Title
The Impact of Ketogenic Diets on Mitochondrial Mass and Healthspan in Aged Mice

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The Impact of Ketogenic Diets on Mitochondrial Mass and Healthspan in Aged Mice

By

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DISSERTATION

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ABSTRACT

There has been increasing interest in the use of ketogenic diets (KD) to manage age-related diseases, and KDs started in early middle age have been shown to improve longevity and healthspan in mice. One of the potential mechanisms underlying the health-promoting effect of KDs is the promotion of mitochondrial biogenesis and prevention of age-related decreases in mitochondrial mass, a hallmark of mammalian aging. This dissertation investigates the impact of an isocaloric KD initiated in 12-month old mice on a panel of markers of mitochondrial mass in whole hindlimb skeletal muscle, brain, liver, and kidney. The dissertation work demonstrates that a KD increased activities of key mitochondrial enzymes in skeletal muscle and brain at an advanced age, but there were no patterns indicative of a widespread increase in mitochondrial content in these tissues or liver and kidneys. Morphological quantification of mitochondria was performed to look into specific regions within tissues, and the results of the study showed an increase in mitochondrial mass in red gastrocnemius muscle, but not prefrontal cortex, hippocampus, or liver left lobe following 1 month of a KD. These results highlight the tissue-specific alterations in mitochondrial content with a KD. This dissertation also investigated the effects on the healthspan of a KD started late in life and determined if an intermittent KD (IKD) could produce similar results to a full KD. At 18 months of age, mice were assigned to a control diet (CD), KD, or IKD (3 consecutive days of KD each week). The KD and IKD improved some measures of cognition, including working memory and spatial learning memory, promoted motor endurance, and prevented a significant decrease in composite healthspan score with aging as observed in control mice. The circulating level of cytokines were also reduced with KD and IKD, and this may contribute to the phenotypic effects observed with these diets. In conclusion, this

dissertation work shows that a KD induces tissue-specific changes in mitochondrial content, and a KD and IKD initiated at late middle age improves measures of healthspan at an advanced age.

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INTRODUCTION

Aging is an inevitable process that increases the risk of many chronic diseases, and interventions that can promote longevity and healthspan could reduce the risk or alleviate the symptoms of various age-associated diseases (1). Calorie restriction (CR), without malnutrition, has been shown to increase lifespan in various species (2) and is associated with a metabolic shift toward a decrease in glycolysis and an increase in fatty acid oxidation (3, 4). A ketogenic diet (KD), which is depleted in carbohydrates and high in fat, has been used to treat drug-resistant epilepsy in children, and recently it there has been emerging interest in the use of KDs to manage a range of age-related diseases (5, 6). KDs mimic some of the metabolic shifts that occur with CR, and this metabolic reprograming with a change from glucose to lipid metabolism may contribute to longevity and healthy aging (7). An elevation in β-hydroxybutyrate, the main circulating ketone body, has also been shown to be involved in health-promoting signaling pathways (8, 9) and associated with anti-aging phenotypes (1, 10-12). We previously demonstrated that mice on a continuous KD have increased median lifespan and improved healthspan measures compared to animals consuming an isocaloric amount of a control diet (13). It has also been reported that mice consuming a KD on alternate weeks have reduced midlife mortality as well as improved cognition compared to control mice (14).

Although the mechanisms through which KDs influence aging are still not entirely known, stimulation and maintenance of mitochondrial content may be one underlying mechanism for the positive effect of KDs on aging (15, 16). Mitochondria are dynamic organelles that play crucial roles in energy metabolism, apoptosis, and intracellular signaling (17), and changes in mitochondrial content are thought to be a significant contributor to mammalian aging (3). By

chronically stimulating pathways in mitochondrial oxidative metabolism, KDs may induce an increase and prevent an age-related decrease in mitochondrial mass. Moreover, β-hydroxybutyrate is associated with pathways that induce mitochondrial biogenesis (8). Some studies have investigated the effects of KDs on measures of mitochondrial content in laboratory rodents. However, these studies have focused on models of disease and injury (18-23), looked at short-term effects in only one tissue (24-27), or used very few markers of mitochondrial mass (28, 29). Thus, relatively little is known about the impact of long-term consumption of a KD diet on mitochondrial content in healthy adult mice, and it is not clear if a KD diet increases mitochondrial content in particular tissues. Part of the dissertation work sought to investigate the impact of a KD on mitochondrial mass in various tissues of healthy middle-aged and advanced-aged mice. Chapter 1 focuses on a panel of commonly used markers of mitochondrial content and evaluated the changes in these markers that occurred with a KD in whole tissue homogenates. Chapter 2 explores morphological quantification of mitochondria in specific regions within a tissue.

In humans, long-term adherence to a strict KD can be difficult, and interventions started later in life or intermittently may be feasible and promote compliance. Several studies have investigated the impact of dietary interventions initiated in late middle-aged mice (> 16 months of age) on aging (30-33). These studies provide evidence that interventions started late in life can mitigate age-related declines in physiological functions, and it remains to be determined if KDs initiated in late middle age are capable of increasing healthspan. Thus, one goal of Chapter 3 was to determine if a KD started at 18 months of age would improve measures of healthspan in aged mice. CR and intermittent fasting have beneficial effects on many indicators of healthy aging and physiological function (34, 35), and animals on these interventions undergo intermittent periods of increased fatty acid oxidation and ketosis between meals. It is possible that intermittent ketosis may be

sufficient to impact functional declines associated with aging. It has been shown that alternate week feeding of a KD improved cognition at an advanced age and some measures of healthspan (14), although mice on this intervention went through weight cycling. However, it is not known if shorter periods of ketosis without weight cycling would generate similar effects. Thus, the other goal of Chapter 3 was to determine if a weekly 3-day isocaloric intermittent KD (IKD) initiated at 18 months of age produces similar aging benefits as a continuous KD.

Chapter 1. A ketogenic diet impacts markers of mitochondrial mass in a tissue-specific manner in aged mice

Declines in mitochondrial mass are thought to be a hallmark of mammalian aging, and a ketogenic diet (KD) may prevent the age-related decreases in mitochondrial content. The objective of this study was to investigate the impact of a KD on markers of mitochondrial mass. Mice were fed an isocaloric control diet (CD) or KD from 12 months of age. Tissues were collected after 1 month and 14 months of intervention, and a panel of commonly used markers of mitochondrial mass (mitochondrial enzyme activities and levels, mitochondrial to nuclear DNA ratio, and cardiolipin content) were measured. Our results showed that a KD stimulated activities of marker mitochondrial enzymes, including citrate synthase, Complex I, and Complex IV in hindlimb muscle in aged mice. KD also increased the activity of citrate synthase and prevented an age-related decrease in Complex IV activity in aged brain. No other markers were increased in these tissues. Furthermore, the impacts of a KD on liver and kidney were mixed with no pattern indicative of a change in mitochondrial mass. In conclusion, results of the present study suggest that a KD induces tissue-specific changes in mitochondrial enzyme activities or structure rather than global changes in mitochondrial mass across tissues.

1.1 Introduction

Decreases in mitochondrial mass or content are observed in aged animals and are thought to be a contributor to mammalian aging (36-38). A ketogenic diet (KD), which is depleted in carbohydrate and high in fat, has been used to manage a range of metabolic and neurologic disorders that are associated with changes in mitochondrial mass, and upregulation in mitochondrial bioenergetic genes and biogenesis has been proposed as one possible mechanism for the therapeutic and health-promoting effects of this diet (39, 40). The main circulating ketone body, β-hydroxybutyrate, is also involved in signaling pathways that may lead to induction in mitochondrial biogenesis (8). Furthermore, previous studies have demonstrated that a KD increases lifespan and improves physiological functions in aged mice when the intervention was started from middle age (13, 14). Thus, an increase in mitochondrial mass might be one possible mechanism for the health-promoting effects of this diet. Although a few studies have provided evidence that a KD may increase mitochondrial content in tissues from some animal models (18, 24, 29), little is known about the effects of KD on mitochondrial mass in healthy middle-aged and aged mice. The goal of the present study was to determine the impact of a KD on markers of mitochondrial mass in tissues from these animals.

Several markers have been used as measures of mitochondrial mass or content in tissues, with some of the markers showing strong correlation with morphological quantification of mitochondrial content or volume using transmission electron microscopy (41). The present study focused on the impacts of a KD on a panel of commonly used mitochondrial markers of mitochondrial mass. These markers were selected to allow measurements in whole tissue homogenates and provide an indication of changes in mitochondrial mass across the entire tissue. The markers selected for our study and can be divided into four categories: 1) mitochondrial

enzyme activities (citrate synthase, complex I, complex IV); 2) mitochondrial protein levels (citrate synthase, complex I protein NDUFB8, complex IV protein MTCO1); 3) mitochondrial to nuclear DNA ratio; 4) cardiolipin content. We decided to use a broad range of markers to determine if a KD alters tissue mitochondrial mass since each marker has limitations and reliance on a single, or few, markers may give an incomplete picture of mitochondrial changes induced by diet. For example, a change in enzyme activity may reflect post-translational regulatory processes rather than an alteration in mitochondrial content and changes in the levels of a subset of mitochondrial proteins may reflect up or downregulation of a particular pathway rather than an overall change in mitochondrial mass. An increase in all, or the vast majority, of the markers would provide a strong indication of a KD-induced increase in mitochondrial mass. However, changes in only a few of the markers would provide evidence of a KD-related alteration in mitochondrial enzyme activity or structure rather than an overt change in mitochondrial mass. To assess the impact of a KD on mitochondrial mass a panel of mitochondrial content markers were measured in four metabolically active tissues from C57BL/6JN mice that were fed a control (CD) or ketogenic (KD) diet from 12 months of age (middle age) to 13 or 26 months of age.

1.2 Materials and Methods

1.2.1 Animals and Diets

Male C57BL/6JN mice were obtained at 11 months of age from the NIA Aged Rodent Colony. Mice were individually housed in polycarbonate cages on racks in a HEPA filtered room maintained on a 12-hour light-dark cycle. Temperature (22–24°C) and humidity (40– 60%) were controlled, and health checks were conducted on all mice at least once daily. Sentinel mice were housed in the same room and exposed to bedding from the study mice on a weekly basis. Health

screens were completed on sentinel mice every three months. Tests included aerobic cultures and serology (MHV, MPV, MVM, M. pul., TMEV [GDVII], Ectro, EDIM, MAD1, MAD2, LCM, Reo-3). All tests were negative throughout the study. All animal protocols were approved by the UC Davis Institutional Animal Care and Use Committee and were in accordance with the NIH guidelines for the Care and Use of Laboratory Animals.

Upon arrival at the UC Davis facility, mice were singly housed and provided ad libitum access to a chow diet (LabDiet 5001; LabDiet, Saint Louis, MO) and food intake was measured. At 12 months of age, mice were randomly assigned to a control (CD) or ketogenic (KD) diet. The control diet contained (% of total kcal) 18% protein, 65% carbohydrate, and 17% fat. The ketogenic diet contained 10% protein, <1% carbohydrate, and 89% fat. The animals were fed 11.2 kcal/day throughout the study. Table 1.1 provides a detailed description of the diet composition. For the control diet, the Envigo (Indianapolis, IN) mineral mix TD.94046 and the vitamin mix TD.94047 were used. The KD included mineral mix TD.79055 and vitamin mix TD.40060 instead, because of their lower carbohydrate content. Thus, calcium phosphate (19.3 g/kg diet) and calcium carbonate (8.2 g/kg diet) supplementation was required for the KD. The vitamin mix added to the KD included choline (choline dihydrogen citrate) for a final concentration of 4.5 g/kg of diet. Nutritional ketosis was confirmed by measuring blood ketone levels using a Precision Xtra glucose and ketone monitoring system (Abbott) through tail snips, and the data were provided in our previously published paper (13). Body weight was measured weekly and there was no significant difference in body weight between CD and KD mice throughout the study (13).

Table 1.1: Experimental diets

g/kg diet	**Control**	**Ketogenic**
Casein	200	181
L-Cysteine	3.0	--
D-methionine	--	2.7
Corn starch	398	--
Maltodextrin	132	--
Sucrose	100	--
Soybean oil	70	70
Lard	0	561
Choline bitartrate	2.5	--
Cellulose	50	85.0
TBHQ	0.014	0.126
Mineral mix	35	60
Vitamin mix	10	13

After 1 or 14 months of dietary intervention, animals were sacrificed with cervical dislocation, and tissues were collected in the morning following an overnight fast. Tissues were collected and snap-frozen in liquid nitrogen. The entire liver, both kidneys, whole brain (including olfactory bulb and medulla), and all the muscles from the hindlimb were harvested.

1.2.2 Mitochondrial enzyme activities

Tissues were powdered under liquid nitrogen using a mortar and pestle. The powdered tissues were homogenized at a 1:10 (w/v) tissue to buffer ratio in a glass homogenizer. Citrate synthase, Complex I and Complex VI activity was measured as described previously (42, 43). Assays were performed in cuvettes with a final volume of 1 mL, using a Perkin Elmer Lambda 25 UV/VIS spectrophotometer (Waltham, MA) equipped with Peltier heating control system and a 9-cell changer. Enzyme activities were expressed as μmol/min/mg protein.

1.2.3 Total cardiolipin content

Lipids were extracted from 6 mg of tissue with methanol and methyl tert-butyl ether. Water was subsequently added for phase separation. After concentrating extracts to complete dryness, samples were reconstituted prior to LC-MS analysis in 110 µL of methanol:toluene (90:10, v/v) with 50 ng/mL CUDA standard.

All measurements were carried out on an Agilent 6530a Q-TOF instrument (1). For positive mode, 10 µL of diluted samples were injected. For negative mode, 1uL of diluted samples were injected. Samples were separated on a Waters Acquity UPLC CSH C18 column (100 × 2.1 mm; 1.7 µm) coupled to an Acquity UPLC CSH C18 VanGuard precolumn (5 × 2.1 mm; 1.7 µm). The column was maintained at 65°C with a flow rate of 0.6 mL/min. The positive ionization mobile phases consisted of (A) acetonitrile:water (60:40, v/v) with ammonium formate (10 mM) and formic acid (0.1%) and (B) 2-propanol:acetonitrile (90:10, v/v) with ammonium formate (10 mM) and formic acid (0.1%). The negative ionization mobile phases consisted of (A) acetonitrile:water (60:40, v/v) with ammonium formate (10 mM) and (B) 2-propnol:acetonitrile (90:10, v/v) with ammonium formate (10 mM). The separation was conducted under the following gradient: 0 min 15% B; 0−2 min 30% B; 2−2.5 min 48% B; 2.5−11 min 82% B; 11−11.5 min 99% B; 11.5−12 min 99% B; 12−12.1 min 15% B; 12.1−15 min 15% B.

The Agilent 6530a QTOF instrument was operated using positive mode electrospray ionization using the following parameters. Acquisition parameters: Mass range, 120-1700 m/z; Acquisition rate, 2 spectra/second; Acquisition time, 500 ms/spectrum; Mode, MS(Seg). Source Parameters: Gas Temp, 325°C; Drying Gas, 8L/min; Nebulizer, 35psig; Sheath Gas Temp, 350°C; Sheath gas flow, 11L/min; VCap, 3500V; Spectrum data type, Centroid. MS TOF parameters: Fragmentor,

120V; Skimmer, 65V; OCT 1 RP Vpp, 750V, Collision Energy, 0V. MSMS were acquired in a separate injection using the following acquisition parameters: MS1 Mass range, 65-1700m/z; MS/MS mass range, 35-1700; MS1 acquisition rate, 4 spectra/second; MS1 acquisition time, 250 ms/spectrum; MS/MS acquisition rate, 8 sepctra/s; MS/MS acquisition time, 125 ms/spectrum. Collision energy depended on m/z and was calculated using the following formula: 3×((m/z)/100)+2.5. Source parameters for MS/MS injections were the same as MS injections.

The Agilent 6530a QTOF instrument was operated using negative mode electrospray ionization using the following parameters. Acquisition parameters: Mass range, 60-1700 m/z; Acquisition rate, 2 spectra/second; Acquisition time, 500 ms/spectrum; Mode, MS(Seg). Source Parameters: Gas Temp, 325°C; Drying Gas, 8L/min; Nebulizer, 35psig; Sheath Gas Temp, 350°C; Sheath gas flow, 11L/min; VCap, 3500V; Spectrum data type, Centroid. MS TOF parameters: Fragmentor, 120V; Skimmer, 65V; OCT 1 RP Vpp, 750V, Collision Energy, 0V. MSMS were acquired in a separate injection using the following acquisition parameters: MS1 Mass range, 65-1700m/z; MS/MS mass range, 35-1700; MS1 acquisition rate, 4 spectra/second; MS1 acquisition time, 250 ms/spectrum; MS/MS acquisition rate, 8 sepctra/s; MS/MS acquisition time, 125 ms/spectrum. Collision energy depended on m/z and was calculated using the following formula: 3×((m/z)/100)+2.5. Source parameters for MS/MS injections were the same as MS injections.

A calibration curve was run for cardiolipin quantification using CL 72:8. The curve was run at the following concentrations: 0.001μg/mL, 0.01μg/mL, 0.1μg/mL, 1μg/mL, 10μg/mL, 20μg/mL, 50μg/mL, 100μg/mL. The LC-MS/MS data was analyzed by MS-DIAL software.

1.2.4 mtDNA to nuDNA ratio

Quantitative PCR of a mitochondrial gene mtND1 (mitochondrially encoded NADH dehydrogenase 1) relative to a single copy nuclear gene, Cftr (Cystic fibrosis transmembrane conductance regulator) was used to measure mtDNA to nuDNA ratio. Total DNA was extracted from tissues using a DNeasy blood & tissue kit (Qiagen, Valencia, CA) and quantified using a NanoDrop 2000c Spectrophotometer (Thermo Scientific, Waltham, MA). A SensiFAST SYBR No-ROX Kit (Bioline, Taunton, MA) was used to perform qPCR in a Roche Lightcycler 480 (Roche Diagnostics, Indianapolis, IN, USA). Primer sequence targeting mtND1 and Cftr includes, mt-Nd1-F: TCCGAGCATCTTATCCACGC, mt-Nd1-R: GTATGGTGGTACTCCCGCTG, Cftr-F:ATGGTCCACAATGAGCCCAG, Cftr-R:GAACGAATGACTCTGCCCCT). The second derivative of the amplification curve was used to determine the cycle threshold, and the data were analyzed by a delta-delta CT calculation.

1.2.5 Western blotting

Powdered tissue samples were homogenized in a RIPA lysis buffer (Cell Signaling, Danvers, MA, USA) with a protease inhibitor cocktail (Roche, Basel, Switzerland). Samples were centrifuged at 16,000 g for 20 minutes, and the supernatant was collected. Protein concentration was measured using a Bradford protein assay kit (Bio-Rad, Hercules, CA, USA), and 30 μg of protein was loaded onto a 4-20% SDS-PAGE gel (Bio-Rad) and run at 200 V for 45 minutes. The protein was transferred to a 0.2 μm nitrocellulose membrane (Bio-Rad) at 100 V for 30 minutes using the plate electrode. Total protein normalization was assayed using a Revert 700 Total Protein Stain Kit (LI-COR, Lincoln, NE, USA). The membrane was blocked using a blocking buffer (LI-COR) then probed with primary antibody overnight at 4˚C. Primary antibodies against MTCO1 (Complex IV)

(ab14705,1:2000; ab203912, 1:1000), NDUFB-8 (Complex I) (ab110242, 1:2000), and Citrate Synthase (ab129095, 1:1000) were obtained from Abcam (Cambridge, United Kingdom). NIR fluorescent secondary antibodies (LI-COR) were applied and membranes were imaged using a LI-COR Odyssey imager.

