THE INFLUENCE OF SUPPLEMENTAL VITAMIN E AND ZINC ON THE RESPONSE OF MOUSE MACROPHAGES TO LIPOPOLYSACCHARIDE

By

MARY RACHEL BELL

Bachelor of Science

Oklahoma State University

2001

Submitted to the Faculty of the Graduate
College of Oklahoma State University in
partial fulfillment of the requirements for the
Degree of
MASTER OF SCIENCE
July 2005

THE INFLUENCE OF SUPPLEMENTAL

VITAMIN E AND ZINC ON THE

RESPONSE OF MOUSE

MACROPHAGES TO

LIPOPOLYSACCHARIDE

Thesis Approved:

Elizabeth Droke
Thesis Advisor

Brenda Smith

Tay Kennedy

A. Gordon Emslie
Dean of the Graduate College

ACKNOWLEDGEMENTS

I would like to thank my graduate committee - Dr. Elizabeth Droke (Advisor), Dr. Tay Kennedy, and Dr. Brenda Smith – for their long lasting support in the completion of my research. I would also like to express gratitude to the staff of the Nutritional Sciences laboratory and to the faculty of the Nutritional Sciences department at Oklahoma State University for all their help in completing this research.

My deepest thanks goes to my wonderful husband and daughter- for their support and understanding while I completed this research, and to my family for always being there to support me.

TABLE OF CONTENTS

Chapter Page

I. INTRODUCTION 1

II. LITERATURE REVIEW 7

Inflammatory Diseases 7
Overview of the Immune System 8
Nutrition and Immunity 10
Zinc 12
Zinc Cellular Uptake 15
Zinc and Immunity 16
Vitamin E 19
Vitamin E Cellular Uptake 20
Vitamin E and Immunity 21
Zinc, Vitamin E and Immunity 24
Pharmacological vs. Physiological Vitamin E and Zinc Concentrations 26
Hypotheses and Expected Results 29

III. METHODOLOGY 36

Cell Culture 36
Media Analysis 37
Determination of the Optimal Cell Density 37
Determination of the Optimal Zinc Supplementation 38
Determination of the Optimal Vitamin E Supplementation 39
Determination of the Optimal Lipopolysaccharide Challenge 41
Determination of the Optimal Combination of Vitamin E and Zinc 42
Determination of the Influence of Vitamin E and Zinc on Mouse Macrophage Response to a Lipopolysaccharide Challenge 43
Statistical Analyses 45

IV. RESULTS 47

Determination of the Optimal Cell Density 47
Determination of the Optimal Zinc Supplementation 48
Determination of the Optimal Vitamin E Supplementation 48
Determination of the Optimal Lipopolysaccharide Challenge 49
Determination of the Optimal Combination of Vitamin E and Zinc 52

Determination of the Influence of Vitamin E and Zinc on Mouse Macrophage Response to a Lipopolysaccharide Challenge....54

V. DISCUSSION....73

Determination of the Optimal Cell Density....73
Determination of the Optimal Zinc Supplementation....73
Determination of the Optimal Vitamin E Supplementation....74
Determination of the Optimal Lipopolysaccharide Challenge....75
Determination of the Optimal Combination of Vitamin E and Zinc....77
Determination of the Influence of Vitamin E and Zinc on Mouse Macrophage Response to a Lipopolysaccharide Challenge....78

VI. SUMMARY, CONCLUSIONS, AND LIMITATIONS....84

VII. BIBLIOGRAPHY....88

LIST OF TABLES

Table Page

1. P-values for the Growth of Macrophages in Vitamin E and Zinc Supplemented Media after 24 and 48 Hours 62

2. Macrophage growth in Vitamin E and Zinc Supplemented medium at 24 Hours..63

3. Macrophage growth in Vitamin E and Zinc Supplemented medium at 48 Hours..65

LIST OF FIGURES

Figure Page

1. Growth of macrophages in zinc supplemented medium.....58

2. Growth of macrophages in vitamin E supplemented medium.....59

3. Growth of macrophages in medium after challenge with Lipopolysaccharide (LPS) from *E. Coli* 055:B5 (A) or *E. Coli* 0111:B4 (B) 60

4. Nitric oxide production by macrophages in response to challenge with Lipopolysaccharide (LPS) from *E. Coli* 055:B5 (A) or *E. Coli* 0111:B4 (B) 61

5. Growth of macrophages in vitamin E supplemented medium irrespective of zinc supplementation 64

6. Growth of macrophages in vitamin E and zinc supplemented medium after a 24 (A) or 48 (B) hour exposure to LPS from *E. Coli* 055:B5.....66

7. Nitric oxide produced by macrophages grown in zinc and vitamin E supplemented medium after a 24 (A) or 48 (B) hour exposure to LPS from *E. Coli* 055:B5 67

8. Nitric oxide produced by macrophages in response to a lipopolysaccharide challenge of E. Coli 055:B5 in supplemented mediums 68

9. Nitric oxide produced by macrophages in zinc and vitamin E supplemented mediums for each lipopolysaccharide concentration.....69

9. Tumour necrosis factor alpha (TNF-α) production by macrophages grown in vitamin E and zinc supplemented medium and challenged with LPS from *E. Coli* 055:B5.....70

10. Interleukin 10 (IL-10) production by macrophages grown in zinc and vitamin E supplemented medium and challenged with LPS from *E. Coli* 055:B5 71

11. Prostaglandin E_2 (PGE_2) production by macrophages grown in zinc and vitamin E supplemented medium and challenged with LPS from *E. Coli* 055:B5 72

CHAPTER I

INTRODUCTION

Inflammatory diseases, such as rheumatoid arthritis, are a health problem in the United States. It is estimated that 0.9 to 1.1% of the North American population suffers from rheumatoid arthritis alone (Alamanos & Drosos, 2005). Sufferers of rheumatoid arthritis not only deal with pain, but also decreased daily functions (Wolfe et al., 1991) and depression (Nagyova et al., 2005). Other inflammatory diseases include atherosclerosis and septic shock. Septic shock is not only associated with death of the patient, but also with increased hospital costs and hospitalization time (Adrie et al., 2005). Atherosclerosis, which is a leading cause of strokes and myocardial ischemia (Sherwood, 1997), can also lead to increased medical costs and death. Due to advances in medicine, mortality rates have declined, however, this means more people are living with this disease for longer periods of time (Reitsma et al., 1999). These inflammatory diseases are associated with increased concentrations of inflammatory mediators in the body (Simkin, 1976; Glauser et al., 1991; Wu et al., 2001).

Inflammatory mediators are released during an immune response. The immune response is triggered when a microbe penetrates the body's first line of defense. The body's first line of defense includes the skin, mucus, bactericidal fluids, and gastric acid. When they penetrate the first line of defense, they are met by phagocytic cells, such as the macrophage (Roitt, 1991) through the innate immune response. The macrophage

destroys the invader through phagocytosis, during which, the macrophage releases several acute inflammatory response mediators, such as prostaglandin-E_2 (PGE_2), nitric oxide, tumor necrosis factor-α (TNF- α), interleukin-1 (IL-1), and IL-6. These mediators are responsible for the redness and swelling associated with an inflammatory response. Increases in these mediators are also associated with inflammatory diseases, such as rheumatoid arthritis (Simkin, 1976), septic shock (Glauser et al., 1991), and atherosclerosis (Wu et al., 2001).

It is well known that vitamin and mineral deficiencies can suppress immune responses, including innate immunity. For example, zinc deficiency affects the immune system; Chandra et al (1984) observed zinc deficiency was linked to decreased macrophage function, while others observed zinc deficiency caused alterations in T-cell function and maturation (Rink & Gabriel, 2000; Prasad, 1998). Supplementation of zinc has been shown to decrease the amount of nitric oxide produced in response to a lipopolysaccharide (LPS) challenge (Abou-Mohamed, et al., 1998). Zinc supplementation has also been shown to decrease the amount of TNF-α and IL-1β produced (Driessen et al., 1995; Prasad, 2004).

Another nutrient studied with the immune system is vitamin E. Meydani and Han (2002) discussed how vitamin E acts as an antioxidant to increase lymphocyte proliferation, increase antibody response, and increase the number of helper T cells. Vitamin E supplementation has also been shown to decrease the amount of inflammatory response mediators released. Supplementation with vitamin E has been shown to decrease the amount of PGE_2, nitric oxide (Wu et al., 1998), IL-6 (Beharka et al., 2000), TNF-α, and IL-1β produced (Han et al., 2000; Devaraj & Jialal, 1998).

Studies have shown over-consumption of a nutrient may have a negative response similar to that of under-consumption; for example, an excess of zinc blocks T-cell function (Rink & Gabriel, 2000). Therefore, research must determine what the optimal amount is to consume. In addition to determining this optimal amount, researchers have to take into account interactions with other nutrients.

Although micronutrients may be able to affect the immune response, the effects of interactions between various vitamins and minerals on the inflammatory immune response have not been thoroughly studied. If two nutrients are known to have similar functions in the body (e.g. zinc and vitamin E both act to decrease nitric oxide produced (Abou-Mohamed, et al., 1998; Meydani et al., 2002)), then it must be determined what effect supplementation or depletion of both nutrients would have on that particular function. To date, research has shown zinc is involved with the absorption of vitamin E (Hatfield et al., 2002; Noh & Koo, 2001; Kim et al., 1998), but has only begun to show how vitamin E and zinc work together in the immune system.

Chandra et al. (1992) determined that supplementation with vitamin A, beta carotene, thiamin, riboflavin, niacin, zinc, copper, selenium, iodine, calcium magnesium and vitamins B_6, B_{12}, C, D, E could increase the antibody response to the influenza vaccine in independently-living elderly as compared to non-supplemented controls, as well as help protect against infection-related illnesses. Girodon et al. (1999) showed in the institutionalized elderly that supplementation with both trace minerals (including zinc) and vitamins (including α-tocopherol) improved the humoral response after influenza vaccination to a greater extent when compared with vitamins alone or the control group. However, it has not been shown how supplementation with both vitamin

E and zinc affect the release of inflammatory mediators due to the innate immune response. The present study examined this relation by studying the influence vitamin E and zinc had on the response of the mouse macrophage to LPS.

The overall goal of the present study was to determine the synergistic effect of vitamin E and zinc supplementation on the response of mouse macrophages (RAW 264.7 cell line) to a LPS challenge. Since LPS is found in the cell walls of bacteria, LPS challenges are used in cellular studies to simulate an infection with a gram-negative bacteria and the subsequent development of inflammation. Therefore, the use of LPS will help to determine vitamin E and zinc supplementation's synergistic effect on immune cells during inflammation. This project will help determine if the combined supplementation of vitamin E and zinc would have a more beneficial effect on the release of pro-inflammatory response mediators (PGE_2, nitric oxide, and TNF-α) and anti-inflammatory response mediators (IL-10) than zinc or vitamin E supplementation alone.

The overarching hypothesis was that the supplementation with both vitamin E and zinc would have a positive synergistic effect on the production of these mediators, and would decrease the release of the pro-inflammatory response mediators more than vitamin E or zinc supplementation alone. It was expected that not only would the PGE_2, nitric oxide, and TNF-α production be decreased by zinc supplementation and vitamin E supplementation alone, but would be decreased to a greater extent by supplementation with both the vitamin E and zinc. It was also expected that IL-10 production would be increased to a greater extent with both zinc and vitamin E, rather than with either alone.

Definitions

Adequacy- refers to an animal, human or cell that has consumed or been provided with an amount of a nutrient to prevent the development of a deficiency.

Deficiency- refers to a human or animal exhibiting overt clinical deficiency symptoms, as well as a measurable deficiency as determined by biochemical measures.

Lipopolysaccharide- refers to a molecule found on the outer cell wall of Gram-negative bacteria (*E. Coli* for example).

Marginal deficiency- refers to a state of deficiency that is only measured by biochemical analyses, and does not produce overt clinical symptoms.

Pathogen- refers to an agent, such as a virus or bacteria, which can cause disease (Sompayrac, 1999).

Pharmacological concentrations- refers to concentrations of nutrients in an organism that are brought about by supplementation of those nutrients that are higher than the normal physiological concentration.

Physiological concentrations- refers to normal concentrations of a nutrient found in an organism that are maintained by adequate nutrient consumption.

Repletion- refers to providing an individual (animal, human or cell) that is deficient in a specific nutrient with that nutrient in order to restore the concentration of that nutrient in the organism or cell.

Supplementation- refers to the provision of an additional quantity of a nutrient to an animal, human or cell whose status for that nutrient is considered to be adequate.

CHAPTER II

LITERATURE REVIEW

Inflammatory Diseases

Inflammatory diseases, such as rheumatoid arthritis, septic shock and atherosclerosis, are major health problems in the United States. The number of people suffering from these diseases is expected to increase over time due to an aging population and advances in health care that allow the person to live longer with the disease (Reitsma et al., 1999).

It is estimated that 0.9 to 1.1% of the general population of the United States suffers from rheumatoid arthritis, but there is a greater prevalence among Native Americans (5.3 to 6.0 %) (Alamanos & Drosos, 2005). Rheumatoid arthritis is caused by an imbalance of cytokines leading to chronic inflammation, which results in cellular damage (Choy & Panayi, 2001). This cellular damage leads to destruction of cartilage and bone, thus leading to the development of rheumatoid arthritis (Choy & Panayi, 2001). Sufferers of rheumatoid arthritis not only suffer pain and functional disability (Wolfe et al., 1991), but suffer from depression and anxiety as well (Nagyova et al., 2005). Rheumatoid arthritis also leads to a decreased life expectancy of up to three to ten years (Alamanos & Drosos, 2005). Treatment of the disease on average costs the patients approximately $5,919 each year, but the more severe the disease, the higher the costs (Yelin & Wanke, 1999).

In addition to rheumatoid arthritis, septic shock is an inflammatory disease that is a major health problem in the United States. Septic shock occurs in 750,000 persons in the United States each year, of which approximately 28.6% of these cases ends in death of the patient (Angus et al., 2001), which makes it one of the major causes of death in critically ill patients (Adrie et al., 2005). Angus et al. (2001) also estimated that the treatment costs per person who suffered septic shock were approximately $22,100 each year.

Septic shock, as well as atherosclerosis and other inflammatory diseases, are associated with increased levels of nitric oxide, and many pro-inflammatory cytokines. In western countries, atherosclerosis is becoming more prevalent due to the higher rate of survival after an acute event (Reitsma et al., 1999). Atherosclerosis is caused by the oxidation of LDL, which when taken up by macrophages causes lipid accumulation and formation of foam cells, and causes inflammatory mediators to be released (Witztum and Steinberg, 1991). This accumulation leads to plaque formation, which can lead to blockage of blood flow in the heart or the brain (Sherwood, 1997), and lead to heart attack or stroke. All of these diseases have been shown to be associated with increases in the pro-inflammatory mediators released by the macrophages (Simkin, 1976; Glauser et al., 1991; Wu et al., 2001); therefore, the present study was designed to determine if supplementation with vitamin E or zinc would decrease the production of pro-inflammatory mediators.

Overview of the Immune System

Innate immunity refers to the bodies' first line of defense, which includes physical barriers, such as skin and mucous membranes. These physical barriers help to prevent

pathogens, such as bacteria, from entering the body. The skin is impermeable to bacteria unless injured, and produces sweat that contains fatty acids, which have a low pH that is unfavorable to bacteria. The mucous membranes secrete mucus, which traps the bacteria, then is removed through coughing. Other lines of defense include tears and saliva, which contain enzymes that damage the pathogens. If a bacteria or other infectious agent penetrates these defenses, they are met by the phagocytes (Janeway et al., 2001).

The phagocytes include macrophages, monocytes, neutrophils, and a variety of other cells. Monocytes are found in the blood, as are neutrophils, and both can move into the tissues. Macrophages are formed when bone marrow promonocytes turn into blood monocytes, then move into the tissues to become macrophages (Janeway et al., 2001).

Macrophages are the first host cells to respond to a pathogen in the body and engulf the pathogen through phagocytosis. During phagocytosis, the pathogen adheres to the macrophage, and the macrophage wraps pseudopodia around the pathogen and forms a phagosome (Parkin & Cohen, 2001). After the macrophage has engulfed the pathogen, the pathogen is joined with granules containing pre-formed mediators (e.g. IL-6 and TNFα) to become a phagolysosome and is then digested (Parkin & Cohen, 2001). After digestion, the degradation products are released back into the body (Janeway et al., 2001). These degradation products signal to other immune cells that there is an invasion in the body, and helps to attract them to the site of the invasion (Sompayrac, 1999). They also function to induce the proliferation of macrophages and natural killer cells (Sompayrac, 2001).

The macrophage also secretes mediators of the acute inflammatory response such as IL-1, TNF, PGE_2, and neutrophil chemotactic factor. Several of these cytokines that

are produced by the macrophage have been shown to produce reactive oxygen species (ROS) (Ozaki et al., 1987), which causes oxidative stress in the body. Prostaglandin-E_2 also decreases T-cell proliferation as well as IL-2 production (Goodwin & Ceuppens, 1982).

The type of immunity previously discussed was innate immunity; the other type of immunity in the human body is specific acquired immunity or adaptive immunity. Innate immunity is quick to destroy invaders but is non-specific and can lead to tissue damage, where acquired immunity is specific but does require a long time to destroy invaders (Parkin & Cohen, 2001). Mediators of innate immunity signal and activate the adaptive response and aid in determining the type of adaptive response that should occur.

Thus, innate and adaptive immunity work together to protect the body from infection. The present study focused on the macrophage (innate immunity), and its secretion of pro-inflammatory mediators in response to LPS. These pro-inflammatory mediators have been linked to various inflammatory diseases, such as septic shock (Glauser et al., 1991) and rheumatoid arthritis (Simkin, 1976); therefore, a decrease in these pro-inflammatory mediators is desired. The present study was designed to determine if zinc and vitamin E supplementation had a synergistic affect on the amount of these pro-inflammatory agents produced.

Nutrition and Immunity

Nutrition in immunology has been studied extensively, but mostly in aspects of how a single nutrient affects the immune system (Kubena & McMurray, 1996). For example, vitamin A supplementation of rats has been shown to increase the phagocytic activity in Kupffer's cells (Olson, 1986). Vitamin A has also been shown to control the

proliferation and differentiation on various other cells in the immune system (Olson, 1986). Supplementation of children with a single large amount (100,000 to 200,000 IU) of vitamin A was found to increase IL-1 production and cytotoxic functions of macrophages (Bhaskaram et al., 1989). Another vitamin that has been studied for its affects on the immune system is vitamin C. Vitamin C supplementation has been associated with enhanced human natural killer cell activity in patients that were exposed to toxic chemicals (Heuser & Vojdani, 1997), as well as enhanced phagocytosis by neutrophils in elderly women (Herbaczynska-Cedro et al., 1994). Vitamin E has also been studied in regards to the immune system, and these results will be discussed in the subsequent sections.

Trace elements have an affect on the immune system as well. For example, iron deficiency has been shown to affect cell-mediated immune function among pregnant women (Prema et al., 1982). Magnesium also affects cell-mediated immune function, as well as humoral immunity (Kubena, 1993). Other trace minerals often studied in regards to the immune system include selenium, zinc and copper. Selenium deficiency has been associated with increased rat macrophage production of PGE_2, as has vitamin E deficiency (Eskew et al., 1989). Copper deficiency, which can be induced by over supplementation with zinc, has been shown to decrease the function of neutrophils in humans (Percival, 1995). Zinc has also been extensively studied, and will be discussed in the subsequent sections.

In addition to vitamin and mineral effects on the immune system, fatty acids have been shown to have effects on the immune system (Kubena & McMurray, 1996), by

changing the fatty acid composition of immune cell membranes, lymphocyte proliferation as well as function can be affected (Calder & Newsholme, 1993).

Some interactions between two nutrients have been studied in regards to immune functions. For example, supplementation with both Vitamin A and vitamin D has been associated with increased phagocytic activity of U 937 human macrophages, as well as enhanced oxidative burst (Tiami et al., 1991). The interactions zinc and vitamin E have with other nutrients will be discussed in subsequent sections.

Zinc

Zinc has several functions in the body, from taste acuity, to reproduction, to immunity. Zinc can be found in body fluids, tissues and organs, including the bone, muscle and skin. Zinc is believed to be involved with over 200 enzymes in the human body, mostly as a part of metalloenzymes (Groff & Gropper, 1999).

These enzymes are involved in tissue and cell growth, vision, digestion, membrane stabilization, metabolism, bone formation and skin integrity. Zinc is also necessary to bind certain proteins to deoxyribonucleic acids (DNA) - zinc provides structure to the "zinc fingers" proteins, thus allowing them to be able to bind with DNA (Groff & Gropper, 1999). Zinc is also needed for structure of cells since it is a component of the microtubules that provide the structure for the cells. (Groff & Gropper, 1999). Zinc is also important in bone metabolism - it has been shown that zinc aids 1,25-dihydroxy vitamin D_3 in bone metabolism, and stimulates protein synthesis (Yamaguchi & Inamoto, 1986). 1,25-dihydroxy vitamin D_3 functions with parathyroid hormone to regulate bone remodeling in the body (Li & Farach-Carson, 2001).

Zinc is also found in superoxide dismutase (SOD), which acts to remove superoxide radicals (such as O_2-) from the body (Groff & Gropper, 1999); therefore, reducing oxidative damage to the body. These superoxide radicals (O_2-), along with H_2O_2 and OH are referred to as reactive oxygen species (ROS), which can lead to oxidative stress in the body. Oxidative stress has been shown to be involved with atherosclerosis, cancer, and immunologic disorders (Prasad, 2004). Zinc reduces the ROS during several steps of their formation. For example, zinc hinders NADPH, which catalyzes the production of O_2- from oxygen (Prasad, 2004). Another way zinc reduces the ROS is through metallothionein, which reduces the amount of OH. Because zinc competes with iron and copper (which catalyze the production of OH), zinc reduces the amount of OH formed through that route also (Prasad, 1998).

Zinc is involved with human digestion through the enzymes carboxypeptidase A and aminopeptidase, which are both involved in protein digestion through the digestion of peptide bonds (Groff & Gropper, 1999). Zinc is also involved with metabolism, and is a part of insulin, which is critical in regulating glucose metabolism (Groff & Gropper, 1999).

