THE EFFECT OF ELEVATED MUSCLE FLUID VOLUME ON INDICES OF MUSCLE DAMAGE FOLLOWING AN ACUTE BOUT OF ECCENTRIC EXERCISE

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Submitted in partial fulfillment
of the requirements for the degree
Master of Science in Applied Health Sciences

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ABSTRACT

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The primary purpose of the current investigation was to develop an elevated muscle fluid level using a human *in-vivo* model. The secondary purpose was to determine if an increased muscle fluid content could alter the acute muscle damage response following a bout of eccentric exercise. Eight healthy, recreationally active males participated in a cross-over design involving two randomly assigned trials. A hydration trial (HYD) consisting of a two hour infusion of a hypotonic (0.45%) saline at a rate of $20mL/min^{-1}/1.73m^{-2}$ and a control trial (CON), separated by four weeks. Following the infusion (HYD) or rest period (CON), participants completed a single leg isokinetic eccentric exercise protocol of the quadriceps, consisting of 10 sets of 10 repetitions with a one minute rest between each set. Muscle biopsies were collected prior to the exercise, immediately following and at three hours post exercise. Muscle analysis included determination of wet-dry ratios and quantification of muscle damage using toluidine blue staining and light microscopy. Blood samples were collected prior to, immediately post, three and 24 hours post exercise to determine changes in creatine kinase (CK), lactate dehydrogenase (LD), interleukin-6 (IL-6) and C-reactive protein (CRP) levels. Results demonstrated an increased muscle fluid volume in the HYD condition following the infusion when compared to the CON condition. Isometric peak torque was significantly reduced following the exercise in both the HYD and CON conditions. There were no significant differences in the number of areas of muscle damage at any of the time points in either condition, with no differences between conditions. CK levels were significantly greater 24hour post exercise compared to pre, immediately and three hours post similarly in both conditions. LD in the HYD condition followed a similar trend as CK with 24 hour levels higher than pre, immediately post and three hours post and LD levels were significantly greater 24 hours post compared to pre levels in the CON condition, with no differences between conditions. A significant main effect for time was observed for CRP ($p<0.05$) for time, such that CRP levels increased consistently at each subsequent time point. However, CRP and IL-6 levels were not different at any of the measured time points when comparing the two conditions. Although the current investigation was able to successfully increase muscle fluid volume and an increased CK, LD and CRP were observed, no muscle damage was observed following the eccentric exercise protocol in the CON or HYD conditions. Therefore, the hypotonic infusion used in the HYD condition proved to be a viable method to acutely increase muscle fluid content in *in-vivo* human skeletal muscle.

ACKNOWLEDGEMENTS

I would like to start off by thanking my supervisor, Dr. Brian Roy for his patience over the past 2 ½ years and for supporting my research interests. Dr. Sandra Peters and Dr. Paul LeBlanc, my committee members who never failed to have a suggestion when I came to them for help, thank you both very much for your assistance. To everyone in the lab, thank you for the laughs and always finding the funny in any situation. Remember, A.B.P (Chris that is for you).

To my friends and family, without their strong shoulders I would never have made it through. Mom and Dad, thank you for the never ending support, love, motivation and for always picking up the phone when I called, even when you knew it was to complain! To my 'little' brother, thank you for your support when I truly needed it. Sean, thank you doesn't even begin to explain my gratitude towards you. You have been my rock, always and truly XO.

To my girlfriends, who have always been able to keep me grounded, with their quirky jokes or reminiscing the good ol' days. Jennifer, we did it – seven years, but we did it!

And finally, I will end with a question for Joleen, what happens when "one day" finally arrives?

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ABBREVIATIONS

ADH – Anti-diuretic hormone, vasopressin
ANOVA – A one-way analysis of variance
ATP – Adenosine triphosphate
ATPase – Adenosine triphosphatase
AQP – Aquaporin

BIA – Bioelectrical impedance analyzer

Ca^{2+} - Calcium
CK – Creatine kinase
Cl^- – Chloride ion
$Cl^-/HCO3^-$ – Chloride carbonate co-transporter/ exchanger
CON – Control trial
CRP – C- reactive protein

DDAVP – Desmopressin

ECF – Extracellular fluid compartment

GD3+ - Gadolinium

H^+ – Hydrogen ion (proton)
H_2O_2 – Hydrogen radical
Hb – Haemoglobin
Hct - Hematocrit
HCO_3^- – Carbonate
HYD – Infusion trial

ICF – Intracellular fluid compartment
IL-1β – Interleukin -1β
IL-6 – Interleukin-6

K^+ – Potassium ion
KCl – Potassium chloride co-transporter

LD – lactate dehydrogenase

$mmol \cdot kg^{-1}$ – Standard international units of osmolality
MVC – maximal voluntary contraction

Na^+ – Sodium ion
NaCl – Sodium chloride

Na^+/H^+ – Sodium proton exchanger
Na^+-HCO_3^- – Sodium carbonate electrogenic co-transporter
Na^+/K^+ ATPase – Sodium potassium ATPase
N-K-2Cl – Sodium potassium two chloride co-transporter

O_2 – Oxygen
O_2^- - Superoxide radical
OH – Hydroxyl radical

PCr – Phosphocreatine
PMN – polymorphoneutrophil

RVD – Regulatory volume decrease
ROS – Reactive oxygen species
RVI – Regulatory volume increase

TBW – Total body water
TNF-α - Tumor necrosis factor
Type I fibres – Slow twitch skeletal muscle fibres
Type II fibres – Fast twitch skeletal muscle fibre

INTRODUCTION

Water comprises 50-60% of total body weight, making it one of the most abundant molecules in the human body(103). One of the integral functions of water is to assist with the maintenance of cellular homeostasis.

Water is transported between the intracellular and extracellular fluid compartments in response to perturbations in the intracellular and extracellular osmolality. An increased extracellular osmolality triggers movement of water out of cells to dilute the increased solute content, leading to shrinkage of cells (112) . Decreased extracellular osmolality leads to water movement into cells and ultimately swelling of the cell occurs (112). There are numerous factors that alter osmolality, including exercise (89), hormones(103), hydration status (58), and others.

There are consequences to alterations in fluid status within the body. An elevated fluid state (hyperhydration) promotes an environment for the synthesis of macromolecules (55) and does not interfere with exercise capacity. Hypohydration is characterized by a reduction of fluid within the body, leading to breakdown of proteins (55), compromised cardiovascular and thermoregulatory mechanisms (93), and decreased exercise capacity (71). There are numerous mechanisms that are activated in response to disturbances in the concentration of fluids or solutes such as activation of ion channels, release of hormones, and the breakdown of macromolecules within the cell, all in an effort to maintain cellular homeostasis.

Skeletal muscle fibres are able to generate and withstand large amounts of force during high intensity activity. The mechanisms involved in completing eccentric contractions leave muscle fibres susceptible to damage within the actin-myosin crossbridges (29). There are two mechanisms underlying skeletal muscle damage, mechanical and oxidative. Mechanical damage occurs as a result of disruption to the sarcolemma and sarcomeres caused by internal or external force. Stretch activated channels located along the sarcolemma that allow the infiltration of Ca^{2+} are also thought to contribute to mechanical damage (3, 83). The second mechanism of muscle damage is oxidative, resulting from elevated levels of reactive oxygen species, which attack structural proteins, lipids and DNA (104). The reactive oxygen species are also released as part of the inflammatory response, but can have detrimental effects on muscle function. To quantify the extent of damage, there are direct and indirect markers that can be measured to quantify damage following exercise.

Both hydration status and exercise have been shown to impact the structure and function of muscle fibres (10-12). Unfortunately, there is limited research that has investigated the effect of an altered hydration state on muscle function during exercise. The research that has been conducted introduces multiple variables that could affect the results, making it difficult to draw specific conclusions. An investigation determining the influence of alterations to muscle cell hydration and its influence on muscle damage during exercise is necessary to better understand the role hydration has in skeletal muscle responses to exercise.

CHAPTER 1: CELL VOLUME

a. Fluid Compartments

Total body water (TBW) makes up 60% of body weight and is sub-divided into two main compartments within the body: the extracellular and intracellular compartments (58). The extracellular compartment (ECF) is composed of interstitial and vascular fluid. The interstitial fluid makes up 80% of the extracellular compartment and includes the lymph fluid. The other 20% of the extracellular compartment is composed of vascular fluid; specifically plasma (103). The intracellular compartment (ICF) accounts for two thirds of total body water; or 40% of total body weight (TBW) (58).

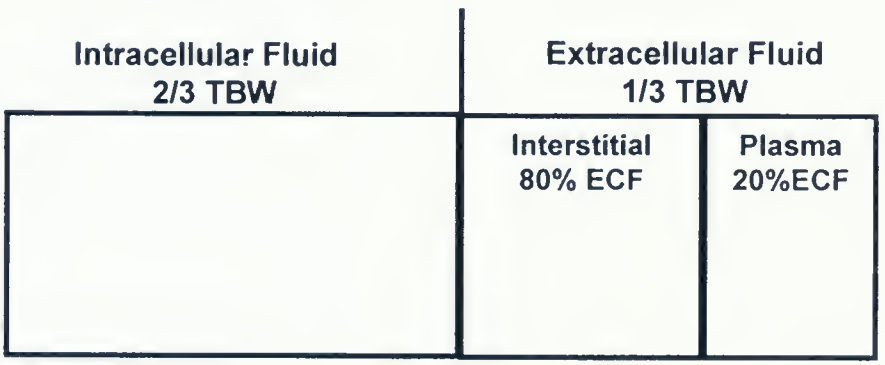


Figure 1. Distribution of Total Body Water in Fluid Compartments.
(TBW – Total Body Water; ECF – Extracellular Fluid).

Each compartment is comprised of various concentrations of ions and fluid volumes. These concentrations remain relatively constant at rest, but are not fixed. There is constant movement of water and solutes between the compartments to maintain homeostatic concentrations of these molecules. Within

the extracellular fluid compartment, there is movement between the interstitial and vascular compartments. The interstitial fluid acts as a reservoir, supplying fluid to the plasma (112).

The intracellular compartment is separated from the extracellular compartment by a plasma membrane barrier. The plasma membrane, also referred to as the sarcolemma in skeletal muscle, assists in regulating the concentration of metabolites and fluid in the cytoplasm of cells (103). The plasma membrane is a semi-permeable barrier, permeable to water and some other molecules (103). The ability of water to move between compartments allows for the extracellular and intracellular compartments to be in a state of osmotic equilibrium (103). Ions are unable to pass freely through the plasma membrane, and therefore the membrane acts as a barrier to maintain specific concentration of ions in each compartment. Sodium (Na^+), chloride (Cl^-), and bicarbonate are most heavily concentrated in the extracellular compartment, while potassium (K^+) is mainly located intracellularly (62). In response to perturbations in cell homeostasis, ion transporters and co-transporters are stimulated to move ions across the plasma membrane.

b. Regulation of Fluid Movement

Hydrostatic and osmotic pressure govern the movement of water between compartments (58). This movement of fluids between compartments occurs without a change in total body water content. Hydrostatic pressure is the pressure the vascular fluid exerts on the vessel walls (58). Hydrostatic pressure

leads to the movement of water from the vascular space into the interstitial compartment (111). Crystalloids and colloids are solutes that create osmotic pressure within the compartments. Crystalloids are solutes such as Na^+, Cl^- and glucose that are able to cross the capillary membrane in solution (112). These solutes produce osmotic pressure within the various compartments when in solution (58). Colloids are proteins and other large molecules in the vascular compartment that are unable to cross the capillary membrane. The pressure created by these colloids stimulate fluid movement in to or out of the cell until an osmotic equilibrium is reached (58). The amount and pressure produced by these solutes is termed osmolality and is measured as osmoles per kilogram of solution (mOsm/Kg) (58). Osmolarity is similar to osmolality, but is expressed as osmoles per litre of solution. Normal serum osmolality ranges from 285-295 mOsm/kg or mOsm/L (28). The osmotic induced shifting of fluid between the ICF and the ECF is directly dependent on the osmolality of the solutes within each compartment (58).

The osmolality of a solution alters the osmotic pressure and stimulates movement of fluids between compartments (58). The tonicity of a solution refers to the osmotic pressure created by the dissolved particles in the solution and directly influences the movement of fluid between compartments (58). The three tonicity states that influence fluid movement are: isotonic, hypotonic and hypertonic. An isotonic solution consists of equal osmotic gradients in the intracellular and extracellular compartment, meaning there is no net change in fluid movement between compartments, the osmolality in an isotonic state ranges

from 285-295 mOsm/kg (112). If the concentration of solutes in the extracellular environment is less than the intracellular compartment (less than 285 mOsm/kg), the cell is in a state of hypotonicity (58). A state of hypotonicity results in a net movement of fluid into the intracellular compartment and swelling of the cell occurs (112). The movement of fluid into the ICF occurs to restore the osmolality to an isotonic state. In a hypertonic state, the extracellular compartment has a greater concentration of solutes than the intracellular fluid and stimulates the movement of fluid out of the cell in an attempt to re-establish osmotic equilibrium, shrinking the cell. Serum osmolality greater than 295 mOsm/kg leads to cell shrinkage (58).

Organic osmolytes are molecules that alter osmolality without affecting cell functioning. These organic ions are found in numerous forms in animal cells and include polyols (glycerol, sorbitol), amino acids (glycine, taurine, proline), and methylamines (45). Of these ions, the most common in mammalian cells are the sugars, sorbitol and inositols; methylamines such as glycerophosphorylcholine; and the amino acid taurine (117). Organic osmolytes can be transported from the ECF or synthesized within the cell (62). The synthesis of organic osmolytes is initiated by the transcription and translation of key enzymes involved in their production and therefore this process is very slow (107). Swelling of the cell leads to a rapid efflux of osmolytes and a down regulation of synthesis within the cell by inhibiting the transcription of the genes responsible for activating the synthesis enzymes, a decrease in mRNA occurs and over a period of hours the number of osmolytes decreases (107). Because the mechanism of shifting

organic osmolytes can be slow, there are more rapid mechanisms that can be activated that result in alterations in ion levels that transport water across the membrane, in response to the osmotic stress.

c. Mechanisms of Water Movement

There are three primary mechanisms underlying the movement of water between the extracellular and intracellular compartments. The first and most inefficient is simple diffusion; the passive movement of water across a membrane, from an area of high water concentration to an area of low water concentration (58). Due to the charge water molecules possess, the cell membrane is not very permeable to these molecules, therefore simple diffusion is a slow and a non-regulated process(59). Co-transport is another mechanism of water movement, where water travels across the membrane during the active transport of ions or solutes(59). During anisosmotic conditions it is important that the movement of water between compartments be rapid and regulated so that the cell may return back to a near equilibrium state. The two methods described above cannot account for the rapid and regulated movement of water that occurs in tissues such as the kidney, red blood cells, and secretory cells(59). The third mechanism of water movement is via water channels, termed aquaporins (AQP).

