Impact of Time-restricted Feeding and Exercise on Immunity in Male C57BL/6 Mice

Martina Faietti

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IMPACT OF TIME-RESTRICTED FEEDING AND EXERCISE ON IMMUNITY IN MALE C57BL/6 MICE

by

Martina Faietti

A Thesis
Submitted in Partial Fulfillment of the Requirements for the Degree of
Master of Science

Major: Nutrition Science

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Acknowledgments

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Abstract

Food intake and exercise are considered modulators of the immune system. Time-restricted feeding (TRF) protocols have been shown to reduce inflammation and alter cytokine responses. The objective of this study was to determine if TRF would alter immune parameters in response to exercise. Thirty-six C57BL/6 mice were divided into three groups; control had access to food ad libitum, TRF groups had access for six hours either immediately after running (TRF-imm) or five hours after running (TRF-del). Mice ran on a treadmill for eight weeks. Overall, TRF-imm gained less weight than control. β-hydroxybutyrate (BHB) was higher in fasted groups but running induced BHB in all groups. Exercise reduced lymphocytes levels and increased granulocytes in all groups. Cytokine IL-6 was low, but post-exercise it was increased in all groups; however, the response was reduced in TRF groups. Our data suggest that TRF does not alter immune composition but reduces exercise-induced IL-6 levels.
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Chapter 1

Background

Time Restricted Feeding

Introduction

It is common to attribute a negative connotation to fasting because the field of nutrition preaches not skipping meals and dividing food into three main meals and some snacks (1). The idea of fasting is associated with the feeling of tiredness, weakness, and mental confusion, but in reality, when we eat less often the body activates a series of metabolic mechanisms able to increase the strength and the ability to withstand fatigue and physical stress. Intermittent fasting is a strategy that alternates periods of fasting and non-fasting. The utility of this diet strategy arises from scientific evidence on the prolonged caloric restriction, which is able to promote the loss of fat mass and the maintenance of the lean mass and at the same time improve the state of health. Intermittent fasting, however, differs from prolonged fasting techniques as it involves the alternation of fasting phases to normal feeding or hyper-feeding phases according to a precise rhythm (2).

The focus of this literature review is to demonstrate the possible benefits of Intermittent Fasting (IF) and Time Restricted Feeding (TRF). IF is a repeated regular cycle of dietary restriction with recognized benefits on the body. Such benefits include reduced obesity, the amelioration or improvement of neurodegenerative disorders, regulation of inflammatory responses, and a reduction of risk factors for cardiovascular disease (CVD) and cancer. Furthermore, IF can promote a longer lifespan and improve brain functions (2). TRF is a type of IF which utilizes a daily schedule allowing a limited number of hours
for feeding and the remaining hours as a fasting. Abstaining from all food during a specific number of hours improves body mass and composition and confers cell protection (3).

In order to determine the advantages of these dietary regimes on the human body, this review was guided by the following research questions. What are the possible benefits of this particular diet protocol? How can it prevent or treat disease? What can be learned from TRF animal trials that is applicable to humans?

**Fasting against obesity**

Time restricted feeding has been proven effective in helping obesity and associated metabolic diseases (4, 5). Obesity is a disorder caused by various genetic and lifestyle factors. In order to fight it, the first line therapy is lifestyle interventions, including changes in diet, reduced caloric intake and increased exercise. However, these changes require constant attention to nutrient quality and quantity, as well as physical activities. Moreover, these methods are successful in a limited percentage of individuals. Therefore, an innovative strategy to prevent and treat obesity and associated metabolic diseases can be the introduction of TRF, where access to food is limited to eight hours during the active phase without altering caloric intake or nutrient composition. Many of the benefits associated with TRF are proportional to the duration of fasting (6). Hatori et al. found that, despite the high caloric intake, mice subjected to TRF demonstrated protection against obesity, hyperinsulinemia, hepatic steatosis, inflammation and better motor coordination (4). During this experiment, mice were fed a high fat diet in a time restricted manner (eight hours per day). The results demonstrated that TRF might function as a non-pharmacological strategy to oppose obesity and associated diseases (4). Another recent study revealed that IF reduces weight gain and fat mass, fasting glucose and insulin levels,
and improves insulin sensitivity and lipid profiles in diet-induced obese mice consuming a high fat diet. This was expressed in terms of lower fat mass accumulation without loss in lean mass. The evidence found in this experiment proposed that both IF and High Intensity Interval Training (HIIT) improve health; the combined action of IF + HIIT led to superior benefits compared to diet or exercise intervention alone regarding body composition and lipid profiles (5).

Interestingly enough, restrictive feeding measures have been proven effective in more than just obesity studies. The aim of one recent study was to determine if IF is a useful method as a restrictive diet for nonobese humans. After three weeks of this diet protocol, the results indicated that subjects lost approximately 2.5% of their initial body weight and 4% of their initial fat mass, with an increase in the fat oxidization; since high fat oxidation is associated with weight loss, it was found that these persons lost more weight. IF was therefore feasible in nonobese subjects, but maintainable only for short periods because of hunger (7).

IF was also investigated in the context of oxidative stress as obesity can increase the risk for degenerative diseases, usually related to oxidative stress as measured by increased Malondialdehyde (MDA) levels in blood (8). In Wistar rats, it has been shown that IF can reduce systemic MDA levels in addition to reducing total cholesterol, triglycerides and LDL, as well as caused increased concentrations of HDL. Moreover, this study also demonstrated that IF is effective in reducing the risk for cardiovascular disease, indicated by a decrease concentrations of plasma lipids and LDL. Besides affecting lipid metabolism, IF has an influence on the brain, memory and other systems (8).
**Fasting against pathologies**

TRF has been shown to alter immune responses in those who suffer from immune pathologies while also improving inflammation and metabolic responses (3). Specifically, TRF can protect against immunosenescence and Alzheimer’s Disease (AD) and can possibly have a preventative role for skin cancer (3, 9, 10).

Immunosenescence is characterized by a decline in immune response and an increase in inflammatory and oxidative profiles. Aging is associated with immunosenescence as well as with a decrease of erythropoietin reserve and an increasing risk of anemia in older adults (3). TRF can affect red blood cells, in fact Gasmi et al. showed that, before TRF protocol, red blood cells were significantly higher in young men compared with older men. After the TRF intervention, it was not found this age effect in red blood cells between young and aged groups, demonstrating that TRF could be an effective strategy to combat the age-associated perturbations to red blood cells and hematocrit levels (3). Furthermore, TRF decreased total white blood cells and lymphocytes, in both young and older men, while monocytes increased. TRF could prevent inflammation by altering the immune cell composition, especially decreasing natural killer (NK) cells. Therefore, TRF could be a lifestyle strategy to reduce systemic low-grade inflammation and age-related chronic diseases linked to aging (3).

Alzheimer’s Disease (AD) is a neurodegenerative disorder of the elderly which results in a progressive loss of memory and behavioral dysfunction (9). Decrease amyloid-β (Aβ) clearance in the brain is considered a trigger for the development of AD. Polarity distribution of the channel aquaporin-4 (AQP4) is important to remove Aβ from brain. Zhang et al. demonstrated that IF resulted in improved cognitive function, prevented Aβ...
deposition in the brain, and restored the AQP4 polarity in a mouse model of AD (APP/PS1). These results are encouraging since they suggest that IF can be beneficial in reducing AD (9).

It was also found that TRF can regulate skin function. Indeed, TRF was demonstrated to be able to alter the expression of many diurnally expressed genes in the skin, including that of the key DNA repair factor Xpa. Moreover, it was found that feeding at non-physiological times can alter the skin’s susceptibility to UVB-induced DNA damage. Therefore, timing of food intake represents a modifier in the regulation of skin health (10).

**Further benefits of fasting**

Intermittent fasting has additional benefits to the human body. It was demonstrated to improve cognitive function against stress by regulation of inflammatory response pathway. Stress, induced by environmental and physiological factors, can cause behavioral changes through altered biochemical reactions. Stress is also an intensive regulator of memory and learning with the potential to remake brain messages to control anxiety, temper, and decision making. Stress is classified as either eustress or distress, depending on if it is beneficial or not. (2). In a study evaluating the effect of IF on the cognitive functions of mice, inflammatory factors associated with chronic stress were measured. One group of mice was subjected to eustress while the other group experienced distress. The study found that the distressed group had a gain in plasma levels of corticosterone (CORT), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) with a considerable brain hypotrophy and adrenal hypertrophy, whereas IF brought about a notable reduction of the plasma inflammatory factors,
especially in IF with distressed mice. IF could regulate inflammatory response by different molecular mechanisms such as stress responses, ketone bodies production, glucose signaling and cytokines (11). It was proved that IF can improve learning, memory and the cognitive process decline in stressful condition (2).

Intermittent fasting has also been demonstrated to increase alertness and the levels of the wake-promoting neurotransmitter orexin. Orexin-A is a wake-promoting neurotransmitter secreted by the hypothalamus (12). In the case of mammals suffering from a decrease in energy due to a reduced food intake, the levels of vigilance and wakefulness are instinctively increased in order to enhance the chances of finding food (12). Almeneessier et al. have shown that, during IF, orexin-A levels are lower at night yet higher in the day in healthy human subjects. Moreover, the levels of orexin-A are affected by the sleep/wake pattern and the circadian rhythms (12).

The last examined study measured the resistance training (RT) with and without time-restricted feeding (TRF) for eight weeks. The purpose was to evaluate the nutrient intake, body composition changes, and the muscular strength in young men. Results indicated that TRF reduced energy intake by 650 kcal per day of TRF, but TRF didn’t affect total body composition during the study. The researchers stated that TRF did not adversely affect muscular improvements or lean mass retention in young males beginning a RT program (13).

**Conclusion**

Further studies are needed to confirm the current findings. One major limitation of most studies mentioned is that they were conducted on animals, specifically mice, with only a few studies confirming the results in humans. The outcomes obtained in these few
human studies show that TRF has had an excellent impact on human health, especially in terms of the fight against obesity. This disorder is one of the greatest health risks of this time period and finding a way to combat it in a non-pharmaceutical way through fasting could be a great revolution in the nutritional field. Furthermore, excellent results have also been obtained by using these particular diet protocols to prevent or combat some types of diseases, bolstering the future of research in this field. Fasting, historically seen as a negative event, could now be the basis of healthy lifestyle.
Immune System

Introduction

The body is considered an excellent habitat for bacteria, viruses, parasites and fungi. The immune system is a complex network designed to defend the human body against invading microbes. White blood cells or leukocytes are the main cells of the immune system and they are produced from hematopoietic stem cells in the bone marrow through a developmental process called hematopoiesis, where cells differentiate into erythroid, myeloid and lymphoid lineages (14).

