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The Role of Exogenous Ketones on Various Aspects of Exercise Performance

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Kinesiology

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Abstract

Routinely, athletes and coaches pursue novel nutritional strategies in an attempt to support exercise training techniques and/or enhance athletic performance. Body carbohydrate (CHO) stores are limited so strategies to enhance fat use and spare CHO during exercise and thus, attenuate the onset of fatigue are commonplace. It has been suggested that oral exogenous ketone administration is ergogenic not only by altering exercise metabolism (sparing CHO), but also by improving exercise cognitive function as well as enhancing post-exercise glycogen resynthesis. However, data supporting these claims are limited and contradictory. Therefore, the purpose of this dissertation was to explore the role of exogenous ketone type, i.e., salts (KS) vs ketone monoester (KME) supplementation on various aspects of performance to provide more insight into the current body of evidence. Study 1 showed that relative to an isoenergetic control, acute ingestion of both a caffeinated KS supplement and the same KS supplement without caffeine improved Wingate peak power output, following a 20 km time trial (TT_{20km}), while only the caffeinated supplement improved the best effort TT_{20km}. Therefore, these performance benefits were likely due to the added caffeine or taurine, not the ketones. Study 2 demonstrated that co-ingestion of KME and CHO after glycogen lowering exercise (GLE) vs isoenergetic CHO alone, resulted in no significant differences in any of the exercise performance parameters suggesting that glycogen resynthesis post exercise was not enhanced with KME. Study 3 revealed that, following induced mental fatigue, KME attenuated the decline in cognitive function during exercise in a complex reaction test, when compared to a non-caloric placebo. Taken together, these data demonstrate that 1) acute KS supplementation is not likely to be ergogenic nor detrimental for intense exercise performance, 2) KME supplementation may improve some aspects of cognitive function during exercise, and 3) KME supplementation has little effect on post-exercise glycogen synthesis following prior GLE. Relative to the ergogenic potential of ketones for athletes, these data are intriguing, but more study is needed to assess fully whether and how ketone supplements are beneficial for athletes.

Keywords: post-exercise recovery, ketosis, ketonemia, cognitive function, mental fatigue.

Summary for Lay Audience

Carbohydrates (CHO - sugars and starches) are an important fuel for brain and muscle during exercise but unfortunately body stores are limited and reduction of these stores is linked to fatigue. Athletes and coaches often seek procedures that can help support exercise training or improve exercise performance, so strategies to increase fat use and/or spare CHO during exercise are common. Some researchers believe that oral ketone administration can help improve exercise performance not only by sparing exercise CHO use, but also by improving exercise cognitive function (decision making, reaction time, etc.), as well as increasing body CHO stores during recovery following exercise. However, evidence to support the veracity of these claims is both modest and inconsistent. Therefore, the purpose of this dissertation was to explore the role of oral ketone type, i.e., salts (KS) vs ketone monoester (KME) supplementation on different aspects of performance to provide more insight into this research area. Study 1 showed that drinking a caffeinated, KS supplement and the same KS supplement without caffeine improved performance of an intense 30-sec sprint, after a 20 km cycling race (TT_{20km}), while only the caffeinated supplement improved the TT_{20km}. These performance benefits were likely due to the added caffeine or taurine, not the ketones. Study 2 demonstrated that drinking KME and CHO after intense exercise resulted in no differences in exercise performance 4 h later, compared to drinking CHO alone. This implies that refilling of CHO stores after exercise was not better with ketone supplementation. Study 3 showed that, after inducing mental fatigue, KME reduced the deterioration in cognitive function during exercise in a complex reaction test. Taken together, these data demonstrate that 1) KS supplementation likely does not improve nor worsen intense exercise performance, 2) KME supplementation may improve some aspects of cognitive function during exercise, 3) KME supplementation has little effect on refilling CHO stores after exercise. Regarding the ability of ketone supplements to improve performance in athletes, these data are intriguing, but more study is needed to evaluate fully if and how ketone supplements are beneficial for athletes.

Co-Authorship Statement

All the data collected in this dissertation was interpreted by the author, Manuel D. Quinones, under the supervision of Dr. Peter W.R. Lemon. Manuel D. Quinones was the first author and Dr. Peter W. R. Lemon was the senior author on all papers included in this dissertation.

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This work is dedicated to my kids Sara, Camila and Juan. Love you guys!

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

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

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List of abbreviations

Ac-CoA	Acetyl coenzyme A
AcAc	Acetoacetate
AMPK	AMP-activated protein kinase
ATP	Adenosine-triphosphate
BBB	Blood Brain Barrier
βHB	Beta-hydroxybutyrate
BDNF	Brain derived neurotropic factor
BD	Butanediol
CHO	Carbohydrates
CRT	Choice reaction test
D-βHB	Beta-hydroxybutyrate (D Isoform)
L-βHB	Beta-hydroxybutyrate (L Isoform)
EDTA	Ethylenediaminetetraacetic acid vacutainer
EK	Exogenous ketone supplements
GLUT- 4	Glucose Transporter 4
HMG-CoA	Beta-hydroxy b-methylglutaryl-coenzyme A
HR	Heart Rate
KB	Ketone body
KD	Ketogenic diet

KE	Ketone ester
KS	Ketone salt
KME	Ketone monoester
MCT	Medium-chain triglyceride
MFT	Mental fatiguing task
mM	millimolar
mTORC1	Mammalian target of rapamycin complex 1
OXCT	Succinyl-Coa: 3-Ketoacid Coenzyme A Transferase
PAR – Q	Physical Activity Readiness Questionnaire
PDH	Pyruvate dehydrogenase
PO	Power output
PRO	Protein
RPE	Rating of Perceived Exertion
TCA	Tricarboxylic acid
TT	Time trial
W_{max}	Peak power output
VAS	Visual analog scale
$\dot{V}O_2\text{max}$	Maximal rate of oxygen consumption

Chapter 1

1

General Introduction

1.1 Introduction

Carbohydrates (CHO) and fat represent important fuels for energy during exercise (Van Loon et al., 2001). The relative contribution of these substrates for energy during exercise depends on factors such as intensity and duration (Van Loon et al., 2001), diet (McSwiney et al., 2018), amongst others (Coggan and Coyle, 1991; Van Loon et al., 1999). Moreover, reduction of CHO stores has been associated with fatigue, which produces a decrement in work rate and may also lead to impaired cognitive function, both important aspects of sports performance (Baker et al., 2014). Unfortunately, CHO stores represent a finite source of energy that can be depleted in 1-2 h of exercise or even more quickly with intense, repeated exercise (Hawley and Lackey, 2015). Therefore, athletes and coaches are constantly pursuing nutritional strategies to support training techniques and/or enhance athletic performance. Specifically, strategies to increase fat use and/or help spare CHO during exercise and thus, attenuate the onset of fatigue are commonplace. Some scientists believe that adapting one's body to utilize a greater amount of fat for exercise energy (fat adapted) may be advantageous, because even in lean individuals, fat represents a virtually unlimited source of energy (Sansone et al., 2018; Volek et al., 2015), unlike CHO.

Over the past decade, there has been a re-emerging interest in adopting high-fat, low-CHO diets or ketogenic diets (KD) with the sole purpose of shifting metabolism to increase fat oxidation during exercise, while reducing the reliance on CHO. Adopting this dietary regime leads to increased ketogenesis (formation of endogenous ketones; acetoacetate, acetone, and β -hydroxybutyrate). These are lipid-derived molecules synthesized in the liver that can be oxidized to replace glucose reductions that occur in response to low CHO diets (Valenzuela et al., 2020). This increased concentration of ketones in the blood is called nutritional ketosis (Evans et al., 2017) and results after ~3 to 6 d on a high fat or low CHO diet (Paoli et al., 2013). Typical concentrations of ketones in blood range from 0-0.5 mM whereas nutritional ketosis is defined as a plasma ketone concentration of ~0.5–3.0 mM, i.e., hyperketonemia (Gibson and Sainsbury, 2017). This is important because ketones can serve as an alternative energy substrate for peripheral tissue such as muscle and brain (Stubbs et al., 2017). Since they are formed

from fat metabolism, this energy reservoir is virtually endless as well and can help spare available CHO. Additionally, the free energy of ATP hydrolysis for ketones has been shown to be greater than CHO, i.e., ketone bodies are energetically more efficient (Evans et al., 2017). However, studies have failed to show a clear benefit in performance when following a ketogenic diet compared to a high CHO diet (Burke, 2021), despite the outlined advantages of ketones over CHO. This may be because in addition to shifting metabolism to allow muscles to utilize more fat during exercise (McSwiney et al., 2018), ketogenic diets also impair rates of muscle glycogenolysis and energy flux (Stellingwerff et al., 2006), resulting in a reduced capacity for high-intensity ATP regeneration. Specifically, studies indicate that with the up-regulation of fat utilization for energy, there is a concomitant reduction in pyruvate dehydrogenase activity (PDH), a key regulatory enzyme responsible for CHO metabolism (Stellingwerff et al., 2006). Even if athletes become fat-adapted and load with CHO a day or two preceding competitions, there is no apparent exercise performance benefit (Burke et al., 2020) likely because of this PDH down-regulation.

Recently, some nutrient supplement manufacturers have developed exogenous ketone (EK) supplements in an attempt to address the exercise performance limitations seen with the ketogenic diet (CHO restriction). Oral administration of EK in healthy adults leads to an acute state of ketosis that can last up to 3 h (Stubbs et al., 2017). Interestingly, this acute ketosis allows the study of ketone use under a unique metabolic scenario, i.e., in combination with high CHO availability. EK have been proposed to have the potential to improve performance not only by altering metabolism during exercise, but also by improving aspects of recovery and even cognitive function (Valenzuela et al., 2021). Consequently, the use of EK has received substantial attention not only from the scientific community (Evans et al., 2017), but also from elite endurance athletes, particularly professional cyclists (Valenzuela et al., 2021).

The first to demonstrate the ergogenic potential of EK was Cox et al. (2016). In a series of studies, these authors showed that supplementing with EK before exercise reduced muscle glycogen utilization with a concomitant increase in fat oxidation during exercise. They also showed that this CHO sparing effect resulted in a 2% improved 30 min time

trial performance (Cox et al., 2016). In contrast, the results of several subsequent studies assessing performance have been equivocal (Evans and Egan, 2018; Waldman et al., 2018; Evans et al., 2019; James and Greer, 2019; Poffe et al., 2020) and any conclusions around the use of EK and exercise performance remain controversial. Several factors have been suggested to explain these differing results. Specifically, the heterogeneity in the literature regarding the ketone type (esters vs salts) and dose (ranging from 106 – 915 mg·kg body mass⁻¹) of the supplement, the small sample sizes (50% of studies using ≤10 participants) studied and the differences in performance tests used have all been implicated (Valenzuela et al., 2020, Margolis and O’fallon, 2020). Due to differences in type and dose of ketone used, various degrees of ketosis are reported in the literature, and this has been identified as a key aspect to determine efficacy of the supplement (Margolis and O’fallon, 2020).

In addition to exercise performance, EK have been proposed to benefit both cognitive function and post-exercise recovery. However, only a few studies have been conducted assessing the efficacy of EK on either cognitive function in exercise or post-exercise recovery. Further, the results are conflicting and, therefore, it is difficult to draw any conclusions at present. Clearly, more research is warranted to elucidate the effects of ketone supplementation.

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Chapter 2

2

Literature Review

2.1 Ketone Metabolism

The process of ketone formation is called ketogenesis and occurs primarily in hepatocytes (Robinson and Williamson, 1980; Laffel, 1999; Evans et al., 2017; Puchalska and Crawford, 2017). Ketone bodies (KB) are lipid-derived molecules synthesized in very small amounts typically, but their synthesis can be upregulated in response to low CHO availability such as during prolonged fasting, starvation, prolonged exercise where CHO stores are depleted, uncontrolled diabetes, and/or dietary manipulations to restrict CHO intake (Robinson and Williamson, 1980; Laffel, 1999; Puchalska and Crawford, 2017). All of the aforementioned scenarios result in a decrease in hepatic glycogen content and an increase in the circulating glucagon-to-insulin ratio (Evans et al., 2017). Both cause an up-regulation of hepatic lipolysis through β -oxidation, a process that results in the formation of Acetyl-CoA (Ac-CoA) to continue feeding the tricarboxylic acid (TCA) cycle and generate ATP (Laffel, 1999). In the first step of the TCA cycle, oxaloacetate (a four-carbon molecule) combines with Ac-CoA (two-carbon molecule) to form citrate (Laffel, 1999). Oxaloacetate is also a gluconeogenic precursor (Laffel, 1999), thus some of it is utilized for gluconeogenesis when CHO availability is low and this leads to an accumulation of Ac-CoA in the mitochondrial matrix of hepatic cells (Evans et al., 2017) (Figure 2.1).

2.1.1 Ketogenesis

In this metabolic process, the excess Ac-CoA molecules are combined to form 4-carbon molecules known as ketone bodies (KB), specifically beta-hydroxybutyrate (β HB), acetoacetate (AcAc) and acetone (Robinson and Williamson, 1980; Laffel, 1999; Evans et al., 2017) (Figure 2.1). This process is called ketogenesis and involves a short sequence of reactions that result in the formation of AcAc initially (Laffel, 1999; Puchalska and Crawford, 2017). There are three possible fates for AcAc after it is formed: 1) the majority of it is reduced to β HB in an NAD^+/NADH -coupled near equilibrium reaction, in which that equilibrium favours the formation of β HB, 2) small amounts of AcAc can enter the circulatory system, and 3) some of it can undergo spontaneous decarboxylation

to form acetone (Laffel, 1999; Puchalska and Crawford, 2017). Once formed, KB, primarily in the form of β HB, are transported out of hepatocytes into the blood stream via the solute ligand carrier (SLC) protein 16A (SLC16A) family of monocarboxylate transporters (Balasse and Fery, 1989; Kahn et al., 2005; Evans et al., 2017), and this leads to a state of ketosis (Figure 2.2).

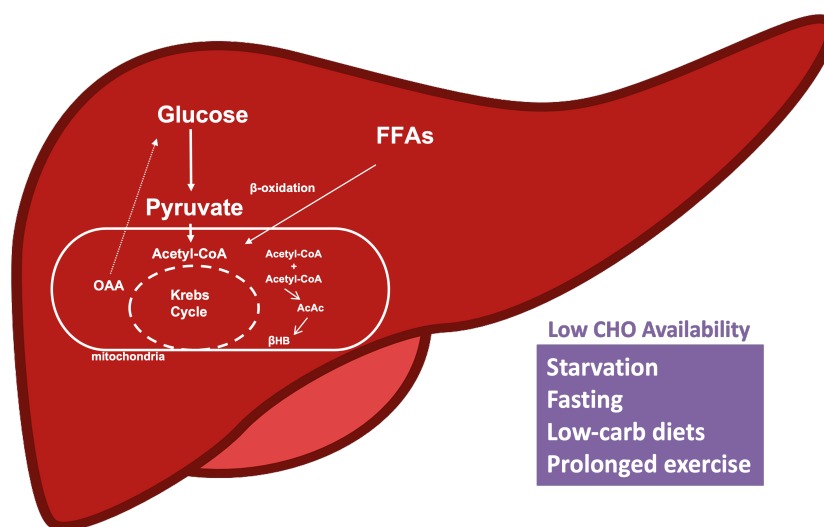


Figure 2.1. Metabolism of Ketone Bodies I. Schematic diagram of the process of endogenous ketone formation (ketogenesis) which occurs in hepatocytes primarily, in response to low glucose availability.

2.1.2 Ketosis

Ketosis refers to an accumulation of KB in blood (Evans et al., 2017). The concentration is determined by the rate of hepatic output or rate of release into circulation and the rate of extra hepatic tissue uptake and utilization (Robinson and Williamson, 1980; Fery and Balasse, 1983; Laffel, 1999) (Figure 2.2). Under normal conditions and with an adequate ingestion of CHO, the concentration of KB in blood ranges from 0-0.5 mM (Robinson and Williamson, 1980; Laffel, 1999; Evans et al., 2017). However, hyperketonemia (A.K.A. ketosis) can occur with the up-regulation of ketogenesis, which leads to a greater-than-normal production of KB (Laffel, 1999; Evans et al., 2017). In ketosis, blood concentrations of KB can range from 0.5 – 8 mM, depending on what is stimulating ketogenesis. For example, blood concentrations of KB may reach concentrations up to 8mM with starvation (Cahill Jr, 1970). Typically, nutritional ketosis or ketosis attained

through dietary manipulations reach blood KB concentrations of ~0.5–3.0 mM (Gibson and Sainsbury, 2017). It is important to note that nutritional ketosis is a safe physiological state, proven to be harmless and associated with a number of therapeutic benefits (Paoli et al., 2013). In contrast, ketoacidosis is a pathological state where blood concentrations of KB can increase up to 20 mM (Laffel, 1999; Puchalska and Crawford, 2017). The problem with this is that KB have a P_{ka} of ~4-5 (Puchalska and Crawford, 2017) which means they are naturally acidic so when they are present in such high concentrations, they become life-threatening since blood pH can be reduced significantly (Laffel, 1999). Ketoacidosis occurs mainly in diabetics (mostly type 1) and severe alcoholics (Laffel, 1999). As alluded to earlier, insulin and glucagon are powerful hormones that regulate ketogenesis (Robinson and Williamson, 1980; Evans et al., 2017). Specifically, insulin inhibits the protein FOXA2, while the hormone glucagon activates it (Wolfrum et al., 2004; Puchalska and Crawford, 2017). This protein will in turn activate the primary regulator of KB production, the enzyme HMG-CoA synthase in the mitochondria of liver cells (Hegardt, 1998; Wolfrum et al., 2004). In type 1 diabetics, insulin production is impaired so there is a risk to accumulate extremely high KB concentrations, reaching a state of diabetic ketoacidosis (Laffel, 1999). Similarly, alcohol metabolism increases Ac-CoA formation and suppresses gluconeogenesis (Laffel, 1999). This coupled with starvation (alcohol taking precedence over food) leads to transient low insulin concentrations that can cause severe alcoholics to reach a state of alcoholic ketoacidosis (Laffel, 1999). In healthy individuals, insulin is secreted as the concentration of KB in blood increases, and this allows ketogenesis to slow down to maintain pH in the normal range (Mullins et al., 2011). This standard feedback control mechanism is important to maintain a state of ketosis and prevent ketoacidosis. Once KB circulate in the bloodstream, they can be transported to extrahepatic tissues where they move intracellularly favoring the concentration gradient and are ultimately oxidised in a metabolic process called ketolysis (Puchalska and Crawford, 2017).

2.1.3 Peripheral Tissue Ketones Utilization (Ketolysis)

Ketolysis refers to the metabolic process involving conversion of KB into energy to fuel metabolic activity of different tissues (Laffel, 1999; Evans et al., 2017). Ketolysis

converts AcAc and β HB back into two molecules of Ac-Coa that can then enter the TCA cycle for ATP regeneration (Laffel, 1999) (Figure 2.2). Acetone, the third KB, is eliminated through respiration and is responsible for the sweet breath odour characteristic of ketosis/ketoacidosis (Paoli et al., 2013). Once KB are synthesized in the liver, they are released into circulation (primarily in the form of β HB) where they travel to fuel other tissues. Although the liver produces KB, it cannot use them for energy because hepatocytes lack an important rate limiting enzyme for ketolysis, Succinyl-Coa: 3-Ketoacid Coenzyme A Transferase (OXCT) (Robinson and Williamson, 1980). The uptake of KB into the mitochondrial matrix of peripheral tissue is mediated by monocarboxylate 1 transporters and is dependent on the rate of appearance of KB in the blood (Evans et al., 2017). Early studies used a variety of fasting protocols as well as infusion of radiolabelled ketone tracers and arteriovenous difference to quantify KB metabolism. These studies showed that extra hepatic tissue such as brain, kidney, as well as both skeletal and cardiac muscle have been identified as sites of KB utilization. Further, skeletal muscle is the major site for ketolysis because a large proportion of the human body (~40% of body mass) is composed of skeletal muscle (Hagenfeldt and Wahren, 1968; Owen and Reichard, 1971, Mikkelsen et al., 2015) despite the fact that heart and kidney displayed the greatest activity of the ketolytic rate limiting enzyme OXCT (Robinson and Williamson, 1980). In addition, Balasse and Fery (1989) demonstrated that skeletal muscle extraction rates saturate between ~1-2 mM, meaning that as the concentration increases above these values, the total relative KB extraction in skeletal muscle decreases. Interestingly, KB have been shown to be more efficient for energy production even when compared to CHO. Specifically, when β HB (main KB involved in ketolysis) is burned in a bomb calorimeter, it yields 31% more energy per C_2 unit compared to pyruvate, the end product in CHO metabolism (Veech, 2004). Furthermore, a 28% increase in the hydraulic efficiency of perfused working hearts was shown when cardiac muscle metabolizes β HB compared to glucose. This increase in efficiency has been explained partly by the greater heat combustion of β HB (Veech, 2004). Importantly, KB utilization in skeletal muscle has been shown to increase as much as fivefold during exercise, when in a state of ketosis (Hagenfeldt and Wahren, 1968; Fery and Balasse, 1983; Balasse and Fery, 1989). Therefore, reaching a state of ketosis

may be an important strategy to improve exercise performance, but how that physiological state is achieved (endogenously or exogenously – Figure 2.2) can have important implications.

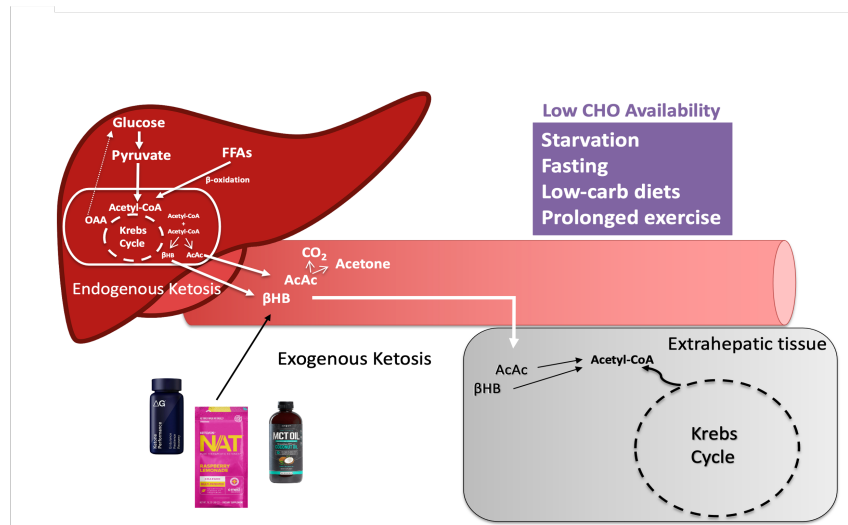


Figure 2.2. Metabolism of Ketone Bodies II. Schematic diagram showing how blood KB can be increased endogenously (ketogenesis) or exogenously via supplementation as well as how ketones can be used for energy by extrahepatic tissue (ketolysis).

2.2 Basis for Ketone Supplementation

In the last few decades, nutrition experts have focused primarily on CHO intake to optimize exercise performance (Hawley and Leckey, 2015). Nonetheless, CHO stores can be depleted within 1-2 h of intense exercise, and this depletion is coincident with the onset of fatigue (Hawley and Leckey, 2015). As such, fatigue becomes an inevitable feature in sports or other activities of moderate to long durations. So, nutrition strategies to attenuate or prevent fatigue-derived decrements in performance are always of interest. As discussed, KB are derived from fat metabolism, and can serve as an alternative fuel for peripheral tissue (Robinson and Williamson, 1980; Veech, 2004). Unlike CHO, fat represents a rather limitless source of energy (hundreds of thousands of calories) and therefore decreasing the reliance on CHO during exercise could be an effective approach to improve athletic performance (Volek et al., 2015). Further, increases in KB blood concentration, i.e., nutritional ketosis, is achieved easily through dietary manipulations such as the use of ketogenic diets (KD) (Volek et al., 2015). However, the KD requires an

individual to consume a very high percentage of calories from fat (70 - 80%) while restricting CHO intake (5 - 10%) (Aragon et al, 2017). Several authors have argued that adaptation to this dietary regime can be beneficial for exercise performance for various reasons: 1) KB can serve as an alternative fuel for peripheral tissue such as brain and muscle (Robinson and Williamson, 1980; Stubbs et al., 2017), so potentially it can improve both physical and cognitive performance during exercise. 2) KB are more efficient energetically, since the free energy of ATP hydrolysis for ketones is greater than CHO (Veech, 2004; Evans et al., 2017). 3) KB can attenuate proteolysis in skeletal muscle, something that might improve recovery from exercise (Robinson and Williamson, 1980; Evans et al., 2017; Vandoorne et al., 2017). However, a major limitation to the KD is that long-term adherence to such a restrictive diet has been reported to be very low (Gibson et al., 2015). Moreover, the micronutrient content of these diets can be as low as 20% of the dietary reference intakes (Zupec-Kania and Zupanc, 2008), something that could become problematic for athletes over time.

