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Exogenous Ketone Bodies as Promising Neuroprotective Agents for Developmental Brain Injury

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Abstract
Ketone bodies are a promising area of neuroprotection research that may be ideally suited to the injured newborn. During normal development, the human infant is in significant ketosis for at least the first week of life. Ketone uptake and metabolism is upregulated in the both the fetus and neonate, with ketone bodies providing at least 10% of cerebral metabolic energy requirements, as well as being the preferred precursors for the synthesis of fatty acids and cholesterol. At the same time, ketone bodies have been shown to have multiple neuroprotective effects, including being anticonvulsant, decreasing oxidative stress and inflammation, and epigenetically upregulating the production of neurotrophic factors. While ketogenic diets and exogenous ketosis are largely being investigated in the setting of adult brain injury, the adaptation of the neonate to ketosis suggests that developmental brain injury may be the area most suited to the use of ketones for neuroprotection. Here, we describe the mechanisms by which ketone bodies exert their neuroprotective effects, and how these may translate to benefits within each of the phases of neonatal asphyxial brain injury.

Introduction

The incidence of hypoxic-ischemic (HI) brain injury following term perinatal asphyxia remains around 1–4/1,000 live births in the Western world [1]. Of these, around 25–50% (0.5–1/1,000) will develop a significantly altered state of cerebral function (encephalopathy), often referred to as HI encephalopathy (HIE). In low- and middle-income countries, rates of HIE are up to 10 times higher than in the Western world and, overall, perinatal asphyxia is thought to account for 23% of neonatal deaths (i.e., 8% of all childhood deaths) worldwide [2]. The etiology and progression of HI brain injury occurs in at least 3 phases over a period of hours to months ([3–5]: for ex-

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tensive reviews on mechanisms of injury and neuroprotection). The initial phase of injury involves the primary failure of energy production due to hypoxia-ischemia, and is characterized by cell swelling, oxidative stress, and mitochondrial dysfunction. This is generally followed by a "latent period" where energy production improves, and during which treatments must be initiated in order to gain maximal benefit. Within 24 h, cellular metabolism deteriorates again due to a secondary energy failure. This is associated with relative cerebral hyperperfusion, seizures, cell death, and inflammation. Once the direct metabolic sequelae of hypoxia-ischemia have resolved, a tertiary injury phase caused by ongoing inflammatory responses is seen. There is also the potential for a quaternary phase of restoration and repair, where environmental inputs may affect long-term development and neuroplasticity.

The current mainstay of treatment for infants with moderate or severe HIE is therapeutic hypothermia (TH), which reduces the risk of a poor outcome (death or severe disability) by around 15% [6, 7]. However, TH may be less efficacious for infants with severe HIE or certain comorbidities [2, 7, 8]. And while therapies such as erythropoietin (Epo) may expand the therapeutic window by promoting neurogenesis and repair [9], interventions that can maintain mitochondrial and cellular function during the latent and secondary phases of injury, either as adjuncts to TH or as standalone therapies in regions where TH may have reduced efficacy [2, 10], are still lacking.

The ketone bodies acetoacetate (AcAc), β-hydroxybutrytate (BHB), and acetone are 3- and 4-carbon end-products of fatty acid metabolism that are naturally produced by the liver under conditions of prolonged dietary carbohydrate restriction and fasting. Following hepatic synthesis, ketone bodies are subsequently released into the blood for transport throughout the body. In adults, basal levels of the most commonly measured ketone, BHB, are low (<0.1 mM), but rise after several days of dietary manipulation, with the target range for "nutritional ketosis" in adults regarded as a BHB level of 0.5–3 mM [11]. Ketone production has historically been associated with starvation and pathological diabetic ketoacidosis; however, endogenous ketone production is also a crucial aspect of normal neonatal brain development and metabolism. Ketone bodies are normally present in significant amounts in the circulation of the healthy newborn infant [12–14], and they are extracted for use both as metabolic substrates and as precursors for fatty acid and cholesterol synthesis in the developing brain [15, 16].

In the setting of a wide range of models of neurological injury, including HI brain injury, ketones have been shown to be mitoprotective, directly and indirectly decreasing cellular oxidative stress [17]. BHB can suppress inflammatory responses, as well as increase the production of neurotrophic factors that may initiate long-term repair [17–19]. Ketogenic diets are also frequently used to increase the seizure threshold in treatment-resistant pediatric epilepsy, with the ketone bodies themselves having pleiotropic antiepileptic actions [18, 20]. As ketone bodies have a wide range of neuroprotective effects, the fact that the neonate is specifically adapted to utilize ketone bodies before and after birth makes these promising candidates for use in neuroprotective therapies for developmental brain injury.

