

EFFECT OF TRACE MINERAL AMOUNT AND SOURCE  
ON IMMUNE FUNCTION AND OXIDATIVE STRESS  
IN DAIRY COWS

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## ABSTRACT

Forty-eight multiparous Holstein cows during early to midlactation were utilized to assess effect of trace mineral amount and source on immune function and oxidative status in dairy cows. Cows were fed a diet formulated to meet or exceed NRC (2001) nutrient requirements for all nutrients except for trace minerals of interest (Zn, Cu, and Mn), which were supplied from basal ration ingredients only. In addition, all cows were fed a preliminary diet for four weeks (week -3 to week 0), containing 0.37% sulfur (dry matter basis, ration addition), 5 ppm of molybdenum from sodium molybdate (topdress), and 250 ppm of iron from iron sulfate (topdress) to decrease trace mineral absorption and status. After this four-week preliminary period, the sulfur content of the basal diet was reduced to 0.30%, the topdress of the Mo and Fe ceased, and cows were assigned to one of four dietary treatments for six weeks (week 1 to week 6) in a randomized complete block design: 1) NRC inorganic (NRC 2001 levels using inorganic trace mineral supplements only); 2) NRC organic (NRC 2001 levels using organic trace mineral supplements only); 3) Commercial inorganic (commercial levels (approximately 2X NRC requirement) using inorganic trace mineral supplements only); 4) Commercial organic (commercial levels using organic trace mineral supplements only). Supplemental inorganic Zn, Cu, and Mn were supplied using zinc sulfate monohydrate, cupric sulfate pentahydrate, and manganese sulfate monohydrate, respectively. Organic trace minerals were supplied as Zn, Cu, and Mn chelated to 2-hydroxy-4-(methylthio)-butanoic acid (HMTBA). All oxidative status parameters from weekly blood samples were not affected by treatments when assessed across the entire treatment period. However, there were tendencies at week 1 of treatment period for decreased glutathione peroxidase activity for cows fed trace minerals at commercial levels, decreased total antioxidant capacity (TAC) in plasma

for cows fed inorganic sources of trace minerals at NRC levels, and decreased concentrations of thiobarbituric acid reactive substances (TBARS) for cows fed organic trace mineral sources. At week 6 of treatment (one week following LPS challenge), cows fed commercial levels of trace minerals tended to have increased TAC and cows fed organic trace minerals had decreased TBARS in plasma. Plasma IgG level was higher in cows supplemented with organic trace minerals over the entire treatment period; responses assessed as differences of before and after *E. coli* J5 vaccination at the end of week 2 of treatment period were not significant. After LPS intramammary challenge at the end of week 5, plasma cortisol concentrations and clinical indices (rectal temperature and heart rate) increased and then decreased to pre-challenge level by 48 h after the challenge. However, the extent and pattern of response of these variables to LPS challenge were not affected by trace mineral level and source. Dry matter intake, milk composition, and milk component yield were not affected by trace mineral level and source, except for a trend for increased milk true protein content for cows fed the commercial level of trace minerals. Body condition score and body weight were not affected by treatment. Overall, varying level and source of trace minerals in the diet resulted in modest effects on parameters related to oxidative stress but did not appear to markedly affect responses of cows to intramammary LPS challenge.

## BIOGRAPHICAL SKETCH

Takashi Yasui was born in Tokyo, Japan. Takashi graduated from Kaijo High school in 1990. He then attended Kitasato University School of Veterinary Medicine and obtained his D. V. M. degree in 1996. Takashi went to work for Hokuraku dairy farming cooperative in Okayama, Japan and was involved in treating dairy cows as a clinical veterinarian. After working for the cooperative for three and half years, Takashi felt the need to know preventive medicine for dairy farmer's benefit. He then started to work for Bussan Biotech Co., Ltd, a feed additive company in 1999. At the company, he had been working on developing combinations of preventative feed additives and promoting the concept of preventive medicine. As continuing working as a technical manager, Takashi felt that more deep and pragmatic knowledge for nutrition and immunology are needed to promote preventive medicine in Japan. Then he attended Cornell University for Master of Science program in Animal Science with a financial support from Bussan Biotech from 2006 to learn dairy nutrition and immunology. Upon completion of this degree, Takashi plans to promote preventive medicine as working for Bussan Biotech. His next goal is to establish a new academic conference in Japan, which is pragmatic and connect industry, academia, and government together to deal with dairy science, problems on farm, and food safety.

To Mayuka, Maron, Shiruku, and all of my friends and benefactors

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## LIST OF ABBREVIATIONS

ADF =	Acid detergent fiber
BCS =	Body condition score
BW =	Body weight
CAT =	Catalase
Con A =	Concanavalin A
CP =	Crude protein
Cp =	Ceruloplasmin
CR =	Calorie restriction
DM =	Dry matter
DMI =	Dry matter intake
ELISA =	Enzyme-linked immunosorbent assay
GSH-px =	Glutathione peroxidase
HIV =	Human immunodeficiency virus
HMTBA =	2-hydroxy-4-(methylthio)-butanoic acid
IL =	Interleukin
Ig =	Immunoglobulin
IGF =	Insulin-like growth factor
LPS =	Lipopolysaccharide
MDA =	Malondialdehyde
MUN =	Milk urea nitrogen
NDF =	Neutral detergent fiber
NEL =	Net energy of lactation
NK =	Natural killer
NRC =	National Research Council

PCV =	Packed cell volume
PEM =	Protein energy malnutrition
PGE =	Prostaglandin E
PHA =	Phyto hemoagglutinin
PUFA =	Polyunsaturated fatty acid
PWM =	Poke weed mitogen
ROS =	Reactive oxygen species
SEM =	Standard error of the mean
SOD =	Superoxide dismutase
TAC =	Total antioxidant capacity
TBARS =	Thiobarbituric acid reactive substances
TMR =	Total mixed ration
TNF =	Tumor necrosis factor

## CHAPTER ONE:

### INTRODUCTION

Immune function and oxidative stress are closely associated. Reactive oxygen species (ROS) such as oxygen ions, free radicals, and peroxides produced through normal cellular activity can destroy cellular membranes, cellular proteins, and nucleic acids (Carroll and Forsberg, 2007). Immune cells are particularly susceptible to oxidative damage not only because of the oxidative respiratory burst during phagocytosis and pathogen kill that generates large amount of ROS, but also because immune cells have a high percentage of polyunsaturated fatty acids in their plasma membrane, which makes them more sensitive to oxidative stress (Chew and Park, 2004). In addition, the immune system uses ROS to maintain signal transduction pathways (Valko et al., 2007a). As a result, the immune system is dependent particularly on high level of antioxidants to protect them from ROS (Carroll and Forsberg, 2007).

Although the body produces a number of endogenous antioxidants, including ceruloplasmin, transferrin, lactoferrin, albumin, haptoglobin, and haemopexin (Sies, 1991), under high oxidative stress conditions their ability to eliminate ROS can be exceeded. A number of vitamins and trace minerals are involved in the antioxidant defense system and a deficiency of any of these nutrients may depress immunity (Spears and Weiss, 2008). In dairy cows, involvement of oxidative stress in etiologies of certain disorders is suggested by reduction in incidence of retained placenta and mastitis when dietary source of antioxidants such as vitamin E and selenium are supplemented (Miller et al., 1993).

Major nutrients and macrominerals have been studied in various ways with focus on optimized performance and health status in dairy cattle (Underwood and Suttle, 1999; NRC, 2001); however, few studies have been conducted that assess the

role of trace minerals in metabolism, oxidative status, or immune function in dairy cows. In fact, there are many trace minerals that contribute to antioxidant defense mainly through components of antioxidant enzymes: Mn, Cu, and Zn for super oxide dismutase (SOD), Se for glutathione-peroxidase (GSH-px), Fe for catalase, and Fe plus Mo for aldehyde dehydrogenase. Therefore, further research for trace minerals supplementation from the standpoint of oxidative balance and immune function may elucidate the opportunity to prevent diseases related to oxidative stress in dairy cows. The purpose of the review of literature to follow is to overview the relationship between major nutrients and immunology and oxidative stress, to review functions of trace minerals for immune system and oxidative stress, and then to review the role of trace minerals in ruminants to understand their role in immune function and oxidative metabolism in dairy cows.



## CHAPTER TWO: REVIEW OF LITERATURE

### OVERVIEW OF IMMUNE FUNCTION AND OXIDATIVE STRESS

Oxidative stress results from an imbalance between the production of reactive forms of oxygen and antioxidant defense (Sies, 1991). It causes the damage of biological macromolecules, disruption of normal metabolism, and dysfunction of normal physiology (Trevisan et al., 2001). Fuente et al. (2002) suggested that immune cells are particularly sensitive to the prooxidant-antioxidant balance because antioxidants maintain the integrity and function of membrane lipids, cellular proteins, nucleic acids, and the control of signal transduction of gene expression in immune cells. In addition, Knight (2000) reported that cells of the immune system have a higher percentage of polyunsaturated fatty acids in their plasma membranes and usually contain higher concentrations of antioxidants than other cells. On the other hand, immune function is fundamentally linked to the generation of ROS (De la Fuente, 2002). For example, ROS produced by respiratory burst, during which oxygen uptake by leukocytes increases severalfold, are used to kill microbes ingested in vacuoles called phagosomes (Kindt et al., 2006). Furthermore, most cell types have been shown to produce a small oxidative burst generating low concentrations of ROS when they are stimulated by cytokines, growth factors, and hormones (Thannickal and Fanburg, 2000) and it is well-known that cells are capable of generating endogenously and constitutively ROS that are utilized in the induction and maintenance of signal transduction pathways involved in cell growth and differentiation (Valko et al., 2007a). Griffiths et al. (2005) reported that low levels of ROS upregulate gene expression in T cells, suggesting that functional activation, proliferation, or programmed cell death of the cells is dependent on the controlled production of ROS. Thus, cellular immune

function appears to be easily influenced by oxidative stress and also is a contributor to oxidant load.

It is not clear how specific aspects of immune system responses are affected by the oxidizing environment caused by reduced antioxidants and/or increased oxidants. One hypothesis is that the enhanced oxidizing environment can lead to a hyperresponsive innate immune system and to enhanced activation of the adaptive immune response, resulting in autoimmunity or allergy (Piganelli et al., 2002; Crapo, 2003). However, Maurice et al. (1997) reported that T cells isolated from the synovial fluid in rheumatoid arthritis showed impaired responses to mitogenic stimulation compared with T cells from the peripheral blood and this hyporesponsiveness correlated with a significant decrease in the level of an intracellular antioxidant, glutathione. The researchers suggested that imbalanced pro-oxidant and antioxidant conditions in rheumatoid arthritis may lead to a chronic dysfunction of the T cells at the site of inflammation and that long-term exposure to oxidants may lead to chronic change in the redox status of T cells (Maurice et al., 1997). Further research is needed to elucidate specific effects of oxidative stress on immune function, although it is clear that this stress causes immune dysfunction.

## NUTRITION INFLUENCES ON IMMUNE FUNCTION AND OXIDATIVE STRESS

The most common interactions between nutrition and immune function have been defined broadly: malnutrition results in increased susceptibility to infection (Keusch, 2003). For example, Chandra (1983) reported that the incidence of postoperative sepsis and mortality in adults were higher among those with protein-energy malnutrition (PEM). In addition, there are substantial available data that suggest that PEM is associated with an increase in the severity and duration of most

infectious diseases (Gershwin et al., 2000). However, the effects of malnutrition on immunity are not confined to PEM because mineral and vitamin deprivation also can have an adverse effect on immune function (Saker, 2006). The state of knowledge regarding specific nutritional interactions with immunocompetence will be discussed in subsequent sections. There are also instances in which decreased immunocompetence can decrease pathology. Hepatic schistosomiasis is one of these examples in which the formation of hepatic granulomas is diminished by the effects of PEM on the cell-mediated immune response, resulting in reduced liver pathology (Keusch, 2003). In this case, because granuloma formation presumably depends on immune response and determines the extent of disease severity, it would appear that PEM partially alleviates pathology.

Other than malnutrition status, it is difficult to explain the mechanisms by which nutrients affect immune system because the immune system is not autonomous from other physiological systems, rather it is influenced by other systems such as the endocrine system and the nervous system (Weigent and Blalock, 1995) and nutrient status also can affect these physiological systems. Klasing et al. (2000) outlined five categories to explain the general effects of nutrition on the immune system: 1) direct regulation by nutrients, 2) indirect modulation mediated by the endocrine system, 3) regulation by availability of substrates, 4) modulation of the pathology caused by an immune antioxidants, and 5) nutritional immunity.

The first mechanism is that nutrients influence regulation of the immune response by modifying intercellular or intracellular communication pathways. Examples include the role of long-chain polyunsaturated fatty acids (PUFA), which modulate the immune system through modifying cellular communication, membrane fluidity, and second messenger signaling resulting in changes in a spectrum of diseases and the incidence of diseases (Klasing et al., 2000). As an example, whereas dietary n-

3 fatty acids supplementation to chick diets significantly decreased the incidence of septicemia of all causes by 25% and the incidence of cellulites by 18%, they increased the incidence of tumors, both spontaneous and from Marek's disease, by 24% (Klasing et al., 2000).