Despite these potential advantages of KD vs high CHO consumption, several investigations where individuals adhered to the KD for a period of 4-12 wk have shown equivocal results relative to exercise performance improvements (Hawley and Leckey, 2015; McSwiney et al., 2018; Burke, 2021). Some researchers argue that this is because athletes compete at CHO dependent intensities (Hawley and Leckey, 2015), so anything that affects CHO metabolism adversely would have a negative effect on exercise performance. For instance, KD have been shown to impair rates of muscle glycogenolysis and energy flux, resulting in a reduced capacity for high-intensity ATP regeneration (Stellingwerff et al., 2006). Furthermore, studies indicate that with the up-regulation of fat utilization for energy, there is a concomitant reduction in pyruvate dehydrogenase activity, a regulatory enzyme responsible for CHO metabolism (Stellingwerff et al., 2006). Therefore, the severe CHO restriction seen with KD might impair performance in sport disciplines that rely heavily on CHO metabolism, despite the potential CHO sparing effect of KB. For this reason, oral KB supplementation in combination with a high CHO intake has emerged as a potential aid for athletic performance (Cox et al., 2016; Evans et al., 2017). Moreover, KB supplementation has become common in professional cycling in recent years (Pinckaers et al., 2017), and its potential as an ergogenic aid is gaining

interest in the scientific community. Several aspects of metabolism have been shown to improve with KB supplementation (Cox et al., 2016; O'Malley et al., 2017; Poffe et al., 2019) including improved exercise performance, enhanced cognitive function, and perhaps even faster recovery after exercise ([details are discussed below] Evans et al., 2017; Valenzuela et al., 2021).

However, the magnitude of ketosis seems to be an important factor to achieve said positive outcomes and this is influenced by the type of exogenous ketone supplement ingested.

2.3 Types of Exogenous Ketones

The most direct way of inducing ketosis is to consume exogenous isolated KB. However, β HB and AcAc in their free acid form can be unstable, expensive, and ineffective at producing sustained ketosis (Poff et al., 2020). Therefore, KB need to be buffered with different electrolytes or bound to other ketogenic precursors to enhance their ability to induce nutritional ketosis (Poff et al., 2020). In general, exogenous ketone supplements can be divided into three main categories, ketogenic agents, ketone salts and ketone esters (Poff et al., 2020). Ketogenic agents do not contain any ketone bodies, but rather are molecules that can be rapidly metabolized in the liver to form β HB, the main KB circulating in blood (Poff et al., 2020). A couple of examples seen in the literature are medium chain triglycerides (MCT) and 1,3 Butanediol (1,3 BD). MCT contain shorter carbon chains (6 to 12 carbons), thus, can be absorbed directly through hepatic circulation and can enter the hepatocyte without requiring a transporter (Van Zyl et al., 1996; Poff et al., 2020; Prins et al., 2020). Once in the mitochondria, they are rapidly broken down to form acetyl CoA and ultimately KB (Van Zyl et al., 1996; Prins et al., 2020). This means that they have the potential to induce nutritional ketosis even in the presence of CHO like any other exogenous ketone supplement. However, caution is recommended when ingesting MCT as these may cause gastrointestinal (GI) problems especially if ingested in large amounts, although strategies such as a 1–2-wk adaptation have been explored to attenuate those symptoms (Thorburn et al., 2006; Poff et al., 2020). Another common agent used to induce ketosis in humans is 1,3 BD. This organic compound is classified as a diol or dialcohol (meaning it contains two hydroxyl groups)

(Poff et al., 2020). 1,3 BD is absorbed passively in the gut (Shivva et al., 2016) and is transported to the liver where it is converted to β HB (Desrochers et al., 1992; Prins et al., 2020). As with other ketogenic agents, large doses should be avoided as these may induce GI distress (Shaw et al., 2019; Prins et al., 2020) and/or symptoms that mimic low level alcohol intoxication such as nausea, euphoria, and dizziness (Shaw et al., 2019).

Typically, these ketogenic agents are ineffective as they induce only modest increases in β HB blood concentrations. i.e., 0.5 to 0.8 Mm (Scott et al., 2019; Shaw et al., 2019; Avgerinos et al., 2020; Poff et al., 2020). The remaining two supplement types, ketone salts (KS) and esters (KE), contain β HB (Stubbs et al., 2017). KS are the most abundant type of ketone supplement in the marketplace. They are made of β HB molecules bound to a mineral ion such as calcium, potassium, or sodium (Poff et al., 2020) that can help buffer hydrogen ions and increase pH (Stubbs et al., 2017). Usually these contain a racemic mixture of two isoforms of β HB, L- β HB and D- β HB (Stubbs et al., 2017). This mixture varies depending on the individual product and some are enriched with D- β HB (Kackley et al., 2020). This mixture is important because the D- β HB, the primary product of endogenous ketogenesis, is believed to contribute mainly to ATP regeneration (Kackley et al., 2020). However, KS supplements induce only modest increases in β HB blood concentrations (Stubbs et al., 2017; Margolis and O'fallon, 2020; Poff et al., 2020). Recently, some authors have combined MCT and KS attempting to induce greater β HB blood concentrations and a more prolonged ketosis state and perhaps mitigating any GI distress (Kesi et al., 2016; Prins et al., 2020). This might be critical because these two factors likely determine effectiveness of the supplement (Valenzuela et al., 2020).

The third type of exogenous ketones are KE, first described by Birkhahn et al. (1977). Recently, these have re-emerged as potential ergogenic/therapeutic agents. KE consist of a ketone body (β HB or AcAc) bound to a ketogenic precursor (1,3 BD, glycerol) through one or more ester bonds (Stubbs et al., 2017; Poff et al., 2020). Several KE are possible, and each differ in terms of tolerability and possibly in physiological effects (Poff et al., 2020). However, the only commercially available type to date are ketone monoesters (KME) and this is the most widely KE supplement type used in human research (Valenzuela et al., 2021). Specifically, KME are composed of a D- β HB molecule bound to 1,3 BD through an ester bond (Stubbs et al., 2017; Poff et al., 2020; Valenzuela et al.,

2021) and appears to induce the most effective nutritional ketosis, displaying β HB concentrations in blood that can be 2-3-fold greater than studies with either KS or ketogenic agents (Stubbs et al., 2017). As mentioned, the magnitude of ketosis can be important in inducing meaningful metabolic changes that can have an impact on performance. Therefore, KME seems to be the best possible option to study and understand the impact of EK supplementation on exercise performance.

2.3.1 Effects of Exogenous Ketone Supplementation on Exercise Performance

As discussed above, exogenous ketone supplementation can induce acute nutritional ketosis (Stubbs et al., 2017). This can shift fuel use during exercise (Cox et al., 2016; O'Malley et al., 2017) resulting in performance benefits (Cox et al., 2016). As demonstrated by Cox et al. (2016) in one of the first investigations evaluating oral ketone supplementation, KME supplementation not only spared CHO during a 60-min pre-fatiguing effort in elite cyclists, but also resulted in a 2% performance increase in a subsequent 30-min time trial. Of course, this improvement in performance is important considering that typically there is <1% difference in performance time between the 1st and the 10th rider in elite races (Valenzuela et al., 2021). In agreement with the results seen by Cox et al. (2016), a few subsequent studies have shown performance improvements (Poffe et al., 2019; Kackley et al., 2020; Poffe et al., 2021a). However, in contrast, most studies have either failed to demonstrate performance benefits (Rodger et al., 2017; Evans and Egan, 2018; Waldman et al., 2018; Dearlove et al., 2019; Evans et al., 2019; James and Greer, 2019; Scott et al., 2019; Shaw et al., 2019; Poffe et al., 2020; Prins et al., 2020; Poffe et al., 2021b) or have reported performance decrements (Leckey et al., 2017; O'Malley et al., 2017). Several recent systematic reviews have concluded that the overall results are equivocal making a conclusion about performance benefits impossible at the present time (Margolis and O'fallon, 2020; Valenzuela et al., 2020; Valenzuela et al., 2021). The main reasons for the discrepancy have been attributed to a high heterogenicity in factors such as ketone supplement type and dose, which can have important implications in the magnitude of ketosis achieved. For instance, some studies have used KE (Cox et al., 2016; Leckey et al., 2017; Evans and Egan, 2018; Dearlove et

al., 2019; Evans et al., 2019; Poffe et al., 2019; Poffe et al., 2020; Poffe et al., 2021a; Poffe et al., 2021b) with doses ranging from 330 – 915 mg·kg body mass⁻¹, while others have used KS (Rodger et al., 2017; O'Malley et al., 2017; Waldman et al., 2018; James and Greer, 2019; Prins et al., 2020; Kackley et al., 2020) with doses ranging from 106 – 610 mg·kg body mass⁻¹, or ketogenic agents (Scott et al., 2019; Shaw et al., 2019) with doses ranging from 350 – 500 mg·kg body mass⁻¹. At the time Cox et al. (2016) conducted the first investigation, KE supplements were not commercially available (only KS), so only laboratory groups that had the ability to synthesize their own KE were able to use them in their investigations. Of course, this added to the discrepancies seen in the literature to date. Another important aspect is that KB pharmacokinetics following a single dose of ketones have also shown that KB are absorbed at a slower rate in a fed state, compared to a fasted state (Stubbs et al., 2017) and this differences also need to be considered. Furthermore, participants in some of these studies have been fed large doses that were reported to cause GI distress (Leckey et al., 2017; Shaw et al., 2019) and that aspect alone has been attributed to be responsible at least partially for the lack of benefits (or even detriments) seen, as it is hard for an athlete to perform while having those symptoms. Moreover, the tests used to assess performance have differed as well. For example, some authors have used a best effort time trial (distance or time) while others have implemented a time to exhaustion test. It is well accepted that best effort time trials are better predictors of performance as they replicate the demands of competitions while time to exhaustion tests are often done at submaximal intensities and fixed workloads, so the interpretation of their results is more limited. In addition, different modes of exercise such as running or cycling have been used, and perhaps the small sample sizes (50% of studies used 10 or less participants) are all factors that contribute to these discrepancies (Margolis and O'fallon, 2020; Valenzuela et al., 2020; Valenzuela et al., 2021). Finally, as mentioned previously, KE supplementation has been proposed to enhance physical and cognitive performance as well as post-exercise recovery. Therefore, all the available evidence regarding each of these aspects of performance is discussed below.

2.3.2 Exogenous Ketones and Physical Performance

Most studies assessing the effectiveness of acute ketone supplementation to date have measured some outcome of physical performance. As mentioned, the first study by Cox et al. (2016) was intriguing as it found an ~2% performance increase in elite cyclists after ingesting KME, however, only a few subsequent studies have been able to show improvements in performance (Poffe et al., 2019; Kackley et al., 2020; Poffe et al., 2021a). For instance, Poffe et al. (2019) had participants train 6 times a wk for 3 wk, consuming either KME or an isoenergetic drink containing MCT after each training session. They measured power output (PO) in the last 30-min of a 2-h standardized endurance session pre- and post intervention and found that PO was 15% greater in the group that consumed KME. Further, Kackley et al. (2020) found that a KS supplement containing caffeine and amino acids was effective at improving time to exhaustion during a staged cycle ergometer test in both Keto-Naïve and in Keto-Adapted participants. Yet, the majority of studies with ketone supplementation to date have shown no changes in performance (Rodger et al., 2017; Evans and Egan, 2018; Waldman et al., 2018; Dearlove et al., 2019; Evans et al., 2019; James and Greer, 2019; Scott et al., 2019; Shaw et al., 2019; Poffe et al., 2020; Prins et al., 2020; Poffe et al., 2021b) and a couple of investigations even showed detriments in performance (Leckey et al., 2017; O'Malley et al., 2017). In the Leckey et al. (2017) study, professional cyclists completed a 31.2 km time trial after consuming ~18g of a ketone diester drink. A ~2% performance decrement was reported, although the authors attributed this decrement in performance to gut disturbances reported by most participants (Leckey et al., 2017). In another study (O'Malley et al., 2017), healthy recreationally active participants had 7% lower PO during a 150 kJ time trial (~10-12-min effort) following 15-min of a staged steady state exercise bout after consuming KS vs a non-caloric placebo.

In an attempt to explain these conflicting data, some authors have suggested that the magnitude of ketosis (1 -2 mM) is important with respect to potential ergogenic effects (Margolis and O'fallon, 2020; Valenzuela et al., 2020; Valenzuela et al., 2021). For instance, in the study by Cox et al. (2016), a KME supplement was used resulting in a blood ketone concentration of ~3 mM whereas in the two studies where detriments were

reported, a ketone diester (Leckey et al., 2017) and a KS supplement (O'Malley et al., 2017) were used, and blood KB concentrations were only ~0.8 mM. In general, KS have been shown to induce a lower level of ketosis compared to KE (Stubbs et al., 2017) and, therefore, are less likely to show any positive performance effects (O'Malley et al., 2017; Rodger et al., 2017; James and Greer, 2019; Waldman et al., 2018; Prins et al., 2020). A recent metanalysis concluded that KS do not enhance exercise performance (Valenzuela et al., 2020), and suggested that research should focus on using ketone monoesters (KME) because they appear to induce sufficient ketosis to cause metabolic changes (Margolis and O'fallon, 2020). Future study should attempt to elucidate the underlying mechanisms responsible for any exercise performance changes. Impaired CHO metabolism has been reported after adopting a KD even if ingestion of CHO returns to adequate amounts prior to the performance (Burke et al., 2020), which occurs because of a reduction in pyruvate dehydrogenase activity (PDH), a key regulatory enzyme responsible for CHO metabolism (Stellingwerff et al., 2006). Although a CHO-sparing effect has been reported with KME supplementation, it is important to elucidate if this CHO-sparing effect of ketones could be an actual impairment on CHO metabolism due to a downregulation in PDH activity as seen with the KD, something that indeed would be contraindicated for exercise performance at high intensities. In addition, some studies have shown that ingestion of KME can lower blood pH by ~0.05–0.10 U at rest (Stubbs et al., 2017) and during submaximal exercise (Dearlove et al., 2019). This could impair high intensity exercise performance because metabolic acidosis has been shown to impair rates of glycolysis, the predominant metabolic pathway during high intensity exercise. Interestingly, Poffe et al. (2021a) tried to address this problem by giving participants bicarbonate. They compared exercise performance with KME, KME + bicarbonate, or bicarbonate alone and found that co-ingestion of KME and bicarbonate improved mean power output during a 15-min time trial at the end of a 3-h cycling race by ~5% compared to the other conditions. However, the same group (Poffe et al., 2021b) also found that when ketosis (~3-5mM) was present during a 30-min time trial, there were no differences in performance independent of bicarbonate use. Clearly, more work needs to be done to elucidate the effects of ketone supplementation on exercise performance.

2.3.3 Exogenous Ketones and Cognitive Performance

Another possible benefit of ketone supplementation during exercise is enhanced cognitive performance (Valenzuela et al., 2021). This could be important, especially late in events when CHO availability is low, because split-second decisions could influence the outcome of an event. It is known that KB can cross the blood brain barrier through monocarboxylate-mediated transport (Evans et al., 2017), and serve as an alternative fuel for the brain in periods of low glucose availability or energy crisis. In fact, KB can supply over 60% of the metabolic energy needs of the brain (Cahill Jr, 1970), and this ability to act as an alternative fuel, i.e., reduce glucose reliance, could help enhance cognitive performance (Evans et al., 2017; Valenzuela et al., 2021). In addition, KB can increase the expression of the protein Brain Derived Neurotropic Factor (BDNF), an important molecule for brain plasticity and regulation of cognitive function (Marosi et al., 2016; Sleiman et al., 2016). Specifically, β HB is believed to inhibit histone deacetylases, enzymes that in turn inhibit the production of BDNF in the brain (Sleiman et al., 2016). Moreover, KB can induce increases in BDNF even when glucose supply is adequate (Hu et al., 2018), suggesting that exogenous ketone supplementation might improve cognitive function or attenuate cognitive impairment during prolonged exercise, regardless of chronic diet composition or macronutrient intake. To date, few studies have evaluated the efficacy of exogenous ketone supplements on cognitive function during exercise (Evans and Egan, 2018; Waldman et al., 2018; Evans et al., 2019; Prins et al., 2020; Waldman et al., 2020). Further, the majority of these studies have measured cognitive function before and after an exercise intervention (Evans and Egan, 2018; Waldman et al., 2018; Evans et al., 2019; Prins et al., 2020). For instance, Evans and Egan (2018) were able to demonstrate that supplementing with KME + CHO before and during a ~90-min Loughborough intermittent shuttle test (5x15-min intermittent exercise at varying intensities to simulate soccer play + 15-min time to exhaustion run) reduced the number of incorrect responses during a cognitive test administered after exercise, when compared to CHO alone. Similarly, Prins et al. (2020) showed that supplementing with KS + MCT, an approach that can induce greater β HB blood concentrations and a more sustained ketotic state, displayed faster response time accuracy prior to a 5k time trial when compared to a non-caloric placebo or KS alone. However, other studies have failed to

show improvements in cognitive function after exercise. For instance, Waldman et al. (2018) reported that KS did not improve cognitive function after performing 4-15s Wingate-type exercise interspersed with 4-min active recovery, compared to a non-caloric placebo. Likewise, Evans et al. (2019) observed no benefits of acute KME supplementation on a multitasking test or on reaction time measured before and after a 10K time trial. Interestingly, neural activation associated with exercise rapidly returns to baseline following the cessation of exercise (Dietrich and Sparling, 2004), therefore, the time at which these cognitive tasks are administered becomes crucial. Lambourne and Tomporowski (2010) observed that there were differences in cognitive function when tests were done during exercise, compared to after exercise (under no supplementation). Specifically, when cognitive function was measured during exercise it led to impairments whereas small improvements were seen when it was measured after exercise. These small methodological differences could, at least in part, explain the discrepancies seen in the literature. There has only been one study assessing the effects of ketone ingestion on cognitive function during exercise (Waldman et al., 2020). In this study Waldman et al. (2020), measured cognitive performance during exercise using a dual stress challenge (DSC) in an attempt to replicate the cognitive demands seen in high-stress occupations. Briefly, participants performed a 30-min steady state exercise session (at 60% $\dot{V}O_2$ peak) while performing two cognitive tests during the last 20 min of exercise. The authors reported no changes in cognitive function when comparing a KS supplement to a non-caloric placebo. These studies indicate that the effects of exogenous ketone supplementation on cognitive function during exercise are mixed so clearly more study is necessary before any conclusion regarding the effectiveness of ketone supplementation on cognitive performance in sports is possible.

2.3.4 Exogenous Ketones and Post-Exercise Recovery

Recovery is another important aspect of performance that takes on more relevance in multistage events like the Tour de France or competitive team sports leagues and tournaments where games can occur with little recovery time like the FIFA World cup. Ketone + CHO supplementation has been proposed to improve recovery after exercise, which could directly improve performance on a subsequent day (Valenzuela et al., 2021).

Several mechanisms suggested to explain this potential ergogenic effect are an increase in glycogen replenishment (Evans et al., 2017; Holdsworth et al., 2017; Takahashi et al., 2019) and/or a decrease in protein oxidation (Evans et al., 2017), something that could favour muscle repair. For example, Takahashi et al. (2019) exposed mice to swimming exercise for 60-min and subsequently isolated epitrochlearis muscle. Using an *in vitro* experimental model, they incubated the isolated muscle with glucose and insulin concentrations set at values representing those recommended in humans for maximal skeletal muscle glycogen recovery ($1.2 - 1.5 \text{ g} \cdot \text{kg body mass}^{-1} \cdot \text{h}^{-1}$) and the only difference was the presence or absence of β HB, which was set at 1, 2, or 4mM when present. The results demonstrated that glycogen replenishment was greater in the presence of β HB at concentrations of 2 and 4mM, with 4mM displaying greater glycogen content. Interestingly, early during incubation, β HB activated the proteins of the insulin-signaling cascade, while downregulating AMPK, changes that resulted in non-AMPK-mediated translocation of GLUT4 and a downregulation of glycogenolysis. These alterations may have contributed to the greater glycogen content in the β HB trials after 120-min, as reported by the authors (Takahashi et al., 2019). AMP-activated protein kinase (AMPK) is known to stimulate the translocation of glucose transporter 4 (GLUT4) (Fisher et al., 2002), and increase the rate of glycogenolysis (Hunter et al., 2011) while decreasing glycogen synthesis, in an attempt to restore muscle energy balance. Unfortunately, only a few investigations assessing the effect of ketone supplementation and recovery have been conducted in humans (Holdsworth et al., 2017; Vandoorne et al., 2017; Poffe et al., 2019). Holdsworth et al. (2017) investigated glycogen repletion in humans using a hyperglycemic clamp to maintain glucose concentrations at $\sim 10\text{mM}$ throughout the experiment and reported that after glycogen depleting exercise, a glucose clamp + KME supplementation increased glycogen content by 50% after two h, compared to a glucose clamp alone. Although exercise performance outcomes were not assessed in this study, this difference in glycogen content would be expected to have a significant impact on many sport disciplines, as often athletes compete at CHO dependent intensities (Hawley and Leckey, 2015). However, Vandoorne et al. (2017) explored the same issue in humans and found conflicting results. In this study, participants performed a bout of intense one-leg glycogen depleting exercise followed by a 5-h recovery period.

During the recovery time, a protein (PRO)-CHO supplement containing adequate amounts ($1.0 \text{ g} \cdot \text{kg body mass}^{-1} \cdot \text{h}^{-1}$ of CHO and $0.3 \text{ g} \cdot \text{kg body mass}^{-1} \cdot \text{h}^{-1}$ of PRO) to obtain optimal muscle protein (Phillips and Van Loon, 2011) and glycogen synthesis (Cermak and Van Loon, 2013) was ingested with or without KME. The authors found that KME + PRO-CHO supplement ingestion increased mTORC1 activation in human skeletal muscle following exercise, and enhanced leucine-mediated muscle protein synthesis using a series of *in vitro* experiments. This may have been because of the observed downregulation of AMPK early during recovery in the KME trial, which in turn would help increase activation of mTORC1 since AMPK may inhibit the mTOR pathway and promote proteolysis (Bolster et al., 2002). Nevertheless, no differences in glycogen resynthesis between both KME and placebo trials were observed. Interestingly, a recent study (Poffe et al., 2019) where participants completed a 3-wk intensive training program designed to induce physiological and perceptual symptoms of overreaching found that, compared to placebo, chronic (3 wk) post exercise and before sleep KME supplementation blunted the physiological symptoms of overreaching vs an isoenergetic MCT. Specifically, KME improved PO during a 30-min time trial performed pre- and post intervention (Poffe et al., 2019). Sprint performance was reduced for all participants due to overtraining, but the KME group marked a tendency to prevent glycogen depletion (resting glycogen content decreased in the control group after 3 wk of training ($p < 0.05$) but not in the KME group [$p = 0.99$]), tolerated greater workloads in the last wk of training and displayed a 15% higher PO during a 30-min time trial at the end of the 3-wk training period (Poffe et al., 2019). However, these data must be interpreted cautiously because food intake during the study was *ad libitum* and based on 2 d food record estimations both energy and CHO was greater in the KME group (wk 2: energy = 3,598 vs 3,981 kcal/d; wk 3: 3,653 vs 4,199 kcal/d; wk 2: CHO = 1,772 vs 2,067 kcal/d; wk 3: 1,773 vs 2,154 kcal/d) vs the control group. Although intriguing, more research needs to be done to elucidate the efficacy of ketone supplements to promote recovery and enhance performance.

In summary, EK supplementation presents as an alternative to reach a state of nutritional ketosis. This state of nutritional ketosis achieved through supplementation has been shown to reduce rates of glycolysis, increase KB utilization for ATP regeneration leading

to a possible CHO-sparing effect, which in turn could benefit exercise performance as demonstrated initially by Cox et al. (2016). Since the pioneering work of Cox et al. (2016) other authors proposed that ketones could help improve not only physical performance but also cognitive performance and post-exercise recovery since KB have been shown to be an important fuel for the brain with an established neuroprotective role in non-exercise contexts as well as an important signaling molecule capable of enhancing the recovery process by increasing glycogen replenishment and reducing proteolysis (Evans et al., 2017). However, subsequent studies have shown equivocal results, with some studies reporting increased exercise performance but the majority of other showing no difference. Unfortunately, the majority of these studies have focused on measuring physical performance so the evidence regarding cognitive function during exercise or post-exercise recovery is lacking. Therefore, the purpose of this dissertation was to confirm possible ergogenic effects of KS supplementation seen in only one study, and also provide more insight to the short body of evidence evaluating cognitive function during exercise and post-exercise recovery, using KME supplementation.

