

**THE EFFECTS OF 1-WEEK AND 8-MONTH KETOGENIC DIETING OR KETONE
SALT SUPPLEMENTATION ON MARKERS OF TISSUE OXIDATIVE STRESS AND
MITOCHONDRIAL QUALITY IN RATS**

By

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ABSTRACT

Purpose: Herein we sought to examine the short-term (i.e., one-week) and long-term (i.e., 8-month) effects of a ketogenic diet (KD) or ketone salt supplementation on multi-organ markers of oxidative stress and mitochondrial function in male Fisher 344 in versus control animals fed standard rodent chow (SC). **Methods:** In AIM 1, 4 month old male Fisher 344 rats were provided isocaloric amounts of a KD (5.2 kcal/g, 23.1% protein, 9.6% carbohydrate, and 65.3% fat, n=10) or SC (3.1 kcal/g, 24% protein, 58% carbohydrate, 18% fat; n=20) and un-supplemented water ad libitum for 7 days. The SC rats were split into sub-groups such that, one group was provided ketone salts in their drinking water in addition to a SC diet (SC+KS ~1.2 g/day, n=10), and one group was un-supplemented (SC, n=10). In AIM 2, 4 month old male Fisher 344 rats were provided the KD (n=8), SC (n=7) and SC+KS (n=7) for 8 months. Following treatment completion for AIMs 1 and 2, rats were sacrificed and blood was obtained and serum was aliquoted and stored, and brain, liver and gastrocnemius muscle were harvested for mRNA and/or protein analyses. In addition for AIM 2, brain, muscle and liver mitochondria were freshly isolated for mitochondrial respiration and reactive oxygen species (ROS) assays. **Results:** Serum ketone levels were greatest in KD rats after one-week and 8-mo interventions relative to the SC and SC+KS groups both suggesting that diet, but not salt supplementation, induced a ketogenic state. Both short-term and long-term KD resulted in blunted weight gain and feed efficiency when compared to both SC and SC+KS rats ($p<0.001$ and $p<0.001$, respectively). Muscle, brain and liver expression of oxidative stress-related genes were not

different between groups after one-week. Similarly, muscle/brain/liver protein expression of glutathione peroxidase, superoxide dismutase 2, and catalase as well as protein carbonyl and 4-hydroxynonenal levels were not different between groups following the 8 mo study. After 8 months, gastrocnemius mitochondrial ROS production was higher in KD animals versus all other treatments ($p=0.007$), and this may have been related to a decreased state III respiration and respiratory control ratio in this tissue relative to other groups ($p=0.072$ and $p=0.018$, respectively). Moreover, gastrocnemius citrate synthase activity (a surrogate of mitochondrial density) was lowest in KD rats versus the SC and SC+KS groups ($p<0.001$). However, despite these gastrocnemius deficits, rotarod performance was greatest in KD rats versus the all other groups after 2 mo, 4 mo and 8 mo of treatment or control diet. **Conclusions:** Our data suggest that ketogenic dieting, but not ketone salt supplementation, reduces feed efficiency and body mass acutely and chronically. No changes in antioxidant muscle/brain/liver gene or protein expression were elicited by short-term or long-term ketogenic dieting or ketone salt supplementation. Interestingly, chronic ketogenic dieting elicits an increase in skeletal muscle mitochondrial ROS formation and, despite the lack of oxidative damage (i.e., protein carbonyl and 4HNE production), this paralleled decreases in mitochondrial quantity and quality markers. Notwithstanding, these skeletal muscle deficits did not translate into a decline in muscular endurance and/or grip strength with long-term ketogenic dieting suggested that a long-term ketogenic diet may result in increased skeletal muscle metabolic efficiency and improved performance in aged (12 month old) rats.

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“This is the way the world ends

This is the way the world ends

This is the way the world ends

Not with a bang but a whimper.”

- T.S. Eliot

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CHAPTER I

INTRODUCTION

The ketogenic diet (KD) is a high-fat, moderate protein, low carbohydrate diet that was initially utilized to treat seizures in children with intractable epilepsy. Many investigations have revealed this dietary approach to be just as, if not more, efficacious than currently available anticonvulsant medications [1, 2]. Beyond being a treatment for epilepsy, the KD has been associated with a myriad of general health benefits. Some of the most robust KD literature has been centered on weight loss and management, but the KD has also been linked to positive outcomes in regards to treating diabetes, polycystic ovaryian syndrome, acne, a wide array of neurological complications, cancer, respiratory and cardiovascular disease, fertility, and increasing longevity [3, 4]. With regard to the latter, the KD has been shown to either confer normal longevity or enhance life span in murine models [5, 6], albeit the specific mechanism of action has not been well elucidated.

One of the prevailing ideas for the numerous benefits of the KD is related to augmented mitochondrial function and protection. Excessive accretion of reactive oxygen species (ROS) have been purported as one of the hallmarks of the aging process as well as reduced functionality of various cellular proteins [7, 8]. Mitochondria bear the brunt of oxidative damage, and lesions to mitochondrial DNA have been reported as much as 10x higher than in the nucleus and accumulates with age [9]. Mitochondrial DNA damage occur in excess because DNA repair is less efficient within the mitochondria, and mitochondrial DNA is not bound my histones or DNA-binding proteins [10]. There is currently data indicating mitochondrial functional decline with age [11, 12]. As such, a manipulation that can reduce or ameliorate mitochondrial oxidative stress may serve to benefit the aging process and general cellular function.

Interestingly, oxidative stress defense and improved mitochondrial quantity/quality are potential mechanisms by which the KD may confer a metabolic advantage [13, 14]. For instance, hippocampal mitochondrial biogenesis in rodents has been reported after four weeks of KD feeding [15]; this being a phenomena which could occur via KD-induced AMPK pathway signaling [16, 17]. KD treatments have also been shown to decrease ROS and hydrogen peroxide production in rodent hippocampi by increasing uncoupling protein mediated proton conductance and increased glutathione biosynthesis [18, 19]. The KD-induced reduction in ROS production is potentially mediated via increased mitochondrial density, uncoupling protein expression and/or an increased glutathione (GSH) biosynthesis and activity [15, 18-20]. With regard to the latter, Milder and Patel [13] theorized that KD-induced ROS defense is related to an acute stimulation of 4-hydroxy-2-nonenal (4-HNE) or hydrogen peroxide which, in turn, stimulates the NF E2-related factor 2 (Nrf2) transcription factor to translocate into the nucleus and prompt the transcription of antioxidant related genes. Namely, Nrf2 stimulates the expression of subunits of glutamate-cysteine ligase (GCL), the rate limiting enzyme in glutathione biosynthesis [21, 22]. In prior work, Milder and colleagues [23] documented that acute KD feeding does in fact stimulate hydrogen peroxide and 4-HNE production in the hippocampus of rodents and, in a temporal fashion, this led to Nrf2 nuclear translocation and an increase in the expression of ROS-protective proteins that promote a REDOX [or reduction in one molecule(s) and the oxidation of another molecule(s)] state.

From this, the current body of literature supports a KD in terms of stimulating a cellular environment that is more resistant to oxidative stress. However, some data indicate that it is not the KD entirely, but rather the metabolites produced during ketogenic dieting that confer the aforementioned benefits. The ketone bodies, beta-hydroxybutyrate (BHB) and acetoacetate

(AcAc), are the consequent metabolites from carbohydrate restriction and lipolysis. These substrates have been introduced in cell culture models with subsequent results of decreased ROS production and resistance to a hydrogen peroxide-induced stress [24, 25]. Thus, it is possible that the ketone bodies have a direct causative effect in terms of reducing production and enhancing handling of ROS.

Based on the aforementioned documented observations, KD and/or ketone treatments seemingly have the potential to reduce ROS production over the lifespan, and this phenomena could potentially lead to improved mitochondrial quality. However, there is currently a dearth of literature detailing the chronic effects of KD or ketone salt ingestion as it relates to mitochondrial function and level of oxidative stress. Therefore, the purpose of this investigation is to examine if KD feeding or BHB salt supplementation affects skeletal muscle, liver or brain measures of oxidative stress and mitochondrial function over the course of 8 months.

CHAPTER 2

REVIEW OF LITERATURE

Background

A brief history of the ketogenic diet

The ketogenic diet (KD) is a high fat, low carbohydrate, adequate protein diet that was initially adopted to treat epileptic seizures. The complications with epilepsy were initially theorized to be related to brain intoxication induced by intestinally derived substances [26]. Thus, a common intervention to control seizures was prolonged fasting. In an attempt to clearly define the mechanistic cause for seizure control, it was hypothesized that a byproduct of fatty acid oxidation (ketone bodies) during times of insufficient carbohydrate acted as a natural anticonvulsant [27]. Prior to these reports, researchers at the Mayo Clinic were investigating different dietary manipulations to mimic fasting and reported that carbohydrate restricted, high fat dietary approaches were satisfactory [28]. This dietary approach for seizure control was pervasive for the following two decades, with great success being reported. Notwithstanding, the advent of phenytoin (a ‘pharmaco’ approach to epilepsy) ushered in the interest of the mechanisms of anticonvulsant drugs over the efficacy of certain diets. For the next few decades, medication was the first-line choice for epilepsy control and fewer doctors prescribed KDs, which led to the under-appreciation of ketogenic dieting as a therapy for seizures.

Even though anticonvulsant medication was the predominant tool used to treat epileptic patients through the mid and late 1990s, there was a cohort of children at Johns Hopkins Hospital that employed the KD approach. In fact, a review of 58 patients found the KD to still have a therapeutic role compared with the anticonvulsant medications [29]. This aforementioned research kept ketogenic dieting in the scientific conversation of viable therapies for seizure

patients. In terms of positive mainstream press, the son of Hollywood producer Jim Abrahams had seizures that were not controlled by medication. Thus, Abrahams explored the potential benefits of the KD as an alternative therapy. Subsequent to trying the diet, Abrahams' son experienced a complete remission of the seizures. Abrahams later began the Charlie foundation which functioned to increase the awareness of the KD as an alternate treatment and a source of funding for research and implementation.

This era in the 1990s led to an increased interest in the KD as a therapeutic treatment leading to numerous studies, reviews, and meta-analysis aimed to delineate their prospective benefit [30-33]. Unrelated to the therapeutic benefits in relation to seizure control, there was a movement running parallel that took note of the benefits associated with weight reduction/maintenance. Dr. Robert Atkins published a book in 1972 praising the benefits of carbohydrate restriction for obese patients in his cardiology practice. At the time he was heavily criticized since his claims were not yet supported by scientific investigations, but succeeding research seems to support at least some of his claims [30, 34-38]. Collectively, the aforementioned research findings regarding the therapeutic and weight loss benefits of ketogenic dieting has spurred curiosity within the scientific community regarding the potential physiological effects of carbohydrate restriction. These aspects will be reviewed in the next section.

The many benefits of ketogenic dieting

Weight loss/maintenance

Currently, there is a multitude of scholarly papers that support the implementation of a KD (at the very least a low-carbohydrate diet) for the purpose of weight loss and maintenance. When compared to other popular dietary approaches (i.e., lower-fat hypocaloric diets), the KD

often promotes a more rapid rate of weight loss, as well as more weight loss over time [32, 39]. Some investigators posit that this benefit is mediated through the satiating effects of increased protein and fat consumption [40], although others posit that there is a distinct advantage related to the unique nature of a ketotic state [41]. While the precise mechanism of action is unclear, subjects lose more weight when eating an ad libitum KD compared to individuals eating a “balanced” diet over a 3-6 month period [39, 42, 43]. One potential mechanism of KD-induced weight loss may be due to the energy cost of gluconeogenesis which has been estimated to be 400-600 kcal/day [44]. While this would in theory increase resting energy expenditure in individuals undertaking this dietary approach, this mechanism of action has not been supported [45]. Another KD-related mechanism of action that has been documented includes leptin and ghrelin being modulated to suppress appetite during nutritionally-induced ketosis [46]. Moreover, others have reported that ketogenic dieting decreases lipogenesis and increases lipolysis [47-49]; an effect which leads to enhanced lipid oxidation [45, 50]. While the precise mechanism of action is likely multi-factorial, the aforementioned research suggests that KD is permissive to weight loss.

Cardiovascular disease

Ketogenic dieting has also been documented to positively modulate factors that contribute to cardiovascular disease. For instance, several studies have reported that the KD can positively modulate blood lipid profiles [42, 43, 51]. The most stark and consistent benefit with ketogenic dieting has been shown with the reduction in blood triglycerides [50, 52]. The reasoning for this has been explained via a metabolic adaptation whereby the body more efficiently oxidizes circulating lipids. What is less understood are KD-induced alterations in blood cholesterol levels. A decrease in total cholesterol has been documented, but more

noteworthy is the consistent increase in high density lipoprotein (HDL) levels in persons that engage in ketogenic dieting [50, 52, 53]. How the KD alters low density lipoprotein (LDL) cholesterol is still subject to debate, as it often remains unchanged in quantity. Nevertheless, low density lipoproteins have been reported to increase in size and become larger, less atherogenic particles in persons engaged in ketogenic dieting [53]. Mechanistically, cholesterol alterations through ketogenic dieting may be due to dietary-induced decrements in insulin given that insulin is a stimulator of the rate limiting enzyme in cholesterol synthesis (cytosolic 3-hydroxy-3-methylglutaryl-CoA reductase) [54]. Notwithstanding, more long term data are required before the KD can be recommended to reduce cardiovascular disease. However, preliminary data monitoring serum biomarkers related to cardiovascular disease supports the efficacy of ketogenic dieting.

Type II Diabetes

The KD has also been shown to be a viable treatment for type II diabetes (T2D). T2D modulation with ketogenic dieting are likely mitigated through improving insulin resistance and altering glucose metabolism. While the characteristics of these problems are still heavily debated, one feature of T2D includes dietary carbohydrate being diverted toward the liver for de novo lipogenesis instead of oxidation in skeletal muscle [55]. Additionally, skeletal muscle becomes resistant to insulin, thus leading to elevated levels of blood glucose. Granted, while de novo lipogenesis has been reported to only account for up to 20% of new triglycerides [56], it is obvious that dietary lipid would not be sufficiently oxidized in a lipogenic state thereby having a greater propensity for storage in adipose tissue. Experimentally, Bistrain et al. [57] utilized a low calorie low carbohydrate diet in a cohort of T2D which resulted in reductions in insulin and bodyweight. Thus, a potential mechanism of action regarding the effects of ketogenic dieting in

mitigating T2D is related to the suppression of insulin, a key anabolic hormone that promotes lipogenesis and inhibits lipolysis [58-60]. A similar study that utilized a very low carbohydrate diet compared to a moderate carbohydrate approach (24 vs 94 g/day) reported a robust decrease hepatic glucose output in the very low carbohydrate group [61]. An in-patient investigation of a KD found improvements in hemoglobin A1C and insulin sensitivity in T2D in just two weeks [62]. The KD has been also been reported to result in weight loss and improvements in a host of metabolic parameters when employed for 14 months in T2D [63]. Arguably, these benefits are simply associated with weight loss. However, when insulin resistant individuals are placed on KD regimens these approaches improve metabolic syndrome markers to a greater extent compared to low fat diets even when total energy intake is controlled [51].

Epilepsy

Perhaps the most abundantly researched benefit to the KD is related to epilepsy control. There are numerous reviews on this topic, especially as it relates to childhood seizures [64-67]. The precise mechanism of action is still up for debate, but the prevailing hypotheses are: the anticonvulsant effects of the ketone bodies [68], reduced neuronal excitability induced by ketones [68], and inhibiting mammalian target of rapamycin pathway signaling in neurons [16]. Overall, carbohydrate restriction and higher lipid consumption leads to a heightened amount of acetyl-CoA in neurons and subsequently high rates of oxaloacetate consumption. In doing this the directionality of the aspartate transaminase reaction is shifted to yield lower quantities of the amino acid aspartate, while supporting the formation of the amino acid glutamate from α -ketoglutarate. The relative overabundance of glutamate primes the conversion the γ -aminobutyric acid, the primary inhibitory neurotransmitter. The aforementioned cascade leads to reduced excitability of neural tissue as well as anticonvulsant effects. Additionally, mTOR

signaling has been implicated in epileptogenesis [69-71], and an initial inhibition of mTOR signaling through KD-induced AMPK activation could theoretically lead to blunted or attenuated seizure events. In summary, the KD certainly has support for treatment of epilepsy with robust mechanistic reasoning.

Cancer

The KD has also been implicated to reduce the growth of certain cancers. Specifically, ketogenic dieting has been theorized to down-regulate insulin and Akt/mTOR signaling in cancer cells [72-74]. As well, a reduction in cancer cell glycolytic activity (known as the Warburg effect whereby cancer cells have a highly accelerated rate of anaerobic glycolysis in order to proliferate), is likely diminished during ketogenic dieting due to the very low amount of carbohydrate being consumed [75]. Mitochondrial dysfunction may also play a role in cancer growth [44, 76, 77] and, thus, potential favorable benefits of ketogenic dieting on mitochondrial physiology may factor into beneficial effects the diet has on cancer. Currently preliminary evidence suggests that the KD can prevent cancer initiation, delay cancer cell growth, and may be an alternate therapy for brain cancer [78-80].

Polycystic ovary syndrome

Polycystic ovary syndrome (PCOS) is a disorder of the endocrine system that is characterized by ovulatory dysfunction, obesity, infertility, and hormonal dysregulation. Interestingly, hyperinsulinemia is a commonly associated symptom [81]. KDs have been tentatively shown to improve some PCOS symptomology [82]. Potential mechanisms are related to insulin being a partial regulator in steroid metabolism. Insulin has been shown to increase ovarian hormonal output, impair progesterone inhibition of gonadotropin-releasing hormone pulse generator, and increase adrenal responsiveness to adrenocorticotrophic hormone [83-85].

Alzheimer's disease

The KD has latent benefits that may ameliorate some problems that are coupled with Alzheimer's disease. Clinical improvements have been documented when Alzheimer's patients have undertaken a KD style of eating [86]. The precise mechanism is, again, not adequately elucidated. Alzheimer's patients do report a higher incidence of seizure and greater neuronal excitability [87-89]. From the literature on epilepsy, amino acid modulation facilitated by ketogenic dieting may create a neuronal environment that is protective to frequent seizures. Additionally, ketone bodies have been reported to induce protective effects to neurons in vitro upon β -amyloid exposure [90].

