EXERCISE RESPONSES TO EXOGENOUS KETONE SUPPLEMENTS IN HUMANS

EXERCISE RESPONSES TO EXOGENOUS KETONE SUPPLEMENTATION IN HUMANS: PHYSIOLOGY, METABOLISM, AND PERFORMANCE

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LAY ABSTRACT

Ketone bodies are molecules that are naturally produced in the body. Ketone body production is increased during periods of fasting or when consuming very small amounts of dietary carbohydrates. This thesis studied the effects of increasing blood ketone levels — achieved by ingestion of commercial ketone supplements — on exercise responses in healthy active people. Drinking a ketone supplement increased the blood concentrations of ketones by several-fold as compared to a ketone-free placebo beverage. The typical stress that occurred during exercise in terms of increased work by the lungs and heart was greater after drinking the ketone supplement. These effects were unrelated to the greater blood acidity that also occurs after ketone supplementation. Simulated race performance by competitive cyclists was impaired after drinking a ketone supplement. These findings advance knowledge regarding the physiological effects of increased blood ketone levels in humans and suggest that supplementation does not enhance exercise capacity.

ABSTRACT

Ketone bodies (KB) are biologically active compounds that are increased during prolonged periods of carbohydrate restriction, e.g., in response to a ketogenic diet or after fasting. Ingestion of KB supplements is a novel method to study the effect of acutely increasing blood KB concentrations without changing diet. This thesis examined physiological, perceptual, and performance responses to acute hyperketonemia or elevated blood KB in humans. Studies 1 and 2 involved ingestion of 0.6 g/kg body mass of a commercial ketone monoester (KE) supplement 30 minutes before a 30-minute cycling bout at an intensity corresponding to ventilatory threshold. KE compared to placebo ingestion increased blood KB concentrations and decreased pH during exercise. Study 1 found that heart rate, ventilation, and rating of perceived exertion during exercise were higher after KE vs placebo ingestion. Performance during a subsequent 3 kJ/kg body mass time-trial duration was not different between treatments. Study 2 reproduced the finding of an increased heart rate and ventilation after KE vs placebo ingestion, but exercise cardiac output was not different between treatments. Peak oxygen uptake (VO_{2peak}) was not different but peak power output at VO_{2peak} was lower after KE vs placebo ingestion. None of the cardiorespiratory effects observed after KE vs placebo ingestion were altered when the KE-associated decrease in blood pH was normalized to placebo levels through bicarbonate co-ingestion. Study 3 showed that mean power output during a 20-min time-trial was lower after ingestion of 0.35 g/kg body mass KE vs placebo in trained cyclists. The lower self-selected workload after KE ingestion was

associated with a lower mean heart rate during the time-trial and a challenge to acid-base homeostasis. In summary, acute hyperketonemia elicited by KB ingestion altered selected physiological, perceptual, and performance responses during exercise in humans.

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LIST OF ALL ABBREVIATIONS AND SYMBOLS

KB	Ketone body
β-HB	β-hydroxybutyrate
ATP	Adenosine triphosphate
O_2	Oxygen
CO_2	Carbon dioxide
ADP	Adenosine diphosphate
KE	Ketone monoester
VO_2	Oxygen uptake
V_E	Ventilation
HR	Heart rate
RER	Respiratory exchange ratio
Q	Cardiac output
VO_{2peak}	Peak oxygen uptake

PREFACE: DECLARATION OF ACADEMIC ACHIEVEMENT

FORMAT AND ORGANIZATION OF THESIS

This thesis is prepared in the "sandwich" format as outlined in the School of Graduate Studies Guide for the Preparation of Theses. It includes a general introduction, three independent studies prepared in journal article format, and a general discussion. The candidate is the first author on all the manuscripts. At the time of the thesis defence, Chapter 2 was published in a peer-reviewed journal, Chapter 3 was accepted for publication in a peer-reviewed journal, and Chapter 4 was submitted to a peer-reviewed journal for consideration for publication. Chapter 4 was subsequently accepted for publication.

CONTRIBUTIONS TO PAPERS WITH MULTIPLE AUTHORSHIPS

CHAPTER 2: Increased cardiorespiratory stress during submaximal cycling after ketone monoester ingestion in endurance-trained adults

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CHAPTER 3: Effect of Acute Ketone Monoester Ingestion on Cardiorespiratory Responses to Exercise and the Influence of Blood Acidosis

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CHAPTER 4: Acute ketone monoester supplementation impairs 20-minute time-trial performance in trained cyclists: a randomized, crossover trial

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CHAPTER 1: Literature Review

1.1 Introduction

For centuries, scientists have tried to manipulate physiological and metabolic processes in an effort to enhance human performance and health (Brown-Séquard 1889). In the early 20th century, epilepsy was treated with dietary manipulations believed to alter fat metabolism. These strategies included prolonged fasting or eating very low amounts of carbohydrate and high amounts of fat (Woodyatt 1921; Peterman 1925; Wheless 2008). The latter strategy is now commonly referred to as a ketogenic diet, named for the associated accumulation of ketone body (KB) molecules in circulation (Peterman 1924; Aragon et al. 2017). The ketogenic diet seemed to have exclusively clinical applications until ~40 years ago when it was first applied to athletes in an attempt to improve endurance performance by enhancing fat oxidation (Phinney et al. 1983).

The very low carbohydrate requirement of a ketogenic diet (i.e., <50 g/d) is associated with an increased reliance on fat for energy provision during exercise (Phinney et al. 1983; Burke 2020). This finding was initially intriguing because endogenous carbohydrate stores are limited and reduced carbohydrate availability during exercise can impair performance (Coyle et al. 1986a; Alghannam et al. 2016). Interest in this topic slowed after several studies suggested that the low dietary carbohydrate inherent to a ketogenic diet did not benefit, and potentially impaired, performance in sports requiring high intensity efforts (Burke and Kiens 2006). Over the last decade, however, research into the efficacy of a ketogenic diet for athletic performance has re-emerged with an emphasis on long-term dietary adherence, performance in longer duration events characterized by lower intensities, and the role of KB (Volek et al. 2014; Burke 2015).

Exogenous KB supplements are a novel method to study the effects of altered blood KB in humans. Ingesting these supplements acutely increases blood KB without otherwise changing diet (Clarke et al. 2012a). From a mechanistic perspective, these supplements can facilitate a more direct assessment of the effects of elevated KB in humans. This is because the potential confounding influences of the profound dietary changes usually required for KB production are largely obviated. Exogenous KB supplementation also induces a unique physiological state to examine exercise responses and performance, characterized by high availability of both carbohydrates and KB. The strategy could potentially enhance endurance performance by inducing the beneficial responses associated with a ketogenic diet (i.e., carbohydrate sparing owing to enhanced fat and/or KB oxidation) without the negative consequences of low dietary carbohydrate intake (i.e., reduced peak carbohydrate oxidation rate and impaired exercise efficiency) (Evans et al. 2017; Pinckaers et al. 2017).

This thesis aimed to examine the role of elevated blood KB, elicited through exogenous KB supplementation, on endurance exercise physiology, metabolism, and performance. This was achieved through three separate studies that examined the effect of acute exogenous KB supplementation on exercise responses and performance. The first project focused on cardiorespiratory stress during submaximal exercise and time-trial performance. The second project further probed the cardiac response to KB supplementation including the potential influence of blood acid-base status. The final project examined endurance performance after KB supplementation in trained cyclists. This opening chapter provides an overview of KB metabolism in healthy humans,

examines the efficacy of exogenous KB supplements for increasing blood KB levels, and considers the effects this intervention on exercise responses.

1.2 Overview of Ketone Body Metabolism

KB are fatty acid-derived hydrocarbon molecules that are produced in the liver and named for containing a ketone functional group (Robinson and Williamson 1980). Many variations of KB theoretically exist but only β-hydroxybutyrate (β-HB), acetoacetate, and acetone are biologically relevant (Robinson and Williamson 1980). β-HB is not technically a KB, but it is metabolically linked to other KB, and it is common practice to consider it a KB (Pinckaers et al. 2017). Of note, only the D enantiomer of β-HB is relevant to metabolism and hereafter β-HB refers to D-β-HB unless stated otherwise (Hagenfeldt and Wahren 1968; Lincon et al. 1987). This subsection will provide an overview of KB production, transportation throughout the human body and their role in muscle substrate oxidation.

1.2.1 KB metabolism and transportation

β-HB, acetoacetate, and acetone are metabolically linked with each other (Woodyatt 1921; Lincon et al. 1987). β-HB and acetoacetate are interconverted by the reversable mitochondrial bound enzyme β-HB dehydrogenase (Lehninger et al. 1960; Hagenfeldt and Wahren 1968). β-HB and acetoacetate exist in an equilibrium that favours higher levels of β-HB during endogenous ketosis (Reichard et al. 1974; Balasse and Féry 1989). This observation can be explained in part by hepatic β-HB dehydrogenase

favouring β-HB production (Evans et al. 2017), mitochondrial redox state (Robinson and Williamson 1980), extrahepatic tissues having a greater capacity to oxidize acetoacetate than β-HB (Winder et al. 1974; Petrick et al. 2020), and spontaneous decarboxylation of acetoacetate to acetone (Reichard et al. 1979). Acetone has received less attention than other KB, which has been attributed to it being excreted through urine and breath (Reichard et al. 1979, 1986; Robinson and Williamson 1980; Pinckaers et al. 2017; Shaw et al. 2020). However, plasma acetone can accumulate in blood and reached ~0.8 mM in 3-d fasted humans, which was comparable to acetoacetate at ~1.2 mM, and only ~16% of the acetone was accounted for in breath and urine samples (Reichard et al. 1979). The other ~84% of the acetone may have been involved in gluconeogenesis, cellular redox state, acetate metabolism, and acid-base balance (Kalapos 2003; Glew 2010). Thus, it is important to consider β-HB, acetoacetate, and acetone during ketosis as all three are elevated simultaneously.

The metabolism of KB involves a series of reversible enzymatic reactions that primarily occur in the mitochondria (Figure 1.2.1). Additional enzymes involved in ketogenesis, i.e., the generation of acetoacetate from acetyl coenzyme A, are exclusive to the liver and thus the liver is the major site of KB production (Robinson and Williamson 1980). After their production in liver, KB can be transported across the mitochondrial membrane to the cytosol via a mitochondrial pyruvate carrier or monocarboxylate transporter and then co-transported with a hydrogen from hepatic tissue to the

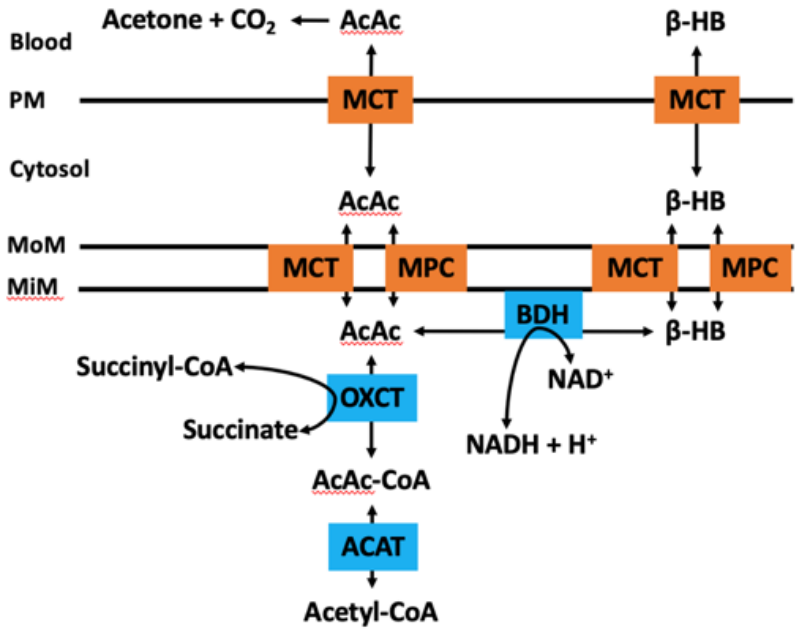

Figure 1.2.1. Overview of ketone body metabolism in extra-hepatic tissues. Note, orange boxes represent transporters and blue boxes enzymes. AcAc indicates acetoacetate; BHB, beta-hydroxybutyrate; CO_2, carbon dioxide; MCT, monocarboxylate transporter; MPC, mitochondrial pyruvate carrier; BDH, BHB dehydrogenase; CoA, coenzyme A; OXCT, succinyl-CoA:3-oxoacid CoA transferace; AcAc-CoA, acetoacetyl-CoA; ACAT, acetyl-CoA acetyltransferase; PM, plasma membrane; MoM, mitochondrial outer membrane; MiM, mitochondrial inner membrane. Modified from Petrick et al. (2020).

bloodstream via bidirectional monocarboxylate transporters (Halestrap 1978; Halestrap and Wilson 2012). KB are soluble in blood and transported in the circulation without a carrier. From the bloodstream, KB can be extracted by extrahepatic tissues such as skeletal and cardiac muscles, kidney, and brain via monocarboxylate transporters, with maximum rates of uptake occurring at ~1 mM β-HB in arterial blood (Martin et al. 2006; Halestrap and Wilson 2012; Mikkelsen et al. 2015). These extrahepatic tissues contain KB enzymes and favour KB oxidation (see section 1.2.3). Thus, KB can be produced in the liver, exported into circulation, and transported throughout the body for extraction and utilization by other tissues.

1.2.2 Nutritional Ketosis

An increase in blood KB concentration above basal levels occurs when rates of KB production exceed breakdown. Nutritional ketosis is typically defined as a blood β-HB > 0.5 mM, though the threshold depends on the blood fraction and analytical method (Krebs 1966; Guimont et al. 2015; Norgren et al. 2020). This state can occur during conditions of low carbohydrate availability and conversely high rates of fatty acid oxidation, e.g., after fasted exercise, prolonged fasting, starvation, and adherence to a ketogenic diet (Figure 1.2.2). The initial rise in circulating KB, i.e., up to ~7 mM, can be primarily attributed to augmented rates of hepatic ketogenesis (Féry and Balasse 1986). Further increases in blood KB are then attributable to a decrease in peripheral tissue utilization and reduced ketogenesis via KB feedback inhibition (Reichard et al. 1974; Balasse and Féry 1989). Together, these may partially explain the plateau in blood KB levels of ~10 mM in non-diabetic ketosis (Balasse and Féry 1989). However, total blood KB levels of ~25 mM have been observed in patients with uncontrolled diabetes,

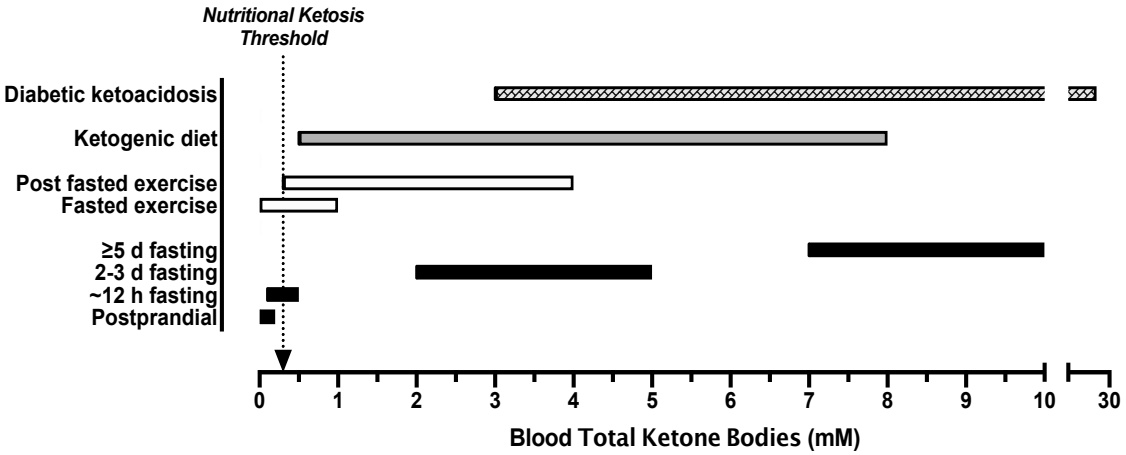

Figure 1.2.2. Total ketone body (i.e., β-hydroxybutyrate plus acetoacetate) concentration in venous blood plasma under various metabolic scenarios. Bars represent ranges. References: Johnson and Walton 1972; Balasse et al. 1978; Robinson and Williamson 1980; Féry and Balasse 1983; Phinney et al. 1983; Balasse and Féry 1989; Pinckaers et al. 2017; Burke et al. 2017, 2020; Evans et al. 2017; McSwiney 2018; and Anderson et al. 2021.

suggesting other mechanisms can be involved (Reichard et al. 1986). The ketogenic diet is unique as it is the only state of nutritional ketosis that allows food intake and is therefore sustainable for longer durations compared to other methods (Pinckaers et al. 2017). On this diet, blood KB concentrations do not usually exceed ~4 mM but can reach 8 mM in some cases (Phinney et al. 1983; Volek et al. 2015; Burke et al. 2017; McSwiney et al. 2017; Pinckaers et al. 2017). Thus, various methods can induce nutritional ketosis and to different magnitudes, which may affect KB metabolism differently.

1.2.3 KB oxidation

KB can be oxidized during exercise under conditions of nutritional ketosis. KB was estimated to account for ~10% of total energy expenditure during moderate intensity exercise in 5-d fasted participants, as determined by stable isotope KB tracer methods (Balasse et al. 1978). KB oxidation also occurred during exercise in postabsorptive and overnight fasted humans when intravenous KB infusion increased circulating KB levels to ~5-6 mM, under the presumption that KB rate of disappearance was equivalent to oxidation (Féry and Balasse 1983, 1986, 1988). There are no KB oxidation data in postprandial participants nor with an interaction of exogenous carbohydrates. Petrick et al. (2020) attempted to mimic exercise in fed humans and KB-carbohydrate interactions at the mitochondrial level by adding physiological levels of pyruvate and KB to isolated mitochondrial preparations derived from human skeletal muscle samples. They demonstrated that KB and pyruvate both independently stimulated oxidative

phosphorylation, but the magnitude of such was several-fold higher with pyruvate. Moreover, when KB were added to a preparation already containing physiologically relevant concentrations of pyruvate, oxidative phosphorylation was not further increased (Petrick et al. 2020). A limitation of this design is it does not account for glycolysis, i.e., pyruvate generation rates, though glycolytic enzymes generally have higher maximal activities than ketolytic enzymes (Winder et al. 1974; Petrick et al. 2020). One exception is hexokinase – the enzyme required before blood glucose enters glycolysis – which may have a lower maximum activity than the enzymes involved in acetoacetate oxidation but not BHB dehydrogenase (Baldwin et al. 1973; Winder et al. 1974). This suggests skeletal muscle may have a greater capacity to oxidize acetoacetate than blood glucose. This effect may also depend on muscle fibre type as more oxidative skeletal muscle fibres had greater KB enzymes contents but lower contents of glycolytic enzymes. Overall, the oxidation of KB in tissues depends on other metabolic substrates and muscle type but represents a low percent of total substrate oxidation.

It has been proposed that KB are more "efficient" fuels than carbohydrates and fats (Veech 2004). This argument is partially based on the higher theoretical energy yield of β-HB vs pyruvate, ~1019 vs 777 kJ per mol of carbon (Veech 2004; Pinckaers et al. 2017). However, comparing β-HB to glucose (993 kJ/mol of carbon) may be more reflective of reality because almost all the pyruvate oxidized by muscles originates as glucose. Alternatively, a better metric from an exercise metabolism lens might be adenosine triphosphate (ATP) produced per oxygen consumed. Oxidative phosphorylation synthesises ATP, which is then used to fuel cellular energetic processes,

but consumes O_2, therefore a higher ATP/O_2 would indicate better substrate efficiency. This metric was not different when β-HB and pyruvate were acutely added to isolated mitochondria (Petrick et al. 2020). Though 24 h exposure of myocytes to β-HB improved mitochondrial coupling and efficiency (Parker et al. 2018). Theoretically, 1 mol of β-HB oxidized yields ~27 mol of ATP while consuming 4.5 mol of O_2 (6.0 ATP/O_2) and 1 mol acetoacetate oxidized yields ~24 ATP and consumes 4 mol O_2 (6.0 ATP/O_2). In comparison, glucose oxidation produces ~38 ATP, ~39 ATP if from muscle glycogen, and consumes 6 mol O_2 (6.3 ATP/O_2). Thus, glucose may be a similarly if not more efficient fuel than KB in addition to producing more ATP per mol substrate.

1.2.4 Effects on carbohydrate and fat oxidation

Assuming KB are a source of ATP, for a given energy demand the oxidation of carbohydrates and fats may be reduced. In non-ketosis scenarios, carbohydrate and fat oxidation are inversely related and can be non-invasively calculated using indirect calorimetry assuming no protein contribution (Frayn 1983; Peronnet and Massicotte 1991). These estimations are also based on the assumption that all carbon dioxide (CO_2) produced is from carbohydrate or fat oxidation, which may be violated in nutritional ketosis since KB produce CO_2 during their oxidation and CO_2 is produced during the decarboxylation of acetoacetate to acetone (Balasse et al. 1978; Reichard et al. 1979). Alternatively, substrate oxidation can be determined using stable isotope tracer molecules and/or delta concentrations of intramuscular substrates determined using biochemical analyses or transmission electron microscopy, and the presence of KB presumably does

not violate the underlying assumptions (Romijn et al. 1993; van Loon et al. 2001; Stellingwerff et al. 2007; Jensen et al. 2020). There are limited data regarding the effects of KB on direct markers of carbohydrate (blood glucose and muscle glycogen) and fat (blood free fatty acids and intramuscular triglycerides) utilization during exercise in humans.

Intravenous KB infusion decreased blood glucose and free fatty acids concentrations, which may affect the oxidation of these substrates (Fajans et al. 1964; Balasse and Ooms 1968). KB reduced hepatic glucose efflux despite increased glucagon-to-insulin ratio (Mikkelsen et al. 2015; Evans et al. 2017). Likewise, adipose tissue lipolysis is reduced by KB, which is mediated in part through KB binding to nicotinic acid receptors on adipose tissue (Taggart et al. 2005; Mikkelsen et al. 2015). During exercise without the presence of KB, lowering blood FFA concentrations was associated with higher blood glucose and muscle glycogen oxidation, and similarly low blood glucose concentration was associated with higher fat oxidation (Coyle et al. 1986b; Stellingwerff et al. 2003; O'Neill et al. 2004). Thus, KB may reduce the oxidation of blood glucose and free fatty acids during exercise by lowering the concentrations of such in circulation, but direct measurements are required to confirm this.

KB may also affect substrate oxidation regardless of changes to circulating concentrations of glucose and free fatty acids. To address this issue, circulating glucose and free fatty acids concentrations were normalized by glucose and heparin intravenous infusions while acetoacetate was simultaneously infused to increased blood KB to 0.4-1.0 mM (Hagenfeldt and Hospital 1979; Beylot et al. 1986; Walker et al. 1991). This model

showed that high KB levels were associated with a ~50% reduction in basal plasma FFA uptake into skeletal muscle, though not statistically significant, and a decrease in basal but not insulin-stimulated glucose uptake. The insulin-stimulated data may be more applicable to exercise because both insulin and muscular contraction promote glucose uptake by augmenting glucose transporter 4 translocation from intracellular stores to the plasma membrane (Bradley et al. 2015). This relationship should also be tested with higher levels of KB as intracellular β-HB of ~3 but not ~1 mM inhibited insulin-stimulated glucose uptake in rodent skeletal muscle as well that plasma β-HB of ~3 mM reduced myocardial glucose uptake (Yamada et al. 2010; Gormsen et al. 2017). Thus, KB may directly inhibit glucose and free fatty acid uptake into tissues, however these mechanistic studies may not reflect what occurs naturally.

The effects of KB per se on intramuscular fuel oxidation in humans are unknown but changes in regulators of rate-limiting enzymes involved in these processes may provide insights. The oxidation of glucosyl units derived from glycogen in skeletal muscle is primarily controlled by three rate-determining enzymes, phosphorylase, phosphofructokinase, and pyruvate dehydrogenase (Holloway and Spriet 2012). Glycogenolysis, which reflects phosphorylase activity, in rodent muscle preparations designed to simulate moderate intensity exercise was unaffected by acetoacetate (Maizels et al. 1977). However, adding acetoacetate to skeletal muscle samples increased muscle citrate content and thereby reduced phosphofructokinase activity, though this effect may be exclusive to highly-oxidative muscle fibres (Berger et al. 1976; Maizels et al. 1977). The addition of acetoacetate to isolated mitochondria also increased acetyl coenzyme A

content resulting in reduced substrate flux through, but not the transformation of, pyruvate dehydrogenase (Ashour and Hansford 1983). This may explain the observed reduced lactate oxidation by acetoacetate to in the perfused hindquarter of exercising rats (Berger et al. 1976). Alternatively, the lower lactate oxidation may relate to a reduced lactate uptake into muscles via monocarboxylate transporter competition with KB since the direct inhibition of pyruvate dehydrogenase by acetyl coenzyme A may not persist during exercise (Hargreaves and Spriet 2020). The KB mediated increase in acetyl coenzyme A may affect phosphorylase and pyruvate dehydrogenase through other mechanisms as augmenting resting muscle acetyl coenzyme A levels via dichloroacetate supplementation reduced skeletal muscle glycogenolysis and pyruvate dehydrogenase flux during exercise (Howlett et al. 1999). This finding may relate to quicker matching of ATP supply to demand at exercise onset and thereby mitigating the increase in adenosine diphosphate (ADP), which is a potent regulator that increases substrate flux through phosphorylase and pyruvate dehydrogenase. In contrast, KB may upregulate intramuscular triglyceride oxidation as adding acetoacetate to rodent skeletal muscle increased resting acetyl-carnitine content (Berger et al. 1976). Increasing muscle carnitine levels via carnitine supplementation was associated with greater fat oxidation during exercise, which was associated with an increased, though not statistically significant, intramuscular triglyceride breakdown (Chee et al. 2021). This effect was related to higher activity of carnitine palmitoyl transferase 1, the rate limiting enzyme in fatty acid oxidation, as well as a lower pyruvate dehydrogenase flux (Wall et al. 2011; Stephens et al. 2013). Overall, KB may alter the contents of intramuscular regulators of key enzymes

involved in intramuscular carbohydrate and fat oxidation. Measurements of such regulators in human tissue and direct measures of intramuscular substrate breakdown are required.

1.2.5 KB metabolism summary

The metabolism of KB contains many regulated processes. KB are produced in the liver, transported across tissue membranes, carried throughout the body in the bloodstream, and may be a fuel and signalling molecule in tissues. KB accumulate in the bloodstream during situations of low carbohydrate availability, a physiological state deemed nutritional ketosis. In this state, KB can be oxidized as a fuel during exercise, but the magnitude of this process is seemingly low when rates of carbohydrate oxidation are high. The presence of KB may also influence carbohydrate and fat oxidation, but this potential interactive effect has not been thoroughly examined in humans. While the mechanistic potential exists for KB to influence the oxidation of individual fuels, direct measures of substrate oxidation are required to determine how potent these effects of KB are during exercise in humans and how all these potential effects of KB will work in concert.

1.3 Exogenous Ketone Body Supplementation

KB supplements contain exogenous KB molecules or compounds that stimulate ketogenesis, e.g., butanediol is converted into β-HB in the liver. Oral ingestion of exogenous KB supplements induces nutritional ketosis without the need to restrict dietary

carbohydrate as required for endogenous ketosis (Clarke et al. 2012a, 2012b). Nutrition ketosis elicited through supplementation differs from KB infusion and endogenous ketosis because the KB must pass through the digestive tract and be absorbed into the bloodstream. It is therefore important to review the digestion and pharmacokinetics of exogenous KB supplements. Since various exogenous KB supplements exist, classified by their biochemical structures, each may exhibit unique digestive and pharmacokinetic effects and will therefore be considered individually.

Table 1.3.1. Chemical composition of exogenous ketone body and ketogenic supplements.

Supplement Type	Chemical Composition
Ketone Body	
Ketone monoester (KE)	D-β-hydroxybutyrate + D-1,3-butanediol
Ketone salt	$Na^+/K^+/Ca^{2+}$ + D/L-β-hydroxybutyrate
Acetoacetate diester	2*(acetoacetate) + D/L-1,3-butanediol
Ketogenic	
Butanediol	D/L-1,3-butanediol
MCT	Fatty acid with a 6-10 carbon chain

1.3.1 Digestion

Ingested KB supplements initially pass through the oral cavity without being subjected to any preliminary metabolism. The inherently bitter flavour of KB and butanediol may serve to simulate the activity of bitter taste receptors in the mouth and throat and impact central nervous function, but direct evidence for this is lacking. Other nutritional manipulations such as carbohydrate and caffeine ingestion stimulate oral taste receptors that have been shown to increase activity of brain centres associated with reward and pleasure (Chambers et al. 2009; Wickham and Spriet 2018).

Gastric emptying of KB supplements from the stomach into the small intestine and absorption from the intestines into the bloodstream are poorly understood. Utilizing

blood acetaminophen concentration as a crude proxy for the sum of gastric emptying and intestinal absorption indicated that ketone monoester (KE) ingestion did not affect the rates of such compared to water (Greaves et al. 2020). Application of general gastric emptying principles suggests that if supplementing beverages with KB increases beverage osmolality, then gastric emptying may be slowed compared to a beverage with a lower osmolality (Vist and Maughan 1992; Horner and Schubert 2015). Thus, exogenous KB supplementation may slow gastric emptying rates, but this effect may not be due to KB per se, and direct measures of gastric emptying are required to confirm this speculation, e.g., double sampling gastric aspiration technique.

