DIETARY FAT AND IRON MODIFY CELL PROLIFERATION AND CYTOKINE PRODUCTION IN GROWING MALE RATS

By

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Bachelor of Science

The University of Tulsa

Tulsa, Oklahoma

1998

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
December 2000

DIETARY FAT AND IRON MODIFY CELL

PROLIFERATION AND CYTOKINE

PRODUCTION IN GROWING

MALE RATS

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ACKNOWLEDGMENTS

I sincerely thank my graduate committee — Dr. Elizabeth Droke (Advisor), Dr. Bahram Arjmandi, and Dr. John Wyckoff – for guidance and support in the completion of this research. My sincere appreciation extends to other departmental staff and faculty – Dr. Brenda Smith and Dr. Edralin Lucas – for their generous provision of help and support throughout the research project.

I would also like to give my special appreciation to my husband, Vince, for his precious suggestions to my research, his strong encouragement at times of difficulty, love, and understanding throughout this whole process. Thanks also go to my parents and brother for their support and encouragement.

Finally, I would like to thank the Department of Nutritional Sciences for support during these two and half years of study.

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CHAPTER I

INTRODUCTION

Scientists have expressed interest in interactions between nutrition and the immune system for many years. Malnutrition is suggested by clinical observations to lead to impaired immune responses associated with specific nutrient deficiencies (Weller et al. 1998). Third-world countries and poorer sections in westernized countries have had a high prevalence of nutrient deficiencies. Additionally, a high prevalence of malnutrition can be found in individuals who show nutritional problems as a result of systemic disorders: cancer, chronic renal disease, burns, multiple trauma and chronic infections (Weller et al. 1998). Ironically, obesity and excesses of nutrient intakes may also result in reduced immune responses (Weller et al. 1998).

Some scientists suggest malnutrition works synergistically with infection to intensify the outcomes of each other. This relation can lead to many altered immune responses with various levels of severity. The nutrient deficiency affects immune function most consistently via changes in cell-mediated immunity and cytokine production. The suppression in cell-mediated immunity is the result of a reduction or a change in T-lymphocyte numbers and their corresponding functions. In detail, T-lymphocyte helper cells ($CD4^+$) will not be available to B-lymphocytes in adequate quantities (Weller et al. 1998). Lymphocyte proliferation in response to stimulation will also decrease (Weller et al. 1998).

Decreased cytokine production indirectly suppressed the rate of wound healing (Weller et al. 1998).

Specifically our laboratory's attention has been directed towards iron deficiency and dietary fat and their interrelationship with regards to immune function. Iron is required for lymphocyte proliferation and for production of antibodies by these cells (Connor and Beard 1997). Many enzymes with vital roles in the immune system also require iron in order to properly function. Studies indicate iron deficiency or overload can suppress immune function (Helyar & Sherman 1992, Berger, Dyck, et al. 2000). Fatty acids are also required for cell-mediated immunity (Gross and Newberne 1980). Diets containing n-6 polyunsaturated fatty acids can suppress immune function; whereas, diets containing n-3 polyunsaturated fatty acids can enhance immune function (Gross and Newberne 1980).

Studies have found complications in identifying causative factors when you not only consider the interactions of individual nutrients with immune function; but, also with the nutrient-nutrient interrelationships in foods and in dietary patterns (Mendola et al. 1995). Many factors that affect the bioavailability of dietary iron have been studied extensively. For instance, factors that enhance non-heme iron absorption are Vitamin C, MFP (Meat, Fish, and Poultry) factor, lactic acid from foods and HCl from the stomach (Hallberg and Hulthen 2000). Factors that reduce iron absorption are phytates and fibers, EDTA (in food additives), calcium and phosphorous (milk) and tannic acid (found in teas) (Hallberg and Hulthen 2000). Studies on factors influencing iron absorption

(especially those trying to determine the meat factor) have generated interest in fatty acids role in iron's bioavailability. There has been an association demonstrated between dietary fat and iron on non-heme iron absorption, iron status, and enterocyte aconitase activity (Droke and Lukaski 1996). The amount of dietary fat and its degree of saturation can either enhance or suppress the absorption and utilization of iron (Bowering et al. 1976) (Amine and Hegsted 1975) (Bowering et al. 1977). Because the relation between iron and immunity may be confounded by dietary fat, it is important to identify correlates of iron and dietary fat interactions on bioavailability to aid in the interpretation of their interactions with the immune system.

The aim of this study was to determine if the type of dietary fat; monounsaturated, polyunsaturated or saturated, altered immune function in growing rats deficient, adequate or overloaded in iron. We fed growing Sprague-Dawley rats one of three dietary iron concentrations; deficient, adequate or high in iron, in combination with one of four types of dietary fat; olive oil, safflower oil, flaxseed oil, or beef tallow. The project determined if the ingestion of these fat sources, typically consumed by humans and fed at a level recommended for decreased risk of chronic diseases, enhanced immune function in iron deficient rats or suppressed immune function in iron adequate or overloaded rats. The study of these interrelationships can lead to insights into mechanisms for the role of iron and fat in immune function.

CHAPTER II

REVIEW OF LITERATURE

Overview

Scientists have expressed interest in interactions between nutrition and the immune system for many years. Malnutrition is suggested by clinical observations to lead to impaired immune responses associated with specific nutrient deficiencies (Weller et al. 1998). The nutrient deficiencies affect immune function most consistently via changes in cell-mediated immunity and cytokine production. Specifically our laboratory's attention has been directed towards iron deficiency and dietary fat and their interrelationship with regards to immune function.

In order to completely investigate the interrelationship between dietary iron and fat with regards to immune function, the review of literature is divided into sections: iron and lipid metabolism, review of immune function and the importance of iron and lipids, and iron and lipid interactions. By looking at each component separately, it will show more clearly the need for further research regarding the roles of dietary iron and dietary fat in the modification of the immune function.

Iron Metabolism

The remarkable and unusual details of iron metabolism have captivated scientists for many years. Although iron is abundant on the earth, the prevalence of iron deficiency is high (Hurrell 1997). Iron deficiency is of grave concern to

many scientists because of its' many biochemical functions in the body. Also, there are negative outcomes associated with a deficiency state and an overload state. Iron deficiency is associated with reduced work performance, greater susceptibility to infections, and impaired neurological function (Marx 1997). Iron deficiency though not desirable, does not have a lethal outcome. On the other hand, iron overload diseases such as hemochromatosis are lethal and are associated with iron-catalyzed oxygen radical damage (Marx 1997). This free iron radical damage has been implicated in lipid peroxidation and ischemic myocardial damage (Ascherio et al. 1994). These disorders stimulate interdisciplinary studies between iron's nutrition, physiology, and biochemistry (Latunde-Dada et al. 1998). To achieve the goal of controlling the incidences of iron-associated disorders, a complete understanding of the chemical and physiologic mechanisms that influence iron bioavailability is necessary (Latunde-Dada et al. 1998).

There are four main aspects to stress when studying iron. First, even if consumption of iron is adequate, only a small portion of it is actually absorbed through the intestine and can be influenced by other dietary components. Second, iron needs to be transported not just to form hemoglobin; but, to multiple body compartments for various metabolic mechanisms. Third, the total storage iron amount can vary widely without noticeable impairment of body function (Yip and Dallman 1996). Lastly, red blood cells have a finite life span and need to constantly be reproduced. The red blood cell turnover is responsible for most iron turnover, but iron is not recoverable if the erythrocyte is lost from the body by

bleeding externally. By briefly reviewing these four aspects of iron metabolism, a strong foundation for the basis of iron's interrelationship with dietary fat can be established.

The body regulates absorption of iron tightly because of potential damage due to extra iron (Monsen 1999). There have been many studies that evaluated the mechanisms that regulate iron absorption. Wein and Van Campen (1991) state that the mechanisms that regulate iron absorption should either be present at the mucosal surface or influence the chemical state of iron before contact with the mucosal surface. Hunt and Roughead (1999) provide further support for the hypothesis made by Ehtechami and Elsenhans (1989) that the mucosal cells in the upper small intestine regulate iron absorption. Proposed mechanisms for uptake of iron at the cell surface include a plasma membrane oxido-reductase mechanism (Thorstenesen 1988). There has also been a proposal of transferrin endocytosis from the serosal side of the intestine (Huebers et al. 1983). Current research indicates iron must be reduced before it can be absorbed (Wollenberg and Rummel 1987). Their findings show the need for a necessary reducing factor, which can be ascorbic acid and/or a reductase enzyme in the apical membrane of the mucosal epithelium. Specific genes involved in the iron uptake and transport by mucosal cells have been identified by experimental studies with genetically anemic mice and anemic Belgrade rats. The gene Nramp2, now referred to as divalent metal iron transporter 1 (DMT1), is located on the apical membrane of the enterocyte (Andrews et al. 1999). It presumably delivers iron to cell compartments where it undergoes endocytosis to be transported to the

basolateral membrane (Fleet 2000). Also, glycosylphosphatidylinositol (GPI)-anchored ceruloplasmin, which acts as a copper-dependent oxidase, has been suggested to work together with a putative basolateral iron transporter to control the exit of iron out of the enterocyte (Wood and Han 1998).

Regardless of the mechanism, researchers agree absorption can be influenced by dietary iron, which can be in two forms, heme and non-heme iron (Hallberg and Hulthen 2000). Heme iron, the most functional and effectively absorbed (Beard et al. 1996) makes up proteins like hemoglobin with an iron-porphyrin prosthetic group (Yip and Dallman 1996). Non-heme iron consists mainly of iron salts (Yip and Dallman 1996). Absorption of non-heme iron is based on the solubility of the iron salt compound (Yip and Dallman 1996). Characteristics of common iron salts are divided into four groups (Hurrell 1997): those that are freely water-soluble; those that are poorly water soluble, but soluble in dilute acids; those that are water soluble, but poorly soluble in dilute acids; and those that the iron compound is protected. Generally, the freely water-soluble salts and the water insoluble but soluble in dilute acids are highly bioavailable in rats (i.e. ferric citrate) (Hurrell 1997). Salts that are poorly soluble in dilute acid have only a low to moderate bioavailability (Hurrell 1997).

Hallberg and Hulthen (2000) suggest the variation in iron absorption from diets is due more to differences in the bioavailability of the iron, which can lead to a >10 fold variation in iron absorption, than to a variation in iron content. Enhancers of iron absorption (e.g. ascorbic acid, vitamin A, β-carotene, meat, fish, and poultry) increase the bioavailability of iron from meals (Reddy et al.

2000). Inhibitors of iron absorption (e.g. inositol phosphates, calcium, and certain structures in polyphenols) decrease the bioavailability of iron from meals (Reddy et al. 2000). Because of the continually expanding list of dietary factors that enhance or inhibit the bioavailability of iron, prediction of iron absorption from a meal is increasingly difficult. Methods that approach the problem include in vitro measurements of food iron bioavailability, such as dialyzable iron or iron uptake in the Caco-2 cell culture model (Reddy, Hurrell, and Cook 2000).

Many studies have observed the effect of specific dietary constituents on iron absorption; but these are single-meal or short-term bioavailability studies. Over a longer period of time the effects of dietary constituents on iron absorption may be negated by adaptive mechanisms (Minihane and Fairweather-Tait 1998). In long-term iron status, the principle determinant of the efficiency of iron absorption is the amount of storage iron. For instance, if iron stores are high, then iron absorption is low. Conversely, if iron stores are low, then iron absorption is high (Fleet 2000). This observation raises speculation that there is genetic regulation of both heme and non-heme receptors and binding proteins (Beard et al. 1996). Others have proposed that intestinal mucosal ferritin stores iron that is not transferred to the plasma and then is lost when the enterocyte is shed (Monsen 1999). Thus, there is a mechanistic explanation for feedback regulation of iron absorption: an adequate iron status increases quantities of iron held by the enterocyte.

Iron absorption is also influenced by the rate of erythrocyte production. Erythrocytes have a mean lifespan of about 120 days in humans (Beard et al.

1996). At the end of the lifespan, they are catabolized by Kupffer cells and spleen macrophages (Beard et al. 1996). Active erythropoiesis enhances iron absorption (Beard et al. 1996) and thus, may be an endogenous signal for iron absorption.

Evaluation of iron absorption in humans is complicated for a variety of reasons (Latunde-Dada et al. 1998). Iron absorption equipment is expensive and is not within the range of most laboratories, even in developed countries. Thus, animal models have been used. Opposing views highlight the differences between iron metabolism in rats and humans. Rats have a greater mucosal cell turnover leading to greater iron losses. Iron absorption in rats is also more dependent on serum iron levels than on internal iron turnover or iron stores, as is the case in humans (Latunde-Dada et al. 1998). However, studies on iron deficiency progression have to subject the patients to repeated phlebotomies because dietary iron deficiency in adults progresses too slowly to lend itself readily to experimental observation (Dallman et al. 1982). Also the collection of solid tissue samples is rarely justifiable in man. Nevertheless, pilot studies with animal models provide the flexibility to manipulate different variables involved in the finding of mechanisms for food iron availability and absorption (Latunde-Dada et al. 1998). The use of growing male rats, in combination with a low iron diet, eliminates female hormone fluxes that may interfere with homeostasis and provides a method for generating iron deficiency via dietary manipulation.

The second aspect to stress when studying iron is it's need to be transported not just to form hemoglobin; but, to various body compartments for

various metabolic mechanisms. To reduce the toxic effects of iron accumulation, iron must move safely from the site of absorption to sites of utilization. The metabolic safety solution is to use the transport protein transferrin (Andrews et al. 1999). Transferrin delivers iron to tissue cells by binding to surface expressed transferrin receptors (Chen et al. 1998). Receptor-mediated endocytosis transports the complex to the cytoplasm where the iron is released and the transferrin is recycled to the plasma membrane (Chen et al. 1998). Studies indicate transferrin receptors are found in abundance on erythroid precursors as well as placental cells (Andrews et al. 1999). If transferrin saturation is low, then iron stores are deficient; and conversely, if there is high transferrin saturation, then there is an indication that iron stores are high (Yip and Dallman 1996).

The third aspect to stress is the amount of total storage iron. Total storage iron amount can vary widely without noticeable impairment of body function (Yip and Dallman 1996). Storage iron is found in two forms: as ferritin, a soluble protein-enveloped core, and as hemosiderin, which is insoluble (Aisen and Listowsky 1980). Ferritin-bound iron is more biologically / biochemically available than hemosiderin-bound iron (Beard et al. 1996). Most of the storage iron is found in the liver, reticuloendothelial cells, and the bone marrow (Beard et al. 1996). It serves mainly as a reservoir to supply cellular iron needs such as hemoglobin production (Yip and Dallman 1996). In the sequence of development of iron deficiency in the rat, iron stores can be depleted before anemia is diagnosed (Dallman et al. 1982).