Amyotrophic lateral sclerosis

Mitochondrial dysfunction may mediate the pathogenesis of amyotrophic lateral sclerosis (ALS). As such, KD may ameliorate some complications associated with the disease via the capacity to augment mitochondrial function. Ketogenic dieting has tentatively been shown to lead to improvements in an ALS rodent model, conferring a higher motor neuron count as well as attenuate motor function loss; however, these findings need to be replicated [91].

Conclusions on the multifactorial therapeutic effects of ketogenic dieting

The KD is commonly utilized as a weight loss/maintenance tool, although the therapeutic effects associated with this diet have been tacitly shown to benefit a myriad of pathogenic conditions. While the exact mechanistic modulation for each clinical condition has yet to be revealed, the convergence of numerous metabolites and differential control mechanisms are likely responsible [92]. Moreover, the drastic and persistent reduction in circulating blood glucose and insulin seems to be a driver for much of the aforementioned effects of ketogenic dieting, and future research should continue to explore the mechanistic link between the KD-

induced improvements in the aforementioned diseases relative to the KD-induced reduction in whole-body glycolytic metabolism.

Ketone bodies, metabolism and utilization

Ketone Bodies

Ketone bodies (KB) are small molecules that are derived from lipid oxidation. Three physiologically-relevant KBs include beta hydroxybutyrate (BHB), acetoacetate (AcAc), and acetone. Upon fasting or severe carbohydrate restriction, liver and muscle glycogen are the first to be depleted. Following sufficient depletion, fatty acids are mobilized from adipose tissue and are transported to the liver (and to a lesser extent the kidneys) for the production of KBs via ketogenic pathways [93]. Following ketogenesis, the KBs (typically BHB) are exported out of the liver and transported via circulation to metabolically active tissues. KBs are then imported via monocarboxylic acid transporters (MCTs) whereby they are converted to acetyl-CoA thereby exerting a glucose and protein-sparing effect [94-96].

Ketogenesis and oxidation

Hepatic ketogenesis is a multi-step process that yields KBs which are, in turn, exported from the liver and circulate in the blood to extrahepatic tissues for oxidation and subsequent ATP production. In general, this process involves the following steps [97]:

KB formation in hepatocyte mitochondria

- 1) Fatty acids are catabolized in the mitochondria via beta-oxidation to form acetyl CoA. During ketogenic dieting high levels of fatty acid influx combined with low amounts of mitochondrial oxaloacetate disproportionately reduces acetyl CoA entry into the TCA cycle. Instead, excessive acetyl CoA is diverted towards KB formation described in steps 2 and 3 below.

- 2) Mitochondrial hydroxymethyl glutaryl (HMG)-CoA synthase (HMG-CS2) adds an acetyl group to acetoacetyl-CoA to form HMG-CoA. It should be mentioned that the acetoacetyl-CoA substrate in the aforementioned reaction is formed by the conjoining of two acetyl-CoAs via the thiolase enzyme.
- 3) Mitochondrial HMG-CoA lyase forms AcAc by removing an acetyl CoA from HMG-CoA. AcAc can spontaneously decarboxylate to form acetone or be converted to BHB through BHB dehydrogenase. It should be noted that greater than 70% of total circulating ketones exist as BHB [98], so the aforementioned fate of AcAc favors the BHB dehydrogenase reaction.

Hepatic KB export, circulation and transport/metabolism in extrahepatic tissues

- 4) KBs cannot be oxidized for ATP production in the liver due to the lack of the mitochondrial succinyl-CoA:3-oxoacid-CoA transferase (SCOT) enzyme in hepatocytes[99]. Thus resultant KBs are exported into the bloodstream from hepatocytes through the SLC16A6 monocarboxylate (MCT) transporter and are metabolized by other tissues containing the mitochondrial SCOT enzyme. Notably, blood KBs in the fed state are less than 0.5 mM but can increase up to 7 mM days following fasting and/or the initiation of ketogenic dieting.
- 5) Blood KBs are transported into extrahepatic tissues (chiefly brain, myocardial cells and skeletal muscle cells) from explicit MCT transporters (i.e., SLC16A1 and SLC16A7). Following cellular uptake and mitochondrial transport, the BDH1 enzyme oxidizes BHB to form AcAc. AcAc is then activated to AcAc-CoA by SCOT. Finally, AcAc-CoA is formed into acetyl-CoA via the mitochondrial thiolase

enzyme. Resultant acetyl-CoA can enter the TCA cycle in order to be oxidized for ATP production.

Notably, individuals engaged in ketogenic dieting still have relatively high levels of blood glucose levels despite taking in very low levels of carbohydrate (e.g., ~90 mg/dL which is only ~10% drop from pre-diet values during a 12-week KD), and this is chiefly due to hepatic gluconeogenesis [51]. Notwithstanding, extrahepatic tissues during ketogenic dieting are thought to be primed towards an oxidative and 'ketone-metabolizing' phenotype rather than a glycolytic phenotype. Specific to the latter form of metabolism, the brain (which cannot oxidize fatty acids) still has a relative supply of blood glucose due to KD-induced gluconeogenesis, and rodent data exists suggesting that brain glucose consumption is reduced by 10% for every 1 mM increase in blood ketone levels due to the ability of brain cells to oxidize KBs [100]. Thus, individuals that commonly present ~2-3 mM blood ketone body levels could have up to a 30% reduction in brain glycolytic activity.

General mitochondrial function

Mitochondria are sometimes referred to as the powerhouse of the cell given that these organelles are responsible for the majority of ATP production in most animal cells.

Mitochondrial density varies among different tissue subtypes within an organism based upon energetic demand [101]. Mitochondria produce energy via oxidation of carbohydrates and lipids, and to a lesser extent amino acids, under typical physiologic circumstances. Substrate oxidation is completed through the TCA cycle which subsequently supplies the electron transport chain (ETC) with electrons carried by the NAD⁺ and FAD coenzymes (redox pairs are NADH and FADH₂, respectively). The ETC consists of complex I (NADH ubiquinone oxidoreductase),

complex II (succinate dehydrogenase), complex III (ubiquinol cytochrome c oxidoreductase), complex IV (cytochrome c oxidase), and complex V (F1F0-ATP synthase). During oxidative phosphorylation complex I accepts electrons from NADH, while complex II accepts electrons from FADH₂. Subsequently, the electrons are transported across the complexes into the inter-mitochondrial membrane space and the resultant electrochemical gradient allows for a proton motive force, or potential energy, to produce ATP at complex V. Along with energy generation, mitochondria are intimately involved in control of apoptosis, intracellular calcium regulation, as well as the turnover of reactive oxygen species (ROS) [102].

Free radical production and control

During the electron transfer process, electrons can escape the respiratory chain and react with molecular oxygen. This produces a free radical, namely superoxide (O₂⁻). Free radical production can be exacerbated by inhibition of complexes I or III. Murine models have elucidated the significance of O₂⁻; specifically, without the mitochondrial enzyme manganese – dependent superoxide dismutase II (SOD2) the accrual of O₂⁻ leads to death in the embryonic state [103]. SOD2 catalyzes the conversion of O₂⁻ to hydrogen peroxide. Catalase is one enzyme that can decompose hydrogen peroxide into molecular water. Another system for oxidative control is the glutathione (GSH) system. Glutathione peroxidase (GPx) reduces hydrogen peroxide thereby yielding glutathione disulfide (GSSH) and water. Following this reaction glutathione reductase consumes an NADPH to reduce GSSH into GSH.

Free radical theory of aging

Harman proposed that aging and related diseases may be due to accumulation of oxidative damage over time, as 2-3% of oxygen consumed by a cell is converted to free radicals [104]. A free radical is simply any chemical that holds an unpaired or odd number of electrons.

An abundant formation of free radicals occurs in the mitochondria, whereby electrons can escape from the respiratory chain and interact with molecular oxygen to form such intermediates as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($\cdot OH$) as mentioned above. Generally all free radicals have a wide propensity to interact with a multitude of macromolecules. Cellular membranes are particularly susceptible to oxidative damage given that free radicals can react with polyunsaturated fatty acids in the lipid bilayer thereby forming lipid peroxides [105]. Lipid peroxides produce an irreparable impairment to membrane fluidity and elasticity which can result rigidity and subsequently rupturing of a cell [106]. This is theorized to be a characteristic problem for post-mitotic cells that have relatively slow turnover rates, like neurons [107]. Moreover, free radicals react with and can cross-link proteins thereby reducing functionality [108]. It has been estimated that oxidized proteins in older animals may constitute 30-50% of total cellular proteins and researchers posit that this may be the etiology for reduced enzymatic catalytic activity with age [8].

Deoxyribonucleic acid (DNA) is another macromolecular target for free radical damage. Specifically, free radicals have the potential to oxidize a base in DNA and, if this occurs in critical coding regions, can be harmful or even lethal to the cell if endogenous DNA repair mechanisms do not mitigate the damage [109]. However, mitochondria bear the brunt of oxidative damage as lesions to mitochondrial DNA have been reported as much as 10x higher than nuclear DNA lesions, and mitochondrial DNA damage from oxidative stress accumulates with age [9]. This is because DNA repair is less efficient within the mitochondria, and is not bound by histones or DNA-binding proteins [10]. It has been posited that less functional mitochondria do not efficiently produce energy for general cellular upkeep, and have heightened free radical production [110]. As such, there is less cellular energy production and general tissue

deterioration with the aging process due to a lifetime culmination in mitochondrial damage via oxidative stress.

Ketogenic dieting and oxidative stress

Within the KD lexicon, there is a claim that ketone bodies are a “clean burning fuel” at least when compared to the complete oxidation of glucose. The specific meaning of “clean burning fuel” is simply a reduction ROS when the substrate of interest (i.e., ketone bodies) are oxidized. In support of the claim, Maalouf and colleagues [24] reported that neocortical neurons treated with BHB and acetoacetate and exposed to glutamate excito-toxicity in vitro were protected from death and had reduced levels of ROS production. This protection was related to greater NADH oxidation in the respiratory chain. There are analogous results when neocortical neurons are exposed to H₂O₂. Kim and colleagues treated neurons with diamide or H₂O₂ and concomitant KB treatments resulted in reduced cell death as well as lowered ROS production [25]. These findings taken together suggest that KBs acutely decrease ROS production in neurons in vitro. Other reports posit that this protection against oxidative injury is through enhanced mitochondrial respiration, especially at complex II, as well as increased ATP production [111, 112]. Another potential mediating factor associated with reduced oxidative stress from a KD is related to an increase expression of uncoupling proteins (UCP) as well as enhanced uncoupling activity. For instance, it has been reported that increased UCP-mediated proton conductance effectively dissipates the proton gradient across the mitochondrial membranes which, in turn, reduces ROS production [18].

While the aforementioned mechanisms may reduce oxidative stress during ketogenic dieting, the augmentation of glutathione biosynthesis via ketogenic dieting may enhance endogenous antioxidant capacity and indirectly reduce ROS formation. In this regard, Milder and

Patel [13] hypothesize that acute ketogenic dieting stimulates the cellular formation of 4-HNE which subsequently leads to the dissociation of Nrf2 from the Keap1 protein. Following this dissociation, Nrf2 translocates into the nucleus and binds to antioxidant responsive elements where Nrf2 acts as a transcription factor to upregulate subunit gene expression of glutamate-cysteine ligase (GCL), the rate limiting enzyme in glutathione biosynthesis [21, 22].

Accumulation of Nrf2 has been document in nuclear fractions of hippocampal cells in KD fed rats [23]. In addition, a KD fed to rodents for 3 weeks has been shown to increase GCL activity and the ratio GSH/GSSH [19]. Thus, ketogenic dieting may provide a chronic benefit whereby: a) endogenous antioxidants (specifically glutathione) are up-regulated, b) there is a reduction in free radical levels via increased cellular glutathione c) the chronic reduction in cellular free radical levels protect mitochondrial integrity and positively augment aging associated with a lifelong reduction in tissue ROS exposure.

Purpose statement and hypotheses

Given the aforementioned literature supporting the potential role of KBs in oxidative stress protection, the primary purpose of this investigation is two-fold (with experimental hypotheses being presented thereafter):

- a) **Purpose 1:** Examine the short-term (1-week) effects of KD feeding, standard chow (SC) feeding with ketone salt supplementation (SC+K) or standard chow feeding alone on muscle/brain/liver antioxidant mRNA expression patterns and total antioxidant capacity.

Hypothesis 1: Given that Milder and Patel [13] hypothesized that KD-induced elevations in blood ketone levels affect multi-tissue endogenous antioxidant mRNA expression within one week, I hypothesize that the KD and SC+K groups will present increases in tissue mRNA levels of antioxidant-related genes due to blood ketone levels being

elevated in these animals. I also hypothesize that tissue total antioxidant levels will be greater in the KD and SC+K groups compared to the SC group.

- b) **Purpose 2:** Examine if long-term (8 months) of KD or SC+K feeding affects muscle/brain/liver antioxidant protein expression patterns, GSH/GSSG levels, and/or total antioxidant levels compared to SC feeding only. Furthermore, this aim will examine if KD or SC+K feeding affects muscle/brain/liver mitochondrial function and mitochondrial ROS production due to the potential improvements in the aforementioned endogenous antioxidants.

Hypothesis 2: Given that a plethora of literature has linked KBs to reducing oxidative stress as well as inducing the expression of endogenous antioxidants, I hypothesize that KD and SC+K groups will present increases in tissue protein levels of antioxidant-related genes, increases in tissue GSH/GSSG levels, and increases in tissue total antioxidant levels. Moreover, I hypothesize that the aforementioned changes in the KD and SC+K groups will translate to reductions in tissue markers of oxidative stress, a reduction in mitochondrial ROS production, and improvements in mitochondrial function.

CHAPTER 3

METHODS

AIM 1 (1-week feedings)

Animals

All experimental procedures were approved by Auburn University's Institutional Animal Care and Use Committee (IACUC, protocol # 2016-2814). Male Fischer rats 4 months of age (~360 g) were purchased (Harlan Laboratories, Indianapolis, IN, USA) and allowed to acclimate in the animal housing facility for 1 week prior to experimentation. During acclimation, animals were provided standard (STD) rodent chow (24% protein, 58% CHO, 18% fat; 114 Teklad Global #2018 Diet, Harlan Laboratories) and water ad libitum in a maintained ambient temperature and constant 12 h light: 12 h dark cycle. For a 1-week period after acclimation, animals were provided isocaloric-isonitrogenous-isofibrous amounts of one of three diets:

- 1) 10 animals (KD) were provided with 12 g/d of a commercially designed KD (Harlan Tekland diet #10787) that was designed to induce nutritional ketosis. The diet specifications were as follows: 5.2 kcal/g, 23.1% protein, 9.6% carbohydrate, and 65.3% fat. Medium chain triglycerides, flaxseed oil and canola oil were prominent fat sources. Casein protein and cellulose powder was added to compensate for between group differences in protein and fiber content.
- 2) 10 animals (SC) were provided with 20 g/d of the aforementioned standard chow given during the acclimation phase.
- 3) 8 animals (SC+K) were provided with 20 g/d of the aforementioned standard chow, along with ad libitum sodium BHB salts added to water bottles. These ketone salts (5.8 kcal/g) were added to water bottles attempting to deliver ~1.2g/d.

Notably, body masses were measured every other day, and food weights were measured daily.

Necropsies and tissue preparation

On the morning of necropsies, rats were food-deprived for 6–10 h but provided water ad libitum. Animals were then transported from the campus vivarium to the School of Kinesiology and allowed to acclimate for 2 h. Thereafter, rats were euthanized under CO₂ gas in a 2-liter induction chamber (VetEquip, Pleasanton, CA). Following euthanasia, a final body mass was recorded, and blood was collected from the heart using a 22-gauge syringe. Collected blood was allowed to clot and placed in a 6-ml serum separator tube, was centrifuged at 3,500 g for 10 min, and resultant serum was aliquoted into 1.7-ml microcentrifuge tubes and stored at -80°C until analysis. The gastrocnemius, brain, and liver were dissected out, and tissue weights (excluding brain) were recorded using a calibrated scale with a sensitivity of 0.0001 g (Mettler-Toledo; Columbus, OH).

Following tissue weights, approximately 50 mg from the right gastrocnemius muscle/liver/brain was immediately placed in RNA/DNA Shield (Zymo Research, Irvine, CA) and stored at 4°C until RNA isolation using Trizol-based methods (described below). The remainder of the tissue was flash frozen in liquid nitrogen and stored at -80°C until total antioxidant analysis.

Tissue RNA isolation, cDNA synthesis and real-time polymerase chain reaction (RT-PCR)

Muscle/brain/liver stored in RNA/DNA Shield described above were placed in 10 volumes Ribozol (Ameresco, Solon, OH) in a 1.7-ml microcentrifuge tube and were homogenized with a tight-fitting pestle. Phase separation (for RNA isolation) was achieved according to manufacturer's instructions. Following RNA precipitation and pelleting, pellets

were resuspended in 30 μ l of RNase-free water, and RNA concentrations were determined in duplicate at an absorbance of 260 nm by using a NanoDrop Lite (Thermo Scientific, Waltham, MA). For cDNA synthesis, 1 μ g of muscle/liver/brain RNA was reverse transcribed into cDNA for rRT-PCR analyses with a commercial qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD). RT-PCR was performed with gene-specific primers and SYBR-green-based methods in a RT-PCR thermal cycler (Bio-Rad, Hercules, CA). Primers were designed with primer designer software (Primer3Plus, Cambridge, MA), and melt curve analyses demonstrated that one PCR product was amplified per reaction. The forward and reverse primer sequences are as follows: Glutamate-Cysteine Ligase Modifier Subunit (Gclm): forward primer 5'-ACATTGAAGCCCAGGAGTGG-3', reverse primer 5'-CGATGACCGAGTACCTCAGC-3'; Glutamate-Cysteine Ligase Catalytic Subunit (Gclc): forward primer 5'-GAGATGCCGTCTTACAGGGG-3', reverse primer 5'-TTGCTACACCCATCCACCAC-3'; NAD(P)H Quinone Dehydrogenase 1 (Nqo1): forward primer 5'-GTTTGCCTGGCTTGCTTTCA-3', reverse primer 5'-ACAGCCGTGGCAGAACTATC-3'; Heme oxygenase 1 (Hmox1): forward primer 5'-GAACTGTGGTCCGGTAGAGGC-3', reverse primer 5'-GGGGAAAGCAGTCATGGTCA-3'. Catalase (Cat): forward primer 5'-TTAACGCGCAGATCATGCA-3', reverse primer 5'-CAAGTTTTTGATGCCCTGGT-3'. Glutathione peroxidase (Gpx): forward primer 5'-TCTGCACACTCCCAGACAAG-3', reverse primer 5'-AGTCACCCATCACCGTCTTC-3'. Superoxide dismutase 2 (Sod2): forward primer 5'-TTAACGCGCAGATCATGCA-3', reverse primer 5'-CCTCGGTGACGTTTCAGATTGT-3'. Fold-change values from SC rats were performed by the Livak method (i.e., $2^{\Delta\Delta CT}$ assuming 100% primer binding efficiency), where $2^{\Delta\Delta CT} = [\text{housekeeping gene}]$

(HKG) CT - gene of interest CT] and $2\Delta\Delta CT$ (or fold-change) = $[2\Delta CT \text{ value for each rat}/2\Delta CT \text{ group average of SC}]$. Of note, 18S ribosomal rRNA (18S) was used as a HKG given that it remained stable across all treatments (primer sequence: forward primer 5'-AAACGGCTACCACATCCAAG-3', reverse primer 5'-CCTCCAATGGATCCTCGTTA-3').