The second mechanism is that dietary factors influence the levels of many hormones that have regulatory influences on the immune system. A change in the profile of hormones originating from the endocrine system is readily induced by dietary factors, including the amount of food consumed, meal pattern, and ratio of protein to energy in the diet (Gershwin et al., 2000). Therefore, dietary factors could affect the immune system indirectly. For example, Savendahl and Underwood (1997) reported that acute starvation in humans caused decreased interleukin-2 (IL-2) production from peripheral blood mononuclear cells (PBMC). In their work, circulating levels of insulin-like growth factor (IGF-I) also decreased. IGF-I is known to stimulate IL-2 production from human peripheral T-cells (Kooijman et al., 1996) and increase lymphocyte number and activity (Clark et al., 1993); therefore, IGF-I can cause major changes in lymphoid tissue that are of potential benefit to immune function (Clark et al., 1993) and decreased IGF-I in fasting status leads to depressed IL-2 production (Savendahl and Underwood, 1997). Calder (2002) reported that nutrients affect the immune system mostly through regulatory mechanisms affecting the expression and production of cytokines. Because the pattern of cytokine response is important for the response to pathogens, nutrient imbalance will completely compromise the development of the future immune response (Calder, 2002). According to the first mechanism, cytokines could be affected directly by nutrition; however, cytokines also could be affected indirectly through hormonal influences.

Although the third mechanism suggests that a steady supply of nutrients is needed for the clonal proliferation of immune cells and their secretory products, the

required amount may not be very high. Klasing et al. (2000) indicated that the amount of substrates resources (nutrients) needed by the immune system is very low relative to needs for growth or milk production or egg production in agricultural animals, illustrating this concept with an example that the weight of new leukocytes and immunoglobulins normally produced each day appears to be less than 1% of the total increase in body weight of a broiler chick at 2 wk of age. However, during inflammation the nutrient requirements for both immune cells and acute-phase responses increase. Acute-phase response cytokines such as interleukin (IL)-1 and tumor necrosis factor alpha (TNF-alpha) exert a stimulatory effect on the synthesis of metallothionein, which facilitates a shift in body zinc from reservoir tissue to tissues in which enhanced cellular activity occurs during inflammation. Therefore, zinc concentration increased in liver, kidney, bone marrow, and thymus, while the concentration decreased in plasma, muscle, skin, and bone, which means that the cytokines change metabolism to provide substrates for the responses (Grimble, 1996). Additionally, during embryogenesis and the neonatal period when the expansion of leukocyte populations are rapid, chronically severe deficiencies of micronutrients are more debilitating to the development and maturation of the immune system than macronutrients such as protein and energy (Gershwin et al., 2000). For example, a study in chicks demonstrated that vitamin E supported normal spleen weight, that both selenium and vitamin E were required for normal bursal weight, and that thymus weight was depressed when both selenium and vitamin E were deficient (Latshaw, 1991). Therefore, the demand or type of nutrients required for a fully functioning immune system varies according to various challenges and the life stage.

The fourth mechanism indicates that some nutrients limit pathology that results from immune responses. Pro-inflammatory cytokines including IL-1, IL-6, IL-8, and TNF-alpha, released during an immune response can exert tissue damage through

hemodynamic change, direct cytotoxicity, and increased release of free radicals and other oxidative molecules (Gershwin et al., 2000). Some nutritional factors, such as n-3 or n-6 PUFAs, which change cell membrane components and modulate these cytokines appear to prevent pathology associated with some types of inflammatory and infectious challenges (Schoenherr and Jewell, 1997). Conversely, deficiencies of nutrients could result in severe tissue damage by inflammatory response. As an example, Sword et al. (1991) demonstrated that deficiencies of vitamin E or selenium led to three-fold increases in lipid oxidation of cell membranes after intraperitoneal LPS challenge in rats.

Finally, the fifth mechanism is that the immune system regulates the concentration of some nutrients in the body. Low levels of nutrients, especially iron, could impair the replication of pathogens because iron is required for pathogens to grow. During the acute phase immune response a variety of iron-binding proteins such as lactoferrin and haptoglobin are produced by liver and by macrophages in an effort to remove iron from the body fluids. In fact, when rabbits were infected with *Pasteurella multocida*, the concentration of iron in their plasma decreased and the growth of *P. multocida* in vitro was inhibited by the low iron concentrations (Kluger and Rothenburg, 1979).

### **Protein and amino acid status and immunity**

Dietary protein content, form of protein delivery as an amino acid or an intact molecule, and the concentration of individual amino acids in the diet have been shown to influence the immune response (Saker, 2006). In a study that examined the effect of increased protein level (16.5% to 23% of the diet) on burned children, the high protein group showed higher complement C3, higher IgG, and higher serum total protein, although the calorie level of the high protein group was lower than the normal protein group (87.1% vs. 77.7%, of desired caloric intake; Alexander et al., 1980). Trocki et al.

(1986) compared the effect of feeding the same amino acid amount and profile as intact protein versus free amino acids in a burn model using guinea pigs and determined that the animals fed the intact protein diet showed statistically significant benefits in C3, serum albumin, and transferrin. Furthermore, many studies have demonstrated that supplementation of single amino acids, especially arginine and glutamine, led to improved immune responses (Saker, 2006). Therefore, a proportion of amino acids in protein may explain the effect of increased protein level on immune function.

There are various plasma proteins that also can have antioxidant activity, including ceruloplasmin, transferrin, lactoferrin, albumin, haptoglobin, and haemopexin (Sies, 1991). The antioxidant defenses provided by those proteins are related to their ability to prevent metal ion-catalyzed free radical reactions from taking place (Sies, 1991). Glutathione, a tripeptide, is also an important antioxidant. Its main protective roles against oxidative stress are: a cofactor of antioxidant enzymes; scavenging hydroxyl radical and singlet oxygen directly; and regenerating vitamin C and E back to their active forms (Valko et al., 2007b). Although imbalanced intakes of protein and amino acids might influence status of antioxidant proteins, few studies have assessed how protein status affects on oxidative stress. Recently, Sanz et al. (2006) reported that methionine restriction in rats profoundly decreased mitochondrial ROS production, decreased oxidative damage to mitochondrial DNA, lowered membrane unsaturation, and decreased protein oxidation markers in mitochondria from heart and liver.

### **Energy status and immunity**

While many studies have assessed the effect of PEM on immune function, few studies have been conducted that have evaluated the effect of energy restriction alone. T cell proliferation induced by mitogens increased with calorie restriction in both adult

and old mice (Weindruch et al., 1986). Fernandes (1995) reported that calorie restriction reduced RNA level of interleukin-6 in tumors and delayed tumorigenesis. The mechanisms whereby calorie restriction modifies immune response and suppresses tumorigenesis remain poorly understood (Gershwin et al., 2000). Calorie restriction reduced oxidative stress, suppressed the production of reactive oxygen intermediates, and slowed aging-related process, presumably by the preservation of the cellular homeostasis function (Yu et al., 1990). Calorie restriction may modulate gene, cell structure, or cell function in immune system.

On the other hand, obesity as a result of excess calorie intake causes immune modulation, which leads to higher inflammation status. In a study in nonelderly women that compared obese with nonobese subjects, obesity was related to higher monocyte and granulocyte phagocytosis and oxidative burst activity along with elevated numbers of leukocytes and lymphocyte and lower T- and B- cell function, although activity of natural killer cells was normal (Nieman et al., 1999). More activated monocytes and granulocytes may induce more production of free radicals and proteolytic enzymes, which leads to damaged tissues and diseases such as arteriosclerosis (Nieman et al., 1999). Moreover, circulating concentrations of pro-inflammatory cytokines such as TNF-alpha and IL-6 increase with obesity (Karagiannides and Pothoulakis, 2007) and the circulating levels of these cytokines are reduced following weight loss (Ryan and Nicklas, 2004). Increased pro-inflammatory cytokines may be associated with inflammatory bowel disease with obesity (Karagiannides and Pothoulakis, 2007). Thus, overnutrition leads to inflammatory change through increased innate immune responses and pro-inflammatory cytokines.

PEM also may cause immune modulation, mainly immunosuppression, indirectly through the immune system modulation mediated by the endocrine system



and regulation by availability of substrates. One unique aspect of PEM is that it could alter virtually every aspect of the immune response (Gershwin et al., 2000). Humoral immunity can be affected as a decline in the production of immunoglobulins, secretory antibodies, and complement (Saker, 2006). Cell-mediated immunity is also affected. The thymus and lymphoid tissues atrophy, peripheral T lymphocytes decrease in number, alterations in cell-mediated delayed cutaneous hypersensitivity and graft-versus-host reactions are apparent, there is an impaired response of lymphocytes to mitogens, and patients exhibit a poor response to contact sensitization or inflammatory reactions as well as a depressed response to vaccines (Saker, 2006). Reduced neutrophil number may occur to varying degrees in patients with PEM (Saker, 2006). Although neutrophils seem to be morphologically normal, cell function is decreased, specifically the capacity of neutrophils to kill phagocytosed bacteria or molds and to secrete chemokines (Saker, 2006). Complement components of innate immunity, lymphokine production, phagocytic function, plasma lysosome production, and acute-phase reactants are affected adversely (Gershwin et al., 2000; Saker, 2006).

As for the relationship between energy and oxidative stress, some studies have been conducted that have focused on calorie restriction (CR) and age-related oxidative stress. CR is hypothesized to decrease mitochondrial electron flow and proton leak to attenuate damage caused by ROS (Lopez-Lluch et al., 2006). Lopez-Lluch et al. (2006) reported that mitochondria under CR conditions had less oxygen consumption, reduced membrane potential, and generated less ROS than controls; however, they remarkably were able to maintain their critical ATP production. The mechanism by which CR modifies oxidative status is still controversial. Possible mechanisms are: that CR can induce proliferation of mitochondria through a peroxisome proliferation-activated receptor coactivator 1-alpha signaling pathway (Lopez-Lluch et al., 2006); that CR may upregulate ROS scavenging enzymes and damage repair enzymes (Merry,

2000); or that the CR effect may result from lowered insulin concentrations and altered insulin signaling (Lambert et al., 2004).

### **Trace mineral status and immunity**

Because minerals are essential to cell function, cell development, and metabolic reactions, their deficiency can cause immune dysfunction. This section focuses on trace minerals that are important for the immune system: zinc, copper, and selenium.

Zinc is an important cofactor for numerous enzymes involved in cell metabolism and zinc deficiency can result in a profoundly immunodeficient state (Saker, 2006). The major abnormalities of immune response are in T cell and neutrophil functions (Chandra, 1990). Depressed delayed type hypersensitivity reactions, decreased CD4 helper T cell and increased CD8 suppressor T cell populations, a reduced proliferative response to mitogens and NK cell activity, and decreased chemotaxis of monocytes and neutrophils result from zinc deficiency (Saker, 2006). Depletion of marrow cells in the B cell compartment ranging from 40 to 90% was observed following a 30-day period of suboptimal zinc intake in adult mice (Saker, 2006). Zinc deficiency results in decreased digestion and phagocytosis in macrophage function, probably because zinc is involved as a cofactor in creating superoxide, which kills bacteria and fungi (Chandra, 1990). Metallothionein plays a role to regulate zinc absorption and partitioning. Mucosal binding of zinc by metallothionein limits zinc absorption at high zinc intakes (Cousin, 1996). Inflammatory cytokines (IL-1 and IL-6) and glucocorticoids induce hepatic metallothionein synthesis, which results in reduced plasma zinc and increased hepatic zinc (Cousin, 1996).

Copper plays a role in the development and maintenance of the immune system and copper status affects several aspects of neutrophil, monocyte, and T cell function (Wintergerst et al., 2007). Copper deficiency exerts deleterious effects on cell-mediated as well as the humoral response (Lukasewycz et al., 1990); in mixed-

lymphocyte reaction, which is an *in vitro* assay of T cell proliferation in a cell-mediated response, the proliferative response of lymphocytes from copper-deficient mice was diminished and copper deficiency led to an impairment of the *in vivo* antibody response in mice to sheep red blood cell antigens. As for cytokines, IL-1 was overproduced and the production of IL-2 was increased (Lukasewycz et al., 1990). Ceruloplasmin (Cp), the major copper-containing protein in serum, reflects copper status. Under marginal copper status, Cp may be better indicator than plasma/serum copper concentration itself (Laven et al., 2007). In copper-deficient cattle, serum Cp activity decreased along with reduced SOD and cytochrome c oxidase activities in leukocyte, which may impair the cell immune function (Cerone et al., 2000). On the other hand, because Cp is also an acute-phase protein, Cp activities increase in inflammatory conditions even under marginally copper deficient status (Strain, 1994).