AQP's are hydrophobic, integral proteins located within the cell membrane of both plants and animals (114). AQP's are expressed in both epithelial and endothelial tissue acting primarily as water transporters (AQP- 1, 2, 4, 5, 8), while some transport glycerol and other small solutes (AQP- 3, 7, 9, 10) (114). AQP

channels have a 10 to 100 fold higher capacity for water permeation than simple diffusion across the plasma membrane (1). To date thirteen AQP's have been detected in mammalian tissue, six of the thirteen are expressed in the kidney, which requires high water permeability to regulate water reabsorption and excretion (66). Mice lacking AQP1 in the kidney demonstrate decreased water permeability and increased serum osmolality following water deprivation (above 500 mOsm/kg) (65). Similarly, humans lacking AQP1 display an inability of the kidney to concentrate urine following fluid deprivation (56).

In the skeletal muscle AQP1 and AQP4 are the main water channels; AQP1 is expressed in skeletal muscle and smooth muscle, while AQP4 is predominantly located in fast-twitch muscle fibres (37, 38). Staining for AQP4 in rat soleus muscle, which is mainly composed of slow-twitch fibres, showed minimal AQP4 expression and fast-twitch fibres expressed a high number of AQP4 channels, suggesting that the role of AQP4 is related to fibre metabolism (37). Fast twitch fibres accumulate high levels of lactate following intense exercise and require rapid, efficient movement of water into the cell to restore muscle volume and decrease cellular fatigue (37). The high expression of AQP4 in the fast twitch muscle supports this hypothesis, but future research needs to confirm this speculation.

d. Ion transport

Anisosmotic conditions stimulate the movement of fluids between compartments in response to elevated or reduced osmolality. Inorganic ions such as Na^+ and Cl^- contribute to the osmolality of the extracellular environment,

while K^+ is found mainly within the intracellular compartment (62). In high concentrations, these inorganic ions can lead to detrimental effects within the compartments. For example, if NaCl is located intracellularly in high concentrations it will cause DNA damage(60). There are numerous co-transporters and channels along the sarcolemma to facilitate rapid movement of these ions following alterations in their concentrations (107).

Following exposure to anisosmotic solutions, the cells attempt to establish their isotonic state quickly, even with continued anisosmotic conditions. This is accomplished by the movement of ions through ion channels along the plasma membrane (45). Ion transport is the most efficient, acute means of altering cell volume following exposure to an anisosmotic environment (62). When exposed to a hypotonic solution a cell swells, but attempts to decrease its volume back to resting levels, this process is termed regulatory volume decrease (RVD). In contrast, exposure to hypertonic solutions result in a decreased cell volume and the cellular processes that attempt to bring the cell volume back up to resting levels is termed regulatory volume increase (RVI) (45). RVD is generally accomplished through the release of K^+ and Cl^- , through K^+ channels, anion channels and the KCl symporter (61). There is also an inhibition of ion uptake mechanisms (62). The accumulation of ions during RVI occurs via the sodium-potassium-chloride (Na^+- K^+- $2Cl^-$) cotransporter, Na^+/H^+ exchanger and Cl^-/HCO_3^- exchanger; leading to a net gain of Na^+ within the cell, increasing cell osmolality (61). This will stimulate the movement of water back into the cell in an

attempt to achieve isotonic conditions. Once isotonic conditions are re-established the Na^+ will be exchanged for K^+ by the Na^+-K^+-ATPase (61).

The mechanisms that stimulate the volume-sensitive channels and co-transporters in response to perturbations in cell volume are poorly understood. Future research would need to isolate each channel or co-transporter to better understand their contribution to the mechanisms of cell volume restoration.

CHAPTER 2: WHOLE BODY FLUID HYDRATION

a. Factors Influencing Whole Body Hydration

There are numerous factors that regulate the movement of water between compartments. Various hormones are involved in the regulation of fluid balance within the body. One such hormone is arginine vasopressin (also called the anti-diuretic hormone; ADH), released from the pituitary gland in response to changes in tonicity, blood volume and/or blood pressure(103). When released, ADH stimulates the reabsorption of water in the kidney in response to decreased ECF concentration or increased plasma Na^+ concentration (hypertonicity). A change in tonicity is the strongest stimulator of ADH, and is monitored by osmoreceptors in the hypothalamus. Resting osmolality of plasma ranges from 285 – 295 mOsm/kg, when osmolality drops below a threshold of 280mOsm, ADH is suppressed; when osmolality exceeds a threshold of 280mOsm ADH is stimulated and released to retain water (103). In addition, receptors in the atria monitor blood volume and stimulate ADH release when blood volume is reduced. The third stimulator of ADH is blood pressure, which is monitored by carotid and aortic baroreceptors (103). A decreased blood volume results in reduced blood

pressure and therefore stimulates ADH release. Furthermore, ADH activates a thirst response in an attempt to facilitate an increased fluid intake (28). Montain et al. (70) observed that increased levels of hyper-tonicity produced a graded increase in the amount of ADH detected in the blood at rest. Hypo-tonicity leads to a decreased thirst mechanism and a suppression of ADH released (102). Without ADH, the kidney's ability to regulate fluid balance is impaired and excretion of water occurs through the bladder.

Angiotensin II is another hormone that is important in fluid homeostasis. Angiotensinogen is a plasma protein that circulates in the blood. When a stimulus such as decreased blood pressure is sensed by the kidney it will lead to the release of renin that activates the conversion of angiotensinogen to angiotgensin I. Angiotensin I is converted to Angiotensin II, that travels to the adrenal gland where it stimulates the production and secretion of aldosterone (103). The main role of aldosterone is to increase the reabsorption of Na^+ in the kidney when Na^+ concentrations are compromised (hypo-osmolality) (103). Angiotensin also stimulates the release of ADH to increase the retention of water, promotes a thirst response and leads to vasoconstriction of vessels; all of these mechanisms are stimulated in an effort to maintain / increase blood volume(103).

Exercise is another potent stimulus for the movement of fluids between compartments. For example, Ploutz-Snyder et al. detected a reduction in plasma volume following a bout of exercise and this was correlated with an increase in the cross sectional area of active muscle (89). This finding supports the idea that exercise leads to the movement of fluid from the plasma to the intracellular and

interstitial space. Nose et al. observed a curvilinear response between increased plasma Na^+ levels and exercise intensity (80). At intensities greater than 70% VO_2 max there was a sharp increase in plasma Na^+ levels, suggesting an increase movement of fluids into the interstitial space (80). During exercise, there is also elevated metabolic activity within the cell, leading to increased osmotic pressure, stimulating an influx of fluid into the intracellular compartment (low solute concentration to high solute concentration) to re-establish an osmotic equilibrium (80). Nose et al., discovered with increased exercise intensity there was a linear decrease in plasma volume and an increased plasma osmolality (80). Following the initial loss of plasma volume to the intracellular compartment, angiotensin and ADH are released, increasing fluid reabsorption in the kidney, and the elevated plasma osmotic pressure stimulates fluid movement from the intracellular and interstitial compartment to the vascular space (24). Following a training protocol, plasma volume has been shown to increase, causing a decrease in ADH released in response to exercise stress (101). Long term exercise training leads to an overall increase in total body water, as demonstrated by Fellman et al. (32). Following a seven day training protocol, participants TBW increased on average 4.2L. This adaptation to training signifies the importance of whole body hydration when performing repeated exercise.

The primary purpose of the regulatory response to perturbations in hydration is to maintain plasma volume. A reduction in plasma volume results in decreased cardiovascular functioning due to decreased cardiac filling and stroke volume (93), and the inability to dissipate heat during exercise in elevated temperatures

(34). Within minutes of the onset of exercise there is a shift of fluid out of the vascular space to the interstitial space. Following this initial efflux, plasma volume stabilizes and is maintained through the increased oncotic pressure caused by plasma proteins and the release of hormones that increase fluid retention and the vasoconstriction of inactive tissue. The vasoconstriction decreases hydrostatic pressure in the capillaries, shifting fluid back to the vascular space (24). When plasma volume is compromised there are detrimental effects on the cardiovascular and thermoregulatory systems.

b. Impact on Cardiovascular and Thermoregulatory Systems

Altered hydration states impact the body's thermal and cardiovascular systems during exercise. During exercise in a hypohydrated state, stroke volume is compromised due to a decreased circulating blood volume, forcing an increase in heart rate and ultimately increased strain on the cardiovascular system leading to a decreased cardiac output over time (93). Due to the increased strain on the cardiovascular system, hypohydration also leads to a decreased time to fatigue during aerobic exercise as the body is unable to maintain cardiac output (71).

The reduction in circulating fluids in the body not only affects cardiovascular functioning, but also thermoregulatory mechanisms. Sweating is one way the body dissipates heat during exercise in response to elevated core temperatures, but exercising in a dehydrated condition (hyper-osmotic state) reduces the circulating fluid in the body. It has been observed that exercising in a dehydrated state decreases the body's sweat rate (34). The reduced sweating is a compensatory mechanism to conserve fluid within the body, but leads to

increased thermal strain. Under normal conditions exercise elevates heat production through increases in metabolism, and as exercise continues heat production exceeds heat loss (103). In response to the rise in heat production, the sweat rate and blood flow to the skin is increased. Exercising in a dehydrated state reduces sweat rate and compromises the ability to complete exercise protocols due to increased core temperature (34). Roy et al. found that exercising in a hypohydrated state increased body temperature to a greater extent than exercising in a euhydrated condition (93). Along with the elevation in body temperature, exercise in a hypohydrated state, can also increase levels of circulating norepinephrine and epinephrine which contribute to the vasoconstriction of some vessels and the redirection of blood to the exercising muscles (93). This vasoconstriction limits the blood flow to cutaneous tissue decreasing the capacity to dissipate heat and thus increasing the risk of heat exhaustion (77).

c. Impact on Metabolism

The tonicity of the extracellular fluid influences the metabolic processes within the cell. Hypo-osmolar extracellular solutions leading to cell swelling, promote an environment for anabolic events, whereas cell shrinkage caused by hyper-osmolality, stimulates catabolic events in the cell (55, 62). Antolic et al. used an in-vitro model to study anisosmotic conditions in skeletal muscle at rest (5). Hyper-osmotic media resulted in a breakdown of larger macromolecules to increase the osmolality of the cell. Farlinger et al. expanded on this work by investigating the effect of anisosmotic conditions on CHO metabolism (30). A

hyper-osmotic condition resulted in decreased glycogen content and increased glycogen synthase activation, while a hypo-osmotic media stimulated glycogen accumulation. From these results it can be concluded that alterations in cell volume influence metabolism within the cell. Keller et al. investigated the effects of anisosmotic conditions on metabolism using an in-vivo model. Extracellular hypo-osmolality led to decreased leucine release (measurement of protein oxidation) from endogenous proteins and decreased leucine oxidation (measurement of protein catabolism) compared to an iso-osmotic state. Keller et al. concluded that hypo-osmolality promoted a protein sparing effect (55). Plasma glucose concentrations were lower in the hypo-osmotic state compared to the hyper- and iso- osmotic states (55). The results by Keller et al. were in accordance with Berneis et al. who found that hypo-osmolality decreased plasma glucose levels and resulted in a positive protein balance (12). Furthermore, in both studies hyper-osmolar conditions resulted in no change in leucine flux or oxidation, while hepatic glucose production and plasma concentration were elevated (12, 55). Based on these studies it would appear that hypo-osmolality promotes a macromolecular anabolic environment, while hyper-osmolality has been shown to stimulate glycogenolysis.

Anisosmotic conditions can be achieved through a multitude of methods ranging from exercise to saline infusion. These alterations in hydration state influences hormone release, macromolecular metabolism, thermoregulatory and cardiovascular functioning at rest and during exercise. There are numerous systems stimulated following alterations in fluid volumes to re-establish cell

homeostasis. The tight control of cell volume indicates the importance of cell volume in proper functioning of the body.

d. Mechanisms of achieving hypo- / hyper-hydration

Anisosmotic conditions can result from exercise, environment, diet and medications, possibly resulting in hypo- or hyper hydration. Hypohydration is a whole body condition where there is a hyper-osmotic imbalance caused by water deficit or an excess amounts of Na^+ (58). This results in a loss of intracellular fluid and a decreased cell size, in an attempt to balance the elevated osmolality of the extracellular compartment. Exercising in a warm environment promotes arteriolar vasodilation to increase skin blood flow and sweat rate in an attempt to cool the body through evaporation. These two mechanisms stimulate the opening of capillary channels and increase hydrostatic pressure in the capillary beds (53). With a prolonged heat load, osmotic pressure results in fluid movement from the intracellular compartment to the extracellular compartment to restore plasma volume and cell shrinkage occurs (44, 81). Exercise induced dehydration has been compared to inactive thermal induced dehydration and it was concluded that both methods resulted in similar levels of dehydration (25). In research, diuretics have been administered to elicit hypohydration (6, 34, 93). Diuretics stimulate increased urine production and a loss of solutes, often resulting in hypohydration (97). Roy et al. achieved hypohydration through administration of a diuretic, called novotriamazide, for four successive days prior to exercise testing and participants decreased plasma volume by 14.6% (93). Similarly,

Fortney et al. elicited an 8.7% decrease in plasma volume following four days of novotriamazide diuretic administration (34).

Hyperhydration is a result of hypo-osmolar ECF conditions leading to swelling of cells, and can be achieved through elevated water intake stimulating a reduction of plasma Na^+ , or infusion of a hypotonic saline (58). This imbalance results in the movement of fluid from the extracellular space into the intracellular space.

e. Methods of Measuring Hydration Levels

An acute change in body weight is a common and non-invasive tool to measure hydration. Using this method, baseline body weight must be quantified preceding alterations in hydration. Limitations to this method of quantifying changes in hydration are that weight can be altered by food or fluid intake, sweating, urinary or bowel excretion (54).

