DIETARY ZINC AND CHROMIUM SUPPLEMENTATION AND EFFECT OF ENDOTOXIN ON THE IMMUNE RESPONSE OF WEANLING PIGS

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LIST OF ABBREVIATIONS

BUN	blood urea nitrogen
Cr	chromium
$CrCl_3$	chromium chloride
CrPic	chromium picolinate
CRP	C-reactive protein
IGF-I	insulin-like growth factor I
i.p.	intraperitoneal
i.v.	intravenous
i.m.	intramuscular
IL-1	interleukin 1
IL-6	interleukin 6
LPS	lipopolysacharide
MT	metalotheinine
PGE_2	prostaglandin E_2
SAS	Statistical Analysis System
TNF-α	tumour necrosis factor alpha
Zn	zinc
$ZnSO_4$	zinc sulfate

CHAPTER I

Introduction

Infection often occurring with malnutrition is major cause of morbidity in all age groups and is responsible for two thirds of all deaths under age 5 years in developing countries (Chandra 1990). The morbidity and mortality rates due to diarrheal disease are particularly severe in young children. In 1993 there were 12 million deaths of infants and children less than 5 years of age, of which one fourth were related to diarrhea (Wapnir 2000). The vast majority occurred in developing countries (Wapnir 2000). Sub-clinical and clinical infections might result in undernutrition and cause growth stunting and a high prevalence of micronutrient deficiencies in children of developing countries (Chandra 1990). The effects of infection on health is also a concern in animal nutrition. Reduced feed intake, and decreased daily gain (Johnson and von Borell 1994, van Heugten et al. 1996), decreased feed efficiency (Johnson and von Borell 1994), and inactivity are behavioral states observed in pigs with an acute bacterial or viral infections leading to an elevation of prophylactic medication, vaccination costs and animal deaths (Normatsu et al. 1995). Chronic infection has recently been attracting more attention than acute epidemic infections in both human and animal nutrition.

The importance of zinc in animal nutrition was first demonstrated by Todd and coworkers (Todd et al. 1934). Since then the importance of zinc in various animal species has been confirmed. Zinc deficiency in humans was first reported by (Prasad et al. 1961). Subsequent studies done in Iran provided data which indicated a beneficial effect of zinc

on growth and sexual development (Sandstead et al. 1967). Zinc deficiency was observed more commonly in males than in females (Abbasi et al. 1980). A manipulation of dietary zinc can produce a measurable zinc deficiency in humans (Rabbani et al. 1987). Zinc deficiency was found to decrease the T helper cell function (Fraker et al. 1977) due to decreased thymulin production with zinc deficiency (Dardenne et al. 1982, Prasad et al. 1988, Prasad 2000). During periods of stress plasma zinc concentrations decrease (Butler and Curtis 1973, McClain et al. 1997), and zinc is redistributed or sequestered in the liver (Butler and Curtis 1973). Increased urinary excretion of zinc was also observed with stress and could lead to increases in nutritional needs during infection. Further, zinc is an important nutritional modulator of immune status; both pharmacological doses (Chandra 1984) and dietary deficiencies (Arthington et al. 1997) can impair the immune system.

Chromium is an important trace element required for carbohydrate and lipid metabolism in animals as well as in humans. Hopkins et al (Hopkins et al. 1968) showed improvement in glucose tolerance in children with protein-calorie malnutrition with the introduction of chromium. Gurson and Saner (Gurson and Saner 1971) supported the findings. Chromium deficiency also can result in impaired glucose tolerance (Mertz W 1993). Stress was found to decrease plasma chromium (Pekarek et al. 1975) and to increase urinary chromium excretion (Borel et al. 1984). Dietary supplementation of chromium during periods of stress was found to decrease cortisol and body temperature (Lee et al. 2000). The changes in chromium balance associated with stress may lead to increases in nutritional needs for this trace mineral during periods of stress.

Disease stress causes profound metabolic changes in the host characterized by shifts in the efficiency of nutrient use for growth and increased catabolism of tissues and

stored nutrients (Elsasser et al. 1995). The acute phase response is a primary host defense mechanism in response to microbial infection (Exton et al. 1995) characterized by elevated temperatures (Elsasser et al. 1995;Wright et al. 2000), endocrine reactions, altered immune cell function (Exton et al. 1995), and release of cytokines especially TNF-α (Elsasser et al. 1996). The production of cytokines to some extent are modulated by dietary zinc (Rink and Kirchner 2000).

Hence, the present study was designed to determine the effects of intraperitoneal administration of lipopolysaccharide (LPS) (Escherichia Coli 0111:B4) on the immune response of weanling pigs fed diets supplemented with different dietary concentrations of zinc or chromium. Due to the structural similarities in the gastrointestinal system of the pig to that of humans (Dodds 1982) weanling pigs were selected as an experimental model. Infection was simulated in the weanling pigs by intraperitoneal (i.p.) injection of LPS E.coli 0111:B4.

Objectives

The following research objectives were developed for the proposed studies:

1) to determine the minimum dose of LPS E.Coli 0111:B4 required to elicit an acute phase response;

2) to determine the effects of endotoxin on the absorption of ^{51}chromium from the gut;

3) to determine the effects of endotoxin, dietary zinc and endotoxin/dietary zinc interactions on the immune response; and

4) to determine the effects of endotoxin, dietary chromium, and endotoxin/dietary chromium interactions on the immune response.

Hypotheses

The following hypotheses were developed for the study:

1. there will be no statistically significant differences between the low dose and high doses of endotoxin on measures of immune response;
2. there will be no statistically significant effect of endotoxin on the absorption of ^{51}chromium ($^{51}CrCl_3$);
3. there will be no statistically significant effect of dietary zinc in protection against LPS induced stress in weanling pigs; and
4. There will be no statistically significant effect of dietary chromium in protection against LPS induced stress in weanling pigs.

Assumptions

1. We assumed that intraperitoneal injection of LPS E.coli 0111:B4 would elicit the immune response.
2. We assumed that the calculated concentrations of the dietary chromium and zinc will be equal to the analyzed concentrations of the minerals in the diet.
3. The animals on each experimental diets will receive the same amount of trace minerals in question.

Limitations

Because of the animal model in this research investigation, the results cannot be extrapolated directly to human infants.

Format of the Dissertation

The four experiments included in the dissertation (chapters IV through VII) are organized as individual manuscripts. Chapter IV was written using the Guide for Authors

from the American Journal of Physiology. Chapter V was written using the Guide for Authors from the British Journal of Nutrition. Chapters VI and VII were written using the Guide for Authors from the Journal of Nutrition.

CHAPTER II

Literature Review

This chapter includes three major sections on zinc, chromium, and endotoxin. A brief review of importance of zinc in human and animal nutrition in regards to growth and immune function are reviewed. The effects of pharmacological doses of zinc supplementation on performance on weanling pigs will be summarized. A review of studies conducted on the effects of chromium on growth, immune and clinical parameters in farm animals will be discussed. The third section will focus on the studies conducted on the effects of endotoxin on acute phase response in different animal models with special emphasis on swine nutrition.

Zinc

Role of Zinc in Animal and Human Nutrition: A Brief History

Animal Nutrition:

Todd and coworkers in 1934 demonstrated the essentiality of zinc in animal nutrition using the rat as an experimental model. Loss of hair was observed when rats were fed diets containing only 1.6 mg zinc/kg diet as zinc oxide. By the 17th week of the experimental period the animals showed the signs of deficiency. Addition of 5 mg zinc/100 g diet improved hair growth and normal fur development in rats (Todd et al. 1934). Since then, the importance of zinc and its essentiality for various animal species has been confirmed.

In 1955, Tucker and Salmon (Tucker and Salmon 1955) observed severe dermatitis in pigs fed a diet containing vegetable protein supplements such as peanut

meal, cottonseed meal and soybean meal. The symptoms of diarrhea, vomiting, anorexia, weight loss were observed. The symptom of dermatitis was cured by alfalfa pasture suggesting the cause of the dermatitis was of nutritional origin. In the same experiment they (Tucker and Salmon 1955) supplemented the basal diets of 6 to 8 weeks old weanling pigs with 0.02% or 0.01% zinc carbonate as part of mineral mix. The pigs were fed for 7 weeks. The animals supplemented with 0.02% or 0.01% zinc carbonate were normal in appearance, and the weight gain was higher than the non-supplemented animals. Also, all the pigs in the group receiving only the basal diet developed dermatitis by the termination of the experiment. The reported zinc content of the basal diet was 34 ppm. The authors concluded that the parakeratosis observed in pigs was due to a nutritional zinc deficiency disorder, and the deficiency symptoms were observed when the pigs were fed a ration containing 34 to 44 ppm of dietary zinc.

Human Studies:

Although the essentiality of zinc for animals was established, zinc deficiency in humans was not considered a problem because of its wide distribution in the human diet. However, the first evidence of zinc deficiency in humans was observed in the Middle East and was reported by Prasad and coworkers (Prasad et al. 1961). A 20 year old, 29.5 kg body weight, 135 cm, patient reported palpitation, fatigability and weakness since he was 11 years. The patient's dietary history revealed only bread from wheat flour with negligible animal protein in the diet. Hemoglobin, hematocrit, and mean cell volume were all low with enlarged liver and spleen. Two weeks after admission oral iron therapy (Ferrosan, one tablet, three times a day) was started. After 10 weeks of hospitalization the patient gained 10 pounds, hemoglobin, hematocrit, and red blood cell count all

increased compared to the pretreatment values. The correction of anemia resulted in decreased liver and spleen size. A follow up visit after 8 months revealed that the patient was eating a well balanced diet with adequate meat, bread, rice and eggs daily. The liver and spleen size decreased, and the presence of pubic hair was observed. The anemia was corrected with iron supplementation. Zinc deficiency was not confirmed in this study. Severe growth retardation, anemia along with hepatosplenomegaly, hypogonadism, rough and dry skin, and geophagia were identified as due to zinc deficiency. The authors suggested that the dietary phytates and phosphates form insoluble complexes that may have decreased the availability of dietary zinc and iron.

Subsequent controlled studies done in Iran provided data which indicated a beneficial effect of zinc on growth and sexual development. They reported the existence of zinc deficiency observed in subjects consuming diets containing only cereal protiens (Sandstead et al. 1967) Sandstead and coworkers conducted a study on 22 nutritional dwarfs during 1961-1963 in Shiraz, Iran. The subjects were supplemented either with oral zinc (30 mg as zinc sulfate), iron (300 mg as ferrous sulfate) or adequate animal protein alone. The supplements were given orally three times a day. Growth was greater in zinc supplemented subjects. Pubic hair appeared in all subjects within 7-12 weeks after zinc supplementation started, genitalia increased to normal size and secondary sexual characteristics developed within 12 to 24 weeks of supplementation with zinc. No such changes were observed in the group supplemented with iron or in the adequate animal protein supplemented group. Anemia due to iron deficiency responded to iron supplementation. Zinc deficiency was reported to be the cause for hypogonadism and growth retardation in these subjects (Sandstead et al. 1967).

In 1977 Halstead and coworkers (Halstead et al. 1977) reported zinc deficiency in two female subjects. In their study 15 males and 2 females were studied for 6-12 months. One group was given a well balanced diet with ample animal protein plus a placebo capsule; the second group was allotted to a nutritious diet plus 27 mg elemental zinc as zinc sulfate/day. The third group received diet plus no additional zinc for 6 months then were supplemented with zinc for another 6 months. Marked increases in growth rates were observed with zinc supplementation. The group of subjects on the nutritious diet alone showed a slow increase in growth with no changes in sexual maturity. When supplemented with zinc, changes in sexual maturity were observed within six weeks. Females exhibited signs of zinc deficiency similar to those males suggesting that zinc deficiency was not limited to males only. Clinical signs of zinc deficiency were found more frequently in males than in females; however, because zinc is sequestered in male genital tract rather than the female ovary, a greater need for zinc was observed in males (Halstead et al. 1977).

Several studies have reported that the frequency of zinc deficiency is greater in males than in females, and even a mild zinc deficiency (2.7 to 5 mg day) can affect testicular function in adult subjects which could be reversed with zinc supplementation (Abbasi et al. 1980). It is believed that zinc deficiency is more prevalent in countries where cereal based proteins are consumed as the major protein source (Prasad 1991).

Acroderamatitis Enteropathica:

During the past 20 years clinical symptoms of zinc deficiency have been recognized. A severe deficiency of zinc was observed in Acrodermatitis Enteropathica (AE), life threatening inherited disorder. The some of the clinical symptoms of AE

include diarrhea, recurrent infection due to cell mediated immune disturbances, growth retardation, and delayed wound healing. Zinc deficiency was also found to increase glucocorticoid levels in the plasma.

Acrodermatatis Enteropathica is an abnormal inherited disorder characterized by symptomatic zinc deficiency, diarrhea, failure to thrive and cutaneous manifestations. Moynahan studied a 2 year girl with severe AE treated with diiodihydroxyquinoline and a lactose deficient synthetic diet. The clinical response to this treatment was not satisfactory. Serum zinc concentrations were below normal. When oral zinc sulfate was administered, the skin lessions and gastrointestinal symptoms cleared completely, and the patient was discharged from the hospital. When zinc was omitted, the child suffered a relapse. The authors attributed the zinc deficiency in this patient to the synthetic diet (Moynahan 1974).

The early observation of Moynahan was confirmed by the case reports on other patients. Sandstrom et al (Sandstrom et al. 1994) studied a 16 year old boy with AE. At the age of 5 months the patient developed vesicles around mouth and anus associated with intense crying. At the age of 6 months the patient was diagnosed with AE by the dermatologist. The patient was treated for watery diarrhea with Enteroviroform. Two years after, the patient returned with the above symptoms along with polydipsia. The patient was given zinc supplementation at 30 mg/d. The symptoms of eczema disappeared with the zinc supplementation. At 9 years of age the dosage of zinc was increased to 65 mg/d. This dose increased the gastrointestinal symptoms. Hence, the daily dose was reduced to 45 mg/d, and after 4 months the dose was further reduced to 34 mg/d. Zinc and copper status and immunological indexes were measured before altering the dose of

zinc. The serum (25 umol/L) and urine zinc (40 umol/L) were very high with treatment of 65 mg zinc /d. When the dose was decreased to 34 mg zinc /d the zinc values returned to normal (9-18 umol/L serum zinc, 1 to 12 umol/L urine zinc). Decreased number of lymphocytes (0.07; normal value 0.1 to 0.2) were observed during the 65 mg/d zinc supplementation period, and the B lymphocytes returned to normal when the dose was decreased to 34 mg zinc /d. The immunoglobulins, IgG2 (0.4 g/L; normal 1.2 to 7.5 g/l) and IgG3 (0.3 g/L; normal 0.4 – 1.3 g/L) were low during the 65 mg/d supplementation period. The authors concluded that 34 mg/d would be a safe upper limit for zinc supplementation during AE (Sandstrom et al. 1994).

Experimental Zinc Deficiency in Humans:

Although the role of zinc in humans has been now defined, and its deficiency has been recognized in several clinical conditions, only recently was the experimental human model developed to study the specific effects of zinc deficiency in man (Prasad et al. 1988, Rabbani et al. 1987).

Rabbani and coworkers (Rabbani et al. 1987) demonstrated that manipulation of zinc in the diet can be used safely for gradual induction of zinc deficiency in humans. Male volunteers, 20-45 years were studied for a period of 56 weeks. The subjects were given a daily hospital diet containing animal protein that provided 10 mg zinc/d for 4 weeks. Then for 8 weeks the subjects received a semi-purified soy protein diet supplemented with zinc sulfate in the form of cookies containing a total of 13.9 mg zinc/day. For the next 28 weeks the zinc supplementation was discontinued (depletion phase). Following depletion phase the subjects consumed a total of 30 mg zinc /day for a period of 20 weeks (repletion phase).

Throughout the study the amounts of all nutrients including protein, vitamins and minerals were kept constant. Body weights were measured three times a week. Blood specimens were drawn and analyzed for zinc, copper, iron, calcium and magnesium by atomic absorption spectrophotometry. Urinary zinc was low (585 μg/24 h) with zinc depletion and increased (910 ug/24 h) during the repletion phase. Plasma zinc fell 25% with depletion and increased to 88% of the original value during the repletion phase. Erythrocyte zinc did not show a significant difference with treatments. Plasma copper was high (170 ug/dl) during the zinc depletion phase and decreased (123 ug/dl) with zinc repletion. Body weight decreased during the depletion phase (6 kg) and increased gradually with zinc repletion. The zinc content of neutrophils and lymphocytes were low during zinc restriction. The results suggest that mild dietary restriction can produce a measurable but safe zinc deficiency (Rabbani et al. 1987).

Zinc and Immunity:

Fraker and coworkers (Fraker et al. 1977) were the first to demonstrate that severe and marginal zinc deficient young adult A/Jax mice have abnormal T helper cell function. Six week old mice were fed ad libitum a diet containing adequate (25 g /kg), marginal (2.5 g /kg), and deficient (0.5 g /kg) zinc diets for a period of 4 weeks. After 22 days the organ weights of four to five mice were recorded. The thymus weights of the zinc deficient mice were 40% lower than the thymus weight of the control mice. The remaining 10 mice were injected with sheep red blood cells (SRBC) and the number of anti-SRBC plasmocytes produced per spleen was determined 7 days later. The zinc deficient mice produced only one fifth of the IgG immunoglobulins compared to the zinc supplemented mice. The IgM number did not differ significantly between the groups.

They suggested that the switch from IgM to IgG was impaired due to impairment of T-helper cell function with zinc deficiency (Fraker et al. 1977).

The function of T helper cells are dependent on the thymulin. Thymulin is a thymic specific hormone which induces several T cell markers and promotes cytotoxicity and suppressor functions (Prasad et al. 1988, Prasad 2000)and is involved in T cell differentiation (Dardenne et al. 1982). Prasad and coworkers (Prasad et al. 1988) assayed thymulin activity in mild zinc deficient subjects, before and after zinc supplementation. The subjects received a regular hospital diet for 4 weeks containing 12 mg zinc /day, followed by 3.0 mg zinc/day for a period of 28 weeks and then were supplemented with 27 mg zinc / day for a period of 12 weeks. Evaluation of thymulin levels in the peripheral blood showed a decreased serum thymulin activity in mildly zinc deficient subjects, that was corrected by supplementation with zinc. The in vitro supplementation of zinc showed the inactive thymulin peptide in serum of zinc deficient subjects was activated by the addition of zinc to the medium. A decrease in the ratio of $T4^+$ to $T8^+$ cells and interleukin-2 activity was observed during the zinc depletion phase and was corrected after repletion of zinc (Prasad et al. 1988).

The separation of the T helper cells into Th1 and Th2 cells according to their function in cell mediated immunity (Th1) and humoral immunity (Th2) is gaining importance in understanding immune mechanisms in humans. Prasad et al (Prasad 2000) studied the effects of experimental human zinc deficiency on T helper cells 1 and 2 (Th1 and Th2) and on cytokine shifts. The human volunteers, 20-45 years, were provided an animal protein based hospital diet, that contained 12 mg of zinc/day for a period of 4 weeks. Then, the subjects received a semi-purified soy protein based zinc deficient diet

(3.0-5.0 mg zinc/day) for a period of 28 weeks, followed by zinc supplements providing 27 mg zinc /d for a period of 12 weeks. Zinc deficiency decreased the production of Th1 products (interluekin-2 and interferon gamma). The Th2 products (interleukin 4 (IL-4), interleukin 6 (IL-6), and interleuin 10 (IL-10)) were not affected by zinc deficiency. Decreased natural killer cell (NK cell) and T cell lytic activity was observed with zinc deficiency. The authors suggested that an imbalance between the Th1 and Th2 in zinc deficiency and the decrease in the NK and T cell lytic activity could be due to decreased IL-2 production (Prasad 2000).

The natural killer (NK) cells play an important role in the host defense against viral infections. Tapazoglou and coworkers (Tapazoglou et al. 1985) reported decreased natural killer (NK) cell activity in patients with sickle cell disease. Twenty-one patients with homozygous sickle cell disease (SCD) were randomly assigned either to zinc supplemented group or placebo. Five patients received 45 mg zinc as zinc acetate/d for more than 24 months, and the remaining 12 subjects served as controls. In addition, two non-SCD male volunteers were included in the zinc restricted diet group (3.0 mg/day for 24 weeks) who then received a zinc repleted diet containing 30 mg/d for 14 weeks. The patients with SCD and with plasma zinc <100 μg /dl and neutrophil zinc <100 μg /10^{10} cells were considered as zinc deficient. The NK cell activity in the patients with SCD and with zinc deficiency was significantly lower than the normal controls. The NK cell activity decreased by week 20 in the normal male volunteers on zinc depleted diet and increased with zinc repletion. This study demonstrates that zinc deficiency alters the NK cell activity, which has an important role in resistance to tumors and microbial infections in the host (Tapazoglou et al. 1985).

Similar observations were made in the patients with sickle cell disease during zinc depletion and repletion phases (Prasad et al. 1988). The decreased NK-cell activity, decreased IL-1 activity, decreased serum thymulin activity and alterations in the lymphocyte subset populations in zinc deficient sickle cell anemia patients were also corrected by supplementation with zinc (Prasad et al. 1988).

Beck and coworkers (Beck et al. 1997) reported an imbalance between cell-mediated and humoral immunity in five young human subjects. The experiment included three phases, baseline (12 mg zinc), zinc depletion (2-3.5 mg zinc plus 1 g phytic acid) and zinc repletion phase (25 mg /day or 50 mg zinc). The experimental periods were baseline (4 weeks), depletion phase (20 –24 weeks), and repletion phase (12 weeks (25 mg zinc) or 8 weeks (50 mg zinc)). During the zinc depletion phase a decrease in TNF-α and interferon gamma was observed. Zinc restriction decreased the precursors to cytotoxic T lymphocytes in the $CD8^+$ subset. A downward trend was observed in the CD4 cells, the precursors of the memory T lymphocytes. All these changes normalized with zinc supplementation (Beck et al. 1997).

Animal Studies:

Gross et al (Gross et al. 1979) reported the first study on the effects of diet induced zinc deprivation on lymphocyte transformation and immune function in rats. The in vitro responses of splenic, thymic and peripheral blood lymphocytes to T-cells mitogens and B cell mitogen were investigated in the zinc deprived rats and in pair fed controls. The 21 day old weanling rats were fed a diet containing 7 ppm zinc (zinc deficient) or 60 ppm zinc (control diet) for a period of four weeks. The in vitro responses to T cell mitogen, and phytohemaglutinin test of spleen, thymus and peripheral blood

lymphocytes were decreased in zinc deficient rats by 50% compared to the control diet fed rats. The response to poke weed mitogen in spleen and thymus were depressed by 50%, and the responses to concanavalin A in spleen and peripheral blood lymphocytes were suppressed in the zinc deficient rats. The results suggested that zinc deficiency might alter lymphopoiesis in bone marrow (Gross et al. 1979).

Dowd et al (Dowd et al. 1986) investigated the effects of zinc deficiency on T-lymphocyte subsets and interleukin-2 production in rats. Six week old rats were fed a zinc deficient diet (1 mg zinc/kg) or a zinc adequate diet (40 mg/kg) as zinc sulfate for a period of 4 weeks. The total lymphocyte counts, neutrophil percents, lymphocyte percents and splenocytes were lower in the zinc deficient rats compared to the zinc adequate pair fed rats. The results suggest that zinc deficiency alters cellular immune responses via a selective effect on the helper T-lymphocytes.

An imbalance in the ratio of T cells to B cells was also noticed during zinc deficiency. King and Fraker (King and Fraker 1991) investigated the phenotypic distribution of splenic lymphocytes in zinc deficient adult mice. The mice were fed a zinc deficient diet (0.8 μg zinc/g diet) or a zinc adequate diet (26-30 μg zinc/g diet) for a period of 30 days. A 50% decrease in the total number of splenic lymphocytes was observed in the zinc deficient rats. The overall ratio of T helper to T suppressor or cytotoxic cells was increased by 20% in the zinc deficient rats (1.75 vs 1.46). The percentage of B cells decreased (5-8%). The results suggest that zinc deficiency caused reductions in the total number but not composition of splenic lymphocytes (King and Fraker 1991)

Shi et al (Shi et al. 1994) reported that zinc deficiency impaires T cell function in mice with nematode infections. Female BALB/c mice were fed zinc deficient diet (0.75 mg/kg), and a zinc sufficient diet (60 mg/kg) or an energy restricted diet with 60mg zinc/kg diet. After four weeks supplementation the mice were exposed to the primary infection with 100 larvae/rat. The immunological parameters were assayed for 5 weeks following the infection. The zinc supplemented mice had only 40% as many worms as zinc deficient mice. Reduced production of IL-4 and IFN-χ, reduced peripheral eosinophilia, and reduced serum levels of IgE and IgG_1 were observed in zinc deficient mice compared to the zinc supplemented mice. The authors concluded that zinc deficiency significantly impaires the functions of Th1and Th2 cell populations (Shi et al. 1994).

A combined effect of zinc deficiency and energy restriction on the immune response during gastrointestinal nematode infection was studied by Shi et al (Shi et al. 1998). The study included female BALB/c mice fed either zinc sufficient (60 mg zinc/kg diet), zinc deficient diet (0.75 mg zinc/kg diet) or pair fed with zinc sufficient diet. The results of the study indicated that parasite numbers were lower in the zinc supplemented group compared to the other groups. Dietary zinc deficiency decreased food intake and growth. Zinc deficiency or energy restriction did not produce a shift in the immune cell populations, even though the spleen size and total number of spleen cells were decreased. The ability of antigen presenting cells to stimulate proliferation of T-cells in response to parasite-specific antigen was decreased with energy restriction and zinc deficiency. Impaired T-cell proliferation and reduced T-cells' cytokine production was observed with

zinc deficiency and energy restriction. The authors attributed these impaired immune responses to the lowered number of lymphocytes due to zinc and energy deficiency.

Zinc Supplementation and LPS Effects:

The host response to LPS endotoxin is characterized by decreases in the serum zinc concentrations. After endotoxin dosing serum zinc decreases in a dose dependent manner. Nakajima and Suzuki (Nakajima and Suzuki 1996) reported that the bone marrow cells in the zinc deficient rats with low metallothionein (MT) levels were adversely affected by repeated i.p. administration of LPS endotoxin, E.coli, 0127:B8. Male rats, 150-200g were allotted to two levels of dietary zinc and two levels of LPS endotoxin. The treatments included either 5.52 mg zinc or 0.06 mg zinc /100 g diet with 0.02 or 2 mg LPS/kg body weight. The peripheral leukocytes and number of bone marrow cells in zinc deficient rats were decreased in the 2 mg LPS/kg group compared to the saline treated group and to the groups treated with 0.2 mg LPS/kg. The authors suggested that the LPS might affect myeloid cell proliferation, thereby decreasing peripheral leukocytes. Metallothionein is a potent radical scavenger that protects against inflammation or infectious disease in various kinds of cells. Supplementation with zinc increases the MT levels in the liver and bone marrow. The increased MT concentrations provide protection against TNF-α induced suppression of progenitor cells proliferation in the bone marrow by scavenging free radicals (DePasquale and Fraker 1979).

Alteration in spleen cellularity and cytokine production was observed in the zinc deficient mice when challenged with LPS. Serushago and Chandra (Serushago and Chandra 1995) reported that low dietary zinc (5 ppm zinc) did not affect weight gain of mice when compared to mice fed a zinc adequate diet (43.3 μg Zn/g diet). After four

weeks on the experimental diets the mice were challenged with 20 μg LPS/kg body weight (strain not mentioned). The zinc deficient mice had a significant reduction in leukocytes in the spleen, and also, the T lymphocyte subsets were significantly reduced in the zinc deficient group. Decreased TNF-α values were observed in the zinc deficient mice, and a delayed peak time was observed in the zinc deficient group (90 minutes PI vs 30 minute in controls). The authors concluded that the active protease involved in TNF-α secretion was a zinc dependent metalloenzyme, that was altered with zinc deficiency (Serushago and Chandra 1995).

Rofe et al (Rofe et al. 1996) reported that zinc supplementation during endotoxemia influenced energy production rather than the acute phase response. The authors suggested that MT had a role in maintaining hepatic glycogen and blood glucose in the endotoxin challenged normal (MT +/+) and MT null (-/-) mice. The MT-I and MT-II null mice were fed commercial pelleted diets and were injected with 1 or 5 mg LPS endotoxin E.coli 0111:B4 /kg body weight. Sixteen hours PI weight loss was significantly higher in the MT null mice injected with 5 mg LPS/kg compared to the other groups. The plasma zinc concentration in normal (MT+/+) mice was lower in both of the LPS treatments compared to the controls and LPS treated normal and MT-/- mice (Rofe et al. 1996).

Bremner et al (Bremner et al. 1987) reported that dietary zinc and endotoxin administration would change MT concentrations in red blood cells in the rat. Twenty male rats, 150 g, were fed a diet containing 5 or 40 mg zinc/kg for a period of 2 weeks. At the end of the second week the rats were injected i.p. with 1 mg LPS endotoxin (E.coli, 026:B6) /kg body weight. Urine, liver and kidney MT was significantly lower in the zinc

deficient rats than in the control rats. Metallothionein was not detected in the blood in the endotoxin group. No differences in MT concentrations were observed in bone marrow cells in the endotoxin challenged zinc adequate and deficient groups. The authors concluded that MT is associated principally with red blood cells and not with leukocytes or platelets.

Parenteral zinc supplementation in adult humans increased the febrile response during the acute phase response. Braunschweing et al (Braunschweig et al. 1997) reported a double blind randomized, placebo controlled clinical trail with supplementation of zinc (30 mg elemental) to patients with sepsis or with pancreatitis and on home parenteral nutrition. The results indicated an increase in body temperature on day 3 of the supplementation. No differences in the cytokine IL-6 or ceruloplasmin concentrations were observed with the zinc supplementation. Serum zinc and urinary zinc were high in the zinc supplemented group compared to the controls. These values increased significantly by day 3 of supplementation. The authors concluded that zinc supplementation during the periods of stress might produce an increased febrile response (Braunschweig et al. 1997).

The importance of zinc in macronutrient metabolism has also been reported. Zinc deficiency reduced hepatic pyruvate kinase gene expression in rats. Kennedy et al studied the effect of adequate dietary zinc (30 mg/kg diet) or deficient levels of dietary zinc (1 mg zinc/kg) on macronutrient selection patterns (Kennedy et al 1998). The duration of the study was 35 days. The authors found that the zinc deficient rats had reduced PK gene expression and that zinc deficient rats prefered a fat containing diet. The authors suggested that this change might be due to decreased insulin production due to zinc

deficiency. They also suggested that PK was regulated by insulin leading to an alteration in hepatic glycolysis (Kennedy et al. 1998).