Despite their promise, the applicability of ketones in acute neurological injury has been limited, as it takes several hours to sufficiently elevate endogenous ketone production [21]. Therefore, multiple ways to bypass endogenous ketone production using "exogenous" sources of ketone bodies have been investigated in adults. This includes the intravenous administration of ketone salts [22–24], as well as the more recent development of orally available ketone salts and esters [25]. Consumption of these compounds results in elevated levels of blood ketones within minutes [25], making them important potential candidates for use after brain injury.

Metabolism of Endogenous and Exogenous Ketone Bodies

Endogenous ketone body production is largely driven by the relative availability of acetyl-CoA and the Krebs cycle intermediate oxaloacetate. In the setting of low circulating insulin (and high glucagon), oxaloacetate in the liver is diverted away from oxidative metabolism for use in gluconeogenesis. At the same time, increased lipolysis from peripheral fat tissues delivers fatty acids to the liver for β-oxidation, resulting in the production of more acetyl-CoA. The simultaneous increase of acetyl-CoA availability, along with the reduced availability of oxacloacetate, leads to the conversion of excess acetyl-CoA to AcAc [26]. AcAc may be converted to BHB through the action of D-β-hydroxybutrytate dehydrogenase (BDH), or spontaneously decarboxylated to form acetone [12, 27]. As the liver lacks the enzyme required to initiate the metabolism of AcAc back to acetyl-CoA, AcAc and BHB are primarily exported in the blood for metabolism in the periph-

Table 1. Exogenous ketone compounds, methods of administration, efficacy, and typical dosages used in adult human subjects

Exogenous ketone compound	Method of administration	Model(s)	Typical dosage for adult human	Typical blood BHB increase	Limitations	Reference
β-Hydroxybutyrate monoester	Oral intragastric	Rodent human	141–714 mg/kg	Up to 5 mM	Poor taste Multiple daily doses required	Clarke et al. [43], 2012 Stubbs et al. [25], 2017
Acetoacetate diester	Oral	Rodent human	not known	1 mM	Poor GI tolerability Only 1 human study to date Multiple daily doses required	D'Agostino et al. [102], 2013 Leckey et al. [37], 2017
Ketone salt	Oral intragastric	Rodent human	10–20 g of BHB per serving	1 mM	Poor GI tolerability at high doses Multiple daily doses required Associated mineral load Low levels of ketosis	Evans et al. [40], 2017 Plecko et al. [35], 2002 Stubbs et al. [25], 2017
Ketone salt	Intravenous	Pig human	20 μmol/kg/min	Up to 3.5 mM	Impractical outside of clinical setting Associated mineral load	Féry and Balasse [22], 1988 Mikkelsen et al. [31], 2015 Muller et al. [33], 1984 Thomsen et al. [32], 2018
Medium chain fatty acids	Oral intragastric	Rodent human	15–30 g of MCT per serving	0.5 mM	Poor GI tolerability at high doses Low levels of ketosis Multiple daily doses required	Henderson et al. [30], 2009 Page et al. [29], 2009

eral tissues. The ratio of AcAc and BHB is largely determined by the redox state (the ratio of NAD+ to NADH) of mitochondria in the liver [28], with BHB usually present at higher levels in the blood than AcAc during fasting [12].

Exogenous ketones typically exist as liquids or dissolvable powders that contain ketone bodies or ketone precursors, including ketogenic medium chain fatty acids (MCFAs) [29, 30], ketone mineral salts [22, 31–35], or BHB or AcAc esterified to a precursor such as butanediol (Table 1) [25, 36, 37]. When ingested, all of these compounds are able to significantly increase circulating ketones without having to significantly decrease levels of insulin or glucose [25, 38]. Ketogenic MCFAs are 6–10 carbon fatty acids that are rapidly absorbed into the portal circulation after consumption, and preferentially converted into ketone bodies in the liver [39]. A typical dose of around 30 g of MCFAs can elevate BHB levels by approximately 0.5 mM in adult humans [29, 30]; however, a further increase in dosage is limited by poor gastrointestinal tolerability [30]. Ketone salts are comprised of a BHB or AcAc molecule bound to an inorganic anion, commonly sodium, potassium, or calcium. The limitations of ketone salts include the relatively low circulating BHB levels (approx. 1 mM) that are achieved with current oral formulations [40, 41], issues with oral tolerability [40, 41], and concerns over the long-term effects of a high mineral load. However, infusions of BHB and AcAc salts can provide sustained elevation of both AcAc and BHB, depending on the formulation used [22–24].