1.2.6 Statistical Analysis

All values are expressed as mean ± SEM unless otherwise indicated. Diet and aging effects on markers of mitochondrial mass were determined using two-way ANOVAs with main effects of diet and age and their interaction, followed by Bonferroni post hoc tests. Significance for all comparisons was set at $p < 0.05$ while controlling for multiple testing by Bonferroni method. All statistical analyses were conducted using GraphPad Prism 8.1 (GraphPad Software Inc., San Diego, CA).

1.3 Results

1.3.1 Ketogenic diet increased activities of mitochondrial enzymes at 26 months of age in hindlimb skeletal muscle without changes in other markers of mitochondrial mass at advanced age

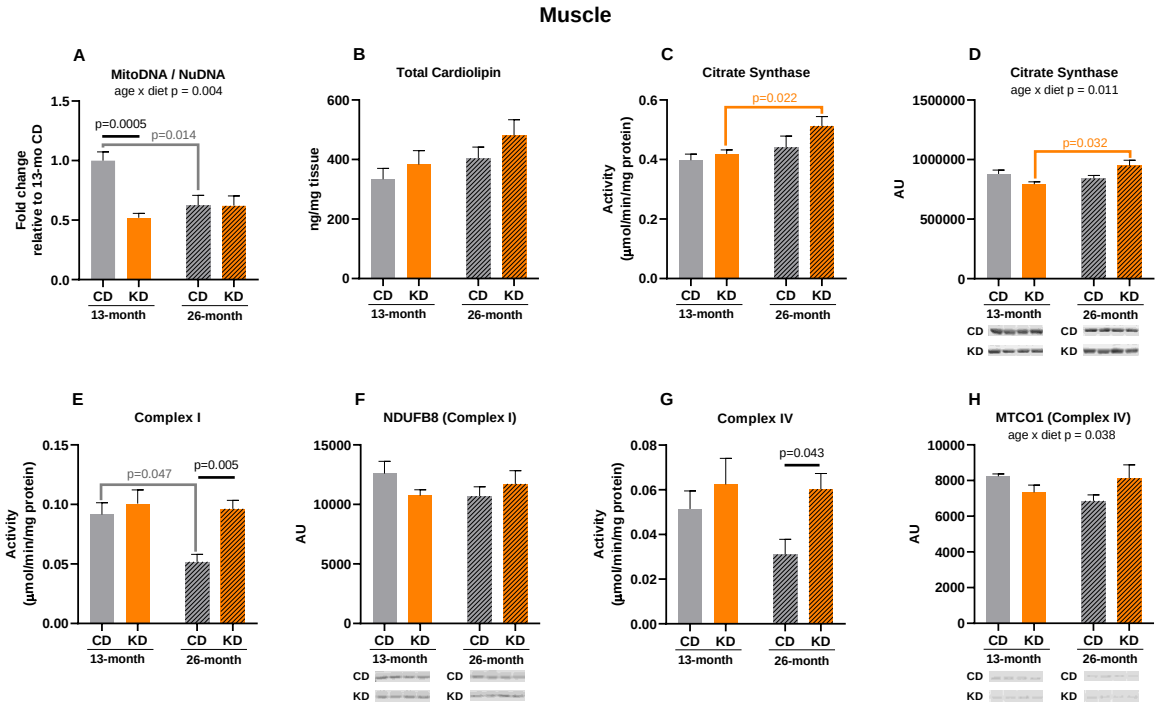

Figure 1.1. Markers of mitochondrial content in hindlimb skeletal muscle of male mice after 1 month (13 months of age) and 14 months (26 months of age) on diet (n=4-6). (A) Mitochondrial to nuclear DNA ratio. Quantification of (B) total cardiolipin. Enzymatic activities for (C) citrate synthase, (E) Complex I, and (G) Complex IV. Quantification of (D) citrate synthase, (F) NDUFB8, and (H) MTCO1 protein levels by western blots. Diets: CD = control, KD= Ketogenic. Ages: solid bar = 13 months, dashed bar = 26 months. Values are expressed as mean ± SEM. 2-way ANOVAs followed by Bonferroni post hoc tests.

After 1 month of KD (13 months of age), mtDNA to nuDNA ratio was decreased ($p = 0.0005$) in the whole hindlimb skeletal muscle (Figure 1.1A). No other mitochondrial markers differed between diet groups at 13 months of age (Figure 1.1B-H).

After 14 months of intervention (26 months of age), KD mice showed a 2-fold increase in Complex I ($p = 0.005$) and IV ($p = 0.043$) activities compared to control mice (Figure 1.1E and 1.1G). These changes in enzyme activities were not accompanied by elevated enzyme protein levels (Figure 1.1F and 1.1H), indicating that the increased activities of these enzymes were likely driven by post-translational mechanisms. There were no diet-related changes in other markers of mitochondrial content (Figure 1.1A-D). Thus, the changes observed in this age group were only increases in mitochondrial enzyme activities.

Complex I activity in control mice decreased by half ($p = 0.047$) at 26 months (Figure 1E) while the aged KD mice maintained the activity of this enzyme compared to their younger cohort, and this was not due to age-related changes in NDUFB8 (Complex I) protein level (Figure 1.1F). There were significant age x diet interactions for mtDNA to nuDNA ratio (Figure 1.1A), citrate synthase protein level (Figure 1.1D), and MTCO1 (Complex IV) protein level (Figure 1.1H), indicating that the diet groups showed a different response to aging. These interactions were accompanied by an age-related decrease in mtDNA to nuDNA ratio ($p = 0.014$) in the control group (Figure 1.1A) and an age-related increase in citrate synthase protein ($p = 0.032$) in the KD mice (Figure 1.1D). Along with this increase in citrate synthase protein level, citrate synthase activity was increased ($p = 0.022$) in 26-month old compared to 13-month old KD mice (Figure 1.1C), and no significant change was observed in old control mice. These results indicate that at the older age KD mice maintained or increased several markers of mitochondrial mass while decreases in some markers were observed in the old control mice. However, neither the KD nor control mice showed a

concerted age-related change in the majority of the markers suggesting that there was not an overt change in mitochondrial mass.

1.3.2 Long-term consumption of a ketogenic diet prevented significant age-related decreases in Complex IV activity and stimulated citrate synthase activity in the brain but induced few changes in other markers of mitochondrial mass

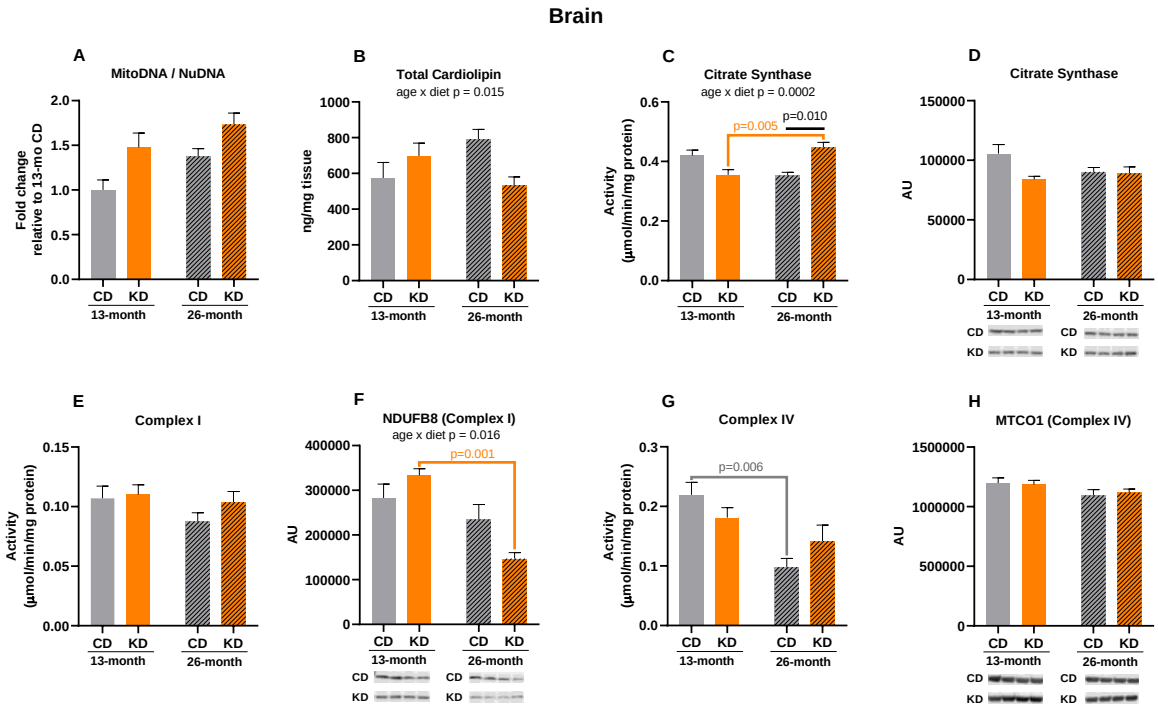

Figure 1.2. Markers of mitochondrial content in the brain of male mice after 1 month (13 months of age) and 14 months (26 months of age) on diet (n=4-6). (A) Mitochondrial to nuclear DNA ratio. Quantification of (B) total cardiolipin. Enzymatic activities for (C) citrate synthase, (E) Complex I, and (G) Complex IV. Quantification of (D) citrate synthase, (F) NDUFB8, and (H) MTCO1 protein levels by western blots. Diets: CD = control, KD= Ketogenic. Ages: solid bar = 13 months, dashed bar = 26 months. Values are expressed as mean ± SEM. 2-way ANOVAs followed by Bonferroni post hoc tests.

After 1 month of KD (13 months of age), there was no significant difference in markers of mitochondrial mass measured between KD and control groups (Figure 1.2A-H).

After 14 months of KD (26 months of age), KD mice showed an increase ($p = 0.010$) in citrate synthase activity compared to the control mice (Figure 1.2C), and the increase was not caused by changes in protein level (Figure 1.2D). No other mitochondrial markers differed between diet groups at 26 months of age (Figure 1.2A-B and 1.2E-H).

Complex IV activity in control mice decreased by half ($p = 0.006$) with aging (Figure 1.2G), while KD mice showed no significant change in the activity of this enzyme with aging. There were also significant age x diet interactions for citrate synthase activity (Figure 1.2C), NDUFB8 (Complex I) protein (Figure 1.2F), and cardiolipin (Figure 1.2B). The interaction for citrate synthase activity was driven by the observation that citrate synthase activity increased ($p = 0.005$) with aging in the KD, but not control, mice (Figure 1.2C). In contrast, the interaction for NDUFB8 (Complex I) was due to a decrease by more than half ($p = 0.001$) in NDUFB8 (Complex I) protein level with aging in the KD, but not control, animals (Figure 1.2F). It should be noted, however, that this change in protein level was not sufficient to alter Complex I enzyme activity in the KD mice (Figure 1.2E). There were no other age-related changes in markers of mitochondrial mass (Figure 1.2A, 1.2D-E, and 1.2H). Some enzyme activities were maintained or increased with aging in the KD mice, but there was no change in mitochondrial markers indicative of an increase in brain mitochondrial mass in these animals.

1.3.3 The effects of ketogenic diet on markers of mitochondrial mass in liver were mixed, indicating no clear pattern of change in mitochondrial mass

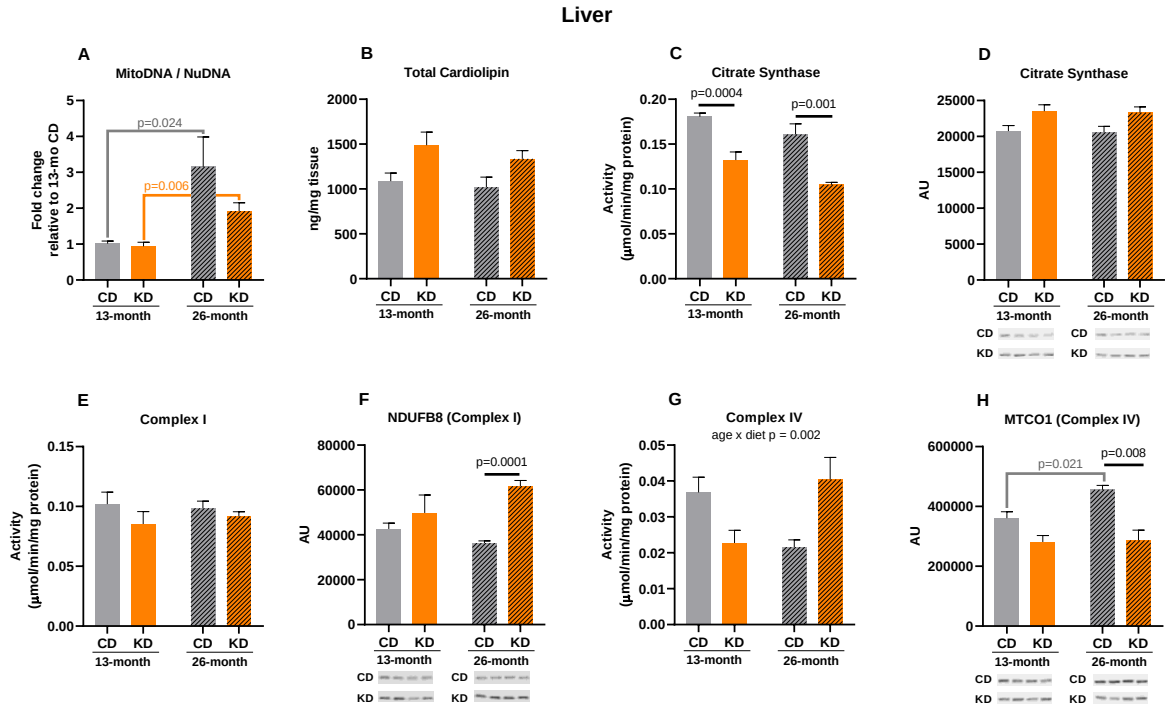

Figure 1.3. Markers of mitochondrial content in the liver of male mice after 1 month (13 months of age) and 14 months (26 months of age) on diet (n=4-6). (A) Mitochondrial to nuclear DNA ratio. Quantification of (B) total cardiolipin. Enzymatic activities for (C) citrate synthase, (E) Complex I, and (G) Complex IV. Quantification of (D) citrate synthase, (F) NDUFB8, and (H) MTCO1 protein levels by western blots. Diets: CD = control, KD= Ketogenic. Ages: solid bar = 13 months, dashed bar = 26 months. Values are expressed as mean ± SEM. 2-way ANOVAs followed by Bonferroni post hoc tests.

Consistent with a chronically activated gluconeogenic pathway in the liver of mice on a continuous KD, citrate synthase activity was decreased ($p = 0.0004$) after 1 month of KD (13 months of age, Figure 1.3C) and this was not accompanied by a decrease in citrate synthase protein level (Figure 1.3D). Other markers showed no significant changes with 1 month of KD (Figure 1.3A-B and 1.3E-H).

After 14 months of KD (26 months of age), citrate synthase activity was decreased ($p = 0.001$) compared to control mice (Figure 1.3C), consistent with the results observed with short-term consumption of a KD. No significant differences were observed in activities of Complex I and IV (Figure 1.3E and 1.3G), but Complex I (NDUFB8) protein level increased ($p = 0.0001$) and Complex IV (MTCO1) protein level decreased ($p = 0.008$) in the KD versus control groups (Figure 1.3F and 1.3H). For the remaining mitochondrial markers, no significant changes were observed (Figure 1.3A-B and 1.3D).

A significant age x diet interaction was observed in Complex IV activity (Figure 1.3G), with the KD mice showing a trend ($p = 0.056$) toward increased Complex IV activity versus decreased (a trend, $p = 0.1$) activity with aging in the control group. This trend toward an increase in Complex IV activity in the KD mice occurred without age-related changes in MTCO1 (Complex IV) protein levels (Figure 1.3H). In contrast, MTCO1 (Complex IV) protein level was increased ($p = 0.021$) with aging in the control animals (Figure 1.3H). Control and KD mice also showed a 3-fold ($p = 0.024$) and 2-fold increase ($p = 0.006$), respectively, in mtDNA to nuDNA ratio with aging (Figure 1.3A). Citrate synthase activity exhibited a trend ($p = 0.053$) toward a decrease in the KD mice with aging (Figure 1.3C). There were no age-related changes in the other markers of mitochondrial mass (Figure 1.3B and 1.3D-F). There was no overall pattern indicative of a change in liver mitochondrial content in aged KD mice or following short or long-term consumption of a KD.

1.3.4 Mitochondrial markers in kidneys did not show concerted changes indicative of an alteration in mitochondrial mass with a ketogenic diet

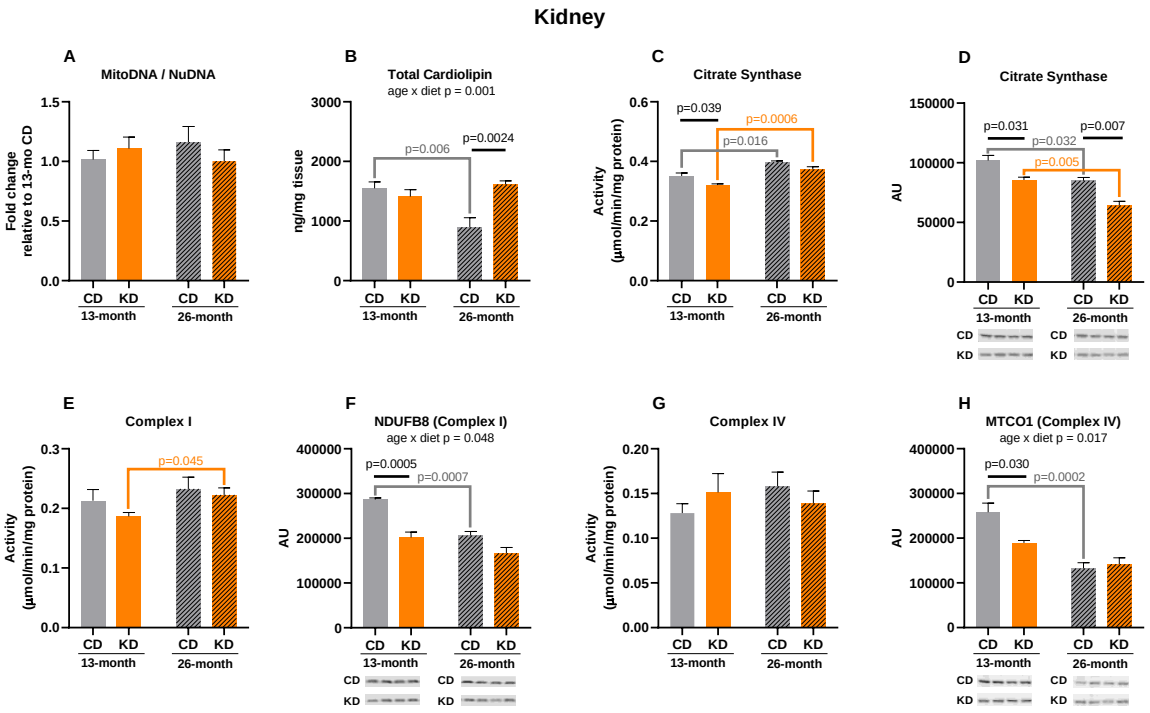

Figure 1.4. Markers of mitochondrial content in the kidney of male mice after 1 month (13 months of age) and 14 months (26 months of age) on diet (n=4-6). (A) Mitochondrial to nuclear DNA ratio. Quantification of (B) total cardiolipin. Enzymatic activities for (C) citrate synthase, (E) Complex I, and (G) Complex IV. Quantification of (D) citrate synthase, (F) NDUFB8, and (H) MTCO1 protein levels by western blots. Diets: CD = control, KD= Ketogenic. Ages: solid bar = 13 months, dashed bar = 26 months. Values are expressed as mean ± SEM. 2-way ANOVAs followed by Bonferroni post hoc tests.