Because selenium is essential for optimum immune response and influences the innate and acquired immune system, deficiency of selenium leads to decreased immunoglobulin titers, impaired neutrophil chemotaxis, and decreased CD8 T cell (Wintergerst et al., 2007). Selenium distributes in the major organs of immune system: bone marrow, thymus, liver, intestines, spleen, and lymph nodes (Spallholz et al., 1990). In tissue and cells in the immune system, selenium has three major functions: reduction of organic and inorganic peroxide, which are formed from the general metabolism and other environmental initiators of free radical chain reactions; metabolism of hydroperoxides, which are produced by in the arachidonic acid cascade and lead to the synthesis of leukotrienes, thromboxanes, prostaglandins, and lipoxins; and modulation of the respiratory burst, which produce superoxide and hydrogen peroxide, resulting from stimulation of the NADPH oxidase activity (Spallholz et al., 1990). The three mechanisms above are exerted through a selenium-containing enzyme, glutathione-peroxidase (GSH-Px). GSH-Px has also shown to alter immunity

through its impact on lymphocyte differentiation and signal transduction (Saker, 2006). Thus, selenium seems to be involved in immune system directly and indirectly. One example demonstrating the extent to which selenium can affect the immune system was a study of coxsackievirus infection, in which the amount of virus was elevated in selenium deficient mice (Gershwin et al., 2000). While antibody production and NK cell activity was not affected with selenium deficiency, T cell proliferative response was greatly diminished (Gershwin et al., 2000). These results indicate that selenium deficiency may cause imbalance of immune system such that helper-2 T cells, which help B cell production of antibody, are favored over helper-1 T cells, which secrete cytokines important for proliferation (Gershwin et al., 2000).

Zinc, copper, and selenium can collectively be related to antioxidative control because they are indispensable components of antioxidant enzymes: zinc and copper for superoxide dismutase (SOD), and selenium for GSH-Px. These enzymes protect sites that are particularly exposed to oxidative stress. For example, these enzymes are present in high concentrations in pulmonary epithelium and erythrocytes (Bourgeois, 2003). Intake of these minerals may affect antioxidant status. Jing et al. (2007) fed rats one of three diets with different zinc levels for 6 weeks, including zinc adequacy (ZA; 34.50 mg/kg, control), zinc deficiency (ZD; 3.30 mg/kg), and zinc overdose (ZO; 345.45 mg/kg) and reported that rats fed the ZD diet had reduced activities of copper-zinc superoxide dismutase (Cu-Zn SOD) and catalase (CAT) and increased concentrations in liver of malondialdehyde and hydrogen peroxide. Rats fed the ZO diet had higher Cu-Zn SOD activity in liver (Jing et al., 2007). The mRNA expression levels of SOD were upregulated in the ZO group, and CAT was downregulated in the ZD group, suggesting that although zinc deficiency impaired antioxidant functions, zinc overdose did not enhance the antioxidant systems of animals (Jing et al., 2007).

Prohaska and Brokate (2001) reported that rats fed a low copper diet for 32 days beginning at 50 days of age had lower Cu,Zn-SOD activity in the liver, heart, and kidney. In addition, there was a significant reduction in Cu,Zn-SOD protein detected by Western immunoblotting that was proportional ( $r = 0.96$ ) to the reduction in Cu,Zn-SOD activity. In the liver, the reduction in Cu,Zn-SOD protein was approximately 50%. The reduction in Cu,Zn-SOD protein was likely related to a post-transcriptional mechanism (Prohaska and Brokate, 2001).

Expression of GSH-Px in most tissues is highly sensitive to dietary levels of selenium, which is bound to the active site as a selenocysteine (Sutphin and Buckman, 1991). When mice were fed diets deficient in selenium and/or vitamin E for 6-8 wk, levels of GSH-Px decreased from 50% in brain to 90% in blood in mice fed the Se deficient diet (Sutphin and Buckman, 1991). No compensatory changes in the activities of the other antioxidant enzymes were reported and addition of vitamin E to the diet did not alter antioxidant enzyme activities or malondialdehyde levels (Sutphin and Buckman, 1991).

### **Vitamin status and immunity**

Although specific mechanisms are not clearly understood, vitamin E may influence innate immune cell activity. Saker (2006) reported that vitamin E inhibits macrophage secretion of PGE<sub>2</sub>, which suppresses IL-1 production. A study in humans suggested that vitamin E supplementation reduces concentrations of proinflammatory cytokines (Webb and Villamor, 2007). Vitamin E deficiency in animals is associated with T cell and B cell dysfunction and suppressed NK cell response (Shikora and Blackburn, 1997). T cell dysfunction induced by vitamin E deficiency was reversed by vitamin E supplementation in another study conducted in humans (Shikora and Blackburn, 1997). Meydani et al. (1990) reported that increased ConA stimulated IL-2 production, lymphocyte proliferation in response to ConA, and delayed type

hypersensitivity response, with vitamin E supplementation in human study, which indicates that vitamin E may specifically enhance a Th1-cell-mediated response (Webb and Villamor, 2007). Also, suppressed PGE2 production by vitamin E enhanced T cell function and proliferation (Meydani et al., 1986; Hayek et al., 2000). As for humoral immunity, there is not strong association with vitamin E supplementation (Webb and Villamor, 2007).

Decreased vitamin C is known to be associated with depressed cell-mediated immunity, poor bacteriocidal activity, and impaired macrophage mobilization (Saker, 2006). Supplementation with vitamin C enhanced T and B cell proliferation and bacterial phagocytosis (Saker, 2006). Among subjects regularly engaged in strenuous physical activity or who live in crowded situations, vitamin C supplementation appears to reduce the incidence of common colds and pneumonia (Webb and Villamor, 2007). Excessive exercise causes Th2-cell proliferation, which suppresses Th1-cell-mediated response and increases susceptibility to virus infection (Lakier Smith, 2003). In addition, physical stress results in free radical production, which leads to impaired motility and function of neutrophils (Webb and Villamor, 2007). Since vitamin C is water-soluble antioxidant important for controlling intra- and extracellular oxidation [Baumgartner in (Shikora and Blackburn, 1997)], it could also suppress PGE2 production as mentioned previously for vitamin E, resulting in enhancing Th-1 cell function. Moreover, Vitamin C as a scavenger of free radicals could directly maintain immune function by protecting membrane lipid receptors and other components of immune cells from oxidation (Shikora and Blackburn, 1997).

Vitamin A deficiency impairs secretory IgA production, decreases mucus production, and leads to keratinization of secretory epithelia (Rombout et al., 1992; Tei et al., 2000; Darwiche et al., 1993). It also has been shown that vitamin A bound to its physiologic carrier retinal-binding protein and chylomicron remnants modulates

B cell activation, cytokine production, and cell differentiation (Shikora and Blackburn, 1997). Beta-carotene, which can be metabolized to vitamin A, has been shown to enhance T cell and B cell generation in animals (Shikora and Blackburn, 1997). In human studies, supplement of beta-carotene improved CD4 T cell counts in populations with chronic illness such as cancer and HIV, and enhanced mitogen-stimulated lymphocyte proliferation in smokers (Kazi et al., 1997; Coodley et al., 1993; van Poppel et al., 1993). Beta-carotene is also an antioxidant vitamin like vitamin E and C, and the protective action of those antioxidant vitamins appears to affect not only cell membrane lipids, but also protein and DNA: however, antitumor action of beta-carotene seems to be independent of their antioxidant activity (Bourgeois, 2003) and immune enhancing effect. The action is related to a gene coding for the structural unit of a gap junction (Bertram and Bortkiewicz, 1995).

With regard to having a specific impact on immune function, there is one vitamin among the vitamin B complex that should be mentioned, pyridoxine (B6) (Saker, 2006). Deficiency is associated with both impaired cell-mediated and humoral responses. In humans with poor vitamin B6 intake, immunological findings include decreased circulating lymphocytes, reduced IgD levels, and a decreased percentage of T helper cells (Shikora and Blackburn, 1997). In human studies, it is suggested that vitamin B6 may be needed in excess of the recommended daily allowance to maintain adequate immune function in compromised patients (Saker, 2006).

Vitamins are representative of the nutrients that can have direct antioxidative effects and contribute to the balance between oxidants and antioxidants. Therefore, the deficiency of those nutrients can result in increased oxidative stress. For example, Bourgeois (2003) suggested that the depletion in vitamin E, vitamin C, and beta-carotene led to an increased n-pentane in exhaled air, which is a biomarker of the oxidation of lipids in cell membrane in vivo. Supplementation of these three vitamins

in the diet decreased of the level of n-pentane exhaled in several animals and humans (Bourgeois, 2003). Moreover, in a study to measure the oxidized nucleoside 8-hydroxy-2'-deoxyguanosine (8-oxo-7,8-dihydro-2'-deoxyguanosine; oxoIdG), a major product of oxidative damage to DNA, in DNA isolated from human sperm provided by healthy subjects and compare oxoIdG to the seminal fluid vitamin C levels, the seminal fluid vitamin C decreased by half and the level of oxo8dG in sperm DNA increased 91% when dietary vitamin C was decreased from 250 to 5 mg/day (Fraga et al., 1991). The protective action of antioxidant vitamins has also been demonstrated on proteins, especially those of the lens (Bourgeois, 2003). It seems that the antioxidant role of vitamin E, vitamin C, and beta-carotene range from lipid to DNA and proteins (Bourgeois, 2003).

## SPECIFIC INFLUENCES OF TRACE MINERALS ON IMMUNITY AND OXIDATIVE STRESS IN RUMINANT

### **Zinc**

Zinc is a component of many metalloenzymes, which affect metabolism of carbohydrates, proteins, lipids, and nucleic acids (NRC, 2001). The earliest and most important clinical signs of zinc deficiency in ruminants, as in other species, are loss of appetite, parakeratosis, and impaired wound healing (Miller et al., 1965). Therefore, it is possible that zinc deficiency weakens the first line of resistance to infection such as skin and also reduces the supply of major nutrients for sustaining the increased basal metabolic rate following an infectious challenge (Suttle and Jones, 1989). In fact, some research suggests that the addition of zinc to typical diets affects disease resistance (Galyean et al., 1995). Chirase (1991) determined that supplementation of zinc as zinc methionine to a basal diet containing 30 mg/kg zinc increased feed intake and reduced body temperature in feedlot cattle challenged with infectious bovine



rhinotracheitis virus (IBRV), which suggested that dietary Zn enhanced the recovery rate of IBRV-stressed cattle. Increasing the level of supplemental zinc from 30 to 100 mg/kg diet slightly reduced morbidity from respiratory diseases in newly weaned calves that had been transported (Spears, 2000).

A genetic disorder (lethal trait A46) of zinc metabolism has been reported in cows that results in severe zinc deficiency (Spears, 2000). The A46 trait is associated with thymic atrophy and increased incidence of numerous infections (Suttle and Jones, 1989). In calves with lethal trait A46, secondary antibody responses to bacteriophage were reduced. In addition, lymphocyte response to PHA, ConA, and PWM stimulation was variably reduced, CD4 T cells were subnormal, and relative numbers of B cells were decreased (Perryman et al., 1989). Because zinc is a component of thymosin, a hormone produced by thymic cells that regulates cell-mediated immunity (NRC, 2001), the reduced hormone by zinc deficiency presumably affected the numbers and functions of lymphocytes.

Effects of dietary zinc deficiency have not been as marked or consistent as zinc deficiency resulting from genetic mutation. Marginal zinc deficiency did not impair cell-mediated or humoral immune response in a controlled cattle study (Droke et al., 1993). The addition of 25 mg/kg of zinc to a control diet that contained 33 mg/kg of zinc did not increase in vitro lymphocyte responses to PHA or PWM stimulation, in vivo cellular response to intradermal PHA administration, or antibody response following vaccination for IBRV (Spears, 2000). Supplementation of 150 or 300 mg/kg of zinc to a control calf starter diet containing 60 mg/kg of zinc fed to Holstein heifer calves did not affect mitogen-induced lymphocyte blastogenesis, IL-2 production by lymphocyte, lymphocyte cytotoxicity, or phagocytic and intracellular killing ability of blood neutrophils (Kincaid et al., 1997).

Zinc could play an important role as an antioxidant through SOD. Activities of SOD were reduced in the testis, intestine, and liver of zinc-deficient goats (Underwood and Suttle, 1999). In addition, zinc metalloenzymes are involved in vitamin A metabolism (Underwood and Suttle, 1999), and low plasma vitamin A value in the presence of adequate dietary vitamin A can occur in zinc-deficient lambs and young goats (Underwood and Suttle, 1999), which may reduce host antioxidant function. Zinc has a potential role in increasing absorption or retention of vitamin E (Hatfield et al., 2002). Although the research was not conducted in ruminants, zinc status in rats has been shown to have a profound effect on intestinal absorption and body status of vitamin E (Kim et al., 1998). As mentioned above, because zinc affects metabolism of carbohydrates, proteins, lipids, and nucleic acids, zinc deficiency may also affect tissue recovery from oxidative damage.

### **Copper**

The authors of the NRC suggested that effects of copper deficiency on immunity are not easily observed (NRC, 2001). Administration of copper to copper-depleted calves increased the ability of isolated peripheral blood granulocytes, primarily neutrophils, to kill ingested *Candida albicans* by over two fold, which indicated that impaired phagocytic killing activity due to copper deficiency can be reversed by copper supplementation (Jones and Suttle, 1981). Marginal copper deficiency in dairy heifers also reduced neutrophil killing of *S. aureus* (Torre et al, 1996). However, Gengelbach et al. (1997) reported that there were no differences in macrophage phagocyte function among calves supplemented with copper or molybdenum compared with controls (Gengelbach et al., 1997).

