NUTRITIONAL IMPLICATIONS FOR OXIDATIVE STATUS, IMMUNE FUNCTION, AND ENERGY METABOLISM IN TRANSITION DAIRY COWS AND RELATIONSHIPS WITH POSTPARTUM PERFORMANCE AND ENDOMETRITIS

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Takashi Yasui

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NUTRITIONAL IMPLICATIONS FOR OXIDATIVE STATUS, IMMUNE FUNCTION, AND ENERGY METABOLISM IN TRANSITION DAIRY COWS AND RELATIONSHIPS WITH POSTPARTUM PERFORMANCE AND ENDOMETRITIS

Takashi Yasui, Ph. D.

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Factors associated with a failure to adapt metabolic and physiological regulation during the transition period include oxidative stress, metabolic disorders, and imbalanced energy status. Those factors are not only correlated to each other, but also related to low performance and immune suppression, which leads to development of periparturient-related diseases such as cytological endometritis (CE). The objectives of this work were to investigate the: 1) effect of source of trace minerals on production, oxidative metabolism, and the incidence of CE; 2) effect of chromium propionate (Cr-Pro) supplementation on production, metabolism, and the incidence of CE; 3) effect of supplemental monensin and varying postpartum fermentable starch level on the incidence of CE and related immune functions; and 4) association of CE with energy metabolism and inflammation. The first experiment found that, compared to inorganic or organic sources, supplemental hydroxy trace minerals (HTM), a recently available source of trace minerals, improved aspects of milk production, modulated plasma indices of oxidative balance, and reduced plasma haptoglobin levels immediately after parturition, suggesting that HTM are more bioavailable and can

affect the body system related to production, oxidative metabolism, and inflammatory responses. In experiment two, feeding Cr-Pro resulted in improved prepartum dry matter intake in addition to reduced prepartum plasma non-esterified fatty acid (NEFA), and decreased incidence of CE along with increased uterine neutrophil influx immediately after parturition. This implies that Cr-Pro can improve periparturient energy metabolism and uterine immunity. The third experiment revealed that innate immune responses were improved during the early postpartum period in cows fed high starch diets for 3 weeks after parturition, and during early lactation in cows fed monensin. Finally, analyses conducted indicated that negative energy balance during the first 3 weeks after parturition was a critical factor to develop CE because cows with CE had higher area under the curve of plasma NEFA and beta-hydroxybutyric acid, and lower calculated energy balance during that period compared to cows without CE. The findings obtained from this work give practical approaches to optimize overall performance in transition dairy cows and provide partial mechanisms for how oxidative status, metabolism, and energy balance interrelate.

BIOGRAPHICAL SKETCH

Takashi Yasui was born in Tokyo, Japan. Takashi graduated from Kaijo High school (Tokyo) 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 (Tokyo), 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 a Master of Science program in Animal Science with a financial support from Bussan Biotech starting in 2006 to learn dairy nutrition and immunology. He earned the M.S. degree in 2008. Takashi and Bussan Biotech agreed that accomplishment of further degree would bring greater benefit to both the company and dairy industry in Japan. In 2010, Takashi attended Cornell University for Doctorate program in Animal Science with further financial support from Bussan Biotech. After completing his Ph.D, he will work for Bussan Biotech to make them a science-technology-based leading company in livestock industry in Japan. His life goal is to establish a new academic conference in Japan, which is pragmatic and will connect industry, academia, and government to deal with dairy and animal science, practical approaches on farm, and food safety.

To Mayuka, and all friends and benefactor

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LIST OF ABBREVIATIONS

ADF	Acid detergent fiber
APP	Acute phase proteins
BCS	Body condition score
BHBA	Beta-hydroxybutyric acid
BW	Body weight
Ca	Calcium
CE	Cytological endometritis
ConA	Concanavalin A
Cp	Ceruloplasmin
CR	Calorie restriction
Cr	Chromium
Cu	Copper
CuZn-SOD	Super oxide dismutase with Cu and Zn
DIM	Days in milk
DMI	Dry matter intake
EB	Energy balance
ECM	Energy corrected milk
FCM	Fat corrected milk
GH	Growth hormone
Gln	Glutamine
GSH-Px	Glutathione-peroxidase

Hp	Haptoglobin
HS	High starch
HTM	Hydroxy trace minerals
IBRV	Infectious bovine rhinotracheitis virus
IDA	Iron deficiency anemia
IFN	Interferon
IGF-1	Insulin-like growth factor-1
IL	Interleukin
ITM	Inorganic sources based upon sulfates of Zn, Cu, and Mn
ITM/OTM	A blend (75:25) of sulfates and chelated sources of Zn, Cu, and Mn
LS	Low starch
LPS	Lipopolysaccharide
MDA	Malondialdehyde
ME 305 milk	Mature equivalent 305 days milk
MFI	Mean fluorescence intensity
Mg	Magnesium
MHC II	Major histocompatibility complex type II
Mn	Manganese
Mn-SOD	Super oxide dismutase with Mn
NDF	Neutral detergent fiber
NDFD	Neutral detergent fiber digestibility
NEFA	Non-esterified fatty acids

NE_L	Net energy for lactation
NF-kB	Nuclear transcription factor kb
PBMC	Peripheral blood mononuclear cells
PCM	Protein-calorie malnutrition
PHA	Phytohaemagglutinin
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear neutrophils
PWM	Poke weed mitogen
ROS	Reactive oxygen species
SCC	Somatic cell count
Se	Selenium
SOD	Super oxide dismutase
SRBC	Sheep red blood cells
TAC	Total antioxidant capacity
TBARS	Thiobarbituric acid-reactive substances
Th1	T helper 1 cell
Th2	T helper 2 cell
TMR	Total mixed ration
TNF-α	Tumor necrosis factor-α
Zn	Zinc

CHAPTER ONE: INTRODUCTION

During the transition period, dairy cows adapt to tremendously increased nutritional demands to support milk production through exquisite homeorhetic control mechanisms (Bauman and Currie, 1980; Bell, 1995). Milk production does not increase gradually; rather, the change in milk yield per day is greatest right after parturition (Ingvartsen et al., 2003). At the same time, dry matter intake (DMI) lags the demands of milk yield; thus, cows enter a period of negative energy balance during early lactation (Bell, 1995). Furthermore, transition dairy cows undergo a period of reduced immunological capacity during the periparturient period (Goff and Horst, 1997; Overton and Waldron, 2004) and, therefore, the time of greatest incidence of infectious and metabolic diseases is overlapped with the time of highest acceleration of milk production (Ingvartsen et al., 2003).

Although the exact mechanism of immune suppression is not clear, there are several factors involved. Oxidative stress can be a significant underlying factor to dysfunctional host immune and inflammatory responses that increase the susceptibility of dairy cattle to a variety of health disorders during the transition period because the oxygen metabolism is accelerated, and the production of reactive oxygen species is increased due to substantial metabolic and physiological adaptations during the transition period (Sordillo and Aitken, 2009). Because trace minerals are involved in the antioxidant defense system, a deficiency in any of them may depress immunity in transition cows (Spears and Weiss, 2008). Also, some evidence implies that oxidative stress is associated with metabolic disorders during the transition period (Bernabucci et al., 2005; Castillo et al., 2005). Furthermore, trace minerals are components of

metabolism for macro nutrients, micro nutrients, and energy (Underwood and Suttle, 1999; NRC, 2001). Therefore, it is also possible that supplementation of these minerals can enhance immune function and improve performance in transition dairy cows.

Chromium is a unique mineral. It is a trace element that functions primarily in glucose and lipid metabolism via its effects on insulin function (Anderson et al., 1988) and has shown positive effects on productive performance in dairy cows (NRC, 2001); however, it is also known to stimulate or modify immune functions in dairy cows such as humoral (Burton et al., 1993; Faldyna et al., 2003) and cell-mediated (Burton et al., 1993; Chang et al., 1996) immune responses. Although innate immune function has not been shown to be affected by chromium in dairy cows (Chang et al., 1996; Faldyna et al., 2003), feeding chromium may contribute to mitigating overall immune depression during the peripartial period by enhancing insulin reactions leading to improved energy metabolism.

Because induced negative energy balance (EB) during the lactation period has minimal impact on immune function (Perkins et al., 2001; Moyes et al., 2009), negative EB per se may not be the predominant factor; however, there is evidence that supports a relationship between negative EB and immune suppression during the transition period. Elevated concentrations of beta-hydroxybutyric acid (BHBA) and non-esterified fatty acids (NEFA), indicators of negative EB, are associated with impaired neutrophil function in dairy cows (Hoeben et al., 1997; Hammon et al., 2006). Blood BHBA level is associated positively with severity (Kremer et al., 1993; Nyman et al., 2008) and incidence (Janosi et al., 2003) of mastitis, suggesting that negative EB

is associated with periparturient immunosuppression, along with the possibility that approaches to alleviate negative EB can modulate immunosuppression. Those approaches include strategies to improve energy intake by cows during the periparturient period and also employing feed additives, such as monensin, that modify ruminal fermentation to increase the overall energetic efficiency of fermentation and increase gluconeogenesis.

Like other infectious diseases, development of uterine diseases is dependent upon the balance between host immunity and the pathogenicity of the bacteria (Sheldon et al., 2009); therefore, minimizing immune suppression may prevent uterine disease. Among these diseases, cytological endometritis (CE) has received significant attention recently because it can be only diagnosed by endometrial cytology, is prevalent in herds, and is linked to significantly decreased reproductive performance in dairy cows (Gilbert et al., 2005; Cheong et al., 2011). Although negative EB status is known to be associated with the incidence of CE (Hammon et al., 2006; Galvão et al., 2010), few studies have been conducted to find its associations with oxidative stress, supplemental chromium, supplemental monensin, and varying postpartum starch level, which may identify practical approaches to reduce the incidence of CE.

The purpose of the review of literature to follow is to highlight the relationships between immune function, oxidative stress, and the potential nutritional influences thereof. Specific attention will be dedicated to describing the state of knowledge relative to these systems and associated nutritional and metabolic interactions in ruminants with particular focus on transition dairy cows. Finally, I will review our knowledge of the mechanisms and implications of uterine inflammation

and immune function in dairy cows to connect uterine disease with aspects of metabolism, immune function, and nutrition.

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CHAPTER TWO: REVIEW OF LITERATURE

OVERVIEW OF IMMUNE FUNCTION AND OXIDATIVE STRESS

Cells that use oxygen and consequently produce reactive oxygen species (ROS) have complex antioxidant defense systems to neutralize ROS and protect themselves against oxidative stress damage (De la Fuente, 2002). Increased oxidative stress, which is caused by imbalance between ROS and antioxidants, has been implicated in the etiology of several human diseases (Devasagayam et al, 2004). In addition, ROS play a role in biologically important processes such as the generation of ATP in mitochondria, detoxification of xenobiotics, apoptosis of effete or defective cells, generation of prostaglandins and leukotrienes, and killing of microorganisms and cancer cells (Devasagayam et al, 2004). Therefore, controlling oxidative metabolism is one of keys to maintainance not only of whole defense system, but also of immune function.

Reactive oxygen species are generated as by-products of cellular metabolism, primarily in mitochondria (Thannickal and Fanburg, 2000). However, ROS are not considered simply as "toxic." They can play an important role in mediating specific cell responses and expression of genes (Aw, 1999). Reactive oxygen species have been implicated in the activation of a variety of kinases (such as protein kinase C and receptor tyrosine kinases) and transcription factors (such as nuclear transcription factor kB (NF-kB). As some enzyme systems evolved to produce ROS for biological signaling, biosynthesis reactions, chemical defense, and detoxification functions, Jones (2006) proposed that the definition of oxidative stress might be changed from "a

disturbance in the prooxidant-antioxidant balance in favor of the former" to "a disruption of redox signaling and control". Regardless of the definition, abnormal accumulation of ROS can be detrimental to physiological systems.

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 (De la Fuente, 2002). In addition, 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 (Knight, 2000). Thus, lipid peroxidation in cell membranes caused by ROS could result in altered immune system functionality because membrane integrity is important for normal membrane metabolic activity, as well as antigen recognition, secretion of cytokines and antibodies, and lymphocyte proliferation (Knight, 2000). Therefore, increased concentrations of ROS could affect the immune system more so than other physiological systems.

At the same time, 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 several-fold, are used to kill microbes ingested in vacuoles called phagosomes (Kindt et al., 2006). Furthermore, 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., 2007). Most cell types have been shown to produce a small oxidative burst that generates low concentrations of ROS

following stimulation by cytokines, growth factors, and hormones (Thannickal and Fanburg, 2000). 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 is a contributor to oxidant load.

It is not clear how specific immune responses are affected by the oxidizing environment caused by reduced antioxidants and/or increased oxidants. Cell membrane-dependent functions are affected by membrane fluidity, which is determined by membrane lipid acyl chain profile (Knight, 2000). Because ROS are known to modify the acyl chain profile, it has been proposed that excess ROS affect integral membrane function, which leads to down-regulation of membrane-bound protein kinase C and membrane NADPH oxidase (Knight, 2000). Another 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 the 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). Although depressed immunocompetence can be reversed by antioxidants in some situations (Knight, 2000), further research is needed to elucidate specific effects of oxidative stress on immune function.

NUTRITIONAL INFLUENCES ON IMMUNE FUNCTION AND OXIDATIVE STRESS

Nutrients can affect the immune system as substrates for the synthesis of macromolecules or through overall supply of energy (Klasing and Leshchinsky, 2000). However, the interactions of nutrition and the immune system are complex. 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 immune antioxidants; and 5) nutritional immunity.

In the first mechanism, nutrients influence regulation of the immune response by modifying intercellular or intracellular communication pathways. As one of the best examples, polyunsaturated fatty acids modulate the immune system through modifying cellular communication, membrane fluidity, and second messenger signaling, which results in changes in a spectrum of diseases and the incidence of diseases (Klasing et al., 2000). As mentioned above, although ROS are involved in intracellular signaling through the activation of various kinases and transcription factors, some nutrients are known to influence the signaling process. Vitamin E blocks NF-kB activation, which is induced by ROS through tumor necrosis factor-α (TNF-α) (Suzuki and Packer, 1993). Zinc (Zn) also inhibits ROS induced NF-kB activation

through super oxide dismutase (SOD) or Zn-finger protein, A-20 (Prasad, 2009). Fat-soluble vitamins such as vitamin A, beta-carotene, and vitamin D also have regulatory actions on leukocytes (Klasing and Leshchinsky, 2000).

The second mechanism is that dietary factors influence the levels of many hormones that have regulatory influences on the immune system. The endocrine, nervous, and immune systems share peptide hormones and cytokines as a common chemical language to communicate with each other (Weigent and Blalock, 1995). Changes in the profile of endocrine hormones are readily induced by dietary factors, including the amount of food consumed, meal pattern, and ratio of protein to energy in the diet (Klasing and Leshchinsky, 2000). Therefore, dietary factors could affect the immune system indirectly. For example, insulin-like growth factor (IGF)-1 is known to stimulate interleukin (IL)-2 production by human peripheral T cells (Kooijman et al., 1996) and increase lymphocyte number and activity (Clark et al., 1993); therefore, increased IGF-1 could cause major changes in lymphoid tissue that are of potential benefit to immune function (Clark et al., 1993). Alternatively, decreased IGF-1, which can be caused by food deprivation, leads to depressed IL-2 production (Savendahl and Underwood, 1997). Nutritional factors also could affect the immune system through modulating neuropeptides. As an example, neuropeptides released from the enteric nervous system in response to gastrointestinal tract feeding are known to stimulate the immune system (Genton and Kudsk, 2003). A study conducted in mice showed that parenteral nutrition with lack of enteral stimulation decreased total T and B cells within gut associated lymph tissue and intestinal lamina propria; in addition, reduced IL-4 and IL-10 mRNA expression in isolated lymphocytes in intestinal lamina propria,

which resulted in decreased intestinal and respiratory tract IgA levels, and loss of established IgA-mediated antiviral and antibacterial immunity (Genton and Kudsk, 2003).

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, milk production or egg production in agricultural animals. In addition, because the immune system seems to have the higher priority for nutrients, it may be difficult to accurately quantify the nutrient requirements of the immune system. Lymphocytes have a glucose transporter similar to that of neurons and erythrocytes (Bushart, 1993), which is constitutively expressed and is not stimulated by insulin or IGF-1, permitting the use of glucose even during periods of starvation (Klasing and Leshchinsky, 2000). Moreover, during inflammation, the nutrient requirements for both immune cells and acute-phase response proteins increase, which indicates that the demand or type of nutrients required for a fully functioning immune system varies according to various challenges and the stage of life. On the other hand, it is clear that the deficiencies of some nutrients impair immune function. There are a number of common aspects when nutritional deficits impact the immune system because immunological changes are similar in humans and other species suffering from protein-calorie malnutrition (PCM) or Zn-deficiency, which have been extensively studied as nutritional-immunological models (Fraker, 2000). Also, glucocorticoids are known to be chronically elevated in both humans and mice with PCM (Fraker, 2000),

which would suggest interplay between nutritional status and endocrine regulation of immune function. Thus, various pathways should be considered when evaluating how the availability of nutritional substrates regulates the immune system.

The fourth mechanism suggests that some nutrients limit pathological responses following immune system activation. Pro-inflammatory cytokines, including IL-1, IL-6, IL-8, and TNF-α, released during an immune response can inflict tissue damage through hemodynamic change, direct cytotoxicity, and increased release of free radicals and other oxidative molecules (Klasing and Leshchinsky, 2000). Some nutritional factors, such as n-3 or n-6 polyunsaturated fatty acids, 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). As an example, the infiltration of inflammatory cells into the lung, which occurs in malnourished rats in response to cytokines, was prevented by the addition of sulfur-containing amino acids to the diet (Grimble, 1994). Conversely, deficiencies of nutrients could result in severe tissue damage by an inflammatory response. As an example, Sword et al. (1991) demonstrated that deficiencies of vitamin E or selenium (Se) led to three-fold increases in lipid oxidation of cell membranes after intraperitoneal lipopolysaccharide (LPS) challenge in rats. On the other hand, the deficiency of nutrients may not always contribute to tissue damage following immune response. For example, inflammatory symptoms in rheumatoid arthritis are exacerbated by intravenous injection of iron dextran (Grimble, 1994).

Finally, in the fifth process involves physiology of the host that modulates nutrient availability can decrease the rate of replication of bacteria and parasites.

During the acute phase of an immune response, a variety of iron binding proteins are produced by liver and by immune cells in an effort to remove iron from the body fluid because iron is required for pathogens to grow (Klasing and Leshchinsky, 2000). An example of iron binding proteins is lactoferrin, which is released by circulating polymorphonuclear neutrophils (PMN) at sites of infection (Chu et al., 2010). The extremely low iron concentrations that are created by the presence of lactoferrin prevent bacteria from aggregating with each other and forming biofilms, thereby keeping bacteria in a planktonic state and rendering them more susceptible to the immune system effector functions (Chu et al., 2010).

Energy status

It has been shown that calorie restriction (CR) influences immune function. T cell proliferation induced by mitogens increased with calorie restriction in both adult and old mice (Weindruch et al., 1986). Calorie restriction led to the preservation of IL-2 production in spleen cells and IL-2 responsiveness in the thymocyte in autoimmune prone mice (Jung et al., 1982). A study using oligonucleotide microarrays showed that long-term CR suppressed the expression of genes encoding inflammatory molecules such as complement components in white adipose tissue in mice (Higami et al., 2006). Calorie restriction also has been shown to ameliorate autoimmune diseases, suppress tumorigenesis, and prolong lifespan; however, the mechanisms whereby calorie restriction modifies immune response and induces the extension of lifespan remain poorly understood (Troyer and Fernandes, 2000; Fernandes, 2008). Hypotheses for the mechanism include attenuation of oxidation damage, insulin signaling, and IGF-1 signaling (Fernandes, 2008). It was reported that CR reduced oxidative stress,

suppressed the production of reactive oxygen intermediates, and slowed aging-related process (Yu et al., 1990). In addition, CR has been shown to reduce plasma insulin concentration in rhesus monkeys and result in markedly lower levels of plasma IGF-1in rats and mice (Masoro, 2005).

