in muscle CSA.

The aim of this thesis was to investigate skeletal muscle changes induced by an acute bout of plyometric exercise before and after plyometric training.

1.2 Human models of muscle damage

Due to ethical reasons inducing different forms of muscle injury in humans is restricted and thus most researchers opt for exercise based methods. The way in which these methods are structured is to have participants perform exercise which places stress on the muscle resulting in 'damage' by using activities which may occur during everyday life or sporting events or training. The models are based on the understanding that eccentric contraction places higher stress on the working muscle than concentric contraction. Protocols are generally subdivided into a whole body exercise models whereby phases of eccentric and concentric contractions are cycled in the movement (usually mimics training and true motion) or an isolated muscle group model system where one specific muscle group is isolated and targeted (highly specific and does not mimic everyday motion), or field models where volunteers are followed during and after sporting competitions that are expected to contain at least a significant amount of eccentric contractions.

- Elbow Flexor Models

This model has been most extensively used by researchers in order to study exercise-induced muscle damage. With this model the elbow flexor muscles are isolated and subjects perform triceps extensions against a maximal externally applied load which forces this muscle group to contract eccentrically, generally resulting in a severe form of muscle damage (Barroso et al. 2010). This type of muscle damage is usually induced using an isokinetic dynamometer. Dynamometers allow for eccentric motion at a constant angular velocity regardless of the applied load (Nikolaidis et al. 2008). The

load, number of repetitions, intensity and angular excursion of muscle actions vary according to the particular protocol used in the specific study.

- Knee Extensor Models

The knee extensor muscle group is another muscle group most frequently used to induce injury, similar to the way injury is induced in the elbow flexors (Byrne and Eston 2002). The injury is induced by eccentric contractions performed using an isokinetic dynamometer or similar devices. The muscle injury is restricted to the knee extensors because the subjects are strapped down in a chair preventing unnecessary movement, while the lower leg is attached to the devices rotating arm. The subject is then told to resist an external load, which acts to bend the knee into flexion, using a maximal effort (Byrne et al. 2001).

- Downhill Running Models

Downhill running forces the hip and knee extensor muscle group to contract eccentrically which causes muscle injury due to these muscles resisting the body mass of the participant down a hill. This type of exercise is usually completed in the laboratory on a treadmill that is set at a particular gradient and at a speed based on the participants' relative maximal oxygen consumption (VO_2 max), peak treadmill speed or maximal heart rate (Nikolaidis et al. 2008; Simpson et al. 2006).

- Plyometric model

Plyometric jumping involves a sequence of rapid jumping movements whereby the large muscle groups of the lower body cycle between eccentric and concentric contractions. In this way concentric contractions create a greater force output when immediately preceded by eccentric contraction of the same muscle (Chimera et al.

2004). Plyometric models differ across studies by the number of sets, the number of jumps per set, time of rest in between sets and the type of jump.

- Prolonged-Racing Events

Prolonged running events such as marathon and ultra-marathon events are also used for inducing skeletal muscle injury (Kim et al. 2007; Knechtle et al. 2011; Koller et al. 1998). However, the disadvantage of this type of model is that athletes competing in these races are generally well-trained to elite athletes whose muscles are adapted to the continuous running motion and hence are relatively injury resistant unless the terrain is extreme or other stressors are added; and it is also more difficult to control external factors.

In summary, between all these unaccustomed exercises, plyometric exercise is both dynamic and easy to standardize. Other exercise protocols including eccentric contraction are too highly controlled hence is not a true reflection of real life situations. Competitive or training models used are difficult to control and impossible standardize.

1.3 Plyometric Training

Plyometric training ('plyometrics') now forms part of many elite athletes' training regimens at least once or twice a week (Close et al. 2005; Twist and Eston 2005). Plyometrics of the lower body is a form of exercise that involves a series of explosive jumping actions. The end goal of this type of training is to increase maximum power of muscular contractions and has, in fact, been shown to result in improved athletic performance in sports involving sprinting and jumping (Chen and Hsieh 2001; Twist and Eston 2005).

Authors of a recent review point out that plyometric exercise improves strength, muscle power, coordination, and athletic performance by greater motor unit recruitment, enhanced reflex potentiation, changes in the elastic properties of muscle as well as connective tissue (Markovic and Mikulic 2010).

Three phases, collectively termed the stretch-shortening cycle (SSC), are observed during plyometric movements (fig. 2.3) (Guerrero et al. 2008; Matavulj et al. 2001). Phase 1 involves an eccentric motion where muscle lengthens while simultaneously producing force. Phase 2 includes the brief transition period from eccentric contraction to concentric contraction. Finally phase 3 is the muscle shortening phase (contraction) which concludes the jumping action (Chen and Hsieh 2001; Matavulj et al. 2001; Twist and Eston 2005).

Twist and Eston 2005 suggested that selective damage of type II muscle fibres was induced because a reduction in force-velocity was observed in the athletes after an acute bout of plyometric exercise. Apart from this indirect suggestion, no studies have reported the effect of an acute bout of plyometric exercise on the sarcomere structure of muscle fibres.

1.4 Structural Muscle Damage

A vast array of literature exists and is growing surrounding the different events occurring during and after muscle damage. In 1902, Hough first described one of the symptoms of exercise-induced muscle damage known as delayed-onset muscle soreness (DOMS) which he noted as being due to micro-tears in muscle. It was only until the early 80's when research in muscle soreness and exercise-induced muscle damage picked up once again, and provided some of the most interesting findings to date. In 1981 Friden et al. first showed evidence of muscle fibre damage in 5 male participants performing eccentric downstairs running. Ultrastructural analysis was then performed using electron

microscopy on muscle biopsies from the soleus muscle up to one week post-exercise. From the images presented, abnormal sarcomeres were observed with myofibre disruptions in the form of Z-line streaming at a subcellular level. In a follow up study by Friden et al. 1983 it was then also found that muscle fibres which were damaged were predominantly type II fibres. The identification of fibre type was based on z-disk width and mitochondrial volume percentage.

Acute muscle damage can be divided into two so-called phases. An early phase in which immediate changes occur in muscle following eccentric exercise. These early events leading up to fibre damage include the inability of fibres to generate the required tension, sarcomere length shifts outside of the optimum range and changes in excitation contraction coupling (Morgan and Allen 1999). Although there are different thoughts as to what could be responsible for the initial reduction in tension following exercise, it is strongly believed that it is mainly due to alterations in sarcomere structural arrangement and changes in excitation-contraction coupling both of which affect tension dependant on the length of muscle (Allen 2001; Davis and Epstein 2007; Morgan and Allen 1999). This exercise-induced sarcomeric damage is sufficient to cause a decrease in muscle functional capacity and performance (Komi 2000; Sorichter et al. 1997). Symptoms following eccentric exercise which can be termed as the late changes which occur in muscle following eccentric exercise (2^{nd} phase) include DOMS; severe myofibre morphological changes and degeneration; decreased muscle strength and power, as well as the release of muscle cytoplasmic and contractile proteins into the circulation and localized inflammation (Chimera et al. 2004; Geronilla et al. 2003; Komi 2000; Sorichter et al. 1997; Sorichter et al. 1998; Yu et al. 2002).

Several parameters in blood are used as indirect markers of muscle damage, the principle being that various muscle proteins will leak into the vasculature and can therefore be

evaluated and related to severity of the injury. The more common proteins include CK, myoglobin (Mb) and lactate dehydrogenase (LDH), with Mb assessed as a concentration, whereas both CK and LDH as enzyme activities. The most common marker is CK activity but this variable does not correlate well with pain, the amount of structural damage or the reduction of muscle function (Chimera et al. 2004; Sorichter et al. 1997). What makes it most difficult to explain is that whatever the acute adaptation is, it is unlikely to be the same kind of adaptation resulting from training; therefore it must be localized at the membrane. This may be due to the escape of this cytoplasmic protein through the sarcolemma after damage followed by membrane closure and is therefore not a good marker of contractile protein damage (Feasson et al. 2002).

The level of C-reactive protein (CRP) in plasma has been proposed as a marker of inflammatory processes occurring as a result of skeletal muscle damage following exercise, since several similarities are shared with the acute phase response of inflammatory diseases. CRP is a member of the class of acute-phase reactants (proteins that increase or decrease their plasma concentrations in response to inflammation) as its levels rise dramatically during inflammatory processes occurring in the body (Miles et al. 2007). CRP rises above normal limits within 6 hours, and peaks at 48 hours (Miles et al. 2007; Phillips et al. 2003). In response to tissue injury white blood cells are recruited to the site of injury and release cytokines such as IL-6. IL-6 triggers the synthesis of CRP by the liver and activates the complement system by binding to phosphocholine present on the surface of dying cells (Thompson et al. 1999).

In addition to membrane damage, which allows enzymes to be released, intramuscular structural and functional proteins that are damaged by the injury may be activated, such as calpains. Calpains breaks down myofibrillar proteins thereby facilitating their release into circulation and allowing them to be detected in blood.

1.5 Calpains and calcium dependant degradation

Two types of Ca^{2+}-dependant proteases are specific to skeletal muscle fibres and are responsible for the breakdown of myofibrillar proteins, namely the ubiquitous calpains (calpain-1 and calpain-2) and calpain-3 (Cagan et al. 1991; Murphy 2010). These proteases seem to be the initial trigger for skeletal muscle degradation (Cagan et al. 1991; Huang and Forsberg 1998). Ca^{2+}-dependant proteases cleave important linking elements of the sarcomere, such as titin, at the start of skeletal muscle degradation. During rest, in the presence of resting intracellular Ca^{2+} concentrations these proteases are mostly present in their inactive full-length form. Activation of the protease function only occurs once part of the molecules structure is cleaved.

Calpain-3 contains three additional insert regions in addition to the domains which are similar to the ubiquitous calpain isoforms. These additional inserts are the NS, IS1 and IS2 regions. In order for calpain-3 to become active the IS1 region must first be cleaved. Importantly, the IS2 region is believed to be attached to the protein, titin under normal conditions (Hayashi et al. 2008; Kinbara et al. 1998).

Interestingly, calpain-3 has been suggested to be involved in sarcomeric remodeling and maintenance of muscle integrity rather than its assumed role in muscle degradation (Beckmann and Spencer 2008). A defect in calpain-3 expression results in limb-girdle muscular dystrophy type 2A (LGMD2A), whereby affected individuals develop the disease later in life (approximately 10 years of age) (Gallardo et al. 2011). Previous studies proposed that the activity of calpain-3 might be regulated by the protein's normal attachment to titin at its c-terminus, situated at the M-band (Hayashi et al. 2008; Murphy and Lamb 2009). Because, thus far in vivo activation of calpain-3 by eccentric exercise has been shown to be the only physiological situation in which this occurs and secondly

because mutations in the titin gene coding for the protein region adjacent to the calpain-3 binding site results in tibial muscular dystrophy. Murphy et al. (Murphy et al. 2007) provided evidence that calpain-3 is autolysed 24hrs after a single maximal knee extension eccentric exercise bout in human *vastus lateralis* muscle. Taken together, this raises the question whether calpain-3 is regulated by increases in resting cytoplasmic Ca^{2+} concentrations or whether it is stretch dependant.

1.6 Calpain-3 Regulation: Small changes in resting Ca^{2+} concentrations or Stretch dependant activation.

It is clear that eccentric exercise results in the loss of calcium homeostasis (Morgan and Allen 1999). Two schools of thought exist as to how calpain-3 is autolysed following eccentric exercise and hence initiating muscle breakdown: 1) due to small changes in resting cytoplasmic Ca^{2+} concentrations following eccentric exercise; and 2) due to mechanical stress placed on muscle during muscle lengthening.

- Ca^{2+} concentration activation

  Numerous studies demonstrate that eccentric contractions result in membrane damage. Both transient muscle cell membrane disruption as well as the disruption of the sarcoplasmic reticulum structure, would allow Ca^{2+} to move down its concentration gradient thus raising intracellular Ca^{2+} concentrations. Increasing intracellular Ca^{2+} concentrations has the potential for activating the calpain proteolytic system which cleaves a wide variety of proteins such as cytoskeletal elements, contractile proteins or regulatory proteins, many of which are reported to be damaged following lengthening contraction. Therefore an influx of intracellular Ca^{2+} could initiate skeletal muscle breakdown following eccentric contraction, immediately after eccentric injury and as the injury progresses (Armstrong 1990).

- Mechanical activation

  Titin is a large abundant protein of skeletal muscle with elastic properties useful during regulation of contraction and relaxation (Kontrogianni-Konstantopoulos et al. 2009; Ojima et al. 2005). The parallel alignment of two Z-disks marks the boundary of a single sarcomere within a muscle cells cytoskeleton. Titin is anchored to the Z-disk and spans half the length of the sarcomere, forming a heterodimer with the protein obscurin (Kontrogianni-Konstantopoulos et al. 2009). Calpain-3 attaches to titin at its c-terminus which attaches at the M-line of the sarcomere (Kontrogianni-Konstantopoulos et al. 2009; Ojima et al. 2005). It is thought that when sarcomeres are overstretched placing strain on titin; calpain-3 may detach from its normal arrangement resulting in its autolysis and thus initiating muscle breakdown.

If calpain-3 is regulated by the above mentioned mechanical activation, one could argue that calpain-3 activity should commence immediately after eccentric contraction induced muscle injury. This does not account for the 24 hr delay in calpain-3 activation following a single eccentric exercise bout observed by Murphy et al. (Murphy et al. 2007). Murphy and Lamb (Murphy and Lamb 2009)provided evidence that calpain-3 in fact is governed by small increases in resting Ca^{2+} concentrations, concluding that calpain-3 remains tightly bound to titin at its c-terminus irrespective of fibre stretching or increased Ca^{2+} concentrations.

1.7 Ultrastructural skeletal muscle damage

The smallest functional unit of skeletal muscle contraction is the sarcomere and repeat sections of sarcomeres make-up the myofibrils. The Z-disks create the boundary of a single sarcomere and are part of the muscle cell cytoskeleton. Cytoskeletal proteins

such as actinin, desmin and dystrophin stabilize the sarcomeres by adding structure and may be injured either by overstretched sarcomeres or by protease activation (Allen 2001).

It is well documented in literature that eccentric or unaccustomed exercise results in skeletal muscle damage (Chimera et al. 2004; Geronilla et al. 2003; Komi 2000; Sorichter et al. 1998). The earliest changes observed following muscle damage induced by eccentric contraction, involve the disruption of sarcomere and cytoskeleton components resulting in structural and mechanical integrity being compromised (Lieber et al. 1996). Membrane disruption, loss of calcium homeostasis, the impairment of muscle contraction resulting in the loss of force production is all included in the early changes after eccentric contraction. Evidence from electron microscopic studies give a visual image of sarcomere disorganization and z-line streaming which can range from moderate to severe with t-tubule and sometimes mitochondrial damage (Lauritzen et al. 2009; Takekura et al. 2001). However, the exact time course and sequence of events surrounding sarcomere disruption remain unknown (Morgan and Allen 1999).

In animal models, Lehti et al. (Lehti et al. 2007) showed that titin; desmin and dystrophin (cytoskeletal proteins) are vulnerable to damage in the early phase after downhill running. Desmin forms a scaffold around the Z-disk of the sarcomere and connects the Z-disk to the subsarcolemmal cytoskeleton. It links the myofibrils laterally by connecting the Z-disks. Their functions during contraction are to maintain the structural and mechanical integrity of the fibre while also helping in force transmission and longitudinal load bearing (Paulin and Li 2004). A recent study by Seto et al (Seto et al. 2011) demonstrated that alpha actinin-3-deficient muscle is more susceptible to damage. They showed that *extensor digitorum longus* muscle from ACTN3 knockout mice had significantly reduced force producing capacity following eccentric contractions induced with a force plate.

Very few studies have considered how vulnerable other myofibrillar or cytoskeletal proteins might be. Still, it has been demonstrated that nebulin (Trappe et al. 2002; Yu et al. 2003) is degraded following high-intensity eccentric resistance exercise in human research participants. Only one study has investigated muscle biopsies from subjects with exercise-induced DOMS, whether or not obscurin is affected. It was either maintained in its position at the M-band level or diffusely spread over the sarcomeres (Carlsson et al. 2008). Collectively, the unique structural properties and subcellular locations of titin, nebulin, and obscurin suggest that they function as molecular scaffolds allowing actin and myosin filaments to remain in place in sarcomeres, providing binding sites for sarcomeric proteins and coordinating the sarcomeric alignment of nearby structures. For a comprehensive review see Kontrogianni-Konstantopoulos et al. (Kontrogianni-Konstantopoulos et al. 2009). Obscurin and calpain-3 both bind titin at its c-terminus along the z-line where Type II muscle fibre damage due to eccentric contraction has been reported over the past 30 years, while the type I fibres have been found to be less affected by this contraction type (Twist and Eston 2005). At first it was thought that the preferential fibre type II biased damage was due to metabolic differences between fibre types. However Patel et al. (Patel et al. 1998) suggested this not to be the case, when the researchers trained rabbit dorsi-flexor muscles (predominant type II fibre composition) with an isometric electrical stimulation protocol to increase muscle oxidative capacity. Results showed that increasing muscle oxidative capacity was not sufficient to protect muscle from a subsequent bout of eccentric contraction induced injury.

Focus then shifted to structural and cytoskeletal differences between different muscle fibre types. Fast twitch fibres are characterized by possessing narrower and weaker z-lines, therefore during muscle lengthening with contraction it is thought that the stronger sarcomeres pull the weaker ones apart resulting in z-line streaming and contractile protein

release (Marginson et al. 2005). Slow oxidative fibres appear to be better protected from repeat mechanical loading because they possess higher levels of certain cytoskeletal proteins which maintain its structural integrity (Tiidus 2008).

1.8 Blood markers of muscle damage

Injury of skeletal muscle frequently occurs during normal life, following unaccustomed exercise and during contact sports (Smith et al. 2008). One symptom includes the release of muscle cytoplasmic and contractile proteins into the circulation. Several parameters in blood are used as markers of muscle damage, because these will leak into the circulation. Markers include proteins such as CK, Mb and LDH.

CK is a key enzyme in muscle metabolism. CK is a dimeric globular protein and has an approximate molecular weight of 43 kDa (Brancaccio et al. 2008; Brancaccio et al. 2007). During muscle contraction it catalysis the transfer of high-energy phosphate between phosphocreatine and adenosine-diphosphate. There are at least five CK isoforms, three of which are present in the cytosol and two in the mitochondria. Eccentric exercise is associated with the greatest increases in serum CK enzyme activity but the time point at which CK peak release into circulation occurs, varies across the literature (Brancaccio et al. 2007). Brancaccio et al. 2008 report that peak CK release following eccentric exercise is at 96 hours post-exercise. However this will differ depending on the level of an individual's training, exercise type, intensity, and duration.

CK-MM is skeletal muscle specific and CK-MB is supposed to be cardiac muscle specific, although an increase in CK-MB can be representative of injury to cardiac or skeletal muscle injury. Physical activity may increase both total CK and CK-MB but using a ratio of CK-MB/total (% relative index) CK one can determine whether cardiac or skeletal muscle

has been damaged. An increased (% relative index) is indicative of cardiac injury as the cause of elevated CK-MB in serum.

Mb release accompanying muscle damage, has also promoted the use of this indirect marker (Brancaccio et al. 2010). However this is also a cytoplasmic protein which causes the same problems of interpretation that exist with CK. Neither gives a clear indication of structural muscle damage; both are not specific to skeletal muscle force production which is possibly why they correlated poorly with the decrease in muscle function after damage (Chimera et al. 2004). Myoglobin is a relatively small protein with a molecular weight of 17.8 kDa and is rapidly released into circulation therefore clearance of myoglobin from the blood is rapid (Sorichter et al. 1999). It functions as an oxygen carrying protein in muscle fibres and is predominantly present in slow oxidative muscle fibres.

The LDH enzyme in recent times has been used less for the diagnosis of muscle fibre injury because of these same problems of not being a structural protein. LDH activity can be measured in a variety of tissues (most tissues). It converts pyruvate to lactate during glycolysis and has its maximal activities at high pyruvate concentrations (Brancaccio et al. 2008). There exist five isoenzymes which are composed of a combination of the M-polypeptide and H-polypeptide. Exercise has been found to induce a significant increase in LDH, the degree of which depends on the intensity and duration of the exercise bout. Eccentric exercise has been found to increase serum LDH levels much more so than concentric exercise and peak values in serum can be observed between three and five days following exercise (Brancaccio et al. 2008).

One of the reasons for the increase in these proteins in the circulation is not structural protein damage, but that the sarcolemma is ruptured and allows the escape of cytoplasmic proteins into circulation (Schiaffino and Partridge 2008). However, the membrane has a

mechanism of ‘self-sealing’ and is able to close itself off as a protective measure, which could prevent further damage to contractile proteins and reduce the loss of muscle functional capacity (Schiaffino and Partridge 2008). Currently this process is unpredictable, and needs to be further investigated.

If the skeletal muscle fibre is unable to seal itself off properly, this causes additional release and greater elevation in circulating levels of Mb especially because it is the smallest of the proteins discussed so far. Due to the relatively simpler assay and lower cost of assessing blood CK levels, it is often used clinically and in applied studies as a surrogate measure of Mb.

1.9 Rhabdomyolysis

Adverse events may occur from eccentric exercise protocols and in the worst case this may lead to death from acute renal failure as a result of a condition called rhabdomyolysis (Moeckel-Cole and Clarkson 2009). This condition may occur in healthy individuals and goes hand in hand with large increases in serum CK and myoglobinurea, which can be exacerbated with heat stress and dehydration.

Rhabdomyolysis occurs in varying levels of severity and consists of the rapid breakdown of skeletal muscle fibres in damaged muscle tissue (George et al. 2010; Moeckel-Cole and Clarkson 2009). Muscle destruction results in the release of muscle cell protein content into the bloodstream, such as CK and Mb (Sorichter et al. 1997). This condition in athletes and healthy populations normally is not dangerous and the symptoms are tolerable, however exertional rhabdomyolysis becomes clinically relevant when muscle damage is too severe (Sayers and Clarkson 2002). Severe muscle damage may result in the continued release in Mb which precipitates in the kidney tubules. There it binds to a protein

called Tamm-Horsefall protein and forms solid deposits which obstruct the normal flow of fluids and may lead to kidney failure and even death (Moeckel-Cole and Clarkson 2009).

Light rhabdomyolysis generally affects athletes who train too strenuously in a mode to which they are unaccustomed, or sedentary subjects who take part in unaccustomed exercise for the first time. DOMS may occur with or without rhabdomyolysis. However, the increase in the muscle proteins (CK, Mb) released is greater with rhabdomyolysis and 5-fold or higher elevations in CK have been proposed as a reasonable cut-off (Brown 2004). However, the definition of the normal range for CK varies e.g. normal range: 55 to 170 U/L) (Brown 2004) or 15-195 U/L (Varsano et al. 2000). A 5-fold increase is also quite vague and possibly not so relevant to eccentric exercise where CK activity can easily be higher than 1 000 U/L. Importantly, although a vast amount of literature is currently available on rhabdomyolysis cases, however it still remains unclear why rhabdomyolysis occurs in some individuals and not in others who performed the same physical activity.

1.10 Responders vs. Non-responders

It has been reported that with eccentric exercise induced damage, protein markers of muscle damage usually increase in circulation after the exercise bout (Brancaccio et al. 2008). However indirect marker variability in response to eccentric exercise exists amongst individuals. The literature reports that in a group of people exercising at a similar intensity and in a similar environment, only a small percentage of the group would eventually develop rhabdomyolysis, with most maintaining a lower CK profile (Clarkson et al. 2005b; Devaney et al. 2007; Yamin et al. 2010). Why selected individuals and not others present with rhabdomyolysis is unclear, although several predisposing conditions have been identified. These include genetics, training status, dehydration, bacterial or viral

infections and alcohol (Braseth et al. 2001; Knochel 1990; Marinella 1998; Vertel and Knochel 1967).

1.11 Single Nucleotide Polymorphisms

Single nucleotide polymorphisms (SNPs) are common variations within the genetic sequence with a frequency of at least 2% in a given population which is not due to new mutations. This DNA variation occurs when a single nucleotide in the genome differs between paired chromosomes in an individual. It is the most frequent form of DNA variation in the genome. SNPs can help explain inter-individual differences in many conditions and is useful in identifying an individual's susceptibility for or protection against various diseases (15817725, 17289909, and 1579488(Clarkson et al. 2005b; Devaney et al. 2007; Wen et al. 2005). But they are also important in other phenotypes besides disease. For SNPs important in exercise physiology a comprehensive review is published regularly in Medicine Science Sport and Exercise (Bray et al. 2009; Hagberg et al. 2011; Rankinen et al. 2002). These reviews suggest that larger sample sizes are required to investigate the influence of SNPs on muscle characteristics. This is true however smaller studies can use the database of SNPs to determine whether subjects fit with the current SNPs put forward.

Several genetic polymorphisms associated with exertional muscle damage variability have been the focus of recent publications. These studies indicated that genes involved in muscle structure (*ACTN3*) or that contribute to growth (*IGF2*), inflammation (*TNFA* and *IL6*), or force production (*MYLK*) can contain polymorphisms that affect baseline CK activity and exacerbate the muscle damage response to eccentric exercise, generally predisposing those with the rare alleles to greater levels of damage.

- Myosin light chain kinase (*MYLK*):

Myosin light chain kinase (MLCK) is expressed by the MYLK2 gene (Stull et al. 2011) in skeletal muscle tissue where it is predominantly expressed in fast twitch muscle (Clarkson et al. 2005b; Stull et al. 2011). It acts to phosphorylate sarcomeric myosin's regulatory light chain (RLC) (Clarkson et al. 2005b), which has an important regulatory function in force development (Clarkson et al. 2005b; Stull et al. 2011). Clarkson et. al. (Clarkson et al. 2005b) suggested that polymorphisms in MLCK would lead to an increase in regulatory light chain (RLC) phosphorylation in some people, allowing for greater tension generation during muscle lengthening and consequently greater muscle damage in those people compared to others without the SNP.

Table 1.1 CK responses and various SNPs suggested as being associated with eccentric exercise-induced muscle damage.

Subject #	Gender	SNP	Genotype	Subject # in genotype groups	Fitness level	Exercise protocol	CK (U/L) at day 4	Mb (ng/ml) at day 4
157	78 men	MYLK (C49T)	CC	97		50 max tricep extensions using non-dominant arm.	5660	316
	79 women		CT	50			8476	433
			TT	6			29063	937
157	78 men	MYLK (C37885A)	CC	121		50 max tricep extensions using non-dominant arm.		342
	79 women		CA	35				494
			AA	1				
157	78 men	ACTN3	RR	35		50 max tricep extensions using non-dominant arm.	106	
	79 women		RX	78			126	
			XX	41			99	
88	60 men	CK-MM	AA	9	physically active	stepping up and down two stairs for 5 min	1048	
	28 women		AG			followed by 15 squats while wearing a backpack		
			GG	79		weighted at 30% of their body weight.	12	
88	60 men	ACE	II	n/a	physically active	stepping up and down two stairs for 5 min	26	
	28 women		ID	n/a		followed by 15 squats while wearing a backpack		
			DD	n/a		weighted at 30% of their body weight.	146	
70	42 males	IL6 G-174C	GG	44	physically active	one set of 50 maximal eccentric movements	2604	
	28 women		GC	24		of the elbow flexors (nondominant arm),	7592	
			CC	2		using a dynamometer.	8403	
70	42 males	TNFA G-308A	GG	58	physically active	one set of 50 maximal eccentric movements	5000	
	28 women		GA	12		of the elbow flexors (nondominant arm),	1500	
						using a dynamometer.		
156	78 females	IGF-II	TT	76		50 maximal isotonic eccentric (muscle lengthening)	5278	333
		T13705C	TC	1		contractions of the elbow flexor muscles of their	21152	1099
						nondominant arm on a modified preacher curl bench.		
	78 males	IGF2AS	GG	16		50 maximal isotonic eccentric (muscle lengthening)	12069	492
		G11711T	TG	38		contractions of the elbow flexor muscles of their	6247	289
			TT	20		nondominant arm on a modified preacher curl bench.	17160	659

CK and Mb values at 4 days post exercise, blank spaces indicate unspecified information, or that a paritícular marker was not used. Some values for CK and Mb are not absolute values but were estmates taken from graphs presented studies.

In the study by Clarkson et al (Clarkson et al. 2005b), participants performed 50 maximal triceps extensions using the non-dominant arm. Two *MYLK* SNPs were tested: C49T (genotypes are CC, CT and TT) and C37885A (genotypes are CC, CA and AA); in 156 male and female subjects (numbers within genotype groups vary see table 2). The authors found that individuals with the homozygous TT rare allele (*MYLK* C49T) had significantly greater increases in CK and Mb compared to the heterozygotes and and subjects with the *MLCK* C37885A heterozygotes (CA) had significantly greater CK levels compared to the homozygous people.

- α-actinin 3 (*ACTN3*):

Alpha actinins functions to bind and anchor actin filaments (Clarkson et al. 2005a; North and Beggs 1996). Clarkson et. al. (Clarkson et al. 2005b) linked genetic variations in the myofibrillar protein ACTN3 which is localized to the z-disk and is fast-twitch specific to explain the variability in response to exercise-induced muscle damage. A polymorphism of ACTN3 has previously been identified in humans and individuals who are homozygous for the *ACTN3* 577X allele cannot produce the ACTN3 protein in their muscle (Clarkson et al. 2005b; Doring et al. 2010). Clarkson et. al. (Clarkson et al. 2005b) tested blood from 157 individuals who completed an elbow flexion eccentric exercise protocol to test whether CK and Mb activity was associated with *ACTN3* genotypes (RR, RX and XX). The authors however found no association with this mutation and increases in CK and Mb; although concluding that the polymorphism may affect resting CK levels in healthy individuals because they observed that subjects with the *ACTN3* 577X polymorphism had lower baseline CK levels compared to heterozygotes.

- Insulin-like growth factor II (*IGF2*) genes:

Insulin-like growth factors are involved in both muscle damage and repair mechanisms (Evans and Cannon 1991; Florini et al. 1996; O'Dell and Day 1998). Devaney et. al. (Devaney et al. 2007) investigated whether there were any associations between various SNP's in the *IGF2* gene region and indicators of muscle damage such as CK, and Mb, using the same study population and exercise protocol as mentioned above (Clarkson et al. 2005b). More significant associations were only found in men where *IGF2* (C13790G, rs3213221), *IGF2* (ApaI, G17200A, rs680), *IGF2* antisense (IGF2AS) (G11711T, rs7924316), and *IGFBP3* (_C1592A, rs2132570) were significantly linked to indicators of muscle damage; they found that men homozygous for the rare *IGF2* G13790C allele (CC) and rare allele for the ApaI G17200A (AA) SNP had high post exercise serum CK activities and those who were homozygous wild type for the IGF2AS G11711T (GG) SNP had greater CK and myoglobin responses to exercise. (Devaney et al. 2007).

- Muscle specific CK:

The muscle specific CK (CK-MM) can be found along the M-line and sarcoplasmic reticulum (Zhou et al. 2006). The CK-MM-encoding gene has been mapped to chromosome 19q13.2–13.3 (Heled et al. 2007; Zhou et al. 2006) where it is positioned in the same region next to two other genes associated with muscle function and specific muscle pathologies. Due to these observations Heled et. al. 2007 hypothesized that different genotypes of the *CK-MM NcoI* polymorphism may be associated with the inter-individual CK responses to exercise. Eighty eight physically active male and female volunteer subjects were subjected to an exercise protocol which included stepping up and down two stairs (for 5 minutes), followed by 15 squats while wearing a backpack weighted at 30% of their bodyweight. Heled et al 2007 classified nine participants as high responders according to their increases in serum CK, which on

average was significantly higher for the high compared to the low responder group, (1,048±421 U/L compared with 12±8 U/L, respectively). However Yamin et. al. (Yamin et al. 2010) argued that CK response following eccentric exercise was not associated with *CK-MM NcoI* polymorphism and that the cut-off point used to define high CK responders and other aspects of the study by Heled et. al. 2007 was flawed.

- Angiotensin-converting enzyme (*ACE*):

ACE is expressed in skeletal muscle tissue and plays an important role in the body's renin-angiotensin system, which mediates extracellular volume and arterial vasoconstriction (Dietze and Henriksen 2008; Jones and Woods 2003). The two main functions of ACE include the generation of angiotensin (ANG) II and degrading kinins during exercise (Coates 2003; Jones and Woods 2003). Since the discovery of an insertion (I)/deletion (D) polymorphism of the ACE gene (Danser et al. 1995), several studies have provided evidence of an association between this polymorphism and human physical performance (Giaccaglia et al. 2008; Rodriguez-Romo et al. 2010; Woods 2009). Due to extensive renin-angiotensin system involvement in skeletal muscle metabolism regulation and injury responses, Yamin et. al. 2007 hypothesized that the *ACE ID* genotype may explain inter-individual differences in CK response. The authors concluded that individuals with the *II* genotype may be more susceptible for developing muscle damage whereas individuals with the *DD* genotype are more protected.

- *TNFA* (-308) and *IL6* (-174) promoter polymorphisms:

Yamin et. al 2008 investigated whether two gene promoter polymorphisms involved in the inflammatory response were associated with individual systemic CK response to eccentric injury using an elbow flexor model. A G/A polymorphism at position -308 of

the *TNFA* gene (Vishnoi et al. 2007) and *G/C* polymorphism at position -174 of the *IL-6* gene (Fishman et al. 1998) were assessed and a stronger association between the *IL-6* gene promoter polymorphism and CK response following eccentric exercise was found compared to a milder association between *TNFA* G-308A genotype and CK activity. Data presented by Yamin et. al. 2008 suggests that individuals expressing homozygosity for the *IL6*-174C allele are at greater risk for exercise induced muscle damage.

1.12 Adaptation to muscle damage (Repeat bout effect)

Exercise-induced muscle damage does not lead to permanent damage but rather adaptation that follows. Adaptation may occur as early as changes from a single bout of exercise, with perhaps immediate subcellular adaptations occurring. The repeat-bout effect is a phenomenon where muscle protects itself from injury, compared to the extent of injury caused by the initial exercise session, the second time around. For the second bout, less damage is evident but it does not prevent muscle damage. This protective mechanism relieves the symptoms of muscle soreness, swelling and inflammation and improves recovery time for muscle strength and range of motion. It also reduces the release of muscle proteins into circulation.

Typical early studies evaluated the repeat bout effect by introducing a single unaccustomed exercise bout and then repeating the same single exercise bout after a week to a month. A study done by Clarkson et al. (Clarkson et al. 1987) is an example of these early studies. The investigators assessed common indicators of muscle damage and found that subjects had both lower pain and CK responses to the exercised ipsilateral limb (same limb) on the second bout of exercise spaced seven days apart. However this phenomenon is more complicated than these earlier studies suggest, with recent studies

presenting even more surprising results since the protective effect is lost if the time between bout 1 and bout 2 is too long. This raises the question, “how long does the protective effect last?” Nosaka et al. 2001 provided evidence that the muscle adaptive protective effect may last for 6 months following the initial exercise bout. In this study high force eccentric exercise of the elbow flexors were performed in 2 bouts in 35 subjects across three groups; group 1, 2 and 3 repeated their second exercise bouts at 6, 9 and 12 months respectively. The protective effects included were lost between months 9 and 12.

Most studies used maximal or close to maximal (80% of the pre-exercise maximal isometric force) eccentric contractions to investigate the repeat bout effect (Chen 2006; Clarkson et al. 1987; Nosaka et al. 2001; Skurvydas et al. 2011). Recently Chen et al. (Chen et al. 2010) showed that by replacing one maximal high force exercise bout with four sub-maximal exercise sessions, would offer the same protection against a subsequent bout of maximal eccentric exercise. Results included no significant differences between both the 100% efforts of the second bouts between the control group and the group that performed the four submaximal bouts. In other words, where the CK, Mb and muscle soreness responses were significantly reduced after the second bout, both groups had adapted to the same extent.

Although the repeat bout effect is extensively demonstrated in literature (Clarkson et al. 1987; Howatson et al. 2007; McHugh 2003), a general consensus as to what the actual underlying mechanisms of this phenomenon are, are yet to be met, although several have been suggested. McHugh (McHugh 2003) categorized three main theories as: the neural adaptation theory of muscle damage; cellular adaptation theory and mechanical adaptation theory; although inflammatory adaptation needs to be considered as well.

Neural adaptation theory: To investigate the central nervous systems involvement as a potential mechanism to protect skeletal muscle from subsequent injury, neural adaptations would have to include one or more of the following: better motor unit recruitment, enhanced motor unit synchronization, the allocation of workload across more fibres and increased recruitment of slow fibres (Tiidus 2008).

Using surface electromyography (EMG), Warren et al. (Warren et al. 2000) provided evidence of a decrease in median frequency in the *tibialis anterior* muscles during the second bout of eccentric contractions suggesting either the recruitment of slow twitch fibres or more efficient synchronisation of motor units. More evidence supporting the neural adaptation theory comes from studies by Howatson and van Someren 2007 and Newton et al. 2007. These authors showed that muscle damage (measured by changes in maximal isometric torque, upper arm circumference and plasma CK) in the repeat bout of eccentric exercise performed by the contralateral arm two weeks post, was significantly reduced, and they attributed to the reduction of some muscle damage markers' responses as a possible learning effect. More recently Dartnall et al. 2011 demonstrated a 57% increase in motor unit synchronization on the second bout of eccentric exercise in human biceps brachii muscle compared to the first bout which was 1 week earlier. However evidence exists suggesting that the repeat bout effect can occur independent of neural adaptation: experiments using electrical stimulation of eccentric contractions in a human elbow flexor model showed that significantly reduced responses were observed for markers of muscle damage following a repeat bout of the same damaging protocol (McHugh 2003; Nosaka et al. 2002; Sacco and Jones 1992). From this it was concluded that the central nervous system's involvement in the repeat bout effect is minimal and peripheral adaptation plays a more important role.