Myeloid cells can be classified in two groups; granulocytes, which contain reactive substances able to destroy microorganism and accentuate inflammation. Neutrophils are the most abundant granulocytes, followed by eosinophils and basophils. The second group of myeloid cells contain monocytes, macrophages and dendritic cells. Monocytes are leukocytes that circulate in the blood, and they are the mobile progenitors of sedentary tissue cells called macrophages, whose main function is the phagocytosis (14). Lymphocytes are identified based on their morphology; natural killer cells (NK cells) are large lymphocytes that are effectors cells of innate immunity and their primary function is the defense against viral infections. They prevent the spread of the infection by killing virus-infected cells and secreting cytokines which block viral replication. Small lymphocytes, including B and T cells, are responsible for the adaptive immune responses. The receptors for B cells are immunoglobulins, whereas for T cells are T-cell receptors, both are products of gene rearrangement during development; thus, each B cell has a unique type of immunoglobulin, and each T cell expresses a unique T-cell receptor. T cells are further subdivided into cytotoxic T cells that kill infected cells, and helper T cells
that secrete cytokines helping other cells of the immune system become fully activated effector cells. For example, helper T cells assist B cells to become plasma cells able to emit antibodies (soluble forms of immunoglobulin) that bind to pathogens and the toxic products they make (15).

The majority of lymphocytes are found in lymphoid tissues that include bone marrow, thymus, spleen, adenoids, tonsils, appendix, and lymph nodes. Bone marrow and thymus are primary lymphoid tissues as these are where lymphocytes develop and mature to the stage at which they are able to respond to a pathogen. B and T cells are generated in the bone marrow, and here B cells complete their maturation, whereas T cells leave the bone marrow at an immature stage and migrate to the thymus where they mature. The others are secondary lymphoid tissues, where mature lymphocytes become stimulated to respond to pathogens (15).

**Immune system and nutrition**

Nutrition is a fundamental factor that regulates the immune system and our body's responses to pathogens. On the one hand, malnutrition reduces immune function and consequently increases the risks of serious infections and mortality; on the other hand, obesity accelerates systemic inflammatory processes that promotes the development of certain forms of autoimmunity, asthma, and insulin resistance leading to type 2 diabetes (16). Growing scientific evidence suggest an interaction between nutritional status and immune function. This interaction can occur through the secretion of adipokines, such as leptin, which can directly transmit the state of availability of nutrients to peripheral lymphocytes and other immune cells. This communication is fundamental to maintain the energy balance in the immunological defense, since the
activated effector lymphocytes have a very high metabolic demand essential for the growth, proliferation and production of proteins necessary for a successful immune response. Leptin deficiency has been shown to decrease immune reactivity, with reduced numbers of circulating CD4+ T cells and impaired T cell proliferation and cytokine release, which demonstrates a link between adipose deposits and the immune system functions (17).

Dietary restriction, in the form of moderate reduction in daily caloric intake while avoiding malnutrition, retards ageing and prolongs life span in test animals (18). Many of the reported beneficial effects are attributed to the dietary restriction's ability to influence and improve the anti-inflammatory qualities of the body, thus changing the reactions of the immune system, which consequently leads to slower aging. Programmed and systematic fasting has also been shown to affect bone marrow function (18). Specifically, this reduction in nutrients causes a decrease in the total B cell population, stopping the early development of B cells and at the same time increasing the number of recirculating mature B cells, thereby optimizing B cell function (18). Dietary restriction also optimizes the functions of the T cells by arresting excessive maturation in the thymus and consequently causing the exhaustion of the mature T cells from the spleen and mesenteric lymph nodes, while simultaneously recruiting them in the bone marrow. In conclusion, dietary restriction has been shown to be effective in reducing the main inflammatory disorders in aging-related diseases and has also been demonstrated to increase stress resistance and suppress organ damage and inflammation following Ischemia (18).

Thousands of years of evolution have led most animals to dictate their habits based on a circadian clock, which regulates different functions, including feeding rhythms. In
this context, feeding is alternated with short periods of fasting which usually coincide with sleep. Fasting in fact induces the body to activate alternate metabolic phases, for instance smaller amount of glucose is consumed compared to more ketone bodies-like carbon sources (19). Growing industrialization has changed human habits disrupting the circadian rhythm and breaking the metabolic homeostasis. Food is constant available throughout the 24h day, facilitating excessive caloric intake. This involves impairing immune functions and chronic diseases, such as obesity and diabetes, due to an imbalance in the food intake. Therefore, the introduction of diets such as IF and TRF could be able to prevent and treat some chronic metabolic diseases and re-establishing the balance between feeding and fasting. Specifically, Cissé et al. have demonstrated that TRF in mice is able to alters the innate immune response (20). In fact, reduction in serum bactericidal capacity in day-fed animals coincide with deficits in proinflammatory cytokine concentrations in serum and production in the spleen. Quite the opposite, night-restricted feeding can increase cytokine production and bacteria killing relative to mice under ad libitum diet (20). However, another study suggested that mice underwent to TRF with a high fat diet have reported benefits in terms of body weight control, but they were not protected against diet-induced systemic inflammation (21).

In a study conducted by Choi et al., fasting diet was impactful in improving demyelination and symptoms in a murine experimental autoimmune encephalomyelitis (EAE) model. This diet diminished clinical severity in all mice, and totally reversed symptoms in 20% of them. These enhancements have been attributed to several factors, such as increased corticosterone levels and regulatory T cells (Tregs) number, reduced levels of pro-inflammatory cytokines, helper T cells Th1 and Th17, and antigen
presenting cells (22).

Furthermore, immune system can be affected by diet through gut microbiome, which is an important determinant in several disorders. It has been shown that IF can improve clinical course and pathology of the multiple sclerosis (MS) animal model, and, as mentioned above, it can also act on EAE, leading to lower levels of inflammation, demyelination and axonal damage (23). Thus, IF is able to influence the gut microbiome leading to an increase in bacteria richness that is inversely correlated with leptin levels. In addition, changes in gut microbiome have enhanced the formation of ketone bodies and the glutathione metabolism, stimulating anti-oxidative pathway. Another aspect to consider refers to the ability of IF to act on the composition of T cells in the gut lamina propria, reducing IL-17 secretion that is usually able to produce T cells, and increasing the number of Tregs. These effects could modulate the immune system response. Given all the elements described before, IF can be considered a very strong immunomodulator and its effects are partially mediated by the gut microbiome (23).

IF can have great effects even on obese subjects suffering from asthmatic events. This diet is able to attenuate the NLRP3 inflammasome and Th2 cell activation in steroid-naïve asthmatics, as well as reduce airway epithelial cell cytokine production. This demonstrates a potential role that nutrition can play in regulating and modifying inflammation in asthma, suggesting a possible development of calorie restriction interventions for the treatment of asthma (24).

**Immune system and exercise**

A chronic state of low grade-inflammation, characterized by raised cytokines levels, is usually present in obesity and inactive population, and it is associated with
cardiovascular and metabolic diseases (25). It is well known that exercise can protect against the development of these chronic diseases, representing an advantageous non-pharmacological intervention thanks to its great anti-inflammatory effects. Indeed, moderate exercise results in lower levels of circulating pro-inflammatory cytokines and increased expression of anti-inflammatory cytokines (26). However, it depends on the amount and intensity of the exercise, as heavy exertion is associated with an increased risk of upper respiratory tract infection (URTI). According to the J-shaped curve, moderate amounts of training could increase immune system response above sedentary levels, while excessive amounts of prolonged high intensity exercise could impair immune function (27). During exercise, interleukin-6 (IL-6) is produced by muscle through a TNF-independent pathway. IL-6 is considered a myokine, that is a cytokine produced and released by contracting skeletal muscle fibers in response to exercise stimulation. IL-6 stimulates the production of other anti-inflammatory cytokines such as IL-1ra and IL-10 in the circulation and inhibits the release of the proinflammatory cytokine TNF-α (28). Therefore, regular exercise is able to hinder the TNF-α secretion and can defend the body against TNF-α-induced insulin resistance. Furthermore, IL-6 increases lipid turnover, encouraging lipolysis and fat oxidation. This study also revealed that IL-6 could have beneficial effects in the protection against chronic diseases like diabetes and CVD that are usually associated with low-grade inflammation (28).

As mentioned earlier, acute exercise causes a pro-inflammatory response with increase in the pro-inflammatory cytokine TNF-α and IL-1β and a dramatic increase in the inflammation responsive cytokine IL-6. Periodic exposure to the pro-inflammatory effects of acute exercise is able to induce an adaptive response, leading to an attenuated exercise-
induced release of cytokines. This is balanced by the release of cytokine inhibitors (IL-1ra, sTNF-r1 and sTNF-r2) and the anti-inflammatory cytokine IL-10 (29). It could be deduced that the variation from the pro-inflammatory effects of a single session of exercise to the anti-inflammatory effects of exercise training is mediated by a change in cytokine secretion in response to repeated prolonged exercise bouts. During acute prolonged exercise, cytokines are secreted from adipose tissue and skeletal muscle, and it is well known that exercise training can alter gene expression in these tissues, which eventually results in modified secretion patterns of cytokines (25). Furthermore, the acute inflammatory response to exercise is intensity dependent. Although obesity influences the basal concentrations of several cytokines, only high intensity interval exercise induces important alterations in IL-8 and IL-10 levels, which could have important implications in the control of chronic low-grade inflammation in obesity (30).

**Immune system and stress**

The functions of the immune system can be altered and damaged by multiple factors, including aging and stress that can interfere with normal functioning. Stress is defined as a state of disharmony and is contrasted by several physiological and behavioral responses in order to establish homeostasis. Stressful experiences and activities can influence both physical and psychological well-being, thus also acting on immune functions (31). Stress can have beneficial effects on humans, so-called eustress, when the response to stressors is positive and it is indicated through feelings of well-being, motivation and above all greater physical stimuli. This is extensively studied and directed in the sports field, where attempts are made to transform stressful moments into positive sources to face competitions. Instead distress is
present when stress factors lead to negative consequences for the whole body acting also on a psychological level. In this case the functions of the immune system can be negatively altered and affect the person's well-being.

There is a well-defined difference in the effects that acute and chronic stress exert on the immune response, being immunostimulant and immunosuppressive, respectively (31). Although acute stress and the events that follow have evolved as an adaptation, and are therefore beneficial, a prolonged exposure to extreme stress can be harmful to the body. An example of these negative effects can be found in the alteration of the synaptic plasticity of adult rats; these ones in fact, exposed to stressors for prolonged periods, have shown negative effects on the synapses (32). Regarding human beings, it was found that psychological stress over time or repeated periodically can be associated with harmful effects on the cardiovascular system which can in turn induce diseases such as obesity and hypertension (33). The responses of the body to stressful environments and situations are manifold and concern various systems. The main one by the neuroendocrine system occurs through the activation of the hypothalamic-pituitary-adrenal axis (HPA) and of the sympathetic nervous system, where the stimulation of the HPA axis increases the glucocorticoid secretion (cortisol) (34). Previously it was thought that the stress response, through the action of glucocorticoid (GC), strictly suppressed immunity. In reality, there are several sources that show how the anti-inflammatory action of GC is very effective in the early stages of acute stress. In fact, initially the immune system is activated rather than suppressed, and following an increase in GC, their anti-inflammatory effects enter the scene, helping the body to recover from the early phases of the stress response. However, if stressful circumstances are prolonged over time or if chronic stress is lengthened, there
will be a suppression of the immune system, which will be mediated in part by the action of the GC (31).

