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# The Effects of the Ketogenic Diet (KD) on Inflammatory Pain

Livia S. Wyss
*Trinity College, Hartford Connecticut*, livia.wyss@trincoll.edu

TRINITY COLLEGE

# THE EFFECTS OF THE KETOGENIC DIET (KD) ON INFLAMMATORY PAIN

BY

Livia S. Wyss

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Inflammatory Pain

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Livia S. Wyss

Honors Thesis Committee

Approved:

_____

Susan Masino, Thesis Advisor

_____

Hebe Guardiola-Diaz, Thesis Committee

_____

Sarah Raskin, Director, Neuroscience Program

Date: _____

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## ABSTRACT

BACKGROUND: Pain is the most common ailment around the world, according to the American Academy of Pain Medicine; 100 million Americans suffer with chronic pain, which is more than any other main disorder and is described by more than 60% as impacted their overall enjoyment of life (AAPA). The ketogenic diet (KD) is a high fat, low carbohydrate dietary regimen, which is described to decrease neuronal excitation, increase ketone bodies and ATP levels, while lowering glucose and proinflammatory cytokines. The KD is an effective therapy for epilepsy; a disorder that arises from either lowered inhibition or increased excitation, similar to pain. The goal of the current study is to establish whether the KD is effective in lowering inflammatory pain in a rat.

METHODS: We investigated whether a strict KD decreases inflammatory pain in adult male rats. Rats were maintained on either the KD or a standard diet for two to four weeks. We obtained both physiological measure and behavioral measures before and after being injected in the right hind paw with heat-killed tuberculosis bacteria (CFA) to cause inflammation. Physiological measures included weight, paw volume, paw weight, blood ketone and blood glucose levels. Tactile sensitivity and spontaneous pain was used to assess behavioral pain.

RESULTS: Ketones were increased in rats after the KD. Our results indicate that the KD may alleviate pain, as there were significant changes indicating lowered swelling from the right paw weight. Paw volume indicated a trend leading us to believe that there may be significance if there were more rats. This was similarly seen in the tactile sensitivity, where there was a trend of KD having lowered pain at 4 hours post-injection compared to CD. Spontaneous pain and myeloperoxidase had no significant changes between the KD and CD groups.

CONCLUSION: The data suggest that KD may alleviate pain as there is a lower inflammatory swelling and a trend towards decreased pain sensitivity. Future research will aim to elucidate whether there is an effect of the KD on inflammatory pain by using lower amounts of CFA and earlier spontaneous pain testing.

# INTRODUCTION

## PAIN

### Background

Pain can be described as a sensory and/or emotional experience that results from physical damage such as tissue injury or nonphysical damage such as emotional pain. Pain is often considered a symptom of another illness and, while often true, pain has a variety of causes and is not fully understood mechanistically. Pain has been classified into various categories but most frequently for clinical assessment and treatment it is separated into: *nociceptive, neuropathic,* and *inflammatory pain* (Pain Management, 2010). This classification illustrates the massive scope the term *pain* encompasses. These three categories, defined in detail below, are defined by the location of the area affected. Researchers believe that there is not one mechanism, which encompasses all three types of pain. Rather it is commonly believed that there is a combination of several mechanisms that cause pain, as it is well known that inflammatory mechanisms are involved in neuropathic pain.

Pain is the most common ailment around the world, a recent study states that more than 1.5 billion people suffer from chronic pain while 3-4.5% suffer from neuropathic pain (Global Industry Analysts, 2011). Throughout history there has been a multitude of methods to treat and understand pain, however there has still been no treatment to successfully treat all types of pain. It is necessary thus to find a safe and effective treatment to alleviate individuals suffering with unrelenting or undertreated pain.

**Treatment and Prevalence**

Pain embodies a wide range of conditions including arthritis, migraines, spinal injuries, cancer, neuropathic pain, and others.

According to the American Academy of Pain Medicine, 100 million Americans suffer with chronic pain, more than any other main disorder. The survey study on chronic sufferers revealed that roughly 60% reported that the chronic pain impacted their overall enjoyment of life (American Pain Foundation, 2006). A survey conducted by the American Pain Foundation (2006) on approximately 300 chronic pain sufferers found that more than half felt they had no control over their pain. Individuals with severe pain have trouble concentrating, they report that their energy level is impacted, and many report an inability to get restful sleep (Niels Becker *et al.,* 1997). These individuals run a risk of developing various comorbidities, may not receive pain relief from medication, and due to cost of medication may develop a financial burden. In the United States the total annual cost of health care due to pain was between $560-635 billion in 2010 (National Academies Press, 2011).

Chronic pain is frequently comorbid with other psychiatric diseases most often with panic disorder, post-traumatic stress disorder and depression (McWilliams, Cox & Enns, 2003). Treatment for chronic pain typically includes NSAIDs, opioids, antiepileptic drugs, and antidepressants. The use of over-the-counter and prescription drugs for both acute and chronic pain is very common in Western culture and yet it is less than ideal as it has several downfalls: it can cause tolerance, dependence and addiction, and it does not always provide the relief necessary to live a normal life. Prescription drug abuse has increased substantially from 1998 to 2008, and is

considered to be a major contributor to drug deaths (SAMHSA, 2010; CDC, 2010). Individuals may not respond positively to pain medicine due to genetic mutations that alter their drug metabolism or may suffer from other issues such as addiction or tolerance.

There is a necessity for more research to understand pain. Recent research has linked the ketogenic diet as providing analgesic effects with little or no side effects. Pain affects individuals' in physical, psychological and economical. Finding an effective and efficient way to manage pain is vital to decrease the burden it places on families and society.

**Pain Etiology**

The pathophysiology of pain has not been completely elucidated, however it is hypothesized that pain is linked to over-excited nerves. Over-excited nerves can arise as a result of glutamate-induced excitotoxicity, increased glutamatergic receptors, or decreased GABA or GABA receptors. Individuals with psychological illnesses such as major depression, somatization disorder and hypochondriasis are much more likely to develop a chronic pain disorder. This connection suggests that there is a common factor among these disorders heightening the probability of comorbidity with pain.

Pain typically arises from injury, inflammation and/or disease and is perpetuated by the interplay of stress responses, deregulated injury signaling and negative emotions. Pain can be beneficial when it acts as a warning but can cause a deleterious impact when it is extended. There are multiple types of pain each with commonalities and differences in the way they are derived and understood.

As mentioned above the three common types of pain are *nociceptive, neuropathic* and *inflammatory*. Here each is discussed briefly related to the etiology of pain. *Nociceptive pain* results from exposure to external stimuli, these neurons express a multitude of ion channels. Changes in these channels can affect the processing of pain. *Neuropathic pain* is a result of tissue damage or disease, which can damage nerve fibers causing pain. Sensory processing abnormality is often altered and individuals may report hyperalgesia, increased pain sensitivity, or allodynia, a condition where non-painful stimuli cause pain. *Inflammatory pain* involves the interplay between the immune system and the central nervous system.

Injury, inflammation and disease can all alter neuronal structure by altering neurotransmitters (inter-neuronal chemical messenger), receptors (a protein which in the presence of specific neurotransmitters transmits a signal to a neuron), ion channels (protein which allows for an influx or efflux of ions in the cell), and connections (number of synaptic terminals) (Stucky, Gold & Zhang, 2001). It is hypothesized that pain is either a result from increased excitatory or decreased inhibitory activity (discussed below; Stucky, Gold & Zhang, 2001).