Lastly, red blood cells have a finite life span (~120 d) and need to constantly be reproduced. The red blood cell turnover is responsible for most iron turnover, but iron is not recoverable if the erythrocyte is lost from the body through external bleeding. Disruptions in iron turnover can be seen during menstruation, pregnancy, and gastrointestinal bleeding (Beard et al. 1996). To meet the needs of tissue, iron must be removed from storage, dietary iron absorption must be enhanced, or recycled from the process of red blood cell turnover (Beard et al. 1996).

Lipid Metabolism

Dietary fat and its role in metabolism has been studied extensively for many years. The high prevalence of studies looking at fat can be contributed to the global relation between nutrition and health. To effectively consider why the influence of fat is important, two different points of view need to be considered: quantity and quality (Bagger et al. 1996). Quantity views are based on developed societies with high levels of dietary fat intake and the health risks associated with high dietary fat intake. Quality views are based on the possible implications of the type of fat on an individual's health (Seiquer et al. 1995). It is these quality views that recent research emphasizes. Studies show the influence of different dietary fat sources on serum lipid levels, lipid plasma concentration, and their relationship to coronary heart disease (Seiquer et al. 1995). The type of dietary fat can also influence the fatty acid composition of different lipid fractions, which is important for eicosanoid synthesis that may influence platelet

aggregation, bleeding time, blood pressure, inflammatory processes and atherogenesis (Seiquer et al. 1995). Because coronary heart disease remains a leading cause of death in developed countries, dietary fat metabolism should be given a high priority in research.

Lipids have important metabolic roles. One such role is their ability to be oxidized to provide energy (Welch and Borlak 2000). Another role for lipids is to serve as precursors for active substances such as prostaglandins, steroid hormones and bile acids (Welch and Borlak 2000). Because of the important roles dietary fat play, a reduced or amplified amount of fat (such as diets high in saturated fat) can lead to problems in lipid metabolism, such as the development of atherosclerosis (Seiquer et al. 1996).

Dietary fat absorption is a multistage process, which is complicated by its insolubility in aqueous medium (Nelson and Ackman 1988). Short chain length fatty acids are usually esterified and released from triacylglycerols in the stomach and small intestine. Then they are bound to albumin to be transported via the portal vein to the liver (Welch and Borlak 2000). Long-chain fatty acids are then absorbed by passive diffusion in the form of a micelle (negatively-charged aggregates of the digested free fatty acids together with the bile salts) into the enterocytes (Welch and Borlak 2000). In the cell, the lipolysis products are then re-esterified to synthesize triacylglycerols, phospholipids or cholesteryl esters (Nelson and Ackman 1988). At this point, the enterocyte transports the lipid out of the cell and into the lymph by binding de novo synthesized apolipoproteins with the lipid to form a lipoprotein (chylomicron) that is aqueously soluble (Welch

and Borlak 2000). The chylomicron can then transport the long-chain fatty acids via the lymphatic system until they reach a point of entry into the bloodstream. The bloodstream then carries the lipids to the rest of the organs (Nelson and Ackman 1988).

The lipoprotein structures that transport the lipids are classified by differences in their hydrated densities: very low density lipoprotein (VLDL); intermediate-density lipoprotein (IDL); low-density lipoprotein (LDL); and high-density lipoprotein (HDL) (Welch and Borlak 2000). The role of HDL is to carry cholesterol from peripheral tissues to the liver where it is catabolized and excreted (Chen et al. 1995). The role of LDL is to carry cholesterol to the peripheral tissues and to regulate de novo synthesis of cholesterol at those sites (Chen et al. 1995). Keep in mind the most important motive for the extensive research on dietary fat is that of hypercholesterolemia as an important co-factor in the development of coronary heart disease (Seiquer et al. 1995). Changes or modifications to the lipoprotein's structure and composition (especially LDL) by the different types of fatty acids making up the chylomicron, are thought to affect their atherogenic potential (Seiquer et al. 1995). Low-density lipoprotein particles made up of cholesterol esterfied with saturated fatty acids, had reduced binding capabilities, internalization and degradation by fibroblast cultures compared with LDL particles made up of cholesterol esterfied with polyunsaturated fatty acids(Seiquer et al. 1995).

The LDL particle may also go through compositional changes that increase its atherogenic potential (Seiquer et al. 1995). The common initiating

step for compositional changes is the peroxidation of polyunsaturated fatty acids that make up the LDL (Seiquer et al. 1995). The more recognizable oxidized LDL can be scavenged by macrophages and accumulate in the lymphoid tissue cells which leads to fatty streak formation. The fatty streak is the earliest recognizable lesion for atherogenesis (Seiquer et al. 1995).

A reduction in serum lipid levels has reduced the amount of lipids available for peroxidation and reduced the risk for atherosclerosis. Even though there have been several drug therapies placed on the market to lower serum cholesterol values, the National Cholesterol Education Program advocates lifestyle dietary modification as the primary treatment for lowering cholesterol values (Ordovas 1999). Currently, the recommended dietary modifications include, lowering the quantity of total fat intake to less than 30% of energy, lowering the amount of saturated fat intake to less than 10% of energy, and lowering the amount of cholesterol intake to less than 200 mg/d along with a greater consumption of dietary fiber and complex carbohydrates (Ordovas 1999). However, controversy in recent research shows lowering of total fat intake may not be the only answer, rather reducing the consumption of saturated and *trans* fats can lead to a regression in atherosclerotic lesions (Ordovas 1999, Conner et al. 1997, Katan et al. 1997). The controversy arises from the variability in response of serum cholesterol to diet between subjects. Because of the variability in responses in human studies, primarily due to the lack of information regarding the heritability of the response to dietary intervention, animal studies are used in today's research of dietary lipids (Ordovas 1999). The variability in

the serum lipoprotein response to dietary manipulation may have a significant genetic component (Ordovas 1999). Despite this uncertainty, studies have focused their attention on the influence of the type of dietary fat on plasma lipid levels.

Epidemiological and clinical study results have helped fuel the awareness that saturated fats are a risk factor for coronary heart disease (Edionwe and Kies 1998). Saturated fats raise serum total cholesterol (TC), and serum LDL and may lower serum HDL (Edionwe and Kies 1998). For some countries; however, unsaturated fats are not affordable by the majority of the people. Saturated fats are cheaper and are still the preferred dietary source (Edionwe and Kies 1998). New studies are thus needed to demonstrate differences in saturated fats. For example, not all saturated fats have similar effects on serum TC or cholesterol in the different lipoproteins (Edionwe and Kies 1998). Such saturated fats contain medium-chain fatty acids like caprylic acid and caproic acid, which have been shown not to raise serum cholesterol levels when compared with palmitic acid (Denke and Grundy 1991). Early evidence suggested and has been confirmed that the same holds true for stearic acid (Bonanome and Grundy 1988). Dietary fats rich in stearic acid are beef tallow and cocoa butter (Denke and Grundy 1991). Hydrogenated vegetable oil contains considerable amounts of *trans* fatty acids, which were recently shown to have adverse effects on serum cholesterol profiles (Sundram et al. 1992). Palmitic acid is considered to raise cholesterol levels causing hypercholesterolaemia (Sundram et al. 1992).

Because polyunsaturated fatty acids (PUFAs) are known to lower blood cholesterol levels, research in PUFAs has increased (An et al. 1997). Most research has focused on n-6 and n-3 PUFAs. N-6 fatty acids, such as those found in safflower oil, lower serum cholesterol levels, but not triacylglycerols (An et al. 1997). N-3 fatty acids, such as those found in flaxseed oil, lower serum cholesterol, and lower serum triacylglycerols even more in experimental animal studies (An et al. 1997). Some n-3 fatty acids may reduce the levels of serum lipids and modify membrane fatty acid composition, thus decreasing the incidence of atherosclerosis and thrombogenesis. Fatty acid composition is also important for oxidative stability of food oils (Lu et al. 1997). Because polyunsaturated fats are high in both linoleic and linolenic acids they are relatively susceptible to oxidation (Lu et al. 1997).

Flaxseed oil contains about 60% α-linolenic acid (ALA) (Kelly et al. 1993). Longer chain n-3 fatty acids (ones with 20 carbons) have a stronger effect on lowering blood lipids and in deposition of longer chain n-3 fatty acids into tissue lipids than ALA (An et al. 1997). Previous studies have shown that humans have a limited capacity to convert ALA to eicosapentaenoic acid (EPA) and docosahexaenoic acid which could explain the lesser effect in reducing blood lipids than longer chain n-3 fatty acids (Kelly et al. 1993). Eicosapentaenoic acid and docosahexaenoic acid are believed to lessen the tendency for platelet aggregation and thrombosis (Allman et al. 1995). A mechanism that is proposed whereby long chain n-3 fatty acids inhibit platelet aggregation is via suppressed production of the platelet prostaglandin thromboxane A_2 (TXA_2), an eicosanoid

(Allman et al. 1995). Arachidonic acid (AA) is the usual substrate for TXA_2 production (Allman et al. 1995); however, EPA can replace AA thus reducing TXA_2 production and thrombotic potential. Some studies suggest ALA is metabolized to EPA by desaturation and chain elongation; but, the efficiency of the conversion limits the ability to also reduce TXA_2 production (Allman et al. 1995). However, an opposing study indicates ALA enhancs platelet EPA sufficiently to alter platelet function in a positive way (Allman et al. 1995). Platelets play an essential role in thrombosis and atherosclerosis, but the confounding research and the mechanisms for the effects of dietary oils on platelet function are not well understood.

The peroxidative capacity of polyunsaturated fatty acids is a concern of nutritionists because free radicals cause tissue damage. Because of this, studies have focused on monounsaturated fatty acids and their lower susceptibility to peroxidation (Adamopouloa et al. 1996, O'Connell and Peters 1997). A reduced susceptibility to peroxidation reduces free radical production (O'Connell and Peters 1997).

Studies also have shown a potential protective role for monounsaturated fatty acids via their ability to lower TC and LDL to a greater extent than polyunsaturated fatty acids (Adamopouloa et al. 1996, O'Connell and Peters 1997). High-density lipoprotein concentrations were greater in volunteers on monounsaturated diets compared with those on polyunsaturated diets (Adamopouloa et al. 1996). Monounsaturated fatty acids also lowered triacylglycerols compared with diets rich in saturated fatty acids (Kris-Etherton et

al. 1998). Triacylglycerols may not be an independent risk factor in coronary heart disease because any effect of triacylglycerols tends to be eliminated once HDL levels are taken into account in multivariate analysis (Adamopouloa et al. 1996). However, triacylglycerols are still considered a risk factor in recent guidelines from the European Atherosclerosis Society (Adamopouloa et al. 1996). Olive oil is the primary food source of monounsaturated fatty acids (Kris-Etherton et al. 1998).

Dietary fatty acid composition and dietary cholesterol influence circulating cholesterol concentrations. The impact of dietary cholesterol on circulating cholesterol concentrations is controversial (Jones et al. 1994). Some studies indicate no effect (Kestin et al. 1989, Keys et al. 1956, Slater et al. 1976) while others indicate a cholesterol raising effect (Jones et al. 1994, Connor et al. 1964, Mattson et al. 1972, Keys 1984). In animal studies, a greater dietary sterol consumption reduced cholesterol synthesis (Jones et al. 1994, Anon 1987, Dietschy and Siperstein 1967). In humans, the degree of saturation of dietary fat had no effect on the synthesis of cholesterol, whereas the addition of dietary cholesterol to the diet resulted in a reduction in fractional absorption and inhibition of cholesterogenesis (Jones et al. 1994, McNamara 1987). Because coronary heart disease remains a leading cause of death in developed countries; dietary fat metabolism should be given a high priority in research.

Immune Function

Scientists have expressed interest in interrelationships between nutrition and the immune system for many years. Inadequate nutrition leads to impaired immune responses associated with specific nutrient deficiencies (Weller, Lockwood, et al. 1998). These nutrient deficiencies affect immune function most consistently via changes in cell-mediated immunity and cytokine production.

A brief overview of the basic concepts of cell-medicated immunity and cytokine production will help to provide plausible explanations for the experimental study to follow. To study the human immune system, one can choose a species of mammal that will reasonably depict humans' immune responses. A rodent model has been suggested to be more similar to human responses (Davis and Hamilton 1998). When comparing mammalian species' immune systems they are similar but not identical (Davis and Hamilton 1998). Significant differences were noted: in the expression of MHC class II molecules, in leukocyte differentiation molecules, and in the composition of the immune system (Davis and Hamilton 1998). The largest differences in immune functions were found in pigs, cats, ferrets, and horses compared with humans and rodents (Davis and Hamilton 1998;Holmes and Lunn 1994;Lunn et al. 1993;Lunney and Pescovitz 1987;Pescovitz et al. 1984). These differences in immune function and composition of the noted species highlight the rodent's immune system as the most analogous to humans for cell-mediated immunity and cytokines.

The cells of the immune system are derived from bone marrow hematopoietic stem cells (Boissonneault 2000). These stem cells develop into

neutrophils, eosinophils, and basophils, monocytes, and B- and T-lymphocytes (Boissonneault 2000). Neutrophils function as efficient phagocytic cells (Boissonneault 2000). Basophils have high quantities of cytoplasmic granules that help to mediate immediate hypersensitivity reactions, including an allergic response (Boissonneault 2000). Upon migration into tissues from the bloodstream, monocytes differentiate into macrophages (Boissonneault 2000). Macrophages remove debris and serum proteins by use of their highly phagocytic nature (Erickson 1998). They can also kill tumor cells, defend against infectious agents and mediate and respond to inflammation (Erickson 1998). More importantly monocytes and macrophages produce regulatory molecules called cytokines (soluble factors), which influence activities of lymphocytes by acting as intercellular mediators of the immunological response (Boissonneault 2000). T-lymphocytes, which mature in the thymus, serve as mediators in immune response regulation and as mediators of delayed-type hypersensitivity and effectors of T-lymphocyte mediated cytotoxicity (Boissonneault 2000). B-lymphocytes, which mature in the bone marrow in adults, serve as producers of antibodies in a humoral immune response (Boissonneault 2000).