Zinc deficiency is a growing world problem due in part to poor zinc intake, and high intake of phytates and phosphates which hinder zinc absorption (Prasad, 1961). Over two billion people may currently be suffering from zinc deficiency worldwide (Prasad, 1998). Because zinc is involved with so many different aspects of the human body, the deficiency of it has been linked to a wide variety of abnormalities in the body. For example, zinc deficiency has been linked to growth retardation, embryonic abnormalities (Hurley & Swenerton, 1966), testicular atrophy (Prasad, 1961), epidermal

hyperkeratinization, chronic diarrhea, delayed wound healing, and loss of both hair and taste (Prasad, 1998).

Zinc deficiency in pregnant women is of major concern due to the growing fetus's demand for zinc and the mother's need for it as well. It has been linked to prolonged gestation periods, poor labor, and increased maternal morbidity, along with the risks to the fetus; however, zinc supplementation was able to decrease all of these risks (Jameson, 1982). In studies performed using rats as the model, it was found that there is an increase in central nervous system malformations among those who are zinc deficient (Hurley & Swenerton, 1966). Women, who have acrodermatitis enteropathica, have a higher incidence of fetuses with congenital malformations as well (Prasad, 1998). Goldenberg et al. (1995) showed that women of lower body mass index (less than 26 kg/m^2), who also had low plasma zinc concentrations, had babies with a greater birth weight and head circumference when supplemented with zinc.

Zinc is very important in human reproduction not only for women, but for men also. Zinc deficient males are subject to decreased serum testosterone levels (Prasad, 1996), as well as hypogonadism (Prasad, 1961).

Ninh et al (1995) reported a decrease in weight gain, insulin-like growth factor-1 (IGF-1), hepatic growth hormone receptors, and serum growth hormone-binding protein (GHBP) in rats that were zinc deficient. Either the decrease in GHBP or the decrease in hepatic growth hormone receptors is believed to have caused the decrease in IGF-1. In a separate study, Ninh et. al. (1996) showed zinc repletion led to an increase in IGF-1, weight and height among zinc deficient Vietnamese children. A zinc deficient diet has

also been shown to lead to a decrease in the basal metabolic rate and in thyroid hormones (Wada & King, 1986).

Zinc supplementation and repletion have been studied in connection with the common cold, respiratory viruses, pneumonia (Bhutta et al., 1999), diarrhea (Hambidge, 2000; Bhutta et al., 2000), aortic aterogenesis (Alissa et al., 2004) and Wilson's disease (Yuzbasiyan-Gurkan et al., 1992). Repletion of body zinc concentrations has also been shown to be both effective in decreasing the duration and severity of both acute and persistent diarrhea, as well as the death rate associated with diarrhea in children in developing countries (Bhutta et al., 2000). The prevalence of pneumonia was also shown by Bhutta et al. (1999) to be decreased in children in developing countries, who had low zinc concentrations, after receiving zinc supplementation. Alissa et al. (2004) showed that zinc or copper supplementation decreased aortic atherogenesis in animals fed a cholesterol diet through a reduction in the aortic lesion area.

Over supplementing with zinc has been linked to copper deficiency in some individuals (Prasad et al., 1978). Since this discovery, there have been studies conducted to see if zinc supplementation could be used to treat Wilson's disease (Brewer et al., 1983; Brewer et al., 1987; Brewer et al., 1990; Hill, et al., 1987), which is an inherited disorder resulting in copper excess (Prasad, 1998). Zinc is able to prevent the copper from being absorbed by activating an intestinal metallothionein, which binds the copper and helps to excrete it from the body (Yuzbasiyan-Gurkan et al., 1992).

Zinc Cellular Uptake

Zinc uptake by cells occurs in two phases, which are rapid and slow uptake (Stacey & Klaasen, 1981). The rapid uptake of zinc by fetal type II pneumocytes occurs

within thirty seconds of adding the zinc treated media to the cells, while the slow uptake still doesn't reach equilibrium within sixty minutes (Ong et at., 1995). Chvapil et al. (1977) demonstrated that rat peritoneal macrophages incubated with 50 μM zinc had zinc concentrations that increased continuously during the entire incubation time of 30 minutes. These macrophages had a zinc content of 2.3 $\mu g/10^8$ cells at zero minutes, and increased to a zinc content of 8.0 $\mu g/10^8$ cells at thirty minutes (Chvapil et al., 1977). This zinc uptake is not only affected by time, but also by zinc concentration (Chvapil et al., 1977) and the zinc status of the cell (McClung and Bobilya, 1999).

The affects of different zinc concentrations on the uptake of zinc to cells have also been studied. When the rat peritoneal macrophages were incubated with increasing amounts of zinc (0 to 800 μM) with and without serum, the zinc uptake of the cells within twenty minutes increased as the zinc concentration increased; however, the presence of serum decreased the zinc content found in the cells (Chvapil et al., 1977).

Zinc status of the cells also affects the uptake of zinc. McClung and Bobilya (1999) demonstrated that zinc status of the porcine endothelial cell also affects zinc uptake; the zinc-deficient porcine endothelial cells uptake zinc faster than the zinc adequate and zinc supplemented porcine endothelial cells. These researchers also showed that zinc supplementation of 5, 25, 50 and 75 μmol/L showed similar zinc uptake rates in cells supplemented with 5, 25, and 50 μmol/L, but a decreased uptake rate in cells supplemented with 75 μmol/L (McClung and Bobilya, 1999).

Zinc and Immunity

Zinc deficiency in known to affect the immune system. Zinc deficiency can be linked to an increase in cases of diarrhea (Bhutta et al., 2000), as well as delayed wound

healing (Lim et al., 2004). Zinc deficiency has also been linked to thymic atrophy, lymphopenia, and anergy (Beck et al., 1997; Prasad, 1998; Rink & Gabriel, 2000).

Zinc is important in innate immunity through a variety of functions. Zinc deficiency has also been linked to decreased macrophage function (Chandra, 1984), as well as the decreased activity of natural killer cells (Rink & Gabriel, 2000; Beck et al., 1997). Although research in zinc deficiency and immune function has been studied for several years, only recently has zinc supplementation become a key focus.

Prasad et al. (2004) demonstrated that in human mononuclear cells, zinc supplementation helped protect the cells against oxidative stress by decreasing the TNF-α induced binding of nuclear factor κB, as well as decreasing the gene expression of TNF-α and IL-1β both of which are associated with oxidative stress. Similar to Prasad et al. (2004), Driessen et al. (1995) found zinc supplementation decreased the amount of TNF-α produced by monocytes and IL-1β after stimulation by staphylococcal enterotoxins A and E in polymorphonuclear cells and whole blood cells of humans. These decreases in pro-inflammatory mediators demonstrate that zinc has anti-inflammatory properties.

Zinc supplementation of 100 and 150 μM has also been shown to decrease the nitric oxide, another pro-inflammatory mediator, produced by isolated rat aortic smooth muscle cells after the rats had been challenged with LPS from *E. Coli* 0127:B8 at 5-mg/kg body weight (Abou-Mohamed, et al., 1998). Because increases in nitric oxide are associated with inflammatory diseases, this decrease in nitric oxide production due to zinc supplementation also demonstrates zinc's anti-inflammatory properties (Abou-Mohamed, et al., 1998).

Research has found zinc supplementation leads to an increase in the amount of IL-1β and TNF-α when polymorphonuclear cells from healthy human donors are stimulated with LPS from *E. Coli* 0111:B4, but not when they were stimulated with phytohaemaglutinin (PHA) (Driessen et al., 1995). This increase in monokine secretion leads to T-cell activation (Driessen et al., 1995). The T-cells then seek out body cells that contain microbes and helps to destroy the cell that contains the microbe, or in the case of macrophages, reactivates the cell to start killing the microbes again (Roitt, 1991).

While zinc supplementation leads to the increase of these monokines, Lastra et al. (2001) has shown that zinc supplementation enhances the macrophage's phagocytic index at six weeks of supplementation. It decreases this index at nine weeks of zinc supplementation in mice, showing that the longer time leads to saturation of the zinc binding sites on the macrophage. This over-saturation may impair the immune system (Lastra et al., 2001). The phagocytic index is an average of the amount of bacteria ingested by each phagosome (http://www.infoplease.com); therefore, the greater the phagocytic index, the greater the amount of microbes destroyed by each phagosome.

Zinc also has been shown to have an effect on LPS, which is found in the walls of gram-negative bacteria, due to a direct interaction with the LPS. Zinc decreases the fluidity of LPS's hydrocarbon chains, which when decreased causes the LPS to induce the formation of less monokines (Wellinghausen et al., 1996).

Zinc also affects cell-mediated immunity. Zinc-deficient humans are known to have a decreased production of IL-2 and tumor necrosis factor-α (TNF-α) (Prasad, 1988); however, the production of IL-4, IL-6, and IL-10 are unchanged by zinc deficiency suggesting that zinc may have more of an affect on T helper cell response-

type 1 (TH1) cells (Beck et al., 1997), which through IL-2 induces T cell proliferation (Parkin & Cohen, 2001).

Zinc is also needed as a part of a cofactor for thymulin, which helps aid T lymphocyte maturation (Shankar & Prasad, 1998), and a deficiency of zinc decreases thymulin activity (Prasad et al., 1988). Zinc deficiency has been suggested to cause a decrease in both T and B lymphocytes by both reducing their ability to function properly and by increasing cell death (apoptosis) (Berger, 2002). In animal studies using mice, Fraker et al. (1978) was able to demonstrate that the T- cell helper function that was impaired due to zinc deficiency, could be restored with zinc supplementation, as could thymus size within two weeks of starting supplementation. King & Fraker (2002) indicated zinc deficiency disrupted erythropoiesis and lymphopoiesis, but not myelopoiesis in mice, which might indicate that the body protects innate immunity more than adaptive immunity in response to a zinc deficiency.

Excessive zinc supplementation, however, has been shown to have detrimental effects on the immune system as well. Lim et al. (2004) found zinc dietary supplementation of 1000 μg/g diet to decrease TNF-α mRNA levels, and to delay wound healing in mice, although zinc supplementation of 500 μg/g diet had beneficial effects. Chandra (1984) found that excessive intakes of zinc in healthy men impaired chemotaxis and phagocytic activity of neutrophils against bacteria.

Vitamin E

Vitamin E is a fat soluble vitamin that acts as an antioxidant in the body. The main function of vitamin E is to maintain membrane integrity of the cells, which it does by preventing oxidation of the unsaturated fats that are found in the phospholipids in the

cell membrane. The cells in the lungs and brain, and erythrocytes have membranes that are high in unsaturated fatty acids, making them more likely to be oxidized (Groff & Gropper, 1999). Vitamin E is a free radical scavenger, which helps protect the integrity of the cell.

As an antioxidant, vitamin E has been thought to aid in the treatment of cataracts since it is believed that cataracts are caused from oxidative damage to the proteins in the eye (Groff & Gropper, 1999). Also, it is believed that vitamin E supplementation can help in the treatment of diabetes mellitus by improving the glucose transport function (Whiteshell et al., 1989).

Vitamin E is also believed to aid in decreasing the risk of atherosclerosis. Gale et al. (2001) showed a diet high in antioxidants, including vitamin C, vitamin E and β-carotene, was associated with a decreased development and growth of atherosclerotic lesions in men. Diets higher in vitamin E have also been associated with higher cognitive function in the elderly (Ortega et al., 2002).

It was found in previous studies that vitamin E deficiency causes red blood cell hemolysis, and causes an increase in natural killer cell activity (Klurfeld, 1993), but this could be decreased by supplementation with vitamin E (Adachi et al., 1997).

A vitamin E deficiency rarely occurs in humans, except for premature infants, but can occur in people with malabsorption diseases and people with abetalipoproteinemia (a genetic disease in which the person lacks apolipoprotein B) (Groff & Gropper, 1999).

Vitamin E Cellular Uptake

Vitamin E uptake by cells varies among types of vitamin E. Gao et al. (2002) showed that in the RAW 264.7 macrophage cell line γ-tocopherol uptake was six times

greater than that of α-tocopherol. Saito et al. (2004) also showed that in Jurkat E6-1 cells (human T-leukemia cells) α-tocotrienol uptake was 70 times higher than that of α-tocopherol; however, the uptake of α-tocotrienol leveled out after only six hours, while the α-tocopherol uptake still had not leveled out after 72 hours. McCormick and Parker (2004) however showed no difference in uptake of α-tocopherol as compared to δ-tocopherol within cell types; however, they showed that after six hours of incubation with these tocopherols, human hepatocytes had a uptake that was 20% of that of the RAW 264.7 mouse macrophages.

Gao et al. (2002) showed that α-tocopherol uptake by the cells increased 0.15 nmoles/well after six hours of incubation with 5.6 μM α-tocopherol; however, Saito et al. (2004) showed uptake levels of α-tocopherol had still not leveled out by 72 hours, so this was a relatively short incubation time to measure.

Vitamin E and Immunity

Vitamin E supplementation has been hypothesized to help in many diseases, such as atherosclerosis, which is influenced by pro-inflammatory mediators, such as PGE_2 and thromboxane B_2 (Wu et al., 2001). Supplementation with vitamin E has been shown to decrease many of these pro-inflammatory mediators (Wu et al., 2001), thus possibly decreasing atherosclerosis development. As stated earlier, other inflammatory diseases are related to increases in a variety of inflammatory mediators.

Some examples of these inflammatory mediators are nitric oxide and PGE_2. Vitamin E supplementation decreased the amount of nitric oxide and PGE_2 produced in mice when stimulated with LPS (Beharka et al., 2000). Another researcher was also able to show this decrease in nitric oxide in a rat in vivo and in vitro study (Khanduja et al.,

2004). It was determined that by decreasing nitric oxide production, vitamin E supplementation decreased LPS-stimulated PGE_2 production through the inhibition of COX activity (Wu D et al, 1998); thus the decrease of nitric oxide production was what lead to the decrease in PGE_2 production. Since, PGE_2, and nitric oxide are known to be involved in the inflammation process by decreasing production of these inflammatory mediators, the risk for inflammatory diseases decreases. Jensen et al. (1988) observed that vitamin E deficiency caused an increase in PGE_2 production, which would be an undesirable outcome.

Another inflammatory mediator is IL-6. Beharka et al. (2000) showed vitamin E supplementation decreased the amount of IL-6 in un-stimulated mouse macrophages, which would be a another desirable decrease.

Other inflammatory mediators shown to be decreased by vitamin E supplementation include IL-1β and TNF-α (Han et al., 2000). These were shown to be decreased in mice fed vitamin E supplemented diets as compared to those fed non-supplemented diets in response to a Con-A challenge (Han et al., 2000). Devaraj and Jialal (1998) were able to determine that the decrease in IL-1β release is due to the inhibition of the 5-lipoxygenase pathway. Increases in IL-1β have been found in the coronary arteries of people with ischemic heart disease (Galea et al., 1996); therefore, a decrease in its levels is desired.

In addition to acting in innate immunity to decrease the amount of pro-inflammatory mediators produced, vitamin E acts in both cell-mediated and humoral immunity. In cell mediated immunity, deficiency of vitamin E has been shown to decrease lymphocyte proliferation after stimulation with concanavalin A (ConA) and

phytohemagglutinin (PHA) (Eskew et al., 1986). This decrease may be due to decreased IL-2 production. IL-2 is needed to induce T cell proliferation; therefore, decreased concentrations of IL-2 would lead to decreased concentrations of T-cells made in response to an antibody. Harris et al. (1980) found this to be true in rats fed a vitamin E deficient diet; the rats produced lower amounts of IL-2 in response to PHA and ConA, and lower levels of levels of lymphocytes. A positive effect is seen with vitamin E supplementation, Sakai & Moriguchi, (1997) showed rats supplemented with vitamin E were able to increase their IL-2 production and lymphocyte proliferation in response to PHA and ConA. Han et al. (2000) also showed an increase in IL-2 production in response to LPS in older mice supplemented with vitamin E.

In cell-mediated immunity, Moriguchi (1998) showed that vitamin E supplementation increased T-cell differentiation in the thymus, thereby, enhancing thymic lymphocyte proliferation. Since supplementation of vitamin E lead to increased T-cell differentiation, Moriguchi (1998) hypothesized that supplementation with vitamin E could result in faster recoveries of patients with bone marrow transplants by increasing differentiation of transplanted bone marrow cells making hospital stays shorter, which would decrease medical costs.

Vitamin E's affect on humoral immunity has been shown as well. When vitamin E is added in place of some of the mineral oil in vaccines, the antibody response is faster than when vitamin E is not used in the vaccine (Franchini et al., 1995); therefore, affecting humoral immunity.

Another way vitamin E affects the immune system is through interferon-γ. Interferon-γ (IFN- γ), which induces macrophage activation (Portillo et al., 1989), was

shown by Han et al. (2000) to be increased in response to a challenge with LPS in vitamin E supplemented older mice. However, Pallast et al. (1999) reported decreased amounts of IFN-γ produced in response to vitamin E supplementation.

Vitamin E also exhibits its antioxidant properties in the immune system by decreasing the amount of reactive oxygen species (ROS) released by macrophages (Pathania et al., 1999). Supplementation with α-tocopherol acetate has been shown to decrease the amount of O_2- and H_2O_2 (known as the ROS) that are released by alveolar macrophages in response to a LPS challenge (Pathania et al., 1999).

Vitamin E works well with other antioxidants as well; for example, supplementation with both vitamin E and vitamin C has been shown to help lower the risk of developing dementia and Alzheimer's disease but not with either one alone (Aubertin, 2004). Supplementation with both vitamin C and vitamin E has also been shown to decrease arachidonic acid production in an *in vitro* study (ElAttar & Lin, 1992). Vitamin E and selenium supplementation was also shown to decrease ethane production due to lipid peroxidation in response to a LPS challenge (Sword et al., 1991).

Zinc, Vitamin E and Immunity

Vitamin E and zinc are known to have several of the same functions, such as membrane stabilization, but their interactions have not been studied thoroughly at this time. Many of the interaction studies have focused on how the deficiency of zinc causes a decreased absorption of vitamin E, as well as other lipid soluble vitamins (Kim et al., 1998), and how a zinc deficient diet can lead to lower levels of α-tocopherol in the liver, kidney, heart, brain and testis (Noh & Koo, 2001). Hatfield et al. (2002) did show that zinc helped to increase the absorption or retention of vitamin E in the pig, but did not find

vitamin E and zinc supplementation to have any interactions other than that. Since both nutrients are important in the immune system, it is important to know if they do act synergistically, and if supplementation is beneficial.

Supplementation studies with both vitamin E and zinc have been done to determine their affects on certain disease states, such as macular degeneration (Jampol & Ferris III, 2001). Supplementation with zinc, vitamin E and other antioxidants was found to help reduce the chances of developing age-related macular degeneration and development of moderate visual acuity loss, as was zinc alone; however, the study did not show if zinc alone was better or if zinc combined with the vitamins was better at reducing these risks (Jampol & Ferris III, 2001).

Studies have also been done to determine if vitamin E supplementation can help to overcome some of the deleterious affects of a zinc deficient diet. Kraus et al. (1997) found that supplementation with antioxidants such as vitamin C, vitamin E, and β-carotene, did improve the osmotic fragility of erythrocytes associated with zinc deficiency, with vitamin C having the most affect. Bettger et al. (1980) found that supplementation with antioxidants (including vitamin E) could improve the skin lesions found in chicks with zinc deficiency, as well as decrease the thiobabituric acid-reactive substances (TBARS).

Zago & Oteiza (2001) also showed a decrease in iron initiated TBARS formation in zinc sufficient animals due to supplementation with both α-tocopherol and zinc, a greater decrease was found with the combination of α-tocopherol and zinc than with either one alone. When combined with a water soluble antioxidant iron induced lipid oxidation was prevented completely.

Chandra et al. (1992) also determined that supplementation with vitamin A, beta carotene, thiamin, riboflavin, niacin, zinc, copper, selenium, iodine, calcium magnesium and vitamins B_6, B_{12}, C, D, and E could increase antibody response to the influenza vaccine as compared to non-supplemented controls, as well as help protect against infection-related illnesses. Girodon et al. (1999) also showed supplementation with trace minerals and vitamins improved the humoral response in the elderly after vaccinations for influenza in groups treated with both trace minerals (including zinc) and vitamins (including α-tocopherol) more than vitamins alone or the control group. Because zinc is believed to be important for the maintenance of natural killer cells, and vitamin E deficiency is associated with an increase in natural killer cell activity, zinc and vitamin E are hypothesized to act together on some functions of the immune system. Investigators have observed supplementation of vitamin E or zinc decrease the amount of PGE_2 and nitric oxide, respectively, produced by macrophages (Wu D et al., 1998 & Abou-Mohamed et al., 1998). However, it is unclear how the interactions between zinc and vitamin E affect the production of PGE_2 and nitric oxide. The present study evaluated the interaction between the zinc and vitamin E in order to determine if the two act synergistically to enhance the reduction of PGE_2 and nitric oxide produced by stimulated macrophages.

Pharmacological vs. Physiological Vitamin E and Zinc Concentrations

In the present study, the media used was already zinc and vitamin E adequate, so the present study only looked at the effects of supplementation with vitamin E and zinc on zinc and vitamin E adequate cells. Combined with the amount of vitamin E and zinc

found in the media, the supplementation levels that were used included both pharmacological and physiological concentrations.

The normal physiological range of zinc in the serum is 0.69 to 1.49 mg/L (Faber et al., 2004), or 10.6 to 22.78 μmol/L. Treatment with pharmacological concentrations of zinc (levels that can only be obtained through consumption of supplements), can lead to the serum zinc concentrations increasing above the normal physiological range. Estefan et al. (1998) showed that in patients with hypopituitarism that consumed a zinc adequate diet (12 mg/day), and consumed 165 mg zinc sulfate supplements had an increased serum zinc concentration of 3.04 ± 0.81 μg/mL after 120 minutes. Faber et al. (2004) showed one week of zinc supplementation with 80 mg zinc sulfate a day did increase zinc serum concentrations, but not above physiological concentrations.

Information from the Third National Health and Nutrition Examination Survey (NHANES III) shows that the average zinc intake from diet is 14 mg/day for men and 9 mg/day for women with the highest amount from food being 24 mg/day. The average intake from both food and supplements for both genders is 13 mg/day (198.75 μmol/day). The upper intake level ranges from 40 mg/day for adults to 7 mg/day for 1-3 year-olds.

The normal physiological range of vitamin E in serum or plasma ranges from 12-42 μmol/L, or 0.5 to 1.8 mg/dL. Gaede et al. (2001) showed vitamin E supplementation of 680 IU combined with vitamin C supplementation of 1250 mg for four weeks in patients with Type 2 diabetes increased serum concentrations of vitamin E, but these serum concentrations were not above the physiological range.