Zinc and IGF-1:

Anorexia and decreased energy intake observed with feeding a zinc depleted diet can influence the plasma insulin-like growth factor-1 (IGF-1). The IGF-1 mediates a diversity of cellular events, including stimulation of amino acids and glucose uptake and regulation of cell cycle (MacDonald 2000). Low IGF-1 concentrations were observed during feed restriction in animals (Elsasser et al. 1995).

Cossack et al (Cossack 1988) studied the effects of zinc deficiency on plasma IGF-1 concentrations in albino male growing rats. The rats were fed a chow diet for 4 days after which they were fasted for 72 h. After fasting for 72 hr the rats were allotted and fed four dietary levels of zinc: a zinc deficient group (ZnD), 30, 90, or 140 ppm of Zn. A group of eight rats were not fasted and were fed the rat chow diet throughout the experimental period. Blood was obtained at intervals from all groups for measurements of plasma Zn and IGF-1. Results showed that plasma IGF-1 decreased at the 3rd day of fasting. At 48 and 72 h of refeeding, the levels of plasma IGF-1 increased significantly in all experimental groups, and no significant differences between the groups were observed. The levels of IGF-1 in rats fed 140 or 90 ppm of zinc continued to increase significantly with refeeding. However, in rats fed 30 ppm or ZnD diets, levels of IGF-1 started to decline after 72 h of refeeding. Levels of plasma zinc followed a similar trend as IGF-1 levels. In addition, the levels of zinc in the tibia were comparable in all groups with somatomedin C (SMC) and plasma zinc levels at the end of the fasting or refeeding

periods. The authors concluded that the level of zinc in the diet should be considered carefully when planning nutritional intervention for severe malnutrition or starvation.

Roth and Kirchgessner (Roth and Kirchgessner 1994) measured IGF-1 levels periodicaly for 32 days in force fed rats. The rats were fed a zinc adequate diet ad libitum, pair fed or rats were fed zinc deficient diet ad libitum. Twenty-four male Sprague-Dawley rats, 108 g, were divided into 2 groups of 12 animals each. The Zn-deficient group (1.3 ppm zinc) and the control group (25 ppm) received a semi-synthetic casein diet for 12 days. All animals were fed 4 times daily by gastric tube. After 12 days, the zinc depleted rats showed reduction of zinc in serum and the femur by 62% and 44% respectively and had a 70% lower serum alkaline phosphatase activity. Zinc deficient rats had elevated serum growth hormone (78%) compared to the control group. Levels of IGF-1 was significantly reduced by 28% in zinc depleted force-fed rats compared with rats fed a zinc adequate diet. Growth depression was observed in the zinc deficient rats even though their energy intake was equal to controls. The results suggest that systemic IGF-1 levels are modulated by dietary zinc.

Impaired growth induced by zinc deficiency in rats has been associated with decreased expression of the hepatic insulin-like growth factor 1 and growth hormone receptor genes. McNall et al (McNall et al. 1995) studied weanling male Sprague-Dawley rats fed zinc deficient diets (no added zinc), pair fed or ad libitum fed for 14 days. Zinc acetate (30 mg/L) was added in the deionized water and was provided to the pair fed and ad libitum fed rats. Zinc deficiency lowered the growth rate by 60% and IGF-1 concentrations were decreased by 46 and 67% compared to pair fed and ad libitum fed rats respectively. The reduction in IGF-1 was associated with a decrease in the IGF-1

gene expression. The authors concluded that the growth retardation due to zinc deficiency was associated with defects in the growth hormone receptor signal pathway, which ultimately causes reduced IGF-1 secretion.

Zinc and IGF-1: Human Studies:

Plasma concentrations of IGF-1 have been reported to respond to zinc supplementation after 3 or 4 weeks (Clark et al. 1999). The first community study done by Xuan et al (Xuan et al. 1996) on zinc and its effects on IGF-1, found that zinc supplementation increased growth and circulating IGF-1 in growth retarded Vietnamese children. Children 4 to 36 months old were supplemented with 10 mg zinc sulfate/day or a placebo for a period of 5 months. The results indicated the probability of at least two episodes of diarrhea and respiratory infections was 2.9 and 3.2 fold higher in the placebo group compared to the zinc supplemented group. Plasma IGF-1 increased in the zinc treated subjects in the first month and at the fifth month; no differences were observed in the placebo group. Zinc supplementation accelerated weight and height gains simultaneously. The authors concluded that zinc deficiency in malnourished children in developing countries is likely to be severe, whereas the studies done in well-nourished children in the US and Canada showed no effect on growth rate with zinc supplementation. In fact, this is the first community study to show that zinc supplementation increased growth and IGF-1 in malnourished children (Xuan et al. 1996).

In a randomized double blind placebo controlled study, the effects of zinc supplementation on serum IGF-1 and on growth was investigated in infants 3 to 9 months old with nonorganic failure to thrive (Hershkovitz et al. 1999). The infants were

supplemented with 2 mg zinc/kg/day for a period of 12 weeks. Serum IGF-1 was similar in all the groups at baseline. After 12 weeks, serum IGF-1 increased in the zinc supplemented group compared to the placebo group (65.0 ± 8 vs 49.4 ± 5 ng/ml). The zinc supplemented group had increased IGF-1 at 12 weeks of supplementation compared to the baseline values (65.8 ± 8 vs 40.3 ± 7 ng/ml). The IGF binding protein-3 (IGFBP-3) did not change significantly. No significant change in growth for age, length for age, or weight for length was observed during the 12 weeks supplementation. The results suggest zinc supplementation for 12 weeks increased IGF-1 but did not have any effect on growth. A longer supplementation period might provide beneficial effects on growth (Hershkovitz et al. 1999).

A shorter zinc supplementation period of 6 weeks did not have any effect on plasma IGF-1 concentrations. Clark et al (Clark et al. 1999) reported no changes IGF-1 in pubertal girls supplemented with 15 mg zinc as zinc citrate /day versus a placebo for 6 weeks. Serum zinc concentrations were higher with zinc supplementation but no changes in height or weight were observed.

Zinc and Cortisol:

Glucocorticoids were shown to alter the intestinal uptake and compartmentalization of zinc in piglets (Wang et al. 1993). The 13-15 day old piglets were injected with 1.5 mg/kg dexamethasone (DEX), a synthetic glucocorticoid, intramuscularly twice a day and were fed a 15.3 mmol/kg zinc diet or control diet (0.3 mmol zinc/kg diet). The maximum zinc uptake in the intestinal brush border membrane vesicles was high in the DEX treated piglets, and the zinc efflux rate was lower in the DEX treated piglets. The liver and the intestinal mucosa zinc were not different between

the DEX treated and control groups but were higher in the high zinc group. It can be concluded that changes in zinc uptake with the DEX treatment may have consequences during infection when glucocorticoids are generally elevated (Wang et al. 1993).

Stress associated with zinc deficiency in the mouse was reported by Depasquale and Fraker (DePasquale and Fraker 1979). The zinc deficient mice had increased levels of plasma corticosterone compared to mice fed zinc adequate diets. A reduction in the T-cell helper function occurred 4 days after the rise in corticosterone associated with zinc deficiency. The authors suggested that zinc deficiency increased corticosterone production, which might have contributed to the loss of immune function (DePasquale and Fraker 1979). Although glucocorticoids inhibit IGF-1 secretion, whether the increases in corticoids induced by Zn deficiency causes the decrease in IGF-1 observed in the other studies will require further elucidation.

Zinc Toxicity

Pharmacological doses of zinc when taken for a prolonged period of time appear to cause several potentially adverse problems. Alterations in the immune response and blood lipid profiles with pharmacological amounts of zinc were reported by Chandra (Chandra 1984). When 11 healthy men were supplemented with 300 mg Zn/day (20 times the RDA) for a period of 6 weeks. The immune response was depressed as indicated by the reduction in lymphocyte stimulation response to phytohemagglutinin as well as chemotaxis and phagocytosis of bacteria by polymorphonuclear leukocytes when compared to the base line values. Serum high-density lipoprotein concentration decreased significantly and low-density lipoprotein level increased slightly with zinc supplementation (Chandra 1984).

Supplementing with lower amounts of zinc 100 or 15 mg Zn/day, (6.66 times the RDA vs RDA) for period of 3 months to 60-89 year old elderly subjects did not have any adverse effects on the immune function. Zinc supplementation did not have any significant effects on the delayed dermal hypersensitivity or in vitro lymphocyte proliferative responses to mitogens and antigens (Bogden et al. 1988).

Zinc is considered non toxic if taken orally (Fosmire 1990). But at amounts well in excess of the recommended dietary allowances for humans (100-300 mg Zn/d vs 1989 RDA of 15 mg Zn/day), evidence of induced copper deficiency along with the symptoms of anemia and neutropenia, impaired immune function and adverse effects on the ratio of low-density-lipoprotein to high-density lipoprotein, cholesterol have been reported (Fosmire 1990).

Hypocupremia, anemia, leucopenia, and neutropenia were observed in a patient with sickle cell anemia treated with 150mg Zn/day for a period of two years. Cessation of zinc supplements normalized the copper status in this patient (Prasad et al. 1978). Zinc supplementation to diets or zinc in the form of supplements need to be monitored with caution.

Zinc and Swine Nutrition

The use of pharmacological levels of zinc has been suggested to have a beneficial effect in controlling E.coli scours in weanling swine. The levels of zinc supplementation reported in the literature are in excess of those recommended by NRC. Feeding pharmacological doses of zinc to weanling pigs did not seem to have detrimental effects. Hahn and Baker (Hahn and Baker 1993) studied the effects of feeding pharmacological

doses of zinc in 28 day old, 8 kg weanling pigs. The study investigated not ony zinc dose but also zinc sources. The diets were supplemented with 0, 250, 500 1000, 3000 and 5000 mg of zinc as zinc oxide/kg diet, 1500 and 2500 mg /kg as zinc sulfate, or zinc lysine complex at 1500 or 2500 mg /kg. The experimental diets were fed for 21 days. Plasma zinc concentrations increased in the pigs fed 1000 mg zinc as zinc oxide and above this level the increase in plasma zinc was linear from all the three dietary sources. During the same time the authors repeated the study by supplementing the diets with either 3000 or 5000 mg zinc/kg as zinc oxide or as zinc sulfate on 21 day old, 8 kg pigs. The daily gain and daily feed intake increased in pigs fed zinc oxide independent of the concentration. The growth performance increased in pigs fed 3000 mg zinc/kg as zinc sulfate only. The authors concluded that supplementing diets between 1000 and 5000mg of zinc/kg is not detrimental to the weanling pigs.

Bobilya et al (Bobiliya et al. 1994)reported loss of bone zinc during dietary zinc deprivation in neonatal pigs. The study included 1-2 week old piglets fed a basal diet with 4 μg zinc/g fed ad libitum, an adequate zinc diet 100 μg zinc/g, and or adequate zinc diet restricted in intake to allow weight gain comparable to the low zinc diet group. Pigs on the adequate zinc diet, fed ad libitum, grew well during the 28 d experimental period. Growth rates of low zinc pigs were impaired such that the pigs' growth in the low zinc group ceased by day 14, and parakeratosis developed at the same time (day 14). During the first weeek plasma zinc dropped in the treatment groups, and those on the low zinc diet continued to have lower plasma zinc compared to the adequate zinc ad libitum fed and adequate zinc restricted intake groups. Bone zinc also decreased significantly in the low zinc group and remained high in the adequate zinc group, compared to the other

groups. The authors concluded that a diet less than 5 μg zinc/kg diet for more than 5 weeks would be detrimental.

Hill et al conducted a study on the effects of dietary sources of zinc on gain, feed conversion and on blood traits. The pigs were fed a diet containing 9 or 12 ppm zinc as zinc sulfate, zinc methionine or zinc methionine with picolinic acid. The pigs fed zinc methionine with picolinic acid showed better gain and feed conversion. Both zinc methionine and zinc sulfate had no effect on weight gain and feed conversion. When the authors repeated the experiment, no differences in the ADG or ADFI were observed between any of the treatments. The results suggest that picolinic acid may not aid in zinc absorption, also the concentrations of zinc in the diet were very low, this might have contributed to the insignificant results (Hill et al. 1986).

Swinkels et al (Swinkels et al. 1996) reported that the soft tissue concentrations of zinc (liver, kidney, brain, and small intestine) were lower in zinc depleted pigs fed 17 mg/kg of zinc compared to pigs fed 45 mg/kg of zinc from zinc sulfate or zinc amino acid chelate. The pigs fed the soy isolate diet containing 17 mg/kg zinc developed parakeratosis by day 24. The average daily gain was lower in the zinc depleted pigs compared to the zinc supplemented pigs regardless of zinc source (zinc sulfate or as zinc amino acid chelate). The authors concluded that zinc amino acid complex and zinc sulfate are equally effective on the growth performance of weanling pigs.

Schell and Kornegay (Schell and Kornegay 1996) studied the growth performance of weanling pigs, 7.5 kg body weight, 28 days of age, fed pharmacological doses of 3000, 2000 or 1000 mg zinc/kg diet as zinc sulfate, zinc methionine, zinc lysine or zinc oxide for a period of two weeks. The growth performance of the pigs was not affected even by

the highest concentration of the zinc (3000 mg/kg diet) independent of the sources. However, higher serum, liver and kidney zinc concentrations were found the pigs fed with 3000 mg/kg diet. The performance was not enhanced with pharmocological doses of dietary zinc supplemented for a period of two weeks.

Cheng et al (Cheng et al. 1998) compared zinc lysine and zinc sulfate utilization by young pigs. Supplementing 100 ppm zinc as zinc sulfate or as zinc lysine improved average daily gain (ADG) and average daily feed intake (ADFI) compared to the pigs fed diets with no added zinc. When the lysine content of the diet was varied (0.8 % vs 1.1 %), the improved ADG and ADFI was observed at 1.1% lysine requirement compared to the 0.8% lysine requirement. The humoral response to sheep red blood cells and to ovalbumin were not influenced by zinc level or source. Pigs fed diets without added zinc had lower liver, kidney, and rib zinc concentrations than pigs fed diets with added zinc. No difference in the zinc tissue concentrations was observed between the two dietary sources of zinc. The authors concluded that supplementing diets with zinc as zinc sulfate or as zinc lysine produced same results in promoting growth performance, zinc absorption and tissue stores.

Chromium

Chromium and its relation to glucose tolerance in animal and in human nutrition gained the attention of nutritionists in the 1960's. However, to date there are less than 150 articles reported in the literature on the effects of chromium on growth. The essentiality of dietary chromium for growth has been demonstrated in numerous species, such as in mice, rats (Schroeder 1968), pigs (Guan et al. 2000, Lee et al. 2000, Lindemann et al.

1995) and humans (Gurson and Saner 1971, Hopkins et al. 1968). Laboratory rats and mice of both sexes when provided 2 or 5 μg/ml of chromium as chromium acetate showed significant increases in growth rates compared to rats or mice with no chromium in their drinking water. Decreased mortality and greater longevity were observed with addition of chromium in the drinking water. The median age of male mice at death was 99 days longer with chromium supplementation; however, this effect was seen only in male rats and mice but not in female rats and mice (Schroeder 1968).

Analytical methods and handling of samples for chromium analysis have improved over the years. The reported values for chromium analysis before 1980 were too high compared to the values reported more recently with improved analytical knowledge and techniques (Anderson 1987). Mertz recommends that analytical values for chromium reported before 1980 should not be accepted (Mertz 1993). However it would be interesting to do a relative comparison of results with the values reported now and then. Chromium concentration of foods and feedstuffs are highly variable. Therefore, it is critical to consider the concentration of chromium in the basal diet while evaluating chromium studies in the literature.

The research on chromium and its effects in farm animals gained attention only in the 1990's and the reported studies are less than 20 in number. Since then, the effect of chromium supplementation on performance of farm animals has been a subject of controversy. Studies done on pigs with different concentrations of supplemental chromium seem to elicit different and inconsistent results. The results seem to depend on weight of the animal, composition of the diet and the environmental stress such as

transportation during the experimental period. The following section presents a review of studies conducted on the effect of chromium on growth in farm animals.

Chromium and Growth:

The effect of feeding chromium as chromium picolinate on growth performance in pigs during the growing-finishing period or during the finishing period alone was reported by Boleman et al (Boleman et al. 1995). They supplemented the diets of twenty-four barrows with an initial body weight of 19.1 kg to a final body weight of 106 kg with 200 μg chromium as chromium picolinate /kg corn soybean meal diet. The pigs were randomly allotted to three dietary treatments. The first group was fed a corn soybean meal basal diet from an initial body weight of 19.1 kg to final body weight of 106.4 kg (growing finishing phase). The second group (finishing phase) was fed basal diet from an initial body weight of 19.1 kg until they weighed 57.2 kg, and then from 57.2 kg to a final weight of 106.4 kg the pigs were supplemented with 200 μg chromium as chromium picolinate /kg diet. The third group of pigs received a basal diet supplemented with 200 μg chromium as chromium picolinate/ kg diet from an initial body weight of 19.1 kg to a final weight of 106.4 kg (growing finishing phase). The chromium concentration in the basal diet was not reported. The growing finishing pigs supplemented with 200 μg chromium as chromium had a decreased average daily gain (ADG) and average daily feed intake (ADFI). The pigs supplemented during the finishing phase had a higher percentage of muscle and lowered 10th rib fat compared to the growing finishing pigs or the controls which had no chromium added to the basal diet. The authors concluded that supplementation of chromium during the finishing phase of pig production may increase the lean body mass.

In contrast Mooney and Cromwell (Mooney and Cromwell 1999) reported no effect of supplementation of 200 μg chromium as chromium picolinate / kg diet. Pigs were fed from an initial body weight of 19.0 kg to a final weight of 109 kg with corn soybean meal combinations that provided 95% (19.5-55 kg) or 80% (55-109 kg) of lysine requirements. They showed no improvement in performance with chromium supplementation at either level of lysine. In the second experiment, pigs were fed a basal diet supplemented with either 0 or 200 μg chromium as chromium picolinate /kg diet with 1.1% of lysine from an initial body weight of 21 kg to a final body weight of 104 kg. Chromium supplementation did not have any effect on the ADG or ADFI. When the authors separated the weight gain into grower (20-55 kg) and finisher (55-109 kg) phases, chromium supplementation improved growth rate and efficiency of feed utilization from 20 to 55 kg body weight. The earlier studies conducted by Mooney and Cromwell (Mooney and Cromwell 1995) also showed an improvement in the ADG in pigs supplemented with 200 μg chromium as chromium picolinate/kg diet when compared to the controls. In these studies, the pigs were fed diets containing 0.90% lysine (27-55 kg) and 0.80% lysine (55-109 kg) with or with out added chromium. The concentration of chromium in the basal diet was not reported, however the authors mentioned the analyzed concentration of chromium to be 208 μg/kg as compared to the calculated concentration of 200 μg/kg (Mooney and Cromwell 1995). The analyzed chromium concentration in the basal diets supplemented with chromium picolinate was reported to be 204 and 205 μg chromium/kg diet which suggested that the chromium concentration in the basal diet with no added chromium was very low (Mooney and Cromwell 1999).

Lindeman et al (Lindemann et al. 1995) studied the effect of higher concentrations of chromium (250 or 500 μg chromium as chromium picolinate/kg diet) on growth performance in crossbred pigs with a mean initial body weight of 40.9 kg fed for a period of 60 days. The authors reported an improvement ($P<0.1$) in gain: feed in both the groups supplemented with chromium regardless of the concentrations. No effect ($P>0.1$) on ADG and ADFI was observed with chromium supplementation. The chromium content in the basal diet was not mentioned (Lindemann et al. 1995).

Contradicting the results of Lindeman et al (Lindemann et al. 1995), Evock-Clover et al (Evock-Clover et al. 1993) reported no beneficial effect of 300 μg chromium as chromium picolinate/kg corn soybean meal diet on performance of pigs with an initial body weight of 30 kg. The pigs were supplemented for a period of 6 weeks, or until they reached 60 kg body weight. The authors suggested that the chromium concentration in the basal diets was 2.5 times higher compared to the total chromium used by Page and coworkers (Page et al. 1993), but the authors did not mention the exact chromium concentration in the diet. The authors found serum insulin and glucose to be decreased as the study progressed, and they suggested on this basis that a study of longer duration might have significant effects on weight gain (Evock-Clover et al. 1993).

Lee and coworkers studied the effect of supplementation of 0 or 400 μg chromium as chromium picolinate/kg diet in 4 week old weanling pigs with a mean body weight of 6.58 ± 0.49, for a period of 38 days (Lee et al. 2000). The authors found no effect of supplementation with chromium on the growth performance. The reported concentration of chromium in the basal diet was 1300 μg chromium/kg corn soybean meal diet.

Some studies have reported that manipulating the ratio of corn and soybean meal in the diet from a deficient to adequate lysine level will improve growth performance of pigs supplemented with chromium picolinate while others have shown no improvements. Page et al (Page et al. 1993) studied the effects of different concentrations of chromium picolinate supplemented diets containing 120% of the lysine requirements for a young pig. The concentrations of 0, 25, 50, 100 and 200 μg chromium/kg diet were supplemented in the diets of growing finishing pigs from an initial body weight of 37.8 kg to a final body weight of 109 kg. Daily gain was increased with 50 and 200 μg chromium supplementation but decreased with 100 μg chromium/kg diet. The authors subsequently supplemented 30.5 kg growing finishing pigs with 0, 100, 200, 400 and 800 μg chromium as chromium picolinate in a diet meeting 120% of the lysine requirement. Both daily gain and ADFI decreased linearly with the increasing concentrations of chromium picolinate supplementation. The results of the study indicated that 100 or 200 μg chromium diet did not significantly reduce growth rate. Addition of 400 or 800 μg chromium as chromium picolinate/kg diet decreased growth rate and feed intake without affecting efficiency of feed utilization (Page et al. 1993).

Amoikon et al (Amoikon et al. 1995) studied 21-25 kg cross bred barrows fed corn-soybean meal diets containing 120% of the lysine requirement supplemented with 0 or 200 μg chromium as chromium picolinate/kg diet. No effect ($P > 0.1$) of chromium supplementation on the ADG or on ADFI was observed in these pigs. The results indicated that chromium supplementation of the diets containing adequate lysine (120%) may not produce significant increases in weight gain. The chromium concentration in the basal diet was not reported (Amoikon et al. 1995).

When the diet provided 100% of the lysine requirement, chromium supplementation was found to increase gain: feed ratio. Lindemann et al (Lindemann et al. 1995) studied the effect of supplementation of 0, 100, 200, 500, and 1000 μg chromium as chromium picolinate/kg corn soybean meal diet with either 100 or 120% of the dietary lysine requirements in cross bred pigs with an initial body weight of 14.5 kg. The authors reported an increase in gain: feed only in the group supplemented with 200 μg chromium and receiving 100% of the dietary lysine recommendation compared to pigs supplemented with 200 μg chromium and fed 120% dietary lysine. No effects of the other concentrations of chromium picolinate were found on performance.

Ward et al (Ward et al. 1997) studied effects on growth performance of pigs fed diets supplemented with either 0 or 400 μg chromium as chromium picolinate/kg corn soybean meal diet containing 80% or 120% of the dietary lysine requirements. Pigs with an initial body weight of 27.2 kg were supplemented for a period of 21 days. The results demonstrated that chromium in the group receiving 80% of the dietary lysine requirement increased ADG and gain:feed. An opposite trend was observed with the diet meeting 120% of the lysine requirement where pigs decreased ADG ($P<0.09$) and the gain: feed ratio ($P<0.05$) with chromium supplementation. The authors concluded that chromium supplementation of diets that are deficient in lysine improved feed efficiency and suggested that it might be due to increased amino acid metabolism or to involvement of chromium in DNA or RNA synthesis (Ward et al. 1997).

The results of the above studies suggest that the beneficial effect on performance with supplementation of chromium was maximal when the quality of protein in the diet

was deficient or marginal. The observed results were not limited to pigs but were also found in rats.

Mertz and Roginski in 1969 (Mertz and Roginski 1969) studied the effects of chromium supplementation on weight gain in rats fed a low protein, low chromium diet. The rats were fed a 10% soy protein diet with sucrose as carbohydrate with or without supplemental chromium (200 μg as chromium chloride/kg diet) for a period of 12 weeks. The concentration of chromium in the soy protein basal diet was 50 μg /kg diet. Raising rats in plastic cages on a low protein, low chromium diet resulted in a moderate depression of growth. When rats were supplemented with chromium they grew better ($P<0.01$) than the controls (Mertz and Roginski 1969). The results suggest that chromium supplementation would be beneficial during periods of protein malnutrition.

Few studies have investigated effects of supplementation with different sources of chromium in diets that contain adequate (1.2 %) lysine. Page et al (Page et al. 1993) compared the effects of chromium chloride and chromium picolinate on the performance of 22.4-kg pigs. The 6 dietary treatments included 1) basal diet with no added chromium; 2) basal diet with 1467 ppb picolinate; 3) basal diet plus 100 μg chromium as chromium picolinate/kg; 4) basal diet plus 200 μg chromium as chromium picolinate/kg diet; 5) basal diet plus 200 μg chromium as chromium chloride /kg diet; 6) basal diet plus 1467 ppb picolinate plus 200 μg chromium as chromium chloride /kg. The authors reported an increase ($P<0.06$) in the feed intake with chromium picolinate supplementation (100 and 200 μg/kg). Daily gain was not affected by treatment. No effect on weight gain was observed with supplementation of 200 μg chromium as chromium chloride / kg diet alone or in combination with 1467 ppb picolinate. The efficiency of feed utilization was higher

in the pigs supplemented with chromium chloride alone (P<0.03) and tended to be lower in pigs supplemented with chromium chloride in combination with picolinic acid (P<0.09). However van Heugten and Spears (van Heugten and Spears 1997)compared the effects of 200 μg chromium/kg diet as chromium picolinate, chromium chloride, or chromium nicotinic acid complex added to diets containing 1.2 % lysine. When three-week-old crossbred weanling pigs (6.35 kg) were supplemented for a period of 34 days, gain and feed intake tended (P<0.10) to be improved with chromium supplementation independent of source. The chromium concentration in the basal diet was reported to be 5.11 ppm (van Heugten and Spears 1997).

Mooney and Cromwell (Mooney and Cromwell 1997) compared varying concentrations of different sources of chromium on the performance of Yorkshire-Hampshire pigs with an average initial body weight of 20 kg. The diets were supplemented with either 0, 200 or 400 μg chromium as chromium picolinate/kg corn soybean meal diet or with 5000 or 25000 μg chromium as chromium chloride /kg corn soybean meal diet for a period of 35 days. The addition of 200 μg chromium increased ADG (P<0.07) and ADFI (P<0.03) but did not affect feed:gain ratio. No difference (P>0.1) in performance was observed with chromium as chromium chloride or with other levels of the chromium as chromium picolinate. Another experiment reported by the same authors compared the effects of either 200 μg chromium as chromium picolinate/kg corn soybean meal diet or 5000 μg chromium as chromium chloride/kg corn soybean meal diet in growing finishing pigs (19-109 kg). No differences in the growth rate, feed intake or feed:gain ratio were observed between the treatment groups; however the chromium

picolinate supplemented pigs tended ($P<0.11$) to gain more rapidly than the chromium chloride group (Mooney and Cromwell 1997).

Studies on the effects of chromium supplementation as high chromium yeast in calves have been equivocal. The first evidence demonstrating beneficial effects for ruminants of supplemental chromium as high chromium yeast was reported by Chang and Mowat (Chang and Mowat 1992). The authors studied the effect of supplemental chromium (400 μg/ kg diet) as high chromium yeast providing 2 mg chromium/g of yeast, for a period of 28 days. The chromium concentration of the basal corn silage diet was reported to be 160 μg/kg. Chromium supplementation increased ADG and ADMI (average dry matter intake). Moonsie-Shageer and Mowat (Moonsie-Shangeer and Mowat 1993) supplemented the diets with 0, 200, 500, and 1000 μg chromium/kg diet as high chromium yeast incorporated into corn-silage diet. Chromium content of the high chromium yeast source was 2 mg of chromium/g of yeast. Eighty-four Charolais-crossed steers with a body weight of 236 kg were fed for a period of 30 days. The ADG at day 30 was increased in calves supplemented with 200 or 1000 μg chromium as chromium picolinate/kg diet compared to the unsupplemented control and the group supplemented with 500 μg/kg diet. The DMI increased in the 200 and 500 μg/kg levels over the 30 day trial period compared to 0 and 1000 μg groups. The results indicate that chromium supplementation at a level of 200 μg/kg diet increased ADG and DMI when compared to 0, 500 and 1000 μg/kg. The authors concluded that the most beneficial effect of chromium supplementation on growth performance was observed at a level of 200 μg/kg diet. The observed pattern, i.e. the improved performance in the 200 μg chromium

supplemented group compared to the 500 and 1000 μg chromium supplemented groups, was not discussed (Moonsie-Shangeer and Mowat 1993).

Arthington et al (Arthington et al. 1997) studied the effect of high chromium yeast supplementation in 6 to 8 week old bull calves with an initial body weight of 85 kg. The calves received 3 mg chromium as high chromium yeast/d for a period of 53 days. The base concentrate mix providing 5% dried molasses, 48.8% finely ground corn, and 46.2% soybean meal was fed to each calf at a rate of 0.11 kg/day. The high chromium yeast was included in the base concentrate replacing finely ground corn at 2.6%. The chromium content of the base concentrate mix was reported to be 610 μg/kg. The chromium concentration of the base concentrate mix with supplemental chromium was 3180 μg/kg (Arthington et al 1997). The authors reported no significant effect of chromium supplementation on growth performance.

Kegley and Spears (Kegley and Spears 1995) compared the effect of supplementation with chromium as chromium chloride ($CrCl_3$), as high-Cr yeast, or as a Cr-nicotinic acid complex in Angus crossbred steers. The steers (initial body weight of 215 kg) were supplemented with 400 μg chromium/kg of diet for a period of 56 days. The concentration of chromium in the basal diet was 660 μg/kg diet. The authors reported no effect of chromium supplementation on performance.

Studies done on Suffolk lambs found no beneficial effects of the supplementation of chromium as chromium picolinate. Concentrations of 400 μg/kg diet (Gentry et al. 1999) or 250 μg/kg diet (Kitchalong et al. 1995) were found to be ineffective on ADG or on DMI in Suffolk lambs. The concentration of chromium in the basal diet was less than 1000 μg chromium/kg diet (Kitchalong et al. 1995).

Guan et al (Guan et al. 2000) studied 20 Yorkshire pigs with an initial body weight of 47.9 kg supplemented with 200 μg chromium as high chromium yeast/kg corn soybean meal diet or a basal corn soybean meal diet. The high chromium yeast was added at a concentration of 0.34% of diet. The concentration of the chromium in the basal diet was 204 μg chromium/kg. The authors reported no effect of the additional 200 μg chromium as high chromium yeast on the ADG or ADFI.

