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The Effects Of Moringa Oleifera On Stress Induced Immune Modulation In Mice

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The Effects of *Moringa Oleifera* on Stress Induced Immune Modulation in Mice

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Biographical Sketch

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Dedication

I dedicate this work to God. I dedicate this work to my grandmother, Blanche Drue, who has always believed that I would accomplish great things and has always loved me. She was a lovely person and a great source of encouragement. My mother and father, Glenn, Sr. and Sharon Drue, who constantly support me and always believe that I will succeed and to my brother Jamar Drue who has always kept me determined. I dedicate this to my family.

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List of Abbreviations

ACTH	Adrenocorticotropic Hormones
BSE	Bovine Spongiform Encephalopathy
CD	Cluster of Differentiation
CDC	Centers for Disease Control and Prevention
CRH	Corticotrophin Releasing Hormones
EDTA	Ethylenediaminetetraacetic Acid
EGCG	Epigallocatechin Gallate
EPA	Environmental Protection Agency
FACS	Fluorescence-Activated Cell Sorting
FDA	Food and Drug Administration
g	Gram
hr	Hour
Ig	Immunoglobulin
IM	Intramuscular
LARU	Laboratory Animal Resource Unit
ml	Milliliter
MT	Moringa Tea
LPS	Lipopolysaccharide
NARMS	National Antimicrobial Resistance Monitoring System
NKT	Natural Killer T-Cells
SGE	*Sarcandra Glabra* Extract
WHO	World Health Organization

Abstract

In both humans and animals, stress causes the body to release adrenaline, cortisol and other stress hormones that cause changes within the body, which include modulation of the immune system. Stress in mammals can cause changes in the profile of immune cells circulating in the blood. Environmental and nutritional stressors can be considered two of the most common animal stressors. In the presence of stress there is an increase in the number of circulating neutrophils and a decrease in the number of lymphocytes within the blood. The physiological changes that occur in animals in response to acute and chronic stress have been shown to have a role in the etiology of many diseases and can lead to enhanced susceptibility to disease. The *Moringa oleifera* tree is a tree that is exceptionally rich in vitamins, minerals and antioxidants that promote health and immunity. The objective of my study was to evaluate the effect of *Moringa oleifera* on stress induced mice and monitor any change on lymphocyte and neutrophil numbers. Using a mouse model, we compared stress induced changes in neutrophil and lymphocyte numbers within the blood of mice that drank Moringa Tea to mice that drank water for 5 days. One 1-hr acute stress and 1 12-hr chronic stress experiments were performed with 1 replication each. It was hypothesized that stressed mice that drank tea would have a higher lymphocyte and neutrophil count than those that drank just water. The data show that after the 12 hr chronic stress period, the lymphocyte count was significantly increased ($t=4.128$, $p=0.0189$) in the stressed Moringa tea group compared to the non-stressed tea group, the neutrophil count also significantly increased in the stressed Moringa tea group when compared to both water/stress ($t=4.083$, $p=0.0126$) and tea/ no stress groups ($t= 4.263$, $p=0.0126$). These data suggest that Moringa may have the potential to be used as a supplemental feed ingredient that when added to the food ration of livestock animals could help alleviate stress induced immune modulation.

CHAPTER 1

Introduction

Stress is defined as an organism's total response to environmental demands or pressures that tend to displace homeostasis (Morgan & Tromborg, 2007). Shown to affect the body's ability to fend off diseases, viruses, and other infectious agents, it is a major problem among humans and animals alike. Stress also impairs the reproductive system, reducing libido and reproductive hormones, which in turn can increase the risk for cardiovascular and other diseases (Shakutsui, 1990). In livestock specifically, stress can affect meat quality, milk production, reproduction and general health. Reducing stress in livestock has been shown to provide advantages of increasing productivity and maintaining production quality (Grandin, 2010). The restraint stress model used in this experiment represents the real life stressors of livestock including transportation, squeeze chutes, overcrowding, handling, nutritional stress and lack of mobility, all of which effects immune modulation.

When the body is stressed, it releases stress hormones, such as Corticotrophin releasing hormones (CRH), Adrenocorticotropic Hormones (ACTH), arginine vasopressin, glucocorticoids, cortisol and corticosterone. ACTH stimulates the release of glucocorticoids and some of the actions of glucocorticoids help mediate the stress response, while other slower actions counteract the primary response to stress and help re-establish homeostasis (Bernabucci, et al, 2010). An imbalance in the stress hormones can causes negative health effects and improper immune modulation (Guo & DiPietro, 2010).

The immune system, which receives messages from the nervous system, is sensitive to stress hormones. The body responds to stress as if it is an infection, and stress hormones facilitate the movement of immune cells such as lymphocytes and neutrophils from the bloodstream and

storage organs, such as the spleen, into tissues where there is a need to defend against infection (Silberman, 2003). Although acute elevations of stress hormones actually facilitate immune function, sustained exposure to moderate to high levels of glucocorticoids acts to suppress immune function. Any imbalance, deficiency, or prolonged exposure to majority of the stress hormones can cause an inadequate immune response, inflammatory responses in the body, slowed wound healing, spikes in blood pressure, improper glucose metabolism, decreased muscle and bone density and many other undesirable effects in animals (Guo & DiPietro, 2010). Therefore chronic stress weakens the immune system, which leads to adverse effects on the body and susceptibility to diseases.

When stressed, people seek relief in many ways, such as exercise and breathing techniques. When livestock and other domestic animals become stressed, it can sometimes be difficult to notice or indicate the source of stress; then production of goods and food is ultimately affected. Animal health must often be monitored for things such as lower than anticipated weights and increased illness. The restraint stress model of this experiment represents several different causes of stress, as mentioned above, and will serve to provide insight on how Moringa tea effects the immune modulation of stress animals. The hypothesis of this experiment should prove *Moringa oleifera's* immune boosting properties.

A strong and balanced immune system is required for health maintenance. The immune system is composed of many interdependent cell types, each with specialized functions that collectively work to protect the body. The cells of the immune system can engulf bacteria, kill parasites or tumor cells, or kill virus-infected cells (Janeway & Travers, 1997).

The immune system protects the body from harmful substances such as viruses, fungi, or bacteria as well as toxins, chemicals, drugs, and foreign inorganic particles that cause diseases

and disorders. Pathogens which include bacteria, parasites, fungi or viruses can breach the bodies' defenses easier with a weakened immune system; they enter through wounds, mucous membranes, and through the respiratory system. Therefore, inefficient or improper immune responses allow diseases to develop or cause immune system disorders (Janeway & Travers, 1997).

Chronic and acute modulation of immune defenses induced by a variety of diseases and conditions places undue stress on the immune system, weakening its capacity to effectively deal with infectious organisms and other immunological requirements elsewhere in the body; such conditions include, anthrax, campylobacter, autoimmune disorders, coccidiosis, dermatitis, acidosis, food and other allergies, bovine spongiform encephalopathy (BSE), and chemical sensitivities. An immune system weakened by stress may lead to further susceptibility to disease.

The biggest problem for animal producers is disease management and any form of disease prevention would cut costs on lost product and maximize efficiency. Farmers often use small amounts of antibiotics to avert these effects. But sustained dosing can accelerate development of antibiotic-resistant bacteria, which may go on to infect humans. As possible alternatives to antibiotics and other chemicals, certain herbal elements can prove to be very beneficial for health and immunity. Using natural plant-based food products, it is possible to help restore immune system imbalance or weakness (Hulbert et al., 2011).

A diet rich in antioxidant vitamins and nutrients can boost immunity to help fight infection by protecting and repairing cells from damage caused by free radicals, which are unstable molecules that react with essential molecules of the body, including DNA, fat and proteins necessary for proper immune modulation. A wide variety of teas are known to have high levels of these immune boosting nutrients. Green, Matcha, and Moringa tea in particular, have become

popularly known as “super teas” mainly for their immunity boosting properties among many other qualities (Hasler, 2002).

The objective of my study was to evaluate the effect of *Moringa oleifera* on stress induced immune modulation using a mouse model and monitor any change on lymphocyte and neutrophil numbers during stress. Based on published data, it was hypothesized that stressed mice that drank water would have a lower lymphocyte count than those that drank tea (Murray, Lallman, Heard, Stenzel-Poore, & Rittenberg, 2001). Using *Moringa oleifera* to prevent infections due to stress could lead to lower disease-management costs, less antibiotic use, and healthier animals. Healthier animals ultimately mean that fewer disease causing microbes make their way to human consumers.

CHAPTER 2

Literature Review

2.1 Effects of Stress on the Body

According to Dantzer, O'connor, Freund, Johnson, & Kelley (2008), stress has usually been conceived as a reflex reaction that occurs ineluctably when animals are exposed to adverse environmental conditions, and which is the cause of many unfavorable consequences, ranging from discomfort to death. The inadequacy of this view is apparent from the new concepts that have been developed from research aimed at understanding the relationships between hormonal and behavioral reactions to stressful situations.

Psychological aspects of environmental stimuli are powerful activators of stress responses. The amount of psychological stress that an animal experiences determines how the immune system will respond. Dantzer & Mormède (1983) stated that the variable of emotional arousal reduces or eliminates responses to other stressors such as heat and cold, which means that one of the most important characteristics of the stress response, its non-specificity, lies in the afferent part of the response, not the efferent. Hormonal and behavioral responses are intimately related in stressful situations. In particular, the perception and ensuing behavior of the subject are critical to the nature and intensity of hormonal and immune response.

