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TRINITY COLLEGE

**EFFECTS OF KETOGENIC DIETS ON AUTISTIC SYMPTOMS OF
FEMALE EL MICE**

BY

Subrina Bisnauth

THESIS SUBMITTED TO
THE FACULTY OF THE NEUROSCIENCE PROGRAM
IN CANDIDACY FOR THE BACCALAUREATE DEGREE
WITH HONORS IN NEUROSCIENCE
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EFFECTS OF KETOGENIC DIETS ON AUTISTIC SYMPTOMS OF FEMALE
EL MICE

By

Subrina Bisnauth

Honors Thesis Committee

Approved:

David Ruskin, Advisor

Charles Swart, Thesis Committee

Hebe Guardiola-Diaz, Director, Neuroscience Program

DATE: _____

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ABSTRACT

The ketogenic diet (KD) is a restricted carbohydrate, high fat and sufficient protein metabolic therapy that elevates ketones as an alternative fuel source, and that reduces seizures in persons with epilepsy which is often comorbid with autism. Autism is characterized by communication deficits, decreased sociability and repetitive behaviours. A restrictive KD reverses symptoms in the BTBR mouse model of autism but its severity is a factor in its clinical applicability. In a study with the EL mouse model of epilepsy and autism, sex-dependent effects were found where only females displayed the behavioural effects of the KD. In the current study, a strict and a milder KD were tested on female EL mice to compare their effects on behavior, blood chemistry and body weight. This study investigated if increased ketones and lowered blood glucose were necessary for behavioural improvement. In order to do so, female EL mice were fed either a standard rodent chow control diet, the restrictive KD or the moderate KD from five weeks of age. At eight weeks of age, behavioral testing, using the 3-chamber test which measures sociability and self-directed repetitive behaviour (grooming), were conducted in order to determine whether autistic symptoms were still present. In addition, the social transmission of food preference test which measures sociability as well was carried out. Weight, blood glucose and ketone levels were also measured. The diets had very similar behavioural effects on the animals, increasing sociability and reducing repetitive behaviours. Interestingly, the moderate KD caused increased weight and did not lower blood glucose yet still improved autistic behaviours. This suggests that caloric restriction and lowered blood glucose may not be necessary for improved behaviours as had previously been thought. Also, a clinical strength KD may possibly be beneficial for autistic children and should be further studied.

INTRODUCTION

Autism

Diagnosis and Prevalence

Autism Spectrum Disorder (ASD) is a neurodevelopmental syndrome that is characterized by deficits in social reciprocity and in communication, as well as by unusual, restrictive, repetitive behaviours (Lord *et al.*, 2000). Social impairments include reduced social-emotional reciprocity, not seeking to share enjoyment, difficulty forming relationships with peers and using non-verbal communication poorly. Communication deficits include improper acquisition of speech with compensation through other methods of communication, using stereotyped speech or delayed echolalia and/or difficulties having conversations. Limited imitative and/or imaginative play also often occur and are associated with abnormal social and communication abilities. Restrictive and repetitive behaviours are characterized by unusual fixations and limited interests, repeated hand and finger movements, whole bodily mannerisms, compulsive ritualistic behaviours, repetitive use of objects and odd sensory seeking behaviours (Lord and Bishop, 2010). Autism usually manifests from infancy, at the latest in the first three years of life (Lord *et al.*, 2000) and is termed a pervasive developmental disorder because many aspects of cognition and behaviour of an affected individual are affected and because symptoms present during development from infancy or even from birth (Walsh *et al.* 2008). Behavioural symptoms of autism may first appear around 1-2 years of age and the diagnosis is made around 2-4 years of age (Courchesne *et al.*, 2007). Currently, there are still no biological markers to help diagnose autism and it remains behaviourally defined (Anagnostou and Taylor, 2011).

Within the revised fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) are several changes to the diagnostic criteria of ASD. The revisions include

only two symptom domains (repetitive, obsessive interests and social-communication), removed subtypes of ASD and describe differences on an individual basis specifically by levels of severity in the two domains relative to levels of development and age. Therefore, an individual diagnosed with ASD would be described on the basis of how severe their symptoms of social-communication and repetitive, restricted behaviours and interests are. This diagnosis could also be linked with other disorders such as genetic and medical conditions (e.g. ASD and Rett Syndrome or ASD and Fragile X), language disorders or other psychiatric conditions (e.g. ASD with intellectual disability or ASD with Attention Deficit Hyperactivity Disorder (ADHD)).

Developmental standards for describing symptoms are also important elements of the revised DSM. Some symptoms like unusual social gaze, facial expressions and gestures are seen in autistics across all age and skill levels. However, some symptoms are expressed differently across development. For instance, a talkative autistic 10 year old girl cannot be diagnosed using the same symptom criteria for a two year old without expressive spontaneous language or for an adult with a better stocked repertoire of functional language. Delineating symptoms by age and development is important for improving understanding of the specificity of deficits in autism. The DSM-V also proposes that autistic individuals would be expected to have a history of each of the sub-domains that define social-communication (significant deficits in non-verbal communication, reduced social reciprocity and peer relationships) and two out of three areas of repetitive behaviours and fixated interests (repeated motor or verbal behaviours that include sensory responses; rituals and routines; obsessive interests). Providing examples that are specific for different age and developmental levels should result in greater sensitivity to diagnoses (Lord and Bishop, 2010).

The Centers for Disease Control (CDC) estimates that 1 in 68 children has been identified with ASD across the US. The most significant risk factor for being autistic is being male; boys are almost five times more likely to have autism than girls: 1 in 42 boys versus 1 in 189 girls. Additionally, white children are more likely to be diagnosed with ASD than are black or Hispanic children (CDC, 2014). Several recent studies, though not always consistent, have found that parents' (both mothers and fathers) advanced age is also correlated with increased risk of ASD. ASDs also incur large financial costs to families and society with affected families estimated to pay more than \$3-5 million dollars beyond the typical cost of raising a child. The cost of ASD in the United States is almost \$90 million dollars per year. In comparison to other children with specialized needs, autistic children are underserved, with inadequate healthcare, less family-oriented care and greater hardships with referrals. Families of autistic children are also under greater financial burden, significantly coordinate their own children's healthcare (greater than 10 hours per week) and will more likely stop or cut back work than other families of children with special needs (Lord and Bishop, 2010).

Abnormal Brain Structure and Etiology of Autism

Presently, one of the most consistent findings of neuropathology of ASD is that of accelerated brain volume growth during early postnatal life. Studies of head circumference, which proxies for brain size, have shown that head circumference is normal or smaller at birth but that growth rate increases at around 12 months. MRI studies have shown that autistic children from ages 18 months to 4 years show a 5%-10% increase in total brain volume compared to age-matched controls. However, it is unclear whether this enlargement continues on into adolescence and adulthood (Amaral *et al.*, 2008). The enlargement appears to occur in both

grey and white matter and studies suggest that white matter contributes disproportionately to this increase in volume (Anagnostou and Taylor, 2011).

A crucial question to address is whether there are region-specific abnormalities in brain volume. The general consensus from the postmortem literature on the older child or adult afflicted with ASD contrasts with that of the postnatal brain overgrowth. Older autistic brains are characterized by neuron loss, degeneration, inflammation and decreased size of the cortical minicolumns (Amaral *et al.*, 2008). Studies that used stereological methods to quantify neurons found that there were fewer neurons in the amygdala which is important for memory, learning and emotion. The amygdala in autistic boys undergoes abnormal enlargement that continues through late childhood. Studies have found a 13%-16% enlargement in the amygdala of autistic children ages 36-56 months (Sparks *et al.*, 2002). Other studies have suggested that enlargement of the amygdala is connected with very severe anxiety (Juraneck *et al.*, 2006) and abnormal sociability and communication (Munson *et al.*, 2006). In typically developing boys, from ages 8-18 years, the amygdala has increased in size by approximately 40% whereas this growth pattern was not seen in autistic boys. Therefore, the amygdala appears to initially be larger in autistic children but is not subject to the same volume increase that occurs in typically developing boys (Schumann *et al.*, 2004). When the cerebellum was examined in autistics across a wide age range, it was found that the total volume was enlarged compared to controls (Amaral *et al.*, 2008). The cerebellum, which is important for its involvement in various cognitive and motor functions, has been found to have fewer Purkinje cells which would result in the cerebellum functioning improperly (Bailey *et al.*, 1998). This finding has yet to be confirmed with stereological studies but studies have observed astrogliosis in the cerebellum which may indicate that glial activation had taken place upon neuronal degeneration or death. Other studies have

found that the neurons within the deep cerebellar nuclei, which is the pathway that leaves the cerebellum, were unnaturally small and pale in autistic adolescents and adults. The cerebellar vermis, which possibly has functions in modulating emotion, sensory perceptiveness and arousal has been found to be smaller or similar in size in autistics than in typically developing children, adolescents and adults (Courchesne *et al.*, 2007). The corpus callosum, which contains interhemispheric axons, has consistently been said to have decreased volume in ASD which may suggest decreased interhemispheric connectivity in this group (Anagnostou and Taylor, 2011). In the frontal cortex which executes higher order functions, increases in pro-apoptotic molecules and decreases in anti-apoptotic molecules were found in adult autistics. Studies have found signs of glial activation in the cerebrum. Both glial activation as well as pro- and anti-inflammatory molecules have also been found in the frontal cortex. In addition, smaller minicolumns have been found in the frontal and temporal cortices of older children, adolescents and adults with ASD. More studies, however, need to be carried out on the pathology of neurons and the numbers of minicolumns in ASD across all ages (Courchesne *et al.*, 2007). Findings have also consistently shown reduced intercolumnar width of layer III in the dorsolateral prefrontal cortex or Brodmann's Area 9. Studies have reported the enlargement of the caudate nucleus which may be associated with repetitive and ritualistic behaviours in autistic adolescents and young adults. Evidence, though somewhat inconsistent, has been found for abnormal volume and shape of the hippocampus with greater cell packing density and smaller neurons being reported (Amaral *et al.*, 2008). Investigations of structure have also reported reduced cingulate size in relation to decreased metabolic activity in autistics. In addition to the abnormalities in volume of the frontal and temporal lobes described above, volumetric abnormalities have also been found in the parietal lobe, thalamus and brainstem (Anagnostou and Taylor, 2011).

