THE EFFECTS OF ACUTE NUTRITIONAL KETOSIS ON MARKERS OF EXERCISE-INDUCED MUSCLE DAMAGE, MUSCLE FUNCTION, AND MUSCLE SORENESS DURING RECOVERY FROM EXERCISE

Patrick William Martin

Department of Kinesiology and Physical Education

McGill University

Montreal, Quebec, Canada

August 2020

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science

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LIST OF ABBREVATIONS

AcAc – Acetoacetate

β-OHB – β-hydroxybutyrate

B2 – Bradykinin 2 receptor

BAM+ – Brief assessment of mood adapted

CK – Creatine kinase

CMJ – Countermovement jump

CRP – C-reactive protein

CON – Carbohydrate supplement

COX-2 – Cyclooxygenase-2

DEXA – Dual-energy x-ray absorptiometry

DOMS – Delayed onset muscle soreness

EC – Excitation-contraction coupling

EIMD – Exercise induced muscle damage

G-CSF – Granulocyte-colony stimulating factor

GDNF - Glial cell-derived neurotrophic factor

IL-1β – Interleukin 1 beta

IL-1ra – Interleukin 1 receptor antagonist

IL-4 – Interleukin 4

IL-6 – Interleukin 6

IL-8 – Interleukin 8

IL-10 – Interleukin 10

IL-12 – Interleukin 12

IL-15 – Interleukin 15

KET – Ketone monoester

M1 – Macrophage 1

M2 – Macrophage 2

MCP1 – Macrophage chemoattractant protein 1

MIVC – Maximal isometric voluntary contraction

NGF – Nerve Growth Factor

NLRP3 - NOD-, LRR- and pyrin domain-containing protein 3

PPT – Pressure pain threshold

RBE – Repeated bout effect

ROS – Reactive oxygen species

TNF-α – Tumor necrosis factor alpha

VAS – Visual analog scale

ABSTRACT

Purpose: The purpose of this study was to evaluate the effects of a ketone monoester supplement on indices of muscle damage during recovery from eccentric exercise.

Methods: In a randomized, double-blind, independent group design, 20 moderately active healthy young adults consumed 360 mg/kg^{-1} bodyweight of a ketone monoester (KET) or energy-matched carbohydrate (CON) supplement twice daily following eccentric exercise (drop jumps). Maximal isometric voluntary contraction (MIVC) torque, counter-movement jump (CMJ) height, and muscle soreness were measured before (PRE), and immediately (POST), 24 h, and 48 h post-exercise. Blood samples were collected for analysis of β-hydroxybutyrate (β-OHB), creatine kinase (CK), and select pro- and anti-inflammatory cytokines.

Results: Peak blood β-OHB concentration after supplement intake was greater ($P < 0.001$) in KET (4.4 ± 0.8 mM) vs. CON (0.4 ± 0.3 mM). Exercise increased CK concentration at 24 h and 48 h vs. PRE (time: $P < 0.001$) with no difference between KET and CON. Exercise reduced MIVC (KET: 19.9 ± 14.6; CON: 22.6 ± 11.1%) and CMJ (KET: 11.0 ± 7.5; CON: 13.0 ± 8.7%) at POST relative PRE; however, there was no difference between KET and CON on the recovery of MIVC at 24 h (KET: 15.4 ± 20.4; CON: 18.7 ± 20.1%) or 48 h (KET: 7.2 ± 21.2; CON: 11.8 ± 20.2%), or CMJ at 24 h (KET: 9.2 ± 11.5; CON: 13.4 ± 10.8) or 48 h (KET: 12.5 ± 12.4; CON: 9.1 ± 11.7). Muscle soreness was increased during post-exercise recovery (time: $P < 0.001$) with no differences between KET and CON. Monocyte chemoattractant protein-1 was greater (group: $P = 0.007$) in CON (236 ± 11 pg/mL) vs. KET (187 ± 11 pg/mL).

Conclusion: Supplementation with a ketone monoester does not expedite the recovery of indices of muscle damage following eccentric exercise in moderately active, healthy young adults.

RÉSUMÉ

But: Le but de cette étude était d'évaluer les effets d'un supplément de monoester cétonique sur les indices des dommages musculaires lors de la récupération après un exercice excentrique.

Méthodes : Dans un groupe randomisé, en double aveugle et indépendante, 20 jeunes adultes en bonne santé modérément actifs ont consommé 360 mg/kg^{-1} d'un supplément de monoester de cétone (KET) ou d'un contrôle des glucides à énergie à équilibre (CON) deux fois par jour pendant la récupération d'un exercice excentrique (sauts excentrique). Le couple maximal de contraction volontaire isométrique (MIVC), la hauteur du saut (CMJ), la douleur musculaire ont été mesurés avant (PRE), immédiatement après (POST), 24h, et 48 h après l'exercice. Des échantillons de sang été prélevés pour l'analyse du β-hydroxybutyrate (β-OHB), de la créatine kinase (CK) et de certaines cytokines pro et anti-inflammatoires.

Résultats: valeurs de crêtede concentration sanguine de β-OHB après la prise de supplément était plus élevé ($P < 0.001$) dans KET (4.4 ± 0.8 mM) par rapport à CON (0.4 ± 0.3 mM). L'exercice a augmenté la concentration de CK à 24 h et 48 h par rapport à PRE (temps: $P < 0.001$) sans différence entre KET et CON. L'exercice a réduit le MIVC (KET: 19.9 ± 14.6; CON: 22.6 ± 11.1%) et le CMJ (KET: 11.0 ± 7.5; CON: 13.0 ± 8.7%) au POST relatif PRE; cependant, il n'y avait pas de différence entre KET et CON sur la récupération de MIVC à 24 h (KET: 15.4 ± 20.4; CON: 18.7 ± 20.1%) ou 48 h (KET: 7.2 ± 21.2; CON: 11.8 ± 20.2%), ou CMJ à 24 h (KET: 9.2 ± 11.5; CON: 13.4 ± 10.8) ou 48 h (KET: 12.5 ± 12.4; CON: 9.1 ± 11.7). Les douleurs musculaires ont augmenté pendant la récupération post-exercice (temps: $P < 0.001$) sans différence entre KET et CON. La protéine-1 chimioattractante des monocytes était plus élevée (groupe: $P = 0.007$) en CON (236 ± 11 pg/mL) par rapport au KET (187 ± 11 pg/mL).

Conclusion: La supplémentation en monoester cétonique n'accélère pas la récupération des indices de dommages musculaires après un exercice excentrique chez de jeunes adultes modérément actifs et en bonne santé.

ACKNOWLEDGEMENTS

First and foremost, I would like to sincerely thank my supervisor Dr. Tyler Churchward-Venne for his mentorship, handwork, support, kindness, understanding, patience, and for his dedication to quality and perfection. Tyler has helped me become a better student, researcher, person, and father. Thank you for all your help, you were always there for me, whether it was during office hours, phone calls in the evenings, emails over the weekends, or coming into the lab during your holidays. You never left me without support, and I am forever grateful.

A special thank you to Prof. Ross Andersen and Dr. Julie Côté for the tremendous support they have given to this research. Thank you for your flexibility and kindness in providing access to some of the research equipment used in this study.

Thank you, Dr. Benoit Gentil for your support and feedback as a member of my advisory committee, as well, thank you for helping me with participant recruitment.

Additionally, thank you to Jamie Lov for your valuable assistance to this research, you were always ready to lend your time and energy. The completion of this research would not have been possible without you. You are dependable, intelligent, thorough and hardworking. There is no one else I would rather count on to be there at 7 am or to review an important protocol.

A special thanks to Ophir Dannenberg for the analysis of our diet records, especially during this unprecedented pandemic. You were dependable, and I am grateful for your work.

Thank you Chloe Fleurent-Gregoire for your help in the early phases of data collection, as well during the analysis of data. You are appreciated.

Thank you to Nina Dai for help during the early phases of research. Your guidance helped me get my ethics approval done.

Finally, I would like to thank my amazing wife Jordan for your constant love, unconditional support, understanding and kindness. You were always there for me, whether it was reviewing my writing, or coming into the lab to help with research when no one else could, all well maintaining our loving home. It was a tremendous challenge for us to have our first child, Callaghan, while both pursuing our master's degrees, both working shift work at the hospitals, and running both of our businesses. I do not know how we survived, but I do know that you were incredible every step of the way. I am confident that our united love, and rock-solid teamwork, is stronger than any obstacle in our path.

CONTRIBUTRITION OF AUTHORS

Patrick W. Martin (first author): conceived and designed the research, conducted the research, analyzed the data, interpreted the results of the experiments, prepared the figures, drafted the thesis, read and approved the final thesis, and holds primary responsibility for the final content along with the principle investigator (Dr. Tyler A. Churchward-Venne).

Jamie Lov: conducted the research.

Dr. Tyler A. Churchward-Venne (principle investigator): secured financial support for the research, conceived and designed the research, interpreted the results of the experiments, edited and revised the thesis, read and approved the final thesis, and holds primary responsibility for the final content.

CHAPTER 1: INTRODUCTION

1. INTRODUCTION

Exercise induced muscle damage (EIMD) can occur in response to strenuous exercise, particularly when it is novel to the individual performing it (1) and involves high-force eccentric muscle actions (2). At the local level, EIMD is characterized by sarcomere disruption and excitation-contraction coupling failure (3). The symptomatic manifestations of EIMD include reduced muscle function (4), increased muscle soreness (2, 3), reduced flexibility/range-of-motion (3), and an increase in markers of inflammation and oxidative stress (5). The response to EIMD can vary from individual to individual (1), and there are many factors that play a role in the individual's susceptibility to EIMD. These factors include the age of the individual (6), their training status (7), the type of exercise performed (8), nutritional status (9), and potentially genetic variance (10).

Although EIMD has been the focus of extensive research, its precise aetiology is not completely understood. However, EIMD is thought to involve two distinct phases: a primary phase and a secondary phase (11). The primary phase of EIMD involves ultrastructural damage to the muscle and/or impairments in the process of excitation-contraction coupling (12). The secondary phase of EIMD occurs in response to the primary phase and involves biochemical changes, the hallmarks of which are inflammation and oxidative stress, that may further exacerbate damage to the muscle and surrounding tissue (13).

Given the temporary detrimental effects of EIMD (i.e., reduced muscle function and increased muscle soreness), there is significant interest among athletes, coaches, and the scientific community on strategies that may help expedite recovery from EIMD. Nutrition plays a key role in facilitating recovery from, and adaptation to, exercise (14). The use of nutritional and/or pharmacological interventions in the treatment of EIMD has been an area of focus for

individuals, athletes and researchers for over 30 years (15). Commonly evaluated nutritional interventions within the context of EIMD include: protein/branched chain amino acid supplements (16, 17), various isolated anti-oxidant compounds (18-20), omega 3 fatty acids (21, 22), and so called 'functional foods' like tart cherry and beetroot (23-27). In terms of pharmacological agents, non-steroidal anti-inflammatory drugs have also been investigated (28, 29). Protein/branched chain amino acid supplements may augment recovery due to their capacity to stimulate increased rates of muscle protein synthesis, and thereby enhance repair and remodelling of tissue (17, 30-32). Alternatively, anti-oxidant supplements and functional foods (e.g., beetroot or tart cherries) may attenuate the effects of inflammation and oxidative stress as a consequence of secondary damage (5, 6, 33).

A novel nutritional intervention which has garnered interest from the athletic and research community is the ingestion exogenous ketone bodies, or more precisely, beta-hydroxybutyrate (β-OHB) (34). Ketone bodies are metabolites that are normally produced endogenously by the liver under periods of prolonged fasting or severe carbohydrate restriction (35). A recently developed ketone monoester supplement has demonstrated the ability to effectively induce nutritional ketosis (defined as a blood ketone concentration > 0.5 mM (36)), without any drastic changes to the diet (37). Some research indicates that ketone monoester supplements improve endurance exercise performance (38, 39). Other research has demonstrated that the ingestion of exogenous ketones post endurance exercise may benefit recovery during a period of overreaching (40). Nutritional ketosis and elevated β-OHB have been suggested to benefit post-exercise recovery via the capacity of β-OHB to stimulate muscle protein synthesis (41), reduce whole-body proteolysis (41, 42), and reduce both oxidative stress (43) and inflammation (44). However, no research to date has explored whether exogenous ketone

monoester supplements, known to increase circulating β-OHB, can expedite recovery following EIMD. Therefore, the overall objective of this thesis was to evaluate the effects of ketone monoester supplementation on various indices of muscle damage during recovery from eccentric exercise in healthy, young adults.

1.1 Purpose and hypothesis

The purpose of this study was to evaluate the effects of supplementation with a ketone monoester, that acutely increases blood β-OHB concentration, on indices of EIMD during recovery from a bout of eccentric exercise in healthy young active adults. It was hypothesized that twice daily supplementation with a ketone monoester (KET) would expedite the recovery of muscle performance, reduce muscle soreness, and alter the concentration of select pro- and anti-inflammatory cytokines when compared to supplementation with an energy-matched carbohydrate supplement (CON).

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

The objective of this literature review is to highlight some of the pertinent research conducted within the area of EIMD and provide the framework that has led to the objectives and hypothesis outlined in this thesis. The literature review begins with an overview of eccentric muscle actions followed by an overview of EIMD. Common markers of EIMD are then discussed with an emphasis on the markers utilized in the present thesis. The literature review finishes with a discussion of ketone bodies and aims to provide an underlying rationale for their use as a novel nutritional intervention to expedite recovery following EIMD.

2.2 Eccentric muscle actions

Muscle contractions can be broken down into three distinct forms; an eccentric contraction (muscle lengthening), concentric contraction (muscle shortening), and isometric contraction (static flexion). The earliest definitions of these types of muscle contractions can be dated back to 1938 (45). The understanding of the forms of muscle contractions has evolved over time. The eccentric contraction was deemed to absorb the external load during the lengthening phase, for instance while walking down stairs, or lowering a weight towards the ground. It is often termed “negative work”. The concentric contraction, or muscle shortening, such as lifting a weight away from the ground, or walking up stairs, is called “positive work” (46). Eccentric and concentric contractions happen many times throughout a day, every day. Examples of predominantly eccentric activities are running downhill, or walking down stairs, where the eccentric load is primarily focused on the knee extensor muscles (47). There are unique differences between each type of contraction, notably, the eccentric contraction can generate a greater force at a lower metabolic cost under similar conditions as concentric contractions (48). The eccentric contraction is more efficient, so it can deliver a mechanical work rate that is up to

four times higher than a concentric contraction for a given metabolic load (49). The benefits of eccentric exercise extend beyond its unique characteristics of high force output at a lower metabolic cost. Eccentric exercise has been shown to have a positive impact on metabolic function including; improving dyslipidemia, glucose tolerance, insulin resistance, reducing inflammation (C-reactive protein), reducing obesity, increasing lean mass, and improving proprioception (14, 50-52). The neurological control of the eccentric contraction is different than both the concentric and isometric contraction, as it is difficult to achieve full voluntary activation in an eccentric contraction in comparison to a concentric contraction (53). Although eccentric muscle actions are common in everyday life and are important to athletic success, unaccustomed high-force eccentric muscle actions can result in exercise-induced muscle damage (EIMD) compared to the other types of muscle contractions (54, 55). The mechanisms of EIMD and its symptomatic manifestation are discussed below.

