Influence of antioxidant supplementation on recovery from muscle damaging exercise

INFLUENCE OF ANTIOXIDANT SUPPLEMENTATION ON RECOVERY FROM MUSCLE DAMAGING EXERCISE

by

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Abstract

Exercise that is both unaccustomed and predominantly eccentric is commonly associated with damage to skeletal muscle that results in a delayed sensation of soreness and period of muscular dysfunction. It is also established that strenuous exercise is associated with a profound elevation in the generation of free radicals. The deleterious effects of these highly reactive molecules have been implicated in exercise-induced muscle damage and are believed to directly contribute to the symptoms of this physiological response to exercise. Although, regular exposure to physical activity results in adaptations to endogenous defence from free radicals this affords limited protection prior to a bout of strenuous or unaccustomed exercise. Subsequently, much attention has focused on the potential ameliorative role dietary antioxidant supplementation may play in reducing the detrimental effects associated with exercise-induced muscle damage. The series of investigations that follows attempts to elucidate this proposed protective role.

It is suggested that damage to skeletal tissue is the result of disruptions to weak sarcomeres that are maximally stressed during force lengthening eccentric contractions and/or predominantly recruited with unaccustomed exercise. The initial investigation showed that downhill running, which is both unaccustomed and eccentric based, resulted in considerable muscle damage and soreness compared to concentric and more familiar level running (Chapter 4). Attempts to reduce this response with vitamin C supplementation prior to downhill running resulted in modest prophylactic effects on some indices of muscle damage (Chapter 5). Therefore, it was hypothesised that a more strenuous mode of exercise would elicit a more profound degree of oxidative damage. Subsequently, vitamin C supplementation showed beneficial effects on indices of muscle damage as well as recovery of normal muscular function following prolonged intermittent shuttle running (Chapter 6). These positive outcomes were not enhanced when a combination of antioxidants were used in the final investigation (Chapter 7). Nevertheless, the evidence from these investigations supports the use of antioxidant supplementation, specifically vitamin C, in reducing the detrimental effects associated with exercised-induced muscle damage.

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Abbreviations

List of abbreviations contained in this thesis. Abbreviations are also defined in the text in the first instance.

AA	ascorbic acid
ADP	adenosine diphosphate
AK	adenylate kinase
ALT	alanin aminotransferase
AMP	adenosine monophosphate
AST	aspartate aminotransferase
AT	anaerobic threshold
A_T	antioxidant
ATP	adenosine triphosphate
Ca^{2+}	calcium
CAT	catalase
CK	creatine kinase
CK-MM	skeletal muscle isoenzyme of creatine kinase
CK-MB	skeletal and cardiac muscle isoenzyme of creatine kinase
CK-BB	brain tissue isoenzyme of creatine kinase
CO_2	carbon dioxide
COX	cyclo-oxygenase
CRP	C-reactive protein

cTnI	cardiac troponin I
cTnT	cardiac troponin T
Cu	copper
DELFIA	dissociation enhanced lanthanide fluorescence immunoassay
DH	downhill
DNA	deoxyribonucleic acid
DOMS	delayed onset muscle soreness
EDTA	ethylenediaminetetraacetic acid
EIMD	exercise-induced muscle damage
ELISA	enzyme linked immunosorbant assay
EMG	electromyography
ESR	electron spin resonance spectroscopy
FABP	fatty acid binding protein
Fe	iron
G-6-P	glucose-6-phosphate
GLUT	glucose membrane transporter
GPX	glutathione peroxidase
GSH	glutathione (reduced)
GSSG	glutathione (oxidised)
GRX	glutaredoxin
H_2O_2	hydrogen peroxide

HOCl	hypochloric acid
HPLC	high performance liquid chromatography
HR	heart rate
HRmax	maximum heart rate
HSP	heat shock protein
IL	interleukin
IMP	inosine monophosphate
IMPE	immediately post exercise
LDH	lactate dehydrogenase
LDL	low density lipoprotein
LFF	low frequency fatigue
LIPOX	lipoxygenase
LIST	Loughborough intermittent shuttle test
LPO	lipid hydroperoxide
LVL	level
Mb	myoglobin
MDA	malondialdehyde
MHC	myosin heavy chain
MNC	mononuclear cells
MPO	myeloperoxidase
MRI	magnetic resonance imaging

MSFT	multi-stage fitness test
MVC	maximal voluntary contraction
NAC	n-acetyl-cysteine
NAD^+	nicotinamide-adenine dinucleotide (oxidised form)
NADH	nicotinamide-adenine dinucleotide (reduced form)
NC	no change
NE	no effect
$NO^{\cdot}$	nitric oxide
NSAIDs	non-steroidal anti-inflammatory drugs
O_2	oxygen
$O_2^{\cdot-}$	superoxide
OH	hydroxyl
$ONOO^-$	peroxynitrite
PE	post-exercise
PGE_2	prostaglandin E_2
P_L	placebo
P-LA_2	phospholipase A_2
PMN	polymorphonuclear cell
Pt	pre-treatment
PUFA	polyunsaturated fatty acid
RDA	recommended daily allowance

RER	respiratory exchange ration
RNS	reactive nitrogen species
ROM	range of motion
ROS	reactive oxygen species
SH	sulphydryl
SOD	superoxide dismutase
SR	sarcoplasmic rectilium
sTn1	skeletal troponin 1
TAS	total antioxidant status
TBARS	thiobarbituric acid-reactive substances
TNF	tumour necrosis factor
TRX	thioredoxin
UA	uric acid
$\dot{V}_E$	minute ventilation
$\dot{V}CO_2$	carbon dioxide production
$\dot{V}O_2$	oxygen uptake
$\dot{V}O_2max$	maximal oxygen uptake
V_C	vitamin C
WBC	white blood cells
XDH	xanthine dehydrogenase
XO	xanthine oxidase

CHAPTER ONE

Introduction

Anyone who has performed physically strenuous exercise is aware of the painful consequences of exertion. Muscular pain can be both acute and delayed, the distinction between both was first made by Hough (1902) who suggested that the pain experienced during and immediately following exercise was linked to fatigue whereas the delayed pain was not. This observation was insightful in recognising the separate mechanisms by which pain or soreness is felt under these two circumstances. Since these early observations it has been established that the delayed soreness is commonly associated with unfamiliar exercise that often contains a predominance of eccentric muscle lengthening actions. This delayed soreness increases during the post-exercise period, peaking in the days following exercise. The exact aetiology underlying this exercise induced soreness is believed to be associated with ultrastructural damage to the contractile apparatus of muscle. Several studies have documented that strenuous, unaccustomed exercise damages muscle cellular structures and disrupts the extracellular matrix (Friden *et al.*, 1983; Newham *et al.*, 1983; Friden, 1984; Jones *et al.*, 1986). These disruptions may initiate a sequence of cellular events that include a loss of membrane integrity and a failure of normal cellular homeostasis resulting from in the influx of extracellular calcium, as well as an impaired ability to remove intracellular calcium stores (Duncan & Jackson, 1987; Allen, 2001). Disturbances in normal cellular function initiate degenerative pathways that ultimately result in muscle autolysis, increased membrane permeability and inhibition of excitation-contraction coupling.

Due to the limitations in directly assessing skeletal muscle damage indices, indirect rather than direct measurement, of these disturbances are monitored. Increases in circulating concentrations of myofibrillar proteins along with changes in muscular function are commonly employed along side subjective ratings of soreness to quantify the extent of injury to contractile tissue (Warren *et al.*, 1999). The characteristic delayed sensation of soreness and associated prolonged muscular dysfunction are thought to be a result on ongoing damage that represents a regenerative process (MacIntyre *et al.*, 1996). Much debate exists on the precise aetiology of this repair and adaptive response, but it is generally accepted that initial

disruptions lead to an acute immune response that is primarily concerned with the repair and regeneration of damaged tissue (Smith, 1991; Pyne, 1994). The by-products of this response are believed to result in the commonly reported symptoms of muscular soreness and also contribute to ongoing damage that is reflected in the delayed recovery of normal muscular function.

Free radicals have been implicated in a variety of pathological disorders (Halliwell & Gutteridge, 1989). Exercise induces large elevations in the production of these highly reactive molecules and direct evidence of this has recently been observed in contracting skeletal muscle (Bailey *et al.*, 2003). It has been proposed that muscle damage is the consequence of an exercise-induced oxidative stress (Sjodin *et al.*, 1990; Alessio, 1993; Jackson, 1996). Furthermore, evidence that the generation of these reactive species is ongoing following exercise has lead authors to suggest that these molecules are responsible for the exacerbation of exercise-induced muscle damage (Zerba *et al.*, 1990; Best *et al.*, 1999). However there is an elaborate endogenous antioxidant defence system that is up regulated with continual exposure to exercise-induced oxidative stress (Powers *et al.*, 1999). It is proposed that free radical generation may overwhelm this endogenous system (Kanter *et al.*, 1993; Child *et al.*, 1998) during and following (Maughan *et al.*, 1989; McBride *et al.*, 1998) a bout of prolonged strenuous exercise. Subsequently, the demand for dietary antioxidants (vitamin C and E) is increased under these conditions (Goldfarb, 1999; Clarkson & Thompson, 2000). Thus, much research has focused on the ameliorative role of these dietary antioxidants on the deleterious effects of free radical generation following strenuous exercise. Vitamin E has been shown to reduce indices of muscle damage following demanding exercise in both animals (Jackson *et al.*, 1983) and humans (Cannon *et al.*, 1990; Meydani *et al.*, 1993; McBride *et al.*, 1998). Its chain breaking properties in lipid peroxidation would appear to support the rationale for inhibiting continued membrane degradation following unaccustomed or eccentric exercise. Vitamin C supplementation has also been investigated as a therapeutic means of abrogating indices of muscle damage and oxidative stress with positive outcomes (Jakeman & Maxwell, 1993; Thompson *et al.*, 2001). Although not specifically located within lipid membranes the hydrophilic nature of this antioxidant along with its proposed role in the regeneration of

vitamin E perhaps highlight a greater potential for attenuating exercise induced oxidative damage (Packer, 1997). It seems, therefore, that the role of free radicals in exercise-induced muscle damage is significant and that there is considerable scope for supplementation with dietary antioxidants to alleviate this disruptive response.

The detrimental effects associated with exercise-induced muscle damage are not just confined to symptoms of soreness and muscular dysfunction. In fact subsequent exercise performance following and initial bout of damaging exercise can be impaired (Gleeson *et al.*, 1995). Also, there is evidence that muscle glycogen replenishment is inhibited following eccentric exercise (O'Reilly *et al.*, 1987). Therefore, there is a potential requirement for a therapeutic modality such as dietary antioxidants in alleviating the symptoms associated with exercise-induced muscle damage, facilitating improved recovery from strenuous exercise and enabling unimpaired performance of subsequent physical activity. The series of investigations that follow attempt to first confirm the precise nature of exercise responsible for muscle damage (Chapter 4) and attempt to abrogate this exercise-induced phenomenon with vitamin C supplementation (Chapters 5 and 6) and a combination of antioxidants (Chapter 7). Initial attempts to identify the potential role of dietary antioxidants focus on vitamin C supplementation prior to the damaging exercise model used in the first investigation (Chapter 5) and a mode of exercise that reflects the demands of more popular sports activities (Chapter 6). The final investigation employed a combination of antioxidants following this intermittent exercise protocol (Chapter 7). How the information obtained from this series of investigations contribute to a better understanding of muscle soreness and muscle damage is reviewed in the General Discussion.

CHAPTER TWO

Review of Literature

2.1 Introduction

The sensation of muscle soreness is perhaps one of the most frequently reported side-effects associated with physical activity. It is not only confined to the sedentary population, who report severe soreness after a novel bout of exercise, but it is often observed in very active, well trained athletes when they perform unaccustomed modes of exercise training. Believed to be a consequence of damage to skeletal muscle, this soreness is often considered a negative factor but perhaps represents an element of the highly complex physiological response to activity and initiates adaptive changes as subsequent exercise results in markedly reduced soreness. Unfortunately, muscle soreness can be a deterrent to those attempting to increase their physical activity levels, and may impair athletic performance in the more highly trained. Hence, a method of alleviating this physiological response to novel activity without inhibiting the adaptive process would be beneficial to all.

2.2 Exercise-induced muscle damage (EIMD)

Exercise that is either unaccustomed or contains a significant element of lengthening 'eccentric' contractions leads to severe muscle soreness and associated impairment of normal muscular function. This phenomenon was first documented by Hough (1902) over a century ago, when he reported a sensation of soreness following repeated eccentric contraction of a finger against a spring. Interestingly, he reported that this soreness was not initially apparent, but developed over the hours following exercise. The delayed soreness has since been established as the most frequently reported symptom following strenuous or unaccustomed exercise, and is commonly referred to as delayed onset muscle soreness (DOMS). The underlying physiological processes that ultimately result in DOMS and associated temporal muscular dysfunction are still to be fully elucidated. Many propose that ultrastructural damage

to the contractile and surrounding connective tissue initiates a sequence of events that result in these symptoms.

2.2.1 Aetiology of exercise-induced muscle damage

Knowledge of the mechanisms attributed to EIMD has progressed considerably from Hough's early work but is still by no means comprehensive. With improved scientific techniques, including muscle biopsy and electron microscopy, research has confirmed the existence of disruptions to the muscle contractile apparatus. However, some suggest this damage is only the precursor to a sequence of events that lead to reductions in normal muscular function along with symptoms of stiffness and pain in the damaged tissue.

2.2.2 Morphological changes

In the early 1950's Asmussen first identified that DOMS was directly associated with the eccentric component of exercise (Asmussen, 1952). Support for his work is found when forced production is assessed during eccentric muscle contraction. During eccentric contractions tension developed by the active muscle produces less torque than that caused by the external resistance and subsequently the muscle is forced to lengthen (Lakomy, 1996). However, as the muscle produces less tension than during concentric actions, the cost of eccentric work is less, and far fewer motor units are recruited (Friden & Lieber, 2001). Therefore, a smaller cross-sectional area of muscle is employed to transfer the tension when compared to concentric actions (Enoka, 1996). Subsequently, failure of the contractile apparatus is more likely to occur under eccentric actions. Thus, most exercise modes employed to investigate the structural changes associated with muscle damage are either eccentric contractions of specific muscle groups (Friden *et al.*, 1983b; Cleak & Eston, 1992b; Brown *et al.*, 1996; Brown *et al.*, 1997; Gleeson *et al.*, 1998; Chen & Hsieh, 2000; MacIntyre *et al.*, 2000) or predominately eccentric based exercise (Armstrong *et al.*, 1983; High *et al.*, 1989; Jakeman & Maxwell, 1993; Meydani *et al.*, 1993; Eston, 1996; Sorichter *et al.*, 2001a).

Evidence to support the predominance of eccentric contractions as a stimulus for muscle damage has been obtained from investigations employing direct comparisons between concentric and eccentric work in the same subjects (Armstrong *et al.*, 1983; Schwane *et al.*, 1983) or using contralateral controlled investigations during which one limb performs eccentric work whilst the other works concentrically (Newham *et al.*, 1983a; Newham *et al.*, 1983b; Newham *et al.*, 1983c). Schwane *et al.* (1983) directly compared running for 45min on a level gradient (concentric/eccentric) with the same duration on a negative gradient (eccentric). Despite the greater metabolic cost of level running greater muscle damage, measured as myofibrillar protein efflux, was observed following the downhill run.

Although skeletal muscle is considered extremely plastic, destructive changes occur in response to unusual demands placed on myofibrils. The majority of morphological studies indicate that the Z disk is the most vulnerable structure to eccentric contraction induced muscle injury (Friden *et al.*, 1981; Armstrong *et al.*, 1983; Friden *et al.*, 1983b; Newham *et al.*, 1983b). Light microscopy of muscle tissue samples taken following eccentric exercise reveals disruption to sarcomeres, specifically at the sites where the thick, myosin, filaments attach to the Z disk. Often referred to as 'Z disk streaming or smearing' the damage is specific to this region as force transduction is considered greatest here during muscle lengthening contractions (Friden *et al.*, 1981; Ebbeling & Clarkson, 1989). Furthermore, animal studies have identified a loss of a fundamental cytoskeleton protein called desmin in the minutes following the onset of eccentric exercise, as well as an influx of the extracellular protein fibronectin (Lieber *et al.*, 1996; Friden & Lieber, 1997). Desmin is of particular importance as it is the connecting intermediately filament between Z-disks of adjacent sarcomeres (Friden & Lieber, 2001). However, recent human studies have failed to demonstrate any change in desmin following eccentric exercise (Yu *et al.*, 2002). Additionally, the intra-sarcomeric protein titin responsible for connecting myosin filaments to the Z disk is another postulated site of ultrastructural damage (Friden & Lieber, 2001). Damage has also been observed in the sarcolemma, T tubules, myofibrils and the cytoskeletal system (Friden *et al.*, 1984; Duan *et al.*, 1990; Byrd, 1992; Sam *et al.*, 2000; Allen, 2001; Takekura *et al.*, 2001). Figure 2.1 shows Z-disk damage following a marathon.

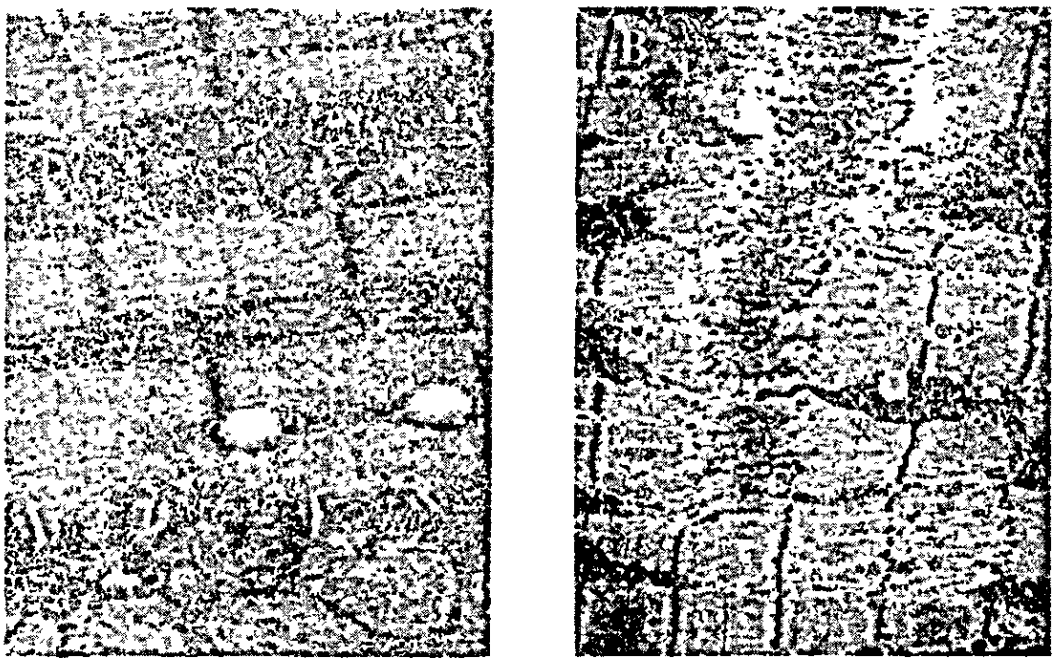


Figure 2.1: Disruptions to normal muscular ultrastructure following a marathon race (Hagerman *et al.*, 1984). Image A shows normal sarcomere structure with intact Z-disk pre-exercise. Image B shows extensive disruption to sarcomeres in the Z-disk region.

Morgan first identified that eccentric contractions lead to an overstretching of sarcomeres termed "sarcomere popping" (Morgan, 1990). He proposed that when A.V.Hill's model for the properties of sarcomeres was applied to muscle contracting on the descending limb of the force-length curve the active lengthening of sarcomeres was non-uniform. Uncontrolled lengthening of individual sarcomeres results in overextension of sarcomeres beyond the point of filament overlap. Most sarcomeres reinterdigitate upon relaxation, however the weakest may become permanently overstretched (Talbot & Morgan, 1996). With repeated contractions the number of weakened or overstretched sarcomeres gradually increases (Allen, 2001). Muscle biopsies taken from humans post eccentric exercise do not reflect a homogenous distribution of damage but a more random pattern (Friden *et al.*, 1983b; Friden & Lieber, 1992; Beaton *et al.*, 2002). This has been attributed to inhomogeneity of sarcomere lengths in myofibrils, which may lead to variations in sarcomere strength. Such small variations are not

noticeable at rest due to the passive force in the myofibrils, but are apparent during eccentric contraction when the force generated is greatest (Katz, 1939). Disruptions to these fundamental components of the excitation-contraction coupling system have pronounced effects on normal muscular function. Katz (1939) was the first to propose that damage to individual sarcomeres did not directly inhibit force production in a series of sarcomeres. Rather, the damaged sarcomeres increased the series compliance which is observed as a rightward shift in the force-length curve (Katz, 1939; Morgan *et al.*, 1996; Proske & Morgan, 2001).

Authors have identified a bias towards type II muscle fibres as being the most susceptible to contractile injury following eccentric exercise (Friden *et al.*, 1983b; Jones *et al.*, 1986; Lieber & Friden, 1993, 1998; McHugh *et al.*, 1999). In the days following damaging exercise fibres that stained heavily with ATPase were predominantly disrupted, indicative of a tendency for type II fibre injury (Lieber & Friden, 1993). Specific techniques are needed to identify the subpopulation of fibres type predisposed to this contraction induced damage (Friden & Lieber, 1997). However, some make the valid point that perhaps the nature of exercise performed is more important when attempting to determine the subpopulation damaged (Proske & Morgan, 2001).

The extent of damage does vary considerably when directly comparing animal and human models (Lieber *et al.*, 1996; Yu *et al.*, 2002). Severe morphological disruption to contractile tissue is most frequently observed in rodents and other animal models. However, ultrastructural damage is inconsistently reported humans (Friden *et al.*, 1983b; Yu *et al.*, 2002). Explanations for these differences are limited but some suggest varying absolute workloads or perhaps an alternative aetiology of ultrastructural damage (Yu *et al.*, 2002). Furthermore, inconsistencies are often observed with varying exercise modes perhaps reflecting a limitation of the muscle biopsy technique when attempting to quantify muscle damage that is not homogeneously distributed. The more strenuous exercise modes often result in severe cellular disruption.

Extensive morphological analysis of muscle tissue has identified severe disruptions to myocellular ultrastructural components and it is generally accepted that mechanical stresses initiate this injury. However, many authors identify this as merely the primary step in a sequence of destructive events that are considered more metabolically based.

2.2.3 Metabolic mechanisms

It has long been established that exercise leads to sizeable perturbations in normal cellular metabolism that are directly linked with exercise duration and intensity. Many authors have identified such metabolic disturbances as the stimulus for ultrastructural damage following strenuous prolonged exercise. Some have reported that hypoxia, ischaemia and waste product accumulation (e.g. lactic acid, Ca^{2+} and hydrogen phosphate) following prolonged or high intensity exercise may lead ATP deficiencies that explain muscle damage (De Vries, 1966; Armstrong, 1984, 1986; Byrnes & Clarkson, 1986; Cleak & Eston, 1992a). Additionally, the lay exercising community commonly attributed the sensation of soreness associated with acute exercise to lactic acid accumulation in the muscles. However, if this were the case then surely concentric exercise, which results in greater post-exercise waste product accumulation, would be more frequently associated with muscle damage as opposed to eccentric work. Direct comparisons between concentric and eccentric based exercise provide support for additional mechanisms other than acute metabolic perturbations for the underlying cause of EIMD (Newham *et al.*, 1983b; Schwane *et al.*, 1983).

Although it is generally accepted that EIMD is mechanical in origin, this injury to the contractile apparatus may initiate a number of metabolic events that exacerbate existing disruptions to cellular homeostasis (Armstrong, 1984). Metabolic stresses characterised by disturbances in cellular mechanisms usually occur after prolonged high intensity or exhaustive exercise. As mentioned, these stresses include insufficient rate of ATP resynthesis, ischaemia, hypoxia, ionic imbalance and accumulation of metabolic by-products. Such stresses may ultimately lead to disruptions in normal cellular function and the most significant implication in muscle cells, a loss of calcium homeostasis. Additionally, ischaemia and subsequent

reperfusion results in temporary hypoxia that may inhibit ATP resynthesis but has also been implicated in free radical production (McCord, 1985; Ambrosio & Tritto, 1999) discussed in section 2.7.

Disruption to the cytoskeletal system of myofibrils has profound consequences on ion concentrations, most significantly calcium (Ca^{2+}). As this particular molecule plays an integral role in excitation-contraction coupling, failure of Ca^{2+} pumps to maintain the concentration gradient either through structural damage or inhibited ATP resynthesis may lead to a loss of Ca^{2+} homeostasis (Jackson *et al.*, 1984; Armstrong *et al.*, 1991). Armstrong (1991) identified this disturbance to normal cellular function as the 'overload phase' during which increases in intracellular Ca^{2+} may initiate a number of indigenous Ca^{2+} proteolytic and phosopholipolytic pathways. Interestingly authors have identified a Ca^{2+} dependent protease called calpain that acts on desmin following eccentric exercise (Belcastro *et al.*, 1998). This provides direct evidence for the underlying cause of sarcomere disruption, namely Z disk streaming.

Damage to the sarcoplasmic rectilium (SR) has been linked to in impaired Ca^{2+} pump function and subsequent disturbances in Ca^{2+} homeostasis in investigations that have controlled intra and extra-cellular Ca^{2+} (Jones *et al.*, 1984; Duncan, 1987). Furthermore, in animal studies increases in mitochondrial and cytosolic Ca^{2+} have been reported with eccentric based exercise modes (Duan *et al.*, 1990; Warren *et al.*, 1995). One interesting point is that increases in intracellular Ca^{2+} are commonly observed as a fundamental mechanism preceding crossbridge formation in excitation-contraction coupling. Under these conditions Ca^{2+} dependent proteases are not activated. It is proposed that these autogenic enzymes are compartmentalized within the SR and that mechanical disruption to sarcomeres may result in increased Ca^{2+} in these specific regions (Armstrong *et al.*, 1991). This theory may also support apparent random distribution of damage to sarcomeres within a myofibril. Recently, Overgaard and co-workers (2002) reported increases in cellular Ca^{2+} following prolonged running (100km) in human subjects. They observed an associated failure of the transmembrane sodium-potassium pumps as well as efflux of the muscle enzyme creatine kinase (Overgaard *et al.*, 2002).

The deleterious implications of elevated intracellular Ca^{2+} included; i) activation of lysosomal and non-lysosomal proteolytic enzymes, such as calpain, ii) inhibition of mitochondrial function, iii) hypercontraction or contracture of sarcomeres and, iv) activation of phospholipases such as phospholipase A_2 (PLA_2). Support for these processes can be obtained from pathological conditions such as muscular dystrophy or malignant hyperthermia, in which loss of Ca^{2+} leads to severe muscle autolysis (Armstrong *et al.*, 1991; McArdle & Jackson, 1997). Interestingly, PLA_2 uses membrane phospholipids as a substrate to produce arachidonic acid which is then metabolised via the cyclo-oxygenase (COX) pathway to produce prostaglandins and thromboxanes. Prostaglandins of the group E_2 are implicated in increased vascular permeability and pain perception by sensitising nerve afferents to chemical and mechanical stimuli. Prostaglandin E_2 is therefore commonly linked to the sensation of soreness following muscle damaging exercise (Smith, 1991; Pedersen & Hoffman-Goetz, 2000). Alternatively, arachidonic acid can be metabolised via the lipoxygenase (LIPOX) pathway to produce leukotrienes that are proposed to increase vascular permeability and via chemotaxis leading to leukocyte infiltration and an acute phase response (Pyne, 1994a; MacIntyre *et al.*, 1995; Pedersen & Hoffman-Goetz, 2000). Also, increased protease and phospholipase activity is directly related to the efflux of muscle specific proteins from the muscle cell due to increased membrane permeability (Balnave & Thompson, 1993; McArdle & Jackson, 1997) discussed in section 2.3. Evidence for the role of these processes in the aetiology of EIMD is obtained from investigations in which non-steroidal anti-inflammatory drugs (NSAIDs) were administered in an attempt to inhibited these pathways and alleviate muscle soreness (see section 2.4.1).

2.3 Indices of muscle damage

The "gold standard" for assessing muscle damage following exercise is still considered to be direct histological evidence obtained from muscle biopsy samples. Unfortunately, this is often impractical due to the invasive nature of tissue sampling and also it is difficult to gain an adequate representative sample due to the inhomogeneous distribution of muscle damage. Also, some argue that this sampling technique results in tissue injury which may be difficult to differentiate from existing damage (Malm *et al.*, 2000). More complex modern medical techniques including magnetic resonance imaging or spectroscopy are probably considered the most accurate methods (Shellock *et al.*, 1991; Mair *et al.*, 1992; Sorichter *et al.*, 2001b), but can be expensive and are therefore not accessible to a majority of investigators. The use of radioisotope uptake to identify muscle damage has been reported, but this technique is consistently associated with artifacts making interpretations difficult (Newham *et al.*, 1986; Cox *et al.*, 1997). Other techniques include simple subjective scales used to assess muscular soreness, or attempting to quantify functional parameters (muscular strength and mobility) and more commonly biochemical parameters, such as measuring the presence of enzymes or other proteins in blood that are specific to skeletal muscle.

2.3.1 Myofibrillar protein leakage

Creatine kinase (CK) is an intramuscular enzyme responsible for maintaining adequate ATP levels during muscle contractions. Its appearance in the systemic circulation is interpreted as an increased permeability or autolysis of the membrane surrounding skeletal or cardiac myocytes (Newham *et al.*, 1983a; Armstrong *et al.*, 1991; Clarkson & Sayers, 1999; Sorichter *et al.*, 2001b). Indeed, increased CK activity is an established indicator of myocardial infarction in patients that are suspected to have suffered a heart attack (Cairns *et al.*, 1983; Noakes, 1987). During initial attempts to quantify changes in CK activity as an index of myocardial infraction, investigators were forced to take caution when interpreting measurements as similar increases were often observed following physical activity. The first

documented evidence that CK activity increased post-exercise was reported in individuals following a variety of activities (Vejjaajiva & Teasdale, 1965).

The efflux of CK and other myofibrillar proteins is predominately influenced by exercise duration as opposed to exercise intensity (Noakes, 1987). Subsequently, prolonged strenuous weight bearing activities result in large changes (Armstrong, 1986; Margaritis *et al.*, 1999; Thompson *et al.*, 1999). However, specific eccentric based exercise is also associated with large increases in serum CK activity (Koller *et al.*, 1998; Sorichter *et al.*, 2001b). The extent of the delayed increase in CK activity depends upon the type of exercise performed. After downhill running, bench stepping, intermittent shuttle running or isometric exercise CK activity significantly increases between 3 to 6h and usually peaks 18 to 24h after cessation of exercise (Newham *et al.*, 1983a; Byrnes *et al.*, 1985; Clarkson *et al.*, 1986; Thompson *et al.*, 1999). Following local muscular eccentric exercise a significant increase in CK activity may not occur until 48h after exercise and may not reach peak values until 7 days post-exercise (Jones *et al.*, 1986; Newham *et al.*, 1987; Clarkson & Tremblay, 1988). The longer delay in the appearance of CK in the blood following high force eccentric contractions has been suggested to be coincident with necrosis of muscle fibers (Clarkson & Tremblay, 1988).

Newham and colleagues (1983) reported a large delayed increase in 8 out of 16 individuals following 20min of bench stepping, during which one leg performed the eccentric phase (step down) whilst the other performed the concentric phase (step up). Increases were greatest between 4 and 5 days post-exercise and were associated with tenderness in the eccentrically worked limb (Newham *et al.*, 1983a). Interestingly, the authors used a mixed subject sample but did not report any effect of gender. Sorichter and colleagues (2001) observed higher plasma concentrations of myofibrillar proteins including CK following a bout of downhill running in males compared to females. However, the authors attributed this variation to differences in muscle mass in male subjects and these differences were removed when CK was expressed relative to body mass (Sorichter *et al.*, 2001a). A prophylactic effect of oestrogen on muscle damage and thus plasma CK activity has been proposed to explain variations in the CK

response to exercise (Munjal *et al.*, 1983). Bar and colleagues (1988) However, some suggest that this effect is only evident at rest (Arnett *et al.*, 2000; Kendall & Eston, 2002).

In a cross-over design Schwane *et al.* (1983) observed a larger increase in CK activity 24h following 45min of running downhill compared to running for the same duration on a level gradient. This particular investigation supports the duration rather the intensity dependent response as the relative exercise intensity for downhill and level running were 57% and 78% $\dot{V}O_2max$ respectively. The variation in intensity was attributed to the lower metabolic cost of the predominantly eccentric downhill running (Schwane *et al.*, 1983). Further evidence to support predominance of eccentric contractions was that the sensation of soreness was only reported in the lower limb muscles in the days following the downhill run. Tiddus and Ianuzzo (1983) presented evidence that the intensity of exercise as opposed to the duration is responsible for the extent of CK release following exercise. However, they varied duration and intensity during eccentric leg extension by controlling the load (percentage of 1 repetition max) and the number of contractions (Tiidus & Ianuzzo, 1983). It could be argued that this is perhaps not comparable to previous prolonged whole body exercise modes where duration is the influencing factor.

One other phenomenon frequently reported when assessing muscle damage using CK activity is profound inter-individual variation. The underlying cause for such variations, that often masks significant changes in CK activity post-exercise, are still not fully established. Some have hypothesised that training status may influence serum CK activity at rest and subsequently exercise induced changes will be more pronounced in the untrained (Noakes, 1987; Balnave & Thompson, 1993). Also, CK response to damaging exercise is reduced with repeated bouts a thus may lead to further artifacts (see section 2.5). Ageing is also associated with an elevated resting serum CK activity that is considered to be linked to muscular atrophy and an increased number of degenerative, damage susceptible fibers (Noakes, 1987). Clarkson and Ebbeling (1988) attempted to identify the existence of CK inhibitors in the serum of individuals classed as "low responders" as a possible explanation for variations between individuals. They mixed sera from these individuals with sera from "high responders"

following eccentric forearm flexion but failed to report any indication of CK inhibitors (Clarkson & Ebbeling, 1988). Inhibitors have however been identified in individuals with pathological muscle disorders (Kagen & Aram, 1987). Whitfield and Martin (1986) identified 60% of the variation as genetic in origin after studying the CK response in 206 twins. They also identified larger variations in male subjects in response to environmental factors. Conversely, recent work discounted genetic factors as a cause for variability in markers of muscle damage following eccentric elbow flexion in identical twins (Gulbin & Gaffney, 2002). It is often overlooked that blood CK activity is merely an indication of the clearance as well as appearance of this protein molecule and also that three isoenzymes exist. Skeletal muscle specific CK-MM accounts from 90 to 100% of the exercise induced increase in total CK activity. CK-MB is also elevated in the blood following prolonged eccentric based exercise and is the isoenzyme used in diagnosis of myocardial infraction as it is present in both skeletal and cardiac muscle. The third form is CK-BB that is exclusive to brain tissue and subsequently is unchanged in plasma following exercise. It may be that variations between investigations could be attributed to the specific isoenzyme measured. Subsequently, the inclusion of more than one muscle protein as an indicator of muscle damage is recommended (Noakes, 1987; Warren *et al.*, 1999).

Myoglobin (Mb) is an oxygen carrying protein that is present in muscle tissue as a source of oxygen for aerobic metabolism. However, research into muscle tissue injury, both skeletal and cardiac, has employed changes in serum concentrations of Mb as another marker of disruptions to the sarcolemma (Cairns *et al.*, 1983; Sorichter *et al.*, 1998). Increases in Mb post-exercise are similar in magnitude to CK but are consistently reported to peak earlier, usually within hours as opposed to days (Byrnes *et al.*, 1985; Thompson *et al.*, 2001a; Dawson *et al.*, 2002). Byrnes and co-workers (1985) were amongst the first to employ this muscle protein's appearance as an indicator of muscle damage. In an investigation into the effect of repeated bouts of downhill running they reported peak increases in Mb concentration 6h following exercise compared with 18 to 42h for CK activity. They attributed the earlier appearance of Mb to a more rapid efflux of the Mb molecule, since Mb is a smaller protein (17,800 Daltons) than CK (80,000 Daltons). Sorichter *et al.* (1998) reported a similar response

following 20min downhill running during which Mb concentrations peaked within 6h and returned to pre-exercise values at 24h at which point CK activity remained elevated. Their findings suggest that Mb, in comparison with CK activity, is a more useful indicator of muscle injury in response to a recently performed exercise bout. Kyröläinen and colleagues (1998) attempted to identify changes in Mb concentrations following stretch-shortening exercise (200 drop jumps) in endurance and power trained individuals. Increases in CK activity and Mb were more pronounced following exercise in the endurance trained individuals. The differences were attributed to the greater proportion of type I fibres in endurance trained individuals. The authors postulate the predominance of type II fibres recruitment during stretch-shortening exercise may elicit greater damage in endurance individuals with more damage susceptible type II fibres. More obviously, the power trained individuals showed a more efficient recruitment of motor units (measured as EMG ratio to eccentric and concentric actions) and therefore may be more accustomed to this model of damaging exercise.

As with CK activity, peak increases are more delayed (24-72h) following local eccentric contractions with specific muscle groups (Rodenburg *et al.*, 1993; Croisier *et al.*, 1996; Nosaka *et al.*, 2001b). This perhaps provides further support for a predominantly mechanical based aetiology of muscle injury with this more localised exercise.

Other myofibrillar proteins measured following exercise include lactate dehydrogenase, myosin heavy chain fragments, skeletal troponin 1, fatty acid binding protein and glutamatic-oxaloacetic transaminase. All increase following strenuous or damaging exercise but vary in the time to peak concentration/activity in the circulation (see Table 2.1). Sorichter *et al.* (1997) directly compared a number of myofibrillar proteins following a variety of exercise protocols. Unsurprisingly they reported the greatest increases following the eccentric based protocols, but also documented increases in muscle specific proteins, myosin heavy chain (MHC) and skeletal troponin 1 (sTn1). Subsequently, they advocated the use of these muscle specific markers in conjunction with the more commonly employed cytoplasmic makers (CK and Mb) when attempting to quantify muscle damage following exercise.

The exact mechanism responsible for the efflux of these myofibrillar proteins into the circulation is still to be fully elucidated. Original research into myofibrillar protein leakage proposed that increases were related to anoxic or hypoxic damage to the sarcolemma. It has since been established that increases are more profound following predominately prolonged aerobic exercise discrediting this early theory. More recent ideas suggest damage to the muscle ultrastructure associated with weight bearing exercise that contains a large component of eccentric contractions. Elevations in serum myofibrillar proteins following exercise that is either non-weight bearing or has a limited eccentric element might be explained by alterations in membrane permeability. Some suggest glycogen depletion, intracellular acetyl-CoA accumulation or possibly lipid peroxidation as potential mechanisms.

Table 2.1 summarises a selection of investigations into EIMD that have employed myofibrillar protein efflux as indices of damage to skeletal muscle following a variety of exercise protocols. This list is by know means comprehensive but reflects the widespread use of myofibrillar proteins as indirect markers of muscle damage with a variety of exercise modes and sampled populations.

Table 2.1: A summary of investigations employing myofibrillar protein efflux as indices for contraction induced muscle damage. (CK, creatine kinase; LDH, lactate dehydrogenase; G-6-P, glucose-6-phosphate dehydrogenase; IMPE, immediately post-exercise, MNC, mononuclear cells; CK-MB, creatine kinase isoenzyme; Mb, myoglobin; PE, post-exercise; cTnI, cardiac troponin I; cTnT, cardiac troponin T, PMN, polymorphonuclear cell; MHC, myosin heavy chain; AST, aspartate aminotransferase; ALT, alanin aminotransferase; Ca^{2+}, calcium; MRI, magnetic resonance imaging; FABP, fatty acid binding protein; NC, no change).

Reference	Sample	Exercise mode	Myofibrillar proteins assessed	Outcomes	Comments
Armstrong *et al.* (1983)	Rodents	90min run on varying gradients (0, +16, -16%)	CK, LDH, G-6-P dehydrogenase	↑ CK, LDH, G-6-P IMPE delayed ↑ with negative gradient (1.5h – 2days)	↑ G-6-P associated with MNC infiltration, predominately type I fibre disruption
Arnett *et al.* (2000)	♀ n=30	eccentric leg extension	CK, CK-MB	↑ CK, CK-MB (24h)	no effect of oestradiol (groups varied in menarchial status).
Bailey *et al.* (2000)	♂ n=19	maximal exercise (sea level, altitude)	CK, Mb, cTnI	↑ CK, Mb at rest & PE at altitude, NC in cTnI	observed relationship between Mb and oxidative stress at altitude
Balnave & Thompson (1993)	♀ + ♂ n=16	40min walking (-25%)	CK, MB	↑ CK (24h), Mb (4h)	repeated bouts showed no PE ↑ CK and Mb response
Blais *et al.* (1999)	♂ n=13	eccentric leg flexion	CK	↑ CK (12h)	reported associated ↑ bradykinin, hormone linked to pain stimulation
Brown *et al.* (1999)	♀ + ♂ n=8	eccentric leg extension	CK, LDH	↑ CK, LDH (72h)	observed associated increase in indices of connective tissue breakdown
Croisier *et al.* (1996)	♂ n=10	eccentric/concentric isokinetic leg contractions	CK, Mb	eccentric ↑ CK (48h), Mb (48h)	eccentric exercise also resulted in ↑ PMN activation
Koller *et al.* (1998)	♂ n=53	varied gradient running, marathon or ultramarathon	CK, MHC, Mb, cTnT	prolonged run;↑ CK (24h), Mb (0-2h), MHC(24-72h) downhill run; ↑ CK, MHC (7days)	evidence of type I fibre damage greater following predominantly eccentric exercise, no evidence for cardiac damage (cTnT)
Mair *et al* (1992)	♂ n=6	eccentric leg extension	CK, MHC (Type I), Mb, cTnI	↑ CK , Mb, MHC	biphasic increase in Mb, evidence for Type I fibre damage
Margaritis *et al.* (1999)	♂ n=17	triathlon	CK, CK-Mb, Mb, LDH, AST, ALT	↑ CK (6h), CK-MB (6h), Mb (IMPE), LDH (IMPE), AST (24h), ALT (48h)	observed poor relationship with changes in muscular function
MacIntyre *et al* (2001)	♂ n=12	eccentric leg extension	MHC	↑ MHC (72h)	relationship between MHC efflux and indices of immune function

Table 2.1: continued.....

Reference	Sample	Exercise mode	Myofibrillar proteins assessed	Outcomes	Comments
Newham *et al.* (1983)	♀ + ♂ n=16	20min stepping exercise	CK	↑ CK (4-7days)	reported large variations in time course of ↑ CK activity amongst subjects
Newham *et al.* (1988)	♀ + ♂ n=8	repeated bouts of eccentric elbow flexion	CK	↑ CK	observed reduction of ↑ CK response with repeated bouts, no effect of muscle dysfunction
Overgaard *et al.* (2002)	♂ n=10	prolonged running (100km)	CK, LDH	↑ CK, LDH (IMPE)	an associated increase in cellular Ca^{2+}
Shave *et al.* (2002)	♂ n=8	maximal exercise & downhill running (~30min)	CK, CK-MB, cTnI, cTnT	↑ CK & CK-MB (48h)	no indication of myocardial damage PE
Sorichter *et al* (1997)	♀ + ♂ n=61	level, downhill running, isokinetic or eccentric leg extension	CK, Mb, MHC, sTnI	↑ CK (24), Mb (2h), sTnI (6h), MHC (24-48h)	greater ↑ with downhill running, moderate changes with leg extension exercise
Sorichter *et al.* (1998)	♂ n=6	20min downhill running	CK, Mb, FABP, cTnI	↑ CK (2-6h), Mb & FABP (30min)	ratio of Mb to FABP indicative of skeletal muscle concentrations
Sorichter *et al.* (2001)	♂ n=13	eccentric leg extension	CK, MHC, cTnI	↑ CK (24h), MHC (24h)	↑ MHC & CK response associated with ↑ T_2 MRI signal
Sorichter *et al.* (2001)	♀ + ♂ n=18	downhill running	CK, Mb, MHC, sTnI	↑ CK, Mb & sTnI (6h), MHC (24h)	no gender difference when accounting for variations in muscle mass
Thompson *et al.* (2000)	♂ n=16	intermittent shuttle running	CK, AST	↑ CK & CK-MB (24h)	no relationship with muscular soreness
Tiidus & Ianuzzo (1983)	♀ + ♂ n=21	dynamic leg extension	CK, GOT, LDH	↑ CK (8h), GOT & LDH (8-24h)	exercise intensity directly influences ↑ muscle protein release
Totsuka *et al.* (2002)	♂ n=15	90min cycling	CK	↑ CK (48h)	suggest high & low CK responder, plus CK activity breakpoint (300-500IU.L^{-1})

2.3.2 Delayed muscular soreness

Perhaps the most obvious method of quantifying DOMS and indirectly EIMD is through the assessment of muscular soreness. Soreness is commonly assessed using a visual analogue or numerical scale for subjective evaluation (Bobbert *et al.*, 1986; Mair *et al.*, 1992; Balnave & Thompson, 1993; Rodenburg *et al.*, 1993; Thompson *et al.*, 2001a) but in some instances a more objective method, i.e. measuring a force applied to a muscle group at the pain threshold, has been employed (Newham *et al.*, 1983c; Eston, 1996; Bailey *et al.*, 2001; Dannecker *et al.*, 2002). Delayed onset muscle soreness is distinguished from acute post-exercise soreness which is primarily thought to be caused by the accumulation of metabolic waste products (Miles & Clarkson, 1994). Unrelated to the temporary effects of fatigue DOMS is characterised by a dull, aching pain combined with tenderness and stiffness, which usually increases in intensity during the first 24h after exercise, peaking between 24 to 72h (Hough, 1902; Abraham, 1977; Friden *et al.*, 1981; Armstrong, 1984; Bobbert *et al.*, 1986). Attempts to identify the specific mechanisms responsible for this sensation in the days following exercise have not provided conclusive evidence and subsequently a variety of models have been proposed (Cleak & Eston, 1992a; Cheung *et al.*, 2003). Despite the lack of a direct link to muscle injury, soreness is the most commonly used marker of damage in research into the aetiology of EIMD (Warren *et al.*, 1999).

The sensation of pain in skeletal muscle tissue is transmitted by myelinated group III and unmyelinated group IV afferent neurons (Armstrong, 1984; Byrnes & Clarkson, 1986; Ebbeling & Clarkson, 1989). Armstrong (1984) proposed that the unmyelinated group IV fibres, which are more greatly distributed in skeletal muscle, were responsible for the dull aching pain associated with DOMS. He also suggested that these fibres were sensitised by both mechanical and chemical stimuli (metaboceptors) as well as noxious agents (nociceptors). Metabolic products including bradykinin, serotonin, histamine, potassium and prostaglandins have all been linked to neural stimulation and the delayed sensation of muscular soreness (Byrnes & Clarkson, 1986; Smith, 1991; Blais *et al.*, 1999). Subsequently, some attribute the delayed onset of soreness following a bout of muscle

damaging exercise to the time required for cells to become necrotic and the accumulation of these substances (Armstrong, 1984; MacIntyre *et al.*, 1995).

Bobbert *et al.* (1986) investigated DOMS muscle soreness following 15min of eccentric dorisflexion of the *gastrocnemius* in one leg, whilst the other leg served as a contralateral control. Increases in perceived soreness in the exercised leg (24-72h) were accompanied by an increase in the leg volume compared to the control leg. It was suggested that the rise in leg volume was reflective of oedema in the damaged tissue which increased intramuscular pressure resulting in soreness. However, the authors failed to report lymphocytosis following exercise making direct link to an inflammatory response causing the oedema difficult. Others have reported similar increases in limb volume following eccentric exercise (Friden *et al.*, 1986; Crenshaw *et al.*, 1994; Chileboun *et al.*, 1998). Chlebourn and co-workers (1998) reported a 26% increase in the cross-sectional area of elbow flexors using ultrasound images peaking 4 days following eccentric contractions. They also reported an immediate increase in passive muscle stiffness (60%) from pre-exercise which was measured as the passive torque in the elbow flexors at a number of given angles. They concluded that stiffness following eccentric exercise was not a result of increased swelling due to the variation in time course, but may play a role in the days post exercise (Chileboun *et al.*, 1998). More recent research has provided support for this conclusion (Whitehead *et al.*, 2001). McHugh *et al.* (1999) conducted a similar investigation with prior assessment of muscle stiffness in the *hamstring* muscles before eccentric contractions. Individuals that were classed 'stiff' prior to muscle damaging exercise reported greater muscle pain and tenderness compared to more compliant individuals (McHugh, 1999). These findings would negate the use of muscle stiffness as an indirect marker of muscle damage, but rather as a predictor of susceptibility to muscle injury and DOMS in less flexible individuals.

In a more controlled investigation Graven-Nielsen *et al.* (1997) infused hypertonic saline into the *tibialis anterior* of subjects to attempt to identify the mechanisms underlying pain with increased intramuscular pressure. Interestingly, they reported no pain with increased intramuscular pressure contradicting the proposed aetiology of compartment syndrome.

Using microdialysis following hypertonic saline infusion they observed increased pain with increasing concentrations of sodium that subsided with a delayed increase in potassium (Graven-Nielsen *et al.*, 1997). Thus, highlighting an ionic imbalance as the mechanism associated with swelling induced muscular pain. These conflicting findings may be due to the compliance of different muscle compartments. The *tibialis anterior* compartment has a relatively low compliance compared to the compartment of the elbow flexors (Newham, 1988).

The time course of DOMS has been described as following an inverted U-shaped curve (Vickers, 2001). However, the exact period at which soreness reaches peak values varies considerably. Typically, following laboratory based protocols authors report a peak between 24 to 48h following bench stepping (Newham *et al.*, 1983a; High *et al.*, 1989), downhill running (Schwane *et al.*, 1983; Byrnes *et al.*, 1985; Maughan *et al.*, 1989) and intermittent shuttle running (Thompson *et al.*, 2001a). Some argue that this time course is not reflective of field based exercise models. Vickers (2001) identified these variations by monitoring muscular soreness following either bench stepping or long distance running. Muscle soreness peaked 36 to 48h following bench stepping compared to an almost immediate increase following long distance running that was reduced over the following 5 days. Unfortunately it is difficult to substantiate these findings as individuals performing the bench stepping were untrained and predominantly female, whereas those who completed a range of running distances (2-50miles) were well trained males. These factors have been shown to contribute to artifacts when interpreting measures of DOMS following exercise (Balnave & Thompson, 1993; Warren *et al.*, 1999; Dannecker *et al.*, 2003). However, the intensity and duration of exercise clearly plays an integral role in muscle damage and the subsequent degree of soreness both during the immediate post-exercise period and in the days following.

2.3.3 Muscular dysfunction

Perhaps the most applicable marker of muscle damage is the temporary reduction in normal muscular function. Such impairments may be more relevant when determining the implications of EIMD on the ability to perform common muscular tasks following eccentric or unaccustomed exercise. Subsequently, assessment of muscular function has been commonly employed when attempting to identify the effects of preventative or therapeutic interventions on EIMD and DOMS (Hasson *et al.*, 1993; Jakeman & Maxwell, 1993; Sayers *et al.*, 2001; Thompson *et al.*, 2001b; Farr *et al.*, 2002).

Most investigations into EIMD report a biphasic reduction in muscle function when measured as maximal voluntary contraction (MVC) and/or range of motion (ROM) (Warren *et al.*, 1999). The initial muscular dysfunction has been attributed to the more acute effects commonly associated with fatigue following exercise. Elevated concentrations of metabolic waste products inhibit the resynthesis of ATP and subsequently effect excitation-contraction coupling (Ebbeling & Clarkson, 1989; Allen, 2001). The secondary loss of muscular strength occurs in the hours or days following the initial damage and is considered to be a direct result of damage to the contractile apparatus. The continuing force loss may also be reflective of further ultrastructural damage during the post-exercise period, that has been previously reported using eccentric exercise models (Friden *et al.*, 1983a; Jones *et al.*, 1986). Force decrements can be as large as 50% of pre-exercise values and are not fully restored until 10 or more days later, although it is acknowledged that progressive ultrastructural damage may develop during the time that strength is recovering (Nosaka *et al.*, 1991; Clarkson *et al.*, 1992). Friden and co-workers (1983b) were amongst the first to relate structural changes to reductions in muscular strength following eccentric exercise. They compared histological changes in biopsy samples to isometric and isokinetic (1.57, 3.14, 5.24 $rad.s^{-1}$) reductions in muscular strength. Knee extensor torque was gradually decreased at all angular velocities 20min following exercise and recovered to pre-exercise values at all but the fastest contraction speed. The extent of structural damage was greatest 3 days following exercise and showed signs of regeneration after 6 days. The slower recovery of muscle torque at

faster angular velocities was associated with a predominance of type II muscle fibre injury.

Extensive research has been conducted, primarily in rodents, on the ultrastructural mechanisms that underlie the reductions in muscular strength following eccentric exercise. Katz (1939) was the first to show a rightward shift in the length-tension curve following eccentric exercise indicative of stretch-shortening damage. More recent attempts to identify the precise aetiology of muscular dysfunction have focused on the observed increase in passive tension as well as the shift in the active length-tension curve (Howell *et al.*, 1993; Morgan *et al.*, 1996; Morgan & Allen, 1999). Whitehead and co-workers (2001) preformed a direct comparison of human and animal muscle properties following eccentric exercise. In both species they observed increases in passive muscle tension and a rightward shift in the length-tension relationship. The authors attributed these observations to mechanisms previously discussed, namely over-stretching and disruption of sarcomeres producing an increased compliance in series with the actively contracting sarcomeres. The uneven distribution of damaged sarcomeres was hypothesised to lead to membrane damage and a loss of Ca^{2+} homeostasis resulting in the development of regions of contracture in the damaged fibres responsible for increases in passive tension. Increases in swelling could not account for these changes 24h post-exercise as oedema was only observed from this point onwards. Subsequently, initial alterations in the contractile properties of damaged muscles are attributed to mechanical disruptions. This investigation provides substantial support for the ultrastructural changes that are believed to initiate the sequences of events that ultimately result in muscular dysfunction as suggested by others (Katz, 1939; Morgan, 1990; Morgan & Allen, 1999; Proske & Morgan, 2001). Figure 2.2 attempts to summarise the sequence of events in muscular dysfunction.