Technologies that have only become recently available allow for the quantification of the grey and white matter. Enlargements have been found in grey and white matter in the frontal, temporal and parietal lobes, with the greatest increases being reported to be in the dorsolateral prefrontal and medial frontal cortex. Evidence on grey matter volume indicate that though grey matter increases may be proportionately smaller than white matter increases in early life, the increases may continue on into later life. Studies spanning childhood to adulthood found a 6%-12% increase in grey matter (Palmen *et al.*, 2005). Some MRI studies have found cortical shape abnormalities within the sylvian fissure, the superior temporal sulcus, the intraparietal sulcus and the inferior frontal gyrus (Nordahl *et al.*, 2007). Two studies investigating cortical thickness found that in 8-12 year olds, there is increased cortical thickness over the entire cerebrum primarily caused by increases in temporal and parietal cortices whereas in adults there was cortical thinning in the frontal, parietal and temporal areas (Hardan *et al.*, 2006). An MRI study involving older and preadolescent autistic children divided white matter into inner and outer compartments and found that the outer portion of white matter, especially in the frontal lobes, was significantly disturbed. Consistent with other cortical data was the fact that the occipital lobes were least deviant (Herbert *et al.*, 2004). These findings suggest that ASD's neuropathology may include increases in short-distance connections, particularly in brain regions that are responsible for higher language, cognitive, social and emotional functions (Courchesne *et al.*, 2007).

There are also several hypotheses regarding the causal factors of autism. Genetic studies in autism are highly focused on mutations of the methyl-CpG-binding protein *mecp2*. Simply put, MeCP2 is a transcriptional repressor protein that has been found to be involved in gene silencing, particularly in the brain. Rare mutations of this gene have been identified in autistic

individuals. *Mecp2* is an X-linked gene which makes it susceptible to X-inactivation and its mutations may result in neurodevelopmental disorders like autism. X chromosome inactivation decreases the number of X chromosomes transcribed to one per diploid set of autosomes which equalizes the distribution between sexes. The high prevalence of autism among males suggested the involvement of X-chromosome linkage which was later evidenced to be true from genome-wide screens (Currenti, 2009). Many other potential genes and their mutations have been identified to be associated with ASD including SHANK3, NLGN3/4, CNTNAP2 and PNET to name a few. Evidence for the genetic role in the etiology of autism has also been derived from studies of twins. The concordance rate for monozygotic (MZ) and dizygotic (DZ) twins differs greatly with an MZ:DZ ratio being approximately 10:1. In twin studies, it was found that estimates of heritability or genetic causes in ASD ranged from 36% to 93% (Ronald and Hoekstra, 2011). These conclusions are consistent with a genetic etiology for ASD (Buxbaum, 2009).

The immune system may also play a role in ASD. Children, in utero, are provided with maternal antibodies, essential for providing the developing fetus with humoral immune system proteins. Some studies show that in mothers of children with ASD, however, maternal immunoglobulin reacts against fetal brain proteins. This hypothesis suggests that transfer of maternal antibodies via placenta negatively affects fetal brain development. Other studies on mice have shown that maternal infections during pregnancy enact changes in pro-inflammatory cytokines in the fetal mouse brain, significantly affecting brain development of the fetus. Moreover, families affected with ASD exhibit with autoimmune disorders. Neurotoxic exposure to heavy metals, such as mercury, has also been linked to ASD. The Food and Drug Association estimates that 16% of women have sufficiently high mercury levels to induce neurological

damage in their children and a reduced ability to excrete mercury is associated with ASD. Another hypothesis, the Redox/Methylation hypothesis, expounds that genetic and environmental factors combine to give a measure of the risk associated with developing autism. The hypothesis postulates that in most cases of autism, environmental exposures (heavy metals, xenobiotics) are clearly seen to be a factor but genetic factors still dictate the population at risk via polymorphisms (Currenti, 2009). Perhaps with further study, clear risk factors and biological markers can be delineated for autism and timely intervention provided for children at risk.

Comorbidities

Apart from its core symptoms, ASD is often accompanied by various psychological and physiological disorders. Epilepsy has been found to be frequently comorbid with ASD with the frequency range being 5% to 38.3% (Tuchman and Rapin, 2002). Epilepsy is defined as two unprovoked seizures of any type. Therefore, any seizures with a specified cause such as trauma, infection, metabolic illness or febrile seizures are not classified as epilepsy. The prevalence of epilepsy among all children is only 2% to 3% compared to the much higher rate among autistic children. Such a high proportion eliminates coincidence and suggests that epilepsy and autism may share underlying neurobiology (Tuchman and Rapin, 2002). The peak risk periods for seizures occur in early childhood before five years of age and in adolescence after ten years of age (Tuchman and Rapin, 2002; Bauman, 2010). Seizures become more apparent after 12 years of age with 66.7% of all seizures that accompany ASD occurring then and 30% occurring by age 20 (Bauman, 2010). Certain gene mutations, such as those governing ligand- and ion-gated channels, have been found in both ASD and epilepsy (Betancur and Coleman, 2013).

Autistic children also commonly suffer several other disorders. Children with ASD frequently suffer from sleep disorders with prevalence rates ranging from 40% to 80%, compared to typically developing children with rates of only 30%. Sleep disorders are also reportedly more severe in ASD with the most common being sleep onset, sleep maintenance and sleep duration. Metabolic disorders have also been associated with ASD including phenylketonuria, creatine deficiency syndromes, Wilson's disease, Lesch-Nyhan syndrome and urea cycle disorders. There is also a growing body of evidence that suggests that mitochondrial dysfunction may play a role in at least a subgroup of autistic individuals. The prevalence is currently unknown but mitochondria supply a very substantial amount of energy to the cell and, therefore, will mostly affect tissues with high energy demands such as the brain (Bauman, 2010). The prevalence of mental retardation in idiopathic autism has been estimated to be ~60% but with the spectrum taken into consideration, the prevalence drops to 30%. Mood and anxiety disorders are also very common in ASD (Amaral *et al.*, 2008).

Treatment

Several treatment options exist for autism including behavioural options. Behavioural interventions which include applied behavior analysis are used to address behavioural problems in autistic youths. An initial behavioural assessment, called a functional analysis, is conducted prior to behavioural intervention. Functional analyses are controlled observational sessions that are performed to objectively figure out the main motivating factor for a child's behavior which may include seeking attention, accessing a favoured item, avoiding doing something or other rewarding characteristics of the behavior. Although behavioural interventions have been successful in decreasing maladaptive behavior in autistic children, timely access to trained personnel and therapists in this intervention is often restricted (Posey *et al.*, 2008).

When behavioural interventions are not fully effective, medication-based treatments are often taken into consideration. A survey of psychotropic drug use in children with pervasive developmental disorders (PDD) found that about half of the subjects are presently being prescribed a psychotropic drug and that 16.5% are taking an antipsychotic drug. Antipsychotics are most frequently used to alleviate mood and behavioural disturbances including irritability, aggression and agitation. Studies have suggested that about 30% of children and adolescents with PDD experience moderate to severe irritability, often accompanied by aggression directed towards others or themselves. Recently, risperidone was approved by the Food and Drug Administration (FDA) for the treatment of irritability in autistic children and adolescents. This is very noteworthy because Risperidone is the first drug to be approved in the treatment of autism and the first atypical antipsychotic to be approved for use in children and adolescents. Risperidone and other atypical antipsychotics, however, had become common in the treatment of autism well before its FDA approval and several published reports had suggested its clinical effectiveness (Posey *et al.*, 2008).

Antiepileptic drugs (AEDs) are also widely administered to autistic individuals. There are several reasons why AEDs are used in ASD, including the high rate of co-occurring epilepsy in these individuals, the anecdotal reports that suggest improved communication and behavior in subjects with autism and epileptic discharges and the knowledge that some disruptive behaviours may be symptomatic of an associated affective disorder. Although the role of AEDs is not currently established, there is evidence that autism, epilepsy and affective disorders are commonly comorbid and that they may share a common neurochemical substrate. This substrate is the common target of the psychotropic mechanism of action of different AEDs (Di Martino and Tuchman, 2001). A survey study of individuals with ASD, conducted in the early 1990s,

found that 15.2% of individuals were medicated with AEDs with the most common for the treatment of epilepsy being carbamazepine, valproic acid and phenytoin. Studies that have examined the behavioural and cognitive effects of AEDs in ASD have found that valproate monotherapy reduced repetitive behaviours and irritability in autistic individuals. Valproate has also been reported to improve core behavioural symptoms in autistic children with and without epilepsy and to significantly improve autistic symptoms in children with subclinical epileptic-like discharges on EEG. Other AEDs that have been reported to improve cognitive effects in autistic children include lamotrigine, levetiracetam, topiramate and carbamazepine (Frye *et al.*, 2013).

The Ketogenic Diet

Background

The Ketogenic Diet (KD) has been successfully used to treat intractable epilepsy since the early 1920s. It is high in fat, low in carbohydrates and provides sufficient proteins for growth (Hartman *et al.*, 2007). Fasting has been known to be an effective antiepileptic treatment for a long time and works for a wide range of seizure disorders. However, because it is an unsustainable method of treatment, KDs were developed to mimic the physiological effects on the body of fasting (Mantis *et al.*, 2004). The KD has been so called because maintenance of the diet results in a sustained state of ketosis in the body (Hallböök *et al.*, 2012). Because the diet provides insufficient carbohydrate to fuel the body's energy needs, energy is largely gained from fatty acid oxidation in the mitochondria. Large amounts of acetyl-CoA are generated during high rates of fatty acid oxidation resulting in the synthesis of the three ketone bodies beta-hydroxybutyrate, acetoacetate and acetone mainly in the liver. The metabolic efficiency of the Krebs cycle is increased and excess acetyl-CoA is diverted into the production of ketone bodies.

Ketone bodies then end up in circulation, raising serum levels substantially, and are utilized by extrahepatic tissues, including the brain, as an energy source. Usually glucose is utilized as fuel for the human brain – fatty acids cannot be used as they do not cross the blood-brain barrier. Ketone bodies enter the brain in direct proportion to ketosis. Normally, ketone utilization by the brain is minimal but while on the KD ketone bodies partly replace glucose as fuel for the brain (Hartman *et al.*, 2007).

The KD has been used to effectively treat various forms of drug-resistant epilepsy (Hallböök *et al.*, 2012). Currently, several types of KD exist for epilepsy treatment with the most common being the traditional KD. It is based on long-chain saturated fats with a low percentage of carbohydrates and proteins in a ratio of 4:1 with there being four times the amount of fats to protein and carbohydrate combined (Hartman *et al.*, 2007). In the clinical setting, patients are usually given 1g of protein per 1kg of body weight, 5-10g of carbohydrates and the remainder of daily calories in fats. The KD minimizes body glucose levels – 55-75mg/dl serum glucose without causing caloric restriction or malnutrition (Hallböök *et al.* 2012). A typical day on the KD for a three year old child is shown below in a table by Zupec-Kania & Spellman (2008).

Breakfast	40g 36% Heavy cream 32g Sausage links 24g Avocado- Haas 5g Canola oil
Lunch	40g 36% Heavy cream 18g Sliced turkey breast 22g Raw cucumber slices & celery sticks 22g Mayonnaise
Dinner	40g 36% Heavy cream 20g Baked cod 43g Roasted cauliflower 23g Butter
Snack	10g Sliced strawberries 28g 36% Whipped heavy cream

Table 1: A typical day on the KD for a 3 year old child. The majority of fats are from heavy cream, vegetable oils, and butter, which represent medium and long chain triglycerides. Adapted from Zupec-Kania & Spellman, 2008.

