# METABOLIC AND STRUCTURAL RESPONSE OF MUSCLE TO EXERCISE

# THE METABOLIC AND STRUCTURAL RESPONSE OF HUMAN SKELETAL MUSCLE TO ACUTE EXERCISE AND NUTRITIONAL MANIPULATION

By

NAOMI M. CERMAK, Hon. B.Sc., M.Sc.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

DOCTOR OF PHILOSOPHY (2010) McMaster University
(Kinesiology) Hamilton, Ontario

TITLE: The metabolic and structural response of human skeletal muscle to acute exercise and nutritional manipulation

AUTHOR: Naomi M. Cermak, Hon.B.Sc. (McMaster University)
M.Sc. (Brock University)

SUPERVISOR: Dr. Martin J. Gibala

SUPERVISORY COMMITTEE: Dr. Mark A. Tarnopolsky

Dr. Gianni Parise

Dr. Michael D. Noseworthy

NUMBER OF PAGES: xvi, 143

## ABSTRACT

The work in this thesis describes the metabolic and structural response of human skeletal muscle to acute exercise and nutritional manipulation. Over a series of three studies, healthy young men performed acute bouts of either endurance or resistance exercise, and a range of invasive and non-invasive techniques were applied to examine the muscle adaptive response during exercise and recovery. Study 1 investigated the hypothesis that co-ingestion of protein with carbohydrate during exercise would improve oxidative energy metabolism and attenuate ultrastructural disruption during prolonged 90 min of cycling at ~70% $VO_2$peak. While protein ingestion increased blood amino acids, there was no difference between treatments in glycogen degradation or the content of TCA cycle intermediates during exercise, or the blood concentration of plasma creatine kinase (CK) after 24 h of recovery. Given the limitations associated with traditional indirect markers of muscle injury, study 2 examined the potential for a non-invasive imaging technique, diffusion tensor magnetic resonance imaging (DT-MRI), to detect exercise-induced changes in skeletal muscle structure. Subjects performed 300 eccentric actions of the leg extensors, a protocol previously shown to induce histological evidence of muscle disruption. DT-MRI revealed changes consistent with muscle disorganization 24 h post-exercise compared to baseline, including decreased fractional anisotropy (FA) and increased tensor eigenvalue $\lambda_3$. The exercise protocol also induced changes in traditional direct and indirect markers of muscle injury, including Z-band

streaming, increased blood CK and a decrease in force-generating capacity. Study 3 examined the potential for DT-MRI to detect structural changes in response to an acute bout of work, previously shown to induce muscle damage that more closely simulated normal endurance exercise. Subjects performed 45 min of downhill running (-10° grade) and DT-MRI revealed increased ADC and tensor eigenvalue $\lambda_3$ 24 h post-exercise compared to baseline, in addition to increased plasma CK and decreased force-generating capacity. The main finding from the thesis is the application of DT-MRI to non-invasively detect exercise-induced changes in skeletal muscle structure as verified using well understood direct and indirect measures of muscle damage.

## ACKNOWLEDGEMENTS

A close friend saw a copy of my thesis lying on a table and asked, "That's it?" Yes my friend, four years of frustration, sacrifice, excitement and failure and "this book" is all you get. I however, feel that your doctoral degree is not just about your thesis but also about the experiences and opportunities made while pursuing your degree. My experiences have been many, my gains in knowledge have been vast, but the opportunities to become a better scientist have been endless, and for that, your sacrifices have not gone unnoticed so please, let me take the time to thank you.

I am forever grateful to my family and my best friends for their unwavering support and encouragement. Mom and Dad, you're awesome. Thank you for pushing me, for guiding me, and for never letting me give up. To all my friends, my teammates, my training partners and my coaches, thank you for providing me with a stress outlet, whether that meant endless laps in the pool or endless rambling while cycling on the road. A special thank you to all my colleagues and skilled technicians at both McMaster and Maastricht University for being so dedicated to 'good science' by giving up countless hours of your own time to teach me, to help me produce higher quality research and overall, to push me to become a better scientist.

To my committee, Drs Mark Tarnopolsky, Gianni Parise and Mike Noseworthy, thank you for your guidance, your patience and your insightful discussion. To my supervisors while abroad, Drs Luc van Loon and Lex Verdijk, thank you for taking me beyond the realm of my thesis and providing me with an outstanding research opportunity which has opened my eyes to exciting future possibilities. I feel so fortunate to have had the opportunity to collaborate with such highly successful researchers; however I feel truly honoured for your friendship.

Finally, a doctoral student is lost without the help and nurturing of their supervisor. I couldn't have wished for a more wonderful, supportive and attentive supervisor than Dr. Martin Gibala. You demanded the best out of me, which constantly made me strive to be better, work harder and challenge my way of looking at research. You provided me with countless opportunities and experiences that went above and beyond supervisor requirements. Your expertise is invaluable, your thirst for knowledge is infectious and your mentorship is unparalleled. You are my mentor, my future collaborator and above all else, my friend. Words cannot express my gratitude, and I can only hope to guide future students with the same patience, expertise and professionalism that you have shown me. Thank you.

## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

## FORMAT AND ORGANIZATION OF THESIS

This thesis has been prepared in the "sandwich format" as outlined in the School of Graduate Studies' Guide for the Preparation of Theses. This thesis is comprised of three original research papers (Chapters 2-4), preceded by a general introduction and followed by a general discussion. Chapter 2 has been published in a peer-reviewed journal with the candidate as first author. Chapters 3 and 4 have been prepared in final formal for submission for publication.

## CONTRIBUTION TO PAPERS WITH MULTIPLE AUTHORSHIP

### Chapter 2

***Publication***

Cermak, N.M., Solheim, A., Gardner, M., Tarnopolsky, M.A., Gibala, M.J. (2009). Muscle metabolism during exercise with carbohydrate or protein-carbohydrate ingestion. *Med Sci Sports Exerc.*, 41(12): 2158-2164.

***Contribution***

The experiments were coordinated and conducted by N.M. Cermak with assistance from the co-authors. This research was conducted as partial fulfillment for the doctoral degree of N.M. Cermak, and fulfillment of a Natural Sciences and Engineering Research Council of Canada (NSERC) Undergraduate Student Research Award (USRA) for A. Solheim. The supervisor for this study was M.J. Gibala. Muscle biopsies were obtained by M.A. Tarnopolsky. M.J. Gibala assisted with the collection of muscle samples while A. Solheim and M.J. Gibala assisted with the collection of blood samples. Blood samples were processed and analyzed by N.M. Cermak and A. Solheim. All muscle samples were analyzed by N.M. Cermak with help from M. Gardner. All statistical analyses were performed by N.M. Cermak. Manuscript preparation was completed by N.M. Cermak with input from M.J. Gibala and the co-authors.

### Chapter 3

***Publication***

Cermak, N.M., Noseworthy, M.D., Bourgeois, J.M., Gibala, M.J. Diffusion Tensor MRI to assess skeletal muscle disruption after eccentric exercise. Manuscript submitted to Medicine & Science in Sports & Exercise, Sept 2010.

***Contribution***

The experiments were coordinated and conducted by N.M. Cermak, with assistance from the co-authors and M.A. Tarnopolsky. The supervisor for this study was M.J. Gibala. Muscle biopsies were obtained by M.A. Tarnopolsky. M.J. Gibala assisted with the collection of muscle and blood samples. Blood samples were processed and analyzed by N.M. Cermak. DT-MRI imaging was conducted by M.D. Noseworthy while all DT-MRI analysis was conducted by N.M. Cermak. Muscle analyses were completed by J. Bourgeois with assistance from N.M. Cermak. All statistical analyses were performed by N.M. Cermak. Manuscript preparation was completed by N.M. Cermak with input from M.J. Gibala and the co-authors.

**Chapter 4**

***Publication***

Cermak, N.M., Noseworthy, M.D., Gibala, M.J. Use of diffusion tensor MRI for assessment of musculoskeletal structure following an acute bout of downhill running. Manuscript to be submitted for publication.

***Contribution***

The experiments were coordinated and conducted by N.M. Cermak, with assistance from the co-authors. The supervisor for this study was M.J. Gibala. M.J. Gibala collected all blood while N.M. Cermak processed and analyzed all blood. M.D. Noseworthy conducted all DT-MRI imaging, while N.M. Cermak performed all DT-MRI analyses. Statistical analyses were completed by N.M. Cermak. Manuscript preparation was completed by N.M. Cermak with input from M.J. Gibala and M.D. Noseworthy.

# CHAPTER 1

# GENERAL INTRODUCTION

## 1.1 INTRODUCTION

An acute bout of strenuous exercise induces numerous metabolic and structural responses within skeletal muscle. Major metabolic changes include a reduction in muscle glycogen and blood glucose availability, the extent of which depends on the relative work intensity and exercise duration. The magnitude of metabolic perturbations in response to acute exercise can be altered by nutritional manipulation. For example, carbohydrate (CHO) ingestion during endurance exercise has been shown to have a beneficial effect on performance as it serves to maintain blood glucose concentration, thereby increasing the time to exhaustion or the intensity one can sustain during the exercise bout. Recently, it was proposed that adding protein (PRO) to a CHO beverage can further augment endurance performance but the mechanistic basis remains unclear. Many studies which have investigated the co-ingestion of carbohydrate and protein (CHOPRO) beverages during endurance exercise have used various beverage concentrations thus making comparisons between studies quite difficult. No study however, has directly examined the mechanistic actions proposed to alter metabolism when PRO is added to a CHO beverage.

Acute exercise can also induce changes in cytoskeletal structure which have been linked to a decrease in muscle-specific force-generating capacity. It

has been proposed that nutritional manipulation and in particular PRO ingestion may attenuate exercise-induced muscle damage (EIMD). The techniques used to quantify EIMD vary considerably and present multiple limitations. One common technique involves blood sampling to measure the concentration of muscle-specific enzymes that can be released following sarcolemma disruption. Measurements of this sort are indirect and do not necessarily correlate with direct measures of muscle damage which is most commonly demonstrated through analysis of skeletal muscle biopsy samples. The biopsy technique in turn is highly invasive and only samples a very small volume of muscle tissue. Further research is needed to determine the potential for other methods, including non-invasive imaging techniques, to accurately assess EIMD.

The purpose of this introductory chapter is to review the metabolic and structural response of human skeletal muscle to acute exercise. The chapter is arranged into four sections that are meant to provide a conceptual framework for the three studies that form the main body of the thesis. The chapter begins with an overview of the metabolic response of skeletal muscle to endurance exercise and is followed by a discussion of nutritional strategies that have been proposed to improve acute endurance exercise performance. The third section considers structural and functional changes in skeletal muscle following acute exercise and the final section discusses common techniques utilized to examine this topic.

## 1.2 Skeletal muscle metabolic response to acute endurance exercise

Endurance exercise is generally used to describe a continuous effort at a work intensity that can be sustained for at least 30 min and up to many hours. The oxidation of fat and CHO are the major contributors to energy production with the relative balance depending largely on the intensity of the exercise. Although fat can provide an almost endless supply of fuel, CHO storage is limited and consequently it is an important determinant of the body's ability to sustain prolonged exercise at relatively high workloads. Depending on the aerobic fitness of an individual, the maximal rate of ATP synthesis from fat corresponds to approximately 55-65% of peak aerobic capacity ($VO_2$) whereas CHO oxidation can contribute to ATP synthesis at up to 100% of $VO_2$peak regardless of training status.

### 1.2.1 Carbohydrate metabolism during an acute endurance exercise bout

CHO is stored primarily in skeletal muscle and liver in addition to the trace amount found in the blood (Spriet & Howlett 1999). Muscle glycogen and blood glucose derived from liver glycogenolysis and gluconeogenesis are important substrates for contracting skeletal muscle as fatigue during endurance exercise is often associated with CHO depletion (Coyle *et al.* 1986). Muscle glycogen utilization is largely dependent on exercise intensity (Figure 1.1) and is most rapidly degraded during the first 30 min of exercise at >60% $VO_2$peak (van Loon *et al.* 2001).

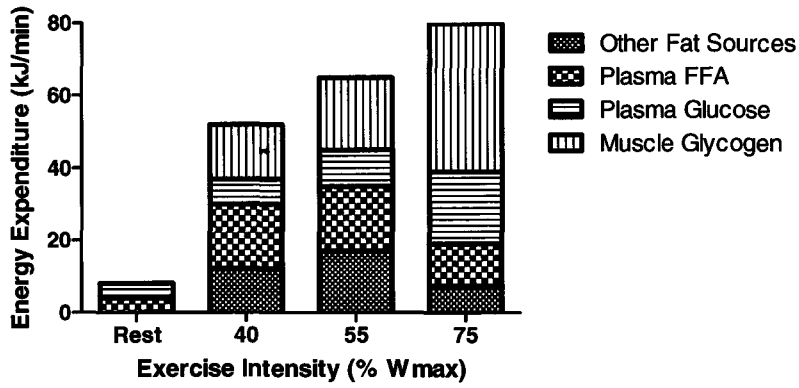



**Figure 1.1**: The relative contribution of intramuscular and extramuscular CHO and FAT to energy metabolism during exercise of increasing intensity. (Adapted from van Loon *et al.* 2001).

As exercise continues, glycogenolysis will begin to decline as a function of reduced muscle glycogen concentration and glycogen phosphorylase (PHOS) activity combined with increased blood glucose availability (Figure 1.2).

Endurance exercise is also a powerful stimulus for muscle glucose uptake, where the utilization of blood glucose is greater at higher intensities and increases during prolonged submaximal exercise with a peak after 90 min (Figure 1.2). Thus, at a given exercise intensity (Figure 1.1), there is an increase in muscle glucose uptake as exercise duration increases which is attributable to a lower glucose-6-phosphate (G-6-P) level due to the decreasing rate of muscle glycogenolysis (Katz *et al.* 1991). The decline in blood glucose uptake after this time is attributable to the increasing availability of plasma free fatty acids (FFA) as fuel, which directly inhibits muscle glucose uptake, and the depletion of liver glycogen stores (Katz *et al.* 1991).

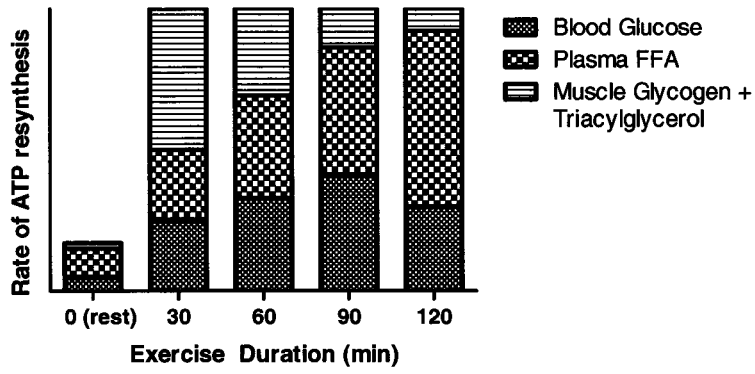



**Figure 1.2**: Changes in the relative contribution of the major fuel sources to ATP resynthesis during prolonged submaximal exercise at an intensity equivalent to ~70% $VO_2$peak (approximately 10x the resting metabolic rate). (Adapted from Gleeson 2000).

### 1.2.2 Protein metabolism during an acute endurance exercise bout

The oxidation of amino acids during endurance exercise likely contributes <5% of total ATP provision (Tarnopolsky 2004) although factors such as training state and CHO availability have been reported to influence the relative energy contribution from PRO (Tarnopolsky 2004; Rennie *et al.* 2006). Koopman and colleagues (2004) evaluated the changes in whole-body PRO metabolism during six hours of continuous moderate-intensity exercise by infusing three stable isotope tracers (Koopman *et al.* 2004). Based on the tracer results, the authors concluded that PRO utilization was not increased during prolonged exercise compared with rest, provided that exogenous CHO was supplied at regular intervals (Koopman *et al.* 2004).

Irrespective of the effects of prolonged exercise on whole-body PRO metabolism, an acute bout of endurance exercise increases the rate of branched-

chain amino acid (BCAA) oxidation by skeletal muscle as evidenced through an increased activation of the rate-determining enzyme branched-chain oxo acid dehydrogenase (BCOAD) (McKenzie *et al.* 2000). In the first step of BCAA catabolism, 2-oxoglutarate is consumed which is an intermediate in the tricarboxylic acid (TCA) cycle. An increased rate of BCAA oxidation during prolonged exercise could potentially impair TCA cycle flux and overall aerobic energy provision by reducing the total concentration of TCA intermediates (TCAi). Gibala and colleagues (2002) tested this theory by combining measurements of muscle TCAi, amino acids and key energy metabolites with direct measurements of limb oxygen uptake during a 90 min bout of moderate intensity exercise. Following a 300% increase during the initial minutes of exercise, the muscle TCAi pool rapidly declined such that the value after 90 min was not different from the resting concentration. Despite the decline in muscle TCAi however, the capacity for aerobic energy provision was not compromised. The authors concluded that changes in muscle TCAi are not causally related to the capacity for aerobic energy provision during prolonged exercise regardless of any effect of BCAA catabolism on muscle TCAi.

## 1.3 Nutritional strategies to improve acute aerobic exercise performance: potential mechanisms

Ingesting mixtures of water, CHO and electrolytes during exercise have been shown to produce physiological benefits typically displayed through

improved performance and/or reduced physiological stress on an athlete's cardiovascular, central nervous and muscular systems (Coyle 2004). A joint position statement by the American College of Sports Medicine, the American Dietetic Association and the Dieticians of Canada recommended that fluid should be consumed at a rate of 150 – 350 mL every 15 – 20 min (600 – 1400 mL per h) depending on personal tolerance (American College of Sports Medicine 2000). Beverages should contain 4% - 8% CHO if exercise is intense or lasting over one hour (American College of Sports Medicine 2000) and the preferential CHO consumed should be glucose as fructose alone is less effective at leaving the gut, possibly leading to gastrointestinal distress (American College of Sports Medicine 2000). Evidence regarding the physiological basis for CHO ingestion and the recent protein dogma will be discussed in the following sections.

### 1.3.1 Classic carbohydrate ingestion

Since the beginning of the 20th century, a great deal of research has accumulated on the effects of CHO feeding during aerobic exercise. Levine and colleagues (Levine *et al.* 1924) measured blood glucose following the 1923 Boston Marathon in a select number of participants. They found that most participants had a low blood glucose concentration following the race, causing speculation that low blood glucose concentration was a source of fatigue. One year later, Levine and colleagues encouraged a few participants to ingest CHO during the race (in combination with a high-CHO diet before the race), which resulted in the prevention of hypoglycemia and significantly improved running

performance. Decades later, after an abundance of research in support of CHO's ergogenic effect when ingested during exercise, sports drinks have become commercially available and are deeply embedded in the "culture" of endurance sports.

Several mechanisms have been proposed to explain CHO's ability to improve endurance performance which includes: 1) maintaining blood glucose and high levels of exogenous CHO oxidation which may spare endogenous glycogen, 2) synthesizing glycogen during low-intensity exercise, or 3) a central effect of CHO (Jeukendrup 2004). Importantly, it should be noted that these mechanisms may be different for relatively short-duration, (~ < 1 h) high-intensity exercise (80% to 85% $VO_2$peak) than for long-duration (>2 h) of low to moderate-intensity exercise (60% to 75% of $VO_2$peak) (Jeukendrup 2004).

CHO feeding during prolonged exercise (>3 h) at 70% $VO_2$peak has been found to prevent the drop in blood glucose observed when only water (placebo) was ingested (Coyle *et al.* 1986). In conjunction with the maintenance of blood glucose levels, CHO oxidation rates were found to be higher when CHO was ingested during the exercise bout and subjects were able to continue exercising for an additional hour at the same intensity (Coyle *et al.* 1986). Although there is a general agreement regarding the importance of plasma glucose acting as a substrate during prolonged exercise, improvements in performance with CHO ingestion have also been found in the absence of any drop in plasma glucose concentration.

CHO feeding may also spare liver glycogen (Bosch *et al.* 1994; Jeukendrup *et al.* 1999) as it has been shown that there is a progressive decrease in endogenous glucose production (liver glycogenolysis and gluconeogenesis) with increasing rates of CHO intake (Jeukendrup *et al.* 1999). There is still considerable debate as to whether CHO feeding during exercise has the ability to spare skeletal muscle glycogen breakdown as there are studies both for (Coutrue *et al.* 2002; Erickson *et al.* 1987; Yaspelkis *et al.* 1993) and against (Coyle *et al.* 1986; Flynn *et al.* 1987) muscle glycogen sparing with CHO feeding. As the evidence for this topic is beyond the scope of this literature review, readers are encouraged to refer to a review by Tzintzas and Williams (1998), summarizing the evidence for a glycogen sparing effect with CHO ingestion during exercise. Lastly, CHO feeding may also have central effects as shown by the ergogenic effect of CHO feeding during relatively short (60 min), high-intensity exercise (>75% $VO_2$peak) (Below *et al.* 1995; Jeukendrup *et al.* 1997; Carter *et al.* 2003), although other studies have shown no effect (Clark *et al.* 2000; McConell *et al.* 2000).

### 1.3.2 Adding protein to a carbohydrate beverage

Recently, it has been suggested that the inclusion of small amounts of PRO (typically 20% of total energy) in a CHO beverage may produce additional benefits over traditional CHO-only beverages (Ivy *et al.* 2002; Williams *et al.* 2003). This proposal is in addition to the daily recommendation of 1.2 g/kg of body weight of dietary PRO for endurance athletes (American College of Sports

Medicine 2000). The first hypothesized mechanism behind PRO's ability to increase time to fatigue (Ivy *et al.* 2003; Williams *et al.* 2003) was an attenuation of muscle glycogen degradation (Ivy *et al.* 2003; Williams *et al.* 2003) through an augmented insulin response (Ivy *et al.* 2003) as following prolonged exercise, the plasma insulin response has been reported to increase when subjects ingest a CHOPRO supplement (Zawadzki *et al.* 1992).

Ivy and colleagues (2003) were one of the first groups to investigate the effects of added PRO to a CHO beverage consumed during exercise on endurance performance. In comparison to the CHO-only, the CHOPRO treatment displayed a significant increase in time to exhaustion (36%), however, there was no difference in the insulin response between the CHO and CHOPRO treatments (Ivy *et al.* 2003) suggesting that insulin is not responsible for any potential glycogen sparing. To account for the performance benefit in the CHOPRO treatment over the CHO-only treatment Ivy and colleagues (2003) suggested other possible mechanisms including: decreased central fatigue and increased TCAi.

In brief, the central fatigue hypothesis is based on the idea that during exercise, branch-chain amino acids (BCAA) decrease and plasma free fatty acids increase thus increasing the unloading of tryptophan from albumin (Davis *et al.* 1992). Tryptophan and BCAA compete for the same transporter across the blood-brain barrier thus an increase in the ratio of plasma free tryptophan to BCAA enhances brain uptake of tryptophan. Tryptophan contributes to fatigue as

it is a precursor to serotonin; which is ultimately responsible for lowering brain activity (Davis & Bailey 1997). In support of this theory, several studies have suggested that the addition of BCAA during exercise will improve endurance performance (Blomstrand *et al.* 1991; Calders *et al.* 1997), however not all studies have been in agreement (Blomstrand *et al.* 1995; van Hall *et al.* 1995). Moreover, supplementing with tryptophan during moderate intensity exercise has shown to have no adverse effects on endurance exercise capacity (van Hall *et al.* 1995) therefore making Ivy's hypothesized central fatigue mechanism unlikely.

Ivy's last hypothesized mechanism suggests that during CHOPRO ingestion, the PRO provides precursors for the anaplerotic reactions required to maintain the TCAi in the skeletal muscle (Ivy *et al.* 2003). At the onset of exercise, there is an initial rapid expansion of the TCAi pool, but as exercise persists, the concentration of these intermediates progressively declines (Bangsbo *et al.* 2006). Although it has been proposed that fatigue during prolonged exercise may result from the depletion of TCAi and thus the inability of the mitochondria to sustain aerobic energy production (Wagenmakers *et al.* 1990) as summarized above, it has been shown that the expansion of the TCAi pool does not enhance oxidative capacity (Dawson *et al.* 2005; Bangsbo *et al.* 2006) thus is an unlikely mechanism for CHOPRO performance improvements.

In support of Ivy's findings however, other research groups have demonstrated a performance benefit when ingesting CHOPRO beverages during exercise over the traditional CHO-only drinks. Saunders and colleagues (2004)

observed a performance benefit with CHOPRO beverages when trained cyclists (≥ 3 d of cycling/wk; $VO_2$peak ≥ 40 mL/kg/min) performed two prolonged bouts of cycle ergometry (one at 75% $VO_2$peak the second bout at 85% $VO_2$peak) to fatigue with a 12-15 h rest period between rides (Saunders *et al.* 2004). In comparison to the CHO-only, time to fatigue was improved in the CHOPRO trial by 29% during the first exercise bout and 40% during the second exercise bout (Saunders *et al.* 2004). According to Saunders, it was speculated that PRO in the CHOPRO beverage may facilitate faster fuel transport across the lining of the intestine as suggested by other hydration studies (Shi *et al.* 1995). Saunders also speculated that performance may have been aided by improved insulin stimulation during exercise however, insulin was not measured during any of the trials and as mentioned, previous work has demonstrated no difference in the insulin response between CHOPRO and CHO-only during exercise (Ivy *et al.* 2003; Van Essen & Gibala 2006).

One of the potential limitations to these early CHOPRO studies surrounds the idea of feeding non-isoenergetically whereby the extra calories supplied in the CHOPRO beverage may be responsible for the improvements in performance and not the added PRO *per se*. Interestingly, when the CHO and CHOPRO drinks were studied iso-energetically, the performance difference was negated (Romano-Ely *et al.* 2006). Another potential limitation revolves around the idea of "time to exhaustion" versus "time to complete a set distance". In an eloquent study by Van Essen and Gibala (2006), highly trained male cyclists

ingested either a placebo, CHO-only or a CHOPRO beverage throughout an 80 km time-trial (Van Essen & Gibala 2006). No difference in performance between the CHO and CHOPRO beverages were observed in addition to blood glucose, blood lactate, plasma free fatty acids (FFA) and plasma ammonia (Van Essen & Gibala 2006). Although the performance results were in agreement with the study by Romano-Ely (2006), this study was unique in that it was one of the first studies to measure performance using a set distance and not time to exhaustion as measured by (Ivy *et al.* 2003; Saunders *et al.* 2004; Romano-Ely *et al.* 2006) and subjects did not undergo an overnight fast (Ivy *et al.* 2003) before the commencement of the 80 km ride. These types of "real world" applicable study designs are important when extrapolating the data to an athletic population as time to exhaustion is not typically how athletes race nor do athletes begin a competition after an overnight fast.

Besides their applicable study design, Van Essen and Gibala (2006) brought forth a number of points concerning the methodology of the beverage design/administration during exercise. A comprehensive review of CHO ingestion during exercise by Jeukendrup (2004) concluded that exogenous CHO oxidation is maximal when a single CHO is ingested at a rate of 60-70 grams per hour, which meets or exceeds the upper limit of the range typically recommended by leading sports nutrition organizations (American College of Sports Medicine 2000; Coyle 2004). Thus when examining the literature on CHOPRO beverages during exercise, Van Essen and Gibala (2006) met the recommendations

administering 60 grams of CHO per hour however, both Saunders et al (2004) and Ivy et al (2003) were under the recommended dosage feeding only 42 and 48 g of CHO per hour respectively. Although subjects were fed CHO within the range supplied by sports nutrition organizations (American College of Sports Medicine 2000; Coyle 2004), the amounts were probably less than optimal for attaining peak rates of exogenous CHO oxidation (Jeukendrup 2004) which makes between-study comparisons difficult.

Regardless of performance improvements and proposed mechanisms hypothesized to occur during the exercise bout, one trend has consistently emerged in the literature whereby ingested PRO may have the ability to attenuate indices of skeletal muscle damage accumulated post endurance exercise (Coombes & McNaughton 2000; Saunders *et al.* 2004; Greer *et al.* 2007; Luden *et al.* 2007; Saunders *et al.* 2007; Skillen *et al.* 2008; Valentine *et al.* 2008). Saunders and colleagues (2004) found that CHOPRO attenuated skeletal muscle damage as measured using plasma CK in comparison to the CHO-only beverage. The observed effect of the CHOPRO beverage was in agreement with an older study published by Ready and colleagues (1999) who observed CK levels to be 36% lower 24 h post a run/cycle duathlon when the co-ingestion of CHO+PRO was consumed versus CHO-only. Additionally, lactate dehydrogenase, another blood enzyme marker, was also found to be lower with the co-ingestion of CHO+PRO versus CHO-only 72 h after exercise (Romano-Ely *et al.* 2006).

In contrast, not all studies have seen a decrease in plasma CK 24 h post exercise after ingesting CHOPRO versus a CHO-only beverage (Millard-Stafford *et al.* 2005; Green *et al.* 2008). Research by Millard-Stafford and colleagues (2005) found ingestion of a CHOPRO, an isocaloric CHO, or a CHO-only (standard 6% CHO) beverage immediately following exercise did not produce any differences in CK values 24 h post exercise. Although beverages were fed for only two hours following an exhaustive run, the discrepancy in the CK finding begs for more extensive research to be conducted in this field of study.

As highlighted, various discrepancies in the methodology such as utilizing beverages that differ in the amount of CHO-content, subjects exercising in the fasted (Ivy *et al.* 2003) versus non-fasted (Van Essen & Gibala 2006) state and various exercise perturbations make it almost impossible to compare/contrast the numerous results. Although the hypothesized theories behind the performance improvements seem unlikely, no study has attempted to directly investigate potential changes in glycogen degradation or TCAi. Future work should focus on designing a study to directly investigate these proposed CHOPRO mechanisms in addition to quantifying indirect measures of EIMD.

## 1.4 Structural and functional changes in skeletal muscle following acute exercise

Strenuous unaccustomed exercise produces an immediate and prolonged reduction in muscle function/force-generating capacity in addition to a delayed

sensation of muscular pain and discomfort. This phenomenon was originally reported by Hough (1902) after studying subjects who experienced a "delayed onset of muscle soreness" (DOMS) following a fatiguing set of rhythmical contractions of the finger flexor muscles (Hough 1902). Hough noted that soreness was not reported until 8-10 h after the exercise and was most severe 48-60 h afterward (Hough 1902). Hough reasoned that the experienced DOMS must be due to some sort of rupture occurring to either the muscle fibres themselves, or to the connective tissue which transmits the pull of the fibre to the tendon (Hough 1902).

Hough's original hypothesis of ruptured fibres occurring as a result of EIMD has been confirmed through histological analysis (Friden *et al.* 1983; Newham *et al.* 1983; Jones *et al.* 1986). Other markers have been shown to change in accordance with EIMD including prolonged reductions in muscle strength, range of motion, and the appearance of muscle proteins in the blood (Newham *et al.* 1983; Clarkson & Dedrick 1988). Although histological and functional markers are rarely correlated, it's important to discuss all measures both structural and functional concerning EIMD as EIMD is a broad concept that the author loosely defines as "any deviation from a skeletal muscle's given homeostasis as a consequence of exercise causing negative structural or practical repercussions". The next two sections will focus on structural disruption and functional changes in skeletal muscle following acute exercise.