After emptying from the stomach, ketone ester and salt supplements are metabolized in the small intestine (Shivva et al. 2016; Shaw et al. 2020). Ketone salt molecules dissociate, producing a molecule of β-HB and mineral (Shaw et al. 2020). Ketone esters are cleaved by non-specific gut esterase enzymes into their components plus a hydrogen ion, i.e., KE + H_2O → D-β-HB + D-1,3-Butanediol + H^+ (Shivva et al. 2016). The conjugate acids of β-HB or acetoacetate produced also dissociate a hydrogen because pKa of these acids is less than that of the small intestine (pKa = 4.7, 3.7, and 6.0 respectively). Thus, the metabolism of ketone esters in the small intestine may impact acid-base balance.

From the intestines, the KB and ketogenic molecules can be transported to, perhaps via sodium-dependent mechanisms, and metabolized in the liver or remain in the gut to be excreted in feces (Martin et al. 2006; Shivva et al. 2016). In the liver, some of the KB will undergo inter-ketone conversion and butanediol is metabolized into β-HB via

alcohol dehydrogenase (Shivva et al. 2016). The latter requires <1 h in most situations but can take up to 4 h after large doses of supplements in some participants (Clarke et al. 2012a). Once in the liver, exogenous KB are metabolized similar to endogenous KB (Shaw et al. 2020). Thus, the liver plays a key role for the ketogenic effect of exogenous KB supplements.

1.3.2 Pharmacokinetics

The limited pharmacokinetic data suggests that exogenous KB supplements enter the bloodstream in <30 min and remain elevated for hours (Table 1.3.2). Obtaining accurate pharmacokinetic data from exogenous KB supplements is challenging because the ingested KB have several potential fates, including inter-KB conversion, oxidation in tissues, passive excretion in breath, urine, and feces (Reichard et al. 1979; Balasse and Féry 1989; Shivva et al. 2016; Stubbs et al. 2017). A stable isotope tracer study using labelled KE could only account for ~23-43% of the ingested KB in breath plus β-HB in urine and fluid compartments (Dearlove et al. 2021). These percentages would likely be higher if acetoacetate was also assessed, but it is unlikely acetoacetate itself makes up the remainder. Since basal blood KB are <0.2 mM under normal feeding conditions and the presence of KB can inhibit endogenous ketogenesis (Balasse and Neef 1985; Lincon et al. 1987), a change in blood KB above basal levels can signal that exogenous KB have entered the bloodstream (Robinson and Williamson 1980; Evans et al. 2017; Norgren et al. 2020). Most studies report an increase in blood KB above basal levels in the first blood sample obtained at 30 min, therefore determining the precise onset of KB entering

circulating is limited by the timing of this sample. Increased sampling frequency after supplementation can increase the precision these measurements.

Table 1.3.2. Effects of ingesting ~350 mg/kg body mass of various exogenous ketone body supplements in fasted humans on blood D-β-hydroxybutyrate (D-β-HB) parameters.

	References	Onset in Blood (min)	[30 min post] (mM)	[Peak] (mM)	Time > basal (h)
Ketone monoester (KE)	(Stubbs et al. 2017)	<10	2.5	2.8	3.0-4.0
Ketone salt	(Stubbs et al. 2017)	<20	0.6	1.0	2.0-2.5
Acetoacetate diester	(Leckey et al. 2017)	<30	0.3	0.5	> 2.5
Butanediol	(Shaw et al. 2019)	<30	0.7	0.8	> 3.5
MCT	(Horowitz et al. 2000)	< 30	0.2	0.2	> 2.0

MCT = medium chain triglyceride.

While all exogenous KB supplements can induce nutritional ketosis, the KE supplement appears to elevate the physiologically relevant blood D-β-HB more than other supplements when matched for dose (Table 1.3.2). This is partially attributable to the KE providing β-HB and butanediol entirely in the D isoform as opposed to racemic compounds in other supplements (Stubbs et al. 2017; Shaw et al. 2020). Additionally, ketone esters contain ~2-3-fold more KB and ketogenic molecules per mol of supplement compared to ketone salts and butanediol (Table 1.3.1). However, comparing blood β-HB between studies is complicated because β-HB is measured in different blood fractions using different determination methods (i.e., lab-based assay vs point-of-care analyzer), both of which affect the blood β-HB determined (Armer et al. 2013; Guimont et al. 2015; Norgren et al. 2020). Future research should compare β-HB determined in different blood fractions using various determination methods to develop methods for comparing blood

β-HB between studies. Nutritional state and fitness may also affect the rise in blood KB after supplementation (Johnson and Walton 1972; Stubbs et al. 2017). Finally, the KB acetoacetate is seldomly measured after exogenous KB supplementation despite blood levels of ~1 mM being reported in some cases (Cox et al. 2016; Stubbs et al. 2017). This is likely because of its degradation in stored blood samples and point-of-care β-HB determination proving an easy, inexpensive, and relatively accurate method (Price et al. 1977; McNeil et al. 2014; Guimont et al. 2015; Norgren et al. 2020). To best determine the efficacy of exogenous KB supplementation for raising blood KB levels acetoacetate must also be considered, i.e., total blood KB = β-HB + acetoacetate. Nonetheless, biochemical structure of KB supplements may influence the rise in KB after ingestion.

Increasing the dose of KB supplement ingested increases the magnitude of ketosis induced but may also increase adverse side-effects. The maximum blood KB after supplementation is unknown, however a higher blood KB level may not be more beneficial (Evans et al. 2017). Ingesting a ~65 g does of KE, which is ~2.5 times higher than the typical commercially recommended dose, raised blood β-HB to 4.5 mM on average during cycling (Poffé et al. 2020a, 2021). This is the highest mean blood β-HB reported to date, and it was associated with minimal side effects, gastrointestinal distress less than mild in severity and mild blood acidosis (Poffé et al. 2020a, 2021). In contrast, ingestion of either acetoacetate diester, medium chain triglyceride, or ketone salt supplement raised blood β-HB <1 mM and was associated with moderate to severe gastrointestinal distress during exercise, including vomiting and diarrhea (Goedecke et al. 1999; Leckey et al. 2017; Evans and Egan 2018; Harvey et al. 2018; Waldman et al.

2018). The small increase in gastrointestinal symptom severity during exercise is commonly reported after ingestion of KE with carbohydrate compared to carbohydrate matched beverages containing lower energy content (Evans and Egan 2018; Greaves et al. 2020; Poffé et al. 2020a, 2020b, 2021). Gastrointestinal symptom severity was however not different when the KE and carbohydrate beverage was compared to an energy-matched carbohydrate-only control (Stubbs et al. 2019). Therefore, beverage energy content and the associated osmolality may play a role in gastrointestinal distress. Indeed, gastrointestinal symptom severity during exercise has been associated with a slowing of gastric emptying rate, which can occur as beverage osmolality increases (Rehrer et al. 1990; Horner and Schubert 2015). Thus, the tolerability of KB supplements may be related to supplement type and dose as well as beverage osmolality, but not the associated rise in blood KB per se.

In summary, the available pharmacokinetic data on exogenous KB supplements suggests these are an effective and non-invasive method to raise circulating KB in humans quickly and for a prolonged duration. The KE supplement is relatively well-tolerated and may induce a magnitude of nutritional ketosis that is greater than other supplements and comparable that achieved during endogenous ketosis (Evans et al. 2017; Stubbs et al. 2017; Shaw et al. 2020). Conversely, the doses of other KB supplements required to induce nutritional ketosis could be associated with moderate-to-severe gastrointestinal distress. Thus, the KE supplement appears to be optimal for studying the effects of acute nutritional ketosis in humans.

1.4 Exercise Responses to Acute Exogenous Ketone Body Supplementation

The ingestion of exogenous KB supplements before exercise serves as a method to increase circulating KB levels, as reviewed in section 1.3. This practice potentially affects exercise responses owing to the physiological and metabolic effects of KB in humans, as reviewed in section 1.2. The utility of exogenous KB ingestion makes this supplementation strategy an attractive method to potentially alter exercise performance particularly in endurance-type events or those that rely on a high rate of aerobic energy metabolism. While some research has examined the effect of more prolonged exogenous KB supplementation and postexercise ingestion to promote exercise recovery (Clarke et al. 2012b; Holdsworth et al. 2017; Vandoorne et al. 2017; Poffe et al. 2019; Evans et al. 2022), these are beyond the scope of the present review. This section will consider the acute effects of exogenous KB ingestion on exercise responses, including cardiorespiratory, oxidation of KB, carbohydrates and fats, and endurance performance.

1.4.1 Cardiorespiratory responses

There is no clear effect of acute ketone body supplementation on pulmonary oxygen uptake (VO_2), which is a marker of exercise efficiency/economy when expressed relative to workload (Margolis and Fallon 2020; Valenzuela et al. 2020). There is some data reporting that butanediol ingestion increased exercise VO_2 at a given absolute intensity, though this observations is not universal (Rodger et al. 2017; Evans et al. 2018; Scott et al. 2018; Shaw et al. 2019). Alternatively, KE ingestion improved markers of exercise economy when plasma β-HB was ~2 mM, but these effects were no longer

present when plasma β-HB was increased to ~4 mM (Dearlove et al. 2019b, 2021). Further, KE ingestion increased exercise VO_2 at a constant workload when plasma β-HB was ~3-4 mM (Poffé et al. 2020a). Thus, exogenous KB supplement type and the associated rise in blood β-HB may independently influence the effects of exogenous KB ingestion on exercise VO_2 and thereby economy. The potential underlying mechanism(s) may relate to O_2 consumption by cardiac, respiratory, and skeletal muscles since pulmonary VO_2 is the sum of these processes.

Peer-reviewed data examining the effects of exogenous KB ingestion on cardiovascular responses during exercise are limited to heart rate (HR). All but one study found that KB supplementation does not affect constant-load exercise HR compared to placebo, which suggests cardiac function is unaffected by this practice (Valenzuela et al. 2020). One study reported that ketone salt ingestion increased exercise HR but used unflavoured water as the control (Evans et al. 2018). This could be due to potential inotropic effects of bitter taste receptor activation on cardiac tissues by KB (Lu et al. 2017; Bloxham et al. 2020). The lack of effects reported in the literature may relate to supplement dose and the associated rise in circulating β-HB as it has been proposed that blood β-HB must exceed 1 mM to affect certain exercise responses (Evans et al. 2017). The only study that achieved this criterion during steady-state exercise reported that KE ingestion increased plasma β-HB to ~1 mM during 1 h of moderate intensity running but heart rate was not different compared to a placebo (Evans et al. 2019). Other KB supplementation studies have reported blood β-HB levels between 2 and 3 mM during intermittent exercise but HR was unaffected (Evans and Egan 2018; Poffé et al. 2020a,

2021). Future research could measure HR during constant load exercise at higher levels of blood KBs and perhaps other measures of cardiovascular function (e.g., blood pressure, stroke volume) as well.

The limited data on indices of respiratory work during exercise suggests it may be increased by high dose KB supplementation. Pre-exercise KE ingestion increased maximal, but not submaximal, ventilation (V_E) by ~8 L/min during an incremental exercise test to exhaustion despite no difference in peak workload achieved (Dearlove et al. 2019b). Similarly, KE ingestion increased V_E by ~9 L/min during an 3 h intermittent exercise protocol (Poffé et al. 2020a). Blood β-HB in both of these studies was ~2-3 mM, which may be important as ketone salt ingestion increased blood β-HB to 0.4 mM but did not affect exercise V_E (Evans et al. 2018). Replicating the higher V_E associated with high blood KB levels during steady-state exercise is necessary to confirm this effect as the current data are limited to intermittent or incremental exercises that may not elicit a physiological steady state.

The mechanism underlying the hyperventilation associated with exogenous KB supplementation may relate in part to acid-base buffering. Mild acidosis is commonly reported after KE ingestion and the magnitude of such is positively correlated with plasma β-HB concentration and thereby the dose of supplement ingested (Dearlove et al. 2021). The additional hydrogen ions may be buffered by blood bicarbonate and, according to the Henderson-Hasselbach equation, increase blood partial pressure of CO_2 and stimulate V_E (Yamashiro et al. 2021). By the same logic, HR and VO_2 should also be increased to reflect the additional O_2 demand to expire excess CO_2. However, this is not

always observed, as previously discussed. It could be that ingestion of higher supplement doses and therefore greater blood acidosis may be required to augment exercise HR and VO_2. This theory was in part assessed through KE and sodium bicarbonate co-ingestion that increased blood β-HB to ~3 mM and maintained a neutral pH ~1 h into exercise, but V_E was still increased compared to control (Poffé et al. 2020a). While this suggests other factors are involved, V_E in that same study was not different than the placebo condition ~3 h into exercise, albeit at this time β-HB was <1 mM and pH was higher in the co-ingestion vs control condition. The intermittent nature of the exercise protocol and carbohydrate ingestion during exercise may further complicate extrapolating these data. Future research is required to confirm the effect of exogenous KB ingestion on indices of cardiorespiratory stress and whether blood acidosis plays a role. Additionally, the literature may benefit from a simpler study design that still uses co-ingestion of KE and bicarbonate as a method to mitigate the confounding effects of pH.

1.4.2 Substrate metabolism

The oxidation of ingested exogenous KB are not well understood. The best examination of this to date reported that ingestion of the U-^{13}C KE resulted in KB comprising <5% of exercise energy expenditure at intensities ranging from 25-75% peak power output when plasma β-HB was ~2-5 mM (Dearlove et al. 2021). This reported relative magnitude of KB oxidation during exercise is comparable to leucine oxidation, which is considered negligible and often ignored when estimating substrate oxidation (Phillips et al. 1993). However, more work may be required to validate the U-^{13}C KE.

Less than 50% of the ingested U-^{13}C KE was unaccounted for in breath and body fluid compartments. While measuring ^{13}C acetoacetate, acetone, and glucose may increase the amount of ingested tracer recovered, it seems unreasonable that these would account for the remainder. Furthermore, the exercise protocol involved 20 min of cycling at each intensity, which may not have been long enough for the ingested tracer to achieve steady-state kinetics when considering that ~1 h of constant-load exercise is required before steady-state calculations can be accurately made in ^{13}C-glucose ingestion studies (Trommelen et al. 2017). Thus, more work using stable isotope traceable KB supplements are required before definitive statements regarding their oxidation can be made, although the current perspective of some researchers is to look beyond the role of KB as a metabolic fuel (Poffé and Hespel 2020).

The current data examining the effects of acute KB supplementation on whole-body substrate oxidation are difficult to interpret. A lack of data in this regard relates in part to the difficulties in extrapolating carbohydrate and fat oxidation from non-invasive gas exchange measures, i.e., respiratory exchange ratio (RER), as the inherent complications associated with KB are furthered by the blood acidosis accompanied by ingesting a large bolus of exogenous KB. Nonetheless, most studies comparing exogenous KB supplement ingestion to a placebo reported no difference in exercise RER, though some studies have reported lower RER and others higher (Valenzuela et al. 2020). Any additional CO_2 produced during the process of mitigating the blood acidosis associated with exogenous KB ingestion may therefore mask a small increase in fat oxidation. That is, acid-base buffering could be increasing RER while a shift towards

higher relative fat oxidation is decreasing RER, therefore resulting on no overall effect. Buffering these excess hydrogens by oral bicarbonate co-ingestion could prove useful for interpreting RER data in this regard. Alternatively, ingestion of ketone salts at a dose that was shown elsewhere to not affect blood pH (Stubbs et al. 2017) raised capillary β-HB to ~0.8 mM and reduced RER, indicative of higher relative fat oxidation, as compared to a placebo (O'Malley et al. 2017). This study is unique in the literature as carbohydrates were not co-ingested with exogenous KB, which also might affect RER (Coyle et al. 1986a), and there was likely no change in blood pH, therefore omitting the potential influence of two confounding variables. Future research should attempt to clarify the influences of exogenous KB and blood pH to exercise RER as it may help interpret the current gas exchange data.

There are limited direct data regarding the effects of exogenous KB ingestion on muscle glycogen and intramuscular triglyceride utilization. Cox et al. (2016) reported KE and carbohydrate ingestion before and during 2 h of constant-load cycling at ~70% of maximum VO_2 reduced muscle glycogen and augmented intramuscular triglyceride breakdown compared to an energy-matched carbohydrate only beverage. In contrast, Poffé et al. (2020b) found that ingestion of 75 g of KE with carbohydrates before and at the beginning of a 3 h intermittent submaximal cycling protocol did not affect muscle glycogen or intramuscular triglyceride breakdown compared to a carbohydrate-matched placebo. These inconsistent findings may be explained by substrate analytical technique, the carbohydrate content of the control beverages, exercise protocol, and KE supplementation strategy. This area of research requires clarification and could benefit

from studies that do not involve KB and carbohydrate co-ingestion to facilitate a more direct examination of exogenous KB.

Exogenous KB ingestion reduces blood glucose and free fatty acid concentrations, but if remains unclear if the oxidation of either of these substrates is changed. As noted earlier, reducing the concentration of either blood glucose or free fatty acids may increase the oxidation of the other. This is difficult to assess though since both blood substrates are generally lower after KB ingestion there may be other effects of supplementation which could in turn influence their oxidation, e.g., blood pH, catecholamine availability, and muscle acetyl coenzyme A and acetyl-carnitine contents (Howlett et al. 1999; Hollidge-Horvat et al. 1999; Watt et al. 2001). Stable isotope tracers are necessary to determine the effects of exogenous KB supplements on blood substrate oxidation.

1.4.3 Exercise performance

Exogenous KB supplements have received considerable interest as a potential ergogenic aid. A seminal paper by Cox et al. (2016) reported that KE ingestion improved performance during a 30-min cycling time-trial that followed a 60-min constant-load bout in endurance-trained cyclists. However, several follow-up studies have been unable to corroborate this finding, including multiple reports of potential ergolytic effects associated with acute exogenous KB supplementation (Leckey et al. 2017; O'Malley et al. 2017; Poffé et al. 2021). These seemingly discrepant findings suggest that the potential effects of exogenous KB supplements depend on several factors, including supplement type, the rise in blood BHB concentration, participant training status, and exercise

intensity and duration (Evans et al. 2017; Stubbs et al. 2018; Dearlove et al. 2019a; Margolis and Fallon 2020). The KE supplement has emerged as the preferable exogenous KB supplement type because it is well tolerated and is the only supplement capable of increasing blood β-HB above a hypothesized ergogenic threshold (Evans et al. 2017; Stubbs et al. 2017, 2018, 2019; Margolis and Fallon 2020). It has been speculated that endurance-trained individuals may have a greater ability to utilize the ingested KB and therefore are more likely to respond to supplementation (Evans et al. 2017).

The potential influence of the various factors noted above is apparent by comparing data from Cox et al. (2016) to others who did not report ergogenic effects of exogenous KB supplementation. Digitization of and post-hoc calculations on the Cox et al. (2016) data revealed the statistical comparison between KE and placebo ingestion achieved a p-value of ~0.05 and ~50% power, i.e., 50% chance of false positive result. In actuality, significant effects that achieved p=0.05 were only replicated in subsequent studies ~50% of the time (Gandevia 2021). Other exogenous KB supplementation studies reported that ingesting similar amounts of KE before ~1 h of exercise did not affect and impaired subsequent 30-min time-trial performance (Evans et al. 2019; Poffé et al. 2021). Additionally, the Cox et al. (2016) study involved a relatively small sample (n=8), did not match carbohydrate content between the experimental and control beverages, did not familiarize the participants with the exercise protocol, and any changes in 30-min time-trial performance may have resulted from a change during the constant-load preceding exercise rather than the time-trial per se. Overall, an ergogenic effect associated with exogenous KB ingestion must be replicated before factors modifying this response should

be discussed. This body of research could benefit from a simpler study design that does not involve carbohydrate co-ingestion, implements a validated time-trial protocol that entirely simulates an endurance sporting event, and is adequately powered.

Leckey et al. (2017) arguably conducted the most externally valid study to date examining the effect of exogenous KB supplement on cycling time-trial performance, but it is also limited by the fact the KB supplement used was acetoacetate diester. Leckey et al. (2017) had 10 professional male cyclists complete a simulated world championship cycling course that was ~31 km in length while adhering to best practice sport nutrition guidelines. Time-trial performance was impaired in the acetoacetate diester compared to placebo condition, however the authors reported that the supplement was associated with gastrointestinal distress in every participant and blood total KB concentration was only ~0.7 mM. Future research in this regard should implement a similar experiment design to Leckey et al. (2017) but consider using the KE supplement to further increase blood KB concentration and avoid gastrointestinal upset.

1.4.4 Exogenous KB supplementation and exercise responses summary

Overall, the effects of acute exogenous KB ingestion on exercise responses are not well understood. More work is required to elucidate any potential effects of this practice on cardiorespiratory, metabolic, and performance responses during exercise. This field of research may benefit from simpler study designs that do not provide carbohydrate during exercise and use steady-state exercise protocols that help determine physiologic and metabolic mechanisms associated with exogenous KB supplementation, which may then

translate to altered athletic performance. While these supplements were initially thought to provide a direct method of examining the effects of KB in exercising humans, the drop in pH associated with these supplements complicates this direct interpretation. A lower pH could increase blood partial pressure of CO_2, stimulating V_E to clear the excess CO_2, then increasing cardiac output (Q), HR, and pulmonary VO_2 to reflect the increased muscular work. Data involving KE and sodium bicarbonate ingestion somewhat support this sentiment, but future work is required to elucidate the influence of blood pH in this regard. Furthermore, blood acidosis was associated with a decrease in skeletal muscle carbohydrate metabolism during exercise and an impairment of exercise performance (Parolin et al. 1999; Carr et al. 2011).

1.5 Purpose of the thesis

The overarching purpose of this thesis was to study the influence of increased KB during exercise on physiological, perceptual, and performance responses in humans. Blood KB concentrations were acutely increased through ingestion of a commercial KE supplement without otherwise changing diet. The purpose of the first study was to examine the effects of KE ingestion on cardiorespiratory exercise responses that could underpin a change in time-trial performance. Endurance-trained adults ingested either 0.6 g/kg body mass of a KE or a flavour-matched placebo and then cycled for 30 minutes at a constant load intensity approximating ventilatory threshold followed by a 3 kJ/kg body mass time-trial (~15-min). Based on the available data at the time (Cox et al. 2016, Evans et al. 2017), it was hypothesized that KE ingestion would reduce markers of

cardiorespiratory and perceived stress and improve time-trial performance. Study 2 sought to examine the effect of KE ingestion on Q during exercise and the influence of KE-associated blood acidosis. Since this study followed up on the results of Study 1, the KE supplementation strategy and 30 min exercise protocol were identical. It was hypothesized that KE ingestion would increase Q at the end of the 30-min constant load exercise bout compared to placebo and that co-ingestion of KE and bicarbonate would attenuate this effect. After the period of constant-load cycling, a peak oxygen uptake (VO_{2peak}) test was performed to further probe the effects on oxygen transport and workload capacity. The purpose of Study 3 was to determine if acute KE supplementation altered performance during a test that closely simulated typical race competition in well trained cyclists. Participants ingested 0.35 g/kg body mass of a KE supplement before completing 20-min time-trial. The supplement dosing strategy was designed based on speculation in the literature that suggested a potential ergogenic effect of KB ingestion at a lower dose than used in Studies 1 and 2 (Evans et al. 2017, Stubbs et al. 2018). Considering the equivocal nature of the studies that had examined performance outcomes (Evans et al. 2022), it was hypothesized that mean power output in the 20-min time-trial would be different after KE and placebo ingestion but a directional change was not specified.

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Increased cardiorespiratory stress during submaximal cycling after ketone monoester ingestion in endurance-trained adults

Devin G. McCarthy, William Bostad, Fiona J. Powley, Jonathan P. Little, Douglas L. Richards, and Martin J. Gibala

Abstract: There is growing interest in the effect of exogenous ketone body supplementation on exercise responses and performance. The limited studies to date have yielded equivocal data, likely due in part to differences in dosing strategy, increase in blood ketones, and participant training status. Using a randomized, double-blind, counterbalanced design, we examined the effect of ingesting a ketone monoester (KE) supplement (600 mg/kg body mass) or flavour-matched placebo in endurance-trained adults (n = 10 males, n = 9 females; $\dot{V}O_{2peak}$ = 57 ± 8 mL/kg/min). Participants performed a 30-min cycling bout at ventilatory threshold intensity (71 ± 3% $\dot{V}O_{2peak}$), followed 15 min later by a 3 kJ/kg body mass time-trial. KE versus placebo ingestion increased plasma β-hydroxybutyrate concentration before exercise (3.9 ± 1.0 vs 0.2 ± 0.3 mM, $p < 0.0001$, d_z = 3.4), ventilation (77 ± 17 vs 71 ± 15 L/min, $p < 0.0001$, d_z = 1.3) and heart rate (155 ± 11 vs 150 ± 11 beats/min, $p < 0.001$, d_z = 1.2) during exercise, and rating of perceived exertion at the end of exercise (15.4 ± 1.6 vs 14.5 ± 1.2, $p < 0.01$, d_z = 0.85). Plasma β-hydroxybutyrate concentration remained higher after KE vs placebo ingestion before the time-trial (3.5 ± 1.0 vs 0.3 ± 0.2 mM, $p < 0.0001$, d_z = 3.1), but performance was not different (KE: 16:25 ± 2:50 vs placebo: 16:06 ± 2:40 min:s, p = 0.20; d_z = 0.31). We conclude that acute ingestion of a relatively large KE bolus dose increased markers of cardiorespiratory stress during submaximal exercise in endurance-trained participants.

Novelty:

- Limited studies have yielded equivocal data regarding exercise responses after acute ketone body supplementation.
- Using a randomized, double-blind, placebo-controlled, counterbalanced design, we found that ingestion of a large bolus dose of a commercial ketone monoester supplement increased markers of cardiorespiratory stress during cycling at ventilatory threshold intensity in endurance-trained adults.

Key words: nutritional ketosis, supplement, exercise, time-trial performance, beta-hydroxybutyrate, perceived exertion.

Résumé : La supplémentation en corps cétoniques exogènes suscite un intérêt croissant pour son effet sur les réponses et la performance à l'exercice. Les études limitées à ce jour présentent des données équivoques probablement dues en partie à des différences dans la stratégie de dosage, à l'augmentation des cétones sanguines et à l'état d'entraînement des participants. En utilisant une conception randomisée, en double insu et contrebalancée, nous avons examiné l'effet de la consommation d'un supplément de monoester de cétone (« KE ») (600 mg/kg de masse corporelle) ou d'un placebo de saveur assortie chez des adultes entraînés en endurance (10 hommes, 9 femmes; $\dot{V}O_{2pointe}$= 57 ± 8 mL/kg/min). Les participants participent à une séance de 30 minutes de pédalage à l'intensité du seuil ventilatoire (71 ± 3 % $\dot{V}O_{2pointe}$) puis, 15 minutes plus tard, ils effectuent un contre-la-montre sollicitant 3 kJ/kg de masse corporelle. La consommation de KE versus le placebo augmente la concentration plasmatique de β-hydroxybutyrate avant l'exercice (3,9 ± 1,0 vs 0,2 ± 0,3 mM, $p < 0{,}0001$, d_z = 3,4), la ventilation (77 ± 17 vs 71 ± 15 L/min, $p < 0{,}0001$, d_z = 1,3), la fréquence cardiaque pendant l'exercice (155 ± 11 vs 150 ± 11 bpm, $p < 0{,}001$, d_z = 1,2) et l'effort perçu à la fin de l'exercice (15,4 ± 1,6 vs 14,5 ± 1,2, $p < 0{,}01$, d_z = 0,85). La concentration plasmatique de β-hydroxybutyrate demeure plus élevée après l'ingestion de KE vs le placebo avant l'essai du contre-la-montre (3,5 ± 1,0 vs 0,3 ± 0,2 mM, $p < 0{,}0001$, d_z = 3,1), mais la performance n'est pas différente (KE : 16:25 ± 2:50 vs placebo: 16:06 ± 2:40 min:s, p = 0,20; d_z = 0,31). Nous concluons que l'ingestion ponctuelle d'une dose bolus de KE relativement élevée augmente les marqueurs de stress cardiorespiratoire pendant l'exercice sous-maximal chez les participants entraînés en endurance. [Traduit par la Rédaction]

Les nouveautés :

- Des études limitées présentent des données équivoques concernant les réponses à l'exercice après une supplémentation ponctuelle en corps cétonique.

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- En utilisant un devis randomisé à double insu, contrôlé par placebo et contrebalancé, nous constatons que la consommation d'une grande dose bolus d'un supplément de monoester cétonique commercial augmente les marqueurs de stress cardiorespiratoire pendant le pédalage à l'intensité du seuil ventilatoire chez les adultes entraînés en endurance.

Mots-clés : cétose nutritionnelle, supplément, exercice, performance au contre-la-montre, bêta-hydroxybutyrate, effort perçu.

Introduction

The ketone bodies beta-hydroxybutyrate (β-HB) and acetoacetate can serve as both fuels and signalling molecules, with the potential to alter tissue-specific and whole-body substrate metabolism (Robinson and Williamson 1980). The venous blood concentration of the major circulating ketone body, β-HB, is <0.2 mM in healthy individuals consuming a mixed diet (Robinson and Williamson 1980; Balasse and Féry 1989). Nutritional ketosis typically refers to a state in which blood β-HB concentration ([β-HB]) is increased to >0.5 mM through dietary intervention (Volek et al. 2015; Norgren et al. 2020). This can be achieved by severely restricting carbohydrate intake, and occurs during fasting or adherence to a ketogenic diet (Phinney et al. 1983; Balasse and Féry 1989). Alternately, commercial exogenous ketone body supplement ingestion acutely induces nutritional ketosis without otherwise changing diet (Clarke et al. 2012; Shivva et al. 2016). There is considerable emerging interest in the effects of this practice on exercise responses and performance (Cox and Clarke 2014; Egan and Agostino 2016; Evans et al. 2017; Pinckaers et al. 2017; Burke and Hawley 2018; Dearlove et al. 2019*a*; Shaw et al. 2020).

