

THE EFFECTS OF NUTRITIONAL SUPPLEMENTATION ON REGENERATION OF MUSCLE FUNCTION AFTER DAMAGE

by

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SUMMARY

It is well established that following eccentric exercise (contractions during which muscles are actively lengthened) and myotoxic injection, disturbances in the regulation of intracellular calcium (Ca^{2+}) homeostasis and increased rate of protein degradation will eventually lead to muscle damage (see Allen et al., 2005). Enhancing the rate at which muscles recover from injury may involve reducing the initial amount of damage by improving the intracellular Ca^{2+} handling ability of the muscle, or increasing the rate of regeneration by enhancing muscle protein synthesis. Two dietary supplements that may accomplish this are creatine monohydrate and whey protein.

Thus, the purpose of this thesis was to examine the effects of creatine monohydrate and whey protein consumption on force recovery after eccentrically-induced muscle damage in healthy individuals. Moreover, to confirm these results by analysing force recovery in two parallel animal studies after controlled, chemically-induced injury. In addition, to determine the mechanisms by which creatine and whey protein exert their effects by examining morphological and biochemical properties of fast-twitch (extensor digitorum longus, EDL) and slow-twitch (soleus) rat skeletal muscle during recovery.

In the human studies, muscle strength was significantly higher in the whey protein- and creatine-supplemented groups compared to those consuming a carbohydrate placebo following eccentrically-induced muscle damage. Lower plasma creatine kinase and lactate dehydrogenase levels in the days after injury was indicative of less muscle damage, and thus, supported the improved functional capabilities observed. In the animal studies, creatine supplementation significantly enhanced functional capacity of the regenerating EDL muscles by reducing the extent of fibre necrosis and augmenting

fibre regeneration following myotoxic injury. These effects occurred as a direct result of increased muscle protein content, regenerating fiber CSA and muscle mass. Whey protein supplementation significantly enhanced functional capacity of the regenerating EDL muscles by reducing the extent of fibre necrosis following myotoxic injury. Similar to creatine supplementation, enhanced functional capacity of the EDL muscles was due to direct increases in muscle protein content, regenerating fiber CSA and muscle mass. However, in contrast to creatine supplementation, improved muscle recovery was only evident in the early stages of muscle regeneration, with no effects from whey protein supplementation observed in the later stages of muscle regeneration. Similarly, only limited effects were observed in the soleus muscles with either supplement.

In conclusion, the results from this thesis have shown the creatine monohydrate and whey protein supplementation may help reduce the extent of, or enhance recovery from, muscle damage. This would not only benefit athletes during intense training phases, competition and during recovery from injury, but may also return people to the workplace earlier. Furthermore, improved functional recovery would also be of considerable benefit to a variety of populations, including those suffering from muscle wasting conditions, weakness associated with aging, neuromuscular disorders, acquired immunodeficiency syndrome, burn injury, cancer cachexia and prolonged sepsis.

DECLARATION

“I, Matthew Cooke, declare that the PhD thesis entitled The Effects of Nutritional Supplementation on Regeneration of Muscle Function after Damage is no more than 100,000 words in length, exclusive of tables, figures, appendices, references, and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work”.

All experimental and analytical work, with the exception of plasma creatine kinase levels, which were analysed by Warren Louey at Melbourne Pathology, was performed by the author, and to the best of my knowledge, has not been previously published by any other author.

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ABBREVIATIONS

½ RT	Half relaxation time
4E-BP1	eIF4E-binding protein 1
ACh	Acetylcholine
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ATP	Adenosine-5-triphosphatase
ATPase	Adenosine triphosphatase
BCAA	Branch chained amino acid
BrdU	Thymidine analogue
BSA	Bovine serum albumin
BV	Biological value
[Ca ²⁺] _i	Intracellular calcium
Ca ²⁺	Calcium ion
Ca ²⁺ -ATPase	Calcium-adenosine triphosphatase
CK	Creatine kinase
CK-PCr	Creatine kinase-creatine phosphate
cAMP	Cyclic-3,5-adenosine monophosphate
CON-INJ	Injured control muscle
CON-NORM	Non-injured control muscle
Cr	Creatine
Cr _{free}	Unphosphorylated creatine
CR-INJ	Injured creatine-supplemented muscle
CrM	Creatine monohydrate
CR-NORM	Non-injured creatine-supplemented muscle
DHPR	Dihydropyridine receptors
DMD	Duchenne muscular dystrophy
DOMS	Delayed onset of muscle soreness
E1	Ubiquitin-activating enzyme
E-C	Excitation-contraction
EDL	Extensor digitorum longus
EDTA	Ethylediniaminetetra-acetic acid

EGTA	Ethylene glycol-bis(β -aminoethyl ether)-N, N'-tetraacetic acid
eIF2B	Eukaryotic initiation factor 2B
FG	Fast, glycolytic
FGF	Fibroblast growth factor (
FL/ML	Fibre Length: Muscle Length ratio
FOG	Fast, oxidative, glycolytic
GK	Glycerol kinase
Glucose-6-P	Glucose- 6- Phosphate
α -GPO	Glycerophosphate oxidase
GSH	Reduced glutathione
HGF	Hepatocyte growth factor
Hz	Hertz
IGF	Insulin-like growth factor
IL-1	Interleukin-1
kDa	Measure of molecular weight or mass
K ⁺	Potassium ions
L _o	Optimal length
LBM	Lean Body Mass
LDH	Lactate dehydrogenase
MAPK	Mitogen-activated protein kinase
MAPR	Mitochondrial adenosinetriphosphate production rate
Mg ²⁺	Magnesium ion
M-CK	Muscle creatine kinase
MHC	Myosin heavy chain
MIP 1- β	Protein 1- β
MM	Muscle Mass
MM:BM	Muscle mass to body mass ratio
MNF	Myocyte nuclear factor
MPO	Myeloperoxide
MRF-4	Myogenic regulatory factor-4
mRNA	Messenger ribonucleic acid
MRFs	Myogenic regulatory factors

MVC	Maximum voluntary contraction
MW	Molecular Weight
N ⁺	Sodium ions
NAC	N-acetyl-L-cysteine
NCAM	Neural Cell Adhesion Molecule
NO	Nitric oxide
NOS-I μ	Nitric oxide synthase-I μ
(NF)- κ B	Nuclear factor
p70 ^{S6k}	70kDa ribosomal protein S6 protein kinase
Pi	Inorganic Phosphate
P _o	Tetanic contraction
P _t	Twitch contraction
PCA	Perchloric Acid
PCNA	Proliferating cell nuclear antigen
PCr	Phosphocreatine
PCSA	Physiological cross sectional area
PDGF	Platelet-derived growth factor
PI-3K	Phosphatidyl inositol 3 kinase
PTP	Permeability transition pore
RM	Repetition Maximum
ROM	Range of motion
ROS	Reactive oxygen species
RyR	Ryanodine receptor
SACs	Stretch activated channels
SEM	Standard error of the mean
SO	Slow oxidative
SOD	Superoxide dismutase
SOL	Soleus
SR	Sarcoplasmic Reticulum
sP _o	Specific Force
TA	Tibialis anterior
TCr	Total Creatine Content
TGF- β	Transforming growth factor- β

Tn	Troponin
Tn-Tm	Troponin-tropomyosin
TNF- α	Tumour necrosis factor- α
TTPT	Time to peak tension
UBI-proteolytic	Ubiquitin-proteosome
(X-A)	Cross-sectional Area
VCAM	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VO _{2max}	The maximum amount of oxygen you can take in and utilize measured in mL.kg.min
WP-INJ	Injured whey protein-supplemented muscle
WP-NORM	Non-injured whey protein-supplemented muscle

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CHAPTER 1

1.0 THESIS SCOPE

From the elite sports-person, to the weekend athlete, or as a result of workplace accidents, muscle injuries are a way of life. Skeletal muscle damage occurs when there is a disruption to the cellular structure of the muscle, which impairs muscle function, usually as a result from trauma; crush injury, excessive exercise or disuse (Proske and Morgan, 2001).

It is well established that skeletal muscle damage is prevalent among individuals who participate in any form of unaccustomed or high intensity exercise, in particular eccentric exercise that involves lengthening of the activated muscles (Brown *et al.*, 1999; Phillips *et al.*, 1999; Friden and Lieber, 2001a; Sbriccoli *et al.*, 2001; Lee *et al.*, 2002; Nosaka *et al.*, 2002). In addition, the micro-damage from eccentric exercise, which leads to minor symptoms that we all experience on occasions may, at times, progress to more major injuries, such as a hamstring tear (Proske and Morgan, 2001). It is currently hypothesized that eccentric exercise leads to an accumulation of intracellular Ca^{2+} causing activation of self-accelerating degradative pathways leading to muscle damage (Gissel and Clausen, 2001). Damaged muscle fibres initiate a cascade of reactions that result in a prolonged and complex interaction between protein synthesis and degradation (Sorichter *et al.*, 1999). However, while protein turnover is elevated substantially, degradation usually exceeds synthesis, and thus, protein breakdown results, leading to muscle atrophy (Wolf, 2006).

The limited ability of damaged muscle to regenerate after gross injuries has been extensively studied (Chambers and McDermott, 1996; Li *et al.*, 2000; Huard *et al.*,

2002; Tidball, 2005). Although the success of regeneration varies with the extent of injury, the basic processes involved are essentially the same for most injuries. These include:

- ◆ initial event
- ◆ autogenic mechanisms
- ◆ phagocytic phase
- ◆ regenerative phase

However, while the mechanisms of muscle damage, cellular degeneration and regeneration have been extensively investigated, there are still many aspects of these processes that remain unclear. Thus, accepted methods of treatment for skeletal muscle injuries are those that have recognised mechanisms of physiological action, such as ice application, massage or ultrasound post-injury (Jarvinen *et al.*, 2000).

The following chapter will review the development of skeletal muscle damage and subsequent repair cycle following eccentric exercise. Though it appears to be a complex network of different mechanisms, which also include inflammatory processes, it is clear from literature that disturbances in the regulation of intracellular Ca^{2+} concentration and changes in the rate of muscle protein degradation will eventually result in skeletal muscle damage and impaired functional capability (Belcastro *et al.*, 1998; Sorichter *et al.*, 1999; Gissel and Clausen, 2001; Allen *et al.*, 2005), and thus will be discussed in detail in section 2.1.2 and 2.1.3, respectively.

It is therefore hypothesized that enhancing the rate at which muscles recover following injury may involve reducing the initial amount of damage by improving the intracellular Ca^{2+} handling ability of the muscle (and thus lessening the initial amount of damage by

reducing the activation of self-accelerating degradative pathways), or increasing the rate of regeneration by augmenting muscle protein synthesis. Although, this is, in no way, the only means of improving muscle recovery from injury, it is nevertheless an integral element in the overall improvement of muscle healing and complete functional recovery.

Two dietary supplements that may theoretically enhance the rate at which muscle recovers from injury via these proposed mechanisms are creatine monohydrate and whey protein isolate. Yet few studies have investigated the efficacy of these dietary supplements in muscle recovery from injury.

Following eccentric exercise, restoration of muscle function will depend on the balance between the rates of protein synthesis and protein breakdown. Enhanced recovery can be achieved by nutrient intake that is capable of producing a positive net muscle protein balance (i.e. one favoring protein synthesis). One dietary supplement that may accomplish this is whey protein isolate. Recently, Cribb *et al.*, (2006) demonstrated that whey protein significantly enhanced the effects of resistance training on muscle strength and performance in humans. In support of these effects, Tipton and colleagues (2004) showed that following acute ingestion of whey protein supplementation after exercise, muscle protein synthesis was increased, resulting in net muscle protein synthesis. Furthermore, unpublished work from our laboratory demonstrated significant increases in muscle force following chronic ingestion of whey protein in rat skeletal muscle, without the aid of exercise training (Cooke *et al.*, 2001). Thus, if these increases occurred as a result of enhanced protein synthesis (as previously reported by Tipton *et al.*, 2004), then whey protein may be useful in increasing the rate of muscle regeneration. Indeed, whey protein was able to increase the oxidative capacity of these

muscles, which is likely to improve regeneration, as all repair processes require oxygen (Smith *et al.*, 2001).

Creatine supplementation, which increases both creatine (Cr) and phosphocreatine (PCr) levels within the muscle, has been extensively studied, and its effects on short term, intense exercise performance and recovery is well documented (for a review see Demant and Rhodes, 1999). However, little is known as to its effects on muscle recovery after injury. Creatine loading may increase the availability of ATP to the sarcoplasmic reticulum (SR), potentially increasing the rate of calcium removal from the cytoplasm (Gissel and Clausen, 2001), thus reducing the extent of muscle damage. In addition, elevated Cr and PCr levels are likely to increase cell volume, and thus may act as an anabolic signal to increase protein synthesis (Volek *et al.*, 1999). Taken together, creatine and whey protein supplementation may be useful in increasing the rate of recovery from muscle injury. Any such improvement will lead to new strategies to improve recovery from muscle injuries, allowing for earlier return to work and play, with the subsequent financial and health benefits.

The overall aim of this thesis is to examine whether dietary supplementation with creatine or whey protein can lessen the damage and/or improve the rate of recovery of skeletal muscles following injury. This chapter will introduce the anatomy and general architecture of skeletal muscle, muscle activation and fibre types. Chapter 2 will review skeletal muscle damage and the regeneration process, and conclude with nutritional intervention that may reduce the extent of, or enhance recovery from, exercise-induced muscle damage.

1.1 INTRODUCTION

1.1.1 Anatomy and Architecture of Skeletal Muscle

In skeletal muscle, the relationship between structure and function is very close. In brief, muscle is a composite structure consisting of muscle cells, organized networks of nerves and blood vessels, and an extracellular connective tissue matrix (Huard *et al.*, 2002). In order for us to understand how our muscles produce joint movement and exert forces, but more importantly for this thesis, facilitate the regeneration process that occurs after injury, we must first examine the detailed framework of human skeletal muscle.

1.1.2 From Whole Skeletal Muscle to Sarcomere

Skeletal muscle is the largest tissue mass in the human body, representing about 40% to 45% of total body weight. Skeletal muscles are attached to bones via connective tissue structures called tendons. Each muscle has an origin (the proximal end) and insertion (the distal end) point of contact with the bone and is therefore intimately involved with limb movement.

Skeletal muscles are a discrete organ, made up of hundreds to thousands of muscle fibres or myofibres, the most basic structural element of the skeletal muscle. Groups of muscle fibres are arranged in bundles called fascicles, and are surrounded by a connective tissue sheath known as the perimysium. These fascicles are also bundled together, surrounded by more connective tissue to form whole muscle (figure 1). The sarcolemma is the plasma membrane that surrounds each myofibre unit. The basal lamina or basement membrane, which constitutes the 100 to 200nm thick external layer, is composed of an inner layer, and intermediate lucida, and outer lamina densa (McComas, 1996). The basement membrane contains a number of proteins, including

collagen, fibronectin, laminin, and many glycoproteins. Each myofibre contains a multitude of nuclei derived from myoblasts located at the periphery of the myofibres. In addition, separate cells called satellite cells are located between the basal lamina and plasma membrane and play a key role in the muscle regeneration process (discussed in detail in section 2.2.1).

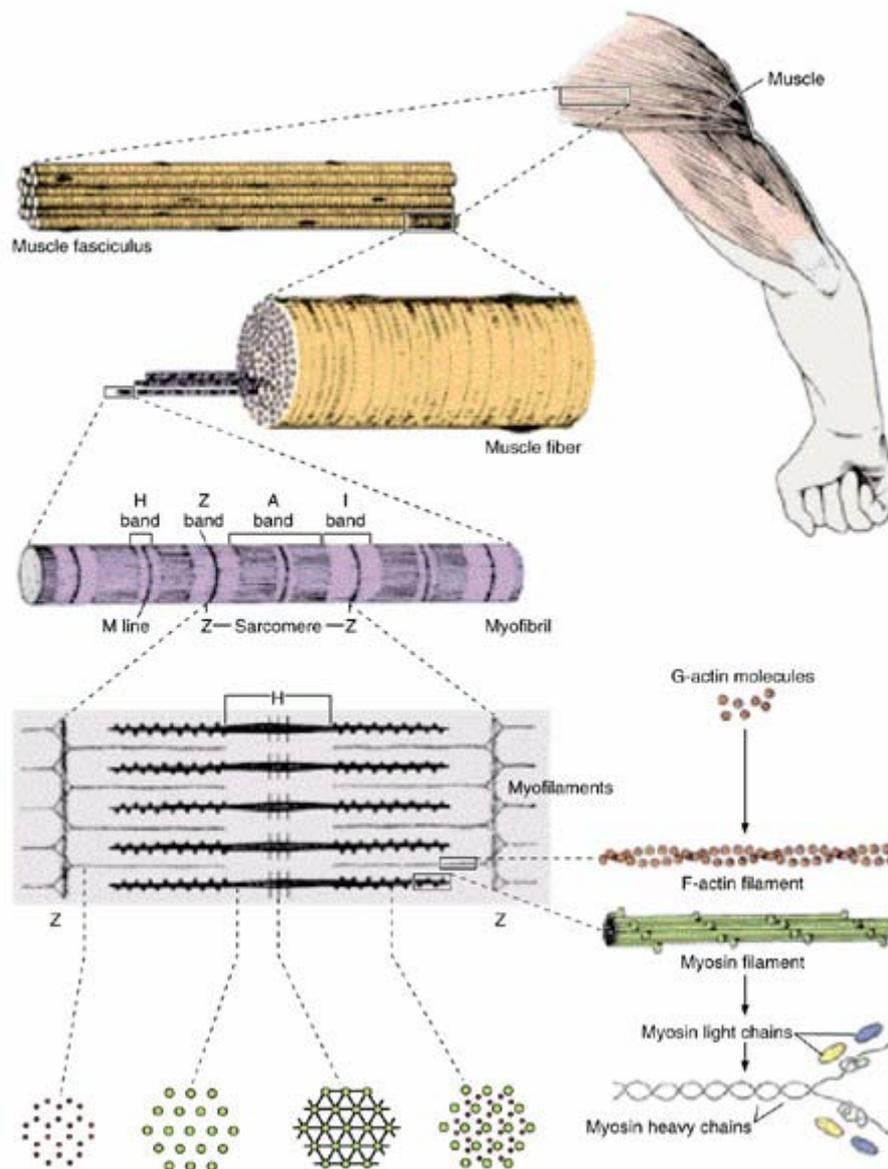


Figure 1. Illustrative diagram of the thick (myosin) and thin (actin) filaments and their organisational within the skeletal muscle (taken from Junqueira *et al.*, 1986).

The well-defined structural hierarchy of filament organization proceeds from the whole muscle all the way to the myofilaments. The arrangement and architecture of muscle fibers within whole muscles are important factors in determining whole muscle functional and contractile properties. Early electron and microscopic techniques were established and used by Hugh Huxley and Andrew Huxley to study intricate muscle fibre structure. Each muscle fibre contained a large number of rodlike myofibrils that ran in a parallel manner and extended the entire length of the muscle fibre. Likewise, Peachy and Eisenberg (1978) showed that myofibrils were arranged similar to the weave in a rope, and are therefore parallel in making up the muscle fibre. The functional consequence of this arrangement is that various myofibrils may not act completely independently during normal contraction (Lieber and Friden, 2000).

The myofibril is the largest functional unit within the muscle fibre. The most distinctive feature of the myofibril is the ordered array of contractile filaments arranged throughout the cell, in what are called sarcomeres, the basic contractile unit of skeletal muscle (figure 1.1). Myofibrils are simply a thread of sarcomeres arranged in series end to end, and are the units responsible for contraction and relaxation of the fibre. Disruption to this arrangement has been implicated in the initial event that leads to muscle damage (see section 2.1.1.1.1). The total number of sarcomeres within a fibre depends on the length and size of the muscle; the larger the muscle fibre, the greater the proportion contractile units.

1.1.2.1 Contractile Proteins – Actin and Myosin

The proteins actin and myosin are the filaments that combine to form the functional unit of muscle – the sarcomere. Sarcomeres are composed of contractile filaments termed “myofilaments”. Two major sets of contractile filaments, thick and thin, exist in the

sarcomere. Actin filaments (also called thin filaments) are found in most cells, and are composed of a long alpha-helical arrangement of actin monomers. These actin filaments are arranged in parallel in skeletal muscle, and are joined by alpha-actinin at each end to form the Z-Line. The distance between adjacent Z-Lines is one sarcomere. The thick filaments represent large polymers of the protein myosin. The myosin filaments are composed of both heavy and light chains, which are arranged in anti-parallel fashion, consisting of a long backbone and a globular head. It is this globular head which binds to the actin filaments to pull the actin filaments toward the centre of the sarcomere during contraction. Thus, active interdigitation of the myosin-containing filaments and the actin-containing filaments, known as the “the sliding filament theory”, produces shortening of the muscle and generation of muscle force (see section 1.1.3.1).

1.1.2.2 Regulatory Proteins – Troponin and Tropomyosin

Actin filaments are associated with two regulatory proteins; tropomyosin and troponin, which are also intimately involved in muscle contraction. Tropomyosin consists of a double helix that lies on the surface of the actin filament, while troponin is a complex of three polypeptides, one of which (troponin T) attaches to tropomyosin (Moss, 1992). The troponin-tropomyosin (Tn-Tm) complex inhibits binding sites on the actin filament to which the myosin heads can become attached, preventing active interdigitation of the thick and thin filaments, and hence sliding of myofilaments.

1.1.2.3 Structural Proteins

In addition to the proteins directly involved in the contraction process, there exist a number of other sarcomeric proteins that are thought to play distinct physiological roles such as membrane stabilization, force transmission, and ion channel anchorage (for a review see Vigoreaux, 1994; Berthier and Blaineau, 1997).

The large proteins titin and nebulin stabilise the highly ordered structure of the sarcomere (Wang and Wright, 1988; Trinick, 1994). At 3000 kDa, titin (also known as connectin) is the largest protein, and is thought to regulate the length and assembly of the filaments by retaining the A-filaments in the centre of the sarcomere during contraction (Horowitz and Podolsky, 1987). Nebulin is a 600 to 900 kDa protein which spans between the Z-Line and A-I-band junction, regulates the assembly of the contractile filaments (Wang and Wright, 1988; Trinick, 1994). Cytoskeletal proteins, such as spectin, vinculin and talin form a bi-dimensional lattice of the cytoplasmic side of the sarcolemma (Small *et al.*, 1992). Transverse connections of this lattice, termed costameres, exist between the myofibrils and the sarcolemma (Pardo *et al.*, 1983), mainly at the level of the Z-Line. Intermediate filaments, composed mainly of the protein desmin, join the Z-Line to the costameres and thus, provide a link between peripheral Z-Lines and the sarcolemma, but may also be intimately involved in transmission of force within the muscle (Granger and Lazarides, 1979; Lovering and Deyne, 2004).

Other proteins also regulate the arrangement of myosin molecules. The M-Line contains 4 proteins, the muscle isoform of creatine kinase (CK), M protein, myomesin and skelemins (see Small *et al.*, 1992), which link the thick filaments into hexagonal arrays. Numerous other proteins have also been observed in skeletal muscle fibres, however to describe them all is beyond the scope of this thesis.

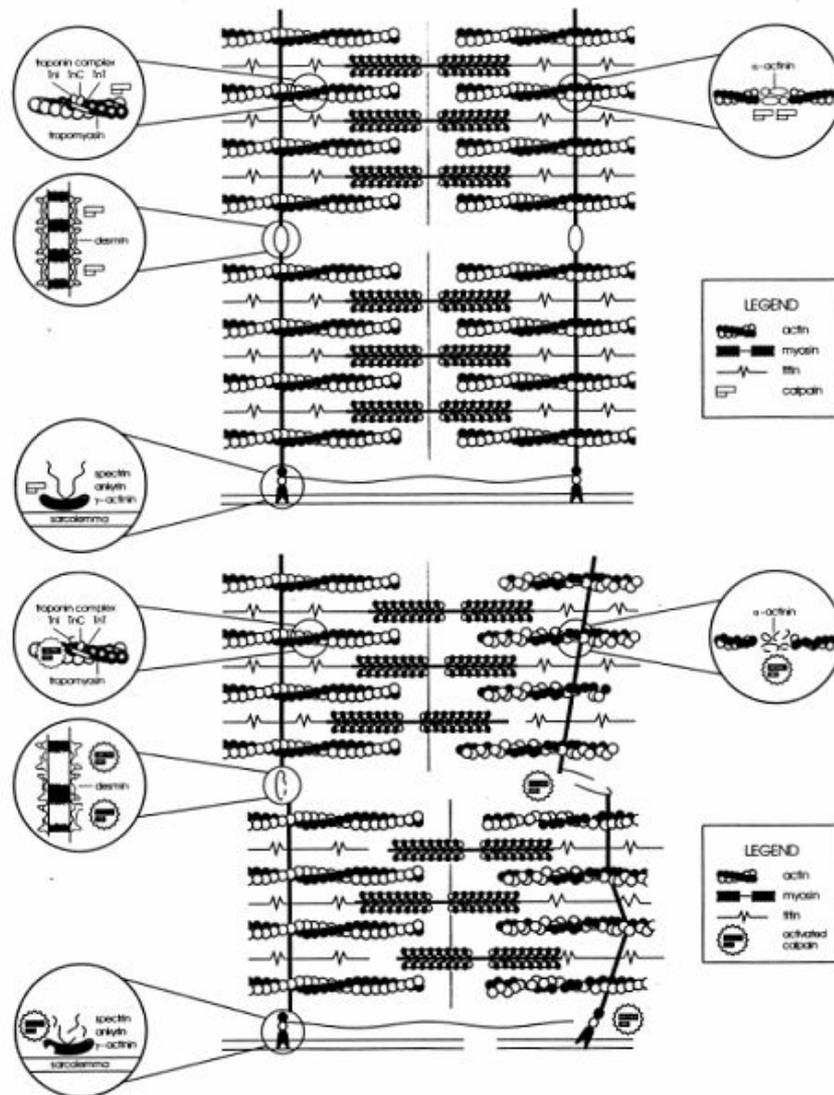


Figure 1.1. Schematic diagram of a sarcomere showing possible organisation of structural proteins at the Z-Line. Proteins and filaments are not drawn to scale (taken from Belcastro *et al.*, 1998). TnI = troponin I; TnT = troponin T; TnC = troponin C.

Myofilaments together with specific contractile proteins help form the cytoskeleton of the muscle fibre, and in turn reinforce the muscle membrane, preventing it from tearing during contraction and relaxation. Damage to, or absence of, key structural proteins can signify structural damage to the muscle, or membrane instability, and therefore lead to muscle degeneration as seen in exercise-induced muscle damage and pathological conditions such as Duchenne’s muscular dystrophy.

Anatomist Carl Gans (Gans, 1982) also pioneered early studies of skeletal muscle architecture over 20 years ago. Gans and his colleagues examined whole muscle through specific experimental methods based on microdissection. Muscles were dissected from the skeleton, their mass and muscle length determined, in addition to their pennation angle (i.e. the fibre angle relative to the force-generating axis). In order to maintain muscle integrity and physiologic length during dissection, muscles were chemically fixed while attached to the skeleton. Determining muscle mass, length and pennation angle; the physiological cross-sectional area (PCSA) can be calculated (Gans, 1982). In short, the PCSA is directly proportional to maximum force output of the muscle. The larger the muscle cross-sectional area (as seen with fibre growth), the greater the number of sarcomere contractile filaments capable of producing force.

In addition to the force generating contractile filaments present in muscle cells, there exists an intricate system for activating these force generators. The two essential components of this system are the transverse tubular system (T-Tubular system) and the sarcoplasmic reticulum (SR) (Figure 1.2). While the T-tubular system is important in transmitting nerve signals deep within the muscle, it is the release and reuptake of calcium (Ca^{2+}) by the SR is an important component, as it may be a potential mechanism by which creatine supplementation may be acting (section 2.4.3).

The SR envelops each myofibril to permit intimate contact between the activation and force generating systems. The SR is an intracellular membrane network whose main function is to release (via the ryanodine receptor) and re-uptake (via the Ca^{2+} -ATPase pump) Ca^{2+} during contraction and relaxation, respectively. Thus, is central to the regulation of excitation-contraction coupling (Endo, 1977).

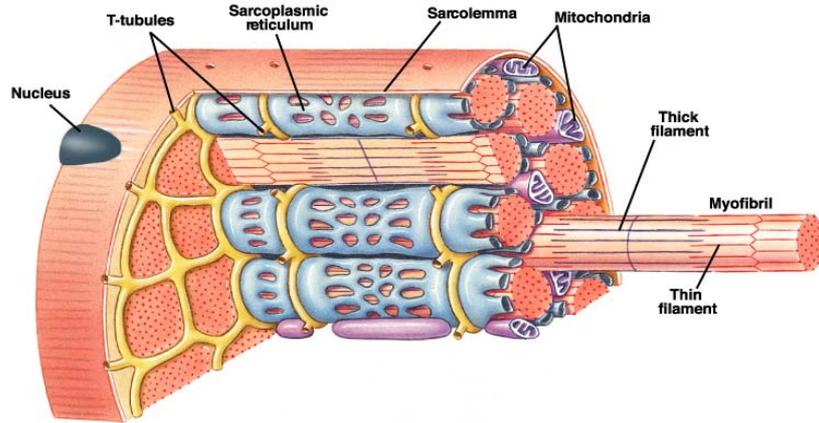


Figure 1.2. Transverse tubule and sarcoplasmic reticulum (taken from Silverthorn, 2004).

1.1.3 Excitation – Contraction Coupling

Excitation-contraction coupling, which was first proposed by Andrew Huxley (Huxley, 1963) is a sequence of events by which transmission of an action potential (nerve impulse) along the sarcolemma leads to the sliding of myofilaments, hence, muscle contraction and muscle force production.

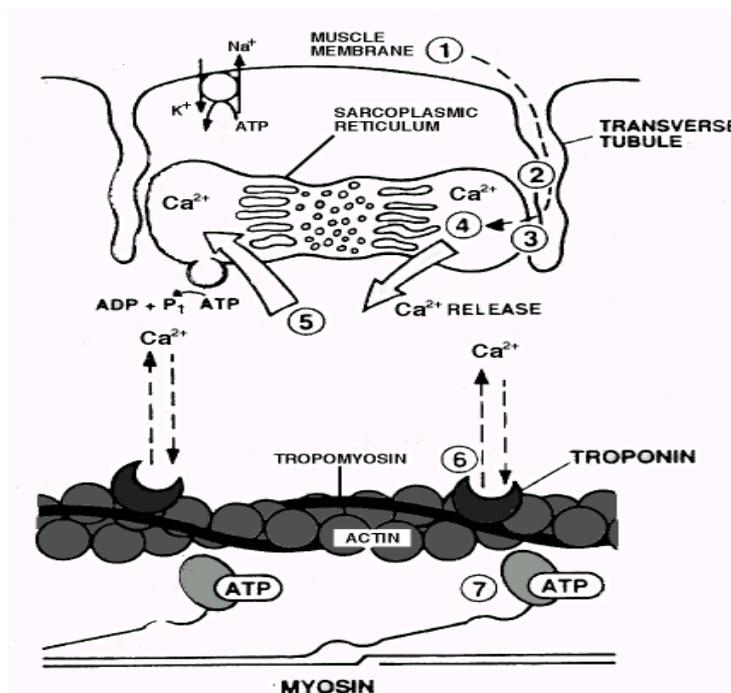


Figure 1.3. Excitation – Contraction coupling (taken from Lamb, 2000).

1.1.3.1 Sequence of Events (see figure 1.3)

An action potential occurs when acetylcholine (ACh) released by a nerve ending at the neuromuscular junction binds to ACh receptors on the motor end plate, causing changes in membrane permeability that allow Na^+ and K^+ ions to flow, thus, depolarizing the membrane (as more Na^+ enters than K^+ exits). Upon depolarization of the muscle, an action potential propagates over the sarcolemma, travels through the T-tubules (2). Voltage-sensing dihydropyridine receptors (DHPR), located in the T-tubules are activated by membrane depolarization, and directly interacts with the ryanodine receptor (RyR), located in the SR. This interaction elicits Ca^{2+} release (4) from the RyR (SR) (1) into the sarcoplasm (cytosol). Ca^{2+} binds to troponin (6) and the inhibition of actin-myosin combination by the troponin-tropomyosin (Tn-Tm) that prevails at rest is lifted, allowing cross-bridge cycling and muscle contraction to proceed (7), and hence, muscle force can be generated. Ca^{2+} ions are then removed from the sarcoplasm. A few bind to special proteins such as calmodulin and parvalbumin, while the majority is pumped across various membranes of the cell, such as sarcolemmal, the inner membrane of the mitochondria, and the membrane enclosing the SR.

The SR Ca^{2+} -ATPase pump is the most important contributor to removing Ca^{2+} from the cytosol into the SR and thus decreasing intracellular Ca^{2+} levels (Martonosi, 1995). The sarcolemmal (SL) Ca^{2+} -ATPase and mitochondrial (M) Ca^{2+} -ATPase also helps pump excess Ca^{2+} from the cytosol back into the extracellular fluid (i.e. out of the cell) and into a small mitochondrial reservoir, respectively. However, their buffering role is at a much lesser extent compared to the SR Ca^{2+} -ATPase pump. When intracellular calcium levels decrease as a result of this re-sequestration of Ca^{2+} , the inhibition of troponin-tropomyosin combination is re-established and myosin ATPase is again inhibited. Therefore, cross-bridge activity ends, and muscle relaxation occurs (Stephenson *et al.*, 1998).

1.1.3.2 Muscle Contraction

Two types of muscle contractions that have been used to investigate the characteristics of muscle force development are twitch and tetanic contractions. Twitch contractions are the response to a single action potential, whereas tetanic contractions occur when impulses are delivered so quickly that there is inadequate time for calcium to be pumped back into the SR and $[Ca^{2+}]_i$ remains elevated. Thus, maximum muscle force output occurs in a tetanic contraction.

The force of tetanic contractions can be influenced by numerous factors, which include intracellular calcium concentration, cross-bridge phosphorylation, elasticity and biomechanics, and as mentioned previously, muscle size (cross-sectional area). Sarcomere length is also an important factor in determining maximal tetanic contraction (maximum muscle strength). It is well acknowledged that force developed by a muscle during a maximum isometric tetanic contraction (where length of muscle remains the same) is a direct function of the magnitude of overlap between actin and myosin filaments (i.e. its starting sarcomere length; Schoenberg and Podolsky, 1972; Schoenberg, 1998). In short, maximal force output can be achieved during the plateau phase of the length-tension curve (figure 1.4) when a muscle fibre is at its optimal length, hence has its greatest myofilament overlap (i.e. maximum number of cross-bridge formation). The descending limb of the length-tension curve indicates stretching of the sarcomeres. Active stretching of the sarcomeres during the descending limb of the length-tension curve (eccentric contraction) is an important concept to this thesis as it is implicated in the initial event that leads to skeletal muscle injury (Section 2.1.1.1.1).

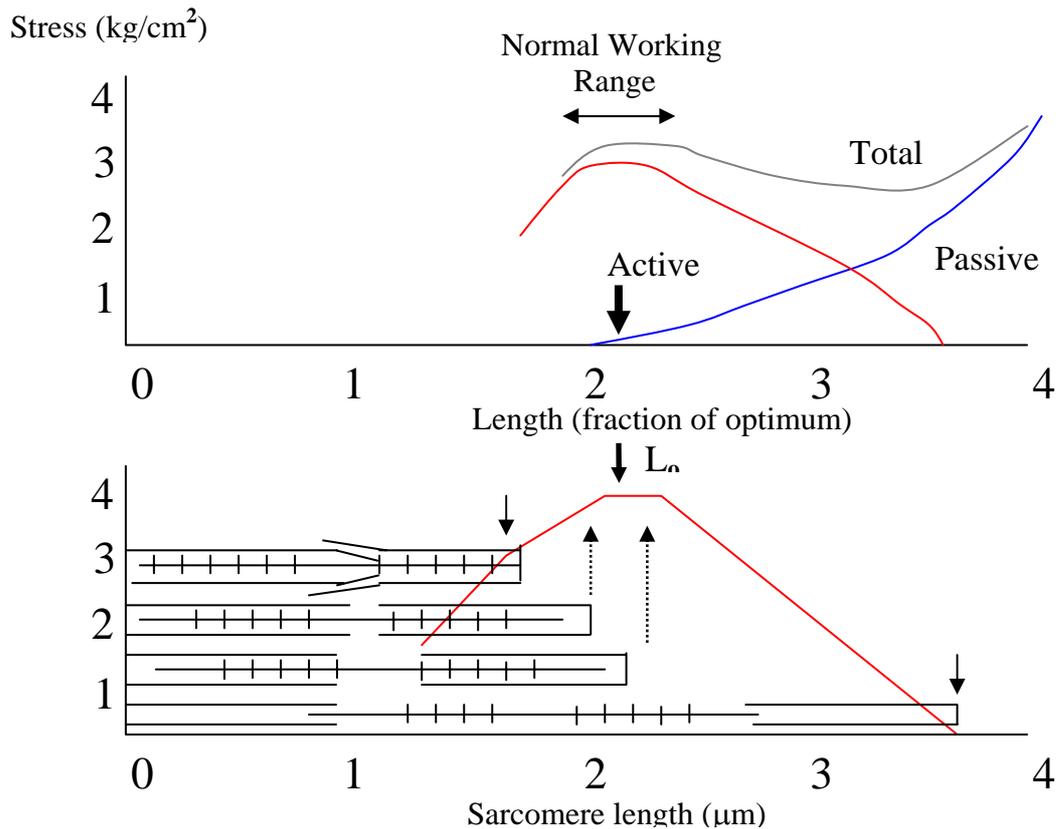


Figure 1.4. Length – Tension curve.

1.1.3.3 Muscle Metabolism

As muscle contraction occurs, ATP provides the energy for contractile events (cross-bridge movement and attachment). Since ATP is the only energy source that can be used directly for contractile activities, it must be generated continuously if contraction is to continue. The conventional view of the energy transfer leading to muscle contraction is depicted in figure 1.5, which shows that ATP is formed in the mitochondria and diffuses to myofibrils. ADP, formed by hydrolysis of ATP by the globular heads of the myosin molecules, diffuses back to the mitochondria, stimulating respiratory activity in the process called respiratory control (Bessman and Geiger, 1981).

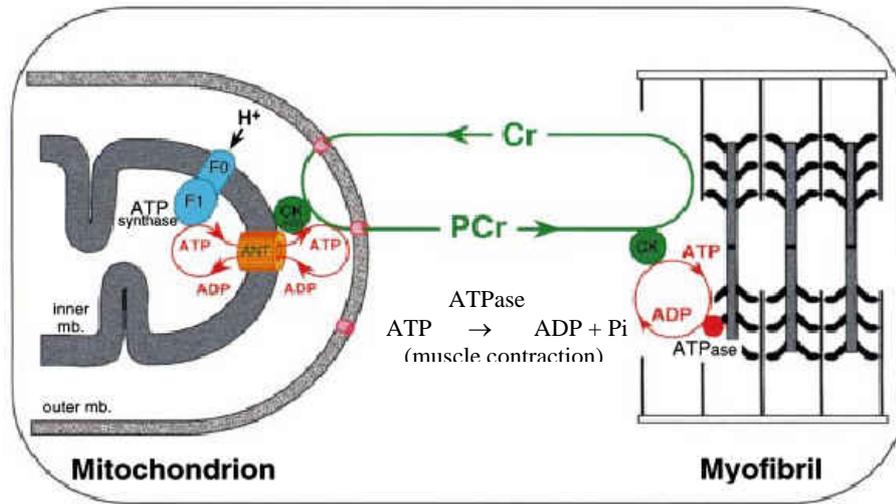
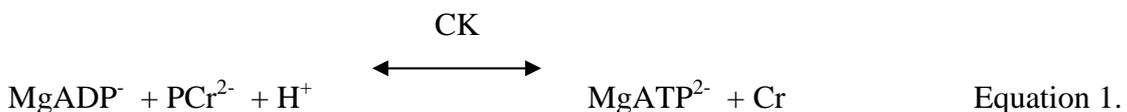


Figure 1.5. The phosphocreatine energy shuttle (taken from Saks *et al.*, 2000).

While ATP resynthesis can occur locally on myofibrils and biomembranes due to activity of CK and glycolytic enzymes as shown above in figure 1.5, it is suggested that their exists site-specific regeneration of ATP, which creates a local pool of ATP in close vicinity of sites of ATP utilization (Korge *et al.*, 1993). An important concept to this thesis is the energy regulation of the SR Ca^{2+} -ATPase pump. The SR Ca^{2+} -ATPase pump is the most energy-demanding pump in the muscle (Rossi *et al.*, 1990). Calcium uptake requires the direct coupling of CK to the SR Ca^{2+} -ATPase, in which CK catalyses the reversible transphosphorylation of the high energy *N*-phosphorly group of phosphocreatine (PCr) to ADP, regenerating ATP (Equation 1; Walliman *et al.*, 1992).



In skeletal muscle, small amounts of CK are bound in an isoenzyme-specific fashion to subcellular structures such as inner mitochondrial membrane (Mi-CK) (Saks *et al.*, 1978), and the M band in myofibrils (Walliman *et al.*, 1977). Interestingly, Rossi *et al.* (1990) demonstrated the existence of a muscle-type MM creatine kinase isoform that is specifically bound to the SR. It is suggested that MM-CK bound to the SR, which

preferentially uses the creatine kinase-creatine phosphate (CK-PCr) system, may be physiologically important *in vivo* for regeneration of ATP directly in the vicinity of the SR Ca^{2+} pump, thus maintaining high ATP/ADP ratios (Rossi *et al.*, 1990). It is hypothesized that while high [ADP] levels in close proximity to the SR Ca^{2+} pump may diminish its efficiency, high [ATP] to [ADP] levels could be essential in the thermodynamic efficiency of ATP hydrolysis, and therefore improved calcium handling ability of the muscle (Rossi *et al.*, 1990).

Indeed, Korge and Campbell (1994) demonstrated that in the presence of creatine phosphate, membrane-bound CK regulated ATP regeneration close to the SR Ca^{2+} pump by monitoring local increases in [ADP], and thus enhancing Ca^{2+} pump function. Isolated SR vesicles were obtained from rabbit fast-twitch skeletal muscles; Ca^{2+} reuptake and efflux, and ATPase activity, thus Ca^{2+} handling ability of the muscle, was measured by monitoring changes in extravesicular $[\text{Ca}^{2+}]$ with a Ca^{2+} -sensitive minielectrode. The efficiency of SR Ca^{2+} pump function was determined by the amount of Ca^{2+} transported per ATP hydrolysed, and thus was expressed as a coupling ratio. A coupling ratio greater than 1 indicated high efficiency for Ca^{2+} transport, while a coupling ratio less than 1 indicated low efficiency. It was hypothesized that local ATP regeneration by the membrane bound CK-CP system is one mechanism by which the cell can significantly improve SR Ca^{2+} pump function, especially when the pump is working at low efficiency, such as those seen during eccentric-induced damage.

1.1.3.4 Fibre Type

An important factor in determining muscles contractile properties is not only the muscle architecture (structure) as mentioned previously, but fibre type, or fibre phenotype predisposition. It has been well established (Close, 1972), that human skeletal muscle is

composed of several fibre types possessing different myosin isoforms (isomyosins) that differ substantially in functional and metabolic characteristics (see table 1). These range from type I, slow-twitch fibres, which produce low forces to type IIX/IIB, fast-twitch fibres, which produce high forces (Essen *et al.*, 1975; Thorstensson *et al.*, 1977). The classification of type IIB fibre in rat skeletal muscle is equivalent to type IIX fibre in human skeletal muscle. Type I fibres are suited to aerobic activities, whereas type IIB/X fibres are better suited to anaerobic, high intensity type activities. Speed of contraction, and hence, force development, is related to how fast the myosin ATPases hydrolysis ATP, with fast fibres having more efficient ATPases (Thorstensson *et al.*, 1977).

Characteristic	Type of Fibre		
	Slow Oxidative (Type I)	Fast Oxidative (Type IIA)	Fast Glycolytic (Type IIB/X)
Myosin ATPase activity	Low	High	High
Speed of Contraction	Slow	Fast	Fast
Force of Contraction	Low	Moderate	High
Fatigue Resistance	High	Moderate	Low
Oxidative Capacity	High	High	Low
Mitochondrial Density	High	High	Low
Myoglobin Content	High	High	Low
Anaerobic Enzyme Content	Low	Moderate	High
Capillary Density	High	High	Low
Glycogen	Low	Moderate	High
Z-line	Intermediate	Wide	Narrow
Fibre Diameter	Small	Moderate	Large

Table 1. Functional and structural characteristics of the three types of skeletal muscle fibres (adapted from Close, 1972).

CHAPTER 2

2.0 REVIEW OF LITERATURE: SKELETAL MUSCLE DAMAGE AND REGENERATION

2.1 SKELETAL MUSCLE INJURY

The area of exercise-induced muscle fibre injury has been discussed in detail in several recent reviews (Allen, 2001; Proske and Morgan, 2001; Huard *et al.*, 2002; Close *et al.*, 2005). It is clear from the literature that unaccustomed or intense exercise such as extended periods of running, strength training or sprinting, especially those incorporating eccentric exercises, can lead to disruption of the normal muscle ultrastructure and impairment of muscle function (for review see Allen, 2001). The terms *concentric* and *eccentric* are used currently to describe contractions in which the muscles are allowed to shorten and lengthen, respectively. However, it has been suggested that the terms *miometric* and *pliometric* contraction are more accurately applicable when describing the active shortening and lengthening of skeletal muscle, and thus a contraction (i.e. *pliometric*) by which muscle damage is induced. However, given that there has been no means to standardize these terms, and that previous literature have used the term 'eccentric contraction' to describe the action of active muscle lengthening, this term will be used throughout this thesis.

Skeletal muscle injury has been studied in both animal (Lieber *et al.*, 1994) and human models (Nosaka and Clarkson, 1996; Brown *et al.*, 1997; Sayers and Clarkson, 2001). Muscle injury can involve primary or secondary sarcolemmal disruption; swelling or interference to the sarcomplasmic reticulum and T-tubular (sarcotubular) system, and disruption of the myofibre and cytoskeleton contractile components, leading to

extracellular myofibre matrix abnormalities and stretching or breaking of intermediate filaments between Z disks (Friden and Lieber, 1992). The most common reported symptom of muscle damage is delayed onset of muscle soreness (DOMS) (Armstrong, 1984; Nosaka and Clarkson, 1996; Lieber and Friden, 2002; Nosaka *et al.*, 2002). Depending on the type and severity of the exercise, DOMS usually appears between 8-24 hours following damage, and usually peaks between 24-48 hours later, but a less intense pain can remain up to 7-10 days (Miles and Clarkson, 1994).

The sensation of pain in skeletal muscle is transmitted via two pathways: myelinated group III, and unmyelinated group IV afferent fibres, which are believed to transmit sharp pain and dull aching pain respectively (Armstrong, 1984). The latter is more commonly associated with DOMS and muscle damage. While not clearly understood, it is believed that chemicals released during muscle damage and regeneration such as prostaglandins, bradykinins, serotonin, histamine and potassium may directly sensitise pain afferent fibres (Armstrong, 1984). Other symptoms of exercise-induced muscle damage include: muscle swelling and increased circumference of injured muscle (Howell *et al.*, 1993; Hirose *et al.*, 2004); decrease in range of motion (ROM); decreased muscle strength; and leakage of myofibre proteins into the blood, the most commonly measured being CK and lactate dehydrogenase (LDH) (Hirose *et al.*, 2004; Zainuddin *et al.*, 2005).

As mentioned in the introduction, muscle injury can be categorised into four stages: (i) initial event; (ii) autogenic phase; (iii) phagocytic stage; and (iv) regenerative phase (Armstrong, 1990). The processes of muscle injury will be discussed in detail using this model as a basis. However, while muscle damage can be divided up into these separate

processes, they overlap enormously, and the exact nature of muscle damage, the mechanisms responsible and processes involved are still not fully understood.

2.1.1 Initial Event

While the initial events that precede skeletal muscle damage are still controversial, it is hypothesised that it may occur as a result of mechanical or metabolic stress (Armstrong, 1990; Byrd *et al.*, 1992; Pyne, 1994)

2.1.1.1 Mechanical Stress

It is clearly evident that there are two prominent signs of damage in a muscle immediately after it has been subjected to a series of eccentric contractions. It is believed that the damage process begins with an overstretching of sarcomeres (Morgan and Allan, 1999; Proske and Morgan, 2001) and damage to the E-C coupling system (Balnave and Allen, 1995; Takekura *et al.*, 2001; Warren *et al.*, 2001). However, which event takes place first; E-C- coupling failure or sarcomere disruption still remains a point of controversy (Proske and Morgan, 2001). Numerous studies have demonstrated that eccentric exercise leads to structural signs of muscle damage, which include sarcomere disruption (Morgan and Allen, 1999) and myofilament and cytoskeleton disorganization often causing to Z-disc streaming (Appell *et al.*, 1992; Waterman-Storer, 1993; Gibala *et al.*, 1995). These changes may be localized or widespread (Newham *et al.*, 1983).

2.1.1.1.1 Sarcomere Disruption

The exact detail of sarcomere disruption following eccentric exercise remains a subject of speculation. However, it has been recognised for many years that sarcomeres are fundamentally unstable on the descending limb of the length-tension curve (i.e. eccentric contraction; Allen, 2001). During an eccentric contraction, the muscle is forced to lengthen while it is activated. As a result, instead of the actin filament being propelled toward the centre of the myosin filament (as seen in normal cross-bridge cycling), the filaments are pulled in the opposite direction by the external forces acting on the muscle (McComas, 1996), leading to overstretching of sarcomeres.

Muscle structural damage after forced lengthening (eccentric contraction) has been reported in a number of studies (Friden *et al.*, 1983; Lieber *et al.*, 1991;1996; Armstrong *et al.*, 1993). An important study by Katz in 1939, examined the relationship between force and speed in muscular contraction of isolated frog muscles (Katz, 1939). It was first demonstrated that there is a difference in the force required for a given rate of stretch than for the same rate of shortening. Furthermore, when force applied to a muscle was greater than 180% of P_o (where P_o is isometric force) as seen during an eccentric contraction, the muscle suddenly yielded and was stretched at a very high velocity. It was also noted that after such rapid stretching of the muscle, fibres became permanently weaker, especially if the stretch exceeded the optimal length of the muscle. These observations led the researcher to suggest that during such rapid stretches some part of the muscle becomes permanently stretched or damaged (Katz, 1939).

Further insight by Morgan (1990) showed that when contracting muscles are stretched on the descending limb, sarcomeres exceed their yield point (the point of contact between the actin and myosin head) and start to extend (elongate) very rapidly, leading

to sarcomere disruption. These observations of non-uniform sarcomere disruption following eccentric contraction lead to the “popping-sarcomere” hypothesis (Morgan, 1990). The “popping-sarcomere” hypothesis describes the process in which sarcomeres become permanently overextended following eccentric contraction, and thus are more liable to damage during the next eccentric contraction. However, early experiments utilizing single eccentric contractions observed little damage to sarcomeres and muscle function, as the majority of overstretched sarcomeres interdigitate with undamaged fibres during relaxation (Talbot and Morgan, 1996).

Thus, increasing the number of eccentric contractions will increase the structural damage to the myofilaments. Talbot and Morgan (1996) demonstrated that there are a greater number of overstretched (disrupted) sarcomeres following repeated eccentric contractions compared to a single eccentric contraction or isometric and concentric contractions. More importantly, while some overstretched sarcomeres are capable of reintegrating during relaxation after repeated eccentric contractions, the likelihood of thick and thin filaments failing to reinterdigitate correctly increases with the number of contractions. Consequently, these extended sarcomeres are more prone to damage in a new contraction. Thus, the number of permanently weakened or overstretched sarcomeres increases, leading to the extensive regions of sarcomere disorganization, as previously reported (Armstrong *et al.*, 1993; Friden and Lieber, 2001a).

Evidence of morphological changes following eccentric-induced muscle damage was first provided by Friden *et al.* (1981). Biopsy samples were examined from human leg muscles following a run down a flight of stairs. While there were no signs of fibre necrosis or rupture by light microscopy, electron microscopy showed characteristic changes in Z-line material through the sarcomere, and hence, the normal uniform

registration of Z-lines across the fibre was disturbed, known as Z-line streaming (see figure 2).

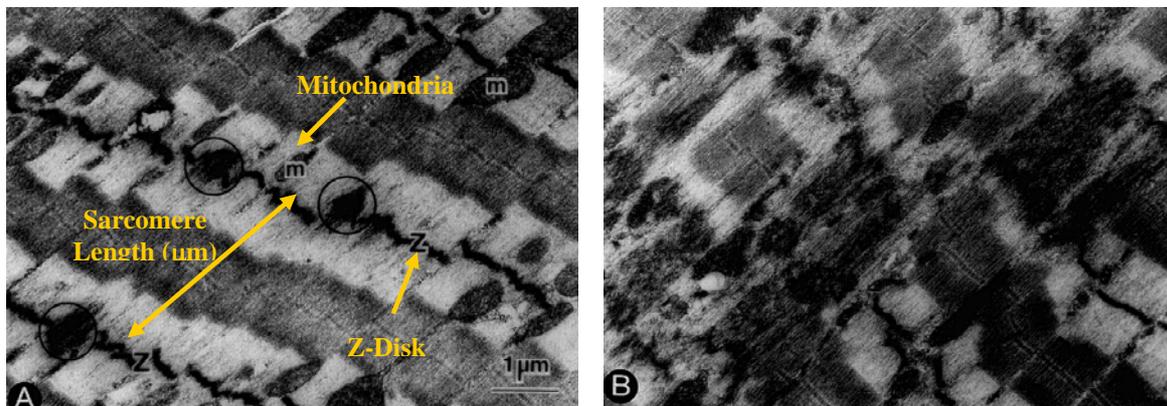


Figure 2. Muscle ultrastructural changes following eccentric contraction. Note Z-line streaming in B (taken from Lieber, 1992).

Researchers have also suggested that repeated eccentric contractions lead to an early loss of cytoskeleton proteins, and therefore may predispose the contractile apparatus to early structural damage and further contribute to sarcomere disruption. In addition, disruption of both sarcolemma and internal cytoskeleton has previously been implicated in loss of force production after injury (McNeil and Khakee, 1992; Sam *et al.*, 2000; Hamer *et al.*, 2002). As mentioned previously, cytoskeleton proteins such as titin, nebulin, desmin and dystrophin have roles in stabilizing the sarcomeric structure and transmission of forces laterally across the fibre, and from fibre to fibre.

One of the early markers of sarcomere disruption (muscle injury) is loss of the cytoskeleton protein desmin. In a series of studies, Lieber and Friden have used antibody-staining techniques to visualize and explore changes in cytoskeletal proteins after exercise. Initially, they showed that loss of desmin staining in rabbit muscle fibres was a feature of eccentric damage. However, such loss could only occur in cells whose membranes were still intact and whose contractile proteins appeared normal (Lieber *et*

al., 1994). Subsequently, in a later study, Lieber and colleagues (1996) established that desmin loss could appear as early as 5-15 min after 30 minutes of eccentric exercise of the rabbit dorsiflexors. Furthermore, a strong linear correlation was established between the magnitude of desmin lost and the decrease in force-generating capacity of the muscle after eccentric contraction. Force is generated within the sarcomeres, and it is generally accepted that this force is transmitted longitudinally along the myofibril as mentioned previously (Patel and Lieber, 1997). However, it is suggested that force is also transmitted radially through major skeletal muscle intermediate filament proteins (such as desmin) of the internal cytoskeleton and outward toward the sarcolemma (Patel and Lieber, 1997). Therefore, it is suggested that high forces generated by eccentric contractions may strain these components of the recognized force-transduction pathway, and thus support the loss of force-generating capacity observed (Lieber *et al.*, 1996).

In contrast, Peters *et al.* (2003) showed that loss of desmin in the rat tibialis anterior (TA) could not explain the precipitous and prolonged loss in muscle force production after repeated eccentric contractions. Eccentric contractions were induced by maximally activating the dorsiflexor muscles via the peroneal nerve and plantarflexing the foot over a ~40 degrees range, which is similar to the methods used by Lieber *et al.* (1996). Immunohistochemical analysis revealed a loss of desmin staining, thus indicating desmin disruption of the cytoskeleton. However, only a small proportion of fibres displayed loss of desmin compared to the average fibre number yielded by the TA and therefore could not explain the measured loss in muscle force production.

Thus, it has been recently hypothesised that compared to desmin loss, reduction of the cytoskeletal protein dystrophin, a large subsarcolemmal protein concentrated at the Z and M lines, may be the vulnerable link in force transmission, and the observed loss in

contractile force after eccentric contraction (Tidball, 1991; Petrof *et al.*, 1993; Patel and Lieber, 1997). In contrast, to the previous methodology that employed repeated eccentric contractions (Lieber *et al.*, 1996; Peters *et al.*, 2003), a single eccentric contraction was performed on the tibialis anterior (TA) muscle of male Sprague-Dawley rats. Contractile force was reduced immediately (15min) following eccentric contraction, and 3 and 7 days thereafter compared to non-injured control muscle. Immunohistochemical staining revealed that within 15 min after the eccentric contraction, dystrophin was preferentially lost compared to the desmin protein, which was also affected, but to a much lesser extent. Therefore, it was concluded that there is a selective vulnerability of dystrophin after single eccentric contraction-induced injury, and it is possible that dystrophin may be the critical component in the loss of muscle strength after injury (Lovering and Deyne, 2004).

Taken together, it is clear that when skeletal muscles are exercised eccentrically in both human and animal models, they exhibit structural signs of muscle damage, which include overstretched sarcomeres and myofilaments and cytoskeleton disorganization, often leading to Z-line streaming of the muscle fibre. Eccentric contractions also cause early loss of specific structural contractile proteins (i.e. dystrophin and desmin), and these reductions may directly contribute to the decline in muscle force after injury.

Interestingly, the presence of overstretched and weakened sarcomeres could place extra strain on specific ionic channels, in particular stretch activated channels (SACs), leading to a local rise in intracellular calcium concentrations $[Ca^{2+}]_i$. SACs were first described in foetal skeletal muscle by Guharay and Sachs (1984). Briefly, these channels release Na^+ , K^+ , and Ca^{2+} , when negative pressure is applied upon them (Franco and Lansman, 1990). While there is no direct evidence that demonstrates an increased opening after

stretched contractions, various blockers of SACs have been implicated in preventing the rise of $[Ca^{2+}]_i$ and intracellular sodium concentrations $[Na^{2+}]_i$, typically seen after stretched contractions. Thus, this provides strong evidence that enhanced calcium influx, hence increased $[Ca^{2+}]_i$, may be mediated through these channels during muscle injury. The concept of $[Ca^{2+}]_i$ and muscle damage will be discussed further in section 2.1.2.

2.1.1.1.2 Excitation – Contraction Coupling

The second most prominent sign of damage in a skeletal muscle is a failure or disruption in the E-C coupling process. While histological evidence suggests that the force deficit associated with eccentric contraction-induced muscle injury is due to structural damage to the contractile elements within the muscle fibres, as previously discussed, an alternative hypothesis is that the force deficit could be explained by an inability to activate contractile proteins, possibly due to a failure or disruption to the E-C coupling process.

The first experimental evidence that the tension deficit after eccentric exercise may be due to changes in excitation-contraction coupling came from studies of caffeine contractures in isolated soleus muscles (Warren *et al.*, 1993). Mouse soleus muscles were isolated, placed in an oxygenated Krebs-Ringer buffer solution, thus mimicking the *in vivo* conditions of the muscle. Muscles performed one of three contraction protocols (20 eccentric; 10 eccentric or 20 isometric contractions). Following the contraction protocol, muscle twitch and tetanic forces were measured in the absence and presence of 50mM caffeine. Caffeine directly releases Ca^{2+} from the SR and bypasses some parts of normal excitation-contraction coupling process. The eccentric exercise protocols caused a substantial fall in maximal isometric tetanic force; however,

caffeine-elicited force was unchanged by such exercise. Though direct SR function and intracellular calcium concentrations were not measured, it was suggested that the measured force deficit in this muscle injury model was from a failure of the E-C coupling process at some step prior to Ca^{2+} release by the SR, not mechanical factors (Warren *et al.*, 1993). Indeed, in a review by Allen (2001), it was suggested that in overstretched sarcomeres, the SR could be directly damaged or its connection with the T-tubules might be impaired, thus resulting in E-C coupling failure. However, how these distortions would affect function is a matter of speculation.

In contrast to Warren *et al.* (1993), a single fibre study examined the role of intracellular free Ca^{2+} concentration in the reduction of muscle force following eccentric contractions (Balnave and Allen, 1995). Single fibres from mouse toe muscles were either stretched by 25% of their optimum length (L_o) and subjected to ten maximal tetanic contractions (protocol I) or were stretched by 50% of their L_o and subjected to between 10 and thirty tetanic contractions (protocol II). Intracellular free Ca^{2+} concentration was measured for each stretch protocol; with each protocol compared to an isometric control. Following both protocols, researchers observed a reduction in tetanic force; a decrease in tetanic $[\text{Ca}^{2+}]_i$ (i.e. the free calcium concentration in the myoplasm after a tetanic contraction), and increased resting $[\text{Ca}^{2+}]_i$ compared to controls. Subsequent addition of caffeine was able to restore tetanic force back to pre-stretch levels after protocol I, thus supporting the results shown by Warren *et al.* (1993). However, caffeine was unable to restore reduced tetanic force levels back to pre-stretch levels following the more severe stretching regime of protocol II, which resulted in a much greater reduction in tetanic force. It was concluded that while E-C coupling disruption is possibly the main contributor to force reduction after a moderate eccentric protocol (protocol I), under more intense stretching protocols, as seen in protocol II,

both mechanical and E-C coupling disruption may be implicated together in the force reduction of the muscle (Balnave and Allen, 1995).

Taken together, whilst it is clear that mild eccentric contraction protocols (such as those used by Warren *et al.*, 1993 and Balnave and Allen, 1995-protocol I) will induce interference to the E-C coupling system, more robust eccentric contractions will induce mechanical damage (i.e. sarcomere disruption as explained in the previous section) of the muscle fibre, and thus also contribute to reduced force of the skeletal muscle.

2.1.1.2 Metabolic Stress: Insufficient Mitochondrial Respiration.

Damage has also been suggested to be a result of metabolic insufficiency in contracted muscles. Mitochondrial respiration is elevated to match ATP synthesis to ATP hydrolysis during exercise. During low to moderate intensity exercise, this match is normally quite good, thus allowing active muscle fibres to maintain ATP concentrations near resting levels. However, there is always some reduction in the concentration of the high energy phosphates during muscular activity (Krisanda *et al.*, 1988), and the possibility that these reductions occur within specific compartments in the fibres makes this a viable hypothesis for the initiating event in muscle fibre injury (Armstrong *et al.*, 1991).

Armstrong and colleagues (1991) suggested that if ATP levels within the vicinity of the Ca^{2+} -ATPase in the SR of sarcolemma are not maintained, removal of Ca^{2+} from the cytoplasm may be compromised, resulting in an elevation in cytosolic Ca^{2+} . The importance of maintaining functional Ca^{2+} pumps to the health of the cell is indicated by experiments using ruthenium red, which inhibits Ca^{2+} -ATPase on sarcolemma, SR and mitochondria (Duncan, 1987). In isolated intact skeletal muscles, ruthenium red

treatment caused rapid and dramatic damage to the ultrastructure of the muscle (Duncan *et al.*, 1980). Also, it has been demonstrated that reduction in energy supply in cells leads to Ca^{2+} release from internal stores (Duchen *et al.*, 1990). Furthermore, a reduction in local ATP and /or a reduction in the free energy from hydrolysis of ATP due to increased ADP could reduce the rate of ATP splitting and slow Ca^{2+} pumping by the sarcoplasmic reticulum pump (Byrd, 1992), thus increasing $[\text{Ca}^{2+}]_i$.

However, since a given force or power output produced with an eccentric contraction is “metabolically cheaper” than that produced by concentric and isometric contractions, and that the buffering capacity of the SR Ca^{2+} -ATPase derives its energy preferentially from coupled enzyme systems as opposed to mitochondrial aerobic respiration (Rossi *et al.*, 1990; Korge and Campbell, 1994; Minajeva *et al.*, 1996), it can be argued that insufficient respiration in the fibres is not the initiating event in muscle injury. Indeed, Aldridge *et al.* (1986) showed this by directly measured high energy phosphate levels in the forearm muscles with ^{31}P -NMR immediately after eccentric exercise that included post-exercise soreness. There were no changes in ATP, creatine phosphate levels or pH in the injured muscles, although 24 hours after exercise there was a significant elevation in inorganic phosphate levels, thus indicating ATP hydrolysis. Therefore, while ATP levels may not be decreased during the initial event, the subsequent increase in $[\text{Ca}^{2+}]_i$ usually seen after eccentric exercise, would indeed require an increased supply of ATP to various Ca^{2+} -ATPase pumps to enhance the buffering capacity of the muscle.

Regardless of the initiating stimulus (metabolic or mechanical stress in nature), the next sequential step in the injury process is an elevation in intracellular Ca^{2+} concentration,

loss of $[Ca^{2+}]_i$ homeostasis and thus a rapid activation of autogenic destructive processes that originate in the muscle fibres (Armstrong, 1990).

2.1.2 Autogenic Phase: Calcium Elevation

There has been increasing interest into the role of free intracellular cytosolic calcium $[Ca^{2+}]_i$ in skeletal muscle cell damage (for a review see Gissel and Claussen, 2001). While low Ca^{2+} levels are necessary for muscle cell function, high Ca^{2+} levels within the muscle have long been associated with cell dysfunction and cell necrosis. Thus, it has been proposed that Ca^{2+} is the primary factor in the degenerative mechanisms underlying the autogenic phase of the injury and repair process (Armstrong, 1990).

There are several intrinsic Ca^{2+} buffering routes that exist in muscle cells – all of which involve the action of specialized membranous pumps that are fuelled by the breakdown of ATP (ATPase pumps). In resting muscle cells, these pumps help maintain cytoplasmic Ca^{2+} at around $10^{-7}M$ (Carafoli, 2004). Under normal physiological conditions, transient elevations of $[Ca^{2+}]_i$ induce mitochondrial respiration, ATPase pump activation, and thus $[Ca^{2+}]_i$ is re-established around resting baseline values. However, repeated eccentric exercise, leads to an influx of Ca^{2+} at a rate that cannot be buffered by the cell's buffering systems. Hence, intracellular Ca^{2+} levels rise, resulting in loss of Ca^{2+} homeostasis, and eventually cellular damage (Armstrong, 1990).

2.1.2.1 Intracellular Calcium Concentration

Numerous studies have showed increases in $[Ca^{2+}]_i$ in skeletal muscle during and after various types of exercise activities. These include: isolated eccentric contractions (Armstrong *et al.*, 1993); downhill running (Duan *et al.*, 1990; Lynch *et al.*, 1997); isometric contractions subjected to electrical stimulation (Gissel and Clausen, 1999;

Mikkelsen *et al.*, 2004) and long distance running of 10-20km (Overgaard *et al.*, 2004) and 100km (Overgaard *et al.*, 2002). It is proposed that the observed elevations in $[Ca^{2+}]_i$ are a direct result of damage to the muscle caused by the various exercises, in particular eccentric exercise.