Several oral ketone esters have been developed, utilizing different compositions of BHB, AcAc, butanediol, and triglyceride esters, all of which have different physical characteristics and physiological effects [42]. Most of the published human data to date describes a BHB-butanediol monoester, which has demonstrated sufficient safety and tolerability to be considered generally recognized as safe (GRAS) as a food ingredient by the FDA [43]. After oral consumption, this ester is hydrolyzed to form BHB and 1,3-butanediol, the latter of which undergoes conversion to BHB via first-pass metabolism in the liver [44]. Consumption of this ketone ester can elevate BHB up to 5 mM within 30 min [44, 45], but limitations for producing sustained ketosis include poor taste and rapid metabolism that necessitate multiple daily doses to maintain blood BHB levels [25]. Importantly, whilst there are some limitations to the use of exogenous ketones, many of these limitations (such as taste and concerns about repeat dosing) are less likely to be problematic in neonates receiving active treatment for HIE in the intensive care setting.

Ketone bodies derived from both endogenous and exogenous sources are metabolized equivalently within the body. Both AcAc and BHB are taken up into target tissues (i.e., the brain, heart, and skeletal muscle) via monocarboxylate transporters (MCTs). These are a family of proton-linked transporters that drive cellular uptake of monocarboxylic acids such as lactate, pyruvate, and the ketone bodies, via facilitated diffusion [46]. Of the MCTs, MCT1 is thought to provide most of the transport across the blood-brain barrier (BBB), with

brain uptake of ketones largely proportional to the concentration of ketones in the blood [16, 21, 47, 48]. MCT1 has the highest in vitro affinity for pyruvate, and slightly lower but similar affinities are seen for AcAc and lactate. The affinity of MCT1 is lowest for BHB, with the affinity constant (Km) for BHB around twice of that AcAc [46]. Once within the cell, BHB is converted back to AcAc via BDH, also generating a molecule of NADH for use in the electron transport chain. AcAc is converted back to 2 molecules of acetyl-CoA, which can enter the Krebs cycle, be diverted into fatty acid synthesis, or enter the mevalonate pathway for the synthesis of cholesterol [15, 16].

Ketones Are Essential Synthetic Precursors, and Their Utilization Peaks in the Newborn

In contrast to the adult brain, which is exposed to ketones as a result of relatively uncommon physiological stressors such as prolonged fasting [12, 49], the healthy neonatal brain appears to depend on some degree of ketosis for normal development and function; ketones act as both an energy source, and are the preferred precursors for fatty acids and cholesterol which make up the majority of the dry matter of the brain and are largely synthesized locally [15, 16, 50]. A degree of continual ketone production is therefore normal in newborn humans. Blood BHB levels increase to around 0.5–2 mM within 12 h of life in healthy newborn infants and remain elevated for at least 1 week [13]. This generation of ketones occurs in both the fed and the fasted state. If fasted, ketones are developed from the lipolysis of subcutaneous fat [21]. The newborn's ample subcutaneous adipose stores, which are a unique feature of humans compared to other primate species [51], act as an important repository for fatty acids, providing the precursors for ketone production. The evolution of significant subcutaneous adipose tissue in the human infant is even thought to have allowed for the increase in cerebral complexity when compared to our prehominid ancestors [51]. In the fed newborn, the MCFAs in breast milk synthesized de novo from blood glucose within the epithelium of the milk duct will also promote the production of ketone bodies in the liver [52].

Many tissues of the human infant exhibit a high rate of ketone uptake and oxidation, especially the brain. For example, in the perfused premature infant brain, the molar utilization of BHB is around 50% greater than that of glucose [53]. The human brain's capacity to oxidize ketone bodies is also greatest at birth, with ketone bodies providing 10–30% of baseline energy requirements [16, 21]. This is one reason why some authors have suggested that glucose alone is insufficient to support the energetic requirements of the newborn human brain, which utilizes nearly 75% of total energy requirements at birth [48, 51].

Significant preclinical evidence also exists to support the importance of ketone bodies in the developing brain of other model organisms, particularly that of the suckling rat [16, 47, 54]. As an in vivo model, the suckling rat provides a vital insight into the developmental changes in ketone metabolism that are intrinsically challenging to study in humans due to the invasive nature of the measurements required. Though the timing and expression of proteins involved in ketone transport and metabolism cannot be assumed to be equivalent in rodents and humans, there are several parallels between the two species. For example, there appears to be a similar pattern of production and utilization of ketone bodies in both newborn humans and rats. As with human infants, newborn rats enter significant ketosis from the beginning of the suckling period, which continues until weaning, due to MCFAs in the dam's milk [55–57]. Furthermore, arteriovenous differences in ketone body levels across the brain are greatest in newborns, with at least 3- to 4-fold greater rates of ketone body extraction, compared to adults, for a given level of BHB and AcAc seen in both suckling rats and humans [21, 47, 58]. This is likely to be largely explained by the BBB expression of MCT1, the transporter responsible for most of the ketone transport into the brain, which peaks during suckling in the rat, and decreases after weaning [15]. A number of enzymes involved in the oxidative and nonoxidative metabolism of ketone bodies are increased 2- to 3-fold in the brain of the suckling rat compared to the adult [47, 54]. Both in vivo and in vitro studies have similarly demonstrated that ^{14}C-labeled AcAc and BHB are readily incorporated into sterols, fatty acids, and phospholipids of both suckling rat and fetal human brain tissue [15, 59, 60].