Acute ketone body supplementation has been reported to enhance (Cox et al. 2016), impair (Leckey et al. 2017; O'Malley et al. 2017), and have no effect on exercise performance (Margolis and Fallon 2019; Poffé et al. 2020*a*, 2020*b*; Prins et al. 2020; Valenzuela et al. 2020). Studies have also reported varying effects on cardiorespiratory variables including heart rate, ventilation, oxygen uptake, and respiratory exchange ratio (RER) during constant load exercise (Cox et al. 2016; Rodger et al. 2017; Evans and Egan 2018; Evans et al. 2018, 2019; Dearlove et al. 2019*b*; Shaw et al. 2019; Prins et al. 2020). The limited and equivocal data to date are likely attributable in part to differences in research design, including specific ketone supplement and dose, participant training status, the nature of the exercise protocol or performance test, and inter-individual differences in responsiveness.

The present study examined the effects of acute ingestion of a commercial (*R*)-3-hydroxybutyl (*R*)-3-hydroxybutyrate ketone monoester (KE) supplement on exercise responses and performance in endurance-trained participants, using a randomized, double-blind, counterbalanced design. A KE supplement was selected as this type can elicit larger increases in blood β-HB as compared with other supplements (Stubbs et al. 2017). It was also administered as a single bolus dose, as it has been postulated that a minimum blood [β-HB] (e.g., ≥2 mM) is needed to elicit a ergogenic effect (Stubbs et al. 2018; Margolis and Fallon 2019). We studied trained participants as they may have a greater capacity to utilize the ingested ketone bodies during exercise, and thus benefit from supplementation (Evans et al. 2017). Exercise responses were assessed during a 30-min bout of constant load cycling performed at individual ventilatory threshold intensity, which is relevant for many endurance-type events (Sanders and van Erp 2020). Performance was subsequently assessed using a 3-kJ/kg body mass time-trial, which was selected to place a high demand on glyco(geno)lysis for ATP provision. We hypothesized that, as compared with a flavour-matched placebo, nutritional ketosis induced by actue KE ingestion would (1) reduce indices of cardiorespiratory and perceived stress, and (2) improve time-trial performance.

Materials and methods

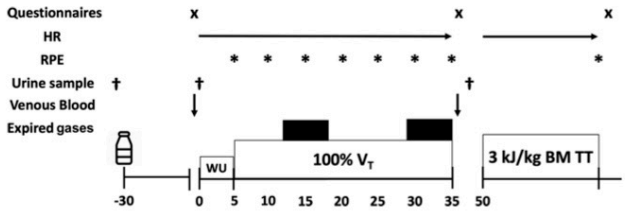
Participants

The study inclusion criteria were: age 18–50 years; regularly engaged in endurance-type exercise for >3 h/wk; habitually consuming >50 g/d of carbohydrate (Aragon et al. 2017); deemed safe to engage in physical activity; and having a peak oxygen uptake ($\dot{V}O_{2peak}$) in the 90th percentile for age and sex (American College of Sports Medicine 2000). A calculation performed using G*Power 3.1 estimated that a total sample size of 15 was required to detect a difference between 2 dependent means (matched pairs), based on a 2-tailed t-test design, with a large effect size (d_z = 0.8) and 80% power at an alpha level of 0.05. To preserve power, N = 20 individuals were recruited. Interested individuals who met the age, activity and dietary criteria were initially screened to assess their capacity to engage in physical activity using a questionnaire (Get Active Questionnaire, Canadian Society for Exercise Physiology). Individuals deemed safe to engage in physical activity subsequently reported to the laboratory and performed a ramp exercise test to volitional exhaustion on an electronically braked cycle ergometer (Excalibur Sport version 2.0, Lode; Groningen, the Netherlands) to determine $\dot{V}O_{2peak}$. Briefly, the ramp test began with a 2-min warm-up at 50 or 100 W and workload was increased 1 W every 2 s until volitional exhaustion or the cadence fell below 60 rpm. Expired gasses and ventilation were continuously recorded with a metabolic cart (Quart CPET, Cosmed Inc., Concord, Calif., USA). $\dot{V}O_{2peak}$ was determined as the mean oxygen uptake ($\dot{V}O_2$) during the last 30 s of the test. Individuals who satisfied the inclusion criterion for $\dot{V}O_{2peak}$ were recruited into the study. Body composition was determined on these individuals using an air displacement plethysmograph for calculations based on fat-free mass (BodPod, Cosmed Inc.). For this measure, participants arrived in the overnight fasted state, and after abstaining from strenuous exercise and water in the morning. One participant sustained a leg injury unrelated to the study and dropped out, and therefore data are reported for the remaining N = 19 (25 ± 5 y, 70 ± 10 kg, 57 ± 8 mL/kg/min, 5–13 exercise h/wk; n = 10 males, 25 ± 3 y, 77 ± 9 kg, 61 ± 7 mL/kg/min, 70 ± 8 mL/kg fat-free mass/min; n = 9 females, 26 ± 7 y, 62 ± 6 kg, 53 ± 6 mL/kg/min, 70 ± 8 mL/kg fat-free mass/min). All participants were informed of the study requirements and potential risks before written informed consent was obtained. The project was approved by the Hamilton Integrated Research Ethics Board (#7209).

Pre-experimental procedures

Following the screening procedures and descriptive measurements, participants performed 2 familiarization trials to become accustomed with the experimental protocol. The protocol involved a 5-min warm-up at 50 W, followed by cycling for 30 min at a workload that elicited individual ventilatory threshold intensity, as determined using the method described by Gaskill et al. (2001). Participants subsequently rested for 15 min and then performed a 3 kJ/kg body mass cycling time-trial. The protocol was modelled after other studies that have utilized a period of steady state exercise, followed by a time trial normalized to body mass, to assess the effect of nutritional interventions on exercise responses and performance (e.g., Wickham et al. 2019). Pilot testing was conducted on n = 5 endurance-trained individuals by having them perform the experimental exercise protocol on 3 occasions ~7 days apart. The coefficient of variation (CV) for heart rate, rating of perceived exertion, and RER during the 30-min bout were 2.6 ± 0.5%, 2.1 ± 0.5%, and 2.3 ± 0.9% respectively. Mean time-trial performance was faster in trial 2 vs 1, but there was no difference in performance between trials 2 and 3, with the CV between the latter 2 trials being ~2.5%. During the first familiarization trial, minor adjustments were made to exercise workload so the measured $\dot{V}O_2$ during

Fig. 1. Schematic overview of the experimental protocol. Endurance-trained participants (n = 19) ingested either 600 mg/kg body mass of the HVMN ketone monoester supplement or a placebo (bottle image) and then cycled at ventilatory threshold (V_T) intensity followed by rest and a time trial (TT). Numbers are time in minutes. HR, heart rate; RPE, rating of perceived exertion; BM, body mass; WU, warm up.



the 30-min steady-state cycle period elicited the predicted ventilatory threshold. For the time trial, the alpha factor on the cycle ergometer was initially set such that a cadence of 80 rpm corresponded to 70% peak power output and adjusted after the first familiarization trial based on participant preference. Participants could view total work completed during the time trial, but no other feedback was provided.

Experimental procedures

A randomized, crossover, double-blind, counterbalanced design was employed. The primary outcome measure was mean time-trial power output. Secondary outcome measures included indices of cardiorespiratory stress (i.e., heart rate, ventilation), expired gases (i.e., $\dot{V}O_2$, carbon dioxide expired ($\dot{V}CO_2$), RER), blood metabolites (i.e., β-HB, acetoacetate, glucose, lactate, free fatty acids), and gastrointestinal distress. Following the familiarization visits, participants performed 2 experimental trials that included expired gas and blood measurements, as detailed below (Fig. 1). Treatment order was determined by a coin flip (block size of 2; stratified by sex). The investigator who interacted with the participants during the experimental trials and collected cardiorespiratory and exercise performance data were blinded to treatment condition. A separate investigator who did not interact with participants performed the randomization and analyses of fingerprick blood samples at the time of sampling. Experimental trials were separated by 7 ± 2 d (range = 3–12 d) and performed at the same time of day within 1 h. Female participants taking oral contraceptives (n = 5) were tested while receiving the same dose of synthetic hormones. The others who were not taking contraceptives were tested in the early follicular phase of their menstrual cycle, except for 1 participant who was amenorrhoeic.

Participants were instructed to maintain their habitual diet and physical activity habits throughout the study. On the day before each experimental trial, participants were instructed to avoid strenuous exercise, ingest the same foods that corresponded to their habitual diet, and avoid alcohol. Caffeine was not restricted, provided the timing and amount of was the same before each trial. Compliance was assessed by a 1-d dietary recall. Subjects ingested a standardized breakfast provided by the researchers 2 h before exercise onset. It consisted of a commercial energy bar (250 kcal, 5-g fat, 44-g carbohydrate, 4-g fibre, 10-g protein; Clif Bar & Company, Calif., USA), and commercial sports drink powder (Gatorade; PepsiCo Canada, Mississauga, Ont., Canada) mixed into 500 mL of water, such that total carbohydrate intake was 1 g/kg body mass. In the 24-h preceding exercise, participants diet contained 2279 [1815–2717] kcal (51 ± 5% carbohydrate, 32 ± 7% fat, 17 ± 3% protein) comprised of 4.1 [3.5–5.2], 1.2 [0.8–1.5], and 1.2 [1.1–1.7] g/kg body mass carbohydrate, fat, and protein respectively.

Participants arrived at the laboratory ∼45 min prior to exercise and completed a questionnaire to assess gastrointestinal symptoms. It contained ten questions pertaining to perceptions symptoms including stomach cramps and burning; nausea; dizziness; flatulence; urge to urinate, defecate, and vomit; and gastric reflux. For each question, participants indicated their perception by marking a 10-cm line. The 0-, 3.3-, 6.6- and 10-cm points on the line corresponded to the ratings "not present", "mild", "moderate" and "severe", respectively. The questionnaire was based on previously published research (Jentjens et al. 2001), but modified to incorporate a visual analog scale as this is the preffered method for assessing gastrointestinal symptoms by gastroenterogolists (Bengtsson et al. 2013). All fluid ingested following arrival and until after the time trial was measured, and fluid ingested during the first trial was matched in the second trial. Starting 35 min prior to exercise, participants were allowed 5 min to ingest either 600 mg/kg body mass of the ketone monoester (KE) supplement ((*R*)-3-hydroxybutyl (*R*)-3-hydroxybutyrate; 120 kcal/25 g KE; Pure ΔG Ketone Ester; HVNM, Calif., USA) or a ketone body free, flavour-matched placebo (PL) (0 kcal; 0.11% bittrex, 0.15% stevia, 1.5% HVMN flavour mix). Both KE and PL were mixed with 25 g of the commercial sports drink powder (Gatorade) and dissolved in water such that total beverage volume was 500 mL. Two participants required ∼15 min to drink the beverages but were allowed to remain in the study since this was consistent between their trials. Approximately 5 min before exercise, body mass was measured, venous and capillary blood samples were obtained, and a supplement tolerability questionnaire was completed.

During the 30-min exercise period, rating of perceived exertion (RPE) was obtained every 5 min with the 20-point Borg scale (Borg 1982), expired gases were collected for ∼5 min over 2 periods starting at ∼7.5 and ∼25 min (Quark CPET; Cosmed Inc.), and heart rate was collected continuously throughout exercise (Model A300; Polar Electro Oy, Kempele, Finland). During the rest period, venous and capillary blood samples were collected in the same order as before exercise, and participants completed the supplement tolerability questionnaire. During the time-trial, heart rate was collected continuously and peak and overall RPE were obtained immediately after time-trial completion. To incentivize and motivate participants, they were made aware during the consent process that a prize (commercial gift card) was given to the participant who achieved the highest mean percentage of their maximum heart rate in the 2 time-trials. The prize was based on this relative marker of subjective effort, as opposed to absolute performance (i.e., time), to better incentive participants and in order that they could be similarly competitive, as previously done in our lab (van Essen and Gibala 2006). After the time trial, participants were weighed and completed a questionnaire to assess blinding effectiveness and supplement tolerability. To assess the effectiveness of blinding participants were first asked if they thought they could distinguish between PL and KE, and if so, which drink they thought they received.

Blood analyses

A total of ~10 mL of venous blood was collected in appropriate BD-vacutainer tubes and was promptly centrifuged for 10 min at 3000 rpm. All samples were kept on ice for ≤60 min until frozen at −80 °C. Commercial colorimetric assay kits were used for the measurements of β-HB and acetoacetate without perchloric acid extraction and within 28 d of storage as per manufacturer instructions (analyzed in triplicate, Abnova, cat no KA1630; Fisher Scientific, Ottawa, Ont., Canada). β-HB was also determined using a point-of-care analyzer (Freestyle Precision Neo; Abbott Laboratories, Ill., USA) and in several blood fractions for comparative purposes (see supplementary file, Supplementary Table S1, Fig. S1[1]). Free fatty acids were determined with a commercial assay kit per manufacturer instructions (Abcam, cat no ab65341; Toronto, Ont., Canada). Glucose and lactate were analyzed by the Hamilton Regional Laboratory Medicine Program. Incomplete blood data was collected on 7 participants owing to difficulty facilitating venous access, or the inability to obtain a post-exercise sample in a timely manner. The blood analyses thus represent data for $n = 12$ participants for whom complete data sets were obtained.

Calculations

Cardiorespiratory data were first averaged into 30-s bins, and then averaged over the recording period. RER was calculated as $\dot{V}CO_2/\dot{V}O_2$ (Frayn 1983). Arterial CO_2 partial pressure (P_aCO_2) was calculated using tidal volume and end-tidal CO_2 as per (Jones et al. 1979). Individual time-trial performance was expressed as % change in power between the KE and PL trials for each participant, relative to individual time-trial CV, based on the method of Burke and Peeling (2018). The time-trial CV was calculated based on the second familiarization and PL trials for each participant, and overall was 3.9 ± 3.0%. Total ketone bodies represent the sum of β-HB and acetoacetate based on the colorimetric assay. A gastrointestinal symptom incidence was a score >0 cm. Gastrointestinal symptom load was the measured distance on the 10-cm scale. Total condition symptom load/incidence was determined for all time points and symptoms, time-point symptom load/incidence for all symptoms at 1 time point, and symptom-specific load/incidence for 1 symptom at the time points specified.

Statistical analyses

Continuous variables were first tested for normality using a Shapiro–Wilks test, and if not normal, then tested for lognormality. If lognormal, then data were log transformed before being tested, and if not, non-parametric tests were performed. Cardiorespiratory and time-trial data were analyzed using 2-tailed paired *t*-tests (condition). Time-trial data were also assessed for time-period effect (familiarization 2 vs experiment trial 1 vs experiment trial 2) with a repeated-measures 1-way analysis of variance (ANOVA). RPE and blood data were analyzed with a 2-way repeated measures ANOVA (time × condition). Significant *F*-tests were followed by a Sidak's post hoc test (both condition and time). A correlation matrix was applied between all ketone body measurements. The percent change in all variables obtained during the 30-min cycle, $\dot{V}O_{2peak}$ and blood β-HB were tested for correlation to individual change in time-trial performance. Significantly correlated variables were then tested for relationship by linear regression. Differences in total condition, time-point, and symptom-specific gastrointestinal symptom incidence were tested with a Chi-squared test (condition × yes/no). Differences in total condition, time-point, and symptom-specific gastrointestinal symptom load were tested with a Wilcoxon test (condition). Statistics were performed with Prism 8 (GraphPad, San Diego, Calif., USA). Significance was accepted at $p = 0.05/3 = 0.0167$ for time-point symptom incidence and load to account for 3 comparisons, and $p < 0.05$ for all other statistical tests. Normal data are presented as mean ± SD, non-normal data as median [interquartile range], and effect size as Cohen's d_z.

Table 1. Plasma metabolites before (Pre-Ex) and after (Post-Ex) a 30-min bout of constant-load cycling at individual ventilatory threshold in the placebo (PL) and ketone monoester conditions (KE).

	PL		KE	
	Pre-Ex	Post-Ex	Pre-Ex	Post-Ex
β-Hydroxybutyrate	0.2±0.3	0.3±0.2	3.9±1.0*	3.5±1.0*,#
Acetoacetate	0.2±0.1	0.3±0.2	1.4±0.5*	2.1±0.7*,#
Total ketone bodies†,‡	0.4±0.2	0.6±0.2	5.3±1.1	5.7±1.2
Glucose	5.8±0.8	4.6±0.6#	4.2±0.9*	4.4±0.6
Lactate‡	2.2±0.9	3.6±1.6	2.6±1.1	3.3±1.4
Free fatty acids	0.22±0.04	0.35±0.12#	0.25±0.03	0.22±0.04*

Note: Values are mean ± standard deviation in mmol/L. Total ketone bodies is the sum of β-hydroxybutyrate and acetoacetate. *Post hoc $p < 0.05$ for KE vs PL at same time point; #Post-Ex vs Pre-Ex within same condition. Post hoc tests were performed after $p < 0.05$ interaction effect for 2-way repeated measures analysis of variance. †$p < 0.05$ KE vs PL (main effect). ‡$p < 0.05$ Post-Ex vs Pre-Ex (main effect).

Results

Blood data

Blood data are summarized in Table 1. Plasma β-HB (interaction, $p = 0.04$) was higher in KE vs PL at Pre-Ex and Post-Ex (post hoc, $p < 0.0001$ for both), with Post-Ex lower than Pre-Ex in the KE trial (post hoc, $p = 0.03$). Plasma acetoacetate (interaction, $p < 0.01$) was also higher in KE vs PL at Pre-Ex and Post-Ex (post hoc, $p < 0.0001$ for both), with Post-Ex higher than Pre-Ex in the KE trial (post hoc, $p < 0.0001$). Total ketone bodies were greater in KE vs PL (condition, $p < 0.0001$) and Post-Ex vs Pre-Ex (time, $p < 0.05$). Plasma glucose (interaction, $p = 0.02$) was lower in KE vs PL at Pre-Ex (post hoc, $p = 0.001$, $d_z = -1.3$). Lactate was higher at Post-Ex vs Pre-Ex (time, $p = 0.03$). Free fatty acids (interaction, $p < 0.01$) were lower at Post-Ex in KE vs PL (post hoc, $p < 0.001$, $d_z = -0.81$).

Cardiorespiratory data

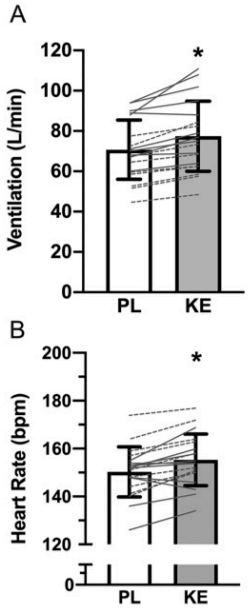
Mean ventilatory threshold intensity was 71 ± 3% $\dot{V}O_{2peak}$, which corresponded to 53 ± 6% of peak power output. Ventilation (71 ± 15 vs. 77 ± 17 L/min, $p < 0.0001$, $d_z = 1.3$; Fig. 2A), heart rate (150 ± 11 vs. 155 ± 11, $p < 0.001$, $d_z = 1.3$; Fig. 2B), breathing frequency and tidal volume (Table 2), were higher during the 30-min exercise period in the KE vs PL condition. End-tidal CO_2 and P_aCO_2 were lower in the KE vs PL condition, while end-tidal O_2, $\dot{V}O_2$, $\dot{V}CO_2$, and RER were unaffected by condition (Table 2). RPE was higher in the KE vs. PL condition at the end of the 30-min bout (15.4 ± 1.6 vs 14.5 ± 1.2; Interaction $p < 0.01$; post hoc, $p < 0.01$, $d_z = 0.85$).

Time-trial performance

Mean time-trial power was not different between conditions (PL = 201 [174–279], KE = 196 [176–295] W, $p = 0.21$, $d_z = -0.32$, Fig. 3A). Time-trial duration was also not different (16:25 ± 2:50 vs 16:06 ± 2:40 min:s, $p = 0.20$; $d_z = 0.31$). Individual change in time-trial power was −0.34 [−0.94 to 0.26]% (Fig. 3B). This was related to the % change in RPE during the 30-min cycle (Fig. 3C), but no other variable including any blood ketone body measure ($p > 0.12$), measures during the 30-min cycling bout ($p > 0.09$), or $\dot{V}O_{2peak}$ ($p = 0.64$). Neither peak (PL = 18.4 ± 1.2, KE = 18.2 ± 1.1, $p = 0.99$) or overall (PL = 17.0 ± 1.5, KE = 17.1 ± 1.7, $p = 0.33$) time-trial RPE were affected by condition. There was no time-period effect for mean power during the time-trial performed during familiarization session 2 (199 [179–266] W), and experimental trial 1 (196 [174–279] W), and experimental trial 2 (201 [176–295] W); ($p = 0.71$).

[1]Supplementary data are available with the article at https://doi.org/10.1139/apnm-2020-0999.

Fig. 2. Expired minute ventilation (A) and heart rate (B) during a 30-min bout of cycling at ventilatory threshold intensity in the placebo (PL) and ketone monoester (KE) conditions. Values are mean ± SD (n = 19), lines connect individual data points (males are solid, females dashed). *, $p < 0.05$ vs PL (paired t-test).



Mean time-trial power was also not different when familiarization trial 2 was compared with the respective placebo trial (p = 0.35).

Gastrointestinal symptoms

No gastrointestinal symptoms were reported on arrival. Total condition symptom incidence (50% vs 37%, $p < 0.0001$) and load (0.8 [0.4–1.3] vs 0.3 [0.3–0.9] cm, $p < 0.01$) were greater in KE vs PL. Time-point symptom incidence and load were unaffected by condition before exercise (p = 0.28 and p = 0.56 respectively) but both metrics were higher in KE vs PL during the 30-min bout (51% vs 33%, $p < 0.01$ and 0.9 [0.4–1.3] vs 0.4 [0.0–0.6] cm, $p < 0.001$ respectively) and time-trial (PL = 35%, KE = 50%, p = 0.01 and 0.7 [0.2–1.3] vs 0.3 [0.0–0.6] cm, p = 0.02 respectively). During exercise and the time-trial, symptom-specific incidence was unaffected by condition ($p > 0.06$ and $p > 0.15$ respectively) but over both time points symptom-specific severity was higher in KE vs PL for stomach cramps, dizziness, gastric reflux, and urge to vomit (Supplementary Table S2[1]).

Discussion

The major novel finding of this study was that acute ingestion of 600 mg/kg body mass of a KE supplement increased markers of cardiorespiratory stress during constant load cycling performed at ventilatory threshold intensity in endurance-trained individuals, as compared with a flavour-matched placebo. Perceived effort was also higher at the end of exercise and there was a small but significant increase in perceived gastrointestinal symptoms. The effect of KE on cardiorespiratory responses was quite consistent, with 17/19 participants having a higher ventilation and

Table 2. Metabolic and respiratory data during 30 min of cycling at ventilatory threshold intensity in the placebo (PL) and ketone monoester (KE) conditions.

	PL	KE	p	d_z
Breathing frequency (breaths/min)	33.2±5.8	34.3±5.4	0.03*	0.52
Tidal volume (L/breath)	2.2±0.5	2.3±0.5	<0.001*	1.01
$P_{ET}O_2$ (mm Hg)	117±3	119±4	0.01*	0.41
$P_{ET}CO_2$ (mm Hg)	30.5±2.2	28.4±3.2	<0.0001*	−0.97
P_aCO_2 (Torr)	32.9±2.0	31.0±2.9	<0.001*	−1.31
$\dot{V}O_2$ (L/min)	2848±659	2879±664	0.09	0.42
$\dot{V}CO_2$ (L/min)	2481±600	2519±636	0.09	0.41
RER	0.87±0.04	0.87±0.04	0.79	0.06

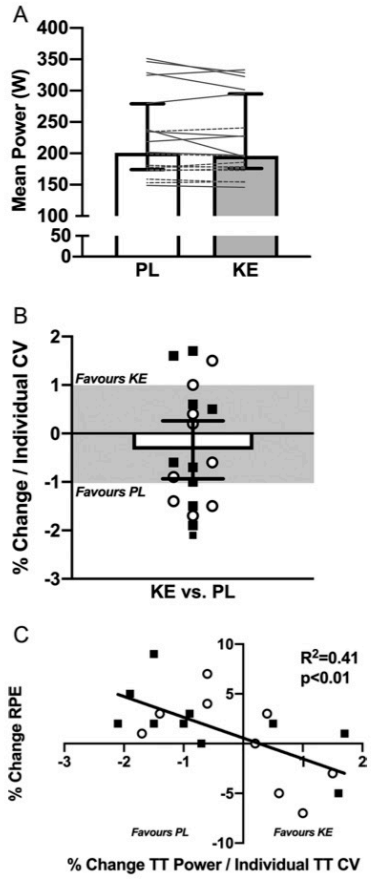
Note: Values are mean ± SD (n = 19). p-values are from paired t-tests. *$p < 0.05$ vs. PL; $P_{ET}O_2$, end tidal O_2; $P_{ET}CO_2$, end-tidal CO_2; P_aO_2, arterial O_2 partial pressure; $\dot{V}O_2$, oxygen uptake; $\dot{V}CO_2$, carbon dioxide expired; RER, respiratory exchange ratio; d_z, effect size.

16/19 having a higher heart rate, as compared with the placebo condition. Performance in a subsequent 3 kJ/kg body mass time-trial, assessed 15 min after the constant load cycling bout, was not different between conditions on average. The individual differences in performance between conditions were related to perceived effort, such that greater perceived effort was associated with reduced performance. These findings were in contrast to our hypotheses, and may be related to the relatively large bolus dose of KE ingested and specific to the exercise conditions studied.

This is the first study to report that KE ingestion elevated heart rate during exercise, in contrast to several previous reports that KE ingestion did not alter exercise heart rate compared with placebo (Evans and Egan 2018; Dearlove et al. 2019*b*; Evans et al. 2019; Poffé et al. 2020*b*). Another study found that ketone salt ingestion increased heart rate during exercise compared with water (Evans et al. 2018), whereas the present study used a flavour-matched placebo. Similar to the present study, Dearlove et al. (2019*b*) showed that ingesting 330 mg/kg body mass KE increased ventilation at maximal exercise workload compared with placebo, and this was associated with reduced blood pH and $P_{ET}CO_2$. We also observed a higher ventilation and reduced $P_{ET}CO_2$ in the KE compared with placebo condition, which were associated with a reduced P_aO_2. Therefore, it is possible that the higher cardiorespiratory stress observed in the KE condition in the present study was related to acidosis. The metabolism of β-HB includes dissociation of H^+, which could increase CO_2 production as predicted by the Henderson–Hasselbach equation, and stimulate increased ventilation in an effort to restore blood pH. However, co-ingesting sodium bicarbonate with KE did not alter the KE-induced increase in ventilation, when measured ∼1 h into a varying-load exercise bout (Poffé et al. 2020*b*). In that study, however, ventilation was lower after ∼3 h of exercise in the bicarbonate supplemented condition compared with KE alone, and similar to the placebo treatment. It was suggested that plasma total ketone bodies must exceed 7 mM to induce acidosis (Robinson and Williamson 1980). In contrast, KE ingestion studies have shown that increasing blood [β-HB] to ∼2–4 mM is associated with reduced blood pH (Stubbs et al. 2017; Dearlove et al. 2019*b*). Thus, there may be differences between acute exogenous β-HB ingestion and endogenous ketosis in terms of the effect on blood acidosis. It is also possible that the elevated cardiorespiratory stress may be explained by direct effects of KE supplementation on cardiac muscle (Robinson and Williamson 1980), sympathetic drive, or as a result of changes in catecholamines (Poffé et al. 2020*a*). Additional work is warranted to examine the effects of KE supplementation on cardiac and neurovascular responses to exercise.

Whole-body $\dot{V}O_2$ and $\dot{V}CO_2$ during exercise were not different after KE ingestion compared with placebo, despite the

Fig. 3. Performance during a 3-kJ/kg body mass time trial (TT) performance in the placebo (PL) and ketone monoester (KE) conditions: mean power (A), percent change KE vs. PL relative to individual TT coefficient of variation (CV) (B), and linear regression between percent change TT power and percent change in rating of perceived exertion (RPE) during the preceding 30-min bout of cycling at ventilatory threshold intensity (C). For panel A, bars are median ± interquartile range (n = 19). For panel B, bars are mean ± 95% confidence interval and the shaded grey area contains data points within 1 day-to-day CV. Lines connect individual data points, open circles and dashed lines represent female participants and squares and solid lines males.



differences in markers of cardiorespiratory stress. Other studies have similarly reported non-significant effects of ketone body supplement ingestion on $\dot{V}O_2$ and $\dot{V}CO_2$ during exercise (Leckey et al. 2017; Evans et al. 2018, 2019; Dearlove et al. 2019*b*; Shaw et al. 2019; Prins et al. 2020). One study found that butanediol ingestion, which increased capillary β-HB to ∼0.5 mM, augmented $\dot{V}O_2$ and $\dot{V}CO_2$ during cycling at ∼73% $\dot{V}O_{2peak}$ as compared with placebo (Shaw et al. 2019). In contrast, KE compared with placebo ingestion has also been reported to reduce $\dot{V}O_2$ during exercise and improve a marker of exercise economy when plasma β-HB was ∼2 but not ∼4 mM (Dearlove et al. 2019*b*). RER was not different between the KE and placebo trials in the present study, but this may not be entirely reflective of substrate oxidation. Interpreting RER was complicated by potential changes in CO_2 production due to acid-base buffering and ketone body contribution to energy expenditure (Balasse et al. 1978; Dearlove et al. 2020). Additional work is necessary to clarify the potential effects of KE ingestion on gas exchange during exercise, including the application of more sophisticated techniques such as stable isotopic tracers to probe underlying physiological processes.