Average vitamin E intake from food according to the NHANES III, was 17.44 μmol per day, and the average intake from both supplements and diet (pharmacological

amounts) was 59.75 μmol per day. The upper intake level ranges from 2325 μmol/day for adults to 465 μmol/day for 1-3 year-olds.

The present study consisted of six separate experiments. The first experiment was determination of the optimal cell seeding density to use for the subsequent experiments, followed by an experiment determining the optimal zinc supplementation concentration and one determining the optimal vitamin E supplementation concentration. An experiment was also done to determine which strain of LPS to use, and which concentration of this LPS to use. A combination study of zinc and vitamin E supplementation was also completed. All of these studies were used to determine different components of the final study that looked at the synergistic effect of supplementation of both vitamin E and zinc on the response of the mouse macrophage to an LPS challenge.

Hypotheses and Expected Results

I. Determination of Optimal Cell Density

A. Hypothesis: The seeding density of 1 x 10^4 cells/well in a 96 well plate will provide the most desired optical density (used as an indicator of growth) of the seeding densities.

1. Expected Results: A greater seeding density will lead to a greater optical density.

II. Determination of Optimal Zinc Supplementation

A. Hypothesis: The 50 μM zinc concentration will provide the most desired optical density (used as an indicator of growth) of the cells as compared to all of the other zinc concentrations (0, 10, 100 and 500 μM).

1. Expected Results: The lower zinc concentrations, 0 and 10, will have a lesser optical density, or amount of growth, than the cells in the 50 μM zinc concentration.
2. Expected Results: The zinc concentrations greater than or equal to 100 μM will have a lower amount of growth, or optical density, than the cells in the 50 μM zinc concentration.

III. Determination of Optimal Vitamin E Supplementation

A. Hypothesis: The 50 μM vitamin E concentration will provide the most desired optical density (used as an indicator of growth) of the cells as compared to the other vitamin E concentrations (0, 25 and 100 μM).

1. Expected Results: The cells in the lower vitamin E concentrations (0 and 25 μM) will have a lesser optical density than the cells in the 50 μM vitamin E concentration.
2. Expected Results: The cells with the highest concentration of vitamin E (100 μM) will have a lesser amount of growth, or optical density, than the cells in the 50 μM vitamin E concentration.

IV. Determination of Optimal LPS Challenge

A. Hypothesis: The LPS from *E. Coli* 055:B5 and 0111:B4 strains will cause the macrophages to produce significantly different amounts of nitric oxide.
    1. Expected Results: LPS from the *E. Coli* 055:B5 will produce a different amount of nitric oxide than LPS from *E. Coli* 0111:B4.

B. Hypothesis: The 100 μg/mL LPS concentration will produce the greatest amount of nitric oxide.
    1. Expected Results: The greater LPS challenge concentration will lead to a greater amount of nitric oxide produced.
    2. Expected Results: The 0 μg LPS concentration will not produce a measurable amount of nitric oxide after any of the time points.

C. Hypothesis: The cells challenged with LPS from *E. Coli* 055:B5 will have a greater optical density (used as an indicator of growth) after 24 and 48 hours as compared to the cells challenged with LPS from *E. Coli* 0111:B4.

D. Hypothesis: The cells challenged with 100 μg LPS/mL will have a lower optical density than the cells challenged with the other concentrations.

1. Expected Results: The cells challenged with the higher LPS concentrations (5, 10, 50, and 100 μg LPS/mL) will have a lower optical density than those challenged with the lower concentrations (0.1 and 1.0 μg LPS/mL).
2. Expected Results: The cells not challenged with LPS will have a lower optical density than those challenged with the lower LPS concentrations (0.1 and 1.0 μg LPS/mL).

V. Determination of the Optimal Combination of Vitamin E and Zinc

A. Hypothesis: The cells supplemented with both 50 μM zinc and 50 μM vitamin E will have a greater optical density (used as an indicator of growth) than the cells supplemented with the other supplementation combinations.

1. Expected Results: The cells supplemented with the greatest amount of zinc (100 μM) and the lower amounts of zinc (0 and 10 μM) will have a lower optical density than those supplemented with the 50 μM zinc concentrations.
2. Expected Results: The cells supplemented with 50 μM vitamin E will have a greater optical density than the cells with the greatest concentration of vitamin E (100 μM) and the lowest amounts of vitamin E (0 and 25 μM).
3. Expected Results: The cells supplemented with both 50 μM zinc and 50 μM vitamin E will have a greater amount of growth than

those supplemented with 0 μM zinc/50 μM vitamin E and 50 μM zinc/0 μM vitamin E.

VI. Determination of the Influence of Vitamin E and Zinc Supplementation on the Response of Mouse Macrophages to a LPS Challenge

A. Hypothesis: The cells supplemented with the combination of 50 μM zinc and 50 μM vitamin E will have a greater optical density (used as an indicator of growth), regardless of LPS challenge, than the cells in all the other treatments.

1. Expected Results: The cells in the 50 μM zinc/50 μM vitamin E treatment will have a greater amount of growth than the cells in the control treatment (0 μM zinc/0 μM vitamin E) regardless of LPS challenge.
2. Expected Results: The cells in the 50 μM zinc/50 μM vitamin E treatment will have a greater amount of growth than the cells in the 50 μM zinc/0 μM vitamin E and the 0 μM zinc/50 μM vitamin E treatments regardless of LPS challenge.

B. Hypothesis: The cells in the highest LPS challenge (1.0 μg LPS/mL) will have the greatest optical density.

1. Expected Results: The cells challenged with 1.0 and 0.1 LPS μg LPS/mL will produce a greater optical density as compared to those cells not challenged with LPS.

2. Expected Results: The cells challenged with 1.0 μg LPS/mL will produce a greater optical density as compared to the cells challenged with 0.1 μg LPS/mL.

C. Hypothesis: The cells supplemented with the combination of 50 μM zinc and 50 μM vitamin E will produce less nitric oxide when challenged with LPS than the cells in all the other treatments, regardless of concentration of LPS challenge.

1. Expected Results: The cells in the 0 μM zinc/50 μM vitamin E treatment will produce less nitric oxide than the cells in the control treatment (0 μM zinc/0 μM vitamin E) regardless of concentration of LPS challenge.
2. Expected Results: The cells in the 50 μM zinc/0 μM vitamin E treatment will produce less nitric oxide than the cells in the control treatment (0 μM zinc/0 μM vitamin E) regardless of concentration of LPS challenge.
3. Expected Results: The cells in the 50 μM zinc/50 μM vitamin E treatment will produce less nitric oxide than the cells in the 50 μM zinc/0 μM vitamin E and 0 μM zinc/50 μM vitamin E treatments regardless of concentration of LPS challenge.

D. Hypothesis: The cells with the greatest LPS concentration (1.0 μg LPS/mL) will produce the greatest amount of nitric oxide regardless of treatment.

1. Expected Results: The cells challenged with 1.0 and 0.1 μg LPS/mL will produce more nitric oxide than those not challenged (0 μg LPS/mL).

E. Hypothesis: The cells in the 50 μM zinc and 50 μM vitamin E treatment will produce less PGE_2 than the cells in all the other treatments, regardless of concentration of LPS challenge.
    1. Expected Results: The cells in the 50 μM vitamin E treatment will produce less PGE_2 than the cells in the control treatment (0 μM zinc with 0 μM vitamin E).
    2. Expected Results: The cells in the 50 μM zinc treatment will produce less PGE_2 than the cells in the control treatment (0 μM zinc with 0 μM vitamin E).

F. Hypothesis: The cells challenged with the greatest amount of LPS (1.0 μg LPS/mL) will produce the greatest amount of PGE_2.
    1. Expected Results: The cells challenged with LPS (1.0 and 0.1 μg LPS/mL) will produce a greater amount of PGE_2 than the cells not challenged with LPS.

G. Hypothesis: The cells in the 50 μM zinc and 50 μM vitamin E treatment will produce less TNF-α than the cells in all the other treatments.
    1. Expected Results: The cells in the 0 μM zinc/50 μM vitamin E treatment will produce less TNF-α than the cells in the control treatment (0 μM zinc/0 μM vitamin E).

2. Expected Results: The cells in the 50 μM zinc/0 μM vitamin E treatment will produce less TNF-α than the cells in the control treatment (0 μM zinc with 0 μM vitamin E).

H. Hypothesis: The cells challenged with the greatest amount of LPS (1.0 μg LPS/mL) will produce the greatest amount of TNF-α.
    1. Expected Results: The cells challenged with LPS (0.1 and 1.0 μg LPS/mL) will produce a greater amount of TNF-α than the cells not challenged with LPS.

I. Hypothesis: The cells in the 50 μM zinc and 50 μM vitamin E treatment will produce a greater amount of IL-10 than the cells in all the other treatments.
    1. Expected Results: The cells in the 0 μM zinc/50 μM vitamin E treatment will produce a greater amount of IL-10 than the cells in the control treatment (0 μM zinc/0 μM vitamin E).
    2. Expected Results: The cells in the 50 μM zinc/0 μM vitamin E treatment will produce a greater amount of IL-10 than the cells in the control treatment (0 μM zinc/0 μM vitamin E).

J. Hypothesis: The cells challenged with the greatest amount of LPS (1.0 μg LPS/mL) will produce the greatest amount of IL-10.
    1. Expected Results: The cells challenged with LPS (0.1 and 1.0 μg LPS/mL) will produce a greater amount of IL-10 than the cells not challenged with LPS.

Chapter III

METHODOLOGY

Cell Culture

The mouse monocyte/macrophage cell line (RAW 264.7) was purchased from ATCC (lot no. 1963180), at passage number 39. This cell line was chosen because it does not produce nitric oxide upon treatment of interferon-α alone, but requires lipopolysaccharide (LPS) for full activation (ATCC 2001). Thus, the cells are more similar to normal mouse macrophages (ATCC 2001). The cells were thawed in a 37°C water bath for two minutes, and then placed in a T-75 flask with 11 mL of complete medium. The complete medium (cDMEM) was Dulbelcco's Moderate Eagle Medium (DMEM with phenol red; Sigma Chemical Co. Catalog no. D5523, St. Louis, MO) with 10% fetal calf serum (Gibco Catalog no. 16000-044, Lot no. 1108708), and 0.5% Penicillin/Streptomycin (Sigma Chemical Co, St. Louis, MO). The cells were counted to determine viability and cell counts were completed using trypan blue exclusion (0.4% Trypan Blue Solution) and a hemacytometer. Cells were incubated with cDMEM in a humidified incubator at 37°C with 5% CO_2/95% air.

The medium was changed every two to three days, and cells were subcultured upon reaching 80-90% confluence. After the fifth subculture, the cells were frozen at a density of 1.0 x 10^6 cells/mL with cDMEM and 5% dimethyl sulfoxide (DMSO). Cryogenic vials were prepared by pipetting 50 μL of sterile dimethyl-sulfoxide into each one, and then adding 950 μL of cells. The cryogenic vials were frozen at -20°C for four

hours, placed in a -80°C freezer overnight, and then transferred to liquid nitrogen for long-term storage. All experiments were performed using these frozen cell aliquots.

Media Analysis

Media was analyzed for zinc concentrations using the Perkin-Elmer 5100 PC Atomic Absorption Spectophotometer with AA Winlab Analyst software.

Determination of the Optimal Cell Density

Experimental Design. In order to determine the optimal cell density to use for the main experiments, a growth experiment with a variety of cell densities was conducted. The basis on which cell density was determined to be optimal for the subsequent experiments was the proliferation of the cells. It was desired to have the cells proliferate as much as possible without surpassing the measurement protocols. These cell densities included: 3 x 10^3 cells/well, 6 x 10^3 cells/well, 1 x 10^4 cells/well, and 5 x 10^4 cells/well in a 96-well Tissue culture treated flat bottomed well plate with lid. Two replications were completed, one replication had six wells per cell seeding density and the second replication had eight wells per seeding density, for a total of fourteen wells per cell density at each time point (24, 48, and 72 hours).

Sample Analyses. The cells were thawed from stock vials by placing 4 mL of cDMEM in a T-25 tissue culture flask and adding the cells from the vial after they had been thawed for 2 minutes in a 37°C water bath. The flask was incubated overnight in a humidified incubator at 37°C with 5% CO_2/95% air, and then 4 mL of spent medium was removed and replaced with new cDMEM. The flask was placed back into the humidified incubator for forty-eight additional hours, after which, the cells were sub-cultured into two T-25 flasks following the previously described method.

For the experiment, the cells were plated into 96-well tissue culture plates at the different cell densities. After 24, 48 or 72 hours of growth, the plates were removed from the incubator, the spent medium removed and replaced with fresh cDMEM. Growth was measured using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma cat. No. M2128) assay, which determines the amount of viable cells in each well of the plate. The MTT is cleaved by the living cells to produce purple formazan crystals. Therefore, only viable cells are measured (Mosmann et al., 1983).

To perform the MTT assay, 50 μL of Hank's Balanced Salt Solution (HBSS) with 5 mg/mL MTT was added to each of the wells and the plates placed back into the incubators. After 4 h of incubation time, the plates were removed from the incubator, and the MTT solution was removed from each well by aspiration. To each well was then added 200 μL of dimethyl-sulfoxide and 50 μL of Sorenson's Glycine buffer (0.1 M glycine, 0.1 M NaCl at a pH of 10.5- adjusted with 1 M NaOH if necessary). The number of viable cells or growth was measured by determining the amount of formazan crystal formation using the BioTek EL_x808 plate-reader and KCJunior software (v1.31.2) at a wavelength of 570 nm. This procedure was based on that previously described by Mosmann (1983).

Determination of the Optimal Zinc Supplementation

Experimental Design. In order to determine the optimal zinc concentration to use in subsequent experiments, five different concentrations of zinc-supplemented medium were used. These concentrations included: 0, 10, 50, 100 and 500 μM Zinc in cDMEM. These concentrations were based upon previous research (Driessen et al., 1995 & Abou-Mohamed et al., 1998). In this experiment, four replications were completed each with

six wells per treatment for a total of 24 wells per treatment at each time point. Optimal zinc concentration was based on how well the cells proliferated in response to the different zinc concentrations; the greater the amount of proliferation, the more optimal the zinc concentration.

Sample Analyses. The cells were started following the same method used in determination of optimal cell density. In order to test for cell growth in response to the different zinc concentrations, a 96-well plate was plated with a cell density of 1.0 x 10^4cells/well (as determined in the previous experiement) with 200 μL of appropriate medium (0, 10, 50, 100, or 500 μM supplemental zinc). The zinc media was prepared by adding zinc sulfate (Sigma Chemical Co, St. Louis, MO) to cDMEM to prepare a stock solution of 1 mM zinc, filter sterilizing with a Millex-GV Durapore syringe driven filter (0.22μM) and then preparing the working dilutions with cDMEM. The plates were incubated in a humidified incubator at 37°C with 5% CO_2/95% air for 24, 48 or 72 hours. The plates were removed from the incubator, and the MTT procedure previously described was used to determine cell growth.

Determination of the Optimal Vitamin E Supplementation

Experimental Design. In order to determine the optimal vitamin E concentrations to use, four concentrations of vitamin E-supplemented medium were used. These concentrations included: 0, 25, 50, and 100 μM vitamin E levels with the cDMEM. These concentrations were based upon previous research (Visioli et al., 2000). In this experiment, two replications were done with six wells used per treatment for a total of twelve wells per treatment at each time point. Optimal vitamin E concentration was based on how well the cells proliferated in response to the different vitamin E

concentrations; the greater the amount of proliferation, the more optimal the vitamin E concentration.

Sample Analyses. The cells were started following the same method used in the previous experiments. The vitamin E medium was prepared by diluting D-α-tocopherol acetate (Sigma Chemical Co, St. Louis, MO, cat no. T1157) with 95% ethanol to make a stock solution. To prepare the working dilutions (0, 25, 50, or 100 μM supplemented vitamin E as D-α-tocopherol and ethanol control), the appropriate amount of stock solution needed for each working dilution was placed into a 5 mL conical tube with an appropriate amount of fetal bovine serum (FBS). The tube was incubated for 15 min, vortexed after every 5 min and replaced in the incubator. Following the 15 min incubation, the appropriate amount of FBS, DMEM, and penicillin/streptomycin were added. By using this method, only 0.0945% of the media added to each well was 95% ethanol meaning less than 0.02 μM of the 200 μM media added to each cell was 95% ethanol, the remainder was medium, FBS or streptomycin. By using doses at this level, an affect of ethanol on the cell functions should not be seen since it is such a small amount compared to the whole.

In order to test for cell growth in response to the different vitamin E concentrations, a 96-well plate was plated with a cell density of 1.0×10^4cells/well with 200 μL of appropriate media (0, 25, 50, or 100 μM supplemented vitamin E as α-tocopherol and ethanol control), and placed into a humidified incubator at 37°C with 5% CO_2/95% air. After 24 and 48 hours of growth, the plates were removed from the incubator, and the MTT assay previously described was used to determine growth. The

72 hour time point was not used since it was shown in the previous experiments that the protocol limitations were surpassed at this time point.

Determination of the Optimal Lipopolysaccharide Challenge

Experimental Design. A complete block design with a 2 x 6 factorial arrangement of treatments was used for this study. To determine the appropriate kind of LPS to use, *E. Coli* 055:B5 and *E. Coli* 0111:B4 strains (Sigma Chemical Co.) were used. In order to determine the appropriate LPS concentrations to use, six different concentrations of LPS medium were used. These concentrations included: 0, 0.1, 1.0, 10, 50, and 100 μg LPS/mL media (DMEM with 10% FBS without penicillin/streptomycin). These levels were based upon previous research (Driessen et al., 1995; Jiang et al., 2000; & Abou-Mohamed et al., 1998). In this experiment, five replications were completed. The 96-well plates contained six wells per treatment (except for the last replication which contained three wells per treatment), whereas the 24-well plates contained two wells per treatment. The basis on which LPS concentration and strain were optimal, was based on how well the cells proliferated with a particular LPS concentration and strain, and how much nitric oxide the cells produced in response to the LPS concentration and strain. The more the cells proliferated and produced nitric oxide, the more desirable, or optimal, the strain and concentration as this would better allow the determination of potential changes by zinc and vitamin E in the subsequent experiment.

Sample Analyses. In order to determine cytotoxicity, the cells were thawed from our stock cells by following the method previously described. The cells were plated on a 96-well plate at a cell density of 1.0×10^4 cells/well with 200 μL of cDMEM added to each well. After 24 h of growth, the spent medium was removed, and the LPS medium added

to each well. The plates were then incubated for an additional 12, 24, 48, or 72 hours, after which they were removed from the incubator, and the MTT assay as previously described was performed.

In addition to the 96-well plates, 24-well plates were plated at a cell density of 6.66 x 10^4 cells/well with 1 mL of cDMEM added to each well. The seeding density of 6.66 x 10^4 cells/well in the 24-well plates was equivalent to the 1.0 x 10^4 cells/well density in the 96-well plate since the 24-well plate has larger well sizes. The 24-well plates were treated in the same manner as the 96-well plates, except after the 12, 24, 48, and 72 hour incubation, the Griess assay (Promega, Madison, WI) was completed. The Griess Assay was done following manufacturer's directions in order to determine nitric oxide production. The Griess assay measures the amount of NO_2- (a product of nitric oxide breakdown) in the sample by using sulfanilamide and *N*-1mapthylethylenediamine dihydrocholoride to produce a purple color that can measured at 520 nm using a Biotek plate reader and KCJunior software (Promega corporation, 1995).

Determination of the Optimal Combination of Vitamin E and Zinc

Experimental Design. A randomized block design with a 2 x 4 factorial arrangement of treatments was used for this study. The combinations included all of the combinations of 0, 25, 50, and 100 μM vitamin E concentrations with 0, 10, 50 and 100 μM Zinc concentrations in cDMEM (without phenol red, Sigma product number D2902), and included an ethanol control. Two replications of this experiment were completed with four wells of each treatment per relplication for a total of eight wells per treatment at each time point. After observing the results from these replications, an additional replication of this experiment was completed using only the combinations of 0 and 50 μM zinc

concentrations with 0 and 50 μM vitamin E concentrations to see if these were the concentrations that were desired. This additional replication had six wells per treatment at each time point. The optimal combination of zinc and vitamin E concentrations was based on how well the cells proliferated in response to the different combinations; the greater the amount of proliferation, the more optimal the combination of those concentrations.

Sample Analyses. The cells were thawed following the method previously described, using cDMEM that did not contain phenol red since the phenol red might decrease the test reliability in this and further experiments. After the cells were allowed to grow for three days, they were subcultured following the method previously described. The cells were plated in 96-well plates at a cell density of 1.0×10^4 cells /well with 200 μL of appropriate zinc and vitamin E supplemented medium. The medium was prepared following the methods previously described for the vitamin E medium, and adding the appropriate amount of zinc stock in place of the same amount of cDMEM.

The plates and T-25 flasks were then placed into a humidified incubator at 37°C with 5% carbon dioxide/95% air, and allowed to grow for 24 or 48 additional hours. After these times were completed, the MTT assay (as previously described) was performed to measure growth.

Determination of the Influence of Vitamin E and Zinc on Mouse Macrophage Response to a Lipopolysaccharide Challenge

Experimental Design. A complete block design with a 6 x 3 factorial arrangement of treatments was used for this study. The treatments consisted of different concentrations of zinc with vitamin E: 0 or 50 μM zinc and/or vitamin E. Indomethacin is a commonly

used non-steroidal anti-inflammatory drug that helps to decrease prostaglandin production (Basini, 2001); therefore, indomethacin (5 or 10 μM) was added as a positive control. The cells were plated with these different mediums, then were allowed to grow for 24 hours. After 24 hours, the zinc and/or vitamin E supplemented cells were incubated with three different concentrations of lipopolysaccharide (*E. Coli* 055:B5): 0 μg/mL, 0.1 μg/mL, and 1.0 μg/mL. These concentrations were based upon the results of the previous experiments.

Sample Analyses. Cells were started in the same manner as previously described.

