KETONE MONOESTER DOSE, CEREBRAL BLOOD FLOW, AND COGNITION IN YOUNG ADULTS

THE EFFECTS OF ACUTE KETONE MONOESTER INGESTION ON RESTING CEREBRAL BLOOD FLOW AND COGNITION IN YOUNG ADULTS

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LAY ABSTRACT

In addition to being an alternative energy source used during fasting or when consuming low amounts of carbohydrates, ketone bodies may act as signals that impact various aspects of brain health. Recent studies suggest that supplementating with a drink that contains ketones is an alternative way to increase ketones in your blood, without dietary modifications. We investigated whether one-time ingestion of a ketone supplement (KME) affects brain blood flow and cognition, as well as whether the size of KME dose differentially impacts these outcomes. Our results show that KME ingestion lowers brain blood flow, to a different extent depending on the dose that was ingested. Despite this, there was no change in memory performance from taking this ketone supplement. These findings were important in demonstrating how KME ingestion impacts the brain, and will inform future guidelines on its potential use for protecting and/or improving brain health.

ABSTRACT

Ketone monoester (KME) supplements are one exogenous ketone intervention which has demonstrated benefits to multiple facets of brain health, including cerebral blood flow (CBF) and cognition. However, it is unknown how KME impact CBF and cognition acutely, and whether the size of KME dose differentially impacts these outcomes. Higher KME doses lower blood pH and arterial CO_2 (P_aCO_2), which are important regulators of CBF. We hypothesized that high-dose KME ingestion would lower CBF via acidosis-induced compensatory reductions in P_aCO_2, whereas low-dose KME ingestion would enhance CBF, mirroring past findings from other exogenous ketone interventions. Changes in cognitive function were also hypothesized to parallel CBF responses. Twenty young adults (age=23±3 years; BMI=23.4±2.2 kg/m^2) participated. In a double-blinded, counterbalanced, crossover design, participants completed 3 separate conditions: 1) high-dose KME (0.6 g/kg); 2) low-dose KME (0.3 g/kg); or 3) placebo. Outcome measures were assessed at fasting baseline, 45-, and 120-min post-ingestion. CBF was assessed using Duplex ultrasound of the internal carotid and vertebral arteries. End-tidal CO_2 ($P_{ET}CO_2$) was measured using a gas analyzer, to approximate P_aCO_2. Hippocampal-dependent function was assessed using the lure discrimination index (LDI) and recognition memory score (REC) from the mnemonic similarity task. Over a 2-hour period post-ingestion, low- and high-dose KME lowered CBF to a different extent relative to baseline (45-min (low-dose, high-dose): Δ10.4%, Δ14.0%; 120-min (low-dose, high-dose): Δ6.2%, Δ18.6%; $P < 0.001$). These reductions were mirrored by dose-dependent reductions in $P_{ET}CO_2$ and changes in CBF were positively correlated with changes in $P_{ET}CO_2$ ($P < 0.001$, $R^2 = 0.219$). Despite reductions in CBF, both LDI ($P = 0.619$) and REC ($P = 0.651$) were unchanged in the KME conditions. These findings provide a foundational

characterization of the acute effects of KME dose on CBF and cognition, which will inform potential therapeutic recommendations on KME for brain health.

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LIST OF ABBREVIATIONS

AUC	Area-under-the-curve
BDNF	Brain-Derived Neurotrophic Factor
BMI	Body Mass Index
β-OHB	Beta-hydroxybutyrate
CA	Cerebral Autoregulation
CBF	Cerebral Blood Flow
CO	Carbon Monoxide
CO_2	Carbon Dioxide
CPP	Cerebral Perfusion Pressure
CPT	Carnitine Palmitoyl Transferase
CSF	Cerebrospinal Fluid
CVC	Cerebrovascular Conductance
CVR	Cerebrovascular Resistance
DBP	Diastolic Blood Pressure
gCBF	Global Cerebral Blood Flow
HR	Heart Rate
ICA	Internal Carotid Artery
IMI	Intrinsic Motivation Inventory
KE	Ketone Ester
KME	Ketone Monoester
KS	Ketone Salt
LDI	Lure Discrimination Index
LMM	Linear Mixed Model
MAP	Mean Arterial Pressure
MCA	Middle Cerebral Artery
MCAv	Middle Cerebral Artery Velocity
MCT	Monocarboxylate Transporters
MCTs	Medium-Chain Triglycerides
MST	Mnemonic Similarity Task
NO	Nitric Oxide
NVC	Neurovascular Coupling
O_2	Oxygen
P_aCO_2	Partial Pressure of Arterial Carbon Dioxide
PCA	Posterior Cerebral Artery
$P_{ET}CO_2$	End-Tidal Carbon Dioxide
REC	Recognition Memory Score
ROS	Reactive Oxygen Species
SBP	Systolic Blood Pressure
SV	Stroke Volume
T2D	Type 2 Diabetes
VA	Vertebral Artery
$\dot{V}O_2$	Rate of Oxygen Consumption
$\dot{V}O_{2peak}$	Peak Rate of Oxygen Consumption
VSMC	Vascular Smooth Muscle Cell

DECLARATION OF ACADEMIC ACHIEVEMENT

Aedan Rourke's Role:

- Obtained ethics approval with the Hamilton Integrated Research Ethics Board
- Completed pre-registrtation of the trial and outcomes on clinicaltrials.gov
- Obtained necessary technical training to conduct the study (phlebotomy and vascular ultrasound)
- Designed study protocol and selected outcome measures to assess
- Developed standard operating procedures for the study
- Set up study materials for data collection
- Trained and supervised undergraduate KIN 3RP3 and 4RR6 research students, who assisted with data collection and analysis
- Led data collection, analysis, and interpretation
- Was primarily responsible for manuscript preparation

Role of co-authors:

- JW assisted AR with ethics application and trial pre-registration
- JW assisted AR with study design and the selection of outcome measures
- JW assisted AR with data interpretation
- JW assisted AR with manuscript writing and editing

Chapter 1: Literature Review: An Overview of Cerebral Blood Flow Regulation, Ketone Body Metabolism, and Cognitive Function

1.1 Major Regulators of Cerebral Blood Flow

The brain requires a constant supply of blood flow to maintain optimal health and function. Despite constituting only 2% of total body mass, the brain receives nearly 15% of total cardiac output and accounts for 20% of total oxygen consumption at rest to support its high energy demands (Williams & Leggett, 1989; Watts *et al*., 2018). Cerebral blood flow (CBF) is tightly regulated to maintain matching of oxygen delivery to cerebral oxygen demand, as insufficient perfusion leads to unconsciousness and excessive perfusion leads to cerebral tissue damage (Bisson *et al.*, 2016). CBF is determined by the combination of cerebral perfusion pressure (CPP) and cerebrovascular resistance (CVR) (Ainslie & Duffin, 2009). CPP is the main driving force of CBF and is defined as the gradient between mean arterial pressure (MAP) at the level of the Circle of Willis and the intracranial pressure. CVR represents the resistance to blood flow and is modulated primarily through alterations in cerebrovascular tone and vessel diameter. Together, there are three main factors which modulate CBF by altering CPP and/or CVR: the partial pressures of arterial blood gases, mean arterial pressure, and cerebral metabolism.

1.1.1 Arterial Blood Gases & Acid-Base Regulation

At rest, cerebral metabolism is the primary driver of CBF but alterations in the partial pressure of carbon dioxide in arterial blood (P_aCO_2) have the strongest influence on changes in CBF (i.e., CBF regulation) (Smith & Ainslie, 2017). Blood oxygen (O_2) concentrations regulate CBF in hypoxia (low atmospheric O_2), but carbon dioxide (CO_2) is the primary regulatory blood gas in normoxic conditions (Smith & Ainslie, 2017). The importance of P_aCO_2 in CBF regulation is

clearly evident in the maintenance of acid-base homeostasis (Hoiland *et al.*, 2019). CO_2 is a byproduct of the blood bicarbonate buffering system in response to changes in pH (i.e., H^+ concentrations) (**Figure 1**), and primarily of cellular metabolism. However, alterations in P_aCO_2 can also mediate changes in blood pH, given that the blood bicarbonate buffer system is a physiological equilibrium (**Figure 1**). CO_2 can freely diffuse across the blood-brain barrier and corresponding changes in CBF occur to retain or remove CO_2 from cerebral tissue and vessels to maintain pH levels within the brain (Carr *et al.*, 2021). Decreases in P_aCO_2 cause cerebral vasoconstriction and decreases in CBF (Querido & Sheel, 2007), leading to the retention of CO_2 to prevent pH levels from becoming alkalotic (Carr *et al.*, 2021). Conversely, increases in P_aCO_2 cause cerebral vasodilation and increases in CBF (Ito *et al.*, 2003), to remove excess CO_2 from the brain and prevent pH levels from becoming acidic (Carr *et al.*, 2021).

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+$$

Figure 1. Blood bicarbonate buffer system.

Cerebrovascular reactivity to CO_2 occurs via the relaxation or contraction of vascular smooth muscle cells (VSMC). CO_2 freely diffuses across the blood-brain barrier where it can alter the cerebrospinal fluid (CSF) pH surrounding cerebral VSMCs. Changes in CSF pH directly impact VSMC intracellular H^+, leading to alterations in cerebral vessel diameter, and therefore CBF (Lassen, 1968; Hoiland *et al.*, 2019). As CO_2 increases in the CSF, the production of H^+ also increases to restore the blood bicarbonate buffer equilibrium (**Figure 1**). This increase in H^+ within the CSF creates a diffusion gradient to drive H^+ flux into the VSMC. Ultimately, the intracellular $[H^+]$ within the VSMC is the primary regulator of VSMC tone (Duffin *et al.*, 2021; Caldwell *et al.*, 2021). When P_aCO_2 is increased, intracellular H^+ will increase within the VSMC and cause a myriad of cellular signalling effects which lead to VSMC hyperpolarization, primarily through

increases in K^+ channel conductance (Hoiland *et al.*, 2019). VSMC hyperpolarization downregulates Ca^{2+} channel activity (Nelson *et al.*, 1990), thus reducing Ca^{2+} influx and intracellular Ca^{2+}, which leads to smooth muscle relaxation and vasodilation (Duffin *et al.*, 2021). When P_aCO_2 is decreased, intracellular H^+ will decrease within the VSMC, which increases intracellular Ca^{2+} and leads to smooth muscle contraction and vasoconstriction (Duffin *et al.*, 2021). Interestingly, the modulation of arterial pH using bicarbonate infusion combined with maintained P_aCO_2 via end-tidal clamping produces no change in CBF in humans, which suggests that CO_2 in the blood ultimately drives corresponding changes in CBF (Lambertsen *et al.*, 1961; Caldwell *et al.*, 2021). Further, animal models suggest that vascular high shear stress may futher contribute to cerebral vasodilation in the presence of elevated P_aCO_2 via the release of vasodilators including nitric oxide (NO) and prostaglandins from endothelial cells (Leffler *et al*., 1994; Parfenova *et al*., 1994).

All of the major cerebral blood vessels are sensitive to changes in CO_2, including the large extracranial vessels in the neck, the internal carotid (ICA) (Willie *et al.*, 2012) and vertebral arteries (VA) (Sato *et al.*, 2012), the intracranial vessels such as the middle cerebral artery (MCA) (Ide *et al.*, 2003; Battisti-Charbonney *et al.*, 2011) and posterior cerebral artery (PCA) (Skow *et al*., 2013), and the smallest pial arterioles within the cerebrum (Wolff *et al.*, 1930). In all vessels, there is a 1-3% reduction in blood flow with hypocapnia (low CO_2) and 3-6% increase in blood flow with hypercapnia (high CO_2), per unit mmHg change in CO_2 below or above eupnoeic P_aCO_2 (Willie *et al.*, 2014). This suggests that the cerebral vasculature is more reactive to a hypercapnic stimulus compared to a hypocapnic stimulus of the same magnitude. Though this sensitivity to CO_2 is present in all cerebral vessels, corresponding changes in CBF also differ between vessels. One study demonstrated that VA reactivity is greater in hypocapnia than the ICA, MCA, and PCA,

whereas hypercapnic reactivity was similar between vessels (Willie *et al.*, 2012). However, reduced reactivity to CO_2 has been observed in the VA relative to the ICA (Sato *et al.*, 2012), as well as in the PCA relative to the MCA (Skow *et al.*, 2013), over a range of P_aCO_2 combining both hypocapnic and hypercapnic stimuli. Despite potential regional differences, extensive sensitivity to CO_2 throughout all cerebral vessels allows for the maintenance of acid-base homeostasis within the brain, despite individual differences between vessels.

1.1.2 Mean Arterial Pressure

Cerebral autoregulation (CA) is a strong contributor to the regulation of CBF at rest and during exercise (Smith & Ainslie, 2017). CA primarily acts to dampen perturbations to CBF caused by acute and/or gradual changes in MAP via adjustments in the vasomotor tone of cerebral vessels (Tzeng & Ainslie, 2014). In general, the pressure-flow relationship between MAP and CBF is pressure-passive, where increases in MAP cause vasodilation and increased CBF, and decreases in MAP lead to vasoconstriction and decreased CBF (Ogoh & Tarumi, 2019). However, over the range of physiological MAP (~90 - 110 mmHg), CBF remains at a relatively constant level despite changes in MAP (Willie *et al*., 2014). This represents the typical range of MAP at rest. CA counteracts these aforementioned pressure-passive mechanisms to prevent large changes in CBF outside of the range of normal MAP. The purpose of CA is to protect the brain from ischemia during instances of systemic hypotension, or hemorrhage in cases of transient or sustained hypertension (Claassen *et al.*, 2021). Thus, increases in MAP stimulate cerebral vasoconstriction to reduce increases in CBF, and decreases in MAP stimulate cerebral vasodilation to prevent large reductions in CBF (Kontos *et al*., 1978). Recent evidence also demonstrates that CA is more efficacious at buffering CBF against increases in MAP compared to decreases, outside of the normal MAP range (Brassard *et al.*, 2017). Modulations in vasomotor tone in response to

fluctuations in MAP are facilitated throughout the entire cerebral vasculature, from the smallest pial arterioles (Lassen, 1959) to the large extracranial vessels in the neck (Willie *et al.*, 2014). The large intracranial and extracranial arteries exert the greatest influence on vascular resistance (Faraci & Heistad, 1990). Overall, vessel reactivity in CA is crucial in the maintenance of adequate cerebral perfusion.

1.1.3 Cerebral Metabolism & Neurovascular Coupling

Neurovascular coupling (NVC) is the tight coupling of local O_2 delivery to local metabolic demand within the brain (Iadecola, 2017). NVC is facilitated by the proximity of excitatory and inhibitory neurons within the cortex, astrocytes, and cortical penetrating arterioles which form the neurovascular unit (NVU) (Willie *et al.*, 2014). When a neuron is activated, the close synapse between astrocytes and arterioles allows for the transmission of neural signals into a series of vascular signals which propagate through adjacent VSMCs via gap junctions (Kawamura *et al.*, 2003). Neuron activation stimulates astrocytes to release vasoactive agents, such as NO and adenosine, onto upstream arterioles to induce vasodilation and increase CBF to that neuronal network (Attwell *et al.*, 2010).

The regulation of NVC responses to neuron activation can be impacted by alterations in substrate provision to the brain, rather than exclusively through neuron oxygen or glucose-sensing mechanisms. Indeed, experimental manipulations which increase blood oxygen and glucose content do not alter NVC responses during somatosensory stimulation (Wolf *et al.*, 1997; Lindauer *et al.*, 2009). Whereas, metabolic shifts induced with the presence of alternative fuel sources to glucose (i.e., pyruvate, lactate, ketone bodies) can directly alter cytosolic redox potential and impact subsequent astrocyte signalling and NVC responses. For example, the lactate/pyruvate ratio within the astrocyte exists in near equilibrium to the cytosolic NADH:NAD^+ ratio (Vlassenko *et*

al., 2006). Studies using intravenous infusion of lactate (Mintun *et al.*, 2004) demonstrate that lactate increases CBF responses to neuron activation in a dose-dependent manner (Hollyer *et al.*, 2019), likely by increasing the astrocyte NADH:NAD^+ ratio which signals for increased blood flow (Ido *et al.*, 2001). Contrarily, intravenous infusion of pyruvate, which lowers the cytosolic NADH:NAD^+ ratio, reduces CBF responses during a visual stimulation task (Vlassenko *et al.*, 2006). As neuronal activity increases, lactate production via pyruvate oxidation within the astrocyte will increase at a similar rate (Vlassenko *et al.*, 2006). The conversion of pyruvate to lactate involves the oxidation of NADH to generate NAD^+. However, the accumulation of lactate within astrocytes via intravenous infusion slows the rate of pyruvate oxidation and leads to excess NADH buildup (Vlassenko *et al.*, 2006). This is a direct example of how alternative fuel sources can alter oxidative glucose metabolism, despite no change in metabolic demand, and modify the sensitivity of NVC responses. Elevations in circulating lactate can also stimulate further increases in CBF though the release of vasodilatory compounds such as NO and adenosine to modulate neuronal excitability and activity, and therefore NVC (Cauli *et al.*, 2023). Although NVC was originally hypothesized to be strictly a coupling of O_2 delivery to demand, changes in substrate provision to the brain can also have large impacts on NVC responses. The following sections of this review will focus on beta-hydroxybutyrate (β-OHB), an alternative substrate that can be used by the brain to support metabolism. The presence of β-OHB directly alters cellular signalling and metabolism, which induces significant alterations to human physiology, especially in the brain.