2.3 Eccentric exercise-induced muscle damage

Novel or unaccustomed exercise, particularly when it involves high-force eccentric muscle actions, can result in EIMD. At the local level, EIMD is characterized by sarcomere disruption and excitation-contraction coupling failure (12, 54). The symptomatic manifestations of EIMD include a temporary decline in muscle strength (i.e. force production), increased muscle soreness, swelling of the affected limb, increased passive tension, and an increase in intramuscular proteins present in the blood (2). The magnitude and duration of muscle damage in response to eccentric exercise may be influenced by several factors including the type and intensity of exercise (3), training status of the individual and their familiarity with the exercise task (1), age (47, 56), and possibly genetics (57) and biological sex (58). Many of the symptoms of EIMD can be problematic, as they can compromise the ability of the athlete or individual to

train or compete on subsequent days (11, 15). Further, the symptoms of EIMD can pose a challenge for individuals trying to develop new exercise routines and adhere to an exercise program (59). This may be particularly true for older adults as they have been shown to have a slower recovery time following EIMD when compared to younger adults (6, 56, 59).

The precise aetiology of EIMD is incompletely understood, however it is thought to occur in a bi-phasic manner (3). The first or primary phase of EIMD occurs as a consequence of the mechanical work performed during a damaging exercise stimulus, but may also involve a metabolic component (60). The secondary phase propagates tissue damage through processes associated with an inflammatory/immune response in the hours and days following the damage-inducing event (2, 5, 11, 60, 61).

2.3.1 Primary muscle damage

Two main theories have been put forth to explain the mechanisms underpinning the initiation of EIMD and the prolonged decline in the force generating capacity of the affected muscle. Morgan (62) put forth the "popping sarcomere hypothesis" that proposes EIMD is caused by direct mechanical stretch of sarcomeres that leads to reduced functional strength after eccentric exercise (63). This theory proposes that half-sarcomere nonuniformity during eccentric lengthening contractions leads to most of the length change being taken up by the weakest half-sarcomeres (63). As stretch persists beyond optimal length, these half-sarcomeres get weaker as they lengthen along the descending limb of the force-length curve (4, 64). Repeated stretch forces these progressively weakening sarcomeres beyond the point of myofibrillar overlap. As eccentric muscle actions are continually performed, there is an increase in the number of nonoverlapped sarcomeres. This ultimately contributes to damage to the myofiber, disruption of

the sarcolemma, and opening of stretch-activated channels. For a comprehensive review of the popping sarcomere hypothesis, see Proske and Morgan (63).

Another theory put forth by Warren and colleagues (65) proposed that failure of the excitation-contraction coupling process contributes to the primary damage phase and is the underlying mechanism explaining the loss of muscle force following eccentric exercise. Warren et al. (66) suggested that force reductions within the first 3 days after muscle damage, before secondary damage ensues, are ~25% due to structural damage and ~75% due to dysfunctional excitation-contraction coupling (66). This theory is primarily based off results that demonstrate that force production in response to caffeine-induced muscle contraction was similar between injured and non-injured muscle in rodents (65). The rationale is based on the fact caffeine interacts directly with the sarcoplasmic reticulum to induce muscle contraction by opening calcium ion channels, thereby bypassing excitation-contraction coupling pathways (11). As discussed by Hyldahl & Hubal in a recent review (11), debate still exists between the mechanical stress theory and the excitation-contraction coupling theory of muscle damage; however, it is likely that both play a role and contribute to EIMD to varying degrees (11).

It has also been suggested that EIMD may be a consequence of metabolic (as opposed to mechanical) stress that arises during exercise (8). Both the metabolic and mechanical models of EIMD rely on disruption of calcium ion homeostasis within the muscle cell. Altered calcium ion homeostasis due to eccentric exercise leads to influx of calcium into the cytosol and activation of calcium dependant proteolytic pathways (8, 60). The metabolic model of EIMD proposes that this calcium disruption is due to cellular energy depletion, rather than ultrastructural damage caused by mechanical loading (60). The metabolic theory of EIMD is less popular than the mechanical theory of EIMD; however, both processes may be involved. Nonetheless, the

primary phase of muscle damage causes a cascade of effects leading to an immune/inflammatory response and the onset of the secondary phase of muscle damage. An overview of the mechanical vs. metabolic theory of EIMD is shown in **Figure 1**.

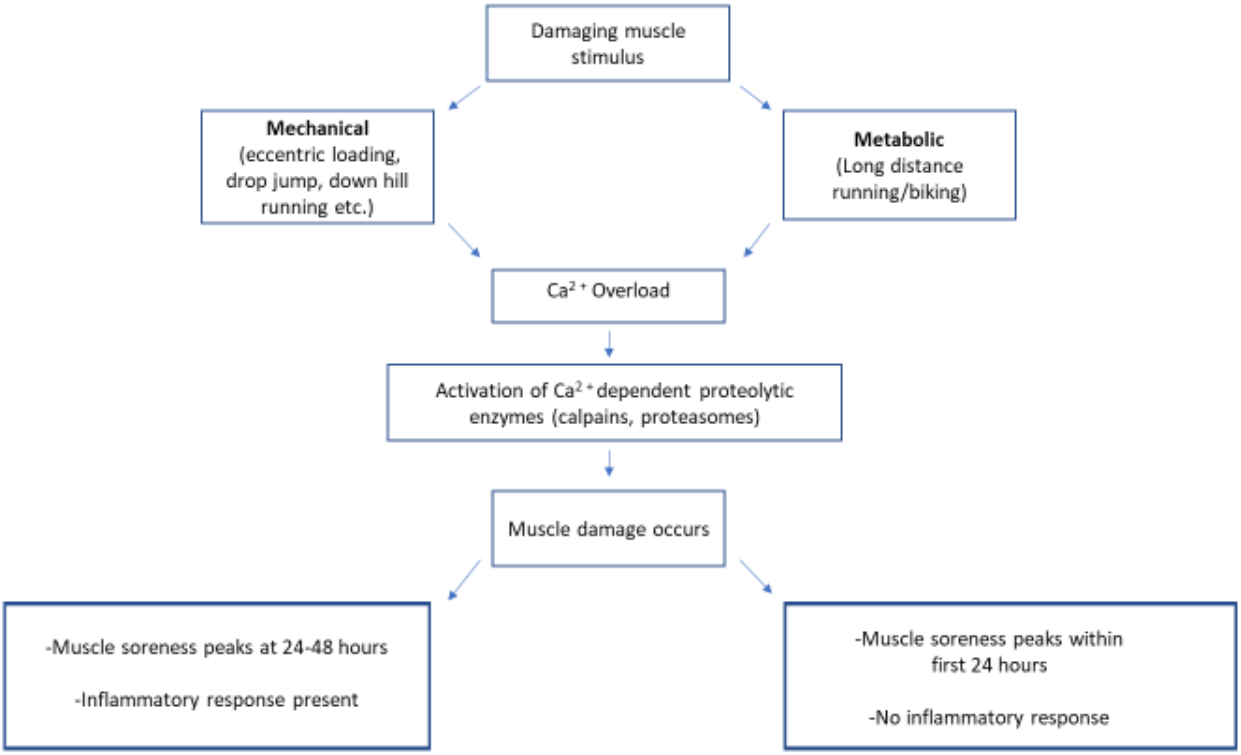

Figure 1. Mechanical vs metabolic theory of muscle damage. Adapted from Tee et al. 2007 (60).

2.3.2 Secondary muscle damage

The mechanical and/or metabolic damage that characterizes the primary phase of EIMD initiates a series of biochemical events that can contribute to further damage (termed secondary damage). The secondary phase of EIMD is believed to be due to the loss of calcium homeostasis as a result of damage to the sarcolemmal membrane and opening of stretch activated channels (11) leading to the influx of calcium into the cytoplasm (67) and the mitochondrial matrix (68). The disturbance in calcium homeostasis triggers an acute phase inflammatory response that first

results in degradation of damaged muscle proteins, followed by regeneration of the damaged tissue (69). For example, increased intracellular calcium stimulates the activation of muscle proteasomes called calpains, which are responsible for the degradation of the structural proteins within the myofiber (70). The increased calpain activity also contributes to the activation of neutrophils and macrophages, which leads to increased production of reactive oxygen species (ROS) and the inflammatory-immune response cascade (71). The inflammatory response is thought to be the main driver of the secondary phase of muscle damage in response to exercise.

2.3.3 Inflammation

The inflammatory response is an important process in the recovery and regeneration of muscle tissue after exercise and is primarily coordinated by signaling molecules known as cytokines (72). Cytokines can bind to receptors on the cell membrane of a target cell. In response to EIMD, damaged muscle cells and other cell types (i.e., leukocytes, endothelial cells), synthesize various cytokines classified as pro-inflammatory (e.g., TNF-α, IL-1β, IL-6, IL-8) or anti-inflammatory (e.g., IL-4, IL-10) depending on their function. Pro-inflammatory cytokines are involved in recruiting immune cells, such as leukocytes (T-regulatory cells, neutrophils, eosinophils, and mast cells) to the site of muscle damage (13). The immune response is characterized by an early invasion of neutrophils to the damaged muscle tissue (13). Neutrophils are a type of phagocyte responsible for the initiation of the pro-inflammatory phase during which they release proteolytic enzymes and cytotoxic molecules (i.e., ROS). Invading neutrophils also release pro-inflammatory cytokines which serve to attract more neutrophils, and thus more cytotoxic molecules to the site of damage (13), which may contribute to further damage and inflammation (13).

Following the invasion of neutrophils into damaged muscle, there is an influx of pro-inflammatory (M1) macrophages in response to the release of chemokines (73). Macrophages are detectable in muscle tissue by approximately 24 hours post-exercise. These macrophages may release pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β, and may reside within the muscle tissue for up to 14 days (13). Although M1 macrophages contribute to the inflammatory process, they can switch phenotype to M2 macrophages depending on their surroundings and the presence of other cell types (74). M2 macrophages can interact with satellite cells and release anti-inflammatory cytokines such as IL-10, IL-15, and MCP-1 that contribute to myogenesis and myofiber remodelling (5).

2.3.4 Oxidative Stress

Oxidative stress refers to an imbalance between the production of ROS (and other nitric oxide derivatives) and antioxidant defence (75, 76). Certain ROS such as the super oxide anion radical are produced by different biochemical reactions within the muscle, but primarily as a product of the electron transport chain within mitochondria (75). Muscle cells produce three main endogenous anti-oxidants to neutralize the ROS; super-oxide dismutase, catalase, and glutathione peroxidase (77). It is now well established that strenuous exercise can accelerate the production of ROS and associated compounds to a degree that exceeds the capacity of antioxidant defence, and thereby results in oxidative stress (78). Excessive production of ROS within muscle cells is associated with muscle fatigue and/or failure, muscle damage, and inflammation (79). Large changes in the concentration of ROS within muscle cells has been shown influence the release of pro-inflammatory or anti-inflammatory cytokines, which signal the immune system to modulate inflammation (79, 80). The immune system itself can become a source of ROS within the muscle cell (74). Certain leukocytes, such as neutrophils, release ROS

such as hydrogen peroxide, as a means for destruction and cleanup of damaged muscle tissue prior to the remodelling of the muscle tissue (74, 81).

Nutrition plays a role in managing oxidative stress within the body. Foods rich in anti-oxidants and polyphenolic compounds have been found to improve exercise performance and/or muscle recovery after exercise (82). Foods such as fruits and vegetables may be a direct source of anti-oxidants or polyphenolic compounds that have the ability to aid the innate anti-oxidant system in neutralizing free radicals produced during exercise, or mitigate the effect of the ROS produced by the immune system during the recovery period (76, 83). The role of nutrition in expediting recovery following EIMD is discussed in more detail below (section 2.5.4).

2.3.5 Mechanisms of muscle soreness

A hallmark symptom of EIMD is muscle soreness that usually peaks 48-72 h following EIMD (3). The exact mechanisms of delayed onset muscle soreness (DOMS) are still not well understood. Armstrong (1984) proposed that DOMS could be caused by 1) structural damage to the muscle, 2) membrane damage, disrupted calcium homeostasis and fiber necrosis, and 3) an inflammatory immune response that sensitizes group IV nerve endings (84). A more recent review puts more emphasis on the influence of nerve growth factor (NGF) release during eccentric exercise and its influence in the generation and potentiation of muscle pain after exercise (14). Peak and colleagues (85) proposed two pathways induced by neurotrophic factors involved in the development of DOMS; 1) the activation of B2 bradykinin receptor via NGF and 2) activation of the cyclooxygenase-2 (COX-2) pathway via glial cell-derived neurotrophic factor (GDNF) (13). These neurotrophic factors may be produced by muscle fibers and satellite cells (86). A study by Nie and colleagues (87) demonstrated that injecting NGF in combination with eccentric exercise increased markers of muscle pain at 24 and 48 hours in comparison to

eccentric exercise alone, demonstrating the role NGF plays in the development of post-exercise muscle soreness (87). It has also been proposed that DOMS is associated with inflammation in the extra-cellular matrix rather than direct myofiber damage (1). Interestingly, myalgia can be induced after eccentric exercise independently of myofiber damage (13). The understanding of DOMS continues to evolve; however, it is likely that structural damage and inflammation to the muscle play a role in the development of muscle soreness.

2.4 Markers of EIMD

In order to assess the presence and magnitude of EIMD both direct and/or indirect measures have been applied (88). Direct assessment of muscle damage in humans requires a needle biopsy so a sample of skeletal muscle tissue can be obtained and examined via histology (light or electron microscopy) for evidence of cellular and subcellular damage. Frequently, indirect measures of EIMD are examined including assessment of strength/force depression of the affected muscle or limb, muscle soreness or pain, flexibility, swelling, and measurement of muscle derived proteins in the blood (e.g., creatine kinase, myoglobin) (3, 89). An overview of direct and indirect measures of EIMD are discussed below along with some of their strengths and limitations.

2.4.1 Cellular and subcellular damage and the skeletal muscle biopsy

A muscle biopsy is commonly performed to obtain a sample of skeletal muscle tissue before and after exercise in order to obtain a direct measure of EIMD. The two most common biopsy needles are the Bergstrom needle and the Weil-Blakesley conchotome (90). Once the muscle sample has been obtained, the individual muscle fibers can be examined under electron microscope (55). Muscle damage can be evaluated based on cellular and subcellular disturbances (e.g., Z-band streaming) (74). Although a skeletal muscle biopsy can provide direct evidence of

EIMD, limitations of the technique include its invasive nature, and the fact that a very small sample is used to estimate damage in an entire muscle (3). There is also evidence that the biopsy procedure itself may produce changes to the muscle mistakenly attributed to EIMD (91, 92). Finally, the procedure may confound accurate assessment of muscle soreness in response to exercise.