The Ketogenic Diet and Autism

The KD has been proposed as a treatment to improve the symptoms of ASD. Many autistic children are medicated with anti-epileptic drugs (AEDs) for the treatment of epilepsy as well as behavioural disturbances. A meta-analysis of children with ASD on AEDs found that 85% of the literature reviewed reported that children on an active AED (valproic acid, carbamazepine and lamotrigine) showed improvement in their behavioural disturbances (irritability and aggressiveness) as well as in their core ASD symptoms of sociability and communication regardless of whether their seizures improved. This suggests shared underlying biology between epilepsy and ASD which means that the KD, an effective anti-epileptic, can potentially be therapeutic against ASD (Di Martino & Tuchman, 2001).

A study was conducted on the island of Crete where 30 epileptic children were treated with KD. The children were between 4 and 10 years of age and displayed autistic behaviour. The KD was administered for six months that included four weeks of continuous administrations followed by a two week diet-free break. Improvement was reported by 18 of the 30 children, who adhered to the diet for a year, in their Childhood Autism Rating Scale scores. Significant improvement (>12 units) was reported by 2 patients, average improvement (>8-12 units) in eight patients and minor improvement (2-8 units) in eight patients (Evangelidou *et al.*, 2002). These results provide further support for the hypothesis that the KD could improve behavioural symptoms and serve as a treatment option for ASD.

Our lab conducts research with an autistic model of mice and the KD that gives further credence to this hypothesis. The BTBR mouse models the three core behavioural deficits of ASD – sociability, communication and repetitive behaviours. Our lab showed, in a recently published study, that juvenile BTBR mice fed a diet constituting a 6.6:1 ketogenic ratio for three weeks

showed improved behavioural symptoms when behavioural tests were carried out at eight weeks of age. Specifically, the BTBR displayed improved sociability in the three-chamber test, reduced self-directed repetitive behaviour in the three-chamber and single-chamber test and improved social communication of food preference (Ruskin *et al.*, 2013). These results are illustrated in Figure 2 below. Based on these promising results, our lab has been motivated to explore the effects of the KD in other rodent models of ASD.

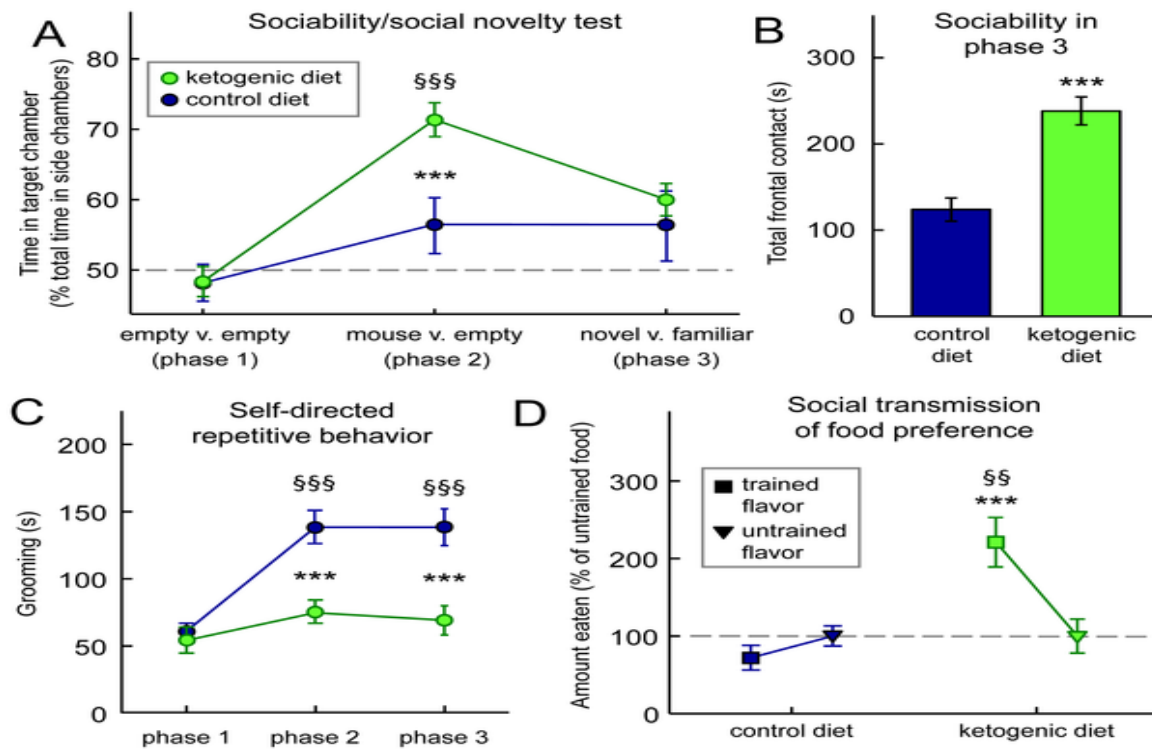


Figure 1: Previous work conducted in our lab on the effect of the KD on behavioral symptoms in the BTBR mouse model of ASD. The KD significantly increased sociability and communication and reduced repetitive behaviors.

The EL Mouse Model

In this project, the EL mouse model of ASD and epilepsy was used. The model was discovered in 1954 to be a mutation from the outbred non-epileptic DDY strain and were validated as a model of epilepsy in 1976. The EL mouse has since been used as a model of

multifactorial idiopathic generalized epilepsy (Meidenbauer *et al.*, 2011). Along with their seizures, EL mice experience incontinence, vocalisations, loss of postural equilibrium among other symptoms (Meidenbauer *et al.*, 2011; Suzuki 1976). EL mice experience handling-induced seizures by post-natal day 50-70. Male EL mice are more susceptible to seizures than females at 60-90 days of age but this disparity disappears by 120 days of age (Meidenbauer *et al.*, 2011). It was also found that EL mice display paroxysmal discharges and abnormal brain plasticity, further strengthening their epileptic phenotype (Suzuki, 2013).

Previous studies have also found abnormal social behaviours in EL mice. One study found that an EL mouse placed in a novel cage with an unfamiliar mouse was less inclined to interact with and explore the unfamiliar mouse (Turner *et al.*, 2007). We have also noticed in our lab that female EL mice exhibit abnormal maternal urges, often consuming her newborn pups. EL mice have also exhibited developmental delays that include abnormal habituation to a new environment and hyperactivity in some tests (McFadyen-Leussis and Heinrichs, 2005).

Another recent study reinforced the fact that the EL mice display an autistic phenotype as well (Table 2). The EL mice were compared to the non-epileptic original DDY strain which served as controls and were subjected to behavioural tests such as the light-dark compartment, social transmission of food preference, open field, myoclonic jumping tests as well as validation of their epileptic phenotype. In all tests, the EL mice were found to exhibit a robust autistic phenotype compared to DDY controls. The EL mouse model is the first model of both epilepsy and autism that consistently demonstrates both phenotypes clearly. Thus, the EL mouse model was determined to be a reliable model for autism and epilepsy for this project.

Component	Characteristics of persons with autism and EL mice	
	Human	Mice
Social interaction ^a	Failure to develop peer relationships appropriate to developmental level Lack of social or emotional reciprocity	Impairment in exploring conspecific, juvenile mice (Lim et al., 2007) Impairment in interacting with conspecific, age-matched mice (Turner et al., 2007)
Communication ^a	Impairment in language development and communication	Impairment in social communication (Fig. 6)
Restricted and repetitive behavior ^a	Stereotypic and repetitive motor mannerisms Preoccupation with a restricted pattern of interest	Myoclonic Jumping (Fig. 4) Excessive self-grooming and head weaving (Fig. 4)
Genetics	Multifactorial (Rutter, 2005)	Multifactorial (Todorova et al., 1999)
Neuroanatomic	Microglial activation (Vargas et al., 2005) Cellular maldevelopment in brain without destructive lesions (Kemper & Bauman, 1993; Casanova et al., 2002)	Microglial activation (Drage et al., 2002) Gliosis observed in hippocampus, but no obvious neuronal loss (Brigande et al., 1992)
Neurochemical	Change in gangliosides (Nordin et al., 1998)	Change in gangliosides (Brigande et al., 1992)
Comorbid traits	Hyperactivity (Ando & Yoshimura, 1979) Epilepsy (Malow, 2006)	Increased locomotor activity and exploratory behavior (Figs. 1 and 5) Model of epilepsy

^aComponents and human characteristics taken from *Diagnostic and Statistical Manual of Mental Disorders, 4th ed.* American Psychiatric Association (2000).

Table 2: A summary of the fidelity of the EL mouse as a model of autism. From Meidenbauer *et al.*, 2011.

We have previously investigated the behavioural effects of our strict KD (6:1 ketogenic ratio) on both male and female EL mice. However, beneficial effects were seen in the females only. Although, our strict KD has been shown to be effective in reversing symptoms in the BTBR and improving those in EL females, its severity limits its clinical applicability. In this study, we compared the behavioural effects of our moderate KD, our strict KD and a control diet on EL females only since no diet effects were seen in the males on the strict diet.

Thesis Overview and Hypothesis

The current study compared the effects of our strict KD (6.6:1 ketogenic ratio), our moderate KD (3:1 ketogenic ratio) and a control diet on female EL mice. Specifically, the diets' effects on autistic behavioural symptoms, weight and blood levels of glucose and beta-hydroxybutyrate of the mice were examined. Based on previous research on the KD in the BTBR mouse model of autism, we predicted that the two KDs would improve autistic behaviors, increasing sociability and communication and reducing repetitive behaviours, as well as increase blood beta-hydroxybutyrate and decrease blood glucose and weight. We wanted to find out specifically how effective our moderate KD was when compared to our strict KD and a control diet.

METHODS

Animals

Female EL mice were weaned at three weeks of age and housed in groups of two to six animals per cage. They were fed standard rodent pellet chow and water *ad libitum* for two weeks and then at five weeks of age, they either remained on the standard rodent chow (n = 18) which became a control diet (CD), or were switched to either the restrictive ketogenic diet (strict KD - 6.6:1 fats to carbohydrates and proteins, BioServ) (n = 18) or to the milder ketogenic diet (moderate KD – 3.2:1 fats to carbohydrates and proteins, BioServ) (n = 18). The mice were fed their respective diets *ad libitum* for another three weeks, after which behavioural testing was performed. Behavioural testing involved the three chamber test which included the three chamber sociability test, social approach test and test for self-directed repetitive behaviours. Blood testing, where blood ketone and glucose levels were measured, was also carried out after behavioural testing, as well as weight measurements. The three chamber tests were video recorded to allow for scoring by two independent viewers in order to validate the data.



Figure 2: Study timeline and testing protocol

Blood Testing

Blood ketone (β -hydroxybutyrate) and glucose levels were measured at eight weeks of age after all behavioural testing but STFP ended. The mice were first generally anaesthetised by carefully placing them in a plastic container that held a small quantity of isoflurane. Tail blood was then taken, after which they were returned to their home cage to recover. The blood was analysed using a Precision Xtra meter and glucose and ketone strips.