Measurement of Growth. Growth and cytotoxicity were determined by plating 96-well plates at a cell density of 1.0×10^4 with 200 μL of appropriate medium (0 and/or 50 μM zinc and 0 and/or 50 μM vitamin E, ethanol control, and 5 μM or 10 μM indomethacin). After 24 h of incubation with the appropriate medium, the medium was removed and replaced with 200 μL of the appropriate treated medium containing the LPS concentrations. Four hours prior to the end of the incubation time with LPS (24 or 48 h), the plates were removed from the incubator. The spent medium was removed and replaced with 200 μL fresh medium of the same kind (containing both the LPS and the treatment), and 50 μL of Hank's Balanced Salt Solution (HBSS) with 5 mg/mL MTT added to each of the wells, and the plates placed back into the incubators. The MTT assay as previously described was then performed. This was replicated two times with fours wells per treatment used in each replication.

Production of Nitric Oxide. To determine the effects of zinc and vitamin E on nitric oxide (NO), PGE_2, Il-10, and TNF-α, the cells were plated in 24-well plates at a cell density of 6.66×10^4 cells/well with 1 mL of the appropriate media (0 and/or 50 μM zinc

and 0 and/or 50 μM vitamin E, ethanol control, and 5 μM or 10 μM indomethacin). The media was prepared following the method previously described, with the exception of not using penicillian/streptomycin, which was not used since it might decrease the macrophage's reaction to the LPS challenge.

After 24 h of incubation with the zinc and vitamin E medium, the LPS treatments were added to the appropriate wells. After 24 or 48 h of incubation with LPS, the plates were removed from the incubator, and placed on ice. The Griess assay (Promega, Madison, WI) was then performed as previously described. Two replications of this with two wells per treatment were performed.

Measurement of Cytokines. After an aliquot of spent medium had been removed for nitric oxide measurement, the plates were centrifuged at 4ºC for 5 min at 1000 rpms, and supernatants collected, and 200 μL alliquoted into cryovials, and stored at -80°C until used for cytokine measurements. Two replications were completed for 24 h and three replications were completed for 48 h, each with two wells per treatment. To determine TNF-α production, the supernatants were thawed, vortexed, and analyzed using the TNF-α enzyme-linked immunosorbent assay kit by BD Pharmingen (Catalog No. 555268). Interleukin-10 production was determined in this same manner using BD Biosciences's BD OptEIA Mouse IL-10 Set (Catalog No. 555252), as was prostaglandin-E_2 production using Amersham Biosciences Prostaglandin E_2 Biotrak Enzymeimmunoassay (EIA) system.

Statistical Analysis

Data was analyzed using SAS (version 8.01, Cary, North Carolina, USA). A mixed model was used with the random effects being the replication of each experiment. Least

square means and differences of least square means were determined then adjusted using Tukey's multiple-comparison to determine treatment effects. Interactions between LPS concentrations and treatments were compared to each other as well as compared together against the main effects. Significance was considered at $p < 0.05$. Data is shown as means ± standard errors of the mean.

Chapter IV

RESULTS

Determination of the Optimal Cell Density

In order to determine the optimal seeding density of the macrophages, we used a variety of cell densities and measured the number of viable macrophages (optical density) by MTT assay after 24, 48 and 72 hours of growth. The optical density was used as a measure of growth of the macrophages in these experiments. After twenty four hours, the optical density (O.D.) or growth of the macrophages seeded at $5x10^4$ cells/well (2.85 ± 0.033) was greater ($p<0.05$) than the growth of the macrophages seeded at 1 x 10^4 cells/well, 3 x 10^3 cells/well or 6 x 10^3 cells/well (0.85 ± 0.027, 0.33 ± 0.022 and 0.44 ± 0.027 respectively). It was also shown that the growth of the macrophages seeded at 1 x 10^4 cells/well was greater than ($p<0.05$) the growth of the macrophages seeded at 3 x 10^3 cells/well and 6 x 10^3 cells/well; however, the growth of the macrophages seeded at 3 x 10^3 cells/well and 6 x 10^3 cells/well were similar ($p>0.05$). After 48 hours, the macrophages seeded at $5x10^4$ cells/well (3.80 ± 0.085) had a greater amount of growth ($p<0.05$) than the other cell densities. The macrophages seeded at 1 x 10^4 cells/well (1.05 ± 0.069) had a greater amount of growth ($p<0.05$) than macrophages seeded at 3 x 10^3 cells/well and 6 x 10^3 cells/well (0.25 ± 0.044 and 0.43 ± 0.054 respectively); however, the growth of the macrophages seeded at 3 x 10^3 cells/well and 6 x 10^3 cells/well were still similar ($p>0.05$). After 72 hours of growth, the macrophages seeded at $5x10^4$ cells/well did not have a growth that was measurable due to the limitations of the MTT

assay, and the KCJunior software. The KCJunior software can only measure optical densities of 4.0 or less in this assay, and the optical density of this seeding density at 72 hours was greater than 4.0. However, the growth was determined for the other seeding densities. These results revealed that the growth of the macrophages at a seeding density of 1 x 10^4 cells/well (2.26 ± 0.201) was greater than ($p<0.05$) the growth of the macrophages at a seeding of 3 x 10^3 cells/well (0.93 ± 0.06) and 6 x 10^3 cells/well (1.49 ± 0.10); also at this time point, the macrophages seeded at 6 x 10^3 cells/well had a greater amount of growth than ($p<0.05$) the macrophages seeded at 3 x 10^3 cells/well.

Determination of the Optimal Zinc Supplementation

At 24 hours, the O. D. or growth was unaffected ($p>0.05$) by zinc supplementation at levels less than or equal to100 μM (Figure 1). Supplementation with 500 μM zinc decreased ($p<0.05$) the growth compared with macrophages supplemented with 100 μM or less of zinc. At 48 hours, the growth of macrophages supplemented with 500 μM zinc was less than ($p<0.05$) that of the macrophages supplemented with 100 μM or less of zinc. Also, at this time point, the growth with 10 μM and 50 μM zinc was greater than ($p<0.05$) with 100 μM zinc (Figure 1). After 72 hours, the macrophages in the 0 μM, 10 μM, 50 μM, and 100 μM zinc supplemented medium had growths greater ($p<0.05$) than the macrophages in the 500 μM zinc supplemented medium, but were similar ($p>0.05$) to each other (Figure 1).

Determination of the Optimal Vitamin E Supplementation

At 24 hours, the O. D. (or growth) was unaffected ($p>0.05$) by vitamin E supplementation (Figure 2). After 48 hours, the growth of the macrophages in the 0 μM, 25 μM, and 100 μM vitamin E supplemented medium was less than ($p<0.05$) the growth

of the macrophages in the 50 μM vitamin E supplemented medium; however, the growth in these concentrations of vitamin E supplementation was similar ($p>0.05$) (Figure 2).

Determination of the Optimal Lipopolysaccharide Challenge

Number of Viable Cells

A significant ($p<0.05$) three way interaction was observed among strain, concentration and time for the optical density or number of viable cells (Figure 3). Therefore, the data was analyzed for a two-way interaction between time and concentration within each strain of LPS.

E. Coli 055:B5. With the *E. Coli* 055:B5, the number of viable macrophages, in all LPS concentrations were similar ($p>0.05$) after 12 hours of LPS challenge (Figure 3A). After 24 hours, the number of viable macrophages in 0 and 0.1 μg LPS/mL media was greater than in 100 μg LPS/mL media ($p<0.05$). The number of viable macrophages in 1.0, 10, 50, and 100 μg LPS/mL media was similar ($p>0.05$), as was the number in 0 and 0.1 μg LPS/mL media ($p>0.05$) (Figure 3A). When the macrophages had been challenged for 48 hours with the *E. Coli* 055:B5, the number of viable macrophages in 0, 0.1, and 1.0 μg LPS/mL media was greater than ($p<0.05$) that of the macrophages in 5, 10, 50 and 100 μg LPS/mL media. It was also determined the macrophages with 0, 0.1, and 1.0 μg LPS/mL media all had similar ($p>0.05$) numbers of viable macrophages. The number of viable macrophages with 5, 10, 50 and 100 μg LPS/mL media were also similar ($p>0.05$). (Figure 3A). After 72 hours of the *E. Coli* 055:B5 challenge, the number of viable macrophages in 5, 10, and 100 μg LPS/mL media was less than ($p<0.05$) that of macrophages with 0, 0.1, and 1.0 μg LPS/mL media. The macrophages in 0, 0.1, and 1.0 μg LPS/mL media wells had a cell viability number that was similar to each other

($p>0.05$), as did the macrophages with 5, 10, 50 and 100 μg LPS/mL media to each other ($p>0.05$). The number of viable macrophages in 50 μg LPS/mL media was similar ($p>0.05$) to that of the macrophages in the 1.0, 5, 10, and 100 μg LPS/mL media. (Figure 3A).

E. Coli 0111:B4. After 12 hours of incubation with LPS from the *E. Coli* 0111:B4 strain, the macrophages challenged with 0 and 0.1 μg LPS/mL media had a greater ($p<0.05$) number of viable macrophages than those challenged with 50 and 100 μg LPS/mL media, but were similar ($p>0.05$) to each other (Figure 3B). After 24 hours of the challenge, the number of viable macrophages in each well was similar ($p>0.05$) for 0 and 0.1 μg LPS/mL media, but was greater ($p<0.05$) in 0 μg LPS/mL media than in 5, 50, and 100 μg LPS/mL media. The macrophages with 0.1 μg LPS/mL media had a similar ($p>0.05$) number as all the other concentrations except for 100 μg LPS/mL media; which had a lesser number ($p<0.05$) of viable macrophages than with 0.1 μg LPS/mL media. The other numbers were similar ($p>0.05$) (Figure 3B). After 48 hours of challenge, the macrophages in 0, 0.1, and 1.0 μg LPS/mL media had a greater ($p<0.05$) number of viable macrophages than the macrophages in 5, 10, 50 and 100 μg LPS/mL media; however, the macrophages in 0, 0.1 and 1.0 μg LPS/mL media had numbers similar ($p>0.05$) to each other, as did ($p>0.05$) the macrophages in 5, 10, 50 and 100 μg LPS/mL media. (Figure 3B). After 72 hours of growth with the *E. Coli* 0111:B4, the number of viable macrophages in the 0 μg LPS/mL media was not able to be determined due to protocol limitations, the growth was too great. The number of viable macrophages in 0.1 and 1 μg LPS/mL media were similar ($p>0.05$) to each other, however, their numbers

were greater ($p<0.05$) than those of the macrophages challenged with 5, 10, 50 and 100 μg LPS/mL media. The macrophages in 5 μg LPS/mL media also had a greater ($p<0.05$) number of viable macrophages than the macrophages in 50 and 100 μg LPS/mL media, but was similar ($p>0.05$) to the number in the 10 μg LPS/mL media. (Figure 3B).

Production of Nitric Oxide. The results of the Griess assay, which was used to determine nitric oxide levels produced by the macrophages in response to the LPS challenge, did not reveal a significant ($p>0.05$) three way interaction (strain, time and concentration) (Figure 4). However, there were significant two way interactions between strain and time, time and concentration, and strain and concentration (data for interactions not shown; refer to Figure 4 for data shown by strain, time and concentration).

When the data was analyzed for strain and concentration, macrophages with 0 and 100 μg LPS/mL media from the *E. Coli* 055:B5 strain produced less ($p<0.05$) nitric oxide than the ones with 0.1, 1.0, 5 and 10 μg LPS/mL media. The macrophages with the 0 μg LPS/mL media from *E. Coli* 0111:B4, produced less ($p<0.05$) nitric oxide than those with 0.1, 1.0, 5, 10 and 100 μg LPS/mL media. Also, the macrophages with 100 μg LPS/mL media from *E. Coli* 0111:B4 produced less ($p<0.05$) nitric oxide than the macrophages with 0.1, 1.0, 5 and 10 μg LPS/mL media.

When the data was analyzed for strain and time, it was shown that macrophages with the LPS from the *E. Coli* 055:B5 strain produced less ($p<0.05$) nitric oxide at zero and 12 hours than at 24, 48 and 72 hours. At 24 hours, less ($p<0.05$) nitric oxide was produced than at 48 and 72 hours also. These macrophages also produced less ($p<0.05$) nitric oxide at 48 hours than at 72 hours. The results were the same in the macrophages

with LPS from the *E. Coli* 0111:B4, except no difference ($p>0.05$) was seen between zero, 12 or 24 hours.

The two way interaction of time and concentration was also analyzed. There were no differences ($p>0.05$) found in nitric oxide produced by any LPS concentration at zero, 12, or 24 hours. After 48 hours, the macrophages challenged with 1.0 μg LPS/mL media produced greater ($p<0.05$) amounts of nitric oxide than those challenged with 0 and 100 μg LPS/mL media, but the same ($p>0.05$) as the other concentrations. It was also found at this time, the macrophages stimulated with 0 μg LPS/mL media produced less ($p<0.05$) nitric oxide than those stimulated with 0.1, 1.0, 5 and 10 μg LPS/mL media, but similar amounts to those macrophages stimulated with 100 μg LPS/mL media.

After 72 hours, the macrophages stimulated with 0 μg LPS/mL media produced less ($p<0.05$) nitric oxide than those stimulated with 0.1, 1.0, 5, 10 and 100 μg LPS/mL media. The macrophages with 0.1 μg LPS/mL media produced a greater amount ($p<0.05$) of nitric oxide than those with 0, 5, 10 and 100 μg LPS/mL media, as did those with 1.0 μg LPS/mL media. The macrophages stimulated with 5 and 10 μg LPS/mL media produced a greater amount ($p<0.05$) of nitric oxide than those with 0 and 100 μg LPS/mL media, however, they produced similar amounts ($p>0.05$) as each other.

Determination of the Optimal Combination of Vitamin E and Zinc

In order to determine the optimal combination of vitamin E and zinc to use in the experiments with LPS, all the possible combinations of four different concentrations of zinc (0, 10, 50 and 100 μM Zinc) and four different concentrations of vitamin E supplementation (0, 25, 50, and 100 μM vitamin E) were used, along with an ethanol control. Macrophages were grown in each of the combinations of the supplemented

media, and growth was assessed after 24 and 48 hours of growth as done in previous experiments. When the data was analyzed, there was a significant ($p<0.05$) interaction among time, vitamin E supplementation and zinc supplementation; therefore, the data was analyzed within each time (Table 1). As time progressed, stronger differences were detected among the vitamin E and zinc treatments (Table 1).

After 24 hours of supplementation, there was not a significant interaction between zinc and vitamin E supplementation (Table 2), however there was a trend ($p=0.074$). The main effect of vitamin E was significant ($p<0.05$). After 24 hours, the macrophages grown in the 0 μM vitamin E media had a greater ($p<0.05$) amount of growth than the macrophages grown in the 100 μM vitamin E media (Figure 5). Growth was not different ($p>0.05$) among 25, 50, or 100 μM vitamin E, nor was it different among 0, 25 or 50 μM vitamin E.

After 48 hours of supplementation, there was a significant ($p<0.05$) interaction between vitamin E and zinc (Table 1). When the data was analyzed for vitamin E effects within each zinc concentration (Table 3), it was found that macrophages with media that contained 0 μM zinc and 0 μM vitamin E had a lower ($p<0.05$) amount of growth than the macrophages in the 0 μM zinc with 50 μM vitamin E. The opposite was seen with the 10 μM zinc supplementation (Table 3), the 10 μM zinc with 0 μM vitamin E had a greater ($p<0.05$) amount of growth than the macrophages in the 10 μM zinc with 25 μM or 50 μM vitamin E. At the 10 μM zinc concentration, it was also shown that macrophages in the 50 μM vitamin E had a lesser ($p<0.05$) amount of growth than the macrophages in the 100 μM vitamin E. Macrophages grown in the 50 μM zinc media (Table 3) with either 0 or 100 μM vitamin E had a greater ($p<0.05$) amount of growth than the macrophages in

the 50 μM zinc media with the 25 or 50 μM vitamin E media. There were no significant ($p>0.05$) differences with 100 μM zinc and any of the vitamin E concentrations (Table 3).

Determination of the Influence of Vitamin E and Zinc on Mouse Macrophage Response to a Lipopolysaccharide Challenge

Optical Density (Growth). There was a significant ($p<0.05$) three-way interaction among media treatment (zinc and/or vitamin E), time and LPS concentration; therefore, the data was analyzed within time for an interactive effect between treatment and LPS (Figure 6).

After 24 hours, the growth was similar ($p>0.05$) across treatments (Figure 6A). After 48 hours differences were observed with treatment and LPS concentration. The macrophages that were subjected to 0 μg LPS/mL media had similar ($p>0.05$) growth across all treatments. For macrophages challenged with 0.1 μg LPS/mL media, the growth of the macrophages treated with 10 μM Indomethachin was less than ($p<0.05$) that of the macrophages in the other treatments. The macrophages that were challenged with 1.0 μg LPS/mL media and incubated in the 0 μM zinc/50 μM vitamin E supplemented media had a greater ($p<0.05$) amount of growth than those in both the 10 μM Indomethachin media and the 50 μM zinc/0 μM vitamin E supplemented media. Also the macrophages challenged with 1.0 μg LPS/mL media that were grown in the 0 μM zinc/0 μM vitamin E supplemented media had a greater ($p<0.05$) amount of growth than that of the macrophages in the 50 μM zinc/0 μM vitamin E supplemented media.

Production of Nitric Oxide. In order to determine nitric oxide produced by macrophages in zinc and vitamin E supplemented medias, macrophages were challenged with different concentrations of LPS from *E.Coli* 055:B5 (0, 0.1, and 1.0 μg LPS/mL media). There was not a significant ($p>0.05$) three way interaction (treatment, LPS concentration and

time) (Figure 7). However, there were significant interactions between LPS concentration and time ($p<0.05$) (Figure 8) and treatment by LPS concentration ($p<0.05$) (Figure 9).

When the data was analyzed looking at LPS concentration by time, it was shown that at 24 hours, the macrophages in 0 μg LPS/mL media produced less ($p<0.05$) nitric oxide than macrophages in 1.0 μg LPS/mL. At 48 hours, the macrophages in 0 μg LPS/mL media produced less ($p<0.05$) nitric oxide than macrophages in both 0.1 and 1.0 μg LPS/mL media.

At 0.1 μg LPS/mL media, the macrophages in the 0 μM zinc/ethanol control supplemented media produced a greater ($p<0.05$) amount of nitric oxide than those in the 0 μM zinc/50 μM vitamin E and 50 μM zinc/50 μM vitamin E supplemented medias. However, the 0 μM zinc/ethanol control was not different ($p>0.05$) from 0 μM zinc/0 μM vitamin E. With macrophages challenged with 1.0 μg LPS/mL media, the 0 μM zinc/ethanol control produced less ($p<0.05$) nitric oxide than the other treatments and indomethacin controls. There were no differences ($p>0.05$) among the zinc/vitamin E treatments and indomethacin controls.

Tumor Necrosis Factor-α (TNF-α). Tumor necrosis factor-α was measured as an indicator of the inflammatory response following the LPS challenge. No differences ($p>0.05$) were seen between treatment and LPS concentrations (Figure 10), but differences ($p<0.05$) among LPS concentrations were seen. The TNF-α produced by macrophages challenged with 0 μg LPS/mL media (14.96 ± 10.42 pg/mL) was less than ($p<0.05$) that produced by the macrophages challenged with 0.1 or 1.0 μg LPS/mL media (4783.19 ± 632.55 and 6036.56 ± 688.14 pg/mL respectively). The macrophages

challenged with 0.1 or 1.0 μg LPS/mL media produced similar ($p>0.05$) amounts of TNF-α.

Interleukin-10. Interleukin-10 (IL-10) an anti-inflammatory mediator was also measured. The results revealed no significant ($p>0.05$) difference in the interaction between treatment and LPS concentration (Figure 11). The data was further analyzed looking for the main effects of treatment and LPS, which did reveal significant ($p<0.05$) effects of the IL-10 produced among treatments and among the LPS concentrations ($p<0.05$) (data not shown).

The treatment effects revealed the IL-10 produced by macrophages grown in the 5 and 10 μg Indomethacin medias was greater than ($p<0.05$) that produced by macrophages in 50 μM zinc/50 μM vitamin E, 50 μM zinc/0 μM vitamin E, 0 μM zinc/50 μM vitamin E, and 0 μM zinc/ethanol control supplemented medias (data not shown). The levels produced by the macrophages in the 5 and 10 μM Indomethacin medias were similar ($p>0.05$) to that produced by the macrophages in the 0 μM zinc/0 μM vitamin E supplemented media, as were the levels produced by the macrophages in the 50 μM zinc/50 μM vitamin E, 50 μM zinc/0 μM vitamin E, 0 μM zinc/50 μM vitamin E, and 0 μM zinc/ethanol control supplemented medias (data not shown).

Prostaglandin- E_2 (PGE_2). Prostaglandin-E_2 (PGE_2) is another inflammatory mediator that is released by the macrophage in response to a LPS challenge. The results revealed a trend ($p=0.0802$) for an interaction between treatment and LPS concentration (Figure 12). Significant main effects did exist among treatment ($p<0.05$), and LPS concentrations ($p<0.05$) (data not shown).

When the data was analyzed for differences among treatments, it was shown that the macrophages in the 0 μM zinc/50 μM vitamin E treatment produced a greater ($p<0.05$) amount of PGE_2 than the macrophages treated with 5 μM or 10 μM Indomethacin and challenged (refer to Figure 12).

When the data was analyzed for differences among LPS concentrations, the macrophages challenged with 1.0 μg LPS/mL media produced a greater ($p<0.05$) of IL-10 than the macrophages challenged with 0 μg LPS/mL media.