Regulatory T cells (Tregs) are fundamental for the control of autoimmune response and for maintaining self-tolerance. A reduction of CD4+ FOXP3+ Tregs in peripheral blood was found in human beings subjected to mental stress; this explains a down-regulation of the inhibiting components of the adaptive immune response, such as Tregs, during periods of acute stress. In case of chronic stress, this situation could therefore lead to the exacerbation of the inflammatory conditions that can be found in autoimmune diseases (35). Another study on rats has shown that a severe acute stress factor can induce an increase in the concentration of inflammatory cytokines and chemokines (36). This shows that exposure to stressors, in the absence of a pathogenic challenge, can stimulate a systematic anti-inflammatory response leading to enhance the formation of these components. On a final note, there is a proven and strong relationship between the immune system and the central nervous system. The latter is in turn susceptible to stressing agents that can alter it to activate or deactivate different functions. Therefore, it is shown how stress can act directly on the brain, causing disorders such as depression, mood swings and even schizophrenia, all in combination with the effects on metabolism and immune functions (36).

**Conclusion**

The outcomes obtained in these studies have shown as immune system is essential for keeping an organism healthy through the defense against pathogens. However, this balance is fragile and susceptible to variations that can happen to the human body, especially because of an imbalance nutrition or chronic stress events. Fortunately, these
variables can be, at least in part, controlled and improved in order to maintain an efficient immune system. IF and TRF can be considered concrete diet therapies able to treat and prevent some chronic disorders as well as they can affect the immune system. It was also demonstrated that exercise can influence the immune system through the secretion of anti-inflammatory and pro-inflammatory cytokines after physical workout. However, further studies are necessary to verify more in-depth way the correlation between these dietary patterns and exercise and what happen to the immune system if they are combined together.
Chapter 2

Purpose and Hypothesis

Purpose

Nutritional status is well known to regulate immune function, as obesity is associated with increased inflammation whereas malnutrition is associated with immune deficiency and increased susceptibility to infection (16). TRF protocols have been shown to reduce inflammation and alter cytokine responses (3). Even a single exercise bout is considered a modulator of immune system, leading to significant changes in the cytokine production (26). Therefore, we are interested in changes in immunity in relation to exercise when following a time restricted feeding protocol. We monitored alterations in leukocyte populations and cytokine levels before and after exercise. We also measured tissue resident immune population at the end of the eighth week TRF protocol.

As a secondary outcome we followed blood glucose and ketone body levels. Ketones are organic compounds derived from lipids and produced in the liver, which are normally present in the blood in small quantities and can act as a source of circulating energy for the tissues during fasting or prolonged exercise (37). The ketone bodies are recognized as a substrate of cerebral energy alternative to glucose; in fact, during the psychosocial stress, the brain requires more glucose from the body to satisfy its growing needs and in case there is not a sufficient reserve of it, the ketone bodies are used as a fuel source. During prolonged fasting, gluconeogenesis leads to the subtraction of intermediates from the Krebs cycle, directing acetyl-CoA towards the production of ketone bodies (37). In this study we determined the ketone body response to fasting protocol and exercise.
**Hypothesis**

Diet and exercise are lifestyle interventions that can help to combat obesity and they are considered modulators of the immune system. Time restricted feeding and physical activity have shown many positive metabolic effects on the body. However, acute exercise is also known to induce stress and increase the release of inflammatory molecules. Therefore, we hypothesize that TRF in combination with exercise alter immune cell composition and effect immunity after exercise. As a secondary outcome, we hypothesize that mice fed ad libitum vs TRF have altered glucose and ketone levels.
Chapter 3

Methodology

Animals

This study was performed in accordance with Institutional Animal Care and Use Committee (IACUC) by the Animal Welfare Act (USDA) and the NIH Public Health Service Policy on the Human Care and Use of Animals. The IACUC considers and approves all experimental animal use on The University of Memphis. All efforts were made to minimize animal suffering and reduce the number of animals used. This study was conducted for three months. Research investigators directed the experiment in the Life Science Department at the University of Memphis Main Campus, where the mice were located. There were two mice per cage, but they were separated into individual cages if there were noticed any signs of fighting or it appeared that one of the mice in a given cage was consuming the majority of the food.

Thirty-six, six-week-old, C57BL/6 male mice were purchased from Envigo. They were housed in a FDA approved animal facility and subjected to a 12:12 hours of light-dark cycle, in a temperature-controlled room that is used for circadian rhythm studies and therefore the light was well-regulated. They were entrained to a reversed light-dark schedule, with the lights off from 6:00 am to 6:00 pm, so that feeding and exercise times coincided with working hours of staff. Mice were acclimated to the treadmill for two weeks prior to the start of the study.

At eight weeks of age, mice were randomly assigned to one of the three groups (n=12 mice per group). All mice were fed with the same diet (Growing Rodent Diet: 21% of protein, 15% of fat and 64% carbohydrates) but had access to food for different time
periods, whereas they had ad libitum access to water throughout the study period: group 1 (control) had access to food ad libitum, group 2 (TRF-imm) had food access immediately after exercise for a period of six hours, and group 3 (TRF-del) had access to food for six hours starting five hours after the end of exercise. The respective protocols are further described in the next section.

![Figure 1. Study schedule. Schematic of the 8-week intervention period.]

**TRF and Exercise**

Time restricted feeding (TRF) occurred as follows: for TRF-imm, the exercise was completed by 8:00 am, after which they immediately had access to food for six hours. TRF-del completed their exercise protocol by 7:00 am but did not have access to food for five hours. After the five hours wait period the mice had access to food for six hours. Control group completed the exercise by 9:00 am, and they had ad libitum access to food. The amount of food consumed was measured every day for each cage. Mice
continued their training and feeding schedules for eight weeks, until all tests were completed. It should be noted that animals in all groups were monitored daily for signs of stress, malnutrition, and impaired health.

![Figure 2. Feeding schedule.](image)

Figure 2. Feeding schedule. Schematic of the three different dietary protocols followed in this study. Mice were entrained to a reversed light-dark cycle. The control group was allowed to feed ad libitum. The time-restricted feeding groups were allowed access to food for 6 hours either immediately after exercise (TRF-imm) or waiting 5 hours after exercise (TRF-del).

All mice were subjected to a training protocol where the training session consisted of running on a treadmill at a speed of 20 m/min at a slope of 10%. A warmup phase was provided for 15 minutes (5 min at 5 m/min; 5 min at 10 m/min, 5 min at 15 m/min). Mice were acclimatized to treadmill running for two weeks before intervention starts. The treadmill exercise was performed daily for five consecutive days with two days rest period (running took place from Monday to Friday, with no running over the weekend). All running occurred at the beginning of their active phase (morning). During the first week, the mice exercised for 30 minutes, in the second week for 45 minutes (15 minutes warm up
and then 30 minutes at 20 m/min), and from the third to eighth weeks, the mice ran on the treadmill for 60 minutes (15 minutes warm up and then 45 minutes at 20 m/min).

Furthermore, animals underwent a treadmill run test to exhaustion using a motorized treadmill to assess the effects of time restricted feeding and exercise on maximal performance. The treadmill test was performed twice, before to start the eight weeks intervention and at the end of the study. Animals ran using a 5% grade at 20m/min for 30 min and 25m/min for the remaining time until they reached exhaustion. A warmup phase was provided for 10 minutes (5min at 10m/min; 5min at 15m/min). Fatigue was defined as the time at which mice were no longer able or willing to keep up with the speed of the treadmill despite gentle hand prodding for a period of 30 seconds.

Materials and Methods

Body weight was measured two times a week and the amount of food consumed was determined daily. Body composition was monitored weekly using an EchoMRITM 100 (Echo Medical Systems, Houston, TX, USA). Glucose and ketone bodies were measured weekly prior to exercise by collecting blood from the tail vein. At the end of the dietary intervention and before sacrifice, glucose and ketones were measured after running. After eight weeks on their respective dietary protocols, mice were sacrificed by CO₂ inhalation and cervical dislocation. Various organs were harvested and frozen in liquid nitrogen and store at -80°C. Prior to euthanasia, a limited cohort (2/3 mice per group) was selected for LPS injection.

Cell isolation

Splenocytes were isolated by forcing the tissue through a 40 μm nylon sterile cell strainer. Red blood cells were lyzed using ACK lysis buffer. In order to obtain cells from
the bone marrow, a needle and a syringe filled with PBS were used to flush the bone marrow out the femurs onto a 40 μm nylon sterile cell strainer.

**Cytokine and white blood cells analysis**

Blood was collected from the facial vein for cytokine analysis and white blood cell profiling at various timepoints during the study. Leukocyte composition was measured from whole blood using a VetScan HM4 Hematology Analyzer (Abaxis, Union City, CA, USA). Plasma was harvested from whole blood by centrifugations and used for cytokines analysis. Levels of IL-6 and TNF-α were measured using a Magnetic Bead Assay (R&D Systems, Minneapolis, MN, USA) as per the manufacturer’s instructions (Millipore) and a Luminex MAGPIX analyzer. Results were analyzed by xPONENT software.

**Flow Cytometry**

Attune NxT Flow Cytometer (Thermo Fisher Scientific, Waltham, MA, USA) was used for immune phenotyping of cells isolated from the blood, spleen and bone marrow. The following fluorescently labeled antibodies were used to distinguish T cell and granulocyte populations: PE-TCR (clone:H57-597, cat: 109207, BioLegend), Pe/Cy7-CD8 (clone: 53-6.7, cat: 100721, BioLegend), FitC-CD4 (clone: GK1.5, cat: 11-0041-82, eBioscience), FitC-Ly6C (clone: HK1.4, cat:128006, BioLegend), PE-Ly6G (clone: 1A8, cat: 127607, BioLegend), PE/Cy7-CD11b (clone: M1/70, cat: 101215, BioLegend). Trustain CD16/32 (clone: 93, cat: 101319) was used to prevent nonspecific binding.
Table 1. Antibodies used for flow cytometry.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Clone</th>
<th>Concentration</th>
<th>Conjugate</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>Stain 1</td>
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<td>1:100</td>
<td>PE</td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>CD8+</td>
<td>53-6.7</td>
<td>1:100</td>
<td>Pe/Cy7</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CD4+</td>
<td>GK1.5</td>
<td>1:100</td>
<td>FitC</td>
</tr>
<tr>
<td>Stain 2</td>
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<td>1:100</td>
<td>FitC</td>
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</tr>
<tr>
<td></td>
<td>CD11b+</td>
<td>M1/70</td>
<td>1:100</td>
<td>PE/Cy7</td>
</tr>
</tbody>
</table>

Statistical Analysis

All statistical analysis was performed with GraphPad Prism software (version 8, GraphPad, San Diego, CA, USA). Data was tested for normality using Shapiro-Wilk test. One-way ANOVA was used to compare group means for differences in organs weight and immune cells percentages (monocytes, lymphocytes, granulocytes). Two-way ANOVA was used when data were collected over time. Paired t-test was used to compared means between two groups before and after exercise. If the data did not meet the assumptions of normality or equal variance, then nonparametric tests (mixed-effect analysis, Kruskal–Wallis, or Wilcoxon tests) were conducted. All data were presented as means ± SEM, and statistical significance was established at p < 0.05.
Chapter 4

Results

All mice in this study gained weight during the eight-week intervention, but the TRF-imm group had significantly less body weight than the control group ($p \leq 0.03$) starting at the 5th week on their respective dietary protocol (figure 3a). This resulted in a weight gain difference between the control and the TRF-imm group over the course of the study that trended to significance ($p=0.06$). The final weight at week 8 for each group were as follows: control; 29.14g±2.23, TRF-imm; 25.78g ±1.70 and TRF-del; 27.15g ±2.00 with a significant difference between control and TRF-imm groups ($p=0.008$).