In contrast, Lowe *et al.* (1994) showed no significant changes in free cytosolic $[Ca^{2+}]$ levels after eccentric and/or isometric contractions. Isolated mouse soleus muscles were subjected to either 20 eccentric or 20 isometric contractions over a 40-minute period in a buffer solution containing varied Ca^{2+} concentrations. While total $[Ca^{2+}]$ was 28-37% higher in muscles that performed eccentric contractions compared to those that performed isometric contractions, free cytosolic $[Ca^{2+}]$ levels were not significantly higher between the 2 groups. It was suggested that muscles subjected to eccentric contractions were able to buffer the increased influx of extracellular Ca^{2+} , maintaining normal free cytosolic $[Ca^{2+}]$, and thus avoid activation of Ca^{2+} -sensitive degradative pathways (Lowe *et al.*, 1994).

However, longer incubation time (i.e. >120 minutes) may be needed to see any increase in cytosolic Ca^{2+} levels. Indeed, Lynch *et al.* (1997) measured $[Ca^{2+}]_i$ in whole mice muscle at 24 hours and 48 hours following downhill running. Resting $[Ca^{2+}]_i$ was only increased at 48 hours, not 24 hours. Furthermore, previous studies have shown increased Ca^{2+} uptake long after muscle activity has ceased (Gissel, 2000). Thus, while resting $[Ca^{2+}]_i$ may not be increased initially after eccentric exercise, damage to muscle structural elements leading to loss of sarcolemmal integrity, as mentioned previously in section 2.1.1.1.1, will allow extracellular Ca^{2+} to leak in, and thus increase $[Ca^{2+}]_i$ (Gissel, 2000).

2.1.2.2 Loss of $[Ca^{2+}]_i$ Homeostasis

While it is clearly evident that exercise that includes a substantial eccentric component leads to an elevation of resting intramuscular calcium content, whether there is subsequent loss of Ca^{2+} homeostasis is still unclear. Nevertheless, it is hypothesised that loss of Ca^{2+} homeostasis may be a result of reduced SR Ca^{2+} -ATPase pump activity (calcium handling ability of the muscle) and thus, negatively influencing the muscles capacity to buffer changes in $[Ca^{2+}]_i$, during and after exercise (Belcastro *et al.*, 1981; Matsushita and Pette, 1992; Carroll *et al.*, 1999). Belcastro and colleagues (1981) reported an 18% reduction in Ca^{2+} -dependant ATPase activity following prolonged running exercise. Furthermore, it was shown *in vivo* that chronic low frequency stimulation (10Hz) of 2-hour duration caused a decrease in SR Ca^{2+} -ATPase pump activity in rat hindlimb muscle (Carroll *et al.*, 1999). With several other studies also reporting loss of SR Ca^{2+} -ATPase pump activity with long-term low frequency stimulation (Dux *et al.*, 1990; Klebl *et al.*, 1998), this provides strong support that reduced SR Ca^{2+} -ATPase activity may eventually lead to loss of Ca^{2+} homeostasis.

Reduced SR Ca^{2+} -ATPase pumping efficiency could be due to a number of reasons. Firstly, increased SR Ca^{2+} -ATPase activity may bring about conformational changes in active binding sites of the SR Ca^{2+} -ATPase, thus making it more prone to degradation by proteolytic enzymes in the cytoplasm during excitation. However, it has been reported that compared to the SR Ca^{2+} -ATPase pump, the sarcolemmal Ca^{2+} -ATPase pump is the preferred substrate of the protease calpain, one of the proteolytic enzymes that is activated by the initial increase in intracellular Ca^{2+} concentration (Salamino *et al.*, 1994). Whether other proteolytic enzymes may degrade the SR Ca^{2+} -ATPase pump such as lysosomal proteases is unknown. Secondly, it is suggested that reactive oxygen species (discussed later in section 2.1.3.3) produced during muscle injury, may inhibit

ATPase activity via modification of sulfhydryl groups on the SR protein, hence, disrupting the normal Ca^{2+} -handling kinetics of the muscle (Favero *et al.*, 1998). Finally, as mentioned previously, high [ADP] levels in close proximity to the SR Ca^{2+} pump may diminish its thermodynamic efficiency to hydrolyse ATP, thus decreasing ATP regeneration directly in the vicinity of the SR Ca^{2+} pump and reducing the calcium handling ability of the muscle (Rossi *et al.*, 1990).

2.1.2.3 Mitochondrial Overload

Increased $[\text{Ca}^{2+}]_i$ may lead to accumulation of Ca^{2+} inside the mitochondria (Duan *et al.*, 1990). While small increases in mitochondrial Ca^{2+} levels in the nm range (as seen with normal contraction) stimulates mitochondrial respiration and ATP synthesis (McMillin and Madden, 1989; Khuchua *et al.*, 1994), accumulation of Ca^{2+} in the μm range may depress mitochondrial function, and therefore decrease ATP production.

Duan *et al.* (1990) employed a 2-hour period of downhill walking, which predominantly utilized stretch contractions, to examine the role of intracellular calcium in muscle damage. Muscles were excised both immediately and 2 days after the exercise protocol. Mitochondria was extracted and $[\text{Ca}^{2+}]$ measured. Mitochondrial $[\text{Ca}^{2+}]$ was increased about 3-fold immediately after exercise and about 6-fold 2 days later. Similarly, treatment of muscles with calcium ionophores, which increase $[\text{Ca}^{2+}]_i$, produced muscle injury with initial contracture, mitochondrial swelling, loss of soluble muscle proteins and eventually degeneration of myofibrils (Duncan, 1987). These studies were first to demonstrate that increased intracellular Ca^{2+} , leading to Ca^{2+} accumulation within the mitochondria, could also be involved in loss of Ca^{2+} homeostasis and development of muscle cell damage.

Hence, whilst initial rises in intracellular Ca^{2+} can be ameliorated by uptake by the mitochondria; further uptake will cause Ca^{2+} accumulation and structural damage within the mitochondria and hence, impair ATP production. It is hypothesized that the ensuing ATP shortage would then interfere with the Ca^{2+} -ATPase mediated Ca^{2+} re-accumulation in the SR, leading to further increases in cytoplasmic Ca^{2+} (Gissel, 2000). However, as mentioned in section 1.1.3.3, ATP for SR Ca^{2+} uptake is reportedly derived favourably from coupled enzyme systems as opposed to mitochondrial aerobic respiration (Rossi *et al.*, 1990; Korge and Campbell, 1994; Minajeva *et al.*, 1996). Thus, reduced Ca^{2+} handling ability of the muscle may be due to diminished ATP regeneration by the CK-PCr system, therefore producing a high [ADP] to [ATP] ratio in close proximity to the SR Ca^{2+} pump (Rossi *et al.*, 1990).

The next sequential step arising from elevated intramuscular calcium levels is activation of the non-lysosomal cysteine protease calpain.

2.1.2.4 Calpain

High levels of Ca^{2+} are known to activate Ca^{2+} -sensitive proteases, in particular calpain, which has been shown to degrade proteins (increase proteolysis), within skeletal muscle (for a review see Belcastro *et al.*, 1998). Muscles contain the ubiquitous u-calpain (calpain 1) and m-calpain (calpain 2), in addition to calpain 3 isoenzyme, which is relatively specific to skeletal muscle (Goll *et al.*, 2003). Calpain 1 and 2 are normally activated by $[\text{Ca}^{2+}]_i$ within the high μM and mM range and hence, are not activated under normal physiological conditions unless additional, as yet unidentified, factors contribute to their activation (Allen, 2005). Recent work has demonstrated that leupeptin, which inhibits calpain 1, 2, and 3 activity is capable of preventing Ca^{2+} -induced uncoupling of proteins, while calpastatin, which only inhibits calpain 1 and 2

activity (Ono *et al.*, 2004) did not prevent this uncoupling. Taken together, these results have reinforced suggestions that compared to calpain 1 and 2; calpain 3 may be central in the activation of Ca^{2+} -dependant proteases, responsible for the initiation of muscle damage (Branca *et al.*, 1999).

Calpain isoenzymes are typically localized throughout the muscle cell and are associated with most organelles, including metabolic and signalling pathways (Johnson, 1990). Within the sarcomere, a small percentage of calpain is closely associated with the I- and Z band regions. Therefore, when calpain is activated by elevated $[\text{Ca}^{2+}]_i$, selective proteolysis of various contractile and/or structural elements occurs. Belcastro and colleagues (1988) demonstrated specific loss/disruption of the Z-line structure in 22% of myofibrils isolated from exercised skeletal muscles in the rat. They observed an extensive loss of two proteins, desmin and α -actinin. In a later *in vitro* study, it was shown that activated calpain promptly degrades desmin, and thus promotes the release of α -actinin (Goll *et al.*, 1991). More recently, calpain 3, which may be activated by sub- μM $[\text{Ca}^{2+}]_i$ is also capable of cleaving titin, another structural protein, as well as disrupting SR Ca^{2+} release in skinned skeletal muscles (Verburg *et al.*, 2005).

2.1.2.4.1 Calpain Activation

While the mechanisms which contribute to the activation of calpain and the subsequent degradation of selected myofibrillar, Z-line, and cytoskeletal proteins remains unclear, it is hypothesised that it may involve mechanically and/or metabolically linked changes in Ca^{2+} homeostasis (for a review see Belcastro *et al.*, 1998). Belcastro (1993) investigated the relationship between exercise and the activation of calpain in rat hindlimb muscle. Results showed that prolonged exercise significantly increased total calpain activity (u-calpain and m-calpain), plasma CK levels (muscle damage indicator), while decreasing

muscle glycogen levels. It was suggested that dysfunctional SR Ca²⁺-ATPase pumps, and thus reduced calcium-buffering capacity of the muscle, leads to increased [Ca²⁺]_i, and activation of calpain activity. While total intracellular calcium levels and SR Ca²⁺-ATPase pump activity was not measured, it was the first study to suggest the relationship between loss of calcium homeostasis; SR pump dysfunction and calpain activation after exercise (Belcastro, 1993).

Taken together, these results suggest that increased [Ca²⁺]_i levels, loss of calcium homeostasis and SR pump dysfunction, leads to maximal calpain activation, which subsequently, due to their close association with the myofibrillar complex, initiates proteolysis of important cytoskeleton proteins such as desmin, α -actinin and titin. Consequently, there is reduced structural stability and increased permeability of the sarcolemma, which leads to further increases in Ca²⁺ influx from the extracellular fluid. In addition to calpain activity, increased [Ca²⁺]_i also activates lysosomal proteases and the phospholipase A2 pathway, inducing further injury to the sarcolemma, possibly due to production of leukotrienes and prostaglandins through free oxygen radical formation, and release of detergent-like lysophospholipids (Byrd, 1992). Thus, a vicious cycle is created where further increases in cytoplasmic Ca²⁺ leads to additional activation of proteases, until at some point the damage to the muscle becomes so severe that large molecules such as CK, LDH, carbonic anhydrase III, myoglobin, and contractile and regulatory proteins (for example skeletal troponin I and myosin heavy chains-MHC) leak out into the extracellular fluid and plasma (Janssen *et al.*, 1989; Duan *et al.*, 1990; Nuviola *et al.*, 1992; Warren *et al.*, 1993; Ferrington *et al.*, 1996; Moran and Schnellmann, 1996; Sorichter *et al.*, 1999; 2001). Subsequently, the muscle cell undergoes necrosis and the inflammatory response and repair phase is initiated (Gissel and Clausen, 2001).

In summary, an increase in $[Ca^{2+}]_i$ is likely to be the central factor in the development of muscle cell damage. Therefore, any intervention that decreases the accumulation of Ca^{2+} in the cytoplasm by enhancing the regulation of cytosolic Ca^{2+} levels, in particular the calcium buffering ability of the SR Ca^{2+} -ATPase pump, may reduce the activity of Ca^{2+} -activated proteases or phospholipases, therefore reducing membrane instability and further Ca^{2+} leakage, but more importantly, reducing/minimizing the extent of muscle damage.

2.1.3 Phagocytic Phase: Inflammatory Process in Muscle Injury and Repair

Recent investigations have explored the relationship between inflammatory cell functions and skeletal muscle injury and repair (for review see Best and Hunter, 2000; Tidball, 2005). These studies have developed a general view in which inflammatory cells dominate both injury and repair processes, through the combined actions of free radicals, growth factors, and chemokines (Tidball, 2002).

The inflammatory response is a coordinated sequence of events that commences with both tissue damage and infection. These early events facilitate antibacterial and antiviral responses before initiating clearance of debris and tissue fragments. While the inflammatory response causes widespread destruction of both damaged cells and healthy cells, it is an integral part of the repair process, preceding the regenerative phase of growth and repair of tissues and restoration of normal muscle function (Kendall and Eston, 2002). It is believed, although still not thoroughly understood, that at least two cell populations respond to muscle injury: inflammatory cells involved in the removal of cellular debris and myogenic cells involved in formation of new muscle cells (Tidball, 1995). Specific cytokines orchestrate the infiltration of both these cells into the muscle (Pyne, 1994).

Previous studies have confirmed that after exercise-induced damage, cytokine interleukin-1 (IL-1) concentration was increased in both serum and muscle samples during recovery, and thus was hypothesised to play a substantial role in promoting muscle inflammation (Tidball, 1995). Researchers suggest that a small group of cytokines, including IL-1, interferon, IL-2, IL-6 and tumour necrosis factor- α (TNF α), are the principle mediators of inflammation (Imura *et al.*, 1996). Cytokines, which are small polypeptide structures, are believed to be a significant link between the immunological and neuroendocrinal systems involved in inflammation, chemotaxis, acute phase response and tumour regression (Pyne, 1994; MacIntyre *et al.*, 1995). In addition, cytokines help regulate the growth, differentiation and functional activities of T and B lymphocytes (Pyne, 1994), which are essential in initiating an immune response.

Of particular interest is cytokine TNF α . Tissue necrosis factor- α has been shown to activate nuclear factor (NF)- κ B pathway in a variety of cell types, including muscle cells (Ghosh *et al.*, 1998), which in turn activates the ubiquitin-proteasome pathway, leading to increased proteolysis (Jackman and Kandarian, 2004; Kandarian and Jackman, 2005).

2.1.3.1 The Ubiquitin-Proteasome Pathway: To Build We Must First Breakdown

The ATP-dependent ubiquitin-proteasome (UBI-proteolytic) system is a major pathway for selective protein breakdown in eukaryote cells, and is thought to degrade the bulk of all intracellular proteins during muscle remodelling. In short, degradation of proteins via this pathway involves two discrete and successive steps: 1) recognizing and labelling damaged or misfolded proteins by conjugation with ubiquitin molecules and 2) degradation of ubiquitin-conjugated proteins by the 26S proteasome, a multicatalytic

enzyme complex (Glickman and Ciechanover, 2002). Activity of the UBI-proteolytic pathway depends upon coordinated interactions among a number of enzyme families (as shown in figure 2.1). The first step uses ATP to combine ubiquitin to an ubiquitin-activating enzyme (E1) via a high-energy thiolester bond. Activated ubiquitin is transferred to an ubiquitin carrier protein (E2). Finally, E2 works together with ubiquitin ligases (E3s), which inturn transfers the activated ubiquitin to the damaged and or misfolded proteins (substrate), thus marking the protein substrate for degradation (Reid, 2005).

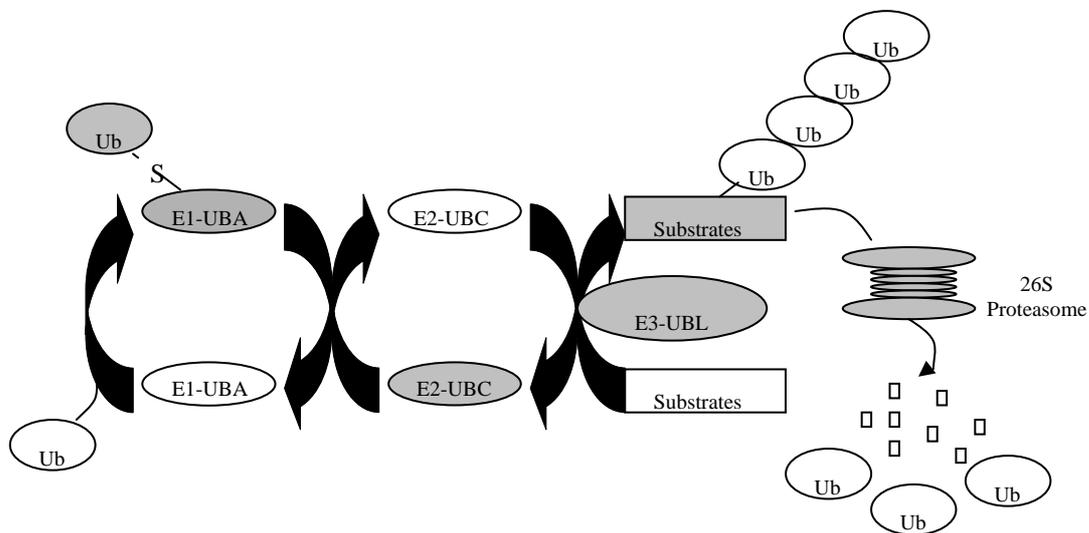


Figure 2.1. The Ubiquitin-Proteasome pathway (modified from Reid *et al.*, 2005).

Ubiquitin upregulation is well documented in response to exercise, in particular eccentric exercise (Thompson and Scordilis, 1994; Willoughby *et al.*, 2000; 2003). Thompson and Scordilis (1994) examined changes in ubiquitin levels in human bicep muscles 2 days following an intense eccentric-isokinetic damaging protocol. Both free and conjugated ubiquitins were significantly increased in the exercised arm compared to control. While specific mechanisms responsible for the observed increase could not be elucidated from this study, it was one of the earliest studies to show that a stress

response involving ubiquitin occurs following eccentric exercise (Thompson and Scordilis, 1994).

In support of this, researchers have shown an increase in serum cortisol (the main stress hormone) after eccentric exercise (Malm *et al.*, 2000). Moreover, these increases lead to an up-regulation of skeletal muscle glucocorticoid receptors, and subsequently, via the ubiquitin-proteasome system, an increase in myofibrillar proteolysis (Kayali *et al.*, 1987; Auclair *et al.*, 1997). Recently, Willoughby and colleagues (2003) demonstrated a decrease in muscle strength and myofibrillar proteins 24-hours post eccentric exercise possibly due to an up-regulation of glucocorticoid receptor-mediated increases in UBI-proteolytic pathway activity.

Therefore, though the principle mechanism contributing to the immediate changes in protein degradation after injury is the activation of the non-lysosomal protease calpain (Belcastro *et al.*, 1998), activation of the UBI-proteolytic pathway may be essential in protein turnover occurring several days post-exercise.

2.1.3.2 Inflammatory Cells: The Role of Neutrophils and Macrophages

Leukocytes, primarily neutrophils and monocytes/macrophages are thought to perform a wide range of functions within the muscle damage and repair cycle (Tidball, 1995).

These include:

- Attack and breakdown of debris (neutrophils and macrophages);
- Removal of cellular debris (macrophages); and
- Regeneration of cells (macrophages)

Circulating neutrophil populations generally peak within 10 hours of the initial injury in humans. The neutrophil is one of the first cells to arrive at the site of injury, where it releases a number of chemo-attractants to enhance the response by recruiting additional neutrophils and mononuclear cells (Kendall and Eston, 2002). Current findings show that neutrophils are capable of direct lysis of muscle cell membranes via respiratory burst, which is mediated through a superoxide-dependent mechanism (Nguyen and Tidball, 2003). Superoxide can be converted to hydrogen peroxide (Hampton *et al.*, 1998) and finally myeloperoxidase (MPO) (Winterbourn, 1986). With limited intrinsic ability to distinguish between foreign and host antigens, both healthy as well as damaged cells and debris are destroyed (Pyne, 1994). Hence, it has been suggested that neutrophils are programmed for mass destruction not caution (McCord, 1995).

It is unlikely that neutrophil-generated superoxide causes direct membrane damage. Superoxide is only a mild oxidant that can be readily removed by reaction with other free radicals or by conversion to hydrogen peroxide by superoxide dismutase (SOD) (Hampton *et al.*, 1998). Hydrogen peroxide is a stronger oxidant than superoxide and has the capacity to peroxidise lipids and damage cell membranes (Hampton *et al.*, 1998). It is suggested that this reduction-oxidation environment within the muscle determines the extent to which neutrophils cause damage (Tidball, 2005). Thus, increasing the antioxidant defences of the muscle may attenuate the effect of the neutrophils in the degeneration process, although results are inconclusive.

At present, the contribution of macrophages to the destruction of damaged tissue after injury or modified muscle use is poorly understood. Compared to neutrophils, macrophages are a greater source of diverse growth factors, free radicals and cytokines, and therefore their role in influencing the course of muscle injury and remodelling are

more complex. Two cell populations have been observed within animal muscle: ED1+ cells, which act as phagocytes, initiating removal of cellular debris; and ED2+ cells, which help regulate the consequent repair process (Tidball, 1995).

Recently, cytotoxicity assays have shown that macrophages break down their target muscle cells by a nitric oxide (NO)-dependent, superoxide-independent mechanism (Nguyen, 2003). The presence of damaged muscle cells increases NO production by macrophages, leading to further muscle cell breakdown, thus, creating a positive feedback mechanism, in which the initial damage promotes increases in NO-mediated toxicity by macrophages (Nguyen, 2003).

It is clear that muscle injury is characterised by neutrophil and macrophage infiltration into the muscle, and subsequent inflammatory processes; all of which promote free radical and reactive oxygen species activity (ROS) (Tiidis, 1998)

2.1.3.3 Free Radicals and Reactive Oxygen Species

Free radicals (species which contain one or more unpaired electrons within their outer orbital) and other reactive oxygen species (ROS) are increasingly gaining recognition for their roles in cellular damage (for a review see Armstrong, 1990). Given the highly reactive nature of ROS and free radicals, the body has developed numerous enzymatic and non-enzymatic antioxidant compounds, which serve to stabilize these reactive species. The major sources of free radicals and ROS include the mitochondrial electron transport system; membrane bound oxidases, infiltrating phagocytic cells, cytochrome P-450, and various catalytic enzymes (Yu, 1994).

It is hypothesised that an important determinant of exercise-induced muscle damage is the increased production of ROS (Jackson and O'Farrell, 1993). During exhaustive physical exercise, flow of oxygen through muscles is greatly increased at high levels of oxygen uptake. At the same time, the rate of ATP consumption exceeds the rate of ATP generation, causing electron leakage from the skeletal muscle mitochondria (Goodman *et al.*, 1997). This leads to rapid increases in the free radical levels, overwhelming the scavenger antioxidant systems, and therefore subjecting the cell to oxidative stress, leading to cellular damage.

However, since eccentric exercise requires less oxygen consumption, it is unlikely free radical and ROS production will be the primary cause of eccentric-induced muscle damage (as mentioned previously in section 2.1.1.2) Nonetheless, it is clear from literature that free radical and ROS production is increased during the muscle degeneration and inflammatory phase (Best *et al.*, 1999). In addition, ROS and free radicals may also indirectly initiate the ubiquitin-proteasome pathway, through activation of NF- κ B pathway, thus leading to increased proteolysis as mentioned in section 2.1.3.1 (Best *et al.*, 1999; Jackman and Kandarian, 2004; Kandarian and Jackman, 2005).

2.1.3.4 Macrophages: Their Role in Muscle Regeneration

While it is established that neutrophils and macrophages are capable of promoting muscle damage *in vivo* and *in vitro* through phagocytosis, release of free radicals, ROS, and digestion of necrotic tissue, it is suggested that macrophages may play a role in muscle repair and regeneration through growth factors and cytokine-mediated signalling (Tidball, 2002).

Lescaudron and colleagues (1999) investigated whether macrophages play a key role in the regenerative process of muscle injury. Treated mice were grafted with muscle transplants that had been conditioned with macrophage inflammatory protein 1- β (MIP 1- β), and vascular endothelial growth factor (VEGF), thus stimulating macrophage infiltration and angiogenesis. Mice treated with both MIP 1- β and VEGF exhibited and amplified monocyte-macrophage infiltration and satellite cell differentiation and/or proliferation compared to controls. Additionally, when mice were depleted with circulating monocyte-macrophages after whole body irradiation, no regeneration occurred in transplanted mice. Thus, it was suggested that the presence of blood-borne macrophages is required for triggering the earliest events of skeletal muscle regeneration (Lescaudron *et al.*, 1999).

Furthermore, Hurme and Kalimo (1992) investigated the onset of muscle precursor cell proliferation in rat muscle after crush injury. Bromodeoxyridine (BrDU), a thymidine analogue, was used as a nonradioactive marker for DNA synthesis. It was shown that DNA synthesis in muscle precursor cells began 24 hours after trauma. This was in agreement with autoradiographical studies of Shultz and Jarzyzak (1985) and Bischoff (1986), who found satellite cells labelled with ³H-thymidine, 15-20 hours and 18 hours, respectively, after crush injury in rats. Interestingly, BrDU positive cells were virtually never identified within the basal lamina cylinders of the disrupted myofibers before phagocytosis of the necrotic cells by macrophages had begun. This delay suggested that the activation of satellite cell proliferation is dependent on certain factors either synthesised or secreted by macrophages (Hurme and Kalimo, 1992).

Therefore, unless invasion of blood-borne macrophages occurs, damaged fibres will remain quiescent in the stage of intrinsic degeneration, and activation of satellite cells and regeneration will proceed no further.

Nonetheless, it still remains to be proven whether macrophage-derived factors are the critical component in muscle repair or regeneration *in vivo*, given that other cells present in muscles are also able to potentially generate regenerative factors. It is clear however, that inflammatory cells, in particular macrophages, are essential in several aspects of successful repair after injury, potentially through secretion of cytokine factors that regulate the satellite cell pool (Tsunawaki *et al.*, 1988; Tidball, 1995). Taken together, it is apparent that with the combined actions of $[Ca^{2+}]_i$ -mediated enzymatic activity and subsequent activation of the inflammatory response following injury, damaged skeletal muscle cells can be destroyed and removed and thus, regeneration can commence.

2.2 SKELETAL MUSCLE REGENERATION

The complexity of the regeneration process in repairing muscle tissue damage and subsequent formation of new muscle fibres has been explored carefully for many years (for a review see Charge and Rudnicki, 2004). Mature muscle is composed of long cells that contain many nuclei. To re-grow or recover from trauma, these mature cells cannot divide; so additional nuclei have to come from another source. Located close to the mature muscle cells are a population of stem cells, known as satellite cells. In response to high-strain exercise or a trauma, satellite cells rapidly replicate and become active to prepare for the formation of new muscle cells (myofibres) or new portions of surviving muscle fibres. The regeneration process begins simultaneously with satellite cell activation and the inflammatory phase (as previously described in section 2.1.3). Studies suggest that muscle regeneration can be considered in four stages (1) satellite-

cell activation (2) myoblast or precursor proliferation, (3) differentiation and, (4) return to quiescence (resting state) (Wozniak *et al.*, 2005). In addition, with subsequent nerve innervation and formation of scar tissue, optimal recovery of contractile function will be achieved (Jarvinen *et al.*, 2005)

2.2.1 Muscle Satellite Cells

Adult muscle cells contain a population of quiescent undifferentiated monocuclear myogenic cells located between the sarcolemma and basal lamina of the muscle fibre (Bischoff, 1994). Satellite cells are found in various species including mammalian (Gamble *et al.*, 1978), reptilian (Kahn and Simpson, 1974), avian (Hartley *et al.*, 1992), and amphibian (Popiela, 1976) skeletal muscles.

Since their first description in the early 1960s, the discovery of muscle satellite cells have confirmed the existence of population of proliferative cells that contribute to postnatal growth, repair of damaged fibres and maintenance of adult skeletal muscle (Mauro, 1961). Satellite cells are present in all skeletal muscles; however, satellite cell population is dependent on species, fibre type composition and age (Hawke and Garry, 2001). For example, researchers have shown that the percentage of satellite cells in adult slow-twitch soleus muscle is two- to three-fold higher than in adult fast-twitch tibialis anterior or extension digitorum longus muscles (Gibson and Schultz, 1982; Snow, 1983).

In mouse skeletal muscle, satellite cells account for 30% of sublaminar muscle nuclei at birth. This number rapidly declines to less than 5% as mice approach 2 months of age (Bischoff, 1994). Following sexual maturity, the total number of satellite cells continues to decrease, although at a greatly reduced rate (Charge and Rudnicki, 2004).

Interestingly, it has been shown that following several cycles of degeneration and regeneration, satellite cell population is maintained in adult muscle, thus suggesting potential mechanisms that may stimulate satellite cell self-renewal following muscle degeneration (Schultz and Jaryszak, 1985). Indeed, Shultz (1996) investigated this phenomenon, by examining the cell cycle time of satellite cells in growing rats. Using a continuous infusion of BrdU, Shultz (1996) showed that approximately 80% of satellite cell population was readily labeled over the first 5 days, indicating mitotic activity. However, only a small portion of the cells labelled with BrdU during the first 5 days could be labelled with a second label ([³H]thymidine) during tandem continuous infusion experiments, and thus suggests that there may be a limited number of mitotic divisions prior to myoblastic fusion (Shultz, 1996). Researchers hypothesized that two satellite populations might exist in growing rats. The first, representing ~80% of satellite cells, characteristically divide rapidly and are responsible for providing myonuclei to growing fibres; while the remaining 20%, known as the “reserve cells”, divide more slowly and thus may replenish the satellite cell pool (Shultz, 1996).

2.2.2 Satellite Cell Activation

The activation of satellite cells upon muscle injury resulting from eccentric contractions, crush injury, or in the course of disease, is well established (for a recent review see Wozniak *et al.*, 2005). Various stimuli have been proposed as initiators of satellite cell activation; such as extracts from injured muscle fibres, factors released from macrophages (as mentioned in section 2.1.3.4), and soluble factors released from damaged connective tissue (Grounds, 1999). Satellite cell activation is not restricted to the damaged site. Indeed, damage at one end of a muscle fibre will activate satellite cells all along the fibre leading to proliferation and migration of satellite cells to the regeneration site (Shultz *et al.*, 1985). Following proliferation, inactivated (quiescent)

satellite cells are restored underneath the basal lamina by the previously mentioned “reserve cells” for subsequent regeneration cycles (Shultz *et al.*, 1985).

2.2.2.1 The Cell Cycle

In short, the cell cycle is a series of changes that a cell undergoes in order for cell division. Satellite cells are in state of quiescence (G_0) under normal circumstances and must be recruited by re-entering the cell cycle in response to injury. With the appropriate signal, quiescent cells (G_0) enter the first growth phase (G_1). The cell then enters the S phase, in which growth continues, in addition to DNA synthesis and replication. G_2 is the final phase preparing the cell for division (M phase).

2.2.2.2 Experimental Methods Used to Identify Activated Satellite Cells

Previously, ultrastructural techniques were used to observe morphological and cell content changes in satellite cells following activation. Quiescent satellite cells are spindle-shaped, have little cytoplasm, and contain few organelles (Shultz and McCormick, 1994); in contrast, once activated, satellite cells display hypertrophied organelles and an extended cytoplasm (Anderson, 2000). Several cell markers have been utilized to detect satellite cells in either their quiescent, activated, or proliferative state. Initial experimental methods used histochemical analysis to investigate satellite cell activity. Uptake of tritiated thymidine (McGeachie *et al.*, 1993), or thymidine analogues such as BrDU (Hurme and Kalimo, 1992; Brotchie *et al.*, 1995), into the DNA of mitotically active cells was used to examine early activity of satellite cells. Although a very simple, quick and useful method, satellite cells need to enter the S phase of the cell cycle for this incorporation to occur, and therefore this method was not sensitive enough to detect initial activation. Similarly, identification of a proliferating cell nuclear antigen (PCNA), which appears during cells in DNA synthesis (Silvestrini

et al., 1995), was also used as a marker for prior activation (Johnson and Allen, 1993). Once more, DNA synthesis was used as a marker of earlier activation (Wozniak *et al.*, 2005), and hence, initial activation of satellite cells was not investigated.

However, researchers are currently using the co-localisation of c-met with its ligand, hepatocyte growth factor (HGF) as a marker of initial satellite cell activation (Anderson, 2000). C-met is a specific marker of satellite cells, independent of its activation state. Binding of HGF with the c-met receptor signals entry of satellite cells into the cell cycle and thus mobilization from G₀ state. Other molecular markers that are expressed in all three states; quiescent, activated, or proliferative include: myocyte nuclear factor (MNF) (Garry *et al.*, 1997); Pax7 (Seale *et al.*, 2000); and adhesion molecules such as M-cadherin (Cornelison and Wold, 1997), NCAM (Covault and Sanes 1986), and VCAM-1 (Jesse *et al.*, 1998). In contrast, molecular markers desmin (Bockhold *et al.*, 1998) myf5 and MyoD (Cornelison and Wold, 1997) are only expressed during activation and proliferation.

Isolating and identifying molecular cell markers such as those mentioned has provided researchers with a greater insight into the role of specific growth factors that are released during muscle regeneration, which ultimately regulate satellite cell activity and proliferation.

2.2.2.3 Growth Factors and Muscle Regeneration.

In vitro studies have implicated an extensive number of trophic factors involved in maintaining a balance between growth and differentiation of satellite cells to restore normal muscle architecture (for a review see Wozniak *et al.*, 2005). These include members of the fibroblast growth factor (FGF) and transforming growth factor- β (TGF-

β) families, insulin-like growth factor (IGF), hepatocyte growth factor (HGF), tumour necrosis factor-α (TNF-α), IL-6 family of cytokines, neural derived factors, nitric oxide (NO) and ATP. In contrast, *in vivo* studies have demonstrated only a few of these factors to have a physiological role in skeletal muscle regeneration.

2.2.2.4 HGF, C-Met and Satellite Cell Activation.

Hepatocyte growth factor is one of the primary effectors of satellite cell activation via the c-met receptor (Allen *et al.*, 1995). HGF is made by fibres and stored or sequestered in the surrounding extracellular matrix (Tatsumi *et al.*, 1998). Early studies used single muscle fibres to investigate satellite cell activation. This model was suggested to be the best, as satellite cells remained in a quiescence state *in vivo* unless stimulated by treatment (Allen *et al.*, 1997). *In vivo*, HGF activates satellite cells and enhances muscle repair (Tatsumi *et al.*, 1998; Miller *et al.*, 2000). The binding of HGF to c-met initiates numerous signalling cascades including the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3 kinase (PI-3K) pathways (Furge *et al.*, 2000), all of which positively influence the transcription of genes required for growth and cell division (Vermeulen *et al.*, 2003).

In contrast, peptide growth factors including IGF-I (Yan *et al.*, 1993; Lee *et al.*, 2004), FGF (Yamada *et al.*, 1989), and potentially platelet-derived growth factor (PDGF) are not demonstrated to induce satellite cell activation *de novo* from the quiescent state, but rather stimulate and influence satellite cell proliferation and differentiation once activated (Johnson and Allen, 1995). However, while IGF-I is often demonstrated to activate satellite cells, those studies in general use cultures of muscle cells that are not in a quiescent state (Papy-Garcia *et al.*, 2002), and thus, are already activated prior to IGF-1 influence.

2.2.2.4.1 Nitric Oxide (NO) and Satellite Cell Activation.

Recently, Anderson (2000) has suggested that NO directly mediates normal activation of satellite cells through the HGF/c-met colocalization. Previous literature has primarily focused on the role of NO as an endothelium-derived relaxation factor (Ignarro *et al.*, 1988). In muscle, NO is produced by nitric oxide synthase-I μ (NOS-I μ), a skeletal muscle-specific isoform of neuronal NOS, as one product of the conversion of L-arginine to citrulline (Moncada and Higgs, 1993). NO synthesis and release are regulated by tissue structure and mechanical forces, such as shear force (where muscle layers shift past one another) (Lancaster, 1996). After injury, satellite cells often stay attached to the external lamina even though the sarcolemma collapses (Schultz and McCormick, 1994). As a result, it is hypothesized that they are ideally positioned to respond to shear-induced NO release from NOS-I μ (Anderson, 2000).

An *in vivo* study in mice has showed that systemic exposure to a non-specific NOS inhibitor, N-nitro, L-arginine methyl ester (L-NAME), just prior to crush injury, prevented immediate injury-induced myogenic cell release and delayed the hypertrophy of satellite cells (Anderson, 2000). The tibialis anterior muscles were sampled from mice injected with either the NOS inhibitor L-NAME or saline (control). Crush injury was applied to the right leg, while the contralateral leg was used as the control. Both legs were excised either immediately after injury (n=2 mice) or 10 min later (n = 2 mice). Results demonstrated satellite cell hypertrophy, colocalization of HGF with c-met and satellite cell activation in the injured muscle of saline treated mice at 0 min and 10 min, but these effects were delayed 10 min or more by the L-NAME treatment. It was concluded that satellite cells show rapid, NO-dependent cell and organelle

hypertrophy, co-localization of HGF and c-met proteins, and reduced adhesion to fibres (Anderson, 2000).

Indeed, other observations, including acute treatment with L-arginine, the NOS substrate and satellite cell cultures subjected to mechanical stretching, support the proposed theory that NO is the preferred pathway to HGF activation (Tatsumi *et al.*, 2002). Conversely, results from NOS^{-/-} mice, which lacks NOS-I μ , have suggested that there may be an alternative pathway in HGF activation (Anderson, 2000). Regardless of whether release of NO initiates the release of HGF from the extracellular matrix, or NO release is bypassed and HGF is released through a NO-independent mechanism, the final result is HGF binding to c-met, satellite cell activation, and thus, cell proliferation, differentiation and formation of new fibres.

2.2.2.5 The Role of Myogenic Regulatory Factors

In addition to trophic factors that indirectly mediate satellite cell activity through specific signalling pathways (for example PI-3K pathway; Coolican *et al.*, 1997), myogenic regulatory factors (MRFs), directly control myogenic cell differentiation and proliferation due to their ability to directly bind to DNA, hence initiating DNA transcription and regulating gene expression. MRFs, which include Myo-D, myogenin, MRF-4, and Myf5, are members of a family of basic helix-loop-helix proteins. In general, Myo-D and Myf5 are involved in the determination of myoblasts during proliferation, whilst myogenin and MRF-4 are involved in the later stages of adult fibre differentiation. Gene knockout experiments have shown that MyoD and Myf5 play a redundant role in myogenesis. Deletion of either gene alone has no effect on muscle development, whereas deletion of both genes results in the complete absence of skeletal muscle (Braun *et al.*, 1992; Rudnicki *et al.*, 1993). Once proliferating myoblasts

withdraw from the cell cycle and become terminally differentiated myocytes, they express the myogenin and MRf4 and subsequent muscle-specific genes such as myosin heavy chains (MHC), myosin light chains, α -actin, troponin-1, and muscle creatine kinase (MCK) (Charge and Rudnicki, 2004). Additionally, Myo-D and myogenin have also been implicated in regulating muscle fibre type, as myogenin and MRF-4 are expressed higher in slow-twitch fibres (Walters *et al.*, 2000), whereas Myo-D is expressed higher in fast-twitch fibres (Lowe *et al.*, 1998).

2.2.2.6 Formation of New Fibres

The extent of proliferation appears to be related to the severity of injury and cell death, as cell numbers must be replaced by satellite cells differentiating into primitive myoblast cells. With the removal of the original muscle fibre as a result of the inflammatory phase, the rapid regeneration of a new muscle fibre begins within the persisting basal lamina of the original muscle fibre. The newly differentiated population of myoblastic cells organise themselves beneath the old basal lamina, and begin to fuse together into elongated multinucleated structures called myotubes (Carlson and Faulkner, 1983), which replace entire damaged fibres. Alternatively, myoblasts can replace damaged portions of surviving fibres by spanning gaps between healthy portions of muscle fibres with removal of cell debris (Hurme *et al.*, 1991).

The multinucleate myotubes then commence synthesis of their own contractile protein filaments and subsequently the assembly of contractile proteins into regularly arranged myofilaments (Fischman, 1970). Eventually, the regenerating myotubes begin to produce their own basal lamina beneath the original one. New nuclei are added to the myotube by fusion of additional myoblasts. The contractile proteins of the myotubes undergo a process of self-assembly into myofibrillar units starting at the periphery of

the myotube. As the myotube matures, and contractile proteins occupy a greater proportion of its volume, the nuclei become more compact, the nucleoli lose their prominence. Eventually, the nuclei become peripherally located, and the differentiating myotubes become organised into the sarcomere structure of mature, functional myofibres, thus completing muscle restoration.

The early stages of muscle regeneration appear to take place in the absence of nervous input. Conversely, non-innervated fibres in the later stages of muscle regeneration lead to atrophy, as shown in animal models (Mong, 1977). Thus, fibre innervation and formation of neuromuscular junctions within myofibres in the later stages will ensure that regenerating fibres mature and develop maximum tension, therefore restoring optimal functional capacity (Carlson and Faulkner, 1983).

Given that regeneration includes the formation of myofilaments, it is clear that regulation of protein synthesis, in particular contractile proteins is essential in myofibre development and thus complete functional recovery of skeletal muscle from injury. The regulation of protein synthesis includes initiation, elongation and termination (Bergmann and Lodish, 1979). However, compared to the latter two potential points of control, initiation appears to be the rate-limiting step in the overall regulation of protein synthesis (Sonenberg, 1994). The rate of initiation of protein synthesis can be regulated directly or indirectly through a number of multiple signal transduction pathways (see figure 2.2). In brief, Akt/PKB serves as a branch point in the P13K-mTOR signalling pathway which in turn leads to rapid phosphorylation of 4E-BP1 and p70^{S6k}, and thus increased translation of mRNA encoding specific proteins (Bolster *et al.*, 2004). In addition, activation of Akt/PKB controls glycogen synthase kinase (GSK), which in turn causes activation of eukaryotic initiation factor (eIF)2B, and thus increased global

protein synthesis, and cell growth. Whilst a comprehensive review of these pathways is beyond the scope of this thesis (for review see Bolster *et al.*, 2004), they need to be mentioned, as certain factors (insulin, exercise and amino acids) appear to activate these signal transduction pathways, and thus up-regulate protein synthesis. Furthermore, activation of these pathways may assist in explanation of results obtained in the studies described in later chapters.

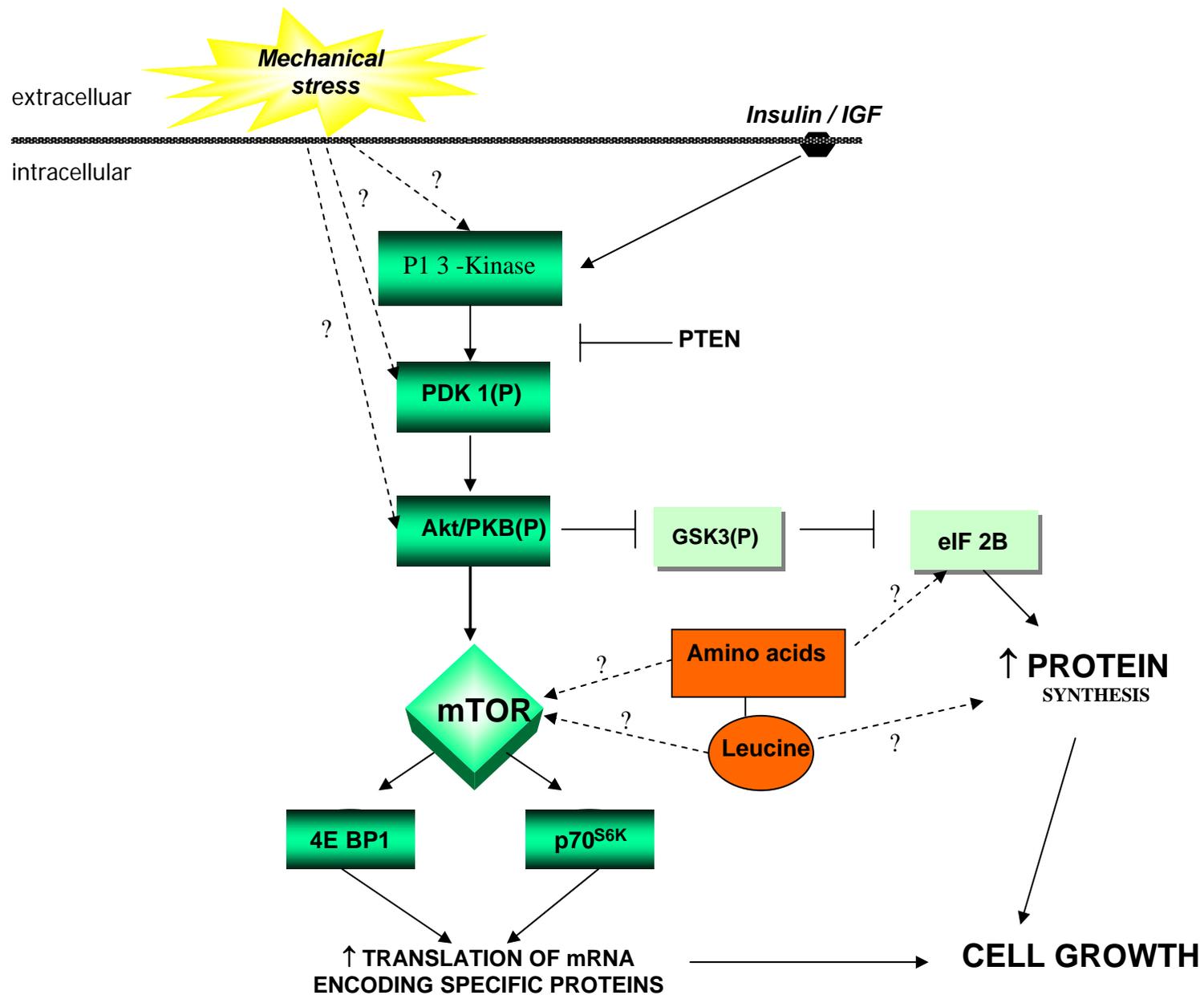


Figure 2.2. Components of the phosphatidylinositol (PI) 3-kinase-mammalian target of rapamycin (mTOR) signalling pathway (modified from Bolster *et al.*, 2004).

Possible pathways by which amino acids activate PI 3-kinase-mTOR signal transduction pathway leading to enhanced muscle protein synthesis and hypertrophy.

← ←---- Activating steps; |—— inhibitory steps in pathway. PDK1, phosphatidylinositol-dependent protein kinase 1; P, phosphate; PKB, protein kinase B; GSK3, glycogen synthase kinase 3; eIF2B, eukaryotic initiation factor 2B; 4E-BP1, eIF4E-binding protein 1; p70^{S6K}, 70kDa ribosomal protein S6 protein kinase.

The processes involved in the muscle damage and repair cycle can be summarised in figure 2.3. Although the area of muscle damage and regeneration has been extensively studied, there still remains many questions unanswered, most likely due to the numerous and complex interactions occurring between processes throughout each stage of the damage and repair cycle, as illustrated in figure 2.3. Nevertheless, it is hypothesised from current literature, that disturbance in the regulation of intracellular Ca^{2+} concentration and changes in the rate of protein degradation will eventually result in skeletal muscle damage and therefore impaired functional capability (Belcastro *et al.*, 1998; Gissel and Clausen, 2001). Thus, enhancing the rate at which muscles recover following injury may involve reducing the initial amount of damage by improving the intracellular Ca^{2+} handling ability of the muscle, or increasing the rate of regeneration by enhancing muscle protein synthesis. Two dietary supplements that may theoretically enhance the rate at which muscles recovery from injury via these proposed mechanisms are whey protein isolate and creatine monohydrate. Whilst these two dietary supplements are well known to athletes and consumed regularly as anabolic ergogenic agents, few controlled scientific studies have investigated the efficacy of these dietary supplements in muscle recovery from injury.

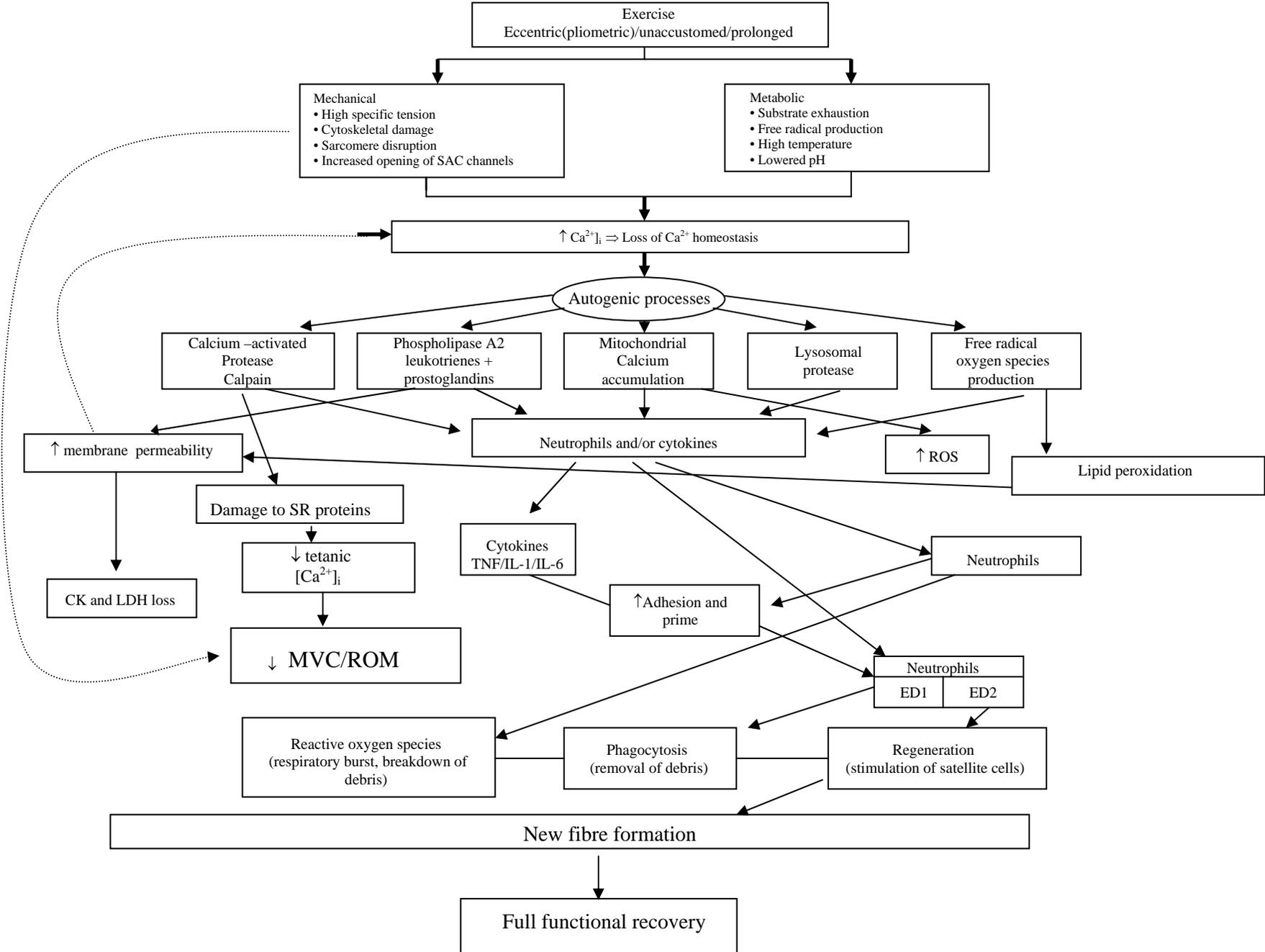


Figure 2.3. Schematic view of the muscle damage and repair cycle (modified from Kendell and Eston, 2002 Allen *et al.*, 2005). TNF/IL-1 =tumor necrosis factor/interleukin-1, MVC/ROM = Maximum voluntary contraction/Range of motion, SAC =Stretch activated channel, CK = Creatine kinase, LDH= Lactate dehydrogenase.

2.3 DIETARY SUPPLEMENT WHEY PROTEIN

Dietary proteins, in particular branched chain amino acids (BCAA), have an important role in the regulation of protein metabolism in skeletal muscle (Evans, 2001; Borsheim *et al.*, 2002; Karlsson *et al.*, 2004). As mentioned previously, it is well established that eccentric exercise produces ultrastructural changes that stimulate muscle protein synthesis and degradation. However, degradation usually exceeds synthesis, thus creating a negative net muscle protein balance, leading to muscle protein breakdown and damage (Evans, 2001). Therefore, a nutritional supplement that can achieve a positive net muscle protein balance after eccentric exercise has the potential to improve muscle recovery after injury.

Whey protein (or as it is sometimes referred to, whey isolate or concentrate) is a collective term for the soluble protein fractions extracted from dairy milk. Whey protein has become an increasingly popular sport supplement; with the estimated total supply market valued at approximately \$470 million in the United States in 2003, and this figure expected to increase each year. As a result, research into dairy protein supplementation as an ergogenic aid has received an increasing amount of attention in recent years (Demling and DeSanti, 2000; Antonio *et al.*, 2001; Burke *et al.*, 2001; Fry *et al.*, 2003; Chromiak *et al.*, 2004; Tipton *et al.*, 2004; Anderson *et al.*, 2005; Cribb *et al.*, 2006). However, few studies have examined the effects of whey protein supplementation on muscle recovery after injury.

2.3.1 Whey Protein and Muscle Protein Synthesis

Stimulating protein synthesis and minimizing protein breakdown (proteolysis) are the two cellular processes that are essential for muscle recovery after damage (Rennie and Tipton, 2000). It is well established that increased protein synthesis rates within the

muscle is vital for muscle regeneration, in particular satellite cell proliferation, which requires an enhanced rate of myofibrillar protein synthesis.

Whey protein is effective at stimulating muscle protein synthesis for a number of reasons. Firstly, whey protein has been shown to exhibit the highest biological value of any known protein (Renner, 1983). Biological value (BV) is the most accurate method of assessing the quality of a protein, as it is a measure of the protein's ability to retain nitrogen in the muscle (Colgan, 1993). Nitrogen metabolism regulates the nitrogen balance in the body through a series of metabolic events. For example, a negative nitrogen balance is associated with muscle catabolism, whereas a positive nitrogen balance is associated with muscle anabolism. Whey protein has a BV score of 104 whereas casein, another milk protein, beef and fish demonstrate a BV score of 77, 75, and 75, respectively (Renner, 1983). Therefore, achieving a positive nitrogen balance after whey protein supplementation may enhance muscle protein synthesis, thus creating an environment that facilitates muscle anabolism, but more importantly improved muscle recovery after injury.

Secondly, compared to other protein sources, whey protein contains a higher dose (45-55mg/100gms) of essential amino acids (those that cannot be synthesized by the body) (Bucci and Unlu, 2000). Essential amino acids are shown to be the most effective at stimulating protein synthesis and therefore promoting muscle growth in adult muscle (Volpi *et al.*, 2003). Furthermore, the ability of a protein to stimulate muscle protein synthesis may reside in the composition of amino acids (Wolfe, 2000). Whey proteins amino acid profile is almost identical to that of skeletal muscle, providing close to all of the correct amino acids in approximate proportion to their ratio in muscle (Wolfe, 2000). Whilst non-essential amino acids contribute little to the overall response to

protein synthesis, one might suggest that this compatibility would position whey as an effective anabolic agent (Ha and Zemmel, 2003).

Finally, there has been increased interest into the branched chain amino acid leucine found in high concentrations in whey protein isolate. Studies have shown that leucine may directly stimulate protein synthesis by activating an additional intracellular signalling pathway, in particular the mTOR pathway (Kimball, 1999). This leads to phosphorylation of p70^{S6K} and thus, increased translation of mRNA encoding specific proteins (Anthony *et al.*, 2001).

Recently, Karlsson *et al.* (2004) investigated the effects of branched chain amino acids (BCAA: leucine, isoleucine and valine) on p70^{S6K} phosphorylation and hence, protein synthesis activity, after resistance training. In a double-blind, crossover test, seven male subjects performed one session of quadriceps muscle resistance training on two occasions. Subjects consumed a solution of BCAA or placebo (flavoured water) during and after exercise. Ingestion of BCAA increased plasma leucine, isoleucine and valine during exercise and throughout the 2 hours post exercise (recovery), whereas placebo had no effect. While resistance exercise led to an increase in p70^{S6K} phosphorylation, which persisted 1 and 2 hours after exercise, BCAA ingestion further enhanced p70^{S6K} phosphorylation 3.5-fold during recovery. The major finding of this study was a significant increase in site-specific phosphorylation and activation of p70^{S6K} in skeletal muscle after ingestion of BCAA during and after acute resistance exercise. Therefore, since the branched chain amino acid leucine is found in high concentrations in whey protein isolate, it could be suggested that the anabolic effectiveness of whey protein may, in part, be positively related to the abundance of leucine.

2.3.2 Muscle Building Properties of Whey Protein

Previous research have also indirectly investigated whey proteins ability to stimulate and/or influence muscle protein synthesis by examining whey's efficacy on muscle building properties, since muscle rebuilding (regeneration) after injury is associated with enhanced protein synthesis rates, in particular myofibrillar proteins, while minimizing protein breakdown. The key to whey's unique muscle building properties seems to be related to a number of factors, which include the amount of protein that remains undenatured during processing, cysteine, and intramuscular glutamine levels.

2.3.2.1 Undenatured Protein During Processing

Aside from amino acid profiles of whey protein, the absorption characteristics and processing methods used during the manufacture of a protein supplement (i.e., degree of isolation and or hydrolyzation) are also thought to have an impact on muscle anabolism and protein synthesis (Lemon *et al.*, 2002). It has been shown that whey peptides are absorbed intact in the upper jejunum and in greater amounts than other proteins due to their different gastrojejunal kinetics (Mahe *et al.*, 1996). Grimbale *et al.* (1986) and Mahe *et al.* (1996) have demonstrated that the absorption and nitrogen retention abilities of whey protein can be enhanced by shortening peptide chain length by specific enzyme-only hydrolysis (Grimble *et al.*, 1986). Similarly, a number of studies have reported an increase in nitrogen incorporation into tissue protein in animals fed hydrolysed whey peptides compared with those receiving the same amount of nitrogen as whole protein or free amino acids (Poullain *et al.*, 1989; Boza *et al.*, 2000). In humans, a partially hydrolysed whey protein was shown to be absorbed faster and induce a higher rate of protein synthesis compared to casein (a whole protein) (Boirie *et al.*, 1997; Dangin *et al.*, 2001; 2003).

However, whether hydrolysis has a practical effect such as faster muscle mass accretion or improved recovery from damage has not been adequately studied (Manninen, 2004). Furthermore, while whey protein increases blood amino acids more rapidly than casein in the first 2 hours, casein has been shown to elevate it for longer than 24 hours, and thus may also have practical implications during days following injury, although this concept was not examined in this thesis.

2.3.2.2 Cystine Levels

Whey protein contains a rich source of cystine. Whey protein generally contains a 3- to 4- fold higher concentration of cyst(e)ine (cysteine and its disulfide twin cystine) compared to other protein sources (Bucci and Unlu, 2000). Per 100gms of protein, whey contains about 2.8gms of cystine, whereas casein contains only 0.3gm (Renner, 1983). An abundant supply of cyst(e)ine in the blood is necessary for hepatic catabolism of cyst(e)ine into protons and sulfate; a process that inhibits carbamoylphosphate synthesis (the first and rate limiting step of urea biosynthesis) (Droge and Holm, 1997). This process down-regulates urea production, promotes glutathione synthesis and shifts whole body nitrogen disposal in favor of preserving the muscle amino acid pool (Hack *et al.*, 1997). In humans, supplementation with whey protein (20grams/day or 1gm/kg/body weight/day) is shown to augment this pathway (Lands *et al.*, 1999; Middleton *et al.*, 2004). In rodents, whey protein is shown to augment this pathway of protein metabolism in a dose-dependant manner (Marriotti *et al.*, 2004).

As a result, it is suggested that cystine levels may be critical in maintaining or increasing muscle mass (Kinscherf *et al.*, 1996). Resistance training is normally associated with gains in muscle mass and strength. Conversely, in a double-blind, randomised trial of 46 athletic men, muscle loss, due to a decrease in plasma cystine and

glutamine levels, was seen in healthy males undertaking an intense resistance-training program for 8 weeks. However, these results were shown to be prevented in an identical group that received an oral NAC (N-acetyl-L-cysteine) supplement. Kinscherf *et al.* (1996) demonstrated an increase in muscle mass, decreased body fat, and maintenance of plasma glutamine and cysteine levels during training after NAC supplementation. In addition, supplements rich in cystine residues are able to prevent muscle loss and trigger increases in muscle mass in conditions of muscle wasting (Akerlund *et al.*, 1996).

2.3.2.3 Muscle Glutamine Levels

Whey protein has also been shown to increase intramuscular glutamine levels, which have been directly linked to influencing muscle cell volume (Low *et al.*, 1996), which enhances protein synthesis, and increases muscle size (Haussinger *et al.*, 1993). This was supported by a recent study by Boza *et al.*, (2000), who demonstrated that plasma and muscle free glutamine concentrations, as well as weight gain, was higher in whey protein diets compared to free amino acid-based diets in starved rats.

Interestingly, plasma glutamine concentrations decline under intense exercise or injury (Keast *et al.*, 1995), as glutamine is utilised at a high rate by lymphocytes and macrophages (Newsholme, 1994) during the inflammatory phase. For this reason, it has been suggested that plasma glutamine is a "metabolic link" between skeletal muscle and the immune system (Newsholme, 1994). More importantly, since inflammation is a necessary component for complete muscle restoration after injury, plasma glutamine may be essential for the muscle cells to regenerate promptly. Therefore, increasing glutamine levels within skeletal muscle after whey protein supplementation may ensure that the inflammatory response will be enhanced, and thus muscle regeneration

improved. Furthermore, since macrophages are associated with satellite cell activation, increased plasma glutamine level, may indirectly increase satellite activation.

2.3.2.3.1 Muscle Glutamine and Oxidative Stress

Glutamine is an important amino acid precursor of glutathione. Glutathione (GSH) is the most abundant short-chain peptide in cells (more than 95% in reduced form GSH). GSH levels appear to play a pivotal role in the metabolic and cell-cycle related functions in virtually all cells, as well as being central in the defence mechanisms against intra- and extra-cellular oxidative stress (Cotgreave and Gerdes, 1998).

A recent study by Micke *et al.* (2001) demonstrated that short-term (14 days) oral supplementation with whey protein increased plasma glutathione levels in glutathione deficient patients with advanced HIV-infection. The study evaluated the effect of oral supplementation with two different cysteine-rich whey protein formulas on GSH levels, parameters of oxidative stress (i.e. the release of superoxide anion by blood monocytes) and immune status in HIV-infected patients. Thirty patients with stable HIV-infection were randomized to a daily dose of 45gms of either protectamin or immunocal (both whey protein formulas with different amino acid content) for two weeks. Results showed that after two weeks of oral supplementation with whey proteins, plasma GSH levels increased in the protectamin group, while the difference in the immunocal group did not reach significance, however was higher (~25%) than the baseline values. However, with low sample numbers, it is difficult to determine the 'biochemical efficiency' of whey protein isolate in a diseased state and whether oral whey proteins can influence glutathione concentrations in muscle tissue. Nevertheless, it was suggested that whey proteins are an effective, well-tolerated means of increasing plasma

glutathione levels in glutathione-deficient patients with advanced HIV-infection (Micke *et al.*, 2001).

2.3.2.3.2 Muscle Glutamine and Ca²⁺ Uptake

In addition to glutathione's role as an antioxidant, researchers have recently suggested that GSH can be intimately involved in Ca²⁺ homeostasis by directly influencing Ca²⁺ release, but more importantly, Ca²⁺ uptake (Belia *et al.*, 2000). It is well known that the presence of mM concentration of GSH in several tissues can positively influence Ca²⁺-ATPase activity (Freitas *et al.*, 1996; Viner *et al.*, 1996). Researchers have suggested that the redox status of glutathione is a fundamental pre-requisite for inhibiting Ca²⁺ release and activating Ca²⁺ uptake (Belia *et al.*, 2000). Belia and colleagues, (2000) demonstrated that GSH enhanced Ca²⁺ pump activity, but more importantly, this effect was not influenced by changes in Ca²⁺ concentration, which is of particular importance during periods of increased [Ca²⁺]_i, as evident during muscle injury. Indeed, unpublished work from our laboratory showed that whey protein was able to increase the rate of Ca²⁺ uptake in SR vesicles isolated from rat skeletal muscle. However, whether this was due to GSH concentration is unknown.

2.3.3 Research into Whey Protein Supplementation

Few research studies have investigated the effects of whey protein on exercise performance, and in particular muscle damage and recovery. Lands *et al.* (1999) demonstrated that an undenatured whey protein supplement was able to increase blood lymphocyte glutathione levels, increase muscle mass, and enhance athletic performance without any fitness training. The study used 18 healthy, untrained subjects to complete a double-blind placebo-controlled three-month study. Subjects supplemented their normal diet with either whey or casein supplements (1mg/kg body weight per day). The

subjects, pre- and post-supplementation completed an anaerobic performance cycling test, with no fitness training permitted between tests. Results showed that the whey supplementation enhanced sprint times and increased power output. At the end of the study, subjects lost body fat but weighed the same. Thus, the researchers suggested that this was evidence of an increase in lean body mass (Lands *et al.*, 1999). This was the first study to demonstrate both an improvement in muscular performance and enhanced antioxidant capacity with any oral supplement.

Similarly, a double-blind study from our laboratory (Cribb *et al.*, 2006) demonstrated that in experienced weight lifters, whey isolate was more effective than casein in enhancing strength and gains in lean body mass during a 10 week resistance training program. The study, which included two groups of equally matched weight trainers, supplemented either casein or whey protein (1.5gm/kg/day) during 10 weeks of resistance training 3 times per week. The results showed both groups significantly enhanced their strength in all exercises assessed. However, the group consuming whey protein supplement increased strength and muscle mass and decreased fat mass significantly more than the group that consumed an equal amount of casein. Although, plasma glutamine levels pre- and post-training showed no change in either supplement group, it was suggested that the supplements prevented a decline in plasma glutamine levels that has been previously reported during intense resistance training (Kinscherf *et al.*, 1996). Although this may argue against glutamine as a mechanism for muscle growth, muscle glutamine levels were not measured. However, it is likely that other mechanisms are involved in the increase in muscle mass, one of which could be enhanced protein synthesis or reduced protein degradation.

In addition, Burke *et al.* (2001) have demonstrated improvement in knee extension peak torque and lean body mass after combining weight training and whey protein supplementation compared to those who just engaged in training alone. Thus, it appears that whey protein is an effective method of increasing muscle mass and strength, most likely by creating an environment of positive nitrogen balance. However, recently it was demonstrated that the combination of protein ingestion and resistance training for a period of 14 weeks had minimal effect on muscle strength and performance (Andersen *et al.*, 2005). Young healthy males were separated into two groups of equally matched weight trainers and supplemented with either protein (16.6g whey protein; 2.8 g of casein; 2.8 g of egg white protein; and 2.8 g of L-glutamine) or carbohydrate maltodextrin. Resistance training was performed 3 times a week, with training loads progressively increased to maintain relative loadings at the intended level.

Muscle strength and performance was measured via vertical jump tests and isokinetic and isometric peak torque measurements. Furthermore, muscle biopsy samples were taken to measure fibre area and proportions. It was demonstrated that resistance training combined with protein supplementation induced greater muscle fibre hypertrophy compared to carbohydrate supplementation; however both groups induced similar gains in muscle strength and performance. Thus, while the protein-supplemented group had significantly bigger muscle fibres, this did not equate to greater improvements in muscle strength or performance compared to carbohydrate supplementation.

