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Metabolic Alteration Induced by Time Restricted Feeding at Different Points in the Circadian Cycle

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METABOLIC ALTERATION INDUCED BY TIME RESTRICTED FEEDING AT DIFFERENT POINTS IN THE CIRCADIAN CYCLE

by

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ABSTRACT

The goal was to determine if a high fat diet given in a time restricted fashion can protect against obesity and inflammatory mediators. 12 week old C57BL/6 male mice were assigned to either a standard chow diet or a diet containing 45% fat. The high fat diet was provided *ad libitum* or in a time restricted fashion, either during the first or the second half of the active phase for 8 weeks. Time restricted meal intake reduced body mass gain, adiposity, glucose intolerance and cholesterol levels in mice fed a high fat diet. Mice fed a high fat diet, even on a time restricted meal pattern, resulted in increased tumor necrosis factor-α, fatty liver development and adipocyte hypertrophy suggesting time restricted feeding can protect against obesity, but does not protect against inflammation. These results suggest that a high fat diet can induce inflammation even in the absence of weight gain.

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Background

Obesity is a major risk factor that affects many people worldwide and contributes to a multitude of diseases including type 2 diabetes, nonalcoholic fatty liver disease, cardiovascular disease and cancer.[1] Many interactions drive this complicated and multifactorial condition and can result from predisposing genetic and metabolic factors, cultural influences, and an ever changing food supply and increasing sedentary environment.[2] Development of obesity is often followed by disruptions of multiple functions affecting many different organ systems, including those that regulate glucose, lipids, cholesterol, and amino acid balance. These disruptions can lead to an increased risk for the development of metabolic syndrome (MetS).[1,3]

MetS is generally associated with central obesity, hypertension, elevated fasting plasma glucose, insulin and cholesterol levels, hypertriglyceridemia, and high density lipoprotein (HDL).[2] For MetS diagnosis, one must exhibit 3 of the following 5 risk factors; abdominal obesity measured in waist circumference (men > 102 cm and women >88cm), triglyceride levels above or equal to 150 mg/dl, HDL cholesterol in men below or equal to 40 and women below or equal to 50, blood pressure more than 130/85 mm/hg and fasting glucose above or equal to 110 mg/dL.[4] Although there are many comorbidities associated with MetS, it is typically associated with patients with an excess of intra-abdominal or visceral adipose tissue.[5]

In the United States, the prevalence of obesity continues to skyrocket with recent reports indicating over 64.1% of American women and 72.3% of American men are categorized as overweight or obese.[6] According to the World Health organization, between the years of 1980 and 2008, obesity worldwide nearly doubled.[7] Based on the

current obesity trend, it is projected that there will be 65 million more obese adults in the United States in 2030.[7]

The prevalence of obesity is due in part to the overconsumption of saturated fat, refined carbohydrates, and low fiber diets.[8,9] Additionally, a sedentary lifestyle is an emerging risk factor for obesity in both adolescent and adult populations. Lee et al. demonstrates that a pediatric sedentary lifestyle correlates to the development of adolescent MetS and elevated child BMI, which could contribute to future obesity in that individual's life. Encouraging the public to reduce this sedentary lifestyle is important and can indirectly aide in the reduction of adiposity in the growing population.[10]

Obesity is associated with many metabolic disruptions and can contribute to an imbalance in inflammatory mediators, one being adipokines. The adipokine Leptin is highly associated with increased adiposity. Leptin is also known as the satiety hormone and functions to regulate energy homeostasis and inhibits appetite and food intake when energy needs are met.[7] Systemic leptin levels increase in proportion to the amount of body fat.[11] In the obese population, constant response to elevated leptin leads to leptin resistance, which in turn leads to the inability to reduce food intake.[11] Leptin also functions as an immune modulator and plays a key role in mediating the pro-inflammatory molecules, specifically in overweight individuals.[6]

Accumulating evidence demonstrates that oxidative stress is present in obese individuals; some of these indications include elevation of biomarkers for reactive oxygen species (ROS) and decreased antioxidant levels.[6] Oxidative stress is a general term that suggests signs of cellular damage caused by an imbalance of antioxidants relative to the amount of reactive oxygen species.[6] Persistent and elevated oxidative

stress leads to inflammation, endothelial cell proliferation and apoptosis, and increased vasoconstriction.[6] Therefore, oxidative stress can damage cellular structures with decreased antioxidant protection, leading to the development of complications associated with obesity.[12]

Inflammation is the body's response to injury to restore tissue homeostasis, structure and physiological function. What starts as acute inflammation can turn into chronic if left unresolved. Persistent low grade inflammation, as seen in obesity, predisposes tissue to cancer development and contributes to many chronic illnesses.[13] Additionally, MetS induces inflammation in the body that leads to a weakened immune system which increases the likelihood of becoming ill.[14,15]

Inflammation helps protect the body by fighting off foreign invaders introduced into the body, whether it is diet induced or by physical insult. Highly complex control elements help control the inflammatory response. During obesity, cell stress leads to the increase in the release of pro inflammatory cytokines resulting in the low-grade inflammation associated with obesity.[16]

In obese individuals, adipose tissue is represented by low-grade inflammation and increased secretion of cytokines.[17] Two cytokines that are frequently seen in obesity-induced inflammation include tumor necrosis factor-α (TNF-α) and Interleukin-6 (Il-6).

TNF-α is a cytokine mainly produced by macrophages and is involved in the metabolic complications of obesity.[17,18] Several studies demonstrate that human fat cells are a significant source of endogenous TNF-α production and is elevated in most models of rodent obesity.[18,19] Recent studies demonstrate that TNF-α is overexpressed in the adipose tissue of humans during obesity.[18,19] Hotamisligil et al. examined 18 control and

19 obese premenopausal women and found that the obese individuals expressed 2.5-fold more TNF-α mRNA compared to the lean controls.[19] Another study showed that TNF-α secretion was enhanced in patients with adipose hypertrophy and decreased in adipose hyperplasia.[17]

In addition to host defense, TNF-α also has multiple actions in the adipose tissue, like altering insulin resistance by inhibiting insulin receptor signaling.[18,19] TNF-α also plays an integral role in lipid homeostasis; several studies suggest that TNF-α suppresses free fatty acid uptake, promotes lipogenesis, induces lipolysis, inhibits lipid-metabolism-related enzyme activity, regulates the metabolism of cholesterol and regulates other adipocyte-derived adipocytokines.[20]

Another cytokine secreted during obesity by many cells including adipocytes is IL-6. Several physiologic factors regulate the secretion of IL-6 including hormones, cytokines, diet, physical activity, stress, hypoxia and others.[21] Similar to TNF-α, IL-6 inhibits lipoprotein lipase (LPL) expression, although it doesn't stimulate lipolysis like TNF-α.[21]