Figure 3. Weight gain during the 8 weeks of intervention. (A) Body weight was monitored weekly during the 8-week intervention. TRF-imm gained less body weight than control from week 5 to 8. Data analyzed using mixed-effects analysis. Results represent mean ± SEM.*$p<0.05$. (B) Weight at 7th week of intervention was subtracted from the weight at the beginning of intervention to calculate weight change (week 7 was chosen as our final data point as various tests including a run-to-exhaustion and glucose tolerance test were performed during the last week). Data analyzed using Kruskal-Wallis test. Results represent mean ± SEM.
Initially both the fasting groups gained more fat mass, with TRF-del having significantly more fat than the control group after 2 weeks on their respective diets (figure 4a, p=0.002). After 2 weeks, the fat mass for both the TRF-imm and TRF-del started to decrease and by the 7th week the fat mass was similar to the control group (p≥0.7). Interestingly, there was a difference in fat mass between the fasting groups from week 4 to week 6 (p≤0.04). Despite the dynamic fat mass change during the 8-week study, there was no significant difference in fat mass gain between week 0 and week 7 (figure 4b).

**Figure 4. Fat mass gain during the 8 weeks of intervention.** (A) Fat mass was measured weekly during the 8-week intervention. Significance identified at: week 2 (#control vs TRF-del), week 3 (#control vs TRF-del), week 4 (#control vs TRF-del and ^TRF-del vs TRF-imm), week 5 (^TRF-del vs TRF-imm), week 6 (^TRF-del vs TRF-imm), week 8 (^TRF-del vs TRF-imm). Data analyzed using mixed-effects analysis. Results represent mean ± SEM. #^p<0.05. (B) Fat mass at 7th week of intervention was subtracted from the fat mass at the beginning of intervention to calculate fat mass gain. No significant differences were detected. Data analyzed using Kruskal-Wallis test. Results represent mean ± SEM.
Lean mass was also monitored during the study. The control group gained consistently more lean mass than the fasting groups with the biggest difference at the 7th week after the start of their respective dietary protocols (figure 5a, control vs TRF-imm; p=0.009, control vs TRF-del; p=0.04). Lean mass gain has shown significant differences between control and both TRF groups (figure 5b, control vs TRF-imm; p=0.01, control vs TRF-del; p=0.01).

Figure 5. Lean mass gain during the 8 weeks of intervention. (A) Lean mass was measured weekly during the 8-week intervention. Significance identified at: week 1 (*control vs TRF-imm), week 2 (*control vs TRF-imm and #control vs TRF-del), week 4 (*control vs TRF-imm), week 5 (*control vs TRF-imm), week 7 (*control vs TRF-imm and #control vs TRF-del). Data analyzed using mixed-effects analysis. Results represent mean ± SEM. *#p<0.05. (B) Lean mass at 7th week of intervention was subtracted from the lean mass at the beginning of intervention to calculate lean mass gain. Significant differences in the gain were detected in the control group compared to the TRF groups (p=0.01). Data analyzed using Kruskal-Wallis test. Results represent mean ± SEM.
Food consumption was measured to determine if the changes in the weight and fat/lean mass were correlated with alterations in food intake. There was no significant difference in the total of energy consumed during the 8-week study between the different groups (figure 6, p>0.5)

**Figure 6. Total food intake.** Total food consumed in grams was used to calculate the energy consumption. Data analyzed using Kruskal-Wallis test. Results represent mean ± SEM. No significance was detected.
Ketone levels have been shown to be affected by both fasting and endurance exercise. During the 8-week study, the ketone body β-hydroxybutyrate (BHB) was measured weekly before daily running (figure 7a). There were variations in the BHB levels from week to week, especially for the TRF-imm group. The TRF-del and control groups had more similar BHB levels during the first few weeks of the study. After week 5, there was a consistent increase in the BHB levels in the TRF-del group resulting in a significant difference from control at 8th week (p<0.05)

Figure 7a. Ketone body BHB during the 8 weeks of intervention. BHB was measured weekly from the beginning to the end of intervention. Significance identified at: week 4 (*control vs TRF-imm, #control vs TRF-del); week 5 (*control vs TRF-imm); week 6 (#control vs TRF-del); week 7 (*control vs TRF-imm, ^TRF-del vs TRF-imm); week 8 (*control vs TRF-imm, #control vs TRF-del). Data analyzed using mixed-effects analysis. Results represent mean ± SEM. *#^p<0.05
At week 8, we measured BHB levels before and after running (figure 7b). Before running (after time-restricted fasting) BHB was found to be slightly, but significantly higher in the TRF groups (TRF-imm vs control; p=0.02, TRF-del vs control; p=0.02). The BHB levels for each group before running were as follows: control; 0.35mM ±0.12; TRF-imm; 0.57mM ±0.14; TRF-del; 0.54mM ±0.14.

After running, BHB was significantly increased in all groups (control; p=0.008; TRF-imm; p=0.008; TRF-del; p=0.002), with no significant difference between groups. The BHB levels for each group were as follow: control; 1.00mM±0.18; TRF-imm; 1.18mM±0.44; TRF-del; 1.05mM±0.23.

**Figure 7b. Ketone body BHB measured before and after running.** Both TRF-imm and TRF-del groups showed significantly higher BHB levels compared to control group (p=0.02) prior to running. Data analyzed using Kruskal-Wallis test. Results represent mean ± SEM. BHB increased in all groups after exercise (p≤0.008), but no significant difference between groups. Data analyzed using Wilcoxon test. Results represent mean ± SEM.
Glucose levels were also monitored weekly (figure 8a). There was extensive variability in glucose levels during the study; at week 1 TRF-del group was significantly lower than control group (control vs TRF-del; *p*=0.02), while at week 2 TRF-del group was significantly higher than the other two groups (control vs TRF-del; *p*=0.008; TRF-imm vs TRF-del; *p*=0.02). However, at week 8 glucose values were similar between all groups (*p*≥0.79): control; 155.90mg/dL±21.83; TRF-imm; 158.10mg/dL±10.09; TRF-del; 150.40mg/dL±9.77.

![Figure 8a. Glucose levels during the 8 weeks of intervention.](image)

Figure 8a. Glucose levels during the 8 weeks of intervention. Glucose was measured weekly from the beginning to the end of intervention. Significance identified at: week 1 (#control vs TRF-del); week 2 (#control vs TRF-del, ^TRF-imm vs TRF-del); week 4 (#control vs TRF-del); week 7 (#control vs TRF-del). Data analyzed using mixed-effects analysis. Results represent mean ± SEM. #^*p*<0.05
At week 8, before sacrifice, glucose levels were measured before and after running (figure 8b). Although exercise induced a decrease in glucose levels in all groups, TRF-imm was the only group significantly lowered after running (p=0.03). The glucose levels for each group after exercise were as follow: control; 128.90mg/dL±32.02; TRF-imm; 110.10mg/dL±31.26; TRF-del; 138.60mg/dL±31.24.

**Figure 8b. Glucose levels measured before and after running.** Glucose levels in TRF-imm group significantly decreased after running (p=0.03). No significant changes in the glucose levels between groups. Data analyzed using Wilcoxon and Kruskal-Wallis tests. Results represent mean ± SEM.
No significant difference was found between groups in the weight of the following organs harvested at the end of the study: epididymal white adipose tissue, heart, spleen, and cecum. There was also no difference in the length of the small and large intestine (table 2). Liver weight of the TRF-imm group was significantly reduced compared to the control group (p=0.02).

**Table 2. Organs weight and length.** Data analyzed using Descriptive statistics. Results represent mean ± standard deviation. No differences were detected between groups. Data analyzed using Kruskal-Wallis test. *control vs TRF-imm; p=0.02.

<table>
<thead>
<tr>
<th></th>
<th>Control (n=8)</th>
<th>TRF-Imm (n=9)</th>
<th>TRF-Del (n=10)</th>
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<tbody>
<tr>
<td>Spleen (g)</td>
<td>0.11 ± 0.03</td>
<td>0.09 ± 0.04</td>
<td>0.09 ± 0.02</td>
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<tr>
<td>Liver (g)</td>
<td>1.15 ± 0.15</td>
<td>0.96 ± 0.10 *</td>
<td>1.08 ± 0.16</td>
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<tr>
<td>Heart (g)</td>
<td>0.17 ± 0.02</td>
<td>0.15 ± 0.03</td>
<td>0.17 ± 0.03</td>
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<tr>
<td>Cecum (g)</td>
<td>0.20 ± 0.05</td>
<td>0.18 ± 0.05</td>
<td>0.22 ± 0.02</td>
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<tr>
<td>Adipose Tissue (g)</td>
<td>0.6 ± 0.25</td>
<td>0.47 ± 0.12</td>
<td>0.53 ± 0.10</td>
</tr>
<tr>
<td>Small Intestine (cm)</td>
<td>35.75 ± 1.51</td>
<td>35.00 ± 1.53</td>
<td>36.05 ± 1.78</td>
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<tr>
<td>Large Intestine (cm)</td>
<td>6.04 ± 0.72</td>
<td>6.19 ± 0.70</td>
<td>6.18 ± 0.68</td>
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</table>
As both nutritional status and exercise can affect immunity, white blood cell populations were monitored before animals ran and also after a run-to-exhaustion test. Fasting did not induce alteration in white blood cell count as there was not difference in total white blood cell numbers or in the composition of the various immune populations after 4 weeks of time-restricted feeding (table 3).

**Table 3. White blood cell populations after 4-weeks of time-restricted feeding.**
Blood was collected in the morning before running. Data analyzed using Descriptive statistics. Results are mean ± standard deviation. No differences were detected between groups at the baseline. Data analyzed using Kruskal-Wallis test.

<table>
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<tr>
<td>WBC</td>
<td>1.36 ± 0.96</td>
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<td>LY%</td>
<td>94.16 ± 3.39</td>
<td>95.04 ± 3.80</td>
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<tr>
<td>MO%</td>
<td>1.29 ± 1.86</td>
<td>1.92 ± 1.60</td>
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</tr>
<tr>
<td>GR%</td>
<td>4.57 ± 3.73</td>
<td>3.04 ± 2.62</td>
<td>9.34 ± 15.02</td>
</tr>
</tbody>
</table>

However, exercise-induced changes were detected in various populations. Running reduced the blood lymphocytes in all groups, but significance was only detected in TRF-del group (figure 9, p=0.008); control and TRF-imm groups trended to significance (control; p=0.06; TRF-imm; p=0.06). At the same time, running increased the granulocyte fraction in all groups but significance was only detected in TRF-del group (figure 10, p=0.008); control and TRF-imm groups trended to significance (control; p=0.06; TRF-
imm; p=0.06). Monocytes levels increased in all groups but not in a significant way (figure 11).