Tissue total antioxidant and serum BHB analyses

Commercial colorimetric assay kits were used to determine muscle/liver/brain total antioxidant capacity (Antioxidant assay kit #709001; Cayman Chemical, Ann Arbor, MI) and serum BHB levels (BHB colorimetric assay kit #700190; Cayman Chemical), respectively, according to manufacturer's instructions. For total antioxidant analyses, approximately 100 mg of frozen muscle/brain/liver was homogenized in assay buffer and centrifuged according to manufacturer's instructions, and supernatants were assayed. Following assay execution, all plates were read in a UV-Vis microplate reader (BioTek Synergy H1 Multi-Mode Reader; BioTek, Winooski, VT) at absorbances according to manufacturer's recommendations.

AIM 2 (8-month feedings)

Animals

All experimental procedures were approved by Auburn University's Institutional Animal Care and Use Committee (IACUC, protocol # 2016-2814). Male Fischer rats 4 months of age (~360 g) were purchased (Harlan Laboratories, Indianapolis, IN, USA) and allowed to acclimate in the animal housing facility for 1 week prior to experimentation. During acclimation, animals were provided standard (STD) rodent chow (24% protein, 58% CHO, 18% fat; 114 Teklad

Global #2018 Diet, Harlan Laboratories) and water ad libitum in a maintained ambient temperature and constant 12 h light: 12 h dark cycle.

For an 8-month period after acclimation, animals were provided isocaloric-isonitrogenous-isofibrous amounts of one of three diets:

- 1) 8 animals (KD) were provided with 17 g/d of a commercially designed KD (Harlan Tekland diet #10787) that was designed to induce nutritional ketosis as described above. Following 8 weeks the food was reduced to 12 g/d to Calorie match them with the SC and SC+K groups described below.
- 2) 8 animals (SC) were provided with 25 g/d of the aforementioned SC. Animals were leaving, on average, ~5 g/d of food uneaten. Therefore, food was reduced to 20 g/d following 8 weeks of feeding in order to better Calorie match groups. Notably, one of these animals developed cancer towards the end of the study resulting in substantial weight loss and, thus, was not included in the analyses.
- 3) 8 animals (SC+K) were provided with 25 g/d of the aforementioned SC, along with ad libitum sodium BHB salts added to water bottles. These ketone salts (5.8 kcal/g) were added to water bottles attempting to deliver ~1.2 g/d for the first week, then 0.6 g/d for the remaining duration. Animals were leaving, on average, ~5 g/d of food uneaten. Therefore, food was reduced to 20 g/d following 8 weeks of feeding in order to better Calorie match groups. Notably, one of these animals inexplicably lost >20% body mass during the first two months of treatment and, thus, was not included in the analyses.

Notably, bodyweights were recorded weekly in these animals and residual food and water was measured daily.

Rotarod performance

Rotarod performance was assessed at 2 mo, 4 mo, 6 mo and 8 mo into the intervention using a single-lane device (Product#: ENV-571R; Med Associates Inc, Saint Albans City, VT, USA). Briefly, all assessments took place during the beginning of the rat light cycle (i.e., 0600-0800) whereby rats were placed on the device and the motorized rotor was initiated at a progressive speed from 4.0-40.0 revolutions per min. An automated timer tracked time spent on the rod and, once the rats fatigued and dismounted from the rod, a laser beam break stopped the timer. Notably, rotarod performance has been used in rodent studies to assess a combination of balance, grip strength, motor coordination and muscular endurance [113].

Necropsies

On the morning of necropsies, rats were food-deprived for 6–10 h but provided water ad libitum. Animals were then transported from the campus vivarium to the School of Kinesiology and allowed to acclimate for 2 h. Thereafter, rats were euthanized under CO₂ gas in a 2-liter induction chamber (VetEquip, Pleasanton, CA). Following euthanasia, a final body mass was recorded, and blood was collected from the heart using a 22-gauge syringe. Collected blood was placed in a 6-ml serum separator tube, blood was centrifuged at 3,500 g for 10 min, and resultant serum was then aliquoted into 1.7-ml microcentrifuge tubes and stored at -80°C until analysis. The gastrocnemius, brain, and liver were dissected out, and weights were recorded using a calibrated scale with a sensitivity of 0.0001 g (Mettler-Toledo; Columbus, OH).

Tissue preparation

The day of necropsies, approximately 800 mg from the right gastrocnemius muscle, 100 mg of liver and 500 mg of whole-brain (without cerebellum) was immediately used for mitochondria isolation as described below. The remainder of the tissue was flash frozen in liquid nitrogen and stored at -80°C until Western blotting, GSH/GSSG and total antioxidant capacity analyses described below.

Mitochondrial isolation, respiration assays, and mitochondrial ROS determination

The day of necropsies, differential centrifugation was used to isolate gastrocnemius, brain, and liver mitochondria from fresh tissue as described previously [114]. Mitochondrial oxygen consumption was measured as described by Messer et al. [115] in a respiration chamber maintained at 37°C (Hansatech Instruments). Isolated mitochondria were incubated with 1 ml of respiration buffer containing (in mM) 100 KCl, 5 KH₂PO₄, 1 EGTA, 50 MOPS, 10 MgCl₂, and 0.2% BSA at 37°C in a water-jacketed respiratory chamber with continuous stirring. Flux through complex I was measured using 2 mM pyruvate and 2 mM malate, whereas flux through complex II was measured using 5 mM succinate. Rotenone (5 µM) was added to prevent electron backflow to complex I in the succinate-driven experiments. The maximal respiration (state 3), defined as the rate of respiration in the presence of ADP, was initiated by adding 0.25 mM ADP to the respiration chamber containing mitochondria and respiratory substrates. State 4 respiration was recorded following the phosphorylation of ADP. The respiratory control ratio (RCR) was calculated by dividing state 3 by state 4 respiration.

Mitochondrial ROS production was determined using Amplex red (Molecular Probes, Eugene, OR). The assay was performed at 37°C in 96-well plates with succinate as the substrate. Specifically, this assay was developed on the concept that horseradish peroxidase catalyzes the

H₂O₂-dependent oxidation of nonfluorescent Amplex red to fluorescent resorufin red, and it is used to measure H₂O₂ as an indicator of superoxide production. SOD was added at 40 U/ml to convert all superoxide to H₂O₂. Using a multiwell-plate reader fluorometer (BioTek Synergy H1 Multi-Mode Reader; BioTek, Winooski, VT), we monitored resorufin formation at an excitation wavelength of 545 nm and a production wavelength of 590 nm. The level of resorufin formation was recorded every 5 min for 15 min, and H₂O₂ production was calculated with a standard curve.

Western blot analysis

Muscle/brain/liver was removed from -80°C storage and crushed on a liquid nitrogen-cooled stage. Approximately 50 mg of tissue from each tissue and was placed in 500 µl of 1x cell lysis buffer (Cell Signaling, Danvers, MA) with added protease inhibitors (1 µg/ml leupeptin) and phosphatase inhibitors (2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate) and was homogenized in microcentrifuge tubes using tight-fitting pestles. Samples were centrifuged at 500 g for 5 min at 4°C. Supernatants were then subjected to a protein assay using a commercial bicinchoninic acid assay (Thermo Scientific) and were prepared for Western blotting using 4× Laemmli reducing buffer at 2 µg/µl. Subsequently, 20 µl of prepped samples were loaded onto precast 12% SDS-polyacrylamide gels (Bio-Rad) and were subjected to electrophoresis (200 V at 75 min) using premade 1x SDS-PAGE running buffer (C.B.S. Scientific, San Diego, CA). Proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad), and membranes were stained with Ponceau S following transfers to ensure even loading and transfer between samples. Membranes were then blocked with 5% nonfat milk powder diluted in TBS with 0.1% Tween-20 (TBST) for 1 h at room temperature. Primary antibodies directed against the proteins of interest were incubated with membranes overnight at 4°C in TBST with 5% BSA

added. The primary antibodies were used to detect whole-tissue 4-hydroxynonenal-conjugated proteins (4-HNE, # ab46545; Abcam, Cambridge, MA), SOD2 (GeneTex), Catalase (GeneTex), glutathione peroxidase (GPx; GeneTex) and protein carbonyls (Oxyblot kit # S7150; Millipore; Bellirica, MA). On the following day, membranes were incubated with anti-rabbit or anti-mouse IgG secondary antibodies diluted in TBST with 5% BSA added (1:2,000; Cell Signaling) at room temperature for 1 h prior to membrane development. Membrane development was accomplished by using an enhanced chemiluminescent reagent (Millipore), and band densitometry was achieved with the use of a digitized gel documentation system and associated densitometry software (UVP, Upland CA). All protein band densities were normalized to Ponceau stain densities. All Western blot analysis data are presented as arbitrary density units.

Citrate synthase activity assays

Muscle/brain/liver tissue homogenate citrate synthase activities were performed as previously described by our laboratory [14]. Briefly, 40 μ g of tissue lysate (obtained from cell lysis described above) were loaded onto 96-well plates. Subsequently, citrate synthase (EC 4.1.3.7) was measured as a function of the increase in absorbance from 5,5'-dithiobis-2-nitrobenzoic acid reduction following the methods described elsewhere [116].

Tissue total antioxidant, serum BHB and serum/tissue GSH/GSSG analyses

Commercial colorimetric assay kits were used to determine muscle/liver/brain total antioxidant capacity (Antioxidant assay kit #709001; Cayman Chemical), muscle/liver/brain GSH/GSSG levels (Glutathione assay kit #703002; Cayman Chemical), and serum BHB levels (BHB colorimetric assay kit #700190; Cayman Chemical), respectively, according to

manufacturer's instructions. For total antioxidant analyses, approximately 100 mg of frozen muscle/brain/liver was homogenized in assay buffer and centrifuged according to manufacturer's instructions, and supernatants were assayed. Following assay execution, plates were read in a UV-vis microplate reader at absorbances according to manufacturer's recommendations.

Statistical Analysis

The independent variable included dietary treatments (SC versus KD versus SC+K).

Criterion dependent variables included the following:

AIM 1 animals (1-week feedings)

- 1) Serum BHB levels (to confirm nutritional ketosis)
- 2) Muscle/brain/liver mRNA expression levels of antioxidant-related genes (Gclc, Gclm, Hmox1, Nqo1, Cat, Sod2, Gpx)
- 3) Muscle/brain/liver total antioxidant capacity

Secondary dependent variables in this aim (not critical to the research question related to oxidative stress) will include 3-day body masses and indices of body composition (i.e., select muscle and fat pad masses).

AIM 2 animals (8-month feedings)

- 1) Serum BHB levels (to confirm nutritional ketosis)
- 2) Muscle/brain/liver protein expression levels of antioxidant-related genes (Cat, Sod2, Gpx)
- 3) Muscle/brain/liver/serum GSH/GSSG levels
- 4) Muscle/brain/liver total antioxidant capacity levels
- 5) Muscle/brain/liver markers of oxidative stress (4HNE and protein carbonyl levels)

6) Muscle/brain/liver mitochondrial ROS levels

7) Muscle/brain/liver markers of mitochondrial function (state III/IV respiration rates)

Secondary dependent variables in this aim (not critical to the research question related to oxidative stress) will include weekly body masses and indices of body composition (i.e., select muscle and fat pad masses).

Statistics will be performed using IBM SPSS version 22.0, and one-way ANOVAs will be performed on dependent variables in AIMS 1&2 with Tukey post hoc tests being performed when the ANOVA $p < 0.05$. All data will be presented as means \pm standard error.

CHAPTER IV

MANUSCRIPT TO BE SUBMITTED

The 1-week and 8-month effects of a ketogenic diet or ketone salt supplementation on multi-organ markers of oxidative stress and mitochondrial function in rats

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ABSTRACT

Purpose: Herein we sought to examine the short-term effects (i.e., one-week) and long-term effects (i.e., 8-month) effects of a ketogenic diet (KD) or ketone salt supplementation on markers of multi-organ markers of oxidative stress and mitochondrial function. **Methods:** In AIM 1, male Fisher rats (4 mo old) were provided isocaloric amounts of KD (5.2 kcal/g, 23.1% protein, 9.6% carbohydrate, and 65.3% fat, n=10), SC (3.1 kcal/g, 24% protein, 58% carbohydrate, 18% fat; n=20) for 7 days. The SC rats were split into sub-groups whereby one group was provided ketone salts in their drinking water (SC+KS ~1.2 g/day, n=10), and one group was un-supplemented (SC, n=10). In AIM 2, male Fisher rats (4 mo old) were provided the KD (n=8), SC (n=7) and SC+KS (n=7) for 8 months. Following respective feeding schemes for AIMs 1 and 2, rats were euthanized, blood was obtained and serum was aliquoted, and brain, liver and gastrocnemius muscle was procured for mRNA and/or protein analyses. In AIM 2, brain, muscle and liver mitochondria were also freshly isolated for mitochondrial respiration and reactive oxygen species (ROS) assays. **Results:** Serum ketone levels were greatest in KD rats in the one-week and 8-mo interventions relative to the SC and SC+KS groups suggesting that the diet, but not salt supplementation, induced a ketogenic state. Both short-term and long-term ketogenic dieting produced blunted weight gain and feed efficiency when comparing KD to SC and SC+KS rats ($p<0.001$ and $p<0.001$, respectively). Notwithstanding, muscle/brain/liver expression of numerous oxidative stress-related genes were not different between groups following the one-week study. Likewise, muscle/brain/liver protein expression of glutathione peroxidase, superoxide dismutase 2, and catalase as well as protein carbonyl and 4-hydroxynonenal levels were not different between groups following the 8 mo study. In the 8 mo study, gastrocnemius mitochondrial ROS production was higher in KD gastrocnemius versus other treatments ($p=0.007$), and this may have been related to a decreased state III respiration

and respiratory control ratio in this tissue relative to other groups ($p=0.072$ and $p=0.018$, respectively). Moreover, gastrocnemius citrate synthase activity (a surrogate of mitochondrial density) was lowest in KD rats versus the SC and SC+KS groups ($p<0.001$). However, despite these gastrocnemius deficits, rotarod performance was greatest in KD rats versus the other groups at 2 mo, 4 mo and 8 mo into the intervention. **Conclusions:** Our data suggest that ketogenic dieting, but not ketone salt supplementation, reduces feed efficiency and body mass acutely and chronically. No changes in antioxidant muscle/brain/liver gene or protein expression were elicited by short-term or long-term ketogenic dieting or ketone salt supplementation. Interestingly, chronic ketogenic dieting elicits an increase in skeletal muscle mitochondrial ROS formation and, despite the lack of oxidative damage (i.e., protein carbonyl and 4HNE production), this paralleled decreases in mitochondrial quantity and quality markers. Notwithstanding, these skeletal muscle deficits did not translate into a decline in muscular endurance and/or grip strength with long-term ketogenic dieting.

Keywords: ketogenic dieting, ketone salts, skeletal muscle, brain, liver, oxidative stress, mitochondria

INTRODUCTION

The ketogenic diet (KD) is a high-fat, moderate protein, low carbohydrate diet initially utilized to treat seizures. Many investigations have revealed this dietary approach to be just as effective as currently available anticonvulsant medications [1, 2]. Additionally, the KD has been associated with a myriad of general health benefits including weight loss and management as well as positive outcomes in regards to treating diabetes, polycystic ovaryian syndrome, acne, a wide array of neurological complications, cancer, respiratory and cardiovascular disease, fertility, and increasing longevity [3, 4]. Concerning the latter, KD has been shown to either confer normal longevity or enhance life span in murine models [5, 6], albeit the specific mechanism of action has not been well elucidated.

One of the prevailing ideas for the numerous benefits of the KD is related to augmented mitochondrial function and protection. Excessive accretion of reactive oxygen species (ROS) have been purported as one of the hallmarks of the aging process as well as reduced functionality of various cellular proteins [7, 8]. Mitochondria bear the brunt of oxidative damage, and lesions to mitochondrial DNA have been reported as much as 10x higher than in nuclear DNA and accumulates with age [9]. Mitochondrial DNA damage occur in excess because DNA repair is less efficient within the mitochondria, and mitochondrial DNA is not bound to histones or DNA-binding proteins [10]. There is currently data indicating mitochondrial functional decline with age [11, 12]. As such, a manipulation that can reduce or ameliorate mitochondrial oxidative stress may serve to benefit the aging process and general cellular function.