Lifestyle interventions such as calorie restriction and increased exercise are popular initial treatment methods used in efforts to defeat obesity and metabolic disease.[1] Unfortunately, there isn't one diet plan that is universally accepted to induce weight loss. There are numerous publications on weight loss diets, some promote minimizing carbohydrate intake without altering fat (e.g. Atkins), many balancing macronutrient balance and glycemic load (e.g. Zone diet), and others restricting fat (e.g. Ornish diet).[22] To ensure a healthy diet, the consumer should be instructed to include complex carbohydrates, which are high in fiber, have food low on the glycemic index, and

consume high water content. Also, one should know about healthy fats and high biological values proteins .[2] Despite the great effort to continue ones certain diet, these lifestyle changes require constant attention to nutrient consumption and quantity. Therefore, their success has been limited to a very small percentage of people. Hence, new interventions are urgently needed to stop this obesity epidemic.[1]

Since obesity is on the continual rise, there has been a lot of research done to determine the best way to decrease weight. For many years, studies of body weight regulation have focused almost completely on caloric intake and energy expenditure.[23] Since the obesity epidemic continues to skyrocket, one diet that has received a lot of attention is Intermittent fasting (IF), or periods of voluntary abstinence from food and drink.[24] In some places, IF has been practiced since the earliest of antiquity depending on culture and lifestyle. IF regimens are linked to certain health outcomes and influence metabolic regulation via effects on the circadian biology.[24] Several cross-sectional and longitudinal studies have shown that IF has positive effects on physical and intellectual performance by affecting various aspects of physiology and biochemistry.[25] Other studies have shown that IF reduces ROS generation and reactive nitrogen species (RNS); it also prevents the expression of oxidative stress genes and clusters of inflammation.[26] There are multiple forms of IF practiced such as alternate day fasting, modified fasting, religious fasting and time restricted feeding.

Alternate day fasting is when no energy containing foods or beverages are consumed on alternating days. Multiple studies concluded that this regimen was as effective as simple calorie restriction in decreasing fasting insulin and glucose concentration; it also reduced total plasma cholesterol and triglyceride level.[24]

A modified fasting regimen generally allows for the consumption of 20-25% energy needs for 2 non-consecutive days during the week, while the rest of the week is one's typical regimen of energy intake.[24] Varady and colleagues have investigated the effects of modified fasting and found this way of IF results in decreased visceral fat, leptin and resistin.[25]

Religious fasting, popular in many denominations, is incorporated into their lifestyle for both spiritual and physical benefits.[24]

Despite IF effectiveness at decreasing weight and lowering the risk of metabolic disease, approximately 20% of people following this diet can't adhere to this rigorous form of dietary restriction. Hence, an alternative form was created and termed "time restricted feeding."[28]

Time restricted feeding is a diet that has gained a considerable amount of attention in the recent years. This type of feeding regimen allows individuals to consume *ad libitum* energy intake within a set window of time (3-4 h, 7-9 h or 10-12 h), which leads to a 12-21 hour fasting window per day.[28] In a murine model, Hatori et al. demonstrated that time restricted feeding, while consuming the same diet and the same number of calories, increased metabolic health when on a high fat diet.[29] Unlike calorie restriction which entails an overall reduction in caloric intake without creating malnutrition, time restricted feeding allows the individual to consume their typical diet, just at specific time intervals.[30]

This form of intermittent fasting has been shown to heighten parasympathetic activity in the autonomic neurons that trigger the gut, heart and arteries. This enhancement results in better gut motility, decreased heart rate and decreased blood

pressure.[25] Evidence from a compilation of multiple studies conclude that in mice, time restricted feeding was associated with reduction in body weight, total cholesterol, glucose, insulin as well as improvements in insulin sensitivity. Furthermore, Chaix et al. showed in mice that time restricted feeding stabilized and reversed the progression of metabolic diseases with preexisting obesity and Type 2 Diabetes.[1]

Since weight gain attributes to an increased risk in metabolic diseases and problems, this form of eating might be the answer to many researchers' questions. Sherman et al. conducted a study using mice on a time restricted eating pattern and found that compared to a high fat *ad libitum* diet, time restricted feeding led to 18% lower body weight, 30% reduced cholesterol levels, 10% decreased TNF-α levels and 3.7-fold improved insulin sensitivity.[31]

Research highlights the potential importance of synchronizing time restricted feeding regimens with the circadian rhythm.[24] Mammals contain a circadian system that controls energy homeostasis by making circadian oscillations of rate limiting enzymes involved in tissue metabolism throughout the day and night.[32] This biological system is made of transcriptional and translational feedback loops that control the expression of clock genes by generating a rhythm of ~24 hours in each cell in the body. The pertinent clock genes include Cry1, Per2, Bmal1, Clock and Rev-erba.[33] Clock genes, along with their circadian rhythm roles, regulate cell proliferation, cell death, immune response, hypoxia, the formation of new red blood cells, tumor genesis, tumor progression and more.[34]

Mammals contain two types of clocks: central and peripheral. Located in the superchiasmatic nucleus of the hypothalamus and entrained by light and dark stimuli, the

central clock aligns and coordinates the independent and self-sustained peripheral clocks found in every cell.[30] Unlike the central clock that is entrained by light, the peripheral clock is partially entrained by feeding patterns.[33] Furthermore, each peripheral oscillator is impacted by its unique stimuli and is capable of being controlled locally.[30] The peripheral clock helps the cell adapt to a daily pattern of food availability by temporarily adapting the expression of multiple genes regulating metabolism.[35] This biological system helps mediate rate limiting steps in glycolysis, fatty acid oxidation and oxidative phosphorylation.[33]

Evidence from multiple studies connects caloric energy regulation and the circadian clock at a molecular, physiological and behavioral level.[36] Emerging evidence suggests that the rhythmic signals attributed to the peripheral clock and time restricted eating may play a positive role in immune and metabolic function.[1] Research indicates that time restricted delivery of nutrients creates rhythmic availability and resets peripheral clocks in a way that potentially positively impacts immune response. It is believed that time restricted feeding provides a time cue that resets the circadian rhythm, leading to better health.[31]

Conversely, Hatori et al. fed mice a high fat diet *ad libitum* and resulted in the mice developing obesity, diabetes and metabolic syndrome. Limiting access to high fat diet during the day or night for up to 6 weeks shows some improvement in body weight fluctuation.[29] A high fat diet fed under *ad libitum* regimen blunts metabolic regulators, including CREB, mTOR, and AMPK, and contributes to metabolic disease.[29] Moreover, research proves that *ad libitum* access to a high fat diet in mice causes obesity, insulin resistance, hepatic steatosis, hypercholesterolemia and dyslipidemia.[37]

This leads to the possibility of exploring biological rhythms relating to time restricted eating versus *ad libitum* eating as targets of intervention strategies to decrease the prevalence of obesity worldwide.