1.2 Ketone Body Metabolism

Glucose is the primary energy source for numerous organ systems within the body, including the brain, which has a near-exclusive reliance on glucose to support metabolic demand (Sims-Robinson *et al.*, 2015). When carbohydrate stores are lowered in scenarios associated with

the suppression of insulin, such as with caloric restrictions, starvation, diabetes, or prolonged exercise, the production of ketone bodies increases as an alternative metabolic fuel for the brain, heart, and skeletal muscle (Puchalska & Crawford, 2017; Masino, 2022). In these scenarios, fatty acids are mobilized and converted into ketone bodies by the liver, where they can be transferred to peripheral tissues to generate ATP through the electron transport chain (**Figure 2**) (Yurista *et al.*, 2021). The main endogenously produced ketone bodies within the body are acetone, acetoacetate, and β-OHB, with β-OHB being the most abundant circulating ketone body in the blood (Lopaschuk & Dyck, 2023).

1.2.1 Endogenous Metabolism of Ketone Bodies

Ketogenesis is the endogenous production of ketone bodies from free fatty acids by the liver (**Figure 2**) (Puchalska & Crawford, 2017). A reduction in available carbohydrate energy stores stimulates lipolysis and fatty acid oxidation, which increases the amount of circulating free fatty acids (Poff *et al.*, 2020). Free fatty acids are brought into the liver through carnitine palmitoyl transferase (CPT). Within the liver mitochondria, free fatty acids are converted to acetoacetate and β-OHB. β-OHB and acetoacetate can be released by the liver into the circulation through monocarboxylate transporters (MCT) where they are taken up by peripheral tissues such as the brain, heart, and skeletal muscle. β-OHB and acetoacetate enter the tricarboxylic acid (TCA) cycle within the mitochondria and are converted to Acetyl-CoA to eventually generate ATP through oxidative phosphorlyation (Yurista *et al.*, 2021).

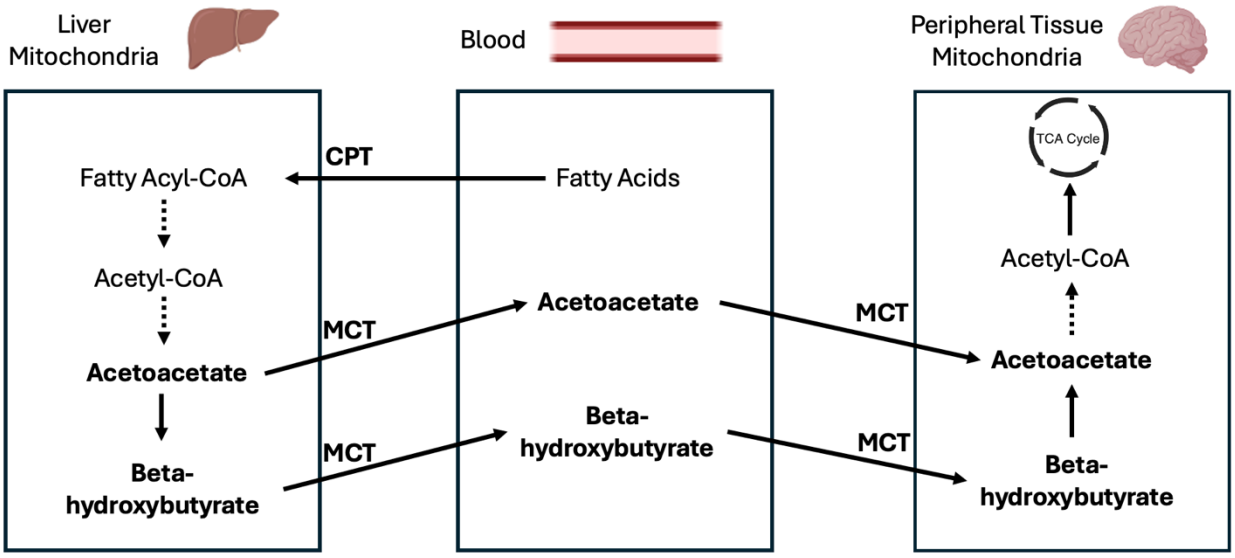

Figure 2. Endogenous ketone body production in the liver and ketone body metabolism in peripheral tissues. Dashed arrows delineate multiple-step metabolic reactions. Modified from Yurista *et al*. (2021).

1.2.2 Nutritional Ketosis and Exogenous Ketone Sources

Nutritional ketosis is the elevation of blood β-OHB concentrations ≥ 0.5 mM and can be achieved either through dietary modifications (i.e., fasting or ketogenic diet) or through exogenous ketone sources (Poff *et al.*, 2020). Exogenous ketone sources can be adminsistered via infusion or oral supplements. Sodium β-OHB is the primary compound used for infusion, whereas the composition of oral supplements include direct ketone bodies (i.e., β-OHB) and indirect ketogenic precursors which stimulate ketone body production (i.e., medium chain triglycerides). The plasma concentration of ketones achieved via dietary modifications differ considerably compared to exogenous interventions. Consumption of a ketogenic (low-carbohydrate, high-fat) diet for 2-4 days will raise blood ketone concentrations beyond 0.5 mM, however blood ketones will generally not rise beyond 1.5 mM in sustained ketogenic diets (Wilson & Lowery, 2017). Similarly, an overnight fast (~12 hours) will achieve blood β-OHB concentrations of 0.5 mM, with prolonged fasting inducing progressive increases in blood ketones (3 days: ~2 mM) (Owen & Reichard,

1971). For comparison, blood ketone concentrations ~ 2 mM can be achieved within 30 minutes of intravenous infusion (Svart *et al.*, 2018) or within 15-30 minutes after supplementation with an oral ketone supplement (Stubbs *et al.*, 2017; Dearlove *et al.*, 2019). As such, nutritional ketosis is achieved much quicker with exogenous ketone sources rather than the endogenous production of ketone bodies through dietary modifications, which requires stimulation by a reduction in available glucose stores in tissue.

Exogenous ketone sources primarily exist in three main forms: medium-chain triglycerides (MCTs), ketone salts (KS), and ketone esters (KE). MCTs contain a glycerol backbone esterified to medium-chain fatty acids and are rapidly digested as their non-polar structure does not require CPT for uptake into tissue mitochondria (Masino, 2022). Once inside the mitochondria, the free fatty acids from the MCTs can be converted into ketone bodies in a similar fashion to endogenous ketogenesis. KS and KE primarily contain the ketone body β-OHB incorporated within its structure. KS consist of a salt (i.e., sodium) attached to β-OHB, whereas KE consist of an ester (i.e., 1,3-butanediol) attached to β-OHB (Masino, 2022). KS are typically administered via intravenous infusion or oral supplements, where they dissociate in solution, before entering the circulation (Stubbs *et al.*, 2017). Alternatively, KE are often ingested orally and cleaved by liver and gut esterases before being rapidly absorbed into the bloodstream (Stubbs *et al.*, 2017). Both KS and KE are absorbed into the circulation as quickly as 15-20 minutes post-ingestion where they can be transported to peripheral tissues for metabolism (Stubbs *et al.*, 2017). Rapid absorption of β-OHB also occurs even in the presence of adequate glucose supply (Svart *et al.*, 2018). However, KE supplementation is more effective than KS at sustaining ketosis and able to achieve higher peak blood ketone concentrations (KS: ~ 1 mM; KE: 3-5 mM) (Stubbs *et al.*, 2017). The remainder

of this review will focus on KE, however studies using alternative exogenous ketone sources will be mentioned throughout.

1.3 Beneficial Effects of Exogenous β-OHB

Beyond its role as an alternative metabolic fuel, there is emerging evidence that β-OHB is also a signaling molecule that has marked effects on human physiology (Newman & Verdin, 2017). These include direct and indirect effects on central and peripheral hemodynamics like cardiac output (CO) and CBF, upregulation of neurotrophins and growth factors critical for the maintenance of brain health, and possibly benefits to cognition. A growing body of evidence from *in vitro* models, rodent models, and some human studies suggest that provision of β-OHB via exogenous sources may have therapeutic applications in conditions like heart failure and neurodegenerative diseases.

1.3.1 Haemodynamic Effects

In general, exposure to exogenous β-OHB sources increases CO, through both increases in stroke volume (SV) and heart rate (HR) (Nielsen *et al.*, 2019; Oneglia *et al.*, 2023). Three-hour infusion of KS increases CO measured via cardiac ultrasonography by 2.0 L/min through both SV (20 ± 2 mL) and HR (7±2 bpm), in both patients with heart failure and age-matched controls (Nielsen *et al.*, 2019). Oral ingestion of a single KE dose also increases CO by 1 L/min measured 60 minutes post-ingestion, primarily driven by increases in HR and small increases in SV (Oneglia *et al.*, 2023). These changes were detected using cardiac magnetic resonance imaging, which can generate detailed structural images and functional measures (i.e., blood flow) of the heart. Collectively, this evidence suggests that β-OHB likely exerts larger effects on cardiac chronotropy via pacemaker cells within the heart, rather than cardiac contractility (Oneglia *et al.*, 2023). In

support of this, a recent trial using KE supplementation observed increased HR, but not CO, during submaximal exercise in the KE group compared to a placebo condition (McCarthy *et al.*, 2023).

Exogenous β-OHB sources may also exert beneficial effects on vascular function. Research in animal models demonstrates that β-OHB may improve vascular function through a myriad of signaling pathways including reductions in oxidative stress and inflammation (Maalouf *et al.*, 2007; Kim *et al.*, 2007; Youm *et al.*, 2015), increased angiogenesis (Weis *et al.*, 2022), or through direct action on the vascular endothelium to cause vasodilation (Han *et al.*, 2018; McCarthy *et al.*, 2021*a*). There are limited studies to date that have demonstrated improved vascular function in humans. One study demonstrated that 14 days of thrice-daily supplementation with oral KE improved flow-mediated dilation of the brachial artery in adults with obesity (Walsh *et al.*, 2021*b*). However, it is unknown whether improved vascular function was due to direct vascular effects of β-OHB, or indirectly through improved glycemic control and/or reduced cellular inflammation with KE supplementation (Walsh *et al.*, 2021*b*).

Exogenous ketone sources containing β-OHB likely do not have a large impact on MAP. β-OHB infusion likely does not alter MAP (Svart *et al.*, 2018). Additonally, findings from studies involving effects of exogenous ketone supplements on MAP are inconsistent. Some studies have reported increases in MAP (Selvaraj *et al.*, 2022), while others have shown decreases in MAP (Myette-Côté *et al.*, 2019). However, multiple acute and chronic studies have reported no change in MAP (O'Connor *et al.*, 2018; Stefan *et al.*, 2021). This is supported by a recent meta-analysis which showed that there is no effect of either acute or chronic exogenous β-OHB supplementation on systolic blood pressure (SBP) or diastolic blood pressure (DBP) (Marcotte-Chénard *et al.*, 2024). Normally, an increase in CO would result in an increase in MAP. However, β-OHB may induce systemic vasodilation and decrease total peripheral resistance (Homilius *et al.*, 2023),

which may explain findings of elevated CO without increases in MAP following exogenous β-OHB exposure. Further, this review included studies that did not have blood pressure as their primary outcome, which could result in trials that were statistically underpowered to detect possible changes in MAP from exogenous ketone interventions. Nonetheless, there is little evidence to suggest that exogenous β-OHB interventions have a large impact on MAP.

1.3.2 CBF and Cerebral Metabolism

β-OHB has been increasingly gaining recognition for its potential to improve brain health. Deleterious alterations to CBF and cerebral metabolism are implicated in neurodegenerative diseases such as Alzheimer's disease (Croteau *et al.*, 2018; Korte *et al.*, 2020). Interventions that elevate plasma β-OHB have demonstrated improvements in CBF and substantial shifts in cerebral metabolism in healthy adults (Hasselbalch *et al.*, 1996; Svart *et al.*, 2018), as well as clinical populations with or at higher risk of developing neurodegenerative diseases (Croteau *et al.*, 2018; Torosyan *et al.*, 2018; Walsh *et al.*, 2021*a*).

β-OHB infusion increases CBF and reduces cerebral glucose metabolism while maintaining oxygen consumption by cerebral tissues (Hasselbalch *et al.*, 1996; Svart *et al.*, 2018). One of the earliest β-OHB infusion studies used the Kety-Schmidt technique to assess global cerebral blood flow in young adults (Hasselbalch *et al.*, 1996). This technique measures cerebral blood flow via serial arterial and venous blood sampling of a diffusible tracer (^{133}Xe). Blood flow is then calculated as the difference in area between the arterial and venous washout curves of the tracer. In this study, intravenous infusion of KS for 3-3.5 hours led to a 39% increase in CBF relative to saline infusion and achieved peak blood β-OHB concentrations of approximately 2.16 mM. Similarly, Svart *et al.* (2018) infused sodium β-OHB intravenously for 4 hours, but used a higher infusion rate compared to Hasselbalch *et al.* (1996) and tested this intervention in middle-

to-older aged adults (mean age = 62 years). Using positron emission tomography, they observed a 30% increase in CBF relative to saline infusion and peak blood β-OHB concentrations of 5.5 mM. Despite different peak blood β-OHB concentrations, both studies demonstrated a reduction in cerebral glucose metabolic rate, with no change in cerebral oxygen metabolism (Hasselbalch *et al.*, 1996; Svart *et al.*, 2018). This suggests that brain ketone uptake with exogenous β-OHB is regulated by the availability of plasma β-OHB and not alterations in glucose availability. The brain appears to readily oxidize β-OHB, despite sufficient carbohydrate supply, when it is present in elevated concentrations in the blood (i.e., greater than 0.5 mM) (Hasselbalch *et al.*, 1996; Svart *et al.*, 2018). Additionally, this increase in oxygen supply through enhanced CBF, but unchanged oxygen utilization, may be contributing to the potential neuroprotective effects of β-OHB (Svart *et al.*, 2018).

Interestingly, these large CBF responses to β-OHB-infusion in humans are not due to alterations in some of the most prominent regulators of CBF, such as in P_aCO_2, pH, or mean arterial pressure (Hasselbalch *et al.*, 1996; Svart *et al.*, 2018). Research conducted in animal models and humans suggests that reductions in oxidative stress and small alterations in cellular redox potential may contribute to increased CBF via β-OHB exposure (Vlassenko *et al.*, 2006; Maalouf *et al.*, 2007; Kim *et al.*, 2007; Shimazu *et al.*, 2013; Xin *et al.*, 2018). Excessive reactive oxygen species (ROS) production can lower resting CBF, which has been linked to the development of neurodegenerative diseases such as Alzheimer's (Zhu *et al.*, 2007). Cell culture (Maalouf *et al.*, 2007; Shimazu *et al.*, 2013) and animal model studies (Kim *et al.*, 2007) have both demonstrated reductions in ROS production with β-OHB exposure. This suggests that a decline in ROS production may be contributing to increases in CBF observed with exogenous β-OHB treatments. Changes to astrocyte redox potential may also help facilitate changes to CBF with β-OHB

interventions (Vlassenko *et al.*, 2006; Xin *et al.*, 2018). The conversion of β-OHB into acetoacetate requires NAD^+ to produce Acetyl-CoA. As β-OHB is used to fuel ATP production, this leads to an increase in the cytosolic NADH:NAD^+ ratio. Increased cytosolic NADH:NAD^+ has been independently linked to increases in CBF (Vlassenko *et al.*, 2006), which suggests that exogenous β-OHB sources could be enhancing CBF by altering astrocyte redox potential.