Human malnutrition and excess nutrient intake can reduce cell-mediated immune responses (Chandra 1997). A very common example is that of protein–energy malnutrition (PEM). The thymus is decreased in size and weight (Chandra 1997). This leads to fewer lymphoid cells, not only in the thymus but also around small blood vessels, in the spleen and in the lymph nodes (Chandra 1997). The reduction in lymphocytes includes mature helper T lymphocytes and

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mature cytotoxic T lymphocytes. Lymphocyte proliferation and DNA synthesis are also suppressed, which may be the result of loss of essential nutrients or inhibitory factors (Chandra 1997). Phagocytosis and the production of several cytokines in humans with PEM are also suppressed (Chandra 1997).

Human malnutrition is usually a complex condition of multiple nutrient deficiencies. Because a single nutrient deficiency is rare, observations of single nutrient interactions with the immune system are limited to laboratory animals deprived of one dietary element (Bendich and Chandra 1990). These observations have confirmed the vital role of several vitamins and trace elements in immunocompetence (Chandra 1997).

Early evidence has established that the cytokines produced by the immune system not only regulate immune function, but also modify the growth process (Spurlock 1997). Now it is also apparent that cytokines mediate a direct regulation of nutrient metabolism and growth (Spurlock 1997). Additionally, cytokines may interact with other modulators of the immune system such as glucocorticoids, prostaglandins, and catecholamines, all of which may affect cellular metabolism (Spurlock 1997). For example, insulin-like growth factor I (IGF-I), is believed to be essential for T lymphocyte proliferation (Savendahl and Underwood 1997). Specific cytokines that make up a cytokine profile when being assessed by researchers are interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-10 (IL-10), and interferon-γ (IFN-γ) (Mosmann and Coffman 1989). Cytokines can control immune responses by influencing and changing the balance of T-helper 1 (Th1) and T-helper 2 (Th2) cells (Von Ruecker and Schmidt-Wolf 2000).

By measuring this cytokine profile, a more accurate measurement of the T helper lymphocyte (Th1/Th2) balance can be determined (Von Ruecker and Schmidt-Wolf 2000). T helper lymphocytes are subsets of T-lymphocytes, which are activated by antigen presenting macrophages, and are essential for further development and organization of the immune response (Von Ruecker and Schmidt-Wolf 2000). T-helper 1 cells function to induce activation of cell-mediated immunity (including activation of cytotoxic T-lymphocytes and delayed-type hypersensitivity reaction) (Von Ruecker and Schmidt-Wolf 2000). T-helper 2 cells function to induce the humoral arm of the immune response (Von Ruecker and Schmidt-Wolf 2000). By determining the Th1/Th2 balance a more precise evaluation of catabolism as well as metabolic stress can be determined (Von Ruecker and Schmidt-Wolf 2000).

Interleukin-2 is an important cytokine in the regulation of the immune system and is produced by T helper-1 (Th1) lymphocytes (Feldmann 1998). Interleukin-2 has enhanced the proliferation of phytohemagglutinin P-stimulated peripheral blood mononuclear cells (Savendahl and Underwood 1997). Interleukin-2 is also known to have a role in stimulating T-lymphocyte proliferation and differentiation, as well as the activation of cytotoxic lymphocytes and macrophages (Feldmann 1998). Recently, a report demonstrated IGF-I-stimulated IL-2 production in human peripheral T lymphocytes (Savendahl and Underwood 1997). A fasting state can depress serum IGF-I production, thus possibly leading to reduced IL-2 production. This suggests a possible role for IL-2

and IGF-I in maintaining normal natural killer cell (NK cells) cytotoxicity (Savendahl and Underwood 1997).

Interleukin-4 is also an important cytokine for the growth of T-lymphocytes, but not to the extent of IL-2. Interleukin-4 is produced by T-helper-2 lymphocytes (Th2) and stimulates B cell proliferation and production of immunoglobulins (Ig), specifically IgE and IgG1 (Feldmann 1998). At the same time, IL-4 stimulates proliferation of TH2 lymphocytes, which will enhance production of IL-10. Interleukin-10 inhibits production of IL-1, TNF-alpha and other pro-inflammatory cytokines (Feldmann 1998).

Interferon-γ (IFN-γ) is a cytokine produced primarily by activated Th1 lymphocytes and NK cells (Feldmann 1998). It helps to induce and enhance the development of more Th1 lymphocytes and suppresses Th2 immune responses (Von Ruecker and Schmidt-Wolf 2000). Interferon-γ does provide antiviral and anitproliferative activities; but, not to the extent of IFN-α and -β (Feldmann 1998). It is however, the most important inducer of macrophage activation, which favors the development and activities of Th1 lymphocytes (Feldmann 1998).

Corticosteroids secreted from the pituitary help to stimulate Th2 lymphocytes to produce IL-10. Interleukin-10's primary role is to control the type of immunological response by T-lymphocytes. It predominantly inhibits the production of IFN-γ and other Th-1 cytokines (Von Ruecker and Schmidt-Wolf 2000). Thus, IL-10 favors Th2 cellular responses and the activation of B-lymphocytes (Feldmann 1998).

Iron and Immunity

Iron deficiency is a worldwide nutrient deficiency, and is consistently associated with morbidity and infectious diseases (Berger et al. 2000). Iron is required for the synthesis of hemoglobin, myoglobin, and certain essential enzymes; a deficiency leads to anemia (Wan et al. 1989). Malnourished patients with anemia and inflammation have greater levels of free iron in conjunction with lower concentrations of serum transferrin, which leads to a greater susceptibility to bacterial pathogens (Wan et al., 1989). Lack of iron reduces an individual's resistance to pathogens through impairment of immunocompetence, more specifically through cell-mediated immunity (Berger et al. 2000).

Iron deficiency causes multiple immune functions to be compromised. One such compromise is the production of fewer NK cells associated with reduced interferon production (Scrimshaw and SanGiovanni 1997). Another compromise is reduced numbers of B-lymphocytes in spleens of anemic mice (Helyar and Sherman 1992). Stimulation and proliferation of lymphocytes are also compromised by iron deficiency (Scrimshaw and SanGiovanni 1997). Both the proportion of T-lymphocyte subsets and the absolute number of T-lymphocytes were reduced with iron deficiency anemia (Walter et al. 1997). The lymphocyte response to concanavalin A (ConA) antigen was impaired in iron deficiency giving rise to a significant correlation between the stimulation index and transferrin saturation (Walter et al. 1997). Another function that is

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compromised by iron deficiency is phagocytic killing power (Scrimshaw and SanGiovanni 1997). Iron serves as the generator of free oxygen radicals in the phagocytic cells (Beisel 1996). Lastly, iron deficiency also suppressed the bactericidal activity of neutrophils (Connor and Beard 1997).

Discussion of iron and immunity would not be complete without an introduction to iron overload and infection. Iron overload is a disorder of iron metabolism resulting from either excess dietary iron absorption or parental iron loading (Wu et al. 1990). In most instances, iron overload is a genetic disorder called hemochromotosis. It leads to the accumulation of iron in tissues, which results in hepatocellular damage and fibrosis leading to cirrhosis (Wu et al.1990). Iron overload can also be toxic, which promotes peroxidative damage to lipid membranes (Wu et al. 1990).

Iron overload, unlike iron deficiency, helps the infectious agent. Iron is not only needed by the host to fight infection, but also by the infectious agent for growth (Scrimshaw and SanGiovanni 1997). A protective effect can be shown by the lack of available iron (iron deficiency) for agent replication (Scrimshaw and SanGiovanni 1997). Ferritin also functions as an iron-withholding mechanism (Scrimshaw and SanGiovanni 1997). High levels of splenic ferritin have been reported to suppress lymphocyte transformation (Wu et al. 1990). For example, adding ferric salts or ferritin to human peripheral blood mononuclear cell cultures suppressed mitogenic responses (Wu et al. 1990). The extent of this effect is also dependent on the chemical structure of iron (Wu et al. 1990). Acid isoferritins have a greater immunosuppressive effect on T-lymphocytes than do

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basic isoferritins (Wu et al. 1990). Transferrin, an iron-binding protein, is found not only in the blood but also in all body fluids and is the standard mechanism for withholding iron from the infectious agent (Scrimshaw and SanGiovanni 1997). The more saturated transferrin is with iron, the less competent it is to provide protection from infection (Connor and Beard 1997). Iron overload induces this high serum ferritin and transferrin level that impairs immunity (Wu, Meydani, Meydani, Burklund, Blumberg, and Munro 1990). To withhold iron may compromise the host's immune function. Wu et al. (1990) state that a high iron diet given to young rats (4 months of age) for four weeks increased ConA-stimulated lymphocyte proliferation. Wu et al. (1990) did not see this same effect for older rats (19-22 months of age). It is vitally important to recognize that iron intake has a narrow range over which the immune system can function normally.

Lipid and Immunity

Numerous studies indicate dietary fat can affect immune function. Dietary fats can influence membrane phospholipid composition (Wu and Meydani 1998) and changes in phospholipid composition are believed to play a large role in alterations of immune function either by membrane-bound enzyme activity or by fatty acid precursors of immune-modulating eicosanoids (prostaglandins, prostacyclines, thromboxanes, leukotrines) (Beisel 1996). For example, studies have shown that polyunsaturated fatty acids modify lymphocyte membrane fluidity resulting in altered receptor function that could influence the generation of intracellular second messengers (Hagve 1988).

T- and B-lymphocyte functions are influenced by dietary fat saturation. B-lymphocyte responses to mitogens or pathogens do not appear to be influenced as greatly as T-lymphocytes by the dietary fat level or degree of saturation (Boissonneault 2000). It is also known that the type and degree of saturation of fat can alter cytokine production. Dietary fat may alter the ability of macrophages to kill tumor cells, possibly by a mechanism that involves prostaglandins (Erickson 1998). Macrophages kill tumor cells by their secretion of the soluble inflammatory mediator tumor necrosis factor-α (TNF-α) (Erickson 1998). Cytotoxic responses are also influenced by dietary fat saturation (Erickison 1984).

It is well known and documented that saturated fats raise serum total cholesterol and LDL and may lower HDL (Edionwe and Kies 1998). These effects of saturated fat lead to the awareness that increased consumption of saturated fatty acids leads to increased risk factors for coronary heart disease and the development of atherosclerosis (Edionwe and Kies 1998). The increased risk for atherosclerosis associated with saturated fatty acids may be the result of a compromised immune function via alterations in macrophage functions (Boissonneault 2000). Macrophages contain receptors for lipoproteins such as LDL and VLDL as well as scavenger receptors that engulf oxidized lipoproteins (Boissonneault 2000). Ironically, macrophages are also partially responsible for oxidative modification of LDL, which is dependent on oxygen radical production (Boissonneault 2000). Patients with atherosclerosis have increased cytokine levels such as IL-2, IL-1, TNF-α and transforming growth factor-β (TGF-β)

(Merendino et al. 1998). There is also evidence indicating the availability of cholesterol is an important determinant for the capacity of lymphocyte proliferation, which directly establishes a relation between cholesterol level and T-lymphocyte proliferation (Merendino et al. 1998). It has been demonstrated that oxidized LDLs significantly reduce and inhibit ConA-induced lymphocyte proliferation (Merendino et al. 1998).

Deficiency of the essential fatty acids (linoleic and linolenic) also alters immune function (Boissonneault 2000). Immune cell phospholipid membranes contain a high accumulation of PUFA (Wu and Meydani 1998). The functions of lymphocytes are highly dependent upon phospholipid membrane associated actions (Wu and Meydani 1998). It is conceivable, therefore, to recognize that changes in the PUFA content of lymphocyte membranes will alter functions such as the secretion of cytokines and lymphocyte transformation (Wu and Meydani 1998).

Dietary n-6 PUFAs, such as those from safflower oil, modify lymphocyte structure and function via changes in membrane fluidity, receptor binding sites, hormonal signal transduction, and the lymphocyte reaction to surface antigen presentation (Wan et al. 1989). N-6 PUFAs also are believed to affect general properties of lymphocytes indirectly by acting as precursors for the production of lipid mediators (eicosanoids) (Wan et al. 1989). Excess dietary consumption of linoleate (n-6 PUFA) may also enhance tumorigenesis and suppress the response of spleen lymphocytes to mitogens, such as ConA (Wan et al. 1989). Furthermore, n-6 PUFAs have been associated with greater production of

prostaglandin E_2 (PGE_2) (Wan et al., 1989). Prostaglandin E_2 can inhibit lymphocyte proliferation, macrophage collagenase synthesis, NK activity, and production of interferon and other lymphokines by T-lymphocytes (Wan et al., 1989).

N-3 PUFAs, from sources such as flaxseed oil, can also alter membrane structure and function of lymphocytes. Cytotoxicity of cytotoxic T-lymphocytes can be enhanced with the incorporation of PUFA into the diet whereas; saturated fatty acids suppress the activity by reducing membrane fluidity and receptors (Fritsche and Johnston 1989). The consumption of diets rich in n-3 PUFA can also reduce the production of PGE_2 (a pro-inflammatory eicosanoid, which depending on the dosage can be quite inhibitory) because of the direct competition for enzymes in the eicosanoid synthesizing pathway (Fritsche and Johnston 1989). In comparison to n-6 PUFAs, n-3 PUFAs, eicosapentaenoic and docosahexaenoic, tend to be more immunosuppressive, which for patients with cardiovascular, autoimmune and inflammatory diseases is beneficial (Jeffery et al. 1996, Kromann and Green 1980, Calder and Newsholme 1992). However, excessive reduction in eicosanoids and cytokines could impair normal host defense mechanisms or homeostasis within the patient (Wu and Meydani 1998). N-3 PUFAs can also suppress the immune response by inhibiting lymphocyte proliferation, IL-2 production, NK cell activity, and antigen presentation (Jeffery. et al. 1996, Jolly, et al. 1997). One study also indicated the administration of fish oil (an n-3 source) suppressed the serum levels of IL-6, IL-8, IL-10, and TNF-α compared with rats fed safflower oil (Tashiro et al. 1998). Another study

supported the results by demonstrating a reduction in IL-4 and IL-10 production in mice fed fish oil compared with mice fed a low-fat diet (Yaqoob and Calder 1995). Although a reduction in the pro-inflammatory eicosanoids and cytokines may be beneficial for patients with cardiovascular, autoimmune and inflammatory diseases, excessive reduction could impair normal host defense mechanisms or homeostasis within the patient (Wu and Meydani 1998).