Citrate synthase activity was decreased ($p = 0.039$) in KD mice after 1 month on diet (13 months of age, Figure 1.4C), and this may in part be due to a decrease ($p = 0.031$) in citrate synthase protein level (Figure 1.4D). After 1 month of KD, Complex I (NDUFB8) and IV (MTCO1) protein levels were lower ($p = 0.0005$, $p = 0.030$) in the KD versus control groups (Figure 1.4F and 1.4H), but the activities of these enzymes were not significantly different (Figure 1.4E and 1.4G) between diet groups. There were no changes in other markers between diet groups at 13 months of age (Figure 1.4A-B).

In the 26-month old group (14 months on diet), cardiolipin content was 2-fold higher ($p = 0.0024$) in old KD versus control mice (Figure 1.4B), implying an increase in inner membrane lipid amount. KD mice showed a decrease ($p = 0.007$) in citrate synthase protein level (Figure 1.4D) consistent with the results observed in the 13-month old group, but this did not translate to a significant decrease in citrate synthase activity (Figure 1.4C). There were no changes in other markers between diet groups at 26 months of age (Figure 1.4A and 1.4E-H).

There were significant age x diet interactions for cardiolipin (Figure 1.4B), NDUFB8 (Complex I) (Figure 1.4F), and MTCO1 (Complex IV) (Figure 1.4H). All of these interactions were driven by a significant ($p < 0.05$) age-related decrease in the control, but not KD, mice. In contrast to the liver, there was an age-related increase in citrate synthase activity in both the control ($p = 0.016$) and KD ($p = 0.0006$) groups (Figure 1.4C), but this was accompanied by a decrease ($p= 0.032$ and 0.005, respectively) in citrate synthase protein abundance with aging in both diet groups (Figure 1.4D). Complex I activity was also increased ($p = 0.045$) with aging in the KD group (Figure 1.4E), and this was not caused by a change in protein level (Figure 1.4F). There were no age-related changes in mtDNA to nuDNA ratio (Figure 1.4A) or Complex IV activity (Figure 1.4G). Similar to the liver, there was no overall pattern indicative of a change in kidney mitochondrial mass in aged KD mice or following short or long-term consumption of a ketogenic diet.

1.4 Discussion

The effects of KDs on measures of mitochondrial mass have been reported in laboratory rodents. However, these studies have almost exclusively focused on models of injury or disease (18-21) or used only one marker of the mitochondrial mass collected from moribund animals (29). Thus, relatively little is known about the impact of long-term consumption of a KD on markers of

mitochondrial mass, and it is not clear if a KD increases mitochondrial mass in particular tissues. This study is the first to report the influence of a KD on a panel of markers of mitochondrial mass in various tissues of healthy middle-aged (13-month) and aged (26-month) mice.

Although there is evidence that KDs increase transcription of proteins involved in mitochondrial energy metabolism (24), increase markers of mitochondrial biogenesis (26), and induce post-translational modification of mitochondrial proteins (14, 44), there is currently no convincing evidence showing an increase in mitochondrial content across multiple tissues with a KD. The results of the present study did not provide evidence of a widespread increase in mitochondrial mass. We did not observe concerted changes in all markers of mitochondrial mass in any tissue implying the intervention may be inducing remodeling of the organelle or impacting only some mitochondrial components rather than inducing a broad increase in mitochondrial mass. This conclusion would be consistent with the fact that animals on a KD did not have increased whole animal energy expenditure (13). Unlike exercise training, where a clear increase in mitochondrial mass has been observed in skeletal muscle (45), there is not a similar drive to increase capacity for mitochondrial energy production in animals consuming a KD. The need to change fuel utilization rather than increase energy production might contribute to the changes observed in the present study where the KD differentially altered markers of mitochondrial content in a tissue-specific manner.

The impact of a KD on mitochondrial mass in skeletal muscle is of particular interest since this post-mitotic tissue is rich in mitochondria and shows an age-related decline in function. Among the markers of mitochondrial mass, activities of ETC enzymes and citrate synthase have shown a strong association with skeletal muscle mitochondrial mass (38). Our results demonstrate that the activities of Complexes I and IV were increased in hindlimb skeletal muscle after 14 months of a

KD and these are consistent with the improved muscle strength observed in our previous study (13). Little is known about the impact of KDs on skeletal muscle ETC enzyme activities, with one study reporting no change in ETC enzyme activities in rats consuming a KD for 6 weeks (25). This finding is consistent with our 1-month results and indicates that duration on diet or age influences the increases in Complex I and IV activities we observed in mice with long-term KD. These increases in enzyme activities on a long-term KD, however, do not appear to be indicative of an increase in mitochondrial content since they were not accompanied by similar changes in Complex I or IV protein levels. Thus, the impact of a KD on these enzymes seems to occur primarily through post-translational mechanisms (46, 47). Increased levels of lysine acetylation and β-hydroxybutyrylation are potential post-translational modifications of mitochondrial enzymes under KD (9), but whether these modifications to citrate synthase, Complex I, or Complex IV impact enzyme activities is not clear (48, 49). More studies are needed to elucidate the mechanisms underlying the changes of mitochondrial enzyme activities under KD. In regard to the influence of a KD on citrate synthase activity, we did not observe a difference between diet groups. Other KD studies in rodents have shown no consensus with gastrocnemius citrate synthase activity decreased (28), increased (29), or unchanged (18, 25) with a KD. These studies differed in animal age, duration on diet, and timing of sample collection in relation to feeding, and there is not sufficient information at this time to determine what factors are driving the differences in citrate synthase activity between these KD studies. The existing citrate synthase data provide little evidence for a clear KD-induced increase in hindlimb skeletal muscle mitochondrial content. In addition to changes in mitochondrial enzymes, the mtDNA to nuDNA ratio has been used as an indicator of mitochondrial mass (50). We found no evidence for an increase in the mtDNA to nuDNA ratio with the KD. In fact, the KD group actually showed a decrease at 1 month of dietary intervention.

An age-related decrease in mtDNA content has been shown in several (51-53), but not all (50) studies using rodent skeletal muscle. In our study, the control mice showed an age-related decrease in mtDNA to nuDNA ratio, while no change was observed in KD mice. Similarly, the KD group did not show age-related decreases in Complex I enzyme activity that was observed in the control group. Long-term consumption of a ketogenic diet increased ETC enzyme activities at the older age and prevented age-related changes in some mitochondrial components that occurred in skeletal muscle in old control mice, although all these changes appeared to be through mechanisms distinct from an overall change in mitochondrial mass. Our results suggest that a KD may promote maintenance of hindlimb skeletal muscle mitochondria with age rather than inducing an overall increase in mitochondrial mass across all hindlimb muscle groups.

The influence of a KD on brain mitochondrial mass was also of interest since we previously observed improved memory in aged mice fed a KD (13). The use of KDs to manage age-related neurological disorders has been actively explored (54) and KDs might provide benefits by stimulating cellular energy metabolism and mitochondrial biogenesis in brain (16). However, relatively little is known about the impact of sustained consumption of a KD on markers of mitochondrial mass. Activities of citrate synthase and ETC enzymes have been reported to decrease with aging in rodent (55-58) and primate (59) brains and KD-induced changes in mitochondrial content could oppose these age-related changes. However, a study in rats reported that consumption of a KD for 8 months did not have an impact on citrate synthase activity in cortex (28). The influence of a KD on brain citrate synthase in our study was complex with one month of diet inducing a trend toward decreased activity and protein level, while long-term consumption of a KD resulted in an increase in citrate synthase activity that was not driven by changes in protein amount. Our results suggest that citrate synthase shows a time-dependent response to a KD that

depends on diet duration and/or age. In regard to ETC enzymes, it was reported that 1 month of a KD did not change the levels of subunits of ETC proteins (60), which is consistent with our findings. Similarly, 14 months of KD did not alter ETC enzyme activities, although it did prevent age-related decreases in Complex IV activity observed in the control group. The KD was able to maintain ETC enzyme activities despite a decrease in Complex I (NDUFB8) protein, providing further evidence that a KD may be altering the activities of some proteins through post-translational modifications. Collectively, our results for brain indicate that long-term consumption of a KD maintained Complex IV activity and increased citrate synthase activity with aging, but these changes do not appear to be due to an overall increase in brain mitochondrial content since similar increases in mtDNA to nuDNA ratio, cardiolipin or protein levels were not observed. The disparate changes in markers of brain mitochondrial mass with consumption of a KD suggest only a partial stimulation of mitochondrial pathways rather than a clear increase in mitochondrial mass.

A limitation of our skeletal muscle and brain work was that the mitochondrial markers were analyzed in homogenates of the entire hindlimb skeletal muscle and brain, and it was not possible to determine the response of specific muscles or brain regions to a KD. Variable impacts of aging on markers of mitochondrial content have been found in different rodent hindlimb muscles (61). Changes in muscle fiber type composition, which can have an impact on mitochondrial mass in muscle, have been reported with aging and calorie restriction (62, 63), and the effect of KD on fiber types should be explored in future studies to elucidate if the changes in markers of mitochondrial mass are correlated to alterations in fiber type composition. In the brain, variations in cell architecture and population can occur with aging and region-specific changes in brain mitochondrial mass have also been observed (64, 65). Studies using transmission electron microscopy (TEM) have shown that KDs may increase mitochondrial abundance under certain

conditions in some neural populations (22, 24, 27, 65). Measurements of markers of mitochondrial mass or mitochondrial quantification using TEM will be needed for future studies in multiple muscles and brain regions to determine if a KD alters mitochondrial mass in specific muscle groups or regions of the brain.

The liver is involved in many metabolic adaptations to a KD, including increased fatty acid oxidation, ketone body production, and gluconeogenesis, and these metabolic alterations might have an impact on some markers of mitochondrial mass. Liver citrate synthase activity was decreased with the KD in our study, which could reflect chronically stimulated gluconeogenesis (66). This could be explained by the fact that citrate synthase is inhibited by palmitoyl-CoA, a metabolite increased during ketosis (67). However, the impact of a KD on citrate synthase activity likely depends on diet composition and physiological state. Rat studies have shown that citrate synthase activity increased (29) or stayed unchanged (28) in liver with a KD. However, the study that reported no change had much higher protein and carbohydrate levels than the diet used in our study, limiting the necessity for gluconeogenesis. The animals on this diet also did not have significantly higher β-hydroxybutyrate level compared to control rats. Animals in the study that found an increased activity were moribund when tissues were collected and this extreme metabolic state makes it hard to compare these results to healthy animals. Another rat study found that a KD combined with voluntary wheel running increased liver citrate synthase activity (26). Liver samples from these rats were collected following a brief fast (3-4 hours) and insulin levels were above typical fasting levels suggesting that gluconeogenesis may have been inhibited at the time of sample collection in this study. Thus, citrate synthase activity is influenced by the metabolic need of the animal, leading to variability in results observed between studies and making the activity of this enzyme a poor marker for liver mitochondrial content. Moreover, a study reported

that a KD differentially altered mitochondrial matrix and inner membrane protein levels in 5-month old mice (44), which are consistent with the mixed changes in citrate synthase, Complex I, and Complex IV protein levels we observed with the consumption of a KD in our study. Previous studies have demonstrated an increase (68, 69) or decrease (70, 71) in mtDNA content in aged rodents, and in this study a significant increase in mtDNA to nuDNA ratio with aging was observed for both control and KD mice, with no diet-related difference. The increase in mtDNA to nuDNA ratio was not likely due to an increase in overall mitochondrial mass as most of the other markers were not changed with aging. Although some significant changes in makers of mitochondrial mass were observed, the lack of a common direction in these changes did not provide support for an alteration in mitochondrial mass between KD and control groups. Overall, liver did not show coordinated changes in mitochondrial markers consistent with an increase in mitochondrial mass, and instead, the mitochondrial changes likely reflected the sustained shift in metabolic pathways induced by long-term consumption of a KD rather than a change in capacity for energy metabolism.

Little is known about the influence of KD and aging on markers of mitochondrial mass in kidneys. Abnormalities in mitochondrial morphology have been observed in kidneys of aged rodents (72), and our results demonstrated that aging decreased cardiolipin content, a marker of mitochondrial membrane content, and the KD prevented the age-related declines observed in the control mice. The results in kidneys also suggest that a KD may help maintain mitochondrial components with aging, but this does not appear to be due to an overall change in mitochondrial mass.

Our data demonstrated that there is no uniform change with a KD in the markers of mitochondrial mass assayed in this study either within or across tissues. Discrepancies in outcomes of dietary interventions may be the result of drawing conclusions from a single marker of mitochondrial mass and the use of different markers between studies. For example, mtDNA content, as mtDNA copy

number or mtDNA to nuDNA ratio, has been commonly used as a major marker of mitochondrial content. However, there are instances when mitochondrial biogenesis is not accompanied with an increase in mtDNA (73), and a study in human skeletal muscle found that mtDNA was a poor marker of mitochondrial content when compared to measurements using TEM (41). Thus, a single or a few markers may not reflect actual changes in mitochondrial mass, and a variety of markers is likely required to thoroughly determine if an intervention increases mitochondrial mass or differentially affects components of mitochondria to induce remodeling of the organelle. Tissue-specific differences were also observed in the study, and more work is needed to determine which markers best reflect changes in mitochondrial mass in each tissue.

1.5 Conclusion

In summary, our study demonstrated that a KD affected markers of mitochondrial mass or content in a tissue-specific manner in male C57BL/6JN mice. Stimulation of mitochondrial enzymes was observed in skeletal muscle and brain, likely due to post-translational modifications of the enzymes rather than an increase in the amount of enzyme or mitochondria. Age-related changes in some markers were also prevented by the KD. The impacts of KD on liver and kidney markers were mixed and likely reflected regulation or remodeling of mitochondria to meet metabolic demands of the animal. The results of the present study suggest that a KD induces tissue-specific changes in mitochondrial enzyme activities or structure rather than global changes in mitochondrial mass across tissues. Moreover, measurements using multiple muscle groups and brain regions are needed for future studies to investigate changes of mitochondrial mass in specific tissue groups or regions in response to a KD.

Chapter 2. A 1-month ketogenic diet increased mitochondrial mass in red gastrocnemius muscle, but not brain or liver in middle-aged mice

Alterations in markers of mitochondrial content with ketogenic diets (KD) have been reported in tissues of rodents, but morphological quantification of mitochondrial mass using transmission electron microscopy (TEM), the gold standard for mitochondrial quantification, is needed to further validate these findings and look at specific regions of interest within a tissue. In this study, the liver left lobe, red gastrocnemius muscle, prefrontal cortex, and hippocampus were used to investigate the impact of a 1-month KD on mitochondrial content in healthy middle-aged mice. The results showed that in red gastrocnemius muscle, both subsarcolemmal (SSM) and interfibrillar (IMM) mitochondrial areas were increased, and this was driven by an increase in the number of mitochondria. Mitochondrial area or number was not altered in the liver, prefrontal cortex, or hippocampus following 1 month of a KD. These results demonstrated tissue-specific changes in mitochondrial mass with a short-term KD and highlight the need to study different muscle groups or tissue regions with TEM to thoroughly determine the effects of a KD on mitochondrial mass.

2.1 Introduction

We have previously investigated the impact of a ketogenic diet (KD) started in early middle-aged (12-month) mice on a panel of commonly used markers of mitochondrial mass were investigated in various tissues (Zhou et al. Aging-US in press). Whole tissue homogenates were analyzed to assess overall changes in mitochondrial mass, and after 1 month of intervention (13 months of age), no concerted changes in markers were observed in whole tissue homogenates. However, it is not known if mitochondrial mass in specific muscle groups or regions within a tissue may be altered with a KD. For example, muscle mitochondrial mass varies depending on fiber type, and muscle regions that are denser in oxidative fibers (type I and IIa) may be impacted by a shift in fuel utilization to a greater extent than other muscle groups. Moreover, transmission electron microscopy (TEM) is considered the gold standard for mitochondrial quantification in tissues (41), and the present study used TEM to perform morphological quantification of mitochondrial mass in regions of skeletal muscle, liver, and brain in middle-aged mice following 1 month of KD.

The impact of KDs on mitochondrial mass has been studied in rodent liver and skeletal muscle using various mitochondrial markers (Zhou et al. Aging-US, in press), but TEM quantification of mitochondrial content in KD animals has not been performed in these tissues. The use of TEM allows the selection of specific regions of interest within a tissue. In the present study, the middle region of the largest liver lobe (the left lobe) was selected as the representative region. Red gastrocnemius muscle was selected as the region of interest for skeletal muscle since this muscle group is widely studied for mitochondrial content and contains a variety of fiber types, including a relatively high level of oxidative fibers (74). TEM has been utilized to quantify changes in mitochondrial content in brain regions with KDs in rodent models of neurological diseases (22, 75, 76), but little is known on how a KD affects mitochondrial mass in healthy adult rodents. A few

studies have been conducted in aged rats to investigate effects of an MCT-supplemented KD on mitochondrial content in the hippocampus and cerebellum (27, 65, 77). However, changes in the prefrontal cortex, which are believed to mediate many of the cognitive declines seen with neurological disorders and aging, have not been studied. Thus, in the present study, morphological quantification of mitochondrial content was performed in both hippocampus and prefrontal cortex since these regions are heavily involved in memory and are susceptible to changes with aging (78).

2.2 Methods and Results

2.2.1 Animals and diets

Male C57BL/6JN mice were obtained at 11 months of age from the NIA Aged Rodent Colony. The housing conditions and diets were same as described in Roberts et al. (13). Mice were randomly assigned to a control (CD) or ketogenic (KD), with body weight counterbalanced in each group, at 12 months of age and were fed 11.2 kcal/day throughout the study. After 1 month of intervention, 13 months of age, animals were sacrificed with CO_2 inhalation in the morning following an overnight fast. Tissues were quickly dissected and cut into cubes in ice-cold PBS before fixation. Body weight was not significantly different between CD and KD mice at the end of the study (CD: 31.85±1.16 g, KD: 32.07±0.52 g).