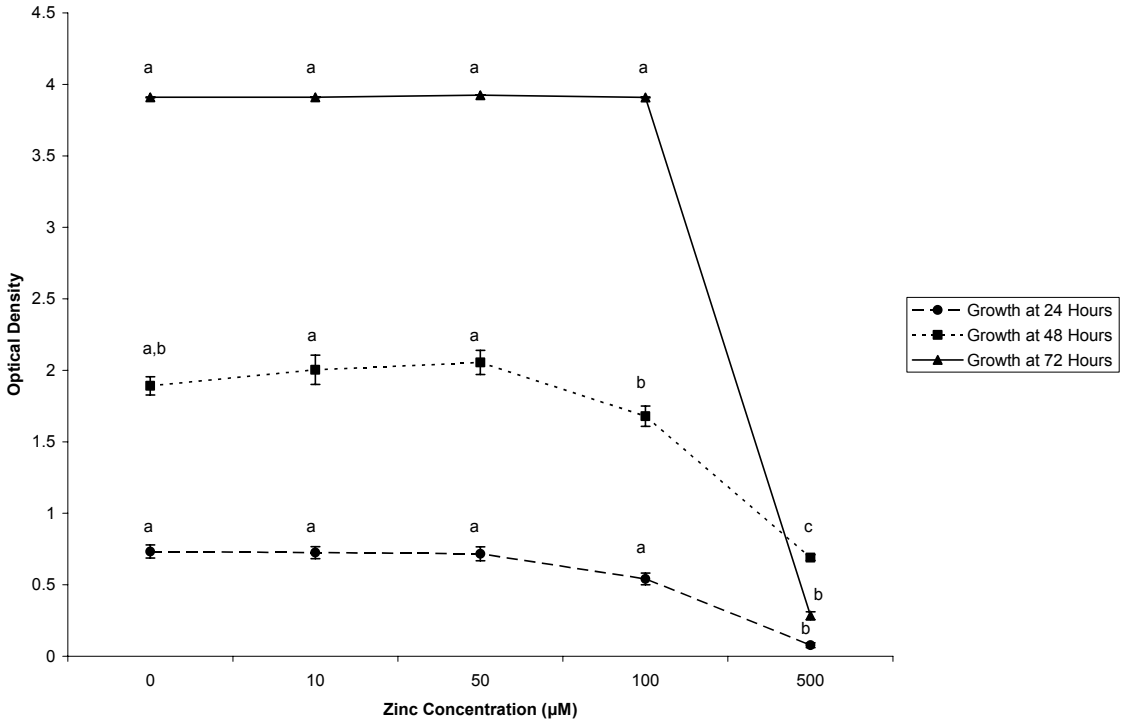

Figure 1. Growth of macrophages in zinc supplemented medium.
Values shown are means ± standard errors. [a,b,c] Means within a time period without common letters are significantly different. ($p<0.05$).

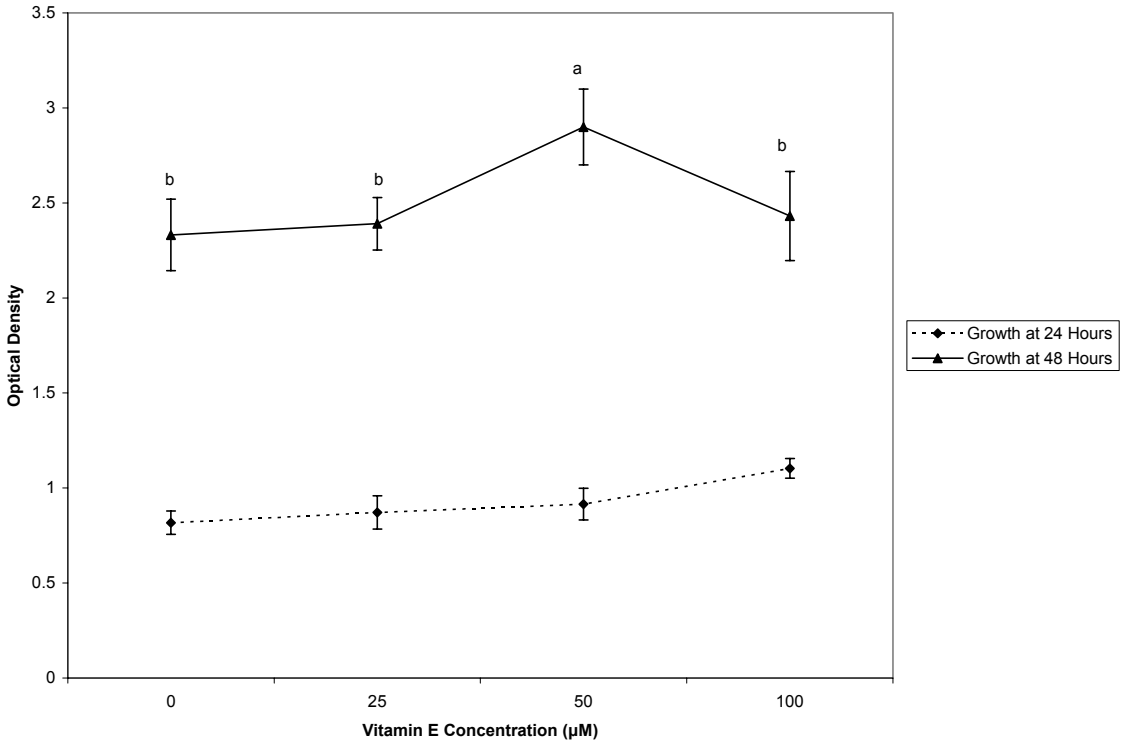

Figure 2. Growth of macrophages in vitamin E supplemented medium. Values shown are means ± standard errors. [a.b] Means within a time period without common letters are significantly different from each other ($p<0.05$).

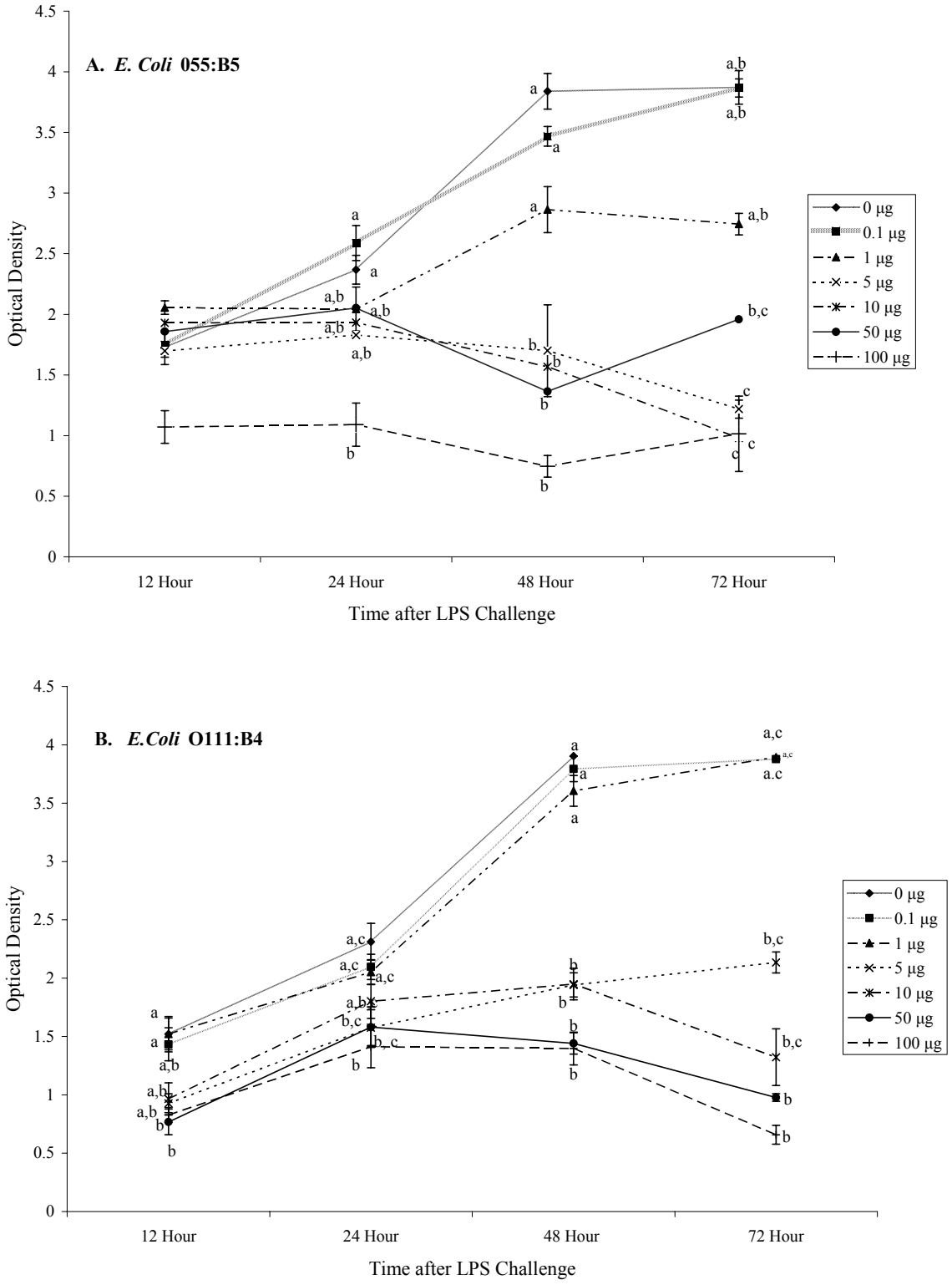

Figure 3. Growth of macrophages in medium after challenge with lipopolysaccharide (LPS) from *E. Coli* 055:B5 (A) and *E. Coli* 0111:B4 (B). Values shown are means ± standard errors. [a,b,c] Means within a time period without common letters are significantly different from each other ($p<0.05$).

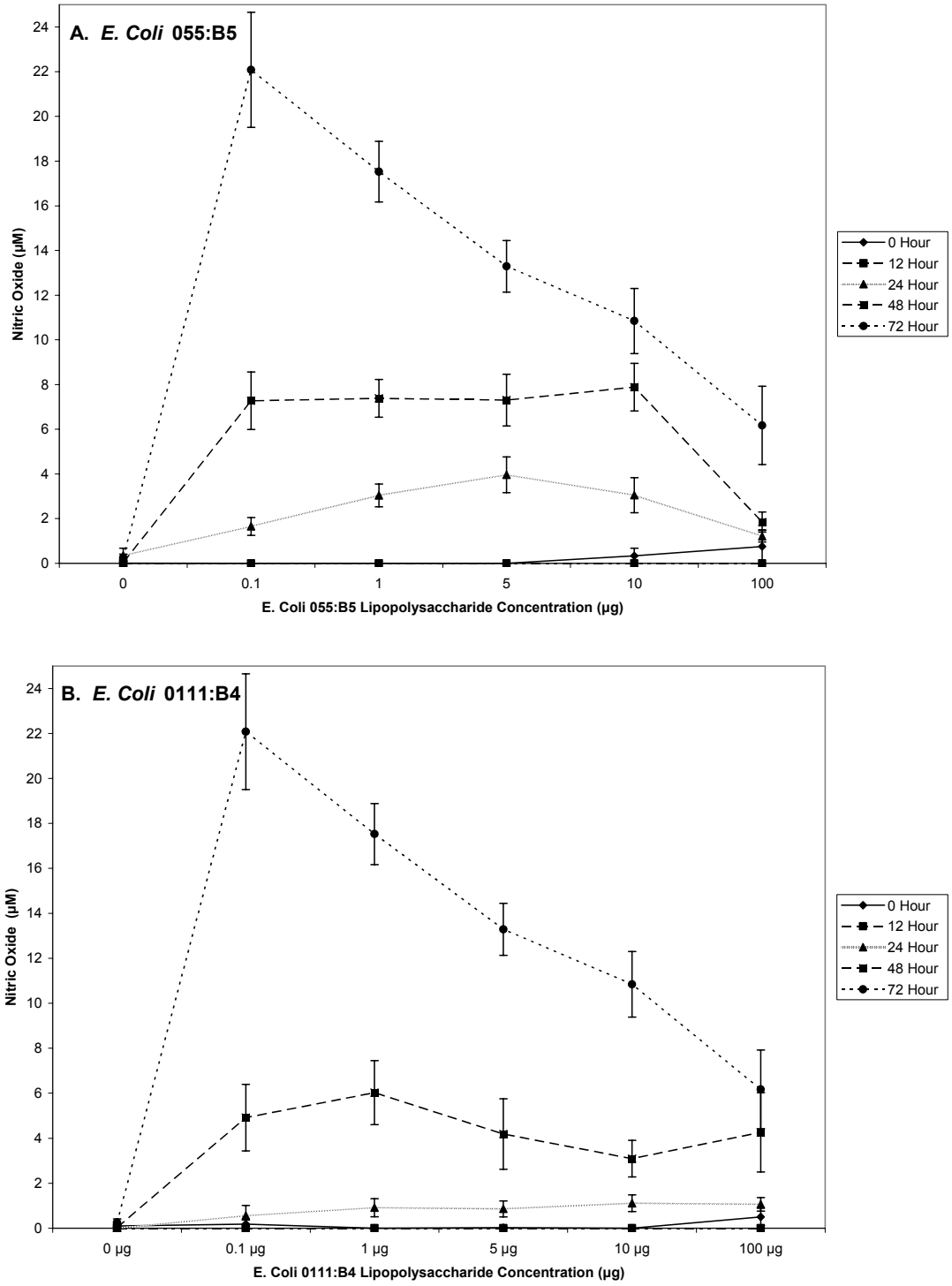

Figure 4. Nitric oxide produced by macrophages after challenge with lipopolysaccharide (LPS) from *E. Coli* 055:B5 (A) and *E. Coli* 0111:B4 (B). Values are means ± standard errors. The data is presented by strain; however, there was not a significant ($p>0.05$) three way interaction among strain, concentration and time.

Table 1. P-values for the Growth of Macrophages in Vitamin E and Zinc Supplemented Media after 24 and 48 Hours

Model	**P-value**
Overall	
Time	<0.0001
Zinc	0.0365
Time x Zinc	0.0011
Vitamin E	0.0001
Time x Vitamin E	0.0002
Zinc x Vitamin E	<0.0001
Time x Zinc x Vitamin E	0.0001
By Time- 24 Hours	
Zinc	0.0822
Vitamin E	0.0441
Zinc x Vitamin E	0.0741
By Time- 48 Hours	
Zinc	0.0091
Vitamin E	<0.0001
Zinc x Vitamin E	<0.0001

Table 2. Growth of Macrophages in Vitamin E and Zinc Supplemented Medium after 24 Hours[1].

Zinc (μM)	Vitamin E (μM)	Optical Density
0	0	0.808 ± 0.034
0	25	0.707 ± 0.018
0	50	0.999 ± 0.101
0	100	0.782 ± 0.054
10	0	0.696 ± 0.023
10	25	0.681 ± 0.042
10	50	0.722 ± 0.036
10	100	0.693 ± 0.046
50	0	0.949 ± 0.070
50	25	0.759 ± 0.040
50	50	0.910 ± 0.076
50	100	0.861 ± 0.040
100	0	0.698 ± 0.054
100	25	0.692 ± 0.027
100	50	0.721 ± 0.040
100	100	0.750 ± 0.028

[1] Values are means ± standard errors.

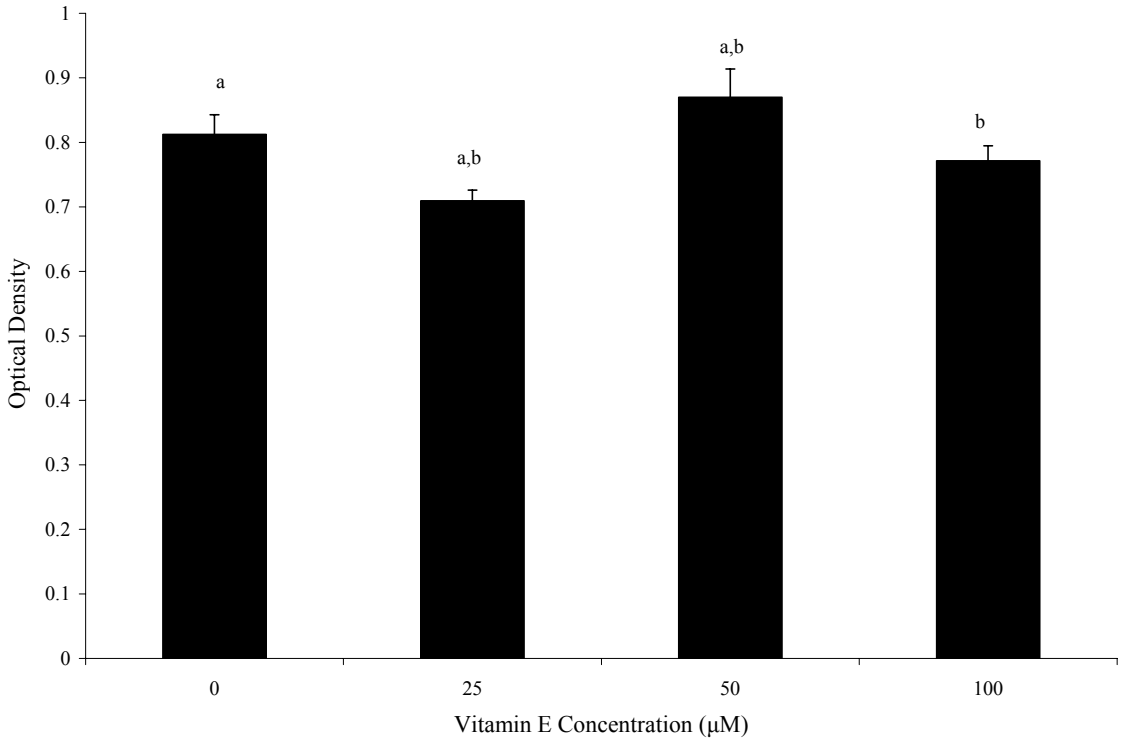

Figure 5. Growth of macrophages in vitamin E supplemented medium irrespective of zinc supplementation. Values shown are means ± standard errors. [a.b] Means without common letters are significantly different from each other ($p<0.05$).

Table 3. Growth of Macrophages in Vitamin E and Zinc Supplemented Medium after 48 Hours[1]

Zinc (μM)	Vitamin E (μM)	Optical Density
0	0	1.973± 0.150[b]
0	25	1.959 ± 0.184[a,b]
0	50	2.331 ± 0.280[a]
0	100	1.904 ± 0.093[b]
10	0	2.194 ± 0.245[a]
10	25	1.624 ± 0.097[b,c]
10	50	1.392 ±0.062[c]
10	100	2.009 ± 0.146[a,b]
50	0	2.474 ± 0.237[a]
50	25	1.268 ± 0.063[b]
50	50	1.885 ± 0.184[b]
50	100	1.955 ± 0.121[a]
100	0	1.548 ± 0.078
100	25	1.495 ± 0.174
100	50	1.198 ± 0.076
100	100	1.789 ± 0.273

[1] Values are means ± standard errors.

[a,b,c] Means within each zinc concentration that do not have common letters are significantly different ($p<0.05$).

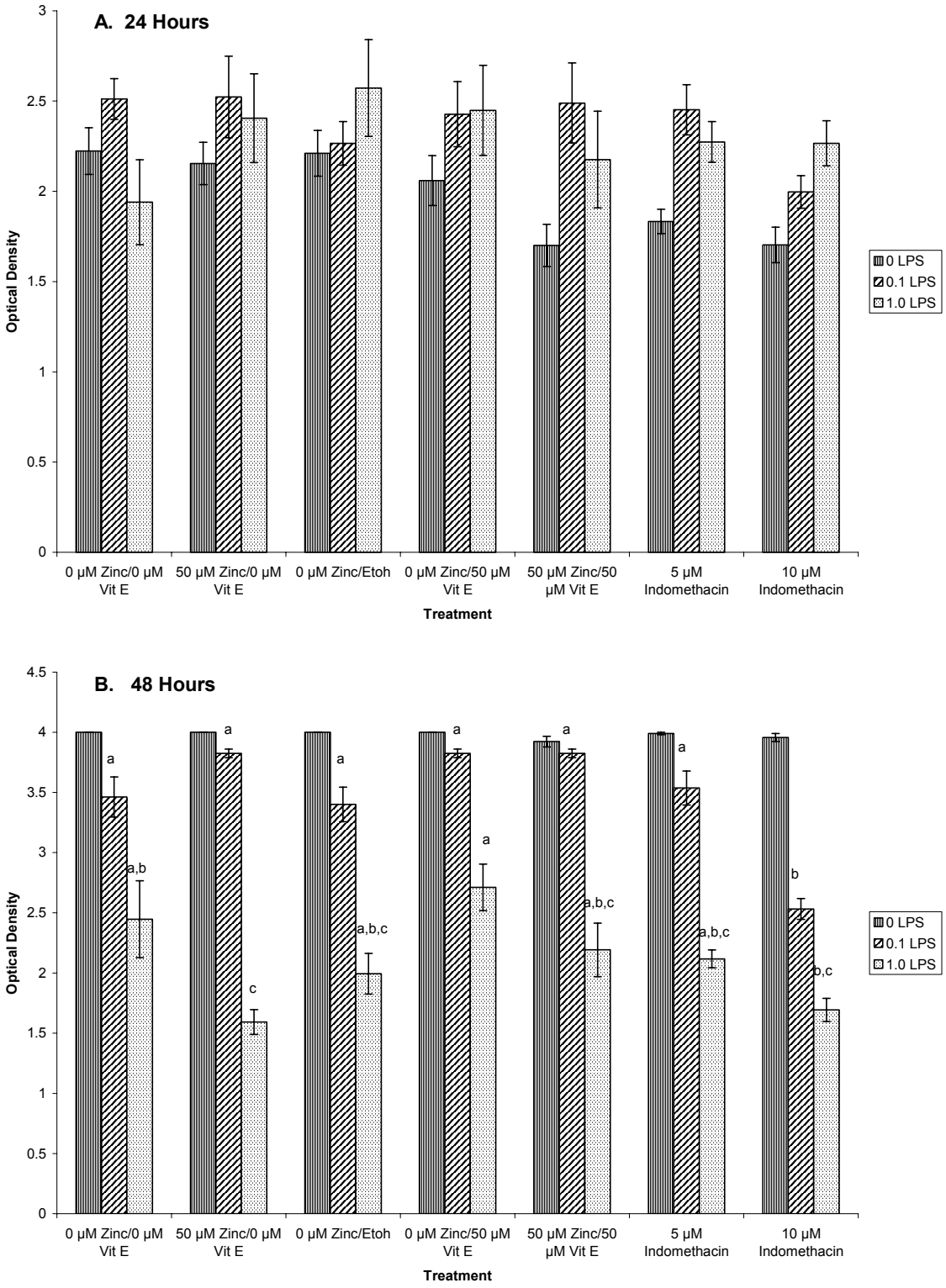

Figure 6. Growth of macrophages in vitamin E and zinc supplemented medium after 24 (A) and 48 (B) hours of challenge with LPS from *E. Coli* 055:B5. Values shown are means ± standard errors. [a,b,c] Means within each *E. Coli* 055: B5 concentration without common letters are significantly different ($p<0.05$).