Bioelectrical impedance (BIA) is another method that has been suggested to be an accurate, quick and non-invasive method to estimate hydration status using electrodes placed on the hand and foot (54). There are numerous factors that influence BIA including postural position prior to measurement, skin and body temperature, food and fluid consumption (54). Postural manipulations alter the hydrostatic pressure in the vessels resulting in fluid movement between compartments (67). Changes in body or skin temperature and food ingestion stimulates fluid movement and ultimately alters the compartment concentrations (54).

There are numerous variables that can be measured in the blood to estimate changes in hydration. Serum osmolality provides an immediate estimate of plasma volume. This method requires a small amount (approximately 50uL) of blood to be drawn. The blood can be measured using an osmometer to determine serum osmolality (55, 63). Hematocrit (Hct) and haemoglobin (Hb) levels in the blood represent plasma volume, but do not reflect total body water (54). Increased levels of Hct and Hb are indicative of decreased plasma volume (15) and decreased levels of Hct and Hb signify increased plasma volume (35).

When muscle biopsies are collected a wet to dry ratio of the muscle tissue can offer a direct method of measuring water content directly in muscle tissue. This method requires weighing muscle following freezing in liquid nitrogen, the muscle is lyophilized and weighed in a dry state. To determine the wet to dry ratio the following equation is used:

$$\frac{WETMASS - DRYMASS}{WETMASS} x100\%$$

A limitation to this method is that the lyophilizing can only be completed after the hydration protocol is completed. This eliminates the opportunity to make adjustments during any hydration protocol. Furthermore, this method cannot differentiate between interstitial or intracellular fluid. To determine the distribution of fluid between the interstitial and intracellular compartment, fibre diameter has been measured in an in-vitro model and results confirmed elevated fibre diameter following muscle incubation in a hypo-tonic bath (5).

There are various methods available to estimate both whole body hydration and tissue fluid levels, each having advantages and disadvantages depending on how the method is carried out and the experimental conditions. Future research is necessary to determine in the accuracy of combining these methods to give a more accurate representation of hydration status in the body.

CHAPTER 3: MUSCLE DAMAGE

a. Composition of Skeletal Muscle

Muscle fibres are multi-nucleated and are comprised of myofibrils encased by sarcolemma membranes. Myofibrils run the entire length of the muscle fibre, parallel to each other and are formed by a series of sarcomeres (95). These sarcomeres contain actin (thin) and myosin (thick) filaments that interact to produce muscle contractions. Light and electron microscopy can distinguish individual sarcomeres by light I bands and dark A bands that run the entire length of the myofibril. The A bands are where the myosin and actin interact with one another. In the middle of the A band is a lighter H band that is composed of only myosin filaments. The 'I' band forms the area where no myosin filaments are located. The area in the I band where actin filaments from adjacent sarcomeres attach is termed the Z-line (95).

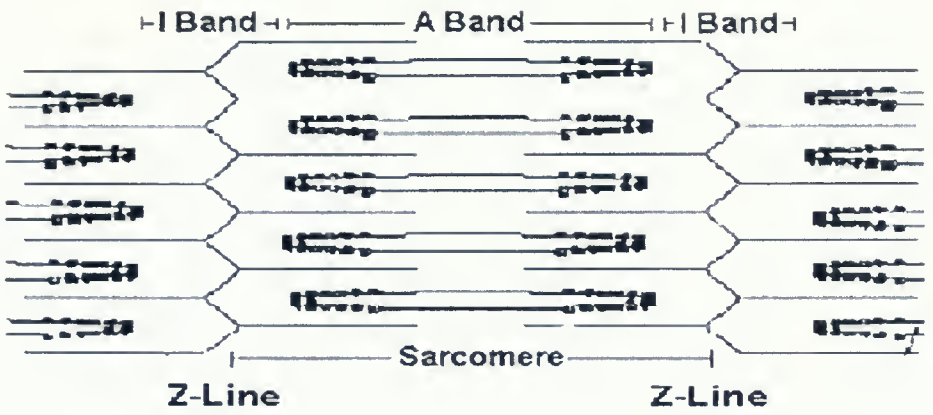


Figure 2. Schematic Diagram of a Sarcomere.

Excitation-contraction coupling (EC) describes the chemical and mechanical events of concentric contractions. Calcium release from the sarcoplasmic reticulum stimulates the binding of the myosin head to the actin filament, this is termed strong binding and leads to the power stroke; myosin pulls the actin until they overlap, shortening the sarcomere and the muscle fibre (29). Following the power stroke, adenosine triphosphate (ATP) attaches to a binding site on the myosin head, dissociating myosin from actin, and are now in a weak binding state (29). During an eccentric contraction, actin is pulled away from the myosin heads in the opposite direction of the power stroke; leading to a lengthening of the sarcomere. Walking downhill is commonly used as a model of eccentric activity. In this model, the quadriceps complete an eccentric contraction, controlling the amount of knee flexion against the force of gravity (29).

Eccentric muscle contractions commonly damage muscle fibres. The increased occurrence of damage during eccentric exercise can be explained in part by the force-velocity relationship (29, 91). When completing a concentric

contraction at a higher velocity, the maximal force that can be produced is decreased. Conversely, during an eccentric contraction the force production plateaus beyond a certain velocity and is therefore unaffected by increased velocity (29). Completing eccentric contractions require less motor unit recruitment, but the activated sarcomere are at their weakest point during these contractions (91). Contrary to the ATP-dependent dissociation of actin-myosin during concentric contractions, eccentric contractions are speculated to mechanically disrupt the filaments, damaging the binding sites on the actin and myosin (29). Following repeated eccentric contractions, the sarcomeres have an increased difficulty re-aligning with one another. This high force and low recruitment leaves the involved muscle fibres highly susceptible to mechanical damage and disruption (29).

b. Mechanical Damage

There are two main theories regarding the mechanisms of mechanical damage in skeletal muscle. Firstly, mechanical damage can occur as a direct effect of eccentric contractions (72). Secondly, damage can result from the metabolic waste products produced during contractions (3) The cross-bridge theory of muscle contraction states that force is generated by the actin filaments being pulled over the myosin filaments, leading to shortening of the sarcomere and the muscle fibre. Once a muscle contraction has occurred, ATP releases the actin and myosin crossbridges (29). However, because eccentric contractions lengthen the sarcomere there is a mechanical disruption of the actomyosin bond (29). Repeated eccentric contractions can lead to mechanical disruption of the

sarcomeres, termed the popping sarcomere hypothesis. This theory suggests that muscle damage resulting from lengthening contractions occurs due to non-uniform lengthening of sarcomeres beyond their optimal length (72). If this lengthening continues, more and more sarcomeres will be stretched past their optimal length, leading to the inability of filaments to overlap and contractions to occur, and thus a reduced capacity to produce force.

The result of repeated eccentric contractions are overstretched sarcomeres (40, 41), z-band streaming (9, 36, 40) and increased membrane permeability(17, 78). Gibala et al. found sarcomere disruption to be greater following eccentric resistance exercise compared to concentric resistance exercise of the biceps brachii in both untrained (40) and strength trained men (41). Mechanical damage is not limited to following resistance exercise, as z-line streaming is also observed following intense aerobic exercise, such as cycle ergometry (36). The extent of damage differs between muscle fibre types; z-line streaming in type II fibres is more severe during high intensity eccentric exercise than type I fibres (36). Type II fast twitch fibres are recruited during high intensity activity and therefore will be more susceptible to damage with this type of contraction. Furthermore, it has been observed that the stability of the z-line differs between fibre types. Type I fibres tend to have wider z-bands, that supports the muscle fibre, making them less susceptible to damage (90).

The direct disruption of the sarcomere structure is a mechanism of muscle damage, but may not be the only explanation of damage. Following repeated stretch contractions micro-tears in the membrane have been detected (3). These

micro-tears increase membrane permeability and disrupt the concentration gradients of metabolites and ions between compartments. For example, membrane tears result in an influx of calcium (Ca^{2+}) into the cell. The mitochondria takes up the excess Ca^{2+} impairing the cellular respiration cycle (3). High Ca^{2+} concentration stimulates proteases, such as calpain, that lead to the breakdown of the myofibrillar proteins desmin and α-actin (9). When compared to resting calpain concentrations, exercised rat muscle showed increased calpain levels that coincided with increased myofibrillar protein damage (11). Increased cytosolic Ca^{2+} concentrations also coincide with elevated creatine kinase (CK) levels in the plasma. CK is an intracellular enzyme that is released due to tears in the membrane, leading to speculation that Ca^{2+} enters the cell via tears in the sarcolemma (83).

Researchers have also observed that in response to repeated stretch contractions there are stretch activated channels that open leading to further disruption of the osmotic gradients. Stretch activated channels are located along the sarcolemma and open in response to the sarcolemma sustaining damage (3). There are non-selective stretch-activated channels that accept Ca^{2+}, Na^{+} and K^{+}, and selective channels that only allow K^{+} and Cl^{-} through (94). Stretch activated channels have been investigated thoroughly in skeletal and smooth muscle using animal models (51, 68, 118). To determine the route of a predominantly extracellular ion, Na^{+}, researchers blocked the Na^{+} stretch activated channels along the plasma membrane with the addition of Gadolinium ($GD3^{+}$), a known stretch activated channel blocker. Following eccentric contractions, they

discovered that the blockage attenuated the influx of Na^+ (118). Furthermore, following exposure to eccentric contractions, rat skeletal muscle demonstrated greater depolarization, due to increased Na^+ influx. When $GD3^+$ was introduced, there was significant membrane repolarization (68). The prevalence of stretch activated channels in human skeletal muscle has not been investigated, although indirect evidence supports the notion that stretch activated channels may be present, and contributes to the responses to contractile activity.

c. Oxidative Damage due to Inflammation

The initial events following exercise induced muscle damage remains uncertain, but what is known is that during exercise local production of pro-inflammatory interleukin-1β (IL-1 β) and tumour necrosis factor- α (TNF-α) occurs (87). IL-1β and TNF-α are classified as pro-inflammatory cytokines because they are released at the site of inflammation and stimulate the migration of inflammatory leukocytes such as neutrophils, macrophages, and lymphocytes from endothelial tissue and lymph nodes (88). These two pro-inflammatory cytokines work together and stimulate the release of IL-6; an inflammatory-responsive cytokine that is thought to be produced locally from the damaged skeletal muscle and also systemically from the endothelial tissue (87). Once neutrophils and macrophages have infiltrated the damaged tissue they begin to break down the tissue through phagocytosis and the release of oxygen free radicals (19).

Reactive oxygen species (ROS) are oxygen radicals produced naturally in the body during increased oxygen consumption and during the inflammatory

response (51). Common ROS include hydroxyl radical ($OH^{\cdot}$), superoxide radicals ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) (105). ROS are produced through the absorption of radiation or by redox reactions such as the electron transfer chain during oxidative phosphorylation (104). In the mitochondria, oxygen is taken up and reduced to produce ATP, during this process some of the oxygen molecules are missed during the reduction process and are left with univalently reduced oxygen intermediates (104). Since ROS are produced even at rest, they do serve positive functions in some cells. ROS are documented to be involved in the respiratory burst that is associated with inflammatory responses (50).

A second source of ROS secretion is during the inflammatory response. Muscle damage is detected in the body and stimulates the migration of polymorphoneutrophils (PMNs). PMN's are white blood cell elements that release lysosomes and ROS to breakdown damaged proteins (51). PMN's are detected in the blood immediately following eccentric exercise, and by 24 hours post exercise they have returned to pre exercise values (86, 110). ROS can also act as cytokines to initiate a migration of inflammatory mediators to the site of damage, termed chemotaxis (51). Infiltration of inflammatory mediators in response to damaged tissue or during exercise can lead to excess amounts of ROS that have detrimental effects on the cell. ROS form covalent bonds with proteins, lipids on the sarcolemma and nucleic acids; leading to the distortion of their structure and function (104). For example, phospholipids that compose the sarcolemma undergo lipid peroxidation when a ROS binds to a polyunsaturated

fatty acid (104). Excess lipid peroxidation can enhance membrane permeability and possibly tissue damage.

In summary, mechanical damage is thought to lead to disruption of the sarcolemma and ultimately disorganization of the sarcomeres; whereas oxidative damage occurs in response to elevated free radicals produced within the cell or are released during the inflammatory response. It is not known whether the mechanical damage leads to oxidative damage or if they are two separate processes that occur in response to exercise induced muscle damage. Future research is necessary to distinguish the two processes and determine the time course of each to understand muscle damage and develop methods to assist in the recovery period.

d. Measuring Muscle Damage: Direct Indicators

Following a bout of exercise, the extent of damage can be determined by visual analysis of muscle using light and/or electron microscopy. Direct indicators of muscle damage after exercise include disruption to the plasma membrane, z-line streaming and unaligned sarcomeres (36, 40, 41). A common method of light microscopy is toluidine blue staining. This method of staining allows for detection of focal z-band streaming. Friden et al. investigated the extent of myofibrillar damage using toluidine blue staining of muscle biopsies following eccentric cycling in humans. The sites of damage were recorded and expressed as a percentage of the total number of fibres viewed under the microscope. They observed focal disturbances in 32% of the fibres at one hour post exercise, 52% of the fibres at three days and 12% of the observed fibres six days following

exercise (36). No specific description was indicated as to what constituted focal damage. Z-line streaming, disruption of myosin filaments and loss of mitochondria in the areas of damage were also observed (36). Beaton et al. evaluated toluidine blue stained samples pre, four hours and 24 hours post exercise and classified the damage to z-lines as moderate (3-10 continuous z-lines with disruption) or extreme (10 or more continuous z-lines with disruption) and expressed the data per mm^2 of muscle quantified (9). Results showed greater z-line streaming at four hours and 24 hours post exercise compared to pre exercise levels. Stupka et al. quantified the amount of skeletal muscle damage and classified the damage as focal damage (encompassing two or less z-lines); or extensive (damage encompassing two or more z-lines) at two time points, pre and 48 hours following exercise (108). Results were expressed as the number of areas of damage (focal or extensive) per fibre. They observed a significant increase in both focal and extensive z-line streaming 48 hours post exercise. To confirm their results from light microscopy, they examined the samples under electron microscopy (108). Gibala et al. also used electron microscopy to compare the damage induced by eccentric contraction exercise and concentric contraction exercise (40, 41). They observed the greatest disruption of muscle fibres in the muscle completing the eccentric protocol compared to concentric contractions. A fibre was considered disrupted if there were any disturbances in the normal myofibrillar patterning (40). If 1-2 adjacent myofibrils showed disruptions it was classified as focal disruption; 3-10 disrupted myofibrils were classified as moderate and if damage was present in more than

10 myofibrils it was classified as extreme disruption. Following the eccentric exercise 81.7% of the fibres sampled were disrupted as compared to 33.2% of fibres following concentric exercise (40).