**Inflammatory Pain**

This study will focus on inflammatory pain and thus the rest of this section is dedicated to the etiology of inflammatory pain. Inflammatory pain can result from both endogenous and exogenous stressors. This change in activity alters the release of various mediators including proinflammatory cytokines (IL-1-alpha, IL-1-beta, IL-6 and TNF-alpha), chemokines, reactive oxygen species, vasoactive amines, lipids, ATP, acid, and other factors (Focus on Pain, 2014). These regulators are typically released by

infiltrating leukocytes, vascular endothelial cells, or tissue resident mast cells (Focus on Pain, 2014).

Sensory information arising from an inflammation typically activates afferent neurons; this signal is relayed to the thalamus and brainstem (Kidd & Urban, 2001). In the beginning stages of inflammation, studies have shown that prostaglandins and bradykinin alter the sensitivity of receptors and reduce the excitatory threshold of neurons (Dray, 1995). Long-term changes due to inflammation occur via cytokines and growth factors causing transcriptional changes such as increasing receptors, ion channels and/or neurotransmitters (Woolf & Costigan, 1999). High stimulus intensity due to tissue damage induces substance P, which increases NMDA receptors and is linked to higher activation via glutamate (Kidd & Urban, 2001). Thus, it is believed that the underlying principal in pain is that the threshold for excitation is lowered, producing more neuronal firing. Furthermore, substance P is secreted by nerves and inflammatory cells and ultimately has been shown to have proinflammatory effects in immune and epithelial cells (O'Connor *et al.*, 2004).

Inflammatory pain is typically manifested by pain, heat, redness and swelling (Maroon, Bost & Maroon, 2010). Pain is considered to arise most frequently from inflammation, which is the type of pain we focus on in this thesis. Current pharmacological intervention for inflammatory pain consists of non-steroidal anti-inflammatory drug (NSAID), which are able to interfere with the inflammatory cascade through inhibiting the production of prostaglandin (Talalay & Talalay, 2001). However, NSAIDs medication has significant side effects and finding alternatives to reducing pain and inflammation would be highly beneficial for many individuals. It has long been known that fasting is anti-inflammatory, however, fasting can be extremely hard

and potentially dangerous for individuals to adhere to (Shibolet *et al.*, 2002). The ketogenic diet is a dietary regimen, which has been found to mimic fasting and is suspected to help reduce pain and inflammation, which is further discussed below.

## KETOGENIC DIET

### Background

The ketogenic diet (KD) is a high fat, adequate protein, low carbohydrate metabolic therapy that has been used to effectively treat epilepsy (Danial *et al.*, 2013). The KD was first recognized by the American Medical Association in 1921 in which Dr. Rawle Geyelin presented the story of Dr. H.W. Conklin who treated his epileptic patients with intermittent starvation and found cure rates up to 90% (Bailey, Pfeifer & Thiele, 2004). Wilder and Winter at the Mayo Clinic were the first to suggest that the effects of altering metabolism may be a result from the presence of ketone bodies and proposed a diet with a higher fat and lower carbohydrate content (Bailey, Pfeifer & Thiele, 2004). The KD mimics the fasting diet originally found to alleviate epileptic seizures. This dietary regimen has since proven to successfully lower seizure rates in both children and adults (Freeman, Kossoff, & Hartman, 2007). However, in the 1940s anticonvulsants were introduced, and the popularity of the KD fell out of favor. The KD was difficult to maintain compared to pharmaceutical interventions such as anticonvulsants. Despite challenges in implementing the KD it has remained a powerful treatment for epilepsy even when anticonvulsants fail. Recent studies suggest that the KD may be effective in alleviating both psychiatric and physiological diseases. Research on the KD is still limited, and a large gap remains on understanding the mechanism of

action. However, drugs that treat epilepsy are often prescribed for pain, suggesting that epilepsy and pain may share molecular elements.

**Biochemical Pathway of the Ketogenic Diet**

The KD reduces carbohydrate consumption while increasing fat intake ultimately lowering glucose metabolism and increasing fat metabolism. The entire mechanism of the KD is still unknown, but it is well known that KD allows for the breakdown of fatty acids in the liver which is the exported to extra-hepatic sites including the brain. The mitochondria in the liver converts β-oxidation-derived acetyl-CoA to ketones via mitochondrial thiolase, HMG-CoA synthase, and HMG-CoA lyase (Danial *et al.*, 2013). The ketone bodies (acetone, acetoacetate, β-hydroxybutyrate) provide an alternative energy source (fig.1) (Danial *et al.*, 2013).

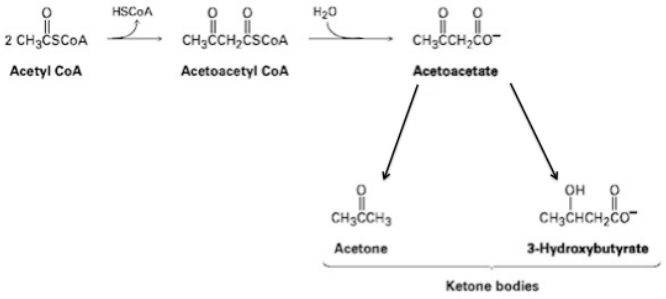



**Figure 1.** Ketone Body Synthesis from Acetyl-CoA

The formation of ketone bodies occurs naturally at a slow level to provide energy to certain organs (particularly the brain) or in fasting or exercise. Ketosis occurs when glucose levels are low, glycogen levels are depleted and thus fatty acids are metabolized. Fatty acids are β-oxidized into acetyl-CoA, which would typically proceed to the tricarboxylic acid (TCA) cycle and would in turn drive the electron transport chain for ATP synthesis. The KD lowers glucose intermediates such as oxaloacetate causing fatty acid levels to exceed the metabolic capacity of the TCA cycle. The

increased level of acetyl-CoA promotes the formation of ketone bodies, noted above are water-soluble molecules that can be used as an energy source throughout the body, including the brain (Masino & Rho, 2012). Ketone bodies are more efficient at making ATP than glucose. Veech (2004) describes that due to a higher heat of combustion in D-β-hydroxybutyrate there is a 28% increase in hydraulic efficiency compared to pyruvate, the end product of glycolysis. Ketones can be synthesized to produce fatty acids in the brain, which provide essential building blocks for biosynthesis of cell membranes and other lipids (Danial *et al.*, 2013). Mechanisms of how the KD is thought to affect the body are described in a later section.

**Dietary Regimen**

The KD is a strict dietary regimen that must be maintained to elicit therapeutic benefits. The "classic" KD is typically described as a ratio of 4:1 of fats to carbohydrates + to protein (Zupec-Kania & Spellman, 2008). Ketosis is typically reached after 72 hours on the diet and when glucose levels are between 55-75 mg/dL and ketone levels range between 4-10 mmol (Zupec-Kania & Spellman, 2008). The "classic" KD uses long-chain triglygerides (LCT), which are 12-18 carbons in length and the predominant fat in the American diet (Bach, Frey & Lutz, 1989). A meal on the "classic" KD may consist of broccoli cooked in butter and ground beef cooked in a cream sauce. The "classic" KD has a very high fat content and can be difficult to adhere to.

The modified KD was introduced as a more palatable alternative to the "classic' KD. The modified KD uses medium-chain triglycerides (MCT), which are composed of 6 to 10 carbons (Bach, Frey & Lutz, 1989). MCTs are hydrolyzed more efficiently in the body than LCTs, producing more ketones per calorie (Kossoff, Zupec-Kania & Rho,

2009). MCTs when metabolized to fatty acids are able to cross into the mitochondrial membrane without the use of a transporter, and thus more quickly converted to fuel (Bach, Frey & Lutz, 1989). The modified KD allows for more proteins and carbohydrates ultimately making it more palatable: a typical meal may be brown rice, a salad with vegetables, and chicken cooked in oil.