2.2.2 Tissue processing for transmission electron microscopy

The left liver lobe, dorsal hippocampus, prefrontal cortex, and red gastrocnemius were selected as the regions for liver, brain, and skeletal muscle. Tissues were quickly dissected and cut into approximately 1 mm^3 cubes in ice-cold PBS. Samples were fixed in 2.5% glutaraldhyde and 2% paraformaldehyde in 0.1M sodium phosphate buffer overnight, then rinsed in 0.1M sodium

phosphate 2 times, each 15 minutes. Tissues were post-fixed in 1% osmium tetroxide. After rinsing in distilled water, samples were dehydrated with a graded ethanol series from 50-100%. The samples were suspended in propylene oxide twice for 15 minutes and then pre-infiltrated overnight in 1:1 propylene oxide: resin (Dodecenyl Succinic Anhydride, Araldite 6005, Epon 812, Dibutyl Phthalate, Benzyldimethylamine) followed by infiltrating for 5 hours in 100% resin. The samples were embedded in fresh resin and polymerized for 24 hours at 70 ˚C. Approximately 100 nm sections were cut using a Leica EM UC6 ultramicrotome and collected on copper grids. The sections were stained with 4% aqueous uranyl acetate followed by 0.3% lead citrate in 0.1N sodium hydroxide and imaged with a FEI Talos L120C transmission electron microscope (Thermo Fisher, Waltham, MA).

2.2.3 Micrograph processing and mitochondrial mass quantification

For hippocamps and prefrontal cortex, 10 images were randomly taken for each sample and only regions of neuropils were included. For red gastrocnemius muscle, 10 images each were taken in the subsarcolemmal (SSM) and interfibrillar mitochondrial (IMM) regions from 3-5 fibers for each sample. Images of whole hepatocytes were taken, and 4-6 cells were used for each sample. The images were then processed through Image J (NIH). The outline of each mitochondrion was precisely drawn using a Surface Pro 6 tablet equipped with a touch pen (Microsoft, Redmond, WA, USA), and filled with a solid bright color. The image with color filled mitochondria was converted into a binary black and white image (Figure 2.5), and then areas of mitochondria were computed using the particle analysis tool. Fractional area was calculated as total mitochondrial area divided by image area for brain tissues, fiber area for muscle tissues, and cytosolic area for liver. Areas of lipid droplets in hepatocytes were measured, and mitochondrial fractional area was calculated with lipid droplets included or excluded, and the results did not significantly differ using either approach.

Mitochondrial number and average mitochondrial area (an indication of mitochondrial size) were also measured.

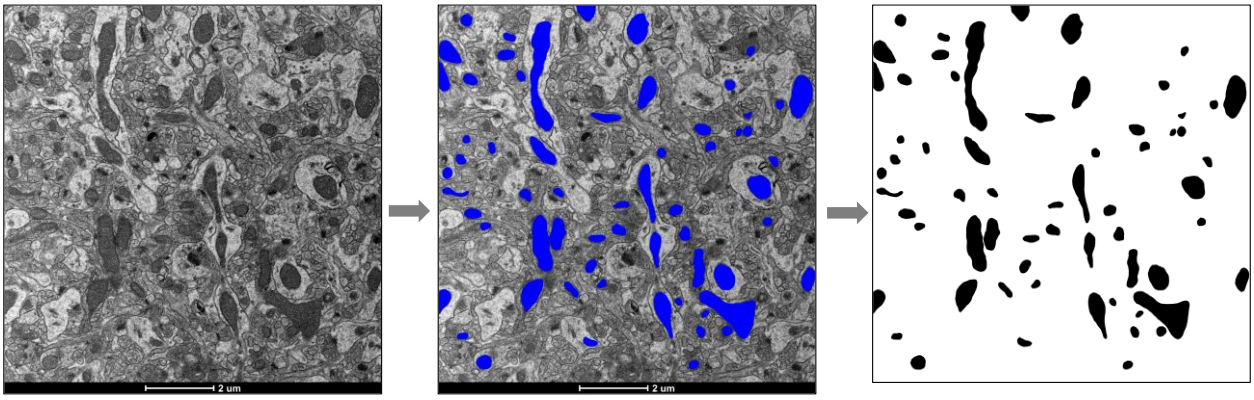

Figure 2.5. Micrographs were processed and analyzed using Image J to calculate mitochondrial fractional area.

2.2.4 Statistical Analysis

All values are expressed as mean ± SEM unless otherwise indicated. Unpaired t-tests were performed to compare mitochondrial fractional area measured by TEM between groups. Significance for all comparisons was set at $p < 0.05$. All statistical analyses were conducted using GraphPad Prism 8.1 (GraphPad Software Inc., San Diego, CA).

2.3 Results

2.3.1 One month of a KD increased both subsarcolemmal (SSM) and interfibrillar (IMM) mitochondrial mass in red gastrocnemius muscle

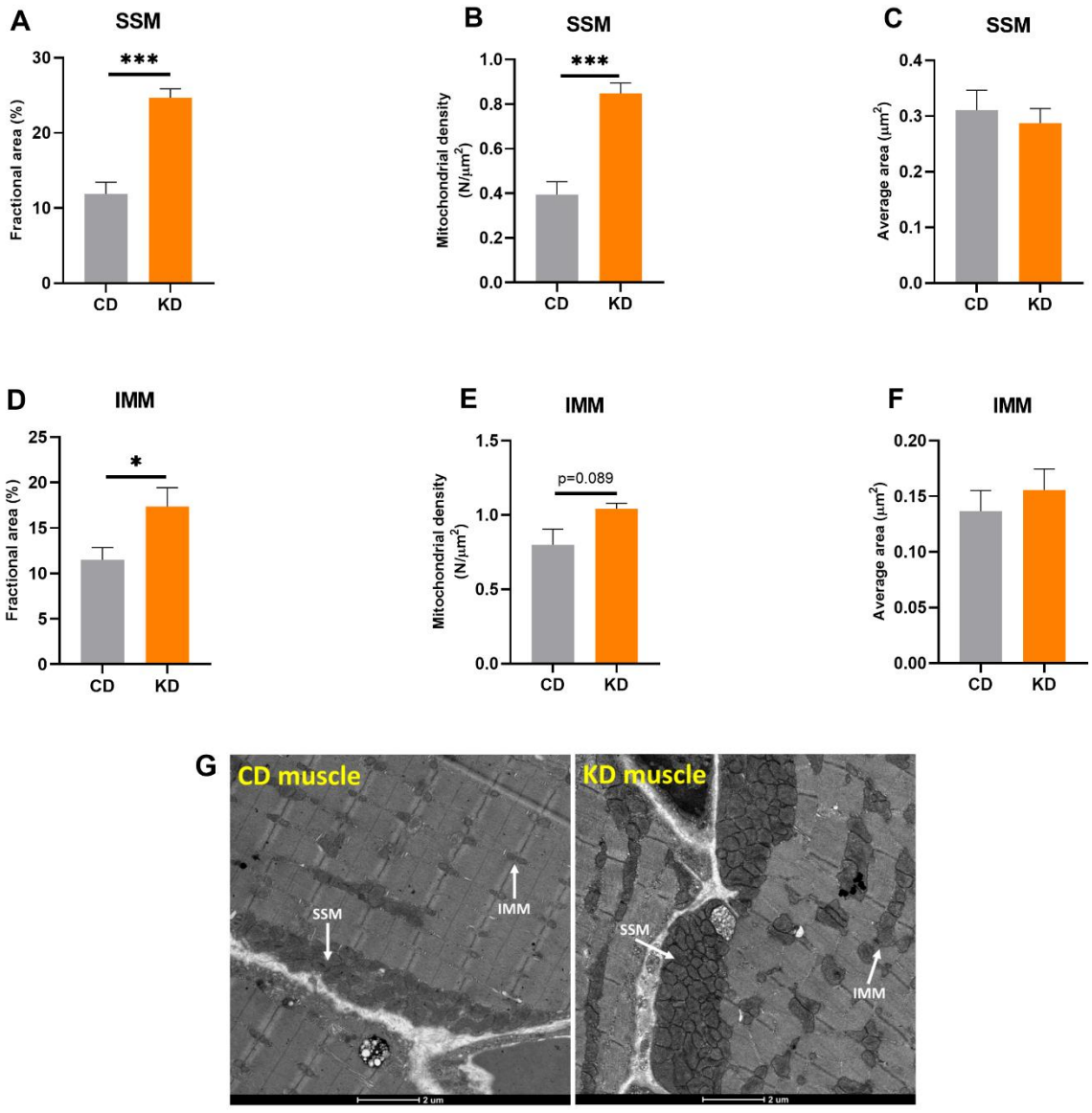

Figure 2.6. Quantification of subsarcolemmal (SSM) and interfibrillar (IMM) mitochondrial mass in red gastrocnemius muscle (n=4-5). Fractional area of (A) SSM and (D) IMM. Number density of (B) SSM and (E) IMM per micrograph. Average mitochondrial area of (C) SSM and (F) IMM. (G)

Representative micrographs of red gastrocnemius muscle of CD and KD animals. Diets: CD = control, KD= Ketogenic. $*p < 0.05$, $***p < 0.001$. All values are presented as mean ± SEM.

To investigate mitochondrial mass in a specific hindlimb muscle, TEM analysis was completed in red gastrocnemius muscle after 1 month of a KD. Results from TEM analysis showed both SSM and IMM areas were significantly higher in KD mice (Figure 2.6A,D), consistent with an increase in mitochondrial content, and this appeared to be driven by an increase in the number of mitochondria in both regions (Figure 2.6B,E). There was no difference in the average size of mitochondria between diet groups (Figure 2.6C,F).

2.3.2 Mitochondrial mass was not altered in prefrontal cortex or hippocampus after 1 month of a KD

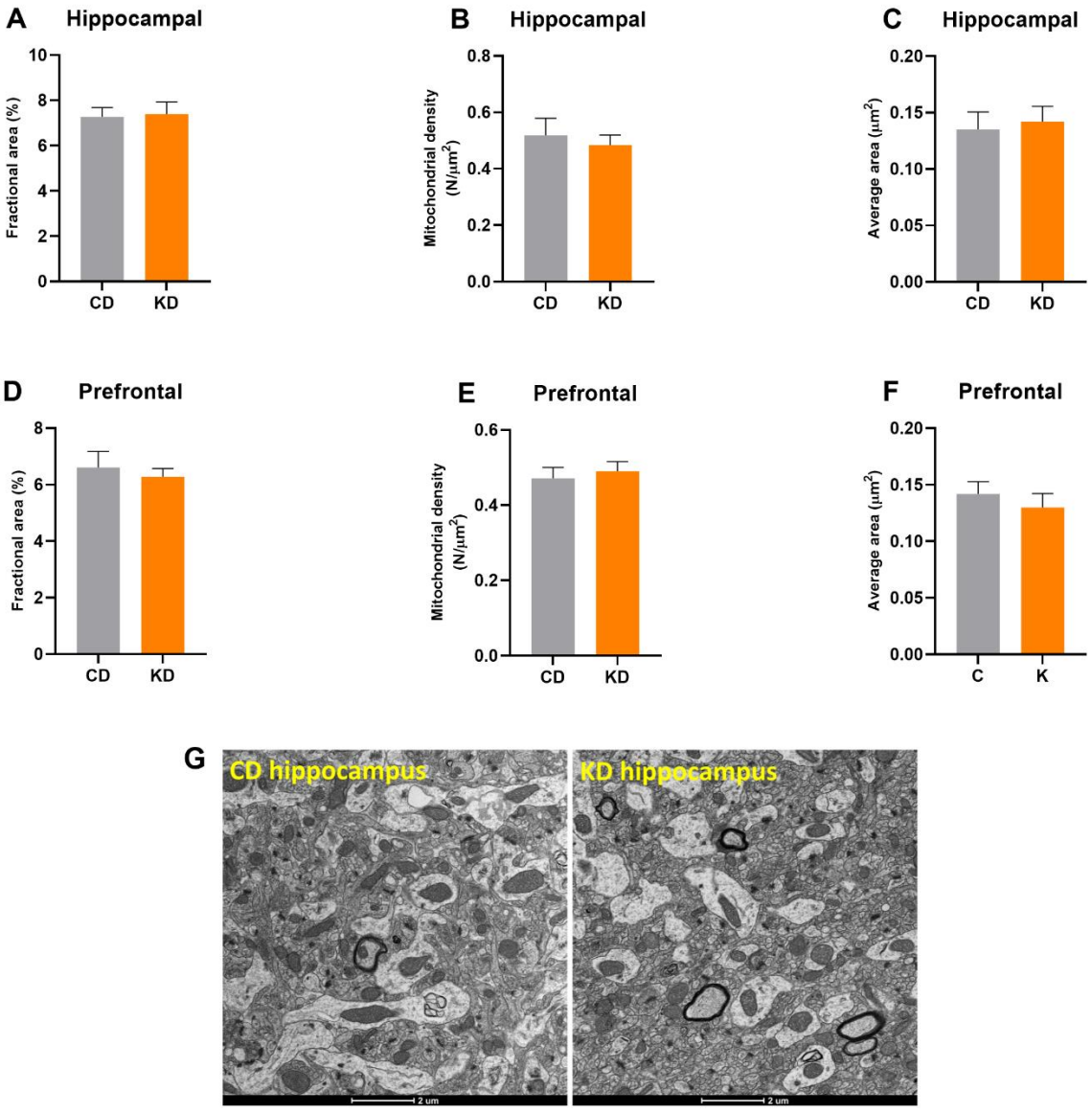

Figure 2.7. Quantification of mitochondrial mass in prefrontal cortex and dorsal hippocampus (n=4-5). Fractional area of (A) hippocampal and (D) prefrontal mitochondria. Number density of (B) hippocampal and (E) prefrontal mitochondria per micrograph. The average area of (C) hippocampal and (F) prefrontal mitochondria. (G) Representative brain micrographs of CD and KD animals. Diets: CD = control, KD= Ketogenic.

Mitochondrial quantification using TEM did not show any changes in prefrontal cortex or hippocampus fractional area after 1 month of a KD (Figure 2.7A-F). Similarly, mitochondrial density and average mitochondrial area were not significantly altered by diet.

2.3.3 Hepatic mitochondrial mass was not altered after 1 month of KD

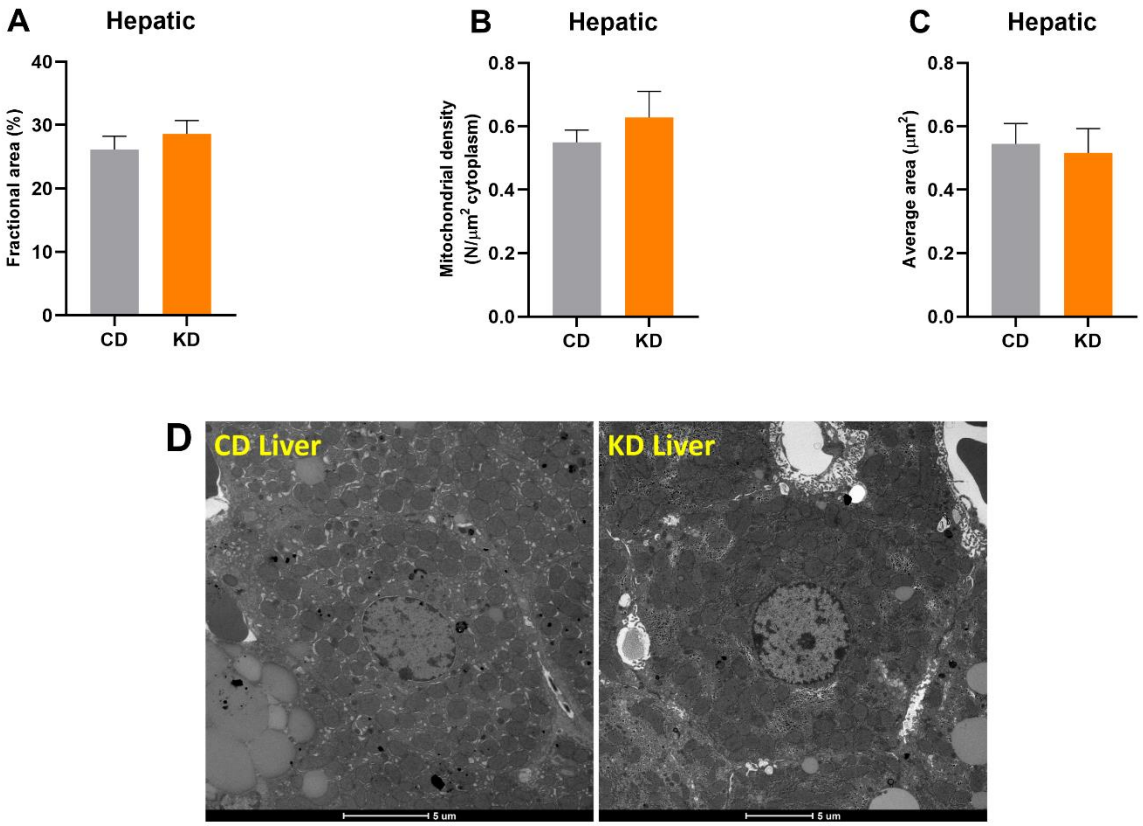

Figure 2.8. Quantification of mitochondrial mass in hepatocytes (n=4-5). (A) Fractional area, (B) number density, and (C) average area of hepatic mitochondria. (D) Representative hepatocyte micrographs of CD and KD animals. Diets: CD = control, KD= Ketogenic. All values are presented as mean ± SEM.

There was no change in mitochondrial area or number in hepatocytes measured with TEM after 1 month of KD (Figure 2.8A-C). No significant differences were observed between diet groups either before or after adjustment for lipid droplet area.

2.4 Discussion

The goal of this study was to morphologically quantify mitochondrial content in regions of skeletal muscle, brain, and liver following 1-month of a KD. Our data indicate that 1-month of a KD results in increased mitochondrial mass in red gastrocnemius muscle, but not liver or brain.

In our previous skeletal muscle work (Zhou et al. Aging-US in press), mitochondrial markers were analyzed in homogenates of the entire hindlimb skeletal muscle, and it was not possible to determine the response of specific muscles to a KD. To explore this issue, we used a separate group of mice and harvested the red gastrocnemius muscle to measure mitochondrial content with TEM. The TEM results demonstrated that 1 month of KD increased both SSM and IMM content in gastrocnemius muscle. Aging is associated with a reduction in mitochondrial content in oxidative muscle fibers, and preservation of mitochondrial mass is thought to alleviate the age-related decline in muscle function and strength (62). The increase in red gastrocnemius muscle mitochondrial mass may contribute to the previous observation that aged mice on a KD perform better in muscle strength and endurance tests than CD animals (13). However, these changes in mitochondrial mass were not reflected in the hindlimb skeletal muscle markers assayed at this same length of time on diet in our previous study (Zhou et al. Aging-US in press). This could indicate the markers used were not sensitive enough to pick up the differences in muscle tissues. However, we think this is unlikely since previous studies have found good correlations between several of these markers and TEM measurements (15). Instead, the TEM results likely indicate that KD-related increases in mitochondrial content in gastrocnemius do not translate to other muscles, and changes in this muscle were blunted by other muscle groups when whole hindlimb homogenates were used to study skeletal muscle. Moreover, a recent study (79) has demonstrated that 2 months of KD in middle-aged mice upregulated mitochondrial biogenesis and increased

citrate synthase activity in gastrocnemius. These results highlight the need for measurements in multiple muscle groups to thoroughly determine the impact of KDs on skeletal muscles.