Behavioural Testing

Three-Chamber Test

The three-chamber test tests for sociability and self-repetitive behaviours in autistic models of mice (Moy *et al.*, 2004). The apparatus was made of Plexiglas and consisted of three equally-sized chambers (42.5 cm x 19.1 cm x 22.2 cm) separated by removable doors. These doors separate the center chamber from the two side chambers. A wire cage (inverted pencil cup) was set against the back wall of each of the two side chambers with cement blocks placed on top. These were present for the entirety of testing.

Before the start of a testing session, the cage of test mice were placed in the testing room, with the cage filter lid removed, to habituate for 30 minutes. Concurrently, the stranger mice (adult, female, albino CD1) were gently placed under the two wire cups in the apparatus and left to habituate for 30 minutes as well (Figure 2A). Afterwards, the stranger mice were returned to their cage and the apparatus was washed with warm water and soap.

Then, in another habituation phase, the subject mouse was placed in the center chamber with the doors down and the lid off for 10 minutes. Following this period, a video-recording camera was set up to record the activity of the test mouse in the three 10 minute phases of the

three chamber test. In the first recorded phase, the doors of the side chambers were removed and the lid placed on top of the apparatus. The mouse was allowed to freely wander into the side chambers (Figure 2B) and this phase tested for side bias. At the end of the first phase and before the start of the second, the test mouse was gently guided back into the center chamber and the doors replaced. A stranger mouse was then placed under one of the wire cages in a side chamber, after which the doors were once again removed to allow the test mouse full access to the side chambers (Figure 2C). This phase tested for social preference. For the third and final phase, the test mouse was again guided into the center chamber and the doors replaced. A second, novel stranger mouse was then put under the remaining wire cup in the other side chamber. The doors were then removed as before (Figure 2D) and this phase tested for preference for social novelty. At the end of the testing session, all mice were returned to their home cage and the entire three chamber apparatus, including the removable doors, wire cages and cement blocks, was washed and dried.

Sociability

Two independent scorers used the recorded video and manual timers to measure how much time the test mouse spent in each chamber, defined as over half of the mouse's body being in a chamber. The measurements were used to compare how much time the test mouse spent in the side chamber containing the stranger mouse (target chamber), in phase two, with the amount of time she spent in the empty chamber. In phase three, the time the test mouse spent with the first stranger mouse was compared to the time she spent in the chamber with the novel stranger mouse (target chamber).

Social Contact

The three chamber test can also be used to test for social contact (Defensor *et al.*, 2011). Social contact is a measure of the test mouse's sociability and how much contact there is between her and the stranger mice. The total amount of social contact was measured in the second and third phases only and was defined as any contact by the front half of the test mouse and the stranger mouse/cage. The behaviour was typified by the test mouse having her paws or face against the stranger mouse's wire cage, climbing the cage, having direct physical contact with the stranger mouse through sniffing with whiskers and/or face and other body parts touching. Social contact was measured because during the three chamber test, the mouse could have been in the target chamber but was not socializing with the stranger mouse. Two independent scorers measured social contact times in all female EL mice undergoing the three dietary treatments.

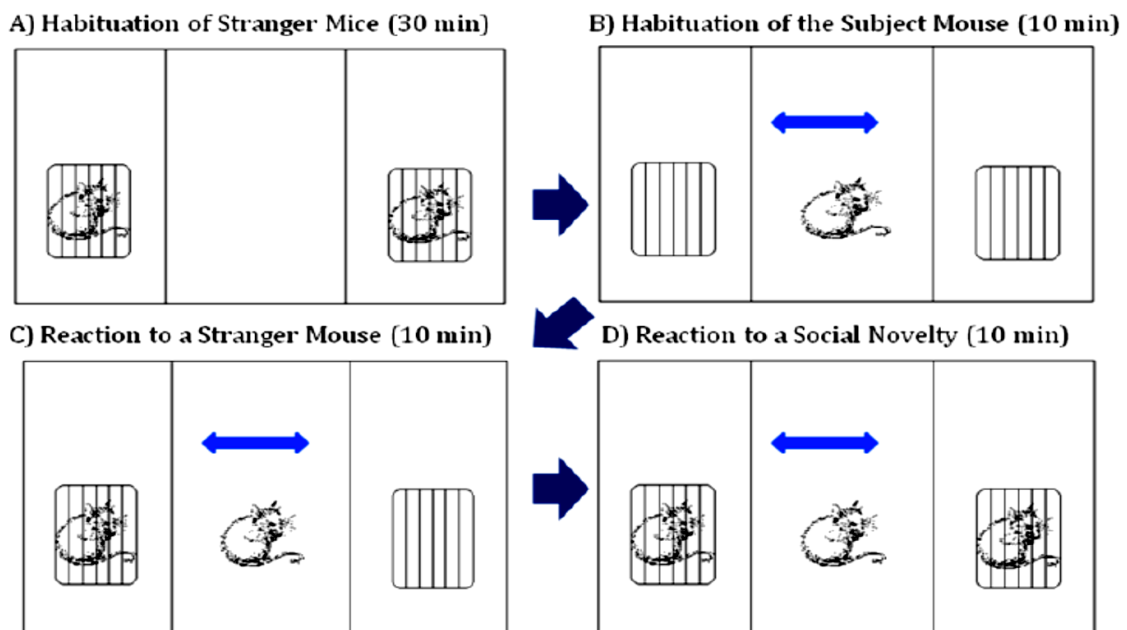


Figure 3: Outline of the Three Chamber Test. (Svedova, 2011)

Self-Directed Repetitive Behaviour (A)

The three chamber test was also used as a test for self-directed repetitive behaviour or grooming. In this case, the amount of time spent grooming by the test mouse in the first two phases was measured in all chambers. Grooming was defined as licking, biting and scratching, including cleaning the face with the paws, biting the hind quarters, licking the tail to name a few. Two independent scorers manually measured grooming time.

Single Chamber Test

Self-Directed Repetitive Behaviour (B)

Before testing began, the home cage of test mice would be placed in the testing room with the filter top off to habituate for 30 minutes. One test mouse was then placed in a clean, empty, transparent enclosure (7.5"x11.5"x5") covered by a lid to habituate for 10 minutes. Afterwards, a video-recording camera was set up to record the test mouse's activity for the next 10 minutes. The time the mouse spent grooming (see Three Chamber: Self-Directed Repetitive Behaviour) was scored by two raters and the scores were averaged. The single chamber test took place on a different day than the three-chamber test.

Social Transmission of Food Preference (STFP)

The STFP measures attention/social awareness of an observer mouse. Twenty-four hours before testing, all chow was removed from the cage and the mice were habituated to eating unflavoured powdered food from a jar. The jars (2" diameter, 1.5" height) were made of glass and had rounded bottoms. After habituation, one mouse was chosen randomly from the cage and designated the demonstrator mouse. The demonstrator mouse was placed in a separate cage,

similar in size and with similar enrichments. She and the observer mice who were left behind in the home cage were deprived of food and fasted for 18 hours.

After the fast, one jar that was filled with bedding and topped by a flavoured food (trained flavour) was placed into the demonstrator mouse's cage for two hours. The flavor used was either cocoa or cinnamon (2% cocoa and 1% cinnamon) and was alternated among test groups. If the test mouse had eaten at least 0.5 grams of food, she was returned to her home cage. If not, she remained in isolation for hour long intervals until she had done so and was then returned to her home cage. The demonstrator mouse interacted with the observer mice for 30 minutes, during which time it is thought that she passively communicated olfactory cues to the observer mice to identify the trained flavour (Wrenn *et al.*, 2003).

Afterwards, the observer mice were placed in separate cages and given a choice between two flavoured foods: the trained flavour and an untrained or novel flavour. Cinnamon and cocoa were alternated as either the trained or untrained flavour depending on which the demonstrator mouse was fed. The observer mice were allowed to feed for two hours and the jars were weighed before and after to determine how much food the mice had eaten. Healthy mice engage in STFP during the 30 minute interaction with demonstrator mice and prefer the trained flavour because they have deemed it safe after recognising it from the still alive demonstrator mice.

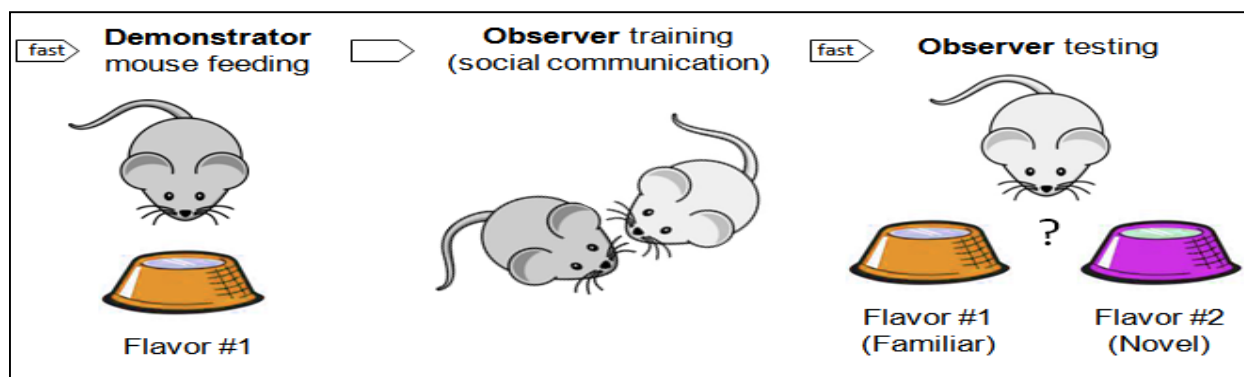


Figure 4: Social transmission of food preference test. The demonstrator mouse is presented with a trained flavour (either cocoa or cinnamon) then interacts with observer mice who are then presented with a choice between the trained and an untrained flavour (from poster by Masino *et al.*).

Statistical Analysis

Data from the three chamber and single chamber tests was scored by two independent raters and at least one scorer was blind to dietary treatment. All data was entered into Excel spreadsheets then analysed using the SigmaPlot software program. Data from all tests - the single chamber and three-chamber tests and blood and weight - were statistically analysed using One Way ANOVA followed by a post-hoc (Student Neuman-Keuls). Data outliers were determined and eliminated using the Grubbs test.

RESULTS

Weight and Blood

Weight

Mice on the moderate KD weighed significantly more than mice on the CD and mice on the strict KD (**Figure 5**). In the past, we have seen that male BTBR and female EL mice on the strict KD lost weight compared to animals on the CD. Here, however, we see that female EL

mice on the strict KD only trend towards losing weight compared to mice on the control diet (p=0.059).