2.4.2 Muscle function

Tests of muscle function are commonly applied to determine the power or force-generating capacity of muscle (4). A prolonged decline in muscle strength following eccentric exercise is considered to be one of the most valid and reliable indirect measures for evaluating EIMD in humans (11, 71, 93). When compared to other measures of EIMD, tests of muscle function are the most relevant to athletes and/or fitness enthusiasts whose principle aim is to expedite the recovery of muscle function as quickly as possible in order to restore optimal performance for training and/or competition. Muscle function is commonly assessed before, and at various time-points after eccentric exercise by monitoring the maximal voluntary contraction (MVC) force of a muscle group (e.g., knee-extensors or elbow flexors) (11). Typically, maximal isometric voluntary contraction (MIVC) force or concentric and/or eccentric muscle torque is assessed using a dynamometer or strain gauge (23-26, 94). Decrements in maximal force output after a bout of eccentric exercise can vary widely among individuals but are typically in the range of 15% to >50% (1, 15). Paulsen and colleagues (74) have suggested that the extent of EIMD be classified as 1) ‘mild’ if the decline in force generating capacity within the first 24 hours after exercise is no more than 20% and/or fully recovers within 48 hours, 2) ‘moderate’ if the decline in force generating capacity after exercise is between 20-50% and/or fully recovers between 48 hours and 7 days, and 3) ‘severe’ if the decline in force generating capacity after exercise is

>50% and/or that recovery of force-generating capacity exceeds 7 days. The magnitude and duration of EIMD can vary depending on factors such as the type and intensity of the exercise task, training status of the subjects, age, biological sex, and genetics (3). Classification of the severity of muscle damage based on the magnitude of decline and time-course of recovery of muscle force generating capacity is shown in **Figure 2**.

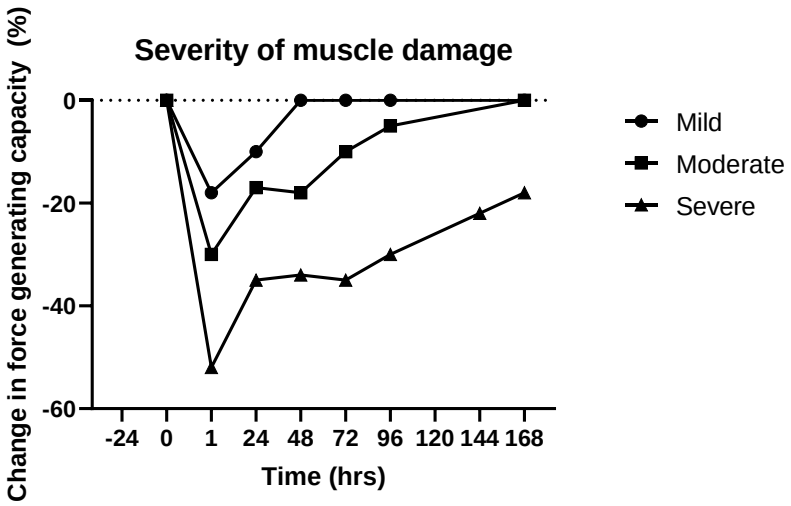

Figure 2. Classification of the severity of muscle damage based on the magnitude of decline and time-course of recovery of muscle force generating capacity. Adapted from Paulsen et al. 2012 (74).

Although MVC is a valid and reliable indirect measure for evaluating EIMD, it is somewhat limited by the fact that contractions are typically performed with isolated muscle groups during isometric contractions and/or at low velocities. As such, they may not accurately reflect the loss of function/performance that may occur with more dynamic sporting activities such as jumping and/or sprinting. Because of this limitation, some studies have applied muscle function tests such as jump tests (e.g., countermovement jump (CMJ) height) ((23, 25, 26, 95)) and/or maximal effort sprints (96, 97). Therefore, a combination of MIVC and dynamic

movement tests such as CMJ height may provide a more complete picture of changes in muscle function is response to eccentric exercise.

2.4.3 Muscle soreness

A common symptom of EIMD is muscle soreness or pain that typically appears in the hours following the damaging bout of exercise and peaks 24-72 hours later (86). Because its appearance is not always present immediately after exercise, it is frequently referred to as "delayed onset muscle soreness" (DOMS) (14). In a review of the literature, Warren (98) determined that subjectively assessed muscle soreness was the most commonly used indirect marker of EIMD in human studies (98).

Common methods for assessing muscle soreness are subjective, as they are determined by what the participant deems as painful, and the sensation of pain varies from individual to individual (99). Muscle soreness is typically measured using a visual analogue scale (VAS) questionnaire (2). A VAS to assess muscle soreness requires the research subject to rate their perceived level of soreness on a scale with wording such as 'no soreness' at one end of the scale and 'unbearably sore' at the other end of the scale (23). A 100 mm VAS has been validated as an effective tool for the assessment of acute and chronic pain, as consecutive pain measurements can be reproduced within 9 mm of accuracy (100). The simplicity and ease of measurement have likely contributed to the VAS assessment being used by many studies as a means to evaluate muscle soreness induced by exercise (16, 19, 24-26, 56, 101-103).

Another approach to quantify soreness is determination of the pressure-pain threshold (PPT) (25, 26). The PPT is defined as the point where the feeling of pressure or force applied to a muscle transitions to the sensation of pain (104). An algometer is a handheld tool that consists of a blunt headed cylindrical probe attached to a strain gauge that is commonly used for the

measurement PPT (101). The algometer is used to apply a constant rate of pressure perpendicular to the muscle belly, and the point at which pain is indicated by the research subject is recorded in Newtons or kPa (101). To date, several studies have reported a pronounced reduction in PPT (25-27, 101) that can last up to 72 hours after exercise (2). Importantly, Fleckenstein and colleagues (101) concluded that PPT is a reliable tool for assessing the degree of muscle pain caused by EIMD. An advantage of PPT over VAS to quantify soreness/pain is that it somewhat less subjective; however, the combination of both measures may represent a useful approach to monitor and quantify muscle soreness in response to EIMD. Muscle soreness can pose a serious problem for the athlete and is important to monitor during the recovery process; however, quantifying muscle soreness in the absence of other indices (e.g., muscle function) is not sufficient to describe EIMD (74).

2.4.4 Muscle derived proteins as markers of damage

As described above, the primary phase of muscle damage is characterized by ultrastructural damage to the muscle, specifically to the myofibers and sarcolemmal membrane (70). The damage to the myofibers induces a host of cascading events modulated by the immune system that lead to further breakdown of muscle tissue followed by the remodelling of muscle fibers (13). The concentration of muscle-derived proteins (e.g., creatine kinase (CK), myoglobin, lactate dehydrogenase (LDH)) in the systemic circulation via blood sampling is commonly measured as a surrogate of EIMD (for a comprehensive review see (13)). One of the most commonly assessed systemic markers of EIMD is CK (105). CK is present within different types of cells such as skeletal muscle, cardiac muscle, and brain tissue, and all are classified as various isoforms (CK-MM: skeletal muscle; CK-MB: cardiac muscle: CK-BB: brain) (106). When the sarcolemmal membrane becomes damaged/disrupted due to EIMD, the permeability of the

membrane increases and muscle proteins normally found in the cytosol of the cell, such as CK and myoglobin, begin to leak into the circulation (3). An increase in CK in the blood following damaging exercise is typically observed within 24 hours but can reach peak concentrations as late as 96-hours post-exercise (13, 106). Although CK is a commonly measured marker of EIMD, circulating muscle specific proteins have been reported to show a poor temporal relationship with muscle function (69). An additional limitation of CK is that it demonstrates high inter-subject variability, even amongst a relatively homogenous subject population (74). Therefore, an increase in CK concentration following eccentric exercise may be best used as an indication that damage has occurred, but should not be used to assess the magnitude of the damage response (15).

2.4.5 Exercise-induced inflammation

Exercise-induced inflammation is commonly assessed using indirect markers such as changes in limb girth (e.g., circumference) due to local swelling/oedema (98), or more directly by measuring the concentration of specific inflammatory biomarkers in blood or muscle samples. Cytokines can be measured either in the blood or in the muscle via muscle biopsy (5). After a bout of muscle damaging exercise, the damaged muscle tissue will begin to release signals that will engage the repair process. The damaged endothelial cells will begin to release cytokines such as interleukin 1β (IL-1β), interleukin 6 (IL-6), granulocyte-colony stimulating factor (G-CSF) and monocyte chemoattractant protein 1 (MCP-1) to signal the immune response (107). G-CSF is noted to peak after 3 hours of exercise and is correlated with an influx of neutrophils (73). Neutrophils begin migration into damaged tissue within the first hours after exercise and begin to produce tumor necrosis factor alpha (TNF-α) and IL-1β (72). The chemokine MCP-1 stimulates the migration and infiltration of monocytes and macrophages into the damaged tissue which

takes place within 24 hours (108). The most commonly measured cytokine produced by cells after the onset EIMD is IL-6 (5). IL-6 is a pro-inflammatory cytokine that is produced by pro-inflammatory macrophages in muscle within 1-4 hours after the damaging stimulus occurs, and is termed “the inflammation response” cytokine (109). IL-6 has been found to modulate the anti-inflammatory cytokines IL-1ra and IL-10 (110). IL-6 also influences the release of pro-inflammatory cytokines (IL-1β, and TNF-α) via communication with macrophages (109). Interlukin-10 (IL-10) has been found to be an anti-inflammatory cytokine that influences the shift of pro-inflammatory macrophages to anti-inflammatory macrophages (107). Other cytokines such as IL-8 and IL-15 are thought to play a role in the repair process of cells, where IL-8 can influence angiogenesis and IL-15 stimulates the anabolic repair of myosin heavy chain fibers (110). The inflammatory process appears to beinitiated by mechanical stress to the muscle fiber tissue.

2.5 Modifiers of EIMD

The extent (i.e., magnitude and/or duration) of EIMD may be influenced by a number of factors including the type of eccentric exercise, training status, biological sex, nutritional supplementation, age, and genetic variation (2, 6, 11, 14, 15, 88). In this section, the influence of exercise type, training status/history, and sex differences are discussed within the context of EIMD as they are relevant to the current thesis. For a review of EIMD and the older adult, the reader is directed to Clifford (6). For a review of the role of genetic variation in the EIMD response, the reader is directed to Hyldahl and Hubal (11).

2.5.1 Exercise type

Various forms of exercise have been applied in EIMD research that may differentially affect the degree of EIMD. Forms of exercise include resistance exercise

(with equal concentric and eccentric loads), eccentrically-biased exercise (e.g., downhill running, running down stairs, drop jumps off an elevated box), maximal single-joint eccentric exercise (e.g., of the quadriceps or biceps using a dynamometer), long-distance running, and eccentric cycling (for review see (74)). In a review of the literature, Peake and colleagues (13) concluded that the magnitude and/or duration of EIMD was greater when 1) exercise is performed at a high vs. low eccentric torque, 2) a greater number (volume) of eccentric contractions are performed, 3) contractions occur at long vs. short muscle lengths, 4) single-joint vs. multi-join exercise is employed, 5) exercise utilizing the arms vs. the legs is employed, 6) exercise involving the knee-flexors vs. knee-extensors is utilized, and 7) a fast vs. slow eccentric velocity is used. In the current thesis, drop jumps (off a 60 cm box), as a form of eccentrically biased exercise targeting the quadriceps was used as it has been applied in numerous EIMD studies (16, 25, 26, 103). Drop jumps have been shown to reliably reduce muscle function (based on MIVC and CMJ), increase muscle soreness, and elevate blood concentrations of CK and LDH; however, the CK response is mild to moderate in comparison to other forms of eccentric exercise (111).

2.5.2 Training status/history

One of the most significant factors in determining the amount of damage induced by exercise is the training status of the individual, and more specifically, their familiarity with the exercise task (7). Skeletal muscle possesses the ability to adapt to a mechanical stress, and by doing so, protect itself from subsequent damage. The repeated bout effect (RBE) refers to the observation that performance of as little as a single bout of eccentric exercise that causes EIMD, results in an adaptation such that EIMD is substantially reduced when the exercise is repeated (112). For example, as compared to the first bout of eccentric exercise, the second bout of eccentric exercise produces significantly less muscle soreness and is associated with a faster

recovery of muscle function (113, 114). The exact mechanisms of the RBE are not well understood, but the adaptions are likely multifactorial (for review see (7)) and include protective neural adaptions (115), mechanical (116), as well as cellular adaptions (117). Interestingly, the RPE may spread beyond the site of damage. An example of the repeated bout effect and contralateral protection was shown in a study by Howatson & van Someren (118). The participants conducted two rounds of 45 unilateral maximal eccentric contractions of the elbow flexors separated by a 2-week period. The initial bout of exercise was performed on only one elbow flexor and induced significant muscle damage as per measured indices (force loss, CK response, reduced range of motion). A second trial was performed two weeks later, this time the muscle damaging exercise was performed on both elbow flexors. The second round of exercise significantly induced muscle damage; however, the degree of damage was significantly less than the initial bout in both elbow flexors. It is important to note that the degree protection experienced by the contralateral arm was less than the arm that underwent the initial bout of damaging exercise (118). Therefore, eccentric exercise may not only induce a protective effect to the direct site of mechanical loading, but also transmit a protective effect to the contralateral limb and potentially throughout the rest of the body. These contralateral limb effects have been replicated in other studies (113, 119).

Given the RBE, selection of research participants with a relatively homogenous training status/background is particularly important, as familiarity with the exercise task will greatly influence the extent of damage (or indices of damage) it produces. Secondly, from a study design perspective, EIMD research may not lend itself well to a within-subject crossover study design where research participants participate in two separate exercise tasks in random order (e.g., one experimental trial and one control trial). In this type of study design the extent of damage would

be expected to be less in the second exercise task as compared to the first. Therefore, depending on the nature of the research question, an independent group study design may be more appropriate than a within-subject crossover study design in order to minimize the potential influence of the RBE.

2.5.3 Sex-differences

One of the most heavily studied potential modifiers of the EIMD response is biological sex. There is some debate as to whether there are sex differences with respect EIMD and its symptomatic manifestations. Animal models of EIMD have provided strong evidence of a protective effect of the hormone estrogen on muscle membranes (120). However, results from studies in humans are more mixed. A study with a relatively large sample size (men: n=82; women: n=83) by Rinard and colleagues (121) demonstrated no difference between men and women with respect to post-exercise muscle soreness, strength loss, and force recovery. Women did however experience a significantly greater loss in range-of-motion (ROM) from 72-168 h post-exercise recovery (121). Another large study (men: n=42; women: n=58) by Sewright and colleagues (58) examined sex differences in response to EIMD for muscle function (MIVC), blood CK and myoglobin concentrations, and muscle soreness. No differences between sexes for soreness and myoglobin were reported; however, women demonstrated a significantly larger relative force loss immediately after exercise while men had a larger absolute response for blood CK (58). Finally, a recent systematic review and meta-analysis incorporating 23 trials demonstrated that there were no significant differences between men and women with regards to muscle soreness, force recovery, and blood CK responses when normalized to body mass, cross-sectional area of muscle, and fat-free mass (122). Overall, the results of this recent meta-analysis

suggest that men and women display similar responses in measures of relative muscle function and muscle soreness following eccentric exercise.

2.5.4 Nutritional interventions to expedite recovery from EIMD

Various approaches have been evaluated for their effectiveness in expediting recovery (e.g., restoring muscle function, reducing soreness, lowering inflammation) from EIMD including physiotherapeutic, pharmacological, and nutritional interventions (15). The focus of this thesis is on nutritional interventions and EIMD; therefore, a brief overview of the mechanisms of how select nutritional interventions may augment recovery from EIMD are discussed. For a detailed overview of post-exercise recovery techniques to reduce markers of muscle damage, muscle soreness, fatigue, and inflammation the reader is referred to Dupuy and colleagues (123).