### 1.4.1 Structural changes following acute exercise

EIMD, typically observed after exercise that involves a large eccentric component, is generally assumed to be initiated by mechanical factors (Armstrong *et al.* 1991; Morgan & Allen 1999; Proske & Morgan 2001). The force per active fibre produced during eccentric actions is greater compared to concentric actions, and the magnitude of the strain (i.e., change in length as a function of initial length [%]) appear to be important factors in determining the extent of EIMD. Other factors however, such as metabolic depletion, calcium influx, generation of reactive oxygen species and musculo-tendonous stiffness, may also initiate or contribute to the damage process (Gissel & Clausen 2001; Kendall & Eston 2002). Initial manifestations of EIMD tend to be directly represented by disrupted sarcomeres and damage to components of the excitation-contraction (E-C) coupling system (Morgan & Allen 1999; Proske & Morgan 2001). The vast majority of morphological studies investigating EIMD demonstrate that the Z-band is the most vulnerable structure to eccentric exercise-induced injury (Friden & Lieber 2001) although damage has also been found in the sarcolemma (Jenkins 1988; Armstrong 1990), T tubules (Friden & Lieber 1996), myofibrils (Armstrong *et al.* 1983) and the cytoskeletal system (Friden *et al.* 1984; Lieber *et al.* 1996). Immediately following a mild form of eccentric exercise, Z-band streaming/disruption with myofibrillar disarray may appear in a single or very few sarcomeres throughout the affected muscle (Figure 1.3). In more severe cases, extensive streaming (wavy appearance and

widening of Z-band stain) and smearing (dispersion of Z-band material into neighbouring sarcomeres) of the Z-band, focal loss of the Z-band and displacement of Z-band material may occur (Friden & Lieber 2001).

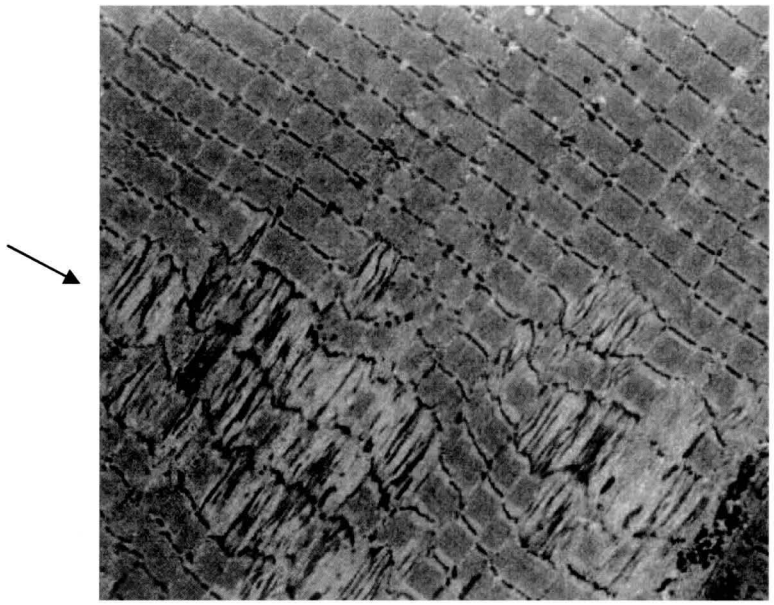


**Figure 1.3**: Moderate Z-band streaming indicated by arrow (x720). (Adapted from Gibala *et al.* 1995).

Some of the first evidence of skeletal muscle ultrastructural damage in humans came from the work of Friden and colleagues in 1981. After subjects performed repeated stair descents, biopsies of the soleus muscle obtained two and seven days post exercise displayed myofibrillar disturbances and Z-band streaming (Friden *et al.* 1981). Friden and colleagues (1983) followed this up with another study using backwards cycling, obtaining muscle biopsies at 1 h, 3 d and 6 d post exercise. They found that 32%, 52% and 12% of the observed fibres demonstrated evidence of focal disturbance corresponding to 1.6%, 2.4% and 0.6% of fibre area. Changes in ultrastructural integrity were also observed as Z-

band steaming, Z-bands out of register, loss of thick myofilaments, loss of mitochondria in areas that showed abnormalities, and disturbed arrangement of filaments at the A-band (Friden *et al.* 1983). Moreover, disturbances predominantly occurred in type II versus type I fibres (Friden *et al.* 1983) and biopsy samples taken greater than 24 h post muscle-damaging exercise illustrated greater damage than those taken immediately following exercise completion (Newham *et al.* 1983).

With respect to metabolic damage, it has been speculated that exercise may induce metabolic damage due to by-products such as free radical production. Although an inflammatory cell response has been shown to occur after acute muscle injury (Tidball 1995; Beaton *et al.* 2002a; Beaton *et al.* 2002b) and the resulting neutrophil infiltration has been positively correlated with CK efflux (Pizza *et al.* 1995; Suzuki *et al.* 1999), it is difficult to ascertain whether inflammation is a response that occurs secondary or independently to fibre damage. In marathon runners, muscle fibre necrosis has been recorded in conjunction with inflammation as biopsy preparations show mitochondria, erythorocytes, leukocytes and other phagocytic cells within the extracellular and extravascular spaces (Hikida *et al.* 1983). As similar characteristics were observed in pre-marathon samples, the authors concluded that both the intensive training as well as the marathon itself induces inflammation and fibre necrosis (Hikida *et al.* 1983). Thus, the actual damaging event (marathon run) may not be responsible for inflammation *per se*. Beaton et al. (2002b) administered a calcium

channel blocker before and after muscle-damaging exercise to determine whether damage could be attenuated by blocking intracellular calcium accumulation. Interestingly, only desmin disruption and Z-band streaming were attenuated with the calcium channel blocker post exercise. Both neutrophil numbers and inflammatory cell (macrophage) infiltration were similar between the control and calcium channel blocker groups, suggesting that inflammation may not be a direct result of fibre disruption. Goodman and colleagues (1997) have suggested that increased muscle-specific proteins in blood post exercise may be the result of free radical induced cell membrane damage, yet studies which have administered Vitamin C and/or E supplementations (Beaton *et al.* 2002a; Dawson *et al.* 2002) have not found any affect on blood enzyme concentrations thus questioning the relationship between concentrations of blood enzymes and oxidative-stress mediated damage.

In conclusion, two separate, but likely not mutually exclusive mechanistic events have been proposed to explain the skeletal muscle response to exercise-induced disruption. The first event is mechanical and involves disruption to the muscle fibres and membrane (Armstrong *et al.* 1991) causing ultrastructural changes such as Z-band streaming and the release of proteins into the blood. The second mechanism includes the infiltration of inflammatory factors such as neutrophils and monocytes (Roxin *et al.* 1986; Sjodin *et al.* 1990; Armstrong *et al.* 1991; Goodman *et al.* 1997) and the production of reactive oxygen species (Armstrong *et al.* 1991; Saxton *et al.* 1994). Whether the two mechanisms act in

conjunction with each other or whether the physiological factors such as the infiltration of inflammatory factors act secondary or act to further damage the muscle ultrastructure remains elusive. In a comprehensive review by Tidball (2005), the inflammatory response is stated to

"...coincide with muscle repair, regeneration, and growth, which involve activation and proliferation of satellite cells, followed by their terminal differentiation. Recent investigations have begun to explore the relationship between inflammatory cell functions and skeletal muscle injury and repair by using genetically modified animal models, antibody depletions of specific inflammatory cell populations, or expression profiling of inflamed muscle after injury. These studies have contributed to a complex picture in which inflammatory cells promote both injury and repair, through the combined actions of free radicals, growth factors, and chemokines".

Thus, the complex network of factors between fibre disruption and inflammatory markers and the ongoing debate as to whether inflammation is secondary to disruption or an independent remodeling event is beyond the scope of this dissertation. For review, see (Smith *et al.* 2008) or (Tidball 2005).

### 1.4.2 Functional changes following acute exercise

From the athlete's perspective, EIMD is only a concern if the loss of muscle function or perceived soreness that generally accompanies muscle damage adversely affects performance. Besides soreness, EIMD tends to induce reductions in muscle strength and power that generally occur immediately following a muscle-damaging bout of exercise and recover quite slowly (Clarkson *et al.* 1992). The following few paragraphs will try and highlight the various aspects of muscle function that have been shown to be negatively affected by EIMD.

The ability to generate power is an important aspect of human muscle performance/function with the understanding that if a selective loss of concentric and eccentric strength at high angular velocities of movement occurs as a result of EIMD, this would in turn, render the affected muscles markedly less powerful at the velocities of movement associated with athletic events. Sherman and colleagues (1984) reported a 47% reduction in work capacity during 50 maximal leg extensions immediately following a marathon running race. Furthermore, Bryne and Eston (2002) reported immediate and prolonged reductions in peak power during a 30 s Wingate test. The reported reductions in power output were the direct result of an inability to achieve a high pedaling frequency since the external load remained constant before and after EIMD. Interestingly, when examining the peak power recovery pattern, further decrements in power were observed at days 1 and 2 post exercise before a linear recovery pattern emerged (Byrne & Eston 2002).

Reductions in peak jumping height have also been found post EIMD and may last for as long as four days after EIMD depending on the type of jump performed (Byrne & Eston 2002). Specifically, jump performance was affected to a greater extent when subjects were instructed to perform a squat jump that does not utilize the stretch-shortening cycle (SSC) than in the countermovement or drop jump (with SSC) (Byrne & Eston 2002) supporting the notion that the eccentric component has a greater determinant on reductions in muscle function/performance than the concentric component.

With respect to sprinting performance following EIMD, Semark et al. (1999) tested the effects of EIMD on a single sprint effort assessed at 5, 10, 20 and 30 m from a standing start. Results suggested that EIMD did not have any detrimental effects on either sprint time or acceleration however, there were also no changes present in the level of CK measured pre and post exercise, suggesting that the EIMD protocol may not have been sufficient enough to negatively influence performance.

When endurance performance is assessed, with the exception of a few studies (Gleeson *et al.* 1995; Braun & Dutto 2003), most available evidence suggests that EIMD has no negative effect on running economy, cardiorespiratory response and/or energy metabolism during standardized submaximal running (Hamill *et al.* 1991; Scott *et al.* 2003; Paschalis *et al.* 2005). It is recommended that these results be interpreted with caution however, as none of these studies included direct measurements of endurance performance such as time to exhaustion at a fixed workload or distance-specific time trials commonly practiced in a real-world athletic setting. When these specific types of direct measures of performance are assessed post EIMD, endurance performance has been shown to decrease (Marcora & Bosio 2007; Twist & Eston 2009). For example post EIMD, 30 min treadmill running (Marcora & Bosio 2007) and 5 min cycling time trials (Twist & Eston 2009) demonstrated slower running speeds and a lower cycling power output production. As expected, these observed decreases in performance occurred without any major effect on heart

rate, ventilatory response, respiratory exchange ratio (RER) or lactate (Marcora & Bosio 2007; Twist & Eston 2009). Perceived effort increased however, suggesting that sense of effort/pain after EIMD *per se*, could be mediating the performance effort.

In summary, researchers have demonstrated that EIMD has not only structural, but also practical repercussions in terms of muscle function and performance. Decreases in muscle power output, vertical jumping height and some measures of endurance performance have all been observed post EIMD. The extent of functional loss likely depends on the mode and intensity used to induce EIMD.

## 1.5 Techniques used to assess exercise-induced changes in skeletal muscle structure

A number of direct and indirect techniques have been employed to evaluate EIMD. Direct techniques include skeletal muscle biopsy samples (Fielding *et al.* 1993; Beaton *et al.* 2002b) and the use of novel sequencing parameters with magnetic resonance imaging (MRI) (Nurenberg *et al.* 1992; Zaraiskaya *et al.* 2006) to assess structural changes post-exercise or post-injury. In contrast, the more commonly used indirect techniques include ratings of delayed onset muscle soreness (DOMS) (Lee *et al.* 2002; Etheridge *et al.* 2008), changes in muscle strength/performance (Etheridge *et al.* 2008; Raastad *et al.* 2010), and measurements of various muscle-specific proteins in blood (Brown *et*

*al.* 1997; Lee *et al.* 2002; Clarkson *et al.* 2006; Etheridge *et al.* 2008). The next few sections will discuss these techniques, highlighting the limitations and any correlation between direct and indirect measurements.

### 1.5.1 Muscle Force

Next to ratings of muscle soreness, a change in maximal voluntary contraction (MVC) is also one of the most practical and commonly used tools to indirectly measure EIMD. Like DOMS, reductions in muscle force do not necessarily correlate to the magnitude of ultrastructural disruption. Furthermore, to increase the accuracy of torque measurements, they need to be standardized at the same joint angle (e.g. knee) so valid comparisons can be made within and among individuals. Often, peak torques are reported and compared without consideration of the joint angle at which they occurred. This is problematic as the same torque produced at two different joint angles does not necessarily require the same amount of muscle force output (Warren *et al.* 1999). Additionally, by duplicating torque measurements at the same joint angle, it is ensured that the ratio of muscle length/optimal muscle length is the same for comparison-wise purposes. Furthermore, torque is also velocity-dependent because of the muscle force-velocity relationship thus ideally, all torque measurements should be made at the same muscle shortening or lengthening velocity which in reality, can only be achieved during isometric contractions (Warren *et al.* 1999). Aside from joint angle and velocity, two major subjective drawbacks exist when using MVC torque as a functional measurement tool for the assessment of EIMD; 1) MVC torque is

affected by fatigue and 2) MVC torque is also affected by an individual's motivation and pain (Warren *et al.* 1999) which makes it difficult to distinguish fatigue-related reductions in torque from EIMD-related reductions, especially when attempting to measure MVC immediately following a muscle-damaging protocol (Fitts 1994). Secondly, even with highly motivated individuals, it is debatable whether maximal effort elicits maximal recruitment of all motor units (Sale 1987; Gibala *et al.* 1995). Although other practical techniques to assess EIMD have been used in the literature (as discussed in a previous section), none of these (vertical jump, power tests etc) have been strongly correlated with ultra-structural events. Furthermore, each practical test may show a different time course for both the extent of performance depression, and the period to full recovery, which may cause confusion and/or be misleading when assessing the severity or duration of experienced EIMD.

### 1.5.2 Blood

Using blood markers as an approach to understanding EIMD has been widely criticized however, some of the more common muscle enzymes analyzed include lactate dehydrogenase (LDH), myoglobin (Mb) and CK (Marcora & Bosio 2007). Although all of the above mentioned markers have been shown to increase after damage-inducing exercise, plasma CK has received the most attention, likely due to the magnitude of the increase and low cost of this particular assay (Clarkson *et al.* 2006).

It was concluded by Janssen and colleagues (Janssen *et al.* 1989) that

the measurement of plasma CK was not a reliable indicator of EIMD, as the expected amount of muscle damage (calculated from enzyme release) was too small to be strongly related to the histological features present in the hours and days following prolonged endurance running. Additional work in the area has demonstrated that the appearance of muscle proteins in the blood are not relevant markers of tissue damage and no correlation has been found between blood CK and Z-band streaming (Fielding *et al.* 1993). Furthermore, CK may be released from other tissues in the body (e.g., cardiac muscle) and may also increase in the blood after exercise without any other signs of muscle damage (Komulainen *et al.* 1995). In order for CK to reach the blood, the disrupted muscle membrane must first allow CK to diffuse into the interstitial fluid followed by release into the circulation, partially through the lymphatic system (Hortobagyi & Denahan 1989). Therefore, an elevated CK may not necessarily increase in correspondence with muscle damage, or may only be a reflection of muscle overload (Komulainen *et al.* 1995). Further complications arise when trying to classify subjects as normal or high CK responders post exercise (Clarkson *et al.* 2006; Heled *et al.* 2007). High CK responders exhibit extreme increases in CK in response to exercise (Heled *et al.* 2007) however a clinical or case definition for high responders compared with normal or low responders does not exist, and reasons for the high responder phenomenon are unknown (Heled *et al.* 2007). Genetics have been hypothesized to be partially responsible, however it remains unclear as to which genes are responsible (Clarkson *et al.* 2005).

Other blood proteins have been found to have similar limitations when used to assess EIMD. Mb for example, has been described as a very non-specific marker as it is found at both the skeletal and cardiac level and is released very early into the blood (Mair *et al.* 1994). Likewise, LDH is also a non-specific marker of skeletal muscle damage because of its wide tissue distribution (Jones *et al.* 1986) in addition to the risk of haemolysis during extraction or handling of the blood which can falsely produce a high LDH result (Olzewski & Engeseth 1985). It has also been suggested (Kyrolainen *et al.* 1998) that protein responses to exercise intensity (power) are curvelinear, implying that a minimum intensity (threshold) of exercise may be required before any leakage of these proteins will take place (Kyrolainen *et al.* 1998).

In summary, when choosing to measure blood markers as an indicator of EIMD, the mode of muscular actions, the intensity and duration of the exercise as well as the training state of the subjects should all be accounted for as each factor has the potential to cause large variations in the concentration of released proteins (Hortobagyi & Denahan 1989). Likely influenced by the training status of the individual, a threshold distance or intensity may exist that must be exceeded in order to induce measureable muscle damage (Janssen *et al.* 1989) as fitness level has been found to be associated with different degrees of muscle breakdown after exercise (Close *et al.* 2005). Thus, researchers should aim for a homogenous subject pool when evaluating EIMD through blood proteins. No study however has measured all these blood markers together, thus it is difficult

to understand how the proteins relate to each other in a time course fashion.

### 1.5.3 Soreness

One of the most commonly used markers in human studies is ratings of DOMS, yet these ratings share a poor temporal relationship with histological evidence of muscle damage and measures of muscle strength/performance (Jones *et al.* 1986). Muscle soreness is generally recorded by asking subjects to rate their perceived pain on a visual analog scale (VAS) from "not sore at all" to "the worst pain ever". Soreness can be assessed at rest or following an action such as a maximal isometric contraction or downhill walking. Regularly referred to as DOMS, it tends to appear around eight hours following typical muscle-damaging exercise (e.g. eccentric) and generally dissipates within 96 h (Jones *et al.* 1987). The sensation of soreness commonly consists of muscle tenderness, pain on palpation as well as mechanical stiffness in the muscle that results in tenderness when the muscle is passively stretched or activated. High subjective ratings of DOMS have been recorded in numerous studies post EIMD (Warren *et al.* 1999) although how DOMS manifests through either ultra-structural or functional alterations remains unknown.

As far as the author is aware, the only other muscle damage marker that has been correlated to DOMS are protein carbonyls (PC), which are thought to be produced as a by-product of oxidative stress (Lee *et al.* 2002). Lee and colleagues (2002) were able to correlate PC with increasing DOMS during the first 24-48 h after eccentric exercise in the non-dominant arm (although DOMS

remained elevated for 72 h) (Lee *et al.* 2002). When DOMS is coupled with a more commonly used blood marker of muscle damage however (for example, plasma CK), it has been demonstrated that the perception of soreness after eccentric exercise is out of phase with CK values (Newham *et al.* 1988). Although useful for the sake of perceived soreness and potential motivation for cognitive function, DOMS is hypothesized to arise from damage and inflammation of non-contractile connective tissue which gives rise to painful sensations when the muscle is palpated, stretched or activated (Jones *et al.* 1989). Thus, quantifying DOMS through ratings of muscle soreness provides little information about the severity of ultrastructural damage.

### 1.5.4 Skeletal muscle

One of the only techniques to directly assess ultrastructural changes following EIMD is histological analysis through a skeletal muscle biopsy technique. Although this is one of the only techniques in which we can directly view any structural alterations post damaging exercise, it is not without its limitations. Firstly, histopathological quantification is rather subjective in nature, thus extreme care must be taken to blind the researcher performing the analysis and often, two researchers will view and quantify the samples in order to obtain inter-individual variability. Secondly, an inherent limitation of this technique is that such a small sample is extrapolated to estimate damage of an entire muscle. Consider for example, that some studies have demonstrated muscle injury including myofibrillar lysis, focal mitochondria degeneration, sarcolemmal

disruption and Z-band streaming immediately to 72 h after a marathon run (Hikida *et al.* 1983; Warhol *et al.* 1985), while others have observed no ultrastructural muscle damage immediately post 15, 25, 30 and 42 km running races (Sjöström *et al.* 1982; Kuipers *et al.* 1989; Goodman *et al.* 1997). These conflicting results may be due to the fact that the damaged muscle fibres represent a very small percentage of the total fibre number in the muscle (Armstrong *et al.* 1991) and could have been missed due to the small amount of sampled tissue. Furthermore, it is also possible for muscle damage to be greater in some areas of the muscle versus other locations, thus the biopsy sample may either over, or under-estimate genuine muscle damage.

It has also been suggested that evidence of ultrastructural damage might be the result of the biopsy procedure itself, specifically the mincing of the biopsied tissue (Roth *et al.* 2000). Strong evidence for this theory is provided by Malm and colleagues (2000) who studied the effect of seven biopsies taken over a period of 7 d in both control subjects and in subjects who performed eccentric cycling exercise. Both conditions displayed similar changes of infiltrating neutrophils and macrophages suggesting that the biopsy technique can produce some changes that can mistakenly be attributed as EIMD (Malm *et al.* 2000; Roth *et al.* 2000).

### 1.5.5 Magnetic Resonance Imaging (MRI) Techniques

One technique that provides a "direct" view of skeletal muscle, but is non-invasive in nature, is MRI. The basis behind MRI is that it uses large volume

radio frequency coils and magnetic field gradients that result in spatial encoding of the amount of hydrogen nuclei (mainly $H_2O$ and fat aliphatic $-CH_2$ groups) allowing two-dimensional and even three-dimensional images to be constructed (Shellock *et al.* 1991). MRI has generally been considered to be an indirect marker of muscle damage when using T1 or T2-mapping, however new pulse sequences have been developed which allow researchers to better quantify direct structural changes. One such imaging sequence - Diffusion Tensor MRI (DT-MRI) measures diffusion of water in biological tissue and has been used to track fibres in the brain as well as demonstrate subtle abnormalities in a variety of diseases (e.g. stroke, dyslexia, multiple sclerosis) (Le Bihan *et al.* 2001).

DT-MRI takes advantage of the fact that cell membranes and other structures restrict the diffusion of molecules in certain directions (Saupe *et al.* 2008). For cells that are elongated (such as skeletal muscle) this diffusion restriction is greater in the transverse axis, relative to longitudinal (Saupe *et al.* 2008) as muscle fibres are filled with protein filaments arranged in a well-known geometry. These filaments act as barriers to diffusion, in addition to nonfilament proteins dissolved in the cytoplasmic water (Cleveland *et al.* 1976), and upon tissue disruption, diffusivity in specific directions will inherently change. More specifically, the overall effect observed from a diffusion MRI image voxel of several $mm^3$ reflects the displacement distribution of water molecules present within that observed voxel (Le Bihan *et al.* 2001). Observing water displacement may provide unique clues to the structure and geometric organization of tissues.

As diffusion is a three-dimensional process, the molecular mobility in tissues may not be the same in all directions. This "anisotropy" may result from a peculiar physical arrangement of the medium or the presence of obstacles that limit molecular movement in some directions (Le Bihan *et al.* 2001). Diffusion is encoded in the MRI signal by using bipolar magnetic field gradient pulses, thus only molecular displacements that occur along the direction of the gradient are visible. Diffusion anisotropy is calculated by observing variations in the diffusion measurements when the direction of the gradient pulse is changed (Le Bihan *et al.* 2001).

After the images are collected along multiple different diffusion encoding directions, a number of quantitative parameters can be used to characterize molecular mobility. The magnitude of diffusivity for each direction (x, y, z), in each voxel is determined (eigenvalues, $\lambda$), and their orientation direction ($\varepsilon$, or eigenvectors) are determined by a mathematical process involving "diagonalization". In other words, the eigenvectors and eigenvalues are simply the main diffusion directions and associated diffusivities (Basser & Jones 2002). To calculate diffusion anisotropy indices, invariant indices are made up of combinations of terms provided from the diagonalized diffusion tensor (i.e., the tensor eigenvalues $\lambda_1$, $\lambda_2$ and $\lambda_3$) (Le Bihan *et al.* 2001). The two most commonly used invariant indices are fractional anisotropy (FA), and the apparent diffusion coefficient (ADC or mean diffusivity) along with the less commonly used relative anisotropy (RA), and volume ratio (VR) (Zaraiskaya *et al.* 2006) which all provide

information describing water diffusivity. Once the eigenvalues are calculated, the ADC is calculated as the average of the three eigenvalues and FA within each voxel is calculated as follows (Basser & Jones 2002):

$$FA = \frac{\sqrt{3}}{\sqrt{2}} \cdot \frac{\sqrt{(\lambda_1 - \langle\lambda\rangle)^2 + (\lambda_2 - \langle\lambda\rangle)^2 + (\lambda_3 - \langle\lambda\rangle)^2}}{\sqrt{\lambda_1^2 + \lambda_2^2 + \lambda_3^2}},$$

where $\lambda_1$, $\lambda_2$ and $\lambda_3$ are the three eigenvalues and $\langle\lambda\rangle$ is the mean of the three eigenvalues. This is done on a voxel-by-voxel basis producing parametric maps of FA and ADC.

Specifically, FA measures the fraction of the "magnitude" that can be ascribed to anisotropic diffusion. FA varies between 0 (isotropic diffusion) and 1 (infinite anisotropy). In skeletal muscle tissue that is damaged for example, the FA value would be closer to 0 in comparison to undamaged muscle tissue (Zaraiskaya *et al.* 2006). To obtain mean FA and ADC, regions of interest (ROI) are generally selected using a high-resolution T1-weighted (anatomical) image, which overlaps the DT imaging.

DT-MRI has already been demonstrated to be capable of evaluating human skeletal muscle injury as Zaraiskaya and colleagues (2006) compared DT images of four subjects with extensive muscle calf trauma to eight healthy controls. A clear reduction in FA was demonstrated in addition to elevated ADC values in injured skeletal muscle, compared with healthy subjects (Zaraiskaya *et al.* 2006). Therefore, diffusion tensor imaging has the potential to be a quantitative method for evaluating EIMD, as the diffusion coefficient is a physical

parameter that directly reflects the physical properties of the tissue in terms of the random translational movement of the molecules under study (Le Bihan *et al.* 2001). Although limitations of this technique include the availability of an MRI machine, qualified technicians, and the cost associated with using MRI, the potential benefits of its non-invasive nature and larger imaging area, compared to a muscle biopsy, suggest that research should focus on whether DT-MRI is a sensitive-enough marker to detect tissue alteration after damaging exercise in comparison to the biopsy technique. In doing so, this technique has the potential to provide further information as to the structural changes proposed to occur in skeletal muscle following acute exercise.

## 1.6 Summary of major objectives and hypotheses

The purpose of this thesis was to examine skeletal muscle metabolic response to acute exercise with nutritional perturbation, and the structural response after acute exercise using a novel MRI technique. The purpose of study 1 was to determine whether co-ingestion of PRO with CHO would alter selected markers of skeletal muscle metabolic control during moderate-intensity exercise as compared to CHO alone. In comparison to CHO alone, CHOPRO ingestion during an acute bout of endurance exercise has been shown to increase time to exhaustion (Ivy *et al.* 2003; Saunders *et al.* 2004; Saunders *et al.* 2007) in addition to attenuating plasma CK (Saunders *et al.* 2004; Saunders *et al.* 2007) measured 24 h following the exercise bout. The purported ergogenic effect of

CHOPRO versus CHO-only has been attributed to several potential mechanisms including attenuated glycogen degradation and less reliance on non-oxidative metabolism secondary to better maintenance of the muscle pool of TCAi. These mechanisms of metabolic control were directly evaluated before and after a 90 min cycling ride at 69% of subjects' $VO_2$ peak. We tested the hypothesis that compared to CHO alone, CHOPRO ingestion during exercise would favourably affect muscle energy metabolism as evidenced by (1) a reduced rate of glycogen catabolism, (2) an increased muscle pool of TCAi, and (3) reduced phosphocreatine utilization.

PRO and CHO co-ingestion during exercise has also been suggested to attenuate ultrastructural disruption as evidenced by a decrease in muscle-specific enzymes. (Saunders *et al.* 2004; Romano-Ely *et al.* 2006; Saunders *et al.* 2007; Valentine *et al.* 2008) Changes in blood enzymes including plasma CK however, do not correlate well with direct measures of ultrastructural disruption based on muscle biopsy sampling (Fielding *et al.* 1993). DT-MRI is a relatively new non-invasive imaging technique that can image a large volume of muscle and quantifies changes in diffusion which may potentially provide insight into structural alterations. Zaraiskaya and colleagues (2006) showed differences in diffusion between injured and healthy muscle however no study had applied DT-MRI to evaluate exercise-induced structural changes in muscle. Therefore, the purpose of study 2 was to determine whether DT-MRI could detect structural disruption 24 h following a bout of high-force eccentric actions of the knee

extensors. For comparison between DT-MRI and traditional measurements of Z-band streaming, skeletal muscle biopsies in addition to DT images were obtained at baseline and 24 h following the unaccustomed exercise bout. We hypothesized that FA, which is a measure of fibre organization, would decrease 24 h following exercise when compared to baseline values, and thereby provide evidence that DT-MRI could be used to non-invasively evaluate exercise induced changes in fibre structure.

Finally, while study 2 applied DT-MRI to evaluate exercise-induced structural changes, the protocol was far from practical or anything commonly experienced in a "real world" setting. Hence, in study 3 we sought to determine whether DT-MRI was capable of evaluating structural disruption following a more "realistic" exercise bout that resembled normal athletic competition. We therefore evaluated structural disruption using DT-MRI 24 h following 45 min of downhill running (-10 degrees) at 70% of maximal heart rate. A similar protocol had already been shown to induce ultrastructural disruption measured by skeletal muscle biopsy samples (Fielding *et al.* 1993) in addition to adversely affecting muscle force-generating potential (Etheridge *et al.* 2008). We tested the hypothesis that, compared to baseline samples, an increase in diffusion would be observed in the knee extensor muscles 24 h after the downhill run, suggesting an alteration in muscle structure.

## 1.7 REFERENCES

American College of Sports Medicine, American Dietetic Association, Dieticians of Canada. 2000. Nutrition and athletic performance. *Medicine & Science in Sports & Exercise* 32: 2130-2145.

Armstrong, RB, RW Ogilvie and JA Schwane. 1983. Eccentric exercise-induced injury to rat skeletal muscle. *Journal of Applied Physiology* 54: 80-93.

Armstrong, RB, GL Warren and JA Warren. 1991. Mechanisms of exercise-induced muscle fibre injury. *Sports Medicine* 12: 184-207.

Armstrong, RB. 1990. Initial events in exercise-induced muscular injury. *Medicine & Science in Sports & Exercise* 22: 429-435.

Bangsbo, J, MJ Gibala, KR Howarth and P Krustrup. 2006. Tricarboxylic acid cycle intermediates accumulate at the onset of intense exercise in man but are not essential for the increase in muscle oxygen uptake. *Pflugers Arch* 452 (6): 737-743.