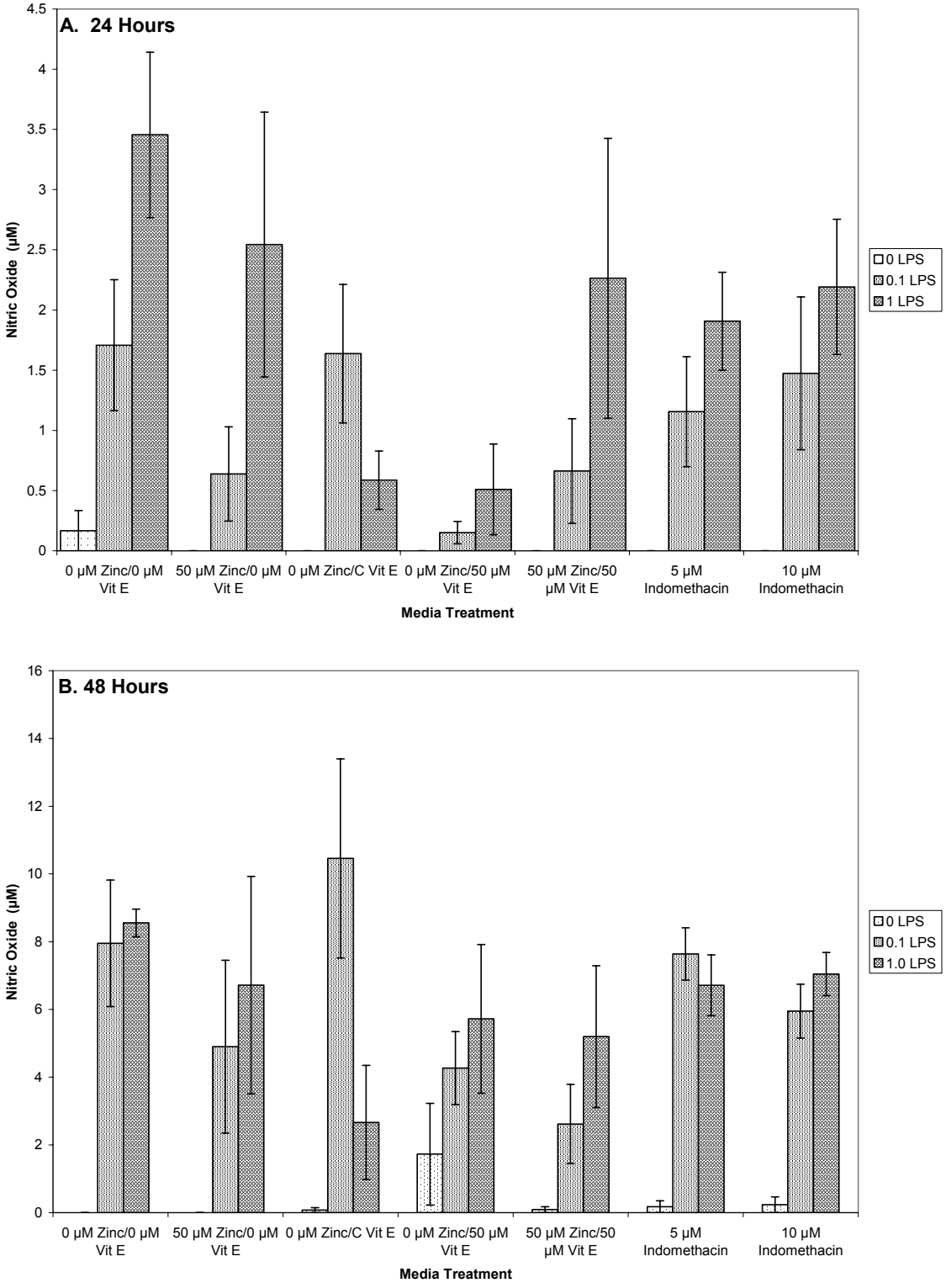

Figure 7. Nitric oxide produced by macrophages in zinc and vitamin E supplemented medium after 24 (A) and 48 (B) hours of challenge with LPS from *E. Coli* 055:B5. Values shown are means ± standard errors.

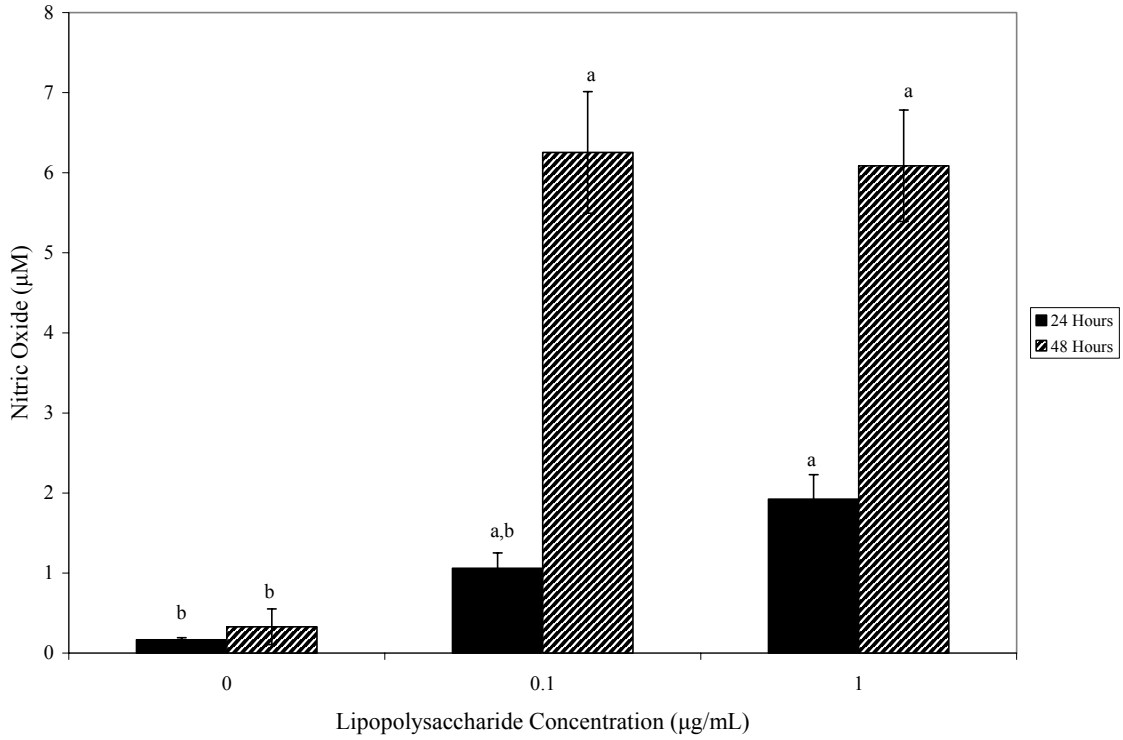

Figure 8. Nitric oxide produced by macrophages in response to challenge with LPS from *E. Coli* 055:B5. Values shown are means ± standard errors. [a,b]Means within each lipopolysaccharide concentration without common letters are significantly different ($p<0.05$).

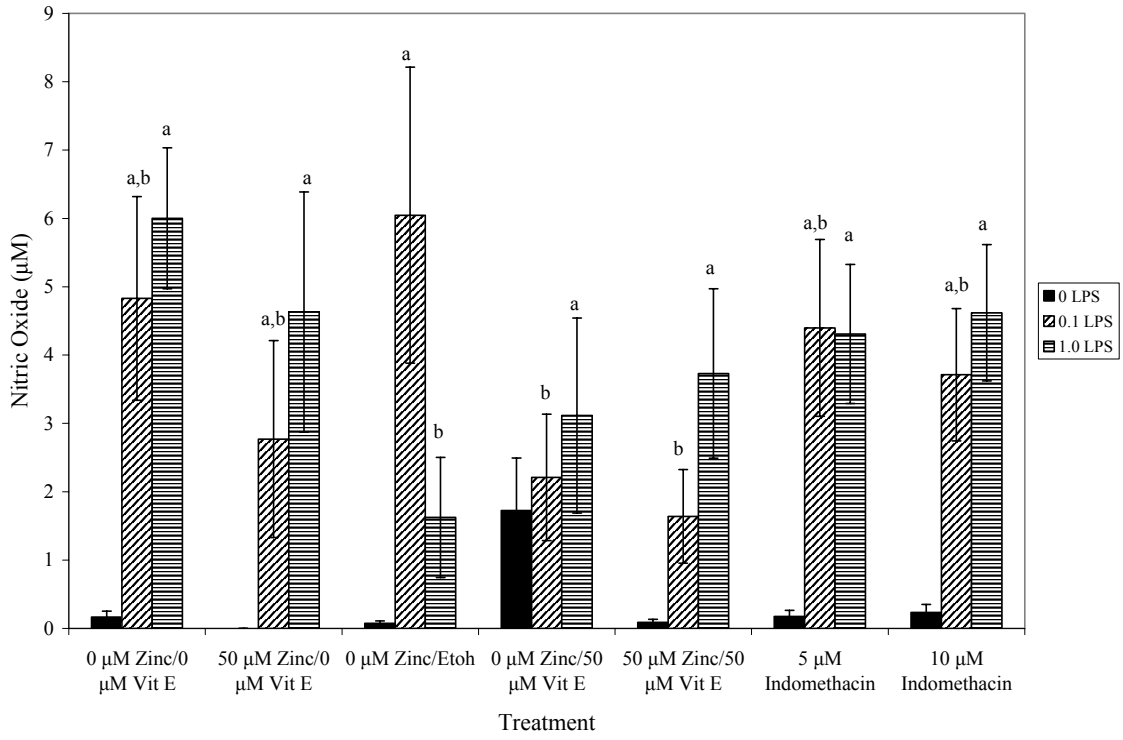

Figure 9. Nitric oxide produced by macrophages grown in zinc and vitamin E supplemented medium and challenged with LPS from *E. Coli* 055:B5. Values shown are means ± standard errors. [a,b]Means within each *E. Coli* 055:B5 concentration without common letters are significantly different ($p<0.05$).

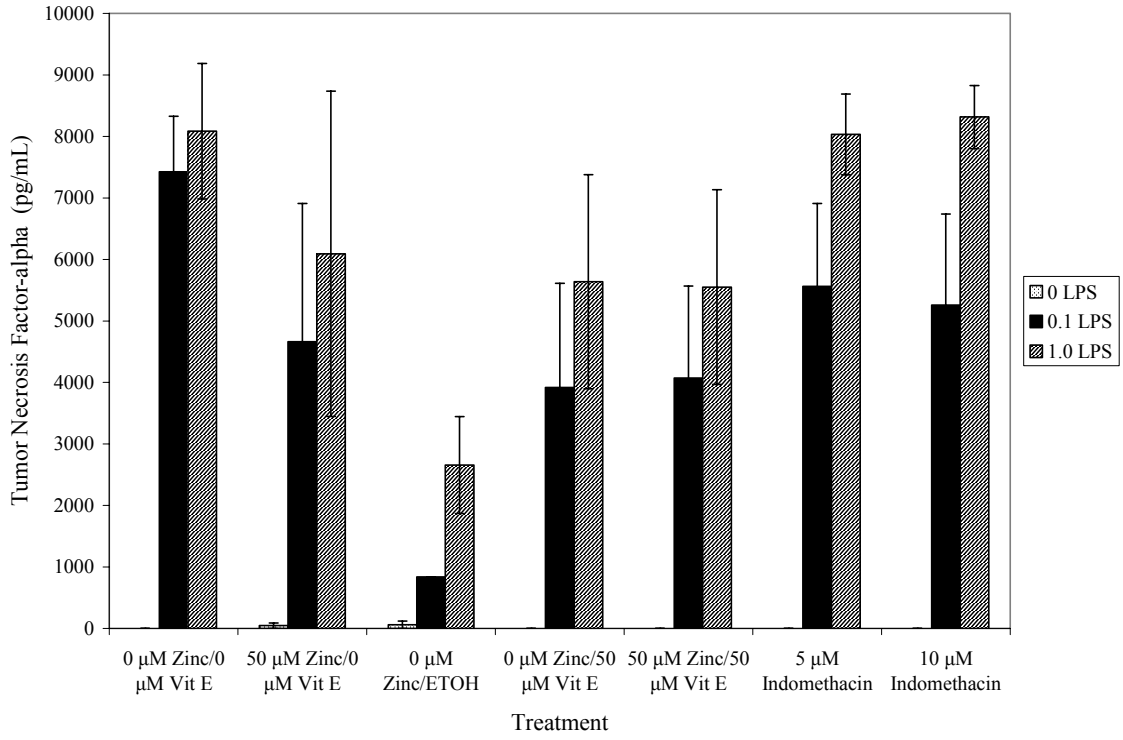

Figure 10. Tumor necrosis factor alpha (TNF-α) produced by macrophages grown in vitamin E and zinc supplemented medium after challenge with LPS from *E. Coli* 055:B5. Values shown are means ± standard errors.

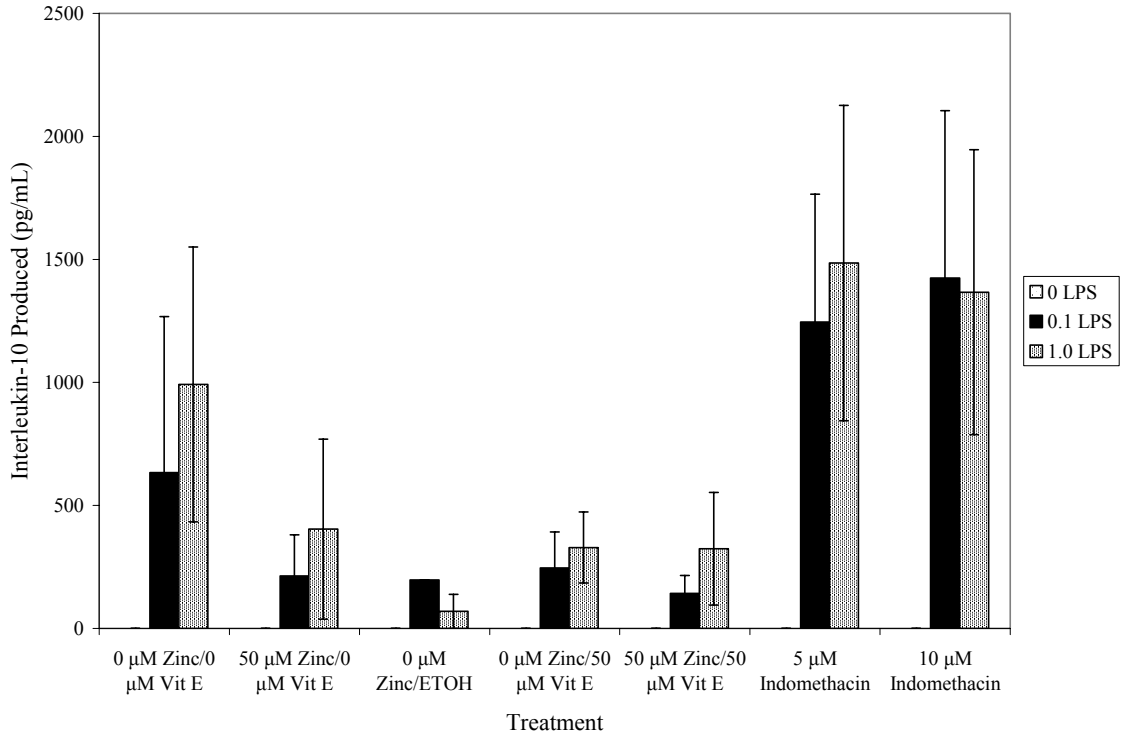

Figure 11. Interleukin 10 (IL-10) produced by macrophages grown in zinc and vitamin E supplemented medium after challenge with LPS from *E. Coli* 055:B5. Values shown are means ± standard errors.

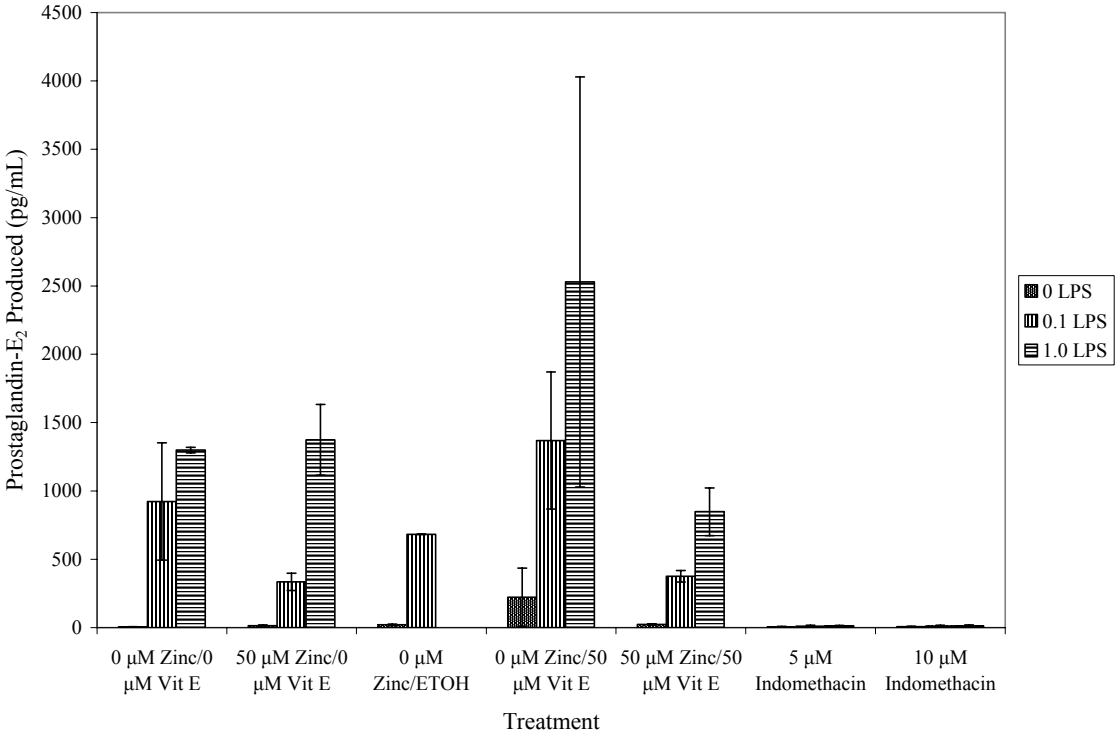

Figure 12. Prostaglandin E_2 (PGE_2) produced by macrophages in zinc and vitamin E supplemented medium after challenge with LPS from *E. Coli* 055:B5. Values shown are means ± standard errors.

CHAPTER V

DISCUSSION

Determination of the Optimal Cell Density

In these experiments, optical density (O. D.) as determined by the MTT assay was used as an indicator of cell growth. This experiment showed the greater the seeding density, the greater the growth after a certain incubation time. The highest seeding density, 5 x 10^4 cells/well had the greatest amount of growth of all the seeding densities at 24 and 48 hours; however, due to the high rate of growth, the O. D. was unable to be measured after 72 hours due to protocol limitations. The protocol used was only able to measure up to an O. D. of 4.0, which was surpassed within 72 hours. Because some of the experiments needed to be carried out to 72 hours, the high rate of growth made this seeding density undesirable for the subsequent experiments.

The 6 x 10^3 cells/well seeding density was not significantly greater than the 3 x 10^3 cells/well density until after 72 hours of growth, which may be due to the lack of needed growth factors for replication due to the small seeding density and less cell to cell contact than the other seeding densities. The seeding density of 1 x 10^4 cells/well had a greater amount of growth than 6 x 10^3 cells/well and 3 x 10^3 cells/well at 24, 48 and 72 hours. This seeding density also had a lesser growth than the 5 x 10^4 cells/well, and did not surpass the protocol limitations making it a favorable choice for the subsequent experiments.

Determination of the Optimal Zinc Supplementation

In this experiment, the optimal zinc supplementation of the cells was determined based on growth. This experiment revealed that the growth of the cells in medium supplemented with 0 μM, 10 μM, 50 μM and 100 μM zinc was greater at all time points than the cells in the 500 μM zinc supplemented medium. This suggests that at this zinc supplementation concentration, the zinc was toxic to the cells leading to the decline in growth. Over supplementation with zinc was shown by Chandra (1984) to impair chemotaxis and neutrophil activity, so it also may impair the rate of growth of the macrophages. Prasad (1978) indicated that humans given long-term zinc supplements became copper deficient, which could also be a factor in the present study. Copper deficiency could lead to a decrease seen in growth, and future studies should assess whether or not copper deficiency is being induced in these cells.

After 48 hours of growth, the cells in the 100 μM zinc supplemented medium had a lesser amount of growth than the cells in the 10 μM and 50 μM zinc supplemented medium, but a similar amount to the cells in the 0 μM zinc supplemented medium suggesting a beneficial effect of the lower zinc concentrations on cell growth; however, this effect was not seen after 24 or 72 hours.

Determination of the Optimal Vitamin E Supplementation

This experiment was performed to determine the optimal vitamin E supplementation based on the growth after 24 and 48 hours in the supplemented medium. This was used to determine what vitamin E supplementation concentrations should be used for subsequent experiments. The growth of the cells in the 0 μM, 25 μM, 50 μM and 100 μM vitamin E supplemented media was not different after 24 hours, which indicates no cytotoxic or cell enhancing effects at these vitamin E levels and this time

point. After an additional 24 hours of growth (48 hours total), growth in all the vitamin E supplementation concentrations was similar with the exception of the 50 μM vitamin E supplemented medium, which was greater than that of the others. This indicates that this level of supplementation was beneficial to the cell, and thus enhanced the growth. Sakai et al. (1997) showed that lymphocyte proliferation could be enhanced with vitamin E supplementation when stimulated with PHA and ConA, and Moriguchi (1998) showed that vitamin E supplementation led to an increase in T-cell differentiation that led to an increase in lymphocyte proliferation. Combined with these studies, the results of the present study suggest an increase in macrophage growth as a result of vitamin E supplementation. Perhaps vitamin E supplementation has a positive effect on growth of many different types of cells in the immune system since it had a positive effect on the growth of the macrophages in this study, and other studies (Moriguchi, 1998; Sakai et al., 1997) have shown its positive effects on proliferation of other types of immune cells. There was no effect from the 100 μM vitamin E supplementation in the present study though, which could suggest that the enhancement is dose dependent and that the 100 μM vitamin E concentration is too much vitamin E to show a positive effect on cell growth.