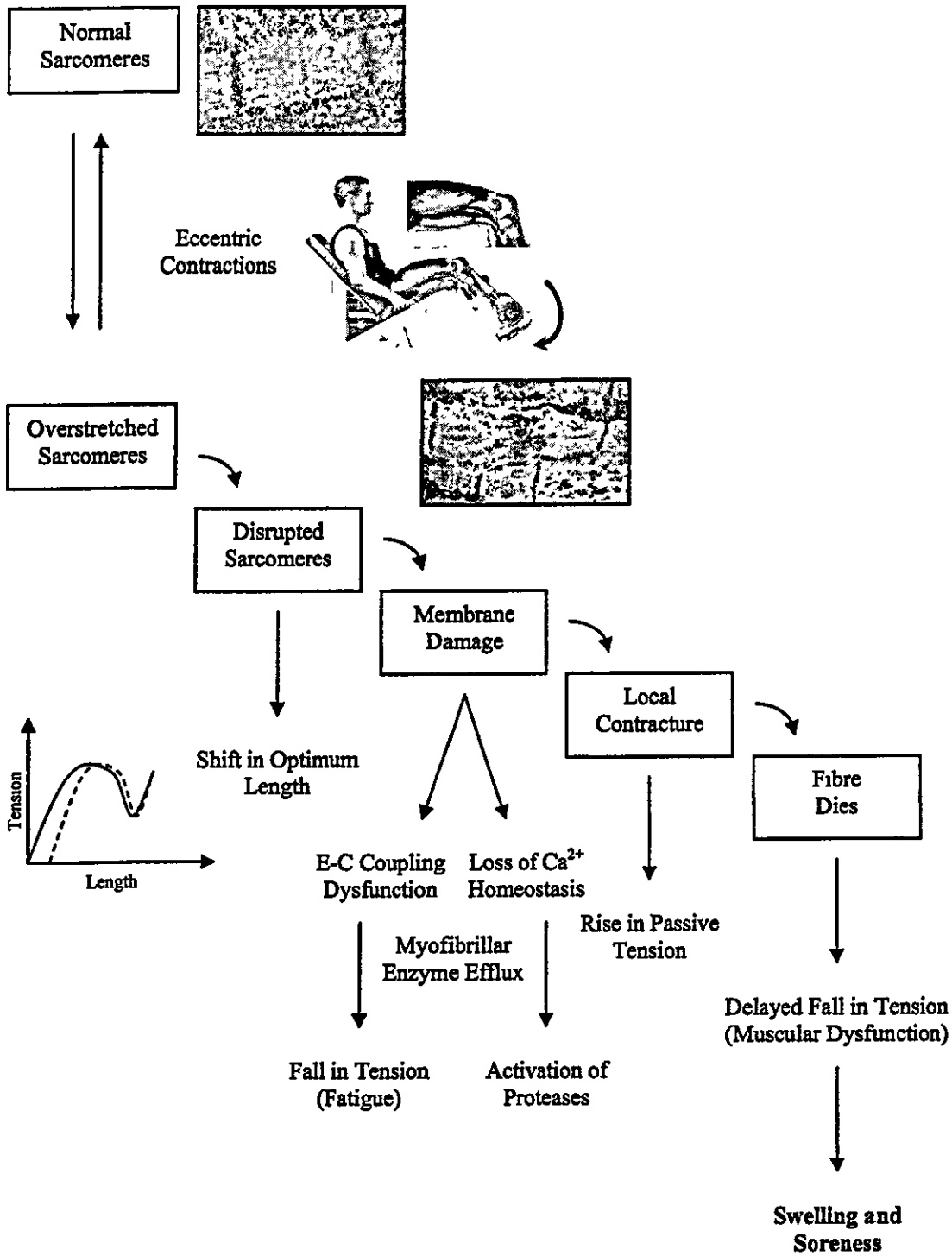


Figure 2.2 Proposed cascades of events leading to muscular dysfunction and muscle soreness following eccentric exercise. Highlighting the initial mechanical disruption to the contractile apparatus and subsequent metabolic alterations to normal cellular function. (Adapted in part from Proske & Morgan, 2001)

Brown *et al.* (1996) examined the changes in human skeletal muscle contractile function following a single bout of stimulated eccentric knee extensions. They reported a delayed onset of contraction with electrical stimulation at 100Hz both immediately and 3 days following the exercise bout, indicative of the biphasic response. Upper limb muscle soreness peaked 3 to 4 days post-exercise during which muscle dysfunction showed signs of recovery. Damaged-induced disturbances in ionic homeostasis in the neuromuscular region were hypothesised to explain the apparent inhibition of excitation-contraction coupling (Brown *et al.*, 1996). They also reported chronic depression of the 20:100Hz force ratio immediately post-exercise suggestive of low frequency fatigue which has previous be linked to impaired Ca^{2+} release from the sarcoplasmic rectilium. Both findings could be attributed to the ultrastructural disruptions commonly reported with histological measurements following similar eccentric models (Friden *et al.*, 1983b). Sargeant and Dolan (1987) reported low frequency fatigue (20:50Hz) following eccentric exercise of the leg extensors (downhill walking, -25%) until collapse as a result of muscle weakness. This response was accompanied with increased plasma CK activity and reductions in isometric MVC and anaerobic power output during isokinetic cycle ergometry up to and beyond 96h post-exercise. The authors also observed a drift in oxygen uptake ($\dot{V}O_2$) from ~45% to ~75% $\dot{V}O_2max$ during downhill walking exercise. This phenomenon has been documented in other investigations employing downhill exercise (Dick & Cavanagh, 1987; Westerlind *et al.*, 1992; Westerlind *et al.*, 1994). The drift in $\dot{V}O_2$ is associated with increased electromyographic (EMG) activity and has been attributed to the requirement for increased motor unit recruitment to maintain tension that is progressively lost in damaged active motor units (Dick & Cavanagh, 1987; Pierrynowski *et al.*, 1987). In fact, this progressive muscle damage observed as increasing electrically activity during downhill running has also been reported following other eccentric exercise (Berry *et al.*, 1990; McHugh *et al.*, 2002). Newham *et al.* (1983) observed increased integrated EMG activity both during eccentric stepping exercise and when pre and post-exercise strength tests were compared. However, some have failed to report eccentric exercise induced increases in EMG activity and attribute strength loss post-exercise to muscle fibre damage alone (Pearce *et al.*, 1998; Kauranen *et al.*, 2001).

Some have linked the delayed reduction in muscle function to DOMS and other indices of muscle damage, although few investigations have reported strong relationships between these markers (Cleak & Eston, 1992b; Croisier *et al.*, 1996). Robenburg *et al.* (1993) reported that the magnitude of muscle soreness following eccentric was moderately correlated with strength loss (r = 0.50-0.63) 48 to 72h following eccentric elbow flexion. They observed stronger relationships with muscular dysfunction and CK activity (r = 0.75 to 0.91) between 48 to 72h post-exercise. The small sample size and subjective nature of assessing DOMS was hypothesised to explain the weaker relationships observed. The causal relationship between muscle dysfunction and DOMS was further investigated by Nosaka and co-workers (2002). They attempted to identify any relationship between indices of muscle damage and DOMS following a varied number of eccentric elbow flexions. Soreness, assed by palpation, was unrelated to indirect markers including plasma CK activity, swelling and changes in MVC leading to the suggestion that DOMS is a poor reflector of eccentric contraction-induced muscle damage. They concluded that individuals do not necessarily experience more soreness when performing exercise protocols that lead to greater changes in indirect markers of muscle damage. Although muscle soreness is commonly associated with EIMD it seems clear that the exact mechanism responsible for this sensation is not directly related to muscle injury per se, but perhaps the final outcome of a sequences of responses.

Byrne and Eston (2002) recently investigated the effect of eccentric knee extension on muscle function assessed with both an isokinetic dynamometer as well as with dynamic exercise. Subjects performed repeated eccentric barbell squats and muscle function was monitored following exercise as either the change isometric muscle strength (MVC) or by measuring vertical jump performance along with power output during a 30s Wingate cycle test. In both investigations MVC was reduced to 65 to 80% of pre-exercise values between 1 and 24h following exercise. Similar reductions in dynamic parameters were observed with peak power during the Wingate test and vertical jump height. The latter was greatest with a squat jump technique which did not include a stretch-shortening phase. This lead the authors to postulate that the method of muscular contraction may be an important factor when determining performance in more applied tests (Byrne & Eston,

2002). Reductions in peak power during the cycling test were suggested to be indicative of a failure in excitation-contraction coupling or force-generating structures (Byrne & Eston, 2002). Additionally, the rate of fatigue, assessed by regression analysis (Balnave & Thompson, 1993), was significantly less following eccentric exercise. Similar findings have been documented (Balnave & Thompson, 1993) as type II fibres are predominantly recruited during eccentric exercise and subsequently damaged (Friden *et al.*, 1983b). Therefore, these more fatigue susceptible fibres will not contribute to post-exercise force generation and thus the post-eccentric exercise fatigue response is diminished due to a predominance of type I fibre recruitment. This damage induced selective recruitment of muscle fibre sub-populations may also explain the loss of force compared with pre-exercise following eccentric contractions.

The use of MVC as an indirect measure of damaged muscle groups has been criticised because even highly motivated individuals may not be able to elicit maximal recruitment of all motor neurons (Warren *et al.*, 1999). Central fatigue has been shown to account for up to 30% of the reductions in force during sustained 60s MVC in rested muscle and with well-motivated individuals (Bigland-Ritchie *et al.*, 1978). Voluntary effort may also be reduced during post-exercise strength assessment because of painful sensations associated with DOMS (Lapointe *et al.*, 2002). However, Newham and colleagues (1987) superimposed electrical stimulation on voluntary isometric action of damaged muscles following eccentric forearm flexion. An additional force was generated by electrical stimulation only if voluntary force was submaximal. Findings indicated that voluntary force generation was maximal throughout (Newham *et al.*, 1987). In light of these inconsistent findings caution is advocated when attempting to interpret exercise-induced alterations in muscle function (Warren *et al.*, 1999).

In an attempt to summarise the time course of the various indices discussed Figure 2.3 represents a selection of reviewed studies showing peak changes at various time intervals following cessation of exercise. The varying peak increases in these indices support the evidence for weak relationships between changes in markers of muscle damage following exercise.

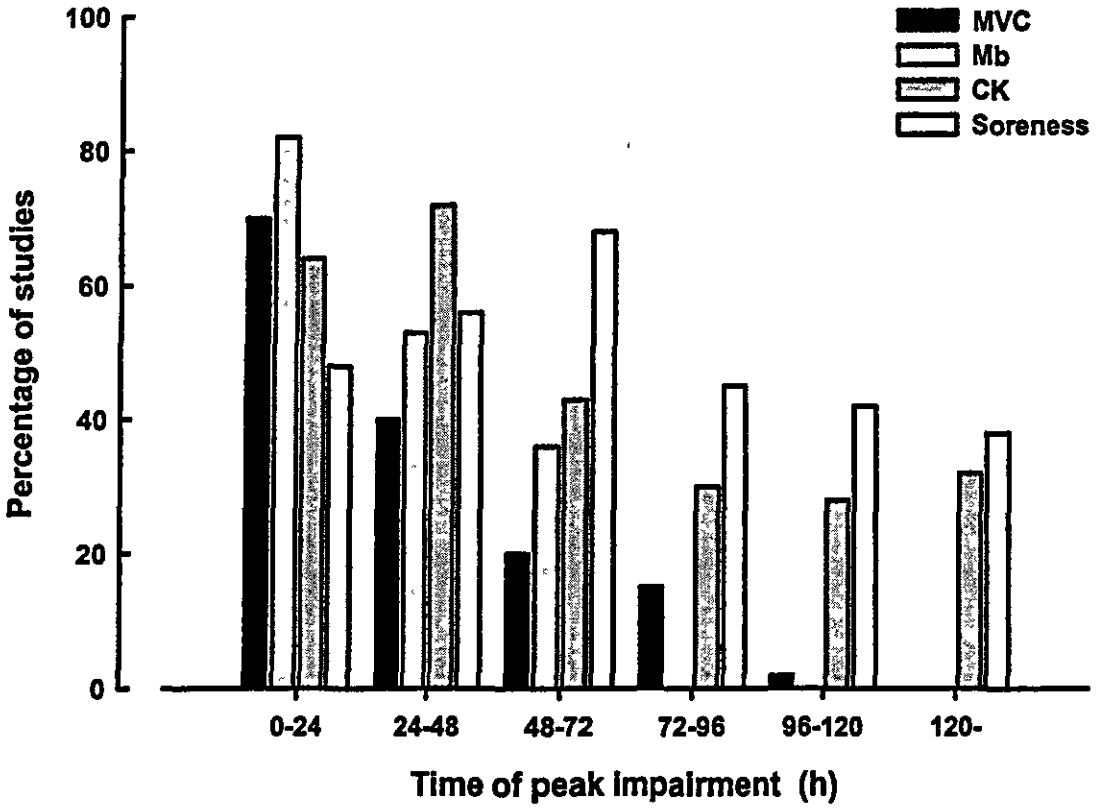


Figure 2.3: Percentage of reviewed studies showing changes in various indices of exercise-induced muscle damage following eccentric or unaccustomed exercise. Data for 4 markers are present; maximal voluntary contraction (MVC) (n = 26 studies), systemic myoglobin (Mb) concentration (n = 18 studies), systemic creatine kinase activity (CK) (n = 33 studies) and perceived soreness (n = 32 studies). Data is taken for changes ≥24h post-exercise. (Where possible changes have been corrected for those observed in a controlled condition.)

2.4 Acute Immune Response

Exercise elicits perturbations in immune function that are both intensity and duration dependent. More commonly established responses include an increased susceptibility to infection following prolonged, strenuous exercise and immunosuppression with heavy training, as well as some positive improvements in innate immune function with regular physical activity. Indeed, exercise is comparable to any stressor and thus is considered a reproducible model for investigating immune responses similar to those associated with infection and disease (Mackinnon, 1999; Shephard, 2001). It is beyond the scope of this review to discuss current knowledge in exercise immunology, as this has been done comprehensively elsewhere (Nieman, 1997; Mackinnon, 1999; Nieman & Pedersen, 1999). Instead, it attempts to highlight the relevant research into the potential role that an acute immune response may play in EIMD.

2.4.1 Inflammatory response

Inflammation can be characterised as the movement of fluid, plasma proteins and leukocytes into tissue in response to injuries, infections or antigens (MacIntyre *et al.*, 1995). The purpose of such a response is to eliminate microbial infections and promote the clearance of damaged necrotic tissue. Acute inflammation is characterised by biphasic changes in vascular permeability and blood flow. There is an initial vasoconstriction followed a few hours later by increased vascular permeability and vasodilation (Smith, 1991). The accumulation of phagocytic cells and the production of acute phase proteins, including C-reactive protein, by hepatocytes follow these vascular responses. Inflammation may become chronic with the infiltration of lymphocytes, neutrophils and monocytes in the following days. It is proposed that this innate immune response is present during recovery from EIMD and may explain the delayed nature of some indices commonly employed to assess the extent of muscular damage.

The sensation of soreness following eccentric exercise is commonly accompanied by localised swelling and heat/redness in the damaged tissue (Newham *et al.*, 1983a;

Bobbert *et al.*, 1986). Indeed, increases in limb volume at the same time as peak soreness have been documented (Cleak & Eston, 1992b; Howell *et al.*, 1993; Chleboun *et al*, 1998). Although some argue that swelling is linked to intramuscular oedema caused by myofibrillar protein leakage (Friden *et al.*, 1983b) direct histological evidence for the infiltration of inflammatory cells provides evidence for an acute immune response (Jones *et al.*, 1986; Fielding *et al.*, 1993). Smith (1991) was amongst the first to fully recognise the potential role of acute inflammation in the aetiology of DOMS and EIMD. She proposed that damage to muscle tissue following exercise provides the stimulus for inflammation with the purpose of healing. The time course of this acute immune response, along with the production of noxious agents and evidence for secondary injury appeared to explain the delayed nature of many of the symptoms of muscle damage.

2.4.2 Leukocyte accumulation

Damaging eccentric exercise is associated with a greater accumulation of neutrophils and other leukocytes compared with non-damaged concentric exercise (Smith *et al.*, 1989; Pizza *et al.*, 1995). Gleeson and colleagues (1995) reported an increase in the number of circulating leukocytes following eccentric box stepping exercise. Initial post-exercise increases were following by further increases in the hours following. However, 2 to 3 days post-exercise circulating leukocytes fell below pre-exercise levels indicative of margination in the initial stages of inflammation (Gleeson *et al.*, 1995). Fielding *et al.* (1993) showed increased accumulation of intra-muscular neutrophils in the *vastus lateralis* following 45min of downhill running which supports the margination observed in other investigations. Further support for the infiltration of inflammatory cells was provided when radioactively labeled leukocytes were re-introduced to individuals prior to eccentric exercise performed in one leg (MacIntyre *et al.*, 1996). Post-exercise radionuclide images showed a significantly greater presence of labeled neutrophils in the exercised leg compared to the control leg at 24h. More recently, MacIntyre and co-workers (2001) showed a relationship between myofibrillar protein efflux and inflammation following damaging exercise. Following infusion of labeled neutrophils they observed increased accumulation of these leukocytes in skeletal muscle along with

elevated concentrations of interleukin-6 (IL-6) in the hours following exercise. This acute inflammatory response was positively correlated with increased concentrations of MHC 2h post-exercise (r = 0.68) and DOMS (r = 0.66) 24h post-exercise (MacIntyre *et al.*, 2001).

Although changes in circulating leukocyte number are indicative of perturbations in immune function post-exercise this only represents systemic changes and are perhaps more reflective of leukocyte distribution and recruitment rather than function. Subsequently, research into EIMD and inflammation has focused on the functional capacity of leukocytes following exercise. Crosier *et al.* (1996) reported a greater increase in plasma polymorphonuclear elastase, a proteolytic enzyme released by neutrophils following degranulation, after eccentric compared with concentric exercise. This was accompanied by decreases in eccentric peak torque and increased serum CK activity and Mb concentration. Similar eccentric induced increases in neutrophil activity, assessed as changes in plasma elastase and myeloperoxidase concentrations, were reported following a 20min bout of downhill running (Camus *et al.*, 1992). Subjects also performed 20min concentric, uphill walking to attempt to highlight the effect of muscle damaging exercise on inflammation. Both modes were followed by an immediate neutrophilia related to the demargination resulting from increases in cardiac output and the secretion of catecholamines commonly linked to leukocytosis following exercise (MacIntyre *et al.*, 1995). The increased neutrophil activity was only reported following downhill running and is suggestive of acute inflammatory response to muscle damage.

The specific signal responsible for the infiltration of leukocytes into injured muscle is still to be elucidated. However, some suggest that tissue fragments and debris may provide the stimulus for resident macrophages and fibroblasts to activate other elements of the immune system in a similar manner to invading pathogens (Pyne, 1994b; Tidball, 1998). The release of vasoactive substances such as nitric oxide, bradykinnin, histamine, leukotrienes and growth factors are also considered integral in the initiation of leukocyte infiltration (Belcastro *et al.*, 1998; Malm, 2002). Systemic counts of circulating monocytes as well as tissue specific macrophages have also been reported to increase

following exercise-induced muscle injury but their accumulation at the site of damage is more delayed peaking between 24 to 72h post-exercise (Armstrong *et al.*, 1983; Armstrong, 1986; Smith *et al.*, 1998). Recently, Pizza and colleagues (2002) showed a pronounced increase in muscle tissue macrophage concentration in rodents 3 days following lengthening contractions compared to passive stretches and isometric contractions. They reported increased neutrophil infiltration following all contractions perhaps suggesting a non-damage related mechanism responsible for post-exercise neutrophilia. However, the damaged-induced increase in macrophages was linked to repair and regeneration of the injured tissue. Investigators have identified two sub-populations of macrophages, $ED1^+$ and $ED2^+$, that increase following eccentric exercise in rodents (St Pierre & Tidball, 1994). It is proposed that $ED1^+$ macrophages act in a similar manner to phagocytic neutrophils to function the removal of cellular debris, whereas $ED2^+$ may regulate the repair and regeneration of necrotic muscle tissue (Tidball, 1995). This provides evidence for the regeneration of muscle tissue following exercise induced injury but caution should be used when extrapolating these findings to humans.

The delayed infiltration of monocytes and macrophages has lead some authors to speculate that they might be primarily responsible for the sensation of DOMS (Smith, 1991). One theory (Smith, 1991) proposes that the release of lysosomes by phagocytic cells may lead to the production of prostaglandins which can stimulate nerve afferents in the injured tissue in a similar manner to that already discussed (section 2.2.3). Indeed increased concentrations of PGE_2 have been associated with increased macrophage accumulation in the days following eccentric exercise (Armstrong *et al.*, 1983). However, much of the evidence for this comes from animal studies and is not supported in human investigations (Kuipers *et al.*, 1985; Croisier *et al.*, 1996). Another theory suggests that phagocytosis and oxidative/respiratory burst of neutrophils and macrophages may exacerbate existing tissue injury. During phagocytosis polymorphonuclear cells engulf necrotic tissue and release lysosomal enzymes as well as oxidative particles (reactive oxygen species; see section 2.7) to degrade this cellular debris. Increased concentrations of these factors have been implicated in ongoing damage to muscle tissue during the post-

exercise period (Best *et al.*, 1999). Lowe *et al.* (1995) showed that increased protein degradation indicative of muscle damage was associated with the infiltration of phagocytic macrophages following eccentric exercise in rodents. Lapointe and colleagues (2002) also linked secondary damage to rodent muscle tissue following eccentric exercise with a delayed accumulation of the $ED1^+$ subpopulation of macrophages. Perhaps either or both theories may help to explain the delayed nature of muscle soreness following the initial EIMD.

2.4.3 Inflammatory mediators

In addition to changes in circulating leukocyte number much attention has focused on increases in pro- and anti-inflammatory cytokines following damaging exercise (Bruunsgaard *et al.*, 1997; Ostrowski *et al.*, 1999; Gleeson, 2000; Nieman *et al.*, 2001; Toft *et al.*, 2002). Cytokines are considered to be an important link between immune and neuroendocrine responses following exercise as they facilitate the infiltration of lymphocytes, monocytes and neutrophils into injured tissue which ultimately results in inflammation and an associated systemic acute phase response (Pedersen *et al.*, 1998). Plasma concentrations of inflammatory cytokines, including interleukin 1 (IL-1) and tumour necrosis factor (TNF-α) have been shown to increase two-fold and interleukin 6 (IL-6) 100-fold following prolonged strenuous exercise (Ostrowski *et al.*, 1998; Ostrowski *et al.*, 1999; Suzuki *et al.*, 2003). Increases in IL-6 are linked with the release of cytokine inhibitors (including IL-1ra) and the anti-inflammatory cytokine interleukin 10 (IL-10) (Pedersen *et al.*, 2001a). Fielding and colleagues (1993) showed increased concentrations of IL-1β and accumulation of neutrophils in skeletal muscle following downhill running. Brunnsgaard *et al.* (1997) were amongst the first to show a delayed increase in systemic concentrations of IL-6 following eccentric compared to concentric exercise. The increase in this inflammatory cytokine was associated with lymphocytosis ($r = 0.72$) and plasma CK activity ($r = 0.75$) in the hours following exercise. Similar increases in IL-6 have been reported following a variety of eccentric based exercise models (Cannon *et al.*, 1991; Croisier *et al.*, 1999; MacIntyre *et al.*, 2001; Willoughby *et al.*, 2003). More recently, evidence for muscle-derived increases in IL-6 following

exercise that is unrelated to muscle damage may make it difficult to support a causal relationship (Jonsdottir *et al.*, 2000; Pedersen *et al.*, 2001b; Steensberg *et al.*, 2001). Additionally, substrate availability has also been implicated in the production of this cytokine perhaps alluding to a more crucial metabolic role other than the mediation of inflammation following eccentric exercise (Keller *et al.*, 2001; Helge *et al.*, 2003). Nevertheless, increased IL-6 production from either leukocytes or muscle tissue may lead to the production of acute phase proteins which are believed to be involved in the repair and regeneration of damaged tissue working together with complement to opsonised necrotic fragments and facilitate removal by phagocytic cells (Pyne, 1994a). Increased levels of serum C-reactive protein (CRP) have been documented following prolonged exercise (Pedersen & Hoffman-Goetz, 2000). Evidence for increases following eccentric exercise remains equivocal (Gleeson *et al.*, 1995; Pyne *et al.*, 1997; Croisier *et al.*, 1999).

Figure 2.4 shows a schematic representation of acute inflammation in relation to the previously mention characteristic responses to muscle damaging exercise. This model was adapted from Smith (1991).

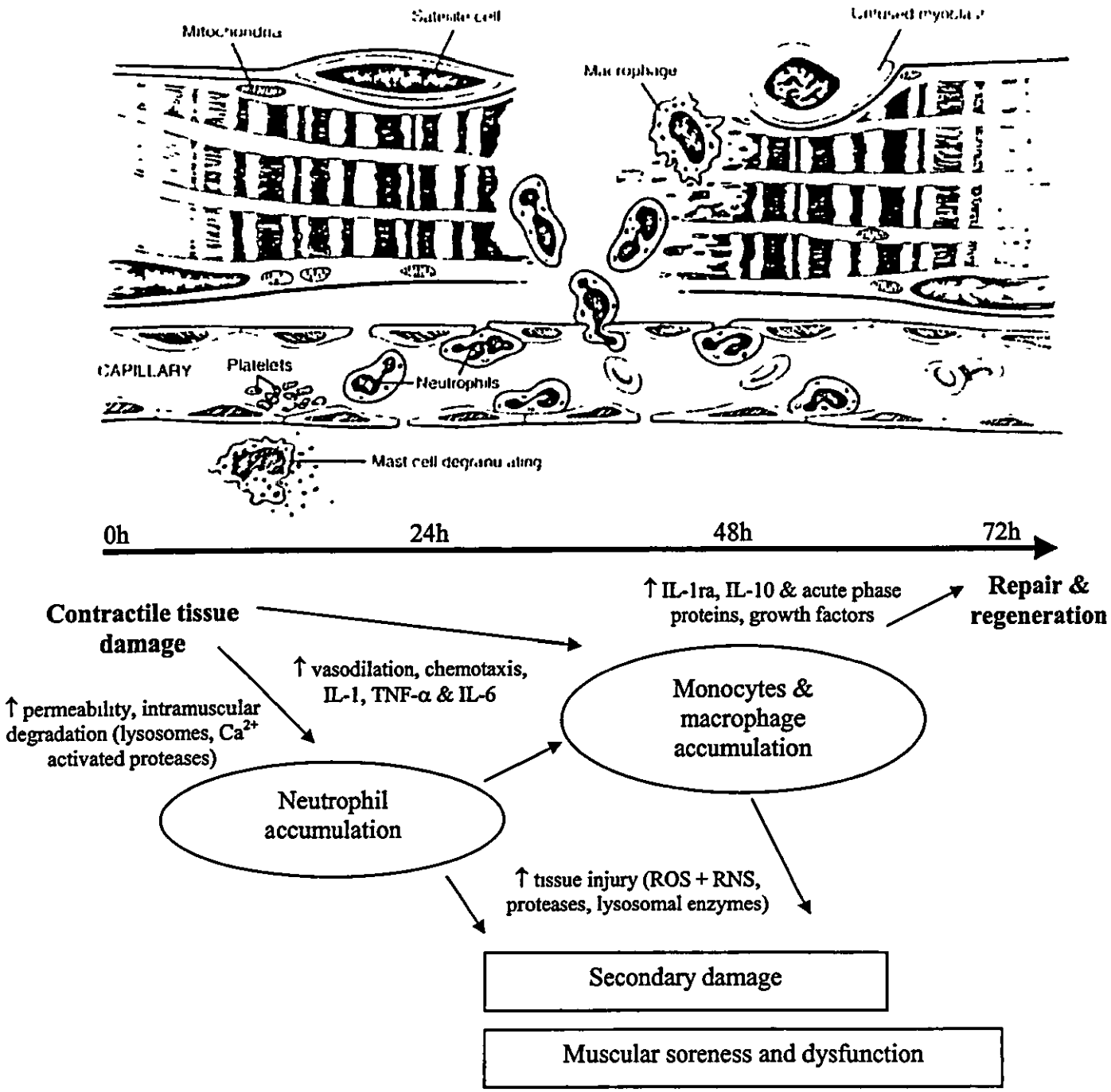


Figure 2.4: A schematic representation of the proposed inflammatory response following exercise induced muscle damage (adapted in part from Smith, 1991 & MacIntyre *et al.*, 1995)

2.4.4 Role of anti-inflammatory agents

Nonsteroidal anti-inflammatory drugs (NSAIDs) have been administered in an attempt to further elucidate the role of acute inflammation in DOMS. These NSAIDs inhibit either the COX enzymes (single action) thus synthesis of PGE_2 or both the COX and LIPOX pathways of arachidonic acid metabolism (dual action) following strenuous exercise. Hasson *et al.* (1993) showed that 400 or 1200mg of ibuprofen ingested 4h before or in the 24h after eccentric exercise, respectively, significantly enhanced recovery of muscle force and reduced DOMS in the days post-exercise. In constrast, Donnelly and co-workers (1990), in a cross-over design, administered ibuprofen (2400mg) prior to and following 45min downhill running. The authors failed to report a prophylactic effect on muscle function, soreness and CK activity (Donnelly *et al.*, 1990). However, findings may have been influenced by the study design which did not account for the repeated bout effect (see section 2.5). Kuipers *et al.* (1985) also reported no effect of NSAIDs on CK activity and DOMS following eccentric cycling, but they too failed to account for the adaptation observed with repeated bouts of eccentric exercise. In a better controlled study Sayers and colleagues (2001) reported enhanced recovery of muscle force and decreased soreness following eccentric exercise with ketoprofen. This study design excluded subjects who failed to report at least moderate soreness following the exercise. They reported administration of ketoprofen 36h post-exercise significantly reduced soreness in over an 8h period (Sayers *et al.*, 2001). These beneficial findings may support the use of dual-action NSAIDs as a temporary treatment for DOMS and muscular dysfunction following eccentric exercise.

2.5 Adaptation and the repeated bout effect

The phenomenon commonly referred to as the repeated bout effect was best defined by Ebbeling and Clarkson (1998) as physical conditioning that results in an adaptation such that all indicators of damage are reduced following repeated bouts of exercise. Reductions in post-exercise efflux of CK with repeated bouts of eccentric exercise were initially attributed to an increased ATP supply in trained subjects (Nuthall & Jones, 1968). However, Friden and colleagues argued against this showing a reduction in soreness and morphological characteristics of skeletal muscle damage following 8 weeks cycle training (Friden *et al.*, 1983a). They attributed the adaptation of skeletal muscle following eccentric exercise to an initial increase in sarcomere length, followed by a sarcomere genesis and increased synthesis of intermediatary filaments to strengthen myofibrils (Friden *et al.*, 1983b). Byrnes *et al.* (1985) observed a reduction in the efflux of myofibrillar proteins (CK, Mb) and muscle soreness following repeated bouts of downhill running 3 and 6 weeks following an initial bout. However, this protective effect was not observed with a repeated bout 9 weeks following the initial exercise (Byrnes *et al.*, 1985).

There is also evidence that even a relatively acute bout of eccentric contractions may stimulate an adaptive response (Paddon-Jones & Abernethy, 2001). Eston and co-workers (1996) reported that a bout of isokinetic eccentric leg contractions performed 2 weeks prior to a bout of downhill running resulted in an attenuation of post-exercise increases in CK activity and muscle tenderness. They also observed a reduction in the exercise-induced decrease in muscle peak torque compared to a control group who only performed the downhill run. Additionally, Clarkson and Tremblay (1988) observed increased serum CK activity, muscle soreness and dysfunction following a bout of 70 maximal eccentric muscle contractions of the elbow flexors. A prior bout of either 12 or 24 maximal eccentric contractions resulted in no CK response and less soreness accompanied by smaller decrements in muscle strength. The authors attributed this adaptation to a strengthening of the muscle membrane and surrounding connective tissue (Clarkson & Tremblay, 1988). Others suggest that an initial bout of exercise may lead to an adaptation

in the motor unit recruitment pattern over the range of motion such that less force is distributed among fibres at any one point during subsequent bouts (Clarkson *et al.*, 1992; Mair *et al.*, 1995; Nosaka & Clarkson, 1995). However, direct assessment (surface EMG) of motor unit recruitment with repeated bouts of isokinetic eccentric exercise provided no evidence for a neural adaptation (McHugh *et al.*, 2001).

Adaptation to an initial bout of muscle damaging exercise has been reported within days (Nosaka & Clarkson, 1994; Mair *et al.*, 1995). Mair and colleagues (1995) identified the characteristic reduction in muscle soreness, myofibrillar protein release and muscular dysfunction with a repeated bout of 70 eccentric knee extensions conducted 13 days following the initial bout. Nosaka & Clarkson (1994) also reported reductions in myofibrillar protein efflux that occurred with repeated bouts of eccentric forearm flexion 3 to 5 days following the original bout, but did not report attenuation in muscle force generation or range of motion. In a subsequent investigation these authors failed to show any exacerbation of indices muscle damage with repeated bouts in the days following the initial eccentric exercise (Nosaka & Clarkson, 1995). Nosaka and co-workers (2001) attempted to identify the duration of the protective effect following eccentric exercise. Subjects performed 2 bouts of eccentric elbow flexion separated by 6, 9 or 12 months. An increased recovery of maximal isometric force, and reduced muscle soreness along with smaller increases in limb volume and plasma CK activity were observed with repeated bouts at 6 months. However, only recovery of muscle force was increased at 9 months, and the 12 month group did not show any repeated bout effect (Nosaka *et al.*, 2001a). These data suggest adaptation may last up to 6 months post-initial damaging exercise. Cellular mechanisms responsible for the adaptation to repeated damaging exercise are still to be fully elucidated but research has identified increased activation of normal quiescent satellite cells following muscle damage induced through prolonged exercise (Dop Bär *et al.*, 1997; Malm *et al.*, 2000; Malm, 2002).

2.6 Other implications of exercise-induced muscle damage

As previously discussed, unaccustomed or eccentric exercise leads a disruption of contractile and connective tissue resulting in a period of muscular dysfunction that is associated with an increased sensation of muscle soreness. The implications of which are profound for both trained individuals performing consecutive exercise bouts within a small time period or more sedentary individuals attempting to increase their physical activity status without such detrimental responses. In addition to these responses, disruptions of the ultrastructure of myofibrils have also been implicated in impaired cellular transport processes. More specifically, evidence exists to show an impaired ability to uptake glucose and replete muscle glycogen following damaging exercise (O'Reilly *et al.*, 1987; Kristiansen *et al.*, 1996). O'Reilly and co-workers (1987) first reported a reduced repletion of muscle glycogen stores following 45min of lower intensity (44% $\dot{V}O_2max$) eccentric cycling. Costill *et al.* (1990) reported a similar reduction in muscle glycogen resynthesis in eccentrically compared to concentrically worked knee extensors. This difference was unaffected by controlling carbohydrate intake during the post-exercise period (Costill *et al.*, 1990). In a similar study, Doyle *et al.* (1993) depleted muscle glycogen store in subjects prior to a bout of either concentric or eccentric exercise in opposite legs. Despite a large carbohydrate intake ($1.6g.kg.h^{-1}$) muscle glycogen stores were 25% lower in the eccentrically compared to the concentrically exercised muscle 3 days following exercise. This delayed glycogen accumulation following eccentric versus concentric exercise in well documented and has been linked to an impaired ability to transport glucose via trans-membrane glucose transports (GLUT 1, 4) (Kristiansen *et al.*, 1996) and/or a transient insulin resistance during the post-exercise period (Kirwan *et al.*, 1992; King *et al.*, 1993). Del Aguilia and colleagues (2000) showed that the apparent inhibition of insulin action following eccentric exercise was related to the increased production of the inflammatory cytokine, TNF-α ($r = 0.77$) in response to tissue injury. Thus, it seems that damage to skeletal muscle initiates a sequence of mechanical, metabolic and inflammatory events that also have profound effects of normal cellular substrate homeostasis.

2.7 Exercise-induced oxidative stress

Define as any species capable of independent existence that contains one or more unpaired electrons (·), free radicals have been implicated in a various degenerative diseases and ageing (Halliwell & Gutteridge, 1989; Jackson, 1996; Niess *et al.*, 1999). Free radicals strive to balance their unpaired electrons by combining with electrons with opposite spins in other substances. They are, therefore, highly reactive and have a short half-life (Sjodin *et al.*, 1990). Increasing evidence suggests that strenuous physical exercise leads to an increased generation of free radicals within skeletal muscle (Jackson *et al.*, 1985; Reid *et al.*, 1992; Ashton *et al.*, 1998; Alessio *et al.*, 2000; Bailey *et al.*, 2003). If free radical production during exercise exceeds endogenous defence mechanisms this may lead to deleterious damage or destruction of cellular macromolecules such as lipids, proteins, nucleic acids, and components of the extracellular matrix collectively known as oxidative stress (Alessio, 1993; König *et al.*, 2001). Oxidative damage by radicals has been associated with decreased performance, fatigue, muscle damage and overtraining (Jackson *et al.*, 1995; Packer, 1997; Tiidus, 1998). Therefore, some authors advocate reducing oxidative stress in order to abrogate these responses and improve exercise tolerance and performance (Dekkers *et al.*, 1996; Goldfarb, 1999; Clarkson & Thompson, 2000). Before such attempts are reviewed it is important to first highlight the potential source of free radicals both during and following exercise and the adaptive role of endogenous antioxidant defense in reducing the mentioned deleterious effects of these highly reactive molecules.

2.7.1 Free radical production during exercise

The most significant free radicals are the superoxide ($O_2^{\cdot-}$), hydroxyl (OH) and nitric oxide radicals ($NO^{\cdot}$). Various biological mechanisms that are often enzymatically controlled or catalyzed by iron and copper are responsible for the generation of these radicals (Table 2.2). Radicals and other highly reactive species are commonly classified as either reactive oxygen species (ROS) or reactive nitrogen species (RNS).

Table 2.2: Examples and generation of reactive oxygen (ROS) and nitrogen species (RNS).

Species	Catalyst	Reaction		
Superoxide		$O_2 + e^-$	→	$O_2^{\cdot -}$
Hydrogen peroxide	(superoxide dismutase)	$2O_2^{\cdot -} + 2H^+$	→	$H_2O_2 + O_2$
Hydroxyl radical	(Fenton reaction)	$Fe^{2+} + H_2O_2$	→	$OH^{\cdot} + OH^- + Fe^{3+}$
	(Fe/Cu – catalyst)	$H_2O_2 + O_2^{\cdot -}$	→	$OH^{\cdot} + OH^- + O_2$
Hypochloric acid	(myeloperoxidase)	$H_2O_2 + Cl^-$	→	$HOCl + H_2O$
Nitric oxide	(NO – synthase)	L-arginine	→	NO + citrulline
Peroxynitrite		$NO^{\cdot} + O_2^{\cdot -}$	→	$ONOO^-$

As mentioned, it has become widely accepted that exercise leads to the production of both ROS and RNS. The majority of evidence for this exercise-induced oxidative stress originates from investigations employing indirect methods of assessing free radical generation, whilst direct evidence of increased ROS formation using electron spin resonance spectroscopy (ESR) has been documented in the muscle of rodents (Davies *et al.*, 1982; Jackson *et al.*, 1985) and serum of humans after exhaustive exercise (Ashton *et al.*, 1998; Groussard *et al.*, 2003). The various mechanisms hypothesised to explain the source of free radicals under physical exercise are discussed extensively elsewhere (Sjodin *et al.*, 1990; Alessio, 1993; Jenkins, 1993; Sen, 1995; Packer, 1997).

The extent of exercise-induced free radical generation varies considerably depending on the duration and intensity of exercise as well as the relative proportion of anaerobic and aerobic metabolism in active muscle (Sjodin *et al.*, 1990; Alessio, 1993). Additionally, the quantity of active muscle mass and the extent of muscle damage should be taken into account when attempting to quantify oxidative stress generated by physical activity (König *et al.*, 2001).

Physical exercise is associated with a 10 to 20 fold increase in whole body oxygen consumption. Oxygen flux through the active skeletal muscle fibres may increase as much as 100 to 200 fold (Sen, 2001). The process of mitochondrial oxidative phosphorylation leads to the reduction of about 90 to 98% of this oxygen consumed (Niess *et al.*, 1999). The remaining oxygen undergoes incomplete reduction, a process that ultimately leads to the generation of the superoxide radical (Halliwell & Gutteridge, 1989). Evidence for the extent of total electron flux in the mitochondria shows that the apparent leakage, probably from the ubiquinone-cytochrome b level (Figure 2.5), of the superoxide generation varies between 2 and 10% (Halliwell & Gutteridge, 1989; Sjodin *et al.*, 1990; Packer, 1997). Although direct evidence for the production of ROS from the mitochondrial electron chain has not been verified in humans under near physiological conditions following exercise (Kaikkonen *et al.*, 1998) it can be concluded that with the increased oxygen consumption in working skeletal muscle during exercise mitochondrial leakage provides a significant site for ROS generation.

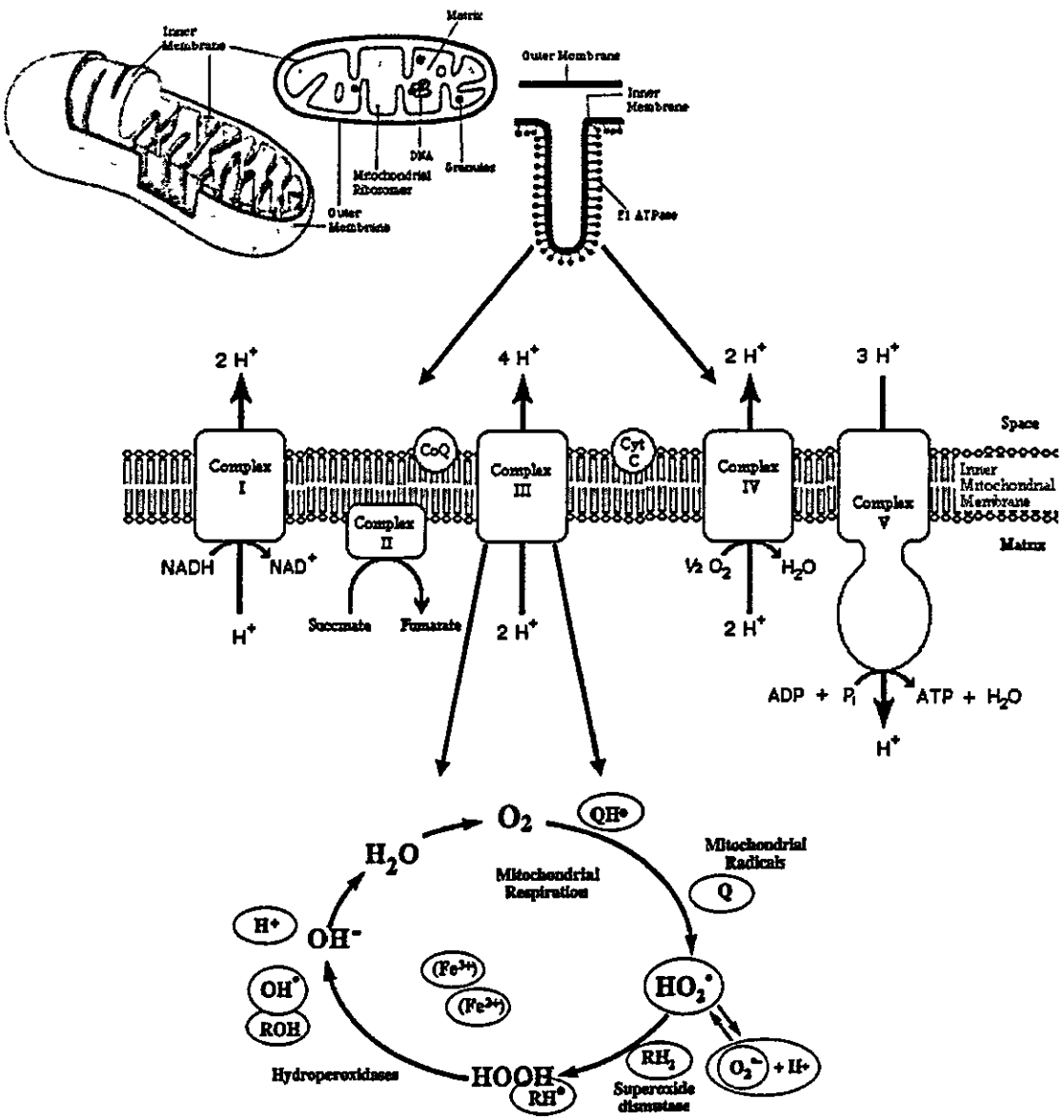


Figure 2.5: A representation of the oxygen cycle in the mitochondria during which oxygen is converted into the hydroxyl radical ($OH^{\cdot}$) and can also produce the superoxide radical ($O_2^{\cdot-}$) (adapted in part from Packer, 1997).

Ischaemic-reperfusion has been associated with increased ROS generation during exercise (Sjodin *et al.*, 1990; Groussard *et al.*, 2003). During ischaemia, adenosine monophosphate (AMP) is formed from ATP via the adenylate kinase reaction. Conversion of AMP to inosine monophosphate (IMP) leads to increased levels of hypoxanthine, which is a substrate for xanthine oxidase (McCord, 1985). Xanthine oxidase predominantly localised in the vascular endothelium, catalyses the conversion of hypoxanthine to xanthine, and xanthine to uric acid. Under normal conditions xanthine dehydrogenase oxidase utilises nicotinamide adenine dinucleotide (NAD^+) as an electron acceptor to produce xanthine and uric acid (Figure 2.6). However, under conditions such as proteolysis caused by disturbances in Ca^{2+} homeostasis, heat stress and oxidation of the thiol groups cause the conversion to the oxidase form which generates the superoxide radical during its catalytic action (McCord, 1985; Sjodin *et al.*, 1990; Pattwell *et al.*, 2003). Endogenous levels of hypoxanthine and uric acid have been shown to increase following high-intensity exercise, suggesting that the xanthine dehydrogenase/oxidase system is active (Hellsten-Westing *et al.*, 1997).

Jenkins and colleagues (1996) showed increased formation of ROS in rats exercised to exhaustion. They attributed this increased oxidative stress to the accumulation of free iron in the skeletal muscle of the rodents. Hydrogen peroxide is converted to the more reactive hydroxyl radical in the presence of iron (Fenton reaction) (Sjodin *et al.*, 1990). Increases in free iron could be explained by the release of myoglobin and/or haemoglobin following exercise-induced muscle damage. Additionally, copper may also contribute to increased formation of the hydroxyl radical via the Haber-Weiss reaction.

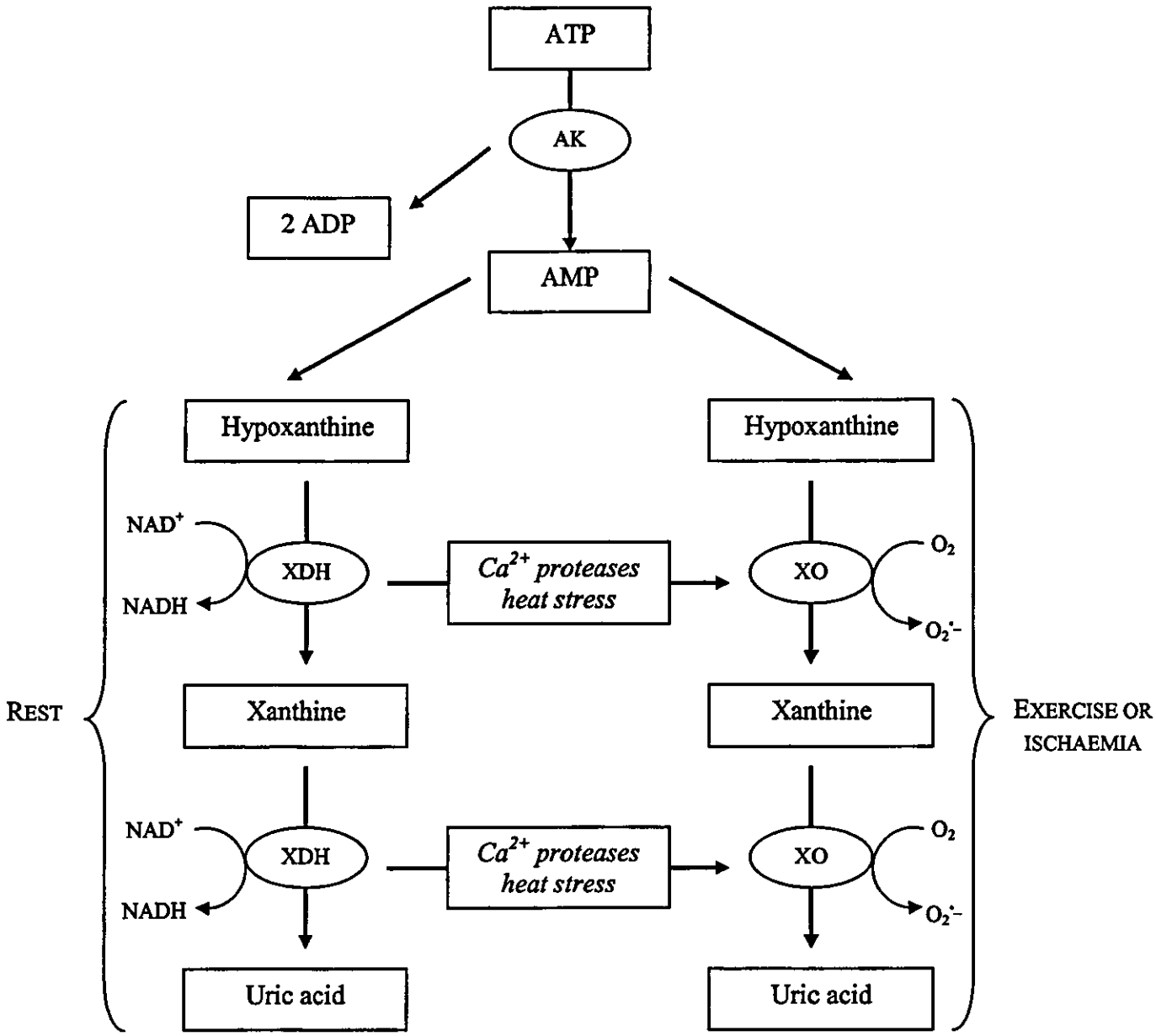


Figure 2.6: The adenylate kinase (AK) reaction and deamination of ATP to AMP and IMP (not shown) and oxidation of hypoxanthine and xanthine to uric acid. Under normal resting conditions the reaction is catalysed by xanthine dehydrogenase (XDH) utilising NAD as an electron acceptor. During intense exercise or ischaemia the reaction is converted by xanthine oxidase (XO) partially proteolysed from XDH regulated by Ca^{2+} activated proteases. XO utilises molecular oxygen as an electron acceptor generating ROS.

As previously discussed, strenuous exercise produces an acute inflammatory response that leads to the infiltration of leukocytes into damaged tissue (see section 2.4). Once activated, neutrophils and macrophages produce the superoxide radical in the NADPH oxidase-catalysed oxidative burst reaction following phagocytosis of cellular debris (Figure 2.4). In further steps this radical in converted to the more reactive hydrogen peroxide and finally HOCl, a reaction catalysed by myeloperoxidase (MPO). These neutrophil-generated ROS are produced in order to destroy either invading bacteria or necrotic tissue (Pyne, 1994b). Activation of neutrophils has been documented following exercise making oxidative burst production of ROS a significant source of free radical generation (Camus *et al.*, 1992; Pyne, 1994b; Croisier *et al.*, 1996; Gleeson *et al.*, 1998). Also, the generation RNS from neutrophils, monocytes and macrophages has been implicated in oxidative stress caused by immunocompetent cells. Specifically nitric oxide, proceeding from arginine via NO-sythase (iNOS), is produced for the same purpose as ROS in oxidative burst activation (Niess *et al.*, 1999). It is proposed that infiltration of neutrophils and monocytes into damaged tissue in response to catecholamine and cortisol secretion as well as cytokine production is not tightly regulated (Niess *et al.*, 1999). Subsequently, activation of phagocytes and increased ROS may exceed endogenous antioxidant defence and contributed to ongoing oxidative damage following exercise. Thus, it proposed that antioxidants may reduce the deleterious effect of ROS generation by inhibiting inflammatory mediators (Peters-Futre, 1996; Tiidus, 1998; Evans, 2000). Also, it has been suggested the superoxide radical generated from the xanthine oxidase pathway is involved in the attraction and activation of leukocytes (Hellsten *et al.*, 1997). Thus, providing further evidence that activated polymorphonuclear leukocytes may be responsible for profound oxidative stress in the post-exercise period.