In the brain, no changes in mitochondrial mass quantification by TEM were observed between diet groups in hippocampus or prefrontal cortex with a KD, and these data are consistent with our previous finding that markers of mitochondrial mass were not altered after 1 month of KD (Zhou et al. Aging-US in press). A study showed similar results in that an 8-week KD had no impact on fractional mitochondrial volume in hippocampus of 21-month-old rats compared to the aged-matched control animals (65). In contrast, one study in rats reported that 22 days on a KD increased mitochondrial number in hippocampus (12), but it should be noted this study used very young animals (P37-41), and the KD could have different effects on mitochondrial content in growing versus adult animals. Moreover, the KD animals were calorie restricted and had a significantly lower body weight compared to the control animals, and it is possible that calorie restriction may have played a role in the changes observed in this study. A couple of studies have also shown that a KD increased hippocampal mitochondrial quantity in mouse models of neurological disorders (75, 76). Furthermore, an 8-week study with KD also found that a KD increased mitochondrial number and area in retinal ganglion cell axons of a mouse model of glaucoma (43). These studies suggest that a KD may increase mitochondrial content under certain conditions in some neural populations, but the results of our study indicate that a KD does not increase mitochondrial content in the prefrontal cortex or hippocampus from healthy, middle-aged mice.

Although some studies have investigated the impact of KDs on markers of mitochondrial content in the liver (26, 28, 29, 44), TEM quantification of mitochondrial mass has not been performed. Also, enzyme markers in liver, especially citrate synthase, can be affected by shifts in metabolism and induction of gluconeogenesis with a KD and may be poor markers of mitochondrial mass in

the liver. Thus, use of TEM to quantify mitochondrial content is important to determine the effects of a KD on mitochondrial mass. The results in liver are consistent with the previous observation that most markers of mitochondrial content were not changed after 1 month of KD (Zhou et al. Aging-US in press). One study showed that lipid droplets were increased in livers of rats fed a KD (80). However, the study used a KD diet that was deficient in choline, which would lead to liver steatosis with a high-fat diet (81). The present study did not observe a change in lipid droplets in hepatocytes with a KD, and mitochondrial fractional area did not differ between CD and KD animals before or after adjusting for areas of lipid droplets.

2.5 Conclusion

Collectively, the results of the present study demonstrate that 1 month of a KD increased mitochondrial mass in red gastrocnemius muscle but had no impact on mitochondrial content in the liver, hippocampus, and prefrontal cortex. These results highlight the tissue-specific effects of a short-term KD on mitochondrial mass and highlight the need for future measurements in specific muscle groups and regions within a tissue to thoroughly determine the impact of KDs on mitochondrial mass.

Chapter 3 A ketogenic diet initiated in late middle age improved cognition and muscle endurance in aged mice

There has been substantial interest in the application of ketogenic diets (KD) to manage disorders associated with aging, and studies have shown KDs started from early middle age improve healthspan and longevity in mice. Thus, KDs might be used to reduce the risk of functional decline at old age. KDs started later in life or intermittently administered may be feasible and promote compliance in an older population. Therefore, this study sought to test if continuous or intermittent KDs started in late middle-aged mice would improve measures of healthspan at an advanced age. 18-month old male C57BL/6JN mice were randomly assigned to an isocaloric control (CD), ketogenic (KD), or intermittent ketogenic (IKD, 3 consecutive days of KD/week) diet. At 20, 23, and 26 months of age, a panel of behavioral tests were performed to assess cognitive and motor functions. Y-maze alternation rate was significantly higher for both IKD and KD mice at 23 months of age and for KD mice at 26 months, indicating an improved working memory. 26-month old KD mice also showed better spatial learning memory as measured by time spent in the target quadrant in the Barnes maze. Improved muscle endurance and strength were observed in aged IK and KD mice as tested by grid wire hang. A significantly increased composite score of all the behavior parameters was observed in KD mice at 26 months of age, and IK mice showed a trend ($p = 0.052$) toward an increased score compared to CD mice. A reduced level of circulating cytokines was observed in aged KD (IL-6, IL-10, and TNF-α) and IKD (IL-6) mice and this may

contribute to the phenotypic improvements observed with these interventions. Overall, KD and IKD initiated in late middle-aged mice improved cognition and motor endurance in aged mice. The KD had the most potent effect on overall healthspan in aged mice, as shown by a higher composite score of all the tests performed with IKD showing results intermediate to other diet groups.

3.1 Introduction

In recent years, there has been substantial interest in the application of ketogenic diets (KD) to improve healthspan and manage age-related diseases, including Alzheimer's disease, type 2 diabetes, Parkinson's disease, and cancer (1, 39, 40). KDs are depleted in dietary carbohydrate and high in fat and can mimic some of the metabolic changes with calorie restriction and fasting, including an increase in fatty acid oxidation and stimulation of endogenous ketogenesis (7). An increased level of β-hydroxybutyrate, the main circulating ketone body, has also been associated with anti-aging phenotypes (1, 10-12) and signaling pathways (8, 9). Studies have shown that a KD started at 12 months of age extended longevity and improved motor function, as well as cognition, in aged mice (13), which further sparked interest in the use of KDs as interventions to promote healthy aging. In humans, long-term adherence to a strict dietary intervention is difficult, and interventions started later in life or intermittently may be more feasible and promote compliance.

Several studies have investigated the impact on aging of interventions initiated in late–middle-aged mice. It has been reported that supplementation of α-ketoglutarate from 18 months of age increased lifespan in female mice and improved frailty in aged female and male mice (32). Rapamycin feeding started at 19 months of age increases lifespan and motor coordination in mice (30). Implementation of wheel running started as late as 19 months of age fostered neurogenesis, and aged mice in the running group performed better in spatial learning memory tasks compared to a sedentary group (31). A diet low in branched-chain amino acids started at 16 months of age promoted metabolic health and robustness in aged mice (33). In regard to a KD, rats fed a MCT supplemented KD from 20 months of age for 3 months showed improved performance in a cognitive dual task which involves both spatial working memory and bi-conditional association

memory (82), although it is not known if other measures of healthspan would also be improved. These studies highlight the fact that aging interventions started in late-middle-age can mitigate age-related declines in physiological functions. Thus, one goal of the present study was to investigate if a KD initiated at 18 months of age would improve measures of healthspan in old mice.

In addition to the effects of a KD started in late-middle-aged mice, the other arm of the study was to test if an intermittent KD (IKD), which consisted of 3 consecutive days of a KD each week, would produce similar aging benefits as the continuous KD. Previous intermittent dietary interventions, including alternate day fasting (34, 35) and adherence to a 4-day fasting mimicking diet twice a month (83), have been reported to increase lifespan and improve physiological functions at old age. These studies involved periods of decreased energy intake, and the present study investigated if intermittent periods of nutritional ketosis without a decrease in energy intake would induce some of the positive effects as seen in these studies. In regard to intermittent interventions of a KD, a weekly cyclic KD was reported to reduce midlife mortality and improve healthspan in aged mice (14) even though the mice during the KD cycles had higher caloric intake and increased body weight. The present study paid particular attention to a shorter 3-day KD paradigm, and mice were fed an isocaloric amount of diet daily to maintain a relatively stable middle-age body weight throughout the study.

This study sought to investigate whether a continuous KD or a 3-day IKD initiated at 18 months of age would improve measures of healthspan in mice. The main component of the study focused on the longitudinal assessment of various mouse behavior tests at 20, 23, and 26 months of age that would elucidate the impacts of these KD interventions on motor and cognitive functions that have been shown to decline in aged mice (84-86). Endpoint measures including levels of serum

markers of metabolism and inflammation, body composition, and weights of muscle groups were collected to provide further information on how a KD and IKD affect other common aging phenotypes.

3.2 Method

3.2.1 Animals and Diets

Male C57BL/6JN mice were obtained from the NIA Aged Rodent Colony at 11 months of age. Upon arrival, mice were group housed in polycarbonate cages on racks in a HEPA filtered room maintained on a 12-hour light-dark cycle and were individually housed from 16 months of age. Health checks were performed daily. Temperature (22–24°C) and humidity (40– 60%) were controlled. Sentinel mice were housed on the same rack and exposed to bedding from the study mice on a weekly basis. Health screens were completed on sentinel mice every three months and tests included aerobic cultures and serology (MHV, MPV, MVM, M. pul., TMEV [GDVII], Ectro, EDIM, MAD1, MAD2, LCM, Reo-3). All tests were negative throughout the study. Mice with dermatitis were treated with weekly nail trims. All animal protocols were approved by the UC Davis Institutional Animal Care and Use Committee and were in accordance with the NIH guidelines for the Care and Use of Laboratory Animals.

When group-housed, mice were provided ad libitum access to a chow diet (LabDiet 5001; LabDiet, Saint Louis, MO). At 16 months of age, the mice were individually housed and provided 11.2 kcal/day of a control diet (CD) (13) and the same amount was fed throughout the study. At 18 months of age, mice were randomly assigned to the control (CD), intermittent ketogenic (IKD), or ketogenic (KD) diet, with body weight counterbalanced in each group. IKD mice were fed KD meals on three consecutive days each week, and the KD mice were fed a KD meal each day. The

control diet contained (% of total kcal) 10% protein, 74% carbohydrate, and 16% fat. The ketogenic diet contained 10% protein, <0.5% carbohydrate, and 89.5% fat. Mouse diets were made in-house. The CD was modified from a AIN93G diet to match the lower protein content in the KD. Table 3.1 provides a detailed description of the diet composition. For both diets, the Envigo (Indianapolis, IN) vitamin mix TD.40060 was used. The vitamin mix used included choline dihydrogen citrate as a source of choline. For the CD the mineral mix TD.94046 was used, while for the KD the mineral mix TD.79055 was used instead due to its lower carbohydrate content. As TD. 79055 is insufficient in calcium and phosphate, calcium phosphate (19.3 g/kg diet) and calcium carbonate (8.2 g/kg diet) supplementation were required for the KD.

Table 3.1: Experimental diets

g/kg diet	**Control**	**Ketogenic**
Casein	111	191
DL-methionine	1.5	2.7
Corn starch	490	--
Maltodextrin	132	--
Sucrose	100	--
Mineral mix	35	20
Vitamin mix	10	18
Soybean oil	70	70
Lard	0	582
Calcium phosphate dibasic	--	19.3
Calcium carbonate	--	8.2
Cellulose	50	85.0
TBHQ	0.014	0.126

At 27 months of age and after 9 months of dietary intervention, animals were euthanized by CO_2 inhalation in the morning following an overnight fast.

3.2.2 Blood ketone measurement

Blood β-hydroxybutyrate level was measured using a Precision Xtra glucose and ketone monitoring system (Abbott) through tail nick.

3.2.3 Body weight and composition

Body weight was measured daily. Body composition was evaluated using NMR relaxometry (EchoMRI-100H, EchoMRI LLC, Houston, TX) at 27 months of age prior to euthanasia.

3.2.4 Serum analysis

Following blood collection by cardiac puncture, blood samples were allowed to clot at room temperature, samples were spun at 1000 g for 10 minutes, and serum was sent to the UC Davis Mouse Metabolic Phenotyping Center for analysis. The following serum assays were completed using kits according to the manufacturer's instructions: free fatty acids (Wako Diagnostics, Richmond, VA), triglycerides (Fisher Diagnostics, Middletown, VA), and total cholesterol, HDL-cholesterol, LDL-cholesterol, and VLDL-cholesterol (Fisher Diagnostics, Middletown, VA) were measured using enzymatic colorimetric assays. IL-6, IL-10, KC/GRO, TNF-α, IGF1, insulin, and FGF21 were determined by ELISA (Meso Scale Discovery, Rockville, MD).

3.2.5 Hepatic lipid analysis

Snap frozen liver samples were sent to the UC Davis Mouse Metabolic Phenotyping Center for analysis. Weighed tissue samples were homogenized in methanol:chloroform. After overnight extraction, 0.7% sodium chloride was added. The aqueous layer was aspirated, and duplicate aliquots of the chloroform/lipid layer are dried under nitrogen gas. The lipid is reconstituted in

isopropyl alcohol and assayed for total triglyceride (TG) and cholesterol (TC) spectrophotometrically using kits according to the manufacturer's instructions (Fisher Diagnostics, Middletown, VA).

3.2.6 Mouse behavior tests

A panel of behavior tests was performed at 20, 23, and 26 months of age. Testing at 20 months of age included: rearing, open field, grid wire hang, grip strength, and novel object recognition tests. Testing at 23 and 26 months of age included: all the tests in the 20-month list plus the Y maze spontaneous alternation and Barnes maze tests. All tests were conducted in the light cycle and the IKD mice were fed the control diet during the testing period.

3.2.7 Open field test

The open field test was conducted in a 40x40x40 cm white acrylic box. Mice were placed at one corner of the box and allowed to freely explore the arena for 15 minutes. Videos were recorded with a camcorder (Sony) at an overhead view. Videos were analyzed and the motion of each mouse was tracked using the Enthovision XT15 software (Noldus, Wageningen, the Netherlands). The center zone was set as a 25x25 cm in the middle of the arena. Total distance traveled and time spent in the center zone were automatically computed using the software.

3.2.8 Novel object recognition test

The novel object recognition test was conducted in the same apparatus as the open field test. The 15-minute open field test was used as the acclimation session for the mice to habituate to the environment. On the next day, in the morning session, two identical objects were placed in the arena, and the mouse was allowed to explore the objects for 10 minutes. In the afternoon session,

6 hours after the morning session, one of the known objects was replaced with a novel object (the novel side was randomized among mice), and the test was performed over 10 minutes. A mouse was considered as exploring an object if the nose of the mouse was pointed toward the object and was within 2 cm from the object. All objects were 5-10 cm in height. The objects used for the 20-month, 23-month, and 26-month tests were: a small glass bottle filled with tissue paper, and a box composed of yellow Lego blocks; an orange plastic cone and a blue plastic bottle; and a small tissue culture flask filled with sand and a clear acrylic bottle filled with glass beads. Time exploring each object was manually scored using two stopwatches.

3.2.9 Rearing test

The apparatus consisted of a clear cylinder 15 cm in diameter. Mice were placed into the cylinder and allowed to explore for 5 minutes. Videos were recorded and scored after the test. The number of rears in 5 minutes was recorded. A "rear" was defined as the mouse putting the forepaws on the side of the cylinder.

3.2.10 Grid wire hang test

The apparatus consisted of a stainless-steel wire mesh screen with 1x1 cm grids and 1 mm wires. The screen was supported on a plastic box and was 40 cm above the surface. Towels were placed at the bottom of the box to cushion the fall. Mice were placed on the top of the screen, and the screen was gently shaken to ensure a firm grip of the mice. The screen was then inverted, and the time till the mice fell was recorded. If the maximal hanging time did not exceed 180 seconds, mice were given another trial after resting in the home cage for approximately 30 minutes (maximum of 3 trials at 20-month and 2 trials at 23 or 26-month). Previous results (data not shown) showed that middle-aged mice did not perform better in the subsequent trials if they already reached 180

seconds in the first trial. At 20 months of age, mice did not perform better in the third trial and the trial was deemed unnecessary at more advanced ages to prevent fatigue or additional stress. Maximal hanging impulse was calculated as (maximum hanging time (s) x body weight (kg) x 9.8 $N \cdot kg^{-1}$).

3.2.11 Grip strength

The grip strength test was performed as previously described (13) using the Imada push-pull force scale and a single metal bar (PS-500N, Northbrook, IL). Mice were given two rounds of three trials each and the maximum grip strength was used.

3.2.12 Y maze spontaneous alternation test

A white acrylic Y-shaped maze was used for this test. Each arm was 120° from each other and 35x8x15 cm (LxHxW) in dimension. Mice were placed in the center of the maze, facing one arm, and allowed to move around the maze for 6 minutes. Videos were recorded with a camcorder at an overhead view. Motion of the mice was tracked using the Enthovision XT15 software (Noldus, Wageningen, the Netherlands). An arm entry was counted when the center point of the mouse travelled to the distal side of the arm (more than 1/3 of the arm length) and returned to the center of the maze. A non-repeating triplet was defined as when the mouse enters three different arms consecutively. The percent alternation was calculated as ((number of non-repeating triplets) ÷ (number of total arm entries - 2)) x 100%.

3.2.13 Barnes maze

The maze consisted of a 92 cm plastic circular disk with twenty 5 cm holes evenly distributed on the periphery, and an overhead LED light source was used to illuminate the maze (700 lux). The

maze was 30 inches elevated from the ground. Under one of the holes, a black escape box equipped with a step and filled with fresh bedding was attached to serve as a shelter for the mice. Signs were placed around the maze to be used as visual cues. During the training trials, mice were placed in the middle of the maze under an inverted bucket. After 10 seconds, the light was turned on, and the bucket was immediately lifted. Mice were allowed to explore the maze for 3 minutes or until they entered the escape box through the target hole. If the mice did not enter the target hole within 3 minutes, the mice were gently directed to the target hole. Mice were trained for 3 trials a day for 3 days and the inter trial interval was 15-20 minutes. On the probe day, the escape box was removed, and the trial lasted for 2 minutes. Videos were recorded using an overhead GigE camera connected to a computer and analyzed using the Enthovision XT15 software (Noldus, Wageningen, the Netherlands). Latency to the target hole, the path traveled to the target hole, errors made before finding the target hole, and time spent in the target quadrant (a quarter of the maze with the target hole in the middle) was calculated automatically using the software.

3.2.14 Statistical analysis

All values are expressed as mean ± SEM and P values <0.05 were considered significant. All analyses were performed using GraphPad Prism 8.1 (GraphPad Software Inc., San Diego, CA). For behavior tests, mixed-effect model of repeated measure two-way ANOVA was performed to test diet and age-related differences. For endpoint results, the differences among diet groups were tested using one-way ANOVA followed by Tukey's multiple comparisons. A z-score for each test was calculated as the number of SDs from the mean of the selected reference group, and the composite score was then computed using the average of z-scores from tests within a category. The directionality of scores was adjusted and a higher positive score indicates better performance.