On the other hand, obesity as a result of excess calorie intake may also be assocated with immune modulation. Clinical and epidemiological data in humans generally indicate that the incidence and severity of specific types of infectious illnesses are higher in obese individuals compared to lean individuals; furthermore, overweight subjects had poor antibody responses to antigens (Martí et al., 2001). However, obesity may augment some immune functions. In a study involving 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 the activity of natural killer cells was not affected by obesity (Nieman et al., 1999). Moreover, circulating concentrations of pro-inflammatory cytokines such as TNF-α and IL-6 increased with obesity (Karagiannides and Pothoulakis, 2007), and the circulating levels of these cytokines were reduced following weight loss (Ryan and Nicklas, 2004). Leptin, an adipokine that generally reflects adipose tissue mass, might play a key role in linking obesity to immune function (Davis and Stern, 2000; Martí et al., 2001). Leptin increases the production of proinflammatory lymphokines such as IL-2 and IFN (interferon)-γ, and inhibits the secretion of IL-4 from the lymphoid cells through the regulation of the proliferation of cells involved in both innate and acquired immune responses (Martí et al., 2001). The effects are presumably mediated by leptin

receptors, which are present on immune cells (Davis and Stern, 2000; Martí et al., 2001). Thus, overnutrition as well as undernutrition may also lead to immune modulation.

As mentioned above, CR is related to the reduction of oxidative stress. Because of the direct relationship between the rate of oxygen consumption and the mitochondorial rate of ROS generation, CR was thought to reduce ROS by lowering the metabolic rate (Sohal and Weindruch, 1996). In fact, Lopez-Lluch et al. (2006) reported that mitochondria under CR conditions consumed less oxygen, had reduced membrane potential, and generated less ROS than controls. However, studies in which metabolic rate was normalized to body weight suggested that the metabolic rate was not decreased by CR and that mitochondrial activity actually was increased during CR (Qiu et al., 2010). Thus, the molecular mechanism underlying CR-induced reduction of oxidative stress remains elusive (Qiu et al., 2010). Proposed mechanisms for a possible relationship between CR and oxidative stress are: 1) CR can induce proliferation of mitochondria through a peroxisome proliferation-activated receptor coactivator 1-alpha signaling pathway (Lopez-Lluch et al., 2006); 2) CR may upregulate ROS scavenging enzymes and damage repair enzymes (Merry, 2000); or 3) CR effect may result from lowered insulin concentrations and altered insulin signaling (Lambert et al., 2004). Recently, Qiu et al. (2010) showed that SIRT3, a mitochondrial deacetylase, which is upregulated in many tissues of CR mice and is an essential mediator of many aspects of the CR response, enhances the ability of superoxide dismutase 2 (SOD2), a major mitochondrial antioxidant enzyme, to reduce cellular

ROS. Activation of SIRT3 in CR animals may be a key step in this defense program against oxidative stress (Qiu et al., 2010).

The combination of protein and calorie malnutrition known as PCM has also been shown to influence the immune system. Protein-calorie malnutrition results in a vast array of immunodeficiencies, increased susceptibility to a wide variety of organism, and increased the rate of infection (Jolly and Fernandes, 2000). Humoral immunity is affected by PCM as a decline in the production of immunoglobulins, secretory antibodies, and complement (Saker, 2006). Cell-mediated immunity and innate immunity also are affected by PCM. Thymus and lymphoid tissues atrophy, decreased number of T cells, neutropenia, and depressed complement components are observed in PCM (Saker, 2006). Although evidence exists suggesting that PCM impairs delayed-type hypersensitivity reactions (Savy et al., 2009), the underlying mechanisms remain poorly understood (Iyer et al., 2012). A recent study in mice showed that PCM specifically impaired the memory function of CD8 T cell population (Iyer et al., 2012). Memory CD8 T cells in PCM demonstrated markedly reduced homeostatic proliferation despite no measurable decrease in cell-surface expression of the gamma-chain cytokine receptors. The memory CD8 T cells also conferred reduced protective immunity as a result of impaired recall proliferation.

Protein and amino acid status

A deficiency of dietary protein or amino acids has long been known to impair immune function and increase the susceptibility of animals and humans to infectious disease (Li et al., 2007). The absolute amount of protein may be important because protein malnutrition has been found to be a major contributor to morbidity and

mortality due to infectious diseases, largely as a result of impaired immune response (Wu et al., 1999). However, the profile of dietary amino acids could be more critical as specific functions of each amino acid have been recognized. Amino acids, including arginine, glutamine, and cysteine, can have roles in: (1) the activation of T lymphocytes, B lymphocytes, natural killer cells and macrophages; (2) cellular redox state, gene expression and lymphocyte proliferation; and (3) the production of antibodies, cytokines and other cytotoxic substances (Li et al., 2007). Arginine promotes insulin release from pancreatic beta-cells by direct depolarization of the plasma membrane at neutral pH in the presence of glucose (Newsholme, 2005). Insulin regulates the metabolism of glucose and amino acids and influences the availability of these nutrients for leukocytes (Li et al., 2007). This represents yet another example of how nutrient supply and the endocrine system interrelate in the overall regulation of the immune system.

The body's natural defense system against oxidative stress consists of several enzymes and non-enzymatic proteins (Da Costa et al., 2012). The major antioxidant enzymes are SOD, glutathione-peroxidase (GSH-Px), catalase, and glutathione S–transferase. The major antioxidant proteins are glutathione, ferritin, lactoferrin, metallothionein, transferrin, and ceruloplasmin (Da Costa et al., 2012). Amino acids also contribute to the antioxidant system as components of antioxidant compounds such as taurine, melatonin, and creatine, which are generated from cysteine, tryptophan, and arginine, respectively (Li et al., 2007). Although imbalanced intakes of protein and amino acids might influence the status of antioxidant proteins, few studies have assessed how these nutrients affect oxidative stress. It has been reported

that protein or methionine restriction decreases mitochondrial ROS production and oxidative DNA damage, whereas these decreases do not occur during lipid or carbohydrate restriction (Caro et al., 2009). The mechanism how this restriction decreases oxidative stresses remains unclear.

Vitamin status

Vitamins have a role in the immune system that extends to both innate and adaptive immune responses. Although some vitamins, such as vitamins C and E, and members of the B complex can act in a relatively nonspecific manner in the immune system (for example as antioxidants), other vitamins, such as vitamins A and D can influence the immune response in highly specific ways (Mora et al., 2008).

Vitamin A modulates innate immunity. All-*trans* retinoic acid, the active metabolite of vitamin A, enhances the activation of the inducible nitric oxide synthase pathway, a component of innate immunity, through retinoic acid receptor-α in rats *in vivo* (Seguin-Devaux et al., 2002). Also, PMN appear to have impaired function during Vitamin A deficiency (Semba, 1998). In vitamin A-deficient rats, the total number of circulating PMN was normal; however, a higher proportion of PMN were hypersegmented (Semba, 1998). Further, vitamin A deficiency compromised mucosal immunity by altering the integrity of mucosal epithelia in various organs and systems (Semba, 1998). Vitamin A can also affect adaptive immune responses (Mora et al., 2008). Retinoic acid, a vitamin A metabolite, enhances cytotoxicity and T cell proliferation; the latter likely is mediated by IL-2 secretion and signaling in T cells (Mora et al., 2008). It has been shown that vitamin A bound to its physiologic carrier retinol-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, supplements of beta-carotene improved CD4 T cell counts in populations with chronic illnesses such as cancer and HIV, and enhanced mitogen-stimulated lymphocyte proliferation in smokers (Coodley et al., 1993; van Poppel et al., 1993; Kazi et al., 1997).

The influence of vitamin D metabolites on the immune system, particularly of 1, 25 $(OH)_2$ vitamin D_3, has been known for more than twenty years (Mora et al., 2008). Most of the known biological effects of 1, 25 $(OH)_2$ vitamin D3 are mediated through the vitamin D_3 receptor, a member of the superfamily of nuclear hormone receptors, and most cells of the immune system express the receptor (van Etten and Mathieu, 2005). Overall, the action of vitamin D_3 on immune system enhances the Th2 (T helper 2 cell) system. It blocks the induction of Th1 (T helper 1 cell) cytokines, particularly IFN-γ and promotes Th2 responses directly by enhancing IL-4 production and indirectly by decreasing IFN-γ production (Mora et al., 2008). The activity of vitamin D_3 on T cell differentiation is enhanced further by its effect on antigen-presenting dendritic cells, in which it suppresses the synthesis of IL-12, a cytokine that promotes Th1 responses (Mora et al., 2008). Vitamin D deficiency also contributes to immune system dysfunction (van Etten and Mathieu, 2005). Subjects with severe vitamin D deficiency were abnormally susceptible to infections such as tuberculosis (van Etten and Mathieu, 2005) and impaired cell-mediated immunity in mice (Yang et al., 1993). Moreover, macrophage activation, chemotaxis, cytokine production, and

hematopoietic stem cell numbers were decreased in vitamin D-deficient mammals (Aslam et al., 1998).

Antioxidant vitamins can affect the immune system, although their effects may not be specific unlike vitamin A and D. It has been known for more than 30 years that some vitamins with antioxidant properties, including vitamin E, vitamin C, and vitamin B6, have protective effects on animal models of atherosclerosis and ischemia reperfusion injury (Mora et al., 2008). Antioxidant vitamins, particularly vitamin E, may influence innate immune cell activity. Vitamin E blocks the release of pro-inflammatory cytokines such as IL-1, IL-6, TNF, and IL-8 by monocytes and macrophages (Mora et al., 2008). Vitamin E also prevents the up-regulation of several adhesion molecules, such as vascular cell-adhesion molecule 1 and intercellular adhesion molecule, on epithelium following induction by oxidized low-density lipoprotein and IL-1 beta (Mora et al., 2008). Vitamin C deficiency is known to be associated with impaired macrophage mobilization (Saker, 2006). Antioxidant vitamins also can modulate adaptive immunity. Vitamin E inhibits macrophage secretion of prostaglandin E2, which results in suppressed IL-1 production (Saker, 2006). Prostaglandin E2 favors Th2-like cytokine secretion profiles in murine and human CD4+ T cells by inhibiting the production of the Th1-associated cytokines (IL-2 and IFN-γ) and up-regulating the production of the Th2-associated cytokines (IL-4 and IL-5) in a dose-dependent way (Hilkens et al., 1995). In addition, Meydani et al. (1990) reported that vitamin E supplementation increased concanavalin A (ConA) stimulated IL-2 production and lymphocyte proliferation, and elevated delayed type hypersensitivity response in humans. Therefore, vitamin E may specifically enhance a

Th1-cell-mediated response (Webb and Villamor, 2007). Vitamin C supplementation appears to reduce the incidence of common colds and pneumonia among subjects regularly engaged in strenuous physical activity or who live in crowded situations (Webb and Villamor, 2007). Excessive exercise causes Th2 proliferation, which suppresses Th1-mediated response and increases susceptibility to virus infection (Smith, 2003); therefore, vitamin C supplementation may suppress PGE2 production, resulting in enhanced Th1 function. Although supplementation with vitamin C is known to enhance B cell proliferation (Saker, 2006), there is not strong association between vitamin E supplementation and humoral immune response (Webb and Villamor, 2007).

Finally, vitamin B6 is considered to have antioxidant properties. Numerous animal studies have found that deficiencies in B6 lead to impaired antibody production; however, many of the studies used sheep red blood cells (SRBC) as an antigen (Rall and Meydani, 1993). Because the immune response to such an antigen requires the initial participation of T lymphocytes, the effects of vitamin B6 deficiency may be mediated by T cells with little direct effect on B cells (Rall and Meydani, 1993).

Trace mineral status

Because of its high rate of cellular turnover, the immune system is a major consumer of essential micronutrients, including the trace minerals (Bogden and Oleske, 2007). In addition, because trace minerals are involved in a variety of physiological, catalytic, regulatory, and structural functions (Underwood and Suttle, 1999), it can be easily imagined that their deficiency could result in immune dysfunction. While there

is little or no evidence that fluoride, iodine, manganese (Mn), or molybdenum may influence immunity, there is considerable evidence that supports the importance of iron, Zn, copper (Cu), and Se for maintaining immune functions (Bogden and Oleske, 2007) and this section focuses on these minerals.

Iron is related to both oxygen metabolism and energy metabolism. Also, hydrogen peroxidases, such as catalase and peroxidase, are iron-dependent enzymes that are critical to controlling ROS (King, 2000). Hence, iron deficiency can affect immune function. Iron-deficient children tend to have impaired lymphocyte and PMN functions, although an increased in the number of infections in children resulting from iron deficiency per se has not been demonstrated nor has cause and effect been established (King, 2000). However, recent studies have provided insight into potential mechanisms mediating iron deficiency effects on the immune system. One study showed that IFN-γ secreted from activated spleen cells from mice was reduced during iron deficiency (Kuvibidila et al., 2010), and another reported that iron deficiency reduced plasma IL-4 level in mice (Kuvibidila et al., 2012). These results suggested that iron deficiency may impair the functions of both Th1 and Th2, although the mechanisms are not known (Kuvibidila et al., 2010; Kuvibidila et al., 2012). On the other hand, iron status may aggravate conditions of infectious diseases because microbial pathogens need iron. It was reported that elevated dietary iron may increase the risk of active pulmonary tuberculosis in humans because *Mycobacterium tuberculosis* utilizes iron to grow (Gangaidzo et al., 2001). McDermid (2007) suggested that elevated iron status could contribute to increase in the mortality in Human Immunodeficiency Virus infection. These varied responses to iron deficiency

or supplementation indicate that evaluating the influence of iron status on immune system is not simple.

The immune system is particularly dependent on a constant availability of Zn; even modest Zn-deficiency can alter immunity (Haase et al., 2006). The immune system has a large turnover of cells, which necessitates a high rate of proliferation, differentiation, and apoptosis, and Zn is involved in all of these processes (Haase et al., 2006). Common hallmarks of Zn-deficiency are lymphopenia and thymic atrophy along with decreased thymic hormone activity (Saker, 2006). Depressed delayed type hypersensitivity reactions, decreased CD4 helper T cell populations, and increased CD8 suppressor T cell populations resulted from Zn-deficiency (Saker, 2006). Zinc deficiency caused significant losses in the proportion of cells of the B-lineage in the marrow that are correlated with the degree of Zn-deficiency, which eventually leads to the reduction of mature B lymphocytes observed in the peripheral immune system as Zn-deficiency advances (King et al., 1995). Zinc deficiency resulted in decreased digestion and phagocytosis in macrophage function, partly because Zn is involved as a cofactor in creating superoxide, which kills bacteria and fungi (Chandra, 1983). Zinc is also a component of superoxide dismutase, SOD, which scavenges damaging ROS in immune cells and also defines Zn as an integral component of phagocytic cell function and viability (Saker, 2006). On the other hand, it has been shown that Zn is vital to endothelial cell integrity as Zn-deficiency caused severe impairment of endothelial barrier function (Hennig et al., 1992), which could partially explain why Zn-deficiency results in reduced disease resistance.

Copper has been shown to play a role in the development and maintenance of the immune system, and a large number of experimental studies have demonstrated that Cu status alters several immune functions (Wintergerst et al., 2007). Among these, neutropenia is a common finding in humans and domestic animals with Cu-deficiency (Minatel and Carfagnini, 2000). Percival (1995) suggested that there is a delay in the process of cellular differentiation in PMN during Cu-deficiency that results in a normal number of progenitor cells and a low level of mature cells in the bone marrow of Cu-deficient humans. Copper deficiency also affects acquired immunity. Copper-deficient mice produced fewer splenic antibody-producing cells in response to sheep red blood cells, and Cu-deficient rats have decreased serum antibody titers to sheep red blood cells (Smith et al., 2008). T-lymphocytes are decreased in humans and rodents with Cu-deficiency, most prominently in the helper T cell population (Minatel and Carfagnini, 2000). The mitogenic reactivity of T cells isolated from the spleen of rats was decreased markedly by dietary Cu-deficiency (Bala et al., 1991). The T cell defects appear to be due to decreased IL-2 mRNA and protein production (Smith et al., 2008).

Selenium is essential for an optimum immune response and influences both innate and acquired immune systems through a key role in redox regulation and antioxidant function, and contributes to membrane integrity and protection against DNA damage (Wintergerst et al., 2007). Polymorphonuclear neutrophils from Se-deficient mice, rats and cattle are able to ingest pathogens *in vitro*, but are less able to kill them compared to PMN from Se-sufficient animals (Arthur et al., 2003). This defective function has been associated with decreased cytosolic GSH-Px activity in the

PMN (Arthur et al., 2003). In Se-deficient macrophages, leukotriene B4 synthesis, which is essential for PMN chemotaxis, is impaired (Arthur et al., 2003). Relative to the acquired immune system, Se-deficient lymphocytes are less able to proliferate in response to mitogens (Arthur et al., 2003). In addition, Se-deficiency decreased antibody production by lymphocytes, titers of IgM and IgG, increased CD4+ T cells, and decreased CD8+ and CD4–/CD8– thymocytes (Wintergerst et al., 2007). On the other hand, supplementation with Se stimulates immune function in subjects with normal Se status. In humans, daily supplementation of Se, given over 8 weeks, induced high affinity IL-2 receptors and enhanced proliferation and differentiation of cytotoxic effector cells, and increased NK cell activity (Wintergerst et al., 2007). This effect seems not to be related to the antioxidant capacity of Se, because it is seen at plasma Se concentrations above those required for saturation of GSH-Px activity, and is related to the potential of Se to upregulate the expression of receptors for the growth-regulating cytokine IL-2 receptors on the surface of activated lymphocytes and NK cells (Wintergerst et al., 2007). Selenium status could have an effect on the genome of a pathogen, changing a normally avirulent virus into a virulent one (Beck, 2000). A study of coxsckievirus infection showed that the amount of virus was elevated in Se deficient mice (Beck, 2000).

Iron, Zn, Cu, and Se are involved in antioxidant capacity because all of them are indispensable components of antioxidant enzymes: iron for catalase, Zn and Cu for SOD, and Se for GSH-Px. Therefore, supply of these minerals may affect antioxidant status. Iron deficiency affects not only the production of hemoglobin but also the production of antioxidant proteins containing ferrous iron such as catalase and

peroxidase (Isler et al., 2002). Accordingly, impairment of the antioxidant defense system has been reported in patients with iron deficiency anemia (IDA) (Acharya et al., 1991; Isler et al., 2002). Iron deficiency also affects non-iron dependent antioxidant enzymes. Olivares et al. (2006) reported that women with IDA presented a 2.9-fold decrease in erythrocyte SOD with Cu and Zn (CuZn-SOD) activity compared to women with normal iron status. Another study conducted in humans showed that erythrocyte SOD activity was significantly lower in anemic patients than in the control group, and oral iron treatment improved the IDA and recovered antioxidant defense system by significantly increasing SOD activity, although erythrocyte GSH-Px activity was maintained at normal levels both in IDA and after oral iron treatment (Isler et al., 2002). Mechanisms involved in decreased erythrocyte SOD activity associated with iron deficiency are not known (Olivares et al., 2006). On the other hand, because iron is required for oxygen transport in hemoglobin, oxygen storage in myoglobin, and electron transport for cytochrome function in aerobic respiration (Johnson and Wessling-Resnick, 2012), excess free iron could generate excess reactive oxygen species, which may result in imbalanced oxidative status. In fact, in beta-thalassaemia, which results in iron overload, increased levels of erythrocyte malondialdehyde, a secondary breakdown product of lipid peroxides, have been reported (Acharya et al., 1991). Given that iron can be an antioxidant or oxidant, clarification of the specific effects of iron status on oxidative stress requires further research.