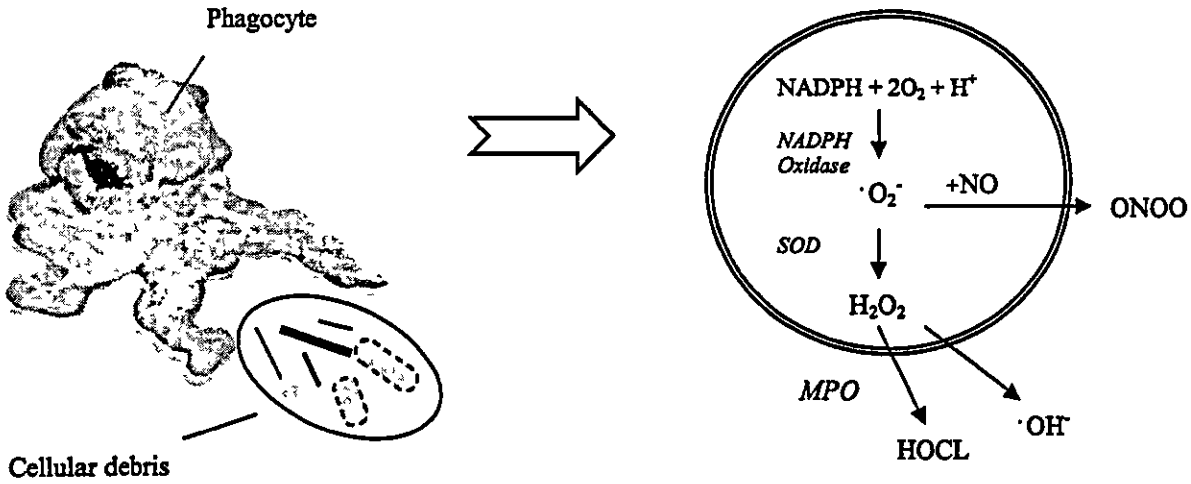


Figure 2.7: Phagocytosis and the generation of ROS/RNS via the respiratory burst. (MPO, myeloperoxidase; SOD, superoxide dismutase)

One other method of ROS formation during exercise is the autoxidation of catecholamines to adenochrome with the generation of the superoxide radical with ischaemic-reperfusion. However, this process is very slow but the addition of the metal ions may catalyse oxidation of catecholamines leading to the production of free radicals (Jewett *et al.*, 1989). As exercise is commonly associated with acute elevations in catecholamines (Mazzeo, 1991), it is apparent that their oxidation might contribute to exercise-induced oxidative stress.

2.7.2 Aetiology of oxidative damage

Although free radicals may play an integral role in a number of biological processes, excessive generation of these highly reactive molecules can lead to oxidation of lipids, proteins and nucleic acids (Alessio, 1993).

Unsaturation points in polyunsaturated fatty acid (PUFA) make these lipid molecules highly susceptible to ROS attack and oxidative damage (Sen, 2001). The uncontrolled and autocatalytic oxidative destruction of PUFA is commonly referred to as lipid

peroxidation. It is initiated by the abstraction of a hydrogen atom from weakly associated methylene carbon molecule. This produces a lipid radical and a bond rearrangement which gives a conjugated diene (Sjodin *et al.*, 1990). The lipid reacts with oxygen to form the peroxide radical which is particularly dangerous as they are able to propagate oxidative damage. These ROS may be carried in the blood to distant targets where new oxidative damage may ensue (Sen, 1995). The newly formed peroxide radical may abstract another hydrogen atom from another PUFA forming a lipid hydroperoxide (LPO) and further carbon-centered radicals. Lipid hydroperoxides are further degraded to aldehydes, including malondialdehyde (MDA), and also hydrocarbons (including ethane and pentane) which are both used in the detection of lipid peroxidation (Alessio, 1993; Packer, 1997). The reactivity of lipid peroxidation means that once initiated the process may proceed until either no further PUFA or oxygen are available to continue the reaction (Halliwell & Gutteridge, 1989). Lipid peroxidation of cellular membranes results in decreased membrane fluidity, an inability to maintain ionic gradients, cellular oedema and tissue inflammation (Alessio, 1993).

Oxidative protein damage is considered widespread in vivo. Some estimated almost 1% of oxygen consumed by a cell contributes to protein oxidation at rest (Halliwell & Gutteridge, 1989). Certain components of protein such as tyrosine, methionine, tryptophan, histidine and sulfhydryl residues are highly susceptible to oxidative damage (Sen, 2001). Proteins damaged by ROS are subsequently vulnerable to proteolytic degradation. The process by which this occurs is not dissimilar to lipid peroxidation, as a hydrogen atom is abstracted for an amino acid carbon group. This is followed by a reaction with oxygen to form the peroxide radical. Following reactive oxygen generation, amino acid residues are converted to carbonyl derivatives, which are used as a common index of oxidative protein damage. Protein thiols are also susceptible to oxidative damage. For example, the hydrogen of the sulphydryl (-SH) group is abstracted forming a sulphur centre radical. These radicals react with other sulpur-centered radicals to form a non-reactive disulphide, which is the fundamental basis for the antioxidant properties of glutathione (Packer, 1997). Oxidative modification of proteins may cause a number of

deleterious effects including receptor modification, disturbances in cellular homeostasis, altered signal transduction and other fundamental cell-regulatory processes (Sen, 2001).

Oxidative damage to DNA happens continuously and has been estimated as high as 10^4 to 10^6 lesions per cell per day (Fehrenbach & Northoff, 2001). Oxidation to nucleic acids is believed to be directly related to metabolic rate making exercise a profound source of oxidative damage to DNA. All components of the DNA molecule are considered susceptible to oxidative damage, one of which is the DNA base guanine, which is oxidised by the hydroxyl radical forming 8-hydroxy-2'-deoxyguanosine (8-OHdG). Damage of this base is assumed to be a potential pathophysiological factor in carcinogenesis (Simic, 1992). Upon repair 8-OHdG is excised and excreted in urine where it is measured as an index of oxidative damage to DNA. Radak *et al.* (1999) investigated human muscle following eccentric exercise and observed increased production of 8-OHdG and nitric oxide.

2.7.3 Evidence for exercise-induced oxidative damage

Due to the highly reactive nature of free radicals they have an extremely short half-life making direct determination of their generation difficult. A few investigations have employed electron spin resonance spectroscopy (ESR) that detects the magnetic field generated by the unpaired electron of a free radical. Jackson and colleagues (1985) used ESR to investigate free radical activity following 30min of stimulated contractile activity. They reported a 70% increase in the ESR signal of freeze-clamped muscle tissue which was associated with increased CK activity in the blood. Ashton *et al.* (1998, 1999) have employed this technique in humans following exhaustive exercise. Increases in ESR signaling were associated with increased lipid peroxidation (MDA & LPO) along with total antioxidant capacity post-exercise. Recently, Bailey and co-workers (2003) were able to provide more direct evidence for radical generation from exercising skeletal muscle by assessing arterial-venous difference in ESR signals at various exercise intensities. Groussard *et al.* (2003) showed increased ESR following 30s of high-intensity exercise. The increase was accompanied by reductions in endogenous antioxidants as

well as thiobarbituric acid-reactive substances (TBARS), an indicator of lipid peroxidation. The contrasting responses of TBARS with the more direct assessment of oxidative stress (ESR) has lead the authors to question the use of TBARS as a marker of oxidative damage (Groussard *et al.*, 2003).

The majority of studies attempting to assess free radical production following exercise have employed indirect markers, primarily due to the inaccessibility of ESR at the time of investigation. Most investigations estimate malondialdehyde (MDA) concentrations through the spectrophotometric determination of plasma TBARS. Kanter and co-workers (1993) reported an intensity dependent production of MDA. Running for 30min at 60% $\dot{V}O_2$max increased TBARS to a lesser extent than running for 5min at 90% $\dot{V}O_2$max. However, significant increases in plasma MDA concentration have been reported following whole body resistance exercise (McBride *et al.*, 1998). These increases were associated with muscle damage and CK activity was also increase 24h following exercise. Maughan *et al.* (1988) identified increases in TBARS following 45min of downhill running. The subjects with the largest increases in TBARS also showed the greatest increases in myofibrillar enzyme release (CK, LDH and AST) suggesting a relationship between lipid peroxidation and exercise-induced muscle injury. As both variables are indicators rather than direct evidence of oxidative damage and muscle injury it is difficult to confidently imply a causal relationship. A similar relationship was reported between MDA and serum CK activity ($r = 0.85$) following an 80km run (Kanter *et al.*, 1988). However, one explanation for this relationship could be the long duration of the exercise (~8.5h). Caution has been advocated when using TBARS to determine MDA as increases in TBARS are often much greater than increases in MDA alone (Ashton *et al.*, 1998; Child *et al.*, 1998). Subsequently the use of more than 2 indirect markers has been advocated when attempting to quantify exercise-induced oxidative stress (Clarkson & Thompson, 2000).

Other techniques have demonstrated increased oxidative stress following exercise. Alessio and co-workers reported increases in lipid hydroperoxides (LPO), protein carbonyls and total antioxidant status following both exhaustive aerobic and isometric

exercise. They attributed the isometric exercise-induced oxidative stress to free radical generation via ischaemic-reperfusion as they reported dramatic changes in blood pressure during muscular contraction. Brickson and colleagues (2001) used a more direct technique of assessing radical production *in vitro* using dichlorofluorescein as a probe. They observed a biphasic increase in oxidant production following acute stretch injury in rabbits that was initially attributed to increased neutrophil activity (MPO). The delayed increase in oxidant production was postulated to be a result of increased xanthine oxidase activity 24h post-injury. Expired pentane has also been reported to increase following cycling (Dillard *et al.*, 1978) and running (Kanter *et al.*, 1993) as well as during exercise under various environmental conditions (Simon-Schnass & Pabst, 1988; Schmidt *et al.*, 2002). However, caution should be taken when interpreting these findings as hydrocarbons, such as pentane, are stored in tissues including muscle (Jackson, 1990) and expired concentrations can be affected by both oxygen concentration *in vivo* and the presence of metal ions (Halliwell & Gutteridge, 1986). Conjugated dienes also increase following exercise (Meydani *et al.*, 1993; Balakrishnan & Anuradha, 1998). Vasankari *et al.* (1998) reported that circulating levels of conjugating dienes were elevated after a 36km, but not a 19km run suggesting exercise duration significantly influenced exercise induced oxidative stress. The use of conjugated dienes as a maker of oxidative stress in humans should be interpreted with caution as they are prevalent in normal diets, thereby confounding whole-tissue peroxidation (Clarkson & Thompson, 2000). Some authors use the ratio of reduced glutathione (GSH) to oxidised glutathione (GSSG) in whole blood as an alternative indication of oxidative stress (Viguie *et al.*, 1993; Groussard *et al.*, 2003). However, circulating levels of GSSG may increase due to release from muscle (Jackson *et al.*, 1995). More recently, assessment of lipid hydroperoxides (LPO) by chemiluminescence has provided a more accurate assessment of lipid peroxidation as well as antioxidant status (Clarkson & Thompson, 2000). Increases in LPO have been documented following a variety of exercise models (Ashton *et al.*, 1998; Schröder *et al.*, 2000; Nieman *et al.*, 2002). Also, F_2-isoprostanes are considered reliable indicators of oxidative stress in vivo, as they reflect the peroxidation of arachidonate during lipid oxidation (Morrow & Roberts, 1999). Recent evidence has shown increased F_2-

isoprostane production following prolonged running (Nieman *et al.*, 2002; Steensberg *et al.*, 2002).

There is evidence that exercise has no effect on indices of free radical activity. Saxton *et al.* (1994) compared indices of free radical mediated damage following maximal eccentric and concentric exercise in leg and arm flexors. Although they observed increased protein carbonyls following concentric knee extension there was no change in plasma levels of TBARS or conjugated dienes following either eccentric or concentric elbow flexion. Lee and co-workers (2002) reported that decreases in muscle strength and increased DOMS following repeated eccentric arm extensions were associated with increased plasma protein carbonyls but observed no change in blood glutathione status. Additionally, marathon running has also been shown to have no effect on immediate or 24h post-exercise plasma TBARS (Rokitzki *et al.*, 1994). However, the failure to report increased free radical activity in these instances are probably due to analytical problems, as the majority of evidence suggests exercise leads to increased indices of oxidative damage.

2.7.3 The protective role of antioxidants

It is evident that free radical generation can lead to uncontrolled damage and degradation of macromolecules that would have profound effects on normal cellular function. Also, oxidative damage can clearly be related to contractile tissue injury both during and following exercise. However, it must be noted that free radicals are essential for a number of biological processes including normal phagocytic function of polymorphonuclear leukocytes (Pyne, 1994b), the biosynthesis of prostanoids and leukotrienes (Halliwell, 1994) and the synthesis of protective and regenerative proteins collectively known as heat shock proteins (HSP) (Fehrenbach & Niess, 1999; Kregel, 2002). Also, free radicals have been implicated in cellular adaptation by influencing gene expression (Jackson, 1999). Secondly, endogenous antioxidants provide a protective function to control free radical production and limit oxidative damage (Goldfarb, 1999).

Antioxidants are substances which can; (i) prevent the formation of radicals, (ii) scavenge the radical species and convert them to less reactive molecules, (iii) assist in repair of damage initiated by radicals, and (iv) assist with other agents to create a favourable environment to protect by supplying reducing equivalents (Goldfarb, 1999). They are separated into enzymatic, superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), glutaredoxin (GRX) and thioredoxin (TRX); and non-enzymatic antioxidants, including nutritionally derived vitamins and provitamins (vitamin E, vitamin C and β–carotene), flavonoids and polyphenols, proteins or peptides containing thiol groups (glutathione) plus various compounds such as ubiquinone and uric acid. The specific functions of these antioxidants are summarised in Table 2.3.

It is important to recognise that although most antioxidants are located in specific cellular sites or compartments antioxidant defence mechanisms involve the interaction of redox-based antioxidant cycles and non-redox-based antioxidants, which act additively and synergistically in an "antioxidant chain reaction" (Sen, 1995). Antioxidant enzymes are located in tissues with high oxidative capacity, including the brain, liver and heart (Kanter, 1994). Research has shown that skeletal muscle antioxidant enzymes are up regulated following exposure to endurance training in humans (Jenkins, 1988; Hellsten *et al.*, 1996) and rodents (Alessio & Goldfarb, 1988; Criswell *et al.*, 1993). This response to exercise training was has been extensively reviewed elsewhere (Powers *et al.*, 1999). Interestingly, the majority of investigations that report increased antioxidant enzyme activity with exercise training suggest elevations in the order of 10 to 15%. However, they also suggest oxidative enzyme activity increases to a much greater extent reducing the ratio of antioxidant protection to oxidative capacity. This highlights the increased demand for nutritional exogenous antioxidants in physically active individuals.

Table 2.3: The location and function of both enzymatic and non-enzymatic antioxidants (adapted from König et al., 2001 and Kanter, 1994).

Antioxidant	Location	Function
Enzymatic		
Superoxide dismutase (SOD)	Mitochondria, cytosol	Dismutases superoxide ($O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2$)
Glutathione peroxidase (GPX)	Mitochondria, cytosol, cell membranes	Reduces hydrogen peroxide ($H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$) and organic hydroperoxides ($ROOH + 2GSH \rightarrow GSSG + ROH + H_2O$)
Catalase (CAT)	Peroxisomes	Reduces hydrogen peroxide ($H_2O_2 + H_2O_2 \rightarrow 2H_2O + O_2$)
Glutaredoxin (GRX)	Cytosol	Protection and repair of protein and non-protein thiols
Thioredoxin (TRX)	Cytosol	Catalyses the reduction of protein S-S bridges Removes hydrogen peroxide Scavenges free radicals
Non-enzymatic		
Vitamin C	Aqueous phase of cells	Scavenges free radicals Recycles vitamin E
Vitamin E	Cell membranes	Break lipid peroxidation chain reactions, reduces several ROS to less reactive forms
Carotenoids	Cell membranes	Scavenges free radicals Protection against lipid peroxidation
Glutathione (GSH)	Ubiquitous non-protein thiols	Scavenges free radicals Removes hydrogen and organic peroxide in a GPX catalysed reaction
Flavonoids/Polphenols	Cell membranes	Scavenges free radicals Metal chelator
Ubiquinones	Cell membranes	Scavenges oxygen radicals and singlet oxygen Recycles vitamin E
Uric acid	Ubiquitous	Scavenges hydroxyl radicals

The principal nutritional antioxidants are the lipid soluble vitamin E and β-carotene and the aqueous vitamin C (ascorbate). Vitamin E is often considered the most important antioxidant because of its association with lipid membranes and role in breaking the chain of lipid peroxidation. Vitamin E is the collective term used for eight naturally occurring lipid soluble nutrients (4 tocopherols and 4 tocotrientols). The derivative α-tocopherol has the greatest biological activity and the greatest free radical scavenging ability (Kanter, 1994) and is the most prevalent in foods (Meydani *et al.*, 1993). When a lipid peroxyl radical collides with vitamin E, the peroxyl radical is converted to a relatively unreactive hydroperoxide, while the vitamin E molecule is converted to the vitamin E radical. This radical is also considered non-reactive and can be reconverted to vitamin E or undergoes further reaction to harmless byproducts (Packer, 1997). Vitamin E is the major change breaking antioxidant present in the mitochondria, thus plays a significant role in reducing radical generation from oxidative phosphorylation. Vitamin E deficiency is extremely rare in humans, probably due to the efficient regeneration of the radical form (chromanoxyl) to its radical quenching form. This occurs through the interaction of a number of non-enzymatic and enzymatic pathways including vitamin C (Figure 2.7). Beta-carotene is one of a class of lipid soluble compounds called carotenoids. Its antioxidant properties include the reduction of singlet oxygen, but β-carotene is also the precursor for vitamin A. In humans, carotenoids are present in adipose, tissue and muscle (Sen, 2001).

Vitamin C (ascorbic acid) has a variety of biological roles including the synthesis of collagen. It is an essential co-factor in a number of hydroxylases responsible for hydroxylation of collagen an essential connective tissue. Vitamin C also acts as a free radical scavenger, neutralising ROS including superoxide, hydrogen peroxide and hypochlorous acid during which it is converted to dehydroascorbic acid. Glutathione is involved in the conversion of dehydroascorbic acid to ascorbate:

$$\text{Dehydroascorbate} + 2\text{GSH} \rightarrow \text{ascorbate} + \text{GSSG}$$

Vitamin C is also reduced to the ascorbate radical during vitamin E resynthesis. This too is converted back to ascorbate by glutathione (Packer, 1997). The majority of vitamin C in foods (80-90%) is in the form ascorbic acid (Rumsey & Levine, 1998). Absorbed in the small intestine vitamin C is transported as dehydroascorbate and reduced to ascorbic acid internally. Evans *et al.* (1992) estimated that in human blood, 38% of vitamin C is in the plasma, 36% in erythrocytes, 16% in platelets, 6% in mononuclear cells and 4% in polymorphonuclear leukocytes. However, the largest *in vivo* stores are found in skeletal muscle (Levine *et al.*, 1999). Dietary requirements for this antioxidant are debated. The current recommended daily allowance (RDA) is 60mg, however in a well controlled investigation a daily dose of 200mg per day was recommended (Levine *et al.*, 1996). This was based of the fact that at this particular dosage bioavailability was 100% and leukocytes appeared saturated. Larger doses have been proposed to act as potent pro-oxidants in the presence of iron (Paolini *et al.*, 1999). This assertion warrants further study in highly active individuals.

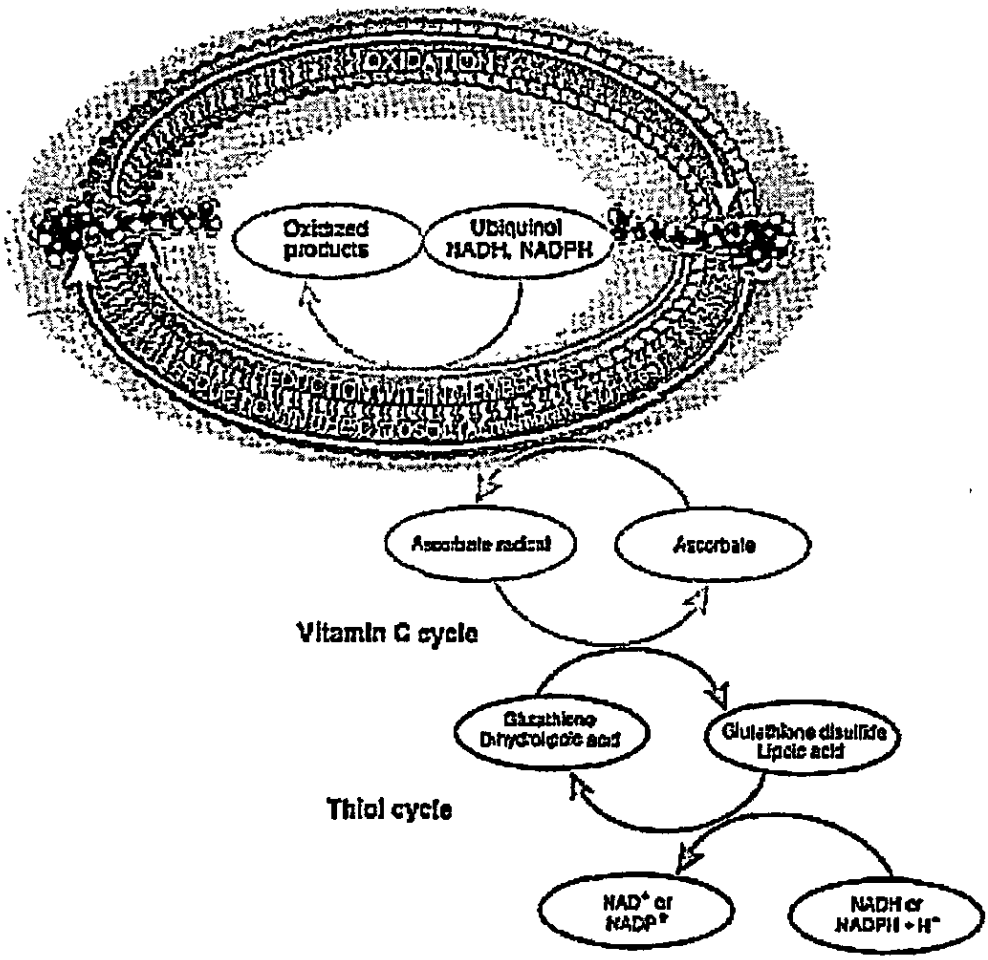


Figure 2.8: The interaction of antioxidants to regenerate vitamin E from its radical form by vitamin C or ubiquinol and indirectly by thiols, glutathione or lipoic acid. (Taken from Packer, 1996)

Gleeson and co-workers (1987) reported increased plasma concentrations of ascorbic acid following a 21km run. However, 24h post-exercise plasma concentrations were decreased to 20% below pre-exercise values, remaining suppressed up to 48h post-exercise. The increase in ascorbic acid was associated with elevations in serum cortisol leading the authors to suggest that increases in ascorbic acid was associated with a concomitant release of cortisol and ascorbic acid from the adrenal glands. Similar increases were reported 5min following a half-marathon, 24h after values returned to pre-exercise levels (Duthie *et al.*, 1990). However, the post-exercise increases may be explained in part by a 6% increase in plasma volume. The authors also reported no change in vitamin

concentrations following exercise. Camus and co-workers (1994) observed contrasting findings with ascorbic acid concentrations following downhill running and uphill walking. Plasma ascorbic acid concentrations were decreased 20min into downhill running, returning to resting values post-exercise. Uphill walking, although at a similar intensity and therefore cortisol production, produced little change in plasma ascorbic acid concentrations. Interestingly, the authors observed increased neutrophil MPO activity following downhill exercise and suggested the increased radical production associated with neutrophil activation might be responsible for reductions in ascorbic acid concentrations. In an earlier investigation, Camus *et al.* (1990) showed increases in plasma α-tocopherol during cycling, peaking at the end of exercise. Unfortunately they failed to account for changes in plasma volume that could have lead to misinterpretations.

2.8 Effect of antioxidants on exercise-induced muscle damage.

Much research has focused on attempts to alleviate the symptoms of EIMD. A variety of techniques have been employed and subsequently advocated or rejected. These include the use of NSAIDs (Almekinders, 1999; Semark *et al.*, 1999; Sayers *et al.*, 2001), massage (Smith *et al.*, 1994; Farr *et al.*, 2002; Hilbert *et al.*, 2003), acupuncture (Lin & Yang, 1999), Cryotherapy (Eston & Peters, 1999), warm-up and stretching (High *et al.*, 1989; Smith *et al.*, 1993; Rodenburg *et al.*, 1994), immobilization versus light exercise (Sayers *et al.*, 2000a; Sayers *et al.*, 2000b) and even hyperbaric oxygen therapy (Mekjavic *et al.*, 2000). However, the majority of investigations into the treatment of EIMD have employed nutritional interventions, most commonly antioxidants. Evidence for the production of free radicals both during and following exercise supports the involvement of oxidative stress in EIMD. Indeed, increased production of ROS by polymorphonuclear leukocytes during inflammation suggests free radicals may significantly contribute to post-exercise muscle damage. Hence, investigations have employed supplementation with dietary antioxidants in an attempt to abrogate exercise-induced oxidative stress and alleviate symptoms of muscle damage. This has been reviewed extensively on a number of occasions (Jenkins, 1993; Kanter, 1994; Dekkers *et al.*, 1996; Goldfarb, 1999; Clarkson & Thompson, 2000; Sen, 2001).

2.8.1 Single antioxidant supplementation

The bulk of investigations employing antioxidant supplementation have used single antioxidants, most commonly vitamin E (Figure 2.8). Dillard and colleagues (1978) were amongst the first to investigate the effects of 2 weeks vitamin E supplementation ($1200IU.day^{-1}$[1]) prior to 60min cycling. Subjects performed the exercise with and without exposure to ozone. Following exercise, increases in expired pentane were reduced with antioxidant supplementation. Francis and Hobbler (1986), showed no effect of a smaller dosage ($600UI.day^{-1}$) on muscle soreness up to 24h following eccentric exercise. Sumida *et al.* (1989) had subjects perform and incremental exhaustive cycling test, ingest vitamin E ($300mg.day^{-1}$) for four weeks and repeat the test. They observed lower MDA and reduced activity of muscle enzymes following the second bout suggestive of a protective effect with vitamin E supplementation. However, they failed to account for the repeated bout effect which could have influenced oxidative markers following the second bout. In a longitudinal study Rokitzski *et al.* (1994) supplemented competitive cyclists with α-tocopherol ($300mg.day^{-1}$) or a placebo for 5 months. Following strenuous cycle training, individuals supplemented with α-tocopherol reported an abrogation of increased serum MDA and CK activity compared to the placebo group. Cannon and co-workers (1990) supplemented young (22-29yrs) and older (55-74yrs) subjects with vitamin E for 48 days prior to a 45min bout of downhill running. They observed a reduction in the CK response during recovery from downhill running compared to a placebo. Additionally, they reported a quicker recovery of CK activity to basal levels as well as a greater neutrophilia and higher peak CK response in the younger subjects. However, supplementation with vitamin E appeared to eliminate these age differences. Peak CK activity was positively correlated with increased neutrophil superoxide release ($r = 0.75$) supporting the hypothesis that neutrophil activity is associated with ongoing membrane damage following exercise. In another study, Cannon *et al.* (1991) investigated the effects of vitamin E supplementation ($800IU.day^{-1}$) for 8 weeks prior to the same downhill running protocol in 21 male subjects. They reported no effect on TNF-α, but a significant reduction in IL-1 secretion from circulating mononuclear leukocytes. This was

[1] 1 IU is approximately equivalent to 0 67mg

accompanied by a reduction in IL-6 secretion, even though this cytokine was unaffected by exercise. The authors suggested that mononuclear leukocytes contributed to muscle proteolysis following exercise and that vitamin E appeared to influence the associated degradation of muscle.

Meydani and co-workers (1993) also reported a beneficial effect of vitamin E supplementation on markers of oxidative stress. In a similar, placebo controlled, study to that of Cannon *et al.* (1990) they reported a reduction in urinary TBARS as well as muscle conjugated dienes during recovery from downhill running (12 days). They suggested vitamin supplementation alleviated exercise-induced free radical mediated muscle damage. McBride and colleagues (1998) investigated the effect of vitamin E supplementation on markers of muscle damage and oxidative stress following resistance exercise. Following two weeks supplementation (1200IU.day^{-1}) resting plasma MDA was significantly greater in the placebo group. Post-exercise increases in CK activity were markedly lower at 24h with vitamin supplementation providing further support for the reduction of membrane disruption following muscle damaging exercise. However, both Beaton *et al.* (2002) and Jakeman and Maxwell (1998) showed no effect of prolonged vitamin E supplementation on muscular dysfunction following eccentric leg exercise. Additionally, Beaton *et al.* (2002) showed no effect of the vitamin supplement on ultrastructural damage (z-disk disruption) or macrophage infiltration following exercise. Recently, Niess *et al.* (2002) observed a temporary reduction in the post-exercise expression of a heat shock protein (HSP72) following 8 days α-tocopherol supplementation (500IU.day^{-1}). The authors suggested exhaustive exercise is associated with increased HSP72 expression by leukocytes, that is transiently abrogated with antioxidant supplementation implying ROS involvement in heat shock protein production.

Table 2.4 includes the majority of investigations employing vitamin E supplementation following strenuous and/or damaging exercise. Findings to date appear to support supplementation with vitamin E, particularly to reduce makers of oxidative stress. However, inconsistencies in exercise modes, supplementation dosage and durations as

well as markers of oxidative and muscle damage make specific recommendations difficult.

Table 2.4: A summary of investigations into vitamin E supplementation and the effects on markers of exercise induced muscle damage, oxidative stress and immune function. (TBARS, thiobarbituric acid reacting substances; PE, post-exercise; PL, placebo; NE, no effect; CK, creatine kinase activity; DOMS, delayed onset muscle soreness; HRmax, maximal heart rate; $\dot{V}O_2max$, maximal oxygen uptake; MDA, malondialdehyde; TAS, total antioxidant status; DNA, deoxyribonucleic acid; WBC, white blood cells; IL, interleukin; HSP, heat shock protein; AT, anaerobic threshold)

Reference	Supplementation strategy	Exercise model	Antioxidant status	Outcomes
Beaton *et al.* (2002)	1200IU.day^{-1} (30 days)	isokinetic eccentric contractions	> plasma vit E. cf. PL	NE on torque, ultrastructural damage, macrophage infiltration or CK
Boyer & Goldfarb* (1996)	800IU.day^{-1} (4 weeks)	eccentric exercise	NE on plasma TBARS	NE on CK & DOMS
Cannon *et al* (1990)	400IU day^{-1} (8 weeks)	45min downhill run (75% HRmax)	> plasma vit. E cf. PL	↓ CK cf. PL (> in elderly subjects >55yrs)
Cannon *et al* (1991)	800IU.day^{-1} (8 weeks)	45min downhill run (75% HRmax)	> plasma vit. E cf. PL	↓ IL-6 & IL-1β, NE on TNF-α
Dillard *et al.* (1978)	1200IU.day^{-1} (2 weeks)	cycling (50% $\dot{V}O_2max$)	-	↓ expired pentane at rest & during exercise
Francis & Hoobler (1986)	600IU.day^{-1} (2 days + 2 days PE)	eccentric exercise	-	NE on DOMS (2days PE)
Hartman *et al.* (1995)	1200mg.day^{-1} (2 weeks)	run to exhaustion	↓ MDA	↓ DNA damage (WBC)
Helgheim *et al.* (1979)	-	strenuous exercise	-	NE on CK & MDA
Jakeman & Maxwell (1993)	400mg.day^{-1} (3 weeks + 7 days PE)	60min stepping	> plasma TAS cf. PL	NE on muscle function
Lewis *et al.* (1992)	800IU.day^{-1} (4 weeks)	3-4h cycling (75% $\dot{V}O_2max$)		NE on CK & DOMS
McBride *et al.* (1998)	1200IU.day^{-1} (2 weeks)	resistance exercise	↓ MDA cf. PL	↓ CK cf. PL

* unpublished data cited in Goldfarb (1999)

Table 2.4 continued.....

Meydani *et al.* (1993)	800IU.day^{-1} (8 weeks)	45min downhill run (75% HRmax)	> plasma vit E cf. PL	↓ TBARS & conjugated dienes
Niess *et al* (2002)	500IU.day^{-1} (8 days)	run to exhaustion (~30min)	> plasma vit. E cf. PL	↓ HSP72 mRNA cf. PL
Rokitski *et al.* (1994)	400IU day^{-1} (5 months)	cycling training	> plasma vit. E cf. PL	↓ CK & MDA
Sacheck *et al.* (2000)	9.8mg or 2.9mg day^{-1} (habitual dietary fat intake)	45min downhill run (75% HRmax)	NE on plasma vit. E	NE on CK, MDA & conjugated dienes PE
Simon-Schnass *et al.* (2000)	400mg day^{-1} (4 weeks)	high altitude exercise	-	↑ AT & ↓ expired pentane
Sumida *et al.* (1989)	300mg.day^{-1} (4 weeks)	run to exhaustion	-	↓ MDA

Research into the effects of vitamin C supplementation is less well documented. This may be due to the specific action of both antioxidants. Vitamin E is perhaps more suitable to positively influence free radical damage due to its site of action. However, the regenerative actions of vitamin C on vitamin E as well as the distribution throughout the aqueous medium in muscle tissue may make it more effective in alleviating oxidative damage. Jakeman and Maxwell (1993) investigated the effects of 3 weeks supplementation with vitamin C ($400mg.day^{-1}$) prior to benching stepping exercise. The combination of supplementation and exercise elevated plasma vitamin C as well as total antioxidant status compared to a placebo. They also observed a reduction in muscle dysfunction in the *triceps surea* following exercise with vitamin C. This was accompanied by a reduction in low frequency fatigue by tetanic stimulation (20:50Hz) compared to the placebo. These data suggest vitamin C may reduce damage from eccentrically based exercise. Kaminski and Boal (1992) examined the effects of ascorbic acid supplementation for 3 days prior and 7 days following eccentric exercise in the calf muscles. It was noted that treatment with ascorbic acid reduced muscle soreness by 33% in approximately half of the subjects. Alessio *et al.* (1997) reported a reduction oxidative stress, measured as the ratio of plasma TBARS to oxygen radical absorbance capacity, following aerobic exercise with 2 weeks vitamin C supplementation ($1g.day^{-1}$). They speculated that short term vitamin C supplementation may act to effectively regenerate vitamin E stores.

Nieman and colleagues (2000, 2002) investigated the effect of large vitamin C dosage ($1.5g.day^{-1}$) on cytokine production and oxidative stress following exhaustive endurance exercise. In their initial investigation the authors reported a reduction the post-exercise increase in a number of inflammatory cytokines (IL-10, IL-1ra and a tendency with IL-6, IL-8). It was suggested that decreased inflammatory cytokine production following exercise was reflective of reduced muscle damage, as injured cells were associated with increased cytokine production. However, in a subsequent investigation the authors reported no effect of the same dosage of vitamin C on a number of cytokines, leukocytes or markers of oxidative damage (LPO and isoprostanes) following exhaustive exercise (Nieman *et al.*, 2002). Ashton and colleagues (1999) observed no effect of an acute dose

(1g) 2h prior to exercise to exhaustion on the direct assessment of ROS activity (ESR) as well as on LPO compared to a placebo. Similarly, Thompson *et al.* (2000) reported no effect of an acute dosage 2h before intermittent shuttle running on markers of muscle damage (CK and AST) and oxidative stress (MDA). However, in another study employing prolonged supplementation (2 weeks) the authors showed modest beneficial effect of vitamin C supplementation ($400mg.day^{-1}$) on IL-6 and MDA as well as muscle soreness following intermittent exercise (Thompson *et al.*, 2001b).

Table 2.5 summarises investigations employing vitamin C supplementation and the effect on muscle damage, oxidative stress and immune function. Collectively, evidence may appear equivocal with regard to vitamin C supplementation. However, as with vitamin E inconsistencies in experimental design and assessment of intervention effects make drawing general conclusions difficult. Also, the is a paucity of good research into vitamin C compared with other antioxidants, thus more research is needed to establish the effects of this antioxidant on EIMD.

Table 2.5: A summary of investigations into vitamin C supplementation and the effects on markers of exercise induced muscle damage, oxidative stress and immune function. (TBARS, thiobarbituric acid reacting substances; PE, post-exercise; PL, placebo; ORAC, oxygen radical absorbance capacity; NE, no effect; ESP, electron spin resonance spectroscopy; DOMS, delayed onset muscle soreness; $\dot{V}O_2max$, maximal oxygen uptake; MDA, malondialdehyde; TAS, total antioxidant status; LFF, low frequency fatigue; MVC, maximal voluntary contraction; IL – interleukin; LPO – lipid hydroperoxides.)

Reference	Supplementation strategy	Exercise model	Antioxidant status	Outcomes
Alessio *et al* (1997)	1g day^{-1} (2 weeks)	30min running (80% $\dot{V}O_2max$)	↓ TBARS PE cf. PL	↓ oxidative stress ratio (TBARS.ORAC) cf. PL
Ashton *et al.* (1999)	1g acute dose (2h pre-exercise)	cycling to exhaustion (~15min)	-	NE on LPO, ESR cf. PL
Kaminski & Boal (1992)	3g.day^{-1} (3 days + 10days)	eccentric leg exercise	-	↓ muscle soreness
Jakeman & Maxwell (1993)	400mg day^{-1} (3 weeks + 7 days PE)	60min stepping	> plasma TAS cf PL	Vit. C = ↑ recovery MVC & LFF (20:50 Hz) cf. PL
Nieman *et al* (1997)	1g day^{-1} (8 days)	2.5h run (75-80% $\dot{V}O_2max$)	-	NE on IL-6, lymphocyte proliferation & leukocyte subsets
Nieman *et al* (2000)	0.5 & 1.5g day^{-1} (7 days + 2 days PE)	ultramarathon	> plasma vit. C cf. PL	↓ IL-6, IL-10, IL-1ra & IL-8 with vit. C (1.5g .day^{-1})
Nieman *et al.* (2002)	1.5g day^{-1} (7 days)	ultramarathon	> plasma vit. C cf. PL	NE on WBC, cytokines, LPO & isoprostanes PE
Peters *et al.* (2001)	1g day^{-1} (9 days)	ultramarathon	> plasma vit C cf. PL	↑ CRP & ↓ cortisol cf. PL
Thompson *et al.* (2001)	1g acute dose (2h pre-exercise)	intermittent running	> plasma vit. C cf. PL	NE on CK, AST & MDA
Thompson *et al* (2001)	400mg.day^{-1} (2 weeks)	intermittent running	> plasma vit. C cf. PL	↓ DOMS, MDA, IL-6 PE
Vasankari *et al.* (1998)	2g pre + 3g PE	27km run	-	↓ conjugated dienes

2.8.2 Mixed antioxidant supplementation

Several investigations have examined combination of antioxidants on exercise-induced muscle and oxidative damage (Table 2.6). A combination of vitamin E (400IU.day^{-1}) and C (200mg.day^{-1}) supplementation for 4.5 weeks was investigated prior to a marathon (Rokitzki *et al.*, 1994). Immediately and 24h post-exercise, MDA was reduced in both groups. However, CK activity was significantly lower at 24h post-exercise with the supplement compared to a placebo, suggesting the supplement offered some protection against EIMD. Kanter *et al.* (1993) examined the effect of a mixed antioxidant supplement, containing vitamin C (1g.day^{-1}), vitamin E (592mg.day^{-1}) and β-carotene (30mg.day^{-1}) on oxidative stress following variable intensity treadmill running. Following 6 weeks supplementation resting serum MDA and expired pentane were lower in the antioxidant supplemented group compared to a placebo group. Exercise resulted in increased serum MDA and expired pentane in both groups but post-exercise levels were lower with the antioxidant supplement. Viguie and co-workers (1989) reported that an antioxidant mixture of 10mg of β-carotene, 1g of vitamin C and 800IU of vitamin E for 8 weeks maintained plasma glutathione status and had beneficial effects on the post-exercise elevation of CK activity following downhill running. Contrastingly, Childs *et al.* (2001) compared the effects of vitamin C (12.5mg.kgBW^{-1}.day^{-1}) and N-acetyl-cysteine (10mg.kgBW^{-1}.day^{-1}) supplementation to a placebo for 7 days following eccentric exercise. Exercise-induced elevations in CK, LDH and Mb were greater following antioxidant supplementation, as were markers of oxidative damage (LPO and F_2-isoprostanes). It was concluded that post-exercise supplementation with this particular antioxidant combination transiently increased tissue damage and oxidative stress. This was attributed to an increased concentration of free iron associated with both acute inflammation and the supplement itself, contributing to a pro-oxidant milieu for vitamin C.

Table 2.6: A summary of investigations into mixed antioxidant supplementation and the effects on markers of exercise induced muscle damage, oxidative stress and immune function. (PE, post-exercise; PL, placebo; NE, no effect; CK, creatine kinase activity; Mb, myoglobin; NAC, N-acetyl-cysteine; TAS, total antioxidant status; MDA, malondialdehyde; LDL, low density lipoprotein; LPO – lipid hydroperoxides; GSH, glutathione; $\dot{V}O_2max$, maximal oxygen uptake)

Reference	Antioxidants	Supplementation strategy	Exercise model	Antioxidant status	Outcomes
Childs *et al.* (2001)	Vit. C + NAC	12.5+ 10mg.kg^{-1}.day^{-1} (7days PE)	eccentric elbow flexion	> plasma TAS cf. PL	↑ CK, LDH & Mb plus LPO & isoprostanes with supplement
Dawson *et al.* (2002)	Vit. C + E	1000mg + 1000IU.day^{-1} (4 wks)	21km run	> plasma vit C & E	NE on CK, Mb, MDA or ultrastructural damage
Dragan *et al.* (1991)	Selenium, vit E, glutathione & cysteine	- (3 weeks)	2h cycling	-	↓ MDA cf. PL
Kaikkonen *et al.* (1998)	Vit. E, coenzyme Q10	13 5 + 90mg.day^{-1} (6 weeks)	marathon	> plasma Q10 & vit. E cf. PL	NE on CK, LDL oxidation
Kanter *et al.* (1993)	Vit. E, C & β-carotene	592 + 1000 + 30 mg.day^{-1} (6 weeks)	35min running (60-90% $\dot{V}O_2max$)	↓ MDA & expired pentane cf. PL	↓ MDA & expired pentane PE
Petersen *et al.* (2001)	Vit. C & E	500 + 400mg.day^{-1} (2 weeks + 1 week PE)	90min downhill run (75% $\dot{V}O_2max$)	> plasma vit. C & E cf. PL	NE on cytokine, lymphocyte & CK changes PE
Rokitski *et al.* (1994)	Vit C & E	200mg + 400IU.day^{-1} (4 5 weeks)	marathon	> plasma vit. C & E cf. PL	↓ CK PE cf. PL
Schroder *et al.* (2000)	Vit. E, C & β-carotene	600 + 1000 + 32 mg.day^{-1} (4.5 weeks)	basketball season	↑ TAS	↓ LPO & LPO.TAS
Viguie *et al.* (1989)	Vit. E, C & β-carotene	800IU + 1g + 10 mg day^{-1} (8 weeks)	downhill run (65% $\dot{V}O_2max$)	maintained plasma GSH	↓ CK & LDH

Evidence obtained from investigations into mixed antioxidant supplementation is at present equivocal (Table 2.6). This may be a result of the diversity of antioxidant combinations employed in addition to inconsistencies in experimental protocols. However, it seems that clarification of the effect of single antioxidant supplementation must be completed prior to assessing the potential role of any combination of antioxidants.

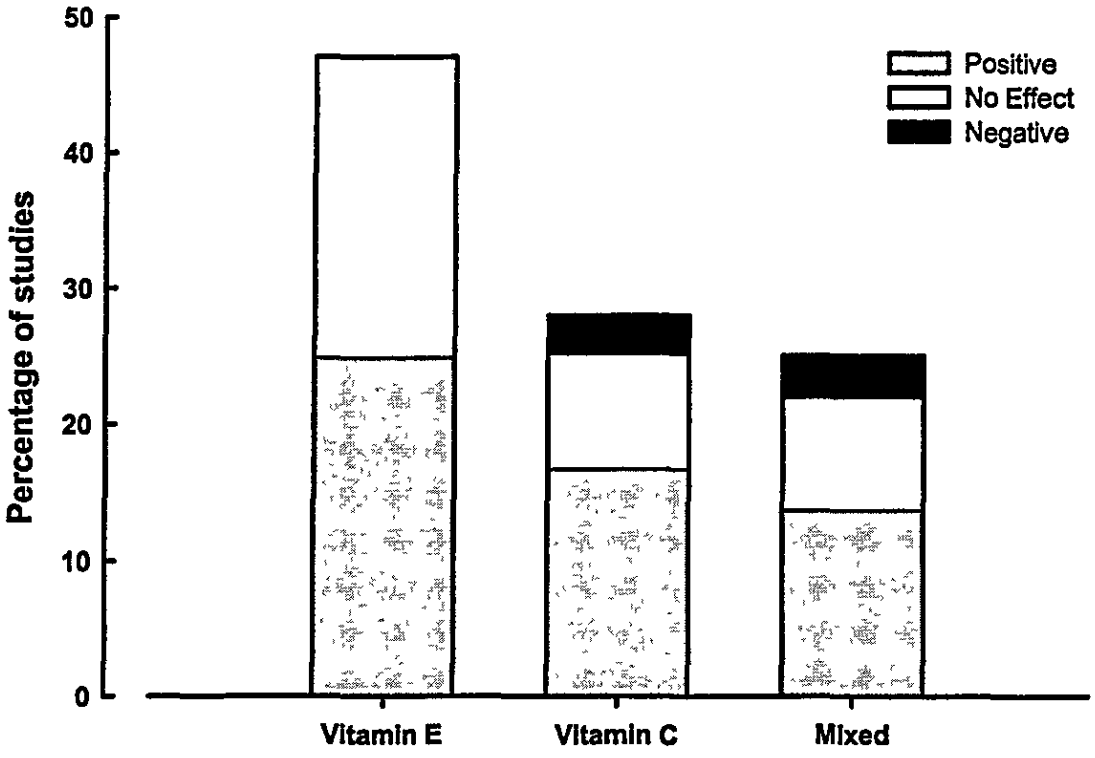


Figure 2.9: A summary of investigations (n = 37) employing dietary antioxidant supplementation and the general outcome of these investigations on markers of oxidative and muscle damage as well as perturbations in immune parameters.

2.9 Summary

In conclusion, the question if and to what extent antioxidants prevent oxidative stress and exercise-induced muscle damage cannot be answered definitively. Moreover, the interactions between antioxidants, inflammatory mediators and oxidative stress remain elusive. There is, however, considerable scope for the involvement of free radicals in the aetiology of exercise-induced muscle damage and soreness. Therefore the administration of antioxidants may have a potential role in the abrogation of deleterious effects associated with oxidative stress and muscle damage. Furthermore, their administration may help to clarify the interaction between the various elements that contribute to exercise-induced muscle damage.

CHAPTER THREE

General Methods

3.1 Introduction

This chapter outlines the methodologies used in the investigations reported in each chapter. All experiments described in this thesis were conducted in the Exercise Physiology Laboratories at Loughborough University and had received approval from the University Ethical Committee before commencement. All immunological measurements were made at Unilever Research, Colworth. A more detailed description of these techniques can be obtained elsewhere (Hurst, 2003 *Ph.D thesis*).

Subjects were all male volunteers (aged 18-30) the majority of whom were undergraduate students at Loughborough University recruited through local advertisements. Prior to participation subjects were informed both verbally and in writing about the exact nature and demands of each investigation. Additionally, subjects were given the opportunity to withdraw from the investigation without reason and at any time. Subjects were required to complete a mandatory medical questionnaire (Appendix I) and give written informed consent before taking part in any investigation. Any subject with a medical condition that might have influenced the investigation or compromised their own health was excluded from taking part in the study. The physical activity status of each subject was self reported before participation in any of the tests.

3.2 Anthropometry

Body mass was measured using a beam balance (Avery Ltd. UK). Subjects were weighed wearing only light clothing during preliminary measurements and nude before and after exercise. The latter was to determine change in body mass through sweating and subjects were asked to dry themselves prior to these measurements.

Height was assessed using a fixed stadiometer (Holtain Ltd., UK). Subjects were measured without footwear, heels together resting against the stadiometer. Whilst the subjects inspired deeply the movable indicator was lower until in contact with the superior point of the head. Measurements were taken to the nearest 0.1cm.

Skinfold thickness was measured as outlined in the ACSM guidelines to exercise testing and prescription (ACSM, 1995). All measurements were made on the right-hand and side of the body using pre-calibrated calipers (Holtain Ltd., UK). Sites for skinfold thickness measurements were initially marked and then measured in sequence, rotating through the sites in order to allow time for skin to regain normal texture and thickness. Sites measured were; i) biceps; a vertical fold taken on the anterior aspect of the arm over the biceps muscle 1cm above the level taken for the triceps, ii) triceps; a vertical fold on the posterior midline of the upper right arm, halfway between the acromion and olecranon process, with the arm held freely to the side of the body, iii) subscapular; a diagonal fold taken at 45° 1 to 2cm below the inferior angle of the scapula and iv) suprailium; an oblique fold in line with the natural angle of the iliac crest taken in the anterior auxiliary line immediately superior to the iliac crest. Duplicate measures were taken at each of the sites described. If the measurements varied by more than 1mm a third measurement was made. The mean skinfold thickness was noted for each site and the sum of all four sites was recorded. Values for skinfold thickness are presented as the sum of these four sites throughout.

3.3 Maximal oxygen uptake

Prior to participation in the main exercise trials subjects were required to complete preliminary exercise tests to determine exercise intensity. For the treadmill protocols individuals completed two tests. The first was designed to determine the oxygen cost ($\dot{V}O_2$) of running at various speeds. Subjects exercised on a treadmill (Technogym, Italy) for approximately 16min during which the treadmill speed was increased every fourth minute. Expired air samples, heart rates, monitored by short range telemetry (Polar 8810, Finland), and ratings of perceived

exertion (RPE) (Borg, 1973) were collected during the final minute of each stage. Maximal oxygen uptake ($\dot{V}O_2max$) was determined using an incremental continuous treadmill test during which the gradient was increased at three minute intervals until the subject reached volitional exhaustion. During this test the speed remained constant (10-13 $km.h^{-1}$) and expired air samples, heart rates and ratings of perceived exertion were collected during the final minute of each stage as well as at volitional exhaustion (Williams *et al.*, 1990). The criteria to determine whether this point was reflective of the 'true' $\dot{V}O_2max$ were; i) a respiratory exchange ratio of greater than 1.15, ii) an increase in $\dot{V}O_2$ less than or equal to 5$ml.kg^{-1}min^{-1}$ with an increase in gradient, and iii) attainment of age predicted maximal heart rate (±10 $b.min^{-1}$) all at termination of the test.. A simple linear regression for $\dot{V}O_2$ against running speed was calculated and a value relative to the $\dot{V}O_2max$ was used to predict exercise intensity at a given speed. Additionally, subjects were required to complete a familiarisation session during which they ran at the calculated speed to ensure it corresponded to the correct intensity (± 2-3%).

Prior to participation in the intermittent shuttle running protocol subjects completed the field based multi-stage fitness test (MSFT). This test required subjects to run between to lines 20m apart to an audio signal from a compact disc player. The intervals between signals progressively shortened throughout the test demanding an every increasing running speed at each successive level. Subjects were verbally encouraged during the test until they either reached volitional exhaustion or were are unable to maintain the required intensity. A value for $\dot{V}O_2max$ was estimated from the level attained. This value was then used to calculate running speeds required to elicit 55% and 95% of $\dot{V}O_2max$ according a predetermined regression equation (Ramsbottom *et al.*, 1988).

3.4 Treadmill protocols

A calibrated motorised treadmill (Technogym, Italy) was reversed for the treadmill protocols to facilitate negative gradients. A safety support rail was constructed around the perimeter of

the treadmill to ensure subjects were able to dismount with ease. A modified clinical goniometer (MIE Medical Research Ltd, UK) was used to monitor the gradient of the treadmill. For the downhill gradients a value of -10% or -9° (0.05 π radians) was employed (Chapters 4 & 5). Individuals were required to run at speeds equal to 70% $\dot{V}O_2$max whilst running on the level for 60min. During treadmill running subject's heart rates and ratings of perceived exertion were recorded at regular intervals. Values for ventilation ($\dot{V}_E$), oxygen uptake ($\dot{V}O_2$) and respiratory exchange ratio (RER) were determined via indirect calorimetry from expired air sample collection during treadmill exercise.

3.5 Intermittent shuttle running

The Loughborough Intermittent Shuttle Test (LIST) is a field test specifically designed to replicate the demands associated with intermittent activity such as soccer (Nicholas *et al.*, 2000). Subjects were required to exercise at varying intensities for a total of 90min, with average exercise intensity equal to 75% $\dot{V}O_2$max. Specifically, subjects completed six times 15min periods of variable-intensity running (55% and 95% $\dot{V}O_2$max), walking and sprinting between two lines 20m apart. Following each 15min set subjects rested for 3min, giving a total rest time of 15min. Variable running speeds were estimated from individual' $\dot{V}O_2$max values, and were dictated by an audio signal from a microcomputer (Hewlett Packard, USA) with software developed for this purpose (H.K.A. Lakomy, Loughborough University). An additional different audio signal was used at 10m to ensure the correct pace was maintained throughout the test. Sprint times were measured using two infra-red photoelectric cells (RS Components Ltd, Switzerland) also interfaced with the computer. The LIST was performed in a sports hall of Loughborough University under constant environmental conditions (ambient temperature ~20-23°C, relative humidity ~50-65%, barometric pressure ~740-780mmHg). Figure 3.1 illustrates the composition of this exercise protocol.