3.3 Results

3.3.1 KDs started in late middle-aged mice distinctively altered body weight and blood ketone level

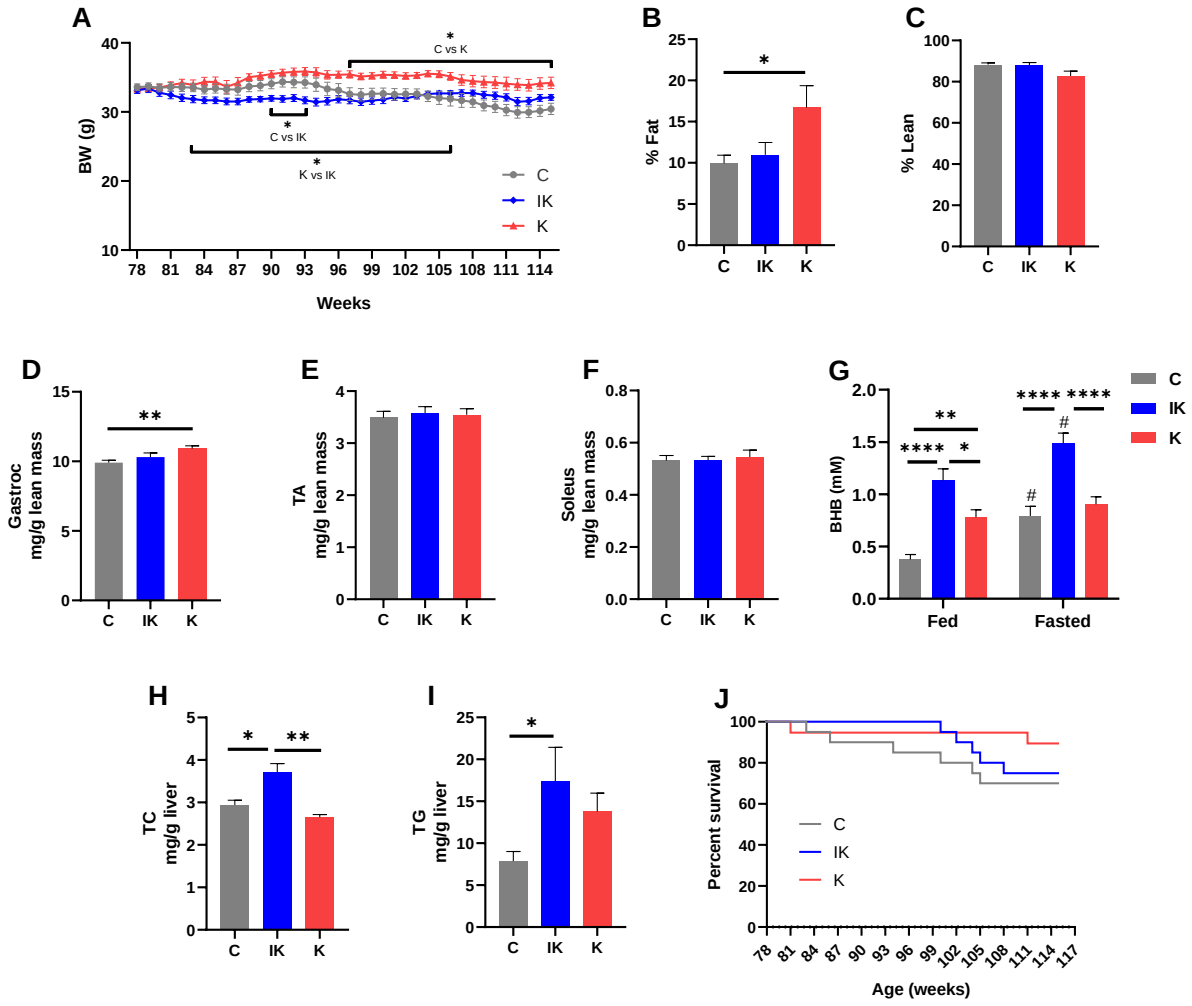

Figure 3.9 Differential adaptation of 18-month male mice to a KD or IKD. (A) Body weights of CD, IKD, and KD mice throughout the study (n=19-20). (B-C) Percent fat (B) and lean (C) mass measured using EchoMRI at 27 months of age (n=10-12). (D-F) Relative mass of gastrocnemius (D), tibialis anterior (E), and soleus (F) muscle to total lean mass (n=10-12). (G) Circulating level of β-hydroxybutyrate (n=19-20). (H-I) Content of liver total cholesterol (H) and triglyceride (I) (n=8). (J) Survival curve of mice throughout the study (n=19-20). Diets: C-control, IK-intermittent

ketogenic, K-ketogenic. $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$. #$p < 0.05$ between fasted vs. fed ketone level. All values are presented as mean ± SEM.

Although all groups were fed an isocaloric amount of diet, KD mice were heavier than the CD mice after 19 weeks on diet (Figure 3.9A) and maintained weight throughout the study. IKD mice had an initial drop in body weight, which was significantly lower than the CD mice after 12 weeks on diet, but then they maintained this weight with aging and it was no longer different from the CD mice after 16 weeks on diet (Figure 3.9A). Body composition measurements at 27 months of age, or after 9 months of intervention, showed that KD mice had a higher fat mass (Figure 3.9B) compared to CD mice but lean mass (Figure 3.9C) was not different among diet groups, consistent with the increase in KD body weight being mostly body fat. Weights of representative muscles from the lower hindlimb were also measured to assess preservation of muscle mass at an advanced age. KD mice had larger gastrocnemius muscles (Figure 3.9D) compared to CD or IKD mice, while the weight of tibialis anterior and soleus was not different among groups (Figure 3.9E-F).

To evaluate the effects of KDs on liver lipid content, triglyceride and cholesterol levels were measured. There was no difference in liver triglyceride or cholesterol between KD and CD mice (Figure 3.9H-I), while the IKD mice had an elevated cholesterol content compared to both the CD and KD mice (Figure 3.9H) and an increased triglyceride content compared to CD mice (Figure 3.9I).

Circulating β-hydroxybutyrate was measured to assess the level of ketogenesis with the consumption of a KD. Both KD and IKD mice had an elevated 3-hours postprandial as well as fasted blood β-hydroxybutyrate level compared to the CD mice (Figure 3.9G). IKD mice also had significantly higher blood β-hydroxybutyrate than KD mice in either the fed or fasted state (Figure 3.9G).

Survival curves were plotted to illustrate the number of mortalities in each group over the course of the study (Figure 3.9J), and the mortality rate was lower in IK and KD group compared to CD by the end of the study.

3.3.2 Serum markers of inflammation were decreased and cholesterol levels were differentially altered with KDs

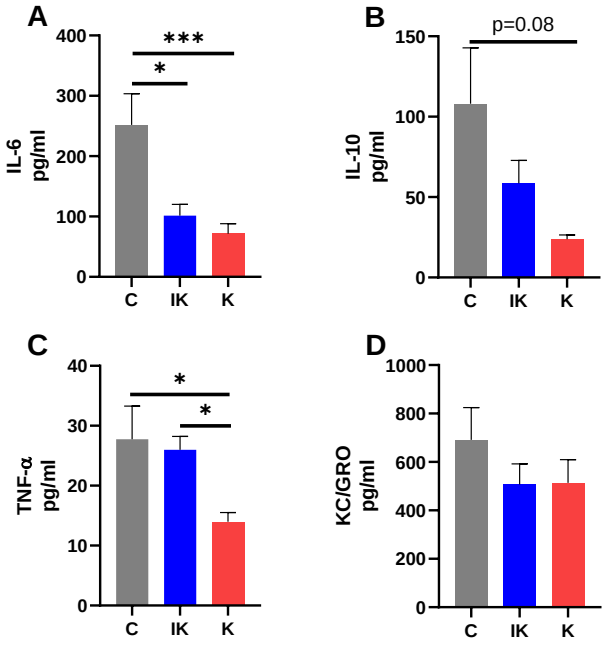

Figure 3.10 Levels of circulating cytokines at 27 months of age in male mice. Circulating levels of (A) IL-6, (B) IL-10, (C) TNF-α, and (D) KC/GRO (n=10). Diets: C-control, IK-intermittent ketogenic, K-ketogenic. ∗p < 0.05, ∗∗∗p < 0.001. All values are presented as mean ± SEM.

To investigate changes in metabolism and inflammation with the KDs, a panel of serum markers was measured. Serum IL-6 was significantly decreased in both KD and IKD group at 27 months of age (Figure 3.10A). KD, but not IKD, mice showed a trend toward decreased IL-10 (Figure 3.10b). KD mice also had a significantly lower level of TNF-α compared to both CD and IKD mice (Figure 3.10C). KC/GRO was not different among diet groups (Figure 3.10D). These results

suggest that continuous KD started at 18 months of age downregulated some markers of inflammation at an advanced age, while old mice on IKD showed a level of serum inflammatory markers intermediate to KD and CD mice.

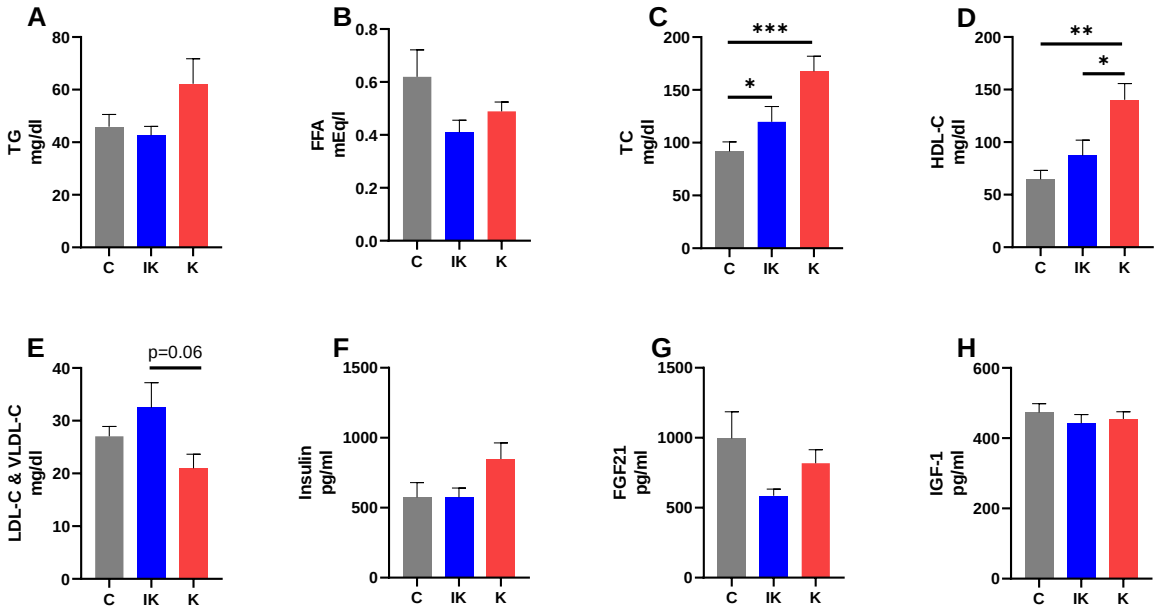

Figure 3.11 Levels of serum markers of metabolism at 27 months of age in male mice. Circulating levels of (A) total triglyceride (TG), (B) free fatty acids (FFA), (C) total cholesterol (TC), (D) HDL-C, (E) LDL-C & VLDL-C, (F) insulin, (G) fibroblast growth factor-21 (FGF21), and (H) insulin like growth factor-1 (IGF1) (n=10). Diets: C-control, IK-intermittent ketogenic, K-ketogenic. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. All values are presented as mean ± SEM.

Serum triglycerides and free fatty acids (FFA) were not different among diet groups (Figure 3.11A). Serum total cholesterol as well as HDL-C levels were elevated in the KD group (Figure 3.11C-D) compared to CD and IKD group. Levels of serum LDL-C and VLDL-C were not increased with the KD (Figure 3.11E), indicating the elevation in total cholesterol was largely due to the increase in HDL-C. IKD mice showed a trend toward increased LDL-C and VLDL-C compared to the KD (Figure 3.11E), but not CD mice. Circulating levels of hormones that regulate metabolic pathways

were also measured. Insulin, fibroblast growth factor 21 (FGF21), and insulin-like growth factor 1 (IGF1) (Figure 3.11F-H) were not significantly altered by any of the dietary interventions.

3.3.3 Age-related declines in measures of motor function were observed in all groups, and diet-associated improvement in grid-wire hang was observed with KD and IKD

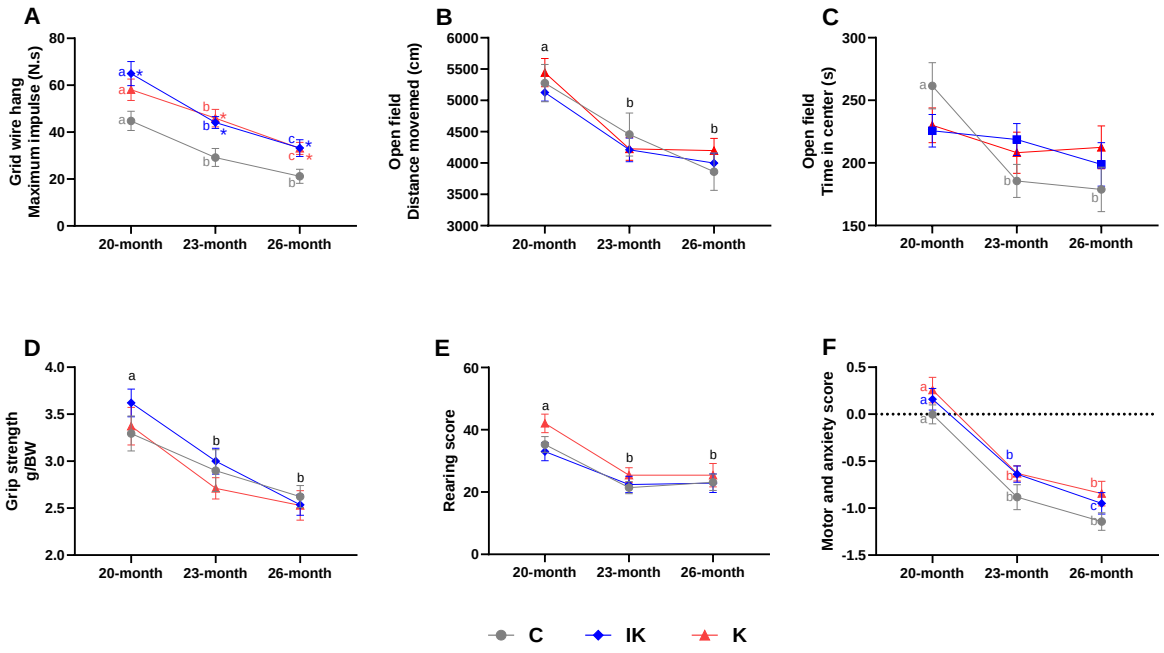

Figure 3.12 Behavior tests of motor function and anxiety in male mice at 20, 23, and 26 months of age. (A) Grid wire hang test: maximum hanging impulse. (B) Open field test: total distance moved as a measure of locomotor activity. (C) Open field test: time spent in the center region as a measure of anxiety. (D) Grip strength test: relative peak force of forelimbs exerted on the force meter. (E) Rearing score: number of wall-contact rears by the front paws of the animal. (F) Composite score of motor function and anxiety. 20-month: n=18-20; 23-month: n=17-18; 26-month: n=14-17. Diets: C-control, IK-intermittent ketogenic, K-ketogenic. $*p < 0.05$. Different letters of a, b, or c denote differences ($p < 0.05$) between age groups. All values are presented as mean ± SEM.

Motor strength and endurance were assessed using the grip strength and grid wire hang tests. Mice fed a KD or IKD were more resistant to falling from the grid wire at 20 (KD showed a trend p=0.09), 23, and 26 months of age (Figure 3.12A). Grip strength performance was not different

among diet groups at any age (Figure 3.12D). Locomotor activity was evaluated using the rearing and open field tests. Neither rearing score nor total distance traveled in the open field was altered with KD or IKD at any age (Figure 3.12B). Time spent in the center region in the open field (Figure 3.12C), which is a measure of anxiety level, was not different among diet groups.

Age-related declines after 20 months of age in rearing score, total distance traveled in the open field, grip strength, and grid wire-hang maximum impulse were observed in all diet groups (Figure 3.12A-B, D-E). Time spent in the center of the open field was decreased in the CD mice after 20 months of age (Figure 3.12C), consistent with an age-related increase in anxiety level, and such a change was not observed in IKD or KD mice with aging. A composite score consisting of the parameters in all the motor function tests was computed to give an indication of motor performance and anxiety level (Figure 3.12F). The motor and anxiety score did not significantly differ between diets. As expected, age-related changes in motor and anxiety scores after 20 months of age were observed in all diet groups.

3.3.4 KD and IKD showed improvements in some measures of cognitive function with age

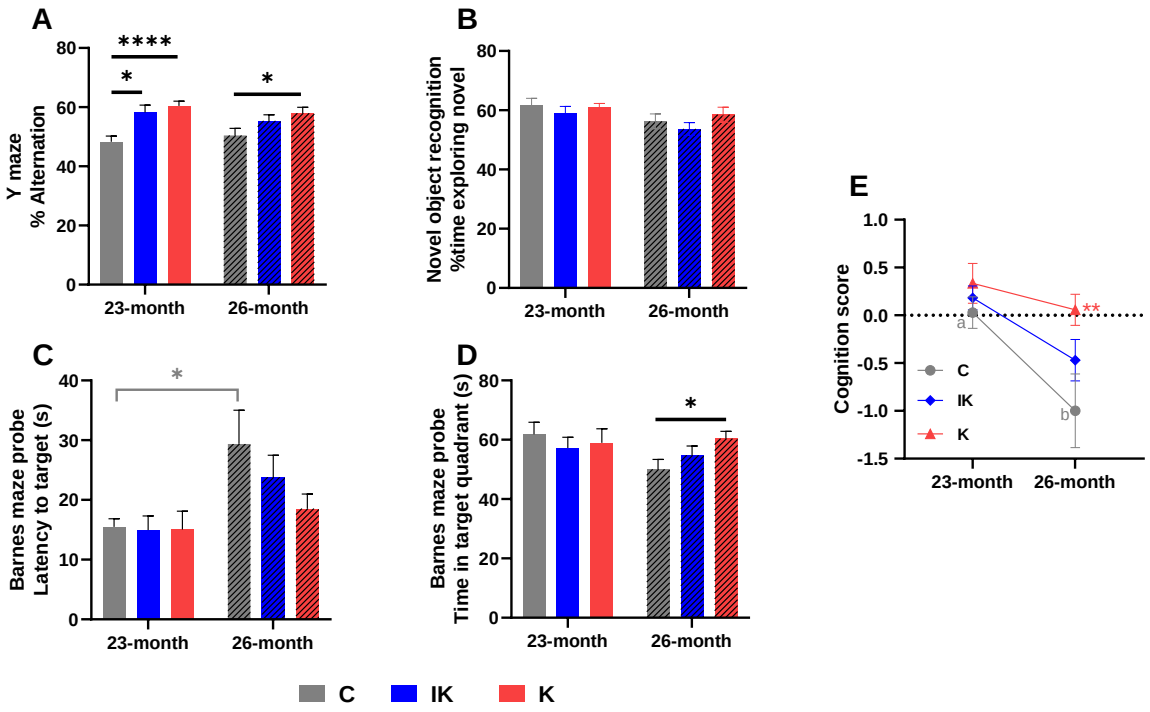

Figure 3.13 Behavior tests of cognitive function in male mice at 20, 23, and 26 months of age. (A) Y maze spontaneous alternation test: percent of alternations. (B) Novel object test: percent time exploring the novel object (C) Barnes maze test: latency to the target hole in the probe trial. (D) Barnes maze test: time spent in the target quadrant in the probe trial. (E) Composite of cognitive tests. 23-month: n=17-18; 26-month: n=14-17. Diets: C-control, IK-intermittent ketogenic, K-ketogenic. $*p < 0.05$, $****p < 0.0001$. Different letters of a or b denote difference ($p < 0.05$) between age groups. All values are presented as mean ± SEM.