Ketone Bodies Have Pleiotropic Neuroprotective Effects

Due to their pleiotropic physiological effects (see below), as well as the fact that the neonatal brain is adapted to uptake and utilize ketone bodies, ensuring adequate ketosis in infants after HIE has the potential to mitigate many of the pathological processes associated with HI brain injury. However, as the process of endogenous ke-

togenesis takes many hours to develop, achieving elevated blood ketone levels within the 6-h latent phase of HI brain injury is not feasible through endogenous ketosis. This leads to the hypothesis that exogenous ketones could be delivered soon after an insult to rapidly induce ketosis to preserve cellular function during the latent and secondary phases of injury. Due to their potential neurotrophic action and function as synthetic precursors, exogenous ketones may also contribute to longer-term repair mechanisms beyond the traditional therapeutic window.

Ketones Directly Regulate Inflammation and Neurotrophic Factors

An increasing body of literature shows that, as well as being energy substrates with a variety of metabolic effects, ketone bodies also have active roles as signaling metabolites ([61]: a comprehensive overview). BHB actively inhibits activation of the innate immune sensor NOD-like receptor protein 3 (NLRP3) inflammasome [19], which controls the release of a number of proinflammatory cytokines and is upregulated in a number of inflammation-associated chronic diseases. NLRP3 activation has been demonstrated in the brain of postnatal day 9 (P9) mice exposed to unilateral hypoxia-ischemia; however, NLRP3 knockout mice do not display decreased brain injury when compared to wild-type (WT) mice [62], and whether neuroprotection can be conferred by directly inhibiting NLRP3 activation after HI injury has yet to be determined.

BHB can also directly act as a specific histone deacetylase inhibitor (HDACi) [63], altering gene expression at the epigenetic level. One pertinent downstream effect of HDAC inhibition by BHB is the upregulation of brain-derived neurotrophic factor (BDNF) [64]. Multiple neuroprotective effects of BDNF after HI brain injury have been described, including the promotion of neuronal regeneration, and decreased circulating BDNF has been shown to be associated with a poor outcome in infants with HIE [65]. Increasing or augmenting the circulating levels of ketone bodies, particularly BHB, after a developmental brain injury may therefore suppress chronic inflammatory responses and upregulate neurotrophic processes.

Ketone Bodies Increase the Seizure Threshold

Since the 1920s, various incarnations of the ketogenic diet (KD) have been used for treatment-resistant pediatric epilepsy. Traditionally, this involves manipulation of the diet to minimize carbohydrate intake to 10–20 g per day, moderating dietary protein, and increasing fat (or MCFA) intake such that there is a 4:1 ratio of total energy from fat to carbohydrate and protein [66]. This results in a state of physiological nutritional ketosis. A large body of research has been compiled to determine the mechanisms by which ketone bodies reduce seizure activity, with BHB, AcAc, and acetone all displaying antiepileptic effects in various seizure models ([20]: an extensive review of the topic).

One of the potential mechanisms underlying the antiseizure effects of ketones is altering synaptic neurotransmission. Though direct effects on γ-aminobutyric acid (GABA) or glutamate receptors has not been demonstrated, a number of potential upstream effects on neurotransmitter balance have been described ([67]: review). Metabolism of ketones decreases aspartate, an inhibitor of the production of GABA from glutamate. AcAc has also been shown to inhibit the uptake of glutamate into presynaptic vesicles as well as decrease in vivo glutamate release in an excitotoxicity model [68]. Ketone bodies may therefore beneficially alter the balance of inhibitory/excitatory neurotransmitter release, which is crucial to long-term neuronal survival after HI brain injury.