A number of nutritional interventions have been investigated in their capacity to expedite recovery following muscle damaging exercise in humans including nutrients such as vitamin D, creatine monohydrate, omega-3 polyunsaturated fatty acids, protein, branched-chain amino acids, and so called 'functional foods' (e.g., beet root, tart cherries, pomegranate) (for reviews see (9, 15)). Nutritional interventions are unlikely to interact with and attenuate the primary phase of EIMD (15, 88); however, some nutrients may interact with components of the secondary phase of EIMD (15). For example, many of the nutritional interventions that have demonstrated some efficacy in expediting the recovery of indices of EIMD (e.g., muscle function, muscle soreness) target inflammation and oxidative stress, processes thought to contribute to secondary damage. Indeed, the consumption of tart cherries (23, 24, 27, 124) and beet root concentrate (25, 26, 125) has been found to improve markers of recovery from EIMD such as soreness, strength, the CK response, and markers of inflammation (23-27, 124). These

foods contain dietary polyphenols, some of which contain antioxidant (18, 126) and anti-inflammatory properties (126-128). Beetroot is a source of nitrates which are metabolised by oral bacteria and are converted to nitrites (127). These nitrites are then metabolised to nitric oxide which improves endothelial function and can improve hypertension (127). Tart cherries have been found to reduce oxidative stress (128), improve lipid profile (128) and sleep quality (128).

Other nutritional interventions such as protein and amino acids, known to augment muscle protein turnover (i.e., the rate of protein synthesis and breakdown), have also shown some efficacy in expediting recovery from EIMD (16, 129). Protein/amino acid supplements may help expedite recovery from EIMD via altering the rate of remodeling and repair of proteins in muscle (5, 32, 130). Overall, nutritional supplements with the potential to modulate inflammatory processes, ROS/oxidative stress, and/or protein metabolism may represent part of an effective nutritional strategy to expedite recovery from damaging eccentric exercise.

2.6 Overview of the ketone bodies

Recently, there has been a surge of interest in the use of ketogenic diets and novel exogenous ketone supplements in athletic populations as well as with military personnel (34, 39, 131) as a means to improve exercise performance. Ketone bodies (beta-hydroxybutyrate (β-OHB), acetoactetate (AcAc), and acetone) are lipid-derived organic molecules that are naturally produced by the body during periods of starvation, prolonged dietary carbohydrate restriction, or prolonged glycogen-depleting exercise (35). Under these conditions, fatty acid mobilization from adipose tissue is increased to supply energy. Some of the acetyl-CoA derived from fatty acids is converted to ketone bodies via hepatic mitochondria (35). Both AcAc and β-OHB are transported in the bloodstream to extrahepatic tissues with a high metabolic demand such as the brain, heart, and skeletal muscle; the majority of acetone is secreted through urine and lost via expiration (35,

36, 132, 133). In extrahepatic tissues, ketone bodies cross the plasma and mitochondrial membranes by monocarboxylate transporters and are converted back to acetyl-CoA and used as an alternative source of energy by the tricarboxylic acid (TCA) cycle (132, 133). In addition to serving as an alternative fuel source, ketone bodies play an important role in regulating carbohydrate, lipid, and protein metabolism (35, 132, 133), cellular signaling and transcription (134, 135), and may have various therapeutic implications (for a review see (39)).

2.6.1 Ketone supplements and exogenous ketosis

Recently, novel exogenous ketone supplements have been developed which can rapidly (within < 30 min) induce nutritional ketosis (blood ketone concentration > 0.5 mM) without the requirement of dietary energy or carbohydrate restriction (36, 37). There are currently two main types of exogenous ketone supplements; ketone salts and ketone esters (36, 37). Ketone salt supplements typically contain β-OHB bound to a cation such as sodium, lead to a very modest rise in blood ketone concentrations (~1 mM), and often contain a racemic mixture of D-β-OHB and L-β-OHB optical enantiomers (36). There are currently two main types of ketone esters; the ketone monoester (R)-3-hydroxybutyl (R)-3-hydroxybutyrate and the 1,3-butanediol acetoacetate diester. These compounds are made up of one or more ketone molecules (AcAc or D-β-OHB) joined with an ester bond to a ketone precursor (glycerol or butanediol) (133). Once orally ingested, the ester bonds are hydrolyzed by esterases found in the gut allowing the subsequent release of ketones into the blood (133). Ketone ester supplements have been shown to safely, rapidly, and reproducibly increase blood ketone concentrations (37), in a dose-dependent manner (36, 37) up to physiological limits of ~7 mM (37). Therefore, as compared to ketone salts, ketone ester supplements can achieve a greater increase in blood ketone concentration (36) and provide the more physiologically relevant D-β-OHB enantiomer. To date, only a limited number of

studies have evaluated the impact of the ketone monoester (R)-3-hydroxybutyl (R)-3-hydroxybutyrate on exercise performance (34, 38-40, 136) and recovery (41, 137, 138). Given the focus of the current thesis on exercise recovery processes, the following section provides an overview on the rationale for the use of exogenous ketone supplements to support exercise recovery processes in athletes and active individuals.

2.6.2 Ketone supplements and exercise recovery

Novel nutritional treatments that can expedite the recovery of muscle function and/or soreness following EIMD are of interest to athletes, coaches and the scientific community. Such treatments may provide a means to improve high-level athletic performance in back-to-back training and/or competition scenarios and may also shed light on the complex mechanisms of EIMD, DOMS, and muscle recovery (15).

Elevated β-OHB has been suggested to influence exercise recovery through mechanisms associated with its ability to regulate protein metabolism (i.e., protein synthesis and breakdown), inflammation, and/or oxidative stress (39). It is well established that protein intake after exercise stimulates increased rates of muscle protein synthesis through activation of the mechanistic target of rapamycin (mTOR) (137). Increased rates of muscle protein synthesis after exercise are critical to support skeletal muscle remodeling and eventually, a change in muscle phenotype with prolonged exercise training (32). Ketone bodies appear to be protein sparing (i.e., anti-catabolic) based on evidence that they can improve nitrogen balance under catabolic conditions (42). In addition, intravenous infusion of β-OHB to ~2 mM has been shown to reduce leucine oxidation and stimulate increased rates of skeletal muscle protein synthesis *in-vivo* in humans (41). Recently, exogenous β-OHB, taken in combination with protein and carbohydrate was shown to stimulate greater mTORC1 pathway activation and protein synthesis in leucine-stimulated

myotubes, in comparison to protein and carbohydrate alone during recovery from intense exercise (137). Collectively, β-OHB appears to possess both anabolic and anti-catabolic properties, which may therefore contribute to the repair/remodeling of proteins following exercise.

As discussed above, oxidative stress refers to a state in which ROS are present in excess, either because of excess production and/or impaired elimination, and contributes to the secondary phase of EIMD (2). Interestingly, anti-oxidant rich supplements that reduce oxidative stress such as beetroot (25, 26, 127) and tart-cherry juice (23, 24, 27, 124) have been found to be effective in improving indices of muscle damage during recovery from eccentric exercise (15). Antioxidant and oxidative stress-mitigating roles of ketone bodies have been described both *in-vitro* and *in-vivo*, although mostly within the context of neuroprotection (139). For example, ketone bodies have been shown to reduce cellular damage, injury, death, and apoptosis in cardiomyocytes and neurons (140-142), with β-OHB able to scavenge ROS such as hydroxyl anion (140). Recently, β-OHB has been shown to be a histone deacetylase inhibitor, which can increase the expression of antioxidant genes and proteins responsible for decreasing cellular oxidative stress (43).

An inflammatory response can also occur during the recovery from EIMD. As described previously, the immune/inflammatory response that occurs after eccentric exercise induces further muscle damage as part of the repair process (e.g., secondary phase of EIMD). Ketone bodies are known to modulate inflammation and immune cell function, with β-OHB exerting a predominantly anti-inflammatory response (139). Various immune system cells including macrophages and monocytes express GPR109A; β-OHB is an endogenous ligand of the GPR109A receptor (143). β-OHB has been reported to lead to anti-inflammatory effects in TNF-

α and lipopolysaccharide-induced inflammation by reducing pro-inflammatory proteins such as iNOS, and COX-2, or cytokines such as TNF- α, IL-1β, IL-6, and MCP-1, partly by inhibiting NF-kB (144, 145) In a recent mouse model study, β-OHB was also shown to decrease inflammation via reducing inflammatory cytokines such as IL-1β (which is elevated due to muscle damage (13, 72)) via blocking the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome pathway (44).

Given the role of β-OHB in regulating protein metabolism, inflammation, and oxidative stress, research is warranted to evaluate the effects of exogenous ketone supplements and elevated β-OHB as a nutritional strategy to expedite the recovery of indices of muscle damage following eccentric exercise. An overview of the potential mechanisms by which β-OHB may influence the recovery of indices of EIMD is shown in **Figure 3**.

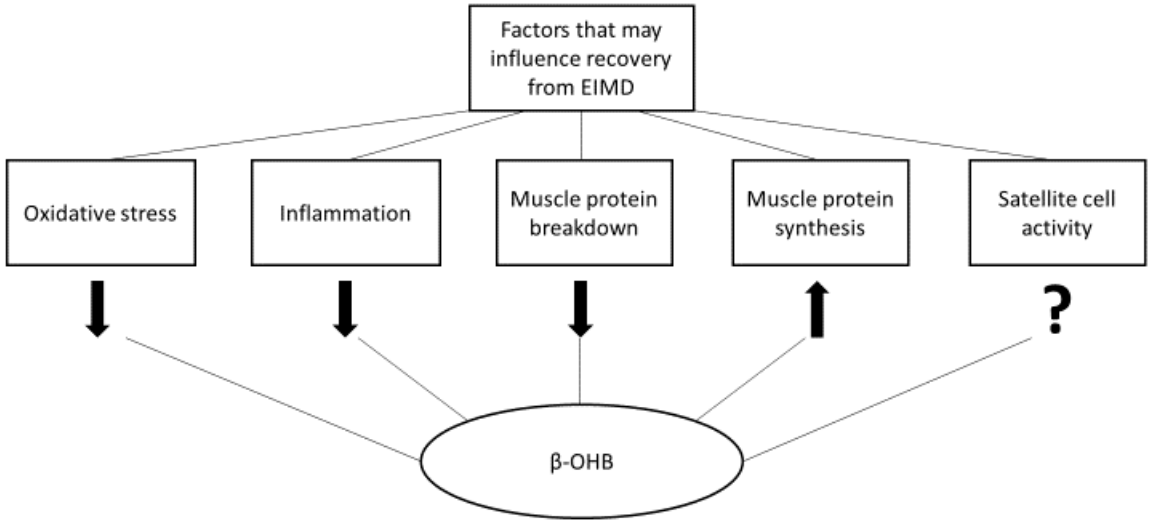

Figure 3. Potential mechanisms by which β-OHB may influence recovery from EIMD. Adapted from Clifford et al. 2019 (6).

CHAPTER 3: MANUSCRIPT

3.1 INTRODUCTION

Exercise-induced muscle damage (EIMD) is a non-pathological condition that occurs in response to unaccustomed exercise involving repeated eccentric (i.e. lengthening) muscle contractions. The main consequences of EIMD are a temporary reduction in muscle function (i.e. force production) and increased muscle soreness accompanied by an increase in intramuscular proteins (e.g. creatine kinase (CK)) in the blood and swelling of the involved muscle group (146). Consequently, EIMD may negatively impact the ability to perform during subsequent training/exercise sessions.

A variety of nutritional interventions have been evaluated in an attempt to alleviate muscle soreness and impairments in muscle function during recovery from eccentric exercise (for reviews see (9, 15, 147, 148)). Many of these interventions focus on nutrients and/or foods thought to target muscle protein metabolism (i.e., muscle protein synthesis and breakdown), oxidative stress, and/or inflammation. For example, protein ingestion stimulates increased rates of muscle protein synthesis after exercise (149) and has been reported in some studies (96, 150-153) to expedite the recovery of indices of EIMD. In addition, fruits such as Montmorency cherries have been reported to expedite the recovery of indices of EIMD (23, 24, 124), and are thought to exert their effects via phytochemicals that possess antioxidant and anti-inflammatory properties, thereby targeting oxidative stress and inflammation and reducing the potential for secondary muscle damage. Given the negative symptoms associated with EIMD, novel nutritional approaches that can effectively reduce indices of EIMD and accelerate recovery after eccentric exercise are of interest to athletes, coaches, and the broader fitness community.

Ketone bodies (i.e. D-β-hydroxybutyrate (β-OHB), acetoacetate, and acetone) are lipid-derived molecules that are normally produced in the body in response to starvation or

carbohydrate restriction (i.e. a ketogenic diet). Under these conditions they serve as an important fuel source for metabolically active tissues including the brain and skeletal muscle. Recently, exogenous ketone 'supplements' have been developed, including the ketone monoester (R)-3-hydroxybutyl (R)-3-hydroxybutyrate (37), that rapidly increases β-OHB to approximately 3-5 mM within ~30 min in healthy humans (137) without the need for dietary restriction. The use of exogenous ketone supplements and increased β-OHB has been suggested to have the potential to augment post-exercise recovery processes through mechanisms related to the capacity of β-OHB to regulate protein turnover, inflammation, and/or oxidative stress (34, 39). For example, β-OHB has been shown to stimulate skeletal muscle protein synthesis in humans (41), and reduce muscle (154) and whole-body protein breakdown (42, 154). Inflammation and oxidative stress-mitigating roles of ketone bodies have been described both *in-vitro* and *in-vivo* (139). Collectively, these actions indicate a potential role for ketone supplements and elevated β-OHB in expediting the recovery of indices of EIMD after eccentric exercise.

The purpose of this study was to evaluate the effects of supplementation with a ketone monoester, that acutely increases blood β-OHB concentration, on indices of EIMD including muscle dysfunction and muscle soreness during recovery from a bout of eccentric exercise in healthy young active adults. We hypothesized that twice daily supplementation with a ketone monoester (KET) would expedite the recovery of muscle function, reduce muscle soreness, and alter the concentration of select pro- and anti-inflammatory cytokines when compared to supplementation with an energy-matched carbohydrate supplement (CON).

3.2 METHODS

3.2.1 Participants

20 healthy young men and women (*n* = 10 men and 10 women) 18-35 years of age, with a BMI >18.5 and <30.0 kg/m^2 participated in this randomized, double-blind, parallel group study. Participants were moderately active (defined as regularly exercising 2-4 times per week) but unaccustomed to high-force plyometric exercise. Participants were excluded if they met any of the following criteria: identified metabolic or intestinal disorder, use of tobacco products, adherence to a ketogenic, vegetarian, or vegan diet, use of certain medications (i.e., corticosteroids, non-steroidal anti-inflammatories, or prescription strength acne medications), musculoskeletal ailments that prevented them from performing the required exercise, pregnancy, asthma, and use of certain dietary supplements (i.e. creatine, fish oil, beta-alanine). Female participants were studied during the early follicular phase of their menstrual cycle (within 5 days of the onset of menses). Participants' characteristics are presented in **Table 1**. All participants were informed about the purpose of the study, the experimental procedures, and possible risks prior to providing informed written consent to participate. The study was conducted in accordance with the ethical standards of the Faculty of Medicine Institutional Review Board at McGill University on human experimentation and in accordance with the Helsinki Declaration of 1975 as revised in October 2013.