Oleic acid an n-9 fatty acid family member found in high concentrations in olive oil also alters membrane structure and function of immune lymphocytes. One study concluded that oleic acid affects immune function in a similar way to n-3 fatty acids (Perez et al. 1989). Oleic acid suppressed the spleen T-lymphocyte proliferation response to cell surface antigens but elevated the response five-fold when cultures were enriched with control macrophages (Perez et al. 1989). The main advantage oleic acid has over PUFAs is oleic acid does not oxidize easily; thus, there is a lower consumption of immunosuppressive oxidized lipids available to damage cells (Boissonneault 2000).

Iron and Lipid Interactions

Research needs not only to consider the interrelationship of nutrients with immune function; but, also with the nutrient-nutrient interrelationships in foods and in dietary patterns (Mendola et al. 1995). Many dietary components can influence iron metabolism; specifically vitamin C, copper and zinc (Hallberg 1981). These interactions of iron with other micronutrients have been studied extensively. However, the interactions of iron and macronutrients are less well

defined (Johnson et al. 1987). In particular, the interaction between iron and dietary fat is vague.

Known interactions of micronutrients with dietary fat can be grouped as to the site at which they occur: luminal, mucosal, and post absorptive events (Lukaski 2000). Interactions of fatty acids with calcium and magnesium occur within the intestinal lumen. Also, interaction between fatty acids and copper and/or zinc in the intestinal lumen or at the cell membrane has been suggested, but not extensively studied (Lukaski 2000). There has been an association demonstrated between dietary fat and iron on non-heme iron absorption, iron status, and enterocyte aconitase activity (Droke and Lukaski 1996). However, the influences of fat are less well defined or understood (Lukaski 2000). Heme-iron is better absorbed by humans than non-heme iron (Hallberg 1981). Efforts to identify the mechanism by which meat promotes heme iron absorption have been extensive (Bjorn-Rasmussen and Hallberg 1979) (Hazell et al. 1978). However, consistent conclusions from these studies have not been made (Lukaski 2000). Increasing the saturation of fat, particularly as stearic acid, tends to favor enhanced absorption of non-heme iron in rats (Bowering et al. 1977). A plausible explanation may be the chelation of stearic acid to iron to form stearate-iron films, which affects the reduction of iron to its more absorbable state, ferrous iron (Wheeler et al. 1971). Iron deficiency in rats can be enhanced by the consumption of unsaturated fat (primarily linoleic acid sources), and retarded by the consumption of saturated fat diets (Johnson et al. 1987). Other research also indicates saturated fat sources such as coconut oil can raise hemoglobin and

liver iron in rats compared with PUFAs sources such as safflower oil (Johnson et al. 1992). Recent findings reveal that olive oil, high in monounsaturated fatty acids, can reduce the blood levels of lipid peroxides and the blood levels of non-heme iron (Chetty et al. 1999). Plausible explanations may be due to olive oil's increased mobilization to the liver, which will lower circulating iron (Chetty, et al. 1999). This reduced level of peroxides in the blood can help to lower the incidences of atherosclerosis in patients that consume high olive oil diets (Chetty, et al. 1999).

Literature Review Conclusions

Because the interaction between iron and immunity may be confounded by dietary fat, it is important to identify correlates of iron and dietary fat interactions on bioavailability to aid in the interpretation of their interactions with the immune system. The aim of this study was to determine if the type of dietary fat; monounsaturated, polyunsaturated or saturated, altered immune function in growing male rats deficient, adequate or overloaded in iron. The study of these interrelationships can lead to insights into mechanisms for the role of iron and fat in immune function.

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CHAPTER III

METHODOLOGY

Experimental Design

A randomized complete block design with a 3 x 4 factorial arrangement of treatments was used for this study. The treatments consisted of different dietary iron concentrations: 10 (iron deficient - FeD), 35 (iron adequate - FeA), or 100 (iron high - FeH) μg/g in combination with different types of dietary fat: safflower oil (SO), flaxseed oil (FO), olive oil (OO) or beef tallow (BT). The fat sources were chosen based on their content of n-3, n-6, and n-9 polyunsaturated fatty acids and stearic acid (Figure 1).

Animals and Diets

A rapidly growing male rat has an increased demand for iron; making it possible to induce iron deficiency with diet. Therefore, male weanling rats were used as the model for this study. One hundred and six male weanling Sprague-Dawley rats (Sasco, Inc., Omaha, NE) were grouped by weight and randomly assigned to one of thirteen groups, (12 treatment groups, 8 rats/group, and one baseline group, 10 rats in group).

The rats were ordered in two groups, with 48 treatment rats and 5 baseline group rats arriving in week 1 and the rest in week 2. Each group was immediately placed on a depletion diet (FeD-SO; Table 1) for 6 days prior to initiation of the respective treatment diets to equalize iron status. Rats in the

baseline group were killed after the 6 d depletion period to obtain initial iron status. The formulation of the diets was based on the AIN-93G Purified Diets for Laboratory Rodents (Reeves et al., 1993). All treatment diets (Table 2) contained 4% safflower oil by weight with an additional 11% consisting of the respective fat source. This supplied 30% of total energy as fat, which is the level, recommended for humans for decreased risk of chronic diseases. However, it is considered a high fat diet for the rat (Reeves et al. 1993). The recommended fat level for rats is 7% fat by weight. The diets were supplemented with ferric citrate (J.T. Baker, Phillipsburg, NJ) to meet the iron requirements of each diet. The recommended dietary iron concentration for growing rats is 35 ppm (Reeves et al. 1993). Upon completion of mixing each diet batch, samples were taken for mineral analyses. Diets were stored frozen in 2.5 kg batches in clean 1-gallon Zip-lock® bags at $-20^{0}C$ to prevent oxidation. An analytical balance was used to measure diets into appropriate diet color-coded polyethylene cups with corresponding sealing lids. The diets were measured with deionized washed measuring spoons accounting for uncertainty error to the hundredth decimal place and kept at $4^{0}C$ until used.

Rats were housed individually in stainless steel suspension cages with free access to deionized water. Rats remained on the experimental diets for 8 weeks. In order to minimize differences in food intake, food intake was measured two times per week. The food intake of all groups was matched to the food intake of the FeD/SO fed rats (Droke and Lukaski 1996). Equalization of food intake was needed to eliminate food intake as a variable for body weight and

immune function differences. Body weight was measured twice weekly accounting for uncertainty error to the tenth decimal place. All procedures involving animals was approved by the Oklahoma State University Laboratory Animal Care Committee (Appendix A).

Sample Collection and Analyses

All reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. Double distilled nitric acid was purchased from GFS (Columbus, OH). Ultrapure 30% hydrogen peroxide was purchased from J.T. Baker (Phillipsburg, NJ).

Rats were killed (1 rat/treatment/d) according to their weight group. Following an overnight fast in which rats were housed in metabolic cages, they were anesthetized with Metofane® (Schering-Plough Animal Health, Union, NJ) and then exsanguinated via the abdominal aorta. Blood samples were collected for hematological and nutritional analyses. An EDTA-coated syringe was used for hematological determinations. A citrate-coated syringe was used for plasma mineral determinations. A syringe with no anticoagulant was used to collect serum. After centrifugation of the serum in the syringe, the serum was aliquoted into tubes and stored at –20°C until analyzed. The spleen was excised, and placed in a pre-weighed tube with 5mL sterile calcium-and-magnesium-free Hanks' Balanced salt solution (HBSS) for splenocyte isolation. Following removal of the spleen, the liver, kidney and heart, were removed, rinsed in 0.9% NaCl and stored at -20^{0}C in sterile bags until mineral analyses were performed.

Before storage, the liver was separated into lobes and the biggest lobe was used for mineral analyses.

Hematocrit (Hct), hemoglobin (Hgb), white blood cell counts (WBC), differential leukocyte counts and red blood cell (RBC) measurements were measured using a ABX-Vega Hematology Analyzer (France). Using the mean values of the baseline group, a change in Hgb and Hct was calculated over time using hemoglobin regeneration as an index of iron utilization (Wienk et al. 1999). Serum iron, unsaturated iron binding capacity (UIBC), and total cholesterol, HDL cholesterol and triacylglycerides parameters were measured using a Cobas Fara II clinical analyzer (Montclair, NJ). Total iron binding capacity (TIBC) and transferrin saturation were calculated from the serum iron/UIBC measurements. Non HDL-cholesterol was calculated from total cholesterol, HDL and triacylglycerides. Plasma and tissue mineral content were determined after wet ashing with ultrapure nitric acid, deionized water and hydrogen peroxide (Hill et al. 1986) and dry ashing using a Lindberg ashing oven (Wisconsin). All materials used for the mineral analysis were acid-washed in a 10% hydrochloric acid solution for twenty-four hours. After washing, materials were then rinsed with deionized water and dried in a clean oven set at 40°C. The mineral concentrations were measured using a Perkin Elmer Model 5100 Atomic Absorption Analyzer (Norwalk, CT). Bovine liver standard (National Institute of Standards and Technology, Gaithersberg, MD) was analyzed for mineral concentrations as a quality control measure. The intra-assay and inter-assay

values were 245.5 ± 26.2 mg Fe per kg sample and 259.3 ± 50.6 mg Fe per kg sample, respectively.

Isolation of Splenocytes, Lymphocyte Proliferation and Cytokine Production

Using aseptic technique in a laminar flow hood, spleens were transferred from the tube into a petri dish and gently teased apart with sterile needles to obtain single cell suspensions. With a sterile polyethylene disposable transfer pipette, the spleen tissue/cell mixture was rinsed with HBSS to further disperse cells into solution. Cells were then filtered (40μm Nylon sterile Falcon filters from Becton Dickinson, Franklin Lakes, NJ) and diluted with 20 mL of HBSS in a 50 mL polypropylene tube. Five-milliliter cell suspensions were layered onto 5 mL of Histopaque and centrifuged for 30 minutes at 1000 rpm at room temperature. Cells at the density interface were removed and washed twice with 12 mL HBSS in a sterile 15 mL polypropylene tube. Cells were counted and resuspended at 2×10^6 viable cells/mL in RPMI 1640 medium containing 100 μ/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine. Cell counts and viability were determined by trypan blue exclusion (0.1% trypan blue, Eastman Kodak, Rochester, NY) and counting in a hemacytometer (Helyar and Sherman 1992).

Isolated splenocytes were then aliquoted in 50 μL RPMI into 96-well tissue culture plates (2×10^5 cells/well). Autologous serum (final concentration, 2%) was used to stimulate in vivo conditions. The cells were then stimulated with either a final concentration of 5 μg/mL or 10 μg/mL ConA; RPMI alone served as

the unstimulated control for basal proliferation and cytokine production. Cells were incubated at 37°C for 48 h in a humidified incubator with 5% CO_2 / 95% air.

Following incubation with mitogen, all wells designated for cell proliferation had 1 μCi of [methyl - ^{3}H]thymidine (6.7 Ci/mmol; NEN Life Science Products, Boston, MA) added and incubated for an additional 24 h **(Calder et al., 1995)**. After incubation cells were harvested onto glass fiber filter paper discs using a Skatron Micro 96 cell harvester unit (Norway). Cellular uptake of ^{3}H-thymidine was quantified through liquid scintillation counting.

Following incubation for 72 hours, the cells designated for cytokine determination were centrifuged to collect supernatant (pooled from 96-well tissue culture plates) and the supernatant samples were then frozen at -20°C until used for cytokine measurements. The supernatant was then thawed, gently vortexed, and analyzed quantitatively for IL-2 using an enzyme-linked immunosorbent assay (ELISA) kit (Pharmingen, San Diego, CA).

Further investigation of cytokine production was performed during a subsequent animal study (approximately one year later). Animals, diets and procedures of the second study were similar to the present study. Splenocytes were isolated, placed into 24-well tissue culture plates (1 x 10^6) and stimulated with concanavalin A (5 μg/mL final concentration) or lipopolysaccharide (5 μg/mL final concentration); RPMI plus serum alone was used as an unstimulated control. Interleukins -2, -4, -10 and IFN-γ were determined by ELISA kits (IFN-γ: R & D Systems, Minneapolis, MN; IL-2, 4, 10: Pharmingen, San Diego, CA).

Statistical Analysis

Data were analyzed for a mixed model using SAS (Version 7.0 SAS Institute Inc., Cary, NC). The random effects variable was the weight group. Lymphocyte proliferation, serum Fe, tissue and plasma mineral values were log transformed prior to statistical analyses. Tukey's multiple-comparison procedure was used to compare individual treatment means. Differences were considered significant at $p < 0.05$. The data are presented as the least square means ± standard error of the mean; log transformed data are presented as the back transformed mean.