Determination of the Optimal Lipopolysaccharide Challenge

This experiment was performed not only to determine the optimal amount of lipopolysaccharide (LPS) to use in subsequent experiments, but to determine which strain of *E. Coli*, either 055:B5 or 0111:B4, to use the LPS from as well. These two factors were determined by macrophage growth and nitric oxide produced by the macrophages. With *E. Coli* 055:B5 strain, the O. D. (i.e. number of viable cells) was similar at all time points (12, 24, 48 and 72 hours after challenge) for macrophages incubated with 0, 0.1

and 1.0 μg LPS/mL media. Similar results were also seen with *E. Coli* 0111:B4; however, with this strain, the number of viable cells was not able to be determined at 0 μg LPS/mL media at 72 hours due to protocol limitations since the O. D. was greater than the maximum measurable O. D. The results also revealed that with LPS from both strains of *E. Coli*, after 48 hours of challenge, the number of viable cells in the 0, 0.1 and 1.0 μg LPS/mL media concentrations was consistently higher than with LPS concentrations greater than or equal to 5 μg LPS/mL. These results could suggest that the 0.1 and 1.0 μg LPS/mL concentrations did not have a cytotoxic effect on the cells, but the greater concentrations may have because they produced significantly lower numbers of viable cells after 48 hours of incubation. Because the decrease in the growth (as compared to the growth of the cells with 0, 0.1 and 1.0 μg LPS/mL) in 5, 10, 50 and 100 μg LPS/mL occurred after 48 hours, the decrease might by due to a lethal build-up of products released by the cells in response to the LPS challenge.

Although no nitric oxide was detected after 0 or 12 hours of challenge by either strain, it was detected after 24, 48 and 72 hours of LPS challenge. This fact could be due to either measurement techniques, or because there were not enough cells to produce enough measurable nitric oxide at the lower time points. It also could have just taken the cells that amount of time to produce nitric oxide in response to the LPS challenge.

The LPS challenge with 0.1, 1.0, 5, and 10 μg LPS/mL of *E. Coli* 055:B5 resulted in a greater amount of nitric oxide than with 0 μg LPS/mL media, which is desirable since the 0 μg LPS/mL media did not have any LPS to induce the formation of nitric oxide. These same concentrations also produced more nitric oxide than 100 μg LPS/mL media, which did not produce significantly more nitric oxide than the control.

This was also seen in the cells stimulated with LPS from the *E. Coli* 0111:B4 strain, the cells with 100 μg LPS/mL produced less nitric oxide than those with 0.1 and 1.0 μg LPS/mL. This may be linked back to the fact that less cells grew in 100 μg LPS/mL media possibly due to the cytotoxic effects of this level of LPS challenge. Because there were fewer cells to produce the nitric oxide, less nitric oxide was produced. The cells stimulated with 0.1 and 1.0 μg LPS/mL media produced the most nitric oxide, which helped to determine that those should be the levels of LPS challenge for the subsequent experiments.

The *E. Coli* 0111:B4 strain did not show any differences in nitric oxide production until after 48 hours of stimulation, while the cells with LPS from *E. Coli* 055:B5 produced more nitric oxide after 24 hours as compared to zero and 12 hours. Because there were more detectable differences after 24 hours of stimulation with the *E. Coli* 055:B5 strain, it was determined this strain should be used for the subsequent experiments.

Determination of the Optimal Combination of Vitamin E and Zinc

This experiment was done to determine the optimal combination of vitamin E and zinc supplementation to use in the subsequent LPS experiments. In this experiment, all the possible combinations of four different levels of vitamin E (0, 25, 50 and 100 μM) and four different levels of zinc (0, 10, 50, and 100 μM) were used. There were no significant differences after 24 hours of growth for any of the supplementation concentrations. However, there were differences after 48 hours suggesting that the effects of supplementation on macrophage growth took more time to develop. After 48 hours of growth, the cells in the 0 μM zinc and 50 μM vitamin E had a greater amount of

growth than the cells in the 0 μM zinc with 0 μM and 100 μM vitamin E. The cells with the 50 μM zinc and 0 μM vitamin E also had a greater amount of growth than the cells with 50 μM zinc with 25 μM and 50 μM vitamin E. Therefore, it was determined that for subsequent experiments, the levels of 50 μM zinc and 50 μM vitamin E should be used since both of these levels had been shown to have a positive effect on the growth of the macrophages.

Of the vitamin E concentrations used in the present study (0-50 μM), the concentrations 50 μM would be considered pharmacological levels since the average intake from food according to the NHANES III, was 17.44 μmol per day, and the average intake from both supplements and diet was 59.75 μmol per day (Centers for Disease Control and Prevention 1988-1994); however, since the normal physiological serum concentrations ranges from 12-42 μmol/L, this dose is much greater than the physiological concentration. The concentrations used were well below the upper intake level that ranges from 2325 μmol/day (for adults) to 465 μmol/day (for 1-3 year-olds) though (Institute of Medicine, 2000)

The highest zinc supplemented (50 μM) media used in this experiment contained 64.526 μmol/L zinc (zinc supplementation combined with levels found in the medium and FBS). This was well above the normal physiological concentrations of 10.6 to 22.78 μmol/L (Faber et al., 2004), and greater than the pharmacological serum concentration seen by Estefan et al. (1998) after subjects were given an acute supplementation of 165 mg of zinc.

Determination of the Influence of Vitamin E and Zinc on Mouse Macrophage

Response to a Lipopolysaccharide Challenge

After 24 hours of growth no significant differences in O. D. (i.e. growth) were seen among the various treatment groups. After 48 hours of growth, a variety of differences were seen, mostly due to a decreased growth with the 10 μg indomethacin. All of the treatments at a LPS challenge of 0.1 μg LPS/mL media had a greater amount of growth after 48 hours than the 10 μg indomethacin media; however, no effects on growth were seen with the 5 μg indomethacin media. Seng et al. (1990) showed that in adjuvant arthritic rats treated with indomethacin (1 or 2 mg/kg twice daily) proliferation of B lymphocytes was suppressed when stimulated with LPS; in the present study, this same effect may be being seen in the macrophages in response to the higher concentration (10 μg) of the indomethacin.

The amount of growth was also lower in the cells in the 10 μg indomethacin media at the LPS concentration of 1.0 μg LPS/mL media as compared to the 0 μM zinc/50 μM vitamin E media, revealing that either the 10 μg indomethacin treatment had a negative effect or the vitamin E supplementation treatment had a positive effect on macrophage growth. Also at this same LPS concentration, the 0 μM zinc/50 μM vitamin E media and 0 μM zinc/0 μM vitamin E media had a greater amount of growth than the cells in the 50 μM zinc/0 μM vitamin E media, which suggests this level of zinc supplementation without vitamin E did not have a protective effect on cell proliferation in response to the higher LPS challenge. The 5 μg indomethacin treatment had the same effects on growth as the vitamin E and zinc supplementations, however, the 10 μg indomethacin treatment caused a lesser amount of growth than all the treatments at 24 hours, and than 0 μM zinc/50 μM vitamin E at 48 hours. This could indicate that the

anti-inflammatory action of the zinc and vitamin E treatments is not the same as the anti-inflammatory action of indomethacin; indomethacin may be acting to decrease macrophages which release inflammatory mediators, and zinc and vitamin E acting to decrease the mediators released.

Increases in nitric oxide and many cytokines produced by macrophages have been linked to inflammatory diseases, such as atherosclerosis (Wu et al., 2001), rheumatoid arthritis (Simkin 1976), septic shock (Glauser et al., 1991), and graft-versus-host disease (Langrehr et al., 1992). Because vitamin E and zinc supplementation have separately been shown to decrease the production of nitric oxide and many of inflammatory cytokines (Prasad et al., 2004; Abou-Mohamed et al., 1998; Wu et al., 2001; Meydani et al., 2002; Wu D et al, 1998; Han et al., 2000), the present study evaluated the possible synergistic effect supplementation with these two nutrients would have.

Abou-Mohamed et al. (1998) found that zinc supplementation would decrease the amount of nitric oxide produced in response to a LPS challenge, and Wu D et al. (1998) reported the same findings for vitamin E supplementation. In the present study it was also observed that cells in the 0 μM zinc/50 μM vitamin E media combination at a LPS challenge of 0.1 μg LPS/mL media produced less nitric oxide than cells in the 0 μM vitamin E. This suggests that the 0 μM zinc/50 μM vitamin E media combination had a beneficial effect in decreasing the amount of nitric oxide produced.

The combination of 50 μM zinc/50 μM vitamin E produced less nitric oxide than the 0 μM zinc/ethanol control (0 μM vitamin E) combination at the LPS concentration of 0.1 μg LPS/mL media. Because the maximal combination of vitamin E and zinc did lower the amount of nitric oxide produced compared to the 0 μM zinc/ethanol control (0

μM vitamin E), this could suggest a beneficial effect of the use of both zinc and vitamin E supplementation. However, the maximal combination of zinc and vitamin E did not produce a significantly lower amount of nitric oxide than the 0 μM zinc/50 μM vitamin E combination, so the effect could be due to just the vitamin E supplementation. The present study was also unable to show a beneficial effect of zinc supplementation alone on decreased amounts of nitric oxide produced, as has been previously shown elsewhere (Abou-Mohamed et al., 1998) possibly due to different measurement techniques or zinc supplementation techniques.

Prasad et al. (2004) indicated that zinc supplementation could reduce the amount of tumor necrosis factor-α (TNF-α) produced in response to a LPS challenge. These results were not reproduced in the present study; there were no significant differences among any of the zinc treatments used. Han et al. (2000) had shown that vitamin E supplementation could decrease the amount of TNF-α produced in vivo, which we were also unable to reproduce in vitro. More TNF-α was produced by the cells that were stimulated with LPS, as is expected, but no differences were found in any of the media treatments possibly due to small sample size.

Another inflammatory mediator, PGE_2, has been indicated to be decreased by vitamin E supplementation (Wu D et al., 1998). The present study did not demonstrate these results possibly due to small sample size or measurement techniques, but did find that the cells in the 0 μM zinc/50 μM vitamin E media produced more PGE_2 than those in the 5 μg and 10 μg indomethacin at the 1.0 μg LPS/mL media concentration, suggesting that the indomethacin did lower the amount of PGE_2 produced, which was expected. Indomethacin acts as an anti-inflammatory medication by inhibiting COX-1 and COX-2

production (Futaki et al., 1994), which causes the decreased formation of PGE_2. It was also revealed that the cells grown in the 0 μM zinc/50 μM vitamin E media that were stimulated with 1.0 μg LPS/mL media produced more PGE_2 than the cells grown in the same media that were not stimulated with LPS, suggesting that the LPS challenge was able to cause the cells to produce more PGE_2.

Interleukin-10 (IL-10) is an anti-inflammatory agent causing the suppression of the production of inflammatory mediators such as TNF-α, IL-1β and IL-6 by monocytes (De Waal Malefyt et al., 1991). An increase in IL-10 would have a positive effect in keeping the inflammatory response in check. The present study revealed no significant differences in IL-10 produced among the vitamin E and zinc supplementation treatments, but did reveal the 5 and 10 μg indomethacin treatments produced more IL-10 than all of the medias except for the control (0 μM zinc/0 μM vitamin E supplemented media). This was also indicated by Rhind et al. (2002) who found IL-10 production to be increased by indomethacin following an intense exercise session, whereas Andreone et al. (2003) found indomethacin to decrease IL-10 production. Because IL-10 is an anti-inflammatory mediator, this decrease is not desired.

The present study demonstrated less nitric oxide was produced by the cells receiving 50 μM of both vitamin E and zinc, which suggests a beneficial effect of the supplementation with both nutrients in reducing the inflammatory response; however, vitamin E supplementation alone also revealed this effect. The addition of 5 and 10 μg indomethacin was able to cause an increase in IL-10 produced, as well as a decrease in PGE_2, in response to a LPS challenge revealing its beneficial effects on the inflammatory immune response. If a greater sample size were used or a different model, such as an *in*

vivo model, a relation among vitamin E and zinc supplementation could have possibly been shown, but with the sample sizes and the *in vitro* model used, it was unable to be shown that a vitamin E and zinc supplementation synergistic relationship decreased the inflammatory response mediators measured. Also, the cells were supplemented with zinc and vitamin E concentrations for 24 hours before receiving the LPS challenge, if the LPS challenge were given at the same time as the zinc and vitamin E treatment, a greater amount of differences may have been observed. Cellular uptake studies with zinc have shown there are two phases to zinc uptake, rapid and slow (Stacey & Klaasen, 1981; Ong et at., 1995), since the LPS was added 24 hours after the media treatments, the rapid uptake had already occurred as well as quite a bit of the slow uptake. A considerable amount of vitamin E uptake had occurred as well, even though Saito et al. (2004) showed α-tocopherol uptake had still not leveled out after 72 hours.

CHAPTER VI

SUMMARY, CONCLUSIONS, AND LIMITATIONS

The first experiments were conducted to determine the appropriate concentrations of vitamin E, zinc, lipopolysaccharide and cell density to use in the final experiment testing the main hypothesis. The main hypothesis of this study was that vitamin E and zinc supplementation would act synergistically to lower inflammatory response cytokines and nitric oxide more than vitamin E or zinc supplementation alone. The first five experiments were successful in determining which levels should be used.

For these experiments, the most appropriate cell density was 1×10^4 cells/per well in a 96 well plate. It was also determined that the most beneficial effects in growth were due to levels of 50 μM vitamin E supplementation and 50 μM or less of zinc supplementation. The optimal results based on nitric oxide production and number of viable macrophages for macrophages challenged with *E. Coli* 055:B5 or *E. Coli* 0111:B4 in this study were from those challenged with *E. Coli* 055:B5 at levels of 0.1 and 1.0 μg LPS/mL media. The data from the first five experiments helped to setup the main experiment.

It is well documented that increases in inflammatory cytokines and nitric oxide are involved in several inflammatory diseases, such as rheumatoid arthritis (Simkin, 1976), atherosclerosis (Wu et al., 2001), and many others. Prostaglandin-E_2, TNF-α, and nitric oxide have all been implicated in these diseases.

The present study indicates that vitamin E supplementation did in fact lower the amount of nitric oxide produced in response to a LPS challenge, as was shown by Wu D et al (1998), and that the supplementation of both vitamin E and zinc lowered nitric oxide but it did not lower it more than vitamin E alone. Therefore, whether or not a synergistic effect existed that decreased nitric oxide more than just supplementation with E alone was unable to be shown.

The present study also did not suggest a decrease in TNF-α due to vitamin E or zinc supplementation, or supplementation with both. The same was true for PGE_2 and IL-10 (no increase). Since other studies have shown differences in these cytokines due to vitamin E supplementation or zinc supplementation, the sample sizes in the present were possibly too small to reveal these same results, or the model used was not satisfactory to reveal these same results.

The macrophages treated with the 5 and 10 μg indomethacin produced more IL-10, an inflammatory response inhibitor, than the macrophages in the other mediums, indicating this medication's anti-inflammatory properties. These same macrophages were also able to lower the amount of PGE_2 produced, which would also suggest indomethacin's anti-inflammatory properties.

Since no significant differences were found in concentrations of pro-inflammatory mediators (TNF-α and PGE_2) in the different treatments, the hypothesis that the treatment with both vitamin E and zinc would reduce these mediators to a greater extent than vitamin E or zinc supplementation has not been proven. It was shown that the macrophages in the vitamin E and zinc supplemented media did produce less nitric oxide than the macrophages in the zinc supplemented and control treatments, but it was not

shown that they produced less than the macrophages in the vitamin E supplemented media; therefore, the hypothesis that supplementation with both vitamin E and zinc would decrease nitric oxide more than supplementation with zinc or vitamin E alone was also not proven. It was also not shown that supplementation with both vitamin E and zinc increased IL-10 production more than vitamin E or zinc supplementation alone.

The present study did not indicate a synergistic effect of vitamin E and zinc supplementation on the response of mouse macrophages to a LPS challenge. However, future studies with larger sample sizes are needed to prove whether or not vitamin E and zinc supplementation can act together to further decrease the amount of nitric oxide and inflammatory cytokines. Other inflammatory mediators, such as IL-1, IL-6, and IL-12, should also be measured in order to determine if there is a synergistic effect of vitamin E and zinc supplementation. Additional models should be used also to determine the effects of these supplementations on the whole immune system, not just the part this study considered.

Limitations

The present study had several limitations since it was an *in vitro* study. In *in vitro* studies, only an isolated cell type is studied, in this case the macrophage; however, *in vitro* studies helps researchers to better understand the action of certain cells, and help to determine if an *in vivo* study would be warranted. The macrophage is just one of many immune cells in the body; therefore, even though the present study did not indicate a synergistic effect of vitamin E and zinc, it does not mean they do not have a beneficial synergistic effect on the immune system's inflammatory response. Also, because zinc has been shown to affect the absorption of vitamin E in the body (Hatfield et al., 2002),

this model is unable to determine just how much of the supplementation would be absorbed by the body or how much would actually be absorbed by the macrophage. Other than the model, the types of cytokines measured also limited this study; if more cytokines were measured an effect could have possibly been indicated.

CHAPTER VII

BIBLIOGRAPHY

Abou-Mohamed, G., Papapetropoulos, A., Catravas, J. D. & Caldwell R. W. (1998) Zn^{2+} inhibits nitric oxide formation in response to lipopolysaccharides: Implication in its anti-inflammatory activity. Eur. J. Pharmacol. 341(2-3):265-272.

Adachi, N., Migita, M., Ohta, T., Higashi, A. & Matsuda., I. (1997) Depressed natural killer cell activity due to decreased natural killer cell population in vitamin-E deficient patient with Shwachman syndrome: reversible natural killer cell abnormality with α-tocopherol supplementation. Eur. J. Pediatr. 156:444-448.

Adrie, C., Alberti, C., Chaix-Couturier, C., Azoulay, E., de Lassence, A., Cohen, Y., Meshaka, P., Chevel, C., Thuong, M., Troche, G., Garrouste-Orgeas, M. & Timsit, J. F. (2005). Epidemiology and economic evaluation of severe sepsis in France: age, severity, infection site, and place of acquisition (community, hospital or intensive care unit) as determinants of workload and cost. J. Crit. Care 20:46-58.

Alamanos, Y. & Drosos, A. A. (2005) Epidemiology of adult rheumatoid arthritis. Autoimmunity Rev. 4:130-136.

Alissa, E. M., Bahijri, S. M., Lamb, D. J. & Ferns, G. A. A. (2004) The effects of coadministration of dietary copper and zinc supplements on artherosclerosis, antioxidant enzymes and indices of lipid peroxidation in the cholesterol-fed rabbit. Int. J. Exp. Path. 85:265-275.

American Tissue Cultures Collection (ATCC) (2001). RAW 264.7. Manassas, VA.

Andreone, P., Gramenzi, A., Loggi, E., Favarelli, L., Cursaro, C., Margotti, M., Biselli, M., Lorenzini, S. & Bernardi, M. (2003) In vitro effect of indomethacin and interferon-α on Th1 and Th2 cytokine synthesis in patients with chronic hepatitis C. Cytokine 26(3): 95-101.

Angus, D. C., Linde-Zwirble, W. T., Lidicker, J., Clermont, G., Carcillo, J. & Pinsky, M. R. (2001). Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. Crit. Care Med. 29(7):1303-1310.

Aubertin, A. (2004) Research still suggests vitamin E may boost immunity and benefit brain. Environ. Nutr. 27(5): 1-4.

Basini, G. & Tamanini, C. (2001) Interrelationship between nitric oxide and prostaglandins in bovine granulosa cells. Prostaglandins 66(3):179-202.
Beck, F. W. J., Prasad, A. S., Kaplan, J, Fitzgerald, J. T. & Brewer, G. J. (1997) Changes in cytokine production and T cell subpopulations in experimentally induced zinc-deficient humans. Am. J. Physio. 35(6):E1002-E1007.

Beharka, A. A., Han, S. N., Adolfsson, O., Wu, D., Smith, D., Lipman, R., Cao, G., Meydani, M. & Meydani, S. N. (2000) Long-term dietary antioxidant supplementation reduces production of selected inflammatory mediators by murine macrophages. Nutr. Res. 20:281-296.

Berger, A. (2002) Science commentary: What does zinc do? B.M.J. 325(7372):1062-1064.

Bettger, W. J., Reeves, P. G., Savage, J. E. & O'Dell, B. L. (1980) Interaction of zinc and vitamin E in the chick. Proc. Soc. Exp. Biol. Med. 163:432-436.

Bhaskaram, P., Jyothi, S. A., Rao, K. V. & Rao, B. S. N. (1989) Effect of subclinical vitamin A deficiency and administration of vitamin A as a single large dose on immune function in children. Nutr. Res. 9:1017-1026.

Bhutta, Z. A., Bird, S. M., Black, R. E., Brown, K. H., Gardner, J. M., Hidayat, A., Khatun, F., Martorell, R., Ninh, N. X., Penny, M. E., Rosado, J. L., Roy, S. K., Ruel, M., Sazawal, S. & Shankar, A. (2000) Therapeutic effects of oral zinc in acute and persistent diarrhea in children in developing countries: pooled analysis of randomized controlled trials. Am. J. Clin. Nutr. 72(6):1516-1522.

Bhutta, Z. A., Black, R. E., Brown, K.H., Gardner, J. M., Gore, S., Hidayat, A., Khatun, F., Martorell, R., Ninh, N. X., Penny, M. E., Rosado, J. L., Roy, S. K., Ruel, M., Sazawal, S. & Shankar, A. (1999) Prevention of diarrhea and pneumonia by zinc supplementation in children in developing countries: pooled analysis of randomized controlled trials. J. Pediatr. 135(6):689-97.

Brewer, G. J., Hill, G. M., Prasad, A. S., Cossack, Z. T. & Rabbani, P. (1983) Oral zinc therapy for Wilson's disease. Ann. Intern. Med. 99:314-320.

Brewer, G. J., Hill, G. M., Prasad, A. S. & Dick, R. D. (1987) The treatment of Wilson's disease with zinc: IV. Efficacy monitoring using urine and plasma copper. Proc. Soc. Exp. Biol. Med. 7:446-455.

Brewer, G. J., Yuzbasyian-Gurkan, V. & Dick, R. (1990) Zinc therapy of Wilson's disease:VIII. Does response studies. J. Trace. Elem. Exp. Med. 3:227-234.