It was suggested that since these participants were untrained, it is likely that the increases in isometric and isokinetic peak torque are a result of adaptive changes in neural function rather than of muscle morphological adaptation, hence, both groups would exhibit similar improvements in muscle strength and performance, regardless of changes in muscle morphology, as shown in the study. Furthermore, a methodological

limitation to this study was lack of dietary analysis during the training period; thus it is difficult to determine whether both groups were consuming equal amounts of protein during the training program. Nevertheless, it was concluded that the ingestion of protein is advantageous when muscle fibre hypertrophy is desirable, again suggesting that whey protein is an effective method of increasing muscle size, possibly due to enhanced protein and contractile synthesis, as evident by increased muscle fibre area. However, whether enhanced protein levels within the muscle can enhance muscle regeneration from injury is still unclear.

2.3.3.1 Whey Protein and Muscle Damage

Recently, Saunders *et al.* (2004) investigated the effect of consuming a carbohydrate and whey protein beverage or a carbohydrate-only beverage on endurance cycling performance and post-exercise muscle damage. Trained male cyclists rode a cycle ergometer at 75% VO_2peak to volitional exhaustion, followed 12-15h later by a second ride to exhaustion at 85% VO_2 peak. Results demonstrated that cyclists who consumed the carbohydrate and whey protein beverage rode 29% longer in the first ride (75% VO_2peak) and 40% longer in the second ride (85% VO_2 peak). Furthermore, peak post-exercise CK levels, an indirect measure of muscle damage, were 83% lower after the carbohydrate and whey protein beverage than the carbohydrate trial. However, given that both studies matched beverages for total carbohydrate content but not total caloric content, it is difficult to determine whether these effects were the result of higher total caloric content of the carbohydrate + whey protein beverage or due to specific protein-mediated mechanisms. Nevertheless, it appears to be the first study to investigate the effects of whey protein on indices of muscle damage.

In contrast to whey protein, Coombes and McNaughton, (2000) examined the effects of branch chained amino acid (BCAA) supplementation on serum indicators of muscle damage after prolonged exercise. Subjects consumed BCAA or normal diet for a period of 14 days. On day 7 of the supplementation period, subjects cycled for 120 minutes on an ergometer at approximately 70% VO_{2max} . Blood samples were taken prior to- and 7 days following the exercise session. BCAA supplementation significantly reduced LDH and CK levels in the days following the exercise session. It was concluded that BCAA supplementation may reduce the muscle damage associated with endurance exercise. However, whether BCAA can improve muscle damage from more high-strain exercises (such as eccentric contractions) is unclear.

In addition, the effects of soy protein on muscle calpain activity and myosin heavy chain degradation has been investigated (Nikawa *et al.*, 2002). It was shown that in rats performing acute running exercise, a 20% soy protein diet significantly suppressed the activation of calpain isoenzymes, fragmentation of MHC in gastrocnemius muscle and the release of CK in plasma; all relevant markers of muscle damage. It was suggested that soy protein isolate might prevent the exercise-induced protein degradation in skeletal muscle through inhibiting the calcium-activated, calpain-mediated proteolysis (Nikawa *et al.*, 2002). Therefore, it could be hypothesized that since whey protein has a higher biological value than soy protein, whey protein supplementation would produce a greater improvement. However, whether whey protein has the potential to elicit similar mechanisms to prevent and/or limit the extent of damage to skeletal muscle in humans has not been extensively investigated.

2.4 DIETARY SUPPLEMENT CREATINE MONOHYDRATE

Creatine (methylguanidine-acetic acid) is a natural compound found predominantly in skeletal muscle. In muscle, 40% of the creatine is Cr_{free}, while the remaining 60% is stored in the form of phosphocreatine (PCr) (Heymsfield *et al.*, 1983). After cellular uptake, creatine (Cr) is phosphorylated to PCr by the CK reaction using ATP. At subcellular sites with high-energy requirements, CK catalyzes the transphosphorylation of PCr to ADP to regenerate ATP, thus preventing a depletion of ATP levels. PCr is therefore available as an immediate energy source, serving not only as an energy buffer, but also as an energy transport vehicle (Guerrero-Ontiveros and Wallimann, 1998). Thus, increasing the availability of PCr would theoretically enhance the ability to maintain power output during intense exercise, as well as promoting recovery between bouts of intense exercise.

Creatine monohydrate (CrM) as a nutritional supplement and ergogenic aid for athletes has been extensively studied over the past 20 years (for reviews see Williams, and Branch, 1998; Kreider, 2003; Rawson and Volek, 2003). Investigations into creatine's role in the energy (ATP) production can be found as early as 1914 (Folin and Denis, 1914). In the early 1990's, Harris *et al.*, (1992) demonstrated that consumption of CrM (20gms/day for 5-6 days) increased muscle Cr concentrations (by approx. 25mmol/kg dry mass). However, despite several problems with the study (i.e. no standardization of dosage based on subjects body weight, age of subjects varied from 20-62yrs, and both males and females were used) it was the first study to demonstrate increased creatine content of muscle following the above supplementation protocol. Numerous studies since have consistently reported that ingestion of CrM at a rate of 20-30 grams per day for 5-6 days increases total muscle creatine concentration (TCr) in both human and animal models by 15–40% (Harris *et al.*, 1992; Balsom *et al.*, 1994; Greenhaff *et al.*,

1994; Casey *et al.*, 1996; Hultman *et al.*, 1996; Volek *et al.*, 1999; Rawson and Volek, 2003; Van Loon *et al.*, 2003).

Furthermore, an expert committee from the American College of Sports Medicine Roundtable meeting (Terjung *et al.* 2000) suggested a subsequent daily maintenance dosage of 2g per day to sufficiently maintain these elevated TCr stores (Hultman 1996). However, Van loom and colleagues, (2003) recently demonstrated that after 6-week maintenance dosage ($2\text{g}\cdot\text{day}^{-1}$) following a standard loading phase (20gms/day for 5-6 days), TCr levels were significantly reduced. Nevertheless, despite the decrease in TCr levels, high intensity sprint performance on a cycle ergometer was improved.

Reduced TCr levels could be due to a number of reasons. Firstly, several researchers have indicated that there appears to be a maximal, or optimal, total intracellular creatine concentration of about 150-160 mmol/kg dry mass (Harris *et al.*, 1992; Greenhaff, 1994; Clark, 1998). In addition, Clark (1998) strongly suggests that there are definite limits to the benefits possible from creatine supplementation, and that once muscle creatine levels have plateaued there will be no further increase. Greenhaff (1994) also notes that muscle uptake of creatine may be saturated after a standard creatine-loading protocol. Both animal and human studies suggest that extracellular creatine induces the expression of a protein that functionally inactivates creatine transporters – a response that may limit maximal levels (Loike *et al.*, 1988), and hence, cause a down-regulation of the creatine transporters during continued supplementation (Guerrero-Ontiveros and Wallimann, 1998; Hespel *et al.*, 2001). Thus, while creatine levels may theoretically decline once they have reached their maximum, the compound is still capable of exerting its effect.

2.4.1 The Effect of Creatine Supplementation on Exercise Performance

A series of investigations have attempted to link CrM supplementation with increased muscle Cr stores and improvements in exercise performance (Greenhaff *et al.*, 1993:1994; Birch *et al.*, 1994; Earnest *et al.*, 1995; Febbraio *et al.*, 1995; Casey *et al.*, 1996; Kreider *et al.*, 1998; Snow *et al.*, 1998; McKenna *et al.*, 1999; Preen *et al.*, 2001). Casey *et al.* (1996) investigated the effect of acute supplementation (20g/day for 5 days) on isokinetic cycle performance (2 x 30 sec at 80 rev/min with 4 min passive rest) in nine healthy males. Creatine supplementation resulted in a significant 19% increase in muscle [TCr], specifically in type II fibres. Following supplementation, significant increases in total work production (joule/kg body mass) was reported for both bouts of exercise. These results suggest that the greater the uptake of creatine by the muscle, the greater the beneficial effect on muscle metabolism (i.e. ATP resynthesis) and performance. In addition, Tesch *et al.* (1989) used young healthy male subjects and found that there was a 13% greater PCr concentration in Type II fast twitch muscles compared to Type I slow twitch muscles. Therefore, it could be suggested that fast-twitch fibres have an enhanced potential to transport Cr into the muscle compared to slow-twitch fibres.

Several reports have shown that the use of oral creatine supplementation can increase performance and recovery during brief, high intensity exercise in humans (Harris *et al.*, 1992; Casey *et al.*, 1996; Volek *et al.*, 1999). In the study by Greenhaff *et al.* (1994), total work production was increased by 4.9% after 2 bouts of 30-second isokinetic cycle ergometer exercises at 80 rev/min with 4min rest interval. Creatine ingestion resulted in a 19% increase in muscle [TCr], measured by muscle biopsy. In contrast, Snow *et al.* (1998) reported that creatine increased muscle total creatine content, but did not induce an improved cycle sprint performance or alterations in anaerobic muscle metabolism.

However, in the study by Snow *et al.* (1998), total muscle creatine concentration was found to increase by a mere 9% after supplementation with 30g of creatine and dextrose for 5 days, rather than the customary total muscle creatine increase of 15-25%, that has been found in studies showing positive effects of creatine supplementation (Greenhaff *et al.*, 1994; Casey *et al.*, 1996). Thus, while total muscle creatine concentration plays a pivotal role in the potential ergogenic benefits from creatine supplementation, increases in the order of 15-25% may be required to detect any improvements in exercise performance. However, whether such increases are needed to improve muscle recovery after injury is unknown.

2.4.2 The Effect of Creatine Supplementation on Muscle Size

Numerous studies have confirmed that supplementation with CrM in conjunction with programmed resistance training is effective for augmenting gains in body and fat free mass, and muscular strength, in both men and women, compared to creatine supplementation alone (Bermon *et al.*, 1998; Chrusch *et al.*, 2001; Earnest *et al.*, 1995; Kreider *et al.*, 1998; Vandenberghe *et al.*, 1997; Kelly and Jenkins, 1998; Francaux and Poortmans, 1999; Volek *et al.*, 1999; Becque, 2000; Willoughby and Rosene, 2001; 2003). However, a clear mechanism that may explain these ergogenic benefits remains elusive.

Previous research has shown that creatine supplementation leads to weight gain within the first few days, likely due to water retention related to the creatine uptake by the muscle (Zeigenfuss *et al.*, 1998; Demant *et al.*, 1999; Terjung *et al.*, 2000). While it has been shown that body mass and fat-free mass increases after creatine supplementation in conjunction with a programmed weight schedule (Volek *et al.*, 1999), it has also been demonstrated that creatine supplementation alone increases muscle mass, without the

influence of weight training (Earnest *et al.*, 1995; Mihic *et al.*, 2000; Warber *et al.*, 2002).

In contrast, Brannon *et al.* (1997) investigated the effect of creatine supplementation separately and in combination with run training, on high intensity running capacity of rodent skeletal muscle. It was shown that there were no significant differences in body weights between control and creatine-supplemented groups (Brannon *et al.*, 1997). Similarly, Rawson *et al.* (1999) demonstrated no significant differences in body mass, body density, or fat-free mass as assessed by hydrostatic weighing in 20 males aged 60–82 years after subjects ingested 20g of creatine for 10 days, followed by 4g of creatine for 20 days. Both studies reported small increases in total creatine concentration. Since the level of creatine increase has been shown to influence whether or not improved performance is observed (Greenhaff *et al.*, 1994; Casey *et al.*, 1996; Snow *et al.*, 1998), it is reasonable to assume that a similar relationship might occur between creatine supplementation and body mass, with an increase in body mass normally associated with large creatine increase after supplementation. However, this is yet to be investigated.

While there is extensive literature on the effects of CrM on exercise performance, in particular high intensity exercise, the efficacy of creatine supplementation on muscle recovery after injury has received little attention to date. Creatine supplementation may theoretically enhance the rate at which muscle recovers from injury by possibly reducing the initial amount of damage by improving the intracellular Ca^{2+} handling ability of the muscle (and thus lessening the initial amount of damage by reducing the activation of self-accelerating degradative pathways), and/or increasing the rate of regeneration by increasing muscle protein synthesis.

2.4.3 SR Ca²⁺ Pump Activity (Intracellular Ca²⁺ Handling Ability of the Muscle)

As previously mentioned in section 1.1.1.4, the SR Ca²⁺-ATPase pump derives its ATP preferentially from PCr via the CK reaction (Rossi *et al.*, 1990). Local rephosphorylation of ADP by the CK-PCr system maintains a low ADP/ATP ratio within the vicinity of the SR Ca²⁺ pump and ensures optimal Ca²⁺ pump function (i.e. removal of calcium from the cytoplasm) (Korge *et al.*, 1993). However, when rates of Ca²⁺ transport are high, there is a potential for an increase in [ADP], thus creating a microenvironment (i.e. high [ADP]/[ATP] ratio) that is unfavourable for ATPase function, and as a consequence, SR Ca²⁺ pump function may be diminished (Rossi *et al.*, 1990; Korge *et al.*, 1993). Furthermore, a decrease in [PCr] below 5mM, which is characteristic of this increased ATPase activity, starts to influence the local ATP regeneration potential of the CK/PCr system (Duke and Steele 1999; 2001).

Korge and colleagues (1993) examined the effect of [PCr] and [ADP] on CK-mediated Ca²⁺ uptake in isolated SR vesicles from horse gluteal muscle and rat fast-twitch skeletal muscle. SR vesicles were placed into a Ca²⁺-uptake buffer solution and Ca²⁺ was monitored by the decrease in extravesicular [Ca²⁺] with a Ca²⁺-sensitive minielectrode. Ca²⁺ uptake was affected by both changes in [PCr] and [ADP]. In particular, when examining the efficacy of CK/PCr system in improving the ADP depression of the SR Ca²⁺-ATPase pump, the addition of PCr to the uptake buffer resulted in significantly longer periods of Ca²⁺ uptake, which presumably resulted in the maintenance of a low ADP/ATP ratio as the ADP is rapidly regenerated to ATP via PCr/CK system. Furthermore, efficient translocation of ATP from PCr/CK system to Ca²⁺-ATPase demonstrated the close localization of CK to the SR Ca²⁺-ATPase. Taken together, these results suggest that while there exists a functional link between CK and

Ca²⁺-ATPase pump, several other factors, in particular [PCr] levels can influence the ability of the CK/PCr system to maintain the low ADP/ATP ratio and fuel Ca²⁺-pump with ATP (Korge *et al.*, 1993).

Similarly, in the absence of PCr or pharmacological inhibition of CK, the rate of Ca²⁺ uptake by the SR Ca²⁺-ATPase pump was diminished, most likely due to the local accumulation of [ADP] and reduction in [ATP] (Duke and Steel, 1999; 2001; Yang and Steele, 2002). However, interestingly PCr withdrawal resulted in a reversal of the Ca²⁺ pump function, and thus further increases in [Ca²⁺]_i, possibly due to a decrease in [ATP] gradient, and Ca²⁺ leaks out of the SR (Duke and Steel, 1999;2001). Therefore, not only may decreased PCr levels result in a reduced rate of Ca²⁺ uptake, it may also lead to further increase in [Ca²⁺]_i due to leakage. However, this concept needs to be further investigation.

Taken together, it is evident that depletion of muscle [PCr] leads to an accumulation of [ADP] and a reduction in SR Ca²⁺-ATPase pump activity, therefore increasing cytosolic [Ca²⁺]. Furthermore, PCr withdrawal may also induce an increase in Ca²⁺ efflux from the RyR receptor of the SR, further contributing to the observed increased [Ca²⁺]_i. As a result, creatine supplementation, which leads to increased PCr levels within the muscle, could theoretically improve the intracellular Ca²⁺ handling ability of the muscle by enhancing the CK/PCr system and increase local rephosphorylation of ADP to ATP, thus maintaining a high [ATP]/[ADP] within the vicinity of SR Ca²⁺-ATPase pump during intense, eccentric exercise. Increasing the rate of Ca²⁺ removal from the muscle cytoplasm would decrease [Ca²⁺]_i accumulation, reduce calpain activation, and thus minimise the extent of muscle damage.

Pulido and colleagues (1998) investigated the effect of creatine supplementation on *in vitro* dystrophic muscle cells from *mdx* mice (a model of muscular dystrophy) that exhibit elevated cytosolic Ca^{2+} concentrations. Primary cell cultures of mouse skeletal muscle cells were prepared from the hind leg muscles of 1-4 day old normal and *mdx* mice. Myotubes were exposed to two types of extracellular stress (a hypo-osmotic solution or high extracellular $[\text{Ca}^{2+}]$) in order to examine changes in and regulation of, cytosolic $[\text{Ca}^{2+}]$. Pre-treatment of *mdx* myotubes for 6-12 days with creatine increased cellular PCr levels and improved regulation of cytosolic $[\text{Ca}^{2+}]$ by improving the SR Ca^{2+} -ATPase pump. Additionally, myotube formation and survival were significantly enhanced by creatine supplementation. This was the first study to show that creatine supplementation appears to lower cytosolic $[\text{Ca}^{2+}]$ and enhance survival of dystrophic skeletal muscle cells (Pulido *et al.*, 1998).

Therefore, since eccentric-induced damage is also characterised by an increase in cytosolic $[\text{Ca}^{2+}]$, it is possible to suggest that creatine supplementation may improve the SR Ca^{2+} -ATPase pumping ability in injured but otherwise normal healthy skeletal muscle. Indeed, researchers have reported a faster rate of muscle relaxation (a calcium removal process) after contraction in creatine-supplemented humans. They suggested that the biochemical adaptations induced by Cr-loading at the level of the SR may allow sarcoplasmic Ca^{2+} -ATPase to operate at a higher thermodynamic efficiency and thereby facilitate muscle relaxation (Van Leemputte *et al.*, 1999).

2.4.4 Creatine and Protein Synthesis

As mentioned, it has been generally reported that the increase in body mass after creatine supplementation is due to water retention (Demant *et al.*, 1999; Terjung *et al.*, 2000). Conversely, it has been recently suggested that creatine supplementation does

not result in water retention, rather an increase in total body water (Powers *et al.*, 2003). However, a limitation to the study by Powers *et al.* (2003) was that caloric and fluid intakes were not monitored, and thus, both may have influenced fluid balance and body mass. Notwithstanding, it has been suggested that an increase in cellular hydration status (i.e. increased cell volume) induced by creatine supplementation may act as an independent anabolic signal, initiating cellular mechanisms needed to create gains in muscle, such as protein synthesis (Volek *et al.*, 1997). In addition, Clark (1998) suggests that elevation of PCr in the muscle cell is also capable of stimulating protein synthesis, somewhat similar to that induced by exercise or insulin.

Cr is also thought to aid in energy transfer from the mitochondria to the contractile proteins by the PCr shuttle (as shown in figure 1.5) to stimulate myofibrillar protein synthesis (Balsom *et al.*, 1994). Increased creatine availability has been reported to stimulate protein synthesis in studies of animal skeletal and cardiac muscle *in vitro* (Ingwall *et al.*, 1974; Ingwall, 1976; Young and Denome, 1984), although other investigators have been unable to confirm this (Fry and Morales, 1980; Parise *et al.*, 2001; Louis *et al.*, 2003).

Recently, Parise *et al.* (2001) and Louis *et al.* (2003) investigated the effects of acute creatine supplementation on muscle protein turnover in both the fasted (Parise *et al.*, 2001; Louis *et al.*, 2003) and fed conditions (Louis *et al.*, 2003) at rest without the influence of resistance training. While both studies showed significantly increased [TCr] levels, there was no effect of creatine in raising myofibrillar protein synthesis, raising leg net amino balance or decreasing leg muscle protein breakdown. Therefore, it was speculated that one explanation for the lack of any effect in resting muscle was that

there needed to be some associated contractile activity for an anabolic effect of creatine to be unmasked (Louis *et al.*, 2003).

However, it was recently demonstrated that creatine in conjunction with acute resistance exercise had no anabolic effect on muscle protein synthesis rates (Louis *et al.*, 2003). Seven healthy men performed 20 x 10 repetitions of leg extension-flexion at 75% 1RM in one leg, on two occasions, 4 weeks apart, before and after ingesting 21g/day creatine for 5 days. Myofibrillar and sarcoplasmic fractional synthetic rates were increased after exercise in both control and supplemented group; however, creatine supplementation had no added effect during the 4 hours post-exercise. While whole body protein breakdown was attenuated in the men after creatine supplementation, no muscle-specific measurements of protein breakdown were made. Therefore, given that muscle protein breakdown represents only a small proportion (25-30%) of whole body protein breakdown, the results may reflect alterations in a rapidly turning over tissue, such as liver proteins (Parise *et al.*, 2001).

As a result, it was concluded that the effect of creatine supplementation on muscle anabolism in normal healthy subjects is probably associated with an increase in exercise performance more so than the stimulation of muscle protein synthesis (Louis *et al.*, 2003). Nonetheless, it is suggested that there may be gene transcriptional changes programmed for muscle anabolism occurring as a result of increased creatine availability and resistance exercise (Louis *et al.*, 2003). However, any increases myofibrillar protein synthesis may not be seen for days to weeks after initial stimulus, could explain the lack of change within the first 4-hours, as shown by Louis *et al.* (2003).

Indeed, recent research has shown that creatine supplementation increases myogenic regulatory factors (for example MRF-4) and MHC synthesis (mRNA expression and protein) following 12 weeks of resistance training in untrained participants compared to non-supplemented controls (Willoughby and Rosene, 2001; 2003). Therefore, the ergogenic benefits of creatine supplementation in terms of acting as a co-regulator, or direct manipulator of gene transcription of amino acid pools (specifically myofibrillar protein synthesis) may require day(s) to weeks after the resistance exercise or eccentric-induced damage.

2.4.5 Creatine and Muscle Damage

The effect of creatine supplementation on muscle recovery after injury has received little attention to date. Few studies have investigated the effect of creatine supplementation on exercise-induced muscle damage (Warren *et al.*, 2000; Rawson *et al.*, 2001; Santos *et al.*, 2004). Rawson and colleagues (2001) supplemented male subjects with creatine monohydrate for 5 days prior to 50 maximal eccentric exercises. The study showed that maximal isometric force of the elbow flexors, serum CK and LDH levels in response to eccentric exercise were not significantly different between the creatine-supplemented and control groups during the 5 days following exercise. Therefore, it was suggested that creatine supplementation does not reduce indirect markers of muscle damage or enhance recovery from high-force eccentric exercise. However, the creatine supplementation period only lasted for 5 days, unlikely to be enough time to induce a cell volume stimulus, nor was the supplementation continued after the exercise (Volek *et al.*, 1999). Thus, it is possible that the supplementation of creatine immediately after exercise and during the recovery phase may have a positive effect, as would a longer supplementation regime prior to the exercise being performed.

In contrast to this statement, Warren *et al.* (2000) demonstrated that recovery of muscle strength after eccentric-induced damage was unaffected following 2-weeks of creatine supplementation. Mouse anterior crural muscle (which is predominantly tibialis anterior) was subjected to an injury-inducing protocol, which consisted of 150 eccentric contractions. Creatine supplementation had no effect on muscle strength during the first 50 eccentric contractions of the protocol, which fell sharply, or on the decline in strength over the entire 150-contraction protocol. Following 3 minutes recovery, there was no effect on isometric strength or on torque loss at any eccentric or concentric angular velocity. However, a number of limitations exist with this study. Firstly, researchers were only interested in how increased muscle Cr influenced peak strength loss and not the recovery of strength *per se* after injury. Therefore, the 3 min recovery period may not be long enough to see any beneficial effect of creatine supplementation on muscle strength loss. Secondly, creatine supplementation may have attenuated other markers of muscle damage such as blood levels of myocellular proteins. However, since injury assessment was only muscle function based, these were not measured. Indeed, one beneficial effect of creatine supplementation shown was a small reduction in swelling (Warren *et al.*, 2000) which may indicate a reduction in inflammation and or an increase in membrane stability, thus, reducing the cascade of events leading to muscle degeneration.

More recently, the effect of a creatine supplementation protocol upon inflammatory and muscle soreness markers were investigated after prolonged running (Santos *et al.*, 2004). Runners with previous experience in marathon races were supplemented for 5 days prior to a 30km race with 4 doses of 5g of creatine and 15g of maltodextrine per day while the control group received the same amount of maltodextrine. Blood samples were collected pre-race, and 24 hours following the end of the test, to measure for CK,

LDH, PGE2 and TNF- α . Athletes from the control group presented an increase in all muscle soreness markers, indicating high level of cell injury and inflammation, while creatine supplementation significantly attenuated these observed changes, with the exception of CK, after the 30km run. All athletes finished the race in a time equivalent to their personal best for that distance (in a 5.8% range). Therefore, it was concluded that creatine supplementation reduced cell damage and inflammation. However, while this Cr supplementation protocol may be an effective strategy in maintaining muscle integrity during and after intense prolonged aerobic exercise, it may not be sufficient to protect muscle fibres from more damaging exercisers, such as those shown by Rawson *et al.* (2001).

Taken together, with such conflicting results from the previous studies (Warren *et al.*, 2000; Rawson *et al.*, 2001; Santos *et al.*, 2004), further research is needed to determine any beneficial effects of creatine supplementation on indices of muscle damage. Furthermore, whether increased intracellular Cr concentration will influence muscle recovery after damage *in vivo* via the proposed mechanisms in section 2.4.3 and 2.4.4 is also unclear.

The proposed mechanisms by which whey protein and creatine supplementation may reduce the extent of, or enhance recovery from, exercise-induced muscle damage can be summarized in figure 2.4 and 2.5 respectively.

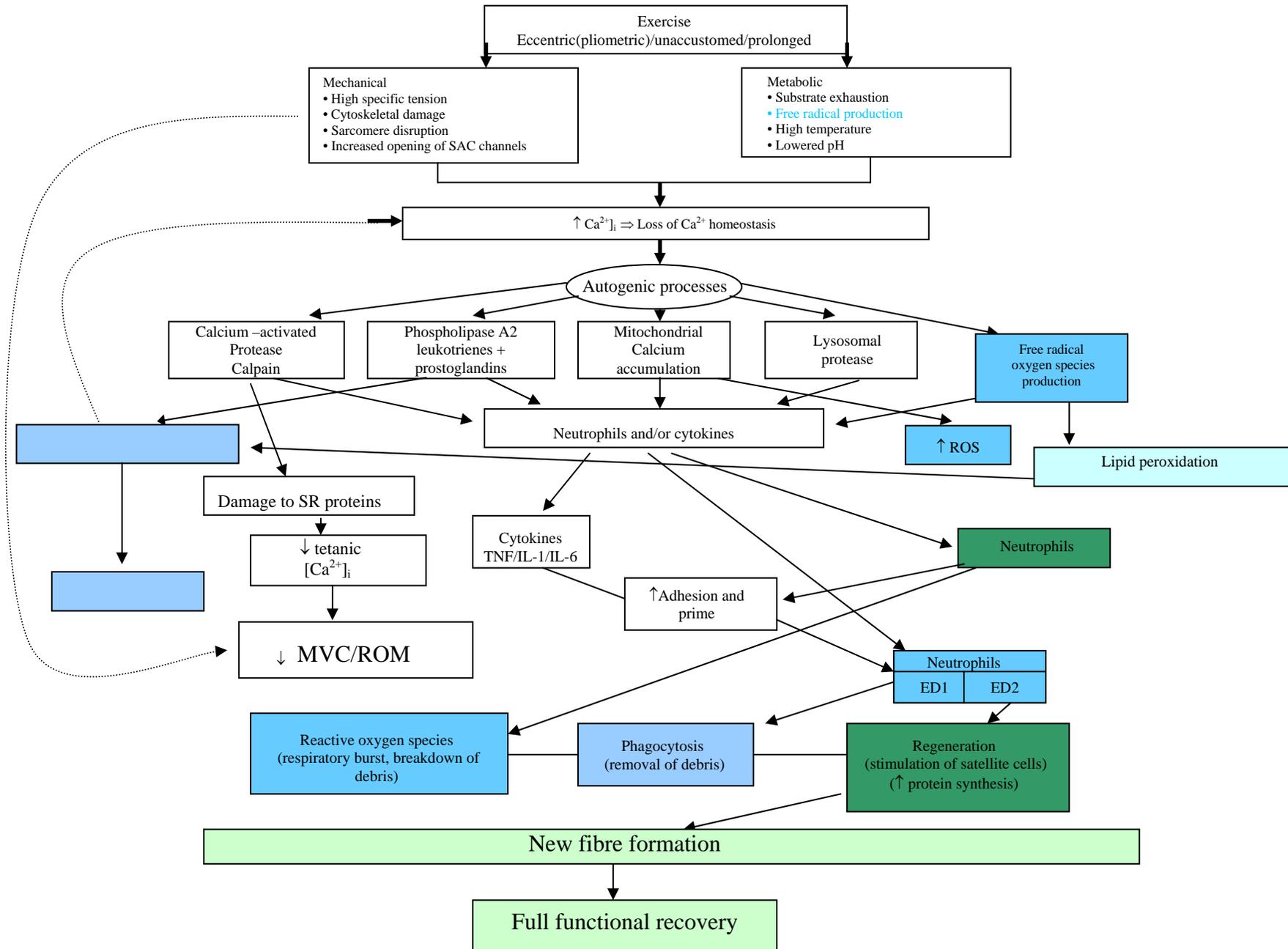


Figure 2.4. Areas where whey protein supplementation may play a potential inhibitory or enhancement role (modified from Kendell and Eston, 2002; Allen *et al.*, 2005). TNF/IL-1 =tumor necrosis factor/interleukin-1, MVC/ROM = Maximum voluntary contraction/Range of motion, SAC =Stretch activated channel, CK = Creatine kinase, LDH= Lactate dehydrogenase. ■ Primary inhibitory effect from whey protein ■ Primary enhancement effect from whey protein ■ Secondary inhibitory effect from whey protein ■ Secondary enhancement effect from whey protein

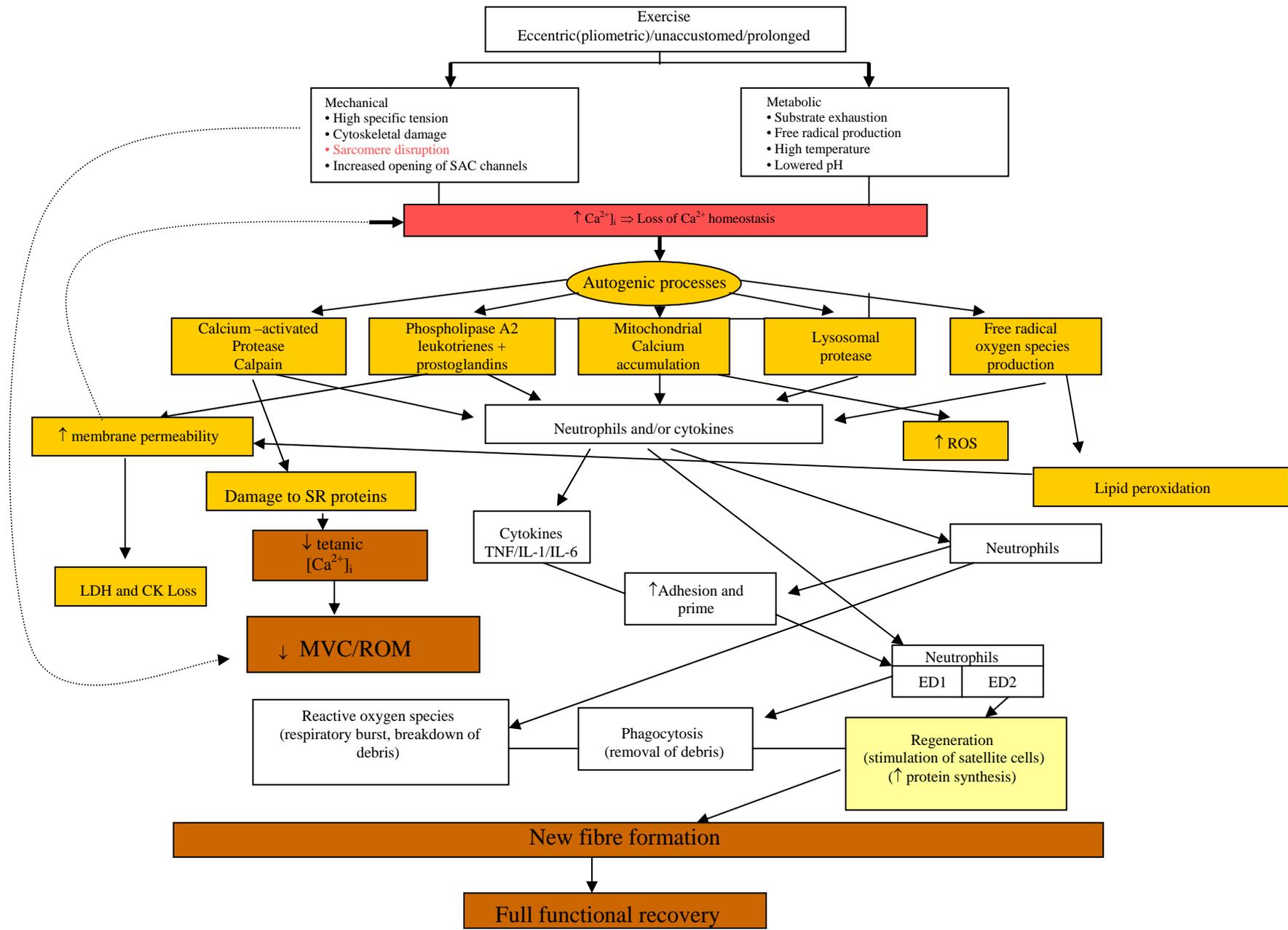


Figure 2.5. Areas where creatine supplementation may play a potential inhibitory or enhancement role (modified from Kendell and Eston, 2002; Allen *et al.*, 2005). TNF/IL-1 =tumor necrosis factor/interleukin-1, MVC/ROM = Maximum voluntary contraction/Range of motion, SAC =Stretch activated channel, CK = Creatine kinase, LDH= Lactate dehydrogenase. ■ Primary inhibitory effect from creatine ■ Primary enhancement effect from creatine ■ Secondary inhibitory effect from creatine ■ Secondary enhancement effect from creatine

2.5 AIMS AND HYPOTHESIS

By the nature of their role and position in the human body, skeletal muscles are frequently subjected to significant physical trauma. While muscle damage can be divided into separate processes (initial event; autogenic phase; phagocytic stage and regenerative phase) they overlap enormously, and the exact nature of the muscle damage, the mechanisms responsible and processes involved are not completely understood. Extensive studies have investigated the effect of creatine supplementation on short-term, intense exercise performance and muscular strength. Very few studies have examined the effect of creatine supplementation on exercise-induced muscle damage. Furthermore, no studies have directly determined whether increased creatine accumulation within the muscle can reduce the initial amount of fibre damage, and/or improve muscle regeneration by enhancing protein synthesis, after chemically-induced muscle damage in rat muscle.

Similarly, some evidence exists to suggest that whey protein is effective at increasing muscle protein concentration, muscle mass and strength. However, again no studies have determined if whey protein supplementation can directly influence intramuscular protein levels and muscle re-growth after eccentric-induced muscle damage in untrained human or rat skeletal muscle, or whether these changes may improve recovery after muscle injury is also unknown.

Thus, the aims of this thesis are as follows

- 1) To examine the effects of short-term dietary consumption of whey protein on muscle proteins and force recovery after eccentrically-induced muscle damage in healthy individuals.
- 2) To examine the effects of dietary creatine supplementation on muscle proteins and force recovery after eccentrically-induced muscle damage in healthy individuals.
- 3) To determine the mechanisms by which the dietary supplements exert their effects by examining morphological, biochemical and contractile properties of rat skeletal muscle during recovery from chemically-induced muscle damage.

It is hypothesized that:

- 1) Following eccentric-induced damage in healthy individuals, loss of muscle strength will be restored earlier and the release of muscle proteins will be decreased, indicative of reduced damage and/or faster rate of recovery following whey protein and creatine supplementation.
- 2) In rat skeletal muscle, creatine supplementation will restore loss of isometric contractile strength at a faster rate; reduce the extent of damage and improve fibre regeneration concomitant with an increased intramuscular ATP, Cr and PCr concentration and protein levels.

- 3) Whey protein supplementation will restore loss of isometric contractile strength at a faster rate; reduce the extent of damage and improve fibre regeneration concomitant with an increased intramuscular protein and contractile levels in rat skeletal muscle.

Several experimental models of muscle injury induce muscle degeneration by causing direct trauma to the structure of the muscle, for example, by laceration, crushing or from eccentric exercises. The advantage of using eccentric exercises as a model of injury in this thesis is that this type of injury is a common occurrence in athletes on and off the sports field and in untrained individuals performing unaccustomed high-strain exercise. Thus, the first two studies will examine the effects of nutritional supplementation on muscle recovery following eccentrically-induced muscle damage in untrained individuals.

However, a limitation to this model is that the level of information regarding the mechanisms is limited due to the vast array of causes (as shown in figure 2.3). Thus, two parallel animal studies will be undertaken using a myotoxic agent bupivacaine, that causes a controlled level of damage specific to muscle fibre to be produced, without causing damage to the basal lamina, blood vessels, or the regenerative capabilities of satellite cell. Furthermore, since the healing processes are essentially the same in both injury models; any improvements in the rate of recovery in the animal model can be directly applicable to muscle injury in humans. To further understand the methodological protocols described in the next chapter, the next section will review models of muscle injury, both quantitative and qualitative measurements of muscle damage.

2.6 MODELS OF MUSCLE INJURY: QUANTITATIVE AND QUALITATIVE MEASUREMENTS OF MUSCLE DAMAGE

Muscle injuries occur through a variety of mechanisms (summarized in table 2). These include external factors such as direct trauma, lacerations and crush injury and internal causes such as muscle tears, ischemia and neurological dysfunction (Carlson and Faulkner, 1983; Armstrong, 1990; Huard *et al.*, 2002). Nevertheless, regardless of the cause, all eventually lead to the same processes of cellular degeneration and regeneration, concluding with restoration of muscle structure and function.

Chemicals and drugs
<ul style="list-style-type: none">• Bupivacaine (Marcaine)• Rifampicin• Quinacrine• Others
Diseases
<ul style="list-style-type: none">• Polymyositis, dermatomyositis• Muscular dystrophy
Exercise, in particular eccentric contractions
Irradiation
Ischemia
Mechanical injuries (stretching, crushing, laceration)
Thermal injuries (heating, freezing)
Biological toxins (venoms, streptococcus A toxin)

Table 2. Causes of muscle fibre degeneration (modified from McComas, 1996).

It is well established that the most common cause of skeletal muscle damage among individuals is any form of unaccustomed or high intensity exercise, in particular eccentric exercise that involves lengthening of the activated muscles (Brown *et al.*, 1999; Phillips *et al.*, 1999; Friden and Lieber, 2001a; Sbriccoli *et al.*, 2001; Lee *et al.*, 2002; Nosaka *et al.*, 2002). As a result, several types of eccentric exercise protocols (i.e. downhill running, stepping and repeated maximal eccentric contractions) have been used in scientific studies to induce and investigate mild to severe forms of muscle injury in both human and animal models.

These diverse protocols share the common outcome of inducing alterations in muscle ultrastructural and thus, similar indicators of muscle injury, which include reductions in muscle strength (maximum voluntary contraction; MVC), muscle soreness (DOMS), changes in range of motion (ROM), and release of myocellular proteins in the plasma (CK and LDH). More than 130 human studies have been published in the past few years alone, confirming the popularity and relative safety of this repairable form of exercise-induced muscle damage (Sayers and Clarkson, 2001).

In functional terms, the most universal method to examine muscle recovery following exercise-induced damage is measurement of isometric strength using a cybex dynamometer (Warren *et al.*, 1999). The method involves the study participant performing a maximal voluntary contraction (MVC) of a muscle group at a fixed joint angle for 2-5 seconds to determine muscle strength. In general, isometric strength is reduced immediately post-eccentric exercise and recovery is sometimes gradual and prolonged, lasting for hours (Davies and White, 1982; Newham *et al.*, 1983), days (Friden *et al.*, 1983; Hortobagyi *et al.*, 1998; Byrne and Eston 2002) or even weeks (Cleak and Eston, 1992; Howell *et al.*, 1993). The magnitude and time course of

strength loss appears dependent on the type of exercise used and the training history of the muscle group, with the greatest reductions in muscle strength usually seen after intense eccentric exercises and participants who are unaccustomed to this type of exercise (Byrne *et al.*, 2004). Reduction in MVC is most likely due to an initial damaging event such as mechanical disruption to the muscle fibre caused by the lengthening contractions. Moreover, a secondary response which occurs in the days following the eccentric exercise, most likely a result of loss of Ca^{2+} homeostasis or the inflammatory response, usually exacerbates the initial damage to the muscle, and thus further contributes to the observed force reduction (Clarkson and Sayers, 1999).

Though examining the recovery of strength loss after damaging exercises through the measurement of MVC is an easy, reproducible method using the cybex dynamometer, the isolated muscle actions that are needed to perform such movements are infrequently utilized alone in normal muscle movements (Komi, 1984). Natural muscle function occurs as a sequence of eccentric stretch, isometric coupling and concentric shortening of muscles, known as the stretch-shortening cycle (Hill, 1970; Komi, 1984). Thus, movements that utilize the stretch-shortening cycle may provide an additional opportunity to study force recovery from injury using a more functional movement that occurs in everyday activities, especially in the sporting context (Byrne and Eston, 2002). The countermovement jump is an example of a vertical jump that utilizes the stretch-shortening cycle by a downward countermovement from an erect standing position, and hence is a convenient model to study force recovery from exercise-induced damage.

In addition to examining both MVC and vertical jump performance as indicators of muscle recovery after injury, a number of indirect approaches have been widely used to

not only examine muscle fibre recovery (DOMS and ROM) but as estimates of the magnitude of exercise-induced damage. A parameter that is often used to estimate the degree of muscle injury after exercise is measurement of serum/plasma CK, and to a lesser extent, LDH (Coombes and McNaughton, 2000; Lavender and Nosaka, 2005; Miliadis *et al.*, 2005).

Numerous studies have evaluated changes in myocellular proteins CK and LDH after exercise and have shown that it differs markedly according to exercise conditions (Nosaka *et al.*, 1992; Newham *et al.*, 1986). It is clear however that peak serum CK and LDH is observed anywhere from 1 -7 days after eccentric-induced exercise (Newham *et al.*, 1986; Nosaka and Clarkson, 1992; Coombes and McNaughton 2000; Santos *et al.*, 2004). It is often understood that the level of CK activity is somehow related to the magnitude of muscle injury, however, Friden and Lieber (2001b) suggested that while serum CK levels may provide a gross indication that skeletal muscle injury has occurred, differences between serum CK levels do not necessarily provide information regarding the extent of muscle damage.

Thus, while indirect measures of eccentric contraction-induced muscle damage, such as serum CK, LDH and loss of force have been used; they are recognized as poor predictors of muscle damage *per se* (Warren *et al.*, 1999). Therefore, the only direct measure of alterations in muscle ultrastructure (in particular the Z-band and myofibrillar apparatus) and possible information regarding the extent of damage and inflammation of injured muscle fibres is through the needle muscle biopsy technique, in which a sample of muscle is taken directly from the injured area, thus, providing direct histological analysis (Gibala *et al.*, 1995). However, it is suggested that the muscle biopsy technique, which only samples a small portion (i.e., 30-200mg) of muscle tissue

from a chosen muscle, may not be representative of muscle as a whole (Warren *et al.*, 1999; Beaton *et al.*, 2002).

Beaton and colleagues (2002) recently demonstrated that eccentric-induced alterations in muscle ultrastructure and inflammatory cell response in muscle biopsy samples from human vastus lateralis muscle is highly variable. Five healthy volunteers performed a “damage protocol” which consisted of 24 sets of 10 maximal isokinetic eccentric muscle actions on the right and left leg one week apart. Needle biopsy samples were taken from the medial portion of the vastus lateralis muscle on two occasions. Two biopsies were taken from within the muscle of the same leg (~14 cm apart), 24 hours following the damage protocol. A week later, a single biopsy was taken from the contralateral leg, 24 hours following the same damage protocol. Biopsies at all three sites showed Z-band disruption and macrophage infiltration after eccentric contractions. However, the results illustrated high variability in both within leg and between leg biopsy samples. It was suggested that although a single biopsy provides a means to directly assess muscle damage, it is not necessarily reflective of the muscle as a whole, in particular the extent of Z-band streaming and cellular infiltration.

Moreover, a number of investigations have reported that intramuscular variability may be in part due to fibre type composition within the sampled portion of muscle (Lexell *et al.*, 1983; Lexell and Taylor, 1991), and it is believed that this intramuscular variability is more prominent than between-leg differences (Lexell and Taylor, 1991). As a result, the use of animal models enables a more extensive and accurate analysis of biochemical and morphological characteristics of whole intact skeletal muscle. In addition, the use of animal tissue provides an opportunity to examine differences in muscle recovery after

injury in both slow-twitch and fast-twitch muscles, than would be possible from human biopsy samples, due to them having a predominance of one fibre type.

As recognized previously, the cause of human skeletal muscle injuries are widespread, with varying severity, often disrupting nerve supply, vasculature and the connective tissue within the muscle, as well as the muscle cells themselves (Hurme *et al.*, 1991). Thus, examining the recovery from muscle injury in a scientifically controlled way is often difficult as the regeneration of nervous, circulatory and fibrous tissue further complicates the processes of muscle cell recovery. Consequently, it is suggested that chemically-induced injury provides a more suitable model for studying muscle regeneration (Louboutin *et al.*, 1996). Although it is rare that myotoxic injury will occur in normal human daily activities, unless exposed to certain venoms or toxins, the use of a myotoxic agent such as bupivacaine hydrochloride (Bupivacaine) allows a controlled level of damage specific to muscle to be produced. More importantly, bupivacaine injection does not damage elements that influence the rate and extent of muscle regeneration, including endomysium, basal lamina, vascular supply, and intramuscular nerves (Halls-Craggs 1974; Louboutin *et al.*, 1996). Therefore, the mechanisms of any enhanced recovery as a result of the dietary supplements, for example, can be analysed.

2.6.1 Local Anaesthetic Bupivacaine

Skeletal muscle toxicity induced by local anaesthetics was first reported in 1959 (Brun, 1959), and has been described in several studies and case reports since then (Halls-Craggs, 1974; Foster and Carlson, 1980; Carlson *et al.*, 1990; Beitzal *et al.*, 2003). Marcain (generic name Bupivacaine) has a proven myotoxic effect when injected intramuscularly, with little or no damage caused by insertion of the needle itself, or the

injection of an otherwise harmless vehicle such as isotonic saline (Rosenblatt, 1992; Rosenblatt and Woods, 1992).

Intramuscular injection of bupivacaine results in reversible myonecrosis, with the extent of muscle damage being dose-dependent, thus allowing a controlled and consistent amount of damage. The histological pattern and the time course of skeletal muscle injury appears uniform; approximately 5 min after intramuscular injection, hypercontracted myofibrils are evident, followed by myocyte oedema and necrosis over the next 1-2 days (Benoit and Belt, 1970; Foster and Carlson, 1980; Kytta *et al.*, 1986). Myoblasts, basal laminae, and connective tissue elements remain intact, which ensures complete muscle restoration within 3 –4 weeks (Foster and Carlson, 1980; Komorowski *et al.*, 1990).

2.6.2 Bupivacaine: Mechanisms of Action

The subcellular mechanisms of local anaesthetic myotoxicity are still not clearly understood. However, as evident in eccentric-induced muscle damage, increased intracellular Ca^{2+} concentrations appear to be the integral part of myotoxic injury, particularly since denervation, inhibition of sarcolemmal Na^+ channels, and direct toxic effects on myofibrils have been excluded as sites of action (Benoit *et al.*, 1980). The increased $[\text{Ca}^{2+}]_i$ can be a result of either specific action of the local anesthetic on Ca^{2+} release channels (ryanodine receptor [RyR]) or a nonspecific increase in Ca^{2+} permeability of SR membranes (Takahashi, 1994). Komai and Lokuta (1999) examined the interactions of bupivacaine with the SR Ca^{2+} release channel of mammalian skeletal muscle and found that clinical concentrations of bupivacaine enhanced RyR activity and hence, Ca^{2+} release. It was suggested that this specific action on RyR, in addition to the nonspecific increase in Ca^{2+} permeability of SR membranes, significantly contributes to

the pronounced myotoxicity of bupivacaine (Foster and Carlson, 1980; Komai and Lokuta, 1999).

In addition to disturbances in SR function, several studies point to other etiologic pathways that are likely to be involved in local anesthetic myotoxicity (Irwin *et al.*, 2002; Zink and Graf, 2004). Bupivacaine has been shown to uncouple oxidative phosphorylation in isolated mitochondria in a dose-dependent fashion (Sztark *et al.*, 2000). Oxidative phosphorylation uncoupling results in intracellular ATP depletion. Recently, it was established that in mitochondria of isolated rat muscle fibres, bupivacaine caused a concentration-dependent depolarization and pyridine nucleotide oxidation of the mitochondria, in particular the activation of a permeability transition pore (PTP) (Irwin *et al.*, 2002). In short, activation of PTP is associated with cell death, possibly via different pathways that include decreased ATP levels, increased myoplasmic $[Ca^{2+}]_i$, and release of apoptotic factors such as cytochrome c, and apoptosis inducing factors: Smac-Diablo and endonuclease G (Irwin *et al.*, 2002). Thus, further contributing to the pathogenesis of bupivacaine.

Taken together, it seems that a variety of experimental findings points to the highly complex pathogenesis of local anaesthetic myotoxicity, which stills remains unclear. Nonetheless, it is apparent that bupivacaine-induced tissue damage is likely to be a function of increased $[Ca^{2+}]_i$.

In summary, the information regarding the magnitude of damage and rate of muscle recovery after injury can be examined directly through needle muscle biopsy technique, in which a sample of muscle is directly taken from the injured site (Gibala *et al.*, 1995), and/or indirectly via measurement of impaired range of motion, loss of MVC and

functional strength, and release of myocellular proteins in serum (CK and LDH). However, since muscle biopsy techniques may not be representative of muscle as a whole (Warren *et al.*, 1999), the use of animal models enables a more extensive and accurate analysis of biochemical and morphological characteristics of whole intact skeletal muscles after chemically-induced damage. Furthermore, whilst there are many causes of muscle damage, the healing processes are identical; therefore any improvement in the rate of recovery in the animal model can be directly applicable to muscle injury in humans.

CHAPTER 3

3.0 EXPERIMENTAL METHODS AND PROCEDURES

This thesis consisted of four separate studies designed to examine the effects of nutritional supplementation on muscle recovery after damage in both human and animal models. The methods described in this chapter are general in nature and relate to common procedures employed in human and animal studies. The primary focus of the thesis was to examine skeletal muscle recovery after injury. Two injury protocols were used to induce muscle damage in both the human and animal models. In the human model, skeletal muscle damage was induced via repeated eccentric muscle contractions. Extent of, and recovery from, damage was evaluated by knee extension/flexion force development (MVC) and vertical jump performance, in addition to plasma CK and LDH analysis. While these are indirect measurements, muscle biopsies were not used because it may not be representative of muscle as a whole, and thus, lead to high variability between biopsy samples analyzed (as discussed in Section 2.6).

The animal model used injection of the local anesthetic bupivacaine to induce muscle damage in Sprague-Dawley rats. The extent of muscle injury and subsequent recovery was examined by isometric force measurements, histological analysis and biochemical measurements of whole skeletal muscle that is predominantly one fibre type. Although it would be ideal to use the same injury protocol in both models, unfortunately our laboratory was not equipped with the apparatus to eccentrically-induce damage to rodents. Nevertheless, since the cause of muscle injury and healing processes are theoretically identical, any improvement in the rate of recovery in the animal model, and more importantly the mechanisms behind the improvements can be directly

applicable to muscle injury in humans (see section 2.6 for justification of the bupivacaine model)

3.1 HUMAN MODEL: ECCENTRIC EXERCISE PROTOCOL FOR LOCAL MUSCLE DAMAGE AND REPAIR

The eccentric damage-induced exercise protocol was used in two studies; study one examined the effects of creatine supplementation on the metabolic aspects of muscle damage (i.e. the calcium handling ability of the muscle), and subsequent damage and repair processors, while the second study examined the effects of whey protein on the regeneration (recovery) aspect of muscle damage.

3.1.1 Participants

A total of thirty-one healthy male participants (aged 18-35) volunteered for the two human studies. Participants were generally students of Victoria University. Brief details of participant characteristics in each study are given in table 3. Further descriptive participant information is provided in subsequent chapters. To meet the criteria as a participant in these studies, the men (a) were non-smokers; (b) had not participated in resistance-training, or any form of structured exercise, for at least six months; (c) had not ingested any ergogenic supplement for a 24-week period prior to the start of supplementation; and (d) agreed not to ingest any other nutritional supplements, or non-prescription drugs that may affect muscle re-growth during the study. In addition, participants agreed to refrain from using any remedy (i.e. massage, ultrasound etc.) for muscle soreness other than consumption of the supplement given; and agreed not participate in any form of physical activity 2 weeks prior to supplementation and during the 2 week recovery period.

All participants were informed verbally, as well as in writing, as to the objectives of the experiments, together with the potential associated risks. All participants signed an informed consent document approved by the Human Research Ethics Committee of Victoria University and the Department of Human Services, Victoria, Australia. All procedures conformed to National Health and Medical Research Council guidelines for the involvement of human subjects for research.

	Study One		Study Two	
Characteristics	Control carbohydrate n = 7	Creatine carbohydrate n = 7	Control carbohydrate n = 8	Whey Protein Isolate N = 9
Age (yrs)	21.7 ± 2.9	22.6 ± 1.6	22.0 ± 3.6	24.2 ± 5.1
Weight (kg)	74.4 ± 6.5	77.9 ± 11.8	77.4 ± 14.1	81.5 ± 7.6

Table 3. Participant characteristics of study one & two. Age and weight characteristics are mean ± SD.

3.1.2 Experimental Design

The study was conducted at the Human Performance Laboratory (L305) at Victoria University. Two weeks prior to baseline strength testing, participants underwent a familiarisation session of the force testing equipment that would be utilized during the study. Participants in study 1 were issued their supplement and instructed on how and when to consume the supplement.

On day 1, baseline measurements of muscle strength were assessed. Subjects were required to be fasted (4 hours) prior to baseline testing and subsequent testing days. Subjects also underwent catheterisation of a forearm vein to allow regular sampling of venous blood prior to and immediately following the resistance exercise session. Subjects then performed an exercise session designed to cause damage to the knee extensor and flexor muscles. Immediately following the session, subjects repeated the

muscle strength tests. Participants in study 2 were issued their supplement and instructed to ingest the first dose immediately. Further blood samples were taken at 30 minutes, 1, 2, and 4 hours following the exercise session. Subjects were instructed to return 24 hours post exercise, and on day 2, 3, 4, 7, 10 and 14 for further blood sampling and muscle strength measurements. Since dietary supplementation protocol for study one and two are different, these will be discussed in detail in subsequent chapters. Therefore only the procedures used in both studies will be discussed in this chapter.

3.1.3 Initial measurements: Muscle strength and Performance

Prior to all muscle strength and performance assessment, participants underwent a familiarisation session of the testing equipment that was utilized to examine muscle recovery during the study.

3.1.3.1 Isokinetic Knee Extension/Flexion and Isometric Knee Extension Measurements

The muscle groups used to examine force recovery after injury were the knee extensors and flexors (i.e. quadriceps and hamstring muscle groups, respectively). Isolated strength measurements were performed on the Cybex Norm[™] Testing and Rehabilitation System (Cybex International Inc. Ronkonkoma, New York), with subsequent analysis using Norm application for Windows. All strength measurements were performed on both legs. The left leg underwent the resistance exercise session (as described later); while the contralateral leg was used as the non-exercised control, in order to account for any improvements during the recovery period as a result of familiarisation. All participants were seated during the testing protocols; the dynamometer axis of rotation was orientated in alignment with their knee. The point of

contact between the participant's lower leg and the padded lever arm was at a fixed distance (relative to the participants lower limb length), slightly above their ankle (see Figure 3.1).

All positions, padded lever arm placement, seat height and position, and lever arm height and position, were recorded during the baseline testing for each participant and subsequently used throughout their recovery-testing periods. All participants were instructed to place their hands on the sidebars, and to work as hard and as fast as possible against the resistance of the dynamometer. Furthermore, participants were verbally encouraged throughout the testing procedures (see figure 3).



Figure 3. Isolated strength measurements were performed on the Cybex Norm[™] Testing and Rehabilitation System.

3.1.3.1.1 Isokinetic Knee Extension and Flexion Strength Measurements

Participants underwent a warm up session (5-minute cycle at 75 W) prior to all muscle strength tests. Measurements of isokinetic knee extension and flexion torque were performed at $60^{\circ}/s$ ($1.57 \text{ rad}\cdot\text{s}^{-1}$) velocity torque in one continuous kicking motion. The range of motion for knee extension and flexion was from 90° to 0° and 0° to 120° , respectively (see figure 3.1 for an example of a knee extension).

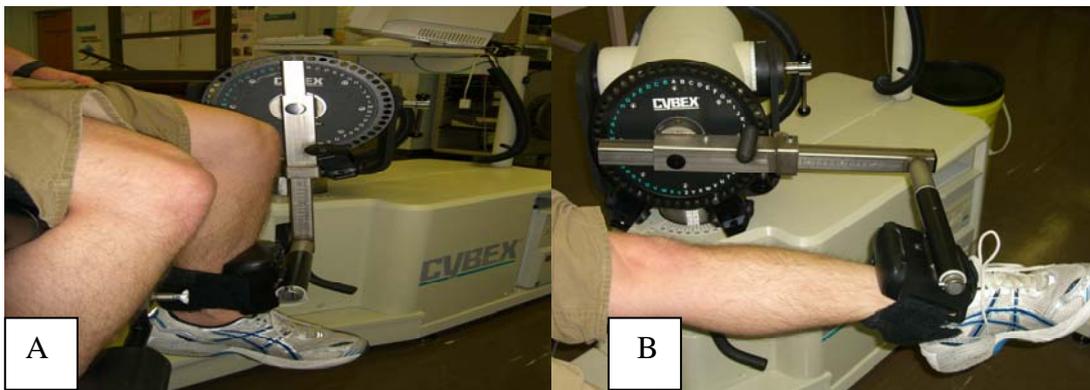


Figure 3.1. Range of motion for the knee extension from 90° (A) to 0° (B)

The participant started in a seated position with their knee aligned with the axis of the dynamometer lever arm and their leg in a resting 90° position. The participant then extended their leg against the resistance provided by the lever arm strapped above their ankle as quickly and forcibly as possible. At the top of the range of motion ($\sim 0^{\circ}$), participants lowered their leg against the resistance provided by the lever arm, as quickly and forcibly as possible, until the end of their range of motion ($\sim 120^{\circ}$). The participants repeated the knee extension/flexion kicking motion for three continuous repetitions.

3.1.3.1.2 Isometric Knee Extension Strength Measurement

Participants performed three maximal isometric knee extension contractions at a knee angle of 60° (0° = full knee extension) and of 5-s duration. A 20 second rest allocated between each isometric contraction, while a 60 sec rest was given between the isokinetic and isometric force measurements. The highest peak torque in the isokinetic knee extension/flexion and isometric knee extension was used as the criterion to measure maximum muscle strength in both forms of assessment. Once both sets were finished, the same strength measurements were performed on the contralateral leg.

3.1.3.2 Vertical (Countermovement) Jump

The countermovement jumps were performed with feet placed on a custom-built strain gauge force platform (Victoria University, Victoria, Australia; see figure 3.2). Participant's feet positions on the force platform were copied to ensure that during subsequent jumps, their feet positions were identical each time. Additionally, participants were instructed to squat with their knees flexed to approximately 90° . The height from their buttocks to the ground was measured and recorded using a purpose-built ruler with a flexible plate perpendicular to the long axis of the ruler. A knee flexion of 90° or more during the downward movement of the countermovement jump was required by participants to be classified as a successful jump. This was ensured by participants needing to feel the touch of the flexible plate prior to performing the jump.

The countermovement jump began with the participant standing in an erect position with knees fully extended (knee = 180°), hands on their hips and feet in the previous measured position. Once in position, the output of the force plate was reset to zero to negate the participant's weight. The countermovement jump commenced after a 3-second visual countdown period. On command, the participant rapidly performed the

descending phase of the countermovement jump, ensuring that they reached a knee flexion of 90° or greater. They then rapidly ascended and pushed vertically off the force platform with maximal effort in one continuous movement. Participants were instructed to keep their hands on their hips and to keep their body erect throughout the jump. Participants were required to complete 3 maximal effort countermovement jumps. A 20 second recovery period was allocated between jumps.



Figure 3.2. The vertical (countermovement) jump performed on a custom-built strain gauge force platform.

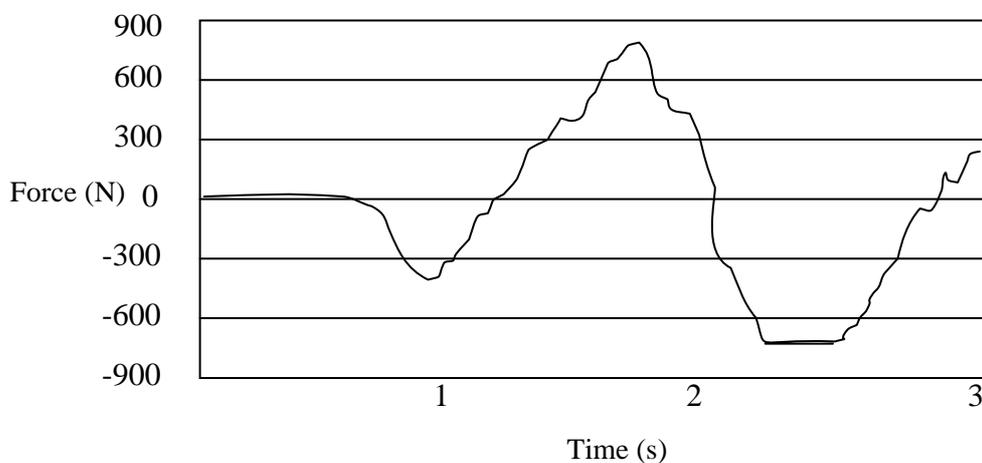


Figure 3.3. An example of a typical force-time curve derived from the force platform during a countermovement jump.

Custom written software (LabVIEW, National Instruments, Austin, Texas) automatically performed computations. Data were sampled at 750Hz for 3 seconds. Figure 3.3 is an example of a force-time graph of the countermovement jump. For this particular graph, the initial ascending portion of the force-time curve denotes the transition from eccentric to concentric contraction (1). The curve then begins to rapidly ascend to a peak, indicating maximum concentric activity, and then descends and reaches a maximum descent (2). This also signifies the concentric phase, most likely involving the toe off phase of the CMJ. Finally, finishing with an airborne (jump) phase and subsequent landing (spike in the curve, 3). The following parameters were derived from the positive portion of the force-time curve: maximum rate of force development (mRFD), peak force, and impulse. The peak of the slope between 1 & 2 indicates mRFD and was determined as the maximum gradient of samples of 10 consecutive data points (Hrysomallis and Kidgell, 2001). Peak impulse refers to the area under the curve between 1 and 2 and was calculated as the integral of force-time (Hrysomallis and Kidgell, 2001). Peak force was simply the highest value on the force time curve. Vertical height is a product of all the previous parameters, and was calculated by the method described previously by Komi and Bosco (1978). The height of rise of centre of mass can be calculated using the following formula:

$$\text{height} = \frac{v^2}{2g}$$

where v = vertical take-off velocity, and g is the acceleration due to gravity ($9.81 \text{ m} \cdot \text{s}^{-2}$) and is calculated by the height of rise from centre of mass, taking into account vertical take-off velocity and acceleration due to gravity (Komi and Bosco, 1978).

The highest value for each parameter was used for subsequent analysis. The way in which power is measured or indicated can vary. Some researchers choose to indicate power by impulse or force (Lyttle et al., 1996), while others find the average force and multiply it by the vertical distance travelled divided by the total amount of time spent on the force platform (Jones et al., 1999). By definition, power is the product of force and velocity. Both force and velocity can be derived from the force platform data, however the velocity data is erroneous. Integrating acceleration-time data can derive velocity. Acceleration data can simply derived from the force data if mass is constant ($a=f/m$). During the execution of a countermovement jump, the mass on the force platform is not constant. For this reason, power will not be directly measured in this thesis. Impulse and RFD will be used as indicators of power.

3.1.3.3 Reliability of data from the Cybex Norm[™] Testing and Rehabilitation

System and Force Platform

Reliability trials were conducted for all parameters of the cybex dynamometer and force platform. Within participant retesting produced a less than 5% coefficient of variance (CV) for each parameter measured.

3.1.4 Resistance Exercise Session: Damage protocol

The resistance exercise session designed to cause muscle damage consisted of three exercises; 1) leg press exercise using a leg press machine (Universal, Cedar Rapids, IA, USA); 2) leg extension exercise using a leg extension machine (Universal, Cedar Rapids, IA, USA); and 3) leg flexion using a leg curl machine (Universal, Cedar Rapids, IA, USA). All three exercises were designed to cause eccentric-induced damage to the quadriceps and hamstring muscles (modified version of Brown et al., 1996; Sorichter et al., 1997).

Two weeks prior to the exercise session, strength assessments (1RM) were performed on the left leg for each participant; the left leg would undergo the damage protocol, while the contralateral leg was used as the control. Thus, only one leg needed to be tested for muscle strength. The strength assessments consisted of the maximal weight that could be lifted once (1RM) in the three of the exercises described above: leg press, leg extension and leg flexion. The 1RM testing protocols followed that prescribed by the National Strength and Conditioning Association (NSCA) (Baechle et al., 2000). In short, the participant's maximal lift was determined within no more than five single repetition attempts following three progressively heavier warm up sets, thus causing minimal damage if any. Participants were required to successfully lift each weight before attempting a heavier weight. Each exercise was completed before the next attempt. Exercise execution guidelines were defined and adhered to for the successful completion of each lift (Baechle et al., 2000). A certified Strength and Conditioning Specialist (NSCA) supervised all lifts.

3.1.4.1 Muscle-Damaging Exercises

After preliminary baseline testing, participants performed a series of resistance exercises designed to elicit muscle damage of the knee extensor/flexor muscle groups. Participants performed all exercises on the left leg while the contralateral leg acted as the non-exercised control (rest). The first set of resistance exercises used a standard leg press machine and required the participant to lower a mass equivalent to 120% of their predetermined unilateral concentric 1RM. To perform an eccentric muscle action, participants were seated with their entire leg at 90° relative to the torso, knees flexed to ~90° and feet rested on the foot pedals. Participants had their weight lifted for them by the investigators, so that the legs finished at ~ 15° of flexion. The participant was then instructed to remove the control leg from the foot pedal, leaving only the left leg on the

foot pedal. From here the participant lowered the weight through an arc of $\sim 75^\circ$ (i.e. back to $\sim 90^\circ$). Each participant performed 40 (4 sets x 10 repetitions per set) eccentric muscle actions using the leg press machine with 3 min rest between each set. Participants were required to lower the weight at a fixed cadence (4 seconds) verbally given by an investigator.

Participants then performed the same number of repetitions and sets using the same leg on a standard knee flexion machine (figure 3.4). Again subjects were required to lower a weight, lifted for them by the investigators; equivalent to 120% of their predetermined unilateral concentric 1RM. Participants lied down on the apparatus, with their stomach facing down, and their thigh slightly flexed $\sim 15^\circ$ relative to their torso and the knee at $\sim 15^\circ$ relative to the thigh. The participant remained in the lying position and had the weight lifted by the investigators so that the knee was flexion at $\sim 90^\circ$ from horizontal. Participants performed extension of the knee, and lowered the weight through an arc of 75° . The same lowering cadence was maintained to assure a relatively constant lowering velocity.