Cellular adaptation theory: Possible cellular adaptations that have been proposed in the literature include the addition of adjacent sarcomeres, better cycling of calcium during excitation contraction coupling, increased protein synthesis (also stress proteins), strengthened plasma membrane, adaptation of the inflammatory response and the removal and replacement of damaged with regenerated fibres.

Proske and Morgan (Proske and Morgan 2001) suggested that the protective effect experienced on the second bout following the initial bout of eccentric exercise may be due to a shift in the length-tension relationship toward a longer muscle length, resulting from the addition of sarcomeres along the length of muscle fibres. Although this theory seems attractive and various animal (Lynn and Morgan 1994; Lynn et al. 1998) and recently human studies (Brockett et al. 2001) lend support to it, reports remain disagreeing. A study by Chen et al. 2007 provides evidence against the notion that the repeat bout effect depends on the shift in optimum angle. The authors compared the effect of four different unaccustomed eccentric exercise intensities (100%, 80%, 60% and 40%) on optimum angle shift and the extent of muscle damage induced by a second bout of eccentric exercise (two to three weeks later) at maximum intensity. They found a significantly greater rightward shift of the optimum angle for the higher intensities compared to the two lower exercise intensities following bout one. All groups however, showed significantly attenuated responses in muscle damage markers following bout two compared to bout one (100%). Therefore it can be deduced that the repeat bout effect cannot merely be explained by an increase in sarcomere number in series.

An impaired excitation contraction coupling system has also been implicated in muscle adaptation following a single bout of eccentric exercise (Warren et al. 2001). Changes in E-C coupling would affect an immediate loss of muscular strength following eccentric exercise (Howell et al. 1993). However if this were the case losses in strength experienced

immediately after the second bout of eccentric exercise would not mimic the initial loss of strength after the first bout as demonstrated by Balnave and Thompson (Balnave and Thompson 1993); Brown et al (Brown et al. 1997) and McHugh and Pasiakos (McHugh and Pasiakos 2004).

Studies demonstrate a blunted inflammatory response following a repeat bout of eccentric exercise compared to the inflammatory response directly after the initial bout (Pizza et al. 1999). This is possible, and could also explain why preconditioning muscle with passive stretching and isometric contractions also offers protection against a subsequent bout of eccentric exercise (Koh and Brooks 2001; Pizza et al. 2002), resulting in a blunted inflammatory response similar to that experienced from an initial bout of eccentric exercise. This led the authors to conclude that the initial inflammatory response to the initial exercise bout (regardless of whether it was concentric preconditioning or an eccentric bout) resulted in a protective effect. The initial bout of exercise causes mechanical disruption to myofibrils resulting in a local inflammatory response which results in secondary damage; however reduced mechanical disruption experienced immediately post-exercise in the repeat bout cannot be explained by an inflammatory adaptation, because inflammation takes time to develop.

Mechanical adaptation theory: Mechanical adaptation to eccentric exercise includes an increase in either passive or dynamic muscle stiffness, cytoskeletal adaptations or an increase in intramuscular connective tissue as described in a review by McHugh (McHugh 2003). Increases in both passive and dynamic muscle stiffness following eccentric training have been previously confirmed, both in human studies (Pousson et al. 1990) as well as animal studies (Reich et al. 2000). These observations could reflect adaptation to cytoskeletal elements responsible for the structure, maintenance and alignment of the sarcomere.

Parcell et al. (Parcell et al. 2009) observed an increase in desmin content after 12 weeks of resistance training in human subjects. This study provides evidence that the cytoskeleton is adaptable with a relatively long and consistent training programme. Barash et al. (Barash et al. 2002) demonstrates a loss of desmin immunostaining (qualitative observation) immediately after a single eccentric contraction bout but a tremendous quantitative increase in desmin content 72-168 h later, in rats. This study indicates a much more dynamic restructuring of the muscle's intermediate filament system and may provide a structural explanation for the protective effects observed in muscle after a single eccentric contraction bout. Contradicting evidence however exists: Sam et al. (Sam et al. 2000) showed in a mouse model that muscles absent of desmin are less susceptible to muscle damage. This finding in the animal model is complemented by findings of McHugh et al. (McHugh et al. 1999) who found that muscle possessing more passive stiffness may in fact, have a greater susceptibility to an initial damaging event. This was demonstrated when comparing individuals classified as having stiff hamstrings with those who had more compliant hamstring muscles (McHugh et al. 1999); they showed that indicators of muscle damage (Strength loss, pain, muscle tenderness, and CK activity) were greater for individuals with stiffer hamstrings.

Other theories attempting to explain the repeated bout effect includes possible protection by heat shock proteins and the ‘stress-susceptible fibre hypothesis’ (Koh 2002; Newham et al. 1987; Thompson et al. 2002; Thompson et al. 2001). Thompson et al. 2001 demonstrated that heat shock proteins are increased by a single bout of elbow flexor eccentric exercise in humans. This observation led to the postulation that heat shock proteins may protect muscle tissue from a second bout of eccentric exercise, however direct evidence is needed. The stress-susceptible fibre hypothesis suggests that within a pool of muscle fibres, some fibres are stress resistant and other stress-susceptible. After a

bout of unaccustomed eccentric exercise the stress-susceptible fibres degenerate and after are regenerated and remodelled becoming stronger, thus the amount of stress-susceptible fibres within the pool of muscle fibres are less resulting in less damage. This may be underlying mechanisms for extensive adaptation in response to training as opposed to a single bout effect.

1.13 Adaptation to muscle following plyometric training

Plyometric training results in various adaptation responses which may improve athletic performance by greater motor unit recruitment, enhanced reflex potentiation, and changes in the elastic properties of muscle as well as connective tissue (Vissing et al. 2008). It incorporates various jumping activities such as: drop, squat, vertical and depth jumps. Currently it forms part of many athletes training regimens at least two or three times per week and is associated with sporting activities such as sprinting, basketball, volley ball, soccer and even rugby.

1.13.1 Musculoskeletal Adaptation

Bone Adaptation

During plyometric jumping the active eccentrically contracting muscle groups can withstand loads in excess of 5 times (well beyond the force which could be voluntarily produced) the athlete's body weight (McMahon and Greene 1979) and this activation occurs in less than 60 ms (Toft et al. 1989). Some of the stressful forces are absorbed by the combined musculoskeletal complex during ground contact time of the jump. Stress transmitted to the bone would be stress that cannot be absorbed by the muscle. Therefore, bone adaptation to plyometric exercise would indicate that the forces applied by the protocol are high and exceed the muscle capacity to absorb the stress. Since the focus of

the thesis is on skeletal muscle only a few studies focussing on bone and tendon adaptation will be considered.

In the short-term, osteocyte activity increases in response to stress placed on bone and acts to maintain bone turnover by signaling osteoblastic activity thus increasing bone formation (Klein-Nulend et al. 1995). An adaptation to bone tissue due to plyometric training would be the increase in bone mass, with most studies assessing the impact of jump activity over period of months. Increases in bone mass have been reported by Guadalupe-Grau et al. (Guadalupe-Grau et al. 2009), they showed a +0.78% relative improvement in whole body bone mineral content using dual-energy X-ray absorptiometry (DXA) when combining plyometric training with strength training over nine weeks. Here 43 males and 23 females were assigned to a training (similar relative fitness levels) or control group, and exercised at a frequency of 3 times per week. Plyometric training included drop jumps and hurdles (40-60jumps) and strength training included inclined leg press (ILP), leg extension (LE), half squat (HS), and leg curl (LC). Guadalupe-Grau et al. 2009 also reported improvements in lumbar spine bone mineral content (+1.2–2%) with the combination of strength and plyometric training. Another study assessing the effects of only plyometric box jumps in children over a period of 12 weeks at a frequency of 5 days per week, found total body and leg bone mineral content to improve significantly compared to controls.

Tendon Adaptation

Linking structures between muscle and bone include the tendonous structures. Plyometric jumping incorporates the stretch shortening cycle motion, which affects stiffness characteristics of the muscle-tendon complex. Interestingly, Kubo et al. (Kubo et al. 2007) reported an increase in joint stiffness and not tendon stiffness following unilateral

plyometric training of plantar flexors in humans after 12 weeks of plyometric training; they concluded that improvements in jumping performance are due to adaptation of mechanical properties of muscle-tendon complex around the joint rather than muscle activation strategies. However this finding is contrasted by Burgess et al. (Burgess et al. 2007) who provided evidence for increased Achilles tendon stiffness following 6 weeks of plyometric training. Kubo et al. (Kubo et al. 2007) in the same article mentioned above assessed changes in the cross-sectional area (CSA) of the Achilles tendon and found no change in CSA after 12 weeks of plyometric training, this finding is supported by Foure et al. 2010 who found no change in CSA following 14 weeks of plyometric training, thus attributing changes in mechanical properties to qualitative changes in tendinous tissues rather than geometrical changes to the Achilles tendon.

1.13.2 Neuromuscular Adaptation

Neural adaptation

It has been previously suggested that neural adaptation rather than change in muscle morphology is responsible for muscle performance gains following plyometric exercise (Wilk et al. 1993); this has been suggested to occur via neuromuscular adaptation to the stretch reflex, elasticity of muscle, and Golgi tendon organs. Potteiger et al. 1999 speculated that improvements in muscle performance observed following 8 weeks of plyometric training could be due to a combination of increased muscle CSA and enhanced motor unit recruitment patterns, although the authors did not measure any neuromuscular outcomes as part of their study. Other studies support this suggestion attributing jump performance improvements to adapted motor unit recruitment patterns and muscle activity of agonists and antagonists (Chimera et al. 2004; Van Cutsem et al. 1998). Using surface electromyography various studies investigated muscle activation strategies during vertical

jumps or maximal voluntary contraction post training intervention. Chimera et al. 2004 investigated the effects of plyometric training on muscle-activation strategies of the lower extremity in females during jumping exercises. They assessed preparatory and reactive activity of the *vastus medialis and lateralis*, medial and lateral hamstrings, and hip abductors before and after six weeks of plyometric training. Surface electromyography revealed preparatory adductor activity to increase along with abductor-to-adductor co-activation, indicating plyometric training resulted in learned pre-programmed motor activation strategies. Kyröläinen et al. 2005 demonstrated that vertical jump height improved significantly as a consequence of 15 weeks of stretch shortening cycle training, and a significant change in muscle activity of the plantar flexors but not the knee extensors were observed.

This does not exclude that muscle itself could also be adapting. Adaptation in fibre-type in response to SSC training has been previously demonstrated in animal models (Almeida-Silveira et al. 1994; Pousson et al. 1991). In humans, only a few studies have assessed fibre-type transitions due to only plyometric training and the results of these studies are contrasting. Both Kyröläinen et al. 2005 and Potteiger et al. 1999 found no significant fibre transitions resulting from 15 weeks of training of the gastrocnemius and 8 weeks of training of the *vastus lateralis* muscles respectively, whereas Malisoux et al. 2006 observed a significant increase in the proportion of type IIa fibres due to 8 weeks of different stretch shortening cycle training.

In humans, Potteiger et al. 1999 and Malisoux et al. 2006 showed that plyometric training induced an increase in the cross sectional area in both type I and type II fibres in strength trained individuals (Potteiger et al. 1999) and in subjects active in sports not involving high-impact jumping (Malisoux et al. 2006b), while Vissing et al. (Vissing et al. 2008) did not observe any increase in muscle fibre CSA of untrained subjects but found significant

increases in whole-muscle cross-sectional area (CSA). This may be due to different techniques, rather than implying hyperplasia. Gains in CSA seem to be greatest when combining plyometric training with strength training as shown by Perez-Gomez et al. 2008, reporting significant muscle hypertrophy (+4.3%). Herrero et al. 2006 also reported on their observation of significant increases in CSA (7.1%) when combining plyometric training with electromyostimulation over a 4 week training period compared to only plyometric training.

In summary the ability of short term lower-body plyometric training in improving muscle strength and explosive power is well known (de Villarreal et al. 2008; Malisoux et al. 2006a; Markovic and Mikulic 2010; Vissing et al. 2008), and the literature suggests that these relative improvements occur regardless of gender or training state (Markovic and Mikulic 2010; Saez-Saez de Villarreal et al. 2010). Studies indicate that improvements in muscle strength and power could be due to both muscular and neural adaptations as demonstrated by studies investigating the mechanisms behind these systems in the same study (Kubo et al. 2007; Kyrolainen et al. 2005; Malisoux et al. 2006b). Potteiger et al. 1999 provides evidence of increased leg extensor power accompanied by a significant increase in CSA of both type I and type II vastus lateralis muscle fibres. Improvements of power and strength seem be to greater when plyometric training is performed in combination with weight training or electromyostimulation. Both strength and muscle CSA were shown to improve in the studies by Herrero et al. (Herrero et al. 2006) and Perez-Gomez et al. (Perez-Gomez et al. 2008) mentioned above.

1.13.3 Jump performance

Improving vertical jump performance is crucial to the success of athletic competition (Potteiger et al. 1999, Kyrolainen et al. 2005). Various jumping activities such as squat jumps, drop jumps and other SSC jumping exercise are used by athletic coaches to

improve muscular strength and power. Studies investigating change in muscle strength and power due to plyometric training generally show that these parameters do result in improved vertical jump height, although some studies report no change or even slight decreases in vertical jump performance (de Villarreal et al. 2008; Herrero et al. 2006; Luebbers et al. 2003; Markovic 2007; Markovic and Mikulic 2010; Turner et al. 2003; Young et al. 1999). Markovic 2007 employed a meta-analytical approach to investigate whether plyometric training did in fact improve vertical jump height. The assessment of the studies showed that inconsistency of reports on vertical jump height performance could be due to training program design, subject characteristics, and the use of different testing methods but mainly due to small sample size since group sizes typically ranged between 8 – 12 participants and concluded that plyometric training does ultimately result in improved vertical jump height.

1.14 Specific Aims

1) To investigate whether rhabdomyolysis induced by an acute bout of plyometric exercise in sedentary individuals was caused by primary mechanical damage or secondary factors.
2) To investigate whether plyometric training will protect the muscle from ultrastructural damage and rhabdomyolysis.

We hypothesize that mechanical damage does not differ but high responders have greater secondary responses, to the same amount of damage.

Chapter 2: Materials and Methods

2.1 Study overview

The study overview is described in figure 2.1. Twenty-six subjects participated in this research study which was divided into: 1) acute study and 2) training intervention. All twenty-six subjects participated in the acute study and only thirteen of those twenty-six subjects continued with the training intervention. The acute study investigated the effects of a single unaccustomed bout of plyometric squat jumping on muscle ultrastructure and blood markers of muscle damage while the training intervention part of the study investigated the effects of 8 weeks of plyometric training on muscle fibre structure and ultrastructure. The acute study and training experimental intervention designs are described individually in figures 2.2 and 2.3 respectively.

2.1.1 Subject recruitment and inclusion criteria

This study was approved by Sub-committee C of the Research Committee of the University of Stellenbosch. The potential subjects, were recruited by means of a flyer and by word of mouth among students at the University. The subjects were informed of the procedures both verbally and in written form. They signed a written consent form before they were allowed to proceed with the study protocol. Subjects were included upon the fulfilment of the following criteria:

Inclusion Criteria:

- Age: 18 – 28 years
- physically active 0 to 2 times per week
- no training of the lower body – other than typical daily activities
- signed informed consent

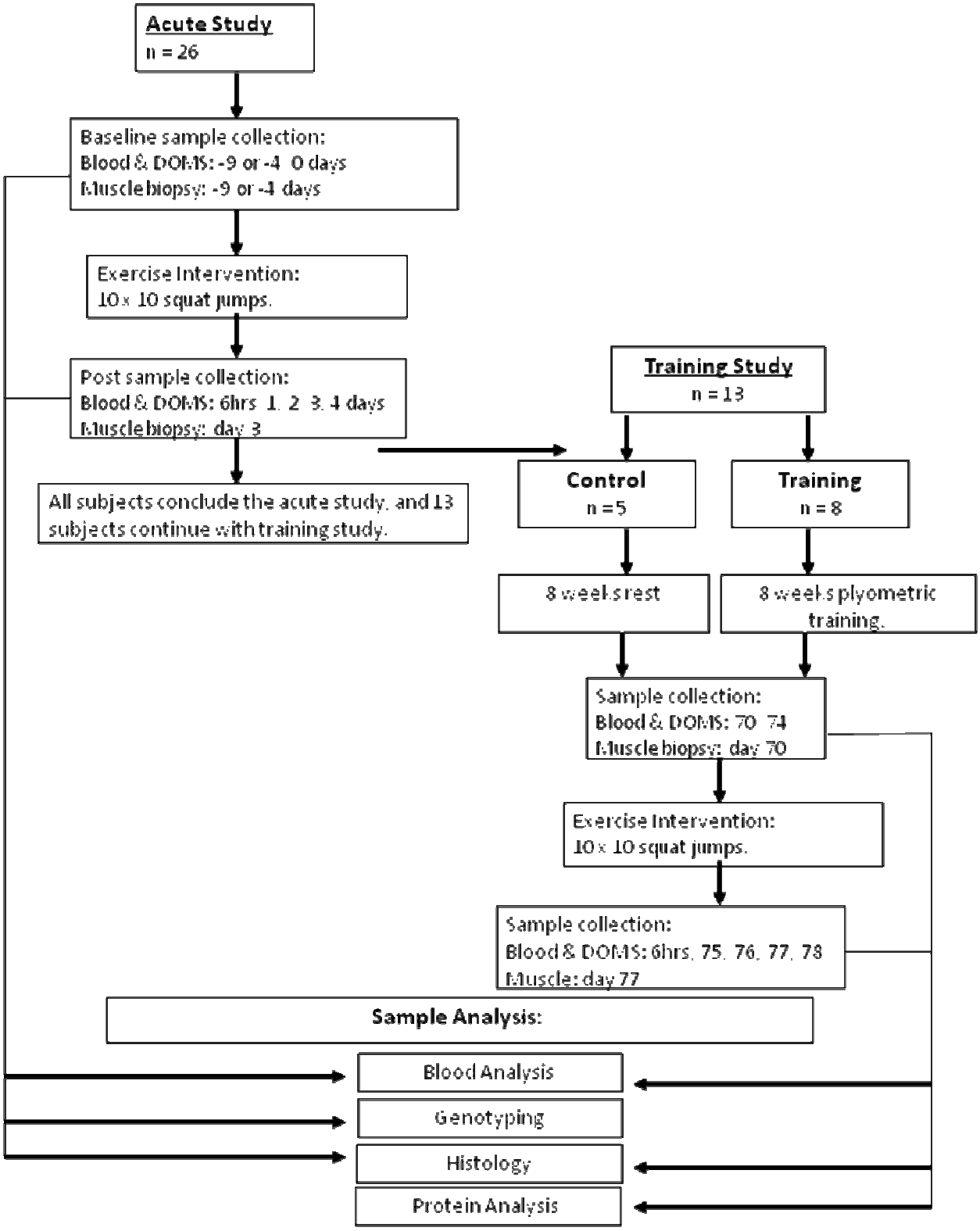


Figure 2.1 Study Overview

The exclusion criteria were:

- trained athletes
- no use of any anti-pain medication 1 week before the study
- subjects currently or chronically treated by any corticosteroid-containing medication (including inhaled forms)
- muscle injury in the previous 2 months (eg. haematoma, strains, sprains, laceration)
- sports injury to the lower limb within the past 6 months (eg. any knee injury, ankle sprains or strains)
- insufficient rehabilitation after any previous sports injury
- a pathologic cardiovascular condition
- informed consent was not obtained
- unwilling to abstain from pain or anti-inflammatory medication during the study

The subject characteristics for the acute study (n = 26) and training intervention (n = 13) are presented in Table 2.1.

Table 2.1 Subject characteristics.

	Acute Study (n = 26)	**Training Study (n = 13)**
Age (yr)	21.5 ± 1.6	21.5 ± 1.7
Height (cm)	175.9 ± 8.9	173.6 ± 10.3
Weight (kg)	73.0 ± 14.1	68.3 ± 17.7
Max Squat Jump height (cm)	222 ± 12	220.2 ± 13.7
95% squat jump height (cm)	210.9 ± 11	209.2 ± 13

Data presented as mean ± sd.

2.2 Study 1: Acute muscle damage study

2.2.1 Study design

After informing the potential subjects of the details of the experiment, and informing them of the request to stop using any anti-inflammatory or anti-pain medication throughout the study and to abstain from heavy/high intensity exercise 1 week before the plyometric exercise intervention and for the duration of the study, until the last blood sample was taken, those who gave informed consent, were entered into the study.

All participants were instructed to report to the testing site at the same time of day; 8 times on specified days (fig. 2.2). On laboratory day -9 baseline blood samples were collected and muscle biopsies obtained. After the initial visit the subjects performed a 10 minute warm-up followed by the exercise intervention. Blood samples were collected as shown in figure 2.2. Thirteen participants' baseline measurements were done on day -9 and the remaining thirteen participants who agreed to the training intervention had baseline samples collected on day -4.

2.2.2 Plyometric exercise intervention

Prior to the exercise intervention subjects completed a brief warm up which consisted of five minutes of backwards and forward running followed by five minutes of general stretching of the leg muscles. The subjects were allowed to perform short periods (<60 sec) of static stretching. This has recently been reported in a systematic review to not compromise maximal muscle performance (Kay and Blazevich 2012) and evidence that this duration of stretching could possibly provide

protection against mechanical injury is poor (McHugh and Cosgrave 2010). Nonetheless, many subjects felt comfortable with this action between sets.

The exercise regime in this study involved a series of jumping which caused the subjects to stretch and shorten their quadriceps muscles consecutively. It is the eccentric component to a depth of a 90° degree knee angle against gravity in one phase of the plyometric jump and the short transition (15-120 ms) from that position to the concentric contraction phase that the subjects were unaccustomed to and which differed from their routine activities.

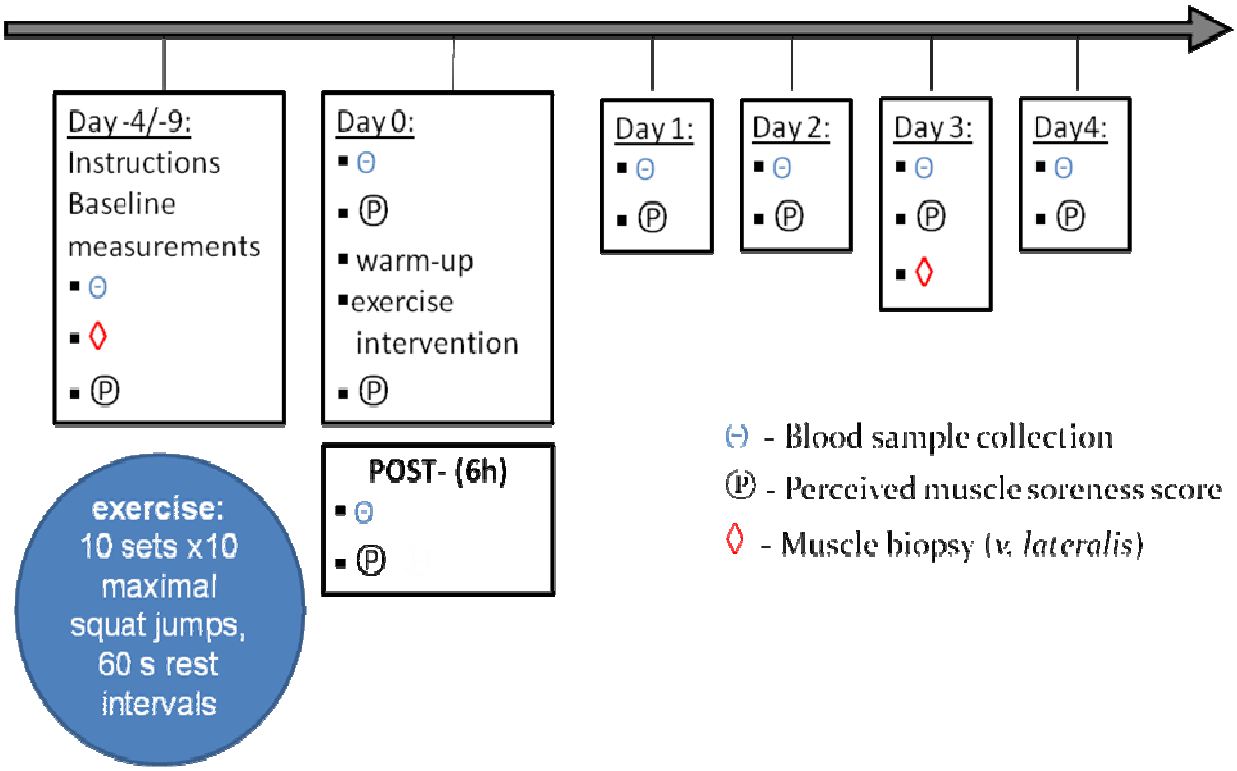


Figure 2.2 Summary of the acute study design.

Maintaining the upper body in the upright position and minimising hip flexion ensured less contribution of the *gluteus* muscle group to the exercise, thus focussing on the anterior thigh muscles, which is also unusual for jumping from a deep position. Subjects performed ten sets of ten maximal vertical jumps, separated by a 60 second recovery time between sets. Prior to starting the exercise, a maximal vertical jump was performed and the maximum height reached by the top of the head was taken as the highest point of the jump. To do this the researcher stood on a chair to see the head height. Ninety-five percent of this height then served as a target height that the participants attempted to maintain for each jump.

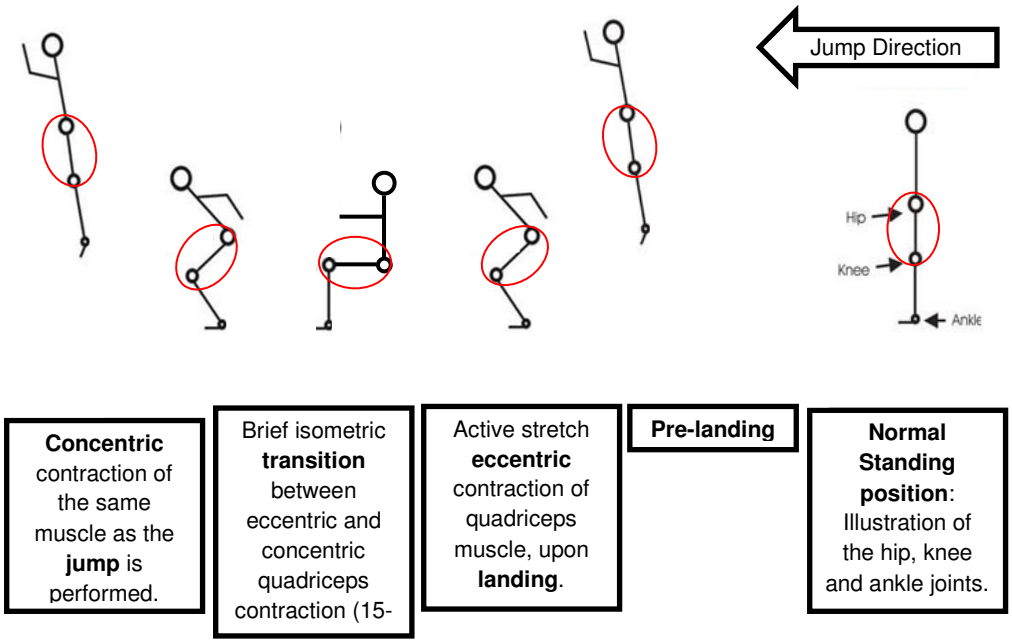


Figure 2.3 Diagram of the plyometric jump and description of the various phases of contraction of the quadriceps muscle.

On landing, participants were instructed to keep the trunk in an upright position minimising hip flexion while adopting a knee joint angle of approximately 90° in

order to promote muscle damage described by Twist *et. al* 2005. Between sets subjects were allowed to move around freely, sit down or do light stretching.

Subjects who could not maintain their target jump height were stopped between sets and given a one minute rest period before having to complete the previous set, thereby completing all of their 100 jumps. The technique was observed by the researcher who stood on a chair to make sure participants maintained their target heights and trunk position. The co-supervisor observed whether their legs reached a 90° angle.

This plyometric exercise protocol induced non-severe transient muscle damage and all subjects were instructed not to exercise for the duration of the study after the plyometric exercise intervention.

2.3 Study 2: Training Intervention

2.3.1 Study design

All participants were instructed to report to the testing site at the same time of day; on specified days (fig. 2.4). On laboratory day 1 baseline blood samples were collected and muscle biopsies obtained by a medical doctor from the *vastus lateralis* (v. lateralis) muscle of a randomly selected leg. The blood samples were taken prior to the skeletal muscle biopsy to prevent any inflammatory related factors released into the blood as a result of the biopsy from affecting the baseline sample. Four days after the initial visit the subjects performed a 10 minute warm-up followed by the exercise intervention (10x10 maximal squat jumps). Blood and muscle samples were collected as described in fig. 2.4.

The training intervention included 3 phases:

Phase 1

Participants were expected to come to the laboratory 6 times on 5 different days. During these visits, the following happened:

Visit 1: Participants were requested to sign the second part of the informed consent form. Then baseline measurements were taken. These included DOMS assessment, blood samples, a maximal vertical jump test (TENDO Weightlifting Analyzer V-206, AMR sport, Queensland, Australia), and a muscle biopsy.

Visit 2: On this day Blood samples were collected and DOMS assessed before and 6 hr after the exercise intervention (visit 3). Participants performed the plyometric exercise regime: a 10-minute warm-up followed by the exercise intervention.

Visits 4, 5, 6 and 7: Blood samples were collected and DOMS was assessed with the final muscle biopsy taken at visit 6.

Training commenced for eight weeks with the total number of jumps increasing progressively. Subjects performed 5x5 and 7x7 maximal squat jumps during the first two weeks and second two weeks respectively. A new target height was established every two weeks of training according to the participant's individual jump improvements. At the start of the first training week and at the end of the last training week, the maximal vertical jump height of participants were recorded using the fitrodyne plyometric jump mat which determines average and peak power and velocity of the jump.

Phase 2:

This phase include 8 weeks of plyometric jump training, over which the number of jumps was progressively increased (see below). The controls were instructed to rest and to refrain from doing any jumping activity for 8 weeks.

Week 1&2: During the first 2 weeks subjects performed a brief 10 minute warm-up followed by 5x5 maximal squat jumps, 3 times each week at the same time of day.

Week 3&4: Subjects performed a brief 10 minute warm-up followed by 7x7 maximal squat jumps, 3 times each week at the same time of day.

Weeks 5 – 8: Participants' performed a brief 10 minute warm-up followed by 10x10 maximal squat jumps, 3 times each week at the same time of day.

Phase 3:

Phase 3 was a repeat of phase 1 and included the control subjects.

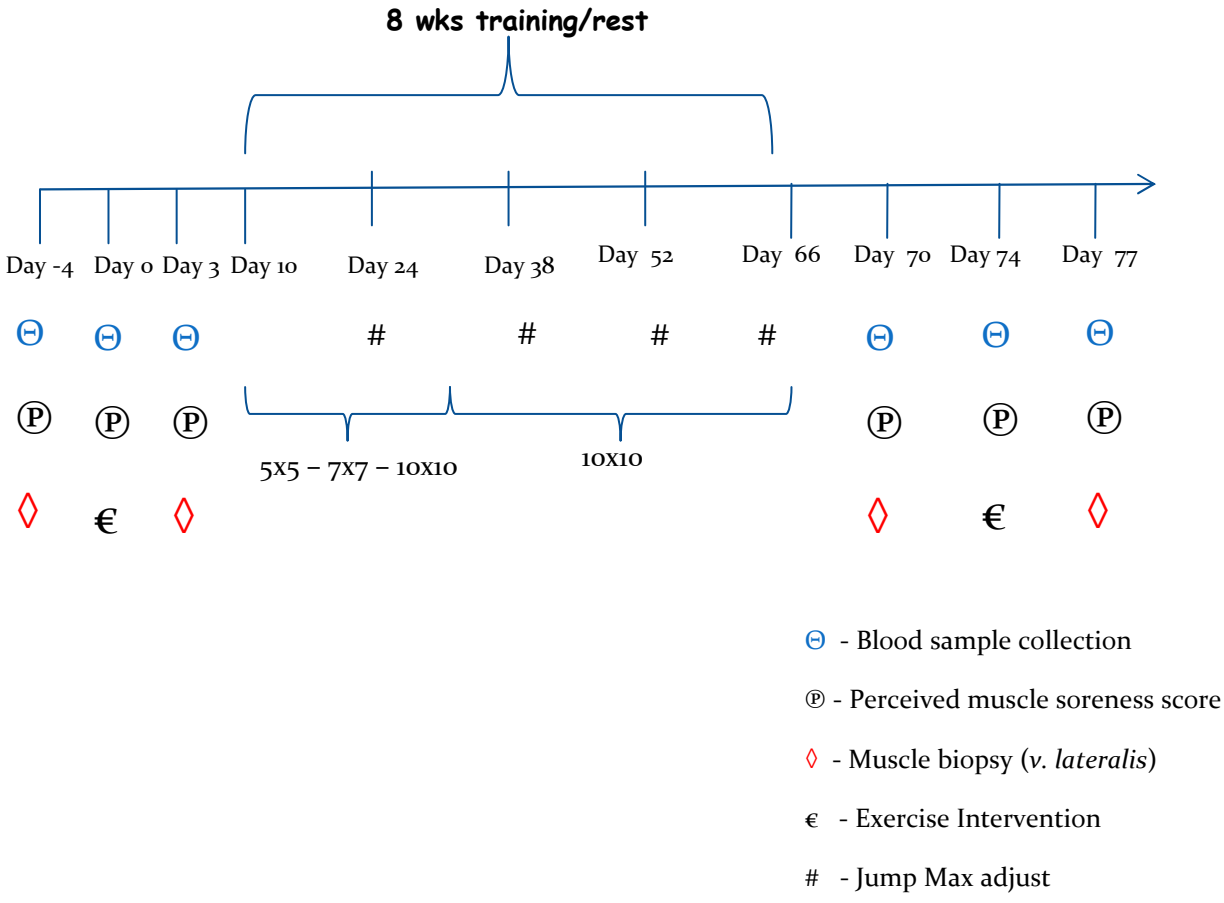


Figure 2.4 Summary of the training study design.

2.4 Testing, sample collection and analysis

2.4.1 Functional test

Participants performed three maximal vertical jumps at baseline and after the plyometric training period with a spring cable attached to their waists, average and peak velocity (m/s) and power (w) of participant's maximal vertical jumps were recorded using a FitroDyne device (TENDO Weightlifting Analyzer V-206, AMR sport, Queensland, Australia), similar to that described by Jennings et al. 2005.

2.4.2 Blood collection

All blood samples were drawn from the forearm vein in serum separating and EDTA tubes (SST and EDTA, BD Vacutainer, Becton Dickinson and Company, New Jersey, USA), with the subjects lying in the supine position for 10 minutes first. On the biopsy days, the blood samples were taken prior to the skeletal muscle biopsy to prevent presence of any acute inflammatory related factors released in the blood as a result of the biopsy. Nine or four days after the initial visit, blood was first drawn before the subjects performed a 10 minute warm-up followed by the exercise intervention and again six hours after the exercise intervention. Blood samples were then collected as described in figures 2.2 and 2.3.

Fifteen ml of blood was collected in 3 separate vacuum sealed tubes. Two intended for serum separation (SST, BD Vacutainer, Becton Dickinson and Company, New Jersey, USA) were inverted 8 times, left to clot and then placed on ice. Thereafter, one tube was sent for independent analysis (see blood analysis) and the other centrifuged within 20 minutes of clotting, at 3500 rpm for 10 min at 4℃. Serum was

collected and stored at -80℃. One tube intended for plasma (EDTA, BD Vacutainer, Becton Dickinson and Company, New Jersey, USA) was inverted 5 times and centrifuged at 3500 rpm for 10 min at 4℃. Plasma was collected and stored at -80℃.

2.4.2.1 Blood analysis

Total serum CK, Mb, LDH and CRP as well as plasma whole blood counts were determined by commercial laboratory *PathCare* pathology laboratory (Stellenbosch Medi Clinic, South Africa) using a one-step sandwich assay (Access CK-MB assay, Beckman Coulter, Inc.).

2.4.2.2 ELISA

A commercially available enzyme-linked immune-sorbent assay (ELISA)-based bead kit system (eBioscience ELISA IL-6 ref: BMS625TWO and lot: 62920021, TNFα ref: BMS622TWO and lot: 62913028, Saint Diego, USA refer to Appendix V for details) was used for the determination of plasma IL-6 and TNF-α concentrations. ELISA kits were stored at 4˚C and lyophilized controls stored at -20˚C, and protocols were carried out as instructed by the manufacturer. All samples were run in duplicate.

Briefly, the absorbance of the 96 well plates was read using Microplate Reader (EL800 Universal, Bio-Tek Instruments INC, Vermont, USA) and a software programme was used for analysis (KC junior, Bio-Tek Instruments INC, Vermont, USA). The system uses multiple colour-coded bead sets, each conjugated by covalent bonding with primary antibodies against IL-6 and TNF-α by. The antibody-coupled beads were then incubated with samples and standard solutions that bind target molecules, non-specific unbound proteins are then washed away. Another

primary antibody (biotinylated detection antibody specific for a different epitope on the cytokine) is added to the reaction solution, which then forms a sandwich of antibodies around the cytokine. Next phycoerythrin labeled streptavidin (streptavidin-PE) is added which binds the biotinylated primary antibodies allowing for detection by fluorescence. Finally, concentrations are determined by fluorescence intensity, after each well content was aspirated by an automated microplate reader (EL800 Universal Microplate (Bio-Tek Instruments INC, Vermont, USA), which identifies the cytokine according to the colour of the bead bound to it (a detailed protocol provided in Appendix IV).