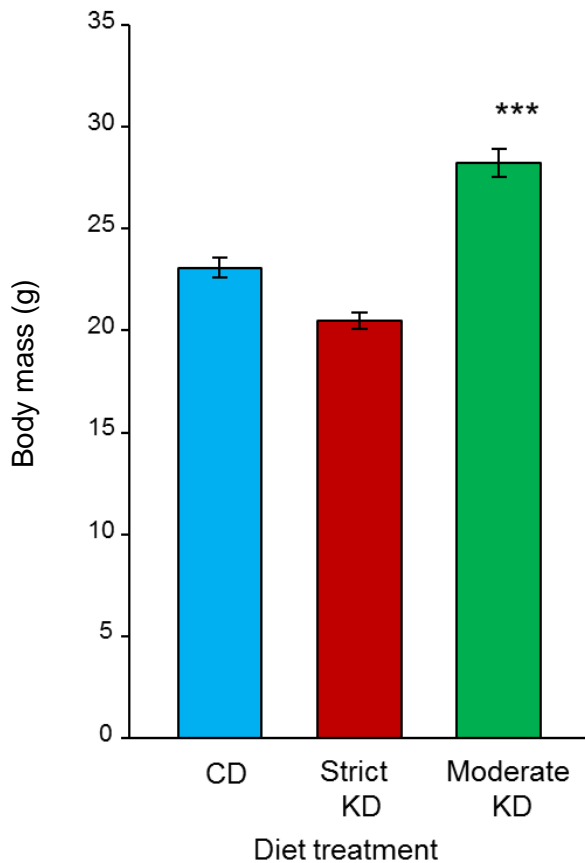


Figure 5: Weight data for female EL mice on CD (n=15), strict KD (n=18) and moderate KD (n=18). Mice on the moderate KD weighed significantly more than mice on the CD and mice on the strict KD. ***=p<0.001

Blood Glucose Levels

Mice on the strict KD had significantly lowered blood glucose levels compared to mice on the CD while no difference was seen in blood glucose levels in animals on the CD and those

on the moderate diet (**Figure 6**). The strict KD, therefore, reduced blood glucose levels more effectively than the moderate KD.

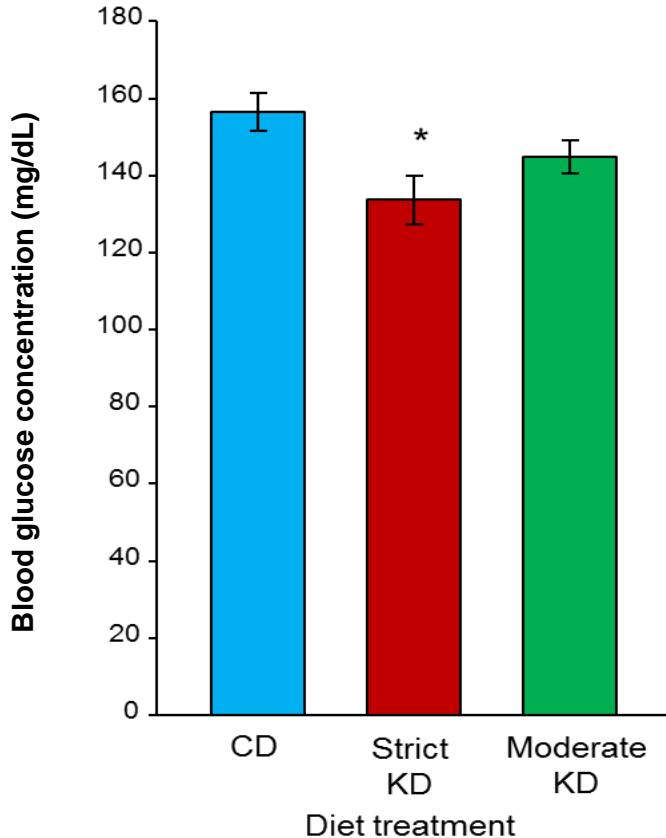


Figure 6: Blood glucose data for female EL mice on CD (n=15), strict KD (n=18) and moderate KD (n=18). Mice on the strict KD had significantly lower blood glucose compared to animals on the CD. *=p<0.05

Blood Ketone (Beta-hydroxybutyrate) Levels

Mice on both KD groups had significantly increased ketone levels compared to mice on the CD (**Figure 7**). Mice on the strict KD also had significantly increased ketone levels compared to mice on the moderate KD. Therefore, the strict KD was more effective at increasing blood ketone levels than the moderate KD.

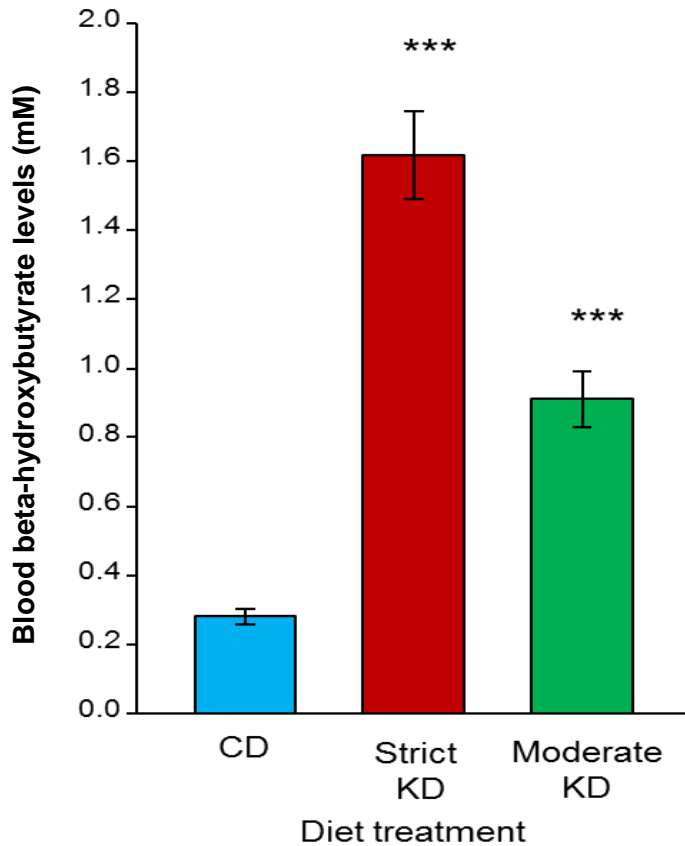


Figure 7: Blood ketone data for female EL mice on CD (n=15), strict KD (n=18) and moderate KD (n=18). Mice on the KDs had significantly elevated blood ketone levels compared to mice on the CD. Mice on the strict KD also had significantly increased ketone levels compared to mice on the moderate KD. ***= $p < 0.001$

Behavioural Testing

Three-Chamber Test

The three-chamber test was used to determine sociability by measuring chamber time and social contact. The three-chamber test was also used to quantify repetitive behavior (grooming).

Chamber Time

In chamber time, mice on both the strict and moderate KDs showed increased sociability in phase 2, the preference for sociability phase, compared to phase 1, the non-social phase (**Figure 8**). Mice on the control diet, however, showed no significant differences between phases which indicates a diet effect. Therefore, both KDs increased sociability in the preference for

sociability phase. There were no significant differences between phases from phase 1 to 3 or from 2 to 3 in any of the diets or between diets in phase 3.

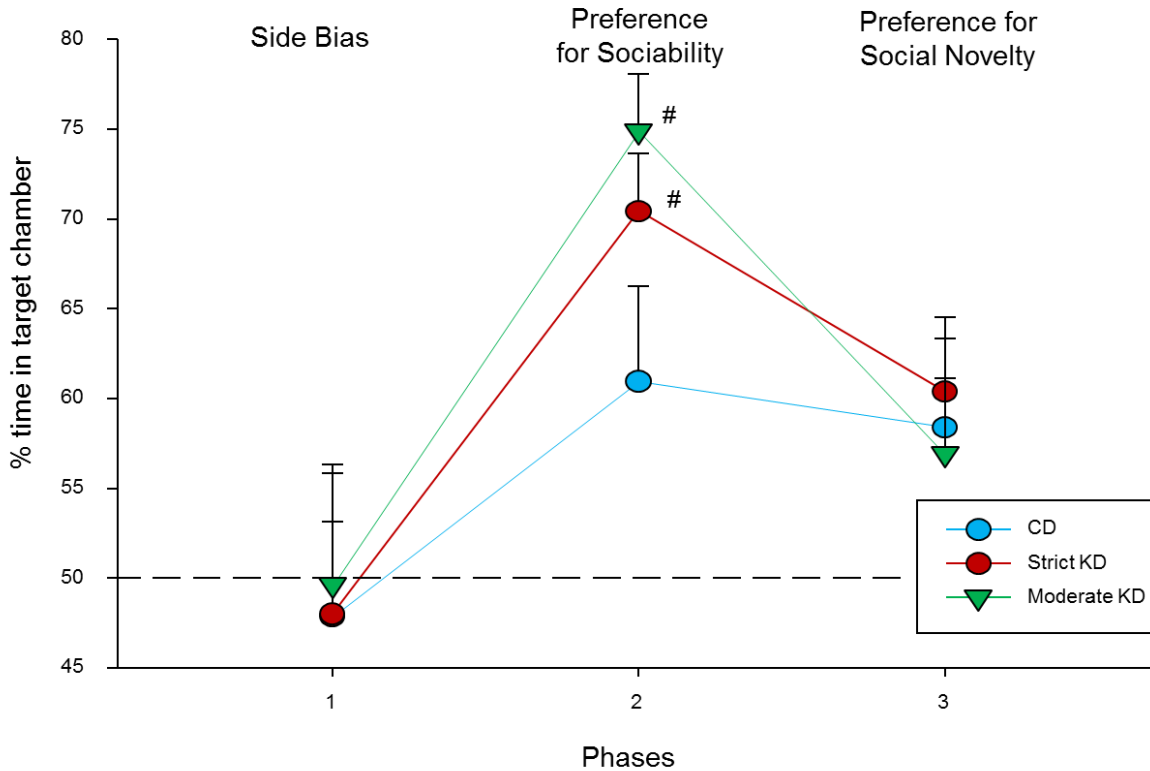


Figure 8: Sociability data from chamber time of the three-chamber test. Female EL mice on the CD (n=18), moderate KD (n=18) and strict KD (n=17) were tested. Mice on the moderate and strict KDs showed increased sociability within phases from phase 1, the non-social phase, to phase 2, the preference for sociability phase. #= $p < 0.05$

Social Approach

The second and third phases of the three-chamber test were also used as a measure of social contact. The total time that female EL mice (n = 18) on CD, moderate KD and strict KD spent in contact with stranger mice was recorded (**Figure 9**). In phase 2, where one stranger mouse was present, mice on both KDs showed significantly increased social contact compared to mice on the CD. In phase 3, the preference for social novelty phase, however, only mice on the

strict KD showed significantly increased social contact compared to mice on the CD.