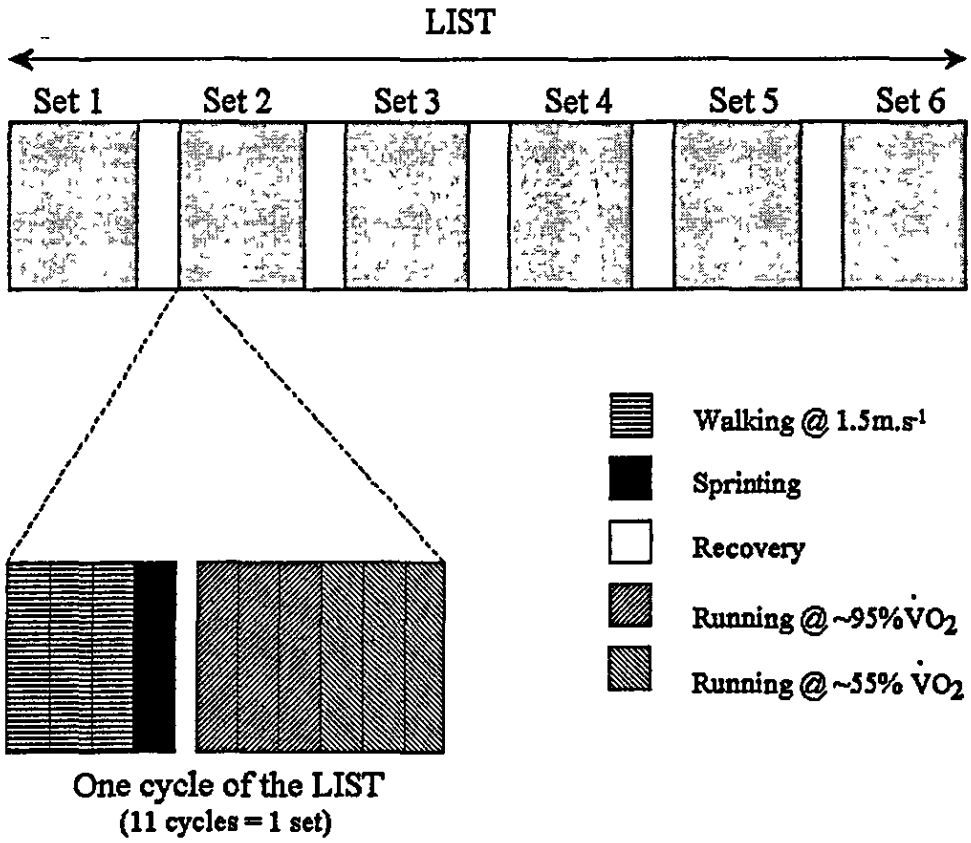


Figure 3.1: A schematic representation of the Loughborough Intermittent Shuttle Test (LIST).

During the completion of the LIST subjects covered 12 km, sprinting approximately 1 km, and changing direction 624 times. Heart rate and ratings of perceived exertion were noted during the final part of each 15min period. Ambient dry and wet bulb temperature were also recorded during each 15min set with a whirling hygrometer (Brannan Thermometers Ltd. UK). Subjects were required to ingest a predetermined volume of water during the LIST equal to 5ml.kg^{-1} pre-exercise, and 2ml.kg^{-1} following each 15min block. This had previously been shown to maintain euhydration (± 1.5% body mass) during intermittent exercise (McGregor *et al.*, 1999).

3.6 Physiological measurements

3.6.1 Expired air analysis

During treadmill exercise, aliquots of expired gas were collected into 200L Douglas bags (Harvard Apparatus, Cambridge) whilst the subject wore a nose clip and breathed through a respiratory value (50ml deadspace) and Falconia tubing. Gas fractions for oxygen and carbon dioxide were measured by sampling through a paramagnetic transducer (Servomex 1440, Crowborough, UK) and infra-red carbon dioxide analyser (Servomex 1440, Crowborough, UK), respectively. The analysers were calibrated prior to and following each test with gases of known composition in the physiological range (British Oxygen Company, London, UK). Expired ventilation ($\dot{V}_E$) was determined using a dry gas meter (Harvard Apparatus, Cambridge, UK) which was checked regularly against a precision 6L Tissot spirometer (Collins Ltd, London, UK) and converted to standard temperature and pressure for a dry gas. Values for oxygen consumption ($\dot{V}O_2$), carbon dioxide production ($\dot{V}CO_2$) and the respiratory exchange ratio (RER) were calculated under the assumptions of the Haldane transformation. Subjects were familiarised with the apparatus to minimise artifacts associated with hyperventilation. The inter-test coefficient of variation for determination of sub-maximal running speed in our laboratory in habituated subjects was 1.7%.

3.6.2 Soreness

Muscle soreness was assessed prior to exercise and then on each subsequent visit to the laboratory. Subjects were asked to rate perceived soreness using a modified Borg CR-10 scale (Borg, 1982) by pointing to a digit and/or verbal descriptor rating muscular soreness from 1 '*not sore*' to 10 '*very, very sore*' (Appendix II). Subjects were encouraged to palpate musculature prior to giving any rating to provoke the sensation of soreness. The intensity of soreness was also rated in specific muscle groups using the 1-10 scale (Chapter 4). Subjects

were also required to make a vertical mark on a horizontal line (100mm) labeled '*not sore*' and '*very, very sore*' at each end when assessing soreness (Chapter 4) (Appendix II).

Previously, it has been demonstrated that the sensation of muscular soreness is often not apparent until subsequent activity is performed (Cleak & Eston, 1992). Thus, subjects were also asked to complete the same procedure following active contraction of the sore muscle groups, termed 'active' soreness. To facilitate this active soreness subjects were required to descend a fixed number of steps (13 steps descending ~2.3m) prior to assessment. (Note; subjects were also required to climb these steps before the assessment of active soreness).

To determine the location of muscle soreness subjects were asked to highlight diagrams of the human musculature prior to rating soreness. Diagrams were simplified into regions of the major muscle groups in the human anatomy and were shown separately as anterior and posterior (Appendix III).

3.6.3 Muscle Function

Changes in muscle function were assessed using a simple trunk flexion text (Chapter 4) and an isokinetic dynamometer (Cybex model 770, LUMEX Inc. USA) interfaced to a computer with CYBEX NORM software (Chapters 5, 6 & 7).

3.6.3.1 Trunk flexion

After a warm-up involving 10min of light callisthenics and a period of stretching, the subjects completed the sit-and-reach test. This test provides a global measure of hamstring, hip and lower back flexibility (Borms & Van Roy, 1996). Subjects were seated on the floor with their bare feet against a sit-and-reach box (HRF Bodycare, UK) about 25-30cm apart. The subjects then slowly reached forward towards their toes while keeping their legs straight and their hands together. The distance from the toes (zero point) was measured in centimetres (positive values were awarded if subjects could reach beyond their toes, and negative values were

awarded if subjects could not reach beyond their toes. The best score from three attempts was recorded

3.6.3.2 Isokinetic dynamometer

Muscle function for leg extension and flexion was determined using an isokinetic dynamometer (Figure 3.2). Prior to assessment on the dynamometer, positional adjustments for leg extension and flexion were made for each subject to ensure movement was restricted to the saggital plane and that the axis of rotation passed through the femoral condyles. Once each subject was seated in the ideal position the positional adjustments were stored on the computer and remained constant throughout testing. The dynamometer was calibrated as outlined by the manufacturer before each study.

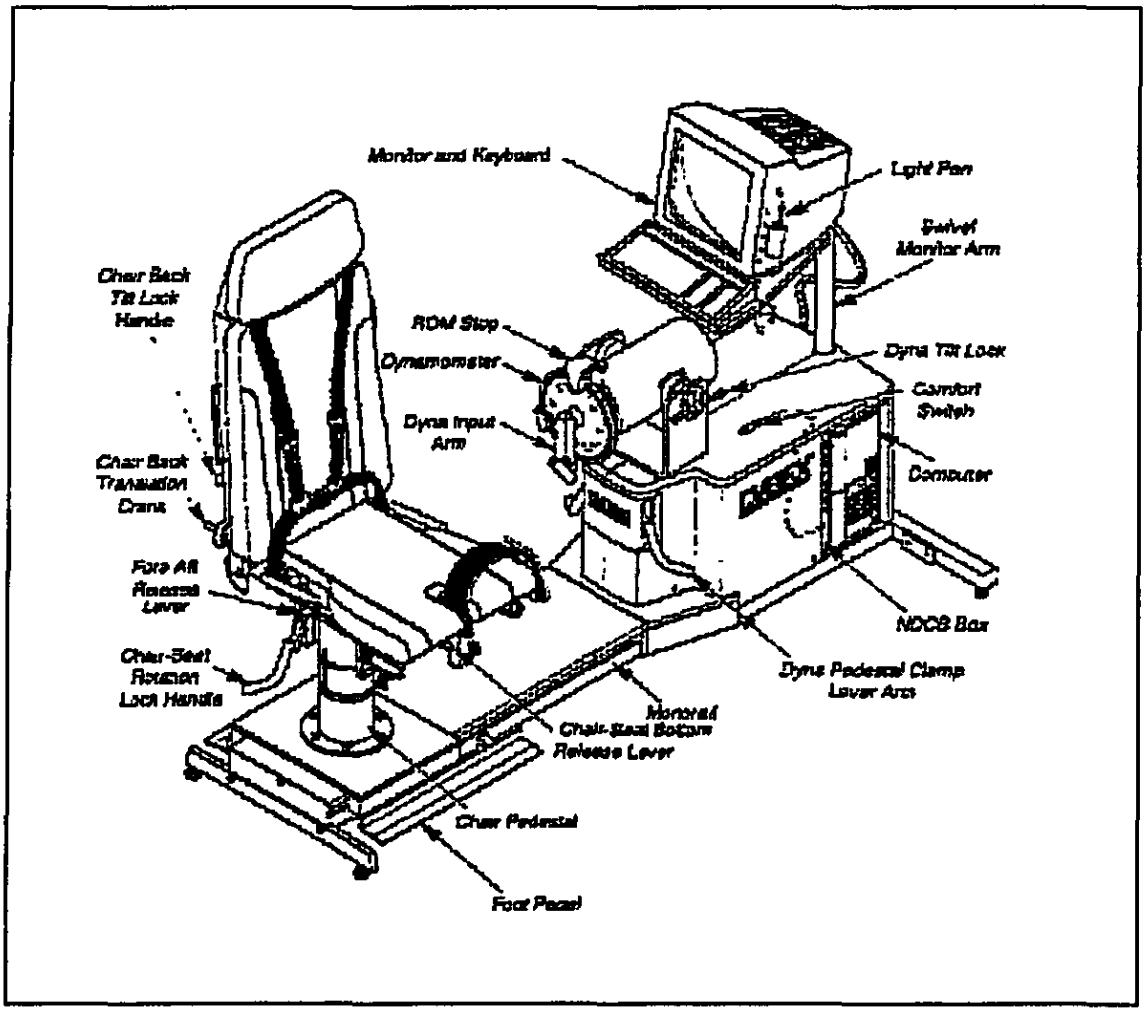


Figure 3.2: Isokinetic dynamometer and associated apparatus (CYBEX^{TM}).

Each assessment was preceded by a standard warm-up, consisting of 5min cycling (Monark, 863e) at 100W and 5min stretching of leg muscle groups. The protocol was specifically designed to measure isometric maximal voluntary contraction (MVC) of the leg extensor and flexor muscles (Chapters 5, 6 & 7), as well as the range of motion (ROM) of the knee (Chapter 7) in the dominant leg. Following a warm-up set of eight repetitions of leg extension and flexion (1.05 rad s^{-1}) subjects completed two repetitions of isometric leg extension for 5s at 60° from anatomical zero and isometric leg flexion for 5s at 20°. MVC were separated by 60s rest periods and subjects were both verbally encouraged and received visual feedback during

each MVC. The highest torque achieved from both repetitions was taken as the peak isometric force at each angle. Prior to assessment of MVC each subject's range of motion (ROM) was determined whilst using the dynamometer (Chapter 7). All measurements were corrected for the effects of gravity.

Due to the novelty of this particular apparatus all individuals were familiarised with the assessment protocol on at least two occasion prior to the initial assessment. To ascertain whether two visits were sufficient to remove any training effect a reproducibility test was conducting in ten (n=10) individuals. These male subjects, who were 22.1 ± 1.2yrs, 1.72 ± 0.1m and 76.9 ± 5.2kg, were initially familiarised with a protocol typical of those used in the investigations on two occasions. Muscle function was assessed on two subsequent occasions, separated by at least two weeks. The data and variation for which are shown in Table 3.1.

Table 3.1: Variation in assessment of muscle function using the protocol employed throughout investigations. Values are means (n=10) and mean difference between tests (± 95% confidence intervals).

Action	Test A	Test B	Difference (± 95% C.I.)	Pearson correlation coefficient
Leg extension ($Nm.kg^{-1}$)	3.78	3.84	-0.6 (± 0.27)	0.96
Leg flexion ($Nm.kg^{-1}$)	2.36	2.40	-0.4 (± 0.15)	0.95
ROM (°)	119	122	-3 (± 1.2)	0.92

The results of this test re-test demonstrate that the muscle function test was reproducible in these individuals. We observed similar correlation coefficients to previous investigations employing isokinetic testing (Pincivero *et al.*, 1997; Warren *et al.*, 1999).

3.7 Dietary analysis

In the studies reported in Chapter 4 subjects were required to weigh and record their food and fluid intake for one day prior to participation in the first main trial. Due to the crossover design, they were then asked to consume the same food and fluid prior to the second main trial. In subsequent studies (Chapters 5, 6) subjects weighed and recorded their food and fluid intake on two occasions each for three consecutive days. On the first occasion the subjects recorded their normal dietary intake an on the second their restricted dietary intake. During supplementation with vitamin C subjects were asked to restrict their intake of this antioxidant by consuming four foods with known vitamin C content per day from a list provided (Appendix V). It was estimated that this would provide approximately 100mg vitamin C per day. In the final study (Chapter 7) both the content of the antioxidant supplement and the duration of supplementation made controlling dietary antioxidant intake difficult. Subsequently subjects were asked to record food and fluid intake for five consecutive days during the supplementation period. Weighed food records were analysed by a registered dietician using a nutritional software package (COMP-EAT version 4.0, Nutritional systems, UK).

3.8 Antioxidant supplementation

In Chapters 5, 6 and 7 subjects were required to complete a period of supplementation prior to participation in the main exercise trial. In studies reported in Chapter 5 and 6 the supplements were soft gelatin transparent capsules containing a white powder (400mg) that was either vitamin C (ascorbic acid) or a placebo (lactose) (Nova Laboratories, Leicester, UK). Subjects were required to ingest two capsules per day (800mg), with food, for a total of nine days. In the investigation reported in Chapter 7 the supplements were soft gelatin black capsules (~1.5g) contained either a mixture of antioxidants (ascorbic acid 400mg, α-tocopherol 400IU, zinc 2.5mg, vitamin B_6 1mg, B_{12} 0.5μg and folic acid 100μg) or a placebo (Soya bean oil) (RP

Scherer Ltd, UK). Supplements were taken twice daily, with food, for a total of 42 days (6 weeks).

3.9 Saliva and urine sampling

In Chapters 6 and 7, saliva samples were collected using plain cotton salivettes (Sarstedt Ltd., UK). Salivettes were placed in the subject's mouth for 1 minute and saturated with saliva. No food or drink except water was consumed within 30min prior to taking this sample. Salivettes were then centrifuged at 4000g for 10min and the salvia was immediately aliquotted and frozen at -80°C. Urine samples were collected upon arrival to the laboratory. Mid-flow samples were collected by subjects directly into sterile urinary collection tubes (Sarstedt Ltd., UK) and frozen at -80°C.

3.10 Blood sampling

Subjects arrived in the laboratory after an overnight fast of at least 10h. Blood samples were taken from a forearm vein using an indwelling cannula (Venflon, 18G, BOC Ohmeda, Sweden) prior to and following exercise (Chapter 4) and at all other times by venipuncture (Chapters 5, 6 & 7). The cannula was inserted under local anaesthetic (1% lignocaine, Antigen Pharmaceutricals Ltd., Ireland), and was flushed with non-heparinised saline solution (0.9% Sodium Chloride, Steripak Ltd., UK). All blood samples were drawn after the subject had been supine for 10min, to minimise the effect of body position on plasma volume. For studies reported in Chapters 4 and 5 samples were drawn into syringes and dispensed into blood collection tubes (Sarstedt Ltd. UK). Monovette collection tubes (Sarstedt Ltd., UK) and butterfly needles (Multifly, 18G, Sarstedt Ltd., UK) were used in subsequent investigations (Chapters 6 & 7). Finger prick capillary blood samples taken during exercise (Chapters 4 & 5) were obtained using a lancet (Autoclix, Roche, Germany) and 20µl micropipettes.

Immediately after sampling approximately 5ml of blood was added to tubes (Chapters 4 & 5) or collected in monovettes (Chapters 6 & 7) containing the anticoagulant ethylenediaminetetraacetic acid (EDTA), where several aliquots of blood (20µl) were removed for determination of haemoglobin, haematrocrit, lactate (Chapters 4 & 5) and glucose (Chapter 5). Tubes were subsequently centrifuged at 4000g for 10min at 4°C (Koolspin, Burkard Scientific Ltd., UK) to obtain plasma which was dispensed and immediately frozen in liquid nitrogen then stored at -80°C. An aliquot of plasma (0.5ml) was added to an equal volume of 10% metaphosphoric acid (Sigma Chemical Co. Ltd., UK), mixed and immediately frozen in liquid nitrogen then stored at -80°C until the supernatant was analysed for vitamin C. For studies reported in Chapters 5, 6 & 7 approximately 2.5ml of blood was collected in tubes containing the anticoagulant lithium-heparin and transported, on ice, on the same day to Unilever R&D, Colworth for further analysis. The remaining blood, either 5ml (Chapter 4) or 10ml (Chapters 5, 6 & 7) was allowed to clot for 30min, and then centrifuged at 4000g for 10min (4°C) to obtained serum. Serum was dispensed and stored at -80°C for further analysis.

3.11 Blood analysis

Analysis of blood samples was principally conducted in the School of Sport and Exercise Science at Loughborough University. However, measurements of immunological parameters (cytokines, immune cell counts and immune cell function) were carried out in the Biosciences Division at Unilever R & D, Colworth. More detailed descriptions of these methods can be obtained elsewhere (Hurst, 2003 *Ph.D thesis*). Vitamin E analysis was conducted at the Rowett Research Institute, Aberdeen the details of which are not commercially available but are outlined in the appendices.

3.10.1 Plasma volume & metabolic markers

Aliquots (20µl) were removed from EDTA treated blood to determine haemoglobin concentrations by the cyanomethemoglobin method (Boehringer Mannheim GmbH Diagnostica, Germany). Haematocrit measurements were determined with a micro-haematocrit reader (Hawksley Ltd, Lancing, UK) following micro-centrifugation (15 min). Changes in plasma volume were assessed using haematrocrit and haemoglobin values (Dill & Costill, 1974).

3.10.2 Blood lactate and glucose

Blood lactate and glucose concentrations were determined from whole blood (20µl) deproteinised in 2.5% prechloric acid (200µl). Prior to analysis samples were mixed, centrifuged at 5000g for 2min (Eppendorf 5414C, Germany) and stored at -20°C prior to analysis. Blood glucose concentrations were determined using a commercially available spectrophotometric technique (Boehringer Mannheim GmbH Diagnostica, Germany). Blood lactate concentrations were measured using a modified fluorimetric method based on that described by Maughan (1982) (Appendix IV).

3.10.3 Markers of muscle damage

Serum creatine kinase activity was determined at 37°C using commercially available spectrophotometric techniques (Randox, UK) designed specifically for use on an automated system (COBAS Mira Plus, Roche Diagnostics Systems, Switzerland). Serum myoglobin concentrations were measured with an immunoturbidimetric assay also specifically developed for the automated system (Randox, UK). Prior to analysis of blood serum each sample the method was checked against a universal quality controls ranging from physiological to pathological values provided by the manufacturers (Randox, UK).

3.10.4 Antioxidant status

Vitamin C analysis was determined by high performance liquid chromatography (HPLC) conducted at the Exercise Biochemistry Research Laboratory at Loughborough University. The apparatus consisted of a pump (Model 302, Gilson, France), autosampler (Basic Marathon, Spark, The Netherlands), ultraviolet detector (Pye Unicam Ltd., UK) connected to an integrator (Model SP4290, Spectra Physics, USA). All vitamin C analysis refers to plasma concentrations of ascorbic acid (Appendix V)

In Chapter 7 vitamin E (α, γ-tocopherol and retinol) analysis was conducted using a similar system as described for vitamin C (Appendix VI). Serum uric acid concentrations were determined at 37°C using a commercially available spectrophotometric method (Randox, UK) using an automated system (COBAS Mira Plus, Roche Diagnostics Systems, Switzerland).

3.10.5 Markers of oxidative stress

In Chapter 7 lipid hydroperoxides (LPO) were measured using a commercially available kit (Cayman Chemicals, UK). This method provides an easy, rapid, sensitive, and complete measure of hydroperoxidation of lipids (Mihaljevic *et al.*, 1996). Equal volumes of plasma and methanol were mixed. Then 1ml cold chloroform was added and mixed. After centrifuging at 1500g for 5min at 0°C, the bottom chloroform layer was carefully transferred to another tube on ice. Absorbance of this layer was measured at 500 nm (Dynex Technologies Inc., USA) The concentration of hydroperoxide values were calculated from a standard hydroperoxide curve and corrected for volume changes during the assay.

In Chapter 7 urinary F_2 isoprostanes were measured at Unilever R&D, Colworth using an antibody developed 'in house'. Briefly, urine was thawed; vortexed and the allowed to stand so that precipitates were not included in the assay. An anti-mouse plate (Perkin Elmer Life Sciences, UK) was pre-washed with a dissociation enhanced lanthanide fluorescence

immunoassay (DELFIA) buffer prior to the addition of standards or samples. Anti F_2 isoprostane monoclonal antibody was diluted with an assay buffer and was then filtered. A tracer (8-iso-PGF2-ovalbumin-europium chelate) was also diluted in assay buffer. A 1 in 100 stock was then made up into storage tracer buffer. The assay protocol was then conducted as with DEFLIA salivary cortisol determination (see section 3.10.6).

3.10.6 Markers of immune function

Serum cortisol concentrations were determined with a commercially available radioimmunoassay (Cort-A-Count, Diagnostics Products Corporation, USA). Radioactivity was measured using an automated gamma counter (Cobra II, Packard Intrustments Co. Inc., USA). All procedures were conducted in the Radiochemistry Laboratories at Loughborough University. Salivary cortisol was determined using a commercially available DELFIA method (Perkin Elmer Life Sciences) and was conducted at Unilever R&D, Colworth.

Serum concentrations of soluble cytokines including interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α) interleukin 10 (IL-10) and interleukin 1 receptor antagonist (IL-1ra) were measured using commercially available enzyme linked immunosorbant assays (ELISA) (R&D Systems Inc., UK). All were based on the same principle of the sandwich enzyme immunoassay technique. Detection of TNF-α and IL-6 required the use of high sensitivity assay kits. All assays were performed as per manufacturer's instructions. Commercially available ELISAs were used to determine C-reactive protein (CRP) (DSLabs Inc., UK), heat shock protein 70 (HSP 70) (Stressgen Biotechnologies Inc., USA) and cell adhension markers (sICAM-1) (R&D Systems Inc., UK). All assays were read on a plate reader (Dynex Technologies Inc., USA) and data was derived using MRX2 software (Dynex Technologies Inc., USA).

Absolute blood cell counts were determined from whole blood XL flow cytometer (Coulter). Total white blood cell (WBC), neutrophil, monocyte and lymphocyte counts are expressed per litre ($x10^9$) throughout. Neutrophil and monocyte phagocytic and respiratory burst activity were assessed using commercially available techniques (ORPEGEN Phama, Germany). The assays are based on either the uptake of or stimulation by opsonised bacteria (Escherichia coli) and determined using flow cytometry (Appendix VIII).

3.10.7 Intra-assay variation

Intra-assay coefficient of variation (SD/mean*100) was determined for each assay and is given in Table 3.2 (excluding F_2-isoprostanes and vitamin E). Each coefficient of variation was determined using at least 20 samples.

Table 3.2: Intra-assay variation

Analyte	Units	Intra assay variation (%)
Lactate	$mmol.L^{-1}$	1.6
Glucose	$mmol.L^{-1}$	1.2
Creatine kinase	$U.l^{-1}$	2 4
Myoglobin	$nmol.L^{-1}$	2 7
Vitamin C	$\mu mol.L^{-1}$	5 1
Lipid hydroperoxides	$\mu mol.L^{-1}$	2.3
Cortisol (serum)	$nmol.L^{-1}$	3.9
Cortisol (saliva)	$nmol.L^{-1}$	3.1
IL-6	$pg.ml^{-1}$	6 9
TNF-α	$pg\ ml^{-1}$	4.9
IL-10	$ng\ ml^{-1}$	3.7
IL-1ra	$pg\ ml^{-1}$	4.8
CRP	$ng\ ml^{-1}$	2.8
HSP 70	$ng.ml^{-1}$	<10%
sICAM-1	$ng\ ml^{-1}$	4 4

CHAPTER FOUR

Delayed onset muscle soreness following level and downhill treadmill running.

4.1 Introduction

Unaccustomed exercise with a significant eccentric component results in muscle damage that is followed by a delayed onset of muscle soreness which peaks between 24 and 48h post-exercise (Armstrong, 1984; Ebbeling & Clarkson, 1989). Although much debate exists on the precise aetiology of muscle damage, the mechanical stresses of eccentric contractions result in ultrastructural damage (Friden *et al.*, 1981) that leads to large increases in circulatory concentrations of myofibrillar proteins and a period of muscular dysfunction (Clarkson & Sayers, 1999). Much of the evidence that eccentric contractions are principally responsible for muscle damage has come from direct comparisons of damaging (eccentric based) versus non-damaging (concentric based) exercise in the same individuals. This has been achieved either through the performance of eccentric contractions in one limb and concentric in the contralateral limb (Newham *et al.*, 1983b) or repeated exercise under both contraction conditions (Schwane *et al.*, 1983; Pizza *et al.*, 1995). However, the prophylactic effect of a single bout of damaging exercise on subsequent bouts makes the latter comparison difficult (Byrnes *et al.*, 1985; Ebbeling & Clarkson, 1989).

During running, leg extensor muscles perform eccentric actions as the foot touches the ground and the centre of mass is decelerated (Armstrong, 1986). Such actions are obviously accentuated during running on a negative gradient (Eston *et al.*, 1995). Previous investigations that have employed downhill running report severe muscle soreness and increased myofibrillar protein efflux following this eccentric based exercise (Schwane *et al.*, 1983; Byrnes *et al.*, 1985; Pizza *et al.*, 1995). Therefore, this preliminary investigation attempts to further explore the mechanisms responsible for EIMD by directly comparing damaging (downhill running) and non-damaging (level running) in the same individuals.

4.2 Methods

Subjects

Eight healthy, active young males volunteered to take part in this investigation. All subjects were habitually active in a variety of sports, but were unfamiliar with the specific mode of exercise used in this investigation. Subject's physiological characteristics are summarised in Table 4.1.

Table 4.1: Physical characteristics of subjects (n = 8)

Characteristics	mean ± *SEM*
Age (years)	21.3 ± 0.4
Height (m)	1.82 ± 0.03
Mass (kg)	81.9 ± 2.6
$\dot{V}O_2max$ ($ml.kg^{-1}.min^{-1}$)	59.2 ± 2.1
HRmax ($b.min^{-1}$)	198 ± 7

Experimental design

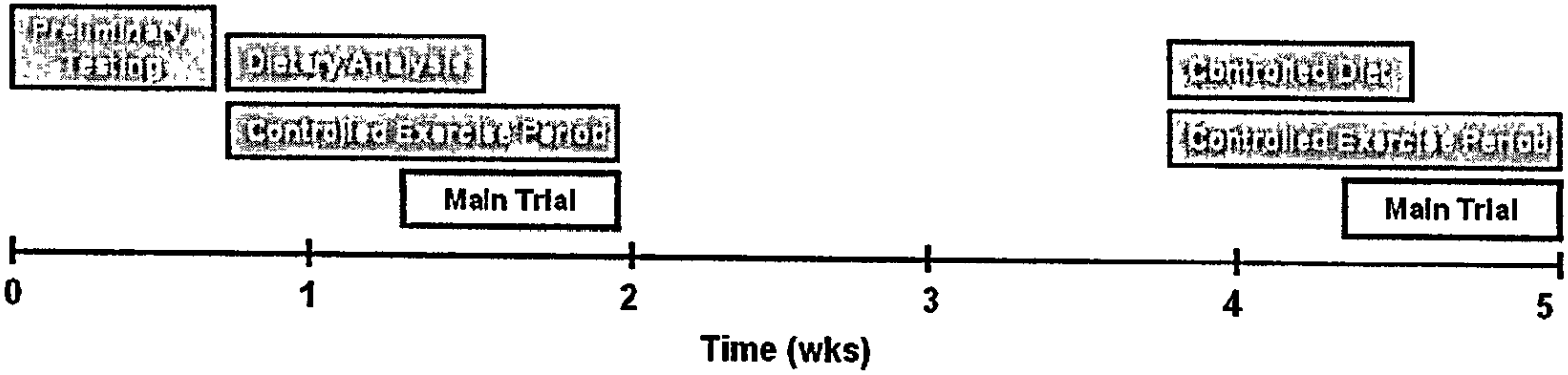
All subjects performed two 60min runs at a speed that elicited 70% $\dot{V}O_2max$ on the level; once on a level gradient (LVL) and once on a downhill (-10%) gradients (DH). Each run was separated by at least 14 days. During the two days prior to each run subjects refrained from strenuous physical activity and recorded their food intake in order to ensure they consumed the same diet before each trial.

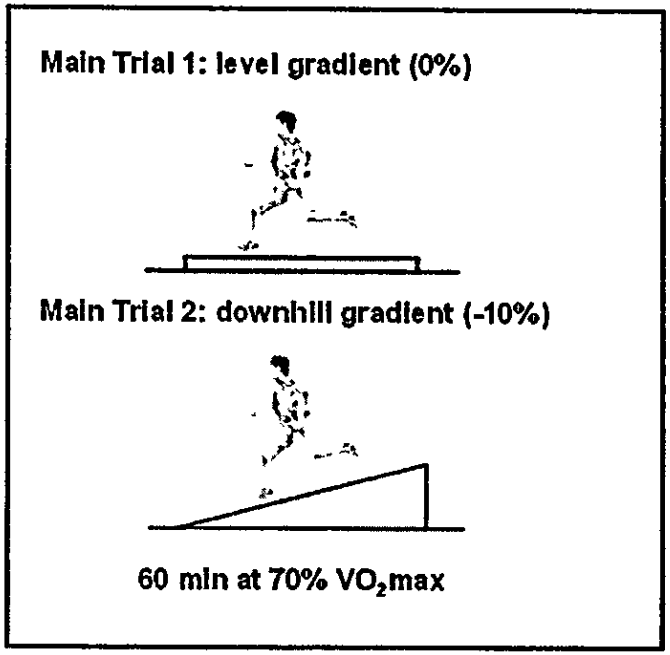
Subjects reported to the laboratory after an overnight fast of 10-12h. A venous blood sample (10ml) was taken prior to and immediately post-exercise (IMPE) and again approximately 1, 6 and 24h after exercise. Ratings of perceived soreness were recorded prior to exercise and again up to 48h post-exercise. Additionally, subjects were required to indicate the regions in which they experienced soreness by assessing soreness in the musculature of the legs (*gluteus maximus, gastrocnemius, hamstrings, quadriceps, and tibialis anterior*) using the 10 point scale. Muscle function was assessed pre-exercise and again approximately 1, 6 and 24h later. Nude body mass was determined before and immediately following exercise. Subjects ingested water *ad libitum* throughout the run and

were encouraged to ingest the same volume during the second trial. Heart rate, expired air samples were collected and ratings of perceived exertion were monitored at regular intervals during exercise. Figure 4.1 illustrates a schematic representation of the experimental design.

Statistical analysis

A two-way (time x gradient) analysis of variance (ANOVA) with repeated measures was used to determine if any differences existed between trials and time during exercise and recovery. For significant F ratios a paired *Students* t-test, with a *Bonferroni* adjustment, was used to locate the differences between means. Pearson product moment correlations were used to examine the relationship between variables. Significance was accepted at the 5% level. Values are expressed as the mean ± standard error of the mean (*SEM*) throughout.





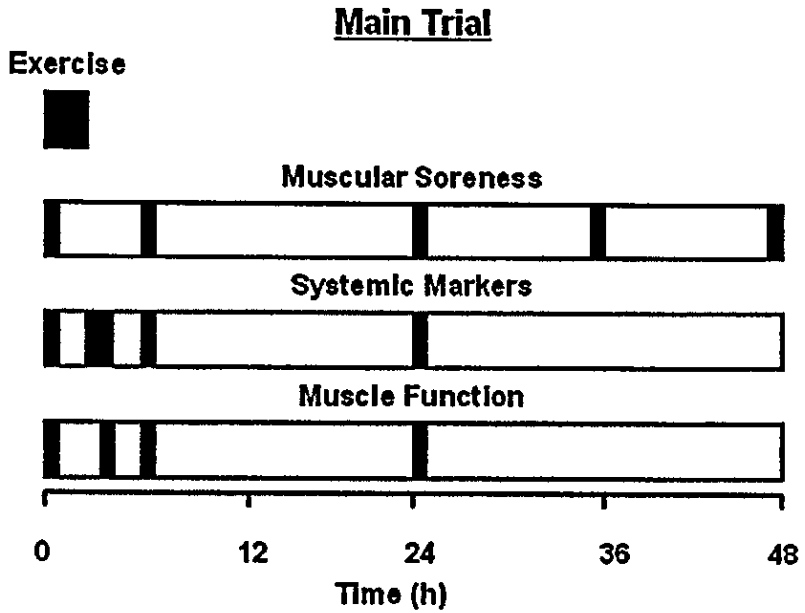


Figure 4.1: A schematic representation of the experimental design.

4.3 Results

Response to exercise

Mean $\dot{V}O_2$ during DH (2.24 ± 0.12 L.min^{-1} or 47.6 ± 0.6 % $\dot{V}O_2$max) was lower than during LVL (3.27 ± 0.17 L.min^{-1} or 66.9 ± 1.1% $\dot{V}O_2$max) ($p < 0.05$). The mean heart rate during DH (149 ± 6 b.min^{-1}) was lower than during LVL (170 ± 4 b.min^{-1}) ($p<0.05$). Heart rate also increased during exercise during both runs ($p<0.05$) (Figure 4.2). Ratings of perceived exertion were increased during DH ($p<0.05$) but were unchanged during LVL. Estimated changes in plasma volume did not differ following either DH (-2.1 ± 0.8%) or LVL (-0.7 ± 1.5%). Blood lactate concentrations increased immediately post-exercise (1.6 ± 0.2 mmol.L^{-1}) from pre-exercise (0.9 ± 0.1 mmol.L^{-1}) values following LVL only ($p<0.05$). Values were different between trials IMPE ($p<0.05$).

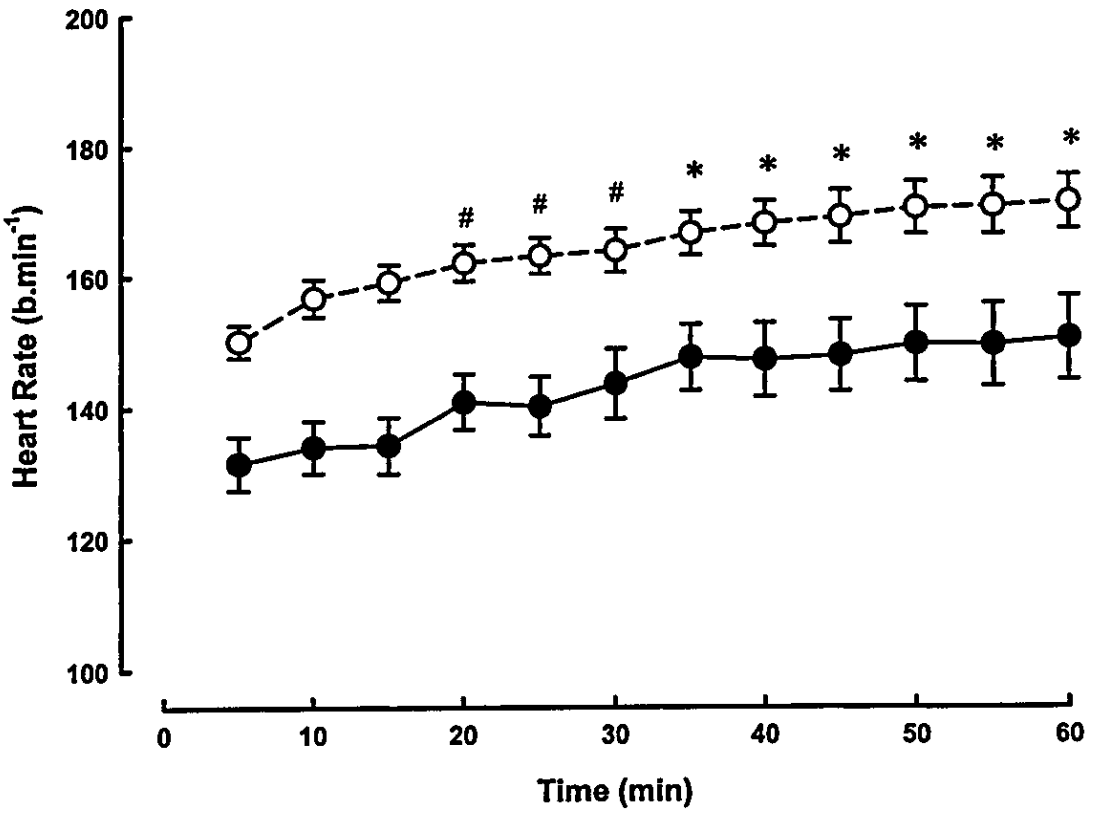


Figure 4.2: Heart rate response to 60min treadmill running on either level (dashed line) or downhill (solid line) gradients (mean ± *SEM*). Values are different ($p<0.05$) from the 5th minute of exercise for LVL ($^{\#}$) and both groups (*).

Muscle soreness

Ratings of perceived passive soreness (both scales) were greater following DH at all time points post-exercise when compared to LVL ($p<0.05$) (Figure 4.3). Values increased (10 point scale) from pre-exercise peak at 24h (7.9 ± 0.6) and remained elevated at 48h (5.7 ± 0.8) post-exercise ($p<0.05$). Values were also increased following LVL but returned to baseline at 48h post-exercise ($p<0.05$). Ratings of perceived active soreness were greater following DH (4.4 ± 0.6) than after LVL (1.7 ± 0.3) ($p<0.05$), peaking at 24h post-exercise ($p<0.05$).

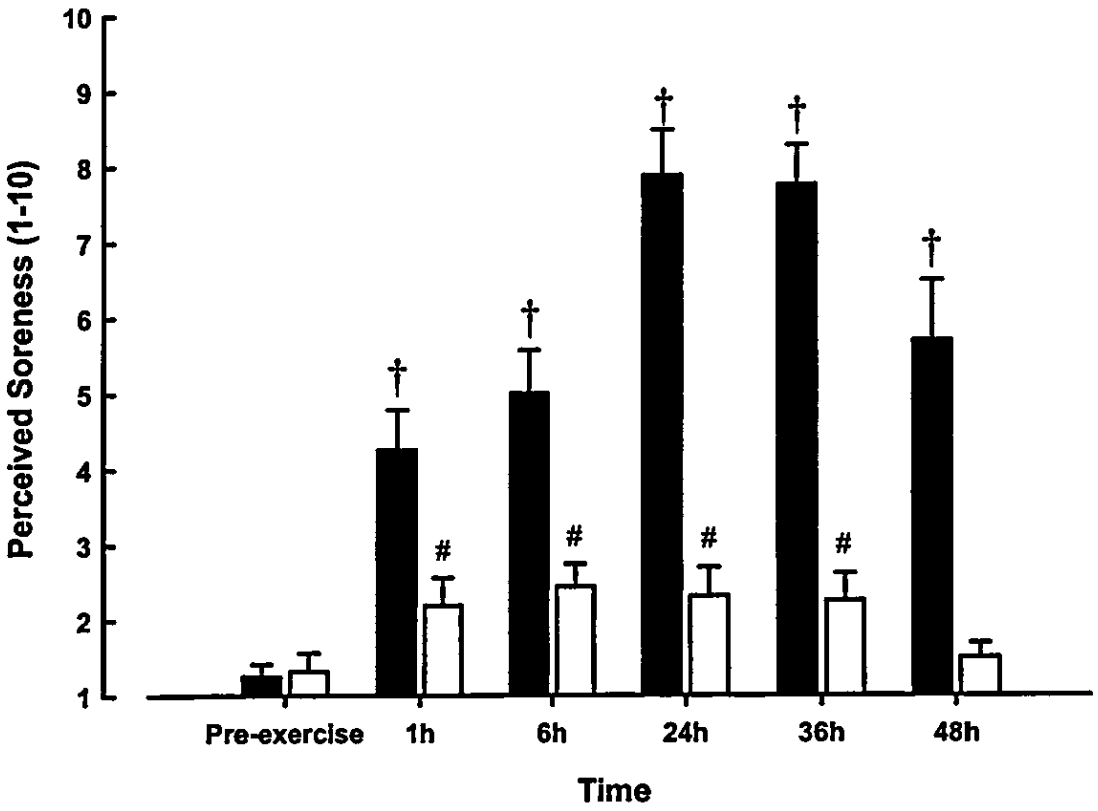


Figure 4.3: Ratings of perceived soreness (10 point scale) following running on either level (clear bars) or downhill (solid bars) gradients (mean ± *SEM*). † different between runs ($p<0.05$). # different ($p<0.05$) from pre-exercise for LVL.

Soreness was most frequently reported in the *trapezius*, *gluteus maximus*, *quadriceps* and *tibialis anterior* in the 24h following DH (Figure 4.4). Ratings of perceived soreness in the leg musculature were greater following DH compared to LVL in the *gluteus maximus* (4.1 ± 0.9; 1.3 ± 0.2), *hamstrings* (3.5 ± 0.8; 1.8 ± 0.5) and *quadriceps* (5.6 ± 0.7; 2.0 ± 0.3) 24h post-exercise ($p<0.05$) (Table 4.2).

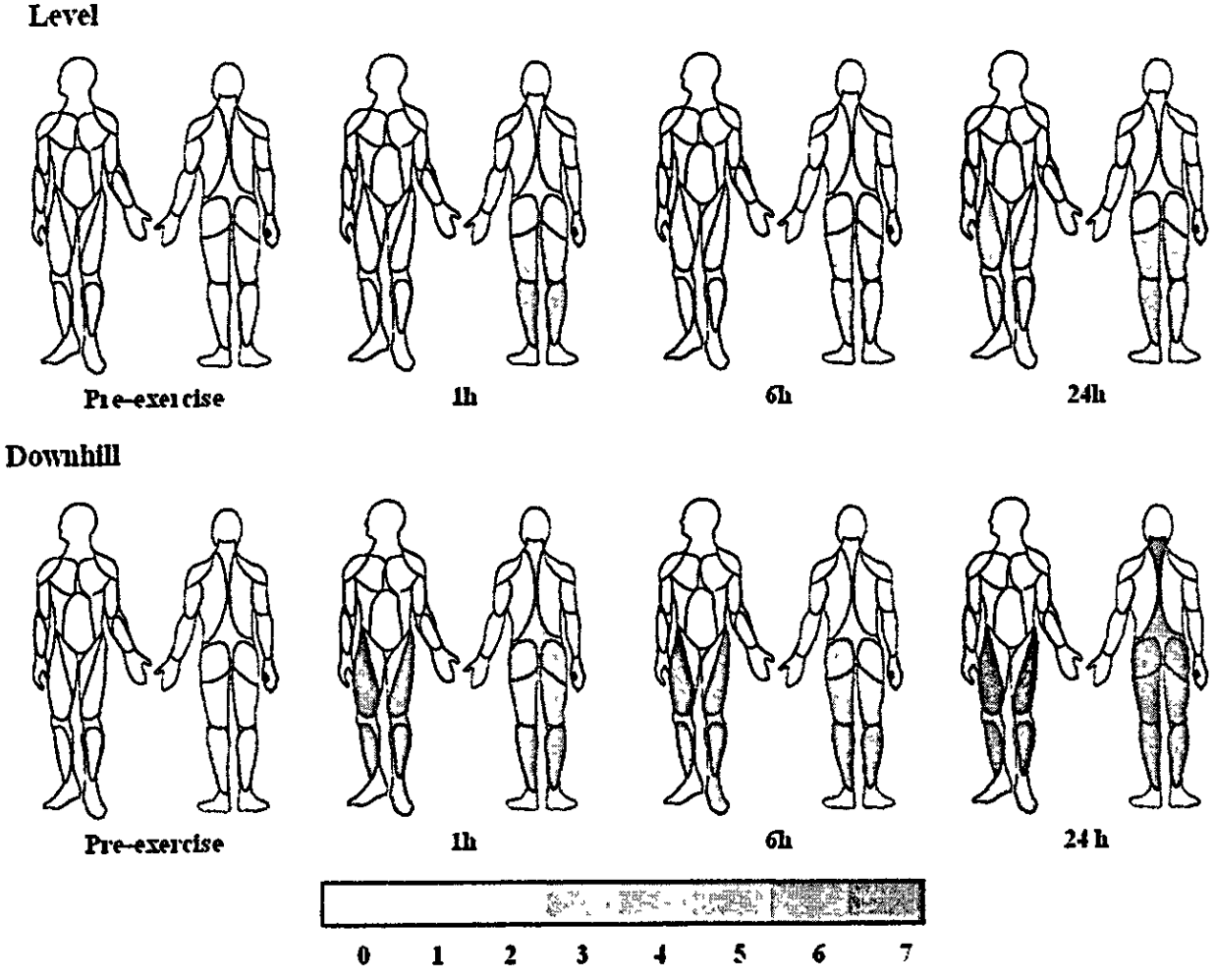


Figure 4.4: Location and frequency of muscle soreness following 60min running on two different gradients. An increase in the density of shading represents an increase in the frequency of soreness in that region (n = 8).

Table 4.2: Location of muscle soreness following either downhill or level running (means ± *SEM*). † different between runs ($p<0.05$).* different from pre-exercise ($p<0.05$).

	Soreness (1-10)			
	Pre-exercise	1h	6h	24h
Level				
Gluteus maximus	1.3 ± 0.2	1.6 ± 0.3	1.8 ± 0.3	1.3 ± 0.2 †
Gastrocnemius	1.1 ± 0.1	1.6 ± 0.5	2.0 ± 0.3 *	2.0 ± 0.5 *
Hamstrings	1.3 ± 0 2	2.0 ± 0 3	1.5 ± 0.2	1.8 ± 0.5 †
Quadriceps	1.3 ± 0 2	1.5 ± 0.2	1.9 ± 0.3 *	2.0 ± 0.3 *†
Tibialis anterior	1.1 ± 0.1	1 8 ± 0.5	1.6 ± 0.2	1.5 ± 0.3
Downhill				
Gluteus Maximus	1.1 ± 0.1	3.2 ± 0.7 *	3.5 ± 0.8 *	5.5 ± 1.1 *†
Gastrocnemius	1.3 ± 0.2	3.2 ± 0.8 *	3 4 ± 0.9 *	4.1 ± 0.9 *
Hamstrings	1.4 ± 0.2	2.9 ± 0.6	3.1 ± 0.8 *	3.5 ± 0.8 *†
Quadriceps	1.6 ± 0.1	3.4 ± 0.5 *	3.9 ± 0.6 *	5.6 ± 0.7 *†
Tibialis anterior	2.0 ± 0.7	2.9 ± 0 9	1.9 ± 0.4	3.3 ± 0.6 *

Muscle function

Trunk flexion was unaffected by either DH or LVL and remained unchanged following exercise.

Markers of muscle damage

Changes in Mb concentration following exercise are shown in Figure 4.5. Serum Mb increased from pre-exercise values following DH and LVL, peaking between 1 and 6h post-exercise, respectively (10.99 ± 2.29 nmol.L^{-1}; 4.88 ± 0.59 nmol.L^{-1}) ($p<0.05$). Peak increases in Mb from pre-exercise values were greater at 1hr following DH (538 ± 112%) compared to LVL (214 ± 49%) ($p<0.05$). Figure 4.5 illustrates that CK activity also increased from pre-exercise values peaking at 24h post exercise following both runs ($p<0.05$). Values were greater at 6 and 24h post-exercise following DH (477 ± 36 U.L^{-1}) than after LVL (297 ± 37 U.L^{-1}) ($p< 0.05$).

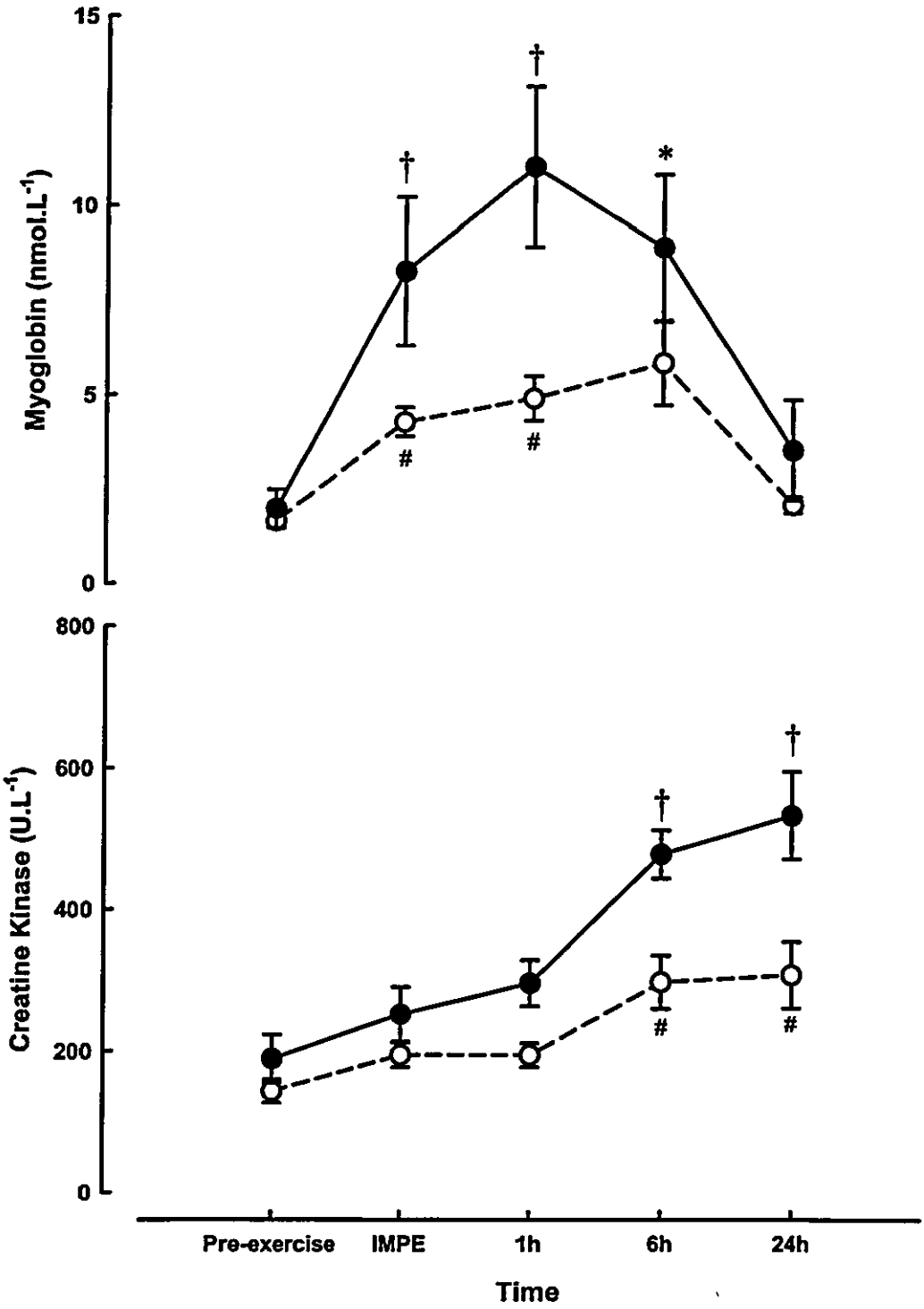


Figure 4.5: Serum myoglobin concentration and creatine kinase activity following running on either level (dashed line) or downhill (solid line) gradients (mean ± *SEM*). † different between runs ($p<0.05$). # LVL different from pre-exercise ($p<0.05$). * both groups different from pre-exercise.

Figure 4.6 shows the interleukin 6 (IL-6) response to both DH and LVL running. Concentrations peaked in the hours post-exercise for both runs (7.97 ± 2.18 $pg.ml^{-1}$; 5.11 ± 1.11 $pg.ml^{-1}$) ($p<0.05$).