Dietary Zn-deficiency can cause increased lipid peroxidation whereas Zn supplementation inhibited this process (Shaheen and el-Fattah, 1995) because Zn

functions as an antioxidant through SOD activity. Zinc-deficiency led to a large increase in the malondialdehyde content of rat blood, liver and pancreas with concurrently decreased glutathione in blood and liver (Shaheen and el-Fattah, 1995). Jing et al. (2007) fed rats one of three diets with different Zn levels for 6 weeks, including Zn-adequate, Zn-deficient, and Zn-overdose and reported that rats fed the Zn-deficient diet had reduced activities of CuZn-SOD and catalase and increased concentrations in liver of malondialdehyde and hydrogen peroxide. Rats fed the Zn-overdose diet had higher CuZn-SOD activity in liver (Jing et al., 2007). The mRNA expression levels of SOD were upregulated in the Zn-overdose group, and catalase was downregulated in the Zn-deficient group, suggesting that although Zn-deficiency impaired antioxidant functions, Zn-overdose did not enhance the antioxidant systems of animals (Jing et al., 2007).

Under conditions of Cu-deficiency, several components of the oxidant defense system can be compromised (Uriu-Adams and Keen, 2005). Activity of SOD was lower in red blood cells of Cu-deficient pigs, rats, chickens, sheep, and humans (West and Prohaska, 2004). Ceruloplasmin (Cp), which is another Cu-dependent antioxidant, also is sensitive to tissue Cu status and low levels of Cp activity are consistently reported in Cu-deficient animals (Uriu-Adams and Keen, 2005). A deficiency of Cu also can decrease the activities of certain non-Cu containing enzymes of the oxidant defense system including catalase and GSH-Px (Uriu-Adams and Keen, 2005). Moreover, Cu-deficiency can alter other ROS scavengers including metallothionein and glutathione (Uriu-Adams and Keen, 2005). Among Cu-related antioxidants, the relationship between SOD activity and Cu status has been well-researched. Prohaska

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

Selenium deficiency is associated with decreased activities of Se-dependent antioxidant enzymes, GSH-Px and thioredoxin reductase, and with changes in the cellular redox status. Expression of GSH-Px in most tissues is highly sensitive to dietary levels of Se, which is bound to the active site of GSH-Px as a selenocysteine (Sutphin and Buckman, 1991). Moriarty et al. (1998) reported that under conditions of Se deprivation, the selenocysteine codon reduces the abundance of cytoplasmic GSH-Px mRNA by a translation-dependent mechanism. Sutphin and Buckman (1991) determined that levels of GSH-Px decreased by 50% in brain and 90% in blood in mice after feeding diets deficient in Se and/or vitamin E for 6 to 8 wk. No compensatory changes in the activities of the other antioxidant enzymes were reported in this study, and the addition of vitamin E to the diet did not alter antioxidant enzyme activities or malondialdehyde levels (Sutphin and Buckman, 1991). On the other hand, other research has showen that Se-deficiency was associated with significant decreases in GSH-Px and thioredoxin reductase activities in both lung and liver of mice, whereas the hepatic SOD activity was higher in Se-deficient than Se-adequate mice, suggesting

that higher SOD activation may be a compensatory response to Se-deficiency (Stýblo et al., 2007).

IMMUNE FUNCTION AND OXIDATIVE STRESS DURING TRANSITION PERIOD IN DAIRY COWS

Nearly all dairy cows experience some degree of immune suppression during the 2 to 3 weeks before and after calving (Goff, 2008). This immune dysfunction is not limited to isolated immune parameters; instead it is broad in scope and affects multiple functions of various cell types involving innate and acquired defense mechanisms (Mallard et al., 1998; Overton and Waldron, 2004). The consequence of immunosuppression is that cows may be hyposensitive to invading pathogens and, therefore, more susceptible to peripartum diseases (Overton and Waldron, 2004).

Innate immunity

The innate immune system can be affected during the transition period. Several studies have shown that PMN function was impaired during the peripartum period (Mallard et al., 1998). Cai et al. (1994) reported that superoxide production by PMN during the prepartum period in cows that subsequently developed metritis, and chemotaxis by PMN from cows that subsequently developed mastitis, were significantly lower than in clinically normal cows, indicating that defects in PMN function may be predisposing factors in the development of these disorders. Another study showed that PMN isolated from blood of cows with retained placenta had significantly lower function in both chemotaxis assay and myeloperoxidase activity before calving, and this impaired function lasted for 1 to 2 wk after parturition

(Kimura et al., 2002). Another interesting finding from this study was that the concentration of plasma IL-8, which is a potent chemoattractant and activator of PMN, was lower at calving in cows with retained placenta than in cows expelling the placenta normally (Kimura et al., 2002). This suggested that the PMN dysfunction may be due to suppressed production of IL-8. Saad (1989) showed that there was a gradual increase in the number of PMN in blood as calving approached followed by a sharp decrease after calving, suggesting that IL-8 may also be decreased right after calving.

Adaptive immunity

Several studies have reported diminished lymphocyte responsiveness around calving (Mallard et al., 1998). The proliferative response of blood and milk lymphocytes upon stimulation with three mitogens was low during the week preceding parturition with the lowest value on the day before calving (Saad et al., 1989). The response of blood lymphocytes returned to a higher level the second week after calving, while that of milk lymphocytes remained at a low level during the first and the second postpartum weeks (Saad et al., 1989). Another study further confirmed a decrease in the lymphocyte blastogenic response to the T cell mitogen concanavalin A around calving (Mallard et al., 1998). In this study, it was found that cortisol, growth hormone (GH), IGF-1, and disease incidence each contributed to the variation in the proliferative response of peripheral blood lymphocytes to mitogen from 3 wk pre-calving to 3 wk post-calving. Burton et al. (1991) showed that lymphocytes from the blood of bovine somatotropin-treated cows responded to mitogen with higher proliferative responsiveness than cells from non-treated control cows during early- to

mid-lactation. This finding supported the concept that hormone fluctuations are involved in the alterations occurring during peripartum lymphocyte blastogenesis (Mallard et al., 1998).

Humoral immunity also is altered during the transition period. Interestingly, cows can be categorized by phenotypes for high or low antibody response, based on the magnitude and kinetics of the response during the peripartum period (Mallard et al., 1998). Mallard et al. (1997) showed that antibody response to ovalbumin immunization at -56, -21, and 0 prior to parturition in dairy cows could be used to partition cows into three groups: animals with sustained measurable antibody response before and after parturition (Group 1); animals that responded poorly to immunization at parturition (Group 2); and animals that did not respond to immunizations at week -3 or parturition (Group 3). Cows with the highest antibody response to ovalbumin (Group 1) had the lowest incidence of disease, particularly mastitis. Correlation analysis indicated that peripartum responses to ovalbumin were associated positively with GH concentration in serum but associated negatively with IGF-1 concentrations (Mallard et al., 1998). As mentioned above, higher proliferative responsiveness of lymphocytes were observed in bovine somatotropin-treated cows (Burton, 1991). These results may reflect certain immunoenchancing properties of GH (Mallard et al., 1998), which can explain hereditarily individual difference of immune response.

Etiology of immune suppression

There seem to be several factors that influence the immune system during the periparturient period. Negative energy balance (EB) could be one of them. In human patients with severe sepsis or major trauma, it was reported that resting energy

expenditure increased progressively over the first week to around 40% above normal, and was still elevated 3 weeks from onset of illness (Plank and Hill, 2000). Therefore, it is possible that negative energy status during peripartum period affects the immune system because the system may need substantial energy to counteract diseases during that period. Kimura et al. (1999) reported that PMN myeloperoxidase activity decreased from baseline prepartum values as parturition approached in both intact and mastectomized cows, suggesting that factors associated with pregnancy and the endocrine changes associated with the transition from pregnancy to lactation are predominant factors causing the loss of PMN function. However, they also reported that myeloperoxidase activity recovered to prepartum values within a week following parturition in mastectomized cows but it remained decreased in PMN obtained from intact cows throughout the first 20 d of lactation (Kimura et al., 1999). This result suggested that the EB shifts to negative as parturition approaches and remains negative in intact cows, which could result in modulating the immune system, such as slowed recovery of PMN function observed in their study. Recently, Moyes et al. (2010a) compared gene expression profiles in blood PMN during a *Streptococcus uberis* intramammary challenge between mid-lactating cows subjected to negative EB and cows fed for ad libitum intake to maintain positive EB, and determined that the gene encoding for major histocompatibility complex type II (MHC II) was downregulated by negative EB. In a similar study (Moyes et al., 2010b) comparing mammary tissue gene expression profiles instead of blood PMN, cows subjected to diet-induced negative EB and intramammary challenge with *S. uberis* had lower expression of genes involved in enhancing cell development, proliferation, and growth.

These results suggested that negative EB is related not only to the impairment of PMN expression of MHC II (Moyes et al., 2010a), but also to the mechanism of increased susceptibility to mastitis (Moyes et al., 2010b) during the transition period.

Among metabolites associated with negative EB, ketone bodies appear to have multiple negative effects on immune functions (Overton and Waldron, 2004). Hoeben (1997) showed that BHBA added *in vitro* had inhibitory effects on the respiratory burst activity of PMN isolated from blood of multiparous cows and concluded that the elevated blood level of BHBA after parturition in high-yielding cows may be partly responsible for the higher susceptibility to local and systemic infections during the postpartum period and during subclinical and clinical ketosis. The concentration of NEFA in blood also may affect immune function, as Hammon et al. (2006) reported that PMN myeloperoxidase activity and NEFA concentrations during the first week after calving were negatively correlated (R = - 0.44).

Oxidative stress during the transition period

It has been reportedthat the considerable increase in oxygen requirements during times of increased metabolic demands resulted in augmented production of ROS (Sordillo and Aitken, 2009). An imbalance between increased production of ROS and the availability of antioxidant defenses needed to reduce ROS accumulation during the transition period may expose cows to increased oxidative stress (Sordillo and Aitken, 2009). Although oxidative stress itself does not exhibit specific clinical symptoms, it has been observed that cows can experience oxidative stress during the transition period, which may contribute to periparturient disorders including metabolic diseases (Sharma et al., 2011). Bernabucci et al. (2005) reported that cows with higher

plasma BHBA and NEFA had higher concentrations of reactive oxygen metabolites and thiobarbituric acid-reactive substances (TBARS), and lower levels of antioxidants in plasma during the transition period.

There are now several studies to support the concept that oxidative stress is a significant underlying factor for dysfunction of host immune and inflammatory responses that result in an increase in the susceptibility of dairy cattle to a variety of health disorders, particularly during the transition period (Sordillo and Aitken, 2009). Whereas Bernabucci et al. (2005) reported higher reactive oxygen metabolites and TBARS in plasma during the transition period, Castillo et al. (2005) reported that there was a peak of total antioxidant status in blood one week after calving, which may be a natural response to reduce oxidative stress. Multiple diseases, including mastitis, mammary edema, metritis, and retained fetal membranes most commonly occur during the transition period (Sordillo and Aitken, 2009). Therefore, it seems that there is a relationship between immune dysfunction during the transition period and oxidative stress. Also, antioxidants such as vitamin E and Se are associated with resistance to mastitis when supplemented during the transition period, suggesting that there is a significant correlation between antioxidant supplementation and decreased incidence of diseases around parturition (Sordillo and Aitken, 2009). Taken together, these findings provide further support for the concept that oxidative stress can modulate immune function and contribute to the occurrence of health disorders during the transition period.

SPECIFIC INFLUENCES OF MINERALS ON IMMUNITY, OXIDATIVE STRESS, AND METABOLISM IN RUMINANTS

Because Zn, Cu, Mn, chromium (Cr), and Se have been comparatively well-studied among trace minerals in ruminants, specific influences of these trace minerals and macro minerals on immunity, oxidative stress, and metabolism will be discussed in this section.

Zinc

Zinc affects metabolism of carbohydrates, proteins, lipids, and nucleic acids (NRC, 2001); therefore, it is logical to conclude that Zn also affects the immune system in ruminants as well as other species. The earliest and most important clinical signs of Zn-deficiency in ruminants are loss of appetite, parakeratosis, and impaired wound healing (Miller et al., 1965). Therefore, Zn-deficiency in ruminants can affect the first line of resistance to infection, i.e., the skin and other stratified epithelia (Suttle and Jones, 1989). In cattle, however, surprisingly little research has been carried out to examine the relationship between dietary Zn and immune function (Spears, 2000). Overall, responses to dietary Zn-deficiency have been inconsistent. One study reported that lymphocyte responses of lymphocytes from heifers given a subcutaneous injection of phytohaemagglutinin (PHA) were impaired by Zn deprivation prior to any loss of appetite (Suttle, 2010), but in finishing steers, Zn-proteinate improved growth without affecting *in vitro* and *in vivo* immune responses (Suttle, 2010). Although Suttle (2010) concluded that there is no evidence that the immune system is more sensitive to Zn deprivation than other Zn dependent functions, Spears (2000) found in controlled studies conducted in cattle and lambs that marginal Zn-deficiency does not

impair cell-mediated or humoral immune responses. As mentioned in this chapter, Zn status is known to affect the immune system in laboratory animals and humans. The reason why ruminants are less sensitive to Zn status is unknown.

However, severe deficiency of Zn appears to have a more definitive influence on immune status. A genetic disorder (lethal trait A46) of Zn metabolism that results in severe Zn-deficiency has been reported in cows (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 pokeweed mitogen (PWM) stimulation were variably reduced, CD4 T cells were subnormal, and relative numbers of B cells were decreased (Perryman et al., 1989). Because Zn is a component of thymosin, a hormone produced by thymic cells that regulates cell-mediated immunity (NRC, 2001), reduced production of thymosin during Zn-deficiency presumably affected the numbers and functions of lymphocytes.

The effects of supplementation of Zn on immune function have been inconsistent. The addition of 25 mg/kg of Zn to a control diet in growing steers that contained 33 mg/kg of Zn did not lead to an increase in *in vitro* lymphocyte responses to PHA or PWM stimulation, *in vivo* cellular response to intradermal PHA administration, or antibody response following vaccination for infectious bovine rhinotracheitis virus (IBRV) (Spears, 2000). Supplementation of 150 or 300 mg/kg of Zn to a control calf starter diet containing 60 mg/kg of Zn fed to Holstein heifer calves did not affect mitogen-induced lymphocyte blastogenesis, IL-2 production by lymphocytes, lymphocyte cytotoxicity, or the phagocytic and intracellular killing

ability of blood PMN (Kincaid et al., 1997). However, a study that summarized 12 trials that evaluated the effects of Zn-methionine complex on lactation performance and udder health, showed that supplementing lactating dairy diets with 180 to 400 mg Zn/d per head of Zn-methionine complex reduced somatic cell count (SCC; 1000/mL) from 294 to 196 (Kellogg et al., 2004).

Although few studies have been conducted to evaluate effects of Zn on oxidative stress, Zn could play an important role as an antioxidant through SOD in ruminants as well as other species. Activities of SOD were reduced in the testis, intestine, and liver of Zn deficient goats (Underwood and Suttle, 1999). In addition, Zn-metalloenzymes were found to be involved in vitamin A metabolism (Underwood and Suttle, 1999), and low plasma vitamin A concentrations in the presence of adequate dietary vitamin A can occur in Zn-deficient lambs and young goats (Underwood and Suttle, 1999), which may reduce host antioxidant function. A study in yearling ewes showed that Zn has a potential role in increasing absorption or retention of vitamin E (Hatfield et al., 2002). Also, because Zn affects metabolism of carbohydrates, proteins, lipids, and nucleic acids (NRC, 2001) as mentioned earlier, Zn-deficiency may also affect tissue repair from oxidative damage.

Considering that Zn is an essential component of numerous metabolic enzymes, it is expected to affect metabolism; however, few studies exist to investigate effects of Zn alone. Cope et al. (2009) reported that cows fed organically chelated Zn at NRC (2001) recommended levels had a higher milk yield than those fed inorganic Zn at the recommended level or organically chelated Zn at a lower level (66% of NRC), but was not different from those fed inorganic Zn at the low level (Cope et al., 2009). However,

it is not known whether of the treatment effect on milk yield was due to modification of metabolism or improvement in udder health because supplementing Zn at the recommended level also reduced somatic cell counts (Cope et al., 2009). Further studies are needed to evaluate effect of Zn on metabolism.

Copper

Studies conducted in cattle have failed to show consistent effects of Cu-deficiency on either cell-mediated or humoral immune responses (Spears, 2000). Also, the authors of the NRC suggested that effects of Cu-deficiency on immunity are not easily observed (NRC, 2001). It is probable that Cu-deficiency affects specific immunity differently in ruminants than in other species, or that Cu-deficiency alters specific immune response of ruminants only after extended periods of time (Minatel and Carfagnini, 2000). Marginal Cu-deficiency in dairy heifers reduced PMN killing of *S. aureus* (Torre et al., 1996a). However, Ward and Spears (1999) reported that Cu deficient calves had higher secondary antibody response to pig erythrocytes than Cu supplemented calves, and cell-mediated response to PHA was decreased by Cu supplementation to Cu-deficient calves. Another study demonstrated that low Cu 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).

Effects of Cu supplementation on immunity also have been inconsistent. Gengelbach et al. (1997) reported that there were no differences in PMN phagocytosis function among calves supplemented with Cu or molybdenum compared with controls. Another study showed that Cu supplementation increased the humoral response to

ovalbumin injected into growing calves, but supplementation decreased antibody titers to porcine erythrocytes in unstressed calves and increased the titers in stressed steers (Ward and Spears, 1999).

Dietary Cu may affect cytokine production in cattle (Spears, 2000). Copper-supplemented calves had higher circulating levels of TNF-α than calves fed additional molybdenum at weaning, and tended to have higher TNF-α than calves fed with iron and molybdenum after IBRV inoculation (Gengelbach et al., 1997). Mononuclear cells isolated from lactating dairy cows that had received a marginal level of Cu (6-7 mg/kg diet) produced less IFN-γ when stimulated with Con A than cell isolated from cows fed adequate levels of Cu, whereas IL-2 production was not affected by treatments (Torre et al., 1996b).

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

Copper status can affect oxidant-antioxidant balance in ruminants because Cu is a component of the antioxidant enzyme SOD. Actually, SOD activity is reduced in PMN from sheep and cattle with Cu-deficiency (Minatel and Carfagnini, 2000). In

addition, production of superoxide by stimulated PMN, which is critical for PMN to kill bacteria, is also impaired in cattle with Cu-deficiency (Minatel and Carfagnini, 2000). Therefore, Cu-deficiency could affect multiple components within oxidative metabolism. Ceruloplasmin may also contribute to antioxidant defenses by scavenging free radical iron and free radicals (Underwood and Suttle, 1999); Cu depletion may increase susceptibility against oxidative damage through lower activity of Cp. Cerone et al. (2000) demonstrated that serum Cu concentrations and activity of ceruloplasmin were decreased by Cu-deficiency caused by dietary supplementation with antagonists to Cu absorption (molybdenum and sulfate) for 9 months in Angus heifers. The form of Cu also may affect oxidative status in different tissues when ruminants are supplemented with Cu. The Cp and erythrocyte SOD activities were higher in Cu supplemented lambs from organic form (proteinate), whereas the liver SOD activity was higher in lambs fed Cu in inorganic form ($CuSO_4$). Copper may be involved in metabolism of vitamin A and E in ruminants. Lower values of vitamins A and E content in heifers without Cu supplementation was observed throughout the period of study when compared to heifers provided with mineral mixture containing Cu sulfate (Sharma et al., 2005b). Overall, it can be speculated that Cu status affects oxidative status in ruminants through Cu dependent enzymes such as SOD and Cp, and Cu related vitamins such as vitamin A and E.