Studies conducted in cattle have failed to show consistent effects of copper deficiency on either cell-mediated or humoral immune responses (Spears, 2000). Ward et al. (1999) reported that copper-deficient calves had higher secondary antibody

response to pig erythrocytes than copper-supplemented calves, and cell-mediated response to phytohemagglutinin was decreased by copper supplementation to copper-deficient calves. Another study demonstrated that low copper status was associated with reduced responses of peripheral blood lymphocytes to stimulation with T cell mitogens, PHA and Con A, following weaning and an IBRV challenge (Spears, 2000). While copper supplementation increased humoral response to ovalbumin injected into growing calves, supplementation decreased antibody titers to porcine erythrocytes (PRBC) in unstressed calves and increased the titers in stressed steers (Ward and Spears, 1999).

Dietary copper may affect cytokine production in cattle (Spears, 2000). Copper supplemented calves had higher circulating levels of TNF-alpha than calves fed additional molybdenum at weaning and tended to have higher TNF-alpha than calves fed with iron and molybdenum after IBRB inoculation (Gengelbach et al., 1997). Mononuclear cells from lactating dairy cows receiving a marginal level of copper (6-7 mg/kg diet) produced less IFN-gamma when stimulated with Con A than cell isolated from cows fed on adequate levels of copper, while IL-2 production was not affected by treatments (Torre et al., 1995).

In spite of inconsistent effects on specific immune responses, copper status can affect disease resistance. A long-term study determined that natural copper deficiency in ewes and lambs caused by improved pasture treatment resulted in a sudden upsurge in infectious mortality (Suttle and Jones, 1986). Because symptoms of copper-deficiency also increased at the same time, susceptibility to infectious diseases was enhanced presumably by copper deficiency (Suttle and Jones, 1986). Although it has not been clearly demonstrated, the dietary copper intake required for optimal immune function may exceed the amount required to prevent more classic signs of copper deficiency (NRC, 2001).

Copper may protect tissues from oxidant stress via two distinct pathways: maintenance of normal iron metabolism and direct action through SOD (Underwood and Suttle, 1999). Dietary copper deficiency depressed liver catalase activity in rats (Taylor et al., 1988). The depressed activity of liver catalase, a heme enzyme, occurred even though the liver concentration of iron was high. Therefore, copper deficiency may cause altered iron metabolism (Taylor et al., 1988). Iron accumulation in rat liver was observed in copper deficiency (Taylor et al., 1988), and the accumulation itself may affect oxidant and antioxidant balance because iron promotes free radical generation (Underwood and Suttle, 1999). Copper-deficient diets fed to rats significantly reduced tissue SOD, which is an important defense enzyme against oxidative stress (Taylor et al., 1988). Since ceruloplasmin, a copper-dependent enzyme, may also contribute to antioxidant defenses by scavenging free radical iron and free radicals (Underwood and Suttle, 1999), copper depletion may increase susceptibility against oxidative damage through lower activity of ceruloplasmin. In addition, significantly higher liver vitamin C concentrations were measured in rats fed a copper-deficient diet (Taylor et al., 1988), which might be compensating metabolic reaction in copper depletion. As mentioned above, although copper status is seemingly related to oxidative status, few studies have been conducted in ruminants to determine the relationships between copper status and oxidative stress.

### **Selenium**

Hogan et al. (1990) reported that intracellular kill of *Staphylococcus aureus* was greater in neutrophils isolated from selenium supplemented cows than in neutrophils from cows not fed supplemental selenium. Neutrophils from selenium-deficient steers were less able to kill ingested *Candida albicans* than ones from the selenium-adequate group, which was associated with non-detectable GSH-Px activity in the selenium-deficient neutrophils (Boyne and Arthur, 1979). While supplemental selenium did not

affect phagocytic ability of neutrophils in dairy cows (Hogan et al., 1990), chemotactic migration of neutrophils in vitro were reduced by selenium deficiency in goats (Spears, 2000). Selenium-deficient bovine mammary artery endothelial cells had significantly enhanced neutrophil adherence when stimulated with TNF-alpha, which may lead to dysfunction exhibiting as either hyper-responsiveness to nominal stimuli or as tight adherence to endothelial cells, with possible hindrance of leukocyte extravasation to the affected tissue (Maddox et al., 1999).

Con A-stimulated lymphocyte proliferation was significantly lower in selenium deficient cows than in cows fed diets supplemented with selenium (Cao et al., 1992). Lipoxygenase pathway products from oxidation of arachidonic acid may be essential for lymphocyte proliferation because GSH-Px plays an important role in the control of the enzymatic oxidation of arachidonic acid; the lymphocytes obtained from selenium deficient cows produced less products from lipoxygenase pathways and partial reversal of the inhibition of lymphocyte proliferation by hydrocortisone was observed when products from lipoxygenase pathways were added (Cao et al., 1992). Sheep supplemented with 0.1 or 0.5 ppm selenium as sodium selenite had higher lymphocyte proliferative responses to phytohemagglutinin, pokeweed, and Con A mitogen than nonsupplemented control sheep, while supplementation with selenomethionine, an organic form of selenium, did not enhance blastogenic (Stabel and Spears, 1993). Further, addition of 1 ppm selenium to the basal diet in either form significantly depressed responses to all three mitogens, indicating a possible cytotoxic effect of high levels of supplemental dietary selenium (Stabel and Spears, 1993). Low dietary selenium does not consistently affect cell-mediated immune response in ruminant (Spears, 2000); therefore, further studies may be needed in ruminants that consider both form and amount.

Humoral immune response has been enhanced by increasing dietary selenium intake in ruminants (Spears, 2000). Calves consuming an average 3 to 6 mg selenium/day had higher antibody titers to chick egg lysozyme than calves ingesting less than 1 mg/day (Stabel and Spears, 1993). Lambs immunized with parainfluenza3 virus, tetanus toxoid, and *Corynebacterium pseudotuberculosis* had higher antibody responses when fed supplemental sodium selenite or selenomethionine, indicating that selenium enhances antibody responses to viral and bacterial antigens as well (Stabel and Spears, 1993).

Selenium status appears to be directly related to disease resistance in ruminants. When selenium injections (0.1 mg Se/kg of BW) were administered 21 d before expected calving, the duration of clinical mastitis symptoms was reduced by 46% compared to controls, although incidence of clinical mastitis was not affected (Smith et al., 1984). Weiss et al. (1990) reported that rates of clinical mastitis and bulk-tank somatic cell count were related inversely to plasma selenium concentrations. Dietary supplementation of 0.14 ppm selenium resulted in less severity and shorter duration for experimental *E. coli* mastitis than 0.04 ppm (Erskine et al., 1989); however, selenium level did not affect induction of experimental mastitis by *S. aureus* (Erskine et al., 1990).

Although selenium may play a role as an antioxidant through selenoproteins in ruminants, the results of studies focused on this role have been inconsistent and its antioxidant function may depend on selenium status and level of other antioxidants in body and diet. It was reported that nutritional muscular dystrophy, which occurred naturally in lambs and calves in part of Oregon state and New Zealand in the 1950s, was caused by selenium deficiency and could be prevented by selenium supplementation (Underwood and Suttle, 1999). Although the lesions are considered to be probably initiated by free-radical damage (Underwood and Suttle, 1999), the

specific selenoprotein responsible has been unclear because there are more than 30 distinctive selenoproteins and these functions are complexly associated each other (Underwood and Suttle, 1999). Furthermore, it is also known that factors involved in the nutritional muscular dystrophy are not constrained to selenium: polyunsaturated fatty acid level in diet may contribute (Underwood and Suttle, 1999).

Selenium supplementation (3 mg/d) during 6-week prepartum period in dairy cows did not affect Thiobarbituric Acid Reactive Substances (TBARS) in red blood cells, while vitamin E supplementation decreased TBARS (Brzezinska-Slebodzinska et al., 1994). Dietary Se supplementation did not affect muscle selenium levels, GSH-px activity, or susceptibility to lipid and oxymyoglobin oxidation in the presence or absence of vitamin E when diets fed to beef cattle were supplemented with organic selenium (0.3 mg/kg) and vitamin E (300 IU/kg feed), for 55 days preceding slaughter (O'Grady et al., 2001). In another study, injection of 1100 IU of Vitamin E and 30 mg of sodium selenite 3 weeks before calving reduced erythrocyte lipid peroxide concentrations and plasma cortisol (Gupta et al., 2005).

### **Manganese**

There are few studies to assess the effect of manganese itself on immune function on ruminants. Crossbred beef females (n = 43 nulliparous heifers; n = 37 primiparous cows) were assigned over a 2-yr period to treatments of either dietary supplementation with organic (50% organic and 50% inorganic manganese, zinc, and copper) or inorganic (100% inorganic CuSO<sub>4</sub>, ZnSO<sub>4</sub>, and MnSO<sub>4</sub>) trace minerals (Ahola et al., 2005). IgG antibody titers specific for porcine red blood cells (PRBC) were greater in heifers supplemented with organic compared to inorganic minerals on day 14 and 21 post PRBC injection (Ahola et al., 2005). Similarly, in a study of crossbred heifer calves, which were shipped 250km, to compare treatment 1: supplemental inorganic trace minerals (106 mg of Zn/kg from ZnO, 58 mg of Mn/kg

from MnO, 37 mg of Cu/kg from CuSO<sub>4</sub>, and 7 mg of Co/kg from CoCO<sub>3</sub>) for 42 d after shipping, and treatment 2: organic form (zinc methionine, manganese methionine, copper lysine, and cobalt glucoheptonate) at a level 3 times that of treatment 1 for the first 14 d after shipping, then switched to the same level of treatment 1 for the remainder of the 42-d trial, parainfluenza-3 virus antibody response was increased more at 14 and 28 d postvaccination and skin swelling response at 12, 24, and 48 h postinjection to intradermal PHA was better in treatment 2 (George et al., 1997). Although those studies did not evaluate the effect of manganese itself, it implies that manganese might contribute to enhancement of immune function.

Manganese can contribute to oxidative balance through manganese super oxide dismutase (MnSOD). Manganese deficiency lowers MnSOD activity in the heart and increases the peroxidative damage caused by high dietary levels of polyunsaturated fatty acids (Underwood and Suttle, 1999). Rams fed the least amount of manganese (13 pg manganese/g of dry matter) had low concentrations of manganese in heart and lung and a lower activity of MnSOD in heart than rams fed the most amount of manganese (45 pg manganese/g of dry matter; Masters et al., 1988). The concentration of manganese in these tissues, and the activity of manganese superoxide dismutase in the heart increased linearly as the concentration of manganese in the diet increased (Masters et al., 1988). Tissue activities of MnSOD are relatively low in lambs at birth (Paynter and Caple, 1984), and it probably reflects the low oxidative stress associated with the protected lifestyle of the fetus (Underwood and Suttle, 1999).

## LPS CHALLENGE AND IMMUNE SYSTEM IN DAIRY CATTLE

As a study model of *E. coli* mastitis, intramammary LPS treatment has been conducted to investigate immune responses in dairy cows. Intramammary infusion of LPS (lipopolysaccharide) causes innate immune responses in dairy cattle. In cows that



were made tolerant to endotoxin by daily intravenous injections, intramammary infusion of one-fifth of the daily dose produced a maximum effect on body temperature, suggesting that inflammatory endogenous mediators were released in the udder and then absorbed into the blood circulation, rather than the absorption of endotoxin (Lohuis et al., 1988). Actually, LPS itself is not chemoattractant for bovine neutrophils (Carroll et al., 1982), and increased inflammatory cytokines such as TNF- $\alpha$ , IL-1, and IL-8 in milk and plasma (Riollet et al., 2000; Waldron et al 2006) and increased neutrophil chemoattractant such as C5a in milk (Riollet et al, 1988) have been recognized after intramammary LPS infusion. Intramammary treatment also causes biological responses including fever, increased plasma cortisol (Waldron et al., 2006), and increased acute phase protein such as plasma haptoglobin (Riollet et al., 2000).

*E. coli* J5 vaccine produces antibodies against core antigens, which consist of lipid A and core polysaccharides (Tyler et al., 1990). The presence of the core antigen compound only forms the basis of cross-protection against a wide range of gram negative bacteria (Dosogne et al, 2002). When dairy cows were vaccinated at 8 weeks and 4 weeks before calving and challenged by intramammary LPS infusion at 8 to 16 days in milk, vaccinated cows had significantly higher J5-specific IgG1 in circulation, trends for higher J5-specific IgG2, and the same level of IgM compared to control cows, along with lower somatic cell counts in milk and less milk yield loss (Wilson et al. 2007). However, even when serum and milk antibody titers are increased after J5 vaccine, effect of the vaccine on incidence and severity of *E. coli* mastitis are not constant (Hill 1991; Hogan et al., 1999). Dosogne et al. (2002) suggested that effects of J5 vaccine are not confined to increased antibodies, but the vaccine also may stimulate Th1 cells through dendritic cells in favor to recruit neutrophils because the most important risk factors for severe *E. coli* mastitis are slow migration of blood

polymorphonuclear neutrophils into the mammary gland and their impaired oxidative burst activity (Dosogne et al., 2002). Therefore, the effects of J5 vaccine may depend on balance of antibody production and Th1 proliferation.