Direct sampling of muscle allows for the visualization of muscle damage and quantification of its occurrence within a sample. Although sampling and visual quantification of skeletal muscle is a direct method of quantification, the identification of sites of damage is subjective. Previous research that has used light microscopy with toluidine blue staining has offered limited visual identification of muscle damage and the classification of degrees of damage was vaguely described. It is important to have multiple independent individuals view and quantify damage to determine test-retest reliability.

e. Measuring Muscle Damage: Indirect Indicators

Indirect measurements of muscle damage include detection of intracellular proteins in the blood (78, 86, 100), inflammatory markers circulating in the blood (14, 84, 110), decreased force production (17, 78) and muscle soreness (78, 100).

The detection of intracellular proteins in the blood is used to indicate increased membrane permeability (86). Creatine kinase (CK), lactate dehydrogenase (LD) and myoglobin (MG) are intracellular proteins that are commonly measured as indicators of muscle membrane damage (17, 31, 78, 83, 86). For example, Feasson et al. found disruption of z-bands and sarcomeres along with increased myoglobin and CK levels in the plasma following a bout of downhill running (31). Similarly, Beaton et al. examined muscle damage following

a bout of eccentric exercise, and the results showed an increased level of CK 24 hours post exercise coincided with increased z-line streaming (9). These observations suggest that indirect markers of muscle damage are a consistent indicator of muscle damage when measured independently or in conjunction with direct markers.

CK is a common indirect measurement of skeletal muscle damage and elevated levels can be detected in plasma in as little as two hours following exercise and has been found to peak 24 – 48 hours post exercise (9, 31, 108). Resting plasma levels of CK range between 50-200 IU (52). Elevated CK has been detected in the blood up to six days post exercise (108). There is large variability in CK detected between studies, for example at 24 hours post exercise researchers have observed CK levels of approximately 1000 IU/L (31, 86) following running protocol, Hirose et al. at 24 hour observed approximately 400 IU/L following eccentric exercise of the elbow flexors (46) and Paulsen et al. at 24 hours detected CK levels of approximately 2500 U/L following eccentric exercise of quadriceps (84). Factors such as muscle mass recruitment, type and intensity of exercise and fitness level have been observed to influence the release of CK (13). Because of the high variability, measuring CK alone does not give an accurate representation of the muscle damage. Thus, measuring CK along with direct quantification or other indirect variables such as maximal voluntary contraction (MVC), range of motion (ROM) or circumference of muscle, along with inflammatory markers will give a more accurate representation of the severity of muscle damage.

Inflammatory markers such as cytokines and neutrophils have been more recently measured in the blood to determine level of inflammation, caused by muscle damage. TNF-α is said to be the first cytokine released (88), although previous literature has not been able to detect TNF-α in the blood following exercise (14, 106, 110). There are numerous speculations to explain why TNF-α is undetectable following these exercise bouts; if the blood is not collected immediately following the exercise the TNF-α might already have been degraded as the half life of TNF-α is very short (27). Petersen and Pedersen suggest that because TNF-α is generally not detected in the blood following exercise that the cytokine response differs between exercise induced muscle damage and infections (88).

IL-6 is a common cytokine measured in the blood (17, 86) because it is released in response to elevated levels of TNF-α and IL-1β. Peak et al. measured the inflammatory response and extent of muscle damage following a bout of downhill running. They observed an elevated neutrophil count, and plasma IL-6 levels coinciding with elevated CK levels immediately following exercise (86). Suzuki et al. observed similar results following a cycling protocol (110). They also observed an interrelationship between CK and neutrophil response following exercise (110). Neutrophil detection occurred before the CK, suggesting that the inflammatory response may further enhance muscle damage following exercise (110). They also observed increased concentrations of IL-6 immediately after, three hours, and 12 hours post exercise (110). These cytokines stimulate further inflammation infiltration to the injured cells to

breakdown and remove damaged proteins. The release of the cytokines occurs as a result of damage to skeletal muscle, consequently making cytokines an indirect indicator of damage.

The function of IL-6 in initiating the acute phase inflammatory response includes stimulating the release of hepatic acute phase proteins (APP) such as CRP and serum amyloid A (39). CRP accumulates at the inflammation site and is thought to be involved in further stimulating the release of IL-6, and TNF-α in monocytes (8). In-vitro research has also observed anti-inflammatory effects of CRP in stimulating the production of tissue factor, a coagulant, in monocytes and inhibiting the effects of neutrophils at the injury site (16, 119). CRP appears to have multiple functions in the inflammatory response and has been used as a marker of inflammation following exercise (2, 26, 84, 85).

Decreased force production in subsequent exercise bouts has been observed following intense exercise (74, 91, 100). It is common to experience a decrease in force production due to muscle fatigue in any exercise protocol, not just eccentric exercise. However, eccentric exercise does leave the muscle more susceptible to disrupted sarcomeres. This alone will not decrease force production, but if the disruption spreads along adjacent myofibrils, the excitation-contraction coupling process can be impaired (91). The timeline for force recovery has been investigated in numerous damage studies (17, 74, 78, 91, 100). Maximal isometric force was reduced immediately following an initial bout of eccentric contractions of the elbow flexors, and although force production increased in the next 48 hours, it still remained lower than baseline values (100).

Similarly, Chen & Hsieh had participants complete a similar protocol and observed decreased force production that remained below baseline at seven days post exercise.

There are numerous mechanisms for detecting muscle damage following a bout of exercise. Both indirect and direct measurements have strengths and weaknesses; muscle biopsies can be variable in detecting muscle damage (10) and although indirect markers are reliable in detecting muscle damage it is not possible to determine the location of the damage within the muscle fibres. Thus, due to the advantages and disadvantages of each method, they are commonly used in conjunction with each other in an attempt to quantify muscle damage.

CHAPTER 4: INTERACTION OF CELL VOLUME AND MUSCLE DAMAGE

To date no direct evidence has demonstrated that cell volume influences damage within muscles resulting from strenuous conditions. However, as discussed previously there is evidence to suggest that cell volume does influence the functioning of cell processes and there is also indirect evidence that suggests that damage to myofibrils is influenced by the hydration state of the cellular compartment.

a. Cell Volume and Muscle Damage: In-Vivo Animal Research

During exercise muscle fibres are placed under great stress leading to the breakdown of structural proteins. This breakdown stimulates the synthesis of new proteins possibly leading to muscle hypertrophy (116). Under resting conditions when the cell volume is elevated, there is a positive protein balance (55). An

increased cell volume may protect the cell from extensive exercise induced damage by altering the structure of the contractile arrangement. The myosin and actin filaments make up a structure that resembles a lattice network (49). Irving et al. placed frog skeletal muscle in a hyper-osmotic media and observed decreased lattice spacing, and increased lattice spacing was observed with hypotonic media (49). In a separate study, Vaughan et al. measured force development in frog skeletal muscle following hypertonic shrinkage and observed decreased isometric tetanus tension and the redevelopment of tension was slower in the hypertonic solution (113). The authors suggested that cell shrinkage caused by exposure to a hypertonic solution decreases the rate of cross bridge formation leading to decreased tension developed. It has not been investigated whether an increased cell volume, resulting in increased lattice spacing alters the damage response in myofibrils following contractions. A possible protective effect of increased cell volume may be caused by the increased lattice spacing allowing more space for the myosin heads to attach to the actin and thus, decreasing the breakage of the myosin-actin interaction during contraction. Future research is required to investigate the influence of increased lattice spacing on contractile induced damage. However, it is clear from previous literature that alterations in cell volume influence the structure and arrangement of myofibrils.

b. Cell Volume and Muscle Damage: In-Vivo Human Research

Indirect evidence of a relationship between cell hydration and muscle damage has come from the symptoms displayed in case reports of heat stroke.

The cause of the muscle damage with heat stroke is likely multifaceted; dehydration, increased core temperature or the exercise itself could each independently or in combination lead to the muscle damage observed. Sports such as weightlifting and wrestling that require athletes to cut weight leave themselves susceptible to heat stroke and muscle damage (109). Future research is required to establish the role of each possible factor and how and if they contribute to muscle damage.

The interaction between altered osmolality and muscle damage is an area of minimal research. Cleary et al. investigated the effect of dehydration on symptoms of delayed onset muscle soreness (DOMS) following a bout of downhill running (22). Participants completed a 45 minute walking protocol in a humid environment to induce dehydration or in a neutral environment to maintain euhydration followed by a 45 minute downhill run in a neutral environment. The symptoms of DOMS such as quadriceps perceived pain, punctuate tenderness, ROM and isometric strength were increased at 24 hours post exercise, however, were not significantly different between the dehydration and euhydration groups (21). There were a number of limitations with the study design. The protocol used to achieve dehydration involved walking in an elevated temperature chamber; following the exercise induced dehydration, participants completed a downhill run. One limitation of this study was that the initial bout of exercise used to dehydrate participants could have caused minor muscle damage, confounding the final results. Secondly, having participants exercise in a hot environment elicits a stress response within the cells (33), potentially exacerbating the

inflammatory response to the exercise. It is difficult to conclude what led to the observed results. More specifically, it is unclear if the dehydration or the elevated environmental temperature confounded the findings of their study.

Hargreaves et al. investigated the effect that water intake had on muscle metabolism during a prolonged exercise trial. Results showed no difference between the fluid and no fluid intake groups in ATP, creatine phosphate and creatine levels. Muscle glycogen was higher and lactate levels were lower in the fluid intake group, suggesting that the fluid consumption promoted alterations in the metabolic response to the exercise (43). Saunders et al. conducted a study with alpine skiers to determine if ingesting carbohydrate and protein drinks would reduce the amount of damage compared to no fluid ingestion. This study showed that consuming a carbohydrate-protein drink decreased levels of CK following the exercise protocol when compared to a carbohydrate only drink (96). The results of this study are not clear, as there are two possible explanations for the results observed; the elevated fluid level alone, or the addition of macronutrients to the fluid led to the reduction in CK levels that were observed. Future research needs to first identify if fluid consumption alone decreases the severity of damage and further investigation is needed to determine how cell hydration influences muscle damage during a bout of exercise.

Taken together, it appears that hydration and fluid levels may have significant metabolic effects during exercise. Unfortunately little information regarding cell volume and muscle damage exists in the literature. What is known is that alterations in cell volume influence the structure of myofibrils (49), and a hypo-

osmolar extracellular state promotes an environment for the production of macromolecules within the cell (12, 55). Following a bout of high intensity exercise in a state of euhydration there is a breakdown of proteins (9, 11). If exercise normally leads to catabolism following exercise in muscle cells, and a hypo-osmolar extracellular state protects the cell during resting conditions one could speculate that exercising in a state of elevated cell volume could possibly protect the muscle cell from damage to structural proteins and ultimately lead to less muscle damage.

CHAPTER 5: STATEMENT OF THE PROBLEM

a. Statement of the Problem

To date no research has investigated the influence cell volume has on the susceptibility of skeletal muscle to damage in response to a bout of eccentric exercise. The literature has shown that both decreased cell volume and eccentric exercise stimulates proteolysis within the cell. No research has investigated the possible influence of elevated skeletal muscle volume has on muscle damage during and following exercise.

The primary purpose of the current investigation is to develop an infusion protocol to achieve an elevated muscle fluid level in a human in-vivo model. The secondary purpose is to establish if an increase in muscle fluid content, resulting from the infusion protocol, can alter the muscle damage response following a bout of eccentric exercise, as indicated through both direct and indirect markers of muscle damage.

b. Hypotheses

Based on the literature it is hypothesized that an elevated muscle fluid level will protect the exercising skeletal muscle leading to less extensive skeletal muscle damage and inflammatory response following a bout of resistance exercise when compared to euhydrated / isotonic conditions.

CHAPTER 6: METHODS

a. Participants

Eight healthy, young males volunteered for this study. All participants were between the ages of 18-30 years with an average body mass of 73.4±10.0kg. All subjects were untrained or participated in aerobic activity less than three times a week; no participants were currently resistance training. The study protocol and any associated risks involved with participation were explained to each participant before written consent was obtained. The study was approved by the research ethics boards at both Hamilton Health Sciences Faculty of Health Sciences Research Ethics Board and Brock University Research Ethics Board.

b. Pre-experimental Protocol

All participants completed a three-day dietary record, consisting of one weekend day and two weekdays prior to the trial. Dietary records were analyzed using Diet Analysis + (Thompson Wadsworth: Florence, KY) to determine habitual dietary intakes. Participants were instructed to include all fluid and solids consumed the amount, and method of preparation. To control for diet, each participant was then given a diet checklist that they were to use as a guide for food and fluid consumption for the days leading up to both trials. Alcohol and caffeine consumption were restricted for 24 hours prior to each trial. Participants were asked to refrain from any physical activity for 48 hours prior to each trial and not change their habitual physical activity levels between trials.

c. Experimental Protocol

To test the hypothesis each participant completed two randomly assigned experimental trials on two separate occasions, separated by a four week rest period: one trial was a hydrated (HYD) condition and the other was a control (CON) condition. Subjects arrived at the laboratory after an overnight fast of approximately 12 hours. Each participant consumed a standardized snack two hours before arriving in the lab on each experimental day (Ensure Meal Replacement: 250 Calories; 38.0g CHO; 9.4g PRO; 6.7g FAT per 235mL bottle). Height and weight of each participant was determined following voiding their bladder.