Interestingly, oxidative stress defense and improved mitochondrial quantity/quality are potential mechanisms by which the KD may confer a metabolic advantage [13, 14]. For instance, hippocampal mitochondrial biogenesis in rodents has been reported after four weeks of

KD feeding [15]; this being a phenomena which could occur via KD-induced AMPK pathway signaling [16, 17]. KD treatments have also been shown to decrease ROS and hydrogen peroxide production in rodent hippocampi by increasing uncoupling protein-mediated proton conductance and increased glutathione biosynthesis [18, 19]. The KD-induced reduction in ROS production is potentially mediated via increased mitochondrial density, uncoupling protein expression and/or an increased glutathione (GSH) biosynthesis and activity [15, 18-20]. With regard to the latter, Milder and Patel [13] theorized that KD-induced ROS defense is related to an acute stimulation of 4-hydroxy-2-nonenal (4HNE) or hydrogen peroxide which, in turn, stimulates the NF E2-related factor 2 (Nrf2) transcription factor to translocate into the nucleus and prompt the transcription of antioxidant related genes. Namely, Nrf2 stimulates the mRNA expression of subunits of glutamate-cysteine ligase (GCL), the rate limiting enzyme in glutathione biosynthesis [21, 22]. In prior work, Milder and colleagues [23] documented that acute KD feeding does in fact stimulate hydrogen peroxide and 4HNE production in the hippocampus of rodents and, in a temporal fashion, this led to Nrf2 nuclear translocation and an increase in the expression of ROS-protective proteins that promote a REDOX state.

From this, the current body of literature supports a KD in terms of stimulating a cellular environment that is more resistant to oxidative stress. However, some data indicate that it is not the KD entirely, but rather the metabolites produced during ketogenic dieting that confer the aforementioned benefits. The ketone bodies, beta-hydroxybutyrate (BHB) and acetoacetate (AcAc), are the consequent metabolites from carbohydrate restriction and lipolysis. These substrates have been introduced in cell culture models with subsequent results of decreased ROS production and resistance to a hydrogen peroxide-induced stress [24, 25]. Thus, it is possible that

the ketone bodies have a direct causative effect in terms of reducing production and enhancing handling of ROS.

Based on the aforementioned documented observations, ketogenic dieting and/or dietary ketone supplementation seemingly have the potential to reduce ROS production over the lifespan, and this phenomena could potentially lead to improved mitochondrial quality. However, there is currently a lack of literature detailing the chronic effects of KD or ketone salt ingestion as it relates to markers of mitochondrial function and oxidative stress beyond what has been extensively reported in the hippocampus. Therefore, the purpose of this investigation is to examine if KD feeding or BHB salt supplementation affects skeletal muscle, brain (entire cortex) and/or liver measures of oxidative stress and mitochondrial function over an acute time frame (i.e., 1 week) or over the course of 8 months.

METHODS

AIM 1 (1-week experiment)

Animals

All experimental procedures were approved by Auburn University's Institutional Animal Care and Use Committee (IACUC, protocol # 2016-2814). Male Fischer rats 4 months of age (~360 g) were purchased (Harlan Laboratories, Indianapolis, IN, USA) and allowed to acclimate in the animal housing facility for 1 week prior to experimentation. During acclimation, animals were provided standard rodent chow (SC; 24% protein, 58% CHO, 18% fat; Teklad Global #2018 Diet, Harlan Laboratories) and water ad libitum in a maintained ambient temperature and constant 12 h light: 12 h dark cycle. For a 1-week period after acclimation, animals were provided isocaloric-isonitrogenous-isofibrous amounts of one of three diets:

- 4) 10 animals (KD) were provided with 12 g/d of a commercially designed KD (Harlan Tekland diet #10787) that was designed to induce nutritional ketosis. The diet specifications were as follows: 5.2 kcal/g, 23.1% protein, 9.6% carbohydrate, and 65.3% fat. Medium chain triglycerides, flaxseed oil and canola oil were prominent fat sources. Casein protein and cellulose powder was added to compensate for between group differences in protein and fiber content.
- 5) 10 animals (SC) were provided with 20 g/d of the aforementioned standard chow given during the acclimation phase.
- 6) 10 animals (SC+KS) were provided with 20 g/d of the aforementioned standard chow, along with ad libitum sodium BHB salts added to water bottles. These ketone salts (5.8 kcal/g) were added to water bottles attempting to deliver ~1.2 g/d.

Notably, body masses were measured every other day, and food weights were measured daily.

Necropsies and tissue preparation

On the morning of necropsies, rats were food-deprived for 6–10 h but provided water ad libitum. Animals were then transported from the campus vivarium to the School of Kinesiology and allowed to acclimate for 2 h. Thereafter, rats were euthanized under CO₂ gas in a 2 L induction chamber (VetEquip, Pleasanton, CA). Following euthanasia, a final body mass was recorded, and blood was collected from the heart using a 22-gauge syringe. Collected blood was allowed to clot and placed in a 6-ml serum separator tube, was centrifuged at 3,500 g for 10 min, and resultant serum was aliquoted into 1.7-ml microcentrifuge tubes and stored at -80°C until analysis. The gastrocnemius, brain, and liver were dissected out, and tissue weights (excluding brain) were recorded using a calibrated scale with a sensitivity of 0.0001 g (Mettler-Toledo; Columbus, OH).

Following tissue weights, approximately 50 mg from the right gastrocnemius muscle/liver/brain was immediately placed in RNA/DNA Shield (Zymo Research, Irvine, CA) and stored at 4°C until RNA isolation using Trizol-based methods (described below). The remainder of the tissue was flash frozen in liquid nitrogen and stored at -80°C until total antioxidant analysis.

Tissue RNA isolation, cDNA synthesis and real-time polymerase chain reaction (RT-PCR)

Muscle/brain/liver stored in RNA/DNA Shield described above were placed in 10 volumes Ribozol (Ameresco, Solon, OH) in a 1.7-ml microcentrifuge tube and were homogenized with a tight-fitting pestle. Phase separation (for RNA isolation) was achieved according to manufacturer's instructions. Following RNA precipitation and pelleting, pellets were resuspended in 30 µl of RNase-free water, and RNA concentrations were determined in duplicate at an absorbance of 260 nm by using a NanoDrop Lite (Thermo Scientific, Waltham, MA). For cDNA synthesis, 1 µg of muscle/liver/brain RNA was reverse transcribed into cDNA for rRT-PCR analyses with a commercial qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD). RT-PCR was performed with gene-specific primers and SYBR-green-based methods in a RT-PCR thermal cycler (Bio-Rad, Hercules, CA). Primers were designed with primer designer software (Primer3Plus, Cambridge, MA), and melt curve analyses demonstrated that one PCR product was amplified per reaction. The forward and reverse primer sequences are as follows: Glutamate-Cysteine Ligase Modifier Subunit (Gclm): forward primer 5'-ACATTGAAGCCCAGGAGTGG-3', reverse primer 5'-CGATGACCGAGTACCTCAGC-3'; Glutamate-Cysteine Ligase Catalytic Subunit (Gclc): forward primer 5'-GAGATGCCGTCTTACAGGGG-3', reverse primer 5'-TTGCTACACCCATCCACCAC-3';

NAD(P)H Quinone Dehydrogenase 1 (Nqo1): forward primer 5'-
GTTTGCCTGGCTTGCTTTCA-3', reverse primer 5'- ACAGCCGTGGCAGAACTATC-3';
Heme oxygenase 1 (Hmox1): forward primer 5'-GAACTGTGGTCCGGTAGAGGC-3', reverse
primer 5'-GGGGAAAGCAGTCATGGTCA-3'. Catalase (Cat): forward primer 5'-
TTAACGCGCAGATCATGCA-3', reverse primer 5'- CAAGTTTTTGATGCCCTGGT-3'.
Glutathione peroxidase (Gpx): forward primer 5'- TCTGCACACTCCCAGACAAG-3', reverse
primer 5'- AGTCACCCATCACCGTCTTC-3'. Superoxide dismutase 2 (Sod2): forward primer
5'- TTAACGCGCAGATCATGCA-3', reverse primer 5'-CCTCGGTGACGTTTCAGATTGT-3'.
Fold-change values from SC rats were performed by the Livak method (i.e., $2^{\Delta\Delta CT}$ assuming
100% primer binding efficiency), where $2^{\Delta CT} = [\text{housekeeping gene}$
(HKG) CT - gene of interest CT] and $2^{\Delta\Delta CT}$ (or fold-change) = $[2^{\Delta CT}$ value for each rat/ $2^{\Delta CT}$
group average of SC]. Of note, 18S ribosomal rRNA (18S) was used as a HKG given that it
remained stable across all treatments (primer sequence: forward primer 5'-
AAACGGCTACCACATCCAAG-3', reverse primer 5'- CCTCCAATGGATCCTCGTTA-3').

Tissue total antioxidant and serum BHB assays

Commercial colorimetric assay kits were used to determine muscle/liver/brain total antioxidant capacity (Antioxidant assay kit #709001; Cayman Chemical, Ann Arbor, MI) and serum BHB levels (BHB colorimetric assay kit #700190; Cayman Chemical), respectively, according to manufacturer's instructions. For total antioxidant analyses, approximately 100 mg of frozen muscle/brain/liver was homogenized in assay buffer and centrifuged according to manufacturer's instructions, and supernatants were assayed. Following assay execution, all plates

were read in a UV-Vis microplate reader (BioTek Synergy H1 Multi-Mode Reader; BioTek, Winooski, VT) at absorbances according to manufacturer's recommendations.

AIM 2 (8-month feedings)

Animals

All experimental procedures were approved by Auburn University's Institutional Animal Care and Use Committee (IACUC, protocol # 2016-2814). Male Fischer rats 4 months of age (~360 g) were purchased (Harlan Laboratories, Indianapolis, IN, USA) and allowed to acclimate in the animal housing facility for 1 week prior to experimentation. During acclimation, animals were provided standard (SC) rodent chow (24% protein, 58% CHO, 18% fat; 114 Teklad Global #2018 Diet, Harlan Laboratories) and water ad libitum in a maintained ambient temperature and constant 12 h light: 12 h dark cycle.

For an 8-month period after acclimation, animals were provided isocaloric-isonitrogenous-isofibrous amounts of one of three diets:

- 4) 8 animals (KD) were provided with 17 g/d of a commercially designed KD (Harlan Tekland diet #10787) that was designed to induce nutritional ketosis as described above. Following 8 weeks the food was reduced to 12 g/d to Calorie match them with the SC and SC+K groups described below.
- 5) 8 animals (SC) were provided with 25 g/d of the aforementioned SC. Animals were leaving, on average, ~5 g/d of food uneaten. Therefore, food was reduced to 20 g/d following 8 weeks of feeding in order to better Calorie match groups. Notably, one of these animals developed cancer towards the end of the study resulting in substantial weight loss and, thus, was not included in the analyses.

6) 8 animals (SC+K) were provided with 25 g/d of the aforementioned SC, along with ad libitum sodium BHB salts added to water bottles. These ketone salts (5.8 kcal/g) were added to water bottles attempting to deliver ~1.2 g/d for the first week, then 0.6 g/d for the remaining duration. Animals were leaving, on average, ~5 g/d of food uneaten. Therefore, food was reduced to 20 g/d following 8 weeks of feeding in order to better Calorie match groups. Notably, one of these animals inexplicably lost >20% body mass during the first two months of treatment and, thus, was not included in the analyses.

Notably, bodyweights were recorded weekly in these animals and residual food and water was measured daily.

Rotarod performance

Rotarod performance was assessed at 2 mo, 4 mo, 6 mo and 8 mo into the intervention using a single-lane device (Product#: ENV-571R; Med Associates Inc, Saint Albans City, VT, USA). Briefly, all assessments took place during the beginning of the rat light cycle (i.e., 0600-0800) whereby rats were placed on the device and the motorized rotor was initiated at a progressive speed from 4.0-40.0 revolutions per min. An automated timer tracked time spent on the rod and, once the rats fatigued and dismounted from the rod, a laser beam break stopped the timer. Notably, rotarod performance has been used in rodent studies to assess a combination of balance, grip strength, motor coordination and muscular endurance [113].

Necropsies and tissue preparation

Necropsies were carried out exactly as detailed in AIM 1 with the exception being that approximately 800 mg from the right gastrocnemius muscle, 100 mg of liver and 500 mg of whole-brain (without cerebellum) was immediately used for mitochondria isolation as described below. The remainder of the tissue was flash frozen in liquid nitrogen and stored at -80°C until Western blotting, GSH/GSSG and total antioxidant capacity analyses described below.

Mitochondrial isolation, respiration assays, and mitochondrial ROS determination

The day of necropsies, differential centrifugation was used to isolate gastrocnemius, brain, and liver mitochondria from fresh tissue as described previously [114]. Mitochondrial oxygen consumption was measured as described by Messer et al. [115] in a respiration chamber maintained at 37°C (Hansatech Instruments). Isolated mitochondria were incubated with 1 ml of respiration buffer containing (in mM) 100 KCl, 5 KH₂PO₄, 1 EGTA, 50 MOPS, 10 MgCl₂, and 0.2% BSA at 37°C in a water-jacketed respiratory chamber with continuous stirring. Flux through complex I was measured using 2 mM pyruvate and 2 mM malate, whereas flux through complex II was measured using 5 mM succinate. Rotenone (5 µM) was added to prevent electron backflow to complex I in the succinate-driven experiments. The maximal respiration (state 3), defined as the rate of respiration in the presence of ADP, was initiated by adding 0.25 mM ADP to the respiration chamber containing mitochondria and respiratory substrates. State 4 respiration was recorded following the phosphorylation of ADP. The respiratory control ratio (RCR) was calculated by dividing state 3 by state 4 respiration.

Mitochondrial ROS production was determined using Amplex red (Molecular Probes, Eugene, OR). The assay was performed at 37°C in 96-well plates with succinate as the substrate. Specifically, this assay was developed on the concept that horseradish peroxidase catalyzes the

H₂O₂-dependent oxidation of nonfluorescent Amplex red to fluorescent resorufin red, and it is used to measure H₂O₂ as an indicator of superoxide production. SOD was added at 40 U/ml to convert all superoxide to H₂O₂. Using a multiwell-plate reader fluorometer (BioTek Synergy H1 Multi-Mode Reader; BioTek, Winooski, VT), we monitored resorufin formation at an excitation wavelength of 545 nm and a production wavelength of 590 nm. The level of resorufin formation was recorded every 5 min for 15 min, and H₂O₂ production was calculated with a standard curve.

Western blot analysis

Muscle/brain/liver was removed from -80°C storage and crushed on a liquid nitrogen-cooled stage. Approximately 50 mg of tissue from each tissue and was placed in 500 µl of 1x cell lysis buffer (Cell Signaling, Danvers, MA) with added protease inhibitors (1 µg/ml leupeptin) and phosphatase inhibitors (2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate) and was homogenized in microcentrifuge tubes using tight-fitting pestles. Samples were centrifuged at 500 g for 5 min at 4°C. Supernatants were then subjected to a protein assay using a commercial bicinchoninic acid assay (Thermo Scientific) and were prepared for Western blotting using 4× Laemmli reducing buffer at 2 µg/µl. Subsequently, 20 µl of prepped samples were loaded onto precast 12% SDS-polyacrylamide gels (Bio-Rad) and were subjected to electrophoresis (200 V at 75 min) using premade 1x SDS-PAGE running buffer (C.B.S. Scientific, San Diego, CA). Proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad), and membranes were stained with Ponceau S following transfers to ensure even loading and transfer between samples. Membranes were then blocked with 5% nonfat milk powder diluted in TBS with 0.1% Tween-20 (TBST) for 1 h at room temperature. Primary antibodies directed against the proteins of interest were incubated with membranes overnight at 4°C in TBST with 5%

BSA added. Primary antibodies were used to detect whole-tissue 4-hydroxynonenal-conjugated proteins (4-HNE, # ab46545; Abcam, Cambridge, MA), superoxide dismutase 2 (SOD2; GeneTex), Catalase (Cat; GeneTex), glutathione peroxidase (Gpx; GeneTex) and protein carbonyls (Oxyblot kit # S7150; Millipore; Bellirica, MA). On the following day, membranes were incubated with anti-rabbit or anti-mouse IgG secondary antibodies diluted in TBST with 5% BSA added (1:2,000; Cell Signaling) at room temperature for 1 h prior to membrane development. Membrane development was accomplished by using an enhanced chemiluminescent reagent (Millipore), and band densitometry was achieved with the use of a digitized gel documentation system and associated densitometry software (UVP, Upland CA). All protein band densities were normalized to Ponceau stain densities. All Western blot analysis data are presented as arbitrary density units.

Citrate synthase activity assays

Muscle/brain/liver tissue homogenate citrate synthase activities were performed as previously described by our laboratory [14]. Briefly, 40 μ g of tissue lysate (obtained from cell lysis described above) were loaded onto 96-well plates. Subsequently, citrate synthase activity was measured as a function of the increase in absorbance from 5,5'-dithiobis-2-nitrobenzoic acid reduction following the methods described elsewhere [116].

Tissue total antioxidant, serum BHB and liver mitochondrial glutathione assays

As described in AIM 1, commercial colorimetric assay kits were used to determine muscle and liver total antioxidant capacity (Antioxidant assay kit #709001; Cayman Chemical), and serum BHB levels (BHB colorimetric assay kit #700190; Cayman Chemical), respectively, according to

manufacturer's instructions. Notably, given that brain tissue was devoted to mitochondrial assays and Western blotting, there was not an adequate amount of tissue remaining for brain total antioxidant capacity determination. Liver mitochondrial oxidized (GSSG) and total glutathione levels were determined using a commercial colorimetric assay (Glutathione colorimetric assay kit #700190; Cayman Chemical) according to manufacturer's instructions, and reduced glutathione (GSH) levels was extrapolated from these values. Notably, muscle and brain glutathione analyses were attempted but not reported due to values being on the lower-end of the assay's sensitivity curve.

Statistical Analysis

Statistics were performed using IBM SPSS version 22.0, and one-way ANOVAs were performed on dependent variables in AIMS 1&2 with Tukey post hoc tests being performed when the ANOVA $p < 0.05$. All data are presented as means \pm standard error.

RESULTS

AIM 1: Effects of 1-wk intervention on body mass change, feed efficiency and serum BHB levels

By day 7, change in body mass was significantly lower in KD versus SC and SC+KS rats ($p=0.036$ and $p<0.001$, respectively) (Figure 1a). Feed efficiency (g body mass gain/kcal consumed) followed a similar pattern whereby KD rats presented significantly lower values compared to SC+KS rats ($p<0.001$), and differences between KD and SC rats approached significance ($p=0.070$) (Figure 1b). Serum BHB levels were greater in KD versus SC+KS rats ($p=0.034$), but not different between KD and SC rats ($p=0.140$) (Figure 1c).