The final exercise consisted of the same number of repetitions and sets using the same leg on a standard knee extension machine. Participants sat on the apparatus with their thigh at 90° relative to their torso and the knee at 90° relative to the thigh. The participant remained seated and had the weight lifted by the investigators so that the knee was extended at $\sim 15^\circ$ from horizontal. As mentioned previously, the weight was equivalent to 120% of their predetermined unilateral concentric 1RM. Participants performed flexion of the knee, and lowered the weight through an arc of 75° . Again the same lowering cadence was maintained to assure a relatively constant lowering velocity,

and all participants were verbally encouraged during each exercise to maintain effort throughout the range of motion.

If subjects were deviating from the required lowering cadence (as a result of fatigue), then a brief (30s) rest was allowed during the set, so that the participant could complete the set while still giving the appropriate effort. A maximum of two rests per set were allowed. Each participant would have experienced some degree of fatigue during the weight lowering, but all participants were able to complete the protocol. While it is possible that the participants could have lowered the weight at slightly different velocities when they were becoming fatigued; a constant timing during the lowering motion was maintained by verbal timing instructions to minimise this. At completion of the resistance exercises, participants rested for 10 minutes before performing the force measurements (as described in Section 3.1.3.1) on both the exercised and non-exercised leg and three vertical jumps (as described in Section 3.1.3.2).



Figure 3.4. An example of a muscle-damaging exercise.

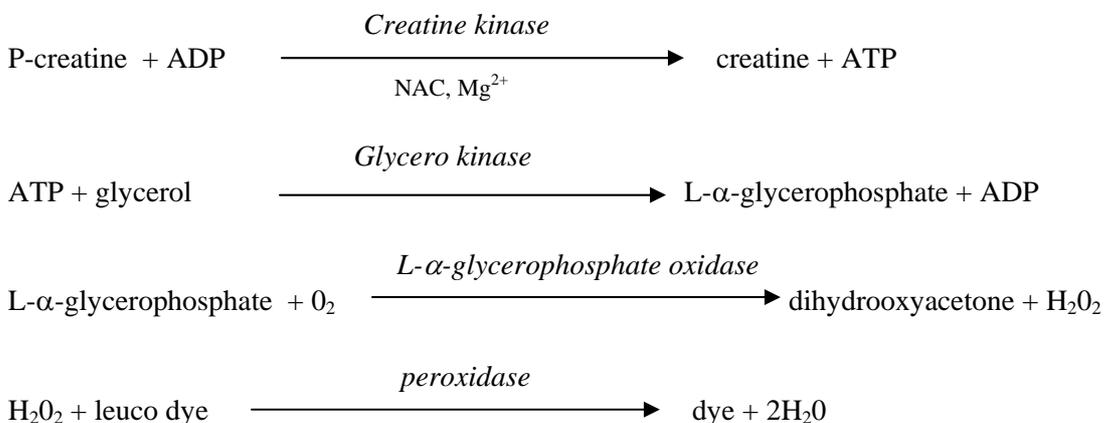
3.1.5 Blood Sampling

Approximately 10mls of venous blood was sampled from the antecubital fossa vein via catheterisation prior to the resistance exercise session (baseline), and 30 minutes, 1, 2, and 4 hours thereafter. Venipuncture technique was used to draw further blood samples at 1, 2, 3, 4, 7, 10 and 14-days after the resistance exercise session. The blood was immediately placed into an ethylenediaminetetra-acetic acid (EDTA) tube, and gently inverted and rolled several times. Blood was then transferred into eppendorf tubes and centrifuged at 3000 rpm for 15 min at 4°C. Plasma was removed and aliquoted into labelled eppendorf tubes and stored at -80°C for subsequent analysis of CK and LDH activity.

3.1.5.1 Creatine Kinase Measurement

Plasma samples were analysed by a 2-step enzymatic colorimetric process using a VITROS Chemistry System (VITROS 750 System, Melbourne Pathology, 103 Victoria Pde, Collingwood, Victoria, 3066). Creatine Kinase activity was determined according to the method of Horder *et al.* (1979).

Principle of analysis:

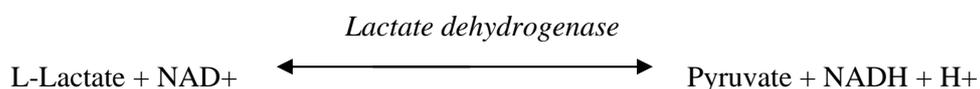


The presence of CK in the plasma sample ensures that the reaction moves from left to right. In the reaction, CK catalyses the conversion of creatine phosphate and ADP to creatine and ATP. In the presence of glycerol kinase (GK), glycerol is phosphorylated to L- α -glycerophosphate by ATP. Oxidation of L- α -glycerophosphate to dihydroxyacetone phosphate and hydrogen peroxide occurs in the presence of L- α -glycerophosphate oxidase (α -GPO). Finally, leuco dye is oxidized by hydrogen peroxide in the presence of peroxidase to form a dye. Reflection densities are monitors during incubation. The rate of change in reflection density is then converted to enzyme activity. The creatine kinase assay was run for 5 minutes at 37°C and at a wavelength of 670nm. Plasma samples were analysed for each time point (baseline, 30, 60, 120, 240min, 24, 48, 72, 96hr, Day 7, 10, 14). Normal reference values for creatine kinase are 22-198 U/L.

3.1.5.2 Lactate Dehydrogenase Measurement

Plasma samples were analysed using a single step enzymatic rate process requiring readings in duplicate on a UV-visible spectrophotometer (SHIMADZU UV-1700, SUZHOU Instrumental manufacturing Co. Ltd, China). Lactate Dehydrogenase activity was determined according to the method of Costill *et al.* (1976).

Principle of analysis:



The presence of LDH in the plasma sample ensures that the reaction moves from left to right. In the reaction, LDH catalyses the reversible oxidation of L-Lactate to pyruvate with the concurrent reduction of β -nicotinamide adenine dinucleotide (NAD) to reduced β -nicotinamide adenine dinucleotide (NADH)

The lactate dehydrogenase assay was run at 25°C using a reaction mixture (pH 7.0) containing 100mM Imidazole, 1.0mM pyruvate, 0.04% BSA and 25µM NADH. Each cuvette contained 900µl of the reaction mixture placed in the spectrophotometer set at 340nm. 100µl of the plasma sample was pipetted quickly into the cuvette and the reaction was monitored every 15 seconds for 3 minutes running Chart V3.6.4 for windows (AD instruments, NSW, Australia). The change in absorbance is directly proportional to the activity of lactate dehydrogenase in the plasma sample and was used to calculate lactate dehydrogenase activity (Refer to Appendix A for detailed methodology). Duplicate samples were performed for each time point (baseline, 30, 60, 120, 240min, 24, 48, 72, 96hr, Day 7, 10, 14). Normal reference values for lactate dehydrogenase are 85-285 U/L.

3.1.6 Statistical Analysis

Subject characteristics are reported as means \pm SD. All other values are reported as means \pm SE. It should be noted that muscle strength values of the exercised leg, performed on the Cybex NormTm Testing and Rehabilitation System, were expressed as percentage of pre-exercise values and normalised to contralateral controls. Vertical jump measurements only expressed as a percentage of pre-exercise values. Statistical evaluation of data was accomplished by using a two-way analysis of variance (ANOVA) with one between groups factor (supplement) and one repeated factor (time), with subsequent Tukey's Post-Hoc analysis. Where an interaction was found, the location of the difference was determined by a students t-test. Difference in participant characteristics and dietary analysis between groups was assessed by students' t-test. A p value of less than 0.05 was accepted for statistical significance.

3.2 ANIMAL MODEL: BUPIVACAINE PROTOCOL FOR LOCAL MUSCLE DAMAGE AND REPAIR

The bupivacaine protocol for local muscle damage and repair was employed in rat skeletal muscle to determine the mechanisms by which the dietary supplements whey protein and creatine may be exerting their effects. Two animal studies were designed to replicate the human experimental design and supplementation protocol. Study three examined the effects of creatine supplementation on the metabolic aspects of chemically-induced muscle damage in rat skeletal muscle (i.e. the calcium handling ability of the muscle), and subsequent damage and repair processors, while the fourth study examined the effects of whey protein on the regeneration (recovery) aspect of chemically-induced muscle damage.

A total of sixty-four male Sprague-Dawley rats (*Rattus norvegicus*) weighing 212.4 ± 3.5 (\pm SEM) were randomly separated into 3 groups: i) control n= 20; ii) creatine-supplemented n=23; and iii) whey protein-supplemented n=21. A total of 9 rats were not included in thesis analysis. 8 rats were directly affected by problems with the heating bath of the contractile testing apparatus, while 1 rat died of anesthetic overdose preceding contractile force testing. Following the same format as the human studies, the experimental design and dietary supplementation protocol for study three and four will be discussed in detail in subsequent chapters. Hence, only the procedures used in both studies will be discussed in this chapter.

3.2.1 Experimental Injury

All rats were lightly anesthetized with a domitor (10 mg.kg^{-1} body weight, Medetomidine Hydrochloride, Novartis Animal Health, NSW, Australia) and ketamine (6 mg.kg^{-1} body weight, Ketamine Hydrochloride, Novartis Animal Health, NSW,

Australia) combination via an intraperitoneal injection (i.p.), such that they were unresponsive to tactile stimuli. The extensor digitorum longus (EDL) and soleus (SOL) muscles of the left hindlimb were surgically exposed, with care taken to avoid damaging the nerve and blood supplies. A total of ~600µl of 0.5% bupivacaine (Bupivacaine Hydrochloride, Pharmacia and Upjoin (Perth) Pty. Ltd, WA, Australia) was injected into both EDL and soleus by two injections in each of the proximal, midbelly, and distal regions of the muscle using a 26 gauge needle (Terumo, USA) (see figure 3.5). This procedure ensured the degeneration of most fibres in the injected muscle (Rosenblatt, 1992). Previous research has shown that injection of Bupivacaine remains contained within the targeted muscle, leaving both nearby blood and nerve supplies intact (Louboutin et al., 1996). This model of injury is specific for muscle fibres (Rosenblatt and Wood, 1992), thereby eliminating the complicating factors of damage to motor nerves and associated blood vessels that occur with other models of muscle injury (Best et al., 2000). The contralateral EDL and soleus muscle served as an uninjured control and was not injected intramuscularly, as previous research has shown that little or no damage is caused by the insertion of the needle itself, or the injection of an otherwise harmless vehicle such as isotonic saline (Rosenblatt, 1992).

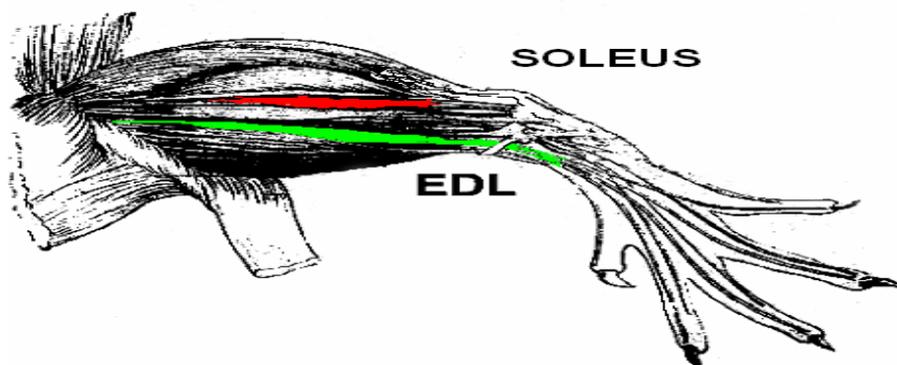


Figure 3.5 Muscles of the left lower hindlimb (taken from Greene, 1963). EDL (green) and soleus (red).

Following injury, the small incision was closed by Michel clips (Fine Science Tools, Nth Vancouver B.C, Canada), and swabbed with Betadine antiseptic (povidone iodine solution, Faulding Pharmaceuticals, SA, Australia). Previous research has shown that in this type of minor operative procedure, michel clips are a preferable option to sutures (Gregorevic et al., 2000). Following the surgical procedure, anesthesia was reversed using Antisedan ($125\mu\text{g}\cdot\text{kg}^{-1}$ body weight, Atipamezole Hydrochloride, Novartis Animal Health , Australasia Pty. Ltd, NSW, Australia) and animals either continued their dietary modification or were randomly separated into a control or whey protein-supplemented group.

At 7 and 14 days post-injury, rats received intra-peritoneal injections of $1\text{ ml}\cdot\text{kg}^{-1}$ body weight Nembutal ($10\text{ mg}\cdot\text{kg}^{-1}$ body weight, Rhone Merieux, QLD, Australia). The level of anaesthesia was monitored via corneal and footpad reflexes, and additional 0.1ml Nembutal injections applied when required. A longitudinal incision was then made into the injured hind leg of the rat. The EDL (predominantly type II fibres) was excised first, and fully tested (Section 3.3.1) before the same dissection procedure as described below was performed on the soleus (predominately type I fibres). The aim of fully testing the EDL muscle while other muscles remained intact in the anaesthetized animal, was to preserve the integrity of these muscles by maintaining blood and nerve supply for the 1-2 hours of experimental testing of each muscle.

3.2.2 Dissection Protocol

The proximal tendon of the EDL is located first and surrounding tissue and muscle was cleared away. Surgical silk size 2.0 (LA-55G Ethicon, Johnson & Johnson, NSW, Australia) was tied securely at the point of proximal tendon insertion of the EDL muscle. The same isolation and tying technique was performed at the point of distal

tendon insertion. The muscle was carefully dissected free from other tissues, starting from the proximal end, and placed into a horizontal custom-built plexiglass bath (see figure 3.6 and 3.7) containing a Krebs Henseleit Ringer solution [NaCl 118mM; KCL 4.75mM; Na₂HPO₄ 1mM; MgSO₄.7H₂O 1.18mM; NaHCO₃ 24.8mM; CaCl₂ 2.5mM and D-Glucose 11.0mM]. This HCO₃⁻ based buffer solution was maintained at a pH of 7.4, by being aerated with carbogen (95% O₂ and 5% CO₂) (BOC gases, Melbourne, Australia). All experiments were conducted at 25°C as isolated muscles do not remain viable for long periods of time if tested at 37°C. These experimental conditions facilitate optimal oxygen diffusion throughout the muscle, and maintain stability of functional measurements *in vitro* (Ryall et al., 2002).

3.3 EXPERIMENTAL VARIABLES

3.3.1 Contractile Properties

The proximal end of the EDL muscle was tied to a micromanipulator. The distal end was attached directly to a sensitive isometric force transducer (Research Grade 60-2999, Harvard Apparatus, South Natick, MA), thus mimicking the direction of force development of the muscle *in vivo*. The muscle was flanked by field-stimulating platinum plate electrodes attached to a stimulator (Grass S11 stimulator, Quincy, MA) coupled to an amplifier (CE-1000, Crown Instruments, Elkhart, IN, USA; see figure 3.8). Deflection of the transducer (which was previously calibrated with a calibration weight of known mass) with a muscle contraction produces a measurable electric signal, proportional to the force produced. Electrical signals were converted to digital signal by Powerlab4510 (ADInstruments, Castle Hill, NSW, Australia) running Chart V5.0.2 for windows.

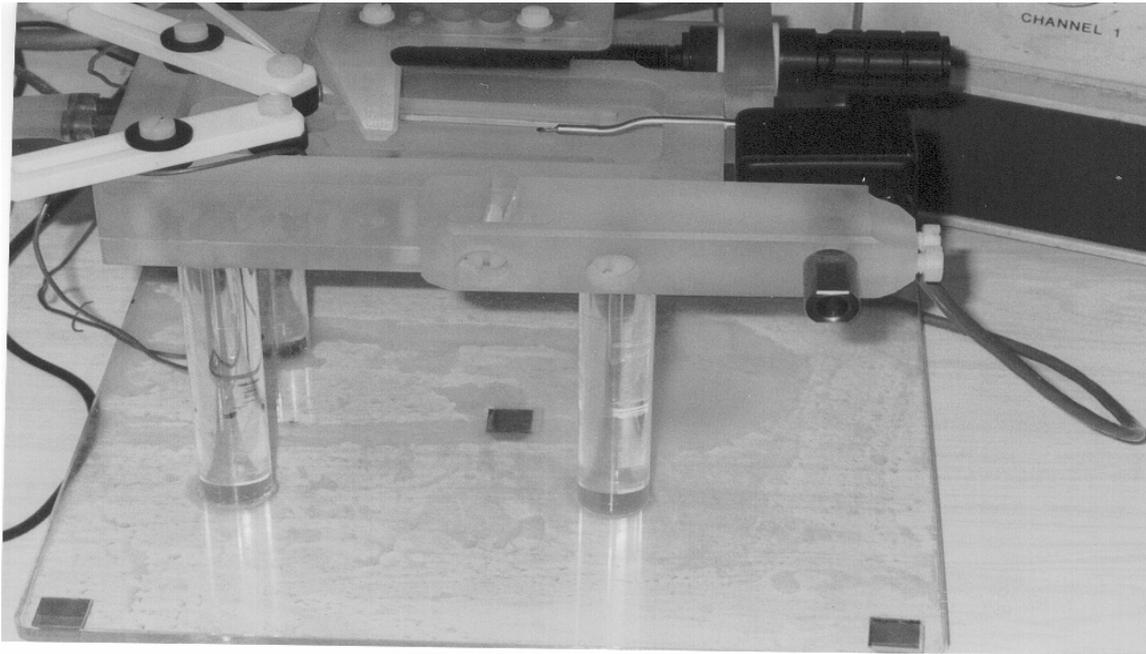


Figure 3.6. Horizontal custom-built plexiglass bath: apparatus used for contractile testing of muscle function

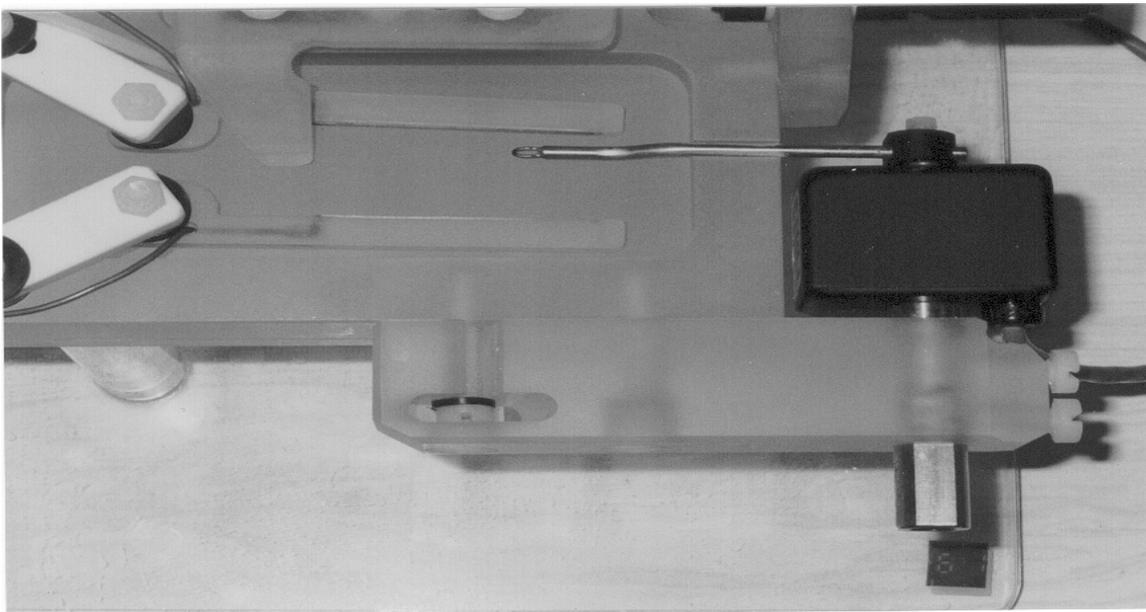


Figure 3.7. The field-stimulating platinum plate electrodes used to stimulate the muscle during contractile testing.



Figure 3.8. The field-stimulating platinum plate electrodes are attached to a (A) stimulator (Grass S11 stimulator, Quincy, MA) coupled to an (B) amplifier (CE-1000, Crown Instruments, Elkhart, IN, USA).

Stimulation of muscle contraction was produced using supramaximal square wave electrical pulses of 0.2msec duration. Optimum contractile length (L_0) of the muscle (the length at which maximal contractile force is produced) was established with a series of twitch contractions, and fine adjustments in muscle length (i.e. slowly stretched out). This ensures maximal activation of each muscle was attained and thus comparisons between muscles could be made. Muscle length at L_0 was measured with a vernier caliper at the point at which the muscle is connected to its tendons.

Having established L_0 , single muscle stimulations (eliciting muscle twitches) were elicited at 40V and increased by 2 volts, until a plateau in twitch force (P_t) was reached (optimal voltage). The idea of these stimulations was to stretch the series elastic elements of the muscle, therefore allowing a good release of calcium and to ensure that the muscle is functioning properly.

A single tetanic stimulation at 100 Hz was performed, with three minutes recovery, followed by a single twitch. The tetanic stimulation is performed to test that the knots are tied correctly, before subsequent experiments can be continued. The single twitch performed at the end of 3 minutes recovery enables us to observe if there is any post-tetanic potentiation, due to sustained phosphorylation of light chains on the myosin heads from the previous tetanic stimulation. This was evident by an increase in the P_t that was previously observed. A lower P_t indicated that the knots had slipped. In this case, the muscle is lengthened out slowly again, until optimal length was re-established.

Force-frequency measurements were used to establish optimal stimulus frequency and maximum isometric tetanic tension (P_o). Single tetanic contractions were stimulated over frequencies ranging from 10Hz to 140Hz, with 3 minutes recovery in-between contractions (see figure 3.9 and 3.10). Optimal stimulus frequency and maximum tetanic force (P_o) was established when complete fused tetanus occurred. The force developed at each frequency was expressed relative to the peak force obtained. The optimal length, voltage and stimulus frequency established was used for the remainder of the experiments.

Three P_t measurements were performed. For each of these twitch contractions, the following measurements were made;

- Peak Force (P_t)
- Time to Peak Tension (TTP)
- Half Relaxation Time ($1/2$ RT)

Time to peak tension (TTP) is an unrefined indicator of the Ca^{2+} release process, the binding to troponin-C and the activation of actin and myosin. Half-relaxation time ($\frac{1}{2}\text{RT}$) is an approximate measure of the ability of the sarcoplasmic reticulum Ca^{2+} -ATPase to re-sequester Ca^{2+} (see figure 3.9 & 3.10).

Following contractile testing, the muscle was removed from the bath. Under a microscope (Lerica, ZOOM 2000TM Z45V, China) surgical silk loops and other debris (including proximal and distal tendons) were removed. The muscle was then blotted dry on filter paper and weighed on an analytical balance (OHAUS, GalaxyTM 160D, Ohaus Corporation, NJ, USA). The muscle was then divided into two sections; one half was snap-frozen in isopentane, cooled in liquid nitrogen. The muscle was placed in a labelled cryule, and stored in liquid nitrogen (-172°C), for later determination of fibre size and proportions, and percentage of damaged area (rate of regeneration). The other portion was immediately snap frozen and stored in liquid nitrogen (-172°C) for later assessment of muscle metabolites and protein analysis. The same procedures were performed on damaged soleus muscle and the contralateral non-damaged EDL and soleus muscles.

Following removal of all muscles required for analysis, rats were killed by overdose of anaesthetic. Peak tetanic forces were expressed relative to the muscles cross-sectional area (specific force-sPo) enabling comparisons between muscles of different area and length. Cross-sectional area (X-A) was calculated by dividing MM (muscle mass) by (fibre-length x density), where fibre length is obtained from the FL/ML (fibre length: muscle length) ratio, which is 0.44 in the EDL and 0.71 in the soleus (Brooks and Faulkner, 1988) and density equals 1.06 g/cm^3 (Close, 1972). The degeneration and regeneration induced by bupivacaine injection does not affect this ratio, because new

fibers grow within the preexisting basal laminae, and therefore the angle of muscle fiber insertion is not altered (Gregorevic et al., 2002). Specific force was calculated by dividing P_o by the X-A (refer to Appendix B for example calculation).

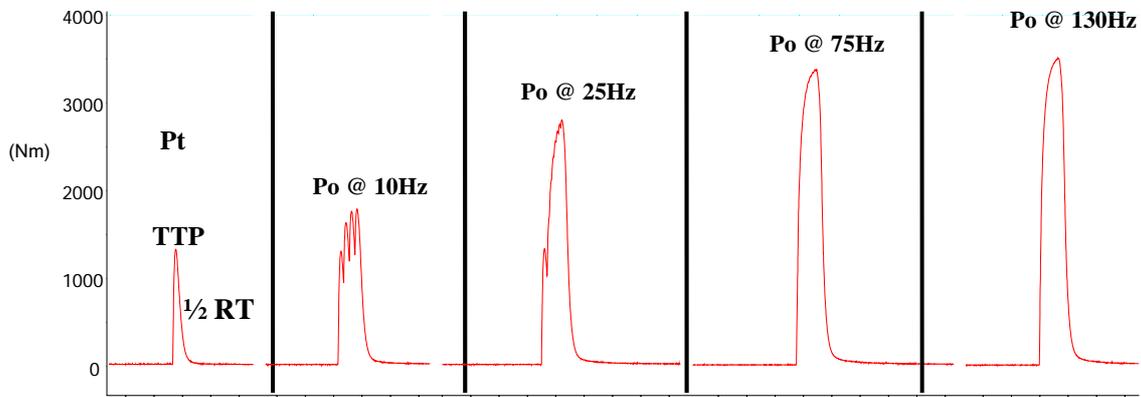


Figure 3.9. A typical example of an EDL twitch contraction (Pt) showing how TTP and $\frac{1}{2}$ RT was measured. TTP = time from the start of the contraction to peak tension; $\frac{1}{2}$ RT = half relaxation time from peak tension. Single tetanic contractions (P_o) were stimulated over frequencies ranging from 10Hz to 130Hz.

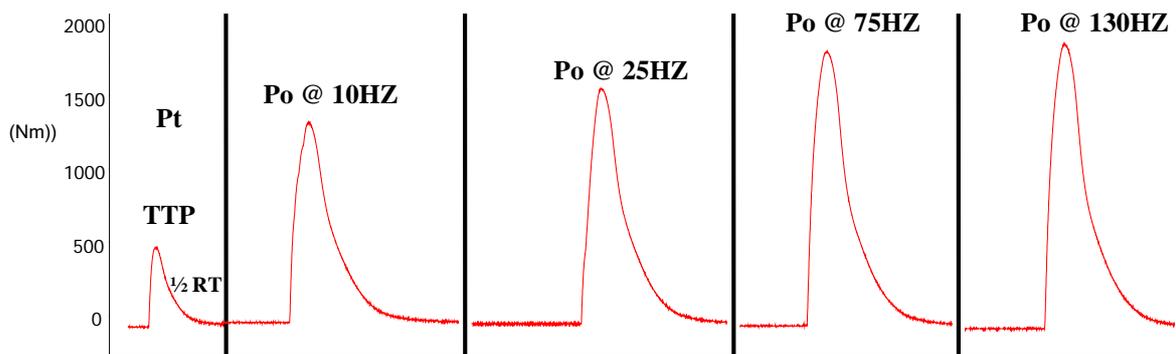


Figure 3.10. A typical example of a soleus twitch contraction (Pt) showing how TTP and $\frac{1}{2}$ RT was measured. TTP = time from the start of the contraction to peak tension; $\frac{1}{2}$ RT = half relaxation time from peak tension. Single tetanic contractions (P_o) were stimulated over frequencies ranging from 10Hz to 130Hz.

3.4 HISTOLOGICAL ANALYSIS OF MUSCLE DAMAGE AND REGENERATION

Histological procedures were performed to examine the effects of dietary supplementation on general tissue morphology of both injured and uninjured muscle, following 7 and 14 days of treatment.

Muscle samples used for contractile experiments were taken out of storage in liquid nitrogen, and placed in a cryostat microtome at -20°C (Microm GmbH D-6900 Heidelberg West Germany). Each muscle sample was mounted onto a chuck with Tissue-Tek (OCT compound). Transverse sections (10 μm thick) were cut from each muscle sample as close to the midbelly as possible and placed onto a microscope slide. Sections were fixed in 10% buffered formol saline solution and stained with routine haematoxylin and eosin (H&E; refer to Appendix C for detailed methodology). Haematoxylin stains the nuclei and other basophilic structures blue, while eosin stains the cytoplasm and acidophilic structures light to dark red. Digital images of muscle sections were obtained using a camera (Zeiss Axiolab; Carl Zeiss GmbH, Jena, Germany) attached to an upright Carl Zeiss light microscope (Zeiss Axiolab; Carl Zeiss GmbH, Jena, Germany) at 5x, 10x, and 20x magnification (see figure 3.12, 3.11 and 3.13, respectively).

Each section was critically examined in a single blinded manner, using an appropriately calibrated Analytical Imaging Station (AIS, v6.0 Imaging Research, Ontario, Canada). Slide inspection by Dr. Alan Hayes ensured that histological analysis was accurate and unbiased. The mean cross sectional (CSA) of individual fibres was calculated by interactive determination of the circumference of at least 150 adjacent fibres from the center of each muscle section.

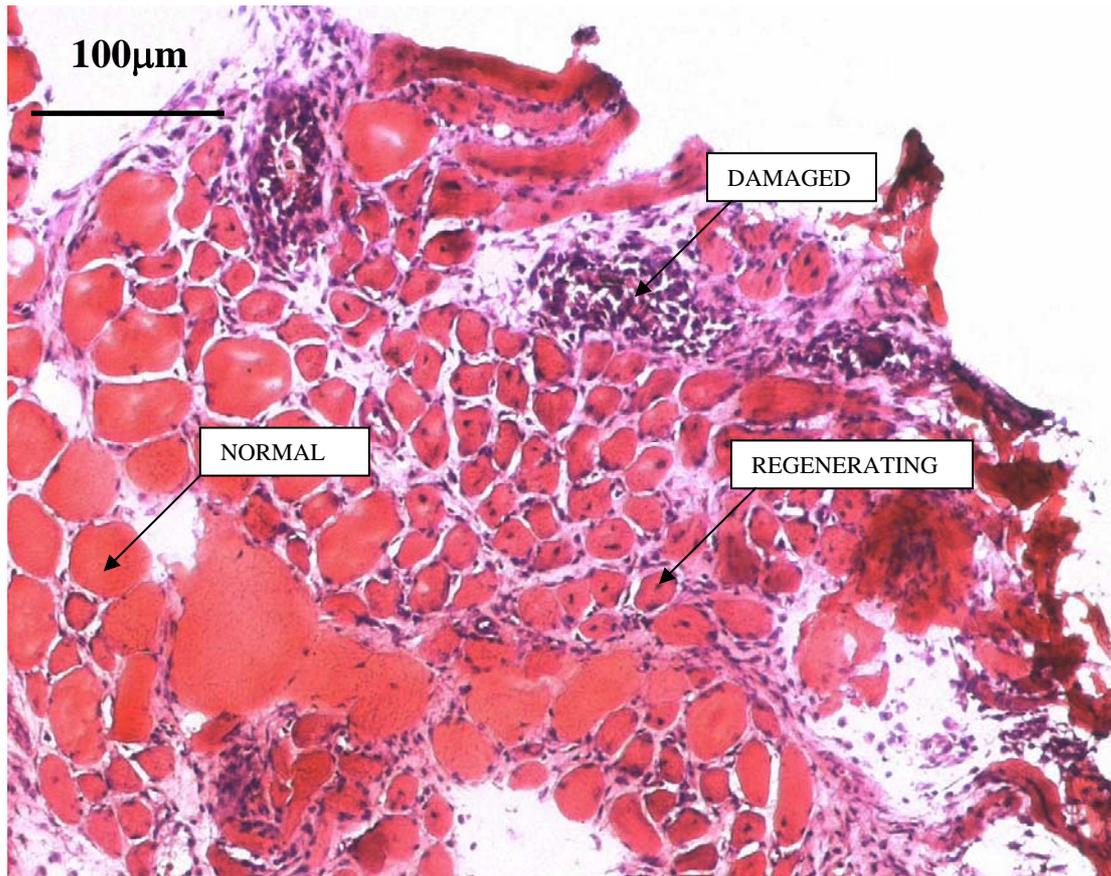


Figure 3.11. A typical cross-sectional area of a damaged soleus muscle fibre at day 7 stained with H & E at 10x magnification displaying all three distinct regions. A) Muscle damage (necrosis, inflammatory cell accumulation and few small-multinucleated regenerating fibres); B) Regenerating fibres (muscles showing larger regenerating fibres with centrally located nuclei); and C) Normal fibres (muscles showing multi-nucleated intact fibres). Note some loss of muscle section morphology is experienced during sectioning owing to extreme fragility of muscle experiencing total population degeneration

A quantitative estimate of relative affected (damaged and/or regenerating) area within cross sections of muscle samples was achieved by using a similar method as described above. Digital image of each muscle sample taken at 10x magnification; modified to enhance the colour of each nuclei (Adobe Illustrator, version 10). Each muscle section was categorised into three distinct regions (see Figure 3.11); A) Muscle damage (necrosis, inflammatory cell accumulation and few small-multinucleated regenerating

fibres), B) Regenerating fibres (muscles showing larger regenerating fibres with centrally located nuclei), and C) normal fibres (muscles showing multi-nucleated intact fibres). Together, these procedures provided a good impartial, qualitative and quantitative method of describing differences between supplemented groups.

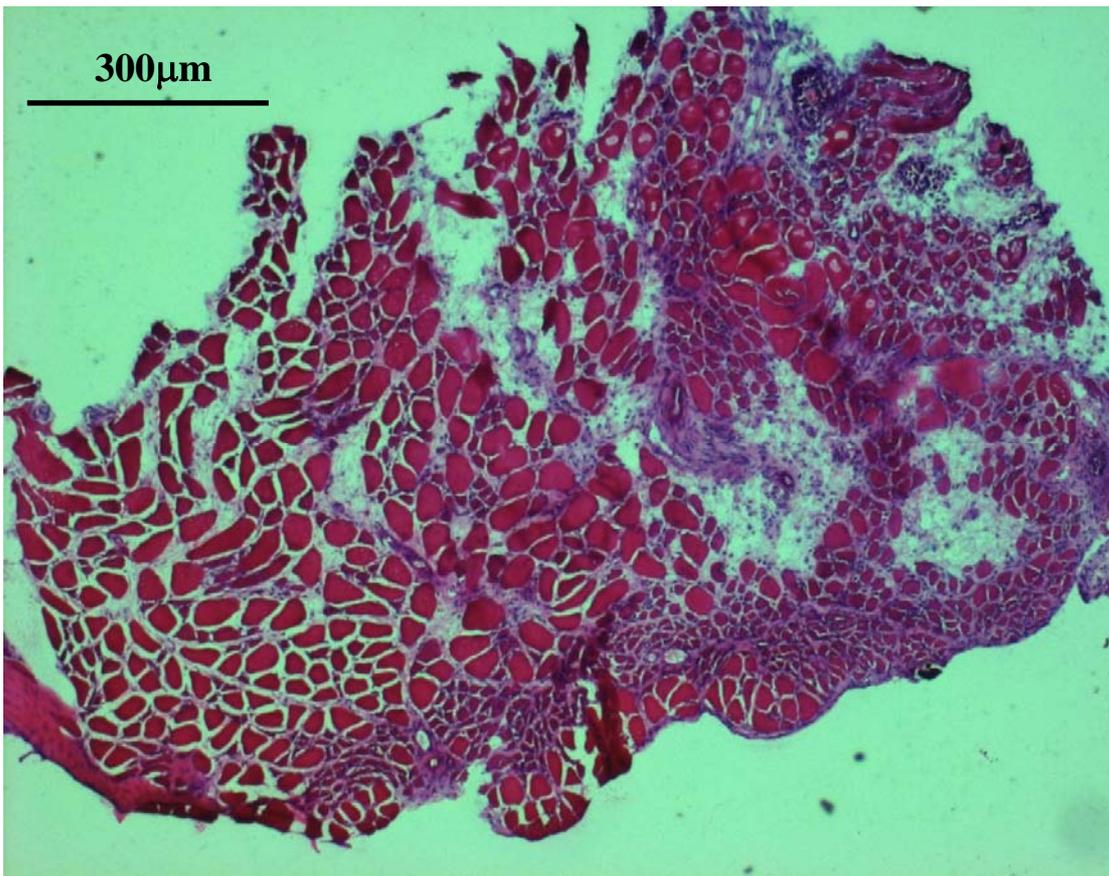


Figure 3.12. A typical cross-sectional area of a damaged EDL muscle fibre at day 7 stained with H & E at 5x magnification. Note widespread muscle fibre degeneration and inflammatory cell accumulation throughout the entire muscle fibre CSA. Some loss of muscle section morphology is experienced during sectioning owing to extreme fragility of muscle experiencing total population degeneration

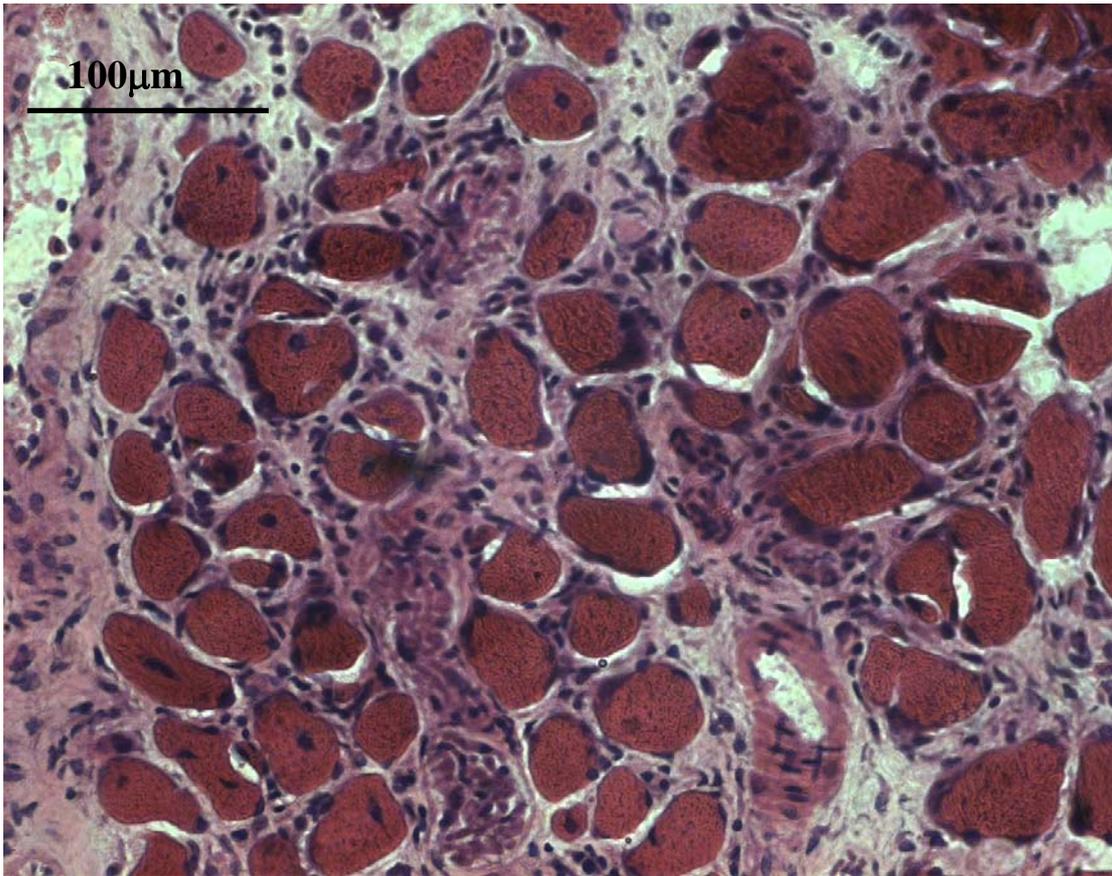


Figure 3.13. A typical cross-sectional area of a damaged EDL muscle fibre at day 7 stained with H & E, at 20x magnification. Note widespread muscle necrosis and inflammatory cell accumulation between regenerating muscle fibres indicated by the centralised nuclei.

3.5 PROTEIN ANALYSIS

The muscle protein content was assessed in both injured and uninjured muscles, following 7 and 14 days of treatment, according to a modified version of methods described by Beitzal et al. (2003). Muscle samples used for contractile experiments were taken out of storage in liquid nitrogen, and allowed to thaw for approximately 3 min at room temperature. The muscle sample was placed on a petri dish over ice, dissected free of visible fat and connective tissue, minced finely and weighed. Approximately 10-20mg of the muscle was placed in a pre-cooled 5 mL glass tissue grinder (Kontes, 885502-0021, NJ, USA). 50µL of ice-cold homogenizing buffer

solution A (containing in mM: KCl 50, KH₂PO₄ 10, MgCl₂·6H₂O 2, EDTA 0.5, DTT 2) was added for every mg of muscle (i.e. a 1:50 wet weight:volume dilution).

A ground glass pestle (Kontes 885501-0021, NJ, USA) was then used to grind the tissue lightly with a rolling motion until no visible fragment of muscle remained. The resulting solution was transferred to an eppendorf centrifuge tube, which was stored on ice. 200 µl of crude homogenate was transferred into a labelled cryule and immediately snap frozen and stored in liquid nitrogen for later assessment of total protein concentration. The remainder of the homogenate was centrifuged at 5°C for 10 minutes at 1000G (Biofuge 28RS, Heraeus Sepatech, West Germany). The supernatant, containing the cytosolic proteins was discarded and the pellet, containing the contractile proteins, was suspended in 200µL of the ice-cold homogenizing buffer solution A. This was then resuspended in the tip of a glass pasteur pipette, ensuring no air bubbles, for 2-3 minutes until there were no remaining lumps. The suspension was then transferred into a labelled cryule and immediately snap frozen and stored in liquid nitrogen for later assessment of contractile protein concentration.

Protein concentration was determined according to methods described by Bradford (1976) using a Bradford Protein Assay (Bio-Rad Protein assay, Bio-rad Laboratories, Hercules, CA, USA). A series of standard protein solutions were prepared from a bovine serum albumin (BSA) stock solution (20mg/mL) in the range 0.1 to 1.2 mg/mL, increasing in 1mg/mL increments, to determine the amount of protein in each sample (mg/ml). Bradford assay dye was prepared by diluting 1 part dye reagent concentrate with 4 parts distilled, deionized water through whatman #1 filter paper (Whatman International, Ltd, Maidstone, England) to remove particulates.

Blanks and standards was analysed in triplicate. Total protein and contractile protein samples (diluted 1:8) was also analysed in triplicate. Blanks, standards and samples were added to appropriately labelled tubes, containing the diluted assay reagent dye. These samples were left to stand for 10 minutes, which is enough time for the reaction to reach completion. At the end of 10 minutes, samples from each kimble tube were transferred to a microcuvette (1941 Kartell, S.p.A, Italy) and read on a UV-visible spectrometer at 550nm against the reagent blank. Spectral readings were recorded for later analysis.

3.6 METABOLITE PROPERTIES

3.6.1 Freeze Drying Process

Muscle samples of both injured and uninjured muscles, following 7 and 14 days of treatment, previously stored at -172°C in liquid nitrogen were removed and cut in a cooled cryostat. Approximately 5 mg of dry muscle is required for the extraction process. The normal dry weight: wet weight ratio is approximately 1 to 4 (23%). Thus, the wet weight was measured to ensure sufficient muscle (approximately 15mg) was obtained. Holes were punctured in the top of eppendorf tubes which were placed in a freeze dryer (Edwards Modulyo, Edwards High Vacuum, Britain, England). Samples were freeze dried for a minimum of 36 hours.

At the completion of the freeze-drying, muscle samples were transferred to a dessicator at room temperature ($20-23^{\circ}\text{C}$). Muscle samples were weighed to determine dry weight and enable the dry weight: wet weight ratio to be calculated. The benefit of freeze-drying is that it allows the muscle samples to be handled at room temperature.

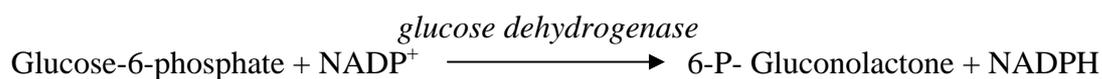
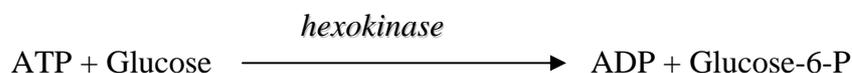
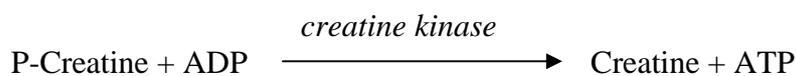
3.6.1.1 Metabolite Extraction Process

The metabolite extraction process was performed in accordance with the method of Harris et al. (1974). Each muscle sample was powdered in a crucible for approximately 15–20 minutes. These are periodically observed under a microscope, with any connective tissue or blood removed from the powder. The extraction process, performed in on ice, began when 250 μ L of ice cold 0.5M perchloric acid (PCA)/1mM ethylenediaminetetra-acetic acid (EDTA) was added to a labelled eppendorf tube containing 2mg of powdered muscle. The perchloric acid is a strong oxidizing agent and extracts all of the acid-soluble metabolites, at the same time denaturing the proteins and causing them to precipitate out. The suspension was vortexed and tapped intermittently for 10 minutes to remove fibres from the wall of the vessel. The samples were then placed in a pre-cooled (0-2 $^{\circ}$ C) centrifuge and spun at 28,000 RPM for 2 minutes. Once finished, the eppendorf tubes were placed back in the ice. 200 μ L of the supernatant was removed by an aliquot, without disturbing the pellet, and placed into a second eppendorf tube. 50 μ L of 2.1M ice cold KHCO_3^- was added to each tube, and left to stand on ice for 5 minutes. The mixtures neutralize, with the potassium and perchloride ions forming another precipitate. The samples were again centrifuged at 28,000RPM for 2 minutes. The supernatant was removed with pasteur pipettes, placed in a labelled cryule, and stored in the ultra-freezer (-172 $^{\circ}$ C) for subsequent metabolite analysis of ATP, PCr, and Cr.

3. 6. 2 ATP and PCr Analysis

The extract was analysed using a 3-step enzymatic process requiring readings in triplicate on a fluorometer (Turner Fluorometer model 112, Sequoia-Turner Corporation, USA). ATP and PCr levels were determined according to the method of Lowry and Passonneau (1972).

Principle of analysis:



An NADH standard curve was recorded on a UV-visible spectrophotometer at 340 nm. The change in fluorescence of the internal ATP, CP standards was then checked against the NADH standard curve (refer to Appendix D for detailed methodology).

Blanks, ATP, CP and NADH standards and samples are analysed in triplicate. A cocktail reagent was made containing everything with the exception of hexokinase and the ADP/creatine kinase solution. The presence of excess ADP and creatine kinase insures that the reaction moves from left to right. The first reading (R1) taken gives an indication of any residual endogenous NADPH that may be present.

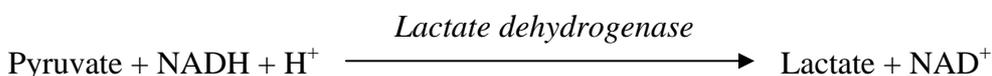
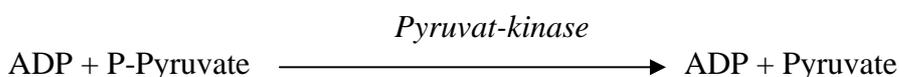
Dilute hexokinase solution was added to each tube. This was the catalyst for reaction 2. This reaction produces glucose-6-phosphate, which is the only thing required for reaction 3 to proceed. The samples are left to run for 30 minutes, which is enough time for equilibrium to be reached. The second reading (R2) is recorded. R1 subtracted from R2 gives the change in NADPH concentration. As all of the NADPH from R2 has come from reaction 2 proceeding, the amount of NADPH gives the value of ATP concentration in the muscle sample.

However, there is still PCr left. The CK/ADP solution is added and this runs the reaction producing ATP. The dilute hexokinase is still present in solution and it catalyses the two-step process of NADPH. A third reading (R3) is taken. The NADPH produced in this step can be directly attributed to the amount of PCr present.

3.6.3 Creatine (Cr) Analysis

The extract was analysed using a 3-step enzymatic process (Turner Fluorometer model 112). Creatine (Cr) levels were determined according to the method of Lowry and Passoneau (1972).

Principle of analysis:



A 15 mM NADH standard was recorded on a UV-visible spectrophotometer at 340 nm. The change in fluorescence of the internal CP standards was then checked against the 15 mM NADH standard (refer to Appendix E for detailed methodology).

Blanks and CP standards were analysed in triplicate, samples were analysed in duplicate. The cocktail reagent made contained everything with the exception of creatine kinase. The presence of excess creatine kinase ensures that the reaction moves from left to right. The first reading (R1) is taken after 15 minutes incubation. This gives an indication of any residual endogenous NADH that may be present.

A creatine kinase/0.05% BSA solution is then added to each tube. The samples are left to run for 60 minutes. This is the catalyst for reaction 2. This reaction produces pyruvate; pyruvate is the catalyst for reaction 3. The second reading (R2) is recorded. R1 subtracted from R2 gives the change in NADH concentration. As all of the NADH from R2 has come from reaction 2 and 3 proceeding, thus, the amount of NADH will give a value of creatine concentration in the muscle sample.

3.7 Statistical Analysis

All values are reported as means \pm SEM. Statistical evaluation for each muscle group (EDL and soleus) and recovery time point (day 7 and day 14) was accomplished by using a two-way analysis of variance (ANOVA) with one between groups factor (supplementation) and one repeated factor (damage vs contralateral. Where an interaction was found, the location of the difference was determined by a one-way ANOVA. Difference in animal morphology characteristics between groups was assessed by students' t-test. Supplementation protocol between groups was assessed by chi-square test. A P value of less than 0.05 was accepted for statistical significance.

While the animal studies will be reported separately in the subsequent chapters to align them with the human studies, the data was compared together as both studies utilized the same control group.

CHAPTER 4

Effects of Creatine Supplementation on Muscle Force Recovery after Eccentrically-Induced Muscle Damage in Healthy Individuals

4.0 INTRODUCTION

Creatine monohydrate (CrM) as a nutritional supplement and ergogenic aid for athletes has been extensively studied over the past 20 years (for reviews see Williams and Branch, 1998; Kreider, 2003; Rawson and Volek, 2003). Creatine is a natural compound found in muscle in both unphosphorylated (Cr) and phosphorylated (phosphocreatine, PCr) forms (Harris *et al.*, 1974). At sub-cellular sites with high-energy requirements, CK catalyzes the transphosphorylation of PCr to ADP to regenerate ATP. Thus, PCr is intimately involved in energy metabolism for muscle contraction, by preventing a depletion of ATP levels.

Several reports have shown that the use of oral creatine supplementation can increase performance and recovery in humans under a variety of different testing conditions, in particular short-term, high intensity exercise (Harris *et al.*, 1992; Casey *et al.*, 1996; Volek *et al.*, 1999), although this has not been shown in all studies (Cooke *et al.*, 1995; Snow *et al.*, 1998). Notwithstanding, creatine supplementation increases intramuscular Cr and PCr levels and therefore may enhance performance by: improving ATP resynthesis via the CK/PCr system (Korge *et al.*, 1993; Rossi *et al.*, 1998); augmenting gains in body mass and lean body mass (LBM) (Vandenbergh *et al.*, 1997; Kelly and Jenkins, 1998); directly influencing protein synthesis (Ingwall, 1976); and increasing muscular strength and power (Tarnopolsky *et al.*, 1997; Willoughby and Rosene, 2001; 2003).

While extensive literature have attempted to link CrM supplementation with increased muscle Cr stores and improvements in exercise performance (Greenhaff *et al.*, 1993; Birch *et al.*, 1994; Earnest *et al.*, 1995; Casey *et al.*, 1996; Febbraio *et al.*, 1996; Kreider *et al.*, 1998; McKenna *et al.*, 1999) the efficacy of CrM supplementation to improve muscle recovery after injury has received little attention to date. Unaccustomed or high-strain exercise, in particular those that incorporate eccentric exercises, can lead to disruption of the normal muscle ultrastructure and altered SR function, which results in an increase in intracellular calcium that can activate degradative pathways (Belcastro *et al.*, 1998). In addition, eccentric exercise causes damage or degradation of muscle contractile proteins, which may contribute to the impairment of muscle function (Allen, 2001). Indirect markers of exercise-induced muscle damage include reduced muscle strength (maximum voluntary contraction; MVC), muscle soreness (DOMS), and release of myocellular proteins, such as CK and LDH into the plasma (Nosaka *et al.*, 1999; Friden and Lieber, 2001).

There is evidence to suggest that depletion of intramuscular [PCr] leads to an accumulation of [ADP] within the vicinity of the SR Ca^{2+} -ATPase pumps, and thus reduces SR pump function to remove intracellular Ca^{2+} from the muscle (Korge *et al.*, 1993). Therefore, increased PCr levels' following creatine supplementation has the potential to delay/prevent the accumulation of [ADP] by re-phosphorylating ADP to ATP via the PCr/CK system. But more importantly, will help maintain the muscles ability to remove intracellular Ca^{2+} from the muscle, and thus reduce the initial amount of damage caused by degradative pathways (for example calpain), which are activated by increased intracellular Ca^{2+} levels.

In addition, creatine may act as a co-regulator, or direct manipulator of gene transcription of amino acid pools following resistance training, and thus, increase protein synthesis during the recovery period post-exercise (Willoughby and Rosene, 2001; 2003). Increased protein synthesis, in particular myofibrillar proteins will therefore enhance the regeneration of new muscle fibres.

Thus, the purpose of this study was to examine the effects of creatine monohydrate consumption on force recovery after eccentrically-induced muscle damage in healthy individuals. We hypothesised that following eccentric-induced damage, muscle strength will be restored earlier and the release of muscle proteins will be decreased, indicative of reduced damage following creatine supplementation.

4.1 METHODS

4.1.1 Participants

Fourteen healthy untrained males volunteered for this study. Subjects were generally students of Victoria University. Descriptive characteristics of the subjects are presented in table 4. Subjects were (a) non-smokers; (b) had not participated in resistance training for at least six months; and (c) had not ingested any ergogenic supplement for a 24-week period prior to the start of supplementation. All participants were informed verbally, as well as in writing, as to the objectives of the experiments, together with the potential associated risks. All participants signed an informed consent document approved by the Human Research Ethics Committee of Victoria University of Australia. All procedures conformed to National Health and Medical Research Council guidelines for the involvement of human subjects for research.

4.1.2 Dietary Supplementation.

Subjects were randomised in a double-blind placebo-controlled fashion into 2 groups: an isocaloric carbohydrate placebo group (n= 7); and creatine-supplemented group (n= 7). The supplements were provided to the participants in identical, unmarked, sealed containers. Supplements were supplied by AST Sports Science, Golden, Colorado USA. Two weeks prior to the resistance exercise session, subjects consumed a loading dose of $20\text{g}\cdot\text{day}^{-1}$ of either the supplement (14% creatine monohydrate and 86% isocaloric carbohydrate) or placebo (100% isocaloric carbohydrate) for a period of 5 days. This was followed by a maintenance dose of $2\text{g}\cdot\text{day}^{-1}$ for a period of 9 days. Following the resistance exercise session, subjects continued to consume the supplements at a dosage rate of $2\text{g}\cdot\text{day}^{-1}$ during the 14-day recovery period. This supplementation protocol has been previously shown to be effective in elevating muscle Cr and PCr levels (Harris *et al.*, 1996; Van Loon *et al.*, 2003).

Subjects were advised to mix the supplement in water and consume the required dosage in 2 equal serves over the period of the day, i.e. breakfast and dinner, 1 hour before eating, during the loading phase. During the maintenance phase and following the resistance exercise session, participants reduced the intake to 1 serve per day, 1 hour before lunch. Nutritional intake was monitored via written dietary diary sheet. Subjects were instructed to record their nutrient intake for a 7-day period. All recordings were assessed using Nutritionist PRO (First Data Bank) software.

4.1.3 Isokinetic/Isometric Strength and Vertical (Countermovement) Jump Measurements

Muscle strength and performance measurements were examined by voluntary isokinetic knee flexion and isokinetic/isometric knee extension using the Cybex NormTm Testing and Rehabilitation System and a vertical jump (CMJ) performed on a custom-built strain gauge force platform (as previously described in section 3.1.3.1 & 3.1.3.2). All strength and performance measurements were performed prior to- and immediately following the exercise session, and on day 1, 2, 3, 4, 7, 10 and 14 post-exercise. It should be noted that muscle strength values of the exercised leg performed on the Cybex NormTm Testing and Rehabilitation System, were expressed as percentage of pre-exercise values and normalised to contralateral controls. Parameters of vertical jump performance were expressed as a percentage change from pre-exercise values. Previous research has shown this to be a successful means of reporting muscle strength and performance data (Rinard *et al.*, 2000; Byrne and Eston. 2002).

4.1.4 Blood Sampling and Analysis

Blood was sampled from the antecubital fossa vein prior to, and 30 min, 1, 2, and 4 hours following, the resistance exercise session and on days 1, 2, 3, 4, 7, 10 and 14 post exercise. Blood was immediately placed into EDTA tubes and centrifuged at 3000 rpm for 15 min at 4°C (as previously described in section 3.1.5). Plasma was stored at -80°C for subsequent analysis of CK and LDH activity.

4.1.5 Statistical Analysis

Subject characteristics are reported as means \pm SD. All other values are reported as means \pm SE. Statistical evaluation of data was accomplished by using a two-way analysis of variance (ANOVA) with one between groups factor (supplement) and one

within group repeated factor (time), with subsequent Tukey's Post-Hoc analysis. Where an interaction was found, the location of the difference was determined by a students t-test. Difference in participant characteristics and dietary analysis between groups was assessed by students' t-test. A P value of less than 0.05 was accepted for statistical significance.

4.2 RESULTS

4.2.1 Participant Characteristics

At baseline there were no differences in the age, body weight or strength level (1RM) between the two groups (see table 4).

Characteristics	Placebo carbohydrate n = 7	Creatine monohydrate n = 7
Age (yrs)	21.7 ± 2.9	22.6 ± 1.6
Weight (kg)	74.4 ± 6.5	77.9 ± 11.8
Leg Press 1RM (kgs)	189 ± 35	184 ± 34
Leg Extension 1RM (kgs)	88 ± 21	80 ± 20
Leg Flexion 1RM (kgs)	59 ± 34	75 ± 28

Table 4. Participants' baseline characteristics. Values are means ± SD of all fourteen males.

4.2.2 Dietary Analysis

One-week dietary analysis (excluding supplementation) revealed no differences in energy, protein, fat and carbohydrate intake between groups throughout the study (see table 4.1).

Characteristics	Placebo carbohydrate n = 7	Creatine monohydrate n = 7
Energy Intake (kcal/kg/day)	32.7 ± 3.9	33.3 ± 4.6
Protein Intake (g/kg/day)	0.92 ± 0.09	0.91 ± 0.13
Fat Intake (g/kg/day)	0.92 ± 0.13	1.08 ± 0.18
Carbohydrate Intake (g/kg/day)	4.33 ± 1.00	4.93 ± 0.81

Table 4.1. Values are means ± SD of a 7-day written dietary recall of all participants submitted during the final week of the recovery period. These values do not include supplementation consumed.

4.2.3 Muscle Strength and Performance Assessment

4.2.3.1 Isokinetic Knee Extension Strength

Pre-exercise absolute values for isokinetic knee extension strength were 206 ± 13 Nm and 197 ± 10 Nm for placebo and creatine-supplemented group, respectively. No differences were detected.

Effect of the resistance exercise session: Reductions in strength (expressed as a percentage of pre-exercise strength) persisted for 4 days and were approximately 12% at 24 hours post-exercise, 22% lower at 48 hours (P<0.05), 15% lower at 72 hours and 13% lower at 96 hours post-injury. Reductions in strength were also observed in the creatine-supplemented group, albeit far smaller % reductions in strength. *Effect of supplementation:* A significant main effect for group was observed (P<0.01), indicating that the creatine-supplemented group had higher isokinetic knee extension peak torque

compared to the placebo carbohydrate group following the resistance exercise session and during recovery (see figure 4).

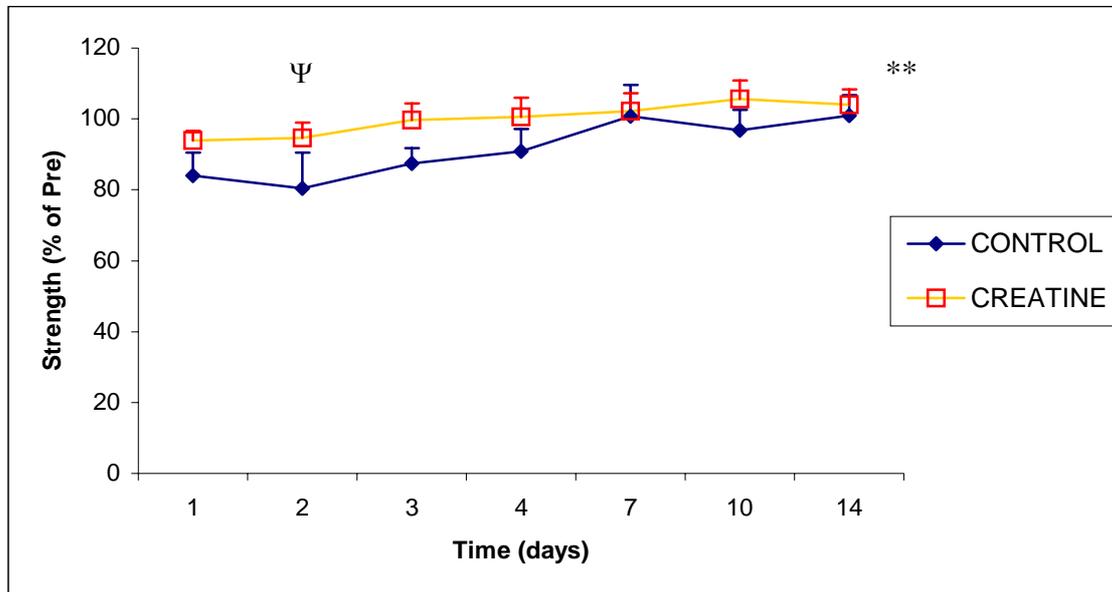


Figure 4. The effect of creatine supplementation on isokinetic knee extension muscle strength after exercise-induced muscle damage, expressed as a percentage of pre-exercise strength (mean \pm SEM). Ψ ($P < 0.05$) significantly different from pre-exercise. $**$ ($P < 0.01$) significant main effect for creatine-supplemented group.

4.2.3.2 Isokinetic Knee Flexion Strength

Pre-exercise absolute values for isokinetic knee flexion strength were 135 ± 9 Nm and 123 ± 9 Nm for placebo and creatine-supplemented group, respectively. No differences were detected.

Effect of the resistance exercise session: Muscle strength (expressed as a percentage of pre-exercise strength) was reduced post-exercise and remained lower during the 14-day period. Muscle strength was approximately 16% lower after 24 hours, 20% lower after 48 hours ($P < 0.05$), 15% lower after 72 hours, 13% lower after 96 hours, 5% lower after 7 days, 4% lower after 10 days and 3% lower after 14 days post-exercise. Similar % changes were observed in the creatine-supplemented group. *Effect of supplementation:*

No significant main effect for group was observed, indicating creatine supplementation had no effect on recovery of isokinetic knee flexion strength after exercise-induced damage (see figure 4.1).

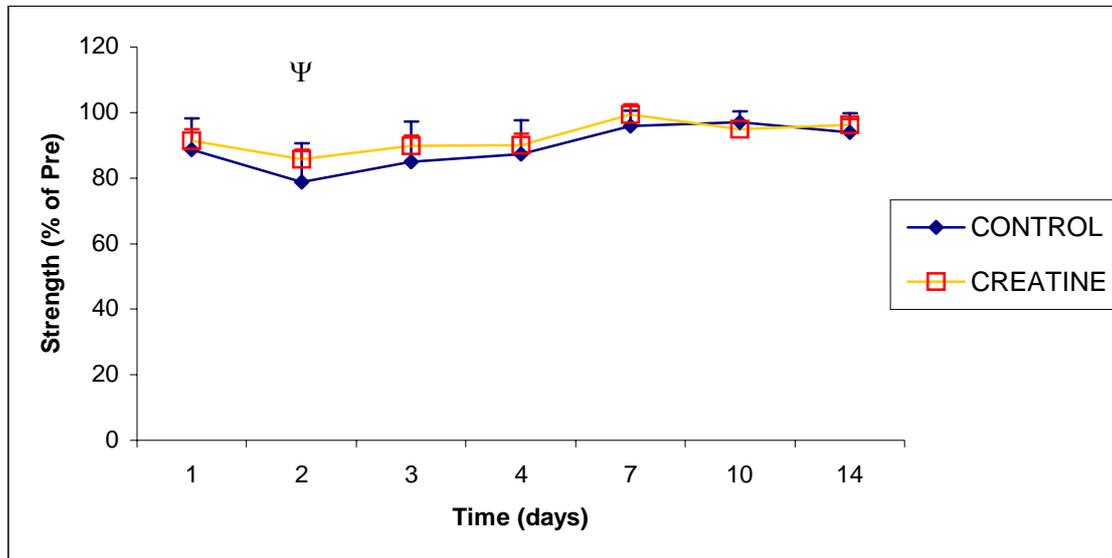


Figure 4.1. The effect of creatine monohydrate supplementation on isokinetic knee flexion muscle strength after exercise-induced muscle damage, expressed as a percentage of pre-exercise strength (mean \pm SEM). Ψ ($P < 0.05$) significantly different from pre-exercise.

4.2.3.3 Isometric Knee Extension Strength

Pre-exercise absolute values for isometric knee extension strength were 234 ± 24 Nm and 210 ± 11 Nm for placebo and creatine-supplemented group, respectively. No differences were detected.

Effect of the resistance exercise session: Reductions in strength (expressed as a percentage of pre-exercise strength) persisted for 14 days and were approximately 21% lower after 24 hrs after exercise ($P < 0.05$), 28% lower after 48 hours ($P < 0.01$), 21% lower after 72 hours ($P < 0.05$), 23% lower after 96 hours ($P < 0.05$), 16% lower after 7 days, 14% lower after 10 days and 8% lower after 14 days post-exercise. Reductions in

strength were also observed in the creatine-supplemented group, albeit far smaller % reductions in strength. *Effect of supplementation:* A highly significant main effect for group was observed ($P < 0.001$), indicating the creatine-supplemented group had higher isometric knee extension strength compared to the placebo carbohydrate group following the resistance exercise session (see figure 4.2).