Table 1: Low Iron – Safflower Oil Depletion Diet

Ingredient[1]	g/kg
Corn starch	397.5
Dextrinized cornstarch	132
Casein, vitamin free	200
Sucrose	100
Alphacel	50
L-cystine	3
Choline bitartrate (41.1% choline)	2.5
Mineral mix (-iron)[2]	35
AIN-93G-VX[3]	10
Safflower oil	70

[1]Dietary ingredients were purchased from Harlan Teklad (Madison, WI).
[2]Mineral mix (g/kg): $CaCO_3$, 357; KH_2PO_4, 196; K Citrate, 70.78; NaCl, 74; KSO_4, 46.6; MgO, 24; $ZnCO_3$, 1.65; $MnCO_3$. 0.63; $CuCO_3$, 0.3; KIO_3, 0.01; Na Selenate, 0.01025; NH_4 Paramolybdate, 0.00795; Na meta-silicate, 1.45; $CrKSO_4$, 0.275; LiCl, 0.0174; Boric Acid, 0.0815; NaF, 0.0635; $NiCO_3$, 0.0318; NH_4 Vanadate, 0.0066; Powdered Sucrose, 218.615.
[3]Vitamin mix

Table 2: Composition of Experimental Diets

Ingredient[1]	10 μg/g Fe	35 μg/g Fe	100 μg/g Fe
	g/kg	g/kg	g/kg
Cornstarch	316.5	314.0	307.5
Dextrinized Cornstarch	132	132	132
Casein, vitamin free	200	200	200
Sucrose	100	100	100
Alphacel	50	50	50
L-Cystine	3	3	3
Choline bitartrate (41.1% choline)	2.5	2.5	2.5
Mineral Mix (-Iron)[2]	35	35	35
AIN-93G-VX[3]	10	10	10
Iron premix[4]	1.0	3.5	10
Safflower oil	40	40	40
Fat source (Safflower oil, Flaxseed oil, Olive oil, or Beef Tallow)	110	110	110

[1] Dietary ingredients were purchased from Harlan Teklad (Madison, WI).
[2]Mineral mix (g/kg): $CaCO_3$, 357; KH_2PO_4, 196; K Citrate, 70.78; NaCl, 74; KSO_4, 46.6; MgO, 24; $ZnCO_3$, 1.65; $MnCO_3$. 0.63; $CuCO_3$, 0.3; KIO_3, 0.01; Na Selenate, 0.01025; NH_4 Paramolybdate, 0.00795; Na meta-silicate, 1.45; $CrKSO_4$, 0.275; LiCl, 0.0174; Boric Acid, 0.0815; NaF, 0.0635; $NiCO_3$, 0.0318; NH_4 Vanadate, 0.0066; Powdered Sucrose, 218.615.
[3]Vitamin mix
[4]Iron premix: Ferric citrate (J.T. Baker; 16.5% Fe), 10 mg Fe/g sucrose

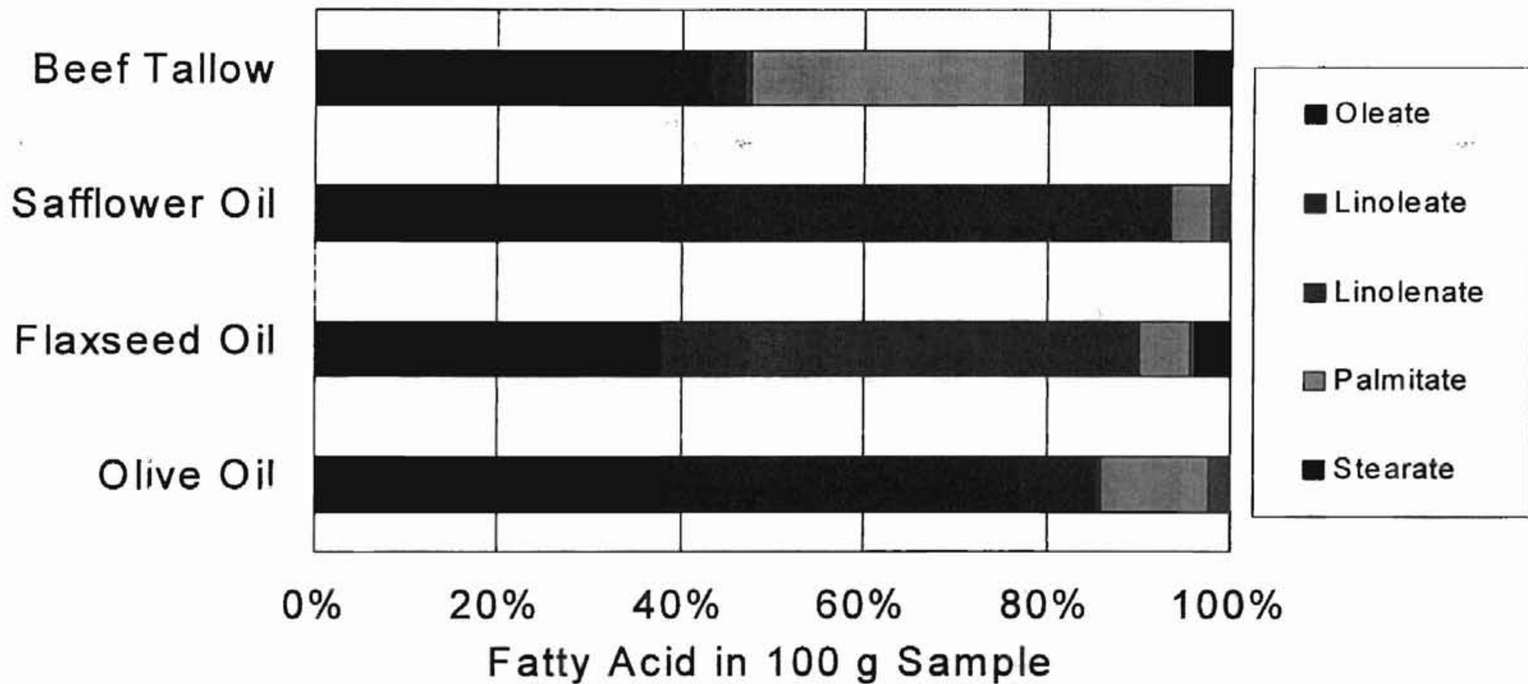

Figure 1: Fatty acid composition of fat sources based on data from USDA Nutrient Database for Standard Reference, Release 13.

CHAPTER IV

RESULTS

After the 8 weeks on treatment diets, rats fed FeD diets had decreased ($p<0.05$) weight gain and food intake compared with rats fed FeA or FeH (Table 3). No differences ($p>0.05$) in weight gain or food intake were found between FeA or FeH or among the various sources of dietary fat. An interaction between iron and fat also was not observed ($p>0.05$) for either weight gain or food intake. Iron intake was affected ($p<0.05$) by dietary iron, which confirms that we fed rats varying levels of iron (0.23 ± 0.01, 0.75 ± 0.01, 1.92 ± 0.01 μg/g; respectively: 10, 35, 100 μg/g Fe). Olive oil and SO fed rats had the lowest iron intake, whereas BT fed rats had the highest iron intake (SO, 0.96 ± 0.014; FO, 1.00 ± 0.016; OO, 0.87 ± 0.015; BT, 1.03 ± 0.015 mg/d). Analysis of diet samples, indicated the OO and SO diets had a lower ($p<0.05$) iron content compared with BT (data not shown).

Spleen weight as a percentage of body weight (Table 3) was greater ($p<0.05$) in FeD rats compared with rats fed FeH, but was not different ($p>0.05$) from rats fed FeA. Kidney weights as a percentage of body weight (Table 3) were reduced ($p<0.05$) in FeD rats compared with FeA rats. Kidney weights were similar ($p>0.05$) between rats fed FeA and FeH. Dietary iron did not affect ($p>0.05$) liver weight (data not shown). Spleen weights in rats fed OO was greater ($p<0.05$) than in rats fed SO (spleen: SO,0.243 ± 0.008; FO, 0.253 ± 0.009; OO, 0.275 ± 0.009; BT, 0.245 ± 0.009 %). Liver weight as a percentage

of body weight was reduced (*p*<0.05) in rats fed SO or FO compared with OO (liver: SO, 2.92 ± 0.17; FO, 2.74 ± 0.17; OO, 3.28 ± 0.17; BT, 3.05 ± 0.17%). Rats fed OO or BT had similar (*p*>0.05) liver weights, as did rats fed BT or SO. Fat did not affect (*p*>0.05) kidney weights. Heart weights were affected (*p*<0.05) by an iron and fat interaction (Table 4). Heart weights were greater (*p*<0.05) in rats FeD with SO, FO or OO compared with rats fed FeH with SO, FO, OO. Heart weights in rats fed BT were unaffected (*p*>0.05).

An FeD diet reduced (*p*<0.05) Hemoglobin (Hgb), Hematocrit (Hct), erythrocyte counts, and mean corpuscular volume (MCV) (Table 5). Reticulocyte counts were greater (*p*<0.05) in rats fed FeD compared with rats fed FeH. Reticulocyte counts in FeA rats was not measured. Safflower oil fed rats had reduced (*p*<0.05) Hgb and Hct compared with BT fed rats (Hgb: SO, 11.7 ± 0.2; FO, 11.9 ± 0.2; OO, 11.9 ± 0.2; BT, 12.2 ± 0.2 g/dL; Hct: SO, 32.0 ± 0.4; FO, 32.6 ± 0.4; OO, 33.2 ± 0.4; BT, 33.6 ± 0.4 g/dL). An FeD diet reduced (*p*<0.05) serum iron concentration, UIBC, TIBC, and transferrin saturation compared with FeA or FeH diets (Table 6). No differences (*p*>0.05) were observed between rats fed FeA or FeH. Effects of dietary fat or the interaction between dietary iron and fat were not observed (*p*>0.05) among treatment groups for these indices of iron status.

Iron utilization (change in Hgb) was decreased (*p*<0.05) in rats fed FeD compared with rats fed FeA or FeH (Figure 2). Rats fed FeD were unable to regenerate Hgb; whereas, rats fed FeA or FeH exhibited a positive change in Hgb. Safflower oil tended (*p*=0.06) to reduce iron utilization (change in Hgb)

compared with BT. Iron utilization was unaffected ($p>0.05$) by a dietary fat and iron interaction.

Liver iron concentrations decreased ($p<0.05$) linearly with decreasing dietary iron (Figure 3). Total liver iron was also reduced ($p<0.05$) with lower dietary iron (1017 ± 189, 3191 ± 172, 4849 ± 170 μg Fe; respectively, for 10, 35, and 100 μg/g). Copper concentrations in the liver was lower ($p<0.05$) in rats fed FeD compared with rats fed FeH. Liver manganese concentrations were lower ($p<0.05$) in rats fed FO compared with rats fed OO (SO, 9.9 ± 1.0; FO, 9.2 ± 1.0; OO, 10.7 ± 1.0; BT, 10.2 ± 1.0 μg/g). No differences ($p>0.05$) were observed among the other fat sources. Dietary iron did not affect ($p>0.05$) liver concentrations of zinc, calcium and magnesium (data not shown). The interaction between dietary iron and fat did not have an affect ($p>0.05$) on liver mineral concentrations.

Kidney iron concentrations were lower ($p<0.05$) in FeD rats compared with rats fed FeA or FeH (Table 7). Kidney copper concentrations were greater ($p<0.05$) in rats fed FeA compared with rats fed FeD. Dietary iron did not affect ($p>0.05$) kidney concentrations of zinc, calcium or magnesium (data not shown). Dietary fat or the interaction between dietary iron and fat did not affect ($p>0.05$) kidney mineral concentrations.

Heart iron concentrations were reduced in ($p<0.05$) in rats fed FeD compared with rats fed FeA or FeH (Table 7). There was no difference ($p>0.05$) between rats fed FeA or FeH. Heart zinc concentrations tended to be greater ($p=0.07$) in rats fed FeH compared with rats fed FeD (56.00 ± 1.04, 59.90 ± 1.03,

62.61 ± 1.03; respectively, for 10, 35 and 100 μg/g Fe). Dietary iron did not affect ($p>0.05$) concentrations of copper, calcium and magnesium. Dietary fat or the interaction between dietary iron and fat did not affect ($p>0.05$) heart mineral concentrations.

Plasma copper concentrations were affected ($p<0.05$) by a dietary iron and fat interaction (Figure 4). When analyzed for iron effects within fat groups, significant iron effects were observed only in rats fed SO. Plasma copper was lower ($p<0.05$) in rats fed FeH compared with rats fed FeD or FeA. Plasma copper was similar ($p>0.05$) between FeD and FeA. When analyzed for fat effects within fat groups, significant fat effects were observed for rats fed FeH. Rats fed SO had lower ($p<0.05$) plasma copper compared with rats fed FO or BT. Plasma zinc concentrations were greater ($p<0.05$) in rats fed BT compared with SO and FO (Figure 5). Plasma zinc was unaffected ($p>0.05$) by dietary iron or the interaction between iron and fat. Plasma calcium concentrations were reduced ($p<0.05$) in rats fed FeA compared with rats fed FeD diets (39.7 ± 1.0, 36.9 ± 1.0, 37.5 ± 1.0 mg/L Fe; respectively, for 10, 35, and 100 μg/g). Rats fed FeH had plasma calcium concentrations that were not different ($p>0.05$) from FeD and FeA. Plasma magnesium concentrations were unaffected ($p>0.05$) by dietary iron and/or fat.

Total white blood cell counts were unaffected ($p>0.05$) by dietary iron and/or fat. An low Fe diet reduced ($p<0.05$) monocyte and eosinophil concentrations compared with FeA or FeH (monocyte: 5.6 ± 0.4, 4.1 ± 0.4, 3.5 ± 0.4 %; respectively for 10, 35, 100 μg/g Fe; eosinophil: 0.63 ± 0.13, 1.33 ± 0.11,

1.23 ± 0.11%; respectively for 10, 35, 100 μg/g Fe). The FeD diet tended to reduce (p=0.06) basophil concentrations compared with FeA or FeH (data not shown). Lymphocyte and neutrophil concentrations were unaffected (p>0.05) by dietary iron. No differences (p>0.05) were found among dietary fat sources or for the interaction between dietary iron and fat on these differential leukocyte counts.

Platelet concentrations were greater (p<0.05) in rats fed FeD compared with rats fed FeA or FeH (Figure 6). No differences (p>0.05) were found among dietary fat sources or for the interaction between dietary iron and fat on platelet concentrations.

Total cholesterol, HDL-cholesterol, and non-HDL cholesterol were unaffected (p>0.05) by dietary iron and/or fat (data not shown). Flaxseed oil lowered (p<0.05) triacylglycerides (TG) compared with OO and BT; however, it was not different (p>0.05) from SO (Figure 7). Safflower oil also lowered (p<0.05) TG compared with BT. Triacylglycerides were not affected (p>0.05) by dietary iron or the interaction between dietary iron and fat.

Splenocyte viability was unaffected (p>0.05) by dietary iron and/or fat (data not shown). Viabilities were greater than 90%. Stimulation of isolated splenocytes with ConA 5μg/mL resulted in higher ^{3}H-thymidine uptake than when splenocytes were stimulated with ConA 10 μg/mL (Figure 8). Rats fed FeD had suppressed (p<0.05) lymphocyte proliferation, in response to both ConA 5 and 10 μg/mL compared with rats fed FeA or FeH. Proliferation was not different (p>0.05) between rats fed FeA or FeH. Splenocyte proliferation in response to concanavalin A (5 μg/mL) was affected (p<0.05) by an interaction between

dietary iron and fat (Figure 9). When analyzed for iron effects within fat groups differences were observed only for rats fed OO. Rats fed FeD had suppressed ($p<0.05$) proliferation compared with rats fed FeH. Lymphocyte proliferation in rats fed FeA was similar ($p>0.05$) to rats fed FeD or FeH. Within iron groups rats fed FeH and SO had suppressed ($p<0.05$) proliferation compared with rats fed FeH and OO (4914.7 ± 1.2, 6974 ± 1.2, 7150 ± 1.2, 4200 ± 1.2 cpm; respectively, for SO, FO, OO and BT).

For both years, IL-2 production by unstimulated cultures was not affected ($p>0.05$) by dietary iron and/or fat. Interleukin-2 production (year 1) in response to ConA 5 μg/mL tended to be greater ($p=0.06$) in rats fed FeD compared with rats fed FeH. Interleukin-2 production in response to ConA 10 μg/mL stimulation was unaffected ($p>0.05$) by dietary iron and/or fat. In year two, production of IL-2 in response to stimulation by ConA (5 μg/mL) lipopolysaccharide was unaffected ($p>0.05$) by dietary iron and/or fat.