3.2.2 Research ethics approval

The study was approved by the Faculty of Medicine Institutional Review Board at McGill University on January 14, 2019 (IRB Study Number: A01-M01-19A). All participants provided written informed consent prior to study participation.

3.2.3 Preliminary testing

Participants underwent an initial screening and familiarization visit during which height (via a wall-mounted stadiometer), weight (via a digital balance), heart rate and blood pressure (Omron 10 series, Model BP786CANN), and body composition (by dual-energy X-ray absorptiometry; GE Healthcare, Madison, WI, USA) were assessed. Participants were also thoroughly familiarized with the exercise testing equipment and procedures. Participants received a demonstration by one of the study investigators and then performed a guided familiarization trial for the performance measures. Participants were deemed healthy based on their responses to a medical questionnaire and screening results. The initial screening and familiarization visit, and onset of the experiment were separated by at least 4 days.

3.2.4 Diet and physical activity

Study participants were asked to refrain from strenuous physical activity and alcohol consumption for 2 days immediately prior to the onset of the experiment and during all experimental test days. In addition, participants were required to complete food intake and physical activity questionnaires during this time. Average dietary intake prior to and during the experiment is shown in *Error! Reference source not found.* and was analyzed using commercially available software (Food Processor version 11.7; ESHA Research; Salem OR, USA). On the evening before each experimental test day, participants were instructed to stop consuming food or beverages other than water or their nutritional treatment (described below) by 20:00 h, after which they remained fasted until testing the following morning.

3.2.5 Overview of study design

The present study utilized a randomized, double-blind, independent group design. One group (KET) received a ketone monoester, while the other group (CON) received an energy-

matched amount of carbohydrate. Participants ingested their assigned treatment twice daily for two days during recovery following an acute bout of eccentric exercise (described below). During the study, participants were required to report to the laboratory on three sequential days (not including the visit for preliminary testing and familiarization) for testing. Testing occurred at baseline (PRE), immediately post-exercise (POST), 24-hours post-exercise (24 h) and 48-hours post-exercise (48 h) and consisted of the following: tests of muscle function (maximal isometric voluntary contraction (MIVC) of the knee-extensors and counter movement jump height (CMJ)), muscle soreness (VAS questionnaire and pressure pain threshold (PPT)), markers of exercise induced muscle damage (serum creatine kinase (CK)), limb girth of the upper leg and calf (as a proxy for swelling/edema), flexibility of the quadriceps (via goniometer during a Modified Thomas Test), and plasma concentrations of select cytokines and chemokines. Testing also consisted of the Brief Assessment of Mood Adapted (BAM+) questionnaire to assess the participants "readiness" for performance (155). The randomization procedure to allocate treatment group was determined via a random-number generator (http://www.randomization.com/). An equal number of men and women were randomized to the KET and CON groups, respectively. An independent person was responsible for the randomization and preparation of the study beverages. The beverages were prepared in non-transparent plastic containers. To limit diurnal and intrasubject variation, all measures were carried out according to a standardized time schedule at the same time of day.

3.2.6 Experimental protocol

On Day #1 of the study, participants reported to the laboratory at ~0800 in the overnight postabsorptive state. Participants underwent a venous blood draw from the antecubital vein via

venipuncture followed by baseline (PRE) testing in the following order: BAM+ questionnaire, VAS of muscle soreness, limb circumference, PPT, and Modified Thomas Test (measures described below). Subsequently, participants performed a 5 min brisk walking warm-up on a treadmill at a speed of 5.0 km/h with an incline of 1.0, followed by assessment of knee extensor MIVC torque (Biodex 4 Pro, Biodex Medical Systems, Shirley, NY, USA) and CMJ height (Dual force plates model 9260AA6, Kistler group, Winterthur, Switzerland). Participants then performed an eccentric exercise protocol consisting of 100 box drops off a 60 cm box (5 sets of 20 repetitions with a 2 min inter-set rest interval). Following 15 minutes of recovery from the eccentric exercise protocol, participants completed post-exercise testing (POST) of the following: BAM+ questionnaire, VAS of muscle soreness, limb circumference, PPT, Modified Thomas Test, knee extensor MIVC, and CMJ. Participants then received and ingested their randomly assigned treatment (KET or CON). Capillary blood samples were obtained via finger prick to assess capillary blood β-OHB concentration (FreeStyle Precision Neo, Abbott Laboratories) immediately following POST testing prior to treatment intake, and at 30, 60, 120, and 180 min following treatment intake while participants rested in a recumbent position on a bed in the laboratory. An independent person obtained the capillary samples to allow the study investigators to remain blinded to the treatment intervention. Participants then received a second treatment drink that they were instructed to consume on their own ~30 minutes before going to sleep. The following morning (Day #2) participants reported to the laboratory in the overnight postabsorptive state and completed the 24 h post-exercise (24 h) testing in a manner identical to PRE (baseline) testing. Participants then received the third serving of their randomly assigned treatment and returned the containers from the treatment consumed the evening prior. Participants then received their fourth and final treatment drink that they were instructed to

consume on their own ~30 minutes before going to sleep. The following morning, participants again reported to the laboratory (Day #3) in the overnight postabsorptive state, completed the 48 h post-exercise (48 h) testing protocol (identical to the 24 h testing outlined above), and returned the containers from the treatment consumed the evening prior. For an overview of the experimental protocol see **Figure 4**.

3.2.7 Venous blood sampling and analysis

Venous blood samples were drawn into a 4-mL vacutainer for serum and two 3-mL vacutainers coated with di-potassium ethylene diamine tetra-acetic acid (EDTA) for plasma during PRE, 24 h, and 48 h time-points. Samples were centrifuged at 3000 g for 15 min at 4 °C. Plasma and serum were then aliquoted and stored at -80 °C until further analysis. Analysis of total CK concentration was carried out on serum samples using a chemistry analyzer (Beckman Coulter Olympus AU5800) at the Clinical Biochemistry Laboratory of the McGill University Health Centre. According to data provided by the laboratory, the coefficient of variation (CV) for total CK is 2.5%. Analysis of circulating granulocyte colony stimulating factor (G-CSF), interleukin-1 beta (IL-1β), interleukin-1 receptor antagonist (IL-1ra), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10), interleukin-12p70 (IL-12(p70)), interleukin-15 (IL-15), monocyte chemoattractant protein 1 (MCP-1), and tumour necrosis factor alpha (TNF-α) was carried out on plasma samples using Luminex xMAP technology for multiplexed quantification of human cytokines, chemokines, and growth factors. The multiplexing analysis was performed in duplicate using the Luminex™ 200 system (Luminex, Austin, TX, USA) by Eve Technologies Corp (Calgary, Alberta). In the first multi-plex experiment, 6 markers were simultaneously measured in the samples using Eve Technologies' Custom Human High Sensitivity 6-Plex Discovery Assay® (MilliporeSigma, Burlington, Massachusetts, USA)

according to the manufacturer's protocol. The 6-plex consisted of IL-6, IL-8, IL-10, IL-12p70, IL-1β and TNF-α. Assay sensitivities of these markers are 0.11 pg/mL, 0.13 pg/mL, 0.56 pg/mL, 0.15 pg/mL, 0.14 pg/mL and 0.16 pg/mL, respectively. In the second multiplex experiment, 4 markers were simultaneously measured in the samples using Eve Technologies' Custom Human Cytokine Panel 1 4-Plex. (MilliporeSigma, Burlington, Massachusetts, USA) according to the manufacturer's protocol. The 4-plex consisted of IL-1ra, IL-15, MCP-1 and G-CSF. Assay sensitivities of these markers are 8.3 pg/mL, 1.2 pg/mL, 1.9 pg/mL and 1.8 pg/mL, respectively. At least one of the two duplicate samples were below the limit of analytical sensitivity in 35% of samples for G-CSF, 42% of samples for IL-1ra, and 47% of samples for IL-15. Therefore, these analytes were removed from analysis. The CV's for the analyzed data were as follows: IL-1β: 9.3%; IL-6: 7.0%; IL-8: 9.5%; IL-10: 8.8%; IL-12p70: 8.4%; MCP-1: 5.7%, and TNF-α 7.8%.

3.2.8 Subjective mood questionnaire (BAM+)

The Brief Assessment of Mood Adapted (BAM +) questionnaire was used to qualitatively assess participants' mood, recovery status and overall performance readiness (155). The questionnaire contains six items from The Brief Assessment of Mood (BAM) and four questions relating to confidence, motivation, muscle soreness and sleep quality. The BAM+ consists of ten questions and participants are asked to mark a line on a 100 mm VAS, anchored with "not at all" and "extremely" at each end. The lines for each question were measured with a ruler, and an overall recovery score was calculated as described by Shearer and colleagues (155).

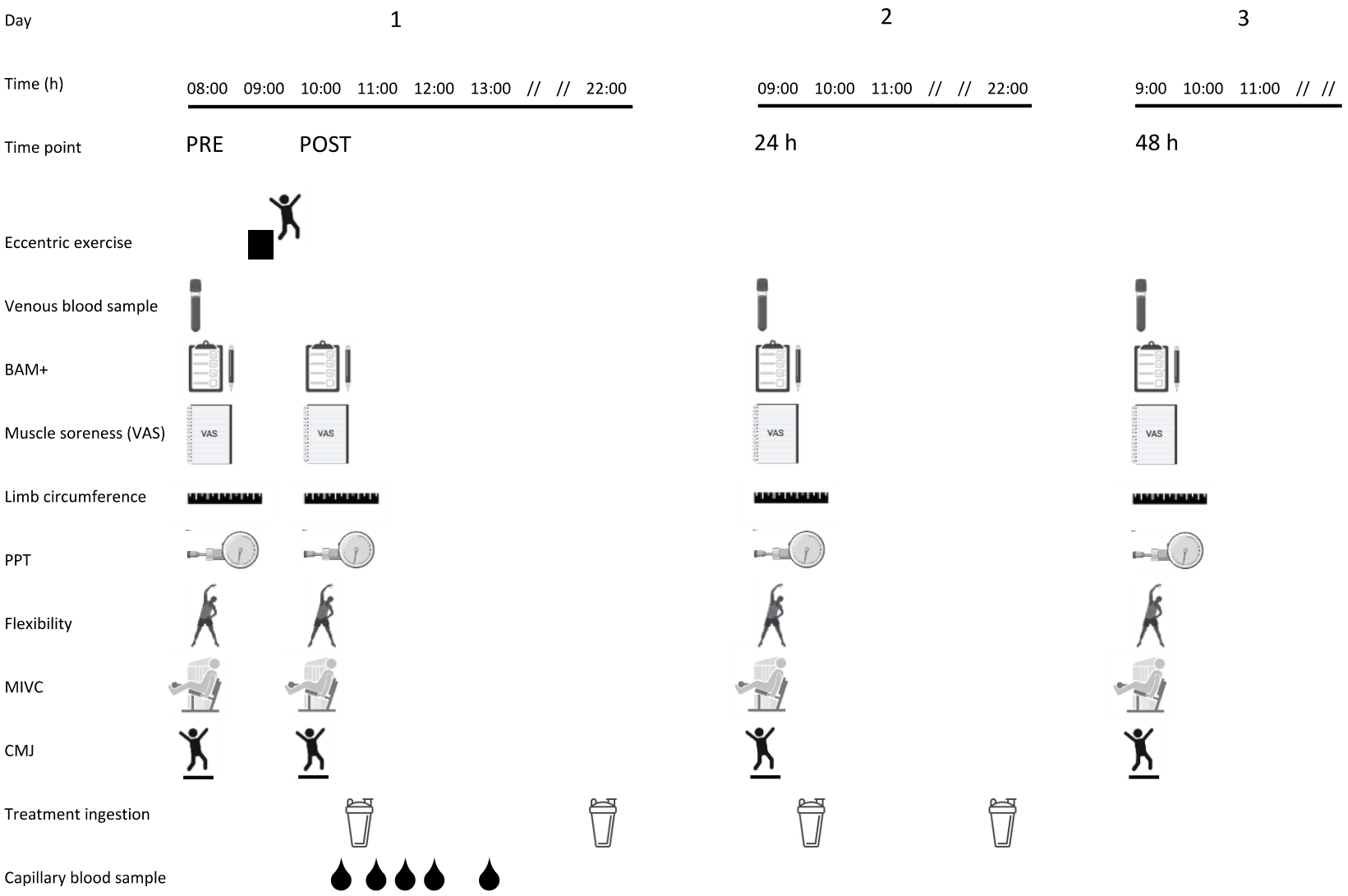
Day
1
2
3
Time (h)
08:00 09:00 10:00 11:00 12:00 13:00 // // 22:00
09:00 10:00 11:00 // // 22:00
9:00 10:00 11:00 // //
Time point
PRE
POST
24 h
48 h
Eccentric exercise
Venous blood sample
BAM+
Muscle soreness (VAS)
VAS
VAS
VAS
VAS
Limb circumference
PPT
Flexibility
MIVC
CMJ
Treatment ingestion
Capillary blood sample

Figure 4. Overview of the experimental protocol. Twenty young adults (10 men; 10 women) performed an acute bout of eccentric exercise and were studied over 48 h of post-exercise recovery. During the recovery period, participants received a ketone monoester (KET) or energy-matched carbohydrate (CON) supplement two times per day (in the morning immediately after testing and in the evening 30 min prior to sleep). Testing occurred at baseline (PRE) before the bout of eccentric exercise, and then immediately (POST), 24 h (24 h), and 48 h (48 h) after exercise and included the following: venous blood sample (except at POST), BAM+, VAS muscle soreness, limb (thigh and calf) circumference, PPT, flexibility, MIVC, and CMJ. Capillary blood samples were taken before (0 min) and after (30, 60, 120, 180 min) intake of the first supplement serving. BAM+: brief assessment of mood adapted; CMJ: countermovement jump; CON: carbohydrate control group; KET: ketone monoester group; MIVC: maximal isometric voluntary contraction; PPT: pressure-pain threshold; VAS: visual analogue scale.

3.2.9 Muscle soreness

Muscle soreness was assessed via VAS questionnaire and determination of PPT. For assessment of muscle soreness using the VAS questionnaire, participants were asked to perform a squat (at ~90° knee flexion) and rate their level of perceived muscle soreness in their lower limbs by marking a 100 mm horizontal line scale. 0 on the VAS represented 'no soreness' and 100 mm represented 'unbearably painful'. The line placement was measured with a ruler and recorded.

PPT was assessed using a handheld digital algometer (Somedic SenseLAB AB, Sodala, Sweden) at three pre-marked sites: the *vastus lateralis* (VL), *rectus femoris* (RF), and *gastrocnemius*. The VL was assessed mid-way between the superior aspect of the greater trochanter and head of the tibia. The RF was assessed mid-way between the anterior patella and inguinal fold. The gastrocnemius was assessed on the medial aspect of the calf at relaxed maximum girth. The sites were marked with semi-permanent ink to ensure consistent measurements between days. PPT was assessed while participants lay supine on a table by a study investigator applying pressure at the sites with the algometer until the participant indicated they felt the sensation of pressure transition to the feeling of pain, at which point the pressure value in kPa was recorded. Each site

was assessed twice unless recordings differed by 100 or more kPa, in which case the site was assessed a third time and the average was recorded.