RPE was higher at the end of constant load cycling in the KE condition compared with placebo, a difference not reported in previous studies that have included this measure (Leckey et al. 2017; Rodger et al. 2017; Evans and Egan 2018; Evans et al. 2018, 2019; Faull et al. 2019; Shaw et al. 2019; Poffé et al. 2020*a*; Prins et al. 2020). One investigation, however, found that ingesting 330 mg/kg body mass of KE increased subjective perceptions of fatigue during exercise compared with a placebo, and these effects were related to blood acidosis (Faull et al. 2019). While speculative, the greater RPE in the present study may have been related to the higher cardiorespiratory stress during the constant-load exercise period, the cumulative effect of which was apparent to the participants near the end of the bout. There was no difference in RPE during the subsequent time trial, although this comparison may have been confounded by the physiological differences during the preceding period of constant-load exercise, as well as the short period of rest in between. The greater RPE in the present study may also be related to the slightly elevated gastrointestinal distress score in the KE compared with placebo condition. These gastrointestinal symptom trends with studies that compared ingestion of a KE plus carbohydrate with carbohydrate-matched beverages (Evans et al. 2019; Poffé et al. 2020*a*). However, it may be the result of the differences in beverage osmolality and potential slowing of gastric emptying as gastrointestinal symptoms were similar between isocaloric KE and placebo beverages (Horner and Schubert 2015; Stubbs et al. 2019). Direct measurements of gastric emptying are necessary to confirm this speculation (Greaves et al. 2021). Thus, gastrointestinal symptoms following KE ingestion may be slightly yet statistically elevated compared with a placebo, perhaps relating to the bolus volume of ketone bodies ingested and/or beverage osmolality; however, the magnitude of gastrointestinal symptoms observed may not be practically meaningful.

Time-trial performance was not different between the KE and placebo conditions. This was in contrast to our hypothesis, and occurred despite plasma β-HB being >2 mM in all participants, a hypothesized ergogenic threshold (Stubbs et al. 2018; Margolis and Fallon 2019). Whereas cardiorespiratory stress responses during constant-load cycling were relatively consistent, individual variability in time-trial performance was apparent. Performance change in the KE compared with placebo condition exceeded individual day-to-day variability in 9 participants, and of those, performance was impaired in 6 and improved in 3 participants (Fig. 3B). These diverse responses may be related in part to perceived effort, as well as the magnitude of nutritional ketosis. A circulating β-HB of ∼1–3 mM was also previously suggested for optimal endurance performance (Evans et al. 2017). In fact, studies involving a submaximal exercise bout followed by a time-trial showed KE supplementation improved performance when BHB was ∼2–3 mM but not when BHB was outside of that range (Cox et al. 2016; Evans et al. 2019; Poffé et al. 2020*b*, 2020*a*, 2020*c*). This optimal range may relate to the physiological mechanisms involved, as exceeding the upper limit was associated with higher cardiorespiratory stress and a mitigation to improvements in markers of exercise economy (Dearlove et al. 2020). The blood β-HB in most participants in the present study prior to the time trial exceeded the upper range (i.e., >3 mM), with the mean value being ∼3.5 mM. However, in the 5 participants whose pre-time-trial β-HB was

<3 mM, performance in the KE vs PL condition was within day-to-day variability in 2 and impaired in 3. This analysis is also limited in that a placebo effect may have been present when calculating individual variability, as it was based on a comparison of the second familiarization session and the PL trial. This would have potentially resulted in more individual changes being classified as within day-to-day variability, when in fact the inherent test–retest variability may have been lower. As noted above, performance in the present study may have also been influenced by the preceding constant-load exercise, because measures of cardiorespiratory stress and perceived effort were different between conditions. Also, the supplements were not matched for total energy, which may have been preferable to assess potential differences in constant-load exercise responses but not performance. Likewise, carbohydrate intake was suboptimal for the exercise challenge and the effects of KE may differ when combined with optimal fueling strategies. Overall, the effects of KE ingestion on endurance performance remains unclear, with dose and exercise context likely being important considerations in this regard. Future research should test endurance performance following KE ingestion at a dose that does not alter blood pH, co-ingesting KE with an antacid such as sodium bicarbonate (Poffé et al. 2020*b*), in combination with other nutritional strategies, and during longer duration exercises.

Both β-HB and acetoacetate were increased after KE ingestion compared with placebo and remained elevated before the time trial. The increase was expected based on the pharmacokinetics of acute KE ingestion (Clarke et al. 2012; Shivva et al. 2016; Stubbs et al. 2017), but the magnitude of such was greater than previous KE and performance studies (Cox et al. 2016; Evans and Egan 2018; Dearlove et al. 2019*b*; Evans et al. 2019). This may be because the pre-exercise meal ingested in this study was smaller than other fed trials and the KE dose was ingested as a single bolus (Evans and Egan 2018; Evans et al. 2019; Poffé et al. 2020*a*, 2020*b*). While remaining elevated in the KE compared with the placebo condition, β-HB decreased over the course of the exercise bout and acetoacetate increased. The KE supplement did not contain acetoacetate, suggesting that some of the ingested β-HB may have been metabolized to acetoacetate, in addition to potentially being oxidized by active tissues (Balasse et al. 1978; Dearlove et al. 2020). Overall, direct measures of gut ketone body absorption, kinetics, and oxidation are required to assess metabolic effects after KE ingestion.

With respect to blood metabolites, the intervention differentially affected the plasma glucose and free fatty acids responses. Plasma glucose was lower in the KE compared with placebo condition prior to exercise, and free fatty acids lower after exercise in KE vs placebo. These trends are generally consistent with other KE supplementation studies (Cox et al. 2016; Myette-Côté et al. 2018, 2019; Dearlove et al. 2019*b*; Evans et al. 2019; Dearlove et al. 2020; Greaves et al. 2021; Poffé et al. 2020*b*, 2020*a*, 2020*c*). The mechanism whereby KE supplementation reduces blood glucose is poorly understood, as discussed by (Dearlove et al. 2020; Greaves et al. 2021), but may partially by explained by a reduction in hepatic glucose output (Mikkelsen et al. 2015). The lower circulating free fatty acids present before the time trial relates to BHB inhibition of adipose tissue lipolysis via nicotinic acid receptor binding. This may have implications for performance in longer duration exercise bouts as reducing free fatty acid availability through nicotinic acid ingestion was associated with increased carbohydrate oxidation and glycogenolysis during submaximal exercise, although this response was highly individualized (Stellingwerff et al. 2003; O'Neill et al. 2004).

While presumably a rise in circulating ketone body concentration after KE ingestion is required to cause physiological effects, the dosing strategy herein induced physiological responses consistent with acidosis. In fact, the blood ketone body levels observed in some participants were elevated to an extent normally seen in clinical conditions. Therefore, ingesting bolus doses of KE greater than 600 mg/kg body mass (~42 g) may present safety concerns related to acidosis. Larger total doses of KE have been well tolerated when the dose was split across multiple ingestion time points occurring over an hour or so (Evans et al. 2019; Poffé et al. 2020*a*). However, ingesting large total doses of KE and thereby inducing high ketone body availability (i.e., blood β-HB > 3 mM) appears to not affect exercise in a way that would benefit performance (Evans et al. 2017; Poffé et al. 2020*c*). It therefore may be worthwhile for future studies to examine the efficacy of lower doses of KE supplementation in this regard.

In conclusion, ingesting 600 mg/kg body mass of a KE supplement before constant-load cycling at ventilatory threshold intensity increased markers of cardiorespiratory stress during exercise as well as perceived effort at the end of the bout in endurance-trained participants. Subsequent performance during a 3 kJ/kg body mass time trial was not significantly different between KE and placebo conditions. Additional research is required to clarify the effect of ketone body supplementation on endurance performance and exercise responses, including studies that directly assess ketone body metabolism and oxidation and that focus on intra- and extra-muscular substrate metabolism.

Conflict of interest statement

Dr. Little is the Chief Scientific Officer for the not-for-profit Institute for Personalized Therapeutic Nutrition and has stock in Metabolic Insights Inc. All other authors declare no competing interests.

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SUPPLEMENTAL MATERIAL

A secondary study purpose was to compare determinations of beta-hydroxybutyrate (β-HB) in various blood fractions based on a point-of-care analyzer and laboratory assays. Previous studies of exogenous ketone body supplementation have largely quantified blood β-HB in different blood fractions using various methods including point-of-care analyzers. Endogenously produced β-HB quantified with point-of-care analyzers do not always align with lab based assays and β-HB may be present in in different quantities in different blood fractions (Guimont et al. 2015; Norgren et al. 2020).

Methods

Blood Analyses

Fingerpick samples were analyzed at the time of collection for [β-HB] using a point-of-care analyzer (Freestyle Precision Neo; Abbott Laboratories, IL, USA). A total of ~10 ml of venous blood for all other measures were collected in appropriate BD-vacutainer tubes. A small aliquot of heparinized blood was immediately analyzed for [β-HB] using a point-of-care analyzer by a researcher unblinded to condition, and the remainder was promptly centrifuged for 10 min at 3000 rpm, except for serum samples which were left at room temperature for 30 min and allowed to clot before centrifugation. All samples were kept on ice for ≤60 min until frozen at -80°C for subsequent analysis within 28 d of storage. Commercial colorimetric assay kits were used for the measurements of β-HB and

acetoacetate without perchloric acid extraction as per manufacturer instructions and in triplicate (Abnova, cat no KA1630; Fisher Scientific, Ottawa, Ontario).

Statical Analyses

Differences in β-HB obtained in different blood fractions and techniques were tested with a repeated measure ANOVA (measurement x time). A correlation matrix was applied between all ketone body measurements. Significantly correlated variables were then tested for relationship by linear regression. Bland-Altman plots assessed method agreement (β-HB in method 1 – method 2) in data from the KE condition only.

Results

Plasma and serum β-HB assessed by point-of-care analyzer were not different from each other but were higher than plasma and serum β-HB determined by colorimetric assay, and capillary and whole blood via point-of-care analyzer ($p<0.0001$ for all, Table S1). Bland-Altman plots revealed poor method agreement between average of plasma and serum β-HB via colorimetric assay and β-HB in capillary, whole blood, and plasma analyzed with point-of-care analyzer (Figure S1). Log(β-HB) in plasma and serum via colorimetric assay were generally correlated to all venous blood fractions at a similar strength and total ketone bodies were correlated to all β-HB measurements. Nothing measured was correlated to acetoacetate in either blood fraction. Log(β-HB), averaged from plasma and serum via colorimetric assay, was linearly regressed to capillary ($R^2=0.20$, $p=0.01$; $y=4.3x + 1.4$), whole blood ($R^2=0.43$, $p<0.0001$; $y=5.6x + 0.82$), and plasma β-HB via

point-of-care analyzer (R^2=0.42, p=0.0001; y=3.9x + 2.9). Plasma total ketone bodies was linearly regressed to capillary, whole blood and plasma β-HB via point-of-care analyzer, and average of plasma and serum log(β-HB) via colorimetric assay (Figure 4B).

Discussion

The method used to determine blood β-HB concentration is also important. Typically, measuring serum ketone bodies is avoided because acetoacetate spontaneously degrades into acetone (Reichard et al. 1979) and our results provide some evidence for this. Thus, acetoacetate and total ketone bodies are best presented in blood plasma. β-HB was similar in plasma and serum fractions, but the point-of-care analyzer yielded higher β-HB compared to colorimetric assay and between-method agreement was poor. Likewise, β-HB quantified in capillary and whole venous blood via point-of-care analyzer were similar to each other but showed poor method agreement with the colorimetric assay despite similar mean values. Some data suggest this poor agreement is in part related to the capillary blood sample rather than the analyzer itself (Armer et al. 2013; Norgren et al. 2020). However, both whole and capillary blood showed similarly poor method agreement in our study, suggesting the point-of-care analyzer also contributes to this difference. Alternately, the point-of-care analyzer and lab-based assay may yield similar results when [β-HB] <3 mM and different results when >3 mM (Armer et al. 2013). Although this may depend on the point-of-care analyzer manufacturer (Guimont et al. 2015; Norgren et al. 2020). Perchloric acid extraction is also postulated to affect [β-HB], but we observed a similar overestimation in point-of-care analyzer vs lab based assay to a

study that used perchloric acid extraction (Guimont et al. 2015). Nonetheless, β-HB obtained with the point-of-care analyzer should be interpreted with caution but may be an economic method, perhaps with adjustment to lab-based assays using the presented linear regressions, to evaluate circulating plasma β-HB in a population sample.

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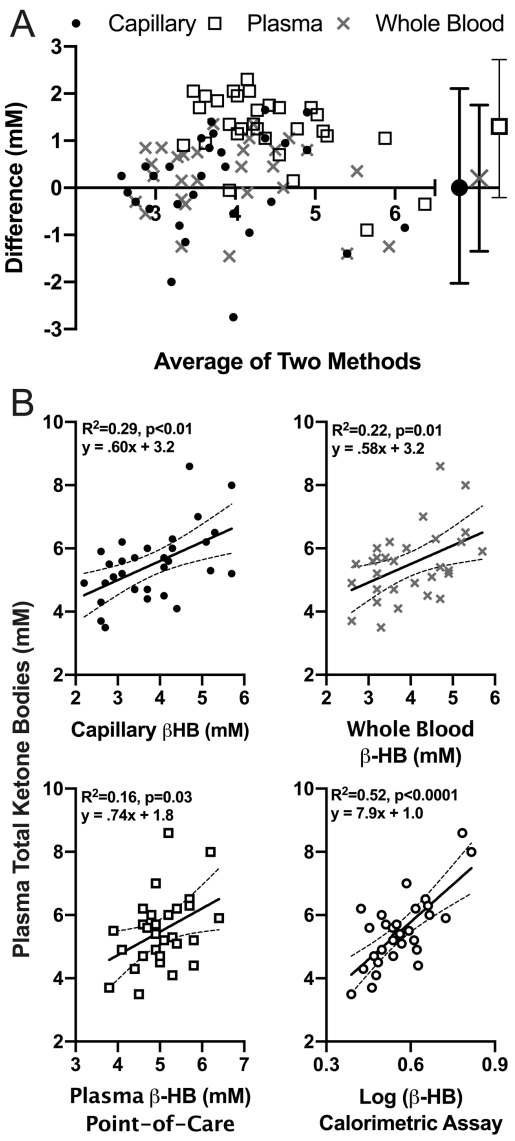

Figure S1. Comparison of blood ketone body analytical methods. (A) Bland-Altman plot comparing capillary, plasma or whole blood beta-hydroxybutyrate (β-HB) determined via point-of-care analyzer to the mean of plasma and serum β-HB determined via colorimetric assay. Symbols with error bars represent mean bias ± 95% confidence intervals. (B) Linear regressions between plasma total ketone bodies (β-HB + acetoacetate) determined via colorimetric assay and capillary, whole-blood, and plasma β-HB determined via point-of-care analyzer and log(β-HB) averaged in plasma and serum samples determined via colorimetric assay. Lines indicate line of best fit ± 95% confidence bands.

Table S1. Blood beta-hydroxybutyrate (β-HB), acetoacetate, and total ketone bodies before (Pre-Ex) and after (Post-Ex) 30 min of cycling at ventilatory threshold intensity in the placebo (PL) and ketone monoester conditions (KE).

	PL		KE	
	Pre-Ex	**Post-Ex**	**Pre-Ex**	**Post-Ex**
β-HB				
POC analyzer				
Capillary [a]	0.1 ± 0.0	0.2 ± 0.1	4.0 ± 1.7*	3.6 ± 0.8*†
Whole blood [a]	0.1 ± 0.1	0.1 ± 0.1	4.4 ± 0.9 *	3.5 ± 0.6*†
Plasma [b]	0.0 ± 0.0	0.0 ± 0.1	5.3 ± 0.6*	4.7 ± 0.4*†
Serum [b]	0.0 ± 0.0	0.0 ± 0.1	5.3 ± 0.6*	4.8 ± 0.4*†
Colorimetric Assay				
Plasma [a]	0.2 ± 0.3	0.3 ± 0.2	3.9 ± 1.0*	3.5 ± 1.0*†
Serum [a]	0.3 ± 0.2	0.3 ± 0.2	4.3 ± 1.0*	3.4 ± 1.0*†
Acetoacetate				
Plasma	0.2 ± 0.1	0.3 ± 0.2	1.4 ± 0.5*	2.1 ± 0.7*†
Serum *	0.2 ± 0.1	0.3 ± 0.1	1.6 ± 0.6	1.8 ± 0.7
Total ketone bodies				
Plasma *†	0.4 ± 0.2	0.6 ± 0.2	5.3 ± 1.1	5.7 ± 1.2
Serum *	0.5 ± 0.3	0.7 ± 0.2	5.8 ± 1.1	5.3 ± 1.3

Values are mean ± standard deviation, units are mmol/L, measurements are from venous blood, and total ketone bodies = β-HB + acetoacetate via colorimetric assay. * indicates *post-hoc* $p<0.05$ KE vs. PL; †, $p<0.05$ Pre-Ex vs. Pre-TT after two-way analysis of variance (condition x time); different letters, *post-hoc* $p<0.05$ between β-HB rows in the KE condition after two-way analysis of variance (β-HB determination x time); POC, point-of-care.

Table S2. Symptom-specific gastrointestinal symptom severity during the 30 min submaximal cycling bout performed at ventilatory threshold intensity and 3 kJ/kg body mass time-trial.

	PL	KE	P value
Stomach cramps	0.0 [0.0-0.4]	0.1 [0.0-1.2]	0.047
Stomach burning	0.0 [0.0-0.4]	0.1 [0.0-1.0]	0.10
Nausea	0.3 [0.0-0.9]	0.9 [0.1-2.4]	0.12
Dizziness	0.2 [0.0-1.4]	1.4 [0.0-3.2]	<0.01
Flatulence	0.0 [0.0-0.4]	0.0 [0.0-0.3]	0.94
Urge to Urinate	0.8 [0.0-2.4]	1.7 [0.3-3.5]	0.33
Urge to Defecate	0.0 [0.0-0.3]	0.0 [0.0-0.2]	0.13
Urge to Vomit	0.0 [0.0-0.4]	0.3 [0.0-1.4]	<0.01
Gastric Reflux	0.0 [0.0-0.4]	0.2 [0.0-1.5]	0.049

Notes: Data were obtained from a 10-cm visual analog scale. Values are median [interquartile range] in cm. Values of 0, 3.3, 6.6, and 10 cm indicate severities of "not present", "mild", "moderate", and "severe" respectively. P-values are from a paired Wilcoxon's test.

CHAPTER 3: Effect of Acute Ketone Monoester Ingestion on Cardiorespiratory Responses to Exercise and the Influence of Blood Acidosis. Published in *Med Sci Sports Exerc*, doi: 10.1249/MSS.0000000000003141.

Effect of Acute Ketone Monoester Ingestion on Cardiorespiratory Responses to Exercise and the Influence of Blood Acidosis

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Abstract

Ketone monoester (KE) ingestion can induce hyperketonemia and blood acidosis. We previously found that acute ingestion of 0.6 g/kg body mass KE increased exercise heart rate (HR) compared to placebo. ***Purpose:*** To examine the effect of KE ingestion on exercise cardiac output ($\dot{Q}$) and the influence of blood acidosis. We hypothesized that KE vs placebo ingestion would increase $\dot{Q}$ and co-ingestion of the pH buffer bicarbonate would mitigate this effect. ***Methods:*** In a randomized, double-blind, crossover manner, 15 endurance-trained adults [peak oxygen uptake ($\dot{V}O_{2peak}$), 60±9 mL/kg/min] ingested either 0.2 g/kg sodium bicarbonate or a salt placebo 60-min pre-exercise, and 0.6 g/kg KE or a ketone-free placebo 30-min before exercise. Supplementation yielded three experimental conditions: basal ketone bodies and neutral pH (CON), hyperketonemia and blood acidosis (KE), and hyperketonemia and neutral pH (KE+BIC). Exercise involved 30 min of cycling at ventilatory threshold intensity, followed by determinations of $\dot{V}O_{2peak}$ and peak $\dot{Q}$. ***Results:*** Blood [β-hydroxybutyrate], a ketone body, was higher in KE (3.5±0.1 mM) and KE+BIC (4.4±0.2) vs CON (0.1±0.0, $p<0.0001$). Blood pH was lower in KE vs CON (7.30±0.01 vs 7.34±0.01, $p<0.001$) and KE+BIC (7.35±0.01, $p<0.001$). $\dot{Q}$ during submaximal exercise was not different between conditions (CON: 18.2±3.6, KE: 17.7±3.7, KE+BIC: 18.1±3.5 L/min, $p=0.4$). HR was higher in KE (153±9 beats/min) and KE+BIC (154±9) vs CON (150±9, $p<0.02$). $\dot{V}O_{2peak}$ ($p=0.2$) and peak $\dot{Q}$ ($p=0.3$) were not different between conditions but peak workload was lower in KE (359±61 W) and KE+BIC (363±63) vs CON (375±64, $p<0.02$). ***Conclusions:*** KE ingestion did not increase $\dot{Q}$ during submaximal exercise despite a modest elevation of

HR. This response occurred independent of blood acidosis and was associated with a lower workload at $\dot{V}O_{2peak}$.

Key words: Nutritional ketosis, Ventilation, Physiology, Acid-base, Metabolism, Inert gas rebreathing

Introduction

There is sizeable interest in the potential for exogenous ketone body supplementation to alter exercise responses in humans (1–4). Many studies have examined the potential ergogenic effects of ketone body supplements (5–7), but less work has focused on the basic physiological and metabolic responses that may underpin changes of exercise performance. The current data regarding the effects of exogenous ketone bodies on cardiorespiratory exercise physiology are limited and equivocal (8–13). We recently found that acute ingestion of a relatively large bolus dose (0.6 g/kg body mass) of a commercial ketone monoester (KE) supplement increased heart rate (HR) and minute ventilation ($\dot{V}_E$), during cycling at individual ventilatory threshold intensity in endurance-trained individuals (10). A higher HR after KE ingestion could be associated with an increased cardiac output ($\dot{Q}$), but this has not been studied during exercise. Ketone body infusion has been reported to increase resting HR and $\dot{Q}$ in older adults (14).

Increased cardiorespiratory stress during exercise subsequent to exogenous ketone body ingestion could potentially be related to the associated blood acidosis (8,15). The higher $\dot{V}_E$ could reflect the additional work of breathing to exhale carbon dioxide to counteract a KE-associated acidosis. This would require more blood flow to ventilatory muscles and therefore a greater $\dot{Q}$, which could be reflected by a higher HR. Interventions that serve to counteract a potential effect of KE ingestion on acidosis (e.g., bicarbonate co-ingestion) might attenuate the increased HR, $\dot{Q}$, and $\dot{V}_E$, during submaximal exercise (11). Others have examined the effects of co-ingestion of exogenous ketone bodies and bicarbonate through an exercise performance lens (11,12,16), but data examining the

potential underlying physiologic mechanisms associated with exogenous ketone bodies are limited.

The primary purpose of this study was to examine the effects of ingesting a 0.6 g/kg body mass KE bolus on indices of cardiorespiratory stress during exercise, and whether these effects were influenced by blood acidosis. Using a randomized, double-blind, crossover design, we measured exercise responses during constant-load cycling performed at individual ventilatory threshold intensity in endurance-trained adults. Our primary hypothesis was that acute KE supplementation would increase $\dot{Q}$ during submaximal exercise compared to a flavour-matched, ketone-free placebo. The higher $\dot{Q}$ would be associated with higher exercise HR and $\dot{V}_E$ following KE ingestion. Our secondary hypothesis was that co-ingestion of bicarbonate with KE will attenuate the increases in $\dot{Q}$, HR, and $\dot{V}_E$ during exercise — due to a less pronounced decrease in blood pH — such that the measures of these variables are similar to the ketone-free placebo trial. We also examined the relationship between exercise $\dot{V}O_{2peak}$ and peak power in response to KE ingestion, as compared to ketone-free placebo and co-ingestion with bicarbonate.

Methods

Participants

This project was approved by the Hamilton Integrated Research Ethics Board (#12811). An *a priori* calculation using an online program (G*Power, version 3.1.9.4) determined that 12 participants provided 80% power at an alpha level of 0.05 with an

effect size f=0.4 for a repeated-measures, within-factors analysis of variance, with 1 group and 3 measurements. The effect size was estimated based on the anticipated change in $\dot{Q}$ during submaximal exercise from HR data in a similar intervention (10) and day-to-day within-participant variability of exercise $\dot{Q}$ as determined in pilot testing. To preserve power, 15 participants were recruited. The study inclusion criteria were: adult aged 18-60 years; deemed safe to engage in physical activity; habitually consuming >50 g/d of carbohydrate (i.e., not following a ketogenic diet); regularly engaging in endurance-type exercise for >3 h/wk; and having a $\dot{V}O_{2peak}$ in the 90th percentile for age and sex (18). Participants were recruited using a printed poster placed around McMaster University campus and shared on social media sites for McMaster University students and local cycling groups. Interested individuals were first informed of the study requirements and potential risks before written informed consent was obtained. Those who met the age criteria were initially asked to complete a Get Active Questionnaire (Canadian Society for Exercise Physiology) to assess general readiness to engage in physical activity, a questionnaire to assess physical activity habits (International Physical Activity Questionnaire), and a custom questionnaire to assess habitual nutrition and menstrual cycle. Their $\dot{V}O_{2peak}$ was then estimated using an online calculator (https://www.worldfitnesslevel.org). Those who satisfied the preliminary inclusion criteria completed a laboratory exercise test to directly measure $\dot{V}O_{2peak}$ as detailed below. If the measured $\dot{V}O_{2peak}$ satisfied the specific inclusion criteria, the participant was fully recruited into the study. The final participant group was comprised of 11 males and 4 females (age 29±12 y, height = 174±10 cm, mass = 73±10 kg, $\dot{V}O_{2peak}$ = 60.2±8.5

ml/kg/min; males, 30±12 y, 179±6 cm, 77±7 kg, 62±9 ml/kg/min; females, 28±3 y, 164±9 cm, 62±10 kg, 55±5 ml/kg/min).

Study Design

Participants performed two familiarization trails followed by three experimental trials in a randomized, crossover, counterbalanced, and double-blinded manner. The three experimental conditions were: ketone-free placebo + sodium chloride [control (CON)]; KE + sodium chloride (KE); and KE + sodium bicarbonate (KE+BIC). Participants were randomized in block sizes of 6, 6, and 3 into 1 of 6 treatment orders (i.e., study arms). The block of 3 participants was randomized in the same way except not all treatment orders were filled. Each treatment order, e.g., CON in trial one, KE+BIC in trial two and KE in trial three, was assigned a number between 1 and 6 and was sequenced using an online random sequence generator (www.RANDOM.org/sequences) to determine the random allocation sequence. The temporal order of participants recruited in each block was assigned to the treatment order that corresponded with the order in the random allocation sequence, i.e., the first participant recruited in a block was assigned to the treatment order that corresponded to the first number in the random allocation sequence. All randomization procedures were performed by an investigator who did not interact with the participants. Both the investigators and participants were blinded to the treatment condition. The investigators were unblinded after analysis of cardiorespiratory data was completed. Data collection occurred in the Human Performance Laboratory at McMaster University from September 2021 through February 2022. The primary outcome measure

was $\dot{Q}$ during submaximal exercise. Secondary outcome measures included peak $\dot{Q}$, peak power output, and VO_{2peak} as well as measurements obtained during submaximal exercise, including indices of cardiorespiratory function (HR, stroke volume, $\dot{V}_E$), gas exchange [$\dot{V}O_2$, carbon dioxide expired, respiratory exchange ratio, gross efficiency, arterial-venous O_2 difference ($\Delta a\text{-}vO_2$)], blood metabolites (β-hydroxybutyrate, glucose, lactate), acid-base balance (blood pH, bicarbonate, and CO_2), and subjective distress markers (rating of perceived exertion and gastrointestinal symptoms).

Pre-Experimental Procedures

Pilot testing was performed to determine the day-to-day variability of $\dot{Q}$ during submaximal exercise. Eight adults completed an exercise bout on two occasions separated by 4-7 d and at approximately the same time of day (±1 h). Exercise involved cycling for 30 min on an electronically-braked ergometer (Excalibur Sport version 2.0; Lode, Groningen, The Netherlands) at a constant-load intensity that elicited a HR between 130 and 150 beats/min. $\dot{Q}$ was measured during exercise the same way as in the experimental trials, i.e., at ~24 and ~29 min into the exercise bout by inert gas rebreathing (Innocor, Innovision, Odense, Denmark). Briefly, participants breathed room air from a rebreathing bag for ~40 s while the device calculated the tidal volume to be used for closed-circuit rebreathing. Participants then inhaled a gas mixture (94% O_2, 5% NO, and 1% SF_6) for 5-6 breaths from the closed-circuit rebreathing bag. To calculate $\dot{Q}$, the Innocor measured the disappearance rate of NO (blood soluble gas) relative to SF_6 (blood insoluble gas) over the course of the rebreathing period using

photoacoustic gas analyzers. Day-to-day variability for exercise $\dot{Q}$ was calculated by comparing the 29 min value only, i.e., single measurement, as well as by averaging the two values recorded on each day, i.e., duplicate, and then comparing between days. The day-to-day coefficient of variation for exercise $\dot{Q}$ was lower (paired 2-tailed t-test: p=0.03) when determined in duplicate (mean[95% confidence intervals: 1.7 [0.9-2.5]%) compared to a single measurement (4.7 [2.4-7.0]%) (Figure S1). To be conservative, we set the day-to-day variation of $\dot{Q}$ during submaximal exercise as the upper limit of the 95% confidence, i.e., 2.5%.