Acetone directly increases the seizure threshold in multiple in vivo models of induced seizures, including the maximal electroshock model of tonic-clonic seizures, the amygdala-kindling model of complex partial seizures with bilateral spread, and the AY-9944 model of chronic atypical absence seizures [20]. Similarly, AcAc (either in its native form or administered as an AcAc-butanediol diester), reduces seizures induced by intrahippocampal 4-aminopyridine (4-AP) infusion in rats, decreases hippocampal seizure activity in an intrahippocampal kainate model of chronic epilepsy, and increases the latency to hyperbaric oxygen-induced seizures [20].

By comparison, BHB has largely failed to replicate the antiepileptic effects of acetone and AcAc in certain adult seizure models. However, BHB does appear to have certain antiseizure effects in the developing brain, as it has recently been shown to decrease glutamate-induced spasms in P12–P15 rats [69]. Blocking ketogenesis in P5–P9 rats also significantly increased susceptibility to flurothyl-induced seizures, and this was reversed by the administration of exogenous BHB [70]. As seizure activity is a common sequela of HI brain injury and seizure burden may contribute to the injury [3, 71], using ketone bodies to decrease the seizure burden in infants with HIE has the possibility of improving outcomes through multiple mechanisms.

Ketones Are Mitoprotective and Can Maintain Redox Homeostasis

Ketone bodies maintain mitochondrial function after injury, which is a significant contributor to their neuroprotective action, and directly relevant to infants with HIE. For instance, both BHB and AcAc have been shown to increase the threshold for activation of the mitochondrial permeability transition (mPT) complex [72], which is a key step in the initiation of both apoptotic and non-apoptotic cell death after HI brain injury.

Free radicals generated after experimental hypoxia-ischemia also directly damage protein complexes within the mitochondrial electron transport chain (ETC), particularly complex I, leading to reduced mitochondrial function once perfusion and oxygenation are restored [73]. In the Vannucci model of unilateral HI brain injury in the rodent, mitochondria ipsilateral to the carotid artery ligation become relatively reduced (a low NAD+/NADH ratio) 1–4 h after hypoxia-ischemia. This implies that, while glycolysis and the Krebs cycle are functioning to provide reducing equivalents for the ETC, respiratory complex dysfunction prevents the use of NADH, which accumulates [74]. This complex I deficit is present in both sexes after unilateral HI but appears to be particularly prevalent in male animals [75]. Exogenous BHB may be able to maintain ETC function in the setting of complex I dysfunction. For instance, in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of Parkinson's disease, BHB administration ameliorated complex I dysfunction, preventing the loss of dopaminergic neurons and restoring motor function [76].

During the metabolism of ketones to produce acetyl-CoA for use in the Krebs cycle, fewer cytosolic NAD+ molecules are reduced to NADH compared to glucose [61, 77]. This allows BHB to spare the cytosolic NAD+ pool, which is already reduced after HI brain injury as indicated by the increased production of lactate, in an attempt to recycle NAD+ from NADH in the absence of adequate oxygen (or in the presence of later mitochondrial dysfunction) [5]. Ensuring adequate NAD+ and an adequately oxidized NAD+/NADH couple is essential for cellular survival, as NAD+ is also required for the activation of sirtuins, which regulate a broad spectrum of antioxidant and prosurvival pathways [78]. Cellular redox state after hypoxia-ischemia is further complicated by the consumption of NAD+ during poly(ADP-ribose) polymerase 1 (PARP-1)-dependent DNA repair, which leads to cell death in rodents after hypoxia-ischemia when NAD+ levels are sufficiently depleted [79, 80]. Additionally, de novo NAD+ synthesis through the kynurenine pathway may be stalled due to the accumulation of reactive oxygen species (ROS) and Krebs cycle intermediates such as succinate, which inhibit quinolinic acid phosphoribosyl transferase and, instead, result in an accumulation of the potentially neurotoxic metabolite quinolinic acid [81]. By providing BHB or AcAc as metabolic fuels for the brain instead of glucose, the cytosolic NAD+ pool may therefore be spared, increasing the activation of sirtuins, maintaining mitochondrial integrity and function, and reducing the likelihood of PARP-1-dependent cell death.

