The effects of eccentric exercise on delayed onset muscle soreness, muscle function and free radical production.

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Abstract

Delayed onset muscle soreness (DOMS) is a muscle strain injury that presents as tender or aching muscles, often felt during palpation or movement. DOMS is associated with unaccustomed, high-force muscular work and is most pronounced if the work involves a significant eccentric component. Despite substantial research into DOMS its aetiology is still unresolved. There have been recent suggestions that reactive oxygen species (ROS) may be involved in the aetiology of DOMS, however the evidence for this association is equivocal. Therefore, the aims of this thesis were to investigate the effects of unaccustomed exercise (downhill running) on DOMS, specifically investigating the role of ROS in its aetiology. Once this had been established, the effects of dietary intervention and antioxidant supplementation on DOMS and ROS were also investigated.

Study 1 demonstrated that downhill running, which resulted in DOMS, also induced the production of ROS, detected by ESR spectroscopy as well as an increases in serum malondialdehyde (MDA) concentration. It was noted that this increase in ROS occurred ~72 h post exercise and was therefore likely to be a result of increased phagocyte activity. Furthermore, the increase in ROS occurred after peak DOMS and when muscle function (as determined by losses of muscle torque assessed by isokinetic dynamometry) was returning to pre-exercise levels, suggesting a temporal dissociation between ROS production and DOMS following downhill running. Circulating levels of ROS were still increasing 72 h post exercise so this study was unable to map the full time-course of ROS production following downhill running.

Study 2 investigated the effects of dietary CHO intake on DOMS, ROS and muscle function. It was found that pre-exercise CHO status had no effect on ROS production, DOMS or losses of muscle function following downhill running. The study demonstrated a bi-phasic increase in creatine kinase (CK), with the second increase corresponding with the post-exercise ROS production. The study extended the post-exercise sampling period to 96 h, however, ROS were still increasing at this time and therefore this study was also unable to conclude a definitive time course of ROS production following downhill running.

Study 3 demonstrated that prolonged ascorbic acid supplementation attenuated the post-exercise increase in ROS production compared to placebo. This attenuation in ROS prevented the secondary peak in CK activity, however it had no effect on DOMS. Interestingly, supplementation with ascorbic acid resulted in more prolonged losses of muscle function. ROS peaked at 96 h post exercise and therefore the time course of ROS production following downhill running was established.

From these studies it was concluded that downhill running did result in post-exercise ROS production. This post-exercise ROS production was associated with secondary muscle damage as measured by plasma CK activity although it had no effect on DOMS. Since supplementation with ascorbic acid attenuated ROS production and prolonged the losses of muscle function, it was suggested that ROS produced by phagocytes in the days following muscle-damaging exercise have a positive role in assisting in the recovery from the trauma.

Dedication

I dedicate this thesis to my parents, for all you have done and all you continue to do for me. Thank-you for supporting me in whatever I choose to do and for always believing in me.

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DECLARATION

I declare that the work presented in this thesis is entirely my own, with the exception of:

The ESR spectroscopy work was measured at Glamorgan University by Dr Tony Ashton.

The leukocytes measured in study 3 were carried out at Liverpool University by the department of haematology.

Some of the work reported in this thesis has already been presented at European conferences and published in European journals (see Appendix A).

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LIST OF ABBREVIATIONS

AA	Ascorbic acid
CHO	Carbohydrate
CK	Creatine kinase
CV	Coefficient of variation
DOMS	Delayed onset muscle soreness
DWN	Downhill run
EDTA	Ethylendiametetra-acetic acid
ESR	Electron spin resonance
FLA	Flat run
GRS	Graphic rating scale
GSH	Total glutathione
H_2O_2	Hydrogen peroxide
Hb	Haemoglobin
HC	High carbohydrate
Hct	Haematocrit
HFMEE	High force maximum eccentric exercise
HOCl	Hypochlorus acid
HPLC	High performance liquid chromatography
LC	Low carbohydrate
LP	Lipid peroxidation
MDA	Malondialdehyde
NEFA	Non-esterified fatty acids
NO	Nitric oxide
$O_2^{\bullet-}$	Superoxide anion
$^{\bullet}OH$	Hydroxyl radical
$ONOO^-$	Peroxynitrite
PBN	α-phenyl-tert-butynitrone (check)
Pl	Placebo
PVC	Plasma volume change
ROS	Reactive oxygen species
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TEP	1,1,3,3-Tetraethoxypropane
VAS	Visual analogue scale
WBC	White blood cells

CHAPTER 1 -General Introduction

"When an untrained muscle makes a series of contractions against a strong spring, a soreness frequently results which cannot be regarded as a phenomenon of pure fatigue"

Hough (1902, p.76)

1.1 Introduction

In 1902, Theodore Hough suspected that the muscular pain observed in the days following unaccustomed exercise was not a direct consequence of fatigue, but was attributable to some other mechanism. This early observation began the research into the phenomenon known as Delayed Onset Muscle Soreness (DOMS). Since Hough's early work, many research groups have attempted to fully understand the causes of DOMS. However despite this century long quest, the exact aetiology of DOMS is still unresolved (Nosaka et al., 2002).

DOMS is a type I muscle strain injury that presents as tenderness or stiffness in response to palpation or movement (Gullick et al., 1996). It is associated with unaccustomed muscular work, especially if the exercise involves a large eccentric component (Newham, 1988). DOMS is therefore experienced by both athletes when starting new training regimen or following a break in training, along with sedentary individuals who begin training.

Many potential mechanisms to account for DOMS have been proposed including: lactate accumulation (Schwane et al., 1983), involuntary muscle spasms (De Vries, 1966), connective tissue damage (Abraham, 1977), muscle tissue damage (Hough, 1902), inflammation (Smith, 1991) and Reactive Oxygen Species (Maughan et al., 1989). It is now generally accepted that unaccustomed exercise results in focal muscle tissue damage (Jackson,1992; Lieber and Friden, 2002; Vincent and Vincent, 1997), although the exact mechanism for this damage and its relationship with DOMS remains unclear (Nosaka et al., 2002). The most recent suggestion is that free radicals may be a potential source for initiating DOMS (Fantone, 1985). The study of free radical production following unaccustomed exercise is new and provides an alternative mechanism for the development of DOMS.

Free radicals are produced following strenuous exercise, although the exact role that radicals play in skeletal muscle damage is not fully understood (McArdle and Jackson, 1997). One reason for this is that definitive models for the detection of radicals are still being assessed and consequently the extant research has predominantly used indirect 'markers' of free radical production, such as malonaldehyde production (Maughan et al., 1989), changes in glutathione levels (Dufaux et al.,1997), or the measurement of diene-conjugated compounds (Saxton et al., 1994). The most direct way to measure free radicals involves Electron Spin Resonance (ESR) spectroscopy (Nieman, 1999). The application of this technique is relatively new and requires considerable expertise. Thus, only a few studies have

utilised ESR with exercising human subjects (Ashton et al. 1998; Ashton et al., 1999; Ashton et al., 2003; Davison et al., 2002; Groussard et al., 2003).

It is still unclear whether the production of free radicals during eccentric exercise is an unwanted consequence of such exercise which can result in significant damage to the skeletal muscle tissue or whether they are a necessity and their production is carefully controlled in an attempt to scavenge damaged or necrotic cells (Petersen et al., 2001). Recently, it has been suggested that free radicals may interact with transcription factors, signalling cells to adapt following the trauma of the exercise (Khassaf et al., 2001). This demonstrates a positive physiological role of free radicals as opposed to the more often cited pathophysiological role (Pattwell et al., 2003).

Since the exact cause of DOMS is still unresolved, there is as yet no known therapeutic intervention that can successfully prevent or attenuate DOMS. There have been numerous postulated interventions including ice (Eston et al., 1999; Yackzan et al., 1994), massage (Farr et al., 2002), stretching (Rodenburg et al., 1994), ultrasound (Tiidus, 1999), anti-inflammatory drugs (Donnelly et al.,1988; Jansen et al.,1983), and antioxidants (Thompson et al., 2001a). The literature examining the effects of antioxidants on DOMS is limited and somewhat conflicting and therefore clearly warrants further investigation.

Free radicals cause oxidative damage and thus antioxidants are any compound that prevent or retard this damage (Halliwell, 1994). They may be categorised as enzymatic and non-enzymatic. Enzymatic antioxidants include superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase, whilst non-enzymatic antioxidants include ascorbic acid, α-tocopherol and β-carotene (Halliwell and Gutteridge, 1999). Antioxidants work by:

- Directly preventing radical formation.
- Scavenging and converting the radical species to a less active molecule.
- Assisting in the repair process following radical damage.
- Assisting in providing a more favourable environment to protect by supplying reducing agents (Goldfarb, 1999).

It has been suggested that DOMS could be prevented through dietary antioxidant supplementation since supplementation may attenuate the peroxidation of lipid membranes (Dekkers et al., 1996) which may be responsible for DOMS. Conversely, if free radicals play a significant physiological role in assisting the recovery from skeletal muscle damage, the supplementation of antioxidants may delay the healing process. Despite this antioxidant paradox, no study has examined the effect of a dietary antioxidant, such as ascorbic acid, in relation to free radical levels directly following a muscle damaging exercise protocol. Also it is presently unknown whether markers of muscle damage in the days following specific muscle damaging exercise in humans are related to free radical concentrations.

Additionally, external factors such as dietary intake may also affect DOMS and this could provide extra information regarding the aetiology. For example, it has been shown that both the ingestion of carbohydrate (CHO) during heavy exercise, and pre-exercise CHO loading are associated with smaller shifts in circulating neutrophil number as well as attenuated changes in neutrophil functional responses (Bishop et al., 1999; Bishop et al., 2001a; Bishop et al., 2001b; Gleeson et al., 1998b). This is potentially important since DOMS-inducing exercise is associated with an acute phase inflammatory response, characterised by the mobilisation and activation of neutrophils (Mackinnon, 2000). These activated neutrophils accumulate at the site of the injury, phagocytosing cellular fragments and micro-organisms. This is achieved both directly by the neutrophils as well as through a sudden stimulus-induced increase in non-mitochondrial oxidative metabolism resulting in the production of superoxide and other free radicals. Such a process is referred to as the oxidative or respiratory burst (Pyne, 1994).

It has been speculated that the secondary damage associated with muscle-damaging exercise may be as a result of the phagocytic oxidative burst and the overwhelming of cellular antioxidant defences (Pizza et al., 1995). If this is the case, then it is possible that pre-exercise CHO loading may attenuate both the oxidative burst and DOMS. As yet, no study has addressed the role of carbohydrate following specific muscle damaging exercise although there has been research on the effects of carbohydrate status on the immune response following an exercise protocol shown to induce muscle damage (Bishop et al., 1999; Bishop et al., 2001b; Bishop et al., 2001c; Gleeson et al., 1998b). Furthermore, it is well established that athletes often consume high

carbohydrate diets in order to maximise endurance performance. It would therefore be interesting to note if such diets influence the extent of DOMS and if it has any effect on the recovery.

Despite a century of research into DOMS, the exact causes remain unclear. The production of free radicals during exercise provides a new and exciting approach to study the aetiology of DOMS in human volunteers and further work in this field is needed (Jackson 1992). The use of nutritional strategies to influence free radical production following muscle-damaging exercise also provides a novel and potentially important model to examine this relationship.

1.2 Aims of thesis

The overall aim of this thesis is to investigate the aetiology of DOMS following muscle-damaging eccentric exercise in human subjects. Furthermore this research intends to address the role that free radicals play in DOMS. Once this is achieved the influence of antioxidant supplementation and dietary intervention on DOMS and ROS production following muscle-damaging exercise will be examined. In order to achieve the overall aim of this research, the following objectives are addressed:

1. To establish the effect of downhill running on DOMS compared to running on the flat (i.e. level running).
2. To investigate the production of ROS during and after downhill running compared to level running.
3. To investigate the effects of dietary intervention on DOMS and metabolic responses following downhill running.
4. To investigate the production of ROS during and after downhill running following dietary intervention.
5. To investigate the effect of ascorbic acid supplementation on DOMS during and after downhill running.
6. To investigate the production of ROS during and after downhill running following ascorbic acid supplementation.

Study 1 will address the differences between downhill and level running in inducing DOMS at the same relative exercise intensity, whilst at the same time investigating the role ROS may have on in the onset and development of DOMS. This study will

therefore address objectives 1 and 2. Study 2 will examine the effects of dietary intervention on DOMS and ROS production thus addressing objectives 3 and 4, and study 3 will investigate the effects of ascorbic acid supplementation on DOMS and ROS production thus addressing objectives 5 and 6.

CHAPTER 2 – Review of the literature

2.1 Introduction

The first reported reference to DOMS was made by Theodore Hough (1902) who stated that when an untrained muscle performed exercise, it often resulted in discomfort that did not manifest until 8-10 hours post-exercise, and concluded that this could not be solely attributed to fatigue. Since Hough's initial observation there has been a proliferation in research into DOMS, and despite this, the exact aetiology remains unclear. This chapter explores the concept of DOMS in relation to the types of exercise, likely causative factors, and methods to assess it. Since ROS are produced during exercise and have been attributed, at least in part to DOMS, an examination of how ROS may be formed as well as the types of free radicals produced will be undertaken. The chapter also reviews the methods of measuring ROS before appraising the role antioxidants and diet on ROS and DOMS.

2.2 Delayed Onset Muscle Soreness (DOMS)

DOMS is classified as a type I muscle strain injury that presents as tender or aching muscles, usually felt during palpation or movement (Cheung et al., 2003) and can affect any skeletal muscle. Following downhill running pain is felt in all of the major flexor and extensor muscle groups of the hip, thigh and leg (Armstrong, 1984).

The temporal sequence of DOMS initiates after exercising for approximately 10-20 minutes when subjects begin to notice problems in the control of their limbs. If the exercise is running, the loss of control is often described a 'heavy' or 'jelly like' legs. Although this feeling is unusual, it is rarely described as being painful (Jones et al., 1997). Pain develops 6-12 hours after the exercise and typically, individuals go to bed with mild discomfort and wake the next morning with severe, sometimes debilitating pain, initially noted when attempting to get out of bed. Peak pain usually occurs between 48-72 hours post-exercise before subsiding, with little or no pain felt 5-7 days after the exercise bout (Armstrong, 1990).

The pain associated with DOMS has been linked to the stimulation of the small myelinated and unmyelinated muscle pain receptors found in muscle tissue (Cleak et al., 1992a) (Table 2.1). These nerve endings are most abundant at the distal myotendinous junction and fascial sheaths, and it is therefore logical that DOMS is often reported to be most pronounced in these areas (Asmussen, 1956). Muscle pain

receptors are polymodal, i.e. they will respond to thermal, mechanical or chemical stimuli, all of which have been postulated as potential mechanisms for inducing DOMS (Armstrong, 1984). These mechanisms will be discussed in greater detail in section 2.3.

Table 2.1 Classification of sensory nerve fibres (Jones and Round, 1990)

2.2.1 Exercise and DOMS

DOMS is associated with unaccustomed, high-force muscular work and is most pronounced if the work involves a significant eccentric component (Armstrong et al., 1983; Byrnes et al., 1985; Newham et al., 1983; Schwane et al., 1983a). However, eccentric muscle actions do not exclusively result in DOMS. Isometric exercise has been shown to induce DOMS (Clarkson et al., 1986; Triffletti et al., 1988), especially if the muscle is exercised in an extended position (Jones et al. 1989). Recently, in a series of experiments employing prolonged multiple sprint exercise, significant DOMS was observed in subjects unaccustomed to that mode of exercise (Thompson et al., 1999; Thompson et al., 2001a; Thompson et al., 2001b).

2.2.1.1 Unaccustomed exercise

The observation that DOMS is caused through unaccustomed exercise was initially made by Hough (1902) and has since been confirmed by many authors (Talag, 1973; Clarkson et al., 1986; Jones et al., 1986). Hough (1902) explained that an untrained muscle was one that had not been making regular contractions. Furthermore, he stated that if a muscle was allowed to detrain over a period of time, even if that muscle still remained strong, then it should still be classified as untrained.

2.2.1.2 Eccentric exercise

Asmussen (1953) first reported that DOMS appeared to be exacerbated by exercise that involves lengthening of the active muscle such as walking downhill or lowering weights, exercise commonly referred to as 'negative work' or 'eccentric exercise'. Despite 'eccentric exercise' being commonly used in the literature to describe such muscle actions, it should be noted that eccentric actually means 'off centre' and therefore lengthening muscle actions is a more accurate description of this event. Furthermore, since contraction actually means 'shortening' which clearly does not occur with lengthening muscle actions, it may also be inappropriate to use this term when referring to lengthening exercise (Faulkner, 2003). However, since the term "eccentric contractions" is commonly used in the literature, for the purpose of this thesis it will be used to describe lengthening muscle actions.

Although it is now accepted that eccentric exercise results in DOMS, the reason for this still remains unclear. One potential mechanism that has received a certain amount of credibility is that fewer motor units are recruited for a given work load than concentric exercise, and therefore the force is activated over a smaller cross sectional area of muscle (Enoka, 1996). It is interesting to note that eccentric exercise requires lower energy costs than concentric exercise, probably due to the fewer motor units being activated.

2.2.1.3 The Repeated Bout Effect

It has been demonstrated that a repeated bout of eccentric exercise reduces the magnitude of DOMS, a process now known as the 'repeated bout effect' (RBE) (Eston et al., 1995). The RBE only occurs following prior eccentric exercise, since prior concentric exercise has been shown to provide no protective effect (Newham et al., 1983). It is still unclear if the RBE protects the muscle from subsequent damage or simply attenuates the magnitude of DOMS. Pierrynowski et al. (1987) reported that the RBE reduces the magnitude of DOMS but has no effect on actual muscle damage.

In contrast Schwane et al. (1983b) demonstrated, through a decreased activity of plasma creatine kinase (CK) following downhill running in rats, that muscle damage was prevented through prior eccentric exercise. Brown et al. (1997) also reported that the RBE protected against DOMS and reduction in force development. The later

study also demonstrated that skeletal muscle adaptation was brought about by a single bout of relatively few eccentric contractions, and concluded that there was no significant prophylactic benefit in increasing the number of eccentric muscle repetitions.

Despite numerous studies investigating the RBE, there is still no definitive model to explain why it occurs. McHugh et al. (1999) reported that the RBE has been attributed to neural, connective tissue, cellular, excitation-contraction coupling, or inflammatory adaptations. It is questionable that one mechanism can explain the RBE and it is likely that the effect occurs through the interaction of multiple mechanisms depending on the form of the eccentric exercise bout and the specific muscle groups involved.

The RBE clearly has significant consequences for studies investigate DOMS, specifically those that utilise a crossover design. Although no definitive time course has been found showing how long the RBE lasts, it appears that this effect lasts for weeks as opposed to days, with several studies reporting the RBE up to 9 weeks (Clarkson et al., 1992; Ebbeling et al., 1989; McHugh et al., 1999). Recently Nosaka et al. (2001) reported that the RBE lasts for at least 6 months and is lost between 9 and 12 months. It must be noted however, that since the study was carried out in humans, it is feasible that the subjects would have performed eccentric contractions in the 6-month period which may have been responsible for the long lasting RBE and therefore these findings should be treated with caution.

In light of this, investigation into DOMS should consider the RBE when designing studies to ensure that adequate time is given between repeated eccentric exercise bouts. Likewise, it has been demonstrated that the RBE occurs after a single bout of eccentric exercise and therefore the selection of subjects to perform DOMS research must also be carefully considered.

2.2.2 Eccentric protocols used in DOMS research

Various exercise protocols have been used in a laboratory setting to induce DOMS in both human and animal populations, as well as on athletic and sedentary individuals (Evans, 1987). Such protocols include downhill running (DHR), plyometric exercise, step tests, and high force maximum eccentric exercise (HFMEE). A summary of some of the studies that have investigated DOMS and the various methods for inducing it can are presented in Table 2.2

Table 2.2 Summary of various methods of inducing DOMS. HFMEE = high force maximum eccentric exercise, DHR = downhill running, LIST = Loughborough Intermittent Shuttle Test.

Author, year	Exercise Protocol	Training Status (Trained/Untrained)	Type of subjects	Subject No & Sex
Abraham (1977)	HFMEE	Both	Human	8 ♂, 3 ♀
Blais et al. (1999)	HFMEE	Trained	Human	13 ♂
Armstrong et al. (1983)	Step Tests	N/A	Animal	140 ♂
Bobbert et al. (1986)	HFMEE	Trained	Human	11 ♂
Brown et al. (1997)	HFMEE	Untrained	Human	1 ♂, 6 ♀
Brown et al. (1999)	HFMEE	Untrained	Human	9 ♂
Byrnes et al. (1985)	DHR	Unspecified	Human	11 ♂ ♀
Childs et al. (2001)	HFMEE	Untrained	Human	14 ♂
Duan et al. (1990)	DHR	N/A	Animal	? ♂ ? ♀
Eston et al. (2000)	DHR	Unspecified	Human	9 ♂, 9 ♀
Evans et al. (1986)	Cycling	Both	Human	9 ♂
Howell et al. (1985)	HFMEE	Untrained	Human	Not stated
Helge et al. (2001)	HFMEE	N/A	Animal	23 ♂
McArdle et al. (1999)	HFMEE	N/A	Animal	15 ♂
Moyna et al. (1996)	Cycling	Untrained	Human	32 ♂ 32 ♀
Ortega et al. (1993)	Cycling	Untrained	Human	10 ♂
Schwane et al. (1983a)	DHR	Both	Animal	Not stated
Sorichter et al. (2001b)	DHR	Unspecified	Human	9 ♂, 9 ♀
Thompson et al. (1999)	LIST	Untrained	Human	16 ♂
Van Der Meulen et al. (1997)	Plyometrics	N/A	Animal	43 ?

Table 2.2 summarises the variety of methods used to induce DOMS in laboratory experiments. It is interesting to note that despite studies reporting significant gender-based differences in skeletal muscle responses to damaging exercise (Komulainen et

al., 1999; MacIntyre et al., 2000), a mixed sex group is still used by many research groups. Tiidus (1995) speculated that the observed gender difference was related to oestrogen and its strong antioxidant properties, which may assist in maintaining post-exercise membrane stability. It is possible that studies utilising mixed gender subject populations may have drawn circumspect conclusions regarding the time course and magnitude of the DOMS response.

The training status of the subjects has also been shown to be important in the magnitude of DOMS (Schwane et al., 1983a), and despite this some authors have not reported the level of fitness of their subjects or have been vague in their description of training status. Vincent and Vincent (1997) reported that the training status of the subjects affected both the magnitude of DOMS and markers of muscle damage as shown through increased plasma CK activity. Trained weightlifters (defined as regular weight training for more than three years) had an attenuated DOMS response following weight-training compared to non-weight lifters despite them being familiarised to the weight training test. It is therefore imperative that the experience of training by subjects is clearly stated, both in terms of level of fitness and experience in eccentric exercise.

Perhaps the most significant inconsistencies can be found by examining the exercise protocols. Research involving high force, maximum eccentric exercise (HFMEE) show considerable variation in their designs, specifically relating to the number of repetitions, the intensity of exercise and the rest periods. Repetitions range from 400 repetitions (Friden and Lieber, 1996) to 60-70 repetitions (Clarkson and Tremblay, 1988), whilst other studies have simply asked subjects to perform as many repetitions

as possible (Howell et al., 1985). It is feasible that DOMS may be related to exercise intensity and as such the variations in protocol could have a significant effect on the magnitude of DOMS.

2.2.2.1 Downhill running

Since Davies and Barnes (1972) demonstrated that downhill running (DHR) involves eccentric contractions, numerous studies have utilised this mode of exercise to induce DOMS in a laboratory setting. Despite DHR now being used by many research groups to induce DOMS, there is still no consensus regarding the optimum exercise duration, intensity or treadmill gradient to induce DOMS. This is highlighted in Table 2.3.

Table 2.3 Variations in downhill running methodologies.

Author	Decline %	Duration	Subjects	Intensity
Kirwan et al (1992)	17%	30 min	Human	60% $\dot{V}O_{2\,max}$
Komulainen et al (1999)	15%	130 min	Animal	17 $m.min^{-1}$
Maughan et al (1989)	13.3%	45 min	Human	75% HR max.
Petersen et al (2001)	5%	90 min	Human	75% $\dot{V}O_{2\,max}$
Pierrynowski et al (1987)	10%	12 min	Human	60% $\dot{V}O_{2\,max}$
Pyne et al (2000)	10%	40 min	Human	52% $\dot{V}O_{2\,max}$
Sharwood et al (2000)	10%	40 min	Human	70% peak run speed
Sorichter et al (2001b)	16%	20 min	Human	70% $\dot{V}O_{2\,max}$
Warren et al (1992)	18.9%	150min	Animal	25 $m.min^{-1}$

Despite the treadmill gradient being a significant factor that induces DOMS, there does not appear to be any consensus regarding what gradient to employ, and some studies have not even reported the angle in their methods. Furthermore, Table 2.3 also highlights the variations in exercise intensity used during downhill running. It has been suggested that the pain observed following eccentric exercise is correlated to the amount of torque produced during the eccentric component of the movement, and hence it is likely that higher intensity exercise results in greater magnitudes of DOMS (Eston, 1995). One of the postulated mechanisms for DOMS is post-exercise infiltration of leukocytes in to the damaged muscle and subsequent free radical production (see section 2.4). Numerous studies have shown that this post-exercise leukocyte infiltration is significantly affected by exercise intensity (Lovlin et al., 1987; Leaf et al., 1997; Mills et al., 1996; Pyne et al., 2000; Robson et al., 1998), intimating that variations in exercise intensities could be a cause of the equivocal findings.

As well as various treadmill gradients and intensities, it is also apparent that varying durations of exercise have been used. Cleak et al. (1992a) documented that the duration of the run is usually 45 minutes, although as can be seen in Table 2.3 many variation of this time have been utilised. The duration of the exercise also affects the magnitude of DOMS, with studies reporting that longer exercise durations not only increased DOMS but also muscle damage, demonstrated through increased plasma CK activity (Tiidus and Ianuzzo, 1983). In a later study McCully and Faulkner (1986) confirmed, through assessing changes in histological appearance, that the degree of muscle injury was directly related to the duration of the exercise protocol employed.

Consequently, due to the extreme variations in downhill running protocols, in terms of intensity, duration and treadmill gradient, comparisons between studies are difficult to make and should therefore be treated with caution.

2.2.2.2 Whole body exercise versus high force maximal eccentric exercise (HFMEE).

Whole body exercise to induce DOMS, such as downhill running or the Loughborough intermittent shuttle test (LIST), results in a different time course of response compared to HFMEE. Plasma CK activity peaks approximately 1 day post-exercise following whole body exercise, compared to approximately 4 days post-exercise following HFMEE (Table 2.3). There is also a significantly greater increase in plasma CK activity following HFMEE than following whole body exercise. For example, Costill et al. (1990) reported maximum plasma CK values of 6988 ± 1913 $U.l^{-1}$ following HFMEE where as Smith et al. (1998) reported maximum plasma CK of 425 $U.l^{-1}$ following DHR.

Table 2.4 Time course of peak DOMS and plasma CK release following whole body exercise and high force maximum eccentric exercise.

Study	Exercise Used	CK peak Days post-ex	DOMS peak Days post-ex
Schwane et al. (1983a)	DHR	1	2
Thompson et al. (2001a)	LIST	1	1-2
Maughan et al. (1989)	DHR	1-2	2
Childs et al. (2001)	HFMEE	3	2
Deschennes et al. (2000)	HFMEE	5	2
Clarkson et al. (1992)	HFMEE	4	2
Byrne et al. (2001)	HFMEE	7	2
Smith et al. (1998)	DHR	Day1 and 5	2

The reason for the differences in the time course of CK release following the two forms of exercise is still unclear (Clarkson et al., 1992). Eston et al. (1995) speculated that the differences may be a result of HFMEE exerting higher muscular forces, which are maintained through a longer strain range compared to downhill running. Although the exact mechanism for these differences is not known, it poses important issues in the design of studies investigating DOMS, and therefore caution should be exercised when comparing whole body and HFMEE studies.

2.2.3 Causes of DOMS

Despite more than 100 years of research into DOMS, there is still no consensus as to the exact events that takes place following the differing forms of exercise that induce

DOMS. Potential mechanisms have been postulated, including lactate accumulation (Asmussen, 1953), involuntary muscle spasms (De Vries, 1966), connective tissue damage (Abraham, 1977; Asmussen, 1956; Komi and Buskirk, 1972), muscle tissue damage (Hough, 1902), inflammation (Smith, 1991), and increased muscle temperature (Davies and Barnes, 1972).

2.2.3.1 Muscle tissue damage

Hough (1902) observed that the delayed pain was closely associated with mechanical tensions within the muscle and suspected that DOMS was caused by actual ruptures within the working muscle tissue. This view was later supported by Friden et al. (1981) who demonstrated that ultrastructural damage occurred to skeletal muscle in a small proportion of fibres following exercise that involved the muscle lengthening during force development. Later studies (Armstrong et al., 1983; Friden et al., 1983; 1983b; Newham et al. 1983; Jones et al., 1986) have supported this view.

It is no longer a matter of controversy that high force, eccentric contractions results in muscle damage, since electron microscopic examinations clearly show disrupted sarcomeres in myofibrils, Z-line streaming, regions of over-extended or half sarcomeres, t-tubule damage and regional disorganisation of the myofilaments (Proske et al., 2001). What is unclear though, is the relationship between this damage and DOMS since it is known that muscle fibre damage is not a predisposition to muscle pain. Studies on patients with primary muscular diseases, such as Duchenne

muscular dystrophy, have demonstrated that despite major disruptions of the myofibrils and sarcomeres the patients report no pain (Lieber and Friden, 2002). Recently, a poor correlation between pain and observed muscle damage has been found (Nosaka et al., 2002).

Although recent studies would suggest that muscle damage does not directly cause DOMS, it is possible that the damage to the muscle may stimulate a cascade of events that ultimately leads to tissue breakdown products stimulating nocioceptors and resulting in the sensation of pain. It is therefore important to identify the cause of the damage following lengthening muscle actions.

Most of the confusion regarding eccentric exercise-induced muscle damage is due to the delay between the end of the exercise and the appearance of soluble intra-cellular constituents from muscle fibres in the circulation. Studies have shown that there is a delay of 0 to 6 days post-exercise in the appearance of such constituents. One explanation for this delay is the concept of 'micro damage'. Micro damage involves the initial exercise resulting in a small amount of damage, which amplifies over the next few days, eventually reaching a critical amount of damage and ultimately causing muscle necrosis.

Ultrastructural muscle damage is evident immediately post-eccentric exercise, and is presented as Z-disc streaming as well as sarcomeres that have the appearance of being

pulled apart (Jones et al., 1997) despite a lack of soluble constituents in the circulation. This damage is likely to be a result of the mechanical trauma of lengthening exercise, presumably due to the high shearing forces developed within the muscle causing damage to the smaller vessels. According to the concept of micro damage, this relatively small amount of damage could be amplified over the next few days. Three common pathways which may result in this amplification of muscle damage have been described by McArdle and Jackson (1997). There is a loss of intracellular calcium homeostasis, a loss of energy supply to the cell and over-activity of oxidising free radical mediated reactions.

2.2.3.2 Connective tissue damage

Although Hough (1902) suspected that DOMS was a result of muscle tissue ruptures, he also suggested that the connective tissue could be the site of these ruptures. This hypothesis was later supported by Asmussen (1956), Komi and Buskirk (1972) and Abraham (1977). The connective tissue consists of the endomysium, perimysium and epimysium of which the endomysium forms a sheath around individual fibres. It is the endomysium that is thought to be damaged following lengthening exercise (Stauber, 1989).

Pain is frequently reported to be most pronounced at the distal myotendinous junction, leading to suggestions that this is the site of injury. However, skeletal muscle pain is difficult to localise especially since it is known that increasing muscular pain results

in pain referral to other site sites including the fascia, tendons, joints, ligaments or even other muscles (Mense and Stahnke, 1983). It would therefore appear unwise to attempt to speculate the origin of the muscular damage from the site of peak pain.

A more rigorous method of identifying the site of muscle damage is the measurement of hydroxyproline (OHP), which is a breakdown product of connective tissue and an indicator of collagen metabolism. Urinary OHP has been used as a marker of connective tissue damage in studies exploring the aetiology of DOMS, although the results of these studies have been inconclusive. Abraham (1977) reported a positive correlation between OHP excretion and DOMS, whereas Horswill et al. (1988) failed to detect any relationship. Gissal et al. (1983) observed an increase in OHP following DOMS-inducing exercise although this was not statistically significant. It is still unclear if damaged connective tissue is responsible for DOMS although recent studies would suggest that, like muscle damage, it is not the connective tissue damage *per se* that results in pain but the production of tissue breakdown products that may sensitise nocioceptors (Barlas et al. 2000b).

2.2.3.3 Lactate

A common misconception is that lactic acid is responsible for DOMS. Prior to 1956, it was assumed that the accumulation of metabolic waste was involved in the aetiology of DOMS. The observation that concentric exercise involves a higher degree of metabolism at a given workload than eccentric work despite no significant

increase in DOMS (Asmussen, 1956) and that eccentric exercise requires lower energy expenditure than concentric exercise (Dick and Cavanagh, 1987) supports the view that lactic acid is not related to DOMS (Sargeant and Dolan, 1987).

Schwane et al. (1983c) tested the theory relating to lactic acid by running subjects either uphill or downhill at the same speed for forty minutes. Downhill running resulted in the subjects running at a lower $\dot{V}O_2$ and producing less lactic acid whilst exhibiting greater DOMS compared to those who ran uphill. This was later confirmed in a study which demonstrated that uphill running resulted in higher lactate concentrations and lower blood pH lower compared to downhill running despite DOMS only being noted after downhill running (Pyne et al., 1997).

2.2.3.4 Increased local muscle temperature

It has been postulated that eccentric exercise results in local elevations in muscle temperature which in turn could cause structural damage to the muscle, necrosis of muscle fibres and the breakdown of connective tissue (Armstrong, 1984). It is known that the small myelinated and small unmyelinated nerve fibres are particularly sensitive to increases in temperature, and therefore local increases in temperature due to inflammation could stimulate nocioceptors and be the cause of DOMS. This suggestion was initially postulated by Davies and Barnes (1972) and later supported by Nadel et al. (1972), both of whom reported that eccentric exercise results in increased muscular temperature compared to concentric exercise.

2.2.3.5 Involuntary muscle spasm

The involuntary muscle spasm theory was initially proposed by DeVries (1966) following a series of experiments in which EMG was used to measure the electrical activity in painful muscles. The theory is based upon the assumption that eccentric exercise results in temporary ischaemia in the active muscle causing the production of a pain substance. This pain substance stimulates pain endings, which in turn results in more muscular spasms thereby prolonging the ischaemia and initiating a vicious cycle. DeVries (1966) noted that following eccentric exercise there was an increase in electrical activity of the muscle suggesting that tonic muscle spasms were occurring. McGlynn et al. (1979) also demonstrated that there was an increase in electrical activity, as demonstrated through EMG, following eccentric exercise although they could not correlate this increase in electrical activity to the perceptions of pain. In contrast, Abraham (1977) reported no increase in electrical activity following eccentric exercise and thus dismissed the muscle spasm theory. It has been proposed that the different results were due to the different electrodes used, as DeVries used unipolar electrodes whilst Abraham used dipolar electrodes. However, Newham et al. (1983) used the same unipolar electrodes as DeVries and also reported no increase in electrical activity.

Cleak et al. (1992a) stated that the results by De Vries (1966) may be explained by the fact that his subjects did not have classical exercise-induced DOMS, but rather had a wide variety of 'accidentally-induced muscle pain'. In summary, the extant data

regarding the muscle spasm theory is equivocal, although it appears that it is unlikely to be a significant factor in DOMS.

2.2.3.6 Inflammation

Following high force eccentric exercise there is enhanced recruitment of leukocytes into the circulation, along with inflammation and subsequent tissue oedema compared to concentric exercise (Pizza et al., 1995). There is growing evidence that DOMS results from this post-exercise inflammatory response (MacIntyre et al., 1996; 2000). The leukocytosis is predominantly a result of transient neutrophilia that normally occurs within the first few hours post-exercise. Some authors had previously reported no change in leukocyte numbers (Bobbert et al., 1986; Schwane et al., 1983b;) although it must be recognised that these studies used a manual counting technique, and also since their first post-exercise sample was 24 hours post-exercise, it is likely that they missed the early leukocytosis (0-6 hrs post exercise). It is now generally accepted that eccentric exercise results in greater leukocytosis than concentric exercise when performed at the same relative exercise intensity (Malm et al., 1999; Smith et al., 1998).

Post-exercise leukocytosis following eccentric exercise initially presents through mobilisation of neutrophils and monocytes into the circulation. Cannon et al. (1994) reported a twofold elevation in circulating neutrophil numbers following downhill running. The accumulation of bradykinin, histamine and prostglandins at the injury

site along with fragments of the damaged skeletal muscle attracts circulating neutrophils and monocytes to the injury site. The accumulation of phagocytes within the injured muscle is presumed to be involved in the degradation of damaged lipid and protein structures. Once the neutrophils are primed at the injury site, they exhibit an oxidative burst that involves the generation of free radicals. It is still of interest if this oxidative burst causes further damage to lipid membranes and intensifies the pain or if it simply assists in the degradation and removal of damaged lipid and protein structures with no significant detrimental effect to the surrounding muscle. This will be discussed in detail in section 2.6.

Along with the accumulation of phagocytes within the muscle there is also infiltration of protein-rich fluid into the muscle that together increases osmotic pressure. It has been proposed that it is the increased osmotic pressure that activates type III and IV nocioceptors within the muscle causing the sensation of DOMS. The accumulation of phagocytes at the site of injury 24-48 hours post exercise has been suggested to sensitise type III and IV nocioceptors and results in DOMS (Smith, 1991).

The theory that increased osmotic pressure caused by inflammation is responsible for DOMS was tested by Mense and Stahnke (1983). The study involved factors that are presumed to be involved in the inflammatory response, including bradykinin, 5-hydroxytryptamine and prostaglandins being injected into the hind limb of cats and subsequently measuring the electrical discharge from isolated nocioceptors. These injections resulted in increased electrical output confirming that inflammation could

be responsible for DOMS. However, it must be noted that this procedure was carried out on cats, and caution must be taken when extrapolating such information into humans.

If inflammation was responsible for DOMS, it would seem logical that administration of anti-inflammatory drugs would provide some degree of attenuation of the magnitude of pain. Numerous studies have examined the effect of non-steroidal anti-inflammatory drugs (NSAID) on DOMS. NSAIDs prevent the production of prostaglandins and endoperoxides by inhibiting arachidonic acid production. This reduced inflammation results in a lesser degree of oedema, combined with lower intra-muscular pressure, and if these are causative factors in DOMS, such drugs should reduce DOMS. Although the role of NSAIDs on DOMS has been extensively researched (Donnelly et al., 1988; Donnelly et al., 1990; Gullick et al., 1996; Hasson et al., 1992; Hasson et al., 1993; Jansen et al., 1983; Pizza et al., 1999) the results are equivocal. Hasson et al. (1992) reported significantly less DOMS 48 hours post exercise following the therapeutic and prophylactic administration of ibuprofen compared to placebo administration whilst other studies have reported no beneficial effects of NSAID supplementation (Donnelly et al., 1988; Donnelly et al., 1990; Gullick et al., 1996).

Inconsistencies in the findings have been attributed to the timing and dosage of the drug administration (Cheung et al., 2003). Since in most studies subjects were given a therapeutic as opposed to a prophylactic dose of NSAIDs, it is likely that the

inflammation will have already occurred prior to the administration of the drug. To investigate this, Hasson et al., (1993) compared the effects of a prophylactic with a therapeutic dose of ibuprofen and reported that the prophylactic dose resulted in the greatest attenuation in DOMS.

Although there are inconsistencies in the results, there does appear to be enough evidence to suggest that inflammation plays a role in the process of DOMS and thus warrants further investigation. It is still unknown if the 'oxidative burst' associated with phagocytes plays a physiological or pathophysiological role and this too requires more research.

The only accepted consensus regarding DOMS is that it is unlikely that one single mechanism can be attributable to the onset of DOMS, and thus various integrated models have been proposed (Armstrong, 1984; Armstrong, 1990; Smith and Jackson, 1990; Smith, 1991; Cheung et al., 2003). Figure 2.1 is an integration of these models.

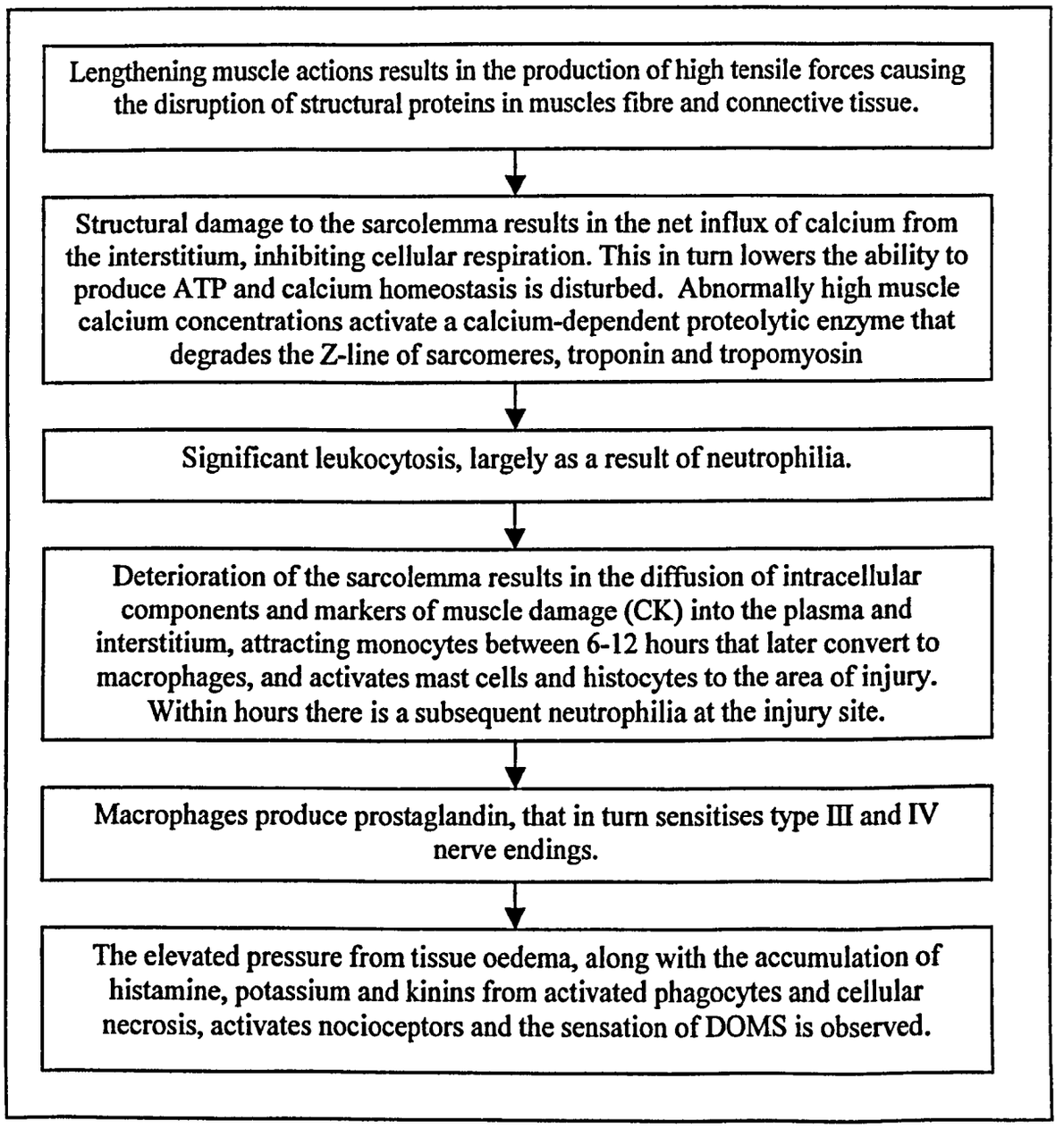


Figure 2.1 Integrated model of the current thoughts regarding the aetiology of DOMS.

Although Figure 2.1 encompasses all of the most recent models proposed, it must be stated that the sequence of events is hypothetical, and more research is needed to substantiate it. It would appear that although muscle damage clearly occurs following eccentric exercise, this muscle damage may not directly cause the sensation of pain.

The observed muscle damage results in subsequent elevations in intra-muscular pressure caused through tissue oedema along with the accumulation of histamines, potassium and kinins, which in turn may activate nocioceptors giving the sensation of pain. Therefore muscle damage could trigger the cascade of events that ultimately results in DOMS.

In spite of several recent investigations, the exact mechanism for this damage and its relationship with DOMS still remains unresolved (Nosaka et al., 2002). The most recent suggestion is that free radicals may be involved in exercise induced muscle damage either initiating or propagating the damage (Fantone, 1985), although current findings are equivocal. Research should focus on investigating and validating the biochemical events that occur in the proposed models. The study of free radical production following unaccustomed exercise is relatively new and provides an alternative mechanism for the post-exercise muscle damage and associated DOMS. The chemistry and involvement of free radicals in DOMS will be discussed in more detail in section 2.4 of this review.

2.2.4 Symptoms and measurement of DOMS

2.2.4.1 Pain

There are two types of pain apparent following exercise, these being an immediate onset and a delayed onset muscular pain. DOMS is specifically concerned with the

second type and it is this type that will be addressed in this thesis. The pain associated with DOMS is most pronounced at the distal myotendinous junction, and has been described as a dull ache that increases in intensity (Hough, 1902). Muscles are painful when palpated or stretched, although there is generally little pain when individuals are resting. For this reason, the pain is often compared to that of a bruise (Jones et al., 1997). The pain develops in the first 24 hours post exercise and increases in intensity usually peaking between 48 and 72 hours post exercise. In most cases the pain has passed by days 5-7 post exercise, usually without any intervention (Lieber and Friden, 2002).

In order to understand the cause and nature of the pain associated with DOMS, it is essential to accurately and reproducibly quantify it. The accurate quantification of pain presents multiple problems since it is inherently subjective and has proven difficult to accurately measure. Studies on animals have overcome this by recording characteristics from the afferent nerve and measuring the firing frequencies in response to graded stimuli (Mense and Stahnke, 1983), However this technique is not possible with human subjects. Human studies have had to rely on asking the subject to report the amount of pain present. This has several drawbacks, none more so than it relies on the subjects being able to accurately quantify a feeling (pain) into words.

Many studies have used a numerical scale to assess DOMS, asking subjects to rate their pain from 0, representing no pain, to 10 representing unbearable pain (MacIntyre et al., 1996). However this method looses accuracy due to the divisions being too

wide to distinguish one level of pain from another. Recent studies have used a visual analogue scale (VAS) in which subjects indicate the level of pain as the distance along a continuous line, with one end of the line representing no pain and the other end unbearable pain (Mattacola et al., 1997; Lenn et al., 2002; Nosaka et al., 2002).

Another scale that has received attention is a Graphic Rating Scale (GRS) (De Conno et al., 1994; Denegar et al., 1988; Denegar et al., 1989; Freyd, 1923). The GRS is similar to the VAS except that it has descriptors placed at equal intervals along the base of the scale, these usually being 'No Pain', 'Dull Ache', 'Slight Pain', 'More Slight Pain', 'Painful', 'Very Painful', and 'Unbearable Pain'. Heft and Parker, (1984) believed that the descriptors used in a GRS lacked sufficient sensitivity to measure the pain experienced, whilst Ohnhaus and Adler (1975) felt the scale lost accuracy by forcing the subject to transform feelings into words. Mattacola et al. (1997) compared the use of a VAS with a graphic rating scale (GRS) for the assessment of pain following DOMS inducing exercise. They reported that providing the subject is familiar with the scales, there was no significant difference between the two. The study also highlighted the importance of familiarisation with either scale and showed that there is a learning effect associated with these measures.

The pain associated with DOMS is similar to that of a bruise and as such it is most noticeable when palpated. Some studies have therefore used pressure tests on or around the affected muscles to quantify the amount of pain (Ritchie et al., 1968; Smith et al., 1994b). This usually involves a fixed weight being applied to the muscle

and the subject reporting how painful the feeling is. It has been documented that there is a need to produce a more reliable and reproducible measurement of pain to pressure application. The pressure algometer is a tool that has been reported to do this, being able to accurately quantify local tenderness on pressure (Hogeweg et al., 1992). The pressure algometer is able to show the amount of pressure applied needed to induce a painful sensation and recent studies have used such a device as a more accurate assessment of muscle pain (Bailey et al., 2000; Barlas et al., 2000a).

Baker and Kelly (1996) investigated site selection for pressure tests following eccentric exercise. They examined 27 sites on the quadriceps muscle using a datum line from mid-patella to lateral iliac crest. The study highlighted two important methodological issues involved in pressure tests. Initially it was reported that some sites were more sensitive to pressure-induced pain and therefore if multiple measurements are to be taken on consecutive days, the identical site must be identified and used repeatedly throughout the trial. A second noteworthy finding was that multiple measurements at one site increased the sensation of local pain. It was therefore recommended that only one measurement should be taken at each site in any one test-session.

2.2.4.2 Impairment of muscle function

DOMS-inducing exercise has repeatedly been shown to cause significant reduction in muscle function, presenting as a loss of static and dynamic muscle force development

characteristics, reduced range of motion, or simply an inability to perform an activity or function within the range considered normal (Cheung et al., 2003).

The assessment of losses of muscle force is common in studies on DOMS and is used as a relatively simple and non-invasive method of assessing muscle damage (Warren et al., 1999). The extant literature has tended to use both the terms strength and force and, therefore in this review force will be synonymous with strength.

Force losses have been frequently demonstrated during DOMS (Clarkson et al., 1992; Cleak et al., 1992b; Donnelly et al., 1990; Eston et al., 1999; 2000; Paddon-Jones et al., 2000) although the magnitude of force losses and the length of time these losses occur are somewhat equivocal.

Following eccentric exercise there is an immediate loss of muscle force which is often attributed to fatigue (Armstrong, 1984). This is followed by a delayed loss of force, which cannot be accounted for by fatigue. It has been demonstrated that losses of muscle force during DOMS can be as great as 60% and these are not always fully restored until 8-10 days post-exercise (Clarkson et al., 1992). The reductions in force are most notable following lengthening muscle actions, although they are still significantly reduced after concentric and isometric contractions. Likewise, the duration of reductions in strength are also greater following eccentric exercise than concentric or isometric exercise. Concentric muscle force has been shown to recover

within 4 days whereas eccentric strength took as long as 10 days post exercise (Evans et al., 1990).

It is generally accepted that there are both intrinsic and extrinsic mechanisms involved in strength losses following DOMS. Extrinsic factors include a reduction in the voluntary effort to produce force due to the increased pain during contraction, whereas intrinsic factors include actual damage to the sarcoplasmic reticulum (Dop-Bar et al., 1997) as well as overstretching of the sarcomeres, reducing the number of cross bridges that can be formed (Newham, 1988). This suggestion that overstretched sarcomeres are responsible for the loss of force is supported by the fact that the greatest losses of strength are exhibited by exercise at longer muscle lengths (Newham, 1988).

It is believed that the intrinsic factors result in the most significant losses of force following DOMS. This is suspected since when the extrinsic factors are removed through percutaneous electrical myostimualtion (PES), losses of force are still significant (Brown et al., 1996; Sargeant and Dolan 1987).