During the first familiarization trial, participants cycled on an ergometer (Excalibur Sport) for 30 min at a workload estimated to elicit individual ventilatory threshold while expired gases were recorded and analyzed with a metabolic cart (Quart CPET, COSMED Inc., Concord, CA). Ventilatory threshold was determined from the screening $\dot{V}O_{2peak}$ test using the mean of three methods as described by Gaskill et al. (19). Workload was adjusted as appropriate to elicit the desired intensity, and then $\dot{Q}$ was determined once near the end of exercise (Innocor). The second familiarization visit involved the exact same exercise procedures and non-invasive measurements as performed in the experimental trials.

Experimental Procedures

Experimental trials were separated by ~7 days for males and ~4 days for females and performed at approximately the same time of day (±1 h). For females, all trials were performed in the same phase of their hormonal cycle. Participants were instructed to maintain their habitual diet and physical activity habits throughout the study. On the day

before each experimental trial, participants were instructed to avoid strenuous exercise, eat their habitual diet, and avoid alcohol. Caffeine ingestion was not restricted, providing the timing and amount was the same before each trial. In the 24-h preceding experimental trials, participants diets contained 2990[2365-3537] kcal (50[44-54]% carbohydrate, 34[30-39]% fat, 18[16-20]% protein). Participants ate a standardized breakfast ~2 h before exercise onset. It contained a commercial energy bar (250 kcal, 5-g fat, 44-g carbohydrate, 4-g fibre, 10-g protein; Clif Bar & Company, CA, USA,), and commercial sports drink powder (Gatorade; PepsiCo Canada, Mississauga, Ontario, Canada) mixed into 400 ml of water, such that total carbohydrate intake was 1 g/kg body mass.

An overview of the trial design is shown in Figure 1. The protocol was modelled after a previous study from our laboratory that the sample size estimation was based on (10). Participants arrived at the lab ~1 h before exercise. Shortly after arrival, participants ingested gelatin-coated capsules (CapsulIN International LLC) containing either 0.20 g/kg body mass of sodium bicarbonate (Pure Baking Soda, Arm & Hammer) or an equimolar sodium dose of sodium chloride (Ionized table salt, Windsor) with ~200 ml of water. The number of capsules ingested, and the appearance of capsules was the same for all trials within a participant, and the total dose of substance administered was roughly split evenly between all the capsules ingested. Participants were allotted a 10-min window to ingest all capsules. One participant required 15-min to ingest all capsules, but this was consistent across all trials and therefore remained in the study. After ingestion of the final capsule, participants rested for 25 min during which they completed the 24-h dietary recall with the researcher using the recording and food estimation methods used

by Vermeulen et al. (20). After the rest period, participants were given up to 5 min to drink either 0.6 g/kg body mass of the KE supplement (120 kcal/25 g KE; Pure ΔG Ketone Ester; HVNM, CA, USA) or a ketone-free, flavour-matched placebo (0 kcal; 0.11% Bittrex, 0.15% stevia, 1.5% HVNM flavour mix). Both KE and placebo were mixed with 25 g of the commercial sports drink powder (Gatorade) and diluted with water such that total beverage volume was 500 ml. Two participants required 10 min to completely ingest the supplements but remained in the study as this was consistent for all trials. Shortly after finishing the drink, an indwelling venous catheter was inserted into an antecubital vein. Participants sat quietly in an upright position in the 15-25 minutes following complete ingestion of the KE or placebo while breath-by-breath expired gases were collected (Quark CPET, COSMED Inc., Concord, CA) and HR recorded (Polar Electro, Model A300; Kempele, Finland) (Table S1). A venous blood sample was drawn 10 min into sitting.

Participants exercised individually under the direct supervision of one investigator and no external motivation was provided. The submaximal exercise bout began 30 min after complete ingestion of the KE or ketone-free placebo and involved cycling on an ergometer (Excalibur Sport) for 5 min at 50 W followed immediately by a 30 min bout at the predetermined individual ventilatory threshold intensity (67±4% VO_{2peak}, 50±5% peak power output). Cycle cadence and ergometer seat and handlebar configuration were consistent within a participant for all experimental trials. During the 30-min cycling bout, rating of perceived exertion was obtained every 5 min with the 20-point Borg scale (21). Expired gases were collected from 10-19 min and analyzed with a metabolic cart (Quark

CPET, COSMED Inc.). HR (Polar Electro, Model A300) was collected continuously throughout. $\dot{Q}$ was measured at ~25 and ~29 min (Innocor) and blood hemoglobin saturation was measured non-invasively from ~21-30 min (Innocor). Approximately 45 s after the second $\dot{Q}$ measurement but before the end of the 30-min bout, a second blood sample was drawn in the same manner as before exercise. Participants then rested for 10 min, during which the indwelling venous catheter was removed and they completed a modified Likard-type scale questionnaire to assess gastrointestinal symptoms perceived during the prior cycling bout (22). The questionnaire results were not shown to any researcher during the trial to preserve blinding.

After the 10-min recovery period, an incremental cycling ramp test to volitional exhaustion was completed using routine procedures in our laboratory to determine $\dot{V}O_{2peak}$ (10,23). Briefly, the ramp test began with a 3-min warm-up at 50 W and workload was increased 1 W every 2 s until the subject's cadence decreased to <60 rpm. Expired gases (Quark CPET) and HR (Polar) were recorded continuously throughout. Participants then rested for 10 min before peak $\dot{Q}$ was determined using a protocol as previously described (23). Briefly, participants cycled for ~1 min at 50 W then ~2-3 min at 90% of peak power output obtained during their baseline $\dot{V}O_{2peak}$ test. HR (Polar) was recorded throughout and $\dot{Q}_{peak}$ (Innocor) measured beginning at the 2 min mark. After each trial, participants completed a questionnaire to assess blinding effectiveness. Participants indicated whether they could distinguish between the (a) KE and ketone-free placebo and (b) bicarbonate and sodium chloride; and if so, indicate which they thought they received and why.

Blood Analyses

A total of ~2 ml of venous blood was collected in a lithium-heparinized syringe during each draw. The sample first underwent blood-gas analysis by a validated point-of-care analyzer (EPOC, Siemens Healthineers; Ontario, Canada) (24). Next, a drop of whole venous blood was applied to point-of-care analyzer to determine [β-hydroxybutyrate] (β-ketone test strips, Freestyle Precision Neo; Abbott Laboratories, IL, USA). To preserve blinding, the point-of-care blood analyses was performed by a researcher who did not interact with the participants.

Calculations

The submaximal exercise $\dot{Q}$ data reported represents the mean of the duplicate measurements. Stroke volume was calculated as $\dot{Q}$ / HR and Δa-vO_2 as $\dot{V}O_2$ / $\dot{Q}$. Expired gas data from the metabolic cart during submaximal exercise represent the mean of data collected from minutes 12-19 of the constant-load exercise period. Respiratory exchange ratio was calculated as CO_2 expired / $\dot{V}O_2$. Gross efficiency was calculated using $\dot{V}O_2$, expired CO_2 and workload as per Moseley & Jeukendrup (2001). Peak power output and HR were the highest values achieved during the $\dot{V}O_{2peak}$ test. $\dot{V}O_{2peak}$ was calculated as the highest $\dot{V}O_2$ attained over 3 consecutive 10-s bins. Gastrointestinal symptom incidence was considered a score >0 for a symptom and severity the sum of scores within a category.

Statistical analyses

Normality was assessed with a Shapiro Wilk's test. All data were normally distributed except for GI questionnaire scores. All data obtained during the incremental test to exhaustion and the 30-min cycling bout were analyzed for differences between conditions (CON vs KE vs KE+BIC) by 1-way repeated measures analysis of variance (RM-ANOVA). A 2-way RM-ANOVA [condition x time (rest, exercise)] assessed differences in blood measurements and rating of perceived exertion. A 1-way repeated-measures Friedman's test (condition) assessed differences in total gastrointestinal symptom severity and incidence. A mixed-effects RM-ANOVA was performed instead of a 1-way RM-ANOVA if a participant was missing one data point (i.e., condition). Significant F-tests were followed by a Tukey's *post-hoc* test (both condition and time as applicable). Correlations were attempted between the individual change scores of blood pH and [β-hydroxybutyrate] in KE vs CON and KE+BIC vs CON and the respective changes of HR, $\dot{V}_E$, and peak power output. A paired 2-tailed t-test compared peak values during the incremental ramp test to exhaustion from the control trial and screening visit. Statistics were performed with Prism 9 (GraphPad, San Diego, CA, USA). Significance was accepted as $p \leq 0.05$ for all analyses. Normal data are presented as mean ± standard deviation, non-normal data as median [interquartile range], change data as mean [95% confidence intervals]. Effect sizes are Cohen's d_z for pairwise comparisons and partial eta squared (η_p^2) for RM-ANOVA unless otherwise stated.

Results

Blood

Blood [β-hydroxybutyrate] (condition $p<0.0001$) was different in all conditions ($p<0.01$ for all) such that KE+BIC (4.4±0.2 mM) > KE (3.5±0.1 mM) > CON (0.1±0.1 mM) (Figure 2A). Blood pH (condition $p<0.0001$) was 0.05[0.04-0.06] units lower in KE compared to both CON (7.30±0.01 vs 7.34±0.01, $p<0.001$) and KE+BIC (7.35±0.01, $p<0.0001$) but not different in KE+BIC vs CON (0.01[-0.1 to 0.2] unit difference, $p=0.6$; Figure 2B).

The remaining blood variables are summarized in Table 3. Venous [glucose] (condition $p<0.01$, rest/exercise $p<0.01$, interaction $p=0.058$) was 0.7[0.2-1.2] mM lower in KE ($p<0.01$) and 0.9[0.4-1.5] mM lower in KE+BIC ($p<0.001$) compared to CON, but not different between KE and KE+BIC (0.2[-0.3 to 0.7] mM difference, $p=0.5$). Venous [lactate] (condition $p=0.03$, rest/exercise $p<0.0001$) was 0.4[0.0-0.7] mM lower in KE compared to both CON ($p=0.04$) and KE+BIC ($p=0.03$) but not different between CON vs KE+BIC ($p=0.9$). Condition did not affect blood hemoglobin concentration ($p=0.17$). Total venous CO_2 (condition $p<0.0001$) and [bicarbonate] (condition $p<0.0001$) were lower in KE compared to CON and KE+BIC, but not different in CON vs KE+BIC.

Submaximal exercise

HR (condition $p<0.01$, $\eta_p^2=0.35$) was 3[1-5] beats/min higher in KE (153±9 beats/min) and 4[1-7] beats/min higher KE+BICARB (154±9) compared to CON (150±9, $p<0.02$ for both) (Figure 3A). $\dot{V}_E$ (condition $p<0.01$, $\eta_p^2=0.35$) was 7[4-10] L/min higher

in KE vs CON (81±16 vs 74±17 L/min, p<0.0001) and 3[1-6] L/min higher in KE vs KE+BIC (77±16, p<0.01) but not different between CON and KE+BIC (4[-1 to 8] L/min difference, p=0.11) (Figure 4A). Tidal volume was higher in KE vs CON (p<0.01) but not in KE+BIC vs CON (p=0.07) or CON vs KE+BIC (p=0.6). Frequency of breathing, stroke volume, $\dot{V}O_2$, $\Delta a\text{-}vO_2$, expired CO_2, and gross efficiency were not different between conditions (Table 2). The individual changes of HR in KE vs CON and KE+BIC vs CON were not correlated with the respective changes of blood pH (p=0.81) or [β-hydroxybutyrate] (p=0.16). $\dot{Q}$ was not different between conditions (CON: 18.2±3.6, KE: 17.7±3.7, KE+BIC: 18.1±3.5 L/min, p=0.4, η_p^2=0.07) (Figure 3B). The individual changes of $\dot{V}_E$ in KE vs CON and KE+BIC vs CON correlated to the respective changes in blood pH (Figure 4B) but not blood [β-hydroxybutyrate] (p=0.14).

Rating of perceived exertion (condition p=0.2) was not different between conditions (CON: 13±1, KE: 13±1, KE+BIC: 13±1). Condition did not affect total gastrointestinal symptom incidence (CON: 2[0-3], KE: 2[1-5], KE+BIC: 1[1-3] of 19, p=0.4) or severity (CON: 3[0-5], KE: 3[1-8], KE+BIC: 2[1-7], p=0.6).

Peak exercise

$\dot{V}O_{2peak}$ (condition p=0.2, η_p^2=0.12) was not different between conditions (CON: 4.55±1.05, KE: 4.38±0.96, KE+BIC: 4.41±0.94 L/min) (Figure 5A). Power output at the end of the $\dot{V}O_{2peak}$ test (condition p=0.02, η_p^2=0.30) was lower in KE (363±61 W, p<0.01) and KE+BIC (367±64 W, p=0.02) vs CON (375±64 W) but not different between KE and KE+BIC (p=0.4) (Figure 5B). HR at the end of the $\dot{V}O_{2peak}$ test (condition p=0.02,

η_p^2=0.24) was lower in KE vs CON (179± 9 vs 181±8 beats/min, p=0.03) and KE+BIC (181±9 beats/min, p=0.03) but not different between CON and KE+BIC (p=0.9) (Figure 5C). $\dot{V}O_{2peak}$ and peak HR were not different in CON compared to the screening visit (p=0.5 and p=0.2 respectively). Peak power output was 13[0 to 26] W higher at the screening visit compared to the CON trial (p=0.04).

Peak $\dot{Q}$, stroke volume, $\Delta a\text{-}vO_2$, and HR determined at the end of 2 min of constant-load cycling at 90% peak power output were not different between conditions (Table 3). HR at the end of the incremental ramp test to exhaustion was higher than the end of the 2-min constant-load bout (181±9 vs 177±8 beats/min, paired t-test p<0.0001). The changes of blood [β-hydroxybutyrate] and pH in KE vs CON and KE+BIC vs CON did not correlate with the respective changes of peak power output (p=0.9 and p=0.11 respectively).

Discussion

The primary finding of this study was that nutritional ketosis induced by acute ingestion of a 0.6 g/kg body mass bolus dose of a KE supplement, with or without sodium bicarbonate, did not increase $\dot{Q}$ compared to the control condition during a constant-load exercise bout performed at individual ventilatory threshold intensity. Exercise HR was nonetheless ~3-4 beats/min higher in both KE supplemented conditions compared to control, which confirms our previous finding under similar exercise conditions (10). We further demonstrate that co-ingestion of bicarbonate and KE did not mitigate the KE-associated increase in exercise HR, which suggests this effect was unrelated to the mild

blood acidosis induced. We also confirmed our previous finding that exercise $\dot{V}_E$ was higher after KE ingestion, and further demonstrate that this effect was partially attenuated by sodium bicarbonate ingestion. Finally, we show that power output at $\dot{V}O_{2peak}$ was lower in the two KE-supplemented trials compared to control.

Submaximal Exercise Responses

Contrary to our primary hypothesis, KE ingestion did not increase $\dot{Q}$ during submaximal exercise compared to control. While we did not use the gold-standard direct Fick method or thermodilution technique to determine exercise $\dot{Q}$, inert gas rebreathing is well correlated with these invasive techniques for exercise measurements in healthy individuals, with similar if not lower day-to-day measurement error (27). We speculated that a higher $\dot{Q}$ would be required to support the extra work of breathing to exhale additional CO_2 resulting from blood pH buffering as potentially suggested by the increased exercise HR. Alternatively, it has been shown that increasing tidal volume but not breath frequency during submaximal exercise via expiratory loading decreased $\dot{Q}$ via a lowered stroke volume (28). However, the lack of effect of condition on exercise $\dot{Q}$ may reflect the regulation of exercise $\dot{Q}$ by arterial O_2 content (29). If arterial O_2 content was not affected by condition, then alteration of $\dot{Q}$ would have been required to maintain O_2 delivery. Future work could examine whether KE ingestion affects exercise $\dot{Q}$ and O_2 delivery in conditions of altered arterial O_2 content such as altitude or cardiorespiratory disease.

Acute KE ingestion increased exercise HR compared to control, and this effect was seemingly not due to blood acidosis. We and others have reported a higher HR after exogenous ketone body infusion and supplementation as compared to control (10,14,30). Though, this finding is not universal (9,11,12,31) and could be related to various factors including ketone supplement dose and exercise intensity. While the mechanisms underlying the KE-associated increased HR remain unclear, possible explanations include nicotinic acid receptor activation (32), myocardial exogenous ketone body uptake being negatively correlated with cardiac ejection fraction (33), and increased blood [norepinephrine] which perhaps is related to a higher work of breathing (34–36). Regardless of the mechanisms involved, the observed KE-associated increase of HR was not associated with a higher $\dot{Q}$. This suggests a reduced stroke volume, but this was not apparent in our study perhaps due to a power limitation. Like in our previous study, HR and $\dot{V}_E$ were higher in most participants after ingestion of KE compared to CON. Other studies have also reported a higher $\dot{V}_E$ after KE compared to ketone-free placebo ingestion (8,10,11). The current study further suggests that the KE-associated hyperventilation was related in part to blood acidosis. This is reflected by exercise $\dot{V}_E$ being not different from control when the blood acidosis associated with KE was mitigated by co-ingestion of KE and bicarbonate. However, blood pH only explained 33% of the variance in exercise $\dot{V}_E$ and the 95% confidence bands of the y-intercept of this linear regression did not include zero. Therefore, the effect of KE on exercise $\dot{V}_E$ seemingly involves blood pH but there may be other factors involved. While the KE-associated HR and $\dot{V}_E$ responses were relatively consistent, indices of exercise economy

were more variable. Studies measuring exercise economy/efficiency after KE compared to placebo ingestion, including this study, report small effect sizes which may contribute to the currently equivocal data in the literature (8–11). Studies involving larger sample sizes are required to examine this potential effect.

Peak Exercise Responses

$\dot{V}O_{2peak}$ and peak $\dot{Q}$ were not different across trials but power output at $\dot{V}O_{2peak}$ was reduced in both KE-supplemented conditions compared to control. The only other study that assessed $\dot{V}O_{2peak}$ after KE ingestion reported no effect of KE on peak power output or $\dot{V}O_{2peak}$ compared to control (8). The present study employed an incremental ramp test which may be more sensitive to small changes in peak power output compared to the step incremental test used by Dearlove *et al.* (8). The lower power output at $\dot{V}O_{2peak}$ suggests that the greater HR and perhaps $\dot{V}O_2$ per power output relationship during submaximal exercise after KE and KE+BIC ingestion compared to control persisted across all intensities and resulted in $\dot{V}O_{2peak}$ being reached at a lower power output. Alternatively, this $\dot{V}O_{2peak}$ and peak power output discrepancy could be reflective of greater day-to-day variability of $\dot{V}O_{2peak}$ compared to peak power output. Peak HR was however not achieved after KE ingestion, as indicated by a lower peak HR compared to control and KE+BIC. This may involve the KE-associated acid-base disturbance and additional work of breathing causing a redirection of the limited blood flow at near-maximal exercise away from locomotor toward respiratory muscles (35,37,38). This presumed decrease of blood flow to the locomotor muscles may have then reduced peak

power output (39). However, $\dot{V}O_{2peak}$ may have remained unchanged owing to a simultaneous increase of respiratory muscle O_2 consumption (37). Overall, more work is required to determine the physiologic effects of ketone bodies at maximal exercise.

Substrate Metabolism

The reduction of blood glucose after KE ingestion compared to control is consistent with the vast majority of studies (40). The current study indicated that the KE-associated lowering of blood glucose was not explained by blood acidosis. The mechanism for this response is not fully understood but may relate to effects on insulin-signalling and hepatic glucose output (40,41). In contrast, mixed results exist with regards to the effects of KE ingestion on exercise blood lactate with some studies reporting no change (9–11,34,42) and others a decrease (8,12,31,43,44). The KE-associated lowering of blood lactate as compared to CON was due to blood pH since lactate was not different between the CON and KE+BIC conditions. Similarly, ingestion of non-ketogenic compounds that induced blood acidosis reduced exercise blood lactate as compared to pH-neutral controls (45–47). Thus, the lower blood lactate after KE ingestion may be attributable to the associated acidosis rather than hyperketonemia per se. Carbohydrate oxidation may have been unaffected by exogenous ketone body ingestion despite alterations to markers of carbohydrate metabolism. Exogenous ketone body ingestion complicates the interpreting of carbohydrate and fat oxidation via the respiratory exchange ratio because of the associated blood acidosis and resulting CO_2 produced from non-oxidative metabolism as well as exogenous ketone bodies potentially serving as an

additional oxidative fuel source (48). However, the influence of these confounding factors may have been negligible when comparing the KE+BIC and CON conditions. First, neither blood pH nor indices of blood CO_2 were different in KE+BIC compared to CON. Second, ketone bodies contributed <5% to total energy expenditure during exercise after KE ingestion, as determined by stable isotope tracer methodologies (9). This relative contribution to total oxidative energy expenditure is comparable to that of leucine oxidation during exercise, which is often deemed negligible when calculating carbohydrate oxidation using indirect calorimetry (49,50). Thus, the lack of effect of condition on respiratory exchange ratio may reflect no direct effect on carbohydrate oxidation. Although, direct measures of carbohydrate oxidation are required to confirm this.

Statistical Power Limitations

The primary study conclusion is limited to that KE ingestion did not increase $\dot{Q}$ during submaximal exercise as compared to CON. This is supported by the 95% confidence intervals of this change (i.e., -0.5[-1.2 to +0.2] L/min) not including the hypothesized increase in $\dot{Q}$ (i.e., +0.5 L/min) nor a +0.4 L/min calculated increase in $\dot{Q}$ based on the 2% increase in HR in KE vs CON and assuming stroke volume did not change between conditions. Also, $\dot{Q}$ during submaximal exercise was lower in KE vs CON. Thus, the increase in HR during submaximal exercise in the KE vs CON conditions likely does not indicate unchanged stroke volume and higher $\dot{Q}$ as hypothesized. However, whether $\dot{Q}$ during submaximal exercise was lowered or unchanged remains to

be determined. This determination would require detecting a small and accurate change in a direct measure of stroke volume, which this study was not designed to achieve.

Since this study was designed to detect an increase in $\dot{Q}$, it is possible that between-condition comparisons on some metrics may have been underpowered if the observed effect contained more variance and/or changed to a smaller magnitude than the hypothesized change in $\dot{Q}$. Regarding magnitude, the effect size achieved for $\dot{V}O_2$ during submaximal exercise (~2% higher in KE and KE+BIC vs CON) would have required ~30 participants to achieve p=0.05 and 80% power using the statistical approach herein. Regarding variability, calculated stroke volume and $\Delta a\text{-}vO_2$ would be expected to contain more variance than $\dot{Q}$ because these calculations incorporated the variance of HR or $\dot{V}O_2$ in addition to $\dot{Q}$. In support of these select and speculated power limitations, exploratory 2-tailed paired t-tests indicated that calculated stroke volume was lower in the KE vs CON condition (p=0.03) and calculated $\Delta a\text{-}vO_2$ (p=0.04) and submaximal $\dot{V}O_2$ (p=0.04) were higher.

Conclusions

Ingestion of a relatively large bolus dose of KE did not increase $\dot{Q}$ during submaximal exercise despite a modest elevation of HR, as compared to ingestion of a ketone-free placebo. In addition to the higher exercise HR, we also confirm our previous finding of increased $\dot{V}_E$ at a fixed submaximal intensity after KE ingestion (10). We also report that peak power output at $\dot{V}O_{2peak}$ was reduced in the KE-supplemented conditions compared to control, which is suggestive of a reduced exercise economy. While the

precise mechanisms underlying the observed cardiorespiratory responses to exogenous ketone body ingestion remain unknown, our data demonstrate that blood acidosis was not the cause of the cardiac responses. Future mechanistic work in this regard could examine potential interactive effects of exogenous ketone bodies, nicotinic acid receptor binding, blood flow distribution, and work of breathing during exercise.

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Conflict of Interest

The authors have no conflicts of interests to declare. The results of this study do not constitute endorsement by ACSM. The results of this study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation.

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Figure Captions

Figure 1. Schematic overview of the experimental protocol. Capsule image represents ingestion of 0.20 g/kg sodium bicarbonate or an equimolar mass of sodium chloride; bottle image, ingestion of 0.6 g/kg ketone monoester or flavour-matched ketone-free placebo beverage; syringe, venous blood sampling; V_T, ventilatory threshold intensity; VO_{2peak}, peak oxygen uptake; RPE, rating of perceived exertion; numbers, time in minutes.

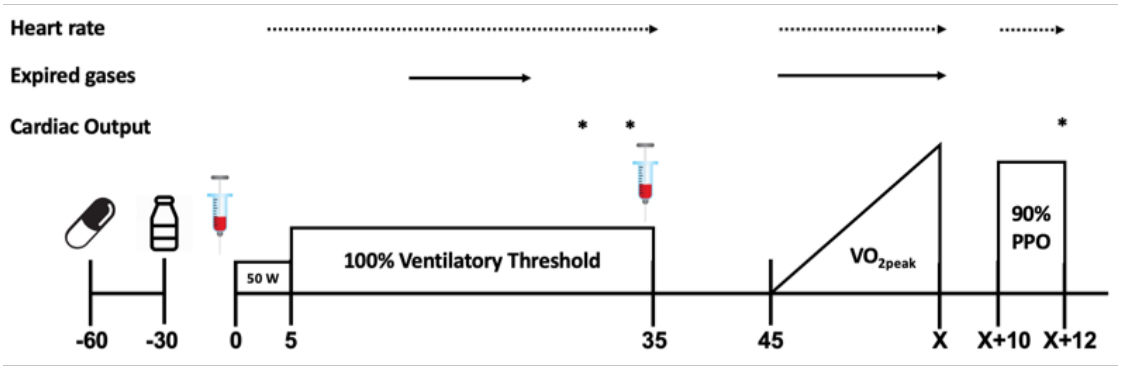
Figure 2. Venous blood [β-hydroxybutyrate] and pH during upright sitting (rest) and at the end of 30 min of cycling at ventilatory threshold intensity (exercise) in the control (CON), ketone monoester (KE), and ketone monoester plus bicarbonate (KE+BIC) conditions. Data are presented as mean ± standard deviation. Statistics were performed on n=15 by repeated measure mixed-effects analysis. *, $p<0.05$ vs CON and †, $p<0.05$ vs KE (*post-hoc* Tukey's test).

Figure 3. Heart rate (A) and cardiac output (B) during 30 min of constant-load cycling at ventilatory intensity threshold in the control (CON), ketone monoester (KE), and ketone monoester plus bicarbonate (KE+BIC) conditions. Bars are mean ± standard deviation (n=15). *, $p<0.05$ vs CON (*post-hoc* Tukey's test).

Figure 4. (A) Expired minute ventilation during 30-min of constant-load cycling at ventilatory intensity threshold in the control (CON), ketone monoester (KE), and ketone monoester plus bicarbonate (KE+BIC) conditions. Bars are mean ± standard deviation (n=15 for CON and KE+BIC, n=14 for KE). *, $p<0.05$ vs CON and †, $p<0.05$ vs KE (*post-hoc* Tukey's test). (B) Delta expired minute ventilation vs venous blood pH during cycling at ventilatory threshold intensity for KE vs CON (open circles) and KE+BICARB vs CON (closed circles) conditions. Solid line is the line of best fit and dashed lines are the 95% confidence intervals of the fit. N=25 owing to a missing blood sample in CON for 2 participants and one missing exercise ventilation in KE for one other participant.

Figure 5. Peak (A) oxygen uptake (VO_{2peak}), (B) power output, and (C) heart rate from an incremental ramp exercise test to exhaustion in the control (CON), ketone monoester (KE), and ketone monoester plus bicarbonate (KE+BIC) conditions. Data are mean ± standard deviation (n=14 in CON, n=15 in KE and KE+BIC). *, $p<0.05$ vs CON (*post-hoc* Tukey's test).