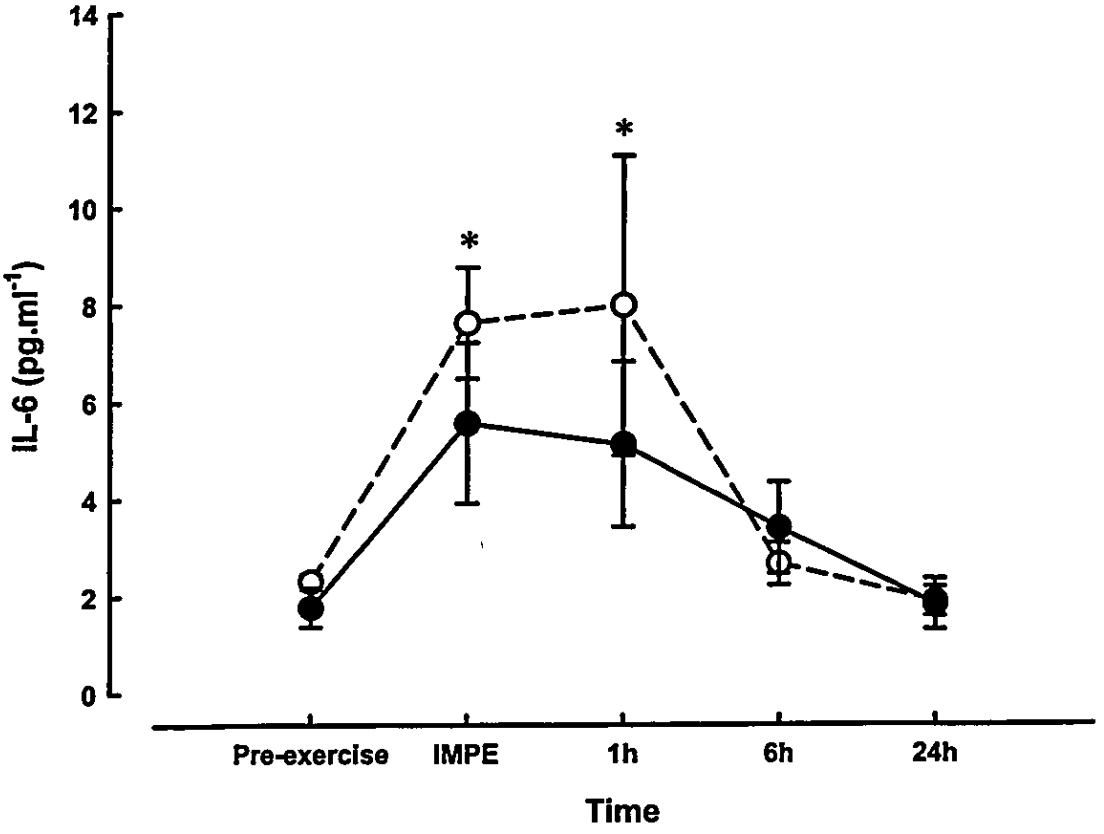


Figure 4.6: Serum interleukin 6 concentration following running on either level (dashed line) or downhill (solid line) gradients (mean ± *SEM*). * different from pre-exercise for both groups ($p<0.05$).

A moderate relationship was observed between peak increases in Mb concentration and CK activity following exercise ($r = 0.74$, $p<0.05$) (Figure 4.7).

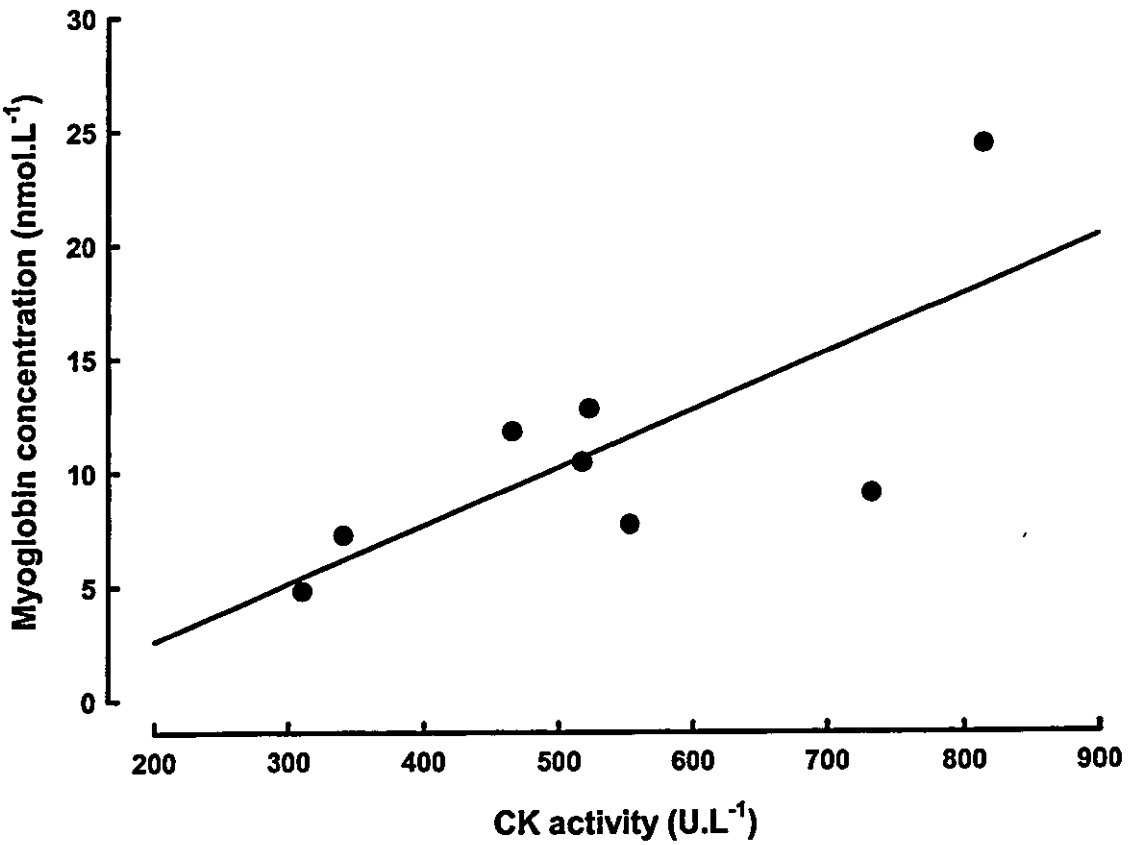


Figure 4.7: Relationship between peaks increases in systemic indices of muscle damage for downhill run only.

4.4 Discussion

The present study demonstrates that downhill running leads to extensive muscular soreness and greater increases in myofibrillar protein leakage when directly compared to level running. These findings are consistent with those of other investigations that have directly compared the response to eccentric and concentric based exercise (Newham *et al.*, 1983a; Schwane *et al.*, 1983; Pizza *et al.*, 1995). Schwane and colleagues (1983) reported similar increases in muscle soreness and CK activity following 45min of running on similar gradients. The peak increases in CK activity (192% at 24h) from pre-exercise values are not as large those observed by these authors (358% at 24h) nor with other investigations employing downhill running as a model for muscle damage (Byrnes *et al.*, 1985; Pizza *et al.*, 1995). This could be due to the small, yet apparent prophylactic effect of the first bout of running. Although level running is considered a concentric based exercise, leg extensors do perform eccentric braking actions during the normal running gait (Eston *et al.*, 1995). The repeated bout effect is characterised as a reduction in muscle soreness and myofibrillar protein leakage following an initial bout of damaging exercise (Ebbeling & Clarkson, 1989). This adaptation has been reported to last up to 6 months following exercise (Nosaka *et al.*, 2001) and thus could have influenced the responses to the second bout of running performed within three weeks of the first. However, the profound increases in the indices of muscle damage following downhill running would imply only a limited effect, similar to that observed by Pierrynowski *et al.* (1987) following a prior bout of uphill running. Recently, Nosaka and Newton (2002) showed that a period of concentric elbow flexion training did not reduce the indices of muscle damage following a bout of maximal eccentric elbow flexions. Interestingly, the authors also showed no apparent effect of eccentric training (submaximal) on the response to maximal eccentric contractions perhaps suggesting specificity is required to achieve this repeated bout effect (Nosaka & Newton, 2002).

The earlier peak in Mb concentration following the downhill run has been attributed to a more rapid efflux of this protein from the circulation, as it is smaller than the CK molecule (Balnave & Thompson, 1993). This gives support for the use of this marker as a reliable indicator of muscle damage because post-exercise increases can be more confidently

attributed to the exercise performed in the last few hours. The rise in CK activity reflects exercise undertaken in the previous days rather than hours. Additionally, the moderate correlation between peak increases in Mb concentration and the more established marker, CK activity, provides further support for its use and has been documented following similar exercise models (Cairns *et al.*, 1983; Kanter *et al.*, 1988). The Mb response in this investigation is similar to other reports using similar eccentric based exercise protocols (Sorichter *et al.*, 1997; Thompson *et al.*, 2000; Sorichter *et al.*, 2001). Although the increase of myofibrillar proteins in the circulation is an established indicator of muscle damage the exact mechanism underlying their efflux is still debated. Some suggest an increased membrane permeability results in their movement across the sarcolemma (Balnave & Thompson, 1993). This explanation would account for the varying time course of Mb and CK as the smaller molecule would be able to pass through smaller lesions. An alternative explanation is that the efflux is simply a result of myofibril degradation and necrosis (Friden *et al.*, 1989). Both are logical but without more direct morphological evidence obtained from muscle biopsies it is difficult to substantiate either explanation to the results of this investigation.

The location of soreness was limited to those muscle groups that performed eccentric actions during the downhill run. This was also reported by Schwane and co-workers (1983). They observed soreness in *gluteal, quadriceps* plus anterior and posterior leg muscles but not in the *hamstrings* and musculature of the lower back as we detected. Others have reported severe soreness in the lower limbs following downhill running (Camus *et al.*, 1992; Smith *et al.*, 1998) but there is no other evidence for this sensation in the musculature of the back. This is perhaps due to the more comprehensive assessment of perceived muscle soreness in the present investigation. The characteristic delayed onset of muscular soreness has been associated with an inflammatory response that facilitates the removal of necrotic tissue (Smith, 1991). The by-products of this process are proposed to stimulate nerve afferents leading to the sensation of soreness (Smith, 1991; Camus *et al.*, 1992; Pizza *et al.*, 1995). Pizza and co-workers (1995) employed a similar comparison of downhill and level running (60min, 70% $\dot{V}O_2max$) and observed a greater leukocyte mobilisation 12h following downhill running. Smith *et al.* (1989) also reported a marked leukocytosis, specifically neutrophilia, in the hours following downhill jogging (40min, 46% $\dot{V}O_2max$) compared to uphill walking.

Camus and colleagues (1992) observed neutrophilia following both downhill running and uphill walking (20min at 60% $\dot{V}O_2$max), but only downhill running was accompanied by an increase in neutrophil activation. We observed an increase in the inflammatory mediator cytokine IL-6 following both level and downhill running which has previously been associated with eccentric exercise-induced muscle damage (Bruunsgaard *et al.*, 1997). However, our findings would appear to support more recent work that eccentric exercise is not solely responsibly for increases in IL-6 (Croisier *et al.*, 1999; Jonsdottir *et al.*, 2000). Therefore, in light of this recent evidence and without more tissue specific measures of leukocyte accumulation it is difficult to support the proposed role of inflammation in this particular investigation. Thus, our findings would support similar investigations that failed to identify an inflammatory response to downhill running (Schwane *et al.*, 1983; Pyne *et al.*, 1997).

Although unaffected by either downhill or level running in this investigation, decreased muscle function has been documented following downhill running using more comprehensive techniques (isokinetic dynamometers and EMG) (Sargeant & Dolan, 1987; Eston *et al.*, 1994). It is obvious that despite being a simple and practical measure of trunk flexion our muscle function test was not specific to those muscles damaged. Others have advocated the use of more specific tests that incorporate measures of maximal voluntary contraction and range of motion when assessing muscle function following eccentric exercise (Warren *et al.*, 1999).

Previous investigations that have employed downhill running have reported an upward drift in $\dot{V}O_2$ during exercise (Byrnes *et al.*, 1985; Dick & Cavanagh, 1987; Pierrynowski *et al.*, 1987). This phenomenon has been observed with an accompanying increase in electrical activity (EMG) of the eccentrically active muscles (Pierrynowski *et al.*, 1987). Subsequently, it is hypothesised that these increases are a result of increased motor unit recruitment caused by muscle damage and local muscular fatigue. We did not observe this upward drift in $\dot{V}O_2$ during either run which fails to support this hypothesis. However, Westerlind *et al.* (1992) argues that the response is unrelated to muscle damage as the $\dot{V}O_2$ drift is observed following repeated bouts of downhill running whilst markers of muscle damage are reduced.

In conclusion the eccentric component of downhill running supports the hypothesis that DOMS is a result of mechanical stress to muscular tissue. The recovery from downhill running was substantially more delayed, and caused more profound damage than that of level running. Muscle damage following downhill running (Kirwan *et al.*, 1992) and eccentric contractions (Doyle *et al.*, 1993; Kristiansen *et al.*, 1996) have previously been associated with impaired glycogen replenishment another limiting factor when performing further exercise. This evidence clearly supports the detrimental responses associated with exercise-induced muscle damage and highlights the potential for treatment or prevention strategies.

CHAPTER FIVE

Recovery from downhill running following vitamin C supplementation

5.1 Introduction

The production of free radicals during exercise has been linked to the initial mechanical disruptions responsible for muscle damage and may also contribute to subsequent muscle injury in the post-exercise period (Pyne, 1994; Goldfarb, 1999). It is proposed that free radical formation during exercise may exceed endogenous antioxidant protection and result in damage to phospholipids through lipid peroxidation (Packer, 1997). This may in turn contribute to the efflux of myofibrillar proteins and facilitate the degenerative proteolytic pathways that lead to ultrastructural damage of muscular tissue. Additionally, the severity of damage has been shown to increase in the post-exercise period (Newham *et al.*, 1983; Kuipers, 1994; Best *et al.*, 1999). Free radicals have also been shown to play a role in the inflammatory response that is linked to this on going damage (MacIntyre *et al.*, 1995). It is therefore clear that free radical production both during and following exercise has a potential role in the aetiology of exercise-induced muscle damage.

Attempts to reduce the formation of these highly reactive molecules and their associated detrimental effects have focused on dietary antioxidant supplementation, mainly with vitamins E and C. Some investigations into vitamin E supplementation have reported beneficial outcomes, including a reductions in myofibrillar protein leakage (Cannon *et al.*, 1990; Rokitzki *et al.*, 1994; McBride *et al.*, 1998) as well as markers of oxidative stress (Sumida *et al.*, 1989; Meydani *et al.*, 1993). However, others have reported no advantageous effects of supplementation with vitamin E (Beaton *et al.*, 2002; Dawson *et al.*, 2002). The ability of vitamin C to regenerate oxidised vitamin E as well as scavenge peroxyl and oxygen radicals has made it an attractive antioxidant with potential to relegate exercise-induced oxidative stress both in phospholipid membranes and in the plasma. Investigations employing vitamin C supplementation have reported protective effects on the ability to generate muscular force (Jakeman & Maxwell, 1993) as well as reducing

lipid peroxidation and markers of inflammation following exercise (Alessio *et al.*, 1997; Ashton *et al.*, 1999; Nieman *et al.*, 2000; Thompson *et al.*, 2001).

We have already established that downhill running leads to moderate degree of muscle damage and soreness. Therefore, the aim of the present investigation was to examine the effect of vitamin C supplementation on recovery from this particular mode of damaging exercise.

5.2 Methods

Subjects

Fourteen healthy male students (n=14) volunteered to take part in this study. Any subject that smoked or used any nutritional supplements was excluded from the investigation. Subjects were assigned, in a double blind manner, to either vitamin C or placebo supplementation. Groups were similar for a number of physical characteristics and activity levels (Table 5.1).

Table 5.1: Physical characteristics of the supplementation groups (mean ± SD).

	Vitamin C (n=7)	**Placebo (n=7)**
Age (years)	23.1 ± 0.9	23.4 ± 2.4
Height (m)	1.79 ± 0.09	1.78 ± 0.07
Mass (kg)	78.1 ± 9.2	76.6 ± 7.9
Sum Skinfolds (mm)	31.3 ± 7.7	31.3 ± 6.3
$\dot{V}O_2max$ ($ml.kg.min^{-1}$)	63.0 ± 5.7	59.8 ± 4.8
Weekly exercise session	5 ± 2	5 ± 2
Daily vitamin C intake (mg)	145 ± 25	161 ± 32

Supplementation

Subjects were randomly assigned, in a double blind manner, to $800mg.day^{-1}$ of either vitamin C (V_C) or a placebo (P_L) supplementation. Supplements were taken in capsule form, twice daily (2 x 400mg) with food, beginning seven days prior to participation in the

main trial, terminating two days after completion. A venous blood sample was taken from subjects before commencing supplementation. Subjects were required to weigh and record all food and fluid intake on two occasions. Prior to supplementation a three day habitual diet was recorded and during the main trial another three day diet was recorded, during which subjects were requested to control their dietary vitamin C intake. The controlled diet (Appendix V) required subjects to consume a number of foods that were estimated to provide a known quantity of vitamin C (~100 $mg \cdot day^{-1}$). Other food intake was not restricted.

Experimental design

On the seventh day of supplementation subjects completed a 60min treadmill run on an incline of -10%. They exercised at an intensity that would elicit 70% $\dot{V}O_2max$ whilst running on the level. Subjects reported to the laboratory having refrained from strenuous physical activity for at least two days and after an overnight fast (10-12h). An 8 ml venous blood sample was taken from a forearm vein prior exercise and again approximately 1, 24 and 48h post-exercise. Ratings of perceived soreness were recorded prior to exercise and again at approximately 12h intervals for the following two days. Muscle function (MVC) was assessed pre-exercise and again approximately 24 and 48h later using an isokinetic dynamometer. Nude body mass was determined before and immediately following exercise. Subjects ingested water *ad libitum* throughout the run. Heart rate, expired air samples and ratings of perceived exertion were monitored at regular intervals during exercise. Figure 5.1 shows a schematic representation of the experimental design.

Statistical analysis

An independent two-way analysis of variance (ANOVA) with repeated measures was used to determine if any differences existed between interventions and time during exercise and recovery. For significant F ratios a paired *Students* t-test, with a *Bonferroni* adjustment, was used to locate the differences between means. Pearson product moment correlations were used to examine the relationship between variables. Significance was accepted at the 5% level. Values are expressed as the mean ± standard error of the mean (*SEM*) throughout.

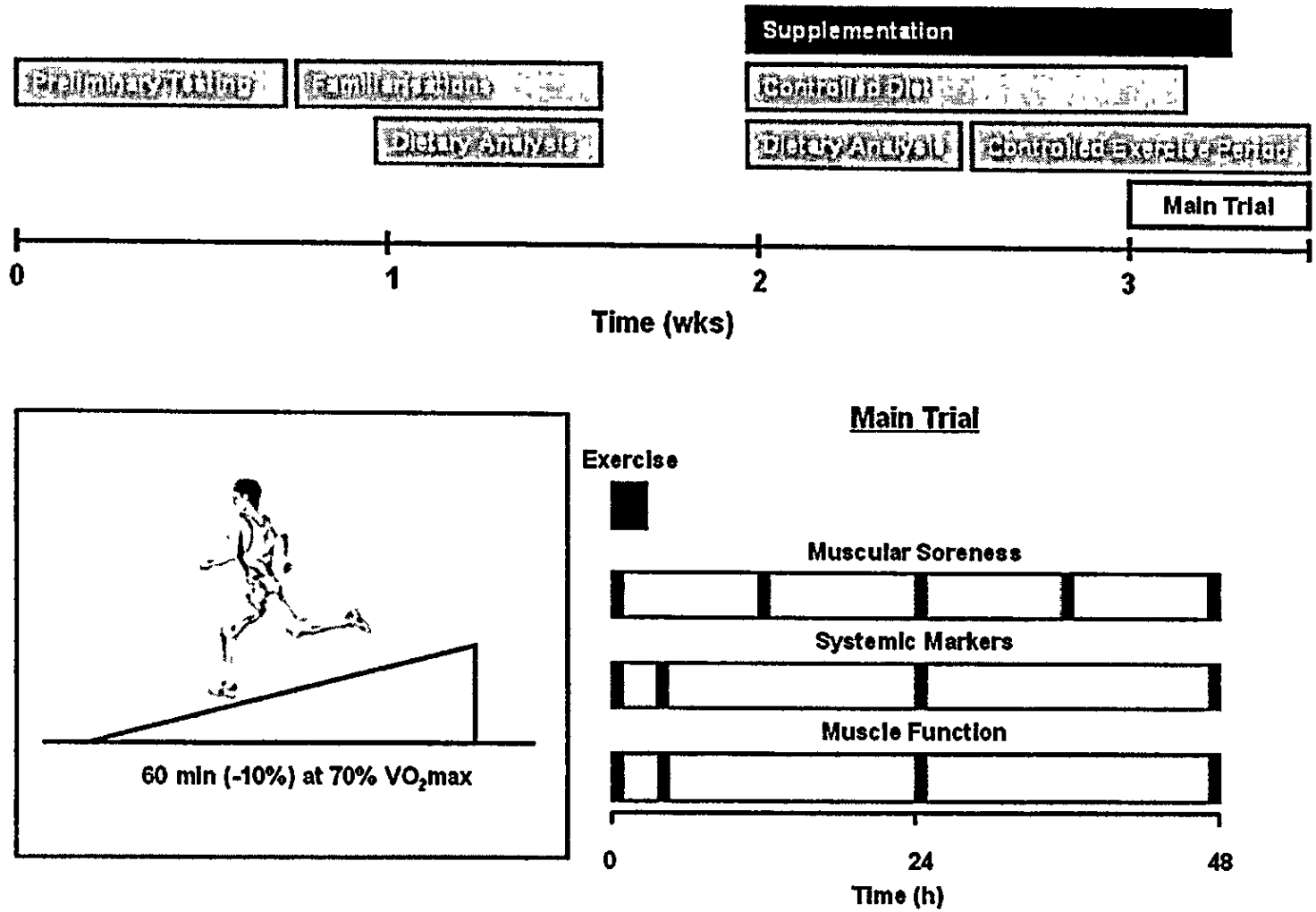


Figure 5.1: A schematic representation of the experimental design

5.3 Results

Response to exercise

Mean $\dot{V}O_2$ (n=14) during the downhill run was not different between groups (V_C 2.35 ± 0.45 $L.min^{-1}$; P_L 2.31 ± 0.27 $L.min^{-1}$ or V_C 48.2 ± 2.4% $\dot{V}O_2max$; P_L 50.9 ± 1.9% $\dot{V}O_2max$) and was increased at 60 min from 15 and 30min during exercise ($p<0.05$). The mean heart rate (n=14) during exercise was (V_C 140 ± 4 $b.min^{-1}$; P_L 137 ± 3 $b.min^{-1}$). Heart rate also increased during exercise ($p<0.05$). Mean ratings of perceived exertion (n=14) were 11 ± 0.5 during exercise. Blood lactate concentrations (n=14) increased immediately post-exercise (3.0 ± 0.3 $mmol.L^{-1}$) from pre-exercise (1.1 ± 0.1 $mmol.L^{-1}$) values following exercise ($p<0.05$). Blood glucose concentrations were unchanged following exercise. Estimated changes in plasma volume did not differ throughout the main trial in either group.

Plasma vitamin C

Following supplementation plasma vitamin C concentration increased with V_C supplementation only. Figure 5.2 shows that plasma concentrations peaked at 1h and remained elevated up to 24h post-exercise compared to P_L ($p<0.05$).

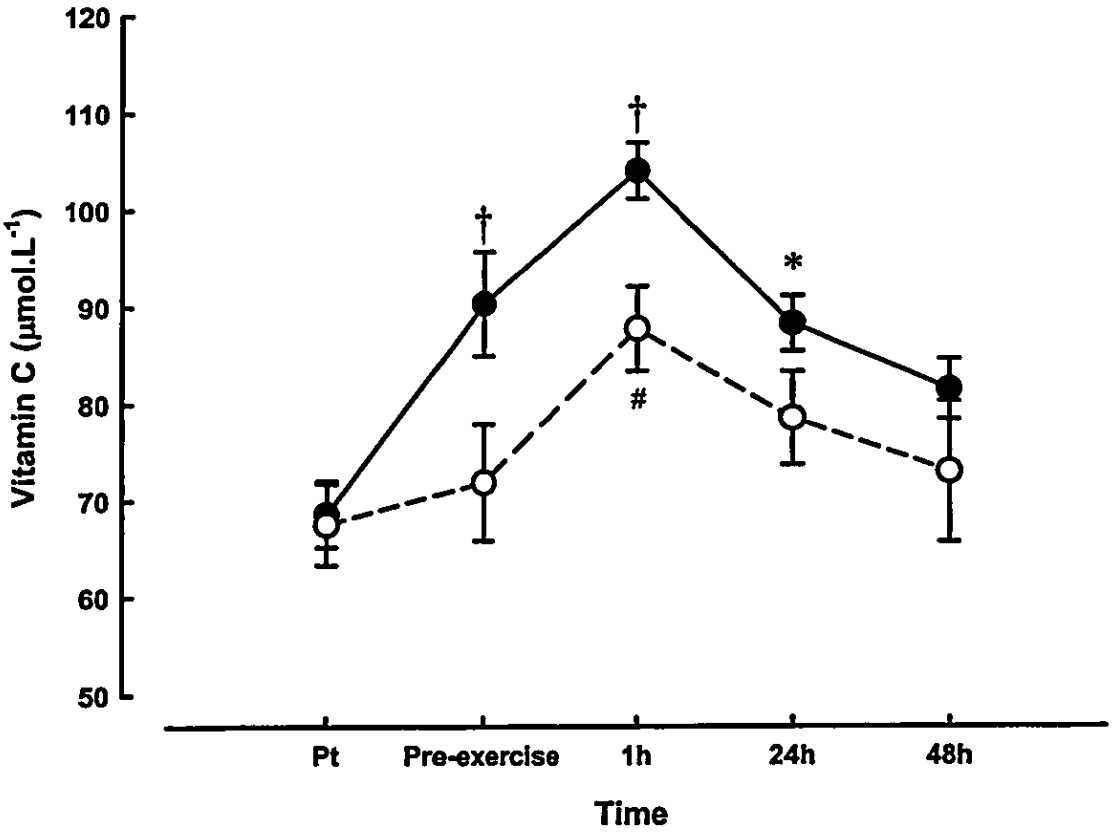


Figure 5.2: Plasma vitamin C concentration pre-treatment (Pt) and following supplementation with vitamin C (solid line) or placebo (dashed line) supplementation (mean ± *SEM*). † different between groups ($p<0.05$). * V_C different from pre-supplementation ($p<0.05$). # P_L different from pre-exercise ($p<0.05$).

Muscle soreness

There was no intervention effect on the perception of soreness prior to and following exercise. Figure 5.3 illustrates that passive soreness was increased ($p<0.05$) in both groups from pre-exercise ratings (1 ± 0) peaking at 36h post exercise (6 ± 1). Active ratings of perceived soreness, assessed whilst descending steps, were also increased ($p<0.05$) in both groups from pre-exercise values (1 ± 0) peaking at 48 h post exercise (5.5 ± 0.5). The location of soreness was most frequently reported in the *hamstrings*, *quadriceps* and *triceps surea* muscle groups (Figure 5.4).

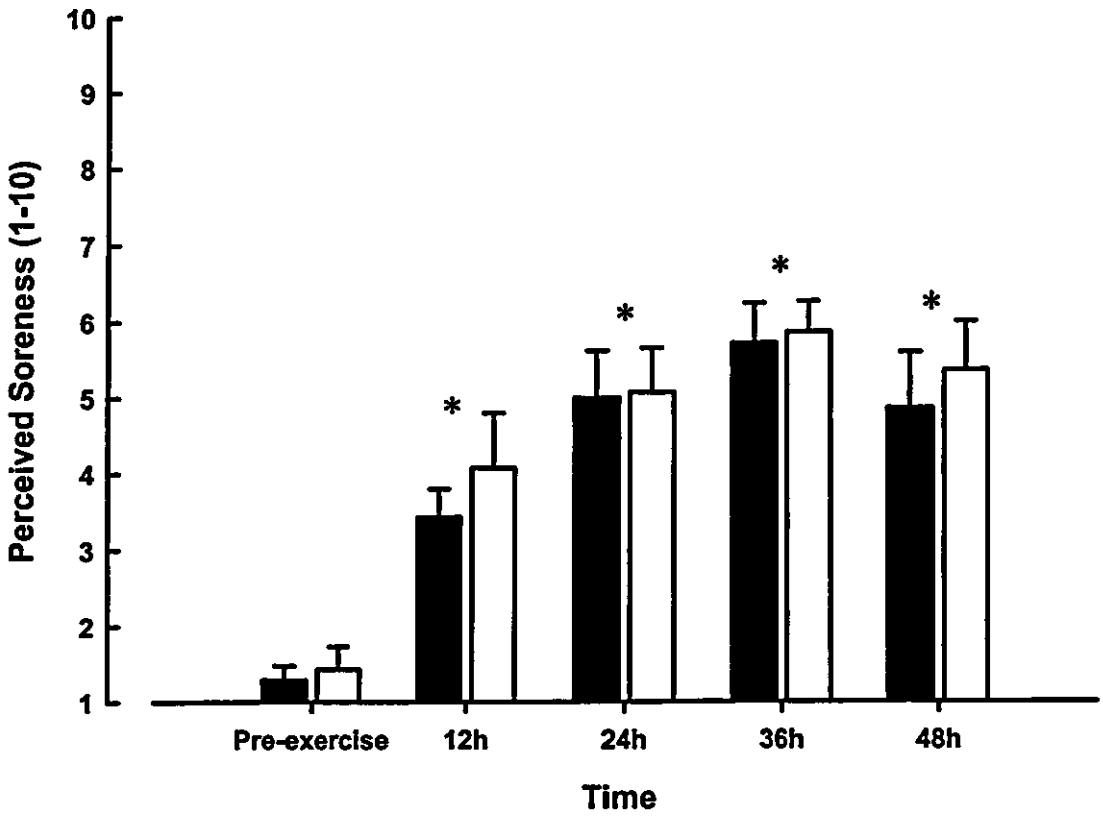


Figure 5.3: Ratings of perceived soreness (10 point scale) following downhill running for the vitamin C (filled bars) and placebo supplemented (clear bars) groups (mean ± *SEM*). * both groups different from pre-exercise ($p<0.05$).

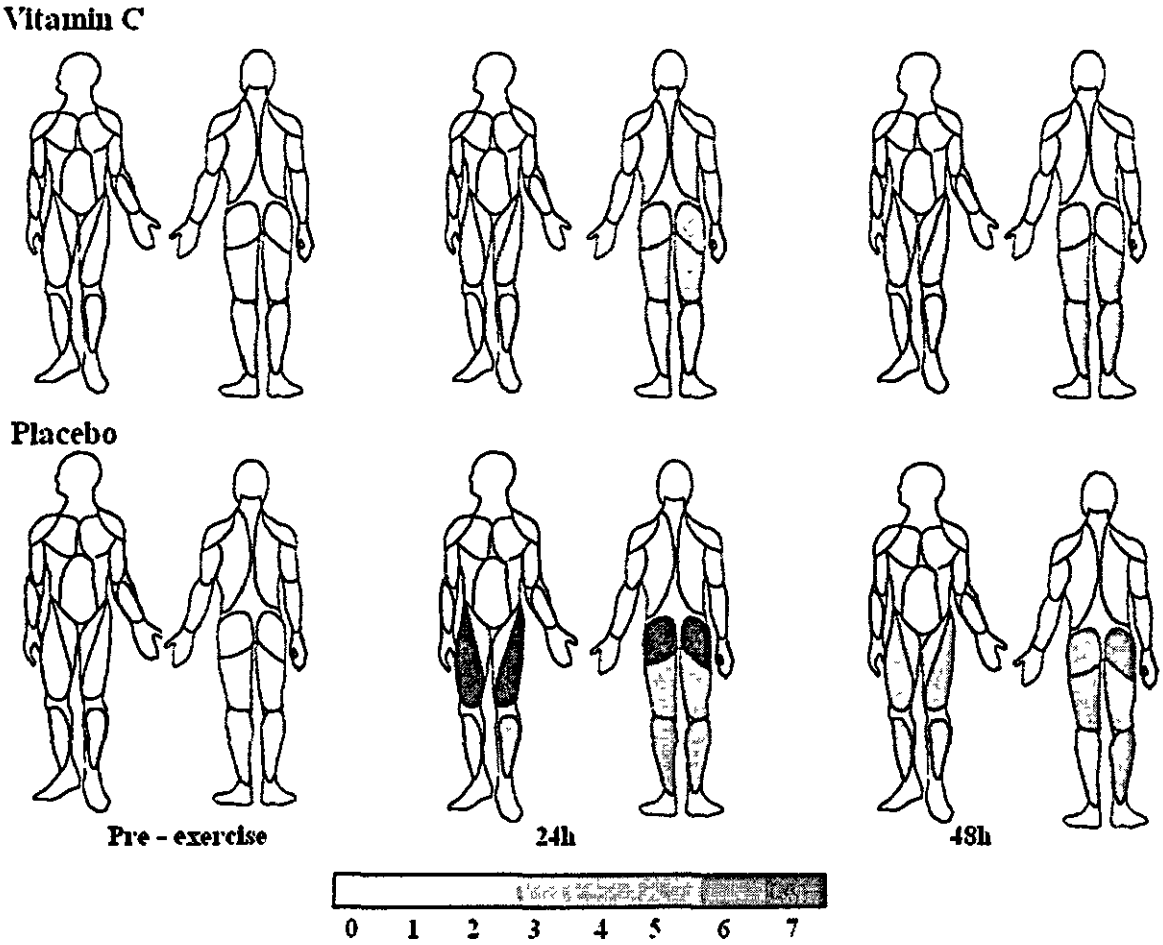


Figure 5.4: Location and frequency of muscle soreness following downhill running for the vitamin C and placebo supplemented groups. An increase in the density of shading represents an increase in the frequency of soreness in that region.

Muscle function

No intervention effect was observed on MVC of the leg flexors or extensor following exercise. Table 5.2 indicates that the MVC of the leg flexors was reduced ($p<0.05$) from baseline at 24h (-7.9 ± 2.4%) and 48 h (-8.9 ± 2.0%) and the leg extensors reduced ($p<0.05$) at 48 h (-7.9 ± 2.7%) in both groups.

Table 5.2: Isometric maximal voluntary contraction force following downhill running with either vitamin C or placebo supplementation (means ± *SEM*). * different from pre-exercise ($p<0.05$).

MVC	Vitamin C	Placebo	Σ (n=14)
Extension ($Nm.kg^{-1}$)			
Pre-exercise	3.63 ± 0.12	3.76 ± 0.24	3.70 ± 0.13
24h	3.54 ± 0.15	3.21 ± 0.33	3.39 ± 0 18
48h	3 47 ± 0.15	3.33 ± 0.26	3 40 ± 0.15 *
Flexion ($Nm.kg^{-1}$)			
Pre-exercise	2.21 ± 0.16	2.36 ± 0.22	2 28 ± 0.50
24h	2.04 ± 0 12	2.14 ± 0 23	2.09 ± 0.12 *
48h	2.05 ± 0.14	2.07 ± 0.17	2.06 ± 0.11 *
Extension (% change)			
24h	-2.5 ± 1.9	-14.9 ± 6 4	-8.7 ± 3.7
48h	-4.3 ± 2.5	-11.5 ± 4 6	-7.9 ± 2.7 *
Flexion (% change)			
24h	-6 6 ± 3.8	-9.2 ± 3.1	-7.9 ± 2.4 *
48h	-6 6 ± 3 0	-11.3 ± 2.7	-8.9 ± 2.0 *

Markers of muscle damage

Changes in Mb concentration following exercise are shown in Figure 5.5. Serum Mb increased from pre-exercise values in both groups peaking at 1h post-exercise ($p<0.05$). Peak increases in Mb from pre-exercise values were lower at 1h in the V_C group (10.12 ± 1.51 $nmol.L^{-1}$) compared to the P_L group (15.11 ± 1.99 $nmol.L^{-1}$) ($p<0.05$). Figure 5.5 also illustrates CK activity also increased from pre-exercise values peaking at 24 h post exercise ($p<0.05$). Peak increases were reduced with V_C (693 ± 65 $U.L^{-1}$) compared to P_L (1440 ± 317 $U.L^{-1}$) ($p<0.05$).

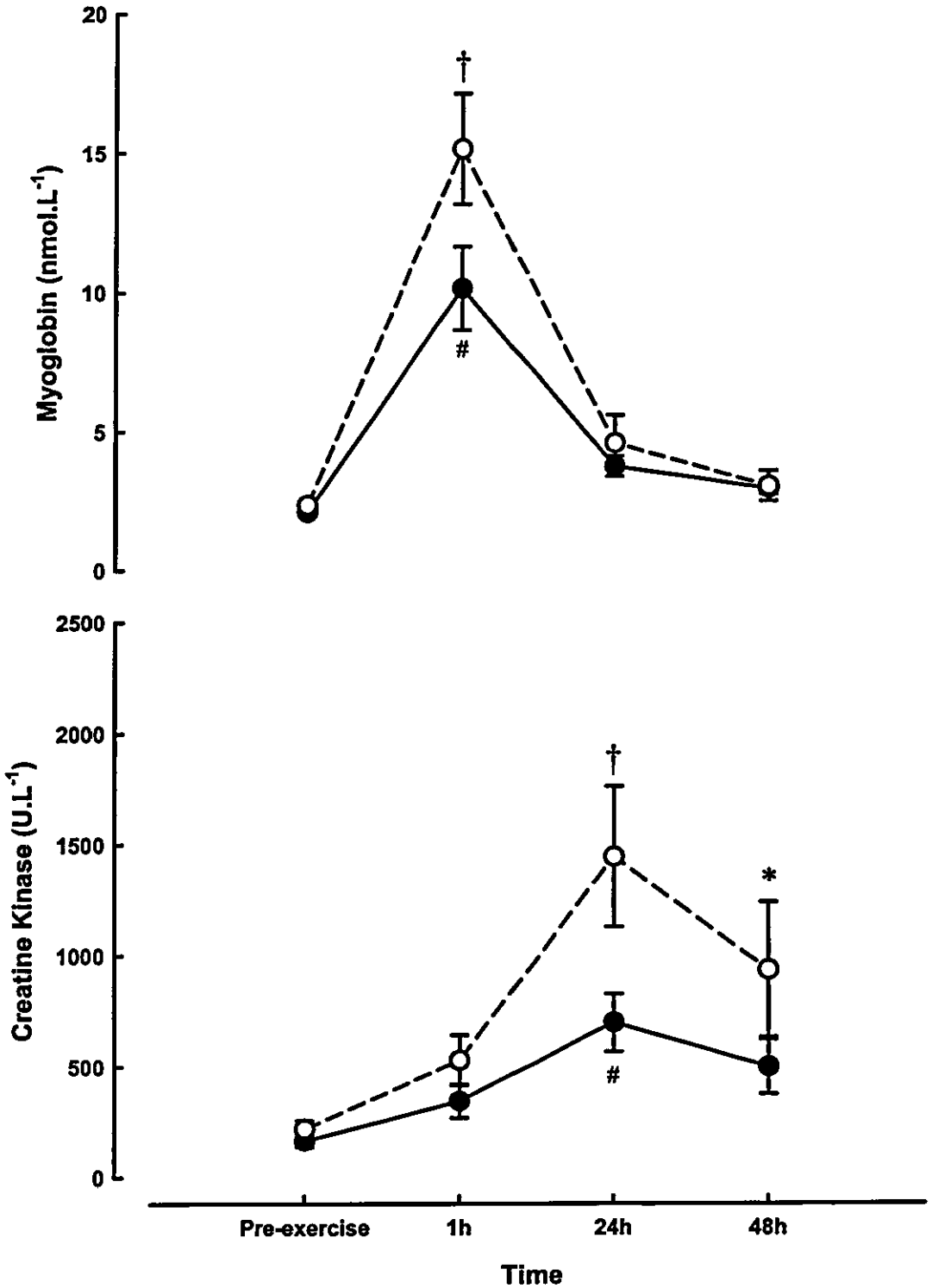


Figure 5.5: Serum myoglobin concentration and creatine kinase activity following downhill running for the vitamin C (solid line) and placebo supplemented (dashed line) groups (mean ± *SEM*). † different between groups ($p<0.05$). # P_L group different from pre-exercise ($p<0.05$). * both groups different from pre-exercise ($p<0.05$).

Uric acid and cortisol

Uric acid (UA) was unaffected by supplementation, but increased 1h post-exercise (V_C 12.01 ± 1.90 $\mu mol.L^{-1}$; P_L 12.36 ± 0.49 $\mu mol.L^{-1}$, $p<0.05$) in both groups. Serum cortisol also showed no intervention effect but did decrease from pre-exercise at 1h post-exercise (V_C 331 ± 52 $nmol.L^{-1}$; P_L 341 ± 41 $nmol.L^{-1}$, $p<0.05$) returning to baseline at 24h in both groups (Table 5.3).

Table 5.3: Serum uric acid and cortisol concentrations following downhill running with either vitamin C or placebo supplementation (means ± *SEM*). * different from pre-exercise ($p<0.05$).

	Vitamin C	Placebo	Σ (n=14)
Uric acid ($\mu mol.L^{-1}$)			
Pre-exercise	316 ± 26	333 ± 18	325 ± 22
1h	367 ± 28	353 ± 20	347 ± 24 *
24h	390 ± 28	355 ± 21	341 ± 24
48h	338 ± 28	349 ± 22	328 ± 25
Cortisol ($nmol.L^{-1}$)			
Pre-supplementation	460 ± 52	521 ± 57	490 ± 54
Pre-exercise	501 ± 73	467 ± 55	484 ± 84
1h	331 ± 52	341 ± 14	336 ± 33 *
24h	541 ± 41	508 ± 59	525 ± 50
48h	429 ± 49	452 ± 41	441 ± 45

Inflammatory markers

Figure 5.6 shows the IL-6 response to exercise for both groups. Systemic concentrations peaked 1h following exercise (V_C 1.6 ± 0.41 $pg.ml^{-1}$, P_L 2.4 ± 0.61 $pg.ml^{-1}$) ($p<0.05$) and returned to pre-exercise values within 24h.

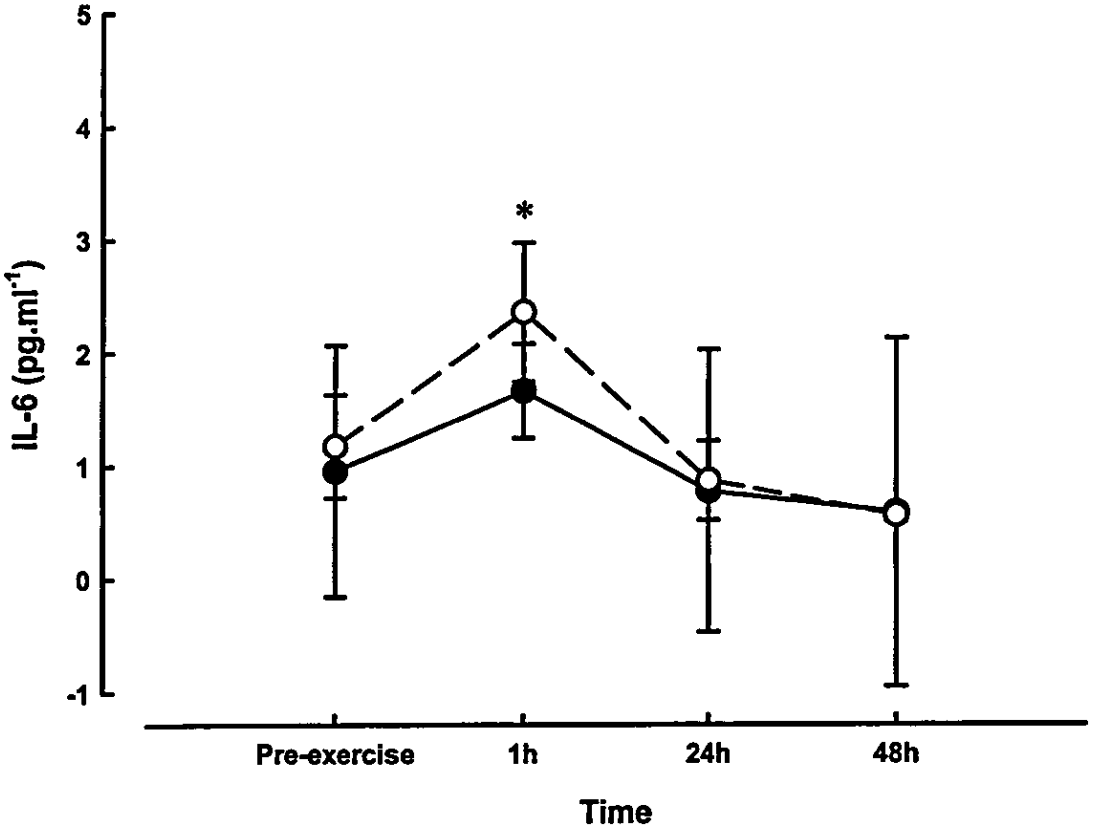


Figure 5.6: Serum interleukin 6 concentration following downhill running for the vitamin C (solid line) and placebo supplemented (dashed line) groups (mean ± *SEM*). * both groups different from pre-exercise ($p<0.05$).

Total white blood cell counts increased at 1h post-exercise in both groups (V_C 5.3 ± 1.0 $x10^9.L^{-1}$, P_L 5.4 ± 0.7 $x10^9.L^{-1}$) ($p<0.05$). Changes in the sub-populations for leukocytes are shown in Table 5.4.

Table 5.4: Change in leukocyte count and relative number following downhill running with either vitamin C or placebo supplementation (means ± *SEM*). * different from pre-exercise ($p<0.05$).

	Vitamin C		Placebo	
	Absolute no ($x10^9.L^{-1}$)	Relative no. (%)	Absolute no ($x10^9.L^{-1}$)	Relative no. (%)
WBC				
Pre-exercise	4.02 ± 0.59	-	3.32 ± 0.28	-
1h	5.30 ± 1.04 *	-	5.46 ± 0.69 *	-
24h	2.62 ± 0.78	-	2.90 ± 0.46	-
48h	2.86 ± 0.54	-	3.41 ± 0.59	-
Neutrophils				
Pre-exercise	1.98 ± 0.39	47.7 ± 4.9	1.45 ± 0.22	46.3 ± 4.9
1h	3.23 ± 0.59 *	60.8 ± 4.6 *	3.44 ± 0.53 *	63.7 ± 2.1*
24h	1.16 ± 0.84	52.8 ± 6.7	1.41 ± 0.29	51.9 ± 3.0
48h	1.25 ± 0.29	42.8 ± 3.0	1.63 ± 0.36	47.8 ± 4.3
Monocytes				
Pre-exercise	0.27 ± 0.17	6.5 ± 2.9	0.26 ± 0.12	8.0 ± 3.1
1h	0.63 ± 0.38	11.7 ± 5.0	0.47 ± 0.04	7.1 ± 0.4
24h	0.60 ± 0.21	3.0 ± 1.5 *	0.30 ± 0.10	9.4 ± 1.9
48h	0.20 ± 0.06	6.8 ± 1.4	0.28 ± 0.13	4.4 ± 3.3
Lymphocytes				
Pre-exercise	1.35 ± 0.13	37.4 ± 5.4	1.14 ± 0.19	37.9 ± 5.6
1h	1.43 ± 0.12	30.7 ± 6.1 *	1.15 ± 0.12	25.5 ± 1.8 *
24h	1.07 ± 0.17	36.8 ± 6.6	1.20 ± 0.11	36.0 ± 3.8
48h	1.19 ± 0.39	40.2 ± 3.5	0.82 ± 0.18	36.1 ± 6.0

Table 5.5 shows polymorphonuclear leukocyte activity assessed as phagocytosis and oxidative burst activity. There was no apparent effect of supplementation on leukocyte function. However, monocyte burst activity was increased 1h post-exercise (84.1 ± 4.9%, $p<0.05$) with P_L and not V_C supplementation.

Table 5.5: Polymorphonuclear leukocyte activity following downhill running with either vitamin C or placebo supplementation (means ± *SEM*). * different from pre-exercise ($p<0.05$).

	Vitamin C		Placebo	
	Phagocytosis (%)	Oxidative Burst (%)	Phagocytosis (%)	Oxidative Burst (%)
Monocyte				
Pre-exercise	70.5 ± 2.5	75.5 ± 3.9	66.3 ± 3.9	77.9 ± 2.6
1h	76.7 ± 4.0	74.6 ± 3 6	77.4 ± 5.0	84.1 ± 4.9 *
24h	60.6 ± 2.7	83.0 ± 6.5	72.1 ± 4.8	77.5 ± 2.1
48h	72.9 ± 2.9	80.1 ± 4.8	63.5 ± 1.8	74.5 ± 4.6
Neutrophils				
Pre-exercise	86.2 ± 2.1	93.0 ± 3.4	85.7 ± 3.2	97.0 ± 0.8
1h	85.2 ± 5.9	87.5 ± 5.9	90.9 ± 4.5	98.3 ± 1.2
24h	82.5 ± 1.9	94.7 ± 3.3	85.6 ± 2.6	97.9 ± 0.5
48h	83.8 ± 3.6	97.3 ± 2.2	87.7 ± 2.7	95.6 ± 1.3

Dietary vitamin C intake

Dietary vitamin C intake was not different between groups during the habitual diet (V_C 145 ± 9 mg.day^{-1}; P_L 161 ± 32 mg.day^{-1}) or the controlled diet (V_C 140 ± 12 mg.day^{-1}; P_L 155 ± 24 mg.day^{-1}). There were also no differences between diets (Table 5.6).

Table 5.6: Daily dietary composition assessed under normal (habitual) and supplemented (controlled) conditions (mean ± *SEM*).

	Habitual Diet		Controlled Diet	
	V_C	P_L	V_C	P_L
Energy Intake (MJ)	11.6 ± 1.3	12.6 ± 1.1	12.1 ± 1.2	13.6 ± 1.3
Carbohydrate (%)	53 ± 2	56 ± 2	55± 4	53 ± 1
Fat (%)	28 ± 3	27 ± 2	29 ± 4	33 ± 2
Protein (%)	19 ± 2	17 ± 2	16 ± 2	14 ± 2
Vitamin C (mg)	145 ± 9	161 ± 32	940 ± 12	155 ± 24
Vitamin E (mg)	6 ± 1	9 ± 2	9 ± 3	11 ± 2

Despite the marked increase in both soreness and markers of muscle damage, the relationship between these parameters was not strong. However, a good relationship was observed between changes in CK and Mb at 24h post-exercise (r = 0.94). Peak increases in Mb were moderately related to pre-exercise plasma concentrations of vitamin C (r = -0.53). Also, relationships were observed between MVC of leg flexors at 48h and active soreness at 48h (r = -0.53) and with MVC of the leg extensors at 48h (r = -0.51) (Figure 5.7).

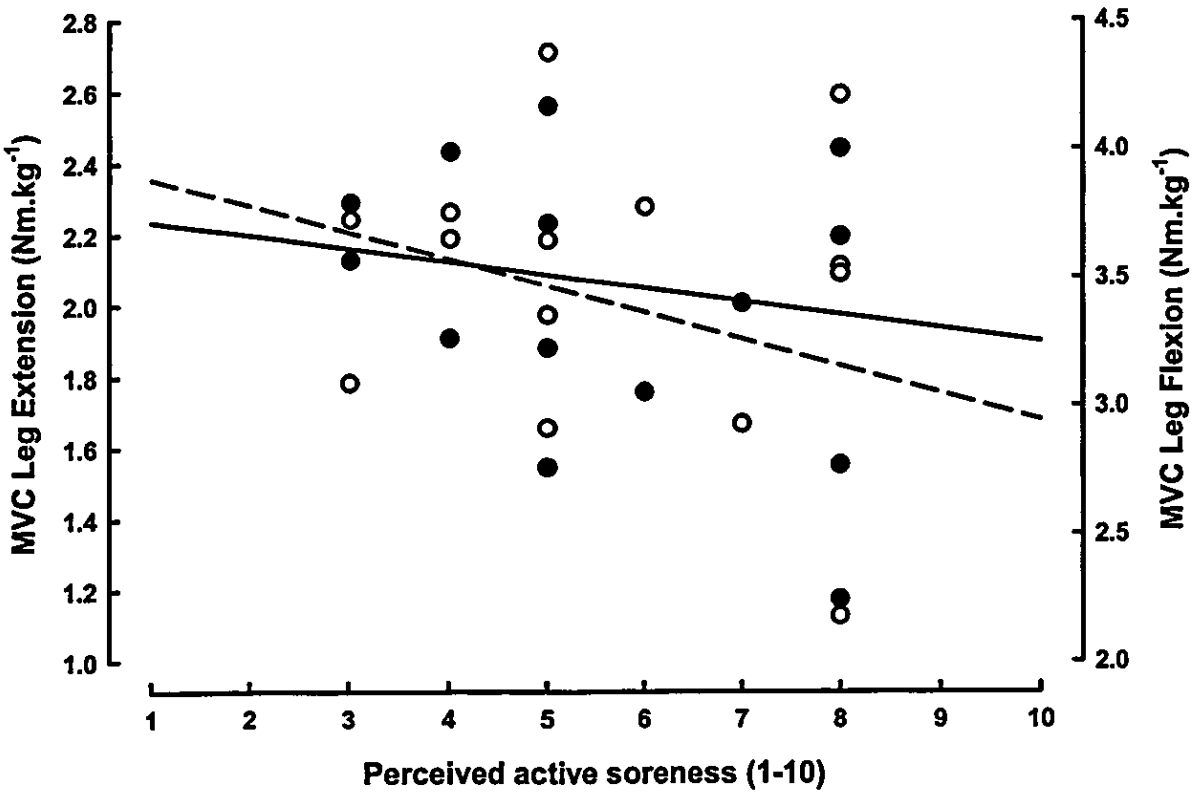


Figure 5.7: Relationships between MVC of leg extensors (solid line, solid points), MVC of leg flexors (dashed line, clear points) and ratings of perceived active soreness all at 48h post-exercise.

5.5 Discussion

The main purpose of this investigation was to evaluate whether vitamin C supplementation had any effect on muscle damage following eccentric based exercise. In line with previous work employing downhill running as a model for exercise-induced muscle damage (Chapter 4) we observed significant increases in myofibrillar protein leakage along with severe muscular soreness. These changes in the days following exercise were similar to other investigations using downhill running as a model for EIMD (Schwane *et al.*, 1983; Byrnes *et al.*, 1985; Pizza *et al.*, 1995). Furthermore, the present study also confirmed previous findings that this eccentric based exercise is associated with reductions in the ability to produce muscular force in the damaged tissue (Sargeant & Dolan, 1987; Balnave & Thompson, 1993).