Basser, PJ and DK Jones. 2002. Diffusion-tensor MRI: Theory, experimental design and data analysis—a technical review. *NMR Biomedicine* 15: 456-467.

Beaton, LJ, DA Allan, MA Tarnopolsky, PM Tiidus and SM Phillips. 2002a. Contraction-induced muscle damage is unaffected by vitamin E supplementation. *Medicine & Science in Sports & Exercise* 34 (5): 798-805.

Beaton, LJ, MA Tarnopolsky and SM Phillips. 2002b. Contraction-induced muscle damage in humans following calcium channel blocker administration. *Journal of Physiology* 544 (3): 849-859.

Below, PR, R Mora-Rodríguez, J González-Alonso and EF Coyle. 1995. Fluid and carbohydrate ingestion independently improve performance during 1 h of intense exercise. *Medicine and Science in Sports and Exercise* 27 (2): 200-210.

Blomstrand, E, S Anderson, P Hassmen, B Ekblom and EA Newsholme. 1995. Effect of branched-chained amino acid and carbohydrate supplementation on the exercise-induced change in plasma and muscle concentration of amino acids in human subjects. *Acta Physiologica Scandinavia* 153: 87-96.

Blomstrand, E, P Hassmen, B Ekblom and EA Newsholme. 1991. Administration of branched-chain amino acids during sustained exercise-effects on performance and on plasma concentration of some amino acids. *European Journal of Applied Physiology* 63: 83-88.

Bosch, AN, SC Dennis and TD Noakes. 1994. Influence of carbohydrate ingestion on fuel substrate turnover and oxidation during prolonged exercise. *Journal of Applied Physiology* 76: 2364-2372.

Braun, WA and DJ Dutto. 2003. The effects of a single bout of downhill running and ensuing delayed onset of muscle soreness on running economy performed 48 h later. *European Journal of Applied Physiology* 90: 29-34.

Brown, SJ, RB Child, H Day and AE Donnelly. 1997. Indices of skeletal muscle damage and connective tissue breakdown following eccentric muscle contractions. *European Journal of Applied Physiology* 75: 369-374.

Byrne, C and R Eston. 2002. Maximal intensity isometric and dynamic exercise performance following eccentric muscle actions. *Journal of Sports Sciences* 20: 951-959.

Calders, P, J-L Pannier, DK Matthys and EM Lacroix. 1997. Pre-exercise branched-chain amino acid administration increases endurance performance in rats. *Medicine & Science in Sports & Exercise* 29: 1182-1186.

Carter, J, AE Jeukendrup, T Mundel and DA Jones. 2003. Carbohydrate supplementation improves moderate and high-intensity exercise in the heat. *Pflugers Arch: European Journal of Physiology* 446 (2): 211-219.

Clark, VR, WG Hopkins, JA Hawley and LM Burke. 2000. Placebo effect of carbohydrate feedings during a 40-km cycling time trial. *Medicine & Science in Sports & Exercise* 32 (9): 1642-1647.

Clarkson, PM and ME Dedrick. 1988. Exercise-induced muscle damage, repair, and adaptation in old and young subjects. *Journal of Gerontology* 43: M91-M96.

Clarkson, PM, EP Hoffman, E Zambraski, H Gordish-Dressman, A Kearns, M Hubal, B Harmon and JM Devaney. 2005. ACTN3 and MLCK genotype associations with exertional muscle damage. *Journal of Applied Physiology* 99: 564-569.

Clarkson, PM, AK Kearns, P Rouzier, R Rubin and PD Thompson. 2006. Serum creatine kinase levels and renal function measures in exertional muscle damage. *Medicine & Science in Sports & Exercise* 38 (4): 623-627.

Clarkson, PM, K Nosaka and B Braun. 1992. Muscle function after exercise-induced muscle damage and rapid adaptation. *Medicine & Science in Sports & Exercise* 24 (5): 512-520.

Cleveland, GG, DC Chang, CF Hazlewood and HE Rorschach. 1976. Nuclear magnetic resonance measurement of skeletal muscle; anisotropy of the diffusion coefficient *Biophysical Journal* 16: 1043-1053.

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

Coombes, JS and LR McNaughton. 2000. Effects of branched-chain amino acid supplementation on serum creatine kinase and lactate dehydrogenase after prolonged exercise. *Journal of Sports Medicine and Physical Fitness* 40: 240-246.

Couture, S, D Massicotte, C Lavoie, C Hillaire-Marcel and F Péronnet. 2002. Oral [(13)c]glucose and endogenous energy substrate oxidation during prolonged treadmill running. *Journal of Applied Physiology* 92 (3): 1255-1260.

Coyle, EF. 2004. Fluid and fuel intake during exercise. *Journal of Sports Sciences* 22: 39-55.

Coyle, EF, AR Coggan, MK Hemert and JL Ivy. 1986. Muscle glycogen utilization during prolonged strenuous exercise when fed carbohydrate. *Journal of Applied Physiology* 61 (1): 165-172.

Davis, JM and SP Bailey. 1997. Possible mechanisms of central nervous system fatigue during exercise. *Medicine & Science in Sports & Exercise* 29: 45-57.

Davis, JM, SP Bailey, JA Woods, FJ Galiano, MT Hamilton and WP Bartoli. 1992. Effects of carbohydrate feedings on plasma free tryptophan and branched-chain amino acids during prolonged cycling. *European Journal of Applied Physiology* 65: 513-519.

Dawson, B, GJ Henry, C Goodman, I Gillam, JR Beilby, S Ching, V Fabian, D Dasig, P Morling and BA Kakulus. 2002. Effect of vitamin C and E supplementation on biochemical and ultrastructural indices of muscle damage after a 21 km run. *International Journal of Sports Medicine* 23: 10-15.

Dawson, KD, DJ Baker, PL Greenhaff and MJ Gibala. 2005. An acute decrease in TCA cycle intermediates does not affect aerobic energy delivery in contracting rat skeletal muscle. *Journal of Physiology* 565: 637-643.

Erickson, MA, RJ Schwarzkopf and RD McKenzie. 1987. Effects of caffeine, fructose, and glucose ingestion on muscle glycogen utilization during exercise. *Medicine & Science in Sports & Exercise* 19 (6): 579-583.

Etheridge, T, A Philp and PW Watt. 2008. A single protein meal increases recovery of muscle function following an acute eccentric exercise bout. *Applied Physiology Nutrition and Metabolism* 33 (3): 483-488.

Fielding, RA, TJ Manfredi, W Ding, MA Fiatarone, WJ Evans and JG Cannon. 1993. Acute phase response in exercise iii. Neutrophil and Il-1 beta accumulation in skeletal muscle. *American Journal of Physiology* 256: R166-R172.

Fitts, RH. 1994. Cellular mechanisms of fatigue. *Physiology Reviews* 74: 49-94.

Flynn, MG, DL Costill, JA Hawley, WJ Fink, PD Neufer, RA Fielding and MD Sleeper. 1987. Influence of selected carbohydrate drinks on cycling performance and glycogen use. *Medicine & Science in Sports & Exercise* 19 (1): 37-40.

Friden, J, U Kjorell and LE Thornell. 1984. Delayed muscle soreness and cytoskeletal alterations. An immunocytological study in man. *International Journal of Sports Medicine* 5: 15-18.

Friden, J and RL Lieber. 1996. Ultrastructural evidence for loss of calcium homeostasis in exercised skeletal muscle. *Acta Physiologica Scandinavia* 158: 381-382.

Friden, J and RL Lieber. 2001. Eccentric exercise-induced injuries to contractile and cytoskeletal muscle fibre components. *Acta Physiologica Scandinavia* 171: 321-326.

Friden, J, M Sjostrom and B Ekblom. 1981. A morphological study of delayed muscle soreness. *Experientia* 37: 506-507.

Friden, J, M Sjostrom and B Ekblom. 1983. Myofibrillar damage following intense eccentric exercise in man. *International Journal of Sports Medicine* 4: 170-176.

Gibala, MJ, JA Gonzalez-Alonso and B Saltin. 2002. Dissociation between muscle tricarboxylic acid cycle pool size and aerobic energy provision during prolonged exercise in humans. *Journal of Physiology* 545: 705-713.

Gibala, MJ, JD MacDougall and MA Tarnopolsky. 1995. Changes in human skeletal muscle ultrastructure and force production after acute resistance exercise. *Journal of Applied Physiology* 78: 702-708.

Gissel, H and T Clausen. 2001. Excitation-induced $ca^{2+}$ influx and skeletal muscle cell damage. *Acta Physiologica Scandinavia* 171: 327-324.

Gleeson, M 2000 *Nutrition in sport,* Ed R Maughn (Oxford: Blackwell Scientific) pp 17-38

Gleeson, M, AK Blannin and B Zhu. 1995. Cardiorespiratory, hormonal and haematological responses to submaximal cycling performed 2 days after eccentric or concentric exercise bouts. *Journal of Sports Sciences* 13: 471-479.

Goodman, C, G Henry and B Dawson. 1997. Biochemical and ultrastructural indices of muscle damage after a twenty-one kilometre run. *Australian Journal of Science Medicine and Sport* 29: 95-98.

Green, MS, BT Corona, JA Doyle and CP Ingalls. 2008. Carbohydrate-protein drinks do not enhance recovery from exercise-induced muscle injury. *International Journal of Sport Nutrition and Exercise Metabolism* 18 (1): 1-18.

Greer, BK, JL Woodard, JP White, EM Arguello and EM Haymes. 2007. Branched-chain amino acid supplementation and indicators of muscle damage after endurance exercise. *International Journal of Sport Nutrition and Exercise Metabolism* 17 (6): 595-607.

Hamill, J, P Freedson, P Clarkson and B Braun. 1991. Muscle soreness during running: Biomechanical and physiological considerations. *International Journal of Sport Biomechanics* 7: 125-137.

Heled, Y, MS Bloom, J Wu, Q Stephens and PA Deuster. 2007. Cm-mm and ace genotypes and physiological prediction of the creatine kinase response to exercise. *Journal of Applied Physiology* 103: 504-510.

Hikida, RS, RS Staron, FC Hagerman, WM Sherman and DL Costill. 1983. Muscle fibre necrosis associated with human marathon runners. *Journal of Neurological Sciences* 59: 185-203.

Hortobagyi, T and T Denahan. 1989. Variability in creatine kinase: Methodological, exercise and clinically related factors. *International Journal of Sports Medicine* 10: 6-80.

Hough, T. 1902. Ergographic studies in muscular soreness. *American Journal of Physiology* 7: 76-92.

Ivy, JL, HW Goforth, BW Damon, TR McCauley, EC Parson and TB Price. 2002. Early post exercise muscle glycogen recovery is enhanced with a carbohydrate-protein supplement. *Journal of Applied Physiology* 93: 1337-1344.

Ivy, JL, PT Res, RC Sprague and MO Widzer. 2003. Effect of a carbohydrate-protein supplement on endurance performance during exercise of varying intensity. *International Journal of Sport Nutrition and Exercise Metabolism* 13: 382-395.

Janssen, GM, H Kuipers, GM Willems, RJ Does, MP Janssen and P Geurten. 1989. Plasma activity of muscle enzymes: Quantification of skeletal muscle damage and relationship with metabolic variables. *International Journal of Sports Medicine* 10 (Suppl 3): S160-S168.

Jenkins, RR. 1988. Free radical chemistry. *Sports Medicine* 5: 156-170.

Jeukendrup, A, F Brouns, AJ Wagenmakers and WH Saris. 1997. Carbohydrate-electrolyte feedings improve 1 h time trial cycling performance. *International Journal of Sports Medicine* 18 (2): 25-29.

Jeukendrup, AE. 2004. Carbohydrate intake during exercise and performance. *Nutrition* 20: 669-677.

Jeukendrup, AE, A Raben, A Gijsen, JH Stegen, F Brouns, WH Saris and AJ Wagenmakers. 1999. Glucose kinetics during prolonged exercise in highly trained human subjects: Effect of glucose ingestion. *Journal of Physiology* 515 (Prt 2): 579-589.

Jones, DA, DJ Newham and PM Clarkson. 1987. Skeletal muscle stiffness and pain following eccentric exercise of the elbow flexors *Pain* 30: 233-242.

Jones, DA, DJ Newham, JM Round and SEJ Tolfree. 1986. Experimental human muscle damage: Morphological changes in relation to other indices of damage. *Journal of Physiology* 375: 435-448.

Jones, DA, DJ Newham and C Torgan. 1989. Mechanical influences on long lasting human muscle fatigue and delayed onset pain. *Journal of Physiology* 412: 415-427.

Katz, A, K Sahlin and S Broberg. 1991. Regulation of glucose utilization in human skeletal muscle during moderate dynamic exercise. *American Journal of Physiology* 260 (3 Prt 1): 411-415.

Kendall, B and R Eston. 2002. Exercise-induced muscle damage and the potential protective role of estrogen. *Sports Medicine* 32 (103-123):

Komulainen, J, TES Takala and V Vihko. 1995. Does increased serum creatine kinase activity reflect exercise-induced muscle damage in rats? *International Journal of Sports Medicine* 16: 150-154.

Koopman, R, DLE Pannemans, AE Jeukendrup, AP Gijsen, JMG Senden, D Halliday, WHM Saris, LJC van Loon and AJM Wagenmakers. 2004. Combined ingestion of protein and carbohydrate improves protein balance during ultra-endurance exercise. *American Journal of Physiology* 287 (4): E712-E720.

Kuipers, H, GM Janssen, F Bosman, PM Frederik and P Geurten. 1989. Structural and ultrastructural changes in skeletal muscle associated with long-distance training and running. *International Journal of Sports Medicine* 10 (Suppl 3): S156-S159.

Kyrolainen, H, TES Takala and PV Komi. 1998. Muscle damage induced by stretch-shortening cycle exercise. *Medicine & Science in Sports & Exercise* 30 (3): 415-420.

Le Bihan, D, JF Mangin, C Poupon, CA Clark, S Pappata, N Molko and H. Chabriat. 2001. Diffusion tensor imaging: Concepts and applications. *Journal of Magnetic Resonance Imaging* 13: 534-546.

Lee, J, AH Goldfarb, MH Rescino, S Hegde, S Patrick and K Apperson. 2002. Eccentric exercise effect on blood oxidative-stress markers and delayed onset of muscle soreness. *Medicine & Science in Sports & Exercise* 34 (3): 443-448.

Levine, SA, B Gordon and CL Derick. 1924. Some changes in chemical constituents of blood following a marathon race. *The Journal of the American Medical Association* 82: 1778.

Lieber, RI, L-E Thornell and J Friden. 1996. Muscle cytoskeletal disruption occurs within the first 15 minutes of cyclic eccentric contraction. *Journal of Applied Physiology* 80: 278-284.

Luden, ND, MJ Saunders and MK Todd. 2007. Postexercise carbohydrate-protein-antioxidant ingestion decreases plasma creatine kinase and muscle soreness. *International Journal of Sport Nutrition and Exercise Metabolism* 17: 109-123.

Mair, J, B Thome-Kromer, I Wagner, P Lechleitner, F Dienstl, B Puschendorf and G Michel. 1994. Concentration time courses of troponin and myosin subunits after acute myocardial infarction. *Coronary Artery Disease* 5 (10): 865-872.

Malm, C, P Nyberg and M Engstrom. 2000. Immunological changes in human skeletal muscle and blood after eccentric exercise and multiple biopsies. *Journal of Physiology* 529: 243-262.

Marcora, SM and A Bosio. 2007. Effect of exercise-induced muscle damage on endurance running performance in humans. *Scandinavian Journal of Medicine and Science in Sports* 17(6): 662-671.

McConell, GK, BJ Canny, MC Daddo, MJ Nance and RJ Snow. 2000. Effect of carbohydrate ingestion on glucose kinetics and muscle metabolism during intense endurance exercise. *Journal of Applied Physiology* 89 (5): 1690-1698.

McKenzie, S, SM Phillips, SL Carter, S Lowther, MJ Gibala and MA Tarnopolsky. 2000. Endurance exercise training attenuates leucine oxidation and BCOAD activation during exercise in humans. *American Journal of Physiology* 278: E580-587.

Millard-Stafford, M, GL Warren, LM Thomas, JA Doyle, T Snow and K Hitchcock. 2005. Recovery from run training: Efficacy of a carbohydrate-protein beverage? *International Journal of Sport Nutrition and Exercise Metabolism* 15 (6): 610-624.

Morgan, DL and DG Allen. 1999. Early events in stretch-induced muscle damage. *Journal of Applied Physiology* 87: 2007-2015.

Newham, DJ, DA Jones, G Ghosh and P Aurora. 1988. Muscle fatigue and pain after eccentric contractions at long and short length. *Clinical Science* 74: 553-557.

Newham, DJ, G McPhail and KR Mills. 1983. Ultrastructural changes after concentric and eccentric contractions of human muscle. *Journal of Neurological Sciences* 61: 109-122.

Nurenberg, P, CJ Giddings, J Stray-Gundersen, JL Fleckenstein, WJ Gonyea and RM Peshock. 1992. MR imaging-guided muscle biopsy for correlation of increased signal intensity with ultrastructural change and delayed-onset muscle soreness after exercise. *Radiology* 184: 865-869.

Olzewski, WL and A Engeseth 1985 *Experimental biology of the lymphatic circulation,* ed M Johnston (Amsterdam: Elsevier Science Publishers) pp 395-422

Paschalis, V, Y Koutedakis, V Baltzopoulos, V Mougios, AA Jamurtas and V Theoharis. 2005. The effects of muscle damage on running economy in healthy males. *International Journal of Sports Medicine* 26: 827-831.

Pizza, FX, JB Mitchell, BH Davis, RD Starling, RW Holtz and N Bigelow. 1995. Exercise-induced muscle damage: Effect on circulating leukocyte and lymphocyte subsets. *Medicine & Science in Sports & Exercise* 27: 363-370.

Proske, U and DL Morgan. 2001. Muscle damage from eccentric exercise: Mechanism, mechanical signs, adaptation and clinical applications. *Journal of Physiology* 537: 333-345.

Raastad, R, S Gylterud Owe, G Paulsen, D Enns, K Overgaard, R Crameri, S Kiil, A Belcastro, L Bergersen and J Hallen. 2010. Changes in calpain activity, muscle structure, and function after eccentric exercise. *Medicine & Science in Sports & Exercise* 42 (1): 86-95.

Ready, SL, JG Seifert and E Burke. 1999. The effect of two sports drink formulations on muscle stress and performance. *Medicine & Science in Sports & Exercise* 31: S119.

Rennie, MJ, J Bobe, K Smith, H Wackerhage and P Greenhaff. 2006. Branched-chain amino acids as fuels and anabolic signals in human muscle. *Journal of Nutrition* 136: S264-268.

Romano-Ely, BC, MK Tod, MJ Saunders and T St. Laurent. 2006. Effect of an isocaloric carbohydrate-protein-antioxidant drink on cycling performance. *Medicine & Science in Sports & Exercise* 38 (9): 1608-1616.

Roth, SM, GF Martel and MA Rogers. 2000. Muscle biopsy and muscle fiber hypercontraction: A brief review. *European Journal of Applied Physiology* 83: 239-245.

Roxin, LE, G Hedin and P Venge. 1986. Muscle cell leakage of myoglobin after long-term exercise and relation to the individual performances. *International Journal of Sports Medicine* 7: 259-263.

Sale, DG. 1987. Influence of exercise and training on motor unit activation. *Exercise and Sports Science Reviews* 15: 95-151.

Saunders, MJ, MD Kane and MK Todd. 2004. Effects of a carbohydrate-protein beverage on cycling endurance and muscle damage. *Medicine & Science in Sports & Exercise* 36 (7): 1233-1238.

Saunders, MJ, ND Luden and JE Herrick. 2007. Consumption of an oral carbohydrate-protein gel improves cycling endurance and prevents postexercise muscle damage. *Journal of Strength and Conditioning Research* 21 (2): 678-684.

Saupe, N, LM White, MS Sussman, A Kassner, G Tomlinson and MD Noseworthy. 2008. Diffusion tensor magnetic resonance imaging of the human calf comparison between 1.5t and 3.0t - preliminary results. *Investigative Radiology* 43: 612-618.

Saxton, JM, AE Donnelly and HP Roper. 1994. Indices of free-radical-mediated damage following maximum voluntary eccentric and concentric muscular work. *European Journal of Applied Physiology* 68: 189-193.

Scott, KE, R Rozenek, AC Russo, JA Crussemeyer and MG Lacourse. 2003. Effects of delayed onset of muscle soreness on selected physiological responses to submaximal running. *Journal of Strength and Conditioning Research* 17: 652-658.

Semark, A, TD Noakes and A St Clair Gibson. 1999. The effect of a prophylactic dose of flurbiprofen on muscle soreness and sprinting performance in trained subjects. *Journal of Sports Sciences* 17: 197-203.

Shellock, FG, T Fukunaga, JH Mink and VR Edgerton. 1991. Acute effects of exercise on MR imaging of skeletal muscle: Concentric vs. eccentric actions. *American Journal of Roentgenology* 156 (4): 765-768.

Sherman, WM., LE Armstrong and TM Murray. 1984. Effect of a 42.2 km footrace and subsequent rest or exercise on muscular strength and work capacity. *Journal of Applied Physiology* 57: 1668-1673.

Shi, X, RW Summers, HP Schedl, SW Flanagan, R Chang and CV Gisolfi. 1995. Effects of carbohydrate type and concentration and solution osmolality on water absorption. *Medicine & Science in Sports & Exercise* 27 (12): 1607-1615.

Sjodin, B, YW Hellsten-Westing and FS Apple. 1990. Biochemical mechanism for oxygen free radical formation during exercise. *Sports Medicine* 10: 236-254.

Sjöström, M, J Fridén and B Ekblom. 1982. Fine structural details of human muscle fibres after fibre type specific glycogen depletion. *Histochemistry* 76 (4): 425-438.

Skillen, RA, M Testa, EA Applegate, EA Heiden, AJ Fascetti and GA Casazza. 2008. Effects of an amino acid-carbohydrate drink on exercise performance after consecutive-day exercise bouts. *International Journal of Sport Nutrition and Exercise Metabolism* 18: 473-492.

Smith, C, MJ Kruger, RM Smith and KH Myburgh. 2008. The inflammatory response to skeletal muscle injury: Illuminating complexities. *Sports Medicine* 38 (11): 947-969.

Spriet, L.L. and R.A. Howlett 1999 *Perspectives in exercise science and sports medicine,* ed D R Lamb and R Murray (Carmel: Cooper) pp 1-51

Suzuki, K, M Totsuka, S Nakaji, M Yamada, S Kudoh, Q Liu, K Sugawara, K Yamaya and K Sato. 1999. Endurance exercise causes interaction among stress hormones, cytokines, neutrophil dynamics and muscle damage. *Journal of Applied Physiology* 87 (4): 1360-1367.

Tarnopolsky, M. 2004. Protein requirements for endurance athletes. *Nutrition* 20 (7-8): 662-668.

Tidball, JG. 1995. Inflammatory cell response to acute muscle injury. *Medicine & Science in Sports & Exercise* 27: 1022-1032.

Tidball, JG. 2005. Inflammatory processes in muscle injury and repair. *American Journal of Physiology* 288 (2): R345-353.

Tsintzas, K and C Williams. 1998. Human muscle glycogen metabolism during exercise. Effect of carbohydrate supplementation. *Sports Medicine* 25 (1): 7-23.

Twist, C and RG Eston. 2009. The effect of exercise-induced muscle damage on perceived exertion and cycling endurance performance. *European Journal of Applied Physiology* 105 (4): 559-567.

Valentine, RJ, MJ Saunders, MK Todd and TG St. Laurent. 2008. Influence of carbohydrate-protein beverage on cycling endurance and indices of muscle disruption. *International Journal of Sport Nutrition and Exercise Metabolism* 18: 363-378.

Van Essen, M and MJ Gibala. 2006. Failure of protein to improve a time trial performance when added to a sports drink. *Medicine & Science in Sports & Exercise* 38 (8): 1476-1483.

van Hall, G, JSH Raaymakers, WHM Saris and AJM Wagenmakers. 1995. Ingestion of branched-chain amino acids and tryptophan during sustained exercise in man: Failure to affect performance. *Journal of Physiology* 486: 789-794.

van Loon, LJ, PL Greenhaff, D Constantin-Teodosiu, WH Saris and AJ Wagenmakers. 2001. The effects of increasing exercise intensity on muscle fuel utilisation in humans. *Journal of Physiology* 536: 295-304.

Wagenmakers, AJM, JH Coakley and RHT Edwards. 1990. Metabolism of branched-chain amino acids and ammonia during exercise: Clues from Mcardle's disease. *International Journal of Sports Medicine* 11: S101-S113.

Warhol, M, A Siegal and WJ Evans. 1985. Skeletal muscle injury and repair i marathon runners after competition. *American Journal of Pathology* 118: 331-339.

Warren, GL, DA Lowe and RB Armstrong. 1999. Measurement tools used in the study of eccentric contraction-induced injury. *Sports Medicine* 27: 43-59.

Williams, MB, PB Raven, DL Fogt and JL Ivy. 2003. Effects of recovery beverages on glycogen restoration and endurance exercise performance. *Journal of Strength and Conditioning Research* 17: 12-19.

Yaspelkis, BB 3rd, JG Patterson, PA Anderla, Z Ding and JL Ivy. 1993. Carbohydrate supplementation spares muscle glycogen during variable-intensity exercise. *Journal of Applied Physiology* 75 (4): 1477-1485.

Zaraiskaya, T, D Kumbhare and MD Noseworthy. 2006. Diffusion tensor imaging in evaluation of human skeletal muscle injury. *Journal of Magnetic Resonance Imaging* 24 (2): 402-408.

Zawadzki, KM, BB Yaspelkis and JL Ivy. 1992. Carbohydrate-protein complex increases the rate of muscle glycogen storage after exercise. *Journal of Applied Physiology* 72(5): 1854-1859.

# CHAPTER 2

## MUSCLE METABOLISM DURING CARBOHYDRATE OR PROTEIN-CARBOHYDRATE INGESTION

(Published in *Med Sci Sports Exerc.* 41(12): 2158-2164, 2009)

## 2.1 INTRODUCTION

It is well established that carbohydrate (CHO) ingestion during prolonged endurance exercise delays fatigue and improves the power output that can be maintained (Colombani *et al.* 1999; Koopman *et al.* 2004). The beneficial effect of CHO supplementation in most instances is attributable to the maintenance of euglycemia and a high rate of CHO oxidation, but other mechanisms may also be involved (Colombani *et al.* 1999; Koopman *et al.* 2004). Based on the wealth of evidence available, sports nutrition experts generally recommend that athletes ingest 30-60 g of CHO per hour during exercise in order to optimize endurance performance and subsequent training adaptations (American College of Sports Medicine 2000). An efficacious way for athletes to meet this recommendation and also satisfy their hydration needs is to drink 600-1400 $mL \cdot h^{-1}$ of a 4-8% CHO solution, preferably in small, frequent doses from the onset of activity (American College of Sports Medicine 2000).

In contrast to CHO ingestion, exogenous provision of protein (PRO) during exercise is not generally regarded as important for endurance athletes (American College of Sports Medicine 2000). However, several studies have reported that

co-ingestion of PRO with CHO during prolonged exercise improved time to exhaustion compared to CHO alone (Ivy *et al.* 2003; Saunders *et al.* 2004; Saunders *et al.* 2007). This is an equivocal area of research, with other studies showing no difference between CHOPRO or CHO alone on endurance performance (Romano-Ely *et al.* 2006; Van Essen & Gibala 2006; Osterberg *et al.* 2008; Valentine *et al.* 2008) and direct comparisons between studies are hampered by differences in research designs. Nonetheless, researchers who have reported an ergogenic effect of CHOPRO compared to CHO alone have identified several potential mechanisms (Ivy *et al.* 2003; Saunders *et al.* 2004; Saunders *et al.* 2007), including a reduced rate of muscle glycogen utilization and less reliance on non-oxidative metabolism, secondary to better maintenance of the muscle pool of tricarboxylic acid (TCA) cycle intermediates (TCAi). However, no study has directly examined this issue and thus mechanisms proposed to explain the improved endurance capacity after CHOPRO ingestion (Ivy *et al.* 2003; Saunders *et al.* 2004; Saunders *et al.* 2007) remain speculative.

The primary purpose of the present study was to determine whether co-ingestion of PRO with CHO would alter selected markers of skeletal muscle metabolic control during moderate-intensity exercise as compared to CHO alone. Using a double-blind, repeated-measures crossover design, we recruited trained cyclists and had them perform 90 min of constant-load cycling at 69±1% of $VO_2$peak while ingesting either a 6% CHO or 6% CHO + 2% PRO solution at a rate of 1 $L \cdot h^{-1}$. This drinking strategy was adopted to ensure a CHO delivery rate of

60 $g \cdot h^{-1}$ in both trials, which is the upper limit generally recommended to improve endurance performance (American College of Sports Medicine 2000). We tested the hypotheses that compared to CHO alone, CHOPRO ingestion during exercise would favourably affect muscle energy metabolism as evidenced by (1) a reduced rate of glycogen catabolism, (2) an increased muscle pool of TCAi, and (3) reduced phosphocreatine utilization. We (Gibala *et al.* 2002b) and others (Bruce *et al.* 2001; Mourtzakis *et al.* 2008) have previously shown that nutritional perturbations can induce measureable changes in glycogen, TCAi and PCr metabolism during exercise at ~70% of $VO_2$peak in human skeletal muscle. A secondary purpose was to examine the influence of CHO versus CHOPRO ingestion during exercise on markers of skeletal muscle recovery. Specifically, we measured the activity of creatine kinase (CK) in blood, an indirect marker of skeletal muscle membrane disruption (Warren *et al.* 1999), and 20 km time trial performance ~24 h following the first exercise bout.

## 2.2 METHODS

### 2.2.1 Subjects

Eight healthy men (29±3 y; 79±3 kg; 181±2 cm) with a background in either road cycling, triathlon or duathlon were recruited. All subjects had been engaged in regular cycle exercise training for at least two years prior to the study (mean: 4.8 ± 0.8 y) and were cycling an average of 12 h/wk, or approximately 300-400 km/wk, at the time of the study. Their peak oxygen uptake ($VO_2$peak)

determined using on-line gas collection system (Moxus Modular VO2 System, AEI Technologies, Inc., Pittsburgh, PA) during a ramp test to exhaustion on an electronically braked cycle ergometer (Lode BV, Excalibur Sport V2.0, The Netherlands), was 55±2 $ml \cdot kg^{-1} \cdot min^{-1}$. After being advised of the purpose and potential risks of the study, all subjects provided written, informed consent. The experimental protocol was approved by the Hamilton Health Sciences/Faculty of Health Sciences Research Ethics Board.