The beneficial effect of chromium supplementation was observed to increase during hormonal stresses leading to greater chromium effects in stressed animals. The effects of chromium supplementation in somatotropin-treated pigs have been reported in the literature and the results are inconsistent. Evock-Clover et al (Evock-Clover et al. 1993) and Anderson et al (Anderson et al. 1997) studied twenty-four castrated male pigs using a 2 x 2 factorial experiment. The diets included a basal diet; basal diet plus 300 μg chromium/kg corn soybean meal diet; basal diet plus 100 μg/kg body wt/d and i.m. of pituitary porcine somatotropin (ppST); and a basal diet in combination with 300 μg chromium as chromium picolinate/kg corn soybean meal diet and 100 μg/kg body wt/d ppST. The pigs were fed from 30-60 kg body weight. The results indicated that ppST improved growth performance in the pigs, but chromium had no effect on the growth performance. No treatment interactions (ppST x Cr) were observed for the growth performance, indicating that the chromium and somatotropin effects were independent (Anderson et al. 1997). Chromium supplementation increased tissue chromium in the liver and kidney but the increase was not observed in the heart or muscle in either the control or ppST treated animals. Somatotropin treatment caused decreased kidney and liver chromium concentrations. The authors suggested this might be due to the increased

tissue size due to somatotropin treatment. The analyzed chromium concentration in the basal diet was reported to be 2,700 μg/kg diet (Evock-Clover et al. 1993).

Myers et al (Myers et al. 1995) conducted a similar experiment on Landrace – Poland China gilts with an initial weight of 20 kg fed to a final weight of 90 kg in a 2 x 2 factorial design. The pigs were fed a basal diet plus 300 μg of supplemental chromium as chromium picolinate/kg corn soybean meal diet. Administration (i.m.) of 100 μg/kg body weight/d of ppST was begun at 60 kg of body weight and was continued through 90 kg. The ppST treatment improved growth performance when compared to controls with no added chromium in the diet, but no effects of chromium supplementation on performance were observed. The chromium concentration of the basal diet was not mentioned (Myers et al. 1995).

Chromium and Immune Response:

Van Heugten and Spears (van Heugten and Spears 1997) investigated the immune response of chromium supplemented pigs challenged with lipopolysaccharide (LPS) endotoxin. Weanling pigs were fed diets containing 200 μg supplemental chromium/kg diet either as Cr-chloride, Cr-picolinate or Cr-nicotinic acid complex or a basal diet with no added chromium for a period of 34 days. On days 7, 10 and 13 of the supplementation period the weanling pigs were challenged with 200 μg LPS (055:B5)/kg body wt. Chromium supplementation either as $CrCl_3$ or chromium picolinate increased in-vitro lymphocyte blastogenic response. Antibody response to sheep red blood cells tended to be increased in Cr-nicotinic acid supplemented pigs on day 14, but antibody response to ovalbumin was decreased on day 14 in pigs supplemented with both organic forms of chromium. No chromium x LPS interaction was observed on cell mediated or on

humoral immune responses. The chromium content in the basal diet was 5.17 ppm. The authors suggested that this high value might be due to processing of the feed ingredients and contamination due to mixing. The authors also thought that the bioavailability of chromium from these feed sources might be low (van Heugten and Spears 1997).

Myers et al (Myers et al. 1997) demonstrated that chromium supplementation provided protection against the adverse metabolic effects of i.v. administration of 20 μg LPS (055:B5) /kg in Poland China X Landrace pigs. Pigs were fed a diet containing 300 μg chromium as chromium picolinate /kg corn-soybean meal diet with or without 100 μg/kg body weight of growth hormone administered intramuscularly. In all pigs an elevation was observed in TNF-α concentration at 2 h postinjection of LPS. Interactive effects of chromium supplementation and ppST lowered TNF-α concentration with growth hormone treatment. No effects of the treatments were observed on IL-6 concentration. Overall the values peaked at 2-6 hours post injection and dropped by 8-h post injection. Earlier Myers et al (Myers et al. 1995) reported that the IL-6 activity at 3 h PI was higher (8130 U/ml) in chromium picolinate treated pigs challenged with LPS (i.v. 0.2 μg LPS/kg) compared to pigs not treated with chromium (1927 U/ml). Also, the peripheral blood mononuclear cells (PBMC) from chromium picolinate treated pigs produced more IL-2 than the PBMC from the pigs fed a basal diet. In this trial, there was no effect of the chromium picolinate treatment on TNF-α concentration. The inconsistency of the results on the effect of chromium supplementation on IL-6 between the two experiments might be due to the different doses of LPS administered, 20 μg/kg (Myers et al. 1997) vs 0.2 μg/kg (Myers et al. 1995). However, the authors did not discuss

the difference in the results. The chromium concentration in the basal diets was not reported.

Lee et al (Lee et al. 2000) investigated the effects of chromium supplementation (400 μg / kg corn soybean meal diet as chromium picolinate) and a repeated challenge of lipopolysaccharide on the immune response of weanling pigs. The 4 week-old weanling pigs were supplemented with either 0 or 400 ppb chromium for a period of 38 days. On day 21 and on day 35 of the supplementation period, the pigs were challenged with 200 μg LPS/kg body weight intraperitoneally. The rectal temperature measured on day 21 before LPS challenge was significantly lower in the chromium supplemented group compared to the controls. Delayed plasma cortisol release was observed with chromium supplementation in weanling pigs measured on day 35. The authors did not report any data on humoral or cell mediated immune responses to chromium supplementation or LPS challenge.

Gentry and coworkers (Gentry et al. 1999) studied the immune response in Suffolk lambs fed 0 or 400 μg/kg diet of supplemental chromium as chromium picolinate for a period of 84 days along with a diet containing low protein (9 to 12.1% crude protein) or high protein (12.8 to 14.4% crude protein). Chromium supplementation with the high protein diet increased blood platelets, fibrinogen and erythrocyte counts on day 66 of the supplementation period. Isolated peripheral lymphocyte counts showed a higher blastogenic response to 4 μg/ml of phytohemagglutinin. The lymphocyte response to pokeweed mitogen (0.2 μg/ml) and plasma cortisol was reduced in lambs supplemented with 400 μg chromium/kg diet. The results of this study indicate that the levels of protein in the diet influence the effectiveness of dietary supplementation with chromium.

Arthington et al (Arthington et al. 1997) studied the influence of chromium on cortisol and immune responses in young calves (6 to 8 weeks old) inoculated with bovine herpesvirus-1 (BHV-1). The calves were supplemented with 3-mg chromium/day as high chromium yeast for a period of 53 days. The concentration of high chromium yeast was 2.6% of the basal diet. On day 53 of the supplementation period all calves were intranasally inoculated with BHV-1. Increased rectal body temperature was observed with BHV-1 administration in all the animals. Chromium supplementation did not have any effect on the lymphocyte proliferative response to mitogens (100 ul/well of pokeweed, phytohemagglutinin, or concanavalin A) or on neutrophil bactericidal function. The acute phase proteins, ceruloplasmin and fibrinogen, were not affected by treatment. Plasma cortisol and TNF-α were not affected by either chromium supplementation or by BHV-1. The lack of TNF-α stimulation to BHV-1 induced fever suggests that BHV-1 induced fever might be due to different mechanisms than endotoxin induced fever.

The effects of supplemental chromium, 500 μg/day as chelated chromium, from 6 week prepartum (wk –6) to 16 weeks postpartum (wk 16) in dairy cows was studied by Burton et al (Burton JL et al. 1993). The humoral response was assessed by immunization of all calves with ovalbumin (OVA) and human erythrocytes (HRBC) at wk –2 (late pregnancy) and wk 2 (early lactation), and the sera were assayed weekly for antigen specific antibodies. The anti-OVA antibody of the IgG response was higher in the chromium supplemented calves. In contrast the anti-HRBC antibody titers in detection of immunoglobulin M (IgM) antibodies were not different in the chromium supplemented group compared to the unsupplemented group. Cell mediated immunity was also assessed in vitro, using antigen (OVA) and mitogen-stimulated peripheral blood mononuclear cell

(PBMC) blastogenesis, with cells collected biweekly from wk –2 to wk 6 (peak milk yield). The mitogen stimulated blastogenic responses of PBMC were higher in the chromium-supplemented group compared to the controls. The authors demonstrated that the supplementation of chromium could alter humoral and cell-mediated immune responses of stressed cattle (Burton et al. 1993).

Moonsie-Shagger and Mowat (Moonsie-Shangeer and Mowat 1993) studied the effect of 0, 200, 500 and 1000 μg/kg diet of supplemental chromium, in the form of high chromium yeast, on the immune response in transportation-stressed feeder calves, fed for a period of 30 days after transportation by truck. Blood was collected at 0, 7, 14, 21 and 28 days of the supplementation period. The primary antibody response to HRBC was higher for chromium treated animals at day 14 compared to day 0. Levels of IgM and IgG_2 were not affected ($P>.05$) by chromium treatment. IgG_1 tended ($P<0.06$) to be higher with chromium supplementation. Chromium supplementation decreased morbidity and rectal body temperature at day 2 and day 5, increased hemoglobin and hematocrit on day 14 and 21, and decreased serum cortisol on day 28. However the effect of chromium supplementation on cell mediated immunity was not reported (Moonsie-Shangeer and Mowat 1993).

Chang and Mowat (Chang and Mowat 1992) investigated the effect of supplementation of 400 μg chromium as high chromium yeast/kg diet on the immune response in Charolais-crossed calves with a body weight of 245 kg. On day 28 of the supplementation period, the calves were vaccinated with infectious bovine rhinotracheitis and with parainfluenza. Two weeks later the steers were re-randomized within chromium treatment groups into soybean meal versus corn silage diet, with supplemental chromium

of 400 μg/kg diet, until day 70 of the experimental period. The chromium content of the diet was between 12.0 to 12.12 ppm on a dry matter basis in all the diets. Chromium supplementation in corn silage or in the soybean meal diet decreased serum cortisol. Chromium had no effect on the immunoglobulins with the corn silage diet but increased ($P<0.05$) serum IgM and total immunoglobulins and tended to increase IgG_1 ($P<0.22$) and IgG_2 ($P<0.17$) in steers fed soybean meal (Chang and Mowat 1992).

In summary, compared to ruminants, the immune response to chromium supplementation of non-ruminants was different. The humoral immune response was improved by chromium supplementation in ruminants. In nonruminants the results were inconsistent. The studies done on calves predominantly used high chromium yeast as the source of chromium, whereas studies conducted on pigs mainly used chromium picolinate. Studies included 200 to 1000 μg chromium/kg diet from different sources.

Chromium and Metabolic Responses:

Animal Studies:

Guan et al (Guan et al. 2000) studied the effect on glucose tolerance of high chromium yeast at a concentration of 0.34% of diet (200 μg chromium as high chromium yeast /kg diet) for a 30 day supplementation period in 47.9 kg Landrace x Yorkshire pigs. A fasting IV glucose tolerance test was conducted with 500 g/L of dextrose infused through the jugular vein at a dose of 0.5 g glucose/kg body weight. The infusion was completed within 6 minutes. Plasma glucose was lower and plasma insulin was higher in the chromium supplemented group compared to the pigs fed the basal diet. Glucose, insulin and C-peptide clearance rates were increased with chromium supplementation.

The authors concluded that the supplementation of chromium in the form of high chromium yeast improved glucose tolerance (Guan et al. 2000).

Evock-Clover et al (Evock-Clover et al. 1993) studied twenty-four castrated male pigs in a 2 x 2 factorial arrangement of treatments. The diets consisting of a basal diet; basal diet plus 300 μg chromium/kg; basal diet plus 100 μg/kg/d of pituitary porcine somatotropin (ppST) (intramuscularly), and basal diet in combination with 300 μg chromium picolinate and 100 μg/kg/d ppST. The pigs were fed for 6 weeks from 30-60 kg body weight. Chromium supplemented pigs had lower serum glucose and insulin levels and higher cholesterol levels compared to the controls. Chromium supplementation normalized the elevated insulin and glucose induced by ppST. However, no significant ppST x Cr interactions on glucose or insulin were observed. Serum somatotropin (pST) increased with ppST treatment. Chromium in combination with ppST further elevated pST levels. The IGF-1 concentrations were also increased by ppST administration. An increase in the IGF-1 concentrations was observed with the duration of treatment or as animal weight increased. No change in IGF-1 was observed with chromium supplementation alone. The authors suggested that change in tissue insulin sensitivity and serum insulin balanced by chromium picolinate supplementation might be the cause of lack of elevation in the IGF-1. Somatotropin treatment increases the IGF binding protein-3 (IGFBP-3), the main storage protein for circulatory IGF-1. Increasing insulin levels would indirectly decrease the IGF binding proteins IGFBP-1 and -2. The IGFBP-1 and -2 carry IGF-1 across the vasculature and into the tissues thereby decreasing the circulating IGF-1. Chromium treatment lowered the high insulin levels caused by the ppST treatment

and indirectly decreased the IGF binding proteins-1 and -2 thereby increasing circulating IGF-1.

Myers et al (Myers et al. 1997) conducted a similar study with twenty-four castrated male pigs, in a 2 x 2 factorial arrangement of dietary treatments. The treatments consisting of a basal diet (-Cr, -ppST); basal diet plus 300 μg chromium/kg; basal diet plus 100 μg/kg body weight/d of pituitary porcine somatotropin (ppST) (intra-muscularly), and basal diet in combination with 300 μg chromium picolinate (CrP) and 100 μg/kg/d ppST. The pigs were fed from 20-60 kg body weight. The ppST treatment was administered to pigs once they reached 60 kg body weight and was continued untill they reached 90 kg body weight. At 90 kg body weight, all the pigs were given an intravenous dose of 200 μg LPS (055:B5)/kg body weight. Chromium supplementation along with somatotropin treatment (+Cr, +ppST) or somatotropin supplementation alone (-Cr, +ppST) increased serum glucose and insulin levels compared to the controls (-Cr, -ppST) or chromium alone (+Cr, –ppST) treated pigs after LPS challenge. Nonesterified fatty acids and urea nitrogen were significantly higher in the controls (-Cr, -ppST) and chromium alone supplemented pigs (+Cr, -ppST) compared to ppST alone (-Cr, +ppST) or in combination with chromium (+Cr, +ppST) treated pigs after LPS challenge. The authors suggested that pST and to a lesser extent CrP provide protection against the adverse metabolic effects of LPS.

Ward et al (Ward et al. 1997) studied lysine requirements and chromium supplementation in 27.2 kg pigs supplemented for a period of 21 days with either 0 or 400 μg chromium as chromium picolinate/kg diet providing 80% or 100% of the lysine requirement. The authors found no significant effect of chromium supplementation

independent of dietary lysine requirement on plasma urea nitrogen, cholesterol, glucose, nonesterified fatty acids, insulin or growth hormone.

Mooney and Cromwell (Mooney and Cromwell 1997) studied the effects of supplementation with different forms of chromium either as chromium chloride (5000 or 25,000 μg chromium/kg diet) or as chromium picolinate (0, 200, 400 μg chromium/kg diet). Hampshire-Yorkshire pigs with an initial body weight of 20 kg were fed supplemented diets for a period of 35 days. The source or the concentration of chromium had no effect on the serum metabolites measured; i.e., urea nitrogen, protein, triglycerides, glucose and nonesterified fatty acids (Mooney and Cromwell 1997).

Amoikon et al (Amoikon et al. 1995) supplemented the diets of 21-25 kg cross bred barrows, fed 120% of their lysine requirement with 200 μg chromium /kg diet as chromium picolinate. No effect of chromium supplementation on glucose or protein was observed. Decreases in cholesterol, nonesterified fatty acids, urea nitrogen and insulin were observed with chromium supplementation. The fasting IV glucose tolerance test (500 mg glucose/kg body wt) showed an increase in glucose disappearance rate and a decrease in glucose half-life with chromium supplementation. No effect of chromium supplementation on the fasting insulin challenge test (0.1 IU of porcine insulin/kg body wt) was observed.

Human Studies:

Beneficial effects of chromium supplementation in various human disease conditions have been reported since the early 1960's. Hopkins et al (Hopkins et al. 1968) reported an improvement in impaired carbohydrate metabolism in malnourished infants in Jordan and in Nigeria with chromium chloride supplementation. Twelve infants, when

administered an oral dose of 250 μg of chromium as chromium chloride, showed an improved glucose clearance rate. The study did not include a control group.

Pekarek and coworkers (Pekarek et al. 1975) studied the relationship between serum chromium concentrations and glucose utilization in normal subjects and in subjects infected with sandfly virus. The subjects were provided with a 300-g carbohydrate diet, which was maintained throughout the study period of three days, and on day 3 the subjects were infected with sand fly virus. The fasting intravenous glucose tolerance test (IVGTT) consisting of 30 g glucose over a five-minute period showed increased glucose and insulin concentrations and decreased serum chromium concentrations in infected subjects compared to the controls. The serum chromium concentration dropped significantly by 15 minutes following the glucose load (1.5 ppb to 0.85 ppb). The decrease in chromium concentration with glucose load indicates the relationship between glucose and chromium metabolism (Pekarek et al. 1975). Although the serum chromium concentrations were erroneously high due to the limited analytical methods available during the period of study (1974), the relative comparisons between the treatments are interesting.

Carters et al (Carters et al. 1968) studied thirty-four children with kwashiorkor who were supplemented with 250 μg chromium (III) as chromium chloride in an aqueous solution daily for 1-3 days. The glucose tolerance curves failed to return to normal following the oral doses of chromium indicating that impaired glucose utilization during protein calorie malnutrition is due to not only chromium deficiency but also due to other disease conditions (Carters et al. 1968).

Gurson and Saner (Gurson and Saner 1971) investigated the effects of chromium supplementation on glucose utilization in children with protein calorie malnutrition. The authors reported that the glucose removal rate was increased following the oral administration of a single dose of 250 μg of $CrCl_3$ (Gurson and Saner 1971).

In summary, the reported studies included various levels of dietary chromium in swine diets. Some studies included levels of dietary chromium from 0 to 1000 μg chromium/kg diet as chromium picolinate. Other studies have included more than 1000 μg chromium/kg diet, but in the form of chromium chloride. The recommended levels of chromium for swine diets have yet to be defined. Reported studies on chromium supplementation and its effect on the immune response in pigs are very scanty. However, supplementation of chromium was reported to be beneficial during disease conditions like protein calorie malnutrition. The pig would be a good model to study the effects of nutrient supplementation on growth and on the immune system during disease conditions. The weanling pig is a useful experimental model in human nutrition because of its anatomical similarities of the gastrointestinal system compared to humans. Thus, disease condition such as infection can be simulated in the pig and the results can be extrapolated.

Endotoxin

The following section describes the structure of endotoxin. A brief review of effects of endotoxin induced infection on cytokine, hormonal, and plasma mineral responses were discussed. Acute phase response to endotoxin was found to depend on the strain of the bacterium, dose of endotoxin, and route/site of administration. Hence studies conducted on pigs, with different doses and strains of endotoxin were summarized including the effects on cytokine, hormonal and macro- and micronutrient metabolism.

Theodor Rietschel and Helmut Brade (Rietschel and Brade 1992), pioneers in endotoxin research, described endotoxins as the toxins present in the integral part of the bacteria that were responsible for cholera, whooping cough, and meningitis. Endotoxin resides on the outer membrane of the Escherichia coli, a gram-negative bacteria. The lipid component, lipid A, is embedded in the outer membrane of bacterial cells and consists of a two layered structure with an oligosaccharide containing an O specific chain present on the outer part and an inner lipid bilayer. The O-specific chain is the most variable segment and is the part that evokes specific immune responses. The structure of O specific chain is differs between strains of E.coli.

Bacterial endotoxins were found to cause an imbalance in the homeostasis of the host metabolism. Endotoxins evoke their effects by stimulating macrophages to produce three groups of mediators: cytokines (Johnson 1998), oxygen free radicals and lipids (Li et al. 1994). These mediators acting independently, together, or in sequence lead to various effects in the host. When the mediators are over-produced harmful effects including high fever, hemodynamic changes, and alterations in gut permeability occur. Numerous dose dependent studies on the endotoxin and acute phase response have been reported in the literature. Elevation in body temperature, cytokines and cortisol, and decreased circulating IGF-1 and food intake were some of the symptoms observed in pigs due to LPS endotoxin.

Endotoxin and Feed Intake:

Metabolic changes associated with infectious diseases or inflammatory processes can result in decreases in gain and feed efficiency. In 1994 Johnson and von Borell (Johnson and von Borell 1994) conducted a study on cross-bred barrows to observe the

behavior of the animals when induced with endotoxin. Pigs, 46. 2 kg body weight, were injected i.p. with E.coli (K-235) at a dose of 0, 0.5, 5 or 50 μg /kg body wt. The authors found decreased food intake in pigs at 1 to 2 h post injection. The barrows injected with 0.5 μg/kg consumed less feed than controls. When compared to the 50 μg LPS/ kg endotoxin group the 0.5 μg LPS/kg consumed more feed. The decreased feed intake in 50 μg /kg group was observed even at 4 to 6 h post injection. A dose dependent effect of LPS was observed on feed intake.

The effect of repeated injections of LPS on feed intake and performance in younger animals was studied by van Heugten and Spears (van Heugten et al. 1994). The 21-d old, 6.0 kg pigs were injected i.m. with 200 μg /kg LPS E.Coli 055:B5 on day 7 and 21 of the experimental period. Body weights recorded over the five week period reaveled that the average daily gain (ADG) and average daily feed intake (ADFI) and gain : feed decreased during week 2 (7 to 14 d post injection) and ADG decreased during week 4 (21 to 28 d post injection) with LPS administration. No effects of LPS were observed during weeks 3 or 5 of the experimental period. The results suggest that pigs recover from infection due to LPS within a week as observed by no differences in the ADG, ADFI or gain: feed during week 3 post injection. Subsequent studies by van Heughten and Spears and Wright et al (van Heugten et al. 1996, van Heugten and Spears 1997), on the effects of repeated challenges with LPS on feed intake and daily gain confirmed the results of the earlier studies that LPS reduces the feed intake and daily gain.

Endotoxin and Body Temperature:

Increases in body temperature were observed with increasing doses of endotoxin. Johnson and von Borell (Green and Vermeulen 1994) injected 46 kg cross-bred barrows

with LPS E.coli K-235, i.p. at a dose of 0, 0.5, 5 or 50 μg /kg body weight. The peak in body temperature was observed at 4 h post injection. The change in body temperature was greatest in the 50 μg /kg pigs compared to 0, 0.5, or 5 μg LPS/kg group. The increase in body temperature was linear with the dose of endotoxin.

More lethal doses of LPS E.Coli 055:B5, were used by Normatsu et al (Normatsu et al. 1995) on two month old, 20-30 kg body weight pigs. The pigs were infused i.v. with varying LPS doses of 2 (n=1), 0.5 (n=2), 0.0625 (n=3), 0.0078 (n=3), 0.001 (n=2), or 0.0001 (n=1) mg/kg body weight. The pigs, infused with 2 or 0.001 mg LPS, had elevated body temperatures (>41°C) between 1 and 3 h post injection. All the pigs injected with more than 0.001 mg LPS/kg body weight showed elevated body temperatures by 24 h post injection. The piglet with highest dose (2 mg LPS/kg) of LPS died at 18 h post injection. The results suggest that 2 mg /kg is a lethal dose of endotoxin. However, the sample size used for each dose needs to be considered while comparing the results.

A lower dose of endotoxin was used by Parrot et al (Parrots et al. 1997)on boars weighing 30 kg. The LPS E.coli 026:B9 was given i.v. at a rate of 0.7 μg /kg body wt and the body temperatures were recorded over 3 h time period. Body temperature was significantly elevated by 60 minutes post injection and started to decrease by 2.5 h post injection. The results suggested that the effect on body temperature with lower doses of LPS is much shorter and that an intravenous dose produces more rapid elevations in body temperature.

More recently, Wright et al (Wright et al. 2000) investigated the immune responses of growing pigs in response to an endotoxin LPS E.coli 055:B5 challenge. Weaned pigs, 21 d old, 10.9 kg, were challenged intraperitoneally with 100 μg LPS/kg

body weight or sterile saline. Rectal temperature was measured until 72 h post injection. Elevated rectal body temperature was observed between 2 and 12 h post injection. No differences in body temperatures were observed at the remaining time periods between the control and the endotoxin groups. The continued elevation in body temperature even at 12 h post injection observed in this study may be due to the administration of a high dose of LPS.

Endotoxin Effects on Cytokines and Hormones:

Cytokines, a class of molecules including interferons and interleukins, are released in response to bacteria, viruses and parasitic organisms. Numerous dose dependent studies on endotoxin and cytokine response have been reported in the literature. Table 1, summarizes a few studies using different experimental models, and different doses and strains of LPS. Because of the differences in the experimental protocols it is difficult to compare the results.

Age related differences in response to endotoxin were studied in 1 to 3 day old and in 2 to 3 week old piglets Li et al (Li et al. 1993). The piglets in both age groups were infused LPS E.coli 0111:B4 /kg body wt at 10 minute intervals. The 1-3 day old piglets, body weight of 1.8 kg, were infused with 10 mg LPS. The second group of pigs, 2 to 3 weeks age, body weight of 4.3 kg, were infused with 5-10 mg LPS/kg body weight. The peak in TNF-α concentration was observed at 60 minutes in the both groups. However the younger pigs had decreased TNF-α concentrations compared to the older pigs. The authors concluded that age related responses are due to the anatomic and physiologic immaturity in the developing piglet.

Normatsu and coworkers (Normatsu et al. 1995) investigated the effects of LPS on the immune system of piglets with special reference to TNF-α and cortisol responses. Two-month old, piglets, 20 to 30 kg body wt. were injected with E.coli (055:B5), i.v. at doses of 2.0 (n=1), 0.5 (n=2), 0.0625 (n=3), 0.0078 (n=3), 0.001 (n=2) or 0.0001 (n=1) mg LPS/kg body wt. Blood samples were collected at 0, 1, 3, 6, 9, 24, 48, and 120 h post injection. The results demonstrated a dose dependent increase of TNF-α and cortisol levels in serum. The TNF-α peak was observed at 1 hr post injection of LPS; however, in the piglet injected with 2 mg LPS/ kg the TNF-α values remained elevated until 6 h post injection. TNF-α values were not elevated in piglets injected with 0.0001 mg of LPS per kg body weight indicating a dose dependent response. Serum cortisol peaked at 1 to 3 hr post injection and returned to normal by 9-h post injection except in the pigs injected with 2 mg of LPS.

Gray et al (Gray et al. 1996) studied the effect of dose on the immune response and persistence of Salmonella choleraesuis infection in weanling pigs, 7 weeks of age. The pigs were exposed to Salmonella choleraesuis intranasally at a dose of 10^9, 10^6 or 10^3 colony forming units/ ml. The pigs in all the groups had elevated serum IgG and IgM titers after exposure to Salmonella choleraesuis polysaccharide and had soluble antigens and lymphocyte response to concanavalin A, as well. The pigs with 10^9 CFU/ml had a lower stimulation index in response to antigens, indicating some form of immunosuppression due to Salmonella choleraesuis infection.

Roth and coworkers (Roth et al. 1993) reported the time course action of interleukin 6 and TNF- α in plasma of guinea pigs injected with 20 μg LPS/kg body wt (strain not mentioned). A biphasic response of IL-6 was observed with endotoxin, with a

peak concentration at 3 and 6 h post injection. The elevation in TNF-α activity in plasma was observed at 1-h post injection and by 3 h post injection the values rapidly declined to 15-20% of the peak. The TNF-α levels were undetected at 5 hr PI.

In contrast Meyers and coworkers (Myers et al. 1999) reported no effect of LPS on cytokines in pigs in different growth stages. The growing (35 kg body weight) and finishing (85 kg body weight) pigs were challenged with endotoxin i.v. (LPS E. coli O55:B5) at a dose of either 2 or 20 μg/kg. A peak in TNF-α was observed in a dose dependent response. The peak TNF-α in plasma was observed 1-2 hr post-injection, and the values returned to basal values by 4 hr post-injection. Peaks in plasma interleukin-6 levels were observed at 1-2 hr post injection and remained elevated through 8 hr post injection. The authors observed no dose or age effect.

More recently, Wright and coworkers (Wright et al. 2000) observed the immune responses of growing pigs in response to a endotoxin (LPS E.coli 055:B5) challenge. Weaned pigs (21 d, 10.9 kg), were challenged intraperitoneally with 100 μg LPS/kg body weight or sterile saline. Rectal temperature was measured and blood samples were collected for 72 h. Daily feed intake also was monitored. Decreased feed intake was observed with LPS challenge compared to control pigs. Cortisol in response to LPS reached a peak at 2 h post injection and remained elevated through 12 h. Circulating TNF-α was increased by LPS between 2 and 4 h post injection. Overall, GH was increased in LPS-treated pigs. Plasma prostaglandin E_2 (PGE_2) increased transiently at 2 h post injection of endotoxin but dropped below baseline levels at 4, 8, and 12 h post injection. This study provided a comprehensive view of systemic effects of LPS on components of the hypothalamo-pituitary-adrenal axis, growth, and immune axes.

Endotoxin and Insulin like Growth Factor (IGF-1):

Serum IGF-1 concentrations are associated with growth rate and feed intake of animals. The decreased feed intake observed with LPS induced infection might affect IGF-1 concentrations. The effects of LPS on weanling, growing and finishing pigs were reported.

The effect of endotoxin on IGF-1 concentrations were studied in weanling pigs (Wright et al, 2000). The objective was to observe the immune responses of growing pigs in response to an endotoxin (LPS E.coli 055:B5) challenge. Weaned pigs, 21 d old, 10.9 kg, were challenged intraperitoneally with 100 μg LPS/kg body weight or sterile saline. The LPS injection suppressed IGF-1 by 2 h post injection and remained reduced relative to controls through 44 h post injection.

Hevener and coworkers (Hevener et al. 1999) conducted a study on the effects of immune challenge on concentrations of serum IGF-1 and growth performance in growing (38.5 kg) pigs. Repeated injections of LPS E.coli, 055:B5, at a dose of 5 mg/kg i.m. were administered two times a day, on day 0 and day 1, and on days 2 and 3 the dose was increased to 10 mg/kg and injected twice a day. Blood samples were collected on day 0, day 4, and weekly thereafter for a period of 8 weeks. No effect of endotoxin on IGF-1 was observed. The authors suggested that the repeated doses of endotoxin might have neutralized the effect of a single dose of endotoxin.

Hevener et al (Hevener et al. 1999) studied the effects of acute endotoxemia on serum IGF-1 in prepubertal gilts. Eight female, finishing pigs (98 kg body wt) were injected i.v. with LPS E.coli 055:B5 (5 μg LPS/kg) or with saline. Blood samples were collected at 1 h intervals for 6 h and at 12, 15, 18, 24, 48, and 72 h. The IGF-1

concentrations decreased at 2 h (130 ± 9 ng/ml) and at 6 h (120 ± 5.5 ng/ml) post injection compared to pretreatment (192 ± 29) values. The LPS group had lower IGF-1 values than the control group at 2 h, and the values remained lower until the end of the experiment (96 h) compared to controls.