2.4 References

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Chapter 3

- 3 Acute ketone salts-caffeine-aurine-leucine supplementation but not ketone salts-aurine-leucine, improves endurance cycling performance

3.1 Abstract

Co-ingestion of ketone salts, caffeine and the amino acids, taurine and leucine has been shown to improve endurance exercise performance. However, there is no study comparing this co-ingestion to the same nutrients without caffeine. We assessed whether ketone salts-caffeine-aurine-leucine (KCT) supplementation was superior to caffeine-free ketone salts-aurine-leucine supplementation (KT), or to an iso-energetic CHO placebo (PLAC). 13 recreationally active men (177.5 ± 6.1 cm, 75.9 ± 4.6 kg, 23 ± 3 y, 12.0 ± 5.1 % body fat; mean \pm SD) completed a best effort 20 km cycling time trial (TT20km), followed 15 min later by a Wingate power cycle test, after supplementing with either KCT (~ 7 g of β HB, ~ 120 mg caffeine, 2.1g leucine, 2.7g taurine), KT (i.e., same supplement without caffeine), or iso-energetic PLAC (11g of dextrose). Blood beta-hydroxybutyrate (β HB) were elevated ($p < 0.001$) after ingestion of both KCT (0.65 ± 0.12 mM) and KT (0.72 ± 0.31 mM) relative to PLAC (0.06 ± 0.05 mM). Moreover, KCT improved TT20km performance (37.8 ± 2.38 min), compared to PLAC (39.4 ± 3.33 min; $p = 0.003$) but not vs KT (38.8 ± 2.87 min; $p = 0.09$). TT20km average PO was greater with KCT (PO = 181 ± 29 W) vs both KT (171 ± 32 W; $p = 0.05$) and PLAC (165 ± 35 W; $p = 0.001$). Peak power output for the Wingate test was also greater for both KCT (1134 ± 137 W; $p = 0.03$) and KT (1132 ± 128 W; $p = 0.04$) vs PLAC (1068 ± 127 W). These data suggest that the observed improved endurance performance effects are likely the result of the co-ingestion of ketone salts, caffeine and taurine rather than ketones alone.

Keywords: exogenous ketones, ketosis, supplement, endurance exercise performance, Wingate

3.2 Introduction

The ketogenic diet is a nutritional strategy characterized by substantial fat (~70 to 80% energy intake) as well as very low CHO (~5% energy intake). This dietary approach has received considerable attention over the past few years as a possible intervention to enhance endurance exercise performance (Volek et al., 2015). The rationale underlying this approach is that fat represents a virtually unlimited energy source even in lean individuals (Sansone et al., 2018; Volek et al., 2015), while the other major muscle fuel, CHO can be depleted during a single exercise bout (~1-2 h of moderate-high intensity exercise; Saltin, 1973; Hawley and Leckey, 2015). Moreover, high dietary fat has significant potential with respect to endurance exercise performance because the resulting upregulated fat mobilization triggers a substantial increased accumulation of circulating ketone bodies. This is called nutritional ketosis (Evans et al., 2017) and is characterized typically by a plasma ketone concentration of ~0.5–3.0 mM, i.e., hyperketonemia (Gibson and Sainsbury, 2017). Importantly, this metabolic state provides a large supply of muscle fuel and is very different than ketoacidosis, a life-threatening condition that can affect diabetics or alcoholics, where the plasma ketone concentration can reach ~10+ mM.

In theory, ketones have at least two metabolic advantages over CHO relative to exercise performance. First, as mentioned, they provide an almost unlimited source of energy and second their free energy of ATP hydrolysis is greater than CHO, i.e., ketone bodies are energetically more efficient vs CHO (Evans et al., 2017). However, despite these theoretical advantages, adopting to a ketogenic diet does not produce a clear performance benefit for endurance exercise compared to a high CHO diet (Burke, 2020). Likely, this is because the associated low CHO intake results in a reduced pyruvate dehydrogenase activity, a key regulatory enzyme in CHO metabolism (Peters et al., 1998). For most athletes, this is critical because they compete at intensities that are largely CHO-dependent (Burke, 2020).

Given the constant pursuit of athletes to find nutritional strategies to enhance exercise performance, manufacturers have developed ketone supplements that induce a state of

mild-modest nutritional ketosis acutely (~1 h). However importantly, this differs from dietary ketosis because these supplements can allow a state of ketosis to be achieved with replete glycogen stores. Therefore, supplemental hyperketonemia has the potential to provide additional muscle fuel (Pinckaers et al., 2017) while sparing CHO. If so, athletic performance could be enhanced. In support of this possibility, Cox et al. (2016) demonstrated a reduction in glycogen use and an increase in fat use during endurance exercise after consuming a ketone ester supplement. In contrast, several subsequent studies assessing the effectiveness of ketone supplements on endurance performance are inconsistent (Margolis and O'fallon, 2020). This may be because of differing experimental methodologies, especially ketone supplement types, and/or dosages used.

Recently, ingestion of a pre-workout supplement containing ketone salts, the amino acids, taurine and leucine, and caffeine improved high intensity exercise performance (Kackley et al., 2020). Unfortunately, the investigators used a water placebo which is problematic here because caffeine is a well-established performance aid (Grgic et al., 2020), even when consumed at low dosages (Pickering and Kiely, 2019). Further, taurine can also improve exercise performance (Warnock et al., 2017). Therefore, the purpose of our study was to determine the endurance exercise performance effects of a ketone salts-caffeine-leucine-aurine drink (KCT) vs both the same drink without caffeine (KT) and an isoenergetic dextrose placebo (PLAC).

3.3 Methods

Participants

Thirteen healthy, young, recreationally active men volunteered to participate (177.5±6.1 cm, 75.9±4.6 kg, 23±3 y, 12.0±5.1 % body fat). All were involved in exercise/sports activities at least twice a wk prior to the study. Each completed a health information form and a physical activity readiness questionnaire (PAR-Q; Thomas et al., 1992) to minimize any potential contraindications to exercise. Potential experimental risks were explained fully prior to any testing and the participants provided written, informed consent of the

study protocol approved previously by the University of Western Ontario's Office of Research Ethics.

Familiarization Session

Prior to the three experimental trials, participants visited the laboratory for a familiarization session. During this visit, body composition (Bod Pod®) was measured by densitometry, as described previously (Noreen and Lemon, 2006) and, to minimize potential experimental learning effects, participants were given an opportunity to practice the exercise tests (20 km cycling time trial [TT_{20km}] and a 30 sec Wingate power test) to be used during the experimental treatments.

Experimental Treatments

A double-blind, repeated-measures crossover research design was implemented involving three experimental treatments, ketone salts (beta-hydroxybutyrate - β HB) with caffeine, leucine and taurine (KCT [KETO//OS – NAT – charged - ~120mg of caffeine; ~7g β HB, ~2.1g of leucine, ~2.7g of taurine), Pruvit Ventures. Inc, Melissa, TX, USA]), ketone salts, leucine, taurine without caffeine (KT [KETO//OS – NAT – caffeine free - ~7g β HB, ~2.1g of leucine, ~2.7g of taurine), Pruvit Ventures. Inc, Melissa, TX, USA]), or iso-energetic dextrose monohydrate (PLAC, 11 g dextrose). Endurance performance was evaluated under each treatment using a best effort cycle TT_{20km} followed 15 min later with a 30 sec maximal Wingate cycle power test. (Figure 3.1; details below).

Each trial was separated by at least one wk and testing occurred during the same time of the day for each participant (\pm 1 h). To avoid order effects, the first participant was randomized to treatment order and each subsequent individual had the experimental treatments rotated systematically. Further, to minimize dietary differences among treatments, each participant maintained a dietary record of all food/drink intake for the two d prior to the first experimental session and replicated this intake for the two d preceding the subsequent trials. Ratings of perceived exertion (RPE; 6-20 scale; Borg and Noble, 1974) and fingerstick blood samples, assessed for β HB and lactate, were obtained before, during, and following the exercise tests (Figure 3.1).

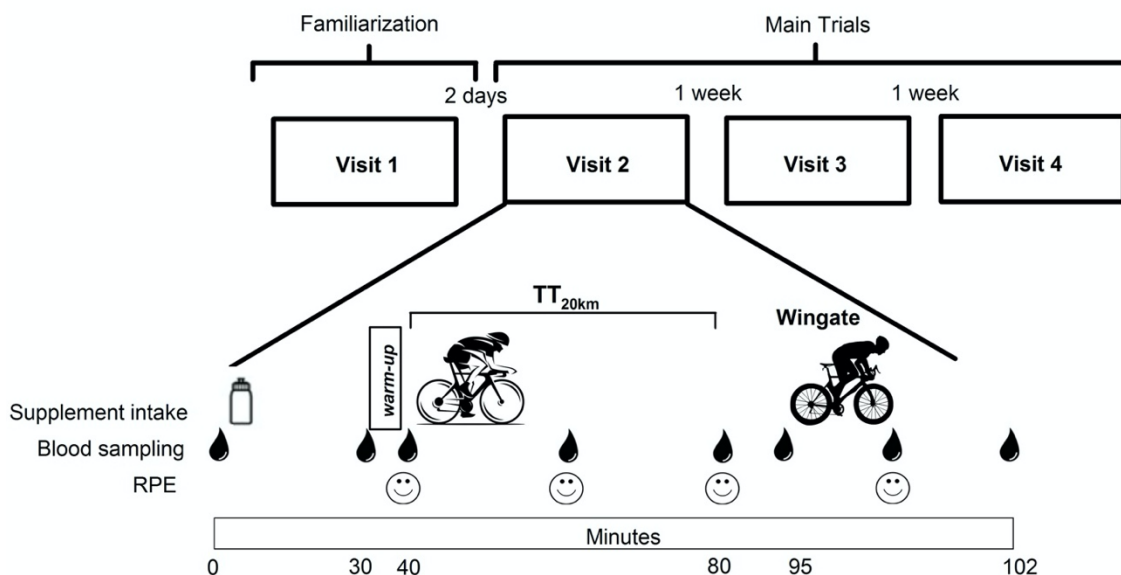


Figure 3.1. Overview of study protocol

Experimental Protocol

Each participant reported to the laboratory 4 h postprandial with limited activity (drive/use of the elevator to get to the lab) as well as having abstained from strenuous exercise, caffeine, or alcohol consumption for 24 h. These controls were utilized not only to standardize conditions among treatments but also to ensure that participants exercised in a fed state, which is typical for most athletes.

Upon arrival at the laboratory, measures of baseline blood β HB (FreeStyle Precision Neo®, Abbott Diabetes Care Limited) and blood lactate (Lactate Scout+, EKF Diagnostics) concentration were obtained from finger stick blood samples. Subsequently, the experimental drink was provided, and participants were given 30 min of rest to allow for digestion/absorption of the supplement. All drinks consisted of 500 ml of water mixed with either KCT, KT, or PLAC. This quantity of ketone salts and the volume of water used to prepare the beverages were based on the manufacturer's guidelines. Water was available ad libitum throughout the first exercise trial and the intake recorded (598 ± 358

ml) so it could be reproduced during subsequent trials. After 30 min, participants performed a standardized (10 min) warm up (cycling at 100 watts) and then completed the best effort TT_{20km} on a Velotron cycle ergometer (CompuTrainer, RacerMate Inc, Seattle, WA). Gearing was kept constant and participants were blinded to feedback such as time, speed, RPM, power output, and heart rate (only distance travelled was provided).

Fifteen min later, the 30 sec Wingate cycle power test was performed. Briefly, following a 25 sec lead in, where participants gradually increased their cadence to ~150 RPM, the load (9% of body mass) was engaged, and participants maintained the greatest power output possible throughout 30 sec. Peak power output, average power output and fatigue index (rate of drop off over the 30 sec) were determined using an online data acquisition system (CompuTrainer, RacerMate Inc, Seattle, WA).

3.4 Statistical Analysis

Statistical analyses were performed using SigmaPlot for Windows (Version 12.0). All data were tested for normality and a non-parametric test (ANOVA on Ranks Test) was used wherever a data set was not normally distributed. Blood metabolite concentrations and rates of perceived exertion were analyzed using a two-way (treatment by time) repeated measures ANOVA. Performance outcomes were analyzed using a one-way repeated measures ANOVA. Post hoc Tukey's HSD testing was used wherever significant main or interaction effects were found. Significance was set at $p \leq 0.05$. Data are presented as means \pm SD.

3.5 Results

Blood Ketones (β HB)

There was a main effect of time ($p < 0.001$), a main effect of treatment ($p < 0.001$), as well as a significant interaction ($p < 0.001$) for blood β HB concentration. Pairwise comparisons indicated that blood β HB were significantly greater ($p < 0.001$) for both KCT and KT groups compared to PLAC at 30 min post treatment ingestion, as well as before, during, and after the TT_{20km} (Figure 3.2). The KCT post TT_{20km} β HB concentration was

also significantly greater ($p= 0.04$) vs KT. Furthermore, KCT β HB concentration before the Wingate test was greater vs both KT ($p= 0.01$) and PLAC ($p< 0.001$).

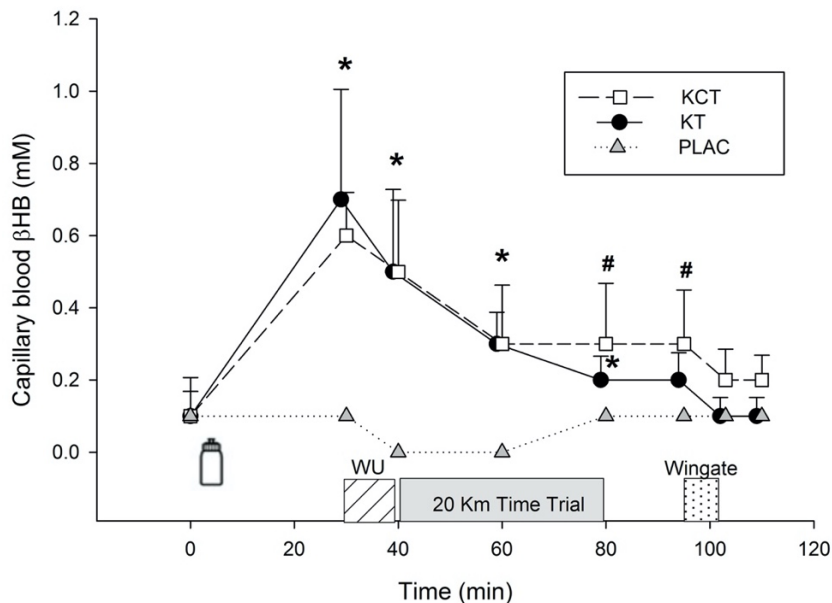


Figure 3.2. Blood β HB concentration. Values are means \pm SD. WU= warm-up; KCT= ketone salts-caffeine-aurine-leucine supplement; KT= ketone salts-aurine-leucine supplement; PLAC= iso-energetic CHO placebo. *Significantly different versus PLAC. # Significantly different versus both KT and PLAC.

Blood Lactate

There was a main effect of time ($p< 0.001$), a main effect of treatment ($p= 0.01$), as well as a significant interaction ($p= 0.02$) for blood lactate concentration. Pairwise comparisons showed that blood lactate was significantly greater during the TT_{20km} for KCT compared to both KT ($p= 0.03$) and PLAC ($p= 0.02$), as well as immediately after the Wingate test (KCT vs KT vs PLAC; $p< 0.001$) (Figure 3.3). Additionally, the blood lactate concentration right after the TT_{20km} was significantly greater ($p= 0.01$) for KCT vs PLAC, but not vs KT ($p= 0.20$).

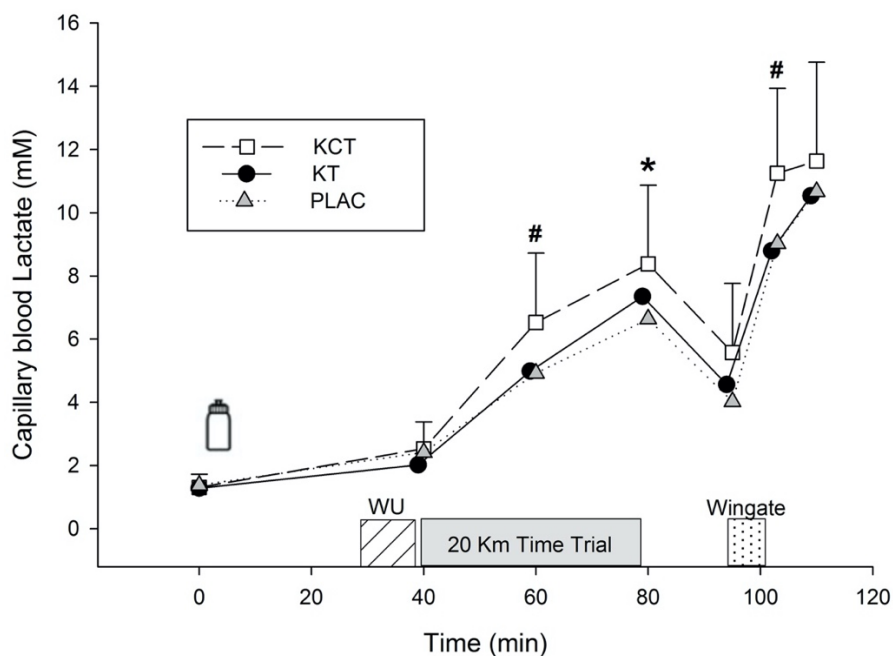


Figure 3.3. Blood lactate concentration. Values are means \pm SD. WU= warm-up; KCT= ketone salts-caffeine-aurine-leucine supplement; KT= ketone salts-aurine-leucine supplement; PLAC= iso-energetic CHO placebo. *Significantly different versus PLAC. # Significantly different versus both KT and PLAC.

Ratings of Perceived Exertion (RPE)

A main effect of time ($p < 0.001$) was detected for RPE (Table 1). As expected, average RPE increased for all treatments as the exercise session progressed. No treatment effect ($p = 0.92$) nor treatment \times time interactions ($p = 0.65$) were observed.

Measures of Exercise Performance

20 Km Time Trial

There were significant differences in both, time to complete best effort TT_{20km} ($p = 0.004$) and in average TT_{20km} power output ($p = 0.002$). Post hoc Tukey's HSD testing revealed that time to complete TT_{20km} was significantly improved ($p = 0.003$) in the KCT trial (37.8 ± 2.28 min) compared to the PLAC (39.4 ± 3.33 min) trial (Figure 3.4). The observed

KCT vs KT differences (38.8 ± 2.87 min; $p = 0.09$) did not attain significance (Figure 3.4). Moreover, average $TT_{20\text{km}}$ power output in the KCT trial was significantly greater (181 ± 29 W) than both KT (171 ± 32 W; $p = 0.05$) and PLAC (165 ± 35 W; $p = 0.001$) trials (Table 3.1).

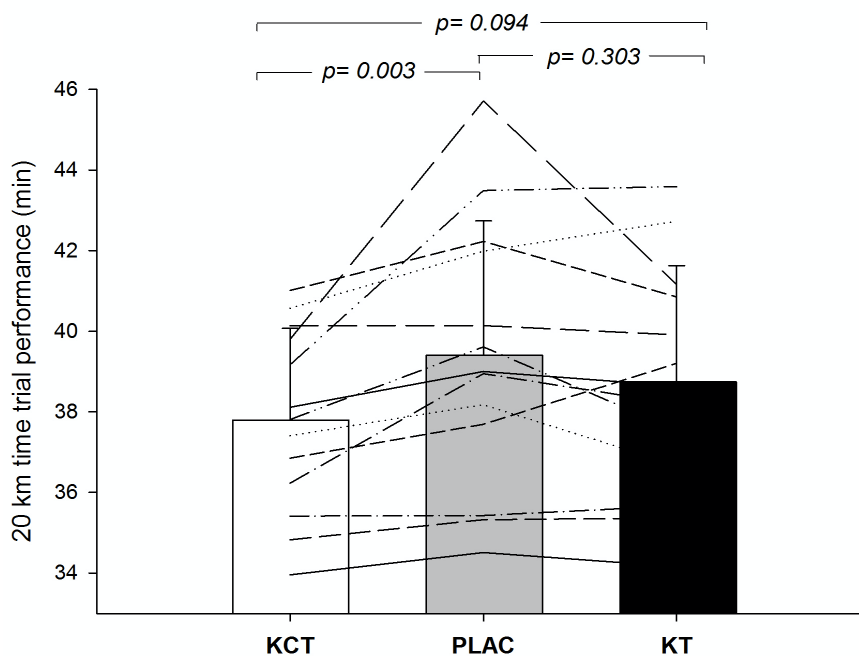


Figure 3.4. Time to complete $TT_{20\text{ km}}$. Values are means \pm SD. KCT= ketone salts-caffeine- taurine-leucine supplement; KT= ketone salts- taurine-leucine supplement; PLAC= iso-energetic CHO placebo. *Significantly different versus PLAC. Lines represent individual responses.

Wingate Test

There were significant differences in Wingate peak power output ($p = 0.02$). Post hoc Tukey's HSD testing revealed that peak power output was significantly greater for both KCT ($p = 0.03$) and KT ($p = 0.04$) compared to PLAC (Table 3.1). Further, both average Wingate power output ($p = 0.08$) and the fatigue index ($p = 0.09$) approached significance for both ketone treatments vs PLAC.

Table 3.1. Performance outcome measures

	KCT	KT	PLAC	One-way RM ANOVA			
				P-value	Post hoc Tukey's HSD test		
					KCT vs PLAC	KCT vs KT	KT vs PLAC
TT_{20km}							
Avg PO (W)	181 ± 29 ^{a,b}	171 ± 32	165 ± 35	0.002	0.001	0.05	0.27
Wingate							
Avg PO (W)	651 ± 70	646 ± 71	634 ± 71	0.08			
Peak PO (W)	1134 ± 137 ^b	1132 ± 128 ^b	1068 ± 127	0.02	0.03	0.99	0.04
Fatigue index (W/S)	25.3 ± 5.7	25.1 ± 4.9	23.5 ± 4.6	0.09			
RPE							
Pre TT _{20km}	8.8 ± 2.1	9.1 ± 2.0	9.2 ± 2.1	Time Effect		p < 0.001	
During TT _{20km}	14.2 ± 1.2	13.8 ± 1.4	14.0 ± 1.2	Treatment Effect		p = 0.92	
Post TT _{20km}	16.8 ± 2.0	16.8 ± 1.3	16.6 ± 2.1	Interaction Effect		p = 0.65	
Post Wingate	18.9 ± 1.2	18.8 ± 1.5	18.7 ± 1.2				

Values are means ± SD. Comparison for Time trial and Wingate power test were done using one-way repeated measures ANOVA. RPE data was analyzed using two-way repeated measures ANOVA. Post hoc Tukey's HSD testing was used to figure out differences between treatments. PO= power output, W= watts, rpm= revolutions per minute, TT_{20km} = 20 km best effort time trial.

^a indicates significant difference from KT group

^b indicates significant difference from PLAC group

3.6 Discussion

The aim of the present study was to assess the effectiveness of acute supplementation with ketone salts-aurine-leucine with (KCT) and without caffeine (KT) vs isoenergetic PLAC (dextrose) on endurance exercise performance. Our results revealed that the time to complete the best effort TT_{20km} was improved with KCT supplementation compared to PLAC. Although, the TT_{20km} time differences between KCT and KT (p= 0.30) did not attain significance, the observed significant average TT_{20km} power output was greater (p= 0.05) with KCT vs KT suggesting that the added caffeine was effective. In addition, peak power output during the Wingate test was greater in both KCT and KT compared to PLAC, while average Wingate power output also favored both ketone groups but not significantly (p= 0.08).

Previous studies evaluating the effects of ketone supplements on exercise performance have been inconsistent, with some studies showing positive effects (Kackley et al., 2020; Cox et al., 2016; Poffe et al., 2019), some others negative effects (Leckey et al., 2017;

O'Malley et al., 2017), while others observed no differences (Rodger et al., 2017; Evans et al., 2019; James and Greer, 2019; Waldman et al., 2018). These conflicting results may be caused by differing methodological practices amongst studies (Margolis and O'fallon, 2020). For instance, previous investigations have used different participant populations, ketone supplement types and performance tests, making definitive recommendations regarding ketone supplementation and exercise performance challenging.

A recent publication reported that vs a water placebo, a pre-workout supplement containing caffeine, ketone salts as well as the amino acids, taurine and leucine extended time to fatigue during high intensity cycling exercise in both keto-adapted and those ingesting a normal diet (Kackley et al., 2020). Caffeine is a well-established ergogenic substance (Grgic et al., 2020), even if ingested in small amounts (Pickering and Kyely, 2019), therefore, the improved performance seen in the study by Kackley et al. (2020) could have been due to the caffeine as the same supplement without caffeine was not evaluated.

In the present study, the comparison between caffeinated and caffeine-free ketone supplementation was made. The ketone supplements used in this study differed only with respect to the presence of caffeine. When compared to PLAC, our TT_{20km} results indicate that caffeine was critical. This indicates that the Kackley et al. (2020) performance enhancement may have been due to the caffeine not the ketones. Further, the observed lack of a significant TT_{20km} improvement in our KT trial vs PLAC suggests that ketone salt supplements have little effect on this type of endurance exercise performance. This conclusion is consistent with several previous studies with ketone supplementation (Rodger et al., 2017; O'Malley et al., 2017; Waldman et al., 2018; James and Greer, 2019) and suggests that the ergogenic effects observed with our KCT, at least for the TT_{20km}, were likely due to the caffeine content.