Copper plays an important biological role as a cofactor for several Cu dependent enzymes (Sharma et al., 2008). The status of Cu level in the body also influences the level of vitamins like A and E, and thyroid hormones (Sharma et al., 2005a). Therefore, the status may influence metabolism. However, few studies have

focused on Cu alone. Sharma et al. (2005) reported that significant improvement was observed in thyroid hormone levels and growth within 60 days after treating hypocuperemic heifers with a mineral mixture containing Cu sulfate, along with significant improvement of the values of vitamin A and E that occured within 30 days of the treatment. A recent study investigated the effects of methionine hydroxy Cu supplementation on lactation performance in lactating cows (Wang et al., 2012). The results showed that milk yield and 4% fat-corrected milk yield of cows in the mixed treatment (Cu-sulfate and Cu-methionine) tended to increase compared with those treated with Cu-sulfate only or Cu-methionine only (Wang et al., 2012). However, the lack of a negative control treatment (no supplementation of Cu) limits interpretability to the form of Cu supplementation.

Manganese

There have been few studies that assess the effect of Mn on immune function on ruminants. In one study where 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 Mn, Zn, and Cu) or inorganic (100% inorganic $CuSO_4$, $ZnSO_4$, and $MnSO_4$) trace minerals (Ahola et al., 2005), IgG antibody titers specific for porcine red blood cells were greater in heifers supplemented with organic compared to inorganic minerals on day 14 and 21 post injection of porcine red blood cells (Ahola et al., 2005). Similarly, in another study that evaluated the effect of source and amount of trace minerals fed following weaning and transport stress (shipped 250 km) on immune response in crossbred heifer calves and compared 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 $CuSO_4$, and 7 mg of Co/kg from $CoCO_3$) for 42 d after shipping, and treatment 2: organic form (Zn-methionine, Mn- methionine, Cu-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 at 14 and 28 d postvaccination; and skin swelling response at 12, 24, and 48 h postinjection to intradermal PHA was greater in calves in treatment 2 than those in treatment 1 (George et al., 1997). Although these studies did not evaluate the effect of Mn itself, the results imply that Mn may contribute to enhancement of immune function.

Manganese can contribute to oxidative balance through super oxide dismutase with Mn (Mn-SOD). Manganese deficiency lowers Mn-SOD activity in the heart and increases the peroxidative damage caused by high dietary levels of polyunsaturated fatty acids (Underwood and Suttle, 1999). Again, there have been few studies to assess the effect of Mn itself on oxidative stress on ruminants. Masters et al. (1998) fed rams 13, 19, 30 or 45 pg Mn per gram of dry matter and demonstrated that that rams fed the least amount of Mn (13 pg) had a lower activity of Mn-SOD in heart than rams fed the most amount of Mn (45 pg), and that the concentration of Mn in heart and lung increased in parallel with increasing the concentration of Mn in the diet. Few studies have investigated the isolated effects of Mn on metabolism.

Chromium

Although the primary role of Cr appears to relate to its ability to enhance the action of insulin (Spears and Weiss, 2008), studies have indicated that Cr

supplementation may affect health and immune response in ruminants (Spears, 2000). Relative to innate immunity, a study showed that supplemental Cr did not affect PMN phagocytic function of dairy cows from 6 wks prepartum to 6 wks postpartum (Chang et al., 1996). However, other studies in periparturient dairy cows indicate that Cr supplementation of practical diets may affect cell-mediated and humoral immune responses (Spears and Weiss, 2008). Cell-mediated immunity was assessed *in vitro* using antigen (ovalbumin)- and mitogen-stimulated peripheral blood mononuclear cells (PBMC) blastogenesis of cells collected from prepartum through early lactation period (Burton et al., 1993), with Cr supplementation leading to increased mitogen-stimulated blastogenic responses of PBMC (Burton et al., 1993). As for humoral immune response in periparturient and early lactation dairy cows, Burton et al. (1993) showed that anti-ovalbumin antibody responses were increased when supplemental Cr was fed and Faldyna et al. (2003) reported that tetanus toxoid-specific antibody titers were higher in cows fed 5 mg Cr per head per day (Faldyna et al., 2003). The mechanism by which Cr modifies immune system is unknown. Burton et al. (1996) demonstrated that *in vitro* concentrations of IFN-γ, IL-2, and TNF-α of the mitogen-stimulated mononuclear cells collected during the peripartum period were decreased in cows supplemented with Cr compared with controls (Burton et al., 1996). Because IFN-γ, IL-2, and TNF-α are produced mainly by Th1 and IFN-γ has potent anti-proliferative effects on Th2, a reduction of these cytokines during Cr supplementation may help to explain enhanced humoral immune responses.

Studies examining the effects of dietary Cr on health in ruminants are also limited. Although one study showed that supplemental Cr had no beneficial effect on

health status and mastitis-related parameters in dairy cows (Chang et al., 1996), another study showed that Cr supplementation during the last 9 wk of pregnancy decreased the incidence of retained placenta observed in unsupplemented cows nearly four fold (Villalobos et al., 1997). Further research is needed to establish the effects of Cr on health status.

The primary role of Cr appears to relate to its ability to enhance the action of insulin (Spears and Weiss, 2008); therefore, it is possible that supplemental Cr influences metabolic status. In fact, several studies have shown effects of Cr on metabolic parameters in dairy cows. A study that evaluated the effects of supplemental Cr (0.8 mg/kg of Cr) on liver triglycerides and blood metabolites in Holstein steers showed that animals supplemented with Cr had lower concentrations of serum triglyceride, plasma BHBA, and liver triglyceride (Besong et al., 2001). Supplementation of 0.5 mg of Cr/kg dietary DM from 6 wk prepartum to 16 wk postpartum resulted in reduced serum BHBA levels in multiparous cows; however, peak values and production rates of cortisol in Cr-supplemented cows tended to be greater than in un-supplemented control cows at 6 wk after parturition (Yang et al., 1996). Hayirli et al. (2001) fed increasing doses of Cr (0, 0.03, 0.06, and 0.12 mg of Cr/kg of$BW^{0.75}$) from 28 d before expected calving date through 28 d of lactation in dairy cows and reported that Cr supplementation did not affect concentrations of blood metabolites and liver triglyceride but decreased serum insulin concentrations. When Cr (10 mg of Cr/d) was fed from 21 d prepartum to 35 d postpartum, adipose tissue lipogenesis in cows treated with Cr was much greater than controls from 14 to 56 d from calving and had returned to prepartum rates by 56 d (McNamara and Valdez,

2005). Smith et al. (2008) determined that Cr supplementation during the transition period resulted in higher plasma glucose and glucagon and lower NEFA prepartum; however, effects on postpartum metabolites and liver composition were not significant. Providing cows with Cr treatment (6 mg Cr/head/day) from 3 weeks prepartum through 12 weeks postpartum under heat stress did not affect serum glucose, Ca and phosphorus concentrations; however, NEFA were decreased at 1 week prepartum and 2 and 4 weeks postpartum (Soltan, 2010). In addition, serum insulin concentrations were increased and cortisol concentrations were decreased compared to controls throughout the whole experimental period (Soltan, 2010). Recently, Sadri et al. (2012) supplemented cows at dosages of 0 or 0.08 mg of Cr/kg of $BW^{0.75}$ from 21 days before anticipated calving through 28 days after calving and determined that plasma metabolites and hormones related to metabolism were not affected by Cr supplementation, but showed trends for decreased BHBA on d 0 after calving, increased cholesterol on d 0, and increased glucagon on d 21. Although Cr supplementation during the transition period appears to result in positive effects on energy metabolism, there is variation in response among experiments and specific reasons for this are not known.

Selenium

Selenium status influences innate immunity in ruminants and Se is a key component of GSH-Px. Neutrophils from Se-deficient steers were less able to kill ingested *Candida albicans* than ones from the Se-adequate group, which was associated with non-detectable GSH-Px activity in the Se-deficient PMN (Boyne and Arthur, 1979). Selenium-deficient bovine mammary artery endothelial cells had

significantly enhanced PMN adherence when stimulated with TNF-α, 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). Also, Maddox et al. (1991) showed that milk from cows fed a Se-depleted diet contained significantly higher eicosanoids concentrations, which are related to functions of innate immunity such as production of PMN hydrogen peroxide and chemotaxis of PMN. Supplementation of Se also affects innate immunity. Hogan et al. (1990) reported that intracellular killing of *S. aureus* was greater in PMN isolated from Se-supplemented cows than in PMN from cows not fed supplemental Se. Although supplemental Se did not affect phagocytic ability of PMN in dairy cows (Hogan et al., 1990), chemotactic migration of PMN *in vitro* were reduced by Se-deficiency in goats (Spears, 2000).

Production of cytokines may be affected by Se status. One study in finishing lambs showed that the concentrations of IL-1 and IL-2 in plasma were increased in Se-supplemented groups, which were provided 0.10 mg/kg Se from sodium selenite, selenized yeast, or Se enriched probiotic containing *Lactobacillus* and *Saccharomyces cerevisiae*, compared with the control group fed a basal diet deficient in Se (Qin et al., 2007).

Acquired immunity in ruminants also is influenced by Se status. ConA-stimulated lymphocyte proliferation was lower in Se-deficient cows than in cows fed diets supplemented with Se (Cao et al., 1992). Sheep supplememted with 0.1 or 0.5 ppm Se as sodium selenite had higher lymphocyte proliferative responses to phytohemagglutinin, pokeweed, and ConA mitogen than non-supplemented control

sheep, while supplementation with selenomethionine, an organic form of Se, did not enhance blastogenesis (Stabel and Spears, 1993). Further, addition of 1 ppm Se 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 Se (Stabel and Spears, 1993). Humoral immune responses have been enhanced by increased dietary Se intake in ruminants (Spears, 2000). Calves consuming an avereage 3 to 6 mg Se/day had higher antibody titers to chick egg lysozome 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 Se enhances antibody responses to viral and bacterial antigens as well (Stabel and Spears, 1993). In addition, beef cows given 120 mg of Se/kg of salt-mineral mix had higher colostral IgG concentration than did Se-deficient animals (Swecker et al., 1995).

Selenium status appears to be directly related to disease resistance in ruminants. When Se 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 Se concentrations. Dietary supplementation of 0.14 ppm Se resulted in less severity and shorter duration for experimental *E. coli* mastitis than 0.04 ppm (Erskine et al., 1989); however, Se level did not affect induction of experimental mastitis by *S. aureus* (Erskine et al., 1990). Numerous studies have also indicated that pre-partum Se supplementation along with vitamin E

can reduce the incidence of retained placenta in dairy cows fed diets low in Se (Spears and Weiss, 2008).

It can be speculated that Se directly affects oxidative status in ruminants as well as in other species due to its antioxidant function. However, the effects of Se supplementation on oxidative status have not been consistent. Selenium supplementation (3 mg/d) during the 6-week prepartum period in dairy cows did not affect TBARS in red blood cells, whereas vitamin E supplementation decreased TBARS (Brzezinska-Slebodzinska et al., 1994). When diets fed to beef cattle were supplemented with organic Se (0.3 mg/kg) and vitamin E (300 IU/kg feed) for 55 days preceding slaughter, dietary Se supplementation did not affect muscle Se levels, GSH-Px activity, or susceptibility to lipid and oxymyoglobin oxidation in the presence or absence of vitamin E (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). These inconsistent results may be due to the different level of Se and other antioxidants in the basal diet used in each study.

Because dairy cows could experience a metabolic load in stressful periods such as early lactation (Graber et al., 2010) or heat stress, Se status can affect metabolism parameters by controlling oxidants resulting from the metabolic load. Recently, Calamari et al. (2011) examined the effects of either dietary Se source or dose on metabolic profiles in heat-stressed lactating dairy cows. The higher NEFA levels and the lower BHBA, along with the trend for lower urea plasma levels, were observed in Se supplemented cows compared with control cows during the hotter period (Calamari

et al., 2011). Although the authors mentioned that the result showed that the metabolic response to heat stress could be partially ameliorated by feeding Se (Calamari et al., 2011), the reason why supplemental Se resulted in high NEFA was not clear. Studies are needed to elucidate the mechanism of how Se affects the metabolic system in dairy cows.

Trace mineral mixtures

Application of the combination of trace minerals can be practical on farm because it is difficult to estimate which mineral level is adequate or not in each herd. Furthermore, practical application commonly involves supplementation of mixtures of various forms of trace minerals. When cows fed organic or inorganic form of Zn, Cu, and Mn for 12 wk postpartum period, PMN functions were unaffected by treatment except for an enhancement in PMN phagocytosis with the organic treatment found for the later days in milk (Nemec et al., 2012); however, rabies antibody titers in cows fed organic forms were 2.8 fold that of cows fed inorganic form (Nemec et al., 2012). The results suggest that organic form may enhance adaptive immune response compared with cows supplemented with inorganic form (Nemec et al., 2012).

Several combinations of trace minerals have been evaluated for performance in dairy cows. When cows received treatments consisting of Zn, Mn, Cu, and Co in either inorganic or organic form from 3 wk prepartum through wk 35 postpartum, supplementation of organic forms of trace minerals resulted in improved lactation performance and claw integrity, but did not affect reproductive performance (Siciliano-Jones et al., 2008). In contrast, Formigoni et al. (2011) determined that partial replacement of inorganic Zn, Cu, and Mn with organic forms during the dry

period and early lactation did not affect performance other than increased milk fat content in cows fed organic forms. In another study, cows fed organic forms had improved conception rate with fewer days in milk to pregnancy compared to cows fed inorganic forms (Uchida et al., 2001). Recently, Ashry et al. (2012) reported that cows fed Zn, Cu, and Mn in organic form from 6 weeks before calving through 3 months after calving had higher milk yield and milk fat content compared to controls fed inorganic sources, although blood metabolites related to kidney and liver metabolism were not affected by treatment (Ashry et al., 2012). Inconsistencies in these data may relate to the period of supplementation. Nocek et al. (2006) reported that long-term fortification of Zn, Mn, Cu, and cobalt with inorganic and organic sources at or above NRC requirements improved both reproductive and productive performance in dairy cows, the experimental duration was much longer than other studies: dry period 1, full lactation 1, dry period 2, and 200 d into the subsequent lactation. Thus, further investigation relative to long-term supplementation strategies is warranted.

Macro minerals

Calcium (Ca) status can affect periparturient immune status. A study investigating more than 2,000 cows revealed highly significant associations between parturient hypocalcemia and retained fetal placenta along with mastitis (Curtis et al., 1983). Several possible mechanisms may explain the associations. Hypocalcemia can be a stressor to the cow (Goff, 2008). Cows typically exhibit a 3 to 4 fold increase in plasma cortisol as part of the act of initiation of parturition; however, subclinically hypocalcemic cows may have 5- to 7-fold increases in plasma cortisol on the day of calving and the typical hypocalcemic cow may exhibit plasma cortisol concentrations

that are 10- to 15-fold higher than pre-calving plasma cortisol concentration (Goff, 2008). Because cortisol is generally considered to be a powerful immune suppressive agent (Goff, 2008), low Ca status may indirectly suppress immune system around parturition. In addition, there may be direct effects of Ca status on the immune system. Kimura et al. (2006) reported that intracellular Ca stores in PBMC significantly decreased at parturition and returned to normal levels as the cows' blood Ca returned to normal levels, indicating that the decrease in PBMC intracellular Ca stores before parturition and the development of hypocalcemia contributes to periparturient immune suppression. They suggested that blunted intracellular Ca release in response to an immune cell activation signal could be attributable to decreased intracellular Ca stores in PBMC (Kimura et al., 2006). Although other macro minerals related to Ca metabolism such as magnesium (Mg) may affect the immune system, there has been little study to examine the association between those minerals and immune status.

Calcium can also affect metabolism due to its requirement for formation of skeletal tissues, transmission of nervous tissue impulses, excitation of skeletal and cardiac muscle contraction, blood clotting, and component of milk (NRC, 2001). It is also involved in the activity of a wide array of enzymes and serves as an important second messenger conveying information from the surface of the cell to the interior of the cell (NRC, 2001). The requirement for Ca increases approximately fourfold on the day of parturition in dairy cows (Overton and Waldron, 2004). Inadequate blood Ca concentrations can cause a cow to lose the ability to rise to her feet, which results in the metabolic disease known as milk fever, though it is more properly termed periparturient hypocalcemia as an elevated body temperature is not typically observed

(Goff, 2008). Also, low Ca status can indirectly affect metabolic status in dairy cows. Hypocalcemia reduces rumen and abomasal motility, increasing the risk of abomasal displacement (Goff, 2008); hypocalcemia reduces feed intake such that greater body fat mobilization occurs in early lactation (Goff, 2008). Van Winden et al. (2003) reported that blood concentrations of Ca were lower, and those of NEFA, BHBA, and aspartate aminotransferase were higher, during the 10 d preceding clinical diagnosis of left displaced abomasum in cows. Furthermore, Kalaitzakis et al. (2010) determined that serum concentrations of Ca, K and Mg were lower in cows that had died within 4 wk of left displaced abomasum surgery than those that survived. In another recent study (Chamberlin, 2011) in which cows were categorized as either normocalcemic or hypocalcemic based on whole blood Ca ion on the day of calving, hypocalcemic cows had higher NEFA concentrations on days 0 (calving) and 21, tended to have higher NEFA concentrations on day 14, and had more lipid in the hepatocytes on day 35, which provided further evidence of an association between Ca status at calving, fat mobilization, and liver lipid infiltration (Chamberlin, 2011).

The importance of Mg on metabolism during the transition period can be explained by the fact that Mg is related to Ca metabolism. Goff (2008) suggested that low Mg status affects Ca metabolism in two ways: 1) by reducing parathormone secretion in response to hypocalcemia and 2) by reducing tissue sensitivity to parathormone. Field evidence suggests that blood Mg concentrations below 0.65 mmol/L in the periparturient cow can increase the susceptibility of cows to hypocalcemia and milk fever (Goff, 2008). As mentioned above, serum Mg as well as

serum Ca was lower in dairy cows, which developed left displaced abomasum and fatty liver (Kalaitzakis et al., 2010).

METABOLISM DURING TRANSITION PERIOD IN DAIRY COWS

As discussed in the former section, negative energy status during the peripartum period can affect the immune system and there are several potential mechanisms (Moyes et al., 2010a; Moyes et al., 2010b). Few studies have evaluated the effects of changing energy level or supplemental energy on the immune system during the transition period. Stabel et al. (1993) reported that dairy cows with Johne's disease fed supplemental energy during the periparturient period had reduced lymphocyte proliferative responses to T cell mitogens during early lactation and increased secretion *in vitro* of immunoglobulin by PBMC after parturition. In contrast, diet energy concentration (high vs. low) and milking frequency (two vs. three) had no effect on the TNF-α responsiveness in early lactation (Røntved et al., 2005). Interestingly, Moya et al. (2008) hypothesized that once-daily milking would improve immune responses as a result of reduced metabolic load; however, immune cells from these cows actually had reduced phagocytosis and decreased production of ROS by PMN and monocytes when compared to those from cows milked twice-daily (Moya et al., 2008). Recently, Graugnard et al. (2012) investigated the effect of prepartum feeding of diets to meet (control, 1.34 Mcal of NE_L per kilogram of dry matter) or exceed (overfed, 1.62 Mcal of NE_L per kilogram of dry matter) dietary energy requirements during the entire dry period on blood PMN function. Phagocytosis capacity was lower prepartum in overfed cows; phagocytosis in the control group

remained constant postpartum, but it was increased at d 7 in the overfed group to levels similar to controls (Graugnard et al., 2012). The mechanism involved in the change of phagocytosis capacity in the overfed treatment is unknown and requires further examination.