The effects of oral β-OHB supplements on CBF and cerebral metabolism have been less researched compared to infusion trials, with most studies employing chronic designs in clinical populations with or at a higher risk of developing neurodegenerative diseases. Thrice-daily supplementation with a 12 g KE supplement for 14 days improves cerebral blood flow (VA: +11%; Common Carotid Artery: +12%) compared to a placebo drink in adults with obesity (Walsh *et al.*, 2021*a*) in the fasted, non-supplemented state (i.e., no β-OHB in the blood). However, ingestion of a single KE (0.482 g/kg body mass) does not alter common carotid artery flow in the two-hour period following an oral glucose tolerance test in adults with obesity (Myette-Côté *et al.*, 2019). This suggests that prolonged and/or repeated exposure to β-OHB through chronic oral β-OHB supplementation interventions may be necessary for improvements in CBF to be observed. Though they are not direct sources of β-OHB, oral MCTs trials in Alzheimer's patients have also demonstrated beneficial effects to both CBF and cerebral metabolism. Specifically, 45 days of daily supplementation with MCTs oil (caprylidene) improved regional cerebral blood flow relative to a placebo in APOε4-negative patients with Alzheimer's disease (Torosyan *et al.*, 2018). Another MCTs trial in Alzheimer's patients demonstrated increases in cerebral metabolism with 30 days of MCTs supplementation (Croteau *et al.*, 2018). The availability of an alternative metabolic fuel (i.e., β-OHB) for the brain may be neuroprotective in Alzheimer's, a pathology where glucose hypometabolism is common and detrimental to brain health and function (Croteau *et al.*, 2018).

Despite these promising results, no research to date has looked at the acute effects of oral β-OHB supplementation, specifically KE, on CBF in young adults. Additionally, both the single dose protocols and heterogeneity in the β-OHB supplement dose used in past studies make it difficult to isolate the relationship between β-OHB supplement dose and CBF. Thus, it remains unknown whether different doses of oral β-OHB supplements differentially impact CBF regulation in young adults.

1.3.3 Effects on Cognitive Function

In addition to exerting effects on cerebrovascular physiology, exogenous β-OHB improves psychological outcomes related to cognition. In particular, interventions with exogenous β-OHB have been associated with improved cognitive performance in domains such as working memory, attention, and executive function in both healthy and clinical populations (Jensen *et al.*, 2020*b*, 2020*a*; Yomogida *et al.*, 2021; Walsh *et al.*, 2021*a*; Quinones & Lemon, 2022; Waldman *et al.*, 2023*a*). KS infusion improves working memory performance on the Wechsler Adult Intelligence Scale letter-number-sequencing task in individuals with type 2 diabetes (T2D) (Jensen *et al.*, 2020*a*). Similarly, acute ingestion of MCTs improves executive function on the N-back test and Go/No-Go task in healthy older adults (Yomogida *et al.*, 2021). Even though MCTs do not raise blood β-OHB concentrations as high as KS (0.65 mM versus 2.4 mM in Jensen *et al*. (2020)), beneficial effects on cognitive performance were still observed.

Improved cognitive performance on a battery of psychological tests may be due to a shift in cerebral metabolism with elevated plasma β-OHB (Hasselbalch *et al.*, 1996; Svart *et al.*, 2018). This metabolic shift has been shown to improve functional connectivity between neuronal networks (Mujica-Parodi *et al.*, 2020). Specifically, a single KE supplement (0.395 g/kg body mass; peak blood β-OHB: 3.5 mM) improves brain network stability, which is a surrogate for

cognition, as quickly as 30 minutes post-ingestion in normoglycemic young adults (Mujica-Parodi *et al.*, 2020). This is likely a result of direct ketone uptake within the brain, as magnetic resonance spectroscopy data from the same study demonstrates that ketone concentrations in the brain reach peak concentrations around 30 minutes after KE ingestion and remain elevated for at least 80 minutes post-ingestion (Mujica-Parodi *et al.*, 2020). Collectively, these results suggest that nutritional ketosis is at least partially responsible for these observed benefits to brain function, rather than merely a compensation for cerebral glucose hypometabolism in T2D (Jensen *et al.*, 2020*a*) and Alzheimer's disease (Szablewski, 2021).

Enhanced cognition may also be facilitated by augmented CBF via exogenous β-OHB interventions (Walsh *et al.*, 2021*a*). Walsh *et al*. (2021) demonstrated that thrice-daily KE supplementation for 14 days improves both CBF and cognitive performance on the Digit Symbol Substitution Test in individuals with obesity. This test primarily assesses the domains of attention, executive function, and processing speed. In particular, there was a positive relationship observed between VA flow and DSST performance. Given that CBF and cognitive function in this trial were assessed in the fasting state, this suggests that persistent increases in CBF may contribute to facilitating improvements in cognitive function with KME supplementation. However, acute changes in CBF do not necessarily cause corresponding changes to cognitive function. In particular, acute decreases in CBF via pharmacological interventions do not lead to decreases in cognitive performance in healthy young and older adults (Shoemaker *et al.*, 2020). This may be due to increased oxygen extraction to compensate for reduced oxygen delivery via CBF, thus matching cerebral metabolic demand and preserving cognitive function. However, these compensations likely only occur to a certain point with acute exposure, as chronic reductions in CBF that occur with increasing age (Leenders *et al.*, 1990) or impaired glucose metabolism (Deery

et al., 2024) are heavily implicated in the development of neurodegenerative pathologies like Alzheimer's disease (Korte *et al.*, 2020). Nonetheless, no studies to date have assessed CBF and cognitive performance concurrently in response to exogenous β-OHB supplementation in healthy adults. Further studies that primarily focus on CBF in a non-rested state (i.e., actively performing a cognitive task) will be needed to uncover if acute changes to CBF directly translate to changes in cognitive performance with exogenous β-OHB sources.

1.4 β-OHB Dose and Metabolic Acidosis – A Threat to CBF?

Despite the encouraging findings of β-OHB increasing CBF from KS infusion trials, there is growing evidence that oral KE supplements may perturb blood pH homestasis and thus threaten CBF regulation. Ingestion of oral KE causes dose-dependent lowering of blood pH at rest, with higher doses reducing pH beyond normal homeostatic levels (7.35-7.45) (Stubbs *et al.*, 2017; Dearlove *et al.*, 2021). A high-dose KE (0.752 g/kg body mass) significantly lowers blood pH (Δ 0.03) compared to a low-dose KE (0.252 g/kg body mass) at rest (Dearlove *et al.*, 2021). Blood acidosis is likely a direct result of KE hydrolysis in the gut. KE supplements are hydrolyzed by esterases in the gut and liver to produce β-OHB and 1,3-butanediol, and 1,3-butanediol further undergoes hepatic metabolism to form β-OHB (Clarke *et al.*, 2012). β-OHB is a weak organic acid and ingestion of oral KE can lower blood pH (Stubbs *et al.*, 2017; Dearlove *et al.*, 2019). Indeed, blood β-OHB concentrations between 2-4 mM have been shown to induce blood acidosis (Stubbs *et al.*, 2017; Dearlove *et al.*, 2019).

Disruptions to blood pH are quickly corrected via respiratory compensations. When blood pH drops, the blood bicarbonate buffer system increases CO_2 production to remove free H^+ ions from the circulation (**Figure 1**). Higher P_aCO_2 is sensed by peripheral chemoreceptors which stimulate the respiratory centers in the medulla oblongata and pons of the brainstem to increase

breathing rate (i.e., hyperventilation) (Ikeda *et al.*, 2017; Gallo de Moraes & Surani, 2019). This is demonstrated in submaximal exercise trials with KE supplementation. In one trial, participants had significantly higher HR and ventilation during exercise (peak blood β-OHB: 3.9 mM) after prior consumption of a 0.6 g/kg KE dose, compared to a placebo beverage (McCarthy *et al.*, 2021*b*). Increased ventilation with KE supplementation quickly removes CO_2 from the blood in an effort to restore acid-base balance (**Figure 1**). In support of this, another exercise trial observed significantly higher ventilation and lower end-tidal CO_2 ($P_{ET}CO_2$) during an incremental cycling test to exhaustion (peak blood β-OHB: 3.7 mM) when consuming a 0.33 g/kg body mass KE before exercise, compared to a placebo drink (Dearlove *et al.*, 2019). Contrarily, these effects have not been observed following consumption of lower KE doses (0.15 g/kg body mass) (Stubbs *et al*., 2017). Further, KS infusion, which increases CBF, does not alter $P_{ET}CO_2$ (Hasselbalch *et al*., 1996). This evidence suggests that specifically bolus KE ingestion lowers P_aCO_2 (represented by $P_{ET}CO_2$), when consumed at doses high enough to lower blood pH. P_aCO_2 is a primary regulator of CBF and reductions in P_aCO_2 cause cerebral vasoconstriction, which subsequently lowers CBF (Querido & Sheel, 2007). This suggests that different oral KE doses of β-OHB may exert differential effects on CBF, where higher KE doses may cause reductions in CBF due to blood acidosis and alterations in P_aCO_2 (**Figure 3**). In turn, lower KE doses which do not perturb acid-base balance may exert beneficial effects on CBF, similar to past β-OHB infusion studies. As such, there is a need for a systematic characterization of how acute oral KE consumption impacts CBF regulation in young adults.

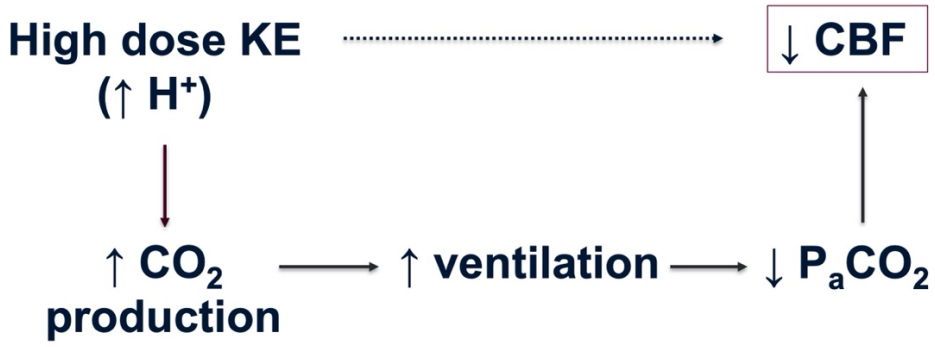

Figure 3. Potential mechanism of the influence of changes in P_aCO_2 via high doses of KE on CBF.

1.5 Summary

Exogenous ketone sources containing β-OHB represent a novel therapeutic intervention that may improve brain health and function through the lifespan. Treatment with exogenous β-OHB may be exerting neuroprotective effects by improving CBF (Hasselbalch *et al.*, 1996; Svart *et al.*, 2018; Walsh *et al.*, 2021*a*), shifting cerebral metabolism (Hasselbalch *et al.*, 1996; Svart *et al.*, 2018), and directly reducing inflammation (Youm *et al.*, 2015; Walsh *et al.*, 2021*b*) and oxidative stress (Maalouf *et al.*, 2007; Kim *et al.*, 2007; Shimazu *et al.*, 2013). These physiological effects may also contribute to improvements in functional outcomes such as neuronal network function (Mujica-Parodi *et al.*, 2020) and cognition (Jensen *et al.*, 2020*a*, 2020*b*; Walsh *et al.*, 2021*a*; Quinones & Lemon, 2022; Waldman *et al.*, 2023*a*). Despite this promising evidence, significant knowledge gaps remain concerning the acute effects of oral KE supplementation on resting CBF and cognitive function in young adults. Future research should build upon the known effects of exogenous β-OHB supplements on cerebral and systemic physiology in order to establish a clear picture of how exogenous β-OHB impacts the brain.

1.5.1 Purpose and Hypothesis for the kCBF Study

The purpose of this study is to investigate the effect of oral KE dose (low versus high) on resting CBF and cognition in normoglycemic adults. We hypothesize that CBF at the timepoint corresponding to the highest mean blood [β-OHB] will be increased after ingestion of 0.3 g/kg body mass KE and decreased after ingestion of 0.6 g/kg body mass KE, as compared to ingestion of a ketone-free placebo. Further, we anticipate that cognitive performance, measured via a hippocampal-dependent memory task, will be positively related to improvements in CBF.

Chapter 2: The Effects of Acute Ketone Monoester Ingestion on Resting Cerebral Blood Flow and Cognition in Young Adults

2.1 Introduction

Production of the ketone body beta-hydroxybutyrate (β-OHB) is increased during periods of energetic stress, including prolonged exercise, fasting, or severe carbohydrate restriction (i.e., ketogenic diets) (Cox & Clarke, 2014; Masino, 2022). In these scenarios, fatty acids are mobilized from adipose tissue and converted into β-OHB by the liver, where it is transported to peripheral tissues including the brain, heart, and skeletal muscle to support metabolism in the presence of low glucose (Puchalska & Crawford, 2017; Poff *et al.*, 2020). Elevated plasma β-OHB is a biological starvation signal that exerts pleiotropic effects throughout the body (Newman & Verdin, 2017), including regulating gene expression via histone deacetylase inhibition (Shimazu *et al.*, 2013), mitigating oxidative stress (Kim *et al.*, 2007), and reducing markers of inflammation (Youm *et al.*, 2015; Walsh *et al.*, 2021*b*). Until recently, appreciably elevating circulating β-OHB levels was only achievable via prolonged fasting or marked carbohydrate restriction. The recent development of oral exogenous ketone supplements provides a novel method of inducing rapid, nutritional ketosis that is safe for human consumption (Soto-Mota *et al.*, 2019), representing an opportunity to investigate the effects of β-OHB *per se* on human physiology and a potentially therapeutic tool for improving human health.

Mounting evidence supports the potential beneficial effects of elevated β-OHB for brain health in humans. Infusion of sodium β-OHB increases cerebral blood flow (CBF) by 39% in young adults, achieving peak blood β-OHB concentrations of approximately 2.16 mM (Hasselbalch *et al.*, 1996). Similarly, sodium β-OHB infusion increases CBF by 30% in middle-to-older aged adults and elevates blood β-OHB concentrations to a peak of 5.5 mM (Svart *et al*., 2018). The mechanisms underlying the rapid modulation of CBF with acute β-OHB exposure are

not well understood and likely stem from a combination of direct and indirect vascular effects, including reductions in inflammation (Youm *et al.*, 2015; Bae *et al.*, 2016; Goldberg *et al.*, 2017; Yamanashi *et al.*, 2017) and oxidative stress (Maalouf *et al.*, 2007; Kim *et al.*, 2007; Shimazu *et al.*, 2013), and direct vasodilatory effects (McCarthy *et al.*, 2021*a*). Changes in cerebral substrate utilization may also increase CBF via alterations in astrocyte redox potential. Cerebral glucose metabolism is reduced by 14 to 33% in the presence of elevated plasma β-OHB, without altering cerebral oxygen metabolism (Hasselbalch *et al.*, 1996; Svart *et al.*, 2018). This shift in cerebral metabolism alters the redox potential within astrocytes and enhances the sensitivity of neurovascular coupling, thereby increasing CBF (Mintun *et al.*, 2004; Vlassenko *et al.*, 2006).

β-OHB infusion studies provide compelling evidence that exogenous β-OHB can increase CBF in humans and a recent trial demonstrates that 14 days of thrice-daily ketone monoester (KME) supplementation increases resting CBF by 12% in the fasted, non-supplemented state in adults with obesity (Walsh *et al.*, 2021*a*). However, it is currently unknown how a single dose of exogenous KME impacts acute CBF regulation. Further, it is unclear whether differences in KME dose differentially affect CBF regulation. Interestingly, recent studies have shown that KME ingestion at higher doses can perturb blood pH, which could negatively impact CBF. Indeed, β-OHB is a weak organic acid and bolus ingestion of an oral KME with a dose greater than 0.33 g/kg body mass lowers blood pH outside of the homeostatic range (Stubbs *et al.*, 2017; Dearlove *et al.*, 2019). Previous work has shown that disturbances to blood pH following KME ingestion are quickly buffered by the blood bicarbonate buffering system and increased ventilation to remove the resultant excess CO_2 from the blood (Dearlove *et al.*, 2019; McCarthy *et al.*, 2021*b*). However, arterial CO_2 (P_aCO_2) is an important regulator of CBF, and reductions in P_aCO_2 lower CBF via cerebral vasoconstriction (Hoiland *et al.*, 2019; Caldwell *et al.*, 2021). This suggests that different

KME doses may exert differential effects on CBF, perhaps due to alterations in P_aCO_2. Thus, there is a crucial need to investigate the impact of different KME doses on CBF, to characterize the basic physiological responses of KME ingestion on CBF and brain health.

Provision of exogenous β-OHB has also been shown to improve performance in numerous domains of cognitive function. Indeed, acute sodium β-OHB infusion in individuals with T2D improves working memory (Jensen *et al.*, 2020*a*) and oral KME ingestion prior to and/or during exercise trials improves attention, reaction time, and executive functions in healthy adults (Evans & Egan, 2018; Quinones & Lemon, 2022; Poffé *et al.*, 2023; Waldman *et al.*, 2023*a*). Acute KME ingestion has also been shown to improve brain network stability, which is a surrogate for cognition (Mujica-Parodi *et al.*, 2020). However, no study to date has investigated the effects of different KME doses on cognitive function at rest in young adults. Acute KME ingestion may improve hippocampal-dependent functions through increases in circulating neurotrophins, such as brain-derived neurotrophic factor (BDNF). Indeed, rodent and *in vitro* models demonstrate that β-OHB exposure increases BDNF gene expression in hippocampal neurons (Sleiman *et al.*, 2016; Marosi *et al.*, 2016; Hu *et al.*, 2018, 2020). Accordingly, assessing the acute effects of KME dose on memory performance is highly warranted to contribute to our understanding of how KME ingestion may impact brain health.