Figures



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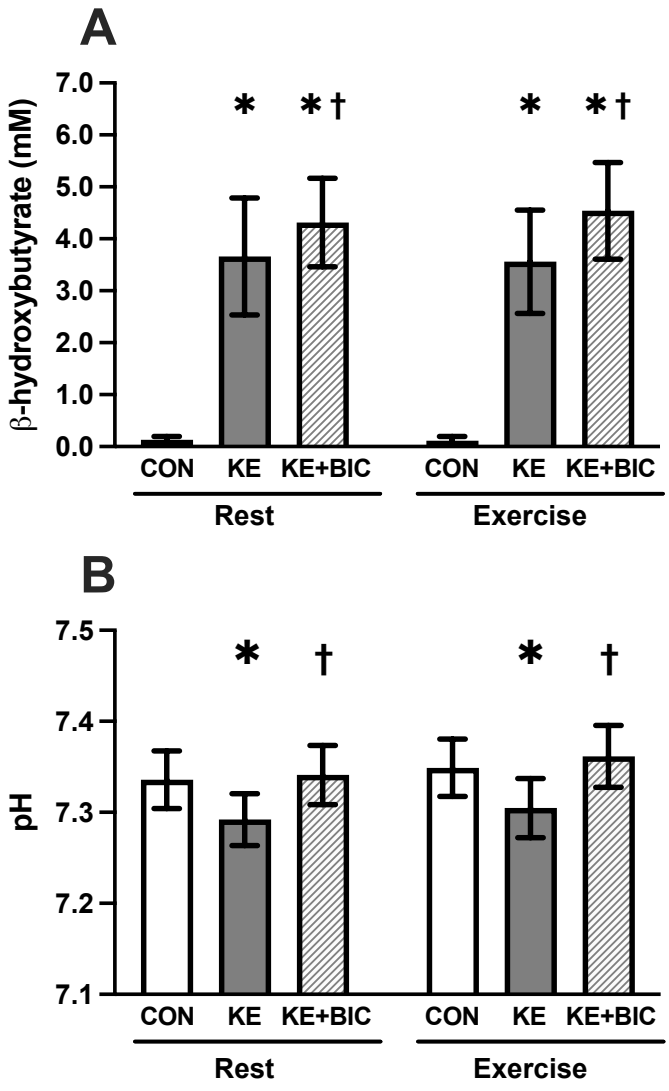

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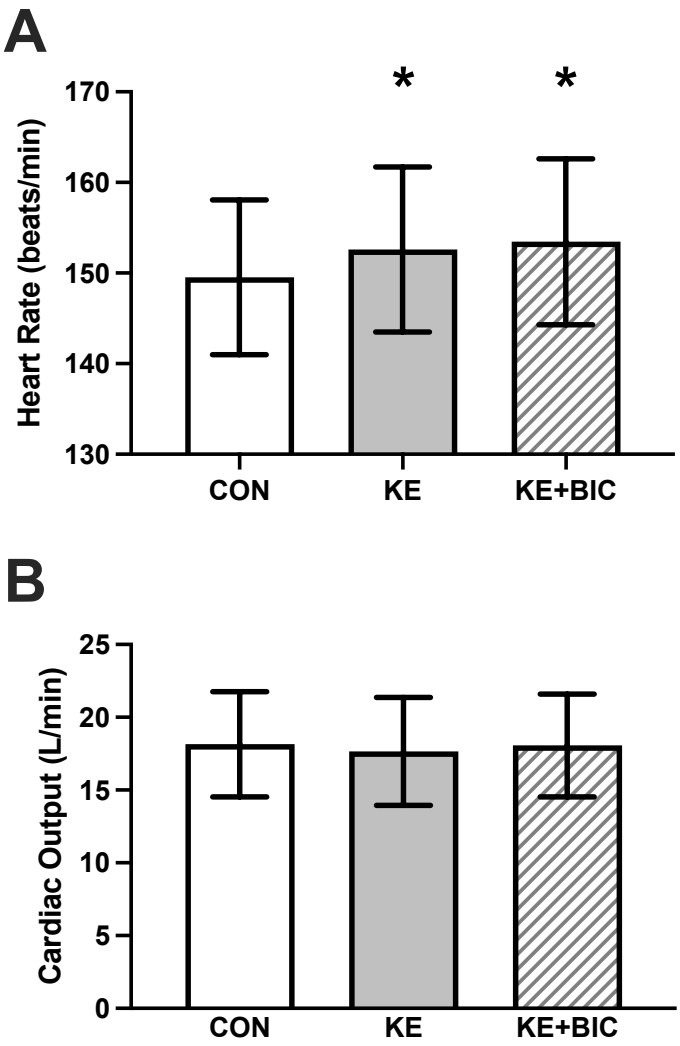

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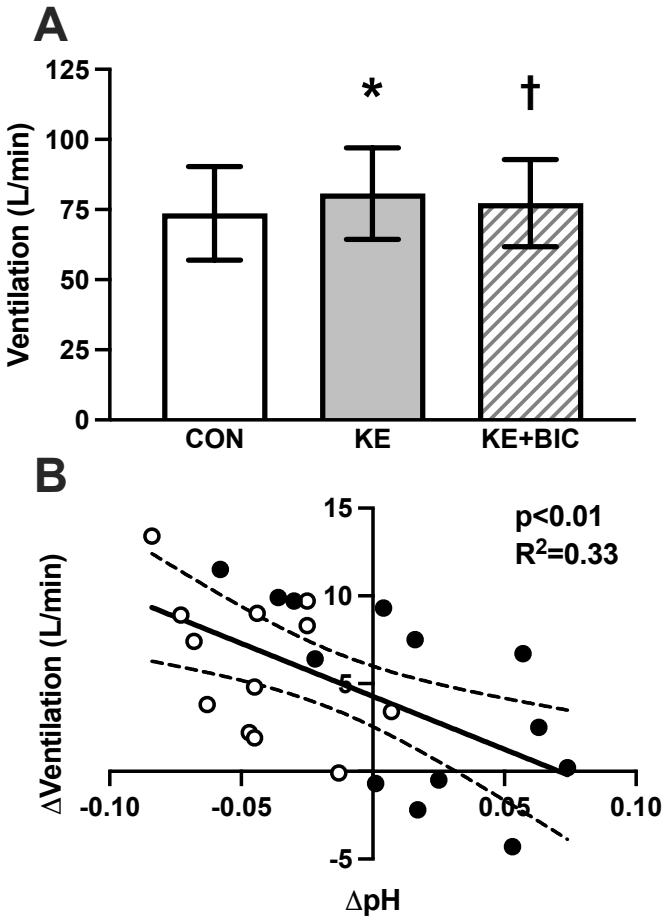

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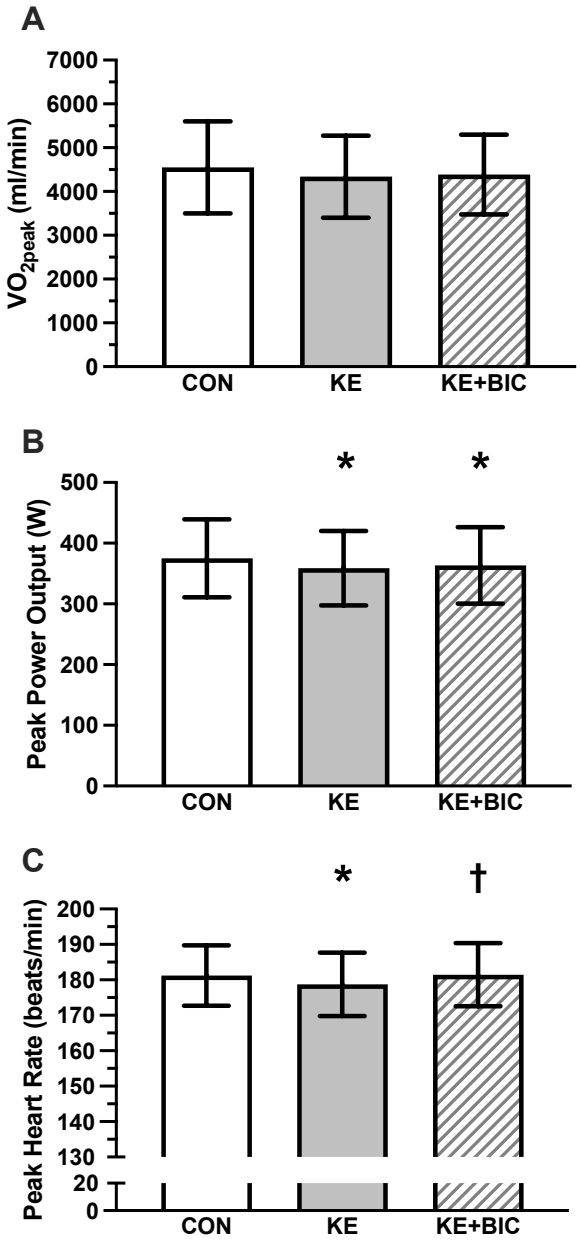

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Tables

Table 1. Blood data during upright sitting (rest) and at the end of 30 min of constant-load cycling at ventilatory threshold intensity in the control (CON), ketone monoester (KE), and ketone monoester and bicarbonate (KE+BIC) conditions.

	Rest			Exercise		
	CON	**KE**	**KE+BIC**	**CON**	**KE**	**KE+BIC**
Glucose (mM) ‡	6.0±1.3[a]	4.9±0.8[b]	4.8±1.5[b]	4.5±0.7[a]	4.2±0.7[b]	3.9±0.6[b]
Lactate (mM) ‡	1.7±0.8[a]	1.4±0.3[b]	1.6±0.3[a]	3.3±1.2[a]	2.9±1.4[b]	3.4±1.5[a]
[Hgb] (g/dL) ‡	14.0±0.8	14.3±1.0	14.5±0.9	14.9±0.8	15.2±1.0	15.1±0.8
Total vCO_2 (mM) ‡	28±2[a]	26±2[b]	29±3[a]	26±2[a]	23±2[b]	26±2[a]
Bicarbonate (mM) ‡	27.9±1.6[a]	24.9±1.7[b]	28.0±3.0[a]	25.4±2.1[a]	21.6±1.8[b]	25.2±1.9[a]

Notes: Data are presented as mean ± standard deviation (n=13). Hgb indicates hemoglobin; a, arterial; v, venous; O_2, oxygen; CO_2, carbon dioxide. $PaCO_2$ was calculated using tidal volume and end tidal CO_2 as per Jones et al. (1979), and CaO_2 assuming [Hgb] was equal in arterial and venous blood and negligible contribution of arterial pressure of O_2. Data were first analyzed by repeated measures 2-way mixed-

effects analysis (condition x time, n=15). Different superscript letters are *post-hoc* Tukey's test $p<0.05$ vs each other (main effect condition); ‡, $p<0.05$ rest vs exercise (main effect time).

Table 2. Cardiorespiratory responses during 30 min of constant-load cycling at ventilatory threshold intensity in the control (CON), ketone monoester (KE), and ketone monoester plus bicarbonate (KE+BICARB) conditions.

	CON	**KE**	**KE+BIC**	**p**	**η_p^2**
$\dot{V}O_2$ (L/min)	3.02 ± 0.75	3.08 ± 0.75	3.08 ± 0.72	0.16	0.12
$\dot{V}CO_2$ (L/min)	2.68 ± 0.67	2.74 ± 0.67	2.75 ± 0.68	0.07	0.16
RER	0.89 ± 0.05	0.89 ± 0.04	0.89 ± 0.05	0.63	0.04
Gross efficiency (%)	18.3 ± 2.4	18.0 ± 2.4	17.9 ± 2.3	0.08	0.15
Δa-vO_2 (ml/L)	164 ± 20	174 ± 24	168 ± 21	0.06	0.19
Stroke volume (ml/beat)	122 ± 24	116 ± 24	118 ± 25	0.17	0.12
Breath frequency (breaths/min)	32.2 ± 5.7	32.2 ± 5.8	32.9 ± 6.4	0.48	0.04
Tidal volume (L/breath)	2.4 ± 0.6	2.6 ± 0.7 *	2.5 ± 0.7	0.02	0.27

Data are presented as mean ± standard deviation from participants with complete datasets only (n=15 for stroke volume, n=14 for all other variables). $\dot{V}O_2$, oxygen uptake; $\dot{V}CO_2$, carbon dioxide expired; RER, respiratory exchange ratio; Δa-vO_2, arterial-venous oxygen difference. P-values are from repeated measures mixed-effects analysis or analysis of variance or one-way analysis of variance (condition, n=15). *, $p<0.05$ vs CON (*post-hoc* Tukey's test). Effect size is partial eta squared (η_p^2).

Table 3. Peak cardiorespiratory data obtained at the end of 2 min of cycling at 90% peak power output in the control (CON), ketone monoester (KE), and ketone monoester plus bicarbonate (KE+BICARB) conditions.

	CON	**KE**	**KE + BIC**	**p**	**η_p^2**
Cardiac output (L/min)	22.1 ± 3.8	21.5 ± 3.7	21.2 ± 3.9	0.30	0.15
Heart Rate (beats/min)	178 ± 8	176 ± 7	178 ± 8	0.09	0.16
Stroke Volume (ml/beat)	124 ± 21	122 ± 20	119 ± 24	0.33	0.14
Δa-vO_2 (ml/L)	205 ± 32	204 ± 28	208 ± 44	0.89	0.005

Data are presented as mean ± standard deviation from participants with complete datasets only (n=15 for heart rate; n=12, Δa-vO2; n=13, everything else). Δa-vO_2, arterial-venous oxygen difference. Stroke volume was calculated as cardiac output / heart rate, and Δa-vO_2 as VO_{2peak} / peak cardiac output. P-values are from one-way ANOVA or mixed-effects analysis (condition, n=14 for cardiac output, n=15 for all other variables). * indicates $p<0.05$ vs CON (*post-hoc* Tukey's test). Effect size is partial eta squared (η_p^2).

CHAPTER 4: Acute ketone monoester supplementation impairs 20-minute time-trial performance in trained cyclists: a randomized, crossover trial. Accepted author manuscript version reprinted, by permission, from *Int J Sports Nutr Exerc Metab*, 2023, https://doi.org/10.1123/ijsnem.2022-0255. © Human Kinetics, Inc.

Title: Acute Ketone Monoester Supplementation Impairs 20-Minute Time-Trial Performance in Trained Cyclists: A Randomized, Crossover Trial

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Running Head: Ketone Monoester Ingestion Impairs Endurance Performance.

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Abstract

Acute ketone monoester (KE) supplementation can alter exercise responses, but the performance effect is unclear. The limited and equivocal data to date are likely related to factors including the KE dose, test conditions, and caliber of athletes studied. We tested the hypothesis that mean power output during a 20-min cycling time-trial (TT) would be different after KE ingestion compared to a placebo (PL). A sample size of 22 was estimated to provide 80% power to detect an effect size dz of 0.63 at an alpha level of 0.05 with a 2-tailed paired t-test. This determination considered 2.0% as the smallest meaningful difference in performance. N=23 trained cyclists (peak oxygen uptake: 65±12 mL/kg/min; mean±SD), who were regularly cycling >5 h/wk, completed a familiarization trial followed by two experimental trials. Participants self-selected and replicated their diet and exercise for ~24 h before each trial. Participants ingested either 0.35 g/kg body mass of (R)-3-hydroxybutyl (R)-3-hydroxybutyrate KE or a flavour-matched PL 30 min before exercise in a randomized, double-blind, crossover manner. Exercise involved a 15-min warm-up followed by the 20-min TT on a cycle ergometer. The only feedback provided was time elapsed. Pre-exercise venous [β-hydroxybutyrate] was higher after KE vs PL (2.0±0.6 vs 0.2±0.1 mM, $p<0.0001$). Mean TT power output was 2.4 [0.6 to 4.1]% (mean[95% CI]) lower after KE vs PL (255±54 vs 261±54 W, $p<0.01$; $dz=0.60$). The mechanistic basis for the impaired time trial performance after KE ingestion under the present study conditions remains to be determined.

Introduction

Nutritional ketosis induced through the ingestion of ketogenic supplements can alter physiological responses to exercise (Evans et al., 2017). This practice has also been purported to enhance performance, at least under selected conditions, although the precise mechanistic basis is unclear (Evans et al., 2022; Pinckaers et al., 2017). As noted by Dearlove et al. (2019), context is key in assessing the potential performance effects of ketone body supplements. Important considerations include supplement type, increase in blood ketone body concentrations, relative exercise intensity, and the training state of participants studied. The ketone monoester (KE) supplement has emerged as the preferred ketone body supplement because, for a given dose, it elicits a greater increase in blood ketone body concentrations and is generally well tolerated (Stubbs et al., 2017, 2019). With regards to exercise intensity, a key consideration is whether acute KE supplementation potentially impairs performance under conditions that necessitate a high rate of carbohydrate oxidation as occurs during some types of athletic competition (Coyle et al., 1986; Hawley & Leckey, 2015). Regarding study participants, the effect of KE on performance in a sample of well-trained athletes compared to recreationally active individuals may increase the ecological validity of the study.

Studies assessing the effect of acute KE ingestion on endurance cycling performance in trained individuals have yielded equivocal data, which may be explained in part by between-study differences in any nutritional controls implemented, KE dosing strategy, and the specific performance test used (Cox et al., 2016; Evans et al., 2019; Margolis & Fallon, 2020; McCarthy et al., 2021; Poffé et al., 2020b, 2020a, 2021). A

seminal study in the field reported that KE ingestion before and during 60 min of cycling at 75% peak power raised blood β-hydroxybutyrate concentration to ~2-3 mM and improved 30-min time-trial performance in eight trained individuals who were studied in the overnight fasted state (Cox et al. 2016). Other studies that have generally found no effect of KE ingestion on performance used test protocols that involved a period of constant-work exercise prior to the performance test (Evans et al. 2019, McCarthy et al. 2021). A recent study by Poffe et al. (2021) reported that ingestion of ~50 g of KE (~0.7 g/kg body mass) during a warmup increased venous blood β-hydroxybutyrate concentration to ~3.5 mM and impaired subsequent 30-min cycling time-trial performance in 12 well-trained cyclists who were studied 2 h after breakfast ingestion. It has been suggested that eliciting a blood β-hydroxybutyrate concentration in the range of 1-3 mM during an exercise may elicit the most favourable conditions for potential performance enhancement, perhaps by limiting the potential gastrointestinal distress, blood acidosis, and increased cardiorespiratory stress associated with relatively larger doses of KE supplements (Evans et al., 2017; Evans et al., 2022). Additional work is warranted to clarify the effect of acute KE supplementation — including strategies that elicit an increase in blood β-hydroxybutyrate concentration to ~1-3 mM — on time-trial performance following a standardized warm-up in trained individuals in the postprandial state, which is the manner in which many athletes typically compete.

The purpose of this study was to determine if acute KE supplementation affects endurance exercise performance in trained individuals. Using a randomized, crossover and triple-blinded design, trained cyclists ingested a (R)-3-hydroxybutyl (R)-3-

hydroxybutyrate KE supplement or a taste- and volume-matched ketone-free placebo, and then performed a laboratory-based 20-min cycling time-trial. Performance using this approach is highly reproducible in trained cyclists, with a reported day-to-day coefficient of variation of 1.4% using the same equipment and procedures as employed in the present study (Macinnis et al., 2019). The test is also strongly correlated with functional threshold power (Macinnis et al., 2019), another performance metric that in turn is strongly correlated with 40-km road race performance (Coyle et al., 1991). Given the equivocal nature of the limited performance studies to date, we tested the non-directional hypothesis that mean 20-min time-trial power output would be different after ingestion of KE and placebo.

Methods

Study design

This randomized, crossover, triple-blinded trial involved four visits to the laboratory for each participant: an initial peak oxygen uptake ($\dot{V}O_{2peak}$) test, a familiarization trial, and two experimental trials. The experimental trials differed only by the supplement ingested before exercise, i.e., KE or ketone-free placebo. Each participant was randomized into one of two treatment orders by coin flip (block size of 1, heads = placebo trial first) by a researcher who was not involved in data collection. The participants and researchers who interacted with the participants and/or were involved with data collection and analysis were blinded to the experimental conditions. This project was approved by the Hamilton Integrated Research Ethics Board (#13837) and registered prior to the start of data

collection on ClinicalTrials.org (NCT05226962). The manuscript was prepared in accordance with the Proper Reporting of Evidence in Sport and Exercise Nutrition Trials guidelines (Betts et al., 2020).

Participants

The inclusion criteria for this study were: (1) healthy adults aged between 18 and 60 years of age; (2) consuming >50 g/d of carbohydrate, i.e., not following a ketogenic diet (Aragon et al., 2017); (3) experience with competitive cycling or time trials or racing; (4) habitually engaging in ≥5 h/week of cycling training over ≥3 d/week; and (5) having an estimated $\dot{V}O_{2peak}$ of ≥55 mL/kg/min for males and ≥48 mL/kg/min for females based on the online calculator available at: https://www.worldfitnesslevel.org. Cycling volume and frequency and the $\dot{V}O_{2peak}$ criteria were based on the characteristics of "trained athletes" described by (De Pauw et al., 2013; Decroix et al., 2016). Interested individuals were first informed of the study requirements, including the inclusion criteria described above, and provided with an electronic copy of the informed consent form. They were also asked to complete a Get Active Questionnaire (Canadian Society for Exercise Physiology) to assess general readiness to engage in physical activity and a custom questionnaire to assess habitual nutrition, physical activity, and menstrual cycle. Those who satisfied all the inclusion criteria were asked to provide written informed consent and were then recruited into the study.

Study sample size was estimated based on the assumption that 2.0% was the minimal important difference. This difference is similar in magnitude to that reported in

the limited number of studies that have reported a significant effect of acute ketone monoester supplementation on time-trial performance (Cox et al., 2016b; Peacock et al., 2022; Poffé et al., 2021). It is also comparable to the improvement observed with other nutritional interventions shown to elicit an ergogenic effect including carbohydrate mouth rinsing and ingestion of carbohydrate, caffeine, and sodium bicarbonate (Brietzke et al., 2019; Carr et al., 2011; Conger et al., 2011). Estimated means (343 W), standard deviations (35 W), and correlation among repeated measures (0.95) were based on data from (Macinnis et al., 2019) for a 20-min time-trial in trained cyclists. A computation using G*Power version 3.1 (Faul et al., 2007) for a paired t-test with 2 tails revealed that an estimated total sample size of 22 participants provided 80% power to detect a change at an alpha level of 0.05 with effect size Cohen's d_z of 0.63. To preserve power, a total of 25 participants were recruited. Recruitment and data collection occurred from February 2022 through August 2022. Posters advertising the study were shared on McMaster University campus, social media platforms, and with local cycling clubs. Thirty-four participants were initially assessed for eligibility, and 23 of the 25 individuals who were recruited and randomized completed the entire study (Figure S1). One participant dropped out due to a knee injury that occurred during a non-study related activity and the other dropped out due to scheduling conflicts. The characteristics of the 23 participants who completed the study are reported in Table 1.

Interventions

The experimental interventions were acute ingestion of either 0.35 g/kg body mass of a KE or ketone-free placebo 30-min before exercise. The commercial (R)-3-

hydroxybutyl (R)-3-hydroxybutyrate KE beverage (deltaG ketone performance, TDeltaS, Thame, Oxfordshire, United Kingdom) contained 120 kcal per 59 ml bottle (4 g carbohydrate, 25 g of (R)-3-hydroxybutyl (R)-3-hydroxybutyrate) and was all purchased in the same order. The placebo beverage was matched to the KE for flavour, volume, and carbohydrate content (16 kcal per 59 ml). It contained the same ingredients as the KE except the (R)-3-hydroxybutyl (R)-3-hydroxybutyrate was replaced by 0.05% Denatonium Benzoate (Bitrex).

Pre-experimental Procedures

$\dot{V}O_{2peak}$ was determined as we have previously described (McCarthy et al., 2021). Briefly, the ramp test began with a 3-min warm-up at 50 W and workload was increased 1 W every 2 s until the participant reached volitional exhaustion and ceased pedalling or decreased the cadence to <60 rpm. Expired gases (Quark CPET, COSMED Inc., Concord, CA) and HR (Polar Electro, Model A300) were recorded continuously throughout. The familiarization trial mimicked the experimental trials except that no supplements were provided before exercise and no blood samples were collected. After the trial, participants ingested ~10 mL of the ketone-free placebo supplement to become accustomed to the bitter flavour but were not informed whether they received the KE or placebo.

Experimental procedures

The two experimental trials were separated by 7 days and performed at the same time of day (±1 h) for a given participant. Owing to scheduling conflicts, the two trials for five participants were separated by 8 to 14 days. For female participants, the two

experimental trials were performed in the same phase of their hormonal cycle. One participant was taking oral contraceptives and completed both trials in the active phase of their cycle, the other completed both trials in the early follicular phase of their hormonal cycle. Participants were instructed to maintain their usual dietary and exercise habits throughout the study. Nutritional strategies outside of the laboratory were self-selected by each participant in accordance with their habitual dietary practices, approved by a researcher, and repeated for all trials, similar to Burke *et al.* (Burke et al., 2017, 2020). The day before and morning of the trials, participants were instructed to eat and drink how they would normally prepare for a race. The intent was for the pattern of nutrition intake to be matched as precisely as possible between trials. Participants were also instructed to avoid alcohol and intense training on the day before and of the trials. A continuous glucose monitoring device (Abbott Libre Sense Glucose Sport Biosensor, Supersapiens, Atlanta, GA) was inserted into the back of the participant's arm, per manufacturer instructions, before the first experimental trial and remained in the participants arm until the second experimental trial was completed.

An overview of the experimental trial is shown in Figure 1. Upon arrival at the laboratory, participants sat upright and ingested 0.35 g/kg body mass of the KE or a flavour- and volume-matched ketone-free placebo beverage from an opaque container. After ingesting the drink, they rested for 30 min, during which they completed a dietary recall questionnaire with the researcher. About 25 min into the rest period, a venous blood sample was drawn from an antecubital vein into a lithium-heparinized syringe and analyzed within 15 min by a researcher who did not interact with the participants and was

not otherwise involved with data analysis to preserve blinding. Point-of-care analyzers determined [β-hydroxybutyrate] (β-ketone test strips, Freestyle Precision Neo; Abbott Laboratories, IL, USA) and [glucose], [lactate], total CO_2 and pH (EPOC; Siemens Healthcare, Ontario, Canada) (Nawrocki et al., 2021) in whole venous blood samples. After the rest period, participants completed a 15-minute self-determined warmup followed by the 20-min time trial on the electronically braked cycle ergometer (Velotron; Racer Mate Inc., Seattle, WA) based on the methods described by Macinnis et al. (2019). The ergometer used in the study allows riders to change power output either by increasing/decreasing cadence or by changing the simulated gear. The time-trial was performed in the presence of two researchers in an otherwise closed laboratory. No food or drink except for water *ad libitum* was provided during the time trial. The only feedback provided to the participant was time elapsed. Bike handlebar and seat configuration as well as fan speed and location were the same for both trials. Heart rate was recorded continuously throughout exercise (Polar A3, Finland). Mean power output was recorded after completion of the time trial. Peak and overall perceived exertion scores were obtained based on the Borg 20-point scale (Borg, 1982) within 1 min of completion of the time trial. Participants then completed a validated gastrointestinal symptom questionnaire for sports nutrition exercise trials to assess perceptions experienced during the time trial (Gaskell et al., 2019). Briefly, participants circled an integer from 1-10 that corresponded to a severity for 19 different gastrointestinal symptoms, 0 was no symptom present, 1-4 mild symptoms, 5-9 severe symptoms, and 10 extremely severe symptoms. Finally, a questionnaire was completed to assess supplement blinding effectiveness as we have

previously described (McCarthy et al., 2021). Participants were provided with no feedback regarding any time-trial data until completion of the study.

Statistical Analysis

Data represent means ± standard deviations for *n*=23 participants based on both experimental trials unless otherwise stated. Blood gas analysis was not performed on *n*=1 blood sample due to technical difficulties with the analyzer. Continuous glucose monitoring data represent *n*=19 as data were not obtained for one or both trials for *n*=4 participants because either the sensor fell off within 24 h of the trial or there were technical errors related to data transmission from the sensor. Rating of perceived exertion data were not obtained for *n*=1 participant. Normality was tested using a Shapiro-Wilks test. Paired 2-tailed t-tests assessed differences between conditions (KE vs placebo) for mean 20-min time-trial power output, mean and peak heart rate, macronutrient and total energy intake, blood [β-hydroxybutyrate], [glucose], [lactate], total CO_2, [bicarbonate] and pH before exercise, and mean [glucose] during exercise. Mean and peak rating of perceived exertion and total gastrointestinal symptom severity and incidence were tested for differences between conditions using a Wilcoxon's test. A time-period effect was assessed for mean time-trial power output by a 2-tailed paired t-test (trial 1 vs 2). Exploratory 2-way repeated-measures analysis of variance (condition x time) tests were performed on power output, heart rate, and glucose via continuous glucose monitoring during the time trial. Statistical significance was accepted as $p \leq 0.05$. All statistical analyses were performed using commercial software (GraphPad Prism v9, San Diego,

CA). Normally distributed data are presented as mean ± standard deviation, non-parametric data as median [interquartile range], change data as mean [95% confidence intervals] and effect sizes as Cohen's d_z.

Results

Time-trial data

Mean 20-min time-trial power output was 6.2 [1.7 to 10.2] W or 2.4 [0.6 to 4.1]% lower after KE vs PL ingestion (255±54 vs 261±54, p=0.01, d_z=0.60) (Figure 2). Mean power output was not different when compared for order effect (Trial 1: 260±53, Trial 2: 257±56 W, p=0.44, d_z=0.03). Mean heart rate during the time trial was lower after KE vs PL ingestion; however, peak heart rate, mean interstitial glucose (*n*=19), and mean and peak rating perceived exertion were not different between conditions (Table 2). Eleven of the 22 participants reported a numerically higher rating of perceived exertion after KE vs placebo ingestion, seven reported the same rating of perceived exertion in both conditions, four reported a lower rating of perceived exertion after KE vs placebo ingestion.

Blood data

The ketone body β-hydroxybutyrate was higher in whole venous blood samples after KE vs PL ingestion (2.0±0.6 vs 0.2±0.1, p<0.0001, d_z=3.1, Figure 3). Other blood measurements sampled before exercise are summarized in Table 3. Venous blood glucose, bicarbonate, and total CO_2 content were lower after KE vs PL ingestion.

Condition did not affect venous blood pH or lactate. Pre-exercise interstitial glucose determined by continuous glucose monitoring was 8[0 to 15] mg/dl lower in after KE vs PL ingestion (75±12 vs 82±17 mg/dl, p=0.048, d_z=0.48).

Questionnaire data

Total gastrointestinal symptom incidence (1[0 to 3] vs 0[0-0] of 19, p=0.0005) and severity (0[0 to 0] vs 0[0 to 1] of 10, p=0.0004) were statistically higher after KE vs placebo ingestion. Upper gastrointestinal symptom incidence (1[0 to 2] vs 0[0 to 0] of 7, p=0.0001) and severity (0.4[0 to 1] vs 0[0 to 0.1] of 10, p<0.0001) were higher after KE vs placebo ingestion, but incidence and severity of lower, other and defecation gastrointestinal symptoms were not different between conditions (p<0.28 for all). Within upper gastrointestinal symptoms, severity of belching (p=0.03), heartburn (p=0.004), bloating (p=0.03), and stomach pain (p=0.03) were higher after KE vs placebo ingestion but urge to regurgitate (p=0.12) was not different and there were no incidences of regurgitation or projectile vomiting. The median severity of any symptom assessed did not exceed a score of 1.