2.4.3 Muscle Soreness

Perceived muscle soreness scores were obtained to indicate soreness of the knee-extensor muscles (*rectus femoris, rectus intermedius, vastus lateralis, and vastus medialis.*). The scores were recorded with the subjects in different positions: during left and right leg squats and with their hands on their hips and squatting to an approximate knee angle of 90°. The participants were asked to describe the level of perceived soreness based upon the visual pain scale which is similar to Borg's rating of perceived exertion scale (Sorichter et al. 1997).The scale is numbered from 0 to 10, with 0 being indicative of no distress of the quadriceps muscles and 10 being agonizing pain (Table 2). Perceived muscle soreness scores were assessed each time the subjects visited the laboratory before blood was collected as described in figures 2.2 and 2.3.

Table 2.2 Rating of perceived soreness.

Score	Worded equivalent
0	No distress
1	
2	Annoying
3	
4	Uncomfortable
5	
6	Dreadful
7	
8	Horrible
9	
10	Unbearable Distress

2.4.4 Muscle biopsies

A total of two biopsies were obtained from each subject during the acute study and two more biopsies were obtained from the thirteen subjects who continued with the training intervention. A baseline biopsy was obtained from each subject from a randomly selected leg nine or four days before the exercise protocol was performed. The next biopsy was obtained on the 3rd day following the acute plyometric exercise intervention on the leg which was not biopsied the first time (figure 2.2). A third biopsy was collected from thirteen subjects after their 8 week training period, followed by a final biopsy 7 days after that (figure. 2.4).

All muscle biopsies were performed by the same medical doctor with extensive experience in this procedure. The researcher provided all the support to the medical doctor before and during the procedure. Using local skin anaesthesia, trephine needle biopsies were used to obtain the sample from the *vastus lateralis* muscle. Although the hip extensor is the prime mover in a vertical jump, evidence exists for muscle damage of the *vastus lateralis* muscle after plyometric exercise (Marginson et al. 2005; Twist and Eston 2005). Moreover the *vastus lateralis* muscle was chosen as the site for the muscle biopsy because amongst all of the muscles

activated (Chimera et al. 2004) during the squat jump it is most easy to access and is routinely biopsied in laboratories around the world. The site of the biopsy was determined with the subjects lying down in a supine position, with their upper backs slightly elevated and with the arms alongside the body in the pronated position. Where the thumb points touched medially towards the upper leg the biopsy was taken.

The biopsy procedure: A biopsy area was cleaned with 70% alcohol before commencing with the procedure. A local anaesthetic, containing 2% Lignocaine HCL, m/v, and 1:80 000 noradrenaline (Xylotox, Adcock Ingram Ltd., Johannesburg, SA), was injected (subcutaneously and then intra-muscularly) before the incision with a scalpel blade was made. The needle biopsy method was used (5 mm Bergström biopsy needle; STILLE, Sweden). Whilst the researcher continued to assist the doctor in the sterile environment, the muscle sample was cut in three pieces by the co-supervisor: one was orientated and mounted in tissue freezing medium (Tissue-Tek, Jung; Leica Instruments GmbH, Germany) and then frozen in isopentane, precooled by liquid nitrogen and stored at -80℃ until further analysis, one was fixed in gluteraldehyde solution for ultrastructural analysis and the third piece was snap frozen in liquid nitrogen for fibre-typing analysis.

2.4.5 Muscle Biopsy Analysis

Baseline muscle samples from each subject were used to determine their muscle fibre-type by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE), immunofluorescence and electron microscopy.

2.4.5.1 SDS-PAGE

Sample preparation

The muscles were frozen in liquid nitrogen, and stored at -80℃. Small pieces of muscle were cut from the frozen muscle samples and placed in 500µl of ice-cold lysis buffer. The cut sections of muscle were subsequently homogenized with a tissue homogenizer (Ultra-Turrax, Germany) in glass test tubes. The homogenates were decanted into eppendorfs and centrifuged at 300 rpm for 5 mins at 4℃. The resulting supernatant was decanted into new eppendorfs and total protein was assayed according to the method by Bradford (Bradford 1976). The amount of protein run on the gel was 120 µg of total protein per lane in sample buffer containing 5% β-MEtOH, 2.5% SDS, 10% glycerol, 62.5 mM Tris, pH 6.8 and 0.1% bromophenol blue. Samples were then boiled for 5 min and allowed to cool prior to electrophoresis.

Separation

The large (Protean IIxi – gel size: 16 x 16 cm) electrophoresis system was used (Biorad Laboratories, Hercules, California, USA) for protein separation. The concentration of the separating gel was 8%. Separation of MHC isoforms was perfomed using a slightly modified method described by Kohn and Myburgh (Kohn and Myburgh 2006). The running conditions for the large-gel apparatus were 75V (constant voltage) for 38 h at 4℃.

The mini electrophoresis systems (Biorad Laboratories, Hercules, California, USA) were used for protein separation. The concentration of the separating gel was 8%. The running conditions for the large-gel apparatus was 100V for 10 min and 200V for 70 min (constant voltage) at room temperature.

Staining

Gels were stained with Coomassie Brilliant Blue. The gels were placed in Coomassie for 10 min at room temperature while gently rocking and then microwaved for 1 min on a high-power setting and left to stain further while rocking at room temperature for another 10 min. Coomassie solution was then removed and the gels placed in a destaining solution for a minimum of 2 hours. Gels were scanned with a computer scanner and relative MHC proportions were determined using ImageJ (1.41 software National Institutes of Health, Bethesda, USA, http//rsb.info.nih.gov/ij).

2.4.5.2 Western Blotting

All mini gels were transferred to PVDF membrane (Immobulon, Millipore) for 1 hour 15 mins at constant 0.5A and 15V, using the mini transblot system (Bio-Rad laboratories). Following the transfer membranes were incubated and blocked in blocking solution containing 5% dry milk in Tris Buffered Saline - Tween (TBS-T) for 1h 30 min at room temperature. Calpain-3 protein levels were detected by immunoblotting using antibody: NCL-CALP-12A2 (calpain 3; dilution 1:1000; mouse monoclonal antibody, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Blots were washed in T-TBS and incubated for 1 hour with a secondary antibody: horse-radish peroxidase-linked donkey anti-mouse (DAKO, Denmark; dilution 2:5000).

The membranes were incubated with luminescence reagent (ECL Plus, Amersham, GE Healthcare, Biosciences, New Jersey, USA), exposed to X-ray film (Hyperfilm, Amersham, GE Healthcare, Biosciences, New Jersey, USA) according to manufacturer instructions, developed and scanned. Band analysis was performed with specialised software (ImageJ 1.41 National Institutes of Health, USA, http//rsb.info.nih.gov/ij).

2.4.5.3 Immunofluorescence

Cross sections of the muscle tissue were cut at 10 µm using a cryostat microtome (Leica CM1100, Leica Microsystem Nussloch GmbH, Germany) at -22ºC and mounted on slides. The following day, the sections were brought to room temperature, and rinsed in 0.01 M phosphate buffered saline (PBS) containing 0.25% detergent (Triton X-100) for 15 min and washed with PBS (3 X 5 min). The sections were incubated in a solution containing MHC II antibodies (1:250; A4.74, mouse monoclonal antibody, Developmental Studies Hybridoma Bank, Iowa City, IA, USA) for 1h, after which the sections were washed with PBS (3 X 5 min), incubated for 1h at room temperature with conjugated secondary antibody (1:250, goat anti-mouse, conjugated to Alexa fluor 488, Invitrogen, Eugene, Oregan, USA), washed again with PBS (3 X 5 min), incubated for 1h at RT with dystrophin (1:250, rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA, USA), washed again with PBS (3 X 5 min) and incubated for 1h at room temperature conjugated secondary antibody (1:250, goat anti-rabbit, Alexa fluor 594, Invitrogen, Eugene). The sections were then washed in PBS (3 X 5 min), and mounted with the fluorescent mounting medium (Dako, Denmark). Samples were observed with a direct fluorescence microscope (Leica DM 5000 B, Wisconson, USA) with x10 and x20 objectives. The researchers observed fibres for positivity to MHCII antibody (MHC I, negative; MHC IIx, faintly positive; MHC IIa, strong positivity) to calculate fibre type percentage whilst also assessing the fibres for loss of dystrophin staining around the fibres to determine which were damaged. When the loss in dystrophin staining was localized in the sarcolemmas of two adjacent fibres and it was impossible to determine with absolute certainty which fibre was damaged, both fibres were considered damaged. For each muscle sample, three sections were

stained for MHCII and dystrophin, and the best section was used to perform the analysis.

2.4.5.4 Electron Microscopy (EM)

Immediately following the biopsy, muscle specimens of eight participants were cut into thin long sections (1mm x 5mm) down with the specimen oriented longitudinally with respect to the fibre direction fixed in 2.5% gluteraldehyde stored in the fridge and transported to a pathology laboratory (Tygerberg hospital) the following day, where the microscopist dehydrated the samples with ethanol 30, 50, 70, 95, and 100% v\v. After dehydration, tissue pieces were put in propylene oxide for 30 min and embedded into resin (EPON 812, Electron Microscopy Sciences, Hatfield, Pennsylvania) 24h before incubating to harden at 50℃ for 48 hours. Ultrathin sections of resin-embedded muscle were examined with a Jeol-Jem 1011 transmission electron microscope (Jeol-Jem 1011 TEM, Leica Microsystem Nussloch GmbH, Germany).

With electron microscopy the researcher confirmed whether structural muscle damage typical of eccentric damage was evident and made certain that the muscle fibre was not damaged due to the muscle biopsy method.

Muscle fibres were typed and studied quantitatively using the morphometric technique of Weibel et al. (Weibel et al. 1966). Ten fibres in longitudinal section were studied per section. For each fibre 3 micrographs (final magnification of 2000; 12,000 and 30 000) were taken for Z-line measurement. The Z-line measurements were made on the region of filament overlap (Salmons et al. 1978), where 10

randomly chosen straight Z-lines were outlined and the mean widths of these were measured.

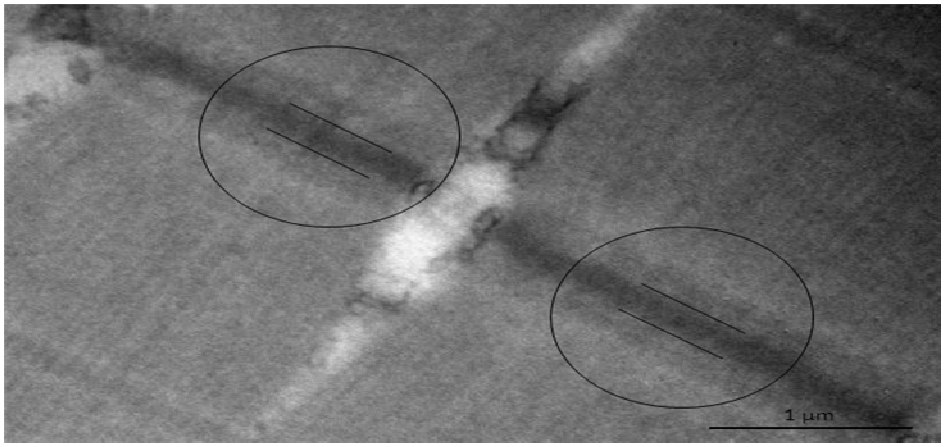


Figure 2.5 Longitudinal section from a human muscle fibre indicating the central, filament overlap zone measured for Z-line width (30 000x).

All fibres were classified as slow-twitch oxidative (SO) or fast-twitch muscle fibres. Wide Z-lines were previously shown to be associated only with slow-twitch fibres (Wroblewski and Jansson 1975).

2.4.5.5 Immunogold

Ultrathin longitudinal sections were cut at 60-70 nm (gold colour of section), and viewed with a transmission electron microscope (Jeol-Jem 1011 TEM, Leica Microsystem Nussloch GmbH, Germany), and placed on nickel grids on 150 mesh (formavar-coated). The sections were then treated with metaperiodate (saturated aqueous solution) to dissolve the resin, washed in TBS 10 x 5mins and placed in citrate buffer pH 6 for 40 min at room temperature. Next the sections were blocked with 1% BSA-C-TRIS buffer for 30 min, incubated with the primary antibody, Titin c-20 (1:10 in 0.1% BSA-TRIS buffer; sc-8724, mouse monoclonal antibody, Santa

Cruz Biotechnology, Santa Cruz, CA, USA) at 4℃ overnight and washed with 0.1% BSA-TRIS buffer 3 x 10 min the next day. Secondary antibody was then added which was diluted 1:50 in 0.1% BSA-TRIS buffer, after which the wash step was repeated. The samples were post-fixed with 2% gluteraldehyde in PBS for 10 min. Sections were then washed with 0.1% TBS-tween 6 x 12 min, stained using uranyl acetate (5 min), dried with filter paper and stained with lead citrate for 2 min in the dark. Finally the sections were washed with distilled water 2 x 5 min, dried with filter paper once more and were then ready to be viewed under the microscope. This was performed by the co-supervisor in Italy (Department of Experimental Biomedicine and Neuroscience Clinical University of Palermo, Palermo, Italy).

2.4.5.6 Genotyping analysis

A piece of snap frozen sample (baseline muscle biopsy) was sent to the Central Analytical Facility (CAF, Stellenbosch University, South Africa). DNA extraction was performed by the CAF technicians and analysed for selected SNPs within the genes of selected muscle structure proteins.

The TNF and IL6 loci were amplified using published primers.

PCR conditions were as follows:

- 1x KAPA ReadyMix (*Kapa Biotech* / *Lasec*)
- 4pmol of each primer
- 20ng DNA template and a final volume of 20µl.

The PCR was performed in a cycler (Verity, Applied Biosystems, Life Technologies, California, USA) with the following cycling conditions: 95℃ for 5 minutes followed

by 35 cycles of 95℃ for 30 seconds, 60℃ (TNF) / 50℃ (IL6) for 30 seconds and 72℃ for 30 seconds and a final extension of 72℃ for 10 minutes.

Post-PCR purification was done using a standard purification system (NucleoFast, Separations, Germany). Sequencing was performed with BigDye Terminator (V1.3, Applied Biosystems, Life Technologies, California, USA) followed by electrophoresis on the DNA Analyser (3730xl, Applied Biosystems, Life Technologies, California, USA). Sequences were analyzed and trimmed using Sequencing Analysis (V5.3.1, Applied Biosystems, Life Technologies, California, USA). Alignments were done using the ClustalW module (BioEdit version 7.0.4.1, Hall, 1999) with the downloaded SND-ID as reference.

Possible polymorphism variants:

- MYLCK C49T genotype (CC, CT, TT)
- TNFA G-308A genotype (GG, GA)
- IL6 G-174C genotype (GG, GC, CC)

2.4.6 Statistical Analysis

Data were assessed for normality by inspecting normal probability plots. A logarithmic transformation (Log10) was applied to non-normally distributed data (CK, Mb, LDH, CRP and DOMS) before analysis. Changes in DOMS (in squat and in standing position) and blood parameters (CK, Mb, LDH and CRP) in responders Vs no-responders over time were analysed by two-way mixed model repeated-measures analysis of variance. If a significant difference was detected, this was further evaluated by the post-hoc Fisher LSD test. Significance was accepted at an alpha of $P \leq 0.05$.

Chapter 3: Results

Acute Study:

3.1 Blood markers of muscle damage (CK & Mb):

The data analysis of CK and Mb levels in the serum showed an immediate rise of these markers after the acute bout of plyometric exercise, although they have a different response over time. In fact, CK continues to rise until 4 days ($P < 0.00001$) after exercise (figure 3.1 A), while Mb presented a double peak, 6hrs ($P < 0.00001$) and 2 days ($P < 0.00001$) after the exercise bout (figure 3.2 A). Closer observation of the data showed large inter-individual variability in both the CK and Mb response to exercise among the 26 subjects (CK range: 153-71,024 U/L at 4 days after; Mb range: (20-2382 ng/ml 4days after). The subjects have been considered high (n=10) or low (n=16) responders based on CK activity after the plyometric intervention, on the base of the cut-off limit (1000 U/L) used to diagnose rhabdomyolysis. From this point forth the groups will be defined as high and low responders.

CK and Mb levels peaked six hours ($P < 0.00001$) and one day ($P < 0.00001$) post exercise, respectively in the low responder group after which values return to baseline the following 24 hours. In contrast, CK levels continue to rise four days post exercise in the high responder group. Mb, interestingly once more presented with a double peak at 6hrs and 3 days post exercise. The levels of CK and Mb were significantly higher in the high responder group compared to the low responders at respective time points (see figures 3.1 and 3.2).

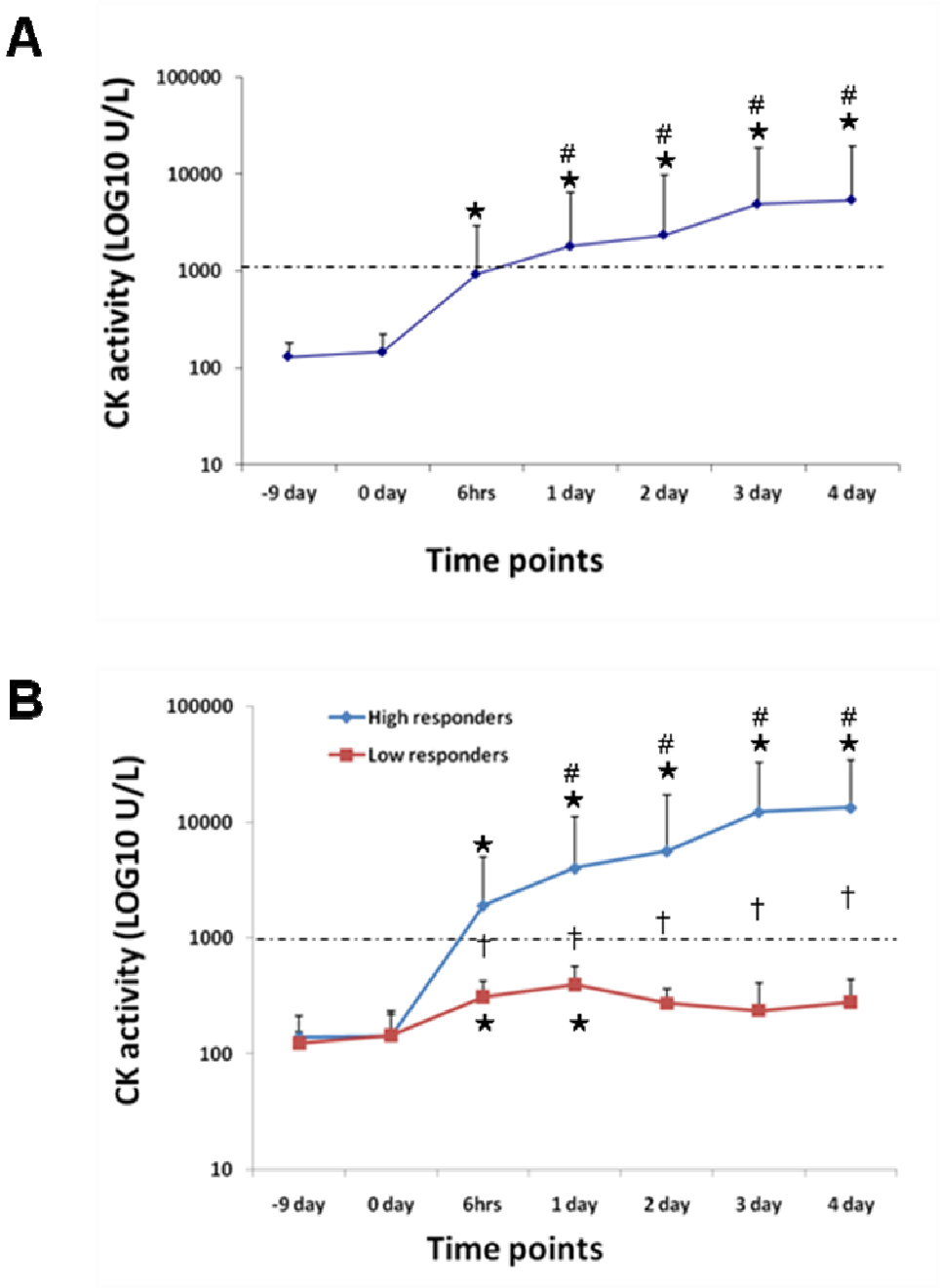


Figure 3.1 Change in serum creatine kinase (CK) over time before and after a single bout of plyometric exercise. A) Average CK activity (Log_{10} U/L) of all subjects (n=26). B) The blue and red lines indicate CK activity (Log_{10} U/L) for the high (n=10) and low responder (n=16) groups respectively. The dashed line indicates the rhabdomyolysis cut-off. * Significantly different from time points -9 and 0, ($P < 0.0001$); # significantly different from time point 6hrs, ($P < 0.05$); † significantly different response between high and low responders, ($P < 0.0001$). Data are expressed as mean ± sd.

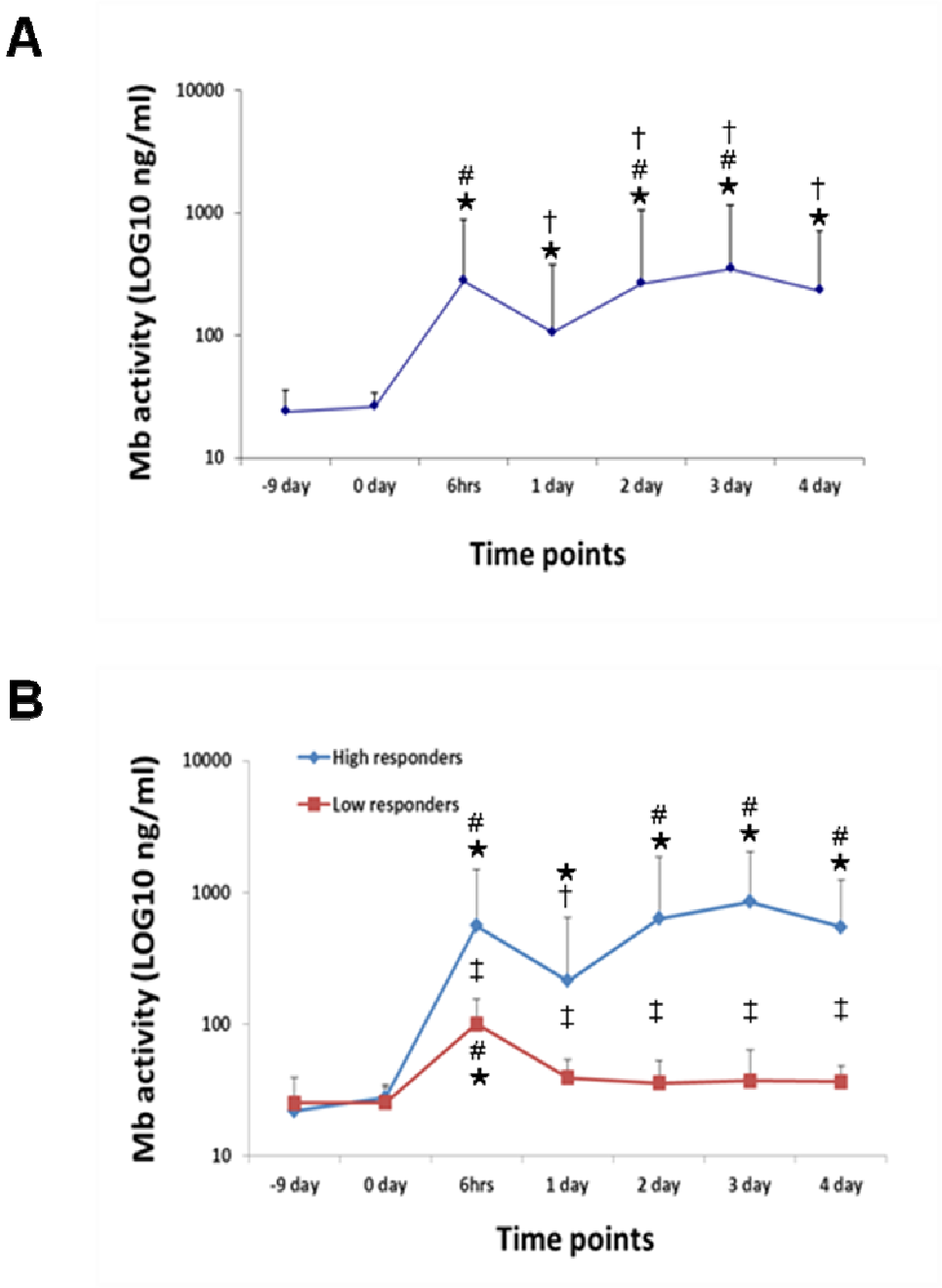


Figure 3.2. Change in serum myoglobin (Mb) over time before and after a single bout of plyometric exercise. A) Average Mb activity (Log_{10} U/L) of all subjects (n=26). B) The blue and red lines indicate Mb activity (Log_{10} U/L) for the high (n=10) and low (n=16) responder groups respectively. * Significantly different from time points 9 and 0, ($P < 0.0001$); # significantly different from day 1, ($P < 0.01$); † significantly different from time point 6 hrs; ‡ significantly different response between high and low responders, ($P < 0.001$). Data are expressed as mean ± sd.

3.2 Subject characteristics before the exercise intervention that may affect the CK response:

3.2.1 Physical characteristics

Neither weight, jump height nor the product of weight x jump height correlated with CK activity at 4 days post exercise, which was the time point of the peak response and all other time points (fig. 3.3 A, B and C).

3.2.2 Muscle characteristics

% Fibre type measured at baseline by immunofluorescence for MHC II (figure 3.19 A) did not correlate with the CK response at any of the time points post- exercise (3.4 A, B, C and D).

Baseline interleukin-6 receptors (IL-6R), was quantified by counting fluorescent positivity along the muscle fibre membrane, in two different ways (figure 3.5) A) IL-6R% cell + (S – section along the membrane expressing fluorescent positivity) and B) IL-6R% cell + (D – fluorescent dots along the membrane) were not found to be associated with the CK response at any time point.

3.2.3 Genetic characteristics (results not shown in a figure)

IL-6 genes (IL6 -174)

Subjects (n=26) were genotyped for the *IL6* G-174C SNP and all samples were found to be homozygous wild type GG without the homozygous CC mutation.

TNFα genes (TNFAα-308)

Subjects (n=26) were genotyped for the *TNFA*-α-308A SNP and all samples were found to be homozygous wild type GG without the homozygous GA mutation.

MLCK C49T

MLCK C49T mutant homozygous CC was not found to be associated with either an increase in CK activity (figure 3.6 A) and Mb concentration (figure 3.6 B) at 4 days post exercise, which was the time point of the peak response and nor with any other time point investigated in this study.

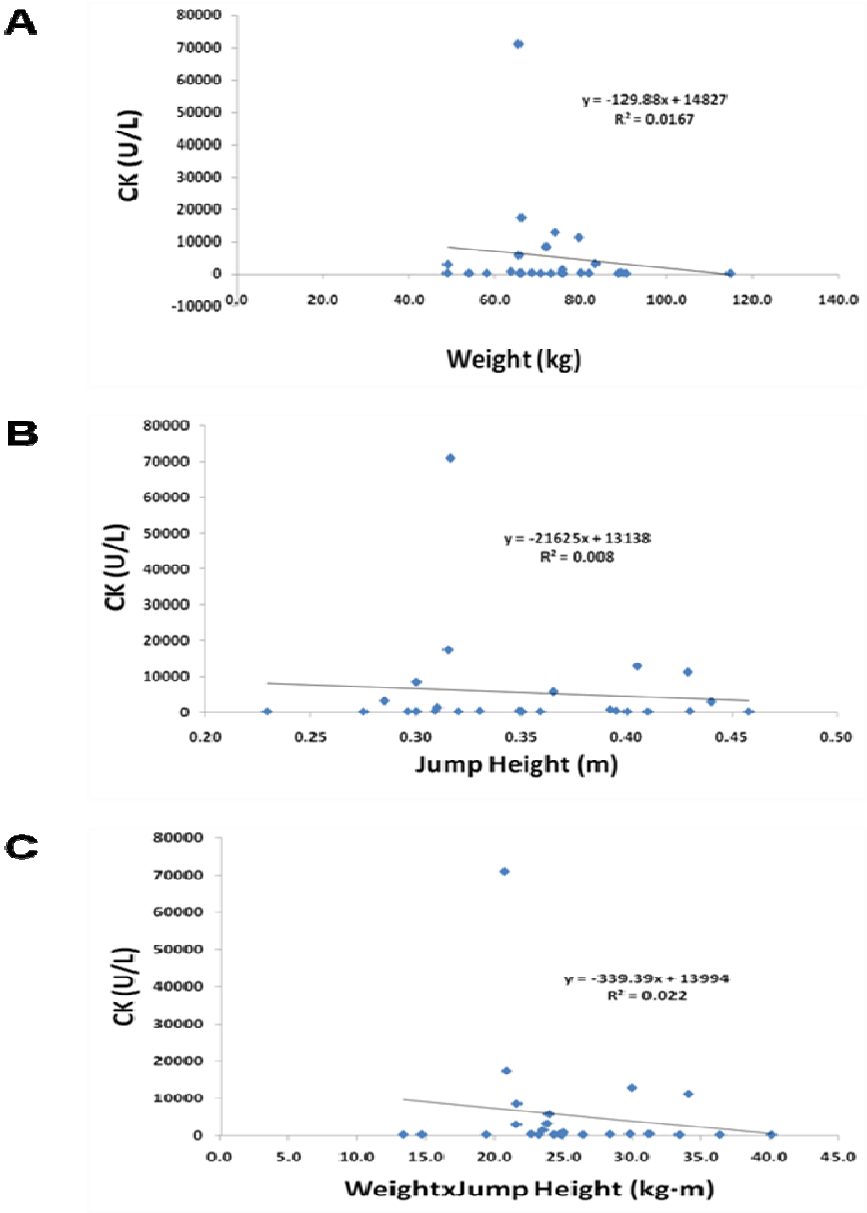


Figure 3.3. Relationship between creatine kinase (CK) activity 4 days after plyometric exercise and (A) weight (kg), (B) jump height (m) and (C) weightxjump height (kg·m). Data are expressed as mean ± sd.

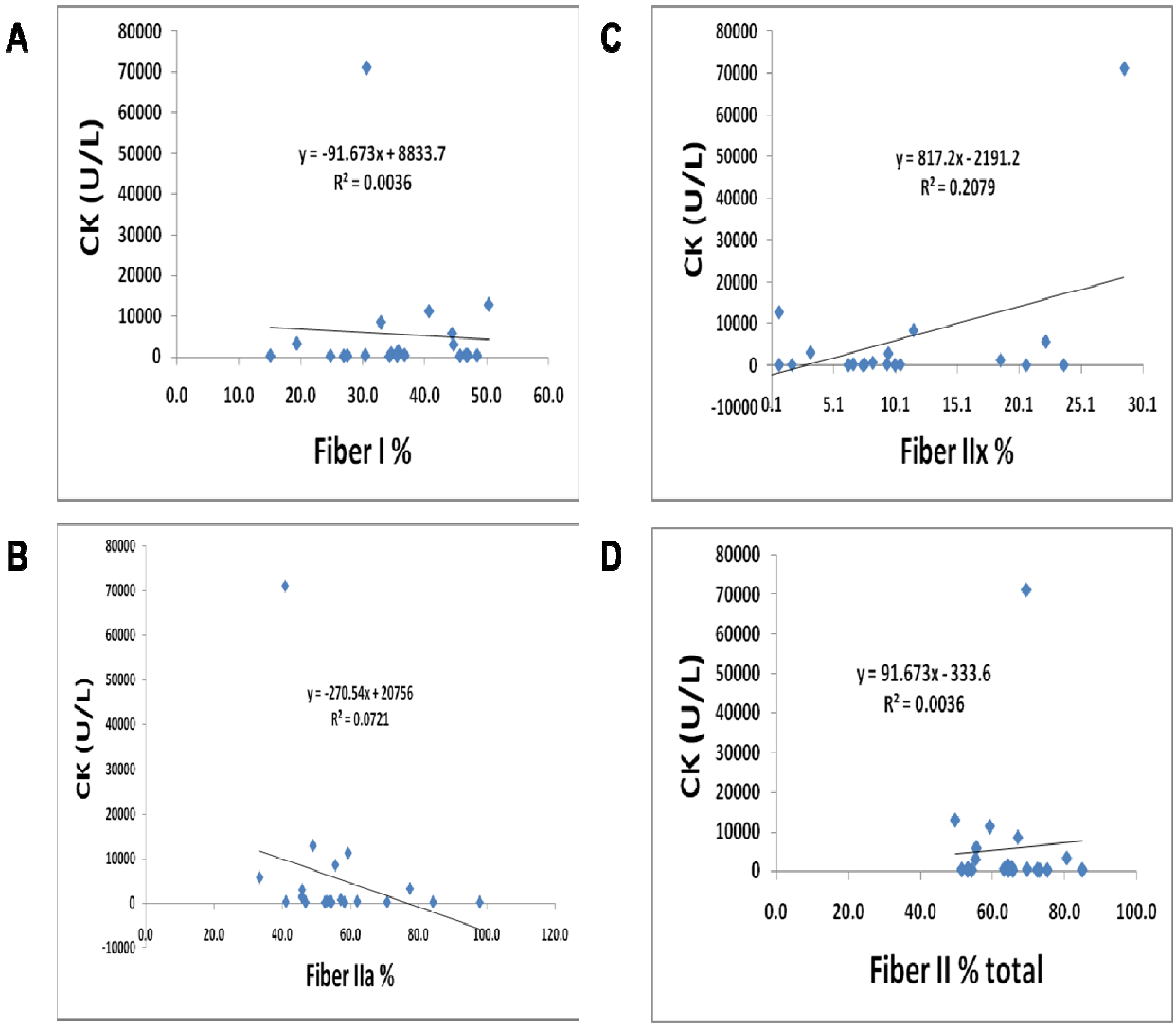


Figure 3.4. Relationship between creatine kinase (CK) activity 4 days after plyometric exercise and individual percentage fibre type (A) fibre I%, (B) fibre IIa% (C) fibre type IIx% and (D) fibre type II total %. Data are expressed as mean ± sd.

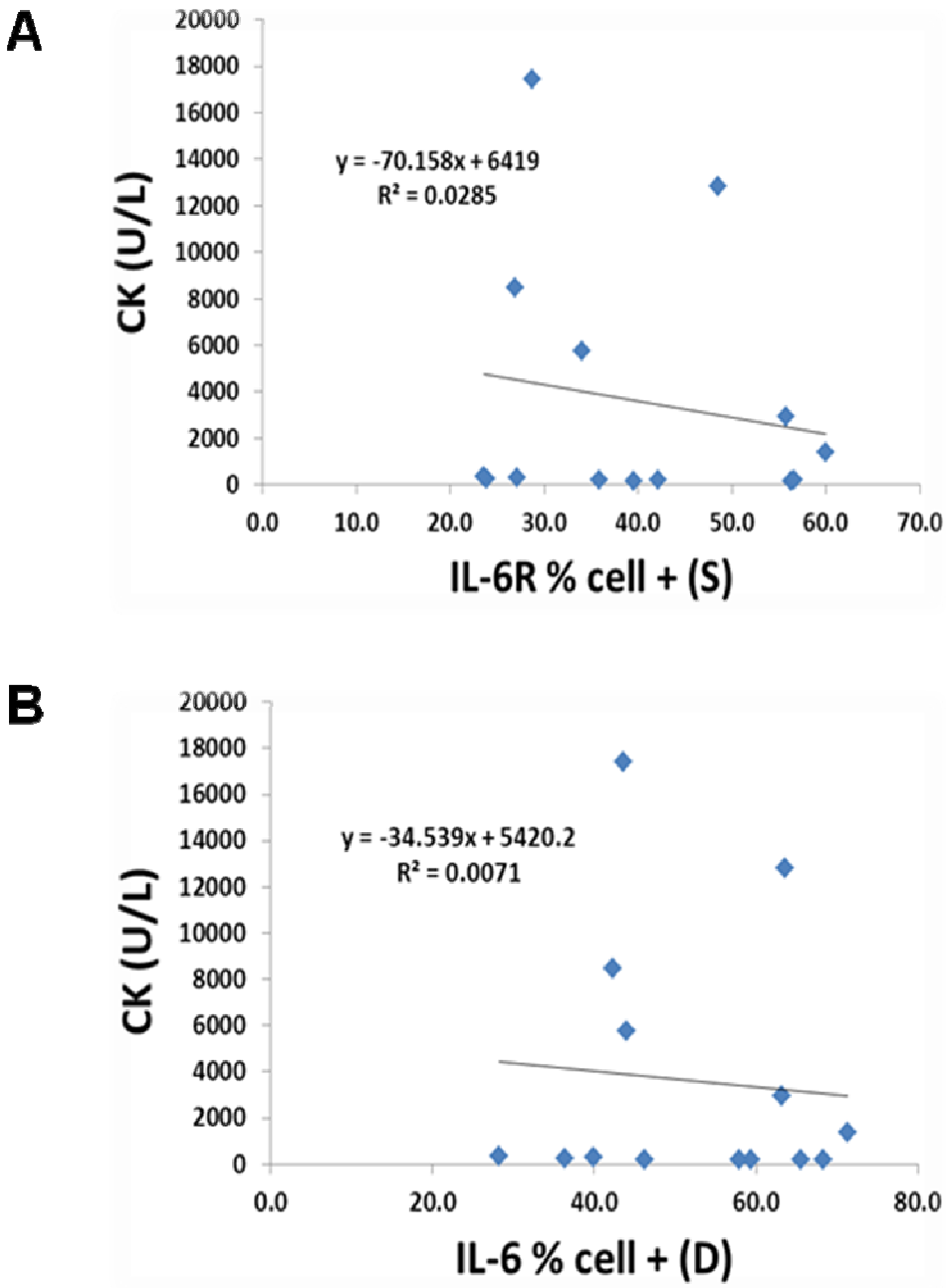


Figure 3.5. Relationship between creatine kinase (CK) activity 4 days after plyometric exercise and (A) IL-6R% cell + (S), (B) IL-6R% cell + (D). Data are expressed as mean ± sd.