Adjustment abilities are limited by genetics and previous experience, and the respective role of each of these factors needs to be delineated more accurately. In addition, most experimental studies have been concerned with acute stress, while chronic multiple stress, which is more likely to be encountered in intensive husbandry, has not received much attention. According to Dantzer et al. (2008), the approach in this field is hampered by the lack of suitable physiological criteria to assess long-term adaptive changes. He further delineates the

opportunities for further research and the need for a more integrated view of stress reactions in farm animals.

Most farm animals will experience some level of stress during their lives. Stress reduces the fitness of an animal, which can be expressed through failure to achieve production performance standards, or through disease and death. Stress in farm animals can also have detrimental effects on the quality of food products. Research assessing stress in animals is important because of the concerns regarding livestock productivity, management practices, and animal welfare. Studies of free-ranging animals allow researchers the opportunity to investigate the effects of stress on individual species and theoretically on whole populations. In many instances when data have been collected on free-ranging animals, stress had been induced by humans and the stressors included fear, capture, handling, restraint, social separation and displacement from optimal habitat (Fowler, 2008).

Current studies of physiological stress range from detailed studies of neuro-anatomical structures and biochemical changes to the interactions of stress related hormones and their effects on other physiological systems (Brenner, Shek, Zamecnik, & Shephard, 1998). To better understand how to combat the effects of stress in the body, M. Wilson, Biscardi, Smith, & S. Wilson (1996) conducted an experiment with female rats, in which he increased the amount of a neurohormone called corticotrophin releasing factor (CRF), thought to be a driving factor of the body's response to stress in the rats' brains. As a result, rats experienced anxious and depressive behavior, such as a reduction in activity, decreased libido and disrupted ovarian cycles. The researchers hoped that by learning about this chemical, they could move toward designing ways for all species to fight the damaging effects of stress.

Stress is the body's reaction to any stimuli that disturb its equilibrium. When the equilibrium of various hormones is altered, the effect of these changes can be detrimental to the immune system. Research has shown a negative effect that stress has on the immune system, mostly through studies where participants were subjected to a variety of pathogens (Khansari, Murgo, & Faith, 1990).

Acute stress is immediate short term stress and quickly diminishes after the stress is alleviated. Short-term stressors that occur during handling and brief transport have been shown to interfere with the biological mechanisms of both reproduction and immune function (Marsland, Bachen, Cohen, Rabin, & Manuck, 2002). In contrast, chronic stress is defined as a state of prolonged tension from internal or external stressors, which may suppress the immune system. Chronic stress takes a more significant toll on the body than acute stress (Frick et al., 2009). It can raise blood pressure, increase the risk of heart attack and stroke, increase vulnerability to anxiety and depression, contribute to infertility, and hasten the aging process (Kort, 1994).

A study revealing the relationship between the immune system and the central nervous system indicate that stress can alter the function of white blood cells involved in immune function. During chronic stress, cortisol is over produced, causing fewer receptors to be produced on immune cells, so that inflammation cannot be ended on its own (Dantzer, 2008). Acute and chronic stresses tend to affect the immune system differently; most often chronic stress leads to an increased suppression of the immune response. Davis et al., (2006) reported that age of weaning, a common stressor in piglets, had no effect on lymphocyte proliferation. Often, acute stress has limited suppressive effects on immune function. Exposure to acute heat and shipping

stresses had no effects on various immune measures, but cold stress caused an increase in Natural Killer T-cell (NKT) cytotoxicity (Hicks et al., 1998).

Chronic stresses have differential effects on various aspects of the immune system. Chronic heat stress had no effect on induced lymphocyte proliferation in pigs, but proliferation was increased in pigs exposed to 14 d of heat and crowding stresses (Sutherland et al., 2006). In pigs, both prenatal stress and social isolation caused a reduction in lymphocyte proliferation (Kanitz, 2004). Furthermore, three days of transportation stress reduced lymphocyte proliferation in steers (Stanger et al., 2005).

Certain stressful events can be anticipated; for example, birth, weaning, handling and transport. Stress causes several undesirable effects, including slow animal growth and lowered immune response. Dietary supplementation beyond normal levels considered adequate might allow for more rapid or complete restoration of immunocompetence (K. Gupta, R. Gupta, Atreja, Verma, & Vishvkarma, 2009).

In addition to environment and nutrition, two of the most common animal stressors include handling and transportation (Dhabhar & McEwen, 2001). Transportation stressors can be physical (changes in temperature, humidity, or noise), physiological (limitation of access to food and water, overcrowding), and psychological (exposure to novel individuals or environments). The restraint stress induced mice used in this experiment represented the similarities of livestock handling and the stress that they may endure.

Much of the work done on handling and transportation stress has been led by Dr. Temple Grandin of Colorado State University. She is considered by many to be the leading researcher in this area and has devoted her career to researching and developing systems that will reduce stress and improve handling of animals during processing, transportation, slaughter, etc.

Research conducted by Grandin (1998), suggests that the stress of handling and transportation is largely induced by fear. Fear is a very strong stressor, and is basically a psychological stress or stress from how animals see, perceive or feel in any given situation. Some examples are restraint, contact with people, or exposure to novelty (anything they are not accustomed to). In cattle, previous experience and genetic factors affecting temperament will work together in complex ways to determine how fearful an animal may become when it is handled, restrained or transported, not only putting a strain on immune response but increasing the possibility of external injuries. The genetic factors are related to observations that certain breed types seem more excitable than others. The squeeze chute, which is a strongly built stall or cage for holding livestock safely while they are examined, marked, or given veterinary treatments, can be perceived as neutral and non-threatening to one animal; to another animal, the novelty of it may trigger intense fear. Novelty is a strong stressor when an animal is suddenly confronted with it. Procedures such as restraint in a squeeze chute do not usually cause significant pain, but fear may be a major psychological stressor in cattle unaccustomed to the handling (Grandin, 1998).

Stress can be harmful when it is long-lasting and animals are unable to adapt successfully to it (McEwen & Lasley, 2002), which further shows that an important distinguishing characteristic of stress is its duration. According to Dhabhar and McEwen (1997), acute stress is defined as stress that lasts for minutes, hours, or a few days; and chronic stress as stress that persists for months or years. Most transportation events last only a few days and are considered acute stress events. Even the transportation of animals from overseas does not take more than a few days, so there is little concern about chronic stress during transportation according to Belcha,

Boyles, & Riley (1984). However, care must be taken to minimize post-trip stress in order to ensure that animals are not chronically stressed.

Zhang et al., (2000), defined chronic stress for mice to be 12-hour daily physical restraint for two days. These mice exhibited a significant reduction in splenocytes, which consist of a variety of cell populations such as T and B lymphocytes, dendritic cells and macrophages, which have different immune functions.

2.2 Immune Response to Stress

Lymphocytes are subdivided into B and T-cells categories and are responsible for the specific recognition of foreign agents and their subsequent removal from the host. T cells multiply and differentiate into helper, regulatory, or cytotoxic T-cells or become memory T-cells. They are then sent to peripheral tissues or circulate in the blood or lymphatic system. B lymphocytes secrete antibodies, which are proteins that bind to foreign microorganisms in body tissues and mediate their destruction, or they can serve as helper cells to assist the production of antibody by B cells (Humphrey, 2002).

Keeping the immune system in balance is crucial for maintaining healthy animals. The immune system is very complex and care should be taken to ensure it stays in balance and strengthened. An underactive or weakened immune system will expose the body to increased susceptibility to infections and disease. Biological agents can harm the immune system by killing off helper T-cells, also called CD4 cells (Segerstrom & Miller, 2004). White blood cells are a cellular component of the blood that defends the body against infection and disease by ingesting foreign materials and cellular debris, by destroying infectious agents and cancer cells, or by producing antibodies (Humphrey, 2002). White blood cells are grouped into three major classes: lymphocytes, granulocytes, and monocytes.

Granulocytes are the most abundant of the white cells and free the body of large pathogenic organisms and are key mediators of allergies and other forms of inflammation. Granulocytes are subdivided into three categories: neutrophils, eosinophils, and basophils based on how their granules take up dye in the laboratory, which makes it easier to identify cells under a microscope for differential analysis (Kuwano & Abo, 2012). The RBCs are biconcave discs stained buff-pink, and the WBCs nucleus and cytoplasmic granules and platelet stain varying degrees of blue and pink (Sobecks & Theil, 2001).

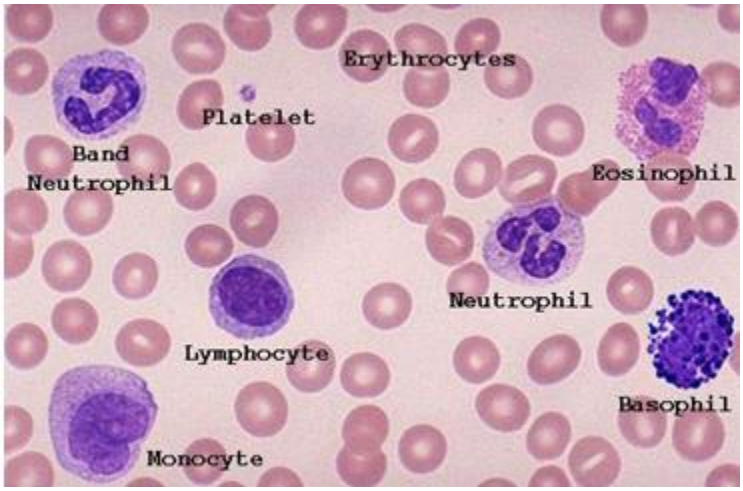

Figure 1. Wright-stained smear of normal blood (x1000) microscopic view of WBC

Neutrophils are the most numerous of the granulocytes making up 50 to 80% of all white blood cells. Neutrophils usually make up 50-70% of circulating white blood cells and serve as the primary defense against infections by destroying bacteria in the blood. They are often one of the first cell types to arrive at a site of infection, where they engulf and destroy the infectious microorganisms through a process called phagocytosis (Humphrey, 2002).