### 2.2.2 Experimental protocol

The main phase of the study was a randomized, double-blind comparison of the effect of ingesting 6% CHO or 6% CHO + 2% PRO (CHOPRO) during a 90 min bout of constant-load cycling that elicited 69±1% of $VO_2$ peak on selected markers of skeletal muscle metabolic control. Subjects arrived at the laboratory in the morning after ingesting a breakfast of their own choosing in order to simulate their habitual pre-race practice. A catheter was inserted into an antecubital vein for blood sampling and the area over one thigh was prepared for the extraction of a needle muscle biopsy sample. Briefly, the lateral portion of one thigh was anaesthetized (1% xylocaine) and a small incision made through the skin and underlying fascia in order to obtain a tissue sample from the vastus lateralis muscle. After removal from the leg, the muscle sample was immediately frozen in liquid nitrogen. After resting blood and biopsy samples were obtained, subjects initiated the cycling bout and ingested either the CHO or CHOPRO drink at a rate of 250 ml every 15 min to ensure a CHO delivery rate of 60 g per h, with or

without 20 g of protein per h. Cardiorespiratory data and ratings of perceived exertion (RPE) were collected and averaged over 5 min intervals beginning at 20, 40, 60 and 80 min into the exercise bout. Rates of whole-body carbohydrate and fat oxidation were calculated based on the equations published by Peronnet & Massicotte (1991). A muscle biopsy and blood sample was also obtained immediately following exercise. Approximately 24 h following the constant load test, subjects returned to the laboratory and performed a simulated 20 km cycling time trial on a Computrainer (RacerMate, Inc., Seattle, WA) using their own bicycle as previously described (Van Essen & Gibala 2006). Subjects received no temporal, verbal or physiological feedback during the time trial. Subjects collected their urine into a 4 L container during the period between the first and second rides, and upon return to the laboratory a blood sample was obtained by venipuncture before the time trial. The two experimental trials were performed in random order separated by at least 7 d. Subjects also visited the laboratory on several occasions prior to the main experiment in order to establish the appropriate workload for the constant-load test and to perform a familiarization time trial.

### 2.2.3 Experimental beverages

The two experimental beverages were formulated by Gatorade (Barrington, IL) and contained the same amount of electrolytes and were similarly flavoured (Table 2.1). The only difference between the beverages was that one contained 6% CHO in the form of sucrose (CHO) and one contained 6% CHO

plus 2 % whey protein (CHOPRO). The source of the whey protein isolate was Lacprodan (Arla Foods, Basking Ridge, New Jersey). The two beverages were delivered as dry powders in sealed packages, identified by code numbers to ensure blinding, and were subsequently stored in sealed and locked containers at room temperature in the laboratory. Aliquots of test beverages were carefully weighed and dissolved in water according to the manufacturer's instructions on the day of each experimental trial. The drinks were stored in translucent containers, each containing 250 ml of fluid, and served slightly chilled.

**Table 2.1:** Composition of experimental beverages

| | Grams of ingredient per L of water | |
|---|---|---|
| Ingredient | CHO | CHOPRO |
| Sucrose | 60 | 60 |
| Whey protein | 0 | 20 |
| Sodium chloride | 0.6730 | 0.6730 |
| Sodium citrate | 0.5963 | 0.5963 |
| Monopotassium phosphate | 0.4447 | 0.4447 |
| Citric acid | 2.7641 | 2.7641 |
| Flavor | 0.5115 | 0.5115 |

CHO = Carbohydrate trial; CHOPRO = Carbohydrate + protein trial.

### 2.2.4 Physical activity and dietary controls

Subjects were asked to keep their weekly training schedule as consistent as possible over the course of the experiment, but given the within-subject design (i.e., each subject served as their own control), training was not standardized across subjects. Subjects were specifically instructed to standardize their final

workout performed 48 h prior to each experimental trial and to perform no physical activity, aside from activities of daily living, for 24 h prior to the start of each trial. Subjects were also advised to maintain their habitual diet over the course of the study, and were required to maintain food records during the 24 h prior to the constant-load test and the 24 h period between the constant-load test and the time trial. Following the first experimental trial, subjects were instructed to replicate their individual nutritional pattern over the course of the second trial and again record food intake, noting any deviations from the first trial. Food records were subsequently analyzed (Nutritionist Five dietary analysis software, First Data Bank, San Bruno, CA) and results confirmed no difference in total energy intake or macronutrient composition between trials (Table 2.2).

**Table 2.2:** Nutritional data

| Macronutrient | CHO | | CHOPRO | |
|---|---|---|---|---|
| | Day 1 | Day 2 | Day 1 | Day 2 |
| Carbohydrate, g | 357 ± 101 | 492 ± 83 | 383 ± 95 | 470 ± 75 |
| Protein, g | 135 ± 20 | 125 ± 14 | 101 ± 16 | 118 ± 12 |
| Fat, g | 96 ± 14 | 75 ± 12 | 92 ± 15 | 83 ± 10 |
| Total Energy, kcal | 2791 ± 488 | 3140 ± 435 | 2723 ± 470 | 3046 ± 359 |

All data are means±SE, n=7. CHO = carbohydrate trial, CHOPRO = carbohydrate + protein trial. Day 1 = 24 h period preceding the experimental exercise trial. Day 2 = ~24 h period between the experimental exercise trial and the time trial. There were no differences between trials for any variable.

### 2.2.5 Muscle analyses

Following initial freezing in liquid nitrogen, muscle samples were subsequently freeze-dried, powdered, dissected free of non-muscle elements and stored at -80°C prior to metabolite analyses using standard methods in our laboratory. For glycogen determination, 2-3 mg aliquot of freeze-dried muscle was incubated in 2.0 N HCl and heated for 2 h at 100°C to hydrolyse the glycogen to glucosyl units. The solution was neutralized with an equal volume of 2.0 N NaOH and analyzed for glucose using an enzymatic assay adapted for fluorometry (Passoneau & Lowry 1993). A second 5-10 mg aliquot of freeze-dried muscle was extracted on ice using 0.5 M PCA containing 1 mM EDTA, neutralized with 2.2 M $KHCO_3$, and the resulting supernatant was analyzed for ATP, phosphocreatine, creatine, lactate and the TCAi citrate and malate using enzymatic assays adopted for fluorometry (Passoneau & Lowry 1993).

### 2.2.6 Blood analyses

Venous blood samples were collected into commercial tubes that contained either no additive or heparin. Non-heparinized blood was allowed to clot, then centrifuged and the serum stored for subsequent analysis of creatine kinase (CK-NAC 2-part liquid reagent set, Pointe Scientific; Canton, MI, USA). Heparinized blood was used for the immediate determination of glucose (Ascensia Contour Blood Glucose Monitor, Bayer Health Care, Toronto, ON) and lactate (Accutrend Lactate, Roche Diagnostics, Mannheim, Germany). The remaining heparinized blood was centrifuged and the resulting supernatant was

removed and stored at -20°C for the subsequent analysis of plasma insulin using an immunoassay kit (Insulin EIA, Alpco Diagnostics; Salem, NH, USA) and plasma amino-acids using an HPLC method described by Wilkinson and colleagues (2007). Briefly, extracts were derivatized prior to injection using Waters ™ AccQ·Fluor ™ reagent kit (Millford, MA) by heating for 10 min at 55°C to form the 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate derivative of all physiologic amino acids. Samples and standards (Sigma, St. Louis, MO) were run on a Waters ™ 2695 HPLC separations module through a 4mm AccQ.Tag column (Water, Nova-Pak $C_{18}$, bonded silica) in order to separate the amino acids. The amino acids were detected using Waters 2475 scanning fluorescence detector excitation and emission wavelengths of 250nm and 395nm, respectively. Amino acid peak areas were integrated, compared with known standards, and analyzed using a Waters Millenium32 software package.

### 2.2.7 Urine analyses

After total urine volume was measured, an aliquot was stored at -86° C for subsequent analysis of urea and creatine using commercial kit assays (Pointe Scientific Inc; Canton, MI, USA).

### 2.2.8 Statistical analyses

All muscle and blood data was analyzed using a two-factor (condition x time) repeated measures analysis of variance (ANOVA) (Sigma Stat 3.1, Point Richmond, California). All diet, time trial performance and urine data was analyzed using a one-way repeated measures ANOVA. The level of significance

for all analyses was set at $P<0.05$ and significant interactions and main effects were subsequently analyzed using a Tukey post hoc test. All data are presented as mean±SE, n=8. One subject did not adhere to the physical activity and nutritional controls following the constant-load test in one trial and hence creatine kinase activity, diet and time trial data are based on n=7.

## 2.3 Results

### 2.3.1 Cardiorespiratory data

Mean $VO_2$, averaged over the 90 min bout of constant-load cycling, corresponded to an intensity of 69±1% of $VO_2$peak in both experimental trials (Table 2.3). There was no difference between treatments in $VO_2$, heart rate, expired ventilation, respiratory exchange ratio, whole-body substrate oxidation or RPE during exercise (Table 2.3).

**Table 2.3:** Cardiorespiratory data during constant-load cycling exercise

| Variable | CHO | CHOPRO |
|---|---|---|
| Oxygen uptake, $mL \cdot kg^{-1} \cdot min^{-1}$ | 38 ± 1 | 38 ± 1 |
| HR, $beats \cdot min^{-1}$ | 149 ± 1 | 148 ± 1.9 |
| Ventilation, $L \cdot min^{-1}$ | 72 ± 3 | 72 ± 2 |
| Respiratory Exchange Ratio | 0.89 ± 0.01 | 0.90 ± 0.01 |
| Carbohydrate oxidation, $kJ \cdot min^{-1}$ | 45±3 | 44.1±4 |
| Fat oxidation, $kJ \cdot min^{-1}$ | 18.1±3 | 18.7±3 |
| Rating of Perceived Exertion | 12.7 ± 0.2 | 12.5 ± 0.2 |

Values are mean±SE, n=8. CHO = carbohydrate trial, CHOPRO = carbohydrate + protein trial.

### 2.3.2 Blood data

The plasma concentration of branched-chain amino acids (Figure 2.1) and total essential amino acids (Table 2.4) were higher after exercise in CHOPRO versus CHO ($P<0.05$). Blood insulin decreased during exercise compared to rest (main effect, $P<0.05$), but there were no differences between treatments. Changes in blood lactate and glucose were also similar between trials. (Table 2.4). Serum CK measured 24 h after the constant-load exercise bout was higher compared to baseline (main effect, $P<0.05$) but there was no difference between treatments (Figure 2.2).

**Table 2.4:** Muscle and blood metabolite data

| Variable | CHO | | CHOPRO | |
|---|---|---|---|---|
| | PreEX | PostEX | PreEX | PostEX |
| Muscle: | | | | |
| ATP, mmol·kg$^{-1}$ d.w. | 23.2±0. | 22.0±1.2 | 26.2±2.3 | 22.3±1.9 |
| Phosphocreatine, mmol·kg$^{-1}$ d.w. | 90.8±7.7 | 84.1±8.0 | 87.7±5.5 | 80.1±7.8 |
| Creatine, mmol·kg$^{-1}$ d.w. | 46.5±6.7 | 53.2±8.1 | 53.1±3.4 | 57.1±5.5 |
| Lactate#, mmol·kg$^{-1}$ d.w. | 17.2±1.4 | 54.0±19.4 | 22.1±4.1 | 54.2±18.6 |
| Blood: | | | | |
| Glucose, mmol·L$^{-1}$ | 4.3±0.4 | 5.1±0.2 | 4.0±0.1 | 4.9±0.3 |
| Lactate#, mmol·L$^{-1}$ | 1.8±0.1 | 2.3±0.1 | 1.7±0.1 | 2.0±0.1 |
| Insulin#, mIU·mL$^{-1}$ | 9.3±3 | 5.0±0.8 | 10.3±3.0 | 5.0±1.3 |
| Essential amino acids, µmol·L$^{-1}$ | 844±95 | 670±62 | 820±96 | 1071±85*+ |

All data are mean±SE, n=8. CHO = carbohydrate trial, CHOPRO = carbohydrate + protein trial. d.w. = dry weight. * $P\leq0.05$ versus Rest within same trial. + $P<0.05$ versus CHO at same time point. # Main effect for time such that Post>Pre ($P\leq0.05$).

### 2.3.3 Muscle data

Muscle glycogen decreased during exercise (main effects, $P<0.05$) but

there was no difference between the CHO and CHOPRO trials (Figure 2.3). The TCAi citrate and malate increased during exercise but there was no difference between trials in either their individual or sum concentration (Figure 2.4). Similarly, there were no treatment effects for muscle ATP, phosphocreatine, creatine or lactate (Table 2.4).

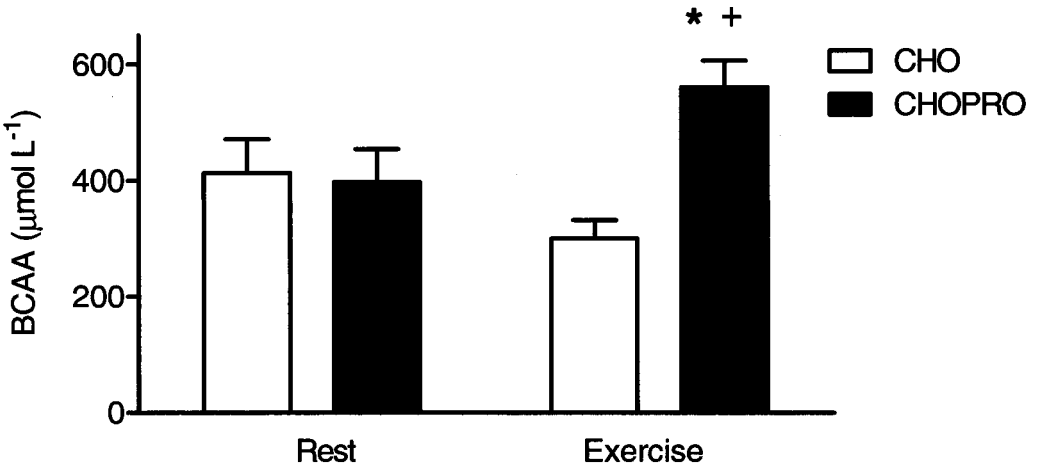



**Figure 2.1**: Plasma branch-chain amino acid (BCAA) concentration before and after 90 min of cycling at 69±1% $VO_2$ peak when subjects ingested a 6% carbohydrate (CHO) or a 6% carbohydrate plus 2% protein solution (CHOPRO). Values are mean±SE, n=8. * P≤0.05 versus Rest within same trial. $^{+}$ P<0.05 versus CHO at same time point.

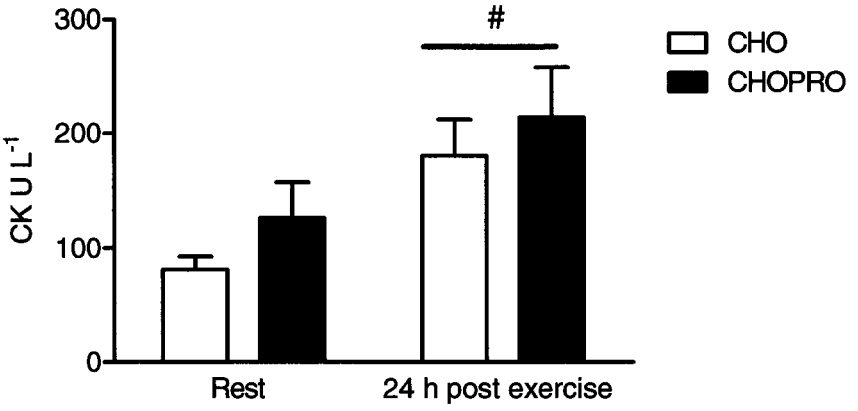



**Figure 2.2**: Serum creatine kinase activity measured before and 24 h after a 90 min bout of cycling at 69±1% $VO_2$ peak when subjects ingested a 6% carbohydrate (CHO) or a 6% carbohydrate plus 2% protein solution (CHO+PRO). Values are mean±SE, n=7. # Main effect for time such that Post>Pre ($P<0.05$).

2.3.4 Urine

There was no difference between treatments in creatinine (CHOPRO: 1.2±0.6 vs CHO: 1.3±0.6 g·24 $h^{-1}$) or urea (CHOPRO: 10.5 ± 1.3 vs CHO: 12.5 ± 1.6 g·24 $h^{-1}$).

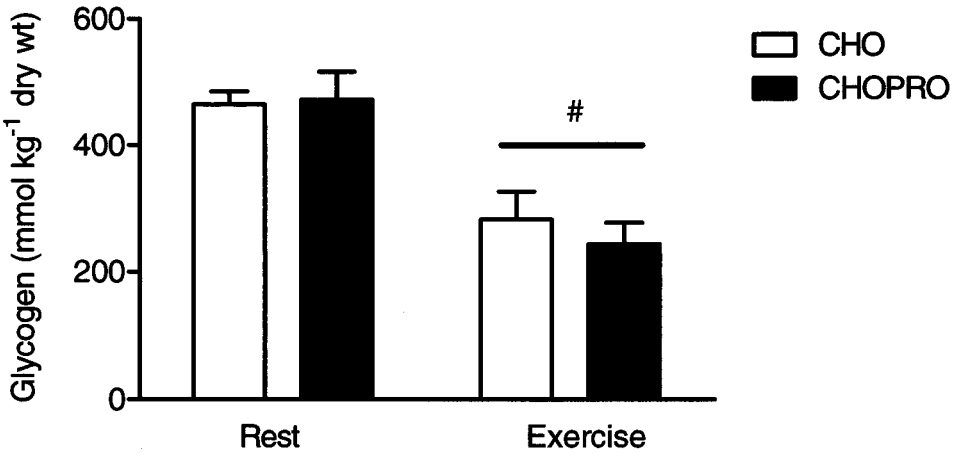


**Figure 2.3**: Muscle glycogen concentration measured in biopsy samples obtained before and after 90 min of cycling at 69±1% $VO_2$ peak when subjects ingested a 6% carbohydrate (CHO) or a 6% carbohydrate plus 2% protein solution (CHOPRO). Values are mean±SE, n=8. # Main effect for time such that Post>Pre (P<0.05).

### 2.3.4 Time-trial performance

Time to complete the 20 km time trial was not different between conditions (Figure 2.5). Average heart rate (CHOPRO 157±3 vs. CHO 158±3 beats·min$^{-1}$) and mean power output (CHOPRO 286±24 versus CHO: 284±22 W) were also similar between treatments.

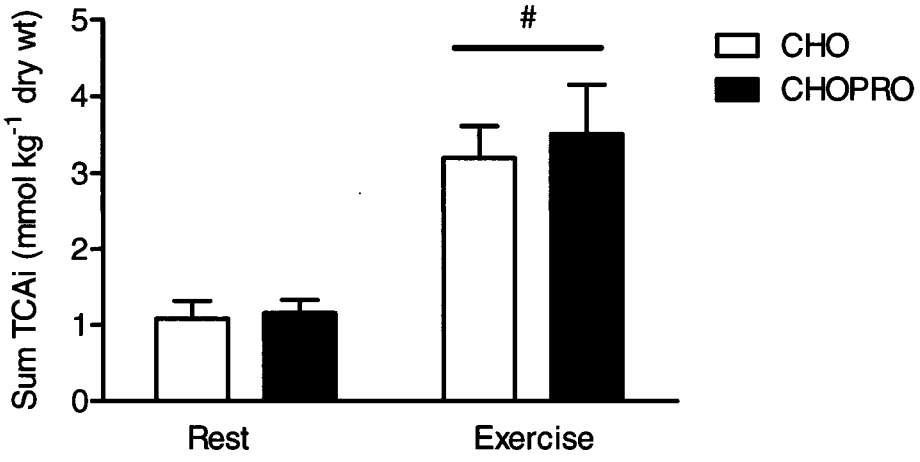



**Figure 2.4**: Sum concentration of the tricarboxylic acid cycle intermediates citrate and malate measured in biopsy samples obtained before and after 90 min of cycling at 69±1% $VO_2$ peak when subjects ingested a 6% carbohydrate (CHO) or a 6% carbohydrate plus 2% protein solution (CHOPRO). Values are mean±SE, n=8. # Main effect for time such that Post>Pre (P<0.05).

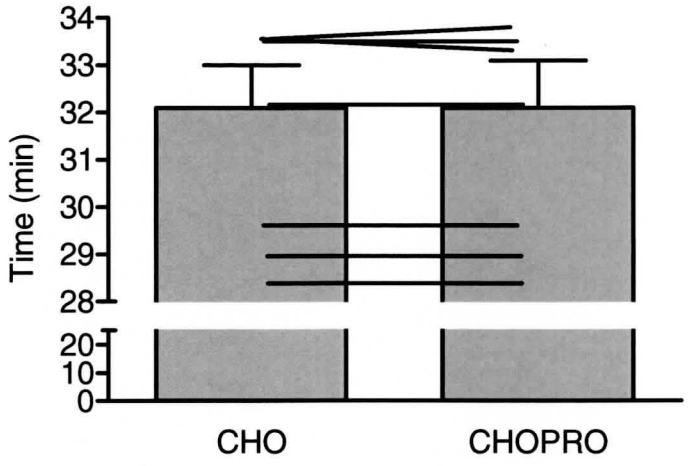



**Figure 2.5**: Individual (lines) and mean time (bars) required to complete a simulated 20 km time trial 24 h following a 90 min bout of cycling at 69±1% $VO_2$ peak when subjects ingested a 6% carbohydrate (CHO) or a 6% carbohydrate plus 2% protein solution (CHOPRO). Values are mean±SE, n=7.

## 2.4 Discussion

The major novel finding from the present study was that co-ingestion of PRO with CHO during 90 min of cycling at 69±1% $VO_2$ peak did not alter selected markers of skeletal muscle metabolic control compared to CHO alone. We also found no effect of the nutritional intervention on the activity of creatine kinase measured in venous blood or next-day time trial performance. The relative concentration of CHO and PRO in the drinks used in the present study was similar to previous studies that have reported CHOPRO ingestion improves endurance capacity compared to CHO alone (Ivy *et al.* 2003; Saunders *et al.* 2004; Saunders *et al.* 2007). However, the overall *rate* of energy intake in the present study was higher owing to this greater volume of fluid ingested, as our goal was to ensure that CHO intake was on the upper end of the range

commonly recommended by sports nutrition experts (American College of Sports Medicine 2000). We also demonstrated that the blood concentration of specific amino acids was increased after CHOPRO ingestion compared to CHO, which is presumably important for the intervention to induce a metabolic effect in skeletal muscle or other tissues (Jentjens *et al.* 2001).

A number of studies have reported that CHOPRO ingestion during exercise improves cycle endurance capacity compared to CHO alone (Ivy *et al.* 2003; Saunders *et al.* 2004; Saunders *et al.* 2007). For example, Ivy et al. (2003) reported that time to exhaustion during cycling at 85% $VO_2$peak was increased by 36% when trained cyclists ingested ~2% PRO with ~8% CHO (at a rate of ~600 $mL \cdot h^{-1}$) during a standardized 3 h variable intensity cycling bout performed immediately prior to the timed ride to fatigue. Similar performance improvements were reported by Saunders and colleagues (2004; 2007) who showed that trained cyclists rode longer to exhaustion at an intensity of 75% $VO_2$peak when they ingested ~2% PRO with ~7% CHO compared to CHO alone (also at a rate of ~600 $mL \cdot h^{-1}$). Various theories have been advanced to explain these observations (Ivy *et al.* 2003; Saunders *et al.* 2004; Saunders *et al.* 2007) and the present study specifically addressed two mechanisms originally proposed by Ivy et al. (2003). Specifically, these authors (Ivy *et al.* 2003) postulated that the improved performance after CHOPRO ingestion "could be related to the sparing or more efficient use of muscle glycogen (or) anaplerotic reactions and the retention of Krebs cycle intermediates."

The precise manner by which PRO ingestion *during* exercise could attenuate the rate of muscle glycogen utilization has not been clearly elucidated. However, the general theory (Ivy *et al.* 2003) is based on the findings from some studies that showed protein ingestion with CHO *following* prolonged moderate-intensity exercise augmented insulin secretion (Jentjens *et al.* 2001; Williams *et al.* 2003; Betts *et al.* 2005; Betts *et al.* 2008) and the rate of muscle glycogen resynthesis (Ivy *et al.* 2002; Williams *et al.* 2003; Berardi *et al.* 2006). In fact, the potential for protein to influence post-exercise glycogen resynthesis is an equivocal topic and appears to depend on the rate and amount of CHO ingested (Burke *et al.* 2004). As summarized by Burke et al. (2004): "Most evidence suggests that feeding a high amount of carbohydrate at frequent intervals negates the benefits of added protein... (but) co-ingestion of protein with carbohydrate will increase the efficiency of muscle glycogen storage when the amount of carbohydrate ingested is below the threshold for maximal glycogen synthesis." Irrespective of data on recovery from exercise, there is little evidence to suggest that ingesting protein with CHO *during* prolonged exercise alters blood insulin concentration compared to CHO alone, and the present results are consistent with other recent studies in this regard (Ivy *et al.* 2003; Van Essen & Gibala 2006). Indeed, as recognized by Ivy et al. in their original study (Ivy *et al.* 2003), an elevation in blood insulin represents a questionable hypothesis to explain the purported glycogen sparing effect of CHOPRO ingestion. The present study is the first to address this topic directly, and we found no difference in the

rate of muscle glycogen utilization during exercise at an intensity of 69±1% of $VO_2$peak when subjects ingested CHO or CHOPRO.

A second mechanism proposed to explain the finding of longer TTE after CHOPRO ingestion compared to CHO alone relates to changes in the skeletal muscle pool of TCAi (Ivy *et al.* 2003). Several investigators have proposed that the increase in muscle TCAi is crucial in order to achieve high rates of mitochondrial respiration during exercise (Sahlin *et al.* 1990; Wagenmakers *et al.* 1990) and a common interpretation is that a given concentration of TCAi is required in order to sustain a given rate of oxidative phosphorylation during exercise (Ivy *et al.* 2003). However, as recently reviewed by Bowtell and colleagues (2007), a substantial body of evidence from both human and animal studies conducted in several laboratories has questioned this theory from a basic science perspective. For example, we (Gibala *et al.* 2002a) combined measurements of muscle TCAi, amino acids and key energy metabolites with direct measurements of limb oxygen uptake during a 90 min bout of moderate-intensity exercise. Following a threefold expansion during the initial minutes of exercise, the muscle TCAi pool rapidly declined such that the value after 60 and 90 min was not different from the resting concentration. Despite the decline in muscle TCAi, the capacity for aerobic energy provision was not compromised, as evidenced by stable limb oxygen uptake during exercise and no change in muscle phosphocreatine content, which is a sensitive indicator of mitochondrial respiration.

In the present study, we found no difference between the CHO and CHOPRO treatments on the total concentration of malate and citrate, which account for ~70% of the muscle TCAi pool in humans (Gibala *et al.* 2002a), or the rate of muscle phosphocreatine degradation. Paradoxically, according to the theory originally proposed by Wagenmakers et al. (1990), one would predict that an increased rate of PRO oxidation during exercise (i.e., secondary to PRO ingestion) could potentially *impair* aerobic energy provision by *reducing* the muscle concentration of TCAi. Regardless, a reasonable interpretation of the available literature is that changes in muscle TCAi are not causally related to the capacity for aerobic energy provision in human skeletal muscle (5).

In addition to our primary focus on skeletal muscle metabolism, we also measured markers of skeletal muscle recovery ~24 h following the first bout. Several studies have reported that PRO ingestion during prolonged exercise attenuates the post-exercise rise in CK (Saunders *et al.* 2004; Luden *et al.* 2007; Saunders *et al.* 2007; Valentine *et al.* 2008), but our data are consistent with other reports that have failed to show a difference in this regard (Millard-Stafford *et al.* 2005; Green *et al.* 2008). Despite the widespread use of these markers (Saunders *et al.* 2004; Millard-Stafford *et al.* 2005; Romano-Ely *et al.* 2006; Greer *et al.* 2007; Luden *et al.* 2007; Saunders *et al.* 2007; Green *et al.* 2008; Valentine *et al.* 2008), blood levels of myofibre enzymes correlate poorly with changes in muscle function and many investigators have recommended de-emphasis on the use of these methods to quantify the magnitude and time course of muscle injury

(Warren *et al.* 1999). Future research that incorporates other techniques including functional measurements, non-invasive imaging, and direct muscle sampling to evaluate histological changes may clarify our understanding of the potential for PRO ingestion to attenuate muscle disruption following exercise. We also found no treatment effect on our other marker of skeletal muscle recovery, 20 km time trial performance. These data are in contrast to a study that showed improved TTE at 85% $VO_2$peak 12-15 h following a bout of exercise when subjects ingested CHOPRO compared to CHO alone, but consistent with recent data from Betts et al (2005), who found no difference between CHO and CHOPRO drinks ingested during recovery on subsequent run performance.

In summary, the present study found that, as compared to CHO alone, co-ingestion of PRO with CHO during cycling exercise at 69±1% $VO_2$ peak did not alter the magnitude of muscle glycogen or phosphocreatine utilization, net expansion of the TCA cycle pool, blood creatine kinase activity, or next-day time trial performance. These data therefore suggest that when trained men ingest CHO at a rate on the upper end of the range generally recommended to improve endurance performance, co-ingestion of PRO does not alter specific markers proposed to reflect an enhanced capacity for skeletal muscle energy delivery. PRO ingestion during exercise has been reported to induce other acute changes that could facilitate exercise capacity, including reduced muscle proteolysis (Matsumoto *et al.* 2007) and improved fluid retention (Seifert *et al.* 2005). It is also possible that the potential metabolic and performance effects of protein

ingestion are influenced by relative exercise intensity, and this could explain some of the equivocal findings in the literature. Regardless, additional work is warranted to establish a viable mechanism to explain the finding by some authors that adding PRO to a CHO-based sport drink improved acute endurance performance.

## 2.5 References

American College of Sports Medicine, American Dietetic Association, Dieticians of Canada. 2000. Nutrition and athletic performance. *Medicine & Science in Sports & Exercise* 32: 2130-2145.

Berardi, JM, TB Price, EE Noreen and Lemon PW. 2006. Postexercise muscle glycogen recovery enhanced with a carbohydrate-protein supplement. *Medicine & Science in Sports & Exercise* 38 (6): 1106-1113.

Betts, JA , E Stevenson, C Williams, C Sheppard, E Grey and J Griffin. 2005. Recovery of endurance running capacity: Effect of carbohydrate-protein mixtures. *International Journal of Sport Nutrition and Exercise Metabolism* 15 (6): 590-609.

Betts, JA, C Williams, L Bobbis and K Tsintzas. 2008. Increased carbohydrate oxidation after ingesting carbohydrate with added protein. *Medicine & Science in Sports & Exercise* 40 (5): 903-912.

Bowtell, JL, S Marwood, M Bruce, D Constantin-Teodosiu and PL Greenhaff. 2007. Tricarboxylic acid cycle intermediate pool size: Functional importance for oxidative metabolism in exercising human skeletal muscle. *Sports Medicine* 37 (12): 1071-1088.

Bruce, M, D Constantin-Teodosiu, PL Greenhaff, LH Boobis, C Williams and JL Bowtell. 2001. Glutamine supplementation promotes anaplerosis but not oxidative energy delivery in human skeletal muscle. *American Journal of Physiology* 280 (4): E669-675.