Obesity and its associated low grade inflammation is a major risk factor for the development of multiple diseases including Type 2 diabetes, nonalcoholic fatty liver disease, cardiovascular disease and cancer.[1] It is also associated with mechanical stress, excess lipid accumulation, abnormalities in intracellular energy fluxes and nutrient availability.[21] In the past years, there have been many interventions for obesity, yet the epidemic has continued to rise. Therefore, many researchers have started looking for better alternatives to attain the weight loss needed for better health. Recently, there has been much research done on intermittent fasting, specifically time restricted feeding. The present study sought to determine if a high fat diet given in a time restricted fashion can protect against obesity and inflammatory mediators.

Materials and Methods

Experimental Animals

Nine week old C57BL/6 male mice were purchased from Harlan Laboratories, Inc., Indianapolis, IN and housed no more than 3 per cage. The mice were housed in a USDA approved animal facility with regulated light-dark cycles. Mice were entrained to a reverse light-dark schedule (12h light: 12h dark), with lights off between the hours of 7am-7pm for 3 weeks. This was done so that the feeding time was done during the active phase ("lights off" phase) of the mice. During entrainment, mice consumed standard rodent chow *ad libitum*. At week 12, experimental mice were switched to a high fat diet

(Research diets, Inc., New Brunswick, NJ) consisting of 45% fat predominantly lard, 35% carbohydrate and 20% protein.

The mice were divided into 4 groups with 10 mice per group. Group 1 (HFD) had access to the high fat diet *ad libitum*. Group 2 (HDF-AM) had access to the high fat diet for 6 hours at the beginning of their active phase (7am-1pm). Group 3 (HFD-PM) had access to the high fat diet for 6 hours at the end of the active phase (1pm-7pm). Group 4 (CHOW) was the control group that consumed rodent chow *ad libitum*. The mice were on their respective diets and feeding schedule for 8 weeks. All animals received water *ad libitum*. Food intake was monitored daily and mice were weighed twice a week. Final body weight and body composition was determined one day prior to sacrifice using an Echo MRI 2100 (Echo MRI LLC, Houston, TX). All experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Experimental animals and were approved by the University of Memphis Institutional Animal Care and Use Committee.

Glucose tolerance test and HOMA-IR

One week before sacrifice, mice were subjected to an intraperitoneal glucose tolerance test. Mice were fasted overnight (10 hours). Baseline fasting blood glucose level was measured from the tail vein using a glucometer (Onetouch Ultra 2 Meter, Bayer Healthcare, Tarrytown, New York). Mice then were administered an intraperitoneal injection of glucose (1g/kg body weight) and blood glucose levels measured every 30 minutes for 90 minutes via the tail vein. Immediately before euthanasia, blood was also collected from the facial vein to determine fasting glucose (Thermo Electron Clinical

Chemistry) and insulin (Mouse Ultrasensitive Insulin ELISA, Alpco, Salem, NH) levels to determine Homeostatic Model Assessment of Insulin Resistance (HOMA-IR).

Tissue Collection and Histology

Mice were fasted for 10 hours before euthanasia. Liver, epididymal adipose tissue and spleen were harvested and weighed for each animal. A portion of the liver and adipose tissue, were placed in a formalin solution (Fisher Scientific Co. LLC), embedded in paraffin, and 4um sections were stained with hematoxylin and eosin. Histological analysis and documentation were performed using an imager M2 microscope (Axiocam MRC, Zeiss, Oberkochen, Germany). The size of individual adipocyte was determined using Axiovision r4.8.2 software. Two to three fields were quantified per mouse, generating data from 300-400 individual adipocytes per group, with slightly more for the chow group as the adipocytes were smaller. A second portion of the liver and adipose tissue, along with the heart, was snap-frozen in liquid nitrogen and stored at -80°. The spleen, mesenteric lymph nodes (MLNs), and remaining adipose tissue were collected and immediately processed for immune cell population analysis. Spleen and MLNs were placed into RPMI 1640 solution containing 2% FBS. Adipose tissue was placed into DMEM (HyClone, GE Healthcare Life Sciences, Logan, Utah).

Blood parameters

Blood was collected from the facial vein immediately before euthanasia. Plasma were collected from whole blood and stored at -80°. Levels of Leptin, TNF-α and IL-6 were measured via magnetic bead assay (R&D Systems, Minneapolis, MN) and read on the Luminex MAGPIX platform. Cholesterol and triacylglycerol levels were measured following standard enzymatic procedures as described by the reagent manufacturer

(Thermo Electron Clinical Chemistry). Levels of insulin were measured via Mouse ultrasensitive ELISA (Alpco; 80-INSMSU-EO1,E10).

Statistical Methods

The data is expressed as the mean ± SEM. Analysis of variance was used to compare groups. Mean ± SD was compared using *Tukey's* test. $P < 0.05$ was accepted as statistical significant.

Results

Time restricted high fat feeding protected against weight gain

We evaluated the effect of time restricted high fat feeding during different times of the circadian cycle on weight gain and body composition. Twelve week old C57BL/6 male mice were entrained on a reverse light-dark cycle (dark from 7am until 7pm). They were subjected to either normal rodent chow or a high fat diet. The high fat diet composition contained 45% fat and resembled the "Western diet; it contained high levels of saturated and polyunsaturated omega 6 fatty acids. Food consumption occurred *ad libitum* or in a time restricted fashion during the animal's normal nocturnal feeding time. For time restricted feeding, mice had access to food from 7am-1pm (HFD-AM) or 1pm-7pm (HFD-PM). There was a small, but significant difference in the amount of food consumed between the HFD group and the HFD-AM group (HFD 13.74 +/- 0.8479 vs. HFD-AM 11.89 +/- 0.4163 ($p=0.007$), with no difference for the HFD-PM group (Figure 1A). Body mass was monitored and recorded twice a week. The body mass gain of mice with constant access to the high fat diet began increasing at week 1 and continued to increase until week 8. However, consuming the same HFD in a time restricted fashion did not cause a similar body mass gain (Figure 1B). At the end of 8 weeks, the HFD

group gained 2-3 times more weight than the time restricted groups. (HFD 16.425 +/- 1.857 vs HFD-AM 5.60 +/- 2.589 ($p<0.0001$) and HFD-PM 8.875 +/- 1.936 ($p<0.001$) (Figure 1C). To determine if body mass gain correlated with energy intake, energy efficiency was calculated as weight gained per kilocalorie consumed. Additionally, the energy stored for the HFD was significantly more than both time restricted groups (HFD 0.018+/-.0004 vs HFD-AM 0.0079 +/- 0.003 ($p=<0.001$) and HFD-PM 0.012 +/- 0.002 ($p=0.004$) (Figure 1D). The results from Figure 1B and Figure 1C indicate that *ad libitum* feeding at least doubles the amount of total body mass gain and body mass gained per kilo calorie consumed.