Coconut oil is an inexpensive MCT that has been receiving a lot of attention for its multitude of beneficial effects. Furthermore, MCTs have been speculated at enhancing the immune system (Bach, Frey & Lutz, 1989). Kono and colleagues (2000) found that MCTs prevented alcohol-induced liver injury in rats by inhibiting reactive oxygen species and TNF-α. TNF-α is a pro-inflammatory cytokine. Due to reduced fat and increased food choice and variety the modified KD is a more palatable dietary regimen that has been found to be just as affective as the "classical" KD.

Dietary treatments such as the KD have long been used as a strategy to combat various clinical conditions. The KD is predominantly used as a treatment for epilepsy, however evidence is growing that the KD could be effective in treating a wide range of diseases and disorders (table 1) (Kossoff, Zupec-Kania & Rho, 2009).

*Table 1.* Uses of the Ketogenic Diets Published in Literature

(Adapted from Barañano & Hartman, 2008)

| **Human Studies** | **Animal Studies** |
|---|---|
| - Seizures (epilepsy)<br>- Metabolic Defects (PDH and PFK deficency, and glycogenosis type V)<br>- *Reduced Astrocytomas*<br>- *Neurological disorders (AD & PD)*<br>- *Autism*<br>- *Migraines and Narcolepsy*<br>- *Glioblastoma multiforme* | - Tumors Reduction (gliomas, prostate cancer, gastric cancer)<br>- Protects against Truma and ischemia<br>- Neurological disorders (AD & PD)<br>- ALS<br>- *Depression*<br>- *Autism* |

**Table 1.** The table above outlines different disorders that have been studied on the KD in either a human or rodent subjects. The italicized have little concrete research or clinical studies in progress.

The worldwide use of the KD has increased with now 73 academic centers in 41 countries advocating it as a therapeutic approach to various illnesses (Kossoff & McGrogan, 2005).

**Ketogenic Diet mechanism of action**

The mechanism of action in the KD is still poorly understood, but it has been linked to reduced neuronal excitability, described below. It is unknown whether the reduced neuronal excitability is caused directly by an increase in ketone bodies and/or a decrease in glucose or the result of another mechanism (Ruskin & Masino, 2012). The potential mechanisms are outlined below (Table 2). There is likely a complex interplay of different networks including the biochemical and physiological systems consequently it may be a combination of more than one mechanism that provides some of the alleviating affects we have seen in the KD. Once this mechanism is understood there are opportunities of easily accessing the vast benefits of the KD without adhering to the constraints of the diet.

*Table 2.* KD Hypothesis for Mechanisms of Action

| |
|---|
| • *Ketone Hypothesis (Glucose Hypothesis)* |
| • *Adenosine Theory* |
| • *Anti-inflammatory and Lowered Reactive Oxygen Species* |
| • *Metabolic hypothesis* |
| • *Amino Acid Hypothesis* |

**Table 2.** The table above outlines the five most common KD hypotheses.

The KD can decrease neuronal excitation, reduce reactive oxygen species, increase adenosine, produce hypoglycemia, alter inflammatory cytokines and

reduce swelling after experimental inflammation (Merry, 2004; Maalouf *et al.*, 2009). The following section elaborates on these mechanisms and how they may underly effects of the KD.

- The ketone hypothesis predicts that the ketone bodies have a direct anticonvulsant effect (Nylen, Likhodii, & Burnham, 2009). Ketone bodies form in excess under low carbohydrate and increased glucose, thus this hypothesis is closely linked to lowered glucose intake from the KD. Acetone, a ketone body, has been shown to have anticonvulsant effects however the specific mechanism is unknown, it is speculated to act through voltage-gated potassium channels (Masino & Rho, 2012). Juge and his colleagues (2010) recently discovered a link between ketone bodies and neuronal excitability. They determined that acetoacetate, a ketone body, inhibits vesicular glutamate transporters (Juge *et al.*, 2010). Juge and colleagues (2010) found that ketone bodies compete with chloride ions, which act as an allosteric activator on the vesicular-glutamate transporter. These transporters are required for the storage of the excitatory neurotransmitter glutamate in vesicles, for later synaptic release. Lowered synaptic glutamate means lowered neuronal excitability, thereby lowering the occurrence of seizures. Furthermore, both acetone and β-hydroxybutyric acid enhance GABA receptor function, illustrating the there is increased inhibition (Yang *et al.*, 2007). Individuals with depression have too much glutamate in their synapses thus the glucose hypothesis offers a potential therapeutic approach for depression; this link is being researched further (Sanacora, Treccani & Popoli, 2012).

- The adenosine hypothesis predicts that the KD increases adenosine, which inhibits neuronal excitability through adenosine $A_1$ receptors ($A_1$Rs) (Masino *et al.* 2009). Adenosine is a neuromodulatory purine; it has been shown *in vivo* and *in vitro* that KD feeding reduces seizure and seizure like activity via $A_1$Rs (i.e. KD effects were blocked by pharmacological or genetic $A_1$R inactivation) (Masino *et al.* 2011; Kawamura *et al.*, 2014). The *in vitro* studies demonstrated that KD-related adenosine was derived from the break down of ATP (core of the ATP molecule) (Masino *et al.* 2011; Kawamura *et al.*, 2014). This ATP was released from intracellular stores from pannexin channels (Masino *et al.* 2011; Kawamura *et al.*, 2014). The KD is known to increase ATP levels in the brain (Pan *et al.* 1999).

- A low carbohydrate high fat diet was shown to significantly reduce proinflammatory cytokines and reactive oxygen species. Proinflammatory cytokines including TNF-α, IL-6, MCP-1, E-selectin, I-CAM, and PAI-1 were reduced on a KD compared to a low fat diet (Forsythe *et al.*, 2007). Lowering proinflammatory cytokines is beneficial in a vast number of diseases and disorders. Ruskin *et al.* (2009) found that the KD reduces experimental inflammation as measured by paw swelling and plasma extravasation. A diet that forces fats to be metabolized reduces the amount of superoxide produced from the electron transport chain, because the beta-oxidation allows for the by-pass of the mechanism that converts glucose to pyruvate (Veech, 2004). This would ultimately reduce the reactive oxygen species throughout the body (Manninen, 2004). Reactive

oxygen species at high concentrations are detrimental to cells and cause apoptosis, whereas evidence suggests reducing reactive oxygen species is beneficial for the body and mind.

- The metabolic hypothesis proposes that the KD is more efficient at providing energy to the brain (Nylen, Likhodii, & Burnham, 2009). Ketone bodies produce more ATP per unit than glucose (elevated ATP production reactive oxygen species, which actually argues against the anti-inflammatory hypothesis above). Furthermore Bough et al. (2006) reported that the KD increases the number of mitochondria in a rat. Many neurological disorders have dysfunctional mitochondria, and thus the KD, which increases their number and efficiency, measured via electron microscopy, with the extra energy from ketone bodies, is ultimately able to improve metabolic function and provide therapeutic relief.

- The fifth hypothesized mechanism is the amino acid theory, which suggests that the KD modifies the balance of amino acid neurotransmitters in the brain. When the brain changes from glucose to ketone body metabolism the ratio of glutamate to GABA decreases, increasing GABAergic inhibition.

Overall the KD is postulated to decrease neuronal excitation by increasing ketone bodies and ATP levels and lowering glucose and proinflammatory cytokines. The KD was also found to increase adenosine and GABA, both inhibitory neuromodulators, and decrease glutamate, an excitatory neuromodulator (Masino and Geiger, 2008; Omote *et al.*, 2011; Juge *et al.*, 2010).

**PROPOSED CONNECTION- Ketogenic Diet and Pain**

As noted, the KD is thought to increase inhibition and/or decrease excitation. The KD is an effective therapy for epilepsy; a disorder that arises from either lowered inhibition or increased excitation, similar to pain. Studies suggest that the KD can be used for pain relief and lowering inflammation. The following evidence described below illustrates converging evidence of the likely effects of the KD influencing pain and inflammation. Masino and Ruskin (2013) describe four postulated mechanisms of the reasoning behind the therapeutic affects of the KD and pain and inflammation *(table 3)*.