**Figure 9. Exercise-induced changes in lymphocyte %**. Changes in blood lymphocyte percentages were determined at 4th week prior to running, and after run-to-exhaustion test. Running significantly reduced the blood lymphocytes levels in TRF-del group (p=0.008). Data analyzed using Wilcoxon test. Results represent mean ± SEM.

**Figure 10. Exercise-induced changes in granulocyte %**. Changes in blood granulocyte percentages were determined at 4th week prior to running, and after run-to-exhaustion test. Granulocytes significantly increased after running only in TRF-del group (p=0.008). Data analyzed using Wilcoxon test. Results represent mean ± SEM.
Immune population from whole blood were further immunophenotyped to determine changes in CD4+ and CD8+ T cells and also CD11b+ cells. Cells were analyzed from whole blood collected at 4th week after the start of the intervention and after run-to-exhaustion test. Representative flow plots are given to demonstrate analysis strategy (Figure 12). Time-restricted feeding did not alter T cell composition in the absence of exercise (table 4; %TCR+/lymphocytes; p>0.96, %CD4+/TCR+; p>0.99, %CD8+/TCR+; p>0.99). Exercise induced a significant increase TCR+ cells in the control and in TRF-del groups (figure 13a, control; p=0.008; TRF-del; p=0.01). Although CD4+ T cells were slightly increased with exercise, only control group trended to significance (figure 13b, p=0.06). No differences were detected in CD8+ T cells after running (figure 13c).
**Table 4. Immune cells phenotyping after 4-weeks of time-restricted feeding.** Blood was collected in the morning before running. Data analyzed using Descriptive statistics. Results represent mean ± standard deviation. No differences were detected between groups at the baseline. Data analyzed using Kruskal-Wallis test.

<table>
<thead>
<tr>
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<th>Control</th>
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<th>TRF-Del</th>
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<td>%TCR⁺/lymphocytes</td>
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<tr>
<td>%CD8⁺/TCR⁺</td>
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<td>43.15 ± 8.84</td>
<td>38.76 ± 7.62</td>
</tr>
<tr>
<td>%CD11b⁺/all cells</td>
<td>7.53 ± 7.74</td>
<td>8.18 ± 10.56</td>
<td>8.25 ± 8.06</td>
</tr>
<tr>
<td>%Ly6c/g/CD11b⁺</td>
<td>65.93 ± 28.96</td>
<td>61.92 ± 26.37</td>
<td>63.88 ± 17.72</td>
</tr>
</tbody>
</table>
Figure 12. **Immune cells population identified by flow cytometry.** Cells were harvested from whole blood and red blood cells removed using ACL lysis buffer. For T cell analysis, lymphocytes were gated followed by a selection of TCR+ cells that was analyzed for CD4+ and CD8+ expression. For CD11b+ cell identification, all the cells population, including monocytes, macrophages, neutrophils were gated followed by a selection of CD11b+ cells that was analyzed for Ly6c/g expression.
Figure 13. T cells composition at 4th week and after run-to-exhaustion test. (A) \%TCR+ cells increased in control (p=0.008) and TRF-del (p=0.01) groups after running. (B) (C) No significance was detected in \%CD4+ T and \%CD8+ T cells after running. Data analyzed using Wilcoxon test. Results represent mean ± SEM.
Whole blood CD11b+ cells were also analyzed. CD11b+ is an integrin family member expressed on various innate immune populations including monocytes, neutrophils, macrophages and granulocytes. No time-restricted feeding-induced differences were detected for CD11b+ cells (table 4). However, the fraction of CD11b+ cells in whole blood were increased with exercise in all groups (figure 14a, p≤0.01). The majority of the CD11b+ cells were also positive for the markers Ly6C and Ly6G, suggesting an increase in the neutrophils, but no differences were detected between groups (figure 14b, p>0.07).

**Figure 14. CD11b+ percentages at 4th week and after run-to-exhaustion test.** (A) Cd11b+ cells significantly increased after running in all groups (p≤0.01). (B) No significant difference was detected in Ly6c/g percentages between groups. Data analyzed using Wilcoxon test. Results represent mean ± SEM.

T cell and CD11b+ populations were also analyzed from spleen and the stem cells compartment. No significant difference was found in any of the immune parameters in either tissue.
To determine if time-restricted feeding affected systemic cytokine levels, IL-6 and TNF-α were measured at week 4 after start of intervention and at sacrifice. Prior to sacrifice mice ran for one hour (normal daily run time). Cytokine IL-6 levels were low in all groups in the absence of exercise (control: 4.41pg/ml±3.36; TRF-imm: 8.34pg/ml±10.54; TRF-del:3.67pg/ml±3.78) with no difference detected between groups (p>0.99). After running, there was an increase in IL-6, the increase in the food restricted groups was small, only trending to significance in TRF-del group (figure 15, p=0.06). The mean increase in the IL-6 for the control group was approximately two times more than the restricted groups, but the increase was not significant due to large variation (p=0.12). The IL-6 levels for each group after exercise were as follows: control; 40.49pg/ml±34.94; TRF-imm; 8.93pg/ml±6.90; TRF-del;18.27pg/ml±15.14. There was no increase in TNF-α induced by exercise (figure 16, p≥0.12).

Figure 15. IL-6 levels at 4th week and at sacrifice time. IL-6 levels were low in all groups in the absence of exercise; IL-6 increase in TRF-del group trended to significance after running (p=0.06). Data analyzed using Wilcoxon test. Results represent mean ± SEM.
To determine if the lower level of exercise-induced cytokines seen with the time-restricted groups also resulted in a lowered immunity in response to an infectious agent, a subset of mice (2-3 animals/group) was exposed to lipopolysaccharide (LPS) in vivo after their last run. Mice received an intraperitoneal injection of LPS, and blood was collected three hours later for cytokine measurement. Both IL-6 and TNF-α increased after LPS exposure in all groups (p<0.5), with no difference between groups (figure 17). IL-6 levels for all groups were above the 1000pg/ml maximal detection limit. The TNF-α levels for each group after LPS exposure were as follows: control; 242.70pg/ml±111.50; TRF-imm; 299.70pg/ml±274.40; TRF-del; 151.00pg/ml±40.07.
Figure 17. IL-6 and TNF-α levels at sacrifice time and after LPS injection. (A)(B) Both cytokines were significantly increased in all groups after LPS exposure. Data analyzed using Mann-Whitney test. Results represent mean ± SEM.
Chapter 5
Discussion, Conclusion and Future Directions

Discussion

People living in the developed world are used to have food available 24 hours per day, typically with at least three principal meals daily. Overconsumption of food usually in combination with sedentary lifestyle leads to overweight and obesity, which are considered key risk factors for the development of some chronic diseases (38). Lifestyle interventions, such as dietary restrictions and physical activity, are at the forefront of the obesity treatment. Intermittent Fasting (IF) is an increasingly popular dietary pattern used for weight management and overall health. Contrary to prolonged periods of fasting that are difficult to perform for people, IF protocol has been shown to produce higher compliance (39). Specifically, a type of IF called time-restricted feeding (TRF) where a person does not have to restrict food type but is restricted to a limited window of food access every day (3). TRF was demonstrated in both animal and human studies to be efficient in decreasing weight without reducing calories, and for this reason TRF could be consider a valuable non-pharmacological strategy against obesity and associated diseases (4, 40). Furthermore, TRF improves cardiometabolic health, reduces cancer incidence, delays aging, and increases lifespan in rodents (4, 19, 39); studies in humans are limited, but similarly suggest that TRF improves clinical outcomes such as body weight, blood pressure, insulin sensitivity, decreases inflammation, ameliorates cognitive functions and reduces the risk factors for cardiovascular disease and cancer (2, 40, 41). According to the study on humans by Jamshed et al., TRF is also able to reduce the mean glucose levels over 24 hours and increases β-hydroxybutyrate (BHB) levels in the morning, proving that even
short-term daily fasting can moderately rise circulating ketones (42).

Many animal studies have now shown TRF to be beneficial, especially when consuming a high fat diet (4, 5). However, what is not known is the physiological effect of TRF when consuming a “healthy” diet and perform daily exercise. To answer this question, we placed male mice on two TRF protocols where the animals had access to food for six hours every day. The first group (TRF-imm) had food access immediately after their daily exercise bout, while the second group (TRF-del) had to wait five hours after exercise before allowing food access. These groups were compared to animals that had ad libitum food access. Consistent with previous studies, there was a decrease in weight gain, but only significantly for TRF-imm group, despite consuming the same amount of energy. In fact, the total food intake was estimated almost identical in all groups, demonstrating that the mice consumed equivalent amounts of calories throughout the duration of the experiment even if TRF groups were forced to fasting. The reduced weight coincided with a significant reduction in lean mass for both the TRF groups. Interestingly, fat mass fluctuated over the course of the study, with an initial increase in the first two weeks for the TRF groups which was then followed by a reduction in fat resulting in no difference in fat mass gain compared to the control group. This was contrary to studies in humans which had shown that TRF is able to decrease fat mass without changing lean mass in young resistance trained men (13, 39). Instead in the study conducted by Gabel et al., after 12 weeks of TRF protocol it was not found any significant change in both fat and lean mass, despite a weight loss (40).

Ketone bodies are lipid-derived organic compounds that can serve as a circulating energy source for tissues during fasting or prolonged exercise (43). Under physiological
conditions, when carbohydrates are sufficiently accessible, circulating ketone body concentrations are relatively low. However, under conditions of limited carbohydrate availability such as fasting and exercise, the body increases its production of ketone bodies; the liver is therefore induced to synthesize ketones through the oxidation of fatty acids which serve as an alternative fuel source to glucose for peripheral tissues including brain, heart and skeletal muscle (44). We observed that TRF resulted in a small induction of the ketone BHB, which was then dramatically increased with exercise in all groups. This is consistent with Evans et al. who demonstrated that physical activity induces ketone bodies that are subsequently oxidized as an alternative fuel source to sustain exercise as carbohydrates ran out (44). Ketones remain elevated also during the post-exercise recovery period. It was also found that the ketone bodies utilization is higher in exercise-trained skeletal muscle (44). Glucose levels varied dramatically over the course of the current study, with no consistent differences between groups. This is in contrast to other studies that reported changes after following TRF protocol (39, 42, 45). The reason for the difference in results is possibly due to the fact that the animals in this study fasted for different time periods, or in the case of the control group, were not fasted prior to glucose measurements. The reason for this was to determine glucose levels prior to the daily exercise bout. Blood glucose levels were reduced with running, but only significantly for the TRF-imm group possibly most likely due to the fact that this group had the longest fasting time prior to running and therefore the lowest carbohydrate stores.

Nutritional status potentially influences immune responses. Malnutrition could be a cause for immunosuppression, a situation where patients are more susceptible to infections, while in contrast overeating leads to chronic inflammation with an associated higher risk of
metabolic and cardiovascular diseases (46). TRF has been demonstrated to affect immune function by reducing systemic low-grade inflammation and age-related chronic diseases linked to immunosenescence (3). In our study we did not see significant differences between the control and TRF groups in overall amounts of various immune populations in blood, spleen or bone marrow.