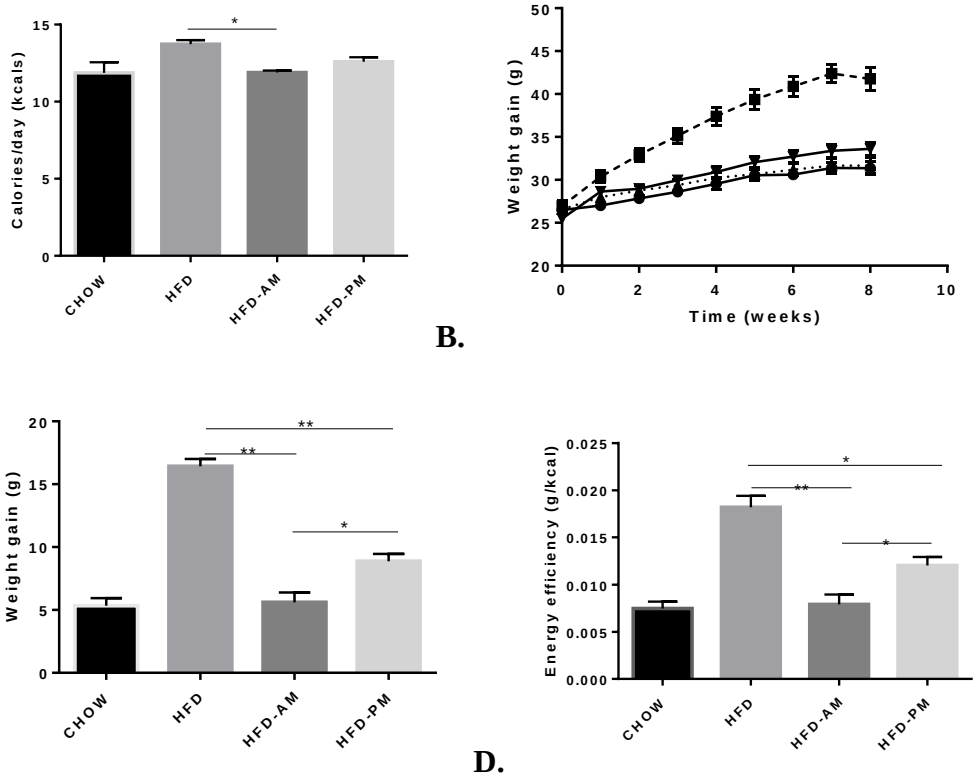


Figure 1. Time restricted high fat feeding protected against weight gain. A.) Average calories consumed per day from chow, HFD, HFD-AM and HFD-PM B.) Weight over time between all groups C.) Total weight gain during 8 week experiment D.) Calories consumed per gram gained for all groups. All values are mean ±SEM. N=10-11 per group. * = $p\leq0.05$, **= $p<0.0001$

Time restricted feeding decrease adiposity and adipose associated parameters

We next examined the effect of time restricted feeding on adiposity, body composition and blood lipid levels. There was no difference in fat free mass between the different groups (22-25g) (Figure 2A). There was a 38-45% increase in overall fat mass in the HFD group when compared to HFD-AM and HFD-PM groups (HFD 17.78 +/- 2.204 vs HFD-AM 9.759 +/- 3.647 ($p=0.0008$) and HFD-PM 10.97 +/- 2.642 ($p=0.0052$) (Figure 2B). There was also a 25-37% increase in epididymal fat pad weight for the HFD vs. HFD-AM and HFD-PM (HFD 1.27 +/- 0.1428 vs HFD-AM 0.8026 +/- 0.3028 ($p<0.0001$) and HFD-PM 0.9527 +/- 0.2158 ($p=0.0080$) (Figure 2C). Total cholesterol correlated with overall fat mass mean HFD being 240 +/- 42.38 while both time restricted feeding groups ranged between 143-165(HFD-AM 142.6 +/- 26.09 ($p<0.0001$) and HFD-PM 165.3 +/- 15.55 ($p<0.0001$) (Figure 2D). Interestingly, there was no significance found with triglycerides among all groups (Figure 2E). The adipokine leptin also correlated with adiposity with HFD having a 28-37% as compared to HFD-AM and HFD-PM. (HFD 18965 +/- 2656 vs HFD-AM 12003 +/- 4717 ($p=0.0011$) and HFD-PM 13675 +/- 3506 ($p=0.0166$) (Figure 2F).