Comparisons between findings in the literature are difficult, largely due to the varying methods of muscle force assessment that have been employed as well as the various time courses that measurements have been taken. Studies have utilised isokinetic/isometric dynamometry (Eston et al., 1995; Eston and Peters, 1999; Paddon-Jones et al., 2000), maximum concentric exercise with free weights (Smith et

al., 1994a), vertical jump height (Farr et al., 2002) and PES (Brown et al., 1996) to demonstrate torque changes following eccentric exercise.

Although there is no consensus as to the best method of assessing loss of force associated with DOMS, in a comprehensive review of the measurement tools used in DOMS research Warren et al. (1999) reported that Maximum Voluntary Contraction (MVC) assessment using an isokinetic dynamometer was the most effective method. It was reported that MVC assessment provided a reliable and reproducible assessment, as well as being highly related to the process of tissue injury and repair.

Muscle function impairment also presents as a reduced range of motion (ROM), demonstrated in terms of an inability to fully flex the joint without pain and a rise in passive tension. Reduced ROM has been shown to occur immediately post-exercise, lasting for up to 10 days post exercise (Clarkson et al., 1992). Reduced ROM is usually described in terms of decreased muscle shortening ability, (Clarkson et al., 1992). It has been postulated that this reduced ROM may be a consequence of overstretched sarcomeres (Kansky et al., 1996) since overstretched sarcomeres may prevent the actin and myosin filaments from producing maximum overlap and ultimately reducing the ability to fully contract the muscle.

An alternative mechanism for reduced ROM involves the disturbance in calcium homeostasis. It is possible that if there is a calcium deficiency in the sarcoplasmic

reticulum, there may also be insufficient calcium to support continuous cross bridge cycling necessary for complete muscle fibre shortening. Howell et al. (1993) speculated that the reduced ROM is due to oedema occurring in the perimusular connective tissue.

Along with a reduced ability to fully flex the joint, reduced ROM also involves a rise in passive tension, this being the relaxed limb adopting and retaining a slightly flexed posture. Howell et al. (1993) reported that passive tension more than doubled following lengthening exercise and remained like this for 4 days post-exercise. The exact mechanism for this is not clear, although similar mechanisms responsible for the inability to fully flex the joint have been proposed.

2.2.4.3 Muscle Damage

Although DOMS is not strictly a symptom of muscle damage, a strong association between muscle damage and DOMS has resulted in the assessment of muscle damage being used as a marker. The muscle damage is typically observed as Z-line streaming, A-Band disruption and mitochondrial abnormality (Friden and Lieber, 1992).

The only true method of assessing muscle damage involves histological verification using an electron or light microscope to visualise the damage and then express this in

quantitative terms. Strictly speaking, this is the only method that allows the justification of the term muscle damage. Histological verification is often reserved for animal studies (Jones et al., 1986) yet there have been studies using a human model (Friden et al., 1983).

Animal studies usually involve the entire muscle being analysed for damage, so ensuring that the site of muscle damage is not missed. This is not possible in human studies and therefore histological verification usually involves muscle biopsies being taken at varying time points before and following the exercise. It is accepted that muscle damage is not distributed homogenously in a given muscle and it is therefore possible that the site of biopsy may not reflect the true amount of damage in the entire muscle. This is demonstrated through studies indicating that more damage is observed in the distal part of the soleus muscle of rats than the proximal part.

Human studies have utilised a variety of methods in an attempt to quantify muscle damage, including the increase in activity of intramuscular proteins in the circulation (Clary and Schwane 1988; Giamkberardino et al., 1996), myosin heavy chain release (Sorichter et al., 2001a), magnetic resonance imaging (Foley et al., 1999), and assessment of muscle function (Lowe et al., 1995).

The most common method of assessing muscle damage following DOMS is the measurement of plasma CK activity. CK is a dimeric enzyme that catalyses the

phosphorylation of ADP to ATP and free creatine. Due to the large molecular weight of CK (~80,000 Da) it cannot pass directly into the circulation. Following skeletal muscle injury, CK passes into the lymph *via* the interstitial fluid and empties into the general circulation (Hortobagyi et al., 1989), and therefore the appearance of CK in the circulation has been used as an indirect marker of skeletal muscle damage.

Although many studies have used CK as an indicator of sarcolemmal permeability (Sorichter et al., 1999; Triffletti et al., 1988; Wojcik et al., 2001), other studies have questioned its use (Nosaka and Clarkson, 1995; Warren et al., 1999). Hortobagyi et al. (1989) reported that CK varies with age, gender, body mass and exercise modality. More importantly it was also reported that the measurement of circulating CK not only reflects CK release into circulation but also clearance from circulation, and thus is affected by changes in blood flow and plasma volume changes. Despite these limitations, CK is still useful in indicating if muscle damage has occurred and is therefore often used as a relatively easy and non-invasive marker of muscle damage following eccentric exercise.

Rodenburg et al. (1993) investigated the inter-relationship between the various 'markers' of DOMS, specifically CK, ROM and MVC. They reported that there was no significant correlation between the measures suggesting that these outcomes are separate components of the process. Likewise, there was no correlation between any of these measurements and DOMS. Recently Nosaka et al. (2002) reported that DOMS did not correlate with other measures of muscle damage and concluded that

DOMS does not reflect the magnitude of exercise-induced muscle damage. Warren et al. (1999) examined the measurement tools used in lengthening muscle action-induced injury and stated that the measurement of blood levels of myofibre proteins should be discouraged for purposes of quantifying muscle injury and/or functional impairment. The authors stated that functional measures such as losses of MVC provided the best non-invasive method of assessing muscle damage, supporting the findings of McCully and Faulkner (1986) who reported a direct correlation between histological verification of muscle damage and loss of maximum force.

2.3 Free Radicals

2.3.1 The chemistry of oxygen

The constant need for oxygen by animals to survive obscures the fact that oxygen is a toxic mutagenic gas (Halliwell and Gutteridge, 1999). In air, oxygen exists as a diatomic molecule at 21%, and strictly should be termed dioxygen (Radak, 2000). In its ground state, dioxygen has two unpaired electrons with identical spins in its outer shell (Halliwell and Gutteridge, 1999). This results in the reduction of oxygen being more complicated than most other elements. According to Pauli's principle, oxygen in this state cannot be reduced in one step and therefore the univalent reduction of oxygen (the reduction of oxygen one electron at a time) occurs (Radak, 2000). The univalent reduction of oxygen can result in the production of reactive intermediates, these being known as 'free radicals' (Halliwell, 1994).

2.3.2 Definition and formation of free radicals

A free radical can be defined as any species capable of independent existence (hence the term free) that contains one or more unpaired electrons (Aruoma, 1994). An unpaired electron indicates one that occupies an atomic or molecular orbital by itself (Halliwell and Gutteridge, 1999) and is conventionally depicted as a heavy superscript dot ($R^{\bullet}$). Free radicals are formed in one of three ways:

1. The addition of a single electron to a non-radical molecule ($A + e^- \rightarrow A^{\bullet-}$).
2. The loss of a single electron from a non-radical ($A - e^- \rightarrow A^{\bullet+}$).
3. Homolytic fission, i.e. the cleavage of a covalent bond resulting in one electron from the pair shared remaining with each atom ($A : B \rightarrow A^{\bullet} + B^{\bullet}$). (Halliwell and Gutteridge, 1999).

Electron transfer (addition or loss of a single electron) is more common than homolytic fission due to the high energy input that is required for homolytic fission to occur (usually through extreme heat, UV light or ionising radiation), (Cheeseman and Slater, 1993). Free radicals are chemically highly reactive because the unpaired electron attempts to stabilize itself by pairing with another electron (Halliwell, 1994). The extent of the reactivity depends upon what the radical is presented with. If the radical meets another radical, a termination reaction occurs and the two radicals become a non-radical ($A^{\bullet} + B^{\bullet} \rightarrow AB$) (Matsuo and Kaneko, 2000). However, if a radical meets a non-radical, a new radical is formed, thus producing a radical chain ($A^{\bullet} + B \rightarrow A + B^{\bullet}$) (Cheeseman and Slater, 1993). This is the most likely scenario since most biological molecules *in vivo* are non-radicals (Matsuo and Kaneko, 2000). The radical chain continues until a termination reaction takes place (Cheeseman and Slater 1993). The termination reaction can be either when the radical meets another radical, or when it meets an antioxidant (any product that prevents damage by molecular oxygen). Antioxidants will be discussed in more detail in section 2.5.

2.3.3 Terminology in the study of free radicals.

Reactive Oxygen Species (ROS) is a term used in the study of free radicals to include both oxygen centred radicals such as the superoxide anion radical ($O_2^{\bullet-}$), nitric oxide (NO), peroxyl ($RO_2^{\bullet}$) and hydroxyl radical ($^{\bullet}OH$) as well as some non-radical derivatives of $O_2^{\bullet-}$ such as, hypochlorus acid (HOCl), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and ozone (O_3) (Halliwell, 1994). Table 2.6 shows examples of ROS and their major sources. The generation of ROS causes 'oxidative stress', which is defined as the harmful influence of *in vivo* oxidation due to an alteration in the bodies' antioxidant:oxidant ratio (Matsuo and Kaneko, 2000). Since this thesis is concerned with the combined effects of radicals and non-radical derivatives, the term ROS will be used throughout.

Table 2.5 Examples of major reactive oxygen species in skeletal muscle and their primary source of origin. (Taken from Lawler and Powers, 1998)

2.3.4 Biological activity of reactive oxygen species (ROS)

ROS are capable of attacking all of the major classes of biomolecules although lipids are particularly susceptible (McBride and Kraemer, 1999). Since cell membranes are rich sources of polyunsaturated fatty acids (PUFA), cell membranes are readily attacked by free radicals (Cheeseman and Slater, 1993). The destruction of PUFA (lipid peroxidation) is said to be especially damaging as it proceeds as a self-perpetuating chain reaction (Halliwell and Gutteridge, 1999). Lipid peroxidation always results in the formation of reactive aldehydes and these aldehydes can diffuse from the original site of damage to other parts of the cell. Consequently ROS have the ability to cause skeletal muscle damage at either the site of origin or elsewhere (Matsuo and Kaneko, 2000)

2.3.5 Potential sources of ROS *in vivo* – implications of eccentric exercise

There are a number of potential sites for ROS generation *in vivo* and muscle in particular is a potentially significant source of ROS, especially during exercise. These sites include the mitochondrial electron transfer system, xanthine oxidase within endothelial cells, and infiltrating phagocytes (Jackson and O'Farrell, 1993).

2.3.5.1 Mitochondrial generation of ROS

Oxidative phosphorylation takes place in the mitochondria where molecular oxygen undergoes a four-electron reduction catalysed by cytochrome oxidase. It has been

estimated that approximately 95% of the total oxygen consumption is reduced in this way (Halliwell, 1994). The remaining 5% may undergo a one-electron reduction, resulting in the formation of $O_2^{\bullet-}$. If $O_2^{\bullet-}$ then undergoes a further one electron reduction, H_2O_2 is produced (Cheeseman and Slater 1993). Therefore, increased oxygen flux through the mitochondria electron transport chain could result in the increased production of ROS. The mitochondria have a well-developed system for protection against ROS, possessing a specific mitochondrial superoxide dismutase (MnSOD) to prevent against $O_2^{\bullet-}$ mediated degeneration of biomolecules (McCord, 1985).

In relation to DOMS, it should be noted that this pathway for ROS production is highly associated with increases in aerobic metabolism (Lovlin et al., 1987). It is known that eccentric muscle actions are metabolically less demanding than concentric exercise despite resulting in greater DOMS (Newham et al., 1983). It would therefore appear unlikely that mitochondrial generation of $O_2^{\bullet-}$ is responsible for initiating DOMS. It is feasible however, that mitochondrial damage as a result of the eccentric contractions could result in $O_2^{\bullet-}$ leakage and subsequent free radical damage, although as yet this has not been fully investigated.

2.3.5.2 Xanthine oxidase derived generation of ROS

Xanthine oxidase is localised in the capillary endothelium of most human tissues including skeletal muscle where it serves to catalyse the oxidation of hypoxanthine to xanthine as well as the oxidation of xanthine to uric acid (Sjodin et al., 1990).

McCord (1985) proposed that during ischaemia there is an activation of a calcium dependent protease that results in the formation of xanthine oxidase from xanthine dehydrogenase as well as the breakdown of ATP with the formation of AMP *via* the adenylate kinase reaction. AMP is then further metabolised to hypoxanthine, this being a substrate for xanthine dehydrogenase and xanthine oxidase. Xanthine oxidase may then use molecular oxygen as an electron acceptor resulting in the formation of xanthine and $O_2^{\bullet-}$.

Jackson (1998) highlighted that for this to occur in exercising muscle three important events must transpire:

1. There must be a significant presence of the enzyme xanthine dehydrogenase/oxidase in the skeletal muscle.
2. There must be a failure in calcium homeostasis to stimulate the calcium-activated protease, and
3. The substrate hypoxanthine must be produced in significant amounts.

Most human tissue has relatively little xanthine oxidase compared to other species, although in skeletal muscle the vascular endothelium is rich in xanthine dehydrogenase which can readily be converted to xanthine oxidase during exercise (McCord and Fridovich, 1969). This provides a potential source for $O_2^{\bullet-}$ production in close proximity to skeletal muscle tissue (Jackson, 1998). Evidence demonstrating that calcium homeostasis is disturbed during exercise is unequivocal (McArdle et al., 2002; McArdle and Jackson, 1997; Jackson et al., 1991) whilst Hellsten-Westing et al. (1993) demonstrated that during high intensity exercise hypoxanthine is readily

released from the exercising muscle to the serum and could therefore provide the necessary substrate for xanthine oxidase within the capillary endothelium (Jackson, 1992).

The above suggests that intramuscular xanthine oxidase could play a key role in $O_2^{\bullet-}$ generation following exercise. It is interesting to note that increased xanthine oxidase activity within the muscle is particularly pronounced following eccentric exercise (Hellsten et al., 1997). Furthermore, it is clear that calcium homeostasis may also be especially disturbed following eccentric exercise (Friden and Lieber, 1996). This would suggest that if there is an increase in ROS production following eccentric exercise, then the xanthine oxidase pathway may be responsible.

2.3.5.3 Leukocyte derived generation of ROS

Several cells in the immune system can produce large quantities of ROS, these include monocytes/macrophages, eosonophils and neutrophils, and interestingly all of these cells demonstrate significant activation during and following eccentric exercise (Malm et al., 1999). Such radicals include $O_2^{\bullet-}$ which can then dismutase into H_2O_2 and then be catalysed into $^{\bullet}OH$ *via* Fenton chemistry (see page 57). The ability of neutrophils to produce ROS and specifically target these against microorganisms is essential in host defence (Smith, 1997). ROS are produced by neutrophils to attack bacteria, viruses and, in the case of exercise-induced muscle damage, to attack degenerated cells (Pyne, 1996). Studies on patients with chronic granulomatous disease (CGD) highlight the importance of neutrophil-derived ROS in host defence.

These patients have genetically defective oxidases that cannot produce $O_2^{\bullet-}$ and consequently H_2O_2 in significant quantities. The neutrophils from these patients show a diminished capacity to deal with and kill bacteria *in vitro* and simply adding H_2O_2 to them restores their bactericidal activity (Halliwell and Gutteridge, 1999).

Following eccentric exercise there is a significant increase in circulating leukocytes (Malm et al., 1999). Chemotactic factors are thought to initiate the invasion of circulating neutrophils into the muscle tissue (Ortega et al., 1993). Such factors include cytokines (TNF-α, IL-1, and IL8) as well as the damaged muscle tissue itself. Basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) are thought to be liberated from damaged skeletal muscle and act as potent chemoattractant agents (Niess et al., 1999).

Once inside the muscle tissue, and when the neutrophils are exposed to a phagocytic stimulus, there follows an 'oxidative' or 'respiratory' burst which is characterised by an increase in oxygen consumption (Halliwell and Gutteridge, 1999). In this oxidative burst, molecular oxygen is initially reduced to $O_2^{\bullet-}$ by the activation of the NADPH oxidase and then further reduced to H_2O_2 (Pyne, 1996). Catalysed by myeloperoxidase (MPO), hypochlorous acid (HOCl) may then be subsequently formed from H_2O_2 (Pyne, 1996). Further reactions can then result in the production of the highly reactive $^{\bullet}OH$ either through Fenton chemistry (see page 57) or from the reaction of $O_2^{\bullet-}$ with HOCl (Niess et al., 1999).

The ROS that are produced through the oxidative burst can react with unsaturated lipids, initiating lipid peroxidation reactions in target cell membranes, specifically assisting in the destruction and removal of damaged or necrotic cells (Moncado and Higgs, 1993). However, it is important to consider that although ROS are only produced at the interface of the phagocyte plasma membrane, it is inevitable that some degree of unwanted leakage of H_2O_2, and $O_2^{\bullet-}$ into surrounding cells occurs that may result in the peroxidation of non-damaged lipid membranes and further muscle damage (Cheeseman and Slater 1993). Moreover, Hellsten et al. (1997) reported that invading leukocytes contain xanthine oxidase. Therefore, the invasion of leukocytes into muscle tissue will increase the level of intramuscular xanthine oxidase, further contributing to increased $O_2^{\bullet-}$ generation after eccentric muscle actions.

To examine the effects of neutrophils on muscle damage, Pizza et al. (2001) cultured skeletal muscle myotubes and subsequently cultured them with human neutrophils. It was reported that human neutrophils injured skeletal myotubes *in vitro* suggesting that neutrophils may exacerbate muscle injury and delay muscle regeneration *in vivo*.

Although it appears likely that leukocyte-derived ROS are produced following eccentric exercise, it must be noted that production of ROS through this pathway is secondary to the muscle damage and thus this pathway cannot be the cause of the initial muscle damage. This secondary ROS production however may result in further lipid peroxidation leading to increased muscle-damage, which could theoretically

result in DOMS. Therefore, attenuation of this secondary ROS production through antioxidant supplementation could prevent the secondary damage and attenuate DOMS. Conversely, the secondary ROS production may be necessary to deal with the initial insult of the eccentric exercise induced muscle damage and it is feasible that antioxidant supplementation will compromise the function of the neutrophils, delaying the recovery from the damage. However, as yet, these hypotheses have yet not been fully tested.

2.3.6 Potentially important ROS in DOMS

There are many different ROS produced *in vivo*, all with varying degrees of toxicity or reactivity. Furthermore, it must be noted that some ROS are metabolically useful in controlled amounts. Perhaps the most significant ROS in an exercise setting especially in relation to exercise-induced muscle damage and DOMS are the superoxide anion radical ($O_2^{\bullet-}$), the hydroxyl radical ($^{\bullet}OH$), hydgrogen peroxide (H_2O_2), and nitric oxide (NO) (Reznick et al., 1998).

2.3.6.1 Superoxide anion ($O_2^{\bullet-}$)

$O_2^{\bullet-}$ is the one electron reduction product of oxygen and is formed in almost all aerobic cells (Halliwell, 1994). It is produced deliberately by phagocytes, assisting in the inactivation of viruses and bacteria (McBride and Kraemer, 1999), although $O_2^{\bullet-}$

can also be formed from 'accidents of chemistry' (Halliwell, 1998). A major cellular source of $O_2^{\bullet-}$ is electron leakage from the mitochondria and endoplasmic reticulum (Halliwell and Gutteridge, 1999). In aqueous solutions $O_2^{\bullet-}$ is not particularly reactive although it is dangerous to biological systems as it can be easily converted to more potent ROS, for example, $O_2^{\bullet-}$ is readily dismutased to H_2O_2 by superoxide dismutase and spontaneous dismutation that can then be converted to the extremely potent $^{\bullet}OH$ (Cheeseman and Slater, 1993).

2.3.6.2 Hydrogen peroxide (H_2O_2)

H_2O_2 is not strictly a free radical although it can react to generate more reactive free radicals. *In vivo*, most of the H_2O_2 generated is produced enzymatically by the dismutation of $O_2^{\bullet-}$,: -

$$2O_2^{\bullet-} + 2H^+ \xrightarrow{\textit{Superoxide Dismutase}} \mathbf{H_2O_2} + O_2$$

H_2O_2 can also be produced by oxidase enzymes *in vivo* including xanthine oxidase (section 2.3.5.2). In its molecular structure, H_2O_2 resembles water and is extremely diffusible within and between cells (Jackson, 2003) (see figure 2.2). Although H_2O_2 is a weak oxidising agent, and has been described as less reactive than $O_2^{\bullet-}$ (Reid, 2001a), it can readily convert to potent radicals such as $^{\bullet}OH$ and is therefore extremely cytotoxic (Halliwell, 1998).

2.3.6.3 Hydroxyl Radical ($^{\bullet}OH$)

$^{\bullet}OH$ is one of the most reactive of all known ROS (Matsuo and Kaneko, 2000). It was originally suspected that $^{\bullet}OH$ was formed directly by the reaction of $O_2^{\bullet-}$ with H_2O_2 in a reaction termed the iron catalysed Haber-Weiss reaction:

$$O_2^{\bullet-} + H_2O_2 \xrightarrow{Fe\text{–}Catalysed} {}^{\bullet}\mathbf{OH} + OH^- + O_2$$

However it has since been shown that the rate constant for this reaction in aqueous solutions is virtually zero (Halliwell, 1994). In *vivo* $^{\bullet}OH$ is more likely to occur via Fenton chemistry as seen below:-

$$O_2^{\bullet-} + Fe^{3+} \rightarrow Fe^{2+}$$

$$Fe^{2+} + H_2O_2 \rightarrow {}^{\bullet}\mathbf{OH} + OH^- + Fe^{3+}$$

$^{\bullet}OH$ radicals have an extremely short half-life (10^{-9} seconds) and thus do not migrate any significant distance within the cell (Reznick et al., 1998). Therefore, when $^{\bullet}OH$ is formed *in vivo* it reacts with and damages any molecule in its immediate vicinity including proteins, carbohydrates, DNA and lipids (Cheeseman and Slater, 1993).

2.3.6.4 Nitric Oxide (NO)

NO is synthesised from the amino-acid L-arginine by vascular endothelial cells, certain cells in the brain, and phagocytic cells (Moncado and Higgs, 1993). It is

continuously generated in skeletal muscle with resting muscle producing low quantities of it and this increasing during contractile activity. Unlike $^{\cdot}OH$ which is always harmful, NO may often be useful in skeletal muscle. NO is a vasodilator and an important neurotransmitter (Reid, 2001b) as well as being involved in the killing of parasites by macrophages (Reid, 2001b). However, over production of NO can be cytotoxic both directly, and by its reaction with $O_2^{\cdot-}$ to form peroxynitrite ($ONOO^-$) a ROS that is more potent than either of its parent species (Halliwell, 1994).

Mitochondria

Figure 2.2 Proposed pathway of ROS formation in skeletal muscle, COQ = Coenzyme Q, $O_2^{\cdot-}$ = superoxide, Mn SOD = manganese superoxide dismutase, Cu,Zn SOD = copper zinc superoxide dismutase, GPx = glutathione peroxidase, H_2O = water, H_2O_2 = hydrogen peroxide, nNOS = neural type nitric oxide synthase, NO = nitric oxide, $^{\cdot}OH$ = hydroxyl radical, Ec SOD = extracellular superoxide dismutase, Fe = iron and $L^{\cdot}$ = long lived lipid radicals. (Personal communication Prof M.J Jackson, Dept of Medicine, Liverpool University).

2.4 Measurement of ROS

Many of the discrepancies in the literature regarding ROS production and DOMS can be attributed to the difficulties and inaccuracies in the measurement of ROS. It is difficult to directly detect ROS in biological systems, largely because they exist in low concentrations and react almost immediately at their formation sites, and therefore do not accumulate (Matsuo and Kaneko, 2000).

There are currently many methods used to identify the production of ROS, although no single method is applicable to all situations. Jackson (1999) stated that since there is no gold standard assay of ROS activity, where possible research should adopt a multi-assay approach, specifically using:-

1. the measurement of endogenous antioxidant levels,
2. the measurement of indicators of ROS activity such as products of lipid peroxidation, and where possible
3. the measurement of direct indicators of free radical activity using electron spin resonance spectroscopy (ESR).

The above methods should only be used to detect an increased presence of ROS and not be used to state whether this increase is primary or secondary to the physiological process. This information can only be gained by subsequent therapeutic interventions, often involving the administration of appropriate antioxidants (Jackson, 1999). It is

possible to postulate the involvement of ROS in the toxicity of many compounds purely on the basis that unspecific measurements reveal ROS appearance at the same time as the toxicity, whereas in many cases the appearance of ROS is secondary to the initial event, a consequence rather than the cause (Cheeseman and Slater, 1993). Data that has attributed ROS as a causative factor, purely on its appearance at a corresponding time, should therefore be treated with caution.

2.4.1 Assessment of lipid peroxidation

Lipid peroxidation (LP) is the oxidative deterioration of polyunsaturated lipids (Halliwell and Gutteridge, 1996). LP is arguably the most widely studied aspect of exercise-induced oxidative stress, largely because peroxidation of membrane lipids is a recognised phenomenon following ROS attack.

LP is initiated by ROS rapidly abstracting hydrogen atoms from esterified polyunsaturated fatty acids found inside the cell membranes and lipoproteins, as well as free fatty acids, forming a carbon centred radical (Oh-ishi et al., 2000). This results in the propagation of peroxidation and the formation of other secondary lipid-derived free radicals, such as peroxyl or alkoxyl radicals. This self-perpetuating chain reaction stops when the radical in halted by a low molecular weight antioxidant, or one radical colliding with another allowing the electrons to pair off and stabilise.

Membrane lipid peroxidation results in the formation of several products including the aldehydes, MDA and 4-hydroxynonenal, conjugated dienes, and lipid hydroperoxides. It is thought that the order of production is firstly conjugated dienes, then lipid hydroperoxides, and finally MDA (Kneepkens, 1994). Furthermore, the volatile hydrocarbons, ethane and pentane, have also been studied in the breath of exercising subjects.

2.4.1.1 Malondialdehyde

The most widely studied marker of LP is malondialdehyde (MDA) in both animal and human models. Most of the studies assessing MDA have measured plasma or serum levels of thiobarbituric acid reactive substances (TBARS). This involves the reaction of the sample with TBA which when heated under acidic conditions produces a red dye by reaction with malonaldehyde (MDA) and other aldehydes. Therefore the extent of the red dye was thought to indicate the extent of MDA production. However, it is now believed that TBARS not only react with MDA, but can also react with carbohydrates, sialic acid and prostaglandins, all of which interfere with the assay and thus the TBARS test lacks the specificity to accurately indicate LP. Furthermore, although some studies have used TBARS as a direct measurement of MDA in the sample, it must be noted that other LP products can also react with TBA, and MDA can be generated during the assay.

To overcome this lack of specificity, many studies now use HPLC-based techniques to measure specific products of lipid peroxidation. One of the most common is the measurement of MDA either by direct analysis or by analysis of the MDA-TBA adduct following the reaction of the sample with TBA (Jackson, 1999). Despite the fact that HPLC is complicated and requires specialist equipment, it is the only method that yields meaningful results and should therefore be the method of choice for assessing MDA production (Oh-ishi et al., 2000).

2.4.2 Electron Spin Resonance (ESR) Spectroscopy.

ESR is also referred to as electron paramagnetic resonance (EPR) spectroscopy. The term EPR is used when transition metal ions are also being studied, whereas ESR is used to denote free radical studies. Both terms are now used interchangeably, although there has recently a trend to use the term electromagnetic resonance (EMR) to bring it in line with nuclear magnetic resonance (NMR) spectroscopy.

In the 1920s, Stern and Gerlag demonstrated that a beam of silver atoms passing through a sufficiently strong inhomogeneous magnetic field would split in two. The reason for this splitting are the electrons in the outermost orbital possessing a property referred to as a 'spin'. The particular spin of an electron (known as its spin quantum number) yields an associated magnetic moment. An unpaired electron has a spin of either + ½ or - ½ and behaves as a small magnet. Quantum restrictions dictate that the spinning electron can only spin in one of two opposite directions. In the absence

of an external magnetic field the electron can adopt either orbit. When an unpaired electron is exposed to an external magnetic field it aligns itself either parallel or antiparallel to that field, creating two possible energy levels. Transition between these different energy levels can be induced by absorption of a photon of appropriate frequency, creating an absorption spectra and this can be detected by ESR (Figure 2.3). Therefore the basic feature of ESR is the ability to detect and characterise the presence of an unpaired electron.

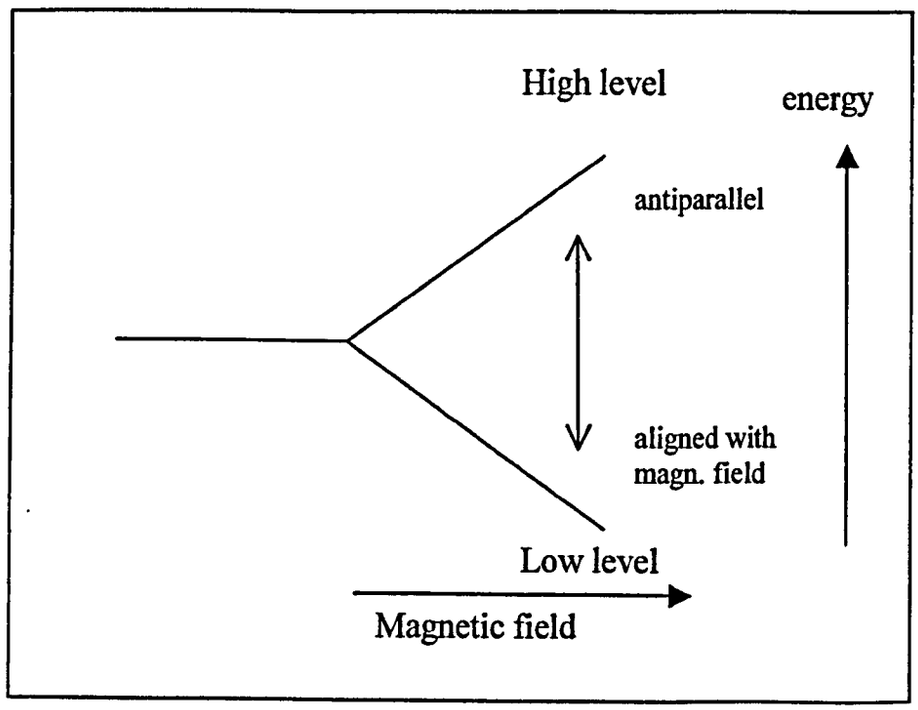


Figure 2.3 Principles of ESR

ESR spectrometers display the first derivative spectra, which show the rate of change of absorbance as opposed to the actual absorbance. Therefore, a point on the derivative curve corresponds to the gradient (slope) at the equivalent point on the absorption plot. The condition to obtain an absorbance is:

$$\Delta E = g\beta H$$

where ΔE is the energy gap between the two energy levels of the unpaired electron,

H is the applied magnetic field,

β is a constant known as the Bõhr magneton (a constant which is the value of the magnetic moment of a free unpaired electron)

g is the splitting factor which for a free electron is 2.0023 and most biologically-important radicals have values close to this. The g value is derived from the formula:

$$g = \frac{h\mu}{H\beta}$$

where μ = frequency (9.54 Ghz)

h = Planck constant (6.6262×10^{-27})

H = Magnetic field

β = Bohr magneton (9.2741×10^{-21})

ESR spectra present as lines referred to as the hyperfine structure (figure 2.5), which is often complex due to free radicals containing many nuclei. One of the advantages of ESR spectroscopy is that specific radicals can be identified by examining the hyperfine structure, the g value and the shape of the line. ESR spectroscopy is extremely sensitive and is able to detect radicals at concentrations as low as 10^{-10}M. This allows the detection of some radical species, for example the ascorbate radical,

although it is still not sensitive to detect more unstable radicals such as $O_2^{\bullet-}$, and $OH^{\bullet}$. In order to detect these, several techniques are available including the use of flow systems, the generation of radicals in a frozen transparent solid matrix, and the widely used spin trapping technique (Halliwell and Gutteridge, 1999).

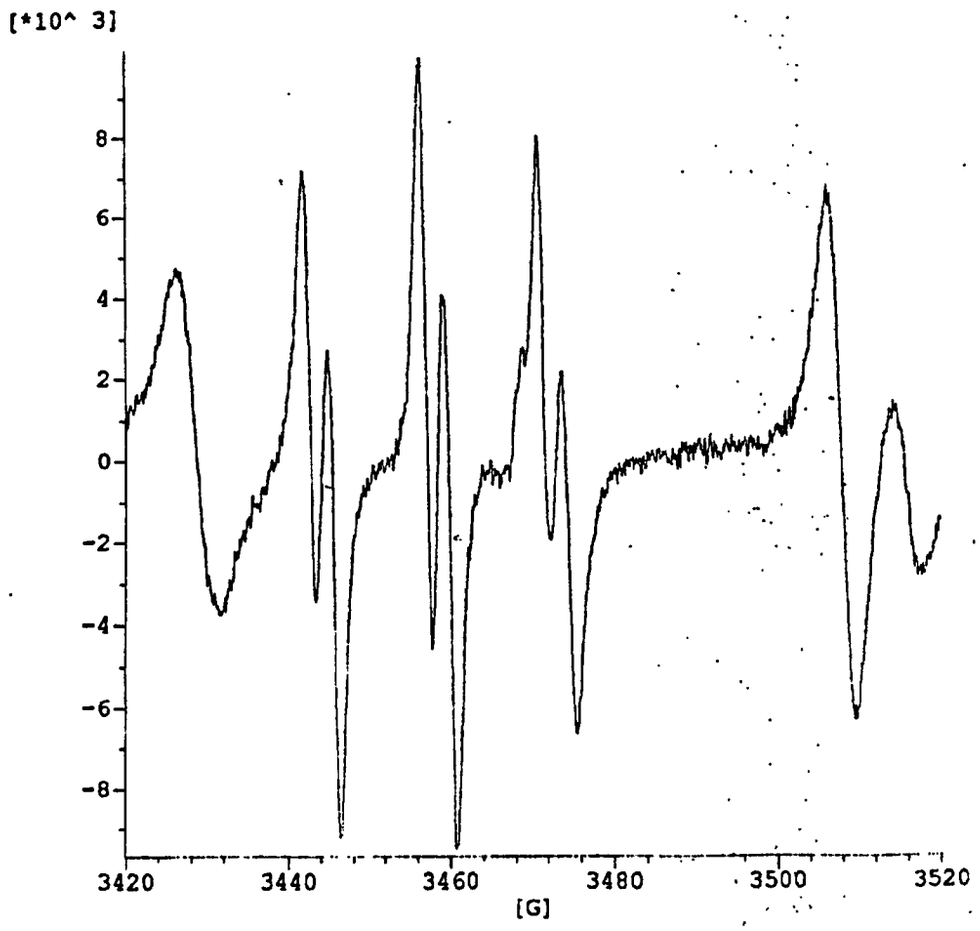


Figure 2.5 Example of an ESR spectra

Despite the known advantages of ESR spectroscopy over other techniques of assessing free radicals, ESR analysis is still scarcely used. The reason for this is that it is expensive, time consuming and requires considerable expertise (Jackson, 1985). Furthermore, because of the difficulties in assessing ROS production in humans, most studies have been carried out on animal models, isolated tissues, cultured cells, cell fractions and even purified biomolecules (Jackson, 1999).

2.4.2.1 Spin trapping

Although theoretically ESR can detect all free radicals, many oxygen-centred radicals generated in biological systems cannot be detected due to their low concentrations, high reactivity, and the high water content. However, with the use of appropriate spin traps, ESR can even detect these radicals. The principle of spin trapping is that highly reactive radical species can be converted to a relatively inert species thus allowing subsequent detection by ESR. A spin trap is a compound that is ESR silent which when rapidly reacted with radicals becomes a unique and relatively stable radical that produces an ESR spectra. Despite the relatively low rate of formation, the spin trapped radical, unlike its parent radical, has the ability to accumulate with time and thus may be readily quantified (Matsuo and Kaneko, 2000).

There are two main categories of spin traps that are used, these being nitrones and nitroso compounds. Nitrones are the most widely used spin traps with α-phenyl-*tert*-butyl nitrone (PBN) often being cited in the literature. Nitrones react rapidly with radicals forming nitroxide radicals that can then be examined by ESR.(figure 2.6)

Figure 2.5 The reaction of a reactive radical with a trap producing a long lived radical. The stability in the long-lived radical occurs through electron delocalisation between the nitrogen and oxygen atoms. (Figure taken from Halliwell and Gutteridge, 1998)

As yet, the application of spin trapping techniques in human studies is somewhat limited with even less attention being given to post exercise spin trapping. Recently, a spin trapping technique combined with ESR has been used post-exercise in human subjects following strenuous exercise, thereby demonstrating that this technique can be applied effectively in human subjects (Ashton et al., 1998; Ashton et al., 1999, Davison et al., 2002) .

In a recent study by Pattwell et al. (2003), the origin of a spin-trapped radical measured in the circulation was examined following ischemic reperfusion injury. The study used PBN to measure trapped radicals in both the general circulation and in the microdialysis fluids from skeletal muscle. Furthermore, 2-3 Dihydroxybenzoic (2-3, DHB) acid was also measured in the microdialysis fluids, as this is a specific marker of $^{\bullet}OH$ activity. It was reported that there was a strong correlation between 2-3, DHB in the microdialysis fluid and the appearance of the PBN trapped species thus suggesting that the rate of production of the trapped radical is regulated by $^{\bullet}OH$

activity. Since the trapped radical in the circulation also correlated with the trapped radical in the microdialysis fluid, it was postulated that the measurement of the circulating trapped radical may provide a relatively non-invasive method of assessing extracellular $^{\bullet}OH$ activity in skeletal muscle. This would support the suggestions of Ashton et al. (1998) postulated that the circulating PBN trapped radical that they observed post exercise was likely to be a secondary radical derived from the reaction of primary radicals such as $^{\bullet}OH$ with membrane lipids.

2.5 Oxidants, Antioxidants and DOMS

The first living organisms on earth survived under an atmosphere containing little oxygen and essentially were anaerobic (Ji and Hollander, 2000). Rising atmospheric oxygen concentration, due to the evolution of photosynthetic organisms, resulted in many of these anaerobes becoming extinct. The few remaining anaerobes have adapted to the current oxygen concentration of 21% by restricting themselves to environments where oxygen could not penetrate. Not all animals did this and a second line of defence was developed, this being the evolution of an antioxidant system to protect them against oxygen toxicity (Halliwell, 1994).

By definition an antioxidant is any compound, usually organic, that prevents or retards oxidation by molecular oxygen (Goldfarb, 1993a) and thus may confer some protection from the damaging effects caused by free radicals (Kent, 1994). Cellular antioxidant defences may be categorised as either enzymatic or non-enzymatic. Primary enzymatic antioxidants include superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (Ji and Hollander, 2000). As well as the primary enzymatic antioxidants, there are also a number of enzymes that do not directly prevent or remove ROS, but play a significant role in the supply of substrates and reducing power for primary enzymatic antioxidants. Such enzymes include glutathione reductase (GR), glucose-6-phosphate dehydrogenase (G6PDH) and glutathione sulphur-transferase (GST) (Halliwell, 1994). The second type of cellular antioxidant are the non-enzymatic antioxidants. These include ascorbate, α-

tocopherol and β-Carotene, and have the ability to directly scavenge certain radicals including $O_2^{\bullet-}$ and the more potent $^{\bullet}OH$ (Ji and Hollander, 2000).

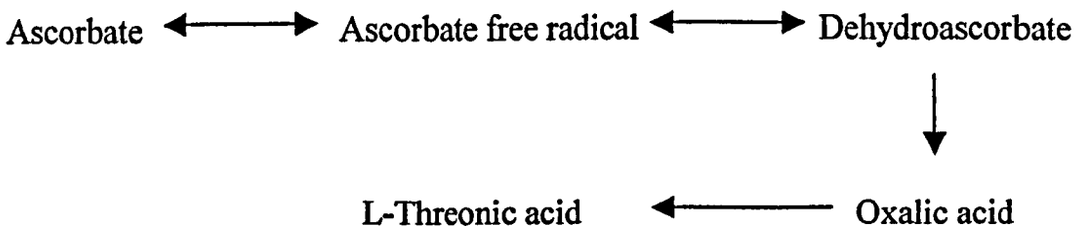
A second system of antioxidant classification involves categorising antioxidants according to where they are synthesised. These include antioxidants that are synthesised endogenously under oxidative stress, and those that cannot be synthesised endogenously and must therefore be taken in through diet. This system of classification emphasises the importance of nutrition on cellular antioxidant stores. Endogenous antioxidants work in concert with their exogenous counterparts, providing defences against ROS (Sen, 2001). Endogenous antioxidant supplies are not able to completely prevent oxidative stress and consequently dietary antioxidants are essential to maintain homeostatic balance (Halliwell, 1994).

It has been suggested that muscle damage could be prevented through dietary antioxidant supplementation (Dekkers et al., 1996). Venditti and DiMeo (1996) reported that ROS-induced muscle damage could be responsible for terminating muscle effort and speculated that greater intramuscular antioxidant concentrations could allow the muscle to withstand oxidative processes more effectively. If ROS are responsible for DOMS then supplementation with the correct antioxidants could feasibly reduce or prevent DOMS.

There has been research on the effect of various antioxidants on post exercise muscle damage, although the results of these studies are equivocal (Surmen-Gur et al., 1999). Furthermore, no study has used direct methods such as ESR spectroscopy to examine ROS production following muscle-damaging exercise. Most of the research into DOMS and antioxidants has been carried out in rats (Armstrong et al.,1983; Jackson, 1992) whilst the effects on humans are still unclear (McArdle and Jackson, 1997).

2.5.1 The chemistry of ascorbic acid

Vitamin C is the generic name for substances that provide the biological activity of L-ascorbic acid ($C_6H_6O_8$). Ascorbic acid is a water-soluble, dietary antioxidant present in the cytosolic compartment of the cell and the extra-cellular fluid, and is known to be a powerful inhibitor of lipid peroxidation in plasma (Sen, 2001). The putative effect of ascorbate occurs though both direct and indirect methods. Ascorbate can function directly as an antioxidant, interacting with $O_2^{\bullet-}$ and $^{\bullet}OH$, although it is better known for its relationship with α-tocopherol (Kanter, 1998). The spatial arrangement of ascorbate allows it to scavenge aqueous phase α-tocopherol radicals generated in the cell membrane during oxidative stress, through its ability to rapidly donate electrons to α-tocopherol radicals (Evans, 2000). After donating an electron, ascorbate can then be oxidised to a semihydroascorbate (SDA) radical, this being a much less reactive substance. SDA radicals are then either converted directly to ascorbate by the enzyme SDA reductase, using nicotinamide adenine dinucleotide (NADH) or indirectly converted into ascorbate in the presence of glutathione (Ji and Hollander, 2000). The antioxidant properties of ascorbic acid can be seen below:



Ascorbate also acts indirectly through its ability to regenerate oxidised α-tocopheryl (Goldfarb, 1999b). Ascorbic acid is specifically important in relation to ROS induced muscle damage and subsequent DOMS, since in response to physical trauma it is able to provide protection against neutrophil-mediated cell injury by scavenging the specific neutrophil derived ROS (Nieman et al., 2002). Despite this fact, the majority of research into ROS and DOMS is still based around α-tocopherol with few studies investigating the relationship between ascorbic acid supplementation, oxidative stress and DOMS.

2.5.2 Ascorbic acid, DOMS and ROS

The supplementation of ascorbic acid to the diet is not a novel idea. As early as the 18th century, British soldiers reportedly added ascorbic acid rich foods to their diets to prevent scurvy (Alessio et al., 1997). Daumezon et al. (1945) published the first reported reference to ascorbic acid supplementation reducing muscle pain. It was claimed that daily supplementation of 300mg ascorbic acid significantly reduced muscle pain in mental patients following shock therapy. In this early stage of investigation it was suspected that ascorbic acid may maintain the cohesiveness of the

cells and blood vessels and may increase the efficiency of tissue respiration, however the link between free radicals, ascorbic acid and muscle pain had not been established.

Following this study, Staton (1952) investigated the effects of ascorbic acid on exercise-induced muscle pain. The study involved 30 days of 100mg ascorbic acid supplementation followed by two bouts of continuous sit-ups for three minutes. Pain was assessed by measuring the fatigue factor between the first and second bouts of sit-ups. It was reported that supplementation minimised the extent of muscle pain compared to a placebo group. It must be stated however, that the assessment of fatigue is not a good measurement of pain and therefore these results should be treated with caution.

Kaminski and Boal (1992) investigated ascorbic acid supplementation on DOMS following isolated calf exercise designed to induce DOMS. Supplementation involved 300mg of ascorbic acid being taken for 3 days prior to the exercise followed by 7 days of supplementation post-exercise. Muscle pain was assessed using a VAS at rest, during movement and following palpation. Ascorbic acid significantly reduced DOMS, with the greatest effects shown when DOMS was at its peak. The study did not however perform any assessment of oxidative stress and also did not attempt to explain the reasons for the observed reductions in DOMS.

Alessio et al. (1997) also reported that ascorbic acid offered some protection from ROS following exercise. The authors administered 1000mg per day of ascorbic acid

for either one day or one week prior to thirty minutes of sub-maximal exercise and examined the ROS response as indicated by plasma thiobarbituric acid reacting substances (TBARS) and oxygen radical absorbance capacity (ORAC). It was reported that the supplementation of ascorbic acid attenuated the increase in exercise induced oxidative stress following both acute and chronic supplementation. The study also demonstrated that loading with ascorbic acid was more effective in reducing oxidative stress than an acute dose, although it must be emphasised that both supplementation groups exhibited a reduced oxidative stress when compared to placebo.

Kearns et al. (2001) examined the effects of a prophylactic dose of ascorbic acid prior to ischemic reperfusion injury in rats. They observed that the ascorbic acid group was able to maintain muscle function whilst at the same time demonstrating reduced tissue oedema and neutrophil infiltration. The authors concluded that a prophylactic dose of ascorbic acid protects against ROS-induced ischemic reperfusion injury and that this may be due to the ascorbic acid attenuating neutrophil respiratory burst activity. However, it must be noted that this was performed on rats and as yet no study has identified this in a human model.

In the first study that directly assessed free radical production (using ESR spectroscopy), as well as measuring indices of lipid peroxidation following exercise in humans, Ashton et al. (1999) demonstrated that acute oral ascorbic acid supplementation (1000mg 2 hrs prior to exercise) was effective in preventing exercise

induced oxidative stress. The study measured ROS production pre and post-exercise following the acute supplementation of ascorbate or without any supplementation. It was observed that the intensity of the PBN adduct (which is proportional to the concentration of radicals in the sample) increased post-exercise without ascorbic acid supplementation whereas there was no significant increase post-exercise with supplementation. The authors concluded that acute supplementation of 1000mg ascorbic acid may be considered an effective antioxidant preventing exercise induced oxidative stress. Recently, these findings have been confirmed by Davison et al. (2003) who also supplemented 1000mg per day of ascorbic acid and examined ROS production using ESR spectroscopy following maximal exercise. It was reported that supplementation with ascorbic acid was effective in reducing ROS production thus confirming the findings of Ashton et al. (1999).

Since Thompson et al. (1999) demonstrated that unaccustomed shuttle running results in significant DOMS, there has been several studies that have used the Loughborough intermittent shuttle test (LIST) to induce DOMS. Recently, several studies have used the LIST to investigate the effects of ascorbic acid on DOMS, although the results of these studies have been conflicting. Wadsworth et al. (2003) administered 1000mg of ascorbic acid pre-exercise and 1000mg 24 h post-exercise. The authors reported that such supplementation had no effect on recovery or muscle pain. In contrast to this, Thompson et al. (2001b) reported that 400mg of ascorbic acid administered each day for 2 weeks prior to the LIST demonstrated modest beneficial effects on recovery and in attenuating post exercise ROS production.

It must be noted though that the exercise protocols used by Ashton et al. (1999), Thompson et al. (2001b), Wadsworth et al. (2003) and Davison et al. (2003) all involved intensive exercise and therefore the likely source of the post-exercise ROS generation would be mitochondrial electron leakage. Leeuwenburgh et al. (2001) reported that following eccentric exercise, markers of ROS dramatically increase in the days following the exercise, peaking approximately 3 days post exercise. These ROS are likely to be produced by phagocytic cells and as yet there has been no study that has investigated the effects of ascorbic acid supplementation on DOMS and ROS production following mechanical muscle damaging exercise such as downhill running.

2.6 Diet ROS and DOMS

Research has failed to agree on a common mechanism involved in DOMS and as such there has been little attention given to external factors that may exacerbate it. If external factors do affect DOMS this may provide extra information regarding its aetiology and one external factor which may play a critical role in DOMS is that of diet.

2.6.1 Carbohydrates, ROS and DOMS

It has been well established that the dietary and exercise habits of an individual can significantly affect carbohydrate stores, both acutely and chronically. There is evidence to suggest that a reduction in blood glucose results in hypothalamic-pituitary-adrenal activation, increased release of cortisol, decreased insulin and a variable effect on blood catecholamine levels (Nieman, 1998). Blood glucose is a vital fuel for cells of the immune system including lymphocytes, neutrophils and macrophages. Blannin et al. (1998) demonstrated the importance of glucose to phagocytes by reporting that when glutamine and glucose are both present in culture medium at normal physiological concentrations, phagocytes utilise glucose at 10 times the rate they utilise glutamine. Therefore maintenance of blood glucose concentration during exercise should attenuate the increases in stress hormones and maintain optimum immune function (Figure 2.6).

Figure 2.6 Interaction between plasma glucose, stress hormones and the immune system. (Taken from Nieman, 1998).

Recently, there have been numerous studies that have investigated the relationship between carbohydrate status and immune function following exercise (Bishop et al., 1999; Bishop et al., 2001a; Bishop et al. 2001b; Gleeson et al., 1998).

Regular supplementation of CHO drinks during strenuous exercise results in a reduced shift in the number of circulating leukocytes and reduces the magnitude of change in immune cell functional responses (Gleeson and Bishop, 2001). Although no study has assessed ROS production and DOMS following CHO supplementation, it does appear that the reduction in the magnitude of changes in immune cell function that has been documented could modify post-exercise ROS production which has been implied in the aetiology of DOMS.

Recently, Bishop et al. (2001b) investigated the effects of a high or low CHO diet on such changes. The diets consisted of 3 days on either 70% (high CHO) or 10% (low CHO) CHO diets. Following the diets, a cycle exercise test consisting of 60 minutes

at 60%W_{max} followed by 30 minutes at 80%W_{max} was performed. The high CHO diet resulted in an attenuated post-exercise increase in plasma cortisol, attenuated circulating neutrophil number as well as attenuated LPS-stimulated elastase release. Since it is likely that ROS produced in the days following eccentric exercise are related to neutrophil activity, it is feasible that dietary manipulation of CHO could also have a direct effect on post-exercise ROS production.

Furthermore, it is known that high muscle glycogen content results in additional water retention in skeletal muscle. It is feasible that this increased water retention, combined with muscle damage and subsequent oedema could increase the magnitude of DOMS.