The above three studies differed in age of animals, route of administration and dose concentrations but used the same strain of E.coli. A lower dose (50 or 100 μg LPS/kg i.m.) in growing pigs did not have any effect on IGF-1 while larger dose (100 μg LPS/kg i.p.) in weanling pigs or an smaller dose (5 μg LPS/kg i.v.) in finishing pigs reduced the serum IGF-1 concentrations. The results suggest that a intramuscular route of administration of LPS might require a dose greater than 100 μg LPS/kg to have significant effect on IGF-1 concentrations.

Endotoxin and Macronutrient Metabolism:

Stress resulting from infection or trauma induces a hypermetabolic state of increased energy expenditure and urinary nitrogen loss. In liver, the immediate metabolic response to infection/inflammation is the shift towards catabolism. Initially, glycogen is metabolized to glucose to sustain normal glycemia in response to reduced food intake (van Heugten and Spears 1997, Gray et al. 1996). Fat oxidation is increased to provide energy through alternative fuels (Burns 1988). After glycogen depletion, glycemia can only be maintained by gluconeogenesis from lactate and amino acids (Wolfe 1997). During infection, priorities for amino acid metabolism must be balanced between albumin synthesis, acute phase protein synthesis and energy metabolism.

Lang et al (Lang et al. 1985), observed that i.v doses of 1000, 100, 10, 1, 0.1 or 0.01 μg LPS, Ecoli, 026:B6/100 g body weight in rats produced a transient but

pronounced hyperglycemia in the 1000 μg LPS group. The response to endotoxin was seen 1-1.5 h after administration. Increases in plasma lactate were also observed in the experimental groups treated with endotoxin at doses of 1000 to 0.1 μg/100 g body weight. However, the increases in lactate values were dose dependent. Hypoinsulinemia and hyperglycemia were observed in the animals at 1.5 and 4 h in 1000 μg/kg endotoxin group. The elevation in body temperature was observed only in rats with the lower doses of endotoxin (10, 1, or 0.1 μg), while the lethal doses (1000 and 100 μg) produced hypothermia. The authors suggested that the elevations in plasma concentrations of norepinephrine following endotoxin might precede changes in the gluco-regulatory hormones (glucagon, insulin, and cortisol) which might lead to hyperglycemia and hyperlactemia.

Previous studies on the changes in muscle protein synthesis in infection are conflicting. Jepson and coworkers (Jepson et al. 1986) reported the effects of endotoxin challenge on protein catabolism in fasted and in fed rats. Rats were challenged with 0.3 mg/100 g body weight of LPS, Ecoli, 0127:B8. Protein synthesis decreased in muscle and increased in liver after endotoxin treatment. Also, the insulin values were elevated with endotoxin treatment, and glucose was decreased with endotoxin challenge. The authors concluded that insulin resistance in muscle could lead to decreased protein synthesis, and the increase in the liver protein synthesis was due to increased synthesis of acute phase proteins.

Gaetke et al (Gaetke et al. 1997)reported a study in which six men and six women received two i.v. doses of 20 units/kg of endotoxin (lot EC-5) 24-h apart. The acute phase

proteins were elevated throughout the study period after LPS injection. But, serum albumin did not change with LPS administration from baseline to 72 h post injection.

Gutterberg et al (Gutteberg et al. 1989) measured plasma lactoferrin (LF) in piglets during gram negative septicemia and endotoxemia. The results indicated that different time periods are required to elicit a host defense mechanism depending on the route of administration. Thirty minutes after an i.v. infusion of 0.25mg of LPS, Ecoli, 026:B6/kg to piglets had increased plasma lactoferrin compared to baseline, and there were no significant changes in the intraperitoneally injected group. In pigs the live infusion of E.coli did not significantly decrease plasma lactoferrin until 120 minutes after infusion.

Endotoxin and Plasma Minerals:

Bacterial infections have been demonstrated consistently to alter plasma levels of various trace elements. The alteration in trace element concentrations was postulated to be an intergral mechanism in the host as an acute phase response (Exton et al. 1995).

Plasma iron in patients or animals with infection decreases rapidly and has been shown to be directed to the liver and spleen instead of the bone marrow (Kampschmidt et al. 1965). Injection of 100 μg E.coli (055:B5) /200g body weight i.v. to rats produced a rapid decrease in plasma concentration of iron within 30 minutes, and the decrease continued until a minimum was obtained at 6 to 12-h after injection. In addition, there was an inhibition of the movement of iron from catabolized red blood cells to plasma transferrin. The authors stated that injection of endotoxin inhibited the reutilization of iron from recently destroyed erythrocytes resulting in a decrease in plasma iron (Kampschmidt et al. 1965).

Small doses of bacterial endotoxins have been shown to produce marked hypoferremia in chickens injected with 1 mg LPS, E.coli, 0111:B4 /kg body weight (Butler and Curtis 1973). Butler and Curtis suggested the hypoferremia might be caused partly by an increase in the rate of plasma iron uptake by the liver and spleen and also by the blockage in iron transfer from the reticuloendothelial system to the plasma, thereby inhibiting the utilization of iron from catabolized hemoglobin. Tufft et al (Tufft et al. 1988) injected chicks with 0.5 and 2.0 mg endotoxin LPS Ecoli, 055:B5 /kg body wt. and found that chicks given endotoxin had decreased iron and zinc levels at 4-h PI independent of dose. Increased serum copper was observed at 8 h PI. Serum iron continued to decrease at 8-h, but plasma zinc started to return to normal by 8-h PI. The authors reported that infection and endotoxin led to redistribution of trace minerals with a decrease in serum levels of zinc and iron and an increase in zinc and iron concentrations in spleen.

Gutterberg and coworkers (Gutteberg et al. 1989) measured plasma iron in piglets during gram negative septicemia and endotoxemia. Thirty minutes after i.v. infusion of 0.25mg/kg body wt. LPS Ecoli, 026:B6, plasma iron decreased compared to baseline; the same trend was observed in the i.p. injected pigs. In pigs, infusion of live E. coli did not significantly decrease plasma iron until 120 minutes after infusion.

Butler and Curtis (Butler and Curtis 1973) reported that effects of LPS on plasma minerals are dose dependent in chicks. Intravascular infusion of LPS Ecoli, 0111:B4 in doses ranging from 0.025 to 3.0 mg/kg, produced a large depression in plasma iron concentration as early as 4-h PI, and the depression was maximum at 12 to 16-h PI. The mean depression of plasma iron in the 0.025, 0.1, 0.5, 1.0, and 3.0 mg/kg groups was

50.1, 61.0, 77.0, 80.7, and 95.6% respectively indicating a direct relationship of drop in plasma iron with dose. Exton et al (Exton et al. 1995) could not demonstrate a dose dependent mechanism in rats injected i.p. with LPS (026:B6) of 100 or 400 μg/kg body weight, but there was a significant drop in plasma iron concentration compared to the control group. Exton et al (Exton et al. 1995) postulated that endotoxin generates acute phase responses through the production of interleukin 1 (IL-1), and, thereby, synthesizing prostaglandins which act on the peripheral organs and the CNS. It can be concluded that the cellular mechanism of iron deficiency due to infection might be partly controlled by molecules other than IL-1 such as prostaglandins.

Chromium:

The effect of administration of IL-1α on 51-chromium absorption in rats was reported by Davis et al (Davis-Whitenack et al. 1999). Ten rats were given an intraperitoneal dose of 1 μg /kg body wt. of IL-1α. After one hour an oral dose of 50 μl (200 μCi, 0.36 μg chromium) of $^{51}CrC_3$ was administered, and blood was collected at .25, .5, 1, 2, and 4 h after ^{51}Cr dose. The rats injected with IL-1α had decreased 51-chromium in blood compared to the control group of rats. The tissue retention of ^{51}Cr was also lower in the IL-1α group of rats compared to controls. The results suggest that endotoxin induced IL-1 can alter the plasma chromium.

Kamath et al (Kamath et al. 1997) observed the effects of prostaglandins and/or indomethacin on ^{51}Cr absorption. Rats were injected with either indometahcin or placebo (5mg /kg body weight). Thirty minutes later the rats were injected with 50 μg /kg of prostaglandin E_1 analog (misoprostol), 7.5 μg /kg body wt of prostaglandin E_2 analogue (16, 16-dimethylprostaglandin E_2) ($dmPGE_2$) or 20 μg /kg body wt of prostacyclin (PGI_1).

Immediately the rats were dosed with $^{51}CrCl_3$ (3.7 mBq). Blood was collected at 15 minute intervals for the first one hour and every one hour thereafter until 6 h of the experimental period. The ^{51}Cr in blood increased in rats treated with indomethacin compared to controls. The control group of rats had higher ^{51}Cr in blood compared to rats injected with PG_{12}, $dmPGE_2$, or misoprostol. The results suggest that prostaglandins decrease the absorption of ^{51}Cr. Treatment with indomethacin increased ^{51}Cr absorption by blocking the cyclooxygenase pathway of prostaglandins synthesis.

Zinc:

Gaetke et al (Gaetke et al. 1997) reported a decrease in plasma zinc levels in human volunteers exposed to LPS endotoxin. Six men and six women received an i.v. dose of 20 units/kg of endotoxin (lot EC-5), two times. The second dose was administered at 24 h following the first dose. The authors found that the plasma zinc decreased during the first 6 h PI; the concentrations started to increase by 12 h PI and reached the baseline values by 24 h PI. When the second dose of LPS was administered 24 h following the first dose, plasma zinc decreased in a similar trend as observed during the first dose suggesting the acute phase response occurred at 6 h PI.

A significant change in zinc metabolism was observed with stressful conditions like trauma or sepsis. Joung et al (Joung et al. 1998), reported decreased plasma zinc values in male trauma patients injured by motor accidents, head injury, open wounds, gun shots, or falls. The authors reported decreased hematocrit, hemoglobin, and increased body temperature and blood glucose. Renal function based on creatinine and urea nitrogen did not change with trauma. Plasma zinc decreased and plasma copper concentrations increased with trauma. The authors concluded that alterations in zinc and

copper concentrations in plasma during trauma might produce susceptibility to moderate zinc and copper deficiency.

Acute disease and ACTH were found to reduce serum concentrations of zinc in humans. Falchuk (Falchuck 1977) studied a total of 156, healthy and diseased subjects. Seven normal subjects were infused with 25 units of adrenacorticotropic hormone (ACTH) in the form of Cortrosyn administered over a 4 hour period. The serum zinc concentrations of the healthy men and women ranged from 90-118 μg/dl compared to the much lower values of 40 to 92 μg /dl in diseased subjects. The subjects whose serum zinc concentrations were greater than 70 μg/dl, decreased to 10 to 66 μg /dl with ACTH administration. The maximum decrease was observed within 4 hours after administration. This decrease was observed in all the patients, normal or diseased. Serum zinc chromatography showed a decrease in zinc fraction II with ACTH administration but the zinc fraction I did not change. Thus, the authors concluded that ACTH might have an important role in the reduction in serum zinc associated with disease states.

In summary it can be concluded that the LPS effects are time and dose dependent. The effects of LPS are varied and involve activation of cytokines, hormones, and liver proteins. Also, LPS induces alterations in plasma mineral concentrations. The effects of LPS were also observed on growth and on growth factors.

CHAPTER III

Methodology

A total of five experiments were conducted to study the effects of dietary supplementation with zinc and chromium and endotoxin challenge on the immune system in weanling pigs . The experiments 1 and 2 were conducted to determine the optimum dose of endotoxin required to produce an acute phase response in weanling pigs. The third experiment was conducted to study the effect of endotoxin on the absorption of $^{51}CrCl_3$ in weanling gilts. Experiments 4 and 5 were conducted using a 2 x 4 factorial arrangements of treatments in a randomized complete block design to study the effects of endotoxin in weanling pigs fed different levels of dietary zinc or chromium on the immune response. The procedures were approved by Oklahoma State University Animal Care and Use Committee (Appendix A).

Experiments 1 and 2

Experimental Designs:

Experiments 1 and 2: Fourteen-day old weanling pigs were obtained from the OSU Swine Research, Teaching and Extension facility. The pigs were matched for weight (5.0 ± 0.5 kg) and were placed in individual pens for a period of 7 d. The pigs were fed a known amount of a segregated early-weaning (SEW) diet (Table 1).

Table 3.1: Composition of Experimental Diets

Ingredients	% weight of feed mixed
AP-420	5.000
Whey, dehydrated	25.000
Lactose	4.750
Soybean oil	3.000
Soy Comil P	3.000
Dried skim milk	5.000
Egg protein	4.000
Corn, ground	29.550
Ethoxiquin	0.030
Fishmeal	8.000
Lysine, HCL	0.140
Methionine	0.150
Neo-Terramycin 10/5	1.000
Berry flavor	0.100
Zinc oxide	0.300
Vitamin premix	0.380
Dical	0.600
Biotin supplement	0.001
Steam rolled oats	10.000

Experimental Protocol

Pigs (5.0 ± 0.5 kg) were randomly assigned to endotoxin treatment or control groups in a completely randomized block design. On day 7 post-weaning, pigs were fasted 4 h and injected intraperitoneally (i.p.) with 9.0 g/L saline solution containing varying doses of LPS (E.coli, 0111:B4, Sigma, Inc., St.Loius, MO).

Blood samples were collected by venipuncture of the jugular vein into dipotassium EDTA treated vaccutainers. The two experiments differed only in endotoxin dose and blood collection time periods.

Experiment 1: Twenty pigs were injected i.p. with 0, 0.25, 0.5, 25 or 50 μg LPS/kg BW on day 7 post weaning. Blood samples were collected for analysis of plasma IGF-1, cortisol, zinc and iron concentrations and rectal body temperatures were recorded at 0, 3, and 6 h post injection (PI).

Experiment 2: This experiment was conducted to determine if a dose >0.5 μg LPS/kg but less than 25 μg LPS/kg would produce as strong an acute phase response as found at a dose of 25 μg LPS/kg body weight. Twelve 7 d post-weaning pigs were injected i.p. with a saline solution containing 0, 12.5 or 25 μg LPS/kg BW. Blood samples were collected for analysis of plasma TNF-α, IGF-1, cortisol, zinc and iron concentrations and rectal body temperatures were recorded at 0, 1.5 and 3 h PI.

Analytical Measures:

Plasma TNF-α was analyzed using a pig TNF-α ELISA Kit (Endogen Inc., Cambridge, MA). Plasma IGF-1 concentrations were analyzed by radioimmunoassay using the method previously described by Clutter et al (1995). Plasma cortisol was

analyzed using a solid phase radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA).

Plasma samples were wet and dry ashed using a modified procedure from Hill et al (Hill et al. 1986). Zinc and iron were analyzed using a Perkin Elmer Model 5100PC atomic absorption spectrophotometer with an air–acetylene flame and deuterium background correction (Perkin Elmer, Norwalk, CT).

Statistical Analyses:

Data were analysed using PROC MIXED in PC SAS (version 8.01 SAS Institute, Cary, NC) to determine the endotoxin effects in a completely randomized design with repeated measures. The model included dose of endotoxin, time, and pig within dose. the slice option was used to test the differences between treatments. Repeated measures were used for the time and dose interactions. An individual pig was considered an experimental unit. Data in this study are presented as Least Squares means. The significant difference was set at $P<0.05$. One pig (25%) succumbed at 6 hr PI with an LPS dose of 50 μg LPS/kg, and its data were excluded from the analyses.

Experiment 3

Experimental Design:

Ten Yorkshire gilts (14 d, 5.0 kg) were weaned and were allotted to the either endotoxin or saline groups in a completely randomized design. The pigs were housed in individual metabolic cages in an off site nursery for 7 days. During this period, pigs were fed SEW diet (Table 1) for 7 days.

Experimental Protocol:

On day 4 of this experimental period, the pigs were fasted for 12 h and anesthetized. A 2 mm silastic jugular catheter was inserted and passed subcutaneously to a dorsal position behind the ears. After the pigs recovered from anesthesia, they were returned to individual metabolic cages. On day 7 of the experimental period, pigs were fasted for 7 hours and injected intraperitoneally with 25 μg LPS/kg body wt (lipopolysaccharide from E.Coli serotype 0111:B4 suspended in saline) or with saline. One hour after the endotoxin or saline dose, the pigs were given an oral dose of 0.7 mCi of $^{51}CrCl_3$. Approximately 0.5 ml of blood was collected from the jugular catheter at 30, 45, 60, 120, 180, 240, 300, 360, 420 and 840 min after ^{51}Cr dosing. Rectal body temperature was recorded at the termination of the experiment. Eight hours after the ^{51}Cr dose, the pigs were given a dose of ketamine HCl (30 mg/kg body weight) (Aveco Co., Inc., Ft.Dodge, IA, USA) and Pentobarbital (1mg/kg body weight) and exsanguinated. Liver, kidney, heart, and spleen were collected, weighed and sampled at necropsy.

Analytical Methods:

Blood and tissue samples were counted in a gamma counter (Packard Instruments Co., Meriden, CT). Samples of the dose were counted repeatedly to allow correction for decay of ^{51}Cr. Total ^{51}Cr in blood was estimated assuming blood was 7% of body weight of pigs (Mount and Ingram, 1971). The ^{51}Cr in each tissue was expressed as percent of the ^{51}Cr dose.

Statistical Analysis:

Log transformations were performed on blood, urine and tissue data to correct for non-homogenity of variance and analyzed by SAS (version 8.01). The general linear model (GLM) procedure was used for analysis of variance of the transformed tissue and urine data. Repeated measures analyses by PROC MIXED (SAS version 8.01) were performed on the transformed blood data. Differences between means were identified using the least significant difference test.

Experiments 4 and 5

Experimental Designs:

Experiment 4: Four dietary zinc levels were set less than NRC level (0 ppm added Zn), Control (100 ppm added Zn) and excessive (1500 and 3000 ppm added Zn) with endotoxin or saline treatment. The animals were allotted to dietary treatments in a 2 x 4 randomized complete block design. Replicates were considered as blocks.

Experiment 5: Four levels of dietary chromium as chromium picolinate were: 0 μg added chromium, 200 μg added chromium, 600 and 1000 μg added chromium/kg diet with or with out endotoxin treatment. The animals were allotted to dietary treatments in a 2 x 4 randomized complete block design. Replicates were considered as blocks.

Animals and Housing:

Crossbred early weaned pigs (14 day old) were obtained from Oklahoma State University Teaching, Research and Extension facility for experiments 4 and 5. A total of 48 animals per experiment were randomly assigned to one of the four dietary treatments.

The early-weaned pigs were individually housed in a controlled environment in plastic metabolic crates during the 14-day experimental period. The temperature in the room was controlled at 85°F, and light was provided 24 hours a day to avoid diurnal variations.

Diets:

Pigs were fed a modified custom made milk replacer formula (no added trace elements at manufacture). The milk replacer was donated by Merick's Inc. (Union Center, WI). The milk replacer formula was analyzed for zinc and chromium concentrations before use. The mineral concentrations in the basal diets were 4.05 ppm zinc and 1.77 μg chromium. For experimental purposes trace mineral mixes were made for each diet level according to the NRC 99 recommendations for growing 5-10 lb weanling pigs except for minerals under study.

Mineral Mixes:

Ingredients for the the trace mineral mixes (Table 3.2 and 3.3) were weighed, combined and then mixed in a burundum-fortified porcelain jar ball mixer on a roller type mill for 2 hours. Four portions of 136.20 gm each of trace mineral mix were weighed and mixed with each of the four levels of zinc and dextrose or chromium and dextrose in a burundum-fortified porcelain jar ball mixer on a roller type mill for 30 min. For mixing diets contaning highest concentration of dietary zinc (3000 ppm) the zinc sulfate and dextrose were added directly to the 68.18 kg milk replacer instead of mixing with trace mineral mix.

Table 3.2: Zinc Mineral Mix Composition

Trace mineral mix with out zinc sulfate: (TM Mix)	
Dextrose	564.830
Ferrous Sulfate[1]	116.90
Copper Sulfate[1]	13.388
Manganese Sulfate[2]	4.308
Calcium Iodate[1]	0.077
Sodium Selenite[1]	0.231
Total (gms)	700.00

Zinc Mixes:	0 ppm	100 ppm	1500 ppm	3000 ppm
Zinc Sulphate[1] (g)	0	19.20	287.72	575.51
TM mix (g) [a]	136.20	136.20	136.20	136.20
Dextrose (g)	681.00	661.80	393.284	105.49
Total	817.20	817.20	817.20	817.20

[1] Prince Agri, Quincy, IL.

[2] Sigma, St.Loius, MO

[a] TM mix provided 100 mg Fe, 10 mg Cu, 4 mg Mn, 0.14 mg I, .30 mg Se / kg diet

Table 3.3: Chromium Mineral Mix Composition

Trace mineral mix without Chromium (TM mix):	
Dextrose	466.716
Zinc Sulfate[1]	98.590
Ferrous Sulfate[1]	116.690
Copper Sulfate[1]	13.388
Manganese Sulfate[2]	4.304
Calcium Iodate[1]	0.077
Sodium Selenite[1]	0.231
Total (g)	700.000

Chromium mixes	0 µg	200 µg	600 µg	1000 µg
Chromium Picolinate (g)	0	34.05	102.15	170.25
TM mix[a] (g)	136.20	136.20	136.20	136.20
Dextrose (g)	681.00	646.95	578.85	510.75
Total (g)	817.20	817.20	817.20	817.20

[1]Prince Agri, Quincy, IL

[2] Sigma, St.Loius, MO.

[a] TM mix provided 100 mg Fe, 100 mg Zn, 10 mg Cu, 4 mg Mn, 0.14 mg I, and 0.30 mg Se / kg diet

Diet Preparation and Storage:

Experiment 4: Milk replacer formula (68.18 kg) and each of the zinc mineral mixes were mixed in a horizontal paddle mixer for 30 minutes. The diets were bagged in plastic containers and stored for animal feeding.

Experiment 5: Each of the chromium mixes (817.20 gm) and milk replacer (2270 gm) were premixed in a plastic bowl using a commercial Hobart mixer for 30 min prior to mixing with the remaining milk replacer formula. Chromium/milk replacer mixes and milk replacer (65.90 kg) were then mixed in a horizontal paddle mixer for 30 minutes. The diets were bagged in plastic containers and stored for animal feeding.

Feeding:

The dry diets were measured with standardized scoops providing 12 g dry diets/100 ml water. The dry diets were mixed with water immediately before feeding. Pigs were fed the respective diets 4 times a day at 8: 00 a.m., 12: 00 p.m., 5: 00 p.m., and 12: 00 a.m. No diet was offered between midnight and 8: 00 a.m. The adaptation diets (0 ppm added Zn or 0 ppm added Cr) were fed for 2 days followed by the experimental diets for 14 days. The amount of formula fed at each feeding increased daily from 200 to 1200 ml by day 14 of the treatment period. Throughout the entire experiment, the total amount of formula provided and the amount left over were recorded.

Experimental Protocol:

In both the experiments, on day 13 the animals were fed at 8:00 a.m., 12:00 noon; 6:00 p.m and at 10:00 p.m. At 12:00 a.m. milk replacer was removed and pigs were provided with deionized water. On the morning of day 14, the animals were weighed and

were randomly assigned to the endotoxin treatment groups. Following the fast the animals were injected intraperitoneally with saline (control) or endotoxin (25 μg/kg body weight lipopolysaccharide, E.coli, 0111:B4 (Sigma Diagnostics, St.Louis, MO). The dose of 25μg LPS/kg body weight was found to be adequate to produce an acute phase response in the preliminary experiments (Chapter IV) conducted in our laboratory. Blood was collected from the jugular vein with K^+ EDTA or trace mineral free sodium heparin vaccutainers (Beckton Dickson) using 20 X 1.5" gauge needles (Sherwood Medicals, St.Loius, MO). Serum was collected in anticoagulant free vaccutainers. Blood samples for serum were kept on ice for at least 30 minutes to clot and then separated by centrifugation for 30 minutes at 2000 x g. Whole blood was analyzed for complete blood counts (CBC), leukocyte differential and reticulocyte counts. Serum was analyzed for glucose, albumin, creatinine, C-reactive proteins, total protein, triglycerides, urea nitrogen and insulin. Plasma samples were analyzed for cortisol, IGF-1, TNF-α and IL-6 and plasma iron, zinc and copper.

Experiment 4: Body temperature was measured at 0, 1.5, 3 and 6 and 24 hr after LPS administration using a rectal thermometer (B-D Flexible™ digital Thermometer). Urine and blood were collected at 0, 1.5, 3, and 6 and 24-h post injection. Body weights were recorded at 0, 6 and 24-h post injection.

Experiment 5: Body temperature was measured at 0, 1.5, 3 and 6 h after LPS administration using a rectal thermometer (B-D Flexible™ digital Thermometer). Blood was collected at 0, 1.5, 3, and 6 h after dosing. Body weights were recorded at 0 and 6 h post injection. At 6-h post injection animals were given sodium pentobarbitol (10 mg/ml)

intravenously. Samples of liver, kidney and muscle were removed, weighed, and stored at –20 °C for subsequent mineral analysis.

Analytical Methods:

The same analytical methods were followed for the samples in all the experiments. The COBAS FARA II clinical analyzer (Roche Diagnostic Systems, Indianapolis, IN) was used to analyze serum samples for glucose, total protein, blood urea nitrogen, albumin, and serum and urinary creatinine, and triglyceride concentrations, and C-reactive proteins. Roche diagnostic kits and reagents were used for all clinical chemistries except C-reactive protein. SPQ antibody reagent set II was used for C-reactive protein (Diasorin, Stillwater, MN).

The analysis of total protein is based on biuret methodology measuring color changes from the formation of a copper protein complex. Divalent copper reacts with the peptide bonds of protein under alkaline conditions to form the biuret complex, which absorbs light at 540-nm wavelength.

The analysis of creatinine depends on the formation of the red colored complex in the presence of alkaline picrate solution. The rate of formation of the red colored complex is directly proportional to the creatinine concentration in the sample and is measured at 520-nm wavelength.

The analysis of blood urea nitrogen is a modification of an enzymatic procedure. Urea in the sample is hydrolyzed by urease to ammonia and carbon dioxide. Ammonia and α– ketoglutarate in the presence of glutamate dehydrogenase and NAD are converted to glutamate, water and NADH. Two moles of NADH are oxidized for each mole of urea

present. The decrease in absorbance at 340 nm is proportional to the urea concentration in the sample.

Albumin was analyzed using the bromocresol green method. The analysis of albumin depends on the Doumas bromocresol green binding assay procedure. Bromocresol green binds to albumin in the sample and produces a shift in the absorbance at 630nm. The change in the absorbance is directly proportional to concentration of albumin in the sample.

Plasma glucose was analyzed by the phosphorylation of glucose by hexokinase in the presence of the adenosine triphosphate and magnesium ions to produce glucose-6-phosphate and adenosine diphosphate. Glucose-6-phosphate dehydrogenase oxidizes G-6-P to 6-phosphonogluconate with the reduction of nicotinade adenine dinucleotide (NADH). One micromole of NADH is produced for each micromole of glucose consumed. The NADH absorbs light at 340 nm and is directly proportional to glucose concentration in the sample.

Triglycerides were analyzed using the principles of the Trinder's reaction method. Triglycerides are hydrolyzed by lipoprotein lipase to glycerol and fatty acids. Glycerol is then phosphorylated to glycerol-3-phosphate by adenosine-5-triphosphate (ATP) in a reaction catalyzed by glycerol kinase (GK). The oxidation of glycerol-3-phosphate is catalyzed by glycerophosphate oxidase (GPO) to form dihydroxyacetone phosphate and hydrogen peroxide. The hydrogen peroxide reacts with 4-chlorophenol and 4-aminophenazone in the presence of peroxidase to form a quinoneimine complex, which can be read at 490-550 nm. The increase in absorbance is proportional to the

concentration of triglycerides in the sample.

The SPQ antibody reagent set II for CRP permits the quantitative determination of CRP in human serum by immunoprecipitin analysis. The sample (antigen) solution and antiserum form an insoluble turbid complex which increases the amount of light scattered by the solution. The absorption of the solution is measured at the wave length of 340 nm.

Cortisol was measured using a Coat-A-Count radioimmunoassay kit (TKCO2) (Diagnostic Products Corporation, Los Angeles, CA). The ^{125}I cortisol competes for a fixed time with cortisol in the sample for antibody sites. The bound antibody is immobilized to the wall of the polypropylene tube. Decanting the supernatant terminates the competition, and the antibody-bound fraction of the radiolabeled cortisol was then counted for one minute on the Cobra II automatic gamma counter (Packard Instrument Co., Meriden, CT).

Insulin was measured using a Coat-A-Count radioimmunoassay kit (TKIN2) for humans (Diagnostic Products Corporation, Los Angeles, CA), The ^{125}I insulin competes for a fixed time with insulin in the sample for antibody sites. The bound antibody is immobilized to the wall of the polypropylene tube. Decanting the supernatant terminates the competition, and the antibody-bound fraction of the radiolabeled insulin was then counted for a minute on the Cobra II automatic gamma counter (Packard Instrument Co., Meriden, CT).

Tumor Necrosis Factor alpha (TNF-α) was measured using an ELISA assay kit (Endogen, Inc Woburn, MA). The TNF-α is an in vitro enzyme-linked immunosorbent

assay for the quantitative measurement of pig TNF-α read at 450 nm on a microplate reader.

Plasma IGF-I was measured by modified radioimmunoassay method (Nichols Institute Diagnostics, San Juan Capistrano, CA) (Appendix B). This technique involved the separation of soluble IGF-1 from binding proteins that are precipitated with acid ethanol. The radiolabeled IGF-1 was then counted for one minute on the Cobra II automatic gamma counter (Packard Instrument Co., Meriden, CT).

Plasma IL-6 was measured by ELISA method described by the R & D Systems (Minneapolis, MN). Ninty-six well flat bottomed high binding Costar plates (Corning Incorporated, Corning, NY) were precoated with capture antibody as described in appendix C. The capture antibody (AF686), detection antibody (BAF686) and the recombinant porcine IL-6 (686-PI) were obtained from the R & D Systems (Minneapolis, MN). The optical density of each well were measured using a 4 parameter log-log curve at a wave length of 450 nm using a micro plate reader .

The ABX Pentra 120 Retic (ABX Diagnostics, Irvine, CA) was used in performing the hematology analyses. The blood components counted or measured were erythrocytes, leukocyte differential counts, red blood cell indices and reticulocyte counts. The detection principle is based on an impedance variation generated by the passage of cells through the calibrated micro-aperture.

All necessary precautions were taken to minimize the contamination of the plasma samples for mineral analysis. Gloves, hair restraints, plastic utensils, distilled deionized water and acid washed glassware were used at all times. When possible, work was done

under a laminar flow hood.