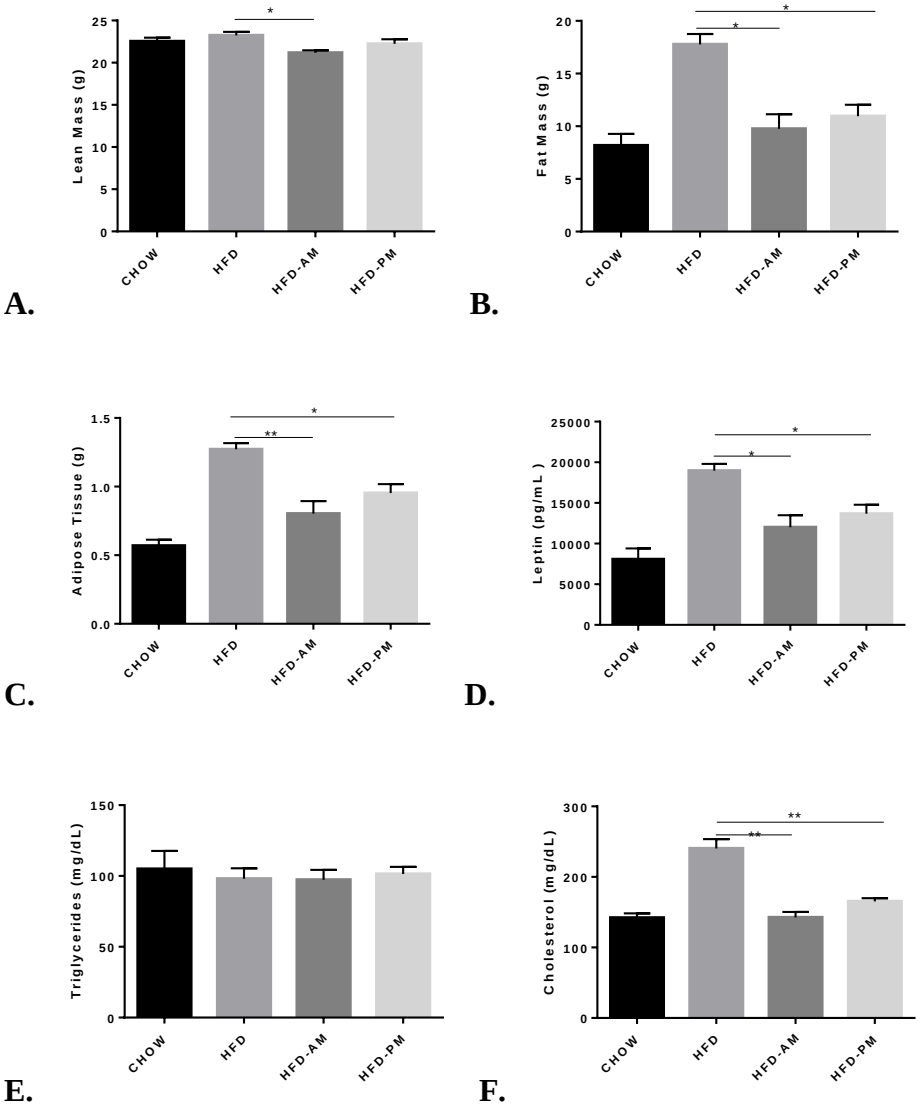


Figure 2. Time restricted feeding decrease adiposity and adipose associated parameters. (A and B) Lean and Fat mass obtained through magnetic imaging resonance. (Chow n=4; HFD, n=5; HFD-AM, n-7; HFD-PM, n=6 (C) Collected Adipose tissue weight, n=10-11 (D) Serum leptin level analyzed, n=10-11 per group (E) Triglyceride levels per group with no significance seen (D) Cholesterol levels with significance seen in HFD compared to HFD-AM and HFD-PM. All values are mean ±SE; *p<0.05 **p<0.0001

Time restricted high fat feeding reduced glucose intolerance and insulin resistance.

High fat diets and obesity induce insulin resistance and glucose intolerance. To test whether time restricted feeding protected against insulin resistance induced by a high fat diet, we subjected the mice to an intraperitoneal glucose tolerance after a 10 hour, one

week before euthanasia. Mice on *ad libitum* high fat feeding were unable to clear glucose, while both HFD-AM and HFD-PM cleared glucose to the same extent as mice consuming a healthy chow diet with glucose levels back to baseline at 90 minutes post glucose injection (Figure 3A). Because the glucose tolerance test suggested that the mice on a high fat diet *ad libitum* induced insulin resistance, we explored whether the same correlation was seen in the fasting glucose levels in mice fed a high fat diet *ad libitum*. Fasting glucose corroborated glucose tolerance test, although significance was lost between HFD and HFD-PM (Figure 3B). Interestingly, fasting insulin levels between the *ad libitum* feeding group and the evening time restricted group differed only by 2% with no significance found (HFD 1.206 +/- 0.3658 and HFD-PM 1.196 +/- 0.4413). There was a 60% reduction in fasting insulin levels when comparing HFD-AM to the *ad libitum* and the HFD-PM (HFD vs HFD-AM 0.4912 +/- 0.2229 (p=0.0024) and HFD-PM vs HFD-AM (p=0.0037) (Figure 3C). HOMA-IR confirmed higher insulin resistance in *ad libitum* and HFD-pm compared HFD-am (Figure 3D).

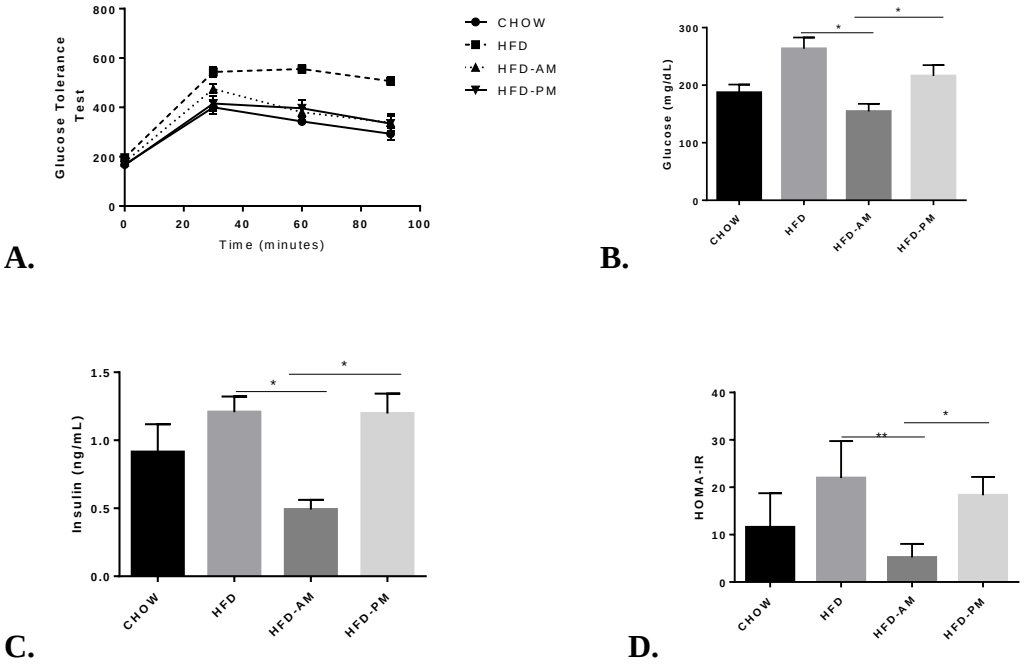


Figure 3. Time restricted high fat feeding reduced glucose intolerance and insulin resistance. A.) Glucose tolerance test over 90 minutes between all groups B.) Average fasting glucose levels C.) Average fasting insulin levels D.) HOMA-IR. All values are mean ±SEM. N=10-11; *p<0.05 **p<0.0001

Time restricted feeding did not protect against liver fat accumulation or adipocyte hypertrophy

Since hepatic steatosis and hypertrophy of adipocytes has become a common topic in clinical practice due to the increasing prevalence of obesity worldwide, we examined if time restricted feeding protected against these metabolic complications.[38] Hepatic steatosis occurs when hepatic fatty acid uptake from plasma and *de novo* fatty acid synthesis is greater than fatty acid oxidation and export.[39] To address if time restricted feeding protected against fatty liver development, we determined liver weight and fat deposition. The mean liver weight of HFD was 1.59 g +/- 0.3597, HFD-AM 0.93+/- 0.1011 and HFD-PM 1.136 +/- 0.09197g, demonstrating a 25-42% reduction in liver weight in the time restricted feeding groups compared to the *ad libitum* group (HFD

vs HFD-AM ($p<0.0001$) and HFD vs HFD-PM ($p<0.0001$) (Figure 4A). The livers of all groups had an elevated amount of intracellular fat deposits (Figure 4C), demonstrating that time restricted feeding does not protect fatty liver development.