Despite this potential relationship between CHO, ROS and DOMS, there has been no studies that have examined the effects of dietary CHO on DOMS following eccentric muscle action or have investigated the effects of such dietary manipulation on ROS generation. The studies that have been performed concentrate on prolonged concentric exercise that is not specifically designed to induce muscle pain (Bishop et al., 1999a; Bishop et al., 1999b; Nieman et al., 1998) and do not make any attempt to monitor the effects of CHO on DOMS.

It is well known that athletes often engage in diets high in CHO intake. Since there is a link between CHO and ROS, it is feasible that such diets could influence the magnitude of DOMS. However, as yet there has been no study that has investigated the effects of high CHO diets on DOMS and muscle function following eccentric exercise.

2.7 Summary

DOMS involves a complex network of events that include an inflammatory response, yet despite considerable research the exact aetiology of DOMS remains unresolved. It is believed that reactive oxygen species are produced following DOMS inducing exercise and the likely source of this is phagocytic cells. However, it is still unknown if these ROS are involved in initiating DOMS or play a role in mediating the recovery.

The review of the literature has highlighted several gaps that this thesis will attempt to fill. In view of the lack of documented research surrounding DOMS, ROS production and muscle function, this thesis was designed to address the following aims, i) to investigate the production of ROS during and after downhill running compared to level running, ii) to investigate the effect of ascorbic acid supplementation on DOMS and ROS during and after downhill running and iii) to investigate the effects of dietary CHO intervention on DOMS and ROS production following downhill running.

CHAPTER 3 - GENERAL METHODS

3.1 Subjects

All of the experiments in this thesis have been carried out using volunteer human subjects, all of whom were male students at Liverpool John Moores University, UK. Subjects were free from any known illness and were excluded if they were taking any vitamin supplements or were regular smokers. The subjects were classified as trained as reflected in their $\dot{V}O_{2\,max}$ values (Table 3.1), although none were classified as elite athletes, and no subjects were familiar with fell running or any other form of exercise that possesses a large eccentric bias. All subjects were informed verbally and in writing about the nature of this study, including all potential risks (Appendix B). Written informed consent was obtained prior to participation and the Liverpool John Moores University Ethics Committee granted ethical approval.

Table 3.1 Mean (±SD) summary of subject characteristics used in this thesis.

Study	*N*	*Design*	*Age (years)*	*Height (cm)*	*Body Mass (kg)*	$\dot{V}O_{2\,max}$ *(L.min^{-1})*	*Body Fat (%)*
1	8	Cross over	24.8 (± 3)	181 (± 5.8)	80.6 (±5.4)	4.5 (± 0.4)	15.6 (±3.2)
2	12	Cross over	23.3 (±3.4)	175 (± 5.4)	76.7 (± 6.0)	4.2 (± 0.5)	14.6 (±3.7)
3	20	Matched pairs	23.4 (±3.8)	179 (± 5.5)	79.0 (± 12.5)	4.4 (± 0.7)	15.2 (±4.2)

3.2 Assessment of $\dot{V}O_{2\,max}$

Prior to each study, all subjects were initially assessed for aerobic capacity by determining their $\dot{V}O_{2max}$ on a motorised treadmill. Subjects started running at 10km.h^{-1} and this was increased by 2 km·h^{-1} every 2 minutes up to 16km·h^{-1}. Thereafter, the treadmill was inclined by 2% every 2 minutes until volitional exhaustion. The $\dot{V}O_{2max}$ was taken as the highest $\dot{V}O_2$ value obtained in any 10-second period, and was stated as being achieved by the following end point criteria: 1. Heart rate within 10 b.min^{-1} of age related maximum; 2. RER >1.15; 3. Plateau of oxygen consumption despite increasing workload <2.0 mL.kg^{-1}.min^{-1}; 4. Blood lactate concentration > 8 mM, (BASES, Physiological testing guidelines, 1997).

Expired fractions of oxygen and carbon dioxide were averaged over each 10-second period and analysed *via* an on-line open circuit spirometry system (Metamax Cortex Biophysic GmbH, Leipzig, Germany) [Figure 3.1]. The volume and composition of the expired gases collected were expressed in standard terms (Standard Temperature and Pressure Dry). Heart rate (HR) was measured continuously by a Polar Accurex Plus heart rate monitor (Kemple, Finland) transmitter fitted to the chest at the V5 position, with heart rates transmitted *via* short range telemetry to a receiver unit and averaged over 5 second intervals for later data retrieval. A fingertip blood sample was taken at the end of the test for assessment of lactate concentration.

Figure 3.1 Metamax gas analyser used for determination of aerobic fitness and used to assess oxygen consumption during the experimental runs

3.3 Downhill running

All three phases of the research involved downhill running with each run lasting for 30 minutes at a gradient of -15%. Previous research using downhill running has used a variety of exercise intensities. For study 1, an exercise intensity of 65% $\dot{V}O_{2\,max}$ was selected, whilst for studies 2 and 3 an intensity of 60% $\dot{V}O_{2\,max}$ was selected.

Throughout the downhill runs, respiratory gases were collected every 10 seconds using the previously described gas analysis system as used in the assessment of $\dot{V}O_{2\,max}$. This allowed the treadmill speed to be continuously altered ensuring the correct exercise intensity was maintained. Furthermore, every 5 minutes, RPE (6-20 Borg scale, (Borg, 1982) and heart rate were recorded.

All of the runs took place on a motorised treadmill (HP Cosmos, Nussdorf-Traunsten, Germany) with all subjects wearing a harness connected to an automatic cut off switch to reduce any potential risks (Figure 3.2). Subjects were verbally motivated throughout and thermal comfort was maintained through the use of two electric fans.

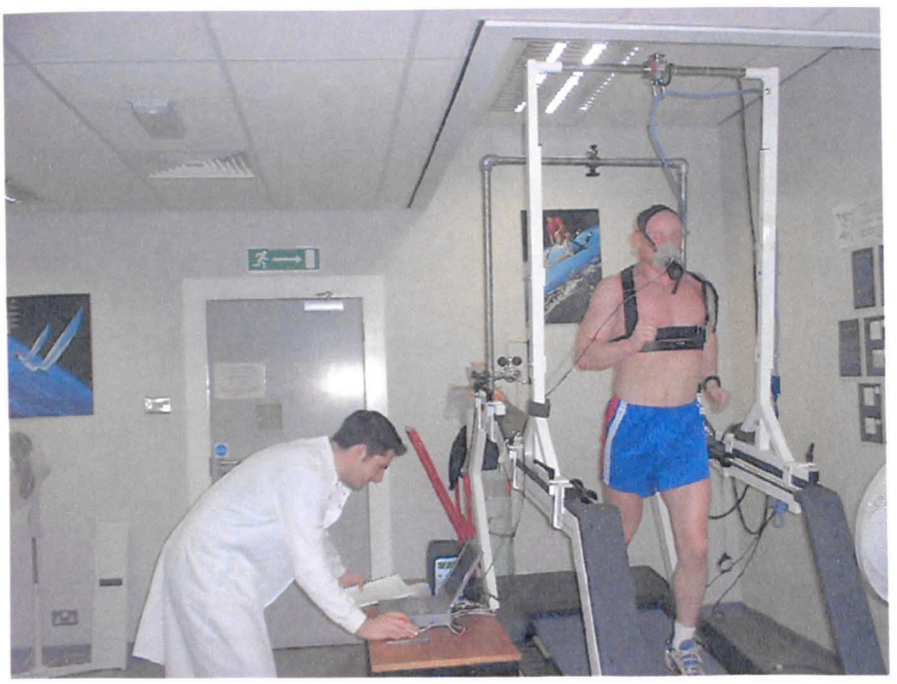


Figure 3.2 One of the subjects performing downhill run.

3.4 Assessment of Muscle Function

Muscle function was assessed using an isokinetic dynamometer (LIDO active, Loredan, California ,USA) on which subjects had been previously familiarised (Figure 3.3). Familiarisation involved each subject performing the battery of tests a minimum of three times, with the last familiarisation 7 days before testing. The test was designed to assess concentric quadriceps torque at slow and fast speeds as well as eccentric quadriceps torque. A summary of the tests used can be seen in Table 3.2.

Table 3.2 Muscle Function assessments on isokinetic dynomometer.

	Concentric 1.04 rad.sec^{-1}	Concentric 5.20 rad.sec^{-1}	Eccentric 2.06 rad.sec^{-1}
Quadriceps	✓	✓	✓

All exercises were carried out using the subject's dominant leg. Subjects were asked to perform each test three times. If the torque was greatest on the third trial, subjects were asked to continue until no further increase in torque was observed. The maximum score was recorded for each of the trials, and verbal encouragement was given throughout. Table 3.3 summarises the time course of muscle function assessment for the 3 studies.

Table 3.3 Time course of muscle function and pain assessment for the 3 studies.

	Time taken (hours)
Study 1	Pre exercise, 0, +24, +48, +72
Study 2	Pre exercise, 0, +24, +48, +72, +96
Study 3	Pre exercise, 0, +24, +48, +72, +96, +168, +336

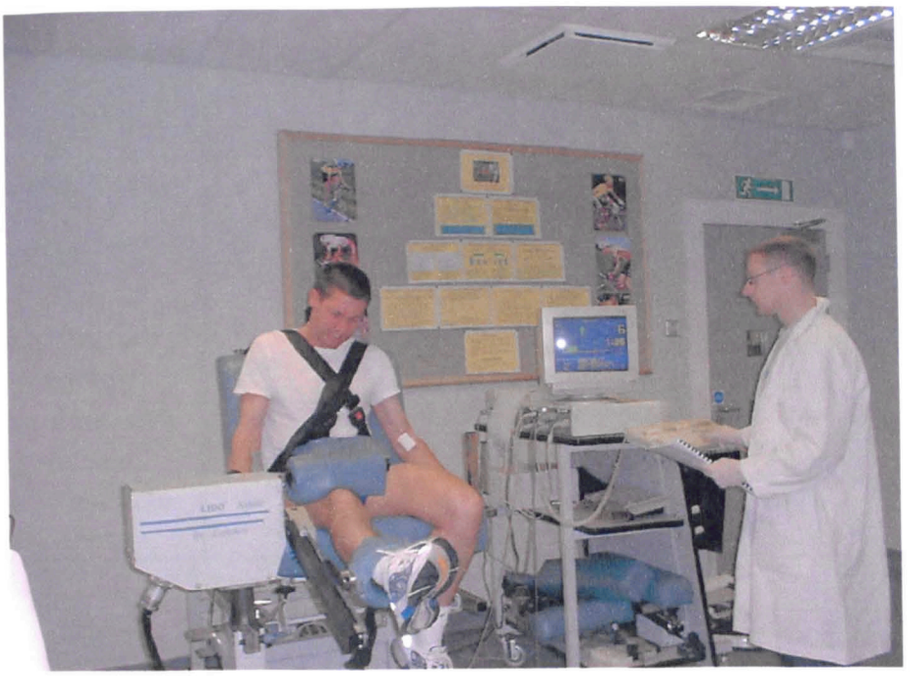


Figure 3.3 Isokinetic Assessment of Muscle Function

3.5 Assessment of DOMS

One of the major problems involved with research into DOMS is the difficulty in assessing muscle soreness. This is largely due to the subjective nature of quantifying soreness, and as such there is no consensus in the literature regarding the most suitable and appropriate methods to employ (Jones and Round, 1999). Animal models are able to measure firing frequencies from the afferent nerve in response to graded stimuli, however this is not possible in human models. Throughout this thesis, various methods have been used to assess muscle soreness and these are summarised in Table 3.4. Table 3.3 summarises the time course of muscle soreness assessment for the three trials.

Table 3.4 Methods of muscle soreness assessment used in this thesis.

	Study 1.	Study 2.	Study 3.
Pain Diagram	✓	✓	✓
VAS	X	X	✓
0-1.8 kg Pressure Algometer	X	✓	X
0-30 kg Pressure Algometer	X	X	✓
Exercise muscle soreness	✓	✓	✓

3.5.1 Pain Diagrams

The pain diagrams involved subjects rating muscle soreness of the gastrocnemious, tibialis anterior, hamstrings, quadriceps, and gluteals, (all being assessed on left and right sides), as well as lower back muscles using a modified Borg Scale, with 0 = no pain, 10 = unbearable pain. Subjects were asked to quantify the amount of pain generally felt in the muscle and write this score into the corresponding box on the pain diagram (Figure 3.4). Scores for the 11 sites were then accumulated and divided by the number of sites assessed and this was then reported as an indicator of muscular pain.

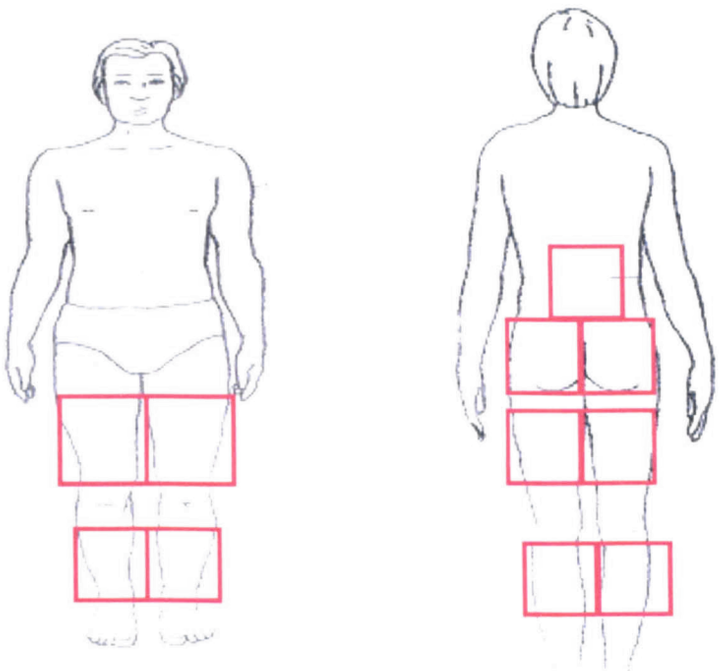


Figure 3.4. Pain diagrams used to assess DOMS.

3.5.2 Visual Analogue Scale (VAS)

The VAS is a common measurement technique used to assess pain. It is a 10 cm line that has polar descriptors at its extremes (Figure 3.5). Unlike a Graphic Rating Scale (GRS) that contains descriptors placed at equal intervals, the VAS allows the individual to rate the amount of pain without having to transform feelings into words (Mattacola et al.,1997) and as such has been suggested to be a more accurate measurement of pain (Ohnhaus, 1975).

Figure 3.5 Visual Analogue Scale (VAS) used to assess muscular pain

Subjects were asked to place a mark on the line that corresponds with the amount of pain felt generally in the legs. The distance from 'no pain' was measured and this distance was used to quantify the amount of soreness.

3.5.3 Pressure Algometer

The pressure algometer is an instrument that can reproducibly quantify local tenderness on pressure (Hogeweg et al., 1992). It consists of a gauge that it attached to a 1cm diameter hard rubber tip that allows the amount of pressure required to initiate a painful sensation to be measured (Figure 3.6). For study 2, a Wagner Force Dial™ FDK2 (Wagner Instruments, Connecticut, USA) was used as described by (Bailey et al., 2000). However, the FDK2 only applies pressure up to 1.8 kg and this was not sufficient to elicit a painful response and so the data were not reported. For study 3, a Wagner Force Dial™ FDK60 that applies up to 30 kg of pressure was used.

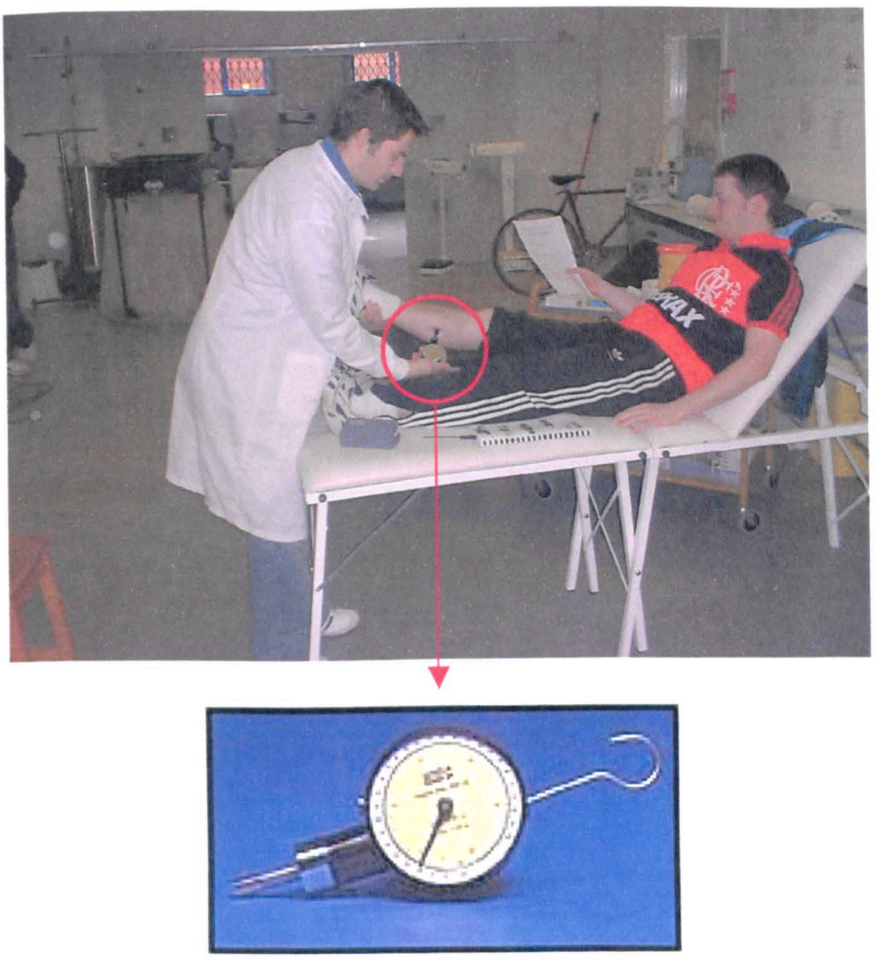


Figure 3.6 Subject being familiarised with the pressure algometer

Pressure was applied over the distal myotendinous junction, as well as the mid belly of the muscle (Bailey et al., 2000). These sites were determined using a datum line from mid-patella to the lateral iliac crest (Baker et al., 1996) and marked using a permanent marker pen to ensure that the identical site was tested on each day during the test. All measurements were taken on the subject's dominant side since significant differences are observed between left and right sides (Hogeweg et al., 1992). All subjects were familiarised to the pressure algometer, which involved 10 practise trials on alternative sites of the body. Pressure was applied at an even rate of approximately 1 kg per second (Fischer, 1987) until the subject indicated that the sensation had changed from 'pressure' to 'discomfort'. The instrument incorporates a

'hold at max' feature that allows the maximum pressure applied to be retained by the algometer.

A total of 3 measurements at each site were taken in sequential order with a minimum of 1 minute between re-tests on sites. The average of the 3 measurements was recorded. The pain was quantified as the summed forces divided by the number of sites assessed (Bailey et al., 2000).

3.5.4 Exercise Muscle Soreness

Muscular soreness was also assessed during the muscle function assessment on the isokinetic dynamometer. Subjects were asked to rate the level of pain felt during the contraction in the muscles being used. The pain was rated on the adapted 0-10 Borg scale as used in the pain diagrams.

3.6 Procurement and storage of blood samples.

Blood samples were drawn immediately before exercise and at varying times post-exercise, up to a maximum of 14 days post exercise. All post-exercise samples on subsequent days were taken at the same time of day as the pre-exercise samples to avoid any circadian variation (Fernandes, 1992).

Blood samples were collected in either Monovette collection tubes (Sarstedt, Germany) or vacutainers (Becton Dickinson, Northampton, UK). All samples were taken by a qualified phlebotomist following an overnight fast from a superficial vein in the antecubital fossa whilst the subjects were supine (including the post-exercise sample) and had been resting for a minimum of 30-minutes (except for the post-exercise sample which was taken immediately upon cessation of exercise). Samples were taken using minimal stasis to prevent any potential increase in oxidative stress due to ischemic-reperfusion injury (Kearns et al., 2001). This was achieved by taking all samples without a tourniquet (Figure 3.7).

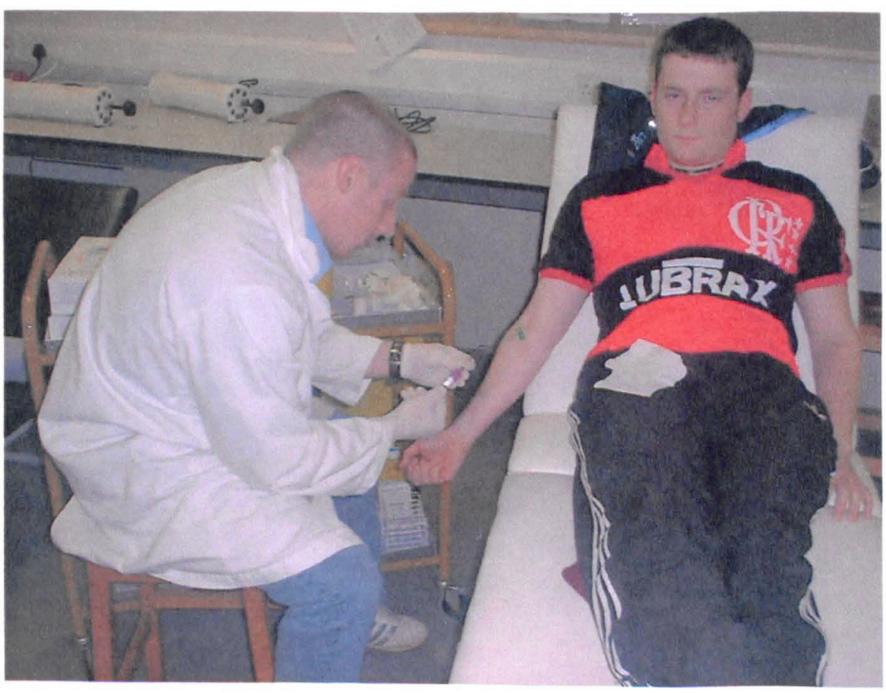


Figure 3.7 Blood being drawn into pre-treated vacutainers without a tourniquet and whilst subject is supine

Serum was obtained by collecting samples in to serum separation tubes (SST). The blood was then stored at room temperature for 30 minutes before being centrifuged at 4°C for 15 minutes at 14,000rpm. Plasma was obtained by collecting the samples into

tubes that had been pre-treated with the anti-coagulant lithium heparin. These samples were then gently mixed and immediately centrifuged at 4°C for 15 minutes at 14,000rpm. All samples (except for those taken for leukocyte analysis) were then stored at –70°C for later analysis.

3.7 Blood analyses

3.7.1 Glucose

Glucose concentrations were determined in serum using a commercially available kit (IL Test™ Glucose Oxidase kit, Instrumentation Laboratory, Warrington, UK) using enzymatic methods on an IL Lab 300 Chemistry Analyser (Instrumentation Laboratory, Warrington, UK), seen in Figure 3.8. A summary of the enzymatic reactions involved in the determination of plasma glucose concentration can be seen below:

Test Principle

$$\beta\text{-D-Glucose} + O_2 + H_2O \xrightarrow{\text{Glucose Oxidase}} \text{Gluconic acid} + H_2O_2$$

$$2\,H_2O_2 + \text{phenol} + \text{4-aminoantipyrine} \xrightarrow{\text{Glucose Peroxidase}} \text{red quinoneimine} + 4H_2O$$

The red quinoneimine dye generated an increased absorbance and this was proportional to the glucose concentration in the sample. Primary measurements were taken at a wavelength of 510nm and CVs were <8.5% at 4 and 10 mM.

Figure 3.8 IL 300 chemistry analyser

3.7.2 NEFA

NEFA was analysed in serum and determined using a commercially available kit using enzymatic methods on an IL Lab 300 Chemistry Analyser.

Test Principle

$$RCOOH + ATP + CoA\text{-}SH \xrightarrow{\text{Acyl-CoA synthase}} \text{Acyl-CoA} + AMP + PPi$$

$$\text{Acyl-CoA} + O_2 \xrightarrow{\text{Acyl-CoA oxidase}} \text{2,3-trans-Enoyl-CoA} + H_2O_2$$

$$2H_2O_2 + \text{4-Aminoantipyrine} + \text{3-methyl-N-ethyl-N-(β-hydroxyethyl)-alanine} \xrightarrow{\text{Peroxidase}} \text{Coloured reaction product} + 4H_2O$$

Primary measurements were taken at a wavelength of 500nm and CVs were <3.4% at 0.2mM and <3.1% at 1.1mM.

3.7.3 Lactate

For the analysis of lactate, blood was collected in tubes that had been pre treated with the anti-coagulant ethylendiaminetetraacetic acid (EDTA). A portion of this blood was immediately de-proteinised using cold perchloric acid. The deproteinised sample was then centrifuged at 4°C for 15 minutes and the supernatant was removed and stored at –70°C for later analysis. Plasma lactate concentrations were determined using a commercially available kit (Randox Lactate PAP, Randox laboratories Ltd, Co. Antrim, UK) using enzymatic methods on an IL Lab 300 Chemistry Analyser.

Test principle

$$\text{Lactate} + O_2 \xrightarrow{\text{Lactate Oxidase}} \text{pyruvate} + H_2O_2$$

$$H_2O_2 + \text{4-aminoantipyrine} + \text{TOOS} \xrightarrow{\text{Peroxidase}} \text{purple product} + 4H_2O$$

(TOOS = N-ethyl-N-(2 hydroxy-3-sulphopropyl) m-toluidine)

The purple product generated an increased absorbance and this is proportional to the lactate concentration in the sample. Measurements were taken at a wavelength of 550nm. The test is linear upto lactate concentrations of 12.21 mM. The CVs were <8 % at 1.2 and 7.8mM.

3.7.4 Creatine Kinase (CK)

Creatine Kinase (CK) analysis was carried out on plasma and determined using a commercially available kit (IL Test™ CK-NAC kit, Instrumentation Laboratory,

Warrington, UK). It was then analysed at room temperature using enzymatic methods on an IL Lab 300 Chemistry Analyser. The principle of the test can be seen below:

Test Principle

$$\text{Creatine phosphate} + \text{ADP} \xrightarrow{\text{Creatine Kinase}} \text{creatine} + \text{ATP}$$

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{Hexokinase}} \text{glucose 6 phosphate} + \text{ADP}$$

$$\text{Glucose-6-phosphate} + \text{NADP}^+ \xrightarrow{\text{G-6-PDH}} \text{gluconate-6-phosphate} + \text{NADPH} + \text{H}^+$$

The rate of increase in absorbance due to the reduction of $NADP^+$ to NADPH is directly related to CK activity in the sample. Absorbance measures are taken at a wavelength of 340nm. The test is linear up to CK activity of 1000 IU/L. Above 1000 IU/L dilutions were made and the results adjusted accordingly. The CVs were <4.8% at 282 and <5.8% at 3890 IU/L.

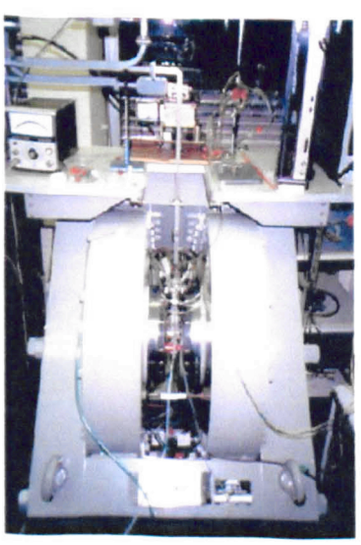
3.7.5 ROS

3.7.5.1 Electron spin resonance spectroscopy

Electron spin resonance (ESR) spectroscopic determination of free radicals was carried out on blood that was collected into a SST containing 1.5 ml of 140mM α-phenyl-*tert*-butylnitrone (PBN) (Sigma, Dorset, UK). The blood was allowed to clot at room temperature for 30 minutes prior to being centrifuged for 15 minutes at 4°C at 14,000rpm. The serum was removed and immediately added to an equal amount of HPLC grade toluene and vortex mixed for 30 seconds. The PBN adduct was then separated and stored at –70°C for later analysis. ESR spectroscopic detection of ROS was carried out as described by (Ashton et al., 1998). Briefly, the PBN adduct was

was carried out as described by (Ashton et al., 1998). Briefly, the PBN adduct was extracted and pipetted into a 5-mm-OD precision-bore quartz ESR-sample tube. The sample was then immediately vacuum degassed using a turbo pump in a freeze thaw procedure.

Room temperature ESR analysis was performed on a JEOL RE2X series X-band spectrometer with 100 kHz frequency modulation, as seen in Figure 3.9. The analysis was performed under the following operating conditions: Microwave frequency, 9.436 GHz; incident microwave power,10mW; scan width ± 4.000 mT; modulation amplitude,0.1000, magnetic field centre, 334.6mT; modulation amplitude, 0.1000 mT; magnetic field centre, 334.6 mT; scan rate, 4.0 min; gain 2.5 x 10^3; time constant, 0.10 or 30 s.



3.7.5.2 Malondialdehyde

Malondialdehyde (MDA) was analysed in serum. Samples were defrosted at room temperature and 40µl of the serum was removed and placed into fresh 0.6ml eppendorf tubes along with 160µl of 2.0M sodium acetate. The samples were then vortex mixed and 3µl of 5% Butylated Hydroxytoluene (BHT) followed by 20µl of 2% Thiobarbituric acid (TBA) was added to all of the samples as well as the prepared standards. The standard was TEP.

The samples and standards were then re-mixed for 15 seconds before being heated for 1 hour on a hot plate at 90°C. After 1 hour the samples were removed from the hot plate and immediately stored on ice. All samples then received 200µl of butanol prior to being mixed again on a whirly mixer. The samples and standards were then centrifuged for 10 minutes at 4°C at 14,000rpm. Samples and standards were removed from the centrifuge and 100µl of the organic solution was drawn into HPLC tubes. All tubes were then analysed using HPLC (Gina 50, Gynkotek, Macclesfield, UK) as seen in Figure 3.10, from a previously reported method (Esterbaur et al., 1984). The CVs were <8% at 0.5µM and <10% at 2.5µM.

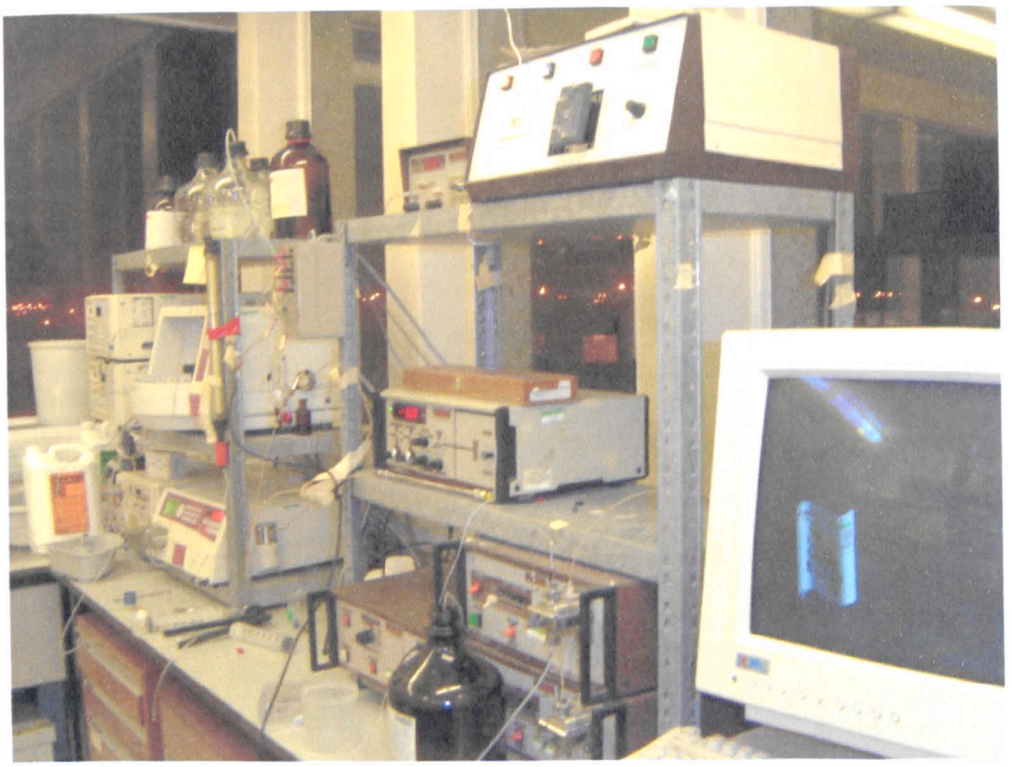


Figure 3.10 Gino 50 HPLC system.

3.7.5.3 Total glutathione (GSH)

Determination of total glutathione (GSH) concentration involved 100µl of EDTA treated blood being immediately deproteinised with 500µl of 10mM hydrochloric acid. This was then vortex mixed for 30 seconds and 300µl of cold 10% 5-sulfosalicylic acid (SSA) was added. The suspension was then mixed on a whirly mixer until it turned brown and the suspension centrifuged for 2.5 minutes at 4°C, 14,000rpm. The protein free supernatant was then retained for the assay of GSH (Anderson, 1985).

GSH concentration was measured using a 96-well micro-plate reader with kinetics (Figure 3.11). Once the samples had been defrosted, 20µl of the sample (or standard)

of this cocktail was then immediately added to each well. The plate was run at 412nm for 10 minutes. The required volumes of the various components of the cocktail varied according to the number of samples being analysed. These volumes can be seen below in table 3.5.

Table 3.5 Required amounts for the GSH cocktail. The daily buffer is the stock buffer (sodium phosphate, 125mM containing 6.3mM disodium EDTA, PH 7.5) plus 0.3mM NADPH (12.5mg/50ml), DTNB is 5,5'-dithiobis-2-nitrobenzoic acid, and the enzyme is glutathione reductase.

# Of Samples	10	20	30	40	50	60	100
Daily buffer	1.75ml	3.5ml	5.25ml	7ml	8.75ml	10.5ml	17.5ml
DTNB	250µl	500µl	750µl	1ml	1.25ml	1.5ml	2.5ml
dH_20	25µl	50µl	75µl	100µl	125µl	150µl	250µl
Enzyme	25µl	50µl	75µl	100µl	125µl	150µl	250µl

Principle

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP$$

$$2GSH + DTNB \rightarrow GSSG + TNB$$

The rate of the highly coloured TNB formation is followed at 412nm and is proportional to the amount of GSH present. The CV for GSH were <8.5% at 40 and 150mM.

Figure 3.11 Microplate reader used for the assessment of GSH

3.7.6 Total and Differential White Blood Cell Counts

For the measurement of white blood cells (WBC), samples were collected in tubes containing EDTA. The samples were kept at room temperature and analysed within 24 hours. WBC were quantified using an automated Coulter counter (Coulter® MAXM Analyser, Coulter Corporation, Miami, Florida) seen in Figure 3.12. The coulter counter counts the number and size of cells by detecting and measuring changes in electrical resistance when a particle in a conductive liquid passes through a small aperture. In order to classify differential WBC's, the coulter counter assesses each individual white cell using low frequency impedance for volume, high-frequency impedance to sense cellular internal content, and light scatter to detect the structure and shape of the cells. The system is then able to process this data and identify the

differential populations. The number of cells counted by the coulter counter is approximately 100 times more than microscope counting, thus significantly reducing the statistical error.

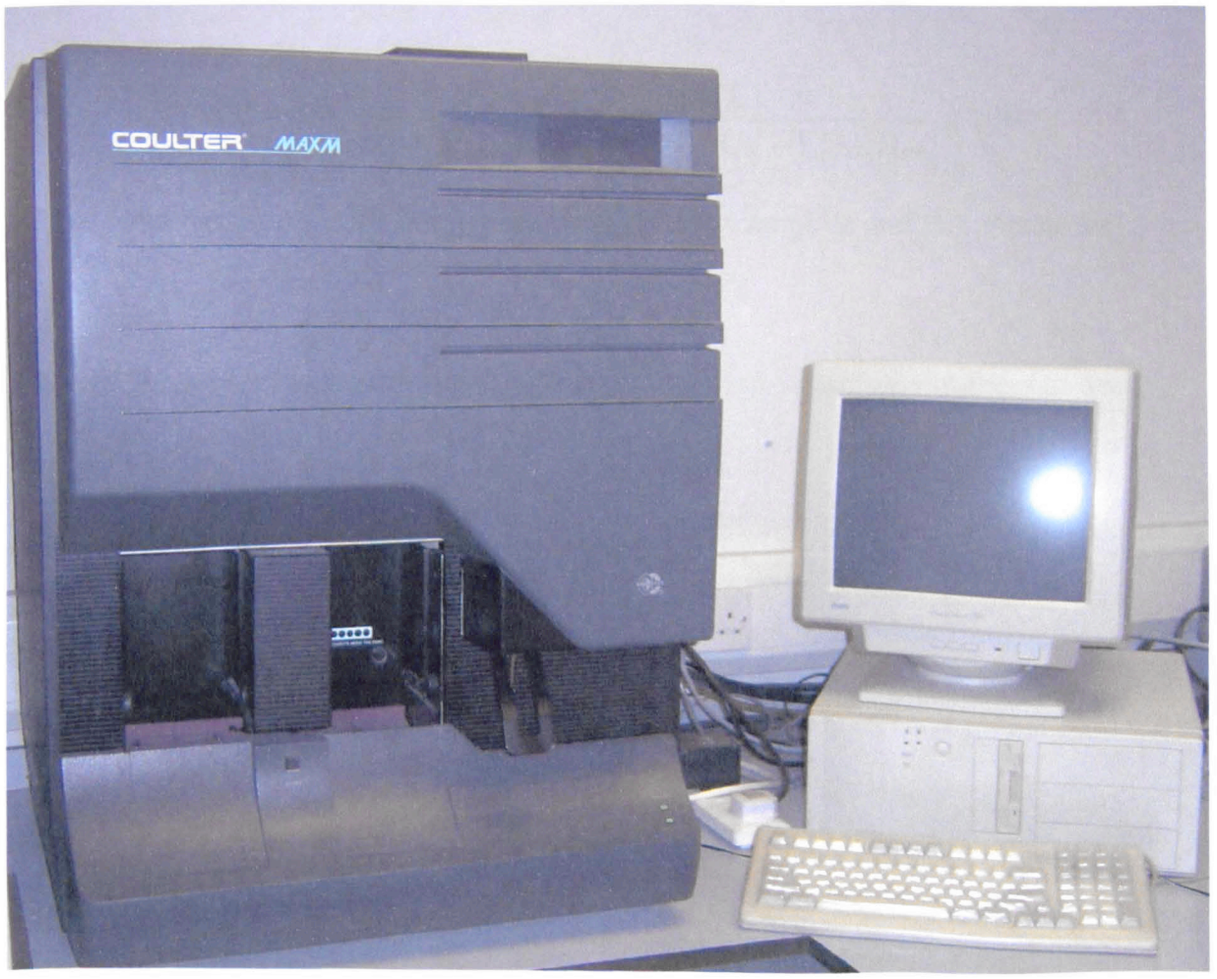


Figure 3.12 Automated coulter counter.

The total number of leukocytes is expressed as the total number of leukocytes measured, multiplied by the calibration constant:

$$WBC = n \times 10^3 \text{ cells}/\mu l$$

The differential counts are expressed as percentages as seen below:

$$NE\% = \frac{\text{Number of cells inside NE area}}{\text{Number of cells inside NE} + \text{LY} + \text{EO} + \text{BA}} \times 100$$

$$WBC = n \times 10^3 \text{ cells}/\mu l$$

The differential counts are expressed as percentages as seen below:

$$NE\% = \frac{\text{Number of cells inside NE area}}{\text{Number of cells inside NE} + \text{LY} + \text{EO} + \text{BA}} \times 100$$

$$LY\% = \frac{\text{Number of cells inside LY area}}{\text{Number of cells inside NE} + \text{LY} + \text{EO} + \text{BA}} \times 100$$

where NE = neutrophil, LY = lymphocyte, EO = eosonophils and BA = basophil.

The coulter counter also expresses differential counts in absolute numbers as seen below:

$$NE\ (10^3)\ \text{cells}/\mu l = \frac{NE\%}{100} \times \text{WBC count}$$

$$LY\ (10^3)\ \text{cells}/\mu l = \frac{LY\%}{100} \times \text{WBC count}$$

The CVs for:

WBC were <7% at 4 and $8x10^9$/L
NE were <9% at 2 and $5x10^9$/L
LY were <5% at 1 and $3x10^9$/L

3.7.7 Plasma Volume Change

All of the post exercise samples were corrected for changes in plasma volume according to the methods of Dill and Costill (1974). Haematocrit (Hct) and Haemoglobin (Hb) values were determined using an automated coulter counter as

described in section 3.7.6. Haematocrit measurements were calculated through summing the electronic volume of the erythrocyte measurements. It is reported that the electronic measurement of Hct is more accurate than traditional centrifugal measures as it does not have the trapped plasma error often associated with centrifugal measures. Haematocrit was calculated in the following way:

$$\text{Hct (\%)} = \frac{\text{Red Blood Cell x Mean Carpuscular Volume}}{10}$$

Haemoglobin was measured at 525nm and calculated in the following way:

$$\text{Hg (g/dl)} = \text{Constant x } \log_{10} \frac{\text{Reference \% T}}{\text{Sample \% T}}$$

Once Hb and Hct had been calculated, plasma volume changes (PVC) were determined using the methods of Dill and Costill (1974). A summary of this method can be seen below:

Change in blood volume (BV):

$$BV_A = BV_B \times \frac{Hb_B}{Hb_A}$$

Change in red cell volume (CV):

$$CVA = BV_A \times Hct_A$$

Change in plasma volume (PV):

$$PV_A = BV_A - CV_A$$

In order to correct the blood samples for changes in PVC, the percentage change in plasma volume had to be calculated. This was done as shown below:

Change in blood volume (%)

$$\Delta BV\ \% = 100\ (BV_A - BV_B) / BV_B$$

Change in red cell volume (%)

$$\Delta CV\% = \frac{CV_A - CV_B}{CV_B} \times 100$$

Change in plasma volume (%):

$$\Delta PV\% = \frac{PV_A - PV_B}{PV_B} \times 100$$

where $_A$ denotes before exercise and $_B$ denotes after the exercise.

The CVs for haematocrit were <6% and for haemoglobin were <4.5%.

3.7.8 Ascorbate

An aliquot of plasma (0.6ml) was immediately added to an equal amount of cold 10% metaphosphoric acid (Sigma Chemical Co. Ltd UK). This was vortex mixed and then immediately frozen at –70°C until the supernatant was analysed for ascorbate

concentration using HPLC as described by Thompson et al. (2001). The CV for ascorbate were <10% at 40 and 150μM.

CHAPTER 4 – Study 1

The effects of downhill running on delayed onset muscle soreness, muscle function and production of reactive oxygen species.

4.1 Introduction

Unaccustomed muscular exercise results in trauma to the contractile components of skeletal muscle (Dop Bar et al., 1997) often causing localised sensations of pain and discomfort. The symptomology of the pain usually manifests itself in the hours and days post-exercise and is termed Delayed Onset Muscle Soreness (DOMS) (Armstrong, 1990). Important corollaries of increased ratings of DOMS include decreased exercise performance, increased muscle stiffness, and loss of force development (Cleak et al.,1992). This type of muscle soreness is particularly pronounced if the exercise involves eccentric muscle actions (Newham, 1988), and poses particular problems for athletes beginning a new training regimen or returning from injury, as well as for sedentary individuals who try to increase their level of physical activity (Jones et al., 1986).

Armstrong et al. (1983) first demonstrated that eccentric exercise induces significantly more DOMS than all other modes of exercise, and since then various forms of eccentric exercise have been employed to induce muscle soreness in both animal (Armstrong et al., 1983; Komulainen et al., 1999) and human (McBride et al., 1999) models. Downhill running involves eccentric exercise and consequently results in greater muscle damage compared to running on the flat. It is therefore an effective and well-documented way of inducing DOMS in a laboratory setting (Clarkson et al., 1986).

The process of DOMS has been well documented, although there is a failure to agree on a common mechanism (Sorichter et al., 1999). The aetiology of DOMS has been attributed to the establishment of an acute phase inflammatory response resulting from metabolic, mechanical or oxidative stress (Pyne, 1994). It has also been shown that intense muscular activity increases the production of Reactive Oxygen Species (ROS) (Maughan et al., 1989) which is associated with skeletal muscle damage (Jackson, 1992), and a subsequent decrease in physical performance (Jakeman and Maxwell, 1993).

There is growing evidence that ROS are involved in the muscular damage observed following strenuous or unaccustomed exercise in both human (Ashton et al., 1999; Fantone, 1985; Maughan et al., 1989; Thompson et al., 2001a) and animal (Duarte et al., 1994; McArdle et al., 1999; Mills et al., 1996; Van Der Meulen et al., 1997) models. However, as yet there is limited literature on the role of ROS production in the aetiology of DOMS.

Most research into ROS and exercise induced muscle damage has centred on post-exercise measurements of ROS, with few studies measuring production beyond 24 hours post exercise (Brickson et al., 2001; Saxton et al., 1994; Warren et al., 1992). In one of the few studies to measure ROS beyond 24 hours post-exercise, Warren et al., (1992) concluded that future studies should measure ROS production beyond 48 hours post-exercise since it is possible that if ROS are involved in the phagocytosis of damaged tissue, they may peak later.

A further problem in research on ROS and DOMS is that most of the studies have used indirect markers of lipid peroxidation such as Malonaldehyde (MDA) production, conjugated diene formation, and expired breath pentane and ethane (Lovlin et al., 1987). Although these techniques are useful, they have been criticised for their lack of specificity and reproducibility (Kanter1998). The most specific and direct method for determining ROS production is Electron Spin Resonance (ESR) spectroscopy, although few studies have utilised this technique in an exercise setting using human volunteers (Ashton et al., 1998; Ashton et al., 1999; Groussard et al., 2003).

The aim of this study was to directly and indirectly assess ROS production immediately and for a period of 72 hours after running on the flat and running downhill, and thereby elucidate the role that ROS play in DOMS. In order to fully investigate the relationship between DOMS and ROS, ratings of pain and functional changes in the muscle should also be observed.

4.2 Methods

4.2.1 Subjects

Eight physically active male subjects, unaccustomed to downhill running volunteered for this study. All were non-smokers and free from any known illnesses as ascertained by a medical questionnaire. Subjects taking antioxidants were excluded. All subjects were informed verbally and in writing about the nature of this study, including all potential risks. Written informed consent was obtained prior to participation and ethical approval was granted by the Ethics committee of Liverpool John Moores University.

4.2.2 Experimental protocol

Each subject was initially assessed for aerobic fitness by determining their $\dot{V}O_{2max}$ as outlined in chapter 3.2. Subjects then participated in two experimental runs, both lasting for 30 minutes at a running speed corresponding to 65% $\dot{V}O_{2max}$. On one occasion the run was performed on a 15% downhill gradient (DWN), whilst the other was performed on the flat (FLA). All subjects were randomly allocated into two groups with one group performing DWN first whilst the other group performed FLA first. The conditions were counterbalanced and there was a 5-week gap between trials to ensure muscle recovery (Harris et al., 1975). For each trial, subjects were required to visit the laboratories on four consecutive days. Day 1 was to perform the experimental run, to have torque and pain measurements taken, and

to give pre and post exercise venous blood samples. The following 3 days were to have muscle function and pain assessed, and to give a resting venous blood sample.

4.2.3 Day 1.

Subjects arrived at the laboratory between 09:00 and 10:30 h. They were instructed to follow their usual dietary pattern prior to the test and to avoid alcohol in the preceding 24 hours. Subjects were also required to record their dietary intake for the 24 hours prior to the test including the morning of the test, and repeat this same diet for the second visit. All subjects were required to avoid exercise in the 24 hours prior to the test and to avoid strenuous exercise for 72 hours after the completion of the run. Following 30-minutes supine rest, a pre-exercise blood sample was taken from an antecubital forearm vein.

Blood samples were collected in Monovette collection tubes (Sarstedt, Germany) following an overnight fast from a superficial vein in the antecubital fossa whilst the subjects were supine and had been resting for a minimal of 30 minutes (except for the post exercise sample which was taken immediately upon cessation of exercise). Samples were taken using minimal stasis to prevent any potential increase in oxidative stress due to ischemic-reperfusion injury (Bailey et al., 2000). This was achieved by taking samples without a tourniquet. A total of four Monovette tubes were used at each sample point.

Tube 1 contained the anticoagulant EDTA and was used for the analysis of leukocytes and glutathione (GSH). Tube 2 contained serum separation gel and was used for the analysis of MDA. Tube 3 (5.5ml) contained serum separation gel along with 1.5ml of the spin trap α-phenyl-*tert*-butylnitrone (PBN) (Sigma, Dorset, UK) and was used for the analysis of ROS using ESR spectroscopy. Tube 4 contained the anticoagulant lithium heparin and was used for the analysis of creatine kinase. Details of the methods used in the blood analysis can be seen in chapter 3.7.

Following the blood sample, subjects were asked to rate their pre-exercise muscle soreness of the gastrocnemious, tibialis anterior, hamstrings, quadriceps, gluteals, trapezius (both left and right sides), and lower back muscles using pain diagrams, with 0 = no pain, 10 = unbearable pain. All subjects had been fully familiarised to the pain diagrams. Details of the assessment of DOMS can be seen in chapter 3.5 Subjects then proceeded to warm up on a cycle ergometer for 5 minutes before completing a series of stretches of their own choice. The stretches were to prepare the subjects for assessment of muscle function.

Muscle function was assessed using an isokinetic leg dynamometer (Lido Active, Loredan) to which subjects had been previously familiarised. Details of the muscle function assessment can be seen in chapter 3.4.

The experimental runs (DWN or FLA) took place on the same motorised treadmill (HP Cosmos Germany) used in the $\dot{V}O_{2max}$ test. The run lasted for 30-minutes at an oxygen consumption corresponding to 65% $\dot{V}O_{2max}$. Throughout the trial, expired gas volumes and concentrations were recorded every 10 seconds. The treadmill speed was continually altered to ensure that the oxygen consumption corresponding to 65% of $\dot{V}O_{2max}$ was maintained. The downhill run was performed at a gradient of -15% with a harness connected to an automatic cut off switch to reduce any potential risks. Every 10 minutes, Ratings of Perceived Exertion (RPE) were recorded using the 6-20 Borg Scale (Borg, 1982), as well as Heart Rate (HR) using a short-range radio telemeter (Polar, Sportstester, PE3000, Kempele, Finland). Laboratory temperature ranged between 18-21°C at a relative humidity of 40-45%. Thermal comfort was maintained throughout the trial by means of two electronic fans (MS16, Amcon).

Immediately following the run, subjects assumed a supine position whilst a second blood sample was taken using the same technique as described previously. Subjects were then re-assessed regarding their perceptions of muscle soreness before completing the muscle function assessment again. This completed day 1 of the trial.