Ketone Bodies Decrease Oxidative Stress

Increased production of ROS and oxidative stress are an important contributor to cellular injury after hypoxia-ischemia [5]. BHB, and to a lesser extent AcAc, has a direct antioxidant capacity, and can act as a scavenger for hydroxyl radicals [82]. Ketone bodies directly reduce oxidative stress in neocortical neurons as well as in isolated cortical mitochondria exposed to hydrogen peroxide [83]. Through epigenetic effects mediated by the inhibition of HDACs, BHB activates the expression of genes in the forkhead box O3 (FOXO3) network, including the upregulation of the antioxidant enzymes catalase and superoxide dismutase 2 (SOD2), reducing oxidative stress burden [17, 84]. In isolated perfused rat hearts, exogenous BHB and AcAc oxidize the coenzyme-Q couple within the inner mitochondrial membrane, reducing the likelihood of reverse-electron transport as well as the transfer of electrons from partially reduced coenzyme Q to oxygen, both of which could otherwise lead to the generation of the highly reactive superoxide species [85, 86]. Finally, ketones may also increase the cytosolic ratio of NADPH to NADP+, by sparing glucose from being used as a metabolic substrate and increasing flux through the pentose phosphate pathway (PPP) [86, 87]. NADPH is normally produced through the metabolism of glucose via the PPP, with a key use of NADPH being the recycling of oxidized glutathione (GSSG) to GSH for the scavenging of free radicals. Depletion of intracellular GSH is a crucial step in the process of oxytosis and ferroptosis (nonapoptotic cell death pathways associated with GSH depletion), and various formulations of the GSH precursors *N*-acetyl-cysteine (NAC) are neuroprotective after HI brain injury and in the setting of neuroinflammation [88–90]. As flux through the PPP and intracellular GSH may both be reduced after HI brain injury [91], increasing the availability of NADPH to recycle GSH could provide another pathway through which oxidative stress can be reduced in the injured brain using

ketone bodies. However, it is worth noting that the capacity to use available GSH via glutathione peroxidase appears to be altered by HI brain injury in the newborn rat in a sex-dependent manner, with males displaying greater impairment [75]. This means that supporting GSH recycling after injury may not be enough to ensure adequate antioxidant function.

Ketones Are Neuroprotective in Multiple Animal Models of Adult Brain Injury

Though the literature supporting the neuroprotective effects of ketone bodies for neonatal brain injury is currently in its infancy [92], a significant body of work has described neuroprotection from the administration of exogenous ketone bodies in a wide range of animal models of adult brain injury. This includes multiple models of Alzheimer's disease, as well as stroke, Parkinson's disease, and traumatic brain injury (TBI) [76, 84, 93–95]. In vitro, 4 mM sodium BHB is neuroprotective in an MPP^+ model of Parkinson's disease, as well as an amyloid precursor protein ($A\beta_{1-42}$)-induced hippocampal neuronal degeneration model of Alzheimer's disease [93]. In the MPTP model of Parkinson's disease, infusions with sodium BHB (1.6 mmol/kg/day) started before the administration of MPTP prevented the loss of dopaminergic neurons and restored motor function [76]. In a triple-transgenic mouse model of Alzheimer's disease, a diet containing 21.5% of calories from the BHB-butanediol ester, started at 8.5 months of age and resulting in an average BHB level of 0.7 mM, provided significant protection of cognition over several months, and increased cytosolic NAD+/NADH [94, 96]. In a controlled cortical impact model of TBI in the adult rat, BHB infusion (30 mg/kg/h) for 3 h after injury maintained the cerebral metabolic rate and ATP production relative to untreated animals [95]. And in a middle cerebral artery occlusion model in the adult mouse, subcutaneous ketone salts (0.4 mmol/kg BHB and 0.45 mmol/kg AcAc) given 30 mins after reperfusion, and repeated hourly for 6 h, significantly reduced infarct and penumbral volume on MRI, as well as improving neurological score 24 h after the injury [84].

With this recent increase in interest in the use of ketosis as a therapy or adjunct in a wide range of neurological diseases, several clinical trials are currently underway. These include investigating the metabolism of exogenous ketones in healthy human adults, and testing the feasibility and efficacy of ketosis to improve the symptoms of Alzheimer's and Parkinson's diseases as well as the outcome of adult stroke and out-of-hospital cardiac arrest. Importantly for clinical translation, a significant body of literature on the metabolism of exogenous ketogenic substrates (ketone salts, ketone esters, and MCFAs) in humans already exists [25, 38, 97].

Ketones in Clinical and Experimental HIE

Two studies using the Vannucci model of unilateral HI in the newborn rat provide promising evidence to support the use of exogenous ketones in infants with HI brain injury and HIE. During the initial characterization of the model in the Vannucci laboratory, Yager et al. [98] described the effects of fasting and insulin-induced hypoglycemia in P7 rats before exposure to unilateral hypoxia-ischemia. Hypoglycemia in insulin-treated animals was correlated with mortality in the hypoxia chamber; however, fasted animals were protected against hypoxia despite having lower glucose levels than the insulin group. This was likely due to the fact that fasted animals had BHB levels more than double that seen in those given insulin (i.e., 1.17 vs. 0.52 mM). More recently, Lee et al. [92] administered exogenous BHB (as an R-BHB sodium salt) to P13 rat pups to maintain BHB >2 mM for 6 h after unilateral HI. They saw significant neuroprotection throughout the brain, including decreased apoptosis and the preservation of hemispheric volume compared to controls, though no difference was seen on behavioral testing. Some potential drawbacks of the therapy were noted. For instance, blood BHB levels were initially raised after HI, and exogenous BHB administration reportedly caused pathological (4–10 mM) BHB levels as well as increased mortality in P7 and P10 rats after injury (these data were not provided in the paper). As humans can readily tolerate, and may see greatest seizure control, at BHB levels >4 mM [99], these issues could be mitigated by using more clinically relevant animal models, or by altering the timing of delivery or the method of inducing ketogenesis.