Few studies have been conducted to examine the association between glucose precursor supply and immune status. One study showed that the incidence of clinical mastitis was reduced in monensin-treated cows, along with decreased the rate of intramammary infection in heifers (Heuer et al., 2001). Duffield et al. (2008) investigated 16 papers to evaluate health outcomes for monensin and found that monensin decreased the risk of mastitis; however, no significant effects of monensin were found for metritis and retained placenta (Duffield et al., 2008). Positive effects of supplemental monensin on bovine paratuberculosis have been reported such as halting and a reversal of the pathological process in histological lesions in ileum, liver, and rectal mucosa in infected cattle (Brumbaugh et al., 2000) and the reduction of fecal shedding of *Mycobacterium avium* subsp. *paratuberculosis* in infected dairy cattle (Hendrick et al., 2006). Considering that the application of a glucose precursor or increased glucose precursor can improve energy status during the transition period, further research is needed to verify its effect on the immune system.

As mentioned in the previous section, a deficiency of dietary protein or amino acids has long been known to impair immune function and increase the susceptibility of animals and humans to infectious disease (Li et al., 2007). However, few studies examine the effects of protein or amino acids on the immune system during transition period in dairy cows. Doepel et al. (2006) examined the effect of an increased

duodenal supply of glutamine (Gln) on immune function and production. Cows received continuous abomasal infusions of water or 300 g/d of Gln for 21 d starting within 48 h of calving. Over the first 3 wk following calving, Gln supplementation had limited effects on immune function as both T cell proliferation and cytokine production from PBMC were not different between the treatments (Doepel et al., 2006). Another study investigated whether administration of Gln would affect mediators of acute phase response in postparturient dairy cows (Jafari et al., 2006) by giving different levels of Gln (0, 106 or 212 g/d) by intravenous infusion for 7 consecutive days starting on d 1 after calving. Cows infused with Gln had greater serum amyloid A, lower haptoglobin, and greater LPS-binding protein in plasma within 21 d postpartum when compared to control, indicating that i.v. administration of Gln modulated acute phase mediators in dairy cows after parturition (Jafari et al., 2006). Although rumen-protected Gln has been already introduced and showed some modulated immune responses in lactating cows (Caroprese et al., 2012), there is little research to examine its effect on immune system during the transition period.

As mentioned in the former section, vitamins have a role in both innate and adaptive immune responses. Therefore, a deficiency of any of these vitamins may depress immunity in transition cows (Spears and Weiss, 2008). Also, supplemental vitamins may improve immune status in dairy cows during the transition period. The relationship between vitamin E and immune status has been well-studied, and its major impact appears to relate to enhanced PMN function (Spears and Weiss, 2008). Improved killing ability of PMN and enhanced function of macrophages, including higher production of IL-1, have been observed in cows that received vitamin E

supplementation (Politis et al., 2001) and vitamin E supplementation prevented the periparturient depression of PMN chemotaxis that occurs in cows that did not receive supplementation (Politis et al., 2001). Because deficiency of vitamin E impairs PMN function, it could explain resistance of cows to mastitis following vitamin E supplementation (Goff, 2006). Indeed increased incidence of mastitis has been observed during the early stages of lactation and coincides with the lowest plasma concentrations of vitamin E (Politis et al., 2001). However, a randomized, controlled field trial with dairy cows demonstrated an adverse effect of vitamin E supplementation during the dry period on mastitis incidence in early lactation (Bouwstra et al., 2010). Although vitamin E supplements increased both absolute vitamin E and the ratio of vitamin E to cholesterol in blood, significantly more cases of subclinical and clinical mastitis occurred in the high supplement group (3,000 IU of vitamin E/cow per day) than the low supplement group, providing135 IU of vitamin E/cow per day (Bouwstra et al., 2010). This result suggests that high amounts of vitamin E may have adverse effects in dairy cows (Bouwstra et al., 2010). Politis (2012) recently reanalyzed data from previously unpublished survey studies and those from published vitamin E feeding trials in which high levels of blood vitamin E were observed. Dairy cows were divided into three groups, depending on blood a-tocopherol levels at dry-off: high (6.25 mg/ml), medium (between 6.25 and 4.25 mg/ml) and low (4.25 mg/ml). Results indicated that there were no differences in the incidence of mastitis between the three groups and the author concluded that supplementation of 3000 IU vitamin E/cow per day in the late dry period remains

recommended because it was generally associated with decreased risk of mastitis (Politis, 2012).

UTERINE INFLAMMATORY DISEASES IN DAIRY COWS

Uterine microbial disease affects half of all dairy cattle after parturition, which could cause infertility by disrupting uterine and ovarian function (Sheldon et al., 2009a). In the last section, the definition and etiology of uterine disease will be discussed, focusing on immunity, nutrition, and metabolism.

Definition

Uterine disease within a week of parturition (metritis) is present in up to 40% of dairy cows (Sheldon et al., 2009a). Subsequently, 15%–20% of cattle have clinical disease that persists beyond 3 wk postpartum (endometritis), and about 30% have chronic inflammation of the uterus without clinical signs of uterine disease, namely cytological endometritis (CE) (Sheldon et al., 2009a).

Definitions of metritis seem to vary. Sheldon et al. (2006) proposed that metritis is defined as an animal with an abnormally enlarged uterus and a fetid watery red-brown uterine discharge, associated with signs of systemic illness (decreased milk yield, dullness or other signs of toxemia) and fever >39.5 C, within 21 days after parturition. Also, it was considered that animals that are not systemically ill, but have an abnormally enlarged uterus and a purulent uterine discharge detectable in the vagina, within 21 days postpartum, may be classified as having metritis (Sheldon et al., 2006). Later, Sheldon et al. (2009a) proposed different criteria: cows are classified as having grade 1 metritis if they have an abnormally enlarged uterus and a purulent

uterine discharge without any systemic signs of ill health; animals with additional signs of systemic illness such as decreased milk yield, dullness, and fever >39.5 C are classified as having grade 2 clinical metritis; animals with signs of toxemia such as inappetance, cold extremities, depression, and/or collapse are classified as having grade 3 metritis, which has a poor prognosis. Clearly, evaluation of metritis requires refined case definitions for appropriate characterization of outcomes.

Endometritis is characterized by the presence of purulent (>50% pus) uterine discharge detectable in the vagina 21 days or more after parturition, or mucopurulent (approximately 50% pus, 50% mucus) discharge detectable in the vagina after 26 days postpartum (Sheldon et al., 2006). A simple grading system based on the character of the vaginal mucus is readily used to evaluate cows with clinical endometritis (Sheldon et al., 2009a). The endometritis grade correlates with the presence of pathogenic organisms associated with uterine disease and is prognostic for the likely outcome of treatment (Sheldon et al., 2009a).

Cytological endometritis (CE) is characterized by inflammation of the endometrium in the absence of signs of clinical endometritis (Sheldon et al., 2009a), which results in a significant reduction in reproductive performance such as lower pregnancy rate, longer median days open, lower pregnancy to first service percentage, and more services before 50% of cows became pregnant compared to cows without CE (Gilbert et al., 2005). Although it was proposed that a cow with CE is defined by >18% PMN in uterine cytology samples collected 21–33 days postpartum, or >10% PMN at 34–47 days, in the absence of clinical endometritis (Sheldon et al., 2006), the cutoff point and optimal time to diagnose CE are still not determined. One study

proposed that CE can be diagnosed if PMN exceeding between 5.5% and 10% of cells in samples collected by flushing the uterine lumen or by endometrial cytobrush, in the absence of clinical endometritis, about 5 wk postpartum (Sheldon et al., 2009a). LeBlanc et al. (2011) mentioned that there is good agreement that more than 5 to 8 % PMN of the total leukocytes and endometrial cells in an endometrial smear at 4 to 5 wk postpartum identifies cows with an undesirable level of inflammation. However, investigating the prevalence of CE 4 h after AI in dairy cows showed that the proportion of PMN had no effect on conception rates in multiparous cows and that first service conception rate was highest for cows with 0%< PMN ≤15% in primiparous cows (Kaufman et al., 2009). Although the incidence of CE is dependent on the cutoff for diagnosis and the time after parturition (Sheldon et al., 2009a), it appears to be in the order of 37%–74% of animals (Gilbert et al., 2005). Further research is required to investigate the appropriate way to diagnose CE.

Etiology

The development of uterine disease depends on the immune response of the cow, as well as the species and number (load or challenge) of bacteria (Sheldon et al., 2006). Uterine defenses rely initially on classical innate immunity and mucosal defense systems rather than adaptive immunity (Wathes et al., 2009). It is reported that cows with the greatest influx of PMN to the uterus on the day of calving had the lowest rates of positive bacterial cultures and prevalence of endometritis later in lactation (Gilbert et al., 2007). One clear contributor to subsequent uterine disease is immune dysfunction around the transition period. Hammon et al. (2006) showed that, for cows that were diagnosed with puerperal metritis and CE, blood PMN functions

were impaired during the periparturient period compared to cows with normal uterine health. Impaired function is not limited to immune cells. The endometrium is known to regulate the inflammatory response after infection by its production and release of cytokines and chemokines (Galvão et al., 2011). Galvao et al. (2011) determined that expression of the TNF-α gene in uterine tissue was decreased in cows with endometritis compared to control cows at calving and at 1 wk postpartum (Galvão et al., 2011). Also, IL-1 gene expression tended to be decreased in cows with endometritis compared to control cows at 1 wk postpartum (Galvão et al., 2011). Therefore, the function of eliminating pathogens in both immune cells and uterus tissue seems to be compromised in cows in uterine disease around the peripartum period.

What causes the immune dysfunction? It may be attributable to hormone change such as increased cortisol (LeBlanc, 2010), prolonged secretion of progesterone (Sheldon et al., 2006), and probably depressed secretion of estrogen (Sheldon et al., 2006; Stephen, 2008). Retained placenta, dystocia, twins and stillbirth can be factors to change the balance between the immune response and the species and numbers of bacteria present (Sheldon et al., 2009b). Cows in the greater lactation may suffer more profound impairment of PMN function than younger cows (Gilbert et al., 1993).

Examining associations between the nutrition and metabolism and uterine disease has become one of the main areas of focus into the causes of the disease. Hammon et al. (2006) reported that cows with CE or metritis had higher NEFA and lower dry matter intake during the periparturient period and higher BHBA during

early lactation compared to cows with normal uterine health. They also demonstrated a negative correlation between PMN function (myeloperoxidase activity) and NEFA concentration during the first week after calving in the study (Hammon et al., 2006). Wathes et al. (2009) found that cows in severe negative balance were still undergoing an active uterine inflammatory response even at 2 wk postpartum, whereas cows in mild negative EB had more fully recovered from their energy deficit with their endometrium reaching a more advanced stage of repair, as evidenced by the finding that inflammatory response genes in endometrium in severe negative EB were upregulated at 2 wk postpartum. Further, Cheong et al. (2011) showed that one of risk factors for CE was ketosis (the odds ratio: 3.83) in a study where 779 cows from 38 herds were used in the analysis. Collectively, these results provide evidence that there is the association between negative EB and uterine health disorders and indicate that negative EB prevents cows from mounting an effective immune response to the microbial challenge experienced after calving, prolonging the time required for uterine recovery and compromising subsequent fertility (Wathes et al., 2009).

Metabolism of oxidants can be a factor for uterine disease. As described above, oxidative stress is increased during the transition period due to high demand of metabolism (Sordillo and Aitken, 2009) and may be a major underlying cause of inflammatory and immune dysfunction in dairy cattle (Sordillo and Aitken, 2009). Therefore, unbalanced oxidative status could contribute to uterine disease; however, few studies have reported any specific associations between the two. One study reported that plasma malondialdehyde concentration, which is an indicator of fat peroxidation, was higher in cows with postpartum metritis than in healthy cows (Kizil

et al., 2010). Although it is not uterine inflammatory disease, another reported that plasma antioxidant capacity was lower at 2 wk prepartum and near calving in cows with retained placenta (Miller et al., 1993). On the other hand, many of micronutrients play a critical role as antioxidants as mentioned in the former section. Therefore, the status of the micronutrients could be the factor for uterine disease. Kizil et al. (2010) reported that GSH-Px activities, beta-carotene, and vitamins (A, E, and C) in plasma were significantly depressed in cows with postpartum metritis. LeBlanc et al. (2004) showed that an increase in α-tocopherol of 1 μg/mL in the last week prepartum reduced the risk of retained placenta by 20%. Those results indicate that micronutrients may affect the development of uterine disease; however, little study has been conducted to examine the correlation between supplemental micronutrients and uterine inflammatory disease.

HYPOTHESES AND OBJECTIVES

The transition period is a critical time to affect performance in dairy cows and directly impacts benefit in dairy farmers. A variety of nutritional strategies have been investigated to optimize health conditions in cows in the period; however, the main topic has been always focused on macronutrient metabolism. Trace elements have started to attract attention because of their potential role in metabolism and immune system function and it is possible that they become an integral part of successful transition period in dairy cows.

Considering the potential role of trace minerals in oxidative status and immune system function, supplementation of those minerals during the transition period could

result in better oxidative balance and improved immune system. To be more specific, oxidative parameters, which are expected to be increased during the transition period, could be changed and the incidence of CE, which can be affected by immune status, could be decreased. If the forms of trace minerals have better bioavailability, the effects could be ever more prominent.

Although there is no required level of Cr, its effect on metabolism and immunity have been demonstrated in dairy cattle. Therefore, supplemental Cr during the transition period, which is the most challenging time for dairy cows, could be beneficial to a cow's health. Metabolic parameters could be improved and the incidence of CE could be affected with Cr supplementation through its modulating effect on immune system.

The associations of energy metabolism with immune functions and subsequent uterine health have been documented. Feeding either higher starch diets or adding ionophores such as monensin during the early postpartum period may improve energy supply and enhance immune functions, thereby decreasing susceptibility to CE.

Therefore, the objectives of the research conducted and described in this dissertation were:

First, to evaluate a new source of trace minerals with potentially higher bioavailability than other inorganic sources and determine whether trace mineral source affects aspects of oxidative metabolism, CE, and performance of cows during the transition period and early lactation;

Second, to evaluate the effects of Cr propionate supplementation to cows during the periparturient period and early lactation on metabolism, performance, and the incidence of cytologically-diagnosed endometritis in dairy cows;

Third, to evaluate the effect of including monensin, an ionophore, in diets of differing starch levels during the immediate postcalving period on incidence of CE and immune function.

And lastly, to evaluate associations of CE with energy metabolism and inflammation during the transition period and early lactation.

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CHAPTER THREE: EFFECTS OF TRACE MINERAL SOURCE ON OXIDATIVE METABOLISM, ENDOMETRITIS, AND PERFORMANCE OF TRANSITION DAIRY COWS[1]

ABSTRACT

Multiparous Holstein cows (n=60) were used to determine effects of trace mineral source on oxidative metabolism, cytological endometritis, and performance of transition cows. After a 1-wk pretreatment period, cows were assigned randomly to one of three dietary treatments from 21 d before expected calving through 84 d post calving. Dietary treatments administered by daily topdress included: 1) Inorganic sources based upon sulfates of zinc (Zn), copper (Cu), and manganese (Mn; ITM); 2) a blend (75:25) of sulfates and chelated sources of Zn, Cu, and Mn (ITM/OTM); and 3) hydroxy trace minerals (HTM) of Zn, Cu, and Mn. The resulting dietary concentrations of supplemental Zn, Cu, and Mn were similar among treatments and averaged 40, 10, and 27 ppm, respectively, before calving and 59, 15, and 40 ppm, respectively, after calving. An interaction of treatment and week existed for milk yield such that cows fed HTM increased milk yield faster than cows fed the other two treatments; a similar interaction was also present for yields of fat-corrected milk and lactose. Cows fed HTM during the prepartum period tended to have higher body weight and had higher body weight during the postpartum period than those fed either of the other two treatments. Plasma antioxidant capacity was lower in cows fed HTM

[1] Research material published in part as abstract in 2012 (Yasui et al., 2012)

than ITM during both prepartum and postpartum periods; cows fed ITM/OTM had intermediate values. Cows fed HTM tended to have lower concentrations of plasma thiobarbituric acid reactive substances than those fed ITM during whole study period. Plasma haptoglobin was lower in cows fed HTM than ITM/OTM at 1 wk postpartum. Endometrial cytology as characterized by low volume uterine lavage at 7 d postcalving and on one day between 40 and 60 d post calving was not affected by treatment. Effects of treatments were not significant for calving to conception within 150 days in milk. In conclusion, supplementation with HTM sources of Zn, Cu, and Mn resulted in evidence of improved productive performance during early lactation along with modulation of plasma variables related to oxidative metabolism and inflammatory response compared to supplementation with sulfate-based sources; however, source of trace minerals did not affect uterine health in this experiment.

INTRODUCTION

Trace minerals are critical for immune function, oxidative metabolism, nutrition and energy metabolism, and reproductive function in dairy cows (NRC, 2001; Spears and Weiss, 2008). The specific roles of zinc (Zn), copper (Cu), and manganese (Mn) have received substantial attention in dairy cows, and studies have been conducted to evaluate effects of Zn, Cu, and Mn on productive and reproductive performance (Siciliano-Jones et al., 2008; Formigoni et al., 2011; Nemec et al., 2012). Furthermore, decreased Zn status was demonstrated to be associated with higher rates of metritis, mastitis, and retained placenta (Enjalbert et al., 2006). Recently, supplementation of organic chelates of Zn, Cu, and Mn in lactating cows was reported

to enhance both innate and humoral immune responses compared to supplementation as inorganic sulfates (Nemec et al., 2012).

Oxidative stress is increased during the transition period due to high demands of metabolism (Sordillo and Aitken, 2009) and likely contributes to periparturient disorders including metabolic diseases (Sharma et al., 2011). Bernabucci et al. (2005) reported that cows with higher plasma BHBA and NEFA had higher concentrations of reactive oxygen metabolites and thiobarbituric acid-reactive substances (TBARS) and lower levels of antioxidants in plasma during the transition period. Furthermore, Sordillo and Aitken (2009) suggested that oxidative stress during the transition period can be a major underlying cause of inflammatory and immune dysfunction in dairy cattle and that a significant relationship exists between antioxidant supplementation and decreased incidence of diseases around parturition. Because of their antioxidant function, trace minerals can contribute to counterbalancing oxidative stress, which could reduce metabolic and immune problems during the transition period.

Cytological endometritis (CE) is characterized by inflammation of the endometrium that results in a significant reduction in reproductive performance in the absence of signs of clinical endometritis (Sheldon et al., 2009). The incidence of CE was reported to be 37–74% of dairy cows (Gilbert et al., 2005; Sheldon et al., 2009). Work conducted in five commercial dairy farms demonstrated that the incidence of CE was high (~53% of cows between 40 and 60 DIM) across those farms and that this had profoundly negative effects on first service conception rate and median days to pregnancy (Gilbert et al., 2005). Given the role of trace minerals in immune function,

it is possible that their interaction with immunity and hence CE may underpin some of the responses of reproduction to trace mineral supplementation outlined above.

Substantial interest has focused on the potential to enhance function of dairy cows by feeding trace mineral sources with greater bioavailability (NRC, 2001; Andrieu, 2008) or to maintain high function by feeding lower overall levels of trace minerals using these more bioavailable sources (Nocek et al., 2006). Indeed, recent research conducted by our research group suggested that trace mineral amount and source can modulate aspects of oxidative metabolism (Yasui et al., 2009). Hydroxy trace mineral sources (Micronutrients, Inc. Indianapolis, IN) have shown higher bioavailability than sulfates in steers (Spears et al., 2004) and weanling pigs (Fry et al., 2012) and recently have become available for potential use in the dairy industry; however, research has not been conducted to determine how these sources compare with either sulfates or currently available chelated sources of trace minerals in dairy cows. In addition, the role of these and other trace mineral sources in the etiology of CE has not been elucidated. Therefore, the objectives of this experiment were to evaluate a new source of trace minerals with potentially higher bioavailability and determine whether trace mineral source affects aspects of oxidative metabolism, CE, and performance of cows during the transition period and early lactation.

MATERIALS AND METHODS

Experimental Animals, Treatments, and Procedures

All procedures involving animals were approved by the Cornell University Institutional Animal Care and Use Committee prior to the onset of the experiment.