4.2.4 Days 2,3 and 4.

Subjects visited the laboratory exactly 24, 48 and 72 hours after their initial visit. They were seated for 30 minutes before a resting blood sample was taken from a prominent vein. The blood test took place at the same time as the pre-exercise blood

test to eliminate any time of day effect (Fernandes, 1992). The samples were treated in an identical manner as described previously. Following the blood tests, subjects were then assessed for DOMS using the same technique as previously described. This was followed by a five minute warm up on a cycle ergometer before leg torque was assessed as previously described. All subjects then undertook 5-weeks recovery before repeating the test using the second condition. A summary of the test protocol can be seen in figure 4.1

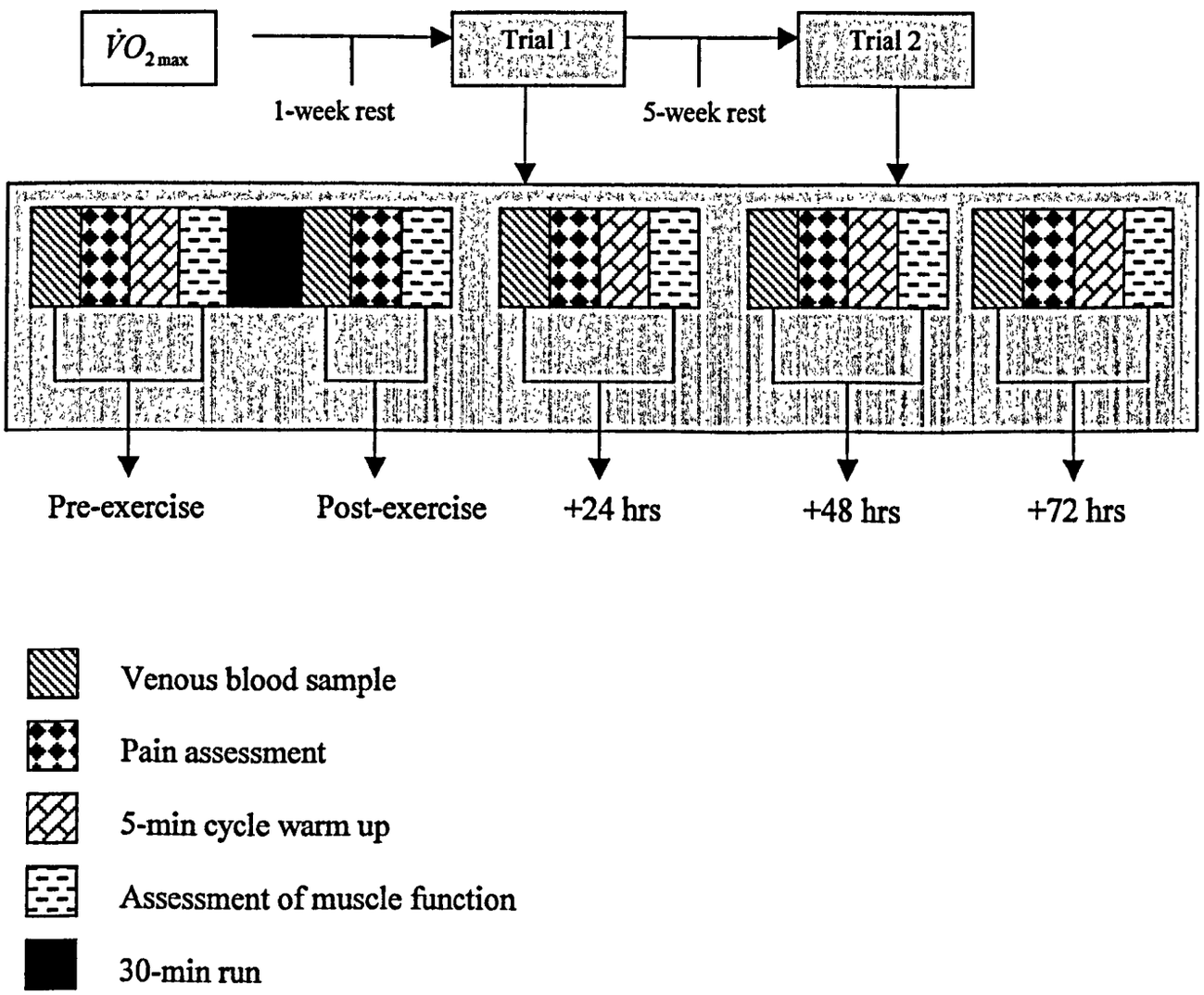


Figure 4.1 Summary of the test protocol

4.2.5 Statistical analyses

Statistical analyses was carried out using the Statistical Package for Social Sciences (SPSS Surrey, UK). All data are presented as means ± SEM. A two way ANOVA with repeated measures was used to analyse all variables. When Mauchley's test of sphericity indicated a minimal level of violation (>0.75) the degrees of freedom was corrected using the Huynh-Feldt adjustment. When the sphericity was <0.75, the Greenhouse Geiser correction was used (Field, 1999). Post Hoc Tukey analysis (Honestly Significant Difference, (HSD)) was performed to identify where the significant differences occurred. Statistical significance was set at $P<0.05$ for all tests.

4.3 Results

4.3.1 Subject characteristics

Prior to the experimental runs, all subjects were initially assessed for $\dot{V}O_{2max}$ as well as having their height, weight and percentage body fat calculated. Subject characteristics can be seen in Table 4.1.

Table 4.1 Mean (±SD) characteristics of the subjects

N	Age (years)	Body mass (kg)	Height (cm)	$\dot{V}O_{2max}$ ($L.min^{-1}$)	Body Fat (%)
8	24.8 (± 3.0)	80.6 (± 5.4)	181 (± 5.8)	4.5 (± 0.42)	15.6 (± 3.2)

4.3.2 Run data

All subjects successfully completed the 30-minute run for both FLA and DWN. Although there was no statistical difference in $\dot{V}O_2$ between the two groups, it was noticed that there was a trend for the $\dot{V}O_2$ to be lower in DWN than FLA. DWN was deemed to be more physically demanding than FLA, reflected in a significantly greater RPE (14.8 ± 2.4 vs. 11.8 ± 1.0 DWN vs. FLAT), greater heart rate (166 ± 4 vs. 158 ± 3), albeit it not statistically significant ($P>0.05$). Furthermore, all subjects exhibited a loss of control of the lower limbs upon completion of DWN, a condition often reported following DOMS inducing exercise. Mean $\dot{V}O_2$, running speed, RPE and heart rates can be seen in figures 4.2-4.5

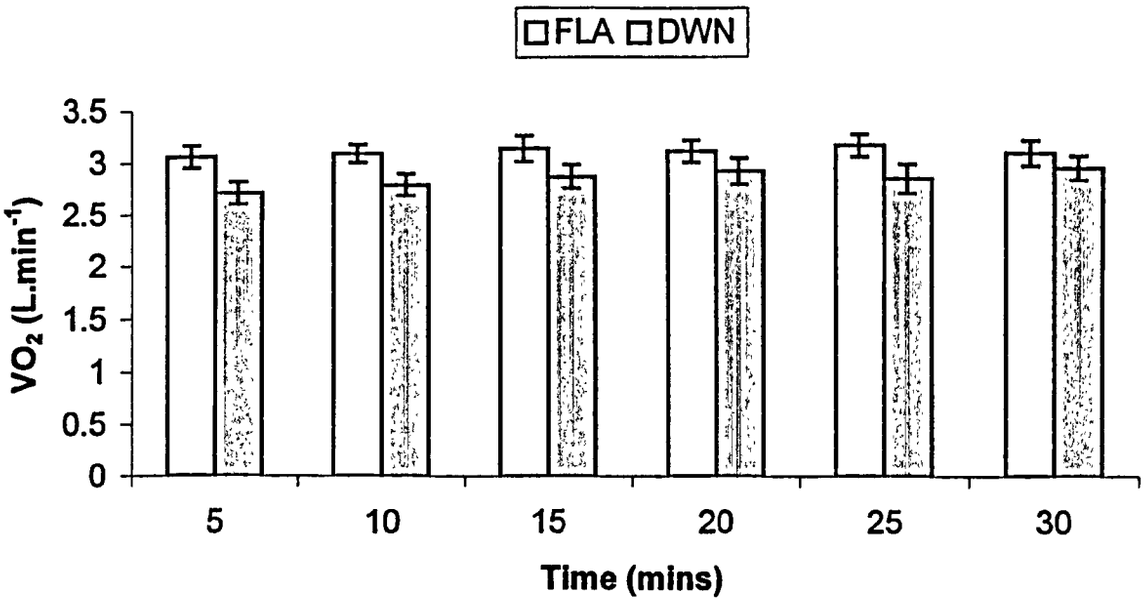


Figure 4.2 Mean (±SEM) oxygen uptake during the experimental runs.

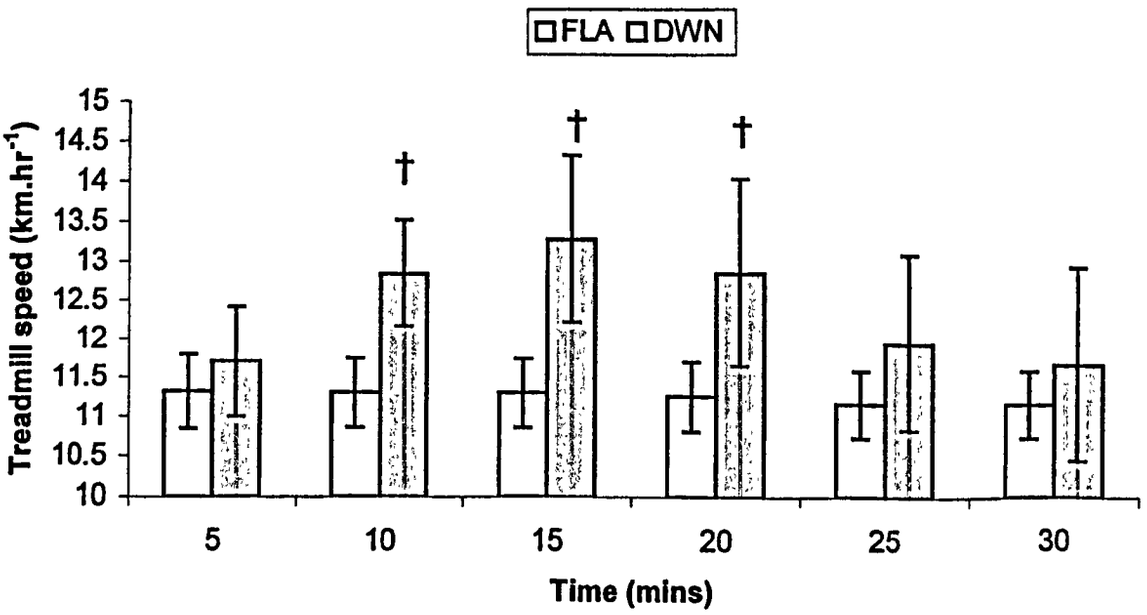


Figure 4.3 Mean (±SEM) treadmill speed during the experimental runs
† indicates significant difference from FLA

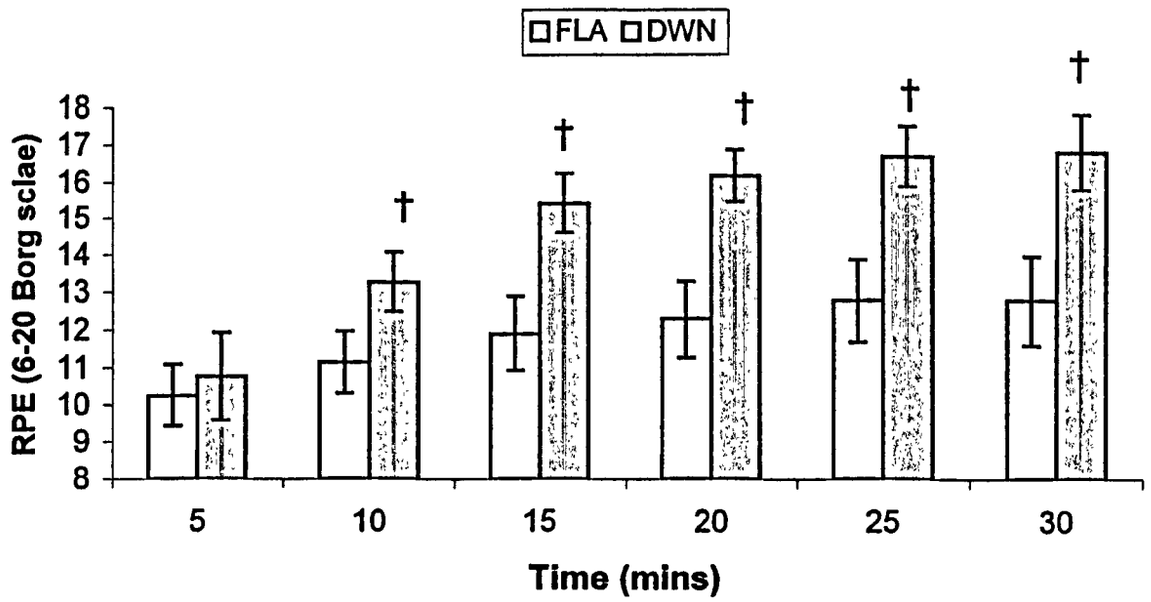


Figure 4.4 Mean (±SEM) ratings of perceived exertion during the experimental runs. † indicates significant difference from FLA

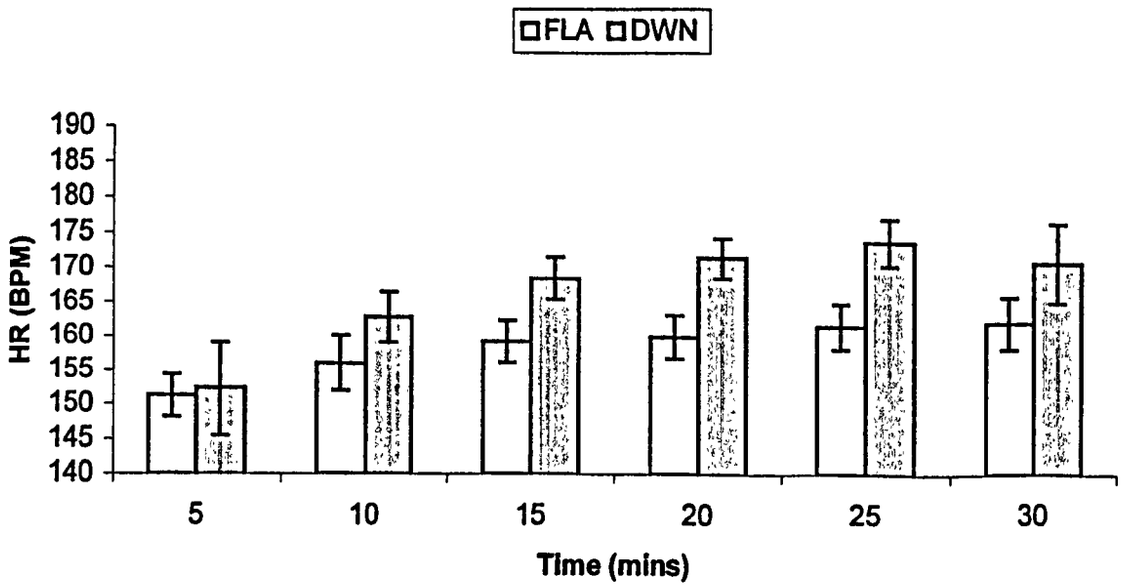


Figure 4.5 Mean (±SEM) heart rates (b.min^{-1}) during the experimental runs

4.3.3 Delayed onset muscle soreness

Mean (±SEM) total ratings of DOMS from the seven assessed sites for FLA and DWN across the time period are reported in Figure 4.6. The downhill run resulted in

a significant increase in DOMS ($P<0.05$) across time, with peak ratings of soreness occurring 48 hours post exercise. Muscle soreness was still significantly elevated at 72 hours ($P<0.05$) although it had significantly reduced from that expressed at 48 hours ($P<0.05$). There were no changes in ratings of DOMS following FLA.

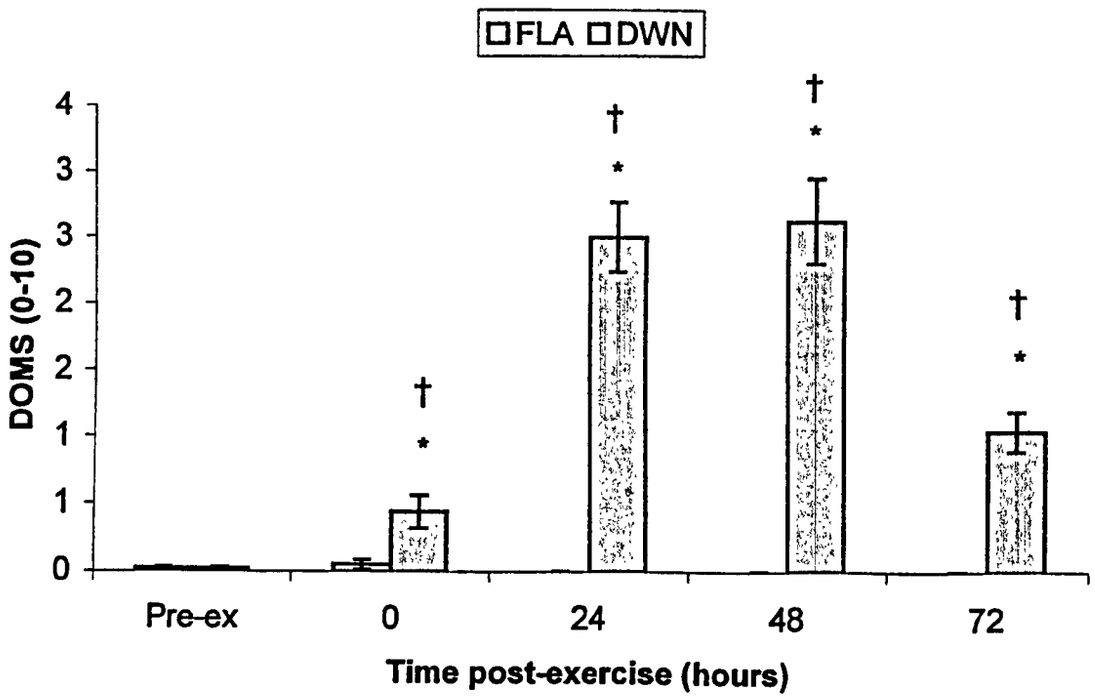


Figure 4.6 Mean (±SEM) total ratings of DOMS using pain diagrams (0-10)
*** indicates significant difference from Pre Exercise (Pre Ex)**
† indicates significant difference from FLA

4.3.4 Reactive Oxygen Species

4.3.4.1 Direct Assessment

Direct assessment of ROS was carried out using ESR spectroscopy combined with a spin trapping technique. There was a significant difference ($P<0.05$) in the signal intensity of the PBN adduct obtained from the ESR spectra between the two conditions (Figures 4.7-4.9). Post Hoc analysis confirmed that the increase observed

following DWN was between 48 hours and 72 hours post exercise ($P<0.05$). There was no significant change ($P>0.05$) at any of the other time points nor was there a significant change in the intensity of the PBN adduct following FLA. The intensity of the PBN adduct was still rising at 72 hours post-exercise so the present study cannot report whether ROS production peaked at 72 h following DWN.

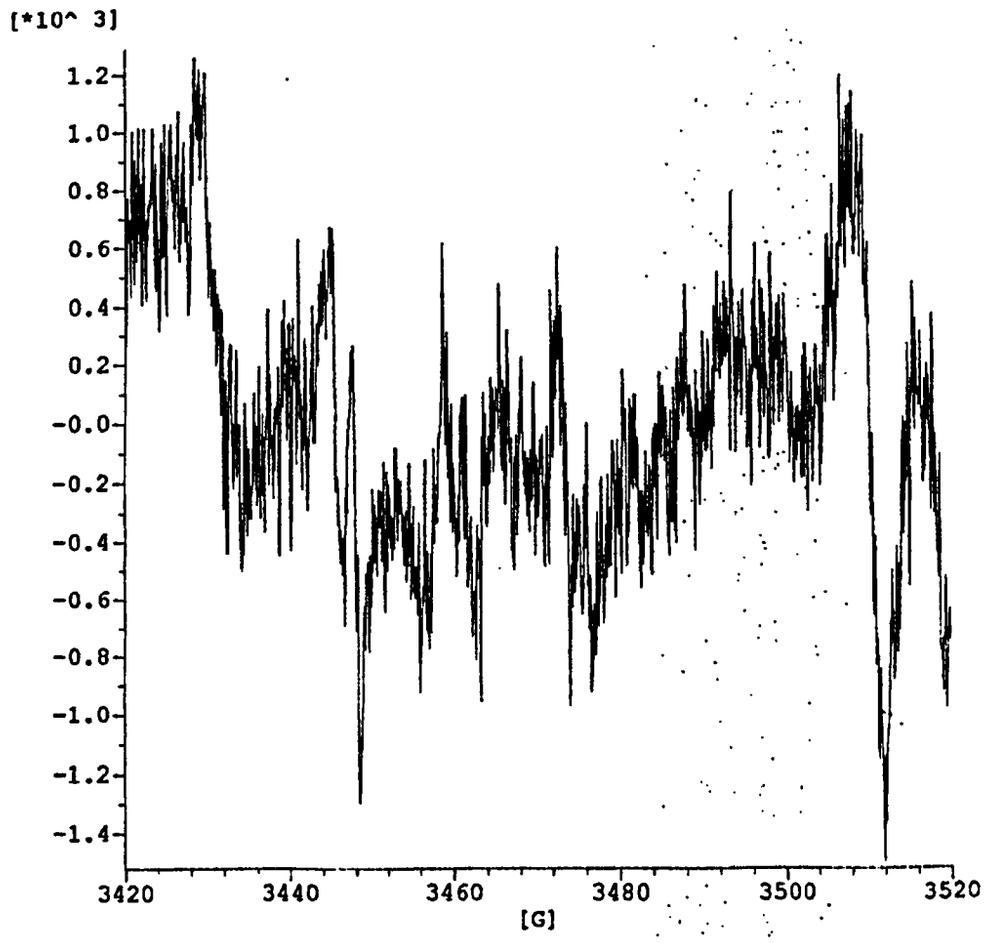


Figure 4.7 Sample ESR spectra taken pre-exercise

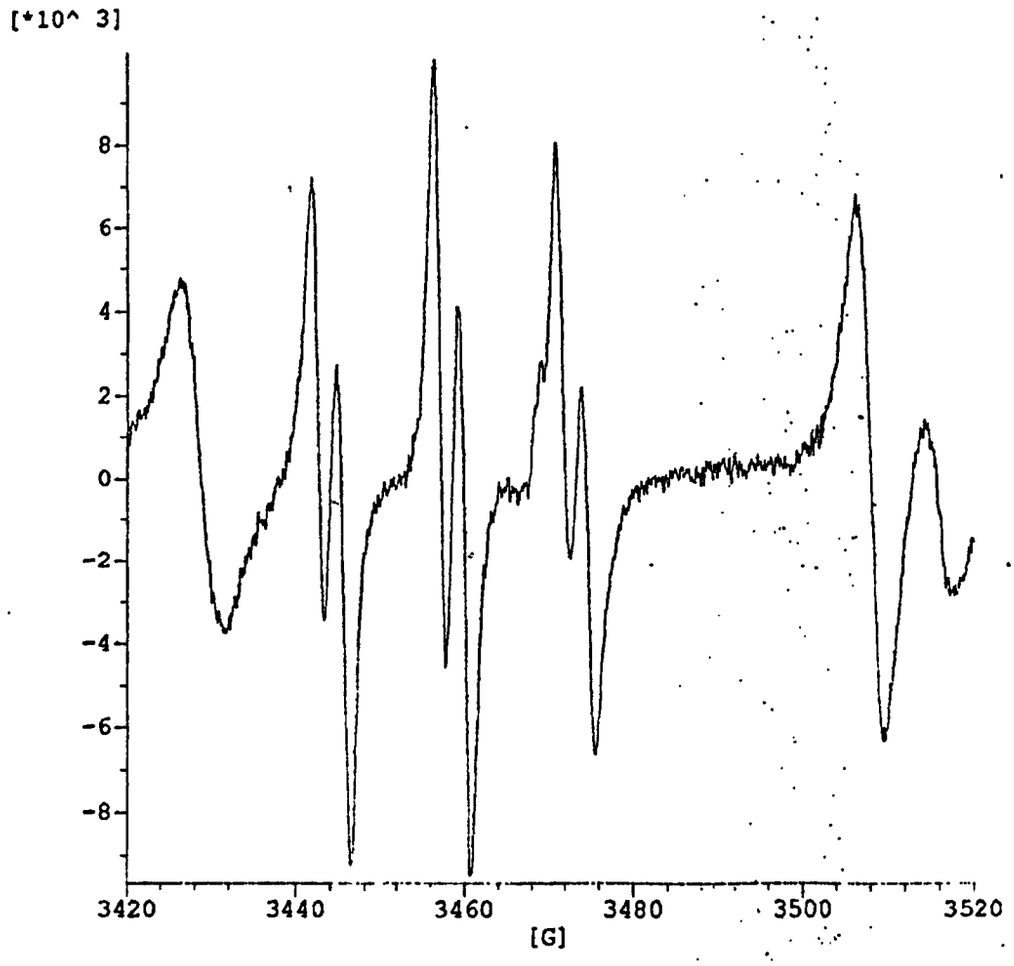


Figure 4.8 Sample ESR spectra taken 72 hours post-exercise showing the characteristic triplet of doublets of nitroxide radical adducts of PBN

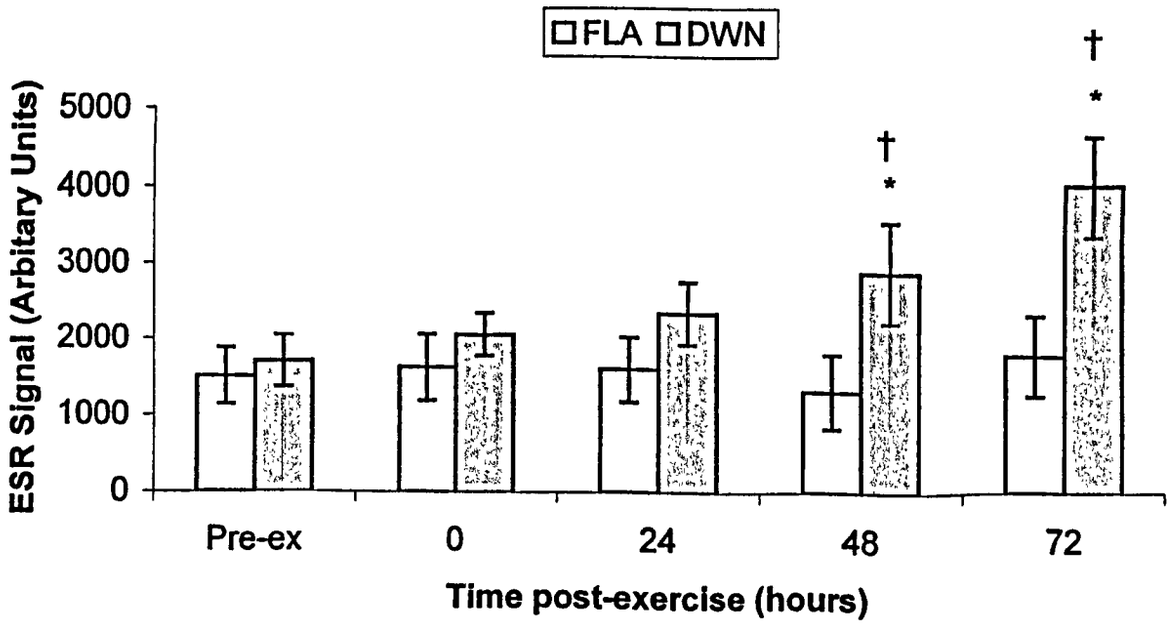


Figure 4.9 Mean (±SEM) intensity of the PBN adduct detected by ESR spectroscopy.
*** indicates significant difference from Pre Exercise (Pre Ex)**
† indicates significant difference from FLA

4.3.4.2 Indirect assessment of ROS

Serum MDA concentrations follow a similar pattern to that evident in the ESR spectroscopy. There was a significant difference in MDA concentration between the two conditions ($P<0.05$) with an increase in the concentration of MDA following DWN ($P<0.05$), occurring at 72 hours post exercise. No significant change in the MDA concentrations was noted at any time point following FLA ($P>0.05$), as seen in Figure 4.10. Observations show that MDA concentrations were increasing at 72 hours post-exercise.

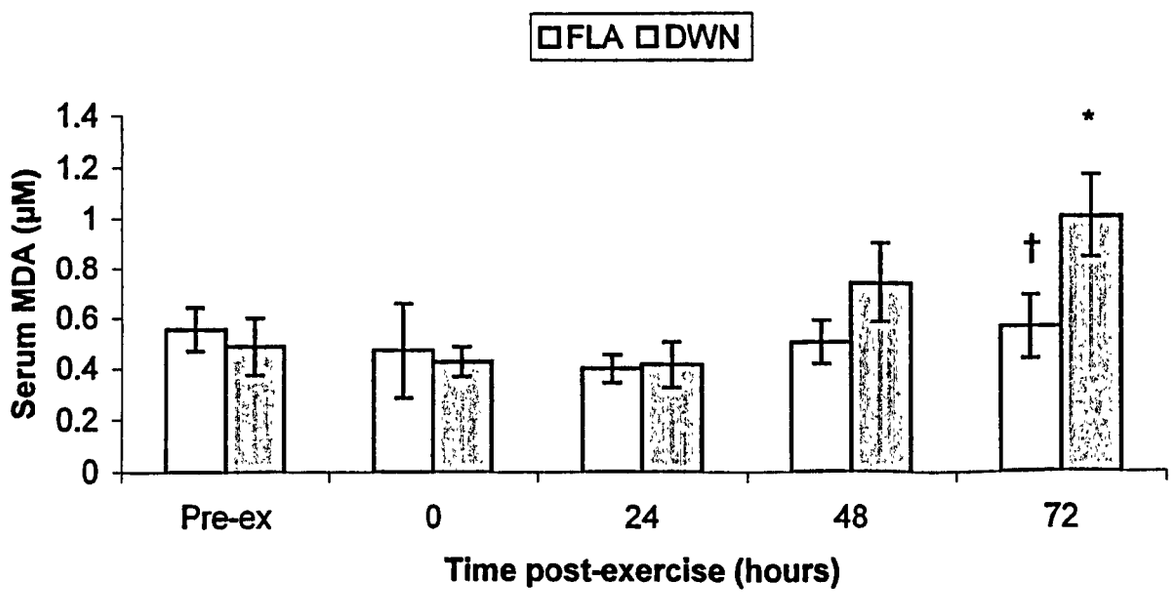


Figure 4.10 Mean (±SEM) serum MDA concentration
*** indicates significant difference from Pre Exercise (Pre Ex)**
† indicates significant difference from FLA

There was no significant difference in total blood glutathione (GSH) across time or between the two groups ($P>0.05$) although there was a trend for GSH to fall 72 hrs post-exercise (Figure 4.11).

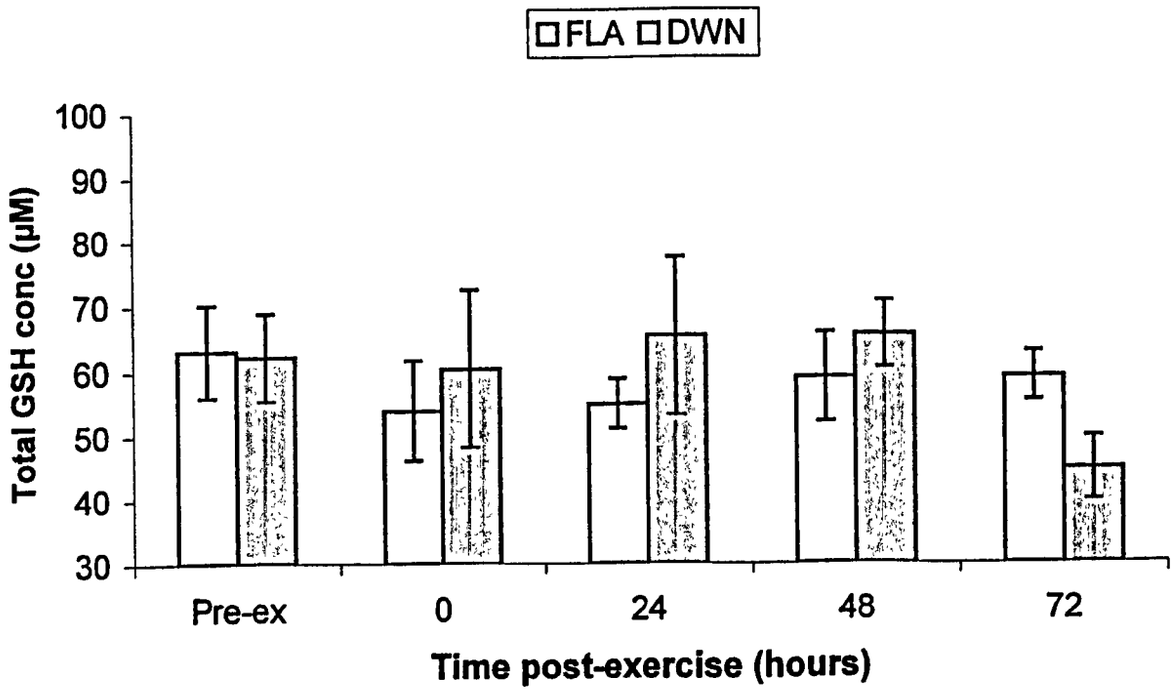


Figure 4.11 Mean (±SEM) total blood glutathione (GSH) concentration.

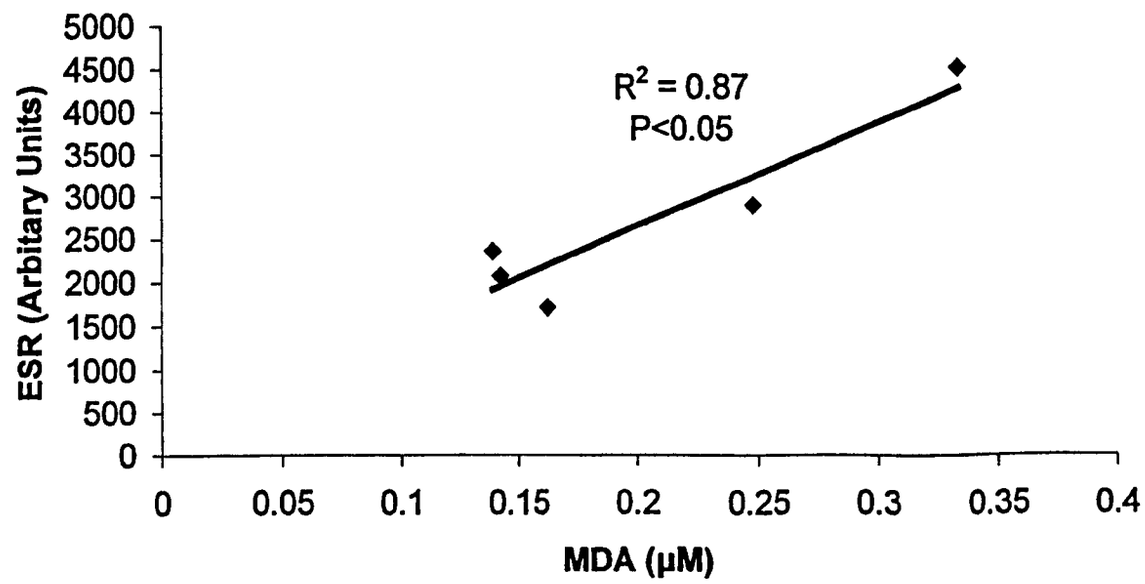


Figure 4.12 Correlation between mean ESR signal and mean MDA concentration at the 5 assessed time points.

Figure 4.12 shows that the correlation between mean MDA concentration and mean ESR signal at the five assessed time points was significant ($R^2 = 0.87$, $P<0.05$).

4.3.5 Muscle damage

Plasma creatine kinase (CK) activity was significantly elevated following DWN compared to FLA ($P<0.05$). Peak CK activities were found 24 hours post-exercise following DWN, and although a lower activity was evident 48 hours post-exercise, this was still significantly greater than pre-exercise ($P<0.05$). No significant changes were noted after FLA ($P>0.05$). Figure 4.13 shows the results in serum CK.

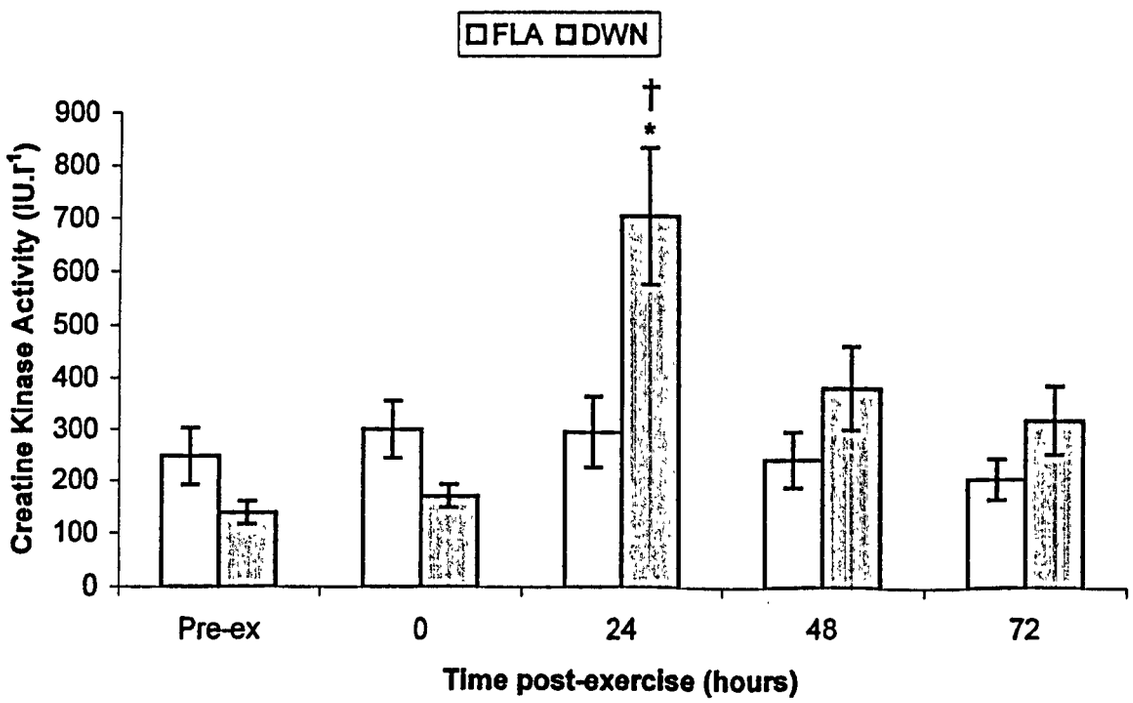


Figure 4.13 Mean (±SEM) plasma creatine kinase (CK) activity.
*** indicates significant difference from Pre Exercise (Pre Ex)**
† indicates significant difference from FLA

4.3.6 Muscle function and DOMS felt during muscle function tests

Isokinetic quadriceps torque for all trials are presented in Figures 4.14 – 4.16. Quadriceps torque was significantly reduced after DWN for all of the measures ($P<0.05$), demonstrating a loss of maximum torque as well as a loss in torque at fast functional speeds. Losses of torque peaked at 24 hours post exercise (~22%, 14% and 12% for concentric 1.04 rad.sec^{-1}, concentric 5.2 rad.sec^{-1} and eccentric 2.08 rad.sec^{-1} respectively) and did not return to pre exercise levels until 72 hours post exercise. There was no significant change in leg torque following FLA.

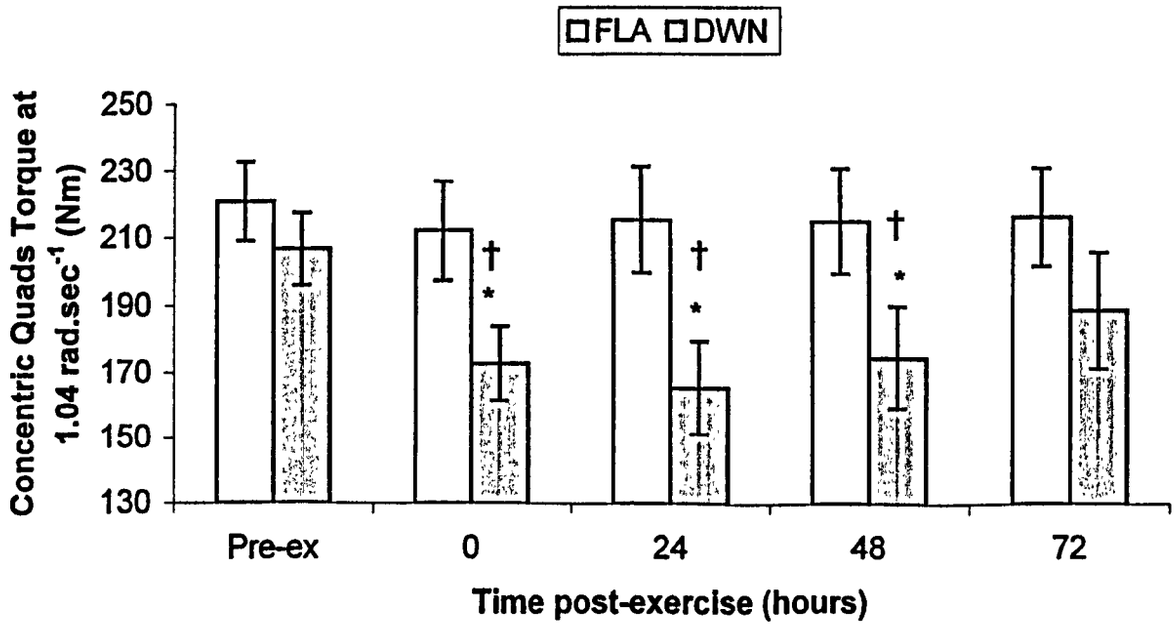


Figure 4.14 Mean (±SEM) concentric quadriceps torque measured at 1.04 rad.sec^{-1}.
*** indicates significant difference from Pre Exercise (Pre Ex)**
† indicates significant difference from FLA

Table 4.2 Mean (±SEM) DOMS felt during concentric contraction at 1.04 rad.sec^{-1}
*** indicates significant difference from Pre Exercise (Pre Ex)**
† indicates significant difference from FLA

Time post-ex (hours)		Pre Ex	0	24	48	72
Pain	FLA	0 (± 0.0)	0.25 (± 0.16)	0.25 (± 0.16)	0 (± 0.0)	0 (± 0.0)
	DWN	0 (± 0.0)	0.75 (± 0.41)*†	1.75 (± 0.52)*†	2 (± 0.68)*†	1 (± 0.37)*†

Tables 4.2 - 4.4 shows the mean (±SEM) ratings of DOMS felt during the muscle function assessment. There was significantly more DOMS reported following all three muscle function assessments in DWN than FLA ($P<0.05$).

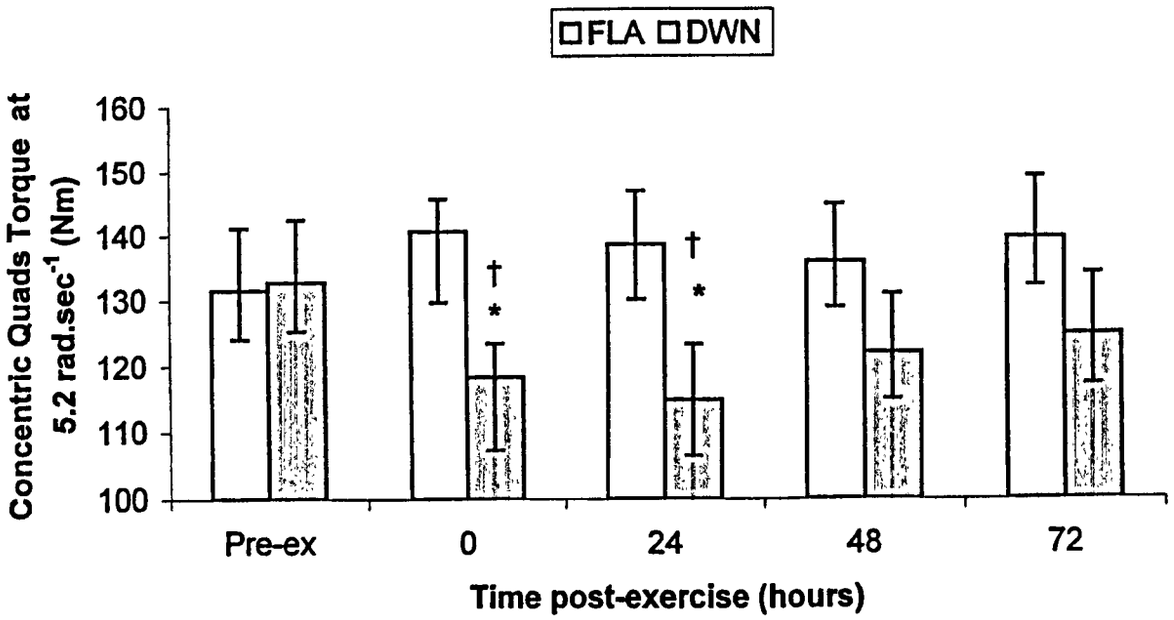


Fig 4.15 Mean (±SEM) concentric quadriceps torque measured at 5.20 rad.sec^{-1}
*** indicates significant difference from Pre Exercise (Pre Ex)**
† indicates significant difference from FLA

Table 4.3 Mean (±SEM) DOMS felt during concentric contraction at 5.20 rad.sec^{-1}
*** indicates significant difference from Pre Exercise (Pre Ex)**
† indicates significant difference from FLA

Time post-ex (hours)		Pre-ex	0	24	48	72
Pain	FLA	0.0 (± 0.0)	0.3 (± 0.5)	0.0 (± 0)	0.3 (± 0.5)	0.0 (± 0)
	DWN	0.0 (± 0.0)	1.1 (± 0.5)*†	1.5 (± 0.5)*†	1.8 (± 0.9)*†	0.8 (± 0.5)*†

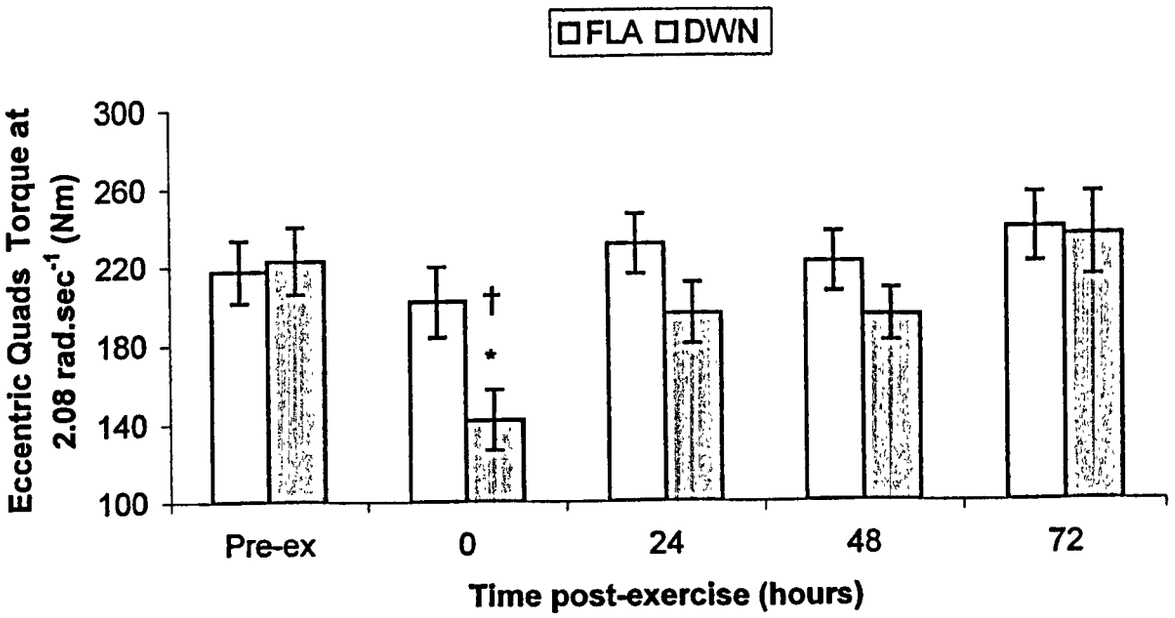


Figure 4.16 Mean (±SEM) eccentric quadriceps torque measured at 2.06 rad.sec^{-1}
*** indicates significant difference from Pre Exercise (Pre Ex)**
† indicates significant difference from FLA

Table 4.4 Mean (±SEM) DOMS felt during eccentric contractions at 2.06 rad.sec^{-1}
*** indicates significant difference from Pre Exercise (Pre Ex)**
† indicates significant difference from FLA

Time post-ex (hours)		Pre-ex	0	24	48	72
Pain	FLA	0.2 (± 0.16)	1.6 (± 0.6)	1.1 (± 0.5)	0.6 (± 0.5)	0.13 (± 0.1)
	DWN	0.2 (± 0.12)	2.4 (± 0.6)*†	3.0 (± 1.1)*†	2.5 (± 1.0)*	1.5 (± 0.7)

4.3.7 Leukocytes

The DWN protocol resulted in a significant leukocytosis ($P<0.05$) which was predominantly due to a transient increase in circulating neutrophil numbers ($P<0.05$), as well as a transient increase in circulating lymphocyte numbers (Figure 4.17 - 4.19). All of the observed increases in leukocyte variables occurred immediately

post exercise, returning to pre exercise levels by 24 hours. There were no significant changes in monocytes, eosinophils, or basophills following DWN, or any significant changes in total or differential circulating leukocyte numbers following FLA ($P>0.05$).