The purpose of this study was to investigate the effect of two separate KME doses (low- and high-dose) on resting CBF and cognition in young adults. We hypothesized that CBF would be increased after ingestion of a low-dose KME and decreased after ingestion of a high-dose KME over a 2-hour period, as compared to ingestion of an inert placebo, due to dose-dependent alterations in P_aCO_2. Further, we hypothesized that performance on a hippocampal memory task would be positively related to changes in CBF after KME ingestion. This systematic

characterization of the dose-dependent effects of KME on these vital components of brain health is critical to establish the foundation of the potential therapeutic effects of this common exogenous β-OHB treatment.

2.2 Methods

This study reports the primary outcomes from a trial registered as 'Ketone Dose and Cerebral Blood Flow Study (kCBF)' (ClinicalTrials.gov Identifier NCT06032156). This trial was approved by the Hamilton Integrated Research Ethics Board (Project ID: 15454) (refer to appendix F for the HiREB approval letter), and all participants provided written informed consent prior to beginning participation in this study. The trial conformed with the standards set by the Declaration of Helsinki and subsequent revisions.

2.2.1 Participants

Healthy, normoglycemic adults between the ages of 18-35 years were recruited from McMaster University in Hamilton, ON and the greater Hamilton area between September 2023 and April 2024. Exclusion criteria were the presence of obesity (body mass index >30 kg/m^2), the presence of any cardiometabolic disease (e.g., T2D, hypertension), having a history of cardiovascular disease or cardiovascular events requiring hospitalization (i.e., heart attack), having a history of concussion(s) with persistent symptoms, and currently following a ketogenic diet or taking ketone supplements. Interested participants who self-reported being eligible based on the above exclusion criteria were invited for an in-person familiarization visit.

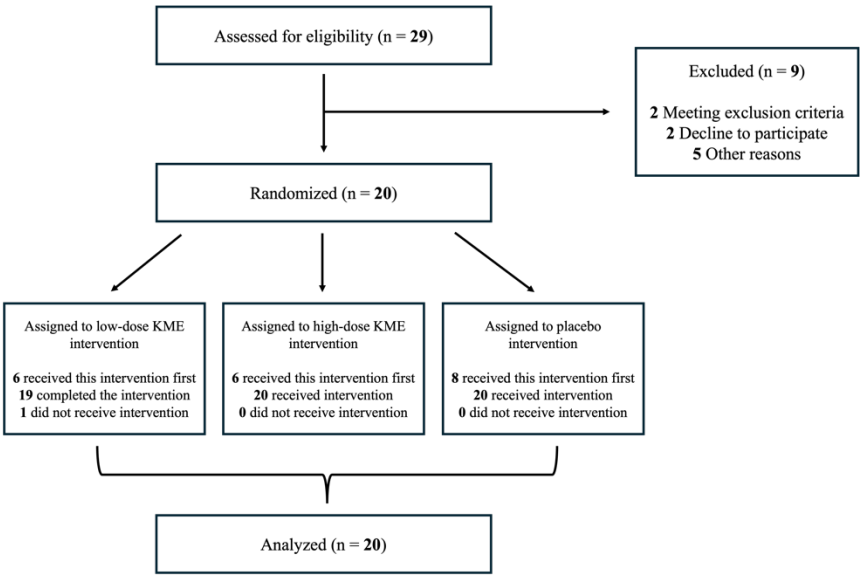

Figure 1. Schematic displaying trial flowchart.

2.2.2 Familiarization and $\dot{V}O_{2peak}$ Testing

Prior to condition randomization, eligible participants completed a familiarization visit to obtain anthropometric measures of height and body mass, practice the mnemonic similarity task (MST) cognitive testing paradigm (Stark *et al.*, 2019), and complete a graded exercise test to exhaustion to measure cardiorespiratory fitness. Participants completed the MST during this familiarization visit to ensure every participant understood how the test was conducted and to minimize possible learning effects. Even though the MST has strong resistance to learning effects from repeated testing (Stark *et al.*, 2023), the MST was still introduced in the familiarization visit to further prevent any impact of repeated testing on the cognitive function results from this trial. As well, resting CBF measurements were obtained using Duplex ultrasound (Terason uSmart 3300; Teratech, Burlington, MA, USA) of the internal carotid and vertebral arteries to ensure that viable scans could be obtained before proceeding to the experimental visits.

To characterize peak rate of oxygen consumption ($\dot{V}O_{2peak}$), participants completed a graded exercise test to volitional exhaustion on a cycling ergometer (Kettler Ergo Race, Kettler,

Virginia Beach, VA). The test consisted of a 3-minute warmup at 50 W, followed by increases of 5 W every 10 s until participants were unable to sustain a cadence above 60 revolutions per minute, upon which the test was terminated. Breath-by-breath expired gases and ventilation were recorded using a metabolic cart (Quark CPET Metabolic Cart, COSMED, Italy) and heart rate was measured using a chest strap monitor (Polar A3, Lake Success, New York). A 'true' $\dot{V}O_{2peak}$ was classified as successfully meeting at least three out of four possible criteria during the test: 1) Respiratory exchange ratio ≥ 1.13; 2) Max heart rate (beats per minute) ≥ (208-0.7*age)*0.93); 3) Perceived exertion ≥ 17 (Borg Scale); or 4) Rate of oxygen consumption ($\dot{V}O_2$) plateau (values ± 0.1 L/min for 3 10-second consecutive recording windows). $\dot{V}O_{2peak}$ was calculated as the mean of the three highest 10-second recording windows of $\dot{V}O_2$ during the test.

2.2.3 Supplement Details

The ketone supplement used in the study (Delta G Ketone, TΔS, Oxford) is commercially available in 1L bottles which contain 1070g of the ketone monoester [*R*]-3-hydroxybutyl [*R*]-3-hydroxybutyrate. For the low-dose KME condition, participants consumed 0.3 g/kg body mass of the ketone monoester. For the high-dose KME condition, participants consumed 0.6 g/kg body mass of the ketone monoester. The mean β-OHB dose delivered was 20.56 ± 3.03 g (low-dose KME) and 41.12 ± 6.06 g (high-dose KME), given a mean participant body mass of 68.52 ± 10.11 kg. The placebo stock contained 0.0007% Bitrex™ (Denatonium Benzoate NF, Johnson Mathey, UK) and 0.25% xanthan gum to match the taste and viscosity of the ketone monoester. Supplements were dispensed into 60 mL opaque bottles. The placebo supplement contained 60 mL of the placebo stock and two drops of calorie-free lemonade flavouring. The remainder of the bottle for the ketone dose conditions was filled with placebo stock and two drops of calorie-free lemon juice flavouring to reach a total volume of 60 mL. Once the supplements were made, the bottles

were labelled with the participant's ID and visit number by a third-party researcher not involved in data collection. Blinding was maintained until the completion of data collection and analysis.

2.2.4 Experimental Visits

A randomized, placebo-controlled, double-blind, counterbalanced, crossover design was used to test three conditions: Placebo, Low-dose KME (0.3 g/kg body mass), and High-dose KME (0.6 g/kg body mass). Participants were randomly assigned to 1 of 6 treatment orders by a third-party researcher not involved in data collection using a random number generator in a counterbalanced manner.

Participants attended the Brain Exercise Enhancement Laboratory at McMaster University three separate times, separated by at least 72 hours to ensure no carry-over of previous experimental visits. Female participants were asked to report whether they take contraceptives using a menstrual cycle questionnaire (see appendix A for a copy of the menstrual cycle questionnaire). Visits for female participants were conducted during days 0-6 of the follicular phase (or placebo phase for contraceptive users) of their menstrual cycle to control for any potential effects of menstrual cycle phase on the outcomes of this study. Experimental visits occurred at the same time of day for a given individual. Participants were instructed to abstain from consuming alcohol, caffeine, food, or drink (excluding water) for at least 8 hours prior, and to abstain from vigorous physical activity for at least 24 hours prior to each experimental visit. During the first visit, participants completed a dietary recall questionnaire to record their food and drink consumption in the 24 hours prior to their first experimental visit (see appendix B for a copy of the dietary recall questionnaire). Participants were asked to replicate the same diet in the 24 hours prior to each subsequent visit as closely as possible. Participants also filled out the 'Consensus Sleep Diary', a questionnaire asking about their sleep quality the night before each

visit (Carney *et al*., 2012), as reductions in sleep quality have been associated with reductions in CBF (Park *et al.*, 2019). The 'Consensus Sleep Diary' can be viewed in Appendix C.

Following completion of intake procedures, participants lay supine and were instrumented with a three-lead electrocardiogram (PowerLab model ML795, AD Instruments, CO, USA) and a non-invasive blood pressure finger cuff (Human NIBP Nano System, model INL382, AD Instruments) for continuous heart rate (HR) and blood pressure (MAP) measurements. An intravenous catheter was inserted into a vein at the elbow for serial blood sampling. Following instrumentation, baseline measures of CBF using Duplex (Terason uSmart 3300 NexGen; Teratech, Burlington, MA, USA) and Transcranial Doppler ultrasound (Neurovision, Multigon Industries), hemodynamics, end-tidal gases, and blood were obtained over a 5-minute collection window. Participants then completed baseline cognitive testing for approximately 15 minutes in a seated position. Upon completion of cognitive testing, participants completed a questionnaire consisting of the intrinsic motivation subscale of the Intrinsic Motivation Inventory (IMI) (Ryan, 1982; Ryan *et al.*, 1983). The intrinsic motivation subscale of the IMI can be viewed in Appendix D.

Following collection of baseline measures, participants were provided with a supplement in an opaque bottle to ingest. The complete ingestion of the drink was considered the 0-minute time marker of the protocol and a stopwatch was started to track elapsed time. Over the subsequent 2-hour period, CBF, end-tidal CO_2, MAP, and HR were collected over 5-minute recording windows at 45- and 120-min post-ingestion. Blood samples were obtained 30-, 45-, 60-, and 120-min post-ingestion. Participants completed post-supplementation cognitive testing at 130-min, following completion of the final physiological measure assessment. Finally, participants completed the intrinsic motivation questionnaire and a questionnaire regarding gastrointestinal

symptoms that may have occurred over the course of the protocol after drink consumption (see appendix E for the gastrointenstinal symptom questionnaire). Figure 2 displays the study protocol for each experimental visit.

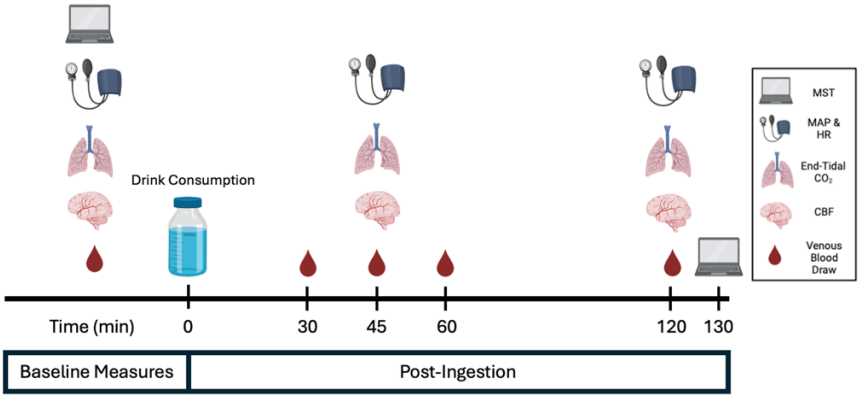

Figure 2. Schematic overview of the experimental protocol. Images created using biorender.com

2.2.5 Haemodynamic and Respiratory Measurements

MAP and HR were collected with an automated brachial cuff sphygmomanometer (OMRON Healthcare Co. Ltd, Kyoto, Japan). The mean value of the three measurements was used for analysis of each variable. End-tidal CO_2 ($P_{ET}CO_2$) was collected using a gas analyzer (PowerLab model ML795, ADInstruments, Colorado Springs, CO, USA). Participants were fitted with a mouthpiece and nose clip before commencing recordings of breath-by-breath gas exchange. The mean value of $P_{ET}CO_2$ during the 5-min recording window was used for analysis. This time period encompassed both the ICA and VA scanning periods.

2.2.6 Middle Cerebral Artery Blood Velocity

Blood velocity of the middle cerebral artery (MCAv) was assessed using a 2 MHz Transcranial Doppler ultrasound (NeuroVision, Multigon Industries, NY, USA) in accordance with localization and signal optimization procedures previously described (Willie *et al.*, 2011). The

ultrasound probe was affixed to the participant's right temporal window using a specialized headband. Recordings included in analysis contained complete waveforms for ≥ 12 consecutive cardiac cycles. The mean value of MCAv during the longest string of consecutive complete waveforms in each recording window was used for analysis. The between-day coefficient of variation for MCAv was 9.8%.

2.2.7 Global Cerebral Blood Flow Assessment

Vessel diameter and blood velocity of the internal carotid (ICA) and vertebral arteries (VA) were measured using a 10 MHz multifrequency linear array Duplex ultrasound (Terason uSmart 3300 NexGen; Teratech, Burlington, MA, USA) following recommendations for location and procedure standardization (Thomas *et al.*, 2015). Peak blood velocity and arterial diameter were viewed simultaneously using B-mode imaging via the pulse-wave mode feature of the ultrasound machine. ICA blood velocity and vessel diameter were measured ≥ 1.5 cm from the carotid artery bifurcation to minimize retrograde flow. Blood velocity and vessel diameter of the VA were measured between C4 and C5 or C5 and C6. The recording location within the vessel was individualized and optimized during each participant's first CBF recording to allow for reliable image acquisition. Recordings were almost exclusively taken from the right ICA and VA, unless image quality was poor, then the left ICA and/or VA was used in substitution. This location was unchanged within participants and between trials. The same insonation angle was used for all recordings (60°). Following acquisition of the first ultrasound image, B-mode gain and dynamic range were unaltered to minimize variability in arterial wall brightness and/or thickness between recordings. ICA and VA diameter and blood velocity were recorded for at least 1 minute using a screen capturing software (Camtasia 2022, TechSmith). Viable recordings included ≥ 12 cardiac cycles. Offline analysis involved the use of custom-edge detection and wall tracking software

(BloodFlow analysis, version 6.1). This analysis software mitigates observer bias by establishing diameter and velocity traces without the need for manual caliper placement by the researcher (Woodman *et al.*, 2001). Mean beat to beat artery flow was sampled at 30 Hz from the selected recording and calculated automatically from these traces (Woodman *et al.*, 2001). The between-day coefficients of variation for ICA and VA diameter were 3.2% and 2.7%, respectively.

Mean arterial pressure (MAP) was calculated as:

$$MAP = DBP + ((SBP-DBP)/3)$$

Blood flow (Q) was calculated as:

$$Q = (\text{mean envelope blood velocity} / 2) \times (\pi \times (0.5 \times \text{diameter})^2) \times 60$$

Global cerebral blood flow (gCBF) was calculated as:

$$gCBF = 2 \times (Q_{ICA} + Q_{VA})$$

Mean shear rate was calculated as:

$$\text{Shear Rate} = 4 \times (\text{mean envelope blood velocity/diameter})$$

Cerebrovascular conductance (CVC) was calculated as:

$$CVC = Q_{ICA}, Q_{VA}, gCBF/MAP$$

2.2.8 Hippocampal-Dependent Function Assessment (Mnemonic Similarity Task)

The mnemonic similarity task (MST) was used to assess high-interference episodic memory, a form of hippocampal-dependent memory (Stark *et al.*, 2019). This test was chosen because of the hippocampal-specific effects of β-OHB observed in rodent and animal models (Sleiman *et al.*, 2016; Marosi *et al.*, 2016; Hu *et al.*, 2018, 2020). The MST is comprised of two main phases, each about 10 min in length and administered via a computer (MST version 0.96). In phase 1, participants engaged in an incidental encoding task, where they made ‘indoor’ or ‘outdoor’

judgments for pictures of everyday objects. Immediately following this task, participants were given a new series of images in phase 2 and were instructed to identify each item as 'old', 'similar', or 'new'. One-third of the images in the test phase were exact repetitions of images presented in the study phase (targets), one-third of the images were new images not previously seen (foils), and one-third of the images were perceptually similar to those seen during the study phase, but not identical (lures). Participants completed the MST a total of 6 times over three separate visits. Each iteration of the test used a different image set of the same length and difficulty (Sets 1-6) to prevent learning effects. The set presentation order was randomized for each participant using a random number generator and counterbalanced.