Neutrophils have a half-life of four to ten hours when not activated and immediate death upon ingesting a pathogen. They are plentiful and responsible for the bulk of an immune response. Neutrophils are present in the bloodstream until directed to a site of infection by

chemical cues in the body. Neutrophils are fast acting, arriving at the site of infection within an hour (Summers et al., 2010). In the presence of stress in a weakened immune system the lymphocyte count will go down while the neutrophil count will increase. However, according to Janeway & Travers (1997) when the body endures chronic stress, neutrophils will likely have died within the first couple of hours and will not be present to defend against infections during a chronic stress period. This means that during the 12-hr stress period, it can be expected that the neutrophil count should be lower when compared to the 1-hr acute stress period.

An imbalance in the immune system will compromise the body's protection from harmful substances such as viruses, fungi, or bacteria as well as toxins, chemicals, drugs, and foreign inorganic particles that cause diseases and disorders which have the potential to spread to human consumers.

2.3 Animal Health and Food Safety

Global demand for animal-based products, such as meat, milk, and eggs is growing due to population growth. Animal-based products play a highly important part in human nutrition. Animal and food production is one of the defining developments of humanity as a species. More than 70% of all human activity is food related (i.e. obtaining, growing, storing, distributing, processing, preparing and cooking, consuming, and waste management) (Bruemmer, 2003). Using animals as food sources is part of human culture, central to family, celebrations, hospitality, community and survival.

According to Blaha (1997), many countries have implemented mandatory meat inspection and strict guidelines for post-harvest food safety measures to improve the hygiene standards during slaughter, meat processing, storage and distribution which have led to a decline of meat related food-borne diseases. Although meat inspection and food hygiene have been

regarded as sufficient to guarantee safe meat, new approaches to food safety and meat quality are becoming necessary, as food borne diseases are still prevalent e.g. 5000 deaths per year in the USA (CDC, 2013). Mandatory meat inspections are indispensable, but unable to control and prevent the emerging food-borne pathogens. Food production has become more dependent on the reliability of the safety and the quality of the food and acceptability of the production procedures than on quantity and price.

Issues of food access, food safety, food security, food knowledge, and food production are of major importance to global stability. With animal production being crucial to the global population, there have been many developments in producing and growing animals quickly to supply the market (Rostagno, 2009). Livestock diseases compromise animal welfare, reduce productivity, and can infect humans. Animal diseases may be reduced through animal husbandry, or reduced through antibiotics and vaccines. It has been believed that the daily administration of antibiotics to animals would make most animals gain as much as 3% more weight than normal (Navarro-Gonzalez, Mentaberre, Porrero, & Serrano, 2012). In an industry where profits are based on quantity the discovery of this increase in weight gain was ground-breaking.

However, the use of antibiotics can create antibiotic resistant bacteria and other microorganisms that can infiltrate a weakened, stress induced immune system and can be passed on to consumers or cause production loss. Natural feed supplements, such as *Moringa oleifera*, have the potential to decrease antibiotic use. Antibiotics have been used in animal feed for years, not only as an anti-microbial agent, but also as a growth-promoting agent and improvement in performance. Tetracyclines, penicillin, streptomycin and bacitracin began to be common additives in feed for livestock and poultry (Verraes et al., 2013). In addition to the common antibiotics, which are of microbial origin, there are other chemically synthesized antimicrobial

agents that are also sometimes used in animal feeds, which include arsenical, Nitrofurantoin, and sulfa compounds, which may be harmless in small amounts, but none of which are natural or healthy and prolonged exposure could possibly have adverse effects on consumers (NARMS, 2014).

Antibiotic use includes efficient conversion of feed to animal products, an increased growth rate and a lower morbidity/mortality rate in general, which is more beneficial financially. The levels of antibiotics are often increased to a few hundred grams/ton when specific diseases are being targeted or known to be spreading (Tenover, 2006). The levels can also be increased in times of weakened immunity such as stress. According to Martinez (2008), after animals have been fed antibiotics over a period of time, they retain the strains of bacteria which are resistant to antibiotics. The bacteria proliferate in the animal. Through interaction, the resistant bacteria are transmitted to the other animals, forming a colonization of antibiotic resistant bacteria. The bacteria flourish in the intestinal flora of the animal, as well as, in the muscle. Transfer of the bacteria from animal to human is possible through many practices. The primary exposure of humans to resistant bacteria occurs in farms and slaughterhouses. Antimicrobial resistant zoonotic pathogens present on food constitute a direct risk to public health (Phillips, 2003).

Hiroshi Nakajima, Director General of the World Health Organization (1988-1996), in his Word Health Report 1996 stated that "Antimicrobials are used in meat production to increase growth, but not usually in sufficient amounts to kill microbes. Drug-resistant bacteria are then passed through the food chain to the consumer". Each time bacteria are exposed to an insufficient dose of antibiotics, they develop stronger defenses (Tenover, 2006).

In addition to the disadvantages of antibiotic use, consumers want products that are raised organically or naturally, without the use of antibiotics or hormones. These ideals create new consumer demands and increase a need for higher quality natural meat and animal products.

2.4 Nutritional Supplements and *Moringa Oleifera*

There are many forms of natural and non-chemical means of improving health and strengthening the immune system naturally. Vitamins C and E, β carotene, selenium and zinc have all been shown to help strengthen the immune system and are present in *Moringa oleifera*. Other immune-boosting foods include fresh garlic, which has claims of antiviral and antibacterial properties. In addition, mushroom varieties such as reishi, maitake, and shiitake may have some influence on immune function (Borchers, Stern, Hackman, Keen, & Gershwin, 1999).

Echinacea is a plant said to stimulate the immune system as well as fight against infection causing germs and bacteria. In combination with other herbs like thyme, uva ursi, garlic, grape root, myrrh, mullein, and colloidal silver, it may be used for any infection or inflammation anywhere in the body. It is often effective against both bacterial and viral attacks (South & Exon, 2001).

Another plant called Astragalus membrane, acts as an immune system stimulant. Garlic strengthens the body's immunity and fights against gastrointestinal infections. Licorice roots are mainly used in Asia, and very beneficial for the rejuvenation of the immune system and for fighting against various illnesses. Probiotics are also very significant for the body as they provide the body with "good" bacteria, which are required for correcting deficiencies and increasing the number of T-cells in the immune system (Madsen, 2006).

Moringa oleifera is well known for its different therapeutic uses and is considered a "super tea" known for super antioxidative properties. Moringa tea is rich in catechin

polyphenols, particularly epigallocatechin gallate (EGCG), which is a powerful anti-oxidant (Wang, 2006). *Moringa oleifera* is the best known and most widely distributed species of *Moringaceae* family. This plant being native to the western and sub-Himalayan tracts, India, Pakistan, Asia, and Africa and is well distributed in the Philippines, Cambodia, America, and the Caribbean Islands (Adejumo, Kolapo, & Folarin, 2012).

For centuries, almost every part of the Moringa tree has been consumed by humans and used for various domestic purposes as for animal forage, alley cropping, bio-pesticides, domestic cleaning agents, fertilizer, foliar nutrient, green manure, gum, honey and sugar cane juice-clarifier (powdered seeds), ornamental plantings, pulp, rope, tannin for tanning hides, water purification, machine lubrication from the oil, manufacture of perfume, and hair care products (Sankhyan, 2013).

Moringa oleifera possesses antitumor, antipyretic, antiepileptic, anti-inflammatory, antiulcer, antispasmodic, diuretic, antihypertensive, cholesterol-lowering, antioxidant, anti-diabetic, and renal- and hepatoprotective activities (Anwar, Latif, Ashraf, & Gilani, 2007). Phytochemicals occur naturally in fruits and vegetables and have the potential counter the effects of imbalances in immune modulation and diseases such as cancer, stroke or metabolic syndrome. (Sharma & Paliwal, 2012). Early studies documented the presence of pytochemicals such as, phenolics, flavonoids, saponins, terpenoids, proanthocyanadins, and cardiac glycosides in the pods of *Moringa oleifera*.

The leaves of *Moringa oleifera* have been reported to be a valuable source of nutrients; it is a rich source of *β*-carotene, protein, vitamin C, calcium, and potassium and act as a good source of natural antioxidants (Sankhyan, 2013). The fresh leaf juice was found to inhibit the growth of human pathogens as *Staphylococcus aureus* and *Pseudomonas aeruginosa (*Rahman et

al., 2009). Various parts of the plant and their active constituents have been shown to possess diverse biological activity, but Sankhyan (2013) further states that little was known scientifically about the antioxidant potential of the leaves of *Moringa oleifera*.

The *in vitro* antioxidant properties and its curative role were investigated in a study by Fakurazi, Sharifudin, & Arulselvan (2012), where *Moringa oleifera* extracts were used in acetaminophen induced toxic liver injury in rats caused by oxidative damage. The results of this study strongly indicated the therapeutic properties of Moringa hydroethanolic extracts against acute liver injury and thereby scientifically support its traditional use.

A comparative analysis of the concentration and dose dependent effect of ethanolic and aqueous extract of *Moringa oleifera* leaves and fruit on markers of oxidative stress, its safety profile in a mice model, and correlation with antioxidant properties using *in vitro* and *in vivo* assays was investigated, established, and explained in a study by Luqman, Srivastava, Kumar, Maurya, & Chanda (2012). The antioxidant capacity of ethanolic extract of both fruit and leaf was higher in the in vitro assay compared to aqueous extract; which showed higher potential in vivo. Safety evaluation studies showed no toxicity of the extracts up to a dose of 100mg/kg body weight. The results support the potent antioxidant activity of aqueous and ethanolic extract of *Moringa oleifera*. Further investigations on isolation, characterization, and identification of active phytoconstituents responsible for the protection of oxidative stress and antioxidant activity are warranted for future work.