Supplementation with vitamin C prior to exercise significantly increased plasma concentrations of this antioxidant compared to the placebo. Previous investigations have reported similar increases with vitamin C supplementation using a range of dosages ($400mg.day^{-1}$ to $1.5g.day^{-1}$) (Nieman *et al.*, 2000; Thompson *et al.*, 2000; Peters *et al.*, 2001a; Peters *et al.*, 2001b). Obviously, those studies employing the larger dosages reported the greatest plasma concentrations post-supplementation. One interesting point is that baseline concentrations appeared greater in this investigation than in others. This may simply reflect the greater dietary intake of vitamin C compared to previous investigations. Peters *et al.* (2001) reported a daily intake of vitamin C of between 83 to $109mg.day^{-1}$ during supplementation prior to ultra endurance running. Thompson and co-workers (2001, 2002) monitored dietary vitamin C intake prior to supplementation and reported daily intakes of ~120 $mg.day^{-1}$ and ~$147mg.day^{-1}$. These authors also attempted to control vitamin C intake during supplementation and subsequently lowered intake to ~$80mg.day^{-1}$ (Thompson *et al.*, 2000). In this investigation dietary intake was reduced from habitual levels during restricted intake, but not to the extent previously reported (Thompson *et al.*, 2000). This may reflect a difficulty in controlling dietary intake in individuals with habitually high dietary vitamin C consumption. Plasma vitamin C concentration increased in response to exercise in both groups. Elevations in blood antioxidant status following exercise are well documented and are proposed to reflect the adaptive response to increased oxidative stress during exercise resulting in a mobilisation from endogenous stores (Gleeson *et al.*, 1987; Alessio *et al.*, 1997).

This may account for the post-exercise elevations in uric acid concentrations which also has antioxidant properties (Sutton *et al.*, 1980).

Vitamin C supplementation reduced the peak increases in myoglobin concentration and creatine kinase activity following exercise compared to placebo supplementation. The presence of these myofibrillar proteins in the blood following exercise is widely accepted as an indication that muscle tissue has been damaged (Clarkson & Sayers, 1999). The apparent protective effect of antioxidants on these markers has been observed elsewhere with vitamin E but not vitamin C (Cannon *et al.*, 1990; Rokitzki *et al.*, 1994; McBride *et al.*, 1998). Vitamin C's ability to regenerate tocopheryl radicals to vitamin E may act to stabilize the vitamin E content in the muscle membrane decreasing the potential for lipid peroxidation. This process possibily inhibited the loss of membrane integrity which leads to myofibrillar protein leakage. The lack of similar findings in studies employing vitamin C supplementation could be a result of inconsistencies in the indices of muscle damage used (Kaminski & Boal, 1992; Petersen *et al.*, 2001). Also, the present investigation employed a relatively large daily vitamin C dosage ($800mg\,day^{-1}$) compared to similar studies (Jakeman & Maxwell, 1993; Thompson *et al.*, 2001). Those investigations employing similar and larger dosages have reported more beneficial effects of vitamin C, but only on indices of immune function (Nieman *et al.*, 2000; Peters *et al.*, 2001a) and oxidative stress (Alessio *et al.*, 1997; Vasankari *et al.*, 1998).

Ratings of perceived soreness peaked at 36h post exercise, which supports the well documented delayed onset of muscular soreness. The apparent benign effect of vitamin C on muscular soreness could be attributed to the limitation of this subjective measure of muscle damage. Soreness was most frequently reported in the lower extremities, namely the *quadriceps*, *hamstring* and *triceps surea* muscle groups. This finding is consistent with the previous investigation into the delayed soreness observed following downhill running (Chapter 4). Soreness was also elevated during active assessment further supporting the rationale that this sensation is often more apparent whilst performing contractions in the damaged muscle groups.

Muscular dysfunction was marked in the days following downhill running using more accurate methods of assessment than previously employed (Chapter 4). Reductions in MVC of the leg extensors and flexors were similar 24 and 48h following exercise perhaps reflecting

damage to the contractile apparatus in these muscle groups. Larger delayed reductions have been reported following other modes of eccentric exercise (Hasson *et al.*, 1992; Sayers *et al.*, 2000). The variation between studies was noted by Warren and co-workers (1999) as a lack of specificity in the testing apparatus used. They argue that to effectively measure the loss in muscle function its assessment should be on the same apparatus that caused the initial damage. This lack of specificity in MVC assessment could have masked any protective effect of vitamin C. However, we did observe moderate relationships between soreness and these delayed reductions in muscular function that might imply a causal effect. Specifically, the delayed loss of MVC has been attributed to ongoing muscle injury linked to the non specific action of phagocytes which migrate to the site of damage as a part of the proposed inflammatory response (Smith, 1991; Pyne, 1994). The byproducts of the actions of these polymorphonuclear leukocytes could elicit a sensation of soreness in addition to the damage of the contractile apparatus in skeletal muscle.

The benign effect of vitamin C supplementation on indices of immune function has been reported elsewhere (Nieman *et al.*, 1997; Petersen *et al.*, 2001; Nieman *et al.*, 2002). These previous investigations attempted to alleviate oxidative and immune changes following ultra endurance exercise during which ROS generation would be profoundly increased compared to downhill running. Thus, it is not unexpected that we did not observe an intervention effect on these parameters. However, exercise-induced muscle damage is believed to initiate an inflammatory response that appears to be corroborated by the marked leukocytosis and elevated IL-6 following exercise. The magnitude of these exercise induced increases are not as great as with more strenuous exercise protocols (Ostrowski *et al.*, 1999; Petersen *et al.*, 2001) but provide evidence for the accumulation of leukocytes in response to downhill running. Fielding and colleagues (1993) reported an infiltration of neutrophils in damaged muscle tissue following 45min downhill running. The increase in circulating neutrophils may reflect a margination and infiltration of these inflammatory cells into the injured musculature in the hours following exercise. The characteristic delayed accumulation of monocytes has lead authors to speculate as to their involvement in both exacerbation of muscle damage as well as DOMS (MacIntyre *et al.*, 1996; Smith *et al.*, 1998). However, we observed a reduction in the relative number of circulating monocytes 24h post-exercise which would appear to contradict this suggestion. Also, the relative contribution of lymphocytes to total WBC was decreased 1h post-exercise. This apparent immuno-suppressive effect is a well

documented response to prolonged exercise (Nieman & Pedersen, 1999; Pedersen & Hoffman-Goetz, 2000). Caution is advised when interpreting changes in circulating leukocytes as a response to muscle damage as increases are documented following non-damaging concentric exercise (Malm, 2002). The functional capacity of these inflammatory cells was unchanged following downhill running except for the oxidative burst activity of monocytes 1h post-exercise. Others have documented more profound increases in polymorphonuclear leukocyte activity following downhill running (Camus *et al.*, 1992). Despite this contrast in findings, the significant increase in oxidative burst activity and hence ROS generation in the placebo group alone might suggest a protective effect of vitamin C supplementation on oxidative stress. Although in the absence of a more reliable indicator of oxidative damage it is difficult to substantiate this.

The physiological responses observed during the downhill running are in agreement with other investigations that have employed this particular mode of exercise. The reduction in the relative exercise intensity from that predicted clearly supports the predominance of eccentric actions during downhill running. The increase in $\dot{V}O_2$ during exercise is reported to be directly related to ongoing muscle damage (Dick & Cavanagh, 1987). It is proposed that this upward drift in $\dot{V}O_2$ is related to the increased muscle fibre recruitment to maintain force production following muscle damage. However, this phenomenon is still observed with repeated bouts of downhill running (Westerlind *et al.*, 1992) and was not evident in the previous investigation (Chapter 4). It is more likely that the increase in $\dot{V}O_2$ is a response to an unaccustomed form of exercise, where the body increasingly employs additional muscle fibres and synergistic muscles to execute the movement (Schwane *et al.*, 1983).

Our rationale for vitamin C supplementation was the prevalence of oxidative stress both during and following exercise. Although we observed an increase in plasma vitamin C and uric acid concentration 1h post-exercise in both groups the lack of a more established marker for oxidative stress makes it difficult to ascertain the existence of free radical production following downhill running. The decrease in serum cortisol concentration in this particular investigation contradicts other findings (Peters *et al.*, 2001a; Petersen *et al.*, 2001). A possible explanation could be that the relative exercise intensity of approximately 50% $\dot{V}O_2$max provided minimal metabolic stress when compared to other more demanding exercise protocols. Indeed, exercise induced oxidative stress is more frequently reported following

marathon running and other exhaustive modes (Alessio *et al.*, 1997; Ashton *et al.*, 1999; Nieman *et al.*, 2002). This may suggest downhill running is not the preferred model for investigating the effect of antioxidant supplementation on muscle damage. Also, the comparatively high dietary intake of vitamin C by the subjects may minimise any effect of vitamin C supplementation as endogenous tissue levels were probably already saturated (Levine *et al.*, 1996).

We did observed a prophylactic effect on established markers of muscle damage with vitamin C supplementation as well as a significant inverse relationship between *in vivo* concentrations of vitamin C and one of these markers. This provides support for both the role of antioxidants in attenuating muscle damage and their use in protection against muscle injury following eccentric based exercise.

CHAPTER SIX

Influence of vitamin C supplementation on the recovery from prolonged intermittent exercise

6.1 Introduction

We have previously shown that two weeks of vitamin C supplementation has some beneficial effects on indices of muscle damage during recovery from downhill running (Chapter 5). It is evident from this and other investigations that a large dose of this particular antioxidant, in excess of current recommended daily amounts, is required to elicit these apparent prophylactic effects. (Kaminski & Boal, 1992; Jakeman & Maxwell, 1993; Nieman *et al.*, 2000). However, large dose vitamin C supplementation is not consistently associated with favourable effects on recovery from prolonged endurance exercise (Nieman *et al.*, 1997; Petersen *et al.*, 2001; Nieman *et al.*, 2002). Recently, Childs and colleagues (2001) suggested vitamin C supplementation (~1g day^{-1}) following eccentric exercise leads to pro-oxidant effects that transiently increase tissue damage and oxidative stress.

This inconsistency in recent reports could be attributed to the variety of exercise models employed as well as the specific indices measured to investigate effects on oxidative stress and subsequent recovery from exercise. One consistent factor is that beneficial effects are more frequently reported following either prolonged or exhaustive exercise (Vasankari *et al.*, 1998; Ashton *et al.*, 1999; Nieman *et al.*, 2000; Thompson *et al.*, 2001b). It is established that the increased whole body oxygen flux during exercise is directly related to an increased production of ROS and an associated state of oxidative stress (Alessio, 1993; Packer, 1997). Additionally, perturbations in immune function are more profound with prolonged exercise. Thus, marked increases in polymorphonuclear leukocyte activity specifically oxidative burst, could provide an additional source of ongoing damage in the post-exercise period (Lapointe *et al.*, 2002).

We have observed positive effects of vitamin C supplementation (800 $mg.day^{-1}$) on indices of muscle damage following 60min downhill running (Chapter 5). This particular exercise

model is considered 'metabolically' less demanding than normal running due to the predominance of eccentric actions. Subsequently, the contribution of ROS to disruption of the sarcolemma and exacerbation of existing contractile tissue damage may be minimal when compared to more exhaustive exercise protocols. Therefore, the aim of the present study was to investigate the effect of this large dose of vitamin C on recovery from prolonged intermittent exercise.

6.2 Methods

Subjects

Thirty two healthy males (n=32) volunteered to participate in this study. All subjects were habitually active in a variety of sports, but were unfamiliar with the mode of exercise to be performed in this investigation. Subjects who smoked or took vitamin supplements were excluded from the study.

Supplementation

Subjects were randomly assigned, in a double blind manner, to 800mg.day^{-1} of either vitamin C (V_C) or a placebo (P_L) supplementation. Groups had similar physical characteristics (Table 6.1). Supplements were taken in capsule form, twice daily (2 x 400mg) with food, beginning seven days prior to participation in the main trial, terminating two days after completion. A venous blood sample was taken from a forearm vein prior to commencing supplementation. Subjects were required to weigh and record all food and fluid intake on two occasions. Prior to supplementation a three day habitual diet was recorded and during the main trial another three day diet was recorded, during which subjects were requested to control their dietary vitamin C intake. The controlled diet required subjects to consume a number of foods that were estimated to provide a known quantity of vitamin C (~100 mg.day^{-1}).

Table 6.1: Physical characteristics of supplementation groups (mean ± SD).

	Vitamin C (n=16)	Placebo (n=16)
Age (years)	21.9 ± 2.6	21.6 ± 1.9
Height (m)	1.80 ± 0.06	1.82 ± 0.07
Mass (kg)	82.4 ± 9.6	83.4 ± 11.5
Sum Skinfolds (mm)	33.4 ± 9.2	34.8 ± 10.2
$\dot{V}O_2max$ ($ml.kg.min^{-1}$)	53.6 ± 4.9	53.3 ± 4.5
Weekly exercise session	6 ± 1	6 ± 1
Daily vitamin C intake (mg)	123 ± 26	119 ± 21

Experimental design

On the seventh day of supplementation individuals completed the Loughborough Intermittent Shuttle Test (LIST). Subjects exercised at an average intensity that was approximately 70% of their maximal oxygen uptake ($\dot{V}O_2max$) for 90min. Subjects reported to the laboratory having refrained from strenuous physical activity for at least two days and after an overnight fast (10-12 h). An 8 ml venous blood sample was taken from a forearm vein prior to exercise and again approximately 1, 24 and 48h post-exercise. Ratings of perceived soreness were recorded prior to exercise, at approximately 12h intervals for the following two days and again at 96 and 168h. Muscle function was assessed pre- and immediately post-exercise (IMPE) and again approximately 24, 48, 96 and 168h later (± 2h). Nude body mass was determined before and immediately following exercise. Subjects ingested water at regular intervals throughout the exercise. Heart rate, RPE and 15m sprint times were monitored at regular intervals throughout the LIST (Figure 6.1).

Statistical analysis

An independent two-way analysis of variance (ANOVA) with repeated measures was used to determine if any differences existed between interventions and time during exercise and recovery. For significant F ratios a paired *Students* t-test, with a *Bonferroni* adjustment, was used to locate the differences between means. Pearson product moment correlations were used to examine the relationship between variables. Significance was accepted at the 5% level. Values are expressed as the mean ± standard error of the mean (*SEM*) throughout.

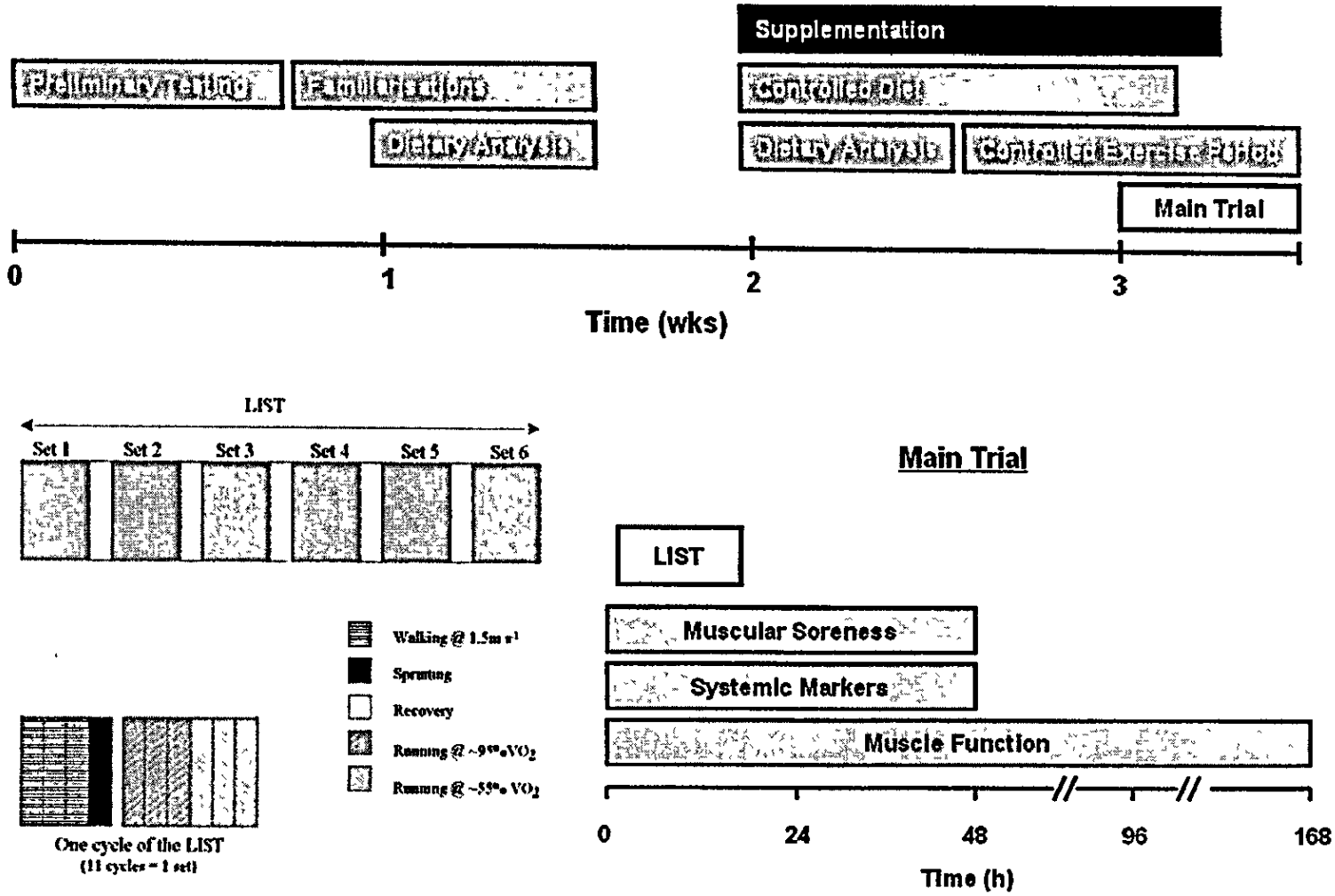


Figure 6.1: A schematic representation of the experimental design.

6.3 Results

Response to exercise

During the LIST, heart rate increased ($p<0.05$) from the onset of exercise at 45, 60, 75 and 90min (166 ± 2 $b.min^{-1}$, $p<0.05$) as did ratings of perceived exertion (17 ± 1, $p<0.05$). Mean sprint times for each 15min block increased from the first block (2.58 ± 0.04s) to the sixth (2.67 ± 0.03s, $p<0.05$) during the LIST but were not different between groups throughout (V_C 2.61 ± 0.02s; P_L 2.66 ± 0.04s). Fluid intake was not different between groups (V_C 1.23 ± 0.04L; P_L 1.24 ± 0.05L) and weight loss was not different (V_C 0.84 ± 0.10%BM; P_L 0.75 ± 0.11%BM). Estimated changes in plasma volume did not differ throughout the testing period in either group.

Plasma vitamin C

Plasma vitamin C concentration increased ($p<0.05$) with supplementation compared to P_L (Figure 6.2) and remained elevated above P_L concentrations until cessation of supplementation 36h post-exercise ($p<0.05$). Figure 6.2 also shows that plasma concentrations also increased 1h post-exercise in both groups ($p<0.05$).

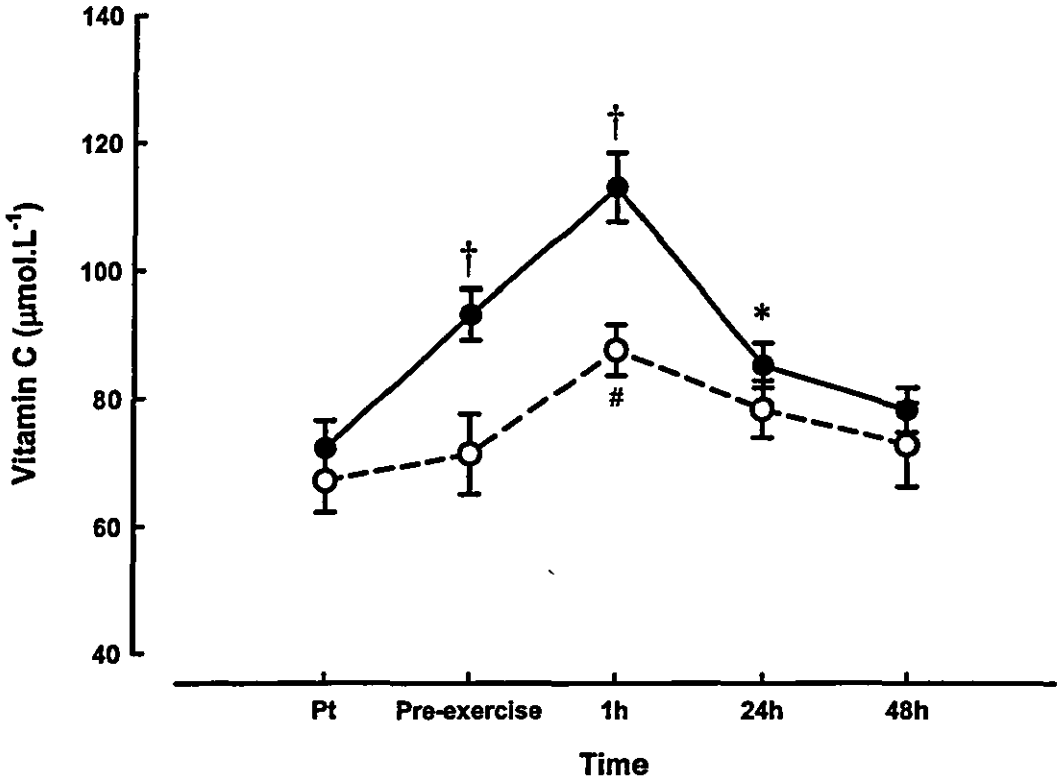


Figure 6.2: Plasma vitamin C concentration pre-treatment (Pt), pre-exercise and following intermittent shuttle running for the vitamin C (solid line) and placebo supplemented (dashed line) groups (mean ± *SEM*). † different between groups ($p<0.05$). # P_L different from pre-exercise ($p<0.05$). *both groups different from pre-exercise ($p<0.05$).

Muscular soreness

Ratings of perceived soreness assessed while at rest (passive) and whilst descending stairs (active) where unaffected by supplementation. Figure 6.3 illustrates that passive soreness increased during the post-exercise period, peaking between 24 and 36h (5.3 ± 0.4, $p<0.05$) and returned to pre-exercise values at 96 and 168h. Active soreness also increased from pre-exercise (2.2 ± 0.2) peaking at 24h (5.1 ± 0.5) ($p<0.05$). The location of soreness was most frequently reported in the *hamstrings*, *quadriceps*, *triceps surea*, *gluteus maximus* and musculature of the lower back (Figure 6.4).

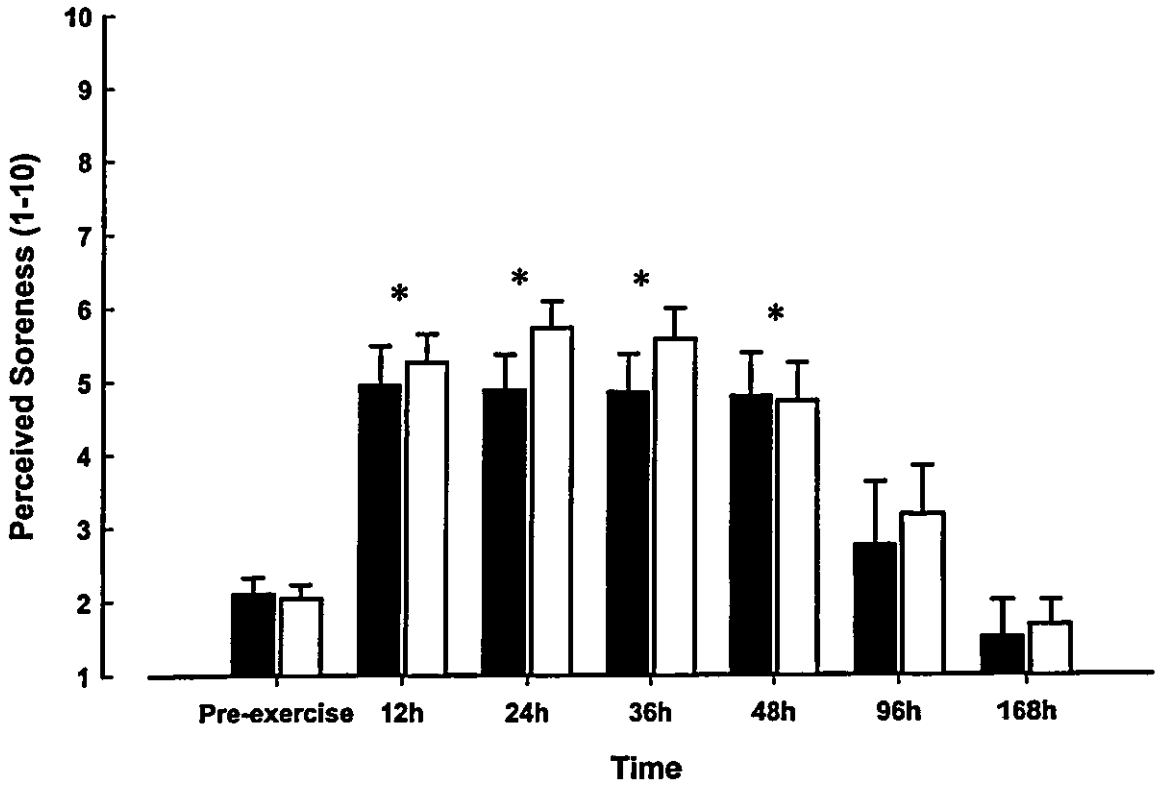


Figure 6.3: Ratings of perceived soreness (10 point scale) following intermittent shuttle running for the vitamin C (filled bars) and placebo supplemented (clear bars) groups (mean ± *SEM*). * both groups different from pre-exercise ($p<0.05$).

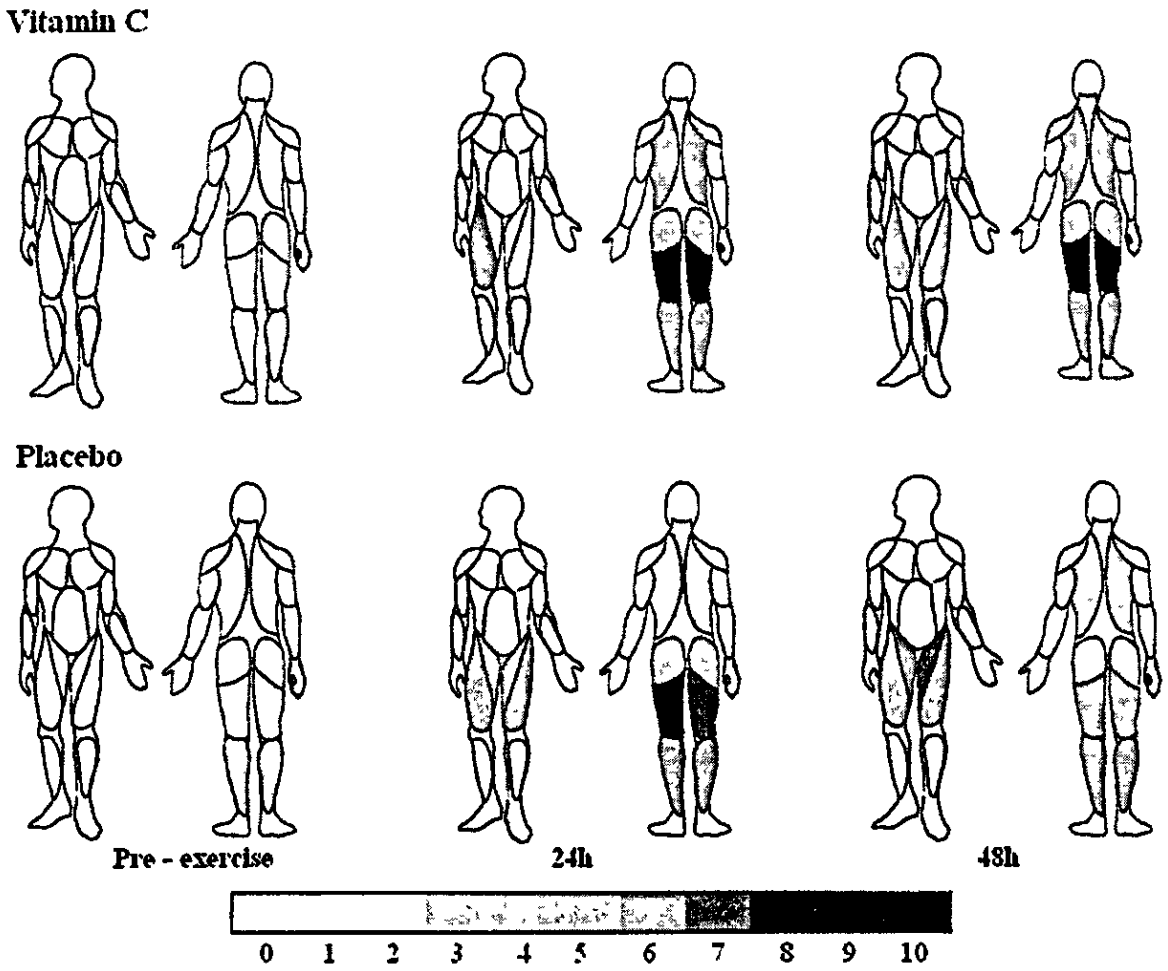


Figure 6.4: Location and frequency of muscle soreness following intermittent shuttle running for the vitamin C and placebo supplemented groups. An increase in the density of shading represents an increase in the frequency of soreness in that region.

Muscle function

Maximal voluntary contraction of the leg flexors was different between groups at 96h (V_C 2.62 ± 0.08 $Nm.kg^{-1}$; P_L 2.16 ± 0.10 $Nm.kg^{-1}$) and 168h (V_C 2.56 ± 0.10 $Nm.kg^{-1}$; P_L 2.11 ± 0.10 $Nm.kg^{-1}$) post-exercise ($p<0.05$). MVC of the leg extensors was not different between groups. Table 6.2 also shows that MVC of both leg flexors and extensors decreased IMPE, 24 and 48h but returned to pre-exercise values at 96 and 168h ($p<0.05$).

Table 6.2: Isometric maximal voluntary contraction following intermittent shuttle running with either vitamin C or placebo supplementation (means ± *SEM*). † different between groups ($p<0.05$). * different from pre-exercise ($p<0.05$).

MVC ($Nm.kg^{-1}$)	Pre-exercise	IMPE	24h	48h	96h	168h
Flexion						
Vitamin C	2.72 ± 0.11	2.04 ± 0.10 *	2.27 ± 0 09 *	2.32 ± 0.10 *	2.62 ± 0 08 †	2.56 ± 0.04 †
(% pre-exercise)	-	(80 ± 4)	(84 ± 2)	(86 ± 3)	(103 ± 2)	(101 ± 4)
Placebo	2.51 ± 0.09	1.92 ± 0.11 *	1.97 ± 0.12 *	2.01 ± 0.12 *	2 16 ± 0.10	2 11 ± 0.10
(% pre-exercise)	-	(83 ± 6)	(78 ± 4)	(80 ± 4)	(90 ± 6)	(88 ± 5)
Extension						
Vitamin C	3.66 ± 0.11	3.26 ± 0.06 *	3.31 ± 0 06 *	3 37 ± 0.15 *	3.70 ± 0.08	3.77 ± 0 07
(% pre-exercise)	-	(89 ± 2)	(90 ± 3)	(92 ± 2)	(101 ± 3)	(103 ± 2)
Placebo	3.57 ± 0.14	3.24 ± 0.16 *	3.32 ± 0.15 *	3.29 ± 0.16 *	3.57 ± 0.19	3 60 ± 0.20
(% pre-exercise)	-	(88 ± 4)	(93 ± 3)	(92 ± 3)	(97 ± 3)	(98 ± 2)

Markers of muscle damage

Changes in Mb concentration following exercise are illustrated in Figure 6.5. Myoglobin concentration increased from pre-exercise values in both groups peaking at 1h post-exercise ($p<0.05$) and returning to baseline at 24 and 48h. Peak increases in Mb were lower at 1h in the V_C group (9.18 ± 0.81 $nmmol.L^{-1}$) compared to the P_L group (17.15 ± 1.58 $nmol.L^{-1}$). Figure 6.5 also shows that CK activity also increased following exercise, peaking at 24h ($p<0.05$). There were no differences between groups for CK activity throughout.

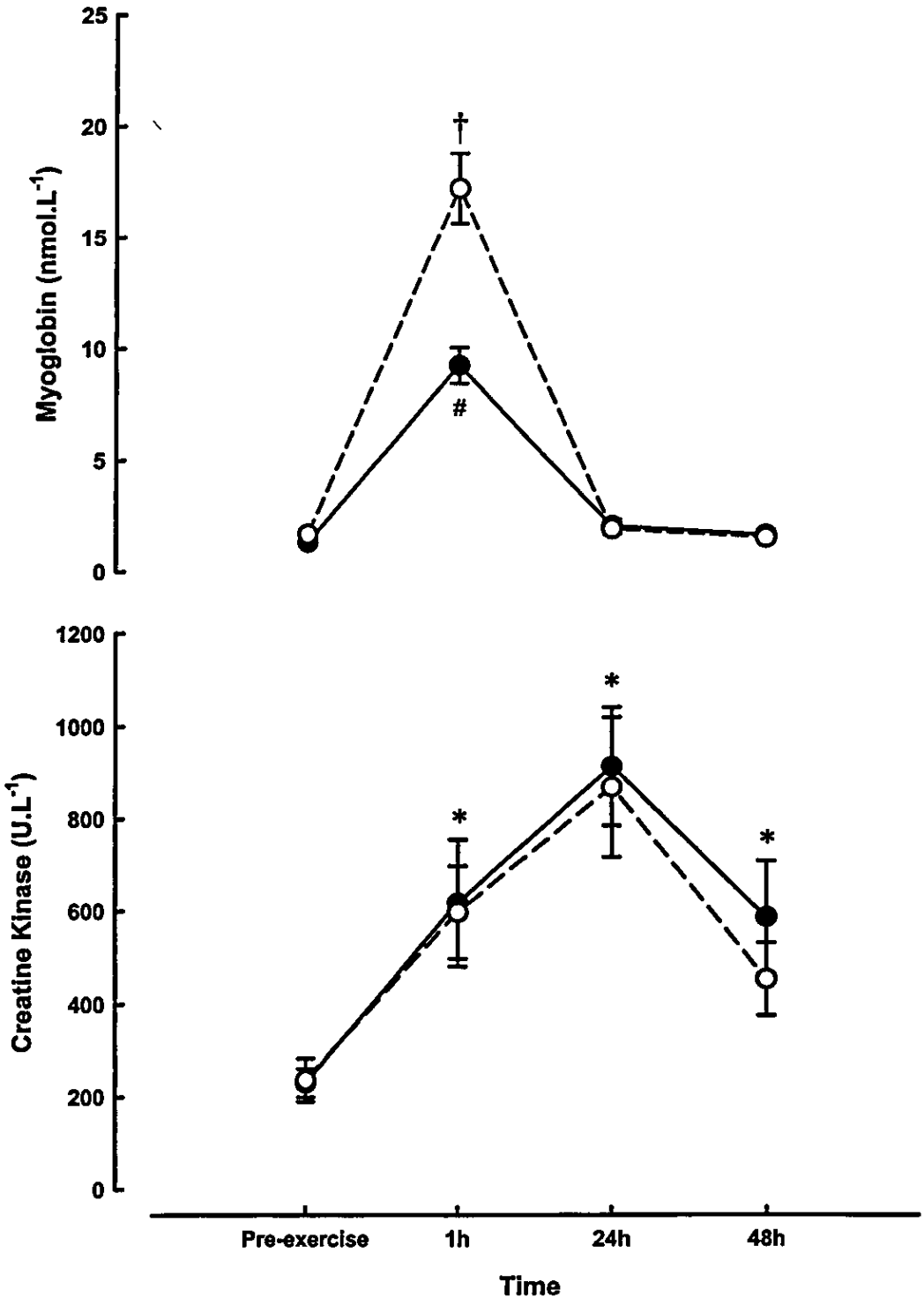


Figure 6.5: Serum myoglobin concentration and creatine kinase activity following intermittent shuttle running for the vitamin C (solid line) and placebo supplemented (dashed line) groups (mean ± *SEM*). † different between groups ($p<0.05$). * both groups different from pre-exercise ($p<0.05$).

Inflammatory markers

Figure 6.6 shows the IL-6 response to exercise for both groups. Systemic concentrations peaked 1h following exercise (V_C 12.6 ± 3.0 pg.ml^{-1}, P_L 8.9 ± 1.6 pg.ml^{-1}) ($p<0.05$) and returned to pre-exercise values within 24h. Changes in other inflammatory mediators following exercise are shown in Table 6.3.

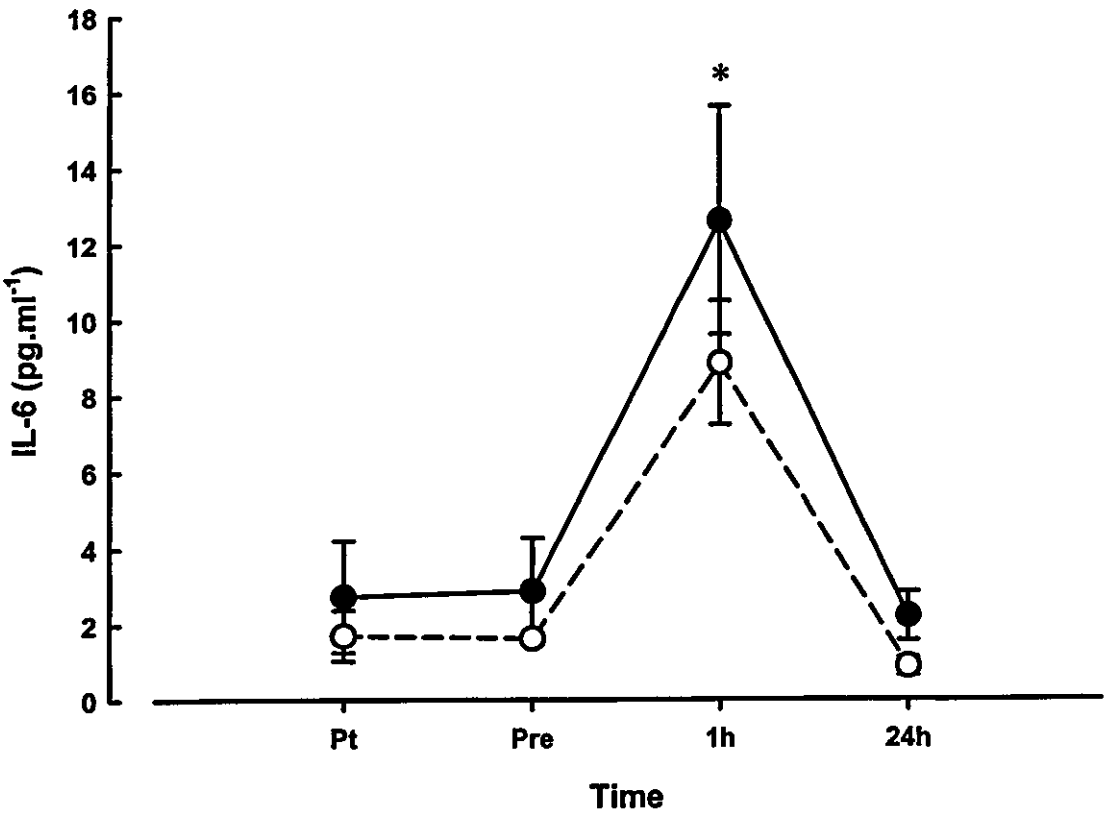


Figure 6.6: Serum interleukin 6 concentration following intermittent shuttle running for the vitamin C (solid line) and placebo supplemented (dashed line) groups (mean ± *SEM*). * both groups different from pre-exercise ($p<0.05$).

Table 6.3: Change in inflammatory mediators following intermittent shuttle running with either vitamin C or placebo supplementation (means ± *SEM*). * different from pre-exercise ($p<0.05$).

	Vitamin C			Placebo		
	Pre-exercise	1h	24h	Pre-exercise	1h	24h
TNF-α ($pg.ml^{-1}$)	1.80 ± 0.42	1.85 ± 0.42	1.68 ± 0.27	1.19 ± 0.16	1.12 ± 0.16	1.16 ± 0.16
IL-1ra ($pg.ml^{-1}$)	803 ± 524	1523 ± 531*	757 ± 528	256 ± 28	1579 ± 627*	295 ± 55
IL-10 ($pg.ml^{-1}$)	3.6 ± 1.3	29.1 ± 11.2*	2.5 ± 0.5	2.3 ± 0.4	24.6 ± 7.3*	2.1 ± 0.3
sICAM-1 ($ng.ml^{-1}$)	301 ± 15	306 ± 17	306 ± 14	271 ± 11	280 ± 12	306 ± 11.2

Total white blood cell counts increased at 1h post-exercise in both groups (V_C 8.8 ± 1.1 $x10^9.L^{-1}$, P_L 8.8 ± 1.1 $x10^9.L^{-1}$) ($p<0.05$). Changes in the sub-populations for leukocytes are shown in Table 6.4.

Table 6.4: Change in leukocyte count and relative number following intermittent shuttle running with either vitamin C or placebo supplementation (means ± *SEM*). * different from pre-exercise ($p<0.05$).

	Vitamin C		Placebo	
	Absolute no ($x10^9.L^{-1}$)	Relative no. (%)	Absolute no ($x10^9.L^{-1}$)	Relative no. (%)
WBC				
Pre-exercise	5.25 ± 1.35	-	5.39 ± 0.81	-
1h	8.75 ± 1.11 *	-	8.77 ± 1.11 *	-
24h	4.11 ± 0.45	-	4.11 ± 0.21	-
Neutrophils				
Pre-exercise	2.74 ± 0.88	49.3 ± 2.8	3.15 ± 0.82	53.2 ± 4.9
1h	6.78 ± 0.12 *	74.8 ± 3.0 *	5.78 ± 0.10 *	76.8 ± 3.5 *
24h	2.59 ± 0.38	55.0 ± 2.8	2.17 ± 0.16	54.3 ± 3.0
Monocyctes				
Pre-exercise	0.51 ± 0.17	8.9 ± 0.5	0.39 ± 0.05	7.7 ± 0.6
1h	0.52 ± 0.07	7.5 ± 0.6	0.54 ± 0.08	6.2 ± 0.4
24h	0.39 ± 0.04	8.8 ± 0.5	0.32 ± 0.03	8.1 ± 0.5
Lymphocytes				
Pre-exercise	1.77 ± 0.28	38.6 ± 3.2	1.60 ± 0.10	36.0 ± 4.5
1h	1.26 ± 0.10	16.9 ± 2.5 *	1.29 ± 0.06	18.5 ± 3.0 *
24h	1.52 ± 0.11	34.7 ± 2.6	1.14 ± 0.13	35.1 ± 2.6

Table 6.5 shows polymorphonuclear leukocyte activity assessed as phagocytosis and oxidative burst activity. There was no apparent effect of the LIST or supplementation on leukocyte function.

Table 6.5: Polymorphonuclear leukocyte activity following intermittent shuttle running with either vitamin C or placebo supplementation (means ± *SEM*).

	Vitamin C		Placebo	
	Phagocytosis (%)	Oxidative Burst (%)	Phagocytosis (%)	Oxidative Burst (%)
Monocyte				
Pre-exercise	48 0 ± 4.3	53.2 ± 5.8	47.4 ± 3.6	54.0 ± 6 8
1h	49.4 ± 6.5	50.6 ± 7.8	54.3 ± 5.0	60.0 ± 7.9
24h	47.8 ± 5.2	51.2 ± 6.0	51.5 ± 3.6	56.1 ± 5.9
Neutrophils				
Pre-exercise	75.6 ± 3.0	89.5 ± 1.8	71.7 ± 2.7	85.2 ± 3.0
1h	70.0 ± 8.3	78.9 ± 5.9	72.5 ± 4.0	80.6 ± 5.3
24h	76.8 ± 2.3	85.9 ± 3.3	82.2 ± 3.2	89.0 ± 2.6

Uric acid, F_2-isoprostanes and cortisol

Serum uric acid concentrations were elevated following exercise at 1h ($p<0.05$) in both groups and were greater in the P_L group at 1h and 48 h post-exercise ($p<0.05$). Table 6.6 shows that values were elevated in the P_L group throughout the post-exercise period ($p<0.05$). Serum cortisol increased 1h post exercise in the P_L group ($p<0.05$), but was unchanged throughout in the V_C group (Table 6.6). However, salivary cortisol increased in both groups 1h following exercise ($p<0.05$).

Table 6.6: Serum uric acid, cortisol, urinary F_2-isoprostanes and salivary cortisol concentration following intermittent shuttle running with either vitamin C or placebo supplementation (means ± *SEM*). † different between groups ($p<0.05$).* different from pre-exercise ($p<0.05$).

	Pt		Pre-exercise		1h		24h		48h	
	V_C	P_L	V_C	P_L	V_C	P_L	V_C	P_L	V_C	P_L
Uric acid ($\mu mol.L^{-1}$)	-	-	323 ± 11	350 ± 10	368 ± 15 *	414 ± 13 *†	345 ± 9	382 ± 14 *	331 ± 9	382 ± 14 *†
F_2-isoprostanes ($ng.ml^{-1}$)	8.2 ± 1.2	12.2 ± 4.5	6.8 ± 1.1	8.8 ± 1.8	-	-	8.8 ± 1.5	8.9 ± 1.8	6 0 ± 1.0	8.9 ± 2.4
Cortisol ($nmol.L^{-1}$)	539 ± 35	555 ± 47	590 ± 45	648 ± 36	665 ± 80	928 ± 98 *†	541 ± 46	581 ± 50	526 ± 46	533 ± 35
Salivary cortisol ($nmol.L^{-1}$)	-	-	17.7 ± 1.4	18.6 ± 1.9	33.6 ± 4.3 *	28.2 ± 3.7 *	-	-	-	-

Dietary vitamin C intake

Dietary vitamin C intake was not different between groups during the habitual diet (V_C 123.0 ± 7.7 mg.day^{-1}; P_L 119.9 ± 8.1 mg.day^{-1}) or the controlled diet (V_C 119 ± 8.4 mg.day^{-1}; P_L 127.8 ± 21.1 mg.day^{-1}). There was also no difference between diets (Table 6.6).

Table 6.7: Daily dietary composition assessed under normal (habitual) and supplemented (controlled) conditions (mean ± *SEM*).

	Habitual Diet		Controlled Diet	
	V_C	P_L	V_C	P_L
Energy Intake (MJ)	14.8 ± 1.2	13.1 ± 1.1	13.2 ± 1.6	13.2 ± 1.0
Carbohydrate (%)	54 ± 2	54 ± 2	53 ± 6	55 ± 2
Fat (%)	31 ± 3	30 ± 2	31 ± 2	29 ± 4
Protein (%)	15 ± 2	16 ± 2	16 ± 3	16 ± 2
Vitamin C (mg)	123 ± 12	119 ± 16	919 ± 8	128 ± 21
Vitamin E (mg)	8 ± 1	8 ± 1	9 ± 1	9 ± 1

Although there were marked changes in the indices of muscle damage following exercise, relationships between these parameters were not strong. Peak increases in Mb were moderately related to plasma concentration of vitamin C pre-exercise (r = -0.57) and 1h post-exercise (r = -0.54). Also, weak relationships were observed between MVC of leg flexors at 24h and pre-exercise plasma vitamin C concentration at (r = 0.47) and between uric acid and plasma vitamin C concentrations 1h post-exercise (r = -0.46).

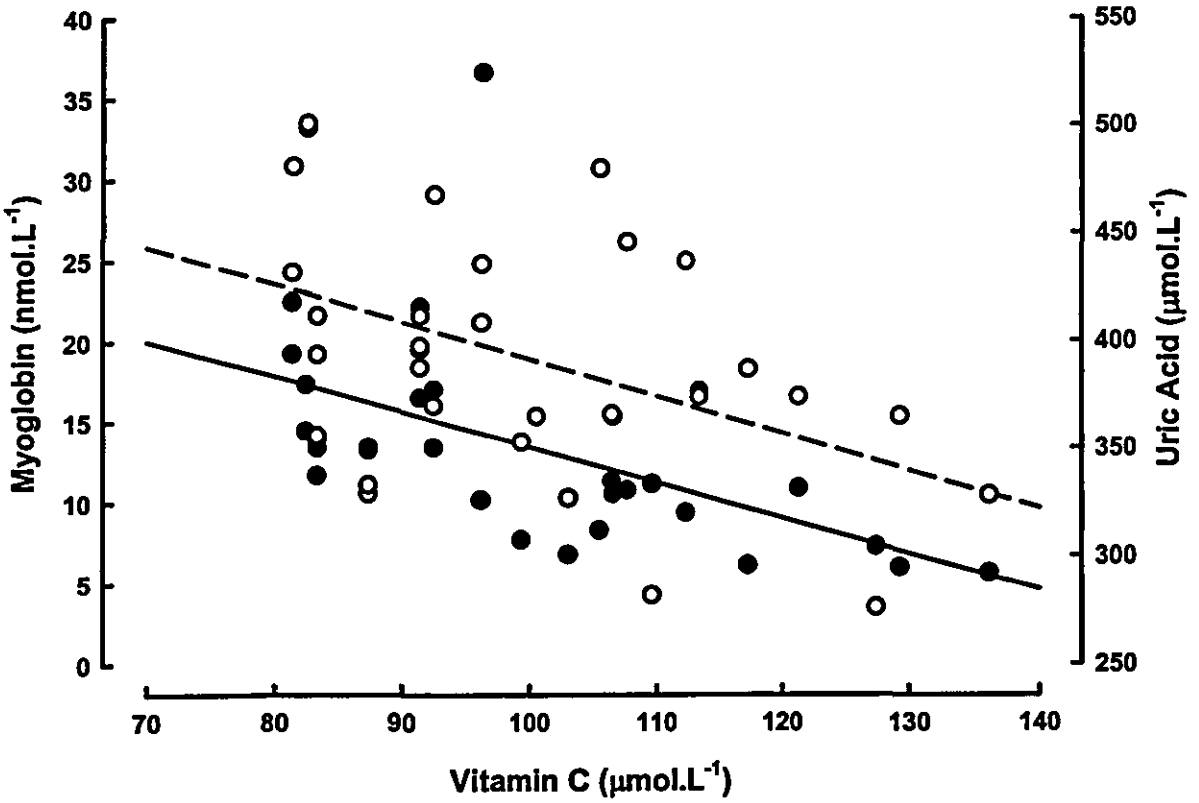


Figure 6.7: Relationships between myoglobin (solid line, solid points), uric acid (dashed line, clear points) and plasma vitamin C concentrations all at 1h post-exercise.

6.4 Discussion

Following supplementation with vitamin C we observed increases in plasma concentrations greater than with the placebo supplement which were similar to those previously reported following the same supplementation strategy (Chapter 5). Based on previous research that suggests oxidative stress and free radical production are involved in the aetiology of EIMD, an elevation in plasma antioxidant status may potentially reduce the severity of this muscle injury (Jakeman & Maxwell, 1993; Dekkers *et al.*, 1996). However, evidence that elevated plasma vitamin C concentration is associated with increased muscle tissue vitamin C concentration is limited to research on smooth and not skeletal muscle (Voskobomik *et al.*, 1998). Thus, increases in plasma vitamin C concentration may not reflect elevations in intracellular cytosolic skeletal muscle quantities.

The major outcome of this investigation was that supplementation with vitamin C reduced the peak increases in myoglobin concentration following exercise compared to placebo supplementation. As already mentioned, the appearance of myofibrillar proteins in the systemic circulation is accepted as an indication of disruption to the sarcolemma and subsequently muscle injury (Dop Bär *et al.*, 1997; Clarkson & Sayers, 1999). Other than evidence from the previous investigation (Chapter 5) the protective effect of vitamin C on this marker of muscle damage is not well documented even though it has been a long-standing marker of skeletal muscle injury (Armstrong, 1984). Creatine kinase activity was increased following exercise in both groups. Others have reported a reduction in post-exercise CK activity with vitamin E (Cannon *et al.*, 1990; Rokitzki *et al.*, 1994a; McBride *et al.*, 1998) and mixed antioxidant supplementation (Viguie *et al.*, 1989; Rokitzki *et al.*, 1994b) following a variety of exercise modes. Additionally, a reduction in the exercise-induced increase of this particular myofibrillar protein was observed with vitamin C following downhill running in the previous investigation (Chapter 5). The large variability between individuals may have masked any effect of the antioxidant supplement in this study. Also, due to the long time course of CK appearance, in our opinion, it is not as reliable an indicator of muscle injury as myoglobin which has a shorter half-life in the circulation (Cairns *et al.*, 1983). Increases in CK activity were not considerably greater than those following downhill running (Chapters 4 & 5). This may support evidence that the duration as opposed to the intensity of exercise is the dependent factor in myofibrillar protein efflux (Schwane *et al.*,

1983; Noakes, 1987). Unfortunately, this is not corroborated with Mb efflux as increases were considerably greater than those observed following downhill running (LIST, >900%; downhill running <500%). Perhaps alluding to a different aetiology of myofibrillar protein leakage for these two molecules. However, the increases in CK activity and Mb concentration were similar to and followed the same time course of those recently reported following intermittent shuttle running (Thompson *et al.*, 2001a; Thompson *et al.*, 2001b).