To assess cognitive performance on the MST, the lure discrimination index (LDI) served as a measure of how well participants discerned between targets and lures. The LDI was calculated as the rate of correctly answering 'Similar' when presented with a lure image subtracted by the rate of incorrectly answering 'Similar' when presented with a foil image. In addition, the recognition memory score (REC) was calculated to quantify the difference between the rate of 'Old' responses for targets and the rate of 'Old' responses for foils, to serve as a measure of recognition memory for repeated items (targets).

Lure discrimination index (LDI) was calculated as:

$$LDI = Similar \mid Lure - Similar \mid Foil$$

Recognition memory score (REC) was calculated as:

$$REC = Old \mid Target - Old \mid Foil$$

2.2.9 Intrinsic Motivation

Intrinsic motivation was assessed immediately following cognitive testing using a subjective questionnaire from the IMI (Ryan, 1982; Ryan *et al.*, 1983). Participants rated their motivation by indicating how well they agreed to 7 different statements about the cognitive test they just performed using an analog scale (1 – not at all true; 4 – somewhat true; 7 – very true).

2.2.10 Plasma β-OHB

A point-of-care analyzer (FreeStyle Libre, Abbott) was used to measure plasma β-OHB in venous blood. If venous blood could not be obtained, capillary blood was used in substitution. Venous blood was drawn in serum separator tubes and K2 EDTA tubes (BD, Franklin Lakes, NJ, USA). For the determination of plasma β-OHB, venous blood obtained at baseline, 30-, 45-, 60-, and 120-min post-ingestion was used. A researcher not involved in data acquisition or analysis recorded venous β-OHB concentrations to maintain blinding.

2.2.11 Sample Size Determination

To date, there has been no previous investigations of the acute effects of KME ingestion on resting CBF nor has there been a characterization of the potential dose-response effects. Previous infusion trials have reported increased gCBF ranging from 30 to 39% (Hasselbalch *et al.*, 1996; Svart *et al.*, 2018), whereas our previous trial found that 14-d of KME ingestion increased gCBF by 12% in the fasted, non-supplemented state in adults with obesity (Walsh *et al.*, 2021*a*). Accordingly, we conducted a sample size estimation based on the hypothesis that dose-dependent differences in gCBF would occur at the measurement time point corresponding with peak plasma β-OHB, which was determined as 45-min post-ingestion based on recent pilot work. A sample size estimation in G*Power (version 3.1) revealed that n =19 provided 80% power at an alpha level of

0.05 with an effect size partial eta squared of 0.23 using a within-factors one-way analysis of variance (3 conditions x 1 time point) (see appendix G for the sample size estimation). The effect size estimation was based on a 12% change in CBF and the associated measurement error, as based on previous published work (Walsh *et al.*, 2021*a*) as well as pilot data from our lab. To preserve power, 20 adults were recruited and randomized (10 male & 10 female).

2.2.12 Statistical Analyses

Statistical analyses were performed using the Jamovi software for Mac (version 2.5.7.0) and R Studio (R Core Team, version 4.3.3). Q-Q plots and Shapiro-Wilk tests were used to assess normality and skewness. All normal values are expressed as mean ± SD. Differences between condition in CBF at 45-min post-ingestion were assessed using a 1-way repeated-measures ANOVA (1 group, 3 measurements). This timepoint was chosen as it was identified as the timepoint of peak β-OHB levels in the blood after KME ingestion in previous pilot work. The "predict" function in R used a linear mixed effects model (LMM) created using the lme4 package (Bates *et al.*, 2015) to predict one gCBF data point that was removed due to poor image quality, for the repeated-measures ANOVA. The LMM contained fixed effects of time, condition, as well as time by condition interaction, and participant ID was included as a random effect. LMM with the same fixed and random effects were used for all experimental variables to assess differences over the 2-hour period post-ingestion. β-OHB area-under-the-curve (AUC) for the low- and high-dose conditions was analyzed using a paired samples t-test. Holm's post-hoc comparisons were conducted for any outcomes with significant ($P < 0.05$) effects of time, condition, or time by condition interaction. Effect sizes are presented as Cohen's d for paired-samples t-test, partial eta squared (n_p^2) for ANOVA, and coefficient of determination (R^2) for linear regression. For exploratory purposes, a correlation matrix was applied between changes in $\dot{V}O_{2peak}$ and changes in

other measured variables significantly affected by time or condition. Significant correlations ($P < 0.05$) were followed by regression analyses.

2.3 Results

2.3.1 Participants

Participant characteristics are displayed in Table 1. A total of 9 males and 10 females completed all experimental visits, and 1 male participant completed 2 out of 3 experimental visits and was included in linear-mixed model analyses. Subjectively reported sleep prior to experimental visits was consistent between visits for individual participants. Subjectively reported gastrointestinal symptoms from drink consumption (placebo, low-dose KME, and high-dose KME) were very low and no major adverse reactions occurred. Participants also correctly predicted the given supplement at a rate of 39%. Finally, all participants achieved a true $\dot{V}O_{2peak}$, as defined by criteria mentioned previously.

Table 1. Participant characteristics.

	Female (n = 10)	**Male (n = 10)**	**Group (n = 20)**
Age (years)	22 ± 2	24 ± 4	23 ± 3
Body mass (kg)	62.47 ± 9.11	**74.57 ± 7.17***	68.52 ± 10.11
BMI (kg/m^2)	22.99 ± 2.50	23.88 ± 1.80	23.43 ± 2.17
$\dot{V}O_{2peak}$ (mL/kg/min) [range]	37.95 ± 5.85 [32.39, 48.89]	**50.91 ± 8.71*** [38.1, 64.15]	44.43 ± 9.82 [32.39, 64.15]
Contraceptive users (%)	50% (5/10)	N/A	N/A
Years of Education	16 ± 2	18 ± 3	17 ± 3

Data are presented as mean ± SD. BMI = body mass index; $\dot{V}O_{2peak}$ = peak rate of oxygen consumption.
* = $P < 0.05$ compared to female.

2.3.2 Plasma β-OHB

Peak plasma β-OHB in the low-dose condition was 2.97 mM and occurred at 45-min post-ingestion (**Figure 3A**). In the high-dose condition, peak plasma β-OHB was 4.72 mM and occurred at 120-min post-ingestion (**Figure 3A**). β-OHB area-under-the-curve (AUC) for the low-dose condition (285 ± 58.9 mM x 120 min) was also significantly lower than the β-OHB AUC for the high-dose condition (458 ± 99.9 mM x 120 min) ($P < 0.001$) (**Figure 3B**).

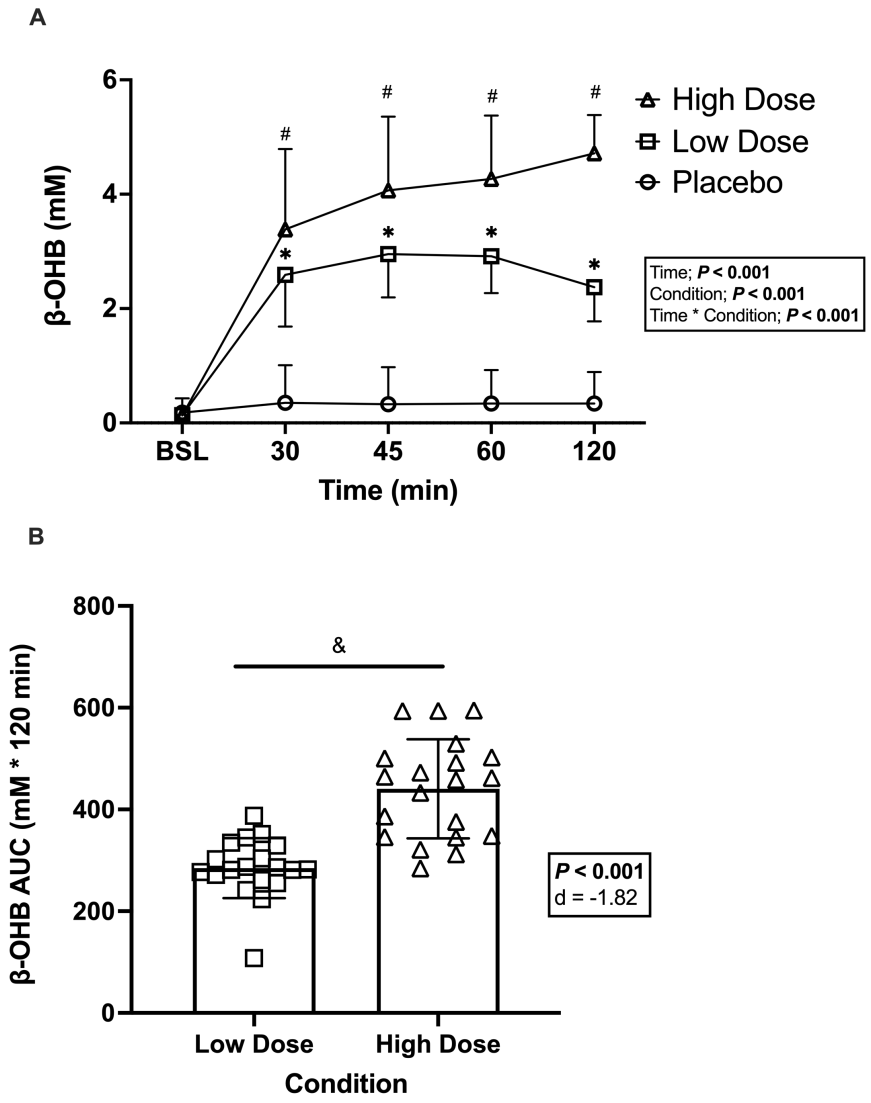

Figure 3. Plasma β-OHB. β-OHB concentrations (**A**) and area-under-the-curve (AUC) (**B**) over the 120-min intervention period. Data are presented as mean ± SD. Individual data are represented as open triangles and open squares in **B**. * = $P < 0.05$ compared to baseline (low-dose KME); # = $P < 0.05$ compared to baseline (high-dose KME); & = $P < 0.05$ compared to low-dose KME.

2.3.3 Cerebral Blood Flow and Cerebrovascular Outcomes

All cerebrovascular measures are presented in Table 2. At 45-min post-ingestion, gCBF was 31.5 mL/min lower ($P = 0.022$) in the low-dose condition and 30.0 mL/min lower in the high-dose condition ($P = 0.168$), relative to the placebo condition (**Figure 4A**). Compared to baseline measures, low-dose and high-dose KME ingestion significantly decreased gCBF over the 2-hour intervention (time * condition interaction, $P < 0.001$) (**Figure 4B**). At 45-min post-ingestion, gCBF was reduced by 10.4% in the low-dose condition ($P = 0.008$), and by 14.0% in the high-dose condition ($P < 0.001$), relative to baseline. At 120-min post-ingestion, gCBF was 6.2% lower compared to baseline in the low-dose condition ($P = 0.393$). In the high-dose condition, gCBF was reduced by 18.6% at 120-min post-ingestion ($P < 0.001$), relative to baseline. Cerebrovascular conductance for gCBF was also significantly reduced with both low-dose and high-dose KME ingestion (time * condition interaction, $P = 0.003$). Further, gCBF remained unchanged at both 45-min and 120-min post-ingestion in the placebo condition.

Regional changes in extracranial artery blood flow are reported in **Figure 6**. There was a main interaction effect of KME ingestion on ICA flow (time * condition interaction, $P < 0.001$) (**Figure 6A**). Post-hoc comparisons revealed that high-dose KME ingestion significantly reduced ICA flow relative to baseline. In the high-dose condition, ICA flow decreased by 14.5% at 45-min post-ingestion ($P < 0.001$) and by 17.8% at 120-min post-ingestion ($P < 0.001$). ICA flow decreased by 9.0% at 45-min post-ingestion ($P = 0.152$) and by 7.0% at 120-min post-ingestion ($P = 0.496$), compared to baseline in the low-dose condition. ICA cerebrovascular conductance was significantly lower in both the low- and high-dose conditions (time * condition interaction, $P = 0.013$). Further, there was an interaction effect of KME ingestion on ICA diameter (time * condition interaction, $P = 0.031$); however, post-hoc comparisons revealed no significant differences in either KME condition relative to baseline. ICA shear rate was not different in the

low-dose and high-dose conditions (time * condition interaction, P = 0.525). All ICA cerebrovascular measures (flow, diameter, and shear rate) did not significantly differ from baseline in the placebo conditions.

VA flow was significantly lower in both the low- and high-dose conditions (time * condition interaction, P = 0.011) (**Figure 6B**). In the low-dose condition, VA flow decreased by 14.6% at 45-min post-ingestion (P = 0.012) relative to baseline and returned to near-baseline values at 120-min post-ingestion. High-dose KME ingestion decreased VA flow by 12.1% at 45-min post-ingestion (P = 0.089), and by 20.1% at 120-min post-ingestion (P < 0.001), relative to baseline. Low-dose and high-dose KME ingestion both significantly reduced VA cerebrovascular conductance (time * condition interaction, P = 0.035). There was also a main interaction effect of KME ingestion on VA diameter (time * condition interaction, P < 0.001), which was significantly reduced in only the high-dose KME condition (45-min: P = 0.006; 120-min: P < 0.001). VA shear rate was not different following KME ingestion compared to baseline (time * condition interaction, P = 0.270), and all cerebrovascular measures (flow, diameter, and shear rate) were unchanged in the placebo condition, relative to baseline.

There was a main interaction effect of KME ingestion on MCAv (time * condition interaction, P = 0.037) (**Figure 6C**). Post-hoc comparisons revealed that high-dose KME ingestion significantly lowered MCAv at 120-min post-ingestion, relative to baseline (Δ15.6%, P < 0.001). Further, cerebrovascular conductance for MCAv was not significantly altered with KME ingestion (time * condition interaction, P = 0.066). All cerebrovascular measures (velocity and cerebrovascular conductance) for the MCA were unchanged in the placebo condition, relative to baseline.

Table 2. Cerebrovascular measures.

	Baseline	45-min	120-min
Global CBF (mL/min)	**Time, $P < 0.001$**; **Condition, $P = 0.042$**; **Interaction, $P < 0.001$**		
LOW	518 ± 105 [471, 565]	**464 ± 72* [417, 511]**	486 ± 87 [439, 533]
HIGH	544 ± 125 [497, 591]	**468 ± 90* [421, 514]**	**443 ± 86* [396, 490]**
PLACEBO	513 ± 110 [466, 560]	491 ± 104 [444, 538]	511 ± 103 [465, 558]
ICA flow (mL/min)	**Time, $P < 0.001$**; Condition, $P = 0.170$; **Interaction, $P < 0.001$**		
LOW	199 ± 50 [178, 220]	181 ± 37 [160, 202]	185 ± 37 [164, 206]
HIGH	214 ± 54 [193, 235]	**183 ± 41* [162, 204]**	**176 ± 37* [155, 197]**
PLACEBO	197 ± 48 [176, 218]	189 ± 46 [169, 210]	200 ± 46 [179, 221]
VA flow (mL/min)	**Time, $P < 0.001$**; **Condition, $P < 0.001$**; **Interaction, $P = 0.011$**		
LOW	60.2 ± 22.0 [51.2, 69.2]	**51.4 ± 17.7* [42.4, 60.4]**	57.9 ± 23.9 [48.9, 67.0]
HIGH	57.8 ± 19.2 [48.9, 66.8]	50.8 ± 18.0 [41.8, 59.8]	**46.2 ± 16.1* [37.3, 55.2]**
PLACEBO	59.5 ± 20.8 [50.5, 68.5]	53.9 ± 19.4 [44.9, 63.0]	55.8 ± 17.7 [46.8, 64.7]
MCA velocity (cm/s)	**Time, $P < 0.001$**; **Condition, $P < 0.001$**; **Interaction, $P = 0.037$**		

LOW	72.4 ± 12.7 [67.0, 77.8]	66.2 ± 11.9 [60.7, 71.7]	68.3 ± 9.2 [62.6, 74.0]
HIGH	66.5 ± 13.5 [61.1, 71.9]	60.8 ± 13.6 [55.3, 66.2]	**56.1 ± 13.2*** **[50.5, 61.7]**
PLACEBO	70.2 ± 8.6 [64.7, 75.6]	66.3 ± 8.5 [60.7, 71.9]	69.8 ± 7.4 [64.3, 75.4]
ICA diameter (cm)	**Time, *P* = 0.030**; **Condition, *P* = 0.026**; **Interaction, *P* = 0.031**		
LOW	0.457 ± 0.040 [0.439, 0.476]	0.455 ± 0.041 [0.436, 0.473]	0.461 ± 0.039 [0.443, 0.480]
HIGH	0.474 ± 0.042 [0.456, 0.492]	0.460 ± 0.040 [0.441, 0.478]	0.461 ± 0.041 [0.442, 0.479]
PLACEBO	0.462 ± 0.040 [0.444, 0.481]	0.459 ± 0.039 [0.441, 0.478]	0.468 ± 0.039 [0.450, 0.487]
VA diameter (cm)	**Time, *P* < 0.001**; **Condition, *P* < 0.001**; **Interaction, *P* < 0.001**		
LOW	0.359 ± 0.045 [0.340, 0.379]	0.349 ± 0.041 [0.330, 0.369]	0.355 ± 0.047 [0.336, 0.375]
HIGH	0.358 ± 0.040 [0.338, 0.377]	**0.345 ± 0.038*** **[0.326, 0.365]**	**0.342 ± 0.041*** **[0.323, 0.362]**
PLACEBO	0.356 ± 0.043 [0.337, 0.376]	0.357 ± 0.041 [0.338, 0.376]	0.360 ± 0.041 [0.341, 0.379]
ICA shear rate (1/s)	**Time, *P* = 0.001**; Condition, *P* = 0.302; Interaction, *P* = 0.431		
LOW	353 ± 100 [314, 393]	329 ± 82 [290, 368]	321 ± 71 [282, 360]
HIGH	344 ± 94 [305, 383]	323 ± 80 [284, 362]	312 ± 71 [272, 351]
PLACEBO	339 ± 81 [300, 378]	335 ± 89 [296, 374]	333 ± 80 [294, 372]