Leg circumference (cm) and a skinfold measurement (mm) halfway between the knee and inguinal line was collected prior to the infusion protocol to determine the cross sectional area of the quadriceps, using the equation developed by Housh et al. (Quadriceps CSA = [(2.52 * leg circumference) – (1.25 * skinfold) – 45.13] (47). Bioelectrical impedance analysis (BIA) was conducted using the Quantum II Bioelectrical Impedance Analyzer (RJL Systems: Clinton, MI) following 15 minutes of lying supine with arms and legs at 45 degree angles from the body. BIA was used to estimate the distribution of fluids between the intracellular and extracellular compartments. Koulmann et al. described a method of measuring BIA where the skin of the right hand and ipsilateral foot are shaved and cleaned with alcohol. Electrodes are then placed on the dorsal side of third finger just below the phalangeal-metacarpal joint and at the mid-point between the second and third metatarsal on the superior side of the foot. The detector

electrodes are then placed on the posterior side of the right wrist and on the anterior ankle and measurements are taken (57).

The volume of 0.45% saline infused during the hydration protocol was based on body surface area ($20mL/min^{-1}/1.73m^{-2}$) (18). To determine the body surface area, height and weight was determined and used in the following equations: (a). √ weight (kg) X height (cm) / 3600 giving a value in meters squared. To determine the mL/min the following calculation was used (b). [$20(ml/min^{-1})$ X x (m^2) / $1.73(m^{-2})$]; where 'x' is the number from calculation (a). The number was then multiplied by 120 minutes to determine the total infusion volume.

Following the initial measurements, a catheter was inserted into an antecubital vein of one arm and the infusion of warmed 0.45% saline began (HYD). During the HYD protocol all participants were instructed to lie supine for the full 120 minutes. A catheter was not inserted during the CON protocol, but participants were also instructed to lie supine for 120 minutes.

After the 120 minute infusion, or rest, a muscle biopsy was collected using the needle biopsy technique from the vastus lateralis under local anaethesia (2% lidocaine). Incisions were made on the lateral portion of the vastus lateralis. The biopsy needle was inserted into the incision and once past the subcutaneous fat and fascia the needle was advanced along the muscle. The needle was parallel with the longitudinal axis of the muscle fibres and a sample collected. A blood sample was then collected from the antecubital vein of the opposite arm used for the infusion into an untreated Vacutainer. Following the infusion, body mass of the participants was determined and a final BIA analysis was conducted.

A single leg model was used for the exercise protocol, where the opposite leg to the one that had been biopsied completed the trial (exercise leg). The leg used for the exercise protocol was randomly assigned for trial one. All exercise and force measurements were completed on a biodex isokinetic dynamometer (Biodex Medical Systems: Shirley NY). Maximal voluntary contraction (MVC) was measured before and two minutes following the eccentric exercise protocol. The eccentric exercise protocol consisted of isokinetic eccentric exercise of the quadriceps. Participants were instructed to gently guide their leg into knee extension and resist the dynamometer moving the leg back into knee flexion. The speed of the exercise was 180°/sec through extension and 60°/sec through flexion. The exercise routine consisted of 10 sets of 10 repetitions were completed with a one minute rest between sets. Participants were actively encouraged to resist the knee flexion.

Immediately after the exercise protocol a second muscle biopsy was collected from the exercise leg. A second blood sample was also collected at this time. The third and final biopsy was collected 180 minutes following the exercise protocol. Blood samples were collected at 180 minutes as well as 24 hours after the exercise protocol (see figure 2).

Following a four week rest period, participants then completed the second exercise trial (HYD or CON), and the control leg from the first trial performed the exercise during trial two.

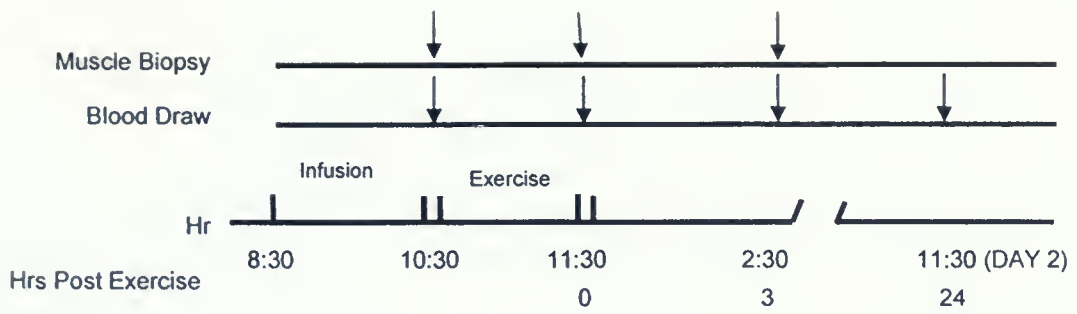


Figure 3. Outline of exercise trial.

CHAPTER 7: ANALYSIS

a. Blood Analysis

Blood samples (7-10mL) were drawn from an antecubital vein into untreated Vacutainers. Blood samples were allowed to clot for 20-25 minutes before being centrifuged for 10 minutes at 3500g. Serum was collected from the centrifuged blood and stored at -80°C in three separate aliquots until analysis could be completed. One aliquot was used for CK and lactate dehydrogenase (LD) analysis. CK and LD samples were taken to the Core lab at McMaster University Medical Center where serum samples were analyzed using commercially available kits manufactured by Roche Diagnostics (Laval, QC). A second aliquot of blood serum was analyzed for interleukin-6 (IL-6) (R&D Systems, Minneapolis, MN) using a quantitative sandwich enzyme immunoassay technique. The third aliquot was used to quantify C-reactive protein (CRP) content in the plasma using a quantitative sandwich enzyme immunoassay technique (Alpha Diagnostics, San Antonio, TX). Serum osmolality was quantified using a Wescor Vapour

Pressure osmometer (Logan, UT). Osmolality determinations were done in triplicate and the average was calculated.

b. Muscle Analysis

A total of six muscle biopsies were collected (pre, immediately post, three hours post, for two trials). Each muscle biopsy was split into two pieces; one section was immediately placed in a chilled fixative (2% gluteraldehyde buffered with 0.1% sodium cacoldylate). Following the gluteraldehyde-fixative, the muscle was postfixed in 1% osmium tetroxide, dehydrated in graded alcohol and embedded in plastic resin. Longitudinal semithin sections (~1μm thick) were cut with a glass knife and stained with toluidine blue (108). These sections were used for quantification of muscle fibre damage using toluidine blue staining. The methodology used for quanitification was similar to the procedure performed by Beaton et al. (9), who classified damage as moderate if it encompassed three to 10 adjacent z-lines and extensive damage consisted of 11 or more adjacent z-lines. The total number of muscle fibres per sample was counted and the amount of focal or extensive damage was expressed per muscle fibre. A second portion of muscle was used for the determination of water content by weighing frozen samples, then lyophilizing and re-weighing. The differences in sample mass were used to estimate muscle water content and expressed as relative water content using the following formula:

$$\frac{WETMASS - DRYMASS}{WETMASS} x100\%$$

c. Isometric Strength

Voluntary isometric peak torque [maximal voluntary contraction (MVC)] of the exercised quadriceps was measured on a Biodex isokinetic dynamometer immediately pre-exercise and two minutes following the isokinetic exercise protocol. MVC was measured to determine the change in maximal force generation following the damage protocol. Subjects completed three MVC isometric knee extension contractions, holding each for five seconds. A rest period of 30 seconds was given between each repetition. The average torque for the three repetitions was recorded.

d. Statistical Analysis

Blood markers, MVC, work output, light microscopy, and BIA data were analyzed using a two way repeated measures analysis of variance (ANOVA) with condition and time as the within factors. If significant interactions were found ($p<0.05$) a Tukey post-hoc analysis was conducted to determine which of the pair wise comparisons were statistically significant. A one-way analysis of variance was conducted on the body weight, muscle weight and plasma osmolality data. If the one-way ANOVA was significant ($p<0.05$), a Tukey post-hoc analysis was conducted to determine which of the pair wise comparisons were statistically significant.

CHAPTER 8: RESULTS

a. Anthropometrics

Body Mass

There were no differences in body mass of the participants before each trial (HYP, 73.4 ± 10.0 kg, CON, 74.3 ± 10.9 kg). Within the HYD condition there was a significantly greater body mass following the infusion (75.1 ± 10.7 kg) compared to the pre (73.4 ± 10.0 kg) mass ($p < 0.05$).

Bioelectrical Impedance (BIA)

There were no significant differences in the total body water (TBW) between the two conditions at the pre time point (see figure 4). When TBW was calculated following the two hour rest period (CON) or the infusion (HYD) there were significantly higher levels of fluid in the HYD trial compared to the CON trial ($p<0.05$). TBW levels Post infusion/rest were greater compared to pre levels in the HYD condition ($p<0.05$). Along with estimating the TBW, the BIA also estimates the distribution of fluid between the intracellular compartment (ICW) and extracellular compartment (ECW). The BIA results showed that the ICW measured prior to (pre) and following (post) the infusion (HYD) or the rest period (CON) was not statistically different between conditions. In contrast, there was significantly greater water content in the extracellular compartment in the CON condition compared to the HYD condition pre infusion ($p<0.05$). Following the infusion protocol (HYD) or rest period (CON) there was significantly greater water content in the HYD condition compared to the CON condition ($p<0.05$). BIA

measurements also demonstrated that the infusion resulted in a significant increase in ECW compared to pre infusion levels in the HYD group.

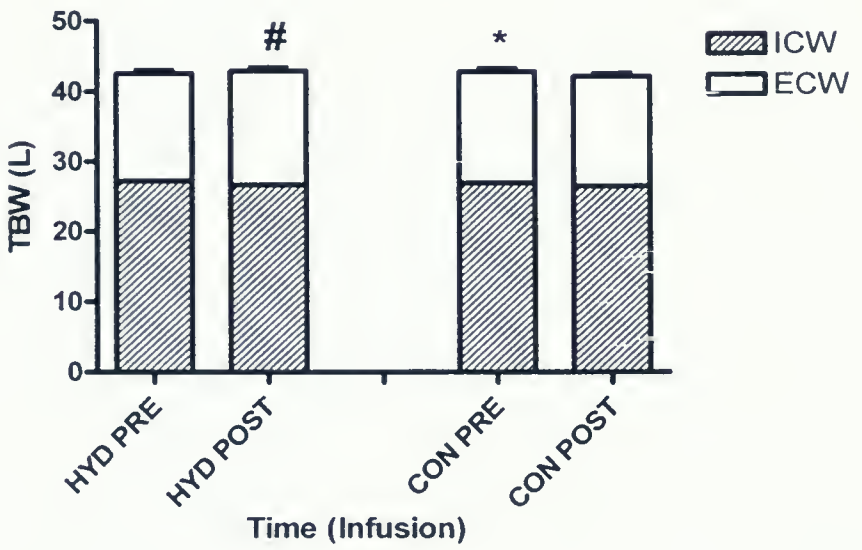


Figure 4. Bioelectrical impedance analysis

Total body water, intracellular (closed bar) and extracellular (open bars) water levels in body pre and post infusion. All values expressed as means ± SE (n = 8). HYD = Hydration condition; CON = control condition; ICW = intracellular water; ECW = extracellular water. # = significantly different than CON post ECW; * = significantly different than HYD pre ECW.

b. Work Output

Isokinetic work was not statistically different between sets in the HYD condition. There was a trend for work output to increase in the first five sets and then decrease in the later sets. In the CON condition there were also no significant differences between sets. The work output followed a similar trend as observed in the HYD condition, but no significant differences were observed

(Figure 5). The HYD and CON condition demonstrated no significant differences in work output, thus there was no treatment effect.

Isometric peak torque was significantly reduced ($p<0.05$) post exercise compared to pre exercise in the HYD condition (Figure 6). Similarly, in the CON condition isometric peak torque significantly ($p<0.05$) decreased from pre exercise to post exercise (Figure 6). The decline in peak torque was similar between the two experimental conditions.

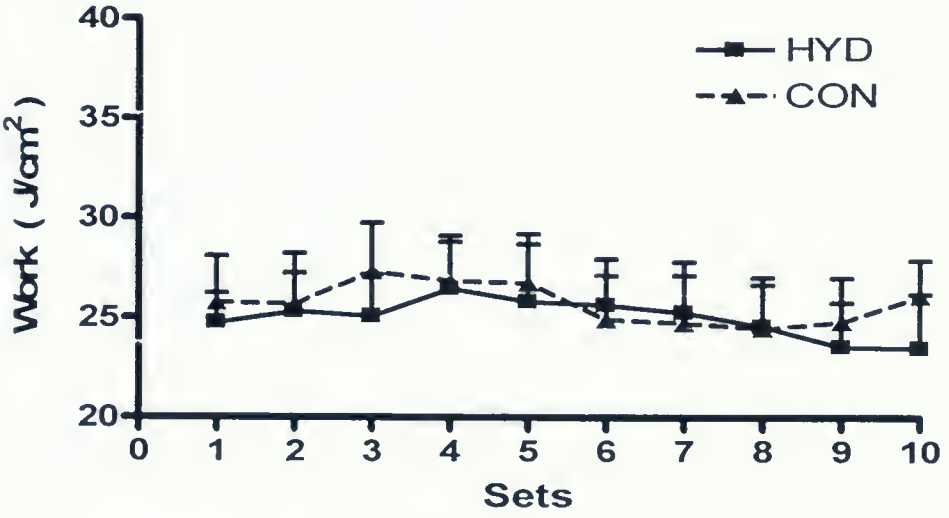


Figure 5. Work output

Expressed in J/cm^2 for each set in hydration (squares) and control (triangles) conditions. All values expressed as means ± SE (n = 8).