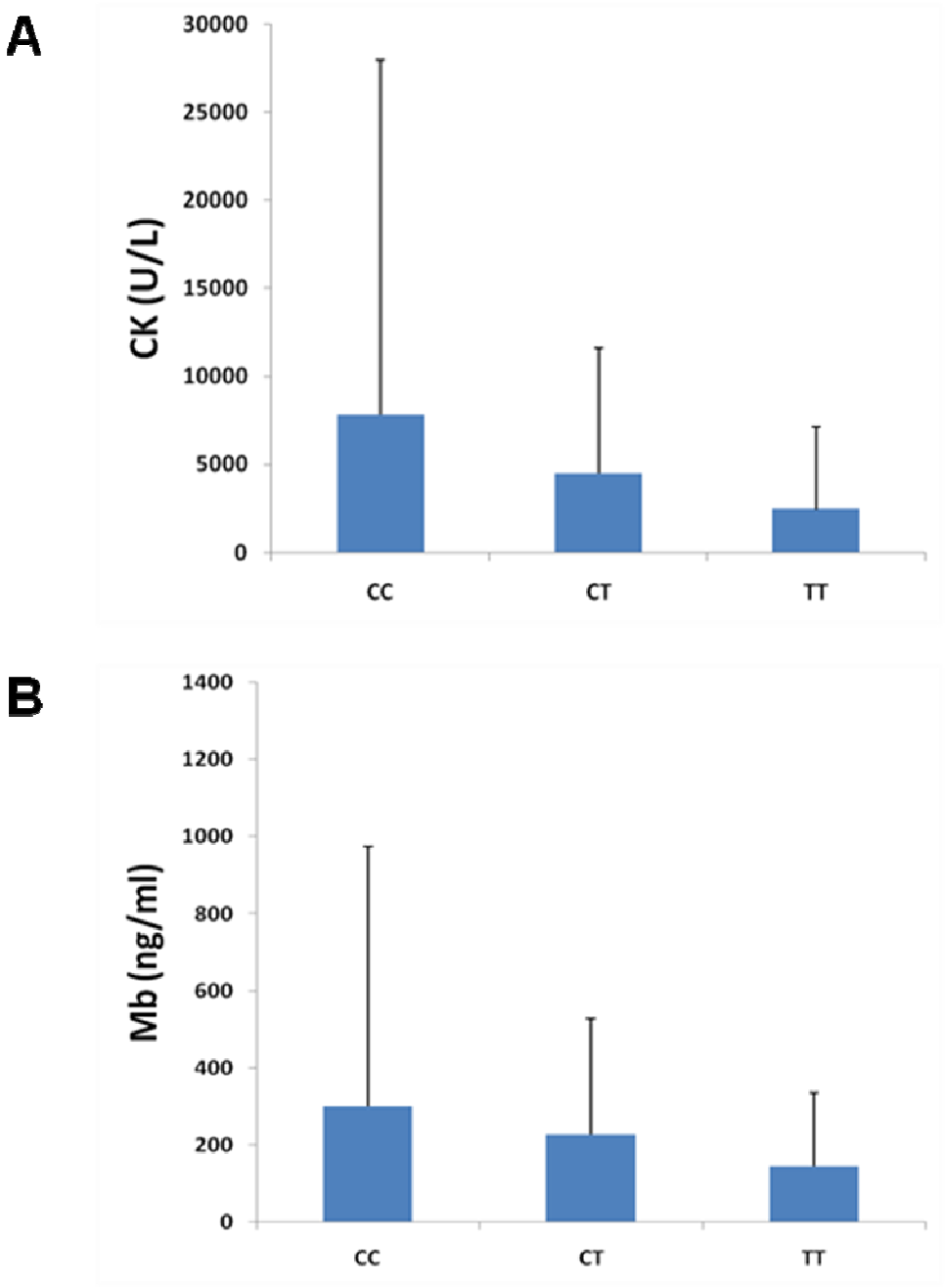


Figure 3.6. Creatine kinase (CK) activity (A) and **myoglobin (Mb) concentration** (B) 4 days after plyometric exercise by MLCK C49T genotype, (CC = wild type homozygous; CT= heterozygous; TT = mutant homozygous). Data are expressed as mean ± sd.

3.3 Response of subjects to the exercise intervention:

3.3.1 Delayed Onset Muscle Soreness

DOMS increased immediately after exercise (6 hrs) and increased until day 2 before gradually returning to baseline values, although soreness was only recorded up to day 4. In all of the subjects, soreness of the quadriceps increased immediately (6 hrs) post plyometric exercise in both positions (standing $P < 0.02$ and squat $P < 0.0002$) compared to baseline and remained high until day 4 for the high responders only. The high responders peaked at day 1 in the standing position ($P < 0.01$) and day 2 ($P < 0.0002$) in the squat position compared to baseline (figure 3.7). The low responders experienced significantly higher soreness scores at 6hrs post-exercise than the high responders in the standing position, but the high responders continued to experience significantly higher muscle soreness after day 3 and 4 compared to the low responders in the squat position (figure 3.8 A).

3.3.2 Damaged fibres

No correlation between the percentage of damaged fibres at day 3, measured by the loss of muscle fibre membrane loss of dystrophin staining, and the CK response was observed for all time points (figure 3.9).

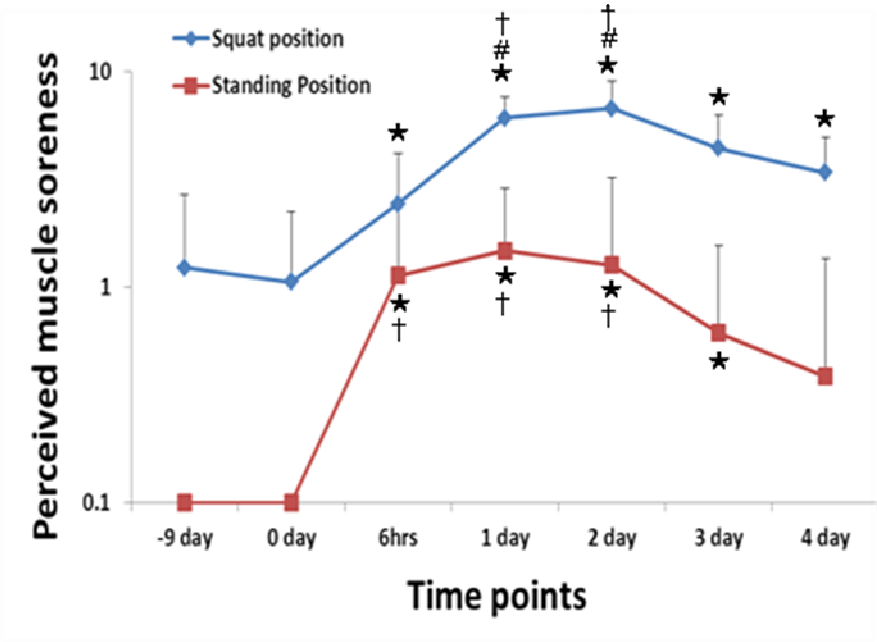


Figure 3.7. Change in perceived muscle soreness over time in two different positions before and after a single bout of plyometric exercise. Blue and red lines indicate perceived soreness (Log_{10}) of all subjects (n=26) in the squat and standing positions respectively. * Significantly different from time points -9 and 0, ($P < 0.0001$); # significantly different from time point 6hrs, ($P < 0.001$); † significantly different from day 3 and 4 ($P < 0.05$). Data are expressed as mean ± sd.

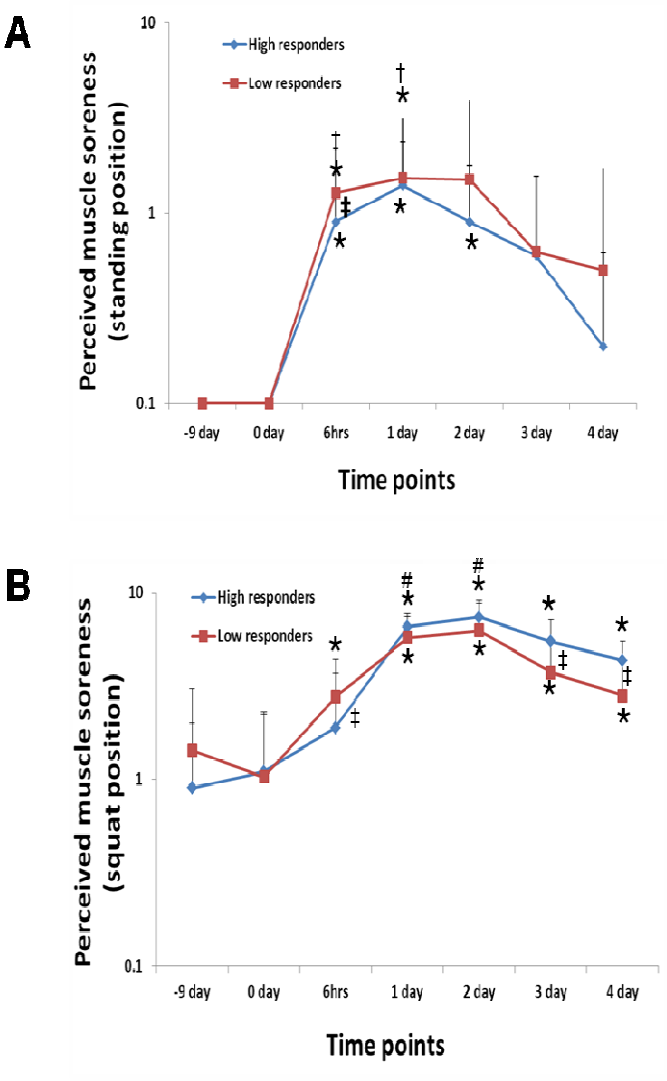


Figure 3.8 Change in perceived muscle soreness over time in two different positions before and after a single bout of plyometric exercise. A) Blue and red lines indicate soreness (Log_{10} U/L) in the standing position for the high (n=10) and low (n=16) responders respectively. B) Blue and red lines indicate soreness (Log_{10} U/L) in the squat position for the high (n=10) and low (n=16) responders respectively. * Significantly different from time points -9 and 0, ($P < 0.0001$); #significantly different from time point 6hrs, ($P < 0.001$); † significantly different from day 3 and 4 ($P < 0.05$). ‡ Significantly different response between high and low responders ($P < 0.05$). Data are expressed as mean ± sd.

No significant differences were observed for DOMS between the high and low responders at any time point in the standing position (figure 3.8 B), except at 6 hours ($P < 0.05$) after exercise in the standing position.

3.3.3 Marker of inflammation (C-reactive protein)

In all subjects six hours after exercise, CRP levels increased significantly and peaked at one day post exercise before returning to baseline values at day 3 (figure 3.10 A). CRP levels for both high and low responders peaked one day post exercise, however CRP values were significantly higher on days 6 hrs, 1 and 2 for the high compared to low responders (figure 3.10 A).

3.3.4 Blood Marker (Lactate dehydrogenase)

Six hours after exercise LDH was significantly higher than day 0 and remained significantly higher at 1, 2, 3 and 4 days post exercise (figure 3.11 A).

LDH values were significantly higher for the high compared to the low responders at time points 6 hours – 4 days (figure 3.11 B).

3.3.5 White blood cell counts

White Blood Cell

White blood cells (WBCs) count peaked 6 hours in the blood for all subjects, after plyometric exercise, after which it decreased significantly lower than baseline values at days 1,2,3 and 4 (figure 3.12 A). The same pattern mentioned before (figure 3.12 A) was observed for both high and low responders, however high responders experienced a significantly greater increase in WBC 6 hours (figure 3.12 B) after plyometric exercise.

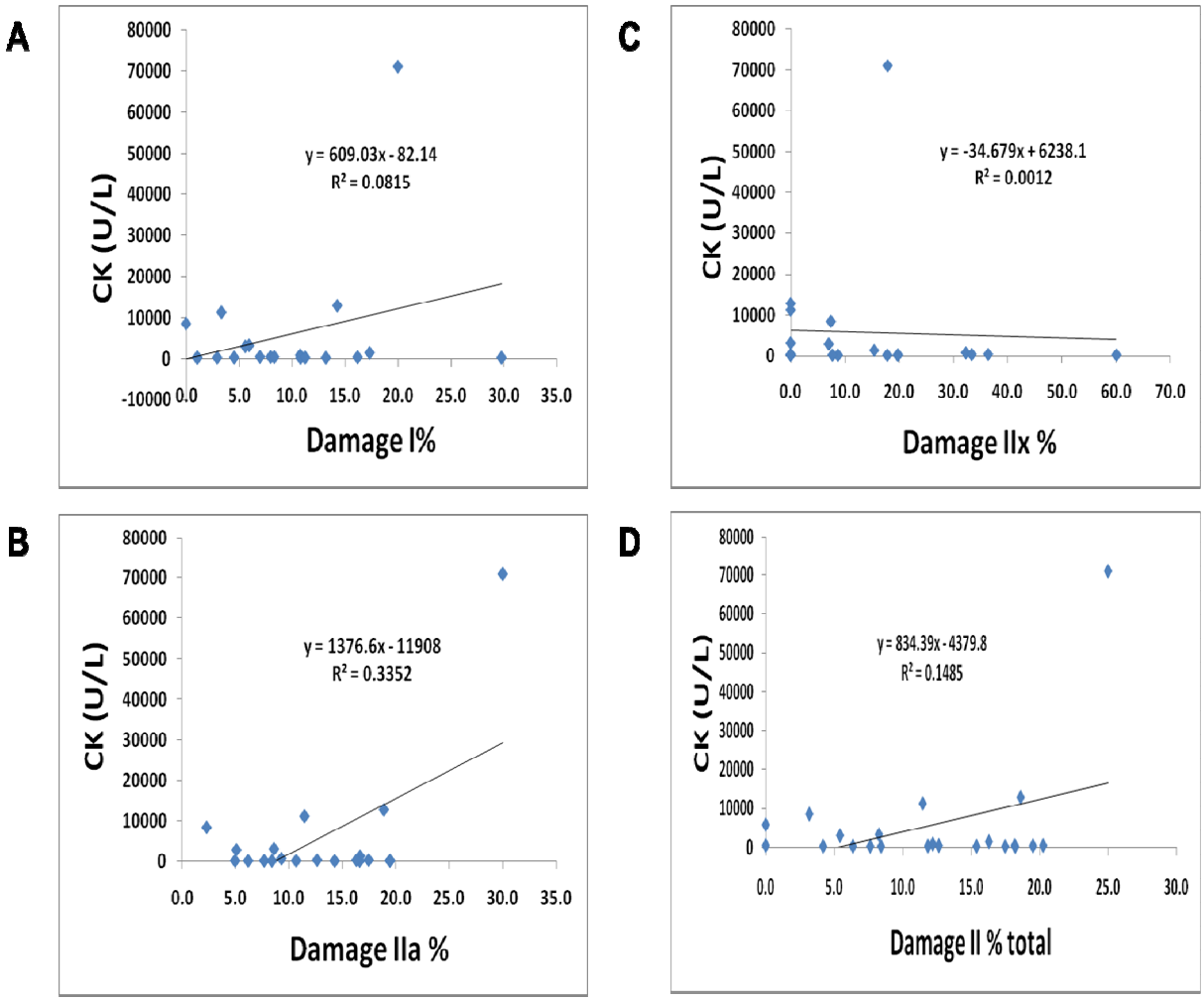


Figure 3.9. Relationship between creatine kinase (CK) activity 4 days after plyometric exercise and damage percentage fibre type 3 days after plyometric exercise (A) fibre I%, (B) fibre IIa% (C) fibre type IIx% and (D) fibre type II total %. Data are expressed as mean ± sd.

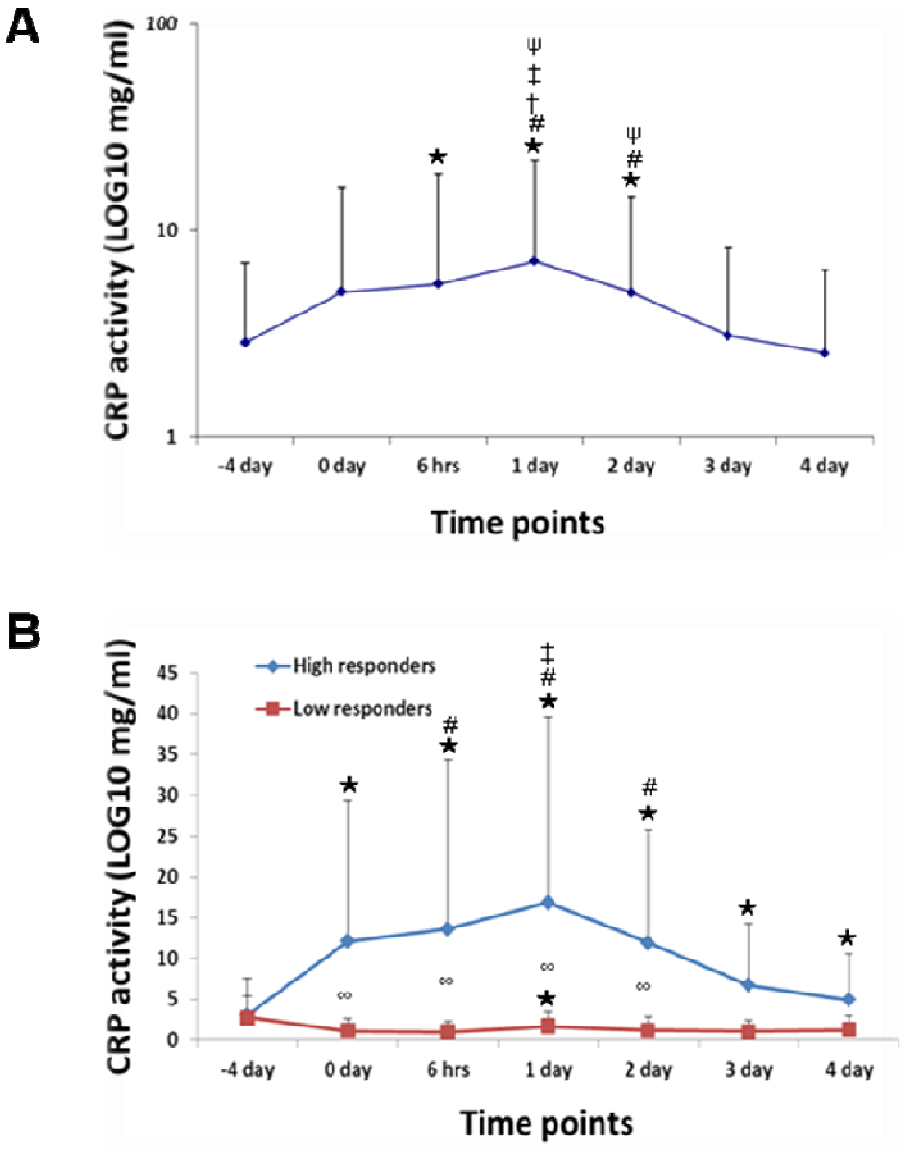


Figure 3.10. Change in serum CRP over time before and after a single bout of plyometric exercise. A) Average CRP activity (Log_{10} U/L) of all subjects (n=14) at different time points. B) The blue and red lines indicate changes in CRP activity (Log_{10} U/L) for the high (n=5) and low (n=9) responders. * Significantly different from time points -4, ($P < 0.01$); # significantly different from time point 0, ($P < 0.01$); † significantly different from time point 6hrs, ($P < 0.01$; ‡ significantly different from time point 3 and 4 ($P < 0.001$); ψ significantly different from time point 4, ($P < 0.01$); ∞ significantly different response between high and low responders ($P < 0.05$). Data are expressed as mean ± sd.

Neutrophils

The neutrophils increased significantly 6 hours after plyometric exercise (figure 3.13 A) for all subjects. The same trend was observed for both high and low responders, with a peak increase in neutrophils 6 hours after plyometric exercise, however high responders experienced a significantly greater increase in neutrophils 6 hours (figure 3.13 B) after plyometric exercise.

Lymphocytes

In all subjects, lymphocyte numbers decreased significantly from day -4 and baseline, 6 hours after exercise and remained significantly lower on days 1, 2, 3 and 4 (figure 3.14 A). No significant differences were observed between groups at any time point. Both high and low groups, lymphocyte numbers decreased significantly at 6 hours post exercise compared to day -4 and 0 and remained significantly lower on days 1, 2, 3 and 4 (figure 3.14 B).

Monocytes

Monocytes peaked 6 hours after plyometric exercise in all subjects and returned to baseline values at day 1 for all subjects (figure 3.15 A). For both high and low responders monocyte numbers peaked 6 hours after exercise (figure 3.15 B). No significant difference was observed in monocyte numbers between the high and low responders.

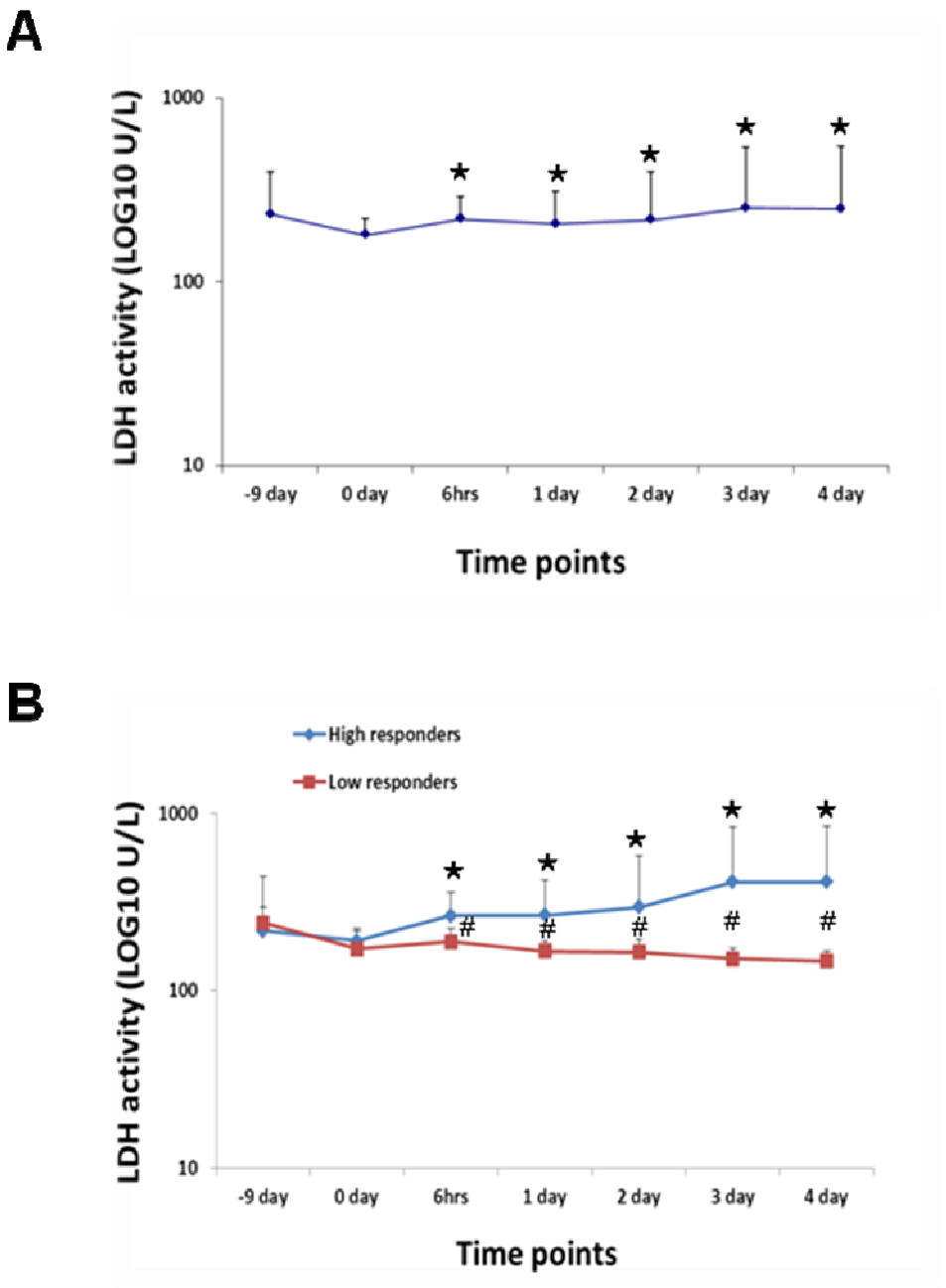


Figure 3.11. Change in serum LDH over time before and after a single bout of plyometric exercise. A) Average LDH activity (Log_{10} U/L) of all subjects (n=26) at different time points. B) The blue and red lines indicate LDH activity (Log_{10} U/L) for the high (n=10) and low (n=16) responder groups respectively. * Significantly different from time point 0, ($P < 0.01$); # significantly different response between high and low responders, ($P < 0.01$). Data are expressed as mean ± sd.

Eosinophils

Eosinophils decreased significantly from baseline values, 6 hours after exercise after which it increased significantly on day 1 compared to 6 hours after exercise (figure 3.16 A). This same trend was apparent for both high and low responders, however eosinophil values were significantly higher for the high compared to the low responders at respective time points, except at day 3 (figure 3.16 B).

Basophils

A significant increase in basophils was observed 6 hours post plyometric exercise from baseline values after which levels decreased significantly at 1, 2 and days post exercise compared to 6 hours after exercise (figure 3.17 A).

In the high group basophil levels increased significantly 6 hours after exercise after which it returned to baseline levels but again increased significantly at day 4 (figure 3.17 B). Low responder basophil levels remained unchanged, although levels were significantly higher day -4 compared to 0, 1, 3 and 4. High responders had significantly greater basophil values compared to low responders at baseline and 6 hours after exercise (figure 3.17 B).

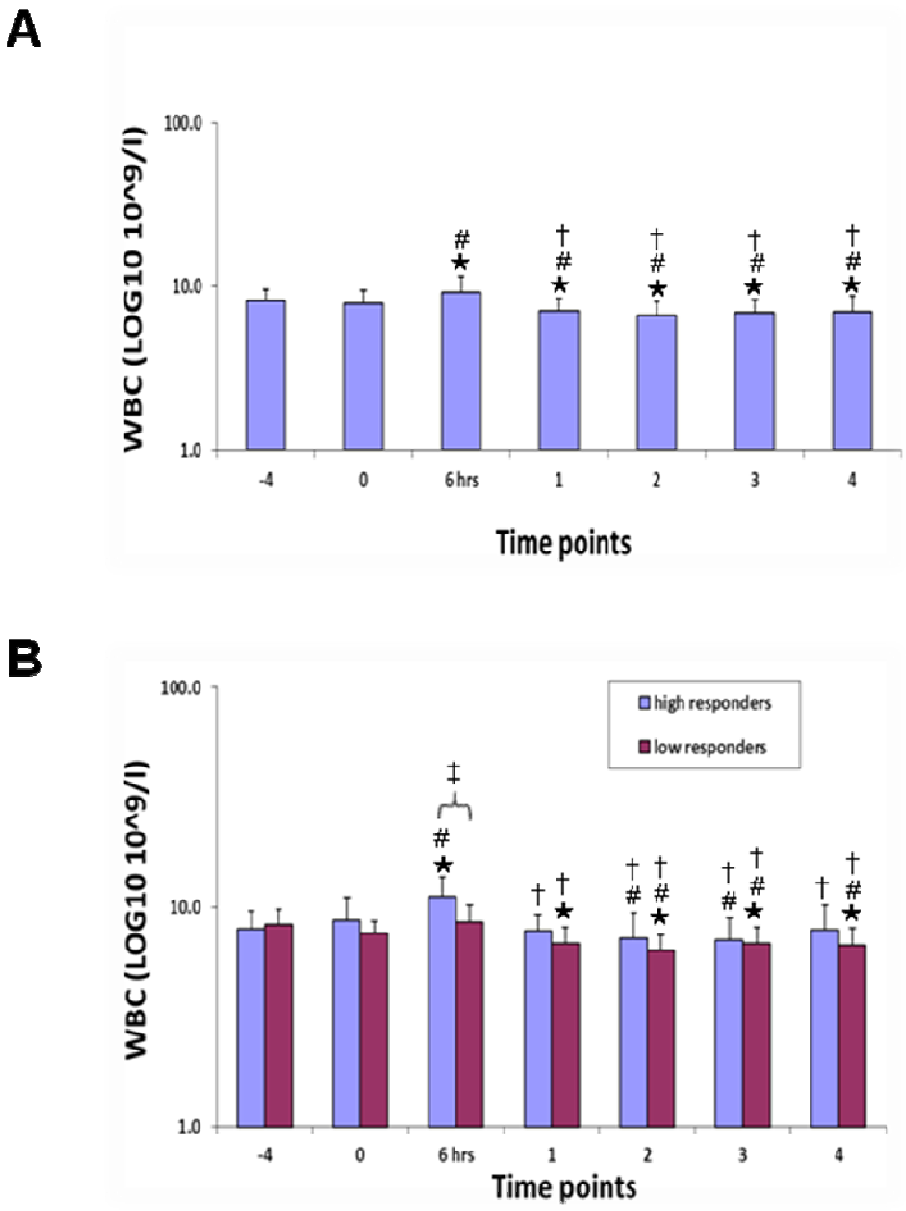


Figure 3.12. Change in White blood cell (WBC) count over time. A) Average WBC activity (Log_{10} 10^9/l) for all subjects (n=14) before and after plyometric exercise. B) The blue and purple bars indicate changes in WBC activity (Log_{10} 10^9/l) for the high (n=4) and low (n=10) responders respectively. *significantly different from day -4, ($P < 0.01$); # significantly different from day 0, ($P < 0.05$); †significantly different from 6hrs, ($P < 0.01$); ‡ significantly different from day 3, ($P < 0.04$); significant difference between high and low responders at respective time points, ($P < 0.01$); Data are expressed as mean ± sd.

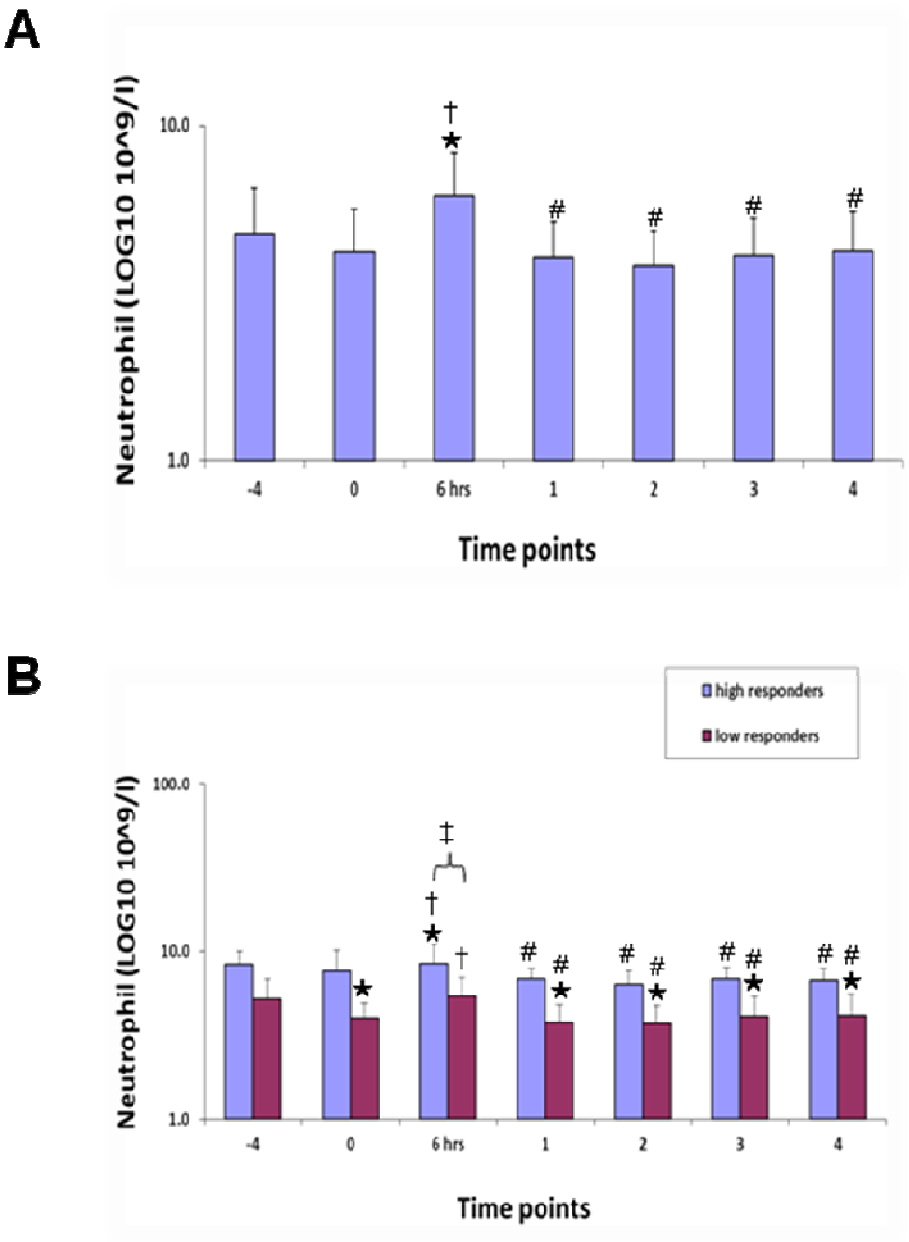


Figure 3.13. Change in Neutrophil count over time. A) Average neutrophil activity (Log_{10} 10^9/l) for all subjects (n=14) before and after plyometric exercise. B) The blue and purple bars indicate changes in neutrophil activity (Log_{10} 10^9/l) for the high (n=4) and low (n=10) responders respectively. *significantly different from day -4, ($P < 0.00001$); † significantly different from day 0, ($P < 0.00001$); # significantly different from 6hrs, ($P < 0.00001$); ‡ significant difference between high and low responders at respective time points, ($P < 0.003$); Data are expressed as mean ± sd.

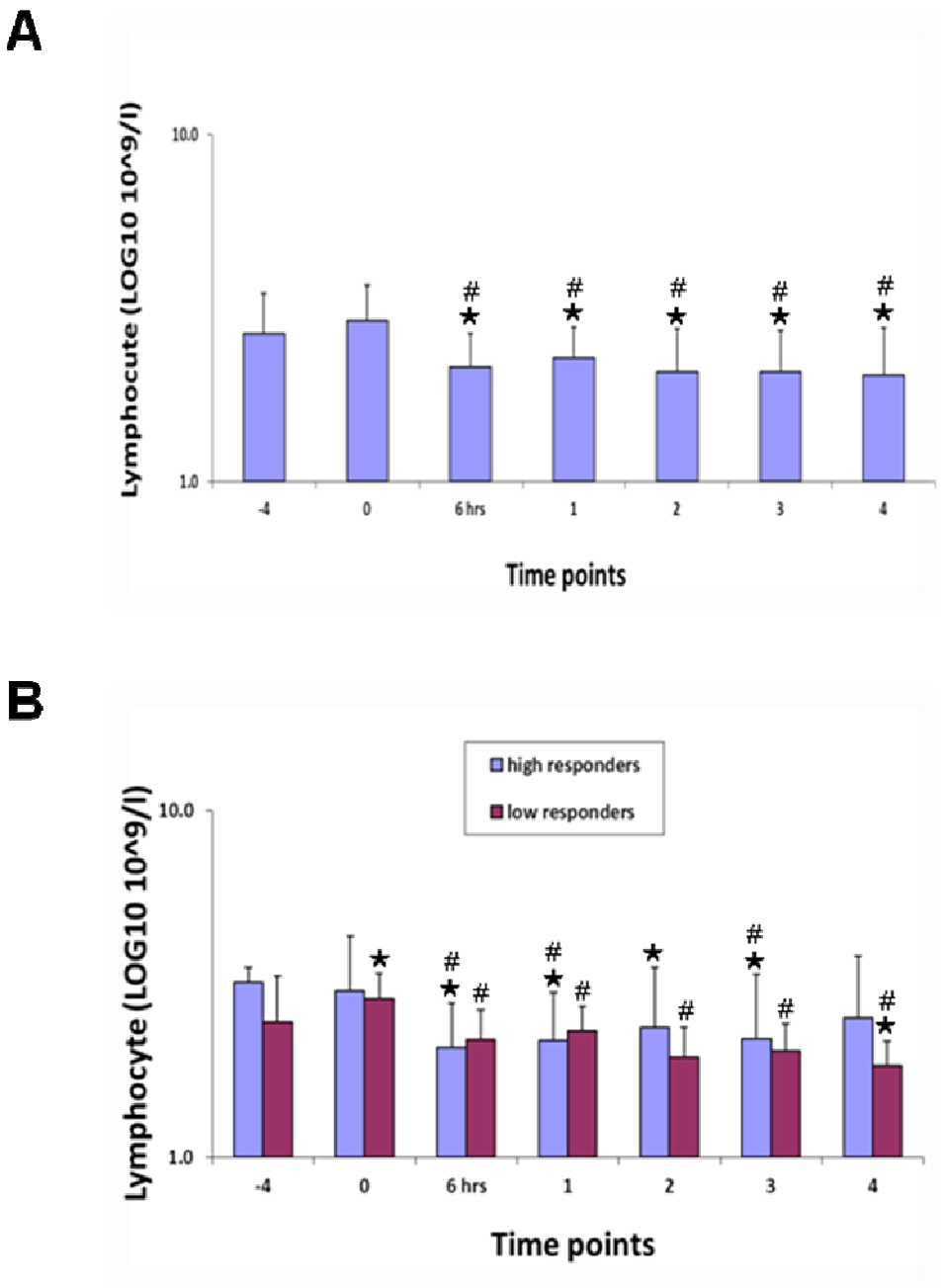


Figure 3.14. Change in Lymphocyte count over time. A) Average lymphocyte activity (Log_{10} 10^9/l) for all subjects (n=14) before and after plyometric exercise. B) The blue and purple bars indicate changes in lymphocyte activity (Log_{10} 10^9/l) for the high (n=4) and low (n=10) responders respectively. * Significantly different from day -4, ($P < 0.05$); # significantly different from day 0, ($P < 0.05$). Data are expressed as mean ± sd.