In contrast, our observed 15 min post TT_{20km}, 30 second Wingate test results cannot be attributed to caffeine as both ketone supplements enhanced performance. For example, both KCT and KT trials resulted in greater ($p= 0.03$ and $p= 0.04$) Wingate peak power output vs PLAC. Moreover, average power output and the fatigue index, which are

arguably more representative of anaerobic performance, approached statistical significance ($p= 0.08$ and $p= 0.09$, respectively) with both KCT and KT trials vs PLAC. While the explanation here is unclear it might involve the amino acid taurine as it was present in both ketone supplements and has been shown to enhance Wingate performance previously (Warnock et al., 2017). More study is needed to determine the magnitude of any performance effects of taurine.

Previously, blood lactate concentration has been shown to be lower during exercise after ingestion of a ketone ester supplement (Cox et al., 2016; Leckey et al., 2017). Of course, lactate is produced during the metabolism of CHO, and therefore, the lower concentrations seen with hyperketonemia may indicate a possible CHO sparing effect, something that could be advantageous for endurance events (Cox et al., 2016). Our blood lactate data were greater consistently with KCT perhaps the result of a greater reliance on CHO as a fuel during either or both exercise performance tests with KCT. Interestingly, the improved performance seen in the KCT trial was not accompanied by an increased perception of effort, as RPE was very similar across all treatments.

Finally, it has been suggested that a minimum blood β HB concentration of ≥ 1 mM is important to elicit positive effects of ketone supplementation (Margolis and O'fallon, 2020). While both ketone supplements we used elevated blood β HB (6-7 fold), neither attained this concentration (peaked at 0.7 mM) perhaps because we used the fed state which enhanced ecological validity but could have slowed supplement absorption, reducing their effectiveness (Stubbs et al., 2017).

In summary, ingestion of KCT and KT elevated blood β HB concentration substantially but the absolute concentration was < 1 mM. KCT improved performance on a best effort TT_{20km} when compared to both KT and PLAC. Further, both KCT and KT improved Wingate peak power output. These data suggest both caffeine and taurine contribute to these performance benefits and that CHO sparing might be involved. With these data, it is difficult to separate the role of ketone salts on performance due to the presence of several components in the supplement studied. Further, study is needed with single ingredients to elucidate any individual or synergistic effects of the ketone salts-caffeine-aurine-leucine

supplement combination but at this time it appears that ketone supplementation alone is unlikely to enhance this type of endurance performance.

3.7 Acknowledgements

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The investigators declare no conflict of interest relevant to the content of this article.

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Chapter 4

- 4 Ketone ester and carbohydrate supplementation does not improve subsequent time trial performance after glycogen lowering exercise.

4.1 Abstract

Relative to CHO alone, exogenous ketones and CHO supplementation have been shown to display greater muscle glycogen content after a 2-h recovery period following glycogen depleting exercise. However, whether this strategy improves subsequent performance is still unknown. We assessed the efficacy of ketone monoester (KME) and CHO supplementation on 20 km (TT20km) and 5 km (TT5km) best effort time trials after a 4-h recovery period following glycogen lowering exercise. 9 endurance trained men (175.6 ± 5.3 cm, 72.9 ± 7.7 kg, 28 ± 5 y, 12.2 ± 3.2 % body fat, $\dot{V}O_{2\max} = 56 \pm 6$ ml · kg $BM^{-1} \cdot \min^{-1}$; mean \pm SD) completed a glycogen lowering exercise session, followed by a 4-h recovery period and subsequent TT20km and TT5km. During the first 2 h of recovery, participants ingested either KME and CHO (KME + CHO) or an iso-energetic placebo and CHO (PLAC + CHO). Blood metabolites during recovery and performance during subsequent time trials were measured. KME + CHO displayed greater blood beta-hydroxybutyrate (β HB) concentration (ranged from 2.7mM at 30-min to 1.2 mM at 120-min; $p < 0.001$) during the 2-h feeding period and lower blood glucose concentration at 30 (5.8 ± 0.6 vs 7.1 ± 0.8 mM, $p = 0.003$) and 60 (6.1 ± 1.2 vs 7.2 ± 2.3 mM, $p = 0.009$) min of the 2-h feeding period. However, no differences in power output (TT20km= 174 ± 22 vs 169 ± 26 W; $p = 0.427$; TT5km= 182 ± 32 vs 186 ± 41 W; $p = 0.524$) nor time to complete the time trials (TT20km= 38.4 ± 2.25 vs 38.9 ± 1.56 min; $p = 0.420$; TT5km= 9.5 ± 0.65 vs 9.5 ± 0.88 min; $p = 0.884$) were seen compared to PLAC + CHO. These data suggest that metabolic changes induced by KME + CHO are not sufficient to elicit any potential benefits in subsequent time trial performance. More study is warranted to assess fully the potential benefits of KME supplementation for athletes during exercise recovery.

Keywords: exogenous ketones, ketosis, glycogen repletion, exercise recovery, insulin.

4.2 Introduction

CHO is considered an important fuel for exercise because the onset of fatigue during prolonged exercise performance at moderate to high intensities is associated with glycogen depletion (Jeukendrup, 2004; Cermak and Van Loon, 2013; Hawley and Leckey, 2015), which occurs typically within ~1-2 h (Saltin, 1973; Hawley and Leckey, 2015). Therefore, greater initial muscle glycogen content has been shown to correlate with better performance not only in intense, intermittent sports (Saltin, 1973; Krustup et al., 2006) but also in prolonged endurance efforts (Coyle et al., 1986). As such, repletion of CHO stores immediately after exercise is an important factor that could determine performance in subsequent events, especially if these events occur within 24 h (Cermak and Van Loon, 2013). Dose-response studies have determined that CHO ingestion of $\sim 1.2 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ is the appropriate acute recovery dose to optimize glycogen repletion with no apparent benefit at greater doses (Cermak and Van Loon, 2013). Protein (PRO) in combination with CHO has also been proposed to enhance glycogen resynthesis when 1 part PRO is provided with ~ 4 parts CHO (Berardi et al., 2008; Upshaw et al., 2016). Both strategies have resulted in better exercise performance a few h after a glycogen lowering exercise bout (Berardi et al., 2008; Cermak and Van Loon, 2013; Upshaw et al., 2016).

Ketone bodies (KB) are water-soluble lipid derived molecules that have been recognized as important because they provide alternative fuel during fasting or exercise, and because they serve as a critical signaling metabolite (Newman and Verdin, 2014). Recently, exogenous ketone supplements have emerged as an effective way to elevate circulating KB that may enhance post-exercise recovery by increasing muscle glycogen content via an insulinotropic response (Holdsworth et al., 2017; Valenzuela et al., 2021) and/or by enhancing protein synthesis (Vandoorne et al., 2017). Nevertheless, evidence in this area of research is scarce and controversial. In animal studies, exogenous KB have been shown to result in enhanced glycogen content when combined with CHO (Maizels et al., 1977; Laughlin et al., 1994; Takahashi et al., 2019). In humans, the first to explore this issue was Holdsworth et al. (2017). These authors demonstrated that glycogen repletion was 50% greater when glucose and a ketone monoester (KME) were taken together, compared to glucose alone. However, in this study they used a hyperglycemic clamp to

maintain glucose concentrations at $\sim 10\text{mM}$ throughout the experiment, something that is impractical for athletes. The only other study that evaluated this issue in humans was conducted by Vandoorne et al. (2017). In their study, participants were given a drink containing $1.0\text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ CHO and $0.3\text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ of PRO while consuming a KME or placebo. No differences in glycogen repletion were observed but the activation of mTORC1, a molecular pathway responsible for protein synthesis was increased. Unfortunately, exercise performance was not assessed in any of these studies, so it is unclear whether or not the positive outcomes observed with concomitant consumption of a KME and CHO is sufficient to enhance subsequent exercise performance. Therefore, the purpose of this study was to assess the efficacy of KME + CHO supplementation on 20 km and 5 km best effort time trials completed after a 4-h recovery period following glycogen lowering exercise.

4.3 Methods

Participants

A total of 13 healthy, endurance trained men were recruited but three participants did not complete the entire experimental protocol due to laboratory closures and restrictions during the COVID-19 pandemic. Another was injured (activities outside of the lab) before completing the study. These four participants were excluded from data analysis and the remaining 9 were included in the study (Table 1). All were involved in some type of endurance training at least 3 d per wk for 6 mo or more prior to the study. Each completed a physical activity readiness questionnaire (PAR-Q+ - Thomas et al., 1992) and a health information form to minimize any potential contraindications to exercise. All potential risks were explained fully prior to any testing, and the participants provided written, informed consent of the study protocol approved previously by the Western University Office of Research Ethics.

Table 4.1. Participant Characteristics and Dietary Intake (n=9)

Characteristics	Value
Height, cm	175.6 ± 5.3
Body mass, kg	72.9 ± 7.7
Age, y	28.0 ± 5.0
Body fat ^a , %	12.2 ± 3.2
\dot{W}_{\max}^b	314.6 ± 42.3
$\dot{V}O_{2\max}^c$, ml·kg ⁻¹ ·min ⁻¹	56 ± 6
Intakes ^d	
Energy, kcal·kg ⁻¹ ·d ⁻¹	32.2 ± 3.5
Carbohydrate, g·kg ⁻¹ ·d ⁻¹	3.5 ± 0.4
Protein, g·kg ⁻¹ ·d ⁻¹	1.3 ± 0.3
Fat, g·kg ⁻¹ ·d ⁻¹	1.6 ± 0.4

Values are means ± SD, n=9. \dot{W}_{\max} , maximum cycle wattage; $\dot{V}O_{2\max}$, maximum oxygen consumption.

^a Body fat was measured using a Bod Pod® (COSMED).

^b Maximum wattage was measured using a ramp incremental test on a Velotron bicycle (Racer Mate, Seattle, WA).

^c Maximum oxygen consumption was measured using breath-by-breath gas analysis during the incremental cycle max test (Vmax Legacy, SensorMedics, Yorba Linda, CA).

^d Energy intake and macronutrient breakdown were based on a 2-day dietary record analysis.

General Study Design

A double blind, randomized, crossover research design was implemented involving two preliminary and two experimental sessions. The preliminary sessions were conducted before any experimental sessions began to collect baseline data as well as to acclimate participants to all the procedures implemented during the experimental sessions. These preliminary sessions were separated by at least two d and the second session was completed at least five d before starting the experiment. There were two experimental sessions, separated by at least one wk and conducted at the same time of the d. These experimental sessions were rotated systematically to prevent order effects. Further, to minimize food intake differences across treatments, participants recorded their entire food/drink intake for the d preceding and the d of the first experimental session and replicated this intake for their second trial. Experimental sessions were comprised of a glycogen lowering exercise protocol followed by a 4h recovery period where participants received either a KME drink (TdeltaS Ltd, Thame Oxfordshire, United Kingdom) + CHO (Dextrose monohydrate) or isoenergetic CHO alone (PLAC - details below).

Subsequently, participants performed a 20 km (TT_{20km}) and 5 km (TT_{5km}) best effort time trials to assess the impact of this recovery nutrition on performance (Figure 4.1).

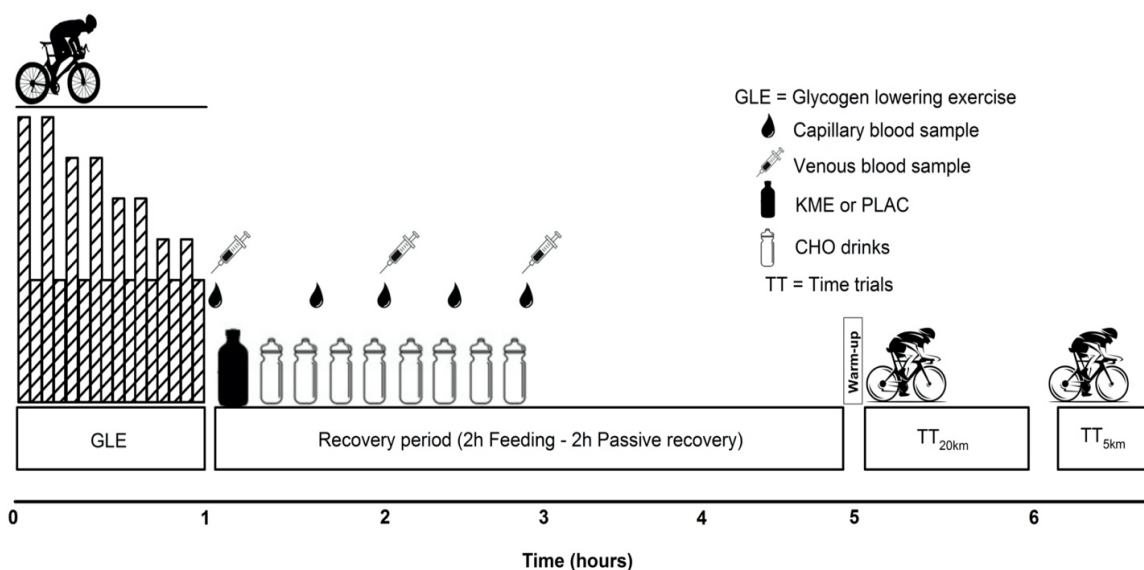


Figure 4.1. Overview of study protocol

Preliminary Sessions

Participants visited the laboratory on two separate occasions prior to any experiments for familiarization sessions. During the initial visit, body composition (Bod Pod®, COSMED, Concord, CA), and an incremental cycle exercise test to volitional fatigue were conducted to determine percent body fat as well as peak cycle power output (\dot{W}_{max}) and $\dot{V}O_{2max}$, respectively. Briefly, body density was measured using the BodPod® and body composition was estimated from body density via the Siri (Siri, 1961) equation. Moreover, $\dot{V}O_{2max}$ and \dot{W}_{max} were determined with an incremental ramp protocol on a Velotron cycle ergometer (Racer Mate, Seattle, WA). Briefly, participants engaged in a 2-min warm up at $100\dot{W}$ followed by an increase of $1\dot{W}$ every 3 sec until volitional fatigue. The greatest wattage was recorded (\dot{W}_{max}). Expired gases were collected via a breath-by-breath collection system (Sensormedics Vmax 29, Yorba Linda, CA), previously calibrated according to manufacturer's guidelines using known gas volumes and composition. The greatest value achieved over a 20-sec collection period was considered max whenever a plateau in $\dot{V}O_2$ occurred (<50% of the expected

increase in oxygen uptake for the increased workload) or when two of the following three criterion measures were attained (± 10 bpm of age predicted maximum HR, $RER > 1.15$ [$RER = \text{volume of CO}_2 \text{ produced} / \text{volume of O}_2 \text{ consumed}$] or volitional fatigue). Heart rate (HR) was monitored throughout the test (Polar RST200TM, Polar Electro Inc., Lachine, Quebec).

At least 48 h following the first visit, participants visited the lab a second time to familiarize themselves with the $TT_{20\text{km}}$ and $TT_{5\text{km}}$ to be used during the experimental sessions. This was done to minimize potential learning effects.

Experimental Sessions

Participants reported to the laboratory at 0800h following a 12h overnight fast, with limited activity (drive/use of the elevator to get to the laboratory) and having abstained from strenuous exercise, caffeine, or alcohol consumption for 24 h. Upon arrival, and after verbal confirmation of a fasted state, participants performed a glycogen lowering exercise bout on a Velotron cycle ergometer. Briefly, the protocol began with a 10-min warm-up at a workload of 50% \dot{W}_{max} . Thereafter, participants engaged in an intermittent exercise protocol that involved cycling in 1-min intervals alternating between workloads of 90% (high intensity effort) and 50% (recovery) of \dot{W}_{max} , respectively. When participants fatigued (inability to maintain cadence at 60 pedal rev/min during the high intensity effort) the greater workload was dropped by 10% of \dot{W}_{max} . Subsequent reductions in workload to 80%, 70%, etc. were done progressively until participants completed 60% of \dot{W}_{max} as the greater workload or could not maintain cadence at 60 rev/min. The recovery workload was 50% \dot{W}_{max} throughout. The duration of the protocol was 56 ± 15.6 min. Water during the glycogen lowering exercise was provided ad libitum and the amount was recorded (640.4 ± 237.2 ml) and replicated in the subsequent experimental trial.

Following the glycogen lowering exercise, CHO recovery drinks and the corresponding treatment were provided. The KME supplement contained 25g of ketone esters, providing a dose of $\sim 0.35 \text{ g} \cdot \text{kg BM}^{-1}$. Blood samples were collected for the first 2 h of a 4-h recovery period. Specifically, capillary blood samples to measure glucose and beta-

hydroxybutyrate (β HB) (FreeStyle Precision Neo®, Abbott Diabetes Care Limited), were obtained immediately after the glycogen lowering exercise and every 30 min during the first 2 h of recovery using a fingerstick method (Figure 4.1). Venous blood samples (~5 ml) to measure serum insulin concentration were collected from an antecubital vein immediately after the glycogen lowering exercise and each h for the first 2h of recovery, using a 21-gauge vacutainer needle and serum separator tubes containing a clot activator. The samples were allowed to clot at room temperature for 30-min and then centrifuged at 3000rpm for 10-min at 4°C (Allegra 21R; Beckman Coulter, Mississauga, Ontario, Canada). Serum supernatants were collected and stored at -80°C until analysis.

As mentioned, the feeding protocol occurred during the first 2h of the 4h recovery period following the glycogen lowering exercise protocol. Specifically, participants were provided with either KME or an isoenergetic appearance-matched placebo after the initial blood sample. Thereafter, recovery drinks containing CHO were supplied every 15 min for 2h of the 4h recovery period at a rate of $1.5 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ (10-12% solution). Participants remained in the lab for the following 2h (passive recovery) before engaging in both 20 and 5 km best effort time trials (TT_{20km} and TT_{5km}) (Figure 4.1).

Time Trials (TT_{20km} and TT_{5km})

After the recovery period ended (4 h following the glycogen lowering exercise), participants performed a 10-min warm-up cycling at 100 W on a Velotron cycle ergometer and then completed a best effort TT_{20km}. Then, after a 5 min recovery period the best effort TT_{5km} was completed. During both TT, participants received only distance travelled feedback. This approach was implemented to minimize pacing strategies. Average power output and time to complete total distance were recorded. Water was available ad libitum throughout the time trial and the intake recorded (750.5 ± 502.3 ml) so it could be reproduced during the subsequent trial.

4.4 Statistical Analysis

Statistical analyses were performed using SigmaPlot for Windows (Version 12.5, SYSTAT, San Jose, CA). Blood metabolites concentrations were analyzed using two-

way (condition by time) repeated-measures ANOVA. Post hoc Tukey's Honest Significant Difference (HSD) testing was used, where necessary. All variables from the TT_{20km} and TT_{5km} were analyzed using paired t tests. Significance was set at $p \leq 0.05$. Data are presented as means \pm SD.

4.5 Results

Blood Ketones (beta-hydroxybutyrate - β HB)

There was a main effect of time ($p < 0.001$), a main effect of treatment ($p < 0.001$), as well as a significant interaction ($p < 0.001$) for blood beta-hydroxybutyrate (β HB) concentration. Pairwise comparisons indicated that blood β HB was significantly greater ($p < 0.001$) for KME + CHO group at 30 ($p < 0.001$), 60 ($p < 0.001$), 90 ($p < 0.001$), and 120 ($p < 0.001$) min post treatment ingestion, compared to PLAC + CHO group (Figure 4.2).

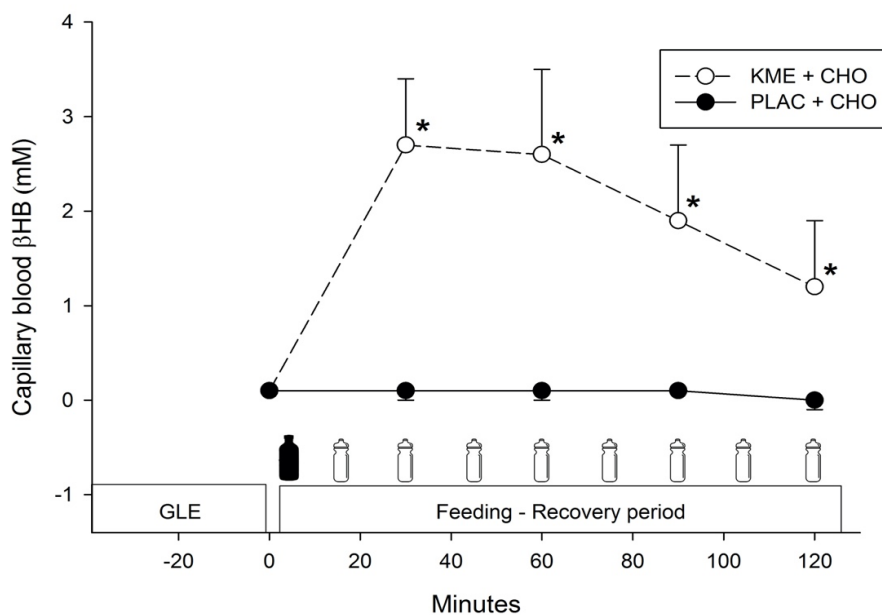




Figure 4.2. Capillary blood β HB concentration. Values are means \pm SD. GLE = Glycogen lowering exercise; KME + CHO = ketone monoester + carbohydrates; PLAC + CHO = iso-energetic placebo + carbohydrates. *Significantly different versus PLAC + CHO.  = treatment ingestion (KME or PLAC).  = CHO drink. β HB = beta-hydroxybutyrate.

Blood Glucose

A significant time x treatment interaction ($p=0.03$) for blood glucose concentration was seen (Figure 4.3). Pairwise comparisons showed that blood glucose was significantly lower at 30-min (5.8 ± 0.6 vs 7.1 ± 0.8 mM, $p=0.003$) and 60-min (6.1 ± 1.2 vs 7.2 ± 2.3 mM, $p=0.01$) for KME + CHO compared to PLAC + CHO. In addition, there was a main effect of time ($p=0.001$) and a main effect of treatment ($p=0.05$).

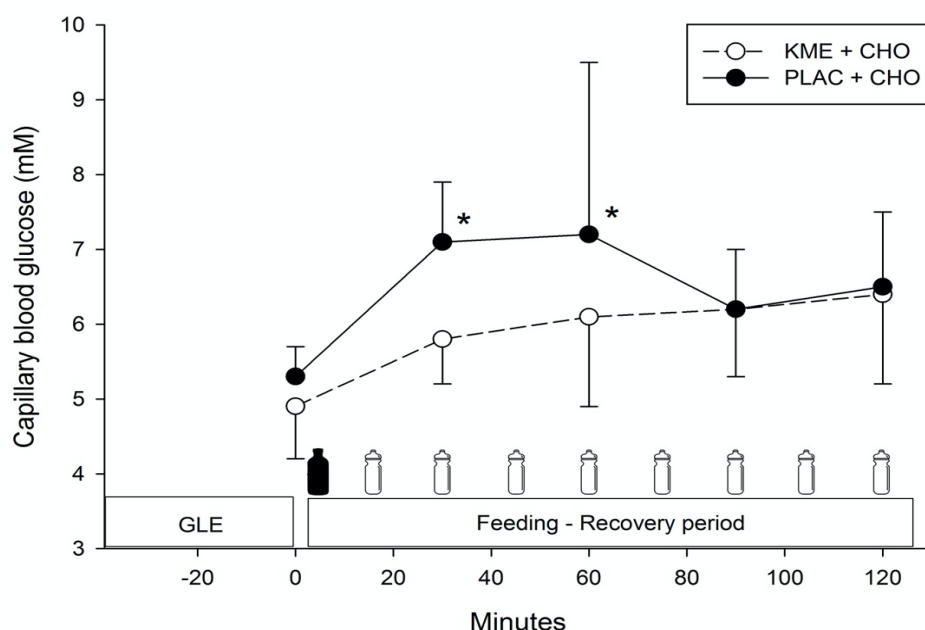




Figure 4.3. Blood Glucose concentration. Values are means \pm SD. GLE = Glycogen lowering exercise; KME + CHO = ketone monoester + carbohydrates; PLAC + CHO = iso-energetic placebo + carbohydrates. *Significantly different versus PLAC + CHO.  = treatment ingestion (KME or PLAC).  = CHO drink.

Measures of Exercise Performance

Time Trials (TT_{20km} and TT_{5km})

There were no significant differences in time to complete the best effort TT_{20km} (38.4 ± 2.25 min vs 38.9 ± 1.56 min; $p=0.420$) (Figure 4.4A) nor the best effort TT_{5km}

(9.5 ± 0.65 min vs 9.5 ± 0.88 min; $p = 0.884$) (Figure 4.4C). Likewise, there were no significant differences in average TT_{20km} power output (174 ± 21.9 W vs 169 ± 25.6 W; $p = 0.427$) (Figure 4.4B) nor average TT_{5km} power output (182 ± 31.8 W vs 186 ± 40.5 W; $p = 0.524$) (Figure 4.4D). between KME + CHO vs PLAC + CHO trials. Average HR during the TT_{20km} and TT_{5km} was 86% and 89% of age-estimated max HR, respectively.