In summary, the functions of major nutrients and trace minerals on immune system and oxidative stress have been investigated and established in human and non-ruminant animals. As for ruminants, however, the mechanism of each trace mineral on immune system and oxidative stress remains unclear. In addition, the relationship between bioavailability and level of dietary trace minerals with function in ruminants has not been well-investigated. The focus of the research described in the rest of this thesis was to further elucidate potential effects of trace mineral amount and source in the diet on immune function and oxidative stress parameters in lactating dairy cows.

**CHAPTER THREE:**  
**EFFECT OF TRACE MINERAL AMOUNT AND SOURCE ON IMMUNE**  
**FUNCTION AND OXIDATIVE STRESS IN DAIRY COWS**

**INTRODUCTION**

Trace minerals are essential to control oxidative stress and modulate immune function (Bendich et al., 1990; Sies, 1991; NRC 2001). Because immune function is influenced strongly by the antioxidant/oxidant balance, the antioxidant levels in immune cells play a pivotal role in maintaining immune cells in a reduced environment and in protecting them from oxidative stress and preserving their function (De la Fuente, 2002). Therefore, it could be important for dairy cow nutrition to consider the balance between antioxidant defense and prooxidant assault and trace minerals have key roles as antioxidants or part of antioxidant proteins. In addition, trace minerals could affect directly or indirectly immune system because they are involved in metabolic enzymes, cell development, and cell function (McArdle and Ashworth, 1999; NRC, 2001). Thus, deficiency and malabsorption of trace minerals impair immune function.

While major nutrients and macrominerals have been studied in various ways with focus on optimized performance and health status in dairy cattle (Underwood and Suttle, 1999; NRC, 2001), few studies have been conducted that assess the role of trace minerals in metabolism, oxidative status, or immune function in dairy cows. Research conducted in our group and others suggests that organic trace minerals are more bioavailable than commonly used inorganic sources (NRC, 2001; Thering et al., 2007); however, whether increased bioavailability translates into increased functional characteristics is not known. Furthermore, the NRC (2001) calculated requirements for major trace minerals of interest (Zn, Cu, and Mn) to be much lower than commonly fed on commercial dairy farms. Hence, the objective of this study was to

determine whether amount and form of trace minerals supplement affect immune function and oxidative stress parameters in lactating dairy cattle.

## **MATERIALS AND METHODS**

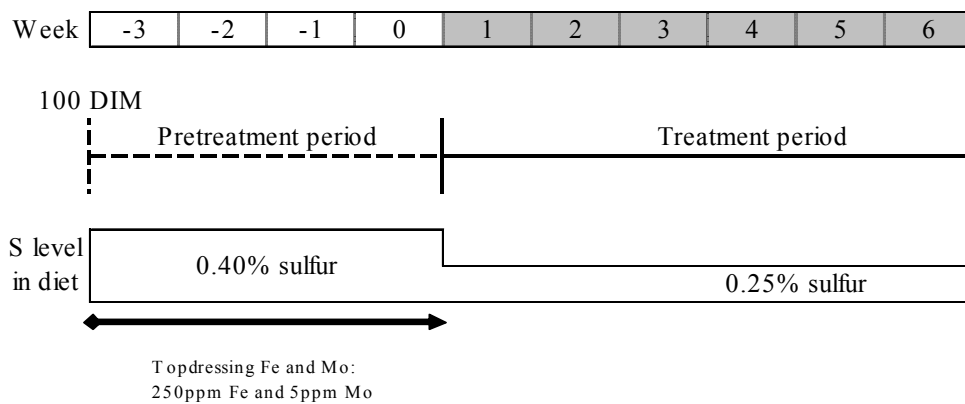
### **Experimental Animals, Treatments, and Procedures**

The Cornell University Institutional Animal Care and Use Committee approved all procedures involving animals. Forty-eight multiparous Holstein cows were utilized in this experiment. Cows were housed in individual tiestalls at the Cornell University Teaching and Research Center beginning at 60 to 140 days in milk and fed a diet formulated to meet or exceed NRC (2001) nutrient requirements for all nutrients except for trace minerals of interest (Zn, Cu, and Mn), which were supplied from basal ration ingredients only. In addition, all cows were fed a pretreatment diet for four weeks (week -3 to week 0), containing 0.40% sulfur (dry matter basis, ration addition), 5 ppm of molybdenum from sodium molybdate (topdress), and 250 ppm of iron from iron sulfate (topdress) to decrease trace mineral absorption and status (Table 3-1; Figure 3-1). After this four-week pretreatment period, the sulfur content of the basal diet was reduced to 0.25% (Table 3-1), the topdress of the Mo and Fe ceased, and cows were assigned to one of four dietary treatments for six weeks (week 1 to week 6; Figure 3-1) in a randomized complete block design: 1) NRC inorganic (NRC 2001 levels using inorganic trace mineral supplements only); 2) NRC organic (NRC 2001 levels using organic trace mineral supplements only); 3) Commercial inorganic (commercial levels (approximately 2X NRC requirement) using inorganic trace mineral supplements only); 4) Commercial organic (commercial levels using organic trace mineral supplements only). Cows were enrolled in groups and blocked by week of enrollment. The groups consisted of five blocks (n=16 for block 1 and n=8 each for blocks 2 through 5). Within blocks, cows were stratified by milk yields and randomly assigned to treatments. The calculated amounts of Zn, Cu, and Mn consumed by

**Table 3-1.** Ingredient and nutrient composition (DM basis)

Ingredient	Pretreatment period	Treatment period
Corn silage, %	46.29	46.29
Hay crop silage, %	15.43	15.43
Corn meal, %	13.37	13.11
Soybean meal (48% CP), %	9.84	9.35
Soy hulls, %	6.01	5.56
Citrus pulp, %	3.76	3.82
Expeller soybean meal	2.41	2.36
Corn germ meal, %	1.90	1.87
Blood meal, %	1.35	1.27
Corn gluten meal, %	0.96	0.88
Calcium carbonate, %	0.56	1.23
Sodium bicarbonate, %	0.67	0.57
Calcium sulfate, %	1.31	0.41
Salt, %	0.36	0.36
Mono dicalcium phosphate, %	0.26	0.26
Magnesium oxide, %	0.18	0.18
Selenium-0.06%, %	0.05	0.05
Vitamin ADE <sup>1</sup> , %	0.02	0.02
Cobalt sulfate, %	< 0.01	< 0.01
Calcium iodate, %	< 0.01	< 0.01
Energy, nutrient, and minerals		
CP, %	16.49	17.38
Soluble Protein % CP	50.51	50.52
ADF, %	21.00	20.27
NDF, %	33.32	32.27
NEL, Mcal/kg	0.76	0.77
Ca, %	0.98	1.07
P, %	0.36	0.36
Mg, %	0.33	0.28
K, %	1.24	1.19
Na, %	0.37	0.32
Fe, ppm	239.22	232.58
Zn, ppm	29.13	26.39
Cu, ppm	6.34	5.25
Mn, ppm	22.45	21.19
Mo, ppm	0.30	0.31
S, %	0.37	0.30

<sup>1</sup>Contained 37,113 IU/kg of vitamin A, 7,216 IU/kg of vitamin D, and 72,165 IU/kg of vitamin E



**Figure 3-1.** Overall schematic of experimental design, indicating pretreatment and treatment periods, changes in dietary sulfur level, and topdress of iron and molybdenum

**Table 3-2. Treatments**

	Treatment1			Treatment2			Treatment3			Treatment4									
	NRC level, Inorganic			NRC level, Organic			Commercial level, Inorganic			Commercial level, Organic									
	Basal diet, mg/day	Inorganic	Sum, mg/day	Basal diet, mg/day	Inorganic	Sum, mg/day	Basal diet, mg/day	Inorganic	Sum, mg/day	Basal diet, mg/day	Inorganic	Sum, mg/day							
Zn	642	700	1342	55.2	647	0	700	1347	54.9	636	1200	0	1836	76.2	663	0	1200	1863	74.2
Cu	128	75	203	8.34	129	0	75	204	8.31	127	275	0	402	16.7	132	0	275	407	16.2
Mn	516	0	516	21.2	519	0	0	519	21.2	511	350	0	861	35.7	532	0	350	882	35.1

cows assigned to the four treatment groups are outlined in Table 3-2. Supplemental inorganic Zn, Cu, and Mn were supplied using zinc sulfate monohydrate, cupric sulfate pentahydrate, and manganese sulfate monohydrate, respectively. Organic trace minerals were supplied as Zn, Cu, and Mn chelated to 2-hydroxy-4-(methylthio)-butanoic acid (HMTBA) as MINTREX™ trace minerals (Novus International, Inc., St. Louis, MO).

Milk yields were recorded at all milkings beginning at week -2 of pretreatment period. Milk samples were collected on one day per week from all three milkings. Samples were composited and stored at 4°C with a preservative (Bronopol tablet, D&F Control System, San Ramon, CA) until analyzed (Dairy One Cooperative Inc., Ithaca, NY) within 24 h for fat, protein, lactose, total solids, milk urea N using infrared analysis (AOAC, 2000; method 972.160), and SCC by an optical fluorescent method (AOAC, 2000; method 978.26).

Ingredient and chemical composition of the diets fed during the experiment are described in Table 3-1. All nonforage ingredients were blended by a commercial feed mill into separate concentrate mixtures, and diet mixing at the farm consisted of mixing the component forages with the appropriate concentrate mixture. Fresh feed was provided each morning at 1000 h, orts were weighed and recorded daily, and water was made available at all times. Samples of the forages and concentrate mixtures were obtained weekly throughout the experiment, and DM content determined by drying at 55°C until static weight. Amounts of individual feed components in the TMR were adjusted weekly based on changes in the DM content of these feed components. Dry matter contents of these TMR were used in calculation of DMI for the corresponding week. The weekly samples of forages, concentrate mixtures, and TMR were composited into 4-wk composite samples and submitted to a commercial laboratory for wet chemistry analysis (Dairy One Cooperative Inc., Ithaca,



NY). Samples were analyzed for DM (method 930.15; AOAC, 2000), CP (method 990.03; AOAC, 2000), ADF and NDF (Van Soest et al., 1991), soluble CP, and macro- and microminerals (Sirois et al., 1994). Body condition scores (BCS) were assessed (1 to 5 scale; Wildman et al., 1982) and body weights (BW) were measured at weeks 0, 3, and 6 of treatment.

### **Blood and Tissue Sampling**

Blood samples were collected from coccygeal vessels once weekly at 1000 h beginning at week -1 of the depletion period and continuing through 6 weeks of treatment. Samples were placed on ice immediately after collection. Two evacuated 10-ml tubes containing heparin as an anticoagulant (Vacutainer®, BD, NJ) were used to draw 14 ml of blood (7 ml per tube) and whole blood was used for hematocrit measurement. Erythrocytes were isolated from 2 ml of whole blood according to the method described by Bernabucci et al. (2005): after centrifugation and pipetting off plasma, remaining erythrocytes were washed 4 times with 0.9% NaCl, lysed using 2 ml of cold water, snap-frozen in liquid nitrogen, and stored at -80°C for later analysis of SOD and GSHPx. Plasma was also harvested as described above and stored at -20°C until analysis for plasma TBARS, plasma total antioxidant capacity (TAC), and plasma IgG.

### **Immunologic Challenges and Sampling**

At the end of week 2 of treatment, all cows were administered a commercially available vaccine for environmental mastitis (J-5 Bacterin, Pfizer, New York, NY). At the end of week 5 of treatment, all cows were fitted with a single indwelling jugular catheter and subjected on the following day to an intramammary lipopolysaccharide (LPS) challenge (100  $\mu$ g of LPS from *E. Coli* serotype 0111:B4 into each of left two homolateral quarters using sterile technique) using procedures described by Waldron et al. (2006). Samples of blood (12 ml) were collected using syringes from the jugular

catheter at 30-min intervals beginning immediately prechallenge and continuing for the 8-h period following LPS administration. Samples were transferred into heparinized test tubes containing 30 IU heparin/ml (source for heparin) as an anticoagulant and placed on ice. Samples were also collected by coccygeal venipuncture at 24 and 48 h postchallenge using heparinized evacuated tubes. Prior to centrifugation, hematocrit was measured on whole blood. Plasma was harvested by centrifugation and two 1-ml aliquots were prepared, snap-frozen in liquid nitrogen, and stored at -20°C until analysis.

Rectal temperatures and heart rates were monitored and recorded at 30-min intervals concurrently with the blood collection.

### **Oxidative Stress Parameters**

SOD activity in erythrocyte lysate preps from the blood samples collected on a weekly basis was measured using a commercial kit (Superoxide DismuTACe Assay kit, Cayman Chemical Company, Ann Arbor, MI). Frozen erythrocyte lysate was thawed and diluted 1:50 with 50 mM Tris-HCL, pH 8.0. The kit utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% inhibition of the superoxide radical. The oxidized tetrazolium salt gives a red formazan dye. The absorbance was read at 450 nm using Versamax tunable microplate reader (Molecular Devices, Sunnyvale, CA) after 45 min incubation at room temperature. SOD was expressed in units per milliliter of packed cell volume (PCV; Bernabucci et al., 2005).