INSERT FIGURE 1 HERE

AIM 1: Effects of 1-wk intervention on muscle/brain/liver oxidative stress-related mRNAs

Gastrocnemius muscle gene expression for both subunits of GCL was similar across groups (Gclc ANOVA $p=0.349$; Gclm ANOVA $p=0.236$). One-way ANOVA analysis indicated a significant difference between treatments regarding muscle Nqo1 mRNA expression patterns (ANOVA $p=0.049$), albeit post-hoc analysis indicated that lower values in KD versus SC rats approached significance ($p=0.060$). Other gastrocnemius antioxidant-related genes were not differentially expressed between groups (Hmox1, ANOVA $p=0.789$; Gsr, ANOVA $p=0.079$; Gpx1, ANOVA $p=0.688$; Cat, ANOVA $p=0.213$; Sod2, ANOVA $p=0.122$; Figure 2a).

Oxidative stress-related genes were not differentially expressed in the brain (Gclc, ANOVA $p=0.831$; Gclm, ANOVA $p=0.505$; Nqo1, ANOVA $p=0.580$; Hmox1, ANOVA $p=0.811$; Gsr, ANOVA $p=0.909$; Gpx1, ANOVA $p=0.194$; Cat, ANOVA $p=0.741$; Sod2, ANOVA $p=0.290$; Figure 2b). Gclc, Gclm and Hmox1 were not expressed differently in liver tissue (ANOVA $p=0.402$, ANOVA $p=0.584$, and ANOVA $p=0.657$, respectively). Liver Nqo1 mRNA was 2.3-fold higher relative to the SC and KD groups ($p=0.047$ and $p=0.053$, respectively; Figure 2c).

Other liver antioxidant-related genes were not differentially expressed between groups (Gclc, ANOVA $p=0.402$; Gclm ANOVA $p=0.584$; Hmox1, ANOVA $p=0.657$; Gsr, ANOVA $p=0.134$; Gpx1, ANOVA $p=0.636$; Cat, ANOVA $p=0.786$; Sod2, ANOVA $p=0.126$; Figure 2c).

INSERT FIGURE 2 HERE

AIM 1: Effects of 1-wk intervention on muscle/brain/liver total antioxidant capacity

Gastrocnemius antioxidant capacity was significantly lower in KD versus SC+KS rats ($p=0.019$), but was not different between the KD and SC groups ($p=0.275$) or SC and SC+KS groups ($p=0.366$; Figure 3). Brain antioxidant capacity was not different between treatments (ANOVA $p=0.920$; Figure 3). One-way ANOVA analysis indicated a significant difference between treatments regarding liver antioxidant capacity (ANOVA $p=0.040$), albeit lower values in the SC group only approached significance compared to KD ($p=0.056$) and SC+KS rats ($p=0.083$) (Figure 3).

INSERT FIGURE 3 HERE

AIM 2: Effects of 8 mo intervention on body masses, feed efficiency and serum BHB

Regarding weekly body masses, KD rats weighed less than SC and SC+KS rats from 10 weeks to 30 weeks ($p<0.05$ at all time points; Figure 4a). Feed efficiency over the 8 mo intervention (g body mass gain/kcal consumed) followed a similar pattern whereby KD rats presented significantly lower values compared to SC+KS rats ($p<0.001$) and SC rats ($p<0.001$) (Figure 4b). Serum BHB levels were greater in KD versus SC rats ($p<0.001$) and SC+KS rats ($p<0.001$) (Figure 4c).

INSERT FIGURE 4 HERE

AIM 2: Effects of 8 mo intervention on rotarod performance

In AIM 2 rats, rotarod performance was assessed at 2 mo, 4 mo, 6 mo and 8 mo into the intervention (Figure 5). At 2 mo, performance was greater in the KD versus the SC ($p < 0.001$) and SC+KS groups ($p = 0.009$). At 4 mo, performance was greater in the KD versus the SC ($p = 0.017$) group, and approached significance in the KD versus SC+KS group ($p = 0.060$). One-way ANOVA analysis indicated no significant difference between treatments at 6 mo (ANOVA $p = 0.085$). At 8 mo, performance was greater in the KD versus the SC ($p = 0.008$) and SC+KS groups ($p = 0.049$).

INSERT FIGURE 5 HERE

AIM 2: Effects of 8 mo intervention on gastrocnemius oxidative stress-related proteins and markers

Gastrocnemius protein expression levels of Cat (ANOVA $p = 0.934$), Gpx (ANOVA $p = 0.655$) and Sod2 (ANOVA $p = 0.368$) were similar between groups (Figure 6a). Likewise, gastrocnemius 4HNE (ANOVA $p = 0.328$) and protein carbonyl levels (ANOVA $p = 0.820$) were similar between groups (Figure 6b).

INSERT FIGURE 6 HERE

AIM 2: Effects of 8 mo intervention on brain oxidative stress-related proteins and markers

Brain protein expression levels of Cat (ANOVA $p = 0.545$), Gpx (ANOVA $p = 0.105$) and Sod2 (ANOVA $p = 0.200$) were similar between groups (Figure 7a). Likewise, brain 4HNE

(ANOVA $p=0.537$) and protein carbonyl levels (ANOVA $p=0.939$) were similar between groups (Figure 7b).

INSERT FIGURE 7 HERE

AIM 2: Effects of 8 mo intervention on liver oxidative stress-related proteins and markers

Liver protein expression levels of Cat (ANOVA $p=0.616$), Gpx (ANOVA $p=0.681$) and Sod2 (ANOVA $p=0.368$) were similar between groups (Figure 8a). Likewise, liver 4HNE (ANOVA $p=0.684$) and protein carbonyl levels (ANOVA $p=0.327$) were similar between groups (Figure 8b).

INSERT FIGURE 8 HERE

AIM 2: Effects of 8 mo intervention on tissue total antioxidant capacity levels

Gastrocnemius total antioxidant capacity levels were not different between groups (ANOVA $p=0.146$). Liver total antioxidant capacity levels were greater in KD versus SC rats ($p=0.028$), but not KD versus SC+KS rats ($p=0.216$). Brain samples were limited due to other assays and were, thus, not performed for this assay.

INSERT FIGURE 9 HERE

AIM 2: Effects of 8 mo intervention on liver mitochondrial glutathione levels

Liver mitochondrial oxidized (GSSG) glutathione (ANOVA $p=0.117$), reduced (GSH) glutathione (ANOVA $p=0.699$) and total glutathione (ANOVA $p=0.321$) were not different

between groups (Figure 10a). Likewise, the redox ratio (GSH/GSSG) was not different between groups (ANOVA $p=0.492$). These assays were attempted in gastrocnemius brain samples, but assay values were on the lower-end of the assay sensitivity curve and, thus, are not presented.

INSERT FIGURE 10 HERE

AIM 2: Effects of 8 mo intervention on tissue mitochondrial ROS production

Gastrocnemius mitochondrial ROS production was higher in KD rats versus the SC ($p=0.014$) and SC+KS groups ($p=0.015$) (Figure 11). Mitochondrial ROS production in the brain (ANOVA $p=0.162$) and liver (ANOVA $p=0.222$) were similar between groups (Figure 11).

INSERT FIGURE 11 HERE

AIM 2: Effects of 8 mo intervention on gastrocnemius mitochondrial function

The 8-mo intervention had a tendency to affect gastrocnemius pyruvate-malate state 3 (ANOVA $p=0.072$) whereby lower values in KD versus SC rats approached significance ($p=0.064$) (Figure 12a). Gastrocnemius pyruvate-malate state 4 was not different between groups ($p=0.119$; Figure 12 b). Gastrocnemius pyruvate-malate RCR values were lower in KD versus SC rats ($p=0.030$) and SC+KS versus SC rats ($p=0.035$) (Figure 12c). Gastrocnemius succinate state 3 (ANOVA $p=0.706$; Figure 12d), succinate state 4 (ANOVA $p=0.500$; Figure 12e) and succinate RCR values (ANOVA $p=0.582$; Figure 12f) were not different between groups.

INSERT FIGURE 12 HERE

AIM 2: Effects of 8 mo intervention on brain mitochondrial function

Brain pyruvate-malate state 3 (ANOVA $p=0.706$; Figure 13a), pyruvate-malate state 4 (ANOVA $p=0.500$; Figure 13b) and pyruvate-malate RCR values (ANOVA $p=0.582$; Figure 13c) were not different between groups. Notably, brain succinate state 3, state 4 and RCR values are not reported given that a reliably state 4 could not be obtained.

INSERT FIGURE 13 HERE

AIM 2: Effects of 8 mo intervention on liver mitochondrial function

Liver pyruvate-malate state 3 (ANOVA $p=0.466$; Figure 14a), pyruvate-malate state 4 (ANOVA $p=0.364$; Figure 14b) and pyruvate-malate RCR values (ANOVA $p=0.714$; Figure 14c) were not different between groups. Liver succinate state 3 (ANOVA $p=0.246$; Figure 14d), succinate state 4 (ANOVA $p=0.552$; Figure 14e) and succinate RCR values (ANOVA $p=0.112$; Figure 14f) were not different between groups.

INSERT FIGURE 14 HERE

AIM 2: Effects of 8 mo intervention on tissue citrate synthase activity

Gastrocnemius citrate synthase activity levels were significantly lower in KD versus SC+KS ($p<0.001$) and SC ($p=0.009$) rats (Figure 15). Brain and liver citrate synthase activity levels in the brain (ANOVA $p=0.386$) and liver (ANOVA $p=0.386$) were similar between groups (Figure 15).

INSERT FIGURE 15 HERE

DISCUSSION

To our knowledge, this is the first study to investigate the impacts of ketogenic dieting or ketone salt supplementation on multi-organ markers of oxidative stress and mitochondrial quality over an acute (1 week) and chronic (8-mo) time frame. Overall, KD-fed rats exhibited weight loss (acute) or an attenuation of weight gain (chronic) compared to the SC and SC+KS groups. Gene expression signatures related to select oxidative stress markers were not acutely modulated in KD- or SC+KS-fed rats compared to SC-fed rats. Likewise, the protein expression of endogenous antioxidants, nor markers of oxidative stress, were affected in KD- or SC+KS-fed rats. In the KD group in AIM 2, there was a markedly higher mitochondrial production of ROS in the gastrocnemius which was coupled with decreased mitochondrial state 3, RCR and citrate synthase activity. However, mitochondrial function nor density were altered in brain or liver tissue of KD-fed rats. Moreover, our rotarod data indicate that the aforementioned gastrocnemius mitochondrial deficits did not translate into a decline in muscular function with long-term ketogenic dieting. These findings are presented in greater detail below.

Ketogenic dieting, but not ketone salt supplementation, elevates serum BHB levels and produces weight loss acutely and an attenuation of weight gain chronically in rodents

We report that ketogenic dieting induces an acute drop in bodyweight and reduced feed efficiency relative to standard chow feeding. Our group has observed a similar outcome in a separate cohort of rats fed the same ketogenic diet over a 6-week period [117]. Likewise, other

rat studies have reported similar findings [118-120]. The particular biochemical cascade and subsequent hormonal milieu that occurs during this timeframe is beyond the scope of the present study; however, it may be due to loss of energy via acetoacetate excretion in the urine or acetone loss in respiration [121]. Notably, ketone salt supplementation did not affect body weight or feed efficiency when comparing SC+KS versus SC-fed rats. Ketone esters, which differ from ketone salts, have been shown to produce anorectic effects in rodents. For instance, ketone ester feedings in mice over a 4-week period has been shown to elevate serum BHB levels, stimulate brown fat activation, elevate resting energy expenditure and lead to weight loss in mice [122]. Kesl et al. [123] also reported 5 and 10 g/kg feedings of ketone esters over a 4-week period in rats attenuated weight gain in rats relative to vehicle-fed rats. This study did not mechanistically address why ketone salt supplementation differs from ketone ester supplementation regarding anorectic effects and remains subject to future investigations.

We also report that KD-fed rats presented elevations in serum BHB levels in the 1-week study (relative to SC+KS rats) and in the 8-mo study (relative to both groups). However, ketone salt supplementation did not elevate serum BHB levels in either aim. Indeed, there is very limited literature regarding the physiological effects of ketone salts; specifically, the Kesl et al. study is the only study to our knowledge that has examined the effects of ketone salt supplementation in rodents. However, our findings are indeed in agreement with the Kesl et al. study who reported that 5 and 10 g/kg of BHB salt feedings (or ~1.7-3.5 g/d which is roughly 2.8-5.8-fold greater than our dosage) also did not alter serum BHB levels in rats over a 12-hour period, whereas the same doses of ketone ester or medium chain triglyceride (MCT) feedings robustly elevated serum BHB levels. Providing a mechanistic explanation as to why serum BHB levels are not responsive to exogenous ketone salt versus MCT or ketone ester supplementation

in rats is difficult to reconcile, and further explanation was also not provided by Kesl et al. Thus, this remains a ripe area for future investigation. Given that ketone salt supplementation did not induce ketonemia acutely or chronically, the remaining portion of the discussion will be mainly focused on discussing the physiological changes that were observed with KD-fed rats versus the SC and SC+KS rats.

Ketogenic dieting or ketone salt supplementation do not acutely alter oxidative stress-related gene expression in muscle/brain/liver tissue

The purpose of AIM 1 was to test the theory posited by Milder and Patel [13] whereby ketogenic dieting is posited to increase the mRNA expression of antioxidant-related mRNAs (i.e. Gclc, Gclm, Nqo1 or Hmox1) after a one-week period leading to an enhanced endogenous antioxidant defense system, particularly in the glutathione system. The data presented herein, does not demonstrate altered mRNA expression of either GCL subunits (the rate limiting step in glutathione synthesis) in skeletal muscle, brain and/or liver tissue after 1-week of KD feeding in rats. Our findings differ from those reported by Jarrett and colleagues [19] who observed increased GCL activity and elevated protein expression in response to a 3 weeks of KD feeding in rat hippocampal tissue. Indeed, we investigated these targets at the mRNA level in different tissues following a 1-week time course which could lead to a discrepancy in the outcomes. Additionally, we observed no alterations in Nqo1 or Hmox1 mRNA expression patterns in muscle, brain or liver tissue in KD-fed rats. Milder and colleagues [23], reported increased activity of Nqo1 in liver and hippocampal tissue, and protein expression of Hmox1 in the liver of Sprague-Dawley rats fed a KD for 3 weeks. Again, this discrepancy could be due differences in time course measurements and assayed tissue types. Additionally, regarding our lack of

significant findings in brain tissue specifically, our discordance with other literature may be related to the region of the brain that was assayed. We performed all assays in the cerebral cortex, while others examining KD-induced alterations in mRNA and protein/enzyme activity have examined the hippocampus. As such future research needs to delve into the variation of neuronal tissue and how it differentially responds to ketogenic dieting. In this regard, Ziegler and colleagues [20] reported that KD feedings over an 8 week period in rats produced a tailored response in the brain based upon region, where Gpx activity increased in the hippocampus, Cat activity decreased in the cerebellum, and the cortex seemed to remain unchanged.

In regard to the other tissues analyzed (i.e. muscle and liver), there are currently no reports to our knowledge that provide indication as to how antioxidant-related mRNAs respond to short-term ketogenic dieting. Thus, our report suggests that ketogenic dieting (or ketone salt supplementation for that matter) does not appreciably affect the mRNA expression signature of oxidative stress-related genes in muscle or liver tissue as it appears to do in the hippocampal tissue.

Chronic ketogenic dieting or ketone salt supplementation does not impact markers of oxidative stress or mitochondrial quality in liver or brain tissue

Regarding our 8-mo feeding experiment, we report that liver and brain protein expression of endogenous antioxidants (i.e., Gpx, Cat, Sod2), liver mitochondrial glutathione levels, markers of oxidative stress (i.e., 4HNE and protein carbonyls) or liver/brain mitochondrial function and density are not affected by ketogenic dieting or ketone salt supplementation. To our knowledge, the longest study investigating a KD intervention in regards to REDOX signaling has been 8 weeks in duration. In this study by Ziegler et al. [20], the authors reported increased Gpx

activity in the hippocampus of KD fed rats which, again, differs from our study given that: a) we investigated the cerebral cortex, and b) our intervention was 6 months longer in duration.

Regarding the effects of ketogenic dieting on liver tissue, a few reports have described increased inflammation and fibroblast growth factor 21 mediated hepatocyte steatosis in rodents [5, 124, 125], although we have reported decreased liver inflammatory signaling and triglyceride accumulation with the KD utilized in the current study [117]. While we did not measure tissue inflammatory markers in the current investigation, reporting lack of alterations in liver oxidative damage markers, liver mitochondrial glutathione levels and/or mitochondrial function in response to long-term ketogenic dieting or ketone salt supplementation is novel to our knowledge. Our findings differ from those of Milder et al. [23] who reported a decrease in liver mitochondrial glutathione levels following three weeks of ketogenic dieting in rats, albeit our data seem to suggest that these levels seem to normalize to levels present in SC-fed rats over an 8-mo feeding period. The lack of elevation in brain/liver citrate synthase activity (a well-validated surrogate of mitochondrial density [126]) reported herein in KD-fed rats is also in discordance with previous literature, as this phenomena has been reported to occur in the hippocampus of KD-fed in response to 3 weeks of feeding [15, 127]. Again, discrepancies in our findings versus the aforementioned literature may be explained by the long-term nature of our study. Specifically, beneficial mitochondrial adaptations in the liver or brain, if they do occur at all, may be transient once the rodents are given an extended period time for dietary adaptation.