*Table 3.* Ketogenic Diet, Pain and Inflammation: Postulated Mechanisms (Masino & Ruskin, 2013)

1. Reduces excitatory and/or increases inhibitory mechanisms
2. Fewer reactive oxygen species has anti-inflammatory effects
3. Adenosine has anti-inflammatory effects
4. Reducing glycolytic metabolism is analgesic

Masino and Ruskin (2013) highlight studies relating high fat and low carbohydrate diets to the perception and inflammation of pain; together these studies suggest a link between these two areas, consistent with postulated mechanisms. These are discussed in detail below.

- Epilepsy and pain have been described as complex conditions that have diverse underlying conditions but both seem to have overexcited nerves.

It is presumed that a treatment that can reduce the excitability of nerves would be a good therapy for pain. Anticonvulsants drugs are often used for neuropathic pain, further exemplifying that if the KD decreases excitatory or increases inhibitory mechanisms in epilepsy then it could produce the same affect in pain (Masino & Ruskin, 2013).

- Reactive oxygen species are a major component of inflammation, and as noted previously the KD reduces reactive oxygen species. Exogenous ketones were shown to reduce reactive oxygen species both *in vitro* and *in vivo* (Kim, Vallejo & Rho, 2010; Masino & Ruskin, 2013). Thus, a diet such as the KD that alter the metabolism and creates increased levels of ketones could be expected to reduce reactive oxygen species. Reactive oxygen species are linked to numerous diseases and disorders, specifically Wiseman and Halliwell (1996) describe that reactive oxygen species have been increasingly linked to inflammatory disease as well as cancer. The KD was found to reduce reactive oxygen species in the brain and ultimately reduce inflammation in a model of multiple sclerosis and in individuals with liver disease (Kim *et al.*, 2012; Tendler *et al.*, 2007).

- Adenosine levels are increased on the KD and adenosine has long been implicated in reducing inflammation and pain, by inhibiting neuronal excitability through the $A_1$Rs (Masino *et al.*, 2009). Schmidt *et al.* (2009) determined, that allopurinol, a non-selective adenosine-receptor that increases adenosine levels induced anti-nociception.

- Reducing glycolytic metabolism is hypothesized to be another mechanism that provides a decrease in excitatory and/or increase in inhibitory

mechanisms. One study found that children on a MCT diet who had their seizures under control when given an infusion of glucose their seizures quickly returned (Huttenlocher, 1976). As discussed previously, pain is related to overly excited neurons and thus if reducing glucose is inhibitory it is expected to lower pain Furthermore when 2-deoxy-D-glucose, which blocks glycolysis, was found to have anticonvulsant effects (Bodnar *et al.*, 1979).

The described mechanisms above suggest a link that should be further analyzed between the KD and different types of pain. Our lab found that an acute model of pain using thermal pain had an increased thermal pain sensitivity when on the KD compared to control rats (Ruskin *et al.*, 2013). These effects were reversed once the KD rats were placed on the control diet (Ruskin *et al.*, 2013). Furthermore, our lab has studied the relationship between the KD and inflammatory pain. One study used local inflammation into the hindpaw with complete Freund's adjuvant to examine swelling and plasma extravasation in juvenile and adult rats (Masino & Ruskin, 2013). Juvenile rats had significantly reduced levels of these two types of inflammation when on the KD; these results were less but still significant in adults (Masino & Ruskin, 2013). Another study on neuropathic pain used the antimitotic cancer drug in which rats were on the KD prior to injection (Masino & Ruskin, 2013). The study found reduced mechanical pain sensitivity on rats fed a strict 6:6:1 diet at the 8-day time point (Masino & Ruskin, 2013). Another study by Piomelli and Sasso (2014) found that an increasing number of lipid molecules are able to suppress the inflammatory process, restore homeostasis in damaged tissue, and attenuate pain sensitivity by

regulating the peripheral nervous system. One study regarding pain and the KD showed a strong trend towards the KD alleviating pain (Yancy *et al.,* 2009). This research suggests that there should be more research to determine whether a strong relationship exists between different types of pain and the KD.

Overall pain is a prevalent and challenging disorder and there are many individuals with unrelenting or unmet need for treatment of pain and inflammation. The KD or a metabolic therapy would be a platform that individuals all around the world could use to tackle their pain. Here we study this intersection between these two fields of research and test the effects of a KD on inflammatory pain.

## Thesis Overview and Hypothesis

The current study aims to test the hypothesis that a ketogenic diet will reduce inflammatory pain. This study will help gather evidence whether the ketogenic diet could be used as a therapy for individuals experiencing pain and reducing inflammation. In the present study we use both behavioral and inflammatory markers to analyze rats on a strict ketogenic diet versus rats on a normal chow after they are injected with CFA (complete Freund's adjuvant) in their right hind paw to cause persistent inflammation.

We expect one of two scenarios: inflammation and inflammatory pain are lowered or only inflammatory pain is lowered. If inflammation and inflammatory pain were lowered on the ketogenic diet, it suggests that the ketogenic diet reduces inflammatory pain by reducing inflammation. Alternatively, if the behavior indicates less pain but there is no significant change in the inflammation, it would suggest this alleviation occurs through an anti-nociceptive mechanism.

# METHODS

## Ethics Statement

Procedures were followed according to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of Trinity College (A3869-01).

## Animals and Method Overview

Male Sprague-Dawley rats were fed a standard chow diet until eight weeks of age. Two to three rats were housed together in cages with water and enrichment toys and kept on a 12 hr light-dark cycle. At eight weeks, half the rats were switched onto a ketogenic diet (KD) (F3666; BioServ, Frenchtown, NJ) for two or four weeks while the other half remained on a standard chow diet (SD). The KD was a "classic" research formulation with a ketogenic ratio of 6:1. All testing occurred two or four weeks after the dietary treatment, at which point rats were 10-16 weeks of age.

Behavior tests consisted of spontaneous pain and tactile sensitivity and were conducted before and after being injected in one hind paw with heat-killed tuberculosis bacteria (CFA) that causes persistent inflammation. Weight and paw volume were recorded throughout the dietary regimen. To verify ketosis glucose and ketone levels are measured via tail blood and trunk blood respectively. Upon sacrificing, two days post injection, blood and paws are collected to assess inflammation. Rats were tested for five consecutive days in the order illustrated (*figure 2*).

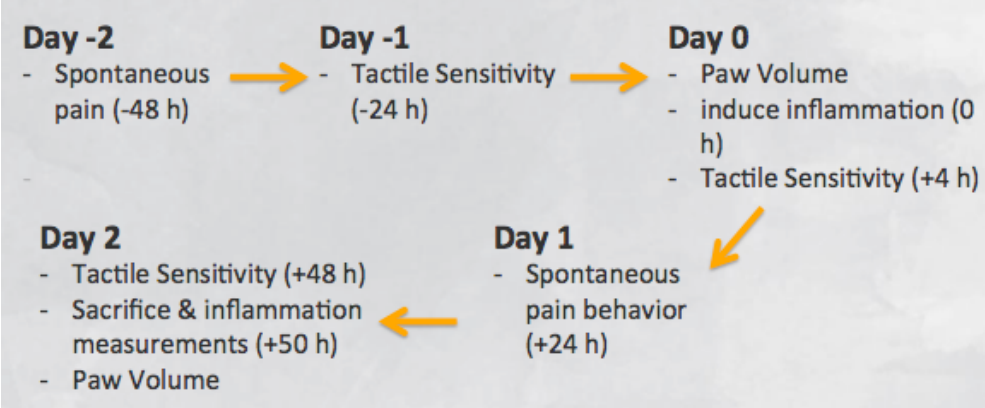
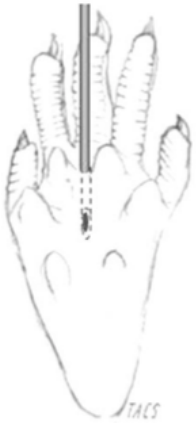



*Figure 2. Testing Schedule*

## Injection

*Complete Freund's Adjuvant (CFA)*



All rats were subjected to 100uL of Complete Freund's Adjuvant (CFA) (thermo scientific) in the right hind paw to induce the inflammation. CFA as mentioned previously is a heat-killed tuberculosis bacteria that causes persistent inflammation.