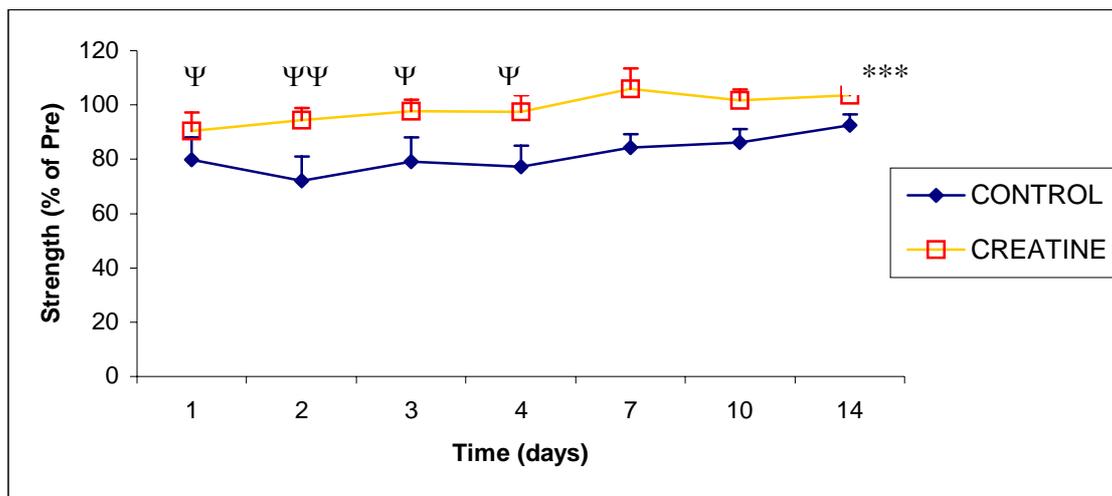


Figure 4.2. The effect of creatine monohydrate supplementation on isometric knee extension muscle strength after exercise-induced muscle damage, expressed as a percentage of pre-exercise strength (mean \pm SEM). $\Psi\Psi$ ($P < 0.01$), Ψ ($P < 0.05$) significantly different from pre-exercise. *** ($P < 0.001$) highly significant main effect for creatine-supplemented group.

4.2.3.4 Parameters of Knee Flexion and Extension

Angle of peak torque (AOPT) was measured for isokinetic extension and flexion. Since the AOPT for isometric extension was set at 60° , time to peak tension (TTPT) was analysed accordingly. AOPT and TTPT were expressed as absolute values and pre-exercise baseline value (100%) subtracted by percentage of pre-exercise value. A negative value for AOPT would represent an increase in angle of peak torque and *vice versa* for a positive AOPT value. In addition, a negative value for TTPT would represent an increase in time to peak tension (i.e. a slower time to reach peak tension), and *vice versa* for a positive value (i.e. faster time to reach peak tension).

Pre-exercise absolute values for AOPT isokinetic flexion were: $55.86 \pm 4.51^\circ$ placebo, $50.71 \pm 4.67^\circ$ creatine; AOPT isokinetic extension: $74.29 \pm 2.62^\circ$ placebo, $70.14 \pm 2.96^\circ$ creatine; and TTPT isometric extension: $3.4 \pm 0.3s$ placebo, $3.5 \pm 0.2s$ creatine. No differences were detected between the creatine-supplemented and carbohydrate group.

Effect of the resistance exercise session: There was no significant main effect for time on angle of peak torque for both knee extension and flexion. In contrast, a strong tendency for main effect for time on time to peak tension ($p=0.06$) was observed in the isometric knee extension. *Effect of supplementation:* A significant main effect for group on isokinetic knee flexion AOPT was observed ($P<0.01$). No other main effects for group were detected (see table 4.2).

Variable (% change from pre exercise value)	Day 1	Day 2	Day 3	Day 4	Day 7	Day 10	Day 14
<u>AOPT Flexion</u>							
Carbohydrate	4.0 ± 8.9	6.3 ± 7.5	12.5 ± 8.9	-1.5 ± 4.3	5.8 ± 2.2	3.4 ± 2.4	-1.2 ± 3.9
Creatine **	-10.8 ± 3.7	-11.7 ± 10.2	-11.4 ± 6.2	-11.3 ± 6.7	-1.5 ± 7	-7.0 ± 6.5	-7.4 ± 10
<u>AOPT Extension</u>							
Carbohydrate	0.5 ± 1.8	-3.8 ± 3.6	-0.7 ± 3.2	-2.5 ± 4.2	-3.9 ± 4.6	-1.6 ± 4.4	-3.2 ± 5.7
Creatine	-7.6 ± 1.8	-3.5 ± 2.1	-0.9 ± 3.2	-3.3 ± 3.0	1.9 ± 3.5	1.6 ± 2.9	0.3 ± 3.10
<u>TTPT</u>							
Carbohydrate	-12.4 ± 13	-20.6 ± 5.7	-19.8 ± 9.6	10.9 ± 8.3	17.8 ± 12.8	1.6 ± 6.2	-8.9 ± 9.1
Creatine	0.1 ± 9.6	-5.9 ± 15.6	21.3 ± 6.9	19.4 ± 10.9	7.3 ± 13.6	0.6 ± 7.5	-14.4 ± 12

Table 4.2. The effect of creatine supplementation of parameters of knee extension and flexion (AOPT and TTPT), after exercise-induced muscle damage, expressed as pre-exercise baseline value (100%) subtracted by % of pre-exercise value (mean \pm SEM).

** ($P<0.01$) significant main effect for creatine-supplemented group.

Variable	24hrs	48hrs	72hrs	96hrs	Day 7	Day 10	Day 14
<u>AOPT Flexion (degrees)</u>							
Carbohydrate	52.1 ± 12	51.9 ± 14	48.9 ± 15	56.7 ± 13	52.4 ± 11	53.9 ± 11	56.4 ± 12
Creatine **	55.6 ± 12	54.7 ± 11	55.3 ± 11	55 ± 10	50.9 ± 12	52.9 ± 6.5	53.9 ± 13
<u>AOPT Extension (degrees)</u>							
Carbohydrate	73.9 ± 7	77.4 ± 13	75.1 ± 12	76.4 ± 13	77.1 ± 11	75.3 ± 9	74.8 ± 9.2
Creatine	75.3 ± 7	72.6 ± 9	71 ± 11	72.1 ± 7.1	68.6 ± 9	72.1 ± 10	70.1 ± 11
<u>TTPT (seconds)</u>							
Carbohydrate	3.67 ± 0.9	4.01 ± 0.6	3.99 ± 1.0	3.03 ± 1.0	2.94 ± 1.7	3.31 ± 0.9	3.60 ± 0.8
Creatine	3.24 ± 1.6	3.59 ± 1.1	3.07 ± 0.9	2.76 ± 0.9	3.16 ± 1.1	3.44 ± 0.8	3.87 ± 0.7

Table 4.2a. The effect of creatine supplementation of parameters of knee extension and flexion (AOPT and TTPT), after exercise-induced muscle damage, expressed as absolute values (mean ± SEM). ** (P<0.01) significant main effect for creatine-supplemented group.

4.2.3.5 Vertical Jump Performance (CMJ)

No significant differences in pre-exercise absolute values between carbohydrate placebo and creatine-supplemented group for peak force (placebo: 896 ± 50 N; creatine: 947 ± 48 N); vertical height (placebo: 17.0 ± 1 cm; creatine: 16.0 ± 1 cm) and maximum rate force development (mRFD) (placebo 6386 ± 861 N s⁻¹; creatine 6070 ± 586 N s⁻¹) were detected. However, peak impulse was significantly higher in the creatine-supplemented group (430.8 ± 22.9 N s) compared to the placebo carbohydrate group (374.8 ± 9.6 N s; P<0.05).

Effect of the resistance exercise session: Indicators of power and performance (peak force, mRFD and peak impulse) of the CMJ test were decreased in both groups following the resistance exercise session, and remained lower throughout the 14-day recovery period. Peak force was significantly lower at 24 hours, 48 hours, 72 hours and 96 hours post-exercise ($P < 0.05$). mRFD was significantly lower at 24 and 48 hours post-exercise ($P < 0.05$). No other significant effects were observed following the resistance exercise session (see table 4.3).

Effect of supplementation: A highly significant main effect for group was observed in the vertical height of the countermovement jump ($P < 0.001$), indicating the creatine-supplemented group were jumping higher compared to the placebo carbohydrate group following the resistance exercise session (see Figure 4.3). Similarly, a significant main effect for group was observed in peak force of the countermovement jump ($P < 0.05$), indicating that in addition to jumping higher, the creatine-supplemented group were also generating higher forces compared to the placebo carbohydrate group following the resistance exercise session (see table 4.3). In addition, there was a highly significant main effect for group on peak impulse ($P < 0.001$). However, unlike the trend seen in vertical height and peak force measurements, peak impulse values were significantly lower in the creatine-supplemented group compared to the placebo carbohydrate group following the resistance exercise session (see table 4.3). Creatine supplementation had no significant effect on mRFD following the resistance exercise session, compared to the placebo carbohydrate group.

Variable (% of pre-exercise value)	Day 1	Day 2	Day 3	Day 4	Day 7	Day 10	Day 14
<u>Peak Force</u>							
Carbohydrate	89.8 ± 2.7	88.8 ± 3.5	89.4 ± 3.3	88.3 ± 3.6	94.2 ± 3.1	93.3 ± 3.3	93.8 ± 2.9
Creatine *	92.9 ± 2.1 Ψ	89.9 ± 2.5 Ψ	91.3 ± 2.6 Ψ	92.4 ± 2.6 Ψ	94.8 ± 2.4	99.8 ± 3.7	96.6 ± 2.1
<u>Peak Impulse</u>							
Carbohydrate	97.4 ± 5.0	92.4 ± 5.4	97.6 ± 5.4	97.7 ± 5.9	97.1 ± 5.1	99.2 ± 2.7	97.5 ± 3.5
Creatine ***	87.9 ± 2.7	86.2 ± 4.1	85.8 ± 3.9	85.9 ± 3.5	85.2 ± 4.9	82.6 ± 7.7	95.8 ± 4.2
<u>mRFD</u>							
Carbohydrate	63.9 ± 4.2	73.1 ± 8.1	83.2 ± 10.1	92.2 ± 12.9	91.7 ± 8.6	94.9 ± 15	92.2 ± 2.7
Creatine	83.3 ± 5.8 Ψ	81.4 ± 6.5 Ψ	80.8 ± 4.6	88.8 ± 8.3	94.2 ± 8.1	86.6 ± 11	93.7 ± 7.5

Table 4.3. The effect of creatine supplementation on indicators of vertical jump performance after exercise-induced muscle damage, expressed as a percentage of pre-exercise performance (mean ± SEM). Ψ (P<0.05) significantly different from pre-exercise. *** (P<0.001), *(P<0.05) significant main effect for creatine-supplemented group.

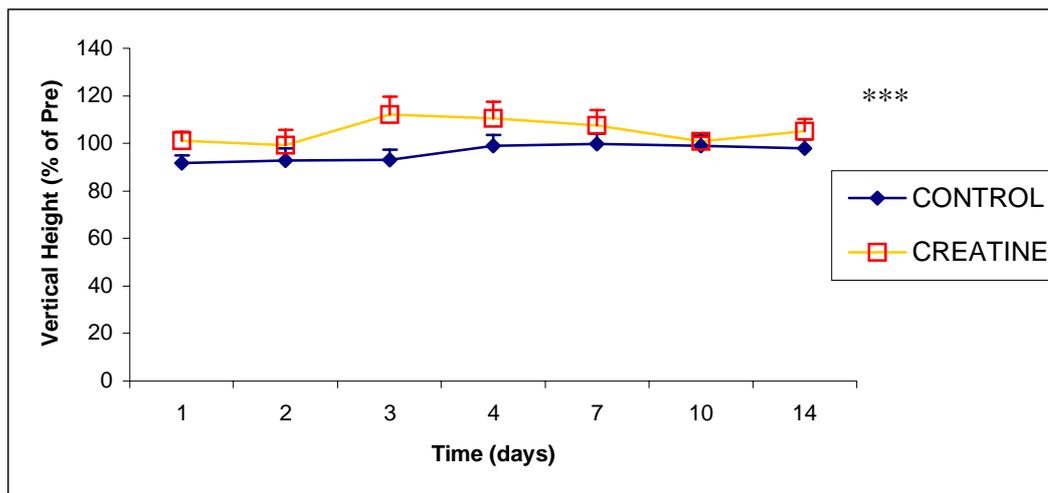


Figure 4.3. The effect of creatine supplementation on vertical height after exercise-induced muscle damage, expressed as a percentage of pre-exercise performance (mean \pm SEM). *** (P<0.001) significant main effect for creatine-supplemented group.

4.2.4 Plasma Creatine Kinase Activity

Pre-exercise CK levels were $176.1 \pm 59.2 \text{ IU} \cdot \text{l}^{-1}$ and $196.4 \pm 37.9 \text{ IU} \cdot \text{l}^{-1}$ (mean \pm SEM) in the placebo carbohydrate and creatine-supplemented group, respectively. No significant differences were detected.

Effect of the resistance exercise session: Figure 4.4. illustrates that CK activity changed significantly over time, being elevated above baseline, at 48 hours (P<0.0001), 72 hours (P<0.0001) and 96 hours (P<0.0001) post-exercise, in addition to a trend towards significance at day 7 (P=0.074). Elevations in plasma CK activity were also observed in the creatine-supplemented group, albeit far smaller increases in plasma CK activity levels.

Effect of supplementation: There was a highly significant main effect for group on plasma CK activity following the resistance exercise session (P<0.0001). However, there was a highly significant group by time interaction for CK activity (P<0.0001),

indicating that participant CK response was not similar, in terms of magnitude, at all recovery time points following the resistance exercise session. Indeed, subsequent analysis with a one-way ANOVA revealed that plasma CK activity was significantly lower at day 2 ($P<0.01$), day 3 ($P<0.001$), day 4 ($P<0.0001$), and day 7 ($P<0.001$) in the creatine-supplemented group compared to placebo carbohydrate post-exercise (see figure 4.4).

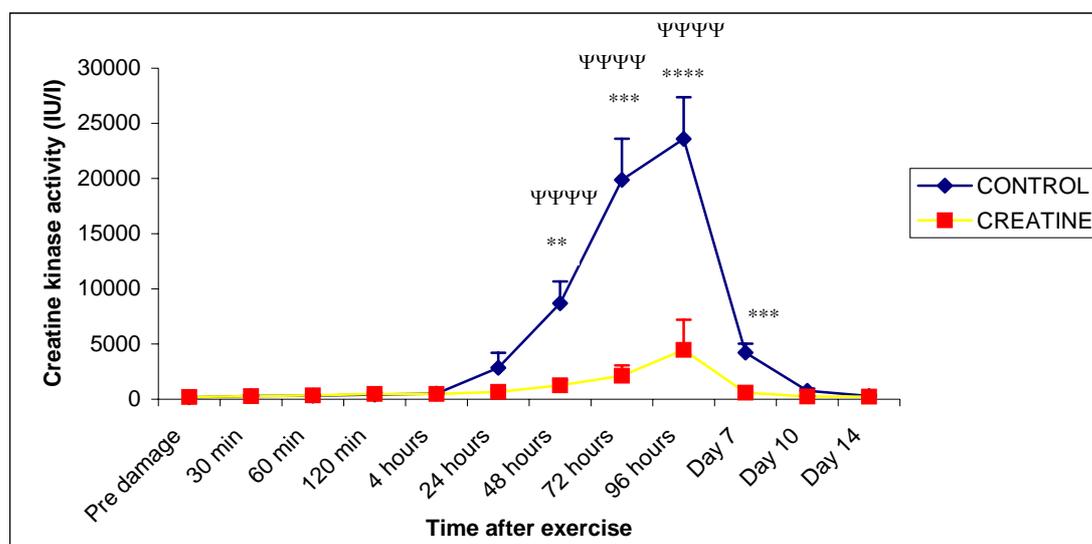


Figure 4.4. The effect of creatine supplementation on plasma CK activity after exercise-induced muscle damage. Values are mean \pm SEM. $\Psi\Psi\Psi\Psi$ ($P<0.0001$) significantly different from pre-exercise. **** ($P<0.0001$), *** ($P<0.001$) ** ($P<0.01$) * ($P<0.05$) significantly different from placebo carbohydrate group.

4.2.5 Plasma Lactate Dehydrogenase Activity

Pre-exercise LDH levels were $156.6 \pm 37.1 \text{ IU}\cdot\text{l}^{-1}$ and $148.0 \pm 31.3 \text{ IU}\cdot\text{l}^{-1}$ (mean \pm SEM) in the placebo carbohydrate and creatine-supplemented group, respectively. No significant differences were detected.

Effect of the resistance exercise session: Figure 4.5. illustrates that LDH activity significantly changed over time, being elevated above baseline at 24 hours ($P<0.01$), 48 hours ($P<0.0001$), 72 hours ($P<0.0001$), 96 hours ($P<0.0001$) and at day 7 ($P<0.05$) post-exercise. Similar elevations in plasma CK activity were also observed in the creatine-supplemented group. *Effect of supplementation:* A trend towards main effect for group was observed ($P=0.093$), indicating plasma LDH activity was generally lower across all time points in the creatine-supplemented group compared to placebo carbohydrate (see figure 4.5).

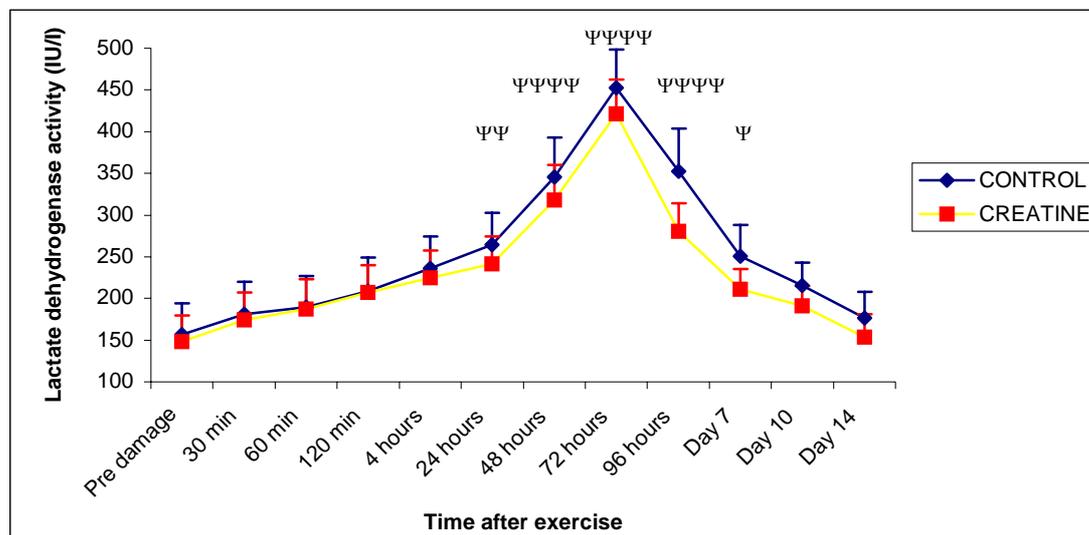


Figure 4.5. The effect of creatine supplementation on plasma LDH activity after exercise-induced muscle damage. Values are mean \pm SEM. $\Psi\Psi\Psi\Psi$ ($P<0.0001$), $\Psi\Psi$ ($P<0.01$) Ψ ($P<0.05$) significantly different from pre-exercise.

4.3 DISCUSSION

The primary objective of this study was to determine whether consumption of creatine monohydrate improves force recovery after exercise-induced damage in healthy individuals. Creatine supplementation significantly improved muscle force recovery (isokinetic and isometric knee extension) compared to placebo, after eccentrically-induced muscle damage. Furthermore, significantly lower plasma creatine kinase levels, and a trend towards lower LDH levels ($P=0.093$) after creatine supplementation in the days following injury support the improved functional capabilities observed. Whether this is due to improved calcium handling ability of the muscle and thus, a reduction in the extent of fibre damage, and/or an enhanced rate of muscle fibre regeneration due to increases in muscle protein synthesis will be discussed in detail later.

Extensive literature has examined the effects of creatine supplementation on exercise performance, in particular high intensity exercise. However, only a few studies have investigated the efficacy of creatine supplementation on the muscle recovery after injury (Warren *et al.*, 2000; Rawson *et al.*, 2001; Santos *et al.*, 2003). Furthermore, with such conflicting results and questions regarding the duration of supplementation period as explained in Chapter 2, it was difficult to determine the beneficial effects of creatine supplementation on indices of muscle damage. Thus, the current study demonstrated that creatine supplementation was able to reduce indicators of muscle damage caused by a resistance exercise session and may have also aided muscle recovery.

Given that the resistance exercise session protocol used in both human studies was the same, the effects of eccentrically-induced damage on muscle strength and performance and release of muscle proteins in the blood in both studies (i.e. Chapter 1 & 2) will be

discussed together. However, any differences, in addition to the effects of supplementation will be discussed in the relevant chapters.

Muscle damage and isometric, concentric and eccentric strength

Isokinetic knee extension and flexion and isometric knee extension peak torque was significantly reduced after repeated eccentric exercises, and remained significantly lower than pre-exercise values for approximately 4 days or longer. It is well established that impairment of muscle function (i.e. loss of muscle strength) following eccentric muscle contractions is a direct result of disruption to the normal muscle ultrastructure, which include: swelling or interference to the sarcoplasmic reticulum and T-tubular (sarcotubular) system; disruption of the myofibre and cytoskeleton contractile components; extracellular myofibre matrix abnormalities; and stretching or breaking of intermediate filaments between Z disks (as discussed in detail in chapter 2). However, it is suggested that subject effort and muscle activation (i.e. the delivery of nerve impulses to neuromuscular junction) may also contribute significantly towards loss of muscle strength and performance (Jakobi *et al.*, 2000).

A reduction in voluntary activation during performance of maximal exercise in the days following eccentric exercise is most likely due to inhibition caused by the presence of muscle soreness, swelling and stiffness (Byrne *et al.*, 2004). Voluntary activation after eccentric exercise has been studied during isometric MVCs by using the twitch interpolation technique (Newhan *et al.*, 1987; Saxton and Donnely, 1996). In brief, this technique involves the delivery of single electrical impulses to the active muscle belly or motor nerve during muscle contraction (i.e. performance of a MVC) and muscle relaxation. Such sensitive force measurement allows for the determination of any additional force increment in response to the electrical impulse. Thus, highlighting the

presence of central fatigue (i.e. delivery of nerve impulse to the neuromuscular junction) if force produced in response to electrical stimuli is greater than that produced by voluntary activation (Allen *et al.*, 1995). However, evidence from studies employing this technique has suggested that full voluntary activation can be achieved during isometric MVCs following eccentric exercise-induced muscle damage (Newhan *et al.*, 1987; Saxton and Donnelly, 1996). These findings suggest that the reductions and subsequent improvements in isometric strength after eccentric exercise are a result of factors at or distal to the neuromuscular junction (i.e. peripheral mechanisms) rather than the delivery of nerve impulses to the neuromuscular junction. Whether central fatigue contributes to the reductions in isokinetic concentric and eccentric strength is unknown, but would be unlikely given that full voluntary activation during isometric MVC is achieved.

Previous research have shown similar decrements in isometric force after fewer eccentric exercisers (Brown *et al.*, 1996), while other studies have demonstrated larger force decrements using a similar (Byrne and Eston, 2002) or a less severe ‘damaging protocol’ as to the one used in the present study (Rinard *et al.*, 2000; Rawson *et al.*, 2001). Such varying responses in the magnitude of strength loss following eccentric exercises are possibly due to the different muscle groups (i.e. elbow flexors of the forearm vs. knee extensor/flexors muscles groups) utilized to induce muscle damage. Arm muscles contain a higher proportion of fast twitch fibres compared to leg muscles. Therefore, since fast twitch fibres are preferentially damaged during eccentric exercise, the severity of the initial amount of damage, and hence, magnitude of strength loss may be different between arms and leg muscles, which have a higher proportion of slow twitch fibres (Rinard *et al.*, 2000; Rawson *et al.*, 2001; Vijayan *et al.*, 2001; Bryne *et al.*, 2004; Olsen *et al.*, 2005).

In addition to different muscle groups utilized, strength loss may be dependent on the angular velocity of movement used during the testing procedures, such that velocity-dependent strength loss may be affected to a greater extent at low angular velocity torque compared to higher angular velocity torque or *vice versa* (Deschenes *et al.*, 2000; Byrne *et al.*, 2004). However, when isometric strength and isokinetic concentric and eccentric strength are tested at a similar angular velocity of movement (as those used in the present study), there appears to be no significant or meaningful difference in the magnitude of strength loss or the rate of recovery across all muscle actions (Byrne and Eston, 2002; Michaut *et al.*, 2002).

It should be noted that muscle strength was expressed as a percentage of pre-exercise strength values and normalised to contralateral controls. This is a common method of analysing loss of muscle strength following exercise-induced damage (Brown *et al.*, 1996; Rinard *et al.*, 2000; Rawson *et al.*, 2001), which therefore not only normalises data by accounting for any improvements during the recovery period as a result of familiarisation, but more importantly reduces the inter-individual variability in muscle strength between participants.

Muscle damage and angle of peak torque and time to peak tension.

It is suggested that high-strain eccentric muscle actions result in redistribution of sarcomere lengths. According to the “popping sarcomere” hypothesis (Morgan and Allen 1999; Proske and Morgan, 2001) lengthening of active muscle does not occur by uniform lengthening of all sarcomeres, but by a non-uniform distribution of sarcomere length change, with some sarcomeres rapidly overextending (“popping”) beyond myofilament overlap and failing to re-interdigitate upon relaxation (Morgan and Allen,

1999). Such over-extended sarcomeres would result in a shift to the right in the length-tension curve, and thus a trend towards longer muscle lengths (Plant *et al.*, 2005). Direct evidence for a shift in the optimal angle for strength (i.e. change in length-tension properties) has been reported following eccentrically-induced damage of the ankle extensors (Whitehead *et al.*, 1998). In a series of studies performed by Morgan and Allen (1999), extensor muscles of the ankle were subjected to supramaximal stimulation, while adjustments in muscle length were made after each isometric contraction. Researchers demonstrated a shift to right in optimal length and a change in optimal angle of peak torque following eccentric-induced muscle damage (Jones *et al.*, 1997; Whitehead *et al.*, 1998).

In the current study, isokinetic knee flexion AOPT expressed as a percentage change from pre-exercise values, was significantly higher than pre-exercise values during the 4 days post-exercise ($P < 0.01$). Hence, indicating that muscles of the knee flexor were required to shorten further in order to produce peak torque. However, whether these observed changes in AOPT in the current study are a direct result of alterations in the length tension curve is unclear, as the shifts in optimal angle reported by Morgan and Allen (1999) were observed in muscles that were isometrically stimulated, while changes in the present study were observed in muscles that performed isotonic contractions. Furthermore, since no changes were observed in the isokinetic knee extension, significance of such results is beyond the scope of this thesis.

In support of reduced isometric knee extension strength following the resistance exercise, the time to peak tension (TTPT) was also prolonged, indicating that not only was isometric peak torque decreased, but the time taken to reach this reduced peak torque was also slower compared to pre-exercise strength and performance values. In

contrast to the observed 4-day loss of muscle strength, TTPT was only prolonged during the initial 48 hours post-injury. Time to peak tension (TTPT) is an unrefined indicator of the Ca^{2+} release process, the binding to troponin-C and the activation of actin and myosin. Reduced isometric peak torque and possibly prolonged TTPT may be attributed a number of factors which include: interference to excitation-contraction (EC) coupling process (Warren *et al.*, 2001); disruption or loss of force-generating structures such as actin and myosin (Thompson *et al.*, 1999); and disruption or loss of force-transmitting structures, such as desmin and dystrophin (Lieber *et al.*, 1994; Barash *et al.*, 2002; Lovering and Deyne, 2004). It is possible that the enhanced restoration of TTPT compared to loss of muscle strength could be due to the either less damage to, or improved recovery of, E-C coupling system and/or contractile proteins such as actin and myosin, while disruption to force-transmitting structures such as desmin and dystrophin remained affected.

Lovering and Deyne (2004) examined contractile function, sarcolemmal integrity, and loss of dystrophin after a single eccentric contraction in the tibialis anterior muscle of male Sprague-Dawley rats. Results showed that within minutes after eccentric-induced damage, dystrophin and to a much lesser extent desmin was preferentially damaged or affected, compared to sarcomeric proteins (actin and myosin heavy chains), integral and extracellular proteins. Whilst these results were observed after a single eccentric contraction, if this selective trend on muscle proteins was to continue following repeated eccentric contractions, than restoration of muscle strength will be gradual, while the time to reach peak tension will return to normal earlier, as seen in the present study.

Muscle damage and vertical (countermovement) jump performance

Similar to the observations in isokinetic and isometric muscle strength; immediate and long lasting reductions in parameters of the CMJ were evident in the current chapter. While numerous studies have employed isolated muscle actions to examine strength loss after muscle damage, limited research has investigated dynamic muscle function using a vertical jumping model after exercise-induced damage (Byrne and Eston, 2002). Byrne and Eston (2002) examined the effects of 100 barbell squats on vertical jumping performance with or without use of stretch-shortening cycle. Reductions in vertical jumping performance were immediate and prolonged (up to 4 days), and was dependent of the type of jump performed (i.e. squat jump, CMJ or drop jump). However, squat jump performance was affected to a greater extent compared to CMJ and drop jump. The main difference between the three types of vertical jump is that the CJM and drop jump incorporates active pre-stretch, whereas squat jump does not. Active muscles that are pre-stretched absorb and store energy in their series elastic elements, which can be later used during the concentric phase (Komi and Bosco, 1978).

Furthermore, pre-stretching enhances the muscles' ability to achieve a high level of force prior to concentric contraction, due to increased potentiation of the contractile machinery and enhanced contribution from reflexes (Van Ingen Schenau *et al.*, 1997). Thus, pre-stretching of activated muscles attenuates the detrimental performance effects of exercise-induced muscle damage (Byrne and Eston, 2002). In the current study, to limit the influence of pre-stretching on the recovery of CMJ performance after resistance training, participants were required to reach a knee flexion of 90° or more (as measured by a purpose-built ruler with a flexible plate) during the downward movement of the CMJ, thus ensuring that each participant was attaining a minimal pre-stretch level. Furthermore, to reduce other variables/factors that may influence the participant's

CMJ performance, participant's feet positions on the force platform were identical for each jump and participants were instructed to keep their hands on their hips throughout the jump.

Peak impulse, maximum rate of force development (mRFD) and peak force were parameters derived from the ascending and descending phase of the force-time curve (positive portion) and were used as indicators of muscle power and force. Reductions in peak force, peak impulse, mRFD and vertical height, expressed as a percentage of pre-exercise values, persisted during the recovery period, following the resistance exercise protocol.

Muscle damage and plasma creatine kinase and lactate dehydrogenase activity

CK and LDH are widely accepted as markers of muscle damage after prolonged exercise (Clarkson *et al.*, 1994; Nosaka and Clarkson, 1995; Gunst *et al.*, 1998; Schwane *et al.*, 2000; Lavender and Nosaka, 2005). Due to the different clearance rates of these enzymes it was decided to measure plasma CK and LDH at 1, 2, 3, 4 hours following exercise and on days 1, 2, 3, 4, 7, 10, and 14 post-exercise. Plasma CK and LDH activity significantly increased during the days post resistance exercise, and remained elevated above baseline until day 10 post-exercise. The time course and magnitude of increased CK and LDH in plasma following the resistance exercise session was in accordance with previous work (Chen and Hsieh, 2001; Rawson *et al.*, 2001), with maximum CK and LDH activity occurring approximately 72 to 96 hours after the resistance exercise. The delay in maximal elevation of CK and LDH activity may be caused by increasing membrane permeability due to secondary or delayed onset damage as a result of increasing Ca^{2+} leakage into the muscle, and thus increased calpain activity and further reductions in membrane integrity (i.e. increased

permeability) (Gissel, 2000). In addition, the difference in the magnitude of CK and LDH present in plasma following eccentric exercise (as seen in figure 4.4 and 4.5) is most likely due to the larger molecular weight of LDH compared to CK and hence a decreased ability to leave from the muscle cell following injury (Fowler *et al.*, 1962).

A review by Clarkson (1997), based on the results by Nosaka and Clarkson (1996), suggested that the higher the plasma peak creatine kinase concentration, the more abnormal MRI (magnetic resonance imaging) is after eccentric muscle actions. These results were later supported by a more recent study that showed a positive correlation between creatine kinase and MRI after eccentric muscle actions in African-American men (Schwane *et al.*, 2000). However, Friden and Lieber (2001) suggested that while serum creatine kinase levels may provide a gross indication that skeletal muscle injury has occurred, differences between serum creatine kinase levels do not necessarily provide information regarding the extent of muscle damage (Friden and Lieber, 2001).

Furthermore, the exercise-induced release of predominantly cytoplasmic creatine kinase can be due to final death of the muscle fibre or to temporary muscle fibre damage accompanied by membrane leakage (McNeil and Khakee, 1992), which does not necessarily reflect myofibrillar disruption. Indeed, any correlation between leakage of muscle enzymes and force production must be a more complex process, as it is well established (Chen and Hsieh, 2001; Rawson *et al.*, 2001; Beaton *et al.*, 2002) that the greatest elevation in plasma enzyme levels occurs in the days following injury (such as 72 and 96 hours as seen in the current study) at a time at which force has considerably recovered.

Dietary Analysis

One-week dietary analysis revealed participants in both the creatine- and carbohydrate-supplemented groups were similar in caloric energy intake, carbohydrate and fat consumption. More importantly protein intake was similar in both groups, as any differences may conflict with the results, due to possible effects of protein intake on recovery from damage (see next chapter). Although supplements were not matched for carbohydrate content, the caloric effect of each supplement was the same.

The effect of creatine supplementation on isokinetic knee extension/flexion and isokinetic knee strength

In the current study, isokinetic and isometric knee extension muscle strength was significantly higher during recovery from exercise-induced muscle damage in the creatine-supplemented group compared to carbohydrate placebo. These results support the work by Santos and colleagues (2003), who also showed beneficial effect from creatine supplementation on indirect markers of muscle damage and inflammation. Although muscle force recovery was not examined, creatine supplementation prior to a 30km run was able to attenuate a number of markers of muscle damage and inflammation (LDH, PGE2 and TNF- α) post-exercise. Therefore, suggesting that creatine supplementation is not only an effective strategy in maintaining muscle integrity during and after intense prolonged exercise, it may also be successful at protecting muscle fibres from more damaging exercises used in the present study.

In contrast, Warren and colleagues, (2000) showed recovery of muscle strength in mice after eccentric-induced damage was unaffected following 2-weeks of prior creatine supplementation. However, a limitation to this study was that the researchers only examined the recovery of the muscle for a 3-minute period following the eccentric

contractions. This period is related to both fatigue and possibly damage and hence, peak damage would not have occurred. Additionally, muscle force related to fatigue would not have recovered in only 3 minutes. Therefore, only effects of increased muscle [Cr] on peak strength loss was examined, as opposed to the recovery of strength *per se* after injury. In the present study, the longer recovery period encompassed both the damage and recovery period following muscle injury, and thus, showed the beneficial effect of creatine supplementation on muscle strength loss after exercise-induced damage.

Furthermore, unlike the results of this study, Rawson *et al.* (2001) demonstrated no beneficial effect from prior consumption of creatine on recovery of isometric strength of the elbow flexors, during 5 days following eccentric exercises. Moreover, serum CK and LDH levels in response to the eccentric exercises were not significantly different between the creatine-supplemented and control groups post-exercise. In contrast to the supplementation protocol used in the present study, Rawson and colleagues (2001) only supplemented for 5 days prior to the eccentric contractions, and thus was not continued during the recovery period post-exercise. It is speculated that in order to unmask the anabolic effects of creatine supplementation there needs to be some associated contractile activity (Parise *et al.*, 2003). Moreover, any transcriptional changes in muscle gene expression programmed for muscle anabolism (i.e. increased myofibrillar protein synthesis) may not be seen for days or maybe weeks after initial stimulus.

Thus, in the present study, it is hypothesised that by continuing creatine supplementation after the resistance exercise (initial stimulus), creatine may act as a co-regulator, or direct manipulator of gene transcription of amino acid pools, thus increasing myofibrillar protein synthesis during the recovery period post-injury (Willoughby and Rosene, 2001; 2003). But more importantly, create a positive protein

balance in which protein synthesis is greater than protein degradation, and thus, reduce further muscle damage post-exercise.

Additionally, a positive protein balance may also improve muscle fibre regeneration by enhancing satellite proliferation. Indeed, following 4-weeks of creatine supplementation, satellite cell mitotic activity was enhanced during compensatory hypertrophy by the removal of synergist muscles of the plantaris rat muscle (Dangott *et al.*, 2000). Thus, supporting the hypothesis that creatine supplementation may also facilitate myotube formation during periods of muscle growth.

In contrast to the previous strength measurements discussed, creatine supplementation had no significant effect of the recovery of isokinetic knee flexion torque post-exercise. While it is understood that 120% of each participants strength (1RM) should illicit the same proportion of damage, and hence, similar decreases in % strength loss, it was evident that the 1RM for the creatine-supplemented participants was approximately 16 kg heavier compared to the 1RM of participants consuming the placebo. Consequently, since 120% of their 1RM was lowered on each exercise machine, the creatine-supplemented group was lowering ~19kg more during the resistance exercise session compared to placebo group. Therefore, further damage may have occurred in the creatine-supplemented group compared to placebo, thus concealing any significant improvements after creatine supplementation. However, this concept requires further investigation.

Interestingly, knee flexion strength did remain lower than pre-exercise values throughout the recovery period (i.e. no recovery as a result of time). While the reason for this observation is not readily apparent in this study, one possible explanation could

be that initial pre-exercise measurements for knee flexion strength may have been high, and thus full recovery would require longer than 14 days. However, as mentioned previously, this concept requires further investigation.

In the current study, AOPT of the knee flexors was significantly higher in the creatine-supplemented group compared to carbohydrate placebo following resistance exercise. This indicates that the creatine-supplemented participants performing isokinetic knee flexion were shortening their muscles further in order to generate peak torque. Direct evidence for a shift in the optimal angle for strength (i.e. change in length-tension properties) has been reported following eccentrically-induced damage of the ankle extensors (Whitehead *et al.*, 1998) and in whole intact muscles recovering from chemically-induced muscle damage (Plant *et al.*, 2005). Though the benefit for having higher AOPT following muscle injury is not clearly, obvious in the present study, it has been demonstrated that when muscles are fully regenerated (mass and force production restored), there is a shift towards the right in length-tension curve (Plant *et al.*, 2005). This rightward shift in the length-tension curve can be attributed to the longitudinal addition of sarcomeres during regeneration (Morgan, 1990).

Thus, in the present study, a right shift in the length-tension curve may represent faster regeneration, and hence support the higher muscle strength in the creatine-supplemented group compared to placebo group. However, the previous observations were shown in muscle's performing isometric contractions, and thus whether such changes in length-tension will influence isotonic contractions is unclear and needs further examination.

The effect of creatine supplementation on vertical (countermovement) jump performance

Peak force of the countermovement jump (CMJ) was significantly higher in the creatine-supplemented group compared to placebo carbohydrate group post-exercise. Peak force signifies maximal concentric muscle activity of the prime movers of knee extension during the early stages of the CMJ. Additionally, vertical height of the CMJ was also significantly higher after creatine supplementation post-exercise, thus supporting the observed higher peak forces. This is in accordance with previous studies (Bosco *et al.*, 1997; Watsford *et al.*, 2003) that have shown an increase in CMJ performance following creatine supplementation, though these improvements were shown in normal, uninjured individuals, rather than after exercise-induced muscle damage.

Nonetheless, it was suggested that such improvements from creatine supplementation could be due to increased PCr levels within the muscle (Hultman *et al.*, 1996), thus increasing ATP supply to the contractile machinery during execution of the CMJ, and/or greater strength gains due to muscular hypertrophy (Dangott *et al.*, 2000). Although, PCr and Cr levels were not measured in the present study, previous research have shown creatine loading in the muscles following similar supplementation protocols as to the one used in the present study (Harris *et al.*, 1992; Balsom *et al.*, 1994; Greenhaff *et al.*, 1994; Casey *et al.*, 1996; Hultman *et al.*, 1996; Volek *et al.*, 1999; Rawson and Volek, 2003; Van Loon *et al.*, 2003) and thus we suggest that creatine loading was achieved in the present study

Several investigators have reported selective damage to type II fast-twitch fibres compared to type I slow-twitch muscle fibres in humans (Friden *et al.*, 1983; Jones *et*

al., 1986) and animal muscle (Friden and Lieber, 1992; Vijayan *et al.*, 2001) after eccentric muscle contractions. Interestingly, it has been suggested that fast-twitch fibres have an enhanced potential to transport Cr into the muscle and thus would benefit more by increases in Cr availability (Casey *et al.* 1996). Therefore, although fibre types were not measured in the present study, any lessening of damage to, and/or faster recovery of type II fibres, those that generate higher forces, as opposed to type I fibres after creatine supplementation would support the observed higher peak forces and vertical height of the CMJ, in addition to the increases in isokinetic and isometric muscle strength, as described earlier.

However, interestingly, indicators of power (peak impulse and mRFD) of the CMJ were not significantly increased after creatine supplementation post-exercise. In fact peak impulse was significantly lower in the creatine-supplemented group compared to the placebo carbohydrate group. These results are in contrast to the changes seen in isokinetic and isometric peak torque following creatine supplementation. It is not readily apparent why peak impulse, which represents the concentric muscle activity averaged over the entire CMJ performance (including the push off toes phase), was lower in the creatine-supplemented group compared to controls. It could be suggested that since the knee flexors of the creatine-supplemented group post-exercise needed to be “shortened” further, as observed in the AOPT measurements, it is possible that over the entire shortening range during the CMJ, RFD and impulse will be lower. However, when the muscle executes the concentric contraction, they produce greater forces and jump higher compared to placebo group.

In addition, other factors aside from AOPT may be influencing peak impulse and mRFD, such as the level of pre-activation prior to performing the CMJ (as explained

previously). A limitation to the measurement of CMJ performance in the current study was the amount of pre-stretching prior to performing the CMJ. Though participants were required to reach a knee flexion of 90° or more during the downward movement of the CMJ, thus ensuring a minimal pre-stretch level was attained for each participant, the exact pre-stretch level was not measured. Furthermore, the CMJ was performed utilizing both legs, as opposed to just using the injured leg. Thus, it is possible that the non-damaged leg may be influencing the affects from creatine supplementation, and thus leading to inconsistent observations.

The effect of creatine supplementation on creatine kinase and lactate dehydrogenase

Plasma CK activity was significantly lower at day 2, 3, 4, 7 and at day 10 in the creatine-supplemented group compared to placebo group following exercise-induced muscle damage. In addition, a trend towards significantly lower LDH levels after creatine supplementation was also observed during recovery (P=0.093), and thus supports lower plasma CK levels and enhanced functional capability in the creatine-supplemented group. Power analysis of the data revealed a power of 0.805. Therefore, while only a trend (P=0.093) was observed in the plasma LDH levels, it could be suggested that with further subjects, this trend towards lower plasma LDH levels in the creatine-supplemented would lead to significance.

These results are in contrast to Rawson *et al.* (2001) who demonstrated no significant changes in plasma CK and LDH levels post-injury following 5 days of creatine supplementation. Whilst it was mentioned previously that continuing supplementation during the recovery period is essential to increase protein synthesis, it may also be critical in minimising protein degradation and thus, muscle fibre damage. Furthermore,

damage to muscle structural elements caused by the initial eccentric exercises leads to further Ca^{2+} leakage into the muscle long after muscle activity has ceased.

Thus, it is hypothesised that reduced SR Ca^{2+} -ATPase pump activity (i.e. reduced calcium uptake) as a result of increased Ca^{2+} influx, leads to loss of Ca^{2+} homeostasis, activation of degradative pathways such as calpain and phospholipase A2, and thus further damage to the sarcolemma of the muscle, as evident by the delayed peaks in plasma CK and LDH activity (Belcastro *et al.*, 1998). Therefore, in the present study creatine supplementation prior to, but also following, eccentrically-induced muscle damage may be improving the Ca^{2+} buffering capacity of the muscle (i.e. the rate of Ca^{2+} removal from the muscle cytoplasm) and thus decrease $[\text{Ca}^{2+}]_i$ accumulation, calpain activation, but more importantly reduce extent of damage and CK and LDH leakage from the muscle into the blood.

Moreover, PCr may be acting as a membrane stabilizer by binding to the polar phospholipid heads of the muscle membrane (Saks *et al.*, 1992), thus reducing membrane fluidity and transforming the membrane into a more ordered state (Saks *et al.*, 1992). Increasing Cr levels, in particular PCr levels, within the muscle after creatine supplementation may additionally enhance the integrity of the sarcolemma by improving the energetics and/or directly acting on lipid structures within the cell (Port *et al.*, 1994). Therefore, further minimising the leakage of muscle proteins into the blood. Indeed Rawson *et al.* (2001) demonstrated lower CK levels during recovery post-exercise, although not significant due to the high variability. If supplementation was continued, perhaps CK levels may have been lower.

In summary, the major finding of this investigation was significantly higher muscle strength following muscle damage after creatine supplementation. In addition, significantly lower plasma creatine kinase levels in the days after injury is indicative of less muscle damage, hence supporting the improved functional capabilities observed. Although the current study did not address the exact mechanisms by which creatine exerts its effects, it could be suggested that creatine supplementation prior to eccentric-induced damage may be enhancing the calcium buffering capacity of the muscle by improving the SR Ca^{2+} -ATPase pump, thus, decreasing intracellular calcium levels and activation of degradative pathways such as calpain. Thus, a reduction in calcium – activated proteases and lipases will minimise additional damage to the sarcolemma, but more importantly, further influxes of calcium into the muscle. Additionally, creatine supplementation post-exercise may have also aided recovery, by minimizing secondary or delayed onset damage, such as increasing protein synthesis, reducing protein degradation, and thus creating an environment that facilitates enhanced satellite proliferation and hence formation of new muscle fibres.

CHAPTER 5

Effects of Whey Protein Supplementation on Muscle Force Recovery after Eccentrically-Induced Muscle Damage in Healthy Individuals

5.0 INTRODUCTION

Dietary proteins, in particular branched chain amino acids (BCAA) have an important role in regulating protein metabolism in skeletal muscle (Evans, 2001; Borsheim *et al.*, 2002; Karlsson *et al.*, 2004). Eccentric exercise produces ultrastructural changes that stimulate muscle protein synthesis and degradation. Protein degradation usually exceeds synthesis and thus a negative protein balance is created, resulting in net muscle protein breakdown and muscle degeneration (Evans, 2001).

Furthermore, within hours of injury, the number of circulating neutrophils can be increased dramatically. Neutrophils migrate to the site of injury where they phagocytize tissue debris and release factors such as lysozymes and oxygen radicals, which also contribute to the increased breakdown and degradation of muscle contractile proteins (Evans, 1986). Taken together, these changes can lead to measurable indirect markers of exercise-induced muscle damage including reduced muscle strength (maximum voluntary contraction; MVC), muscle soreness (DOMS), and release of myocellular proteins such as CK and LDH into the plasma (Nosaka *et al.*, 2000; Friden and Lieber, 2001).

Following eccentric exercise-induced damage, muscle regeneration induces local accumulation of several peptide growth factors, including insulin like growth factor (IGF-I) (Yan *et al.*, 1993), fibroblast growth factor (FGF) (Yamada *et al.*, 1989), and potentially platelet-derived growth factor (PDGF). All three growth factors stimulate

satellite cell proliferation. Satellite cells are involved in the process of repairing injured muscle fibres, and subsequent formation of new muscle cells *in vivo*; all processes which require an enhanced rate of protein synthesis (Evans. 2001).

Therefore, a nutritional supplement that can achieve a positive net muscle protein balance is likely to increase the rate of protein synthesis, satellite cell proliferation, and thus improve muscle fibre regeneration. Indeed, Tipton *et al.* (1999) and more recently Borsheim (2002), have shown that net muscle protein balance is negative post-exercise when individuals are maintained in the post-absorptive state during recovery. In contrast, those who ingested protein had a positive protein balance.

There is evidence to suggest that compared to regular protein supplements, whey isolate is more effective at increasing blood amino acids and protein synthesis due to its different absorption kinetics and amino acid profile (Mahe *et al.*, 1996; Bucci and Unlu, 2000). Hydrolysed whey proteins are rapidly absorbed in the upper jejunum (Mahe *et al.*, 1996) and contain the highest concentration of the essential amino acids, including BCAA's (Bucci and Unlu, 2000). Furthermore, whey protein exhibits the highest biological value (BV) of any known protein (Renner, 1983). Whey protein has a BV score of 104 whereas casein, another milk protein, beef and fish have BV scores of 77, 75, and 75, respectively (Renner, 1983). Biological value is the measure of the protein's ability to retain nitrogen in the muscle (Colgan, 1993), and a positive nitrogen balance is associated with muscle anabolism (protein synthesis).

Thus, the purpose of this study was to examine the effects of short-term consumption of dietary supplement whey protein on muscle proteins and force recovery after eccentrically-induced muscle damage in healthy individuals. We hypothesised that

following eccentrically-induced damage: loss of muscle strength will be restored earlier; and release of muscle proteins will be decreased, indicative of reduced damage and/or faster recovery following whey protein supplementation.

5.1 METHODS

5.1.1 Participants

Seventeen healthy untrained males volunteered for this study. Subjects were generally students of Victoria University. Descriptive characteristics of the subjects are presented in table 5. Subjects were (a) non-smokers; (b) had not participated in resistance-training for at least six months; and (c) had not ingested any ergogenic supplement for a 24-week period prior to the start of supplementation. All participants were informed verbally, as well as in writing, as to the objectives of the experiments, together with the potential associated risks. All participants signed an informed consent document approved by the Human Research Ethics Committee of Victoria University of Australia. All procedures conformed to National Health and Medical Research Council guidelines for the involvement of human subjects for research.

5.1.2 Dietary Supplementation.

Subjects were randomised in a double-blind placebo-controlled fashion into 2 groups: an isocaloric carbohydrate placebo group (n= 8); and whey protein-supplemented group (n= 9). The supplements were provided to participants in identical, unmarked, sealed containers. The macronutrient content of the supplements was as follows; approx. 90gms protein, 8gms isocaloric carbohydrate, 2gms fat/100gms for Whey protein supplement (VP2™ Hydrolyzed Whey Isolate) and 100gms isocaloric carbohydrate/100gms for placebo control. Supplements were supplied by AST Sports Science, Golden, Colorado USA. The whey protein was independently assessed on two

separate occasions (Naturalac Nutrition LTD, Level 2/18 Normanby Rd Mt Eden, New Zealand) and met label ingredients on both occasions. Subjects were instructed to consume 1.5 grams of either the supplement or placebo per kilogram of body weight for a period of 14 days following a resistance exercise session, while maintaining their habitual daily diet. Resistance-trained athletes widely use protein supplements to achieve high protein intakes (Marquart *et al.*, 1998). Therefore, we chose a supplement dose that was characteristic of this population (Marquart *et al.*, 1998), even though the participants in this study were untrained individuals.

Subjects were advised to mix the supplement in water and consume the required dosage in 3 equal serves over the period of the day, i.e. breakfast, lunch and dinner, 1 hour before eating. Nutritional intake was monitored via written dietary diary sheet. Subjects were instructed to record their nutrient intake for a 7-day period. All recordings were assessed using Nutritionist PRO (First Data Bank) software.

5.1.3 Isokinetic/Isometric Strength and Vertical (Countermovement) Jump Measurements

Muscle strength and performance measurements were examined by voluntary isokinetic knee flexion and isokinetic/isometric knee extension using the Cybex NormTm Testing and Rehabilitation System and a vertical jump (CMJ) performed on a custom-built strain gauge force platform (as previously described in section 3.1.3.1 & 3.1.3.2). All strength and performance measurements were performed prior to- and immediately following the exercise session, and on day 1, 2, 3, 4, 7, 10 and 14 post-exercise. It should be noted that muscle strength values of the exercised leg, performed on the Cybex NormTm Testing and Rehabilitation System, were expressed as percentage of pre-exercise values and normalised to contralateral controls. Parameters of vertical jump

performance were expressed as a percentage change from pre-exercise values. Previous research has shown this to be a successful means of reporting muscle strength and performance data (Rinard *et al.*, 1999; Byrne and Eston. 2001).

5.1.4 Blood Sampling and Analysis

Blood was sampled from the antecubital fossa vein prior to and 30 min, 1, 2, and 4 hours following, the resistance exercise session and on days 1, 2, 3, 4, 7, 10 and 14 post exercise. Blood was immediately placed into EDTA tubes and centrifuged at 3000 rpm for 15 min at 4°C (as previously described in section 3.1.5). Plasma was stored at -80°C for subsequent analysis of CK and LDH activity.

5.1.5 Statistical Analysis

Subject characteristics are reported as means \pm SD. All other values are reported as means \pm SE. Statistical evaluation of data was accomplished by using a two-way analysis of variance (ANOVA) with one between groups factor (supplement) and one repeated factor (time), with subsequent Tukey's Post-Hoc analysis. Where an interaction was found, the location of the difference was determined by a students t-test. Difference in participant characteristics and dietary analysis between groups was assessed by students' t-test. A P value of less than 0.05 was accepted for statistical significance.

5.2 RESULTS

5.2.1 Participant Characteristics

At baseline there were no differences in the age, body weight or strength level (1RM) between the two groups (see table 5).

Characteristics	Placebo carbohydrate n = 8	Whey protein isolate n = 9
Age (yrs)	22.0 ± 3.6	24.2 ± 5.1
Weight (kg)	77.4 ± 14.1	81.5 ± 7.6
Leg Press 1RM (kgs)	274 ± 112	283 ± 87
Leg Extension 1RM (kgs)	88 ± 26	84 ± 25
Leg Flexion 1RM (kgs)	40 ± 8	46 ± 22

Table 5. Participants' baseline characteristics. Values are means ± SD of all seventeen males.

5.2.2 Dietary Analysis

Based on supplement dosage of 1.5g/kg.bw/day, there was no difference in the amount of supplement ingested between the placebo carbohydrate and whey protein-supplemented group during the 14-day recovery period (see table 5.1). One-week dietary analysis (excluding supplementation) revealed no differences in energy, protein, fat and carbohydrate intake between groups throughout the study.

<u>Variable</u>	Placebo Carbohydrate n = 8	Whey Protein n = 9
Supplement consumption (g/kg/day)	115.92 ± 21.2	122.21 ± 11.4
Energy Intake (kcal/kg/day)	30.14 ± 7.3	29.43 ± 5.1
Protein Intake (g/kg/day)	0.82 ± 0.09	0.85 ± 0.06
Fat Intake (g/kg/day)	0.94 ± 0.18	0.97 ± 0.18
Carbohydrate Intake (g/kg/day)	4.58 ± 1.45	4.32 ± 0.95

Table 5.1. Values are means ± SD of 7-day written dietary recall of all participants submitted during the final week of the recovery period. These values do not include supplementation consumed.

5.2.3 Muscle Strength and Performance Assessment

5.2.3.1 Isokinetic Knee Extension Strength

Pre-exercise absolute values for isokinetic knee extension strength were 234 ± 18 Nm and 238 ± 9 Nm for placebo and whey isolate group, respectively. No differences were detected.

Effect of the resistance exercise: Reductions in strength (expressed as a percentage of pre-exercise strength) persisted for 7 days and were approximately 16% lower at 24 hours post-exercise (P<0.01), 20% lower at 48 hours (P<0.01), 18% lower at 72 hours (P<0.01), 11% lower at 96 hours (P<0.05) and 7% lower at day 7. Reductions in strength were also observed in the whey protein-supplemented group, albeit smaller % reductions in strength. *Effect of supplementation:* A significant main effect for group

was observed ($P < 0.01$), indicating that the whey protein-supplemented group had higher isokinetic knee extension peak torque compared to the placebo carbohydrate group following the resistance exercise session (see figure 5).

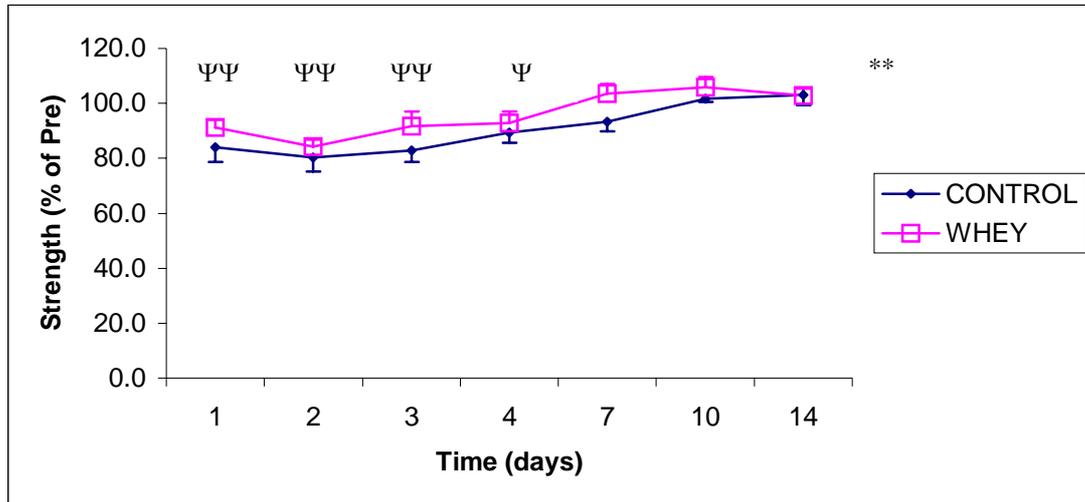


Figure 5. The effects of whey protein supplementation on isokinetic knee extension muscle strength after exercise-induced muscle damage, expressed as a percentage of pre-exercise strength (mean \pm SEM). $\Psi\Psi$ ($P < 0.01$), Ψ ($P < 0.05$) significantly different from pre-exercise. ** ($P < 0.01$) significant main effect for whey protein-supplemented group.

5.2.3.2 Isokinetic Knee Flexion Strength

Pre-exercise absolute values for isokinetic knee flexion strength were 132 ± 8 Nm and 138 ± 5 Nm for placebo and whey isolate group, respectively. No differences were detected.

Effect of the resistance exercise: There was no significant main effect for time on the isokinetic knee flexion strength, indicating no significant change from pre-exercise strength values. *Effect of supplementation:* A significant main effect for group was observed ($P < 0.05$), indicating that although minimal decrements in force recovery were evident after the resistance exercises, whey protein-supplemented group still had higher

isokinetic knee flexion peak torque compared to the placebo carbohydrate group following the resistance exercise session (see figure 5.1).

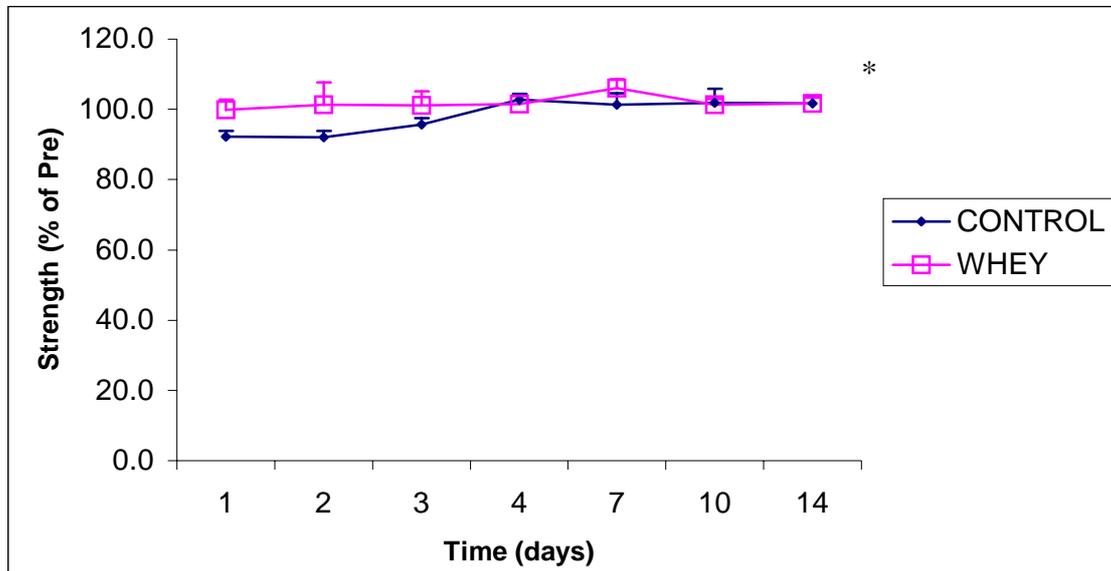


Figure 5.1. The effects of whey protein supplementation on isokinetic knee flexion muscle strength after exercise-induced muscle damage, expressed as a percentage of pre-exercise strength (mean \pm SEM). * ($P < 0.05$) significant main effect for whey protein-supplemented group.

5.2.3.3 Isometric Knee Extension Strength

Pre-exercise absolute values for isometric knee extension strength were 314 ± 3 Nm and 290 ± 2 Nm for placebo and whey protein-supplemented group, respectively. No differences were detected.

Effect of the resistance exercise: There was a highly significant main effect for time on isometric knee extension strength ($P < 0.001$). Similar to isokinetic knee extension, reductions in strength (expressed as a percentage of pre-exercise strength) persisted for 7 days and were approximately 21% lower after 24 hours post-exercise ($P < 0.001$), 14% lower after 48 hours ($P < 0.001$), 16% lower after 72 hours ($P < 0.01$), 13% lower after 96

hours ($P<0.05$), and 7% lower at day 7 ($P=0.063$) post-injury. Reductions in strength were also observed in the whey protein-supplemented group, albeit smaller % reductions in strength. *Effect of supplementation:* Similarly, a highly significant main effect for group was observed ($P<0.001$), indicating the whey protein-supplemented group had higher isometric knee extension strength compared to the placebo carbohydrate group following the resistance exercise session (see figure 5.2).

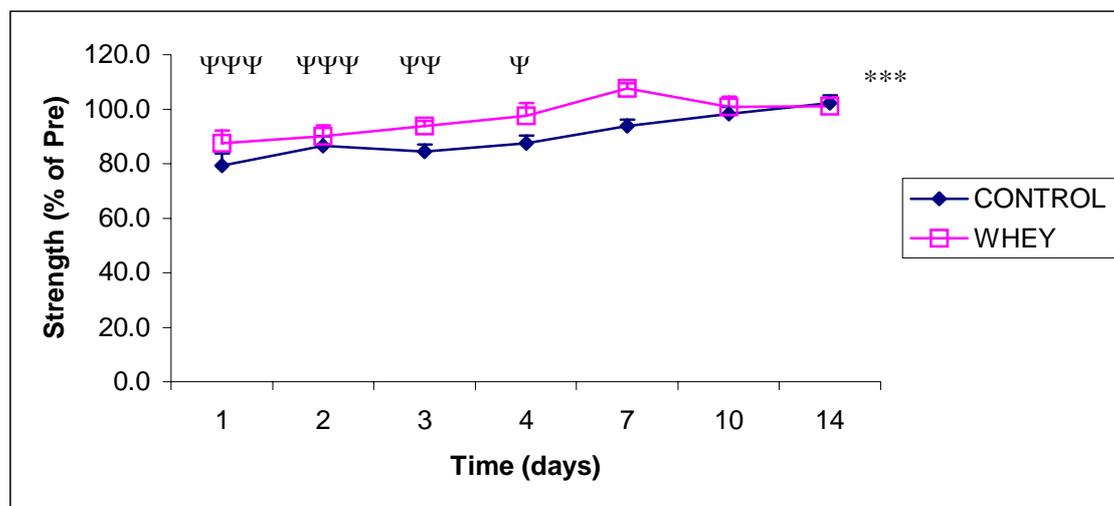


Figure 5.2. The effect of whey protein supplementation on isometric knee extension muscle strength after exercise-induced muscle damage, expressed as a percentage of pre-exercise strength (mean \pm SEM). $\Psi\Psi\Psi$ ($P<0.001$), $\Psi\Psi$ ($P<0.01$), Ψ ($P<0.05$) significantly different from pre-exercise. *** ($P<0.001$) highly significant main effect for whey protein-supplemented group.

5.2.3.4 Parameters of Knee Flexion and Extension

Angle of peak torque (AOPT) was measured for isokinetic extension and flexion. Since the AOPT for isometric extension was set at 60° , time to peak tension (TTPT) was analysed accordingly. A negative value for AOPT would represent an increase in angle of peak torque and *vice versa* for a positive AOPT value. In addition, a negative value for TTPT would represent an increase in time to peak tension (i.e. a slower time to reach peak tension), and *vice versa* for a positive value (i.e. faster time to reach peak tension).

Pre-exercise absolute values for AOPT isokinetic flexion were: $31.88 \pm 2.64^\circ$ placebo, $35.44 \pm 5.24^\circ$ whey; AOPT isokinetic extension: $56.25 \pm 2.5^\circ$ placebo, $64.67 \pm 2.12^\circ$ whey; and TTPT isometric extension: 2.7 ± 0.3 s placebo, 3.9 ± 0.1 s whey. Significant differences for AOPT isokinetic knee extension and TTPT isometric knee extension pre-exercise values were detected between whey protein-supplemented and carbohydrate group ($P < 0.05$). *Effect of the resistance exercise:* There was no significant main effect for time on angle of peak torque and time to peak tension, indicating a minimal effect on parameters of knee flexion and extension following the resistance exercise session.

Effect of supplementation: A significant main effect for group on isokinetic knee extension AOPT and isometric knee extension TTPT was observed ($P < 0.01$). Following the resistance exercise session, the whey protein-supplemented group exhibited decreases in AOPT in comparison to their pre-exercise values, in contrast to an increase in AOPT, observed in the placebo carbohydrate group post-exercise. Furthermore, whey protein-supplemented group exhibited faster TTPT in comparison to their pre-exercise values post-exercise, whereas the placebo carbohydrate group demonstrated slower TTPT post-exercise (see table 5.2). No other supplementation effects were observed.

Variable (% change from pre-exercise value)	Day 1	Day 2	Day 3	Day 4	Day 7	Day 10	Day 14
<u>AOPT Flexion</u>							
Carbohydrate	-28.9 ± 16.7	-21.3 ± 19.3	-20.4 ± 14.6	-31.5 ± 13	-28.9 ± 19.9	-40.5 ± 18.2	-24.9 ± 13
Whey Protein	3.4 ± 9.7	-3.1 ± 9.7	-12.0 ± 12.9	-18.1 ± 13	-17.2 ± 9.9	-22.1 ± 18.4	-34.2 ± 17
<u>AOPT Extension</u>							
Carbohydrate	-5.1 ± 3.6	-11.4 ± 3.9	-13.4 ± 5.4	-10.1 ± 4.8	-9.9 ± 6.8	-10.0 ± 4.6	-3.6 ± 7.2
Whey Protein**	3.9 ± 3.7	3.2 ± 3.3	0.6 ± 4.3	-0.3 ± 3.7	0.8 ± 2.7	-2.9 ± 4.6	1.2 ± 3.9
<u>TTPT</u>							
Carbohydrate	-25.3 ± 19.1	-52.4 ± 21.4	-30.7 ± 18.0	-19.7 ± 12.4	-32.4 ± 18.2	-6.2 ± 13.5	-11.2 ± 10.5
Whey Protein**	-4.4 ± 9.3	9.1 ± 11.1	-11.4 ± 10.9	4.6 ± 9.4	12.9 ± 8.8	8.9 ± 9.9	15.7 ± 7.0

Table 5.2. The effect of whey protein supplementation of parameters of knee extension and flexion (AOPT and TTPT), after exercise-induced muscle damage, expressed as pre-exercise baseline value (100%) subtracted by % of pre-exercise value (mean ± SEM). ** (P<0.01) significant main effect for whey protein-supplemented group.