Moringa tea leaves are freeze dried, which prevents the EGCG compound from being oxidized, unlike other teas, such as black or oolong leaves which are made from fermented leaves (Shankar, 2008). The fermentation results in the EGCG being converted into other compounds that are not nearly as effective in preventing and fighting various diseases

(Cambridge, 2013). *Moringa oleifera* is considered a "super food", or a nutrient-rich food considered to be especially highly beneficial for health and well-being.

Brunswick Laboratories performed an independent study (Ou, Hampsch-Woodill, & Prior, 2001) using Moringa to test its Oxygen Radical Absorbance Capacity, or ORAC value score. The ORAC value score is an accepted measurement of antioxidants in foods and supplements. Receiving a higher score than other foods known to be considered "super foods", moringa received a score of 157,000 µmol TE/100g (hydrophilic and lipophilic). Matcha tea is a powered version of traditional green tea and the only version of green tea in which the whole leaf is consumed to provide the most health benefits. Matcha tea scored 134,000, turmeric at 127,000 and acai at 102,700. The green tea that is bought at the local grocer and is renowned for its antioxidant health benefits only scored 1,240.

Moringa tea leaves are also abundant in both essential and non-essential amino acids such as arginine and cystine. Building up amino acids would improve a weakened immune system especially if the body is not producing non-essential amino acids properly. For example, arginine, a non-essential amino acid, has been shown in studies (Collier, Casey, & Kanaley, 2005) to cause the release of the growth hormones considered crucial for optimal muscle growth and tissue repair. It also improves immune responses to bacteria, viruses, and tumor cells while promoting the healing of body's wounds (Guo, 2010). Cystine, another non-essential amino acid, functions as an antioxidant and is a powerful aid to the body in protecting against radiation and pollution. It deactivates free radicals, neutralizes toxins, aids in protein synthesis and essential for the formation of new skin cells, which aids in recovery (Kroll, Becker, & Schneider, 1974).

All of the nutritional benefits of Moringa tea show to be a possible replacement for antibiotics and other chemical additives to animal feed and could possibly help to boost

immunity. To supply the demand of more natural and organic materials, Moringa tea has the potential to be a safe alternative to antibiotics and other chemicals that strengthen immune function and prevent infection and disease in animals.

Restraint stress in mice has been shown to cause high levels of plasma adrenocorticotropic releasing hormone (ACTH), corticotrophin releasing hormone (CRH) and changes in the levels of immune cells in the blood of Balb/c mice (Flint & Tinkle, 2001). One hour of restraint in mice has been shown to enhance dendritic cell, neutrophil, macrophage, and lymphocyte trafficking, maturation, and function. It has also been shown to augment innate and adaptive immune responses and to cause an increase in neutrophils and a decrease in lymphocytes (Dhabhar, 2008). Twelve hour restraint is shown to weaken the immune system, not only does it make the body more vulnerable to catching illnesses but can also impair their immune system's ability to respond to its own anti-inflammatory signals that are triggered by certain hormones, possibly altering the course of an inflammatory disease, strain the heart and damage memory cells in the brain (Miller et al., 2002).

Research conducted by Dhabhar (1997) states that different strains of rodents respond differently to the effects of restraint. BALB/c mice appear to be highly responsive to stressors behaviorally, as they exhibit more anxiety producing high levels of ACTH and CRH. Therefore Balb/c mice were used as a model to monitor the effects of Moringa tea on modulation of lymphocytes and neutrophils in the blood of mice under acute and chronic stress.

In the literature, there are different definitions for the length of time in acute and chronic stress studies in mice. The majority of the literature using mice utilized a 12 hour time frame to induce chronic stress. In a study by Zhang (2000), it was reported that mice subjected to chronic 12-hour daily physical restraint for two days exhibited a significant reduction in splenocytes.

Recently, in a study by Voorhees et al. (2013) 6 hours daily stress was used for the span of 28 days. Another study by Bitgul, Tekmen, Keles, & Oktay (2013) 6 hours daily stress was used for the span of 32 days. On the other hand, acute stress defined by Maroun et al. (2003), was defined between 1 and 5 hours. It was decided to use 1 hour stress as a model for acute stress and 12 hour stress as a model for chronic stress to be consistent with previous studies.

CHAPTER 3

Methodology

3.1 Mice

All experimental procedures in this study involving animals have been approved by the North Carolina A&T State University Institutional Animal Care and Use Committee. Forty eight female Balb/c mice between 5 to 7 weeks of age at the onset of each experiment, purchased from Harlan Laboratory in Indianapolis, Indiana were used in this study. A total of 2 experiments with 1 replication each were run with 12 mice in each experiment and 12 in each replication. All mice were maintained in the Laboratory Animal Resource Unit of North Carolina A&T State University and were used in accordance with applicable regulations. All mice were on a 12 light/12 dark cycle and the temperature of the mouse room was consistently 68-70^{O}F with consistent 58-60% humidity. Ear notching was used for identification purposes. During the 5 days, prior to stress, mice were fed *ad libitum.* Mice were minimally handled during recording of weights using an electronically balanced scale.

3.2 Treatments

Both experiments had 1 replication, all utilizing groups of 12 mice divided into 4 treatment groups consisting of 3 mice per cage. One 1-hr acute stress experiment and 1 12-hr chronic stress experiment were performed with 1 replication each.

Experiments and their replications were performed during 4 separate weeks, one week per replication for each group of 12 mice. Experiment 1 compared 12 mice receiving no stress with 12 mice receiving 1-hr stress, with half of the mice in each stress condition receiving either tea or water as shown in table 1. Weeks 1 and 2 represent replications with 12 mice per replication. Data were combined for both repetitions in both experiments for statistical analysis.

Experiment 2 compared 12 mice receiving no stress with 12 mice receiving 12-hr stress, using the same design as in experiment 1.

Table 1

Experiment 1 Treatment Groups

		No Stress	**1 hour Stress**
Water	**Wk1**	Water	Water
	Wk2	No Stress	1-hr Stress
Tea	**Wk1**	Tea	Tea
	Wk2	No Stress	1-hr Stress

Table 2

Experiment 2 Treatment Groups

		No Stress	**12 hour Stress**
Water	**Wk3**	Water	Water
	Wk4	No Stress	12-hr Stress
Tea	**Wk3**	Tea	Tea
	Wk4	No Stress	12-hr Stress

3.3 Moringa Tea Preparation and Consumption

Approximately, 50g of dried moringa oleifera leaves were steeped in 5 L of boiling distilled water at 95^0C under for 30 minutes. The resulting tea was poured through sterile cheese cloth into 3 L sterile beakers to filter out the steeped leaves, pouring 2.5 L at a time. The cheese cloth filtered tea was then poured through a filter paper lined funnel into a vacuum flask. Once all of the tea had been filtered into the vacuum flask, a final filtration was performed using a sterile 250ML 0.22µM PES vacuum filter system (Celltreat, Largo FL.). The resulting tea was

aliquoted into 50ml conical tubes under a laminar flow hood and stored at 4^0C until used. Each group of 12 mice was given a fresh 100 ml of distilled water or Moringa tea every morning for 5 days. The consumption of water or tea was calculated each day by subtracting the amount of liquid remaining from the amount of liquid offered using a graduated cylinder.

Figure 2. Mice divided in cages according to treatment group

3.4 Stress

3.4.1 1-hr stress. On the morning of day 5, mice were placed into narrow cylinder restraints prepared from 33mm polyethylene flat-top with screw caps for 1 hour (figure 3). The restraint tubes allowed breathing but no further movement to induce stress for 1 hour with no food or water. The restraint was performed in a room adjacent to the experimental room to ensure quiet conditions. One hour stress trials were completed during 9:00am – 10:00am. Feed and water was removed from the non-stressed animals during the 1 h stress period.

Figure 3. Mice in restraint tubes during stress period

3.4.2 12-hr stress. On day 5, the stress treatment mice were placed into 33mm polyethylene flat-top with screw cap restraints as described, for 12 hours with no food or water. The restraint was performed in a room adjacent to the experimental room to ensure quiet conditions from 6:00am-6:00pm. Feed and water was removed from the non-stressed animals during the 12 h stress period.

3.5 Red Blood Cell Collection

Immediately following either stress period, mice were placed in an anesthesia chamber and anesthetized using Isoflurane (Baxter, Deerfield, IL). Approximately 200- 500ul of blood was collected from each mouse through cardiac puncture for analysis by flow cytometry and a drop of blood was used to prepare blood smear for staining and differential counts. Death was assured by cervical dislocation. To prevent coagulation of the blood, 50 µl of 0.5 EDTA was mixed into each blood sample used for flow cytometry.

3.6 Differential Cell Staining

A WBC differential cell count was performed to determine the percentage of each type of white blood cell present in the mouse blood. The slides were made using fresh mouse blood smeared onto the slides. The prepared slides were dried, fixed and were stained using HEMA 3 Stain Set (Protocol, Pittsburgh, PA), utilizing a three-step staining procedure comparable to the Wright-Giemsa method (Appendix D). Cover slips were affixed to each slide and analyzed using light microscopy under 100x magnification in oil to manually count the number of each WBC type. The WBCs were counted based on color and morphologic criteria. Approximately 100 WBC were counted per slide.