Recent studies have shown that there is an interaction between exercise and immune system. Moderate exercise was observed to reduce the risk of developing various chronic disorders, such as cardiovascular disease, metabolic syndrome, type 2 diabetes and even cancer (47, 48, 49). Intense exercise can also induce adverse effects on health, by increasing the risk of upper respiratory tract infections (URTI). The relationship between the amount and intensity of physical exercise and risk of illnesses can be interpreted with a J-shaped curve. This means that the absence or very low physical activity is associated with a higher risk of illness compared to moderate activity that decreases the relative risk of infection (50). Instead performing prolonged, high-intensity exercise is associated with the above average risk of infection, resulting from a decrease in the immune system functionality (51). Neutrophils, macrophages and natural killer (NK) cells are the major cellular type of innate immunity susceptible to changes caused by exercise. Our findings confirm the results found by Peake et al., who demonstrated that exercise is able to increase circulating neutrophils and monocytes, and at the same time reduce circulating lymphocyte counts in the body (52). In our study we showed that running induced an increase in the granulocyte percentages at the expense of lymphocyte levels in the blood. The cause of this change in the immune cell composition could be related to elevated circulating stress hormones, and alterations in the pro/anti-inflammatory cytokine balance.
in response to exercise, but the clinical significance of these changes is still unknown (53). CD11b+ is usually expressed on the surface of monocytes, neutrophils, granulocytes and macrophages; in our study the attune flow cytometer showed an increase of CD11b+ cells in all groups, confirming the above-mentioned results. Despite a fractional reduction in lymphocytes with exercise, T cells percentage went up, specifically CD4+ cells slightly increased, but the increase only trended to significance in the control group, probably due to the fact that the number of samples in this analysis was relatively low. Since the lymphocyte percentage takes into account not only T cells but also B and NK cells, the decreased lymphocytes levels are most likely due to a decrease in these population; it was demonstrated that lymphocyte subpopulations increase during exercise but decline during recovery, and in addition the functions of NK and B cells are inhibited (54). No differences were detected in splenic and bone marrow immune populations after physical activity, including CD4+ and CD8+ T cells, and CD11b+ cells. Conversely, previous studies have shown that cytotoxic T cells increase in the spleen after exercise, but not other differences in various immune cells were identified (55, 56).

After intense exercise, there is a window of reduced immunity while having increased in inflammatory molecule secretion (for example cytokine IL-6). Specifically, Pedersen et al. demonstrated that physical activity induces a low concentration of lymphocytes, followed by a concomitant secretion of circulating proinflammatory and anti-inflammatory cytokines (54). The mechanisms underlying exercise associated immune changes include neuroendocrinological factors such as cortisol, adrenaline, which exert effects on lymphocytes and neutrophils (57). Typically, IL-6 is the first cytokine released into the circulation during exercise, increasing more dramatically than any other
cytokine (58). IL-6 is considered a myokine, that is a cytokine produced and released by contracting skeletal muscle fibers in response to exercise stimulation (28). The first thought was that IL-6 response to exercise was associated to muscle damage (59); however, several studies discovered that IL-6 levels increase without muscle damage after exercise (60, 61, 62). Rather, intensity and duration of the exercise determine the magnitude of the exercise-induced increase of plasma IL-6 (58). IL-6 exerts its effects locally within the muscle through the activation of adenosine monophosphate activated protein kinase (AMPK) to increase fat oxidation and glucose uptake; when it is released in the circulation, IL-6 can act as a hormone. (63). Our data showed that the levels of both IL-6 and TNF-α were low in all groups before activity. Physical exercise, running in this particular study, resulted in IL-6 increase, but not TNF-α. Previous studies confirm our findings, declaring that IL-6 is involved in the regulation of TNF-α levels, in particular IL-6 is able to inhibit TNF-α pro-inflammatory effects and induce the production of anti-inflammatory cytokines IL-1ra and IL-10 (28, 64, 65). However, exercise is likely to suppress TNF-α also via IL-6-independent pathways (66).

Of particular interest was the fact the IL-6 response was reduced in the fasting groups compared to the control group. This suggests that TRF might result in an immune dysfunction after exercise. To test if there is an overall loss of immune function, a subset of mice was challenged with the immune stimulant, LPS. All mice had a robust immune response with an increase in both IL-6 and TNF-α levels. This suggest that TRF, and specifically fasting prior to exercise, attenuates the IL-6 response to intense exercise. This result is in accordance with Faris et al. and others who found that TRF is able to attenuate inflammatory status of the body by suppressing proinflammatory cytokine expression,
especially IL-6 (67) (68). Further studies are required to evaluate the reduction in IL-6 and muscle adaptation.

**Conclusion**

In summary, this study has evaluated the effects of time-restricted feeding (TRF) on body composition, metabolic parameters and immune functions in combination with exercise in C57BL/6 male mice.

Mice belonging to TRF groups gained less weight than ad libitum group and the difference in weight is mostly a consequence of the reduced lean mass gained by the TRF groups. The production of ketone bodies was higher in the fasting groups and induced after running, consistent to the fact that during these times the body needs to synthetize ketones as fuel source.

At rest, the immune system was not particularly affected by TRF, and exercise-induced change in immune population was similar between the control and TRF groups. Interestingly, TRF resulted in attenuated exercise-induced IL-6 levels, while no difference was observed in cytokine levels after an immune stimulus. This result suggests that TRF, and most probably running after extended fasting, alters the myokine response in muscle.

**Limitations and Future Directions**

The novel findings presented here highlight the necessity for further investigation in many directions. Firstly, the number of mice for certain measurements was found to be not sufficient; larger experimental groups would provide greater statistical power and allow for a better understanding of association between various factors where effect size is small. Second, since the combination of diet and exercise could be stressful for mice, there was a lack of a control group who never exercise. The suggestion for future studies would
be to have also a group who just follows the TRF diet. Third, further studies are needed to investigate the effects of TRF when consuming a “healthy” diet, because the majority of studies on mice so far have examined the consequences of TRF when mice are fed with a high fat diet. Fourth, further investigations are required to evaluate if the production of IL-6 in this study mainly comes from skeletal muscle and to explore more in depth the correlation between this cytokine and TRF protocol.
References


44. Evans M, Cogan KE, Egan B. Metabolism of ketone bodies during exercise and training: physiological basis for exogenous supplementation. J Physiol. 2017 May 1;595(9):2857-2871.


Appendix

IACUC Protocol
for Use of Live Vertebrates for Research, Teaching or Demonstration University of Memphis

Date submitted to Attending Veterinarian for pre-review: 11/5/18

IACUC Protocol #0833

Date Submitted to IACUC: 12/3/18

Dates Protocol will be in effect: from 1/1/19 to 12/31/19

(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):

Impact of time restricted feeding and exercise on body composition and associated measures in male C57BL/6 mice

I. Personnel

Investigator/Instructor: Richard Bloomer and Ryan Wible

Department: Health Studies

Academic Rank: Dean and Research Assistant Professor

Campus phone: 678-5638 and 678-1424  Emergency phone: 901-438-7250 and 602-214-34
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.

Matt Butawan, BS: 3 years of rodent handling experience.

Ryan Wible, PhD: 4 years of experience in laboratory rodent handling, including breeding, general husbandry, gavage, and dissection.

Marie van der Merwe, PhD: More than 10 years of experience using mice as a research model.

Melissa Puppa, PhD: 10 years of experience working with mice including breeding, exercise training/testing, injections, surgery, dietary interventions, gavage, GTT, electroporation, blood collection, and dissection/necropsy.

Yufeng Zhang, PhD: 8 years of experience working with vertebrate including husbandry, breeding, dissection, surgery, training, injections, oral gavage, blood collection, metabolic measurements.

Brandt Pence, PhD: 10+ years of experience with mice, including breeding, exercise training, behavioral tests, injections/inoculation (intraperitoneal, intramuscular, intradermal, intranasal), gavage, glucose tolerance testing, implantation surgeries, blood collection, dissection.

Richard Bloomer, PhD: 3 years of prior experience using rodents in research.

Kyley Rose (undergraduate student): No experience with animals and will attend Laboratory Animal Training; training will be provided during experiment.

Martina Faietti (graduate student): No experience with animals and will attend Laboratory Animal Training; training will be provided during experiment.

Randi East (undergraduate student): No experience with animals and will attend Laboratory Animal Training; training will be provided during experiment.

Aaron Persinger (graduate student): 2 years of experience using mice as a research model including exercise training/testing and necropsy

Ashley Pyrke (undergraduate student): No experience with animals and will attend 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
- [x] No
- [ ] Not Applicable

Will be done by the time the study is initiated.

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

- [x] 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):

Obesity has become an epidemic in the Western world, largely due to poor dietary habits. While multiple dietary programs exist to combat obesity, one that has received a great deal of attention recently is Time Restricted Feeding (TRF). This program involves a block of time each day when feeding is allowed, typically over a 4-8 hour period, with the remainder of the day involving food restraint. In our recent mouse studies using TRF protocols, a 6-hour feeding window has been met with very favorable outcomes with regards to cardio-metabolic parameters of health.

While our prior studies of TRF have demonstrated promise, we have yet to include exercise training within the research design. With regards to humans, many exercise enthusiasts have expressed interest in utilizing a TRF protocol, with the typical goal of reducing body fat and maintaining lean body mass. The problem for many is that they have difficulty scheduling the feeding block at a time of day that allows for the desired effects but does not greatly interfere with the social aspects of eating. That is, most exercise enthusiasts have been led to believe that they must consume nutrients within 30-60 minutes following acute exercise—for purposes of fueling muscle tissue. Most working adults exercise either early morning (e.g., 5:00am) or early evening (e.g., 5:00pm). If attempting to start feeding 30-60 minutes post exercise and adopting a TRF regimen, the morning exerciser will close their window by lunch time. The evening exerciser will need to go the entire work day without
food. Both scenarios are not ideal, for a variety of reasons.

It is possible that the ingestion of food within the one hour post exercise is unnecessary. If so, exercise enthusiasts would be able to begin their TRF regimen whenever they desired, making this much more manageable for the majority of those who exercise and are interested in utilizing this approach.

The goal of the present study is to compare two different TRF approaches with regards to body mass/body fat, physical performance, insulin sensitivity, inflammation, oxidative stress, and related variables in C57BL/6 male mice. Animals will run on a treadmill five days per week for 30-60 minutes. One TRF group will receive food for 6 hours immediately following the cessation of exercise. Another TRF group will receive food for 6 hours beginning five hours following the cessation of exercise. A third group will be provided food ad libitum, 24 hours per day. The food provided to all animals will be the Growing Rodent Diet, consisting of 21% protein, 15% fat, 64% carbohydrate.

Selected outcome measures will be determined before, throughout, and after 8 weeks of assignment to the dietary programs, with a total of 36 animals assigned to one of three diet groups (n=12 per group).

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.

Six week old C57BL/6 male mice will be purchased from Envigo or another vendor. After arrival, mice will be pair-housed in the animal facility on the University of Memphis campus. Mice will be entrained under a 12h light:12h dark schedule for two weeks with 24-hour access to food (Growing Rodent Diet [21% protein, 15% fat, 64% carbohydrate]). During the two-week entrainment period, mice will begin the reverse light-dark schedule, with lights off between the hours of 6am-6pm. This will be done so that the exercise and feeding time will be during the active phase (“lights off” phase) of the mice. Mice will be housed in Life Sciences in an area that is used for studies of the circadian rhythm and therefore the light is well-regulated. During the two weeks, mice will be acclimated to the treadmill.