## Behavior Tests

*Spontaneous Pain*

Spontaneous pain was assessed using the spontaneous foot-lifting model. Rats were habituated to the testing room for fifteen minutes prior to being placed in an empty plastic cage for thirty minutes. The behavior test was recorded by a video camera from underneath the cage. In a blinded analysis of the video each hind paw was separately scored on a scale of 0-5 at 10, 15, 20 and 25 minutes for

one minute.

*Table 3. Scoring Paradigm for Spontaneous Pain*

| | |
|---|---|
| **0** | paw pressed normally on floor |
| **1** | paw rests lightly on floor, toes ventroflexed |
| **2** | only internal edge of paw pressed to floor |
| **3** | only heel pressed to floor, hind paw in inverted position |
| **4** | whole paw is elevated |
| **5** | animal licks paw |

If there was locomotion or grooming at the time intervals listed then the time scored was delayed. The spontaneous pain baseline measurement was at 48 hours prior to the CFA injection, and compared to the 24 hours post injection. (Djouhri *et al., 2006).*

*Tactile Sensitivity - Electrical*

Nociceptive pain was quantified with an electrical measure of pressure using the electronic von Frey (Almemo 2390-5, IITC). The device recorded the maximum amount of pressure applied to each hind paw. This was repeated to achieve two recordings, which were averaged.

## Physiological Measures

*Paw Displacement*

Paw volume was measured prior to the CFA injection and at 48hours, just prior to sacrifice. The paw volume was measured via water displacement using a digital analytical balance (Mettler-Toledo) to determine the difference. Paw

volume provides a measurement on the level of physical inflammation of the paw.

*Procedures at Sacrifice*

Animals were sacrificed at 48 hours post CFA injection; the rats were anesthetized by being exposed to isoflurane in a large glass container. The blood glucose level was measured from tail blood by puncturing the tail with a hypodermic needle, while ketone (beta-hydroxybutyrate) level was measured from trunk blood after decapitation. The blood glucose and ketone levels were analyzed using a Precision Xtra glucose/ketone meter strips (Abbott Laboratories, Bedford, MA).

Trunk blood was collected and centrifuged to isolate the plasma and frozen for later analysis on the inflammatory markers present. After sacrifice the hind paws are amputated at the ankle joint. Paws are weighed on a digital analytical balance (Mettler-Toledo). Hindpaw pads are dissected, frozen in liquid nitrogen and crushed, and sonicated and repeatedly freeze/thawed to disrupt all membranes. After centrifugation, supernatant was tested with an enzyme assay for myeloperoxidase, an enzyme contained in neutrophils, the most common type of leukocyte. The change of absorbance at 460nm was measured spectophotometrically using the myeloperoxidase assay (Bradley *et al*. 1982). The assay combined 0.1mL of the supernatant to 2.9mL of 50mM phosphate buffer, pH 6.0, which contained 0.167 mg/mL o-dianisidine dihydrochloride and 0.0005% hydrogen peroxide (Bradley *et al*. 1982).

## Statistical Analysis

Three independent researchers with two of the three blinded to the diet-treatment, scored the spontaneous pain. Statistical tests were performed using t-tests and ANOVA, except in cases of non-normality where we used Wilcoxon test. There were no statistical differences in paw weights between rats that received two weeks of diet treatment to those who received four weeks, thus the data was collapsed across these two groups. $P<0.05$ was considered significant.

# RESULTS

Rats underwent physiological measures (ketone and glucose levels, weight, and leukocyte levels) as well as behavioral tests (tactile sensitivity and spontaneous pain) to determine whether there was a difference in inflammatory pain between KD and CD rats.

## Physiological Results

### *Blood Analysis: β –hydroxybutyrate (ketone) & Glucose*

Trunk blood was collected after decapitation and the *β- hydroxybutyrate* levels were determined by Precision Xtra meters. β-hydroxybutyrate is a ketone that indicates ketosis, and was found to be significantly higher in the KD group compared to the CD group (Fig. 3A). The glucose levels between the two diet-groups had no significant difference (Fig. 3B). In prior studies and unpublished data we have noticed that as rats age and the longer they adhere to the KD diet the more comparable their glucose levels are to rats on a CD, often where they difference is insignificant. This data highlights that the rats were in ketosis but due to their age on diet onset there is often no change in glucose levels.

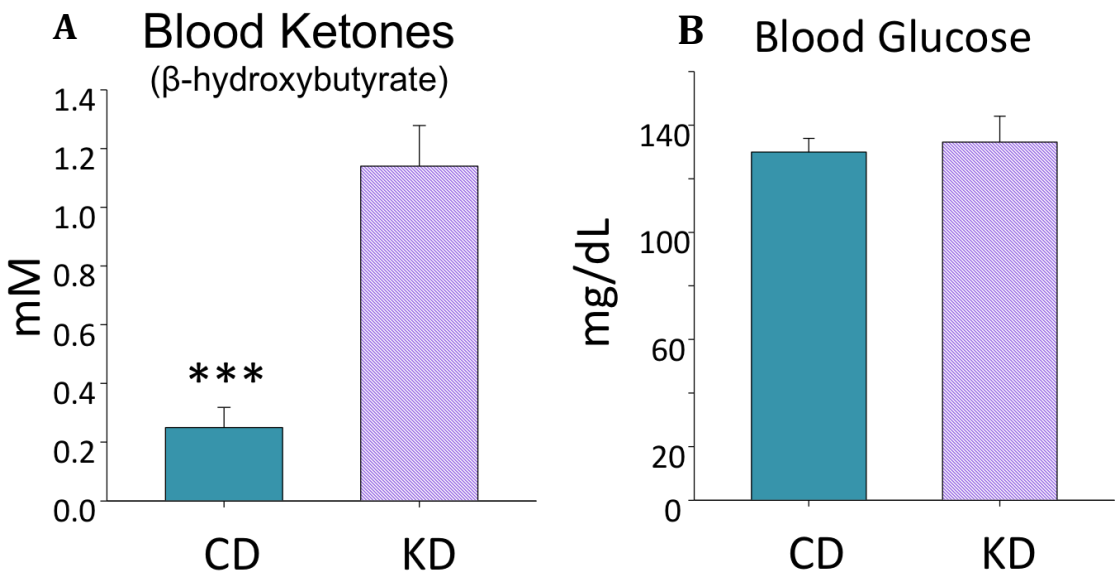



**Figure 3. (A)** KD rats had significantly higher levels of β-hydroxybuturate than the CD rats ($p<0.001$). **(B)** Glucose levels were not significantly altered in either group. (Male rats, KD n=14; CD n=10 here and in every subsequent figure). Measurements were made as described in methods.

### *Body Weight*

Rats were weighed prior to the CFA injection, if there was a significant difference between the body weights of the KD rats versus the CD rats that would need to be taken into account for analysis. As depicted (Fig 4) there was no significant difference between the body weights and thus the dose of CFA for the rat size is consistent as is the paw volume.