VA shear rate (1/s)	**Time, *P* = 0.012**; Condition, *P* = 0.778; Interaction, *P* = 0.270		
LOW	211 ± 47 [190, 232]	198 ± 39 [178, 218]	208 ± 42 [187, 229]
HIGH	212 ± 49 [191, 233]	205 ± 51 [184, 226]	191 ± 46 [171, 212]
PLACEBO	218 ± 47 [197, 238]	197 ± 43 [176, 218]	203 ± 46 [183, 224]
ICA CVC (mL/min/mmHg)	**Time, *P* < 0.001**; Condition, *P* = 0.515; **Interaction, *P* = 0.013**		
LOW	2.47 ± 0.69 [2.20, 2.74]	**2.16 ± 0.49*** **[1.89, 2.43]**	2.20 ± 0.48 [1.94, 2.47]
HIGH	2.63 ± 0.68 [2.36, 2.89]	**2.20 ± 0.50*** **[1.93, 2.46]**	**2.13 ± 0.44*** **[1.86, 2.40]**
PLACEBO	2.42 ± 0.65 [2.15, 2.68]	2.22 ± 0.56 [1.96, 2.49]	2.36 ± 0.56 [2.09, 2.62]
VA CVC (mL/min/mmHg)	**Time, *P* < 0.001**; **Condition, *P* = 0.002**; **Interaction, *P* = 0.035**		
LOW	0.740 ± 0.268 [0.632, 0.849]	**0.610 ± 0.211*** **[0.502, 0.719]**	0.683 ± 0.265 [0.574, 0.791]
HIGH	0.708 ± 0.229 [0.599, 0.816]	**0.608 ± 0.212*** **[0.499, 0.716]**	**0.553 ± 0.175*** **[0.444, 0.662]**
PLACEBO	0.729 ± 0.272 [0.621, 0.837]	0.635 ± 0.247 [0.526, 0.743]	0.659 ± 0.216 [0.551, 0.767]
gCBF CVC (mL/min/mmHg)	**Time, *P* < 0.001**; Condition, *P* = 0.330; **Interaction, *P* = 0.003**		
LOW	6.42 ± 1.39 [5.84, 7.00]	**5.54 ± 0.93*** **[4.96, 6.12]**	**5.77 ± 1.02*** **[5.19, 6.35]**
HIGH	6.67 ± 1.51 [6.09, 7.25]	**5.61 ± 1.08*** **[5.03, 6.19]**	**5.35 ± 1.03*** **[4.76, 5.93]**

PLACEBO	6.30 ± 1.49 [5.72, 6.87]	5.76 ± 1.28 [5.17, 6.34]	6.04 ± 1.27 [5.42, 6.59]
MCAv CVC (cm/s/mmHg)	**Time, $P < 0.001$**; **Condition, $P < 0.001$**; Interaction, $P = 0.066$		
LOW	0.899 ± 0.155 [0.830, 0.969]	0.791 ± 0.136 [0.720, 0.861]	0.816 ± 0.096 [0.743, 0.889]
HIGH	0.822 ± 0.170; [0.753, 0.891]	0.736 ± 0.181 [0.666, 0.807]	0.677 ± 0.152 [0.605, 0.750]
PLACEBO	0.865 ± 0.135 [0.795, 0.935]	0.775 ± 0.125 [0.703, 0.847]	0.827 ± 0.085 [0.756, 0.898]

Data are presented as the estimated marginal means ± SD; [95% Confidence Interval]. * = $P < 0.05$ compared to baseline within condition.

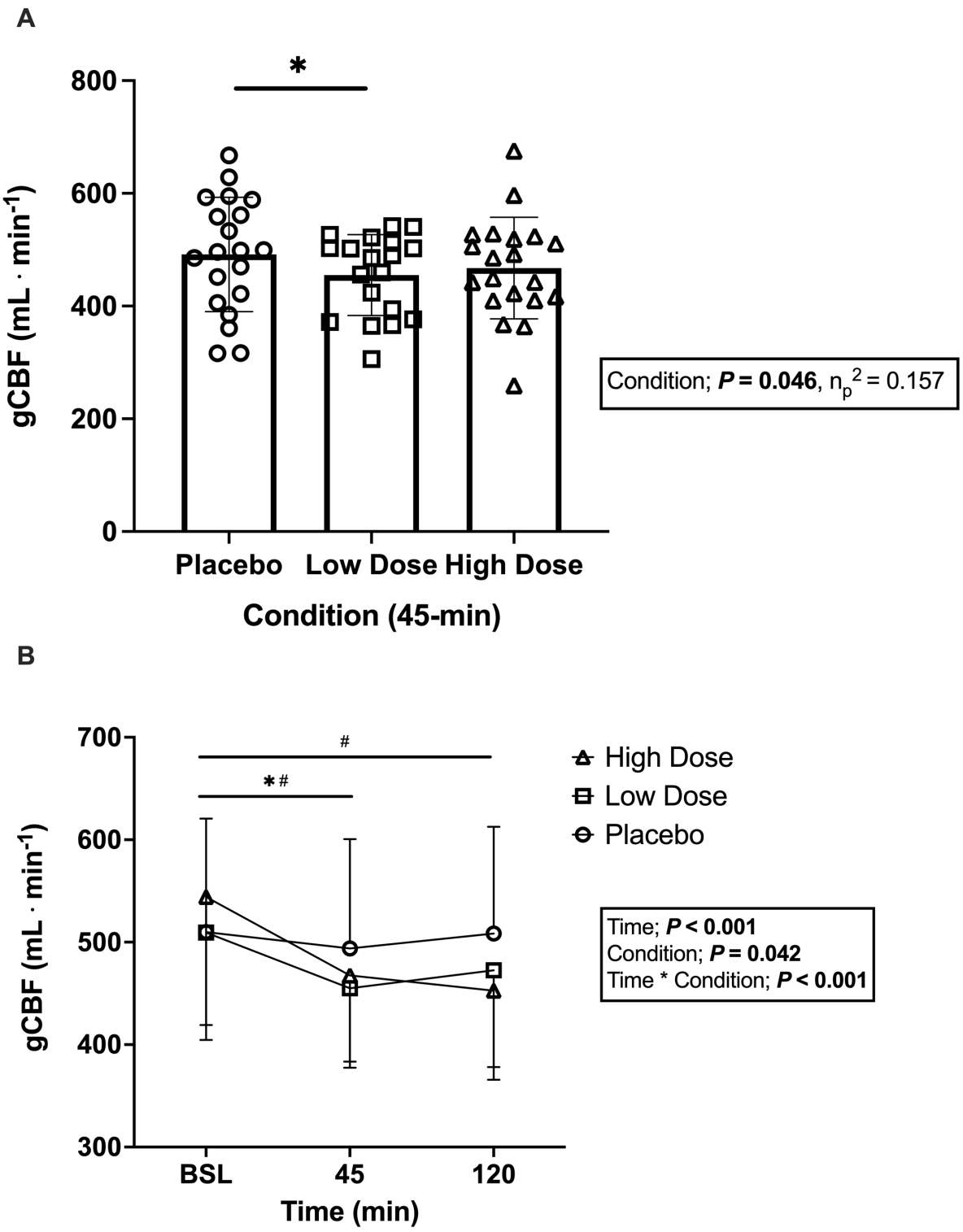

Figure 4. Group global CBF (gCBF) responses. Global CBF at 45-min post-ingestion (**A**) and over the 120-min intervention (**B**) for the high-dose KME (open triangles), low-dose KME (open squares), and placebo (open circles) conditions. Data are presented as mean ± SD. Individual data are represented as open symbols in **B**. * = $P < 0.05$ compared to baseline measures (low-dose KME); # = $P < 0.05$ compared to baseline measures (high-dose KME).

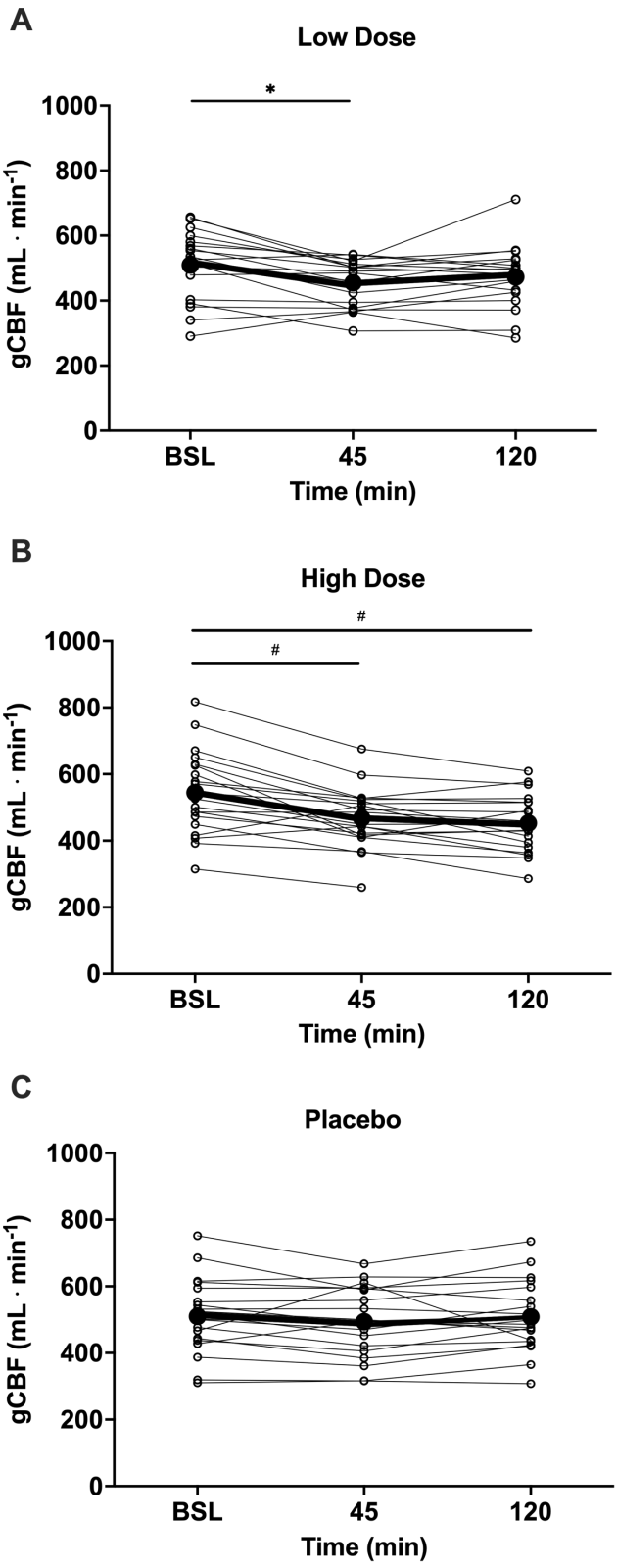

Figure 5. Individual global CBF (gCBF) responses in the low-dose KME (**A**), high-dose KME (**B**), and placebo (**C**) conditions. Group means are enlarged in black. * = $P < 0.05$ compared to baseline measures (low-dose KME); # = $P < 0.05$ compared to baseline measures (high-dose KME).

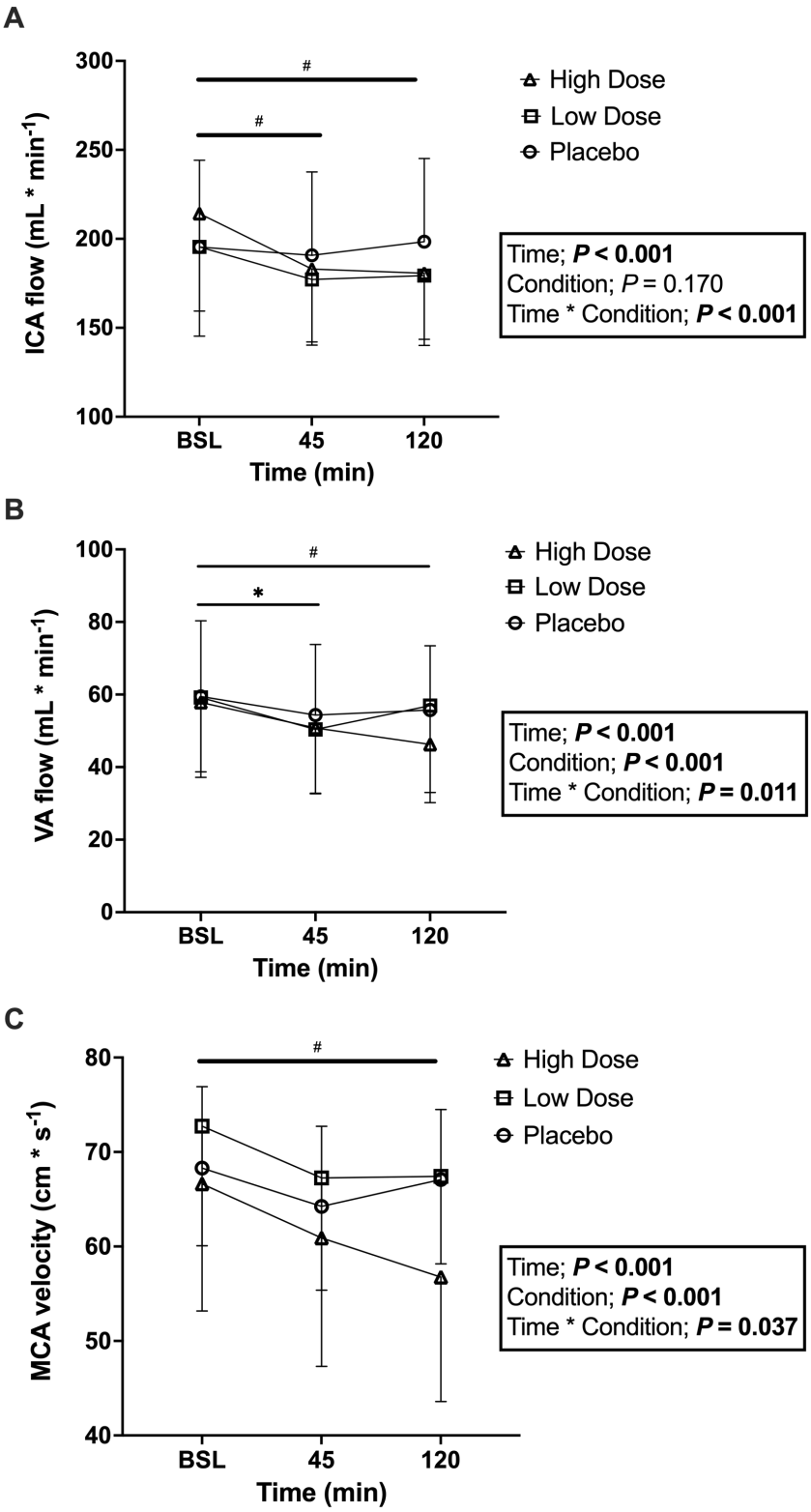

Figure 6. Regional CBF responses. ICA flow (**A**), VA flow (**B**), and MCA velocity (**C**) for the high-dose KME (open triangles), low-dose KME (open squares), and placebo (open circles) conditions. Data are presented as mean ± SD. * = $P < 0.05$ compared to baseline measures (low-dose KME); # = $P < 0.05$ compared to baseline measures (high-dose KME).

2.3.4 Haemodynamic and Respiratory Responses

Haemodynamic and respiratory measures are displayed in Table 3. Relative to baseline, KME ingestion significantly elevated HR by 9.8% and 19.2% at 45-min post-ingestion in the low ($P = 0.002$) and high-dose conditions ($P < 0.001$), respectively. At 120-min post-ingestion, HR increased by 6.1% relative to baseline in the low-dose condition ($P = 0.480$). In the high-dose condition, HR remained significantly elevated relative to baseline at 120-min post-ingestion (Δ19.5%; $P < 0.001$). HR did not change at either 45-min or 120-min in the placebo condition. No changes in MAP were observed with KME ingestion (time * condition interaction, $P = 0.701$). There was a main effect of condition for MAP ($P = 0.031$), and post-hoc analyses revealed a significant difference between the high-dose and placebo conditions, likely driven by an increase in MAP in the placebo condition.