Chronic ketogenic dieting may negatively impact skeletal muscle mitochondrial quantity or quality

While chronic ketogenic dieting did not alter markers of brain/liver mitochondrial quality or quantity in the current study, our data suggests that it may negatively impact these variables in skeletal muscle. In KD-fed rats, we observed that gastrocnemius citrate synthase activity was lowest, mitochondrial ROS production was greatest and pyruvate-malate respiratory control was impaired; notably the latter variable was also impaired in ketone salt-supplemented rats relative to SC controls. Our laboratory has previously investigated an assortment of endogenous antioxidant proteins, mitochondrial density and mitochondrial quality in skeletal muscle following six weeks ketogenic dieting in 3 mo-old rats [14]. Specifically, we reported ketogenic dieting increased succinate respiratory control compared to rats fed a Western diet suggesting that shorter-term ketogenic dieting may benefit lipid oxidation. However, our current data indicates that this mitochondrial alteration does not persist with 8 months of ketogenic dieting in rats. Regarding the pyruvate-malate respiratory control ratio in skeletal muscle, the depression KD-fed rats maybe related to increased concentrations of uncoupling proteins in the gastrocnemius mitochondria as this phenomena has also been reported by Srivastava and colleagues [128] and Sullivan and colleagues [18] who reported KD-induced increases in uncoupling protein in brown adipose and neural tissue, respectively. Likewise, the KD-induced decrease in the pyruvate-malate respiratory control ratio may be related to the increase in ROS production observed in the skeletal muscle mitochondria. Indeed, increases in skeletal muscle ROS generation have been implicated in catalyzing reductions of mitochondrial function [129-131]. Following this line of reasoning, the KD-induced decrease in gastrocnemius citrate synthase activity observed herein may be related to KD-induced decrements in skeletal muscle mitochondrial quantity, and this all may have occurred due to the observed increases in mitochondrial ROS. Regarding the KD-associated mechanisms that facilitate declines in

mitochondrial density and function, past literature has suggested that ketonemia, high fat diet feeding and/or fasting in humans and rodents down regulates oxidative phosphorylation, lowers skeletal muscle mitochondrial density and/or lowers the efficiency mitochondrial respiration [132-134]. Therefore, while short-term ketogenic dieting may enhance mitochondrial biogenesis or physiology in certain tissues (such as the hippocampus or even skeletal muscle as we have reported in our past study), skeletal muscle may be highly susceptible to KD-induced decrements in mitochondrial physiology following chronic feeding regimens. Notwithstanding, it is notable the KD-fed rats generally performed better on rotarod testing throughout the long-term intervention relative to SC- and SC+KS rats. Notably, while rotarod testing does not test maximal aerobic capacity such as a progressive treadmill test, rotarod testing does provide a surrogate of muscle strength and endurance. Therefore, we posit that the KD-induced skeletal muscle mitochondrial deficits reported herein did not translate into an appreciable decline in muscular function.

Limitations

Indeed this study possesses certain limitations. First, due resource limitations we were constrained to smaller groups n-sizes (n=7-8 per group) in AIM 2. In this regard, longer-term studies with greater n-sizes studying both genders are needed in order to replicate our findings. Second, it is notable that only mRNA expression was assayed in AIM 1, albeit this was intentional given that others have posited that ketogenic dieting may alter the genes that we assayed herein after a 1-week feeding period [13]. Lastly, all experiments were performed on sedentary animals. In this regard, and as stated previously, we have examined the effects of 6 weeks of ketogenic dieting in sedentary animals versus animals that were given access to running

wheels and have reported that skeletal muscle mitochondrial density increases in KD-fed animals that exercised [14]. Hence, given that ketogenic dieting may negatively impact skeletal muscle mitochondrial function in sedentary animals, it would be interesting to examine how longer-term ketogenic dieting affects skeletal muscle mitochondrial physiology in animals or humans that are concurrently engaged in exercise training. In spite of these limitations, we posit that the data presented herein provide a comprehensive evaluation as to how short-term and long-term ketogenic dieting affect oxidative stress markers, antioxidant gene and protein expression and mitochondrial function in multiple tissues. Moreover, given that much of the positive data in this regard related to ketogenic dieting has been reported to occur in the hippocampus, these data can serve as a template to guide future research aiming to examine how ketogenic dieting and/or exogenous ketone salt supplementation affect similar markers in other rodent or human tissues.

Conclusions

These data provide a comprehensive description regarding the short-term and long-term effects of ketogenic dieting or supplemental ketone salt feeding on body mass, multi-tissue oxidative stress-related markers and markers of mitochondrial physiology. Consistent with other literature, we report that ketogenic dieting, but not ketone salt supplementation, induces ketonemia and facilitates acute weight loss while attenuating chronic weight gain. Notwithstanding, and contrary to other prevailing hypotheses, our data suggests that ketogenic dieting or ketone salt supplementation in rodents does not appreciably alter the mRNA expression of genes related to glutathione synthesis or endogenous antioxidant defense systems in rodent muscle, brain and liver tissue. Likewise, long-term ketogenic dieting or ketone salt supplementation in rodents does not appear alter the protein expression of endogenous

antioxidants or markers of oxidative damage in these tissues. The potentially negative impact that long-term ketogenic dieting has on skeletal muscle mitochondria function and ROS production was an unanticipated finding and requires further examination.

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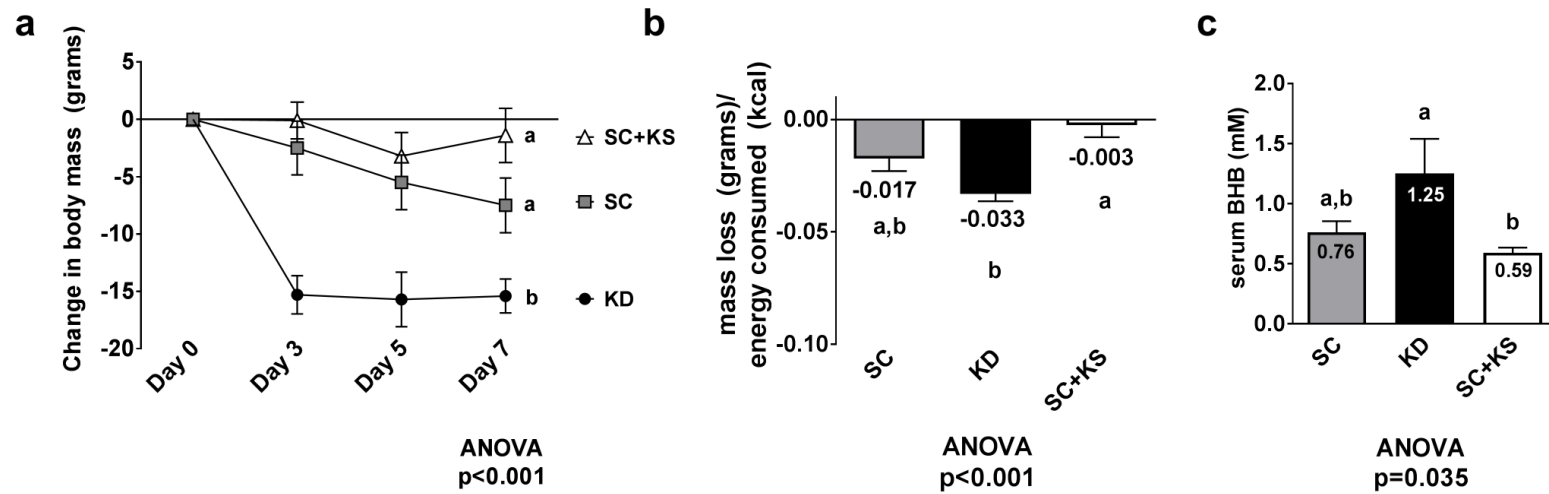
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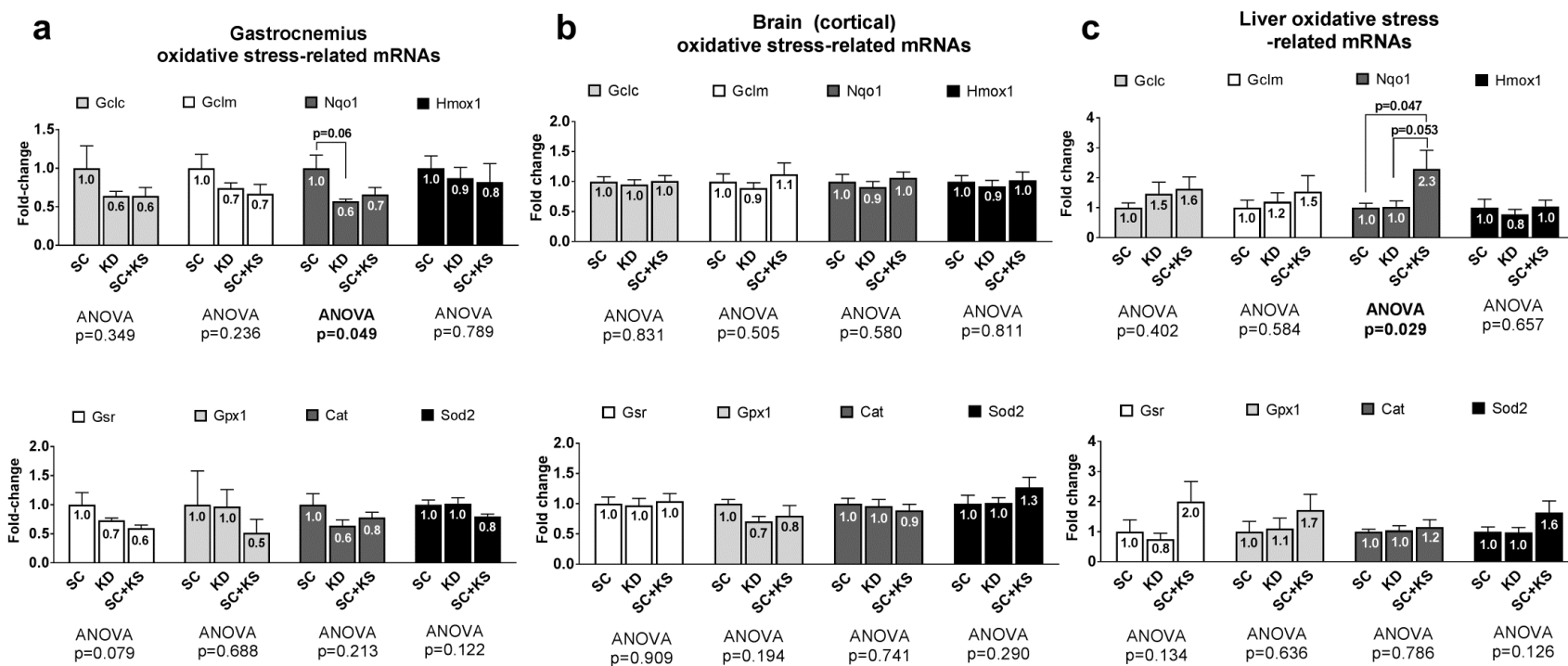
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Figure 1. Change in body mass, overall feed efficiency and serum BHB levels in 1-wk fed rats



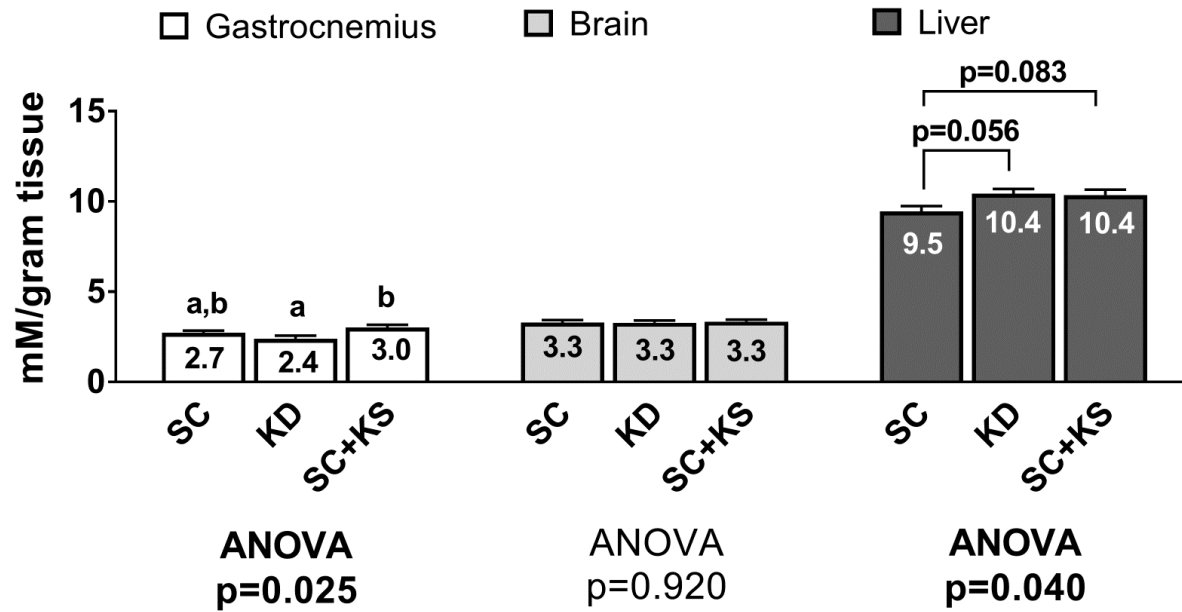
Legend: Change in body mass is presented in panel A. Feed efficiency is presented in panel B. Serum BHB levels are presented in panel C. Data in panel A are presented as mean±standard error values. In panels B and C, all bars are presented as mean+standard error values and group means are indicated within each bar. For all panels, different lettering indicates post-hoc differences (p<0.05) and similar lettering notations indicates lack of significant differences between treatments. Abbreviations: SC, standard chow-fed rats; KD, ketogenic diet-fed rats; SC+KS, standard chow-fed rats with supplemental NaBHB (sodium beta hydroxybutyrate)

Figure 2. Oxidative stress and endogenous antioxidant related mRNAs in 1-wk fed rats



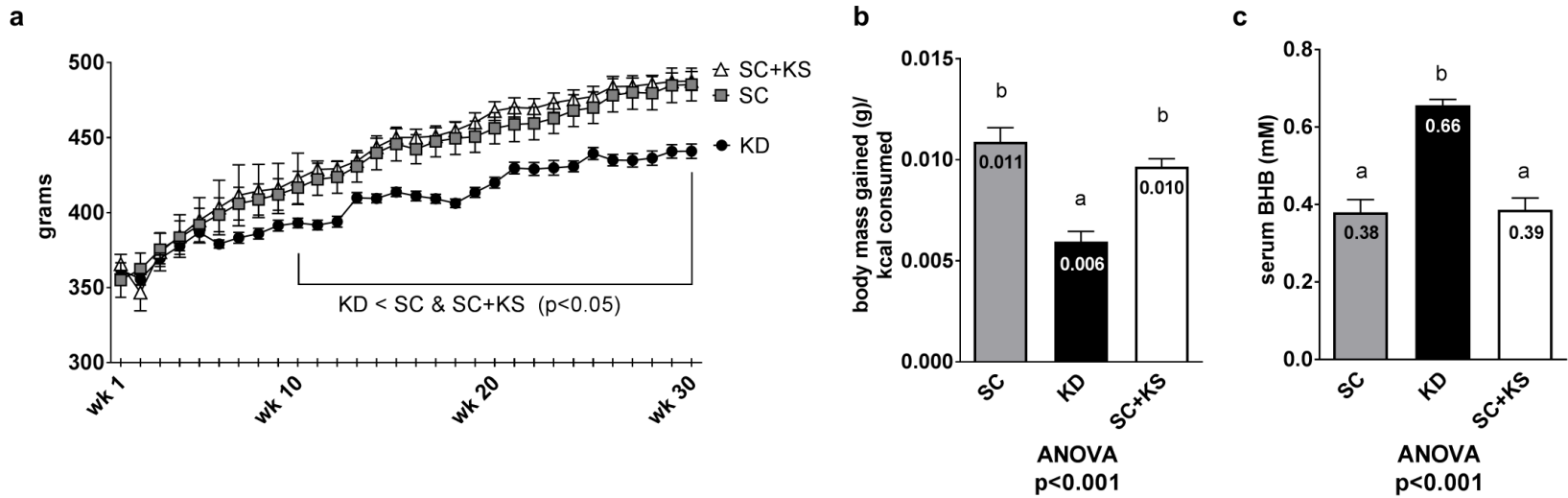
Legend: Oxidative stress and endogenous antioxidant related mRNAs are shown for gastrocnemius (panel A), brain (panel B), and liver (panel C). All bars are presented as mean+standard error values and group means are indicated within each bar; different lettering indicates post-hoc differences ($p < 0.05$) and similar lettering notations indicates lack of significant differences between treatments. Abbreviations: SC, standard chow-fed rats; KD, ketogenic diet-fed rats; SC+KS, standard chow-fed rats with supplemental NaBHB (sodium beta hydroxybutyrate)

Figure 3. Tissue total antioxidant capacity in 1-wk fed rats



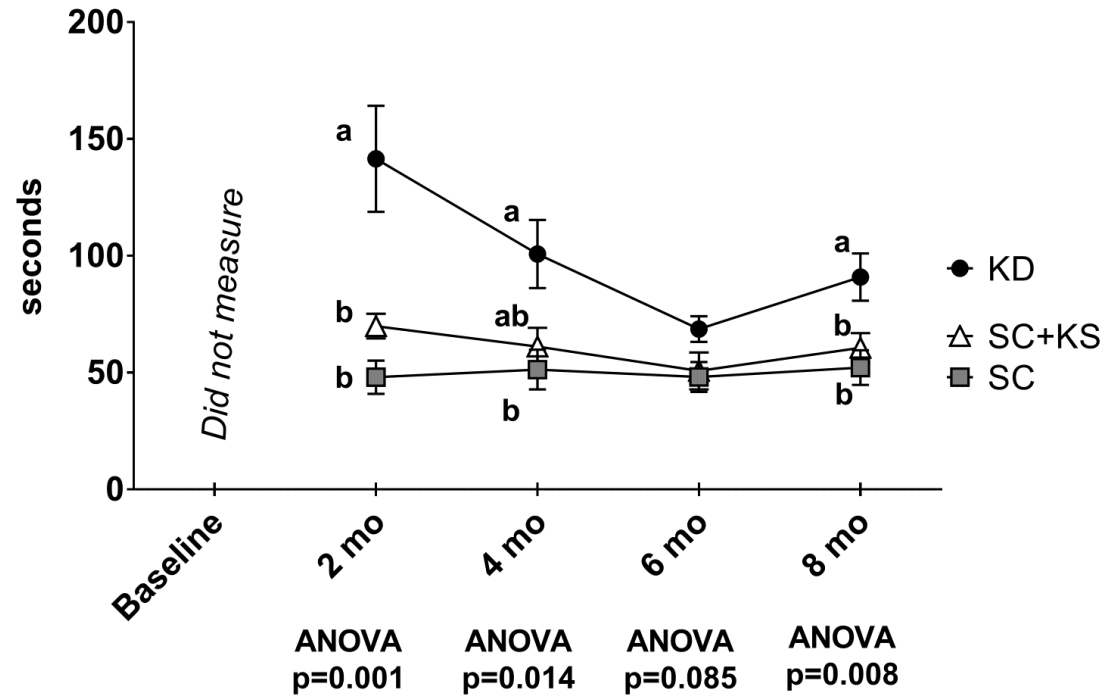
Legend: Tissue total antioxidant capacity levels are presented. All bars are presented as mean+standard error values and group means are indicated within each bar; different lettering indicates post-hoc differences ($p < 0.05$) and similar lettering notations indicates lack of significant differences between treatments. Abbreviations: SC, standard chow-fed rats; KD, ketogenic diet-fed rats; SC+KS, standard chow-fed rats with supplemental NaBHB (sodium beta hydroxybutyrate)