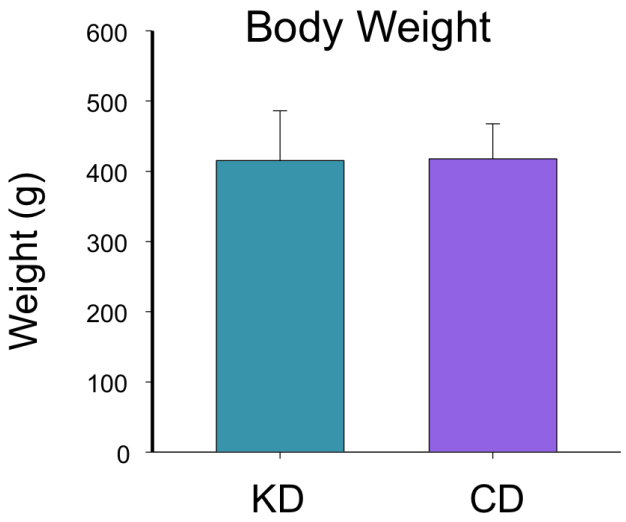



**Figure 4.** There was no significant difference in body weight of rats on the CD versus KD diet at the time of CFA injection.

### *Paw Weight*

Paws were obtained and weighed on the day of sacrifice, at 48 hours post CFA injection. The KD right paws weighed significantly less than the CD (Fig. 5). Left paws showed no significant difference between the two diet groups (Fig. 5).

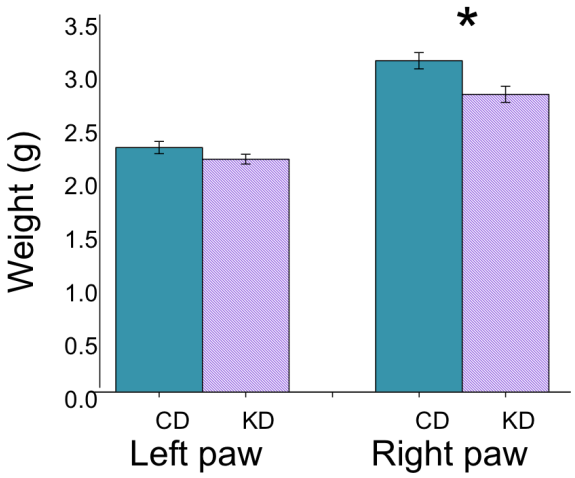



**Figure 5.** Rats fed the KD had significantly higher right paw weights than CD ($p<0.05$).

*Paw Volume*

Paw volume was measured using water displacement prior to CFA injection, -24 hours, and post CFA injection, +48 hours. A trend indicating that KD had less swollen paws is illustrated; however it did not quite reach significance (Fig. 6A). There was no difference found between the left hind paw of both diet groups suggesting that the difference is not due to size of the rats but due to the KD treatment (Fig. 6B).

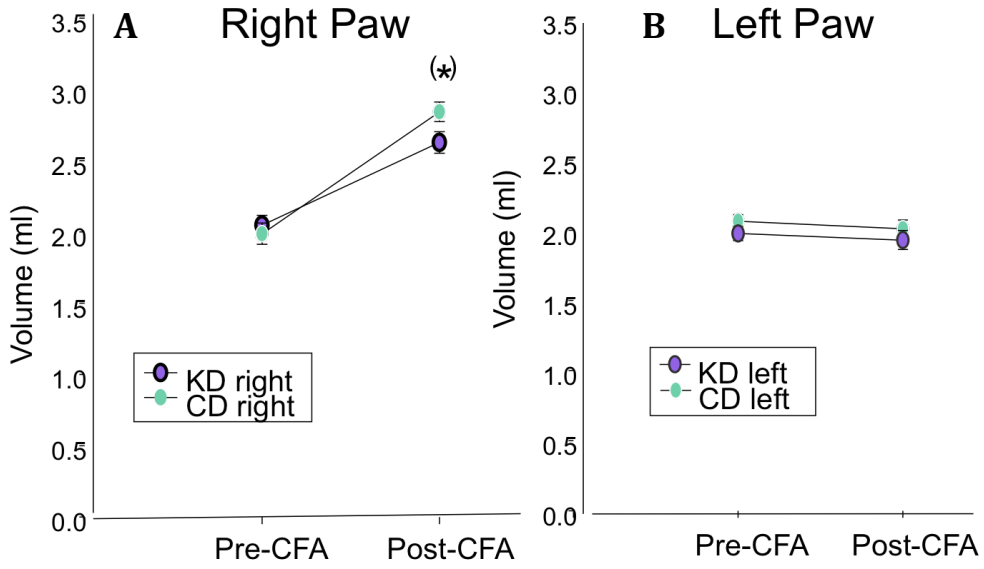



**Figure 6. (A)** KD rats had slightly lowered paw volume at 48 hours post CFA, the level was not significant (p=0.055). **(B)** No difference in paw volume between KD and CD.

*Leukocyte Levels*

Leukocyte levels where measured using the myeloperoxidase assay. There was no significant difference between the KD and CD in the right paw (fig. 7).

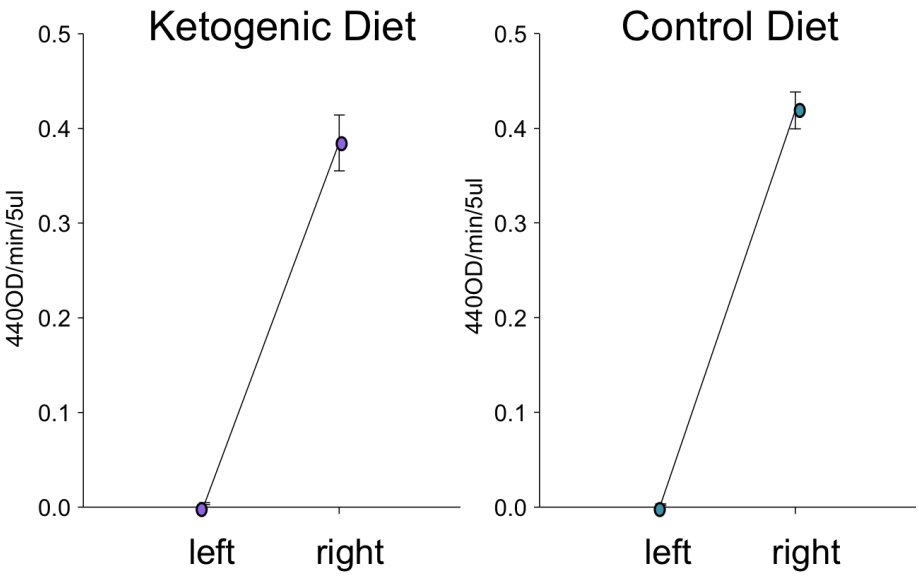



**Figure 7.** No significant difference between the left and/or right paw between the KD and CD groups in myeloperoxidase.

## Behavioral Results

### *Tactile Sensitivity*

Rats went through tests of tactile sensitivity before and after being injected in the right hind paw with heat-killed tuberculosis bacteria to cause inflammation. Tactile sensitivity using the electronic von Frey probe did not differ between treatment groups before injection, but was strongly increased at 4 hours post-injection in both treatment groups. In the inflamed paw (right) there was a lowered amount of pressure that the KD rats could withstand compared to the CD rats. At 48 hr post-injection, sensitivity was no longer significant between the KD and CD rats (fig 8A). There were no significant differences in tactile sensitivity between the KD and CD in the left paw over the three time periods (fig 8B).