3.2.10 Limb circumference

Circumference at the mid-thigh and calf was assessed as a proxy of limb swelling using an anthropometric tape measure. Both sites were measured with the participant in a standing position. The mid-thigh measure was determined as the mid-point between the inguinal crease and superior aspect of the patella. The calf measurement was made at the widest part of the calf. Both sites were marked with semi-permanent ink to ensure consistent measurements between days (16, 94).

3.2.11 Flexibility

The Modified Thomas test was used to assess flexibility about the thigh region (including hip and knee joint) using a goniometer. The test was performed by having the participant lay supine on the edge of a treatment table, hold his or her non-testing knee to the chest, while letting the opposite (tested) thigh and leg hang freely off the table. The goniometer was lined up to the greater trochanter, the lateral epicondyle of the femur and the lateral malleolus. Hip extension angle (X° - 90°) and knee flexion angle (180° - X°) were then determined.

3.2.12 Maximal Isometric Voluntary Contraction (MIVC)

MIVC of the knee extensors was assessed using a dynamometer (Biodex 4 Pro, Biodex Medical Systems, Shirley, NY, USA). Participants were seated in an upright position, securely fastened with the knee angle set at 90-degrees and instructed to perform a maximal force knee extension for 3 seconds. Each participant performed five contractions separated by 1 min of rest. Force was recorded in Torque (Nm). The peak value from five maximal contractions was used for analysis.

3.2.13 Countermovement Jump (CMJ)

A force plate system (Dual force plates model 9260AA6, Kistler group, Winterthur, Switzerland) was used to measure CMJ height (cm). Participants were required to descend into a squat position (to a ~ 90° knee angle) before jumping vertically with maximum effort. During testing, participants' hands remained on the hips. Each participant performed three maximal effort jumps separated by 30 seconds of rest. The average value of the three jumps was used for analysis.

3.2.14 Capillary blood samples

β-OHB concentration in capillary blood was determined using a handheld monitor (FreeStyle Precision Neo, Abbott Laboratories, Witney, UK). Fingertip capillary samples were collected using a lancet following cleaning with alcohol and allowing to air dry. The first blood droplet sample was discarded with a cotton swab and the subsequent droplet samples were used for analysis. Capillary blood β-OHB concentration was assessed immediately following POST testing prior to treatment intake, and at 30, 60, 120, and 180 minutes after treatment intake.

3.2.15 Eccentric exercise protocol

The eccentric bout of exercise consisted of 100 drop jumps off a 60 cm high box with a non-slip rubber top (Northern Lights Fitness Products Inc. Cornwall, Ont, Canada). Each repetition of the drop jump exercise was performed by having the participant first step onto the box (alternating between legs during each step-up), then "drop" off the box onto the floor and land on two feet, while immediately descending to a ~90º knee angle and jumping vertically with maximal effort. Participants performed 5 sets of 20 repetitions with a 2-min inter-set rest period. The technique was first demonstrated by one of the study investigators on two occasions before participants performed the exercise bout. Each participant was given corrective feedback and

strong verbal encouragement to ensure maximal efforts. This protocol has previously been utilized in several muscle damage research studies and has been demonstrated to induce significant lower limb muscle soreness and declines in muscle function (16, 95, 103, 111, 156).

3.2.16 Nutritional treatments

The KET group received the ketone monoester (R)-3-hydroxybutyl (R)-3-hydroxybutyrate (Pure ΔG® Ketone Ester; HVMN, CA, USA) at a dose of 360 mg/kg^{-1} body weight per serving. The CON group received an energy-matched amount of carbohydrate as a combination of Glacier Cherry Gatorade (G2 - Gatorade Company, Inc., Chicago, IL, USA) and Dextrose powder (NOW foods, Inc., Bloomingdale IL, USA) with 10 drops of liquid Vanilla Stevia (Stevia Select Inc, USA). All treatments were volume-matched and prepared in opaque drinking containers. Participants ingested their assigned treatment twice daily (morning and evening) for two days (48 hours) during recovery following an acute bout of eccentric exercise (described above). The morning treatments were consumed at the laboratory under direct supervision of one of the study investigators, while participants consumed the evening treatments on their own ~30 min before going to bed. Participants were asked to return the used bottles at the end of the study.

3.2.17 Statistical analysis

Due to technical issues, analysis of plasma cytokine/chemokine concentration was not performed in 2 subjects, therefore plasma cytokine/chemokine data represent $n = 9$ per group. Participants' baseline characteristics and peak β-OHB concentration were tested with independent sample *t*-tests. All other dependent variables were tested using a 2-factor (group × time) repeated-measures ANOVA. When a statistically significant main effect for time or group × time interaction was observed, Bonferroni-corrected post hoc comparisons were performed.

Assumptions of the ANOVA models were assessed using Mauchley's test and the D'Agostino–Pearson omnibus normality test at a significance of $P < 0.05$. If a significant Mauchley's test was determined, the Greenhouse-Geisser correction factor was used to adjust the degrees of freedom accordingly. For data that did not pass normality, values were transformed with the ln of the value. The statistical analysis was performed on transformed data, but non-transformed data are presented in graphic or tabular form for clarity. Sample size was determined by completing a power analysis (power = 0.9, $\alpha = 0.05$) based on differences in isometric strength (MIVC) data from Bowtell and colleagues (24) during recovery from eccentric muscle damaging exercise. This determined a sample size of 6 in each group would provide statistical power at 90%, with an alpha level of 0.05. However, to preserve power and account for dropouts, we recruited n=10 per group. Statistical analysis was performed with use of the Statistical Package for the Social Sciences (SPSS, Version 24. IBM Corp., Armonk, NY, USA). For all analyses, differences were considered statistically significant at $P < 0.05$. All data are expressed as means ± SD.

3.3 RESULTS

3.3.1 Participants' characteristics

The were no differences between KET and CON groups for any of the participants' characteristics (**Table 1**).

Table 1. Participant characteristics

	KET (*n* = 10)	**CON (*n* = 10)**	***P***
Age, yr	25 ± 5	24 ± 4	0.42
Body mass, kg	72.6 ± 12.8	70.7 ± 11.3	0.73
Height, cm	169 ± 10	172 ± 7	0.39
BMI, kg/m^2	24.3 ± 2.2	23.3 ± 2.3	0.32
Body fat, %	27.3 ± 8.1	26.1 ± 7.2	0.74
Lean mass, kg	49.5 ± 11.1	49.4 ± 9.2	0.98
Heart rate, bpm	65 ± 15	68 ± 10	0.68
Systolic BP, mmHg	113 ± 7	113 ± 7	0.92
Diastolic BP, mmHg	71 ± 9	70 ± 8	0.77

Values are mean ± SD. *n* = 10 per group. Data were analyzed with an independent samples t test. CON: carbohydrate control group; KET: ketone monoester group.

3.3.2 Dietary intake

Average total energy, fat, and protein intake, as well as protein intake relative to bodyweight were not different between the pre-study phase compared to the EIMD recovery phase (all $P > 0.05$) and were not different between KET and CON groups ($P > 0.05$). Average carbohydrate intake was lower during the EIMD recovery phase compared to the pre-study phase

(time; $P = 0.03$) but was not different between KET and CON groups ($P > 0.05$). Average dietary intake of study participants is shown in **Table 2**.

Table 2. Average dietary intake of study participants over 2 days prior to the onset of the experiment (pre-study phase) and during the experiment (EIMD recovery phase) who ingested a ketone monoester supplement (KET) or energy-matched carbohydrate supplement (CON).

	KET (*n* = 10)		**CON (*n* = 10)**	
	Pre-study phase	EIMD recovery phase	Pre-study phase	EIMD recovery phase
Energy, kcal·d^{-1}	2548 ± 941	2319 ± 634	2464 ± 831	2020 ± 565
Carbohydrate, g	334 ± 162[a]	276 ± 98[b]	289 ± 99[a]	193 ± 56[b]
Fat, g	88 ± 37	91 ± 37	98 ± 35	96 ± 39
Protein, g	107 ± 35	104 ± 27	112 ± 49	114 ± 43
Protein, g·kg^{-1}·d^{-1}	1.5 ± 0.6	1.5 ± 0.5	1.6 ± 0.6	1.6 ± 0.5

Values are mean ± SD. $n = 10$ per group. Phases without a common letter differ. Data were analyzed with a two-factor repeated measures (within subject factor: time; between-subject factor: treatment) ANOVA. Energy, kcal· d^{-1}; time effect: P =0.053; group effect: $P = 0.526$; time × group interaction: $P = 0.518$. Carbohydrate; time effect: $P = 0.03$; group effect: $P = 0.124$; time × group interaction: $P = 0.263$. Fat; time effect: $P = 0.934$; group effect: $P = 0.644$; time × group interaction: $P = 0.762$. Protein; time effect: $P = 0.875$; group effect: $P = 0.646$; time × group interaction: $P = 0.670$. Protein, g · kg^{-1} · d^{-1}; time effect: $P = 0.848$; group effect: $P = 0.753$; time × group interaction: $P = 0.619$. CON: carbohydrate control group; KET: ketone monoester group.

3.3.3 Capillary blood β-OHB concentration

Capillary blood β-OHB concentrations (***Figure 5***) increased (Group × Time interaction; $P < 0.001$) following ingestion of the ketone monoester and were significantly higher in KET compared to CON at 30 (KET: 4.0 ± 1.3; CON: 0.1 ± 0.03 mM), 60 (KET: 4.1 ± 0.8; CON: 0.1 ± 0.00 mM), 120 (KET: 2.7 ± 0.6; CON: 0.3 ± 0.2 mM), and 180 (KET: 1.3 ± 0.5; CON: 0.4 ± 0.3 mM) min after intake (all $P < 0.001$).

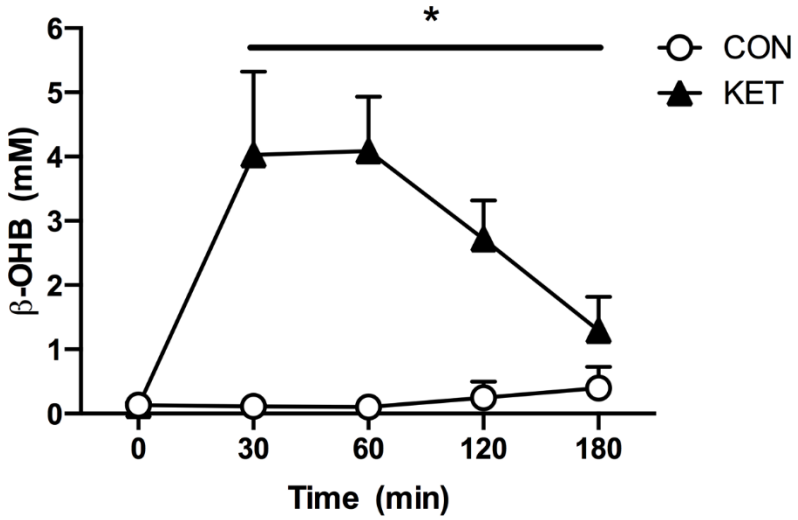

Figure 5. Capillary blood β-OHB concentrations (mM) immediately following POST testing prior to treatment intake (t = 0 min) and 30, 60, 120, and 180 min after treatment intake. Values represent means ± SD. * Indicates difference between KET and CON. Data were analyzed with a two-factor repeated measures (within subject factor: time; between-subject factor: treatment) ANOVA. Bonferroni-corrected post hoc comparisons were performed following a significant main effect for time or time x treatment interaction. Time effect: $P < 0.001$; group effect: $P < 0.001$; time × group interaction: $P < 0.001$. β-OHB: beta-hydroxybutyrate. CON: carbohydrate control group; KET: ketone monoester group.

3.3.4 Serum creatine kinase concentration

Serum CK concentration (**Figure 6**) was increased (Time; $P < 0.001$) at 24 h (319 ± 315 IU•L^{-1}; $P < 0.001$) and 48 h (198 ± 155 IU•L^{-1}; $P = 0.037$) after eccentric exercise when compared to PRE (136 ± 122 IU•L^{-1}). There were no differences between KET and CON (Group; $P = 0.918$).

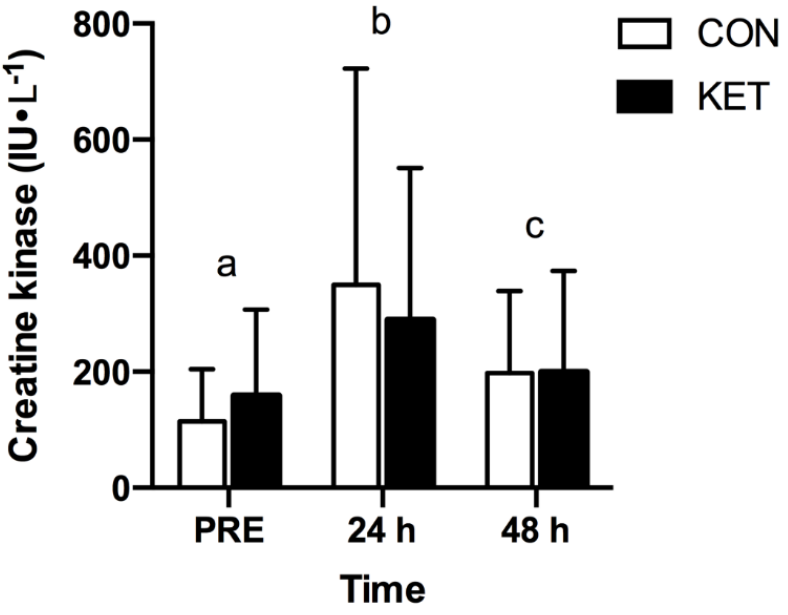

Figure 6. Serum creatine kinase concentrations (IU•L^{-1}) at baseline (PRE) prior to exercise, and 24 h, and 48 h after exercise. Values represent means ± SD. Times without a common letter differ. Data were analyzed with a two-factor repeated measures (within subject factor: time; between-subject factor: treatment) ANOVA. Bonferroni-corrected post hoc comparisons were performed following a significant main effect for time or time × group interaction. Time effect: $P < 0.001$; group effect: $P = 0.918$; time × group interaction: $P = 0.313$. CON: carbohydrate control group; KET: ketone monoester group.

3.3.5 Maximal isometric voluntary contraction and countermovement jump height

MIVC torque (***Figure 7, Panel A***) was decreased (Time; $P < 0.001$) at POST (-21.2 ± 12.7%; $P < 0.001$) and 24 h (-17.1 ± 19.8 %; $P = 0.003$) compared to PRE following the bout of eccentric exercise. At 48 h, MIVC was decreased (-9.5 % ± 20.3) but not significantly different from PRE ($P = 0.232$). There were no differences in MIVC between KET and CON (Group; $P = 0.612$). Countermovement jump height (***Figure 7, Panel B***) was decreased (Time; $P < 0.001$) at POST (-12.0 ± 8.0 %; $P < 0.001$), 24 h (-11.31 ± 11.1%; $P = 0.003$), and 48 h (-10.8 ± 11.9%; $P = 0.010$) compared to PRE following the bout of eccentric exercise. There were no differences in CMJ between KET and CON (Group; $P = 0.571$).