Plasma samples were prepared for mineral analysis by a modified procedure following the method of Hill and coworkers (Hill et al.1986). Samples of 0.5 - 1 ml were pipetted into acid washed borosilicate glass tubes. The tubes containing the samples were placed in a heating block and wet ashed at 105°C repeatedly with 100 μl each of double distilled concentrated nitric acid (GFS Chemicals, OH), type 1 water and 30% Ultrex hydrogen peroxide (Baker Scientific). The tubes were dried in the heating block and dry ashed in a muffle furnace ramped at 0.5°C per minute to the ashing temperature of 375°C for a duration of 48 hours. This cycle of wet and dry ashing was repeated until a soluble, colorless and clear solution was obtained. The samples were then analyzed using a Perkin Elmer 5100PC atomic absorption spectrophotometer equipped with an air-acetylene flame and deuterium background correction and a graphite furnace with Zeeman background correction for chromium analysis. Flame analysis was used for zinc, iron, and copper.

Statistical Analyses:

The experiment's were randomized complete block design with factorial arrangement of treatments. PC-SAS (version 8.01) was used for the analyses. The data were analyzed using PROC MIXED with sources of variation including diet, endotoxin, time, diet x endotoxin (error term for diet and endotoxin) time x diet x endotoxin interaction and residual. If the diet x endotoxin interaction were significant ($P<0.05$), simple effects of diet x endotoxin were analyzed using the SLICE option for the LSmeans statement. If diet x endotoxin were not significant ($P>0.05$), then the main effects were

analyzed for significance using LSmeans with PDIFF option (SAS 1989). Comparisons between treatments at individual blood collection times were analyzed using the PROC GLM procedure. The single degrees of freedom contrasts were used for diet and endotoxin main effects and the interactions. The coefficients for unequally spaced dietary levels of chromium were derived using the integrative matrix language (PROC IML) procedure of SAS (Version 8.01)

CHAPTER IV

The Optimum Dose Of Endotoxin Required To Produce An Acute Phase Response In Weanling Pigs[a,b,c]

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Experimental Biology Meetings, 97. S. Mandali, B.Z de Rhodas, E. Lucas, L.J. Spicer, A.B. Arquitt, B.J. Stoecker, S. Carter and C. Maxwell. Effect of endotoxin on the plasma IGF-1 in weanling pigs. FASEB 97, A241.

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Abstract

Two experiments were conducted to determine the minimum dose of lipopolysaccharide (LPS), E.coli 0111:B4, required to produce an acute phase response in segregated early-weaned (SEW). After weaning (d 12-13), (5.0 ± 0.5 kg body weight (BW)) pigs were housed in an off-site nursery in individual pens for 7 d. The pigs were fed a known amount of an early weaning diet (SEW). In Experiment 1, twenty pigs were randomly allotted to 5 experimental groups; On d 7 post-weaning, pigs were fasted 4 h and injected intra-peritoneally (i.p.) with a 9 g/L saline solution containing 0, 0.25, 0.50, 25 or 50 μg LPS (0111:B4)/kg BW. Blood samples were collected and rectal body temperature was recorded at 0, 3, and 6 h post injection (PI). At 6 h PI an increase in body temperature and a drop in plasma zinc ($P<0.01$) were observed in the 25 μg LPS group and plasma iron decreased ($P<0.05$) in all the groups compared to the baseline values. At 3 h PI, a decrease in plasma IGF-1 ($P<0.01$) and a six-fold elevation in plasma cortisol were observed in groups injected with 25 or 50 μg/kg BW. In Experiment 2, twelve early weaned pigs were fasted 4 h and injected i.p. with saline solution containing 0, 12.5 or 25 μg LPS/kg BW. Blood samples were collected and rectal body temperatures were recorded at 0, 1.5 and 3 h PI. Plasma zinc or iron concentrations were not affected at any

time periods. At 1.5 h PI, TNF-α peaked in the 25 μg LPS/kg group. Body temperature was significantly elevated at 3 h PI in the 25 μg LPS/kg group. These results suggest that optimum dose of 25 μg LPS (E.coli 0111:B4)/kg BW is required to produce an acute phase response in weanling pigs, and timed blood draws for 6 h PI are required to observe changes in plasma minerals.

Key Words: Cortisol, IGF-1, Fever, Zinc, Iron

Introduction

Endotoxic lipopolysaccharide (LPS), the principal component of gram-negative bacteria, is a major contributing factor in the pathogenesis of bacterial infections. Endotoxins, potentially lethal molecules produced by many bacteria including those responsible for cholera, whooping cough, and plague, continue to attract the attention of many researchers (17). Disease stress causes profound metabolic changes in the host, characterized by shifts in the efficiency of nutrient use for growth and increased catabolism of tissues and stored nutrients (5, 9). The acute phase response is a primary host defense mechanism in response to microbial infection (7) characterized by fever (6), endocrine reactions, altered immune cell function (7, 24) and the release of cytokines, especially tumor necrosis factor α (TNF-α) (6, 24).

LPS is known as potent stimulant of the host immune system (22). TNF-α, one of the pro-inflammatory cytokines produced by macrophages and monocytes, (12, 14) is thought to be critically important for coordinating host defense and immune responses, which include fever, anorexia, hypersomnia, acute phase protein synthesis, changes in trace metals, neutrophilia and antibody production (15).

The pig is a recognized experimental animal model for human diseases because it provides relevant information about growth, development, and aging due to its similarities with humans in physiology, anatomic structure, nutritional needs, and metabolism (4). Further, scours, associated with E.coli infection in weanling pigs, is a major health concern for pork producers. Hence, the weanling pig was selected as an experimental model.

It is difficult to compare results from previous studies on endotoxin effects on the immune response because of the differences in the strain of the bacterium from which the endotoxin was obtained, the dose and route of administration of endotoxin as well as the stage of development of the animal. The present study was undertaken to determine the optimum dose of LPS endotoxin (E.coli, 0111:B4) required to produce an acute phase response.

Research Design and Methods

Fourteen-day old weanling pigs were obtained from the OSU Swine Research, Teaching and Extension facility. The pigs were matched for weight (5.0 ± 0.5 kg) and were placed in individual pens for a period of 7 days. The pigs were fed a known amount of a segregated early-weaning (SEW) diet. Pigs (5.0 ± 0.5 kg) were randomly assigned to endotoxin treatment or control groups in a completely randomized block design. On day 7 post-weaning, pigs were fasted 4 h and injected intraperitoneally (i.p.) with 9.0 g/L saline solution containing varying doses of LPS (E. coli, 0111:B4, Sigma, Inc., St. Loius, MO). Blood samples were collected by venipuncture of the jugular vein into dipotassium EDTA treated vaccutainers. Two experiments were conducted that differed only in

endotoxin dose and blood collection time periods.

Experiment 1: Twenty pigs were injected i.p. with 0, 0.25, 0.5, 25 or 50 μg LPS/kg BW on day 7 post weaning. Blood samples were collected for analysis of plasma IGF-1, cortisol, zinc and iron concentrations and rectal body temperatures were recorded at 0, 3, and 6 h post injection (PI).

Experiment 2: This experiment was conducted to determine if a dose >0.5 μg LPS/kg but less than 25 μg LPS/kg would produce as strong an acute phase response as found at a dose of 25 μg LPS/kg body weight. Twelve 7 d post-weaning pigs were injected i.p. with a saline solution containing 0, 12.5 or 25 μg LPS/kg BW. Blood samples were collected for analysis of plasma TNF-α, IGF-1, cortisol, zinc and iron concentrations and rectal body temperatures were recorded at 0, 1.5 and 3 h PI.

Plasma TNF-α was analyzed using a pig TNF-α ELISA Kit (Endogen Inc., Cambridge, MA). Plasma IGF-1 concentrations were analyzed by radioimmunoassay using the method previously described by Clutter et al (3). Plasma cortisol was analyzed using a solid phase radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA).

Plasma samples were wet and dry ashed using a modified procedure from Hill et al. (11). Zinc and iron were analyzed using a Perkin Elmer Model 5100PC atomic absorption spectrophotometer with an air--acetylene flame and deuterium background correction (Perkin Elmer, Norwalk, CT).

Statistical Analyses:

Statistical analyses were performed using the PROC MIXED in PC SAS (SAS Institute, Cary, NC version 8.01) to determine the endotoxin effects in a completely randomized design with repeated measures. The model included dose of endotoxin, time, and pig within dose. Slice option was used to test the differences between treatments. Repeated measures were used for the time and dose interactions and are presented in the tables. An individual pig was considered an experimental unit. Data in this study are presented as least squares means. The significant difference was set at $P<0.05$. One pig (25%) succumbed at 6 hr PI with LPS dose of 50 μg LPS/kg and its data was excluded from the analysis.

Results

Experiment 1: At 6 h post injection, body temperatures in all the treatment groups were elevated ($P < 0.001$) compared to pre-injection baseline values (Table 4.1). Compared to the control group the endotoxin groups tended ($P<0.07$) to have higher body temperatures. However, a dose of 25 or 50 μg LPS/kg produced a greater elevation in body temperature at 6 h PI compared to the rest of the treatment groups.

A dose of ≥25 μg LPS/kg produced a significant (Dose $P<0.02$) elevation in concentrations of plasma cortisol at 3 h and at 6 hr PI (Figure 4.1). Plasma cortisol concentrations did not change ($P>0.05$) between 0 and 6 h in the 0, 0.25, or 0.5 ug LPS/kg groups (Figure 1). Plasma IGF-1 concentrations decreased by 60% over time regardless of dose of LPS and were lowest at 6 h PI (Time, $P<0.001$) (Table 4.1).

Dose of LPS had no effect ($P>0.05$) on plasma zinc concentrations (Table 4.1).

A significant decrease in plasma zinc concentration was observed during the 6 h experimental period in experiment 1 (Time, $P < 0.0001$) (Table 4.1) regardless of LPS dose. Plasma iron concentrations tended to be lower with the 25 μg/kg dose at 3 h PI and the magnitude of drop in plasma iron concentrations were greater with a LPS dose of 50 μg/kg compared to rest of the experimental groups (Table 4.1). At 6-h PI plasma iron concentrations decreased significantly with all the doses of LPS; however, the magnitude of decrease was greater with LPS doses of 25 μg/kg or more (Dose x Time, $P<0.03$) (Table 4.1).

Experiment 2: An elevation in body temperature was observed in the endotoxin groups. At 3 h PI the magnitude of elevation was higher with the 25 μg/kg dose compared to rest of the groups (Dose x Time, $P<0.004$) (Table 4.2). By 1.5 h, LPS administration significantly increased TNF-α (Dose x Time, $P<0.03$), but the increase was greater with the 25 μg/kg versus 12.5 μg/kg dose(Figure 4.2). The TNF-α concentrations reached baseline values (0 h) by 3-h PI in the 0 and 25 μg/kg groups. Plasma cortisol concentrations at 3 h PI (Time $P<0.001$) were by a dose of 25 μg LPS/kg (Figure 4.3). No significant alterations were observed in concentrations of plasma IGF-1, zinc or iron with LPS administration during the 3 h experimental period (Table 4.2).

Discussion

The results of the present study indicate that an intraperitoneal injection of LPS endotoxin in weanling pigs produced a dose dependent acute phase response over time. With doses lower than 25 μg LPS/kg the effect on body temperature, cortisol, IGF-1, TNF-α, and plasma minerals was either not significant or showed minor changes over

time. In general, 25 μg LPS/kg body weight produced more rapid and greater changes than the 0.25, 0.5, and 12.5 μg/kg dose and was not lethal.

In the present study fever occurred in all experimental groups. The magnitude of hyperthermia was not significant for the 0.25 LPS at 3 and 6 h PI, whereas a higher fever was observed in piglets injected with 0.5 (Table 4.1) 12.5 (Table 4.2), 25 or 50 (Table 4.1) μg LPS group at 3 or 6 h PI. The results indicated that 25 μg or above of LPS produced an acute febrile response to endotoxin. However, in the present study untreated controls also experienced a rise in body temperature, which may be due to the stress effects of handling and blood sampling. Studies of endotoxin effects on the acute phase response in pigs showed dose dependent linear increases in body temperature (12). Green and Vermeulen (8) hypothesizes that hyperthermia (41 °C) prevents gram-negative bacteria from synthesizing their protective LPS coat, thereby allowing the host defense system to multiply and kill the pathogens, even before the production of host antibodies. In the present study, only a dose of ≥ 0.5 μg LPS consistently produced an elevation in the body temperature (>40.0 °C).

In support of earlier findings, increased TNF-α due to endotoxin peaked at 1.5 h and returned to baseline by the end of 3 h (Figure 4.2). The effect of LPS is dose and time dependent and also varies with the strain, route of administration, and age or weight of pigs. For instance, Wright and coworkers (24) administered a dose to growing pigs 5 wk-old that was twofold higher (E.coli 055:B5, 100 μg LPS/kg body wt) compared to the highest LPS dose in the present study, and reported a peak response of TNF-α at 2 h PI

that remained elevated even at 4 h PI. On the other hand, a tenfold increase in TNF-α was observed at 2 h and remained elevated at 4 h in pigs weighing 12 kg injected i.p. with 5 μg of LPS/kg BW (0111:B4) (23). Thus age or body weight of pigs may influence its immune response. Takaki and coworkers (19) demonstrated an increase in proinflammatory cytokine IL-6 with immobilization stress. We saw no significant increase in TNF-α concentrations in the saline treated control pigs indicating that the stress, if any, associated with immobilization during phlebotomy had minor impact on the pigs of the present study.

The IGF-1 concentrations decreased by 25 to 50% at 3 hour PI and by 70% at 6 hr PI in weanling pigs in the present study. Several studies have documented a fasting-induced decrease in plasma IGF-1 (3, 20). Because we saw no significant effect of LPS on IGF-1 concentrations, the decrease in plasma IGF-1 is likely due to the fact that all pigs were fasted 4 h before LPS injection. Previous reports in pigs (24), cattle (5), and humans (13) indicate that LPS induces a decrease in IGF-1 over time but the decrease is not observed until after 24 h.

The results of the present study showed only doses >25 μg LPS 0111:B4/kg body weight produced a peak response in plasma cortisol at 3 h PI in response to endotoxin confirming the role of the hypothalamo-pituitary-adrenal axis (HPA axis) response to stress (2, 17, 18). Concentrations of plasma cortisol in experiment 1 at 3 h PI were much higher than the concentrations of plasma cortisol in experiment 2 at 3 h PI at a dose of 25 ug/kg. With more animals in the experiment 1 than experiment 2 (20 vs 12), there was more acoustic disturbance in the laboratory during the experiment 1 than experiment 2,

which may have contributed to this difference.

The results of the present study showed a decrease in plasma iron concentrations 6 h after LPS (endotoxin) injection whereas LPS had no effect on plasma zinc concentrations. Previously, decreases in iron and zinc concentrations in serum of chickens (1, 21), mice (7), and pigs (10) with endotoxin treatment have been observed. However, besides species differences (7, 10, 21) previous studies included different doses and strains of endotoxin and route of administration compared with the present study. Because administration of recombinant human TNF-α was found to depress serum iron in mice (18), elevation in the TNF-α with endotoxin administration observed in the present study might have induced the decrease in the plasma iron concentrations. Further research will be required to verify this suggestion. The decreases in the plasma zinc with time observed in the present study can be attributed to the diurnal variations or fasting (16).

In the present study the magnitude of acute phase response was time and dose dependent. Time dependent effects of LPS were observed on TNF-α at 1.5 hr, on body temperature and plasma cortisol at 3 to 6 h PI and on plasma minerals at 6 h PI. A dose of 25 μg LPS consistently produced a non-lethal acute phase response in weanling pigs that was not found consistently with lower doses.

Conclusion

The results of experiments with endotoxin treatments of 0, 0.5, 5.0, 12.5, 25, 50 μg LPS/kg BW demonstrated that a dose of 25 μg was sufficient to elicit an immune response in weanling pigs. With blood collections at 0, 1.5, 3 and 6 h post injection, we demonstrated that blood collection at 0, 3 and 6 h are necessary to demonstrate the effects

of endotoxin on plasma minerals, and cortisol. Blood collections at 0, 1.5 and 3 h are also necessary to record TNF-α peaks. Elevation in plasma cortisol, TNF-α, and body temperature indicate an interrelationship between hormone-immune- and acute phase protein response. Investigations on the immune response in early-weaned cross-bred pigs can expect an i.p. dose of 25 μg LPS (E.coli, 0111:B4) /kg BW will elicit an acute phase response.

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Table 4.1: Acute Phase Response to Different Dose of LPS Endotoxin, 0111:B4, in Weanling Pigs (Experiment 1)*

Time	Dose of LPS endotoxin (μg/kg body weight)						P-Value			
(h)	0	0.25	0.5	25	50	Mean†	Pooled SEM	Dose	Time	Dose x Time‡
Temperature (°C)										
0	38.61	39.11	39.27	39.11	38.22	38.86§	0.28			
3	39.05	39.55	39.72	39.77	40.16	39.65§§	0.56	0.07	0.0001	0.89
6	39.27	39.83	40.00	40.38	40.55	40.06§§§	0.56			
Mean	38.97	39.49	39.66	39.75	39.64					
IGF-1 (ng/ml)										
0	81.79	118.85	83.64	93.75	106.72	96.95§	23.80			
3	65.43	65.61	54.26	47.1	44.65	55.41§§	23.80	0.50	0.001	0.78
6	45.99	50.06	43.42	25.72	29.91	39.05§§§	23.80			
Mean	64.40	78.17	60.44	55.52	60.42		23.80			
Plasma Zinc (mg/L)										
0	1.55	1.97	1.75	2.04	1.82	1.82§	0.21			
3	1.41	1.68	1.81	1.88	1.25	1.61§	0.21	0.44	0.0001	0.3
6	1.08	1.29	1.27	0.85	0.81	1.06§§§	0.21			
Mean	1.34	1.64	1.61	1.59	1.29					
Plasma Iron (mg/L)										
0	1.45^{a}	2.40^{b}	2.26^{b}	2.75^{b}	2.64^{b}	2.30	0.31			
3	1.48^{a}	1.82^{a}	1.70^{a}	2.31^{b}	1.78^{c}	1.82	0.31	0.57	0.0001	0.03
6	1.35^{a}	1.46^{b}	0.99^{c}	0.83^{d}	0.64^{e}	1.05	0.31			
Mean	1.43	1.89	1.65	1.96	1.68					

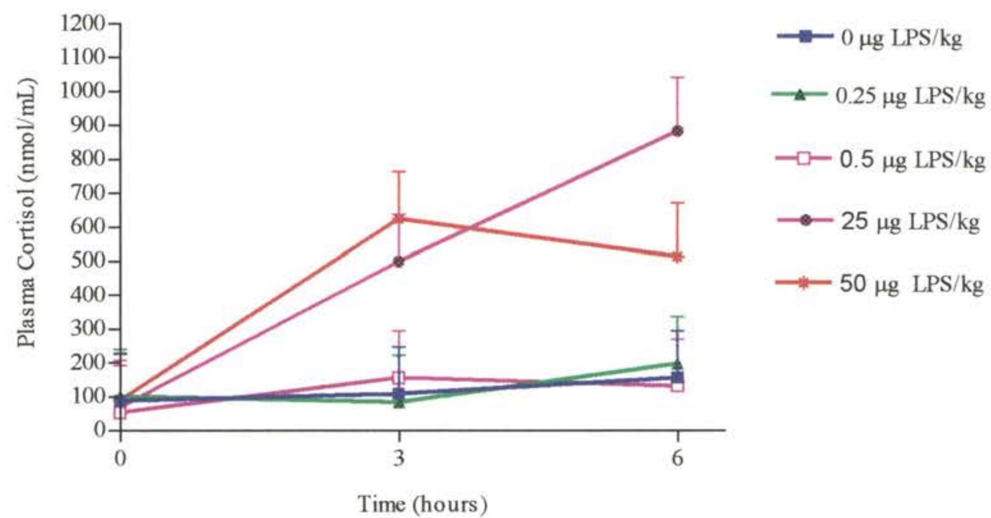
*Data are the Least Square Means ± SEM (n=4)
† Means not sharing a common symbol in a column are significantly different
‡ Means not sharing a common symbol in a row are significantly different

Table 4.2: Acute Phase Response to Different Dose of LPS E.coli 0111:B4 in Weanling Pigs (Experiment 2)*.

	Time (h)	Dose of Endotoxin (µg/kg BW) 0	12.5	25	Mean†	Pooled SEM	P - Value Dose	Time	Dose x Time‡
Temperature (°C)									
	0	39.35[a]	39.44[a]	39.33[a]	39.37	0.28	0.0001	0.03	0.004
	1.5	39.13[a]	39.50[a]	38.83b[a]	39.15	0.28			
	3	38.93[a]	39.94[b]	40.33[c]	39.73	0.28			
	Mean	39.13	39.62	39.49					
IGF-I (ng/mL)									
	0	110.11	116.63	122.19	116.3	12.97	0.6	0.13	0.4
	1.5	142.52	115.93	98.73	119.00	12.97			
	3	93.06	102.11	91.44	95.53	12.97			
	Mean	115.23	111.55	104.12					
Plasma Zinc (mg/L)									
	0	1.83	1.27	1.67	1.59	0.14	0.5	0.50	0.4
	1.5	1.64	1.60	1.63	1.62	0.14			
	3	1.67	1.38	1.46	1.50	0.14			
	Mean	1.71	1.41	1.58					
Plasma Iron (mg/L)									
	0	2.47	2.17	1.67	2.10	0.27[§]	0.3	0.08	0.8
	1.5	2.20	2.33	1.96	2.16	0.27[§]			
	3	1.78	1.84	1.16	1.59	0.27[§§]			
	Mean	2.15	2.11	1.59					

* Data are the Least Square Means and pooled SEM (n=4)
† Means not sharing a common symbol in a column are significantly different
‡ Means not sharing a common letter in a row are significantly different

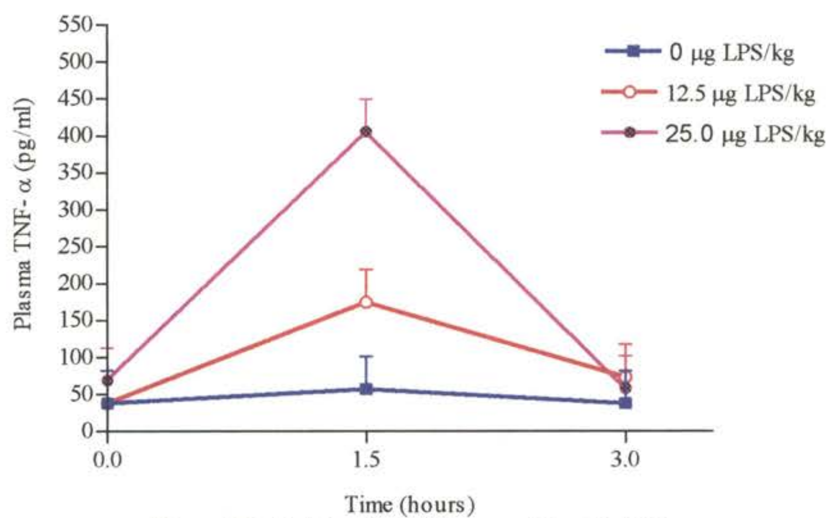
Figure 4.1: Plasma Cortisol Concentrations in Weanling Pigs Injected With Different Doses of Endotoxin (LPS E.coli 0111:B4) (Experiment 1).



*Dose (P<0.02) Time (P<0.003) D x T (P<0.09)

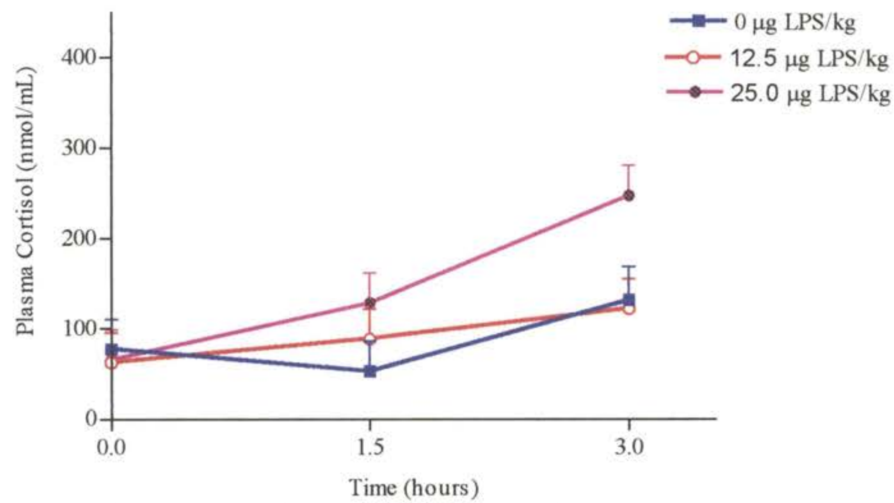
[1]Data are the LSmeans ± SEM (n=4)

Figure 4.2: Plasma TNF-α Concentrations of Weanling Pigs Injected with Different Doses of Endotoxin (LPS E.coli 0111:B4) (Experiment 2)*[1]



*Dose ($P<0.1$), Time ($P<0.01$), Dose x Time ($P<0.03$)
[1]Data are the LSmeans ± SEM (n=4)

Figure 4.3: Plasma Cortisol Concentrations of Weanling Pigs Injected with Different Doses of Endotoxin (LPS E.coli 0111:B4) (Experiment 2)*[1]



*Dose (P<0.2), Time (P<0.001), D x T (P<0.14)

[1]Data are the LSmeans ± SEM (n=4)

CHAPTER V

Endotoxin Decreases Absorption, Tissue Retention and Urinary Excretion of $^{51}CrCl_3$ in Weanling Gilts[a,b]

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Running Title: $^{51}CrCl_3$ in Endotoxin Injected Weanling Gilts

Key Words: • LPS endotoxin • Pigs • $^{51}CrCl_3$

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[b]Data were presented at the Experimental Biology 97 meetings, as a poster session- Mandali S, Arquitt AB, Stoecker BJ, Maxwell CV, de Rodas BZ, & Morgan G (1997) Endotoxin decreases absorption, tissue retention and urinary excretion of $^{51}CrCl_3$

in weanling gilts. *Federation of American Society of Experimental Biology Journal* 11, 2349.

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Abstract

Effects of endotoxin on absorption of Cr were investigated in Yorkshire gilts using $^{51}CrCl_3$. Gilts were weaned at 14 d of age. Four days later, the pigs were anesthetized and a 2 mm silastic jugular catheter was inserted and passed subcutaneously to a dorsal position behind the ears. At 21 d of age, pigs were deprived of food for 7 h and injected intraperitoneally with saline or 25 μg endotoxin (Lipopolysaccharide from E.Coli serotype 0111:B4) per kg body wt suspended in 9 g/L saline. One hour after the endotoxin dose, an oral dose of 0.7 mCi of $^{51}CrCl_3$ was given by micropipette. Blood was sampled from the catheter at intervals until necropsy at 8 h after the $^{51}CrCl_3$ dose. Rectal temperature at necropsy was 39.4°C in the endotoxin-treated compared to 37.9°C in the control pigs. Blood and tissue samples were counted in a gamma counter. $^{51}CrCl_3$ in blood was lower at 3 ($P<0.03$), 4 ($P<0.01$), 5 ($P<0.007$), 6 ($P<0.03$) and at 8 ($P<0.04$) h after dosing in the endotoxin-injected pigs compared to the controls. Eight hours after $^{51}CrCl_3$ dosing, $^{51}CrCl_3$ retention was less in liver ($P<0.01$), heart ($P<0.02$), and kidney ($P<0.04$) in endotoxin-treated pigs and tended to be lower in spleen ($P<0.06$) and in urine ($P<0.16$) with endotoxin treatment. These data suggest that during times of infection there might be decreased Cr absorption and retention.

Introduction

When lipopolysaccharide (LPS), a common component of the cell wall of E.coli gram negative bacteria, is injected into animals and humans, it produces similar pathophysiological and immunological alterations as the live bacteria (Myers et al. 1997). Alterations in the barrier function of the gastrointestinal mucosa were observed to be one of the pathophysiological alterations due to bacterial challenge. The reaction of the intestinal mucosa to bacterial challenge showed villous atrophy and creation of intercellular spaces (Kenworthy, 1970). Lipopolysaccharide is also known to activate macrophages, resulting in the production of cytokines, interleukin-1 (IL-1α), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) (Myers et al. 1999). The cytokines IL-1α, IL-6 and TNF-α enhance prostaglandin production (Grimble, 1989) and also act on liver to produce acute phase proteins. TNF-α is a potent mediator of sepsis resulting in a marked inflammatory response. Administration of recombinant murine TNF-α to rats (125 μg/kg body wt) depressed the gut absorptive capacity of D-xylose (Singh et al. 1993).

Acute stress alters mineral metabolism. Intraperitoneal injection of IL-1α decreased absorption, retention and urinary excretion of ^{51}Cr from $^{51}CrCl_3$ (Davis-Whitenack et al. 1999) in rats. In preliminary investigations (Mandali et al. 1997), both plasma Zn and Fe decreased significantly with intraperitoneal injections of 25 or 50 μg LPS/kg body wt. At this dose body temperature in experimental animals increased significantly by 3 h. Further, TNF-α was significantly elevated by 1.5 h after 25 μg/kg of LPS injection (Mandali et al. 1997). Also, increased urinary Cr excretion (Borel et al.

1984), and decreased plasma Zn concentration (Joung et al. 1998) were observed in trauma patients. Therefore, stress induced by LPS infection might have an impact on status of the trace mineral Cr. Hence, the present study was undertaken to determine the effects of LPS on the absorption, tissue retention and urinary excretion of $^{51}CrCl_3$ in weanling pigs.

Experimental Methods

Ten Yorkshire gilts (14 d, 5.0 kg) were weaned and were allotted to endotoxin or saline groups in a completely randomized design. The pigs were housed in individual metabolic cages in an off site nursery for 7 d. During this period, pigs were fed a segregated early weaning (SEW) diet.

On day 4 of this experimental period, the pigs were fasted for 12 h and anesthetized. A 2 mm silastic jugular catheter was inserted and passed subcutaneously to a dorsal position behind the ears. After the pigs recovered from anesthesia, they were returned to individual cages. On day 7 of the experimental period, pigs were fasted for 7 h and injected intraperitoneally with 25 μg LPS/kg body wt (lipopolysaccharide from E.Coli serotype 0111:B4 (Sigma, St.Louis, MO). suspended in saline) or with saline. One hour after the endotoxin or saline dose, pigs were given an oral dose of 0.7 mCi of $^{51}CrCl_3$. Approximately 0.5 ml of blood was collected from the jugular catheter at 0.5, 0.75, 1, 2, 3, 4, 5, 6, 7 and 8 h after ^{51}Cr dosing. Rectal temperature was recorded at the termination of the experiment. Eight hours after the ^{51}Cr dose, the pigs were given a dose of ketamine HCl (30 mg/kg body wt) and pentobarbital (1 mg/kg body wt) and exsanguinated. Liver, kidney, heart, spleen, bile, bladder urine and blood were sampled at necropsy.