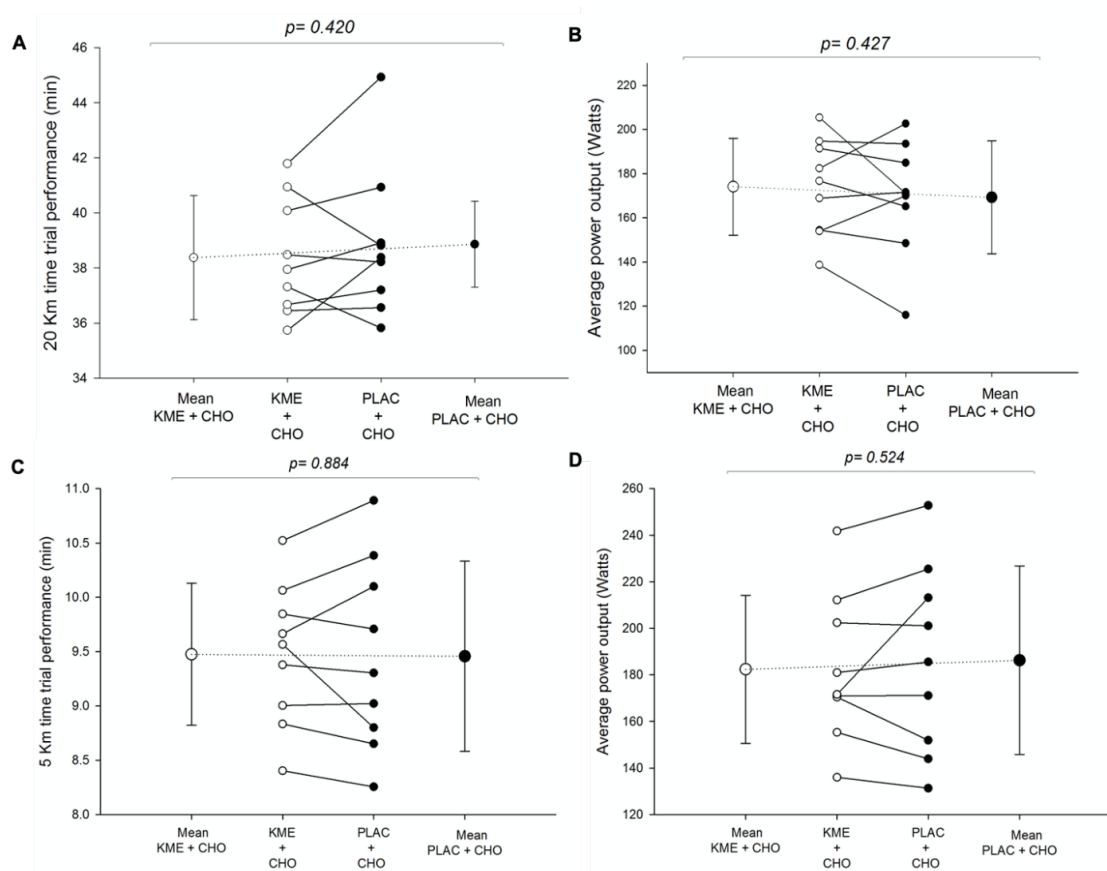


Figure 4.4. Group means and individual data for A) time to complete and B) Average PO during TT_{20km} as well as C) time to complete and D) Average PO during TT_{5km}. Values are means \pm SD. KME + CHO = ketone monoester + carbohydrates; PLAC + CHO = iso-energetic placebo + carbohydrates.

4.6 Discussion

The aim of the present study was to assess the efficacy of acute glucose-ketone ester supplementation on exercise performance after a 4-h recovery period following glycogen lowering exercise, i.e., whether exercise recovery KME + CHO supplementation vs PLAC + CHO might enhance time trial performance by improving glycogen resynthesis

following glycogen lowering exercise. Our results indicate that KME + CHO displayed greater β HB blood concentration throughout the recovery period and lower blood glucose concentration during the first h of the recovery period, compared to the PLAC + CHO trial. However, there were no significant differences in any of the exercise performance parameters assessed during the TT_{20km} and TT_{5km} completed four h after glycogen lowering exercise. These data suggest that glycogen resynthesis during our 4 h recovery period was similar between treatments.

These data add to our understanding of KME supplementation on performance because only a few studies have evaluated the role of exogenous ketone supplementation during post-exercise recovery on muscle glycogen resynthesis in humans (Holdsworth et al., 2017; Vandoorne et al., 2017) and those results are conflicting. For instance, Holdsworth et al. (2017) found that muscle glycogen replenishment was superior (50% greater content) when combining KME supplementation and glucose compared to glucose alone, while Vandoorne et al. (2017) found no differences in muscle glycogen replenishment after five h of recovery when using a CHO-PRO mix drink with and without KME following glycogen depleting exercise. Although muscle glycogen content was not measured in the present study, we did not observe any differences in exercise performance after the recovery period (Figure 4.4), which suggests that both experimental treatments resulted in similar rates of glycogen resynthesis. However, it is possible that the TT_{20km} and TT_{5km} completed in this study were not long enough to challenge glycogen stores, i.e., under both conditions, glycogen stores were sufficient, even if differences existed. Consequently, a longer time trial and/or alternatively the use effort independent measures such as muscle glycogen concentration might be needed to reveal differences, if any.

Several mechanisms have been proposed to upregulate glycogen resynthesis after exercise when using ketone supplementation. First, in both animal and human studies β HB can activate proteins in the insulin-signaling cascade while downregulating AMPK (Vandoorne et al., 2017; Takahashi et al., 2019). This results in non-AMPK-mediated translocation of GLUT4 transporters and a downregulation of both glycogenolysis and glycolysis, which could facilitate glucose uptake by the muscle cell as well as directing

more muscle glucose toward glycogenesis (Takahashi et al., 2019). Second, elevated (> 4mM) blood β HB concentrations may lead to hyperinsulinemia especially if combined with high glucose availability, and this could in turn, facilitate glycogen resynthesis. For instance, Takahashi et al. (2019) demonstrated greater increases in glycogen content in mice muscle after 120 min when the β HB concentration was set at 4mM, compared to 2mM and 1mM. In humans, Holdsworth et al. (2017) found increases in muscle glycogen content after a single dose of 573 mg·kg⁻¹ KME (peak blood β HB concentration of 5.3mM), compared to a glucose alone trial. In contrast, Vandoorne et al. (2017) found no difference in muscle glycogen content when providing an initial dose of 500 mg·kg⁻¹ KME and four subsequent doses of 250 mg·kg⁻¹ KME at 1-h intervals (blood β HB concentration of ~2.9mM after the first h of recovery and a peak concentration of 4.3mM at four h of recovery). In our study, we provided participants with a single dose of ~350 mg·kg⁻¹ KME and peak β HB concentration was only 2.7mM (Figure 4.2), something that might have contributed to the lack of difference seen in our time trials. Alternatively, the discrepancy in muscle glycogen content reported in these human studies could be influenced by glucose availability because Biden and Taylor (1983) found that β HB does not stimulate insulin release in the absence of glucose but can promote insulin secretion in the presence of 5mM of glucose, at least in isolated rat pancreatic islets. These observations suggest that insulin release from beta cells in response to β HB requires a concurrent high-normal blood glucose. For example, using a hyperglycemic clamp in humans to maintain glucose concentrations at ~10mM throughout the experiment, Holdsworth et al. (2017) found a 50% increase in muscle glycogen content in a KME trial after 2 h of recovery. This was associated with a twofold greater insulin concentration by the end of the clamp and a 32% greater whole-body glucose uptake with KME. In contrast, Vandoorne et al. (2017) did not observe differences in muscle glycogen content nor blood insulin with KME supplementation even with blood glucose concentration at ~6.5mM throughout a 5-h recovery period. In the present study, CHO was provided at 15-min intervals to ensure a constant blood glucose response with KME (~6.1mM glucose throughout the 2-h feeding period). Unfortunately, our blood insulin data are not yet available due to COVID-19 restrictions but, given the exercise performance results obtained, as well as β HB and glucose concentrations during the feeding period, we can

speculate that insulin concentrations were similar between the two trials. These data suggest that glucose bioavailability needs to be abundant for β HB to stimulate an insulinotropic response. Moreover, glucose transport across the GI tract is a key factor in absorption and the capacity of the glucose transporter (sodium-dependent glucose transporter 1), requires ~ 1.3 to $1.7 \text{ g CHO} \cdot \text{min}^{-1}$ (Duchman et al., 1997). Importantly, it has been shown that these transporters can be upregulated with a chronic high CHO diet, i.e., $\sim 8.5 \text{ g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ (Cox et al., 2010; Jeukendrup, 2017). In our study, participants consumed only modest amounts ($3.5 \text{ g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) of CHO (Table 4.1) and, of course, this could have limited glycogen synthesis during the feeding/recovery period of this study. Clearly, more research is warranted to elucidate the possible benefits of KME supplementation in recovery and future investigations should ensure both high β HB concentration and high glucose availability.

Interestingly, in our study the KME + CHO trial displayed lower glucose concentrations during the first h of recovery. This observation is in agreement with the glucose response seen by Vandoorne et al. (2017) where glucose was also lower at 60 and 90 min of recovery. Moreover, this apparent hypoglycaemic action of β HB has also been reported during exercise after KME ingestion (Cox et al., 2016; Evans and Egan, 2018; Dearlove et al., 2019) as well as during β HB infusion studies in humans where the blood β HB concentration was set $\sim 2\text{mM}$ (Miles et al., 1981; Mikkelsen et al., 2015). Often these differences in circulating glucose are explained partly by hyperinsulinemia, however, this response can still occur even in the absence of high insulin, as observed in the Vandoorne et al. (2017) study. Consequently, this hypoglycemic response might be explained by a decrease in hepatic glucose production (gluconeogenesis) and hepatic output because β HB infusion studies have shown a 20% decrease in endogenous glucose production in healthy males (Miles et al., 1981; Mikkelsen et al., 2015). Of course, this response can have important therapeutic implications. For instance, Soto-Mota et al. (2021) found recently that 3 times a d KME supplementation for 4 wk was safe, well tolerated, and resulted in improved glycaemic control in patients with type 2 diabetes.

In summary, KME + CHO supplementation resulted in a reduced blood glucose concentration during the first h of recovery period, as well as greater β HB blood

concentration throughout the recovery period, when compared to the PLAC + CHO trial. However, no significant differences in any of the exercise performance parameters measured during the TT_{20km} and TT_{5km} were observed. These data suggest that the metabolic changes induced by the CHO and KME provided in this study is perhaps not sufficient to elicit any potential benefits in subsequent exercise performance trials. In the future, it is important to consider strategies to improve key factors such as feeding protocols, CHO and β HB doses that ensure adequate glucose and ketone bioavailability, as well as longer performance trials and/or effort independent assessment measures.

4.7 Acknowledgements

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The investigators declare no conflict of interest relevant to the content of this article.

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Chapter 5

- 5 Exogenous ketone ester supplementation may improve some aspects of cognitive function during a simulated soccer match after induced mental fatigue

5.1 Abstract

Exogenous ketone supplementation has been proposed as a way to enhance cognition during exercise. However, whether these benefits are seen during intermittent exercise after induced mental fatigue is unknown. We assessed whether a ketone monoester supplement (KME) was superior to a non-caloric placebo (PLAC) in maintaining cognitive performance during a 45-min simulated soccer match after induced mental fatigue. Nine recreationally active men (174.3 ± 4.2 cm, 76.6 ± 7.4 kg, 30 ± 3 y, 14.2 ± 5.5 % body fat, $\dot{V}O_{2\max} = 55 \pm 5$ ml \cdot kg $BM^{-1} \cdot \min^{-1}$; mean \pm SD) completed a 45-min simulated soccer match consuming either KME (25g of β HB) or PLAC, after a 40-min mental fatiguing task. The soccer match consisted of 3 blocks of various intensity exercise. Cognitive function (Stroop test and Choice Reaction Task [CRT]) and blood metabolites were measured during exercise. KME displayed lower concentrations of both blood glucose (block 2: 4.6 vs 5.2 mM, $p = 0.02$; block 3: 4.7 vs 5.3 mM, $p = 0.01$) and blood lactate (block 1: 4.7 vs 5.4 mM, $p = 0.05$; block 2: 4.9 vs 5.9 mM, $p = 0.01$) during exercise, compared to PLAC, perhaps indicating a CHO sparing effect. Although both treatments resulted in a decrease in %correct answers for the CRT during exercise relative to baseline, KME displayed a smaller decrease compared to PLAC (1.3 vs 3.4 %, $p = 0.02$). No other differences in cognitive function were seen. These data suggest that KME supplementation can improve some aspects of cognitive function during high intensity intermittent exercise. However, more study is warranted to assess fully the potential cognitive/physical benefits of KME for athletes.

Keywords: exogenous ketones, ketosis, soccer, intermittent exercise.

5.2 Introduction

In team sports of moderate to long duration such as soccer, a high cognitive and physical demand is imposed as athletes are required to make numerous split-second decisions that can influence the outcome of the competition while performing high intensity actions such as sprinting, jumping, tackling, etc (Smith et al., 2018). Often, the onset of fatigue occurs towards the end of a match and is associated with depletion of glycogen stores (Krustup et al., 2006; Cermak and Van Loon, 2013). During this time, athletes may experience physical fatigue evidenced by a decrement in work output (Bangsbo et al., 1991; Rampinini et al., 2007), as well as increased mental fatigue exhibited as poor decision making or deterioration of skills (Baker et al., 2014; Smith et al 2016; Quinones and Lemon, 2019). This is important as the abovementioned factors are critical determinants of game play. In light of this, nutrient supplementation strategies to avoid or delay the onset of fatigue are of considerable interest to many.

Ketone bodies (KB) are lipid derived metabolites produced in the liver during periods of low CHO availability such as starvation, prolonged exercise, uncontrolled diabetes, or dietary manipulations (Laffel, 1999; Puchalska and Crawford, 2017). Aside from glucose, KB are the only energy substrate that can cross the blood brain barrier through monocarboxylate-mediated transport (Evans et al., 2017). This allows them to act as an alternative fuel for the brain while reducing glucose reliance, something that might help enhance cognitive performance (Evans et al., 2017; Valenzuela et al., 2021). In addition, KB can increase the expression of the protein Brain Derived Neurotropic Factor (BDNF), an important molecule for brain plasticity/regulation of cognitive function (Marosi et al., 2016; Sleiman et al., 2016). Recently, exogenous ketone supplements have emerged as an alternative to a ketogenic diet in order to induce hyperketonemia acutely (Cox et al., 2016; Stubbs et al., 2017) and are available commercially in two forms, ketone salts (KS) and ketone esters (KE). These supplements have been shown to help spare CHO (Cox et al., 2016) and to provide an alternative fuel for the brain (Cunnane et al., 2011). However, only a few studies have evaluated the efficacy of exogenous ketone supplements on cognitive function during exercise with some studies showing positive effects (Evans and Egan, 2018; Prins et al., 2020) while others found no change

(Waldman et al., 2018; Evans et al., 2019; Waldman et al., 2020) when compared to CHO or a non-caloric placebo. This discrepancy has been attributed, in part, to the use of KE (Evans and Egan, 2018; Evans et al., 2019) vs KS (Waldman et al., 2018; Prins et al., 2020; Waldman et al., 2020), as the former can induce greater circulating KB (Stubbs et al., 2017), as well as other procedural differences (Margolis and O'fallon, 2020). For example, most studies have measured cognition before and after exercise as opposed to during exercise. The latter would have greater ecological validity as cognitive changes are likely rapid in recovery from exercise. Further, it has been shown that cognitive function displays greater impairments when the tests and physical exercise are performed concurrently (Lambourne and Tomporowski, 2010), possibly due to a dual-task interference. To our knowledge, only one ketone supplementation study has measured cognitive function during steady state exercise, it used KS, a supplement that only induces slight to modest increases in circulating KB (Waldman et al., 2020), and it found no changes in cognition when compared to a non-caloric placebo. Whether the effects of exogenous KE are similar during high intensity, intermittent exercise, like that performed in a soccer match, is unknown. Therefore, the purpose of the present study was to assess whether ketone monoester (KME) supplementation can enhance/maintain cognitive performance during a 45-min simulated soccer match after induced mental fatigue. We hypothesized that KME supplementation would enhance/maintain cognitive function during the simulated soccer match.

5.3 Methods

Participants

A total of 14 healthy, male soccer players were recruited but five participants did not complete the entire experimental protocol due to laboratory closures and restrictions during the COVID-19 pandemic. These five participants were excluded from data analysis and the remaining nine were included in the study (height = 174.3 ± 4.2 cm, body mass = 76.6 ± 7.4 kg, age = 30 ± 3 y, body fat = $14.2 \pm 5.5\%$, and $\dot{V}O_2$ max = 55 ± 5 ml · kg BM⁻¹ · min⁻¹). All were experienced, recreational soccer players from a range of outfield playing positions who had been involved in soccer training/play at least 2 d per

week for 6 mo prior to the study. Each participant completed a physical activity readiness questionnaire (PAR-Q+ - Thomas et al., 1992) and a health information form to minimize any contraindications to exercise. All potential risks were explained fully prior to any testing, and the participants provided written, informed consent of the study protocol approved previously by the Western University's Office of Research Ethics.

General Study Design

A double blind, randomized, crossover research design was implemented involving two preliminary and two experimental sessions that comprised two treatments, KME (TdeltaS Ltd, Thame Oxfordshire, United Kingdom) or a non-caloric appearance-matched placebo (PLAC) (Crystal Light, Kraft Ontario, Canada). The preliminary sessions were conducted before any experimental sessions began and were used to collect baseline data needed to design the running protocol to be implemented in the experimental sessions, as well as to acclimate participants to all the cognitive function tests and measurements utilized during the experimental sessions. These preliminary sessions were separated by at least two d and the second session was completed at least five d before starting the experiment. The two experimental sessions were separated by at least one wk, were conducted at the same time of the day, and were rotated systematically to prevent order effects. Further, to minimize food intake differences across treatments, participants recorded their entire food/drink intake for the d preceding and the d of the first experimental session and replicated this intake for their second trial. Experimental sessions were comprised of a 40-min mental fatiguing task (MFT), followed by a 45-min simulated soccer match (Figure 5.1). Participants performed both sessions while receiving either 25g (329 ± 34 mg·kg⁻¹) of ketones (KME) or placebo (PLAC). Both drinks were 125ml in volume and were provided in plastic cups. Cognitive function (Stroop test and Choice Reaction Test (CRT) – see details below), blood metabolites, rates of perceived exertion, mental fatigue and mental effort were measured before, during and after exercise (Figure 5.1).

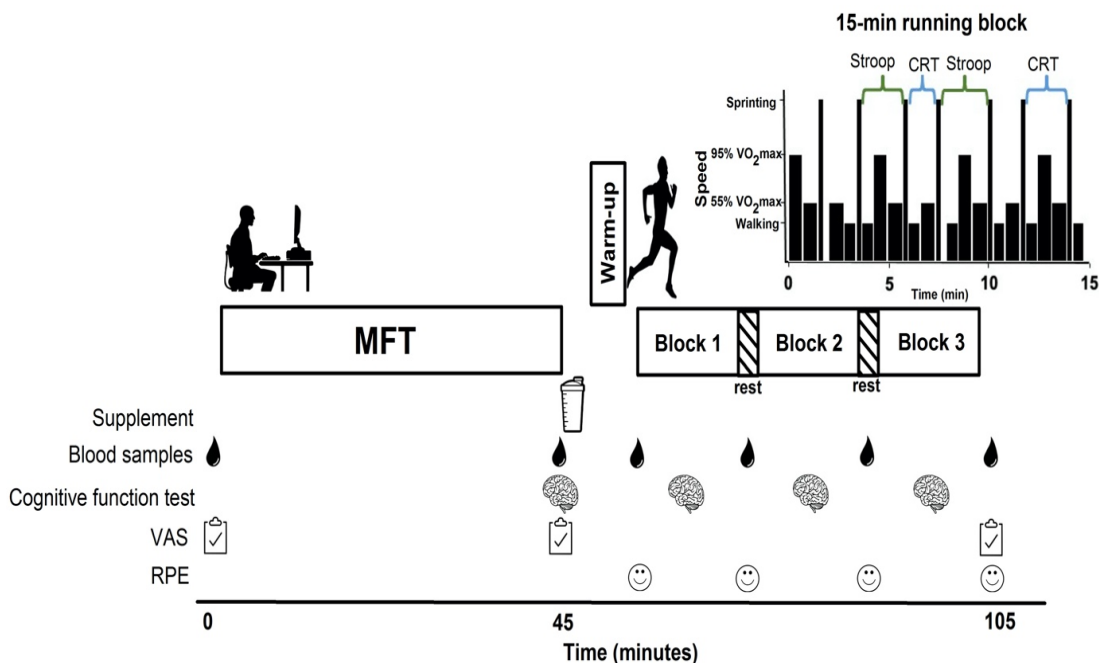


Figure 5.1. Overview of study protocol. MFT = mental fatiguing task. CRT = choice reaction test.

Preliminary Sessions

As mentioned, participants visited the laboratory on two separate occasions prior to any experiments for familiarization sessions. During the initial visit, body composition (Bod Pod®, COSMED, Concord, CA) $\dot{V}O_{2\max}$ (running treadmill test), and maximal sprinting speed (treadmill test) were determined. Briefly, body density was measured using the BodPod® and body composition was estimated from the measured body density with the Siri (Siri, 1961) equation. $\dot{V}O_{2\max}$ was determined via an incremental speed protocol on a treadmill (Desmo Pro, Woodway®, Wisconsin, USA). Participants started running at $9.7 \text{ km} \cdot \text{h}^{-1}$ with the treadmill set at a constant grade of 1%. Subsequently, increases in speed of $0.16 \text{ km} \cdot \text{h}^{-1}$ every 12 sec were applied until volitional fatigue. Heart rate (HR) was monitored throughout the test (Polar RST200™, Polar Electro Inc., Lachine, Quebec) and expired gases were analyzed via a breath-by-breath collection system (SensorMedics Vmax 29, Yorba Linda, CA), calibrated according to manufacturer's guidelines using known gases volumes and composition. The greatest value achieved over a 20-s collection period was considered max whenever a plateau in $\dot{V}O_2$ occurred

(<50% of the expected increase in oxygen uptake for the increased workload) or when two of the following three criterion measures were attained (± 10 bpm of age predicted maximum HR, $RER > 1.15$ [$RER = \text{volume of CO}_2 \text{ produced} / \text{volume of O}_2 \text{ consumed}$] or volitional fatigue). Furthermore, peak sprint speed was determined using a 10-s all-out effort on a non-motorized treadmill (Desmo Pro, Woodway®, Wisconsin, USA). Briefly, at a 2% incline with a disengaged belt, each participant propelled the belt themselves and the greatest speed shown on the treadmill display board during the test was recorded as peak speed.

At least 48 h following the first visit, participants returned the lab a second time to familiarize themselves with both the cognitive tests and the high intensity intermittent running protocol used to simulate the second half of the soccer match. This was done to eliminate potential learning effects during the experimental sessions.

Experimental Sessions

Participants reported to the laboratory 4 h postprandial with limited activity (drive/use of the elevator to get to the laboratory), having abstained from strenuous exercise, caffeine, or alcohol consumption for 24 h. This dietary control was utilized not only to standardize conditions between treatments but also to ensure that participants were able to exercise in a fed state, as this is how people engage in competition typically. Upon arrival, capillary blood samples for baseline glucose, beta-hydroxybutyrate (βHB) (FreeStyle Precision Neo®, Abbott Diabetes Care Limited) and lactate (Lactate Scout+, EKF Diagnostics) concentration were obtained using the fingerstick method. Subsequently, baseline ratings of mental fatigue were collected using a 100-mm visual analog scale (VAS) and then, the mental fatiguing task (MFT) commenced. For the MFT, a longer version of the Stroop task (40 min) was implemented as this approach has been shown to induce a state of mental fatigue previously (Smith et al., 2016). Following the MFT, pre-exercise cognitive function was assessed using both the Stroop test and CRT. Ratings of mental fatigue and mental effort were collected using a 100-mm VAS. Next, capillary blood samples to assess blood metabolites were collected and the corresponding treatment (KME or PLAC) was given. Five min after drink ingestion, a 5-min warm-up was completed,

followed by a 45-min simulated soccer match (Figure 5.1). The simulated soccer match was comprised of 3 x 15-min intermittent running blocks interspersed with 3-min of passive recovery. The intensities and times used during each block were full stopping (15 s), walking (35 s), jogging (46 s at 55% $\dot{V}O_2\text{max}$), cruising (42 s at 95% $\dot{V}O_2\text{max}$) and sprinting (17 s at 90% of peak sprint speed). This protocol has been used in our laboratory previously (Quinones and Lemon, 2019) and has been shown to replicate the physiological demands of soccer play (Drust et al., 2000). Heart rate was monitored throughout using a Polar RST200TM (Polar Electro Inc., Lachine, Quebec). Capillary blood samples to measure glucose, βHB and lactate, as well as ratings of perceived exertion (RPE – 6-20 Borg scale) were taken before commencing the simulated match and during each 3-min passive recovery throughout.