In response to “could you tell whether you received the ketone monoester or placebo?”, *n*=9 indicated “no” after both trials, *n*=10 indicated “yes” after one trial, and *n*=4 indicated “yes” after both trials. Of the 18 questionnaires that “yes” was selected, n=11 follow-up responses to "did you receive ketone monoester or placebo?” matched the solution received (i.e., correct guess) and n=7 did not match (i.e., incorrect guess). *N=3* participants correctly guessed the solution received in both trials.

Exploratory time-based analyses

Mean power output in every quarter of the time trial was lower after KE vs PL ingestion (condition p=0.01, interaction p=0.92, Figure 3A). Heart rate at 5, 10, 15 and 20 min of the time trial was lower after KE vs PL ingestion (condition p=0.04, interaction p=0.69, Figure 4B). Neither condition (p=0.09) nor condition x time (p=0.17) affected glucose via continuous glucose monitoring throughout the time trial (Figure 4C).

Discussion

The primary finding of this study was that mean power output during a 20-min cycling time-trial was lower after acute ingestion of a 0.35 g/kg body mass of a KE supplement ingested 30-min before exercise as compared to a ketone-free placebo drink in trained cyclists who were studied in the postprandial state. The difference in time-trial power output was ~2.4% lower after KE vs placebo ingestion, which exceeded the minimal important difference that was established prior to data collection and for the purposes of estimating sample size.

Our findings agree with recent work by Poffe et al. (2021) who studied trained cyclists 2 hours after ingestion of a carbohydrate-rich breakfast. These authors reported that acute ingestion of 50 g (~0.7 g/kg body mass) of KE with co-ingestion of carbohydrates elevated blood β-hydroxybutyrate to ~3.5 mM and impaired 30-min cycling time-trial performance by ~1.4% compared to carbohydrates alone. In that study, participants manually adjusted workload at 5-min intervals throughout the time trial, whereas in the present study participants adjusted workload by altering the simulated

"gear" and/or cadence. Both of these studies are also in agreement with the work of Leckey et al. (2017), who reported that acetoacetate diester ingestion impaired 31-km time-trial (~50 min) performance in professional cyclists who were studied under conditions that simulated sport nutrition guidelines. The reported increase in blood β-hydroxybutyrate concentration in that study was <1 mM (Leckey et al. 2017), and all participants reported gastrointestinal distress potentially owing to the nature of the supplement. Overall, while the available body of work remains limited, it appears that ketogenic supplement ingestion impairs relatively short duration, high intensity cycling performance.

It has been proposed that ketogenic supplement ingestion may be ergogenic if the exercise test is performed under conditions that result in a circulating β-hydroxybutyrate concentration of 1-3 mM (Evans et al., 2017). Venous β-hydroxybutyrate concentration after KE supplement ingestion in the current study was ~2 mM but this was associated with impaired time trial performance. These data therefore do not support the notion that increasing blood β-hydroxybutyrate concentration to a range of 1-3 mM is ergogenic, at least under the present study conditions. The potential effect of KE dose and the corresponding rise of blood β-hydroxybutyrate concentration on endurance performance in various tests requiring 1-2 h of moderate-to-high intensity efforts is less clear (Cox et al. 2016, Evans and Egan 2018, Evans et al. 2019, Peacock et al. 2022).

The lower heart rate during the time-trial after KE ingestion may relate to effects of KE ingestion on peak heart rate and/or perceived exertion or reflect the lower time-trial power output. Interpreting these heart rate data are challenging because ingestion of 0.6

g/kg KE increased heart rate during submaximal cycling at a constant workload that was equivalent to ventilatory threshold intensity (McCarthy et al., 2021, 2023). It is unknown whether the comparatively lower dose of KE used in the current study would similarly affect exercise heart rate or whether heart rate responses to KE ingestion differ depending on relative exercise intensity. This is further complicated by the fact that mean power output was different during the two time trials in the present study. Despite the lower power output and heart rate achieved during the time trial after KE ingestion, ratings of perceived exertion were not statistically different between conditions. We previously reported that ingestion of 0.6 g/kg body mass KE increased perceived exertion during exercise at a fixed workload and this response was related to subsequent performance in a ~15-min time-trial (McCarthy et al., 2021). Thus, these data suggest that perceived exertion and heart rate remained affected by a relatively smaller dose of KE, but more work is needed to fully understand these effects.

Ingestion of 0.35 g/kg body mass of KE (~25 g) did not affect pre-exercise blood pH but affected other metabolites involved in blood acid-base balance. In contrast, blood pH was lowered in fasted participants after ingestion of ~0.3 g/kg body mass of KE (Dearlove et al., 2019; Stubbs et al., 2017) as well as in fed participants after ingestion of 45-50 g of KE (~0.7 g/kg body mass) (Poffe et al., 2021; Poffé et al., 2020a, 2020b, 2021). A stress to blood acid-base balance by KE ingestion in the current study is suggested by a lowering of blood [bicarbonate] and total CO_2. These changes could indicate that excess hydrogen ions, presumably from the KE drink, required buffering by bicarbonate, which would then result in less free blood [bicarbonate] and more CO_2 in the

blood, trigger a rise in ventilation to compensate, and then the hyperventilation would reduce blood CO_2. Thus, blood acid-base status was presumably still challenged in the current study before exercise, and it is possible that the hydrogen ions produced during exercise would have had a bigger effect on blood pH after KE compared to placebo ingestion.

As seen in some studies (Evans & Egan, 2018; McCarthy et al., 2021; Poffé et al., 2020b, 2021), KE ingestion slightly elevated the incidence and severity of gastrointestinal distress, however the magnitude of change is unlikely to be practically relevant. Notably, a study primarily examining gastrointestinal symptoms during exercise after KE ingestion determined the incidence and severity of such to be no different than carbohydrate intake (Stubbs et al., 2019). The marginally higher gastrointestinal distress observed after KE vs placebo ingestion in the current study was specific to the upper gastrointestinal tract and corresponded to an overall mean score of 1 vs 0 on a 10-point scale. The KE supplement is proposed to be largely absorbed in the upper gastrointestinal tract when provided in a low dose (Shivva et al., 2016) and, therefore, the lack of reported symptoms beyond the upper gastrointestinal tract may be because they were never exposed to the KE. Nonetheless, the gastrointestinal symptoms in response to KE ingestion were minimal.

A reduction in blood glucose at rest and during exercise is observed in many studies after KE ingestion (Falkenhain et al., 2022). A previously study from our laboratory reported that ingestion of 0.6 g/kg body mass KE reduced blood glucose when measured at rest but not immediately postexercise (McCarthy et al. 2021). In the present study, blood glucose before exercise was lowered by KE ingestion as determined by both

venous sampling and continuous glucose monitoring, but exercise glucose determined by continuous glucose monitoring was not different between conditions. The mechanisms underlying the effect of KE on blood glucose during exercise are unclear and studies involving stable isotope tracers are needed to comprehensively examine glucose kinetics during exercise.

A strength of the present work was that, in designing and conducting the study, we sought to meet best practice guidelines including those related to minimizing the risk of bias in reporting (Betts et al., 2020). The study protocol was registered prior to data collection and included information related to our hypotheses, sample size, and key outcomes. The final sample size (n=23) exceeded our *a priori* estimate in this regard, which in turn was based on the difference observed from nutritional interventions generally considered to be ergogenic and day-to-day repeatability of the performance test used. A limitation of the present study is that the real-world extrapolation of the outcome is specific to the nature of the 20-min time trial, i.e., high-intensity continuous type exercises. Additionally, the 15-min self-determined warm-up does not necessarily mimic the longer, variable-intensity strategies that are often used by elite cyclists prior to competition. While 20-min time-trial performance is correlated with functional threshold power and both tests involve similar metabolic and physiologic demands, more work is required to determine the effects of KE ingestion on longer duration endurance events. Additionally, this study focused on performance and provides little insight into potential mechanisms that may explain the observed differences in performance. A comprehensive recent review by Evans et al. (2022) considers some of the potential mechanisms and

additional research is needed to advance the field in this regard.

In conclusion, we show that acute ingestion of 0.35 g/kg body mass KE impaired 20-min time-trial performance in trained cyclists when compared to a placebo. Our data suggest that this effect may relate to the effect of KE ingestion on blood acid-base status, heart rate, and/or perceived exertion. This KE-associated impairment of performance occurred despite meaningful gastrointestinal distress during the time trial and [β-hydroxybutyrate] being elevated to a previously hypothesized ergogenic range. More work is required to elucidate the underlying physiological responses to acute KE ingestion and how these in turn may be linked to changes in exercise capacity.

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Authorship: DGM, MF, PP, LVL, and MJG contributed to conceptualization; DGM, MF, and MJG to methodology; DGM and MF to validation; DGM, MF, JB, BB, and PP to investigation; MJG to resources; DGM, MF, and JB to data curation; DGM to writing – original draft; all authors to writing – review and editing; DGM to visualization; LVL and MJG to supervision; DGM to project administration; and MJG to funding acquisition. All authors approved the final version of this paper.

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Protocol: This trial was registered as a clinical trial before data collection was initiated (NCT05226962).

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Figures & Tables:

Table 1. Participant characteristics.

	All (n=23)
Age (years)	31 ± 9
Weight (kg)	76 ± 11
Height (m)	1.77 ± 0.07
$\dot{V}O_{2peak}$	
(L/min)	4.9 ± 1.0
(mL/kg/min)	65 ± 12
Peak Power Output (W)	390 ± 60
HR_{peak} (beats/min)	185 ± 13
VT1 (%$\dot{V}O_{2peak}$)	64 ± 8
RCP (%$\dot{V}O_{2peak}$)	85 ± 7

Data are presented as mean ± standard deviation for n=23 participants except *n*=21 for HR_{peak} and n=22 for VT1. $\dot{V}O_{2peak}$, peak oxygen uptake; HR_{peak}, peak heart rate; VT1, ventilatory threshold 1; RCP, respiratory compensation point.

Table 2. Physiological and perceptual measurements during the 20-min time-trial performed 30 min after ingestion of 0.35 g/kg ketone monoester (KE) or a ketone-free placebo (PL).

	PL	**KE**	**Δ**	**p**	**d_z**
Mean Glucose (mg/dl)	88±17	81±12	-7 [2 to -16]	0.12	0.38
Heart Rate (beats/min)					
Mean	167±15	165±14	-2 [0 to -4]	0.03	0.48
Peak	181±13	178±13	-2 [1 to -5]	0.11	0.34
RPE (/20)					
Mean	17 [16-18]	17 [16-18]	0.5 [0 to 1]	0.11	---
Peak	19 [19-20]	19 [19-20]	0 [0 to 1]	0.75	---

Data are presented as mean ± standard deviation for n=23 participants for heart rate and n=19 for mean glucose. Data are presented as median [interquartile range] for n=22 for RPE as this was not normally distributed. Change scores of KE – PL (Δ) are presented as mean [95% confidence interval] for glucose and heart rate and median [95% confidence intervals] for RPE. Analyses is based on a 2-tailed pared t-test or Wilcoxon's test as appropriate for KE vs PL. d_z, Cohen's effect size. RPE, rating of perceived exertion; * $p<0.05$ vs PL.

Table 3. Resting venous blood metabolites and acid-base status 30-min after ingestion of 0.35 g/kg body mass of ketone monoester (KE) or a ketone-free placebo (PL).

	PL	KE	Δ	p	d_z
β-hydroxybutyrate (mM)	0.2 ± 0.1	2.0 ± 0.6*	1.8 [1.6-2.0]	<0.0001	3.1
Glucose (mM)	5.2 ± 0.6	4.7 ± 0.9*	0.5 [0.1-1.0]	0.02	0.57
Lactate (mM)	2.0 ± 1.1	1.8 ± 0.9	0.2 [-0.4-0.7]	0.56	0.13
pH	7.32 ± 0.04	7.31 ± 0.03	0.001[-0.02-0.03]	0.65	0.10
Bicarbonate (mM)	29.9 ± 1.9	28.2 ± 1.8*	1.7 [0.8-2.6]	0.0007	0.87
Total CO_2 (mmHg)	30.5 ± 2.0	28.9 ± 2.0*	1.7 [0.7-2.6]	0.001	0.85

Data are presented as mean ± standard deviation for n=23 participants β-hydroxybutyrate and n=22 for other measures. Change scores (Δ) for KE - PL are mean [95% confidence interval]. Analyses is based on a 2-tailed pared t-test for KE vs PL. d_z, Cohen's effect size. RPE, rating of perceived exertion; * $p<0.05$ vs PL.

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as mean ± standard deviation (n=23 for power output and heart rate, n=19 for glucose). *, $p<0.05$ main effect condition (KE vs PL) from exploratory 2-way repeated measures analysis of variance.

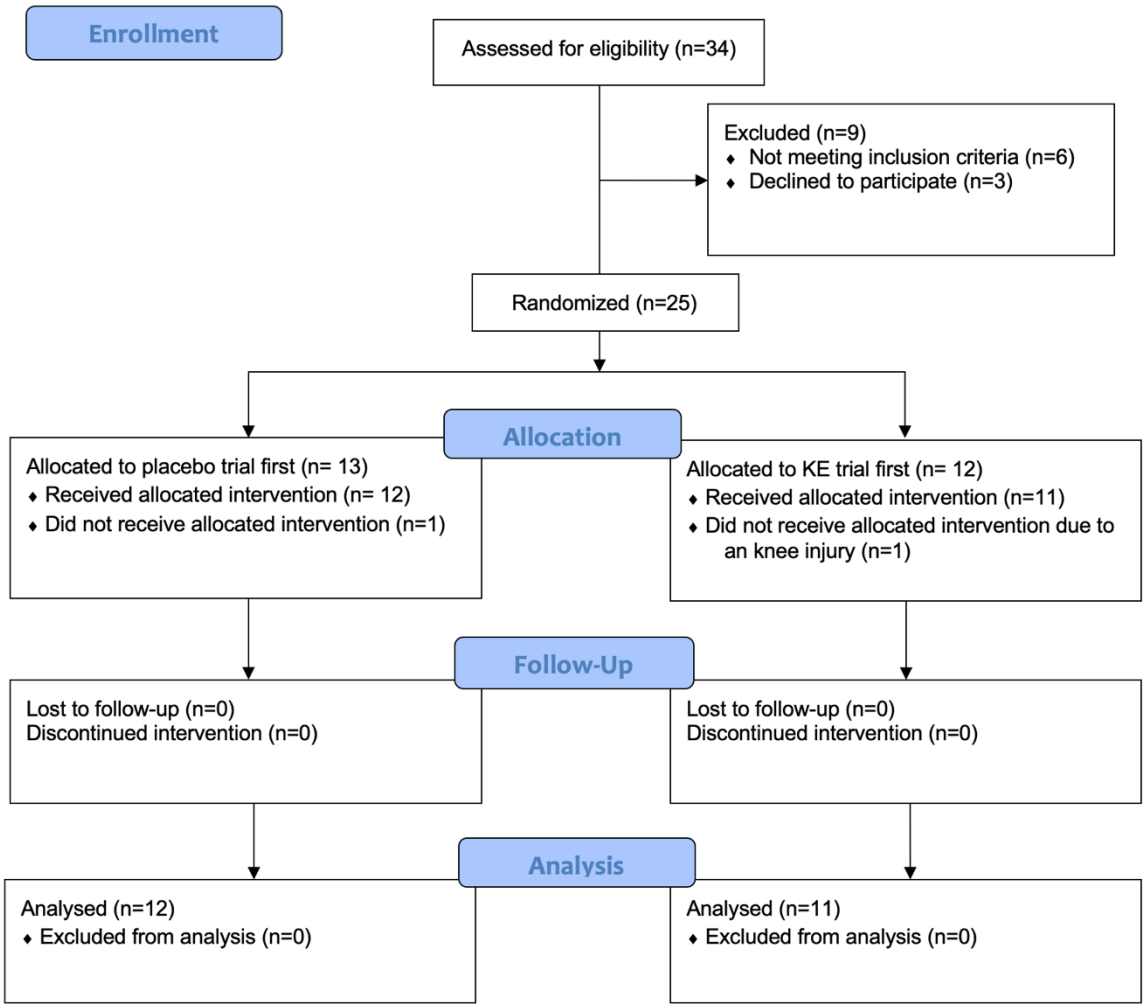

Figure S1. CONSORT participant flow diagram.

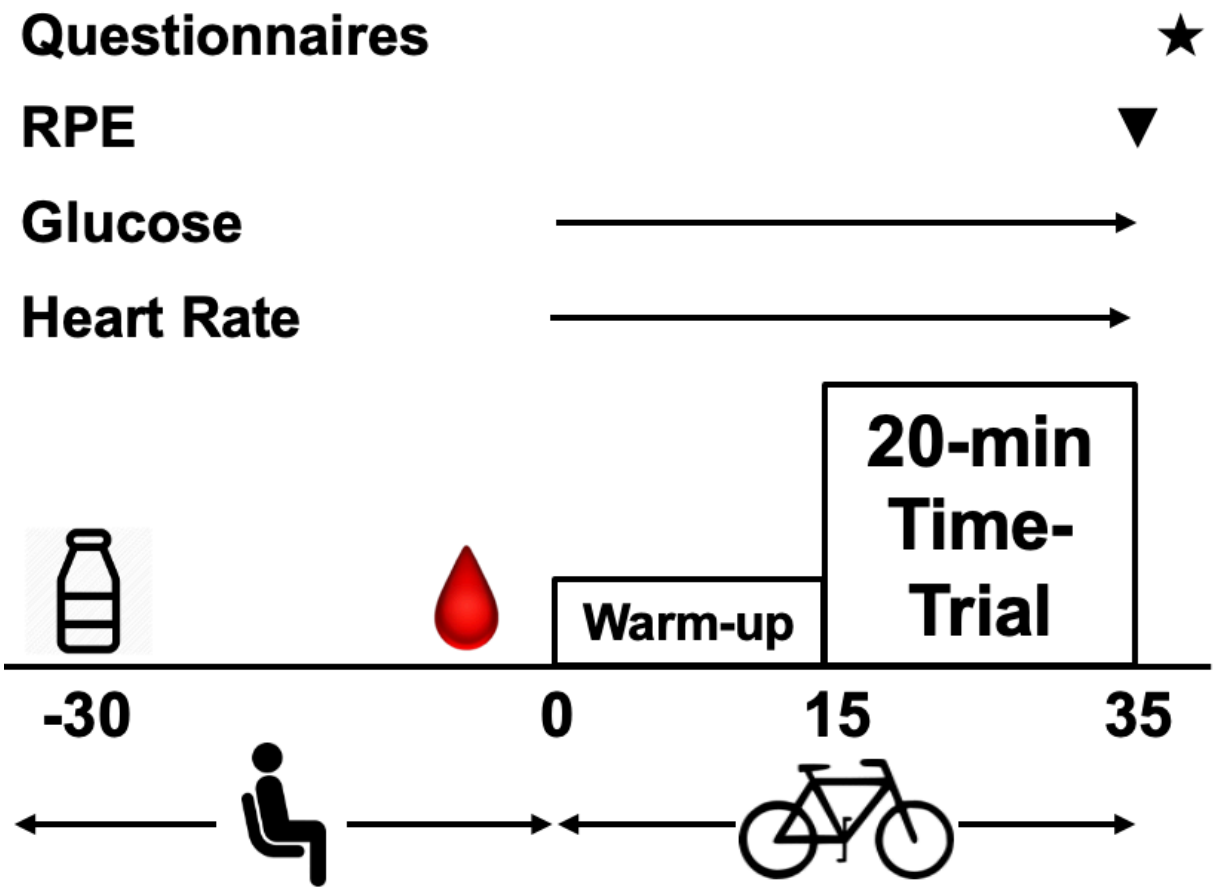

Figure 1. Schematic overview of the experimental protocol. Twenty-three trained cyclists ingested 0.35 g/kg body mass of a ketone monoester supplement (KE, DeltaG Ketone Performance) or a ketone-free placebo (PL) in a randomized, crossover, double-blinded manner (bottle image). Glucose was measured via continuous glucose monitoring. The blood drop represents venous blood sampling; RPE, rating of perceived exertion; numbers, time in minutes

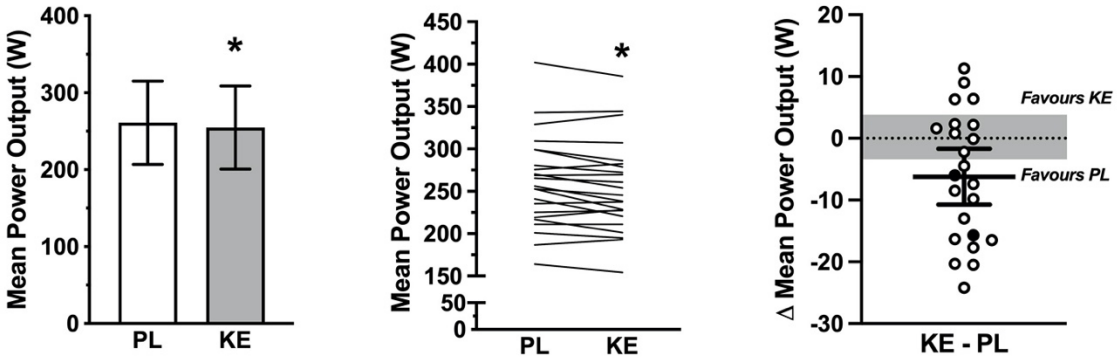

Figure 2: Mean power output during the 20-min cycling time-trial after ingestion of 0.35 g/kg ketone monoester (KE) or a ketone-free placebo (PL). Left: Bars are mean ± standard deviation (n=23). Middle: lines connect individual participant data points. Right: Long line represents the mean change, error bars are the 95% confidence interval of the change, and dots are individual participants (open=male, closed=female). * $p<0.05$ KE vs PL (paired 2-tailed t-test).

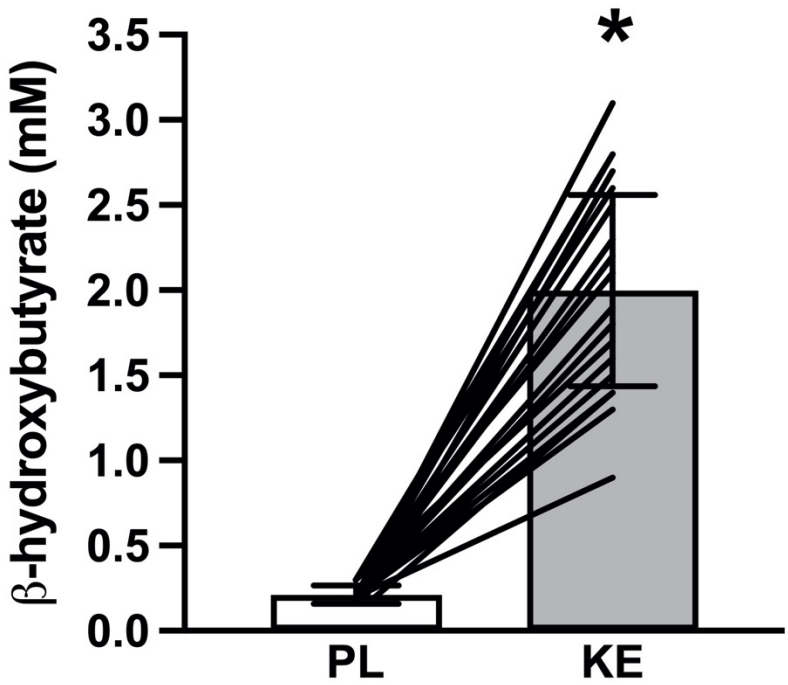

Figure 3: Pre-exercise whole venous blood β-hydroxybutyrate concentration after ingestion of 0.35 g/kg ketone monoester (KE) or a ketone-free placebo (PL). Bars are mean ± standard deviation (n=23) and lines connect individual participant data points.

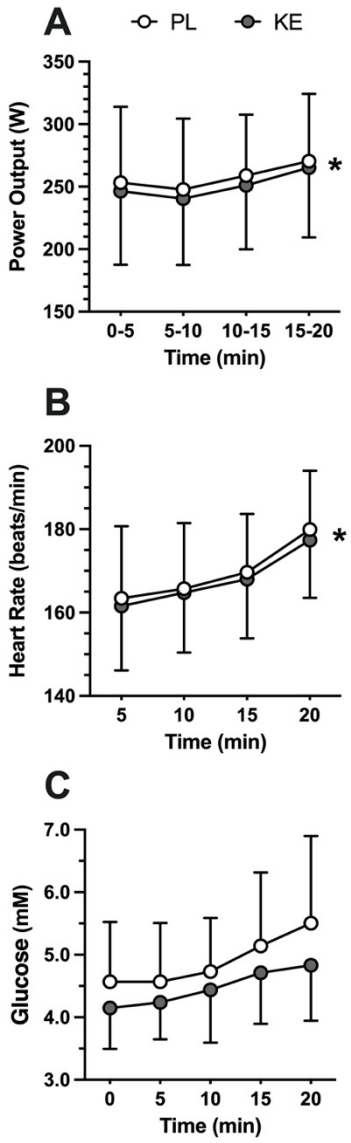

Figure 4: (A) Power output, (B) heart rate, (C) and glucose determined from continuous glucose monitoring during the 20-min time-trial after ingestion of 0.35 g/kg ketone monoester (KE, grey circles) or a ketone-free placebo (PL, open circles). Data are presented as mean ± standard deviation (n=23 for power output and heart rate, n=19 for glucose). *, $p<0.05$ main effect condition (KE vs PL) from exploratory 2-way repeated measures analysis of variance.

CHAPTER 5: General Discussion

5.1 Introduction

This thesis aimed to advance knowledge regarding the role of KB in the integrative physiological response to exercise in healthy active humans. This was probed by elevating blood KB above typical basal levels (i.e., the induction of hyperketonemia) through acute ingestion of commercial KE supplements. This approach to increase circulating blood KB permitted a more direct evaluation of the effects of KB as it did not otherwise involve dietary manipulation.

Owing to the limited research on exogenous hyperketonemia when this thesis work was initiated, the first study (Chapter 2) examined the effects of acute hyperketonemia on cardiorespiratory stress and indices of substrate utilization during a bout of constant-load submaximal cycling followed by a short time trial to test exercise performance. The results showed that V_E, HR, and perceived exertion were elevated after KE ingestion compared to a flavour-matched, ketone-free placebo. Expired gas estimations of substrate oxidation were unchanged, as was performance during the time-trial. However, the study also suggested that blood acidosis associated with KE ingestion (Dearlove et al., 2019; Stubbs et al., 2017) may have impacted on the exercise responses and the data might not reflect an effect of elevated KB per se. The interpretation of the performance data was also potentially confounded by individual perceived exertion responses during the preceding controlled exercise period.

The results from the first study led to two follow-up projects. One attempted to parse the physiologic impacts of blood acidosis from acute hyperketonemia (Chapter 3). The other assessed the effect of KB supplementation on endurance performance under

conditions that more closely simulated typical athletic competition (Chapter 4). This follow-up work in Chapter 3 reproduced some of the main findings associated with acute hyperketonemia that were identified and described in Chapter 2. It also suggested that the higher HR and perhaps O_2 requirement of exercise associated with acute hyperketonemia were not associated with a higher Q. By administering a blood pH buffer with the KE supplement, it was also determined that blood acidosis could not entirely account for the cardiorespiratory effects of exogenous KB supplementation. The final study showed that acute ingestion of KE, at a lower dose than used in the initial studies, impaired 20-min cycling time-trial performance in trained cyclists. Overall, this thesis demonstrates that acute exogenous hyperketonemia can alter the integrative physiological response to exercise in humans, and it highlights several areas that warrant future investigation.

5.2 Isolating the potential effects of increased KB

One goal of this work was to isolate for the effects of increased KB availability on exercise physiology and metabolism in humans. This had traditionally been achieved through long-term fasting or adherence to a ketogenic diet, interventions that involve the manipulation of both dietary carbohydrate intake and KB availability. KB supplements provided a feasible tool to elevate KB in humans without otherwise changing diet or requiring intravenous infusion. Despite this acute intervention overcoming the potential confounding effects of additional dietary manipulation, KB supplementation alters several other factors in addition to KB availability. This limits the potential to attribute some purported effects to KB per se. For example, KE ingestion lowered blood glucose

concentration, free fatty acid concentration, pH, and partial pressure of CO_2 (Chapters 2 and 3), all of which could have had implications for physiological responses independent of alterations to blood KB levels (Coyle et al., 1986; Kowalchuk et al., 1984; Parolin et al., 1999; Stellingwerff et al., 2003). Additionally, the mechanisms that could be attributed to acute hyperketonemia throughout this thesis are under conditions of high-carbohydrate availability. It is possible that KB may elicit different effects depending on the availabilities of carbohydrates. However, acute KE ingestion did not alter cardiorespiratory exercise responses in participants that adhered to a ketogenic diet for ~3 weeks (Whitfield et al., 2020). Therefore, the data contained in thesis regarding acute elevations of exogenous KB could translate to how KB work when elevated through other dietary manipulations.