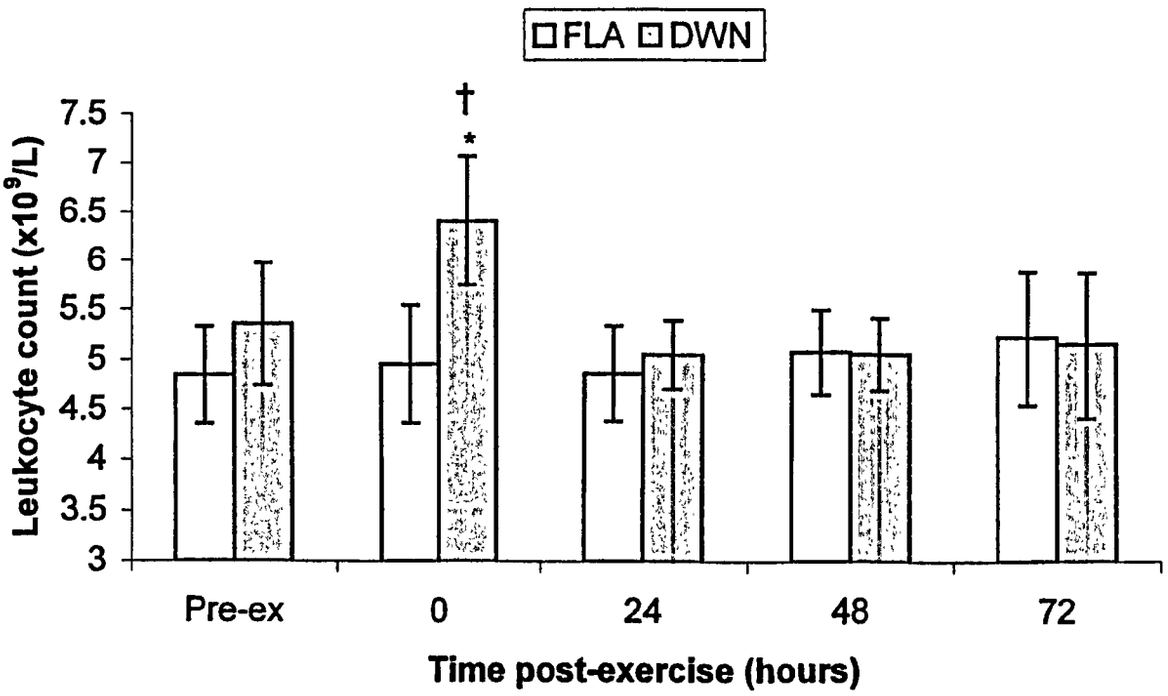


Figure 4.17 Mean (±SEM) leukocyte count (x10^9/L).
*** indicates significant difference from Pre Exercise (Pre Ex)**
† indicates significant difference from FLA

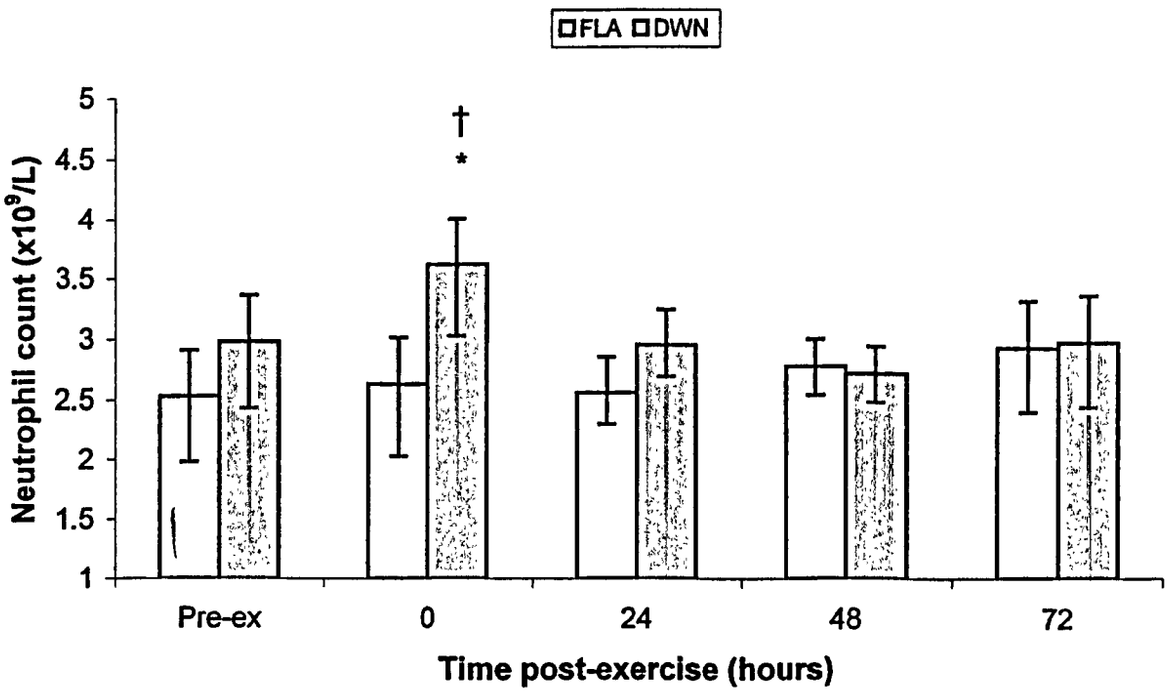


Figure 4.18 Mean (±SEM) neutrophil count (x10^9/L).
*** indicates significant difference from Pre Exercise (Pre Ex)**
† indicates significant difference from FLA

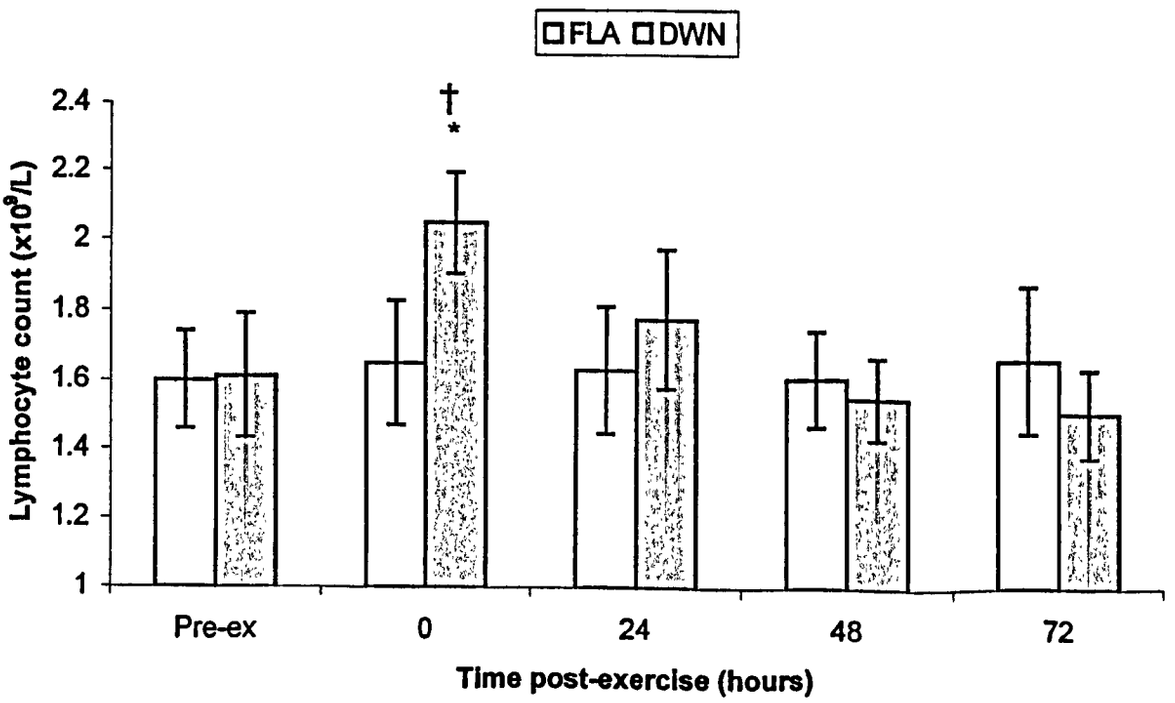


Figure 4.19 Mean (±SEM) lymphocyte count (x10^9/L).
*** indicates significant difference from Pre Exercise**
† indicates significant difference from FLA

4.4 DISCUSSION

The present research confirmed that 30-minutes of downhill running induces significantly greater DOMS and loss of muscle function compared to 30-minutes running on the flat at an equivalent oxygen uptake. However, the major novel finding from this research is that downhill running results in an increase in ROS production where as FLA fails to do so. The increase in ROS following DWN is observed between 48 and 72 hours post exercise and this increase occurs when mean ratings of DOMS are subsiding, and also when muscle torque has returned to near pre-exercise levels. One interpretation of this finding is that the production of ROS are not solely responsible for the DOMS and/or the observed loss of torque, although it is also possible that a "washout" effect may occur as part of muscle cell regeneration causing the radicals to be detected in increasing amounts over time.

The major aim of this study was to investigate the role of ROS in the onset and development of DOMS. It has been proposed that since ROS have the ability to induce skeletal muscle damage, a condition observed following eccentric exercise (Dop Bar et al., 1997), they may be involved in the onset of DOMS (Brown et al., 1997). Despite this fact there is lack of consensus regarding the role of ROS in exercise induced muscle damage, and the extant data is conflicting (McArdle et al., 2002).

A significant increase in the intensity of the ESR spectra as well as a significant increase in serum MDA concentration following DWN were observed, whilst there was no change following FLA. This demonstrates increased lipid peroxidation and an up-regulation of ROS production. Furthermore, there was also a fall (albeit not a statistically significant fall) in total GSH concentration at 72 hours. GSH is a sacrificial antioxidant and therefore a fall in GSH implies an antioxidant response to an oxidative challenge. It is possible that the observed decrease in GSH level could be due to its use in regenerating ascorbic acid and α-tocopherol, as well as being used to directly scavenge certain ROS (Ji, 1995). This is the first time that an up-regulation of ROS production has directly been assessed following downhill running, although it has been previously reported following other forms of exercise (Davies et al.,1982; Jackson et al.,1985).

It is possible to identify the free radicals detected by ESR spectroscopy through a detailed analysis of the spectra. The ESR spectra presented as six peaks of equal intensity, characteristic of nitroxide radical adducts of PBN (Figure 4.6). Hyperfine coupling constants recorded from the ESR spectra were $a_{\beta H}$: 1.9 gauss and a_N: 13.7 gauss. This would identify the species as alkoxyl radicals presumably derived from peroxide decomposition and/or oxidatively damaged membrane lipids. The values for the hyperfine coupling constants are consistent with literature reports using similar subjects during exercise (Ashton et al., 1998; Ashton et al., 1999; Davison, 2002).

Pattwell (2003) used a microdialysis technique to measure the ESR-detectable species in the extracellular fluid as well as in the circulation. The same species were being detected in both the extracellular fluid and the circulation, suggesting the measurement of the circulating ESR-detectable species is an accurate and relatively non-invasive method of assessing extra-cellular ROS generation.

A second signal of two lines was apparent at either end of the six line spectrum with a splitting factor of approximately 80 gauss. This is attributed to copper (Personal Communication, Prof. MCR Symons), and is the first report of a copper derived radical being detected in human serum during exercise. It is proposed that this species is derived from caeruloplasmin, possibly indicating liberation of copper from albumin as a consequence of the damaging exercise (Personal Communication, Dr T Ashton).

The increase in ROS production following DWN occurs after 48 and 72 hours post-exercise. It is possible that the time course of elevation in ROS concentration may have extended beyond the test period of this study, and future studies in this thesis will identify this temporal sequence. Brickson et al. (2001) reported a similar increase in ROS production in the days following lengthening exercise, although it must be emphasised that this was in male New Zealand rabbits. Previous studies have implied that ROS produced following eccentric muscle actions may result in peroxidation of lipid membranes and contribute to DOMS, whereas our data reveal

that there is a temporal dissociation between ROS and the onset or development of DOMS.

The results from this study show that DWN induces DOMS whereas FLA fails to do so and thus confirms previous research (Byrnes et al., 1985; Schwane et al., 1983; Smith et al., 1998). In a review of the measurement tools used in the assessment of muscle damage following eccentric exercise induced muscle damage, Warren et al. (1999) reported that the use of histology and blood myofibre proteins should be discouraged when attempting to quantify muscle injury, and that greater emphasis should be placed on functional measures such as maximal voluntary contraction torque. In the current study, torque losses occurred immediately after exercise as has been reported previously (Lowe et al., 1995), although peak losses generally occur between 24 and 48 hours post exercise. Along with the increase in CK at 24 and 48 hours post exercise following DWN, this confirms that DWN successfully induces muscle damage.

It is possible to postulate that ROS are directly responsible for the damage of many compounds, whereas in many cases the production of ROS is secondary to the initial damaging mechanism. Cheeseman and Slater (1993) emphasised that it is vital that the role of ROS in the causation of disorders is separated from the production of ROS as a consequence of the disorder. In this study, ROS were not produced until 48-72 hours post exercise and at this time muscle torque, serum CK and DOMS had all returned to near pre-exercise levels. This implies that the production of ROS is

not likely to cause any further muscle soreness, but it could be involved in mediating the recovery from DOMS.

The findings in the present study are substantiated by Van Der Meulen et al. (1997) who administered acute vitamin E supplementation in an attempt to prevent post-exercise ROS production. They reported that although supplementation attenuated increases in ROS production, it failed to provide any protection against functional strength losses or structural changes within the muscle. The authors concluded that ROS production following exercise is not responsible for strength losses or muscle damage and thus supports the current findings.

It is possible that ROS damages myocytes 72 hours post-exercise and that the level of production is not sufficient to cause any significant increase in muscle soreness or losses of torque. Such a view is in agreement with the work of McBride and Kraemer (1999). It is also possible that the production of ROS at this time is essential for cellular regeneration and repair, although this hypothesis at this time remains speculative.

Although the present study did not attempt to elucidate the source of ROS production, the results intimate that the production of ROS following DWN are not a consequence of electron transfer leakage but are likely to be a result of inflammatory mediators specifically involving the infiltration of phagocytic cells

into the damaged muscle (McBride and Kraemer, 1999). This is proposed since no increase in ROS production immediately post-exercise was observed, as well as there being no increase in oxidative stress following FLA despite the same metabolic stress of FLA compared to DWN. Furthermore, the leukocyte data confirmed that DWN induced significant leukocytosis compared to FLA. Gleeson et al. (1998) reported a rise in plasma elastase concentration 3 days post-eccentric exercise. It was suggested that this increase reflected an increase in the activity of phagocytic neutrophils. This would support the suggestion of the present study that the ROS originate from phagocytic cells in response to the initial trauma.

Hellsten et al. (1997) reported that the production of ROS in the days following eccentric exercise is the result of a secondary inflammatory process occurring over a four-day period after exercise, and an invasion of leukocytes containing xanthine-oxidase. This appears to be the most likely mechanism for the production of ROS observed in the present study, implying that inflammatory or mechanical factors as opposed to metabolic factors are the predominant source of ROS production following DWN. Data from the present study may therefore imply that the production of ROS following DWN does not induce DOMS but may play a key role in the removal of damaged and necrotic cells which is necessary before muscle regeneration can occur (Lowe et al.,1995).

Ashton et al. (1998) reported that the most likely mechanism for the production of the ESR-detectable species would involve primary ROS, such as $^{\bullet}OH$ and $O_2^{\bullet-}$,

reacting with lipid membranes. This suggestion is supported by Pattwell et al. (2003) who assessed $^{\bullet}OH$ activity through the measurement of 2,3 and 2,5 dihydroxybenzoate (DHB) in the extracellular fluid. It is known that the hydroxylation of salicylate results in the formation of 2,3 and 2,5 DHB and therefore the authors infused salicylate into the microdialysis probes and measured the formation of 2,3 and 2,5 DHB used HPLC. A strong correlation between the ESR-detectable radical and 2,3/2,5 DHB in the microdialysis fluid was reported, and therefore concluded that the rate of production of the circulating ESR radical is directly related to $^{\bullet}OH$ activity.

The current data therefore shows that ROS are produced in the days following eccentric exercise, and these are likely to be a consequence of $^{\bullet}OH$ attack on lipid membranes. Furthermore, it is possible that such radical species may play a physiological, as opposed to a pathophysiological, role specifically acting as key cell signalling molecules to aid in the repair of the damaged myocytes.

The current study demonstrated a correlation between the ESR signal and MDA concentration, a correlation that has also been documented by Davison et al., (2002). It is therefore feasible that MDA can be used as an inexpensive, non-invasive method of assessing $^{\bullet}OH$ attack on lipid membranes following muscle-damaging exercise.

In conclusion, the most significant findings of this study are that 30-minutes of downhill running results in a significant increase in ROS production and lipid peroxidation. Furthermore, although DWN is successful in inducing DOMS compared to FLA it appears that the increase in ROS may not be responsible for inducing DOMS. Future studies in this thesis will employ a longer time-course than 72 hours post-exercise to measure ROS and thus establish the temporal sequence of ROS production following downhill running. Furthermore, the thesis will now explore dietary interventions, such as carbohydrate manipulation, and antioxidant supplementation to further clarify the role of ROS in DOMS following downhill running.

CHAPTER 5 – Study 2

The effects of pre-exercise carbohydrate loading on delayed onset muscle soreness, muscle torque, and production of reactive oxygen species following downhill running.

5.1 Introduction

It is known that plasma glucose is a vital fuel for several cells of the immune system including phagocytes. It has recently been shown that pre-exercise CHO status and CHO ingestion during exercise is associated with smaller shifts in the number of circulating leukocytes, as well as attenuation of many immune cell functional responses (Bishop et al., 1999a; Bishop et al., 1999b; Bishop et al., 2001a; Bishop et al., 2001b; Gleeson et al., 1998; Henson et al., 1999; Nieman et al., 1998). Elevation of pre-exercise CHO status through a high CHO diet is designed to maximise liver and muscle glycogen stores, thereby maintaining blood glucose and ultimately minimising any immuno-suppression (Gleeson et al., 2000). Conversely, depletion of pre-exercise CHO stores through the intake of a low CHO diet should result in decreased liver glycogen and thus reduced availability of blood glucose during exercise, resulting in a greater stress response and an associated immuno-suppression.

It is feasible that alteration of pre-exercise CHO status through dietary manipulation affects post-exercise immune response and therefore impacts upon phagocyte-derived ROS production. This information would further highlight the contribution of phagocytes in post-exercise ROS production as well as aiding in the clarification of the role of ROS in DOMS and losses of muscle function.

As well as the mechanistic aims, this study also attempted to clarify the effects of dietary CHO manipulation on DOMS and muscle function. It is well documented that athletes often attempt to alter pre-exercise CHO status to maximise performance. This often-involves CHO depletion and intensive training in order to deplete CHO stores followed by several days on a high CHO diet to maximally load CHO (Goforth et al., 2003). If training on a low CHO diet results in greater DOMS and increased ROS production, then it is essential for athletes to be aware of this.

Study 1 clearly demonstrated that there was a significant increase in DOMS following 30 minutes of running downhill. It was also shown that this increase in DOMS was accompanied by a transient increase in ROS, although whether this production was physiological or pathophysiological could not be established. It was suggested that the ROS produced came from a phagocytic pathway, and was likely to be $O_2^{\bullet-}$ release from phagocytic cells forming H_2O_2 and $^{\bullet}OH$ *via* Fenton chemistry. However, since no attempt was made to intervene with the $O_2^{\bullet-}$ production, these suggestions were purely speculative. Therefore, one of the aims of this study was to further examine the contribution of phagocytes to the production of ROS, DOMS and losses of muscle function following 30 minutes of downhill running.

Study 1 also demonstrated that the increase in ROS occurred after peak losses of torque and when ratings of DOMS were subsiding, implying that post-exercise increases in ROS may not be responsible for DOMS. However, since there was no attempt to attenuate ROS production, it was not possible to prove this disassociation (Jackson, 1999). Therefore, this study also intends to further investigate the

association between ROS and DOMS through attenuating the delayed increase in ROS production.

Study 1 raised several issues which this study has to address. In order to fully understand the role of ROS in DOMS, it is important that the time-course of production following damaging exercise is fully understood. In study 1, ROS were still elevated at 72 h post-exercise, therefore measurement of ROS will be undertaken for an extra 24h (i.e. 96 h). It was also apparent that despite 65% $\dot{V}O_{2\,max}$ being a common exercise intensity reported in the literature, many of the subjects found it difficult to maintain this for the entire 30-minute run. Since a low CHO diet may attenuate exercise capacity, it was decided that this study would use a lower intensity, this being 60% $\dot{V}O_{2\,max}$.

Study 1 demonstrated a correlation between plasma MDA and the lipid radical detected by ESR spectroscopy. This correlation has since been reported in the literature in a separate study (Davison et al., 2002). Therefore, due to the expense and time associated with ESR spectroscopy, it was decided that the use of MDA along with total blood GSH was sufficient to demonstrate an increase in ROS production and consequent peroxidation of lipid membranes.

Finally, it was also observed that the statistical power for some of the tests was low and thus there was a chance of an α or β error. It was decided that the subject numbers should increase by 50% from n=8 to n=12 for this study.

5.2 Methods

5.2.1 Subjects

Twelve physically active male subjects, unaccustomed to downhill running volunteered for this study. All were non-smokers and free from any known illnesses as ascertained by medical questionnaire. Subjects taking antioxidants, or following any carbohydrate modified diet were excluded. All subjects were informed verbally and in writing about the nature of this study, including all potential risks. Written informed consent was obtained prior to participation and ethical approval was granted by the Liverpool John Moores University Ethics Committee.

5.2.2 Experimental protocol

Each subject was initially assessed for aerobic fitness by determining their $\dot{V}O_{2max}$ as described in chapter 3.2. The assessment of aerobic fitness was carried out one week prior to the first experimental run. All subjects then participated in two experimental runs, both lasting for 30 minutes at a running speed corresponding to 60% $\dot{V}O_{2max}$. On one occasion the run was performed following two days on a high carbohydrate diet (HC), whilst the other was performed following two days on a low carbohydrate diet (LC). All subjects were randomly allocated into two groups with one group performing LC first whilst the other group performed HC first. The conditions were counterbalanced and there was a 5-week interval between trials.

For each trial subjects were required to visit the laboratories on five consecutive days. Day 1 was to perform the experimental run, to have leg torque and pain measurements taken, and to give pre and post-exercise venous blood samples. The following 4 days were to have torque and pain assessed, and to give a resting venous blood sample.

5.2.3 Dietary intervention

All subjects were instructed to follow a given diet for 48 hrs prior to the experimental trials. The diets were: HC - 77% Carbohydrate, 12% Protein, 11% Fat, or LC – 11% Carbohydrate, 12% Protein, 77% Fat. Furthermore, the diet was specifically designed for each individual ensuring that the HC contained ~8g.kg^{-1}.bw carbohydrate whilst the LC contained ~1g.kg^{-1}.bw. Diets were produced in conjunction with the subjects to ensure they were palatable for the individual. An example of the two diets can be seen in Tables 5.2 and 5.3. Fluids were consumed *ad libitum,* although drinks containing sugar were excluded. The diets were iso-energetic with both diets providing ~14MJ of energy. A summary of the characteristics of the two diets can be seen below in Figure 5.1 and 5.2 and in Table 5.1.

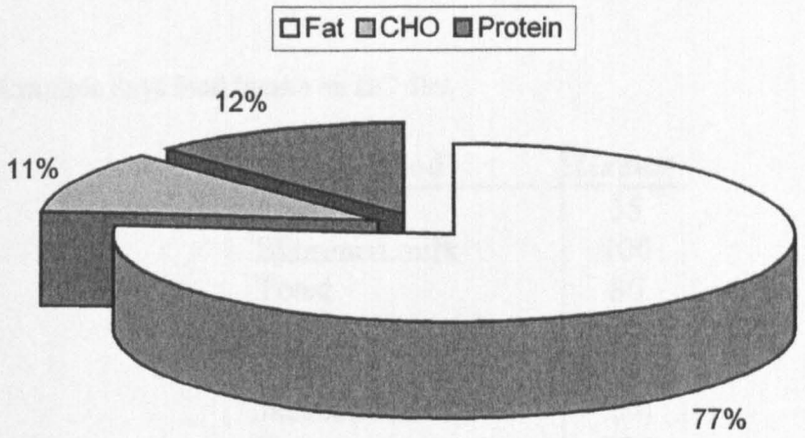


Fig 5.1 Percentage carbohydrate, fat and protein from the LC diet

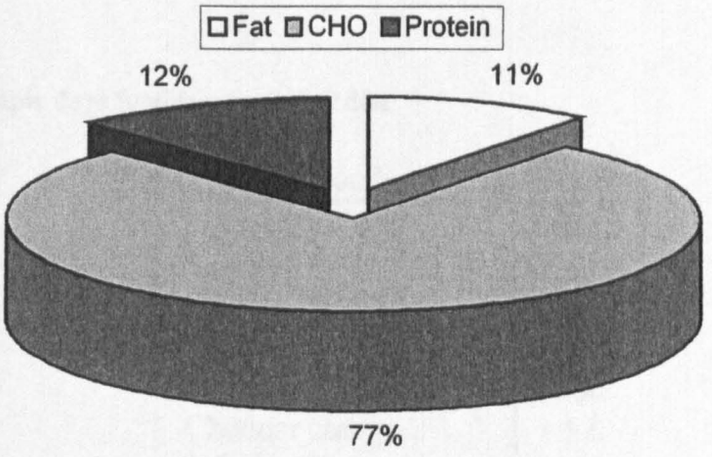


Fig 5.2 Percentage carbohydrate, fat and protein from the HC diet

Table 5.1 Energy content of the two diets

	Fat KJ	CHO KJ	Protein KJ	Total KJ	% CHO
HC	1536	11,214	1760	14510	77
LC	11,126	1619	1783	14528	11

Table 5.2 Example days food intake on HC diet

Food	Grams
Cornflakes	35
Skimmed milk	100
Toast	36
Jam	32
Raisons	40
Jacket potato	220
Beans	200
Toast	72
Jam	32
Super noodles	80
Toast	36
Apple	120
Fruit cake	70
Mars	65

Table 5.3 Example days food intake on LC diet

Food	Grams
Eggs scrambled	60
Sausage	60
Bread wholemeal	30
Butter	55
Lasagne/mac chee	300
Cheddar cheese	57
Whole milk	180
Lard	20
Callogen	50
Mayonaise	32
Ice cream	84

5.2.4 Day 1.

Subjects arrived at the laboratory between 0800 and 0930 h following an overnight fast. Following 30-minutes supine rest, a pre-exercise blood sample (16.4ml) was taken from an antecubital forearm vein.

All blood samples were collected in vacutainers as outlined in chapter 3.6 and 3.7. A total of three vacutainers were used at each sample point. Tube 1 contained EDTA and was used for the analysis of leukocytes, GSH and lactate. Tube 2 contained serum separation gel and was for the analysis of MDA. Tube 3 contained the anticoagulant lithium heparin and was used for the analysis of NEFA, glucose and CK.

Following the blood sample, subjects were asked to rate their pre-exercise muscle soreness of the gastrocnemious, tibialis anterior, hamstrings, quadriceps, gluteals, (both left and right sides) and lower back muscles using pain diagrams as described in chapter 3.5. Subjects then proceeded to warm up on a cycle ergometer before completing a series of stretches of their own choice. The stretches were to prepare the subjects for assessment of muscle function.

Muscle function was assessed using an isokinetic leg dynamometer as outlined in chapter 3.4.

The experimental run took place on the same motorised treadmill as used in the $\dot{V}O_{2max}$ test. The run lasted for 30-minutes at an oxygen consumption corresponding to 60% $\dot{V}O_{2max}$. Specific details of the run can be seen in chapter 3.3. RER and $\dot{V}O_2$ were assessed every 10 seconds throughout the run using on-line gas analysis.

Immediately following the run, subjects assumed a supine position whilst a second blood sample was taken using the same technique described previously. Subjects were then re-assessed regarding their perceptions of muscle soreness before completing the force assessment again. This completed day 1 of the trial.

5.2.5 Days 2,3,4 and 5.

Subjects visited the laboratory exactly 24, 48, 72 and 96 hours after their initial visit. They were seated for 30 minutes before a resting blood sample was taken from a prominent vein. The blood test took place at the same time as the pre-exercise blood test to eliminate any time of day effect (Fernandes, 1992). The samples were treated in an identical manner as described previously. Following the blood tests, subjects were then assessed for DOMS. This was followed by a five minute warm up on a cycle ergometer before leg force was assessed as previously described. All subjects then undertook 5-weeks recovery before repeating the test using the second condition. A summary of the test protocol can be seen in figure 5.1

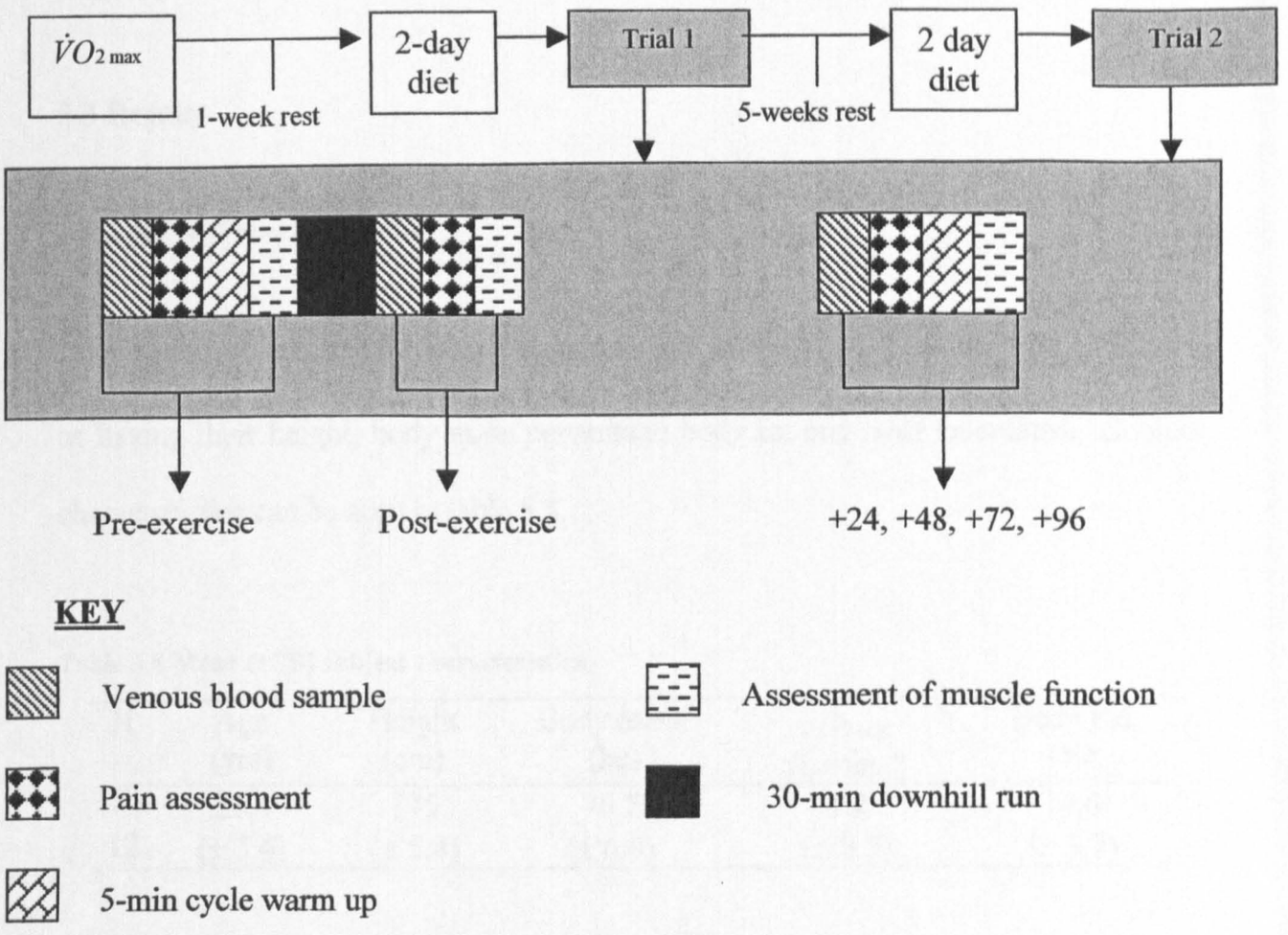


Figure 5.3 Schematic representation of the test protocol

5.2.6 Statistical analyses

Statistical analyses was carried out using the Statistical Package for Social Sciences (SPSS Surrey, UK). All data are presented as means ± SEM. A two way ANOVA with repeated measures was used to analyse all variables. When Mauchley's test of sphericity indicated a minimal level of violation (>0.75) the degrees of freedom was corrected using the Huynh-Feldt adjustment. When the sphericity was <0.75, the Greenhouse Geiser correction was used (Field, 1999). Post Hoc Tukey analysis (Honestly Significant Difference, (HSD)) was performed to identify where the significant differences occurred. Statistical significance was set at $P<0.05$ for all tests.

5.3 Results

5.3.1 Subject characteristics

Prior to the experimental runs, all subjects were initially assessed for $\dot{V}O_{2max}$ as well as having their height, body mass percentage body fat and BMI calculated. Subject characteristics can be seen in table 5.4.

Table 5.4 Mean (±SD) subject characteristics

N	Age (yrs)	Height (cm)	Body mass (kg)	$\dot{V}O_{2max}$ ($L.min^{-1}$)	Body Fat (%)
12	23.3 (± 3.4)	175 (± 5.4)	76.7 (± 6.0)	4.2 (± 0.5)	14.6 (± 3.7)

5.3.2 Run data

All subjects successfully completed the 30-minute run on both occasions, and were able to maintain 60% $\dot{V}O_{2max}$ throughout. A summary of the run data can be seen in Table 5.5. There was no significant difference in mean $\dot{V}O_2$, RPE, HR, VE and run speed between the two groups ($P>0.05$).

Table 5.5 Mean (±SEM) summary of the run data.

		Time (mins)						
	Group	Pre-ex	5	10	15	20	25	30
Heart Rate *b.min^{-1}*	HC	149 (± 6.7)	151 (± 4.7)	156 (± 3.9)	163 (± 4.1)	164 (± 3.6)	164 (± 3.6)	166 (± 3.2)
	LC	142 (± 3.0)	153 (± 2.7)	163 (± 2.8)	169 (± 2.8)	170 (± 3.4)	173 (± 3.0)	174 (± 2.9)
$\dot{V}O_2$ *L.min^{-1}*	HC	2.18 (± 0.1)	2.53 (± 0.09)	2.60 (± 0.08)	2.71 (± 0.07)	2.63 (± 0.09)	2.58 (± 0.11)	2.58 (± 0.10)
	LC	2.22 (± 0.07)	2.48 (± 0.15)	2.51 (±0 .07)	2.58 (± 0.06)	2.5 (± 0.09)	2.61 (± 0.11)	2.56 (± 0.08)
RPE *6-20*	HC	10 (± 1.0)	11 (± 1.0)	12 (± 0.0)	13 (± 0.0)	14 (± 0.0)	15 (± 1.0)	15 (± 1.0)
	LC	10 (± 1.0)	11 (± 0.0)	12 (± 0.0)	13 (± 0.0)	14 (± 1.0)	15 (±1.0)	15 (± 1.0)
VE *L.min^{-1}*	HC	54.7 (± 2.0)	68.9 (± 2.5)	69.9 (± 1.5)	75.4 (± 1.8)	74.7 (± 1.4)	71.3 (± 1.8)	75.4 (± 2.5)
	LC	59.2 (± 2.5)	65.4 (± 3.5)	70.6 (± 1.7)	73.3 (± 1.9)	71.3 (± 2.1)	72.9 (± 2.9)	73.8 (± 2.4)
Run Speed *km.hr^{-1}*	HC	12.7 (± 0.5)	13.0 (± 0.4)	13.0 (± 0.4)	12.7 (± 0.5)	11.9 (± 0.7)	11.5 (± 0.7)	11.5 (± 0.7)
	LC	12.3 (± 0.5)	12.8 (± 0.4)	12.7 (± 0.4)	12.1 (± 0.5)	11.7 (± 0.5)	11.4 (± 0.5)	11.1 (± 0.6)

5.3.3 Metabolite data

Figure 5.4 shows the non-protein respiratory exchange ratios (RER) during the runs under both dietary conditions. It can be seen that HC resulted in a significantly higher RER pre-exercise and this remained elevated throughout the duration of the run. Mean RER throughout the runs were 0.91 (±0.01) and 0.85 (±0.01) for HC and LC respectively.

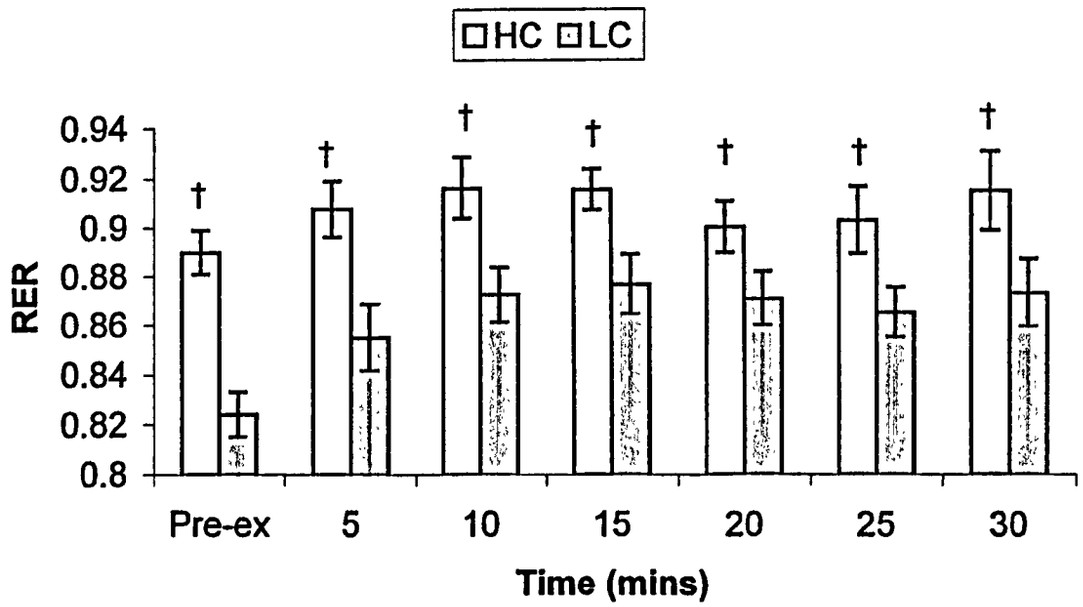


Figure 5.4 Mean (±SEM) respiratory exchange ratio (RER).
† indicates significant difference from LC

Plasma glucose, NEFA and lactate concentrations were measured immediately pre and post-exercise. There was no significant difference in pre-exercise blood glucose between HC and LC ($P>0.05$). However, HC demonstrated a significant increase in plasma glucose immediately post-exercise ($P<0.05$) where LC failed to do so ($P>0.05$). There were significantly higher pre-exercise NEFA concentrations in LC compared to HC ($P<0.05$). Furthermore, both groups exhibited a post-exercise increase in NEFA concentration ($P<0.05$) however the rise in NEFA in LC was significantly greater than in HC ($P<0.05$).

Pre-exercise lactate concentrations were significantly greater in HC than in LC ($P<0.05$). Both groups demonstrated a significant increase in lactate post exercise ($P<0.05$) and there was no significant difference in post-exercise lactate concentrations between the two groups ($P>0.05$). Plasma glucose, NEFA and lactate concentrations can be seen in figures 5.5, 5.6 and 5.7 respectively.

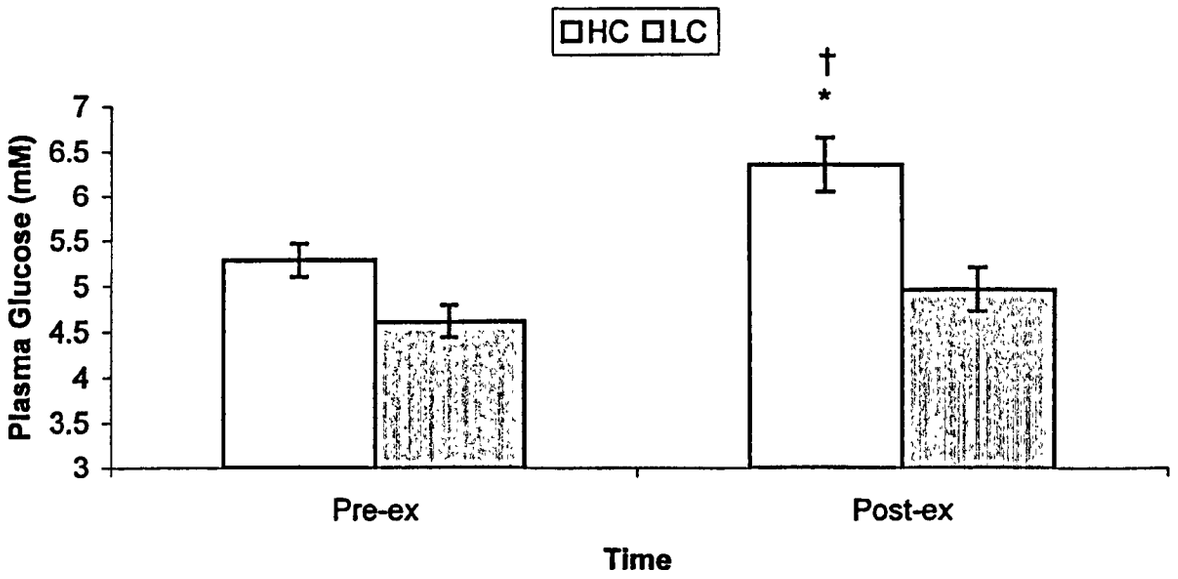


Figure 5.5 Mean (±SEM) plasma glucose concentrations
*** indicates significant difference from pre exercise**
† indicates significant difference from LC

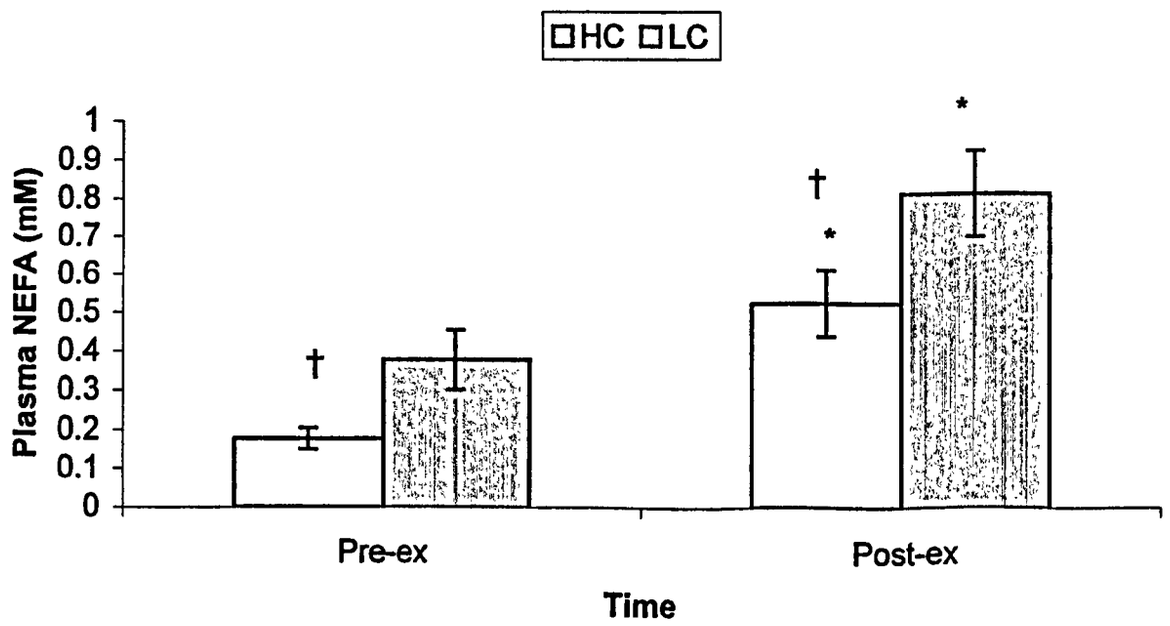


Figure 5.6 Mean (±SEM) plasma NEFA concentrations
*** indicates significant difference from pre exercise**
† indicates significant difference from LC

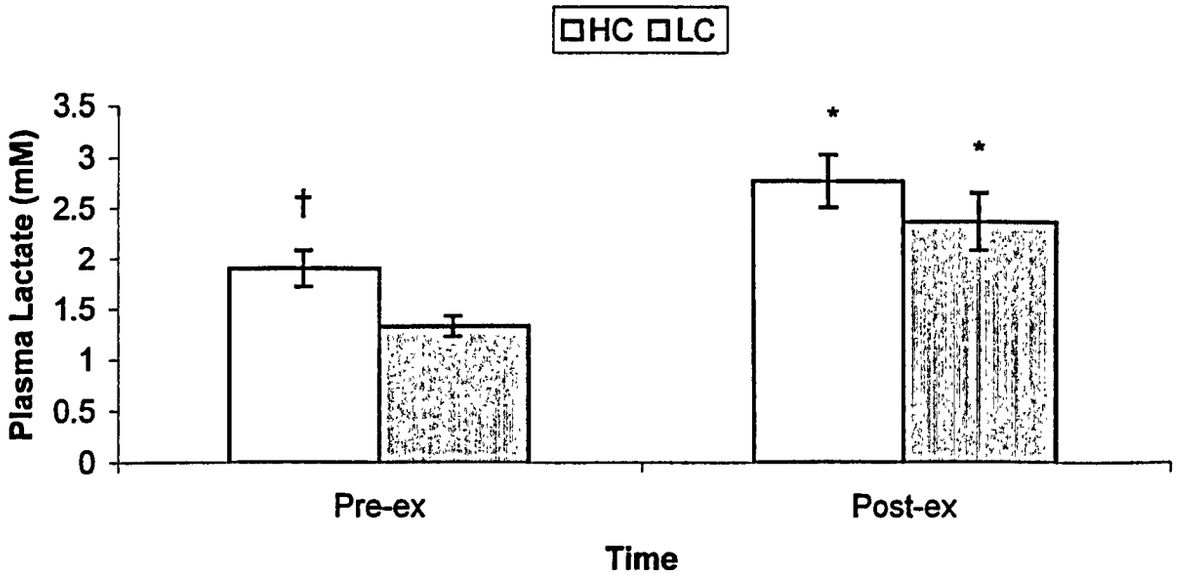


Figure 5.7 Mean (±SEM) plasma lactate concentrations
*** indicates significant difference from pre exercise**
† indicates significant difference from LC

5.3.4 Delayed onset muscle soreness

There was a significant increase in ratings of muscle soreness immediately post-exercise in both groups ($P<0.05$) which cannot be classed as DOMS. However there was a further significant increase 24 h post exercise and this remained elevated up to 96 h post exercise ($P<0.05$). There were no significant differences in perceptions of DOMS between the two groups ($P>0.05$). Ratings of DOMS can be seen in figure 5.8.

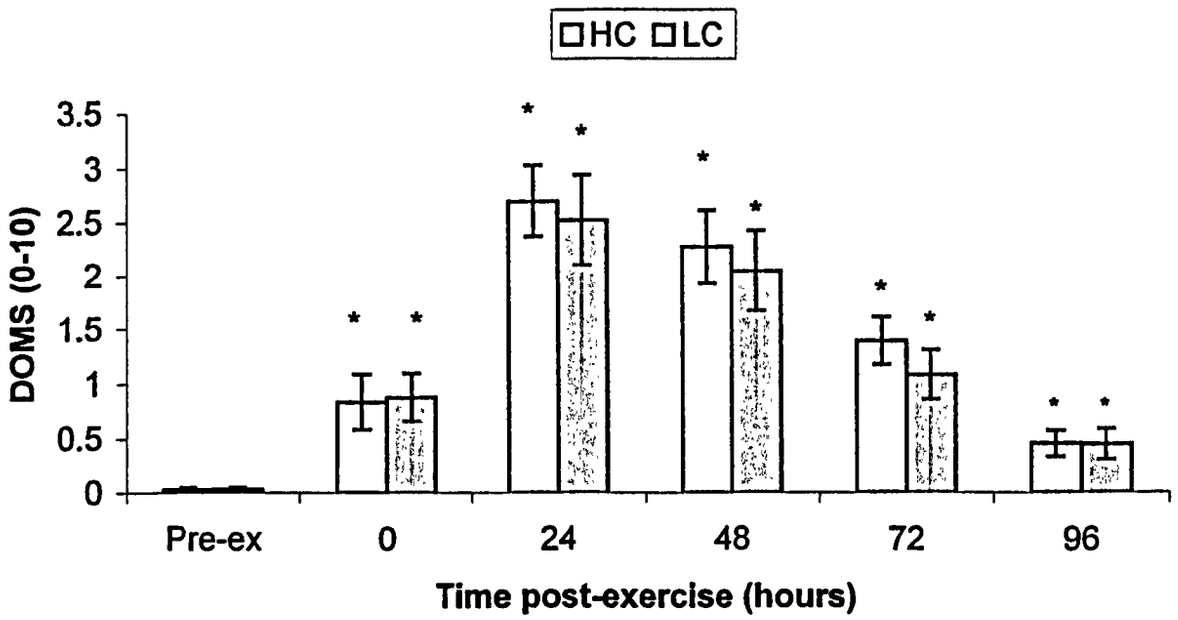


Figure 5.8 Mean (±SEM) ratings of muscle soreness using pain diagrams in conjunction with an adapted Borg Scale (0-10).
*** indicates significant difference from pre-exercise**

5.3.5 Reactive Oxygen Species

Data from serum concentrations of MDA can be seen in figure 5.9. There were no significant increases in MDA concentrations post-exercise ($P<0.05$) although MDA concentrations increased significantly in both groups 72 h post exercise and remained elevated at 96h post exercise ($P<0.05$). There were no significant differences in MDA between HC and LC ($P>0.05$).

Total blood glutathione concentrations (GSH) fell at 72 and 96 h post-exercise suggesting an antioxidant response to an oxidative stress. However, possibly due to the large SEMs associated with the measurement of total blood GSH, the difference was not statistically significant ($P>0.05$). There was also no significant difference in total blood GSH concentration between the two groups ($P>0.05$). Total blood GSH concentrations can be seen in figure 5.10.

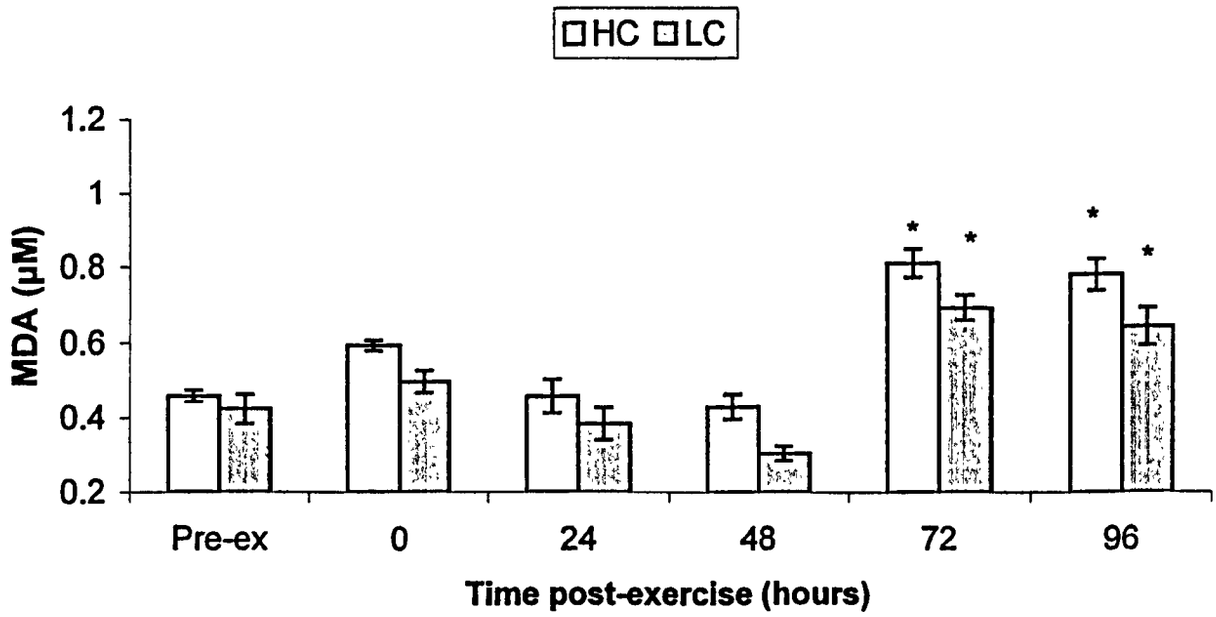


Figure 5.9 Mean (±SEM) serum MDA concentrations
*** indicates significant difference from pre exercise**

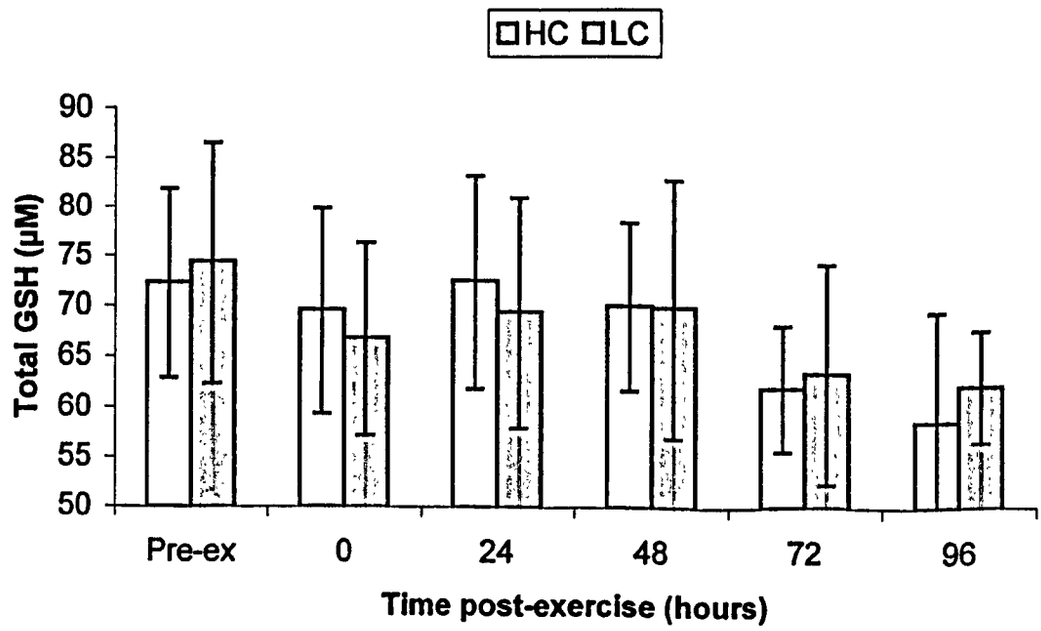


Figure 5.10 Mean (±SEM) total blood glutathione concentrations

5.3.6 Muscle damage

Plasma CK activity was used to assess muscle damage (Figure 5.11) There was a significant increase in plasma CK activity 24 h post-exercise in both groups ($P<0.05$). This had fallen by 48 h and remained at baseline levels at 72 h post exercise. There was however, a second significant peak in CK 96 h post exercise ($P<0.05$). There was no significant difference in the biphasic change in plasma CK between the two conditions ($P>0.05$).