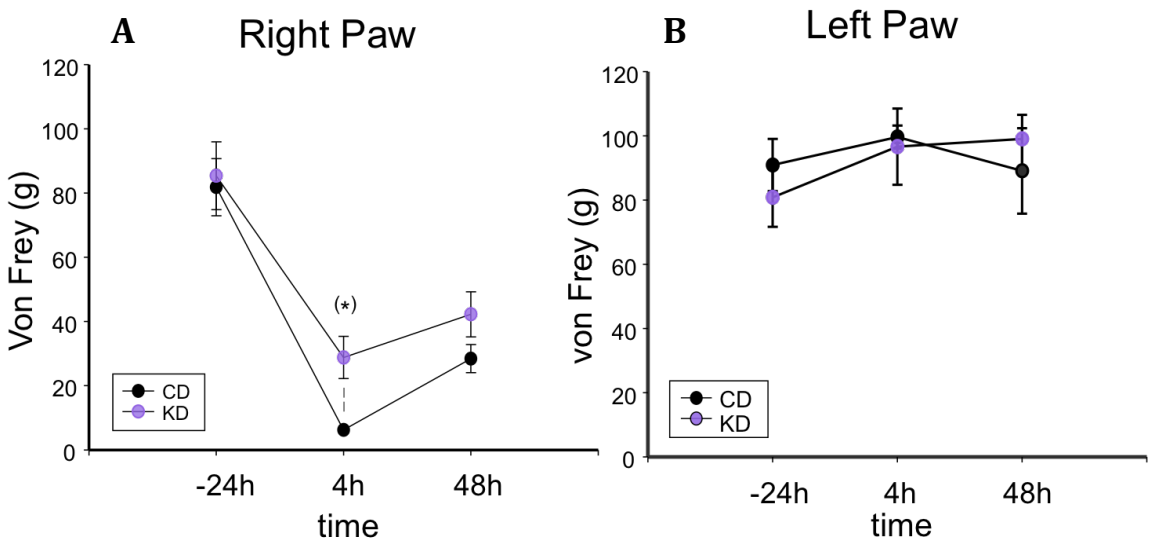



**Figure 8.** There was no significant difference in the tactile sensitivity prior to the CFA injection. **(A)** At 4hr post CFA injection there was a trend for the KD rats to be able to withstand more pressure to the inflamed paw (right) than the CD rats (p=0.053). **(B)** Left paw showed no significant changes in response.

***Spontaneous Pain***

Spontaneous pain data illustrates an expected change between each specific diet group and the pre and post CFA for the right paw (Fig 9A & 9B). This significance illustrates that the CFA was causing a severe inflammation in the right paw that debilitated the rats' usual motor movement. However, there was no significant difference across the groups for both prior and post CFA injection (Fig. 9A & 9B). There was no significance between the left paw treatments (Fig. 9C & 9D). The graphs illustrate the distribution of scores using a box and whiskers plot. The top and bottom lines indicate the range of scores, while the second line from the top and second line from the bottom illustrate the first and third quartile of the data set. The average is the centerline in each box plot.

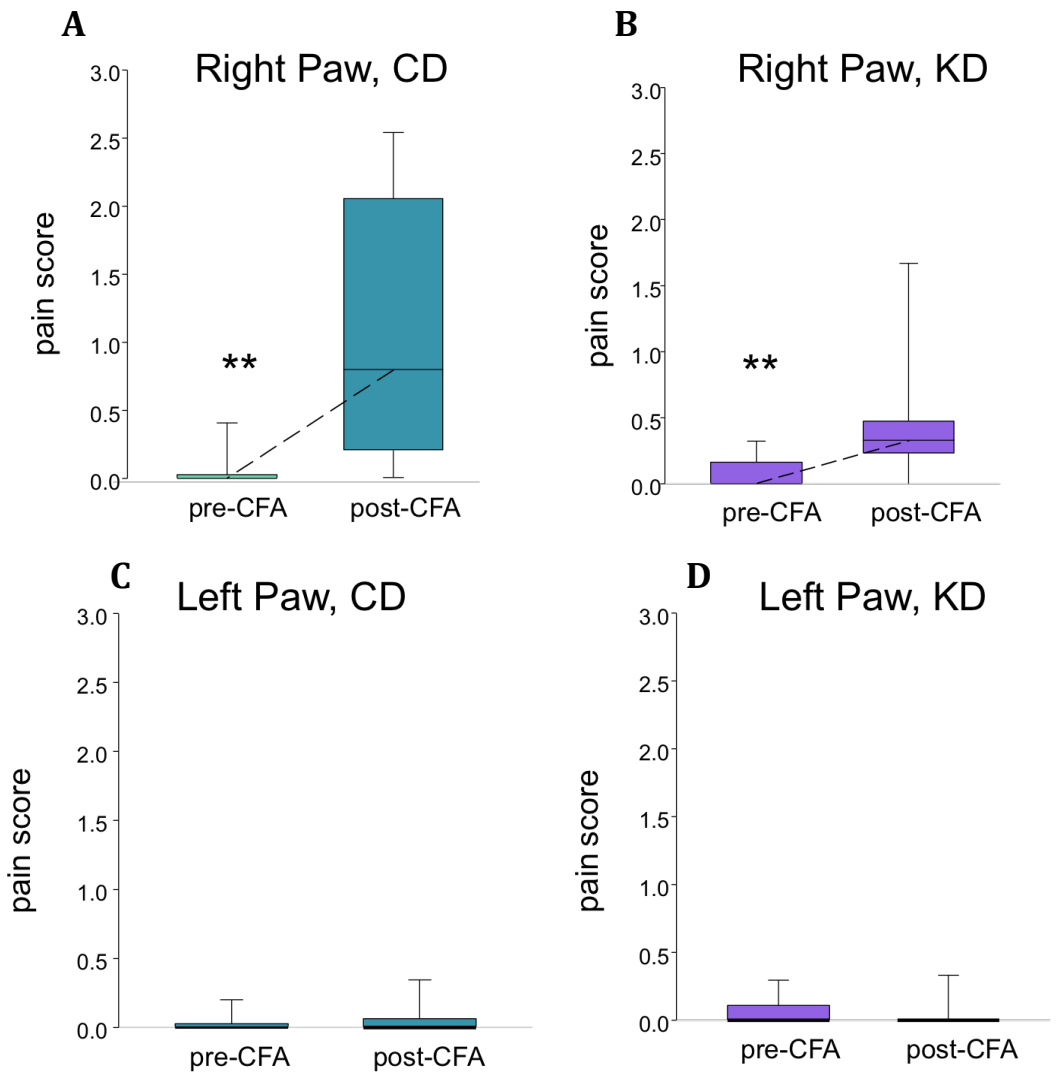



**Figure 9.** There was no significant difference in the spontaneous pain between KD and CD in either the right paws **(A & B)** or between the left paws **(C & D). (A)** There was a significant difference between pre-CFA and post-CFA in the right control paw ($p<0.01$). **(B)** Similarly there was a significant difference seen between pre-CFA and post-CFA in the right control paw ($p<0.01$). **(C & D)** No significance seen in the left paw between the pre-CFA and post-CFA in either treatment group.

# DISCUSSION

The effects of the KD on inflammatory pain were analyzed using physiological and behavioral techniques. The KD feeding promotes recovery after inflammatory insult, however spontaneous pain did not illustrate a reduction in nociceptive pain. Paw weights and volumes suggest that the paw is less swollen and healing more quickly in rats fed a KD.

## Physiological Effects

*Ketone, Glucose and Body Weight*

Our study found significant changes in the impact of KD on ketone levels (fig.3A). This was expected, since the rats had been on the KD diet two to four weeks. Glucose levels were not significantly altered (fig.3B). Masino and Ruskin (2013) report a similar finding that rats had lowered blood glucose shortly after starting the KD but at ten weeks there was no noticeable difference in blood glucose levels between the KD and control. Previous studies indicate that there are strong effects of the KD even when glucose is not significantly different (Masino & Ruskin, 2013; Ruskin *et al.*, 2013). Since the body weight was consistent between the CD and KD (fig. 4), their paw displacement and paw weight is also comparable to determine whether there was a difference of inflammation due to diet.