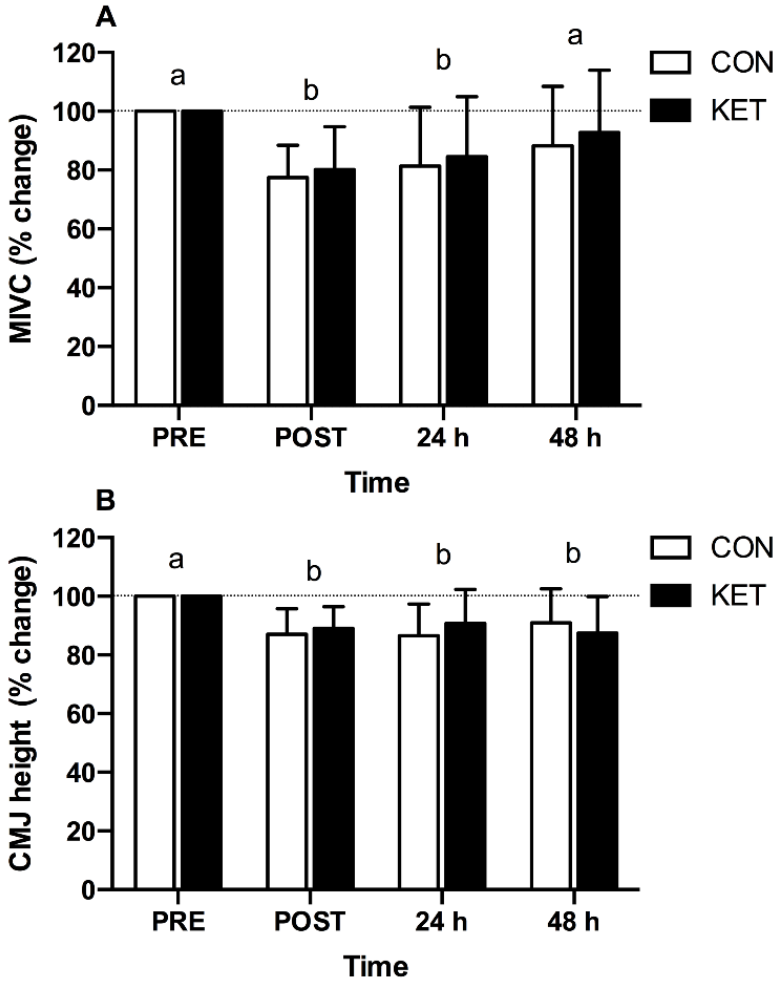

Figure 7. Percent change in maximal isometric voluntary contraction (A) and countermovement jump (B) relative to baseline (PRE) prior to exercise immediately (POST), 24 h, and 48 h after exercise. Values represent means ± SD. Times without a common letter differ. Data were analyzed with a two-factor repeated measures (within subject factor: time; between-subject factor: group) ANOVA. Bonferroni-corrected post hoc comparisons were performed following a significant main effect for time or time × group interaction. Maximal isometric voluntary contraction; time effect: $P < 0.001$; group effect: $P = 0.612$; time × group interaction: $P = 0.594$. Countermovement jump; time effect: $P < 0.001$; group effect: $P = 0.571$; time × group interaction: $P = 0.378$. CMJ: countermovement jump; CON: carbohydrate control group; KET: ketone monoester group; MIVC: maximal isometric voluntary contraction.

3.3.6 Muscle soreness and pressure pain threshold

Ratings of muscle soreness (***Figure 8, Panel A***) were increased (Time; $P < 0.001$) after eccentric exercise at POST, 24 h, and 48 h compared to PRE (all $P < 0.001$). Ratings of muscle

soreness were also greater at 24 h (P = 0.010) compared to POST. There were no differences in ratings of muscle soreness between KET and CON (Group; P = 0.896). The PPT (***Figure 8, Panel B***) was reduced (Time; P < 0.001) at 24 h (P = 0.002) following eccentric exercise compared to PRE. There were no differences in PPT between KET and CON (Group; P = 0.113).

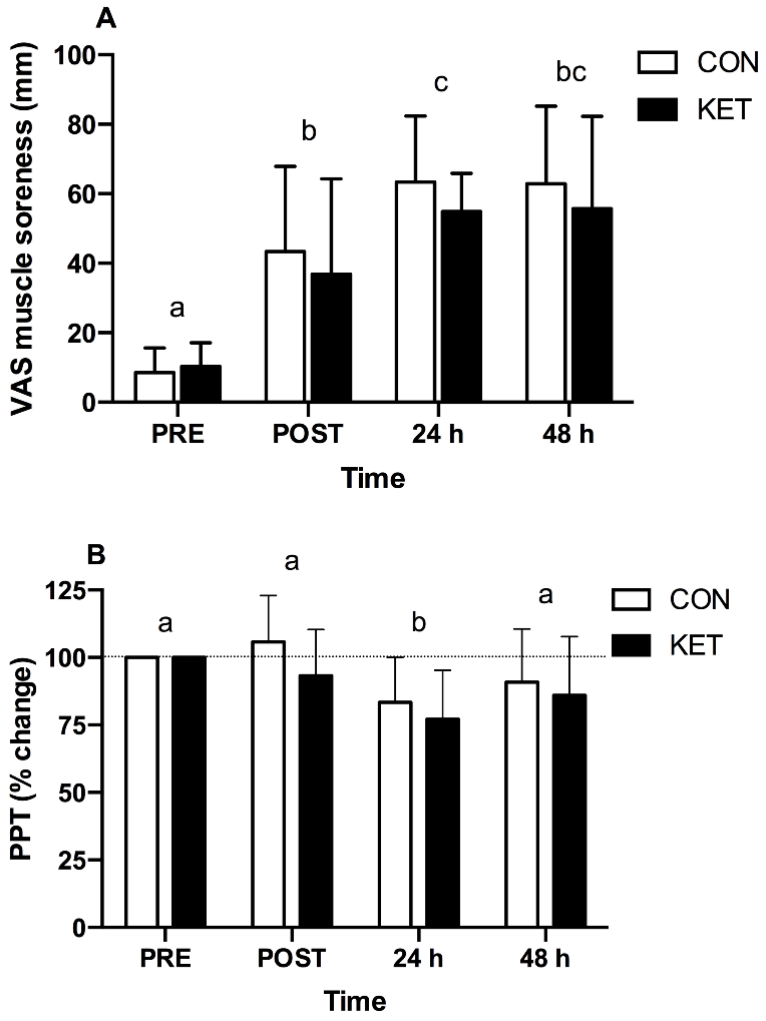

Figure 8. Visual analogue scale derived muscle soreness (A) and percent change in pressure-pain threshold (B) relative to or at baseline (PRE) prior to exercise, immediately (POST), 24 h, and 48 h after exercise. Values represent means ± SD. Times without a common letter differ. Data were analyzed with a two-factor repeated measures (within subject factor: time; between-subject factor: group) ANOVA. Bonferroni-corrected post hoc comparisons were performed

following a significant main effect for time or time × group interaction. Visual analogue scale derived muscle soreness; time effect: $P < 0.001$; group effect: $P = 0.896$; time × group interaction: $P = 0.517$. Pressure-pain threshold; time effect: $P < 0.001$; group effect: $P = 0.113$; time x group interaction: $P = 0.492$. CON: carbohydrate control group; KET: ketone monoester group; PPT: pressure-pain threshold; VAS: visual analogue scale.

3.3.7 Thigh circumference, calf circumference, and flexibility

Thigh circumference (***Figure 9, Panel A***) was increased (Time; $P < 0.001$) at POST ($P < 0.001$) and 24 h ($P = 0.015$) compared to PRE. There were no differences in thigh circumference between KET and CON (Group; $P = 0.584$). Calf circumference (***Figure 9, Panel B***) was increased (Time; $P = 0.001$) at POST compared to PRE ($P < 0.001$) following the bout of eccentric exercise. There were no differences in calf circumference between KET and CON (Group; $P = 0.771$). Flexibility (***Figure 9, panel C***) was not different over time (Time; $P = 0.467$), or between KET and CON (Group; $P = 0.594$).

3.3.8 Brief Assessment of Mood Adapted

BAM + scores (***Figure 10***) were reduced (Time; $P = 0.001$) at POST ($P = 0.011$) and 24 h ($P = 0.008$) following the bout of eccentric exercise compared to PRE. There was a trend ($P = 0.060$) for BAM+ scores to be lower at 48 h compared to PRE. There were no differences in BAM+ scores between KET and CON (Group; $P = 0.793$).

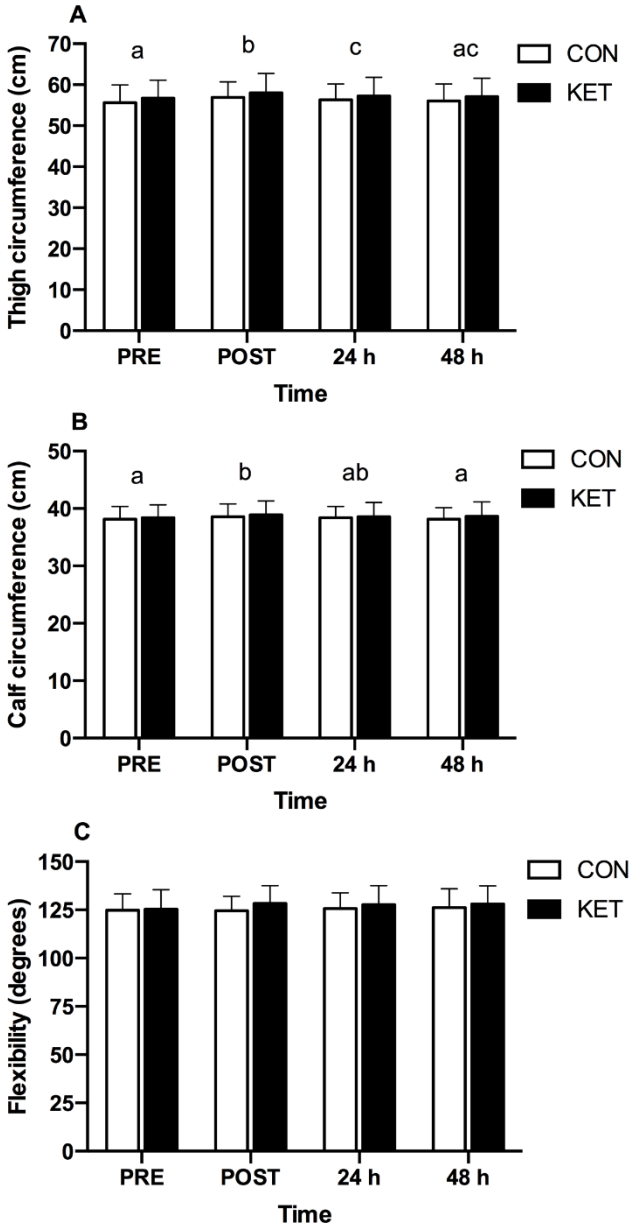

Figure 9. Thigh circumference (A), calf circumference (B), and flexibility (C) at baseline (PRE) prior to exercise, and immediately (POST), 24 h, and 48 h after exercise. Values represent means ± SD. Times without a common letter differ. Data were analyzed with a two-factor repeated measures (within subject factor: time; between-subject factor: group) ANOVA. Bonferroni-corrected post hoc comparisons were performed following a significant main effect for time or time × group interaction. Thigh circumference; time effect: $P < 0.001$; group effect: $P = 0.584$; time × group interaction: $P = 0.960$. Calf circumference; time effect: $P = 0.001$; group effect: $P = 0.771$; time x group interaction: $P = 0.443$. Flexibility; time effect: $P = 0.428$; group effect: $P = 0.594$; time x group interaction: $P = 0.603$. CON: carbohydrate control group; KET: ketone monoester group.

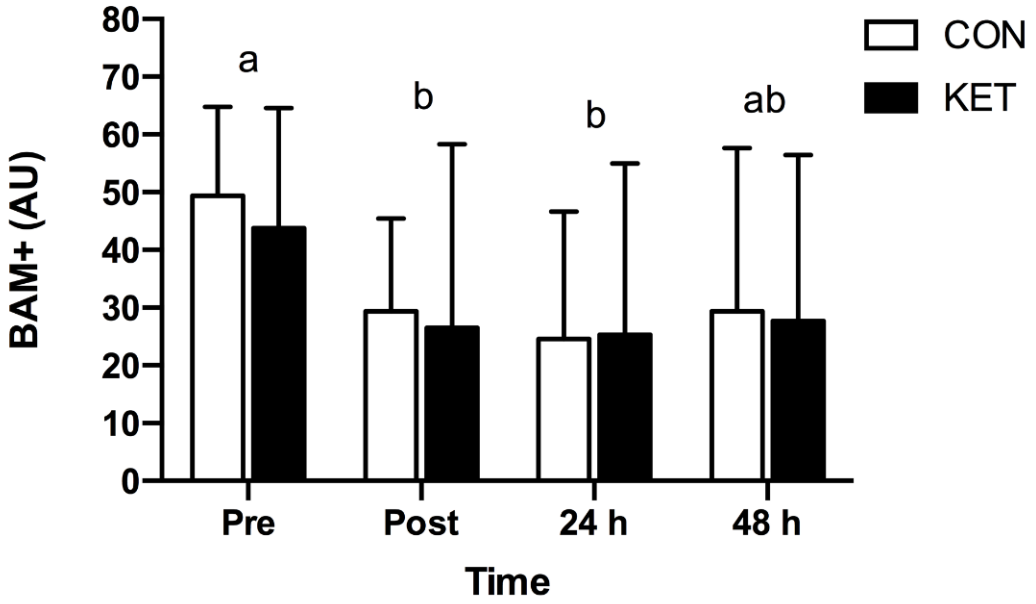

Figure 10. Brief assessment of mood adapted at baseline (PRE) prior to exercise, and immediately (POST), 24 h, and 48 h after exercise. Values represent means ± SD. Times without a common letter differ. Data were analyzed with a two-factor repeated measures (within subject factor: time; between-subject factor: group) ANOVA. Bonferroni-corrected post hoc comparisons were performed following a significant main effect for time or time × group interaction. Time effect: $P = 0.001$; group effect: $P = 0.793$; time × group interaction: $P = 0.945$. AU: arbitrary units; BAM+: brief assessment of mood adapted; CON: carbohydrate control group; KET: ketone monoester group.

3.3.9 Plasma cytokines & chemokines

Plasma cytokines and chemokines are shown in **Table 3**. Plasma IL-1β showed a group × time interaction ($P = 0.027$); however, Bonferroni corrected pairwise comparisons showed no differences between KET and CON at any time-point (all $P > 0.05$). Plasma IL-6 was not different over time (time; $P = 0.650$) or between KET and CON (group; $P = 0.166$). Plasma IL-8 was not different over time (time; $P = 0.677$) or between KET and CON (group; $P = 0.998$). Plasma IL-10 was not different over time (time; $P = 0.259$) or between KET and CON (group; $P = 0.594$). Plasma IL-12(p70) was not different over time (time; $P = 0.185$) or between KET and

CON (group; $P = 0.287$). Plasma MCP-1 was greater in CON compared to KET (group; $P = 0.007$). Plasma TNF-α was not different over time (time; $P = 0.619$) or between KET and CON (group; $P = 0.153$).

TABLE 3. Plasma concentration of IL-1β, IL-6, IL-8, IL-10, IL-12(p70), MCP-1, and TNF-α at baseline (PRE) prior to exercise, and 24 h and 48 h after exercise in participants who ingested a ketone monoester supplement (KET) or energy-matched carbohydrate supplement (CON).