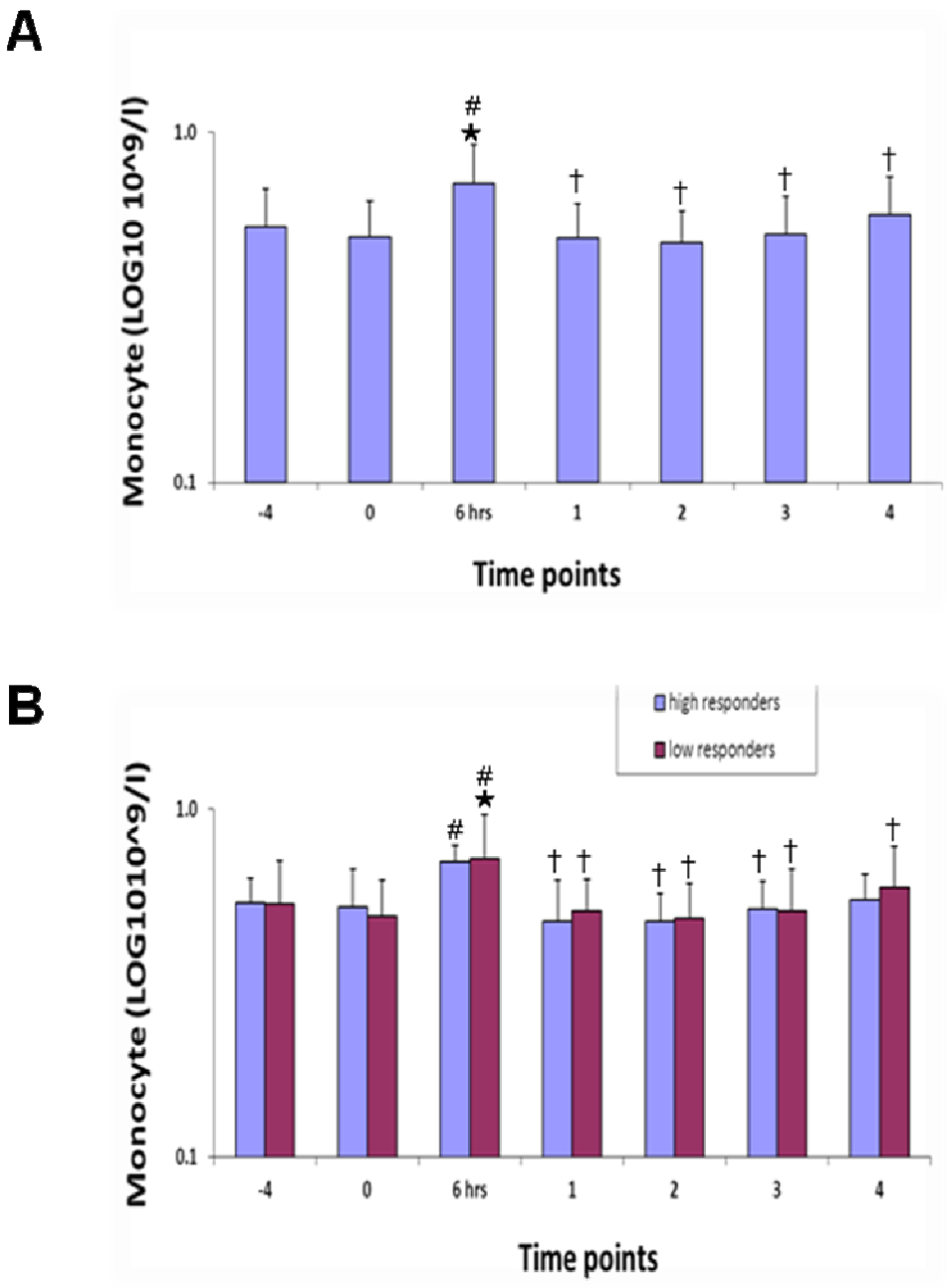


Figure 3.15. Change in Monocyte count over time. A) Average monocyte activity (Log_{10} 10^9/l) for all subjects (n=14) before and after plyometric exercise. B) The blue and purple bars indicate changes in monocyte activity (Log_{10} 10^9/l) for the high (n=4) and low (n=10) responders respectively. * Significantly different from day -4, ($P < 0.02$); # significantly different from day 0, ($P < 0.05$). †significantly different from 6hrs ($P < 0.05$), Data are expressed as mean ± sd.

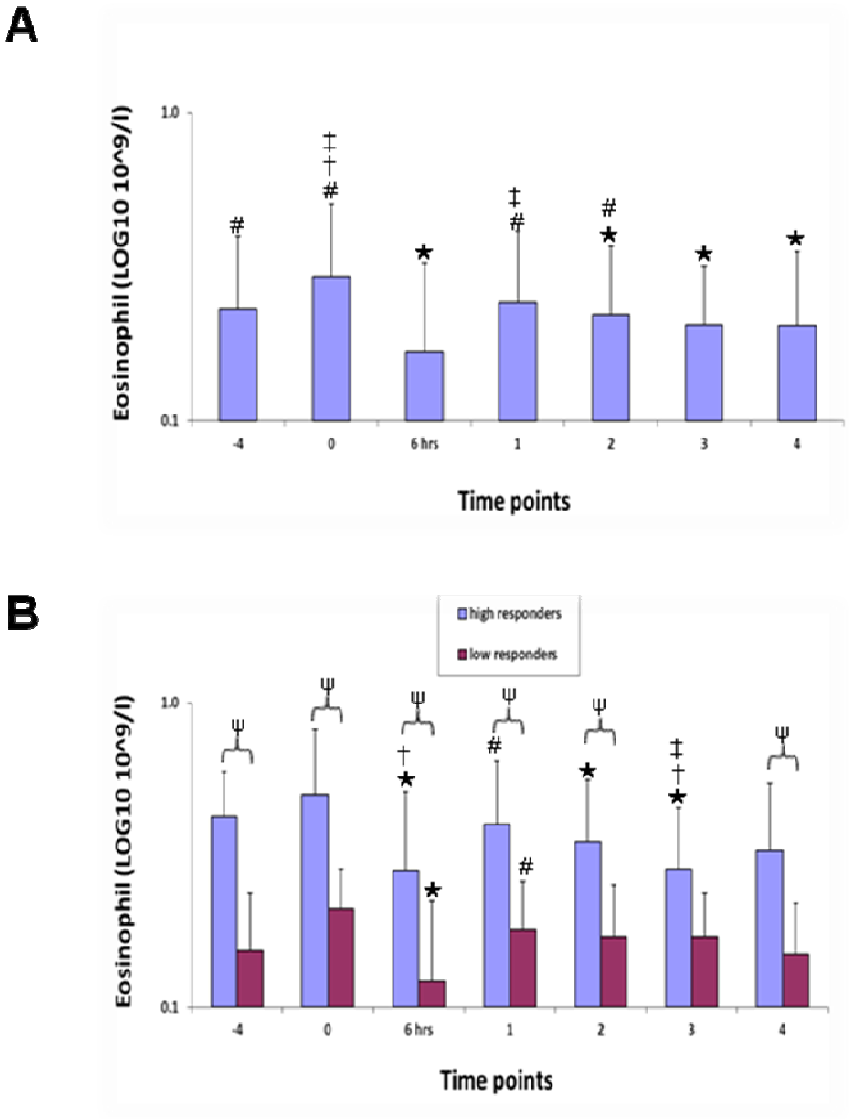


Figure 3.16. Change in Eosinophil count over time. A) Average eosinophil activity (Log_{10} 10^9/l) for all subjects (n=14) before and after plyometric exercise. B) The blue and purple bars indicate changes in eosinophil activity (Log_{10} 10^9/l) for the high (n=4) and low (n=10) responders respectively. † Significantly different from day -4, ($P < 0.03$); * significantly different from day 0, ($P < 0.02$); #significantly different from 6hrs, ($P < 0.03$); ‡ significantly different from day 3, ($P < 0.04$); ψ significant difference between high and low responders at respective time points, ($P < 0.04$); Data are expressed as mean ± sd.

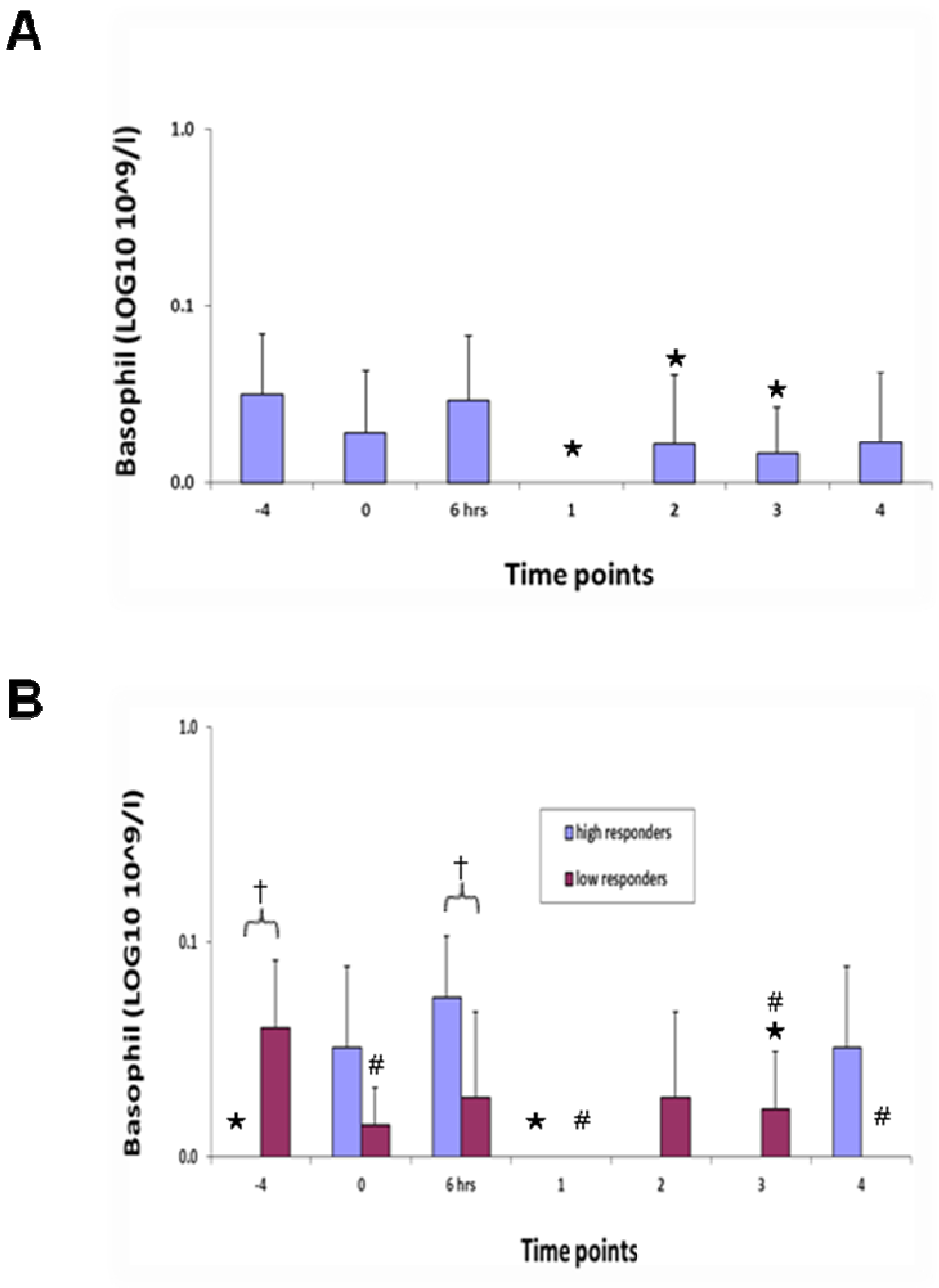


Figure 3.17. Change in Basophil count over time. A) Average basophil activity (Log_{10} 10^9/l) for all subjects (n=14) before and after plyometric exercise. B) The blue and purple bars indicate changes in basophil activity (Log_{10} 10^9/l) for the high (n=4) and low (n=10) responders respectively. * Significantly different from 6hrs, ($P < 0.05$); # significantly different from day -4, ($P < 0.04$); † significant difference between high and low responders at respective time points, ($P < 0.04$); Data are expressed as mean ± sd.

3.3.6 Cytokines

TNF-α

TNF- α level was undetected in serum of all subjects at all time points.

IL-6

Serum IL-6 levels peaked six hours after plyometric exercise in all subjects and returned to baseline values by day 1 (figure 3.18 A), this same trend was apparent for both high and low responders. However no significant difference was observed between high and low responders at respective time points although serum IL-6 levels were slightly higher in the high responders (figure 3.18 B).

3.3.7 Damaged fibres

Sarcolemmal damage was quantified by the loss of dystrophin staining (figure 3.19), this was indicative of membrane damage.

Muscle damage in the form of z-disk streaming was apparent in all subjects from electron micrographs taken of biopsy 2. Damage ranged from no damage to severe damage as illustrated in figure. 3.20 B. Visible differences of structural and ultrastructural muscle damage were not apparent when comparing electron micrographs of high and low responders.

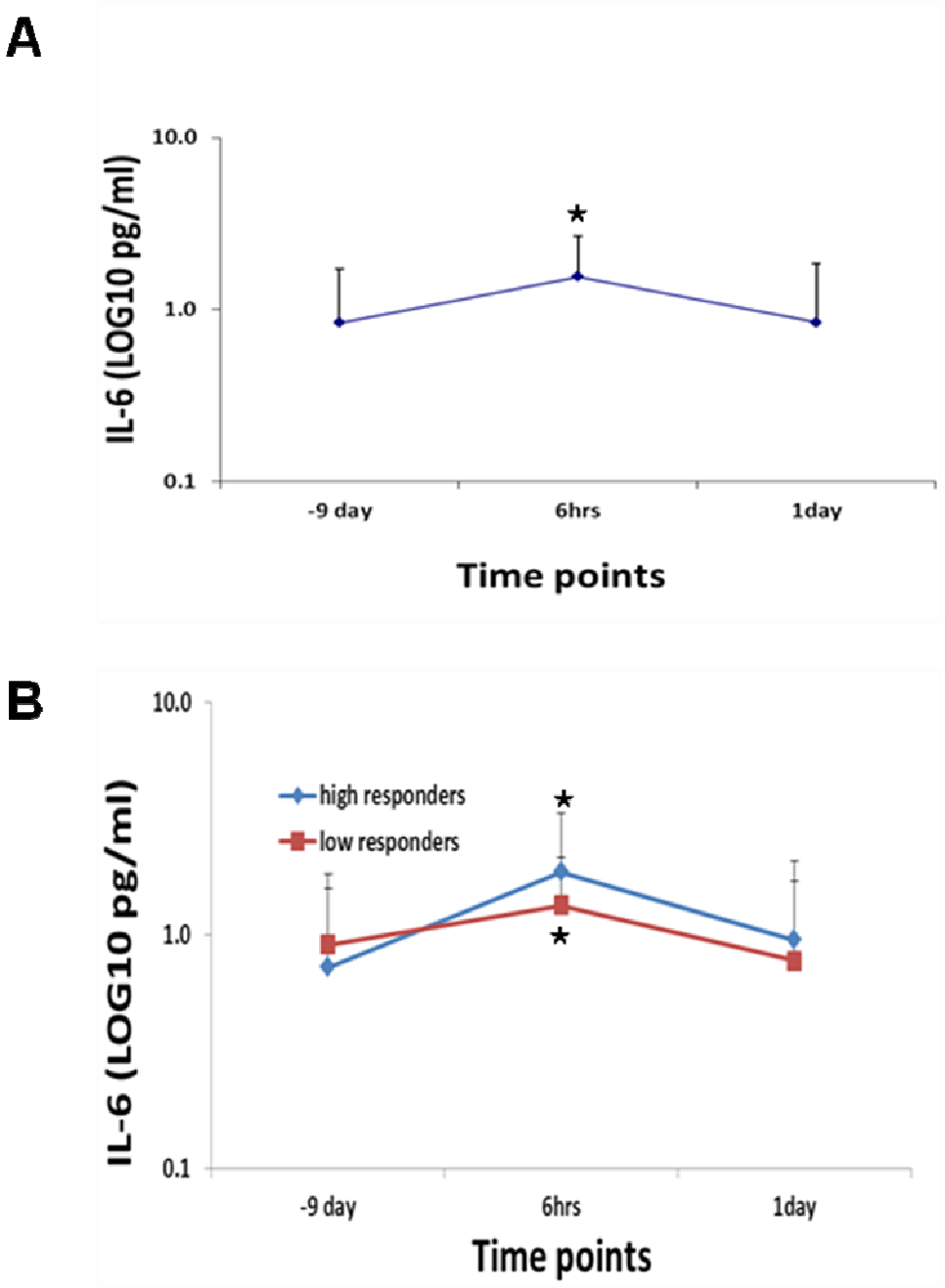


Figure 3.18. Change in serum IL-6 over time. A) Average serum IL-6 activity (Log_{10} U/L) for all subjects (n=26) before, 6hrs and 1 day after plyometric exercise. B) The blue and red lines indicate changes in serum IL-6 activity (Log_{10} U/L) for the high (n=10) and low (n=16) responders respectively. * Significantly different from -9 and day 1, ($P < 0.03$). Data are expressed as mean ± sd.

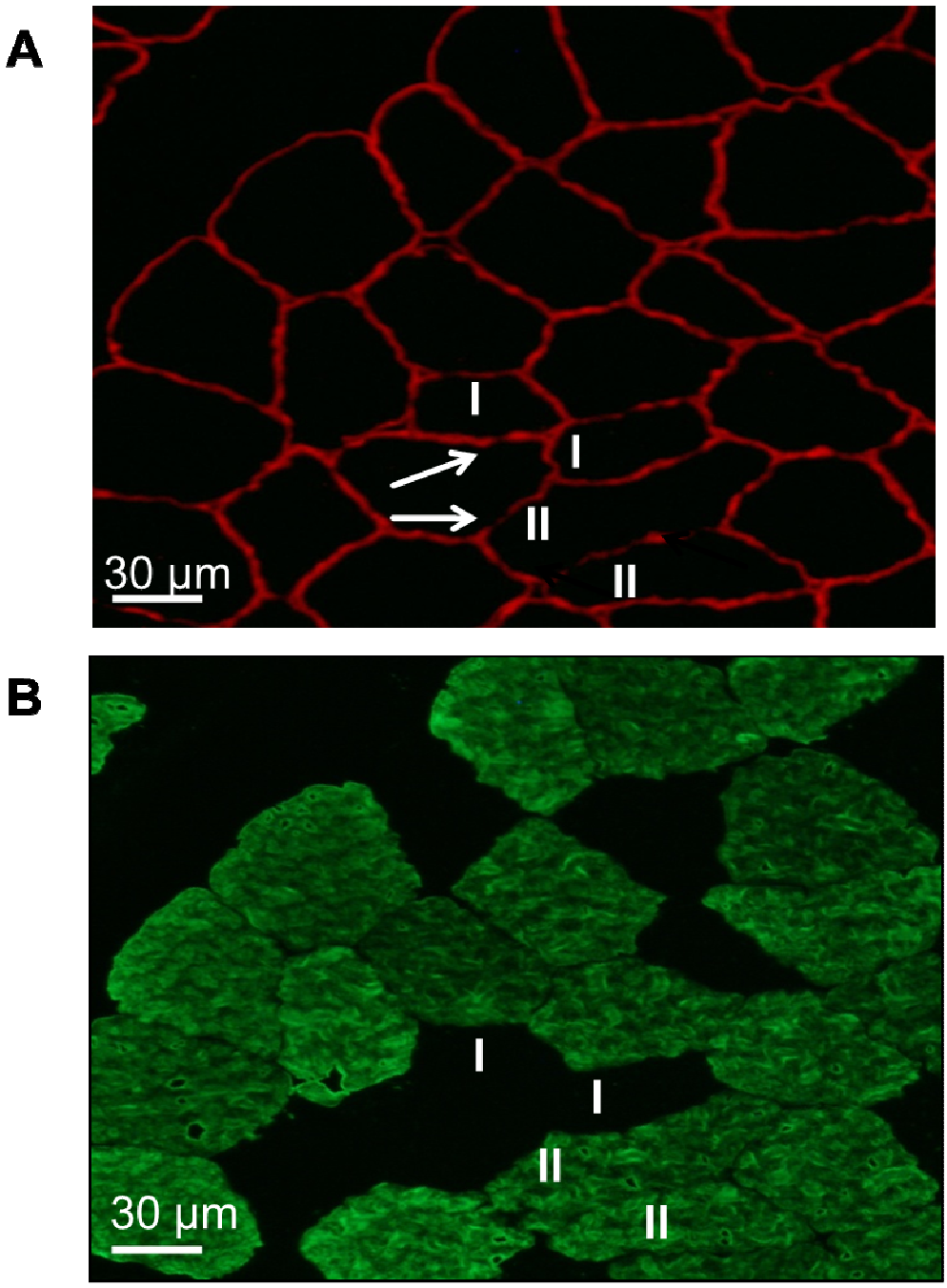


Figure 3.19 Immunofluorescence of cross-sections of muscle fibres from a biopsy taken 3 days after the plyometric exercise intervention, double immunostaining with anti-dystrophin (A) and anti-myosin heavy chain II (B). Arrows show the loss in dystrophin staining; II indicates MHC-II positive fibres; I, MHC-I fibres. Scale bars 30 µm.

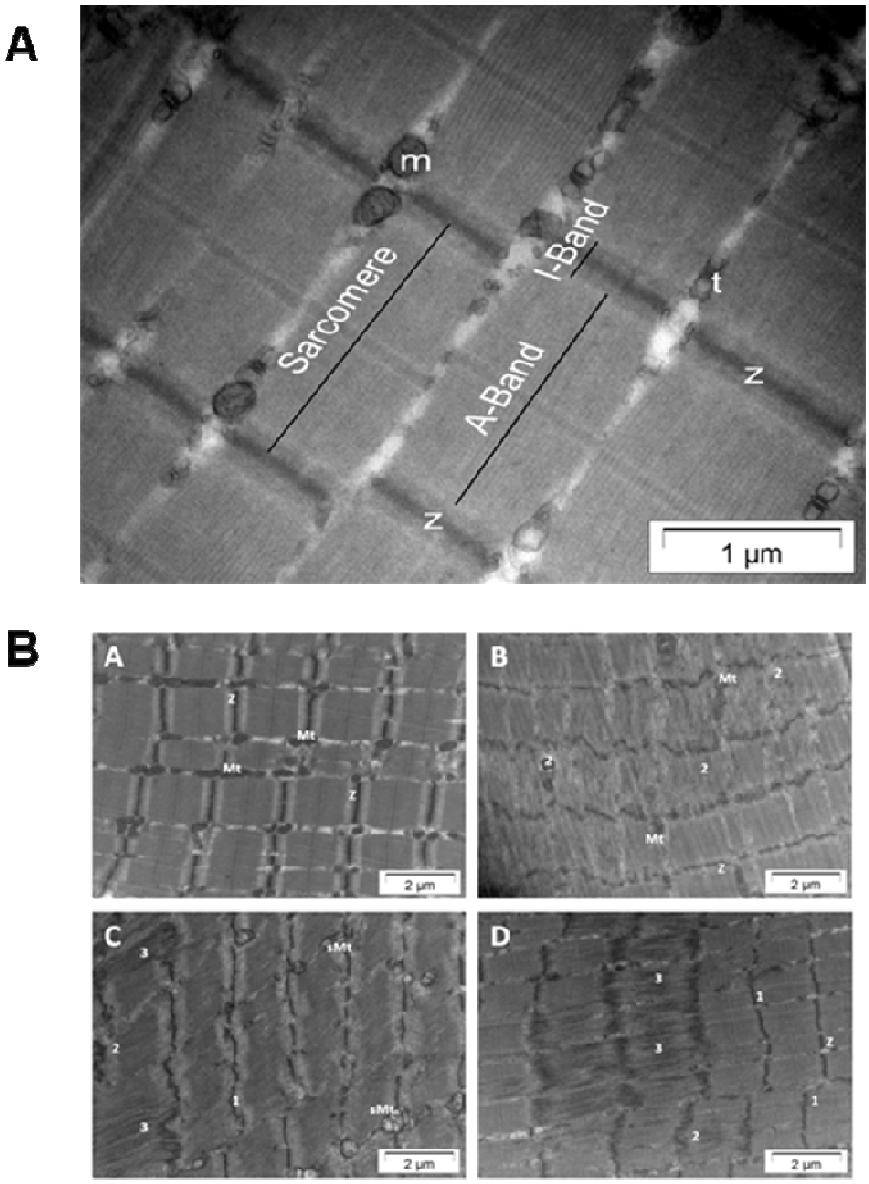


Figure 3.20. A) Longitudinal section of human vastus lateralis muscle. Calibration bar corresponds to a distance of 1 µm (30 000x). (m-mitochondrion, z-Z-band, and t-Transverse tubular system). **B) Electron micrographs of longitudinal sections** (1µm) illustrating different stages of Z-disk disruption/damage following eccentric exercise in human skeletal muscle, A-no damage, B-wavy Z-disks, D-strongly waved Z-lines and D-severe damage (absence of Z-lines). Z-normal z-disk, Mt-mitochondria, sMt-swollen mitochondria, 1-wavy z-disk, 2-mild z-disk streaming, 3-disintegration of z-disk. No difference in ultrastructural damage was observed between high and low responder groups.

Training Intervention Study:

3.1 Cross-sectional area

No significant change in individual muscle fibre CSA was observed following 8 weeks of plyometric training. Although fibre type IIa (3453-4168 μm^2) and total fibre type II (3434-4118 μm^2) CSA in the training group showed a trend toward (P=0.07) an increase in CSA following 8 weeks of plyometric training (figure 3.21). No significant changes in CSA for the control group were observed.

3.2 Fibre shift

No significant change in individual muscle fibre type percentage was observed following 8 weeks of plyometric training. However, the training group showed a trend (P=0.06) toward increased type IIa fibres (46.6-57.5%) following 8 weeks of plyometric training (figure 3.22). No significant changes in fibre percentage for the control group were observed.

3.3 Performance results

3.3.1 Average and Peak power

Following 8 weeks of plyometric training, average and peak power increased significantly in the training group $P < 0.0003$ and $P < 0.001$ respectively (figure 3.23 A and B). While no significant differences were observed for the control group.

Both average and peak velocity increased significantly ($P < 0.003$ and $P < 0.03$ respectively) in the training group post plyometric training, however a significant increase in peak velocity (0.05) was observed in the control group following 8 weeks of rest (3.24 A and B).

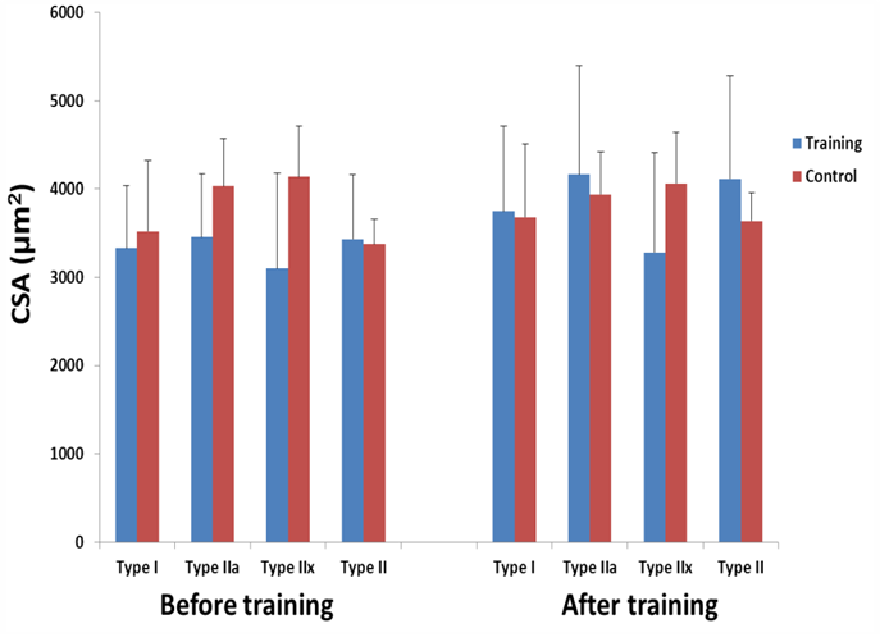


Figure 3.21 Individual muscle fibre cross-sectional area before and after 8 weeks of rest or plyometric training. Fibre type IIa (3453-4168 µm2) and total type II fibre CSA (3434-4118 µm2) in the training group showed a trend toward (P=0.07) an increase in CSA following 8 weeks of plyometric training

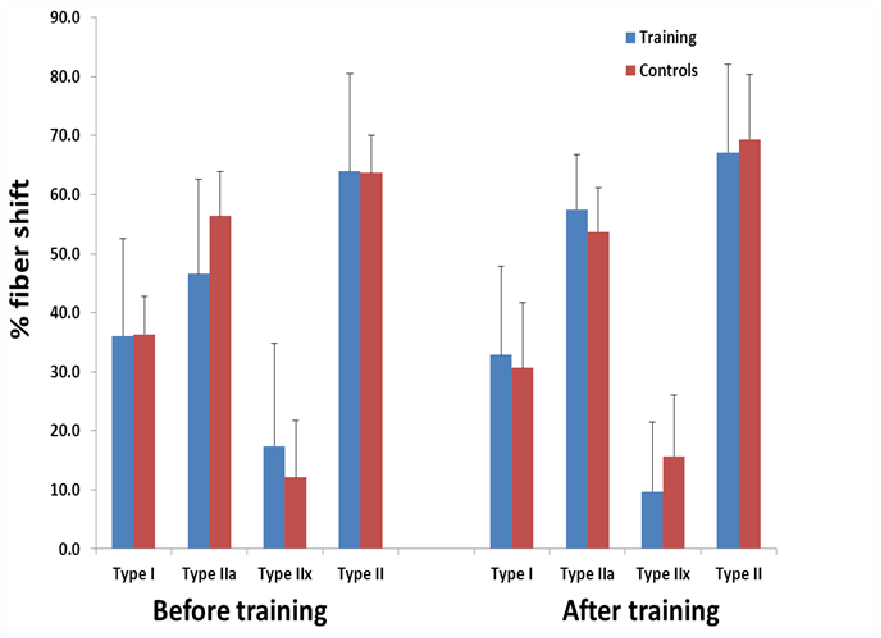


Figure 3.22 Individual muscle fibre transitions before and after 8 weeks of rest or plyometric training. The training group showed a trend (P=0.06) toward increased type IIa fibres (46.6-57.5%) following 8 weeks of plyometric training

3.3.2 Maximum vertical jump height

Significant increases in vertical jump height were noted after plyometric training in the training as well as control group (figure 3.25) $P < 0.02$ and $P < 0.05$ respectively.

3.4 Blood marker response

CK levels increased immediately 6 hours ($P < 0.00001$) following the first acute bout of plyometric exercise in the training group and continued to rise until day 4 ($P < 0.00001$). Following plyometric training CK levels were significantly lower than pre training values at all time points in the training group ($P < 0.001$). CK levels in the control group peaked one day after the first acute bout of plyometric exercise and pre training values at days 1, 2, 3 and 4 were significantly lower than pre training values at the same days of the training group. Following 8 weeks of plyometric training, the CK response was blunted and CK levels were significantly lower than pre training values at respective time points in the training group (3.26 A). The control group had significantly lower CK levels compared to the training group following the first acute bout of plyometric exercise; however the training group had significantly lower CK levels in response to the second acute bout of exercise compared to the control group at day 3 post exercise. Mb peaked at 6 hours following the first acute bout of plyometric exercise in both control and training group (figure 3.26 B). At this time point the training group had significantly higher Mb levels compared to the control group. Following training the second acute bout of plyometric training, elicited a significantly lower Mb response in comparison with the first acute bout of exercise in both training and control groups. The peak increase at 6 hours following the second acute bout of exercise was significantly lower than the 6 hour time point following the first acute bout of exercise for both

groups. In fact, this was true for all time points following the second acute bout of exercise.

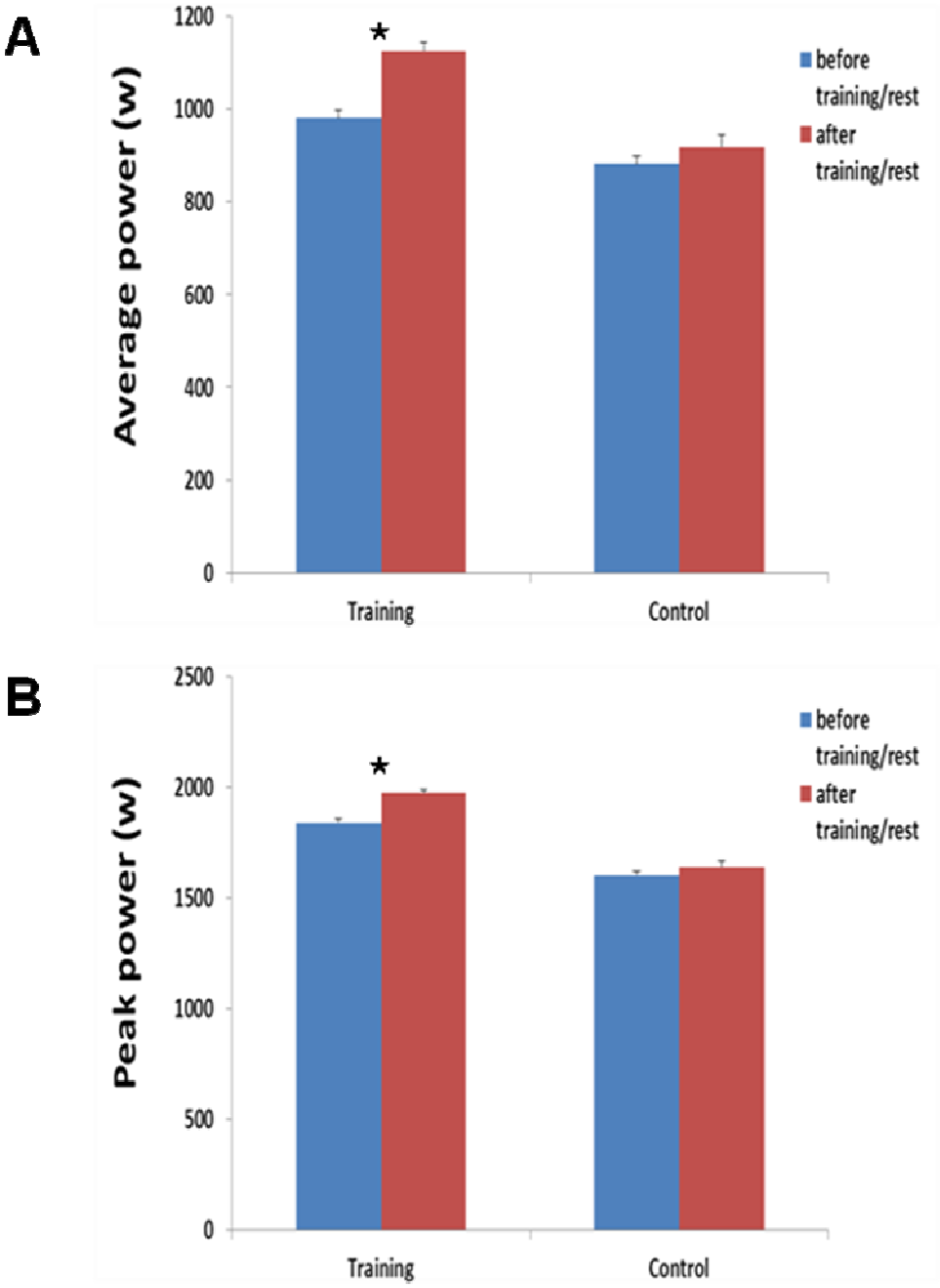


Figure 3.23 Average (A) and Peak (B) power before and after 8 weeks of rest or plyometric training. * Significantly different from pre jump values within groups ($P < 0.001$).