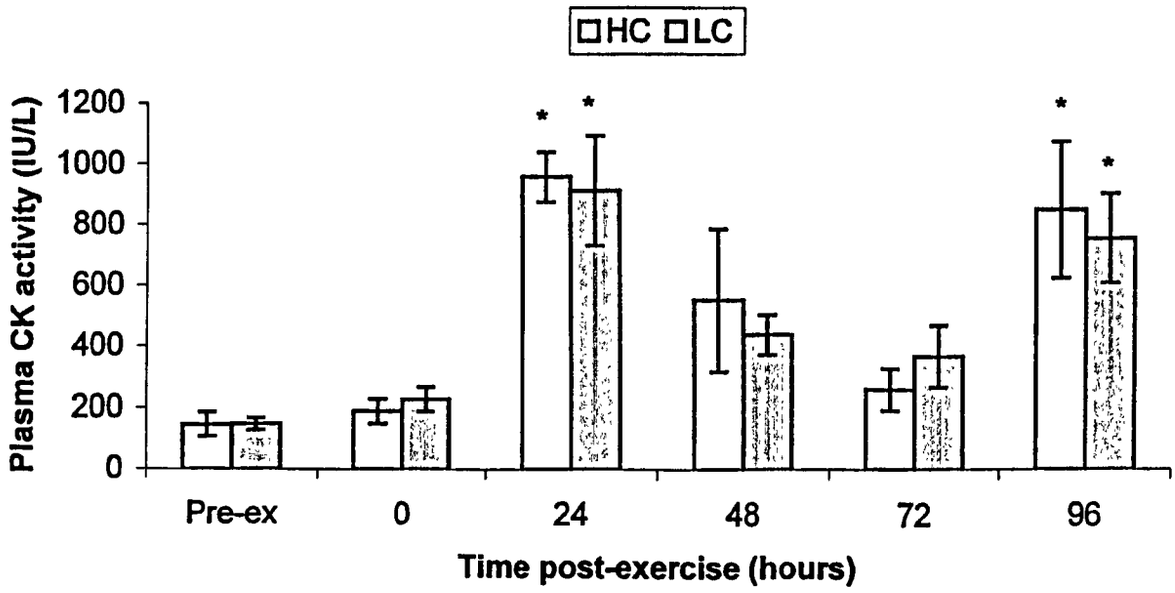


Figure 5.11 Mean (±SEM) plasma CK activity
*** indicates significant difference from pre exercise**

5.3.7 Muscle Function and DOMS during muscle function test

Figure 5.12 shows the change in concentric torque at 1.04 rad.sec^{-1} following both dietary interventions. There was a significant reduction in muscle torque 24 h post-exercise and this remained suppressed up to 72 h post exercise. There was no significant difference in muscle torque measured at 1.04 rad.sec^{-1} between the two conditions ($P>0.05$). Table 5.6 shows the magnitude of DOMS reported during

concentric torque assessment at 1.04 rad.sec^{-1}. There was a significant increase in ratings of DOMS post-exercise ($P<0.05$) and this remained elevated up to 96 h post exercise. There was no significant difference in DOMS between the two groups ($P>0.05$)

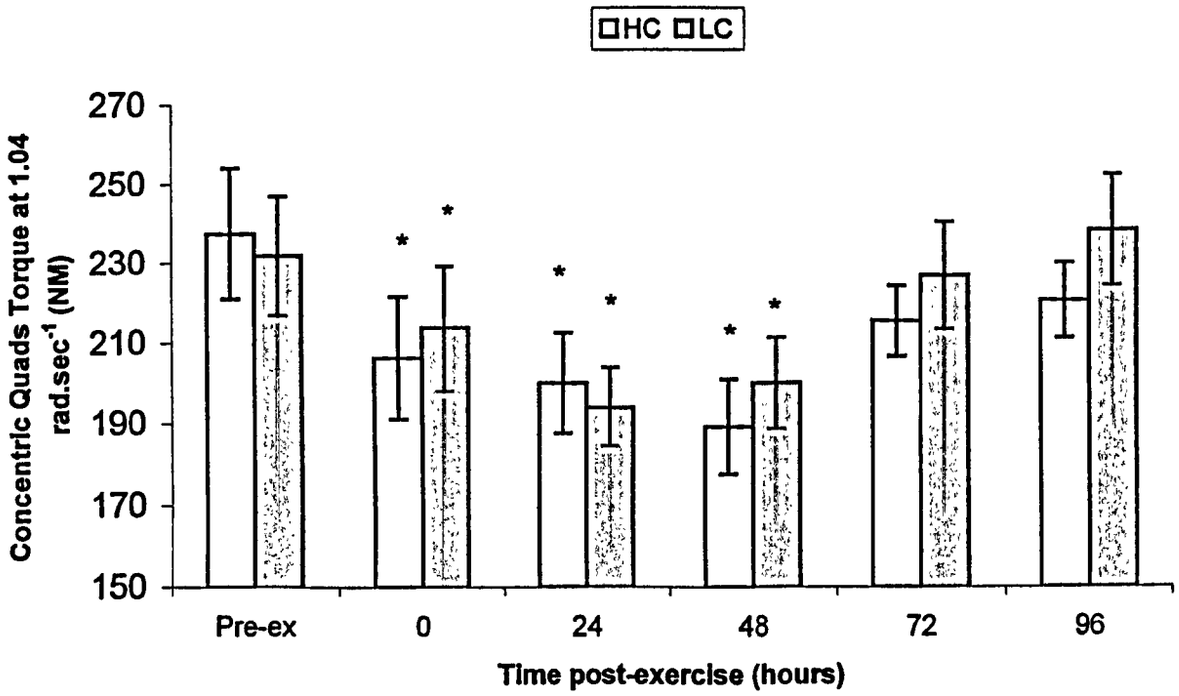


Figure 5.12 Mean (±SEM) concentric quadriceps torque measured at 1.04 rad.sec^{-1}
*** indicates significant difference from pre-exercise**

Table 5.6 Mean (±SEM) DOMS felt during concentric contractions at 1.04 rad.sec^{-1}
*** indicates significant difference from pre-exercise**

Time Post-ex (hrs)		Pre-ex	0	24	48	72	96
Pain	HC	0.0 (± 0.1)	1.1* (± 0.5)	3.7* (± 0.7)	4.8* (± 0.7)	1.6* (± 0.4)	0.3 (± 0.1)
	LC	0.0 (± 0.2)	1.8 * (± 0.4)	3.8* (± 0.5)	4.6 * (± 0.6)	1.4* (± 0.3)	0.4 (± 0.3)

Concentric quadriceps torque was also assessed at the faster more functional speed of 5.20 rad.sec^{-1} (Figure 5.13). There were significant torque losses post-exercise and 24

h post exercise (P<0.05) although muscle torque returned to baseline by 48 h post exercise (P>0.05). Ratings of DOMS during this assessment of muscle function increased post-exercise and remained significant up to 48 h post-exercise. There was no significant difference in ratings of DOMS between the two groups (P>0.05).

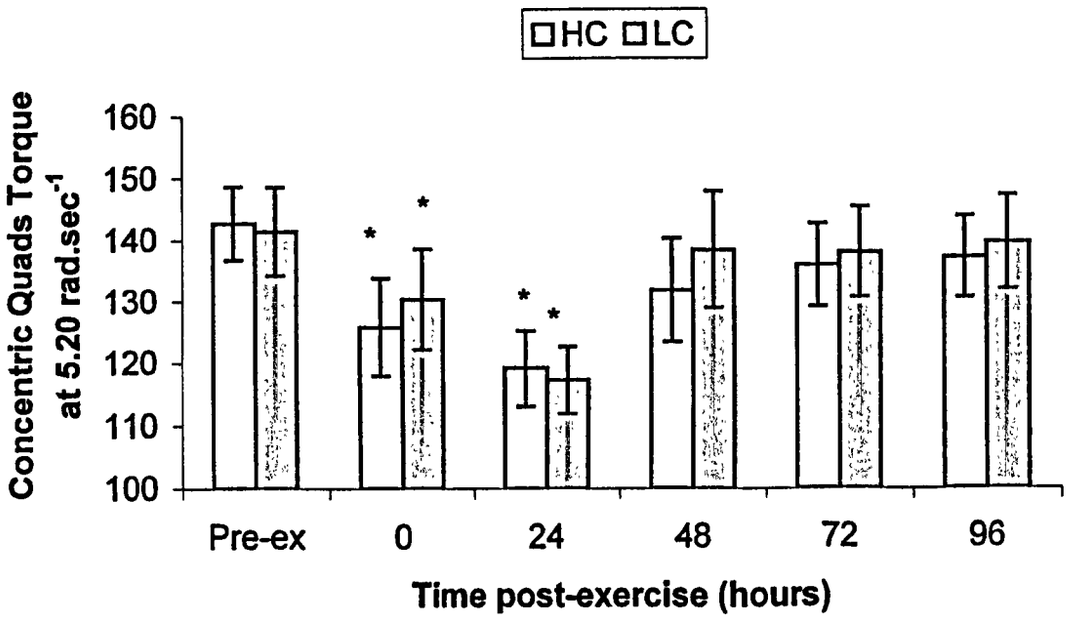


Figure 5.13 Mean (±SEM) concentric quadriceps torque measured at 1.04rad.sec-^{1}
*** indicates significant difference from pre-exercise**

Table 5.7 Mean (±SEM) DOMS felt during concentric contractions at 5.20 rad.sec^{-1}
*** indicates significant difference from pre-exercise**

Time Post-ex (hrs)		Pre-ex	0	24	48	72	96
Pain	HC	0.0 (± 0.0)	0.5 (± 0.3)	1.7* (± 0.6)	2.0* (± 0.7)	0.7 (± 0.3)	0.5 (± 0.2)
	LC	0.0 (± 0.0)	0.4 (± 0.3)	1.3 * (± 0.5)	1.8 * (± 0.5)	0.5 (± 0.4)	0.2 (± 0.2)

Muscle torque was also assessed using an eccentric protocol (Figure 5.14). There was a significant reduction in muscle torque post exercise (P<0.05) and up to 72 h post exercise (P<0.05). There was no significant difference in muscle torque between the two groups (P>0.05).

DOMS assessed during the eccentric protocol can be seen in table 5.8. There was significant DOMS post-exercise and up to 96 h post exercise ($P<0.05$). There was no significant difference between the two groups ($P>0.05$).

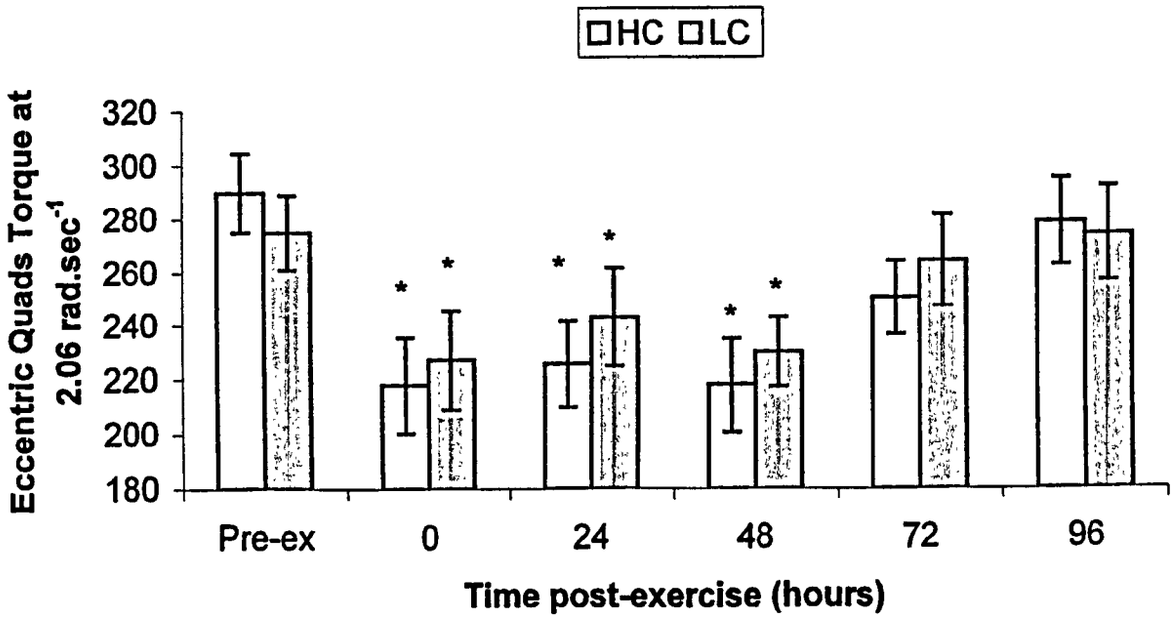


Figure 5.14 Mean (±SEM) concentric quadriceps torque measured at 5.20 rad.sec^{-1}
*** indicates significant difference from pre-exercise**

Table 5.8 Mean (±SEM) DOMS felt during eccentric contractions at 2.06 rad.sec^{-1}
*** indicates significant difference from pre-exercise**

Time Post-ex (hrs)		Pre-ex	0	24	48	72	96
Pain	HC	0.4 (± 0.3)	1.9* (± 0.5)	3.2* (± 1.0)	4.1* (± 0.7)	2.2* (± 0.4)	1.0 (± 0.7)
	LC	0.3 (± 0.3)	1.7* (± 0.7)	3.0* (± 0.7)	3.8* (± 0.7)	2.1* (± 0.5)	0.7 (± 0.5)

5.3.8 Leukocytes

Downhill running resulted in a transient leukocytosis ($P<0.05$) occurring immediately post-exercise following both diets. This was largely due to transient neutrophilia

(P<0.05) as well as a transient increase in lymphocytes (P<0.05) [Figures 5.15-5.17]. There was no change in circulating eosonophils or basophils counts following either dietary condition (P>0.05). Furthermore, there was no significant difference in total or differential leukocyte counts between the two diets (P>0.05).

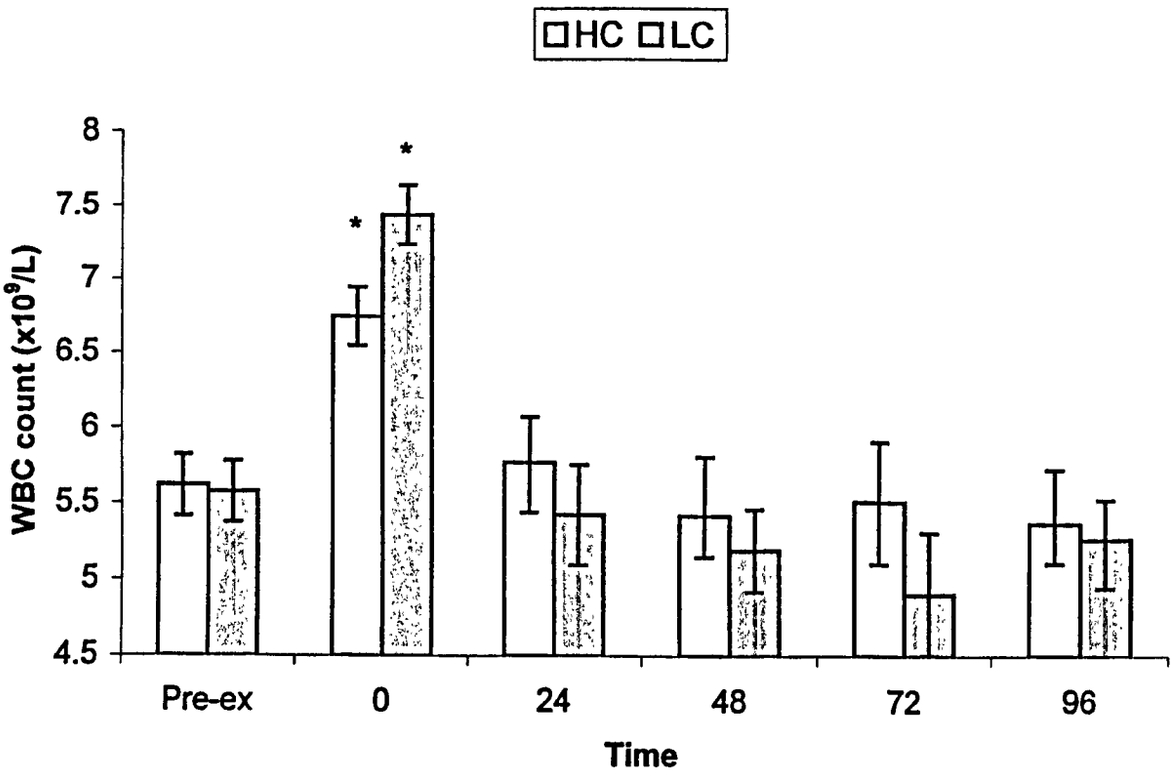


Figure 5.15 Mean (±SEM) leukocyte count (x10^9/L)
*** indicates significant difference from pre-exercise**

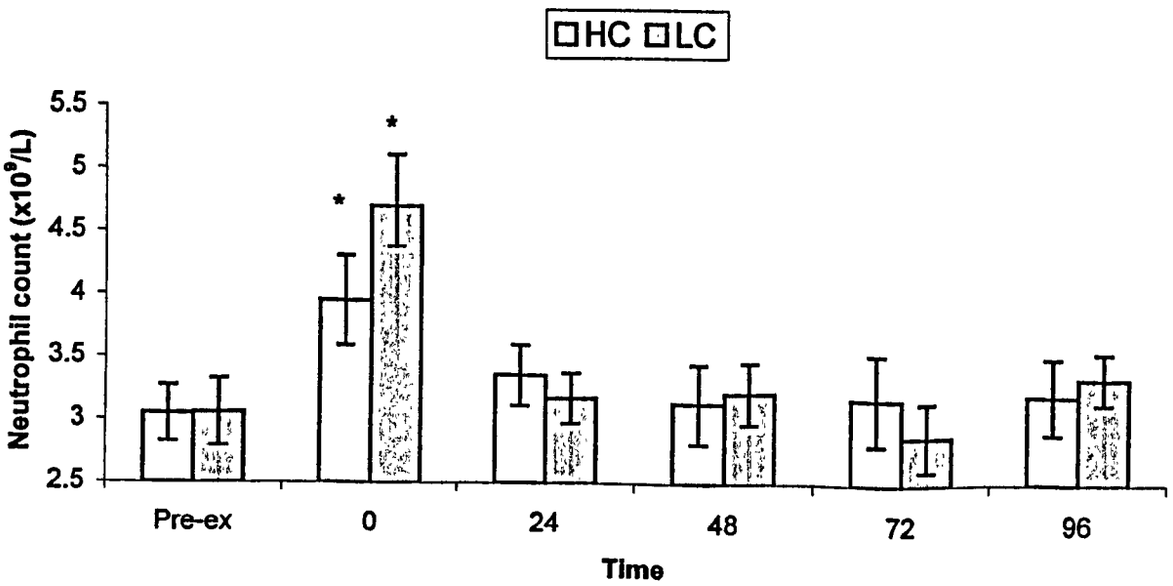


Figure 5.16 Mean (±SEM) neutrophil count (x10^9/L)
*** indicates significant difference from pre-exercise**

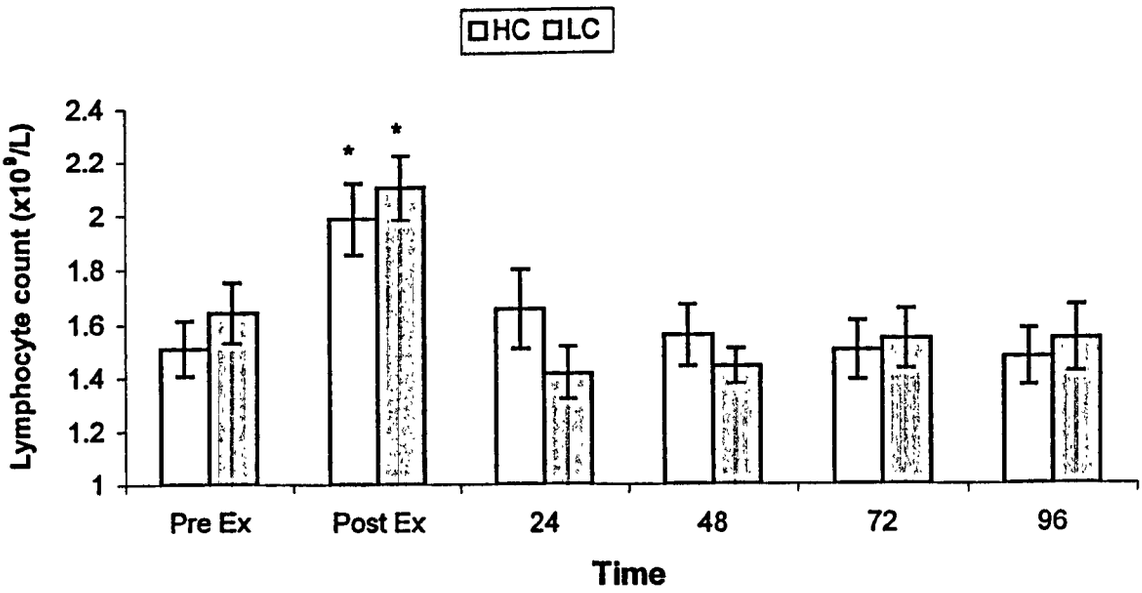


Figure 5.17 Mean (±SEM) lymphocyte count (x10⁹/L)
*** indicates significant difference from pre-exercise**

6.4 Discussion

The present study investigated the effects of pre-exercise dietary CHO status on DOMS, ROS and muscle function following 30 minutes of downhill running. Two days of a high CHO diet significantly increased resting RER and lactate concentrations, as well as decreased pre-exercise NEFA concentrations, suggesting that the diets were successful in altering pre-exercise CHO status. Since the diets were not combined with a bout of strenuous exercise, it is likely that muscle glycogen stores were not significantly affected although there was likely to have been a significant reduction in liver glycogen stores. However, despite these changes in pre-exercise liver glycogen status, there were no observed differences between the two groups in regards to DOMS, ROS or changes in muscle function.

All subjects chose their own foods from a comprehensive list. Food was purchased and pre-packed for the subjects and consequently the metabolite data suggested good adherence to the diets since RER was significantly higher following HC than LC, plasma lactate and glucose were higher on HC, and NEFA was lower on HC. The diets were also iso-energetic and therefore any differences observed were due to the macronutrient differences and not total energy intake.

Circulating leukocytes demonstrated a similar response to that reported by Bishop et al. (2001b) following 3 days on a CHO manipulated diet. There was a trend (albeit not statistically significant) for attenuation in post-exercise leukocytosis following HC compared to LC, which was largely due to attenuated neutrophilia. Interestingly

despite this attenuation in neutrophilia, there was no significant difference in ROS responses between the two conditions. Serum MDA and total blood GSH suggested oxidative stress and lipid peroxidation were occurring 72 and 96 h post exercise. One interpretation of these findings is that ROS produced in the days following downhill running is not related to post-exercise neutrophilia.

However as reported by Bishop et al. (2001b), this type of conclusion could only be made if the functional capacity of the blood compartment is assumed to be directly related to the tissue in question, in this case the damaged muscle cells. This is highly questionable. In response to physical trauma, neutrophils migrate to the site of injury where they then release chemotactic factors (Gleeson et al., 2000) which subsequently recruit other neutrophils to the damaged tissue. It is known that following eccentric exercise, there is compartmentalisation of muscle damage (Evans, 1987) and therefore the site of injury will have a finite size. Therefore, not all of the neutrophils in the circulation will need to infiltrate the damaged tissue and hence the circulating neutrophil count may not be a good indicator of neutrophil functional activity.

Bishop et al. (2001b) reported that despite the attenuated neutrophilia associated with a high carbohydrate diet, when changes in neutrophil were expressed as LPS-stimulated elastase release per neutrophil, there was no difference between a high carbohydrate and low carbohydrate diet. Therefore, in relation to the present study, it would appear that despite the attenuation in circulating neutrophils, there were still sufficient neutrophils in the circulation to migrate to the site of injury and exhibit a

respiratory burst, as demonstrated through increased MDA production. If the role of neutrophils is to be examined in ROS production following muscle-damaging exercise, then a more direct inhibitor/scavenger of the neutrophil oxidative burst should be employed, such as vitamin C.

This study also attempted to map the time course of ROS production following downhill running. Study 1 demonstrated an increase in ROS 72 h post-exercise. Since these parameters had not returned to baseline by 72 h post-exercise it was decided that an extra day would be added in this study. However, in the present study it was noted that markers of ROS were still significantly elevate 96 h post-exercise and therefore it is still not possible to state the peak in ROS production following downhill running. Recently, Childs et al. (2001) reported that markers of ROS (MDA, isoprostanes and lipid hydroperoxides) peaked 72 h post-exercise following eccentric arm exercise. However, it is known that plasma CK follows a difference time course of response following eccentric arm exercise compared to downhill running and therefore it is feasible that ROS production may also follow a different time course between the two exercise protocols. Future studies should therefore measure ROS production for longer than 96 h post downhill running to fully map this temporal increase in ROS.

Muscle damage was assessed through plasma CK activity. It was observed that there was no difference in plasma CK between the two dietary conditions. This would suggest that pre-exercise liver glycogen depletion has no effect on post-exercise muscle damage. It was interesting to observe that there was a biphasic response in

CK following both dietary conditions. As in study 1, there was an initial increase in CK observed 24 hours post exercise. This probably reflects muscle damage caused by the mechanical trauma of the eccentric contraction (Clary et al., 1988). However, there was also a secondary increase in CK observed 96 h post exercise. Although this is unusual it is not the first time that a biphasic CK response has been reported in the literature (Smith et al., 1998). Since there was an increase in ROS production at 72 and 96 h post exercise, it is possible that this secondary increase in CK represents free radical attack on lipid membranes resulting in secondary muscle damage.

There was however no relationship between this secondary increase in CK activity and DOMS. DOMS had fallen significantly by 96 h post exercise and had almost returned to pre-exercise levels. This suggests that the secondary muscle damage caused by up regulation of ROS production does not prolong the sensation of DOMS. One of the aims of this study was to determine if the production of ROS played a pathological or physiological role in the aetiology of DOMS. Since ROS have now been shown to be associated with secondary muscle damage, the data could be interpreted as ROS play a pathological role. However, the increase in ROS is not associated with further losses of muscle function or increased DOMS and therefore the role of ROS in DOMS is still unresolved. There were also no significant difference in ratings of DOMS between the two dietary conditions, demonstrating that pre-exercise CHO status also does not effect muscle soreness.

The present study also sought to determine the effects of dietary CHO manipulation on muscle function following downhill running. There were significant losses of muscle function following both diets, however there were no significant differences between the two dietary conditions. It was interesting to note that the losses of muscle function demonstrated a different temporal sequences at the varying test speeds. At the slower speeds (1.04 rad.sec^{-1}) muscle function was impaired for 24 h longer than at the faster speeds (5.20 rad.sec^{-1}). This implies that at the faster functional faster speeds there is a more rapid recovery of muscle function than at the slower speeds, which are designed to look at maximal torque.

It is therefore important to note what speeds muscle function is assessed at when using changes in muscle torque to track losses of muscle function in relation to DOMS. This may explain some of the discrepancies in the literature. Several studies have used jump height (Farr et al., 2002), and sprint speeds (Thompson et al., 1999) to assess muscle function. According to the present studies, such measurements would recover much faster than assessments that involve maximal force production and therefore caution should be made when comparing muscle function tests. Ideally, electrical stimulation of the muscle, to remove any neural input, would be the method of choice and it is speculated that using this method of assessment would result in the longest losses of muscle function.

It has previously been suggested that high fat diets, especially those high in n-3 PUFA may inhibit neutrophil chemotaxis and the subsequent generation of $O_2^{\bullet-}$ by an up-regulation of superoxide dismutase (Luostarinen et al., 1996). Although the amount of PUFA was not determined in this study, it can be concluded that 2 days on a 77% high fat diet does not inhibit the production of $O_2^{\bullet-}$. Furthermore, this study can also conclude that high fat diets offer no protection from DOMS as suggested by Lenn et al. (2002). Furthermore, high fat diets have no effect on recovery of muscle function following downhill running.

It was noted that all subjects were able to maintain running at an intensity corresponding to 60% $\dot{V}O_{2\,max}$ during this study and furthermore, this intensity resulted in significant DOMS, loss of muscle function, and ROS production. It can therefore be concluded that 60% $\dot{V}O_{2\,max}$ is a suitable exercise intensity to run downhill in studies on DOMS and will hence be the exercise intensity used for the rest of this thesis.

In summary, 30 minutes of downhill running at 60% $\dot{V}O_{2\,max}$ results in a delayed increase in ROS production that is associated with secondary increases in muscle damage as seen by increased plasma CK activity. However, although a high CHO diet resulted in a trend for attenuated leukocytosis, this had no effect on ROS production, DOMS or muscle function. Although this could be interpreted as neutrophils having no effect on ROS production following downhill running, it was speculated that circulating neutrophil levels may not represent what is happening at the injury site. It was also concluded that in agreement with Bishop et al. (2001a)

when changes in immune function are low, pre-exercise CHO status has little effect on immune function and thus has no effect on ROS production. In order to further investigate the relationship between neutrophil derived ROS and DOMS, a specific neutrophil derived ROS inhibitor should be used, such as ascorbic acid.

CHAPTER 6 – Study 3

The effects of ascorbic acid supplementation on delayed onset muscle soreness, muscle torque, and production of reactive oxygen species following downhill running.

6.1 Introduction

In study 1, 30 minutes of downhill running was found to result in a significant increase in ROS production in the days following the exercise. The increase in ROS was speculated to be due to phagocyte-derived $O_2^{\bullet-}$ production resulting in the subsequent formation of the more potent $^{\bullet}OH$. Furthermore, peroxidation of lipid membranes by $^{\bullet}OH$ resulted in the production of the secondary alkoxyl radical which was detected by ESR spectroscopy.

Study 2 further investigated the relationship between ROS and DOMS by manipulating dietary CHO in an attempt to attenuate the $O_2^{\bullet-}$ release by phagocytic cells. It was concluded that using the chosen exercise protocol, the interaction between pre-exercise carbohydrate status and the phagocytic oxidative burst was not strong enough and therefore the next study should investigate the effects of specific ROS inhibitors on DOMS and muscle function, such as ascorbic acid.

In response to physical trauma, ascorbic acid is able to provide protection against phagocyte-mediated cell injury by scavenging the specific phagocyte-derived ROS (Nieman et al., 2002). Ascorbic acid is able to scavenge ROS by rapid aqueous-phase electron transfer and thereby reduce the adhesion of phagocytes to the endothelium, attenuating their respiratory burst and preventing lipid peroxidation (Kearns et al., 2001). Despite this, the majority of research into antioxidant supplementation on

ROS and DOMS is based around α-tocopherol with few studies investigating the relationship between ascorbic acid supplementation, oxidative stress and DOMS.

It was not possible to conclude from studies 1 and 2 if the production of ROS caused further damage and prolonged DOMS, or assisted in recovery from the initial trauma. In the first two studies, ROS concentrations were at their highest when muscle function was returning to pre-exercise value, suggesting that the production of ROS in the days following exercise-induced muscle damage may assist the recovery. Despite this, the presence of ROS can only indicate that ROS are being produced and cannot determine if their production is involved in the observed pathology (Jackson, 1999). The only way that ROS can be confirmed to be involved in the pathology is through specific antioxidant intervention. The aim of this study was therefore to examine the effects of prolonged ascorbic acid supplementation on ROS production following downhill running, and investigate the effects of this supplement on DOMS and muscle function. Furthermore, since the first two studies did not establish the time course of ROS production following downhill running, the post-exercise test period was extended to 14 days.

6.2 Methods

6.2.1 Subjects

Twenty physically active male subjects, naïve to downhill running, volunteered for this study. All were non-smokers and free from any known illnesses as ascertained by medical questionnaire. Subjects taking vitamin supplementation were excluded. All subjects were informed verbally and in writing about the nature of the study, including all potential risks. Written informed consent was obtained prior to participation and ethical approval was granted by the Liverpool John Moores University Ethics Committee.

6.2.2 Experimental protocol

Each subject was initially assessed for aerobic fitness by determining their $\dot{V}O_{2\,max}$, as described in chapter 3.2. The assessment of $\dot{V}O_{2\,max}$ was carried out one week prior to the downhill run. The downhill run lasted for 30 minutes at a running speed corresponding to 60% $\dot{V}O_{2\,max}$. All subjects were randomly allocated into two groups, placebo (Pl n=10), and ascorbic acid (AA n=10).

For each trial subjects were initially required to visit the laboratories on five consecutive days. Day 1 was to receive the supplement, have muscle function and pain measurements taken, provide pre and post-supplement venous blood samples, perform the downhill run and to give a post-exercise venous blood sample. The

following 4 days were to have muscle function and pain assessed, and to give a resting venous blood sample. Furthermore, subjects were required the visit the laboratory again on day 7 and day 14 to have muscle function and pain assessed, and to give a resting venous blood sample. Subjects were instructed to avoid any strenuous exercise for 72 h prior to the downhill run, and to continue to abstain from any strenuous exercise throughout the 14 day duration of the test. Subjects were also instructed to avoid any form of therapeutic intervention to alleviate the muscle soreness including, drug, ice or massage therapy.

6.2.3 Day 1

On the day of the experimental run, subjects visited the laboratory 2 h prior to their allocated run time. On arrival subjects were seated for 30 minutes and then a pre-supplement blood sample was taken. Subjects were then given 1000mg of either ascorbic acid (Roche, UK) or 1000mg of a visually identical lactose placebo (Roche). Subjects were randomly assigned into the two groups and supplementation was administered double blind. Following the supplementation, subjects were given a 2 h break to allow time for the supplement to be absorbed.

Subjects returned to the laboratory 2 h post-supplementation to give a second venous blood sample and were then asked to rate their pre-exercise muscle soreness of the gastrocnemious, tibialis anterior, hamstrings, quadriceps, gluteals, (both left and right sides), and lower back muscles using a visual analogue scale (VAS), pain diagrams

and pressure algometry as described in section 3.5. Subjects had been previously familiarised to all of the methods of pain assessment to eliminate any learning effects. Subjects then proceeded to warm up on a cycle ergometer for 5 minutes before completing a series of stretches of their own choice. The stretches were to prepare them for the assessment of muscle function on an isokinetic dynamometer, as described in section 3.4.

All blood samples were collected in vacutainers following an overnight fast from a superficial vein in the antecubital fossa whilst the subjects were supine and had been resting for a minimal of 30-minutes (except for the post exercise sample which was taken immediately upon cessation of exercise). Samples were taken using minimal stasis to prevent any potential increase in oxidative stress due to ischemic-reperfusion injury. This was achieved by taking all samples without a tourniquet. A total of four vacutainers were used at each sample point.

Tube 1 (5ml) contained the anti-coagulant EDTA and was used for the analysis of total and differential leukocyte numbers, total blood GSH, haematocrit and haemoglobin. Tube 2 (6ml) contained serum separation gel and was used for the determination of MDA. Tube 3 (6ml) contained lithium heparin and was used for the analysis of plasma CK and ascorbate.

The downhill run took place on the same motorised treadmill used in the $\dot{V}O_{2max}$ test. The run lasted for 30-minutes at a decline of –15% and at an oxygen consumption corresponding to 60% $\dot{V}O_{2max}$. Throughout the trial, expired gas volumes and concentrations were recorded every 10-seconds. The treadmill speed was continually altered to ensure that the oxygen consumption corresponding to 60% of $\dot{V}O_{2max}$ was maintained. Every 10 minutes, ratings of perceived exertion (RPE) were recorded using the 6-20 Borg Scale, as well as heart rate (HR) using a short-range radio telemeter (Polar, Sportstester, PE3000, Kempele, Finland). Thermal comfort was maintained throughout the run by means of two electronic fans (MS16, Amcon).

Immediately following the run, subjects assumed a supine position whilst a third blood sample was taken using the same technique as described previously. Subjects were then re-assessed regarding their perceptions of muscle soreness before completing the force assessment again. This completed day 1 of the trial. All subjects were then instructed to continue to take 1000mg per day of their allocated supplement for 14 days. Supplements were taken in the form of 2 x 500mg tablets, one to be taken in the morning and one at night.

6.2.4 Days 2,3,4,5, 7 and 14.

Subjects visited the laboratory exactly 24, 48, 72, 96 h after their initial visit and again at 7 and 14 days post-exercise. They were supine for 30-minutes before a resting blood sample was taken from a prominent vein. The samples were treated as

described above. Following the blood tests, subjects were then assessed for DOMS. This was followed by a five minute warm up on a cycle ergometer before muscle function was re-assessed. A summary of the test protocol can be seen in figure 6.1.

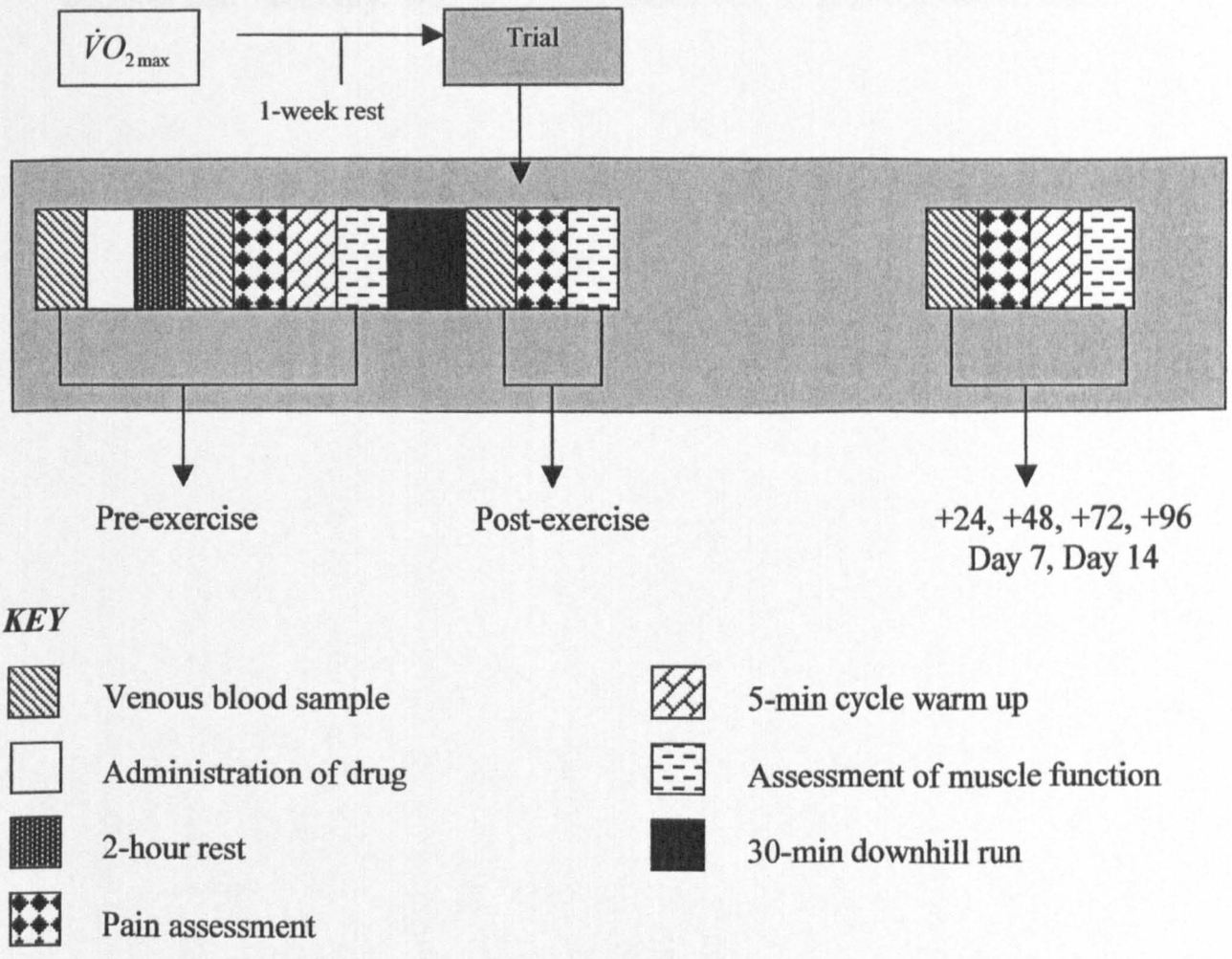


Figure 6.1. Schematic representation of the test protocol.

6.2.5 Statistical analysis

Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS Surrey, UK). All data are presented as means ± SEM. A two-way ANOVA with repeated measures was used to analyse all variables. When Mauchley's test of

sphericity indicated a minimal level of violation (>0.75) the degrees of Freedom was corrected using the Huynh-Feldt adjustment. When the sphericity was less than 0.75, the Greenhouse Geiser correction was used. Post-Hoc Tukey analysis (Honestly Significant Difference, (HSD)) was performed to identify where the significant differences occurred. Student's unpaired t-tests were used to compare individual variables were necessary. Statistical significance was set at $P<0.05$ for all tests.

6.3 Results

6.3.1 Subject characteristics

Prior to the experimental runs, subjects were initially assessed for $\dot{V}O_{2max}$ as well as having their height, weight, and percentage body fat calculated. There was no significant difference between the two groups for any of the characteristics (unpaired t-test P>0.05). A summary of the subject characteristics can be seen in table 6.1.

Table 6.1 Mean (±SD) characteristics of the subjects.

	Age (yrs)	*Height (cm)*	*Weight (kg)*	*$\dot{V}O_{2max}$ ($L.min^{-1}$)*	*Body Fat (%)*
Group PI	22.1 (± 1.8)	180 (+ 5.1)	78.4 (+ 14.6)	4.3 (± 0.6)	14.2 (± 3.4)
Group AA	24.2 (± 4.8)	179 (± 5.9)	78.3 (± 7.3)	4.4 (± 0.5)	13.6 (± 2.8)

6.3.2 Run data

Subjects successfully completed the 30-minute run at the desired work intensity. There were no significant differences (P>0.05) in heart rates, $\dot{V}O_{2max}$, RPE, RER, VE and run speed between the two groups (Table 6.2).

Table 6.2 Mean (±SEM) summary of the run data.

		Time (mins)						
	Group	Pre	5	10	15	20	25	30
Heart Rate *b.min^{-1}*	Pl	156 (± 6.1)	164 (± 4.7)	171 (± 3.3)	174 (± 3.6)	176 (± 3.3)	181 (± 3.4)	182 (± 3.4)
	AA	158 (± 3.6)	159 (± 4.9)	167 (± 5.1)	169 (± 4.5)	172 (± 4.7)	173 (± 5.1)	176 (± 5.8)
$\dot{V}O_2$ *L.min*	Pl	2.48 (± 0.1)	2.65 (± 0.1)	2.73 (± 0.1)	2.78 (± 0.1)	2.77 (± 0.2)	2.65 (± 0.2)	2.73 (± 0.2)
	AA	2.41 (± 0.1)	2.66 (± 0.1)	2.69 (± 0.2)	2.65 (± 0.2)	2.73 (± 0.2)	2.65 (± 0.2)	2.73 (± 0.2)
RPE *6-20*	Pl	10.8 (± 0.5)	12.5 (± 0.6)	13.3 (± 0.7)	14.2 (± 0.6)	14.6 (± 0.7)	15.3 (± 0.6)	15.5 (± 0.6)
	AA	10.7 (± 0.6)	11.8 (± 0.4)	12.7 (± 0.4)	13.2 (± 0.4)	13.6 (± 0.6)	14.1 (± 0.7)	14.2 (± 0.8)
RER	Pl	0.82 (± 0.01)	0.88 (± 0.01)	0.90 (± 0.01)	0.89 (± 0.01)	0.88 (± 0.01)	0.89 (± 0.01)	0.90 (± 0.01)
	AA	0.84 (± 0.01)	0.91 (± 0.01)	0.89 (± 0.01)	0.91 (± 0.01)	0.90 (± 0.01)	0.90 (± 0.01)	0.90 (± 0.01)
VE *l.min^{-1}*	Pl	58.9 (± 4.9)	68.2 (± 5.0)	69.9 (± 5.4)	70.9 (± 5.4)	72.9 (± 5.5)	74.4 (± 5.7)	76.9 (± 5.5)
	AA	56.1 (± 4.0)	64.9 (± 1.4)	69.8 (± 2.5)	73.3 (± 2.7)	73.2 (± 2.8)	72.6 (± 3.0)	74.9 (± 3.2)
Run Speed *km.hr^{-1}*	Pl	13.7 (± 0.8)	13.5 (± 0.8)	13.1 (± 0.9)	12.6 (± 1.1)	12.2 (± 1.2)	12.1 (± 1.2)	12.0 (± 1.0)
	AA	13.8 (± 0.6)	14.0 (± 0.6)	14.0 (± 0.6)	13.5 (± 0.6)	13.1 (± 0.6)	12.4 (± 0.4)	12.2 (± 0.5)

6.3.3 Plasma Ascorbate concentration.

There was no significant difference ($P>0.05$) in baseline levels of plasma ascorbate concentration between the two groups. However 2 h post-supplementation, plasma concentrations of ascorbate had significantly increased in AA compared to Pl ($P<0.05$). Furthermore, plasma ascorbate concentrations remained significantly elevated above basal values for the two-week period in AA ($P<0.05$). There was however a significant increase ($P<0.05$) in plasma ascorbate concentration in Pl

immediately post-exercise. Plasma ascorbate concentrations for AA and Pl throughout the trial can be seen in Figure 6.2.

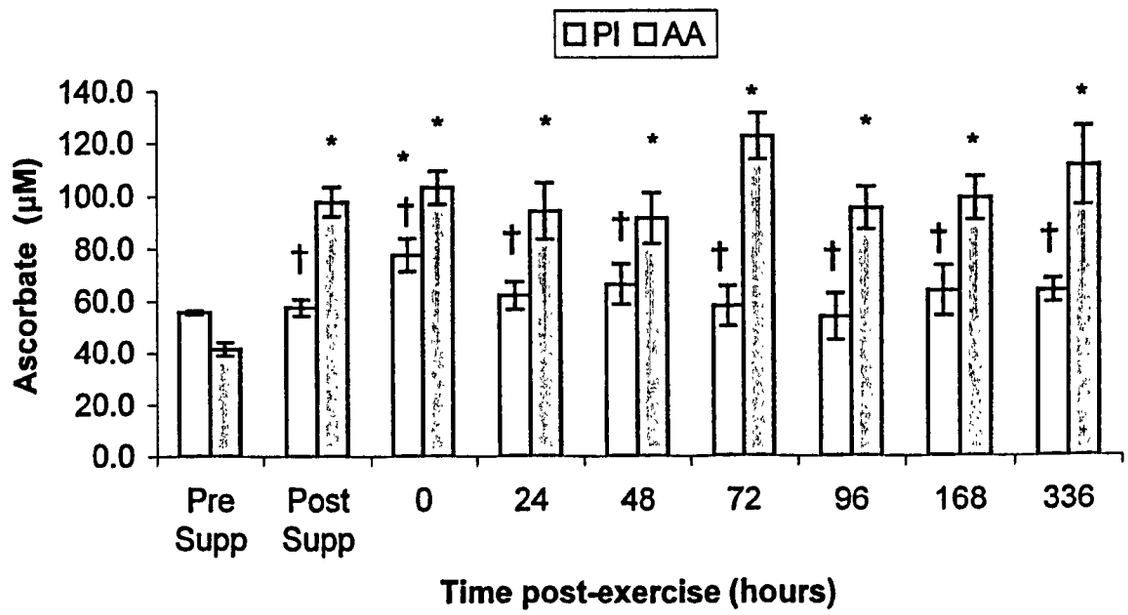


Figure 6.2. Mean (±SEM) plasma ascorbate concentrations.
*** indicates significant difference from pre-supplement**
† indicates significant difference from AA

6.3.4 Delayed onset muscle soreness

The downhill runs were successful in eliciting significant muscle soreness in both AA and Pl. Peak soreness occurred 48 h post-exercise in both groups and was still significantly elevated 96 h post exercise ($P<0.05$). This was evident in the VAS (Figure 6.3), the pain diagrams (Figure 6.4), and pressure algometry (Figure 6.5). All perceptions of DOMS had returned to baseline by 14 days post-exercise and there was no significant difference between the two groups ($P>0.05$). There was a significant correlation (Pearson's product moment correlation, $R^2 = 0.98$; $P<0.05$) between the pain diagram and the VAS (figure 6.6), and there was also a significant correlation ($R^2 = 0.93$; $P<0.05$) between the change in pressure applied to gain a painful response (pressure algometry) and the pain diagrams (Figure 6.7).