3.7 Fluorescence Activated Cell Sorter (FACS) Staining

The collected blood was temporarily stored on ice until blood was collected from all animals in that replication. The red blood cells were incubated with 2ml of 1X multi-species RBC lysis buffer for 5 min at room temp (eBioscience, San Diego, CA) to lyse RBC with minimal effect on WBC. The samples were centrifuged at 1000 RPM for 5 minutes.

The supernatant was poured off and the pellets were resuspended with 5 µl of purified rat IgG_{2b} anti-mouse CD16/CD32 monoclonal antibody (Cat. No. 553141) (Fc) Block for 10 minutes on ice to prevent non-specific binding of FACS antibodies to mouse Fc receptors. All FACS antibodies used in this study were obtained from BD Biosciences.

Anti- CD3 FITC was used to identify T-cells, anti- CD4 PE was used as a marker for T helper cells, anti- CD8 APC was used to detect killer T-cells, Anti- CD19 APC and PerCP cy 5.5 were used to detect B cells, CD45 PerCP Cy 5.5 was used to detect all white blood cells and anti-Ly6G PE was used to detect neutrophils. Cells were stained in 96 well U bottom plates and incubated with antibody on ice in the dark for 30 minutes. Two combinations of antibody were

used. Plate 1 – comparing T cells, neutrophils and B-cells (CD3 FITC, Ly6G PE, CD45 PerCP Cy 5.5, CD19 APC) Plate 2- comparing (Helper- T-cells, killer T-cells and B-cells (CD3 FITC, CD4 PE, CD8 APC, CD19 PerCP cy 5.5). After 30 minutes, the cells then were washed by adding 200 µl of FACS wash and centrifuged at 1500 RPM for 2 minutes. Supernatant was removed, the pellet was resuspended, and the wash was repeated. The cells then were fixed with 100 µl of 1% paraformaldehyde incubated 10 min on ice followed by an additional wash as described. Finally cells were resuspended in 200 µl of FACS buffer. Accuri C6 Flow Cytometer (BD Biosciences, San Jose, CA). All samples were analyzed immediately after FACS staining protocols or refrigerated and analyzed the following day.

3.8 Statistical Analysis

A Completely randomized one-way ANOVA with Bonferroni post hoc tests was used to perform statistical analysis on the data generated for differential cell analysis and overall flow cytometry of lymphocyte and neutrophil counts in all stress groups. A paired student t-test was used on data generated for weight, consumption and T-cell flow cytometry. All data were analyzed to discover if any differences existed within each set of data. Data from replications 1 & 2 were combined, as were the data from replications 3 & 4 for statistical analysis Threshold was set at p-value of 0.05 or below via paired student t-tests, and for ANOVA Bonferroni post hoc tests using Graphpad Prism Version 5.00 (La Jolla, CA).

CHAPTER 4

Results

4.1 Mice Show a Higher Consumption of Moringa Tea than Water

We have previously shown that mice consume tea at a similar rate or higher than water (Minor & Smith (2013). Here we showed that tea groups drank more fluid than the water groups ($p<0.05$, $df=3$) in Figure 4. The tea group performed significantly different than the water groups at $p= 0.0027$, across 5 days, showing that the mice preferred to drink the tea versus the water. In addition, the water groups consumed more feed across 5 days, and the tea group consumed significantly less feed than the water group at $p = 0.0291$.

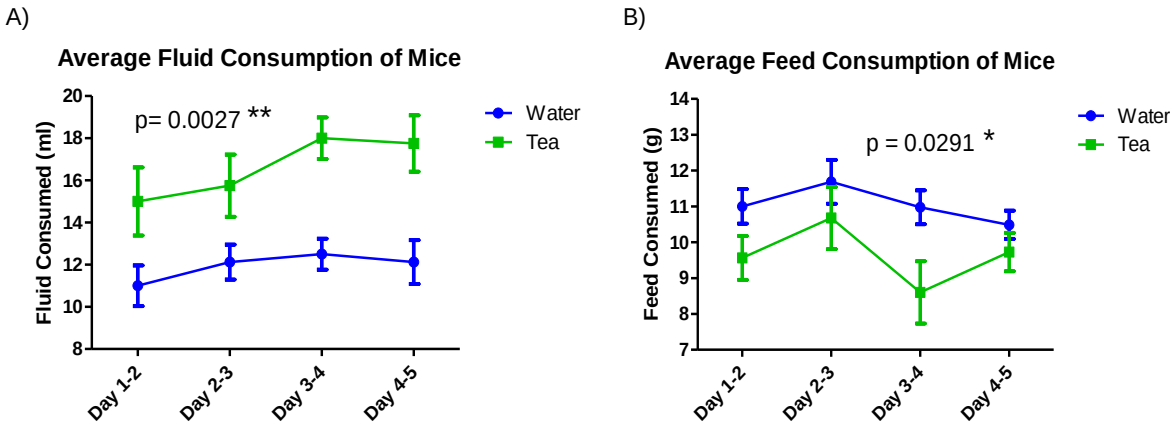

Figure 4. Average feed and fluid consumption of mice over 5 days.

4.2 Consumption of Moringa Tea Does Not Cause Changes in Weight

Although the water groups consumed feed at a significantly greater rate than the tea group, weight gain between the groups was not significantly different at $p>0.05$, $t=1.395$, $df=4$ (Figure 5). This suggests that, if the nutrient rich Moringa tea was to replace water as a daily supplement, it would not offset the normal expectancy of animal weight. Mice were minimally handled during recording of weights using an electronic balanced scale for 5 days during normal feeding and the consumption of Moringa tea or Water.

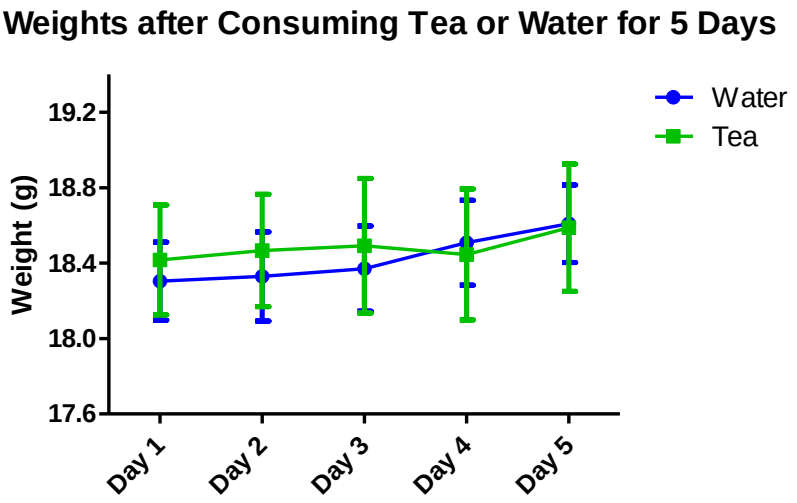

Figure 5. Average weight of mice consuming moringa tea or water.

4.3 Moringa Tea Consumption Mitigates the Immune Modulation in Mice Stressed for 1 Hour but Not 12 Hours.

The results show that Moringa tea consumption may mitigate the immune modulation in mice Stressed for 1 hr but not after 12 Hrs. Previous studies in the lab have shown that mice that consume Moringa tea have decreased neutrophil infiltration into the lung after inhalation challenge with lipopolysaccharide (LPS), a potent inflammatory mediator (Minor & Smith, 2013). Stress causes changes in the levels of neutrophils and lymphocytes in the blood (Hulbert et al., 2011) therefore; for this study we were specifically interested in investigating if Moringa consumption has an effect on the stress induced modulation of neutrophil and lymphocytes in the blood following 1 hr and 12 hr restraint induced stress.

As expected, differential cell analysis of blood collected from mice after the 1 hour stress period showed a significant decrease ($t=4.032$, $p= 0.0006$) in the lymphocyte numbers within the blood of mice that drank water and were restrained for 1 hr as compared to mice with water/no stress to be consistent with method group using a Completely Randomized One Way ANOVA (Figure 6A). Furthermore, there was a non-significant trend toward an increase in the number of

neutrophils (Figure 6B) in the blood of mice that consumed water. In contrast, mice that consumed Moringa tea showed an even smaller decrease in lymphocytes and no change in neutrophil count within the blood (Figure 6A and B).

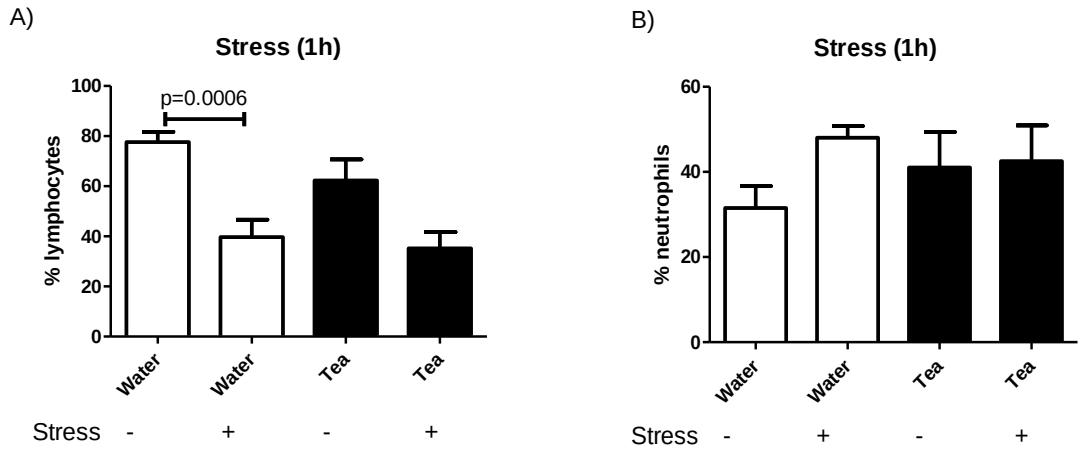

Figure 6. Differential analysis of lymphocytes (A) and neutrophils (B) in mouse blood samples lymphocytes (A) and neutrophils (B) under 1 hour stress.