Production of IL-4 (year 2) in unstimulated cultures was not different ($p>0.05$) among groups (data not shown). Interleukin-4 production (year 2) in response to ConA (5 μg/mL) stimulation tended to be enhanced ($p=0.05$) in rats fed OO compared with rats fed BT or FO. There was a trend ($p=0.08$) for an iron and fat interaction in the ConA stimulated cells.

Interleukin-10 production (year 2) was reduced ($p=0.05$) in unstimulated cells of rats fed FeD compared with unstimulated cells of rats fed FeH diets (data not shown). Production of IL-10 (year 2) in ConA stimulated cultures was not different ($p>0.05$) among groups. There was also a strong trend ($p=0.07$) for IL-

10 production of lipopolysaccharide stimulated cells to be reduced in rats fed BT, compared with rats fed FO.

Production of IFN-γ (year 2) in unstimulated cultures was not different ($p>0.05$) among groups (data not shown). Interferon-γ production in response to ConA tended ($p=0.06$) to be greater in rats fed FO compared with rats fed SO (Figure 10). Interferon-γ production in response to ConA stimulation was unaffected ($p>0.05$) by dietary iron or an interaction between iron and fat. Interferon-γ production in response to lipopolysaccharide stimulation was similar ($p>0.05$) among treatment groups.

Table 3: Weight gain, organ weights, and food intake in rats fed low, adequate or high iron[1]

Dietary Iron	Weight gain	Spleen weight per body weight	Kidney weight per body weight	Heart weight per body weight	Food Intake
(µg/g)	(g)	(%)	(%)	(%)	(mg/d)
10	241 ± 5[a]	0.272 ± 0.009[a]	0.62 ± 0.01[a]	0.394 ± 0.008[a]	14.3 ± 0.2[a]
35	254 ± 5[b]	0.248 ± 0.008[a,b]	0.66 ± 0.01[b]	0.335 ± 0.008[b,c]	14.9 ± 0.1[b]
100	266 ± 5[b]	0.242 ± 0.008[b]	0.64 ± 0.01[a,b]	0.323 ± 0.008[c]	15.2 ± 0.1[b]

[1]Values are presented as the lsmean ± SE (n=7-8).
[a,b,c]Means within a column with unlike letters are different ($p<0.05$).

Table 4: Heart weights, in rats fed low, adequate or high iron with different sources of fat[1]

Dietary Fat Source	10 µg/g Fe	35 µg/g Fe	100 µg/g Fe
Safflower oil	0.409 ± 0.013[a]	0.325 ± 0.012[b]	0.322 ± 0.012[b]
Flaxseed oil	0.385 ± 0.014[a]	0.342 ± 0.013[a,b]	0.321 ± 0.013[b]
Olive oil	0.418 ± 0.014[a]	0.339 ± 0.013[b]	0.307 ± 0.013[b]
Beef Tallow	0.366 ± 0.016	0.333 ± 0.013	0.341 ± 0.013

[1]Values are presented as the lsmean ± SE (n=7-8).
[a,b,]Means within a row with unlike letters are different ($p<0.05$).

Table 5: Hematological data in rats fed low, adequate or high iron[1]

Dietary Iron	Hemoglobin	Hematocrit	Erythrocyte Count	Mean Corpuscular Volume	Reticulocyte Count
(μg/g)	(g/dl)	(%)	(10^6/mm^3)	(μm^3)	(10^6/mm^3)
10	8.2 ± 0.2[a]	24.0 ± 0.4[a]	7.98 ± 0.09[a]	29.9 ± 0.4[a]	1.17 ± 0.04[a]
35	13.7 ± 0.2[b]	36.9 ± 0.4[b]	7.39 ± 0.08[b]	50.0 ± 0.4[b]	ND[2]
100	13.9 ± 0.2[b]	37.5 ± 0.3[b]	7.25 ± 0.08[b]	51.7 ± 0.3[b]	0.19 ± 0.04[b]

[1]Values are presented as the lsmean ± SE (n=7-8).

[2]ND = not determined

[a,b,c]Means within a column with unlike letters are different ($p<0.05$).

Table 6: Iron status indicators in rats fed low, adequate or high iron[1]

Dietary Iron	Serum Iron[2]	UIBC[3]	TIBC	Tf_{sat}
(μg/g)	(μg/dL)	(μg/dL)	(μg/dL)	(%)
10	29 ± 1[a]	480 ± 12[a]	511 ± 8[a]	6 ± 2[a]
35	156 ± 1[b]	290 ± 11[b]	453 ± 7[b]	36 ± 2[b]
100	169 ± 1[b]	259 ± 12[b]	431 ± 8[b]	40 ± 2[b]

[1]Values are presented as lsmean ± SE (n=7-8).
[2]Serum iron is presented as the back transformed lsmean ± SE.
[3]UIBC, unsaturated iron binding capacity; TIBC, total iron binding capacity; and, Tf_{sat}, transferrin saturation.
[a,b,c]Means within a column with unlike letters are different ($p<0.05$).

Table 7: Kidney and heart mineral concentrations in rats fed low, adequate or high iron[1]

Organ	Dietary Iron	Iron	Copper
	(μg/g)	(μg/g)	(μg/g)
Kidney	10	124 ± 1[a]	26 ± 1[a]
	35	254 ± 1[b]	32 ± 1[b]
	100	276 ± 1[b]	27 ± 1[a,b]
Heart	10	203 ± 1[c]	17 ± 1
	35	287 ± 1[d]	18 ± 1
	100	301 ± 1[d]	18 ± 1

[1]Values are presented as the back transformed lsmean ± SE (n=7-8).
[a,b]Kidney means within a column with unlike letters are different ($p<0.05$).
[c,d]Heart means within a column with unlike letters are different ($p<0.05$).

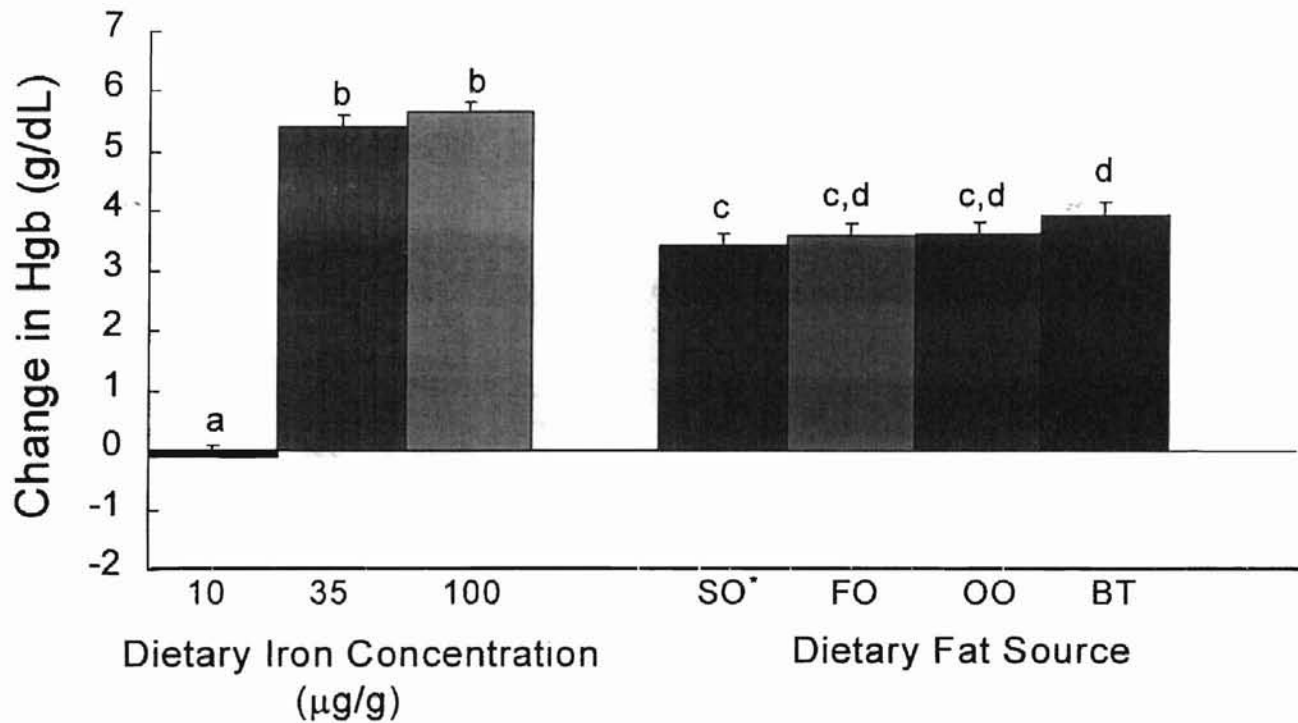

Figure 2: Iron utilization with different concentrations of dietary iron or sources of dietary fat. *Dietary fat source: SO, safflower oil; FO, flaxseed oil; OO, olive oil; and BT, beef tallow. [a,b]Dietary iron concentration (n=7-8): bars with unlike letters are different ($p<0.05$). [c,d]Dietary fat source (n=7-8) : bars with unlike letters are different ($p=0.06$)

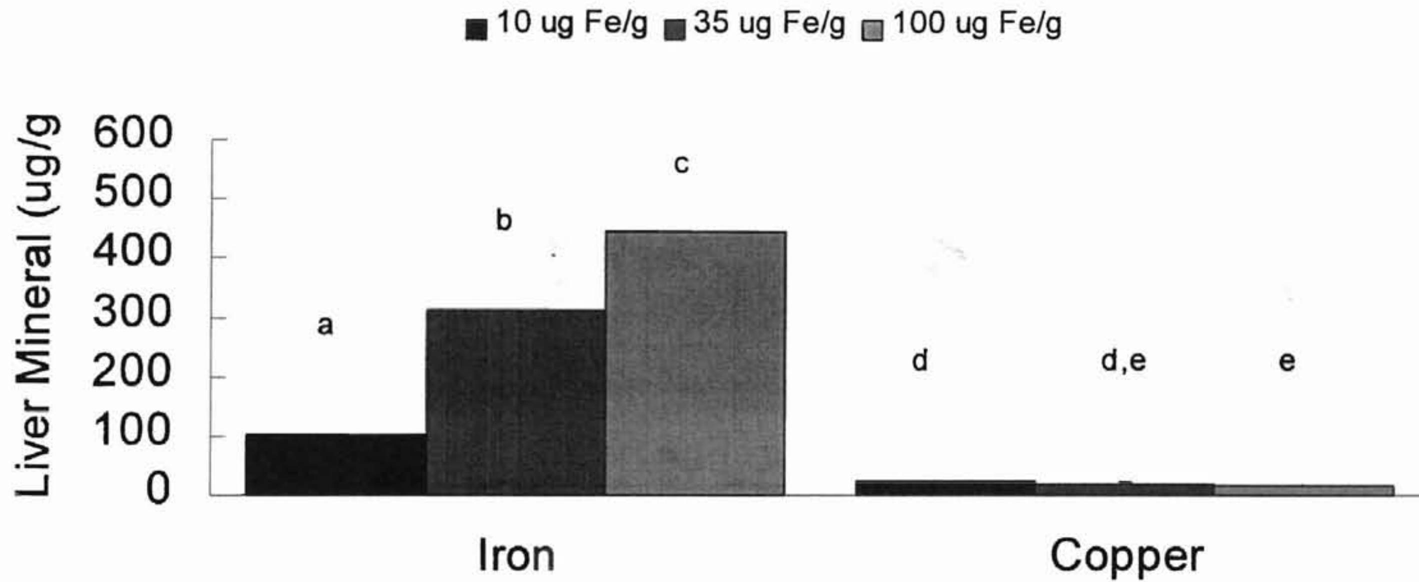

Figure 3: Liver mineral concentrations in rats fed low, adequate or high dietary iron. Values are presented as the back transformed lsmean with SE. [a,b,c]Liver iron concentration (n=7-8): bars with unlike letters are different ($p<0.05$). [d,e]Liver copper concentrations (n=7-8): bars with unlike letters are different ($p<0.05$).

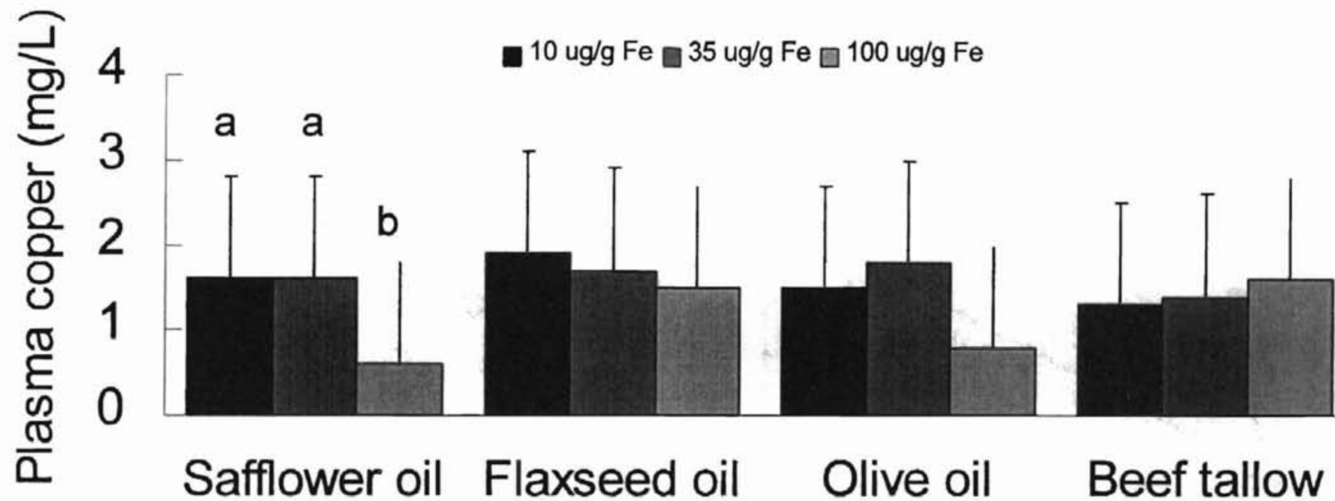

Figure 4: Plasma copper in rats fed low, adequate or high with different sources of dietary fat (n=7-8). Values are presented as the back transformed lsmeans with SE. Dietary Fe and fat interaction, (p<0.05). [a,b]Bars with unlike letters are different (p<0.05).