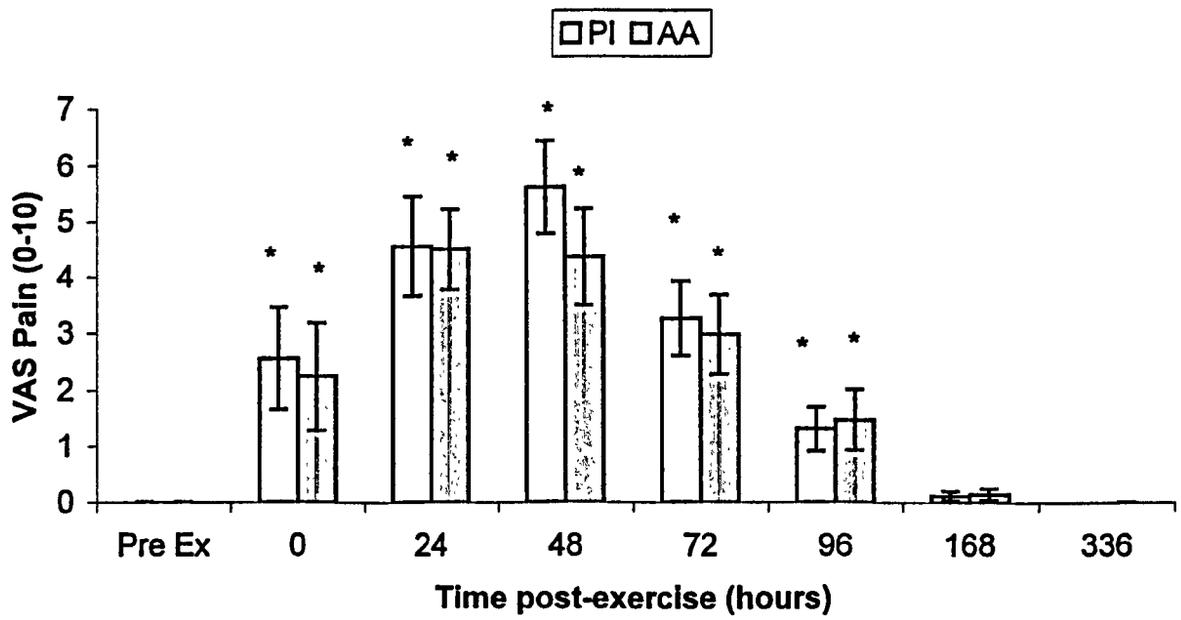


Figure 6.3. Mean (±SEM) ratings of muscle soreness assessed using a visual analogue scale
*** indicates significant difference from pre-exercise**

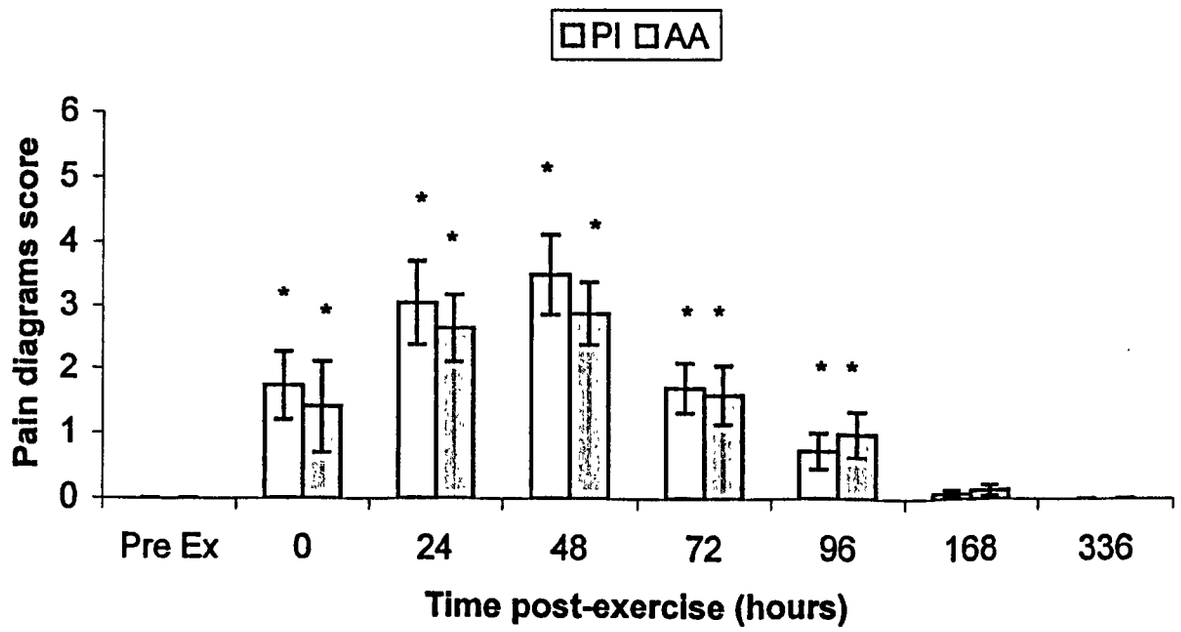


Figure 6.4. Mean (±SEM) ratings of muscle soreness assessed using pain diagrams.
*** indicates significant difference from pre-exercise**

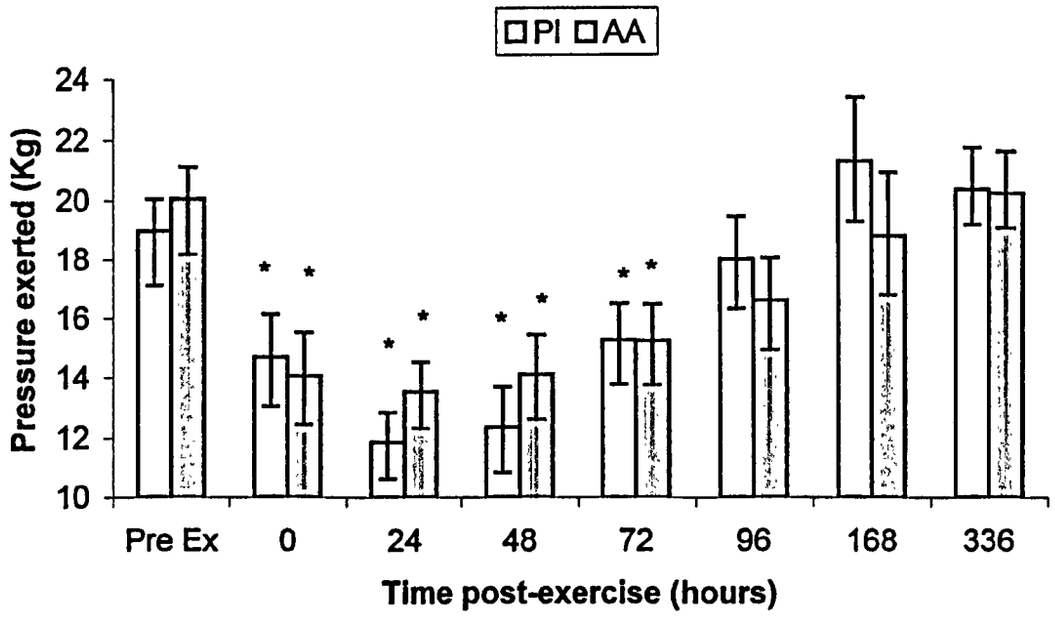


Figure 6.5 Mean (±SEM) pressure exerted (Kg) using a pressure algometer to achieve a painful response.
*** indicates significant difference from pre-exercise**

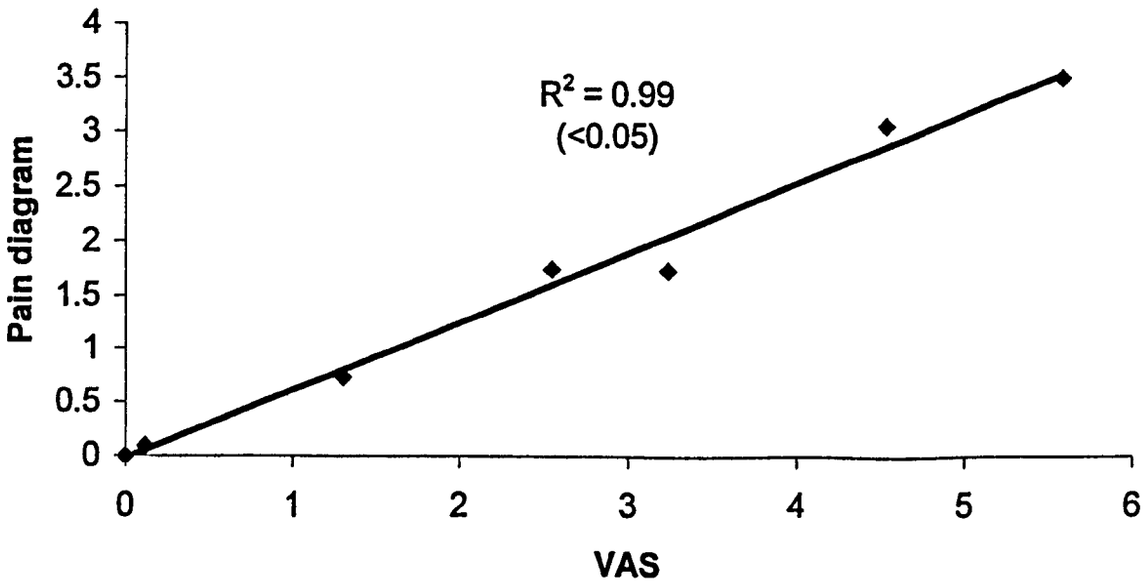


Figure 6.6 Correlation between mean scores from pain diagrams and mean scores from the VAS. Data are combined scores from PI and AA.

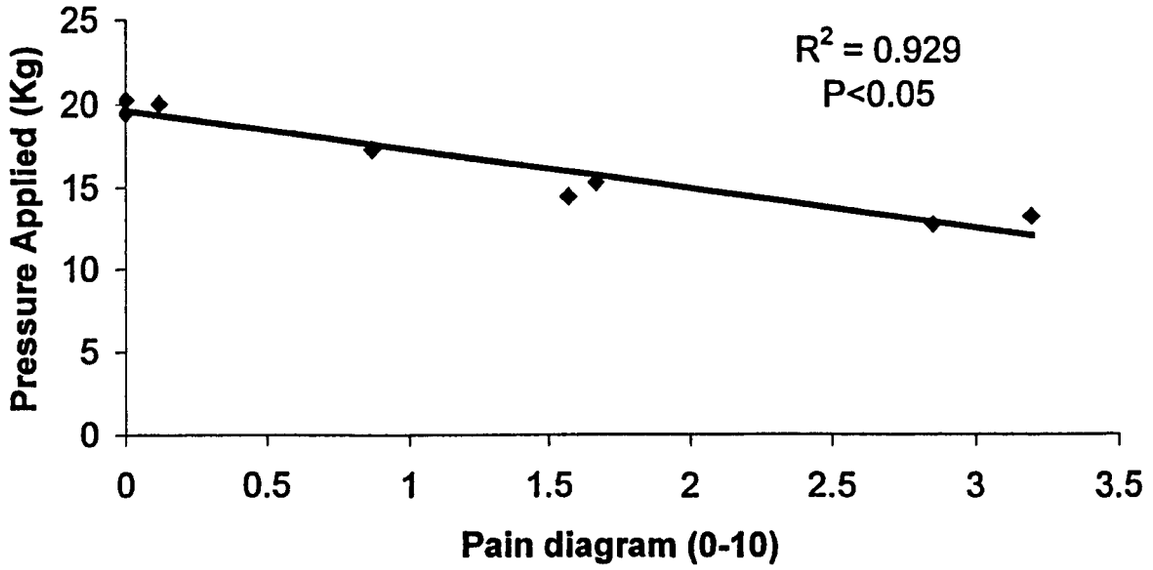


Figure 6.7 Correlation between mean scores from the pain diagrams and mean scores from the pressure algometer. Data is combined scores from Pl and AA.

6.3.5 Reactive oxygen species

Plasma concentrations of MDA can be seen in figure 6.8. There was no increase in MDA concentration in both groups immediately post-exercise (P>0.05). There was a significantly elevated serum MDA concentration in Pl compared to AA at 72 and 96 h post exercise (P<0.05). There was no significant change in total GSH concentration across time or between the two groups (P>0.05). There was however, a trend for GSH concentration to fall in Pl at 48 and 72 h post exercise, although this was not deemed to be statistically significant (Figure 6.9).

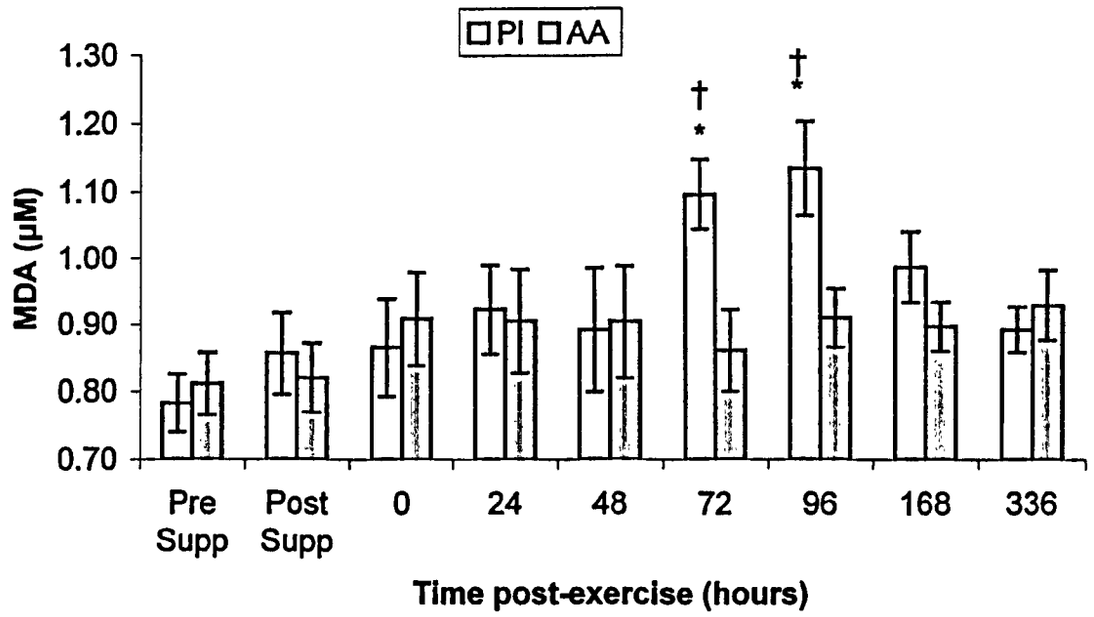


Figure 6.8 Mean (±SEM) serum malondialdehyde concentrations.
*** indicates significant difference from pre-exercise (post supp)**
† indicates significant difference from AA

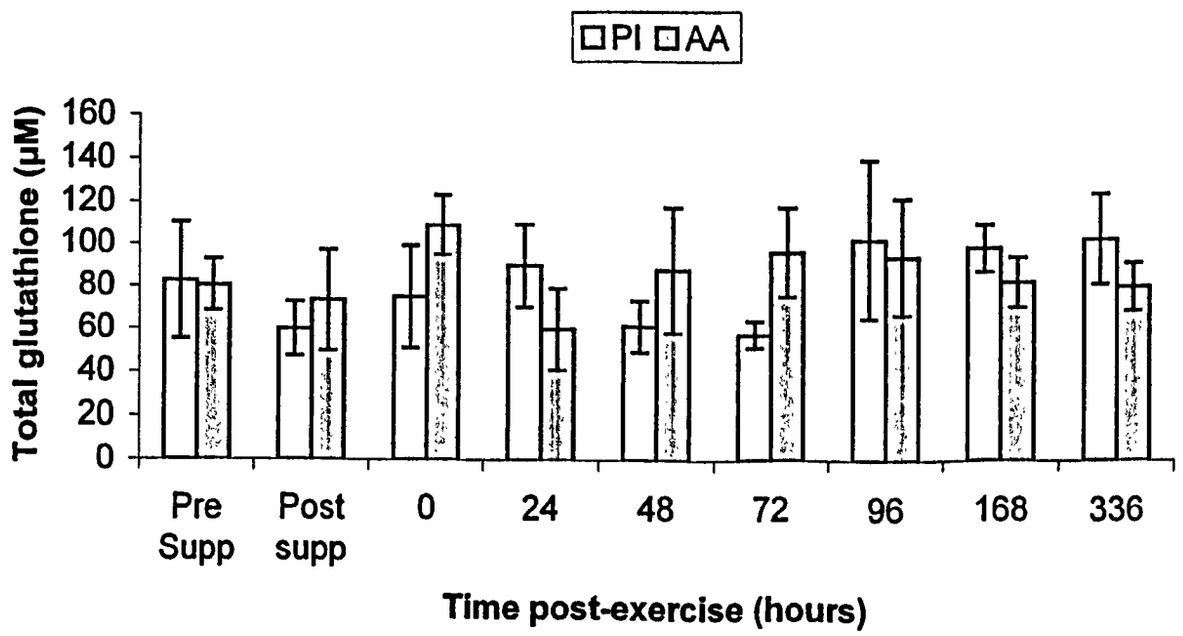


Figure 6.9 Mean (±SEM) total blood glutathione concentrations.

6.3.6 Muscle damage

Figure 6.10 shows plasma CK activity for the two groups. A significant increase in CK activity for both groups was observed 24 h post exercise ($P<0.05$). There was however, a second significant peak in CK activity 96 h post-exercise in the placebo group that was not evident in the supplement group ($P<0.05$). There were no differences in CK activity between the two groups at any other time point ($P>0.05$).

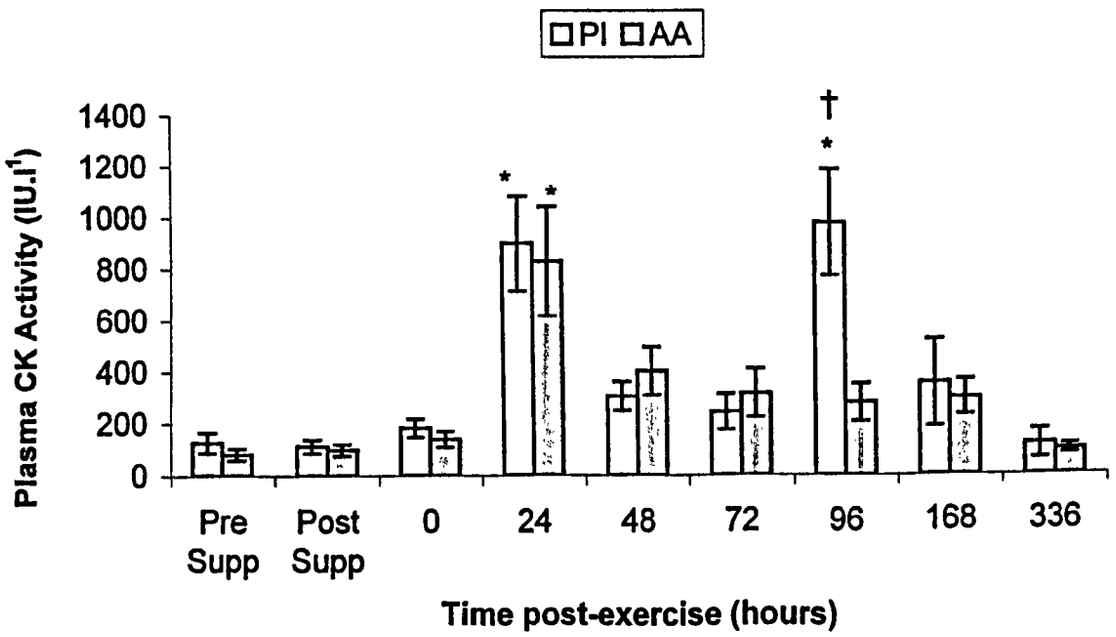


Figure 6.10 Mean (±SEM) plasma CK activity.
*** indicates significant difference from pre-exercise (post supp)**
† indicates significant difference from AA

6.3.7 Muscle function and DOMS felt during muscle function tests

Muscle function is expressed as percent change in torque from pre-exercise scores. Figure 6.11 shows the change in concentric quadriceps torque at 1.04 rad.sec^{-1}. A significant loss of peak torque was observed post-exercise in both groups ($P<0.05$). In Pl muscle function returned to basal levels by day 7 ($P>0.05$), although in AA

muscle function was still significantly impaired at both 7 and 14 days post-exercise ($P<0.05$) and had not retuned to basal levels by the end of the test period.

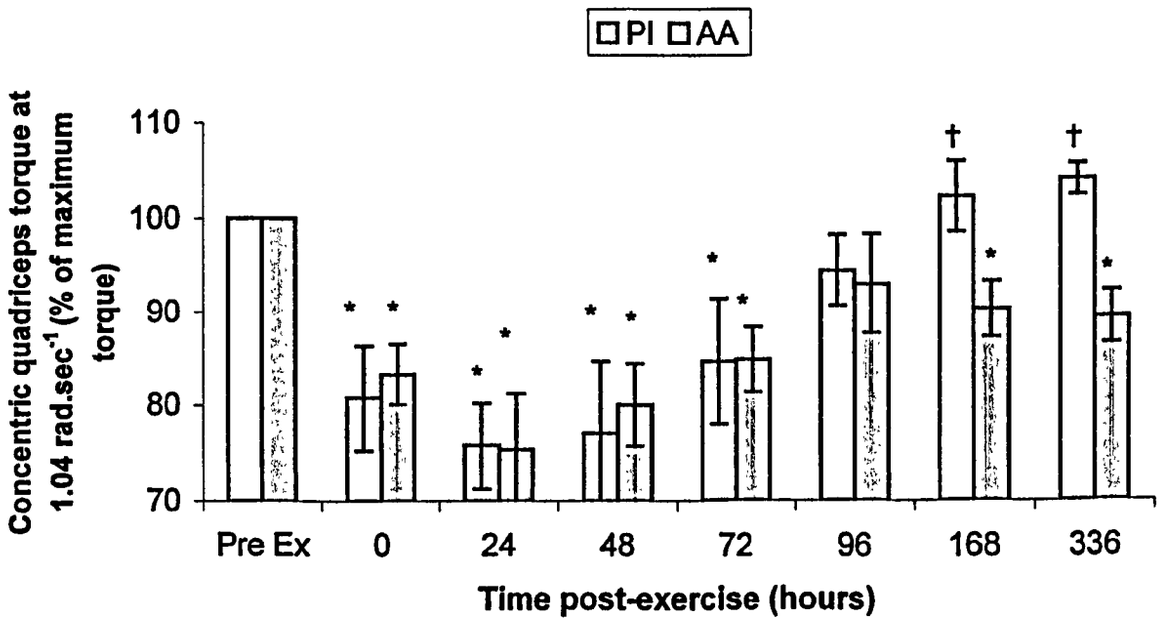


Figure 6.11 Mean (±SEM) concentric quadriceps torque at 1.04 rad.sec^{-1}, expressed as percent of pre-exercise value.
*** = significant difference from pre-exercise**
† = significant difference from AA

Tables 6.3 – 6.5 represents the mean (±SEM) ratings of DOMS felt during the muscle function assessment. There was a significant increase in DOMS post-exercise in both conditions and this remained elevated up to 96h post exercise ($P<0.05$). There was no significant difference in DOMS felt during the contraction between the two groups ($P>0.05$).

Table 6.3 Mean (±SEM) DOMS felt during concentric contraction at 1.04 rad.sec^{-1}
*** = significant difference from pre-exercise**

Time Post-Ex (hours)		Pre Ex	0	24	48	72	96	168	336
DOMS	PI	0.0 (± 0.0)	2.1 * (± 0.8)	5.6 * (± 0.9)	5.0 * (± 0.8)	2.9 * (± 0.5)	1.4 * (± 0.4)	0.2 (± 0.1)	0.0 (± 0.0)
	AA	0.0 (± 0.0)	2.0 * (± 1.1)	5.0 * (± .6)	4.5 * (± 0.8)	2.5 * (± 0.9)	1.4 * (± 0.7)	0.1 (± 0.1)	0.0 (± 0.0)

Muscle function was also impaired in both groups post-exercise when measured at 5.20 rad.sec^{-1}. There was however no significant difference between the two groups (P>0.05) with muscle function returning to basal levels at 96 h (Figure 6.12).

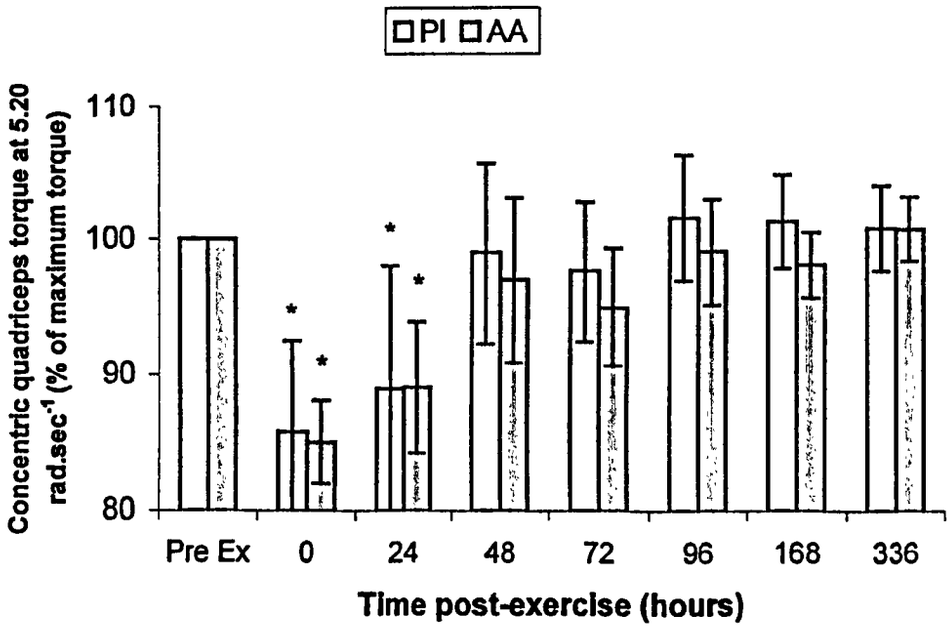


Figure 6.12 Mean (±SEM) concentric quadriceps torque at 5.20 rad.sec^{-1} expressed as percent change from pre-exercise.
*** = significant difference from pre-exercise**
† = significant difference from AA)

Table 6.4 Mean (±SEM) DOMS felt during concentric contraction at 5.20 rad.sec^{-1}
*** = significant difference from pre-exercise**

Time Post-Ex (hours)		Pre Ex	0	24	48	72	96	168	336
DOMS	PI	0.0 (± 0.0)	1.6 (± 0.5)	4.1 * (± 0.8)	3.7* (± 1.0)	2.5* (± 0.6)	1.1 (± 0.6)	0.2 (± 0.1)	0.0 (± 0.0)
	AA	0.0 (± 0.0)	1.0 (± 0.7)	3.8* (± 0.8)	2.8* (± 0.7)	1.9* (± 0.7)	1.1 (± 0.5)	0.2 (± 0.2)	0.0 (± 0.0)

Eccentric muscle torque at 2.06 rad.sec^{-1} was also assessed. Figure 6.13 shows that there was a significant loss in muscle torque post-exercise in both groups (P<0.05).

In both groups this was still apparent up to 72 h post exercise. However, although muscle torque had returned to basal levels by 96 h in Pl (P>0.05), muscle torque was still impaired at day 7 and day 14 post-exercise in AA.

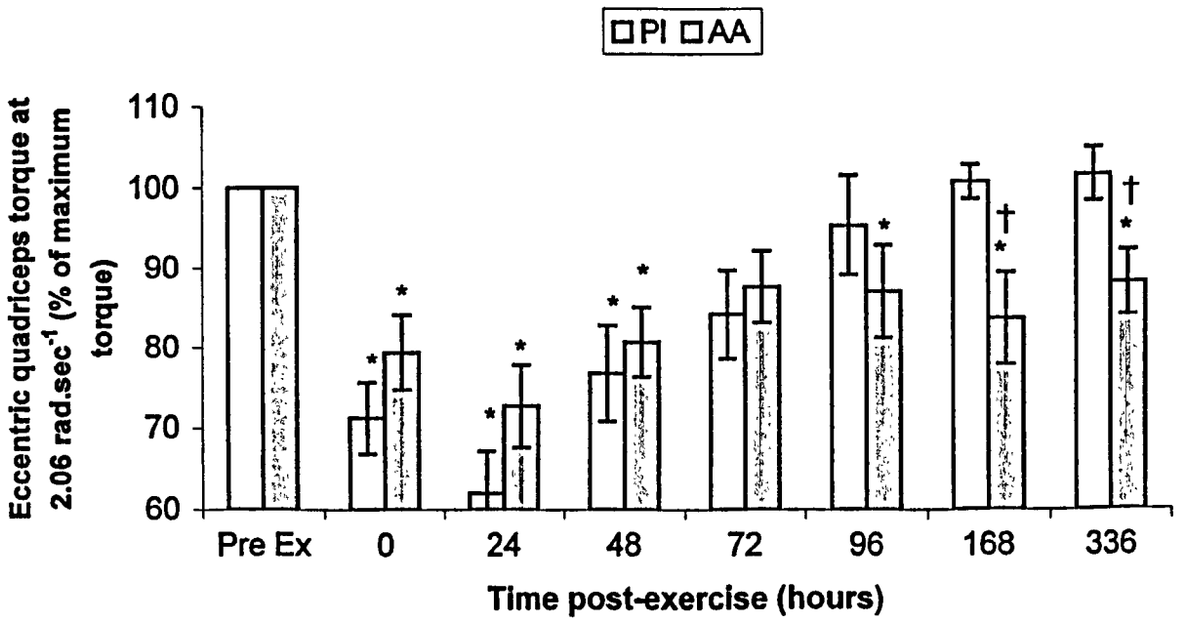


Figure 6.13 - Mean (± SEM) eccentric quadriceps torque at 2.06 rad.sec^{-1} expressed as percent change from pre-exercise.
*** = significant difference from pre-exercise**
† = significant difference from AA

Table 6.5 DOMS felt during eccentric contraction at 2.06 rad.sec^{-1}
*** = significant difference from pre-exercise**

Time Post-Ex (hours)		Pre Ex	0	24	48	72	96	168	336
DOMS	Pl	0.0 (± 0.0)	3.2* (± 1.1)	6.1* (± 0.9)	6.2* (± 0.7)	4.6* (± 1.0)	2.0* (± 0.7)	0.5 (± 0.4)	0 (± 0.0)
	AA	0.0 (± 0.0)	1.4* (± 0.9)	4.7* (± 0.8)	4.1* (± 0.8)	2.6* (± 0.8)	0.8 (± 0.7)	0.3 (± 0.3)	0.0 (± 0.0)

6.3.8 Leukocytes

There was significant leukocytosis (P<0.05) immediately post-exercise following both supplements. This was predominantly due to a transient increase in circulating

neutrophil counts ($P<0.05$) as well as a transient increase in lymphocyte counts, (Figures 6.14-6.16). There was however no significant difference in total or differential leukocyte counts between the two groups ($P>0.05$).

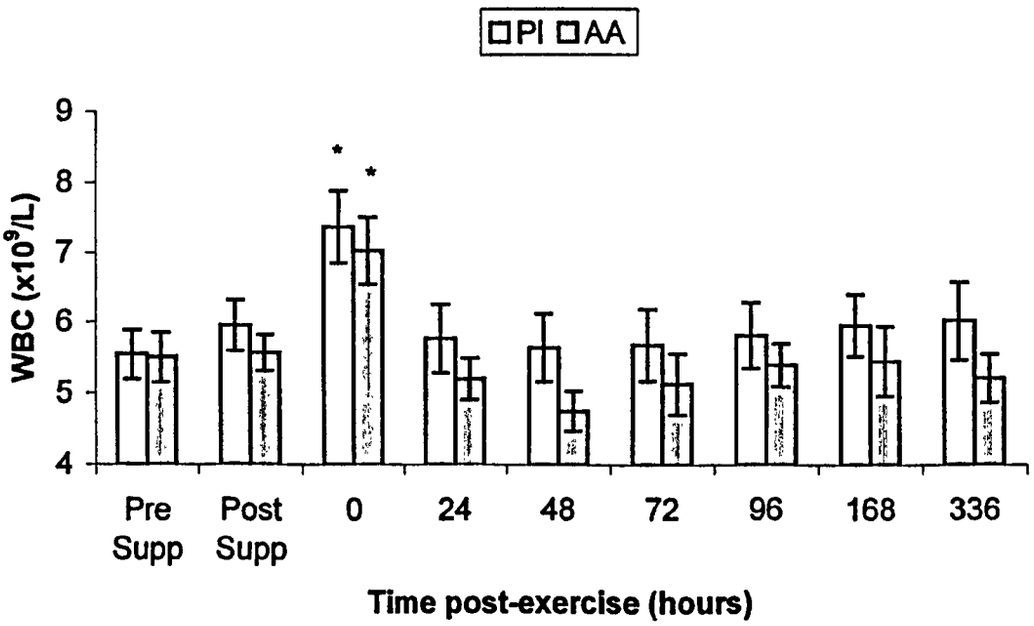


Figure 6.14 Mean (±SEM) leukocyte count ($x10^9$/L)
*** indicates significant difference from pre-exercise (post supp)**

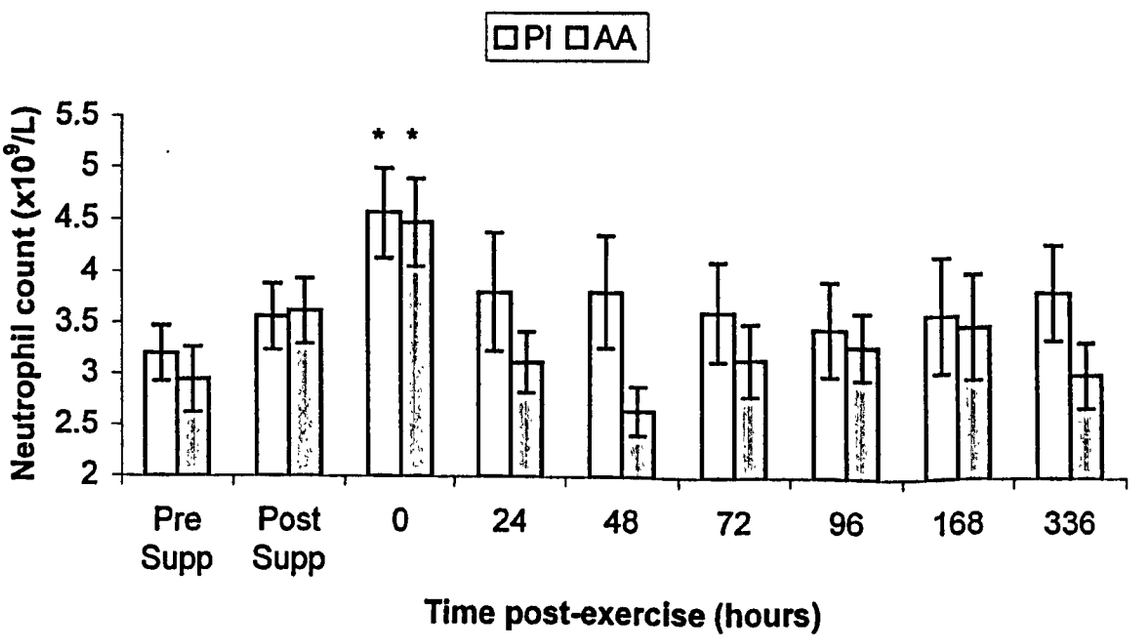


Figure 6.15 Mean (±SEM) neutrophil count ($x10^9$/L)
*** indicates significant difference from pre-exercise (post supp)**

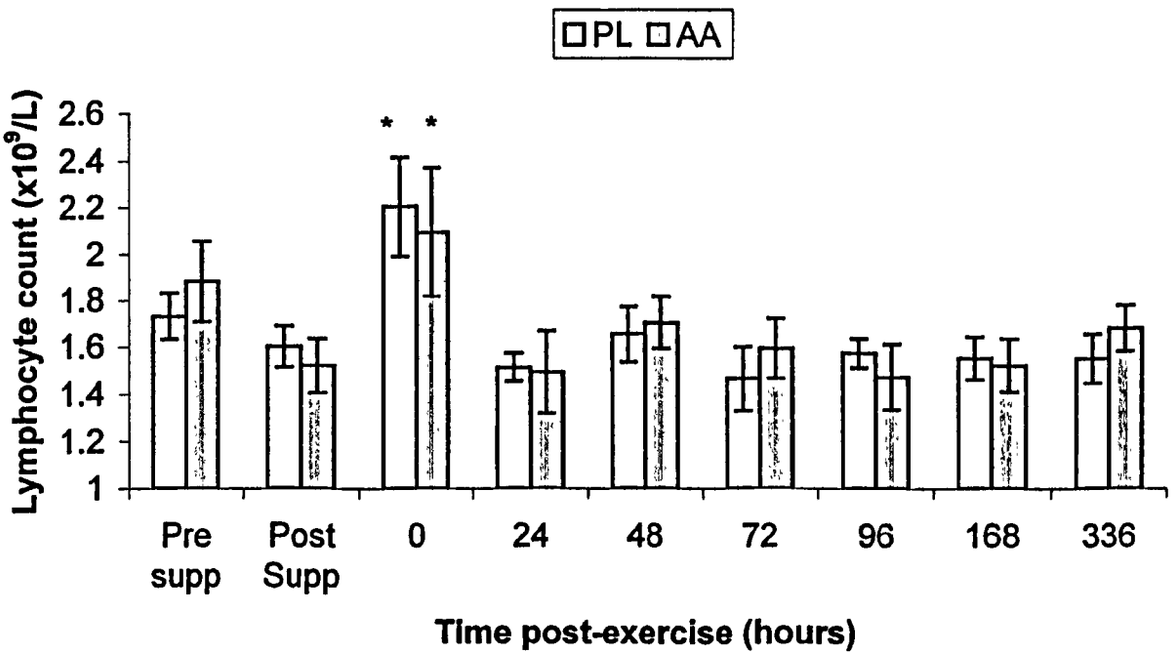


Figure 6.16 Mean (±SEM) lymphocyte count (x10^9/L)
*** indicates significant difference from pre-exercise (post supp)**

6.4 Discussion

The purpose of this study was to examine the effects of a therapeutic and prophylactic dose of ascorbic acid supplementation on DOMS and exercise-induced ROS production. Supplementation with ascorbic acid significantly increased plasma levels of ascorbate, this being observed pre-exercise and throughout the duration of the trial. The downhill run resulted in an increase in ROS production 96 h post exercise, as indicated by elevated serum MDA concentrations and a trend for a fall in total blood GSH. Interestingly, the ascorbic acid supplementation reduced the increase in ROS and attenuated secondary increases in plasma CK activity. However despite the reduction in ROS by the ascorbic acid supplementation, there was no significant difference in DOMS between the two groups and furthermore, ascorbic acid supplementation resulted in a more prolonged loss of muscle function.

Plasma ascorbate concentrations increased significantly following the supplementation and remained elevated for the two-week supplementation period. Peak plasma ascorbate concentration was ~120μM which is similar to values reported in the literature following similar supplementation (Thompson et al., 2001b). Two hours post-supplementation, plasma ascorbate concentration were elevated to a comparable extent as reported following several weeks of 1000mg doses of ascorbic acid (Kanter et al., 1993). Ascorbate rapidly reaches a steady state in humans when administered in amounts greater than 200mg per day (Levine et al., 1996) and it would therefore appear that prolonged supplementation with ascorbic acid is not necessary to achieve plasma ascorbate saturation.

In agreement with Thompson et al., (2001a; 2001b) there was an increase in plasma ascorbate in Pl immediately post-exercise, demonstrating an endogenous release or decreased clearance of ascorbate in response to the exercise. In contrast to Thompson et al. (2001a; 2001b) the present study did not demonstrate any further increase in ascorbate concentration post-exercise in AA. This may be due to the longer and more metabolically strenuous exercise protocol used in the afore-mentioned studies and would suggest that the exercise protocol used in the present study was not intense enough to stimulate the same level of endogenous ascorbate release.

This study aimed to elucidate the time course of ROS production following downhill running. Studies 1 and 2 have both demonstrated that following 30-minutes of downhill running there is a significant increase in ROS production, although the time course employed in these studies failed to determine peaks in ROS. In the present study it can be seen that plasma MDA concentration began to increase 72 h post exercise and peaked 96 h post exercise. Plasma MDA concentrations began to fall by day 7, and by day 14 there was no significant difference in plasma MDA from baseline. This is the first time that the time course of ROS production has been fully reported in humans following downhill running.

The primary aim of the study was to investigate the relationship between ascorbic acid supplementation, ROS production, DOMS and muscle function. The results clearly show that ascorbic acid supplementation attenuates increases in serum MDA

concentration compared to the placebo group. It is known that MDA is a specific marker of ROS mediated lipid peroxidation, and therefore suggests that ascorbate is able to scavenge the excess production of ROS prior to lipid peroxidation occurring. Although attenuation of ROS following ascorbic acid supplementation has been demonstrated immediately post-exercise following high intensity concentric exercise (Allesio et al., 1997; Ashton et al., 1999; Davison et al., 2003, Thompson et al., 2001a,), this is the first report of ascorbic acid attenuating ROS in the days following muscle-damaging exercise.

It is known that MDA correlates with alkoxyl radical production as detected by ESR spectroscopy (observation from study 1 and Davison et al., 2002), and furthermore the alkoxyl radical is thought to represent $^{\bullet}OH$ attack on lipid membranes (Pattwell et al., 2003). The increase in MDA in this study is therefore likely to be related to phagocytic derived $O_2^{\bullet-}$ production leading to $^{\bullet}OH$ generation through Fenton chemistry. Ascorbate is able to specifically protect against phagocytic derived $O_2^{\bullet-}$ through its ability to rapidly donate an electron to $O_2^{\bullet-}$(Ji, 1995). It would therefore appear that 1000mg of ascorbic acid given prior to and following downhill running successfully saturates phagocytes with ascorbate and scavenges the ROS that are produced. Alternatively, 1000mg supplements of ascorbic acid may cause an increase in plasma ascorbate concentrations that is sufficient to effectively deal with the increased ROS production. This ultimately prevents the peroxidation of lipid membranes and accounts for the decreased serum MDA production.

Plasma CK activity was measured to indirectly assess skeletal muscle damage. In both groups, plasma CK activity significantly increased 24 h post exercise as seen in studies 1 and 2. However, in the placebo group, there was a secondary peak in CK activity 96 h post exercise that was not seen in the supplemented group. This secondary peak in CK activity occurs at a time when serum MDA concentrations are elevated and suggests that ROS mediated secondary muscle damage is occurring, probably as a result of $^{\bullet}OH$ attack on lipid membranes. The lack of a second CK peak in the AA group confirms this suggestion and shows that ascorbate is able to scavenge the neutrophil-derived ROS thus preventing lipid peroxidation occurring. This finding of a secondary peak in CK activity has been previously observed by Smith et al., (1998) following downhill running.

Despite a secondary peak in CK, there was no evidence of further declines in muscle function or increased DOMS which suggests that the increase in ROS that stimulates a secondary increase in CK is not sufficient to cause any further decline in muscle function or stimulate pain receptors.

The present study sought to clarify the effect of ascorbic acid supplementation on DOMS. There was no significant difference in DOMS between the two groups. As in studies 1 and 2, peak pain occurred 48 h post-exercise and all perceptions of DOMS had gone by day 7 post-exercise. The present study demonstrated that although antioxidant therapy did reduce the secondary increase in ROS and may have prevented secondary muscle damage, supplementation with ascorbic acid had no effect on DOMS, further suggesting a dissociation between ROS and DOMS.

It was also feasible that supplementation with ascorbic acid may delay muscle function recovery as assessed through changes in muscle torque. Concentric quadriceps torque at 1.04 rad.sec^{-1} fell significantly in both groups post-exercise. In the placebo group, muscle torque returned to pre-exercise levels at 96 h post exercise, however in the ascorbic acid-supplemented group, muscle torque was still significantly less than pre-exercise at day 7 and day 14 post-exercise. An identical pattern was also seen when muscle torque was assessed eccentrically. These results suggest that the ROS produced as a consequence of muscle damaging exercise may play a vital role in muscle regeneration. These findings are supported by Thompson et al. (2001a), who also reported poorer muscle function in the leg flexors following ascorbic acid supplementation when assessed at 1.04 rad.sec^{-1}. The authors could not confirm the mechanisms for this finding although they suggested it could be related to pro-oxidant properties of ascorbic acid, specifically as a result of ascorbic acid's ability to recycle the ferrous form of iron. However, the authors did state that the most recent suggestions regarding ascorbic acid as a pro-oxidant are that it demonstrates poor pro-oxidant properties and that this was therefore an unlikely explanation of their observation. Furthermore, if ascorbate was acting as a pro-oxidant then higher circulating MDA would have occurred which was clearly not the case.

There are a number of reasons why the suppression of ROS by ascorbate in the days following damaging exercise delays the recovery. It is known that phagocytosis occurs following muscle damaging exercise, specifically involving the infiltration of neutrophils and macrophages into the damaged site. Once microbes, particulate

material or cytokines perturb the membrane of the phagocyte, these cells then produce $O_2^{\bullet-}$, a process referred to as the oxidative burst (Leeuwenburgh and Heinecke, 2001). During the oxidative burst, molecular oxygen is reduced by single electrons to form $O_2^{\bullet-}$ which is then dismutased by superoxide dismutase into H_2O_2. If the contents of secondary granules are then secreted, myeloperoxidase can then react with H_2O_2 in the presence of a halide chloride to form Hypochlorous acid (HOCl) and singlet oxygen ($^1O_2$). Furthermore, the more potent radical $^{\bullet}OH$ can also be formed through iron catalysed Fenton chemistry.

It is suspected that HOCl and $^{\bullet}OH$ are the important end points of this oxidative burst (Niess et al., 1999) although this is still not fully established. What is clear though, is that the oxidative burst plays an essential role in the killing and subsequent removal of damaged and necrotic tissue. The present study has clearly shown that 1000mg of ascorbic acid supplementation is able to attenuate post-exercise ROS production, and this is likely to be due to suppression of the oxidative burst. It would appear that this attenuation of the oxidative burst hinders the phagocytes in dealing with the trauma and it may be that this prolongs the damage and delays recovery.

An alternative mechanism involves the association between ROS and Heat Shock Proteins (HSPs). McArdle et al. (2003) have recently demonstrated that following eccentric contractions in a mouse model, the production of ROS results in a rapid increase in HSPs (specifically HSP70). It has been previously shown that HSP70 is a necessary component of the cellular repair machinery (McArdle et al., 2002) and therefore attenuation of ROS by ascorbic acid may attenuate the production of HSP70

and delay recovery. However since HSPs were not assessed in this study, this remains speculative and future work should be carried out to measure HSPs.

The findings of the present study regarding muscle function and ROS have significant implications for both scientists and athletes. Contrary to popular belief, ROS produced in the days following muscle-damaging exercise are not responsible for the prolonged losses of muscle function and pain although they may play a key role in mediating the recovery. Moreover, supplementation with ascorbic acid to prevent post-exercise ROS production does not attenuate DOMS or preserve muscle function, but may hinder recovery. Athletes would therefore be advised that supplementation of ascorbic acid is not only unnecessary but may be detrimental to future performance. It is known that HSPs play a vital role in protecting the muscle from subsequent damage (McArdle, 2002), and therefore attenuation of ROS and hence attenuation of HSPs may prevent the muscle from becoming protected from a repeated bout of muscle damaging exercise.

Based on these results, it is concluded that 30-minutes of downhill running results in a delayed increase in ROS production that peaks at 96 h post exercise. This increase in ROS production results in secondary muscle damage as indicated by increases in CK activity. Prolonged ascorbic acid supplementation attenuates the increase in ROS production and prevents secondary muscle damage, although ascorbic acid supplementation has no effect on DOMS. Furthermore, the present study concludes that the increase in ROS is necessary for muscle recovery and attenuation in this response significantly delays the recovery of muscle function as demonstrated through prolonged losses of muscle torque.

CHAPTER 7 - Synthesis of findings

7.1 Synthesis of findings

The studies performed in this thesis were designed to investigate the relationship between DOMS, loss of muscle function, and ROS. Furthermore, the studies were also designed to provide an insight into the role that CHO and antioxidant supplementation may play in attenuating DOMS.

Study 1, was designed to identify if ROS are produced following muscle damaging exercise. Downhill running was used as a method of inducing DOMS and this was compared to running on the flat. The results showed that following 30 minutes of downhill running there was a significant increase in the sensation of DOMS compared to running on the flat. ROS were produced following downhill running, however they were not detectable in the circulation until 72 h post exercise. It was observed that at this time, ratings of DOMS and muscle function had started to return to pre-exercise levels.

These findings implied that as opposed to the ROS causing DOMS and losses of muscle function, ROS may be involved in mediating the recovery. However, this was purely speculative as to make such a judgement, some form of ROS inhibition would be necessary (Jackson, 1999). This therefore raised several interesting questions that would be addressed in the ensuing studies.

Free radicals were assessed directly using ESR spectroscopy combined with a spin trap (PBN). It is often reported in the literature that ESR spectroscopy should be the method of choice as it is the only way to directly measure free radicals. ESR works by its ability to detect and characterise the presence of an unpaired electron. It has recently been reported that the ESR detectable radical is likely to be a secondary lipid radical formed through the peroxidation of lipid membranes by $^{\bullet}OH$ (Pattwell et al., 2003). This conclusion was made as the ESR detectable radical correlates well with the hydroxylation of salicylate to form 2-3 DHB (a method of assessing $^{\bullet}OH$ activity). Therefore, our results would suggest the presence of $^{\bullet}OH$ 72 h post downhill running. We speculated that the origin of $^{\bullet}OH$ was phagocytic derived $O_2^{\bullet-}$ being dismutased to H_2O_2 and finally forming $^{\bullet}OH$ *via* Fenton chemistry. It was therefore concluded that following downhill running there is an increase in phagocyte derived $O_2^{\bullet-}$ which in turn leads to peroxidation of lipid membranes. However it could not be concluded from this study if this was a physiological or pathological phenomenon.

In study 1 ROS were also measured using indirect methods, these being the production of MDA and total circulating GSH levels. It was noted that there was a strong relationship between ESR spectroscopic detection of free radicals and MDA. This has since been reported in the literature (Davison et al., 2002). Given the expense and time consuming nature of ESR spectroscopy (up to 2hr analysis per sample), it was decided that it would be justified to continue with MDA and GSH analysis alone for the remainder of the thesis.

It became apparent from study 1 that although 72 h was a time course often used in the literature to study DOMS, 72 h was not long enough to fully explore the relationship between DOMS and ROS. It was observed that ROS were at their highest 72 h post exercise and this was when we stopped measuring. Future studies in this thesis would therefore measure ROS for a longer time course in an attempt to fully map the temporal sequence of events.