Variable	24hrs	48hrs	72hrs	96hrs	Day 7	Day 10	Day 14
<u>AOPT Flexion (degrees)</u>							
Carbohydrate	38.5 ± 10	36.2 ± 14	36.8 ± 12	40.8 ± 13	39.4 ± 18	42.3 ± 15	38.9 ± 13
Whey Protein	36.6 ± 16	37.8 ± 13	39.9 ± 13	41.4 ± 14	41.1 ± 12	42.3 ± 17	44.1 ± 14
<u>AOPT Extension (degrees)</u>							
Carbohydrate	58.9 ± 8	62.5 ± 9	63.4 ± 9	61.2 ± 5	61.1 ± 8	61.5 ± 8	57.3 ± 8
Whey Protein**	61.9 ± 8	62.5 ± 9	64.8 ± 9	64.9 ± 11	64.1 ± 9	67.1 ± 13	64.1 ± 10
<u>TTPT (seconds)</u>							
Carbohydrate	3.28 ± 1.0	3.43 ± 1.0	3.68 ± 0.7	4.21 ± 0.4	2.21 ± 0.4	2.69 ± 0.3	3.43 ± 0.6
Whey Protein**	4.01 ± 0.9	3.58 ± 1.2	4.26 ± 0.9	3.66 ± 0.9	3.33 ± 0.8	3.61 ± 1.0	3.39 ± 0.9

Table 5.2a. The effect of whey protein supplementation of parameters of knee extension and flexion (AOPT and TTPT), after exercise-induced muscle damage, expressed as absolute values (mean ± SEM). ** (P<0.01) significant main effect for whey protein-supplemented group.

5.2.3.5 Vertical Jump Performance (CMJ)

No significant differences in pre-exercise absolute values between placebo carbohydrate and whey protein-supplemented group for peak force (placebo: 944 ± 80 N; whey: 1007 ± 62 N); peak impulse (placebo: 414.9 ± 34.4 N·s; whey: 429.4 ± 31.3 N·s); vertical height (placebo: 20 ± 2 cm; whey: 18 ± 2 cm) and maximum rate force development (mRFD) (placebo: 5610 ± 844 N·s⁻¹; whey: 6090 ± 636 N·s⁻¹)

Effect of the resistance exercise: The resistance exercise session had no significant effect of indicators of power and performance of the CMJ test, as indicated by no significant main effect for time. *Effect of supplementation:* Though the resistance exercise session had little effect on the vertical jump performance, maximum rate of force development was significantly higher in the whey protein-supplemented group compared to placebo carbohydrate group post-exercise ($P < 0.05$, see figure 5.3). Whey protein supplementation had no other significant effects on indicators of power and performance of the vertical jump test compared to placebo carbohydrate group (see table 5.3).

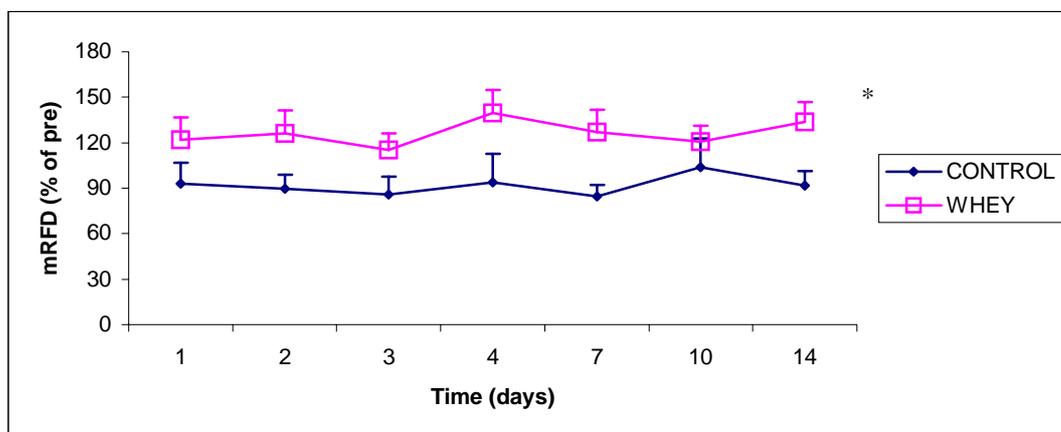


Figure 5.3. The effect of whey protein supplementation on maximum rate of force development (mRFD) after exercise-induced muscle damage, expressed as a percentage of pre-exercise performance (mean \pm SEM). * ($P < 0.05$) significant main effect for whey protein-supplemented group.

Variable (% of pre-exercise value)	Day 1	Day 2	Day 3	Day 4	Day 7	Day 10	Day 14
<u>Peak Force</u>							
Carbohydrate	98.1 ± 5.5	100.7 ± 6.1	97.3 ± 5.7	99.4 ± 5.1	101.2 ± 5.8	101.8 ± 5.5	102.6 ± 6.6
Whey Protein	97.5 ± 4.2	99.9 ± 4.6	99.6 ± 4.7	102.9 ± 5.8	106.9 ± 4.6	105.2 ± 4.3	109.2 ± 4.6
<u>Peak Impulse</u>							
Carbohydrate	85.9 ± 6.5	103.6 ± 7.9	100.4 ± 9.9	101.9 ± 9.9	101.5 ± 6.6	103.4 ± 6.6	92.4 ± 5.5
Whey Protein	96.5 ± 2.8	94.3 ± 4.1	101.2 ± 3.6	102.7 ± 3.1	101.5 ± 6.1	101.7 ± 3.8	96.5 ± 3.6
<u>Vertical Height</u>							
Carbohydrate	91.2 ± 3.7	91.2 ± 5.1	103.4 ± 6.6	102.7 ± 3.6	97.8 ± 2.8	100.5 ± 5.1	104.2 ± 3.5
Whey Protein	100.9 ± 6	93.6 ± 4.6	100.6 ± 5.7	100.3 ± 3.4	95.6 ± 2.6	110 ± 5.4	97.7 ± 3.1

Table 5.3. The effect of whey protein supplementation on parameters of vertical jump performance after exercise-induced muscle damage, expressed as a percentage of pre-exercise performance (mean ± SEM).

5.2.4 Plasma Creatine Kinase Activity

Pre-exercise CK levels were $225.4 \pm 50.1 \text{ IU}\cdot\text{l}^{-1}$ and $198.1 \pm 50.3 \text{ IU}\cdot\text{l}^{-1}$ (mean ± SEM) in the placebo carbohydrate and whey protein-supplemented groups, respectively. No significant differences were detected.

Effect of the resistance exercise: Figure 5.4 illustrates that CK activity changed significantly over time, being elevated above baseline at 48 hours ($P < 0.05$), 72 hours ($P < 0.05$) and 96 hours ($P < 0.05$) post-exercise. Elevations in plasma CK activity were also observed in the whey protein-supplemented group, albeit far smaller increases in plasma CK activity levels. *Effect of supplementation:* Plasma CK activity was significantly lower at 24 and 72 hours post-exercise in the whey-supplemented group compared to carbohydrate placebo ($P < 0.05$, figure 5.4).

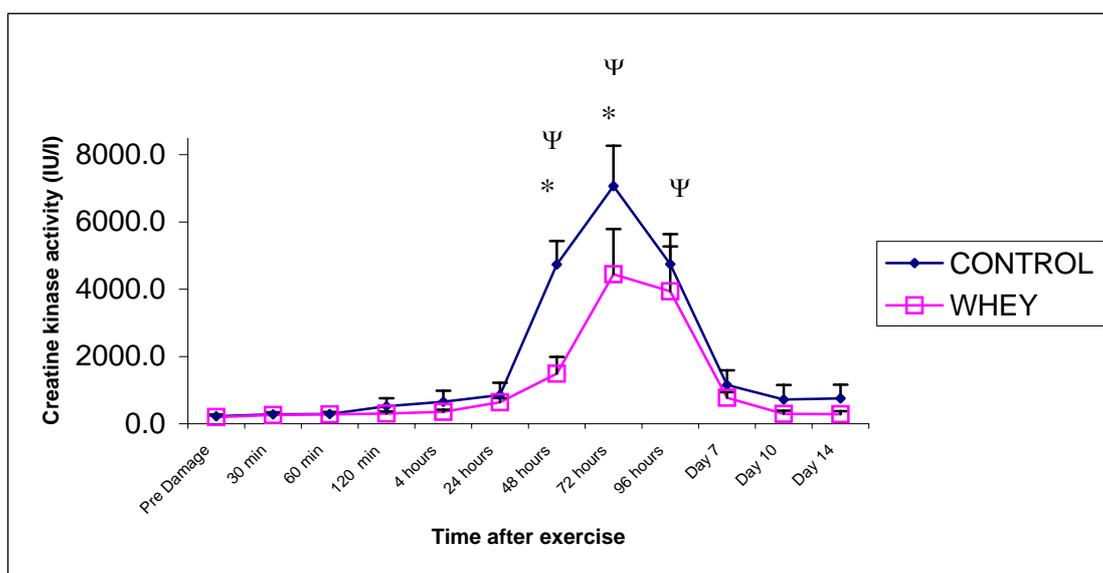


Figure 5.4. The effect of whey protein supplementation on plasma CK activity after exercise-induced muscle damage. Values are mean \pm SEM. Ψ ($P < 0.05$) significantly different from pre-exercise. * ($P < 0.05$) significantly different from placebo carbohydrate.

5.2.5 Plasma Lactate Dehydrogenase Activity

Pre-exercise LDH levels were $154.9 \pm 11.0 \text{ IU}\cdot\text{l}^{-1}$ and $152.2 \pm 9.9 \text{ IU}\cdot\text{l}^{-1}$ (mean \pm SEM) in the placebo carbohydrate and whey protein isolate supplemented groups, respectively. No significant differences were detected.

Effect of the resistance exercise: Figure 5.5 illustrates that LDH activity significantly changed over time being elevated above baseline at 24 hours ($P < 0.01$), 48 hours ($P < 0.0001$), 72 hours ($P < 0.0001$), 96 hours ($P < 0.0001$) and at day 7 ($p < 0.01$) post-exercise. Similar elevations in plasma CK activity were also observed in the whey protein-supplemented group. *Effect of supplementation:* There was a significant main effect for group on plasma LDH activity following the resistance exercise session ($P < 0.05$), indicating LDH activity was significantly lower in the whey protein-supplemented group compared to placebo carbohydrate post-exercise.

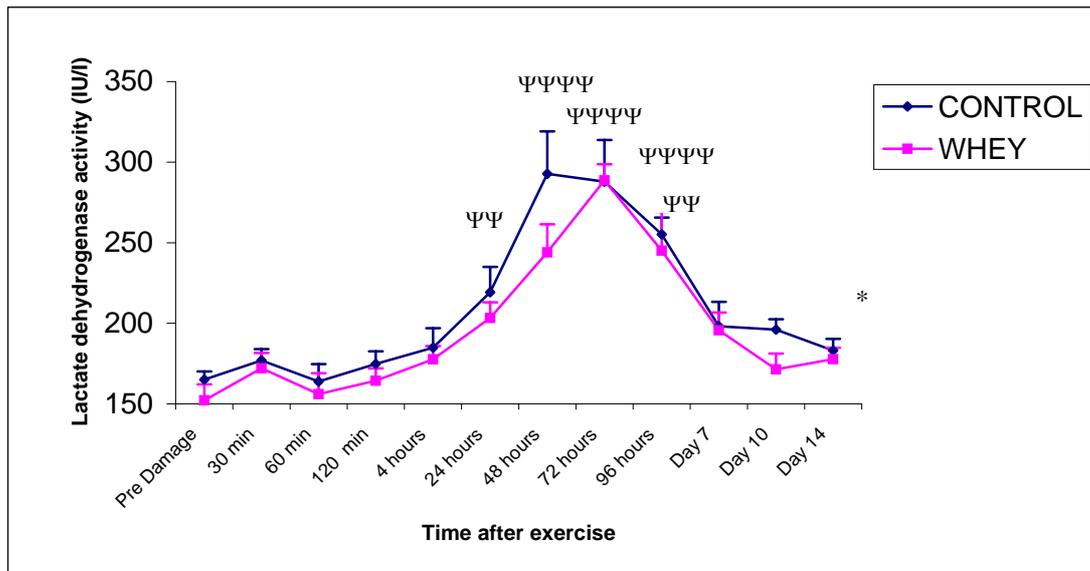


Figure 5.5. The effects of whey protein isolate supplementation on plasma LDH activity after exercise-induced muscle damage. Values are mean \pm SEM. $\Psi\Psi\Psi\Psi$ ($P<0.0001$), $\Psi\Psi$ ($P<0.01$) significantly different from pre-exercise. * ($P<0.05$) significant main effect for whey protein-supplemented group.

5.3 DISCUSSION

The primary objective of this study was to determine whether short-term consumption of whey protein isolate improves force recovery in healthy individuals after exercise-induced muscle damage. The resistance exercise protocol resulted in an immediate and prolonged impairment of muscle function and a release of the muscle proteins CK and LDH. Similar to chapter 4, reductions in isokinetic and isometric knee extension peak torque persisted for 4 days or longer following the resistance exercise session. However, isokinetic knee flexion peak torque was not significantly affected by the resistance exercise session. It should be noted that two different types of leg flexion exercise machines were used in chapter 4 and 5.

The current chapter utilized a leg flexion machine that required participants to lie face down on the apparatus, and thus perform an eccentric contraction in a prone position (as described in section 3.1.4.1). However, due to unforeseen circumstances, the previous chapter utilized a leg flexion machine that required the participants to perform knee extension in a seated position. The participant began seated with their knee extended at $\sim 15^\circ$ from horizontal. The weight was lowered to $\sim 90^\circ$, so that their knee was at 90° relative to the thigh. The participant performed extension of knee through an arc of 75° , whilst resisting a weight equivalent to 120% of their predetermined unilateral concentric 1RM. Similarly to the current study, participants were required to apply resistance against the weight (eccentric contraction) at a fixed cadence (4 seconds) verbally given by an investigator.

It could be suggested that the design of the knee flexion machine used in chapter 5 limited the ability to lift heavy weights, thus lower 1RMs were evident (i.e. approximately 24kg) compared to those used in the current chapter. Consequently, participants performing extension of the knee on the knee flexion machine in the current chapter, lowered less weight compared to participants in chapter 4. Thus, it is possible that this absolute workload was not sufficient to cause extensive muscle damage of the knee flexor muscle group.

In addition, parameters of the CMJ performance were not significantly affected following the resistance exercise session. Although both the exercised and non-exercised leg are utilized during the execution of a CMJ, it is clear that reductions in jumping performance can be attributed to the reductions in strength of the knee extensors and flexors of the exercised leg. Thus, in the current chapter, since minimal decrements in force were observed in the knee flexor muscle groups post-exercise, it is

likely that to achieve any decreases in CMJ performance, injury to both muscle groups would be required (as shown in the previous chapter). Interestingly, the magnitude of CK and LDH release in the current study was much lower than the magnitude response observed in chapter 4, and thus, taken together with the minimal decrements in isokinetic knee flexion strength and CMJ performance, may be indicative of less damage.

Nonetheless, whey protein supplementation significantly improved the rate of muscle force recovery (isokinetic and isometric knee extension) compared to placebo, after exercise-induced muscle damage. While previous research into whey protein supplementation have examined its effects on muscle strength gains after resistance training (Burke *et al.*, 2001; Anderson *et al.*, 2005; Cribb *et al.*, 2006), no studies have determined whether whey protein can improve the recovery from such exercises. Thus, to our knowledge, this is the first study to demonstrate that whey protein supplementation may reduce the extent of damage, and/or enhance recovery from, intense training, in particular eccentric exercise such as weight training.

The effect of whey protein supplementation on isokinetic knee extension/flexion and isokinetic knee strength

In the current study, isokinetic and isometric muscle strength was significantly higher during recovery from exercise-induced muscle damage in the whey protein-supplemented group compared to placebo carbohydrate. Interestingly, although minimal decrements in muscle strength were observed in the isokinetic knee flexion after the resistance exercises, whey protein-supplemented group still had higher isokinetic knee flexion peak torque (close to 100% of pre-exercise values) compared to the placebo

carbohydrate group. Thus, providing evidence that whey protein supplementation is also beneficial to less severe cases of muscle injury.

Recent studies have confirmed that resistance exercise stimulates an increase in myofibrillar and sarcoplasmic proteins (Louis *et al.*, 2003; Mittendorfer *et al.*, 2005) as well as connective tissue proteins (Miller *et al.*, 2005). A single bout of resistance exercise results in the acute stimulation of muscle protein synthesis (up to 50-100% above basal values) that peaks within 3-24 hours, and can remain elevated, although at a diminishing rate, for up to 48 hours post-exercise (Chesley *et al.*, 1992; Biolo *et al.*, 1995; Phillips *et al.*, 1997). Studies that have assessed both the rate of muscle protein breakdown and synthesis in response to a bout of resistance exercise have demonstrated that in a fasted state (Biolo *et al.*, 1995; Phillips *et al.*, 1997) the net muscle protein balance remains slightly negative. It is suggested that recovery of muscle protein synthesis, and hence a positive protein balance after exercise would require intake of protein or amino acids (Anthony *et al.*, 1999). In the present study, oral ingestion of whey protein after the resistance exercise session most likely increased delivery of amino acids to the muscle, thus, augmenting muscle protein synthesis and minimising protein degradation; creating a positive protein balance. While increased muscle protein synthesis is possibly due to increased delivery of amino acids, decreased protein degradation is possibly due to the insulin effect from the carbohydrate. However, insulin levels were not measured in the current study.

Furthermore, muscle protein synthesis and breakdown rates were not measured in the present study, notwithstanding, previous studies have shown that providing exogenous amino acids, especially within the first 4 hours after resistance exercise (as implemented in the present study), enhances protein synthesis, reduces protein breakdown, and

maintains a positive protein balance (Tipton *et al.*, 1999; Biolo *et al.*, 2003). Although these results were not observed following ingestion of whey protein, a more recent study has confirmed the positive impact from whey protein supplementation, in addition to casein (another milk protein) on protein metabolism after resistance training exercise (Tipton *et al.*, 2004). Participants were randomly assigned to either a whey, casein or placebo group. Results showed that a 20gram bolus dose of whey or casein provided a positive effect on muscle protein balance after resistance training. While both proteins had different effects on blood amino acid responses, it was clear that whey protein (and casein) were able to increase muscle protein net balance following resistance training.

Thus, in the present study, the higher strength values in the whey protein-supplemented group compared to placebo group following the resistance exercise session, may be reflecting not only an increase in protein synthesis, but equally as important it may be indicating reduced protein breakdown, and thus lessening the amount of muscle degeneration and loss of muscle mass. Moreover, since satellite cell proliferation requires a positive net muscle protein balance (Evans, 2001), muscle fibre regeneration is likely to be enhanced following whey protein supplementation. Recent studies in humans (Cuthbertson *et al.*, 2005) have confirmed *in vitro* (Christie *et al.*, 2002) and *in vivo* (Yoshizawa *et al.*, 1997; 2004) that amino acids stimulate muscle protein synthesis directly via activation of the Raptor- mTOR complex (Hara *et al.*, 1998) and increase phosphorylation of the p70S6k and eIF4-BP1 complexes (Liu *et al.*, 2002). The large (289 kDa) Raptor- mTOR complex is expressed more in muscle than other tissues (Kim *et al.*, 2002), is nutrient sensitive, and contains multiple binding sites.

The effect of whey protein supplementation on angle of peak torque, time to peak tension and CMJ performance

In the current study, the resistance exercise session had no significant effect on angle of peak torque in both isokinetic knee extension and flexion. However, an interesting observation was that time to peak tension for isometric extension was significantly quicker after whey protein supplementation compared to placebo group. Time to peak tension (TTP) is an unrefined indicator of the Ca^{2+} release process, the binding to troponin-C and the activation of actin and myosin. Thus, it is possible that faster TTP after whey protein supplementation may be a result of increased protein synthesis or reduced protein degradation, in particular E-C coupling proteins and/or contractile proteins such as actin and myosin.

Similarly, the rate of force development (mRFD) of the CMJ was increased after whey protein supplementation compared to placebo. It is suggested that contractile mRFD may be influenced by muscle size and fibre type composition (Aagaard *et al.*, 2002). It was recently demonstrated that whey protein is an effective method of increasing muscle mass, most likely due to enhanced protein and contractile synthesis, as evident by increased muscle fibre area after supplementation (Anderson *et al.*, 2005). However, these observations were demonstrated in combination with resistance training, and thus, whether whey protein increases protein levels within the muscles, or directly increases muscle fibre size, without the added influence of strength or athletic training, is unclear. However, Lands *et al.* (1999) did show performance enhancement (sprint times and increased power output) following whey protein supplementation without additional training. Nonetheless, increased muscle mass of regenerating fibres post-injury following whey protein supplementation would improve force producing capabilities as shown in the measurements of isokinetic and isometric strength in the current study.

Although both muscle size and fibre type composition were not measured in the present study, whey protein supplementation may be increasing muscle CSA and/or muscle mass of the injured muscle fibres possibly due to its ability to influence net muscle protein balance within the muscle (Ha and Zemel, 2003), however, muscle protein synthesis and/or degradation were not measured in the current study, and hence, we can only speculate this as a possible mechanism. Furthermore, whey protein may also be influencing the regeneration of specific fibre types (i.e. fast-twitch vs slow twitch fibres). Any improvements in the regeneration of fast-twitch fibres, which are known for producing explosive forces compared to slow-twitch muscles, following whey protein supplementation would result in greater force producing capacity (i.e. mRFD, TTPT and muscle strength) as seen in the present study. This potential mechanism was further investigated in chapter 7.

The effect of whey protein supplementation on creatine kinase and lactate dehydrogenase

Plasma CK and LDH levels were generally lower during recovery in the whey-supplemented group compared to carbohydrate placebo group, which may be indicative of less muscle fibre damage. The pattern of change in CK and LDH in the current study was similar to that following high force, eccentric exercise reported by Nosaka and Clarkson (1996). Whey protein supplementation had no significant main effect on plasma CK response after exercise, however, subsequent one-way ANOVA analysis revealed significantly lower CK activity at day 2 and 3 post-exercise in the whey protein-supplemented group compared to placebo carbohydrate. Plasma LDH activity was significantly lower during the recovery period compared to the placebo carbohydrate, as evident by a significant main group effect.

One possible explanation for different effects of whey protein supplementation on plasma CK and LDH activity could be due to the extreme variability in CK response after exercise compared to the LDH response. Although CK is used as an indirect marker of muscle damage, there is a larger inter- and intra-participant variability in the CK response after exercise because blood concentrations reflect what is being released from damaged tissue as well as what is taken up by the reticuloendothelial system (Clarkson *et al.*, 1992; Clarkson and Newham, 1995). In a study by Gunst *et al.* (1998), it was shown that serum CK activity was related to serum glutathione activity, in that low glutathione levels were associated with higher CK responses. Researchers suggested that glutathione served as a CK-preserving agent during the lifetime of the enzyme in the plasma circulation. As a consequence, plasma CK activity following exercise-induced damage may be influenced by factors other than damage to the muscle (Rinard *et al.*, 1999). In the present study, whey protein supplementation is likely to enhance the antioxidant defences of the plasma, and thus preservation of plasma CK activity, by increasing plasma glutathione levels. However, even given this potential preserving of plasma CK in the whey protein-supplemented group, the plasma CK activity levels were still generally lower after whey protein supplementation compared to carbohydrate placebo in the present study, and significantly so 2 and 3 days post-exercise. Therefore, strongly suggesting a reduction in muscle fibre damage, and diminished leakage of muscle proteins to the plasma following whey protein supplementation.

In addition, researchers have suggested that glutathione (reduced form-GSH) may also be intimately involved in Ca^{2+} homeostasis by reducing the free radical damage of the SR Ca^{2+} -ATPase pump, following exercise-induced damage, and thus improve the Ca^{2+} handling ability of the muscle. However, since ingestion of whey protein occurred

following the resistance exercise session in the present study, it is unlikely that whey protein supplementation reduced the influx of calcium that occurs during the initial stages of muscle damage. Nevertheless, secondary increases in Ca^{2+} influx due to damage to structural elements, loss of sarcolemmal integrity and increased opening of SACs (Allen *et al.*, 2005) may be diminished by the improved buffering capacity. Improved intracellular Ca^{2+} handling ability will reduce $[\text{Ca}^{2+}]_i$ accumulation and activation of degradative pathways that leads to increased membrane permeability. Consequently, leakage of myocellular proteins from the muscle would be lower as observed in the current study.

Dietary Analysis

One-week dietary analysis revealed participants in both the whey protein – and carbohydrate – supplemented groups were similar in caloric energy, carbohydrate and fat intake, but more importantly protein consumption. The macronutrient content of each supplement was 90gms protein; 3gms carbohydrate, 1.5gms fat per 100gms for whey protein isolate, and 100gms carbohydrate for the placebo control. The macronutrient content of the whey protein isolate supplement was used to ensure that the whey-supplemented group consumed 1.5g/kg of protein per day. Although it is clear that supplements were not matched for carbohydrate and/or fat content, the caloric effect of both supplements were the same as isocaloric supplements were used.

There is no evidence to suggest that the carbohydrate alone supplement may have contributed to the observed benefits. Previous research has been shown that a carbohydrate drink with the same energy content as the protein supplement produces dramatic increases in blood glucose and insulin, but fails to stimulate protein synthesis (Anthony *et al.*, 1999; Gautsch *et al.*, 1998). Indeed, Borsheim *et al.* (2002)

demonstrated that essential amino acids alone (without addition of carbohydrate) are an effective method at stimulating muscle protein synthesis following resistance training. Furthermore, in a later study by the same laboratory (Miller *et al.*, 2003) an addition of 35 grams of carbohydrate to the amino acid mixture, did not cause a greater stimulation of net muscle protein synthesis than compared to the amino acids alone (Miller *et al.*, 2003). These results suggest that stimulation of protein synthesis is firstly not a caloric effect, as an addition of 35g of carbohydrate to the amino acid mixture had no extra effect of protein synthesis. Furthermore, the muscle's ability to build protein after exercise requires intake of amino acids, and that carbohydrate supplements alone are not sufficient to stimulate recovery of protein synthesis.

Thus, in the current study, the beneficial effect from the whey protein supplement is likely due to its amino acid content, in particular the high EAA content, as opposed to any other constituents in the supplement. Interestingly, since both groups were consuming the current recommended dietary allowance (RDA) for protein (0.8 g/kg/day) in sedentary individuals, the improvements in force recovery and reduced extent of damage can be attributed to the extra protein provided by the whey protein supplement. It should also be suggested that the dietary analysis data for both whey protein-supplemented and control groups are not representative of the population, since the protein and energy intakes of these subjects are so low.

In summary, the major finding of this investigation was significantly higher muscle strength following eccentrically-induced muscle damage after whey protein supplementation. In addition, significantly lower plasma creatine kinase and lactate dehydrogenase levels in the days after injury suggested less damage to the muscle, and thus supports the improved functional capabilities observed. Though the current study

did not address the exact mechanisms by which whey protein may exert its effects, it has been suggested that providing amino acids to the intracellular pool within the first few hours following eccentric exercise, and in days thereafter during recovery, whey protein supplementation is likely to maximise muscle protein synthesis, while ameliorating the need to increase protein degradation. Thus, creating a positive protein balance which is essential for the anabolic recovery of muscle fibres following injury.

CHAPTER 6

Effects of Creatine Supplementation on Contractile, Morphological and Biochemical Properties of Rat Skeletal Muscle during Recovery from Chemically-Induced Muscle Damage

6.0 INTRODUCTION

Skeletal muscle damage occurs when there is a disruption to the cellular structure of the muscle, which impairs muscle function, usually as a result from trauma; crush injury, excessive exercise or disuse (Proske and Morgan, 2001). The use of the human muscle biopsy technique allows direct examination of the damaged muscle area after injury. However, it has been suggested that this may not be representative of muscle as a whole (Warren *et al.*, 1999), and therefore could lead to high variability between biopsy samples analyzed (Beaton *et al.*, 2002). Thus, animal models allow a more extensive and accurate analysis of the magnitude of damage and rate of muscle recovery of whole intact skeletal muscles after chemically-induced muscle damage, than would be possible from human biopsy samples. Furthermore, since the healing processes are identical, any improvements in the amount of damage or rate of recovery in the animal model can be directly applicable to muscle injury in humans.

Chemically-induced (myotoxic) injury provides a suitable model for studying muscle regeneration. When injected into skeletal muscle, bupivacaine hydrochloride (bupivacaine), causes rapid degeneration by disrupting intracellular Ca^{2+} homeostasis (Takahashi, 1994; Zink *et al.*, 2002). The use of a myotoxic agent allows a controlled level of damage specific to muscle to be produced, but more importantly, does not damage elements that influence the rate and extent of muscle regeneration, including

endomysium, basal lamina, vascular supply, and intramuscular nerves (Halls-Craggs 1974; Louboutin *et al.*, 1996;).

Few studies have investigated the effect of creatine supplementation on muscle recovery after injury (Warren *et al.*, 2000; Rawson *et al.*, 2001; Santos *et al.*, 2003). As mentioned previously, these studies have used indirect markers of muscle damage to determine the efficacy of creatine supplementation on muscle recovery. Thus, no studies to date have determined the mechanisms by which increased intracellular Cr concentration will influence muscle recovery after injury. In our human study (see chapter 4), creatine supplementation was able to significantly improve force generation and decrease the leakage of intracellular proteins such as CK, indicative of less damage and/or faster regeneration. Therefore, the purpose of this study was to confirm these results by analysing force recovery of rat skeletal muscle after controlled, chemically-induced injury. Furthermore, to determine the mechanisms by which dietary supplement creatine exert its effects by examining morphological and biochemical properties during recovery. We hypothesised that creatine supplementation will restore loss of isometric contractile strength at a faster rate; reduce the extent of damage and improve fibre regeneration concomitant with increased intramuscular ATP, Cr and PCr concentration and protein levels.

6.1 METHODS

6.1.1 Experimental Design and Dietary Treatment

Thirty-five male Sprague-Dawley rats (*Rattus norvegicus*) weighing 203.5 ± 3.8 (mean \pm SEM) were randomly separated into 2 groups: i) control n= 20; ii) creatine-supplemented n= 15. Control rats were fed normal chow. Creatine-supplemented rats were fed creatine monohydrate (CrM) (AST sports science, CO USA) as a 0.02%

(0.02g CrM/100g normal rat chow) mixture for 5 days (loading phase) and a maintenance dosage of 0.002% (0.002g CrM/100g normal rat chow) mixture for 9 days prior to myotoxic injury. This is equivalent to a dose of approximately 1.35g/kg/day and 0.135g/kg/day, respectively. Following injury, rats continued to consume the maintenance dosage (0.002%) for either 7 or 14 days.

The doses and method of administration of creatine was chosen to mimic those used in chapter 4, scaled for the higher metabolic rates of rats. Animals were acquired from Monash Animal Services (Monash University, Melbourne, Australia) and housed at the animal holding facility at Werribee campus, Victoria University. All procedures described below received ethical approval from the Victoria University Animal Experimentation Ethics Committee and conformed to the Australian code of practice for the care and use of animal for scientific purposes.

Animals were housed at a constant temperature (22°C), under a 12:12-h light-dark photoperiod. Animals were housed in pairs prior to muscle injury in accordance with ethical approval. While a more accurate measurement of the level of creatine supplementation would be obtained if the rats were housed separately, there was no evidence to suggest that there was an uneven distribution of supplementation, as rats were fed *ad libitum*, and similar increases in body mass were seen in both rats in each cage. All animals were housed separately following muscle injury in accordance with ethical approval with access to normal or supplemented chow *ad libitum*.

6.1.2 Experimental Protocol

All rats were lightly anesthetized with a domitor (10 mg.kg^{-1} body weight) and ketamine (6 mg.kg^{-1} body weight) combination via an intraperitoneal injection (i.p.), such that they were unresponsive to tactile stimuli. The EDL and soleus muscles of the left hindlimb were surgically exposed and injected with 0.5% bupivacaine (as described in section 3.2.1), causing degeneration of most fibres in the injected muscle (Rosenblatt, 1992). Following injury, the small incision was closed by Michel clips, and swabbed with Betadine antiseptic (povidone iodine solution).

At day 7 and 14 post-myotoxic injury, rats were deeply anaesthetized with Nembutal (pentobarbitone sodium 60 mg.ml^{-1} , i.p.). The level of anesthesia was monitored via corneal and footpad reflexes, with additional doses administered when required. In order to maintain blood flow and nerve supply to the last possible moment, the injured EDL muscle was surgically exposed first and immediately tested for isometric contractile properties (as described in section 3.3.1), followed by the injured soleus muscle and contralateral non-damaged EDL and soleus muscles. At the completion of contractile measurements, each muscle was immediately blotted dry, weighed and divided into 2 sections; one half was snap-frozen in isopentane, cooled in liquid nitrogen and stored for later histological analysis (as described in section 3.4). The other portion was immediately snap frozen in liquid nitrogen for later biochemical analysis (as described in section 3.5 & 3.6). All muscle samples were stored at -80°C .

6.1.3 Statistical Analysis

All values are reported as means \pm SEM. Statistical evaluation for each muscle group (EDL and soleus) and recovery time point (day 7 and day 14) was accomplished by using a two-way analysis of variance (ANOVA) with one between groups factor (supplementation) and one repeated factor (damage vs contralateral). Where an interaction was found, the location of the difference was determined by a one-way ANOVA. Difference in animal morphology characteristics between groups was assessed by students' t-test. Supplementation protocol between groups was assessed by chi-square test. A P value of less than 0.05 was accepted for statistical significance. It should be noted that while the animal studies will be reported separately (chapters 6 for creatine & 7 for whey protein, respectively) in order to align them with the previous human studies (chapters 3 & 4), the data was compared together as both studies utilized the same control group.

6.2 RESULTS

6.2.1 Supplementation and Morphometric Measurements

6.2.1.1 Supplementation Protocol

Based on their food consumption, rats supplemented with creatine monohydrate prior to myotoxic injury consumed 1.41 ± 0.3 g.kg⁻¹ body weight per day for 5 days and 0.129 ± 0.04 g.kg⁻¹ body weight per day for 9 days. These supplement intakes were similar to the expected target dosage of 1.35 and 0.135 g.kg⁻¹ body weight per day, respectively. However, following myotoxic injury, rats consumed 0.119 ± 0.07 and 0.123 ± 0.04 g.kg⁻¹ body weight per day, for 7 and 14 days, respectively. These observed supplement intakes were lower than the expected target dosage of 0.135 g.kg⁻¹ body weight per day.

6.2.1.2 Morphometric Measurements: Body Weight

In order to obtain an accurate measurement of body weight (BW) changes prior to, and following, chemically-induced damage, rats were categorised into 2 groups: those that consumed either normal or creatine-supplemented rat chow for a period of 21 (Group 1) or 28 days (Group 2), respectively. Furthermore, body weights for both control and creatine-supplemented groups were measured 2 weeks prior to the injury protocol (initial BW), on the day of injury protocol (BW after 14 days) and on either day 7 post-injury (BW after 21 days) or day 14 post-injury (BW after 28 days) (see table 6). As expected, rats increased their BW during the 21 and 28-day period.

Effect of supplementation: There were no significant differences in body weights between control and creatine-supplemented group at all time points (see Table 6.2.1.2). However, when expressed as a change from initial body weight, creatine-supplemented rats gained approximately 6g more body mass compared to controls after 2-weeks supplementation in Group 1 ($P < 0.05$, see figure 6). A similar trend was observed in Group 2, although this was not significant.

Group 1	Initial BW (g)	BW (g) after 14 days	BW (g) after 21 days
Control (n = 10)	184.8 ± 7.9	209.4 ± 9.3	222.5 ± 8.1
Creatine (n = 7)	201.4 ± 6.2	232.3 ± 7.6	248 ± 7.6
Group 2	Initial BW (g)	BW (g) after 14 days	BW (g) after 28 days
Control (n = 10)	217.3 ± 5.2	243.7 ± 6.6	259.2 ± 7.2
Creatine (n = 8)	210.6 ± 5.2	242.0 ± 6.7	260.1 ± 6.9

Table 6. Rat body weights (BW) (g) for both normal (control) and creatine-supplemented group measured 2-weeks prior to injury (initial BW), on the day of injury protocol (BW after 14 days) and on either day 7 post-injury (Group 1: BW after 21 days) or day 14 post-injury (Group 2: BW after 28 days).

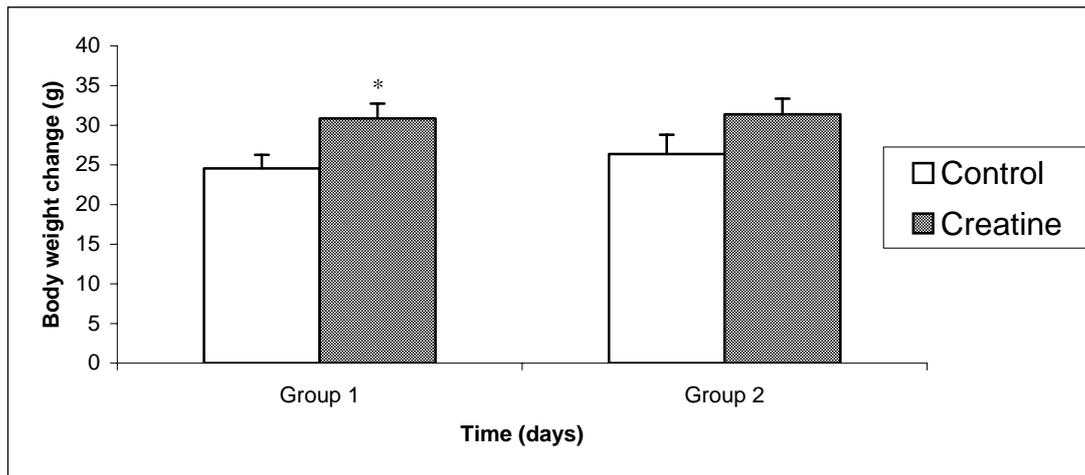


Figure 6. Changes in rat body weight (the difference in grams between initial BW and BW after 14 days) for both normal (control) and creatine-supplemented animals in group 1 (those consuming normal or creatine-supplemented rat chow for a period of 21 days) and group 2 (those consuming normal or creatine-supplemented rat chow for a period of 28 days). * ($P < 0.05$) significantly different from control in group 1.

6.2.1.3 Morphometric Measurements: Muscle Mass

Muscle mass changes in normal- and creatine-supplemented EDL and soleus muscles at day 7 and 14 post-injury are summarized in Figure 6.1a & 6.1b. Only the non-supplemented animals will be used when describing the effects of myotoxic injury. Unless an interaction occurred, similar effects from myotoxic injury in the supplemented groups also occurred and are thus shown in the relevant figures. However, the magnitude of these changes was not necessarily the same, due to the significant effects of supplementation, which will be subsequently discussed.

Effect of myotoxic injury: There was a highly significant decrease in muscle mass in both the EDL ($P < 0.01$) and soleus ($P < 0.001$) muscles at day 7 post-injury. However, by day 14, no differences were observed, as muscle mass was nearly recovered to uninjured muscle mass values. *Effect of supplementation:* Creatine-supplemented muscles were significantly heavier than control muscles at day 7 post-injury ($P < 0.05$ see figure 6.1a & 6.1b), indicating that not only were injured muscles of creatine-supplemented animals heavier than control animals during recovery, the contralateral uninjured muscles were also significantly heavier in the creatine-supplemented animals compared to control animals. Similarly by day 14, creatine-supplemented EDL muscles were again heavier than controls ($P < 0.05$), indicating that while muscle mass was nearly fully recovered to uninjured values, creatine-supplemented muscles were still heavier than the control muscles. However, only the EDL muscles were found to be significantly heavier ($P < 0.05$) A similar trend towards significance occurred in the soleus muscles, although not significant ($P = 0.085$).

When muscle weights were expressed relative to body mass (MM:BM), myotoxic injury caused a significant decrease in both the EDL and soleus muscles at day 7 ($P < 0.01$) and day 14 ($P < 0.01$) post-injury (table 6.1a & 6.1b). *Effect of supplementation:* No differences in MM:BM were observed between creatine-supplemented and control at day 7 post-injury. However, by day 14 post-injury, creatine-supplemented animals demonstrated significantly higher MM:BM in the soleus muscle ($P < 0.01$, see table 6.1b), while only a strong trend for higher MM:BM was evident in the EDL muscles ($P = 0.07$).

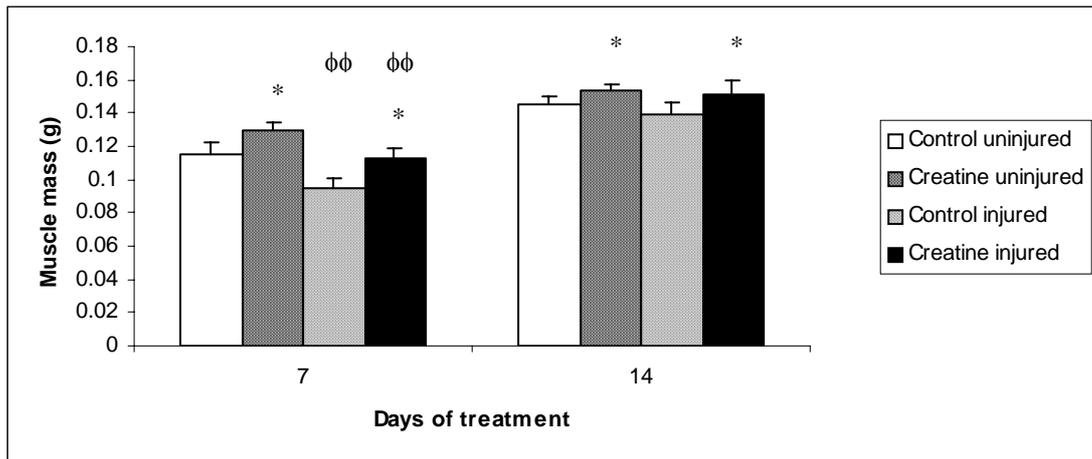


Figure 6.1a. Muscle mass changes of uninjured and injured EDL muscles from rats treated with normal- or creatine-supplemented rat chow at day 7 and 14 post-injury. Values are mean \pm SEM. $\phi\phi$ ($P < 0.01$) significantly different from contralateral values. * ($P < 0.05$) significantly different from control animals.

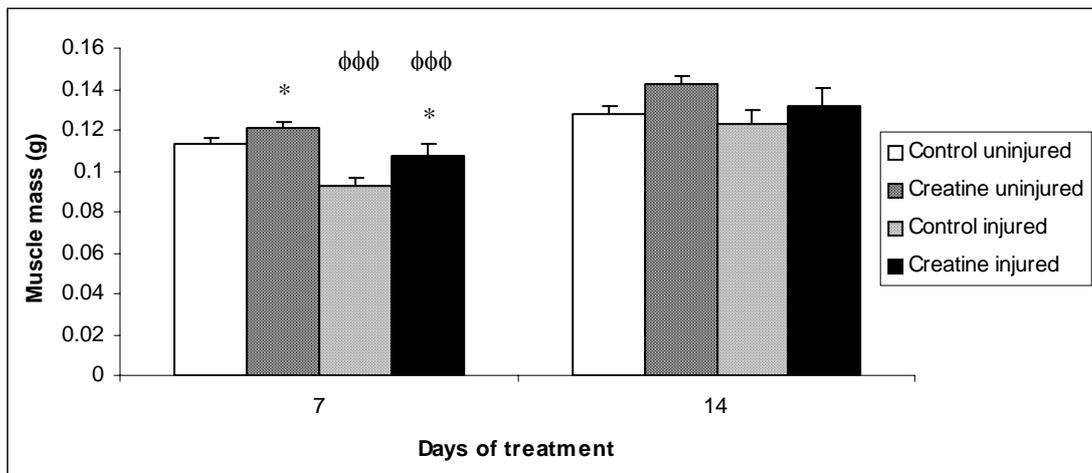


Figure 6.1b. Muscle mass changes of uninjured and injured soleus muscles from rats treated with normal- or creatine-supplemented rat chow at day 7 and 14 post-injury. Values are mean \pm SEM. $\phi\phi\phi$ ($P < 0.001$) significantly different from contralateral values * ($P < 0.05$) significantly different from control animals.

6.2.2 Contractile Measurements

Contractile properties of all muscles are summarized in Table 6.1a & 6.1b. Creatine-supplemented muscles will be categorized as CR-INJ and CR-NORM for injured and uninjured muscles, respectively, while control muscles will be categorized as CON-INJ and CON-NORM for injured and uninjured muscles, respectively.

Effect of myotoxic injury: Without supplementation, optimal lengths for both CON-INJ EDL and soleus muscles were not significantly different from CON-NORM at day 7 and 14 post-injury. Time to peak twitch tension (TTPT) was significantly slower in the EDL muscles at day 7 post-injury ($P < 0.05$), while no effects from injury were evident at day 14 post-injury. One-half relaxation time ($\frac{1}{2}$ RT) was significantly prolonged in the CON-INJ EDL muscles at day 7 and 14 post-injury compared with time-matched CON-NORM EDL muscles ($P < 0.001$ see Table 6.1a). However, similar effects from myotoxic injury were not observed in the creatine-supplemented group, as evident by a significant group by damage interaction ($P < 0.05$). No significant effects from myotoxic injury were observed on TTPT in CR-INJ EDL muscles at day 7 post-injury, and $\frac{1}{2}$ RT in the CR-INJ EDL muscles at day 7 and 14 post-injury (see table 6.1a).

In the soleus muscles, $\frac{1}{2}$ RT was significantly faster in the CON-INJ soleus muscles at day 7 ($P < 0.001$) and 14 ($P < 0.05$) post-injury, compared to CON-NORM. However, as seen in the EDL muscles, no significant effect from myotoxic was observed in $\frac{1}{2}$ RT in the CR-INJ soleus muscles at day 7 post-injury. No significant effects from myotoxic injury were observed in TTPT of the soleus muscles at any time point.

Treatment duration		CON-INJ	CR-INJ	CON-NORM	CR-NORM
	n	10	7	10	7
	MM:BM (g)	0.042 ± 0.003	0.048 ± 0.002	0.051 ± 0.003	0.054 ± 0.002
7 days	Opt Length (cm)	2.92 ± 0.1	3.11 ± 0.07 *	2.95 ± 0.07	3.1 ± 0.08 *
	Pt (mN)	122.4 ± 16.3 φφφφ	220.7 ± 38.1 φφφφ *	521 ± 83.5	508.7 ± 68.2
	TTP (ms)	55 ± 5.2 φ	45 ± 6	47 ± 4.2	44 ± 4.2
	½ RT (ms)	92 ± 9.4 φφφ	53 ± 4.3 ***	60 ± 5.1	52 ± 6.3
	n	10	8	10	8
	MM:BM (g)	0.053 ± 0.003	0.058 ± 0.002	0.056 ± 0.002	0.059 ± 0.001
14 days	Opt Length (cm)	3.14 ± 0.04	3.23 ± 0.06 *	3.14 ± 0.04	3.28 ± 0.06 *
	Pt (mN)	459.7 ± 20.2 φφφφ	560.4 ± 28.0 φφφφ *	713.2 ± 86.9	805.9 ± 25.3 *
	TTP (ms)	49 ± 5.9	44 ± 3.1	46 ± 3.1	38 ± 1.8
	½ RT (ms)	103 ± 30.2 φφφ	59 ± 4.8 *	55 ± 4.4	46 ± 2.3 *

Table 6.1a. Morphometric and contractile properties of uninjured and injured EDL muscles from rats treated with normal- or creatine-supplemented rat chow at 7 and 14 days post-injury. n = number of animals in each group, MM:BM muscle mass: body mass ratio. Values are mean ± SEM. φφφφ (P<0.0001), φφφ (P<0.001), φ (P<0.05) significantly different from contralateral values. * (P<0.05) ***(P<0.001) significantly different from control muscles at day 7 and/or day 14 post-injury.

As expected with the observed changes in muscle mass, absolute force (Po) was significantly lower in both the CON-INJ EDL and soleus muscles at day 7 post-injury, with the Po only ~27% (P<0.0001) and ~43% (P<0.001) of CON-NORM values of the EDL and soleus muscles, respectively. The recovery of Po was steady, but slow, such that Po values were still only ~69% (P<0.001) and ~74% (P<0.01) of uninjured muscle values by day 14 post-injury in the EDL and soleus muscles, respectively (see figure 6.2a & 6.2b). When taking into account muscle cross-sectional area (i.e. Po was normalized for muscle size), the specific forces (sPo) of both the CON-INJ EDL and soleus muscles was still significantly lower than CON-NORM sPo values by day 7

($P < 0.01$) and 14 ($P < 0.05$) post-injury (see figure 6.2c & 6.2d). Similarly, the sPo of CON-INJ EDL muscle at day 7 and 14 post-injury was 32% and 71% of CON-NORM muscle values, respectively. The sPo of CON-INJ soleus muscles at day 7 and 14 post-injury was 51% and 76% of uninjured muscle values, respectively

Treatment duration	CON-INJ	CR-INJ	CON-NORM	CR-NORM
n	10	7	10	7
7 days MM:BM (g)	0.042 ± 0.001	0.045 ± 0.002	0.050 ± 0.002	0.051 ± 0.002
Opt Length (cm)	2.89 ± 0.1	2.94 ± 0.04	2.93 ± 0.03	2.95 ± 0.04
Pt (mN)	78.7 ± 8.5 φφφ	102.6 ± 29.1 φφφ	211.0 ± 41.5	186.3 ± 51.4
TTP (ms)	135 ± 20	138 ± 9	150 ± 13	123 ± 14
½ RT (ms)	197 ± 16 φφφ	216 ± 17	272 ± 23	210 ± 28
n	10	8	10	8
14 days MM:BM (g)	0.047 ± 0.003	0.050 ± 0.003 **	0.049 ± 0.001	0.055 ± 0.006 **
Opt Length (cm)	3.09 ± 0.04	3.10 ± 0.05	3.19 ± 0.03	3.15 ± 0.05
Pt (mN)	164.4 ± 15.1 φφφ	200.4 ± 29.8 ** φφφ	188.1 ± 32.4	316.1 ± 20.2 **
TTP (ms)	150 ± 12	152 ± 7	146 ± 13	130 ± 20
½ RT (ms)	238 ± 12 φ	236 ± 18 φ	277 ± 28	253 ± 46

Table 6.1b. Morphometric and contractile properties of uninjured and injured soleus muscles from rats treated with normal- or creatine-supplemented rat chow at day 7 and 14 post-injury. n = number of animals in each group, MM:BM muscle mass: body mass ratio. Values are mean ± SEM. φφφ ($P < 0.001$) φ ($P < 0.05$) significantly different from contralateral values. ** ($P < 0.01$) significantly different from control muscles at day 14 post-injury.

Similarly, peak twitch tension (Pt) was significantly lower in both CON-INJ EDL and soleus muscles at day 7 ($P < 0.0001$) and 14 ($P < 0.001$) post-injury (see table 6.2a & 6.2b). The Pt of CON-INJ EDL was 23% and 64% of CON-NORM muscle values at

day 7 and 14 post-injury, respectively. However, similar to the observed changes in Po and sPo post-injury, the Pt values for the CON-INJ soleus muscles, although lower than CON-NORM values, were higher than CON-INJ EDL muscles (37% and 87% of CON-NORM values at day 7 and 14 post-injury).

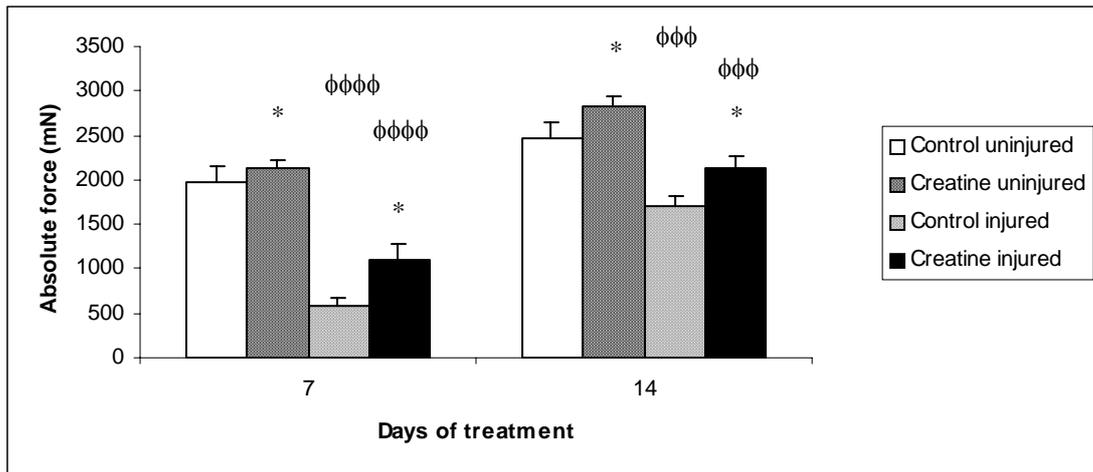


Figure 6.2a Absolute forces (Po) of uninjured and injured EDL muscles from rats treated with normal- or creatine-supplemented rat chow at day 7 and 14 post-injury. Values are mean \pm SEM. $\phi\phi\phi\phi$ ($P<0.0001$) $\phi\phi\phi$ ($P<0.001$) significantly different from contralateral values. * ($P<0.05$) significantly different from control muscles at day 7 and 14 post-injury.

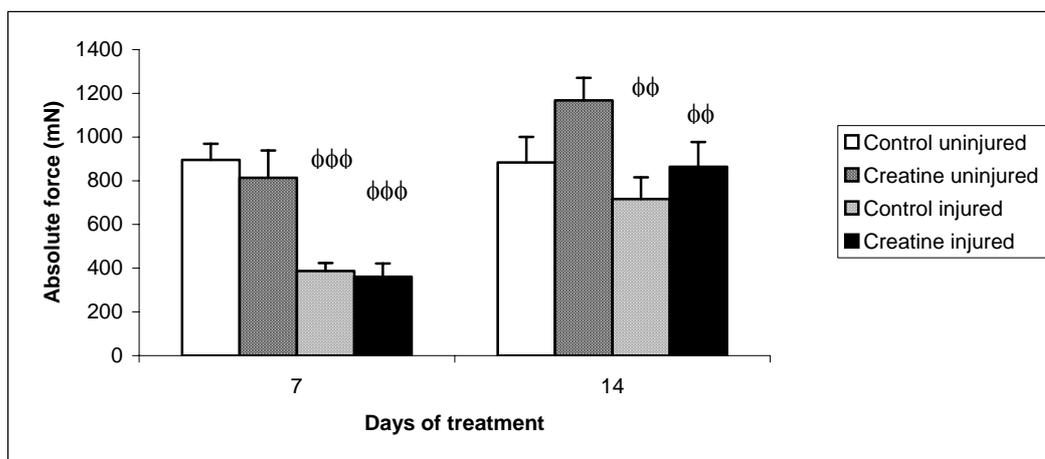


Figure 6.2b Absolute forces (Po) of uninjured and injured soleus muscles from rats treated with normal- or creatine-supplemented rat chow at day 7 and 14 post-injury. Values are mean \pm SEM. $\phi\phi\phi\phi$ ($P<0.001$) $\phi\phi$ ($P<0.01$) significantly different from contralateral values.

Effect of supplementation: Optimal length (L_o) was significantly longer in the creatine-supplemented EDL muscles compared to control muscles at day 7 and 14 post-injury ($P < 0.05$, see table 6.1a). Furthermore, $\frac{1}{2}$ RT was significantly quicker in the creatine-supplemented EDL muscles compared to control muscles at day 7 ($P < 0.001$) and 14 ($P < 0.05$) post-injury, although this was only evident in the regenerating EDL muscles (CR-INJ). Creatine supplementation had no significant effect on TTPT. Similarly, no significant changes in L_o , $\frac{1}{2}$ RT and TTP were observed in the soleus muscles following creatine supplementation at any time point.

Absolute forces (P_o) were significantly higher in the creatine-supplemented EDL muscles at day 7 and 14 post-injury ($P < 0.05$), indicating that both CR-INJ and CR-NORM were generating higher forces than CON-INJ and CON-NORM, respectively. Moreover, when P_o of regenerating muscles was expressed as percentage of contralateral values, these values were still higher than time matched control values. Following creatine supplementation, P_o was ~52% (vs 27% CON-INJ) and ~76% (vs 69% CON-INJ) of contralateral values at day 7 and 14 post-injury, respectively, which is consistent with the significant increases in muscle mass in the creatine-supplemented muscles (see figure 6.2a). No significant effects of supplementation were observed in the soleus muscles at day 7 post-injury, however a trend towards higher P_o was observed at day 14 post-injury, following supplementation ($p = 0.093$ figure 6.2b).

In addition, when P_o was normalized for muscle size, specific forces were still significantly higher ($P < 0.001$) in the CR-INJ EDL muscles compared to CON-INJ EDL muscles at day 7 post-injury (see figure 6.2c), indicating that for a given muscle size, CR-INJ EDL muscles were producing greater forces compared to CON-INJ muscles at day 7 post-injury (see figure 6.2c). In contrast, no differences in sP_o were observed

between creatine-supplemented EDL muscles and control EDL muscles at day 14 post-injury. Similarly, creatine supplementation had no significant effect on sPo in the soleus muscles at day 7 or at day 14 post-injury (figure 6.2d)

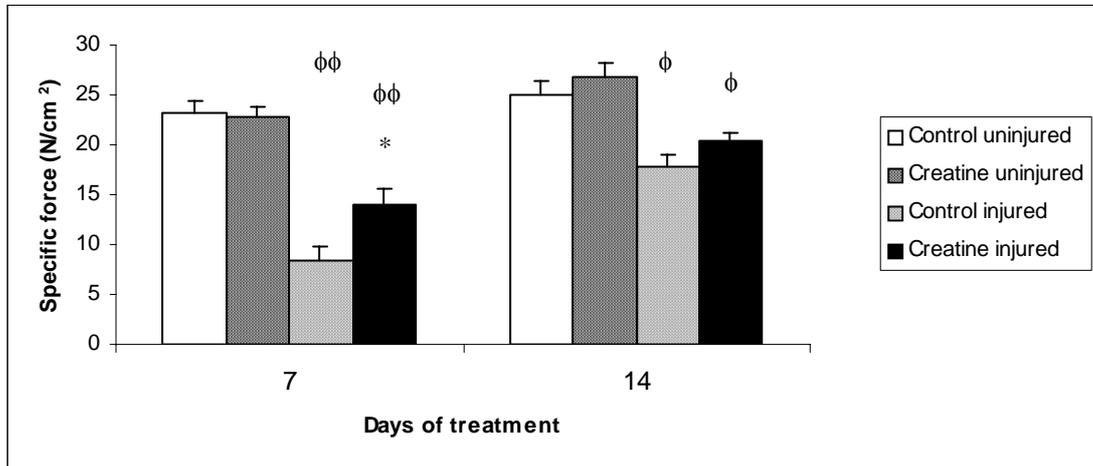


Figure 6.2c Specific forces (sPo) of uninjured and injured EDL muscles from rats treated with normal- or creatine-supplemented rat chow at day 7 and 14 post-injury. Values are mean \pm SEM. $\phi\phi$ ($P < 0.01$) ϕ ($P < 0.05$) significantly different from contralateral values. * ($P < 0.05$) significantly different from control muscles at day 7 post-injury.

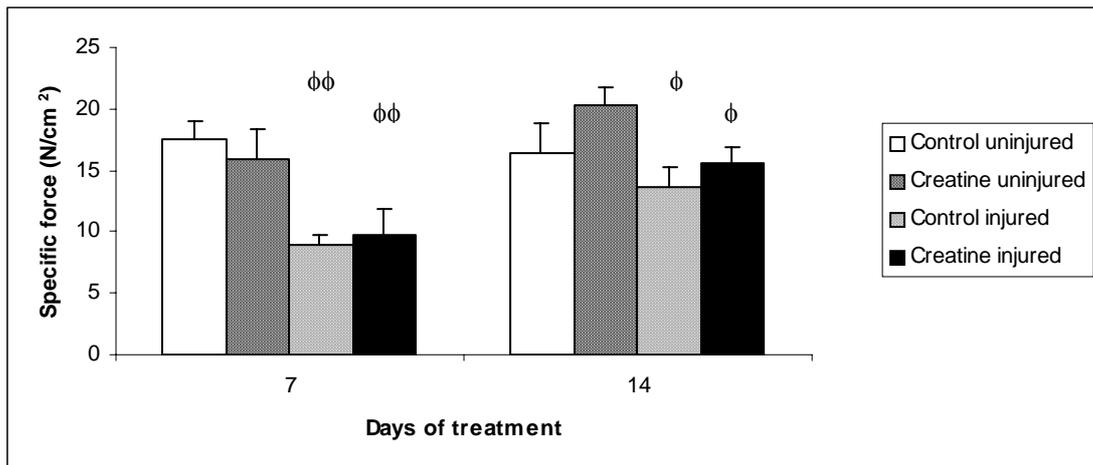


Figure 6.2d Specific forces (sPo) of uninjured and injured soleus muscles from rats treated with normal- or creatine-supplemented rat chow at day 7 and 14 post-injury. Values are mean \pm SEM. $\phi\phi$ ($P < 0.01$) ϕ ($P < 0.05$) significantly different from contralateral values.

Peak twitch forces were significantly higher in the CR-INJ EDL muscles at day 7 post-injury ($P < 0.05$), while no effects were observed in the contralateral EDL muscles and/or either soleus muscles at day 7 post-injury. At day 14 post-injury, peak twitch forces were significantly higher in the creatine-supplemented EDL ($P < 0.05$) and soleus ($P < 0.01$) muscles compared to control muscles (see table 6.1a & 6.1b).

6.2.3 Intramuscular Metabolite Levels

The metabolite concentrations of ATP, creatine (Cr), phosphocreatine (PCr) and total creatine ($\text{TCr} = \text{Cr} + \text{PCr}$) determined for all muscles are displayed in table 6.2a & 6.2b.

Effect of myotoxic injury: ATP and TCr levels were significantly lower in both the injured EDL and soleus muscles at day 7 and 14 days post-injury ($P < 0.05$). Cr levels in the EDL were significantly decreased after myotoxic injury at day 7 ($P < 0.05$). No effects on Cr levels were observed at day 14 post-injury. PCr levels were significantly decreased following myotoxic injury at day 7 and 14 ($P < 0.05$). In contrast, no effects of myotoxic injury were observed on PCr levels in the CR-INJ EDL muscles at any time point, as evident by a significant group by time interaction ($P < 0.05$)

Cr levels were only lower at day 14 post-injury in the soleus muscle ($P < 0.01$), as no changes in Cr levels were seen at day 7 post-injury (table 6.2a & 6.2b). However, interestingly, Cr levels were significantly lower in the CR-INJ soleus muscles at day 7 post-injury. PCr levels were significantly decreased following myotoxic injury at day 7 ($P < 0.05$), while no significant changes were observed at day 14 post-injury. However, a significant group by damage interaction was revealed ($P < 0.05$), as PCr levels were decreased in the CR-INJ soleus muscles at day 14 post-injury ($P < 0.05$). No significant changes were observed at day 7 post-injury (table 6.2b).

Treatment duration	CON-INJ	CR-INJ	CON-NORM	CR-NORM	
	n	8	7	8	7
7 days	ATP (mmol/kg dw)	10.3 ± 2.1 φ	14.0 ± 1.2 φ	17.6 ± 2.2	19.6 ± 0.5
	PCr (mmol/kg dw)	19.2 ± 4.8 φ	31.0 ± 4.9	27.5 ± 8	27.2 ± 3.8
	Cr (mmol/kg dw)	54.9 ± 6.9 φφ	50.9 ± 4.0 φφ	64.8 ± 3.6	79.9 ± 10.7
	TCr (mmol/kg dw)	74.2 ± 9.4 φ	81.9 ± 4.0 φ	92.3 ± 7.2	107.2 ± 13
	n	8	8	8	8
14 days	ATP (mmol/kg dw)	12.9 ± 1.1 φ	12.9 ± 1.4 φ	18.4 ± 1.8	21.9 ± 1.2
	PCr (mmol/kg dw)	33.8 ± 6.5 φ	44 ± 3.8	46.1 ± 6.2	36.3 ± 3
	Cr (mmol/kg dw)	52.4 ± 4.8	53 ± 3.8	55.6 ± 4.2	67.8 ± 3.1
	TCr (mmol/kg dw)	86.2 ± 3.8 φ	97 ± 4.1 φ	101.7 ± 4.4	104.1 ± 3.1

Table 6.2a. Muscle metabolite levels of uninjured and injured EDL muscles from rats treated with normal- or creatine-supplemented rat chow at day 7 and 14 post injury. n = number of animals in each group. Values are mean ± SEM. φφ (P<0.01), φ (P<0.05) significantly different from contralateral values.

Treatment duration	CON-INJ	CR-INJ	CON-NORM	CR-NORM	
	n	8	7	8	7
7 days	ATP (mmol/kg dw)	4.8 ± 1.6 φ	7.4 ± 0.7 φ	7.7 ± 1.6	12.6 ± 0.7
	PCr (mmol/kg dw)	4.9 ± 0.5 φ	15.2 ± 3	11.8 ± 4.6	16.4 ± 3.5
	Cr (mmol/kg dw)	59.7 ± 8.4	42.5 ± 2.7 φ	54 ± 2.9	55.8 ± 7.2
	TCr (mmol/kg dw)	64.6 ± 8.1 φ	57.7 ± 3.7 φ	65.8 ± 5.2	72.2 ± 8.7
	n	8	8	8	8
14 days	ATP (mmol/kg dw)	8.0 ± 1.1 φ	9.0 ± 0.6 φ	10.8 ± 1.8	12.6 ± 0.7
	PCr (mmol/kg dw)	25.3 ± 5.3	14.3 ± 3.6 φ	28.1 ± 5.1	26.4 ± 3
	Cr (mmol/kg dw)	44.6 ± 5 φφ	45.9 ± 2.3 φφ	53.9 ± 2.7	55.5 ± 2.8
	TCr (mmol/kg dw)	69.8 ± 6 φ	60.3 ± 5.9 φ	82 ± 2.7	81.9 ± 4.6

Table 6.2b. Muscle metabolite content of uninjured and injured soleus muscles from rats treated with normal- or creatine-supplemented rat chow for 7 and 14 days. n = number of animals in each group. Values are mean ± SEM. φφ (P<0.01), φ (P<0.05) significantly different from contralateral values.

Effect of supplementation: No significant effects of supplementation on ATP levels were observed in the EDL and soleus muscles at day 7 and 14 post-injury. Despite TCr levels being ~16% and ~10% higher at day 7 post-injury in the CR-NORM EDL and CR-NORM soleus muscles, respectively; this was not significant. However, a trend towards higher TCr levels in the CR-INJ EDL muscles was shown at day 14 post-injury (P=0.09).

6.2.4 Intramuscular Protein Levels

Muscle protein content was measured to confirm whether alterations in muscle mass was due to alterations in functional material (see table 6.3a & 6.3b).

Effect of myotoxic injury: Total protein and contractile protein concentrations were significantly lower in the injured EDL muscles at day 7 and 14 post-injury (p<0.05). However, when contractile protein concentration was expressed as a percentage of protein concentration (% contractile protein), there was no significant effect from myotoxic injury. Similarly in the injured soleus muscles, protein concentrations were lower at day 7 and 14 post-injury (p<0.05). However, in contrast to the injured EDL muscles, contractile concentrations, in addition to % contractile protein were not significantly different from contralateral soleus muscles (table 6.3a & 6.3b).