Sixty Holstein cows entering second lactation or greater from the Cornell University Teaching and Research Center Dairy were enrolled in this experiment at 28 d before expected calving and assigned to one of three topdress treatments with randomization restricted by previous lactation 305-d mature-equivalent milk production. Treatments were initiated 21 d before expected calving and continued through 84 d post calving: 1) Inorganic sources based upon sulfates of Zn, Cu, and Mn (ITM); 2) a blend (75:25) of sulfates and chelated sources of Zn, Cu, and Mn (ITM/OTM); and 3) hydroxy trace minerals (HTM) of Zn, Cu, and Mn (IntelliBond; Micronutrients, Inc., Indianapolis, IN). The blend ratio for the ITM/OTM treatment was typical of a common strategy for trace mineral supplementation in the dairy industry. The target concentrations of supplemental Zn, Cu, and Mn across treatments were the same for both prepartum and postpartum periods: 60, 15, and 40 ppm, respectively. Based upon actual DMI of cows during the prepartum and postpartum periods, actual concentrations of supplemental Zn, Cu, and Mn for the prepartum period were essentially the same among treatments: 40, 10, and 27 ppm, respectively. Actual concentrations of supplemental Zn, Cu, and Mn for the postpartum period were similar among treatments: 59, 15, and 39 ppm for ITM treatment; 61, 15, and 41 ppm for ITM/OTM treatment; and 58, 14, and 39 ppm for HTM treatment. Cows were housed in tiestalls for the duration of the experiment. Ingredient and chemical composition of the diets fed during the experiment are described in Table 3-1. Formulated dietary ingredients and composition were typical of those fed in the Northeastern U.S., except that the mineral supplements fed omitted Zn, Cu, and Mn as minerals targeted for treatment and also omitted Se, Co, and I. All three experimental topdress premixes also contained Se, Co, and I at targeted

Table 3-1. Ingredient and chemical composition (DM basis, % of DM unless otherwise noted) of experimental diets.

Item	Prepartum diet	Postpartum diet
Corn silage, processed	42.40	39.16
Wheat straw	25.12	2.04
Legume silage	-	16.31
Ground shelled corn	-	7.51
Soybean meal	-	5.10
Wheat middlings	-	4.52
Corn germ meal	8.18	3.60
Distillers grain (with solubles)	6.99	5.12
Citrus pulp	-	2.99
Cereal fines	-	2.94
Soy Chlor[1]	3.26	-
Amino Plus[2]	3.22	-
Soybean hulls	3.15	1.79
Canola meal	3.21	2.59
Blood meal	-	1.31
Molasses	1.04	1.19
Bypass fat[3]	-	1.10
Alimet[4]	-	0.10
Urea	-	0.33
Calcium carbonate	2.18	0.84
Calcium sulfate	0.56	-
Sodium Bicarbonate	-	0.82
Potassium-magnesium-sulfate[5]	0.23	-
Potassium chloride	-	0.08
Mono dicalcium phosphate	0.20	-
Salt	0.11	0.40
Magnesium oxide	0.09	0.11
Vitamin A, D, E premix[6]	0.05	0.03
Vitamin E premix[7]	0.02	-
Rumensin[8]	0.01	0.01
Biotin 1%	-	0.06
Chemical composition (±SD[9])		
CP	12.4 (1.02)	16.8 (0.52)
Soluble protein (% of CP)	32.7 (5.34)	43.3 (3.84)
ADF	29.1 (1.96)	21.4 (0.71)
NDF	47.6 (3.9)	33.9 (1.1)
Starch	18.1 (1.9)	24.7 (1.3)
Sugar	4.38 (0.89)	5.70 (0.80)
Ether extract	2.95 (0.33)	3.80 (0.16)
Ash	7.30 (0.44)	6.50 (0.47)
Ca	1.25 (0.10)	0.84 (0.06)

Table 3-1 (Continued)

P	0.39 (0.03)	0.42 (0.01)
K	1.12 (0.05)	1.47 (0.06)
Mg	0.35 (0.01)	0.32 (0.02)
Na	0.14 (0.03)	0.45 (0.04)
Cl	0.52 (0.02)	0.48 (0.07)
S	0.29 (0.03)	0.24 (0.01)
Fe (ppm)	232.2 (21.8)	205.1 (26.0)
Zn (ppm)	39.4 (3.7)	43.1 (6.7)
Cu (ppm)	6.0 (1.4)	7.7 (1.3)
Mn (ppm)	35.0 (4.3)	35.1 (5.2)
DCAD, mEq/100g DM[10]	2.0 (2.2)	29.0 (1.6)

[1]Anionic feed supplement; West Central, Ralston, IA.
[2]Rumen undegradable protein supplement; AGP Inc., Omaha, NE.
[3] Prilled saturated fatty acid; Cargill, Minnetonka, MN.
[4]D,L-2-hydroxy-4-methylthiobutanoic acid; Novus International Inc., St. Louis, MO.
[5]Contained 18.8% potassium, 11.5% magnesium, and 22.9% sulfate.
[6]Contained 30,464 IU/kg of Vitamin A, 5,862 IU/kg of Vitamin D, and 93,784 IU/kg of Vitamin E.
[7]Contained 499,400 IU/kg of Vitamin E.
[8]Contained 176 g/kg of monensin sodium, Elanco Animal Health, Greenfield, IN.
[9]Standard deviation calculated from 5 and 7 samples for prepartum and postpartum diet, respectively.
[10]Calculated as mEq [(Na + K) - (Cl + S)] / 100 g DM (NRC, 2001).

concentrations in the final TMR of 0.3 ppm (added), 0.4 ppm (added) and 0.8 ppm (added), respectively. 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 0800 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 Cornell Net Carbohydrate and Protein System/Cornell-Penn-Miner profiles by a commercial laboratory (Cumberland Valley Analytical Services, Hagerstown, MD). Samples were analyzed for DM (method 930.15; AOAC, 2000), CP (method 990.03; AOAC, 2000), soluble protein (Krishnamoorthy et al., 1982), ADF (method 973.18; AOAC, 2000), NDF (Van Soest et al., 1991), starch (Hall, 2009), sugar (DuBois et al., 1956), ether extract (method 2003.05; AOAC, 2006), ash (method 942.05; AOAC, 2000), and minerals (method 985.01; AOAC, 2000).

Cows were milked twice daily (0900 and 2100 h) and milk yields were recorded at all milkings for the 84-d postpartum treatment period. Milk samples were collected on the same day each week from both 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, true protein, lactose, total solids, and MUN using infrared analysis (method 972.160; AOAC, 2000), and SCC by an optical fluorescent method (method 978.26; AOAC, 2000). Body condition scores (BCS, 1 to 5 scale; Wildman et al., 1982) and body weights (BW) were measured weekly from the preliminary period through the whole treatment period. Gait scores were measured once during the week before assignment to treatment and at 4, 8, and 12 wk postpartum using a five-point scale (1 = normal to 5 = severely lame; Sprecher et al. 1997).

Plasma Sampling and Analyses

Samples of blood were obtained from each cow via coccygeal blood vessel puncture on one day before assignment to treatment and once weekly from 21 d prepartum through 84 d postpartum. Plasma was harvested following centrifugation (2,800 × g for 15 min at 4°C), snap-frozen in liquid N_2, and stored at −20°C until analyses. Plasma samples collected from -3 wk prepartum through 12 wk postpartum were analyzed for total antioxidant capacity (TAC) and TBARS. Plasma samples collected from 1 wk postpartum through 8 wk postpartum were analyzed for concentrations of haptoglobin (Hp). Plasma TAC was measured colorimetrically using a commercial kit (Antioxidant Assay kit, Cayman Chemical Company, Ann Arbor, MI). The assay relied on the ability of antioxidants present in the samples to inhibit the oxidation of ABTS (2, 2′-azino-di-[3-ethylbenzthiazoline sulfonate]). The capacity to prevent ABTS oxidation was compared with a water-soluble tocopherol analogue and was expressed as millimolar concentrations. Plasma TBARS was measured fluorometrically using a commercial kit (TBARS Assay kit, Cayman Chemical Company, Ann Arbor, MI). In the

assay, an adduct formed by the reaction of malondialdehyde in the samples and thiobarbituric acid under high temperature and acidic conditions was measured and expressed as micromolar concentrations. Plasma concentrations of Hp were measured by enzymatic analysis (Phase Range Haptoglobin Assay, Tridelta Diagnostics Ltd., Morris Plains, NJ). In the assay, haemoglobin peroxidase activity preserved by the bound of Hp in the samples to haemoglobin, which was directly proportional to the amount of Hp, was converted to milligrams per milliliter of Hp by comparison to a standard curve. Spectrophotometric and fluorometric measurements were conducted using a Versamax tunable microplate reader (Molecular Devices, Sunnyvale, CA) and Synergy HT Multi-Mode microplate reader (BioTek instruments, Inc., Winooski, VT), respectively.

Endometrial Cytology and Reproductive Outcomes

Evaluation of endometrial cytology by low volume lavage (Gilbert et al., 2005) was determined on all cows at 7 d postcalving (1st lavage) and on one day between 40 and 60 d (2nd lavage) post calving as previously described (Cheong et al., 2011). The perineum of the cow was cleansed and a 64-cm Flex Tip sterile plastic infusion pipette (Exodus Breeders Corp., York, PA) was manipulated through the cervix into the uterus. Sterile saline solution (20 mL) was injected into the uterus and agitated gently via the rectum; then a sample of the fluid was aspirated. The recovered fluid was centrifuged using a cytocentrifuge directly onto a glass slide. After drying, the slides were fixed and stained using a rapid Romanowsky-type staining procedure and examined at 400X magnification. Two hundred cells were counted from each slide, and results were expressed as the percentage of polymorphonuclear neutrophils (PMN) in total cells (excluding erythrocytes). All the slides were read masked to

treatment by the same investigator (TY). The percentage of PMN was compared among treatments as a continuous variable for both 1st and 2nd lavage. Incidence of CE diagnosed with cut-off point of 10% PMN (Cheong et al., 2011) was dichotomously analyzed in 2nd lavage. Time to conception within the first 150 DIM was analyzed using Kaplan-Meier approaches as described below.

Statistical Analyses

Data from several cows were removed prior to data analysis because of health reasons. One cow assigned to ITM/OTM was removed from the entire study because she stepped on two teats during the prepartum period; therefore, the number of cows available for the prepartum analysis was 20, 19, and 20 for ITM, ITM/OTM, and HTM, respectively. In addition, 5 cows were removed from the postpartum dataset due to severe health problems following parturition: 1 cow from ITM was removed because of the development of retained placenta, ketosis, and displaced abomasum; 3 cows were removed from ITM/OTM (one cow because of ketosis and hypocalcaemia; one cow due to ketosis, mastitis, and displaced abomasum; and one cow because of retained placenta and displaced abomasum); and 1 cow from HTM because of dystosia, retained placenta, and fever.

Data for DMI and milk yield were reduced to weekly means prior to analysis. Baseline values collected during the pretreatment week prior to assignment to treatment were used as covariates for DMI, BW, BCS, gait score, plasma TAC, and plasma TBARS. Previous 305-d mature equivalent milk yields were used as covariates for milk yield and milk composition. Weekly variables were analyzed using the MIXED procedure of SAS version 9.1 (SAS Institute, Cary, NC) for a completely randomized design with repeated measures. Model terms

were the fixed effects of covariate, treatment, week, and the interaction of treatment and week. The random effect was cow nested within treatment. For each weekly 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. Covariates were retained in the model if $P < 0.10$.

The percentage of PMN in the total cells in low volume lavage was analyzed as a continuous variable for data collected during the 1st and 2nd lavage. Incidence of CE was analyzed as a dichotomous variable using the cutpoint described above for 2nd lavage. Kaplan-Meier survival analysis (Kaplan and Meier, 1958) was conducted to produce survival curves of treatments effects on calving to conception within 150 DIM. Statistical significance was declared at $P < 0.05$ and trends were discussed at $0.05 < P < 0.10$. Least squares means and standard error of the mean were reported throughout.

RESULTS

Production results are presented in Table 3-2. Overall effects of treatment on DMI, milk yield, and milk components were not significant; however, an interaction of treatment and week existed ($P = 0.02$) for milk yield such that cows fed HTM

Table 3-2. Least squares means for productive performance and gait score in cows fed varying sources of Zn, Cu, and Mn during the periparturient period and early lactation.

Item	Treatment			SEM[4]	*P*–value		
	ITM[1]	ITM/OTM[2]	HTM[3]		Trt[5]	Wk[6]	Trt x Wk
DMI, kg/d							
Prepartum	15.0	14.8	14.8	0.3	0.87	<0.001	0.68
Postpartum	25.4	24.5	25.8	0.5	0.18	<0.001	0.93
Milk,[7] kg/d	45.3	45.7	46.6	1.1	0.65	<0.001	0.02
Fat. %	3.35	3.29	3.29	0.06	0.67	<0.001	0.92
Fat, kg/d	1.48	1.45	1.49	0.05	0.80	<0.001	0.11
Protein, %	3.01	2.96	3.01	0.05	0.75	<0.001	0.71
Protein, kg/d	1.34	1.33	1.37	0.04	0.66	0.002	0.19
Lactose, %	4.71	4.70	4.66	0.04	0.53	<0.001	0.94
Lactose, kg/d	2.14	2.16	2.18	0.05	0.83	<0.001	0.05
SCC (x 1000)	347	285	382	67	0.56	0.71	0.74
Urea N, mg/d	11.0	11.8	11.2	0.3	0.14	<0.001	0.84
Total solids, %	12.0	11.9	11.9	0.1	0.65	<0.001	0.67
Total solids, kg/d	5.38	5.35	5.46	0.11	0.74	<0.001	0.16
3.5% FCM,[8] kg/d	43.6	43.3	44.3	1.2	0.80	<0.001	0.03
ECM,[9] kg/d	43.2	42.9	44.0	0.9	0.62	<0.001	0.18
BW, kg							
Prepartum	719[b]	740[ab]	765[a]	14	0.07	0.69	0.48
Postpartum	638	644	682	14	0.04	<0.001	0.19
BCS							
Prepartum	3.34	3.44	3.37	0.07	0.56	0.02	0.99
Postpartum	2.86	2.79	2.86	0.05	0.59	<0.001	0.84
Gait score	1.8	1.8	1.6	0.1	0.31	0.51	0.93

[a-b]Means within a row with different lower-case superscripts differ (P < 0.05).

[1]ITM = inorganic sources based upon sulfates.

[2]ITM/OTM = a blend (75:25) of sulfates and chelated sources.

[3]HTM = hydroxy trace minerals.

Table 3-2 (Continued)

[4]SEM = standard error of the mean.
[5]Trt = treatment.
[6]Wk = week.
[7]Represents milk yields collected daily from parturition through 84 d postpartum and then reduced to weekly means prior to analysis.
[8]FCM = (0.4324 × kg of milk) + (16.216 × kg of milk fat).
[9]ECM = [(0.323 × kg of milk) + (12.82 × kg of fat) + (7.13 × kg of protein)].

increased milk yield faster than cows fed the other two treatments (Figure 3-1). This interaction was also present and similar for yields of 3.5% FCM ($P = 0.03$) and lactose ($P = 0.06$; data not shown). Content and yield of milk fat, true protein, and total solids were not affected by treatment, and neither somatic cell count nor concentrations of milk urea N were affected by treatment (Table 3-2). Cows fed HTM had higher BW than those fed ITM during the prepartum period ($P = 0.02$) and than those fed other two treatments during the postpartum period ($P = 0.04$; Table 3-2). BCS were not affected by treatment during either the prepartum or postpartum periods. Gait scores were not affected by treatment.

Analyses conducted in plasma suggested that trace mineral source modulated aspects of oxidative metabolism. Plasma TAC was lower in cows fed HTM than ITM treatment during whole, prepartum, and postpartum study period ($P = 0.03$, 0.03, and 0.04, respectively; Table 3-3). Although effects of treatment on plasma TBARS were not significant when assessed separately by prepartum and postpartum periods, cows fed HTM tended ($P = 0.07$) to have lower plasma TBARS than those fed ITM when analyzed across the entire study period (Table 3-3). Although effects of treatments on plasma Hp were not significant when assessed using all weekly data from wk 1 through 8 postpartum, there was a tendency ($P = 0.10$) for lower plasma Hp in cows fed HTM than those in ITM/OTM treatment during wk 1 postpartum (Table 3-3).

The percentages of PMN in both 1st and 2nd lavage were not affected by treatments (Table 3-4). One 2nd lavage from one cow fed the ITM/OTM treatment was not obtained because of cervix deformity for unknown reason, which resulted in 15 cows total for the ITM/OTM treatment. There were no treatment effects on

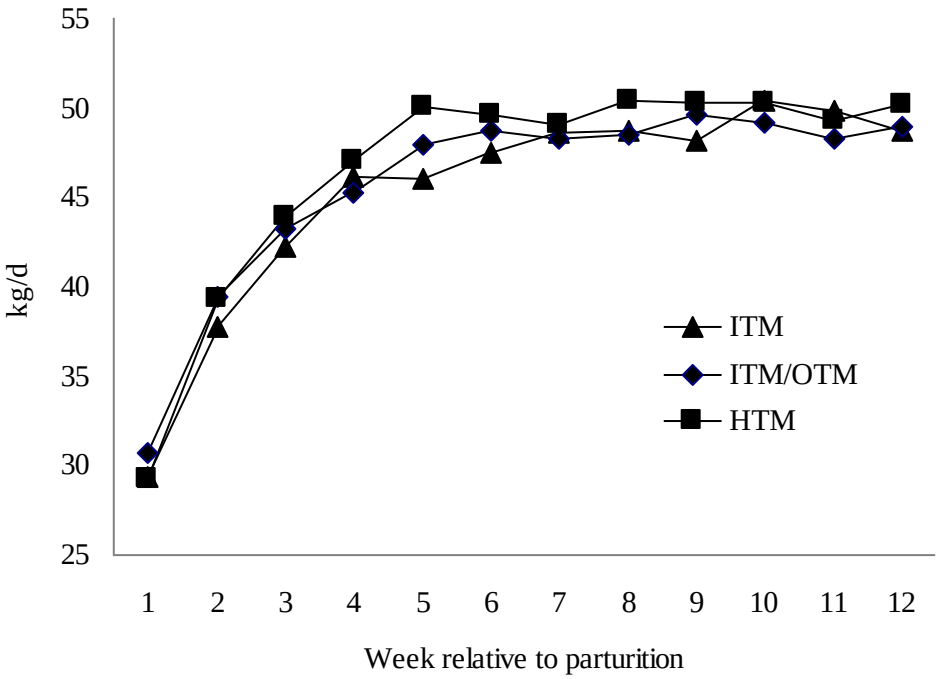

Figure 3-1. Milk yield for cows fed three sources of trace minerals during the transition period and early lactation. Values are least square means, n = 19 for ITM (inorganic sources based upon sulfates), n = 16 for ITM/OTM (75:25 blend of sulfates and chelated sources), and n = 19 for HTM (hydroxy trace minerals); SEM averaged 1.7 kg/d; the *P*-value for the interaction of treatment × week was 0.02.

Table 3-3. Plasma total antioxidant capacity (TAC), thiobarbituric acid reactive substances (TBARS), and haptoglobin (Hp) in cows fed varying sources of Zn, Cu, and Mn during the periparturient period and early lactation.