Blood and tissue samples were counted in a gamma counter (Packard Instruments Co., Meriden, CT). Samples of the dose were counted repeatedly to allow correction for decay of ^{51}Cr. Total ^{51}Cr in blood was estimated assuming blood was 7% of body weight of pigs (Engelhardt, 1965). The ^{51}Cr in each tissue was expressed as percent of the ^{51}Cr dose.

Data were analyzed using the Statistical Analysis System (SAS, Cary, NC, version 8.1). Log transformations were performed on blood, urine and tissue data to correct for non-homogenity of variance. The general linear model (GLM) procedure was used for analysis of variance of the transformed tissue and urine data. Repeated measures analyses were performed on the transformed blood data by PROC MIXED procedures of SAS. Significant differences were set at $P<0.05$.

Results and Discussion

Gilts injected with LPS endotoxin had significantly lower ($P<0.05$) blood ^{51}Cr at 3 ($P<0.03$), 4 ($P<0.01$), 5 ($P<0.007$), 6 ($P<0.03$) and at 8 ($P<0.04$) h compared to the controls (Figure 5.1). Nine hours after the endotoxin dosing ^{51}Cr retention was significantly lower in liver ($P<0.01$), kidney ($P<0.02$) and heart ($P<0.04$)in endotoxin-treated pigs and tended to be lower in spleen ($P<0.06$) compared to control group (Table 5.1). Infection associated with LPS endotoxin significantly decreased the ^{51}Cr retention in the tissues. Total ^{51}Cr in urine collected from the bladder varied considerably within treatment, but tended to be lower in the gilts injected with endotoxin (0.36 vs 1.40 % of the dose). The results suggest that the ^{51}Cr absorption was low in the endotoxin treated pigs. No significant effect of endotoxin on the bilary Cr concentration was observed.

Rectal body temperature was higher ($P<0.05$) in endotoxin injected pigs (39.4°C) compared to control pigs (37.9°C) at 9 h post injection.

One of the mechanisms of acute phase response to LPS endotoxin is the activation of prostaglandin E_2. Prostaglandins were found to decrease the gut absorptive capacity and also prostaglandins were shown to increase mucosal alkaline secretion in rats. Studies conducted in our laboratory have shown that administration of an analog of prostaglandin E_2 (16,16-dimethyl-PGE_2 at 7.5 μg/kg body wt) (dm PGE_2) (Kamath et al. 1997) and injection of interleukin - 1α (1 μg/kg body wt) (Davis-Whitenack et al. 1999) significantly decreased ^{51}Cr absorption and tissue retention in rats. Administration of 5-30 μg/kg dmPGE_2 increased mucosal alkaline secretion in rats (Takeuchi et al. 1986; Takeuchi et al. 1988; and Leung et al. 1989). In the present study a decrease in the absorption of the ^{51}Cr may be due to the prostaglandins produced in response to endotoxin infection which may have contributed to the increased alkaline medium in the mucosa of the duodenum. Cr is poorly absorbed from the alkaline environment (Mertz & Roginski 1969).

Indomethacin inhibits the cyclooxygenase pathway of prostaglandin synthesis. Johnson & von Borell (1994) reported decreased effects of LPS challenge in pigs pretreated with indomethacin (5mg/ kg body wt). Pretreatment with indomethacin (5mg/ kg body wt, i.p.) in rats 30 minutes before the ^{51}Cr dose increased ^{51}Cr concentrations in the blood compared to the placebo group (Kamath et al. 1997).

Singh et al. (1992) reported a significant decrease in gut absorptive capacity at 2 and 4 h after the initiation of sepsis by cecal ligation and puncture in rats. Also

administration of TNF-α was found to depress the gut absorptive function which might be due to the release of PGE_2 which decreases the gut absorptive capacity (Singh et al. 1993). In the present study the decreased absorption and tissue retention of $^{51}CrCl_3$ might be due to several factors. Cytokines, expressed by LPS activated leukocytes, induce prostaglandin synthesis that would lead to altered gut absorptive capacity. Another possibility for LPS induced decreased absorption might be due to the decreased blood flow to the intestinal microvilli. Schmidt and coworkers reported a reduction in the villous blood flow in rats when injected i.v. with either 1.5 mg LPS/kg or 15 mg LPS /kg body wt. The authors suggested it might be due to the effect of endotoxin on the microcirculation of intestinal villi (Schmidt et al. 1996).

Conclusion

In the present study LPS endotoxin significantly decreased the ^{51}Cr concentrations in the tissues and tended to decrease bladder urine suggesting that LPS endotoxin may have decreased the absorption of Cr from the intestinal mucosa.

Acknowledgements

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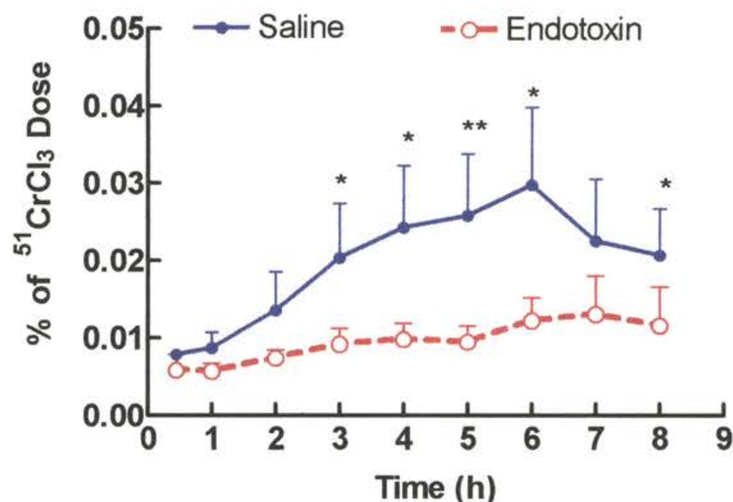

Figure 5.1 ^{51}Cr in blood of weanling gilts following an oral dose of $^{51}CrCl_3$. Gilts were injected intraperitoneally with LPS endotoxin or control 1 h prior to the ^{51}Cr dose. Data represent time-points after the ^{51}Cr dose and are expressed as percentage of total ^{51}Cr *p<0.04; **p<0.007.

Table 5.1: Tissue ^{51}Cr 9 h after Intraperitoneal Injection of Saline or Endotoxin and 8 h after Oral Dosing with $^{51}CrCl_3$ in Weanling Pigs* †

Tissue	Saline	Endotoxin	P-value‡
Liver	0.0412 ± 0.0147	0.0215 ± 0.0105	P<0.01
Kidney	0.0443 ± 0.0157	0.0269 ± 0.0163	P<0.04
Heart	0.0060 ± 0.0021	0.0033 ± 0.0019	P<0.02
Spleen	0.0018 ± 0.0004	0.0013 ± 0.0007	P<0.06
Bile	0.0015 ± 0.0006	0.0012 ± 0.0006	P<0.19
Bladder Urine	1.4000 ± 0.8202	0.3607 ± 0.2603	P<0.16

*Mean ± SEM (standard error of mean). †Data are expressed as percentage of ^{51}Cr dose per gram tissue. ‡Data were log transformed for statistical analyses.

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CHAPTER VI

Dietary Zinc Supplementation and Endotoxin Challenge in Weanling Pigs: Effect on Hematology, Clinical Chemistry and Hormone Profile[a,b]

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Running Title: Dietary Zinc and Endotoxin in Weanling Pigs

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[a]The project was supported by Targetive Research Intiative Program (TRIP) and Oklahoma Agricultural Experiment Station (OAES)- Hatch 2041, 2132

[b]Part of the data were presented at the American Society of Animal Sciences, Mid-West Meetings, S.L. Mandali., S.D. Carter., A.B. Arquitt., E.A. Droke., B.J. Stoecker., L.J. Spicer and M.J. Rincker. Effects of dietary zinc and endotoxin challenge on the acute phase response in weanling pigs. Journal of Animal Science, 2001, 79 (Suppl 1): and at the Society for Experimental Biology Meetings. S.L. Mandali., A.B. Arquitt., S.D. Carter., E.A. Droke., B.J. Stoecker., L.J. Spicer and M.J. Rincker. Effects of dietary zinc

and endotoxin on immune function and clinical parameters in weanling pigs fed milk based formula. FASEB J, 2000, 14, A239.

Abstract

A 2 x 4 factorial arrangement of treatments was used to determine the effects of dietary Zn concentration and endotoxin challenge on weanling pigs. Forty-eight weanling pigs (5.5 kg; 15 d) were allotted randomly to four liquid diets. Dietary treatments were prepared by adding $ZnSO_4$ to a customized commercial milk replacer (MR, 5 ppm Zn) (Merick's Inc., Union Center, WI) to provide 0, 100, 1,500, or 3,000 ppm added Zn. Diets were mixed prior to feeding by adding 12 g of MR to 100 mL of deionized water. The diets were fed 4 times/d for 14 d. On d 14, pigs were fasted 10 h and injected i.p. with saline or 25 μg LPS (E.coli 0111:B4)/kg BW. Blood samples and temperature were collected at 0 h (before injection), and at 3, 6 and 24-h post injection (PI). Growth performance from d 0-14 was not affected ($P>0.10$) by dietary Zn. On d 14 (prior to LPS), plasma Zn concentrations for the 4 dietary treatments were, respectively: 0.36, 1.09, 3.50, and 4.30 mg/dL (linear, $P<0.01$). Increasing dietary Zn decreased C-reactive protein (CRP), albumin, and cortisol (linear, $P<0.01$), but increased triglycerides and urea nitrogen (linear, $P<0.01$). A biphasic response was observed over time for temperature, leukocyte, neutrophil, and lymphocyte counts in pigs injected with LPS, but no response was noted in control pigs (LPS x time, $P<0.01$). Body temperature and cortisol increased up to 6 h in LPS-injected pigs, and then decreased to baseline values by 24-hr PI (LPS x time, $P<0.01$). A Zn x time interaction ($P<0.01$) was observed for plasma Zn and CRP; the response to dietary Zn was linear at 0 and 24-h PI, but quadratic responses were noted at 6 h. Increasing Zn decreased insulin in control pigs, but increased it in LPS-injected

pigs (Zn x LPS, $P<0.01$). These results suggest that endotoxin challenge has a marked effect on the acute phase response of weanling pigs. Dietary Zn affected cortisol and C-reactive protein independent of endotoxin challenge. This suggests that dietary Zn at concentrations of more than 100 ppm decreases the acute phase response.

Key Words: • Pigs • CBC • Insulin • Body Temperature • Cortisol • C-reactive protein • Zinc • Endotoxin

Introduction

The importance of zinc in human and animal nutrition has been recognized for more than 40 years. Zinc deficiency is common in young children in the developing world and is associated with reduced immunocompetence and increased rates of serious infectious conditions (Yamey 1999). Zinc is essential for the normal development of immune system function because of its importance in the activation of the thymic hormone required for the maturation of the T cells (Dardenne et al. 1982). Zinc deficiency has been shown to decrease T lymphocyte production in animals. Apart from being an essential modulator of immune system, zinc also plays a role in protein, carbohydrate, and lipid metabolism. Prasad et al (Prasad et al. 1961) found that growth failure and arrested sexual maturity was due to the deficiency of zinc in malnourished children in Iran and that the symptoms were reversed by dietary supplementation of the zinc. The importance of zinc in swine nutrition was first demonstrated by Tucker and Salmon's classical study done on parakeratosis in swine (Tucker and Salmon 1955).

Wasting of peripheral tissues, fever, and loss of appetite are features of infection. The response to acute infection is characterized by protein and fat catabolism (Lin et al. 1998), increased nitrogen loss, insulin resistance (Leininger et al. 2000), increased

synthesis of the hepatic acute phase proteins and antioxidants, and increased energy expenditure (Lin et al. 1998, Grimble 1998). Rapid increases in plasma concentrations of non-esterified fatty acids (Leininger et al 2000) and glycerol occur with infection due to increased hepatic lipogenesis and enhanced lipolysis in adipose tissue (Wolfe 1997).

Feeding pharmacological doses of zinc has been shown to improve growth performance in weanling pigs. Growth stasis is commonly observed in pigs during the weaning transition, which causes profound loss to pork producers. Strategies to improve production such as early weaning in piglets might impose physiological stress (Myers et al. 1999). It is postulated that under extreme production conditions, immune and endocrine responses may be altered to affect the ultimate health status of the animal (Borgs and Mallard 1998). The effect of feeding pharmacological amounts of dietary zinc on the immune system in weanling pigs has not been reported previously. Hence, the present study was undertaken to determine the effects of feeding pharmacological doses of zinc alone or in combination with LPS treatment on the clinical and immunological parameters in early-weaned pigs.

Materials and Methods

Exprimental Design: Forty-eight, 14 d old boar pigs (BW 4.5 ± 0.3 kg) were weaned, individually housed in plastic metabolic cages, and were assigned randomly to four dietary zinc concentrations in a randomized complete block design.

Dietary Treatment: Dietary treatments included basal diet with no added zinc 0, 100, 1500, and 3000 ppm added zinc as zinc sulfate (Prince Agri, Quincy, IL). The diets were prepared by addition of trace minerals to a customized commercially prepared milk replacer formula (Merick's Inc. Union Center, WI) containing no added trace minerals.

The diets were mixed before feeding with 12 g of diet plus 100 ml of de-ionized water. Pigs were fed 4 times a day. After 2-d on the basal diet, pigs were fed the experimental diets for 14 d. The amounts fed each feeding increased daily from 200 to 1200 ml by day 14 of the treatment period. Deionized water was provided ad libitum.

Endotoxin treatment: On day 14 of the experimental period, pigs were deprived of food for 10-h and injected intraperitoneally (i.p.) with saline or 25 μg/kg body weight endotoxin (Lipopolysaccharide, E.coli 0111:B4) (Sigma Chemicals, St.Loius, MO).

Analyses: Rectal body temperature measurements and fasting blood samples were collected prior to LPS injection at 0 h, and at 1.5, 3, 6, and 24 h after LPS injection. Hematological parameters were analyzed (0, 1.5, 3, 6 and 24 h) using ABX-Pentra 120 Retic (ABX Diagnostics, Irvine, CA). Serum was analyzed for clinical chemistries (0, 6, 24 h) using commercially available reagents (Cobas Fara II Clinical Analyzer, Roche Diagnostic Systems, NJ). Plasma Cortisol (0, 3, 6 and 24 h) and insulin (0, 6, and 24 h) were measured using solid-phase RIA kits (Diagnostic Products Corporation, Los Angeles, California).

Statistical analyses: The experiment was a randomized complete block design. The data were analyzed using PROC MIXED with sources of variation including diet, endotoxin, time, diet x endotoxin (error term for diet and endotoxin), time x diet x endotoxin interaction and residual. If the diet x endotoxin interaction were significant ($P<0.05$), simple effects of diet x endotoxin were analyzed using the SLICE option for the LSmeans statement. If diet x endotoxin were not significant ($P>0.05$), and if main effects were significant, these were analyzed using LSmeans with the PDIFF option (PC SAS version 8.01, SAS Institute, Cary, NC). The contrasts were used to compare unequally

spaced coefficients using interactive matrix language (PROC IML) with single degrees of freedom for linear, quadratic and cubic effects. Significance was set at P<0.10

Results

Experimental diets: The analyzed concentration of zinc was 5.21, 129.40, 1544.0, 2859.0 ppm respectively for 0, 100, 1500, and 3000 ppm added zinc.

Weight gain and Performance: The initial weight of the weanling pigs was 5.08 ± 0.17 kg. The Average daily gain (ADG), average daily feed intake (ADFI) and gain:feed (G:F) did not significantly differ among dietary treatments during the 14-d supplementation period.

Body Temperature: The increase in body temperature with endotoxin treatment was considered evidence of infection. Body temperature was elevated (LPS x Time, P<0.0001) with endotoxin over time. Body temperature did not differ significantly between the diet groups during the 6 h experimental period (Zn x Time, P>0.1).

Hematology: Significant (LPS x Time, P<0.05) decreases in lymphocytes, and monocytes (Table 6.1), and increases in leukocytes (WBC) (Table 6.3), neutrophils, and eosinophils (Table 6.1) were observed with endotoxin treatment over time. Leukocytes and neutrophils increased within first 6 h PI and then decreased to baseline values by 24 h PI. Lymphocytes decreased during the first 6 h of endotoxin exposure but by 24 h PI the values recovered to preinjection baseline values. No effect of diet was observed on the hematological indicators over the 6 h experimental period (Zn x Time, P>0.1)

(Table 6.1).

Clinical Chemistry: Increased muscle breakdown was observed at 6 h after endotoxin administration indicated by the elevated serum creatinine and urea nitrogen in

endotoxin treated pigs fed 1500 and 1000 ppm dietary zinc compared to the pigs in the other groups (Table 6.2). Signficant decrease in albumin ($P<0.07$) and glucose ($P<0.08$), and a linear increase in urea nitrogen ($P<0.001$), and triglycerides ($P<0.009$) were observed with increasing dietary zinc supplementation (Table 6.2).

Hormone Profile: Increased plasma cortisol ($P<0.005$) and C-reactive protein (LPS x Time, $P<0.05$) (Table 6.3) was observed with endotoxin treatment over time. A linear increase was observed for plasma cortisol (Zn, $P<0.05$), and C-reactive protein (Zn, $P<0.01$) with linear increase in the dietary concentrations of zinc. A linear effect of endotoxin with diet (Zn x LPS, $P<0.04$) was observed for the serum insulin (Figure 6.1). Elevated acute phase protein secretion was observed in zinc deficient group with inceased C-reactive protein ($P<0.01$) that remained elevated at 24 h PI (Table 6.3).

Discussion

In the present experiment, growth performance, hematology, and clinical parameters were evaluated in weanling pigs supplemented with different concentrations of zinc and challenged with the endotoxin, LPS E. Coli 0111:B4. Leukocyte counts, neutrophils (%) and body temperature increased within the first 3 h of the endotoxin challenge, and the values continued to be elevated at 6 h PI. The values reached the baseline values by 24 h PI. Lymphocytes, monocytes, eosinophils and basophils decreased by 3 h PI and continued to be depressed at 6-h PI and returned to the baseline values by 24 h PI. The results are consistent with infection resulting from the endotoxin challenge. Kornegay and coworkers (Kornegay et al. 1993) reported decreased total leukocytes and lymphocytes, hemoglobin, hematocrit, serum glucose and urea nitrogen with stress created by inadequate pen space.

Circulating lymphocytes are sensitive to glucocorticoids such that even a minor increase in the cortisol was found to deplete the thymus derived circulating lymphocytes (Kornegay et al 1993). Glucocorticoid elevations result in thymocyte death by activating endonuclease that rapidly degrades DNA causing cell death (Cohen and Duke 1984). In the present study cortisol increased significantly with endotoxin treatment and resulted in decreased lymphocytes in the endotoxin group.

In the present study pigs fed zinc deficient diets had elevated plasma cortisol concentrations compared to the pigs fed zinc adequate or excess diets confirming previous reports (Quarterman and Humphries 1979, Kornegay et al. 1993). Serum creatinine and urea nitrogen increased at 6 h PI indicating skeletal muscle breakdown in response to endotoxin; however, those fed 3000 ppm dietary zinc fed pigs had elevated urea nitrogen and triglycerides significantly greater than the other groups suggesting that the breakdown of protein and fat was highest in the 3000 ppm group.

Hyper-metabolic state increases serum glucose concentrations, in the present study hypoglycemia was observed at 24 h PI in the endotoxin group independent of the level of dietary zinc. Fasting during the experimental period might have contributed to the decrease in serum glucose. Non-significant decreases in glucose concentrations were observed in the 3000 ppm zinc supplemented group and can be related to the increased insulin concentrations in 3000 ppm group. Droke et al (Droke et al. 1993) also reported numerical decreases in plasma glucose and increases in serum insulin in lambs supplemented with adequate (40 mg/kg diet) dietary zinc compared to the lambs fed deficient (3.7 mg/kg) and marginal (5 mg/kg) dietary zinc. The transcriptional control of phosphoenolpyruvate carboxykinase (PEPCK), a key rate limiting enzyme in the

gluconeogenic pathway is reduced by zinc deficiency (Rofe et al. 1996, Kennedy et al. 1998). Insulin and glucagon, often elevated in endotoxemia, are found to be opposing the effect of PEPCK (Rofe et al. 1996). In the present study the joint effect of endotoxin and dietary zinc in the 3000 ppm and 0 ppm group resulted in elevated insulin at 6 h PI. The results suggest a reduced hepatic extraction of insulin from the portal blood into the liver both by zinc excess and zinc deficient diets.

In the present study an increase in C-reactive protein was observed at 6 h and remained elevated at 24 h PI of endotoxin. Similarly Gaetke et al (Gaetke et al. 1997) reported a significant elevation in C-reactive proteins after LPS injection. The elevation in CRP was highest in the zinc deficient group of the present study indicating that increase in stress-induced acute phase protein synthesis may be due to a zinc deficiency.

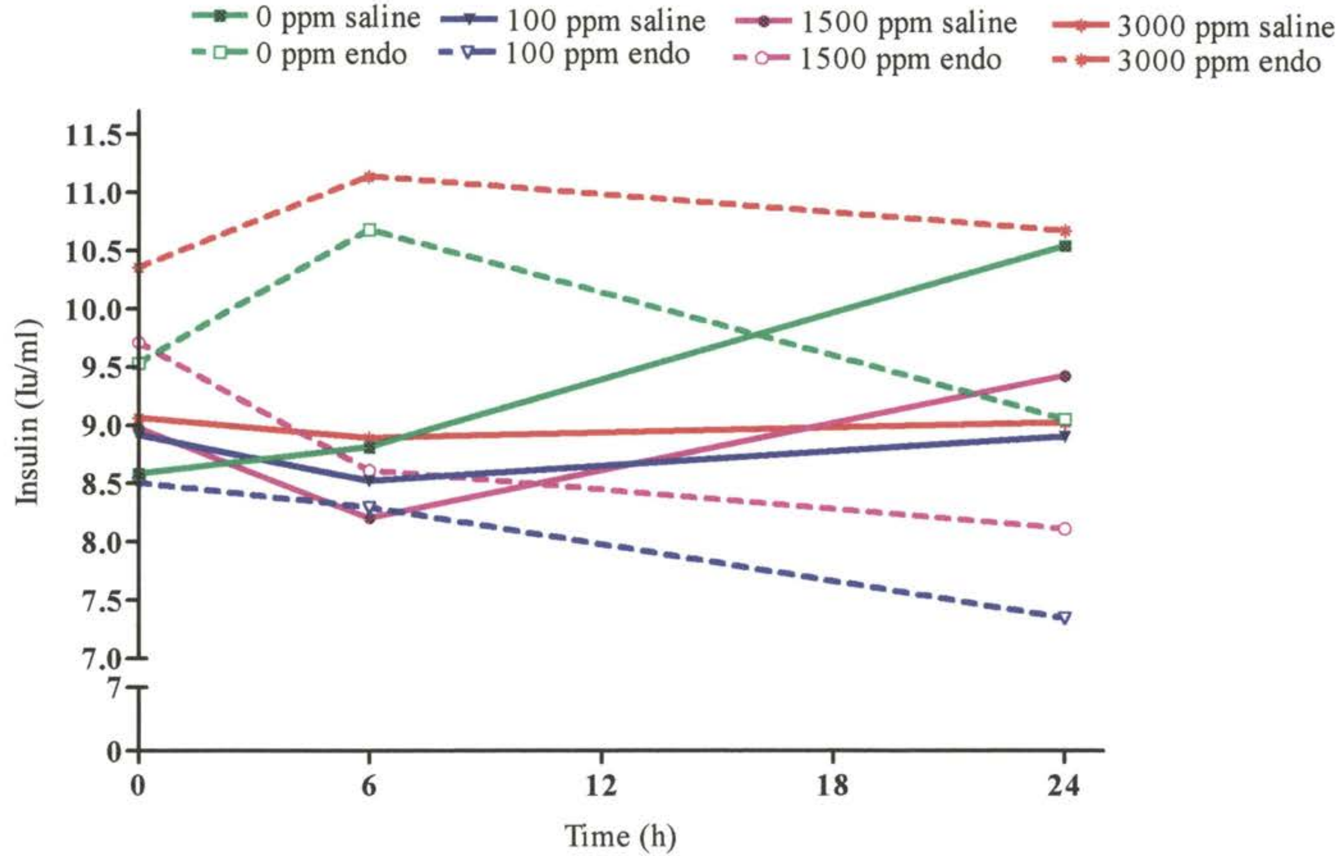
Conclusions

In the present study the 3000 ppm dietary zinc fed groups had decreased plasma cortisol and C-reactive protein compared to the rest of the groups. The results indicate that dietary zinc supplementation above the NRC requirement (100 ppm) may be beneficial during endotoxin-induced stress. The NRC recommended zinc level was sufficient to promote adequate growth, but during periods of stress this level of dietary zinc may not be beneficial. Levels more than 100 ppm might be needed to meet the demands to mount an acute phase response.

The current study is the first to evaluate the time course and magnitude of immune response after LPS administration in weanling pigs supplemented with deficient, adequate and pharmacological levels of dietary zinc. Further research on the effects of varying

concentrations (100 - 3000 ppm) of zinc on the immune response of weanling pigs during bacterial infections is recommended.

Figure 6.1: Effect of Endotoxin and Dietary Zinc on Serum Insulin in Weanling Pigs Over Time.*



*Pooled SEM 0.55, D x E (P<0.04)

Table 6.1: Main Effects of Endotoxin and Dietary Zinc on the Hematological Parameters in Weanling Pigs[1]

Time	Endotoxin (ug/kg)		P-Value[3]	Dietary Zinc (ppm)					P-value[3]		
(h)	0	25	E x T	0	100	1500	3000	SEM[2]	D	E x D	D x T
Lymphocyte %											
0	48.69	47.78	<0.05	47.62	46.11	50.24	48.99	6.52	0.90	0.90	0.8
1.5	44.34	50.17		43.97	50.30	47.97	46.79	8.09			
3	42.79[a]	30.24[b]		38.10	38.49	36.19	33.30	6.44			
6	47.29[a]	21.76[b]		37.89	34.43	30.64	35.14	6.34			
24	55.06	51.46		57.22	54.08	49.82	51.95	5.91			
Neutrophil %											
0	47.07	47.62	<0.05	47.63	49.61	45.71	46.45	6.72	0.90	0.90	0.9
1.5	51.82	46.34		52.04	46.42	48.68	49.25	8.13			
3	53.61[a]	65.17[b]		58.59	57.44	58.26	63.26	6.53			
6	49.28[a]	74.76[b]		59.28	61.71	66.53	60.57	6.5			
24	39.16	41.64		35.70	39.31	43.95	42.69	5.89			
Monocyte %											
0	2.29	2.45	>0.05	2.56	2.72	2.17	2.03	0.25	<0.05	0.60	0.3
1.5	1.87	1.51		2.03	1.64	1.53	1.55	0.27			
3	2.30	1.73		2.09	2.19	1.95	1.82	0.46			
6	2.02	1.80		2.12	2.19	1.47	1.93	0.41			
24	3.25	3.29		3.78	3.93	2.81	2.57	0.62			
Eosinophil %											
0	2.10	2.20	<0.05	2.26	2.01	1.80	2.51	0.4	0.70	0.70	0.7
1.5	1.85	1.81		1.58	1.66	1.75	2.31	0.65			
3	1.56	2.26		1.72	1.96	2.00	1.98	0.64			
6	1.39	1.20		1.01	1.28	1.35	1.52	0.34			
24	2.35[a]	3.66[b]		3.50	2.62	3.29	2.62	0.61			
Basophil %											
0	0.15	0.13	>0.05	0.14	0.15	0.12	0.15	0.03	0.40	0.70	0.7
1.5	0.16	0.13		0.14	0.13	0.13	0.17	0.03			
3	0.11	0.12		0.13	0.13	0.09	0.10	0.02			
6	0.12	0.10		0.10	0.09	0.10	0.10	0.02			
24	0.14	0.18		0.20	0.19	0.12	0.15	0.03			

[1] Data are the LSmeans n=6 per treatment. [2] pooled SEM n=6. [3] Means with in a row with a different superscript differ significantly.

Table 6.2: Main Effects of Endotoxin and Dietary Zinc on the Serum Chemistry in Weanling Pigs [1]

	Time	Endotoxin (ug/kg)		P-Value[3]	Dietary Zinc (ppm)					P-Value[3]		
	(h)	0	25	E x T	0	100	1500	3000	Pooled SE[2]	D	E x D	D x T
Creatinine												
(mg/dL)	0	1.12	1.19	>0.1	1.15	1.12	1.16	1.20	0.06	0.1	0.07	>0.1
	6	1.23	1.32		1.28	1.24	1.24	1.34	0.05			
	24	1.13	1.22		1.15	1.16	1.19	1.20	0.06			
Glucose												
(mg/dL)	0	117.43	112.80	>0.1	110.91	118.00	120.33	111.20	7.92	0.08	0.19	>0.1
	6	105.67	104.98		112.95	99.77	108.41	100.17	10.13			
	24	121.09	111.92		120.91	119.25	117.45	108.42	8.05			
Urea Nitrogen												
(mg/dL)	0	9.23	9.80	>0.1	8.88^{a}	6.78^{b}	8.15^{a}	14.23^{c}	1.73	0.001	0.07	>0.1
	6	11.22	11.02		10.26^{a}	9.68^{a}	10.74^{a}	13.80^{b}	1.93			
	24	9.33	10.44		7.63^{a}	7.27^{a}	11.27^{b}	13.36^{c}	1.6			
Albumin												
(mg/dL)	0	3.16	3.27	>0.1	3.45	3.31	3.07	3.03	0.14	0.07	>0.1	>0.1
	6	3.16	3.04		3.21	3.13	3.08	3.00	0.15			
	24	3.01	3.10		3.23	3.07	2.88	3.03	0.16			
Protein												
(mg/dL)	0	5.05	5.27	>0.1	5.56	5.04	4.99	5.07	0.27	0.1	>0.1	>0.1
	6	4.91	4.95		5.05	4.84	4.99	4.84	0.16			
	24	4.83	4.83		5.10	4.70	4.69	4.85	0.22			
Triglycerides												
(mg/dL)	0	30.56	32.57	<0.01	32.33^{a}	25.16^{b}	31.06^{a}	37.7^{c}	3.75	0.009	>0.1	>0.1
	6	37.31	34.60		32.89^{a}	32.5^{a}	33.86^{a}	44.5^{b}	4.36			
	24	27.94^{a}	39.31^{b}		32.58^{a}	27.58^{b}	37.9^{c}	36.44^{b}	6.15			

[1] Data are the LSmeans n=6 per treatment.
[2] pooled SEM n=6.
[3] Means with in a row with a different superscript differ significantly.