The ecological validity of isolating for the effects of KB per se in humans is debatable. KB are signalling molecules that affect the availability and potential influence other metabolites. In terms of metabolic substrates, the decrease in blood free fatty acid concentration associated with KE ingestion can be attributed to signalling effects of KB on adipose tissue nicotinic acid receptor activation and the lowered blood glucose after KE ingestion could be due to effects on hepatic tissue, blood insulin concentration, and/or the insulin-signalling pathway (Mikkelsen et al., 2015; Taggart et al., 2005). Therefore, an elevation of KB and concomitant drop in blood free fatty acid and glucose concentrations may provide better representation of the integrative physiological responses that occur during acute hyperketonemia in humans, as compared to elevating blood KB per se. Other consequences of KE ingestion that are potentially associated with

the signalling effects of KB include but are not limited to: suppressed rates of hepatic KB production (Robinson and Williamson, 1980); decreased blood insulin concentration (Falkenhain et al., 2022); increased activity of the mammalian target of rapamycin target complex 1 which potentially facilitate muscle protein synthesis (Vandoorne et al., 2017); increase nuclear concentration of the regulator of mitochondria biogenesis, i.e., peroxisome proliferator-activated receptor-gamma co-activator 1-alpha (Gomora-Garcia et al., 2023); augmented erythropoietin synthesis (Evans et al., 2023). Although, the outcomes associated with the signalling effects of KB may depend on the situation examined. For example, blood free fatty acid concentration at rest and during exercise are elevated during situations of endogenous ketosis, such as 3-4 weeks of adhering to a ketogenic diet, as opposed to the lower blood free fatty acid concentration observed after acute KE ingestion (Burke et al., 2017; Phinney et al., 1983). Thus, isolating for the effects of KB per se in humans is difficult, but the studying the interactions between KB and other metabolites can be useful for understanding how these molecules interact in nature.

In addition to acute exogenous hyperketonemia via KE ingestion impacting on other metabolites, such effects could not be attributed to a single KB, i.e., β-hydroxybutyrate (β-HB), acetoacetate or acetone. Chapter 2 showed that blood acetoacetate levels were increased after KE ingestion despite the KE supplement not containing any acetoacetate. Likewise, in a state of endogenous ketosis β-HB and acetoacetate exist at a ratio of ~3:1 (Robinson & Williamson, 1980). This becomes further complicated as acetone can be metabolized into other molecules such as glucose

(Reichard et al., 1979) (Reichard et al. 1979). Future work could attempt to isolate for the effects of β-HB by inhibiting the enzyme β-HB dehydrogenase but isolating for acetoacetate and acetone would be more complicated. While the increases of β-HB, acetoacetate, and presumably acetone after acute KB supplementation prohibits attributing a mechanism of action to a specific KB, this situation does resemble what occurs in nature.

The blood acidosis associated with KE ingestion may not represent what occurs in nature because the source of the hydrogen ions is likely due to the biochemistry of the KE molecule. KE is thought to be cleaved into β-HB and butanediol in the small intestine, ultimately resulting in the conjugate acids of such molecules to be absorbed into the bloodstream and then dissociating a hydrogen ion (See section 1.3 for more details). In contrast, ketoacidosis associated with high levels of endogenous KB can be attributed to excess fatty acid metabolism (Green & Bishop, 2019). Therefore, isolating or removing the influence of blood acidosis after KE ingestion could improve our understanding of the effects of acute hyperketonemia in humans by removing the confounding influence specific to the KB supplement. Chapter 3 showed that the KE-associated drop in blood pH was prevented by co-ingestion of the pH buffering agent bicarbonate. Contrary to our hypotheses, changes in pH did not entirely explain the cardiorespiratory responses. In fact, the only response that was entirely attributable to pH was the KE-associated decrease in blood lactate. Thus, it appears that something associated with KE ingestion other than the blood acidosis is the underlying mechanism for most of the observed

effects, and that these observations are likely associated with an effect related to acute hyperketonemia rather than an unintended consequence of the supplement.

In summary, isolating for the physiologic and metabolic effects of KB is challenging and more work is required to fully understand the role(s) of KB in humans. Inducing acute hyperketonemia through KB supplementation affects other biologically relevant metabolites and compounds that should be considered when attempting to study physiologic and metabolic responses in this physiological state. These considerations regarding the relevance of isolating for KB are important when studying mechanisms, but the practical application of KB supplements would encompass all the consequences of acute KB supplementation, e.g., blood glucose regulation (Myette-Côté et al., 2018) and exercise performance (Evans et al., 2017, 2022; Pinckaers et al., 2017).

5.3 Cardiorespiratory Exercise Responses

One of the main knowledge contributions of this thesis is characterizing the basic exercise physiological responses to acute ingestion of a ketone monoester supplement in healthy humans. The increased V_E, HR, and perceived exertion after KE vs placebo ingestion during exercise at a fixed workload are suggestive that the physiological stress that normally occurs during exercise was potentiated in this condition. The data also demonstrated that these responses were associated with hyperketonemia and not the mild blood acidosis associated with KE ingestion. Other work corroborates this statement by showing that the greater VO_2 and V_E after ingestion of KE vs a ketone-free control was still observed with blood pH was normalized via co-ingestion of bicarbonate (Poffé et al.,

2020a). Altogether, this suggests that acute hyperketonemia may have caused the elevation of physiological stress during exercise. However, it is possible that factors other than hyperketonemia are induced by ketogenic supplementation that have not yet been identified that could explain the associated increase in physiological stress. For example, Stubbs et al. (2017) showed that the blood acidosis and increased blood potassium concentration after KE ingestion was restored to pre-supplementation levels after 2 h of resting. All studies to date have examined exercise responses ~30-60 minutes after KE ingestion, and therefore other factors in addition to blood KB could be manipulated in concert. Future work could employ longer rest periods between KB supplement ingestion and assessment of physiologic and metabolic exercise responses.

While many physiological stressors were increased after KE ingestion, the integration of these physiological responses and underlying mechanisms remain unclear. The lowered peak power output at VO_{2peak} and unchanged VO_{2peak} after KE ingestion (Chapter 3) is suggestive that exercise economy was impaired by KE ingestion. This suggestion is further supported by an increased VO_2 during fixed workload exercise after KE vs placebo ingestion as determined by a retrospective analysis of data from Chapters 2 and 3 (Appendix A). Altogether, these data could indicate that the O_2 cost of exercise was increased, which could require a greater V_E and HR, if reflective of greater respiratory and cardiac muscle work, respectively. These elevated cardiorespiratory stress markers could then manifest as greater perceptions of effort (Robertson, 1982). Nonetheless, a study primarily designed to assess whether acute ingestion impairs exercise economy and efficiency would be a valuable addition to the literature.

Presuming acute hyperketonemia resulted in a modest elevation of the O_2 requirement of exercise, then compensation via augmented O_2 delivery and/or extraction would occur in concert per the Fick equation (i.e., VO_2 = Q * arterial-venous O_2difference). We tested the hypothesis that exercise Q would be augmented after KE ingestion which would reflect an increased O_2 delivery (i.e., Q * arterial O_2 content) to active tissues, however the results suggested that Q was not elevated (Chapter 3). O_2 delivery could have alternatively been augmented after KE ingestion through increased arterial O_2 content. While the hyperventilation, blood acidosis, and lower estimated arterial CO_2 associated with hyperketonemia each could have independently influenced arterial O_2 content, these are unlikely to have induced a meaningful effect during submaximal exercise at sea level in healthy humans due to the sigmoidal nature of the oxyhemoglobin curve, already near maximal saturation of hemoglobin with O_2, and relatively little contribution of O_2 dissolved in arterial blood to arterial O_2 content. Some data suggest hyperketonemia could increase arterial O_2 content during exercise in hypoxic conditions, presumably because this arterial O_2 content was not near maximal levels in the control condition (Poffe et al., 2021). Thus, O_2 delivery to tissues in healthy humans during exercise at sea level was likely not augmented by acute hyperketonemia.

The data in this thesis, while not precisely measuring or accounting for all factors potentially involved, suggest that O_2 extraction was increased by acute hyperketonemia. Calculated arterial-venous O_2 difference in Chapter 3 did not reach statistical significance ($p=0.06$), however exploratory post hoc testing releveled it was higher after KE vs placebo ingestion (Tukey's test $p=0.03$). This presumed elevation of whole-body arterial-

venous O_2 difference is likely reflecting O_2 extraction by skeletal muscles since a large majority of blood flow during exercise is directed towards such (Calbet et al., 2007). An increased O_2 consumption by skeletal muscle during exercise may be explained in part by alterations of substrate oxidation such that less efficient fuels (as assessed by a lower ATP production per O_2 consumed) were oxidized to a relatively greater extend, a shift towards greater ATP provision from oxidative phosphorylation instead of substrate level phosphorylation or altered efficiencies of ATP consuming enzymes. Some research has reported that KB contributed ~5-10% of total energy expenditure during exercise performed with acute hyperketonemia (Balasse et al., 1978; Dearlove et al., 2021). Even this small relative contribution of KB to oxidative phosphorylation could increase the O_2 cost of muscular contraction independent of alterations of ATP requirement if glucose oxidation was replaced by the oxidation of KB. Indeed, theoretical calculations using the data in Chapter 4 determined that an additional ~30 ml of O_2 per minute min would be required during exercise to support a 5% contribution of KB to energy expenditure assuming a 5% reduction in glucose oxidation. This additional VO_2 plus the calculated ~10 ml/min to support the KE-associated hyperventilation (Coast et al. 1993) is similar to the ~40 ml/min increase of whole body VO_2 after KE vs placebo ingestion calculated in Appendix A. Alternatively, acute hyperketonemia has been suggested to increase fat oxidation and decrease carbohydrate oxidation in vitro and in exercising humans, though not a universal finding (Valenzuela et al., 2020). This change could alternatively explain a greater O_2 cost per ATP synthesized owing to fatty acids being less efficient than glucose

in this regard (Chapter 1.2). More work is required to determine the effects of acute hyperketonemia on exercise economy and the underlying mechanisms.

In summary, acute hyperketonemia via KE ingestion was seemingly associated with a greater physiological stress during exercise. It could be speculated that acute hyperketonemia increased the O_2 cost of exercise through a greater extraction by tissues, presumably skeletal muscle, due to the different efficiencies of KB and glucose as metabolic fuels. This greater O_2 cost of exercise would then require a greater V_E, and these changes altogether could be interpreted by the exercising human as more difficult. However, the greater HR but not Q associated with acute hyperketonemia does not fit this theory and suggests these responses are regulated through other mechanisms.

5.4 Substrate Metabolism

There are several barriers in interpreting the effects of acute hyperketonemia on substrate metabolism in humans. While some studies reported an altered respiratory exchange ratio after KB supplementation, most studies report no effect (Cox et al., 2016; Evans et al., 2018; Valenzuela et al., 2020). As previously discussed in Chapter 3, the blood acidosis and CO_2 metabolism from non-oxidative sources associated with KE ingestion complicates the interpretation of substrate utilization using classic equations based on expired gas data. Preventing the drop in blood pH and total venous CO_2 associated with KE ingestion by bicarbonate co-ingestion may overcome these limitations and allow for the respiratory exchange ratio to be a valid proxy for substrate oxidation.

Respiratory exchange ratio was nonetheless unaffected after KE ingestion independent of changes in venous CO_2 and pH (Chapter 3, Poffe et al. 2020).

Despite some promise that expired gases could still serve as a proxy for substrate oxidation during exercise in a state of acute hyperketonemia via KB supplementation if pH is normalized, this thesis provides evidence that future work in this regard should consider controlling for blood pH and use direct measurements. Data from Chapter 3 demonstrated that the KE-associated decrease in exercise blood lactate concentration was not present when pH was neutralized via co-ingestion of bicarbonate. This is corroborated by classic literature examining the role of pH on substrate metabolism (Kowalchuk et al., 1984; Sutton et al., 1981). Therefore, KE ingestion may have altered substrate metabolism via altering blood pH and not KB per se. However, this change in blood lactate concentration was not associated with any change in the respiratory exchange ratio which may indicate a discordance between lactate metabolism and oxidative phosphorylation during acute hyperketonemia. Thus, changes in expired gases and/or blood lactate concentration after KE ingestion may not be reflective of substrate oxidation. More direct measurements of intramuscular fuel utilization during exercise after KE ingestion in healthy adults reported conflicting results (Cox et al., 2016; Poffé et al., 2020b). An alternate approach to probe this issue could involve stable isotope tracer determinations of substrate metabolism in a state of acute hyperketonemia.

5.5 Endurance Performance

This thesis advanced knowledge regarding the effect of KE ingestion on exercise performance. The first two studies in this thesis sought to isolate for the basic physiologic and metabolic effects of KB and therefore did not use co-ingestion of carbohydrates during exercise due to potential nutrient interactions. To improve the ecological validity of the last study that focused on exercise performance, we employed an exercise test that would not normally necessitate carbohydrate intake during exercise per best practice sport nutrition guidelines, i.e., short duration events of <60 minutes (Jeukendrup, 2014). Furthermore, most studies in this regard employed a controlled period of exercise before an endurance performance test, though data from Chapter 2 suggests that a moderate-to-heavy intensity (approximating ventilatory threshold) bout of exercise may introduce confounding influences in a subsequent high-intensity performance test. This consideration may have implications for some KE performance studies (Cox et al., 2016; Evans et al., 2019), but others have employed a lower intensity exercise (50-70% of ~ventilatory threshold intensity) which may better resemble a warm-up (Poffé et al., 2021). Thus, the performance impairment induced by acute KE ingestion in Chapter 4 allows for a clear interpretation of the data from a simple study design.

This thesis was limited in its ability to provide potential mechanisms associated with the endurance performance impairment induced by acute KE ingestion. This limitation is assuming that the dose of KE ingested shortly before exercise impacts the resulting exercise responses. There has been speculation regarding potential dose-dependent effects of KE (Dearlove et al., 2021; Evans et al., 2017; Stubbs et al., 2018)

though more primary research in this regard is needed to elucidate this potential response. Assuming KE dose does in fact influence exercise responses, then the data herein are limited to suggesting altered perceived exertion and a challenge to acid-base status as potential mechanisms underlying the performance impairment by KE ingestion. If KE dose is not a relevant consideration in this situation, then impaired exercise economy and efficiency and a greater cardiorespiratory stress may additionally be involved. The potential effects induced by KE ingestion that could impact endurance performance are covered in detail by Evans et al. (2022), which includes alterations of substrate metabolism and efficiency of oxidation, gastrointestinal upset, and increased cardiorespiratory stress. Future research in this area of research could examine potential dose dependent effects after KE ingestion.

Whether KE supplementation can provide an ergogenic effect for athletic performance is still interesting to consider but it may be beneficial to examine situations that are not KE ingestion alone within 30 minutes of exercise. For example, KE have shown beneficial effects during recovery from exercise (Poffe et al., 2019; Vandoorne et al., 2017) and with co-ingestion of KE and other ergogenic aids such as carbohydrates and bicarbonate (Poffé et al., 2020a). Future research is required to further understand the performance responses to co-ingestion of KE and other supplements, and such research could perhaps benefit from examining the effects on physiology and metabolism associated with these supplementation strategies.

In summary, this thesis demonstrated that strict assessments of endurance exercise performance are beneficial in the examination of the ergogenic potential of KE

supplementation, and that acute KE ingestion was ergolytic to high-intensity endurance-type performance. The mechanisms associated with this performance impairment remain to be determined and might depend on potential dose-dependent effects of acute KE ingestion before exercise. Future research examining the potential benefits of KE supplements should consider different dosing strategies (i.e., longer duration between KE ingestion and exercise, repeated ingestion at rest, or post exercise) and/or lower intensity athletic events. Alternatively, the potential benefits of KE ingestion may not be in athletes but rather clinical populations with compromised basal metabolism, i.e., diabetes, heart failure, and cognitive impairments.

5.6 Potential influence of biological sex

Like many other fields of exercise physiology and sports nutrition, the exercise responses to acute hyperketonemia via KB supplementation are relatively understudied in females compared to males. A recent meta-analysis that examined the performance effects of KB supplementation reported that 3 of 80 participants included in studies published between 2016 and 2020 were female. While this thesis did not directly examine sex-based differences in response to an acute hyperketonemia, the data in Chapter 2 shed light on this potential interactive effect. Study 1 involved 9 female and 10 male participants that had similar cardiorespiratory fitness, as determined by VO_{2peak} normalized to kilogram fat-free mass. Exploratory sex-based statistical analyses (repeated measures two-way ANOVA [sex x condition]) and visual inspection of the data indicated that sex did not mediate any of the observed responses, or lack thereof, of KE ingestion

on exercise cardiorespiratory and performance responses (interaction $p>0.14$ for all). Therefore, these data suggest that the effects of acutely elevated exogenous ketone availability may not be different between the sexes.

This indication of no sex differences in response to KE ingestion informed the two subsequent projects in this thesis such that equal numbers of males and females were not recruited. This led to considerably fewer female participants examined in the two subsequent chapters. However, as anticipated, the female responses in Chapters 3 and 4 of the thesis were not strikingly different than the male responses. It is important to consider that the scope of this thesis is narrow as compared to the broader field of exercise physiology and these data suggests sex did not affect the physiological response of KE ingestion in a very specific setting. Therefore, it is possible that other responses to KB supplementation are sex dependent, and more work is required to determine whether sex influences any of these responses. Future research could match male and female participants during recruitment and examine the potential role of hormonal cycle phase on these responses.

5.7 Conclusions

In conclusion, KB supplementation induced acute hyperketonemia – a physiological state involving elevated KB availability and alterations of other metabolites – without otherwise changing diet enabling a more direct examination of KB on the physiologic, metabolic, and performance responses during exercise. Acute hyperketonemia, independent of blood acidosis, was associated with greater

cardiorespiratory stress and perhaps an increased O_2 requirement of exercise and perceived exertion in humans. KE ingestion, which involved a stress to acid-base homeostasis, impaired high-intensity endurance exercise performance in trained athletes. The observed alterations of basic exercise responses may explain the impaired high-intensity endurance performance when KE are consumed immediately prior to exercise.

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APPENDIX A: Acute ketone monoester supplementation increases the oxygen cost of submaximal exercise in endurance-trained adults. Abstract published in *Appl Physiol Nutr Metab* 47(10): S81, 2022 and presented at the Canadian Society of Exercise Physiology Annual General Meeting 2022. This work is based on the analysis of combined data collected as part of the projects described in Chapters 2 and 3.

Nutritional ketosis induced by acute ketone monoester supplementation (KE) affects exercise responses. There are limited and equivocal data regarding the effect of KE on exercise economy. We probed this issue by combining data from two separate studies from our lab that used the same supplementation and exercise protocol. Analysis included 27 endurance-trained adults (17 males, 10 females; VO_{2peak}: 58±8 ml/kg/min, mean±SD). In a randomized, crossover, and double-blinded manner, participants ingested either 0.6 g/kg body mass KE or a ketone-free placebo. After, they rested for 30 minutes and then cycled at a constant workload that elicited ventilatory threshold 1 (~70% VO_{2peak}) for 30 minutes. Venous [β-hydroxybutyrate], the major circulating ketone body, was higher during exercise after KE vs placebo ingestion (3.6±1.0 vs 0.2±0.2 mM, $p<0.0001$). Steady-state VO_2 was 41[12-70] ml/min (mean[95% CI]) higher during exercise after KE vs placebo ingestion (2933±725 vs 2892±722 ml/min, $p=0.01$, $d_Z=0.54$, 77% post-hoc power). Exercise heart rate ($p<0.0001$, $d_Z=1.2$) and minute ventilation ($p<0.0001$, $d_Z=1.3$) were also higher after KE vs placebo. We conclude that ingestion of a large bolus dose of KE increased the oxygen cost of submaximal cycling and thereby impaired exercise economy. This effect was associated with a higher cardiorespiratory stress after KE ingestion. (Supported by NSERC).

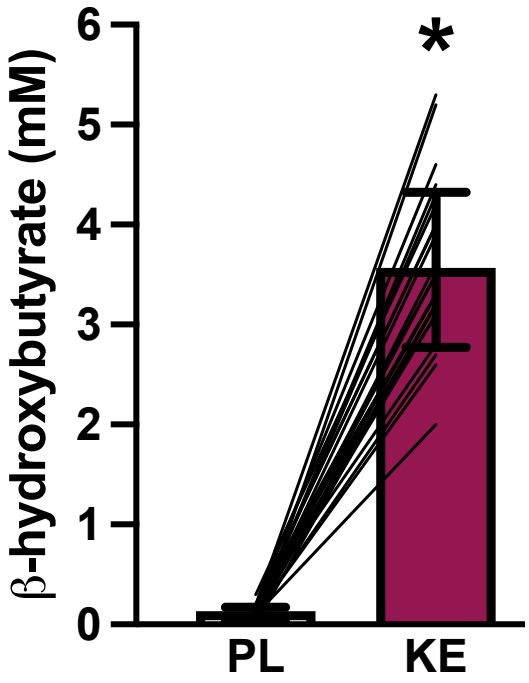

Figure 1. Pre-exercise venous [ketone body] after ketone monoester (KE) and placebo (PL) ingestion. *, $p<0.05$ (paired t-test). Data are mean±SD, lines connect individual data points.

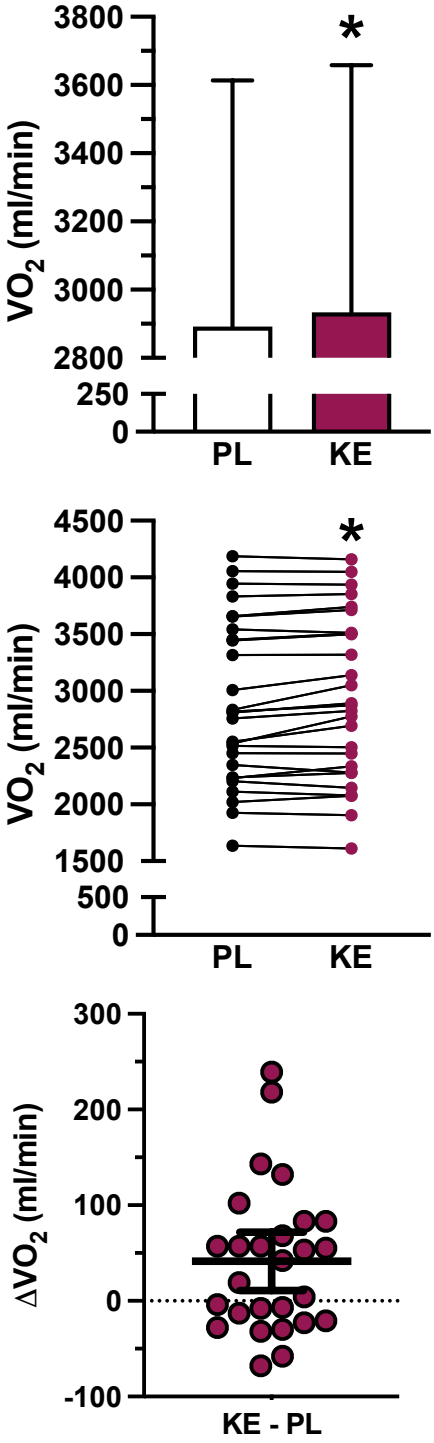

Figure 2. O_2 uptake ($\dot{V}O_2$) during cycling after ketone monoester (KE) vs placebo (PL) ingestion. (Top) Bars are mean± SD (n=27), (middle) lines connect individual data points, (bottom) thick horizontal lines are mean ± 95% CI of change scores, dots are individual change scores. *, $p<0.05$ (paired t-test).

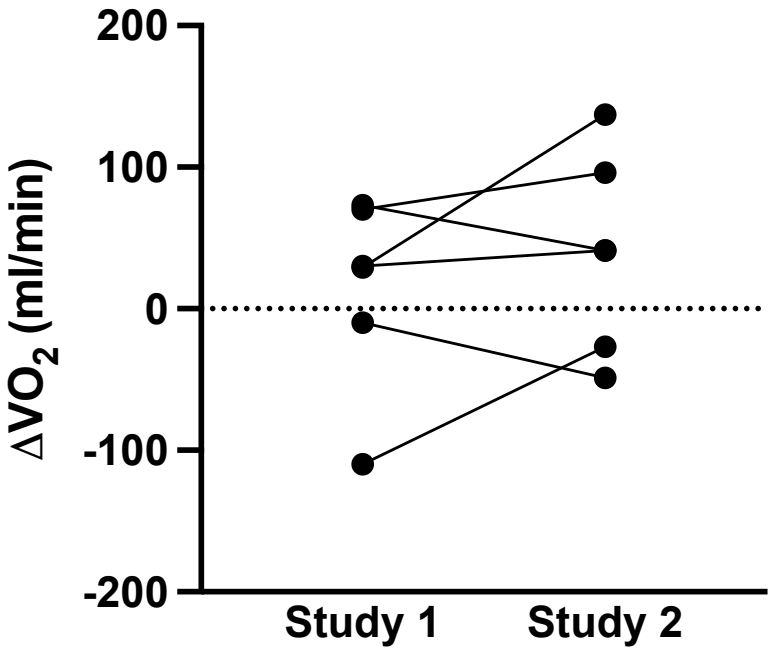

Figure 3. The difference in exercise O_2 uptake ($\dot{V}O_2$) after ketone monoester (KE) vs placebo (PL) ingestion for participants that completed both studies. Dots are individual change scores (KE vs PL) and lines connect individual data points.

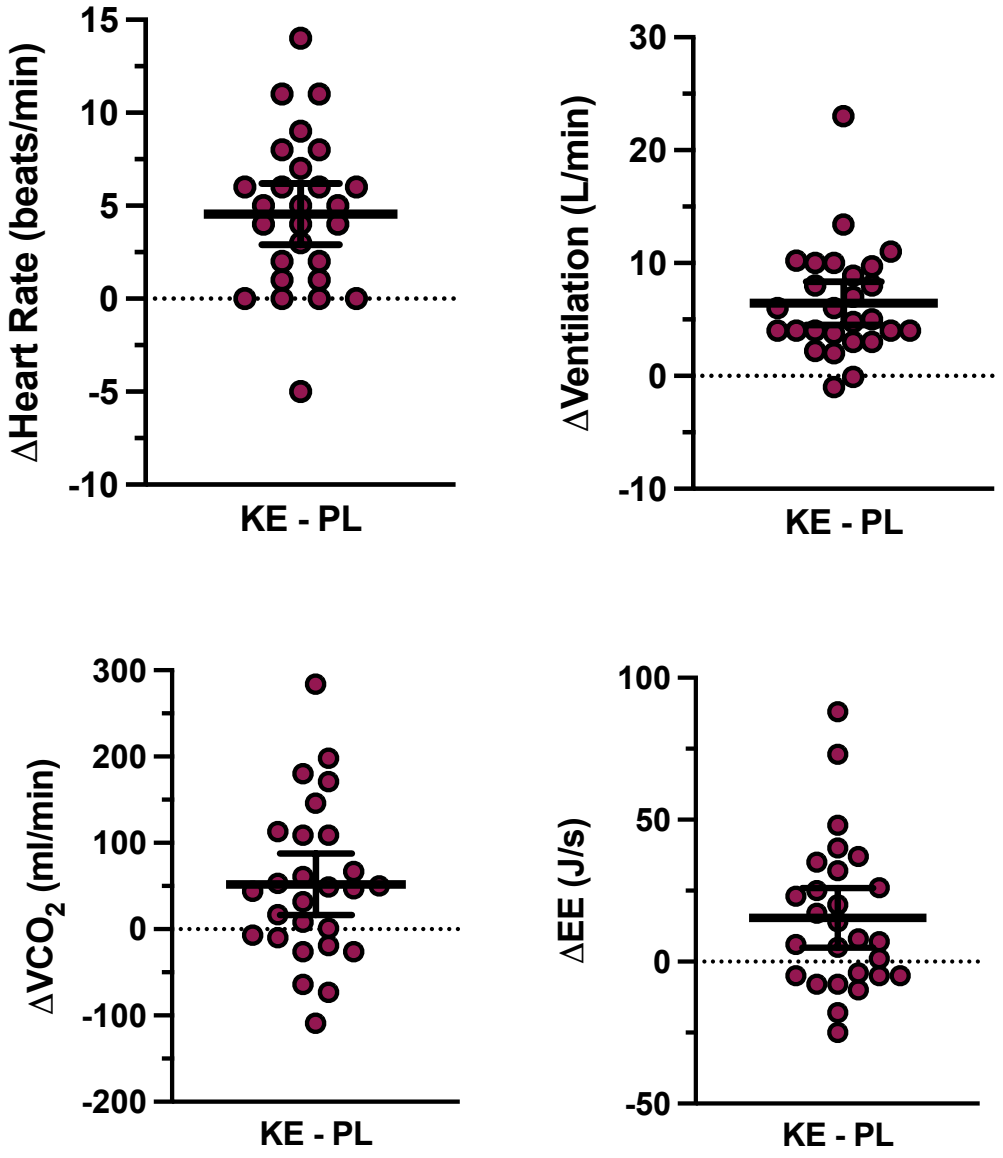

Figure 4. Submaximal exercise responses after ketone monoester (KE) vs placebo (PL) ingestion. Horizontal lines represent mean ± 95% CI and dots are individual participant change scores. VCO_2, expired CO_2; EE, energy expenditure.

Table 1. Participant Characteristics.

	All (N=27)	Males (N=17)	Females (N=10)
Age (years)	27±8	27±9	27±6
Body Mass (kg)	71±11	77±7	61±7
$\dot{V}O_{2peak}$ (mL/kg/min)	59±8	62±8	53±6

Notes: Data are mean ± SD. VO_{2peak}, peak oxygen uptake.

Table 2. Submaximal exercise responses.

	PL	KE	p
Heart rate (beats/min)	150±9	154±154*	<0.0001
Ventilation (L/min)	72±16	79±17*	<0.0001
$\dot{V}CO_2$ (L/min)	2.54±0.65	2.59±0.67*	0.006
RER	0.89±0.04	0.89±0.04	0.82
Energy Expenditure (J/s)	992±249	1008±251*	0.006

Notes: Data are mean ± SD (n=27). PL, placebo; KE, ketone monoester; RER, respiratory exchange ratio; VCO_2, expired CO_2. P values are from paired t-tests (KE vs PL). *, $p<0.05$ vs PL.