GSH-px activity in erythrocyte lysate preps from the blood samples collected on a weekly basis was measured by commercial kit (Glutathione Peroxidase Assay kit, Cayman Chemical Company, Ann Arbor, MI). Frozen erythrocyte lysate was thawed and diluted 1:50 with 50 mM Tris-HCL, pH 7.6, containing 5 mM EDTA and 1 mg/ml

BSA. Glutathione peroxidase catalyzes the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidized glutathione is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP<sup>+</sup>. The decrease in absorbance was followed at 340 nm for 3 min. Enzyme activity was reported in units per milliliter of PCV in erythrocytes and in units per milliliter in plasma. The absorbance was read once per minute for 5 consecutive minutes at 340 nm using a plate reader at room temperature. GSH-px activity was expressed in nM per minute per milliliter of PCV (Bernabucci et al., 2005).

Plasma TBARS was measured in samples collected on a weekly basis using a commercial kit (TBARS Assay kit, Cayman Chemical Company, Ann Arbor, MI). Thiobarbituric acid in the kit reacts with lipid peroxidation in plasma samples and forms Malondialdehyde-TBA adduct under high temperature (100°C) and acidic conditions. The adduct was measured colorimetrically at 530-540nm. TBARS was expressed in Malondialdehyde (MDA) µM concentration. Plasma TAC was also measured in the same samples using a commercial kit (Antioxidant Assay kit, Cayman Chemical Company, Ann Arbor, MI). Plasma samples were diluted 1:20 with assay buffer (5 mM potassium phosphate, pH 7.4, containing 0.9% sodium chloride and 0.1% glucose) before assay. The assay relies on the ability of total antioxidants, including aqueous and lipid-soluble antioxidants, in the samples to inhibit the oxidation of 2,2'-Azino-di-3-ethylbenzthiazoline sulphonate (ABTS) by metmyoglobin. The amount of oxidized ABTS was read at 750 nm. TAC was expressed in antioxidant mM concentration.

### **Immune Function Parameters**

Plasma IgG was measured in samples collected on a weekly basis by commercial ELISA kit (Bovine IgG ELISA Quantitation Kit, Bethyl Laboratories, Bethyl TX). All processes were conducted at room temperature. ELISA assay plate (NUNC-IMMUNE plate, NUNC, Denmark) was coated with 100  $\mu$  l of 1  $\mu$  l/ml capture antibody (sheep anti-bovine IgG) per well. The coated plate was incubated for 60 minutes. After incubation, the capture antibody was dumped and each well was washed with 200  $\mu$  l of Wash Solution. Washing was repeated 3 times. 200  $\mu$  l of Blocking solution was added to each well. After 30 minutes incubation, the solution was dumped and each well was washed 3 times. 100  $\mu$  l of plasma samples, which were diluted 1:200,000, and 100  $\mu$  l of standard IgG, which ranged from 7.8 to 500 ng/ml, were transferred to assigned wells. After 60 minutes incubation, the samples and standards were dumped and each well was washed 5 times. 100  $\mu$  l of detection antibody (1 mg/ml of sheep anti-bovine IgG- Horseradish-peroxidase conjugated), which was diluted 1:12,500, was transferred to each well. After 60 minutes incubation, the detection antibody was dumped and each well was washed 5 times. 100  $\mu$  l of substrate solution (1-Step™ Ultra TMB-ELISA, PIERCE, IL) was transferred to each well. The plate was incubated for 20 minutes. To stop substrate reaction, 100  $\mu$  l of 2 M sulfuric acid was transferred to each well. The wavelength was read at 450 nm and the concentration of IgG (mg/dl) was calculated.

Plasma cortisol was measured in samples collected during the LPS challenge using a commercial radioimmunoassay kit (COAT-A-COUNT Cortisol, SIEMENS, CA). Total counts, nonspecific binding, and calibrators were counted in triplicates while plasma samples were in duplicates. Calibrators included 0, 0.5, 1, 5, 10, 20, and 50  $\mu$  g/dL and plasma cortisols were calculated from the calibrators and expressed in  $\mu$  g/dL. The intra-assay coefficient of variation was 13.7%.

## **Statistical Analysis**

Data for milk yield and DMI were reduced to weekly means prior to analysis. Effects of mineral treatments on weekly variables (milk yield, milk components, DMI, oxidative stress parameters, and IgG) were evaluated using the MIXED procedure of SAS version 9.1 (SAS Institute, Cary, NC) for a randomized complete block design with a 2 x 2 factorial arrangement of treatments. Model terms were block, level, source, week, and two- and three-way interactions of the main effects with week. Data obtained during the last week of the pretreatment period were used as covariates during their respective analysis and retained in the model if  $P > 0.20$ . The random effect was cow nested within the interaction of level and source of trace mineral. For each variable, cow was subjected to four covariance structures (first-order autoregressive, heterogeneous first-order autoregressive, compound symmetry, and heterogeneous compound symmetry) and the structure yielding the smallest Akaike's information criterion was selected. The method of Kenward-Rogers was used for calculation of denominator degrees of freedom.

In addition, statistical analysis was conducted using data from wk 1 only (represents immediate effects of trace mineral supplementation) and wk 6 only (represents one week following LPS challenge) using the MIXED procedure of SAS. Terms in the model included block, level, source, and the interaction of level and source.

Effects of mineral treatments on responses after LPS challenge were evaluated as above using SAS for both area under the curve analysis (calculated using cubic spline interpolation and the trapezoidal rule using the EXPAND procedure of SAS) and repeated measures analysis. Values at time 0 were used as covariates. One cow was removed from the dataset of weekly variables due to hip dislocation, which was

allocated in treatment 2. Significance was declared at  $P < 0.05$  and trends toward a significant difference at  $0.05 < P < 0.15$ . Least squares means are presented throughout.

## **RESULTS AND DISCUSSION**

Analyses of the experimental diets (Table 3-1) indicated that the profiles of macro- and micronutrients were similar during the pretreatment and treatment periods with the exception of sulfur, which was higher during the pretreatment period as designed. Because iron and molybdenum were topdressed during the pretreatment period and not during the treatment period, the smaller difference of sulfur than intended between the pretreatment and treatment periods likely did not affect the results of the present study. Tolerance level for sulfur in ruminants has been reported to be 0.4% of diet (Kandylis, 1984; NRC 1980). Excessive sulfur can interfere with absorption of other elements, especially copper and selenium (NRC, 2001). Iron can also interfere with the other minerals, primarily copper and zinc. 250 ppm of iron has been implicated as a cause of copper depletion in cattle (Bremmer et al., 1987; Phillippo et al., 1987). Molybdenum and sulfur can interact and synergistically inhibit copper absorption (NRC, 2001). As for molybdenum itself, as little as 5 ppm of molybdenum has been demonstrated to cause copper depletion in heifers (Bremmer et al., 1987; Phillippo et al., 1987). Thus, the combination of 0.40% of sulfur, 250 ppm of iron, and 5 ppm of molybdenum used in our study was considered sufficient for practical purposes to lower status. The original target concentrations of Zn, Cu, and Mn in the diet were 48, 11, and 14 ppm, respectively, for the NRC (2001) level and 68, 19, and 33 ppm, respectively, for the commercial level. Based upon actual analysis of the forage and concentrate ingredients fed and dividing the topdressed supplement amounts across the actual DMI for cows assigned to the different treatment groups, actual concentrations for the NRC level averaged 55, 8, and 21 ppm for Zn, Cu, and Mn, respectively, and 75, 16, and 36 ppm for the commercial level.

**Table 3-3.** Oxidative stress parameters measured in blood sampled collected weekly throughout the experiment

Whole treatment period <sup>1</sup>								
Item	NRC		Commercial		SEM	P-value		
	Inorganic	HMTBA-chelate	Inorganic	HMTBA-chelate		Level	Source	Level x source
Number per treatment	12	11	12	12	--	---	--	--
SOD, u/ml of PCV	777	760	785	775	23	0.57	0.52	0.86
GPX, nmol/min/ml of PCV	29,030	28,101	27,921	27,576	833	0.30	0.41	0.71
TBARS, MDA $\mu$ M	5.04	4.58	5.04	4.92	0.32	0.57	0.35	0.58
TAC, mM	1.53	1.58	1.63	1.65	0.09	0.29	0.67	0.85

Week 1								
Item	NRC		Commercial		SEM	P-value		
	Inorganic	HMTBA-chelate	Inorganic	HMTBA-chelate		Level	Source	Level x source
Number per treatment	12	11	12	12	--	---	--	--
SOD, u/ml of PCV	774	761	786	754	31	0.93	0.46	0.74
GPX, nmol/min/ml of PCV	29,215	28,650	27,217	28,179	898	0.15	0.81	0.36
TBARS, MDA $\mu$ M	5.52	4.56	4.99	4.22	0.60	0.45	0.13	0.87
TAC, mM	1.25	1.59	1.58	1.55	0.12	0.20	0.20	0.15

Week 6								
Item	NRC		Commercial		SEM	P-value		
	Inorganic	HMTBA-chelate	Inorganic	HMTBA-chelate		Level	Source	Level x source
Number per treatment	12	11	12	12	--	---	--	--
SOD, u/ml of PCV	729	702	737	740	24	0.29	0.59	0.51
GPX, nmol/min/ml of PCV	29,424	28,873	27,436	28,862	1,076	0.34	0.68	0.35
TBARS, MDA $\mu$ M	4.36	3.46	3.96	3.75	0.29	0.83	0.05	0.21
TAC, mM	1.34	1.35	1.45	1.50	0.17	0.09	0.67	0.77

1: Two- and three-way interaction of main effects within week were not significant.

SOD = super oxide dismutase; GPX = glutathione peroxidase; PCV = packed cell volume; TBARS = thiobarbituric acid reactive substances; MDA = malondialdehyde; and TAC = total antioxidant capacity

The effects of trace mineral level and source on oxidative stress parameters measured in erythrocyte lysate or plasma on a weekly basis are presented in Table 3-3. Because the main effects within week were not significant, overall results were presented as overall main effects. Effects of treatment on activities of GSH-Px and SOD in erythrocyte lysate and concentrations of TBARS and TAC in plasma were not significant when evaluated across the entire experimental period. Although activity of SOD was not affected by treatment when evaluated during only week 1 or 6 after assignment to treatment, a trend ( $P = 0.15$ ) existed for decreased GSH-Px activity in erythrocyte lysate for cows fed the commercial level of trace minerals during week 1 of treatment. Differences among treatments in GSH-Px activity evaluated during week 6 of treatment only were not significant.

As described by Sies (1991), SOD and GSH-Px are enzymatic antioxidants and both of them are mineral-dependent enzymes. Therefore, the activities can be affected by mineral status. Strain (1994) indicated that copper deficiency resulted in lower hepatic mRNA concentrations of GSH-Px, which shows that minerals that are not directly related to the antioxidant enzyme component can affect the activity as GSH-Px is primarily considered to be a selenium-dependent enzyme. However, a trend for increased activity of GSH-Px at week 1 in our study suggested higher activity at NRC level compared to the higher commercial levels. Reasons for this trend are not known. Effects of dietary mineral level on antioxidant enzymes have been reported in several studies. Cerone et al. (2000) determined that feeding 30 ppm of molybdenum and 225 ppm of sulfate to induce copper deficiency decreased SOD levels in leukocytes and macrophages, along with decreased serum copper level. Likewise, calves had higher activity of erythrocyte SOD when fed copper compared with molybdenum (Gengelbach and Spears, 1998). Another study suggested that plasma copper level has a negative relationship with DNA damage in cattle, which also indicates that high



oxidative stress caused by hypocupremic cattle can impair genetic information (Picco et al., 2004). In our study, however, effects of trace mineral level and source on SOD activity were not significant, despite previous reports that the organic source utilized has higher bioavailability than inorganic sources (Thering et al., 2007). Bengoumi et al. (1998) reported that no correlation was detected between plasma copper concentration and erythrocyte SOD activity, while there were positive relationships between plasma zinc concentration and activity when feeding 1289 mg/d of zinc and 310 mg/d of copper for three months, which are twice the daily calculated requirements for the cows. One explanation for the lack of correlation between plasma copper and erythrocyte SOD is that three-month supplementation may be not enough to increase SOD activity (Bengoumi et al., 1998). The same possibility may exist in the present study, as minerals were only supplemented for six weeks.

Effects of treatment on Total Antioxidant Capacity (TAC) were not significant when evaluated across the entire treatment period (Table 3-3). However, a trend ( $P = 0.15$ ) existed for an interaction of trace mineral level and source during week 1 of treatment such that cows fed inorganic trace mineral sources at NRC (2001) recommended levels had lower TAC than cows fed organic trace mineral sources at NRC levels or cows fed either source at commercial levels. To our knowledge, this is the first study to directly evaluate the NRC recommendations for trace mineral supplementation; these results suggest that feeding more bioavailable sources of trace minerals may enhance antioxidant capacity of plasma when trace minerals are supplemented at NRC levels.