Figure 4. Change in body mass, overall feed efficiency and serum BHB in 8-mo fed rats



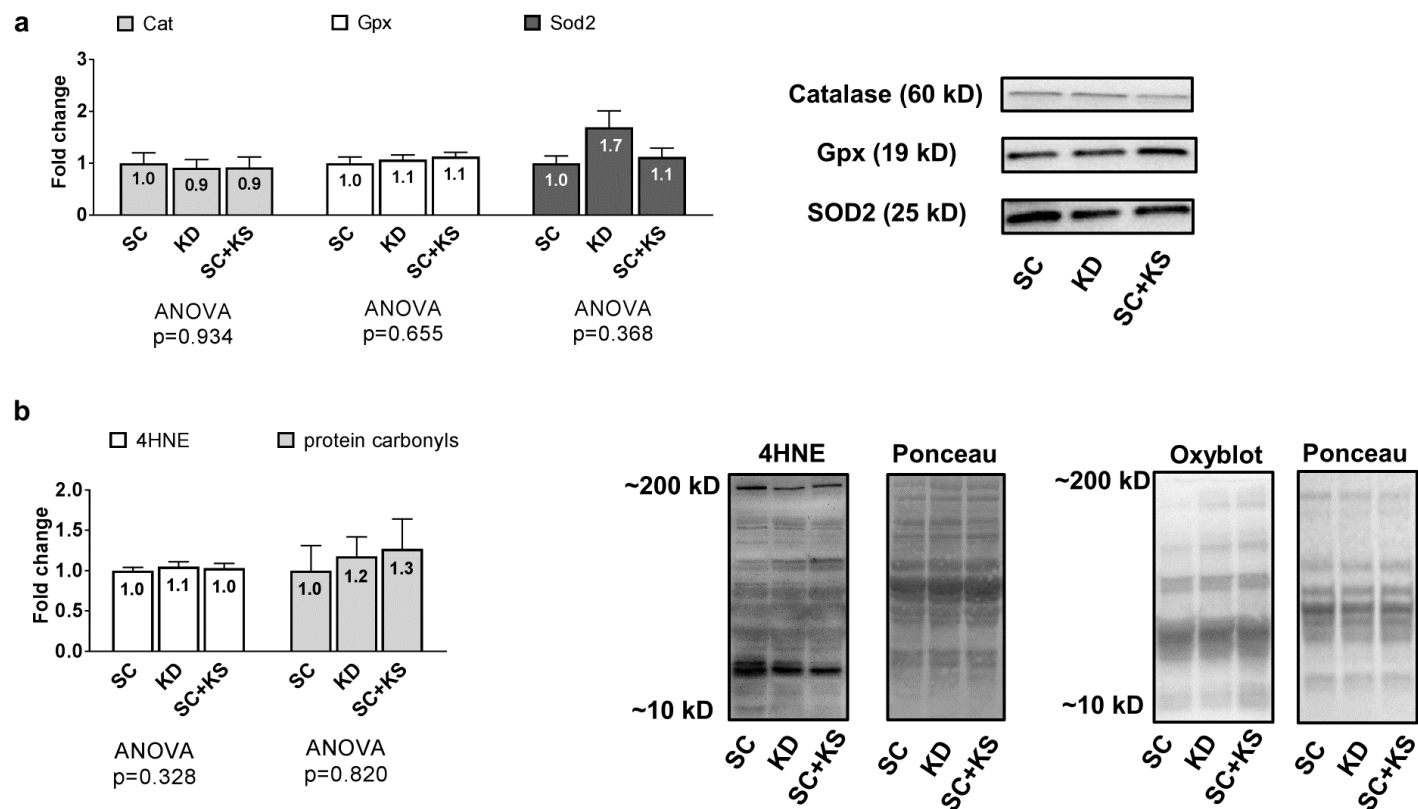
Legend: Change in body mass is presented in panel A, feed efficiency is presented in panel B and serum BHB levels are presented in panel C. Data in panel A are presented as mean±standard error values. In panels B and C, all bars are presented as mean+standard error values and group means are indicated within each bar; different lettering indicates post-hoc differences ($p < 0.05$) and similar lettering notations indicates lack of significant differences between treatments. Abbreviations: SC, standard chow-fed rats; KD, ketogenic diet-fed rats; SC+KS, standard chow-fed rats with supplemental NaBHB (sodium beta hydroxybutyrate)

Figure 5. Change in rotarod performance in 8-mo fed rats



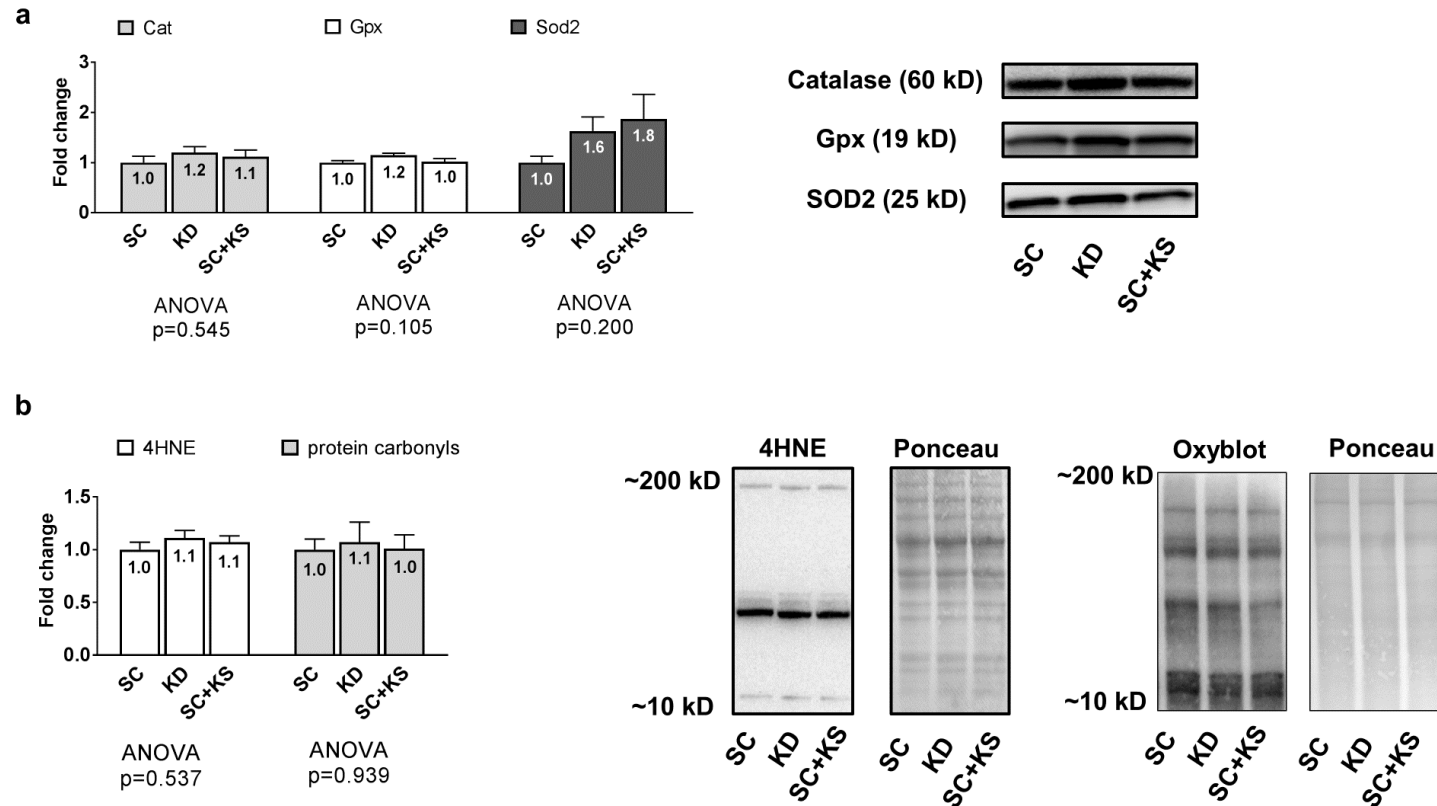
Legend: Rotarod testing indicates potential changes in motor behavior and/or muscular endurance. All data are presented as mean±standard error values. Different lettering indicates post-hoc differences (p<0.05) and similar lettering notations indicates lack of significant differences between treatments. Abbreviations: SC, standard chow-fed rats; KD, ketogenic diet-fed rats; SC+KS, standard chow-fed rats with supplemental NaBHB (sodium beta hydroxybutyrate)

Figure 6. Gastrocnemius oxidative stress-related proteins and markers in 8-mo fed rats



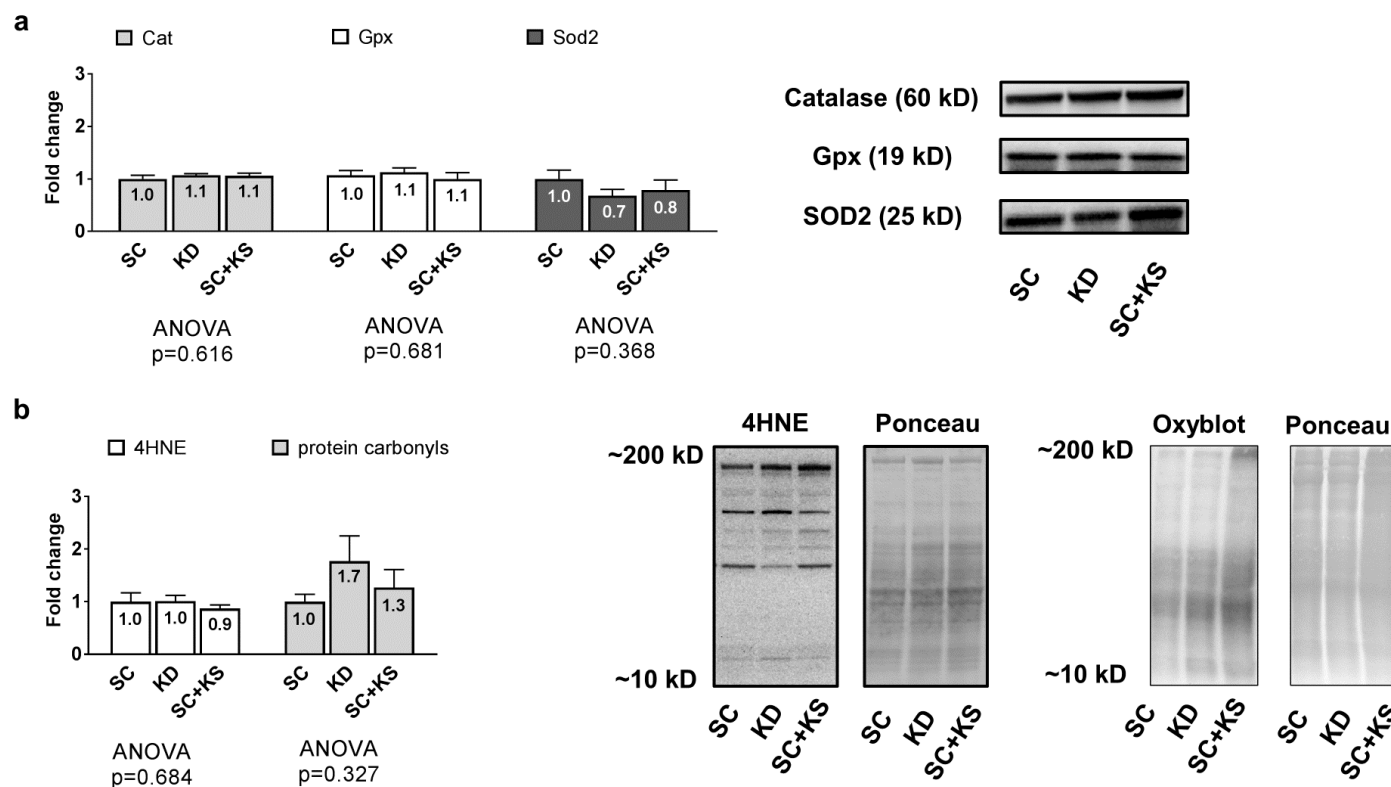
Legend: Gastrocnemius catalase (Cat), glutathione peroxidase (Gpx), and mitochondrial superoxide dismutase (Sod2) protein levels are presented with representative images in panel A. In panel B, tissue 4-Hydroxynonenal (4HNE), and protein carbonyls (Oxyblot) are presented with representative images. All bars are presented as mean+standard error values and group means are indicated within each bar. Abbreviations: SC, standard chow-fed rats; KD, ketogenic diet-fed rats; SC+KS, standard chow-fed rats with supplemental NaBHB (sodium beta hydroxybutyrate)

Figure 7. Brain oxidative stress-related proteins and markers in 8-mo fed rats



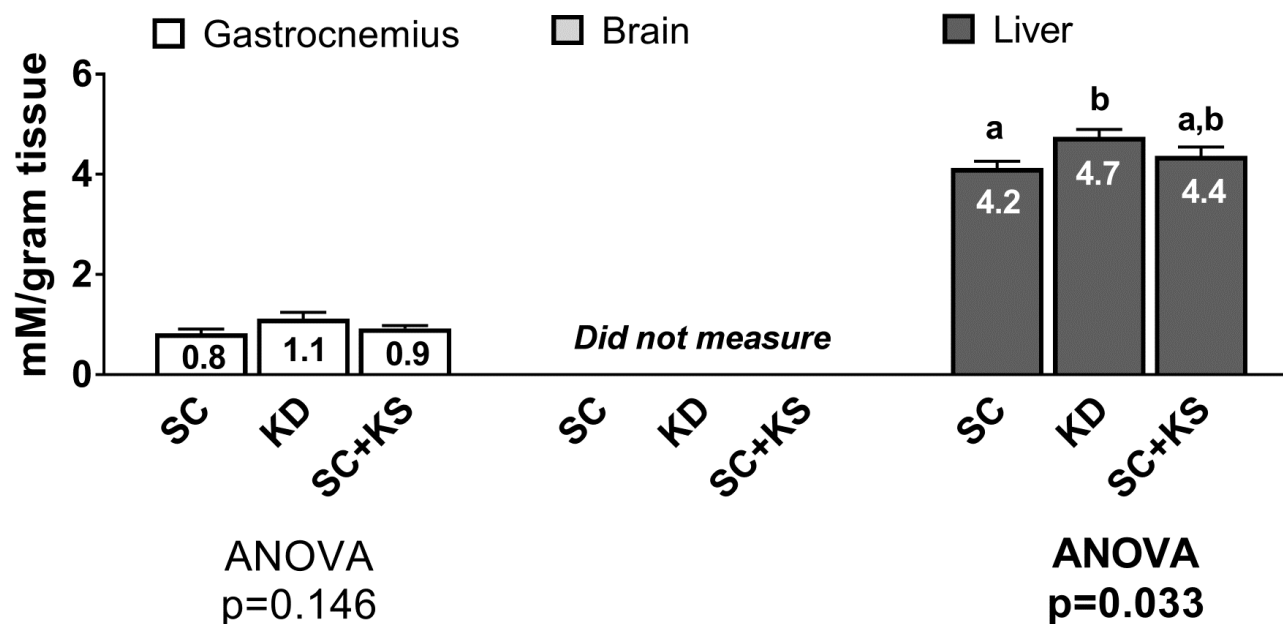
Legend: Brain catalase (Cat), glutathione peroxidase (Gpx), and mitochondrial superoxide dismutase (Sod2) protein levels are presented with representative images in panel A. In panel B, tissue 4-Hydroxynonenal (4HNE), and protein carbonyls (Oxyblot) are presented with representative images. All bars are presented as mean+standard error values and group means are indicated within each bar. Abbreviations: SC, standard chow-fed rats; KD, ketogenic diet-fed rats; SC+KS, standard chow-fed rats with supplemental NaBHB (sodium beta hydroxybutyrate)