Calder, P. C. & Newsholme, E. A. (1993) Influence of antioxidant vitamins on fatty acid inhibition of lymphocyte proliferation. Biochem. Mol. Biol. Int. 29:175-183.

Centers for Disease Control and Prevention (CDC). National Cemter for Health Statistics (NCHS). (1988-1994) National Health and Nutrition Examination Survey Data. (Department of Health and Human Services) Hyattsville, MD.

Chandra, R. K. (1984) Excessive intake of zinc impairs immune response. JAMA 252:1443-1446.

Chandra, R. K. (1992) Effect of vitamin and trace-element supplementation on immune responses and infection in elderly subjects. Lancet 340:1124-1127.

Choy, E. H. & Panayi, G. S. (2001) Cytokine pathways and joint inflammation in rheumatoid arthritis. N. Engl. J. Med. 344:907-916.

Chvapil, M., Stankova, L., Bernhard, D. S., Weldy, P. L., Carlson, E. C. & Campbell, J. B. (1977). Effect of zinc on peritoneal macrophages in vitro. Infection and Immunity 16(1):367-373.

De Waal Malefyt, R., Abrams, J., Bennet, B., Figdor, C. G. & De Vries, J. E. (1991) Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. J. Exp. Med. 174: 1209-1220.

Devaraj, S. & Jialal, I. (1998) α-Tocopherol decreases interleukin-1β release from activated human monocytes by inhibition of 5-lipoxygenase. Arterioscler. Thromb. Vasc. Biol. 19:1125-1133.

Driessen, C., Hirv, K., Kirchner, H. & Rink, L. (1995) Zinc regulates cytokine induction by superantigens and lipopolysaccharide. Immmunoloy 84:272-277.

ElAttar, T. M. A. & Lin, H. S. (1992) Effect of vitamin C and vitamin E on prostaglandin synthesis by fibroblasts and squamous carcinoma cells. Prostaglandins Leukot. Med. 47: 253-257.

Eskew, M. L., Scheuchenzuber, W. J., Scholz, R. W., Reddy, C. C. & Zarkower, A. (1986) The effects of ozone inhalation on the immunological response of selenium- and vitamin E-deprived rats. Environ. Res. 40:274-284.

Eskew, M. L., Zarkower, A., Scheeuchensuber, W. J., Burgess, J. R., Scholz, R. W., Hildenbrandt, G. & Channa Reddy, C. (1989) Effects of inadequate vitamin E and/or selenium nutrition on the release of arachidonic acid metabolites in rat alveolar macrophages. Prostaglandins 38: 79-89.

Estefan, V., Mendonca, B. B., Arnhold, I. J. P., Nicolau, W. & Brandao-Neto, J. (1998) Effects of acute and chronic zinc administration on growth velocity in patients with hypopituitarism. Nutr. Res. 18(11):1865-1877.

Faber, C., Gabriel, P., Ibs, K. H. & Rink, L. (2004) Zinc in pharmacological doses suppresses allogenic reaction without affecting the antigenic response. Bone Marrow Transplant. 33(12):1241-1246.

Fraker, P. J., DePasquale-Jardieu, P., Zwickl, C. M. & Luecke, R. W. (1978) Regeneration of T-cell helper function in zinc-deficient adult mice. Proc. Natl. Acad. Sci. USA 75(11):5660-5664.

Franchini, A., Bertuzzi, S., Tosarelli, C. & Manfreda, G. (1995) Vitamin E in viral inactivated vaccines. Poult. Sci. 74:666-671.

Futaki, N., Takahashi, S., Yokoyama, M., Arai, I., Higuchi, S. & Otomo, S. (1994) NS-398, a new anti-inflammatory agent, selectively inhibits prostaglandin G/H synthase/cyclooxygenase (COX-2) activity in vitro. Prostaglandins 47:55-59.

Gaede, P., Poulsen, H. E., Parving, H. H. & Pedersen, O. (2001) Double-blind, randomized study of the effect of combined treatment with vitamin C and E on albuminuria in Type 2 diabetic patients. Diabetic Med. 18:756-760.

Gale, C. R., Ashurst, H. E., Powers, H. J. & Martyn, C. N. (2001) Antioxidant vitamin status and carotid atherosclerosis in the elderly. Am. J. Clin. Nutr. 74:402-408.

Galea, J., Armstrong, J., Gadson, P., Holden, H., Francis, S. E. & Holt, C. M. (1996) Interleukin-1β in coronary arteries of patients with ischemic heart disease. Arterioscler. Thromb. Vasc. Biol. 16:100-1006.

Gao, R., Stone, W. L., Huang, T., Papas, A. M. & Qui, M. (2002) The uptake of tocopherols by RAW 264.7 macrophages. Nutr. J. 1:2.

Girodon, F., Galan, P., Monget, A., Boutron-Ruault, M., Brunet-Lecomte, P., Preziosi, P., Arnaud, J., Manuguerra, J. & Hercberg S. (1999) Impact of trace elements and vitamin supplementation on immunity and infections in institutionalized elderly patients. Arch. Intern. Med. 159:748-754.

Glauser, M. P., Zanetti, G., Baumgartner, J. D. & Cohen, J. (1991) Septic shock: Pathogenesis. Lancet 338: 732-736.

Goldenberg, R. I., Tamura, T., Neggers, Y., Copper, R. L., Johnston, K. E., DuBard, M.B. & Hanth, J.C. (1995) The effect of zinc supplementation on pregnancy outcome. JAMA 274: 463-468.

Goodwin, J. S. & Ceuppens, J. (1982) Regulations of the immune response by prostaglandins. J. Exp. Med. 155:943-948.

Groff, J.L. & Gropper, S. S. (1999) Advanced Nutrition and Human Metabolism 3rd Edition. Belmont, California, USA.

Hambidge, M. (2000) Human zinc deficiency. J. Nutr. 130:1344S-1349S.

Han, S. N., Wu, D., Ha, W. K., Beharka, A., Smith, D. E., Bender, B. S. & Meydani, S. N. (2000) Vitamin E supplementation increases T helper 1 cytokine production in old mice infected with influenza virus. Immunology 100:487-493.

Harris, R. E., Boxer, L. A. & Baehner, R. L. (1980) Consequences of vitamin-E deficiency on the phagocytic and oxidative functions of the rat polymorphonuclear leukocyte. Blood 55:338-343.

Hatfield, P. G., Robinson, B. L., Minikhiem, D. L., Kott, R. W., Roth, N. I., Daniels, J. T. & Swenson, C. K. (2002) Serum α-tocopherol and immune function in yearling ewes supplemented with zinc and vitamin E. J. Anim. Sci. 80:1329-1334.

Herbaczynska-Cedro, K., Wartanowicz, M., Panczenko- Kresowska, B., Cedro, K., Klosiewicz-Wasek, B. & Wasek, W. (1994) Inhibitory effect of vitamins C and E on the oxygen free radical production in human polymorphonuclear leucocytes. Eur. J. Clin. Invest. 24: 316-319.

Heuser, G. & Vojdani, A. (1997) Enhancement of natural killer cell activity and T and B cel function by buffered vitamin C in patients exposed to toxic chemicals: the role of protein kinase-C. Immunopharmacol. Immunotoxicol. 19: 291-312.

Hill, G. M., Brewer, G. J., Prasad, A. S., Hydrick, C. R. & Hartman, D. E. (1987) Treatment of Wilson's disease with zinc: I. Oral zinc therapy regimens. Hepatology 7:522-528.

Hurley, L. S. & Swenerton, H. (1966) Congenital malformations resulting from zinc deficiency in rats. Proc. Soc. Exp. Biol. Med. 123: 692-696.

Institute of Medicine (IOM) (2000) Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc. National Academies Press.

Institute of Medicine (IOM) (2000) Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium, and Carotenoids. National Academies Press.

Jameson, S. (1982) Zinc status and pregnancy outcome in humans. In Clinical applications of recent advances in zinc metabolism (Prasad, A.S., Dreosti, I. E., & Hetzel, B. S. eds), pp: 39-52. New York.

Jampol, L. M. & Ferris III, F. L. (2001) Antioxidants and zinc to prevent progression of age-related macular degeneration. Arch. Ophthalmol. 119:1417-1436.

Janeway, C. A., Travers, P., Walport, M. & Shlomchik, M. (2001) Immunobiology. Garland Publishing, New York, NY.

Jensen, M., Fossum, C., Ederoth, M. & Hakkarainen, R. V. (1988) The effect of vitamin E on the cell-mediated immune response in pigs. J. Vet. Med. 35:549-555.

Jiang, Q., Elson-Schwab, I., Courtemanche, C. & Ames, B. N. (2000) γ-Tocopherol and its major metabolite, in contrast to α-tocopherol, inhibit cyclooxygenase activity in macrophages and epithelial cells. Proc. Natl. Acad. Sci. USA 97(21):11494-11499.

Khanduja, K. L., Avti, P. K., Kumar, S., Pathania, V. & Pathak, C. M. (2005) Inhibitory effect of vitamin E on proinflammatory cytokines-and endotoxin-induced nitric oxide release in alveolar macrophages. Life Sci. 76(23): 2669-2680.

Kim, E., Noh, S. K. & Koo, S. I. (1998) Marginal zinc deficiency lowers the lymphatic absorption of α-tocopherol in rats. J. Nutr. 1289:265-270.

King, L. E. & Fraker, P. J. (2002) Zinc deficiency in mice alters myelopoiesis and hematopoieses. J. Nutr. 132:3301-3307.

Klurfield, D. M. (1993) Vitamin E and human immune functions. Nutrition and Immunology 8th Edition. pp 217-228.

Kraus, A., Roth, H. P. & Kirchgessner, M. (1997) Supplementation with vitamin C, vitamin E or beta-carotene influences osmotic fragility and oxidative damage of erythrocytes of zinc-deficient rats. J. Nutr. 127(7):1290-1296.

Kubena, K. S. (1993) The role of magnesium in immunity. J. Nutr. Immunol. 2:107-126.

Kubena, K. S. & McMurray, D. N. (1996) Nutrition and the immune system: a review of nutrient-nutrient interactions. J. Amer. Diet. Assoc. 96(11): 1156-1164.

Langrehr, J. M., Murase, N., Markus, P. M., Cai, X., Neuhaus, P., Schraut, W., Simmons, R. L. & Hofman, R.A. (1992) Nitric oxide production in graft-versus-host reactions in the rat. J. Clin. Invest. 90:679-683.

Lastra, M. D., Pastelin, R., Camacho, A., Monroy, B., & Aguilar, A. E. (2001) Zinc intervention on macrophages and lymphocytes response. J. Trace Elem. Biol. 15:5-10.

Li, W. & Farach-Carson, M. C. (2001) Parathyroid hormone-stimulated resorption in Calvaria cultured in serum-free medium is enhanced by the calcium-mobilizing activity of 1,225-Dihydroxyvitamin D_3. Bone 29(3):231-235.

Lim, Y., Levy, M. & Bray, T. M. (2004) Dietary zinc alters early inflammatory responses during cutaneous wound healing in weanling CD-1 mice. J. Nutr. 134:811-816.

McClung, J. P. & Bobilya, D. J. (1999) The influence of zinc status on the kinetics of zinc uptake into cultured endothelial cells. J. Nutr. Biochem. 10:484-489.

McCormick, C. C. & Parker, R. S. (2004) The cytotoxicity of vitamin E is both vitamer- and cell- specific and involves a selectable trait. J. Nutr. 134:3335-3342.

Meydani, S. N. & Han, S. N. (2002) Nutrient regulation of the immune response: The case of vitamin E. In: Present Knowledge in Nutrition (eds Bowman, B. & Russel, R. M., eds.), pp. 449-462. Washington, D. C., USA.

Moriguchi, S. (1998) The role of vitamin E in T-cell differentiation and the decrease of cellular immunity with aging. Biofactors 7(1/2): 77-87.

Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J. Immunol. Methods 65:55-63.

Nagyova, I., Stewart, R. E., Macejova, Z., van Dijk, J. P. & van den Heuvel, W. J. A. (2005) The impact of pain on psychological well-being in rheumatoid arthritis: the mediating effects of self-esteem and adjustment to disease. Patient Educ. & Counseling 58(1):55-62.

Ninh, N. X., Thissen, J. P., Collette, L., Gerard, G., Khoi, H. H. & Ketelslegers, J. M. (1996) Zinc supplementation increases growth and circulating insulin-like growth factor I (IGF-I) in growth-retarded Vietnamese children. Am. J. Clin. Nutr. 63: 514-519.

Ninh, N. X., Thissen, J. P., Mailer, D., Adam, E., Mulumba N. & Ketelslegers, J.M. (1995) Reduced liver insulin like growth factor-I gene expression in young zinc-deprived rats is associated with a decrease in liver growth hormone (GH) receptors and serum GH binding protein. J. Endocrinology 144: 449-456.

Noh, S. K. & Koo, S. I. (2001) Feeding of a low-zinc diet lowers the tissue concentrations of α-tocopherol in adult rats. Bio. Trace Elem. Res. 81:153-167.

Olson, J. A. (1986) Physiological and metabolic basis of major signs of vitamin A deficiency. In: Vitamin A deficiency and its control (Bauernfeind JC), pp. 19-67. Academic Press, New York, NY.

Ong, T. J., Kemp, P. J., Olver, R. E. & McArdle, H. J. (1995) Characterization of zinc uptake and its regulation by arachidonic acid in fetal type II pneumocytes. Am. J. Physio. 269:L71-L77.

Ortega, R. M., Requejo, A. M., Lopez-Sobaler, A. M., Andres, P., Navia, B., Perea, J.M.& Robles, F. (2002) Cognitive function in elderly people influenced by vitamin E status. J. Nutr.132(7): 2065-2068.

Ozaki, Y., Ohashi, T. & Kume, S. (1987) Potentiation of neutrophil function by recombinant DNA-produced interleukin-1α. J. Leukocyte Biol. 42: 621-627.

Pallast, E. G., Schouten, E. G., deWaart, F. G, Hannie, C. F., Doekes, G., von Blomberg, B. M. & Kok, F. J. Effect of 50- and 100-mg vitamin E supplements on cellular immune function in noninstitutionalized elderly persons. Am. J. Clin. Nutr. 69:1273-1281.

Parkin, J. & Cohen, B. (2001) An overview of the immune system. Lancet 357:1777-1789.

Pathania, V., Syal, N., Pathak, C. M. & Khanduja, K. L. (1999) Vitamin E suppresses the induction of reactive oxygen species release by lipopolysaccharide, interleukin-1β and tumor necrosis factor-α in rat alveolar macrophages. J. Nutr. Sci. Vitaminol. 45:675-686.

Phagocytic index [Online]. http://infoplease.com [Accessed June 25, 2005].

Percival, S. S. (1995) Neutropenia caused by copper deficiency: possible mechanisms of action. Nutr. Rev. 53: 59-66.

Portillo, G., Turner, M., Chantry, D. & Feldmann, M. (1989) Effect of cytokines on HLA-DR and IL-1 production by a monocytic tumour, THP-1. Immunology 66:170-175.

Prasad, A. S., Bao, B., Beck, F. W. J., Kucuk, O. & Sarkar, F. H. (2004) Antioxidant effect of zinc in humans. Free Rad. Biol. & Med. 37(8): 1182-1190.

Prasad, A. S., Brewer, G. J., Schoomaker, E. B. & Rabbani, P. (1978) Hypocupremia induced by zinc therapy in adults. JAMA 240:2166-2168.

Prasad, A., Halsted, J. A. & Nadimi M. (1961) Syndrome of iron deficiency anemia, hepatosplenomegaly, hypogonadism, dwarfism, and geophagia. Am. J. Med. 31: 532-546.

Prasad, A. S., Meftah, S., Abdaliah, J., Kaplan, J., Brewer, G. J. & Bach, J. F. (1988) Serum thymulin in human zinc deficiency. J. Clin. Invest. 82: 1202-1210.

Prasad, A.S. (1998) Zinc in human health: An update. J. Trace Elem. in Exper. Med. 11: 63-87.

Prasad, A. S. (1996) Zinc: The biology and therapeutics of an ion. Ann. Intern. Med. 125(2):142-144.

Prema, K. Ramalakshmi, B. A., Madhavapeddi, R. & Babu, S. (1982) Immune status of anaemic pregnant women. Br. J. Obstet. Gynae. 89(3): 222-225.

Reitsma, J. B., Dalstra, A. A. A., Bonsel, G. J., Van der Muelen, J. H. P., Koster, R. W., Gunning-Schepers, L. J. & Tijssen, J. G. P. (1999) Cardiovascular disease in the Netherlands, 1975-1995: Decline in mortality, but increasing numbers of patients with chronic conditions. Heart 82(1): 52-56.

Rhind, S. G., Gannon, G. A., Shepard, R. J. & Shek, P. N. (2002) Indomethacin modulates circulating cytokine responses to strenuous exercise in humans. Cytokine 19(3):153-158.

Rink, L., & Gabriel, P. (2000) Zinc and the immune system. Proc. Nutr. Soc. 59:541-552.

Roitt, I. (1991) Essential Immunology, 7th edition. Oxford, England.

Saito, Y., Yoshida, Y., Nishio, K., Hayakwa, M. & Niki, E. (2004) Characterization of cellular uptake and distribution of vitamin E. Ann. N. Y. Acad. Sci. 1031:368-375.

Sakai, S. & Moriguchi, S. (1997) Long-term feeding of high vitamin E diet improves the decreased mitogen response of rat splenic lymphocytes with aging. J. Nutr. Sci. Vitaminol. 43:113-122.

Seng, G. F., Benenshon, J. & Bayer, B. M. (1990) Changes in T and B lymphocyte proliferative responses in adjuvant-arthritic rats: antagonism by indomethacin. Eur. J. Pharmacol. 178(3):267-273.

Shankar, A. H. & Prasad, A. S. (1998) Zinc and immune function: the biological basis of altered resistance to infection. Am. J. Clin. Nutr. 68(2S):447S-463S.

Sherwood, L. (1997) Human Physiology: From Cells to Systems. Wadsworth Publishing, Belmont, CA.

Simkin, P. A. (1976) Oral zinc sulphate in rheumatoid arthritis. Lancet pp. 539-542.

Sompayrac, L. (1999) How the Immune System Works. Blackwell Science, Malden, MA.

Stacey, N. H. & Klaasen, C. D. (1981) Zinc uptake by isolated rat hepatocytes. Biochem. Biophys. Acta 640:693-697.

Sword, J. T., Pope, A. L. & Hoekstra, W. G. (1991) Endotoxin and lipid peroxidation in vivo in selenium- and vitamin E-deficient and –adequate rats. J. Nutr. 121(2):251-257.

Tiami, H., Chatau, M. T., Cabanne, S. & Marti, J. (1991) Synergistic effect of retinoic acid and 1,25 dihydroxyvitamin D_3 on the differentiation of the human monocyte cell line U937. Leuk. Res. 15: 1145-1152.

Visioli, F., Marangoni, F., Moi, D., Rise, P. & Galli, C. (2000) In vitro differentiation of human monocytes to macrophages results in depletion of antioxidants and increase in *n*-3 fatty acid levels. FEBS Letters 471:75-77.

Wada, L. & King, J. (1986) Effect of low zinc intakes on basal metabolic rate, thyroid hormones and protein utilization in adult men. J. Nutr. 116:1045-1053.

Wellinghausen, N., Schroom, A. B., Seydel, U., Brandenberg, K., Luhm, J., Kirchner, H. & Rink, L. (1996) Zinc enhances lipopolysaccharide-induced monokine secretion by alteration of fluidity state lipopolysaccharide. J. Immunol. 157(7):3139-3145.

Whiteshell, R., Reyen, D., Beth, A., Pelletier, D. & Abumrad, N. (1989) Activation energy of slowest step in the glucose carrier cycle: correlation with membrane lipid fluidity. Biochemistry 28:5618-5625.

Witztum, J. L. & Steinberg, D. (1991) Role of oxidized low density lipoprotein in atherogenesis. J. Clin. Invest. 88: 1785-1792.

Wolfe, F., Hawley, D. J. & Cathey, M. A. (1991) Clinical and health status measures over time: prognosis and outcome assessment in rheumatoid arthritis. J.Rheumatology 18(9): 1290-1297.

Wu, D., Hayek, M. G. & Meydani, S. N. (2001) Vitamin E and macrophage cyclooxygenase regulation in the aged. J. Nutr. 131:382S-388S.

Wu, D., Mura, C., Beharka, A. A., Han, S. N., Paulson, K. E., Hwang, D. & Meydani, S. N. (1998) Age-associated increase in PGE_2 synthesis and COX activity in murine macrophages is reversed by vitamin E. Am. J. Physiol. 275:C661-C668.

Yamaguchi, M. & Inamoto, K. (1986) Differential effect of calcium regulating hormones on bone metabolism in weanling rats orally administered zinc sulfate. Metabolism 35: 1044-1047.

Yelin, E. & Wanke, L. A. (1999) An assessment of the annual and long-term direct costs of rheumatoid arthritis: the impact of poor function and functional decline. Arthritis Rheum. 42(6):1209-18.

Yuzbasiyan-Gurkan, V., Grider, A., Nostrant, T., Cousins, R. J. & Brewer, G. J. (1992) The treatment of Wilson's disease with zinc: X. Concurrent induction of intestinal metallothionein and suppression of copper absorption by zinc therapy. J. Lab. Clin. Med. 120: 380-386.

Zago, M. P. & Oteiza, P. I. (2001) The antioxidant properties of zinc: Interactions with iron and antioxidants. Free Radical Biol. & Med. 31(2):266-274.

VITA

Mary Rachel Bell

Candidate for the Degree of

Master of Science

Thesis: THE INFLUENCE OF SUPPLEMENTAL VITAMIN E AND ZINC ON THE RESPONSE OF MOUSE MACROPHAGES TO LIPOPOLYSACCHARIDE

Major Field: Nutritional Sciences

Biographical:

Personal Data: Born in Jefferson City, Missouri, on February 1, 1979, the daughter of Donald and Mary Ann Bell, wife of Michael Bryant and mother of Kira Bryant.

Education: Graduated from Morris High School, Morris, Oklahoma in May 1997; received Bachelor of Science degree in Human Nutrition-Dietetics from Oklahoma State University in August 2001. Completed the requirements for the Master of Science Degree with a major in Nutritional Sciences at Oklahoma State University in July, 2005.

Experience: Employed by Oklahoma State University, Department of Nutritional Sciences Laboratory as an undergraduate, 1998-2001 and as a graduate assistant, 2001-2003.