KME ingestion significantly decreased $P_{ET}CO_2$ (time * condition interaction, $P < 0.001$). $P_{ET}CO_2$ decreased by 5.7% (Δ-2.16 ± 1.65 mmHg) at 45-min post-ingestion ($P < 0.001$) and by 4.42% (Δ-1.74 ± 1.44 mmHg) at 120-min post-ingestion ($P = 0.003$), relative to baseline in the low-dose condition. In the high-dose condition, $P_{ET}CO_2$ decreased by 8.8% (Δ-3.45 ± 1.75 mmHg) at 45-min post-ingestion ($P < 0.001$) and 10.0% (Δ-3.94 ± 1.85 mmHg) at 120-min post-ingestion ($P < 0.001$), relative to baseline. Further, the percent change in $P_{ET}CO_2$ was significantly positively correlated with the corresponding percent change in gCBF ($P < 0.001$, $R^2 = 0.219$) (**Figure 7C**). $P_{ET}CO_2$ did not change at either 45-min post-ingestion or 120-min post-ingestion in the placebo condition.

Table 3. Hemodynamic and respiratory measures.

	Baseline (BSL)	45-min	120-min
$P_{ET}CO_2$ (mmHg)	**Time, $P < 0.001$; Condition, $P < 0.001$; Interaction, $P < 0.001$**		
LOW	38.5 ± 2.6 [37.1, 39.9]	**36.3 ± 2.7* [34.9, 37.8]**	**36.8 ± 2.9* [35.3, 38.2]**
HIGH	38.8 ± 2.6 [37.4, 40.2]	**35.4 ± 3.0* [34.0, 36.8]**	**34.9 ± 2.7* [33.5, 36.3]**
PLACEBO	38.4 ± 3.7 [37.0, 39.8]	38.3 ± 3.3 [36.9, 39.7]	38.6 ± 3.8 [37.2, 40.0]
HR (bpm)	**Time, $P < 0.001$; Condition, $P < 0.001$; Interaction, $P < 0.001$**		
LOW	61.0 ± 6.2 [57.4, 64.7]	**67.0 ± 7.7* [63.3, 70.6]**	64.7 ± 6.9 [61.0, 68.4]
HIGH	59.9 ± 8.5 [56.3, 63.6]	**71.4 ± 11.9* [67.8, 75.1]**	**71.6 ± 8.5* [68.0, 75.3]**
PLACEBO	60.8 ± 7.5 [57.2, 64.5]	58.8 ± 7.4 [55.1, 62.4]	59.1 ± 6.0 [55.5, 62.8]
MAP (mmHg)	**Time, $P < 0.001$; Condition, $P = 0.031$;** Interaction, $P = 0.701$		
LOW	81.2 ± 6.6 [78.1, 84.2]	84.2 ± 6.8 [81.1, 87.3]	84.5 ± 6.7 [81.4, 87.5]
HIGH	81.9 ± 5.9 [78.8, 84.9]	83.7 ± 5.5 [80.6, 86.7]	83.5 ± 6.5 [80.4, 86.5]
PLACEBO	82.4 ± 8.1 [79.4, 85.5]	85.8 ± 7.2 [82.7, 88.8]	85.1 ± 6.6 [82.1, 88.2]

Data are presented as the estimated marginal means ± SD; [95% Confidence Interval]. ***** = $P < 0.05$ compared to baseline within condition.

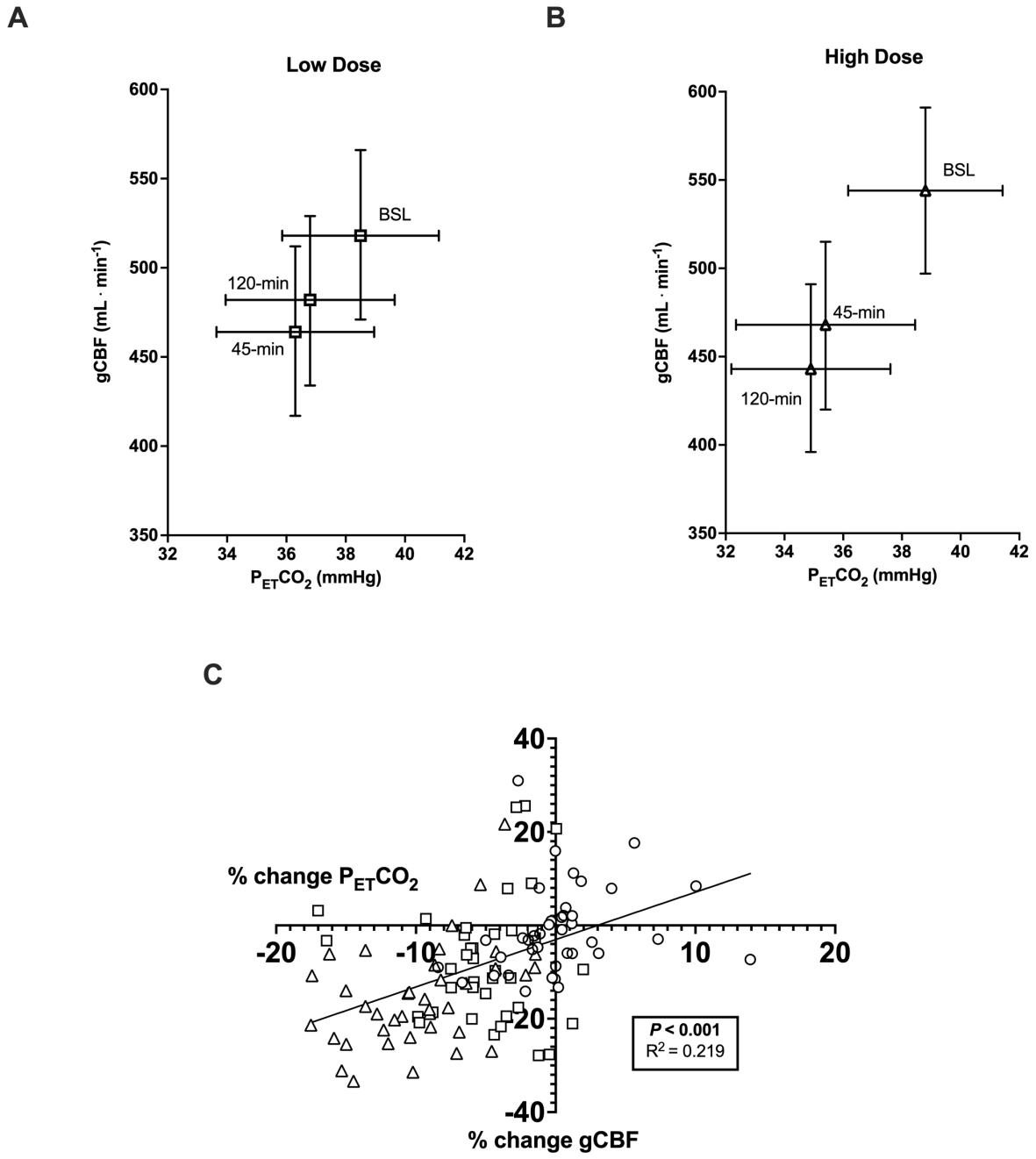

Figure 7. Global CBF (gCBF) versus end-tidal CO_2 ($P_{ET}CO_2$) in the low-dose KME (**A**) and high-dose KME (**B**) conditions. Data are presented as estimated marginal means for gCBF and $P_{ET}CO_2$ for baseline (BSL), 45-min post-ingestion (45-min), and 120-min post-ingestion (120-min) ± SD ($P_{ET}CO_2$ – horizontal error bars) and ± 95% confidence interval (gCBF – vertical error bars). **C)** Percent change in $P_{ET}CO_2$ versus percent change in gCBF in the low-dose (open squares), high-dose (open triangles), and placebo (open circles) conditions.

2.3.5 Hippocampal-Dependent Function

Hippocampal-dependent function, represented by the lure discrimination index (LDI) and recognition memory score (REC) from the MST, did not change after acute KME ingestion (time * condition interaction, $P = 0.915$) (**Figure 8**). Intrinsic motivation also did not differ between conditions (time * condition interaction, $P = 0.795$).

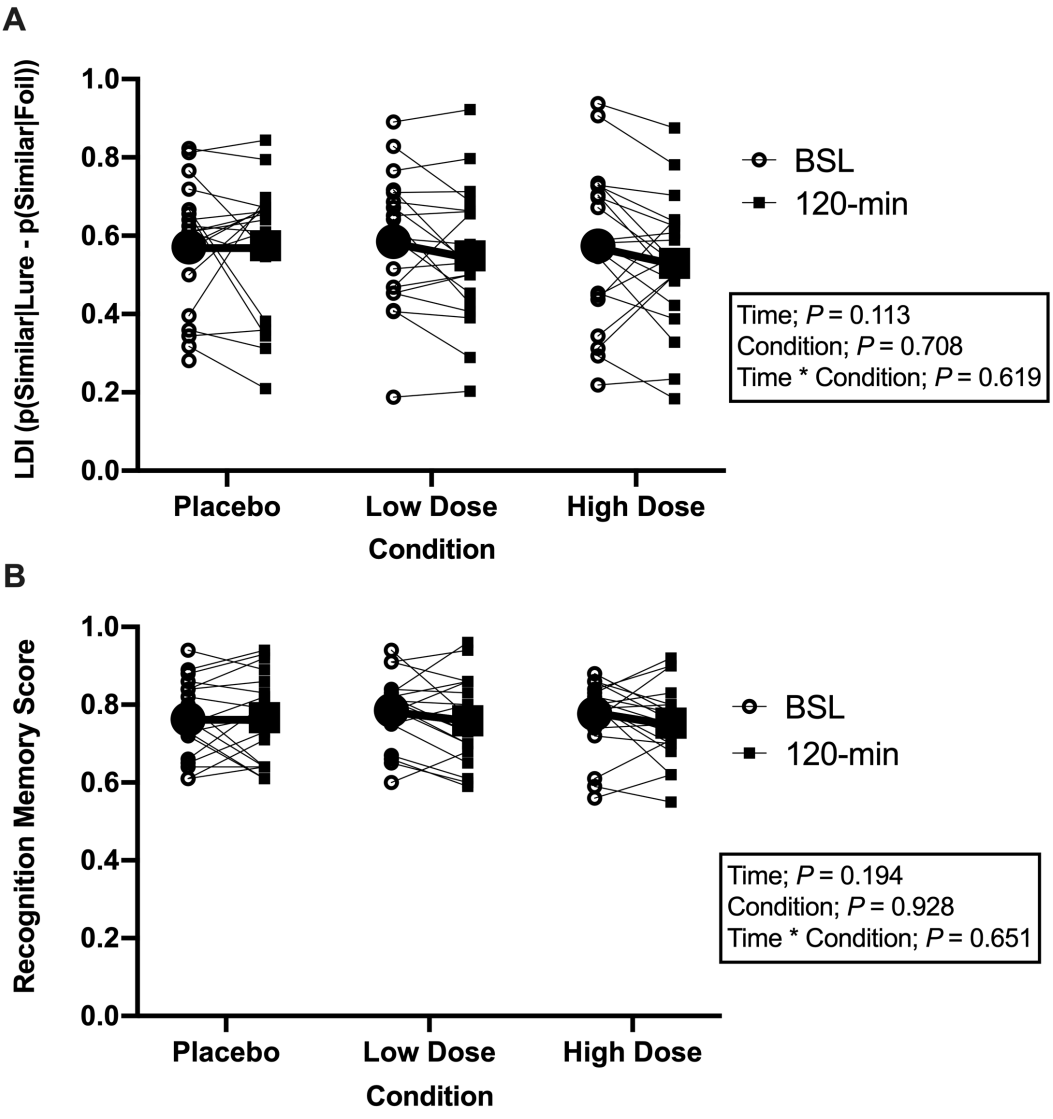

Figure 8. Hippocampal-dependent memory performance. Individual responses in lure discrimination index (LDI) (**A**) and recognition memory score (REC) (**B**) from the MST in the low-dose KME, high-dose KME, and placebo conditions. Responses are shown as the LDI (**A**) or REC (**B**) at 120-min post-ingestion relative to baseline measures within conditions. Group means are enlarged in black.

2.3.6 Exploratory Correlations

We explored relationships between β-OHB parameters and individual participant characteristics. $\dot{V}O_{2peak}$ was significantly correlated with changes in plasma β-OHB at 120-min post-ingestion in the low-dose condition ($P = 0.002$; $R^2 = 0.453$). Participants with higher $\dot{V}O_{2peak}$ tended to have lower blood β-OHB concentrations after low-dose KME ingestion. $\dot{V}O_{2peak}$ was also significantly associated with β-OHB AUC in the low-dose condition ($P = 0.011$; $R^2 = 0.323$), where higher $\dot{V}O_{2peak}$ was correlated with lower β-OHB AUC over 120 min (**Figure 9A**). However, this relationship was not present in the high-dose condition ($P = 0.452$) (**Figure 9B**).

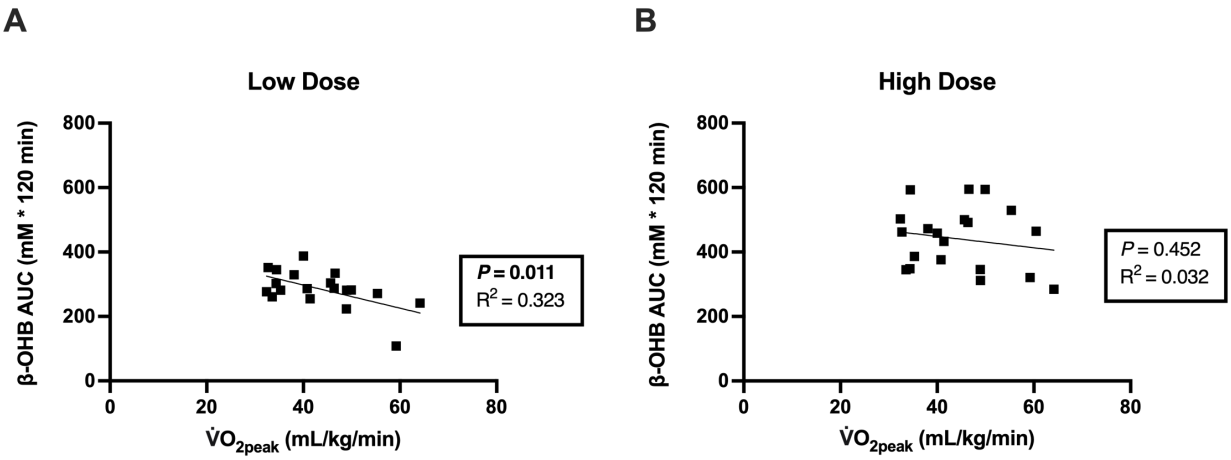

Figure 9. Exploratory correlations. β-OHB area-under-the-curve (AUC) versus peak rate of oxygen consumption ($\dot{V}O_{2peak}$) in the low-dose KME condition (**A**) and the high-dose KME condition (**B**). Individual data are represented by black squares.

2.4 Discussion

The purpose of this trial was to characterize the effect of different doses of an oral KME on resting CBF and cognition over a 2-hour period in young adults. We hypothesized that low-dose KME ingestion would improve CBF and cognitive performance, whereas acute high-dose KME ingestion would reduce CBF and cognitive performance. The main findings were: 1) low-dose and high-dose KME lower CBF similarly at 45-min post-ingestion; 2) CBF returns closer to baseline levels with low-dose KME, whereas reductions are sustained with high-dose KME at 120-min post-ingestion; 3) $P_{ET}CO_2$ was reduced in a dose-dependent manner, which mirrored reductions in CBF; and 4) both low-dose and high-dose KME ingestion do not impact performance on a task of hippocampal-dependent memory. Collectively, these findings suggest that acute KME ingestion significantly alters CBF regulation, which may be due to reductions in P_aCO_2. However, reductions in CBF following KME ingestion did not impact cognitive performance.