Isometric MVC of both the leg flexors and extensors was reduced immediately following and up to 48h post-exercise. Similar reductions have been reported with intermittent exercise (Thompson *et al.*, 2001b) and previously with more eccentric based exercise (Chapter 5). The dysfunction observed IMPE could be attributed to exercise-induced muscle injury but is perhaps more indicative of the acute effects of fatigue following prolonged exercise (Clarkson *et al.*, 1992; Allen, 2001). The further reduction in strength during the days following exercise has been attributed to ongoing damage (Jakeman & Maxwell, 1993; Thompson *et al.*, 2001b). Individuals supplemented with vitamin C recovered MVC of the leg extensors to pre-exercise values within the assessment period (7 days), unlike those taking the placebo supplement. The eccentric actions performed by this muscle group during decelerations and turning in the intermittent exercise would provide a logical explanation for the existence of muscle injury. Thompson and colleagues (2001) observed an improved recovery of leg flexor isometric strength with vitamin C supplementation (400mg.day^{-1}) 24h following the same intermittent exercise, although this was not accompanied by the reductions in myofibrillar protein appearance in the circulation. Jakeman and Maxwell (1993) reported improved muscle function following less demanding exercise with vitamin C supplementation (400mg.day^{-1}), which was associated with an improved low-frequency fatigue immediately following exercise. Thus, it appears that vitamin C may offer some protection that reduces the extent of muscle damage and facilitates a more rapid recovery of normal muscular function. Whether this prophylactic mechanism influences damage imposed during exercise or in the post-exercise period remains to be elucidated.

The characteristic delayed reduction in muscle function following muscle damaging exercise has been linked to ongoing oxidative damage induced by phagocyte activity in the days post-exercise (MacIntyre *et al.*, 1995; Lundberg, 2001; Lapointe *et al.*, 2002). Although there was a significant increase in circulating neutrophils post-exercise there was no apparent increase in the phagocytic activity of these leukocytes making it difficult to directly link the delayed

reductions in muscular function with an increased in free radical generation from active polymorphonuclear leukocytes. Additionally, intermittent exercise induced elevations in a number of inflammatory cytokines that could be associated with neutrophilia. Increases following this particular exercise mode were not as marked as those reported with more prolonged endurance based exercise (Ostrowski *et al.*, 1999; Nieman *et al.*, 2000; Suzuki *et al.*, 2003). Changes in IL-6 were not has pronounced as previously documented following intermittent exercise (Thompson *et al.*, 2001b) which may be linked to the different training status of subjects in each investigation. The cytokines and cytokine inhibitor that were significantly elevated following exercise in this study have been categorised as either inflammatory responsive (IL-6) or anti-inflammatory (IL-1ra and IL-10). Thus, it may be that any acute phase response and subsequent chronic inflammation was abrogated by the actions of these cytokines, or alternatively, this exercise model was not severe enough to augment pro-inflammatory cytokines, including TNF-α. Pedersen and colleagues (2001) have proposed that following exercise increases in systemic concentrations of TNF-α and IL-1β facilitate an acute inflammatory response. However, exercise is also accompanied by an increase production of IL-6, specifically from contracting skeletal muscle, that directly affects IL-10 and IL-1ra which act as anti-inflammatory mediators (Pedersen *et al.*, 2001). It is beyond the scope of this investigation to attempt to corroborate this sequence of events without more comprehensive assessment.

Changes in uric acid concentration following exercise were greatest in the placebo group. These increases were greater than those observed in the vitamin C group and might indicate a reduction in oxidative stress with vitamin supplementation and an inhibition of endogenous antioxidant production, such as uric acid. Similar investigations that have also observed reduction in markers of lipid peroxidation have reported attenuation of the post-exercise increase in uric acid (Duthie *et al.*, 1990; Maxwell *et al.*, 1993). Recently, Nieman *et al.* (2002) reported diminished uric acid levels following an ultramarathon in individuals supplemented with vitamin C (1.5g.day-1). In this case, these reductions had no influence on oxidative or immune markers post-exercise. The weak inverse relationship between uric acid and vitamin C concentrations 1h post-exercise might reflect an increase in blood antioxidant status that reduced the release of endogenous antioxidants such as vitamin C and uric acid.

Changes in serum cortisol were only observed with placebo supplementation, remaining unchanged from pre-supplementation values with vitamin C. Peters and co-workers (2001) reported a similar reductions in ultramarathon runners supplemented with vitamin C ($500mg.day^{-1}$ or $1000mg.day^{-1}$) suggesting that supplementation blunts the mobilisation of this antioxidant from the adrenal cortex, inhibiting cortisol secretion and facilitating an acute inflammatory response. They speculate that this abrogation of vitamin C may lead to temporal immunosuppression as increases in cortisol are commonly associated with leukocytosis following exercise (Nieman *et al.*, 1989; Gabriel *et al.*, 1992). Unfortunately, Peters and co-workers (2001) didn't control carbohydrate intake during exercise. Carbohydrate has been shown to affect exercise-induced changes in stress hormones and cytokines during exercise (Nehlsen-Cannarella *et al.*, 1997; Bishop *et al.*, 2002; Nieman *et al.*, 2003). Recently, Bishop and co-workers (2002) observed a reduction in the IL-6 and cortisol response, as well as neutrophil accumulation and activity with carbohydrate ingestion during the same intermittent exercise protocol employed in this investigation. As subjects were fasted prior to and throughout exercise an explanation for the reduced cortisol response with vitamin C supplementation in this investigation remains unclear. Salivary cortisol, unaffected by supplementation, was increased in both groups following exercise. It is proposed that salivary assessment of cortisol gives a more accurate indication of free (active) cortisol following exercise and is influenced to a lesser extent by emotional status compared to serum measurements (Stupnicki & Obminski, 1992). Neither salivary nor serum cortisol was associated with the neutrophilia following exercise which supports previous observations using a similar intermittent protocol (Gray *et al.*, 1993).

Although perception of soreness increased following intermittent exercise, as reported previously (Thompson *et al.*, 1999; Thompson *et al.*, 2001b), there was no effect of supplementation. Kaminski and Boal (1992) reported reductions with vitamin C following eccentric exercise using a more comprehensive assessment of soreness, but similar findings are not well documented. This may be due to the subjective nature of perceived muscle soreness, where individuals rate pain on previous experiences rather than an arbitrary scale. Others have employed more comprehensive methods of assessing muscle soreness through the use of pressure tolerance tests on sore and damaged muscles (Bailey *et al.*, 2001). This is still somewhat subjective and it might simply be a limitation to the direct assessment of exercise induced muscle damage.

The marker of oxidative stress employed was apparently unaffected by either supplementation or exercise in this investigation. Vitamin C supplementation, at a lower dosage, has been associated with modest beneficial effect on a less specific product of lipid peroxidation (MDA) following intermittent exercise (Thompson *et al.*, 2001b). Nieman and co-workers (2002) also reported a no effect of vitamin C on plasma F_2-isoprostane response to exercise. They did however observe an increase following exercise in the placebo supplemented group. Thus, assessment of this oxidative stress marker should be made earlier than 24h following exercise to clarify the extent of exercise induced oxidative damage.

In summary, the reduction in markers of muscle damage as well as the more rapid recovery of the ability to generate force observed with vitamin C supplementation would advocate the use of this antioxidant as a nutrition therapy for exercise-induced muscle damage. However, without more established evidence for the existence of oxidative stress following exercise, the exact mechanisms underlying the protective role of vitamin C remain unclear.

CHAPTER SEVEN

Influence of mixed antioxidant supplementation on the recovery from prolonged intermittent exercise

7.1 Introduction

It was previously demonstrated that supplementation with vitamin C (800mg.day^{-1}) prior to prolonged intermittent exercise leads to reductions in markers of muscle damage and a decrements in muscle function commonly reported following strenuous exercise (Chapter 6). The exact mechanisms underlying the protective effect of antioxidants still remain unclear, but there is considerable growing support for their use as a nutritional therapy following demanding exercise (Sumida *et al.*, 1989; Cannon *et al.*, 1990; Meydani *et al.*, 1993; Rokitzki *et al.*, 1994; Hartmann *et al.*, 1995; Alessio *et al.*, 1997; Thompson *et al.*, 2001b). Although the effects of single antioxidant supplementation are well documented, there are only a small number of studies that have employed a mixed supplement that is perhaps more applicable to commercially available multivitamin supplements. To date evidence is equivocal, but this may be a result of the paucity of research in mixed antioxidant supplementation. As previously, mentioned *in vivo* radical formation both during and following exercise is not confined to one specific tissue. The multitude of sources of these highly reactive molecules would appear to advocate a combination of antioxidants as the preferred technique. Elevating endogenous concentrations of antioxidants in a number of tissues should provide more effect protection from oxidative damage. In fact, some have advocated their use, arguing that a single nutrient may not be physiologically relevant when considering the myriad of factors involved (Pedersen & Hoffman-Goetz, 2000). Therefore, the aim of this investigation was to determine whether a mixture of antioxidants could influence recovery from demanding exercise.

7.2 Methods

Subjects

Thirty eight males (n=32) volunteered to participate in this study. All subjects were habitually active in a variety of sports. Subjects who smoked or took vitamin supplements were excluded from the study.

Supplementation

Subjects were randomly assigned, in a double blind manner, to either a mixed antioxidant (A_T) or a placebo (P_L) supplementation. The constituents of the antioxidant supplement are shown in Table 7.1.

Table 7.1: Composition of antioxidant mixture.

Antioxidant Mixture	per tablet	per day	UK RDA
Vitamin C (ascorbic acid)	400mg	800mg	60mg
Vitamin E (α-tocopherol)	400 IU	800 IU	10mg
Zinc	5mg	10mg	15mg
Vitamin B_6	2mg	4mg	2mg
Vitamin B_{12}	1μg	2μg	1μg
Folic acid	200μg	400μg	200μg

Groups were matched for a number of physical characteristics (Table 7.2). Supplements were taken in capsule form, twice daily with food, for six weeks terminating two days after completion of the exercise. A venous blood sample was taken from a forearm vein prior to commencing supplementation. Subjects were required to weigh and record all food and fluid intake for 5 days during the 6 weeks supplementation.

Table 7.2: Physical characteristics of supplementation groups (mean ± SD).

	A_T (n = 20)	P_L (n = 18)
Age (years)	22 ± 1	22 ± 2
Height (m)	1.79 ± 0.07	1.80 ± 0.07
Mass (kg)	77.4 ± 10 1	79.4 ± 8.3
BMI ($kg.m^{-2}$)	24.1 ± 2.6	24.5 ± 1.8
Sum skinfolds (mm)	32.5 ± 7.9	35.5 ± 8.7
$\dot{V}O_2max$ ($ml.kg.^{-1}.min^{-1}$)	55.1 ± 4.0	54.1 ± 4.8
Training session (per wk)	6 ± 3	5 ± 3

Experimental design

On the fortieth day of supplementation individuals completed the Loughborough Intermittent Shuttle Test (LIST). Subjects exercised at an average intensity that was approximately 70% $\dot{V}O_2max$ for 90min. Subjects reported to the laboratory having refrained from strenuous physical activity for at least two days and after an overnight fast (10-12h). An 8ml venous blood sample was taken from a forearm vein prior to exercise and again approximately 1, 24 and 48h post-exercise. Ratings of perceived soreness were recorded prior to exercise and again at approximately 12h intervals for the following two days. Muscle function was assessed pre- and immediately post-exercise (IMPE) and again approximately 24, 48, 96 and 168h later (± 2h). Nude body mass was determined before and immediately following exercise. Subjects ingested water at regular intervals throughout the exercise. Heart rate, ratings of perceived exertion and 15m sprint times were monitored at regular intervals throughout exercise (Figure 7.1).

Statistical analysis

An independent two-way analysis of variance (ANOVA) with repeated measures for time was used to determine if any differences existed between interventions and time during exercise and recovery. For significant F ratios a paired *Students* t-test, with a *Bonferroni* adjustment, was used to locate the differences between means. Pearson product moment correlations were used to examine the relationship between variables. Significance was accepted at the 5% level. Values are expressed as the mean ± standard error of the mean throughout.

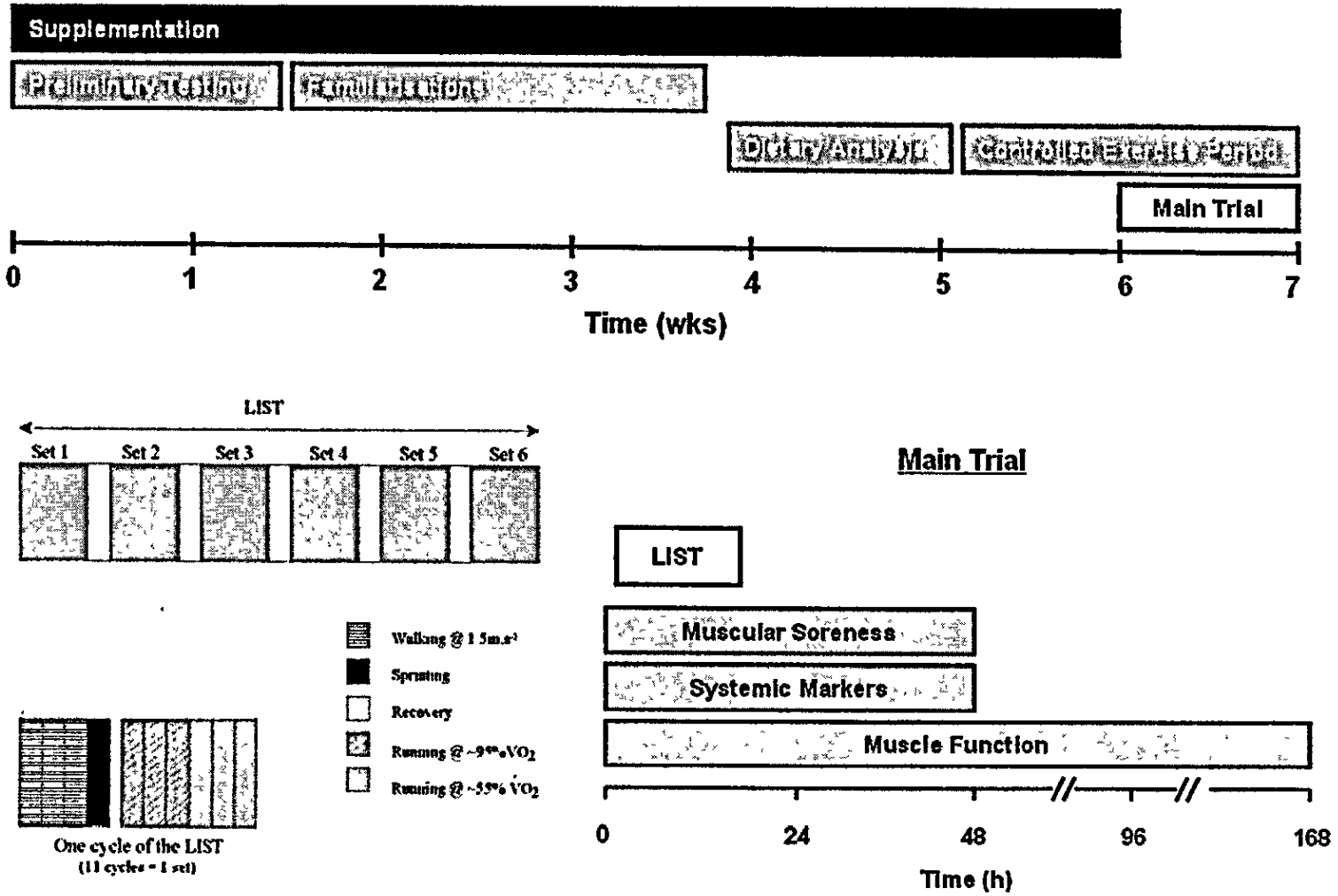


Figure 7.1: A schematic representation of the experimental design

7.3 Results

Response to intermittent shuttle running

During the 90min intermittent running heart rate increased ($p<0.05$) from the onset of exercise peaking during the final 30min ($167 \pm 4 b.min^{-1}$) as did ratings of perceived exertion (16 ± 1, $p<0.05$). Mean sprint times for each 15min block increased from the first block (2.65 ± 0.04s) to the sixth (2.77 ± 0.03s, $p<0.05$) during the LIST but were not different between groups throughout (A_T 2.70 ± 0.04s; P_L 2.74 ± 0.07 s). Fluid intake was not different between groups (A_T 1.13 ± 0.03L; P_L 1.16 ± 0.04L) and subsequently weight loss was not different (A_T 0.52 ± 0.11%BM; P_L 0.40 ± 0.12%BM). Estimated changes in plasma volume did not differ throughout the testing period in either group.

Plasma vitamin C & E

Plasma vitamin C concentration increased ($p<0.05$) with supplementation compared to P_L (Figure 7.2) and remained elevated above P_L concentrations until cessation of supplementation 48h post-exercise ($p<0.05$). Figure 7.2 also shows plasma concentrations also increased 1h post-exercise in both groups ($p<0.05$). Changes in plasma tocopherol (vitamin E) and carotenoids are shown in Table 7.3.

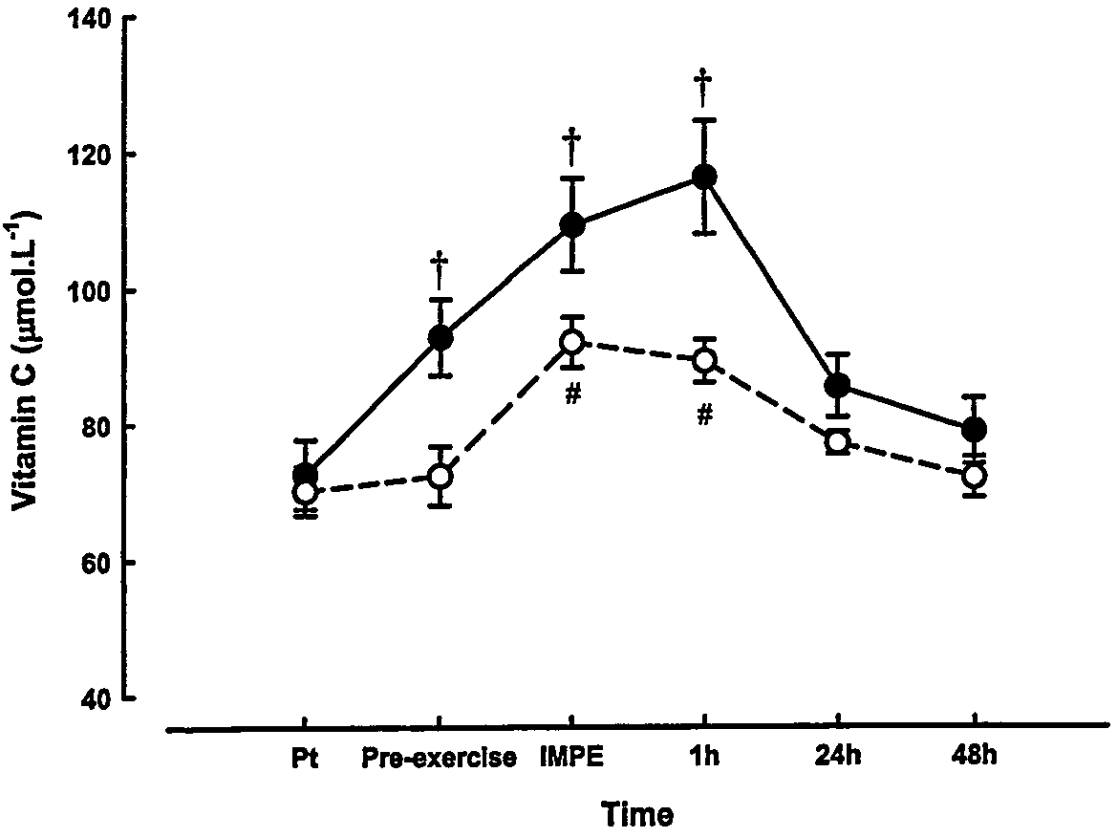


Figure 7.2: Plasma vitamin C concentration pre-treatment (Pt), pre-exercise and following intermittent shuttle running for the antioxidant (solid line) and placebo supplemented (dashed line) groups (mean ± *SEM*). † different between groups ($p<0.05$). # P_L group different from pre-exercise ($p<0.05$).

Table 7.3: Plasma vitamin E (α, γ-tocopherol) and retinol concentration pre-treatment (Pt), pre-exercise following antioxidant and placebo supplementation (mean ± *SEM*). † A_T different from pre-exercise and different between groups ($p<0.05$).

	A_T (n = 20)		P_L (n = 18)	
	Pt	Pre-exercise	Pt	Pre-exercise
α-tocopherol (µmol.l^{-1})	6.89 ± 0.46	11.30 ± 0.71 †	7.38 ± 0.51	7.87 ± 1.01
γ-tocopherol (µmol l^{-1})	0.32 ± 0.03	0.19 ± 0.02 †	0.43 ± 0.04	0.39 ± 0.03
Retinol (µmol.l^{-1})	0.59 ± 0.02	0.59 ± 0.03	0.62 ± 0.03	0.57 ± 0.02

Muscular soreness

Ratings of perceived soreness assessed while at rest (passive) and whilst descending stairs (active) where unaffected by supplementation. Figure 7.3 illustrates that passive soreness increased during the post-exercise period, peaking at 24h (5.2 ± 0.2, $p<0.05$). Active soreness also increased from pre-exercise (2.2 ± 0.2) peaking at 24h (5.4 ± 0.4). As previously observed (Chapter 6) the specific location of soreness was in the *hamstrings*, *quadriceps*, *triceps surea*, *gluteus maximus* and musculature of the lower back (Figure 7.4).

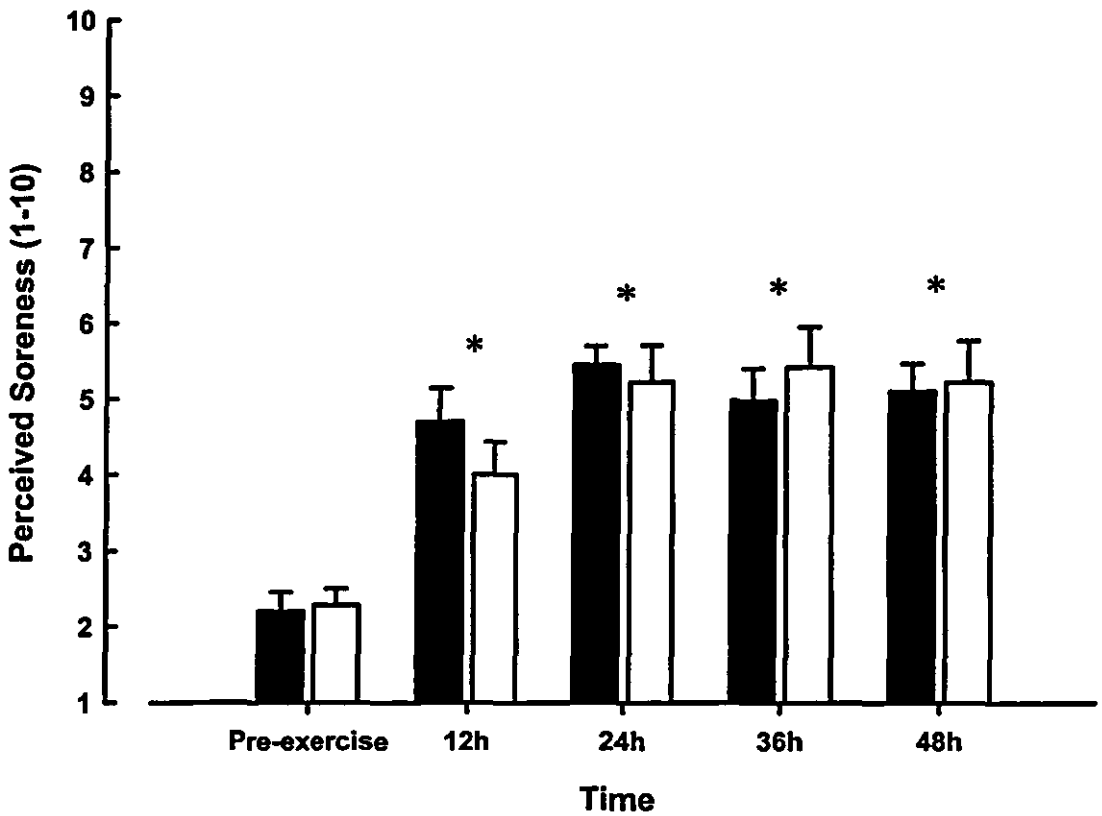


Figure 7.3: Ratings of perceived soreness (10 point scale) following intermittent shuttle running for the antioxidant (solid bars) and placebo supplemented (clear bars) groups (mean ± *SEM*). * both groups different from pre-exercise ($p<0.05$).

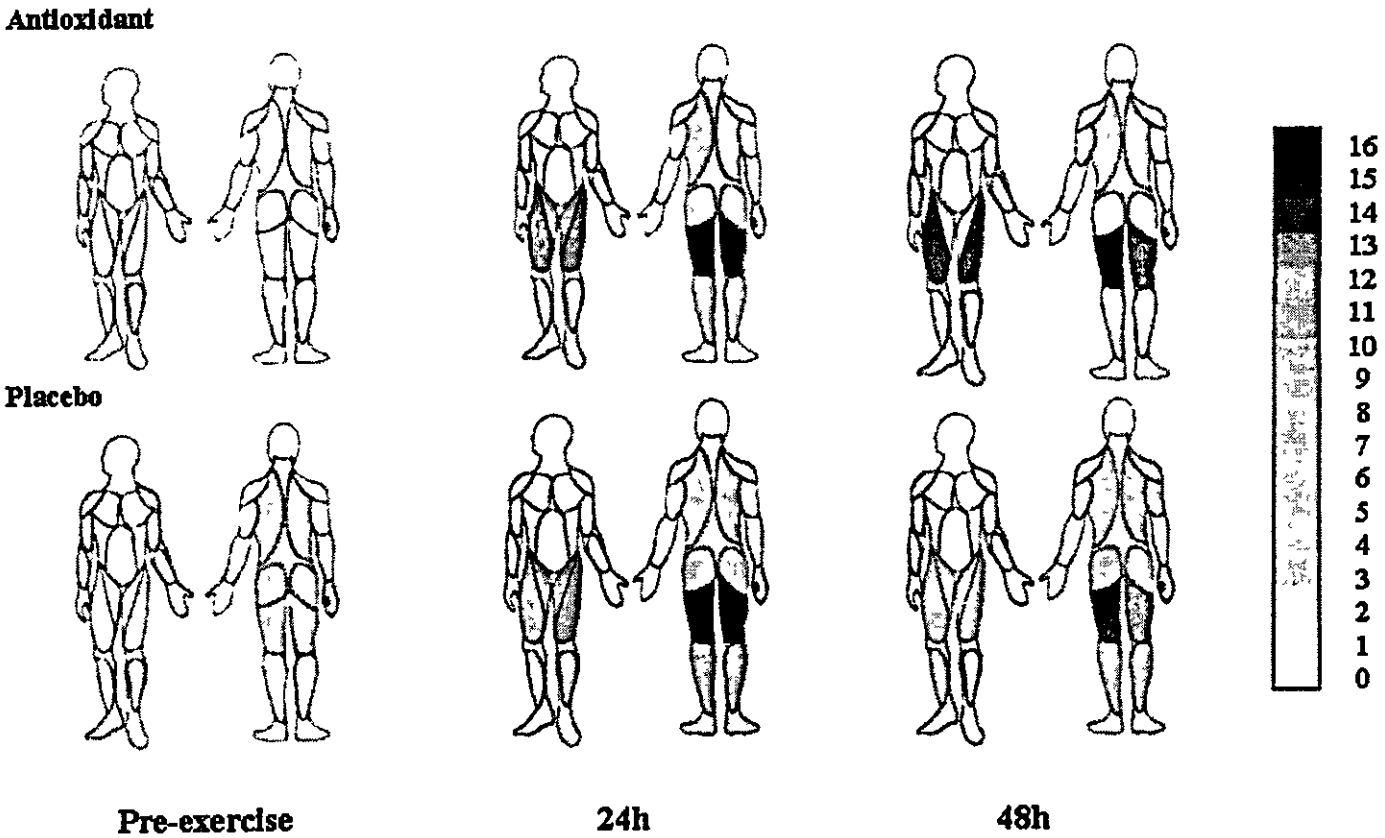


Figure 7.4: Location and frequency of muscle soreness following intermittent shuttle running for the antioxidant and placebo supplemented groups. An increase in the density of shading represents an increase in the frequency of soreness in that region.

Muscle function

Table 7.4 shows changes in MVC and ROM following exercise. Maximal voluntary contraction of the leg flexors decreased IMPE, 24 and 48h in both groups ($p<0.05$) but returned to pre-exercise values at 96 and 168h with A_T only ($p<0.05$). Peak torque (MVC) of the leg extensors was also reduced IMPE, 24 and 48h following exercise in both groups, but remained below pre-exercise values in both groups up to 168h ($p<0.05$). Range of motion was reduced following exercise ($p<0.05$) but returned to pre-exercise levels at 168h with A_T only ($p<0.05$).

Table 7.4: Isometric maximal voluntary contraction and range of motion following intermittent shuttle running with either antioxidant or placebo supplementation (means ± *SEM*). † different between groups ($p<0.05$). * different from pre-exercise ($p<0.05$).

MVC ($Nm.kg^{-1}$)	Pre-exercise	IMPE	24 h	48 h	96 h	168 h
Leg Flexion						
A_T	4.09 ± 0.16	3.47 ± 0.13 *	3.64 ± 0.15 *	3.64 ± 0.17 *	4.05 ± 0.14	4.16 ± 0.11 †
(% pre-exercise)	-	(85 ± 2)	(89 ± 2)	(89 ± 2)	(90 ± 1)	(110 ± 3)
P_L	3.88 ± 0.12	3.25 ± 0.09 *	3.47 ± 0.13 *	3.66 ± 0.13 *	3.71 ± 0.13 *	3.43 ± 0.14 *
(% pre-exercise)	-	(84 ± 2)	(90 ± 2)	(95 ± 2)	(95 ± 1)	(88 ± 3)
Leg Extension						
A_T	2.62 ± 0.09	2.20 ± 0.08 *	2.17 ± 0.10 *	2.18 ± 0.10 *	2.07 ± 0.10 *	2.20 ± 0.17 *
(% pre-exercise)	-	(84 ± 2)	(83 ± 2)	(83 ± 3)	(73 ± 2)	(78 ± 2)
P_L	2.57 ± 0.12	2.12 ± 0.11*	2.04 ± 0.11 *	2.05 ± 0.12 *	1.83 ± 0.14 *	1.95 ± 0.16 *
(% pre-exercise)	-	(83 ± 3)	(80 ± 3)	(80 ± 4)	(71 ± 3)	(75 ± 2)
ROM						
A_T	117 ± 2	106 ± 2 *	106 ± 2 *	105 ± 2 *	102 ± 2 *	108 ± 2 †
(% pre-exercise)	-	(90 ± 1)	(91 ± 1)	(90 ± 2)	(91 ± 1)	(97 ± 2)
P_L	120 ± 2	106 ± 1 *	105 ± 2 *	106 ± 2 *	102 ± 3 *	103 ± 2 *
(% pre-exercise)	-	(88 ± 1)	(88 ± 1)	(88 ± 2)	(86 ± 2)	(87 ± 2)

Markers of muscle damage

Changes in Mb concentration following exercise are illustrated in Figure 7.5. Serum myoglobin increased from pre-exercise values peaking 1h post-exercise in both groups (A_T 36.20 ± 6.69 $nmol.L^{-1}$; P_L 35.52 ± 7.32 $nmol.L^{-1}$, $p<0.05$) and returning to baseline at 24 and 48h. There was no effect of antioxidant supplementation on Mb response following exercise. Figure 7.5 also shows that CK activity also increased following exercise, peaking at 24h (A_T 1357 ± 254 $U.L^{-1}$; P_L 1374 ± 231 $U.L^{-1}$, $p<0.05$). There were no differences between groups for CK activity throughout.

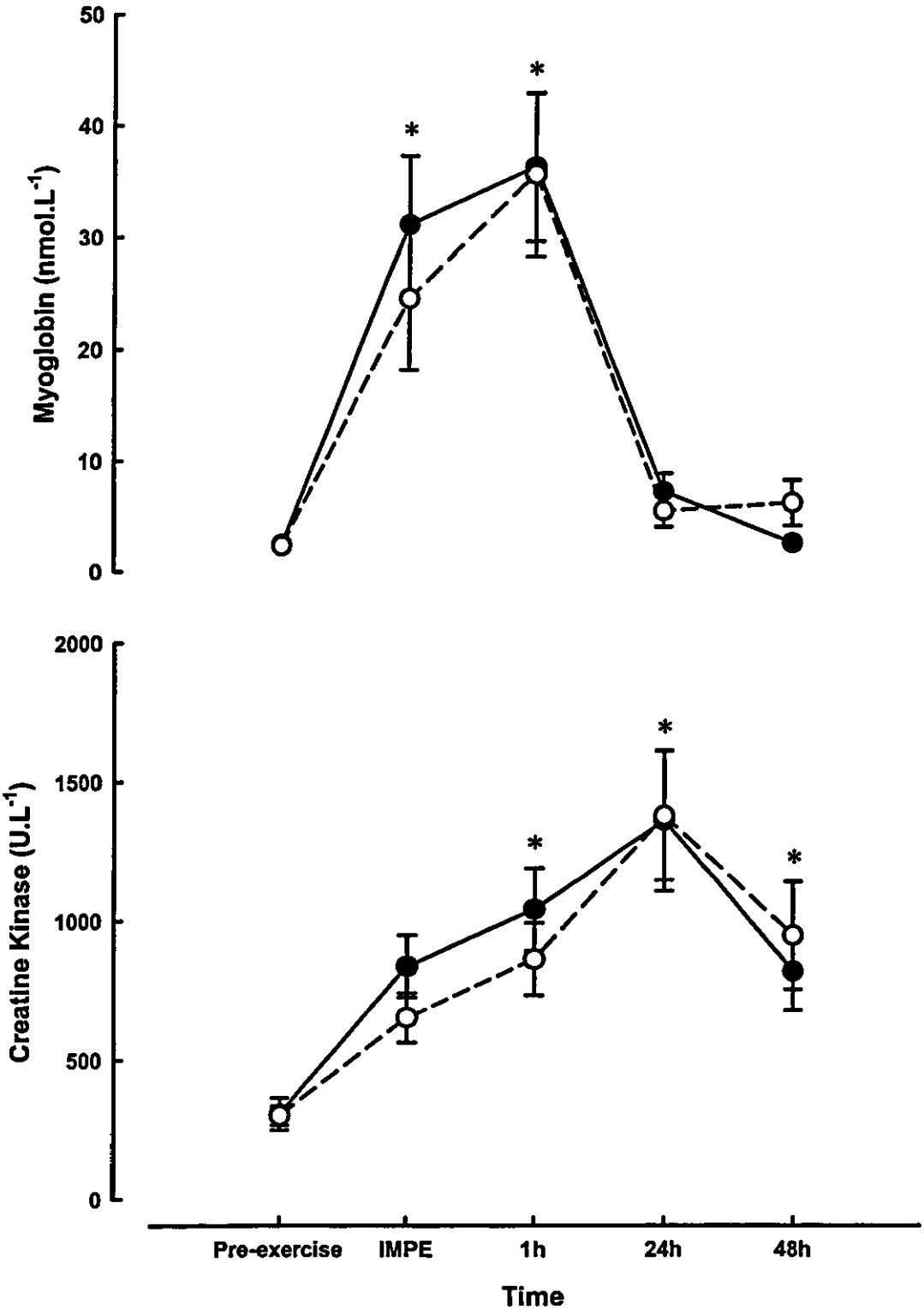


Figure 7.5: Serum myoglobin concentration and creatine kinase activity following intermittent shuttle running for the antioxidant (solid line) and placebo supplemented (dashed line) groups (mean ± *SEM*). * both groups different from pre-exercise ($p<0.05$).

Inflammatory markers

Figure 7.6 shows the IL-6 response to exercise for both groups. Systemic concentrations peaked IMPE (A_T 11.5 ± 1.6 pg.ml^{-1}, P_L 8.9 ± 1.9 pg.ml^{-1}) ($p<0.05$) and remain elevated 1h following exercise. Changes in other inflammatory mediators following exercise are shown in Table 7.5.

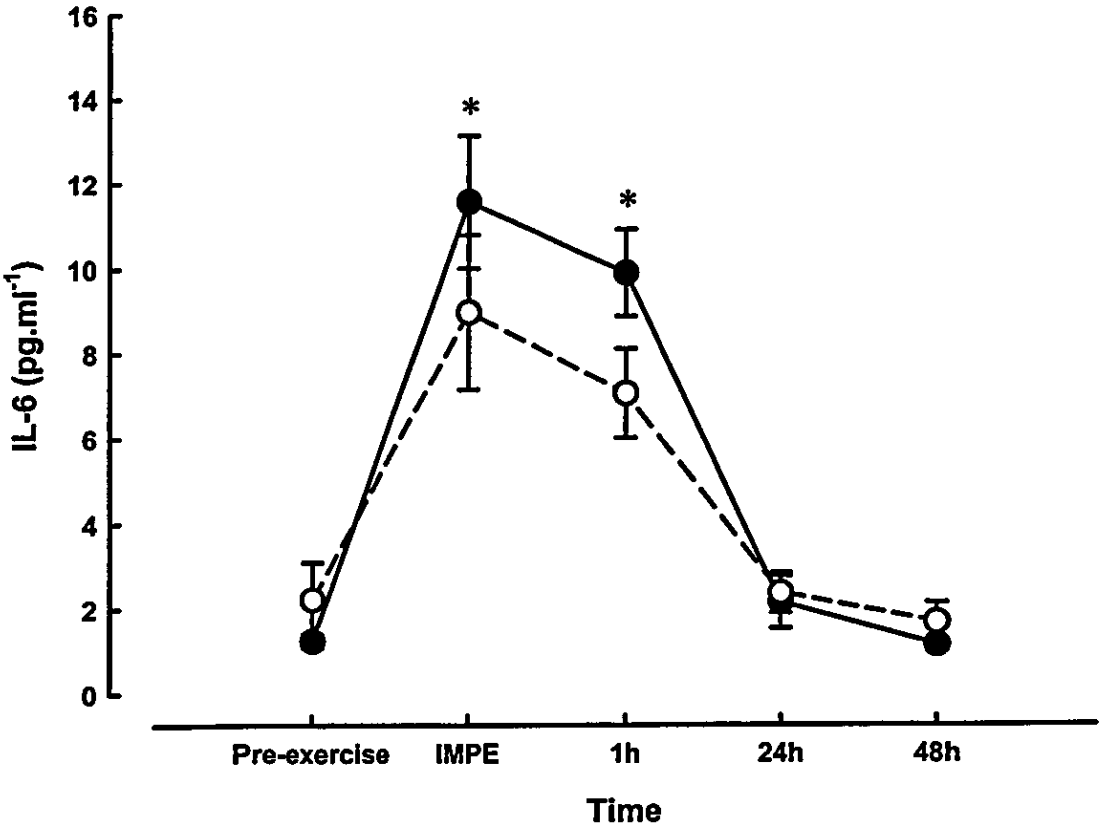


Figure 7.6: Serum interleukin 6 concentration following intermittent shuttle running for the antioxidant (solid line) and placebo supplemented (dashed line) groups (mean ± *SEM*). * both groups different from pre-exercise ($p<0.05$).

Table 7.5: Change in inflammatory mediators following intermittent shuttle running with either antioxidant or placebo supplementation (means ± SE). * different from pre-exercise ($p<0.05$).

Variable ($pg.ml^{-1}$)	Pre-exercise	IMPE	1h	24h	48h
TNF-α					
A_T	1.17 ± 0.42	1.33 ± 0.34	1.08 ± 0.31	1.06 ± 0.31	1.09 ± 0.16
P_L	1.15 ± 0.27	1.35 ± 0.19	1.38 ± 0.18	1.16 ± 0.18	1.26 ± 0.16
IL-1ra					
A_T	205 ± 24	559 ± 129 *	1523 ± 322 *	270 ± 45	224 ± 25
P_L	204 ± 16	435 ± 90 *	1137 ± 263 *	247 ± 21	388 ± 154
IL-10					
A_T	0.0 ± 0.3	15.6 ± 5.2 *	9.5 ± 8.9 *	0.0 ± 0.4	0.0 ± 0.4
P_L	0.1 ± 0.5	7.7 ± 4.3 *	5.9 ± 4.5 *	0.0 ± 0.4	0.0 ± 0.2

Exercise also induced increases in circulating acute phase protein concentrations (C-reactive protein) as well as heat shock proteins (HSP70), although neither were different between groups (Figure 7.7).

Total white blood cell counts increased immediately post-exercise in both groups (A_T 11.1 ± 1.5 $x10^9.L^{-1}$, P_L 7.6 ± 1.1 $x10^9.L^{-1}$) and remained elevated 1h following exercise ($p<0.05$). Changes in the sub-populations for leukocytes are shown in Table 7.6.

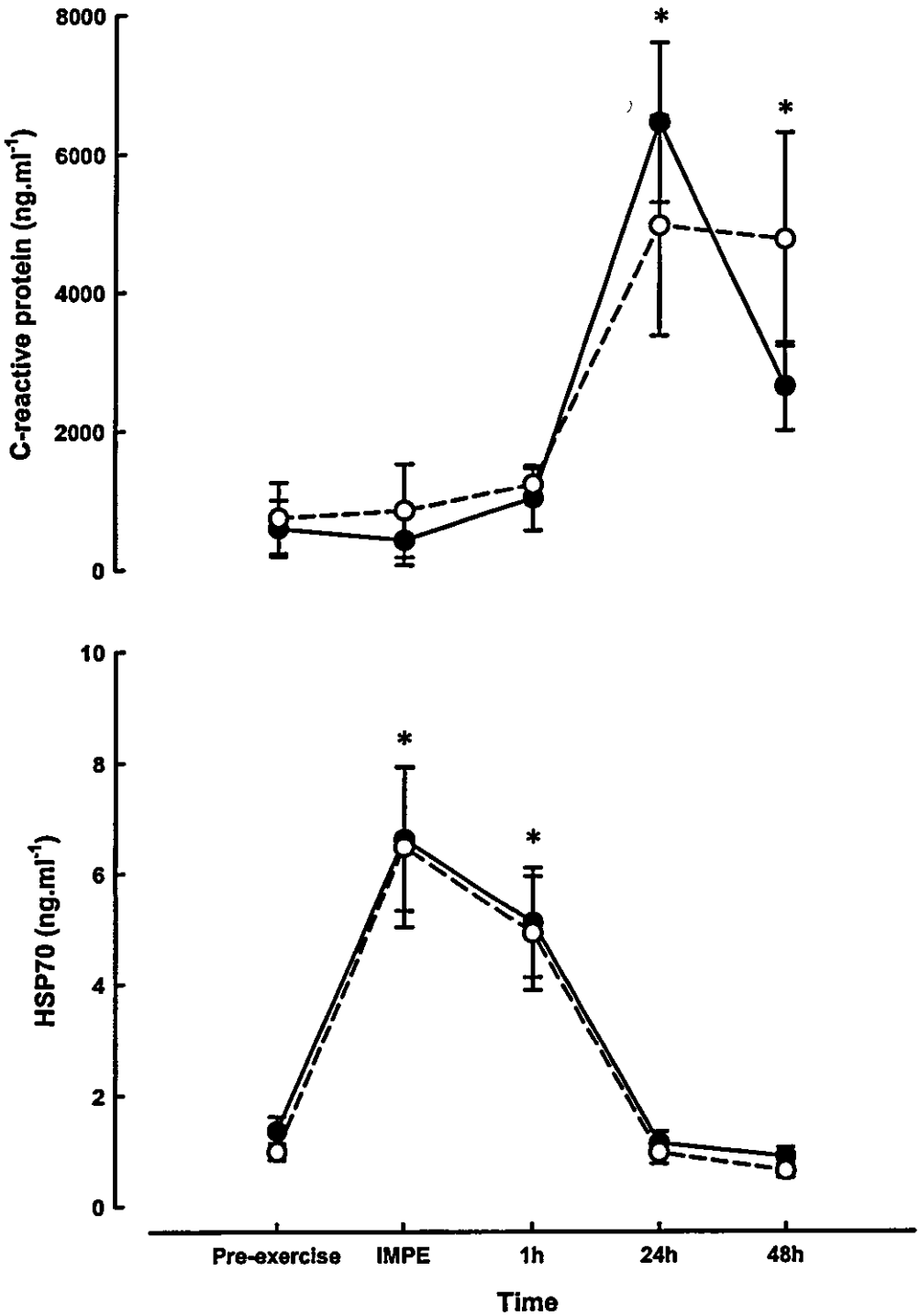


Figure 7.7: Plasma C-reactive protein and HSP70 concentration following intermittent shuttle running for the antioxidant (solid line) and placebo supplemented (dashed line) groups (mean ± *SEM*). * both groups different from pre-exercise ($p<0.05$).

Table 7.6: Change in leukocyte count and relative number following intermittent shuttle running with either antioxidant or placebo supplementation (means ± *SEM*). * different from pre-exercise ($p<0.05$).

	Antioxidant		Placebo	
	Absolute no ($x10^9.L^{-1}$)	Relative no. (%)	Absolute no ($x10^9.L^{-1}$)	Relative no. (%)
WBC				
Pre-exercise	4.81 ± 0.35	-	3.58 ± 0.48	-
IMPE	11.09 ± 1.51 *	-	7.62 ± 1.14 *	-
1h	14.00 ± 2.09 *	-	12.10 ± 1.29 *	-
24h	4.53 ± 0.54	-	4.20 ± 0.49	-
Neutrophils				
Pre-exercise	2.29 ± 0.21	64.9 ± 5.4	2.12 ± 0.28	53.2 ± 4.9
IMPE	6.55 ± 1.06 *	68.8 ± 5.5	4.93 ± 0.78 *	56.6 ± 5.1 *
1h	7.71 ± 1.45 *	59.2 ± 3.9	7.58 ± 0 93 *	66.9 ± 3.9
24h	2.49 ± 0.33	62.0 ± 5.1	2.26 ± 0.31	59.5 ± 4.4
Monocyctes				
Pre-exercise	0.37 ± 0.18	7.0 ± 0.9	0.27 ± 0.10	7.7 ± 1.2
IMPE	0.34 ± 0.05	7.8 ± 0.4	0.50 ± 0.07	8.7 ± 0.9
1h	0.49 ± 0.15	6.9 ± 0.8	0.35 ± 0.09	7.4 ± 0.6
24h	0.63 ± 0.11	7.8 ± 0.9	0.49 ± 0.09	6.4 ± 0.6
Lymphocytes				
Pre-exercise	2.08 ± 0.16	41.0 ±3.1	1.46 ± 0.11	39.5 ± 1.7
IMPE	1.79 ± 0.15	19.8 ± 4.5 *	1.54 ± 0.09	21.4 ± 1.9 *
1h	1.45 ± 0.21 *	13.4 ± 3.6 *	1.79 ± 0.14	11.8 ± 2.6 *
24h	1.93 ± 0.13	40.1 ± 3.3	1.33 ± 0.03	37.5 ± 2.9

Table 7.7 shows polymorphonuclear leukocyte activity assessed as phagocytosis and oxidative burst activity. Monocyte phagocytic activity was elevated 1h following cessation of exercise (A_T 53.3 ± 1.9%, P_L 51.6 ± 1.7%, $p<0.05$) but was unaffected by supplementation.

Table 7.7: Polymorphonuclear leukocyte activity following intermittent shuttle running with either antioxidant or placebo supplementation (means ± *SEM*). * different from pre-exercise ($p<0.05$).

	Antioxidant		Placebo	
	Phagocytosis (%)	Oxidative Burst (%)	Phagocytosis (%)	Oxidative Burst (%)
Monocyte				
Pre-exercise	42.1 ± 2.1	49.7 ± 3.3	45.2 ± 1.9	46.1 ± 3.0
IMPE	49.5 ± 3.7	45.0 ± 3.9	46.7 ± 2.8	47.7 ± 3.7
1h	53.3 ± 1.9 *	42.0 ± 4.2	51.6 ± 1.7 *	49.0 ± 3.6
24h	42.5 ± 2.6	41.7 ± 4.9	46.3 ± 2.2	45.2 ± 3.1
Neutrophils				
Pre-exercise	65.8 ± 3.0	88.2 ± 2.6	68.6 ± 3.0	85.9 ± 1.5
IMPE	67.1 ± 3.9	88.3 ± 4.0	69.4 ± 2.1	87.2 ± 1.4
1h	69.9 ± 1.5	80.2 ± 3.7	71.6 ± 1.6	85.8 ± 1.9
24h	62.8 ± 4.0	87.1 ± 3.8	65.4 ± 2.7	84.8 ± 2.3

Uric acid, F_2-isoprostanes, lipid hydroperoxides and cortisol

Serum uric acid concentrations, shown in Table 7.8, were elevated following exercise peaking at 1h in both groups ($p<0.05$) and returned to pre-exercise values 48h post-exercise. Plasma lipid hydroperoxides (LPO) increased IMPE ($p<0.05$), but were unaffected by supplementation. Serum cortisol was also elevated immediately and 1h following exercise ($p<0.05$).

Table 7.8: Serum uric acid, cortisol, plasma lipid hydroperoxides, urinary F_2-isoprostanes and salivary cortisol concentration following intermittent shuttle running with either antioxidant or placebo supplementation (means ± *SEM*). † different between groups ($p<0.05$). * different from pre-exercise ($p<0.05$).

	Pt		Pre-exercise		IMPE		1 h		24 h		48 h	
	A_T	P_L	A_T	P_L	A_T	P_L	A_T	P_L	A_T	P_L	A_T	P_L
Uric acid ($\mu mol.L^{-1}$)	-	-	258 ± 9	284 ± 9	280 ± 10 *	313 ± 13 *	295 ± 8 *	316 ± 15 *	293 ± 13 *	307 ± 11 *	275 ± 11 *	284 ± 12
F2-isoprostane ($ng.ml^{-1}$)	14.0 ± 1.4	7.0 ± 2.3	9.2 ± 14.3	4.4 ± 0 8	-	-	-	-	9.2 ± 5.7	6.9 ± 0.9	20.3 ± 4.1	3.7 ± 0.8
LPO ($\mu mol.L^{-1}$)	-	-	2.1 ± 0.4	1.9 ± 0.5	7.0 ± 1.3 *	7.2 ± 1.1 *	-	-	-	-	-	-
Cortisol ($nmol.L^{-1}$)	459 ± 13	401 ± 18	498 ± 25	453 ± 23	595 ± 26 *	564 ± 44 *	734 ± 71 *	868 ± 97 *	534 ± 23	565 ± 47	528 ± 38	520 ± 32
Salivary cortisol ($nmol.L^{-1}$)	26.1 ± 2.4	25.0 ± 2.2	22.0 ± 9.1	24.3 ± 2.3	23.8 ± 4.6	22.6 ± 5.6	17.4 ± 5.6	16.4 ± 2.8	20.9 ± 2.7	15.9 ± 3.1	22.0 ± 2.2	17.2 ± 1.6

Dietary vitamin intake

Dietary vitamin C intake was not different between groups during the supplementation period (A_T 119.8 ± 6.8mg.day^{-1}; P_L 122.7 ± 18.6mg.day^{-1}). This was also the case with dietary vitamin E intake (A_T 6.7 ± 1.0mg.day^{-1}; P_L 7.7 ± 0.7 mg.day^{-1}). There was also no difference between diets (Table 7.9).

Table 7.9: Daily dietary composition assessed over the five day period of the main trial in antioxidant and placebo supplemented groups (mean ± *SEM*). *Note*; values for vitamin C and E for the antioxidant group do not include the additional intake from the antioxidant supplement.

	Antioxidant	Placebo
Energy Intake (MJ)	13.4 ± 1.1	13.4 ± 0.9
Carbohydrate (%)	53 ± 3	54 ± 2
Fat (%)	31 ± 1	30 ± 2
Protein (%)	16 ± 1	16 ± 1
Vitamin C (mg)	120 ± 7	123 ± 18
Vitamin E (mg)	9 ± 1	8 ± 1

Peak increases in Mb were related to peak changes in CK activity (r = 0.89). Also, peak Mb concentration was positively related to peak soreness (r = 0.41) and C-reactive protein concentrations at 24h following exercise (r = 0.61) (Figure 7.8). Weak relationships were observed between peak IL-6 response and peak C-reactive protein concentration (r = 0.58).