Table 6.3: Main Effects of Endotoxin and Dietary Zinc on the Acute Phase Response in Weanling Pigs [1]

	Time	Endotoxin (ug/kg)		P-Value[3]	Dietary Zinc (ppm)					P-Value[3]		
	(h)	0	25	E x T	0	100	1500	3000	SEM[2]	D	E x D	D x T
White Blood Cells ($10^3/mm^3$)												
	0	14.63	18.10	<0.05	16.38	15.57	18.78	14.73	3.06	0.90	0.90	0.1
	1.5	14.86	14.71		15.21	12.87	18.25	12.32	3.09			
	3	14.49^{a}	21.12^{b}		19.05	16.95	21.95	13.27	4.27			
	6	13.77^{a}	27.21^{b}		20.28	20.28	22.78	18.63	3.92			
	24	13.05^{a}	17.19^{b}		14.41	13.50	18.49	14.07	2.23			
C-Reactive Protein												
(mg/dL)	0	4.43	5.08	<0.05	6.84^{a}	4.95^{b}	4.49^{b}	2.74^{c}	0.93	<0.01	0.89	<0.05
	6	4.21	5.18		4.99	4.67	4.08	5.04	0.91			
	24	3.59^{a}	6.89^{b}		7.08^{a}	5.32^{b}	4.65^{b}	3.77^{c}	0.72			
Cortisol	0	116.07	133.92	<0.005	149.53^{a}	125.68^{b}	130.14^{a}	94.50^{c}	17.62	<0.05	0.94	0.1
(nmol/mL)	3	119.75^{a}	475.93^{b}		319.64^{a}	331.37^{a}	285.03^{b}	255.40^{b}	82.45			
	6	158.59^{a}	260.56^{b}		253.42^{a}	194.79^{a}	212.18^{a}	177.92^{a}	42.4			
	24	110.44^{a}	89.17^{b}		121.68^{a}	110.79^{a}	89.50^{b}	77.20^{c}	14.17			
IGF-I	0	74.02	50.24	0.90	68.64	91.9	54.51	34.21	12.4	0.90	0.90	0.1
(ng/mL)	6	49.12	48.35		52.71	63.38	48.90	29.91	12.4			
	24	50.19	29.94		50.66	48.39	26.18	34.98	11.24			

[1]Data are the LSmeans n=6 per treatment.

[2] pooled SEM n=6.

3 Means with in a row with a different superscript differ significantly.

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CHAPTER VII

Immune Response and Hormone Profile of Endotoxin Challenged Weanling Pigs Fed Different Levels of Dietary Chromium as Chromium Picolinate

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[c]Part of the data were presented at the Society for Experimental Biology Meetings, 2001 S. L. Mandali., A.B. Arquitt., S.D. Carter., B.J. Stoecker, E.A. Droke., L.J. Spicer and M.J. Rincker. Dietary chromium and endotoxin affect hormone and clinical chemistry profiles in weanling pigs. FASEB. J.

Abstract

A 2 x 4 factorial arrangement of treatments was used to determine the effects of dietary chromium (Cr) concentration as chromium picolinate (CrPic) and endotoxin (LPS) challenge in weanling pigs. Forty-eight weanling pigs (5.5 ± 0.5 kg; 16 d) were allotted randomly to four diet groups. Dietary treatments were prepared by adding CrPic to a customized commercial milk replacer (MR*, 1.77 ppb Cr) to provide 0, 200, 600 or 1000 μg Cr/kg dry wt MR. Diets were mixed prior to each feeding (12 g of MR/100 ml de-ionized water) and were fed four times/d for 14 d. On d 14, pigs were fasted 10 h and injected i.p. with saline or 25 μg LPS (E.coli 0111:B4)/kg body wt. Blood samples were collected and rectal body temperatures were measured at 0 h (before LPS injection), and at 1.5, 3, and 6 h post injection (PI). Urea nitrogen, white blood cells, neutrophil, and were increased; lymphocytes counts decreased in LPS injected pigs (LPS x time $P<0.01$). C-reactive protein, and urea nitrogen increased linearly with dietary chromium and LPS challenge (Cr x LPS $P<0.05$). Cortisol was higher in pigs fed 600 ppb Cr injected with LPS (Cr x LPS $P<0.05$). Serum insulin increased quadratically with dietary Cr and LPS challenge peaking at 6 h PI (Cr x LPS x Time $P<0.05$). Serum IGF decreased over time independent of dietary treatment or LPS challenge (Time $P<0.01$). Data suggest that dietary chromium (600 ppb) alters selected clinical and hormonal parameters as well as the response to endotoxin challenge in weanling pigs.

Key Words: • Pigs • Chromium Picolinate• TNF-α • LPS • Body Temperature • Cortisol • C-reactive protein

Introduction

Literature from the past 40 years demonstrates that chromium is an essential trace nutrient in human nutrition, and its effects on glucose tolerance, lipids, and lean body growth have been reviewed extensively (Anderson 1987, Mertz 1993). Recently the influence of chromium on the stress response has gained attention. Elevated plasma cortisol was observed in chromium depleted guinea pigs during stress (Seaborn and Stoecker 1990). Chromium supplementation has been reported to decrease circulating cortisol during stress in calves (Moonsie-Shangeer and Mowat 1993). Impaired carbohydrate metabolism during periods of stress was found to be improved with chromium supplementation in animals (Chang and Mowat 1992, Lee et al. 2000, Lindemann et al. 1995, Page et al. 1993) and in humans with various pathological conditions (Carters et al. 1968,Gurson and Saner 1971, Hopkins et al. 1968, Pekarek et al. 1975).

Effect of chromium supplementation on the immune system in animals is controversial. Chromium supplementation modulated immune response in pigs (Lee et al. 2000, Myers et al. 1995, Myers et al. 1997), in calves (Burton et al. 1993, Chang and Mowat 1992, Kegley et al. 1996, Moonsie-Shangeer and Mowat 1993) and in dairy cows (Burton et al. 1993) in some studies. Other studies found no beneficial effect of supplementation of different sources of chromium on the immune response in endotoxin challenged pigs (van Heugten and Spears 1997) or in the young calves supplemented with chromium as high chromium yeast followed by inoculation with Bovine Herpevirus-1 (Arthington et al. 1997).

Lipopolysaccharide-induced macrophage stimulation and the elevations in cytokines in response to endotoxin have been reported previously (Lee et al. 2000, Myers et al. 1995, Myers et al. 1997). Also it was observed that stress results in an increase in cortisol, and a reduction in the lymphocyte proliferative responses to mitogens (Minton et al. 1992).

Elevated cortisol during stress acts antagonistically to insulin, preventing the entry of glucose into muscle and adipose tissue. If chromium increases action of insulin, then the increased insulin action may decrease the metabolic effects of cortisol during stress. The role of chromium on the immune response during periods of stress has yet to be answered. Hence the present study was undertaken to determine the effects of dietary supplementation of varying levels of chromium on immune response in weanling pigs during periods of stress induced by bacterial infections.

Materials and Methods

Animals: Forty-eight, 14 day old boar pigs (BW of 4.5 ± 0.5 kg) were weaned, individually housed in plastic metabolic cages, and randomly assigned to four dietary chromium concentrations in a randomized complete block design. Replications were considered as blocks. The diets were prepared by addition of trace minerals to a commercially prepared milk replacer containing no added trace mineral source (Merick's Inc, Union Center, WI). Dietary treatments included basal diet with a) no added chromium b) 200, c) 600 or d) 1000 μg added chromium as chromium picolinate/kg diet (Prince Agri, Quincy, IL). The dry diets were mixed with water immediately before every feeding. For every 100 ml of deionized water, 12 g dry diet was added and mixed thoroughly. Pigs were fed 4 times a day: 8.00 a.m., 12:00 p.m., 5:00 p.m., and 12:00 a.m

and after 2 d on the basal diet, pigs were fed the experimental diets for 14 d. The amounts fed at each feeding were increased daily from 200 to 1200 ml by day 14 of the experimental period. On day 14, pigs were deprived of food for 10 h and injected intraperitoneally with saline or endotoxin (25 μg LPS, *E.Coli*, 0111:B4/kg body weight) (Sigma Chemicals, St.Louis, MO). Water was provided ad libitum.

Blood collection and analysis: Rectal body temperature measurements and fasting blood were collected before (h 0) and at 1.5, 3 and 6 h after LPS injection. Blood was collected from the jugular vein using a Monoject vaccutainer with a 20 x 1 needle {Sherwood Medical, St.Louis, MO). Whole blood was analyzed for complete blood counts and differential counts using the ABX Pentra 120 (ABX Diagnostics, Irvine, CA). Blood samples were centrifuged (4°C) at 2000 X g for 30 minutes, and serum and plasma were aliquoted and stored at –20 °C. Serum was analyzed for glucose, albumin, total protein, urea nitrogen, creatinine, and triglycerides using commercially available reagents by COBAS FARA II clinical analyzer (Roche Diagnostic Systems, NJ). Serum insulin was measured (at 0 and 6 h) by radioimmunoassay methods (Diagnostic Products Corporation, Los Angeles, CA) using human controls as calibrators. Insulin like growth factor 1 (IGF-1) (Nichols Institute Diagnostics (NID), San Juan Capistrano, CA) and cortisol (Diagnostic Products Corporation, Los Angeles, CA) were measured at 0, 3, and 6 h by radioimmunoassay method using human kits and serum calibrators as controls for inter-assay variation. Tumor necrosis factor-α (TNF-α) was measured (at 0, 1.5, 3 and 6 h) using an ELISA kit for pig TNF-α (Endogen, Inc., Woburn, MA).

Statistics: The experiment was a randomized complete block design with a 2 x 4 factorial arrangement of treatments. The data were analyzed using PROC MIXED with

sources of variation including diet, endotoxin, time, diet x endotoxin (error term for diet and endotoxin), time x diet x endotoxin interaction and residual. If the diet x endotoxin interaction was significant ($P<0.05$), simple effects of diet x endotoxin were analyzed using the SLICE option for the LSmeans statement. If diet x endotoxin was not significant ($P>0.05$) then main effects were analyzed for significance using LSmeans with the PDIFF option (SAS 1989). The single degrees of freedom contrasts were used for diet and endotoxin main effects and the interactions. The coefficients for unequally spaced dietary levels of chromium were derived from using the integrative matrix language (PROC IML) procedure of SAS (1985).

Results

Weight Gain and Growth Performance:

No difference in average daily gain (ADG) was observed with chromium supplementation (Table 7.1). However the pigs fed 200 μg chromium as chromium picolinate/kg diet showed a numerically higher ADG compared to groups supplemented with 0, 600 and 1000 μg chromium/kg diet. A significant ($P<0.05$) increase in average daily feed intake (ADFI) was observed for the 200 μg chromium supplemented group (Table 7.1). Gain: feed was highest ($P<0.008$) in the group supplemented with 1000 μg chromium. The pigs fed 1000 μg chromium as chromium picolinate/kg diet exhibited greater improvements in feed conversion efficiency compared to 0, 200 and 600 μg chromium-supplemented group. The feed intake was numerically higher in pigs supplemented with 200 μg chromium as chromium picolinate/kg diet, and lower in the 1000 μg chromium group, when compared to the groups supplemented with 0 and 600 μg chromium /kg diet.

Body Temperature:

Endotoxin increased body temperature (LPS x Time P<0.05) at 3 h PI. No effect of diet (Cr) or the treatment combination (Cr x LPS) was observed for the body temperature at any of the time periods (P>0.1) (Table 7.2)

Hematology:

Endotoxin significantly decreased white blood cells, and monocytes at 1.5 h. White blood cells increased at 3 h experimental period. At 3 h post injection lymphocytes and monocytes decreased and neutrophils increased with endotoxin. At 6 h post injection endotoxin significantly increased white blood cells, neutrophils and decreased monocytes and lymphocytes (Table 7.2).

Clinical Chemistry:

Serum urea nitrogen increased and triglycerides decreased with endotoxin at 6 h post injection (LPS x Time P<0.05). Significant effect of chromium and endotoxin were observed for serum creatinine (Cr x LPS P<0.05) at 6 h post injection (Table 7.3). C-reactive protein increased linearly with dietary chromium and endotoxin challenge (Cr x LPS P<0.05) (Figure 7.1).

Hormone Profile:

Serum insulin (Figure 7.2) and plasma cortisol (Figure 7.3) showed a quadratic effect of treatment with time (Cr x LPS x Time P<0.05). A decrease in serum insulin was observed with or without endotoxin in 0 and 1000 compared to 200 and 600 μg chromium supplemented group. Plasma cortisol increased quadratically with time (Cr x LPS x Time P<0.05). Plasma IGF-1 concentrations decreased over time (P<0.05),

however no effect of treatment (diet or endotoxin) was observed (Cr x LPS P>0.1) on plasma IGF-1 (Table 3).

Tumor Necrosis Alpha-α:

TNF-α alpha showed a quadratic effect of the treatment combination (Cr x LPS P<0.05) (Figure 4). The pigs fed with 600 ppb chromium showed a peak TNF-α activity compared to rest of the dietary group.

Discussion

In the present study, no differences were observed in growth performance in weanling pigs fed milk replacer diet, with added concentrations of 0, 200, 600, or 1000 μg chromium as chromium picolinate/kg diet, for a period of 14 days. However the gain:feed was higher in the group supplemented with 1000 μg chromium than in other groups. The ADFI was higher in pigs fed 200 μg chromium /kg diet compared to other groups. Studies reported previously on the effect of chromium supplementation on growth performance have demonstrated variable and inconsistent results. The duration of the study and the age of the animals were different in the studies reported in the literature compared to the age of the animal and duration of the experiment in the present study. In growing pigs Mooney and Cromwell reported that addition of 200 μg chromium as chromium picolinate/kg diet increased ADG and ADFI but did not increase gain: feed, and no effect of 400 μg chromium as chromium picolinate/kg diet was observed on the ADG, ADFI or gain: feed (Mooney and Cromwell 1997). But when the study was repeated using only 200 μg chromium as chromium picolinate/kg diet, authors found no effect on ADG, ADFI and on gain:feed (Mooney and Cromwell 1999). Lindemann et al

(Lindemann et al. 1995) observed an improvement in gain:feed in pigs (starting wt, 40.9 kg) supplemented with either 200 or 500 μg chromium as chromium picolinate/kg diet. In contrast Boleman et al (Boleman et al. 1995) reported a reduction in the ADG and ADFI with supplementation of 200 μg chromium as chromium picolinate/kg diet. Kornegay et al (Kornegay et al. 1997) found no effect of 200 μg chromium as chromium picolinate on the growth performance in growing finishing pigs. Lee et al (Lee et al. 2000) reported that feeding 400 μg chromium as chromium picolinate/kg diet did not have any effect on the growth performance in 4 weeks old weanling pigs fed for a period of 38 days. However in the earlier studies the analyzed concentrations of the chromium in the corn soybean meal basal diets were very high, hence addition of 100 or 200 ug chromium to the basal diet may not have produced any effect on the growth performance. The results of the present study are in accordance with the results reported by van Heugten and Spears. The study was conducted for a period of 34 days, addition of 200 ug chromium to the basal diet as chromium picolinate increased ADG, but the increase in the daily gain was observed only during 16 to 32 day of the experimental period (van Heugten and Spears 1997).

No effect of diet on serum glucose, triglycerides, total protein, albumin or creatinine was observed in the present study when the pigs were fed either 0, 200, 600, or 1000 μg chromium as chromium picolinate/kg diet. Similar findings on the effects of chromium supplementation (200 – 1000 ug/kg diet) on serum metabolite concentrations in pigs (growing, or growing-finishing phase) were reported in the literature (Lindemann et al. 1995, Mooney and Cromwell 1997, Ward et al. 1997). Evock-Clover et al (Evock-Clover et al. 1993) found a decrease in glucose and insulin in a group supplemented with

300 μg chromium as chromium picolinate /kg diet. However, in their study the chromium concentrations in basal diets were 2.5 times higher than the chromium concentrations in the experimental diets like in the present study. Blood glucose values were not affected in endotoxin-challenged pigs when supplemented with 300 μg chromium as chromium picolinate/kg diet (Evock-Clover et al. 1993).

During stress, one of the integrated responses is the elevation in energy metabolism and an increase in the breakdown of skeletal muscle. Glucose levels in the blood are elevated due to increased synthesis and decreased uptake of glucose by the cells. In the present study we failed to see the hyperglycemic response in glucose with dietary chromium supplementation independent of endotoxin treatment. The results are similar to the results reported in the literature (Evock-Clover et al. 1993, Myers et al. 1997, Ward et al. 1997).

In the present study urea nitrogen, the end product of protein metabolism, and the primary method of nitrogen excretion tended to be lower with 600 and 1000 μg chromium supplementation. The results suggests a more efficient utilization of absorbed nitrogen for protein synthesis with chromium picolinate supplementation, which would explain lean gain with chromium picolinate supplementation reported in earlier studies. Over time, the urea nitrogen increased indicating possible effects of endotoxin on the skeletal muscle breakdown.

In the present study, endotoxin decreased triglycerides over time, these results are in contrast to the reported increase in triglycerides during stress (Burns 1988). In the present study 1000 μg chromium supplemented group had lowered (41.91 mg/dl) triglycerides compared to rest of the chromium supplemented groups with or without

endotoxin. The which may indicate the effects of dietary chromium on lipid metabolism. The effect of endotoxin and dietary chromium on lipid metabolism need to be addressed in the future.

An increase in the serum insulin over time (0 to 6 hr) was observed in the endotoxin challenged pigs fed 200 and 600 μg chromium as chromium picolinate/kg diet. The insulin concentrations at 6 h post injection were similar in the chromium deficient group (0 ug) and in the high chromium (1000 ug) group.

All the weanling pigs exposed to LPS endotoxin (0111:B4) displayed signs of infection, but the duration and the intensity of the effects of the endotoxin varied with the dietary treatments. From the results of the study, it can be clearly shown that the immune system activation was gradual and transient with endotoxin challenge. As an early acute phase response, decreases in white blood cells, and an increase in TNF-α, were observed at 1.5-h post injection. Over time white blood cells started to rise and an increase in neutrophils was observed at 3-h post injection along with continued decrease of monocytes. By 3-h post injection, TNF-α started to decline and reached baseline values by 6 h post injection. At 3 h, followed by the elevation of TNF-α concentration, white blood cells and the neutrophils increased and also an increase in cortisol, and body temperature were observed at 3-h post injection, indicating host's acute phase response to endotoxin. Further as time progressed, continued decreases in TNF-α, and monocytes, and continued increases in white blood cells and neutrophils were observed between 3 and 6-h post injection. Lymphocytes decreased from 3 to 6-h post injection.

The effects observed for the clinical chemistries were most significant at the 6 h post injection, indicating the sequence of events beginning with TNF-α activation

between 0 and 1.5 h and followed by the increase in cortisol and the body temperature at 3 h post injection.

Elevation in plasma cortisol in response to endotoxin was lower in the 1000 μg chromium picolinate group compared to the 600, 200, and 0 μg group at 3-hour post injection. The magnitude of change in plasma cortisol over time was also low in the group supplemented with 1000 μg chromium as chromium picolinate. Previously reported results on the effect of the chromium supplementation in pigs during stress response included only ≤ 400 μg of dietary chromium picolinate. The highest dietary chromium level (1000 μg)included in the weanling pig diets in the present study was two and one half times higher than the level of dietary chromium reported in the literature. The findings of Moonsie-Shageer and Mowat (Moonsie-Shangeer and Mowat 1993) support the results of the present study. Calves supplemented with 1000 μg chromium as high chromium yeast tended to have decreased plasma cortisol when compared to 0, 200, and 500 μg chromium supplementation. In the present study decreased cortisol levels in pigs supplemented with 1000 μg chromium might suggest a role for chromium in modulating the stress response to endotoxin. How chromium might modulate cortisol production is unknown.

Inflammation produces an acute rise in pro-inflammatory cytokines. Elevated TNF-α was reported in endotoxin-challenged pigs by several authors (Leininger et al. 2000, Myers et al. 1995, Myers et al. 1997). In the present study a lowered activity of TNF-α was observed for the 1000 μg endotoxin group compared to the 0, 200 and 600 μg chromium-supplemented endotoxin-challenged pigs. The peak response in TNF-α was

observed for the 600 μg chromium picolinate supplemented endotoxin group compared to the endotoxin groups supplemented with 0, 200 and 1000 μg. The pigs fed 1000 μg of supplemental chromium when injected with endotoxin had no significant change in the TNF-α compared to the control group supplemented with 1000 μg chromium. Myers et al (Myers et al. 1997) reported a lowered TNF-α concentration in pigs supplemented with chromium (300 μg/kg diet) as tripicolinate, when compared to the controls at 1.5 h after an i.v. infusion of 20 μg LPS (055:B5)/kg body weight. But chromium concentration of the basal diets was unknown. A moderate TNF-α production aids local tissue defenses against bacterial infections. Also TNF-α is known to stimulate the production of neutrophils from the bone marrow (Miyan et al. 1998). In the present study a significant increase in neutrophils was not observed until after the TNF-α peak. TNF-α may have stimulated the neutrophil production between 1.5 and 3-h post injection.

In the present study, body temperature increased with time in the endotoxin treated pigs independent of the dietary treatments. However the pigs fed 1000 μg chromium as chromium picolinate showed an early rise in body temperature by 1.5-h post injection remained elevated at 3 and 6 h post injection. The groups supplemented with 0, 200 and 600 μg chromium did not show an elevation in body temperature until 3 h post injection. The magnitude of elevation in the body temperature from 1.5 to 3-h was highest in the 0 μg group, followed by 600, 200, and 1000 μg chromium supplemented group. Moonsie-Shageer and Mowat (Moonsie-Shangeer and Mowat 1993) reported a decrease in morbidity and in body temperature at day 2 and day 5 of the treatment period in calves supplemented with 200, 500 and 1000 μg chromium as high chromium yeast.

Temperature >40°C was considered as the criterion for morbidity (Moonsie-Shangeer and Mowat 1993).

In the present study, numerically higher values were observed for IGF-1 in pigs fed 600 μg chromium picolinate compared to the group of pigs supplemented with 0, 200, and 1000 μg chromium, and injected with or without endotoxin. The concentrations of IGF-1 dropped at 3 h and started to rise towards baseline at 6 h post injection in all the treatment groups. Endotoxin treatment decreased IGF-1 concentrations in the groups supplemented with 0 or 200 μg chromium. However pigs fed 600 and 1000 μg, the IGF-1 concentrations increased with the endotoxin treatment at 3 h post injection but were not statistically significant.

Evock-Clover et al (Evock-Clover et al. 1993) reported no effect of 300 μg of chromium supplementation on the IGF-I concentrations in growing finishing pigs. A change in the tissue insulin sensitivity and serum insulin balance is found in response to chromium picolinate supplementation. Somatotropin treatment directly increases IGFBP-3, the main storage protein for IGF-1. Somatotropin also increases serum insulin. Increase in serum insulin indirectly lowers IGFBP-1 and IGFBP-2. The IGFBP-1 and IGFBP-2 transports IGF-I across the vasculature and into tissues. Chromium picolinate treatment reduces insulin and indirectly increases IGFBP-1 and IGFBP-2 concentrations and increases the tissue availability of IGF-1, thus decreasing the circulating levels of IGF-I.

Conclusions

Short term feeding (14 day) of different dietary levels of chromium as chromium picolinate during the early weaning period did not have significant affect on the average daily gain. Supplementation of 1000 μg chromium as chromium picolinate/kg diet

improved gain: feed. However, a study of longer duration would be recommended to see the effects of chromium supplementation on the growth performance.

Dietary chromium protection against endotoxin-induced damage was seen in some of the variables examined in this study. The 1000 μg group showed an early acute phase response as seen by the increase in the body temperature, but the recovery from endotoxin treatment was slow compared to the other groups. TNF-α activity was blunted in the 1000 μg group; cortisol response also was low at 3 h PI and continued to maintain the same concentration even at 6 h post injection when compared to the other groups of pigs supplemented with 0, 200 or 600 μg chromium/kg diet. Perhaps supplementation with 1000 μg chromium /kg diet suppressed the immune system.

A stress-modulating function for dietary chromium is still inconclusive. A single dose of LPS endotoxin was not sufficient to modulate all of the immunological functions measured, even though the pituitary-adrenal axis was clearly activated as shown by the increased cortisol.

Further research on the effects of varying levels of chromium as chromium picolinate (200 to 1000 ug) on the immune response in weanling pigs during periods of bacterial infections is recommended.

Figure 7.1. Effect of Dietary Chromium and Endotoxin on C-reactive Protein

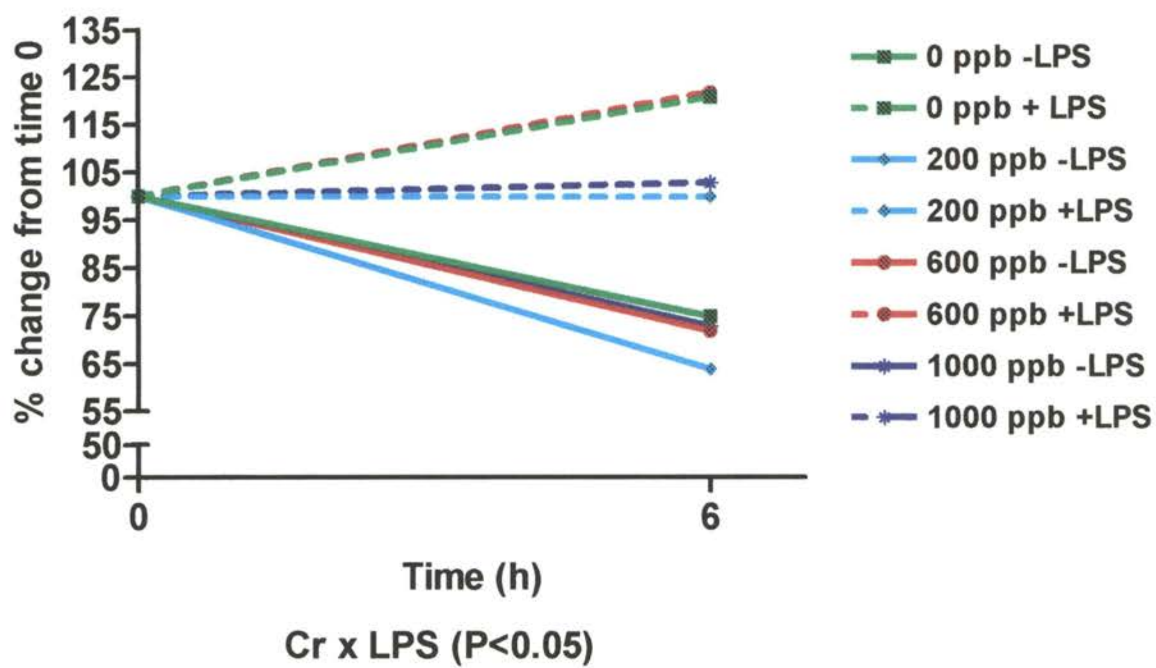

Figure 7.2: Effect of Dietary Chromium and Endotoxin on Serum Insulin

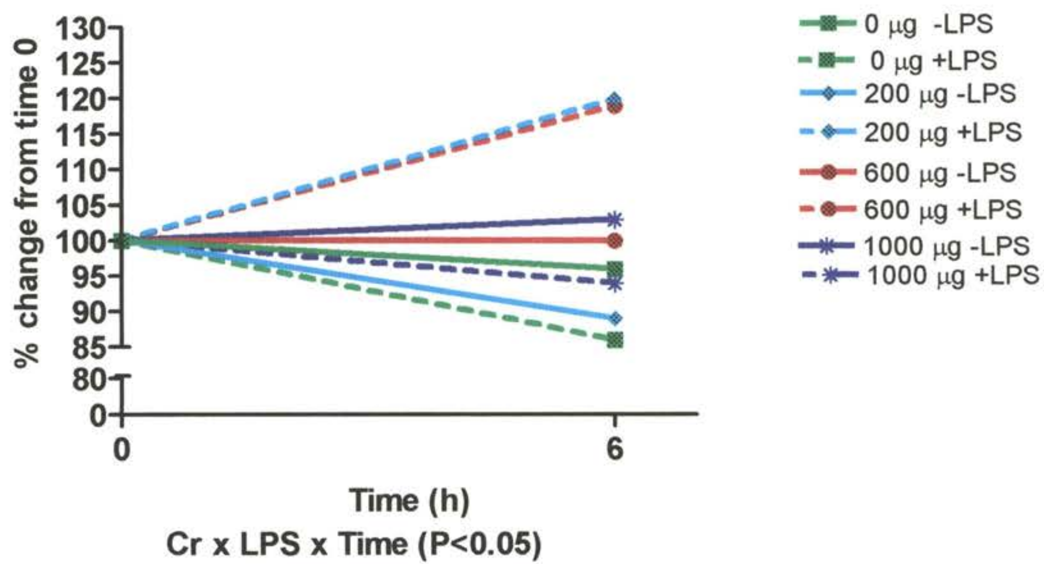

Figure 7.3: Effect of Dietary Chromium and Endotoxin on Plasma Cortisol

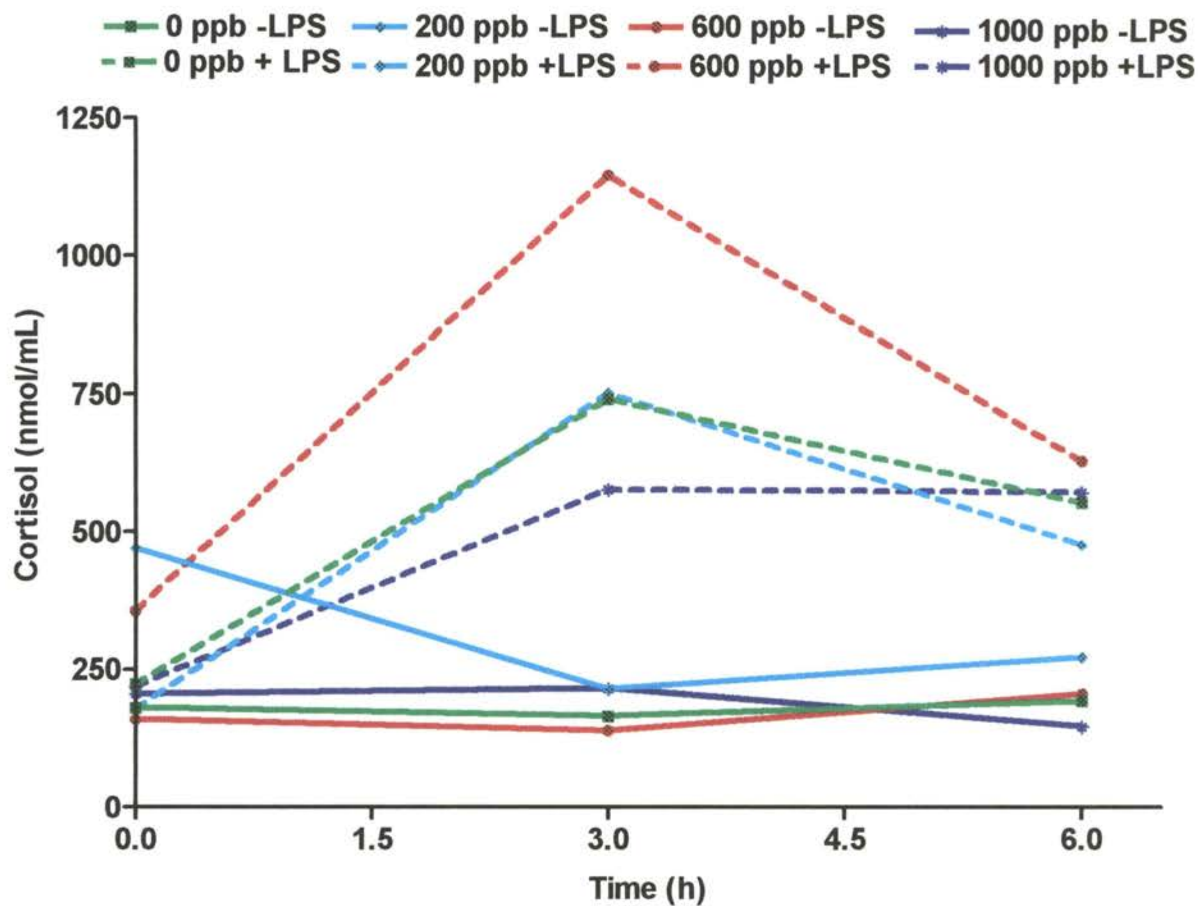

Figure 7.4: Effect of Dietary Chromium and Endotoxin on Plasma TNF- α Concentrations in Weanling Pigs