At eight weeks of age, mice will be assigned to one of the three groups. They will begin exercising on the treadmill five days per week as follows:
Week 1: 30-45 min
Weeks 2-8: 60 min

The three diet groups will be:
TRF immediate phase (start feeding immediately post exercise; early active phase of cycle) TRF delayed phase (start feeding at ~5 hours post exercise; late active phase of cycle) Ad libitum feeding (food available 24 hours per day)
Mice will continue on their diets until all testing is completed (during early week 9). Water will be provided ad libitum throughout the study period. The amount of food consumed will be measured daily. It should be noted that animals in all groups will be monitored daily for signs of stress, malnutrition, and impaired health. Body weight will be recorded three times weekly and should not cause undue stress to the animals due to frequency of handling.

There will be two mice housed per cage. From our previous studies we know that genetically similar mice eat basically a constant volume of food. We can therefore pair-house the mice and determine an average amount of food consumed. Previous studies have used a similar set up where 3-5 mice were co-housed (Hatori et al.). If there are any signs of fighting or it appears that one of the mice in a given cage is consuming the majority of the food, those specific mice will be separated into individual cages.

For fasting glucose blood collection, mice will be placed on a flat surface and restrained by gently holding onto the tail without pulling. 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 once per week for measurement of glucose. Isoflurane cannot be used, as it increases blood glucose levels independent of treatment. We have used this exact procedure in two recent studies. Immediately before the weekly blood collection, the heart rate of the mice will be recorded using the ECGenie. Heart rate may be collected again after the conclusion of an exercise bout. To collect heart rate mice will be placed on an elevated platform on the device to acclimate for ~5 minutes. After acclimation, heart rate signal will be captured through a sensor under the paws for approximately 10 seconds and the mouse will be returned to its home cage.

Stool samples will be collected weekly, immediately prior to exercise to determine changes in intestinal microbiome composition. Mice will be placed in a sterile container for 1-5 minutes. This is sufficient to collect a stool sample.

In addition to weekly glucose and heart rate monitoring and stool collection, prior to commencing the specific diet plans, animals will undergo a MRI for determination of body mass/body fat. Body composition will be determined using an EchoMRI™ 100. The MRI is housed in room 115 in Life Sciences, the same location that we would like for our mice to be housed in. For scanning, animals are placed within cylindrical tube holders and movement restricted to the bottom 7.5 cm as stated in the instrument manual and the study by Jones et al. The animals are scanned without anesthesia, sedation or restraint and are free to move within the holder. The smallest possible holder is used to limit the movement of the mouse (without constraining them) in order to reduce measurement errors induced by motion. Scanning time is approximately 40 seconds. There is no prior training required for the animal. We have used this system in a recent experiment involving approximately 60 mice, without incident. The MRI will be done before and after the 8 week intervention.

Animals will undergo a treadmill run test to exhaustion using a motorized treadmill to assess the effects of time restricted feeding and exercise on maximal performance. Specifically, mice will be acclimated to the treadmill prior to testing. The treadmill test will be performed twice by each mouse; once prior to starting the 8 week intervention and once at the end of the 8 week intervention.
Animals will run using a 5% grade at 20m/min for 30 min and 25m/min for the remaining time until they reach exhaustion. A warm up phase will be provided for 10min (5min at 10m/min; 5min at 15m/min). Fatigue will be defined as the time at which mice are no longer able or willing to keep up with the speed of the treadmill despite gentle hand prodding for a period of 30 seconds. Signs that the animal is nearing fatigue include trouble reaching the front of the treadmill and the tail falling. Very mild electric shock (an intensity to deliver a feeling of “mild tingling” only) will only be used if mice do not respond well to gentle hand prodding. Mice quickly learn that the grid provides an aversive stimulus and will promptly move away from it when placed on the treadmill and maintain some distance from it when running. Our past and current work using running protocols demonstrates that mild shocking is preferable to obtain the best running performance. The frequency and amplitude of shock will be as low as possible to motivate the animals to remain on the treadmill belt, without causing unnecessary distress (again, an intensity to deliver a feeling of “mild tingling” only). We have used small electric shock in many prior studies and this is well-accepted in rodent running studies. To ensure that mice reach true physiologic exhaustion, the criterion for exhaustion is often defined as laying on top of the shock grid and failing to continue running in the face of repeated aversive stimuli. We use gentle hand prodding and determine time of fatigue when mice are not able to keep up with the treadmill despite this and mild shock because we would like to analyze physiological capacity and not just fatigue-like behavior.

Equipment will be cleaned upon the completion of testing with ethanol solution. All urine and feces will be cleaned off of the device and the surrounding area.

Blood will be collected for cytokine analysis and blood profile at the beginning of the study at week 4 and at the time of sacrifice. The facial vein, which does not require the use of anesthesia, will be the primary means of blood collection. The facial vein will be punctured with a very quick prick with a needle. Approximately 50-100ul of blood will be drawn into a capillary tube and used for analysis. An alternative to facial vein blood withdraw is to use the lateral saphenous vein in the leg. This method does not require anesthesia. The hind leg will be gently immobilized in the extended position and punctured with a quick prick with a needle. Approximately 50-100ul of blood will be drawn into a capillary tube and used for analysis. A second alternative is to use retro-orbital blood draw. This will only be used if we are not able to collect blood from the facial or saphenous vein, as it is a more invasive method. Animals will be lightly anesthetized by isoflurane inhalation (2-5%/1L O2/min) and waste gases will be scavenged using carbon filters that will be weighed after use and disposed of and replaced after increase of 50g is observed. Approximately 50-100ul of blood will be drawn with a capillary tube and used for analysis from one orbit. If the retro-orbital method is used for blood draw and an incident occurs such as the eye dislocates, or the eye becomes damaged or infected, euthanasia will be used immediately. No more than one blood draw will be conducted on any single day. All three blood draws are separated by 4 weeks and performed by trained investigators in this technique (i.e., Drs. Puppa and van der Merwe).

Gastrointestinal permeability will be measured in mice at week 6 of the diet and exercise intervention immediately after exercise. GI permeability is measured using a fluorescent dextran (FD; 4000 Da; Sigma Aldrich, St. Louis) (600 mg/kg; 125 mg/ml) administered by gavage as has been previously
done (Puppa et al, 2011). Approximately 120ul of blood will be collected from the tip of the tail vein before mice are gavaged with the dextran. After 1 hr, 120 µL of blood will be collected from the tip of the tail vein into a heparinized capillary tube.

At the end of the dietary intervention and following the completion of treadmill and MRI tests, prior to sacrifice, blood will be collected from the facial vein for the determination of lipids, glucose, insulin, oxidative stress markers, cytokines, immune cell populations, and other variables. Puromycin (0.04µmol/g) will be injected intraperitoneally 30 min before tissue collection to measure protein synthesis rates. Tissue collection will be completed with the mouse anesthetized with isoflurane (2-5%). Mice will be euthanized by removal of the heart while anesthetized. Cervical dislocation will be used as a secondary means of euthanasia. These methods will be used at the end of non-survival surgery since the excision of muscle to measure muscle protein synthesis requires the mouse to be alive as lack of oxygen to the tissue will interfere with basal protein synthesis rates. Organs to be harvested are liver, spleen, intestine, lymph nodes, adipose tissue, skeletal muscle, and heart.

If an animal exhibits signs of illness or injury (including excessive weight loss over short period of time, inability to exercise, scruffy looking coat or visible infection) the animal will be removed from the protocol.

References


The goal of this experiment is to study a very popular feeding regimen with regards to multiple health-related outcomes. Results will provide evidence for or against a TRF pattern of eating, specifically in conjunction with regular exercise. We know from our prior work in mice that the TRF model can yield favorable results, but we have yet to study this in the context of regular exercise. We are unaware of any literature that describes the effectiveness of TRF combined with exercise. This is important to both scientists and those engaged in exercise for the purpose of improving their health. As obesity is becoming more of a problem in the Western world, learning more about the potential effectiveness of a TRF dietary program is of importance.

The C57BL/6 mouse model has been used previously to study the effect of diet on health-related outcomes. As we are interested in the interaction between the immune system, oxidative stress, physical performance, and other organs, we cannot use isolated cell lines or model organisms such as yeast. Additionally, many reagents have been developed for the use of mouse tissues, especially antibodies that will be used to identify certain outcome measures. As we are focusing on the effect of the dietary program on multiple organ systems, it is only feasible to use an animal model.

There will be 12 mice per group for a total of 36 mice. This number is the norm for similar studies of dietary-induced changes in our health-specific parameters. This number should be sufficient to determine statistical significance for the tests planned during this study.

C. Rationale for Involving Animals and the Appropriateness of Species and Number Used.

Indicate (here) briefly the short and/or long-term benefits (to humans and/or other animals) of this use of animals for research, teaching or demonstration. Provide rational for and the number of animals to be used. In addition, state briefly why living animals are required for this study, rather than some alternative model.

The goal of this experiment is to study a very popular feeding regimen with regards to multiple health-related outcomes. Results will provide evidence for or against a TRF pattern of eating, specifically in conjunction with regular exercise. We know from our prior work in mice that the TRF model can yield favorable results, but we have yet to study this in the context of regular exercise. We are unaware of any literature that describes the effectiveness of TRF combined with exercise. This is important to both scientists and those engaged in exercise for the purpose of improving their health. As obesity is becoming more of a problem in the Western world, learning more about the potential effectiveness of a TRF dietary program is of importance.

The C57BL/6 mouse model has been used previously to study the effect of diet on health-related outcomes. As we are interested in the interaction between the immune system, oxidative stress, physical performance, and other organs, we cannot use isolated cell lines or model organisms such as yeast. Additionally, many reagents have been developed for the use of mouse tissues, especially antibodies that will be used to identify certain outcome measures. As we are focusing on the effect of the dietary program on multiple organ systems, it is only feasible to use an animal model.

There will be 12 mice per group for a total of 36 mice. This number is the norm for similar studies of dietary-induced changes in our health-specific parameters. This number should be sufficient to determine statistical significance for the tests planned during this study.

D. Do the procedures described in B above, have the potential to inflict more than momentary pain or distress (this does not include pain caused by injections or other minor procedures)?

Yes ☐ No ☒
I have considered alternatives to procedures that might cause more than momentary or slight pain/distress, and I have not found such alternatives. As such, I have used one or more of the following methods and sources to search for such alternatives: (check below each method used)

- Agricola Data Base
  - ☑ Medline Data Base
- TOXLINE
- BIOSIS
- Lab. Animals Journal
- Lab Animal
- ATLA (Alternatives to Laboratory Animal Journal)
- Lab Animal Welfare Bibliography (QL55L27311988)
- CAB Abstracts
- Animal Welfare Info Center
- Quick Biblio. Series
- "Benchmarks"
- "Alternatives to Animal Use in Research, Testing and Education"
- Current Contents
- CARL
- Direct contact with colleagues (if selected, you MUST document this below)

List search words for the literature search:

```
Time restricted feeding, intermittent fasting, dietary restriction, caloric restriction, obesity, inflammation, oxidative stress, treadmill test, EchoMRI, insulin resistance, insulin sensitivity, fatty acids, body composition, glucose, insulin, blood sugar (words used in isolated and in combination in PubMed and Google Scholar).
```
What is the length of time that the literature search covers?