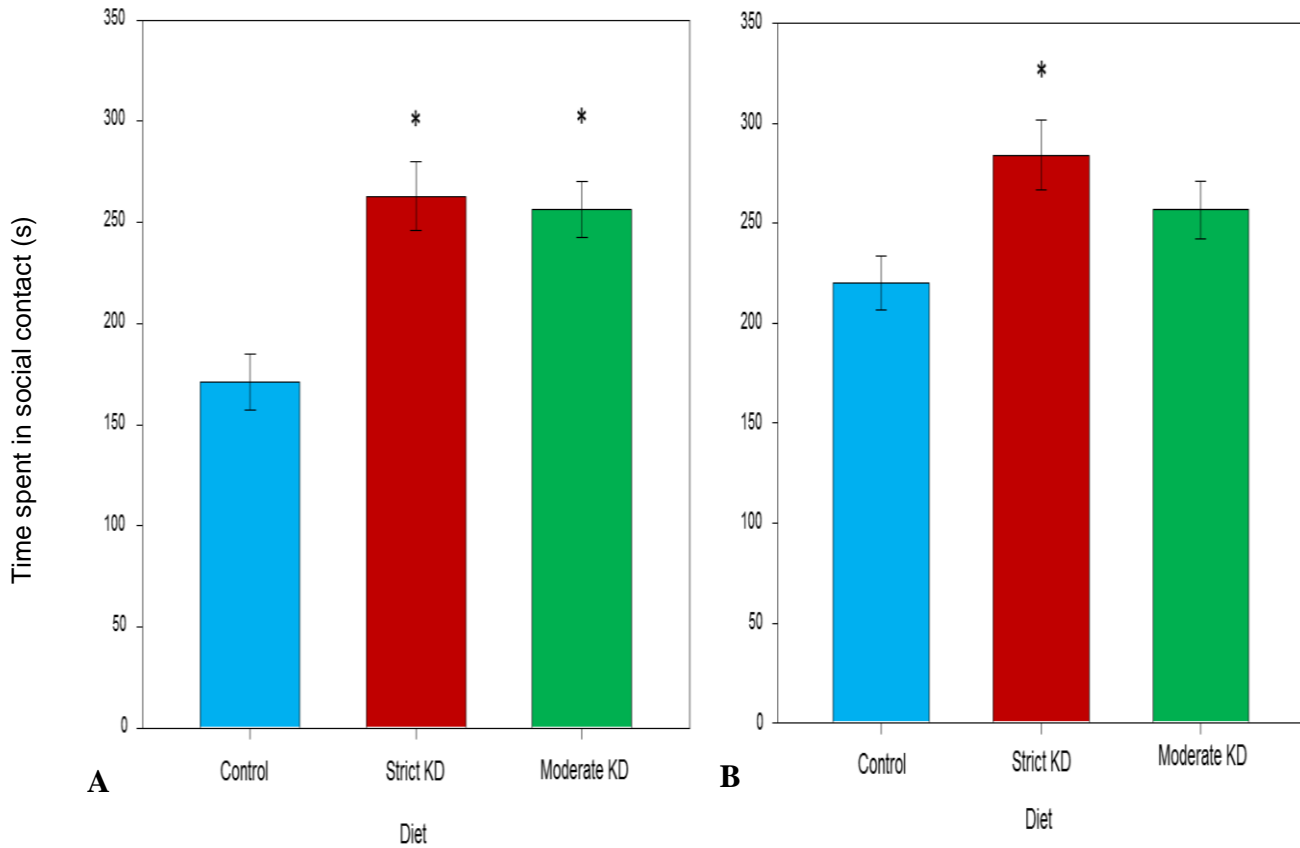


Figure 9: Sociability data from social approach in the three-chamber test. Data was obtained from female EL mice (n=18) on the CD, strict KD and moderate KD during the **A**) second and **B**) third phases of the test. In the second phase, mice on both the moderate and strict KDs showed increased social contact compared to mice on the CD. In the third phase, however, only mice on the strict KD showed significantly increased social contact compared to mice on the CD. *=P<0.05

Self-Repetitive Behaviour

The first two phases of the three-chamber test were used to determine the amount of time spent grooming in a social versus a non-social setting. Time spent grooming by female EL mice on the CD, strict and moderate KDs was recorded (**Figure 10**). In phase 1, the non-social phase, mice on both the strict and moderate KDs showed decreased grooming compared to mice on the

CD. However, in phase 2, the social phase, mice on the moderate KD increased their grooming compared to phase 1.

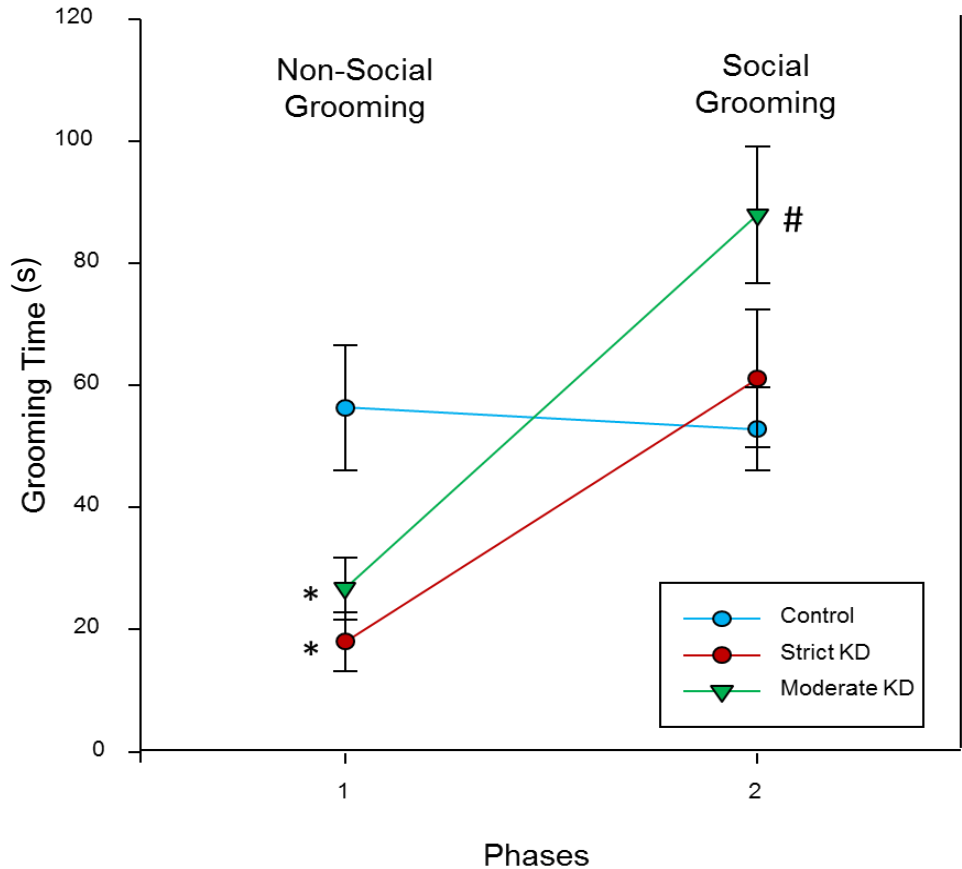


Figure 10: Results from the three-chamber test of self-repetitive behavior (grooming). Female EL mice on the CD (n=18), moderate KD (n=17) and strict KD (n=17) were tested. Mice on the KDs groomed significantly less in phase 1 than mice on the CD. However, mice on the moderate KD groomed significantly more in phase 2 than in phase 1.

Single Chamber Test

The amount of time spent grooming in the single chamber test was also used as an indicator of self-directed repetitive behavior. Grooming time was measured from female EL mice on the CD, moderate and strict KDs (**Figure 11**). No significant diet effects were seen in animals on any diet.

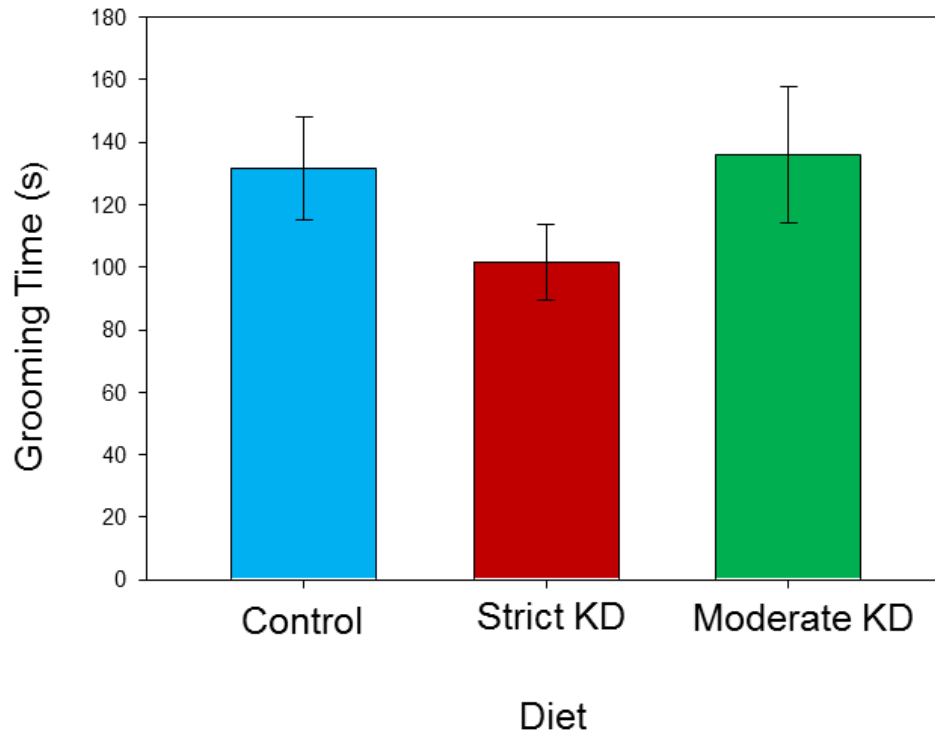


Figure 11: Results from the single chamber test of self-repetitive behavior (grooming). Female EL mice on the CD (n=18), moderate KD (n=13) and strict KD (n=18) were tested. No diet differences were observed.

Social Transmission of Food Preference

The STFP experiment was used to test communication in female EL mice on the CD, strict and moderate KDs. There was no dietary effect on the amount of trained food consumed by mice on the KDs compared to mice on the CD.