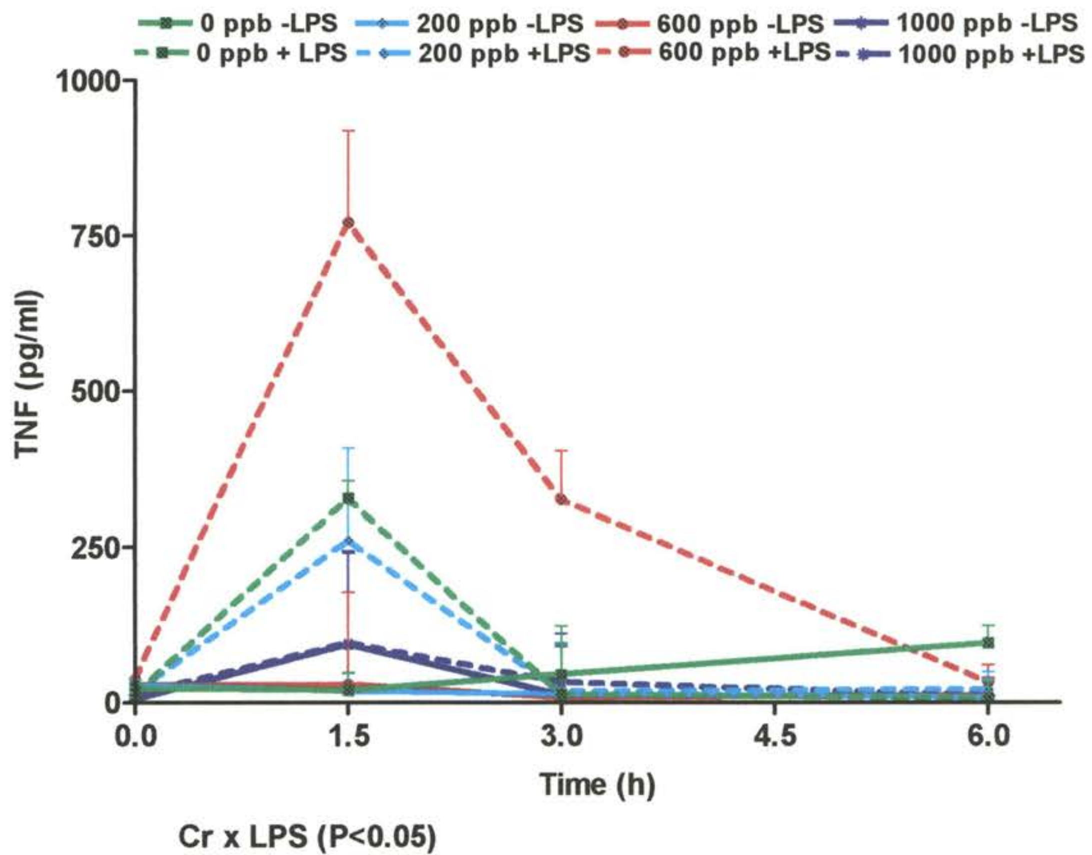

Table 7.1: Effect of endotoxin (LPS 0111:B4) and dietary chromium on hematology[a]

Time (h)	Endotoxin (μg/kg BW) 0	25	P- Value[c]	Dietary Chromium (μg/kg diet) 0	200	600	1000	SEM[b]	P-Value D[c]	D x E[c]
White Blood Cells ($10^3/mm^3$)										
0	13.00	13.00	<0.05	11.68	13.24	13.61	13.45	1.53	NS	NS
1.5	12.43[a]	9.76[b]		11.01	10.87	10.30	12.20	1.33		
3	11.81[a]	16.54[b]		12.73	16.64	13.00	14.42	3.6		
6	11.80[a]	21.71[b]		16.84	15.84	14.74	19.23	2.56		
Lymphocyte (%)										
0	54.87	55.47	<0.05	56.61	55.56	55.15	53.34	4.14	NS	NS
1.5	50.38	52.98		50.97	51.73	55.12	48.90	5.54		
3	48.67[a]	33.67[b]		38.67	39.39	47.12	39.59	4.57		
6	49.38[a]	18.07[b]		32.47	32.80	35.32	34.10	3.75		
Neutrophil (%)										
0	39.07	38.31	<0.05	37.41	38.40	38.34	40.63	4.15	NS	NS
1.5	44.78	43.30		45.03	43.95	40.55	46.65	5.59		
3	46.86[a]	62.69[b]		57.75	56.42	48.72	56.21	4.59		
6	46.30[a]	75.75[b]		57.30	63.83	60.22	62.36	5.89		
Monocyte (%)										
0	3.81	4.32	<0.05	4.06	3.90	4.24	4.05	0.57	NS	NS
1.5	3.26[a]	2.01[b]		2.49	2.70	2.80	2.54	0.41		
3	2.82[a]	1.79[b]		2.25	2.13	2.54	2.32	0.42		
6	2.81[a]	1.79[b]		2.15	2.03	2.72	2.31	0.37		
Temperature (°F)										
0	101.95	101.65	<0.05	101.56	102.25	101.76	101.60	0.3	NS	NS
1.5	101.58[a]	102.22[b]		101.58	102.20	101.70	102.10	0.48		
3	101.50[a]	104.15[b]		103.03	102.63	102.83	102.80	0.5		
6	101.55[a]	104.11[b]		102.84	103.01	102.86	102.59	0.54		

[a] Data are the LSmeans of 6 pigs per treatment.

[b] Pooled standard error n=6 . [C] P values indicate significance of difference in treatment means between treatments within time points.

Table 7.2: Effect of dietary chromium and endotoxin on serum chemistry in weanling pigs

Item	Time	Endotoxin (μg/kg)		P-Value[3]	Dietary Chromium (μg/kg)				Pooled	P-Value[3]		
	(h)	0	25	LPS x Time	0	200	600	1000	SEM[b]	Cr	Cr x LPS	Cr x Time
Creatinine (mg/dL)	0	1.06	1.07	P<0.01	1.05	1.01	1.08	1.12	0.05	>0.1	>0.1	>0.1
	6	1.13[a]	1.33[b]		1.23	1.14	1.24	1.29	0.04			
Albumin (mg/dL)	0	3.05	3.18	P>0.1	2.99	3.12	3.19	3.16	0.12	>0.1	>0.1	>0.1
	6	2.95	2.97		2.81	3.01	3.01	3.00	0.13			
Protein (mg/dL)	0	5.01	5.11	P>0.1	5.02	4.98	5.05	5.18	0.15	>0.1	>0.1	>0.1
	6	4.78	4.70		4.68	4.80	4.69	4.80	0.14			
Urea Nitrogen (mg/dL)	0	7.18	7.12	P<0.01	7.35	6.73	7.80	6.73	0.59	>0.1	>0.1	>0.1
	6	7.97[a]	9.32[b]		9.11	7.75	8.87	8.85	0.87			
Triglycerides (mg/dL)	0	47.33[a]	41.16[b]	P<0.05	44.25	44.16	46.08	42.50	3.74	>0.1	>0.1	>0.1
	6	47.13[a]	38.35[b]		43.51	42.26	46.00	41.33	3.59			
Glucose (mg/dL)	0	107.45	114.12	P>0.1	107.08	114.08	107.83	114.16	8.48	>0.1	>0.1	>0.1
	6	110.85	112.62		109.33	117.83	104.90	114.83	7.18			

[1] Data are the Lsmeans n=6

[2] Pooled SE n=6

[3] Means within a row with different superscript differ significanlty

TABLE 7.3: Effect of endotoxin (LPS 0111:B4) and dietary chromium on hematology[a]

	Time	Endotoxin (μg/kg BW)		P- Value[c]	Dietary Chromium (μg/kg diet)						P-Value
	(h)	0	25		0	200	600	1000	SEM[b]	D[c]	D x E[c]
IGF-1	0	89.02	75.79	>0.05	65.49	97.89	89.67	76.57	20.05	>0.05	>0.05
(ng/mL)	3	71.4	57.17		54.6	68.9	87.14	46.55	30.5		
	6	71.43	82.19		72.8	73.77	102.21	58.59	25.5		

[a] Data are the LSmeans of 6 pigs per treatment.

[b] Pooled standard error n=6 . [C] P values indicate significance of difference in treatment means between treatments within time points.

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CHAPTER VIII

Summary, Conclusions and Recommendations

Summary

The purpose of this research was to investigate the effects of zinc and chromium on the immune response in the weanling pigs. The weanling pig was selected as a experimental model due to its physiological similarities with that of the human gastrointestinal system. Lipopolysaccharide (LPS) endotoxin, E.coli, 0111:B4 was used to simulate E.coli associated infections in weanling pigs.

A series of experiments were conducted to investigate the effects of endotoxin on the immune response in the weanling pigs. In experiment 1, we were able to demonstrate that a minimum dose of 25 μg/kg LPS endotoxin, E.coli, 0111:B4 was sufficient to elicit the immune response in weanling pigs and that endotoxin effects are time and dose dependent. Blood collections at 0, 1.5, 3, 6 h were nessecary to observe the gradual and transient changes in the host due to endotoxin.

In experiment 2 the results provided sufficient evidence that a 25 μg/kg LPS endotoxin E.coli 0111:B4, significantly decreased ^{51}Cr absorption in the blood. Tissue retention, and urinary excretion of ^{51}Cr were also low with endotoxin administration. It was observed that trace minerals were decreased during the periods of stress. Hence, the beneficial effects of dietary supplementation with trace minerals during periods of stress may be of importance.

Experiments 3 and 4 were conducted to investigate the effects of endotoxin on weanling pigs supplemented with different concentrations of zinc (experiment 3) and chromium (experiment 4). The results demonstrated that feeding weanling pigs for 14 days with 0, 100, 1500 or 3000 ppm zinc as zinc sulfate did not affect growth performance. Supplementing with 3000 ppm did not prove to be beneficial for the immune response during endotoxin challenge in weanling pigs. Dietary zinc at a level of 100 ppm was found to be sufficient to maintain normal growth, but during periods of stress a level of more than 100 ppm is needed.

In experiment 4, weanling pigs were fed diets containing 0, 200, 600 or 1000 μg chromium as chromium picolinate/kg diet for period of 14 days. Feed efficiency ratio was highest in the 1000 μg chromium supplemented pigs compared to the rest of the groups. The immune response was low in the 1000 μg chromium supplemented group as evidenced by the decreased cortisol, TNF-α and a continued elevation in body temperature. Supplementing 1000 μg chromium/kg diet was found to suppress the immune system. Feeding 600 μg chromium/kg diet for 14 d was observed to be a safe and adequate level of dietary chromium both for growth and for immune system function.

Conclusions

A dose of 25 μg of LPS/kg body weight was sufficient to produce an immune response in weanling pigs. Endotoxin significantly decreases the absorption and retention of ^{51}Cr in the blood and tissues. Feeding pharmacological levels of zinc-3000 ppm (30 times NRC) or 1000 μg chromium as chromium picolinate may not be beneficial to weanling pig's immune system during stress induced by the LPS endotoxin.

Recommendations

Since LPS was found to decrease the absorption of chromium, studies on the effect of LPS on the intestinal morphology and gut permeability in weanling pigs need to be revisited. Investigation on the effect of LPS on intestinal growth and development in weanling pigs with different levels of trace minerals is of importance. Also the mechanisms of how trace elements affect gut morphology during periods of stress need to be addressed.

The present study was conducted with a single LPS challenge dose. Hence repeated doses of LPS need to be examined closely for evaluating the effects of sustained infection on growth and the immune response in weanling pigs fed different levels of zinc. The range of dietary levels of zinc in the present study was (0, 100, 1500 and 3000). The effects of dietary zinc between 100 – 1500 and between 1500 - 3000 on the immune response in weanling pigs during the periods of stress are still not explored. In future studies the effects with supplementation of dietary levels of zinc between 100 and 1500 ppm on growth and immune response in weanling pigs need to be considered.

The present study was a short term study, in order to understand the role of chromium on growth and development, long term supplementation of the weanling pig with different chromium concentrations in the diet needs further investigation. The range of dietary chromium included in the present study was broad (0 – 1000). The effects of dietary chromium at more points in the range between 200 and 1000 ppb on the immune response need to be investigated.

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Appendix A

Animal Care and Use Form

OKLAHOMA STATE UNIVERSITY

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

Memorandum

DATE: June 1, 1998

TO: Dr. Andrea Arquitt
Nutritional Science

FROM: Dr. Archie Clutter
IACUC Chairman

SUBJECT: Protocol Approval

Your protocol, #737, entitled "effects of Endotoxin and Trace Minerals on Immune Function and Lean Body Mass in Weaning Pigs", has been approved for 144 pigs by the Institutional Animal Care and Use Committee. The protocol is approved through May 31, 2001.

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

Institutional Assurance number A3722-01

The Campaign for OSU

Appendix B

Modified procedure of IGF-1

Modified Radioimmunoassay for Insulin Like Growth Factor 1
(Procedure for Nichols Institute Diagnostics (NID)
Acid Ethanol Extraction:
Prepacked IGF-1 Acid-Ethanol Solvents purchased from NID catalog #40-2101
Solvent Solution:
12.5% 2N HCL/87.5% Ethanol:
21.6 ml of 1.6M HCL (concentrated)
103.4 ml distilled water or deionized H2O (chilled)
875 ml absolute ethanol (chilled)

Buffer Solution: 0.855M Tris Base (Final pH = 11.0)
103.54 g Trizma Base
1 liter distilled or deionized water

Acid-Ethanol Extraction Procedure:
1) Pipette 200 µl of plasma sample or human controls to the bottom of appropriately labeled plastic tubes
2) Add 700 µl of acid ethanol solution (12.5% /87.5% v/v) to all tubes.
3) Vortex and incubate all tubes at room temperature for 30 minutes. Parafilm well to prevent evaporation.
4) Centrifuge at 4 °C for 30 minutes, at 3000 RPM.
5) After centrifugation, pipet 200 µl of supenatant to the corresponding labelled tubes without any delay or disturbance of the precipitate.
6) Vortex throughly, and incubate for 30 minutes at room temperature. Centrifuge at 4 °C for 30 minutes, at 3000 RPM. During centrifugation, label final sample dilution tubes and add exactly 1.4 ml of phosphate buffer (NID catolog # 30-3046)
7) After centrifugation carefully transfer 100 µl of supernatant and to appropriately labeled tubes containing phosphate buffer. This makes a final dilution of 1:225.
Mix diluted samples. The sample are refrigerated overnight (stable for 2 weeks at 2-8 °C) for the RIA procedure

RIA Procedure:
The RIA procedure was followed as described in the IGF-1 extraction procedure by NID

Appendix C

IL-6 ELISA Protocol

Standards and Antibody:

Purchased from (R & D Systems, Inc, Minneapolis, MN)

Anti-porcine IL-6 Antibody (AF 686)

Biotinylated Anti-porcine IL-6 Antibody (BAF 686)

Recombinant Porcine IL-6 (PI 686)

Solutions Required:

Wash Buffer-0.05% Tween 20 in PBS, pH 7.4

Diluent- 1% BSA in Phosphate-buffered saline, pH 7.4

Substrate Solution- 1:1 mixture of color Reagent A (H_2O_2) and Color reagent B (Tetramethyl benzindine) (R & D Systems catalog # DY 999)

Stop Solution- 1 M $H_2 S0_4$

Streptavidin HRP (R & D systems catalog # DY998)

Plate Preparation:

Transfer 100 µl /well of the capture antibody to an ELISA plate. Seal plate and incubate overnight at room temperature (12-16 h)

Aspirate each well and wash with wash buffer, repeating the process two times for a total of three washes. Wash by filling each well with wash buffer (400 µl) using a autowasher. After the last wash invert the plate and blot against the clean paper towel.

Block plates by adding 300 µl of PBS containing 1% BSA, 5% sucrose and 0.05% sodium azide to each well. Incubate at room temperature for 60 minutes.

Repeat aspiration. (Step 2).

Standards:

Standards were serial diluted to a concentration of 0,76.29, 152.59, 305.18, 610.35, 1220.70, 2441.41 and 4882.81 pg/ml

Assay Procedure:

Add 100 μl of samples or standards in an appropriate diluent (1% BSA in phosphate-buffered saline, pH 7.4).

Mix by gently tapping the plate frame for 1 minute. Cover with an adhesive strip and incubate 2 hours at room temperature.

Wash plate 3 times by using 400 μl of wash buffer /per well for each wash.

Add 100 μl biotinylated detection antibody, to each well. Cover with new adhesive strip and incubate 2 hours at room temperature.

Wash plate as in step 3.

Add 100 μl Streptavidin HRP to each well. Cover plate and incubate for 20 minutes at room temperature.

Repeat wash (Step 3).

Add 100 μl substrate solution to each well. Incubate for 20 –30 minutes at room temperature in the dark.

Add 50 μl of stop solution to each well. Gently tap.

Determine the optical density within 30 minutes using microplate reader set at 540-570 nm and a 4 parameter log log curve with wave length correction.

APPENDIX D

Supporting Material For Chapter VI

Table D.1: Effect of dietary zinc on the weight gain and growth performance in weanling pigs[1]

	DIETARY ZINC (ppm)					
	0	100	1500	3000	SEM[b]	P- Value
Weight (kg)						
d-0	5.06	5.00	5.08	5.17	0.37	NS
d-14	6.75	7.10	7.11	6.81	0.38	NS
ADG (kg)	0.48	0.51	0.51	0.48	0.02	NS
ADFI (l)	1.406	1.474	1.228	1.316	0.1	NS
Gain (kg)/Feed (l)	0.35	0.35	0.43	0.41	0.036	NS

[a]Data are the LSmeans n=12 pigs for each treatment

[b]Data are the pooled SEM, n=6

Table D.2: Effect of endotoxin and dietary zinc on body temperature of weanling pigs

Time (h)	Endotoxin (μg/kg)		P-Value	Dietary Zinc (ppm)				P-Value	
	0	25	E x T	0	100	1500	3000	Zn x Time	E x Zn
Temperature (0F)									
0	101.91	101.82	NS	101.92	101.75	101.96	101.84	NS	NS
1.5	101.52	103.70	<0.0001	102.26	102.30	102.40	102.07		
3	101.40	103.77	<0.0001	102.85	102.75	102.29	102.45		
6	101.65	103.51	<0.0001	102.65	102.55	102.59	102.53		
24	101.62	101.83	NS	102.05	101.35	101.83	101.68		

Table D.3: Effect of endotoxin and dietary zinc on the hematology, clinical chemistry, body temperature and hormone profile in weanling pigs[1]

	Endotoxin (μg/kg)		P-Value	Dietary Zinc (ppm)					P-Value
	0	25	E	0	100	1500	3000	SEM[b]	D
White Blood Cells ($10^3/mm^3$)	14.14	6.56	0.02	17.07	15.83	20.05	14.60	3.26	NS
Lymphocytes (%)	47.58	40.26	0.05	45.08	44.56	42.7	43.28	6.49	NS
Monocytes (%)	2.328	2.138	NS	2.47	2.55	1.95	1.95	0.4	NS
Neutrophil (%)	48.23	55.15	0.07	50.58	51.09	52.88	52.39	6.61	NS
Eosinophil (%)	1.856	2.269	0.04	2.04	1.93	2.01	2.25	0.48	NS
Basophil (%)	0.139	0.139	0.5	0.15	0.139	0.115	0.133	0.02	NS
Temperature (°F)	101.62	102.78	0.0001	102.35	102.14	102.21	102.11	0.42	NS
Cortisol (nmol/mL)	29.02	33.48	0.00	211.07	190.66	179.21	151.26	39.16	.03,L
Insulin (IU/mL)	8.93	9.34	0.40	9.09	9.16	8.92	9.37	0.55	0.8
IGF-1 (ng/mL)	57.78	42.84	NS	57.34	67.89	43.20	33.03	12.24	.05, Q
Triglycerides (mg/dL)	22.62	22.39	0.10	0.00	0.00	0.00	0.00	4.75	.009,L
Total Protein (mg/dL)	4.93	5.02	NS	5.24	4.86	4.89	4.92	0.22	.03,Q
Albumin (mg/dL)	3.11	3.13	NS	3.30	3.17	3.01	3.02	0.16	.05,L
Urea nitrogen (mg/dL)	9.93	10.42	NS	8.92	7.91	10.05	13.80	1.75	.0001,L
Glucose (mg/dL)	114.73	109.90	0.09	114.92	112.34	115.40	106.60	8.7	.08,L
Creatinine (mg/dL)	1.16	1.24	0.02	1.19	1.17	1.20	1.25	0.055	NS
C-reactive protein (mg/dL)	2.88	3.42	0.00	1.66	1.56	1.36	1.68	0.85	0.009

[a] Data are the LSmeans n=6 per treatment.

[b] Data are the pooled SEM n=6.

Table D.4: Main effect of time on hematology, serum chemistry, and hormonal profile of weanling pigs supplemented with dietary zinc and injected with endotoxin[a]

	Time						P-Value		
	0	1.5	3	6	24	SEM[b]	T	D x T	E x T
White Blood Cells ($10^3/mm^3$)	16.32	14.53	17.85	20.52	15.02	3.26	0.0001	NS	0.0001
Lymphocytes (%)	48.03	47.18	36.15	34.82	53.83	6.49	0.0001	NS	0.0001
Monocytes (%)	2.39	1.736	1.834	1.91	3.28	0.4	0.0001	NS	0.0001
Neutrophil (%)	47.57	49.52	59.95	61.73	40.26	6.61	0.0001	NS	0.0001
Eosinophil (%)	2.12	1.92	1.91	1.38	2.97	0.48	0.0001	NS	0.0001
Basophil (%)	0.14	0.14	0.11	0.1	0.16	0.02	0.0001	NS	0.0001
Temperature (°F)	101.86	102.25	102.58	102.59	101.72	0.42	0.0001	NS	0.0001
Cortisol (nmol/mL)	124.97		297.85	209.57	99.79	39.16	0.0001	NS	0.0001
Insulin (IU/mL)	9.15			9.13	9.12	0.55	NS	NS	NS
IGF-1 (ng/mL)	62.25			48.72	40.05	12.24	NS	NS	0.02
Triglycerides (mg/dL)	31.56			35.18	33.62	4.75	NS	NS	0.003
Total Protein (mg/dL)	5.16			4.93	4.83	0.22	0.004	NS	NS
Albumin (mg/dL)	3.21			3.1	3.05	0.16	NS	NS	NS
Urea nitrogen (mg/dL)	9.51			11.12	9.88	1.75	0.07	0.0004	NS
Glucose (mg/dL)	115.11			105.32	116.5	8.7	NS	NS	NS
Creatinine (mg/dL)	1.156			1.27	1.17	0.055	0.0001	NS	0.05
C-reactive protein (mg/dL)	4.75			4.69	5.21	0.85	0.03	0.03	0.0001

[a] Data are the LSmeans n=48 pigs for each time period.

[b] Data are the pooled SEM, n=6.

T- time effect; D xT- diet x time interaction, E x T- endotoxin x time interaction.

APPENDIX E

Supporting materials for Chapter VII

Table E.1: Main effects of endotoxin and dietary chromium on serum chemistry in weanling pigs[a]

Main Effects	Albumin (mg/dL)	Creatinine (mg/dL)	Glucose (mg/dL)	Insulin (IU/mL)	Protein (mg/dL)	Triglyceride (mg/dL)	Urea nitrogen (mg/dL)
Time (h)							
0	3.11	1.06	110.79	10.80	5.06	44.25	7.15
6	2.96	1.23	111.72	10.84	4.74	43.44	8.65
Endotoxin (μg/kg)							
0	3.01	1.09	109.12	10.63	4.9	47.42	7.58
25	3.07	1.2	113.37	11.01	4.91	40.27	8.22
Dietary Chromium (μg/kg)							
0	2.90	1.14	108.19	10.56	4.85	44.06	8.23
200	3.07	1.08	115.95	11.19	4.89	43.36	7.24
600	3.10	1.16	106.37	10.84	4.87	46.04	8.33
1000	3.08	1.20	114.50	10.68	4.99	41.91	7.79
Sources of Variation							
Endotoxin	>0.05	<0.02	>.1	>0.1	>.10	<0.02	>.10
Diet	>0.05	>.08	>.1	>0.1	>.10	>.10	<0.07
Endotoxin X Diet	>0.05	<0.005	>.1	>0.1	>.10	>.10	<.09
Time	<0.0001	<0.0001	>.1	>0.1	<0.0001	>.10	<0.0001
Endotoxin x Time	<0.0001	<0.0001	>.1	>0.1	<.01	>.10	<.01
Diet x Time	>.05	>0.05	>.1	>0.1	>.10	>.10	>.10
Diet x Endotoxin x Time	>.05	>0.05	>.1	<0.04	>.10	>.10	>.10

[a]Data are the Lsmeans n=6 and pooled SE.

Table E.2: Main effects of endotoxin and dietary chromium on the hematology, body temperature, and cytokine profile in weanling pigs[a]

	Endotoxin (µg/kg BW)			Dietary Chromium (µg/kg diet)						
	0	25	P- Value	0	200	600	1000	SE[b]	D	D x E *
White Blood cells	12.26	15.25	<0.02	13.07	14.15	12.91	14.83	2.26	NS	NS
Red blood cells	6.65	6.63	NS	6.88	6.51	6.71	6.47	0.24	NS	0.07
Hemoglobin	10.32	10.61	NS	10.66	10.28	10.42	10.47	0.40	NS	0.03
Hematocrit	30.79	31.97	<0.02	31.87	30.75	31.76	31.14	0.823	NS	0.03
Mean Cell Volume	46.44	48.40	<0.03	46.57	47.32	47.46	48.25	5.57	NS	NS
Mean Cell Hemoglobin	15.57	16.24	<0.03	15.57	15.84	15.96	16.25	0.49	NS	NS
Platelets	664.69	488.95	<0.0004	610.58	630.15	537.86	528.55	64.64	NS	0.04
Lymphocyte %	50.83	40.05	<0.0007	44.68	44.87	48.18	43.98	4.50	NS	NS
Neutrophil %	44.25	55.01	0.001	49.37	50.65	46.96	51.46	5.06	NS	NS
Monocyte %	3.18	2.48	0.01	2.74	2.69	3.08	2.81	0.44	NS	NS
Eosinophil %	1.57	1.60	NS	1.40	1.66	1.65	1.33	0.36	NS	NS
Basophil %	0.19	0.19	NS	0.19	0.18	0.23	0.16	0.21	NS	NS
Reticulocyte %	3.59	3.61	NS	2.91	4.37	3.65	3.45	0.69	NS	0.01
Interleukin-6	46.67	333.23	0.09	329.99	28.34	114.80	278.66	254.98	NS	NS
Tumor Necrosis Factor-α	27.95	124.97	0.005	67.96	48.25	155.83	34.37	66.56	0.04, Q	0.03, Q
Insulin like Growth Factor -1	57.96	53.79	NS	48.22	60.14	69.76	45.43	12.24	NS	NS
Cortisol	143.79	386.28	0.0001	247.08	264.67	322.27	226.12	59.51	.02,Q	0.02, Q
Temperature	101.65	103.03	0.0001	102.25	102.52	102.29	102.27	0.46	NS	NS
C-reactive protein	2.08	2.25	NS	4.25	4.00	4.52	4.54	0.555	NS	.03L

[a] Data are the LSMeans; [b] Data are the pooled SEM n=6

Table E.3: Effect of time on hematology, body temperature and cytokine profile in weanling pigs[a]

Measures	TIME (h)					
	0	1.5	3	6	SE[2]	P-Value[3]
White Blood cells ($10^3/mm^3$)	13	11.09	14.28	16.81	2.26	<0.0001
Red blood cells ($10^6/mm^3$)	7.03	6.72	6.57	6.19	0.24	<0.0001
Hemoglobin (g/dL)	10.96	10.66	10.47	9.75	0.40	<0.0001
Hematocrit (%)	33.31	31.8	31.12	29.24	0.822	<0.0001
Mean Cell Volume (mm^3)	47.41	47.53	47.45	47.47	5.57	>0.1
Mean Cell Hemoglobin (Pg)	15.96	15.94	15.95	15.88	0.49	>0.1
Platelets ($10^3/mm^3$)	617.89	580.77	542.64	557.89	64.64	<0.0001
Lymphocyte %	55.17	51.68	40.99	34.07	4.50	<0.0001
Neutrophil (%)	38.69	44.04	55.02	60.24	0.44	<0.0001
Monocytes (%)	4.06	2.63	2.38	2.3	5.06	<0.0001
Eosinophil %	1.85	1.43	1.46	1.5	0.36	<0.002
Basophil %	0.24	0.18	0.178	0.158	0.21	<0.08
Reticulocyte %	3.62	3.58	3.75	3.39	0.69	<0.08
Temperature (°F)	101.79	101.89	102.82	102.82	0.46	<0.0001
Tumor Necrosis Factor-α (pg/ml)	20.15	202	58.57	25.28	66.56	<0.0001
Interleukin-6 (μg/ml)	76.62	173.6	274.29	229.93	256.0	<0.05
Cortisol (nmol/ml)	215.1		475.49	369.29	59.51	<0.002
Insulin like Growth Factor -1 (ng/mL)	82.405		64.29	76.83	12.54	<0.02
C-reactive protein (mg/dL)	4.43		4.24		0.55	<.08

[a]Data are the main effects of time; [b]Pooled SEM n=6

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VITA

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