Burke, LM, B Keins and JL Ivy. 2004. Carbohydrates and fat for training and recovery. *Journal of Sports Sciences* 22: 15-30.

Colombani, PC, E Kovacs, P Frey-Rindova, W Frey, W Langhans, M Arnold and C Wenk. 1999. Metabolic effects of a protein-supplemented carbohydrate drink in marathon runners. *International Journal of Sport Nutrition and Exercise Metabolism* 9: 181-201.

Gibala, MJ, JA Gonzalez-Alonso and B Saltin. 2002a. Dissociation between muscle tricarboxylic acid cycle pool size and aerobic energy provision during prolonged exercise in humans. *Journal of Physiology* 545: 705-713.

Gibala, MJ, N Peirce, D Constantin-Teodosiu and PL Greenhaff. 2002b. Exercise with low muscle glycogen augments TCA cycle anaplerosis but impairs oxidative energy provision in humans. *Journal of Physiology* 540 (3): 1079-1086.

Green, MS, BT Corona, JA Doyle and CP Ingalls. 2008. Carbohydrate-protein drinks do not enhance recovery from exercise-induced muscle injury. *International Journal of Sport Nutrition and Exercise Metabolism* 18 (1): 1-18.

Greer , BK, JL Woodard, JP White, EM Arguello and EM Haymes. 2007. Branched-chain amino acid supplementation and indicators of muscle damage after endurance exercise. *International Journal of Sport Nutrition and Exercise Metabolism* 17 (6): 595-607.

Ivy, JL, HW Goforth, BW Damon, TR McCauley, EC Parson and TB Price. 2002. Early postexercise muscle glycogen recovery is enhanced with a carbohydrate-protein supplement. *Journal of Applied Physiology* 93: 1337-1344.

Ivy, JL, PT Res, RC Sprague and MO Widzer. 2003. Effect of a carbohydrate-protein supplement on endurance performance during exercise of varying intensity. *International Journal of Sport Nutrition and Exercise Metabolism* 13: 382-395.

Jentjens, RL, LJ van Loon, CH Mann, AJ Wagenmakers and AE Jeukendrup. 2001. Addition of protein and amino acids to carbohydrates does not enhance postexercise muscle glycogen synthesis. *Journal of Applied Physiology* 91 (2): 839-846.

Koopman, R, DLE Pannemans, AE Jeukendrup, AP Gijsen, JMG Senden, D Halliday, WHM Saris, LJC van Loon and AJM Wagenmakers. 2004. Combined ingestion of protein and carbohydrate improves protein balance during ultra-endurance exercise. *American Journal of Physiology* 287 (4): E712-E720.

Luden, ND, MJ Saunders and MK Todd. 2007. Postexercise carbohydrate-protein-antioxidant ingestion decreases plasma creatine kinase and muscle soreness. *International Journal of Sport Nutrition and Exercise Metabolism* 17: 109-123.

Matsumoto, K, M Mizuno, T Mizuno, B Dilling-Hansen, A Lahoz, V Bertelsen, H Munster, H Jordening, K Hamada and T Doi. 2007. Branched-chain amino acids and arginine supplementation attenuates skeletal muscle proteolysis induced by moderate exercise in young individuals. *International Journal of Sports Medicine* 28: 531-538.

Millard-Stafford, M, GL Warren, LM Thomas, JA Doyle, T Snow and K Hitchcock. 2005. Recovery from run training: Efficacy of a carbohydrate-protein beverage? *International Journal of Sport Nutrition and Exercise Metabolism* 15 (6): 610-624.

Mourtzakis, M, TE Graham, J Gonzalez-Alonso and B Saltin. 2008. Glutamate availability is important in intramuscular amino acid metabolism and TCA cycle intermediates but does not affect peak oxidative metabolism. *Journal of Applied Physiology* 105 (2): 547-554.

Osterberg, KL, JJ Zachwieja and JW Smith. 2008. Carbohydrate and carbohydrate + protein for cycling time-trial performance. *Journal of Sports Sciences* 26 (3): 227-233.

Passoneau, JV and OH Lowry. 1993. *Enzymatic analysis: A practical guide.* (Totowa: Humana Press)

Péronnet, F and Massicotte D. 1991. Table of nonprotein respiratory quotient: An update. *Canadian Journal of Sport Sciences* 16 (1): 23-29.

Romano-Ely, BC, MK Tod, MJ Saunders and T St. Laurent. 2006. Effect of an isocaloric carbohdyrate-protein-antioxidant drink on cycling performance. *Medicine & Science in Sports & Exercise* 38 (9): 1608-1616.

Sahlin, K, A Katz and S Broberg. 1990. Tricarboxylic acid cycle intermediates in human muscle during prolonged exercise. *American Journal of Physiology* 259: C834-C841.

Saunders, MJ, MD Kane and MK Todd. 2004. Effects of a carbohydrate-protein beverage on cycling endurance and muscle damage. *Medicine & Science in Sports & Exercise* 36 (7): 1233-1238.

Saunders, MJ, ND Luden and JE Herrick. 2007. Consumption of an oral carbohydrate-protein gel improves cycling endurance and prevents postexercise muscle damage. *Journal of Strength and Conditioning Research* 21 (2): 678-684.

Seifert, JG, RW Kipp, M Amann and O Gazal. 2005. Muscle damage, fluid ingestion, and energy supplementation during recreational alpine skiing. *International Journal of Sport Nutrition and Exercise Metabolism* 15: 528-536.

Valentine, RJ, MJ Saunders, MK Todd and TG St. Laurent. 2008. Influence of carbohydrate-protein beverage on cycling endurance and indices of muscle disruption. *International Journal of Sport Nutrition and Exercise Metabolism* 18: 363-378.

Van Essen, M and MJ Gibala. 2006. Failure of protein to improve a time trial performance when added to a sports drink. *Medicine & Science in Sports & Exercise* 38 (8): 1476-1483.

Wagenmakers, AJM, JH Coakley and RHT Edwards. 1990. Metabolism of branched-chain amino acids and ammonia during exercise: Clues from Mcardle's disease. *International Journal of Sports Medicine* 11: S101-S113.

Warren, GL, DA Lowe and RB Armstrong. 1999. Measurement tools used in the study of eccentric contraction-induced injury. *Sports Medicine* 27: 43-59.

Wilkinson, SB, MA Tarnopolsky, MJ MacDonald, JR MacDonald, D Armstrong, SM Phillips. 2007. Consumption of fluid skim milk promotes greater muscle protein accretion after resistance exercise than does consumption of an isonitrogenous and isoenergetic soy-protein beverage. *American Journal of Clinical Nutrition* 84 (4): 1031-1040.

Williams, MB, PB Raven, DL Fogt and JL Ivy. 2003. Effects of recovery beverages on glycogen restoration and endurance exercise performance. *Journal of Strength and Conditioning Research* 17: 12-19.

# CHAPTER 3

## DIFFUSION TENSOR MRI TO ASSESS SKELETAL MUSCLE DISRUPTION AFTER ECCENTRIC EXERCISE

(Submitted Sept 2010 to Medicine & Science in Sports & Exercise)

### 3.1 INTRODUCTION

Strenuous unaccustomed exercise, especially activity that involves a large eccentric component, produces structural changes in skeletal muscle that are often associated with a temporary decline in force-generating capacity (Clarkson & Hubal 2002). Traditionally, the only direct structural assessment of exercise-induced muscle disruption involves the histological analysis of tissue obtained from a needle biopsy sample. Morphological studies investigating muscle disruption using this technique have demonstrated that the Z disk is the most vulnerable structure to exercise, especially one characterized by intense eccentric muscle actions (Friden *et al.* 1983; Newham *et al.* 1983; Friden & Lieber 2001). A limitation of needle biopsy sampling however is the invasive nature of the technique, and the fact that it may over-or under-estimate tissue disruption owing to the non-uniform pattern of Z-band streaming and the relatively small "snapshot" provided by the biopsy. It has also been suggested that the biopsy procedure itself might induce tissue damage (Malm *et al.* 2000) and thus it is desirable to identify non-invasive methods that might provide a

more representative assessment of exercise-induced muscle disruption.

Non-invasive magnetic resonance imaging (MRI) methodologies have been applied to examine the gross anatomy of individual muscles and can also be used to quantify the directional diffusion of water in tissues (Galban *et al.* 2004; Zaraiskaya *et al.* 2006; Deux *et al.* 2008; Karampinos *et al.* 2009; Schwenzer *et al.* 2009). As human skeletal muscle has a highly hierarchical organization involving a complex fibre architecture, high resolution *in vivo* probing of skeletal muscle would be essential for establishing the connection between structure and function (Galban *et al.* 2004; Zaraiskaya *et al.* 2006; Karampinos *et al.* 2009). Diffusion tensor magnetic resonance imaging (DT-MRI) is a useful technique for the non-invasive structural characterization of various anisotropic tissues (Basser *et al.* 1994; Schwenzer *et al.* 2009). This technique measures the self diffusion of water in biological tissues and has been applied to detect neural abnormalities in a variety of diseases including stroke, dyslexia and multiple sclerosis (Yamada K 2009). Unlike other common MRI-accessible parameters such as T1 or T2 which reflect local magnetic properties of nuclear spins and their interaction with their biochemical environment, DTI is an intrinsic physical property that reflects microstructure organization and is not magnetic in origin (Le Bihan *et al.* 2001). After the DT images are collected along multiple different diffusion encoding directions, a number of quantitative parameters can be used to characterize molecular mobility. The magnitude of diffusivity for each direction (x, y, z), in each voxel is determined (eigenvalues, $\lambda$), and their orientation

direction (ε, or eigenvectors) are determined by a mathematical process involving "diagonalization". In other words, the eigenvectors and eigenvalues are simply the main diffusion directions and associated diffusivities (Basser & Jones 2002).

As skeletal muscle fibres are filled with protein filaments arranged in a well-known geometry, these filaments, in addition to nonfilament proteins dissolved in the cytoplasmic water act as barriers to diffusion (Cleveland *et al.* 1976), creating a diffusion restriction greater in some directions than in others (Saupe *et al.* 2008). DT-MRI in the skeletal muscle is therefore based on the assumption that the primary eigenvector (and first eigenvalue) of the diffusion tensor coincides with local fibre orientation and magnitude of diffusion (Basser *et al.* 1994; Le Bihan *et al.* 2001; Damon *et al.* 2002; Sinha *et al.* 2006) while the secondary and tertiary eigenvalues have been suggested to be representative of diffusion within the endomysium, and throughout the fibre radius (Tseng *et al.* 2003; Galban *et al.* 2004). Although DT-MRI has a number of different outcome parameters, one of particular interest is fractional anisotropy (FA) which is a measure of organization/disorganization within an imaged area and calculated on a scale from 0-1, with greater disorganization (i.e., more isotropic structure) characterized by a lower value.

Previous research has used DT-MRI to evaluate human calf muscle in patients presenting with traumatic injury such as hematomas and muscle tears (Zaraiskaya *et al.* 2006). In that work, clear differences in FA, apparent diffusion coefficient (ADC or mean diffusivity), and eigenvalues were demonstrated in

injured skeletal muscle when compared with healthy controls (Zaraiskaya *et al.* 2006) suggesting modified fibre structure/cell membrane integrity, known to occur following a muscle tear (Hough 1902). Few studies have applied DT-MRI to examine healthy skeletal muscle structure, although tensor eigenvalues and ADC have been shown to increase following contraction (Deux *et al.* 2008) or passive muscle shortening (Schwenzer *et al.* 2009) in human calf muscles. However, to our knowledge no study has utilized DT-MRI to assess *exercise-induced* skeletal muscle disruption as commonly investigated through the histological analysis of skeletal muscle biopsies or the measured concentration of blood proteins. If DT-MRI is sensitive enough to detect exercise-induced skeletal muscle disruption, DT-MRI could be a unique, novel technique for studying skeletal muscle structural impairment following intense, unaccustomed exercise.

The primary purpose of the present study was to evaluate the potential for DT-MRI to non-invasively detect structural changes in skeletal muscle after an acute bout of strenuous unaccustomed exercise. Subjects performed an eccentric leg extension protocol that was previously shown to induce ultrastructural evidence of skeletal muscle disruption and decrease force-generating capacity 24 h following exercise (Beaton *et al.* 2002a). In addition to DT-MRI measurements, we also quantified structural disruption through the analysis of needle biopsy samples as well as changes in blood, performance and muscle soreness ratings performed before and 24 h after exercise. We hypothesized that, consistent with greater structural disorganization, FA would

decrease and ADC would increase, 24 h following exercise compared to baseline. We also hypothesized that muscle disruption would correlate with changes in the DT-MRI parameters FA and ADC. A secondary purpose of the study was to examine the potential influence of the biopsy procedure itself on ultrastructural indices of muscle disruption.

## 3.2 METHODS

### 3.2.1 Subjects

Ten healthy active men (23 ± 1 yr; 74 ± 3 kg; 180 ± 1 cm) were recruited for the study. All subjects were habitually engaged in a variety of recreational exercise pursuits but none were trained in any particular sporting event. After being advised of the purpose and potential risks of the study, all subjects provided written, informed consent. The experimental protocol was approved by the Hamilton Health Sciences/Faculty of Health Sciences Research Ethics Board and the St. Joseph's Healthcare Research Ethics Board.

### 3.2.2 Overview of experimental protocol

Subjects initially reported to the laboratory on several occasions in order to become familiar with the experimental procedures and measurement devices. At least one week following familiarization, subjects returned to the laboratory for a series of measurements that constituted pre-exercise baseline testing. The measurements included, in order: a blood draw, a needle biopsy sample from the vastus lateralis muscle of one leg, a MRI scan of both legs, and functional

performance tests of leg strength and vertical jump height. At least one week following baseline testing, subjects performed an acute bout of high-force eccentric exercise using a protocol (Beaton *et al.* 2002a) previously shown to induce ultrastructural evidence of muscle disruption and reduced volitional force-generating capacity. Twenty-four hours following exercise, subjects repeated the battery of measurements that were performed at baseline, in the same order, except that a needle biopsy sample was obtained from the vastus lateralis muscle of each leg. The twenty-four hour post-exercise time point was selected based on previous research showing extensive Z-band disruption at this time (Faulkner *et al.* 1993).

3.2.3 Exercise protocol

The exercise protocol consisted of 300 eccentric actions of the knee extensors performed on an isokinetic dynamomter (Biodex-System 3, Biodex Medical Systems, Inc., NY, USA). The protocol was based on the work of Beaton and colleagues (Beaton *et al.* 2002a) and was divided into 15 sets of 20 repetitions at a speed of 0.52 rad/s with 1 min rest between sets. The protocol was performed twice, once with each leg.

3.2.4 Measurements and analyses

Venous blood draw. Venous blood samples were collected into Vacutainers (Franklin Lakes, New Jersey, USA) that contained no additive. The non-heparinized blood was allowed to clot, then centrifuged and the serum was stored for subsequent analysis of creatine kinase (CK-NAC 2-part liquid reagent

set, Pointe Scientific; Canton, MI, USA).

Needle muscle biopsy sample. During the baseline sample, the area over one thigh was prepared for the extraction of a needle muscle biopsy sample, while during the 24 h post testing; each leg was prepared for the extraction of a needle muscle biopsy sample. Briefly, the lateral portion of the thigh to be biopsied was anaesthetized (1% xylocaine) and a small incision made through the skin and underlying fascia in order to obtain a tissue sample from the vastus lateralis muscle. After removal from the leg, the muscle sample was embedded in optimal cutting temperature (OCT) compound, and cryopreserved in liquid nitrogen-cooled isopentane. All muscle biopsies were analyzed for muscle disruption by assessing Z-band streaming under light microscopy. After initial fixation, the tissue samples were post-fixed in osmium tetroxide, dehydrated in graded baths of ethyl alcohol and embedded in an epoxy resin with fibres oriented longitudinally. Each block was then sectioned (0.5 μm) and stained with toluidine blue. Individual fibres from each longitudinal muscle section were studied under 1000x magnification and examined for extensive Z-band streaming, defined as 3 or more consecutive and/or adjacent disrupted Z-bands (Gibala *et al.* 1995). Muscle disruption was expressed as both per area ($mm^2$) and per the number of fibres analyzed.

Magnetic Resonance Imaging (MRI). The DT-MRI measurements were performed using a 3T MRI scanner (GE Healthcare; Milwaukee, WI, USA) and a standard quadrature knee coil using 15 diffusion encoding directions. The lower

thigh was positioned in the coil in such a way that the distal quarter of the thigh was at the centre of the coil and the biopsy site was located between 2-5cm from the centre. A routine diffusion-weighted spin-echo echo-planar imaging (EPI) pulse sequence was applied to collect a series of axial 2D images through the lower leg of each subject using parameters optimized for skeletal muscle: b= 300 s/mm$^2$, TE=67 s, TR = 6000 s, field of view (FOV) = 20 cm, number of excitations (NEX) = 8, matrix size = 64x64, 4 mm thickness and zero spacing. The acquisition time for each DT-MRI measurement was about 10 min. In addition to the DT-MRI scans, the anatomical scans consisted of a 3-plane localizer (30 s), multi-slice axial 2D STIR (short tau inversion recovery) (5 min), and geometry matched proton density weighted, fat saturated (PD-FS) (5 min) which were required to view the anatomical structure of the lower thigh for region of interest (ROI) analysis and to rule out any gross pathology. All scans were performed on each thigh (total scan time per leg = 40 min) with a brief rest in between. Following image collection, a number of quantitative diffusion parameters were used to characterize molecular diffusion of water. After diagonalization of the diffusion tensor at each voxel, only non-zero elements (i.e. eigenvalues ($\lambda_1$, $\lambda_2$, $\lambda_3$) remain along the diagonal. Each eigenvalue is associated with an eigenvector ($\varepsilon_1$, $\varepsilon_2$, $\varepsilon_3$) describing its spatial orientation. It is assumed the eigenvector associated with the largest eigenvalue ($\lambda_1$) is oriented along the direction of the muscle fibre bundle, while $\lambda_2$ and $\lambda_3$ are perpendicular directions. The diffusion tensor allows calculation of diffusion rotationally invariant indices

(Le Bihan *et al.* 2001). We calculated both FA and ADC which are the most commonly used invariant indices, each providing voxel-wise information describing water diffusivity. ROI analysis of DT-MRI parametric images of individual eigenvalues ($\lambda_1$, $\lambda_2$, $\lambda_3$), FA, and ADC was accomplished using AFNI (Cox 1996). Briefly, ROIs were drawn on high resolution anatomic PD-FS images of vastus lateralis, medialis and intermedius and the mean of each diffusion parameter was calculated.

Leg extensor torque. Isokinetic testing consisted of three maximal concentric contractions performed at 1.05 rad/s. Maximal isokinetic tests were recorded as the highest torque value (Nm) obtained during the three kicking attempts.

Vertical jump height. Vertical jump height was assessed using a Vertec jumping system (Sports Imports, Hilliard Ohio, USA). After peak-standing height was recorded with subjects standing flat-footed with their dominant arm outstretched above their head, subjects were asked to perform three counter-movement vertical jumps. To obtain vertical jump height, the standing height was subtracted from the highest maximal jump height achieved.

Muscle Soreness. Ratings of soreness were assessed using a 10 cm visual analog scale (VAS). Subjects were instructed to indicate a soreness rating by drawing a mark on the line between 0 (not sore at all) and 10 (extreme soreness) while at rest.

### 3.2.5 Physical activity and nutritional controls

Subjects were instructed to maintain their habitual diet over the course of the experiment. Subjects kept a diet record for 24 h prior to and over the course of the baseline measurements and were asked to replicate their pattern of food intake prior to and during the post-exercise measurement period. Subjects were also instructed to perform no physical activity aside from activities of daily living for 48 h prior to baseline measurements and the eccentric exercise trial. Baseline and post-exercise measurements for a given subject were collected at the same time of day.

### 3.2.6 Statistical analyses

Vertical jump, muscle soreness and CK data were analyzed using a 1-factor (time: pre- and post-exercise) repeated-measured analysis of variance (ANOVA). Muscle Z-band streaming data were analyzed using a Friedman non-parametric test with three time points: pre-exercise, post-exercise (same leg as biopsied at rest); post-exercise (opposite leg as biopsied at rest) in an effort to assess the relative impact of the pre-exercise biopsy on ultrastructural measures of muscle disruption. All DT-MRI parameters and isokinetic torque, were analyzed using a 2-factor (time x leg) repeated measures ANOVA. Significance was set at $P<0.05$ and significant main effects or interactions were further analyzed using a Tukey's honestly significant post hoc test. Pearson product moment correlations were used to examine the relationship between muscle disruption measured in biopsy samples, and muscle disruption calculated by DT-

MRI parameter FA. All data are presented as mean ±SE, n=10 unless otherwise stated. Statistical analyses were performed using Sigma Stat 3.5 (Chicago, IL).

## 3.3 RESULTS

### 3.3.1 Performance, muscle soreness and blood data

Isokinetic torque (P=0.004) and vertical jump height (P=0.003) were lower 24 h after exercise compared to baseline (Table 3.1). Muscle soreness (P=0.001; Table 3.1) and serum CK activity (P=0.004; Table 3.1) were higher 24 h post-exercise compared to baseline.

**Table 3.1:** Performance, muscle soreness and blood data

| Variable | Pre | Post |
|---|---|---|
| Isometric (Nm) | 215 ± 19 | 210 ± 15 |
| Isokinetic (Nm) | 195 ± 8.0 | 168 ± 6.5* |
| Soreness (cm) | 0.6 ± 0.9 | 5.6 ± 1.1* |
| Vertical Jump (cm) | 49 ± 1 | 46 ± 1* |
| Creatine Kinase (U/L) | 54 ± 8 | 133 ± 18* |

All values are mean ±SE, n=10. Pre = Pre-exercise. Post = 24 h following exercise that consisted of 300 eccentric actions of the knee extensors. *Pre>Post (P<0.05).

### 3.3.2 Ultrastructural data

Skeletal muscle disruption was higher in both 24 h post-exercise samples compared to baseline, whether expressed per area (P=0.001, Figure 3.1A) or per total number of fibres analyzed (P=0.003, Figure 3.1B). The extent of fibre disruption in the leg that was subjected to the pre-exercise biopsy was numerically higher than in the leg that was not biopsied pre-exercise, but there

was no significant difference between the post-exercise samples. Individual data for all subjects are presented in Figure 3.1.

3.3.3 DT-MRI data

FA was lower in the vastus lateralis 24 h post-exercise compared to baseline (P=0.02, main effect for time, Figure 3.2), whereas ADC was increased (P=0.04, main effect for time; Figure 3.3). Tensor eigenvalues $\lambda_2$ and $\lambda_3$ were both higher 24 h post-exercise compared to baseline (P=0.003 and 0.009, respectively, main effects for time, Figure 3.4) whereas there was no change in tensor eigenvalue $\lambda_1$. Muscle fibre disruption quantified as the number of extensive areas of Z-band streaming per number of fibres analyzed and per sample area was negatively correlated with FA (r= -0.512; P=0.02 and r= -0.453; P=0.04 respectively, Figure 3.5A and 3.5B). There were no exercise-induced changes in FA, ADC or tensor eigenvalues in vastus medialis or vastus intermedius, except for tensor eigenvalue $\lambda_3$ which was higher 24 h post-exercise compared to baseline in the former (0.53 ± 0.01 vs. 0.56 ± 0.01 $x10^{-9}$ $m^2/s$; P=0.009).

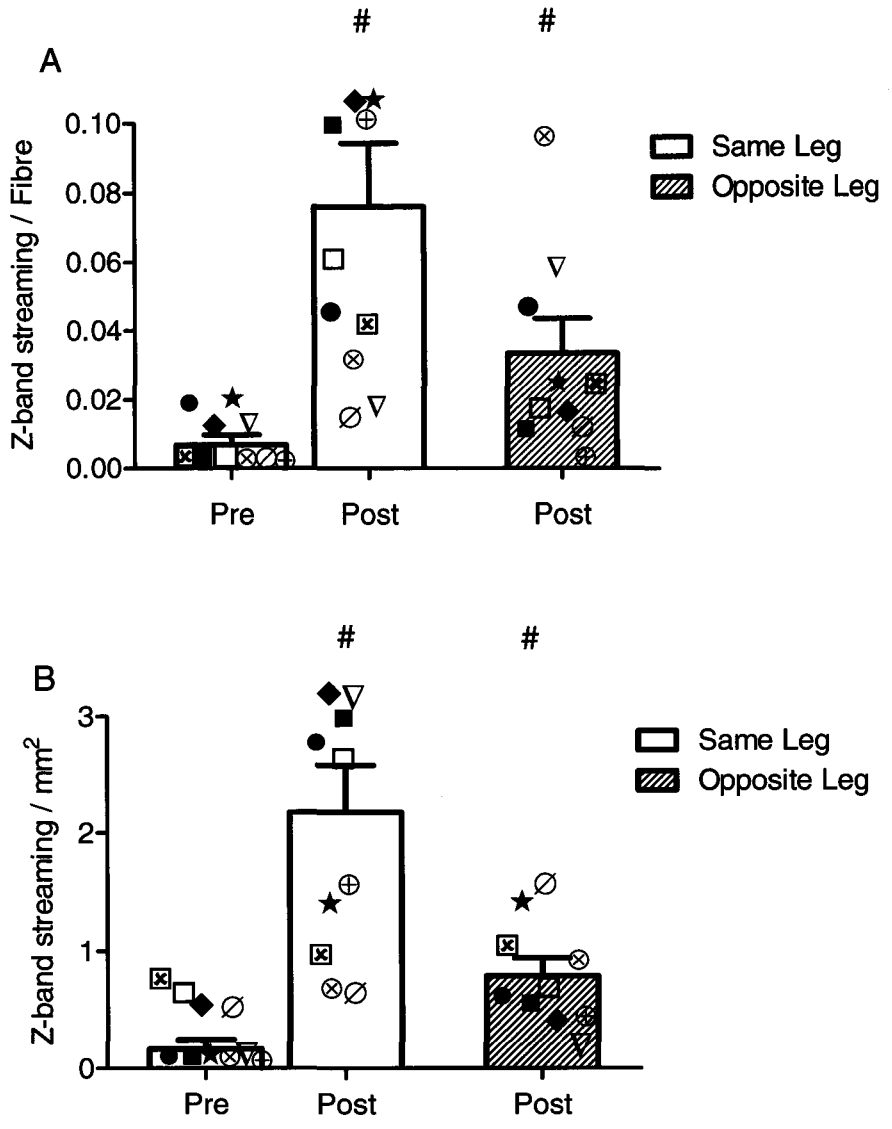



**Figure 3.1:** Individual as well as mean values of ultrastructural changes expressed as disrupted Z-bands per fibre (Panel A) and disrupted Z-bands per area (Panel B) of the vastus lateralis measured before and 24 h after 300 eccentric actions. Values are mean ±SE, n=10. #Significantly different than Pre ($P<0.05$). All other symbols (▲, ■, etc.) are individual data points.

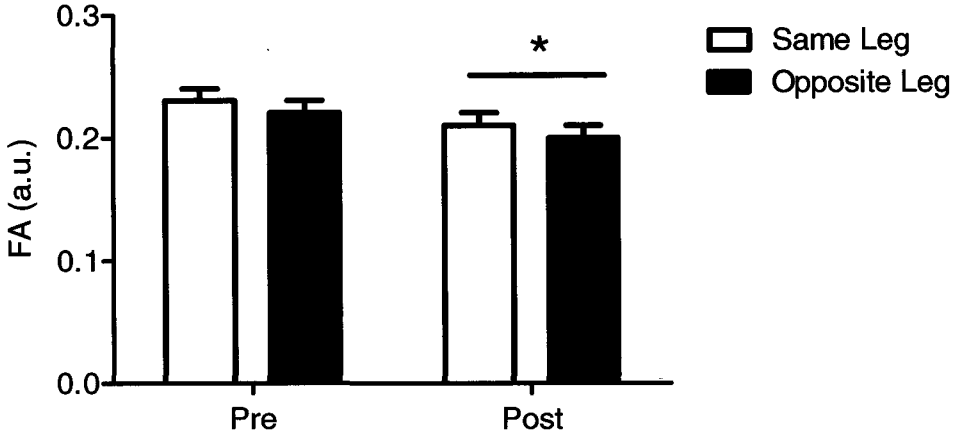



**Figure 3.2:** Fractional anisotropy in arbitrary units (a.u.) measured before and 24 h after 300 eccentric actions of the knee extensors. Values are mean ±SE, n=10. *Main effect for time such that Post>Pre (P<0.05).

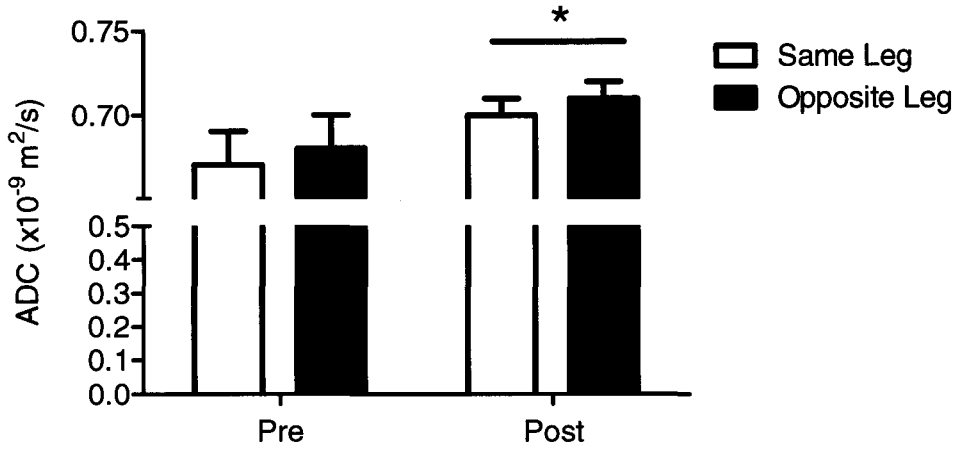
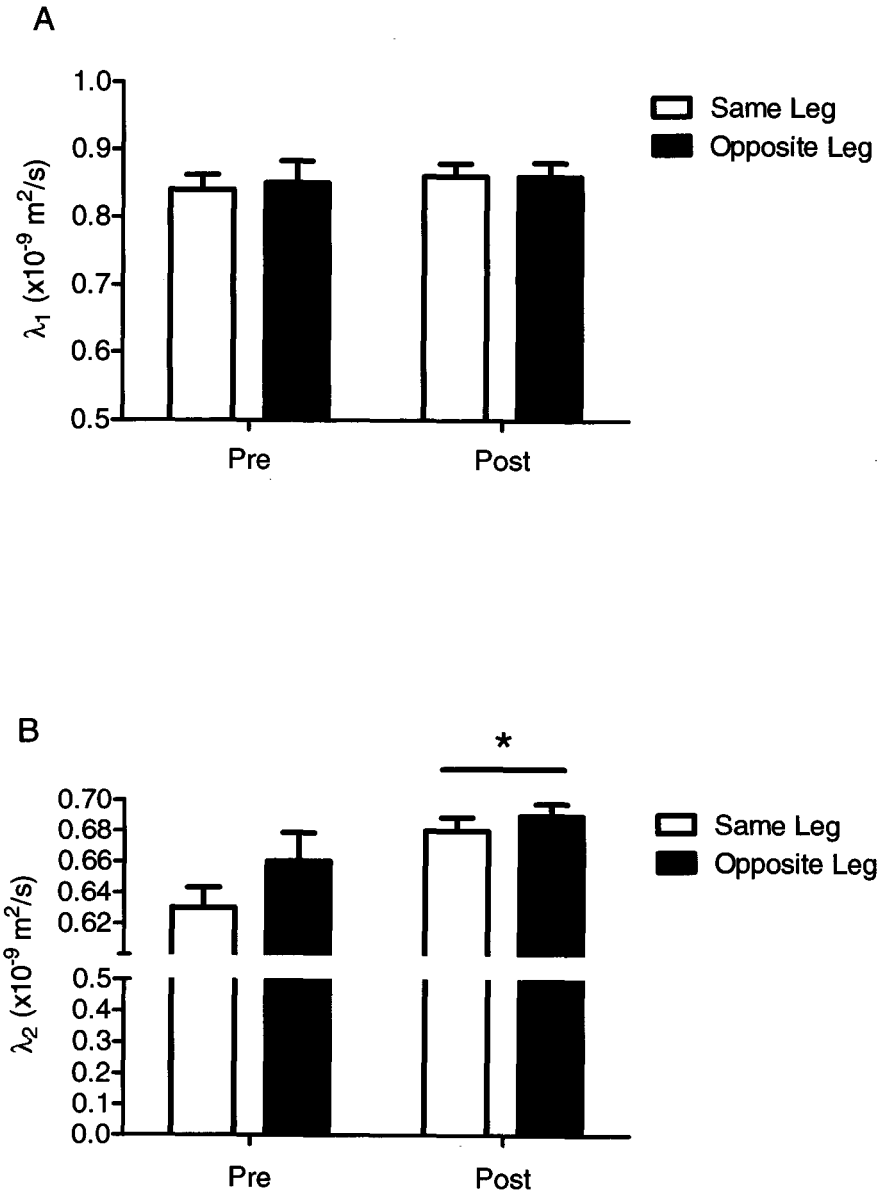



**Figure 3.3:** Apparent diffusion coefficient (ADC) or mean diffusivity measured before and 24 h after 300 eccentric actions of the knee extensors. Values are mean ±SE, n=10. *Main effect for time such that Post>Pre (P<0.05).