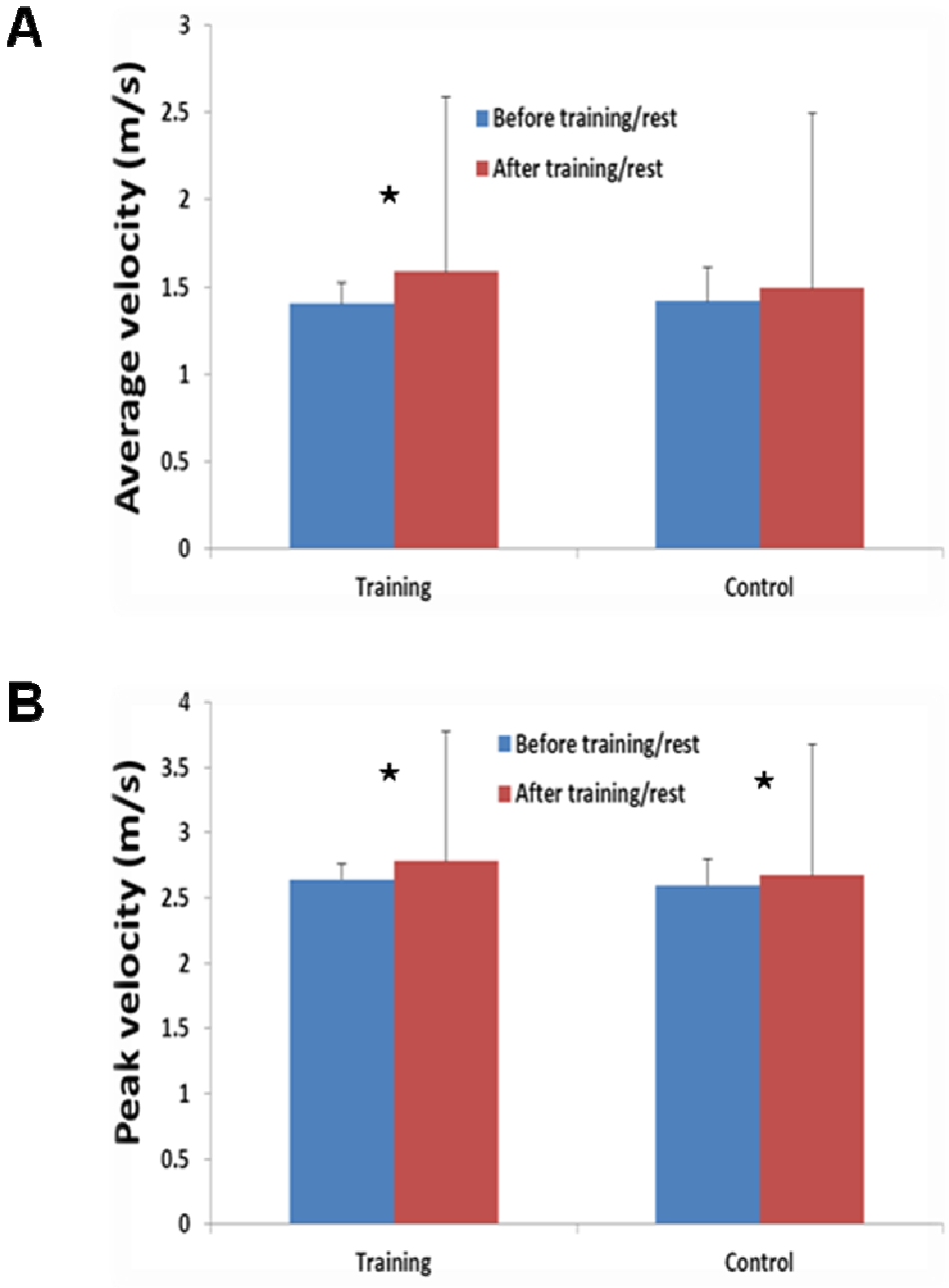


Figure 3.24 Average (A) and Peak (B) velocity before and after 8 weeks of rest or plyometric training. * Significantly different from pre jump values within groups, (Training $P < 0.003$, Control $P < 0.03$).

3.5 Delayed Onset of Muscle Soreness

In the squat position, soreness increased significantly from baseline to 6 hours ($P < 0.02$, $P < 0.01$) following the first acute bout of plyometric exercise, peaked at day 2 and decreased gradually in both groups (figure 3.28 A). Following 8 weeks of plyometric training/rest soreness was significantly lower in the training group compared to the control group in response to a second acute bout of plyometric training (figure 3.28 B). The control group experienced less soreness after 8 weeks of rest in response to the second acute bout of exercise compared to first acute bout of exercise. In the standing position DOMS peaked at 6 hours ($P < 0.002$) and day 1 ($P < 0.00001$) in the training and control groups respectively, however this peak increase was significantly lower than compared to the pre training value of the same time points.

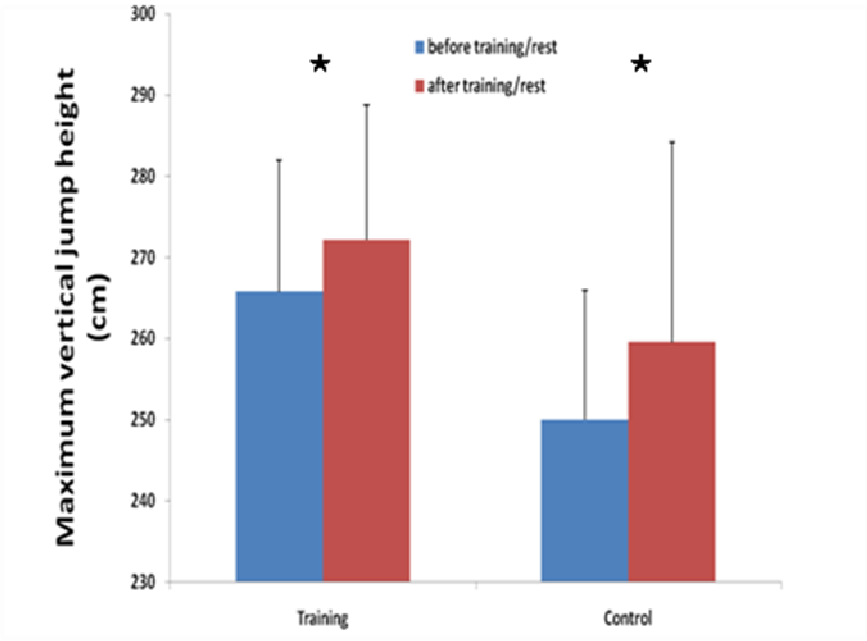


Figure 3.25 Maximum vertical jump height before and after 8 weeks of rest or plyometric training. * Significantly different from pre jump values within groups, (Training $P < 0.02$ and Control $P < 0.05$).

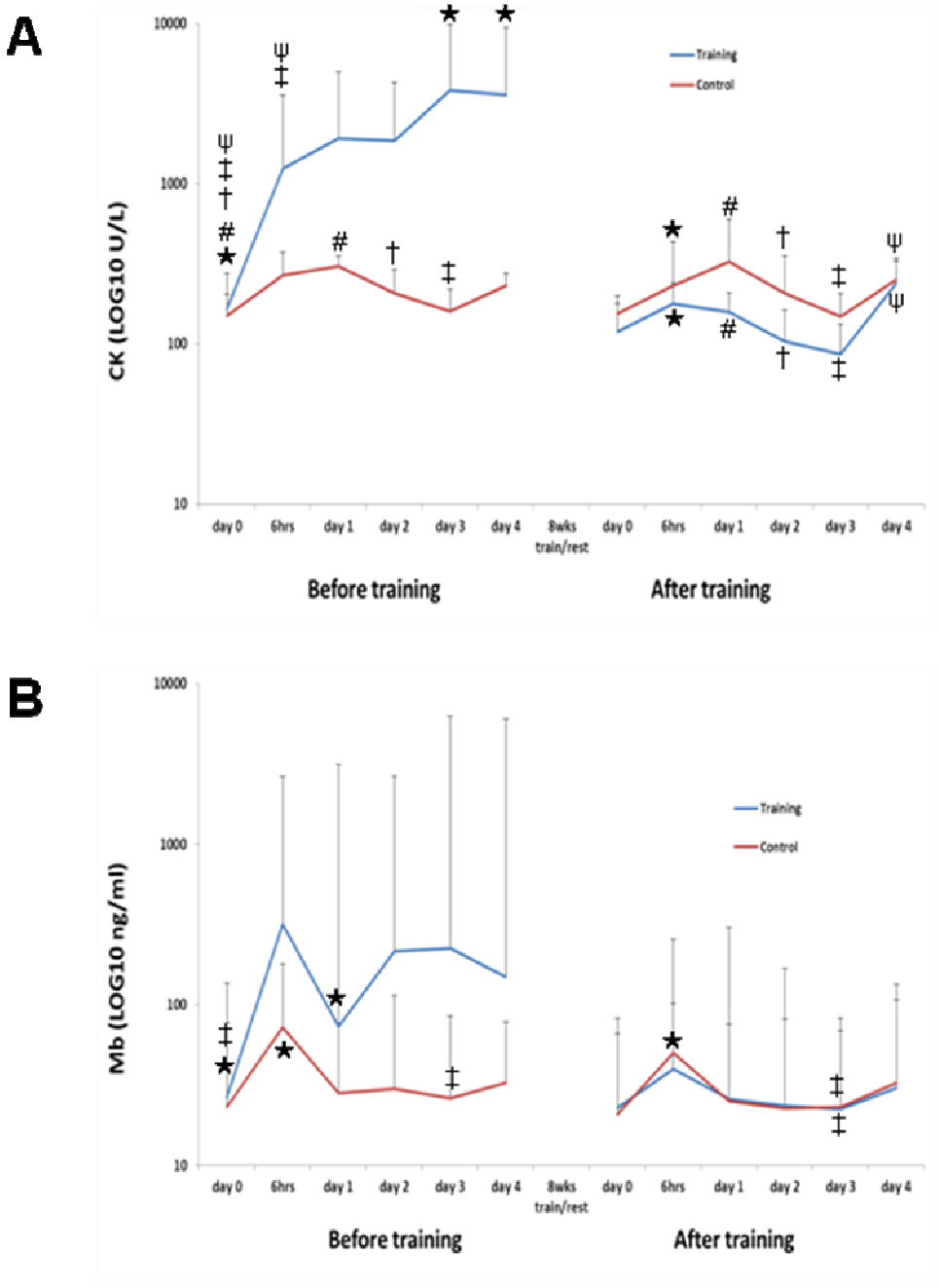


Figure 3.26 Change in serum creatine kinase CK and myoglobin Mb over time before and after plyometric training. The blue and red lines indicate CK (A) and Mb (B) activity (Log_{10} U/L) for the training (n=8) and control (n=5) groups respectively. * Significantly different from time point 6 hrs before training, ($P < 0.04$); # significantly different from day 1 before training, ($P < 0.01$); † significantly different from day 2 before training, ($P < 0.01$); ‡ significantly different from day 3 before training, ($P < 0.01$); ψ significantly different from day 4 before training, ($P < 0.01$). Data are expressed as mean ± sd.

3.6 Inflammatory response (C-reactive protein)

In the training group, CRP peaked one day ($P < 0.01$) following the first acute bout of plyometric exercise compared to baseline and returned to baseline values on subsequent days. Following 8 weeks of training no significant changes were observed in CRP values in the training group in response to a second acute bout of plyometric exercise. No significant changes in CRP levels were observed for the control group following the first and second acute bouts of plyometric exercise.

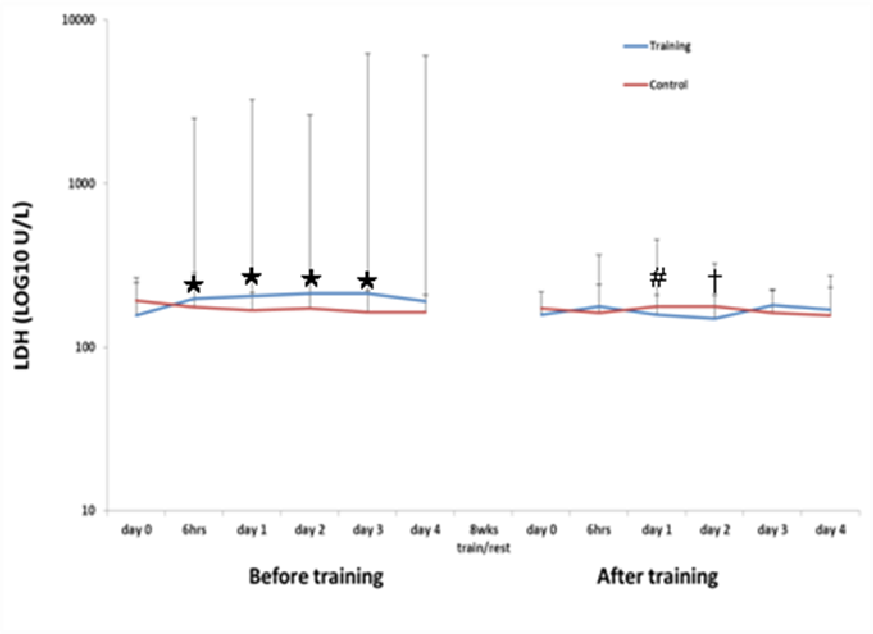


Figure 3.27 Change in serum Lactate dehydrogenase (LDH) over time before and after plyometric training. The blue and red lines indicate LDH activity (Log_{10} U/L) for the training and control groups respectively. * Significantly different from day 0, ($P < 0.004$) before training; # significantly different from day 1, before training ($P < 0.001$); † significantly different from day 2 before training, ($P < 0.001$). Data are expressed as mean ± sd.

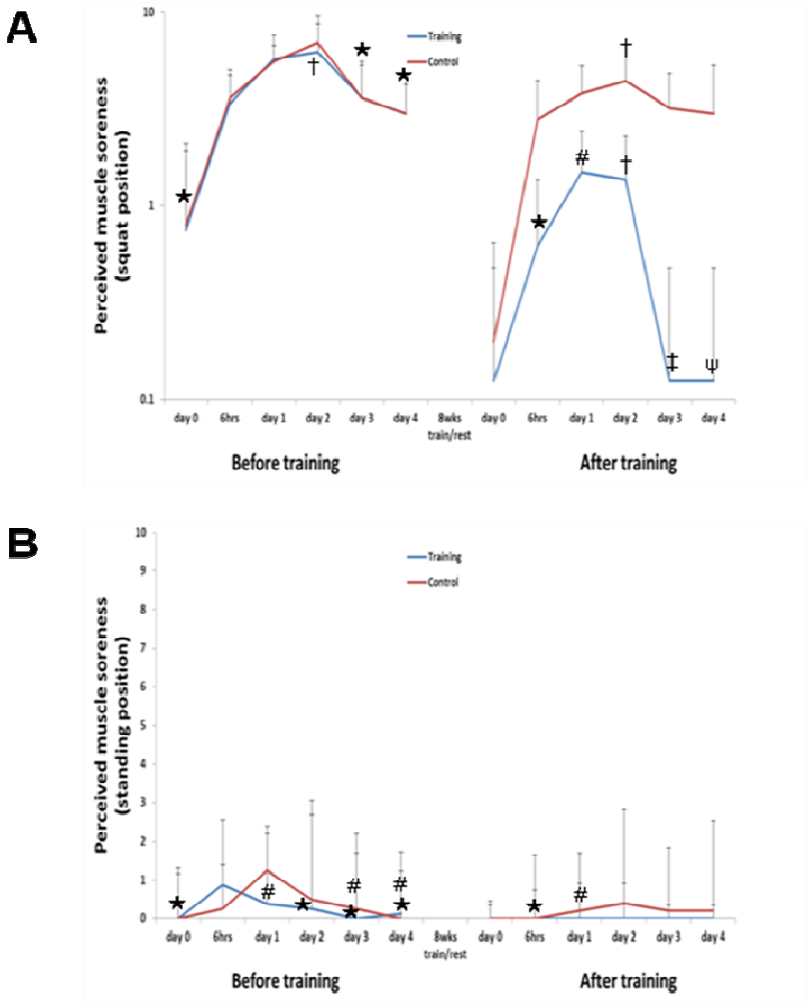


Figure 3.28 Change in perceived muscle soreness before and after 8 weeks of plyometric training. A) Blue and red lines indicate soreness (Log_{10} U/L) in the squat position for the training and control groups respectively. B) Blue and red lines indicate soreness in the standing position for the training and control groups respectively. Note normal values are used to indicate soreness in the standing position because LOG10 statistical analysis cannot be performed on zero values. * Significantly different from time points 6 hrs, ($P < 0.05$) before training; # significantly different from day 1, ($P < 0.01$) before training; †significantly different from day 2 ($P < 0.04$) before training. ‡ Significantly different from day 3 ($P < 0.00001$) before training; ψ significantly different from day 4 ($P < 0.00001$) before training. Data are expressed as mean ± sd.

3.7 White blood cell counts

White blood cells (WBCs) peaked 6 hours ($P < 0.002$) in the blood (figure 3.30) compared to baseline for the training group following the first acute bout of plyometric exercise after which it decreased significantly to baseline levels on days 1, 2,3 and 4 ($P < 0.01$). This peak was significantly higher than the control group at the same time point, following the first acute bout of plyometric exercise, and all time points before and after training ($P < 0.01$). In the control group white cells remained at baseline values after the first acute bout and second acute bout of training.

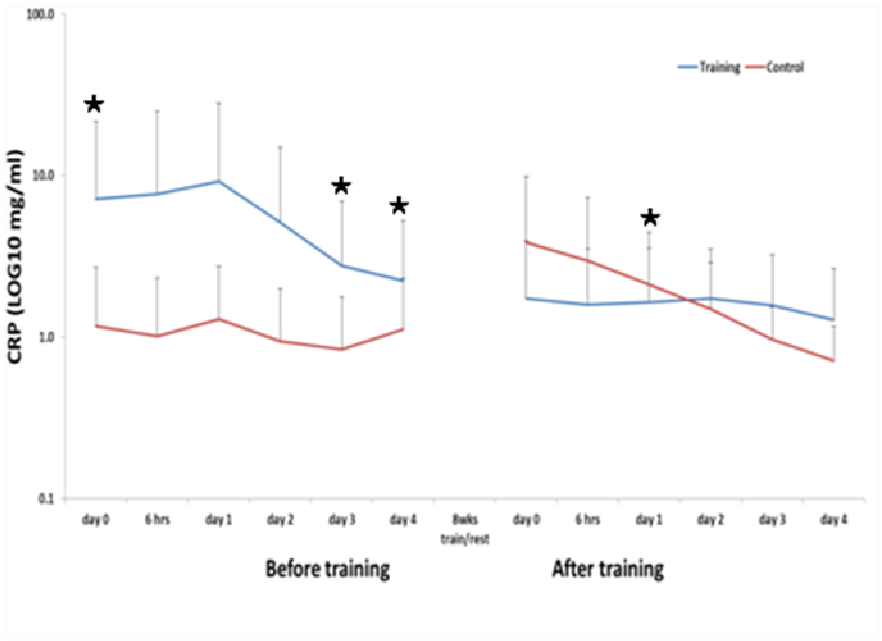


Figure 3.29 Change in serum CRP over time before and after 8 weeks of plyometric training. Average CRP activity (Log_{10} U/L) of the training and control groups. * Significantly different from day 1 before training, ($P < 0.03$). Data are expressed as mean ± sd.

Neutrophils

There was a peak in neutrophils 6 hours following the first acute bout of plyometric exercise in the training group ($P < 0.00001$). This peak was significantly greater ($P < 0.01$) than all-time points (training and control) before and after plyometric training (figure 3.31).

Lymphocytes

Lymphocyte counts was significantly lower at time points 6 hours, 1, 2, 3 and 4 ($P < 0.01$) compared to day 0 in the training group (figure 3.32), before plyometric training. Day 0 (training group) before the first acute plyometric exercise was not significantly different from pre-training values compared to the control group at the same time point and post-training values of the training group at day 0, but was significantly different from post training control group values at the same time point ($P < 0.04$) .

Monocytes

Monocyte levels for both training and control groups were not significantly different 6 hours following the first acute bout of plyometric exercise (figure 3.33), neither were these values significant compared to post training values at the same time point. However 6 hours after the first acute bout of plyometric exercise in the training group, monocyte levels were significantly different to days 0, 1, 2, 3 and 4 ($P < 0.04$).

Eosinophils

No significant differences were observed for eosinophil levels between any time point's pre and post training (figure 3.34).

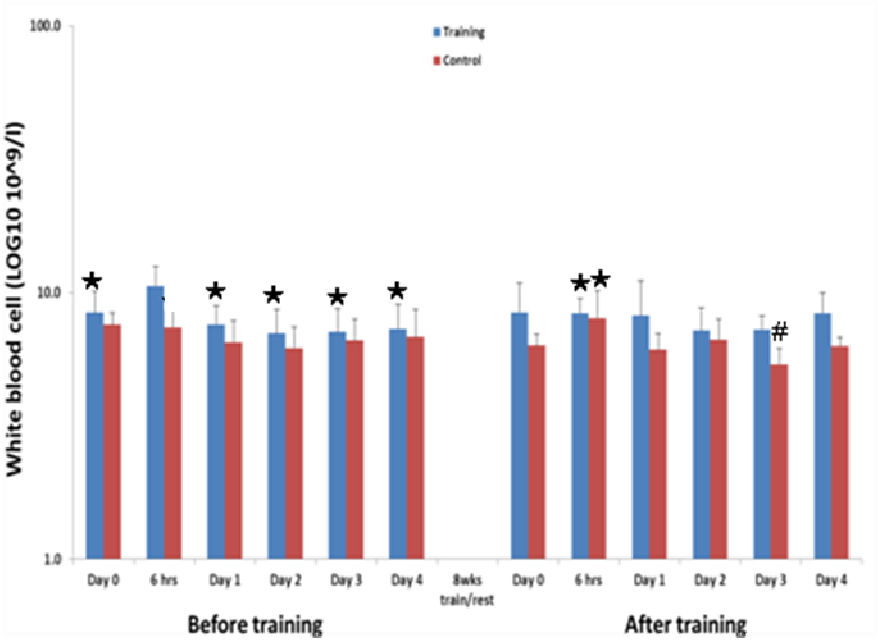


Figure 3.30 Change in White blood cell (WBC) count over time. WBC activity (Log_{10} 10^9/l) before and after 8 weeks of plyometric training for the training and control (n=10) groups respectively. * Significantly different from time point 6 hrs before training, ($P < 0.01$); # significantly different from day 3 before training, ($P < 0.01$). Data are expressed as mean ± sd.

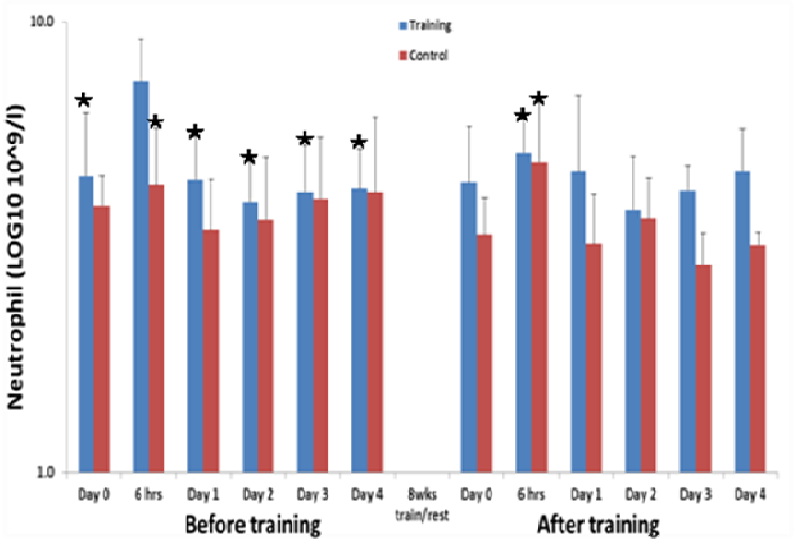


Figure 3.31 Change in neutrophil count over time. Neutrophil activity (Log_{10} 10^9/l) before and after 8 weeks of plyometric training for the training and control groups respectively. * Significantly different from time point 6 hrs before training, ($P < 0.01$). Data are expressed as mean ± sd.

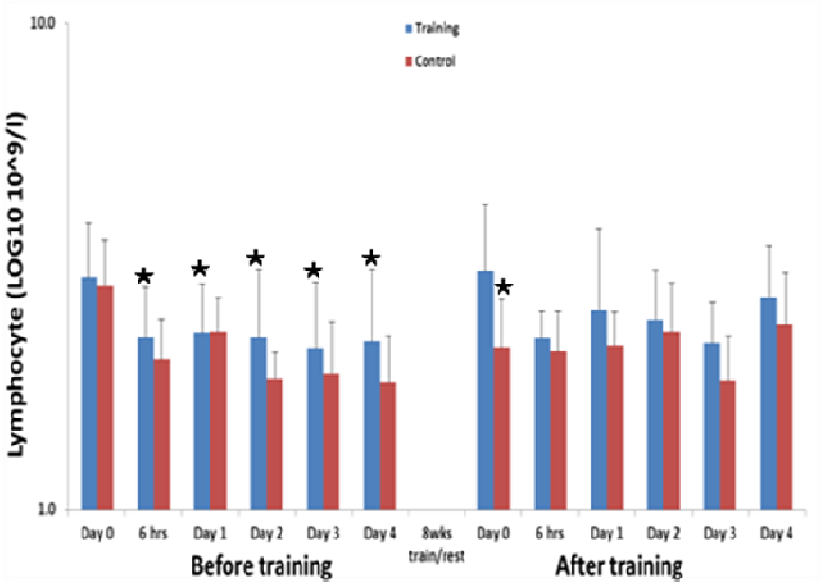


Figure 3.32 Change in lymphocyte count over time. Lymphocyte activity (Log_{10} 10^9/l) before and after 8 weeks of plyometric training for the training and control groups respectively. * Significantly different from day 0 before training, ($P < 0.05$). Data are expressed as mean ± sd.

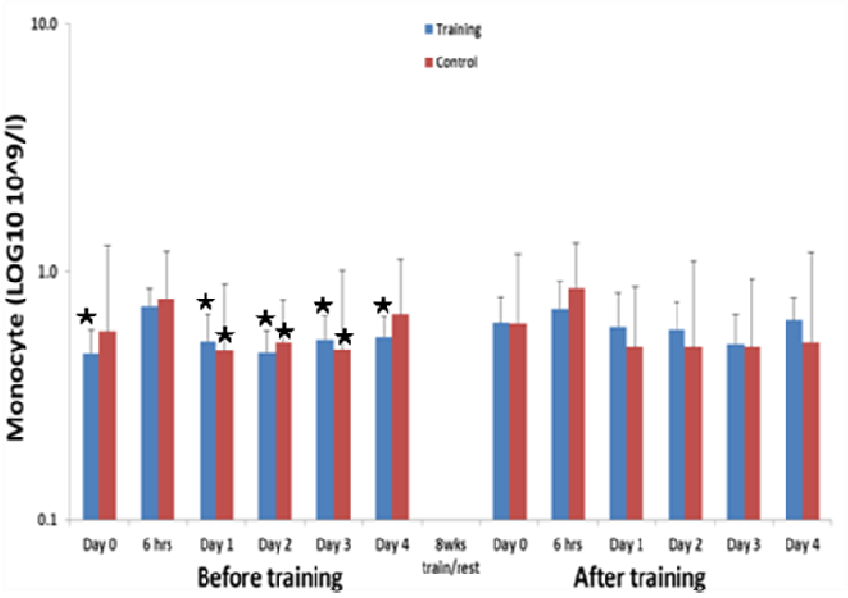


Figure 3.33 Change in monocyte count over time. Monocyte activity (Log_{10} 10^9/l) before and after 8 weeks of plyometric training for the training and control groups respectively. * Significantly different from time point 6 hrs before training, ($P < 0.04$). Data are expressed as mean ± sd.

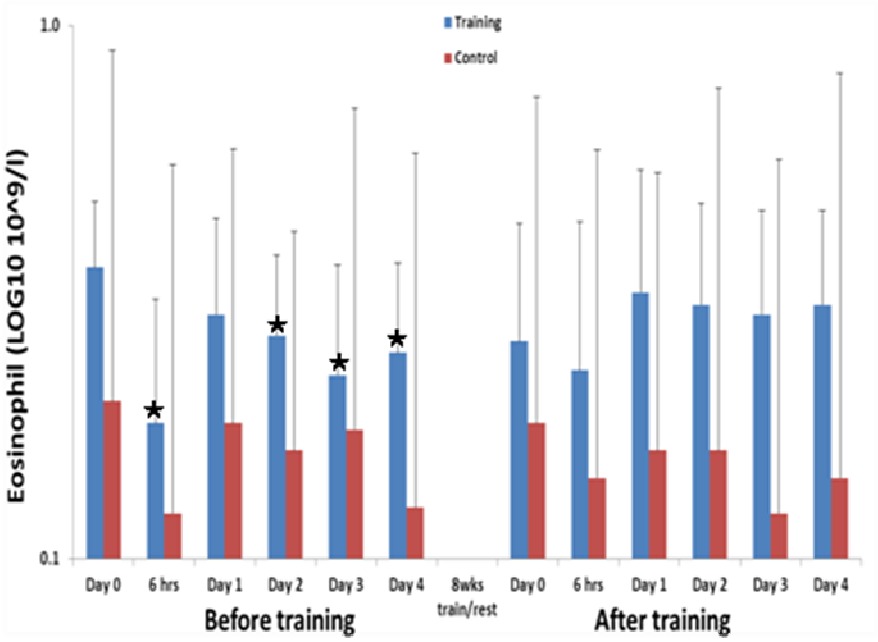


Figure 3.34 Change in eosinophil count over time. Eosinophil activity (Log_{10} 10^9/l) before and after 8 weeks of plyometric training for the training and control groups respectively. * Significantly different from day 0 before training, ($P < 0.03$). Data are expressed as mean ± sd.

3.8 Titin localization by Immunogold

With transmission electron microscopy, in the baseline muscle biopsy, immuno-reactivity for titin was observed prevalently along the M-line region of sarcomere, while the negative control showed relatively low non-specific binding. The muscle biopsies obtained 3 days after the first plyometric intervention showed a higher level of immuno-reactivity compared to the baseline biopsy. The same levels of immuno-reactivity were observed in the muscle biopsies obtained before and after the second acute bout of plyometric exercise. No qualitative differences were observed between the training and the control group at any time points.

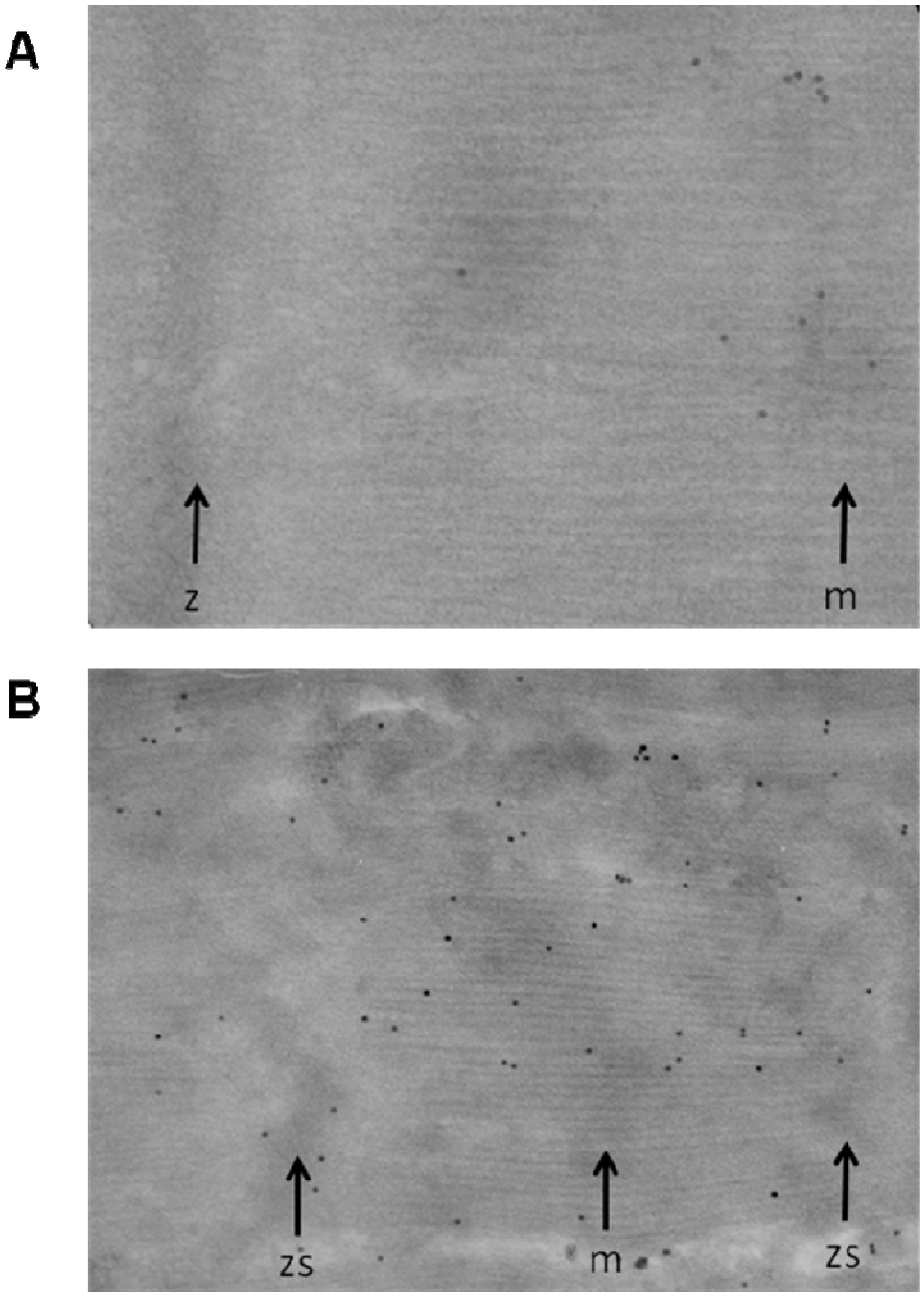


Figure 3.36 Immuno-electron microscopic detection of anti-titin binding sites on ultrathin sections of LR-white resin embedded *vastus lateralis* muscle (z-Z-band, m-M-line and zs-Z-disk streaming). A) Baseline muscle biopsies show, colloidal gold particles distributed along the M-line; B) An example of a muscle biopsy analysed after the first acute bout of plyometric exercise, colloidal gold particles are distributed throughout the sarcomere.

3.9 Calpain-3 activation

There was an increase in calpain-3 autolysis after the exercise intervention at day 3 for both the training and control group. The ratio between “total autolysed calpain-3” and the autolysed calpain-3 remain constant within subjects, however the ratio in the post plyometric exercise biopsy was higher after the first acute bout of exercise for both groups compared to the second acute bout.

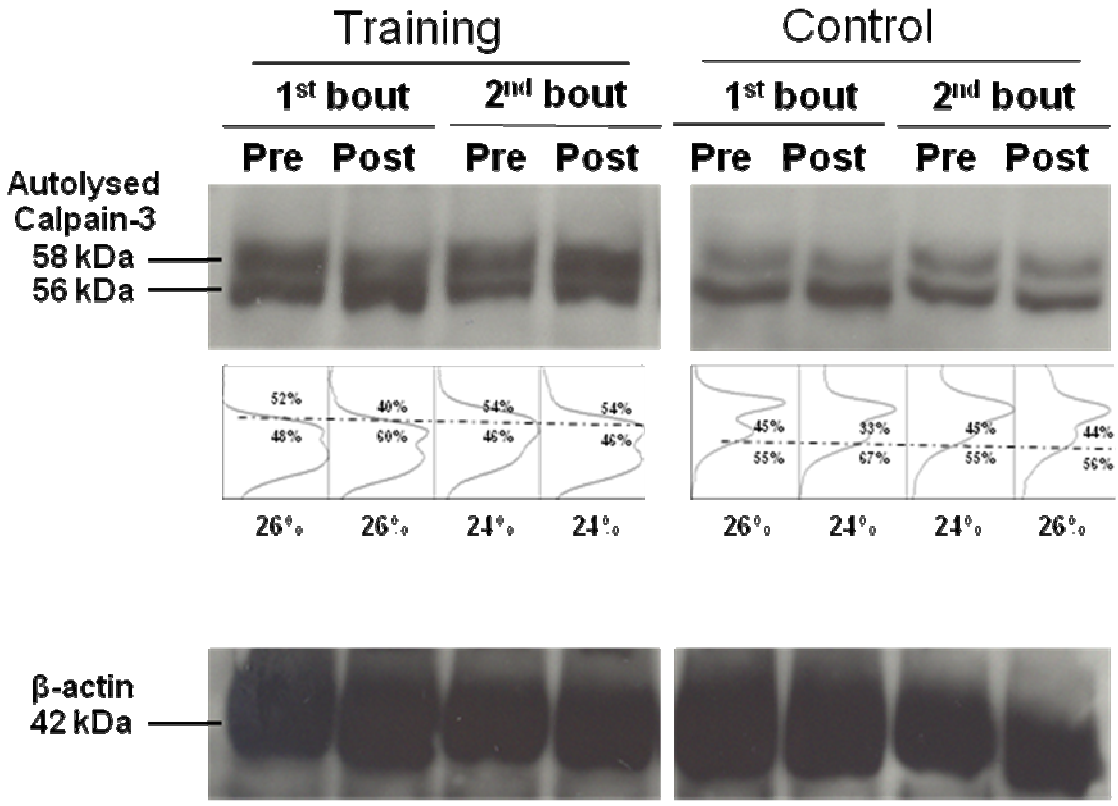


Figure 3.37 Effect of plyometric training on the extent of calpain-3 autolysis. Western blots of calpain-3 for 4 subjects n=2 training group; n=2 control group), before and after training. Figure shows autolysed calpain-3 58- and 56-kDa bands before and after training; and β-actin indicates the relative loading of the samples.