	Treatment				*P*-value		
Item	ITM[1]	ITM/OTM[2]	HTM[3]	SEM[4]	Trt[5]	Wk[6]	Trt x Wk
TAC, mM							
Whole study period[7]	2.14[a]	2.07[ab]	1.93[b]	0.07	0.09	<0.001	0.94
Prepartum period[8]	2.09[a]	1.93[ab]	1.84[b]	0.08	0.09	0.01	0.85
Postpartum period[9]	2.16[a]	2.09[ab]	1.95[b]	0.08	0.12	<0.001	0.87
TBARS, uM							
Whole study period[7]	2.11[A]	1.98[AB]	1.95[B]	0.07	0.16	<0.001	0.40
Prepartum period[8]	1.47	1.49	1.38	0.06	0.37	0.16	0.74
Postpartum period[9]	2.26	2.11	2.10	0.09	0.29	<0.001	0.23
Hp, mg/mL							
Postpartum period[10]	0.83	0.91	0.78	0.09	0.55	<0.01	0.66
1 wk postpartum[11]	1.05[AB]	1.26[A]	0.90[B]	0.16	0.25	-	-

[a-b]Means within a row with different lower-case superscripts differ ($P < 0.05$).
[A-B]Means within a row with different upper-case superscripts tend to differ ($P < 0.1$).
[1]ITM = inorganic sources based upon sulfates.
[2]ITM/OTM = a blend (75:25) of sulfates and chelated sources.
[3]HTM = hydroxy trace minerals.
[4]SEM = standard error of the mean.
[5]Trt = treatment.
[6]Wk = week.
[7]Represents plasma samples collected weekly from 3 wk prepartum through 12 wk postpartum.
[8]Represents plasma samples collected weekly from 3 wk prepartum through 1 wk prepartum.
[9]Represents plasma samples collected weekly from 1 wk postpartum through 12 wk postpartum.
[10]Represents plasma samples collected weekly from 1 wk postpartum through 8 wk postpartum.
[11]Represents plasma samples collected from 1 wk postpartum only.

Table 3-4. Percentages of neutrophils in 1st and 2nd uterine lavage and incidence of cytological endometritis (CE) in 2nd uterine lavage from cows fed varying sources of Zn, Cu, and Mn during the periparturient period and early lactation.

	Treatment			SEM[4]	*P*-value
Item	ITM[1]	ITM/OTM[2]	HTM[3]		Trt[5]
% of PMN[6]					
1st lavage[7]	40.4	40.2	41.2	6.2	0.99
2nd lavage[8]	12.5	9.9	8.9	2.4	0.48
Cows with CE, n[9]	7	5	7	-	0.97
Cows without CE,	12	10	12		

[1]ITM = inorganic sources based upon sulfates.
[2]ITM/OTM = a blend (75:25) of sulfates and chelated sources.
[3]HTM = hydroxy trace minerals.
[4]SEM = standard error of the mean.
[5]Trt = treatment.
[6]Percentage of polymorphonuclear neutrophils (PMN) in total 200 cells, excluding erythrocytes.
[7]Lavage at 7 d postpartum.
[8]Lavage at one day between 40 and 60 d postpartum.
[9]Number of cows that developed CE (cytological endometritis diagnosed positive if more than 10% PMN were counted in 2nd lavage).

incidence of cytological endometritis assessed at 2nd lavage (Table 3-4). The risk for conception from calving through 150 DIM was not affected by treatment ($P = 0.31$; Figure 3-2).

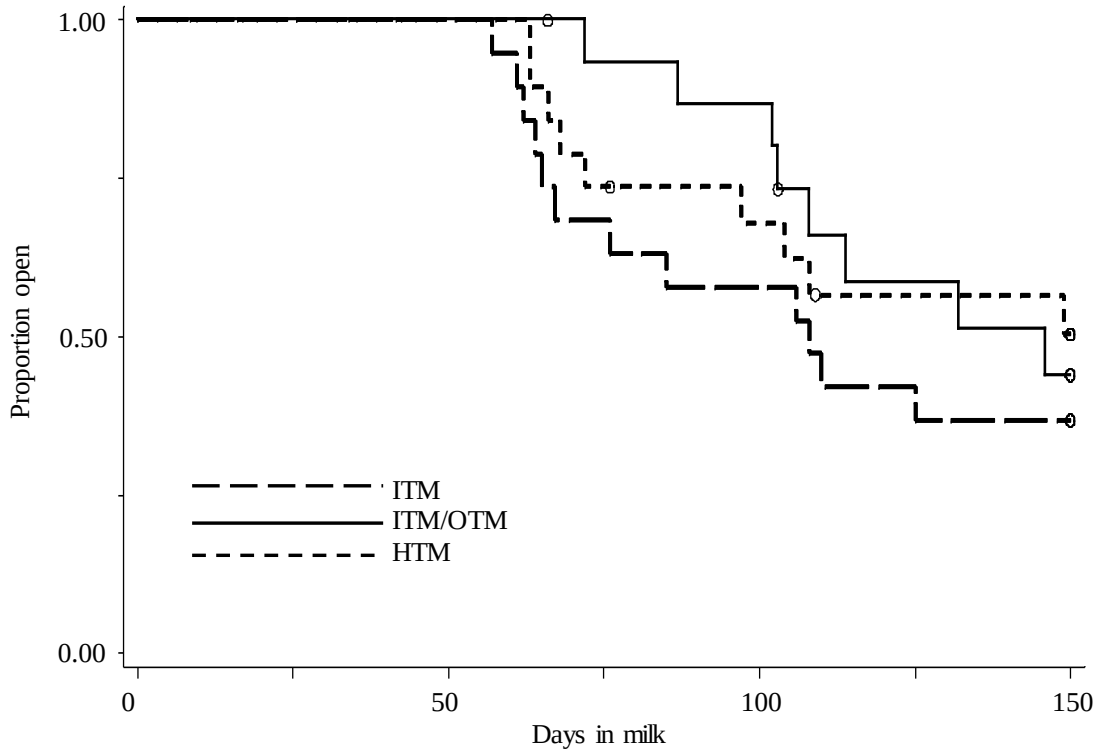

Figure 3-2. Kaplan-Meier survival analysis for treatments effects on calving to conception within 150 days in milk for cows fed three sources of Zn, Cu, and Mn during the periparturient period and early lactation. ITM = inorganic sources based upon sulfates, ITM/OTM = 75:25 blend of sulfates and chelated sources, and HTM = hydroxy trace minerals. Circles show censored cows, which came off from the reproduction record or had not been pregnant within 150 days in milk. Treatments did not affect calving to conception interval ($P = 0.31$).

DISCUSSION

Target and final concentrations of supplemental Zn, Cu, and Mn were essentially identical across the three treatments during the postpartum period. During the prepartum period, the final concentrations of supplemental Zn, Cu, and Mn were lower than targeted because cows consumed more of the prepartum diet than anticipated. However, prepartum DMI was essentially identical across the three treatments; therefore, concentrations of the trace minerals of interest were decreased proportionally for cows fed all three treatments.

Cows fed HTM increased both milk (Figure 3-1) and FCM yield (data not shown) faster than cows fed the other two treatments. The trace minerals Cu, Mn, and Zn, are incorporated into a variety of proteins and enzymes that are involved in a wide range of physiological processes (Nemec et al., 2012). Therefore, the deficiency or supplementation of those minerals can affect productive performance in dairy cows. Also, because total amount of individual trace minerals accumulated in fetus generally increase as fetal development increases during the late pregnancy in dairy cows (House and Bell, 1993) and Zn, Mn, and Cu status of cattle is lowest during late gestation and early lactation (Siciliano-Jones et al., 2008), effects of trace minerals could be more prominent during the transition period. The different response among treatments immediately after calving such as faster increased milk production in cows fed HTM may support the assumption. In addition, our results suggest greater overall responses to HTM than sulfate-based sources or the blend of chelated- and sulfate-based sources chosen in this study. Bioavailability is important factor to determine

effect of trace minerals. The organic forms are considered to have greater bioavailability than inorganic forms (Ammerman et al., 1995) and have been used in dairy cow experiments, resulting in significant improvements in udder health, lameness, and productive and reproductive performance (Andrieu, 2008). Indeed, both Ballantine et al. (2002) and Nocek et al (2006) reported that cows fed organic forms of Zn, Cu, Mn, and Co had increased milk yields during early lactation. One difference between these studies was treatment period; the period in the former study was from 21 d prior to calving through 250 d of lactation (Ballantine et al., 2002), whereas experimental periods in the latter study were dry period 1, full lactation 1, dry period 2, and 200 d into the subsequent lactation (lactation 2); the increased lactational performance was recognized in lactation 2 (Nocek et al., 2006). Because change in milk performance was recognized in early lactation when treatments started even from 3 wk before parturition in both Ballantine et al. (2002) study and the current study, supplementation of trace minerals during the prepartum period may be important to affect milk performance in the early postpartum period. Another difference among these studies was the level of supplemental non-inorganic trace minerals fed. Levels in the current study were similar to those of Nocek et al. (2006); however, those fed by Ballantine et al. (2002) were much lower (13, 5, and 7 ppm for Zn, Cu, and Mn, respectively) than the current study, although total levels of trace minerals (basal diets plus supplements) were similar among the three studies. However, total levels of trace minerals may also affect productive response; among studies, where the same portion of the inorganic Zn, Cu, and Mn was replaced with organic trace mineral, the responses of productive performance were different: significantly increased milk

production (Ballantine et al., 2002); increased milk production only at peak period (Kincaid and Socha, 2004); and tendency for increased milk yield (Siciliano-Jones et al., 2008). The total levels of trace minerals for Zn, Cu, and Mn for those studies were: 155, 23, and 119 ppm; 75, 20, and 67 ppm; and 102, 21, and 86 ppm, respectively. Therefore, the different response might have resulted partially from difference of total intake of trace minerals. Although our results in the present experiment indicate that HTM had favorable effects on milk yield during early lactation, further research is needed to investigate effects of total and supplemental levels of trace minerals as well as varying levels of different forms of trace minerals on productive performance.

Percentages and yield of milk components generally were not affected by treatments in the current study; however, interactions of treatment and week existed for both 3.5% FCM and lactose yields consistent with the same interaction for milk yield. Few studies have reported effects of supplemental trace minerals on milk composition. The responses to feeding a mixture of organic trace minerals vary across studies: no response for percentages and yield of milk components (Uchida et al., 2001); increased percentage of protein (Kincaid and Socha, 2004); increased yield of protein (Siciliano-Jones et al., 2008); and increased yield of fat and protein (Ballantine et al., 2002; Kellogg et al., 2003; Griffiths et al., 2007). The reason for the discrepancy among studies is unknown. A meta-regression analysis showed that milk fat and protein percentages were not significantly influenced by supplementation with a mix of organic trace minerals, whereas protein and fat yields in milk were increased by the mix (Rabiee et al., 2010); however, those production outcomes were heterogeneous, indicating that the responses to supplementation with organic trace minerals are not

uniform (Rabiee et al., 2010). Factors that created the inconsistency included the use of other supplements such as monensin and duration of treatment before and after calving (Rabiee et al., 2010). Therefore, those factors may affect responses of milk components to trace mineral supplementation among studies.

The HTM treatment affected BW such that it increased BW during the prepartum period compared to ITM treatment and during the postpartum period compared to other two treatments (Table 3-2), even though DMI and milk yield were not affected by treatments. Few studies have shown effects of trace mineral status on BW in dairy cows. Neither Nocek et al. (2006) nor Kincaid and Socha (2004) showed that supplements of organic trace minerals changed BW. Further investigations are needed to elucidate the effects seen in the current study.

Plasma TAC, which represents gross antioxidant capacity including both water- and fat- soluble antioxidants, was lower in cows fed HTM than cows fed ITM during the whole, prepartum, and postpartum study periods (Table 3-3). Furthermore, plasma TBARS, which is considered to represent a composite number of lipid oxidative end products including malondialdehyde (MDA; Bernabucci et al., 2005), tended to be lower in cows fed HTM than cows fed ITM during whole study period (Table 3-3). Oxidative stress is increased during the transition period due to high demand of metabolism and can be one of major causes for metabolic and immune dysfunctions (Sordillo and Aitken, 2009; Sharma et al., 2011). Because several trace minerals are required for proper function of enzymes involved in the antioxidant defense system (Spears and Weiss, 2008), their supplementation during the transition period could modulate oxidative status. Therefore, we anticipated that the

bioavailability of trace minerals would influence indices of oxidative stress and there would be a negative relationship between TAC and TBARS. In fact, Bernabucci et al. (2005) found that antioxidant index, such as erythrocyte superoxide dismutase and plasma thiol groups concentration, were decreased after calving, whereas plasma TBARS and reactive oxygen metabolites were increased during the same stage. The reason why both TAC and TBARS showed the same trend in HTM treatment is not known. The tendency of lower TBARS in cows fed HTM might be explained by better bioavailability of HTM, which may contribute to reducing lipid peroxidation through antioxidant capacity of trace minerals. Indeed, our research group found that feeding organic source of Zn, Cu, and Mn at NRC or commercial (2X of NRC) level decreased plasma TBARS right after intramammary lipopolysaccharide challenge in mid-lactation cows when compared to inorganic source (Yasui et al., 2009). Lower plasma TAC in cows fed HTM may be due to less demand of antioxidant as a result of low plasma TBARS. In contrast to Bernabucci et al. (2005), Castillo et al. (2006) reported that plasma Total Antioxidant Status (TAS), which represents the dynamic equilibrium between pro-oxidants and antioxidants in the plasma compartment, peaked 1 wk postpartum and decreased as lactation progressed in parallel with plasma MDA concentrations. They speculated that decreased TAS parallel to lower levels of MDA than levels right after parturition indicates that cows have lower levels of lipoperoxide production as lactation progress due to appropriate metabolic adaptations initiated in early lactation (Castillo et al., 2006). Further research is needed to elucidate the relationship between antioxidant status and oxidative parameters.

In the current experiment, effects of source of Zn, Cu, and Mn on plasma Hp were not significant over the entire postpartum period; however, cows fed HTM tended to have lower plasma Hp than those in ITM/OTM treatment during the first week after parturition. Acute phase proteins (APP) such as Hp, serum amyloid A, and C-reactive protein are plasma proteins that increase in concentration following stimulus conditions such as infection, inflammation or trauma (Eckersall, 2000). Haptoglobin is a major APP in ruminants such that it has a negligible circulating level in normal animals, but increases over 100-fold on stimulation (Eckersall, 2000). Because cows fed HTM tended to have both lower plasma TBARS than cows fed ITM and lower plasma Hp during the first week postcalving than cows fed ITM/OTM, it suggests that cows fed HTM had decreased systemic inflammation during the first week postcalving. Although it has been shown that serum Hp concentration within a week after parturition can be an indicator or risk factor to predict development uterine diseases such as metritis and CE in dairy cows (Huzzey et al., 2009; Chan et al., 2010; Dubuc et al., 2010), the tendency of decreased Hp in cows fed HTM was not associated with the extent of uterine PMN influx in the current study. Because PMN function also relies on other peripartum factors such as calcium status (Kimura et al., 2006; Martinez et al., 2012) and energy balance (Hammon et al., 2006), uterine immune status might have been influenced by these factors and therefore might not have shown any relationship with systemic inflammation in the current study. Studies have indicated that the higher Hp during the periparturient period is associated with both decreased milk yield and poorer reproductive performance in dairy cows (Chan et

al., 2010; Huzzey, 2012); however, the current study was not of appropriate scale to assess reproductive endpoint outcomes.

Although this was the first experiment to investigate effects of sources of Zn, Cu, and Mn on the extent of influx of PMN to the uterus at 7 d and one day between 40 and 60 d postpartum, the effects were not significant (Table 3-4). We chose the range (40 to 60 d postpartum) as the sampling time point for 2nd lavage because prevalence of CE, which is diagnosed by percentage of PMN in uterine lavage, decreases over time after calving and remains nearly unaffected after around 35 d postpartum (Sheldon et al., 2009) and it was found that CE diagnosed late in the voluntary waiting period (40 to 60 d postpartum) was highly prevalent in commercial herds in New York (Gilbert et al., 2005; Cheong et al., 2011). The development of uterine diseases depends on the immune response of the cow as well as the species and number of bacteria (Sheldon et al., 2006). Uterine defenses rely initially on innate immunity and mucosal defense systems rather than adaptive immunity right after parturition (Wathes et al., 2009) and PMN are the main leukocyte type involved in bacterial clearance after uterine infection (Galvao et al., 2011). Indeed, Gilbert et al. (2007) reported that PMN proportion in uterine lavage on the day of calving was negatively correlated with rates of positive bacterial culture at calving and the PMN proportion on 49 d postpartum. Therefore, we evaluated percentages of PMN in both the first and second lavage and hypothesized that these percentages are negatively associated each other. In addition, because studies have indicated that high oxidative metabolites such as MDA (Kizil et al., 2010) and malfunctions of immune responses (Hammon et al., 2006; Galvao et al., 2011) are critical factors to uterine inflammatory

disease, we also hypothesized that supplemental Zn, Cu, and Mn would affect PMN dynamics in the uterus. The reason for the lack of response of uterine cytology to various sources of trace minerals in the current study is unknown. Our result showed around 40% of PMN influx at 7 d postpartum among all treatments. Gilbert et al. (2007) showed the median proportion of PMN at 0, 7, and 21 d postpartum was 37%, 20%, and 41%, respectively. Therefore, all of the cows in the current study may have had originally high percentage of PMN compared to normal range, which may confine bacterial infection to the same extent immediately after calving among all treatments and result in the same percentages of PMN at 40 to 60 d postpartum. Because few studies have shown the extent of the PMN influx to the uterus, further research is needed to clarify the normal percentage of PMN.

Consistent with the result of PMN percentage, treatments effects on incidence of CE at 40 to 60 d postpartum were also not significant (Table 3-4). Likewise, the proportion of non-pregnant cows by days in milk was not affected by source of trace minerals in the current study (Figure 3-2). Although the incidence of CE has been determined in several studies (Kasimanickam et al., 2004; Gilbert et al., 2005; Gilbert et al., 2007; Cheong et al., 2011), the current study was the first to evaluate effects of source of Zn, Cu, and Mn on the incidence of CE, which is known to have detrimental effects on subsequent reproductive performance (Gilbert et al., 2005), such as prolonged days to pregnancy and low conception rates (Kasimanickam et al., 2004; Gilbert et al., 2005; Cheong et al., 2011). The collective results indicates that source of trace minerals may not have significant impact on endometrial cytology and the following reproductive performance; however, the results were not consistent with

modulating effects of HTM treatment on plasma oxidative parameters as mentioned above. As mentioned above, PMN function may be affected by calcium status (Kimura et al., 2006; Martinez et al., 2012) or energy balance (Hammon et al., 2006), these factors may be more predominant to affect uterine immune status than oxidative stress status. Further research is warranted to more comprehensively evaluate the roles of level and source of supplemental trace minerals with oxidative status and uterine PMN infiltration.

Because of involvement of trace minerals in a wide range of physiological processes (Nemec et al., 2012), it was also expected that their supplementation improved reproductive outcome independent of uterine immune status; however, effect of source of trace minerals on the proportion of non-pregnant cows by days in milk was not significant in the current study as mentioned above. The result was consistent with other studies such that reproductive outcomes were not affected by source of trace minerals (Siciliano-Jones et al., 2008; Formigoni et al., 2011). However, several studies including a meta-analysis study have shown that cows fed 100% organic form or a mix of organic and inorganic forms of trace minerals had improved reproductive performance such as fewer days to conception (Uchida et al., 2001; Rabiee et al., 2010), fewer number of services per conception (Uchida et al., 2001; Rabiee et al., 2010), and fewer days to first estrus (Nocek et al., 2006) compared to cows fed 100% inorganic form. The discrepancy may imply reproductive outcomes are affected by other factors than source of trace minerals. As discussed for the result of Hp, the unresponsiveness in the current study could be also attributable to the undersized scale.

CONCLUSIONS

Supplementation of HTM, a newly available source of trace minerals, resulted in improvements in milk production compared to both ITM and ITM/OTM treatments. Feeding HTM modulated markers related to oxidative metabolism during the periparturient period; cows fed HTM had lower plasma TAC than those fed ITM along with a tendency for decreased plasma TBARS in cows fed HTM compared to ITM. Plasma Hp was lower in cows fed HTM at 1 wk postpartum than those fed ITM/OTM. Although the effects of trace mineral sources on both endometrial cytology and days to pregnancy were not significant in this experiment, the results indicate that aspects of production and oxidative metabolism can be modulated by trace mineral source during the periparturient period.