Figure 8. Liver oxidative stress-related proteins and markers in 8-mo fed rats



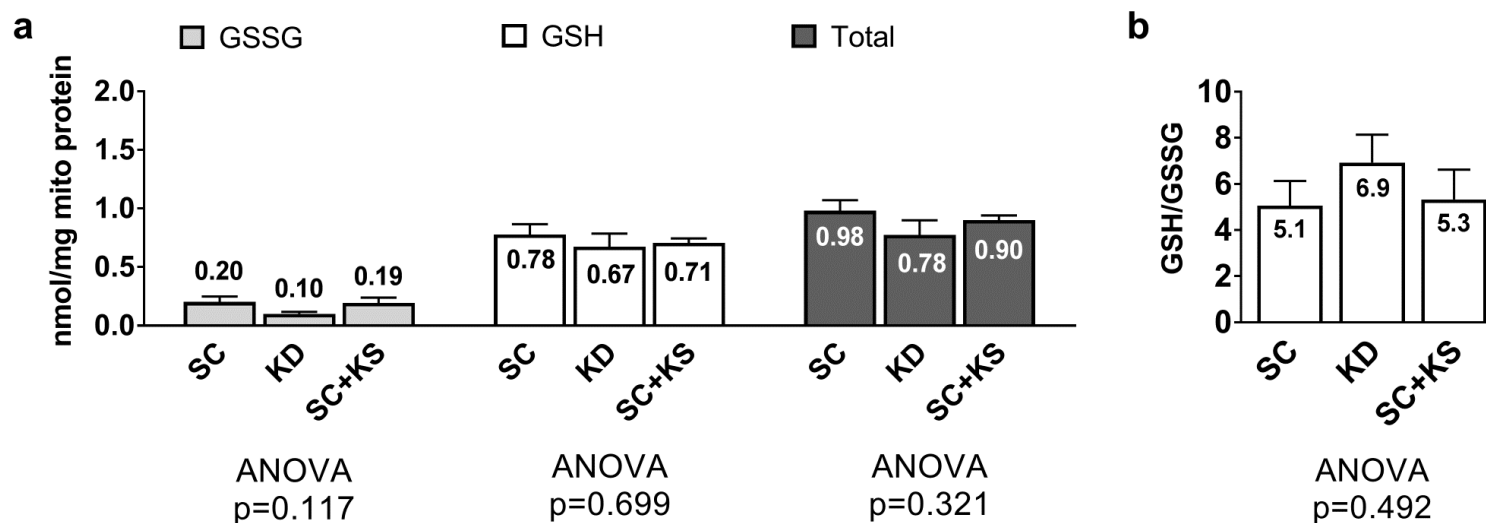
Legend: Liver catalase (Cat), glutathione peroxidase (Gpx), and mitochondrial superoxide dismutase (Sod2) protein levels are presented with representative images in panel A. In panel B, tissue 4-Hydroxynonenal (4HNE), and protein carbonyls (Oxyblot) are presented with representative images. All bars are presented as mean+standard error values and group means are indicated within each bar. Abbreviations: SC, standard chow-fed rats; KD, ketogenic diet-fed rats; SC+KS, standard chow-fed rats with supplemental NaBHB (sodium beta hydroxybutyrate)

Figure 9. Tissue total antioxidant capacity levels in 8-mo fed rats



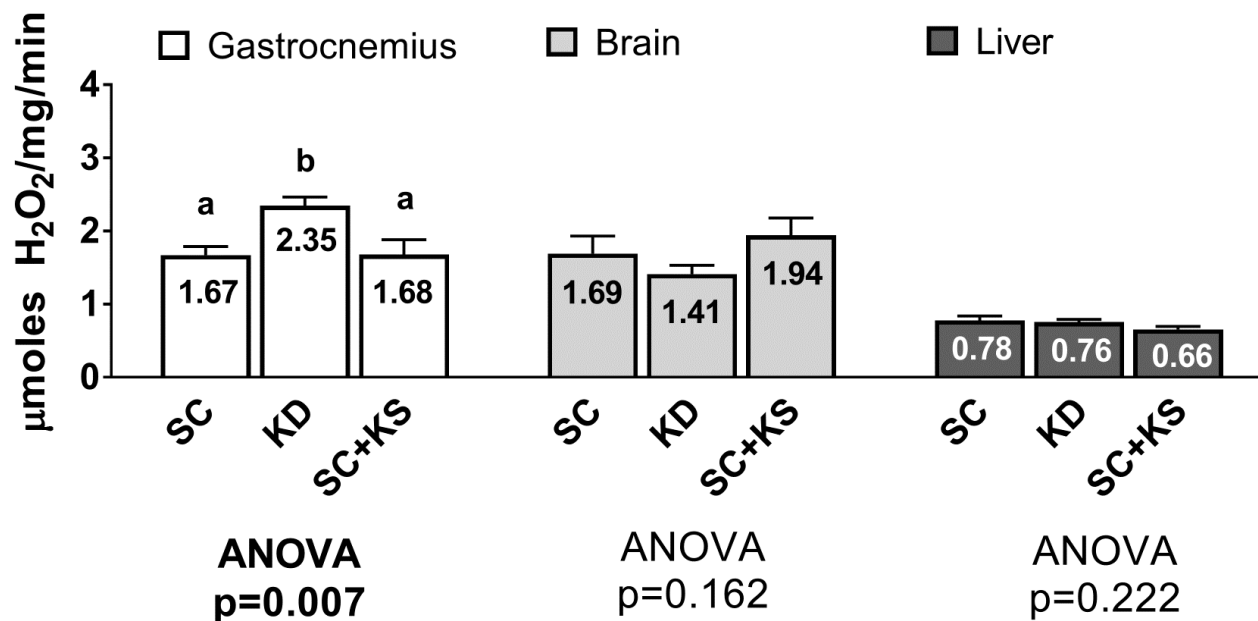
Legend: Tissue total antioxidant capacity levels are presented. Brain samples were limited due to other assays and were, thus, not performed. All bars are presented as mean+standard error values and group means are indicated within each bar; different lettering indicates post-hoc differences ($p < 0.05$) and similar lettering notations indicates lack of significant differences between treatments. Abbreviations: SC, standard chow-fed rats; KD, ketogenic diet-fed rats; SC+KS, standard chow-fed rats with supplemental NaBHB (sodium beta hydroxybutyrate)

Figure 10. Liver mitochondrial glutathione levels in 8-mo fed rats



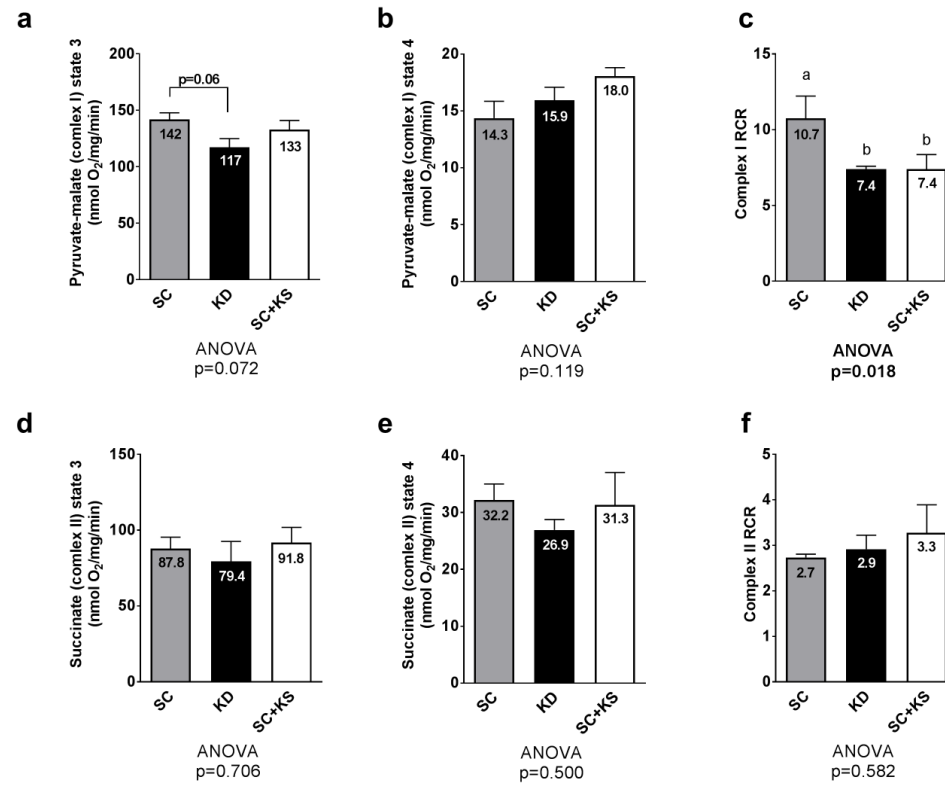
Legend: Legend: Liver mitochondrial oxidized (GSSG), reduced (GSH) and total glutathione are presented in panel A. Redox ratio values (GSH/GSSG) are presented in panel B. These assays were attempted in gastrocnemius brain samples, but assay values were on the lower-end of the assay sensitivity curve and, thus, are not presented. All bars are presented as mean+standard error values and group means are indicated within each bar. Abbreviations: SC, standard chow-fed rats; KD, ketogenic diet-fed rats; SC+KS, standard chow-fed rats with supplemental NaBHB (sodium beta hydroxybutyrate)

Figure 11. Tissue mitochondrial ROS production in 8-mo fed rats



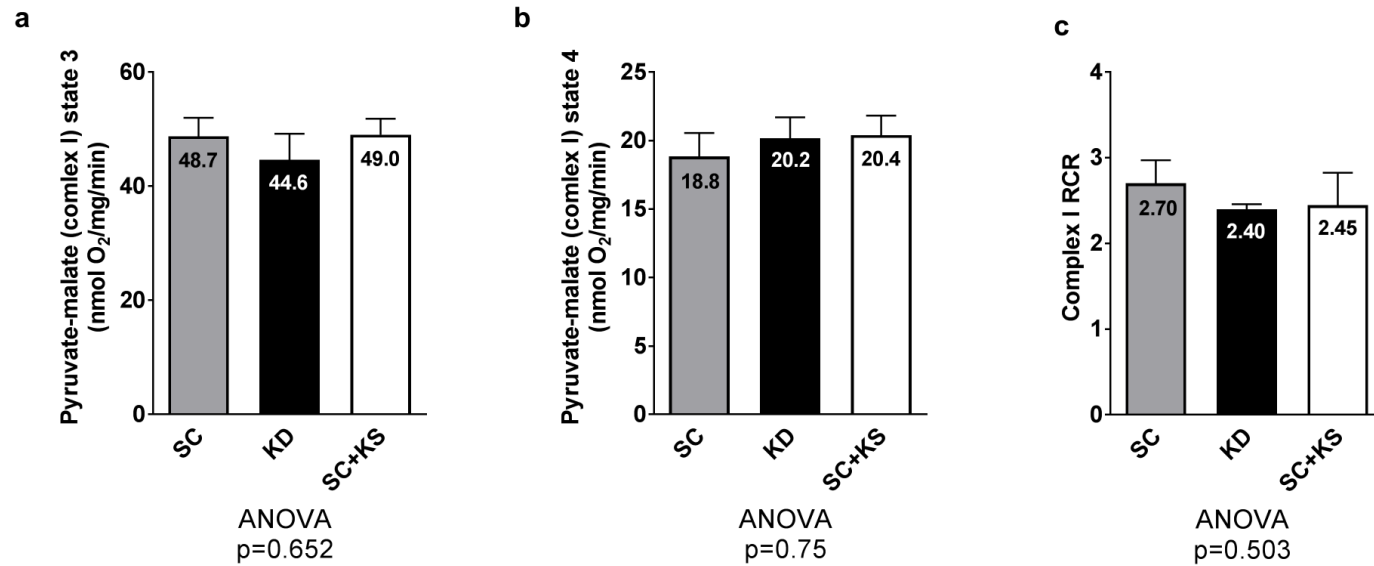
Legend: Tissue mitochondrial reactive oxygen species production levels are presented. All bars are presented as mean+standard error values and group means are indicated within each bar; different lettering indicates post-hoc differences ($p < 0.05$) and similar lettering notations indicates lack of significant differences between treatments. Abbreviations: SC, standard chow-fed rats; KD, ketogenic diet-fed rats; SC+KS, standard chow-fed rats with supplemental NaBHB (sodium beta hydroxybutyrate)

Figure 12. Gastrocnemius mitochondrial function in 8-mo fed rats



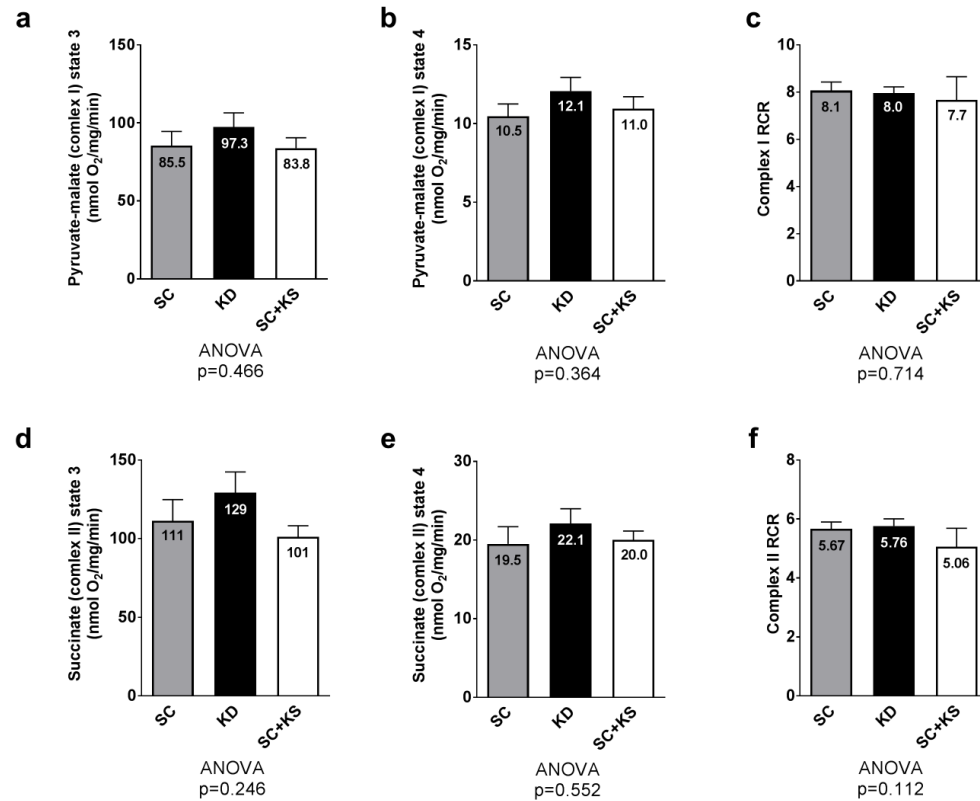
Legend: State 3 pyruvate-malate is presented in panel A. State 4 pyruvate-malate is presented in panel B. Pyruvate-malate RCR is presented in panel C. State 3 succinate is presented in panel D. State 4 succinate is presented in panel E. Succinate RCR is presented in panel F. All bars are presented as mean+standard error values and group means are indicated within each bar; different lettering indicates post-hoc differences ($p < 0.05$) and similar lettering notations indicates lack of significant differences between treatments. Abbreviations: SC, standard chow-fed rats; KD, ketogenic diet-fed rats; SC+KS, standard chow-fed rats with supplemental NaBHB (sodium beta hydroxybutyrate); RCR, respiratory exchange ratio

Figure 13. Brain mitochondrial function in 8-mo fed rats



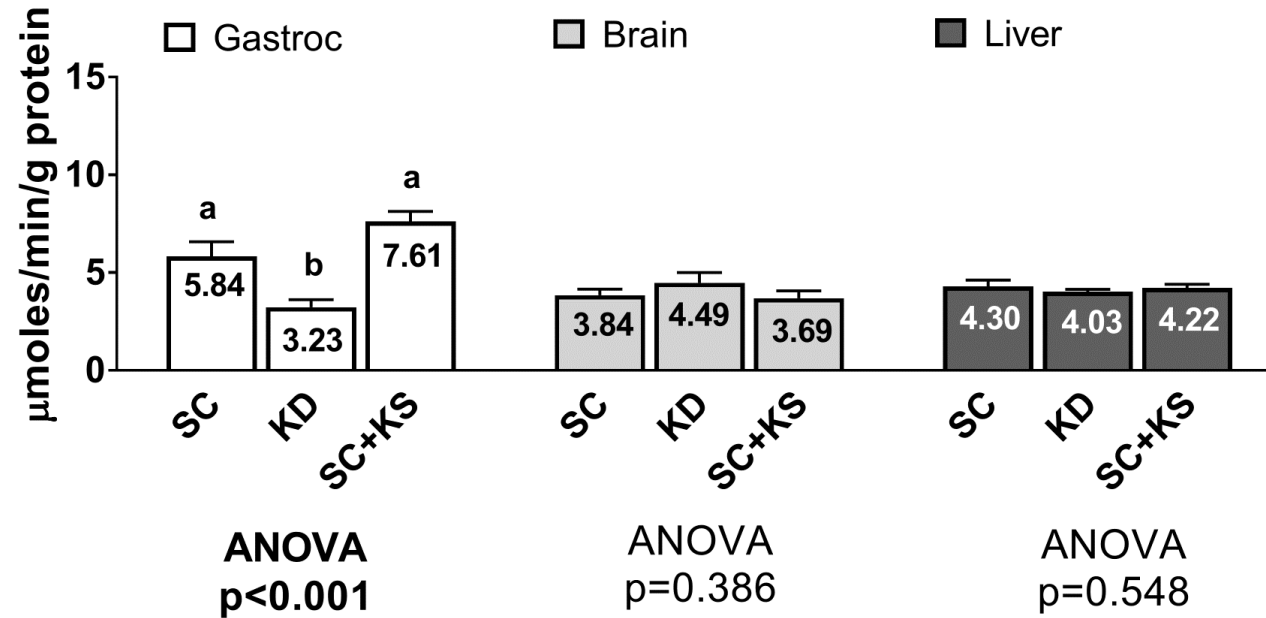
Legend: State 3 pyruvate-malate is presented in panel A. State 4 pyruvate-malate is presented in panel B. Pyruvate-malate RCR is presented in panel C. All bars are presented as mean+standard error values and group means are indicated within each bar. Abbreviations: SC, standard chow-fed rats; KD, ketogenic diet-fed rats; SC+KS, standard chow-fed rats with supplemental NaBHB (sodium beta hydroxybutyrate); RCR, respiratory exchange ratio

Figure 14. Liver mitochondrial function in 8-mo fed rats



Legend: State 3 pyruvate-malate is presented in panel A. State 4 pyruvate-malate is presented in panel B. Pyruvate-malate RCR is presented in panel C. State 3 succinate is presented in panel D. State 4 succinate is presented in panel E. Succinate RCR is presented in panel F. All bars are presented as mean+standard error values and group means are indicated within each bar. Abbreviations: SC, standard chow-fed rats; KD, ketogenic diet-fed rats; SC+KS, standard chow-fed rats with supplemental NaBHB (sodium beta hydroxybutyrate); RCR, respiratory exchange ratio

Figure 15. Tissue citrate synthase activity in 8-mo fed rats



Legend: Tissue citrate synthase activity is presented. All bars are presented as mean+standard error values and group means are indicated within each bar; different lettering indicates post-hoc differences (p < 0.05) and similar lettering notations indicates lack of significant differences between treatments. Abbreviations: SC, standard control; KD, ketogenic diet; SC+KS, standard control with supplemental NaBHB (sodium beta hydroxybutyrate)