Ketone levels in infants with HIE are less well understood, other than in a small group of patients described by Reinke et al. [100] and Ahearne et al. [101]. In cord blood from asphyxiated infants who did not have evidence of significant clinical HIE, increased levels of BHB were seen compared to in healthy controls. Similar to in the rat after unilateral HI, ketogenesis may be initially upregulated during or after asphyxia due to increased glucose utilization and the release of catecholamines and cortisol, both of which can increase lipolysis and fatty acid

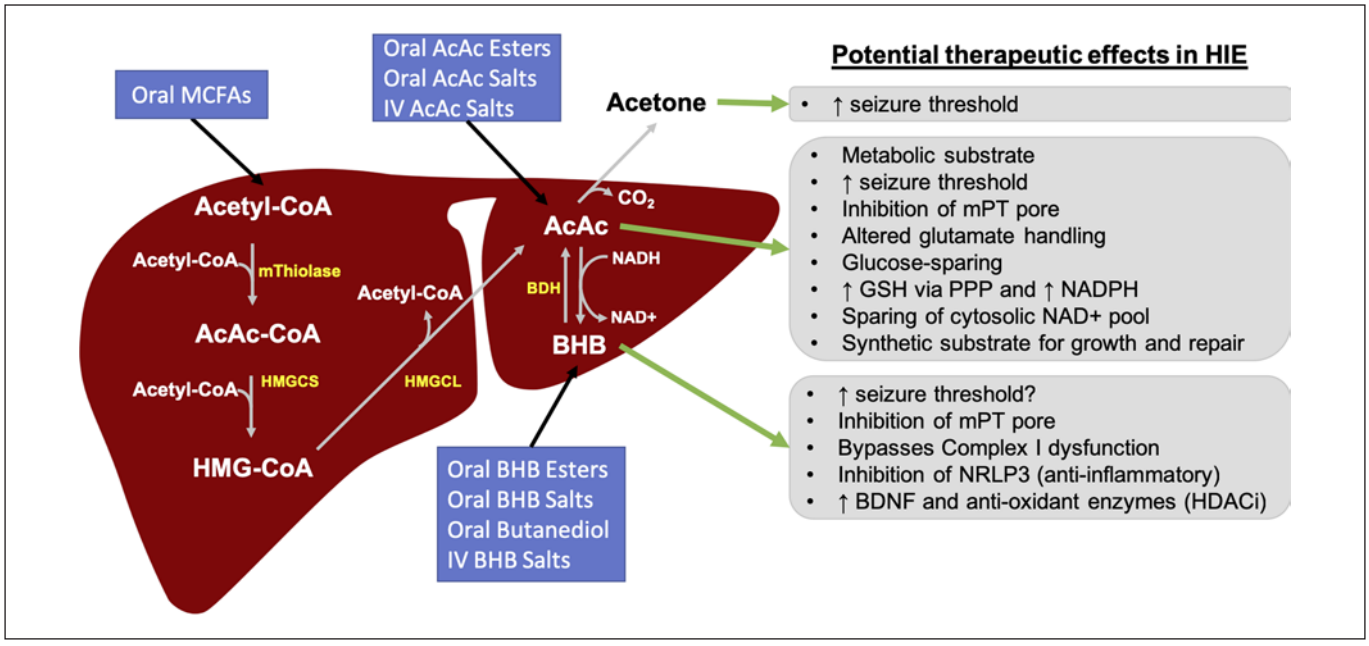

Fig. 1. Exogenous ketones and potential neuroprotective effects of ketone bodies in neonatal brain injury. Salts and esters of AcAc and BHB, as well as MCFAs, are a potential source of exogenous ketones for the injured newborn brain. MCFAs are delivered to the liver via the portal circulation, and this results in excess hepatic mitochondrial acetyl-CoA relative to available oxaloacetate. Three rounds of acetyl-CoA condensation result in HMG-CoA, which is then converted to AcAc. AcAc is converted to BHB in a process governed by the mitochondrial redox state (NAD+/NADH ratio), and both AcAc and BHB are released into the blood where AcAc can be decarboxylated to acetone. AcAc and BHB salts are directly absorbed into the circulation, with esters and butanediol rapidly converted to AcAc and BHB in the liver. All 3 ketone bodies are readily taken up into the developing brain and have unique and overlapping neuroprotective effects relevant to infants with hypoxic-ischemic encephalopathy (HIE). AcAc, acetoacetate; BHB, β-hydroxybutyrate; MCFAs, medium chain fatty acids; AcAc-CoA, acetoacetyl-CoA; HMG-CoA, hydroxymethylglutaryl-CoA; mThiolase, mitochondrial thiolase; HMGCS, HHMG-CoA synthase; HMGCL, HMG-CoA lyase; BDH, beta-hydroxybutrytate dehydrogenase; NAD, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; mPT, mitochondrial permeability transition; GSH, glutathione; PPP, pentose phosphate pathway; NLRP3, NOD-like receptor protein 3 inflammasome; HDACi, histone deacetylase inhibitor, BDNF, brain-derived neurotrophic factor.

delivery to the liver. By comparison, infants with severe HIE were found to have decreased levels of both acetone and BHB, and low BHB was part of a metabolite model that predicted the infants in the cohort that later died [101]. The timing and mechanism of any changes in ketone production after HIE, as well as any associations with severity of injury, therefore remain to be fully elucidated.

Exogenous Ketones for Neonatal Neuroprotection: Future Directions

Ensuring adequate ketosis after neonatal HI through exogenous administration of ketogenic substrates is a promising neurotherapeutic avenue. Ultimately, determining the best methods to apply to neonates with HIE will ideally require direct comparisons across multiple species and models of HIE. The timing and duration of ketone administration must be further investigated in animal models, including the most use-appropriate ketogenic compound, or combination of compounds. Early evidence indicates that different ketone bodies provide different benefits (Fig. 1), with data thus far indicating that AcAc (and associated acetone) provides a greater reduction of seizure activity than BHB [20, 102]. Therefore, salt or esters of AcAc may be more beneficial in the early stages of HIE, as they can result in more equal increases in circulating AcAc and BHB compared to exogenous BHB [22, 25, 102]. By comparison, greater relative increases in BHB may result in more of a mitoprotective or epigenetic effect to increase the production of neurotrophic factors and bolster antioxidant defenses [61]. Sustained ketosis may also be beneficial for several days or