Because adipocyte hypertrophy is highly correlated with cell stress and inflammation, we determined if time restricted feeding protected from adipocyte hypertrophy. Similar to what was seen in liver histology, no significant difference was seen between all high fat groups (HFD – 6928 μm^2 +/- 1138, HFD-AM 5923 μm^2 +/- 1574 and HFD-PM 5997 μm^2 +/- 1369) (Figure 4B and 4D).

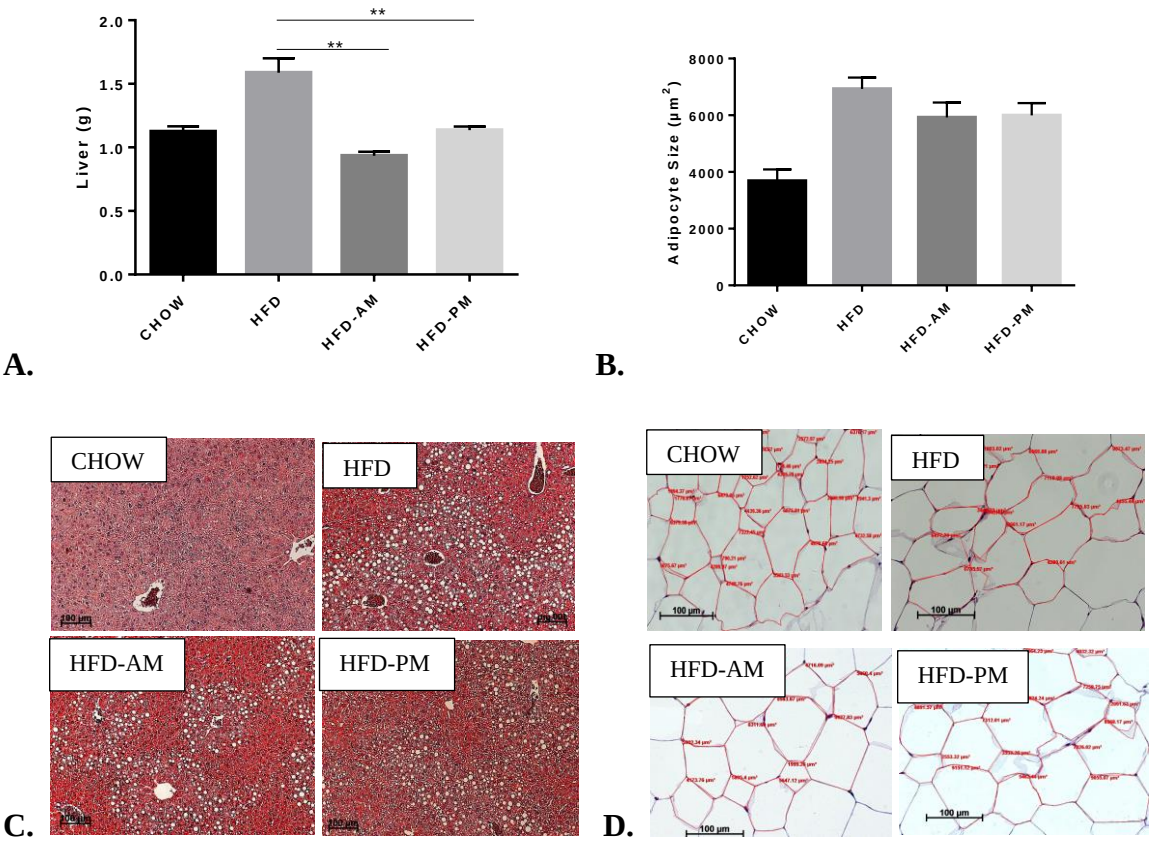
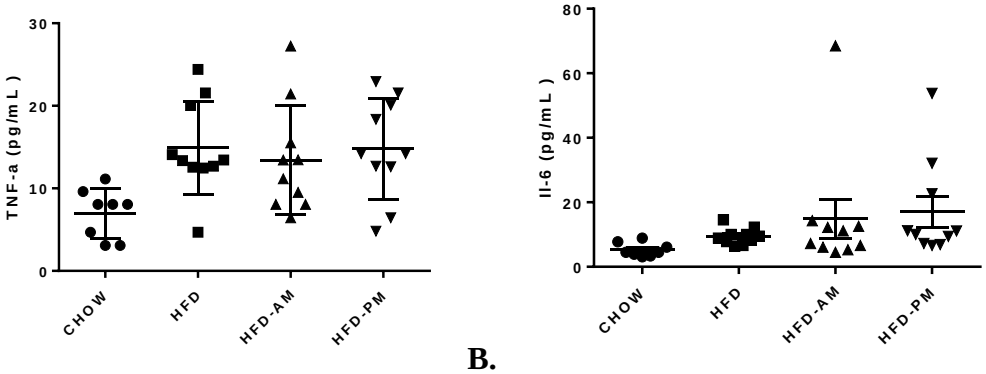


Figure 4. Time restricted feeding did not protect against liver fat accumulation or adipocyte hypertrophy A.) Average liver weight collected at harvest B.) Average adipocyte size C.) Liver histology indicating fatty liver development in all high fat groups D)Adipocyte histology and increased hypertrophy of adipocytes when on a high fat diet. All values are mean ±SEM. N=10-11; *$p<0.05$ **$p<0.0001$

Increased inflammatory markers found in all high fat diets markers

Despite the endless amounts of data on local and systemic inflammatory networks associated with obesity, the exact triggers that cause inflammation in obesity are still unknown.[40] One hypothesis postulates that an overload of nutrients in metabolic cells such as adipocytes induces intracellular stress resulting in the release inflammatory molecules.[41] Our data demonstrate that time restricted feeding decreased total adiposity, but interestingly, there was no reduction in the inflammatory molecule TNF-α. This cytokine was increased in all groups consuming the high fat diet (51-54% compared to chow) with no difference between *ad libitum* and the time restricted feeding groups (HFD 14.93 +/- 5.645, HFD-AM 14.03 +/- 6.653 and HFD-PM 14.79 +/- 6.071) (Figure 5A). IL-6 was not increased in any group (Figure 5B).