Discussion

Acute Intervention

In a recent publication we reported that the plyometric squat jump protocol used in this acute study, induced muscle damage that was visible by electron microscopy, alongside the often described increased perceived pain and high levels of blood markers of muscle damage. We were also the first to provide direct morphological evidence that plyometric exercise results in preferential damage to fast twitch muscle fibres (Macaluso F, Isaacs AW and Myburgh KH. Plyometric exercise induces damage preferentially of type II muscle fibres. Journal of Athletic Training 2012; in press). Two subjects developed rhabdomyolysis in response to the protocol. To determine if any indirect markers outside of CK are higher with rhabdomyolysis, two subjects are insufficient. Therefore the design of the acute study was to include 26 subjects while focusing more on potential physiological and structural mechanisms induced by a single bout of plyometric exercise. Studies in the literature generally focus on a single factor that could be responsible for the difference in increased CK susceptibility amongst individuals. The value this thesis offers is that various factors (physical characteristics, muscle characteristics, genetic characteristics and the response of subjects to the exercise intervention) were considered when investigating this phenomenon, since a single event cannot explain the complexities of muscle damage. It is clear that adaptations occur. Some adaptations to a single bout of plyometrics are not likely to be the same as those that occur in response to weeks of training where gene expression can cause the accumulation of specific proteins in response.

It still remains unclear why within a group of people exercising at a similar intensity and in a similar environment, only a small percentage would develop rhabdomyolysis (Clarkson et al. 2005b; Devaney et al. 2007; Yamin et al. 2010). The clinical significance is that rhabdomyolysis may lead to renal damage, when excess myoglobin released into the bloodstream precipitates in the nephron (Moeckel-Cole and Clarkson 2009). In this study we investigated whether indirect factors occur to a greater extent in the subjects who respond with very high CK levels.

Plyometric jumping is perfect for studying mechanical muscle damage compared to other damage models such as downhill running which is more suited for the study of the immune response. The immune response has received a lot of attention by investigators in literature over the past decade and is thus not the focus of this thesis. Plyometric jumping could impart forces of up 5 times an individual's body weight on the active muscle groups (McMahon and Greene 1979), therefore physical factors which could contribute to the condition of rhabdomyolysis, considered were individual weight, jump height and weight x jump height. The rationale behind this was that the weight of the subject can be considered the mass of an object that is dropped from a height equal to the jump height of the subject. Considering that gravitational acceleration is constant ($9.8 m/s^2$), the greater the subject's mass the greater the acceleration will be. Thus the force of impact can be considered. However these characteristics all showed no association with the CK response. As mentioned above plyometric jumping results in mainly fast twitch muscle damage, differences in individual % fibre type or the % of damaged fibres were not associated with the CK response therefore we can deduce that individuals with more type II fibres do not present with greater damage.

Muscle soreness increased immediately (6 hr) after the exercise intervention and was higher in the squat compared to the standing position in all subjects. On days 3 and 4 following the plyometric exercise the high responders reported significantly higher soreness scores compared to the low responders. This could indicate that low responders perhaps recover faster than high responders. By analysing muscle structure and ultrastructure using light and electron microscopy, it was possible to observe muscle membrane damage and ultrastructural damage in the form of z-disk streaming. However no visible differences were observed when comparing electron micrographs of high and low responders. This does not indicate with any certainty that there are no differences, because one should remember that the muscle biopsy is representative of only a tiny section of the entire muscle. We observed that damage included sarcolemmal disruption as observed by the loss of dystrophin staining which allowed the leakage of muscle proteins such as CK and Mb into circulation. CK and Mb levels showed wide variability amongst subjects ranging from 153 U/L to 71 024 U/L and 20 ng/ml to 2382 ng/ml 4 days post plyometric exercise respectively.

An interesting observation was that high responders had a significantly greater inflammatory response to exercise compared to low responders. The inflammatory marker CRP peaked one day after plyometric exercise in both groups and remained significantly greater at time points 6 hours, day 1 and day 2, this corresponded with the earlier peak release of WBCs, neutrophils and IL-6 all at 6 hours after the exercise intervention. A possible reason for the more pronounced inflammatory response could be that high responders have a significantly higher number of circulating neutrophils than low responders. Neutrophils accumulate in the muscle injured area within two hours following mechanical damage, acting to phagocytise

cellular debris by producing oxygen free radicals (Pizza et al. 2005; Toumi and Best 2003). Literature suggests that neutrophils may damage surrounding non-injured tissue by rapidly releasing reactive oxygen species known as respiratory burst (Brickson et al. 2001). Brickson et al. (Brickson et al. 2003) used a single stretch injury model in rabbit *tibialis anterior* muscle to demonstrate that by inhibiting neutrophils respiratory burst with an antibody the extent of muscle fibre damage was reduced and the muscle structural proteins desmin and dystrophin were preserved. We propose that initial mechanical injury would cause a greater inflammatory response in high responders; neutrophils thus migrate to the injured area via chemotactic signals produced by damaged skeletal muscle cells. High responders also have slightly higher circulating levels of IL-6 at 6 hours after exercise and IL-6Rα at baseline. Muscle is known to produce IL-6 after a strenuous exercise bout (Tomiya et al. 2004; Willoughby et al. 2003) and not TNF-α. IL-6 can act as both inflammatory and anti-inflammatory mediator (Tilg et al. 1997). In this instance IL-6 would perform pro-inflammatory duties since the muscle is 'damaged' and recruit neutrophils to the compromised areas. Neutrophils would then produce more IL-6 and recruit more neutrophils to remove cell debris. The number of accumulating neutrophils will thus be higher in the high compared to the low responders, resulting in the over production of oxygen free radicals which target the proteins desmin and dystrophin resulting in further muscle membrane damage. This could be the reason for the continued rise in CK up to 4 days after the exercise event, the second peak in Mb release and the blunted CK response seen in the low responders. Should this be the case researchers need to add to the question "Why do only some individuals exercising at a similar intensity and in a similar environment, develop rhabdomyolysis" by asking an alternative question which may

well provide the answer to the first question: “Why do certain individuals exercising at a similar intensity and in a similar environment, develop a greater inflammatory response”. Recently, researchers have attempted to approach both of these questions using a genomic approach.

Recent studies report that a series of SNPs are responsible for an individual’s predisposition to high increases in CK and Mb values post eccentric exercise. These studies have already established the genotypes of the different SNPs described in this thesis using larger study populations. In this thesis a preliminary check on how the subjects fitted into the published genotypes was done. We considered three of these SNPs namely *MYLK* (involved in muscle force production) and *TNFA* and *IL6* (involved with inflammation) genes. Clarkson et al. and Yamin et al. (Clarkson et al. 2005b; Yamin et al. 2008) showed that individuals who possess the MYLCK (TT) and TNFA (GA) and IL6 (CC) alleles are more susceptible to exercise induced muscle damage than peers with similar characteristics exercising at the same intensity. However in this study, individuals who were genotyped with the rare MYLCK TT allele were not necessarily more susceptible to muscle damage. In fact the subject with the highest CK value (71024 U/L) 4 days after exercise was genotyped as wild type homozygous CC. A point to make was that in the study by Clarkson et al. (Clarkson et al. 2005b) CK values between subjects (n=6) in the mutant group were largely varied and thus one individual with a really high CK level may increase the average CK value considerably. All individuals genotyped for the IL6 G-174C SNP were found to be homozygous wild type GG without the homozygous CC mutation and moreover all individuals were found to be homozygous wild type GG without the homozygous GA mutation when genotyped for the TNFA-α-308A SNP. These results clearly contrast

each other since the GG genotype IL6 G-174C and TNFA-α-308A has been shown to be associated with a low CK response profile and high CK response profile respectively. Controversially a 2008 publication stated that the reference numbers of the SNP IDs used by Yamin et al. (Yamin et al. 2008) were incorrect, thus allowing us to focus our attention to other possible SNPs. However, results from the training study show that high responder individuals are able to protect themselves from being susceptible to damage, since post training CK and myoglobin values were blunted post plyometric training. Surely individuals with a genetic mutation should on subsequent bouts of exercise still elicit a similar response as before, since ones genetic make-up remains the same.

Responses to the Plyometric Training Intervention

Next we investigated whether plyometric training would protect the muscle from the ultrastructural damage and rhabdomyolysis seen after a single unaccustomed bout.

Muscle adaptations resulting from plyometric training which could potentially affect damage include fibre type shifts and increasing muscle fibre size exist. Similar to the protocol used in this thesis, Potteiger et al. 1999 subjected individuals to 8 weeks of plyometric training including vertical, bounding and depth jumping activities. The authors reported that individuals doing only plyometric training increased both type I (4.4%) and type II (7.8%) fibre cross-sectional area. In the current study training resulted in no significant increases in fibre CSA or a percentage fibre type shift toward an increased type II fibre profile, although the training group showed a trend toward increased CSA of the type IIa (P=0.07) fibres and percentage fibre shift toward type IIa (P=0.06) fibres. This finding is consistent with the data presented in this thesis that shows preferential damage of type IIa

fibres, therefore indicating significant recruitment. A reason that only a trend was observed could be that subjects performed less jumps during training than in the Potteiger protocol. The trend toward increased type IIa muscle fibre CSA area and percentage fibre shift is consistent with jump performance improvements obtained by the training group. The control group however also improved their vertical jump height, but this can be subscribed to improved coordination of the jump performed the second time around. However, despite the improvement in controls (which was taken into account by the statistical analysis), the improvement in the training group was still very significant.

Soreness of the quadriceps peaked 2 days after the initial bout of plyometric exercise in both groups in the squat position. After the 8 week period of training or rest in the case of controls, soreness was significantly less for the training group compared to the control group indicative of muscle adaptation. Soreness was also less in the control group following 8 weeks of rest compared to the first acute bout of exercise. Interestingly, the blood markers of muscle damage CK and Mb as well as the inflammatory marker CRP, levels were all significantly lower after 8 weeks of training. The control group had no significant differences in these markers. This could be either because all of the subjects in the control group were low responders and that they are somehow protected, or the lower perceived muscle soreness in the control group could perhaps be due to the repeat bout effect. The ‘repeated bout effect’ phenomenon, has been characterized by reduced negative changes to muscle morphology, improvements in muscle function and less severe changes in markers of muscle damage. This has been documented in numerous studies, however; no clear adaptation mechanisms have been shown (Lynn and Morgan et al. 1994; McHugh 2003). The improvement in jump performance by the control

individuals could perhaps be due to improved neural co-ordination during the jump following the first acute bout of exercise and the reduction in biochemical indicators may be due to the remodeling effect of calpain-3 (Kramerova et al. 2007). Nosaka et al. (Nosaka et al. 2001) provided evidence that the protective effect of the repeat bout may last for 6months following the initial exercise bout.

Accompanying the blunted inflammatory response post training was a significant reduction in circulating WBC and neutrophil levels. This is an important finding since this provides a mechanism for exercise induced rhabdomyolysis, suggesting that an inflammatory adaptation occurred resulting in less membrane damage. The electron microscopy results indicated that membrane damage probably was not a result of mechanical influences. Intracellular mechanisms are also activated in the time period following the jumping.

Currently two schools of thought exist as to how muscle specific calpain-3 is activated following eccentric exercise, since there is uncertainty on how this protein is regulated and what its function is. Murphy et al. (Murphy et al. 2007) provided evidence that calpain-3 is autolysed 24 hours following a single bout of eccentric exercise and in a subsequent article showed that calpain-3 is regulated by small increases in resting Ca^{2+} levels rather than mechanically via the stretch of titin (Murphy and Lamb 2009). We did not observe any difference in membrane damage following the first and second acute bouts of exercise (before and after training) by the loss of dystrophin staining. Using transmission electron microscopy we observed that all subjects with the exception of three control subjects presented with no z-disk streaming after training/rest, although still observing the stretch of titin in muscle biopsies taken from trained individuals. When blotting for calpain-3 we did not observe the 94 kDa band showed by Murphy et al. although the ubiquitous

calpains (at 53 and 54 kDa) showed total autolysed expression did not change. However, the second band showed higher expression in biopsy 2 in both training and control groups. Therefore we can speculate that only the first acute bout of exercise (before training) will stimulate the activation of calpain-3.

Titin has been proposed to perform two main functions: it acts as a molecular scaffold for the myofilament thus contributing to the development of the sarcomere and has been termed a 'molecular spring' that stores and re-uses elastic energy thus contributing to passive and active force production by skeletal muscle (Trappe et al. 2002, Lehti et al. 2007, Kontrogianni-Konstantopoulos et al. 2009). In an animal downhill running model Lehti et al. (Lehti et al. 2007) trained rats over 5 sessions and observed that titin expression increased significantly following repeated exercise although immunohistochemical observation showed normal titin staining. The results of this study indicate that this increase in titin expression is in fact a stretch of titin protein as seen 3 days following an acute bout of plyometric exercise (figure 3.36).

All the results taken together lead to the following proposal:

After training membrane damage occurs as described in the acute study, however the myofibrils do show disorganization regardless of the stretch of titin. We considered titin to be stretched and not over expressed as other studies indicate since the antibody Titin c-20 (1:10 in 0.1% BSA-TRIS buffer; sc-8724, mouse monoclonal antibody, Santa Cruz Biotechnology, Santa Cruz, CA, USA) binds and recognizes the C-terminus of titin as observed in fig. 3.36A along the M-line of the sarcomere. Following eccentric exercise this protein will stretch (protein unfolds) and expose a greater surface area for antibody binding as observed in fig. 3.37B

between the M-lines of adjacent sarcomere. Proteins such as CK can only be released if the sarcomere structure is compromised. A single eccentric bout of exercise is enough to desensitize calpain-3 to Ca^{2+} activation on a subsequent bout, suggesting calpain-3 is part of the remodelling effect (Kramerova et al. 2007) rather than degrading muscle proteins for breakdown.

Conclusion

The findings of this thesis suggest rhabdomyolysis could be caused by a combination of factors which increase the inflammatory response in the damaged area causing “secondary inflammatory damage”. These factors may include training state, myokine IL-6 response to acute exercise, neutrophil recruitment and ROS production. All of these factors may determine the extent of myofibre membrane damage experienced following eccentric exercise. Directly linking these factors to membrane damage specifically remains to be researched. This damage may be limited by training, although not necessarily by promoting less membrane disruption but by remodelling sarcomeric proteins via calpain activation. This allows a more stable and fixed assembly of proteins in the sarcomere preventing the release of proteins, independent of membrane damage. The data suggest that titin is stretched rather than overexpressed following eccentric exercise since the protein is found between the M-line of adjacent sarcomeres after plyometric exercise. Finally, the results lend support to the idea that calpain-3 activation is not due to the stretch of titin because biopsies post training still show the stretch of titin, without calpain-3 activation present.

References

Allen DG (2001) Eccentric muscle damage: mechanisms of early reduction of force. Acta Physiol Scand 171: 311-319

Almeida-Silveira MI, Perot C, Pousson M, Goubel F (1994) Effects of stretch-shortening cycle training on mechanical properties and fibre type transition in the rat soleus muscle. Pflugers Arch 427: 289-294

Armstrong RB (1990) Initial events in exercise-induced muscular injury. Med Sci Sports Exerc 22: 429-435

Barash IA, Peters D, Friden J, Lutz GJ, Lieber RL (2002) Desmin cytoskeletal modifications after a bout of eccentric exercise in the rat. Am J Physiol Regul Integr Comp Physiol 283: R958-963

Barroso R, Roschel H, Ugrinowitsch C, Araujo R, Nosaka K, Tricoli V (2010) Effect of eccentric contraction velocity on muscle damage in repeated bouts of elbow flexor exercise. Appl Physiol Nutr Metab 35: 534-540

Beckmann JS, Spencer M (2008) Calpain 3, the "gatekeeper" of proper sarcomere assembly, turnover and maintenance. Neuromuscul Disord 18: 913-921

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254

Brancaccio P, Lippi G, Maffulli N (2010) Biochemical markers of muscular damage. Clin Chem Lab Med 48: 757-767

Brancaccio P, Maffulli N, Buonauro R, Limongelli FM (2008) Serum enzyme monitoring in sports medicine. Clin Sports Med 27: 1-18, vii

Brancaccio P, Maffulli N, Limongelli FM (2007) Creatine kinase monitoring in sport medicine. Br Med Bull 81-82: 209-230

Braseth NR, Allison EJ, Jr., Gough JE (2001) Exertional rhabdomyolysis in a body builder abusing anabolic androgenic steroids. Eur J Emerg Med 8: 155-157

Bray MS, Hagberg JM, Perusse L, Rankinen T, Roth SM, Wolfarth B, Bouchard C (2009) The human gene map for performance and health-related fitness phenotypes: the 2006-2007 update. Med Sci Sports Exerc 41: 35-73

Brickson S, Hollander J, Corr DT, Ji LL, Best TM (2001) Oxidant production and immune response after stretch injury in skeletal muscle. Med Sci Sports Exerc 33: 2010-2015

Brickson S, Ji LL, Schell K, Olabisi R, St Pierre Schneider B, Best TM (2003) M1/70 attenuates blood-borne neutrophil oxidants, activation, and myofiber damage following stretch injury. J Appl Physiol 95: 969-976

Brockett CL, Morgan DL, Proske U (2001) Human hamstring muscles adapt to eccentric exercise by changing optimum length. Med Sci Sports Exerc 33: 783-790

Brown TP (2004) Exertional rhabdomyolysis: early recognition is key. Phys Sportsmed 32: 15-20

Burgess KE, Connick MJ, Graham-Smith P, Pearson SJ (2007) Plyometric vs. isometric training influences on tendon properties and muscle output. J Strength Cond Res 21: 986-989

Byrne C, Eston R (2002) The effect of exercise-induced muscle damage on isometric and dynamic knee extensor strength and vertical jump performance. J Sports Sci 20: 417-425

Byrne C, Eston RG, Edwards RH (2001) Characteristics of isometric and dynamic strength loss following eccentric exercise-induced muscle damage. Scand J Med Sci Sports 11: 134-140

Cagan S, Hatala R, Kucharova L (1991) A proposed classification of changes in the electrocardiogram in acute myocardial infarction. Cor Vasa 33: 1-10

Carlsson L, Yu JG, Thornell LE (2008) New aspects of obscurin in human striated muscles. Histochem Cell Biol 130: 91-103

Chen TC (2006) Variability in muscle damage after eccentric exercise and the repeated bout effect. Res Q Exerc Sport 77: 362-371

Chen TC, Chen HL, Lin MJ, Wu CJ, Nosaka K (2010) Potent protective effect conferred by four bouts of low-intensity eccentric exercise. Med Sci Sports Exerc 42: 1004-1012

Chen TC, Hsieh SS (2001) Effects of a 7-day eccentric training period on muscle damage and inflammation. Med Sci Sports Exerc 33: 1732-1738

Chimera NJ, Swanik KA, Swanik CB, Straub SJ (2004) Effects of Plyometric Training on Muscle-Activation Strategies and Performance in Female Athletes. J Athl Train 39: 24-31

Clarkson PM, Byrnes WC, Gillisson E, Harper E (1987) Adaptation to exercise-induced muscle damage. Clin Sci (Lond) 73: 383-386

Clarkson PM, Devaney JM, Gordish-Dressman H, Thompson PD, Hubal MJ, Urso M, Price TB, Angelopoulos TJ, Gordon PM, Moyna NM, Pescatello LS, Visich PS, Zoeller RF, Seip RL, Hoffman EP (2005a) ACTN3 genotype is associated with increases in muscle strength in response to resistance training in women. J Appl Physiol 99: 154-163

Clarkson PM, Hoffman EP, Zambraski E, Gordish-Dressman H, Kearns A, Hubal M, Harmon B, Devaney JM (2005b) ACTN3 and MLCK genotype associations with exertional muscle damage. J Appl Physiol 99: 564-569

Close GL, Kayani A, Vasilaki A, McArdle A (2005) Skeletal muscle damage with exercise and aging. Sports Med 35: 413-427

Coates D (2003) The angiotensin converting enzyme (ACE). Int J Biochem Cell Biol 35: 769-773

Danser AH, Schalekamp MA, Bax WA, van den Brink AM, Saxena PR, Riegger GA, Schunkert H (1995) Angiotensin-converting enzyme in the human heart. Effect of the deletion/insertion polymorphism. Circulation 92: 1387-1388

Davis JS, Epstein ND (2007) Mechanism of tension generation in muscle: an analysis of the forward and reverse rate constants. Biophys J 92: 2865-2874

de Villarreal ES, Gonzalez-Badillo JJ, Izquierdo M (2008) Low and moderate plyometric training frequency produces greater jumping and sprinting gains compared with high frequency. J Strength Cond Res 22: 715-725

Devaney JM, Hoffman EP, Gordish-Dressman H, Kearns A, Zambraski E, Clarkson PM (2007) IGF-II gene region polymorphisms related to exertional muscle damage. J Appl Physiol 102: 1815-1823

Dietze GJ, Henriksen EJ (2008) Angiotensin-converting enzyme in skeletal muscle: sentinel of blood pressure control and glucose homeostasis. J Renin Angiotensin Aldosterone Syst 9: 75-88

Doring FE, Onur S, Geisen U, Boulay MR, Perusse L, Rankinen T, Rauramaa R, Wolfahrt B, Bouchard C (2010) ACTN3 R577X and other polymorphisms are not associated with elite endurance athlete status in the Genathlete study. J Sports Sci 28: 1355-1359

Evans WJ, Cannon JG (1991) The metabolic effects of exercise-induced muscle damage. Exerc Sport Sci Rev 19: 99-125

Feasson L, Stockholm D, Freyssenet D, Richard I, Duguez S, Beckmann JS, Denis C (2002) Molecular adaptations of neuromuscular disease-associated proteins in response to eccentric exercise in human skeletal muscle. J Physiol 543: 297-306

Fishman D, Faulds G, Jeffery R, Mohamed-Ali V, Yudkin JS, Humphries S, Woo P (1998) The effect of novel polymorphisms in the interleukin-6 (IL-6) gene on IL-6 transcription and plasma IL-6 levels, and an association with systemic-onset juvenile chronic arthritis. J Clin Invest 102: 1369-1376

Florini JR, Ewton DZ, Coolican SA (1996) Growth hormone and the insulin-like growth factor system in myogenesis. Endocr Rev 17: 481-517

Friden J, Seger J, Ekblom B (1988) Sublethal muscle fibre injuries after high-tension anaerobic exercise. Eur J Appl Physiol Occup Physiol 57: 360-368

Friden J, Sjostrom M, Ekblom B (1983) Myofibrillar damage following intense eccentric exercise in man. Int J Sports Med 4: 170-176

Gallardo E, Saenz A, Illa I (2011) Limb-girdle muscular dystrophy 2A. Handb Clin Neurol 101: 97-110

George M, Delgaudio A, Salhanick SD (2010) Exertional rhabdomyolysis--when should we start worrying? Case reports and literature review. Pediatr Emerg Care 26: 864-866

Geronilla KB, Miller GR, Mowrey KF, Wu JZ, Kashon ML, Brumbaugh K, Reynolds J, Hubbs A, Cutlip RG (2003) Dynamic force responses of skeletal muscle during stretch-shortening cycles. Eur J Appl Physiol 90: 144-153

Giaccaglia V, Nicklas B, Kritchevsky S, Mychalecky J, Messier S, Bleecker E, Pahor M (2008) Interaction between angiotensin converting enzyme insertion/deletion genotype and exercise training on knee extensor strength in older individuals. Int J Sports Med 29: 40-44

Guadalupe-Grau A, Perez-Gomez J, Olmedillas H, Chavarren J, Dorado C, Santana A, Serrano-Sanchez JA, Calbet JA (2009) Strength training combined with plyometric jumps in adults: sex differences in fat-bone axis adaptations. J Appl Physiol 106: 1100-1111

Guerrero M, Guiu-Comadevall M, Cadefau JA, Parra J, Balius R, Estruch A, Rodas G, Bedini JL, Cusso R (2008) Fast and slow myosins as markers of muscle injury. Br J Sports Med 42: 581-584; discussion 584

Hagberg JM, Rankinen T, Loos RJ, Perusse L, Roth SM, Wolfarth B, Bouchard C (2011) Advances in exercise, fitness, and performance genomics in 2010. Med Sci Sports Exerc 43: 743-752

Hayashi C, Ono Y, Doi N, Kitamura F, Tagami M, Mineki R, Arai T, Taguchi H, Yanagida M, Hirner S, Labeit D, Labeit S, Sorimachi H (2008) Multiple molecular interactions implicate the connectin/titin N2A region as a modulating scaffold for p94/calpain 3 activity in skeletal muscle. J Biol Chem 283: 14801-14814

Heled Y, Bloom MS, Wu TJ, Stephens Q, Deuster PA (2007) CK-MM and ACE genotypes and physiological prediction of the creatine kinase response to exercise. J Appl Physiol 103: 504-510

Herrero JA, Izquierdo M, Maffiuletti NA, Garcia-Lopez J (2006) Electromyostimulation and plyometric training effects on jumping and sprint time. Int J Sports Med 27: 533-539

Howatson G, Van Someren K, Hortobagyi T (2007) Repeated bout effect after maximal eccentric exercise. Int J Sports Med 28: 557-563

Howatson G, van Someren KA (2007) Evidence of a contralateral repeated bout effect after maximal eccentric contractions. Eur J Appl Physiol 101: 207-214

Howell JN, Chleboun G, Conatser R (1993) Muscle stiffness, strength loss, swelling and soreness following exercise-induced injury in humans. J Physiol 464: 183-196

Huang J, Forsberg NE (1998) Role of calpain in skeletal-muscle protein degradation. Proc Natl Acad Sci U S A 95: 12100-12105

Jennings CL, Viljoen W, Durandt J, Lambert MI (2005) The reliability of the FitroDyne as a measure of muscle power. J Strength Cond Res. 19:859-63.

Jones A, Woods DR (2003) Skeletal muscle RAS and exercise performance. Int J Biochem Cell Biol 35: 855-866

Kay AD, Blazevich AJ (2012) Effect of acute static stretch on maximal muscle performance: a systematic review. Med Sci Sports Exerc 44: 154-164

Kim HJ, Lee YH, Kim CK (2007) Biomarkers of muscle and cartilage damage and inflammation during a 200 km run. Eur J Appl Physiol 99: 443-447

Kinbara K, Sorimachi H, Ishiura S, Suzuki K (1998) Skeletal muscle-specific calpain, p49: structure and physiological function. Biochem Pharmacol 56: 415-420

Klein-Nulend J, van der Plas A, Semeins CM, Ajubi NE, Frangos JA, Nijweide PJ, Burger EH (1995) Sensitivity of osteocytes to biomechanical stress in vitro. FASEB J 9: 441-445

Knechtle B, Knechtle P, Mrazek C, Senn O, Rosemann T, Imoberdorf R, Ballmer P (2011) No effect of short-term amino acid supplementation on variables related to skeletal muscle damage in 100 km ultra-runners - a randomized controlled trial. J Int Soc Sports Nutr 8: 6

Knochel JP (1990) Catastrophic medical events with exhaustive exercise: "white collar rhabdomyolysis". Kidney Int 38: 709-719

Koh TJ (2002) Do small heat shock proteins protect skeletal muscle from injury? Exerc Sport Sci Rev 30: 117-121

Koh TJ, Brooks SV (2001) Lengthening contractions are not required to induce protection from contraction-induced muscle injury. Am J Physiol Regul Integr Comp Physiol 281: R155-161

Kohn TA, Myburgh KH (2006) Electrophoretic separation of human skeletal muscle myosin heavy chain isoforms: the importance of reducing agents. J Physiol Sci 56: 355-360

Koller A, Mair J, Schobersberger W, Wohlfarter T, Haid C, Mayr M, Villiger B, Frey W, Puschendorf B (1998) Effects of prolonged strenuous endurance exercise on plasma myosin heavy chain fragments and other muscular proteins. Cycling vs running. J Sports Med Phys Fitness 38: 10-17

Komi PV (2000) Stretch-shortening cycle: a powerful model to study normal and fatigued muscle. J Biomech 33: 1197-1206

Kontrogianni-Konstantopoulos A, Ackermann MA, Bowman AL, Yap SV, Bloch RJ (2009) Muscle giants: molecular scaffolds in sarcomerogenesis. Physiol Rev 89: 1217-1267

Kramerova I, Kudryashova E, Venkatraman G, Spencer MJ (2007) Calpain 3 participates in sarcomere remodeling by acting upstream of the ubiquitin-proteasome pathway. Hum Mol Genet 16: 1006

Kubo K, Morimoto M, Komuro T, Yata H, Tsunoda N, Kanehisa H, Fukunaga T (2007) Effects of plyometric and weight training on muscle-tendon complex and jump performance. Med Sci Sports Exerc 39: 1801-1810

Kyrolainen H, Avela J, McBride JM, Koskinen S, Andersen JL, Sipila S, Takala TE, Komi PV (2005) Effects of power training on muscle structure and neuromuscular performance. Scand J Med Sci Sports 15: 58-64

Lauritzen F, Paulsen G, Raastad T, Bergersen LH, Owe SG (2009) Gross ultrastructural changes and necrotic fiber segments in elbow flexor muscles after maximal voluntary eccentric action in humans. J Appl Physiol 107: 1923-1934

Lehti TM, Kalliokoski R, Komulainen J (2007) Repeated bout effect on the cytoskeletal proteins titin, desmin, and dystrophin in rat skeletal muscle. J Muscle Res Cell Motil 28: 39-47

Lieber RL, Thornell LE, Friden J (1996) Muscle cytoskeletal disruption occurs within the first 15 min of cyclic eccentric contraction. J Appl Physiol 80: 278-284

Luebbers PE, Potteiger JA, Hulver MW, Thyfault JP, Carper MJ, Lockwood RH (2003) Effects of plyometric training and recovery on vertical jump performance and anaerobic power. J Strength Cond Res 17: 704-709

Lynn R, Morgan DL (1994) Decline running produces more sarcomeres in rat vastus intermedius muscle fibers than does incline running. J Appl Physiol 77: 1439-1444

Lynn R, Talbot JA, Morgan DL (1998) Differences in rat skeletal muscles after incline and decline running. J Appl Physiol 85: 98-104

Macaluso F, Isaacs AW and Myburgh KH (2012) Plyometric exercise induces damage preferentially of type II muscle fibres. J of Athletic Training; in press

Malisoux L, Francaux M, Nielens H, Renard P, Lebacq J, Theisen D (2006a) Calcium sensitivity of human single muscle fibers following plyometric training. Med Sci Sports Exerc 38: 1901-1908

Malisoux L, Francaux M, Nielens H, Theisen D (2006b) Stretch-shortening cycle exercises: an effective training paradigm to enhance power output of human single muscle fibers. J Appl Physiol 100: 771-779

Marginson V, Rowlands AV, Gleeson NP, Eston RG (2005) Comparison of the symptoms of exercise-induced muscle damage after an initial and repeated bout of plyometric exercise in men and boys. J Appl Physiol 99: 1174-1181

Marinella MA (1998) Exertional rhabdomyolysis after recent coxsackie B virus infection. South Med J 91: 1057-1059

Markovic G (2007) Does plyometric training improve vertical jump height? A meta-analytical review. Br J Sports Med 41: 349-355; discussion 355

Markovic G, Mikulic P (2010) Neuro-musculoskeletal and performance adaptations to lower-extremity plyometric training. Sports Med 40: 859-895

Matavulj D, Kukolj M, Ugarkovic D, Tihanyi J, Jaric S (2001) Effects of plyometric training on jumping performance in junior basketball players. J Sports Med Phys Fitness 41: 159-164

McHugh MP (2003) Recent advances in the understanding of the repeated bout effect: the protective effect against muscle damage from a single bout of eccentric exercise. Scand J Med Sci Sports 13: 88-97

McHugh MP, Connolly DA, Eston RG, Kremenic IJ, Nicholas SJ, Gleim GW (1999) The role of passive muscle stiffness in symptoms of exercise-induced muscle damage. Am J Sports Med 27: 594-599

McHugh MP, Cosgrave CH (2010) To stretch or not to stretch: the role of stretching in injury prevention and performance. Scand J Med Sci Sports 20: 169-181

McMahon TA, Greene PR (1979) The influence of track compliance on running. J Biomech 12: 893-904

Miles MP, Pearson SD, Andring JM, Kidd JR, Volpe SL (2007) Effect of carbohydrate intake during recovery from eccentric exercise on interleukin-6 and muscle-damage markers. Int J Sport Nutr Exerc Metab 17: 507-520

Moeckel-Cole SA, Clarkson PM (2009) Rhabdomyolysis in a collegiate football player. J Strength Cond Res 23: 1055-1059

Morgan DL, Allen DG (1999) Early events in stretch-induced muscle damage. J Appl Physiol 87: 2007-2015

Murphy RM (2010) Calpains, skeletal muscle function and exercise. Clin Exp Pharmacol Physiol 37: 385-391

Murphy RM, Goodman CA, McKenna MJ, Bennie J, Leikis M, Lamb GD (2007) Calpain-3 is autolyzed and hence activated in human skeletal muscle 24 h following a single bout of eccentric exercise. J Appl Physiol 103: 926-931

Murphy RM, Lamb GD (2009) Endogenous calpain-3 activation is primarily governed by small increases in resting cytoplasmic [Ca2+] and is not dependent on stretch. J Biol Chem 284: 7811-7819

Newham DJ, Jones DA, Clarkson PM (1987) Repeated high-force eccentric exercise: effects on muscle pain and damage. J Appl Physiol 63: 1381-1386

Nikolaidis MG, Jamurtas AZ, Paschalis V, Fatouros IG, Koutedakis Y, Kouretas D (2008) The effect of muscle-damaging exercise on blood and skeletal muscle oxidative stress: magnitude and time-course considerations. Sports Med 38: 579-606

North KN, Beggs AH (1996) Deficiency of a skeletal muscle isoform of alpha-actinin (alpha-actinin-3) in merosin-positive congenital muscular dystrophy. Neuromuscul Disord 6: 229-235

Nosaka K, Newton M, Sacco P (2002) Responses of human elbow flexor muscles to electrically stimulated forced lengthening exercise. Acta Physiol Scand 174: 137-145

Nosaka K, Sakamoto K, Newton M, Sacco P (2001) How long does the protective effect on eccentric exercise-induced muscle damage last? Med Sci Sports Exerc 33: 1490-1495

O'Dell SD, Day IN (1998) Insulin-like growth factor II (IGF-II). Int J Biochem Cell Biol 30: 767-771

Ojima K, Ono Y, Hata S, Koyama S, Doi N, Sorimachi H (2005) Possible functions of p94 in connectin-mediated signaling pathways in skeletal muscle cells. J Muscle Res Cell Motil 26: 409-417

Parcell AC, Woolstenhulme MT, Sawyer RD (2009) Structural protein alterations to resistance and endurance cycling exercise training. J Strength Cond Res 23: 359-365

Patel TJ, Cuizon D, Mathieu-Costello O, Friden J, Lieber RL (1998) Increased oxidative capacity does not protect skeletal muscle fibers from eccentric contraction-induced injury. Am J Physiol 274: R1300-1308

Paulin D, Li Z (2004) Desmin: a major intermediate filament protein essential for the structural integrity and function of muscle. Exp Cell Res 301: 1-7

Phillips T, Childs AC, Dreon DM, Phinney S, Leeuwenburgh C (2003) A dietary supplement attenuates IL-6 and CRP after eccentric exercise in untrained males. Med Sci Sports Exerc 35: 2032-2037

Pizza FX, Cavender D, Stockard A, Baylies H, Beighle A (1999) Anti-inflammatory doses of ibuprofen: effect on neutrophils and exercise-induced muscle injury. Int J Sports Med 20: 98-102

Pizza FX, Koh TJ, McGregor SJ, Brooks SV (2002) Muscle inflammatory cells after passive stretches, isometric contractions, and lengthening contractions. J Appl Physiol 92: 1873-1878

Pizza FX, Peterson JM, Baas JH, Koh TJ (2005) Neutrophils contribute to muscle injury and impair its resolution after lengthening contractions in mice. J Physiol 562: 899-913

Potteiger JA, Lockwood RH, Haub MD, Dolezal BA, Alumzaini KS, Schroeder JM, Zebas CJ (1999) Muscle power and fiber characteristic following 8 weeks of plyometric training. J Strenght and Cond Res 13: 275-279.