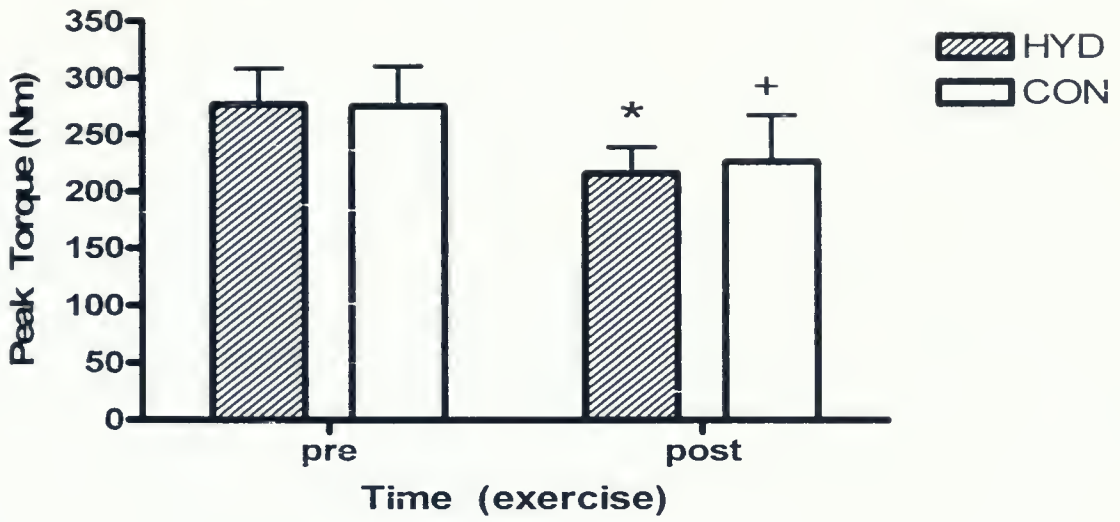


Figure 6. Peak isometric torque

Pre and post exercise hydration (closed bars) and control (open bars) conditions. All values expressed as means ± SE (n = 8). HYD = hydration condition; CON = control condition. * Significantly different from pre values in HYD condition. + Significantly different from pre values in CON condition.

c. Blood Analysis

Plasma Osmolality

Plasma osmolality was significantly different between HYD and CON conditions ($p<0.05$). Following the infusion during the HYD condition plasma osmolality was 268.5 ± 2.7 $mmol \cdot kg^{-1}$. Whereas, serum osmolality in the CON condition was significantly greater at 271.5 ± 2.8 $mmol \cdot kg^{-1}$.

Creatine Kinase

The CON condition had significantly ($p < 0.001$) greater levels of CK at 24 hours (531.4 ± 118.9 IU) compared to pre (183.0 ± 61.6 IU), immediately post

(201.1 ± 64.4 IU) and three hours post (216.37 ± 66.6 IU) exercise (Figure 7). In the HYD condition, there was a significant ($p<0.001$) increase in CK levels in the blood 24 hours (440.3 ± 51.4 IU) after exercise compared to pre (85.1 ± 6.0 IU), immediately post (94.87± 6.9 IU) and three hours (128.7 ± 12.5 IU) post exercise (Figure 7). There were no significant ($p>0.05$) differences between the HYD and CON condition at any of the four measured time points.

Lactate Dehydrogenase

Similar to the CK results, LD values in the control condition had significantly ($p<0.05$) greater values 24 hours post exercise compared to pre exercise values (Figure 8). LD in the HYD condition was significantly greater 24 hours post exercise compared to pre ($p<0.001$), post ($p <0.05$) and three hours post ($p <0.05$) exercise (Figure 8). Post exercise LD levels were significantly ($p<0.05$) higher in the CON condition compared to the HYD condition. However, LD concentrations were similar between conditions at all other time points.

C-Reactive Protein and IL-6

There was a significant main effect ($p<0.05$) for time, such that CRP levels increased consistently at each time point in both conditions. There were no significant differences between the conditions (Table 1). Similarly, for IL-6 there were no significant differences ($p>0.05$) in IL-6 concentration between conditions at any time point. Furthermore, there were no significant differences between time points (Table 1).

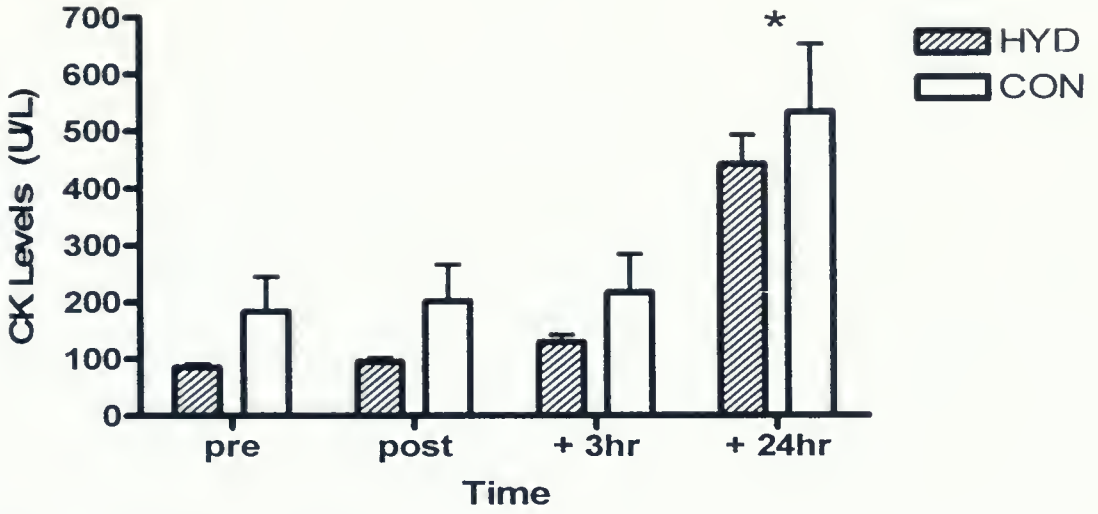


Figure 7. Creatine Kinase

CK levels in serum during hydration (closed bars) and control (open bars) pre, immediately post, 3hr and 24hr post exercise. All values expressed as means ± SE (n = 8). CK = creatine kinase; HYD = hydration condition; CON = control condition. * 24hr significantly different from pre, post, 3hr in HYD and CON conditions.

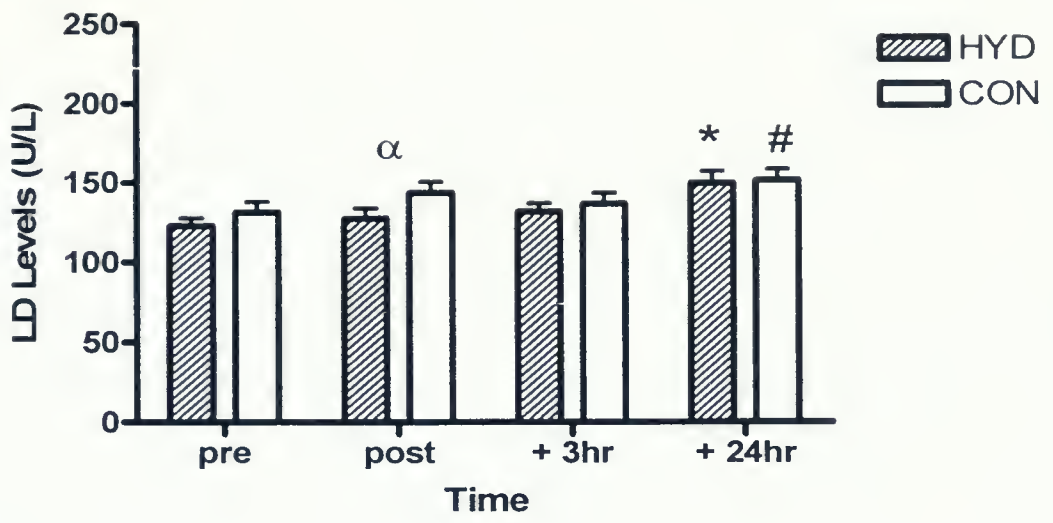


Figure 8. Lactate dehydrogenase

LD levels in serum during hydration (closed bars) and control (open bars) pre, immediately post, 3hr and 24hr post exercise. All values expressed as means ± SE (n = 8). LD = lactate dehydrogenase; HYD = hydration condition; CON = control condition. * = 24hr significantly different from pre, post, 3hr in HYD condition; # = 24hr significantly different from pre in CON condition; α = significant difference between CON and HYD condition post.

Table 1. Blood Concentrations of IL-6 and CRP

Blood Marker	Condition	Pre	Immed. Post	3hr. Post	24hr. Post
IL-6 (pg/ml)	CON	0.131 ± 0.008	0.139 ± 0.007	0.137 ± 0.006	0.134 ± 0.007
	HYD	0.123 ± 0.002	0.138 ± 0.009	0.136 ± 0.006	0.135 ± 0.006
C-RP (ng/ml)	CON	548.21 ± 234.75	604.76 ± 385.68	593.02 ± 313.28	2691.80 ± 1043.33
	HYD	728.81 ± 331.66	679.32 ± 391.47	684.55 ± 340.83	2506.70 ± 1324.60

Note: Values are expressed as means ± SE; n = 8. CON = control group; HYD = Hydration group; IL-6 = interleukin-6; C-RP = C- reactive protein. Main effect for time ($p<0.05$), for CRP, such that 24hr higher than pre, immediately post, and 3hr post exercise.

d. Muscle Analysis

Water Content

Following the infusion during the HYD condition relative muscle water content (80.1 ± 0.8%) was significantly greater than during the CON (77.9 ± 0.6%) condition prior to exercise (Figure 9). The muscle water content immediately following exercise was not statistically different ($p>0.05$) from pre levels in both the HYD and CON condition. Although not statistically significant ($p=0.056$), there was a trend for the HYD condition to have greater water content (79.6 ± 0.4%) immediately post exercise compared to the CON condition (78.1± 0.6%). There were no significant differences ($p>0.05$) three hours post exercise between conditions or compared to pre levels.

Light Microscopy

An average of 57.7 fibres (range 29-115) per sample were counted and analyzed. Samples were blindly assessed by two people on two separate occasions, to test inter- and intra-variability of the quantification. Pearson r value for intra variability was 0.97 and the inter variability was 0.93. The number of areas of damage was expressed per muscle fibre (see Table 2). There were no differences in the number of areas of moderate damage in the CON condition at the measured time points. Similarly, there were no differences in the number of areas of moderate damage observed at any of the time points during the HYD condition. Furthermore, there were no significant differences in areas of moderate muscle damage between the HYD and CON group (Figure 10).

The CON condition resulted in no significant differences in extreme areas of damage between pre, post and three hour time points. Similarly, within the HYD condition there were no significant differences between pre, post and the three hour time points. There were no significant differences in extreme muscle damage between the HYD and CON groups. An example of a muscle stained with toluidine blue is provided in figure 11.

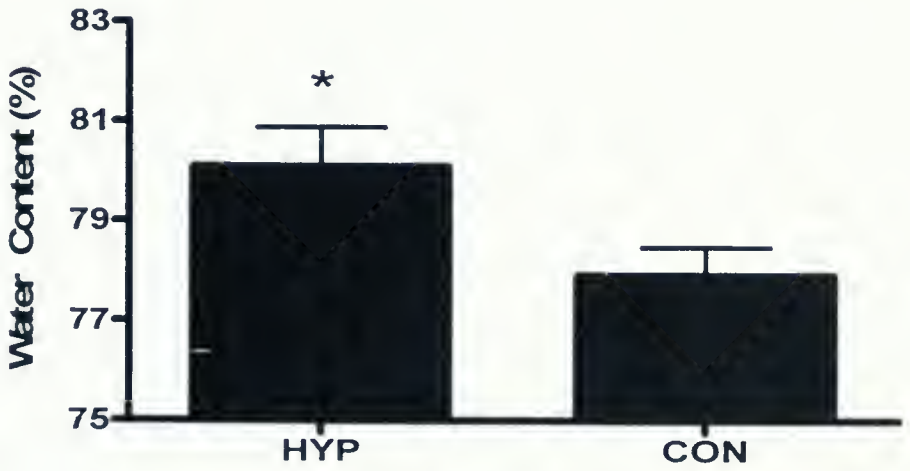


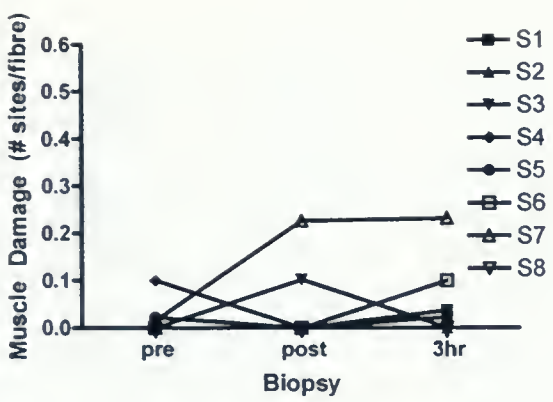
Figure 9. Percent water content post infusion

All values expressed as means ± SE (n = 8). * Significantly different from CON condition.

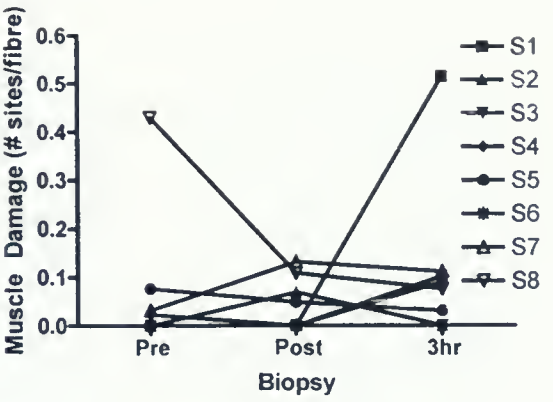
Table 2. Average Sites of Damage.

Muscle Damage	Condition	Pre	Immed. Post	3hr. Post
Moderate	CON	0.017 ± 0.012	0.047 ± 0.033	0.049 ± 0.029
	HYD	0.070 ± 0.052	0.045 ± 0.019	0.115 ± 0.059
Extreme	CON	0.003 ± 0.003	0.002 ± 0.002	0.011 ± 0.004
	HYD	0.010 ± 0.009	0.014 ± 0.008	0.006 ± 0.004

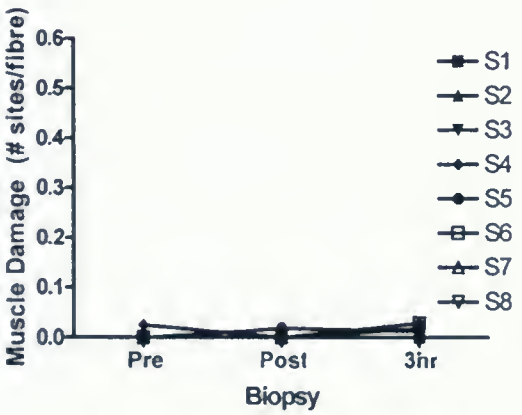
Note: Values are expressed number of sites of damage per muscle fibre. Values are expressed as means ± SE; n = 8.
CON = control group; HYD = Hydration group.