Cognitive Function Tests

Two cognitive function tests were used, the Stroop test and Choice Reaction test (CRT). The Stroop test was used for the MFT (40-min) to induce mental fatigue, and together with the CRT during the exercise bout to assess cognition. A computer screen was set up in front of the treadmill and a box with four buttons to respond to the stimuli presented on the screen was placed in front of the participant.

The Stroop Test

The Stroop test evaluates selective attention and ability to inhibit a learned skill consciously (Stroop, 1935; MacLeod, 1991). For this test, words of four colours (red, blue, yellow, and green) were displayed in the centre of the computer screen written in either congruent (i.e., word ‘blue’ written in blue ink) or incongruent ink (i.e., word ‘blue’ written in green ink). Each test was comprised of 44 random trials (4 practice trials where on-screen feedback [correct / incorrect] was given + 20 congruent and 20 incongruent trials with no feedback). Using the 4-button box, participants responded by pushing the button that matched the color displayed on the computer screen, not the word. This test was performed twice within each exercise block for a total of 264 trials (24 practice trials, 120 congruent and 120 incongruent) during the soccer match

simulation. The Stroop effect was quantified as the difference in mean choice-reaction time between congruent and incongruent trials. The task was scored as the difference in mean choice reaction time (ms), accuracy (%) and Stroop effect (ms) from baseline (pre-exercise) relative to exercise.

Choice Reaction Test (CRT)

For this test, the computer screen was divided into four equal-area quadrants and each area was assigned a specific response key in the 4-button box located in front of the participants. Stimuli sporadically flashed on the screen and participants were instructed to respond as quickly as possible by pushing the button on the box that corresponded to the location of the stimuli on the screen. This test was performed twice within each exercise block, with each test comprised of 80 random trials, for a total of 480 trials during the soccer match simulation. The task was scored as the difference in mean choice reaction time (ms) and accuracy (%) from baseline (pre-exercise) relative to exercise.

5.4 Statistical Analysis

Statistical analyses were performed using SigmaPlot for Windows (Version 12.5, SYSTAT, San Jose, CA). Blood metabolites concentrations as well as ratings of perceived exertion, mental fatigue and mental effort were analyzed using two-way (condition by time) repeated-measures ANOVA. Post hoc Tukey's Honest Significant Difference (HSD) testing was used, where necessary. The Stroop test and CRT were analyzed using a paired t test. Significance was set at $p \leq 0.05$. Data are presented as means \pm SD.

5.5 Results

Blood Metabolites

There was a significant treatment \times time interaction ($p < 0.001$) for blood β HB (Figure 5.2A). Pairwise comparisons showed that blood β HB concentration was greater for KME after warm-up (0.97 ± 0.48 vs 0.1 ± 0.09 mM, $p < 0.001$) and during all 3 exercise blocks

- block 1 (1.48 ± 0.32 vs 0.09 ± 0.06 mM, $p < 0.001$), block 2 (1.63 ± 0.50 vs 0.12 ± 0.08 mM, $p < 0.001$) and block 3 (1.61 ± 0.60 vs 0.16 ± 0.08 mM, $p < 0.001$), compared with PLAC. In addition, there were main effects of both time ($p < 0.001$) and treatment ($p < 0.001$) for blood β HB concentration. As expected, blood β HB was greater at every time point after supplement ingestion, compared to both baseline and post-MFT.

There was a significant treatment \times time interaction ($p = 0.02$) for blood glucose (Figure 5.2B). Pairwise comparisons showed that blood glucose was greater for PLAC in block 2 (5.2 ± 0.6 vs 4.6 ± 0.6 mM, $p = 0.02$) and block 3 (5.3 ± 0.8 vs 4.7 ± 0.9 mM, $p = 0.01$), compared with KME. In addition, no main effect of treatment was found ($p = 0.58$) but there was a main effect of time ($p = 0.02$) for blood glucose concentration showing that blood glucose was lower post-MFT, compared to baseline.

A main effect of time ($p < 0.001$) was also detected for blood lactate (Figure 5.2C). As expected, lactate concentrations were greater during exercise relative to baseline and post-MFT. No treatment effect ($p = 0.47$) was observed but there was a treatment \times time interaction ($p = 0.02$). Pairwise post hoc comparisons indicated that blood lactate concentration was greater for PLAC for exercise block 1 (5.4 ± 1.8 vs 4.7 ± 1.4 mM, $p = 0.05$), and block 2 (5.9 ± 2.5 vs 4.9 ± 1.9 mM, $p = 0.01$), compared with KME.

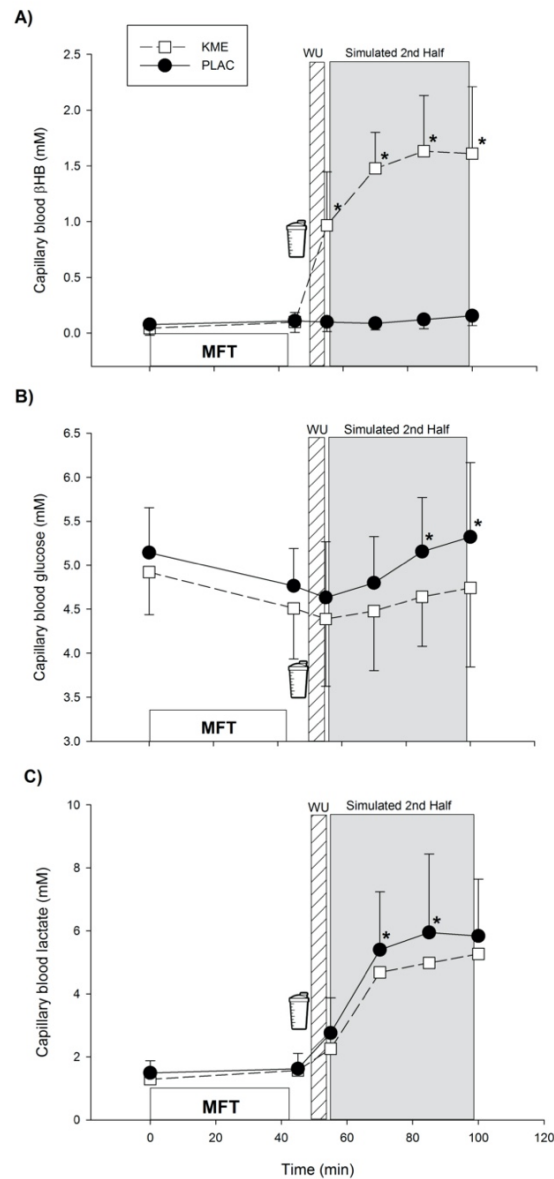


Figure 5.2. Effects of KME ingestion on blood metabolites during exercise after induced mental fatigue. A) blood β HB concentration B) blood glucose concentration C) blood lactate concentration. Values are means \pm SD. MFT = mental fatiguing task; WU = warm-up; KME = ketone monoester supplement; PLAC = non-caloric placebo. *Significantly different versus PLAC.

Visual Analog Scales

Subjective ratings of mental fatigue increased significantly in both trials after the mental fatiguing task ($p = 0.003$) and after exercise ($p < 0.001$), compared to baseline. However,

no differences were found between KME and PLAC at any time point ($p= 0.53$) (Figure 5.3A).

Subjective ratings of mental effort were calculated as the difference between pre- and post-exercise ratings. A paired t test showed no difference in mental effort under both KME and PLAC conditions ($p= 0.47$) (Figure 5.3B).

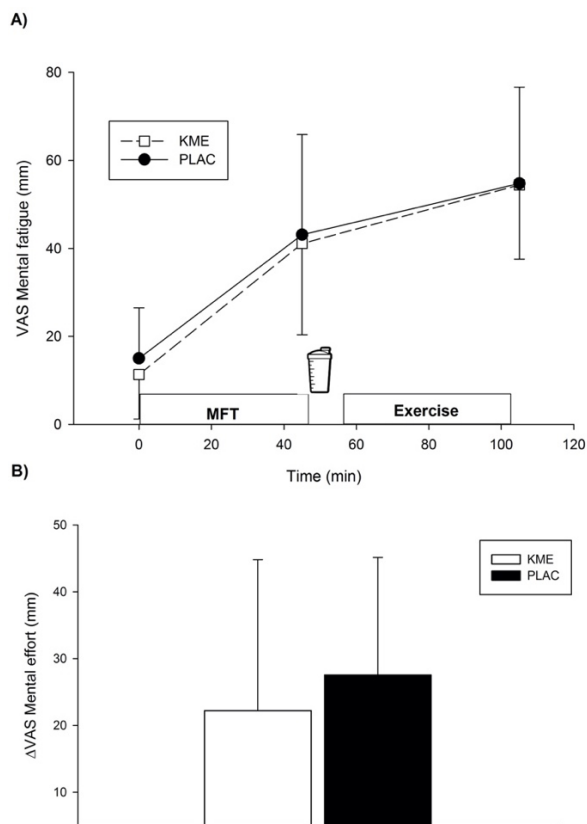


Figure 5.3. Effects of KME supplementation on subjective ratings of mental fatigue and mental effort A) Subjective ratings of mental fatigue B) difference in subjective ratings of mental effort, relative to pre-exercise (Post MFT). Values are means \pm SD. MFT = mental fatiguing task; KME = ketone monoester supplement; PLAC = non-caloric placebo. VAS = Visual Analogue Scale

Ratings of Perceived Exertion (RPE)

A main effect of time ($p < 0.001$) was detected for RPE (Table 1). As expected, average RPE for block 2 and block 3 were greater than both pre-exercise and block 1. No treatment effect ($p = 0.90$) nor treatment \times time interactions were observed ($p = 0.13$).

Table 5.1. Ratings of perceived exertion (RPE)

	KME	PLAC	Two-way RM ANOVA	
Pre-SSM	9.3 ± 1.0	9.9 ± 1.1	Time Effect	p < 0.001
Block 1	13.2 ± 1.0 ^a	12.8 ± 1.1 ^a	Treatment Effect	p = 0.90
Block 2	14.7 ± 1.4 ^b	14.8 ± 1.4 ^b	Interaction Effect	p = 0.13
Block 3	15.9 ± 1.8 ^b	15.8 ± 1.6 ^b		

Values are means ± SD. RPE data was analyzed using two-way repeated measures ANOVA. Post hoc Tukey's HSD testing was used to figure out differences between treatments. There were no significant differences between KME and PLAC (p= 0.13).

SSM = Simulated Soccer Match

^a indicates significant difference from Pre-SSM

^b indicates significant difference from Pre-SSM and Block 1

Cognitive Function

The Stroop test was used to assess cognition before and during exercise. The difference between all variables pre-exercise vs during exercise were calculated and compared using paired t testing. Results showed that there were no differences in reaction time for congruent (p= 0.78) or incongruent (p= 0.43) trials, as well as no differences in the percent of correct answers for congruent (p= 0.38) and incongruent (p= 0.16) trials. Lastly, there were no differences in Stroop effect (p= 0.44) when comparing KME to PLAC group (Table 5.2).

Moreover, a choice reaction test (CRT) was used as well to measure cognition before and during exercise. Similarly, the difference between all variables pre-exercise vs during exercise were calculated and compared using paired t tests. Results revealed that compared to PLAC, the KME group displayed a reduced decrease in the percent of correct answers during exercise (p= 0.02), while maintaining a similar increase in reaction time (p= 0.11), indicating a better maintenance of cognitive function with KME (Figure 5.4).

Table 5.2. Stroop test

Variable measured	Treatment	Δ Score	P-value
Congruent	KME	26.7 \pm 60.4	0.78
	PLAC	22.4 \pm 50.2	
Percent Correct (%)	KME	3.6 \pm 4.7	0.38
	PLAC	1.9 \pm 2.2	
Incongruent	KME	24.9 \pm 111.4	0.43
	PLAC	0.31 \pm 62.0	
Percent Correct (%)	KME	4.4 \pm 5.4	0.16
	PLAC	5.4 \pm 5.6	
Stroop effect	KME	-1.8 \pm 68.3	0.44
	PLAC	-22.1 \pm 71.6	

Values are means \pm SD. The difference in pre-exercise Stroop task (post MFT) and during exercise were calculated for all variables (Δ Score). Data was analyzed using a paired t test. There were no significant differences between KME and PLAC.

5.6 Discussion

The purpose of the present study was to assess the efficacy of acute KME supplementation on cognitive performance during a 45-min simulated soccer match after induced mental fatigue. Interestingly, our results revealed that relative to pre-exercise, the number of correct answers was better maintained with KME during exercise compared to PLAC, while reaction time remained similar in the CRT. In contrast, no differences were seen with the Stroop test when comparing both treatments. As expected, KME supplementation elevated blood β HB, lowered blood glucose the last 30 min of exercise, and lowered blood lactate during the first 30 min of exercise perhaps indicating a shift in exercise fuel use away from CHO.

Previous investigations evaluating the effects of ketone supplements on cognitive function using exercise have produced mixed results, with a few studies showing positive outcomes (Evans and Egan, 2018; Prins et al., 2020), and others showing no benefits (Waldman et al., 2018; Evans et al., 2019; Waldman et al., 2020). Part of the discrepancy has been attributed to factors such as the type of supplement used (KS vs KE) or differing methodology (Margolis and O'fallon, 2020). For example, KE have been shown to induce a greater ketonemia compared to KS (Stubbs et al., 2017). This is likely important

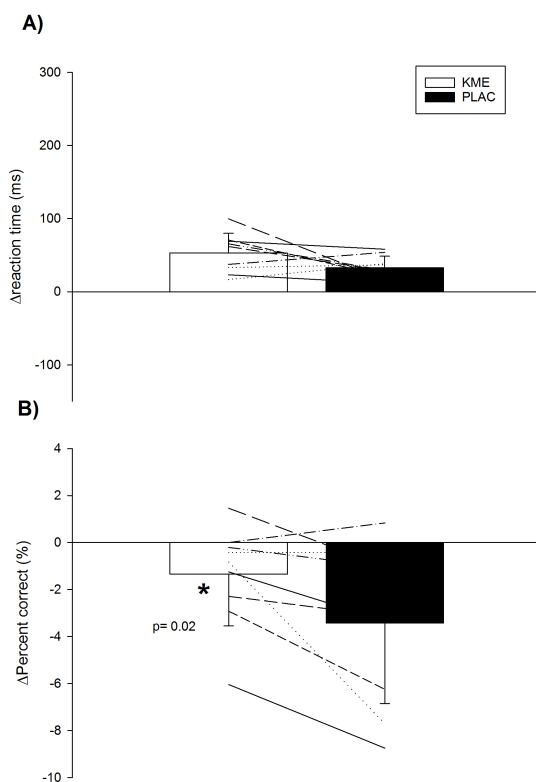


Figure 5.4. Effects of KME supplementation on cognitive function (CRT) during exercise after induced mental fatigue, compared to PLAC. A) Δ reaction time during exercise, relative to pre-exercise (Post MFT) B) Δ % correct answers during exercise, relative to pre-exercise (Post MFT). Values are means \pm SD. MFT = mental fatiguing task; KME = ketone monoester supplement; PLAC = non-caloric placebo.

because the magnitude of ketosis (in the range of ~ 1 to 3 mM) has been deemed essential to induce certain physiological changes (Evans et al., 2017). In the present study, participants ingested KME and reached a blood β HB concentration of ~ 1.6 mM. A previous study that also found cognitive function improvements provided KME at a greater dose ($750 \text{ mg} \cdot \text{kg}^{-1}$) and generated blood β HB concentrations of ~ 2.6 mM (Evans and Egan, 2018). In contrast, Walmand et al. (2018; 2020) provided KS and reported no benefit on cognitive function. In these studies, participants achieved a blood β HB concentration of ~ 0.5 mM (Walmand et al., 2018) and ~ 0.8 mM (Walmand et al., 2020). Furthermore, the difference in the mode of exercise (intermittent vs continuous) as well as the time when the cognitive tasks are administered may also affect the outcomes. For

instance, Evans and Egan (2018) demonstrated that supplementing with KME and CHO before and during a ~90-min intermittent exercise session reduced the number of incorrect responses during a cognitive test administered after exercise, when compared to CHO alone. However, the same authors found no benefits when these cognitive tasks were performed after a 10 km time trial (Evans et al., 2019). Perhaps exercise mode and duration contributed to these differing results. In the present study, participants performed only 45-min of intermittent exercise, although it was done in a state of pre-induced mental fatigue. Our findings are consistent with Evans and Egan (2018), since participants ingesting KME in our study were able to reduce the number of incorrect answers relative to baseline during the CRT, compared to PLAC. This is likely important for team sports because CRT has been shown to provide insight on individual decision-making under pressure (Ozdemir et al., 2010). Further, Evans and Egan (2018) administered their cognitive task before and after exercise, while in the present study it was done concurrently with exercise, something that enhances ecological validity and this timing difference may be important. For example, Lambourne and Tomporowski (2010) observed small improvements in cognitive function when tests were completed after exercise cessation whereas a slight impairment was seen when tests were done during exercise, something that would make any cognitive task more sensitive to detect a potential difference. However, this apparent benefit of KME supplementation was not detected with the Stroop test in the present study. A possible explanation for this could be that participants had accrued some learning during the mental fatiguing task, perhaps making the treatment test less sensitive to detect any differences. As mentioned, the Stroop test was used during the initial 40-min of the study protocol to induce a state of mental fatigue. Consistent with Smith et al. (2016) this approach was effective at increasing subjective ratings of mental fatigue (Figure 5.3A) but given the duration of the task, it could be that our participants were accustomed to the stimuli presented, making it less likely to find an effect during exercise, if any. Nonetheless, these results should be interpreted with caution and more study is needed to confirm the present findings.

Interestingly, metabolic responses appeared to differ in our study between the two treatments. As expected, KME ingestion elevated blood β HB during exercise vs PLAC, an effect that has been consistent across studies using ketone supplementation (Margolis

and O'fallon, 2020; Valenzuela et al., 2020; Valenzuela et al., 2021). Furthermore, KME lowered blood glucose in the last 2 blocks of exercise (block 2: 4.6 vs 5.2 mM, $p= 0.02$; block 3: 4.7 vs 5.3 mM, $p= 0.01$), and reduced blood lactate during the first 2 blocks of exercise (block 1: 4.7 vs 5.4 mM, $p= 0.05$; block 2: 4.9 vs 5.9 mM, $p= 0.01$). These metabolic responses are consistent with the majority of studies using KME supplementation during exercise (Cox et al., 2016; Evans and Egan, 2018; Dearlove et al., 2019), particularly for the one that measured cognitive function and found small improvements as well (Evans and Egan, 2018). Interestingly, one KME study examining cognitive function did not observe such metabolic responses nor any differences in cognition (Evans et al., 2019). Originally, it was argued that these metabolic responses are indicative of a potential CHO sparing effect, because the first study reporting these metabolic changes found improvements in physical performance following KME ingestion (Cox et al., 2016). However, subsequent investigations have suggested that high blood β HB concentrations also lowers pH and these perturbations to acid-base homeostasis could reduce the rate of glycolysis and thus lactate production (Dearlove et al., 2019). Of course, this could affect soccer performance adversely. However, it is important to note that these perturbations have been reported only when blood β HB concentrations is raised substantially. For example, Dearlove et al. (2019) found that power output did not differ at high exercise intensities despite a blood β HB concentration of ~ 3.7 mM. In our study, average blood β HB concentration was ~ 1.6 mM during exercise, so it is plausible that acidosis-induced detriments would be minimal. If so, the present data may be caused by a CHO sparing effect. Unfortunately, physical performance was not assessed directly in the present study so whether the aforementioned differences might enhance exercise performance must await further experimentation.

In conclusion, KME supplementation vs a non-caloric placebo resulted in fewer cognitive errors relative to baseline in the CRT, while no differences were observed in any other cognitive variable measured during intermittent exercise. Furthermore, KME supplementation elevated circulating β HB and produced lower concentrations of both glucose and lactate during exercise. These data suggest that KME supplementation has the potential to improve some aspects of cognitive function during sports characterized

by repeated, intense, intermittent exercise. However, more study is needed to assess fully the possible cognitive/physical benefits of KME for athletes.

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The investigators declare no conflict of interest relevant to the content of this article.

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Chapter 6

6

Discussion

6.1 General discussion

Exogenous ketone (EK) supplementation has emerged recently as a potential aid for exercise performance. Oral administration of EK in healthy adults leads to an acute state of ketosis or hyperketonemia that can last up to 3 h (Stubbs et al., 2017). Ketosis is defined as a plasma ketone concentration of $\sim 0.5\text{--}3.0$ mM (Gibson and Sainsbury, 2017). This transient elevation of ketone bodies (KB) promote important metabolic changes proposed to aid both exercise performance and post-exercise recovery (Valenzuela et al., 2021). For instance, EK have not only been shown to reduce muscle glycogen utilization while increasing fat oxidation during exercise (Cox et al., 2016) but also to improve some aspects of cognitive function during exercise (Evans and Egan, 2018; Prins et al., 2020). The latter could be important for success in many competitive sports because split-second decisions are required throughout games. Moreover, EK in combination with CHO have been shown to be better than CHO alone for post-exercise glycogen repletion (Holdsworth et al., 2017). However, not all EK studies have observed these potential ergogenic effects (Rodger et al., 2017; Leckey et al., 2017; O'Malley et al., 2017; Waldman et al., 2018; Evans et al., 2019; James and Greer, 2019; Poffe et al., 2020; Poffe et al., 2021). Consequently, it is difficult to determine the efficacy of these supplements on competitive sports performance. As mentioned previously, several factors such as type of EK supplement (esters vs salts), dose of supplement, exercise modality, as well as the exercise tests used, appear to contribute to the discrepancies seen in the literature to date.

In this dissertation, the goal was to explore the role of EK supplementation and help elucidate the potential ergogenicity of EK. In study 1, the results are consistent with the literature indicating that ketone salts (KS) are likely not ergogenic (Margolis and O'fallon, 2020; Valenzuela et al., 2020; Valenzuela et al., 2021) possibly because they induce only modest increases in blood ketones. Further, the experiments that have shown KS supplements to improve exercise performance (Kackley et al, 2020) are likely due to several other added ingredients such as caffeine and taurine. Specifically, in the first study, the comparison amongst a caffeine-containing KS supplement vs the same supplement without caffeine and an iso-energetic placebo was made. Results showed that

the caffeine-containing group improved performance on a best effort TT_{20km} when compared to the other two supplements, and that both KS supplements improved Wingate peak power output. Both caffeine (Grgic et al., 2020) and taurine (Warnock et al., 2017) have been shown to be ergogenic so it is likely that both contributed to these performance benefits. The presence of these other components makes the role of KS on exercise performance difficult to determine based on the data gathered in this dissertation. . Importantly, it has been suggested that a minimum blood β HB concentration of ≥ 1 mM is important to elicit positive effects of ketone supplementation during exercise (Margolis and O'fallon, 2020) and neither of the two KS supplements used in study 1 attained this concentration (peaked at 0.7 mM). This may have been because of the supplement type, i.e., KS and/or because of ingesting them in the fed state, which has been shown to slow supplement's absorption (Stubbs et al., 2017).

This dissertation also explored the potential for ketone monoesters (KME) to improve important aspects of performance such as post-exercise recovery and cognitive function during exercise. KME can induce a greater degree of ketonemia, and, because of this, have been considered more effective than KS. Specifically, KME, the type of supplement used in studies 2 and 3 of this dissertation, contain a β HB molecule bound through an ester bond to 1,3 Butanediol, a ketogenic precursor that can be absorbed and converted to β HB in the liver to induce a greater degree of ketonemia. In animals, infusion (Laughlin et al., 1994) and incubation (Maizels et al., 1977; Takahashi et al., 2019) studies have demonstrated that ketones (both acetoacetate and β HB) and CHO together results in greater glycogen resynthesis vs CHO alone. A recent animal study showed that when mice muscles were incubated with optimal glucose and insulin availability in a β HB environment (1 vs 2 vs 4 mM), the greatest concentration displayed more glycogen repletion after exercise (Takahashi et al., 2019). In humans, only two studies have explored this issue with conflicting results. During recovery from glycogen depleting exercise, Holdsworth et al. (2017) found a 50% increase in muscle glycogen content with oral KME and intravenous CHO supplementation (glucose clamp maintaining concentration at ~ 10 mM), compared to a glucose clamp alone, whereas Vandoorne et al. (2017) observed no difference in glycogen content with oral KME and PRO + CHO (glucose concentration achieved was ~ 6.5 mM) or PRO + CHO alone. Of course, greater

initial glycogen content is associated with better performance not only in high intensity intermittent sports (Saltin, 1973; Krustup et al., 2006) but also in prolonged endurance efforts (Coyle et al, 1986). In study 2 of this dissertation, exercise performance was compared with KME and CHO supplementation vs isoenergetic CHO alone, following glycogen lowering exercise and the data indicate that KME and CHO supplementation did not improve time trial performance. Based on these results and the methodology of the previous two investigations (Holdsworth et al., 2017; Vandoorne et al., 2017), it appears likely that a β HB concentration of $>4\text{mM}$ and a high glucose availability ($\sim 10\text{mM}$) is necessary to enhance exercise recovery glycogen storage. In the future, doses should be optimized to ensure sufficient glucose and ketone bioavailability.