Pousson M, Perot C, Goubel F (1991) Stiffness changes and fibre type transitions in rat soleus muscle produced by jumping training. Pflugers Arch 419: 127-130

Pousson M, Van Hoecke J, Goubel F (1990) Changes in elastic characteristics of human muscle induced by eccentric exercise. J Biomech 23: 343-348

Proske U, Morgan DL (2001) Muscle damage from eccentric exercise: mechanism, mechanical signs, adaptation and clinical applications. J Physiol 537: 333-345

Rankinen T, Perusse L, Rauramaa R, Rivera MA, Wolfarth B, Bouchard C (2002) The human gene map for performance and health-related fitness phenotypes: the 2001 update. Med Sci Sports Exerc 34: 1219-1233

Reich TE, Lindstedt SL, LaStayo PC, Pierotti DJ (2000) Is the spring quality of muscle plastic? Am J Physiol Regul Integr Comp Physiol 278: R1661-1666

Rodriguez-Romo G, Ruiz JR, Santiago C, Fiuza-Luces C, Gonzalez-Freire M, Gomez-Gallego F, Moran M, Lucia A (2010) Does the ACE I/D polymorphism, alone or in

combination with the ACTN3 R577X polymorphism, influence muscle power phenotypes in young, non-athletic adults? Eur J Appl Physiol 110: 1099-1106

Sacco P, Jones DA (1992) The protective effect of damaging eccentric exercise against repeated bouts of exercise in the mouse tibialis anterior muscle. Exp Physiol 77: 757-760

Saez-Saez de Villarreal E, Requena B, Newton RU (2010) Does plyometric training improve strength performance? A meta-analysis. J Sci Med Sport 13: 513-522

Salmons S, Gale DR, Sreter FA (1978) Ultrastructural aspects of the transformation of muscle fibre type by long term stimulation: changes in Z discs and mitochondria. J Anat 127: 17-31

Sam M, Shah S, Friden J, Milner DJ, Capetanaki Y, Lieber RL (2000) Desmin knockout muscles generate lower stress and are less vulnerable to injury compared with wild-type muscles. Am J Physiol Cell Physiol 279: C1116-1122

Sayers SP, Clarkson PM (2002) Exercise-induced rhabdomyolysis. Curr Sports Med Rep 1: 59-60

Schiaffino S, Partridge T (2008) Skeletal Muscle Repair and Regeneration. Amsterdam, The Netherlands: Springer pg. 222-224

Seto JT, Lek M, Quinlan KG, Houweling PJ, Zheng XF, Garton F, MacArthur DG, Raftery JM, Garvey SM, Hauser MA, Yang N, Head SI, North KN (2011) Deficiency of alpha-actinin-3 is associated with increased susceptibility to contraction-induced damage and skeletal muscle remodeling. Hum Mol Genet 20: 2914-2927

Simpson RJ, Florida-James GD, Whyte GP, Guy K (2006) The effects of intensive, moderate and downhill treadmill running on human blood lymphocytes expressing the adhesion/activation molecules CD54 (ICAM-1), CD18 (beta2 integrin) and CD53. Eur J Appl Physiol 97: 109-121

Skurvydas A, Brazaitis M, Kamandulis S (2011) Repeated bout effect is not correlated with intraindividual variability during muscle-damaging exercise. J Strength Cond Res 25: 1004-1009

Smith C, Kruger MJ, Smith RM, Myburgh KH (2008) The inflammatory response to skeletal muscle injury: illuminating complexities. Sports Med 38: 947-969

Sorichter S, Mair J, Koller A, Gebert W, Rama D, Calzolari C, Artner-Dworzak E, Puschendorf B (1997) Skeletal troponin I as a marker of exercise-induced muscle damage. J Appl Physiol 83: 1076-1082

Sorichter S, Mair J, Koller A, Pelsers MM, Puschendorf B, Glatz JF (1998) Early assessment of exercise induced skeletal muscle injury using plasma fatty acid binding protein. Br J Sports Med 32: 121-124

Sorichter S, Puschendorf B, Mair J (1999) Skeletal muscle injury induced by eccentric muscle action: muscle proteins as markers of muscle fiber injury. Exerc Immunol Rev 5: 5-21

Stull JT, Kamm KE, Vandenboom R (2011) Myosin light chain kinase and the role of myosin light chain phosphorylation in skeletal muscle. Arch Biochem Biophys 510: 120-128

Takekura H, Fujinami N, Nishizawa T, Ogasawara H, Kasuga N (2001) Eccentric exercise-induced morphological changes in the membrane systems involved in excitation-contraction coupling in rat skeletal muscle. J Physiol 533: 571-583

Ten Broek RW, Grefte S, Von den Hoff JW (2010) Regulatory factors and cell populations involved in skeletal muscle regeneration. J Cell Physiol 224: 7-16

Thoenes M. (2005) Rhabdomyolysis: when exercising becomes a risk. J Pediatr Health Care 24: 189-193

Thompson D, Pepys MB, Wood SP (1999) The physiological structure of human C-reactive protein and its complex with phosphocholine. Structure 15:169-77

Thompson HS, Clarkson PM, Scordilis SP (2002) The repeated bout effect and heat shock proteins: intramuscular HSP27 and HSP70 expression following two bouts of eccentric exercise in humans. Acta Physiol Scand 174: 47-56

Thompson HS, Scordilis SP, Clarkson PM, Lohrer WA (2001) A single bout of eccentric exercise increases HSP27 and HSC/HSP70 in human skeletal muscle. Acta Physiol Scand 171: 187-193

Tiidus TM (2008) Skeletal muscle damage and repair. United States of America: Human Kinetics pg. 5

Tilg H, Dinarello CA, Mier JW (1997) IL-6 and APPs: anti-inflammatory and immunosuppressive mediators. Immunol Today 18: 428-432

Toft E, Sinkjaer T, Andreassen S (1989) Mechanical and electromyographic responses to stretch of the human anterior tibial muscle at different levels of contraction. Exp Brain Res 74: 213-219

Tomiya A, Aizawa T, Nagatomi R, Sensui H, Kokubun S (2004) Myofibers express IL-6 after eccentric exercise. Am J Sports Med 32: 503-508

Toumi H, Best TM (2003) The inflammatory response: friend or enemy for muscle injury? Br J Sports Med 37: 284-286

Trappe TA, Carrithers JA, White F, Lambert CP, Evans WJ, Dennis RA (2002) Titin and nebulin content in human skeletal muscle following eccentric resistance exercise. Muscle Nerve 25: 289-292

Turner AM, Owings M, Schwane JA (2003) Improvement in running economy after 6 weeks of plyometric training. J Strength Cond Res 17: 60-67

Twist C, Eston R (2005) The effects of exercise-induced muscle damage on maximal intensity intermittent exercise performance. Eur J Appl Physiol 94: 652-658

Van Cutsem M, Duchateau J, Hainaut K (1998) Changes in single motor unit behaviour contribute to the increase in contraction speed after dynamic training in humans. J Physiol 513 (Pt 1): 295-305

Varsano S, Gershman M, Hamaoui E (2000) Pergolide-induced dyspnea, bilateral pleural effusion and peripheral edema. Respiration 67: 580-582

Vertel RM, Knochel JP (1967) Acute renal failure due to heat injury. An analysis of ten cases associated with a high incidence of myoglobinuria. Am J Med 43: 435-451

Vishnoi M, Pandey SN, Choudhury G, Kumar A, Modi DR, Mittal B (2007) Do TNFA -308 G/A and IL6 -174 G/C gene polymorphisms modulate risk of gallbladder cancer in the north Indian population? Asian Pac J Cancer Prev 8: 567-572

Vissing K, Brink M, Lonbro S, Sorensen H, Overgaard K, Danborg K, Mortensen J, Elstrom O, Rosenhoj N, Ringgaard S, Andersen JL, Aagaard P (2008) Muscle adaptations to plyometric vs. resistance training in untrained young men. J Strength Cond Res 22: 1799-1810

Warren GL, Hermann KM, Ingalls CP, Masselli MR, Armstrong RB (2000) Decreased EMG median frequency during a second bout of eccentric contractions. Med Sci Sports Exerc 32: 820-829

Warren GL, Ingalls CP, Lowe DA, Armstrong RB (2001) Excitation-contraction uncoupling: major role in contraction-induced muscle injury. Exerc Sport Sci Rev 29: 82-87

Weibel ER, Kistler GS, Scherle WF (1966) Practical stereological methods for morphometric cytology. J Cell Biol 30: 23-38

Wen SJ, Wang ZG, Chen GH, Liu Y, Liu JL, Luo Y, Tang J (2005) [Relationship of the HSG gene single nucleotide polymorphism and hypertension]. Zhonghua Yu Fang Yi Xue Za Zhi 39: 15-18

Wilk KE, Voight ML, Keirns MA, Gambetta V, Andrews JR, Dillman CJ (1993) Stretch-shortening drills for the upper extremities: theory and clinical application. J Orthop Sports Phys Ther 17: 225-239

Willoughby DS, McFarlin B, Bois C (2003) Interleukin-6 expression after repeated bouts of eccentric exercise. Int J Sports Med 24: 15-21

Woods D (2009) Angiotensin-converting enzyme, renin-angiotensin system and human performance. Med Sport Sci 54: 72-87

Wroblewski R, Jansson E (1975) Fine structure of single fibres of human skeletal muscle. Cell Tissue Res 161: 471-476

Yamin C, Amir O, Sagiv M, Attias E, Meckel Y, Eynon N, Amir RE (2007) ACE ID genotype affects blood creatine kinase response to eccentric exercise. J Appl Physiol 103: 2057-2061

Yamin C, Duarte JA, Oliveira JM, Amir O, Sagiv M, Eynon N, Amir RE (2008) IL6 (-174) and TNFA (-308) promoter polymorphisms are associated with systemic creatine kinase response to eccentric exercise. Eur J Appl Physiol 104: 579-586

Yamin C, Oliveira J, Meckel Y, Eynon N, Sagiv M, Ayalon M, Alves AJ, Duarte JA (2010) CK-MM gene polymorphism does not influence the blood CK activity levels after exhaustive eccentric exercise. Int J Sports Med 31: 213-217

Young WB, Wilson GJ, Byrne C (1999) A comparison of drop jump training methods: effects on leg extensor strength qualities and jumping performance. Int J Sports Med 20: 295-303

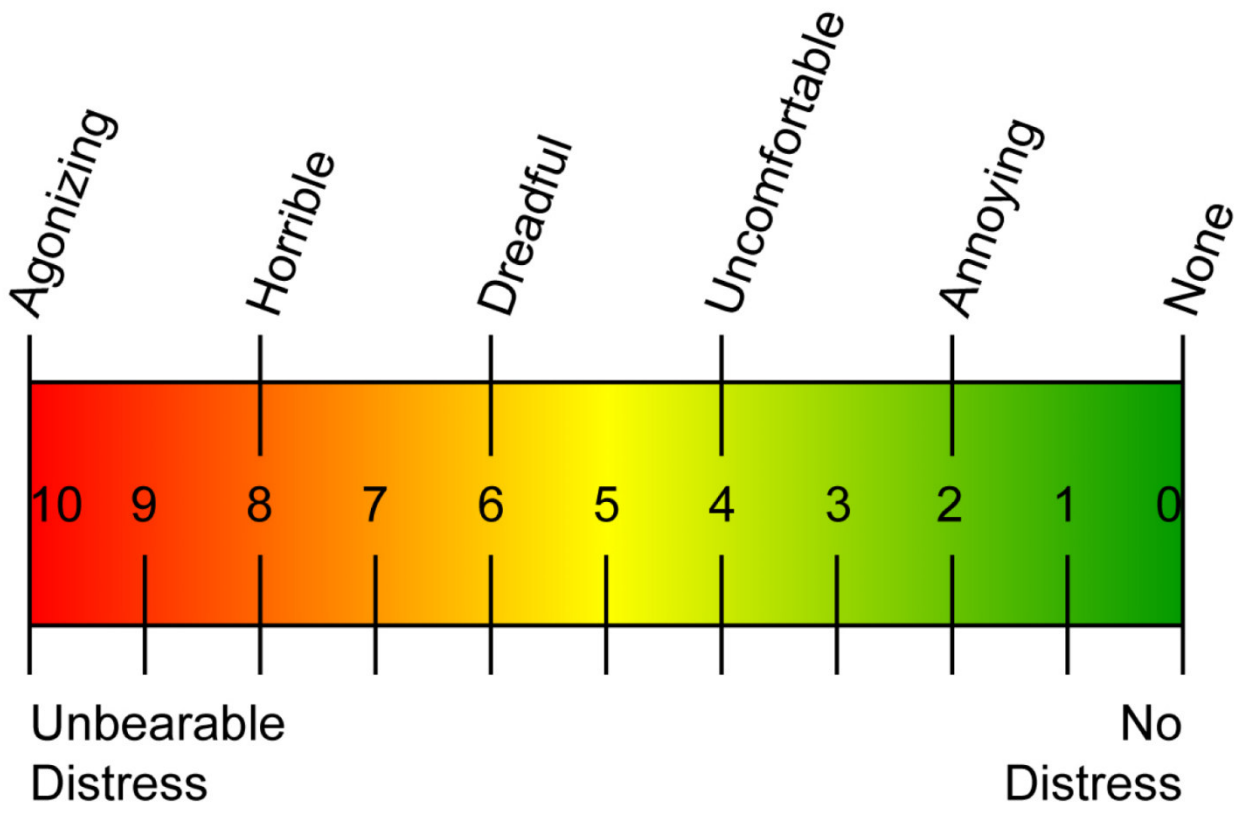
Yu JG, Furst DO, Thornell LE (2003) The mode of myofibril remodelling in human skeletal muscle affected by DOMS induced by eccentric contractions. Histochem Cell Biol 119: 383-393

Yu JG, Malm C, Thornell LE (2002) Eccentric contractions leading to DOMS do not cause loss of desmin nor fibre necrosis in human muscle. Histochem Cell Biol 118: 29-34

Zhou DQ, Hu Y, Liu G, Gong L, Xi Y, Wen L (2006) Muscle-specific creatine kinase gene polymorphism and running economy responses to an 18-week 5000-m training programme. Br J Sports Med 40: 988-991

Appendix I

Visual Pain Scale



Subject Questionnaire

Name: Surname: US Number:

Cell no: Age: Height: Weight:

Physical activity and Injury questionnaire:

Physical Activity:

Did you participate in any sports during high school? | Y | N |

If yes, which sporting activity/ies?

……

At what level did you compete?

Recreational: Regional Competition: National:

Did you, or are you participating in any competitive sport at university? | Y | N |

If yes, what sport did/are you participating in?

How long ago?

currently: <2 months ago: 2 months ago: 6 months ago:

1 year ago: > 1 year ago:

Do you currently participate in any recreational physical activity? | Y | N |

If yes, what?

How often?

< twice a week twice a week 3 times a week > 3 times a week

In the past 4 weeks?

< twice a week twice a week 3 times a week > 3 times a week

At what intensity?

Low: moderate: high:

What is your current level of fitness currently?

Unfit: Moderately fit: Highly trained:

Injury history:

Have you had any injury to your lower extremities?	Y	N

If yes, where?

Hip: knee: Ankle: upper leg: lower leg:

Of what nature?

Sprain: Strain: Contusion: injury to bone:

Dislocation:

How long ago?

< 2 months ago: 3 months ago: 6 months ago:

1 year ago:

> 1 year ago:

Code:

Name: Height:

Surname: Weight:

Student Number: Vertical jump height ave:

Friday 21st Aug

Leg Dominance:

Leg Of first Biopsy:

Perceived Muscle Soreness Score:

Standing Position: Squat: LL squat: RL squat:

Monday 31st Aug

Perceived Muscle Soreness Score:

Standing Position: Squat: LL squat: RL squat:

Perceived Muscle Soreness Score (6hrs post-exe):

Standing Position: Squat: LL squat: RL squat:

Tuesday 1 Sept

Perceived Muscle Soreness Score:

Standing Position: Squat: LL squat: RL squat:

Wednesday 2 Sept

Perceived Muscle Soreness Score:

Standing Position: Squat: LL squat: RL squat:

Thursday 3 Sept

Perceived Muscle Soreness Score:

Standing Position: Squat: LL squat: RL squat:

Friday 4 Sept

Perceived Muscle Soreness Score:

Standing Position: Squat: LL squat: RL squat:

Appendix II

S - dilution series
H – High
L – Low
Blank

	PRE SAMPLES IN BLACK
	DAY 0 SAMPLES ARE IN RED
	DAY 1 SAMPLES IN GREEN

Kit 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	s1	s1	L	L	EvS	EvS	WD	WD	TR	TR	RH CON	RH CON
B	s2	s2	H	H	DP CON	DP CON	JL	JL	PL	PL	PD CON	PD CON
C	s3	s3	JM	JM	GA CON	GA CON	CvN1	CvN1	CA8	CA8	EvS	EvS
D	s4	s4	TT4	TT4	WP	WP	LA2	LA2	JK	JK	DP CON	DP CON
E	s5	s5	DL	DL	JF7	JF7	SA10	SA10	JM	JM	GA CON	GA CON
F	s6	s6	GV11	GV11	RS CON	RS CON	WR	WR	TT4	TT4	WP	WP
G	s7	s7	RH CON	RH CON	SB9	SB9	ZN6	ZN6	DL	DL	JF7	JF7
H	blank	blank	PD CON	PD CON	SR	SR	NB	NB	GV11	GV11	RS CON	RS CON

Kit 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	s1	s1	L	SB9*	ZN6	ZN6	DL	DL	JF7	JF7	SA10	SA10
B	s2	s2	H	SR*	NB	NB	GV11	GV11	RS CON	RS CON	WR	WR
C	s3	s3	WD	WD	TR	TR	RH CON	RH CON	SB9	SB9	ZN6	ZN6
D	s4	s4	JL	JL	PL	PL	PD CON	PD CON	SR	SR	NB	NB
E	s5	s5	CvN1	CvN1	CA8	CA8	EvS	EvS	WD	WD	TR	TR
F	s6	s6	LA2	LA2	JK	JK	DP CON	DP CON	JL	JL	PL	PL
G	s7	s7	SA10	SA10	JM	JM	GA CON	GA CON	CvN1	CvN1	CA8	CA8
H	blank	blank	WR	WR	TT4	TT4	WP	WP	LA2	LA2	JK	JK

Appendix III

- 38 samples of TNF-α and IL-6
- Washed twice times slowly (wash buffer)
- Do not allow wells to dry

- Standard dilution:
    - (External) Label eppies 7 (S1-S7)
    - Make up Standard's plus Control
    - 50ml standard plus 950ml sample Diluent
    - Control plus 200ml dH_2O (10-30 minutes)
    - Swirl gently
    - Dilute: 50µl control in 950 sample Diluent
    - 225µl sample Diluent (25ml bottle)
    - 225µl Diluted Standard in S1 and mix
    - 225µl S1 in S2 and mix
    - 225µl S2 in S3
    - 225µl S3 in S4
    - 225µl S4 in S5
    - 225µl S5 in S6
    - 225µl S6 in S7
- Added 100µl S1-S7
- Added 100µl Control 1-2
- Added 100µl Sample Diluent in blank 1-2
- Added 50µl Sample(duplicate)
- 60µl Biotin-Conj plus 5940µl Sample Diluent

- Added 50µl Diluted Biotin-Conj in ALL
- Cover and incubate [2hours at RT on 100pm]
- 20 minutes prior to completion after nr 8
    - Added 120ml Strep-HRP plus 11880ml Assay Buffer
- Washed 6 times
- Added 100µl Diluted Strep-HRP in ALL
- Incubate for 1 hour
- 6000µl Amp Diluent (2×) plus 6000µl dH_2O (1×)
    - Centrifuge vial to collect liquid in Amp. Sel. 1
- 24ml Amp Reagent 1 plus 11976 Amp Diluent (1×)
- Washed 6 times
- Added 100µl Amp Solution 1 to ALL
- Incubated for 15minutes
    - Centrifuge 2 vials Amp Solution 2
- 15+9=24µl Amp. Reagent 2 plus 11976 Assay buffer
- Washed 6 times
- Added 100µl Amp. Reagent 2 to ALL
- Incubate for 30 minutes
- Washed 6 times
- Added 100µl TBS substrate to ALL
- Incubate 10-20minutes in dark/shade
    - If highest standard is dark blue, add 100µl STOP solution
- Read at 450nm plus 620nm as reference

Reagent provided

- Aluminium pouch with a Micro well plate coated with monoclonal antibody to human TNF-α
- 1 vial (100µl) Biotin-conjugate anti-human TNF-α polyclonal antibody
- IL-6 monoclonal antibody
- 1 vial (150µl) Streptavidin-HRP
- vial human TFN-α Standard lyophilized, 1 ng/ml upon reconstitution
- 2 vial human IL-6 Standard lyophilized, 200 pg/ml upon reconstitution
- 1 vial Control high, lyophilized
- 1 vial Control low, lyophilized
- 1 bottle (12ml) Sample Diluent
- 1 vial (5ml) Assay Buffer Concentrate 20× (PBS with 1% Tween 20 and 10% BSA)
- 1 vial (5ml) Assay Buffer Concentrate 20× (PBS with 1% Tween 20 and 10% BSA)
- 1 vial (25ml) Sample Diluent
- 1 vial (7ml) Amplification Diluent Concentrate (2×)
- 2 vials (75µl) Amplification Reagent 1
- 1 vial (75µl) Amplification Reagent 1
- 1 vial (15µl) Amplification Reagent 2
- 2 vials (15µl) Amplification Reagent 2
- 2 bottles (50ml) Wash Buffer Concentrate 20× (PBS with 1% Tween 20)
- 1 vial (15ml) Substrate Solution (tetramethyl-benzidine)
- 1 vial (15ml) Stop Solution (1M Phosphoric acid)
- 8 Adhesive Films

TNF/ IL6

Some for both

Materials required but not provided

- 5ml and 10ml graduated pipettes
- 5µl to 1000µl adjustable single channel micropipettes with disposable tips
- 50µl to 300µl adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- Microplate shaker
- Microwell strip reader capable of reading at 450nm (650nm as optional reference wave length)
- Glass-distilled or deionized water
- Statistical calculator with program to perform regression analysis
- Paper towels

TNF-α

Streptavidin-HRP

Streptavidin-HRP must be used within 30minutes after dilution

Make a 1:400 dilution of the concentrated Streptavidin-HRP solution with Assay Buffer(1×) in a clean plastic tube as needed according to the following table:

Number of strios	Streptavidin-HRP (ml)	Assay Buffer(1×) (ml)
1-12	0,030	11,970

Amplification Solution 1

Make a 1:300 dilution of Amplification Reagent 1 in Amplification Diluent (1×) as needed according to the following table

Number of strips	Amplification Reagent 1 (ml)	Amplification Diluent (ml)
1-12	0,04	11,96

Amplification Solution 2

Make a 1:2000 dilution of Amplification Reagent 2 in Assay Buffer(1×) as needed according to the following scheme:

Number of strip	Amplification Reagent 2 (ml)	Assay Buffer (1×) (ml)
1-12	0,006	11,994

Reagent Preparation Summary

Wash Buffer(1×)

Add Wash buffer Concentrate 20× (50ml) to 950ml distilled water.

Number of Strips	Wash Buffer Concentrate (ml)	Distilled Water (ml)
1-12	50	950

Assay Buffer(1×)

Add Assay Buffer Concentrate 20× (5ml) to 95ml distilled water.

Number of strips	Assay Buffer Concentrate (ml)	Distilled Water (ml)
1-12	5,0	95,0

IL6

Streptavidin-HRP

Streptavidin-HRP must be used within 30minutes after dilution

Make a 1:400 dilution of the concentrated Streptavidin-HRP solution with Assay Buffer(1×) in a clean plastic tube as needed according to the following table:

Number of strips	Streptavidin-HRP (ml)	Assay Buffer(1×) (ml)
1-12	0,12	11,88

Amplification Solution 1

Make a 1:500 dilution of Amplification Reagent 1 in Amplification Diluent(1×) as needed according to the following table

Number of strips	Amplification Reagent 1 (ml)	Amplification Diluent (ml)
1-12	0,024	11,976

Amplification Solution 2

Make a 1:500 dilution of Amplification Reagent 2 in Assay Buffer(1×) as needed according to the following scheme:

Number of strip	Amplification Reagent 2 (ml)	Assay Buffer (1×) (ml)
1-12	0,024	11,976

Appendix IV

- Defrost the primers in ice
- Perform nano drop test on the samples of DNA(tests that the samples are in a correct concentration for the experiment, dilute if not in correct concentration)
    - Clean pedestal between samples
    - Use water as a blank
    - Use clean tip
    - Add sample ID
    - Dilute if its more than 20ng
- Primer dilution:
    - Received in 100 mmol
    - Make up 10 µmol solution, dilute with PCR water or distilled water
    - Make up 20-30 ml
- PCR reaction:
    - Keep cold but do not allow the tray to get wet.
- Do not touch the bottom of the tube anything o this surface will interfere with the readings
- This is a 20 µl reaction:
    - 10 µl of HRM mix containing:
    - 4 µl of reverse primer
    - 4 µl of forward primer
    - 2 µl of DNA
- Place in Rotogene(Corbett) and balance

DNA Extraction

1. Tissue was placed in 1.7ml tube and homogenise with the TissueLyzer (*Qiagen*).
2. Add 400µl of Buffer PL2 (NucleoSpin Plant II Kit, *Separations*) with 2µl of proteinase K (10mg/ml; *Sigma-Aldrich*).
3. The sample was incubated overnight in a waterbath at 60℃.
4. The protocol of the manufacturer was followed.
5. The extraction was performed on the Tecan TMP 2000 Liquid Handling Platform.

Appendix V

Staining procedure 1

- Leave 3min at room temperature
- Place in Paraformaldehyde(2%) in PBS for 8min
- Place in 0.25% Triton X-100 in PBS for 15 min
- Place in PBS 3x5min
- Dry around section and mark with wax pen
- Pipette blocking serum onto section area(20% goat in PBS) allow to sit for 30 min
    - 5ml PBS
    - 2% BSA(0,1g)
    - 0.2% milk powder (0.01g)
- Wash in PBS
- Pipette Ab IL6 Rα (rabbit monoclonal) allow to sit overnight
    - 8µl IL6 Rα +1600µl PBS
- Wash in PBS
- Pipette Alexa flour 594(goat anti-rabbit) allow to sit for 1 hour
    - 8µl Alexa flour 594 + 160 µl PBS
- Wash in PBS
- Pipette Laminin (mouse polyclonal) allow to sit for 2 hours
    - 8µl Laminin + 160 µl PBS
- Wash PBS
- Pipette Alexa flour 488(goat anti-mouse) allow to sit for 1 hour
    - 8µl Alexa flour 488 + 160 µl PBS
- Wash in PBS

- Pipette Hoescht and allow to sit for 15 min
- Wash in PBS
- Mount coverslips onto slide with mounting media
- Cover slide in foil
- Store in freezer (-20℃)

Staining procedure 2

- Leave 3min at room temperature
- Place in Paraformaldehyde(2%) in PBS for 8min
- Place in 0.25% Triton X-100 in PBS for 15 min
- Place in PBS 3x5min
- Dry around section and mark with wax pen
- Pipette blocking serum onto section area(20% goat in PBS) allow to sit for 30 min
    - 5ml PBS
    - 2% BSA(0,1g)
    - 0.2% milk powder (0.01g)
- Wash in PBS
- Pipette Ab MHC II (mouse monoclonal) incubate 1 hour
    - 8µl IL6 Rα +1600µl PBS
- Wash in PBS
- Pipette Alexa flour 488 (goat anti-mouse) incubate for 1 hour
    - 8µl Alexa flour 594 + 160 µl PBS
- Wash in PBS
- Pipette Dystrophin (rabbit polyclonal) incubate for 1 hours
    - 8µl Laminin + 160 µl PBS
- Wash PBS

- Pipette Alexa flour 594 (goat anti-rabbit) incubate for 1 hour
    - 8µl Alexa flour 488 + 160 µl PBS
- Wash in PBS
- Pipette Hoescht and allow to sit for 15 min
- Wash in PBS
- Mount coverslips onto slide with mounting media
- Cover slide in foil
- Store in freezer (-20℃)

Appendix VI

Materials

- 2-Mercaptoethanol – Sigma- Aldrich, M7154- 100ML
- 50ml Falcon tubes - BD Biosciences, Adcock- Ingram Scientific group, 0420964AAd
- Acrylamide: N,N' – Methylethylene – Disacrylamide 37:5:1 – FLUKA, 01709
- Ammonium persulfate (APS) – Sigma, A3678 – 25G
- Bradford reagent[1]
- BSA (1mg/ml)
- Chemiluminescence film – Amersham, 28906836
- Chromatography paper (3mm) – Whatmann, 3030917
- ECL detection reagents – Amersham, RPN2209
- Electrotransfer apparatus – TRANS-BLOT SD. Semidry transfer cell , BIO-RAD
- Eppendorf tubes (1.5ml) – QSP, 509-GRD
- Ethanol
- Milk powder
- Milli.q. water
- N,N.N',N' – Tetramethylethylene – diamine (TEMED) – Sigma, T9281- 50ML
- Plastic containers

[1] APPENDIX II

- Primary antibodies – A4.74 (MHCIIa; dilution 1:1000; Developmental Hybridoma Bank, Iowa) and A4.951 (MHC I; dilution 1:1000; Developmental Hybridoma Bank, Iowa)
- Protein markers – Precision plus protein standards dual colour, 161-0374. peqGOLD prestained protein marker IV, 27- 2110
- PVDF membrane – Immubilon- P, Millipore, IPVH000010
- Lysis buffer*
- Running buffer[2]
- Sample buffer*
- Secondary antibody – Anti-mouse IgG, horseradish peroxidase (from donkey), NA934V
- Shaker - The Belly Dancer', STOVALL LIFE SCIENCE incorporated. 4,702,610
- Sodium dodecylsulfate (10%) – Sigma, L3771- IKG
- Table-top centrifuge – LAB NET International, Spectrafuge 16M- C0160-230
- TBS*
- Tris-HCl , 1.0M (pH6.8)*
- Tris-HCl ,1.5M (pH 8.8)
- Tween 20 – Saarchem MERCK chemicals , SAAR6164500RF
- U-shaped adaptor cassette and loading system – BIO- RAD

[2] APPENDIX II

- Vortex – Thermolyne, Type 16700 Mixer, Maxi-Mix 1
- Western blotting apparatus – BIO- RAD
- X-ray developer – AXIM , FDC-DEVRU0010BB
- X-ray fixative – AXIM, FFC- F1XRUHS10BB

Technique

Sample Preparation

1. Test tubes and Eppendorf tubes were marked and placed on ice to chill
2. Muscle samples were removed from the -80℃ freezer and kept in liquid nitrogen
3. 500µl lysis* buffer was added to marked test tubes
4. Small sections of muscle were cut from each sample and placed into specifically marked test tubes
5. The samples in the test tubes were homogenized up and down 5 times or until tissue appears homogenized completely
6. The samples were left on ice for 5 min and then decanted into marked epis
7. Centrifuge at 300 rpm, 5 min, 4℃
8. The supernatant was decanted into newly marked eppendorf tubes, ready for use or storage at – 80° C.

Protein Determination

1. A 1:5 dilution of Bradford reagent was made up and filtered twice with filter paper.

2. A standard curve was obtained using a 200µg/ml BSA solution using dilution stated below:

200µg/ml BSA	(=µg protein)	H2O	Bradford reagent
100µl	20	0µl	900µl
80µl	16	20µl	900µl
60µl	12	40µl	900µl
40µl	8	60µl	900µl
20µl	4	80µl	900µl
10µl	2	90µl	900µl
0µl	0	100µl	900µlBLANK

3. Samples were vortexed and allowed to stand for 5 min.

4. The spectrophotometer was zeroed with the blank and absorbencies were read within 30min at 595nm.

5. 5µl of each sample was pipetted into an eppendorf tube with 95ml dH_2O and 900µl Bradford reagent.

6. Samples were vortexed and read at 595nm.

7. A standard curve was plotted to determine how much of each sample must be used for Western blot analysis.

Protein separation by SDS-PAGE

1. Glass was cleaned with 70% alcohol and placed into assembly
2. Assembly was placed onto rubber to center, with corner on foot rubber, and clipped into place while pushing down.
3. Separating gel* was then added to assembly with a plastic squeeze pipette
4. A few drops of butanol was added to prevent oxidation of gel and to ensure a straight line with no meniscus
5. It was allowed to set for 30min or overnight
6. Butanol was washed off with dH_2O
7. Stacking gel[3] was made up and added on top of the already set separating gel
8. Combs were placed in at an angle to prevent bubbles from forming
9. It was allowed to set for about 30 min
10. 16µl sample buffer* (850µl + 150µl mercaptoethanol) was added to each sample
11. Samples were denatured by boiling for 5min, after making a hole in each lid of the eppendorf tubes.
12. Samples were centrifuge for 1min at 468g
13. Combs were removed carefully and acrylamide was washed off with dH2O
14. Gels were taken off assembly stand and clicked into U-shaped core-latch

[3] APPENDIX II

15. The U-shaped assembly was placed into tank and inside running buffer* was added in the middle compartment so that it just overflowed into the wells.

Loading the samples

1. A molecular marker was added into the first well on the left- 10µl
2. Outside running buffer was placed in outer compartment to ½ way up
3. Electrodes were attached and the gel run was done at 75V, for 38 hrs

Electrotransfer

12 Whatmann 3MM papers and I PVDF per gel = 8.8cm x 5.3cm were used

1. PVDF membranes were soaked in methanol for 15sec and then rinsed with water and soaked in anode buffer 2*
2. 3 papers were soaked in anode buffer 1*
3. 1 paper was soaked in anode buffer 2*
4. 4 papers were soaked in cathode buffer[4]
5. On anode plate: 3 papers soaked in anode 1
6. 1 paper from anode 2 was placed on top of the previously placed papers
7. PVDF membrane was placed and rolled flat
8. Stacking gel was cut off and gel was placed on PVDF membrane with marker on the left

[4] APPENDIX II

9. 4 papers soaked in cathode buffer were then added and rolled flat
10. Power supply: limit 0.5A, 15 volts, 7 hours.

Specific binding of proteins

1. Membranes were washed in TBS –Tween[5] (washing buffer) x3
2. Milk (5%) in TBS-T used to block membranes and placed on belly dancer on lowest setting for 1.5 to 2 hrs.
3. 5µl Ab in 5ml TBS-Tween (primary antibodies) in 50ml Falcon tubes
4. Membrane was placed in falcon tube with tweezers, with marker at the bottom and proteins to inside
5. It was mixed on rotator in fridge for 5 hrs or overnight
6. Membranes were washed well with TBS-Tween 5 times for 5min.
7. Secondary antibody was added: 2.5µl Ab in 5ml TBS-Tween
8. Membrane was put on rotator for 1 hour
9. Membranes were washed 3 x 5min in TBS-tween and keep wet at all times

Exposure

1. ECL detection reagents were used at room temperature
2. 2x transparencies were cut and taped together on the left side
3. 500µl of each reagent was added into a falcon tube
4. Membrane was placed in tub and exposed to ECL for 1min

[5] APPENDIX II

5. Membrane was placed in between transparencies
6. 2 pieces X-ray film were cut to fit area were proteins are expected to be
7. The bottom X-ray is placed first and top X-ray second
8. After 5-10min, top film was developed for 5min and fixed
9. After another 5min the bottom film was developed

Rapid Coomassie Staining Procedure

1. Make up the following solutions

   Staining solution: 40% methanol; 10% acetic acid; 50% water; 0.1% (w/v) Coomassie Brilliant Blue R250.

   De-stain solution: 40% methanol; 10% acetic acid; 50% water.

2. Commence staining procedure (with the mini-gels results can be seen in 10 minutes; however use longer incubation times for thicker gels).
   a. Place gel in a glass staining container with lid
   b. Add enough stain to cover gel well.
   c. Microwave on high for 1 minute.
   d. Stain while rocking gently for up to 10 minutes.
   e. Pour out stain (may be re-used), and add a large amount of de-stain.
   f. Destain on belly dancer gently at room temperature.
   g. Leave for 2 hours to overnight for complete destaining.

Appendix VII

Solution Composition

1. Anode Buffer 1
   - 18.2g Tris + 380ml water
   - pH
   - 100ml methanol
   - Completed to 500ml water
2. Anode buffer 2
   - 1.51g Tris + 380ml water
   - pH
   - 100ml methanol
   - Completed with 500ml water
3. Bradford reagent
   - 500mg Coomassie Brilliant Blue G
   - 250ml 95% EtOH
   - 500ml Phosphoric acid
   - Made up to 1L with dH_2O
   - Filtered
   - Stored on ice/ in fridge

4. Cathode buffer

- 1.51g Tris-base
- 2.6g ε- aminohexanoic acid
- pH
- 100ml Methanol
- Completed to 500ml with water

5. Lysis Buffer

- 50 mM Tris pH 7.4
- 150 mM NaCl
- 1% Triton X-100
- 1% IGEPAL (NP-40)
- 0.1% SDS
- DIH_2O
- Prior to use add Protease inhibitor cocktail (Sigma) 20µl

6. Inside Running Buffer

- 11.25g Glycine
- 12.1g Tris
- 1g SDS
- 1000 ml Distilled water

7. Outside Running Buffer

- Dilute 1 part inside running buffer with 1 part distilled water

8. Sample buffer

- 0.0625M Tris-HCl ph 6.8
- 2% SDS
- 5% β-Mercaptoethanol
- 10% Glycerol
- 0.01% Bromophenol Blue

9. 8% Separating gel

	1 x large gel	2 x small gels
100% Glycerol	7.5ml	3ml
Millipore H20	3.75ml	1.5ml
1.5M Tris-HCl,pH8.8	3.35ml	1.33ml
10%SDS stock	1ml	400µl
Glycine	2.5ml	1ml
30% Acrylamide	6.7ml	2.7ml
10% APS(0.1g/ml)	250µl	100µl
Temed	20µl	5µl

10. 5% Stacking gel

	1 x large gel	2 x small gels
100% Glycerol	1.5ml	500µl
Millipore H20	1.68ml	560µl
0.5M Tris-HCl,pH6.8	700µl	235µl
10%SDS stock	200µl	65µl
0.1M EDTA pH 7.0	200µl	65µl
30% Acrylamide	667µl	220µl
10% APS(0.1g/ml)	50µl	15µl
Temed	10µl	3µl

11. TBS (10 X)

- 121g Tris base
- 40g NaCl
- 5L H_2O

- pH 7.6

12. Tris-HCl (pH 8.8, 1.5M)

- 181g Tris
- 800ml dH_2O
- HCl to pH 8.8
- Made up to 1L

13. Tris-HCl (pH 6.8, 0.5M)

- 120g Tris
- 800ml H_2O
- HCl to pH 6.8
- Made up to 1L