Effect of supplementation: Following 7 days recovery, a trend for higher protein (P=0.08) and contractile protein (P=0.09) levels were observed in the CR-INJ EDL muscles compared to CON-INJ EDL muscles. By day 14 post-injury, only protein concentration was significantly higher in the CR-INJ compared to CON-INJ EDL muscles (P<0.05), with a strong trend for higher contractile proteins (P=0.06) following creatine supplementation.

Interestingly, a similar effect was observed in the contralateral muscles, with significantly higher protein levels shown in CR-NORM compared to CON-NORM ($P < 0.05$, see table 6.3a). Creatine supplementation had no significant effects on % contractile protein at any time point.

The soleus muscles demonstrated significantly higher protein levels in both the CR-INJ and CR-NORM compared to CON-INJ and CON-NORM, respectively, at day 7 post-injury. Furthermore at day 14 post-injury, protein levels were significantly higher in the CR-NORM compared to CON-NORM ($P < 0.05$, see table 6.3b), while no effects from creatine supplementation were observed in the injured soleus muscle. Creatine supplementation had no significant effect on contractile and % contractile protein at any time point.

Treatment duration		CON-INJ	CR-INJ	CON-NORM	CR-NORM
	n	9	7	9	7
	[protein] (mg/g)	115.34 ± 10.3 ϕ	149.4 ± 14.6 ϕ	161.3 ± 14.5	163.8 ± 14.4
7 days	[contractile protein] (mg/g)	30.7 ± 3.7 ϕ	39.8 ± 4.7 ϕ	46.9 ± 5.7	52.4 ± 3.2
	% Contractile protein	26.6 ± 2.7	26.6 ± 3.5	29.1 ± 4.4	32 ± 2.1
	n	9	8	9	8
	[protein] (mg/g)	123.7 ± 12.2 ϕ	154.5 ± 10.1 ϕ *	162.1 ± 11.1	169.3 ± 6.6 *
14 days	[contractile protein] (mg/g)	32.4 ± 4.3 ϕ	45.4 ± 5.4 ϕ	45.9 ± 4.1	56.3 ± 5.6
	% Contractile protein	26.2 ± 1.9	29.3 ± 2.1	28.3 ± 2.8	33.3 ± 3.3

Table 6.3a. Muscle protein content of uninjured and injured EDL muscles from rats treated with normal- or creatine- supplemented rat chow at day 7 and 14 post-injury. n = number of animals in each group. Values are mean ± SEM. ϕ ($P < 0.05$) significantly different from contralateral values. * ($P < 0.05$) significantly different from control muscles at day 14 post-injury.

Treatment duration		CON-INJ	CR-INJ	CON-NORM	CR-NORM
	n	8	7	8	7
	[protein] (mg/g)	112.2 ± 16.4 ϕ	122.2 ± 16.5 ϕ *	136.9 ± 15.7	201.5 ± 34.3 *
7 days	[contractile protein] (mg/g)	37.3 ± 5.5	35.4 ± 2.8	41.7 ± 5.4	46.7 ± 4.9
	% Contractile protein	33.2 ± 2.7	28.9 ± 2.6	30.5 ± 2.3	23.2 ± 3.4
	n	10	8	10	8
	[protein] (mg/g)	124.6 ± 11.7 ϕ	121.2 ± 6.1 ϕ	141.5 ± 5.6	195.9 ± 20.6 *
14 days	[contractile protein] (mg/g)	36.1 ± 4.6	29.8 ± 1.8	42.9 ± 3.7	46.7 ± 8.1
	% Contractile protein	29 ± 1.8	24.6 ± 1.3	30.3 ± 3.2	23.8 ± 4.2

Table 6.3b. Muscle protein content of uninjured and injured soleus muscles from rats treated with normal- or creatine- supplemented rat chow for 7 and 14 days. n = number of animals in each group. Values are mean ± SEM. ϕ (P<0.05) significantly different from contralateral values. * (P<0.05) significantly different from control muscles at day 7 and/or day 14 post-injury.

6.2.5 Muscle Fibre CSA

Cross-sectional area of both intact (non-damaged) and regenerating muscles was measured to examine that increases in muscle strength were due to changes in fibre CSA (see figure 6.3a & 6.3b).

Effect of myotoxic injury: As expected, CSA of regenerating EDL and soleus muscle fibres were significantly smaller compared to CSA of intact fibres at day 7 and 14 post-injury (P<0.001, see figure 6.3a & 6.3b). *Effect of supplementation:* At day 7 post-injury, regenerating fibres of both creatine-supplemented EDL and soleus muscles demonstrated significantly larger fibre CSA compared to control EDL and soleus muscles (P<0.05 see figure 6.3a & 6.3b). Furthermore, CSA of intact fibres was significantly larger in both the EDL and soleus muscles following creatine

supplementation ($P < 0.05$ see figure 6.3a & 6.3b). However, by day 14 post-injury, no effects of supplementation were evident.

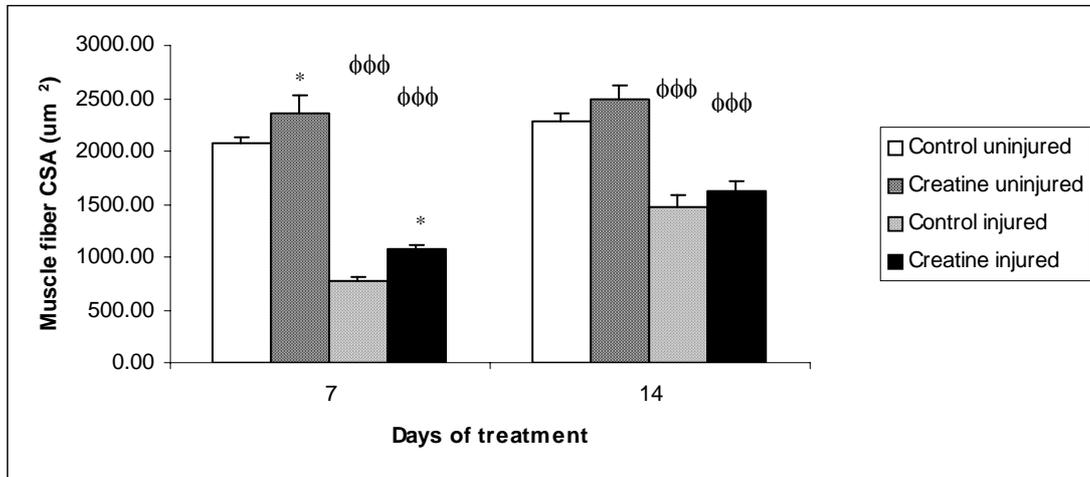


Figure 6.3a. Muscle fibre CSA of regenerating and intact EDL muscles from rats treated with normal- or creatine- supplemented rat chow at day 7 and 14 post-injury. Values are mean \pm SEM. $\phi\phi\phi$ ($P < 0.05$) significantly different from contralateral values. * ($P < 0.05$) significantly different from control muscles at day 7 post-injury.

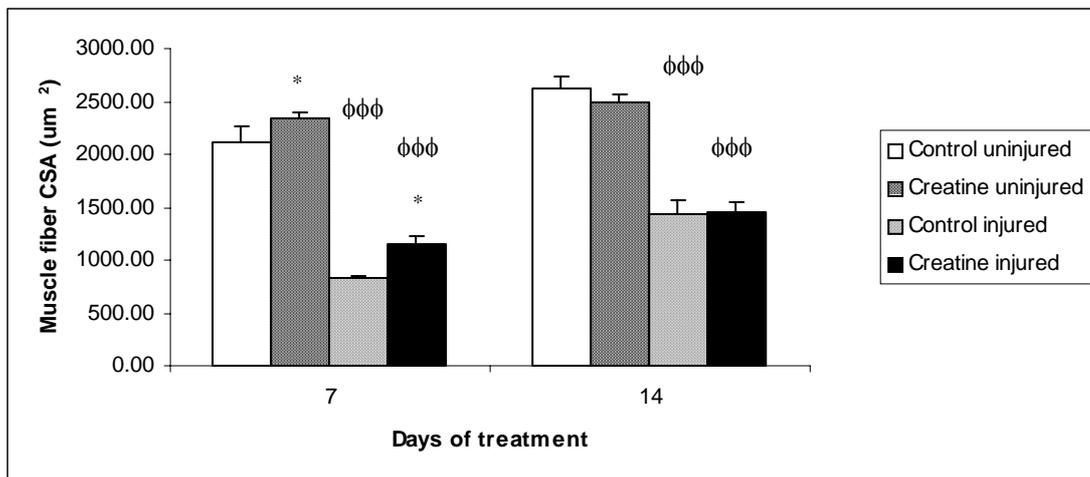


Figure 6.3b. Muscle fibre CSA of regenerating and intact soleus muscles from rats treated with normal- or creatine- supplemented rat chow at day 7 and 14 post-injury. Values are mean \pm SEM. $\phi\phi\phi$ ($P < 0.05$) significantly different from contralateral values. * ($P < 0.05$) significantly different from control muscles at day 7 post-injury.

6.2.6 Percentage of Damaged, Regenerating and Intact Fibres

Effect of myotoxic injury: Large amounts of fibre necrosis were clearly observed in both EDL and soleus muscles at day 7 post-injury, as evident by a large reduction in intact muscle fibres and an increase in damaged and regenerating fibres (see figure 6.4a, 6.4b & 6.4d). By day 14 post-injury, restoration of muscle fibres was apparent with an increase in intact muscle fibres and a reduction in regenerating and damaged fibres (see figure 6.4b & 6.4e). *Effect of supplementation:* Creatine supplementation significantly reduced the extent of fibre degeneration and improved muscle fibre regeneration in the EDL muscles at day 7 post-injury as evident by a ~9% reduction in the extent of fibre damage and a ~17% increase in intact muscle ($P < 0.05$, see figure 6.4a & 6.4b). No effects from creatine supplementation were demonstrated at day 14 post-injury, or in the soleus muscles at both any time point (see figure 6.4c, 6.4d & 6.4e).

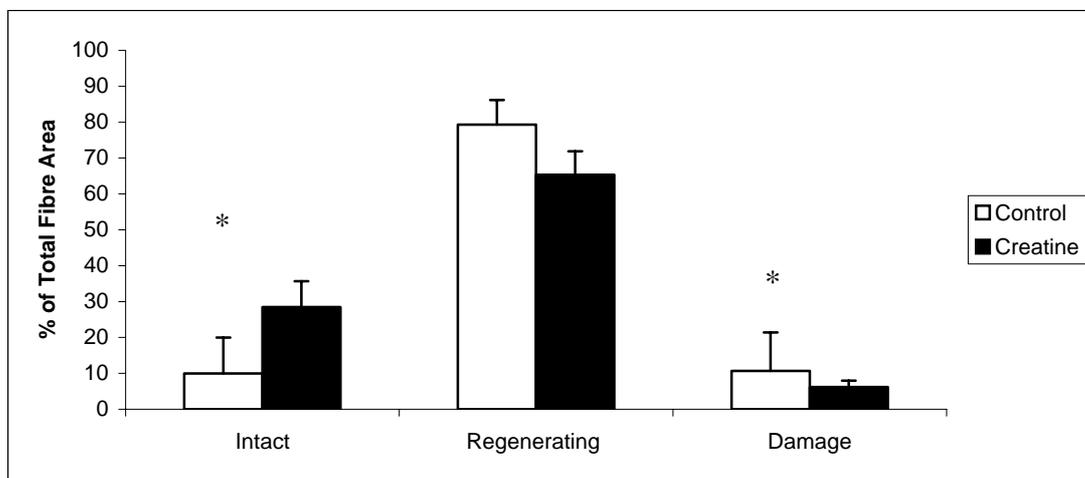
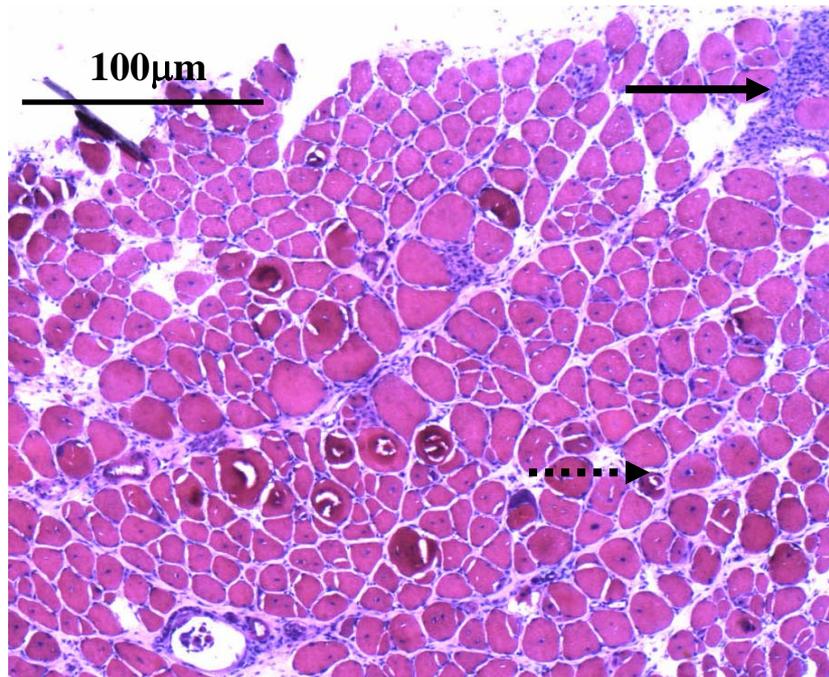


Figure 6.4a. Muscle fibre recovery (expressed as a % of muscle CSA) of injured EDL muscles from rats treated with normal- or creatine- supplemented rat chow at day 7 post-injury. Values are mean \pm SEM expressed as either % of damaged fibres, regenerating fibres, and undamaged (intact) fibres. * ($P < 0.05$) significantly different from control muscles.

A).



B).

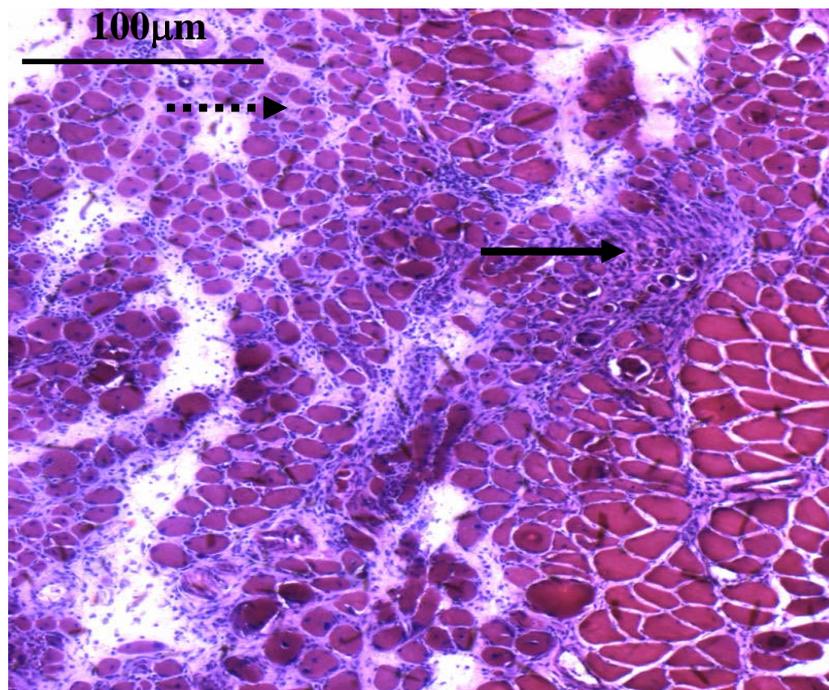


Figure 6.4b Creatine significantly reduced fibre necrosis and improved muscle fibre regeneration in the EDL muscle. Muscles were isolated from (A) creatine-supplemented and (B) control rats at day 7 post-injury. ← Necrosis identified by infiltrating lymphocytes and ←..... regenerating fibres presenting centrally nucleated fibres shown on a representative cross-sectional muscle section stained with H&E. Note widespread necrosis in the control muscles compared to creatine-supplemented EDL muscles at day 7 post-injury. In addition to larger regenerating fibres in the creatine-supplemented EDL muscles compared to control EDL muscles.

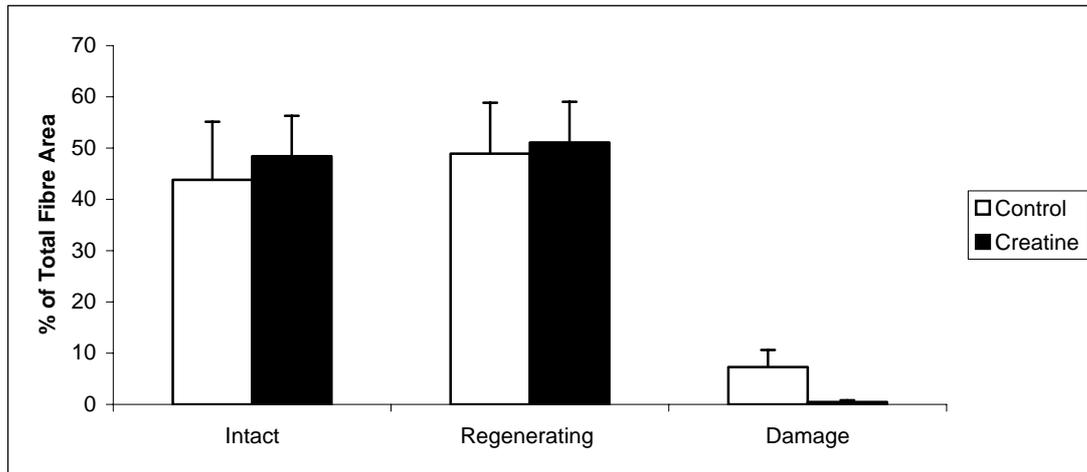


Figure 6.4c. Muscle fibre recovery (expressed as a % of muscle CSA) of injured EDL muscles from rats treated with normal- or creatine- supplemented rat chow at day 14 post-injury. Values are mean \pm SEM expressed as either % of damaged fibres, regenerating fibres, and undamaged (intact) fibres.

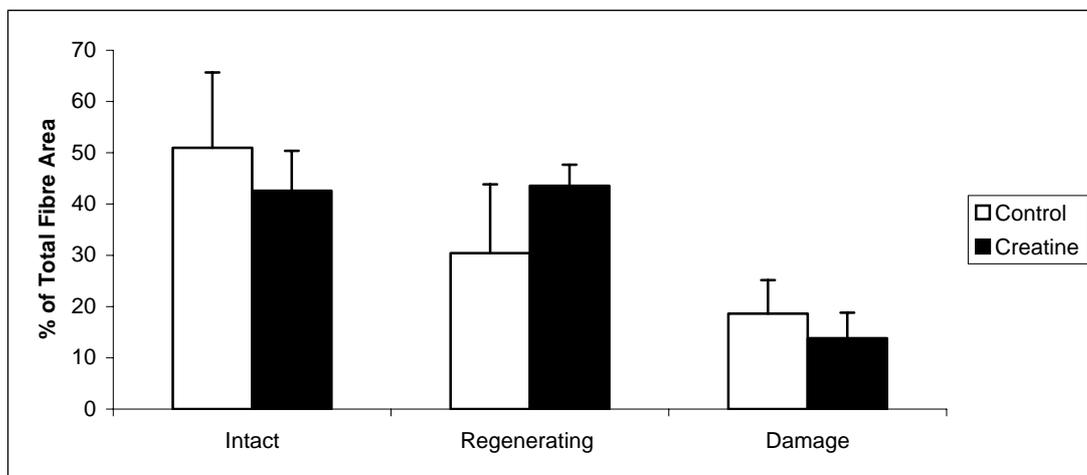


Figure 6.4d. Muscle fibre recovery (expressed as a % of muscle CSA) of injured soleus muscles from rats treated with normal- or creatine- supplemented rat chow at day 7 post-injury. Values are mean \pm SEM expressed as either % of damaged fibres, regenerating fibres, and undamaged (intact) fibres.

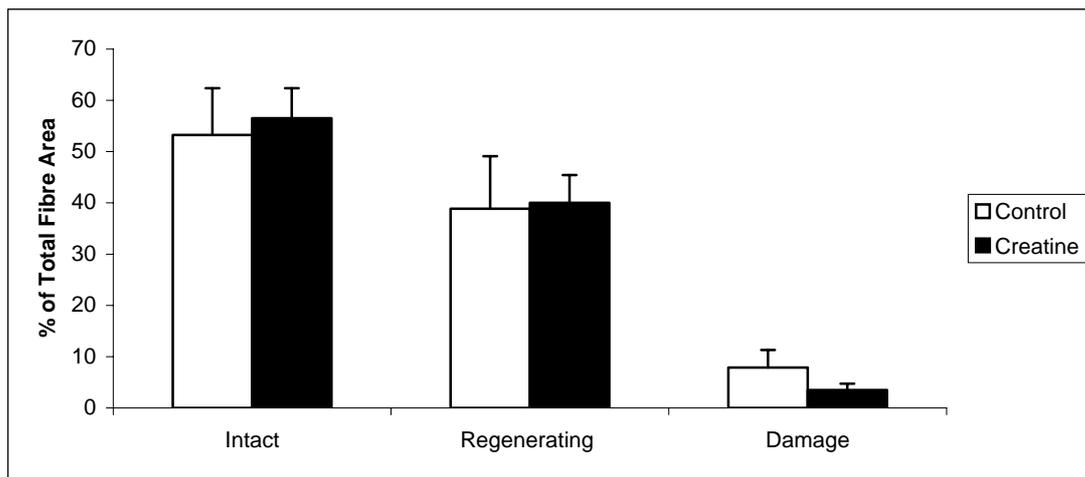


Figure 6.4e. Muscle fibre recovery (expressed as a % of muscle CSA) of injured soleus muscles from rats treated with normal- or creatine- supplemented rat chow at day 14 post-injury. Values are mean \pm SEM, expressed as either % of damaged fibres, regenerating fibres, and undamaged (intact) fibres.

6.3 DISCUSSION

The major objective of this study was to determine the morphological and biochemical mechanisms by which dietary supplementation with creatine may increase contractile function and hence, force of rat skeletal muscle during recovery from chemically-induced muscle damage. Creatine supplementation significantly improved muscle strength (both absolute and specific force) in the fast-twitch EDL muscle following myotoxic injury. Enhanced functional capacity of regenerating muscles occurred because of direct increases in muscle protein content, fiber CSA and muscle mass. While numerous studies have examined the ergogenic potential of creatine supplementation in both humans (Casey *et al.*, 1996; Febbraio *et al.*, 1996; Greenhaff *et al.*, 1993:1994; Birch *et al.*, 1994; Earnest *et al.*, 1995; Kreider *et al.*, 1998; Snow *et al.*, 1998; McKenna *et al.*, 1999) and animals (Brannon *et al.*, 1997; Robinson and Loiselle, 2002; O.Eijnde *et al.*, 2003), only a few studies have investigated the efficacy of creatine supplementation on the muscle recovery after injury (Warren *et al.*, 2000;

Rawson *et al.*, 2001; Santos *et al.*, 2003). Additionally, to the authors knowledge, no studies have determined the mechanisms by which creatine may exert its effect during muscle recovery after myotoxic injury. Thus, the present study provides information on the means by which creatine supplementation may affect the biochemical, structural and functional aspects of muscle fibre recovery following chemically-induced muscle damage.

Given that the bupivacaine protocol used in both animal studies was the same, the effects of chemically-induced damage on morphological, biochemical and contractile properties of rat skeletal muscle in both studies (i.e. chapter 3 & 4) will be discussed together. However, the effects of supplementation will be discussed in the relevant chapters.

Myotoxic injury and muscle mass and intramuscular protein levels

Local myotoxic injury with bupivacaine caused extensive damage to the EDL and soleus muscles. It is well established that within 15 minutes after the injection, there is disruption of the sarcolemma, hypercontraction of the myofibrils, and swelling of the sarcoplasmic reticulum (Rosenblatt, 1991). During the first few days following chemically-induced damage, there is loss of myofilament banding pattern, lysis of the Z-band, rupturing of the myofibrils, and fragmentation of the cytoplasm (Halls-Craggs, 1974; Nonaka *et al.*, 1983). Macrophages infiltrate the injured site, breakdown necrotic fibres and phagocytose cellular debris, thus, further contributing to the degeneration phase of the muscle (Nonaka *et al.*, 1983). This sequence of events lead to a marked decrease in muscle mass, strength, intramuscular total proteins, percentage of intact (non-damaged) fibres, and metabolite levels. In the present study, there was a highly significant decrease in muscle mass in both the EDL and soleus muscles at day 7 post-

injury, which was supported by a decrease in total proteins in both muscle types. However, by day 14 post-injury, only a marked reduction in total proteins was evident, as mass of both injured EDL and soleus muscles was near complete restoration. These results indicate that some of the increase in muscle mass was not associated with contractile material, but infiltrating cells, necrotic areas etc.

Previous studies have shown similar decreases in muscle mass and protein levels in the EDL muscle following myotoxic injury with bupivacaine (Beitzel *et al.*, 2003). While other studies have shown greater reductions in muscle mass in the EDL and soleus muscle (Plant *et al.*, 2005), it is well established that the extent of damage caused by bupivacaine is dose-dependant (i.e. the more injected the greater the damage). Though the dosage of bupivacaine used in the study by Plant and colleagues (2005), higher amounts of bupivacaine injected would result in further injury to the muscle.

Myotoxic injury and contractile properties

The decrease in muscle proteins and muscle mass was accompanied by large reductions in absolute forces. Without supplementation, recovery of Po was steady, but slow, such that Po values were still only ~70% and ~77% of uninjured muscle values by day 14 post-injury in the EDL and soleus muscles, respectively. The disparity between the rate of recovery of muscle mass and muscle force-producing capacity at day 14 post-injury could be attributed to the expression of developmental isoforms of contractile and regulatory proteins during muscle regeneration. These isoforms are known to have reduced functionality compared to the more mature isoforms (Gregorevic *et al.* 2002). In addition, increases in non-contractile muscle mass, as opposed to contractile mass will also influence the ability to produce force based on a given muscle size. Indeed, when Po was normalized for muscle size, specific force (sPo) of injured EDL and soleus

muscles at day 14 post-injury was significantly lower than uninjured contralateral EDL and soleus muscles. Therefore, indicating that although mass of both EDL and soleus muscles was close to uninjured contralateral values, force-producing capacity was still significantly lower than contralateral values.

While it is evident that the muscles' internal structure was still undergoing remodelling and maturation processes at day 14 post-injury, it could be suggested that by day 21 both the EDL and soleus muscle would be fully recovered, although, this was not measured in the present study.

Previous studies have noted full recovery of morphometric properties within 3-4 weeks following intramuscular bupivacaine injection (Benoit and Belt, 1970; Halls-Craggs, 1974). However, the recovery of muscle function following injury has been inconsistent. Rosenblatt (1991) reported that isometric twitch and tetanic tension reached control levels within 21 days, whereas other studies suggest that at least 60 days is required for restoration of function following bupivacaine injection (Carlson and Faulkner, 1996). Again, the time taken for full restoration would depend on the initial amount of damage caused, which further depends on the amount of bupivacaine injected. Nonetheless, results from the present study indicate that force-producing capacity of the both the EDL and soleus muscles was close to contralateral levels at day 14 post-injury, and thus would likely reach full restoration by day 21 post-injury.

Following myotoxic injury, time to peak tension (TTPT) and one-half relaxation time ($\frac{1}{2}$ RT) was significantly prolonged in the INJ-CON EDL muscles following myotoxic injury. In contrast, the INJ-CON soleus muscle demonstrated significantly faster $\frac{1}{2}$ RTs post-injury, while no significant effects of myotoxic injury were observed in TTPT.

Time to peak tension is an unrefined indicator of the Ca^{2+} release process, the binding to troponin-C and the activation of actin and myosin. One-half relation time is an approximate indicator of SR Ca^{2+} -ATPase function, as Ca^{2+} ions are removed from the sarcoplasm via a number of mechanisms, which include the SR. The effects of myotoxic injury in the EDL muscle in the present study are consistent with previous studies (Beitzel *et al.*, 2003; Plant *et al.*, 2005), and are most likely due to interference and/or damage to E-C coupling, reduced energy available to the SR Ca^{2+} -ATPase pump, and/or disruption to the contractile apparatus (for example loss of contractile proteins actin and myosin).

However, since limited research have examined the effects of myotoxic injury in the soleus muscles, the contrasting results observed could be due to different fibre composition of the soleus compared to the EDL muscles, and thus different quantities of SR Ca^{2+} pumps. However, this concept requires further investigation.

It has been established that during the early stages of muscle regeneration, there is a shift in the length-tension curve towards the left, and thus shorter optimal lengths (Plant *et al.*, 2005). Conversely, when muscles are fully regenerated (i.e. muscle mass and force restored), length-tension curve shifts towards the right, and thus longer optimal lengths (Plant *et al.*, 2005). In the present study, optimal lengths for both EDL and soleus muscles were shorter (although not significant) than the uninjured contralateral optimal lengths at day 7 and 14 post-injury. Therefore, suggesting that there was a shift in length-tension curve towards the left during muscle regeneration, as observed by Plant *et al.* (2005).

Myotoxic injury and metabolic properties

Following myotoxic injury, there is an increased influx of Ca^{2+} into the muscle which leads to loss of calcium homeostasis and thus an increase in $[\text{Ca}^{2+}]_i$. While various ATPase pumps within the cell endeavour to buffer the Ca^{2+} (i.e. remove from the cell), the mitochondria also uptake some of the Ca^{2+} , to further help these buffering systems. Small increases in mitochondrial Ca^{2+} levels in the μm range (as seen with normal contraction) stimulates mitochondrial respiration and ATP synthesis (McMillan and Madden, 1989), however, accumulation of Ca^{2+} within the nm range causes structural damage to the mitochondria and as a result reduces mitochondrial function and decreases ATP production.

Furthermore, increased ATP hydrolysis within the vicinity of the Ca^{2+} -ATPase pumps due to the increased $[\text{Ca}^{2+}]_i$ will further contribute to the reduction in intramuscular ATP levels. Furthermore, increased permeability of the muscle membrane as a result of damage, may lead to increased leakage of Cr and/or PCr out of the muscle, and thus, it is likely that TCr levels; in addition to ATP levels will be decreased following injury. In the current study, ATP and TCr levels were significantly lower in both CON-INJ EDL and soleus muscles after 7 and 14 days post-injury. Cr levels were also significantly decreased in the CON-INJ EDL, although only at day 7 post-injury. Conversely, Cr levels were only lower at day 14 post-injury in the CON-INJ soleus muscles, while no changes were observed at day 7 post-injury. PCr levels were significantly lower in the CON-INJ EDL muscles at day 7 and 14 post-injury. However, PCr levels were only significantly lower at day 7 post-injury in the CON-INJ soleus muscles.

However, caution is taken when interpreting the metabolite results in both chapter 6 and 7, as number of other factors may be influencing intramuscular ATP and PCr (TCr) levels within the muscle rather than supplementation alone. Muscles analysed in both chapters were not frozen *in situ* as both the injured and uninjured EDL and soleus muscles were first placed in a krebs ringer solution and tested for contractile properties, lasting for approximately 40 minutes. Once tested, muscles were removed quickly, weighed, cut in half, and then snap frozen in liquid nitrogen. While there is no evidence to suggest that this method will cause further variation in metabolite levels between each muscle, as muscles were subjected to very similar *in vitro* testing conditions and procedures, it is evident that the metabolite levels in the uninjured muscles were generally lower compared to typical resting muscle samples (Op t Eijnde *et al.*, 2001), and thus, it is possible that some ATP hydrolysis took place during the contractile testing period.

Myotoxic injury and histological measurements

Substantial areas of necrosis identified by infiltrating lymphocytes and regenerating fibres as evident by centralised nuclei was observed at day 7 post-injury. The degeneration of skeletal muscle after intramuscular injection with 0.5% bupivacaine injection is well characterised (Halls-Craggs, 1979; Rosenblatt, 1991; Gregorevic *et al.*, 2000; Plant *et al.*, 2005), with rapid myonecrosis leaving less than 20% of fibres intact usually seen at 2 day post-injection (Foster and Carlson, 1980). Cross-sectional area of regenerating fibres increases during recovery until full maturation is complete (indicated by peripheral nuclei). In the current study, regenerating fibre CSA of the both EDL and soleus was significantly smaller at day 7 post-injury compared to uninjured fibre CSA. Although regenerating fibre CSA of both EDL and soleus muscles increased by day 14 post-injury, this was still significantly lower than uninjured fibre CSA. Furthermore, the

number of intact (non-damaged) fibres was still lower than 100%, thus providing further evidence that both the EDL and soleus muscles were still undergoing regeneration at day 14 post-injury.

The effect of creatine supplementation on morphometric measurements

While numerous studies have confirmed that supplementation with CrM in conjunction with programmed resistance training is effective for augmenting gains in body and fat free mass, and muscular strength in both men and women (Bermon *et al.*, 1998; Earnest *et al.*, 1995; Kreider *et al.*, 1998; Vandenberghe *et al.*, 1997; Kelly and Jenkins, 1998; Francaux and Poortmans, 1999; Volek *et al.*, 1999; Becque, 2000; Chrusch *et al.*, 2001; Willoughby and Rosene, 2001; 2003), it has also been demonstrated that creatine supplementation alone may increase muscle mass, without the added influence of weight training, which is a potent anabolic signal in itself (Earnest *et al.*, 1995; Mihic *et al.*, 1998; Warber *et al.*, 1998). It is generally reported that the mechanisms responsible for increased body mass after creatine supplementation is water retention (Demant *et al.*, 1999; Terjung *et al.*, 2000). However, an increase in cellular hydration status (i.e. increased cell volume) induced by creatine supplementation may also act as an independent anabolic signal, initiating cellular mechanisms needed to create gains in muscle, most likely via an increase in protein synthesis (Volek *et al.*, 1999).

In the present study, following 3 weeks of creatine supplementation, animals gained more body weight (approximately 5-6g) than animals consuming normal rat chow, although this trend did not continue following an extra 7 days of creatine supplementation. In addition, both injured and uninjured muscles were significantly heavier than control muscles following creatine supplementation at day 7 and day 14 post-injury. Thus, since muscle mass continued to significantly increase from day 7 till

day 14 post-injury, with no significant change in body mass, it was likely that creatine-supplemented animals showed an increase in LBM at day 14 post-injury compared to control animals. Indeed, an increase in MM:BM was evident at day 14 post-injury (28 days supplementation) in the creatine-supplemented soleus muscles, with a strong trend for higher MM:BM in the EDL muscles ($P=0.07$).

Taken together, these results support previous studies that have shown an increase in muscle mass and lean tissue following creatine supplementation (Francaux and Poortmans, 1999; Bembien *et al.*, 2001). While this is likely the case for the uninjured muscles, a significant increase in muscle mass in the injured muscles following creatine supplementation, is most likely due to a reduction in muscle fibre damage and/or faster recovery following injury.

The effect of creatine supplementation on contractile, histological and biochemical properties

The major finding of the current study is that at day 7 post-injury, CR-INJ EDL muscles exhibited greater restoration of force-producing capacity than CON-INJ EDL muscles. CR-INJ P_o was ~52% of uninjured muscle values after creatine supplementation, whereas with the control muscles, P_o was only ~27% of uninjured muscle values. Furthermore, by day 14 post-injury, P_o was significantly higher in the CR-INJ EDL muscles, which were almost fully restored (~87% of uninjured values) compared to CON-INJ muscles (~69% of uninjured values).

When corrections were made for cross-sectional area, specific force was also significantly greater in the CR-INJ EDL muscles at day 7 post-injury, thus indicating that for a given muscle size, injured EDL muscles were producing greater forces

compared to injured control muscles following creatine supplementation. This is consistent with an increase in contractile tissue. Contractile protein levels were ~30% higher ($P=0.08$) in the Cr-INJ EDL muscles compared to CON-INJ EDL muscles at day 7 post-injury. Furthermore, larger CSA of the regenerating EDL muscle fibres was also observed. It should be noted that specific forces, while an indicator of force based on a given muscle size is influenced, especially during the early stages of muscle regeneration, by inflammation and muscle swelling which is still evident within the muscle. Therefore, despite the fact that mass is increased, this may not influence force, which is why sPo was still lower by day 14 post-injury.

However, it is clear that the increase in specific force in the CR-INJ EDL muscle was due to the increase in contractile mass (and to a lesser extent, increased CSA of regenerating fibres), as muscle swelling and inflammation would have increased size but not force.

Nevertheless, at day 7 post-injury, CR-INJ EDL muscles exhibited heavier muscles, larger absolute forces, and specific forces, in addition to larger regenerating fibres, perhaps as a result of reduced damage to the muscle and or greater protein accretion within the muscles. Indeed, histological analysis revealed a significant reduction in the extent of fibre necrosis, in addition to a significant increase in intact (uninjured) muscle fibres in the EDL following creatine supplementation at day 7 post-injury. Very few studies have examined histologically the effects of creatine supplementation on the fibre necrosis and/or regeneration in normal healthy muscles following injury. However, a recent study has shown a reduction in skeletal muscle degeneration in *mdx* mice following creatine supplementation (Passaquin *et al.*, 2002). The sequence of events that lead to muscle necrosis and/or apoptosis in Duchenne's muscular dystrophy (*mdx*

mice) and chemically-induced muscle damage are very similar, in that both are characterized by increase in cytosolic calcium concentrations. Therefore, reduced fibre damage following creatine supplementation may be due to its local effects on intracellular calcium regulation.

Three cytoplasmic (MM-CK) and two mitochondrial (Mi-CK) isoforms of CK have been identified in skeletal muscle (Walliman *et al.*, 1992). These isoforms, together with their substrates creatine and PCr, represent an intricate cellular energy buffering and transport system, connecting sites of energy production with sites of energy consumption (Passaquin *et al.*, 2002). The SR Ca^{2+} -ATPase pump (SERCA) derives its ATP preferentially from PCr via SR-bound CK, and thus due to their close association, maintains a high ATP/ADP ratio within the vicinity of the SERCA pump, thus allowing the energetically demanding pump to function optimally (Korge *et al.*, 1993). Therefore, an important mode of action from creatine supplementation is to improve the calcium handling ability of the muscle. This concept was fully supported by findings in transgenic CK null mutant mice that show severely altered Ca^{2+} homeostasis and slower relaxation times in skeletal muscle (Steeghs *et al.*, 1997).

In the present study, creatine supplementation significantly increased the rate of muscle relaxation in the injured EDL muscles at day 7 and day 14 post-injury. Furthermore, relaxation times were also faster in the uninjured EDL muscles at the same recovery time points following creatine supplementation, although this difference was not significant. Whilst SR Ca^{2+} -ATPase function was not directly measured in the current study, previous research from our laboratory has shown an increase in Ca^{2+} uptake rate by SR vesicles when fuelled by PCr rephosphorylation of ADP rather than just ATP directly. In addition, SR vesicles from creatine-supplemented EDL muscles also showed

faster Ca^{2+} uptake. Taken together, these observations of improved force recovery and reduced damage may suggest that creatine supplementation is improving SR Ca^{2+} -ATPase function, and the intracellular Ca^{2+} handling ability of the muscle. Thus, Ca^{2+} accumulation within the muscle will be reduced, as well as the activation of self-accelerating degradative pathways such as calpains and phospholipases, which leads to muscle fibre damage. Furthermore, these results would also be consistent with the higher forces and less damage (as measured by plasma CK and LDH levels) observed after creatine supplementation in human participants in chapter 4.

In addition to the observed increases in muscle mass, strength, and increased intact muscles fibres in the injured EDL muscles following creatine supplementation, optimal length in the creatine-supplemented EDL muscles was also significantly longer compared to control injured muscles at day 7 post-injury.

It is established that 7 days after bupivacaine injection when muscles are undergoing regeneration, there is length-tension curve shift towards the left. When muscles are fully regenerated (mass and force production restored), length-tension curves shift towards the right. The rightward shift in the length-tension curve of the muscle can be attributed to the longitudinal addition of sarcomeres during regeneration (Morgan, 1990). An increase in muscle fibre length is mediated by sarcomeres added serially at the ends of the fibres (Williams and Goldspink, 1981). Therefore, in the present study, a shift to longer optimal length in the injured EDL muscle may be evidence of “faster regenerated” muscles, due to less damage and a greater number of intact fibres (i.e. greater number of sarcomeres) as shown in the histological analysis. These results are consistent with the higher AOPT values (shift to the right in length-tension) of the knee flexors observed in the creatine-supplemented participants post-injury in chapter 4.

Thus, further supporting the suggestion that the improved force recovery, and lower plasma CK and LDH levels observed in the human participants following creatine supplementation was due to a reduction in the extent of damage and enhanced muscle regeneration.

However, it is interesting that the uninjured EDL muscle also exhibited a shift in the right in the length-tension curve (i.e. longer L_0). Again this is likely due to an increase in sarcomere numbers, although in contrast to regenerating muscle fibres, the mechanism for increased sarcomeres in the uninjured EDL muscle is most likely due to a cell volume effect caused by increased creatine within the muscle. Increased cell volume will not only lead to an increase in muscle mass, as previously mentioned, but may indirectly signal satellite cells to proliferate by fusing with the enlarging myofibres (Dangott *et al.*, 1999), thus increasing formation of new fibres (sarcomeres). However, this is purely speculative and needs further investigation.

Although protein levels were significantly increased in the soleus muscles at day 7 and in the uninjured soleus muscles at day 14, it is clear from the observation in muscle strength, morphometric and histological measurements that the EDL muscles were more responsive to the creatine supplementation in both the injured and uninjured muscles at day 7 and 14 post-injury. While the causes for such differential responses between fast-twitch and slow-twitch fibres following creatine supplementation are not readily apparent, it has been shown that slow-twitch muscles are slower to recover from injury, compared to the fast-twitch muscles, with incomplete restoration of mass and force observed as long as 60 days after chemically-induced muscle damage (Plant *et al.*, 2005). This is likely due to the more “plastic” nature of fast-twitch fibres, and thus is more adaptable, when compared to the more “postural” slow-twitch fibres.

Indeed, a limitation in the present study and also in the next chapter was that rats were able to walk freely within their cage during the recovery period following myotoxic injury, and thus the results did not differentiate between the mechanical aspects of loading and of regeneration. Therefore, since the fast-twitch and slow-twitch muscles have different functions during walking, they are subject to different stimuli during regeneration, and thus their regenerative capacity and effects from supplementation may vary (Plant *et al.*, 2005).

Notwithstanding, the differential effects from creatine supplementation are consistent with Passaquin and colleagues (2002) who demonstrated a significant protection of EDL muscle against necrosis after creatine supplementation, while no reduction in necrosis was observed in the soleus muscles following supplementation.

It has been suggested that the predominant protective effect of creatine may be in fast-twitch, glycolytic EDL muscles, which differs in its metabolism, compared to slow-twitch, oxidative soleus muscles. Furthermore, since fast-twitch fibres have an enhanced potential to transport Cr into the muscle, it is likely that they would benefit immensely from creatine supplementation compared to slow twitch muscle fibres (Casey *et al.* 1996). Certainly in the present study, EDL muscles showed a ~16% increase in TCr levels following 21 days supplementation, while soleus muscles demonstrated a lower increase of ~10% following the same supplementation protocol. Although these increases were not significant, it does not necessarily indicate that creatine loading was not achieved, as other studies have reported similar increases and shown beneficial effects from supplementation (McConnel *et al.*, 2005). No other effects on metabolite levels were observed following supplementation.

Nevertheless, it could be suggested that since less creatine was transported into the soleus muscle, this could translate into less elevation of PCr for the PCr/CK system which rephosphorylates ADP to ATP. As a result, there is less ATP available and a decrease in SR Ca²⁺-ATPase function (reduced Ca²⁺ buffering capacity). Thus, reduced protection of the soleus against necrosis. However, because damage has occurred, there still is the beneficial effect from the satellite cell proliferation and therefore, an increase in protein levels, but not specially contractile proteins (as shown), which may require an extra stimulus for example, myogenic transcription factors. Indeed, regenerating fibres of the soleus muscles exhibited larger CSA at 7 days post-injury following creatine supplementation. Thus, although protein levels and CSA of regenerating fibres was higher, no significant changes in contractile proteins occurred, thus there was no force benefit in the soleus muscles over the time frame investigated.

Interestingly, while creatine intake prior to injury was similar to the target dosage of 1.35g g.kg⁻¹ body weight per day, it was evident that observed supplement intake following injury was lower than the expected target dosage of 0.135 g.kg⁻¹ body weight per day at day 7 and at day 14 post-injury. These observations may reflect that there may have been some form of discomfort to the rats when trying to reach and eat their food, and thus food intake was reduced. Furthermore, this reduction in food intake reduced creatine supplementation below the target creatine dose, and thus may have contributed to the lower than expected 'loading'. Notwithstanding this, it is clear that the beneficial effects from creatine supplementation were still apparent in the present study, despite the reduced creatine intake during recovery post-injury. This could be due to the creatine loading prior to damage; however, we can only speculate since creatine levels prior to damage were not measured in the current study.

In summary, results from the current study and from chapter 4 have clearly shown that creatine supplementation significantly enhances functional capacity of regenerating muscles following damage. These results occurred because of significant reduction in fibre necrosis and enhanced fibre regeneration. Reduced fibre necrosis following creatine supplementation is possibly due to improved SR Ca²⁺-ATPase pump function, thus enhancing the calcium buffering capacity of the muscle. Any improvements in SR function will reduce the extent of calcium accumulation within the muscle, but more importantly will minimise the degradative pathways such as calcium-activated proteases and lipases, which leads to further muscle damage. Enhanced fibre regeneration as evident by increased CSA of regenerating fibres following creatine supplementation is a direct result of increased muscle protein content (possibly due to a reduction in protein degradation), thus creating an environment that facilitates enhanced satellite proliferation and hence formation and maturation of new muscle fibres. As a result, the force developed by the muscle increases, concomitant with increased fibre area and protein content.

CHAPTER 7

Effects of Whey Protein Supplementation on Contractile, Morphological and Biochemical Properties of Rat Skeletal Muscle during Recovery from Chemically-Induced Muscle Damage

7.0 INTRODUCTION

Several studies have explored the effect of whey protein supplementation in combination with resistance training (Burke *et al.*, 2001; Tipton *et al.*, 2004; Andersen *et al.*, 2005; Cribb *et al.*, 2006). While it appears that whey protein may enhance adaptations from resistance training, due to its favourable effect of protein metabolism and lean body mass (LBM) accretion, whether whey protein supplementation can enhance muscle regeneration from injury has received little attention to date. Moreover, no studies have examined the mechanisms by which whey protein influences muscle recovery after damage.

In our human study (see chapter 5), whey protein supplementation was able to significantly improve force generation and decrease the leakage of intracellular proteins such as CK and LDH, indicative of less damage and/or faster regeneration. However, since only indirect measurements of muscle damage and recovery was made, we could only speculate on the mechanisms by which whey protein may be exerting. Consequently, the use of an animal model (as mentioned in chapter 6) allows a more extensive and accurate analysis of the magnitude of damage and rate of muscle recovery of whole intact skeletal muscles after injury than would be possible from human biopsy samples. Furthermore, since the healing processes are essentially the same, any improvements in the rate of recovery in the animal model can be directly applicable to muscle injury in humans.

Thus, the purpose of this study was to confirm human results by analysing force recovery of rat skeletal muscle after controlled, chemically-induced injury. Furthermore, to determine the mechanisms by which dietary supplement whey protein exerts its effect by examining morphological and biochemical properties during recovery. We hypothesised that whey protein supplementation will restore loss of isometric contractile strength at a faster rate; and reduce the extent of damage and improve fibre regeneration concomitant with increased intramuscular total and contractile protein levels.

7.1 METHODS

7.1.1 Experimental Design and Dietary treatment

Adult male Sprague-Dawley rats (*Rattus norvegicus*) weighing 214.9 ± 4.7 (mean \pm SEM) were randomly separated into 2 groups: i) control n= 20; ii) whey-protein-supplemented n=20. Control rats were fed normal chow. Whey protein-supplemented rats were fed whey protein isolate (AST sports science, CO USA) at 5g/kg/day in the chow. Rats were fed normal or whey protein-supplemented chow for a period of 14 days following chemically-induced damage. The doses and method of administration of whey protein was chosen to mimic those used in chapter 5, scaled for the higher metabolic rates of rats.

Animals were acquired from Monash Animal Services (Monash University, Melbourne, Australia) and housed at the animal holding facility at Werribee campus, Victoria University. All procedures described below received ethical approval from the Victoria University Animal Experimentation Ethics Committee and conformed to the Australian code of practice for the care and use of animal for scientific purposes. Animals were housed at a constant temperature (22°C), under a 12:12-h light-dark photoperiod. All

animals were housed separately following muscle injury in accordance with ethical approval with access to normal or supplemented chow *ad libitum*.

7.1.2 Experimental Protocol

All rats were lightly anesthetized with a domitor (10 mg.kg^{-1} body weight) and ketamine (6 mg.kg^{-1} body weight) combination via an intraperitoneal injection (i.p.), such that they were unresponsive to tactile stimuli. The EDL and soleus muscles of the left hindlimb were surgically exposed and injected with 0.5% bupivacaine (as described in section 3.2.1), thus causing degeneration of most fibres in the injected muscle (Rosenblatt, 1992). Following injury, the small incision was closed by Michel clips, and swabbed with Betadine antiseptic (povidone iodine solution).

At day 7 and 14 post-myotoxic injury, rats were deeply anaesthetized with Nembutal (pentobarbitone sodium 60 mg.ml^{-1} , i.p.). The level of anaesthesia was monitored via corneal and footpad reflexes, with additional doses administered when required. In order to maintain blood flow and nerve supply to the last possible moment, the injured EDL muscle was surgically exposed first and immediately tested for isometric contractile properties (as described in section 3.3.1), followed by the injured soleus muscle and then the contralateral EDL and soleus muscles. At the completion of contractile measurements, each muscle was immediately blotted dry, weighed and divided into 2 sections; one half was snap-frozen in isopentane, cooled in liquid nitrogen and stored for later histological analysis (as described in section 3.4). The other portion was immediately snap frozen in liquid nitrogen for later biochemical analysis (as described in section 3.5 & 3.6). All muscle samples were stored at -80°C .

7.1.3 Statistical Analysis

All values are reported as means \pm SEM. Statistical evaluation for each muscle group (EDL and soleus) and recovery time point (day 7 and day 14) was accomplished by using a two-way analysis of variance (ANOVA) with one between groups factor (supplementation) and one within group factor (damage/contralateral). Where an interaction was found, the location of the difference was determined by a one-way ANOVA. Difference in animal morphology characteristics between groups was assessed by students' t-test. Supplementation protocol between groups was assessed by chi-square test. A P value of less than 0.05 was accepted for statistical significance. It should be noted that while animal studies will be reported separately in order to align them with the previous human studies, the data was compared together as both studies utilized the same control group.

7.2 RESULTS

7.2.1 Supplementation and Morphometric Measurements

7.2.1.1 Supplementation Protocol

Based on their food consumption, rats supplemented with whey protein for 7 days and 14 days following muscle injury consumed 3.01 ± 0.12 and 2.94 ± 0.15 g.kg⁻¹ body weight per day, respectively. This observed supplement intake was lower than the expected target dosage of 5 g.kg⁻¹ body weight per day ($p < 0.001$ day 7, $p < 0.05$ day 14), indicating rats were not consuming the amount of whey protein that was required.

7.2.1.2 Morphometric measurements: Body weight

In order to obtain a more accurate measurement of body weight (BW) changes following chemically-induced damage, rats were categorised into 2 groups: those that consumed either normal or whey protein-supplemented rat chow for a period of 7 (Group 1) or 14 days (Group 2), respectively. *Effect of supplementation:* There were no significant differences in body weights prior to chemically-induced muscle damage or at day 7 and 14 post injury, between control and whey-supplemented animals (see table 7). Furthermore, when expressed as a change from initial body weight, no significant differences were evident.

Group 1	Initial BW (g)	BW (g) after 7 days
Control (n = 10)	209.4 ± 9.3	222.5 ± 8.1
Whey protein (n = 10)	224.8 ± 8.3	234.4 ± 6.1
Group 2	Initial BW (g)	BW (g) after 14 days
Control (n = 10)	243.7 ± 6.6	259.2 ± 7.2
Whey protein (n = 10)	232.9 ± 7.4	249.1 ± 9.5

Table 7. Rat body weights (BW) (g) for both normal (control) and whey protein-supplemented groups at day 7 and 14 post-injury.

7.2.1.3 Morphometric Measurements: Muscle mass

Muscle mass changes in normal- and whey protein-supplemented EDL and soleus muscles at day 7 and 14 post-injury are summarized in Figure 7.1a & 7.1b.

Effect of myotoxic injury: There was a highly significant decrease in muscle mass in both the EDL (P<0.01) and soleus (P<0.001) muscles at day 7 post-injury. However, by day 14 no differences were observed, as muscle mass was nearly recovered to uninjured

muscle mass values. Expressed relative to body mass (MM:BM) muscle weights were significantly lower in both the EDL and soleus muscles at day 7 ($P<0.01$) and 14 ($P<0.01$) post-injury (table 7.1a & 7.1b). *Effect of supplementation:* Whey protein-supplemented EDL muscles were significantly heavier than control EDL muscles at day 7 post-injury ($P<0.05$, see figure 7a), indicating that not only were injured EDL muscles of whey protein-supplemented animals heavier than injured control muscles during recovery, the contralateral uninjured EDL muscles were also significantly heavier in the whey protein-supplemented animals compared to control animals. However, this trend did not continue by day 14 post-injury, as no differences in muscle mass were observed between whey protein-supplemented EDL muscles and control EDL muscles. Whey protein supplementation had no significant effect on the soleus muscles at day 7 and 14 post-injury.

No significant changes in MM:BM were observed between whey protein-supplemented muscles and control muscles at day 7 and day 14 post-injury. However, a strong trend ($P=0.075$) for a higher muscle mass to body mass ratio (MM:BM) was revealed in the whey-supplemented EDL muscles at day 7 post-injury compared to time matched EDL control muscles.

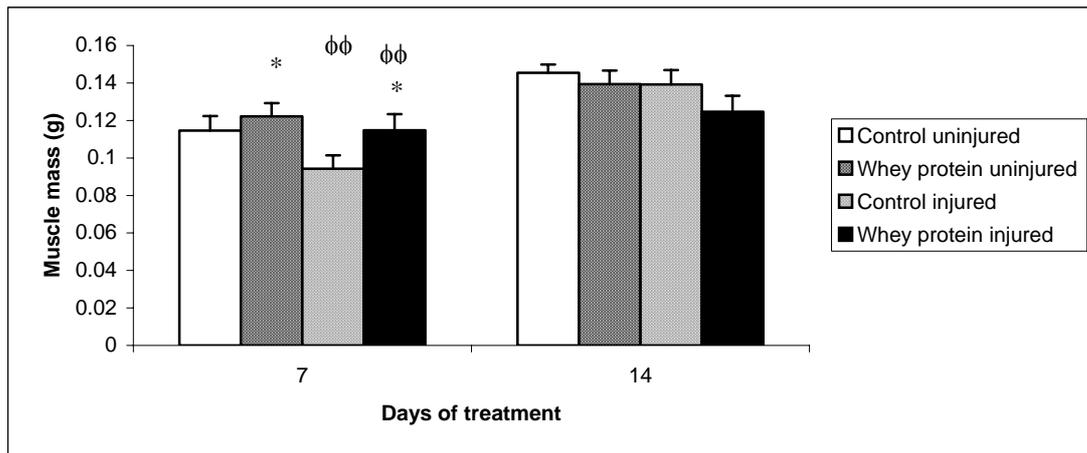


Figure 7a. Muscle mass changes of uninjured and injured EDL muscles from rats treated with normal- or whey protein-supplemented rat chow at day 7 and 14 post-injury. Values are mean \pm SEM. $\phi\phi$ ($P < 0.01$) significantly different from contralateral values. * ($P < 0.05$) significantly different from control animals.

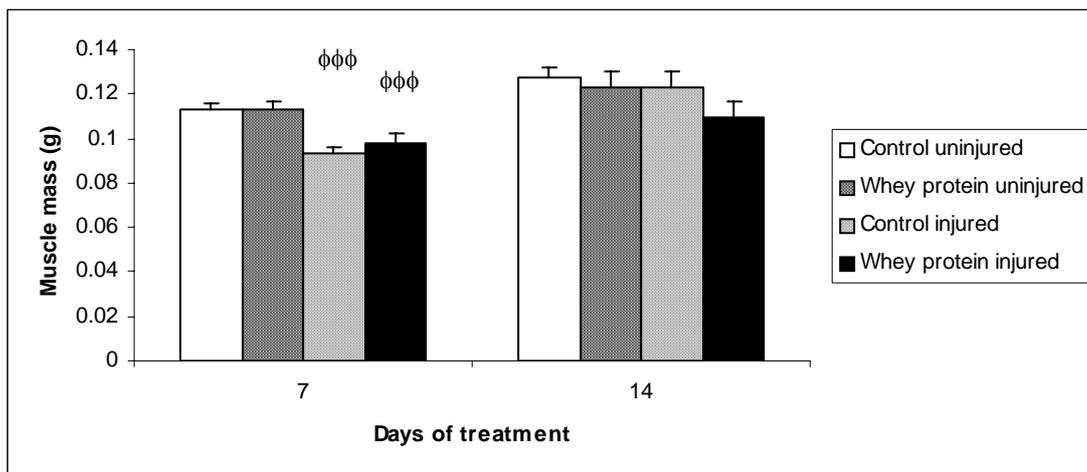


Figure 7b. Muscle mass changes of uninjured and injured soleus muscles from rats treated with normal- or whey protein-supplemented rat chow at day 7 and 14 post-injury. Values are mean \pm SEM. $\phi\phi\phi$ ($P < 0.001$) significantly different from contralateral values.

7.2.2 Contractile Measurements

Contractile properties of all muscles are summarized in table 7.1a & 7.1b. Whey protein-supplemented muscles will be categorized as WP-INJ and WP-NORM for injured and uninjured muscles, respectively, while control muscles will be categorized as CON-INJ and CON-NORM for injured and uninjured muscles, respectively.

Effect of myotoxic injury: Optimal lengths for both CON-INJ EDL and soleus muscle, were not significantly different from CON-NORM at day 7 and 14 post-injury. Conversely, time to peak twitch tension (TTPT) was significantly slower at day 7 post-injury ($P < 0.05$) however no effects from injury were evident at day 14 post-injury. One-half relaxation time ($\frac{1}{2}$ RT) was significantly prolonged in the injured EDL muscles at day 7 and 14 post-injury compared to contralateral EDL muscles ($P < 0.001$, see table 7.1a). In contrast to the EDL muscle, $\frac{1}{2}$ RT was significantly quicker in the injured soleus muscles at day 7 ($P < 0.001$) and 14 ($P < 0.05$) post-injury, compared to contralateral soleus muscles. No significant effects from injury were observed in TTPT of the soleus muscles at any time point.

Treatment duration		CON-INJ	WP-INJ	CON-NORM	WP-NORM
	n	10	10	10	10
7 days	MM:BM (g)	0.042 ± 0.003	0.049 ± 0.003	0.05 ± 0.003	0.053 ± 0.002
	Opt Length (cm)	2.89 ± 0.1	3 ± 0.06 *	2.93 ± 0.03	2.98 ± 0.03 *
	Pt (mN)	122.4 ± 16.3 φφφφ	288.8 ± 60.1 φφφφ *	521 ± 83.5	563.69 ± 69.2
	TTP (ms)	55 ± 5.2 φ	55 ± 3.1	50 ± 4.2	47 ± 4.2
	$\frac{1}{2}$ RT (ms)	92 ± 9.4 φφφ	77 ± 7.9 φφφ *	60 ± 5.1	56 ± 5.8 *
	n	10	10	10	10
14 days	MM:BM (g)	0.05 ± 0.003	0.050 ± 0.002	0.06 ± 0.002	0.056 ± 0.002
	Opt Length (cm)	3.09 ± 0.04	3.09 ± 0.04	3.19 ± 0.03	3.18 ± 0.04
	Pt (mN)	459.7 ± 20.2 φφφφ	383.92 ± 46.9 φφφφ	713.2 ± 86.9	668.5 ± 59.4
	TTP (ms)	49 ± 5.9	47 ± 4.2	46 ± 3.1	38 ± 2.8
	$\frac{1}{2}$ RT (ms)	103 ± 30.2 φφφ	69 ± 5.1 φφφ	55 ± 4.4	46 ± 3

Table 7.1a. Morphometric and contractile properties of uninjured and injured EDL muscles from rats treated with normal- or whey protein-supplemented rat chow at 7 and 14 days post-injury. n = number of animals in each group, MM:BM muscle mass: body mass ratio. Values are mean ± SEM. φφφφ ($P < 0.0001$), φφφ ($P < 0.001$) significantly different from contralateral values. * ($P < 0.05$) significantly different from control muscles at day 7 post-injury.

Treatment duration	CON-INJ	WP-INJ	CON-NORM	WP-NORM	
	n	10	10	10	10
7 days	MM:BM (g)	0.042 ± 0.001	0.042 ± 0.002	0.050 ± 0.002	0.049 ± 0.001
	Opt Length (cm)	2.92 ± 0.06	2.95 ± 0.04	2.95 ± 0.07	2.99 ± 0.03
	Pt (mN)	78.7 ± 8.5 φφφ	105.9 ± 17φφφ	211 ± 41.5	184.3 ± 30.4
	TTP (ms)	135 ± 20	146 ± 12	150 ± 13	148 ± 11
	½ RT (ms)	197 ± 16 φφφ	224 ± 23φφφ	272 ± 23	264 ± 18
	n	10	10	10	10
14 days	MM:BM (g)	0.047 ± 0.003	0.044 ± 0.002	0.049 ± 0.001	0.050 ± 0.001
	Opt Length (cm)	3.14 ± 0.04	3.02 ± 0.05 *	3.14 ± 0.04	3.08 ± 0.05 *
	Pt (mN)	164.4 ± 15.1 φφφ	177.4 ± 12.7 φφφ	188.1 ± 32.4	231.1 ± 28.1
	TTP (ms)	150 ± 12	146 ± 15	146 ± 13	142 ± 12
	½ RT (ms)	238 ± 12 φ	250 ± 23 φ	277 ± 28	293 ± 29

Table 7.1b. Morphometric and contractile properties of uninjured and injured soleus muscles from rats treated with normal- or whey protein-supplemented rat chow at 7 and 14 days post-injury. n = number of animals in each group, MM:BM muscle mass: body mass ratio. Values are mean ± SEM. φφφ (P<0.001), φ (P<0.05) significantly different from contralateral values. * (P<0.05) significantly different from control muscles at day 14 post-injury.

As expected with the observed changes in muscle mass, absolute force (Po) was significantly lower in both the CON-INJ EDL and soleus muscles at day 7 post-injury, with the Po only ~27% (P<0.0001) and ~43% (P<0.001) of CON-NORM values of the EDL and soleus muscles, respectively. The recovery of Po was steady, but slow, such that Po values were still only ~69% (P<0.001) and ~74% (P<0.01) of uninjured muscle values by day 14 post-injury in the EDL and soleus muscles, respectively (see figure 7.1a & 7.1b). When taking into account muscle cross-sectional area (i.e. Po was normalized for muscle size), the specific forces (sPo) of both the CON-INJ EDL and

soleus muscles was still significantly lower than CON-NORM sPo values by day 7 ($P<0.01$) and 14 ($P<0.05$) post-injury (see figure 7.1c & 7.1d), indicative of injured muscles being made up of not only contractile material, but also infiltrating cells, necrotic areas etc.

The sPo of CON-INJ EDL muscle at day 7 and 14 post-injury was 32% and 71% of CON-NORM muscle values, respectively. Similarly, the sPo of CON-INJ soleus muscles at day 7 and 14 post-injury was 51% and 76% of uninjured muscle values, respectively.

Peak twitch tension (Pt) was also significantly lower in both CON-INJ EDL and soleus muscles at day 7 ($P<0.0001$) and 14 ($P<0.001$) post-injury (see table 7.1a & 7.1b), the Pt of CON-INJ EDL was 23% and 64% of CON-NORM muscle values at day 7 and 14 post-injury, respectively. However, similar to the observed changes in Po and sPo post-injury, the Pt values for the CON-INJ soleus muscles were higher than time-matched CON-INJ EDL muscles, exhibiting 37% and 87% of CON-NORM values at day 7 and 14 post-injury.

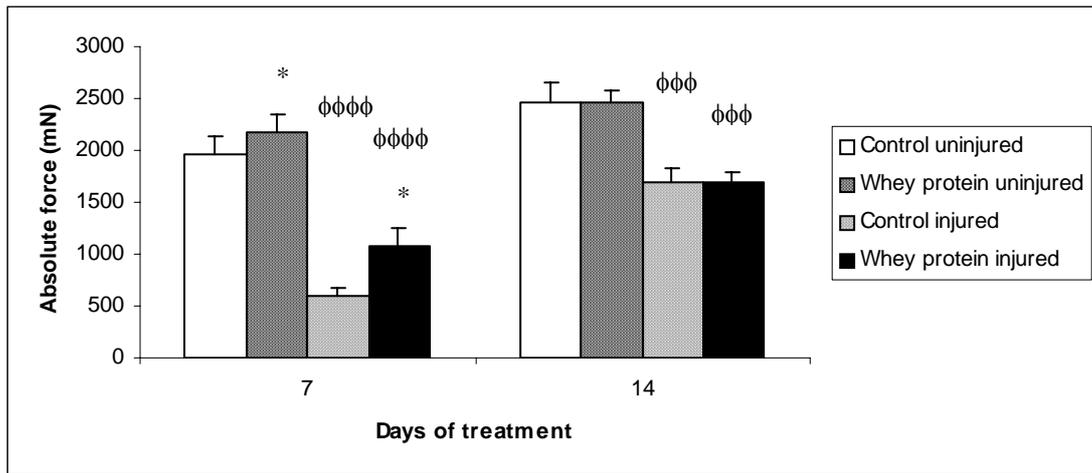


Figure 7.1a Absolute forces (P_o) of uninjured and injured EDL muscles from rats treated with normal- or whey protein-supplemented rat chow at day 7 and 14 post-injury. Values are mean \pm SEM. $\phi\phi\phi\phi$ ($P<0.0001$) $\phi\phi\phi$ ($P<0.001$) significantly different from contralateral values. * ($P<0.05$) significantly different from control muscles at day 7 post-injury.

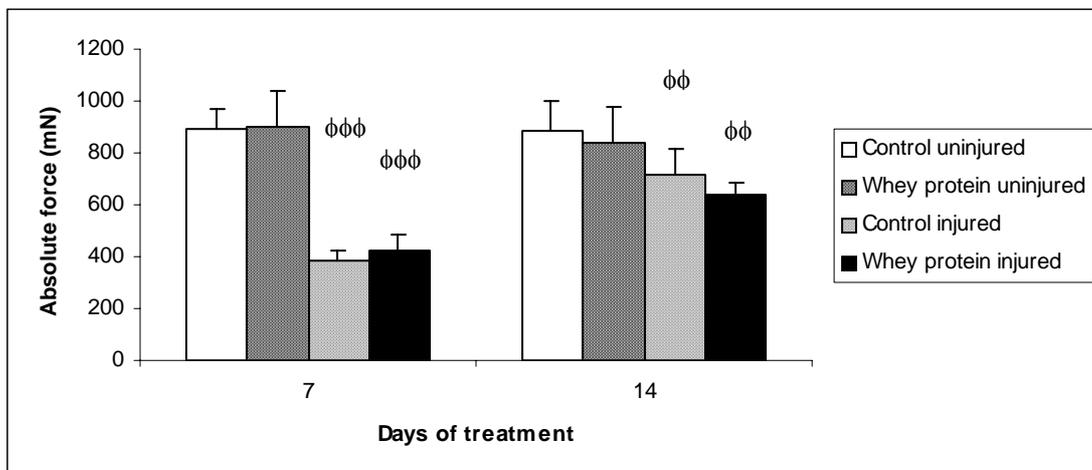


Figure 7.1b Absolute forces (P_o) of uninjured and injured soleus muscles from rats treated with normal- or whey protein-supplemented rat chow at day 7 and 14 post-injury. Values are mean \pm SEM. $\phi\phi\phi$ ($P<0.001$) $\phi\phi$ ($P<0.01$) significantly different from time matched contralateral values.