A. **B.**

Figure 5. Increased inflammatory markers found in all high fat diets markers A.) Increased TNF-α in all high fat groups B.) Il-6 with no significance seen among all groups. All values are mean ±SEM. N=10-11; *p<0.05 **p<0.0001

Discussion

Obesity in the United States continues to increase with recent reports indicating over 64.1% of American women and 72.3% of American men categorized as overweight/ or obese.[6] There is accumulating evidence demonstrating the association between obesity

and the increased risk of metabolic diseases such as insulin resistance, Type 2 diabetes, dyslipidemia and nonalcoholic fatty liver disease.[7] In the current study, we introduce time restricted feeding, a lifestyle intervention that could prevent obesity in the future without altering caloric intake. Time restricted feeding is a potential behavioral intervention that de-emphasizes caloric intake, thus making it a more attainable lifestyle modification.[1]

Findings from the present investigation indicate that time restricted feeding protected against body weight gain when the animals were subjected to a similar high fat diet. When the high fat diet was consumed in an *ad libitum* fashion, the mice exhibited weight gain as soon as one week after the start of the diet, while both the time restricted feeding groups gained weight at the same rate as the group consuming chow. Interestingly, the group consuming their meal at the end of the active phase did have a slight but significant increase as compared to mice consuming their food early in their active phase. At the end of the 8th week on their specific feeding regimen, the mice consuming the high fat diet in an *ad libitum* fashion gained 2-3 times more weight and had 38-45% more body fat than the time restricted mice, despite the fact there was no significance in food intake during the 8-week period. Since feeding and fasting drive daily rhythms in the key regulators of nutrient homeostasis, there is extensive coupling between circadian oscillator components and the feeding-fasting driven metabolic regulators.[29,35] There is emerging evidence suggesting that constant calorie intake leads to disruption of certain circadian rhythms leading to obesity.[29] It has been hypothesized that a high fat diet under *ad libitum* access disrupts the circadian and feeding rhythms thereby perturbing metabolic processes. This disruption as well as nutrient quality can

increase the risk for the development of obesity and metabolic syndrome.[29] As our feeding schedule included both a group that consumed their food at the beginning of their active phase (coupled with the central circadian clock) and one that ate towards the end of their active phase, we examined the effect of timing of feeding on various metabolic parameters.

Like the trend seen in overall fat mass, there was an increase in adipose tissue weight, cholesterol and leptin in the group consuming the high fat diet *ad libitum*, with a similar decrease in both time restricted groups. Interestingly, there was no difference in triacylglycerol level in any of the groups, suggesting that the excess lipids are primarily stored in tissue. The time restricted feeding did not seem to protect the mice against fat accumulation in the liver as the fatty deposits were just as abundant as in the *ad libitum* high fat diet group. Despite development of the fatty liver, the time restricted groups did not develop glucose intolerance or insulin resistance as seen by their fasting glucose levels, glucose tolerance test and HOMA-IR. It has been suggested that coordination among the components of the circadian oscillators and metabolic regulators aides in maintaining glucose homeostasis and anabolic metabolism. Reducing the feeding period likely decreases the net daily anabolic effect of insulin on fatty acid synthesis and storage, thus increasing the fasting duration supporting fatty acid utilization.[1] Therefore, increasing fat consumption instead of glucose in one's diet for an energy source likely contributes to increased insulin sensitivity and the observed weight loss seen in the mice on the time restricted regimen.

Excessive weight gain can led to accumulation of adipose tissue since it is the main site of storage of excess energy and is now recognized as a bona fide endocrine organ.[7] Hypertrophy of the adipocytes induces cell stress that triggers the release of proinflammatory molecules.[7] Although previous studies demonstrated reduced inflammation in the time restricted feeding groups, findings from the current study revealed in all high fat diet regimens regardless of the time of day and fasting period, hypertrophy of the adipocytes and an increase in the inflammatory molecule, TNF-α. This result is in accord with previous research that demonstrates a lipid-rich diet is associated with oxidative stress.

Although the current study does not mirror previous studies, it may be due to diet composition and macronutrient distribution between studies. In some diets, increased consumption of selected micronutrients may increase antioxidant defense leading to decreased oxidative stress and inflammation, while a diet high in saturated fats may lead to the activation of the cascade of inflammatory signals leading to the release of cytokines.[42] While saturated fatty acids promote inflammation, other classes of fatty acids, such as long chain polyunsaturated omega 3 fatty acids, exert anti-inflammatory benefits.

Conclusion

In summary, our findings show that consuming a high fat diet in a time restricted fashion protects against obesity, but it does not protect against the diet-induced inflammation. These results suggest that a high fat diet can induce inflammation even in the absence of dramatic weight gain. Future studies should explore the role of increased omega 3 fatty acids, like DHA and EPA, on mice fed in a time restricted fashion to

determine if this could decrease inflammation associated with the high fat diet thereby mitigating the inflammatory reaction induced by the high fat diet.

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Appendix

A. IACUC Approval

IACUC PROTOCOL

FOR USE OF LIVE VERTEBRATES FOR RESEARCH, TEACHING OR DEMONSTRATION

UNIVERSITY OF MEMPHIS

Date submitted to Attending Veterinarian for pre-review: 1/28/2016

IACUC Protocol # 0776 Date Submitted to IACUC 1/31/2016

Dates Protocol will be in effect: from 2/19/2016 to 2/18/2017

(not to exceed three years including two yearly renewals)

Is this protocol related to an external grant or contract application? Yes ☐
No ☒

If yes, complete the following:

Agency: _____ Date Submitted _____

Grant #

University account for Animal Care Facility per diem charge:

If the protocol is not related to an external grant or contract application, complete the following:

University account for Animal Care Facility per diem charge: 211700

Project Title: (If project relates to a grant or contract application, give that title; if multiple protocols relate to one grant, give unique titles for each protocol; if the project is related to a class, give the course name and number):

Metabolic alteration induced by time restricted feeding at different points in the circadian cycle

I. Personnel

Investigator/Instructor: Marie van der Merwe

Department: Health and Sport Sciences

Academic Rank: Assistant Professor

Campus phone**:** 901 678 3476 Emergency phone: 901 406 7458

Attending Veterinarian: Karyl Buddington

Phone: 901-678-2359 Emergency phone: 901-258-1232

List all individuals that will handle animals using this protocol, their affiliation, and their level of expertise (e.g. relevant qualifications). If the protocol applies to a class then so specify.

Marie van der Merwe – PhD (Molecular Pharmacoly), Postdoctoral Fellowship (Bone Marrow Transplantation): More than 10 years of experience using mice as a research model.

Brooks Crone – 1 month mouse handing, training will be provided during experiment

Jackie Wyman- 6 months mouse handing, breeding

Jessica Hill-6 months mouse handing, breeding

Ryan Moran.- 2 months rat handling

Matt Butawan- 2 years mouse and rat handling

Emily Beatty- No experience with animals, but has been to Laboratory Animal Training; training will be provided during experiment

If additional personnel become involved in handling animals used in this protocol, it is the responsibility of the principal investigator to notify the Animal Care Facility in writing before they start.