To determine the concentration of lymphocytes and neutrophils, a WBC differential was performed which determined the cell count of each type of white blood cell present in the mouse blood. The slides were made using fresh blood smears and were stained using HEMA 3 Stain Set to show the percentage of lymphocytes (A) and neutrophils (B) cells per slide.

A study by Murray, Lallman, Heard, Stenzel-Poore, & Rittenberg (2001) showed that 12 hours of stress also result in decreases in lymphocytes in the blood. Therefore, it was expected that stressed mice that drank water may have a lower lymphocyte count following 12 hour stress period. Furthermore, based on research by Oliver (2001), it was also expected that the neutrophil numbers would decrease after 12 hour stress period.

We report that mice that were placed into the restraints for 12 hours showed a similar, but non-significant decrease in the percentages of circulating lymphocytes in mice that drank water

as compared to tea (figure 7A). However, while there was no change in the percentages of neutrophils in the blood of mice that drank water, there was a non-significant trend toward an increase neutrophil level within the mice that drank Moringa tea (figure 7B). This suggests that Moringa tea may not mitigate the immune modulation of lymphocytes and neutrophils after 12 hours of stress.

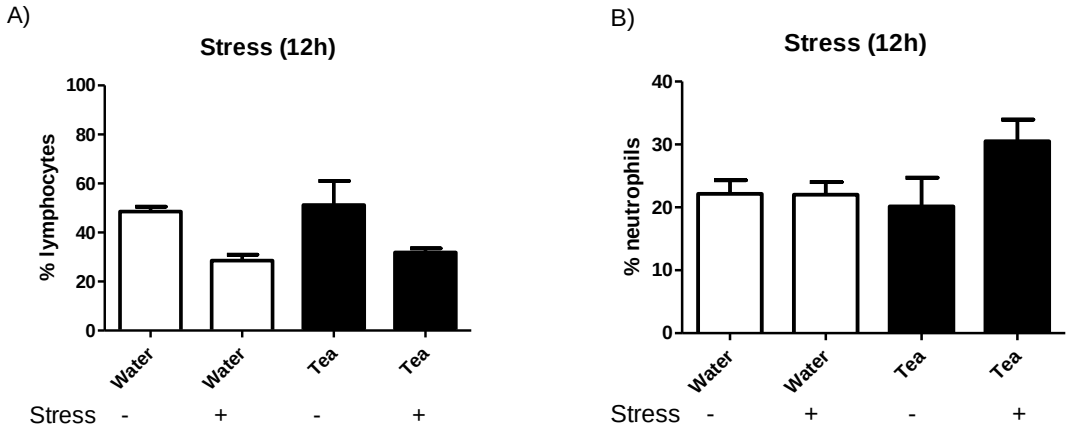

Figure 7. Differential analysis of lymphocytes (A) and neutrophils (B) in mouse blood samples under 12 hour stress.

4.4 The Effect of Moringa Tea Consumption on Lymphocyte and Neutrophil Counts During 12 Hour Stress.

The data in figures 6 and 7 show that the lymphocyte populations within in the blood of mice that experience stress for both 1 and 12 hours exhibit some differences as a result of tea or water consumption. However, because lymphocytes are a category of white blood cells that includes specific cells types such as T-cells (CD4+ and CD8+) and B-cells that cannot be distinguished by differential cell staining and counting, we performed flow cytometry on the mouse blood to examine the effects on these specific cell types. Flow cytometry allows for cell types to be distinguished and counted using antibodies that recognize cell-specific surface markers. Therefore, we used flow cytometry analysis to determine if there were changes in the

number of specific cell types, focusing specifically on T-cells; (CD3+), helper T-cells (CD4+), killer T-cells (CD8+), and B cells (CD19+). We also stained cells with (LY6G+) to compare the changes in neutrophils. Although, flow cytometry was performed for trial 1 and trial 2, the cell staining for trial 1 was not optimal and, therefore, we do not have data to report. For trial 2, we report that, similar to differential cell counts shown in figure 7B, stressed mice that drank tea had a greater modulation of granulocytes in the blood as compared to the non-stress tea group (Figure 8A). Furthermore, for the granulocytes, their total percentage of neutrophils increased significantly as compared to the mice that drank water (t=4.263, p=0.0094) (Figure 8B).

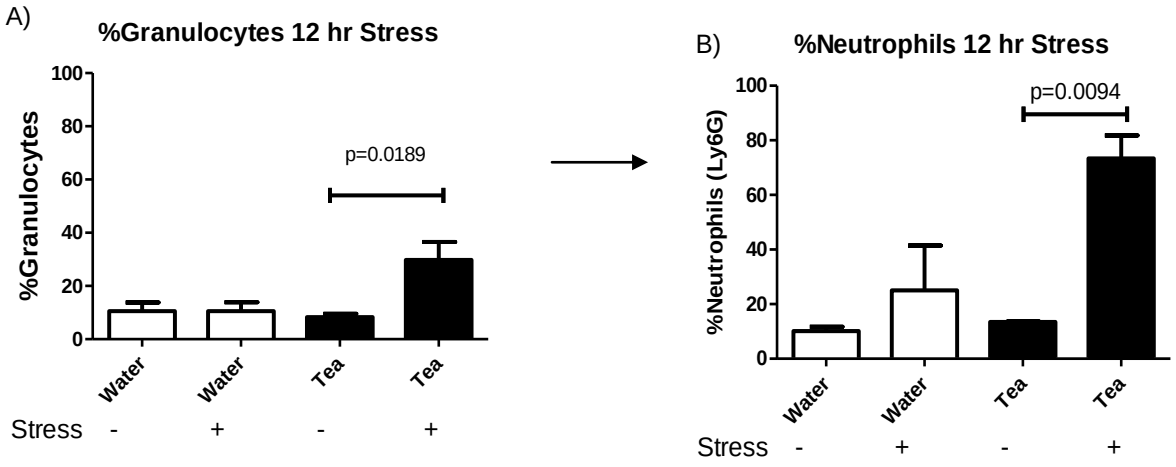

Figure 8. The percent granulocytes and neutrophils after 12 hour stress period using flow cytometry.

The data above indicate that there was a significant increase in the number of neutrophils for tea after the 12 hour stress. In addition, by flow cytometry analysis the data show that the lymphocytes count was significantly increased (t=4.128, p=0.0189) in the stressed Moringa tea group compared to the non-stressed tea group (Figure 9A). Again, using flow cytometry we sought to further characterize the lymphocytes, focusing specifically on B-cells and T-cells. We report that there were no significant differences in both types of lymphocytes as a function of

stress for the tea groups. A one-way ANOVA, coupled with Bonferroni's multiple comparison tests to evaluate differences in total WBC counts of helper (CD4) and killer (CD8) T cells showed that a there was no change in the number of helper T-cells or killer T-cells. (Figure 9B and C).

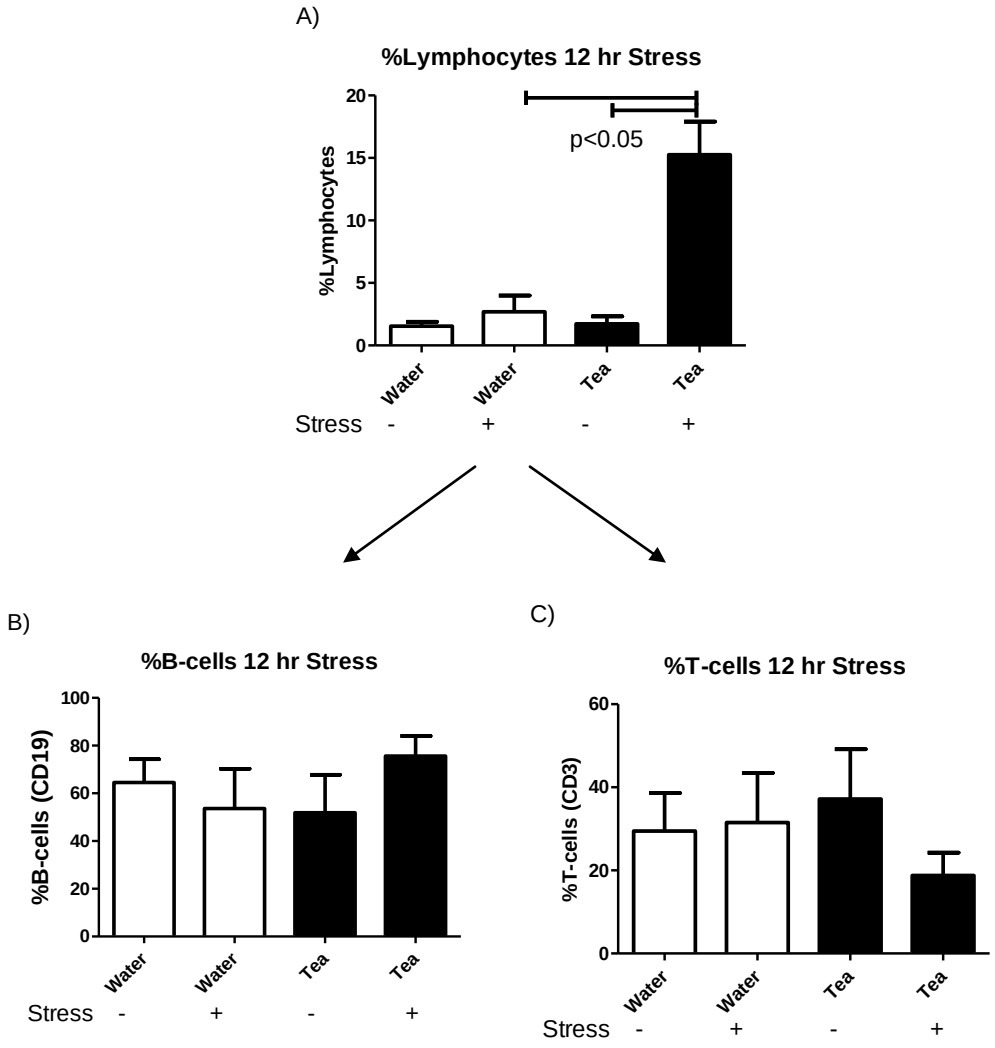

Figure 9. The percent T and B-cells after 12 hour stress period using flow cytometry.