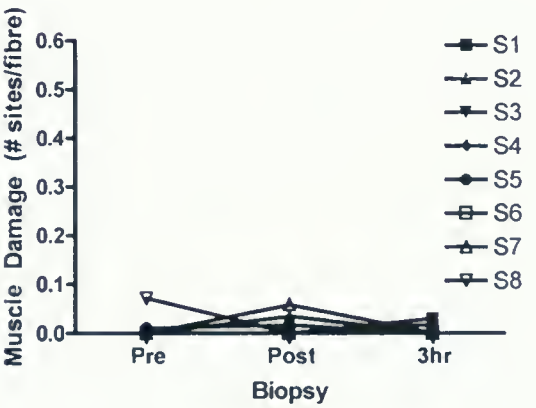
A. HYD – moderate



B. CON - moderate



C. HYD - extreme



D. CON - extreme

Figure 10. Muscle damage per fibre, by subject

A, HYD = hydration trial, moderate damage; B, CON = control trial, moderate damage; C, HYD = hydration trial, extreme damage; D, CON = control trial, extreme damage.

Figure 11. Toluidine blue stained muscle.

(A) undamaged muscle; (B) moderately damaged muscle; (C) extreme damage.

CHAPTER 9: DISCUSSION

a. Development of Model

A novel finding of the current investigation was that the implemented infusion protocol resulted in a significant increase in muscle fibre water content evidenced by the increased relative water content ratio in the HYD condition compared to the CON condition. Muscle from the HYD condition had a relative water content of 80.1 ± 0.8% and the CON condition had a relative water content of 77.9 ± 0.6%.

The current study is to our knowledge, the first to attempt to isolate fluid alterations and investigate their influence on a bout of exercise. The current infusion protocol was adapted from Claris-Appiani et al. who infused participants with a 0.45% hypotonic NaCl solution for two hours at a rate of 20mL/min^{-1}/1.73m^{-2} to investigate kidney function (18). They observed a urine clearance rate lower than the infusion rate, indicating an accumulation of fluid within the body (18). The infusion of hypotonic saline stimulates the movement of fluid into the muscle fibres and other tissue from the plasma due to the osmotic gradient created between compartments. It has been well documented that when alterations in total body water occur, plasma volume is generally well protected (24, 34, 81). The principle that determines fluid movement between compartments assists in explaining the protection of plasma volume. Hydrostatic pressure is the pressure the vascular fluid exerts on the vessel walls (58). The increased pressure leads to a shift in fluid from the vascular space to the interstitial space (111). Applying these principles to an infusion protocol suggests

that in the situation where there is an excess of fluid infused (hyperhydration), fluid will move to the interstitial space to return the plasma volume to resting levels.

There has been limited research to date that has attempted to achieve an elevated muscle cell volume in-vivo. Previous research has attempted to investigate alterations in cell volume on exercise performance (22, 23, 99) but has mainly focused on using dehydration as a method to alter TBW. A major limitation in the design of the dehydration protocols is the use of exercise in hot environments to achieve dehydration. This confounds the results as it is unclear whether the elevated environmental temperature or the fluid alteration has contributed to the development of muscle damage.

Previous research has used various hydration protocols to achieve hyperhydration. Oral consumption of a water-glycerol mixture at rest has been found to increase plasma osmolality and reduce urine volume, ultimately achieving hyperhydration (92). When compared to water ingestion alone, consumption of a glycerol solution significantly decreased urine flow and increased the release of anti-diuretic hormone (ADH) (35, 42, 73). However, there are some negative side-effects of glycerol consumption: including nausea, bloating and light-headedness when glycerol is consumed prior to exercise (73).

Another model that has been used is an infusion of a 0.45% hypotonic saline combined with administration of desmopressin (DDAVP), an anti-diuretic drug (12, 55). Desmopressin mimics the actions of arginine vasopressin, which stimulates reabsorption of water in the renal tubule (7).

Although both of these methods were effective at increasing fluid retention and both resulted in a state of hyperhydration, the relative distribution of the excess fluid between the various body compartments was not determined. In the current research, relative skeletal muscle water content was calculated using a wet-dry muscle ratio. Previous research completed in our laboratory using an isolated in-vitro model observed an elevated wet-dry ratio in skeletal muscle tissue that coincided with increased muscle fibre diameters when muscle was incubated in a hypotonic solution (5, 30). The relative water content in the current study was lower (80.1%) than the levels observed by Farlinger et al. who observed 83.0% relative water content in an hypo-osmotic extracellular condition, using an isolated in-vitro model (30). One of the advantages of using an in-vitro model is the ability to manipulate the extracellular media to simulate either physiological or non-physiological conditions. In their study muscle was incubated in hypo-osmotic media with an osmolality of 190 ± 10 $mmol \cdot kg^{-1}$. Although in the current research, attaining these levels was not possible, the infusion of 0.45% saline did result in a reduction in serum osmolality and an elevated relative water content in muscle that was similar to what was previously observed using an in-vitro hypo-osmotic condition (5, 30). A limitation in using a wet-dry ratio to determine water content is the inability to distinguish the distribution of fluid between the intracellular compartment and the interstitial fluid. Antolic et al. observed a correlation between elevated fibre diameter and increased wet-dry ratio in muscle incubated in a hypotonic extracellular media (5). These observations suggest that fluid is moving into the intracellular

compartment; however, this method needs to be established in an in-vivo model. Future research could investigate the distribution of fluid between the interstitial and intracellular compartment following an infusion.

Another method used to estimate the distribution of fluid in the body is bioelectrical impedance (BIA) (57). However, in the current study BIA results were not consistent with the wet-dry ratio results in quantifying changes in fluid volume between compartments following the infusion protocol, leading to speculation that the BIA system used may not be sensitive enough for these experimental conditions. The BIA results in the current investigation detected significant differences in the extracellular compartment volume between the HYD and CON condition that was not observed using the wet-dry ratio. Diet, timeline of measurements and methodology between trials were standardized to minimize the possibility of these variables interfering with the results. Therefore, the inconsistency of results between the wet-dry ratio and the BIA are likely caused by the insensitivity of the BIA methods under the current experimental conditions. Koulmann et al. evaluated the use of BIA in estimating changes in fluid compartments with varying levels of hydration and determined that BIA results were accurate in determining total body water levels, but was unable to accurately determine the changes that occurred following dehydration. They concluded that further research needs to be conducted to determine the validity of BIA (57).

Although the method of determining muscle water content used in the current research was unable to determine the distribution of water within the intracellular

compartment, we can conclude that infusion of a 0.45% saline does increase relative water content of muscle, similar to levels observed using an in-vitro model. Furthermore, we can also conclude the BIA is not sensitive enough to determine the changes in fluid distribution observed in the current protocol.

b. Work Output

There was a trend with the eccentric exercise protocol for work output to increase in the first five sets and then decrease in the last five sets, with no significant differences between sets observed. This is similar to what was previously observed by Beaton et al. who observed that following 30 sets of 10 repetitions of a isokinetic knee eccentric exercise, participants displayed significantly lower work output at set 10, 15, 20, 25, and 30 compared to set one (9). Within the current study, the HYD and CON conditions followed a similar non-significant trend for both conditions. The similarity between the conditions in work output suggests that any changes in the markers of damage were likely the result of our experimental intervention, rather than differences in work output.

A possible reason for the trend of work output to increase during the first five sets could be due to a learning effect occurring in the first half of the exercise protocol. To avoid a learning effect during the experiment, previous studies have had participants come in for a familiarization session prior to the actual experimental protocol (9, 100). This was not done in the current study to avoid the possibility of a protective effect from the familiarization exercise. Howatson et al. found a protective effect following a second exercise bout completed by the contralateral and ipsilateral arm 14 days after the first bout (48). Similarly,

Nikolaidis et al. investigated the effect of a repeated bout of knee extension exercise three weeks following an initial bout of exercise. They found a significant attenuation of muscle damage following bout two compared to bout one (76). The trend observed in the current study could be a result of a learning effect, but the steady decline in work output from set six through 10 suggests the protocol was successful at altering normal function of the active muscle.

Isometric strength was measured before and immediately following the exercise protocol. Previous literature has measured isometric torque before and following eccentric exercise as an indirect measure of muscle damage (9, 40, 41). When muscle damage is quantified directly using electron microscopy or light microscopy, the decreased isometric torque coincides with elevations in damage to the z-lines (9, 40, 41). When isometric torque is measured along with other indirect markers of damage such as range of motion, muscle soreness, and CK; they all display significant alterations following eccentric exercise. Hirose et al. observed elevated CK in the blood, decreased isometric strength and range of motion following eccentric elbow exercise (46). Saxton et al. observed elevated CK and decreased strength and flexed joint angle following a bout of eccentric exercise of the elbow flexors (98).

In the current study isometric peak torque was reduced ($p<0.05$) compared to pre-exercise values immediately following the exercise protocol. Post isometric torque was 79.2 and 79.1% of pre exercise levels for HYD and CON, respectively. Gibala et al. observed immediately post isometric torque levels of 67.7% of pre torque following a bout of exercise of the elbow flexors. Peak torque

continued to decrease at 24 hours post and was 60.8% of pre levels 48 hours post exercise (40). Beaton et al. had participants complete an isokinetic exercise protocol consisting of 300 eccentric knee contractions, and led to post isometric torque to be 54% of pre torque levels (9). The observed declines in torque are greater than the results observed in the current research project, but the exercise protocols involved a significantly greater amount of work as compared to the current protocol. However, our results are consistent with previous research (9, 17, 40, 41, 78, 100) with regards to decreased isometric peak torque immediately following eccentric exercise.

The results of the current study demonstrate that the exercise protocol was successful at reducing total work output and isometric torque similar to what has been observed in previous research (9, 40, 46, 98).

c. Blood Analysis

CK and LDH are commonly used as an indirect measurement of skeletal muscle damage (9, 17, 74, 86, 100), as elevated levels detected in the plasma are indicative of cellular membrane disruption. CK is located intracellularly and is responsible for catalyzing the reversible cellular reaction between phosphocreatine (PCr) and ADP to produce creatine and ATP (103). Similarly, LDH is an enzyme that converts pyruvate to lactate within the cell (103).

CK levels at 24 hours post exercise increased 5.4 and 3.8 fold in the HYD and CON condition respectively, compared to pre exercise levels. LD levels followed more of a linear increase with peak values of 149 U/L and 151 U/L for the HYD and CON condition respectively at 24 hours. The CK results in the present study

at 24 hours post exercise are similar to Beaton et al. who observed a 5.0 fold increase 24 hours following 300 isokinetic contractions of the quadriceps compared to pre levels, and when a 48 hour blood sample was taken there was an even further increase in CK levels detected (9).

When CK has been measured over a seven day period in succession, peak CK levels have been observed between day three and four following exercise with values at 24 hours similar to values in the current study (46, 78); suggesting that with time points at 72 and 96 hours post exercise the current study may have observed similar results.

There is large variability between participants' CK levels at rest and following exercise, as evidenced in this study by the large standard error values. This trend in large inter-subject variability has been observed in previous literature (79). The inconsistency in CK has been observed by Clarkson et al. who compared CK levels following eccentric, concentric or isometric exercises. There was a similar increase with all three types of exercise with no significant differences between groups (20). It has been well documented that eccentric exercise leads to elevated muscle damage compared to concentric exercise (40, 41, 78). Thus, the measurement of CK and LDH is indicative of muscle damage, but cannot be relied on to determine severity of damage.

Inflammatory cytokines are commonly measured in the blood following exercise as an indirect indicator of damage as they are the precursors to neutrophil and macrophage infiltration to repair damaged tissue (4). IL-6 is considered an anti-inflammatory cytokine as it inhibits the production of pro-

inflammatory cytokines TNF-α and IL-1β that precede IL-6 in the inflammatory response (87). IL-6 also promotes the synthesis of other anti-inflammatory cytokines such as IL-1 receptor antagonist and IL-10 (82, 88, 106). This initiates the acute-phase in the inflammatory response. The timeline for elevated IL-6 detection in the blood following exercise ranges anywhere from immediately post to six hours post exercise (82, 84, 86) and appears to be strongly influenced by the type and intensity of the exercise. Nieman et al. compared the IL-6 response following a running and a cycling protocol and observed elevated IL-6 levels following the running exercise compared to the cycling (75). Running requires more muscle recruitment compared to cycling and they suggested that IL-6 levels are dependent on the amount of muscle recruited. Paulsen et al. had participants complete an eccentric exercise protocol of the quadriceps that consisted of 300 maximal contractions; IL-6 peaked at six hours post exercise and returned to baseline by 24 hour (84). Although the muscle mass recruited was smaller, the intensity of work was high and therefore there was a strong response of IL-6, and may explain the results of the current study that found no significant changes in IL-6 at any of the measured time points or between conditions. In the current study, the work intensity may not have been high enough to elicit IL-6 release, or the amount of active muscle may have been too small to influence elevated circulatory concentration of IL-6.

The concentration of CRP appears to be influenced by the same factors that influence IL-6, specifically exercise intensity, type and muscle mass recruited. In the current study, a significant main effect for time was observed, such that the

CRP levels at 24 hours post exercise were significantly greater than pre, post and three hour post. Similar to the current results, Paulsen et al. observed elevated CRP levels 23 hours following 300 eccentric contractions of the quadriceps, and the levels of CRP peaked 47 hours post exercise (84). They also observed elevated IL-6 that peaked six hours post exercise (84). Their data supports the idea that IL-6 stimulates the release of CRP. Contrary to these results, Croisier et al. observed elevated levels of IL-6 that peaked 30 minutes following 90 eccentric contractions of the quadriceps, but there was no change in CRP levels compared to resting levels at any of the measured time points (30 minute post – 96 hours post exercise) (26). Similarly, Milias et al. observed no significant changes in CRP levels at 24-96 hours following an eccentric elbow flexors protocol, even though there were significant decrements in isometric torque following the protocol (69). Akimoto et al. compared a marathon run, a cycling exercise and a downhill treadmill run and observed elevated CRP levels in both the running activities 24 hours post and no change in the cycling exercise (2). The increased level of CRP detected at 24 hours in the current study coincides with previous research that has detected CRP following exercise, and further suggests inflammatory processes where occurring by 24 hours following the exercise.