Effect of supplementation: Optimal length (L_o) was significantly longer in both the WP-INJ and WP-NORM EDL muscles compared to CON-INJ and CON-NORM, respectively, at day 7 post-injury ($P < 0.05$, see table 7.1a). At day 14 post-injury, no significant effects from whey protein supplementation were observed on L_o . Similarly, in the soleus muscle, no significant changes in L_o were observed at day 7 post-injury, following supplementation. However, by day 14 post-injury, in contrast to the results observed in the EDL L_o at day 7 post-injury, L_o was significantly shorter in whey protein-supplemented soleus muscles compared to control muscles ($P < 0.05$, see table 7.1b).

Whey protein supplementation resulted in significantly quicker $\frac{1}{2}$ relaxation times in muscles at day 7 post-injury ($P < 0.05$, see table 7.1a). In addition, a very strong trend towards significance in both injured and contralateral muscles following whey protein supplementation was observed at day 14 post-injury ($P = 0.06$). Conversely, whey protein supplementation had no significant effect on TTPT in the EDL muscles at any time point. Furthermore, no differences in $\frac{1}{2}$ RT and TTPT were observed between whey protein-supplemented and control soleus muscles at either any time point.

Absolute forces (P_o) were significantly increased in the whey protein-supplemented EDL muscles at day 7 post-injury ($P < 0.05$), indicating that both WP-INJ and WP-NORM were generating higher forces than CON-INJ and CON-NORM, respectively, which is consistent with the significant increases in muscle mass in the whey protein-supplemented muscles (see figure 7.1a). Moreover, when P_o of regenerating muscles was expressed as percentage of contralateral values, these values were higher than time-matched control values. Following whey protein supplementation, P_o was $\sim 44\%$ (vs 27% CON-INJ) of contralateral values at day 7 post-injury, which is consistent with the

significant increases in muscle mass in the whey protein-supplemented EDL muscles. No significant changes in P_o were observed at day 14 post-injury, following supplementation. Furthermore, no differences in P_o were observed between whey protein-supplemented and control soleus muscles at either any time point.

When P_o was normalized for muscle size, specific forces were significantly higher in the whey protein-supplemented EDL muscles compared to control EDL muscles at day 7 post-injury ($P < 0.05$, see figure 7.1c), although this was far more pronounced in the injured than the uninjured muscles. No other effects were observed in sP_o following whey protein supplementation in the EDL muscles at day 14 post-injury or in the soleus muscles at any time point.

Peak twitch forces were significantly higher in the WP-INJ EDL muscles at day 7 post-injury ($P < 0.05$), while no effects were observed in the contralateral EDL muscles. Furthermore, whey protein supplementation had no significant effect on P_t at day 14 post-injury, or in the soleus muscles at day 7 and 14 post-injury (table 7.1a & 7.1b).

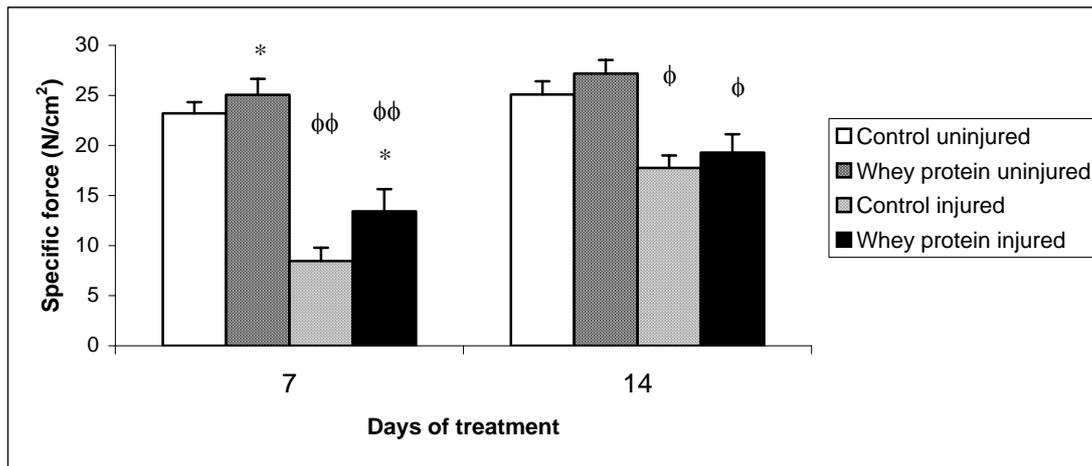


Figure 7.1c. Specific forces (sPo) changes of uninjured and injured EDL muscles from rats treated with normal- or whey protein-supplemented rat chow at day 7 and 14 post-injury. Values are mean \pm SEM. $\phi\phi$ ($P < 0.01$) ϕ ($P < 0.05$) significantly different from contralateral values. * ($P < 0.05$) significantly different from control at day 7 post-injury.

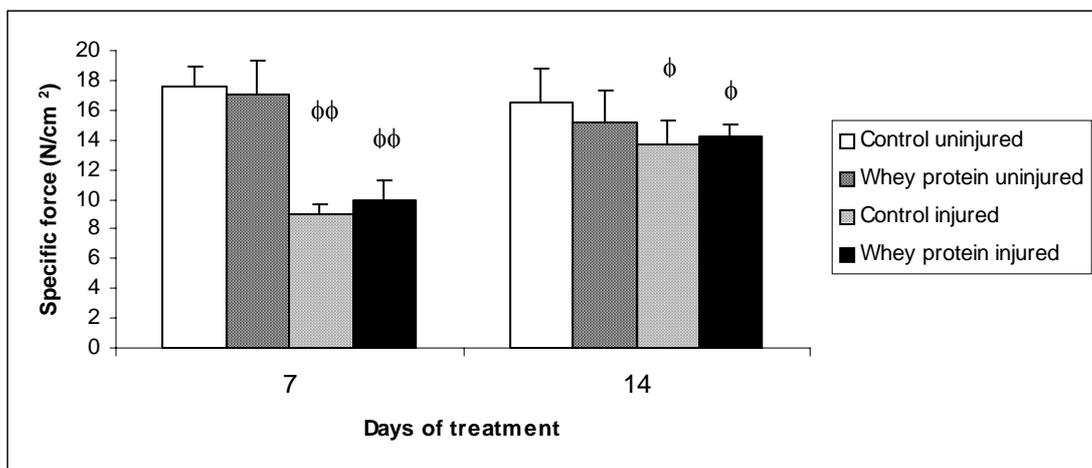


Figure 7.1d. Specific forces (sPo) changes of uninjured and injured soleus muscles from rats treated with normal- or whey protein-supplemented rat chow at day 7 and 14 post-injury. Values are mean \pm SEM. $\phi\phi$ ($P < 0.01$) ϕ ($P < 0.05$) significantly different from time matched contralateral values.

7.2.3 Intramuscular Metabolite Levels

The metabolite concentrations of ATP, creatine (Cr), phosphocreatine (PCr) and total creatine (TCr = Cr + PCr) are displayed in table 7.2a & 7.2b.

Effect of myotoxic injury: ATP and TCr levels were significantly lower in both the injured EDL and soleus muscles at day 7 and 14 days post-injury ($P < 0.05$). Similarly, Cr levels in the EDL were significantly decreased after myotoxic injury at day 7 ($P < 0.01$) and day 14 ($P < 0.05$). Conversely, Cr levels in the WP-INJ EDL muscles were higher at day 14 post-myotoxic injury, as revealed by a significant group by damage interaction ($P < 0.05$).

Cr levels were only lower at day 14 post-injury in the soleus muscle ($P < 0.01$), as no changes in Cr levels were seen at day 7 post-injury (table 7.2a & 7.2b). PCr levels were significantly decreased in the injured EDL and soleus muscles at day 7 post-injury. However, a significant group by damage interaction was detected ($P < 0.05$), as evident by no effect of myotoxic injury on PCr levels in the WP-INJ EDL muscles at day 7 post-injury. PCr levels were significantly decreased in the injured EDL muscles at day 14 post-injury ($P < 0.05$, table 7.2a & 7.2b).

Treatment duration	CON-INJ	WP-INJ	CON-NORM	WP-NORM	
	n	8	8	7	7
7 days	ATP (mmol/kg dw)	10.3 ± 2.1 φ	13.4 ± 2.1 φ	17.6 ± 2.2	18.2 ± 0.8
	PCr (mmol/kg dw)	19.2 ± 4.8 φ	28.1 ± 6.3	27.5 ± 8	28.8 ± 5.6
	Cr (mmol/kg dw)	54.9 ± 6.9 φφ	54.8 ± 3.7 φφ	64.8 ± 3.6	64.8 ± 5.2
	TCr (mmol/kg dw)	74.2 ± 9.4 φ	82.9 ± 5.9 φ	92.3 ± 7.2	93.7 ± 2.9
	n	8	8	8	8
14 days	ATP (mmol/kg dw)	12.9 ± 1.1 φ	13.2 ± 1 φ	18.4 ± 1.8	17.8 ± 0.8
	PCr (mmol/kg dw)	33.8 ± 6.5 φ	29.5 ± 4.2 φ	46.1 ± 6.2	45.9 ± 4.8
	Cr (mmol/kg dw)	52.4 ± 4.8	61.9 ± 4.7	55.6 ± 4.2	53.7 ± 3.1
	TCr (mmol/kg dw)	86.2 ± 3.8 φ	91.4 ± 4.9 φ	101.7 ± 4.4	99.7 ± 3.9

Table 7.2a. Muscle metabolite levels of uninjured and injured EDL muscles from rats treated with normal- and whey protein-supplemented rat chow at day 7 and 14 post injury. n = number of animals in each group. Values are mean ± SEM. φφ (P<0.01), φ (P<0.05) significantly different from contralateral values.

Treatment duration	CON-INJ	WP-INJ	CON-NORM	WP-NORM	
	n	8	8	8	8
7 days	ATP (mmol/kg dw)	4.8 ± 1.6 φ	9.1 ± 0.6 φ *	7.7 ± 1.6	11.9 ± 1.6 *
	PCr (mmol/kg dw)	4.9 ± 0.5 φ	22.6 ± 3.8	11.8 ± 4.6	18.3 ± 4.2
	Cr (mmol/kg dw)	59.7 ± 8.4	41.1 ± 2.4	54 ± 2.9	52.2 ± 2.7
	TCr (mmol/kg dw)	64.6 ± 8.1 φ	63.6 ± 3.7 φ	65.8 ± 5.2	70.5 ± 6.1
	n	8	8	8	8
14 days	ATP (mmol/kg dw)	8 ± 1.08 φ	8.6 ± 0.9 φ	10.8 ± 1.8	10.6 ± 0.7
	PCr (mmol/kg dw)	25.3 ± 5.3	23.2 ± 4.5	28.1 ± 5.1	23.5 ± 6.1
	Cr (mmol/kg dw)	44.6 ± 5 φφ	40.9 ± 3.4 φφ	53.9 ± 2.7	47.4 ± 3.3
	TCr (mmol/kg dw)	69.8 ± 6 φ	64 ± 4.9 φ	82 ± 2.7	70.8 ± 5.7

Table 7.2b. Muscle metabolite levels of uninjured and injured soleus muscles from rats treated with normal- and whey protein-supplemented rat chow at day 7 and 14 post injury. n = number of animals in each group. Values are mean ± SEM. φφ (P<0.01), φ (P<0.05) significantly different from contralateral values. *(P<0.05) significantly different from control animals.

Effect of supplementation: ATP levels were significantly increased in the whey protein-supplemented soleus at day 7 post-injury ($P < 0.05$, see table 7.2b). No other effects of whey protein supplementation were observed on metabolite levels.

7.2.4 Intramuscular Protein Levels

Muscle protein content was measured to confirm whether alterations in muscle mass was due to alterations in functional material (see table 7.3a & 7.3b).

Effect of myotoxic injury: Contractile and total protein concentrations were significantly lower in the injured EDL muscles at day 7 and 14 post-injury ($p < 0.05$). However, no significant effect of myotoxic injury was observed in the WP-INJ EDL muscles at day 14 post-injury. Furthermore, when contractile protein concentration was expressed as a percentage of protein concentration (% contractile protein), there was no significant effect from myotoxic injury. Similarly, in the injured soleus muscles, total protein concentrations were lower at day 7 and 14 post-injury ($p < 0.05$). However, in contrast to the injured EDL muscles, contractile protein concentrations were not significantly different from contralateral soleus muscles (table 7.3a & 7.3b), as was also the case for % contractile protein.

Effect of supplementation: Interestingly, whey protein supplementation had no significant effects on total or contractile protein levels in either muscle types at any time point (see table 7.3a & 7.3b).

Treatment duration	CON-INJ	WP-INJ	CON-NORM	WP-NORM
n	9	8	9	9
[protein] (mg/g)	115.3 ± 10.3 ϕ	136.4 ± 12.8 ϕ	161.3 ± 14.5	163.8 ± 22.2
7 days [contractile protein] (mg/g)	30.7 ± 3.7 ϕ	32.9 ± 3.6 ϕ	46.9 ± 5.7	43.8 ± 4.2
% Contractile protein	26.6 ± 2.7	24.1 ± 1.3	29.1 ± 4.4	26.7 ± 3.8
n	9	8	9	9
[protein] (mg/g)	123.7 ± 12.2 ϕ	126.4 ± 12.5 ϕ	162.1 ± 11.1	144.1 ± 11.9
14 days [contractile protein] (mg/g)	32.4 ± 4.3 ϕ	38.8 ± 4.6	45.9 ± 4.1	37.2 ± 3.2
% Contractile protein	26.2 ± 1.9	30.1 ± 1.1	28.3 ± 2.8	25.8 ± 2.7

Table 7.3a. Muscle protein content of uninjured and injured EDL muscles from rats treated with normal- or whey protein-supplemented rat chow for 7 and 14 days. n = number of animals in each group. Values are mean ± SEM. ϕ (P<0.05) significantly different from contralateral values.

Treatment duration	CON-INJ	WP-INJ	CON-NORM	WP-NORM
n	8	8	8	8
[protein] (mg/g)	112.2 ± 16.4 ϕ	116.6 ± 12.9 ϕ	136.9 ± 15.7	135.1 ± 10.2
7 days [contractile protein] (mg/g)	37.3 ± 5.5	30.9 ± 3.5	41.7 ± 5.4	39.8 ± 4.5
% Contractile protein	33.2 ± 2.7	26.5 ± 1.4	30.5 ± 2.3	29.4 ± 2.4
n	8	8	8	8
[protein] (mg/g)	124.6 ± 11.7 ϕ	123.1 ± 14.6 ϕ	141.5 ± 5.6	162.8 ± 15.5
14 days [contractile protein] (mg/g)	36.1 ± 4.6	30.4 ± 3.2	42.9 ± 3.7	44.8 ± 4.1
% Contractile protein	29 ± 1.8	24.9 ± 1.8	30.3 ± 3.2	27.5 ± 2.5

Table 7.3b. Muscle protein content of uninjured and injured soleus muscles from rats treated with normal- or whey protein-supplemented rat chow for 7 and 14 days. n = number of animals in each group. Values are mean ± SEM. ϕ (P<0.05) significantly different from contralateral values.

7.2.5 Muscle Fibre CSA

Cross-sectional area of both intact (non-damaged) and regenerating muscles was measured to examine whether alterations in muscle strength were due to changes in fibre CSA (see figure 7.2a & 7.2b).

Effect of myotoxic injury: As expected, CSA of regenerating EDL and soleus muscle fibres were significantly smaller compared to CSA of intact fibres at day 7 and 14 post-injury ($P < 0.001$, see figure 7.2a & 7.2b). *Effect of supplementation:* At day 7 post-injury, muscle fibre CSA was significantly greater in the WP-INJ EDL muscles compared to CON-INJ muscles ($P < 0.05$), indicating that the regenerating EDL fibres of the whey protein-supplemented animals were larger than the regenerating EDL fibres of the control animals ($P < 0.05$, figure 7.2a). No significant differences in muscle fibre CSA were observed after whey protein supplementation at day 14 post-injury or in the soleus muscles at either recovery time point.

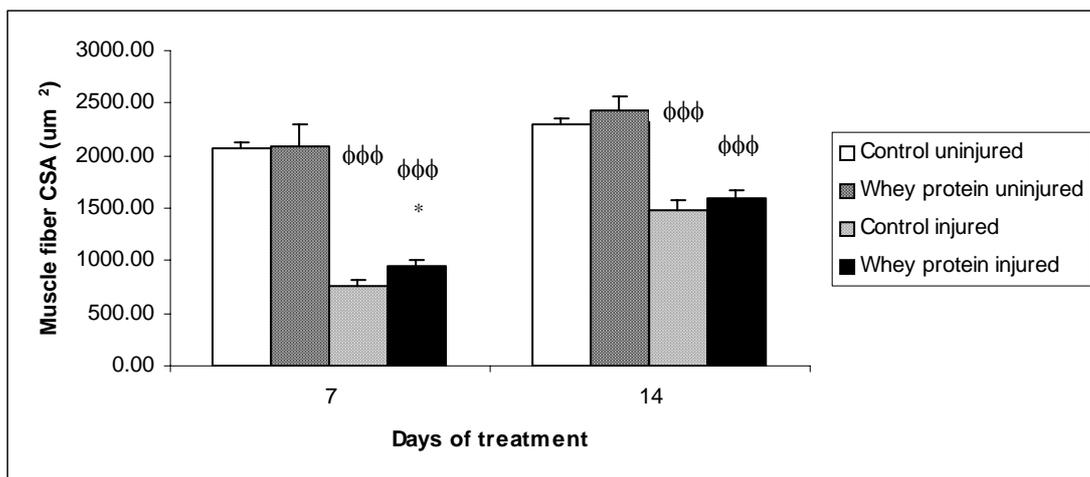


Figure 7.2a. Muscle fibre CSA of uninjured and injured EDL muscles from rats treated with normal- or whey protein- supplemented rat chow at day 7 and 14 post-injury. Values are mean \pm SEM. $\phi\phi\phi$ ($P < 0.01$) significantly different from contralateral values. * ($P < 0.05$) significantly different from control animals.

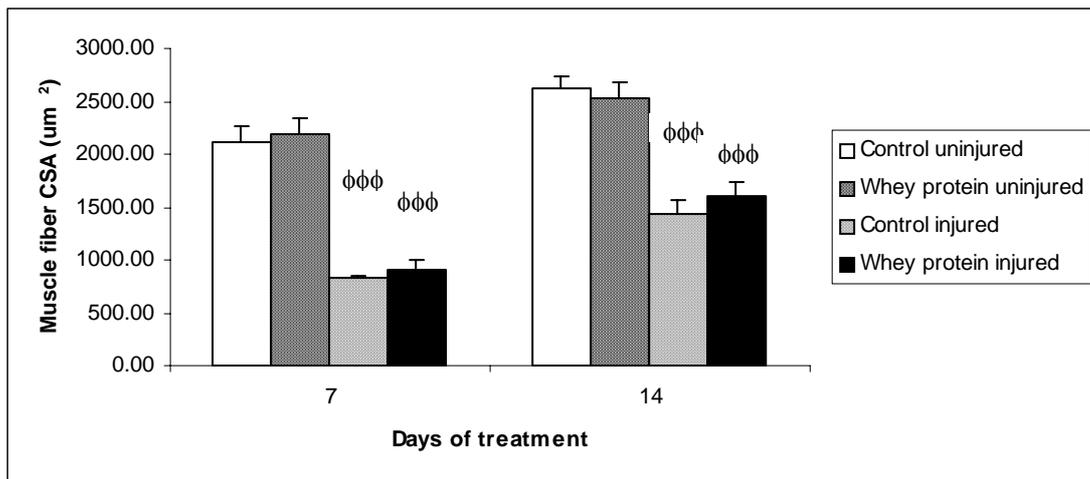


Figure 7.2b. Muscle fiber CSA of uninjured and injured soleus muscles from rats treated with normal- or whey protein- supplemented rat chow at day 7 and 14 post-injury. Values are mean \pm SEM. $\phi\phi\phi$ ($P < 0.01$) significantly different from contralateral values.

7.2.6 Percentage of Damaged, Regenerating and Intact Fibres

Effect of myotoxic injury: Large amounts of fibre necrosis were clearly observed in both EDL and soleus muscles at day 7 post-injury, as evident by a large reduction in intact muscle fibres and an increase in damaged and regenerating fibres (see figure 7.3a & 7.3c). By day 14 post-injury, restoration of muscle fibres was apparent with an increase in intact muscle fibres and a reduction in regenerating and damaged fibres (see figure 7.3b & 7.3d). *Effect of supplementation:* Whey protein supplementation significantly reduced the amount of fibre degeneration in the EDL muscles at day 7 post-injury, as evident by a ~10% reduction in the extent of fibre damage ($p < 0.05$, see figure 7.2.6a). No effects from whey protein supplementation were demonstrated at day 14 post-injury, or in the soleus muscles at any time point (figure 7.3b, 7.3c & 7.3d).

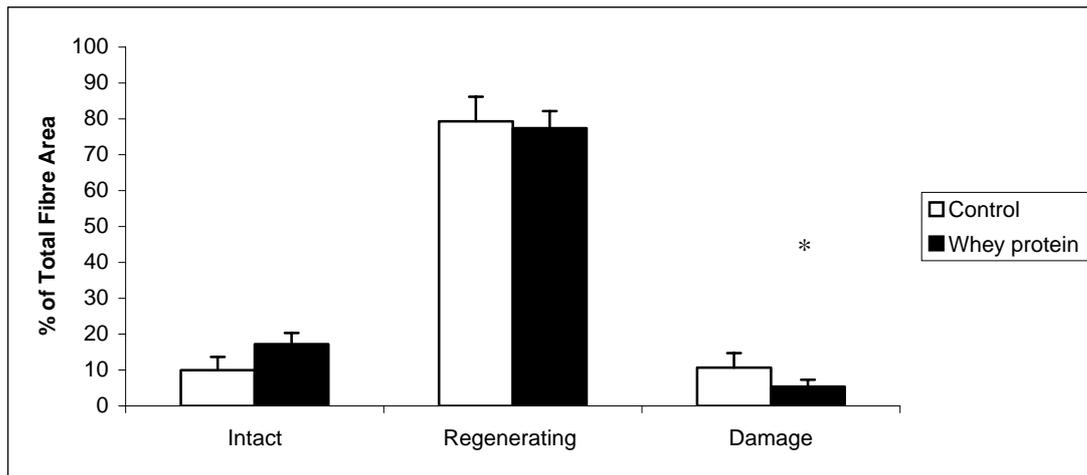


Figure 7.3a. Muscle fibre recovery (expressed as a % of muscle CSA) of injured EDL muscles from rats treated with normal- or whey protein- supplemented rat chow at day 7 post-injury. Values are mean \pm SEM expressed as either % of damaged fibres, regenerating fibres, and undamaged (intact) fibres. * Significantly different ($P < 0.05$) from injured control values.

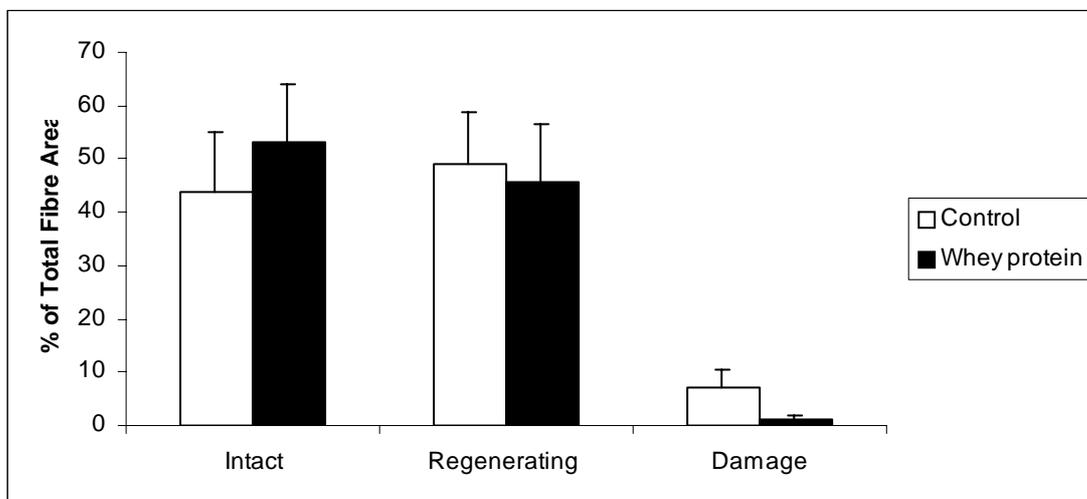


Figure 7.3b. Muscle fibre recovery (expressed as a % of muscle CSA) of injured EDL muscles from rats treated with normal- or whey protein- supplemented rat chow at day 14 post-injury. Values are mean \pm SEM expressed as either % of damaged fibres, regenerating fibres, and undamaged (intact) fibres.

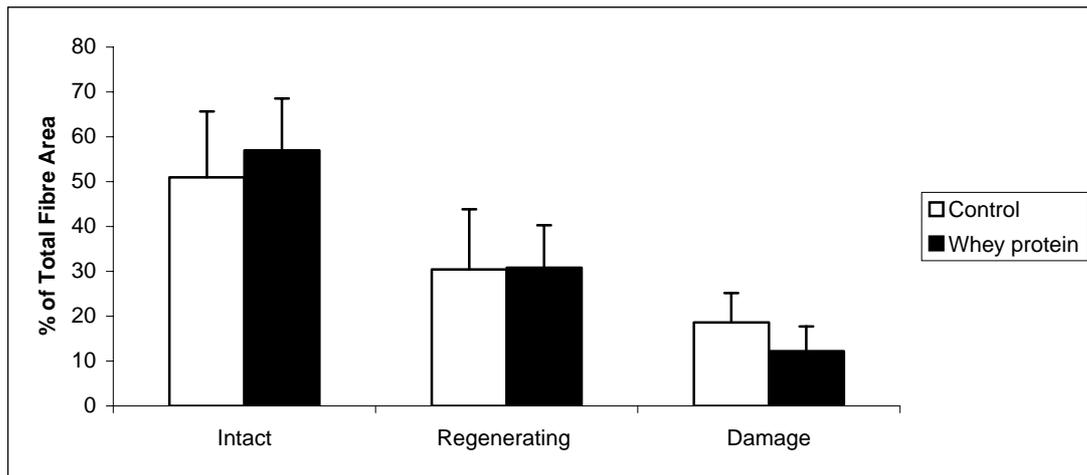


Figure 7.3c. Muscle fibre recovery (expressed as a % of muscle CSA) of injured soleus muscles from rats treated with normal- or whey protein- supplemented rat chow at day 7 post-injury. Values are mean \pm SEM expressed as either % of damaged fibres, regenerating fibres, and undamaged (intact) fibres.

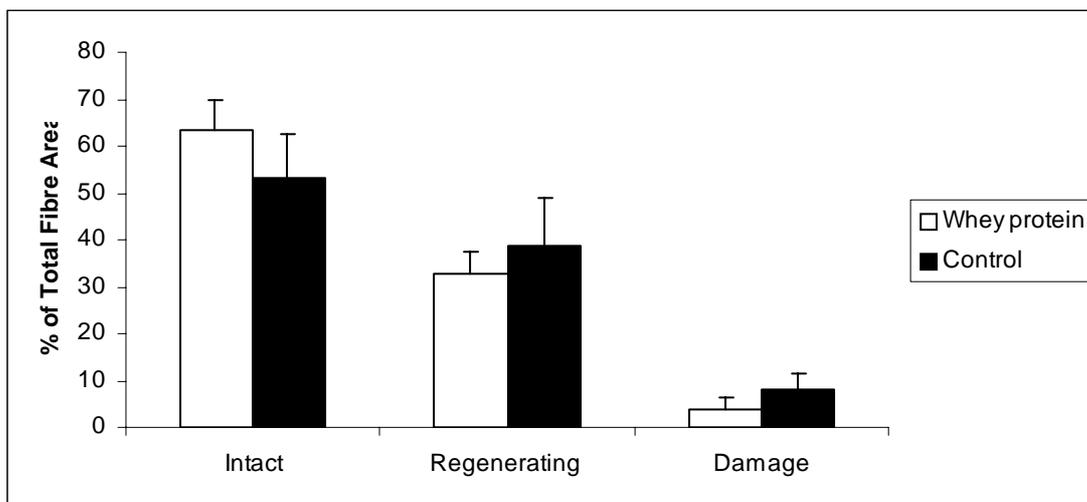


Figure 7.3d. Muscle fibre recovery (expressed as a % of muscle CSA) of injured soleus muscles from rats treated with normal- or whey protein- supplemented rat chow at day 14 post-injury. Values are mean \pm SEM expressed as either % of damaged fibres, regenerating fibres, and undamaged (intact) fibres.

7.3 DISCUSSION

The major objective of this study was to determine the mechanisms by which dietary supplement whey protein exerts its effect by examining morphological, biochemical and contractile properties of rat skeletal muscle during recovery from chemically-induced muscle damage. Whey protein supplementation significantly improved muscle strength (both absolute and specific force) in the fast-twitch EDL muscle at day 7 following myotoxic injury. Enhanced functional capacity of regenerating muscles occurred because of direct increases in fiber CSA and muscle mass. While several studies have explored the effect of dairy protein supplementation in combination with resistance training (Demling and DeSanti 2000; Antonio *et al.*, 2001; Burke *et al.*, 2001; Fry *et al.*, 2003; Chromiak *et al.*, 2004; Tipton *et al.*, 2004; Cribb *et al.*, 2006), few studies have investigated the efficacy of whey protein supplementation on the muscle recovery after injury (Saunders *et al.* 2004; see chapter 5). Additionally, to the authors' knowledge, no studies have determined the mechanisms by which whey protein may exert its effect during muscle recovery after injury. Thus, the present study provides information on the means by which whey protein supplementation may affect the biochemical, structural and functional aspects of muscle fibre recovery following chemically-induced muscle damage.

The effect of whey protein supplementation on morphometric measurements

Previous studies investigating human dietary supplementation have indicated gains in body mass are associated with whey protein supplementation (Lands *et al.*, 1999). However, such an effect in rats was not seen in this study, as no differences in body mass were observed between whey protein-supplemented animals and control animals. Furthermore, both groups gained similar body mass during the study. In addition, researchers consider the primary effect of whey protein is an increase in lean body mass

(Lands *et al.*, 1999; Cribb *et al.*, 2006). An increased lean body mass would be observed if muscle mass was maintained and there is a decrease in body mass. In the present study, increases in both muscle mass and body mass were observed following whey protein supplementation and thus, no significant changes in lean body mass (as estimated by MM:BM) were observed. However, it is possible that the supplementation period utilized in the present study was not long enough to create gains in lean body mass, as other studies have shown increases in LBM following chronic protein supplementation (Lands *et al.*, 1999; Burke *et al.*, 2001).

The effect of whey protein supplementation on intramuscular protein levels and muscle strength.

It is well established that following bupivacaine injection, there is a significant decrease in absolute proteins (muscle proteolysis) and thus, loss of muscle mass and force (Rosenblatt and Woods, 1992). In the present study, whey protein significantly enhanced recovery of muscle mass and absolute force of the injured EDL muscles at day 7 post-injury. Furthermore, protein levels were ~18% higher in the WP-INJ EDL muscles compared to CON-INJ EDL muscles at day 7 post-injury. When corrections were made for cross-sectional area, specific forces were significantly higher in the whey protein-supplemented injured EDL muscles at day 7 post-injury, thus indicating that for a given muscle size, injured EDL muscles following whey protein supplementation were producing greater forces compared to injured control muscles.

The increased muscle mass and absolute forces observed in the injured EDL muscles at day 7 post-injury suggests that following whey protein supplementation, muscle protein synthesis was enhanced and thus, protein breakdown was attenuated. Although muscle protein synthesis and breakdown rates were not measured in the present study, it is clear that loss of muscle protein (i.e. degradation) usually associated after bupivacaine

injection was reduced in the present study, as evident by ~18% higher total protein levels in the WP-INJ EDL muscles compared to the CON-INJ EDL muscles, although not significant. In support of this, the extent of muscle fibre necrosis in the injured EDL muscles was also significantly lower in the whey protein animals compared to the control animals. Furthermore, CSA of regenerating EDL muscle fibres was significantly larger in the whey protein supplemented animals compared to control animals at day 7 post-injury.

These results clearly demonstrate possible mechanisms by which whey protein is improving muscle functional recovery from injury. Moreover, the reduced damage and increased regeneration, observed in the whey protein-supplemented EDL muscles helps explain the improved force recovery, and lower plasma CK and LDH levels observed in the human participants following whey protein supplementation (chapter 5).

It is established that due to its unique amino acid profile and rapid absorption kinetics (Biorie *et al.*, 1997; Bucci and Unlu, 2000; Dangin *et al.*, 2001; 2003), whey protein is the most effective at stimulating protein synthesis, and thus provides further possible mechanisms by which whey protein supplementation improved functional capacity of the injured EDL muscles at day 7 post-injury. In addition to whey's ability to effectively stimulate muscle protein synthesis, whey may also enhance inflammatory processes and improve the antioxidant status of the muscles.

Following intense exercise or muscle injury, glutamine is utilised at a high rate by lymphocytes and macrophages during the inflammatory phase (Newsholme, 1994). It is suggested that plasma glutamine may be a "metabolic link" between skeletal muscle and the immune system (Newsholme, 1994), but more importantly, since inflammation is a

necessary component for complete muscle restoration after injury, plasma glutamine may be essential for the muscle cells to regenerate promptly. In the present study, although glutamine levels were not measured, it is likely that since whey protein is a good source of glutamine, intramuscular glutamine levels would be increased following supplementation. Thus, any increases in glutamine in the present study may have improved the function of macrophages and neutrophils, and hence, enhanced and/or increased the rate of inflammation (i.e. decreasing the inflammation period and reducing the extent of fibre necrosis).

Furthermore, since macrophages may play a role in the activation of satellite cells, increased glutamine levels could indirectly enhance satellite activation, and thus, new fibre formation. Indeed, both these effects (reduced fibre damage and enhanced regeneration) were observed in the present study, and therefore provides further possible mechanisms by which whey protein is improving muscle recovery after injury not only in this study, but also in the human study (chapter 5).

In addition to glutamine's role as an integral component during the inflammatory phase, it is also an important amino acid precursor of glutathione. Glutathione (GSH) is one of the most abundant low molecular weight peptides in eukaryotic cells and the most prevalent intracellular thiol (Zable *et al.*, 1997). In the cell, more than 95% is in the reduced form GSH. Glutathione acts as both a reducing agent and antioxidant, and thus is central in the defence mechanisms against intra- and extra-cellular oxidative stress (Cotgreave and Gerdes, 1998). An increase in the cellular antioxidant system could have pathophysiological relevance by reducing excess protein oxidation and degradation following myotoxic injury. Oxidative stress, due to excess oxidation production and/or insufficient antioxidant capacity, increases protein degradation (Reid, 2005). In

particular the ubiquitin proteolysis system, which is now believed to be critical in degradation of myofibrillar proteins, is sensitive to oxidative stress (Strong and Pattee, 2000; Reid, 2005), and hence may be upregulated in the presence of reactive oxygen species (ROS). Thus, by increasing glutathione levels, the antioxidant defenses of the muscle would be enhanced and the activity of the ubiquitin proteolysis system during muscle degeneration may be reduced, and thus the amount of protein degradation also diminished. Indeed, this is supported, at least in the EDL, by a significant reduction in the extent of muscle damage and ~18% higher total protein levels following whey protein supplementation at day 7 post-injury.

Reduced fibre necrosis following myotoxic injury could also be due to enhanced calcium uptake by the SR Ca^{2+} -ATPase pump, and thus improved calcium handling ability of the muscle. Although SR Ca^{2+} -ATPase function was not directly measured in the current study, previous research from our laboratory has shown an increase in Ca^{2+} uptake by SR vesicles of EDL muscles following whey protein supplementation. Indeed, in the present study whey protein supplementation significantly increased muscle relaxation times in the both the injured and uninjured EDL muscles at day 7 post-injury, indicative of faster Ca^{2+} removal from the cytosol.

It is suggested that ROS produced during and/or following muscle injury may inhibit ATPase activity via modification of sulfhydryl groups on the SR protein, hence disrupting the normal Ca^{2+} handling kinetics of the muscle (Favero *et al.*, 1998). Thus, increasing glutathione levels within the muscle after whey protein supplementation may reduce the free radical damage of the SR Ca^{2+} pumps, thus improving the intracellular Ca^{2+} handling ability of the muscle. Improved Ca^{2+} handling ability following muscle injury will reduce further increases in intracellular calcium, and hence, reduce the

activation of self-accelerating degradative pathways such as calpains and phospholipases, which lead to further damage to the muscle. Indeed, the magnitude of muscle fibre damage was significantly reduced in both the human and current study in the days after muscle damage as evident by a reduction in CK and LDH levels and extent of fibre necrosis, respectively, following whey protein supplementation.

Increased antioxidant defences of the muscle due to increased glutathione levels may also reduce free radical damage of the mitochondria and thus improve the ATP levels within the muscle. In the current study, ATP levels were significantly higher in the WP-INJ soleus muscles and higher (although not significant) in the WP-INJ EDL muscles compared to CON-INJ muscles, at day 7 post-injury. These results may suggest that increased GSH within the muscle following whey protein supplementation may reduce free radical damage to the mitochondria as a result of inflammation and muscle degeneration, and thus improve mitochondrial function.

However, a number of other factors may be influencing intramuscular ATP levels rather than supplementation alone, and thus caution is taken when interpreting these results. As mentioned, muscles were not frozen *in situ* as both the injured and uninjured EDL and soleus muscles were first placed in a krebs ringer solution and tested for contractile properties, lasting for approximately 40 minutes. Once tested, muscles were removed quickly, weighed, cut in half, and then snap frozen in liquid nitrogen. While there is no evidence to suggest that this method will cause further variation in metabolite levels between each muscle, as muscles were subjected to very similar *in vitro* testing conditions and procedures. However, it is evident that the metabolite levels in the uninjured muscles were generally lower compared to resting muscle samples (Op t Eijnde *et al.*, 2001), and thus, it is possible that some ATP hydrolysis took place during

the contractile testing period. Although ATP levels were higher in the soleus muscles following whey protein supplementation, no other effects from supplementation were observed in the soleus muscles.

It is clear that whey protein supplementation significantly influenced the recovery of fast-twitch EDL muscles following myotoxic injury, compared to the slow-twitch soleus muscles, as evidence by significantly heavier muscles and greater strength in the WP-INJ EDL muscles compared to CON-INJ muscles, in addition to a significant reduction in the extent of muscle fibre damage and larger CSA of INJ EDL muscle fibres. While the causes for such differential responses between fast-twitch and slow-twitch fibres following whey protein supplementation are not readily apparent in the current study, as mentioned in the previous chapter, it has been shown that slow-twitch muscles are slower to recover from injury, compared to the fast-twitch muscles, with incomplete restoration of mass and force observed as long as 60 days after chemically-induced muscle damage (Plant *et al.*, 2005). This is likely due to the more “plastic” nature of fast-twitch fibres, when compared to the more “postural” slow-twitch fibres.

Indeed, a limitation in the present study was that rats were able to walk freely within their cage during the recovery period following myotoxic injury, and thus the results did not differentiate between the mechanical aspects of loading and of regeneration. Therefore, since the fast-twitch and slow-twitch muscles have different functions during walking, they are subject to different stimuli during regeneration, and thus their regenerative capacity and effects from supplementation may vary (Plant *et al.*, 2005).

Interestingly, while muscle mass and strength were significantly enhanced in the injured EDL muscles following whey protein supplementation, the uninjured contralateral EDL

muscle also demonstrated the same increases following 7 days of whey protein supplementation. These results support the improved isokinetic knee flexion in the whey protein-supplemented group, following minimal decrements in muscle strength after the resistance exercises, in our human studies (chapter 5). It was suggested that whey protein supplementation may be increasing muscle strength in the uninjured muscle fibres, in addition to the injured fibres.

Previous research into the effects of whey protein in humans has reported significant increases in muscular strength. However, these studies have been in combination with programmed strength or athletic training. In the current study, rats did not undergo any training, thus suggesting that whey protein was able to increase strength without the influence of weight training. This supports the work of Lands *et al.* (1999) who demonstrated that subjects supplementing their diets with whey protein were able to generate greater power, and increase the amount of work they could achieve during an all out exercise program, without the influence of any fitness training.

Taken together, these results may reflect whey proteins ability to increase nitrogen retention in the uninjured muscles fibres, and thus create a positive nitrogen balance, which is associated with muscle anabolism. Also, whey protein contains a high concentration of cysteine; an increase in cysteine levels may be critical in maintaining or increasing muscle mass (Kinsharf *et al.*, 1996; Bucci & Unlu 2000). Although nitrogen balance and cysteine levels were not measured in the present study, any increases would lead to muscle anabolism, as evident in the uninjured EDL muscles following 7 days of supplementation. Another possible mechanism, though highly speculative, is that because injury was induced in the EDL and soleus muscles of the left hind limb, the right hind limb may have to accommodate the decreased functional

capacity of the contralateral leg, and as a result, there may be some compensatory hypertrophy in the right hind limb which is improved by the increase protein availability following supplementation, although this concept required further investigation.

Another interesting observation is that supplement intake was significantly lower than the supplement target dosage of 5 g.kg⁻¹ body weight per day at day 7 and 14 post-injury. This reduced food intake was also evident in chapter 6, with creatine intake significantly lower than the target doses at day 7 and 14 post-injury. These observations may reflect that there may have been some form of discomfort to the rats when trying to reach and eat their food, and thus food intake was reduced. Notwithstanding this, the supplement dosage used in the present study was chosen to mimic those used by resistance-trained individuals. Therefore, although the whey protein intake was lower than the target dosage, the rats would still be receiving high amounts of protein in the days following myotoxic injury as they were not undertaking any training.

In summary, whey protein supplementation significantly enhanced functional capacity of regenerating EDL muscles in the early stages of muscle recovery following injury, as evident by a significant reduction in muscle fibre necrosis, increased CSA of regenerating fibres, and heavier muscle weights. In the absence of feeding following muscle damage, there is a transient fall in intracellular amino acids, as the requirement for an increased protein synthesis leads to increases in both protein breakdown and/or inward amino acids transport to maintain the intracellular amino acid pool (Tipton and Wolfe, 1998). Consequently net muscle protein balance remains negative (Biolo *et al.*, 1995; Phillips *et al.*, 1997). In the present study, whey protein supplementation may have provided the necessary exogenous amino acids to maintain the intracellular amino acids pool within the injured muscles, thus creating a positive protein balance as

synthesis of new proteins would be derived from the exogenous amino acids, as opposed to the amino acids from the increased degradation of muscle proteins. Although protein synthesis and breakdown rates were not measured in the present study, the ~18% higher protein levels in the injured EDL muscles at day 7 post-injury, may be reflecting the ‘anticatabolic effects’ of whey protein supplementation, rather than its ability to increase sarcomeric protein synthesis *per se*, as contractile proteins were not significantly increased, nor was the rate of muscle fibre recovery, as similar contractile, morphological, and histochemical properties were observed at day 14 post-injury between whey protein-supplemented and control animals. Finally, whey protein’s ability to improve the recovery of fast-twitch EDL muscles compared to the slow-twitch soleus muscles may reflect the “plastic” nature of the EDL muscles, and thus their enhanced adaptability, compared to the more postural soleus muscles.

CHAPTER 8

8.0 CONCLUSIONS AND FUTURE DIRECTIONS

In summary, the studies reported in this dissertation have shown that the nutritional supplements creatine monohydrate and whey protein can play an important physiological role in the recovery from muscle injury by reducing the extent of fibre necrosis and improving muscle regeneration. In the human studies, muscle strength was significantly higher in the whey protein- and creatine-supplemented groups compared to those consuming the placebo carbohydrate following eccentrically-induced muscle damage. Lower plasma creatine kinase and lactate dehydrogenase levels in the days after injury was indicative of less muscle damage, and thus, supported the improved functional capabilities observed. However, a limitation to these studies was the use of indirect measurements to determine the magnitude of muscle damage and rate of muscle recovery. Therefore, we could only speculate on how creatine and whey protein may be potentially exerting its positive effects. Thus, the use of the animal model in this dissertation allowed a more extensive and accurate analysis of muscle damage and regeneration following injury, but more importantly, since the healing processes are essentially the same in both models of injury; the mechanisms by which whey protein and creatine supplementation were improving recovery from injury in the animal model could be directly applicable to the improvements observed in the human studies.

In the animal studies, creatine supplementation significantly enhanced functional capacity of the regenerating EDL muscles by reducing the extent of fibre necrosis and augmenting fibre regeneration following myotoxic injury. These effects occurred as a direct result of increased muscle protein content, regenerating fiber CSA and muscle mass. Whey protein supplementation significantly enhanced functional capacity of the

regenerating EDL muscles by reducing the extent of fibre necrosis following myotoxic injury. Similar to creatine supplementation, enhanced functional capacity of the EDL muscles was due to direct increases in muscle protein content, regenerating fiber CSA and muscle mass. However, in contrast to creatine supplementation, improved muscle recovery was only evident in the early stages of muscle regeneration, with no effects from whey protein supplementation observed in the later stages of muscle regeneration.

Direct comparisons between the effects of whey protein and creatine supplementation on muscle recovery after damage revealed creatine supplementation showed greater improvements in various aspects of recovery measurements compared to whey protein supplementation. Creatine-supplemented EDL muscle demonstrated significantly higher specific forces at day 7 post-injury compared to whey protein-supplemented EDL muscle. Thus, while both supplements increased specific forces compared to control muscles, creatine-supplemented EDL muscles were generating greater forces for a given muscle size. These observations are most likely due to the higher total and contractile proteins in the creatine-supplemented EDL muscle compared to whey protein-supplemented muscles – albeit these differences were not significant; any increases in contractile mass would produce higher forces for a given muscle size.

Furthermore, at day 14 post-injury, creatine-supplemented injured EDL muscles were significantly heavier, demonstrated higher absolute forces and total proteins compared to whey protein-supplemented muscles. This supports the improved recovery in the later stages of muscle regeneration in the creatine-supplemented animals compared to the whey protein-supplemented animals.

The observed effects of creatine supplementation in both the human and animal models (chapter 4 and 6), could be due to creatine's dual mode of action to improve muscle

recovery from injury. Firstly, we suggest that increasing intramuscular PCr and Cr levels will improve the ATP regenerating capacity of the SR Ca^{2+} -ATPase pump function by maintaining a high ATP/ADP ratio within the vicinity of the ATPase pump via the PCr/CK system. Thus, improving ATP regeneration will enhance the calcium buffering capacity of the muscle during and following exercise- or chemically-induced muscle damage. Such improvements will reduce the extent of calcium accumulation within the muscle, but more importantly will minimise the degradative pathways such as calcium-activated proteases and lipases, which lead to further damage to the muscle. In chapter 6, SR Ca^{2+} -ATPase function and intracellular calcium levels were not directly measured, especially during the initial stages following bupivacaine injection. However, it could be suggested that since $\frac{1}{2}$ relaxation time, which is an approximate indicator of SR Ca^{2+} -ATPase function, was significantly faster in the creatine-supplemented EDL muscles (both injured and uninjured) at day 7 and 14 post-injury compared to control muscles, Ca^{2+} handling ability may have been improved following creatine supplementation. Thus, this may have contributed to the reduced muscle damage and enhanced functional recovery observed in both human and animal models. Indeed, given that it was also suggested as the cause of less damage in *mdx* myotubes (Pulido et al., 1998), then this seems a likely mechanism.

The second mode of action of creatine could be its ability to directly manipulate, or act as a co-regulator of, muscle gene transcription of the amino acid pool (Willoughby and Rosene, 2001; 2003). Thus, an up-regulation of mRNA may lead to increased myofibrillar protein synthesis during recovery post-injury. Increasing protein synthesis greater than protein degradation will result in a positive protein balance that will facilitate satellite cell proliferation and new fibre formation. Furthermore, while this may hold true for injured muscle fibres, it was clear in the animal study (and perhaps in

the human study, although not measured) that mass of uninjured muscles was significantly increased following creatine supplementation. It is suggested that increased creatine levels within the myofibres will increase the cellular hydration status of the muscle fibres (i.e. increased cell volume). Increased cell volume not only causes an increase in muscle mass, but may also be indirectly signaling satellite cells to proliferate and fuse with the enlarging myofibres (Dangott et al., 1999), thus increasing protein synthesis.

The ability of whey protein supplementation to improve muscle recovery after damage in both the human and animal studies (study 4 and 7), may reflect whey proteins 'anticatabolic characteristics' as opposed to its capacity to increase the rate of muscle regeneration *per se*. Increasing the appearance of exogenous amino acids to the intracellular pool following exercise- and chemically-induced muscle damage enables protein synthesis rates to increase without the necessity for increasing breakdown of muscle proteins to obtain the AA's. Thus, protein degradation is minimised, but more importantly, muscle fibre degeneration is reduced. Increasing protein synthesis and reducing protein breakdown will result in a positive protein balance, hence facilitating satellite cell proliferation and muscle fibre regeneration. Furthermore, since the amino acid composition of whey proteins is very similar to that of skeletal muscle, whey protein supplementation may not only be facilitating satellite cell proliferation and muscle fibre regeneration, it may also be providing the amino acids that are essential for muscle remodelling, although this concept has yet to be investigated.

Nonetheless, it is clear that whey protein is an effective method of stimulating protein synthesis due to its amino acid composition and rapid absorption kinetics (Biorie et al., 1997; Bucci and Unlu, 2000; Dangin et al., 2001; 2003). However, the exact

mechanism(s) for this increase are not completely clear. The mTOR-signaling pathway has been well characterized in the regulation of protein synthesis, via the phosphorylation of two key proteins, ribosomal protein S6 and eukaryotic initiation factor (eIF)4G. It is established that the mTOR pathway can be influenced by cellular amino acid concentrations (Beugnet et al., 2003), in particular the branched chain amino acid leucine, which has been shown to directly enhance phosphorylation (i.e. activation) of the 70-kDa ribosomal protein S6 kinase, thereby up-regulating protein synthesis in skeletal muscle by enhancing both activity and synthesis of proteins integral to mRNA translation (Karlsson *et al.*, 2004). Therefore, since whey protein contains a high concentration of leucine, it is likely that plasma leucine levels may have increased following whey protein supplementation. Although, plasma leucine levels were not measured in either study (chapter 5 & 7) any increases would produce an anabolic effect that would reduce the extent of damage and improve functional recovery as shown in both animal and human studies.

An interesting observation in both animal studies (chapter 6 and 7) was the ability of whey protein and creatine to better enhance the recovery of fast-twitch EDL muscles compared to the slow-twitch soleus muscles post-injury. While the causes for such differential responses between fast-twitch and slow-twitch fibres following whey protein and creatine supplementation was not readily apparent in this dissertation, it has been shown that slow-twitch muscles are slower to recover from injury, compared to the fast-twitch muscles, with incomplete restoration of mass and force observed as long as 60 days after chemically-induced muscle damage (Plant et al., 2005), and thus perhaps a longer recovery period was required to observe any beneficial effects from both supplements.

Furthermore, given the “plastic” nature, and thus more adaptable properties of the fast-twitch fibres, compared to the more “postural” slow-twitch fibres, it is possible since both muscles have different functions during walking, they will be subjected to different stimuli during the regeneration period, and thus, their regenerative capacity and effects from supplementation may vary. Nevertheless, the greater improvement in the fast-twitch compared to slow-twitch muscles further supports the improved functional recovery in both human studies. It is well established that fast-twitch fibres are more susceptible to eccentric exercises due to their preferential recruitment during high strain exercise compared to slow twitch muscles. In addition, narrower Z bands, which may represent lower amounts of thick and thin filaments, would place greater stress on the fast fibres during lengthening contractions (Vijayan et al., 2001). Thus, damage to the fast-twitch fibres, which produce high forces compared to slow-twitch fibres will lead to marked reductions in muscle strength as shown in both human studies (chapter 4 and 5). Therefore, since whey protein and creatine enhanced the recovery of fast-twitch EDL muscles compared to the slow-twitch soleus muscles post-injury in both chapter 6 and 7, it is likely that this preferential recovery of the fast-twitch fibres, which are known to produce high forces, was also occurring in both human studies (chapter 4 and 5). Thus, providing strong support to the higher forces produced in the days following exercise-induced damage after creatine and whey protein supplementation.

In conclusion, the results from this thesis have shown the creatine monohydrate and whey protein supplementation can improve muscle functional capacity during the days following muscle injury. Although, it is clear that these observations were a direct result of reduced muscle necrosis and/or increased fibre regeneration, in addition to increased protein levels, regenerating fibres CSA, and muscle mass; there are still questions remained unanswered. Thus, in addition to studying intact muscle fibre preparations *in*

vitro, further work utilizing single muscle fibre preparations to directly assess the contractile apparatus may provide evidence of any changes at an intramuscular level in response to creatine and whey protein supplementation. To further investigate creatine's mechanisms of action, additional work directly examining SR Ca²⁺-ATPase function and intracellular Ca²⁺ concentrations in the early stages of muscle damage and regeneration would determine whether creatine supplementation improved the calcium handling ability of the muscle during, and following muscle injury. Examining muscle protein synthesis/breakdown rates and muscle transcription factors that control for myofibrillar proteins will help further elucidate whey protein's mechanism of action. Moreover, in addition to examining the release of cytosolic proteins in plasma, which may not necessarily reflect the amount of structural damage, investigating the release of structurally bound proteins such as myosin heavy chains and skeletal troponins will provide a more accurate measurement of the extent of muscle damage.

In terms of significance to the wider community, whey protein and creatine supplementation may help reduce the extent of, or enhance recovery from, exercise-induced muscle damage, and thus not only benefit athletes during intense training phases, competition and during recovery from injury, but may also return people to the workplace earlier. Furthermore, research into promoting functional recovery is also of considerable benefit to a variety of populations, including those suffering from muscle wasting conditions, weakness associated with aging, neuromuscular disorders, acquired immunodeficiency syndrome, burn injury, cancer cachexia and prolonged sepsis. Further research on how to manipulate muscle mass and muscular strength in such a way as to enhance recovery from sports injuries, or influence the conditions listed above is desirable and would provide subsequent financial and health benefits to the individual and/or community.

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APPENDICIES

APPENDIX A

DETERMINATION OF LACTATE DEHYDROGENASE (LDH) ACTIVITY

PREPARATION OF SOLUTIONS

1. 1M KH₂PO₄

Dissolve 6.8045g of KH₂PO₄ (BDH 10203) in 40ml milli-Q water. Bring up to 50ml with Milli-Q water.

2. 10% Bovine Serum Albumin

Dissolve 1g BSA (CSL 06711701) in 5 ml Milli-Q. Bring up to 10 ml with milli-Q.

3. 1M 2-Mercaptoethanol

Add 701.3ul ME (Biorad 161-0710) to 10ml Milli-Q water.
(MW=78.13g; Density =1.1143)

4. Homogenisation Buffer

Reagent	Stock	Working	Volume for 25ml	Volume for 50 ml	Volume for 100ml
KH ₂ PO ₄	1M	170mM	4.25 ml	8.5 ml	17 ml
BSA	10%	0.02%	50 ul	100 ul	200 ul
2-Mercaptoethanol	1M	5mM	125 ul	250 ul	500 ul

5. 1M Imidazole

Dissolve 6.808 g of Imidazole (Merck 1.04716.0050) in 90ml Milli-Q water. Bring volume up to 100 ml with Milli-Q water. Imidazole is located on the chemical shelf. Store in freezer once made.

6. 100mM Pyruvate

Dissolve 0.1100 g of Pyruvate (B&M 128 147) in 10 ml Milli-Q water. Make fresh on the day.

7. 25mM Nicotinamide adenine dinucleotide (NADH)

Dissolve 17.74 mg of NADH (B&M 837075) in 1 ml Milli -Q water

8. Reaction Cocktail

Reagent	Stock	Working	Volume for 25ml	Volume for 50 ml	Volume for 100ml
Imidazole	1M	100mM	42.5 ml	5.0 ml	10 ml
Pyruvate	100mM	1mM	500 ul	1 ml	2 ml
BSA	10%	0.04%	100 ul	200 ul	400 ul

Adjust pH to 7.0

10 ml of the reaction cocktail is then pipetted off and stored on ice. The appropriate volume of 25 mM NADH is then added to the remaining reaction cocktail to generate a final reaction concentration of ~25 uM

	Stock	Working	Volume for 15ml	Volume for 40 ml	Volume for 90ml
NADH	25mM	25 uM	60 ul	160 ul	360 ul

ASSAY PROCEDURE

1. Make up Reagent Cocktail and adjust pH to 7.0
2. Pipette off 10ml of the reagent cocktail and store on ice. This will be used as your 'reagent blank'.
3. Add the appropriate volume of 25 mM NADH to the remaining reaction cocktail.
4. Set up 1941 cuvettes (analyse samples in duplicate)
5. Add 1ml of your reagent blank to a single cuvette (this will be used to set the spectrophotometer to 0).
6. Add 900 ul of the reagent blank/NADH solution to the appropriate cuvettes
7. Place 'reagent blank' cuvette into the spectrophotometer set at 340nm. Set ref to 0.
8. Place cuvette containing the reagent blank/NADH solution into the spectrophotometer.
9. Add 100 ul of your plasma sample to the appropriate cuvettes
10. Once added quickly begin chart windows and analyse the gradient of the slope for 3 minutes.
11. Repeat for all plasma samples

CALCULATION OF LDH ACTIVITY

$$\frac{\text{SLOPE X TOTAL VOLUME}}{6.22 \text{ X VOLUME OF PLASMA}}$$

APPENDIX B

EXPRESSING MUSCLE FORCES ACCORDING TO CROSS-SECTIONAL AREA.

To compare maximum tetanic contraction (P_o) between animals independent of muscle size, measured forces were expressed relative to the muscles' cross-section area (CSA). An estimate of CSA was achieved using established techniques described by Close et al. (1972).

An example of the calculations for force per cross section area is provided here.

The EDL muscle is tested and produces the following results;

Optimal length (L_o)	27mm
Muscle mass (MM)	164gms
Peak tetanic contraction	2423

According to Brooks and Faulkner. (1998) fibre length (fibre length: muscle length ratio) is 0.44 in the EDL and density equals $1.06\text{g}/\text{cm}^3$ (Close, 1972).

Cross-sectional area (X-A) is calculated by muscle mass/ fibre length therefore;

$$\begin{aligned}\text{CSA} &= 0.164\text{g} / 2.7 \times 0.44 \times 1.06\text{g}/\text{cm}^3 \\ &= 0.130\end{aligned}$$

Specific force was then calculated by dividing P_o by the X-A

$$\begin{aligned}\text{SPo} &= P_o / \text{X-A} \\ &= 2423 / 0.130 / 1000 \\ &= 18.64 \text{ N}/\text{cm}^2\end{aligned}$$

Calculating soleus SPo is the same as for the EDL, except according to Brooks and Faulkner. (1998) fibre length (fibre length: muscle length ratio) is 0.71 in the soleus.

APPENDIX C

HAEMOTOXYLIN & EOSIN STAINING SOLUTIONS

10% Buffered Formol Saline (Fixative)

9g	NaCl
100mL	40% Formaldehyde
900mL	Tapwater

Harris' Haemotoxylin

Ready-made solution (Merck OB356029; 2.5L)

OR

2.5 g	Haemotoxylin Crystals
25mL	100% EtOH
50g	Ammonium OR Potassium Alum
500mL	Distilled Water
1.25g	Mercuric Oxide (red)

1. Dissolve the haemotoxylin in alcohol
2. Dissolve the alum in water and heat
3. Remove from heat and mix two solutions
4. Bring to a boil as rapidly as possible (limiting this heat to less than 1 minute and stir often)
5. Remove from heat and add mercuric oxide slowly
6. Reheat to a simmer until it becomes dark purple
7. Remove from heat immediately and plunge the vessel into a basin of cold water until cool
8. Ready to use when cool
9. Add 2-4mL glacial acetic acid per 100mL solution to increase precision as a nuclear stain
10. Filter before use

1% Stock Alcoholic Eosin

1g	Eosin Y, water soluble
20mL	Distilled water
80mL	95% EtOH

1. Dissolve Eosin in water
2. Add EtOH to complete stock solution

Alcoholic Eosin Working solution

1. Dilute 1% alcoholic stock 1:3 (i.e 1 part stock to 2 parts EtOH) with 80% EtOH just prior to use
2. Add 0.5ML glacial acetic acid to each 100mL stain and stir before use

Scott's Tap Water

3.5g	Sodium Bicarbonate (sodium hydrogen carbonate)
20g	Magnesium Sulphate
1L	Tap water

HAEMOTOXYLIN & EOSIN STAINING METHOD

1. Air dry frozen sections for at least 30 seconds (if samples have been sitting in air while cutting, this time should be enough).
2. Fix Tissue in 10% buffered formol saline for 2 minutes.
3. Wash in tap water x5 washes/dips.
4. Place in Haemotoxylin for 30 seconds.
5. Wash in tap water x5 washes/dips.
6. Place in Scott's Tap Water for 3:30 minutes.
7. Wash in tap water x5 washes/dips.
8. Place slides in Eosin for 3 minutes.
9. Dehydrate sections in:
 - 90% EtOH (dip)
 - 100% EtOH (dip)
10. Clear in Xylene.
11. Mount with DPX and coverslips.

APPENDIX D

DETERMINATION OF ATP AND PCr

Preparation of Dilute Reagent

<u>Reagent</u>	<u>Stock Conc.</u>	<u>Final Conc.</u>	<u>Volume for (ml)</u> <u>50 ml</u>
1. Tris Buffer (pH 8.1)	1.0 M	50 mM	2.5 ml
2. MgCl ₂	1.0 M	1 mM	50 µl
3. D.T.T	0.5 M	0.5 mM	50 µl
4. Glucose	100 mM	100 µM	50 µl
5. NADP ⁺	0.05 M	50 µM	50 µl
6. G-6-P-DH (B&M 127175)			5 µl

Reagents 1 to 5 were added together (in a 50ml volumetric flask), this was brought up to volume with milliQ water. Reagent 6 was then added.

Preparation of Dilute Enzyme

1. Dilute Hexokinase. 25µl of Hexokinase was added to 12 x 75mm kimble tubes containing 1 ml of reagent (or 50 µl hexokinase –2ml of reagent). They were then mixed by inversion.
2. To the 1ml of reagent, 10µl of 10% BSA, 2mg ADP, and 2 mg CK were added (this is preferably made after the first incubation step).

Procedure for Assay

1. 10 x 75 kimble tubes were used to run the assay, the tubes were labelled on the front
2. Blank, standards, NADH standards, and samples were analysed in triplicates
3. 1 ml of cocktail was added into the kimble tubes.
4. Before commencing the assay, a set of NADH (50µM, 100µM, 200µM, 400µM) standards (from the freezer) were read on the spectrophotometer at 340nm.
5. 10µl of milliQ water, standards (ATP: 200µM & 100µM; CP: 500µM & 250 µM) and samples were added to their appropriately labelled tubes.
6. They were then mixed on vortex.
7. These were read on the previously warmed up Turner Fluorimeter 365nm absorption, 455 nm emission. Sensitivity was set on number 3 (R1).
8. After reading, 25 µl of dilute hexokinase was added to each 10 x 75 mm culture tube, then mixed
9. They were then placed in dark for 30 minutes at room temperature.
10. After incubation the kimble tubes were read on fluorometer (on same setting-‘R2’).
11. 20 µl of dilute ADP-CK was next added to each 10 x 75 mm kimble tube and mixed.
12. After addition of the dilute ADP-CK the kimble tubes were placed in dark for 60 minutes at room temperature.
13. After incubation, they were read on fluorimeter at same setting (R3).

APPENDIX E

DETERMINATION OF MUSCLE CREATINE

Preparation of Reaction solution

<u>Reagent</u>	<u>Stock Conc.</u>	<u>Final Conc.</u>	<u>Volume for (ml)</u> 50 ml
1. Imadazole (pH 7.4)	1.0 M	50mM	2.5 ml
2. MgCl ₂	1.0 M	5mM	250 µl
3. KCl	1.0 M	30mM	1.5 ml
4. PEP	10 mM	0.1mM	480 µl
5. ATP	solid	0.2mM	6 mg
6. LDH (B&M 106984)	5 mg/ml	1ug/ml	10 µl
7. PK (B&M 128155)	10 mg/ml	5ug/ml	25 µl
8. NADH	15mM		
9. Cr standard	200uM		
10. Cr standard	500uM		
11. BSA	0.05%		

Reagents 1 to 7 were added to 15 ml of milli-Q water (in a 50ml volumetric flask). This was brought up to volume with milliQ water and mixed.

1 ml of the cocktail reagent is taken out and added to a 10 x 75 mm kimble tube labeled 'reagent blank'. This was used later to set the Turner Fluorimeter at 0, before reading blanks, standards, and samples.

100ul of the concentrated 15mM NADH solution was added the remaining 49 ml of the cocktail (200ul into 99 ml), and mixed well.

Preparation of Creatine Kinase Solution

1. 10 mg of creatine kinase (Sigma C3755) (located in freezer) was added to an 12 x 75 mm kimble tube. 1 ml of 0.05% BSA was added, and mixed by inversion.

NADH calibration

1. An aliquot of 15mM NADH was fully thawed out. 200ul of the 15mM NADH was added to 15 ml of milli-Q water (in a 100ml volumetric flask). This was brought up to volume with milliQ water and mixed.
2. A sample of the diluted NADH solution is added to a microcurvette (1941). This was read on the spectrophotometer @ 340 nm, with water used as the blank. From the absorbance NADH concentration is determined. This must not be below 11mM.

Assay Procedure

1. 10 x 75 kimble tubes were used to run the assay, top of tubes were labeled
2. Blanks (H₂O & reaction mix), creatine standards (200 and 500 μ M) were analysed in triplicates.
3. Samples were analysed in duplicates.
4. 1 ml of the cocktail was added into each tube.
5. 30 μ l of Milli-Q water, standards, and samples was then added to the appropriately labeled tubes and mixed on vortex.
6. Each tube was placed in dark for 15 minutes at room temperature.
7. After incubation, tubes were read on previously warmed up Turner Fluorimeter 365nm absorption, 455 nm emission. Sensitivity setting number 1 (R1).
8. After the reading, 20 μ l of Creatine Kinase solution was added to each kimble tube, and mix on vortex.
9. Each tube was placed in dark for 60 minutes at room temperature.
10. After incubation they were all read on fluorometer on same setting (R2)
11. Each tube was read again 15 minutes later (R3). This is necessary, as the reaction is not at end point reaction. That is blank may tend to run.