In summary, study 1 demonstrated that 30 minutes of downhill running resulted in muscle damage (demonstrated through increased plasma CK activity), impaired muscle function and a transient leukocytosis. Interestingly, 72 h post exercise there was an increase in ROS production resulting in $^{\bullet}OH$ attack on lipid membranes. The study could not determine if this observed increase in ROS was causing further muscle damage and prolonging DOMS or alternatively was necessary in muscle regeneration. A schematic of the main findings of this study leading into the questions to be answered in later studies can be seen in figure 7.1

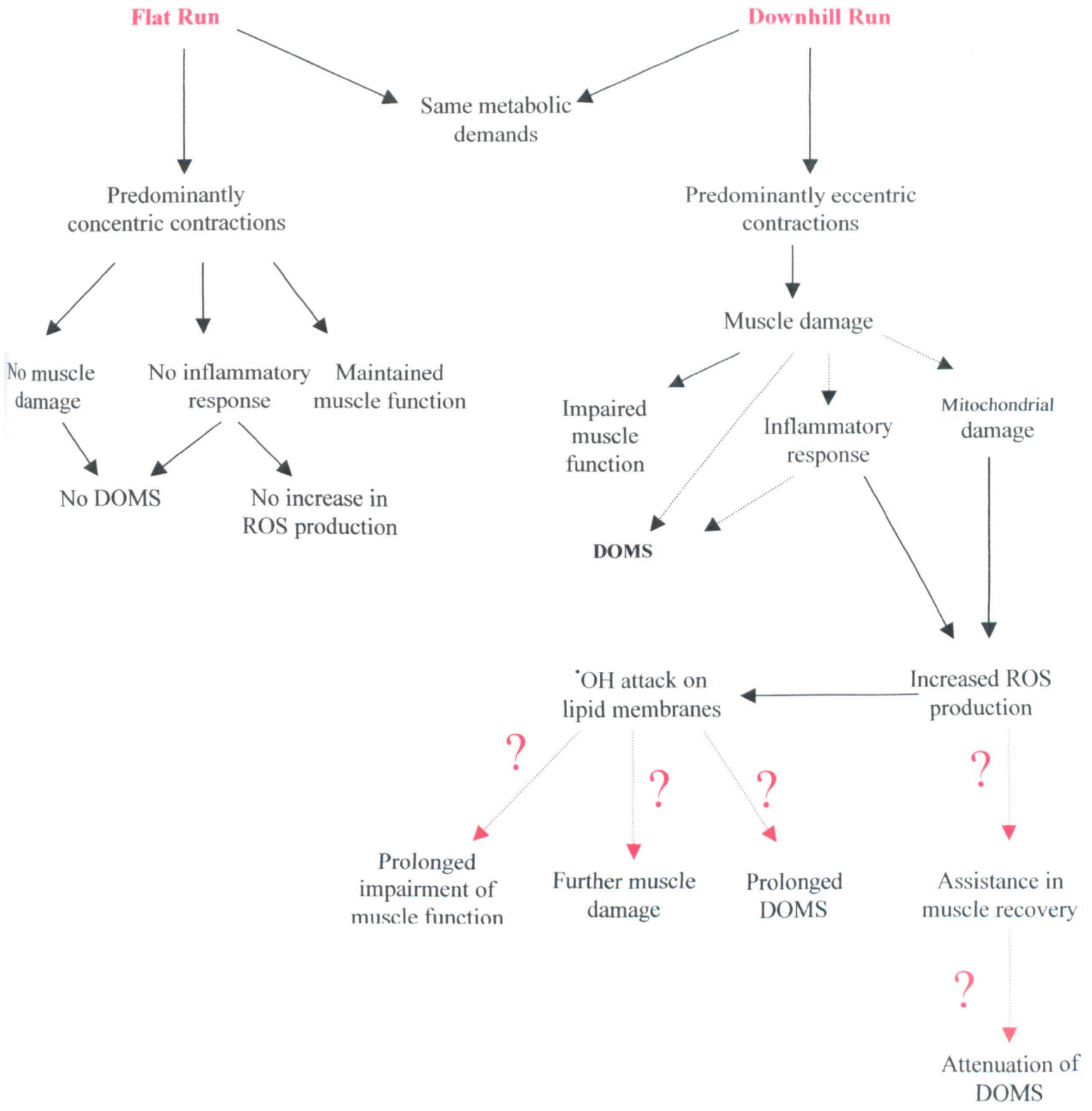


Figure 7.1 Schematic of the main findings from study 1

Study 2 sought to further investigate the relationship between ROS and DOMS though dietary manipulation. It was thought that if the ROS are being produced from phagocytic cells, then dietary CHO could influence the magnitude of this response. It was seen that despite the diets being successful in altering pre-exercise metabolite status, and post-exercise leukocytosis, the diets had no effect on DOMS, ROS production or muscle function. It was suggested that despite the slight attenuation in post exercise neutrophilia, circulating neutrophil counts were unlikely to represent what is happening at the injury site and therefore it would be unlikely that this dietary manipulation would have affected ROS production. The study concluded that to fully investigate the relationship between phagocyte derived ROS and DOMS, a specific ROS inhibitor should be used and this was the basis for study 3.

It was interesting to note that in this study, ROS were still significantly elevated at 96 h post-exercise and therefore the full temporal sequence of ROS production had still not been fully established. It was decided that the next study should add two extra measurement points, one being at day 7 and a final one at day 14.

Study 2 also demonstrated a biphasic response in CK activity. There was an initial peak at 24 h post exercise, as seen in study 1, but there was also as second peak observed at 96 h post-exercise. It was suggested that this secondary increase might reflect secondary damage to lipid membranes by $^{\bullet}OH$ generated from phagocytic derived $O_2^{\bullet-}$. This did not affect ratings of DOMS or muscle function; however to

fully establish this relationship an antioxidant intervention study would be necessary.

A schematic of the main findings from study 2 can be seen in figure 7.2

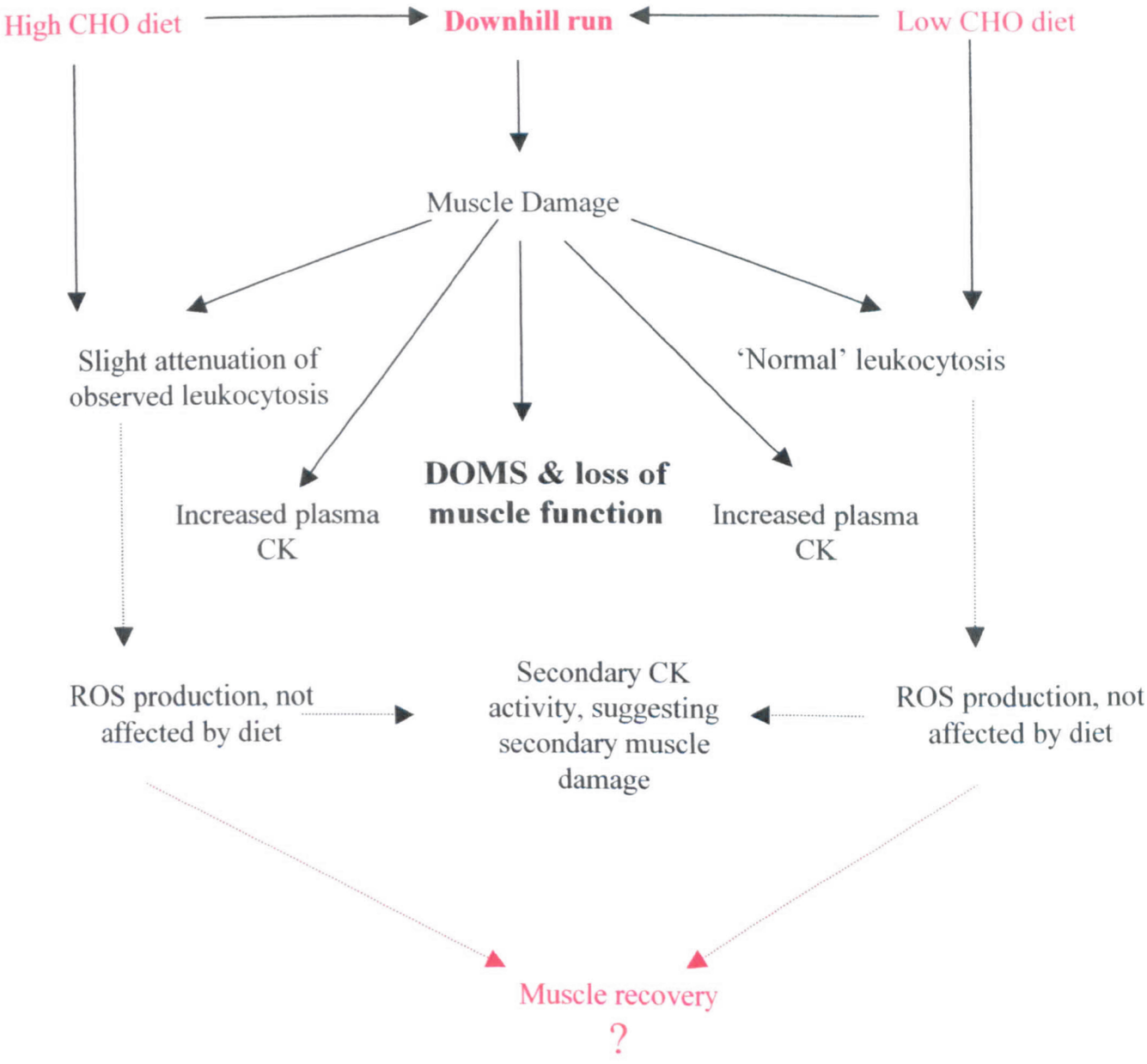


Figure 7.2 Schematic of main findings from study 2

The final study was introduced into the thesis in an attempt to answer some of the questions raised by the first two studies. Ascorbic acid was chosen as the ROS inhibitor because of its effectiveness in quenching neutrophil derived $O_2^{\bullet-}$.

It was seen that supplementation of ascorbic acid was successful in attenuating ROS production as witnessed in the first two studies. The increases in ROS presented as an attenuated increase in MDA as well as maintenance of total blood GSH. The study concluded that since ascorbic acid attenuated the increase in ROS, it was likely that the ROS observed where formed from phagocytic $O_2^{\bullet-}$ production.

The main finding from this study though was that despite this attenuation of ROS production, DOMS was still observed and there was no improvement in muscle function recovery. Conversely, supplementation with ascorbic acid may have delayed muscle recovery demonstrated by a prolonged loss of muscle function. By 96 h post exercise muscle function was back to pre-exercise levels in the placebo group, however it was still attenuated up to 14 days post-exercise following ascorbic acid supplementation.

It was therefore concluded that ROS produced in the days following muscle-damaging exercise are necessary for muscle regeneration and inhibiting their production delays recovery. Future research should look further into this, attempting to establish the exact methods by which ROS assist in muscle recovery. In a series of

recent experiments, McArdle et al. (2003) has identified that ROS stimulate the production of HSPs that assist in muscle regeneration and help in protecting the muscle from future damage. It would be interesting to investigate this relationship between ROS, HSP's and DOMS following downhill running.

Ascorbic acid supplementation also attenuated the secondary increase in CK activity. This suggested that the secondary increase in CK activity was due to ROS production, however this damage was not of sufficient magnitude to cause further DOMS or losses of muscle function.

Finally, the extra two time points used in study 3 allowed the temporal changes in ROS to be established. It was seen that ROS peaked 96 h post exercise and this had returned to baseline by day 7. There was no sign of any increase in ROS 14 days post-exercise.

A schematic of the main findings from study 3 can be seen in figure 7.3

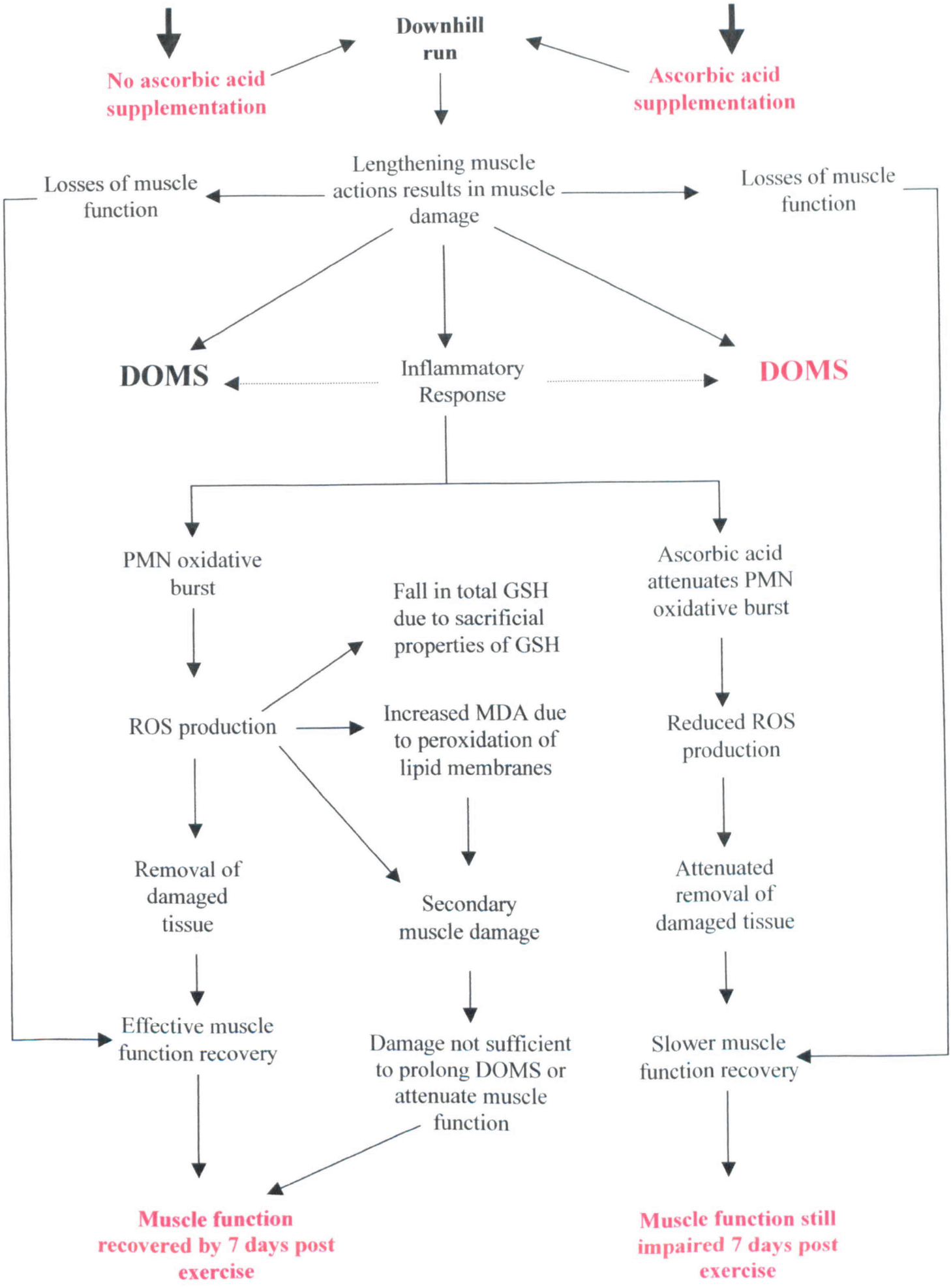


Figure 7.3 Schematic of the findings from study 3.

7.2 Conclusion

From the studies carried out in this thesis, the conclusions are that downhill running results in significant ROS production. The production of ROS occurs between 48 and 96 h post exercise and returns to basal levels by day 7 post-exercise. It is likely that ROS produced following downhill running originates from phagocytic derived $O_2^{\bullet-}$ resulting in the subsequent production of $^{\bullet}OH$, which in turn damages lipid membranes. This results in the production of secondary alkoxyl radicals detectable by ESR spectroscopy, and lipid peroxidation being detected by increased blood levels of MDA. Due to the disassociation between losses of muscle function, muscle soreness and ROS production, this thesis also suggests that ROS are not responsible for DOMS following downhill running and may in fact be necessary for recovery.

Along with the mechanistic conclusions that can be drawn from this thesis, practical recommendations to athletes can also be made. Study 2 demonstrated that pre-exercise CHO status has no effect on DOMS or losses of muscle function. This implies that training in a CHO depleted or elevated state has no effect on DOMS and will therefore have no effect on subsequent muscle torque production. However, study 3 demonstrated that ascorbic acid supplementation delayed recovery from the initial trauma. Athletes should therefore be advised that when starting a new training regime, it is not advisable to take 1000mg doses of ascorbic acid in an attempt to prevent DOMS. Figure 7.4 summarises the cascade of events that occurs following downhill running, highlighting the relationship between ROS, DOMS and muscle function.

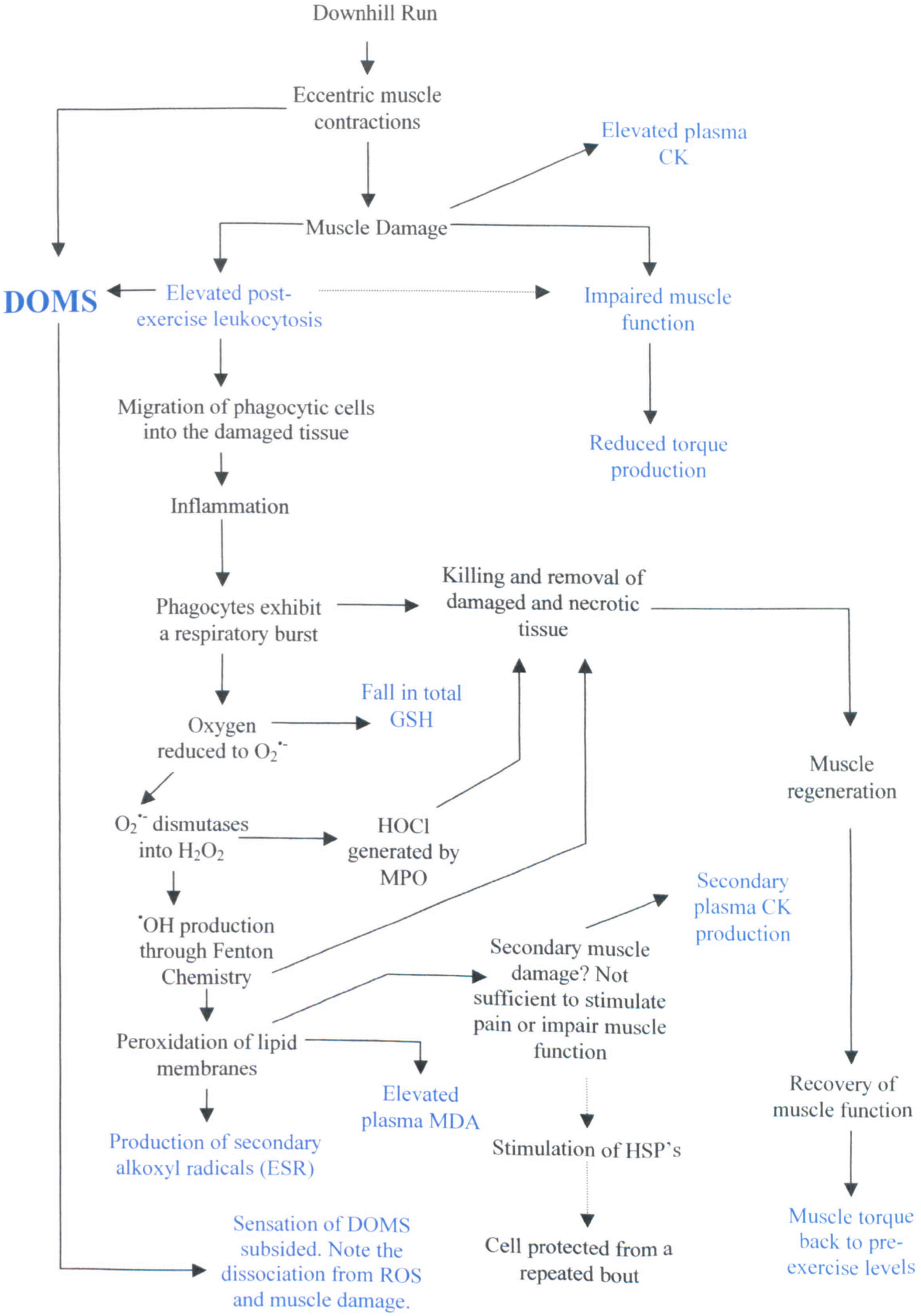


Figure 7.4 Model to illustrate the relationship between DOMS, ROS and losses/recovery of muscle function
Blue indicates actually assessed variable

7.3 Recommendations for future research

Having carried out the research in this thesis, and from the comprehensive review of the literature, the following recommendations for future research can be made:

- It would be interesting to carry out histological verification (either through muscle biopsies or in an animal model) to examine the extent of the secondary muscle damage caused by ROS production in the days following downhill running.

- The iron chelator, deferoxamine (DEF) is able to attenuate $^{\bullet}OH$ activity. Administration of DEF could be used to further examine the specific ROS that are produced following downhill running and examine which free radicals are playing the physiological role and which have a pathological role.

- It was suggested in study 3, that the reason that ROS may help with muscle recovery was due to their interaction with HSPs. A study that measured muscle HSPs following downhill running would be very beneficial.

- Finally, following an initial bout of eccentric exercise, the damaged cells are then protected from a repeated bout. It would be worthwhile carrying out an experiment that performed a repeated bout of downhill running 1 month after the initial bout in which ascorbic acid had been taken. The researcher proposes that the protective effect would be diminished following an initial bout in which ascorbic acid was supplemented.

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APPENDIX A – PUBLICATIONS

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APPENDIX B – SUBJECTS INFORMED CONSENT FORMS & INFORMATION SHEETS

Participant Information Sheet: Phase1.

Experiment Title: The effects of downhill (eccentric) and flat (concentric) running on delayed onset muscle soreness, muscle and immune function.

Project Title: The effect of environmental temperature and diet on muscle function following downhill running.

Project Researcher: Graeme L. Close, BSc. (Hons).

Supervisors: Dr D. MacLaren, BSc. (Hons), Ph.D.
Deputy Head of the School of Human Sciences

Dr N.T Cable BSc. (Hons), Ph.D
Reader & Head of Centre of the School of Human Sciences

Mr D. Doran B.A. (Hons) DIS. Mmed Sci, P.G.cert
Lecturer, School of Human Sciences

Purpose

The purpose of the present study is to examine the effect of downhill running on the markers of muscle damage and immune function. The findings from this research will allow a greater understanding of the immune response to high intensity exercise and ultimately develop ways of reducing and/or preventing exercise induced muscle damage and immunosupression. This will benefit recreational athletes as well as professional sportspeople. Furthermore the results from this investigation will be used in the submission of a thesis, hopefully resulting in a PhD.

Procedures

The study will take place in the exercise physiology laboratories of Liverpool John Moores University. The test will involve 3 phases with phases 2 and 3 being the experimental trials.

Phase 1. Perform an initial VO_{2peak} test on a motorised treadmill

The VO_{2peak} test is a maximal incremental exercise test performed until volitional exhaustion. The test will take place on a motorised treadmill. After a thorough warm-up, you will commence running at 10 m/s. Every two minutes the treadmill speed will increase by 2 m/s. This will continue until you can no longer maintain the required pace. Throughout the test, oxygen consumption and heart rate will be continuously monitored.

Phase 2. 30-minute downhill / flat run on a motorised treadmill.
Phase 3. 30-minute downhill / flat run on a motorised treadmill.

On arrival at the laboratory you will be asked to rest in a seated position for 15 minutes. Following this resting period a 20ml venous blood sample will be taken. The sample will be taken from a prominent vein in the arm using aseptic techniques. A **qualified phlebotomist** will take **all** blood samples. The blood samples will be later analysed for CK, CRP, cortisol, catecholamines, glucose, lactate, NEFA haemoglobin, haematocrit, lymphocytes, and neutrophils populations, MDA, and glutathione.

Following the pre-exercise blood sample muscle soreness will be assessed by means of by means of oral questioning and pressure tests on lower muscles. You will then warm up before performing a series of leg strength tests on an isokinetric leg dynamometer.

Subjects will then perform the run. The run will involve you running for 30 minutes at 65% of your own maximum oxygen uptake. Heart rate and oxygen uptake will be monitored throughout the run.

You will perform the run **twice** with a three-week gap between tests. On one occasion the run will be downhill, whilst on another occasion the run will be on the flat.

Following the run you will be asked to sit down whilst a second blood sample is taken. Immediately following this a second series of leg strength tests will be performed.

The whole process should take approximately 1 hour.

You will also be required to revisit the laboratory 24, 48 and 72 hours post exercise. The purpose of these visits will be to assess muscle strength and soreness as well as to give a 20ml venous blood sample. This visit should last approximately 30 minutes.

PLEASE NOTE: If you sign this document it **does not** affect your right to withdraw from the study at **any time** without prejudice.

Finally, thank-you very much for your participation in the study. Your time and effort is very much appreciated.

Participant Information Sheet: Phase 2.

Experiment Title: The effect of environmental temperature on the markers of immune response and muscle damage following high intensity downhill running.

Project Title: The effect of environmental temperature and diet on muscle function following downhill running.

Project Researcher: Graeme L. Close, BSc. (Hons).

Supervisors: Dr D. MacLaren, BSc. (Hons), Ph.D.
Deputy Head of the School of Human Sciences

Dr N.T Cable BSc. (Hons), Ph.D
Reader & Head of Centre of the School of Human Sciences

Mr D. Doran B.A. (Hons) DIS. Mmed Sci, P.G.cert
Lecturer, School of Human Sciences

Purpose

The purpose of the present study is to examine the role of environmental temperature on the markers of muscle damage and immune function following downhill running.

Procedures

The study will take place in an environmental chamber in the exercise physiology laboratories of Liverpool John Moores University. The test will involve 3 phases with phases 2 and 3 being the experimental trials.

Phase 1. Perform an initial VO_{2peak} test on a motorised treadmill

The VO_{2peak} test is a maximal incremental exercise test performed until volitional exhaustion. The test will take place on a motorised treadmill. After a thorough warm-up, you will commence running at 10 m/s. Every two minutes the treadmill speed will increase by 2 m/s. This will continue until you can no longer maintain the required pace. Throughout the test, oxygen consumption and heart rate will be continuously monitored.

Phase 2. 30-minute downhill run on a motorised treadmill in either 30 or 20°C heat.
Phase 3. 30-minute downhill run on a motorised treadmill in either 30 or 20°C heat.

On arrival at the laboratory you will be asked to rest in a seated position for 15 minutes. Following this resting period a 20ml venous blood sample will be taken. The sample will be taken from a prominent vein in the arm using aseptic techniques. A qualified phlebotomist will take all blood samples. The blood samples will be later

analysed for CK, CRP, cortisol, catecholamines, glucose, lactate, NEFA haemoglobin, haematocrit, lymphocytes, and neutrophils populations, MDA, and glutathione.

Following the pre-exercise blood sample muscle soreness will be assessed by means of by means of oral questioning and pressure tests on lower muscles. You will then warm up before performing a series of leg strength tests on an isokinetric leg dynamometer.

Subjects will then perform the run. The run will involve you running for 30 minutes at 65% of your own maximum oxygen uptake. Heart rate and oxygen uptake will be monitored throughout the run. Furthermore core temperature will be monitored by means of a rectal probe.

You will perform the run twice with a three-week gap between tests. On one occasion the run will be performed in the heat (~30°C), whilst on another occasion the run will be performed under a normal environmental temperature (~20°C).

Following the run you will be asked to sit down whilst a second blood sample is taken. Immediately following this a second series of leg strength tests will be performed.

The whole process should take approximately 1 hour.

You will also be required to visit the laboratory 24, 48 and 72 hours post exercise. The purpose of these visits will be to assess muscle strength and soreness as well as to give a 20ml venous blood sample. This visit should last approximately 30 minutes.

PLEASE NOTE: IF YOU SIGN THIS DOCUMENT IT **DOES NOT** AFFECT YOUR RIGHT TO WITHDRAW FROM THE STUDY AT **ANY TIME** WITHOUT PREJUDICE TO ACCESS OF SERVICES WHICH ARE ALREADY BEING PROVIDED OR MAY SUBSEQUENTLY BE PROVIDED TO THE PARTICIPANT.

Finally, thank-you very much for your participation in the study. Your time and effort is very much appreciated.

FORM OF CONSENT TO TAKE PART AS A SUBJECT IN A MAJOR PROCEDURE OR RESEARCH PROJECT

Title of project/procedure: Factors influencing muscle function following downhill running

I, ...agree to take part in
(**Subject's** full name)*
the above named *project/procedure,* the details of which have been fully explained to me and described in writing.

Signed..... Date .....
(Subject)

I, ...certify that the details of this
(**Investigator's** full name)*

project/procedure have been fully explained and described in writing to the subject named above and

have been understood by him/her.

Signed..... Date .....
(Investigator)

I, ...certify that the details of this
(**Witness'** full name)

project/procedure have been fully explained and described in writing to the subject named above and

have been understood by him/her.

Signed ..... **Date**.....
(Witness)

NB The witness must be an independent third party.

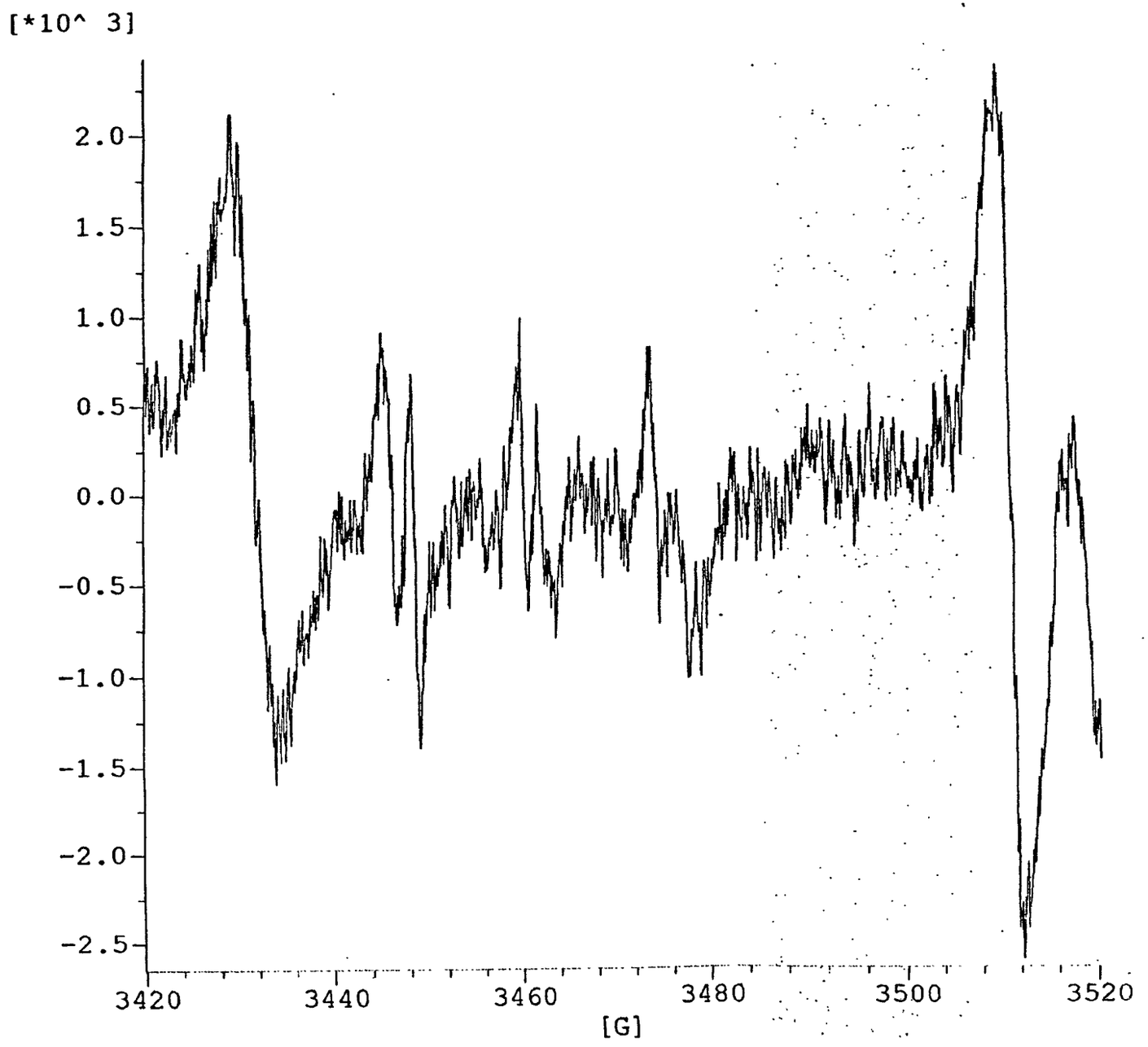
* Please print in block capitals

APPENDIX C – SAMPLE ESR SPECTRA

WIN-EPR Acquisition

Datum: 01.Nov.2000 Zeit: 17:02

FileName: C:\Tony Ashton 1\Don's work\RC Pre-Ex Flat.par
Comment: Richard Chapman Pre-Ex Flat



Parameter List

Operator:	Tony
Resonator:	c:\...\tm_9808.cal
Acqu. Date:	01.Nov.2000
# of Scans:	3

Field

Center Field:	3470.000 G
Sweep Width:	100.000 G
Resolution:	2048 points

Microwave

Frequency:	9.712 GHz
Power:	20.068 mW

Receiver

Receiver Gain:	1.00e+005
Phase:	0.00 deg
Harmonic:	1
Mod. Frequency:	100.00 kHz
Mod. Amplitude:	1.50 G

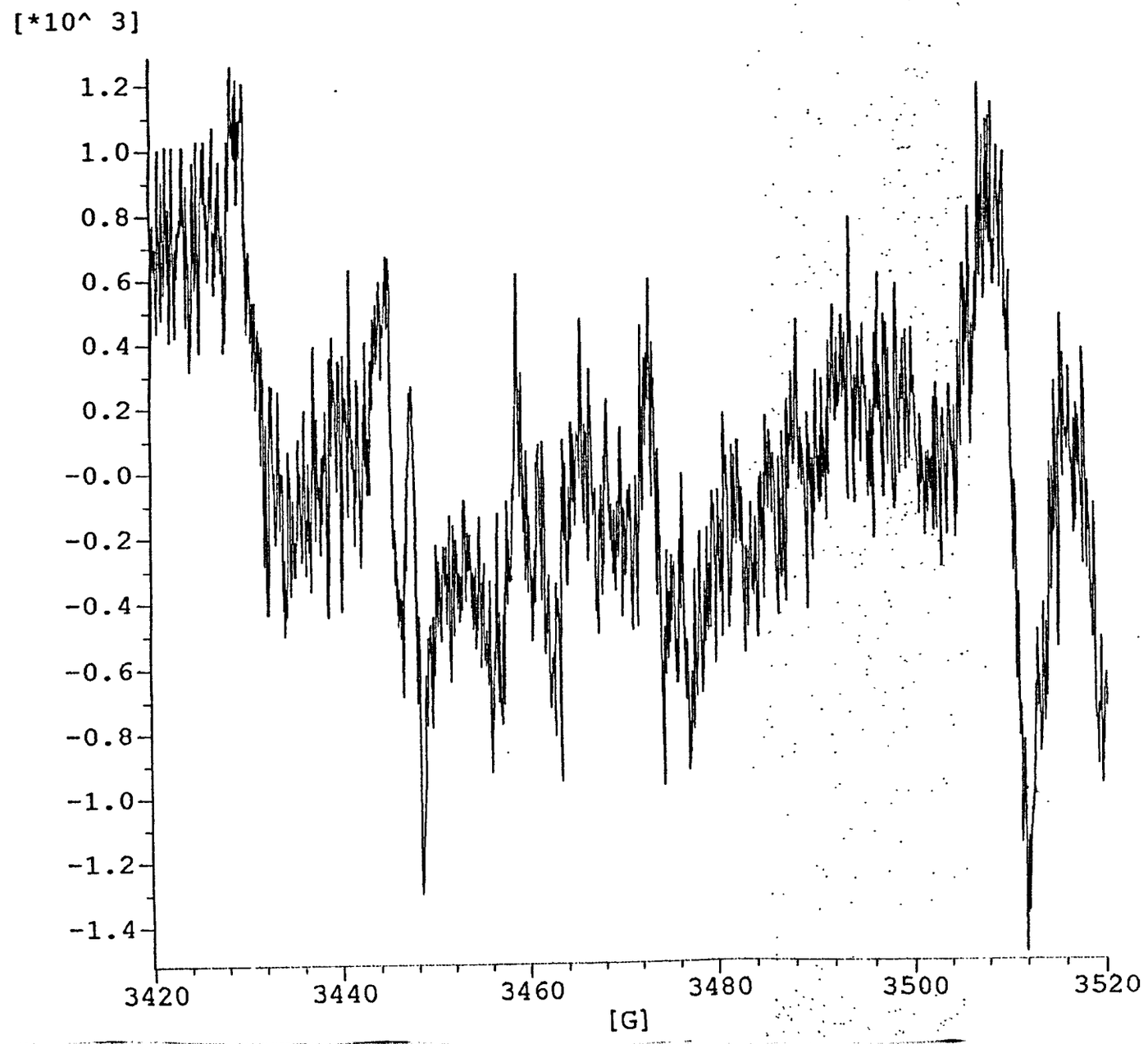
Signal Channel

Conversion:	81.920 ms
Time Constant:	163.840 ms
Sweep Time:	167.772 s

WIN-EPR Acquisition

Datum: 01.Nov.2000 Zeit: 16:51

FileName: C:\ ...\RC Post-Ex, Downhill.par
Comment: Richard Chapman Post-Ex Downhill



Parameter List

Operator:	Tony
Resonator:	c:\...\tm_9808.cal
Acqu. Date:	01.Nov.2000
# of Scans:	4

Field

Center Field:	3470.000 G
Sweep Width:	100.000 G
Resolution:	2048 points

Microwave

Frequency:	9.712 GHz
Power:	20.068 mW

Receiver

Receiver Gain:	1.00e+005
Phase:	0.00 deg
Harmonic:	1
Mod. Frequency:	100.00 kHz
Mod. Amplitude:	1.50 G

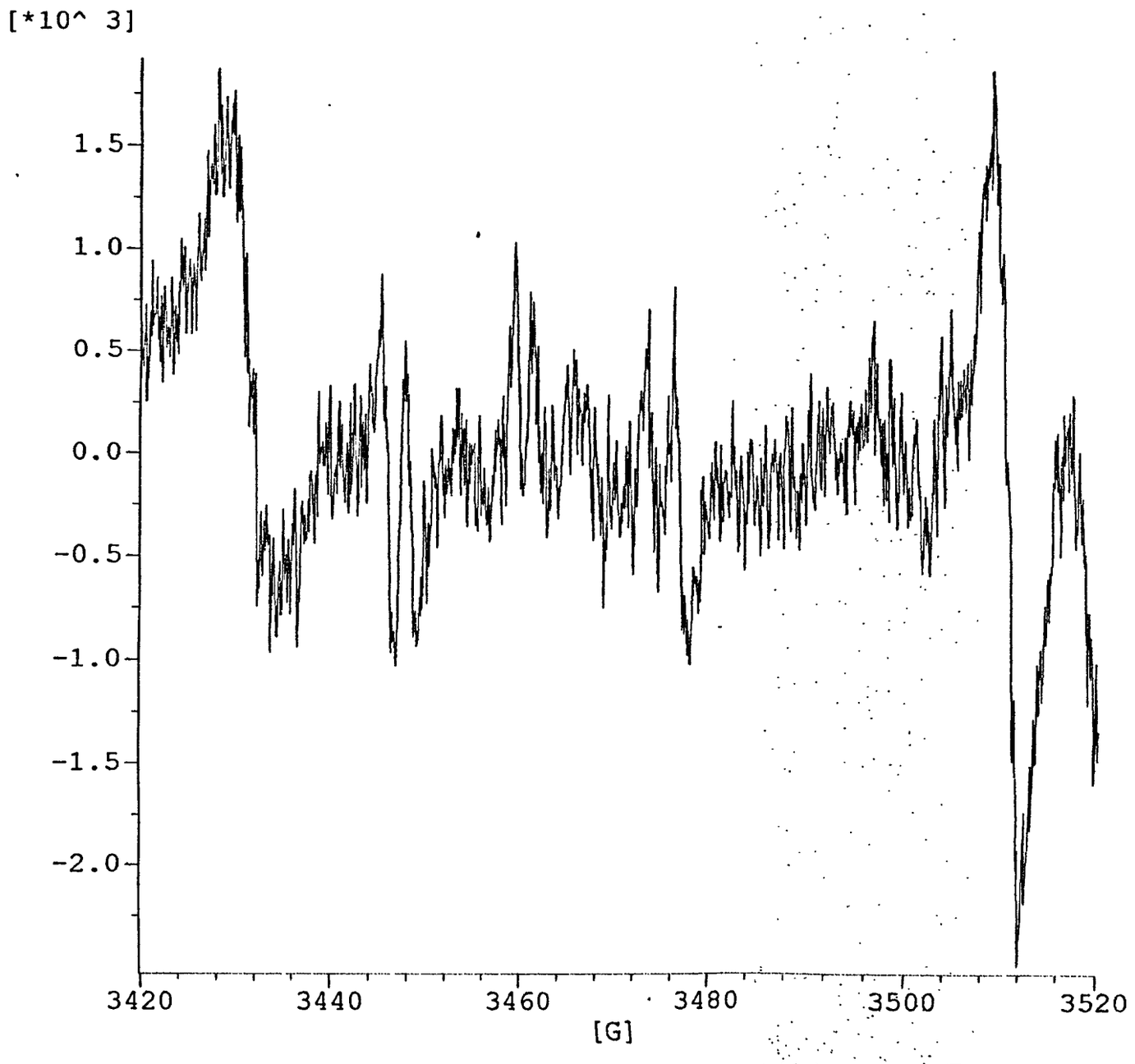
Signal Channel

Conversion:	81.920 ms
Time Constant:	163.840 ms
Sweep Time:	167.772 s

WIN-EPR Acquisition

Datum: 01.Nov.2000 Zeit: 15:59

FileName: C:\ ...\RC +24 hrs Downhill.par
Comment: Richard Chapman +24 hrs Downhill



Parameter List

Operator:	Tony
Resonator:	c:\...\tm_9808.cal
Acqu. Date:	01.Nov.2000
# of Scans:	4

Field

Center Field:	3470.000 G
Sweep Width:	100.000 G
Resolution:	2048 points

Microwave

Frequency:	9.717 GHz
Power:	20.068 mW

Receiver

Receiver Gain:	1.00e+005
Phase:	0.00 deg
Harmonic:	1
Mod. Frequency:	100.00 kHz
Mod. Amplitude:	1.50 G

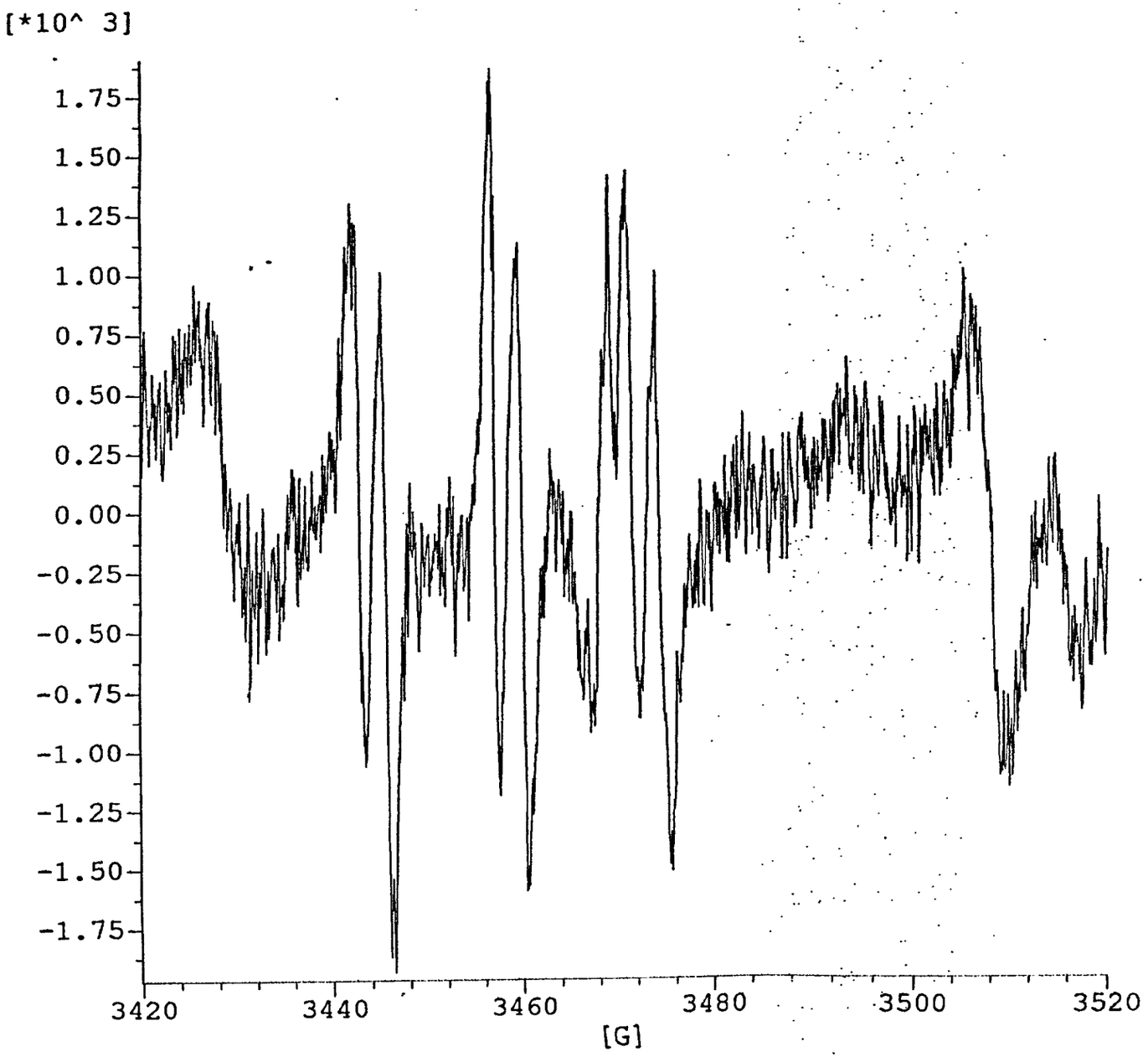
Signal Channel

Conversion:	81.920 ms
Time Constant:	163.840 ms
Sweep Time:	167.772 s

WIN-EPR Acquisition

Datum: 01.Nov.2000 Zeit: 15:45

FileName: C:\ ...\RC +48 hrs Downhill.par
Comment: Richard Chapman +48 hrs Downhill



Parameter List

Operator:	Tony
Resonator:	c:\...\tm_9808.cal
Acqu. Date:	01.Nov.2000
# of Scans:	3

Field

Center Field:	3470.000 G
Sweep Width:	100.000 G
Resolution:	2048 points

Microwave

Frequency:	9.702 GHz
Power:	20.068 mW

Receiver

Receiver Gain:	1.00e+005
Phase:	0.00 deg
Harmonic:	1
Mod. Frequency:	100.00 kHz
Mod. Amplitude:	1.50 G

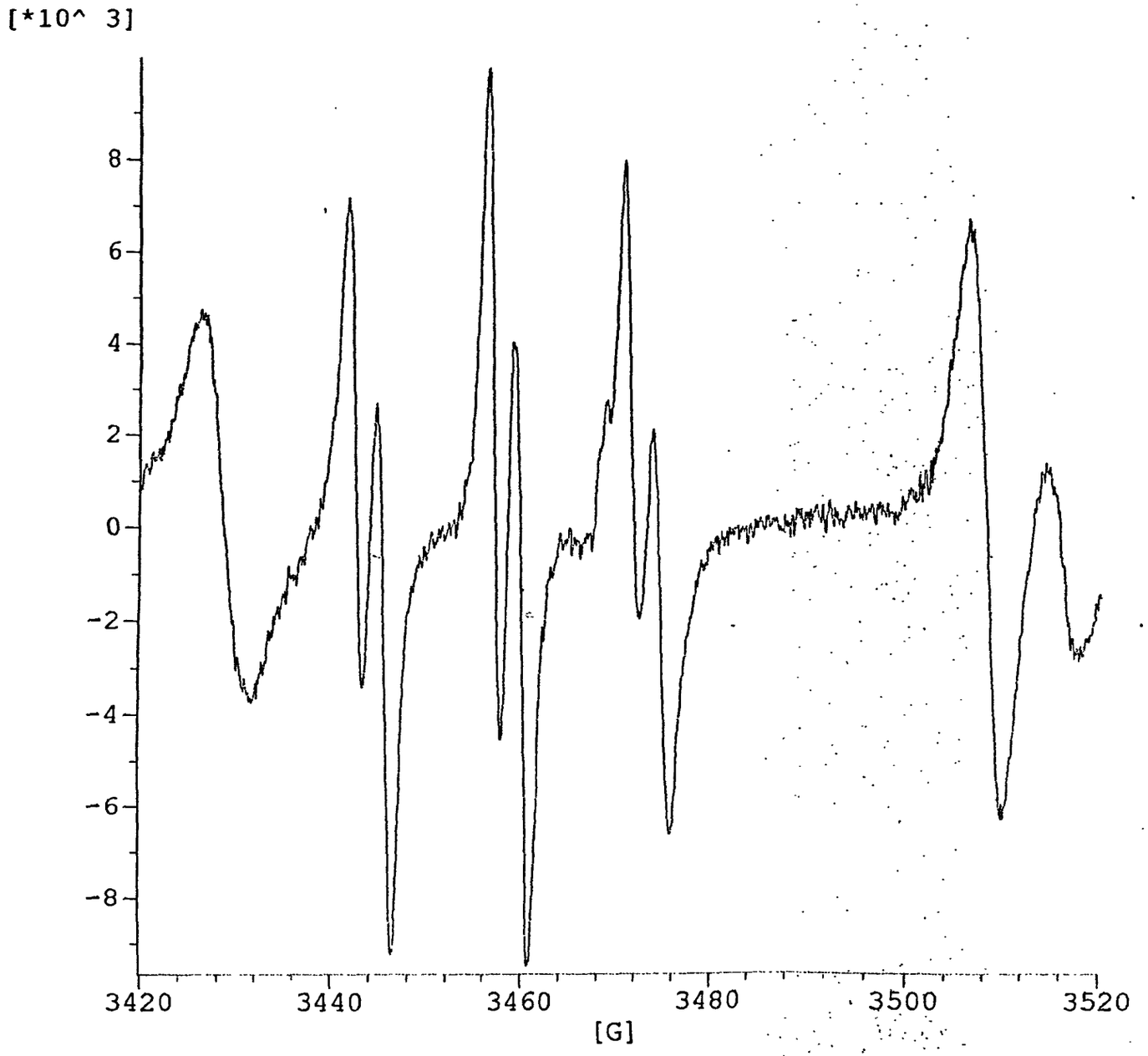
Signal Channel

Conversion:	81.920 ms
Time Constant:	163.840 ms
Sweep Time:	167.772 s

WIN-EPR Acquisition

Datum: 01.Nov.2000 Zeit: 16:37

FileName: C:\ ...\RC +72 hrs Downhill.par
Comment: Richard Chapman + 72 hrs Downhill (Re-done)



Parameter List

Operator:	Tony
Resonator:	c:\...\tm_9808.cal
Acqu. Date:	01.Nov.2000
# of Scans:	3

Field

Center Field:	3470.000 G
Sweep Width:	100.000 G
Resolution:	2048 points

Microwave

Frequency:	9.702 GHz
Power:	20.068 mW

Receiver

Receiver Gain:	1.00e+005
Phase:	0.00 deg
Harmonic:	1
Mod. Frequency:	100.00 kHz
Mod. Amplitude:	1.50 G

Signal Channel

Conversion:	81.920 ms
Time Constant:	163.840 ms
Sweep Time:	167.772 s