In addition, the dissertation plan included an exploration of the potential of KME supplementation to enhance or attenuate decrements in cognitive function observed often during prolonged exercise (Study 3). The underlying rationale is that KB have been shown to serve as an alternative fuel for the brain (Cunnane et al., 2011) as well as serving as important signalling molecules increasing the expression of the protein Brain Derived Neurotropic Factor (BDNF), known to influence brain plasticity and the regulation of cognitive function (Marosi et al., 2016; Sleiman et al., 2016). In fact, several previous studies had shown positive effects of KME (Evans and Egan, 2018) and KS + MCT (Prins et al., 2020) supplementation on cognitive function. In study 3, this dissertation evaluated whether KME supplementation could help attenuate cognitive decline during intermittent exercise after induced mental fatigue, compared to a non-caloric placebo. As expected, the results indicated that mental fatigue decreased the number of correct answers in both supplement groups with exercise, relative to baseline. However, KME vs a non-caloric placebo attenuated the cognitive decline, although no other cognitive benefits were observed during the intermittent exercise studied.

6.2 Limitations

This dissertation aimed to explore the role of ketones on various aspects of exercise performance. While some of the results obtained in the three investigations help elucidate the role of EK supplementation, several limitations need to be noted. For example, in

study 1, both supplements studied contained other ingredients such as leucine and taurine, and differed only with respect to the presence of absence of caffeine. This, of course, makes it difficult to reach a definitive conclusion regarding KS. Future studies need to isolate these other ingredients. In study 2 and 3, a more effective type of EK (KME) was used. The dose administered was based on manufacturer's guidelines and while some positive effects were seen in cognitive function during exercise (β HB concentration >1 mM) the resulting ketonemia may have been suboptimal, i.e., <4 mM β HB concentration, for post-exercise glycogen resynthesis. Furthermore, studies 2 and 3 were ongoing during the COVID-19 pandemic and, due to laboratory closures, the number of participants was less than originally planned. While some important effects were revealed, this reduced number of participants may have produced a statistical type II error, relative to the potential benefits of EK supplementation. Finally, a glycogen lowering exercise treatment based on previous literature was implemented in study 2 in order to assess any performance benefits but glycogen measures were not taken. Consequently, the degree of glycogen depletion could have differed amongst participants and, if so, this could have affected the glycogen repletion.

6.3 Summary and Conclusion

In summary, study 1 demonstrated that ingestion of both a caffeinated KS supplement and the same KS supplement without caffeine induced only a modest elevation in blood β HB concentration (~ 0.7 mM). Both supplements improved Wingate peak power output, following a 20 km time trial (TT_{20km}); however, only the caffeinated KS supplement improved performance on a best effort TT_{20km}. Further, study 2 found that, despite reduced blood glucose and greater β HB concentration, co-ingestion of KME and CHO after glycogen lowering exercise (GLE) vs isoenergetic CHO alone, resulted in no significant differences in any of the exercise performance parameters. These exercise performance data are consistent with similar glycogen replenishment in both trials. Finally, study 3 observed that, following induced mental fatigue, KME supplementation resulted in fewer cognitive errors during exercise in a complex reaction test, compared to a non-caloric placebo.

In conclusion, the data from this dissertation indicate that 1) acute KS supplementation is not likely to be ergogenic nor detrimental for exercise performance, 2) KME supplementation may improve some aspects of cognitive function during exercise, and 3) KME supplementation has little effect on post-exercise glycogen synthesis following prior exercise. Finally, there are limited studies investigating these issues in the literature, and, therefore, more study is needed to assess fully the possible benefits of KE for athletes.

6.4 Future studies

As indicated, the degree of ketonemia appears to be a key factor promoting any physiological/biochemical benefit. Therefore, dosage studied is critical. For example, it appears that ketonemia needs to be $\sim 4\text{mM}$ to induce important effects during post-exercise recovery such as hyperinsulinemia. Further and importantly, this same degree of ketonemia may affect exercise performance negatively due to perturbations to acid-base homeostasis. One of the main challenges in the ketone supplementation area of study is the conflicting results observed to date perhaps due to high heterogeneity of experimental methodology. Important considerations include: 1) ketone supplement type and dose as both determine the magnitude of ketosis achieved and 2) whether acute ingestion occurs in the fed vs fasted state because the former slows absorption significantly (Stubbs et al., 2017). Moreover, several other factors to consider are the tests studied and the mode of exercise used to evaluate performance as these have differed across studies published to date. Finally, it is recommended that future ketone supplementation studies 1) use KME, 2) document the degree of induced ketosis so that any possible effect threshold can be established, and 3) for post exercise glycogen resynthesis studies, CHO availability be quantified.

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Appendices

Appendix A. Human ethics approval study 1



Date: 7 May 2019

To: [REDACTED]

Project ID: 113474

Study Title: The effects of pure therapeutic ketones/caffeine free supplementation vs caffeinated pure therapeutic ketones on a 20 km cycling time trial and a Wingate test exercise performance.

Application Type: HSREB Initial Application

Review Type: Full Board

Meeting Date / Full Board Reporting Date: 12/Mar/2019

Date Approval Issued: 07/May/2019

REB Approval Expiry Date: 07/May/2020

Dear Dr. Peter Lemon

The Western University Health Science Research Ethics Board (HSREB) has reviewed and approved the above mentioned study as described in the WREM application form, as of the HSREB Initial Approval Date noted above. This research study is to be conducted by the investigator noted above. All other required institutional approvals must also be obtained prior to the conduct of the study.

Documents Approved:

Document Name	Document Type	Document Date	Document Version
Borg 20 point Scale	Recruitment Materials	06/May/2019	2
Data Collection Form - Ketones - May 6, 2019	Other Data Collection Instruments	06/May/2019	2
dietary diary form - version 2 - May 6, 2019	Other Data Collection Instruments	06/May/2019	2
Facebook Recruitment - Ketones	Recruitment Materials	06/May/2019	2
LOI Ketone supplementation version 3 - clean copy	Written Consent/Assent	06/May/2019	3
PARQ Plus 2019	Other Data Collection Instruments	06/May/2019	2
Participant Health Information Form 2019	Recruitment Materials	06/May/2019	1
Protocol - Manuel - ketones - version 2	Protocol	06/May/2019	2
Recruitment advertisement poster - Ketones	Recruitment Materials	06/May/2019	2
Recruitment Email - Ketones	Email Script	06/May/2019	2
Recruitment Email - Ketones	Recruitment Materials	06/May/2019	2

Documents Acknowledged:

Document Name	Document Type	Document Date	Document Version
Flow Diagram - Version 2 - May 6, 2019	Flow Diagram	06/May/2019	2
Rational References - version 1 - May 6, 2019	References	06/May/2019	1

No deviations from, or changes to, the protocol or WREM application should be initiated without prior written approval of an appropriate amendment from Western HSREB, except when necessary to eliminate immediate hazard(s) to study participants or when the change(s) involves only administrative or logistical aspects of the trial.

Appendix B. Human ethics approval study 2



Date: 11 September 2019

To: [REDACTED]

Project ID: 113713

Study Title: Effect of ketone supplementation on glycogen replenishment and time trial performance following a glycogen lowering exercise

Application Type: HSREB Initial Application

Review Type: Full Board

Meeting Date: 16/Jul/2019 13:00

Date Approval Issued: 11/Sep/2019 09:37

REB Approval Expiry Date: 11/Sep/2020

Dear Dr. Peter Lemon

The Western University Health Science Research Ethics Board (HSREB) has reviewed and approved the above mentioned study as described in the WREM application form, as of the HSREB Initial Approval Date noted above. This research study is to be conducted by the investigator noted above. All other required institutional approvals must also be obtained prior to the conduct of the study.

Documents Approved:

Document Name	Document Type	Document Date	Document Version
Borg 20 point Scale	Other Data Collection Instruments	23/Jul/2019	2
Data Collection Form - Ketones 2 - Jun 25, 2019	Other Data Collection Instruments	25/Jun/2019	1
dietary diary form - version 1 - Jun25, 2019	Other Data Collection Instruments	25/Jun/2019	1
Facebook Recruitment - Ketones	Recruitment Materials	26/Jun/2019	1
LOI Ketone supplementation glycogen repletion version 1	Written Consent/Assent	03/Jul/2019	1
PARQ Plus 2019	Other Data Collection Instruments		
Participant Health Information Form 2019	Recruitment Materials	29/Jun/2019	1
Recruitment advertisement poster - Ketones	Recruitment Materials	27/Jun/2019	1
Recruitment Email - Ketones	Email Script	26/Jun/2019	1
STUDY OUTLINE Ketone ester glycogen repletion Ver 1	Protocol	26/Jun/2019	1

No deviations from, or changes to, the protocol or WREM application should be initiated without prior written approval of an appropriate amendment from Western HSREB, except when necessary to eliminate immediate hazard(s) to study participants or when the change(s) involves only administrative or logistical aspects of the trial.

REB members involved in the research project do not participate in the review, discussion or decision.

The Western University HSREB operates in compliance with, and is constituted in accordance with, the requirements of the TriCouncil Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2); the International Conference on Harmonisation Good Clinical Practice Consolidated Guideline (ICH GCP); Part C, Division 5 of the Food and Drug Regulations; Part 4 of the Natural Health Products Regulations; Part 3 of the Medical Devices Regulations and the provisions of the Ontario Personal Health Information Protection Act (PHIPA 2004) and its applicable regulations. The HSREB is registered with the U.S. Department of Health &

Appendix C. Human ethics approval study 3



Date: 26 November 2020

To: [REDACTED]

Project ID: 116711

Study Title: The effect of exogenous ketone supplementation on cognitive function during exercise after induced mental fatigue

Application Type: HSREB Initial Application

Review Type: Full Board

Meeting Date: 03/Nov/2020 13:00

Date Approval Issued: 26/Nov/2020 14:44

REB Approval Expiry Date: 26/Nov/2021

Dear Dr. Peter Lemon

The Western University Health Science Research Ethics Board (HSREB) has reviewed and approved the above mentioned study as described in the WREM application form, as of the HSREB Initial Approval Date noted above. This research study is to be conducted by the investigator noted above. All other required institutional approvals must also be obtained prior to the conduct of the study.

Documents Approved:

Document Name	Document Type	Document Date	Document Version
Ketones and mental fatigue protocol	Protocol	21/Sep/2020	1
VAS	Other Data Collection Instruments	28/Sep/2020	1
dietary diary form	Other Data Collection Instruments	28/Sep/2020	1
Data Collection Form	Other Data Collection Instruments	29/Sep/2020	1
Par Q+ 2020	Other Data Collection Instruments		
Borg 20 point Scale	Other Data Collection Instruments	29/Sep/2020	1
Participant Health Information Form 2020	Recruitment Materials	29/Sep/2020	1
Facebook Recruitment - Ketones	Recruitment Materials	12/Oct/2020	1
Recruitment Email - Ketones	Email Script	12/Oct/2020	1
LOI ketones and mental fatigue	Written Consent/Assent	19/Oct/2020	1
Recruitment Email - Ketones 3 Version 3 tracked copy	Recruitment Materials	24/Nov/2020	3
Recruitment Email - Ketones 3 Version 3 clean copy	Recruitment Materials	24/Nov/2020	3

No deviations from, or changes to, the protocol or WREM application should be initiated without prior written approval of an appropriate amendment from Western HSREB, except when necessary to eliminate immediate hazard(s) to study participants or when the change(s) involves only administrative or logistical aspects of the trial.

REB members involved in the research project do not participate in the review, discussion or decision.

The Western University HSREB operates in compliance with, and is constituted in accordance with, the requirements of the TriCouncil Policy Statement: Ethical

Appendix D. Letter of Information and Informed Consent study 1



Title of Study: The effects of pure therapeutic ketones/caffeine free supplementation vs caffeinated pure therapeutic ketones on a 20 Km time trial and a Wingate test exercise performance.

Principal Investigator: [REDACTED]
 Graduate Student: [REDACTED]

[REDACTED]

LETTER OF INFORMATION AND CONSENT

INVITATION TO PARTICIPATE

You are being invited to participate in a research study at the Exercise Nutrition Research Laboratory (Room 2235, 3M Centre) investigating the effects of pure therapeutic ketones/caffeine free supplementation vs caffeinated pure therapeutic ketones on a 20 Km time trial and a Wingate test exercise performance.

PURPOSE OF THE LETTER

The purpose of this letter is to provide you with information required for you to make an informed decision regarding participation in this research.

There are no conflicts of interest to declare related to this study.

PURPOSE OF THIS STUDY

Ketosis is a physiological state where the concentration of circulating blood ketones is elevated. Some researchers support that a state of ketosis, reached through the use of ketogenic diet (very high fat – low carbohydrate diet), has the potential to improve athletic performance. Recently, many endurance type athletes have engaged in ketogenic diets, claiming that this dietary change has led to improvements in exercise performance. A state of ketosis has been shown to be beneficial in many aspects of health, however its effectiveness in athletic settings is rather controversial. One of the biggest challenges to achieve and maintain ketosis is complying with the diet due to its

restrictive nature. Consequently, manufacturers have developed supplements that can induce a state of ketosis within 1 hour. Therefore, if ketosis is responsible for performance improvements, these supplements should help individuals improve performance. Unfortunately, only a handful of studies have used these supplements so there is very little evidence supporting the use thereof to enhance athletic performance. High blood ketones concentration seems to increase the use of ketones for energy during physical activity. These changes in energy production may allow individuals to spare important energy substrates, something that would be represent an advantage. Ketone salts have been shown to elevate the concentration of ketones in blood and as such they may allow individuals to enhance performance. Manufacturers now provide ketones salts combined with other ingredients such as caffeine. However it is unknown whether or not the addition of these ingredients has any positive or negative effect on athletic performance. Caffeine alone has been shown to improve athletic performance in several studies. Therefore, the purpose of this study is to compare the effectiveness of a caffeine free ketone salt supplement vs a caffeinated ketone salt supplement.

INCLUSION CRITERIA

In order to be eligible to participate in this study you must be a healthy male or female recreationally active individual, 18-35 year old, non-regular caffeine user.

EXCLUSION CRITERIA

You will be excluded from this study if you:

- Are not involved on regular exercise (at least 2x week)
- Have symptoms or take medication for respiratory, cardiovascular, metabolic, neuromuscular disease
- Use any medications with side effects of dizziness, lack of motor control, or slowed reaction time
- Are taking part in other research study
- For women, if you are pregnant or become pregnant during the study.
- Have a history of concussion/head injuries.
- Have an excessive alcohol intake (>2 drinks/day)

STUDY OUTLINE:

All study activities will be completed in the Exercise Nutrition Research Laboratory (Room 2235, 3M Centre). *****For the test days, you will be asked to arrive at the laboratory at least 4 hours after ingesting your last meal. You will also be asked to refrain from exercise and from consuming caffeine or alcohol for 24 hours prior to your study visits. You will repeat food intake for 24 hours before each test day to equalize energy intake for all three testing days *****

STUDY PROCEDURES

If you volunteer to participate in this study, we will ask you to do the following things:

1. You will visit the lab for one familiarization session prior to the study days. The familiarization session will be held at least 2 days before the first test day. This session will involve filling out forms to ensure your safe participation in the study, a measurement of body composition, and an acclimation to the exercise performance tests to be used during test days. The total time required for this session will be ~2h.
2. For the study sessions you visit the laboratory on three additional occasions. All visits will involve the exercise testing and the consumption of one experimental drink (drink containing ketones, drink containing ketones/caffeine or drink containing water) 30 minutes before starting to exercise. The order of these three experimental drinks will be randomized for each participant and they will complete all trials. The exercise tests for all study days consists of a 20 km time trial followed by a Wingate test.
3. We will measure blood ketones and blood lactate before ingesting the drink, 15 and 30 minutes after ingesting the drink, before starting, at 10 km and at the end of the 20km time trial, and before, right after and 6 minutes after the Wingate test. Therefore, we will collect a total of 9 samples during each study visit for a total of 27 samples over a 3-week period.
4. We will also measure rates of perceived exertion during the exercise tests.

Familiarization session: Before your inclusion in the study sessions, you will be asked to fill out a physical activity readiness questionnaire and a participant information form for personal and familial health history. This evaluation session will be held at least 2 days before the first test day. Additionally, during the familiarization session you will have your body composition determined via BodPod® and will practice both the 20km time trial and Wingate test. The BodPod® is a chamber which determines body volume by measuring the space your body takes up and together with your body weight allows us to calculate lean and fat content of the body. The 20 km time trial is essentially a race so you would have to complete the distance as fast as possible. Finally, the Wingate test is an all-out 30 second sprint on a bike ergometer. In this test you would have to bike as fast as possible for 30 seconds.

Test day 1 (Total duration is approximately 2 hours): You will arrive at the exercise nutrition lab at least 4 hours after your last meal with limited activity (drive/use of the elevator to get to the lab). This strategy is used to ensure that you are in a rested physiological state and thus will not affect any baseline measurements of blood ketones or lactate concentrations. Upon arrival, the first fingerpick blood lactate and blood ketone samples will be collected and the corresponding drink will be provided immediately after. You will be allowed 30 minutes to read/study. The drink is a solution containing ketones and caffeine. During those 30 minutes two more blood samples will be taken (at 15 and 30 minutes). After this, you will perform a 10- minute standardized warm-up and at the end we will take another fingerpick blood lactate and blood ketone

samples. Subsequently, exercise performance will be evaluated. The first exercise performance test is a 20 km time trial. Blood samples and rates of perceived exertion will be taken at 10 km and at the end of the test. After concluding this test, a 15-minute break will be given before doing a Wingate test. Blood samples and rates of perceived exertion will be taken at before, immediately after and 6 minutes after the end of the test.

Test day 2 and test day 3 (Total duration is approximately 2 hours): These visits will take place at least one week after the previous study visit. You will perform the same study procedures you did during the first study visit.

POSSIBLE RISK AND HARMS

This study involves strenuous exercise that may pose a risk of minor muscle injury, discomfort or soreness. All exercise involves some health risk (primarily cardiovascular or hydration-related) and you may experience symptoms of fatigue while participating in this study. Importantly, similar exercise to that used in this study is completed by kinesiology students and Mustang athletes daily. Further, the risks of cardiovascular complications are much reduced in young, healthy individuals. Finally, you will be encouraged to drink enough water to stay hydrated.

Blood Collection: Fingerprick: this method may result in some bleeding or bruising. Pressure on the puncture site upon removal will diminish this risk.

POTENTIAL BENEFITS

Participating in this study may give you some information about your body composition, exercise capacity, strategies to maximize performance through nutrition interventions and if requested, you will receive you own data as well as the mean of the group.

COMPENSATION

You will not be compensated for your participation in this study. You will not be reimbursed for additional costs such as parking or transportation.

VOLUNTARY PARTICIPATION

Participation in this study is voluntary. You may refuse to participate, refuse to answer any questions or withdraw from the study at any time. The investigator may withdraw you from this research if circumstances arise which warrant doing so.

RIGHTS OF A PARTICIPANT (in the event of a study related injury)

If you suffer any study related injury during your participation in this study care will be provided to you at no cost.

CONFIDENTIALITY

If you agree to join this study, only members of the study team will look at your personal information (e.g., name) and collect only the information they need for the study.

The information that is collected for the study will be kept in a locked and secure area by the study doctor for 5 years. Only the study team or the people or groups listed below will be allowed to look at your records.

Representatives of the University of Western Ontario Health Sciences Research Ethics Board may look at the study records and at your personal health information to check that the information collected for the study is correct and to make sure the study followed proper laws and guidelines.

All information collected during this study, including your personal health information, will be kept confidential and will not be shared with anyone outside the study unless required by law. You will not be named in any reports, publications, or presentations that may come from this study.

If you decide to leave the study, the information about you that was collected before you leave the study will still be used in order to answer the research question. No new information will be collected without your permission.

CONTACT FOR FURTHER INFORMATION

If you have any questions about this research project, feel free to call us [REDACTED] for clarification.

Further, if you have any questions about the conduct of this study or your rights as a research subject you may contact the Office of Research Ethics at Western University at [REDACTED]

Consent Form

The effects of pure therapeutic ketones/caffeine free supplementation vs caffeinated pure therapeutic ketones on a 20 Km time trial and a Wingate test exercise performance.

Investigators: Dr. Peter W.R. Lemon and Manuel Quinones, MSc.

I have read the accompanying "Letter of Information", have had the nature of the study explained to me and I agree to participate. All questions have been answered to my satisfaction.

If you wish to participate in future studies in the Exercise Nutrition Research Lab, the research team will collect your contact information.

I wish to be contacted for future studies in the Exercise Nutrition Research Laboratory.

Yes _____ (check mark), No _____ (check mark) Date: _____

By signing below, I agree to participate in this study.

Name of Participant (please print): _____

Signature of Participant: _____

Date: _____

Name of Person Obtaining Informed Consent: _____

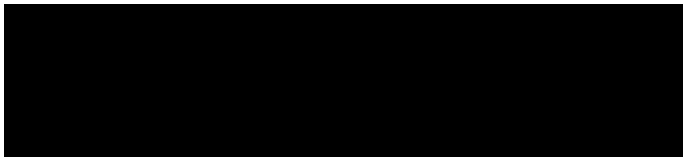
Signature of Person Obtaining Informed Consent: _____

Date: _____

You will receive a copy of the consent form after it has been signed. You do not waive any legal rights by signing the consent form.

This letter is for you to keep for future references.

Sincerely,



Appendix E. Letter of Information and Informed Consent study 2



Title of Study: Effect of ketone supplementation on glycogen replenishment and time trial performance following a glycogen lowering exercise

Principal Investigator: [REDACTED]

Graduate Student: [REDACTED]

The costs incurred in this study will be covered with laboratory funds.

LETTER OF INFORMATION AND CONSENT

INVITATION TO PARTICIPATE

You are being invited to participate in a research study at the Exercise Nutrition Research Laboratory (Room 2235, 3M Centre) investigating the effects of ketone supplementation on glycogen replenishment and 20 Km cycling time trial performance following glycogen lowering exercise.

PURPOSE OF THE LETTER

The purpose of this letter is to provide you with information required for you to make an informed decision regarding participation in this research.

There are no conflicts of interest to declare related to this study.

PURPOSE OF THIS STUDY

Carbohydrates are important for exercise at moderate to high intensities. Low glycogen content (stored form of carbohydrates) is associated with the onset of fatigue. Thus, greater initial glycogen content has been correlated to better performance not only in high intensity intermittent sports, but also in prolonged endurance sports. Dose-response studies have determined that ingestion of 1.2 g·kg⁻¹·h⁻¹ of CHO is the appropriate acute recovery dose to optimize glycogen repletion, with no apparent benefit at higher doses. Protein in combination to CHO has also been proposed to enhance glycogen resynthesis when 1 part protein is provided with ~4 parts CHO. Both

strategies, optimal CHO intake and CHO in combination with protein for glycogen resynthesis, have resulted in better performance a few hours after a depleting exercise bout. A recent study provided athletes with ketone esters after depleting exercise and found that in the presence of high glucose availability, ketone esters increased glycogen stores by 50% compared to a no ketone high carbohydrate treatment. However, authors in this study provided carbohydrates using a clamp method to maintain glycaemia at 10Mm/L so it is unknown whether or not ingesting glucose at recommended dosages would have the same effect. Furthermore, it is unknown whether or not this difference would translate into improved performance in efforts of moderate duration. Therefore, the purpose of this study would be to assess the effect of glucose-ketone supplementation on a 20 km time trial after a 2-hour feeding / 4-hour recovery period following glycogen depleting exercise.

INCLUSION CRITERIA

In order to be eligible to participate in this study you must be a healthy male or female trained active individual (at least 1y training experience – at least 3x week), 18-40 year old.