Previous trials have shown that acute infusion of sodium β-OHB elevates CBF by 30 (peak plasma β-OHB: 5.5 mM) to 39% (peak plasma β-OHB: 2.19 mM) (Hasselbalch *et al.*, 1996; Svart *et al.*, 2018). Contrary to these findings, we observed that ingestion of either a high- or low-dose KME significantly reduces resting gCBF within 45 min post-ingestion, which closely corresponded with peak plasma β-OHB. At 45-min post-ingestion in the low-dose condition, gCBF was 10.4% lower compared to baseline, corresponding with plasma β-OHB of 2.97 mM. Interestingly, gCBF displayed a return closer to baseline levels at 120-min, which was paralleled by a reduction in plasma β-OHB to 2.37 mM. In the high-dose condition, gCBF was reduced by 14.0% at 45-min, with plasma β-OHB at 4.07 mM. At 120-min post-ingestion, further reductions in gCBF (Δ18.0%) and increases in plasma β-OHB concentrations occurred (peak: 4.72 mM), relative to baseline. These changes persisted when accounting for MAP, as cerebrovascular conductance was significantly reduced in both the low- and high-dose KME conditions. We

attribute these observations of reduced CBF to the dose-dependent lowering of P_aCO_2 (represented as $P_{ET}CO_2$) following KME ingestion. $P_{ET}CO_2$ significantly decreased at the same timepoints as gCBF after ingestion of low- and high-dose KME, and $P_{ET}CO_2$ at 120-min post-ingestion was significantly different between the low and high-dose KME conditions. Changes in P_aCO_2 have a strong influence on cerebral vessel tone and resistance at rest (Smith & Ainslie, 2017). In support of this, we observed that there was a moderate positive correlation between percent changes in $P_{ET}CO_2$ and percent changes in gCBF following KME ingestion. Collectively, these results suggest that increases in cerebrovascular resistance and/or alterations in vessel tone are contributing to the observed reductions in blood flow in both the low- and high-dose KME conditions.

β-OHB delivered via KME supplements acts as a weak organic acid in the blood, leading to declines in pH at rest (Clarke *et al.*, 2012; Stubbs *et al.*, 2017; Dearlove *et al.*, 2019, 2021; McCarthy *et al.*, 2023). A recent trial demonstrates that KME ingestion (0.33 g/kg body mass) elevates ventilation at rest, as well as during an incremental cycling test to exhaustion (Dearlove *et al.*, 2019), likely to compensate for the blood pH disturbance imposed by KME. Similarly, consumption of a 0.6 g/kg body mass KME prior to exercise also elevates ventilation during sub-maximal cycling compared to a placebo drink (McCarthy *et al.*, 2021*b*). Initially, the blood bicarbonate buffer system will increase CO_2 production when blood pH is reduced. This is sensed by peripheral chemoreceptors which stimulate the medulla and pons in the brainstem to increase ventilation (Ikeda *et al.*, 2017; Gallo de Moraes & Surani, 2019). Ultimately, hyperventilation reduces $P_{ET}CO_2$ (Dearlove *et al.*, 2019; McCarthy *et al.*, 2021*b*). As CO_2 can readily cross the blood-brain barrier, reductions in P_aCO_2 decrease the intracellular $[H^+]$ within vascular smooth muscle cells (Hoiland *et al.*, 2019; Duffin *et al.*, 2021; Caldwell *et al.*, 2021). This initiates a cascade of signalling events which ultimately increases intracellular $[Ca^{2+}]$, causing cerebral

vasoconstriction and reduced CBF (Duffin *et al.*, 2021). Although arterial pH was not measured in the current study, the modulation of arterial pH with maintained P_aCO_2 via end-tidal clamping produces no change in CBF in humans, which suggests that CO_2 levels in the blood ultimately drive corresponding changes in CBF (Lambertsen *et al.*, 1961; Caldwell *et al.*, 2021). In summary, this trial provides strong evidence for the potential involvement of arterial CO_2 in mediating CBF responses to KME ingestion.

KME ingestion also impacted factors that contribute to elevated CBF such as HR and MAP. Indeed, we observed that peak HR relative to baseline was 6 bpm higher in the low-dose condition and 11 bpm higher in the high-dose condition. This is consistent with previous studies, as a single KME dose (0.714 g/kg) increases HR by 9 bpm in young adults (Selvaraj *et al.*, 2022). Another study demonstrated an increase in CO of 2.0 L/min with acute KME ingestion (0.483 g/kg), likely due to β-OHB exerting signaling effects on cardiac chronotropy via pacemaker cells within the heart (Oneglia *et al.*, 2023). We also observed no effect of KME ingestion on MAP in this trial, which is supported by a recent meta-analysis combining both KS and KME trials which demonstrated no effect of exogenous ketone supplementation on either SBP or DBP (Marcotte-Chénard *et al.*, 2024). However, β-OHB may cause systemic vasodilation and decrease total peripheral resistance (Selvaraj *et al.*, 2022; Homilius *et al.*, 2023), which may explain our finding of increased HR, and presumably CO, without any change in MAP after KME ingestion. CBF regulation is sensitive to changes in MAP, and somewhat sensitive to CO, in the resting state (Willie *et al.*, 2014). In general, increases in CO lead to increases in CBF (Ogoh *et al.*, 2005); however, we likely did not observe these effects due to the lowering of P_aCO_2. Nonetheless, it is possible that β-OHB-induced elevations in HR may be mitigating the observed reductions in gCBF in this trial. The current evidence from this trial suggests that CBF responses from KME ingestion

are likely driven by compensatory physiological responses to acute stressors, which may mask the proposed beneficial effects of β-OHB signalling on CBF in humans.

We assessed hippocampal dependent memory because evidence from rodent and *in vitro* models have demonstrated that β-OHB exposure improves hippocampal neuron function, particularly through increasing BDNF expression (Sleiman *et al.*, 2016; Marosi *et al.*, 2016; Hu *et al.*, 2018, 2020). Contrary to our hypotheses, low- and high-dose KME ingestion do not impact cognitive performance despite reductions in CBF. This finding is in contrast to past studies which have shown improvements to cognitive function, relative to a placebo condition, using a wide variety of KME doses; 0.75 g/kg (Evans & Egan, 2018), 25 g initial + 12.5 g every 30 min during an ultra-marathon (Poffé *et al.*, 2023), 25 g (Quinones & Lemon, 2022), and 0.375 g/kg + 6% carbohydrate solution (Waldman *et al.*, 2023*a*). Our findings are also disparate from a recent trial displaying that a single KME dose (0.395 g/kg) improves neuronal network function at rest compared to a placebo (Mujica-Parodi *et al.*, 2020). However, one past trial has reported no change in cognitive function with acute KME ingestion (0.188 g/kg) prior to exercise (Waldman *et al.*, 2023*b*). Differences in the cognitive domain tested and experimental protocol (i.e., prolonged exercise or mental fatiguing tasks) may contribute to these disparate findings. Cognitive improvements in past KME studies were observed in domains such as attention and executive function, which were not specifically assessed in this study. This trial employed a single cognitive test which primarily assesses hippocampal-dependent functions, such as pattern separation and high-interference memory (Stark *et al.*, 2019). We also plan to analyze plasma and serum BDNF concentrations at a later date, in an effort to uncover possible mechanisms behind these findings.

This present study is also not the first to demonstrate no changes to cognitive function, despite reductions in CBF. Our findings are consistent with a recent study showing that

pharmacologically induced reductions in CBF do not alter cognitive performance on a variety of tasks in healthy young and older adults (Shoemaker *et al.*, 2020). However, this relationship is likely not persistent beyond acute exposure, as chronic reductions in CBF are heavily implicated in the development of neurodegenerative pathologies like Alzheimer's disease (Korte *et al.*, 2020).

We also conducted exploratory analyses to examine whether cardiorespiratory fitness (i.e., $\dot{V}O_{2peak}$) and/or biological sex were correlated to changes in outcomes significantly affected by KME ingestion. Peak β-OHB concentrations at 45- and 120-min post-ingestion, as well as β-OHB AUC, after low-dose KME ingestion were negatively correlated with $\dot{V}O_{2peak}$. It is speculated that higher cardiorespiratory fitness could be contributing to lower β-OHB exposure via greater metabolic flexibility and/or removal capacity, thus contributing to increased clearance of β-OHB from the blood. However, this may not be the case with higher β-OHB exposure, as this relationship was not present in the high-dose KME condition. The exact physiological mechanisms responsible for this observation remain unknown.

2.4.1 Strengths, Limitations, and Future Directions

This double-blinded, crossover, counterbalanced, placebo-controlled trial provides valuable insight on CBF regulation and cognitive function after acute KME ingestion. The current study addresses crucial knowledge gaps regarding the effects of KME supplements on CBF and cognitive function. Most importantly, this trial provides a foundational characterization of the relationship between KME dose and CBF regulation at rest with KME, which had not been done previously. However, this study does contain a few limitations worth addressing. This study employed resting CBF as the only measure of cerebrovascular physiology, which does not fully represent functional aspects of brain health. Future studies are needed to uncover how acute KME ingestion impacts cerebrovascular function, such as with a cerebrovascular reactivity to CO_2 or

visual stimulation (i.e., neurovascular coupling) tasks. Further, it is unclear whether CBF is only lowered beyond the doses chosen in the study due to the absence of an additional lower KME dose (i.e., 0.15 g/kg body mass). The investigation of lower KME doses should be conducted to clarify this characterization of CBF responses to acute KME ingestion. Finally, the observed relationship between $P_{ET}CO_2$ and CBF in this experimental design does not definitively establish the causal link between changes in P_aCO_2 and changes in CBF. Protocols involving end-tidal clamping of CO_2 should be used to corroborate evidence from this trial to quantify the involvement of arterial CO_2 in the mediation of CBF responses to acute KME ingestion. Future studies should also focus on developing strategies to prevent perturbations to acid-base homeostasis with oral KME ingestion, to determine if the same oral KME doses from this study can exert beneficial effects on CBF and cognition. This could be further supplemented with longer supplementation protocols to determine the chronic impact of oral KME on brain health and function. Despite these limitations, this rigorous and well-controlled study design provides confidence in the observed effects from this study.

2.4.2 Conclusion

In conclusion, acute KME ingestion lowered resting CBF in a manner where responses differed between the low- and high-dose conditions, in young adults. These reductions were likely due to corresponding reductions in P_aCO_2 that were dependent on β-OHB dose. Despite reductions in CBF, cognitive performance was not altered with acute KME ingestion. The recent advent of KME supplements provides an exciting potential tool for protecting and improving brain health. The findings from this study are crucial to first understand how different doses of KME impact resting CBF and cognition before we can recommend the therapeutic use of KME supplements.

This research provides novel and significant insight for informing the foundation of therapeutic effects of exogenous β-OHB for protecting and improving brain health in humans.

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Appendices

Appendix A – Menstrual Cycle Questionnaire

McMaster University

Date: _____ PARTICIPANT ID: _____

Menstrual Cycle Questionnaire

Acute Ketone Dose & Cerebral Blood Flow Study

Please read through the following questions and answer as best as possible.

1. When was your last period? _____

2. How long do your periods last? _____

3. If you are not currently having a menstrual cycle, when was the last time you menstruated?

_____

4. Are you taking oral contraceptive pills (birth control pills), hormonal contraception, or other hormones?

_____

a. If so, how long have you been taking them? _____

b. What is the brand name of the contraceptive? _____

Appendix B – Dietary Recall Sheet

McMaster University | SCIENCE

24-Hour Dietary Recall

Participant ID: _____ Date: _____ RA: _____

Day of the week: Monday Tuesday Wednesday Thursday Friday Saturday Sunday

Does this day represent your typical eating habits (excluding provided meals)? Yes No

Please be as specific and honest as possible for review with the Research Assistant. Thank you.

Food Item	Quantity	Notes
Example: Breakfast		
Large brown eggs	2	Scrambled eggs
Folgers coffee	1 cup	With crème and sugar
Whole grain bread	2 slices	Dempsters
Butter	1 tbsp	

1

Version 1, June 26th, 2023

Appendix C – Consensus Sleep Diary

kCBF Dose Study Nightly Sleep Diary Participant ID:

Today's date			
1. What time did you get into bed?			
2. What time did you try to go to sleep?			
3. How long did it take you to fall asleep?			
4. How many times did you wake up, not counting your final awakening?			
5. In total, how long did these awakenings last?			
6. What time was your final awakening?			
7. In total, how long was your sleep?			
8. How would you rate the quality of your sleep?	o Very poor o Poor o Fair o Good o Very Good	o Very poor o Poor o Fair o Good o Very Good	o Very poor o Poor o Fair o Good o Very Good
9. Comments (if applicable) Ex. "I have a cold"			

Version 1: August 24, 2023 (HIREB #15454)

Appendix D - Intrinsic Motivation Subscale of the Intrinsic Motivation Inventory

Version 1: November 7th, 2023

McMaster University

COGNITIVE TASK EVALUATION QUESTIONNAIRE

Participant ID: _____ Visit Number _____ Date:_____

For each of the following statements, please indicate how true it is for you, using the following scale:

1 2 3 4 5 6 7

not at all true somewhat true very true

1. I enjoyed doing this activity very much. _____
2. This activity was fun to do. _____
3. I thought this was a boring activity. _____
4. This activity did not hold my attention at all. _____
5. I would describe this activity as very interesting. _____
6. I thought this activity was quite enjoyable. _____
7. While I was doing this activity, I was thinking about how much I enjoyed it. _____

Appendix E - Gastrointestinal Symptoms Questionnaire

Date: **Participant ID:** **Visit # (1,2,3):**

kCBF Dose Study Gastrointestinal Symptoms Scale

DAY 1

Instructions: Please mark a vertical line through the horizontal line to indicate your response to each question.

No nausea _____ Worst imaginable nausea

No urge to vomit _____ Worst imaginable urge to vomit

No bloating _____ Worst imaginable bloating

No belching _____ Worst imaginable belching

No cramps _____ Worst imaginable cramps

Which dose do you think you received? **PLACEBO** **KETONE-LOW** **KETONE-HIGH**

How confident are you in that response? Please mark a vertical line through the horizontal line.

Not confident at all _____ I am 100% confident

Appendix F – HiREB Approval Letter

HiREB
Hamilton Integrated Research Ethics Board

Date: Mar-09-2023

Local Principal Investigator: Dr. Jeremy Walsh

Participating HiREB Centre(s): McMaster University

Project ID: 15454

Project Title: The effect of acute ketone monoester ingestion on cerebral blood flow and cognition

Review Type: Delegated

Date of Final Approval: Mar-08-2023

Ethics Expiry Date: Mar-08-2024

The Hamilton Integrated Research Ethics Board (HiREB) Panel B has reviewed and approved the above-mentioned study.

The following documents have been approved:

Document Name	Document Date	Document Version
Ketone Dose_deidentifier key	Dec-05-2022	1
Ketone Dose_DCS_V1	Dec-05-2022	1
kCBF_Menstrual Questionnaire_V1	Dec-09-2022	1
GetActiveQuestionnaire_CSEP	Jan-09-2023	1
GetActiveQuestionnaire_CSEP_ReferenceDocument	Jan-09-2023	1
kCBF_Protocol_V2_clean	Jan-21-2023	2
kCBF_Recruitment Poster_V2_clean	Jan-21-2023	2
kCBF_Email Recruitment Script_V2_clean	Jan-21-2023	2
kCBF_Consent_Form_V2_clean	Jan-21-2023	2

The following documents have been acknowledged:

Document Name	Document Date	Document Version
Walsh_GCP certificate	Dec-09-2022	1

While HiREB has reviewed and approved this application, the research must be conducted in accordance with applicable regulations and institutional and/or public health requirements.

We are pleased to issue final approval for the above-named study until the expiry date noted above. Continuation beyond that date will require further review and renewal of HiREB approval. Any changes or revisions to the original submission must be submitted on a HiREB amendment form for review and approval by the Hamilton Integrated Research Ethics Board.

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

The Hamilton Integrated Research Ethics Board (HiREB) provides ethical review and ongoing ethical oversight on behalf of Hamilton Health Sciences, St. Joseph's Healthcare Hamilton, Research St. Joseph's Hamilton, the Faculty of Health Sciences at McMaster University and Niagara Health. HiREB operates in compliance with and is constituted in accordance with the requirements of: The Tri-Council Policy Statement on Ethical Conduct of Research Involving Humans (TCPS 2); The International Conference on Harmonisation of Good Clinical Practices Guideline (ICH GCP); Part C Division 5 of the Food and Drug Regulations, Part 4 of the Natural Health Products Regulations; Part 3 of the Medical Devices Regulations and the provisions of the Ontario Personal Health Information Protection Act 2004 and its applicable Regulations. For studies conducted at St. Joseph's Healthcare Hamilton, HiREB complies with the Health Ethics Guide of the Catholic Alliance of Canada. HiREB is qualified through the Clinical Trials Ontario (CTO) REB Qualification Program and is registered with the U.S. Department of Health and Human Services (DHHS) Office for Human Research Protection (OHRP).

Sincerely,

Frederick A Spencer

Dr. Frederick A. Spencer, MD
Chair, Hamilton Integrated Research Ethics Board

Hamilton Integrated Research Ethics Board (HiREB)
237 Barton Street, Suite C1-205 Hamilton, ON L8L 2X2
Telephone: 905-521-2100, Ext. 42013

Appendix G – Sample Size Determination