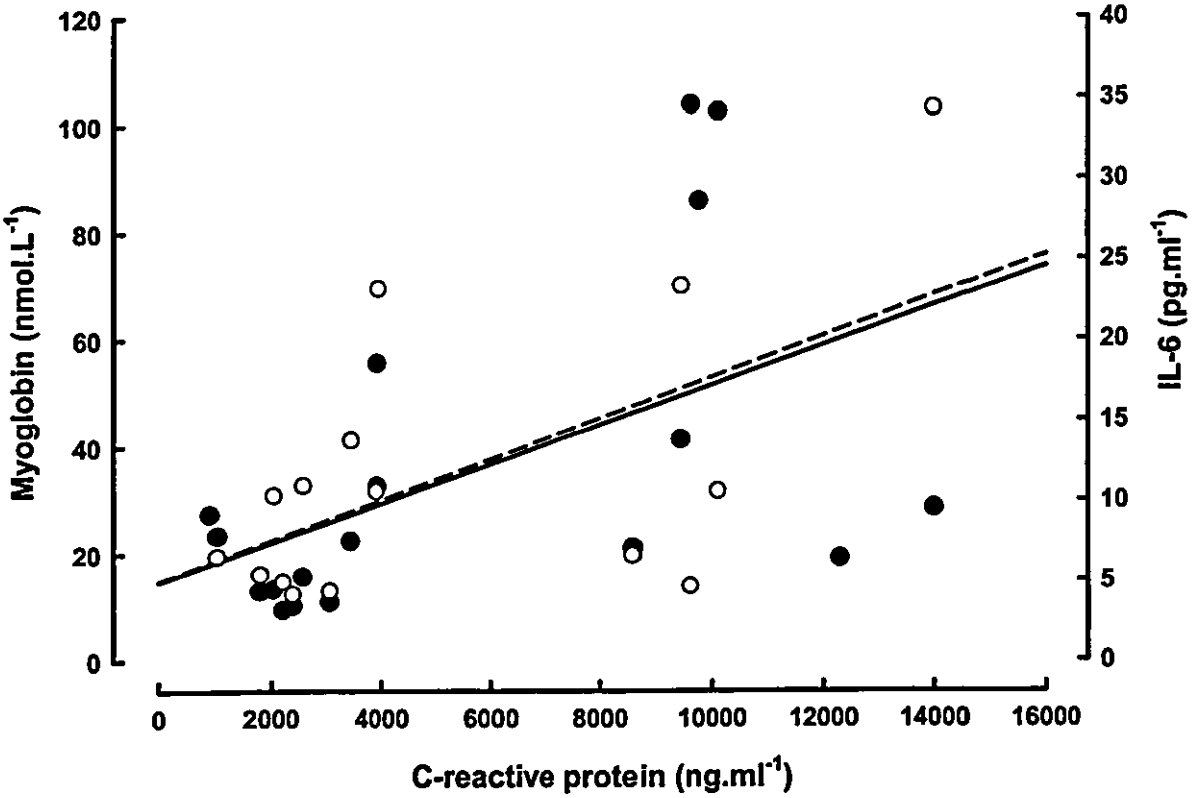


Figure 7.8: Relationships between peak myoglobin (solid line, solid points), IL-6 (dashed line, clear points) and C-reactive protein concentrations following exercise.

7.4 Discussion

The purpose of the current investigation was to examine the effect of mixed antioxidant supplementation on markers of muscle damage as well as muscle function following prolonged intermittent exercise. Plasma concentrations of the major antioxidant constituents increased following supplementation. Increased plasma concentrations of vitamin C were similar to those previously reported following the same dosage but shorter supplementation period (Chapters 5 and 6). The relatively half-life of vitamin C (ascorbate) in the circulation is suggested to be 7-14 days depending on initial plasma concentrations (Rumsey & Levine, 1998). Thus, either this high turnover or the saturation of *in vivo* ascorbate could explain the benign effect of 6 weeks compared to 1 week supplementation. Plasma vitamin E concentrations were also elevated to similar levels reported following either single (Cannon *et al.*, 1990; Meydani *et al.*, 1993) or mixed supplementation (Petersen *et al.*, 2001). However, the chain breaking properties of this antioxidant on free radical formation are specific to lipid membranes. Thus, tissue rather than plasma concentrations are more important when attempting to alleviate oxidative skeletal muscle damage (Jackson *et al.*, 1983). The rationale for the inclusion of zinc in the antioxidant supplement was focused on it's role as a co-factor for endogenous antioxidant enzymes, including superoxide dismutase, (Halliwell & Gutteridge, 1989) as well as it's effect on immune function (Singh *et al.*, 1994; Shephard & Shek, 1998). Vitamin B_6 and B_{12} are also reported to be vital for normal immune function although in excess may have detrimental effects (Gleeson *et al.*, 2001).

The favourable effects observed with antioxidant supplementation on the recovery of MVC in leg flexors 168h post-exercise were comparable to those observed with vitamin C supplementation in the previous investigation (Chapter 6) and have been previously reported with this single antioxidant (Jakeman & Maxwell, 1993; Thompson *et al.*, 2001b). Improvements in the recovery of ROM with antioxidant supplementation, to our knowledge, have not been previously reported. Jakeman and Maxwell (1993) observed improved recovery of both MVC and 20:50Hz tetanic tension of the triceps surea with vitamin C supplemented individuals following bench stepping exercise. The reductions in low frequency fatigue were proposed to be a result of the inhibition of excitation-contraction coupling, specifically through the failure of calcium release from the sarcoplasmic reticulum

(SR). Damage to the SR following exercise has been linked with oxidative stress (Kagan *et al.*, 1989). This provides a basis for the recovery of muscle function observed with antioxidant supplementation, as the combination of vitamin C and E may have provided defence against lipid peroxidation induced by the exercise. However, Jakeman and Maxwell (1993) did not report these beneficial effects on the recovery of muscle function following the same exercise in individuals supplemented with vitamin E ($400mg.day^{-1}$). It could be argued that supplementation period (7 days) was insufficient to increase tissue concentrations of this lipid soluble antioxidant, despite the increase in total antioxidant capacity of plasma. Despite this, the regenerative properties of vitamin C on vitamin E could account for the site specific action and thus substantiate the ultrastructural mechanisms proposed to facilitate recovery from low frequency fatigue.

The established increase in myofibrillar protein leakage have been observed following intermittent exercise (Chapter 6) (Thompson *et al.*, 1999). Increases in myoglobin during the post-exercise period followed the same time course previously reported but were considerably greater peaking at $35.52 \pm 7.7.3nmol.L^{-1}$ as compared to $25.67 \pm 7.01nmol.L^{-1}$ (Thompson *et al.*, 2001b) and $17.15 \pm 0.81nmol.L^{-1}$ (Chapter 6), for placebo groups. One possible explanation for this variation could be linked to the pro-oxidant properties of vitamin C (Jenkins *et al.*, 1993; Paolini *et al.*, 1999). This antioxidant does have the potential to recycle ferrous iron to produce the hydroxyl radical which may result in oxidative damage linked to myoglobin efflux. Childs and co-workers (2001) recently provided evidence for this when they observed increased muscle damage with antioxidant supplementation (vitamin C and N-acetyl cysteine) following eccentric exercise. However, previous research has employed vitamin C supplementation without pro-oxidant effects following intermittent exercise (Thompson *et al.*, 2001a; Thompson *et al.*, 2001b). Differences could therefore be attributed to inter-individual variability. Creatine kinase, however, showed similar increases to those reported before (Chapter 6) (Thompson *et al.*, 2001b).

Another possible explanation for the modest effects of mixed supplementation on recovery from exercise might be related to the dietary intake of antioxidants. Although, vitamin C and E intake was not different between groups the mean intake was far in excess of the current recommendations. The average daily intake of vitamin C (≥ 120 mg day^{-1}) was over twice the UK recommendation. Sufficient dietary intake could therefore have masked any effect of the

antioxidant supplement. This increase in dietary antioxidant intake has been positively correlated with total energy intake (Carbajal *et al.*, 1996). Individuals participating in this investigation were physically active partaking in approximately six training session per week and subsequently had high total energy intake (~13 $MJ.day^{-1}$). Although unfamiliar with the exercise performed the activity status of the subjects could have incurred a prophylactic effect similar to the well documented 'repeated bout effect', in which an adaptive response to subsequent damaging exercise is observed (Ebbeling & Clarkson, 1989). This, along with increased dietary antioxidant intake, may have reduced the potential protective effects of supplementation.

Schröder and colleagues (2000) reported beneficial effects on markers of lipid peroxidation and total antioxidant status with mixed antioxidant supplementation (vitamin C, E and β-carotene) in elite basketball players during a competitive season. However, they reported marginal plasma vitamin C concentrations in the placebo supplemented players during the season and suggested the associated metabolic demands of repeated intermittent exercise could be responsible for this reduction. This increased susceptibility to oxidative damage in the placebo group meant that antioxidant supplementation had a more profound effect on total antioxidant status compared with this investigation. Subsequently, there is evidence to support the use of mixed antioxidant supplementation even in individuals accustomed to damaging exercise. Perhaps future research should focus on the more chronic effects of antioxidant supplementation as this study clearly shows deficiencies induced by training and competition.

Intermittent exercise induced significant increases in lipid hydroperoxides (LPO) but not urinary F_2-isoprostanes. Both are considered reliable indirect indices of lipid peroxidation (Sen, 2001), particularly when assessed together (Clarkson & Thompson, 2000) or in conjunction with more direct markers (Ashton *et al.*, 1999). Previous investigations employing LPO assessment as an indication of oxidative damage have reported greater increases following ultramarathon exercise (Nieman *et al.*, 2002) and lower changes with shorter, less strenuous exercise models (Alessio *et al.*, 2000; Schröder *et al.*, 2000). The similar response of urinary F_2-isoprostanes to the previous investigation (Chapter 6) may indicate that this particular measure is not precise enough to detect exercise-induced changes i.e. serum measures might be more sensitive to change compared with urine. Alternatively,

assessment at 24h post-exercise is not early enough to observe the significant increases previously reported closer (<1h) to cessation of exercise (Nieman *et al.*, 2002; Steensberg *et al.*, 2002).

The marked increase in leukocyte accumulation was analogous to the changes observed in the previous investigation (Chapter 6) and can be attributed to a striking neutrophilia, despite the lymphopenia in the hours following exercise. Increases in cortisol have been proposed to have a causal effect on circulating leukocyte number following exercise (Nieman *et al.*, 1989; Gabriel *et al.*, 1992; Venkatraman & Pendergast, 2002). Although serum cortisol was increased following exercise there were no apparent relationships between these parameters. Gray *et al.* (1993) observed similar changes in leukocytes following 90min intermittent exercise but did not report correlations with elevated total and free cortisol concentration both immediately and 1.5h post-exercise. These findings are supported by evidence from more damaging eccentric based exercise (Nieman *et al.*, 1995; Pizza *et al.*, 1995). Interestingly, although serum cortisol increased following exercise salivary concentrations remained unchanged. Similar differing responses have been documented following exercise and are suggested to be the result of the specific form of cortisol measured (Stupnicki & Obminski, 1992; del Corral *et al.*, 1994). Stupnicki and Obminski (1992) suggested that changes in serum cortisol, a measure of total cortisol, are larger than salivary cortisol which represents free or active cortisol. These authors advocated the use of salivary measures not only for its versatility but also that it is considered more reflective of the physiological stress imposed by exercise and not other emotional factors (Stupnicki & Obminski, 1992).

Increases in inflammatory mediators post-exercise were comparable to those previously observed (Chapter 6) although these changes were unaffected by supplementation. Exercise-induced elevations of IL-6 were less following intermittent exercise with vitamin C supplementation (Thompson *et al.*, 2001b). Cannon and co-workers (1991) also observed a reduction in post-exercise concentrations of IL-6 and IL-1β with vitamin E ($800 IU.day^{-1}$) but reported no effect on exercise-induced elevations of plasma TNF-α. These authors noted that changes in cytokines following downhill running were associated with increased urinary makers of muscle proteolysis (3-methylhistidine) providing direct evidence for a link between inflammation and muscle autolysis (Cannon *et al.*, 1991). Nieman *et al.* (2000) observed similar effects with vitamin C supplementation ($1.5g.day^{-1}$) on circulating cytokines

immediately following an ultramarathon. These authors propose that the extreme demands associated with prolonged endurance events such as ultramarathon provided the ideal model for investigating the effects of antioxidant on exercise-induced changes in immune function (Nieman *et al.*, 2002). However, in their initial investigation supplementation was not randomised and carbohydrate intake, known to influence cytokine production (Bishop *et al.*, 2001), was not controlled. These confounding factors make it difficult to advocate the proposed beneficial effects of vitamin C on the immune parameters assessed. Petersen and colleagues (2001) reported no influence of a mixed antioxidant supplement on cytokine production and changes in leukocyte counts following exercise that supports the findings in this investigation. They suggested that the benign effect of vitamin supplementation does not support the role of free radicals in exercise-induced immune changes. However, as they did not include a measure of oxidative stress it is difficult to rule out free radical induced damage following exercise. In addition to the exercise-induced increase in LPO we observed an increase in heat shock proteins (HSP70) following intermittent exercise. These stress proteins have been implicated in the protection of damaged cells from various inflammatory mediators including ROS and cytokines (Kelly *et al.*, 1996). Recently, Neiss *et al.* (2002) investigated the effect of vitamin E supplementation following exhaustive exercise on heat shock protein (HSP72) expression in leukocytes. Exercise induced significant increases in HSP72 mRNA that were more profound in the placebo group, but did not reveal a significant treatment effect. However, these findings support the potential role of ROS in inflammation following exercise.

Although the antioxidant mixture had minimal affects on the indices of muscle damage following exercise we did observe relationships between these markers that go some way to support current theories in the aetiology of exercise-induced muscle damage. Firstly, there was a strong relationship between peak increases in CK activity and Mb concentrations providing further evidence for the causal mechanisms for myofibrillar protein leakage. Also, a weak relationship between peak increases in the sensation of soreness with myoglobin concentration 1h post-exercise. The subjective nature of assessing muscular soreness makes it an inaccurate method of quantifying muscle damage, but this relationship with a more reliable marker of muscle injury provides further evidence for the association of DOMS with disruptions to skeletal muscle ultrastructure. Finally, much debate exists on the proposed role of inflammation in exercise-induced muscle damage (Malm, 2001). Increased IL-6

concentrations are proposed to facilitate the release of acute phase proteins involved in repair and regeneration of injury muscle tissue leading to a more chronic inflammatory response (Pyne, 1994). The relationships between peak changes in myoglobin with C-reactive protein and IL-6 and C-reactive protein support the role of this innate immune response in the aetiology of muscle damage following exercise.

In summary, the modest effects of mixed antioxidant supplementation provide some support for protection against muscular dysfunction following demanding exercise. However, the lack of strong relationships between markers of oxidative stress and muscle damage make it difficult to identify the underlying mechanisms responsible for this improved recovery from intermittent exercise and also to support the role of free radicals in exercise-induced muscle damage.

CHAPTER EIGHT

General Discussion

The major outcomes of this series of investigations are that; i) unaccustomed strenuous exercise results in severe muscular damage, dysfunction and soreness, ii) this is accompanied by perturbations in immune function and increased levels of oxidative stress, iii) supplementation with antioxidants prior to such exercise may abrogate some of these deleterious responses, iv) the prophylactic effects of these micro-nutrients are more marked when supplemented as a single antioxidant rather than in combination.

8.1 Evidence for the aetiology of muscle damage

Since Hough's (1905) early documentation of exercise-induced muscle damage extensive research conducted over the past century has lead to a plethora of evidence to support his original proposals. Research has identified the nature of exercise responsible for damage to skeletal muscle, the symptoms associated with this damage as well as the regenerative processes and the adaptation to repeated bouts of exercise that protects muscle from subsequent injury. This general summary fails to include the discrepancies in proposed mechanisms and evidence provided from a myriad of investigations into EIMD. Nevertheless, it can be concluded that exercise that is either unaccustomed or contains predominantly eccentric contractions results in damage to the contractile apparatus. These initial disruptions instigate a sequence of events that bring about muscular soreness and dysfunction that may be noticeable for a number of days following exercise. It is the precise mechanisms that are responsible for these effects that remain unclear. The investigations conducted in this thesis attempted to provide further support for the proposed aetiology of EIMD.

8.1.1 Muscle damaging exercise

Muscle damage is consistently reported following eccentric exercise. Indeed the bulk of research focused on elucidating the aetiology of this damage employs eccentric contractions to

specific muscle groups (Clarkson & Sayers, 1999; Allen, 2001) as this provides excellent control of extraneous variables and results in the greatest amount of muscle injury. Assuming the early events in EIMD are morphological, it follows that eccentric contractions of skeletal muscle are most likely to induce injury to the contractile apparatus if muscle contains inhomogeneous regions of weaker sarcomeres (Friden & Lieber, 2001; Proske & Morgan, 2001). Thus, in an attempt to establish the role of eccentric contractions we employed a mode of exercise that was both eccentric based and yet more comparable to commonly performed exercise. Our findings clearly support the well documented response when directly comparing eccentric based downhill running to more familiar concentric running on the level (Chapter 4). However, it could be argued that the contribution of eccentric contractions to general physical activity is relatively minimal. Although eccentric actions predominated in the initial investigations on downhill running (Chapters 4 and 5) some muscle groups reported as sore following the intermittent exercise (Chapters 6 and 7) were not exercised eccentrically throughout. Others have reported muscle damage and soreness following concentric and isometric actions that may help to explain this observation (Clarkson *et al.*, 1986; Moreau *et al.*, 1995; Prou & Marini, 1997; Philippou *et al.*, 2003). It seems that unaccustomed exercise which requires the activation of more vulnerable muscle groups containing weaker sarcomeres do not require the high forces associated with eccentric contractions to induced disruptions. This might also explain why moderate eccentric exercise often fails to protect skeletal muscle from a subsequent bout of maximal eccentric exercise (Prou *et al.*, 1999; McHugh, 2003). Intermittent exercise resulted in comparable muscle soreness and dysfunction to the more eccentric downhill running. Although this was more noticeable in muscle groups recruited during deceleration and turning movements. However, indices of muscle damage were often elevated to a greater extent than following downhill running highlighting the prevalence of EIMD in this more common mode of exercise.

8.1.2 Limitations of indices of muscle damage

The assessments of markers of muscle damage following exercise are indirect methods of quantifying the extent of muscle injury. Nevertheless it follows that the changes in these

markers post-exercise should be related if they reflect the events previously outlined. However, relationships between these markers were at best weak making causal effects difficult to substantiate. Others employing similar techniques have also found similar weak relationships (Newham, 1988; Gray *et al.*, 1993; Camus *et al.*, 1994; Chleboun *et al.*, 1998; Nosaka *et al.*, 2002), but some report stronger associations between markers of muscle damage and indices of inflammation (Cannon *et al.*, 1991; Lowe *et al.*, 1995; Bruunsgaard *et al.*, 1997; MacIntyre *et al.*, 2001), oxidative stress (Maughan *et al.*, 1989; Groussard *et al.*, 2003) and muscle function (Rodenburg *et al.*, 1993). It has been proposed that individual variability may be the primary cause for these inconsistencies (Warren *et al.*, 1999). However, some argue that certain markers employed for assessing muscle damage are unrelated to the mechanisms proposed (Croisier *et al.*, 1999; Malm, 2001; Nosaka *et al.*, 2002). It is clear from the investigations conducted in this thesis that there is large variability not only between individuals but also in the time course for the appearance of these markers. Inconsistencies in exercise protocols as well as specific markers employed are probably responsible for the lack of good associations and support for proposed mechanisms. Thus, until a large meta-analysis is conducted or more direct indices are used it will be difficult to accumulate considerable evidence for the precise aetiology of muscle damage induced by exercise.

8.1.3 Assessment of muscle function

We consistently reported a biphasic reduction in muscle function following both downhill running and intermittent exercise. This temporary and delayed response is well documented following eccentric based exercise that results in injury to the contractile apparatus. In an attempt to follow recommendations outlined by Warren (1999) we assessed alteration in muscle function via changes in isometric MVC (Chapters 5 and 6) as well as ROM (Chapter 7). Investigations into the specific nature of the disruptions to muscle ultrastructure conclude that reductions in muscular strength at optimal lengths may not be indicative of dysfunction but rather a change in optimum length reflective of a shift in the length-tension curve (Katz, 1939; Allen, 2001). Inclusion of isometric MVC at a range of muscle lengths would better represent the extent of muscular dysfunction. However, as we attempted to identify the

influence of antioxidant interventions on indices of EIMD within subjects this phenomenon was considered negligible. More problematic is the nature of assessing muscle function following whole body exercise. Warren (1999) recommended specificity when measuring post-exercise reductions i.e. apparatus used for assessment should be similar to the mode of exercise that induced damage. Comprehensive muscle function assessment following running exercise is both time consuming and complex hence our focus on the major muscle groups subject to eccentric contractions during both protocols. Nonetheless, the significance of impaired muscle function following muscle damaging exercise can not be ignored. The inability to directly assess muscle damage without conducting invasive techniques and the limitations of other indirect measures make assessment of muscular dysfunction the most applicable procedure for quantifying the extent of muscle injury along with its implications on normal muscular function.

Additionally, a prior bout of damaging exercise can directly affect physiological responses to subsequent submaximal exercise that may limit the level and duration of performance (Gleeson *et al.*, 1995). Also, glycogen replenishment is delayed following damaging exercise that will have profound implications on subsequent exercise performance (O'Reilly *et al.*, 1987; Kristiansen *et al.*, 1996). These findings, in conjunction with the more apparent reduction in muscle function following unaccustomed or eccentric exercise have provided the rationale for a variety of attempts to provide treatment for or prevent exercise-induced muscle damage (Connolly *et al.*, 2003).

8.1.4 Evidence for an inflammatory response

The role of inflammation in exercise-induced muscle damage is still under much debate (Malm, 2001). Initial attempts to explain the characteristic delayed symptoms of muscle soreness and dysfunction were attributed to this acute immune response (Smith, 1991). However, recent evidence is equivocal (Malm *et al.*, 2000; Beaton *et al.*, 2002; Nosaka *et al.*, 2002). The parameters assessed and reported in previous chapters of this thesis are typical of those reported in the vast majority of exercise studies that have attempted to identify this

response. Increased circulating numbers of neutrophils and monocytes, accompanied by changes in cytokine production are characteristic of an acute immune response. However, these changes are documented following non-damaging exercise and may simply reflect perturbations in immune function directly related to the intensity and/or duration of exercise, rather than injury to skeletal muscle (Mackinnon, 1999; Pedersen & Hoffman-Goetz, 2000). Indeed, changes in circulating numbers of leukocytes and plasma cytokine concentration were more profound following intermittent exercise compared to downhill running. For example, increases in the inflammatory responsive cytokine IL-6 were considerably greater following intermittent exercise. Also, it is important to highlight the fact that only presence of neutrophils in the interstitium of muscle truly reflects significant inflammation (MacIntyre *et al.*, 1996; Beaton *et al.*, 2002). Obviously, this can only be determined histologically so caution is advised when assessment of inflammation is monitored as changes in systemic concentrations of neutrophils (MacIntyre *et al.*, 1995). Recent evidence suggests that attempts to quantify histological changes in leukocyte accumulation via muscle biopsy may in fact have similar effects to the damaging exercise itself (Malm *et al.*, 2000). Thus, efforts to clarify such changes with biopsies are not advised and interpretation of previous investigations employing this technique should be treated with prudence (Friden *et al.*, 1983; Cannon *et al.*, 1989; Smith, 1991; Friden & Lieber, 1992; Fielding *et al.*, 1993). This limitation obviously makes elucidation of the potential role of inflammation in EIMD and subsequent adaptation difficult. Until there is an introduction of more accurate less invasive techniques then current evidence should be interpreted with caution.

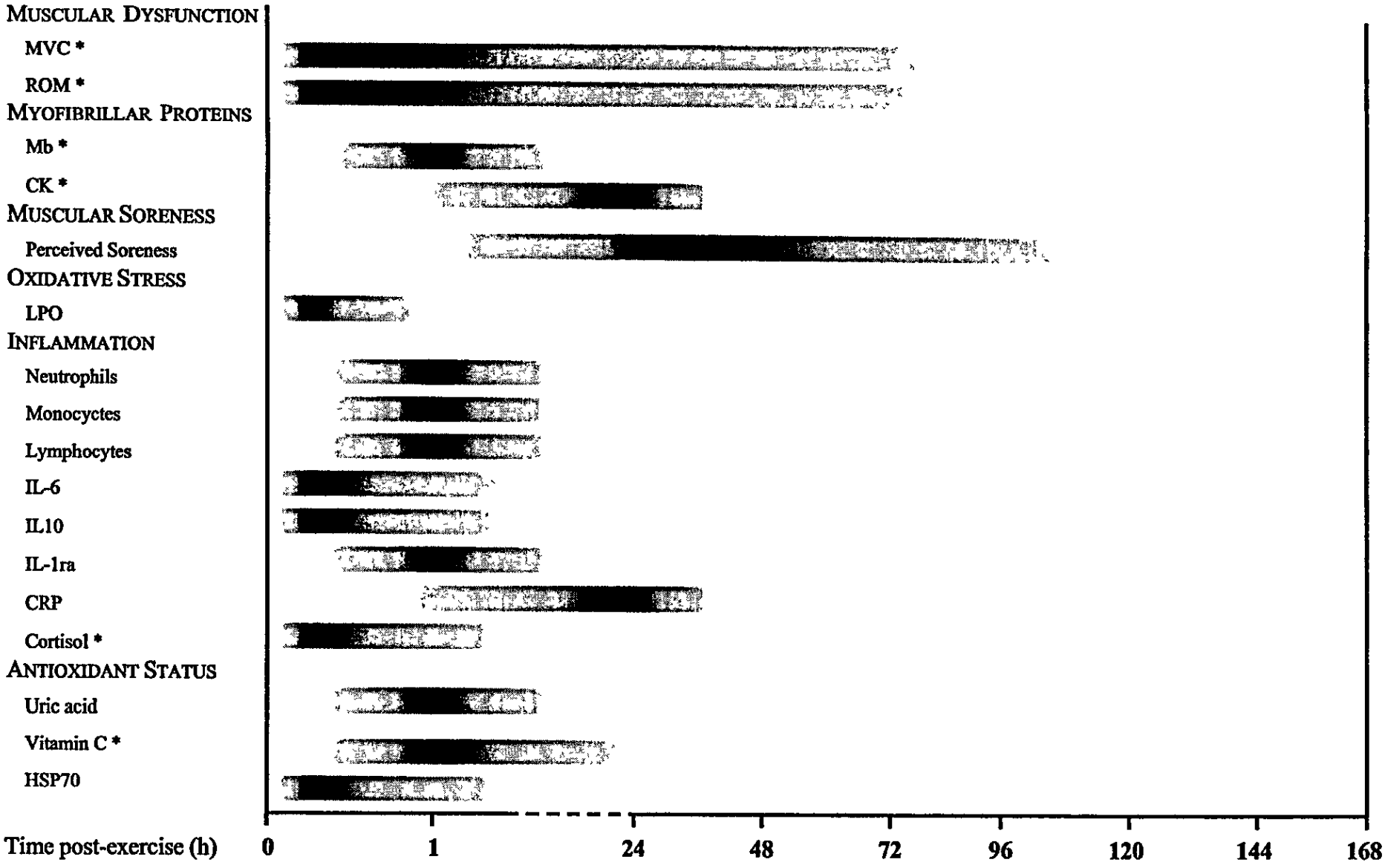
8.1.5 Contribution from oxidative damage

With the advancement of specific techniques for quantifying free radical activity it has been established that exercise induces profound levels of oxidative stress that may be a direct result of skeletal muscle radical generation (Reid *et al.*, 1992; Bailey *et al.*, 2003). To what extent these muscle generated radicals are involved in muscle damage both during and following exercise remains unclear. However, both evidence from the final investigation (Chapter 7) and previously documented work has established that muscle damaging exercise is associated with

increased levels of markers of lipid peroxidation (Kanter *et al.*, 1993; Meydani *et al.*, 1993; Saxton *et al.*, 1994; McBride *et al.*, 1998; Thompson *et al.*, 2001). We also reported increased ROS generation from active phagocytes post-exercise supporting their proposed role in post-exercise muscle damage (Camus *et al.*, 1992; Best *et al.*, 1999; Lapointe *et al.*, 2002). This considerable evidence would certainly explain the potential protective role of vitamin E and C.

Figure 8.1 illustrates the changes in indices of muscle damage assessed following both modes of exercise in order to highlight evidence attained from the investigation in this thesis supporting the aetiology of exercise-induced muscle damage.

Figure 8.1: Time course for changes in indices of muscle damage, oxidative stress and immune function following exercise. Peak changes are indicated by the darkest region of shading. * indicates indices influenced by antioxidant supplementation.



8.2 Antioxidant interventions

8.2.1 Single antioxidant supplementation

In an attempt to establish the potential role of antioxidant supplementation we investigated the effects of vitamin C on muscle soreness and markers of muscle damage following downhill running (Chapters 5). Although there were some beneficial effects on indices of muscle damage it was assumed that based on the established mechanisms of free radical generation during exercise (Sjodin *et al.*, 1990) the relatively low oxygen consumption observed during downhill running might explain the minimal intervention effects. Thus, in order to continue to maintain an exercise protocol that both provides sufficient oxidative insults to contractile tissue and is comparable to commonly performed activities we investigated the effect of the same supplement on intermittent shuttle running exercise (Chapter 6). This particular mode of exercise has been shown to produce muscle damage and associated changes in muscle function and soreness (Thompson *et al.*, 1999). Also, recent evidence shows a potential but modest effect of vitamin C supplementation at a lower dosage (Thompson *et al.*, 2001). The investigation reported in Chapter 6 identified the greatest therapeutic role of antioxidant supplementation on both indices of muscle damage and recovery of muscle function. These outcomes provided evidence for a free radical mediated role in the aetiology of muscle damage both during and following intermittent exercise.

Previously, vitamin C supplementation was shown to positively influence the post-exercise response of the inflammatory mediator IL-6 following muscle damaging exercise (Thompson *et al.*, 2001). As this cytokine has been implicated in EIMD (Bruunsgaard *et al.*, 1997) its reduction with antioxidant supplementation may suggest a protective role that reduces the extent of inflammation following exercise. However, recent research suggests a more significant biological role of this cytokine that is unrelated to muscle damage (Ostrowski *et al.*, 1998; Pedersen *et al.*, 2001b). Evidence from the first investigation showed a tendency for IL-6 production to be greater with concentric exercise (Chapter 4). Additionally, we observed a benign effect of antioxidant supplementation on the post-exercise IL-6 response (Chapters 5,

6 and 7). Furthermore, others have failed to observe the repeated bout effect with IL-6 that is well documented with other indices of EIMD (Croisier *et al.*, 1999; Ostrowski *et al.*, 2000). Also, it appears that IL-6 production is not limited to leukocytes and may in fact be directly produced from contracting skeletal muscle (Ostrowski *et al.*, 1998). The glycogen content of skeletal muscle has been shown to directly influence IL-6 production during exercise (Keller *et al.*, 2001; Steensberg *et al.*, 2001). This evidence has lead some authors to propose that IL-6 plays a major regulatory role in substrate metabolism, unrelated to EIMD, opening up an exciting new aspect to exercise immunology and potential for better understanding pathological conditions such as obesity (Pedersen *et al.*, 2001a; Pedersen *et al.*, 2001b; Wallenius *et al.*, 2002).

8.2.2 Mixed antioxidant supplementation

In light of the positive outcomes observed with vitamin C supplementation (Chapter 6), we attempted to exploit the protective role of this vitamin by using a mixture of antioxidants in quantities previously associated with positive effects on the recovery from strenuous exercise (Viguie *et al.*, 1989; Cannon *et al.*, 1991; Kanter *et al.*, 1993; Meydani *et al.*, 1993; Alessio *et al.*, 1997). Unfortunately, the intervention effects were minimal perhaps highlighting the need for a more comprehensive assessment of single antioxidant supplements before attempting to combine their free radical scavenging properties.

The minimal effect of mixed antioxidant supplementation (Chapter 7) compared to vitamin C supplementation (Chapter 6) could be explained by an alternative mechanism. Vitamin C is associated with the regulation of collagen synthesis (Murad *et al.*, 1981). Demanding exercise leads to increased connective tissue turnover (Brown *et al.*, 1997) which may be influenced by vitamin C supplementation. It is unlikely that this process would affect myofibrillar protein efflux, but damage to connective tissue could be implicated in the sensation of muscle soreness. Thus, vitamin C may have an additional effect independent of its antioxidant role (Darr *et al.*, 1993). Unfortunately this can only be substantiated with more direct assessment of connective tissue damage post-exercise not included in these investigations. However,

assuming this alternative role contributed to the beneficial effects reported in Chapter 6 there is evidence that shows vitamin E inhibits the collagen synthetic role of vitamin C (Geesin *et al.*, 1991). Although speculative, this could explain the modest effect observed with the mixed antioxidant supplement on indices of muscle damage following intermittent exercise.

Several investigations report similar beneficial outcomes with vitamin C (Kaminski & Boal, 1992; Jakeman & Maxwell, 1993; Thompson *et al.*, 2001) and vitamin E (Cannon *et al.*, 1990; Rokitzki *et al.*, 1994; McBride *et al.*, 1998) supplementation on indices of muscle damage. Evidence for the effects of mixed antioxidant supplementation remains equivocal (Schröder *et al.*, 2000; Childs *et al.*, 2001; Petersen *et al.*, 2001). However, it was recently highlighted that single antioxidant supplementation may not have physiologically relevant effects on all exercise-induced modulations, therefore mixed supplementation may ultimately present the greatest effects (Pedersen & Hoffman-Goetz, 2000). In light of the findings in these investigations and similar outcomes in the relevant literature it certainly seems that scavenging of free radicals appears to afford some protection against the commonly reported side-effects following muscle damaging exercise.

8.3 Summary

Inconsistencies are commonly reported when attempting to relate findings from investigations on EIMD and antioxidant supplementation, making general conclusions difficult to substantiate. Variations have been mainly linked to differing exercise protocols as well as supplementation strategies. Perhaps different modes of eccentrically based exercise result in differing magnitudes of oxidative stress, inflammation and muscle damage that is either alleviated or unaffected with a particular antioxidant. With-in investigation variations are well documented in exercise modes that facilitate muscle damage. Such variations have been attributed to the use of indirect assessments of muscle damage and oxidative stress rather than direct measures. The use of such indices is generally accepted in this particular aspect of exercise physiology due to the limitations of direct measures. The subjective nature of assessing muscle soreness will obviously make interpretations of findings difficult as does the

training status of individuals. The adaptation observed with prior exercise can be controlled for, but in individuals performing regular physical activity abstention from this routine is often difficult to impose. Some studies have attempted to reduce these inter-individual factors by implementing cut-off points for soreness and muscle function (Clarkson & Ebbeling, 1988; Sayers *et al.*, 2001; Totsuka *et al.*, 2002). Although this may lead to a sample of 'responders' to muscle damaging exercise it is difficult to substantiate, particularly when attempting to generalise findings. Also, investigations employing supplementation with dietary antioxidants consistently report habitual intakes in excess of national recommended daily amounts. Therefore, current dietary habits may mean that only individuals deficient in antioxidants will benefit from exogenous supplementation.

An alternative explanation for the delayed nature of muscular soreness could be linked to research focused on pain. That is, the increased sensation of any pain following injury is more commonly reported over a chronic time course. Wall (1979) proposed that the immediate phase following injury is more commonly painless and that pain is more an awareness of need-state rather than a sensation. He advocated the comparison of pain to hunger or thirst rather than sight or hearing implying a strong connection to body state. Thus, DOMS may serve to promote healing and avoid further injury by inhibiting or limiting an individual's capacity to perform damage inducing exercise. Attempts to alleviate these sensations and associated responses may not be as beneficial in the long term.

In summary, muscle damage is commonly associated with unaccustomed activity that includes a significant contribution of eccentric contraction. The precise aetiology for this exercise-induced phenomenon is still under much debate. However it is clear that free radicals play an integral role in perturbations following exercise that are highly likely to exacerbate existing mechanical damage of skeletal muscle. Thus, supplementation with dietary antioxidants showed some beneficial responses on indices of muscle injury following different modes of damaging exercise that provide evidence for the role of free radicals in EIMD. Effects were more profound with single antioxidant supplementation perhaps indicating that a clearer understanding of the individual actions of antioxidants is needed before attempting to

investigate combined antioxidant supplements. Nevertheless, these results provide considerable evidence for the use of antioxidants as a therapeutic treatment for this commonly documented response to strenuous physical activity and support a continuation of research in this field to further elucidate their precise role.

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APPENDICES

Appendix I **Medical History Questionnaire**

HEALTH SCREEN FOR STUDY VOLUNTEERS

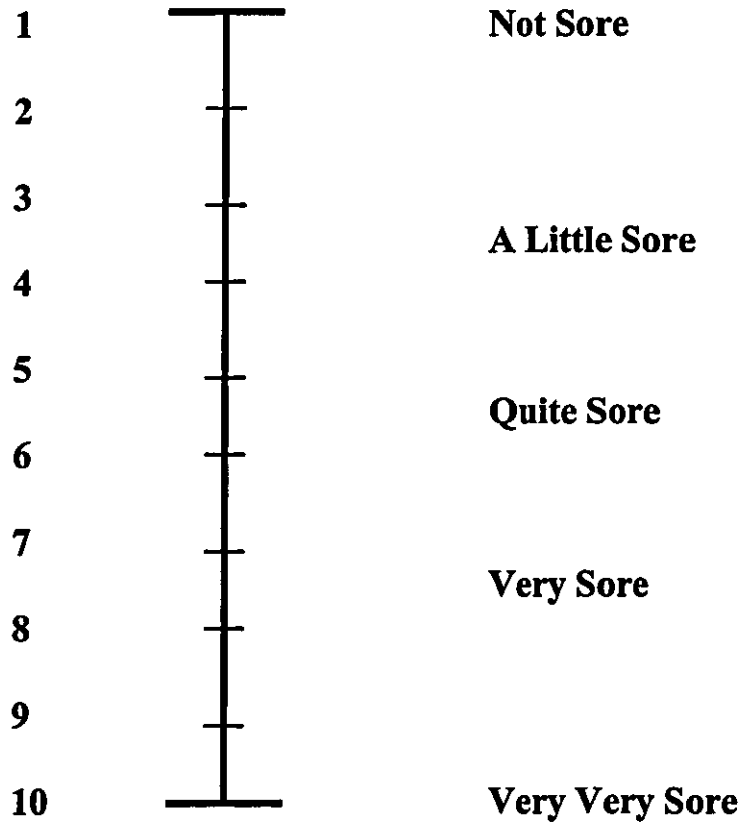
It is important that volunteers participating in research studies are currently in good health and have had no significant medical problems in the past. This is to ensure (i) their own continuing well-being and (ii) to avoid the possibility of individual health issues confounding study outcomes.

Please complete this brief questionnaire to confirm fitness to participate:

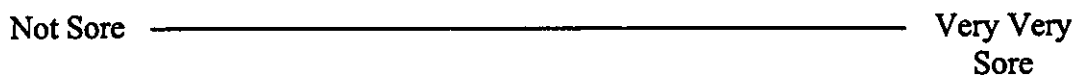
1. **At present,** do you have any health problem for which you are:
   - (a) on medication, prescribed or otherwise..... Yes ☐ No ☐
   - (b) attending your general practitioner ..... Yes ☐ No ☐
   - (c) on a hospital waiting list ..... Yes ☐ No ☐
2. **In the past two years,** have you had any illness which require you to:
   - (a) consult your GP ..... Yes ☐ No ☐
   - (b) attend a hospital outpatient department..... Yes ☐ No ☐
   - (c) be admitted to hospital ..... Yes ☐ No ☐
3. **Have you ever** had any of the following:
   - (a) Convulsions/epilepsy ..... Yes ☐ No ☐
   - (b) Asthma ..... Yes ☐ No ☐
   - (c) Eczema ..... Yes ☐ No ☐
   - (d) Diabetes..... Yes ☐ No ☐
   - (e) A blood disorder..... Yes ☐ No ☐
   - (f) Head injury..... Yes ☐ No ☐
   - (g) Digestive problems..... Yes ☐ No ☐
   - (h) Heart problems ..... Yes ☐ No ☐
   - (i) Problems with bones or joints ..... Yes ☐ No ☐
   - (j) Disturbance of balance/coordination..... Yes ☐ No ☐
   - (k) Numbness in hands or feet ..... Yes ☐ No ☐
   - (l) Disturbance of vision ..... Yes ☐ No ☐
   - (m) Ear / hearing problems ..... Yes ☐ No ☐
   - (n) Thyroid problems ..... Yes ☐ No ☐
   - (o) Kidney or liver problems ..... Yes ☐ No ☐
   - (p) Allergy to nuts..... Yes ☐ No ☐
4. **Has any,** otherwise healthy, member of your family under the age of 35 died suddenly during or soon after exercise? ..... Yes ☐ No ☐

Appendix II **Rating of Perceived Soreness**

Scale A

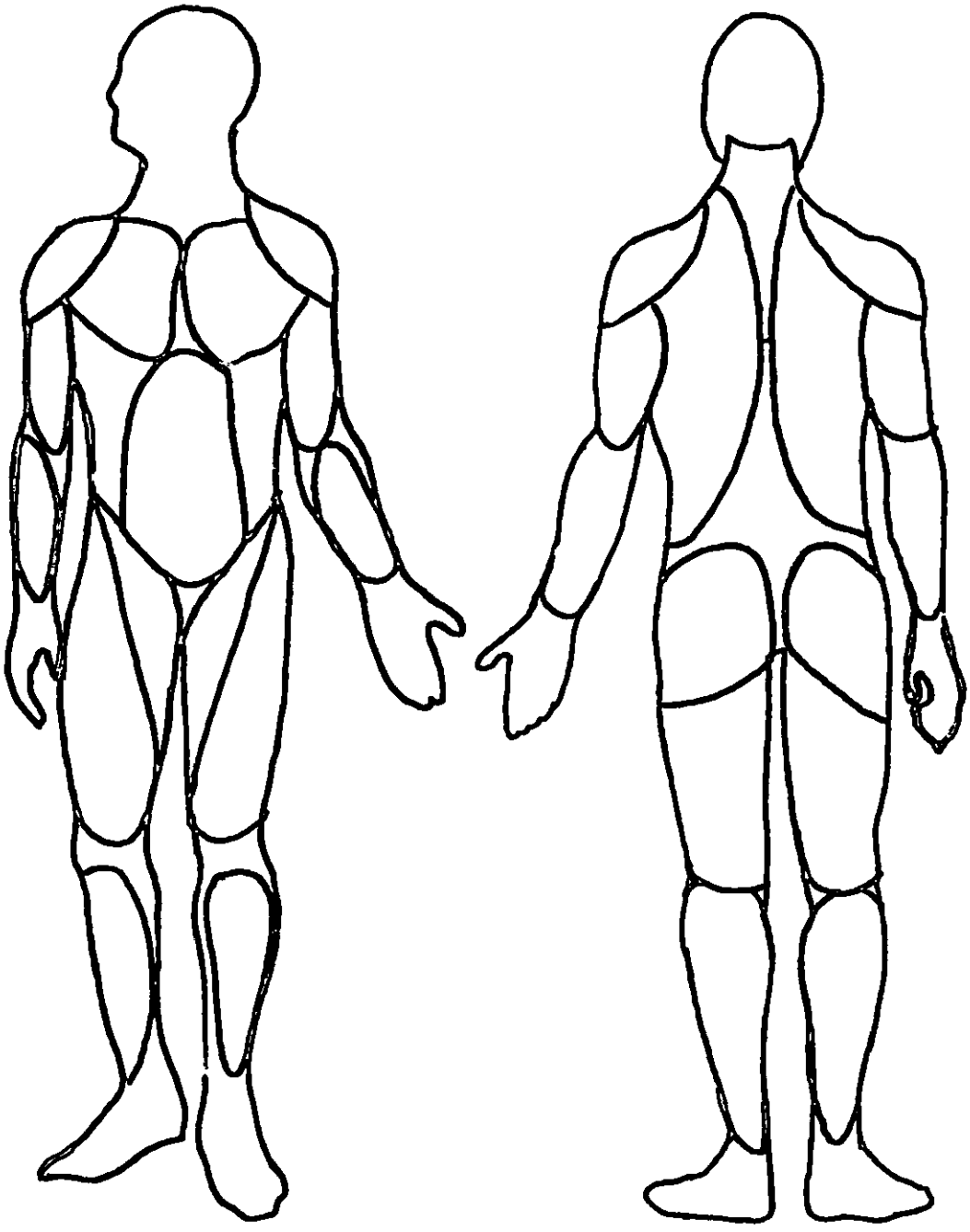


Scale B



Appendix III **Location of Muscle Soreness**

PLEASE HIGHLIGHT THE PARTS OF THE BODY WHERE YOU FEEL SORE.



Appendix IV **Lactate Analysis**

Whole blood lactate concentrations were determined according to the method modified from that described from Maughan (1982). This assay is based on the conversion of lactate to pyruvate in the presence of lactate dehydrogenase (LDH).

$$\text{Lactate} + NAD^+ \xrightarrow{\text{LDH}} \text{Pyruvate} + NADH + H^+$$

The amount of NADH produced is proportional to the amount of lactate present. The reaction is allowed to proceed by the removal of pyruvate due the addition if hydrazine (to form pyruvate hydrazone).

Reagents and chemicals

- 1.1mol.L^{-1} Hydrazine Buffer (pH 9.0)
- 70 mmol.L^{-1} Hydrochloric acid (Lactate Diluent)
- 1.0mol.L^{-1} Lactate (Sigma Co., UK)
- NAD^+ (Boehringer Mannheim GmbH, Germany)
- Lactate dehydrogenase (Boehringer Mannheim GmbH, Germany)
- Sigma metabolite control (Sigma Co., UK) (Quality control)

Procedures

1. Standards were prepared using the 1.0mol.L^{-1} lactate solution in the range 0-10mmol.L^{-1}, diluted to the same extent as the samples (1:10), and frozen until needed.

2. Frozen, deproteinised samples (plus standards and quality controls) were allowed to defrost at room temperature, and were subsequently vortexed and centrifuged for 3min.

3. A reaction mixture was made, constituting 10µl LDH and 2 mg NAD^+ per ml hydrazine buffer. A total of 200µl of reaction mixture was required per tube.

4. Twenty (20) µl of supernatant was placed into a glass fluorimeter test tube.

5. Two hundred (200) µl of reaction mixture was added to the tube, vortexed, and allowed to incubate for 30-40min.

6. One (1) ml of lactate diluent was added to the tube, and mixed well.

7. The fluorescence of the sample, quality controls, and standards were read using a fluorimeter (Model 8-9, Locarte, UK). The calibration range was established using the top and bottom standards.

8. The concentration in each sample was determined according to the linear regression equation established for the standards.

Appendix V **Vitamin C portions**

The following food portions contain 20mg of vitamin C. We would like you to try to eat 4 of these portions a day (no more than 5 portions). **You are allowed to eat as much of all other foods as you would like.** You do not have to record you diet over the 2 weeks of supplementation, but try and take in 4 portions of these foods. Over the period of the main trial you must record your diet and continue to stick to these guidelines.

50ml ($^1/_4$ glass)	Orange Juice (unsweetened)
250ml (1 bottle)	Lucozade sport (NGR or Energy)
25ml (5 teaspoons)	Ribena with 100ml water (1/2 glass or carton)
80ml (16 teaspoons)	Orange Squash with 320 ml water (2 glasses)
30g ($^1/_2$)	Kiwi Fruit
55g ($^1/_2$ small)	Grapefruit
100g ($^1/_2$ small tin)	Mandarins tinned in juice/fruit salad in juice
75g ($^1/_2$ slice)	Cantaloupe Melon (weighed without skin)
70g (1 medium)	Tangerine/satsuma (weighed without skin)
15g ($1^1/_2$ slices)	Green, yellow or red pepper - raw
120g (2 small)	Tomatoes
70g (4)	Cherry tomatoes
100g (2 tablespoons)	Coleslaw
150g (small)	Jacket potato
200g (20)	Chips
200g (4 small)	Roast/boiled potatoes
200g (2 small)	Bananas
150g (4 tablespoons)	Mixed vegetables
45g (heaped tablespoon)	Broccoli
75g (2 tablespoons)	Cauliflower
30g (3)	Sprouts

Appendix VI **Vitamin C analysis**

Plasma vitamin C concentrations were determined using a method adapted from a previously outlined technique (D. Thompson 1999, Phd Thesis)

Chemicals

- Degassed mobile phase = perchloric acid (Fisher Scientific, UK) adjusted to pH 1.2 at room temperature.
- Metaphosphoric acid 5% (Sigma Chemical Co., UK).
- Ascorbic acid (Sigma Chemical Co., UK).

Column and operating conditions

- Five (5) μm, 250mm * 4.6mm C18 Luna column (Phenomenex, UK).
- Spectrophotomeric detection: wavelength = 241nm
- Flow rate was set at 1.2ml.min^{-1}, which gave a retention time of approximately 3.4min.

Procedures

1. Plasma suspensions were defrosted on ice and kept chilled on the day of analysis (stored with 10% metaphosphoric acid 1:1).

2. Standards were prepared on a daily basis in the range 0-300μmol.L^{-1}, by dissolving ascorbic acid in 5% metaphosphoric acid.

3. Once samples had defrosted, they were vortexed and centrifuged at 4°C for 10min in order to obtain a clear supernatant.

4. Plasma samples and standards were diluted (1:1) in chilled 5% metaphosphoric acid, and 50μl used for injection via an autosampler.

5. The concentration of ascorbic acid in the samples was determined as the area under the ascorbic peak in relation to a calibration curve determined for the standards.

Appendix VII **Vitamin E analysis**

Plasma concentrations of vitamin E were determined using reversed phase high-performance liquid chromatography (HPLC) as described by Duthie (1999).

Chemicals

- Mobile phase = acetonitrile-tetrahydrofuran-methanol containing BHT-ammonium acetate ($10g.L^{-1}$).
- Hexane containing 500mg $BHT.L^{-1}$.
- 1,4-dioxan-ethanol-acetonitrile (20:20:40, by volume, DEA)
- ammonium acetate ($10g.L^{-1}$ H_2O)

Column and operating conditions

- Beckman Ultrasphere ODS5 μm, 250mm x 4.6 mm column (Beckman, High Wycombe, UK) set at 29° was used
- Flow rate was set at $1.05ml.min^{-1}$ and run time was 30min.
- Wavelengths were changed during each run
    - visible detection; 0-11.9min at 450nm; 12-17.4min at 472nm; 17.5-30min at 450nm
    - fluorescence detection; 0-5.1min, 330 & 470 (excitation/and emission); 5.2-14.6min, 298 & 328; 14.7-30min, 349 & 480nm.

Equipment

- Waters 470 scanning fluorescence detector (Water, Watford., UK).
- 486 tuneable absorbance detector 600E system controller 712 WISP (Water, Watford., UK).
- Jones choromatograph column chiller model 7955 (Jenes Hengued, Mid Glamorgan., UK)

Procedures

1. Plasma samples were defrosted on the day of analysis.

2. Standards were prepared in the range 0-$100\mu mol.L^{-1}$ and included every sixth sample in the run. Echinone blanks were also included in each run.

3. Plasma (200μl), 200μl water and 400μl ethanol were added to a 2ml microtube, vortexed and with 700μl hexane (containing BHT) and 100μl echinone for 10s. The microtube was shaken for 10min on the vortex then centrifuged for 5min. Of the hexane layer, 600μl was removed and taken to dryness on the speed vac for 9min. The dry sample was dissolved in 200μl DEA and shaken for 5-10min before application to the HPLC column.

4. The concentration of α-tocopherol in the samples was determined as the area under the α-tocopherol peak in relation to a calibration curve determined from the standards.

Appendix VIII **Immune cell function**

Phagocytic and respiratory burst activity of polymorphonuclear leukocytes was determined using commercially available techniques (ORPEGEN Pharma, Germany) outlined below;

Phagocytosis

The Phagotest testkit (ORPEGEN Pharma, Germany) was used for the investigation of the phagocytic function of neutrophils and monocytes in whole blood. The assay was based on the uptake of FITC (fluorescein) fluorescent labelled *Escherichia coli* (10^9 per ml) opsonised with immunoglobulin and complement from pooled human sera.

- 100µl of heparinised whole blood per tube was temperature equilibrated for 10min in iced water.
- 10µl of pre-cooled labeled bacteria was added to the assay tube at 37°C, a negative control sample remained on ice, and tubes were then vortexed.
- Phagocytosis was stopped after 10min by addition of 100µl of ice-cold quench solution, 3ml of ice cold wash buffer (Instamed-Salts) and by placing the tubes in iced water (permitting discrimination between attachment and internalisation of bacteria by quenching any FITC fluorescence of surface-bound bacteria leaving the fluorescence of internalised bacteria unaffected).
- All tubes were vortexed and centrifuged for 5min at 1400g (-4°C) the supernatant was removed.
- Another 3ml of wash buffer was added, tubes vortexed and the cells centrifuged.
- The supernatant was decanted and 2ml of room temperature lysing solution (lysis of erythrocytes and simultaneous fixing of leukocytes) added for 20min. Tubes were centrifuged, washed and centrifuged again.
- 100ul of DNA staining solution (propidium iodide) and 0.5ml of wash buffer added (DNA staining allowed discrimination between cells and artifacts).
- Tubes were vortexed and incubated for 10min in ice, in the dark.

Following preparation, samples were read on XL flow cytometer (Coulter) within 60min due to decomposition of fluorescence signal over time. Relative values were obtained for the amount of internalised fluorescence (i.e. the number of ingested bacteria per cell), the percentages of each cell type which had undergone phagocytosis (i.e. ingested bacteria), the percentage of each cell type of the total and any changes in morphology by side scatter changes.

Respiratory burst

The Bursttest (Phagoburst) test kit (ORPEGEN Pharma, Germany) was used to determine oxidative burst in heparinised whole blood. The kit contained unlabelled opsonised *E. coli* bacteria as particulate stimulus (10^9 bacteria per ml). Dihydrorhodamine (DHR) -123 was the fluorogenic substrate.

- 100µl of heparinised whole blood was placed in each tube and temperature equilibrated for 10min in iced water.
- 10µl of bacteria was added to the assay tube, 10µl of washing solution to another paired tube to act as a negative control.
- All tubes were vortexed and incubated for 10min at 37°C in a water bath.
- After incubation 10µl of DHR was added to each tube, vortexed and returned to the water bath for a further 10min (DHR-123 is converted to rhodamine-123 by reaction with oxidant products released by the phagocytic cells).
- 2ml of lysing buffer was added to each tube, vortexed and left at room temperature for 20min.
- Cells were centrifuged and the supernatant discarded. All tubes were washed by the addition of 3ml of cold washing buffer, vortexed and centrifuged again.
- The supernatant was again decanted. 100µl of DNA staining solution and 1ml of wash buffer was added, vortexed and incubated for 10min, on ice, in the dark.

The samples were read on XL flow cytometer (Coulter) within 30min due to decomposition of fluorescence signal. Data for relative burst, percentage burst of each cell type, the percentage of the relative cell groups and any morphological changes as seen via changes in side scatter were recorded.

(*T.Hurst, personal communication*)