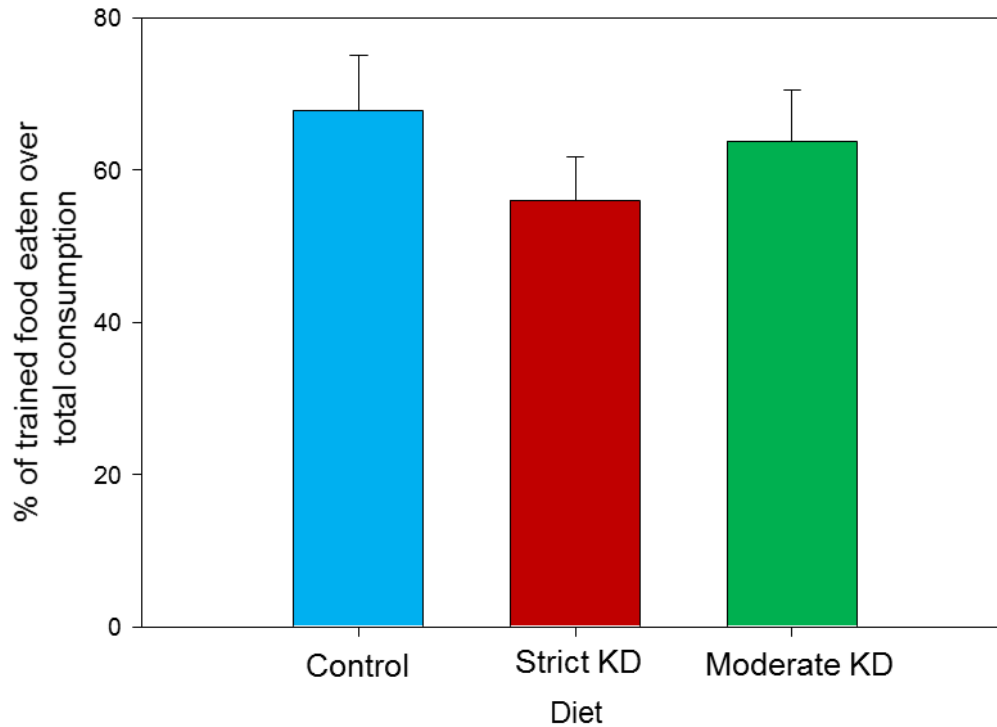


Figure 12: Data from the STFP test in female EL mice on the CD (n=14), strict (n=13) and moderate (n=14) KDs. No dietary effect was observed on the amount of trained food consumed by the mice.

DISCUSSION

According to the data collected, this study found that the moderate or clinical strength KD had very similar behavioural effects in the female EL mice compared to the strict KD. These results represent the first time that the efficacy of the moderate KD has been compared with that of the strict KD in an autistic mouse model with favourable results in terms of behavioural benefits. We had hypothesized that both diets would improve autistic behaviors, increasing sociability and communication and reducing repetitive behaviours, as well as increase blood beta-hydroxybutyrate and decrease blood glucose and weight. Our hypothesis was largely supported with the key exception being that blood glucose was not reduced as an effect of either of the KDs.

Previous Studies and Inconsistencies

A previous study was conducted in this lab by Ruskin *et al.*, 2013 on another autistic mouse model, the BTBR. In this study, the effects of the strict KD on blood chemistry and autistic behaviours were also investigated. Based on the blood chemistry effects of this study where blood ketones were significantly increased, inducing ketonemia, it was expected in the current study that such a significant increase would also occur with both the strict and moderate diets which indeed took place. It was also predicted, based on the results of this study that the strict diet significantly lowered blood glucose, that both the strict and moderate diets would have similar effects in the EL mice in the current study. However, only the strict diet significantly reduced blood glucose in the EL mice while the moderate diet had no effect. This is of particular interest because reduced blood glucose is supposed to be one of the hallmark effects of the KD

and this has been hypothesized to play a role in the anti-seizure mechanism of the diet and potentially in the mechanism for alleviating autistic behaviours (Ruskin *et al.*, 2013).

In the BTBR study by Ruskin and colleagues, it was found that the strict diet reduced autistic behavioural symptoms. In this study, BTBR mice on the strict KD spent significantly more time in the chamber with a stranger mouse in the second phase of the three chamber test compared to mice on the control diet in the same phase. There was also increased sociability within phases from phase 1 to phase 2. In the current study, both diets increased sociability in phase 2, the preference for sociability phase, from phase 1 but did not differ significantly from mice on the CD in phase 2. Also, in the BTBR study, the mice on the strict KD showed a very significant increase in social contact in phase 3. In the current study, both diets showed a significant increase in social contact in phase 2 but only the strict diet showed a significant increase in phase 3. Furthermore, the strict KD very significantly reduced self-repetitive behaviour in phases 2 and 3 of the three chamber test in BTBR mice while in the EL mice, significant grooming time reductions were only seen in phase 1 for mice on both diets. Finally, KD feeding in BTBR mice improved social communication in the social transmission of food preference test as mice ate significantly more of the trained flavor than the untrained flavor while no diet effects were observed in the EL mice. Thus, there are similarities between the strains for certain autistic traits but differences for other traits. These differences reflect the great variation of symptoms present in ASD which fall on a spectrum.

In this lab, we have previously found that the EL mice exhibited autistic behaviors in some tests but not others. Specifically, we have seen sex differences in autistic symptoms. Unexpected results include that EL male and female mice were able to perform social transmission of food preference, considering a previous study found that EL mice showed

significantly less preference for the trained flavor (Meidenbauer *et al.*, 2011). The animals in the previous study were fed the strict KD. When the males in that study were compared with the females in the current study on the strict diet, we saw that males on the diet showed no significant weight changes or differences in blood glucose levels, sociability or self-repetitive behaviours compared to mice on the CD. They did, however, show increased ketone levels as expected. Sex differences have also been seen in BTBR mice (Defensor *et al.*, 2011) where females were seen to have higher levels of sociability in social proximity tests than males. The males, however, consistently displayed autistic behaviours including avoidance of contact, increased self-grooming and less time spent engaged in social interactions to name a few. Autism is a heterogeneous condition (Lord *et al.*, 2000) where no two individuals present with the same symptoms and symptoms are varied and fall on a spectrum. The autistic symptoms of the EL and BTBR models could therefore fall on such a spectrum which may explain the differences seen in the effects of the strict KD on autistic symptoms in the two models.

Very interesting also is the fact that contrary to our hypothesis, the moderate diet did not reduce blood glucose but animals on this diet still showed improved sociability in the preference for sociability phase in the three chamber test as well as improved social contact in the same phase. Grooming time was also reduced in phase 1 of this test. These results indicate that a more moderate ketogenic ratio may not result in lowered blood glucose as has previously been seen in a more strict version of the diet (Ruskin *et al.*, 2013) and more importantly, that lowered blood glucose may not be necessary for improvement in autistic behaviours to occur as had been previously hypothesized. Furthermore, the moderate diet caused a very significant increase in weight in animals, indicating an absence of caloric restriction. Studies have shown that the KD manages epilepsy best when administered in restricted amounts and since fasting lowers blood

glucose levels, Seyfried and colleagues suggested that caloric restriction might contribute to the antiepileptic and anticonvulsant effects of the KD (Greene *et al.*, 2001; Seyfried *et al.*, 2004; Mantis *et al.*, 2004; Eagles *et al.*, 1999). However, in the current study beneficial behavioural effects were seen in the absence of caloric restriction where a very significant weight increase was seen in the 3:1 moderate KD, suggesting that caloric restriction is not necessary for the beneficial effects of the KD. The study by Seyfried *et al.* tested a 3.4:1 KD in female EL mice as well while the study by Eagles *et al.* tested a 7.7:1 KD in male Sprague-Dawley rats. The differences in sex, animal species and ketogenic ratio in these studies versus the current study may or may not have influenced the difference in results regarding the necessity for caloric restriction in the efficacy of the KD, and more studies are needed to determine if a relationship exists between these variables. It is also possible that ASD and epilepsy may have different neurobiological mechanisms so that caloric restriction may be highly beneficial for the KD's antiepileptic effects but unnecessary for its autistic behavioural effects.

Proposed Mechanisms of the Ketogenic Diet

Autistic spectrum disorders and epilepsies are heterogeneous disorders that have diverse etiologies and pathophysiologies. The high rate of co-occurrence of these disorders suggest potentially shared underlying mechanisms (Di Martino and Tuchman, 2001). Although the efficacy of the KD against epilepsy has been acknowledged, its antiepileptic mechanism is still unknown. It is proposed that the behavioural effects of the KD are due to the very high levels of blood ketones which are thought to have neuroprotective effects. One theory proposes that beta-hydroxybutyrate may provide a more efficient energy source unit oxygen than glucose (Veech *et al.*, 2001). The diet also appears to induce mitochondrial biogenesis as electron micrographs of the dentate/hilar area of the hippocampus exhibited a 46% increase in mitochondrial profiles in

rats fed the KD (Bough *et al.*, 2006). The combination of these factors may account for neurons' increased ability to withstand metabolic challenges that would normally deplete the resilience of neurons resulting in their demise (Gasior *et al.*, 2006). Acetoacetate has also been shown to be neuroprotective against glutamate-mediated apoptosis, a major mechanism that occurs upon cell injury (Gasior *et al.*, 2006). Additionally, the KD may be neuroprotective by increasing GABA levels which consequently increases GABA-mediated inhibition (Yudkoff *et al.*, 2001). The diet may also confer neuroprotection by enhancing antioxidant mechanisms by reducing free radical production (Veech, 2004) and by controlling Reactive Oxygen Species (ROS) formation (Ziegler *et al.*, 2003; Freeman *et al.*, 2006). The diet may also protect against different forms of cell death. For example, the diet protected against apoptosis in mice induced by the glutamate receptor agonist and excitotoxin kainate, shown by reductions in apoptosis markers (Noh *et al.*, 2003). Inflammation has also been hypothesized to contribute to the development of chronic epilepsy and fasted rats have shown increased expression of the cytokine interferon- γ in the hippocampus which has been shown to protect against excitotoxic cell death (Lee *et al.*, 2006). The role of decreased carbohydrates as a neuroprotective mechanism has also been studied and was found to be protective against hippocampal damage and functional neurological deficits caused by the seizure-inducing excitotoxin kainite as well as against glutamate- and oxidative stress-induced neuronal death in culture (Lee *et al.*, 1999). It has also been found that the KD can reduce seizures in mice by increasing activation of adenosine A₁ receptors (Masino *et al.*, 2011).

Figure 13 summarises these proposed mechanisms.