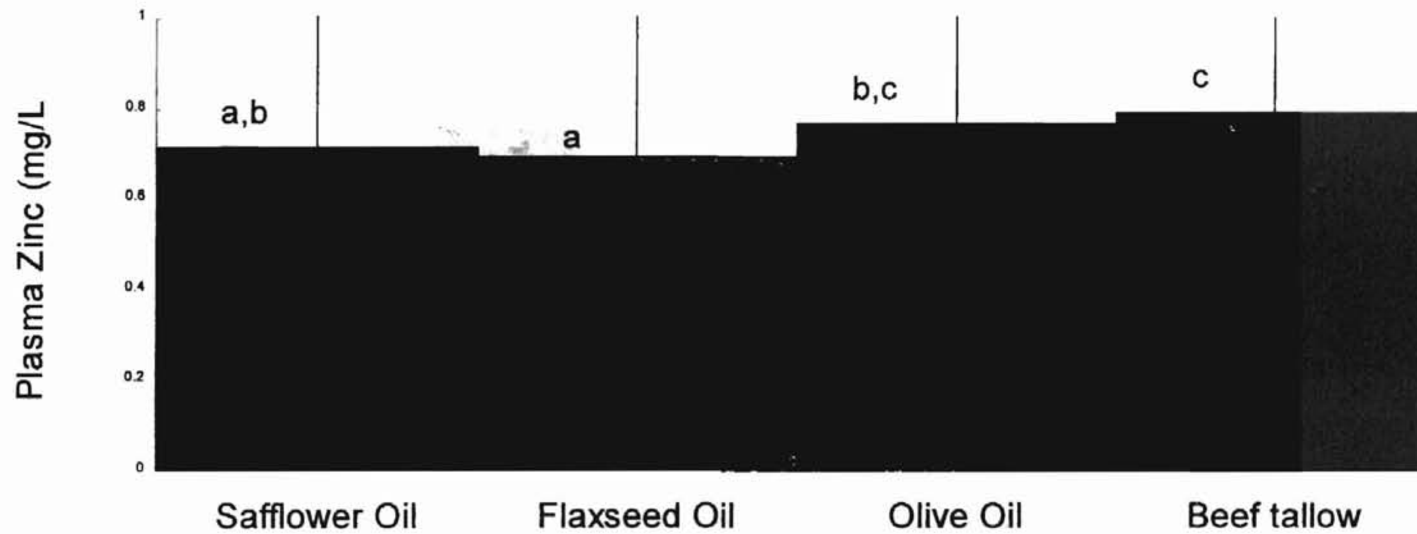

Figure 5: Plasma zinc in rats fed different sources of dietary fat. Values are presented as the back transformed lsmean with SE (n=7-8). [a,b,c]Bars with unlike letters are different (p<0.05).

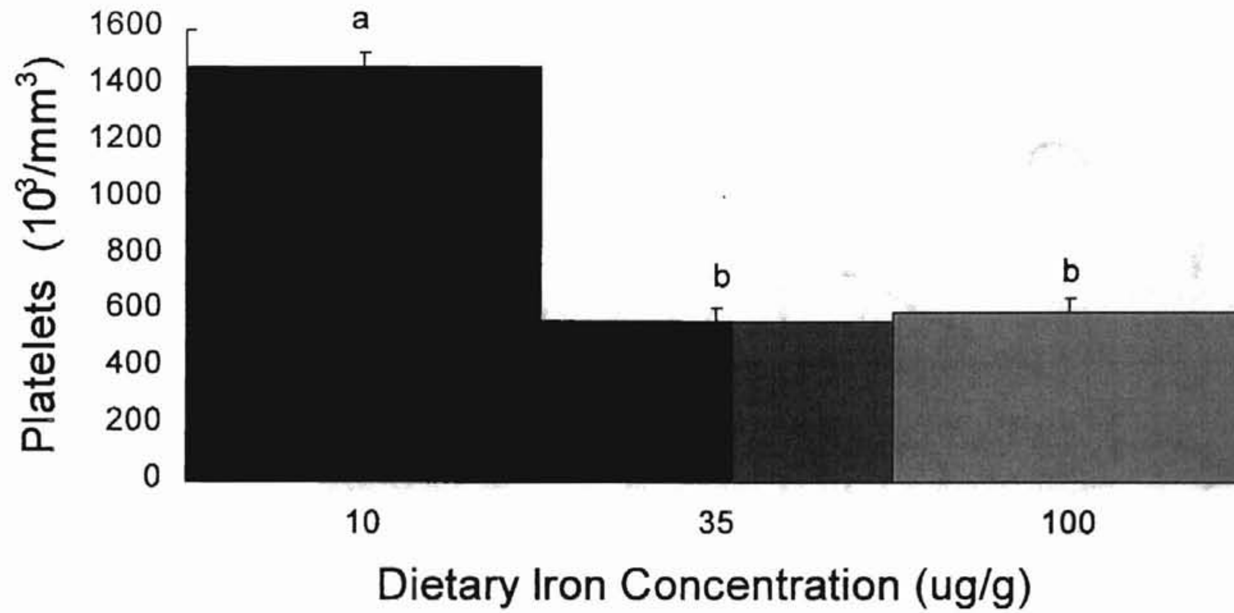

Figure 6: Platelets in rats fed low, adequate or high dietary iron (n=7-8). [a,b]Bars with unlike letters are different (p<0.05).

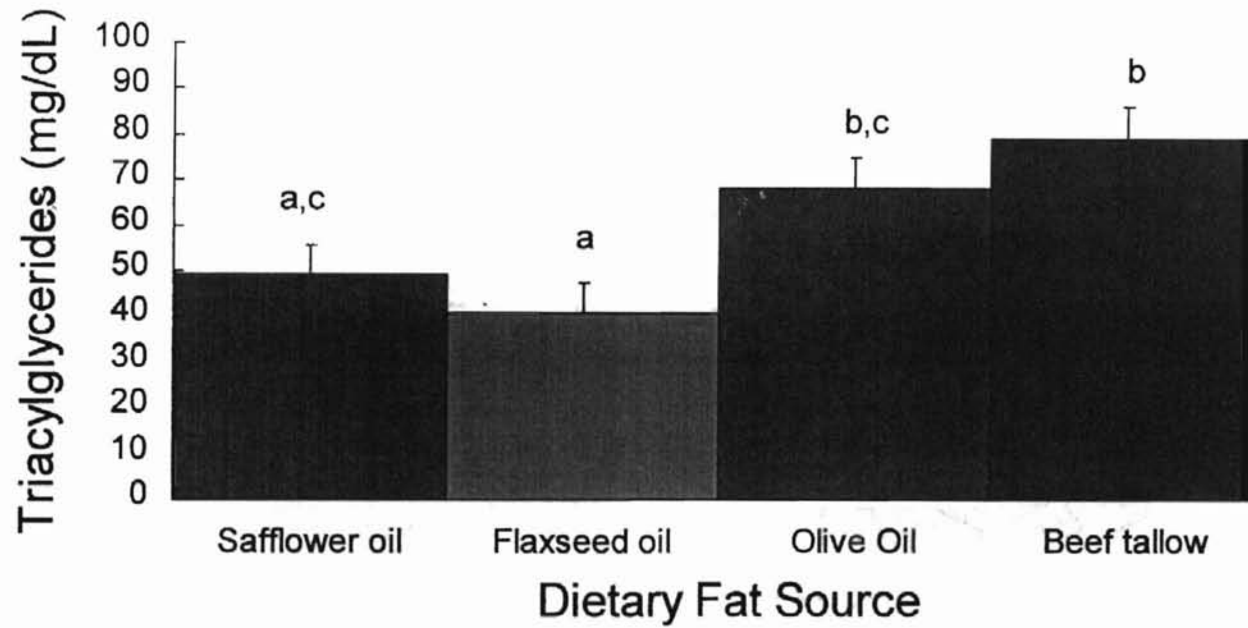

Figure 7: Triacylglyceride concentrations in rats fed different sources of dietary fat (n=7-8). [a,b,c]Bars with unlike letters are different ($p<0.05$).

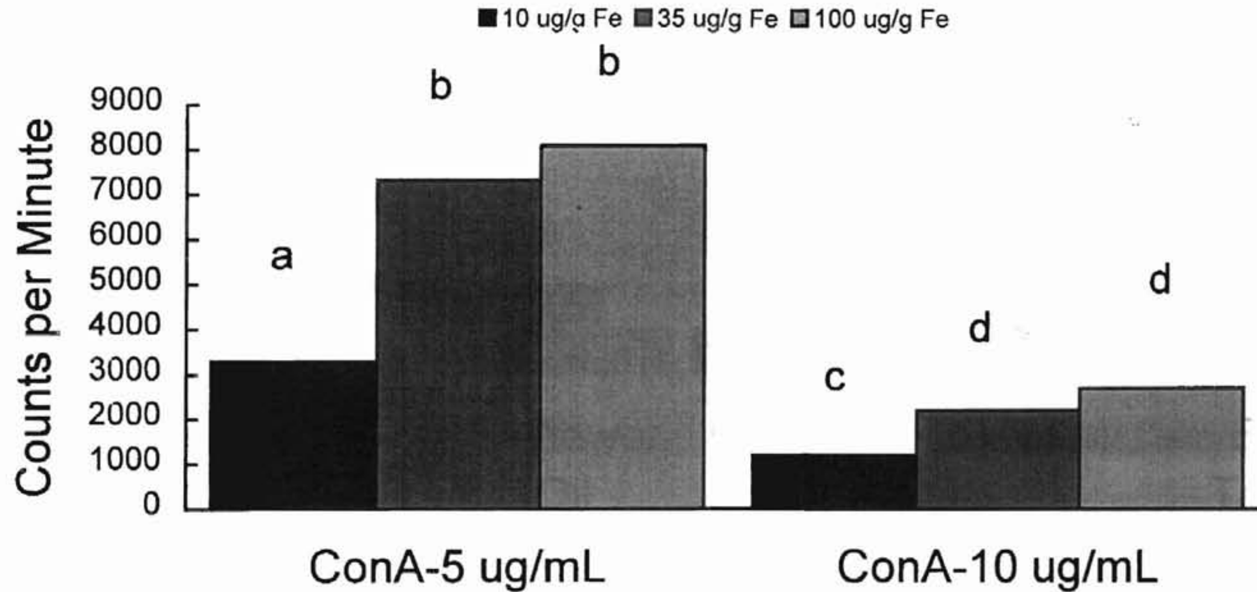

Figure 8: Mitogen-induced lymphocyte proliferation in rats fed different concentrations of dietary iron. Cultures were stimulated with Concanavalin A. Values are presented as the back transformed lsmeans with SE (n=7-8). [a,b]ConA-5 ug/mL: bars with unlike letters are different (p<0.05). [c,d]ConA-10 ug/mL: bars with unlike letters are different (p<0.05).

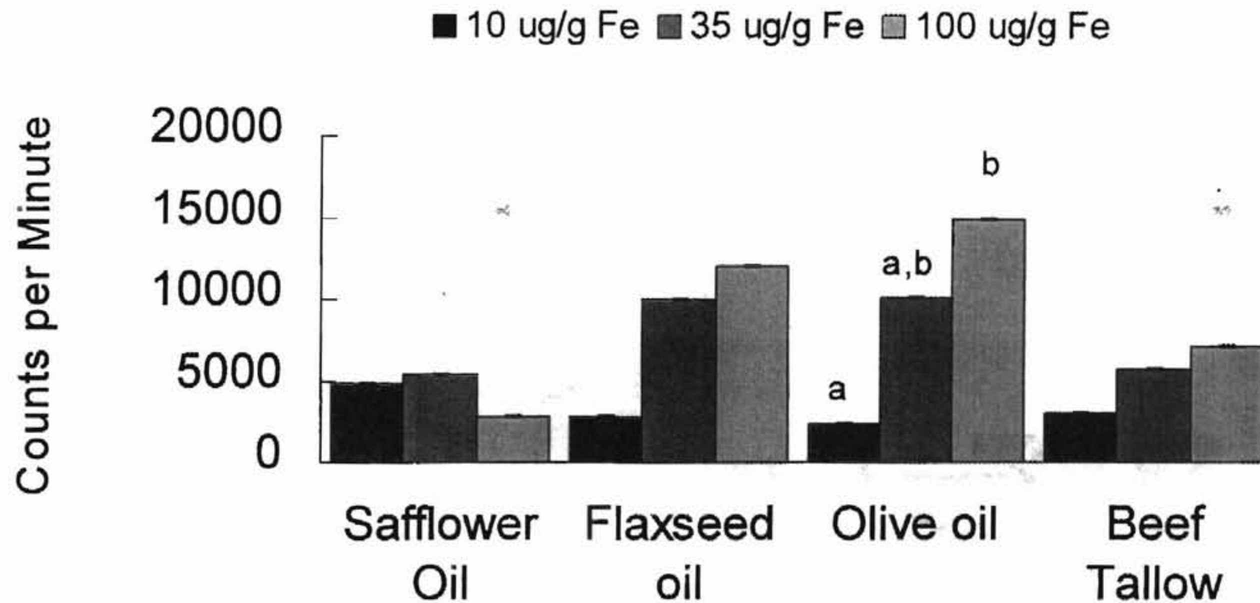

Figure 9: Mitogen-induced lymphocyte proliferation in rats fed different sources of dietary fat. Cultures were stimulated with 5ug/mL concanavalin A. Values are presented as the back transformed lsmean with SE for ^{3}H-thymidine uptake in counts per minute (n=7-8). Dietary iron and fat interaction ($p<0.05$). [a,b] Bars with unlike letters are different ($p<0.05$).