	KET (*n* = 10)			**CON (*n* = 10)**		
	PRE	24 h	48 h	PRE	24 h	48 h
IL-1β (pg/mL)	1.8 ± 1.1	1.9 ± 0.9	1.8 ± 0.8	2.3 ± 1.8	1.6 ± 0.8	1.8 ± 0.9
IL-6 (pg/mL)	1.8 ± 0.7	1.8 ± 0.6	1.8 ± 0.4	2.3 ± 0.8	2.1 ± 0.5	2.0 ± 0.4
IL-8 (pg/mL)	5.9 ± 2.2	6.3 ± 2.3	6.3 ± 2.2	6.9 ± 2.6	5.7 ± 1.9	5.9 ± 2.1
IL-10 (pg/mL)	5.1 ± 2.4	5.1 ± 2.5	5.2 ± 2.5	7.5 ± 7.1	4.9 ± 2.6	5.3 ± 2.7
IL-12(p70) (pg/mL)	4.3 ± 1.0	4.3 ± 1.1	4.3 ± 1.4	4.9 ± 4.2	3.4 ± 1.3	3.5 ± 1.3
MCP-1 (pg/mL)*	134.1 ± 28.6	125.2 ± 21.0	131.9 ± 52.1	155.0 ± 21.9	196.7 ± 63.7	153.0 ± 25.7
TNF-α (pg/mL)	6.1 ± 1.3	5.8 ± 1.2	5.7 ± 1.3	7.5 ± 4.3	7.5 ± 2.3	7.0 ± 3.0

Values represent mean ± SD. * Indicates difference between KET and CON. Data were analyzed with a two-factor repeated measures (within subject factor: time; between-subject factor: group) ANOVA. Plasma IL-1β; time effect: $P = 0.130$; group effect: $P = 0.931$; group × time interaction: $P = 0.027$. Plasma IL-6; time effect: $P = 0.650$; group effect: $P = 0.166$; group × time interaction: $P = 0.317$. Plasma IL-8; time effect: $P = 0.677$; group effect: $P = 0.998$; group × time interaction: $P = 0.145$. Plasma IL-10; time effect: $P = 0.259$; group effect: $P = 0.594$; group × time interaction: $P = 0.232$. Plasma IL-12(p70); time effect: $P = 0.185$; group effect: $P = 0.287$; group × time interaction: $P = 0.241$. Plasma MCP-1; time effect: $P = 0.253$; group effect: $P = 0.007$; group × time interaction: $P = 0.104$. Plasma TNF-α; time effect: $P = 0.619$; group effect: $P = 0.153$; group × time interaction: $P = 0.849$. CON: carbohydrate control group; IL-1β: interleukin-1 beta; IL-6: interleukin-6; IL-8: interleukin-8; IL-10: interleukin-10; IL-12(p70): interleukin-12 p70; KET: ketone monoester group; MCP-1: monocyte chemoattractant protein 1; TNF-α: tumour necrosis factor alpha.

3.4 DISCUSSION

In the current study, we evaluated the impact of twice daily (morning and evening 30 min prior to sleep) supplementation (360 mg/kg^{-1} bodyweight per serving) with a ketone monoester (KET) as compared to an energy-matched carbohydrate control (CON) on indices of EIMD during recovery from a single bout of eccentric exercise in healthy, moderately active, young adults. MIVC was reduced by 19.9 ± 14.6% and 22.6 ± 11.1%, while CMJ was reduced by 11.0 ± 7.5% and 13.0 ± 8.7% at POST in KET and CON groups respectively, following eccentric exercise. Acute ingestion of the ketone monoester supplement following POST testing markedly elevated circulating blood β-OHB concentration from 30-180 min after intake, demonstrating acute nutritional ketosis in the KET group. However, twice daily ketone monoester supplementation (KET) did not alter the exercise-induced increase in serum CK concentration, or expedite the recovery of MIVC or CMJ at 24 h or 48 h after exercise compared to CON. Similarly, there were no differences on measures of muscle soreness (VAS and PPT), limb girth, or the concentration of select pro- and anti-inflammatory cytokines in response to the supplements. Overall, twice daily ingestion of a ketone monoester supplement that acutely elevates blood β-OHB concentration does not expedite the recovery of indices of muscle damage following eccentric exercise in moderately active, healthy young adults.

Consistent with previous work (37), acute ingestion of the ketone monoester (KET) led to a rapid (within 30 min) and pronounced (peak: 4.4±0.8 mM) increase in blood β-OHB concentration that was sustained for at least 180 minutes (Figure 5). Changes in blood β-OHB concentration were assessed in response to initial (first) supplement intake on Day 1 following POST testing; blood β-OHB concentration following supplement intake in the evening was not assessed as participants were instructed to ingest their respective treatment at home ~30 min

prior to sleep. It has previously been reported that ketone monoester intake increases the blood β-OHB:acetoacetate concentration in a 5:1 ratio (133). Therefore, peak total blood ketone body concentrations of ~5.3 mM may have been achieved post-exercise; concentrations similar to those achieved with fasting (157).

Consistent with other studies utilizing the same exercise protocol (25, 26, 158) drop-jump exercise resulted in increases in serum CK concentration when assessed at 24 h and 48 h post-exercise (Figure 6). It has been suggested that a ketogenic diet may reduce EIMD based on the observation that blood CK was reduced 24 h post-exercise in rodents fed a ketogenic vs. control diet (159). However, the lack of difference between KET and CON on serum CK concentration in the present study suggests that ketone monoester supplementation after exercise might not be beneficial for attenuating damage to the cell membrane resulting from eccentric exercise.

The decline in muscle force production following eccentric exercise is considered one of the most reliable indirect markers of EIMD (74, 98). Although not unequivocal, a number of nutritional interventions including use of branched-chain amino acids, protein, creatine, omega-3 polyunsaturated fatty acids, vitamin D, beetroot, pomegranate, and cherries have been reported to expedite impaired force production and/or performance after exercise (for reviews see (9, 15, 147, 148)). In the present study, we hypothesized that ketone monoester supplementation and elevated β-OHB may accelerate the recovery of indices of muscle damage via mechanisms related to the reported capacity of β-OHB to stimulate protein synthesis (41, 137) and suppress proteolysis (42), and/or to modulate inflammation (44) and oxidative stress (43, 135, 141). Eccentric exercise led to a similar decline in MIVC at POST, indicating a similar initial response to the exercise stimulus in both the KET and CON group (Figure 7, panel A). However, the recovery of MIVC did not differ between KET and CON groups when assessed 24 h or 48 h after

exercise. Similarly, the eccentric exercise protocol led to a similar early decline in CMJ height at POST (Figure 7, panel B); however, the recovery of CMJ height was not expedited in KET compared to CON at 24 h or 48 h post-exercise. To our knowledge, this is the first study to evaluate the impact of ketone monoester supplementation as a nutritional strategy to accelerate the recovery of impaired muscle function after eccentric exercise. Vandoorne and colleagues (137) recently reported that co-ingestion of a ketone monoester with protein and carbohydrate after strenuous exercise enhanced the post-exercise activation of mTORC1 and protein synthesis rates (in C_2C_{12} cells) compared to protein and carbohydrate only; however, post-exercise recovery was evaluated for 5 hours and changes in muscle function were not assessed. Huang and colleagues (159) reported that a ketogenic diet accelerated post-exercise recovery of performance in rodents based on measures of locomotion time taken 24 h after a bout of exhaustive exercise. However, there are substantial differences between a ketogenic diet and ketone supplements (160) and the translation of these findings to humans is unclear. Overall, twice daily supplementation with a ketone monoester that acutely raises circulating β-OHB does not expedite the recovery of muscle function based on static (MIVC) or dynamic (CMJ) measures of performance over the initial 2 days after eccentric exercise.

Muscle soreness is a common symptom of EIMD and is the most utilized marker of muscle injury in muscle damage research (98). Recent evidence indicates that intramuscular generation of molecules such as nerve growth factor, bradykinin, and prostaglandin E2, likely play an important role in muscle soreness due to EIMD (11, 161). These substances can be synthesized by immune cells and act to excite and sensitize local muscle nociceptors, resulting in sensations of pain and soreness in the muscle belly and connective tissues (11). A number of nutritional interventions have been reported to reduce muscle soreness following eccentric

exercise (for reviews see (9, 15, 147, 148)). In the present study, muscle soreness was increased in response to eccentric exercise based on both VAS and PPT measures, but there was no difference between KET and CON groups (Figure 5, panel A and B). Therefore, ketone monoester supplementation does not appear to be an effective strategy to reduce muscle soreness that manifests over the initial 2 days after eccentric exercise.

Nutritional solutions to expedite the recovery of indices of muscle damage are thought to interact with the secondary damage cascade that is characterized by inflammation and oxidative stress, thereby attenuating exacerbation of damage and aiding subsequent recovery (15). Ketone bodies appear to modulate inflammation and immune cell function, with β-OHB exerting a predominantly anti-inflammatory response (139). β-OHB has been reported to lead to anti-inflammatory effects in TNF-α and lipopolysaccharide-induced inflammation by reducing pro-inflammatory proteins such as inducible nitric oxide synthase and cyclooxygenase-2, or cytokines such as TNF- α, IL-1β, IL-6, and MCP-1, partly by inhibiting NF-κB (144, 145). In a recent animal study, β-OHB was also shown to decrease inflammation via reducing inflammatory cytokines such as IL-1β via blocking the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome pathway (44). Antioxidant and oxidative stress-mitigating roles of ketone bodies have also been described (139). For example, ketone bodies have been shown to reduce cellular damage, injury, death, and apoptosis in cardiomyocytes and neurons (140-142), with β-OHB able to scavenge reactive oxygen species such as hydroxyl anion (140). Recently, β-OHB has been shown to be a histone deacetylase inhibitor, which can increase the expression of antioxidant genes and proteins responsible for decreasing cellular oxidative stress (43). In the present study, we measured plasma concentrations of select pro- and anti-inflammatory cytokines (IL-1β, IL-6, IL-10, IL-12(p70), and TNF-α) and chemokines (IL-8

and MCP-1) at baseline (PRE), 24 h, and 48 h post-exercise recovery (Table 3). We observed no difference over time or between KET and CON groups on all markers except MCP-1, which was lower in KET vs. CON (group: $P = 0.007$). A review by Peake and colleagues (162) examining the impact of various types of exercise on cytokine expression and secretion, highlighted that plasma concentrations of IL-1β, IL-6, IL-8, IL-10, TNF-α, and MCP-1 have been reported to increase in response to exercise. However, plasma cytokine responses to exercise are variable between individuals and dependent on the combination of mode, intensity, and duration of exercise (162). Therefore, a different type and/or intensity of exercise may have led to a more robust systemic cytokine response. Alternatively, many systemic cytokine responses appear to occur <24 h post-exercise (74). Given that the first post-exercise blood sample was taken 24 h after exercise, we may not have captured early time and or group-dependent differences in circulating cytokines. To better capture the exercise-induced inflammatory response, early post-exercise blood sample collection, evaluation of additional markers of inflammation, and assessment of intramuscular cytokine mRNA/protein expression may be necessary.

In summary, results of the present study demonstrate that twice daily ingestion of a ketone monoester supplement that acutely elevates blood β-OHB concentrations does not enhance the recovery of indices of muscle damage following eccentric exercise in moderately active, healthy young adults.

CHAPTER 4: CONCLUSION AND SUMMARY

4.0 OVERALL CONCLUSION AND SUMMARY

There is currently substantial interest in the role of ketone bodies in exercise performance and recovery (34). Recently, novel orally ingested ketone supplements have been introduced that result in acute elevations in circulating β-OHB (38). Unlike a ketogenic diet which requires carbohydrate restriction, the delivery of exogenous β-OHB via ketone supplements creates a novel physiological state where nutritional ketosis can be achieved with fully replete carbohydrate stores (39). Given that ketones provide an alternative fuel source for the brain and skeletal muscle and can regulate the mobilization and utilization of carbohydrate and fat (38), ketone supplements have been investigated as a means to enhance endurance exercise performance (38, 40, 163). However, elevations in β-OHB through acute nutritional ketosis have also been suggested to potentially accelerate exercise recovery (39) through mechanisms related to stimulation of increased rates of muscle protein synthesis, reduced protein breakdown, lowering of oxidative stress, and reducing inflammation (39). This led to the hypothesis that ingestion of a ketone monoester supplement after eccentric exercise may expedite the recovery of indices of EIMD including muscle force generating capacity and muscle soreness. Consequently, the purpose of this thesis was to evaluate the effects of ketone monoester supplementation and acute elevation in blood β-OHB concentration on indices of EIMD including muscle function, muscle soreness, serum CK, and the concentration of select cytokines and chemokines during recovery from a bout of eccentric exercise in healthy young active adults. Contrary to the hypothesis, ketone monoester supplementation and acute increases in circulating β-OHB did not accelerate the recovery of muscle function (MIVC and CMJ) or muscle soreness (VAS and PPT), and did not alter the concentration of circulating CK, or the concentration of select pro- and anti-inflammatory cytokines or chemokines. Collectively, the results presented

demonstrate that ketone monoester supplements do not accelerate the recovery of common indices of EIMD in healthy young adults.

4.1.1 Limitations and future directions

This study was a tightly controlled investigation examining the impact of a nutritional intervention on the recovery of common indices of EIMD in healthy young adults over 48 hours (i.e., 2 days). However, aspects of muscle function (i.e., CMJ) and muscle soreness (i.e., VAS ratings) had not returned to baseline (PRE) values by 48 hours. It is possible that continued supplementation with the ketone monoester and evaluation of these outcomes at 72 hours or to the point of full recovery may have yielded a difference between KET and CON treatments. For example, Clifford and colleagues (25) reported differences in the recovery of CMJ performance and PPT with beetroot supplementation vs. an isocaloric control as late as 72 hours after a bout of eccentric exercise. Thus, future studies should aim to monitor signs and symptoms of EIMD (e.g., 'damage markers') repeatedly (daily) until full recovery.

Another consideration within the present study relates to the dose and timing of the ketone monoester supplement. The ketone monoester supplement was provided at a dose of 360 mg/kg^{-1} body weight per serving in the morning following exercise testing and in the evening prior to sleep. While the dose provided is similar to that provided in previous research (164) and induced a marked rise in circulating β-OHB concentration (Figure 5), the half-life of the β-OHB ketone monoester is relatively short (165). More frequent consumption (e.g., every 180 minutes during the day) of the ketone monoester to sustain elevated blood β-OHB concentrations over the day may have led to a different result. The safety and tolerability of the β-OHB ketone monoester has been demonstrated in healthy volunteers who received 3 servings per day for 28 days (166).

The amount of muscle damage experienced by the majority of the research participants in the present study based on the decline in MIVC would be considered mild to moderate (74). Whether ketone monoester supplementation may facilitate recovery following a more strenuous bout of eccentric exercise and more severe EIMD (i.e., a decline in force-generating capacity >50% and/or recovery of force-generating capacity that exceeds 7 days (74)) is unclear and requires investigation. More severe damage that is accompanied by a greater inflammatory response and delayed regeneration may represent an environment where any potential benefit of ketone monoester supplementation can be better unmasked.

In conclusion, twice daily ingestion of a ketone monoester supplement that acutely elevates blood β-OHB concentrations does not expedite the recovery of indices of muscle damage following eccentric exercise in moderately active, healthy young adults.

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