A
1.0
0.9
0.8
0.7
0.6
0.5
λ1 (x10-9 m2/s)
Pre
Post
Same Leg
Opposite Leg
B
*
0.70
0.68
0.66
0.64
0.62
0.5
0.4
0.3
0.2
0.1
0.0
λ2 (x10-9 m2/s)
Pre
Post
Same Leg
Opposite Leg

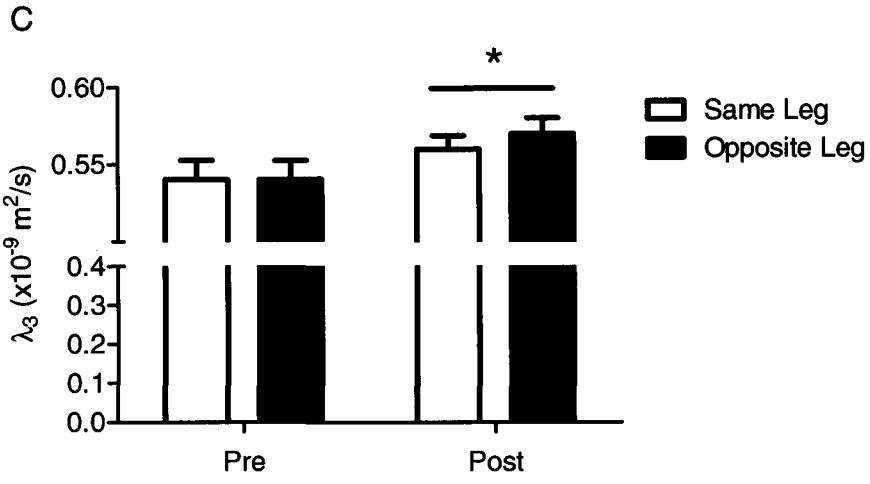
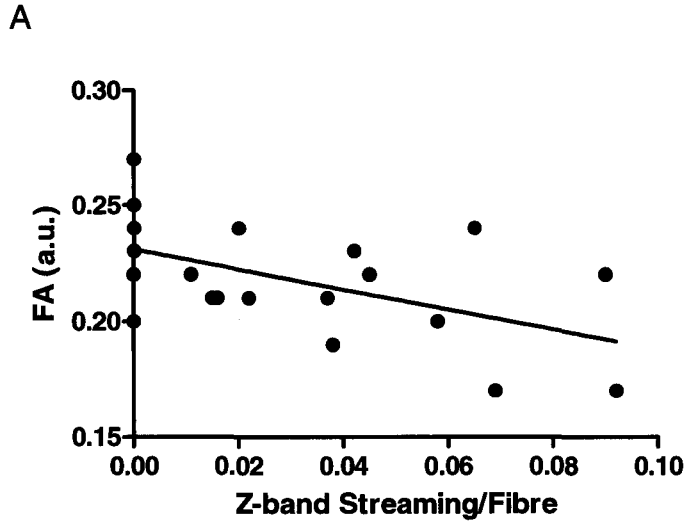



**Figure 3.4:** Eigenvalue $\lambda_1$ (Panel A), $\lambda_2$ (Panel B) and $\lambda_3$ (Panel C) measured before and 24 h after 300 eccentric actions of the knee extensors. Values are mean ±SE, n=10. *Main effect for time such that Post>Pre ($P<0.05$).



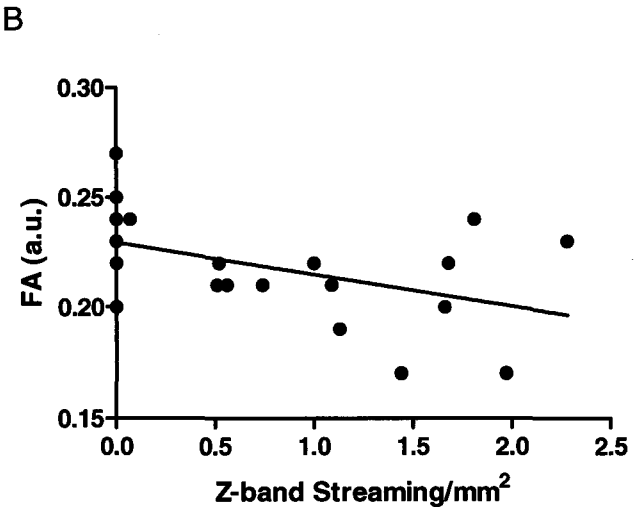



**Figure 3.5:** Negative relationship between muscle disruption expressed as disrupted Z-bands per fibre (Panel A) and disrupted Z-bands per area (Panel B) and FA of the vastus lateralis (r= -0.512; P=0.02 and r= -0.453; P=0.04).

## 3.4 Discussion

The major novel finding from the present study was the potential for DT-MRI to non-invasively detect changes in human skeletal muscle structure following an acute bout of high-force eccentric actions of the quadriceps. FA decreased and ADC increased at 24 h compared to baseline in the vastus lateralis muscle, which reflects changes in fibre geometry consistent with more disorganized structure. Our protocol of 300 eccentric actions of the knee extensors on an isokinetic dynamometer to provoke skeletal muscle disturbances has previously been shown to induce ultrastructural disruption in the muscle as assessed by Z-band streaming using light microscopy (Beaton *et al.* 2002a; Beaton *et al.* 2002c; Raastad *et al.* 2010). We confirmed using needle biopsy

samples that the exercise protocol produced Z-band streaming, as well as changes in other traditional markers of disruption, including plasma CK activity, muscle performance and soreness (Brown *et al.* 1997; Beaton *et al.* 2002a; Lee *et al.* 2002; Raastad *et al.* 2010). Furthermore, correlation analysis revealed a significant negative relationship between muscle disruption expressed as both the number of extensive areas of Z-band streaming per total number of fibres analyzed and per sample area with FA. This relationship suggests that as ultrastructural disruption increases as evaluated by muscle biopsy histology, fibre structure assessed by DT-MRI parameter FA becomes more disorganized.

Previous work that has utilized an MRI sequencing technique to non-invasively assess muscle disruption has been focused on T1 (Nurenberg *et al.* 1992) and T2-mapping (Black & McCully 2008). Like Z-band streaming, these sequencing techniques are subjective as changes in T1 or T2 are not able to detect variations in diffusion measurements when the direction of the gradient pulse is changed (Le Bihan *et al.* 2001). Currently, DT-MRI is considered the only method available to measure water diffusion *in vivo*. This technique is related to the physical process that involves random movement of water molecules within tissue (Brownian motion) and is influenced by the cellular microstructure of the muscle tissue through the presence of hydrophobic cellular membranes. Although DT-MRI has been widely used to study brain white matter and cardiac muscle (Damon *et al.* 2002; Tseng *et al.* 2003), its suitability for assessing human skeletal muscle structure and function has not been extensively explored.

Our current study observed a decrease in FA in the vastus lateralis muscle 24 h following unaccustomed exercise. FA measures the fraction of the "magnitude" that can be ascribed to anisotropic diffusion and varies between 0 (isotropic diffusion) and 1 (infinite anisotropy). The lack of significant FA findings in the vastus intermedius and vastus medialis is not surprising given that FA may not readily divulge subtle muscle changes as it is more of a global mixing of the three eigenvalues. If one eigenvalue is increasing while another is decreasing, the overall FA change is negligible. Given that we were able to not only show a decrease in the stringent calculation of FA in the vastus lateralis, but were also able to correlate this change with muscle fibre disruption as quantified by Z-band streaming, suggests that FA is perhaps one of the best, and most rigorous indicators of muscle disruption when using DT-MRI. In the calf muscles, FA values have also been shown to decrease following either 30 or 60 minutes of isometric contraction by repeated plantar flexion exercise with a gradual return to baseline values after 1 week (Okamoto *et al.* 2008). This study demonstrated that the effects of exercise can alter FA long after the cessation of the exercise protocol, suggesting that some altered muscle structure may persist following unaccustomed exercise. Unfortunately, the study was limited by its small sample size with only two subjects, who each performed different durations of exercise loading (30 and 60 min) and no tensor eigenvalues or ADC values were reported, making comparisons between this and our current study very difficult.

Besides the change in FA, the most common finding in our current study

between the vastus lateralis and v. medialis was the increase from baseline to 24 h post in tensor eigenvalue $\lambda_3$, which is thought to be indicative of the muscle changing its morphology from longitudinal to more spherical in shape (Karampinos *et al.* 2009; Schwenzer *et al.* 2009). A parallel relationship between DT-MRI eigenvectors and symmetry axes of the myocardial architecture has been shown in cardiac tissue (Tseng *et al.* 2003), whereby the first, second and third eigenvectors correspond to the fibre, sheet, and sheet normal directions respectively (Tseng *et al.* 2003). In skeletal muscle, although relationships between the eigenvectors and anatomical structures have been proposed, no study has been able to specifically link each eigenvector to a specific anatomical structure. Instead, focus has been on testing the relationship between DT-MRI parameters and the functional state of the muscle (Galban *et al.* 2004; Deux *et al.* 2008; Okamoto *et al.* 2008; Karampinos *et al.* 2009; Schwenzer *et al.* 2009). Eloquent studies investigating the ability of DT parameters to reflect changes in skeletal muscle architecture have shown that both passive and active muscle shortening produce similar changes in tensor eigenvalues, ADC and FA in the human calf (Deux *et al.* 2008; Schwenzer *et al.* 2009). For example, during dorsi-flexion when the tibialis anterior is either passively shortened (Schwenzer *et al.* 2009) or actively contracted (Deux *et al.* 2008), tensor eigenvalues and ADC increase, while FA decreases in comparison to a passively lengthened joint position, suggesting that changes in DT parameters are related to the current functional state of the muscle. More specifically, as the cross-sectional area of

the muscle fibre increases with muscle shortening and decreases with muscle lengthening, the cell membrane becomes an important barrier for water diffusion as diffusivity changes with altered fibre cross-sectional area. Increased fibre radius due to muscular shortening allows for facilitated diffusion of water in the radial directions, resulting in an increase in tensor eigenvalues $\lambda_2$ and $\lambda_3$. Changes in tensor eigenvalue $\lambda_1$ are generally smaller, or less significant, as anatomically, it has been shown that the first eigenvalue represents diffusion along the main direction of the muscle fibres (Sinha *et al.* 2006; Heemskerk 2007). In comparison to the eigenvalues running in the perpendicular direction ($\lambda_2$ and $\lambda_3$), the primary eigenvalue does not change to the same extent with changes in muscle length, as observed in our current study where there was no change in the magnitude of diffusion in tensor eigenvalue $\lambda_1$. With respect to tensor eigenvalues λ's 2 and 3, work by Galban and colleagues (Galban *et al.* 2004) in the human calf have shown a strong correlation between the tensor eigenvalue $\lambda_3$ and the physiological cross-sectional area of a muscle, which has been mathematically shown to be related to the average fibre radius of the muscle, which in turn is proportional to the maximum muscle force. This observation coincides well with the theoretical assumption of increased water diffusion within the muscle tissue due to muscle tears (Hough 1902) as observed in the current study by the increase in the perpendicularly ordered tensor eigenvalues $\lambda_2$ and $\lambda_3$. Additionally, our current study found a positive relationship between tensor eigenvalue $\lambda_3$ and plasma CK, an indirect indicator of cell

membrane "leakage" further supporting the notion that changes in $\lambda_3$ may indicate structural disruption.

Although our observed changes in tensor eigenvalues from pre to 24 h post unaccustomed exercise were not as large as compared to Zaraiskaya and colleagues (2006), their study was a between-subject design comparing healthy subjects to subjects presenting with severe athletic injuries (hematomas etc.). Furthermore, in the current study, the entire cross-section of the vastus lateralis was selected as the region of interest (ROI), which differs from previous work (Zaraiskaya *et al.* 2006) in which the ROI was selected from the damaged area only, thus favouring relatively high tensor eigenvalues and decreased FA as compared to healthy controls. As we still detected differences after selecting an ROI of the entire muscle cross-section instead of favouring selection of damaged areas, this provides further support for the sensitivity of DT-MRI to detect exercise-induced changes in skeletal muscle.

In addition to determining whether we could non-invasively detect structural changes following unaccustomed exercise using DT-MRI, we investigated the potential influence of the biopsy procedure itself on estimates of exercise-induced changes in muscle ultrastructure. It has been suggested that the invasive nature of the biopsy technique could induce ultrastructural damage that is unrelated to exercise *per se* (Malm *et al.* 2000).We designed our study to measure any potential biopsy induced-structural changes by biopsying only one leg during the baseline trial and both legs following the bilateral leg-kicking

exercise. With this design, any residual effects from the first biopsy procedure during the baseline trial should be evident in the second biopsy taken from the *same* leg 24 h following the eccentric exercise and observed by the difference in muscle disruption between the leg that was only biopsied once (post-only) and the leg that was biopsied twice (baseline/post). Regardless of whether the leg was biopsied once or twice, Z-band streaming increased when compared to the baseline sample. In an absolute sense, Z-band streaming was lower in the leg that did not undergo the baseline biopsy, but this was not significantly different from the other leg post-exercise. Although there was a high-degree of variability between the leg that was biopsied once versus twice, Beaton and colleagues (2002b) performed a similar eccentric protocol on each leg one week apart to assess biopsy variability within and between legs. The within leg coefficient of variation that was biopsied twice 24 h following the leg-kicking bout was 41 ± 30%, and the between-leg coefficient of variation was 57 and 68 ± 36% (Beaton *et al.* 2002b), which falls well within the variation found in the present study. This high variability found in the Z-band streaming response may potentially be due to the small sample provided by the biopsy or from the variability in the response of eccentric exercise on the involved muscles (Nurenberg *et al.* 1992). Nurenberg and colleagues (1992) correlated T2 –signal intensity images with Z-band streaming after a downhill running protocol in nine subjects and found that the injured muscles varied between subjects despite the controlled exercise. Overall in the current study, calculated DT parameters were not different between the leg

that was biopsied only once, versus twice, supporting the muscle biopsy technique as a structural indicator of muscle disruption however, the high degree of variability should be taken into consideration when evaluating study results.

In summary, the present study examined the potential for DT-MRI to non-invasively detect exercise-induced skeletal muscle disruption following an acute bout of high-force eccentric actions of the quadriceps. In addition to increases in CK activity, muscle soreness, and a decrease in muscle performance, analyses of needle biopsy samples showed increased Z-band streaming. Diffusion parameters calculated using DT-MRI showed that FA decreased and ADC increased 24 h post exercise compared to baseline. There was a significant negative relationship between Z-band streaming and FA, such that greater fibre disruption was associated with more disorganized structure measured by DT-MRI. Overall, these data suggest that DT-MRI is a sensitive tool to non-invasively detect exercise-induced changes in muscle structure, however much more work using DT-MRI is warranted. Future work should examine the time course of changes in diffusion parameters following exercise and whether DT-MRI can be applied to detect potential changes in skeletal muscle following other types of exercise that more closely simulate normal athletic activity.

## 3.5 References

Basser, PJ and DK Jones. 2002. Diffusion-tensor MRI: Theory, experimental design and data analysis—a technical review. *NMR Biomedicine* 15: 456-467.

Basser, PJ, J Mattiello and D Le Bihan. 1994. MR diffusion tensor spectroscopy and imaging. *Biophysical Journal* 66: 259-267.

Beaton, LJ, DA Allan, MA Tarnopolsky, PM Tiidus and SM Phillips. 2002a. Contraction-induced muscle damage is unaffected by vitamin E supplementation. *Medicine & Science in Sports & Exercise* 34 (5): 798-805.

Beaton, LJ, MA Tarnopolsky and SM Phillips. 2002b. Variability in estimating eccentric contraction-induced muscle damage and inflammation in humans. *Canadian Journal of Applied Physiology* 27 (5): 516-526.

Beaton, LJ, MA Tarnopolsky and SM Phillips. 2002c. Contraction-induced muscle damage in humans following calcium channel blocker administration. *Journal of Physiology* 544 (3): 849-859.

Black , CD and KK McCully. 2008. Muscle injury after repeated bouts of voluntary and electrically stimulated exercise. *Medicine & Science in Sports & Exercise* 40 (9): 1605-1615.

Brown, SJ, RB Child, H Day and AE Donnelly. 1997. Indices of skeletal muscle damage and connective tissue breakdown following eccentric muscle contractions. *European Journal of Applied Physiology* 75: 369-374.

Clarkson, PM and MJ Hubal. 2002. Exercise-induced muscle damage in humans. *American Journal of Physical Medicine and Rehabilitation* 81 (11 (Suppl)): S52-S69.

Cleveland, GG, DC Chang, CF Hazlewood and HE Rorschach. 1976. Nuclear magnetic resonance measurement of skeletal muscle; anisotropy of the diffusion coefficient *Biophysical Journal* 16: 1043-1053.

Cox, RW. 1996. AFNI: Software for analysis and visualization of functional magnetic resonance neuroimages. *Computers and Biomedical Research* 29 (3): 162-173.

Damon, BM, Z Ding, AW Anderson, AS Freyer and JC Gore. 2002. Validation of diffusion tensor MRI-based muscle fiber tracking. *Magnetic Resonance Medicine* 48: 97-104.

Deux, JF, P Malzy, N Paragios, G Bassez, A Luciani, P Zerbib, F Roudot-Thoraval, A Vignaud, H Kobeiter and A Rahmouni. 2008. Assessment of calf muscle contraction by diffusion tensor imaging. *European Radiology* 18 (10): 2303-2310.

Faulkner, JA, SV Brooks and JA Opiteck. 1993. Injury to skeletal muscle fibers during contractions: Conditions of occurrence and prevention. *Physical Therapy* 73 (12): 911-921.

Friden, J and RL Lieber. 2001. Eccentric exercise-induced injuries to contractile and cytoskeletal muscle fibre components. *Acta Physiologica Scandinavia* 171: 321-326.

Friden, J, M Sjostrom and B Ekblom. 1983. Myofibrillar damage following intense eccentric exercise in man. *International Journal of Sports Medicine* 4: 170-176.

Galban, CJ, S Maderwald, K Uffmann, A de Greiff and ME Ladd. 2004. Diffusive sensitivity to muscle architecture: A magnetic resonance diffusion tensor imaging study of the human calf. *European Journal of Applied Physiology* 93: 253-262.

Gibala, MJ, JD MacDougall and MA Tarnopolsky. 1995. Changes in human skeletal muscle ultrastructure and force production after acute resistance exercise. *Journal of Applied Physiology* 78: 702-708.

Heemskerk, A.M., and Damon, B.M. 2007. Diffusion tensor MRI assessment of skeletal muscle architecture *Current Medical Imaging Reviews* 3 (3): 152-160.

Hough, T. 1902. Ergographic studies in muscular soreness. *American Journal of Physiology* 7: 76-92.

Karampinos, DC, KF King, BP Sutton and JG Georgiadis. 2009. Myofiber ellipticity as an explanation for transverse asymmetry of skeletal muscle diffusion MRI in vivo signal. *Annals of Biomedical Engineering* 37 (12): 2532-2546.

Le Bihan, D, JF Mangin, C Poupon, CA Clark, S Pappata, N Molko and H. Chabriat. 2001. Diffusion tensor imaging: Concepts and applications. *Journal of Magnetic Resonance Imaging* 13: 534-546.

Lee, J, AH Goldfarb, MH Rescino, S Hegde, S Patrick and K Apperson. 2002. Eccentric exercise effect on blood oxidative-stress markers and delayed onset of muscle soreness. *Medicine & Science in Sports & Exercise* 34 (3): 443-448.

Malm, C, P Nyberg and M Engstrom. 2000. Immunological changes in human skeletal muscle and blood after eccentric exercise and multiple biopsies. *Journal of Physiology* 529: 243-262.

Newham, DJ, G McPhail and KR Mills. 1983. Ultrastructural changes after concentric and eccentric contractions of human muscle. *Journal of Neurological Sciences* 61: 109-122.

Nurenberg, P, CJ Giddings, J Stray-Gundersen, JL Fleckenstein, WJ Gonyea and RM Peshock. 1992. MR imaging-guided muscle biopsy for correlation of increased signal intensity with ultrastructural change and delayed-onset muscle soreness after exercise. *Radiology* 184: 865-869.

Okamoto, Y, A Kunimatsu, S Miki, S Masashi, M Niitsu and M Minami. 2008. Fractional anisotropy values of calf muscles in normative state after exercise: Preliminary results. *Magnetic Resonance in Medical Sciences* 7 (3): 157-162.

Raastad, R, S Gylterud Owe, G Paulsen, D Enns, K Overgaard, R Crameri, S Kiil, A Belcastro, L Bergersen and J Hallen. 2010. Changes in calpain activity, muscle structure, and function after eccentric exercise. *Medicine & Science in Sports & Exercise* 42 (1): 86-95.

Saupe, N, LM White, MS Sussman, A Kassner, G Tomlinson and MD Noseworthy. 2008. Diffusion tensor magnetic resonance imaging of the human calf comparison between 1.5t and 3.0t - preliminary results. *Investigative Radiology* 43: 612-618.

Schwenzer, NF, G Steidle, P Martirosian, C Schraml, F Springer, CD Claussen and F Schick. 2009. Diffusion tensor imaging of the human calf muscle: Distinct changes in fractional anisotropy and mean diffusion due to passive muscle shortening and stretching. *NMR in Biomedicine* 22: 1047-1053.

Sinha, S, U Sinha and VR Edgerton. 2006. In vivo diffusion tensor imaging of the human calf muscle. *Journal of Magnetic Resonance Imaging* 24: 182-190.

Tseng, WY , VJ Wedeen, TG Reese, RN Smith and EF Halpern. 2003. Diffusion tensor MRI of myocardial fibers and sheets: Correspondence with visible cut-face texture. *Journal of Magnetic Resonance Imaging* 17 (1): 31-42.

Yamada K, Sakai K, Akazawa K, Yuen S, Nishimura T. 2009. MR tractography: A review of its clinical applications. *Magnetic Resonance in Medical Sciences* 8 (4): 165-174.

Zaraiskaya, T, D Kumbhare and MD Noseworthy. 2006. Diffusion tensor imaging in evaluation of human skeletal muscle injury. *Journal of Magnetic Resonance Imaging* 24 (2): 402-408.

# CHAPTER 4

## USE OF DIFFUSION TENSOR MRI FOR ASSESSMENT OF MUSCULOSKELETAL STRUCTURE FOLLOWING AN ACUTE BOUT OF DOWNHILL RUNNING

(Manuscript to be submitted for publication)

### 4.1 INTRODUCTION

Strenuous unaccustomed exercise, especially activities that involve a large eccentric component, produce ultrastructural changes in skeletal muscle that are often associated with a temporary decline in force-generating capacity (Clarkson & Hubal 2002). Traditionally, the only direct ultrastructural assessment of skeletal muscle disruption following a bout of unaccustomed exercise is from a muscle biopsy sample. Besides the various methodological limitations and assumptions however, obtaining athletes to participate in these types of biopsy studies often proves difficult due to the invasive nature of this technique. Various other indirect and less invasive measurements exist including the measurement of blood enzymes, muscle strength and perceived muscle soreness but these have all been shown to correlate poorly with ultrastructural results (Jones *et al.* 1986; Fielding *et al.* 1993).

Diffusion tensor magnetic resonance imaging (DT-MRI) is a useful technique for the non-invasive structural characterization of various anisotropic tissues (Basser *et al.* 1994). Initially used for brain neuronal fibre tracking (Pierpaoli & Basser 1996), DT-MRI has proven to be a powerful, non-invasive

tool for providing information about tissue characteristics and pathology. The technique is based on the physical process of Brownian motion that involves the random movement of water molecules within tissue. Due to the presence of cellular membranes and other structures that restrict molecular diffusion, water movement exhibits preferential diffusion along certain directions within organized tissue. For example, water molecules would diffuse more rapidly along the length of a skeletal muscle fibre compared with its perpendicular direction. It is this directional dependence that is referred to as anisotropic diffusion (Zaraiskaya *et al.* 2006) and diffusion anisotropy can be exploited to gain information about tissue organization at a microscopic level (Le Bihan *et al.* 2001). DT-MRI involves the estimation of the within-voxel diffusion tensor using a series of diffusion-weighted images and once estimated the eigenvalues ($\lambda_1$, $\lambda_2$, $\lambda_3$) and eigenvectors ($\varepsilon_1$, $\varepsilon_2$, $\varepsilon_3$) are calculated providing information concerning the magnitude of diffusivity along the three orthogonal directions (Basser & Jones 2002). These eigenparameters are independent of tissue orientation in the laboratory frame of reference and can provide information about local fine tissue structure and anatomy.

Only a few studies have been reported on DT-MRI applications involving skeletal muscle tissue. The limited work has shown the feasibility of *in vivo* fibre tracking of muscle fibres (Sinha *et al.* 2006), the relationship between diffusive and architectural properties of muscle tissue (Galban *et al.* 2004; Karampinos *et al.* 2009; Schwenzer *et al.* 2009) and the evaluation of muscle injury based on

disturbed muscle structure in injured calf muscles (Zaraiskaya *et al.* 2006). We recently demonstrated that DT-MRI could be applied to evaluate structural changes induced by a strenuous bout of unaccustomed knee-extensor exercise (Cermak *et al.* 2009). Our study included needle biopsy sampling to confirm ultrastructural evidence of fibre disruption including an increase in Z-band streaming 24 hours post-exercise (4). DT-MRI revealed a decrease in fractional anisotropy (FA) in the vastus lateralis 24 hours after exercise compared to baseline, suggesting that the structure was more 'disorganized' or less anisotropic in nature. Furthermore, we found a negative relationship between increased Z-band streaming and decreased FA, suggesting that as histological evidence of fibre disruption increases, muscle organization decreases as calculated through DT-MRI parameter FA. Collectively, these studies indicate that DT-MRI could play an important role in understanding skeletal muscle organization and how unaccustomed exercise may alter structural pathology. Further research is warranted however, specifically concerning whether structural disruption can be observed using DT-MRI following a more practical, real-world exercise setting.

The primary purpose of the present study was to determine whether DT-MRI could be applied to non-invasively detect ultrastructural changes in skeletal muscle after an acute bout of downhill running. The exercise protocol was similar to that which has previously been shown to induce ultrastructural evidence of skeletal muscle disruption and an acute decrease in force-generating

capacity (Fielding *et al.* 1993; Feasson *et al.* 2002). We tested the hypothesis that 24 h following downhill running, diffusion would increase in the knee extensor muscles (vastus lateralis, v.intermedius and v.medialis), relative to baseline which is suggestive of altered tissue architecture. In addition to DT-MRI images, we also quantified changes in blood, performance and perceptual markers associated with skeletal muscle disruption before and 24 h after exercise.

## 4.2 METHODS

### 4.2.1 Subjects

Ten healthy active men (25 ± 1 y; 74 ± 4 kg; 175 ± 2 cm) were recruited for the study. All subjects were habitually engaged in a variety of recreational exercise pursuits but none were trained in any particular sporting event (Table 4.1). Their peak oxygen uptake ($VO_2$peak), determined using on-line gas collection system (Moxus Modular $VO_2$ System, AEI Technologies, Inc., Pittsburgh, PA) during a step-wise test to exhaustion on a treadmill (Life Fitness 95Ti, Schiller Park, IL), was 52 ± 3 $ml \cdot kg^{-1} \cdot min^{-1}$. After being advised of the purpose and potential risks of the study, all subjects provided written, informed consent. The experimental protocol was approved by the Hamilton Health Sciences/Faculty of Health Sciences Research Ethics Board and the St. Joseph's Healthcare Research Ethics Board.

**Table 4.1:** Characteristics of the subjects' physical activity levels

| Subject No. | Sport | Activity Level | Hours per wk |
|---|---|---|---|
| 1 | Squash | moderate | 2 |
| 2 | Swimming | moderate | 2 |
| 3 | Squash, resistance training | moderate | 2, 1 |
| 4 | Cycling | low | 1 |
| 5 | Cycling, squash | moderate | 5, 1 |
| 6 | Walking | low | 1 |
| 7 | Squash | low | 1 |
| 8 | Baseball, resistance training | moderate | 2, 3 |
| 9 | Squash, resistance training | moderate | 2, 3 |
| 10 | Running | low | 1 |

Physical activity levels of each subject during an average week. Subject No. = Subject number. Activity levels: low, less than two exercise bouts per week; moderate, two to four exercise bouts per week; high, more than four exercise bouts per week. Hours per wk = Average number of hours playing each particular sport/exercise per week.