Has the investigator/instructor and all personnel listed above received the appropriate vaccinations (tetanus, rabies)? Yes ☒ No ☐
Not Applicable ☐

Is it necessary for personnel listed on this protocol to be tested for TB?

Yes ☐ No ☒

If you have questions about the kind of vaccination or about TB, call the Animal Care Facility at 678 2359.

All U of M personnel involved in this protocol must complete the animal care and use training program before animals can be procured or before experiments/teaching or demonstration. In submitting this protocol, I, as Principal Investigator/Instructor accept the responsibility for compliance with this requirement.

In addition, the Principal Investigator/Instructor must be willing to provide appropriate supervision for all persons working on this protocol. In the case of a class, the Instructor must be responsible for training any students in classes involved prior to using animals.

II. Project Description

A. Summary **(Enter a brief description below of your project, using lay terminology)**:

The 24 hour light/dark cycle that we as humans have evolved in, has produced cyclical biological (circadian) rhythms that regulates many of our physiological processes including eating behaviors and metabolic programs. These biological rhythms are regulated by gene products that are generated in the brain (central clock) and peripheral organs (peripheral clock) by light and dietary intake, respectively. Previous data demonstrated that mice fed a high fat diet consumed on an *ad libitum* regimen have dampened diurnal rhythms, increase in inflammatory molecules, and various dysfunctions associated metabolic syndrome (such as obesity and diabetes).[1] When the same diet was consumed in a restricted fashion (food access allowed for 8 hours during the active phase), mice ate the same amount/calories of food as their *ad libitum* counterparts, but the restricted group did not have the same amount of weight gain and had better metabolic outcomes.

Due to the connection between inflammatory molecules, metabolism and the circadian rhythm, an unanswered question remains – is there an ideal time to consume a high fat diet in a restricted fashion to protect against high-fat induced obesity, inflammation and metabolic syndrome?

The goal of this study is therefor to determine if a high fat diet given at the beginning or end of the murine active cycle has the same protective affects against inflammation and the diet-induced metabolic syndrome phenotype.

9 week old C57BL/6 male mice will be entrained under a 12h light: 12h dark schedule for 3 weeks with rodent chow available *ad libitum*. During the entrainment period, mice will begin the reverse light-dark schedule, with lights off between the hours of 7am-7pm. This will done so that the feeding time will be during active phase ("light off" phase) of the mice. Mice will be housed in Life Sciences in an area that is currently used studies of the circadian rhythm and therefore the light is well regulated. At week 12, the mice will be switched to their specific diets. The high fat diet will consist of 45% fat with lard as the fat source. Mice will be divided into 4 groups: Group 1 will have access to the high fat diet *ad libitum*. Group 2 will have access to the high fat diet for 6 hours at the beginning of their active phase (7am-1pm). Group 3 will have access to the high fat diet for 6 hours at the end of the active phase (1pm-7pm). Group 4 (control group) will consume rodent chow ad libitum. The mice will be on their particular diets for eight weeks. There will be a total of 40 mice.

The amount of food consumed will be measured daily and the weights of the mice will be taken twice per week. Blood will be drawn from the facial vein to determine glucose, insulin, cytokine and cortisol levels three times during the 8 week period. A glucose tolerance test will be performed by administering glucose intraperitoneally and then measuring blood glucose every 30 minutes for 90 minutes via the tail vein. All mice will be sacrificed by CO2 inhalation and cervical dislocation. Tissues will be harvested immediately. This form of euthanasia does not affect the outcome measures as determined by our previous studies. Organs to be harvested are liver, spleen, small intestine, lymph nodes, adipose tissue, and heart. Blood will be drawn through the hepatic vein after death to measure triglyceride levels, cytokines, immune cell populations, and leptin levels.

B. Describe IN DETAIL the procedures you will follow. Include accompanying documentation and reference to previously published work in the box below. Provide a complete bibliographic citation and describe any variations from the published technique. The bibliography may be included in the box below or appended to this protocol.

Mice: C57BL/6 male mice will be used for this study and housed at the animal facility on the University of Memphis campus.

Diets and treatments: All of the mice will be given a normal, or chow diet for 3 weeks. At 12 weeks, the mice will be separated into 4 groups. Three of the groups will be on the high fat diet with 45% lard and 41% carbohydrate (20% sucrose, 9% corn starch, and 12% Maltodextrin). Group 1 mice will be fed the high fat meal *ad libitum* to resemble continuous eating of the western diet throughout the day/night. Group 2 will be fed the high fat meal between 7am-1pm to resemble a time restricted feeding during the morning. Group 3 will be fed the high fat meal between 1pm-7pm to resemble a time restricted feeding during the latter part of the active day. Previous data from Sherman et al. reported a 3 hour time restricted feeding over a 16 week period resulted in better rest-immune function along with decreased disease markers. This data demonstrate that the time restriction we propose (6h) should not have negative health consequences (weight loss and death) as none was seen with a shorted (3h) restriction time. [1] (Multiple students will be involved in removing and replacing food at various time points.) Group 4 will be fed a normal meal *ad libitum* as the control to resemble eating a healthy diet continuously throughout the day, also known as grazing. The diets will be purchased from Research Diets, which has experience in producing the western diets for rodent studies.

Experimental Design: This study will require 40 male mice. The 40 mice will be divided into 4 groups as indicated above. There will be two mice per cage. From our previous studies we know that genetically similar mice eat the basically a constant volume of food. We can therefore pair house the mice and determine an average amount of foofd. consumed. Previous studies used the used a similar wet up where 3-5 mice were co-housed. (Hatori et al.) Food will be weighed daily. Mice will be weighed 2 times per week. Glucose tolerance test will be performed at the beginning of the experiment (when mice are put on their respective diets), at 4 and 8 weeks after the start of the diet. For the glucose tolerance test, mice will be fasted and blood glucose levels determined by blood from tail vein. Mice will be given a 1g glucose/kg body weight intraperitoneally and blood (10ul) collected every 30 minutes for 90 minutes to measure glucose levels. For blood collection, the tip of the tail will be snipped – 1mm region. This part of the tail has little nerve innervation and does not cause the animal any distress. By "milking" the tail, blood can be collected at multiple time points without having to cut again. Isoflurane cannot be used as it increases blood glucose levels independent of treatment. Blood will also be collected from the facial vein for measurement of systemic insulin, cytokine, leptin, and cortisol levels. Isoflurane will be used during this collection to anesthetize the mice. At 8 weeks, mice will then be euthanized (CO2 inhalation) to collect liver, spleen, adrenal glands, heart, mesenteric lymph nodes, small intestine, and adipose tissue for histology, biochemistry, and immune cell harvest.

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