At 23 months of age, both KD and IKD mice showed a higher percent alternation in the Y maze spontaneous alternation test (Figure 3.13A), consistent with an improved working memory. At 26 months of age, KD mice still performed significantly better in Y maze compared to CD mice, while the performance of IKD mice was not significantly different from CD or KD mice at this age. An age-related difference was not observed in the Y maze test from 23 to 26 months of age with any of the dietary interventions.

Improved spatial learning memory, which was assessed using the Barnes maze, was observed for the KD mice at 26 months of age (Figure 3.13C-D), represented as a significant increase in time spent in the target quadrant and lower mean values for latency, although not statistically significant due to a large variance of latency to target hole in the probe trial. Spatial learning memory was not altered at 23 months of age among any diet groups. Although a significant difference in latency to target hole was not observed at 26 months of age among diet groups, an age-related increase in latency was detected in CD, but not IKD or KD mice, indicating preservation of spatial learning memory with IKD and KD at an advanced age.

No diet-related difference in percent time exploring novel objects was observed for the novel object recognition test at all ages (Figure 3.13B), consistent with no change in recognition memory with any of the dietary interventions. Also, no age-related changes were detected for the novel object recognition.

To evaluate overall effects of age or diet on cognition, parameters of the cognitive tests were combined to calculate a composite cognition score normalized to the mean performance of 23-month CD mice (Figure 3.13E). At 26 months of age, KD mice had a higher cognition score compared to CD mice. IKD mice showed a cognition score intermediate to KD and CD. CD mice showed a decrease in cognition score from 23 to 26 months of age, while both the IKD and KD mice showed no significant difference with aging.

Overall, these results suggest that a KD improved cognition at old age compared to CD mice. IKD mice improved working memory compared to CD mice at 23 months of age, and at 26 months of age showed measures of memory intermediate to KD and CD mice. Both IKD and KD preserved

significant decreases in memory at advanced age, while an age-related difference was observed with the CD.

3.3.5 Composite healthspan score was higher with KD and IKD at 26 months of age

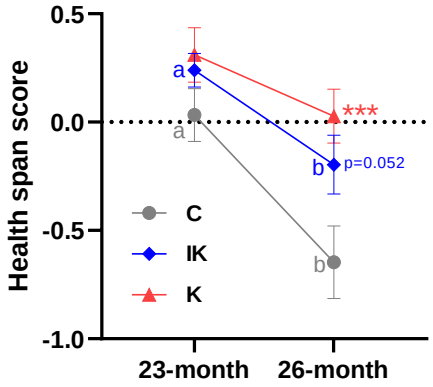

Figure 3.14 Composite score of healthspan calculated using parameters form all the behavior tests conducted. 23-month: n=17-18; 26-month: n=14-17. Diets: C-control, IK-intermittent ketogenic, K-ketogenic. ***p < 0.001. Different letters of a or b denote difference (p < 0.05) between age groups. All values are presented as mean ± SEM.

A composite score computed from representative parameters of all the behavior tests performed can be used to give an indication of overall healthspan (Figure 3.14). At 23 months of age, no difference was observed among dietary interventions. At 26 months of age, KD mice had a higher score and IKD mice showed a strong trend toward an increased score compared to the CD mice. An age-related decline in healthspan score was detected for both the CD and IKD groups, but not the KD group. Altogether, our results show that a KD started in late middle-aged mice improved and prevented theage-related decline in healthspan score, and IKD had an impact intermediate to KD and CD.

3.4 Discussion

The effects of KDs on longevity and healthspan have been studied in early middle-aged (12 month old) laboratory mice (13, 14). Protective effects of KDs against age-related diseases have also been demonstrated (1). However, it is not known if KDs initiated at late middle age would confer similar effects. In addition to a continuous KD regime, a KD administrated intermittently for a brief period is also of particular interest as a strategy to mimic changes that occur with intermittent fasting. An intermittent metabolic switch between glucose and ketone body utilization has been proposed to optimize cognitive functions in aging (87) and mice cycled on a weekly KD had reduced mortality and enhanced healthspan (14). The goal of this study was to induce continuous or intermittent ketosis (3 days per week) in late middle-aged mice through KDs and assess whether these dietary interventions improve measures of healthspan at old ages. Our results demonstrate that a KD started at 18 months of age improved composite healthspan score at 26 months of age, and a weekly 3-day IKD showed a health-promoting effect intermediate to KD and CD. This increase in healthspan score is primarily driven by an improvement in cognitive functions. Also, healthspan measures were carried out when the IKD mice were on a CD, and this might be particularly important as calorie restriction or intermittent fasting does not produce continuous ketosis. Furthermore, mice on a regular CD manifested an age-dependent decline in cognitive function, while the KD and IKD mice showed no significant change in the cognition score.

Aging is associated with a gradual decline in cognitive function, and the cognitive health-promoting effects of KDs have been demonstrated in studies using aged animals. Although previous studies showed that KDs started in 12-month old mice improved recognition memory in novel object recognition test (13, 14), performance in novel object test was not altered with KD or IKD in this study, and this suggests that KDs initiated at early late middle age may be necessary

to show an improvement in recognition memory when fed isocalorically to a CD. Spatial learning memory and working memory have also been used to study cognition in aged animals as aging is also accompanied by a decline in these memory functions (88, 89). Our results demonstrate that spatial learning and working memory were improved in mice fed a KD at 26 months of age, and mice fed an IKD had improved spatial working memory at 23 months of age. Although KDs have been shown to improve spatial learning and working memory in rodents, most of the studies focused on models of diseases or used younger animals (90-96), and relatively little was known about how KDs affect these types of memories at advanced age. Results of the present study highlight the potential therapeutic effects of KDs on attenuating age-related decline in spatial learning and working memory. Regional differences in the brain have been observed for the effects of KDs and this may explain why certain memory tasks were improved while others were not. It has been found that spatial memory is more susceptible to neurodegeneration in the hippocampus and recognition memory is impacted to a much less extent (97). Studies have also shown that a KD differentially alters synaptic morphology in rat hippocampus (65). Moreover, it has been reported that in old mice calorie restriction improved spatial learning memory evaluated through the Barnes maze but not recognition memory measured with the novel object test (98), consistent with our findings that memory tasks can be variably impacted by diet. More studies are needed to investigate the differential effects of KDs on cognitive tasks and determine if brain regions responsible for specific types of memory are variably affected by KDs. The mechanisms responsible for KD-related changes in cognitive function are also not entirely known. Several mechanisms associated with the effects of KDs on cognition in aged animals have been proposed, including an elevation in acetylation level of hippocampal histone proteins (1, 96), the attenuation of age-related decline in synaptic plasticity (99), an upregulation in BDNF signaling (87), and an

increase in mitochondrial abundance in some neuronal populations(27). Additional work is needed to determine if any of these mechanisms contribute to the cognitive changes observed in the present study.

A decline in motor function and muscle strength also occurs with aging (85) and our results showed that locomotor activity, grip strength, and wire hang performance were reduced with age in all diet groups. Although age-related decline in motor function was not prevented with a KD or IKD, a significant improvement in grid wire hang performance was observed in KD and IKD mice. This suggests motor endurance and coordination, measured by the grid wire hang, was more profoundly affected by KDs when started in late middle age, while grip strength, a measure of maximal isometric strength of the forelimb, was not significantly impacted. Moreover, hindlimb muscles were involved in the grid wire hang but not the grip strength test, and the increase in gastrocnemius muscle weight in the aged KD might contribute to the improved grid wire hang performance. Recent studies have also demonstrated KD started in early middle age preserved hindlimb muscle mass better than the control mice at an advanced age (13), and a ketone ester (KE) mitigated muscle loss in a mouse model of muscle atrophy (100). Low carbohydrate diets have recently gained popularity with endurance athletes, and studies have reported that KDs and KEs may improve performance in some endurance sports (101). Our findings were consistent with the notion that KDs may promote endurance.

Our results also show that CD mice showed an increased level of anxiety at 23 and 26 months of age in the open field test, while the anxiety level of KD and IKD mice was not impacted by aging. These results are consistent with previous studies that reported anxiolytic effects of KDs in laboratory rodents (102).

Age-related diseases are associated with chronic inflammation and are accompanied by an elevation in proinflammatory cytokines. Our results support the notion that a metabolic shift from glucose to ketone body utilization decreased the level of inflammation (87). Of particular interest, the level of serum IL-6 was significantly decreased in both aged IKD and KD mice, and a lower level of IL-6 has been associated with a reduced risk of experiencing a cognitive decline with aging (103, 104). Moreover, increased circulating levels of IL-6 can influence skeletal muscle redox balance (105) and is associated with a decline in muscle strength and function (106). A reduced level of circulating IL-6 in KD and IKD mice is consistent with our results that cognition and muscle endurance were improved in aged mouse. To study the effects of KDs started in late middle age on metabolism, a panel of circulating metabolites and hormones was measured. KD and IKD started at 18 months of age increased total circulating cholesterol, and this change was not observed with a KD started at 12 months of age (13). The increase in circulating cholesterol in the KD mice was primarily due to an increase in HDL-C, which is associated with anti-inflammatory effects and enhanced endothelial wall function (107). The slight elevation in total circulating cholesterol in IKD mice seems to be a combination of moderately increased HDL-C, VLDL-C, and LDL-C, although these markers were not significantly different compared to CD mice. The slight increase in circulating LDL-C and VLDL-C is consistent with the fact that liver triglyceride and cholesterol levels were increased in IKD mice. Although a significant increase in liver triglyceride and cholesterol were detected, the levels in the IKD mice were still within the range of normal aged C57BL/6J (108) and C57BL/6JN (109) mice, and were far below the level of high fat fed aged animals (110, 111). The mild elevation in liver triglyceride and cholesterol may be an indication that the IKD mice were going through adaptation phases under the dramatic cyclical changes in the metabolism induced by their feeding strategy. After the IKD mice were refed the high-

carbohydrate CD, the lipogenesis pathways in liver may be upregulated, as seen in animals during the refeeding phase of an intermittent fasting regime (112), and at the end of the CD phase (time of euthanasia) higher level of triglyceride was accumulated. On the other hand, when the IKD mice were in the KD phase, a significantly higher circulating β-hydroxybutyrate level was observed in both fasted and fed stages, suggesting either upregulated ketogenesis or reduced ketone uptake by peripheral tissues. However, future studies are needed to determine the specific metabolic changes that occur with intermittent KD approaches.

We recognize limitations to the present study, including the fact that the Y maze and Barnes maze tests were not conducted at ages earlier than 23 months. Since working memory was improved at 23 months of age with both IKD and KD, it is possible that these interventions would improve Y maze performance at 20 months of age and it is important to determine how quickly working memory is altered with KD approaches. Another limitation is the lack of baseline healthspan measurements in the study. C57BL6/J mice show heterogeneity in aging and age-associated functional decline (32), and a baseline healthspan measurements would be useful in determining age-related changes in healthspan for each diet group. Furthermore, diet assignment based on a baseline measurement at late middle age would ensure that mice with various levels of healthspan performance were evenly distributed in different dietary interventions.

3.5 Conclusions

Overall, our results demonstrate that the neuroprotective and health-promoting action of KDs are effective when started in late middle age. Both a KD and IKD initiated in late middle-aged mice improved cognition and motor endurance in aged mice. A KD had the most potent effect on

measures of healthspan in aged mice, with IKD showing results intermediate to the KD and CD diet groups.

CONCLUSION

One goal of this dissertation work was to investigate the impact of a KD on mitochondrial mass in tissues of middle-aged and aged mice. Chapter 1 reports tissue-specific changes in markers of mitochondrial mass and does not provide evidence of a widespread increase in mitochondrial content across tissues. In whole tissue homogenates of hindlimb muscle and brain, a KD stimulated activities of key mitochondrial enzymes in 26-month old mice (after 14 months of KD), while no clear pattern indicative of a possible change in mitochondrial content was observed in liver or kidneys. There were no concerted changes in all markers of mitochondrial content in any tissue suggesting the KD may be impacting components of the mitochondria or prompting remodeling of the organelle rather than inducing a broad increase in mitochondrial content across tissues. To take a step further, morphological quantification of mitochondrial mass with TEM was performed to examine regions of interest within tissues. The results of TEM quantification demonstrated that mitochondrial mass was increased in red gastrocnemius muscle in 13-month old mice (after 1 month of KD), and this was accompanied by an increase in the number of mitochondria. This indicates that mitochondrial content in different skeletal muscle groups may be variably impacted by a KD and the mitochondrial alterations that occurred in red gastrocnemius were blunted by analyzing markers of mitochondrial mass in all the muscles of the hindlimb. A 1-month KD did not alter mitochondrial mass in the prefrontal cortex, hippocampus, or the liver left lobe, consistent with the findings in Chapter 1 that most markers of mitochondrial mass were not changed after the same duration of intervention. These results demonstrate that a KD may stimulate components of the mitochondria in the brain and skeletal muscle at old age, and increase mitochondrial mass in certain skeletal muscle groups, supporting the notion that KD may alleviate the age-associated decline in mitochondrial content and

function in some tissues or tissue regions. These studies also highlight the need of using multiple markers of mitochondrial mass or TEM quantification across different regions of various tissues to thoroughly determine the impact of a KD on mitochondrial mass.

Since health-promoting effects of a KD started at early middle age have previously been reported (13), the other objective of the present work was to determine if a KD, or intermittent KD, started in late middle age (18 months of age) would have beneficial effects on healthspan in aged mice. This study showed that both a continuous KD and intermittent KD (3 days a week) initiated at 18 months of age improved working memory in aged mice. The continuous KD also improved spatial learning memory at advanced age compared to control mice. In addition, while a decline in composite cognition score from 23 to 26 months of age was observed in control mice, KD and IKD mice did not show significant age-related changes in cognition score. In regard to motor function, age-related declines were observed in all dietary groups, but mice on the KD and IKD performed better in a motor endurance test compared to the control mice. Control mice showed an age-related increase in anxiety level, and this was not observed in mice on KDs. A composite healthspan score was computed as an overall indication of healthspan. A continuous KD started at 18 months of age showed a significantly higher composite healthspan score compared to control mice at 26 months of age, and an IKD showed an effect intermediate to the CD and KD. In addition to the improvements in measures of healthspan, KDs also reduced levels of circulating cytokines compared to control mice at an advanced age, and this may contribute to the improvements in health span measures observed with these diets in older animals.

This dissertation highlights the potential of using KDs to manage the age-related decline in mitochondria and provides evidence supporting the notion that stimulation of mitochondrial

content in some tissues may be one of the underlying mechanisms of KDs' therapeutic effects. A KD started late in life and used continuously or intermittently may also have health-promoting actions, including improvements in cognition and motor endurance.

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Appendix: Mouse behavioral testing protocols

The appendix includes protocols for the behavior tests used in the study. Some of the protocols are modified to be specifically used in aged mice where it is important to minimize fatigue or stress.

Grip Strength

Purpose

To determine forelimb grip strength

Time

<5 min/animal x 2 rounds

Materials

- Push-pull scale, manufacturer: Imada
    - Including a small metal grid, small rubber bands, and plastic sheet
- Paper and pen to record grip strength
- 5% bleach and 70% EtOH
- Timer, pen and recording sheet

Before the Test

Bleach any material entering the testing room. Mice should not be fed before the test. Attach the small metal grid to the force gauge. The piece of plastic must prevent the mouse from grabbing the metal fork instead of the bar. Use small rubber bands to attach the bar to the fork. Try to minimize the mobility of the accessories.

Recommended Work Flow

Optimal number of people: 1

- One person to handle the mouse and record pulling force

1. Clean Grid and meter with 70% ethanol and attach grid to meter. Ensure hooks are facing upward.

2. Tape meter to bench with grid hanging over edge. Turn meter on and check that Peak (P) force is being measured. This will record the highest value for any attempts rather than a "real-time" measurement.
3. Allow the mouse to reach the metal grid using the forelimbs only. Try to keep the body at a 45° angle (tail above head) and in line with the force gauge. Avoid lateral angles.
    a. Make sure animal does not use hind limbs during pull.
    b. Optional: gently moving the mouse back and forth tends to increase the grip.
4. Allow the animal to pull for 30 sec (45 sec for older mice)
5. Repeat 2 more times, recording the maximum value.
6. Allow each mouse to rest at least 10 minutes (this is a good time to proceed testing the rest of the mice).
7. Repeat the test and record the highest value. (2 sets of 3 trials)

2- and 4-Limb Wire Hanging Test

Purpose

To test muscle function, endurance, and coordination

Time

≤ 3 min/mouse (2 limb); 3-8 min/mouse (4 limb)

Materials

- Clear plastic box (16”x16”16”)
    - Two small holes drilled in the center, ~1 inch from the top, on two opposite sides
- Metal bar (2mm) (2 limb test)
- Metal grid (1mm thickness and 1 cm grid) and 2 blockers (4 limb test)
- Tape
- Video camera (optional) and tripod
- Towels to cushion the bottom of the box
- Stopwatch
- Paper and pen to record scores

Before the Test

Mice may be fed before the experiment. Clean all material with 70% EtOH and bleach any material entering the testing room. Do not use bleach on the metal bar or grid.

2-Limb Wire Hang:

Attach the 2 mm bar to the box by sliding it through the two holes. Use tape to secure. Place the towel(s) at the bottom of the box to cushion the mice after a fall. If recording, place the camera on the tripod and position so that the length of the wire can be viewed.

Two initial scores are established: falling and reaching. The initial falling score is 10 and the initial reaching score is 0. Each fall will decrease the falling score by 1 and each reach will increase the reaching score by 1. If a mouse manages to balance/stand on the wire, correct the behavior by gently pulling the mouse down by the tail.

Recommended Workflow

Optimal number of people: 2

- One person to handle the mouse
- One person to time the test and record falls or reaches

1. Start the video camera (if recording)
2. Open the cage and say the mouse ID out loud
3. Begin the timer
4. Place the mouse in the center of the metal wire, only allowing the animal to grab the wire with the forelimbs. Let the mouse hang.
5. Wait until the mouse falls or reaches one of the sides of the box.

   Note: Before reaching the sides, the mouse may explore for few seconds. Only physical contact with the side of the box is considered a reach.

   Stop the timer when the mouse obtains either a fall or reach.
6. Place the mouse immediately in the original position in the center of the wire and begin the timer again.
7. Repeat until the falling score is 0 or the total hanging time is 3 minutes.
8. If the mouse reaches a falling score of 0 before 3 minutes, record the stopwatch time.

The main outcome of the test is the maximum hanging time and number of falls/reaches in each minute.