In addition to responses during week 1, plasma TAC tended ( $P = 0.09$ ) to be higher during week 6 of treatment for cows supplemented with the commercial levels of trace minerals, regardless of source. Because LPS challenge was conducted at week 5, cows at week 6 might have had relatively high demand for antioxidants,

which might have led to increases in antioxidants than SOD and GSH-Px. TAC represents total endogenous antioxidant capacity including enzymatic antioxidants, non-enzymatic proteinaceous antioxidants, and non-enzymatic small molecular antioxidants. To our knowledge, there are not studies to evaluate the relationship between TAC and minerals in ruminants. In human, TAC increased when selenium and vitamin E were supplemented for pulmonary tuberculosis patients (Seyedrezazadeh et al., 2008). Zinc deficiency can reduce catalase activity (Jing et al., 2007), cause low plasma vitamin A (Underwood and Suttle, 1999), and decrease utilizing vitamin E (Kim et al., 1998). Copper deficiency can also depress catalase activity (Taylor et al., 1988), cause iron accumulation (Taylor et al., 1988), and decrease ceruloplasmin activity (Laven et al., 2007). Therefore, high levels of antioxidant-related minerals could contribute to increasing TAC.

Trace mineral level and source did not affect plasma TBARS as assessed over the 6-week supplementation period (Table 3-3); however, cows supplemented with the organic forms of trace minerals tended ( $P = 0.13$ ) to have lower TBARS during week 1 of treatment and had lower ( $P = 0.05$ ) TBARS during week 6 of treatment. Given that TBARS have been considered to represent a composite index of lipid oxidative end products including malondialdehyde (MDA) and to be a good general indicator of oxidative stress (Bernabucci et al., 2005), these results suggest that cows fed the organic trace mineral supplement, regardless of level, had decreased oxidative stress than cows fed the inorganic supplements. Dairy cows with mastitis and calves with diarrhea showed increased erythrocyte MDA level, along with increased blood copper concentration and decreased blood zinc concentration (Ranjan et al., 2005; Ranjan et al., 2006). In addition, dairy cows exposed to moderate heat stress conditions (Bernabucci et al., 2002) and after calving (Bernabucci et al., 2005) had higher circulating concentrations of TBARS. Decreased plasma TBARS at week 6 might be

due to better bioavailability of the organic minerals, which might have contributed to decreasing oxidative stresses after LPS challenge at week 5 as mentioned above. However, there was difference between TBARS and TAC responses since the former responded only to mineral source, while the latter responded to mineral level. The reason for the difference is unknown. Because TBARS is an indicator mainly for lipid peroxidation and TAC represents whole antioxidants activity, organic minerals and methionine contained in organic mineral sources in the present study may be used only for lipid antioxidants, while every antioxidant responding to level of minerals may reflect TAC level. In addition, the relationship of MDA/TBARS and antioxidants are not constant. In human patients with childhood asthma, plasma MDA level was higher and TAC level was lower than healthy subjects and there was negative relationship between MDA and TAC (Kocyigit et al., 2004). During the peripartum period, dairy cows showed increased erythrocyte SOD, decreased erythrocyte GSH-px, and no changes in plasma TBARS before calving, while decreased SOD, increased GSH-px, and increased TBARS after calving (Bernabucci et al., 2005). Further study is needed to fully determine the relationships of each oxidative stress indicator and each antioxidant with oxidative stress in ruminants.

Results for plasma IgG concentrations over both the entire experimental period and by individual weeks of the treatment period are described in Table 3-4. Cows fed the organic sources of trace minerals had higher plasma concentrations of IgG during the entire period, which was reflected by responses or trends toward responses during many of the individual weeks. Because treatment differences were apparent before J5-Bacterin vaccination, data were also presented for weeks 3 to 6 expressed as a difference from week 2 (Table 3-4, lower panel). When expressed in this manner, differences in plasma IgG among treatments were not significant. Reasons for the increased circulating IgG levels in cows fed the organic trace mineral sources

**Table 3-4. IgG level (mg/dl) from weekly blood samples**

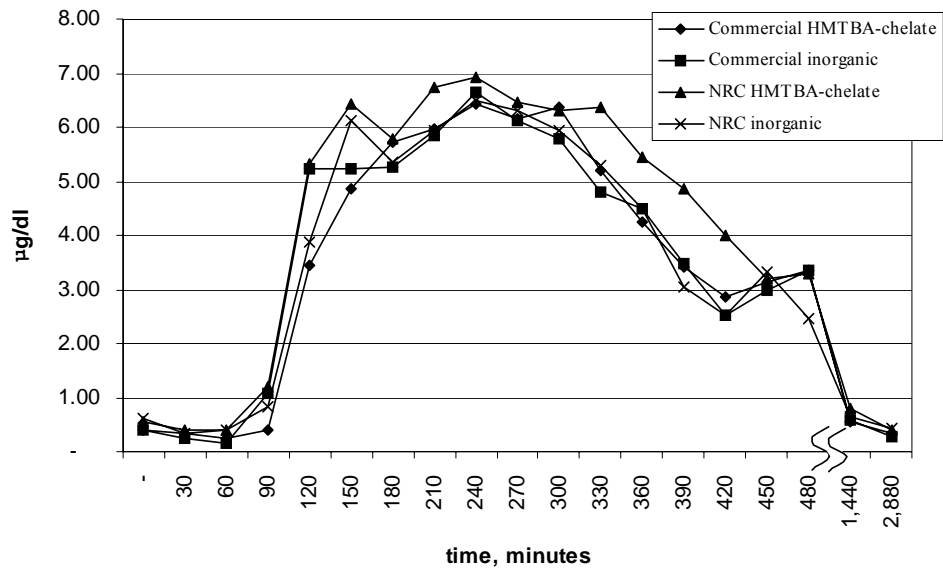
	NRC						Commercial						P-value			
	Inorganic			HMTBA-chelated			Inorganic			HMTBA-chelated			SEM	Level	Source	Level x source
	12	11	12	12	11	12	12	11	12	12	11	12				
Number per treatment	2,378	2,609	2,321	2,741	127	0.76	0.01	0.43	---	---	---	---	---	---	---	---
Whole treatment period	2,184	2,322	2,187	2,541	169	0.49	0.13	0.50	---	---	---	---	---	---	---	---
Week 1	2,185	2,502	2,259	2,596	169	0.61	0.05	0.95	---	---	---	---	---	---	---	---
Week 2	2,395	2,789	2,629	2,971	183	0.24	0.04	0.88	---	---	---	---	---	---	---	---
Week 3	2,688	2,805	2,477	2,760	164	0.42	0.21	0.59	---	---	---	---	---	---	---	---
Week 4	2,442	2,646	2,314	2,793	196	0.96	0.07	0.46	---	---	---	---	---	---	---	---
Week 5	2,372	2,588	2,062	2,784	192	0.76	0.01	0.17	---	---	---	---	---	---	---	---
Week 6																

	NRC						Commercial						P-value			
	Inorganic			HMTBA-chelated			Inorganic			HMTBA-chelated			SEM	Level	Source	Level x source
	12	11	12	12	11	12	12	11	12	12	11	12				
Number per treatment	291	221	104	227	158	0.55	0.86	0.52	---	---	---	---	---	---	---	---
Week 3 to 6	210	289	369	375	208	0.54	0.83	0.85	---	---	---	---	---	---	---	---
Week 3	510	328	209	160	164	0.14	0.47	0.67	---	---	---	---	---	---	---	---
Week 4	265	173	44	192	208	0.61	0.89	0.55	---	---	---	---	---	---	---	---
Week 5	186	83	-197	189	215	0.50	0.49	0.24	---	---	---	---	---	---	---	---
Week 6																

throughout the treatment period are not known. The production of antibodies for some specific antigens other than J5 antigen might be stimulated by organic mineral treatments. Nemeč et al. (1990) reported that vitamin E supplementation in beef heifers showed a tendency for higher titers against *Salmonella typhimurium* even prior to vaccination challenge of *Brucella abortus* than no supplementation and selenium supplemented groups. Presumably, vitamin E supplementation enhanced the production of antibody in several cows whose humoral system had been activated by previous exposure to *S. typhimurium* (Nemeč et al., 1990). Although we did not investigate the type of pathogen exposure of the cows prior to enrollment in our study, organic minerals might affect immune system to enhance the production of antibodies to the pathogens in a manner analogous to vitamin E. On the other hand, humoral responses to copper or zinc status in cattle are not consistent (Spears, 2000). Zinc is related to the metabolism of vitamin A and the absorption of vitamin E (Underwood and Suttle, 1999; Hatfield et al., 2002). Deficiencies of vitamin A and E in cattle are associated also with copper deficiency (Sharma et al., 2005). Since vitamin A is related to B cell activation and cytokine production (Shikora and Blackburn, 1997) and vitamin E may enhance humoral immune response as mentioned above (Nemeč et al., 1990), the effect of zinc and copper on production of antibodies may depend on vitamin A and E status. Further studies are needed to investigate whether organic minerals enhance production of antibodies specific to J5 antigen as well as previous exposure and whether the effect of organic minerals on humoral immune system varies with vitamin A and E status.

Patterns of response of variables assessed following LPS challenge at the end of week 5 of treatment are presented in Figures 3-2 through 3-5. As expected, LPS challenge elicited significant increases in plasma cortisol (Figure 3-2), which peaked at 240 minutes postchallenge. Differences among treatments, assessed either by



**Figure 3-2.** Least squares means for plasma cortisol after LPS challenge. Values at time 0 represent an average from each treatment. P-values:level = 0.24; source = 0.31; level x source = 0.21. SEM=0.42.

**Table 3-5.** Area Under the Curve for cortisol and clinical parameters after LPS challenge

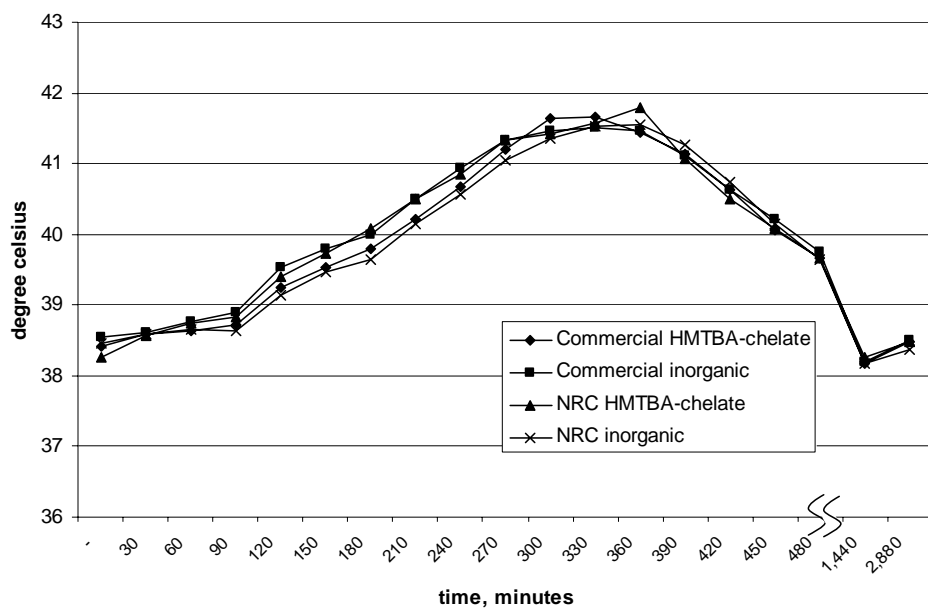
Item	NRC		Commercial		SEM	Level	P-value	
	Inorganic	HMTBA-chelate	Inorganic	HMTBA-chelate			Source	Level x source
Number per treatment	12	11	12	12	---	---	---	---
Cortisol	1,817	2,136	1,817	1,794	172	0.30	0.37	0.30
Body temperature	19,253	19,310	19,324	19,275	46	0.68	0.93	0.23
Heart rate	47,651	49,352	47,349	46,654	972	0.11	0.59	0.20

treatment by time interactions in Figure 3-2 or as calculated area under the curve (Table 3-5) were not significant. Previous research demonstrated that injection of 30 mg of sodium selenite and 1100 IU of vitamin E 3 weeks before calving decreased plasma cortisol level in crossbred dairy cattle (Gupta et al., 2005). This implies that reducing reactive oxygen metabolites is related to reducing cortisol as well (Gupta et al., 2005), although the mechanism is unknown. If reducing oxidative stress decreases cortisol level, better bioavailability or higher dose of trace minerals may lead to lower cortisol. In our study, however, the form and level of minerals did not result in differential responses of plasma cortisol to LPS challenge. Erskine and Bartlett (1993) reported that during *E. coli*-induced mastitis, serum zinc, copper, and iron level decreased to 28, 52, and 35% of pre-treatment level. The response of withholding zinc and iron is non-specific host defense to limit availability of those minerals by pathogenic bacteria (Middleton et al., 2004). Therefore, whether demand for the minerals becomes high or low after inflammatory challenge is not clear. Further study is needed to evaluate the dynamics of circulating mineral concentrations during the inflammatory process. A previous study, which administered 25  $\mu$ g of endotoxin to two contralateral quarters in lactating Holstein cows in midlactation also resulted in similar peak timing (270 minutes after the challenge) of plasma cortisol concentrations (Jackson et al., 1990). Waldron et al. (2006) infused the same amount of LPS into cows at 7 d postcalving and determined that plasma cortisol concentration was increased and remained elevated for the duration of the experimental period (480 minutes) without showing clear peak. The different responses of cortisol among studies may be due to the differences in the levels of inflammatory cytokines. Increased cortisol after the challenge implies that administration of LPS increased the activity of the hypothalamic-pituitary-adrenal (HPA) axis (Wang et al., 2007), and in rats, TNF-alpha stimulates the axis directly through hypothalamus and adrenal, and