Although the current study had participants perform unilateral eccentric exercise using a large muscle group, there were no significant changes in IL-6, but for CRP a main effect for time was observed. The current exercise protocol did lead to reductions in isometric torque, and elevated CK both indicative of

muscle damage. The elevated levels of CRP indicate that the inflammatory response was activated as CRP is released in response to cytokines (39); suggesting that the IL-6 release may have occurred during exercise or prior to the blood sample following the exercise protocol.

d. Quantification of Muscle Damage

Contrary to what was hypothesized, there were no significant difference ($p>0.05$) in the number of areas of moderate or extreme damage at any of the measured time points (pre, post, three hours). More unexpectedly, there was no damage observed in the CON condition or the HYD condition. Previous literature quantifying sites of damage using light or electron microscopy have concluded that following a bout of eccentric exercise there was significantly greater damage (9, 40, 108). There are a number of possible explanations for the contradictory results found in the current research. The first speculation is that the exercise protocol was not damaging enough. An eccentric exercise protocol was implemented because previous research has compared eccentric exercise to concentric exercise protocols and found greater damage in the eccentric bout of exercise compared to the concentric (40, 64). The force velocity relationship of muscle demonstrates that completing a concentric contraction at a higher velocity decreases the maximum force it can exert (29). The force generated during an eccentric contraction is not affected by changes in velocity and requires smaller fibre recruitment. These two factors combined lead to greater stress placed on the active fibres and likely contribute to the increased damage during eccentric contractions (72). Beaton et al. had participants complete eccentric isokinetic leg

exercise consisting of 30 sets of 10 repetitions with a one minute break between sets. Toluidine blue staining revealed significantly greater moderate and extreme z-line streaming at four and 24 hours post exercise (9). Their exercise protocol consisted of 200 more contractions than the current protocol, supporting the speculation that the current exercise protocol was not damaging enough. Feasson et al. implemented a downhill running protocol to induce muscle damage and observed mild muscle damage in two participants immediately post exercise and more extensive damage 24 hours post exercise in eight participants (31). The purpose of the current research project was to induce muscle damage following a bout of eccentric exercise that was more realistic to common exercise routines; the goal was not to cause excessive amounts of damage. Similarly, we recruited untrained participants and were concerned with their ability to complete a running protocol or a strenuous eccentric protocol. Therefore, the design of the current protocol may have limited the amount of damage.

Another factor that influences the amount of damage is the muscle groups recruited to complete the exercise protocol. Gibala et al. had untrained participants perform an eccentric exercise protocol of the biceps consisting of eight sets of eight repetitions resulting in a significant increase in muscle damage immediately and 48 hours post exercise. When the protocol was repeated with trained participants, there was significantly greater damage at 21 hours post exercise compared to baseline values (40). The significant damage observed by Gibala et al. can be explained in part by the muscle group exercised. The biceps brachii muscles consist of two muscle bellies. In contrast to this, the current

research utilized the quadriceps muscle group for exercise, consisting of four separate muscles. The biceps brachii muscle could be considered a more isolated muscle, requiring lower repetitions to induce damage compared to a larger muscle group such as the quadriceps (9, 40).

A third possible explanation for the results observed was the time points implemented were too acute to detect damage. Feasson et al. observed damage using electron microscopy immediately post exercise in only two participants (31). In the same study, biopsies collected 24 hours post exercise revealed eight participants displayed extensive damage (31). There was no reference to the absolute number of sites of damage in the study and no significance was discussed. Quantification of damage in the current research study revealed damage in six participants three hours following the exercise. There is high variability in the total number of fibres in each sample, thus we felt it was necessary to express the total number of sites of damage per fibre. Previous research has focused on the effects of exercise 24 hours and 48 hours post exercise when the inflammatory process is activated, leaving the researchers unable to determine if damage was caused by the mechanical events or the oxidative events that follow. Due to the gap in literature regarding acute changes following exercise, the current investigation assessed damage immediately post and three hours post exercise to quantify muscle damage with hopes to determine if the exercise protocol alone leads to the myofibrillar damage. However, no damage was observed at the chosen time points, leading to the speculation that the muscle damage observed in the previous literature at time

points of 24 hours and 48 hours may be induced mainly by the inflammatory process and to a lesser degree the mechanical events of the exercise protocol.

The mechanical damage caused by eccentric exercise is caused by the sarcomeres being stretched past their optimum length; called the popping sarcomere hypothesis, introduced by Morgan and Proske (72). Once past their optimal length the sarcomeres begin to 'pop' and tear. This tearing leads to the disruption of myofibrils and sarcolemma, and opening of stretched activated channels (118). This disrupts the balance of ions between the intracellular and extracellular compartments. Elevated levels of Na^+ have been observed following eccentric exercise (118) as well as elevated Ca^{2+} concentrations within the cell (11). In an animal model, it has been observed that elevated Ca^{2+} disrupts excitation-contraction coupling by stimulating the activation of calpain proteases that attack myofibrillar proteins and the sarcolemma (11). This may lead to increased membrane permeability, and in turn an increase in the release of intracellular proteins such as CK and LD to stimulate the inflammatory response. Although there is no research investigating the time course of the above events, it is plausible that this process could take longer than our last time point of three hours post exercise. If this is the case, an additional biopsy at 24 hour post exercise might have revealed more extensive myofibrillar disruption.

The results demonstrate that no skeletal muscle damage occurred in the CON trial; therefore, we are unable to explore the possibility of the HYD condition having a protective effect because damage did not occur. It is clear that hyperhydration offers protection to the cell under resting conditions (55, 71, 93,

115). Antolic et al. used an isolated animal model to investigate the effect of aniosomotic conditions on resting metabolism and observed decreased levels of adenosine triphosphate and phosphocreatine, and an increased level of lactate in cells incubated in a hyperosmolar solution, suggesting a catabolic effect of hyper-osmotic incubations (5). In a human model, Keller et al. manipulated whole body hydration and observed in the hypo-osmolality condition protein breakdown and plasma glucose levels were reduced compared to iso-osmolality. Hyper-osmolality led to elevated hepatic glucose production (55). Roy et al. examined the effect of whole body hypohydration on cardiovascular and thermal responses during exercise. They concluded that diuretic induced hypohydration placed elevated cardiovascular and thermal strain on the body evidenced by the increased rectal temperature and circulating norepinephrine, while stroke volume and cardiac output decreased (93). Hargreaves et al. compared the effect of fluid ingestion during exercise to no fluid on exercise performance and metabolism. The researchers observed higher muscle glycogen levels and lower glycogen utilization in the group that ingested water throughout the exercise compared to no fluids (43). It is apparent from previous research that alterations in hydration level influences metabolism and exercise performance, yet the current study was the first to investigate the possibility of a protective effect of an elevated muscle cell volume on muscle damage.

Despite the success of increasing muscle fibre volume in the current study, it is unclear whether a protective effect exists. Further research is necessary to investigate skeletal muscle damage under euhydrated conditions and then

compare the results of the euhydrated to the results of an increased muscle fibre volume condition to determine if there is a protective effect.

CONCLUSIONS

The most significant finding of the current study is that the infusion of hypotonic saline (0.45%) led to a significant increase in muscle fluid content in human skeletal muscle, similar to what has been observed in previous research in our laboratory using an isolated model (5, 30). To our knowledge there has been no other research attempting to quantify elevated muscle fibre volume and thus, this finding is novel in the field of cell hydration and provides a viable model for future in-vivo studies.

Although the current study was able to achieve elevated skeletal muscle fluid volume, there was no muscle damage observed following the eccentric exercise protocol in the CON condition, leaving the researchers unable to discuss the possibility of a protective effect of the elevated fluid content of the muscle. However, the protocol did result in increased CK, LD and CRP but no differences were observed between the two trials.

There are numerous plausible reasons to explain the lack of damage observed following the exercise protocol; the exercise protocol may not have been intense enough to cause damage to the active muscle. Previous literature that has observed elevated inflammatory markers such as IL-6 and CRP have completed whole body exercise such as running or cycling (2, 75, 82). The current study utilized a unilateral exercise protocol that may not have stimulated

a systemic release of IL-6 or CRP into the blood but may have been more isolated to the active quadriceps muscle.

Secondly, the time points used for the collection of muscle and blood were acute. If muscle samples were collected at later time points (24-48 hours post) and blood was collected in the week following, there may be signs of skeletal muscle damage and / or a protective effect of the HYD condition during the recovery period. Previous literature has focused on time points 24-48 hours following exercise (9, 36, 40, 41), leaving them unable to determine if the muscle damage observed was due to the mechanical events of the exercise or the inflammatory response. Thus, current investigation focused on the acute response to a damaging bout of exercise and was unable to identify muscle damage; suggesting that the muscle damage observed at later time points may be induced by the inflammatory response. Future research is necessary to determine the time course of muscle damage following eccentric exercise.

Due to these contradictory results further research is necessary to determine if additional time points following the exercise would display damage or if the exercise protocol was not of sufficient intensity to cause damage. Once this is determined, future research can determine if elevated muscle cell volume protects exercising muscle from damage.

FUTURE DIRECTIONS

Using an in-vitro model, a future study could investigate the influence of altering osmotic conditions (both hypo- and hyper- osmotic) prior to lengthening

contractions and determine the effects on the excitation contraction coupling and structure of cytoskeleton. The current study provided a good basis for future work with the development of the hypotonic saline infusion model, however a number of questions still remain. For example, a follow-up study is necessary to determine if the infusion protocol implemented in the current investigation was successful at achieving an increased intracellular volume within skeletal muscle cells, through fibre diameter analysis. The current investigation was able to confirm elevated muscle fibre fluid volume, but it is crucial to identify the exact distribution of fluid between the interstitial and intracellular compartments.

Future work should also examine the time course of damage following the exercise protocol. The addition of muscle biopsies at 24 / 48 hours following the eccentric exercise would give a more complete understanding of the time course of muscle damage as the current investigation determined the acute response to the exercise protocol.

Future work could also investigate changes in inflammatory cytokines such as IL-6 directly within exercised and control skeletal muscle tissue at various time points. This would provide insight into the time course of the inflammatory response and the sequence of events during the inflammatory process following exercise. Combining the measurements at later time points with the results of the current investigation would give a more complete understanding of the relative contributions of the mechanical and oxidative events and time course of muscle damage.

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APPENDIX I: ETHICS APPROVAL

Hamilton
Health Sciences

RESEARCH ETHICS BOARD

REB Office, 1057 Main St. W., Hamilton, ON L8S 1B7
Telephone: 905-521-2100, Ext. 42013
Fax: 905-577-8379

February 15, 2007

PROJECT NUMBER: **07-024**

PROJECT TITLE: Impact of skeletal muscle hydration on contractile induced muscle damage and gene expression

PRINCIPAL INVESTIGATOR: **Dr. M. Tarnopolsky**

This will acknowledge receipt of your letter dated February 8, 2007 which enclosed a copy of the revised protocol, the revised Subject Information and Consent Form, the revised Participant Information Sheet for Tissue Storage and the revised REB application for the above-named study. These issues were raised by the Research Ethics Board at their meeting held on January 15, 2007. Based on this additional information, we wish to advise your study has been given ***final*** approval from the full REB. The protocol submission, including the Subject Information and Consent Form, the Participant Information Sheet for Tissue Storage and the recruitment poster were found to be acceptable on both ethical and scientific grounds. ***Please note*** attached you will find the Information Sheet/Consent forms with the REB approval affixed; all consent forms and recruitment materials used in this study must be copies of the attached materials.

We are pleased to issue final approval for the above-named study for a period of 12 months from the date of the REB meeting on January 15, 2007. Continuation beyond that date will require further review and renewal of REB approval. Any changes or amendments to the protocol or information sheet must be approved by the Research Ethics Board.

We wish to advise the Research Ethics Board operates in compliance with ICH Good Clinical Practice Guidelines and the Tri-Council Policy Statement.

Investigators in the Project should be aware that they are responsible for ensuring that a complete consent form is inserted in the patient's health record. In the case of invasive or otherwise risky research, the investigator might consider the advisability of keeping personal copies.

A condition of approval is that the physician most responsible for the care of the patient is informed that the patient has agreed to enter the study. Any failure to meet this condition means that Research Ethics Board approval for the project has been withdrawn.

PLEASE QUOTE THE ABOVE-REFERENCE PROJECT NUMBER ON
ALL FUTURE CORRESTPONDENCE

Sincerely,

F. Jack Holland, MD, FRCP, FRCP (C)
Chair, Research Ethics Board

DATE: March 1, 2007

FROM: Linda Rose-Krasnor, Chair
Research Ethics Board (REB)

TO: Brian Roy, PEKN
Mark Tarnopolsky, Rosemarie Harrison

FILE: 06-259 ROY

TITLE: Impact of skeletal muscle hydration on contractile induced muscle damage and gene expression

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The Brock University Research Ethics Board has reviewed the above research proposal.

DECISION: Accepted as is.

This project has received ethics clearance for the period of March 1, 2007 to December 31, 2007 subject to full REB ratification at the Research Ethics Board's next scheduled meeting. The clearance period may be extended upon request. ***The study may now proceed.***

Please note that the Research Ethics Board (REB) requires that you adhere to the protocol as last reviewed and cleared by the REB. During the course of research no deviations from, or changes to, the protocol, recruitment, or consent form may be initiated without prior written clearance from the REB. The Board must provide clearance for any modifications before they can be implemented. If you wish to modify your research project, please refer to http://www.brocku.ca/researchservices/forms to complete the appropriate form **Revision or Modification to an Ongoing Application.**

Adverse or unexpected events must be reported to the REB as soon as possible with an indication of how these events affect, in the view of the Principal Investigator, the safety of the participants and the continuation of the protocol.

If research participants are in the care of a health facility, at a school, or other institution or community organization, it is the responsibility of the Principal Investigator to ensure that the ethical guidelines and clearance of those facilities or institutions are obtained and filed with the REB prior to the initiation of any research protocols.

The Tri-Council Policy Statement requires that ongoing research be monitored. A Final Report is required for all projects upon completion of the project. Researchers with projects lasting more than one year are required to submit a Continuing Review Report annually. The Office of Research Services will contact you when this form ***Continuing Review/Final Report*** is required.

Please quote your REB file number on all future correspondence.

LRK/law

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