Note: for 26 month-old mice, total test time remained 3 minutes, however falls/reaches for each minute were recorded separately. Often the older mice achieved a falling score of 0 between minutes 1 and 2 of the test.

4-Limb Wire Hang:

Place the metal grid on top of the clear plastic box. Use blockers to mask the edges of the metal grid to prevent mouse from escaping around the edges of the grid. Place towels at the bottom of the box to cushion the mouse.

Recommended Workflow

- Optimal number of people: 1

1. Place the mouse in the center of the metal grid, allowing the mouse to grab the wire with all 4 limbs. Gently shake the grid to ensure a firm grip. Flip over grid so the mouse is hanging.
2. Immediately start the timer.
3. Wait until the mouse falls and stop the timer. Record the time in seconds.
4. If the maximal hanging time exceeds 3 min, the test is finished for that mouse.
5. If not, return the mouse to the home cage, continue with the remaining mice, and then go back to repeat that mouse. A maximum of 3 trials can be performed. For advanced aged mice (over 26 months old), only a total of 2 trials may be performed.
6. Weigh the mice shortly after the test and record BW.
7. Wipe and clean any urine on the metal grid between mice. Remove feces from the towel.

8. At the end of the test, carefully clean the grid with 70% EtOH to prevent rust formation.

Note: The main outcome of the test is maximum hanging impulse and is calculated as (hang time (sec) × body weight (kg) × 9.8 m/s^2).

Reference

"The use of hanging wire tests to monitor muscle strength and condition over time". Maaike van Putten, Leiden University Medical Center, Department of Human Genetics, Leiden, the Netherlands.

Rearing Test

Purpose

To test locomotor activity and exploratory behavior

Time

5 min/mouse

Materials

- Clear acrylic cylinder (20cm diameter)
- A clean surface
- Paper towels
- 70% EtOH
- Timer
- Video camera and tripod
- Mirror (optional but recommended)

Before the Test

Mice may be fed before the experiment (ensure consistency between groups). Clean all material with 70% EtOH and bleach all materials. Paper towels may be used as a clean surface under the cylinder and replaced between mice. Place the camera on the tripod. Position the mirror so that the observer can determine if the mouse is touching the wall of the cylinder when not facing the camera.

Recommended Workflow

Optimal number of people: 1

1. Start video camera
2. Open the cage and say the mouse ID out loud

3. Begin the timer (5 min)
4. Place the mouse in the cylinder and walk away
5. After 5 min, stop the video recording
6. Return the mouse to the cage and clean the materials before the next mouse.

Analysis

Watch the video at normal speed and record rearing scores (number of rears in 5 min) as well as latency time (time before the first rear). A rear is one in which the mouse touches the wall of the cylinder with forepaws. If the mouse touches the wall more than once but does not return to the resting position (all paws on the ground), it is considered a single score. In some cases, mice may jump while in the cylinder. This is an optional parameter to be recorded.

Y Maze Spontaneous Alternation Test

Purpose

To test working memory

Time

6 minutes/mouse (AM)

Materials

- White plastic Y-maze – each arm 35 x 8 x 15cm
- Camera and Noldus Enthovosion XT software
- 70% EtOH and 5% bleach

Before the Test

This test is accomplished in one day and mice can be fed before the experiment. Clean all testing material with bleach and bleach any material entering the testing room. Turn on the white noise machine and transport the mice at least 30 minutes prior to the testing to acclimate. Activate software and open saved template for the experiment. Adjust the settings in the software as necessary.

Recommended Workflow

Optimal number of people: 1

1. Start acquisition
2. Place the first mouse in the center of y-maze facing a random corner and allow it to explore the environment for 6 minutes. Acquisition should automatically end.
3. Return the mouse to its cage and clean the inside of the box with 70% EtOH.
4. Repeat for additional mice.

Troubleshooting

- Be as quiet as possible during the recording time. You can step into the ancillary room and close the door.

Analysis

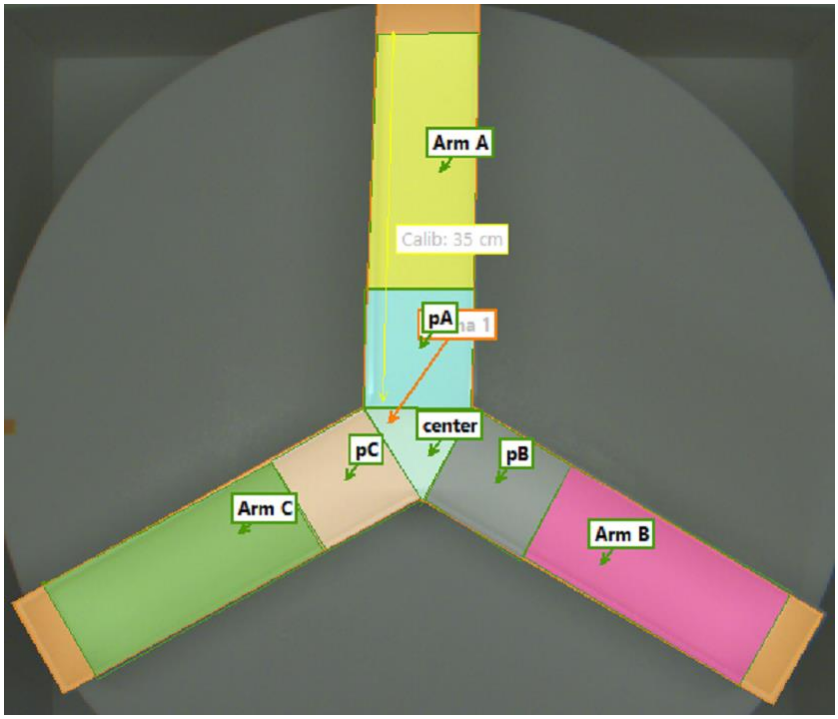


Figure: Arena setup for the Y maze

The arena consists of a center zone, 3 proximal zones covering the first third of the arm (pA, pB, and pC), and 3 arm zones (Arm A, Arm B, or Arm C). An arm entry is defined as when the center point of the mouse enters the distal arm zone followed by an entry into the center zone. An entry is not complete without the mouse returning to the center zone. For instance, the following sequence of mouse movements "center – pA – ArmA – pA – center" is translated as an Arm "A" entry, and the sequence "center – pA – ArmA – pA – ArmA – pA – enter – pB – ArmB" is translated into arm entries of "AB".

The center point of the mouse is tracked automatically via Enthovision XT15. After trial acquisition, the Enthovision XT15 software generates an Excel file showing the zone of the mouse center point at each time point (0.04 sec increments) in a binary format, which indicates presence or absence of the animal in any of the zones. The data file was analyzed using MATLAB codes. The data is run through the codes and the sequence of mouse movements is first translated into arm entries. Then, non-repeating entry triplets were extracted (e.g. “ABC”, “CBA”, “ACB”, etc).

The percent alternation was calculated as ((number of non-repeating triplets) ÷ (number of total arm entries - 2)) x 100%.

References:

Proceedings of Measuring Behavior 2010 (Eindhoven, The Netherlands, August 24-27, 2010) 144 Eds. A.J. Spink, F. Grieco, O.E. Krips, L.W.S. Loijens, L.P.J.J. Noldus, and P.H. Zimmerman

Miedel, C. J., Patton, J. M., Miedel, A. N., Miedel, E. S., Levenson, J. M. Assessment of Spontaneous Alternation, Novel Object Recognition and Limb Clasping in Transgenic Mouse Models of Amyloid-β and Tau Neuropathology. J. Vis. Exp. (123), e55523, doi:10.3791/55523 (2017).

Open Field (OF) and Novel Object Recognition (NOR) Test

Purpose

OF-To test locomotor activity and anxiety

NOR-To test recognition memory

Time

Environment adaptation/Open field test = 15 minutes/mouse (day 1)

Test #1 (2 identical objects) = 10 minutes/mouse (day 2 AM)

Test #2 (1 identical and 1 novel object) = 10 minutes/mouse (day 2 PM)

Materials

- White plastic box – 4 boxes 2x2 (40 cm^2)
- Pairs of objects similar in size (number of pairs = number of mice being tested)
    - Example: Small orange cones and small cell culture flask filled with sand
- Camera and Noldus Enthovision XT software
- 70% EtOH and 5% bleach
- White noise machine

Before the Test

This test is accomplished over two consecutive days (see time needed above). Mice can be fed before the experiment. Wash the objects with an unscented strong detergent (e.g. Alconox), dry with paper towels and wipe with 70% EtOH. Clean all testing material with bleach and bleach any material entering the testing room. Turn on the white noise machine and transport the mice at least 30 minutes prior to the testing to allow acclimation. Activate software and open saved template for the experiment. Adjust the settings in the software as necessary. For OF, the center zone is set as a 25x25 cm square in the middle of each arena.

Recommended Workflow

Optimal number of people: 1

A. **Environment adaptation/Open field test: Day 1**

5. Start acquisition
6. Place the first four mice in the corner of each empty arena and allow them to explore the environment for 15 minutes.
7. Return the mouse to the home cage and clean the inside of the box with 70% EtOH.
8. Repeat for additional mice.

B. **Test #1, Identical Objects: Day 2- AM**

1. In the morning, place two identical objects, selected at random (i.e. boxes or bottles) in the box. They should be placed in the same half of the box, in a symmetrical fashion, around 10 cm away from any wall.
2. Start acquisition.
3. Place 4 mice individually inside each box, facing the wall and opposite to the objects.
4. Allow the mice to explore for 10 minutes without any interference,
5. Return the mice to their home cage.
6. Clean the objects and inside of the box with 70% EtOH.
7. Begin again with the next set of 4 mice.

C. **Test #2, Novel Object: Day 2- PM**

1. Six hours later (after the first mouse completed test #1 above), place one original and one novel object into the box. Randomize position (left / right) of the novel object
2. Start acquisition.

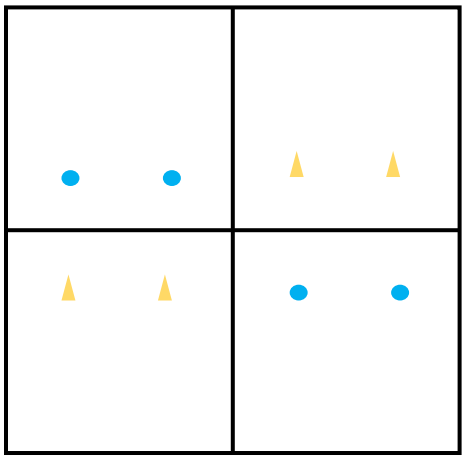
3. Place 4 mice individually inside each box, facing the wall and opposite to the objects.
4. Allow mice to explore for 10 minutes without any interference (keep room as quiet as possible).
5. Return the mice to their home cage.
6. Clean the objects and inside of the box with 70% EtOH.
7. Begin again with the next mouse, alternating which two identical objects are used each time.

Troubleshooting

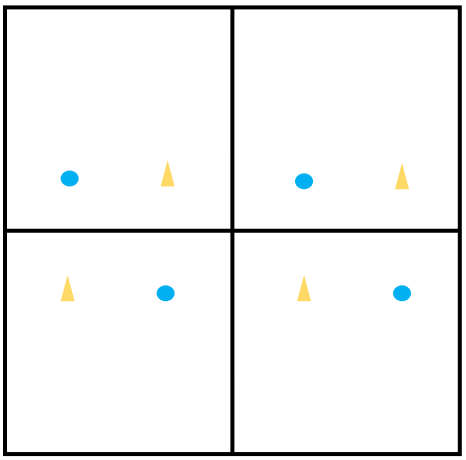
- Be as quiet as possible during the recording time. You can step into the ancillary room and close the door.

Recommended positioning of objects

Day 2 AM:



Day 2 PM:



Analysis

OF

The center point of the mouse is tracked automatically via Enthovision XT15.. The major outcomes are: 1) Total distance travelled and 2) Time spent in the center zone.

NOR

Videos are recorded and analyzed manually, reproducing them at 0.5X speed. Slow motion allows for a more accurate quantification. While viewing the videos, two manual online stopwatches are used in the same screen. Each stopwatch is used to record the time of exploration dedicated to each object by the mouse (defined as left and right objects). Alternatively, time exploring each object can be scored using the manual scoring tool in Enthovision XT.

It is important that the viewer analyze the videos **blind** (i.e. he/she shouldn't know which is the novel object or to which diet group the mouse belongs).

Behavior is considered "exploratory" only when the nose is pointing towards the object, at most 2 cm away. Sometimes exploration may be brief (half a second or less). Climbing or sitting on an object does not necessarily constitute exploration. The best indication of exploration is when the mouse sniffs the object. Additionally, the mouse may settle itself very close to an object for many seconds without actively exploring. This should not be counted as exploration time.

While 10 minutes of video are recorded, only the first 20 seconds of total exploration time (including both objects) is used for analysis. That is, all stopwatches are stopped when the sum of the exploration time for both objects is 20 seconds (or 40 seconds due to 0.5X speed viewing). At this time, the video is also stopped and the total time elapsed recorded.

The major outcome is percent time exploring the novel object.

Note 1: Aged mice may not reach 20 seconds of total exploration in 10 minutes of recording. In this case it may be necessary to record for 12 minutes.

Note 2: If several mice do not reach 20 seconds of total exploration even with the extended trial time, represent exploration time of each object as a percentage of the total time spent in the box.

<u>Reference</u>

Leger M, et al. 2013. Object recognition test in mice. Nature Protocols 8:2531-7.

Barnes Maze Test

Purpose: To test spatial learning memory

Time: 15 min/mouse

Materials

- Barnes maze (92 cm plastic circular disk with twenty 5 cm holes evenly distributed on the periphery)
- Maze stand (30 inches high)
- Black escape box that can be attached under the holes
- 5% bleach & 70% ethanol spray
- Bedding and 2 empty cages
- Tripod
- LED light
- Extension cords
- Small opaque bucket
- Timer

Before the test:

1. Set up the maze and overhead camera. The maze should be leveled and in the middle of the camera view.
2. Set up the panels such that each panel is around 25 cm from the maze.
3. Mice may be fed before the experiment. Transport 6 mice to the testing room 30 min before the test (n=5-7 mice per cohort as the inter trial interval should be around 15-25 min).

4. Set up lighting (~700 lux) and place visual cues on the panels.
5. Wipe and clean the maze, box, and bucket with bleach (first day only) and then 70% ethanol.
6. Place the escape box underneath the target hole.
7. Set up the experiment in Noldus Enthovision.

<u>Recommended workflow:</u>

Optimal number of people: 2

1. Place bucket upside-down in the middle of maze and then place mouse inside the bucket.
2. Turn on light.
3. Wait for 10 seconds.
4. Start software tracking and recording, and then quickly lift bucket.
5. Experimenter moves quickly to designated area.
6. Wait until mouse enters the escape box or when 3 min is reached.
    a. If mouse has not entered the escape box, gently guide the mouse to the target hole, and encourage the animal to enter the box.
7. Stop tracking (click manually if software does not stop automatically).
8. Turn off light and let the mouse stay in the box for 1 min.
9. After 1 min is reached, place the mouse back in the home cage. For training day 2 and 3, 30-45 secs may be sufficient.
10. Change bedding in escape box, wiping with 70% ethanol.
11. Wipe maze with 70% ethanol.
10. Repeat for each mouse: 3 trials per day for 3 days.

11. After cohort 1 has finished trial 2, transport another 6 mice into the room. This way, the second cohort will have time to acclimate while the first cohort is doing trial 3.
12. At test day, remove the escape box, and track the mouse for 120 seconds.

Analysis

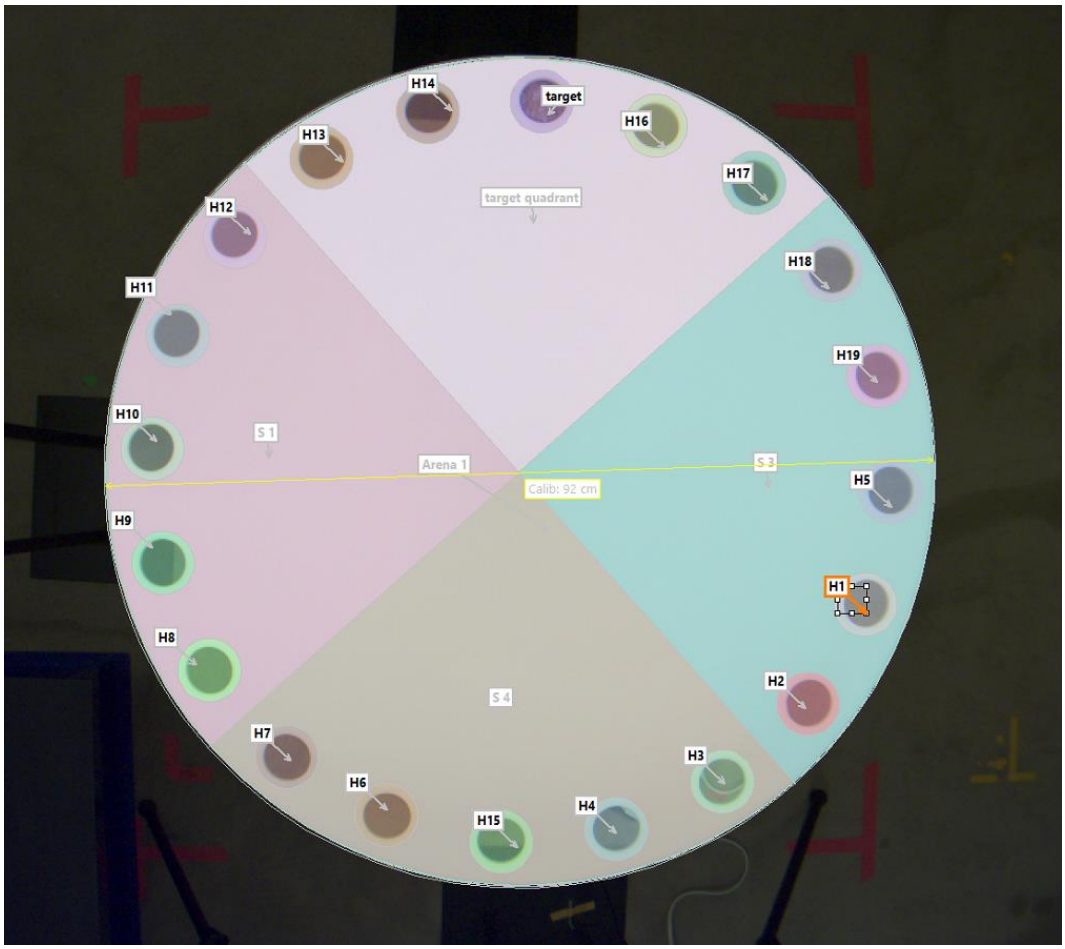


Figure: Arena setting of the Barnes maze

Nose and center point of the mouse are tracked automatically via Enthovision XT15 software. A hole visit is defined when the nose point is within 1 cm from a hole by setting hole entry zones as 7 cm circles centered on the holes. During training trials, latency and pathlength to the target hole and errors before target visit are computed. In Probe trials, in addition to those parameters, time

spent (center point) in the target quadrant (a quarter of the arena with the target at the center) was also computed.