weeks after injury to ensure the availability of precursors for the synthesis of lipids that will be essential for structural repair after HI brain injury [15, 16]. Infusions of exogenous ketones during or prior to at-risk births should also be considered, as ketones cross the placenta and are more efficient substrates than glucose in terms of oxygen utilization [85, 103]. Ensuring adequate ketone levels before or during HI may reduce cellular oxygen demands, as well as maintaining the cellular redox state, both of which would contribute to the promotion of neuronal survival.

Importantly, especially considering the prevalence of HIE in low-resource settings, all the potential methods to increase ketosis in neonates are inexpensive, stable at room temperature (they do not require refrigeration), and easy to monitor. For instance, blood BHB can be measured using the same point-of-care monitors used to monitor blood glucose, with 1 unit recently validated to detect BHB levels >0.4 mM in newborns [104]. To increase the understanding of how ketone bodies are associated with outcomes in HIE, prospective studies tracking blood BHB levels alongside blood glucose in asphyxiated infants would be simple to perform in any neonatal intensive care unit.

While it is important to note that not all the putative benefits of ketosis are likely to be seen in neonates with HIE, current evidence suggests that adequately supporting ketosis after asphyxia in neonates is a promising future area for neuroprotection research that directly supports healthy neonatal physiology.

Conclusion

There remains a significant need for therapies that can augment current therapeutic strategies for infants with HIE. Exogenous ketones have the potential to be neuroprotective across the 3 main phases of injury after hypoxia-ischemia by preserving mitochondrial function, and reducing seizure burden, inflammation, and oxidative stress, and providing the synthetic precursors required for repair and growth. As newborn infants are uniquely adapted to the uptake and utilization of ketone bodies, they also constitute the group of patients most likely to benefit from the strategies available to induce ketosis for neuroprotection. Due to the wide-ranging potential benefits of ketone bodies in the injured developing brain, as well as the ease of the storage, administration, and monitoring of exogenous ketone salts and esters, the investigation of ketone bodies as therapeutic agents for term HIE, as well as other forms of developmental brain injury, is a promising field for future preclinical research.

Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

B.J.S. is an employee of HVMN Inc., which sells exogenous ketone products. She has the option to purchase stock in HVMN Inc.

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