When tea was consumed, there was no change in the number of helper T-cells or killer T cells by flow cytometry after 12 hours of restraint stress (Figure 10 A and B). The co-receptors CD4 and CD8 showed no significant differences using a one-way ANOVA coupled with

Bonferroni's multiple comparison tests. This could be an indication that the rise in lymphocytes in stressed mice that consumed tea may not have resulted from a rise in T-cells or in B-cells. This also shows that there was no change in the number of helper T-cells or killer T-cells when tea was consumed after 12 hours of restraint stress. This may mean that the rise in lymphocytes in stressed mice that consumed tea may not have resulted from a rise in helper or killer T-cells.

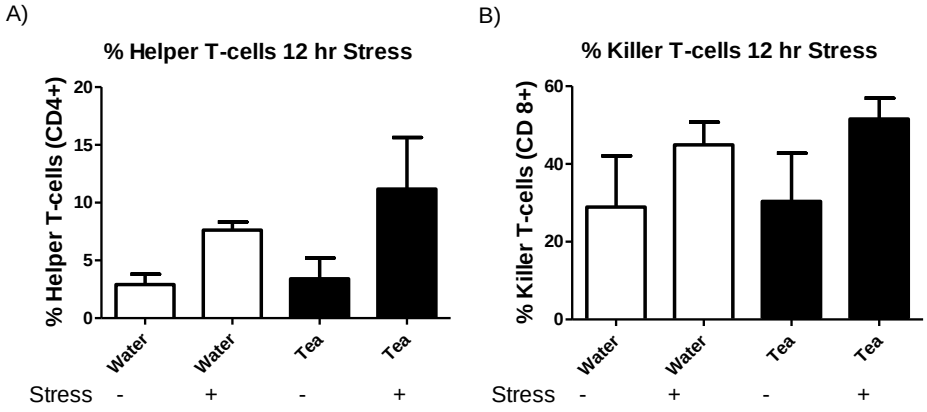

Figure 10. The percentage of helper T-cells and killer T-cells after 12 hour stress period using flow cytometry.

The objective of this study was to by using Moringa tea, monitor any change in the percentage of lymphocytes and neutrophils. The data show that after the 12 hr stress period, the lymphocyte count was significantly increased ($t=4.128$, $p=0.0189$) in the stressed Moringa tea group compared to the non-stressed tea group, the neutrophil count also significantly increased in the stressed Moringa tea group when compared to both water/stress ($t=4.083$, $p=0.0126$) and tea/ no stress groups ($t= 4.263$, $p=0.0126$).

CHAPTER 5

Discussion and Future Research

The long-term goal of this lab is to find solutions for current and emerging agricultural health and disease issues by identifying immune enhancing nutritional supplements that promote the health of feed and fiber animals. There are many factors that may influence immune responses in an animal confronted with stress; stressor types, duration of the stressors, chronic or acute, genetics, age, and many others, such as time of sample in relation to circadian rhythms, time of sample relative to the onset of stress, the activation of stress hormones, and pathogen exposure.

Many factors influence the stress responsiveness and the immune response of an animal to stress. Stress can suppress the immune response of an animal and can allow pathogens to cause disease affect meat quality, milk production, egg production, reproduction and general health. Neutrophils, one indication of stress, have a half-life of 4 to 10 hours when not activated, and immediate death upon ingesting a pathogen. Neutrophils are present in the bloodstream until signaled to a site of infection by chemical and hormonal cues in the body. Neutrophils have a quick response and arrive at the site of infection within an hour (Butterfield, Best, & Merrick, 2006). When the body endures chronic stress, the half-life of neutrophils will likely have expired. Further research is required to determine if new neutrophils are activated during chronic stress or if they will have died and will not be present to defend against infections during a longer periods of stress.

The objective of my study was to determine if Moringa tea had any effect on lymphocyte and neutrophil numbers during stress. Based on published data it was expected that stressed mice that drank water would have a lower lymphocyte count than those that drank tea (Murray,

Lallman, Heard, Stenzel-Poore, & Rittenberg, 2001). It was also expected that neutrophil numbers would decrease after a 12 hour chronic stress period, based on Oliver, Mathew, Wilder, & Cronstein (2011).

Figure 4A shows that the mice preferred to drink the tea versus water showing that it would increase fluid intake if Moringa tea was supplemented in the water supply. The water groups consumed significantly more feed than the tea groups $p < 0.05$; however there were no changes in weight gain. This suggests that if the nutrient rich Moringa tea was to replace water as a daily supplement, it would not offset the normal expectancy of animal weight. Given the nutritional value of Moringa tea, it may be accurate to say that the Moringa tea groups significantly received more immunity boosting nutrients than the water groups, while the water groups that consumed more feed were just getting regular nutrition.

The results in figure 7B show that neutrophils were increased in the blood of mice that drank the tea, but our data does not resolve whether these cells have an increased half-life and survive longer or if there is an increase production of new neutrophils being released from the bone marrow to respond to the stress. We report that mice that were placed into the restraints for 12 hours had similar changes in the percentages of circulating lymphocytes by differential cell counting (Figures 7A). However, we detected increases in the number of lymphocytes in the blood (Figure 9A) by flow cytometry. It has been reported by Murray et al. (2001) that the lymphocyte numbers severely decrease during chronic stress. Our data suggests that supplementation with *Moringa oleifera* may increase overall granulocytes and lymphocyte counts during longer stress periods.

There is a difference between the number of cells counted between the differential cell analysis and flow cytometry, which may explain our different findings with different techniques.

Differential cell analysis is done manually under light microscopy and Flow cytometry is a more objective technique that allows for cell types to be identified and counted using fluorescently tagged antibodies that transmits the data onto a computer and is more accurate as it count thousands more cells than can be counted by differential cell analysis. Our manual counting may not have been sensitive and accurate enough to detect these subtle changes.

In previous studies, Frick et al. (2009) found that restraint stress markedly reduced the total number of spleen cells and killer T-cell activity, and also altered the balance of CD4/CD8 T-cells by decreasing the ratio of CD4 helper cells. CD4 cells are "helper" cells that alert "killer" to the site of infections. For example, if CD4 cells become depleted after the 12-hour stress period, the body is left vulnerable to a wide range of infections that it otherwise would have been able to fight. In this same study it was concluded that water extract of *Sarcandra glabra*, another plant supplement with high nutrient value, showed good efficacy and safety in anti-stress treatment and modulated stress-attenuated immunologic response by improving anti-oxidative capacity.

In another study, Cao et al. (2012) employed a restraint stress mouse model to investigate the effect of *Sarcandra glabra* Extract (SGE) against influenza. Mice were infected with influenza virus three days after restraint, while SGE was orally administrated for 10 consecutive days. Results showed that SGE had a crucial effect on improving susceptibility marker levels to recover the balance of host defense systems. These studies, along with the data reported in this Moringa study show that the use of plant supplements can have positive effects on immune modulation. The data in this study showed that after the 12 hr stress period, the lymphocyte count was significantly increased ($t=4.128$, $p=0.0189$) in the stressed Moringa tea group compared to the non-stressed tea group, the neutrophil count also significantly increased in the

stressed Moringa tea group when compared to both water/stress (t=4.083, p=0.0126) and tea/ no stress groups (t= 4.263, p=0.0126).

Based on T-cell subset depletion studies and the analysis of gene knockout mice (Harty, Tvinnereim, & White, 2000); it is evident that CD8 (+) T cells contribute to resistance against intracellular infections with certain viral, protozoan, and bacterial pathogens. Although they are known primarily for their capacity to kill infected cells, CD8 (+) T cells elaborate a variety of effector mechanisms with the potential to defend against infection. We found that mice that consumed Moringa tea showed no significant difference in CD8 (+) T cells when compared to the water groups.

According to the National Research Council (US) Committee (2006), thousands of animals are transported to facilitate research, teaching, and training, and for breeding-colony establishment and maintenance. Stress during transportation is unavoidable and can affect the quality of ensuing research activities. However, when science-based good practices in animal handling and transport are identified and implemented, the transportation experience can be made less stressful by implementing simple measures such as the addition of Moringa tea to drinking water as demonstrated in this study.

Stress causes several undesirable effects, including slow animal growth and lowered immune response. Implementation of stress reduction for animals will produce calmer, easier to handle animals and will increase not only productivity, but animal welfare, health and of course, profit. Certain stressful events can be anticipated, like birth, weaning, handling and transport. Many parallels can be drawn between the restraint stress utilized in this study and the handling of animals, such as squeeze chutes, overcrowding, transportation, cages, etc.