1960-2018

III. Animal Use

A. List all animal species to be used (example below).

<table>
<thead>
<tr>
<th>Species</th>
<th>Number</th>
<th>Age</th>
<th>Sex</th>
<th>Weight</th>
<th>Where Housed (Bldg./Rm#)</th>
</tr>
</thead>
<tbody>
<tr>
<td>e.g. Hooded Wistar rats</td>
<td>45</td>
<td>2 months</td>
<td>male</td>
<td>250-350 gm</td>
<td>Psychology Bld./422I</td>
</tr>
<tr>
<td>C57Bl/6 mice</td>
<td>36</td>
<td>6 weeks</td>
<td>Male</td>
<td>15-20 gm</td>
<td>Life Sciences/115 (desired)</td>
</tr>
</tbody>
</table>

1Individuals using ectotherms need to only approximate numbers.
2Individuals using fish or other ectotherms need not answer this question.

Is any species threatened or endangered? Yes ☐ No ☑

B. Source of animals

☑ Commercial vendor (Source________Envigo Labs_________________________)

☐ Bred at The University of Memphis
☐ Captured from wild. Identify method of capture: _____________________________
☐ Transferred from another study (IACUC Protocol Number ______________________)
☐ Donated (Source ____________________________)
☐ Tennessee Wildlife Resources Agency

Is the supplier a USDA approved source? Yes ☑ No ☐

If not, explain why:

☐ Animals are already in residence at U of M
C. Will surgery be conducted on animals?  

Yes ☐  No ☒

If yes, complete this section:

☐ Non Recovery Surgery  ☐ Recovery Surgery  ☐ Multiple Survival Surgery (if the latter is checked, complete section F)

Surgeon(s) (Name/Job/Title/Academic Rank)  Location of Surgery (Bldg. & Room #)

D. Will Anesthetic(s), Analgesic(s), or Tranquilizing agents be administered?  Yes ☒  No ☐

If yes, complete this section (example below).

<table>
<thead>
<tr>
<th>Species &amp; Sex</th>
<th>Agent</th>
<th>Dose</th>
<th>Route</th>
<th>Performed by (Name/Title/Academic Rank)</th>
</tr>
</thead>
<tbody>
<tr>
<td>e.g. male Hooded Wistar rats</td>
<td>sodium pentobarbitol</td>
<td>50 mg/kg</td>
<td>i.p.</td>
<td>Mr. Smith/Research Technician/B.S.</td>
</tr>
<tr>
<td>Male C57BL/6 Mice</td>
<td>Isoflurane</td>
<td>2-5%/L O2/min</td>
<td>Inh</td>
<td>Matt Butawan/Research Associate Ryan Wible (Research Assistant Professor) Marie van der Merwe/ Assistant Professor Melissa Puppa/ Assistant Professor</td>
</tr>
</tbody>
</table>

E. Will euthanasia be carried out?  Yes ☒  No ☐

If yes, complete this section (example below).
Mice will be provided restricted access to food, either 8am-2pm or 12pm to 6pm each day. They will be allowed to eat as much food as they would like during these time periods. The food provided to all animals will be the *Growing Rodent Diet*, consisting of 21% protein, 15% fat, 64% carbohydrate.

<table>
<thead>
<tr>
<th>Species &amp; Sex</th>
<th>Agent</th>
<th>Dose</th>
<th>Route</th>
<th>Performed by</th>
</tr>
</thead>
<tbody>
<tr>
<td>e.g. male Hooded Wistar rats</td>
<td>sodium pentobarbitol</td>
<td>150 mg/kg</td>
<td>i.p.</td>
<td>Mr. Smith/Research Technician/B.S.</td>
</tr>
<tr>
<td>C57BL/6 mice</td>
<td>cervical dislocation</td>
<td></td>
<td></td>
<td>Matt Butawan/Research Associate Ryan Wible (Research Assistant Professor) Marie van der Merwe/ Assistant Professor</td>
</tr>
<tr>
<td>C57BL/6 mice</td>
<td>Cardiac removal</td>
<td></td>
<td></td>
<td>Matt Butawan/Research Associate Ryan Wible (Research Assistant Professor) Marie van der Merwe/ Assistant Professor Melissa Puppa/ Assistant Professor</td>
</tr>
</tbody>
</table>

If no, describe disposition of animal(s) at conclusion of this study in box below.

F. Will special housing, conditioning, diets or other conditions be required? Yes ☒ No ☐

If yes, please explain in box below.

Mice will be provided restricted access to food, either 8am-2pm or 12pm to 6pm each day. They will be allowed to eat as much food as they would like during these time periods. The food provided to all animals will be the *Growing Rodent Diet*, consisting of 21% protein, 15% fat, 64% carbohydrate.
G. Will animals be removed from the U of M campus at any time? 

<table>
<thead>
<tr>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
</table>

If yes, please indicate to where and for how long in box below.


H. If they are to be housed for more than 24 hours outside approved facilities at U of M, provide a scientific justification in box below.


IV. Toxic and Hazardous Substances

A. Check off any of the following below that will be used in these experiments?

- Infectious agents (Fill out a, b)
- Radioisotopes (Fill out a, b, e)
- Toxic chemicals or carcinogens (Fill out a, b)
- Recombinant DNA (Fill out a)
- Experimental drugs (Fill out a)
- Malignant cells or hybridomas (Fill out a, c)
- Adjuvants (Fill out a)
- Controlled substances (Fill out a, d, e)

For each checked off category, answer the questions indicated below:

a. Identify the substance(s) and completely describe their use, including how will be injected or given to the animal(s):


b. Describe all procedures necessary for personnel and animal safety including biohazardous waste, carcass disposal and cage decontamination:


c. If transplantable tumors or hybridoma cells are to be injected into the animals, have the tissues/cells been tested for inadvertent contamination by viruses or mycoplasma? 

<table>
<thead>
<tr>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
</table>

If yes, what was the result (indicate in box below).
d. In the box below, provide a complete list of these substances, and if their use is not explicitly explained in the materials already provided, explain their use and role in the research.

Provide DEA license # covering the use of these substances: ________________________________

To whom (or what entity) is the license issued? ________________________________

e. Provide Radioisotope License Number: ________________________________

To whom is the license issued? ________________________________

V. Categories of Animal Experimentation Based Upon Level of Manipulation and Pain: (check off each category that is applicable to this application)

☐ A. Animals will be involved in teaching, research, experiments or tests involving no pain, distress, or use of pain-relieving drugs.

☒ B. Animals will be subject to mild stress only (e.g., food or water deprivation of less than 24 hours for use in behavioral studies such as operant conditioning; physical restraint for less than 30 minutes), and will not be subject to surgery, painful stimuli, or any of the other conditions described below. Procedures described in this protocol have the potential to inflict no more than momentary or slight pain or distress on the animal(s) that is, no pain in excess of that caused by injections or other minor procedures such as blood sampling.

☐ C. Animals will have minor procedures performed, blood sampling, etc. while anesthetized.

☐ D. Live animals will be humanely killed without any treatments, manipulations, etc. but will be used to obtain tissue, cells, sera, etc.

☐ E. Live animals will have significant manipulations, surgery, etc. performed while anesthetized. The animals will be humanely killed at experiment termination without regaining consciousness.

☒ F. Live animals will receive a painful stimulus of short duration without anesthesia (behavior experiments with flight or avoidance reactions--e.g., shock/reward) resulting in a short-term traumatic response. Other examples in this category are, blood sampling, injections of adjuvants, or drugs, etc. Injection for glucose tolerance test; possible low grade shock while on treadmill.
G. Live animals will have significant manipulations performed, such as surgery, while anesthetized and allowed to recover. Such procedures cause post-anesthetic pain/discomfort resulting from the experiment protocol (e.g., chronic catheters, surgical wounds, implants) which cause a minimum of pain and/or distress. Also included are mild toxic drugs or chemicals, tumor implants (including hybridomas), tethered animals, short-termed physically restrained animals (up to 1 hour), mother/infant separations.

H. Live animals will have significant manipulations or severe discomfort, etc. without benefit of anesthesia, analgesics or tranquilizers. Examples to be included in this category are: toxicity testing, radiation sickness, irritants, burns, trauma, biologic toxins, virulence challenge, prolonged: restrictions of food or water intake, cold exposure, physical restraint or drug addiction. All use of paralytic agents (curare-like drugs) must be included in this category. Describe any abnormal environmental conditions that may be imposed. Describe and justify the use of any physical restrain devices employed longer then 1 hour.

VI. Justifications for Category G Studies and Deviations from Standard Techniques

Describe in the box below any steps to be taken to monitor potential or overt pain and/or distress during the course of this study and how such pain or distress will be alleviated. Be as detailed as necessary to justify your procedure.

VII. Certifications

(By submitting this protocol, I am acknowledging that I comply with the certifications included in Section VII.) (check one)

☐ Animal Use for Research. I certify that the above statements are true and the protocol stands as the original or is essentially the same as found in the grant application or program/project. The IACUC will be notified of any changes in the proposed project, or personnel, relative to this application, prior to proceeding with any animal experimentation. I will not purchase animals nor proceed with animal experimentation until approval by the IACUC is granted.

☐ Animal Use for Teaching/Demonstration. I certify that the information in this application is essentially the same as contained in the course outline and a copy of the laboratory exercises using animals is on file in the IACUC office. The IACUC will be notified of any changes in the proposed project, or personnel, relative to this application, prior to proceeding with any animal experimentation. I will not proceed with animal experimentation until approval by the IACUC is granted.
Estimate the cost of maintaining animals used in this protocol based on current per diem charge at University of Memphs.

Please specify cost per unit of time: $7.20/day ($0.24/cage/day)

Specify anticipated total costs for project duration: $605 (12 weeks)

As supervisor of this project it is required that you inform your department chair concerning any animal per diem costs related to this project that are to be paid by the department.

By submitting this protocol, the Principal Investigator/Course Director indicates that the following have been considered:

1. Alternatives to use of animals.
2. Reduction of pain and stress in animals to the lowest level possible.
3. The proper needs of the animals with respect to housing and care.
4. The lowest number of animals used that will give the appropriate experimental results.
5. Use of the most primitive species that will give the appropriate experimental results.
6. Proper training of all personnel in the care and handling of the species used and in the procedures called for in this protocol before beginning the experiment/teaching or demonstration.
7. That this protocol is not an unnecessary repeat of results already in the literature or in the case of teaching/demonstrations, results that can be demonstrated using models or video material.

Principal Investigator/Course Director (Type Name) Richard Bloomer and Ryan Wible

e-mail address rbloomer@memphis.edu and rswible@memphis.edu

Date 12/3/2018

Federal Law requires that members of the IACUC be given adequate time to read and review protocols including any changes or revisions in them.

Pre-review of protocols by the Attending Veterinarian is required before submission to the IACUC. New protocols or modifications or renewals to protocols must be submitted to the IACUC Chair by the 1st business day of the month to be considered for review during that month. Incomplete protocols will be returned to the principal investigator.

E-mail the completed protocol to the IACUC Chair, Dr. Amy de Jongh Curry, adejongh@memphis.edu

February, 2015