### 4.2.2 Overview of experimental protocol

Subjects initially reported to our laboratory for several pre-study visits in order to become familiar with the experimental procedures and measurement devices. At least one week following familiarization, subjects returned to the laboratory for a series of measurements that constituted pre-exercise baseline testing. The measurements included, in order: a blood draw, MRI scan of both legs, and functional performance tests of leg strength. At least one week following baseline testing, subjects performed an acute bout of downhill running previously shown to induce ultrastructural evidence of muscle disruption and reduced volitional force-generating capacity (Fielding *et al.* 1993). Twenty-four hours following exercise, subjects repeated the battery of measurements that

were performed at baseline, in the same order.

4.2.3 Exercise protocol

The exercise protocol consisted of a 45-minute downhill run on a treadmill (Life Fitness 95Ti, Schiller Park, IL) at negative 10 degrees. The subjects ran at a speed that elicited 70-75% of their maximal heart rate (HR) which was calculated by using the following equation:

(Maximal HR – resting HR) x 70% + resting HR

4.2.4 Measurements and analyses

Venous blood draw. Venous blood samples were collected into Vacutainers (Franklin Lakes, New Jersey, USA) that contained no additive. The non-heparinized blood was allowed to clot, centrifuged, and the serum was stored for subsequent analysis of creatine kinase (CK-NAC 2-part liquid reagent set, Pointe Scientific; Canton, MI, USA).

Magnetic Resonance Imaging (MRI). The MRI measurements were performed using a 3T MRI scanner (GE Healthcare; Milwaukee, WI, USA) and standard transmit/receive quadrature knee coil. The lower thigh was positioned in the coil in such a way that the distal quarter of the thigh was at the centre of the coil. A routine diffusion-weighted spin-echo echo-planar imaging (EPI) pulse sequence was applied to collect a series of axial 2D images through the lower leg of each subject using parameters optimized for skeletal muscle: b= 300 s/mm$^2$, TE=67ms, TR = 6000ms, field of view (FOV) = 20 cm, number of excitations (NEX) = 4, matrix size = 64x64, 4 mm thickness and zero spacing, 15

diffusion encoding directions. The acquisition time for each DT-MRI measurement was about 10 min. In addition to the DT-MRI scans, anatomical scans consisting of geometry matched multislice axial 2D STIR (short tau inversion recovery) (5 min), and proton density weighted, fat saturated (PD-FS) (5 min) were acquired to rule out any gross pathology (Figure 4.1) and for region of interest (ROI) analysis. All scans were performed on each leg (total scan time per leg = 40 min) with a brief rest in between.

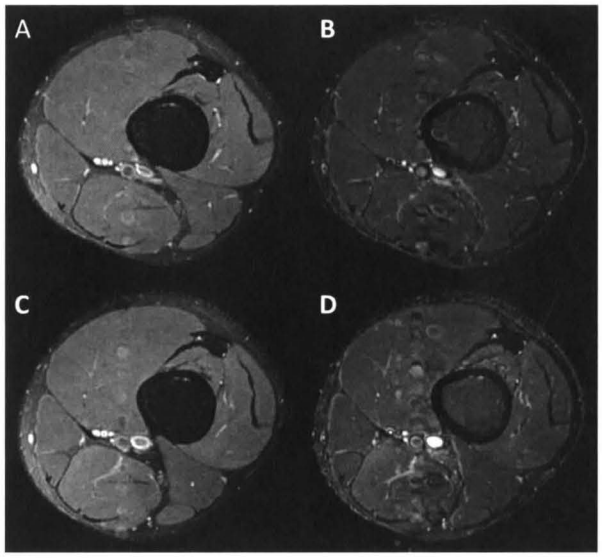



**Figure 4.1:** Sample of anatomical images from 1 subject pre and post. No areas of disruption are apparent when using PD-FS or STIR.

A = proton density weighted, fat-saturated (PD-FS) Pre
B = short tau inversion recovery (STIR) Pre
C = proton density weighted, fat-saturated (PD-FS) Post
D = short tau inversion recovery (STIR) Post

After the images were collected, a number of quantitative diffusion

parameters were used to characterize molecular diffusion of water. After diagonalization of the diffusion tensor, at each voxel, only non-zero elements (i.e. eigenvalues $\lambda_1$, $\lambda_2$, $\lambda_3$) remain along the diagonal. Each eigenvalue is associated with an eigenvector ($\varepsilon_1$, $\varepsilon_2$, $\varepsilon_3$) describing its spatial orientation. It is assumed the eigenvector associated with the largest eigenvalue ($\lambda_1$) is oriented along the direction of the muscle fibre bundle, while $\lambda_2$ and $\lambda_3$ are perpendicular directions. The diffusion tensor allows calculation of diffusion rotationally invariant indices (Le Bihan *et al.* 2001). We calculated both FA, and the apparent diffusion coefficients (ADC) which are the most commonly used invariant indices, each providing voxel-wise information describing water diffusivity. ROI analysis of DT-MRI parametric images of individual eigenvalues ($\lambda_1$, $\lambda_2$, $\lambda_3$), FA, and ADC was accomplished using AFNI (Cox 1996). Briefly, ROIs were drawn on high resolution anatomic PD-FS images of the vastus lateralis, medialis and intermedius and the mean of each diffusion parameter was calculated.

Leg extensor torque. Isometric testing consisted of three maximal isometric contractions performed at 1.57 radians (0 radians = fully extended leg) with 5 s rest between each contraction. Isokinetic testing consisted of three maximal concentric contractions performed at 1.05 rad/s. Maximal isometric and isokinetic tests were recorded as the highest torque value (Nm) obtained during the three kicking attempts.

Muscle Soreness. Ratings of soreness were assessed using a 10 cm visual analog scale (VAS). Subjects were instructed to indicate a soreness rating

by drawing a mark on the line between 0 (not sore at all) and 10 (extreme soreness) while at rest.

4.2.5 Physical activity and nutritional controls

Subjects were instructed to maintain their habitual diet over the course of the experiment. Subjects kept a diet record for 24 h prior to and over the course of the baseline measurements and were asked to replicate the pattern of food intake prior to and during the post-exercise measurement period. Subjects were also instructed to refrain from physical activity, aside from activities of daily living, for 48 h prior to baseline measurements and the downhill running exercise trial. Baseline and post-exercise measurements for a given subject were collected at the same time of day.

4.2.6 Statistical analyses

Muscle soreness and blood data were analyzed using a 1-factor (time: pre- and post-exercise) repeated-measured analysis of variance (rmANOVA). All DT-MRI data, isometric and isokinetic torque, were analyzed using a 2-factor (time x leg) repeated measures ANOVA. Significance was set at $P<0.05$ and significant main effects or interactions were further analyzed using a Tukey honestly significant post hoc test. All data are presented as mean ± standard error (SE), n=10 unless otherwise stated. Statistical analyses were performed using Sigma Stat 3.5 (Chicago, IL).

## 4.3 RESULTS

### 4.3.1 Performance, muscle soreness and blood data

Isometric (P=0.006) and isokinetic torque (P=0.036) were lower 24 h after exercise compared to baseline (Table 4.2). Muscle soreness (P<0.001; Table 4.2) and serum CK activity (P=0.005; Figure 4.2) were higher 24 h post-exercise compared to baseline.

**Table 4.2:** Performance data

| Variable | Pre | Post |
|---|---|---|
| Isometric (Nm) | 238 ± 12 | 209 ± 11* |
| Isokinetic (Nm) | 194 ± 7.7 | 178 ± 5.3* |
| Soreness (cm) | 0.6 ± 0.1 | 5.2 ± 0.7* |

All values are mean ±SE, n=10. Pre = Pre-exercise. Post = 24 h following exercise that consisted of 45 minutes of downhill running (-10 degrees) at 70% of maximal heart rate. *P<0.05 vs. Pre.

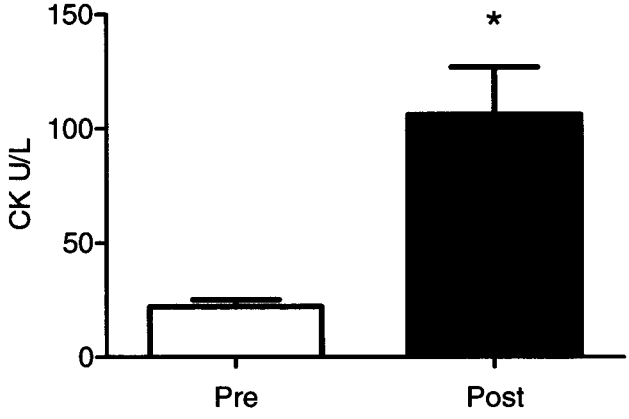



**Figure 4.2**: Serum creatine kinase activity measured before and 24 h after exercise that consisted of 45 minutes of downhill running (-10 degrees) at 70% of maximal heart rate. Values are mean ±SE, n=10. * P<0.05 vs. Pre.

### 4.3.2 DT MRI data

In the vastus lateralis, ADC or mean diffusivity increased 24 h post-exercise compared to baseline ($P<0.05$; Figure 4.3). There was also a statistically significant increase in tensor eigenvalues $\lambda_2$ and $\lambda_3$, indicating an increased diffusion in those two directions ($P<0.05$; Figure 4.4) while no difference was detected for $\lambda_1$ (Figure 4.4). In the vastus intermedius, ADC ($P<0.05$; Figure 4.5) and tensor eigenvalue $\lambda_3$ increased 24 h post-exercise compared to baseline ($P<0.05$; Figure 4.6). In the vastus medialis, ADC ($P<0.05$; Figure 4.7) and tensor eigenvalues $\lambda_1$ and $\lambda_3$ increased 24 h post-exercise compared to baseline ($P<0.05$; Figure 4.8). No other statistical differences were found. No ultrastructural changes were evident when viewing standard anatomical MR imaging (Figure 4.1).

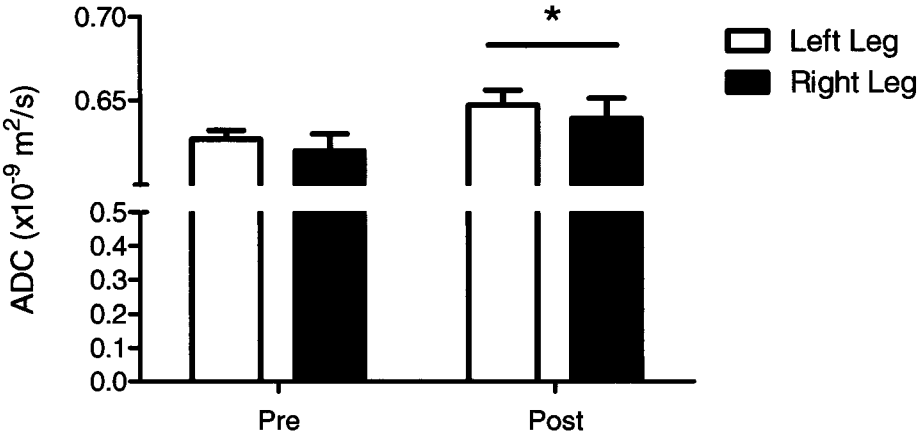
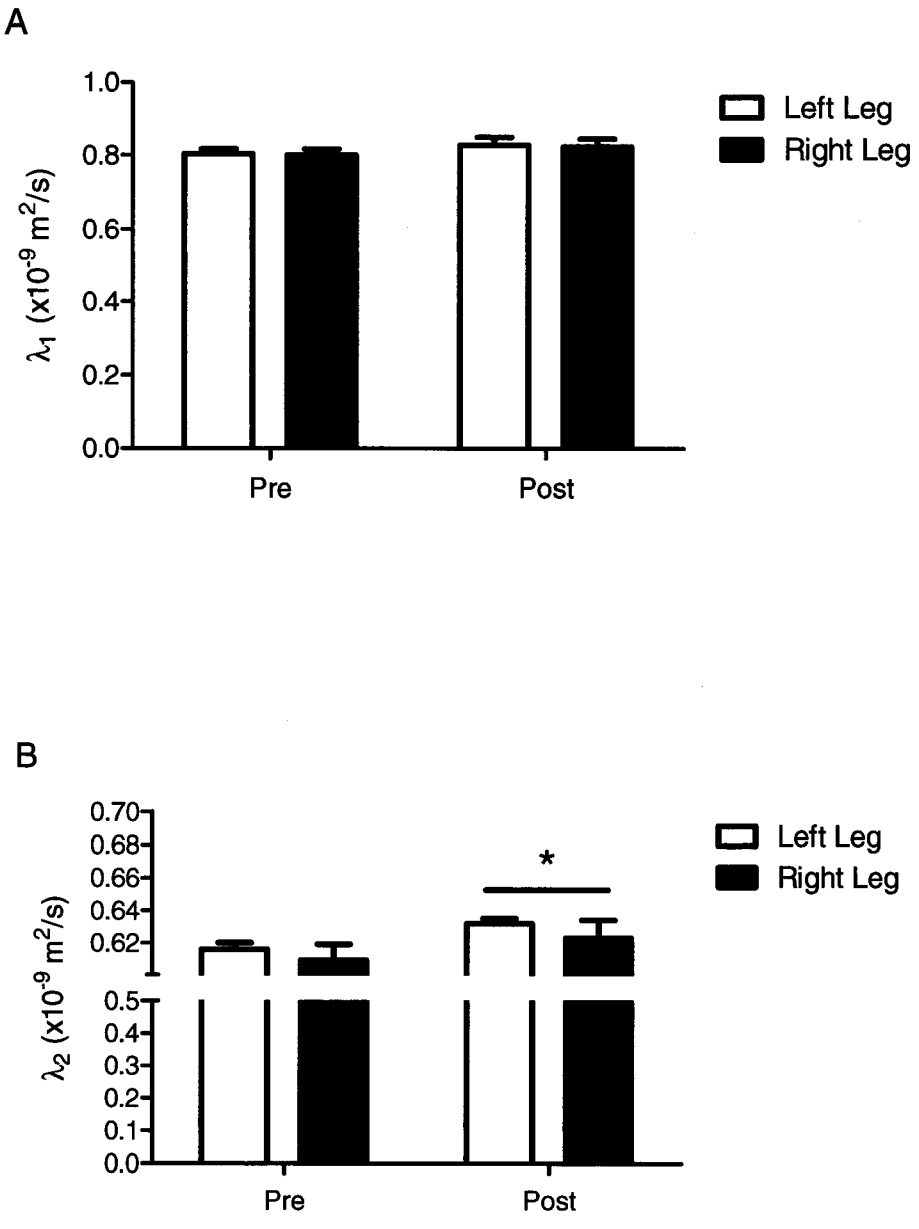



**Figure 4.3**: Apparent diffusion coefficient (ADC) of the vastus lateralis expressing the mean diffusion measured before and 24 h after 45 min of downhill running (-10 degrees) at 70% of maximal heart rate. Values are mean ±SE, n=10. *Main effect for time such that Post>Pre ($P<0.05$).


A
1.0
0.8
0.6
0.4
0.2
0.0
λ1 (x10-9 m2/s)
Pre
Post
Left Leg
Right Leg
B
0.70
0.68
0.66
0.64
0.62
0.5
0.4
0.3
0.2
0.1
0.0
λ2 (x10-9 m2/s)
*
Pre
Post
Left Leg
Right Leg

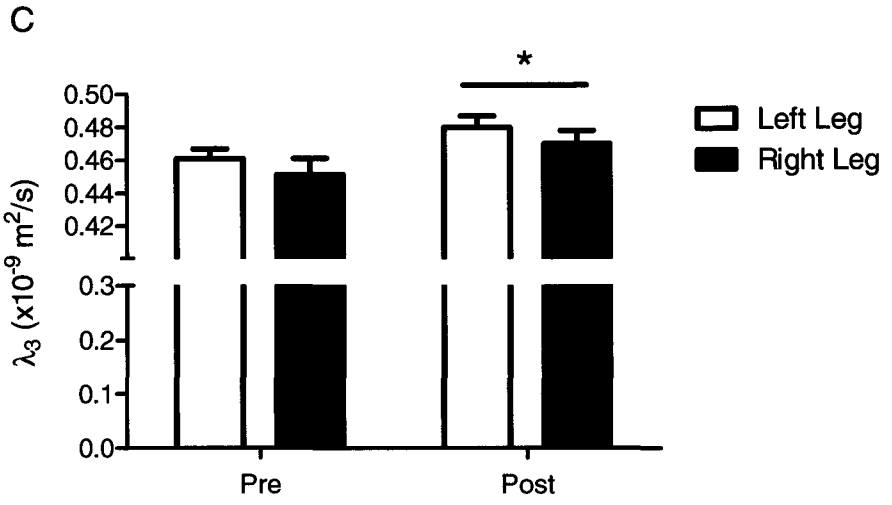



**Figure 4.4**: Tensor eigenvalues $\lambda_1$ (Panel A), $\lambda_2$ (Panel B) and $\lambda_3$ (Panel C) of the vastus lateralis measured before and 24 h after 45 min of downhill running (-10 degrees) at 70% of maximal heart rate. Values are mean ±SE, n=10. *Main effect for time such that Post>Pre (P<0.05).

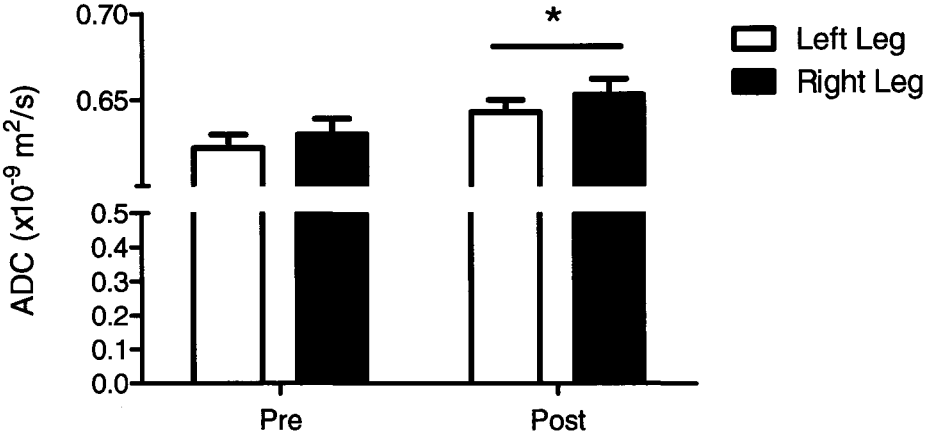
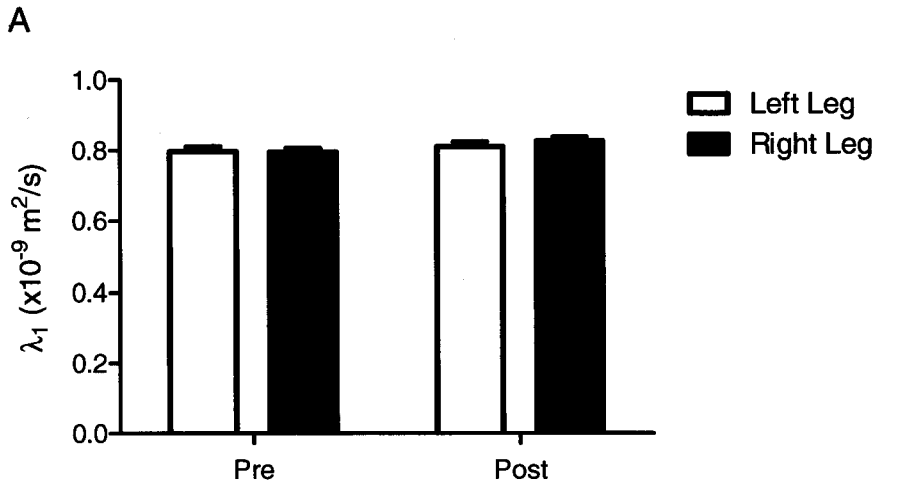
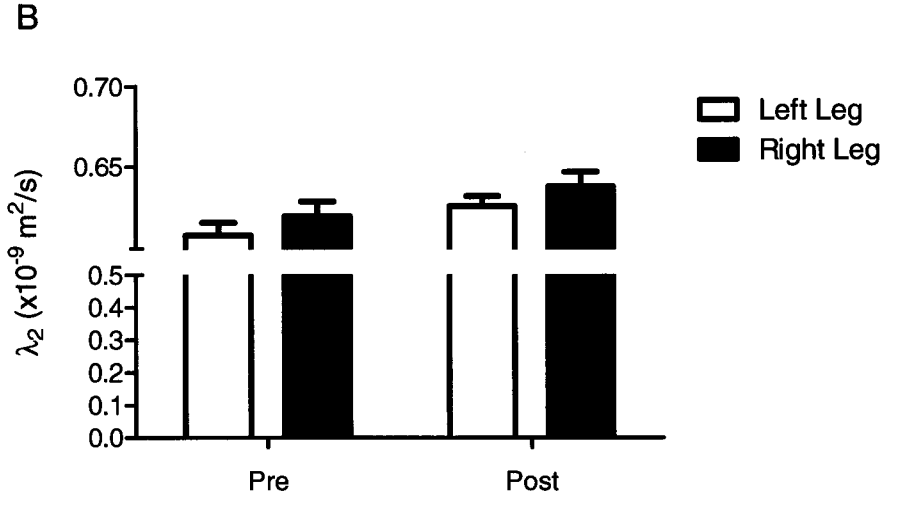



**Figure 4.5**: Apparent diffusion coefficient (ADC) of the vastus intermedius expressing the mean diffusion measured before and 24 h after 45 min of downhill running (-10 degrees) at 70% of maximal heart rate. Values are mean ±SE, n=10. *Main effect for time such that Post>Pre (P<0.05).


A
1.0
0.8
0.6
0.4
0.2
0.0
λ1 (x10-9 m2/s)
Pre
Post
Left Leg
Right Leg


B
0.70
0.65
0.5
0.4
0.3
0.2
0.1
0.0
λ2 (x10-9 m2/s)
Pre
Post
Left Leg
Right Leg

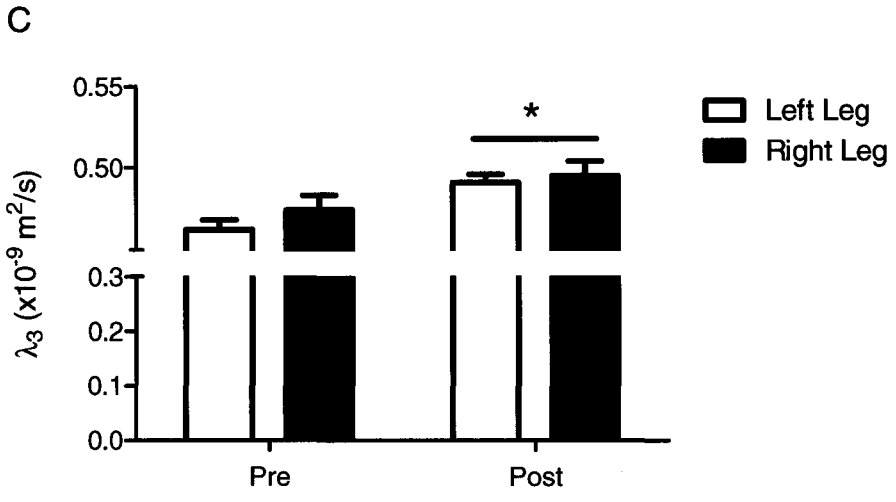



**Figure 4.6**: Tensor eigenvalues $\lambda_1$ (Panel A), $\lambda_2$ (Panel B) and $\lambda_3$ (Panel C) of the vastus intermedius measured before and 24 h after 45 min of downhill running (-10 degrees) at 70% of maximal heart rate. Values are mean ±SE, n=10. *Main effect for time such that Post>Pre (P<0.05).

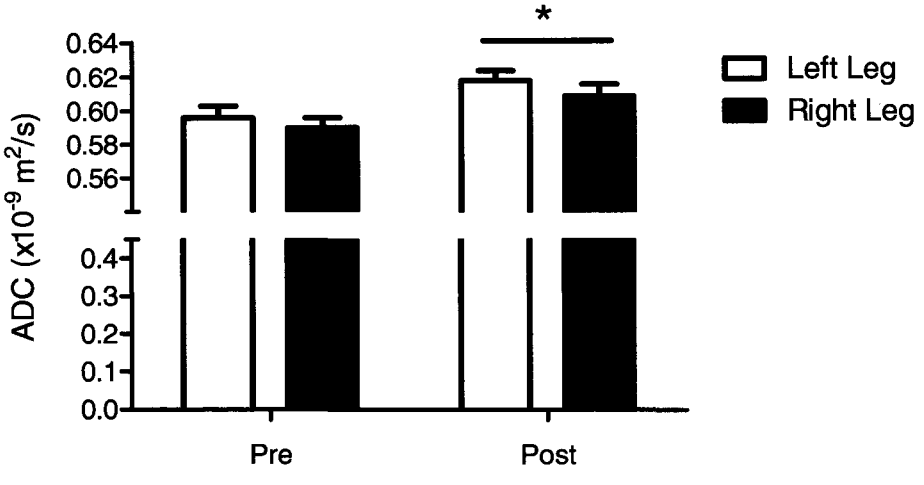
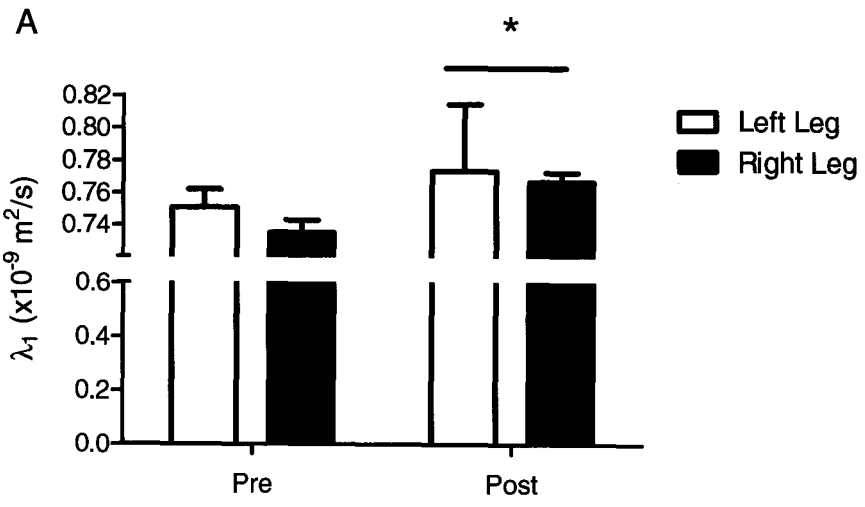
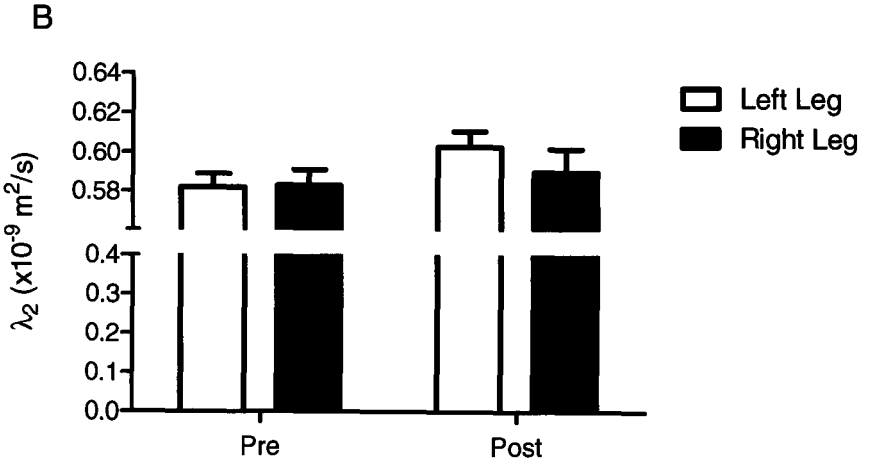



**Figure 4.7**: Apparent diffusion coefficient (ADC) of the vastus medialis expressing the mean diffusion measured before and 24 h after 45 min of downhill running (-10 degrees) at 70% of maximal heart rate. Values are mean ±SE, n=10. *Main effect for time such that Post>Pre (P<0.05).


A
*
0.82
0.80
0.78
0.76
0.74
0.6
0.4
0.2
0.0
λ1 (x10-9 m2/s)
Pre
Post
Left Leg
Right Leg


B
0.64
0.62
0.60
0.58
0.4
0.3
0.2
0.1
0.0
λ2 (x10-9 m2/s)
Pre
Post
Left Leg
Right Leg

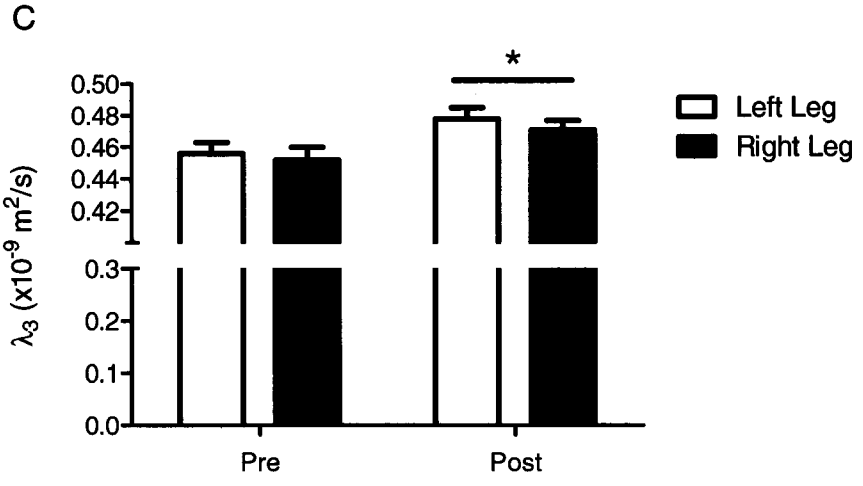



**Figure 4.8**: Tensor eigenvalues $\lambda_1$ (Panel A), $\lambda_2$ (Panel B) and $\lambda_3$ (Panel C) of the vastus medialis measured before and 24 h after 45 min of downhill running (-10 degrees) at 70% of maximal heart rate. Values are mean ±SE, n=10. *Main effect for time such that Post>Pre ($P<0.05$).

## 4.4 Discussion

The major novel finding from the present study was the application of DT-MRI to non-invasively detect structural changes in skeletal muscle 24 h following a bout of downhill running. Compared to baseline, evaluation of the knee extensor muscles 24 h following the cessation of exercise revealed an increased mean diffusivity in the vastus lateralis, v.intermedius and v.medialis, in addition to a significant increase in tensor eigenvalue $\lambda_3$. Indirect evidence of muscle disruption 24 h post-exercise included increased plasma CK activity and muscle soreness and a decrease in maximal isometric and isokinetic torque. Based on the magnitude of these changes and comparisons with previous work that included needle biopsy sampling (Betts *et al.* 2009; Nunan *et al.* 2010), it is

reasonable to assume that our downhill running protocol most likely induced skeletal muscle ultrastructural changes. Specifically, similar to the protocol used in the current study, downhill running has been shown to induce ultrastructural changes as evidenced by an increase in the percentage of damaged Z-bands and neutrophil accumulation observed at both 45 min and 5 days post running (Fielding *et al.* 1993).