The immune system defends the body against disease causing micro-organisms and depends a great deal on healthy, regular and nutritious nourishment. Various deficiencies in nutrient which have all been shown to be plentiful in *Moringa oleifera*, such as of zinc, selenium, copper, iron, potassium, vitamins A, B, C, E and more, can deteriorate immune responses. Dietary supplementation beyond normal levels considered adequate will allow for more rapid or complete restoration of immunocompetence. Regularly receiving good amounts of antioxidants, amino acids, and other nutrients would prevent a large majority of the problems that animal producers face. Adding a food supplement like Moringa may generate a lot of health benefits, not just a healthier immune system.

Reducing stress on livestock during handling in combination with supplements such as Moringa tea additives may help reduce sickness, strengthen immune systems and increase productivity. Any conflicting findings reported maybe partially explained by the types and durations of the stressors. Moreover, the aspect of the effects of *Moringa oleifera* on immune modulation on stress induced mice has been assessed. A more in depth understanding of the complexity of these relationships will help to improve animal health and well-being.

Future studies should use a much larger sample of subjects and increase the length of the chronic stress period. Although the majority of the literature has considered 12 hours a sufficient length of time to induce chronic stress in mice, a lengthened chronic stress model could possibly yield higher WBC counts and display an even larger difference in lymphocyte and neutrophil counts. The number of days of tea consumption could also be increased to show longer term effects of the tea in conjunction to the longer stress periods.

One weakness of this study was that samples were obtained at two different times of day, morning and evening for the two stress groups. Time of day has been shown to affect cortisol

levels, which are indicators of stress in humans and in rodents (Young, Abelson, & Lightman (2004). As mentioned earlier, stress has been shown to have an immunosuppressive impact on the immune system, mediated through cortisol. The body is then more susceptible to the proliferation of pathogens which could possibly alter the WBC counts (Bernabucci, et al, 2010). Future research should alter the laboratory day-dark cycle to accommodate sample collection at the same time each day for each condition.

Future use of these findings can further explore how many antioxidants and/or other nutrients the body can actually absorb over a given period of time and what is the most effective level of those consumed antioxidants? Perhaps the dried leaves could be added directly to the animal feed. The best nutrient delivery method also needs to be further explored to ensure the highest levels of bioavailability. The results of this study can be used as a foundation for new studies that could focus on the development of natural supplements that may help better the animal industry.

5.1 Conclusion

Many parallels can be drawn between the restraint stress utilized in this study and the handling of animals. This and previous work done on using Moringa tea or other plant based supplemental feeds has displayed immune modulating properties of *Moringa oleifera*, and our data show that it possibly could be used as a daily supplement to aid in production of healthier animals and in reduction of the use of sub-therapeutic antibiotics that are thought to contribute to the problem of antibiotic resistant pathogens. Many animal stressors cannot be avoided, i.e. birth, weaning, handling, transport etc. The results of this study could possibly be used as a foundation for new studies that could focus on the development of natural supplements that may help better the animal industry.

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Appendix A

Moringa Contains Very High Antioxidants and Anti-inflammatory Compounds

Antioxidants and anti-inflammatory compounds work best when combined with other antioxidants and anti-inflammatory agents. Moringa contains the following antioxidants and anti-inflammatory compounds or compounds with antioxidant and anti-inflammatory characteristics.
Antioxidants: Vitamin A, Vitamin C, Vitamin E, Vitamin K, Vitamin B (Choline), Vitamin B1 (Thiamin), Vitamin B2 (Riboflavin), Vitamin B3 (Niacin), Vitamin B6, Alanine, Alpha-Carotene, Arginine, Beta-Carotene, Beta-sitosterol, Caffeoylquinic Acid, Campesterol, Carotenoids, Chlorophyll, Chromium, Delta-5-Avenasterol, Delta-7-Avenasterol, Glutathione, Histidine, Indole Acetic Acid, Indoleacetonitrile, Kaempferal, Leucine, Lutein, Methionine, Myristic-Acid, Palmitic-Acid, Prolamine, Proline, Quercetin, Rutin, Selenium, Threonine, Tryptophan, Xanthins, Xanthophyll, Zeatin, Zeaxanthin, Zinc.
Anti-inflammatory compounds: Vitamin A, Vitamin B1 (Thiamin), Vitamin C, Vitamin E, Arginine, Beta-sitosterol, Caffeoylquinic Acid, Calcium, Chlorophyll, Copper, Cystine, Omega 3, Omega 6, Omega 9, Fiber, Glutathione, Histidine, Indole Acetic Acid, Indoleacetonitrile, Isoleucine, Kaempferal, Leucine, Magnesium, Oleic-Acid, Phenylalanine, Potassium, Quercetin, Rutin, Selenium, Stigmasterol, Sulfur, Tryptophan, Tyrosine, Zeatin, Zinc.

Appendix B

Animal Stressors

There are many factors that can cause an animal stress (Morgan & Thomberg, 2007).

- Danger – This triggers the fight or flight response.
- Illness – This causes and is caused by stress.
- Pain – unpleasant feeling often caused by intense or damaging stimuli.
- Accidents – A form of danger and pain.
- Synthetic chemicals – From household products, vaccinations, commercial pet food and many other products and veterinarian drugs
- Inappropriate diet – The wrong nutrients and/or chemicals.
- Weaning – Sudden weaning and being separated from its mother early
- Confinement – A cause of boredom.
- Isolation – An unnatural situation for a pack or herd animals.
- Over-crowding – Even pack animals need their own space.
- Boredom – which can cause anxiety and stereotypic movements
- Changes in routine/environment – Animals are more comfortable with routine.
- Over-stimulation – The opposite of boredom which, at times, can be too much for an animal to handle.

Appendix C

Moringa Oleifera Nutritional Value of Leaves

Analysis of Moringa dried leaf powder contains the following per 100 grams of edible portion

Moisture % 7.5	
Calories 205	**Oxalic acid** (mg) 1.6%
Protein (g) 27.1	**Vitamin A-B** carotene (mg) 16.3
Fat (g) 2.3	**Vitamin B** - choline (mg)
Carbohydrate (g) 38.2	**Vitamin B1** - thiamin (mg) 2.64
Fiber (g) 19.2	**Vitamin B2** - riboflavin (mg) 20.5
Ca (mg) 2,003	**Vitamin B3** - nicotinic acid (mg) 8.2
MG (mg) 368	**Vitamin C** 0 ascorbic acid (mg) 17.3
P (mg) 204	**Vitamin E** tocopherol acetate mg) 113
K (mg) 1,324	
Cu (mg) 0.57	**Arginine** (g/16gN) 1.33%
	Histidine (g/16gN) 0.61%
Fe (mg) 28.2	**Lysine** (g/16gN) 1.32%
S (mg) 870	**Tryptophan** (g/16gN) 0.43%
	Phenylanaline (g/16gN) 1.39%
	Methionine (g/16gN) 0.35%
	Threonine (g/16gN) 1.19%
	Leucine (g/16gN) 1.95%
	Isoleucine (g/16gN) 0.83%
	Valine (g/16gN) 1.06%

Appendix D

Red Blood Cell Lysis Protocol

Purpose

To lyse red blood cells from mouse blood samples

Materials

0.5M EDTA

FACS Buffer

15ml conical tubes

2- 96 well U-bottom plates

FC block/ (CD16/32)

Pipettes

1X multi-species RBC Lysis Buffer

1.5ml tubes

Centrifuge

Container of ice

Pipette tips

Procedure

1. Collect 200 – 500µl blood from each mouse into 1.5ml tubes with 50µl 0.5M EDTA

2. Transfer 200µl of blood from each mouse into 15ml tubes and add 2.0ml of 1X multi-species RBC Lysis Buffer.

3. Incubate for 5 minutes at room temperature

4. Spin down 1100 RPM for 5 minutes in centrifuge and remove supernatant

5. Resuspend pellet, add 5µl of FC block and incubate 10 minutes on ice

6. Add 225 µl of FACS buffer

7. Transfer 100µl to 2 96 well U-bottom plates

8. Spin down at 1300 RPM for 2 minutes

9. Remove supernatant and resuspend by tapping gently

FACS Staining Protocol

Purpose

To stain the surface of the cells to differentiate the different types during flow cytometry

Materials

1% Paraformaldehyde	Container of ice
Pipettes	Pipette tips
Aluminum foil	FACS Buffer
Anti- CD3 FITC	Anti- CD4 PE
CD45 PerCP Cy 5.5	Ly6G PE
Anti- CD8 APC	CD19 PerCP cy 5.5
CD19 APC	Centrifuge

Procedure

1. Add 100µl single stain controls to the first row of both plates according to the following:

Plate 1		Plate 2	
CD3 FITC	0.25µl	CD3 FITC	0.25µl
Ly6G PE	0.15µl	CD4 PE	0.15µl
CD45 PerCP Cy5.5	0.31µl	CD19 PerCP Cy 5.5	0.31µl
CD19 APC	0.15µl	CD8 APC	0.31µl

2. Add 100µl single of staining cocktails to each mouse sample according to the following:

Plate 1		Plate 2	
CD3 FITC	3.1µl	CD3 FITC	3.1µl
Ly6G PE	1.8µl	CD4 PE	1.8µl
CD45 PerCP Cy5.5	3.9µl	CD19 PerCP Cy 5.5	3.9µl

CD19 APC	1.8 µl	CD8 APC	3.9 µl

3. Incubate 30 minutes on ice and cover with aluminum foil

4. Wash cells by adding 200µl of FACS buffer. Spin 1500 RPM for 2 minutes

5. Discard supernatant by shaking once into the sink,

6. Resuspend pellets and repeat wash

7. Resuspend pellets

8. Fix cells with 100µl 1% Paraformaldehyde for 10 minutes on ice

9. Repeat wash

10. Resuspend pellets in 200µl of FACS buffer

11. Perform Flow analysis or place in the refrigerator for next day analysis