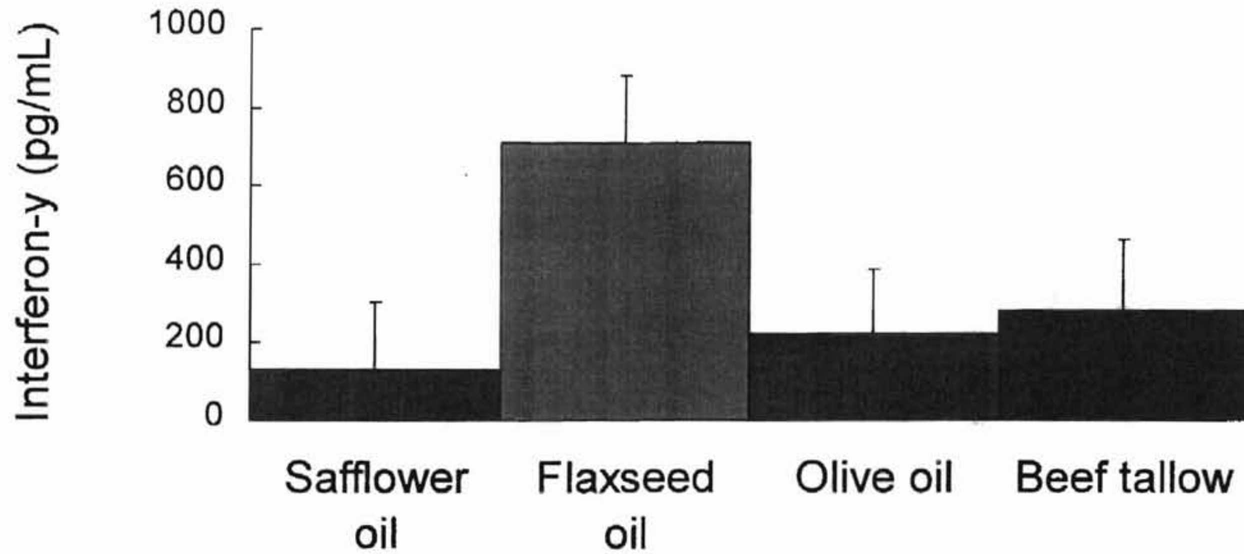

Figure 10: Interferon-γ production in response to concanavalin A stimulation (5 ug/mL) in rats fed different sources of dietary fat. (n=7-8). Dietary fat (p=0.06).

CHAPTER V

DISCUSSION

Poorer growth rates and food intakes were observed in rats fed a diet deficient in iron. Even though food intake was restricted to the intake of the FeD/SO group, differences in food intake and thus iron intake occurred. Previous studies have indicated iron deficiency can reduce growth (Helyar and Sherman 1992). Analyses of diets for iron content indicate BT contained greater amounts of iron, which may explain why BT fed rats also had a higher iron intake compared with SO within iron groups. This opposes another study that found poor weight gain in rats fed high amounts of stearic acid compared to SO (Johnson, Lukaski, et al. 1992). However, they fed their source of stearic acid to rats in the form of free fatty acid; our study fed rats a source of stearic acid in the form of BT. Beef tallow, extruded from animal fat, has been shown to favor increased absorption of non-heme iron in rats (Bowering et al. 1977). This mechanism may reflect other studies evaluating the meat factor for promotion of iron absorption in rats (Bjorn-Rasmussen and Hallberg 1979, Gordon and Godber 1989). Reduction of food intake, iron intake and growth could be a factor that could complicate immunological results.

Organ weights as a percentage of body weight varied in rats fed low, adequate or high iron with different sources of fat. Spleen weights were enlarged in rats fed low iron compared to high iron fed rats. An enlarged spleen size might implicate increasing the total number of spleen cells compared with normal iron

status. Corresponding to another study that looked at moderate and sever iron deficiency in mice our data indicated no significant differences in liver weight among groups (Helyar and Sherman 1992). Kidney weight was reduced in rats fed low iron compared to the control rats fed adequate iron. An interaction between iron and fat was observed in heart weight. Low iron within all fat treatments resulted in larger heart weight compared to adequate and high iron treatments. Enlargement of the heart weight in rats fed low iron could be a factor that could complicate immunological results.

The various indices of iron status indicate that rats fed FeD had iron deficiency anemia. The rats fed FeH did not show hematological signs nor tissue mineral concentrations indicative of iron overload. Hemoglobin, Hct and iron utilization were reduced with SO (compared with BT) or with FeD diets. This is similar to another study indicating that enhancement of iron availability by a stearic acid source is most important when dietary iron is limiting (Johnson et al. 1992). Safflower oil contains a higher proportion of linoleic acid; whereas BT contains a higher proportion of stearic acid. Linoleic acid is known to enhance iron deficiency compared with stearic acid (Johnson et al. 1987, Amine and Hegsted 1975). The reduction of iron retention enhanced by safflower oil diets is thus confirmed by the present study. In contrast to the previous research, iron utilization was unaffected by an interaction between dietary fat and iron. The previous research (Droke and Lukaski, 1996; Johnson et al. 1987) used the AIN-76A diet whereas the present study used the AIN-93G diet formulation, which may be a factor in the differences between studies. Only one level of fat was

used in the present study (30% of total energy); use of a lower level may have allowed for a detection of an interactive effect on iron utilization.

High iron has been known to reduce both copper and zinc status because of known competitions during absorption and metabolism (Yu et al. 1994, Cousins 1985). Plasma and tissue zinc concentrations were not consistently affected by dietary fat or iron. Plasma, liver and kidney copper concentrations were decreased with the high iron diet. An iron-copper interaction has previously been observed in other studies. Increasing intakes of iron reduced status, absorption and biliary excretion of copper in rats (Yu et al. 1994). This may be reflective of the proposed mechanism indicating ceruloplasmin, which acts as a copper-dependent oxidase, works together with a putative basolateral iron transporter to control the exit of iron out of the enterocyte (Wood and Han 1998). However, in the plasma, not only were the copper concentrations suppressed in the high iron fed rats, but specifically in the high iron/safflower oil fed rats, suggesting that safflower oil may promote the reduction in copper status. Safflower oil, a polyunsaturated fatty acid may be altering cells membrane fluidity resulting in altered receptor function that could influence the generation of intracellular second messengers (Hagve 1988). Changes in copper metabolism could cause difficulties measuring immune responses. Copper deficiency is associated with impaired proliferation of T lymphocytes in response to mitogens (Prohaska and Failla 1993). Evidence suggests that copper may affect the T cell activation process by altering the transition of the target cell to the competent stage, which ultimately progresses to its proliferative stage (Failla 1998).

Total cholesterol, HDL-cholesterol, and non-HDL cholesterol were unaffected by dietary iron and/or fat. This is unexpected because our study varied the type of fat to include an n-9, n-6, n-3, and a saturated fat. Variations in the degree of saturation should result in varied effects on plasma lipids. No significance differences may resulted from high standard error bars associated with the lsmeans for each treatment group. On the other hand, triacylglycerides were significantly different by fat treatments. Flaxseed oil, an n-3 fatty acid had the lowest TG concentration. Corresponding to other polyunsaturated fatty acid versus saturated fatty acid studies, this is to be expected (An et al. 1997, Lu et al. 1997). Some experimental animal studies also suggest that n-3 fatty acids not only lower serum TG levels, but modify the membrane fatty acid composition, which implicates the reducing effect on the incidence of atherosclerosis and thrombogenesis (An et al. 1997, Lu et al. 1997).

Other studies indicate that iron deficiency suppressed lymphocyte proliferation (Keown et al. 1984, Helyar and Sherman). Corresponding to these studies, our data did indicate a lowering effect on proliferation from iron deficiency. In terms of our hypothesis, our observed data did indicate that iron deficiency will suppress immune function via suppression of lymphocyte proliferation. Effects of high iron on lymphocyte proliferation tend to be more controversial. One study has indicated iron overloading for four weeks on young rats can increase ConA-stimulated lymphocyte proliferation (Wu et al. 1990). Our data correspond to this study indicating a high iron diet increases proliferation responses to ConA.

Splenocyte mitogen-induced lymphocyte proliferation was also significantly affected by an interaction between dietary iron and fat. A FeH/SO reduced proliferation compared with a FeH/OO diet. This suggests that cell proliferation in rats fed FeH is enhanced with OO. Olive oil is a monounsaturated fatty acid whereas; safflower oil is a polyunsaturated fatty acid. One possible mechanism that dietary olive oil does not suppress lymphocyte proliferation when combined with high iron is that olive oil does not suppress the diacylglycerol or ceramide, which appear to promote T-lymphocyte proliferation (Jolly et al. 1997). The high iron may promote the synthesis of arachdonic acid from linoleic acid. Studies indicate safflower oil or fish oil may suppress the formation of prostaglandins formed from arachidonic acid. (Jolly et al. 1997) Others studies have shown that polyunsaturated fatty acids modify lymphocyte membrane fluidity resulting in altered receptor function that could influence the generation of intracellular second messengers (Hagve 1988). Perhaps safflower oil alters splenocyte membrane fluidity so ceramide or diacyglycerol receptors are formed when high iron is available.

Cytokines that make up the profiles for T-helper-1 and T-helper-2 lymphocytes can be used to measure immune responses. Those cytokines that represent T-helper-1 (Th1) lymphocytes or IL-2 and INF-γ. Interleukin-4 and IL-10 represent T-helper-2 (Th2) lymphocytes (Von Ruecker and Schmidt-Wolf 2000). Interleukin-2 function to stimulate cell proliferation. Interferon-γ stimulates production of Th1 lymphocytes and suppresses the production of cytokines originating from Th2 lymphocytes. Interleukin-4, similar to IL-2.

enhances the proliferation of T lymphocytes, but not to as great of an extent. Interleukin-10 suppresses the production of cytokines and functions of Th1 lymphocytes. Any alterations to one or more of the cytokines can modify whether a Th1 or Th2 response predominates. These responses from cytokine could be a factor in the lymphocyte proliferation results.

Concanavalin A and lipopolysaccharide stimulate not only splenocyte proliferation but also cytokine production. Dietary iron and fat gave varied results in response to mitogen stimulation. Iron deficiency did not appear to suppress cytokine production. A higher iron diet also did not suppress cytokine production. Flaxseed oil appeared to enhance production of IFN-γ, which would suggest a diminished response from Th2 lymphocytes. Interleukin-4 production appeared to be enhanced by OO. However, lymphocyte proliferation was similar between FO and OO. Varied production of cytokines may suggest that diet altered Th1/Th2 balance, which is influenced by cytokines in order to control immune response.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Weight gain, food intake and various indices of iron status were reduced by low iron and unaffected by fat and an interaction between dietary iron and fat. From this data, we can conclude that iron deficiency was established in the animals fed low iron. However, high iron fed rats did not have indicators of iron overload except for increased liver iron. Reduction of food intake, iron intake and growth in animals fed low iron could complicate immunological results.

Iron utilization was reduced with SO compared with BT. The enhancement of iron utilization by beef tallow has implications for human diets, because beef tallow is a source of stearic acid, which is the principal fatty acid in most meat fats (Johnson et al. 1992). Agreed, there are differences in iron utilization between rats and humans; rats absorb less heme iron compared with non-heme iron, which is contradictory to the relationship found in humans. Nevertheless, a significant correlation in non-heme iron using the meat factor has been found in both rats and humans. Unlike previous research, there were no observed differences in iron utilization for the interaction between dietary iron and fat.

Identical to other studies, plasma copper was affected by iron deficiency. However, it was also affected by the interaction between dietary iron and fat. Within SO treatments, high iron reduced copper compared with low and adequate iron. This interaction could influence the generation of intracellular secondary messengers, which also could complicate immunological results.

Liver Manganese concentrations were affected by dietary fat, thus warranting further investigation into the interaction between dietary fat and manganese.

Similar to other studies, iron deficiency did suppress lymphocyte proliferation. However, splenocyte mitogen-induced lymphocyte proliferation was also significantly affected by an interaction between dietary iron and fat. Within olive oil treatments, low iron reduced proliferation compared with high iron. This might suggest that olive oil synergistically with low iron further suppresses proliferation compared with high iron. Within high iron treatments, SO reduced proliferation compared with OO. This might suggest that OO enhances immune responses of high iron fed rats compared to SO; suppression of proliferation was not observed with high iron compared with adequate iron. Generally, FO, OO, and BT fed rats had greater enhancement of mitogen-induced lymphocyte proliferation than SO.

Although lymphocyte proliferation was affected by the interaction between dietary iron and fat, other immune measurements were not. There was a great deal of variation in the effects on mitogen-induced cytokine production by isolated splenocytes. Little is known about the interaction between dietary fat, iron and cytokines. However, the finding that cytokines are involved in cellular proliferation and our findings, suggest that interactions between dietary iron and fat could be involved in alteration of the cytokines produced by T-helper cells.

Our present findings indicate further research is needed in the area of nutrient-nutrient interactions and their effect on immune function. Research in

this area with an immunologically challenged animal would provide further knowledge on Th1/Th2 cellular activity. In addition, additional immune measurements should be taken throughout the study to eliminate any adaptation affect. Since iron overload was not established with the concentration of high iron used, then there is implication for a study to increase high iron treatments above 100 μg/g of iron in diets. Fat levels might also be adjusted to compare our findings for 15% fat by weight to the AIN93-G standard of 7% fat by weight. Identification of other immunological measurements such as interleukin receptors will also provide advancement in nutrient-nutrient interactions and their effects on immune function.

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Appendix

Oklahoma State University

Animal Care Approval Form

OKLAHOMA STATE UNIVERSITY

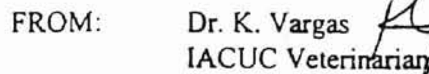
College of Veterinary Medicine
Laboratory Animal Resource's Unit
Stillwater, Oklahoma 74078-2002
405-744-7631

Memorandum

DATE: February 3, 1998

TO: Dr. Elizabeth Droke
Nutritional Sciences

FROM: Dr. K. Vargas
IACUC Veterinarian



SUBJECT: Protocol Approval

Your protocol, #710, entitled "Dietary Fat/Iron: Role in Immune Function", has been approved for 220 rats by the Institutional Animal Care and Use Committee. The protocol is approved through January 31, 2001.

A modification must be submitted to the committee for approval prior to any changes in the protocol.

Institutional Assurance number A3722-01

VITA

Andrea Dawn Shotton

Candidate for the Degree of

Master of Science

Thesis: DIETARY FAT AND IRON MODIFY CELL PROLIFERATION AND CYTOKINE PRODUCTION IN GROWING MALE RATS

Major Field: Nutritional Sciences

Biographical:

Personal Data: Born in Gainsville, Texas, On June 9, 1976, the daughter of Carl and Karen Barnes, wife of Vincent Shotton.

Education: Graduated from Broken Arrow Senior High School, Broken Arrow, Oklahoma in May 1994; received Bachelor of Science degree in Chemistry from The University of Tulsa, Tulsa, Oklahoma in May 1998. Completed the requirements for the Master of Science Degree with a major in Nutritional Sciences at Oklahoma State University in December, 2000.

Experience: Employed by DaPro Rubber Inc. and by The University of Tulsa as an undergraduate and as a chemistry stock room assistant, 1997-1998; employed by Northeastern Oklahoma College as a chemistry laboratory teacher, 1998; employed by Oklahoma State University, Department of Nutritional Sciences as a graduate research assistant, 1998 to present.

Professional Memberships: American Chemical Society, American Society of Nutritional Sciences.