Various techniques (Fielding *et al.* 1993; Feasson *et al.* 2002; Etheridge *et al.* 2008) have been used to evaluate ultrastructural disruption following downhill running however the only direct measurement of skeletal muscle ultrastructure following unaccustomed exercise involves a muscle biopsy sample. There are many limitations associated with this invasive procedure, including the fact that the results are based on a small (50-100 mg) sample that must be extrapolated to represent the whole muscle, which can result in an over or under-estimation of actual ultrastructural disruption. Other indirect measurement techniques such as quantifying the amount of enzyme release in the blood, subjective ratings of muscle soreness, strength measurements and even various MRI sequencing parameters (e.g. T1, STIR) cannot accurately evaluate the extent of ultrastructural changes and most indirect methods are poorly correlated with ultrastructural results (Jones *et al.* 1986).

Traditional MRI sequencing parameters are limited in their potential to evaluate structural changes following unaccustomed exercise. Nurenberg and colleagues (Nurenberg *et al.* 1992) used MRI-guided muscle biopsies obtained

30 minutes following a bout of downhill running at 8 km/h at a fixed grade of -8% to evaluate whether a correlation existed between an increase in signal-intensity (SI) and ultrastructural injury. At 48h post-exercise, muscle biopsy locations were determined based on varying SIs found during the MRI. Linear regression analysis revealed a high correlation between ultrastructural injury and the observed SI increase on T1-weighted, spin-density and STIR images (Nurenberg *et al.* 1992) suggesting that MRI is a potent tool for non-invasively evaluating ultrastructural changes following unaccustomed exercise. Perhaps somewhat surprising, was the lack of correlation between areas of soreness and areas of SI increase (Nurenberg *et al.* 1992), in addition to the variability in SI increase between subjects, suggesting variations in muscle recruitment exist even during a controlled exercise that were not previously recognized (Fleckenstein *et al.* 1989).

One major drawback of the traditionally used MRI sequencing parameters however, is their high degree of subjectivity and lack of a measurable unit if measured by the degree of signal increase as per Nuremburg and colleagues (1992). As such, DT-MRI would seem superior as it eliminates any subjective bias in addition to having the capacity to quantify diffusion changes on a measureable scale. After extensive research in brain tissue using DT-MRI, Zaraiskaya and colleagues (2006) established DT-MRI's ability to distinguish injured from healthy skeletal muscle in the human calf. Recently, we utilized DT-MRI to determine whether anisotropic diffusion decreases at 24 h following 300

eccentric actions of the knee extensors, in addition to obtaining muscle biopsies from the vastus lateralis to assess markers of ultrastructural disruption (Cermak *et al.* 2009). Briefly, in comparison to baseline sampling, FA decreased in the vastus lateralis 24 h following the unaccustomed exercise protocol which is suggestive of skeletal muscle fibre disorganization (Cermak *et al.* 2009). Additionally, the magnitude of diffusion in tensor eigenvalue $\lambda_3$ (perpendicular to the greatest magnitude of diffusion) increased 24 h post exercise, implying that the skeletal muscle fibre structure appeared more "spherical" in shape versus the structured cylindrical, healthy fibre orientation (Karampinos *et al.* 2009). Likewise, light microscopy examination for Z-band streaming found an increase in this marker of ultrastructural disruption 24 h post exercise, supporting the ability for DT-MRI to non-invasively assess ultrastructural changes. In the current study, we wanted to determine whether DT-MRI had the ability to distinguish ultrastructural differences following a more traditional bout of endurance exercise. As per our initial DT-MRI study, significant changes in diffusion were observed compared to baseline values. Specifically across all muscles imaged, tensor eigenvalue $\lambda_3$ increased from baseline to 24 h post exercise. This observation is in agreement with our initial study whereby in the vastus lateralis and v.medialis, tensor eigenvalue $\lambda_3$ increased, providing consistency between two very different muscle disruption protocols. Unlike our first study or the research led by Zaraiskaya and colleagues (2006) however, no significant findings were found upon calculation of the FA in each imaged muscle

tissue. This null finding may have been due to the two different exercise protocols whereby our initial study used a severe muscle disruption protocol that is very unlike any real-world application while Zaraiskaya and colleagues (2006) compared two different subject pools (healthy versus traumatic muscle injury). Furthermore, use of FA may not readily divulge subtle muscle changes as it is more of a global mixing of the 3 eigenvalues. If one eigenvalue is increasing while another decreasing the overall FA change is negligible.

DT-MRI and its relationship with skeletal muscle has also been investigated at varying muscle lengths (Schwenzer *et al.* 2009). Muscle shortening (dorsiflexion) and lengthening (plantar flexion) of the human calf was examined for any differences between FA, ADC and tensor eigenvalues. As hypothesized, muscle shortening through dorsiflexion caused a decrease in FA and increase in ADC, while muscle lengthening resulted in increased FA and decreased ADC. These findings were attributed to the knowledge that the cross-sectional area of the muscle fibre increases with muscle shortening and decreases with muscle lengthening (Schwenzer *et al.* 2009). Therefore, a decrease in FA during muscle shortening is a result of the muscle pathology becoming more spherical in shape. This study was essential in affirming the notion that the cell membrane is an important barrier for water diffusion and that DT-MRI is sensitive to the shape and position of the skeletal muscle fibres.

In accordance with previous research (Damon *et al.* 2002) there was a significant difference between the secondary ($\lambda_2$) and tertiary ($\lambda_3$) eigenvalues,

which characterize diffusion in the plane perpendicular to the myofibre orientation (Karampinos *et al.* 2009). Karampinos and colleagues (2009) demonstrated that myofibre ellipticity is explained by the transverse asymmetry found in the tertiary eigenvalue ($\lambda_3$). Myofibre ellipticity can be extremely useful in the characterization of morphological changes in skeletal muscle with age, hypertrophy or following physical inactivity (Karampinos *et al.* 2009). The authors stated that although muscle quality has been conventionally defined in the aging or obesity literature via whole limb measurements (i.e., knee extension strength or muscle mass), it has been suggested that it can also be assessed in terms of the tertiary eigenvalue ($\lambda_3$) of the diffusion tensor. The human calf, which was imaged by these researchers, has a strong linear correlation to the effective muscle physiological cross-sectional area, which in turn is proportional to the maximum muscle force. Therefore, connecting water diffusional anisotropy to muscle fibre architecture can help elucidate the connection between structure and muscle quality.

Although an expensive tool, there are many advantages to using DT-MRI to assess structural disruption. Besides the non-invasive aspect and lack of discomfort for the subjects, DT-MRI gathers information over an extensive volume of the muscle. In the present study, 25 slices of 4 mm each were obtained which essentially gathered 10 cm of information along subject's thighs. This large volume is essential for avoiding any over or under-estimation of the damaged areas as experienced when utilizing the skeletal muscle biopsy

procedure. Additionally, if using an athletic population, subjects often show some minor areas of Z-band streaming/neurosis or inflammatory markers at baseline, which makes comparing baseline to the intervention quite difficult. With the larger surface area covered when using DT-MRI, it is anticipated that a better overall picture of health of the skeletal muscle will be observed. Lastly, DT-MRI has the capability to image multiple muscles at once, providing the researcher with a complete story of how various muscle groups react to an exercise intervention. As previously mentioned, this is extremely important when muscle use varies between subjects during a given exercise intervention.

In summary, mean diffusion values and tensor eigenvalue $\lambda_3$ increased 24 h following 45 minutes of downhill running (-10% grade) at 70% of subjects' maximal heart rate in addition to observing changes in traditional muscle disruption markers such as CK and torque measurements. Across all imaged muscles, the increase in the magnitude for the tensor eigenvalue $\lambda_3$ provides evidence for an altered muscle structure as a result of unaccustomed endurance exercise. Overall, we suggest, based on these results, that diffusion tensor MRI has the ability to non-invasively evaluate structural disruption following eccentrically focused endurance exercise. In conclusion, this study has provided the framework to further utilize this novel non-invasive technique to investigate various other muscle perturbations. Some of these potential applications include investigating changes in muscle fibre structure with hypertrophy or atrophy in either the healthy or aging skeletal muscle, examining potential differences in the

structural response with concentric versus eccentric-based exercise and investigating any structural response with the "repeated-bout" effect. In the immediate future, researchers may wish to explore the time course of diffusion following unaccustomed exercise to grasp a better understanding of any time-sensitive structural responses found when using DT-MRI.

## 4.5 References

Basser, PJ and DK Jones. 2002. Diffusion-tensor MRI: Theory, experimental design and data analysis—a technical review. *NMR Biomedicine* 15: 456-467.

Basser, PJ, J Mattiello and D Le Bihan. 1994. MR diffusion tensor spectroscopy and imaging. *Biophysical Journal* 66: 259-267.

Betts, JA, RJ Toone, KA Stokes and D Thompson. 2009. Systemic indices of skeletal muscle damage and recovery of muscle function after exercise: Effect of combined carbohydrate-protein ingestion. *Applied Physiology Nutrition and Metabolism* 34 (4): 773-784.

Cermak, NM, MD Noseworthy, MA Tarnopolsky and MJ Gibala. 2009. Use of diffusion tensor magnetic resonance imaging for assessment of musculoskeletal structure following high-force eccentric exercise. *Applied Physiology Nutrition and Metabolism* 34 (Suppl1): S16.

Clarkson, PM and MJ Hubal. 2002. Exercise-induced muscle damage in humans. *American Journal of Physical Medicine and Rehabilitation* 81 (11 (Suppl)): S52-S69.

Cox, RW. 1996. AFNI: Software for analysis and visualization of functional magnetic resonance neuroimages. *Computers and Biomedical Research* 29 (3): 162-173.

Damon, BM, Z Ding, AW Anderson, AS Freyer and JC Gore. 2002. Validation of diffusion tensor MRI-based muscle fiber tracking. *Magnetic Resonance Medicine* 48: 97-104.

Etheridge, T, A Philp and PW Watt. 2008. A single protein meal increases recovery of muscle function following an acute eccentric exercise bout. *Applied Physiology Nutrition and Metabolism* 33 (3): 483-488.

Feasson, L, D Stockholm, D Freyssenet, I Richard, S Duguez, JS Beckmann and C Denis. 2002. Molecular adaptations of neuromuscular disease-associated proteins in response to eccentric exercise in human skeletal muscle. *Journal of Physiology* 543 (1): 297-306.

Fielding, RA, TJ Manfredi, W Ding, MA Fiatarone, WJ Evans and JG Cannon. 1993. Acute phase response in exercise iii. Neutrophil and Il-1 beta accumulation in skeletal muscle. *American Journal of Physiology* 256: R166-R172.

Fleckenstein, JL, LA Bertocci, RI Nunnaly, RW Parkey and RM Peshock. 1989. Exercise-enhanced MR imaging of variations in forearm muscle anatomy and use: Importance in MR spectroscopy. *American Journal of Roentgenology* 153: 693-698.

Galban, CJ, S Maderwald, K Uffmann, A de Greiff and ME Ladd. 2004. Diffusive sensitivity to muscle architecture: A magnetic resonance diffusion tensor imaging study of the human calf. *European Journal of Applied Physiology* 93: 253-262.

Jones, DA, DJ Newham, JM Round and SEJ Tolfree. 1986. Experimental human muscle damage: Morphological changes in relation to other indices of damage. *Journal of Physiology* 375: 435-448.

Karampinos, DC, KF King, BP Sutton and JG Georgiadis. 2009. Myofiber ellipticity as an explanation for transverse asymmetry of skeletal muscle diffusion MRI in vivo signal. *Annals of Biomedical Engineering* 37 (12): 2532-2546.

Le Bihan, D, JF Mangin, C Poupon, CA Clark, S Pappata, N Molko and H. Chabriat. 2001. Diffusion tensor imaging: Concepts and applications. *Journal of Magnetic Resonance Imaging* 13: 534-546.

Nunan, D, G Howatson and KA van Someren. 2010. Exercise-induced muscle damage is not attenuated by beta-hydroxy-beta-methylbutyrate and alpha-ketoisocaproic acid supplementation. *Journal of Strength and Conditioning Research* 24 (2): 531-537.

Nurenberg, P, CJ Giddings, J Stray-Gundersen, JL Fleckenstein, WJ Gonyea and RM Peshock. 1992. MR imaging-guided muscle biopsy for correlation of increased signal intensity with ultrastructural change and delayed-onset muscle soreness after exercise. *Radiology* 184: 865-869.

Pierpaoli, C and PJ Basser. 1996. Toward a quantitative assessment of diffusion anisotropy. *Magnetic Resonance Medicine* 36: 893-906.

Schwenzer, NF, G Steidle, P Martirosian, C Schraml, F Springer, CD Claussen and F Schick. 2009. Diffusion tensor imaging of the human calf muscle: Distinct changes in fractional anisotropy and mean diffusion due to passive muscle shortening and stretching. *NMR in Biomedicine* 22: 1047-1053.

Sinha, S, U Sinha and VR Edgerton. 2006. In vivo diffusion tensor imaging of the human calf muscle. *Journal of Magnetic Resonance Imaging* 24: 182-190.

Zaraiskaya, T, D Kumbhare and MD Noseworthy. 2006. Diffusion tensor imaging in evaluation of human skeletal muscle injury. *Journal of Magnetic Resonance Imaging* 24 (2): 402-408.

# CHAPTER 5

# GENERAL DISCUSSION

## 5.1 SUMMARY OF FINDINGS

Nutritional ingestion has a potent influence on metabolic control during an acute endurance exercise bout (Jeukendrup 2004). With specific focus on CHO feeding *during* exercise, a strong case has been made for the benefits of CHO ingestion (Jeukendrup 2004). Much less is known however, about any positive metabolic effect(s) of the co-ingestion of PRO and CHO consumed during endurance exercise. The few investigations that have found positive effects of CHOPRO ingestion on endurance performance have been varied in their methodology including feeding below the recommended intake of CHO per hour, performing exercise after an overnight fast, and using time to exhaustion (Ivy *et al.* 2003; Saunders *et al.* 2004; Saunders *et al.* 2007), versus a set distance or workload which is more typical of how athletes prepare and race. Although researchers have proposed mechanisms by which CHOPRO may improve muscle metabolism over CHO-only, no study has directly investigated these potential metabolic mechanisms.

Therefore, study 1 (Chapter 2) directly examined markers of skeletal muscle metabolic control that were hypothesized to be responsible for any augmentation in endurance performance. Regardless of whether CHO-only or CHOPRO was ingested during a 90 min bout of cycling exercise at 69% $VO_2$peak

no difference in muscle glycogen degradation or concentration of key TCAi was found. Additionally, no change in next-day aerobic performance as assessed by a 20 km cycling time-trial, nor any variation in plasma CK, an indirect measure of muscle disruption, was noted. This novel work suggested that if fed at the recommended nutritional intake of 60 g CHO per hour plus added PRO, there is no added metabolic or structural benefit of CHOPRO over CHO-only.

After study 1, questions remained over whether added PRO really has the ability to attenuate EIMD. Although study 1 found no difference in plasma CK between CHOPRO versus CHO-only, methodological concerns arose over the correct technique to evaluate EIMD. Traditionally, the only direct way to assess ultrastructural disruption is through the analysis of skeletal muscle biopsy samples, as all other indirect techniques poorly correlate with ultrastructural results. Therefore, we chose to investigate a relatively new MRI technique in musculoskeletal imaging called diffusion tensor for studies 2 and 3 to examine whether DT-MRI is sensitive enough to non-invasively evaluate skeletal muscle disruption. Study 2 demonstrated that 24 h following 300 eccentric actions of the knee extensors, FA decreased in the vastus lateralis which is indicative of muscle fibres becoming more disorganized or less uniform in structure. With the confirmation of direct ultrastructural changes post exercise using Z-band streaming, it was suggested that DT-MRI could evaluate structural changes in skeletal muscle following high-force eccentric exercise. Study 3 applied the non-invasive DT-MRI technique used in Study 2 to investigate whether changes in

diffusion were apparent following more "real-world" applicable exercise. Twenty-four hours following 45 min of downhill running, increased mean diffusion and tensor eigenvalue $\lambda_3$, were observed. Collectively, these studies have provided a platform for further investigative work using DT-MRI to assess structural changes in skeletal muscle following various exercise perturbations.

The present chapter collectively summarizes the important findings from these studies to provide novel insight regarding both the metabolic and structural response of skeletal muscle following acute exercise. Additionally, limitations of this work are discussed along with recommendations and insight for future research.

## 5.2 New insights regarding DT-MRI and human skeletal muscle

As this work progressed, an emerging novel aspect and focus of the research became the use of DT-MRI in skeletal muscle, with specific emphasis on evaluating the potential for DT-MRI to assess structural disruption 24 h following unaccustomed exercise. Although changes in diffusion were observed in both studies 2 and 3, little is known whether these small changes in diffusion relate to alterations in tissue morphology. If shifts in diffusion, as measured through DT-MRI, reflect some sort of modification to skeletal muscle tissue, then it would seem logical to assume that changes in diffusion should be observed after both acute exercise and chronic training. These observations would help to support the findings from this thesis whereby changes in diffusion characteristics

from baseline to 24 h post strenuous unaccustomed exercise reflect altered tissue morphology.

It was briefly mentioned in study 3 (Chapter 4) that perturbations in diffusion can be observed during passive lengthening or shortening of the human calf muscles (Schwenzer *et al.* 2009). With shortening, muscle cross-sectional area became larger, consequently increasing tensor eigenvalues λ's 2 and 3 that are perpendicular to the main direction of diffusion. With passive muscle lengthening, the opposite effect occurred whereby muscle cross-sectional area was reduced, resulting in a decrease in the magnitude of tensor eigenvalues λ's 2 and 3. With such dramatic changes in diffusion through relatively small passive changes in muscle length, this research would suggest that DT-MRI is extremely sensitive to joint angle and directly reflects changes in muscle morphology. In studies 2 and 3, we observed increased magnitude of diffusion in tensor eigenvalue $\lambda_3$. As we measured the lower thigh at the same joint angle (neutral hip/knee position), our effective change in diffusion 24 h post exercise cannot be attributed to a change in passive muscle length. Instead, it is possible that some sort of fibre rupture/disruption occurred as evidenced with an increase in Z-band streaming. This does not imply that DT-MRI measures Z-band streaming *per se*, but rather with strenuous, unaccustomed exercise, cellular barriers to diffusion become leaky or rupture altogether, causing diffusion to be less restricted perpendicular to the main direction of diffusion.

Changes in diffusion also become apparent immediately following an

exercise bout. Although there is limited research available using DT-MRI in skeletal muscle, one study measured diffusion immediately following a calf-loading exercise as briefly mentioned in study 2. Okamoto and colleagues (2008) obtained bilateral DT-MRI images of the anterior tibialis, gastrocnemius and soleus muscle of 10 healthy males at rest and then subjected only two of the ten subjects to exercise loading in their right calves. The exercise consisted of either 30 or 60 min of isometric contraction by repeated plantar flexion and dorsal extension of the right ankle (tiptoe position). FA values were recorded immediately post exercise-loading, 24 h, 72 h and 1 week post exercise and expressed as a FA ratio of right (exercised calf) over left (non-exercised calf). This acute bout of plantar flexion induced similar results from the passive lengthening/shortening study by Schwenzer *et al.* (2009) whereby, plantar flexion exercise caused no change in the FA of the tibialis anterior however, FA ratios between the right/left gastrocnemius decreased immediately post exercise in both the 30 and 60 min subjects and remained below baseline until 1 week post exercise. In the soleus, both subjects recovered to their baseline values by 72 h post exercise. Although this data is from only two subjects and no other diffusion characteristics (tensor eigenvalues, ADC etc) were reported, it is interesting to note that not only are diffusion changes observed immediately post exercise, but diffusion is affected by the extent of the involved muscle group (gastrocnemius involved in plantar flexion vs. tibialis anterior), and the intensity or duration of the exercise bout (i.e., the subject that performed the calve exercise for 60 min had

much lower FA values in contrast to the 30 min exercising subject).

If DT-MRI is truly sensitive to structural changes in skeletal muscle, a modification in diffusion would also be expected following chronic exercise training. Nakai and colleagues (2008) found increased FA after one month of walking exercise with training equipment. Fourteen women wore training equipment designed to exert stress on the quadriceps, biceps femoris and gluteus maximus muscles while 7 women wore "normal" equipment with no added weight stress. All subjects were required to walk at least 10 000 steps a day as monitored by a pedometer. In the walking equipment group, one month of walking, increased FA in all imaged muscles compared to the "normal" or no added walking weight group (Nakai *et al.* 2008). The increased FA implies that stressed skeletal muscles became more anisotropic or uniform in structure which is the opposite effect observed following unaccustomed exercise.

To summarize, although there is limited literature in which DT-MRI was used to assess human skeletal muscle, the available research suggests that DT-MRI is sensitive to joint angle, exercise intensity/duration and chronic training. Furthermore, these diffusion changes after various perturbations are likely due to altered structure and morphology, providing the framework for future structural studies using DT-MRI.

## 5.3 Limitations and Future Work

Although findings in study 1 suggest that CHOPRO does not alter selected

markers of metabolic control during exercise compared to CHO alone, the results are specific to the nature of the study design. Firstly, the study was designed to mimic conditions athletes normally face including performing a set distance (20 km time trial), feeding CHO at the upper end of recommended exogenous CHO consumption (Jeukendrup 2004) and negating an overnight fast. As liver glycogen stores are depleted after an overnight fast, it seemed logical to allow subjects to eat identical breakfasts before each exercise trial in order to refuel their system and mimic real-world exercise procedures. Additionally, to truly investigate whether CHOPRO induced metabolic advantages over CHO-only it was necessary to feed at the upper limits of exogenous CHO oxidation (Jeukendrup 2004) therefore, taking full advantage of the benefits of exogenous CHO consumption to investigate whether added PRO would produce any *additional* metabolic or performance benefits. With this 'real-world applicable' study design, we may have 'hid' any potential benefit of PRO, however, this particular design allowed us to avoid acknowledging that any measurable effect could be due to the added calories per se, and not the PRO-only. Furthermore, it is possible that our length of aerobic exercise [90 min versus 3 h (Ivy *et al.* 2003)] was not long enough or intense enough based on previous exercise intensities [69% versus 75-85% $VO_2$peak (Ivy *et al.* 2003; Saunders *et al.* 2004; Saunders *et al.* 2007)] to produce enough metabolic stress to elicit any potential performance benefits of the added PRO. For example, some threshold of intensity may need to be reached before any EIMD symptoms are displayed

(Janssen *et al.* 1989). Regardless, even after 90 min of exercise, no metabolic trends emerged in the CHOPRO versus the CHO-only group implying a lack of any potential metabolic benefit of CHOPRO versus CHO-only.

Future work should focus on chronic training to observe whether CHOPRO versus CHO-only has any metabolic benefit after cumulative bouts of varying intensity aerobic exercise. It is possible that perhaps a positive benefit of PRO ingestion occurs after the body has been repeatedly stressed such as during a very intensive training week, or in events such as a multi-day cycling stage race (e.g. Tour de France), where nutritional intake levels may fall short, putting the athlete at risk for negative protein balance. In this regard, CHOPRO ingestion may help the athlete reach their nutritional needs by achieving positive protein balance for protein synthesis and ultimately attenuating muscle breakdown and increased muscle recovery as observed by Etheridge and colleagues (2008) after a 30 min downhill run. Immediately post-exercise, 100 g of protein or placebo were fed to the subjects and measurements were recorded for up to 72 h post downhill run. Although blood measurements (CK and protein carbonyl) were not different between the two feeding conditions, peak power output assessed during 5 s repeated sprints on a cycle ergometer and peak isometric contractions remained within baseline values of the PRO group, but significantly decreased in the placebo, suggesting that some sort of structural mechanism may exist with PRO consumption post aerobic exercise (Etheridge *et al.* 2008).

We also found no attenuation of plasma CK measured 24 h following the aerobic cycling exercise in the CHOPRO versus CHO-only group. As mentioned during the assessment of metabolic control, it is possible that our exercise was not difficult enough to elicit a potent response in plasma CK as some sort of threshold may exist before any potential differences in plasma CK are observed. Perhaps more importantly however, is the technique used to assess EIMD. Measuring blood enzymes produces a host of limitations, while a muscle biopsy is extremely invasive in nature thus often making recruitment in athletic population circles difficult. Therefore, we aimed at testing an MRI technique to determine whether we could evaluate ultrastructural disruption non-invasively and avoid prior limitations experienced when using blood, strength or soreness analysis. Although studies 2 and 3 demonstrated that DT-MRI was extremely valuable with ultrastructural assessment, the cost and accessibility associated with using an MRI cannot be overlooked. Technical considerations must also be kept in mind as muscle DTI has an inherently low signal-to-noise ratio (SNR) due to the short $T_2$ of the muscle (Karampinos *et al.* 2009). As such, the dependence of the tensor eigenvalues on SNR should be taken into account when interpreting any muscle DT-MRI results. Additionally, movement of the subject within the MRI and replicating image areas between trials must all be considered when attempting to utilize a highly sensitive MRI technique as even passive shortening/lengthening of the calf can alter diffusion (Schwenzer *et al.* 2009).

Although DT-MRI is sensitive, the observed diffusion changes are very

small (typically <10%). Ideally, when we first started investigating DT-MRI and its potential to evaluate structural changes, we had hopes of it becoming a sensitive enough tool to detect changes in fibre disruption following nutritional perturbations. Due to small diffusion differences observed in both studies 2 and 3, where our goal was to induce a measureable degree of structural disruption, we question whether DT-MRI is sensitive enough to detect nutritional attenuations in structure if they were present. After performing a sample size calculation based on a power of 0.8 and an alpha level of 0.05, at least 36 subjects would be required to detect potential differences, which from the cost of using MRI, seems highly unpractical.

As very little work has been done using DT-MRI to image skeletal muscle however, there are endless possibilities for future research including a time course study to evaluate changes in structure following unaccustomed exercise. As previously discussed, the changes in DT-MRI are relatively small from pre to post strenuous unaccustomed exercise. Currently, as no time course study has been completed (using an appropriate number of subjects and reporting a full-range of diffusion characteristics), we are unaware of whether we missed the most opportune time to measure diffusion. If there is a large response at a given time point, which is the case for CK, whereby 72 h post eccentric leg-kicking has a higher value than when measured 24 h post, it provides a better chance to detect any statistically significant attenuation in diffusion through various perturbations.

Additionally, as most work using DT-MRI has investigated the human calf muscles (Zaraiskaya *et al.* 2006; Okamoto *et al.* 2008; Schwenzer *et al.* 2009) it is possible that various muscle groups are more or less sensitive to exercise or nutritional perturbations. Smaller relative changes in FA and eigenvalues were observed in the human quadriceps used in the current research, than what had previously been found in the human calf (Zaraiskaya *et al.* 2006; Schwenzer *et al.* 2009). Irrespective of investigating EIMD per se, DT-MRI could also prove useful in other areas of work including structural changes with aging and hypertrophy. As DT-MRI has proven to be a sensitive enough tool to detect structural changes following disruption, acute, and chronic exercise training, DT-MRI may be a useful tool in the assessment of skeletal muscle structure and function with aging and impending sarcopenia.

Finally, as changes in FA were observed immediately post an acute exercise bout (Okamoto *et al.* 2008), DT-MRI may provide a useful tool for investigating any potential structural differences between work-matched concentric versus eccentrically-based exercise, perhaps shedding light on the theory that structural disruption is observed after unaccustomed eccentrically-biased versus concentric exercise only (Newham *et al.* 1983).

## 5.5 General Conclusions

Collectively, these studies demonstrated that there is no known metabolic benefit of ingesting CHOPRO over CHO-only. As no negative metabolic

consequences were found however, nutrition during exercise is likely a personal preference. While tackling the skeletal muscle structural side of the nutritional debate, we found that many markers of EIMD have a host of limitations. Thus, the pursuit of a non-invasive tool to investigate whether DT-MRI could evaluate structural changes following unaccustomed exercise was chosen. Although changes in diffusion were apparent 24 h following exercise, these observed changes were quite small suggesting that a large number of subjects would be needed in order to use this technique to assess nutritional perturbations on skeletal structure. Regardless, if the appropriate financial and technical resources are available, DT-MRI is a sensitive, useful, non-invasive tool for the assessment of skeletal muscle fibre structure.

## 5.6 References

Etheridge, T, A Philp and PW Watt. 2008. A single protein meal increases recovery of muscle function following an acute eccentric exercise bout. *Applied Physiology Nutrition and Metabolism* 33 (3): 483-488.

Ivy, JL, PT Res, RC Sprague and MO Widzer. 2003. Effect of a carbohydrate-protein supplement on endurance performance during exercise of varying intensity. *International Journal of Sport Nutrition and Exercise Metabolism* 13: 382-395.

Janssen, GM, H Kuipers, GM Willems, RJ Does, MP Janssen and P Geurten. 1989. Plasma activity of muscle enzymes: Quantification of skeletal muscle damage and relationship with metabolic variables. *International Journal of Sports Medicine* 10 (Suppl 3): S160-S168.

Jeukendrup, AE. 2004. Carbohydrate intake during exercise and performance. *Nutrition* 20: 669-677.

Karampinos, DC, KF King, BP Sutton and JG Georgiadis. 2009. Myofiber ellipticity as an explanation for transverse asymmetry of skeletal muscle diffusion MRI in vivo signal. *Annals of Biomedical Engineering* 37 (12): 2532-2546.

Nakai, R, T Azuma, M Sudo, S Urayama, O Takizawa and S Tsutsumi. 2008. MRI analysis of structural changes in skeletal muscles and surrounding tissues following long-term walking exercise with training equipment. *Journal of Applied Physiology* 105 (3): 958-963.

Newham, DJ, G McPhail and KR Mills. 1983. Ultrastructural changes after concentric and eccentric contractions of human muscle. *Journal of Neurological Sciences* 61: 109-122.

Okamoto, Y, A Kunimatsu, S Miki, S Masashi, M Niitsu and M Minami. 2008. Fractional anisotropy values of calf muscles in normative state after exercise: Preliminary results. *Magnetic Resonance in Medical Sciences* 7 (3): 157-162.

Saunders, MJ, MD Kane and MK Todd. 2004. Effects of a carbohydrate-protein beverage on cycling endurance and muscle damage. *Medicine & Science in Sports & Exercise* 36 (7): 1233-1238.

Saunders, MJ, ND Luden and JE Herrick. 2007. Consumption of an oral carbohydrate-protein gel improves cycling endurance and prevents postexercise muscle damage. *Journal of Strength and Conditioning Research* 21 (2): 678-684.

Schwenzer, NF, G Steidle, P Martirosian, C Schraml, F Springer, CD Claussen and F Schick. 2009. Diffusion tensor imaging of the human calf muscle: Distinct changes in fractional anisotropy and mean diffusion due to passive muscle shortening and stretching. *NMR in Biomedicine* 22: 1047-1053.

Zaraiskaya, T, D Kumbhare and MD Noseworthy. 2006. Diffusion tensor imaging in evaluation of human skeletal muscle injury. *Journal of Magnetic Resonance Imaging* 24 (2): 402-408.