EXCLUSION CRITERIA

You will be excluded from this study if you:

- Are not involved on regular exercise (at least 3x week)
- Have symptoms or take medication for respiratory, cardiovascular, metabolic, neuromuscular disease
- Use any medications with side effects of dizziness, lack of motor control, or slowed reaction time
- Are taking part in other research study
- For women, if you are pregnant or become pregnant during the study.
- Have a history of concussion/head injuries.
- Have an excessive alcohol intake (>2 drinks/day)
- Smoker

STUDY OUTLINE

All study activities will be completed in the Exercise Nutrition Research Laboratory (Room 2235, 3M Centre). ***For the test days, you will be asked to arrive at the laboratory at 7:30 am following an overnight fast (no food or drink except water after 7:30pm). You will also be asked to refrain from exercise and from consuming caffeine or alcohol for 24 hours prior to your study visits. You will record everything you eat and drink 2 days before the experimental day in order to duplicate both nutrient and energy intake for the second experimental day. ***

STUDY PROCEDURES

If you volunteer to participate in this study, we will ask you to do the following things:

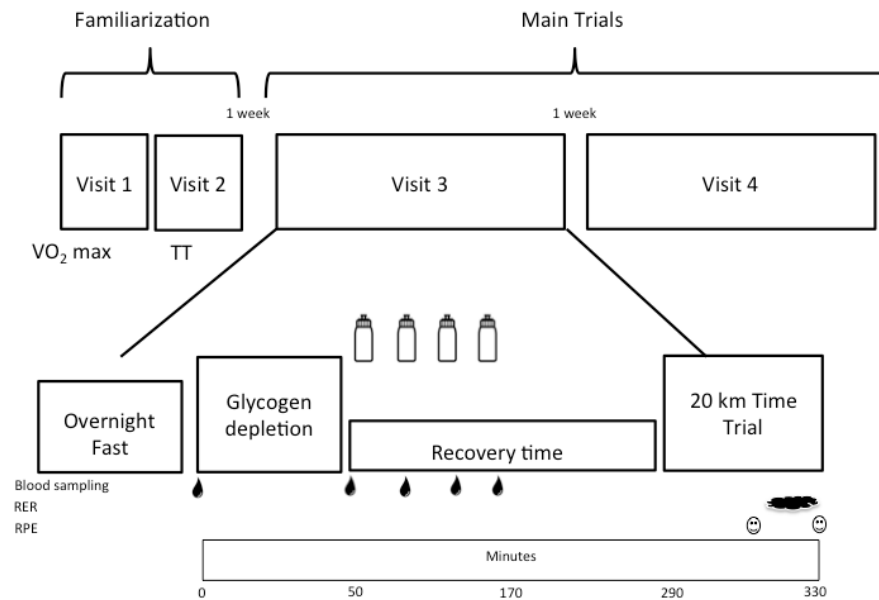
1. You will visit the lab for two familiarization sessions prior to the two study days. The familiarization sessions will be held on separate days and at least 2 days before the first test day. The first session will involve filling out forms to ensure your safe participation in the study, a measurement of body composition, and an incremental cycling test to determine maximum oxygen consumption and maximum power output. On a second visit, you will be familiarized with the exercise testing to be used in the experimental days. The total time required per session will be ~2h.
2. For the study sessions you visit the laboratory on two additional occasions. Both visits will involve a glycogen depletion cycling protocol, the exercise testing and the consumption of one experimental drink (drink containing ketones and carbohydrates or drink containing carbohydrates alone). The order of these experimental drinks will be randomized for each participant and they will complete all trials. The exercise test to be used in study days consists of a 20 km cycling time trial.
3. We will take blood samples to measure blood ketones and insulin before ingesting the drink, and every 30 minutes for a period of 2 hours. Therefore, we will collect a total of 5 samples during each study visit for a total of 10 (5 per experiment) samples over a 2-week period. All sampling will be obtained using a catheter from antecubital vein into EDTA containing tubes and only requires 5ml of blood (1 teaspoon).
4. We will also measure rates of perceived exertion and gas exchange during the 20 km cycling time trial.

Familiarization session: Before your inclusion in the study sessions, you will be asked to fill out a physical activity readiness questionnaire and a participant information form for personal and familial health history. This evaluation session will be held at least 2 days before the second familiarization session. Additionally, during this session you will have your body composition determined via BodPod® and will perform an incremental cycle exercise test to volitional exhaustion to determine the individual maximum power output (W_{max}) and maximal oxygen consumption ($VO_2 max$). The BodPod® is a air filled chamber (~volume = 600 litres) which determines body volume by measuring the air your body displaces. Together with your body weight we calculate body density which enables us to estimate your lean and fat body content. For this test, you will remain seated, keeping still and breathing normally. The test takes 45 seconds and is usually done several times for accuracy. You will be asked if you are okay after each test. There is a window that allows you to see outside which eliminates claustrophobic issues for most. Further, there is a panic button so you can end the test at any time. The incremental cycle exercise test will be performed on an electromagnetically braked cycle ergometer (Velotron, RacerMate, Inc., Seattle, Washington, USA) with cycling starting at 95 W for 3 min, followed by incremental steps of 35 W every 3 min until exhaustion. Breath-by-breath on line analysis of O_2 and CO_2

will be carried out using open-circuit metabolic cart (Vmax Legacy, Sensor Medics, CA, USA). Heart rate (HR) will be recorded using a HR monitor (Polar RS200TM, Polar Electro Inc., Lachine, Canada). Wmax will be calculated from the last completed work rate, plus the fraction of time spent in the final non-completed work rate multiplied by the work rate increment. In a second familiarization session, you will practice the 20 km time trial to be used in testing days to assess performance. The 20 km cycling time trial is essentially a race so you complete the 20km, as fast as possible.

Test day 1 (Total duration is approximately 7 hours): You will arrive at the exercise nutrition lab at 7:30 am following an overnight fast, and with limited activity (drive/use of the elevator to get to the lab). This strategy is used to ensure that you are in a rested physiological state and thus will not affect any baseline measurements. Upon arrival, a fingerpick blood ketone sample will be collected. Then, you will engage in a standardized glycogen depleting exercise protocol. This protocol begins with a 10 minute warm up period at a workload of 50% Wmax. Thereafter, you will be instructed to cycle 2-min block periods at alternating workloads of 90% and 50% of Wmax, respectively. This will be continued until you are no longer able to complete the 2 min at 90% Wmax. That moment will be defined as the time at which you are unable to maintain cycling speed at 60 revolutions/min. At that moment the high-intensity block will be reduced to 80% Wmax. Again, you will cycle until you are unable to complete a 2-min block at 80% Wmax, after which the high-intensity block will be reduced to 70% Wmax. Similarly, when unable to maintain 70%, the workload will be reduced to 60%. You will be allowed to stop when pedalling speed could not be maintained at 60% Wmax. Water will be provided ad libitum during the exercise protocol. The corresponding drinks will be provided during a 2-hour feeding period immediately after cessation of the depleting exercise. The first drink consist of either ketone supplementation or isocaloric carbohydrate supplementation, followed by a carbohydrate containing drink every 15 minutes for the duration of the 2 hour feeding period (9 drinks in total). Immediately before the start of the recovery drinks and every 30 min throughout the 2h feeding period 5 ml blood (1 teaspoon) will be collected by certified personnel via venipuncture using a catheter from antecubital vein into EDTA containing tubes and processed to measure plasma ketones (mmol/L) and insulin (pmol/L) using FreeStyle Precision Neo® ketone meter (Abbott Diabetes Care Limited, Saint-Laurent, Quebec) and a standard insulin radioimmunoassay kit, respectively. After this, you will be allowed a 2-hour recovery period before initiating a performance evaluation. Exercise performance will be assessed with a 20 km cycling time trial. Gas exchange and rates of perceived exertion will be collected during this test.

Test day 2 (Total duration is approximately 7 hours): This visit will take place at least one week after the previous study visit. You will perform the same study procedures you did during the first study visit.



POSSIBLE RISK AND HARMES

This study involves strenuous exercise that may pose a risk of minor muscle injury, discomfort or soreness. All exercise involves some health risk (primarily cardiovascular or hydration-related) and you may experience symptoms of fatigue while participating in this study. Importantly, similar exercise to that used in this study is completed by kinesiology students and Mustang athletes daily. Further, the risks of cardiovascular complications are much reduced in young, healthy individuals. Finally, you will be encouraged to drink enough water to stay hydrated.

Blood Collection: There will be some risk or discomfort involved in this study as a catheter will be inserted in your forearm by a certified professional in order to draw about 1 teaspoon of blood. This method may result in some bleeding or bruising. Pressure on the puncture site upon removal will diminish this risk.

POTENTIAL BENEFITS

No direct benefits are likely from participating in this study.

COMPENSATION

You will not be compensated for your participation in this study. You will not be reimbursed for additional costs such as parking or transportation.

VOLUNTARY PARTICIPATION

Participation in this study is voluntary. You may refuse to participate, refuse to answer any questions or withdraw from the study at any time with no effect to your academic status. You can also choose to remove your data from the study should you wish to do so. The investigator may withdraw you from this research if circumstances arise which warrant doing so. For example, if you cannot comply with the study protocol, or if you start/follow a ketogenic diet or for women, if you become pregnant during the study.

RIGHTS OF A PARTICIPANT (in the event of a study related injury)

If you suffer any study related injury during your participation in this study care will be provided to you at no cost.

CONFIDENTIALITY

If you agree to join this study, only members of the study team will look at your personal information (e.g., name) and collect only the information they need for the study.

The information that is collected for the study will be kept in a locked and secure area by the study doctor for 7 years. Only the study team or the people or groups listed below will be allowed to look at your records.

Representatives of the University of Western Ontario Health Sciences Research Ethics Board may look at the study records and at your personal health information to check that the information collected for the study is correct and to make sure the study followed proper laws and guidelines.

All information collected during this study, including your personal health information, will be kept confidential and will not be shared with anyone outside the study unless required by law. You will not be named in any reports, publications, or presentations that may come from this study.

If you decide to leave the study, the information about you that was collected before you leave the study will still be used in order to answer the research question. No new information will be collected without your permission.

CONTACT FOR FURTHER INFORMATION

If you have any questions about this research project, feel free to call us [REDACTED] for clarification. Further, if you have any questions about the conduct of this study or your rights as a research subject you may contact the Office of Research Ethics at Western University at [REDACTED]

Consent Form

Effect of ketone supplementation on glycogen replenishment and time trial performance following a glycogen lowering exercise

Investigators: Dr. Peter W.R. Lemon and Manuel Quinones, MSc.

I have read the accompanying "Letter of Information", have had the nature of the study explained to me and I agree to participate. All questions have been answered to my satisfaction.

If you wish to participate in future studies in the Exercise Nutrition Research Lab, the research team will collect your contact information.

I wish to be contacted for future studies in the Exercise Nutrition Research Laboratory.

Yes _____ (check mark), No _____ (check mark) Date: _____

By signing below, I agree to participate in this study.

Name of Participant (please print): _____

Signature of Participant: _____

Date: _____

Name of Person Obtaining Informed Consent: _____

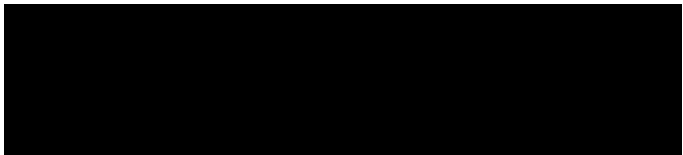
Signature of Person Obtaining Informed Consent: _____

Date: _____

You will receive a copy of the consent form after it has been signed. You do not waive any legal rights by signing the consent form.

This letter is for you to keep for future references.

Sincerely,



Appendix F. Letter of Information and Informed Consent study 3



Title of Study: The effect of exogenous ketone supplementation on cognitive function during exercise after induced mental fatigue

Principal Investigator: [REDACTED]
 Graduate Student: [REDACTED]

[REDACTED]

This study is a student project and the costs incurred in this study will be covered with laboratory funds.

LETTER OF INFORMATION AND CONSENT

INVITATION TO PARTICIPATE

You are being invited to participate in a research study at the Exercise Nutrition Research Laboratory (Room 414 – Health Science Building) investigating the effects of ketone (breakdown products of fat metabolism) supplementation on cognitive function during exercise after induced mental fatigue.

PURPOSE OF THE LETTER

The purpose of this letter is to provide you with the information required for you to make an informed decision regarding participation in this research.

There are no conflicts of interest to declare related to this study.

PURPOSE OF THIS STUDY

Carbohydrates (starches & sugars) are important muscle and brain fuel for exercise at moderate to high intensities. Low content of carbohydrates (CHO) is often associated with fatigue. Thus, greater amount of CHO before or during activity has been associated with better performance not only in high intensity intermittent sports, but also in prolonged endurance sports. Soccer specifically is a very demanding sport, not only physically, but also cognitively. Therefore, it is not surprising to see a deterioration in decision making and skill performance towards the end of a soccer match. Exogenous

ketone esters are a commercially available supplement that have been shown to potentially help preserve CHO as well as to serve as an alternative fuel for the brain. Research investigating the effects of ketones on performance in general are rather controversial. This inconsistency in the results has been attributed to differences in supplements (esters vs salts), methodology, as well as the dose administered in those studies. Some positive results in cognitive function have been observed with the use of ketone esters during endurance exercise. However, whether the effects of exogenous ketone esters are similar during high intensity, intermittent exercise, like that performed in a soccer game, is still unknown. Therefore, the purpose of the present study is to assess whether ketone ester supplementation can enhance cognitive performance later in a simulated soccer game after induced mental fatigue.

INCLUSION CRITERIA

In order to be eligible to participate in this study you must be a healthy male or female recreationally active individual, 18-35 year old.

EXCLUSION CRITERIA

You will be excluded from this study if you:

- Are not involved in regular exercise (at least 2x week)
- Have symptoms or take medication for respiratory, cardiovascular, metabolic, neuromuscular disease
- Use any medications with side effects of dizziness, lack of motor control, or slowed reaction time
- Are taking part in another research study
- For women, if you are pregnant or become pregnant during the study.
- Have a history of concussion/head injuries.
- Have an excessive alcohol intake (>2 drinks/day)
- Are consuming a ketogenic diet (for at least 2 weeks)
- Are a smoker
- ** Cannabis use is not an exclusion criteria; however, it must be avoided on experimental days.**

STUDY OUTLINE

All study activities will be completed in the Exercise Nutrition Research Laboratory (Room 414 – Health Science Building). ***For the test days, you will be asked to arrive at the laboratory at least 4 hours after ingesting your last meal. You will also be asked to refrain from exercise and from consuming caffeine or alcohol for 24 hours prior to your study visits. Also, you will need to record everything you eat and drink (type and quantities) 24 hours before the experimental day in order to repeat both food and fluid intake for the second experimental day. ***

STUDY PROCEDURES

If you volunteer to participate in this study, you will do the following:

1. Visit the lab for two familiarization sessions prior to the two study days. The familiarization sessions will be held on separate days and at least 2 days before the first test day. The first session will involve filling out forms to ensure your safe participation in the study, a measurement of body composition, a maximum oxygen consumption test and a sprint test to determine the treadmill speeds to be used during study days. The second familiarization session will be used to acclimate you to the running exercise intensities and to practice the cognitive tests. The total time required for the two familiarization sessions will be ~3h.
2. For the study sessions you visit the laboratory on two additional occasions. Both visits will involve a mental fatiguing task (MFT), ingestion of one experimental drink (drink containing ketone esters or iso-caloric drink containing carbohydrates alone) and executing cognitive tests while running. The running exercise for both study days consists of a 45 - minute half simulated soccer game divided into 3 x 15-minute blocks running at varying intensities that replicate the activity pattern of soccer such as sprinting, jogging, cruising, walking etc. The order of these experimental trials will be randomized, and you will complete both trials.
3. Visual analog scales (VAS) to assess mental fatigue will be used before and after the MFT. Also, motivation to exercise and mental effort will be assessed using VAS at the end of the MFT.
4. Capillary blood samples for ketones, glucose and lactate will be taken before and after the mental fatiguing task, right before starting the running exercise, and every 15 minutes at the end of each exercise block. i.e., 6 fingerprick samples will be taken during each study visit for a total of 12 samples over a 2-week period.
5. Perceived exertion ratings will be taken at the end of each exercise block and cognitive function will be assessed within each block during the 45- minute running exercise.

Familiarization session 1: You will be asked to fill out a physical activity readiness questionnaire and a participant information form for personal and familial health history. You will also have your body composition determined via BodPod® and will perform an incremental running exercise test to voluntary fatigue to determine your maximal oxygen consumption (VO_2 max).

The BodPod® is a air filled chamber (~volume = 600 litres) which determines body volume by measuring the air your body displaces. Together with your body mass we calculate body density which enables us to estimate your lean and fat body content. You will remain seated, keeping still and breathing normally. The test takes 45 seconds and is usually done several times for accuracy. There is a window that allows you to see outside which eliminates claustrophobic issues for most. Further, there is a panic button so you can end the test at any time.

The maximal oxygen consumption test involves a continuous, incremental test on a treadmill until you reach volitional fatigue. Breath-by-breath online analysis of O_2 and CO_2 will be carried out using an open-circuit metabolic cart (Vmax Legacy, Sensor Medics,

CA, USA). Heart rate (HR) will be recorded using a HR monitor (Polar RS200TM, Polar Electro Inc., Lachine, Canada).

Familiarization session 2: You will be running one 15-minute block exercise to acclimate to the running intensities that will be used during the study, and will practice the cognitive tests to remove any learning effect.

Test day 1 (approximately 2 hours): You will arrive at the exercise nutrition lab at least 4 hours after your last meal with limited activity (drive/use of the elevator to get to the lab). This strategy is used to ensure that you are in a rested physiological state and thus will not affect any baseline measurements of blood glucose, ketones or lactate concentrations. Upon arrival, the first fingerpick blood sample will be collected. Then, mental fatigue will be assessed and right after the MFT will begin. This task has a duration of 30 minutes and involves performing the Stroop test during this time. At the end of the MFT, mental fatigue, mental effort and motivation will be assessed using VAS. Next, cognitive function will be assessed using both the Stroop test and the Complex reaction time test. A fingerpick blood sample will be collected and the corresponding drink will be provided immediately after. You will be given 10 minutes before the warm-up starts. The drink is a solution containing either ketone esters or sugar (dextrose). After this, you will perform a 5-minute standardized warm-up and begin the 45-minute half simulated soccer match. Right before starting the soccer match simulation, a fingerpick blood sample and rate of perceived exertion (RPE) will be collected. Cognitive function will be assessed during the 45-minute running exercise. Blood samples and RPE will be collected at the end of each 15-minute exercise block (Figure 1).

Test day 2 (approximately 2 hours): This visit will take place at least one week after the previous study visit. You will perform the same study procedures you did during the first study visit but with the other drink.

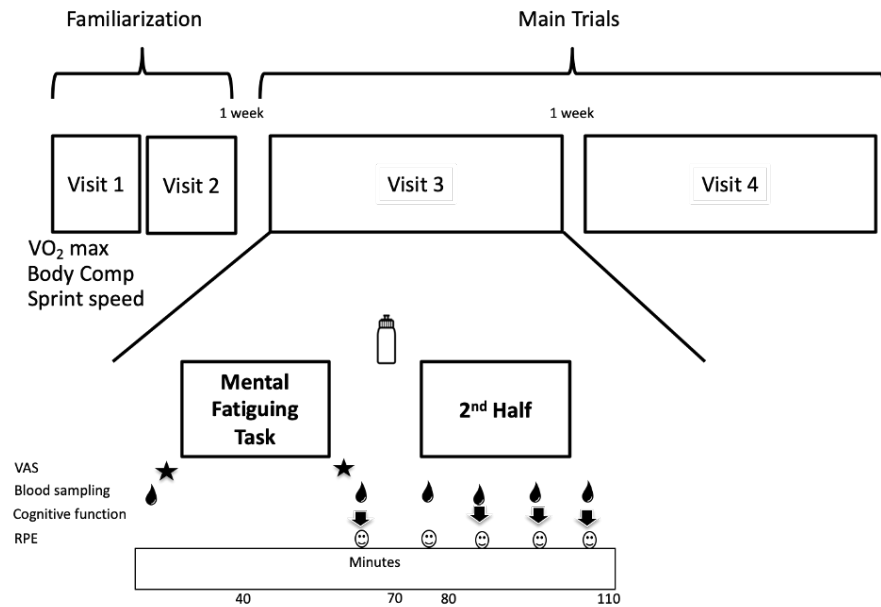


Figure 1. Experimental protocol. VO_2 max = maximal oxygen consumption, (💧) = blood sampling, (↓) = Cognitive function measures, RPE (😊) = rating of perceived exertion, (🍷) = ketone ester supplement.

POSSIBLE RISK AND HARMES

This study involves strenuous exercise that may pose a risk of minor muscle injury, discomfort or soreness. All exercise involves some health risk (primarily cardiovascular or hydration-related) and you may experience symptoms of fatigue while participating in this study. Importantly, similar exercise to that used in this study is completed by kinesiology students and Mustang athletes daily. Further, the risks of cardiovascular complications are much reduced in young, healthy individuals. Finally, you will be encouraged to drink enough water to stay hydrated.

Blood Collection: Fingerprick: this method may result in some bleeding or bruising. Pressure on the puncture site upon removal will diminish this risk.

POTENTIAL BENEFITS

No direct benefits are likely from participating in this study.

COMPENSATION

You will not be compensated for your participation in this study nor will you be reimbursed for any additional costs such as parking or transportation.

VOLUNTARY PARTICIPATION

Participation in this study is voluntary. You may refuse to participate, refuse to answer any questions or withdraw from the study at any time with no effect to your academic or work status. You can also choose to remove your data from the study should you wish to do so. The investigator may withdraw you from this research study if circumstances arise which warrant doing so, i.e., if you cannot comply with the study protocol.

RIGHTS OF A PARTICIPANT (in the event of a study related injury)

If you suffer a study related injury during your participation in this study care will be provided to you at no cost.

CONFIDENTIALITY

Only members of the study team will look at your personal information (e.g., name) and collect only the information they need for the study.

All information that is collected for the study will be kept in a locked and secure area by the study doctor for 7 years. Only the study team (listed below under the Contact for Further Information) will be allowed to look at your records. Further, you will be identified with a study number so your records will not be easily connected to you. Finally, the master file identifying you will be encrypted and stored separately on a University server behind a firewall.

Representatives of the University of Western Ontario Health Sciences Research Ethics Board may look at the study records and at your personal health information to check that the information collected for the study is correct and to make sure the study followed proper laws and guidelines.

All information collected during this study, including your personal health information, will be kept confidential and will not be shared with anyone outside the study unless required by law. You will not be named in any reports, publications, or presentations that may come from this study.

If you decide to leave the study, the information about you that was collected before you leave the study will still be used in order to answer the research question. No new information will be collected without your permission.

CONTACT FOR FURTHER INFORMATION

If you have any questions about this research project, feel free to call us [REDACTED] for clarification.

Further, if you have any questions about the conduct of this study or your rights as a research subject you may contact the Office of Research Ethics at Western University at [REDACTED]

Consent Form

The effect of exogenous ketone supplementation on cognitive function during exercise after induced mental fatigue

Investigators: Dr. Peter W.R. Lemon and Manuel Quinones, MSc.

I have read the accompanying "Letter of Information", have had the nature of the study explained to me and I agree to participate. All questions have been answered to my satisfaction.

If you wish to participate in future studies in the Exercise Nutrition Research Lab, the research team will collect your contact information.

I wish to be contacted for future studies in the Exercise Nutrition Research Laboratory.

Yes _____ (check mark), No _____ (check mark) Date: _____

By signing below, I agree to participate in this study.

Name of Participant (please print): _____

Signature of Participant: _____

Date: _____

Name of Person Obtaining Informed Consent: _____

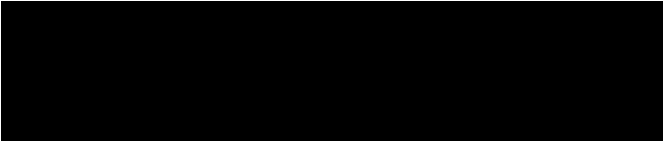
Signature of Person Obtaining Informed Consent: _____

Date: _____

You will receive a copy of the consent form after it has been signed. You do not waive any legal rights by signing the consent form.

This letter is for you to keep for future references.

Sincerely,



Curriculum Vitae

Name: Manuel Quinones

Post-secondary Education and Degrees: University of Guelph-Humber
Toronto, Ontario, Canada
2014 B.A.Sc

The University of Western Ontario
London, Ontario, Canada
2014-2016 M.Sc.

The University of Western Ontario
London, Ontario, Canada
2016-2021 Ph.D.

Honours and Awards: Lippincott Williams & Wilkins/Wolters Kluwer Health Book Prize
for outstanding undergraduate abstract
OEP (Ontario Exercise Physiology) 2014

Western Research Graduate Scholarship
2014 to 2019

Related Work Experience Teaching Assistant
The University of Western Ontario
2014-2019

Professor, Fanshawe College

- Part-time Instructor in the Fitness and Health Promotion and Massage Therapy programs.
- Instructor for various courses including:
Nutrition, Exercise Physiology, Training for sports,
Pathology

2017 – present.

Publications/Presentations:

Quinones, M.D., Vecchione, J. and Upshaw, A. Endurance and high intensity interval training similarly affect subsequent leg power and strength in young trained men. *Ontario Exercise Physiology Conference, 2014. (Winner of the Lippincott Williams & Wilkins/Wolters Kluwer Health Book Prize for outstanding undergraduate abstract).*

Joseph Vecchione, **Manuel Quinones**, Omar Choque, Adam N. Upshaw. Acute effect of two aerobic exercise modes on subsequent strength and power in young endurance trained subjects. *Bodies of Knowledge Conference – University of Toronto. June 2014*

M.D. Quinones and P.W.R. Lemon. Effect of modified corn starch ingestion on repeated sprint and soccer skill performance during a simulated soccer game. *CSEP Annual Conference, Winnipeg Manitoba Canada, 2017.*

Quinones, M. D., and Lemon, P. W. R. (2019). Hydrothermally modified corn starch ingestion attenuates soccer skill performance decrements in the second half of a simulated soccer match. *International Journal of Sport Nutrition and Exercise Metabolism.* 29(5), 498 – 504.

Quinones, M. D., and Lemon, P. W. R. (2021). Low glycemic CHO ingestion minimizes cognitive function decline during a simulated soccer match. *International Journal of Sport Science and Coaching.* doi.org/10.1177/17479541211028578