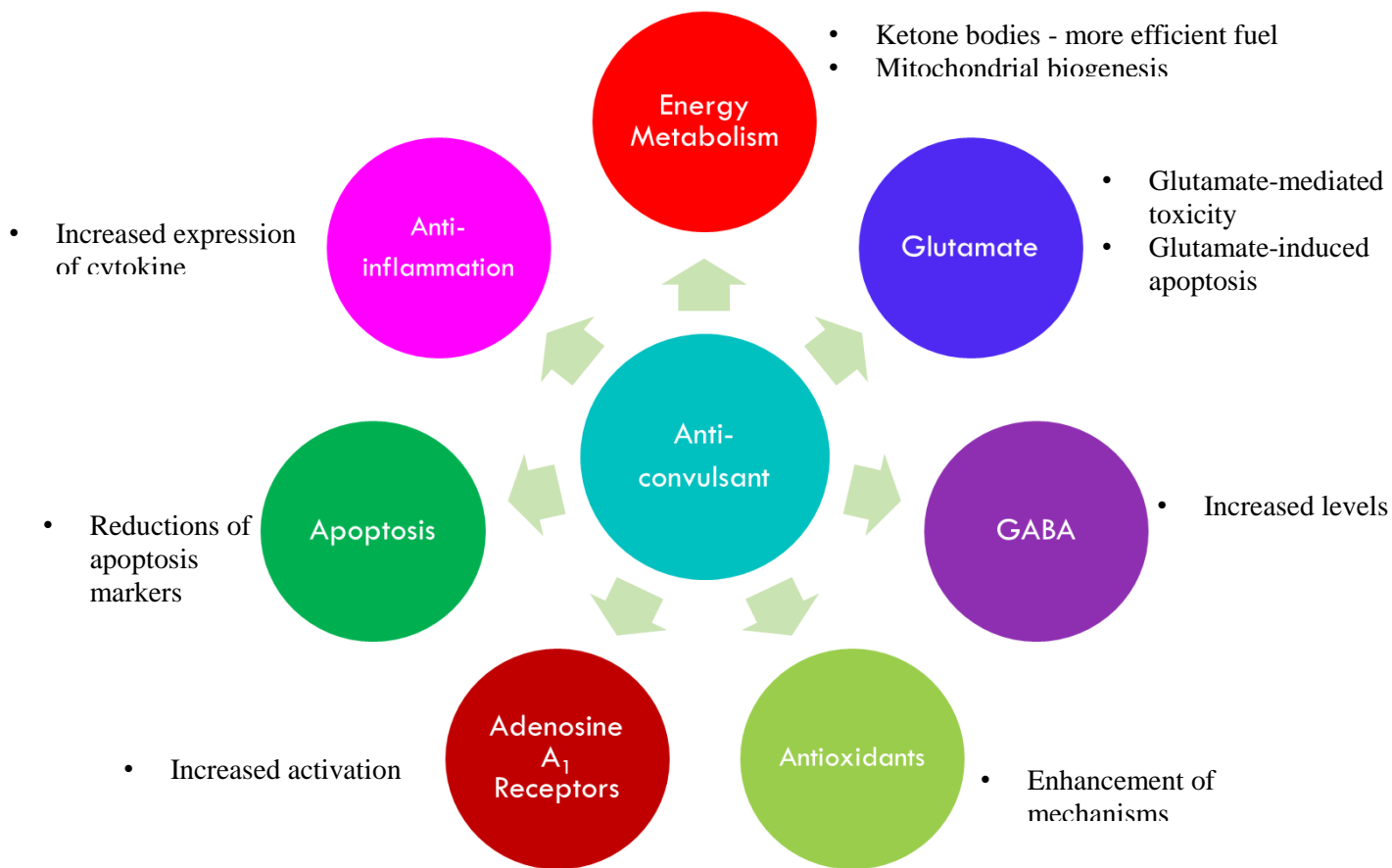


Figure 13: Schematic showing proposed anti-convulsant mechanisms of the Ketogenic Diet.

Looking at proposed mechanisms of autism, evidence of parallels with epilepsy were found. Several lines of evidence suggest that an impairment of GABAergic transmission contributes to the development of ASD. In particular, it has been hypothesized that at least some forms of autism result from an imbalance between excitation and inhibition in circuits involved in sensory, mnemonic, social and emotional processes. Changes in GABA_A and GABA_B receptors have been found in brain samples from ASD patients. The resulting hyperexcitability, due to impaired GABAergic transmission, could thus disrupt the normal formation of cortical maps leading to a relatively unstable state. This altered GABAergic function may also reduce the threshold for developing seizures (Pizzarelli and Cherubini, 2011). Such excitatory-inhibitory defects have also been found in mouse models of autism (Gogolla *et al.*, 2009). Urakubo and

colleagues also found that maternal exposure to high-dose lipopolysaccharide, a well-characterized model of infection in rodents, in pregnant rats decreases TNF- α in the fetal brain. Given the mounting evidence that cytokines play important roles in the development of neurons and glial cells, changes in levels of these pro-inflammatory cytokines in the fetal environment may contribute to abnormal brain development associated with prenatal exposure to infection (Urakubo *et al.*, 2001). Mitochondrial dysfunction has also been theorized to play a part in the mechanism of autism. One study conducted with autistic children found a high prevalence of mitochondrial dysfunction in children presenting with full syndrome autism. Mitochondrial dysfunction could greatly amplify and propagate brain dysfunction, such as that found in autism, given that the highest levels of mtDNA abnormalities are observed in post-mitotic tissues with high energy demands (e.g., brain) (Giulivi *et al.*, 2010). Given these hypothesized mechanisms of autism, the proposed therapeutic mechanisms of the KD that may cause improved autistic behavioural effects are summarized in **Figure 14** below.

Potential for Use as Diet Therapy

The strict KD has been shown exhaustively to be neuroprotective in many ways but particularly in epilepsy and more recently autism. Autism is often co-morbid with epilepsy suggesting a shared underlying mechanism which prompted investigations into the KD's efficacy against autism. Our lab has now shown that our strict KD is efficacious in two mice models of autism, one also being a model for epilepsy. However, the severity of our strict KD makes it clinically inapplicable which further prompted the development and use of our moderate KD. We have now also shown that the moderate KD has very similar efficacy as the strict KD in alleviating autistic behavioural symptoms in an animal model. This moderate KD, being

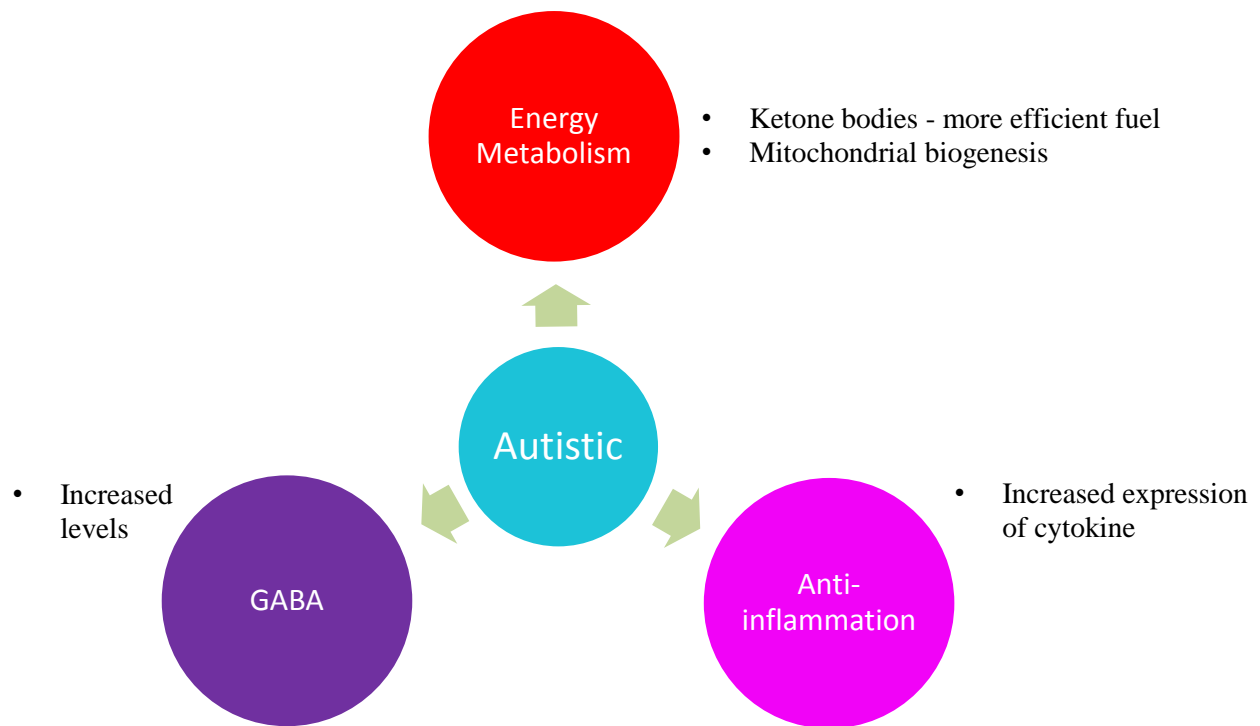


Figure 14: Schematic showing proposed autistic-relieving mechanisms of the Ketogenic Diet. These overlap with its therapeutic anti-convulsant mechanisms and may provide evidence for shared mechanisms of the two disorders.

clinically relevant, can be potentially beneficial in children with ASD in improving core behavioural symptoms. To date, no drug has been shown to have significant impacts on the core symptoms which has been problematic, for without improved social relatedness and communication and reduced repetitive behaviours, children with autism would not be able to benefit from social services, educational programs and behavioral interventions, according to experts (Anthes, 2014). Risperidone and apiprazole are the only drugs currently approved by the FDA to treat irritability associated with ASD and not any of the core symptoms (Anthes, 2014; McDougle *et al.*, 2005). As it stands, the ketogenic diet is an established, effective non-pharmacologic treatment for intractable childhood epilepsy (Kossoff *et al.*, 2008) with very manageable initial side effects. Due to the evidence that it may improve the core symptoms of

ASD, where other pharmacological and non-pharmacological treatments have failed, further studies are warranted into the efficacy of the moderate KD in children with ASD.

SUMMARY AND CONCLUSIONS

In summary, we found that the moderate KD was similarly efficacious to the strict diet in improving behavioural symptoms in the EL mouse model of autism. This is particularly important because it suggests that the clinical strength KD, the moderate KD, can be potentially beneficial for children afflicted with ASD. The KD has shown efficacy in treating the core behavioural symptoms of ASD and is an established treatment option for intractable childhood epilepsy. Therefore, future studies are warranted to determine its efficacy in treating core behavioural symptoms in children. In addition, we found that the moderate KD did not reduce blood glucose, one of the hallmark blood chemistry changes associated with the diet, but still caused improved behavioural symptoms. Because of this associated blood chemistry change, it was hypothesized that this change along with increased blood ketones is necessary for the improved behavioural effects of the diet. However, the findings of this study suggest that decreased blood glucose may not be needed but just increased levels of blood ketones. One way to further investigate whether increased ketones and not blood glucose is necessary for autistic behavioural effects would be to have patients elevate their ketone levels through consumption of ketone body esters such as 1,3-butanediol monoester of beta-hydroxybutyrate and glyceryl-tris-3-hydroxybutyrate (Hashim and VanItallie, 2014). Ketone ester-induced hyperketonemia is robust, convenient, and safe, and the ester can be taken regularly as a food supplement without a need to change the habitual diet (Newport *et al.*, 2015). Data collected also showed that the moderate KD resulted in weight gain which indicates an absence of caloric restriction. Previous

studies have showed that the KD best manages epilepsy, which is hypothesized to share a common underlying mechanism with autism, when administered in restricted quantities. However, the findings of the current study suggest that caloric restriction may not be necessary for its beneficial effects in autism without epilepsy.

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