*Paw Weight and Paw Displacement*

Paw weight illustrates a correlation between lowered paw weight and the KD (fig. 5), which suggests that the KD reduces swelling in the right hind paw.

Paw displacement almost reached significance (fig. 6) further suggesting that the KD reduces swelling in the right hind paw. The body weight as mentioned previously was not significantly different between the two diet groups, and thus we can attribute this difference to paw volume and paw weight, and not to body size. Paw size and paw weight depict the amount of swelling that occurred, the trend towards lowered paw size and paw weight illustrates the KD may reduce inflammation. These results are consistent with a previous study from our lab, which found reduced inflammatory swelling and pain in juvenile and adult rats on the KD. The specific study conducted by Ruskin *et al.* (2009) triggered nociceptive pain and found a decrease in hind paw swelling in the KD compared to the CD.

Our lab performed a similar study previously using CFA; only hind paw swelling and plasma extravasation was analyzed in both juvenile and adult rats (Masino & Ruskin, 2013). Masino and Ruskin (2013) found a larger significance between the juvenile rats compared to the adult rats. The results from this experiment may have not reached significance but based on this previous study we cannot assume there was no effect.

*Leukocyte Levels*

Leukocyte levels were expected to be elevated in the KD right hind-paw compared to the CD right hind-paw (fig. 7). A stronger anti-inflammatory response is correlated with higher myeloperoxidase levels (Bradley *et al*. 1982). The effect in myeloperoxidase levels between the groups because the CFA dose might have been too high creating a ceiling effect that the KD could not rescue

the inflammation. Trying a lower dose would help elucidate whether there is stronger anti-inflammatory response in one diet group over the other.

## Behavioral Effects

*Tactile Sensitivity – Electronic Von Frey Probe*

The responsiveness to tactile pressure was measured using an electronic von Frey probe. There was a trend towards reduced nociception four hours post CFA injection in the KD rats, as indicated by the very low pressure scores in tactile sensitivity (fig. 8). Suggesting that the KD may help reduce pain after inflammatory insult, but more testing would need to be performed. The trend seen at four hours did not continue at 48-hours post CFA injection (fig. 8). It is likely that the inflammatory pain is too strong and that a dietary treatment is not able to cause a substantial difference in such a strong effect, thus we suggest lowering the CFA dose in future experiments.

It is important to note some of the caveats with the electronic von Frey. Prior to testing the tactile sensitivity with the electronic von Frey, we attempted to quantify nociceptive pain using manual force of a von Frey hair. Six probes with forces of 2.5, 5.93, 7.79, 21.92, 37, and 51.7g were applied to determine the withdrawal reflex (Data not shown). Due to this sequential measure of tactile sensitivity using a mechanical von Frey it is suspected that there was likely increased sensitivity in both the manual and electronic von Frey measurements. Increasing the amount of time between hind paw pokes may decrease the variability and the risk of becoming sensitized. If we assume that this probing

occurred regularly to the same degree and strength on each paw and animal, another caveat that should be considered is the location of the pressure on the paw. The location would alter the pressure; although the sensitivity was meant to be at the same place each time there will be some variance. Furthermore, another caveat is that depending on how the rat is positioned the pressure needed to lift the paw varies and thus a lighter pressure may be recorded that is not dependent on pain.

Many of the caveats listed above are problems inherent in manual and electronic von Frey testing. However, in future studies we will likely remove manual tactile sensitivity, as results were highly variable likely due to increased sensitivity. The electronic von Frey seemed to give more consistent measures of force.

*Spontaneous Pain*

Spontaneous pain was filmed and later analyzed on a five-point scale depending on the movement of each hind paw. Within the KD there was a significant difference before and after the CFA injection, similarly seen in the CD before and after the CFA injection, exemplifying that the CFA produced a strong inflammatory response (fig. 9). The lack in significance may result from the difficulty of differentiating between the criteria between the scores in the middle. The scoring criteria for 2, 3 and 4 on the spontaneous pain measure differentiated between the areas in which pressure was placed on the paw. Distinguishing the location of pressure may have caused the scoring to not strongly differentiate between the scores for the different diet groups. The results had many zeroes and overall the pain scores were relatively low, which caused the graphs to have their

distribution close to the x-axis. Finding a clearer way to discern the spontaneous pain could illustrate new significance in further studies. Furthermore we suggest an earlier time point, as there were more significant trends seen closer to the CFA injection. In the future a time point at roughly 6 hours post CFA injection should be tried.

**Effects of the KD on Inflammatory Pain**

Rats were in ketosis, and any effects seen that differed between the KD and CD rats could be attributed to the effects of the KD. Our results indicate that the KD may alleviate pain, as there were significant changes indicating lowered swelling from the right paw weight. Adding more rats to this study could alter the trend to a significant difference between the KD and CD. Specifically, in paw displacement and tactile sensitivity we saw a strong trend that may reach significance with added rats.

Though the data on nociception is not quite as definitive, there is a trend towards the KD decreasing nociception. Our lab aims to repeat this study by adding more rats and altering some of the tests. As mentioned previously we believe lowering the amount of CFA and pushing the spontaneous measurement forward in the time should be tried in the future. It would be interesting to look at juvenile rats, as Ruskin *et al* (2009) found more consistently significant differences in pain in the juvenile KD compared to the adult KD.

It is likely that multiple mechanisms account for these changes in nociception and inflammation, highlighted in the introduction. Our results more strongly support a inflammatory mechanism as we see that swelling is reduced

through the paw weight and paw displacement than a nociceptive mechanism as spontaneous pain had no change, and tactile only had a trend towards significance.

Of the postulated mechanisms of the KD to influence pain (or seizures) these data do not disprove most of them. For instance here we have not measured GABA levels, vesicular glutamate uptake, mitochondrial profiles, etc. We do disprove the glycolytic hypothesis, because of the KD effects that are present with a lack of glucose. Our results do seem to support the anti-inflammatory hypothesis regarding how the KD might be anti-convulsant or anti-epileptic.

# CONCLUSION & FUTURE DIRECTIONS

The work included in this thesis demonstrates a trend that the KD may lower inflammatory pain.

Our data illustrated that the KD rats had a lowered inflammatory pain at four hours post inflammatory insult in the tactile sensitivity measure. There was no reduction in nociceptive pain as there were no significant differences in the spontaneous pain measure. Physiological measures indicated that rats were in ketosis and appeared to have lowered paw volume and weight while body weight did not alter suggesting that there may be a reduction in swelling in the KD right hind paw. Increasing the number of rats could result in significance in some of the tests that had close significance.

In an attempt to further discern whether the KD may aid in pain relief, other models of pain should be studied. An arthritic model or an inflammatory bowel disease model could be used to further study the effect of KD on inflammatory diseases. Studying a different diet such as an MCT diet, which has been shown to be easily metabolized and therapeutically effective in epilepsy could be very beneficial to determine whether there are stronger effects than seen in the KD. Another dietary regimen that should be pursued is calorie restriction, which is anti-inflammatory (Shibolet *et al.,* 2002).

The present results suggests that more data is needed to determine whether the KD can help alleviate pain and reduce inflammation, however taken together with previous studies it offers a promising avenue for pain alleviation that should be pursued.

Clinical work with the KD is still very minimal but could offers a wide set of benefits as there are no side effects and very minimal cost. Alleviating pain through an effective and non-pharmaceutical approach is extremely valuable for millions of individuals suffering from either chronic pain or who would like to use alternative medicine. Thus, focusing research on alleviating pain through a dietary regimen or metabolic strategy could be extremely advantageous.

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