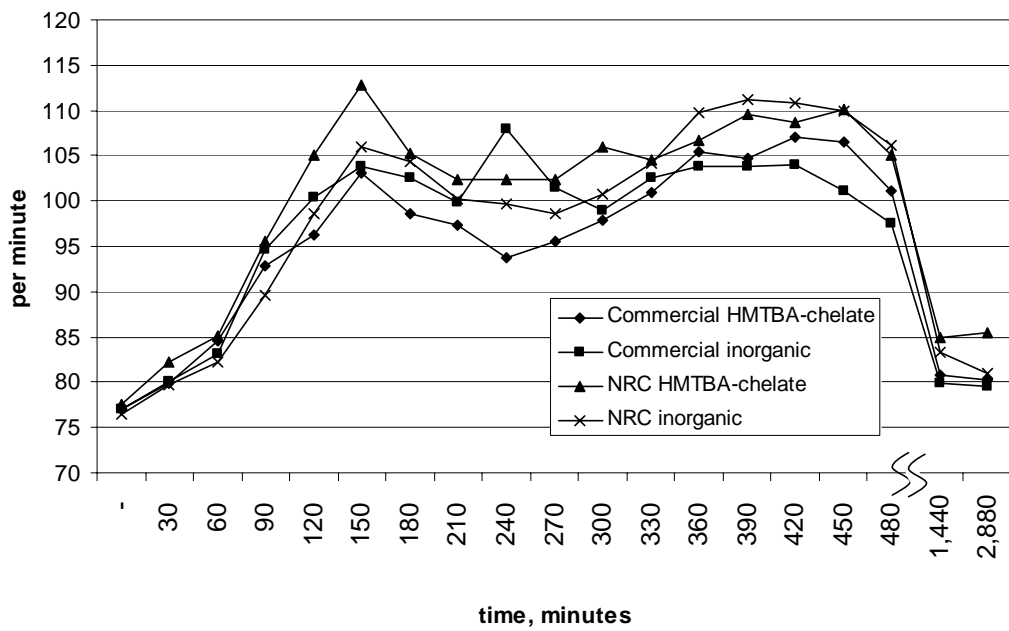


indirectly by stimulating local secretion of IL-1, IL-2, or IL-6 (Bernardini et al., 1990). Subcutaneous injection of TNF-alpha in lactating cows induced an increase in plasma cortisol along with increased haptoglobin and growth hormone (Kushibiki et al., 2003). Therefore, during a period such as right after calving, where inflammatory response is dominant, inflammatory cytokines keep circulating, which may lead to constantly high level of cortisol. On the other hand, intravenous administrations of LPS/endotoxin in cows relatively show sooner and higher peak of plasma cortisol than intramammary administration (Jackson et al., 1990; Waldron et al., 2003). TNF-alpha is released from peripheral blood monocytes and liver Kupffer cells by intravenous treatment (Kushibiki et al., 2003) and from mammary leukocytes by intramammary treatment (Jackson et al., 1990). Intravenous treatment may produce greater cytokine response because the LPS is administered directly to the systemic circulation (Jackson et al., 1990).

Rectal body temperature started increasing at 30 minutes after LPS challenge in all treatments and peaked at 330 to 360 minutes after the challenge (Figure 3-3). Differences among treatments, evaluated either as a treatment by time interaction or as area under the curve (Table 3-5) were not significant. The times of peaks are similar to other studies that conducted intramammary LPS injection, even though the doses of LPS are the same (Waldron et al., 2006) or less (Jackson et al., 1990). In addition, the peaks were reached after plasma cortisol peaks, which is also concordant with other studies (Jackson et al., 1990; Waldron et al., 2006). When LPS was administered intravenously, the temperature peak varied according to doses (Jackson et al., 1990; Jacobsen et al., 2005; Waldron et al., 2006). The reason why intramammary treatment shows relatively stable increasing pattern and intravenous treatment shows different patterns is unknown. It may result from differences in the magnitude and kinetics of



**Figure 3-3.** Least squares means for body temperature after LPS challenge. Values at time 0 represent an average from each treatment. P-values: level = 0.66; source = 0.84; level x source = 0.15. SEM=0.14.

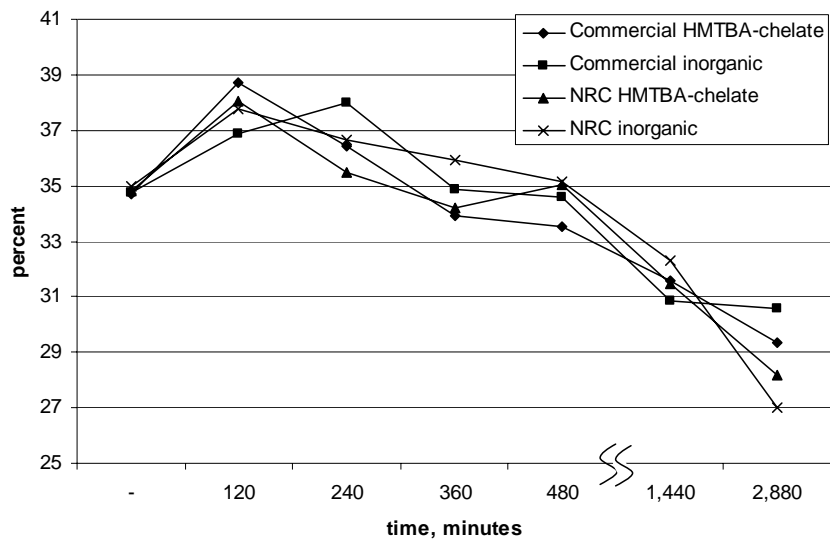


**Figure 3-4.** Least squares means for heart rate after LPS challenge. Values at time 0 represent an average from each treatment. P-values: level = 0.02; source = 0.36; level x source = 0.10. SEM=2.9.

cytokines production or the subsequent absorption from the mammary gland, or differences in the synthesis of different cytokines (Jackson et al., 1990).

Heart rate started to increase at 30 minutes after LPS challenge in all treatments (Figure 3-4) and generally remained increased for 480 minutes. The extent of increased heart rate did not significantly differ among treatments (Table 3-5). Waldron et al. (2006) reported that heart rate of LPS-infused cows showed a similar pattern to rectal temperature and remained moderately elevated even at 44 hours after the challenge. The study used the same dose of LPS as ours. On the other hand, another study showed that intravenous-LPS treatment resulted in that heart rate was highly variable and was not affected by LPS infusion (Waldron et al., 2003). The reasons for the different patterns in intramammary treatment and the highly variable response in the case of intravenous treatment remains unclear.

Hematocrit values increased from prechallenge to 2 hours after LPS challenge and continued to decrease even at 2440 minutes after the challenge (Figure 3-5). Differences among treatments were not significant. The early slight increase is consistent with results from a study conducted in swine, which showed increased hematocrit after intravenous *E. coli* challenge (Jesmok et al., 1992), and might be due to increased exudates from blood vessels by inflammatory response. One of the major hallmarks of the inflammatory response is adherence of peripheral blood leukocytes to the endothelial lining of blood vessels, which is modulated by TNF-alpha (Jesmok et al., 1992). However, an aberrant extension effect of TNF-alpha under some inflammatory condition may cause compromising microvascular endothelial barrier, leading to edema (Jesmok et al., 1992). Edema was observed of LPS challenged quarters in all treated cows in our study. The mechanism of the decreased hematocrit response remains to be elucidated.



**Figure 3-5.** Least squares means for hematocrit after LPS challenge. Values at time 0 represent an average from each treatment. P-values: level = 0.75; source = 0.38; level x source = 0.95. SEM=0.99

**Table 3-6.** Milk yields, DMI, and milk components

Item	NRC				Commercial				P-value	
	Inorganic	HMTBA-delta	Inorganic	HMTBA-delta	Inorganic	HMTBA-delta	SEM	Level	Source	Level x source
Number per treatment	12	11	12	12	12	12	---	--	---	---
DMI, kg/d	24.7	24.9	24.4	25.1	24.4	25.1	0.46	0.94	0.31	0.55
Milk, kg/d	40.9	41.3	41.0	41.5	41.0	41.5	0.61	0.81	0.43	0.96
Fat, %	3.38	3.33	3.23	3.30	3.23	3.30	0.10	0.35	0.92	0.48
Fat, kg/d	1.38	1.34	1.32	1.34	1.32	1.34	0.04	0.39	0.71	0.40
True protein, %	2.93	2.95	2.98	2.98	2.98	2.98	0.03	0.08	0.74	0.68
True protein, kg/d	1.20	1.21	1.22	1.22	1.22	1.22	0.02	0.30	0.61	0.99
SCC, linear score	1.71	1.65	1.59	1.62	1.59	1.62	0.11	0.44	0.87	0.65
MUN, mg/dl	9.83	9.88	10.16	10.13	10.16	10.13	0.35	0.38	0.97	0.92
Lactose, %	4.75	4.75	4.78	4.72	4.78	4.72	0.03	0.89	0.22	0.24
Lactose, kg/d	1.95	1.97	1.96	1.96	1.96	1.96	0.03	0.90	0.75	0.76
Total solids, %	11.99	11.99	11.94	12.00	11.94	12.00	0.10	0.80	0.77	0.77
Total solids, kg/d	4.91	4.92	4.89	4.95	4.89	4.95	0.07	0.94	0.58	0.71

Performance data (DMI, milk yield and milk components; Table 3-6) were analyzed using wk 1 to 4 only because LPS intramammary challenge was conducted at week 5, which was considered to affect production performance. Dry matter intake averaged 24.8 kg/d across the four treatments and differences among treatments were not significant. Similarly, neither level nor source of trace mineral supplementation affected overall milk yield. Milk composition and milk component yield was not affected by trace mineral level and source, except for a trend ( $P = 0.08$ ) for increased milk true protein content for cows fed the commercial level of trace minerals.

Thering et al. (2007) also showed that organic mineral (Mintrex) treatment, which provided 322 mg Zn, 150 mg Cu, 130 mg Mn, 3.78 mg Se, and 20 mg of biotin per day for 4 weeks, did not affect milk yield and DMI compared to Met and Lys complexes of trace minerals (AA-complex treatment). In their study, however, Mintrex significantly increased milk fat, true protein, and total solids (Thering et al., 2007). Since Mintrex contains HMTBA as a potential methionine source as the chelate, the increment of milk protein and fat in the previous study might be partly due to the methionine effect. Application of rumen-protected methionine has shown increased milk protein and fat with increased methionine supply (NRC, 2001). Differences in responses between the current study and that of Thering et al. (2007) are not certain, but suggest an interaction of basal diet with milk component response to chelated trace mineral sources. Recent studies showed that AA-complex treatment generally improved lactational performance. Nocek et al. (2006) reported that mixed treatment with inorganic and the complex mineral sources at 100% NRC level produced more milk, more energy-corrected milk, and more fat than inorganic source at 100% NRC level. In the study, the complex mineral treatment at 75% NRC level had the same performance as only inorganic mineral treatment at 100% NRC level. In addition, Siciliano-Jones et al. (2008) reported that 360 mg Zn, 200 mg Mn, 125 mg Cu, and 12

**Table 3-7. Body condition scores and body weight**

Item	NRC		Commercial		SEM	Level	P-value	
	Inorganic	HMT/BA- <sub>cholate</sub>	Inorganic	HMT/BA- <sub>cholate</sub>			Source	Level x source
Number per treatment	12	11	12	12	---	---	---	---
BCS	2.7	2.6	2.7	2.7	0.0	0.67	0.41	0.53
BW	638	628	635	637	6	0.57	0.45	0.35



mg Co as AA-complex source produced more milk, more energy-corrected milk, more fat, and more protein and solids than sulfate form of minerals. Treatments of those studies were fed beginning prepartum and continuing through the lactation period. Therefore, more demanding conditions around the peripartum period may cause more performance differences between mineral sources than mid lactation period, where our study conducted. BCS and BW were not affected by treatments for whole treatment period (Table 3-7). To our knowledge, there are few studies which show that trace minerals change body condition and weight in lactating dairy cows. In beef cattle, the effect of trace minerals is inconsistent. Nunnery et al. (2007) reported that supplementation of inorganic or organic zinc did not affect growth and Spears (2002) determined that supplementation of both organic- and inorganic-zinc sources increased average daily gain (ADG) during growing period for steers, while only organic zinc supplementation resulted in greater ADG during finishing period.

## **CONCLUSIONS AND IMPLICATIONS**

Feeding levels of Zn, Cu, and Mn either at commercial levels or from more bioavailable sources resulted in modest improvements in some parameters of oxidative status in this experiment. Although activities of important enzymes (GSH-Px and SOD) were not responsive to level or source of trace minerals, decreases in plasma TBARS for cows fed organic sources of trace minerals and increased TAC activity of plasma at week 1 posttreatment for cows fed either source of trace minerals at commercial levels or the organic source at NRC levels compared to the inorganic sources at NRC levels. Although cows had a vigorous response to LPS administration as evidenced by changes in clinical signs and plasma cortisol concentrations, trace mineral feeding level and source did not affect responses of these variables to LPS challenge.

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