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CHAPTER FOUR: EFFECTS OF CHROMIUM PROPIONATE SUPPLEMENTATION DURING THE PERIPARTURIENT PERIOD AND EARLY LACTATION ON METABOLISM, PERFORMANCE, AND ENDOMETRITIS IN DAIRY COWS[1]

ABSTRACT

Multiparous Holstein cows (n=61) were used to determine the effects of chromium propionate (Cr-Pro) supplementation during the periparturient period and early lactation on metabolism, performance, and the incidence of cytological endometritis (CE). After a 1-wk preliminary period, cows were assigned randomly to one of two treatments from 21 d before expected calving through 63 d post calving: 1) control (n=31) and 2) Cr-Pro (n=30) administered by daily topdress at a rate of 8 mg/d of chromium. There was a tendency for increased dry matter intake (DMI) during the prepartum period for cows fed Cr-Pro. Effects of Cr-Pro supplementation on postpartum DMI and milk yield were not significant; however, cows fed Cr-Pro tended to have higher urea N concentrations in milk. Cows fed Cr-Pro tended to have lower plasma concentrations of nonesterified fatty acid (NEFA) during the prepartum period. An interaction of treatment and day existed during the postpartum period such that cows fed Cr-Pro had lower plasma glucose concentrations during the immediate postpartum period compared to controls. Plasma haptoglobin was not affected by treatments during the postpartum period. Blood neutrophil glycogen concentrations

[1] Research material published in part as abstract in 2012 (Yasui et al., 2012)

were not affected by treatment when sampled both at 7 d postcalving and on one day between 40 and 60 d postcalving. Evaluation of endometrial cytology by low volume lavage at 7 d postcalving (1st lavage) and on one day between 40 and 60 d (2nd lavage) post calving revealed that cows fed Cr-Pro tended to have higher percentage of neutrophil at 1st lavage and had a 50% reduction in the incidence of CE at 2nd lavage. Effects of treatments on the calving to 1st ovulation within 56 days in milk (DIM) and the calving to conception within 150 DIM were not significant. In conclusion, supplementation with Cr-Pro resulted in trends for increased DMI and lower NEFA prepartum. Postpartum production and reproductive performances were not affected by treatment; however, Cr-Pro supplementation affected the postpartum influx of neutrophils into the uterus and the incidence of CE, suggesting positive effects of Cr-Pro supplementation on uterine health.

INTRODUCTION

Chromium (Cr) is essential to normal carbohydrate, lipid and protein metabolism (Vincent, 2004; Pechova and Pavlata, 2007). Also, Cr probably affects energy metabolism because chromodulin has been found to bind to activated insulin receptor, stimulating its kinase activity (Vincent, 2004). The demand for Cr is typically increased during different forms of nutritional, metabolic, and physical stress (Pechova and Pavlata, 2007). Also, cattle demonstrate a favorable response to Cr supplementation, especially if the animals are under physiological demands (NRC, 2001). Several studies conducted during the transition period and early lactation have demonstrated that cows fed supplemental Cr have increased milk yield (Hayirli et al.,

2001; Smith et al., 2005; Sadri et al., 2009) and improved energy metabolism as measured by lower circulating concentrations of NEFA or BHBA (Smith et al., 2008; Soltan, 2010; Sadri et al., 2012) when fed supplemental Cr.

In addition to its effects on nutritional metabolism, several studies suggest that Cr may also have immunomodulatory effects in cattle. Chromium supplementation has affected cell-mediated immunity such that mitogen-stimulated blastogenic responses in peripheral blood mononuclear cells were elevated in cows fed Cr (Burton et al., 1993). Cows fed Cr had higher humoral immune responses such as increased anti-ovalbumin antibody (Burton et al., 1993) and tetanus toxoid-specific antibody (Faldyna et al., 2003) responses compared to control. Positive effects of Cr on innate immunity have not been demonstrated to date (Chang et al., 1996; Spears, 2000); however, there is still the possibility that Cr affects innate immune responses because level of serum cortisol was significantly decreased in stressed calves fed Cr (Mowat et al., 1993), and neutrophil function is known to be negatively affected by glucocorticoids in cattle (Burton et al., 1996).

Recent work has established linkages between energy metabolism during the periparturient period and reproductive performance (Butler et al., 2006; Roche et al., 2009; Ospina et al., 2010). Elevated concentrations of NEFA and BHBA in blood during the transition period were associated with decreased reproductive performance in a large field study (Ospina et al., 2010). We speculate that these associations can be explained partly by the potential development of cytological endometritis (CE) because the odds of having CE were 3.9 times higher in cows with ketosis (Cheong et al., 2011). Further, the development of CE has been shown to negatively affect

reproductive outcomes: lower first service conception rate and longer days to pregnancy in a study conducted in five commercial dairy farms (Gilbert et al., 2005); and lower odds for pregnancy at first insemination and longer median days open in another study conducted in 779 cows from 38 herds (Cheong et al., 2011) in cows with CE compared with cows without CE. Uterine defenses are known to initially rely on innate immunity rather than adaptive immunity (Wathes et al., 2009). Therefore, it is possible that chromium supplementation affects incidence of CE through its effects on either energy metabolism or immune function.

Given the potential effects of Cr supplementation on both energy metabolism and immune function, we hypothesized that Cr supplementation during the periparturient period and early lactation would improve aspects of metabolism and performance and decrease the incidence of cytological endometritis in cows. Therefore, the objectives of this experiment were to evaluate the effects of Cr supplementation to cows during the periparturient period and early lactation on performance, metabolism, inflammatory response, and the incidence of CE in dairy cows.

MATERIALS AND METHODS

Experimental Animals, Treatments, and Procedures

All procedures involving animals were approved by the Cornell University Institutional Animal Care and Use Committee prior to the onset of the experiment. Holstein cows (n=61) entering second lactation or greater from the Cornell University Teaching and Research Center Dairy were enrolled in this experiment at 28 d before expected calving and assigned to one of two topdress treatments with randomization

restricted by previous lactation 305-d mature-equivalent milk production. Treatments were administered from 21 d before expected calving through 63 d post calving: 1) control; and 2) Chromium propionate (Cr-Pro, KemTRACE Chromium Propionate, Kemin Industries, Des Moines, IA) administered by daily topdress at a rate of 8 mg/d of chromium. All cows were housed in individual tiestalls and fed the same basal diet during the prepartum period and also fed the same basal diet during the postpartum period.

Ingredient and chemical composition of the diets fed during the experiment are described in Table 4-1. Both prepartum and postpartum diets were typical of those fed in the Northeastern U.S. 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 0800 h, orts were weighed and recorded daily, and water was made available at all times by individual water cups. 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 content of each TMR was 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 Cornell Net Carbohydrate and Protein System/Cornell-Penn-Miner profiles by a commercial laboratory (Cumberland Valley Analytical Services, Hagerstown, MD). Samples were analyzed for DM (method 930.15; AOAC,

Table 4-1. Ingredients and chemical composition (DM basis, % of DM unless otherwise noted) of experimental diets.

Item	Prepartum diet	Postpartum diet
Corn silage, processed	42.83	51.29
Wheat straw	27.59	2.04
Legume silage	-	14.37
Ground shelled corn, finely	-	2.65
Soybean meal (47.5% CP)	-	4.08
Wheat middlings	-	4.04
Corn germ meal	8.01	2.76
Distillers grains (with solubles)	1.23	5.39
Soy Chlor[1]	2.67	-
Amino Plus[2]	5.39	-
Soybean hulls	3.54	4.45
Canola meal	5.86	1.78
Blood meal	-	1.55
Dextrose	-	1.14
Bypass fat[3]	-	1.25
Urea	-	0.33
Calcium carbonate	1.07	0.75
Calcium sulfate	0.70	0.14
Sodium bicarbonate	-	0.82
Mono dicalcium phosphate	0.23	0.08
Salt	0.16	0.41
Magnesium oxide	0.23	0.15
Copper sulfate	< 0.01	-
Selenium, 0.06%	0.04	0.05
Trace mineral premix[4]	0.01	0.02
Vitamin A, D, E premix[5]	0.03	0.02
Vitamin E premix[6]	0.01	-
Condition Aid[7]	0.38	0.46
Chemical composition (±SD[8])		
CP	13.6 (0.59)	16.9 (0.34)
Soluble protein (% of CP)	32.2 (3.76)	37.7 (2.71)
ADF	28.4 (0.62)	21.9 (0.71)
NDF	47.7 (0.94)	37.2 (0.88)
Starch	19.9 (1.67)	25.3 (1.27)
Sugar	3.18 (0.46)	3.00 (0.30)
Ether extract	2.89 (0.08)	4.20 (0.34)
Ash	6.92 (0.35)	6.70 (0.33)
Ca	1.03 (0.11)	0.89 (0.10)
P	0.39 (0.01)	0.40 (0.01)
K	1.04 (0.06)	1.27 (0.07)
Mg	0.37 (0.02)	0.34 (0.02)

Table 4-1 (Continued)

Na	0.14 (0.01)	0.45 (0.06)
Cl	0.45 (0.04)	0.37 (0.08)
S	0.30 (0.04)	0.26 (0.03)
Fe (ppm)	468.5 (63.9)	407.6 (29.0)
Zn (ppm)	63.2 (7.25)	73.7 (7.70)
Cu (ppm)	17.3 (2.34)	16.0 (2.00)
Mn (ppm)	62.8 (5.56)	64.1 (5.64)
DCAD, mEq/100g DM[9]	1.50 (3.73)	25.6 (3.15)

[1]Anionic feed supplement; West Central, Ralston, IA.
[2]Rumen undegradable protein supplement; AGP Inc., Omaha, NE.
[3] Prilled saturated fatty acid; Cargill, Minnetonka, MN.
[4]Contained 30,317 mg/kg of Cu, 136,466 mg/kg of Mn, 3,393 mg/kg of Cot, 3,040 mg/kg of I, and 153,916 mg/kg of Zn.
[5]Contained 30,464 IU/kg of Vitamin A, 5,862 IU/kg of Vitamin D, and 93,784 IU/kg of Vitamin E.
[6]Contained 499,400 IU/kg of Vitamin E.
[7]Flow agent; Oil-Dri Corporation of America, Chicago, IL.
[8]Standard deviation calculated from 6 and 7 samples for prepartum and postpartum diet, respectively.
[9]Calculated as mEq [(Na + K) - (Cl + S)] / 100 g DM (NRC, 2001).

2000), CP (method 990.03; AOAC, 2000), soluble protein (Krishnamoorthy et al., 1982), ADF (method 973.18; AOAC, 2000), NDF (Van Soest et al., 1991), starch (Hall, 2009), sugar (DuBois et al., 1956), ether extract (method 2003.05; AOAC, 2006), ash (method 942.05; AOAC, 2000), and minerals (method 985.01; AOAC, 2000).

Cows were milked twice per day (0900 and 2100 h) and milk yields were recorded at all milkings for the 63-d postpartum treatment period. Milk samples were collected on the same day per week from all 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, and MUN using midinfrared analysis (AOAC, 2000; method 972.160), and SCC by an optical fluorescent method (AOAC, 2000; method 978.26). Body condition score (1 to 5 scale; Wildman et al., 1982) was assessed and BW were measured weekly from the preliminary period throughout the entire treatment period.

Plasma Sampling and Analyses

Samples of blood were obtained from each cow via coccygeal blood vessel puncture on one day before assignment to treatment and three times per week from 21 d prepartum through 56 d postpartum. Plasma was harvested following centrifugation (2,800 × g for 15 min at 4°C), snap-frozen in liquid N_2, and stored at −20°C until analyses. Plasma samples collected from the start of the study through 21 d postpartum were analyzed for plasma metabolites (glucose, NEFA, BHBA, and insulin). Plasma concentrations of glucose were determined by enzymatic analysis

(glucose oxidase) using a commercial kit (kit 510-A; Sigma Chemical, St. Louis, MO). Plasma concentrations of NEFA were analyzed by enzymatic analysis (NEFA-C; Wako Pure Chemical Industries, Osaka, Japan). Plasma concentrations of BHBA were determined by enzymatic analysis (BHBA dehydrogenase; kit no. 310, Sigma Chemical). Plasma concentrations of insulin were determined by double-antibody RIA (Porcine Insulin RIA cat. no. PI-12K, Linco Research, Millipore, St. Charles, MO), with a reported specificity to bovine insulin of 90%. Intra- and interassay CV for the insulin RIA were 11.1 and 15.3%, respectively. Plasma samples collected from 1 wk postpartum through 8 wk postpartum were also analyzed for concentrations of haptoglobin (Hp). Plasma concentrations of Hp were measured by enzymatic analysis (Phase Range Haptoglobin Assay, Tridelta Diagnostics Ltd., Morris Plains, NJ). All spectrophotometric measurements were conducted using a Versamax tunable microplate reader (Molecular Devices, Sunnyvale, CA).

Blood Neutrophil Glycogen

Samples of blood were obtained from each cow via coccygeal blood vessel puncture at 7 d postcalving and on one day between 40 and 60 d postcalving. Blood polymorphonuclear neutrophils (PMN) were isolated as previously described (Flaminio et al., 2002) and frozen for subsequent determination of glycogen content. Glycogen content of neutrophils was determined as described previously (Galvão et al., 2010). Briefly, glycogen was hydrolyzed to glucose using amyloglucosidase; available glucose was determined by reacting 50 μL of supernatant with a 1-mL mixture of 1 mM ATP, 0.9 mM NADP, 5 μg of glucose-6-phosphate dehydrogenase, 0.3 M triethanolamine, and 4 mM MgSO4 and recording the appearance of NADPH after the

addition of 5 μL of hexokinase (2 mg/mL) as change in optical density at 340 nm on a spectrophotometer (Versamax tunable microplate reader, Molecular Devices, Sunnyvale, CA). This change in optical density was compared with a standard curve of glycogen assayed in similar fashion, and results were expressed as micrograms of glycogen/10^6 PMN.

Endometrial Cytology

Evaluation of endometrial cytology by low volume lavage (Gilbert et al., 2005) was determined on all cows at 7 d postcalving (1st lavage) and on one day between 40 and 60 d (2nd lavage) post calving as previously described (Cheong et al., 2011); the perineum of the cow was cleansed and a 64-cm Flex Tip sterile plastic infusion pipette (Exodus Breeders Corp., York, PA) was manipulated through the cervix into the uterus. Sterile saline solution (20 mL) was injected into the uterus and agitated gently per rectum; then a sample of the fluid was aspirated. The recovered fluid was centrifuged using a cytocentrifuge directly onto a glass slide. After drying, the slides were fixed and stained using a rapid Romanowsky-type staining procedure and examined under 400X magnification. Two hundred cells were counted from each slide, and results were expressed as the percentage of PMN in total cells (excluding erythrocytes). All the slides were read masked to treatment by the same investigator (TY). The percentage of PMN was analyzed subsequently as a continuous variable for both 1st and 2nd lavage. Incidence of CE diagnosed with cut-off point of 10% PMN (Cheong et al., 2011) in 2nd lavage was analyzed subsequently as a dichotomous variable.

Reproductive Parameters

Plasma samples collected from calving through 56 d postpartum were analyzed for progesterone by RIA (Staigmiller et al., 1979; Nara and First, 1981). Intra- and interassay CV for the progesterone RIA were 11.2 and 13.2%, respectively. Ovulation was assumed to have occurred 3 d before concentrations of plasma progesterone in two consecutive samples were greater than or equal to 1 ng/mL. In addition, the proportion of non-pregnant cows within 150 DIM was analyzed as another parameter for reproductive performance.

Statistical Analyses

The number of cows enrolled in each treatment was 31 and 30 for control and Cr-Pro, respectively. However, four cows were removed from the postpartum or specific DIM dataset due to health problems: one cow from control after parturition because of retained placenta, ketosis, and displaced abomasum; one cow from Cr-Pro after 28 DIM because of mastitis and injury of a teat; one cow from control after 28 DIM because of mastitis and injury of a teat; and one cow from Cr-Pro after 40 DIM because of bloated rumen and mastitis.

Data for DMI and milk yield were reduced to weekly means prior to analysis. Baseline values collected during the pretreatment week prior to assignment to treatment were used as covariates for DMI, BW, BCS, and plasma metabolites. Previous ME 305 milk yields were used as covariates for milk yield and milk composition. Weekly (DMI, BW, BCS, milk yield, and milk composition) and 3-time-per-week (plasma metabolites) variables were analyzed by MIXED procedure of SAS version 9.1 (SAS Institute, Cary, NC) for a completely randomized design with

repeated measures. Model terms were the fixed effects of covariate, treatment, week or day, and the interaction of treatment and week or day. The random effect was cow nested within treatment. For each weekly or 3-time-per-wk 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. Covariates were retained in the model if $P < 0.10$.

The percentage of PMN in the total cells in low volume lavage was analyzed as a continuous variable for both 1st and 2nd lavage. Incidence of CE was dichotomously analyzed for 2nd lavage. Kaplan-Meier survival analysis (Kaplan and Meier, 1958) was conducted to produce survival curves of treatments effects on calving to conception within 150 DIM and calving to 1st ovulation within 56 DIM. Statistical significance was declared at $P < 0.05$ and trends were discussed at $0.05 < P \leq 0.15$. Least squares means and standard error of the mean were reported throughout.

RESULTS

The effects of Cr-Pro supplementation during the prepartum period and early lactation on performance are described in Table 4-2. Supplementation with Cr-Pro tended to increase DMI during the prepartum period (16.5 vs. 15.8 kg/d; $P = 0.07$); however, Cr-Pro did not affect postpartum DMI. Milk yield averaged 42.2 kg/d during the 9-wk postpartum period and was not affected by treatment. Chromium supplementation did not affect yields and percentages of milk fat, true protein, lactose,

Table 4-2. Least squares means for dry matter intake (DMI), milk yield, milk composition, and body weight (BW) and body condition score (BCS) for cows fed control or chromium propionate (Cr-Pro) during the periparturient period and early lactation.

Item	Treatment		SEM[1]	*P*–value		
	Control	Cr-Pro		Trt[2]	Wk[3]	Trt x Wk
DMI, kg/d						
Prepartum	15.8	16.5	0.3	0.07	<0.001	0.26
Postpartum	22.9	22.9	0.3	0.98	<0.001	0.33
Milk,[4] kg/d	42.0	42.5	0.9	0.74	<0.001	0.97
Fat. %	3.73	3.80	0.07	0.46	<0.001	0.94
Fat, kg/d	1.53	1.56	0.04	0.57	<0.001	0.85
Protein, %	3.01	3.07	0.05	0.37	<0.001	0.17
Protein, kg/d	1.24	1.26	0.02	0.36	0.60	0.84
Lactose, %	4.81	4.80	0.02	0.91	<0.001	0.26
Lactose, kg/d	2.02	2.05	0.05	0.74	<0.001	0.49
SCC (LS[5])	2.0	2.6	0.3	0.12	<0.001	0.14
Urea N, mg/d	10.6	11.2	0.2	0.08	0.07	0.52
Total solids, %	12.5	12.6	0.1	0.40	<0.001	0.99
Total solids, kg/d	5.19	5.27	0.11	0.62	<0.001	0.98
3.5% FCM,[6] kg/d	43.0	43.7	0.9	0.59	<0.001	0.63
ECM,[7] kg/d	42.0	42.7	0.9	0.58	<0.001	0.92
BW, kg						
Prepartum	710	731	9	0.12	0.002	0.19
Postpartum	610	630	10	0.14	<0.001	0.18
BCS						
Prepartum	3.35	3.40	0.06	0.59	0.45	0.97
Postpartum	2.89	2.89	0.05	0.97	<0.001	0.14

[1]SEM = standard error of the mean.
[2]Trt = treatment.
[3]Wk = week.
[4]Represents milk yields collected daily from parturition through 63 d postpartum and then averaged for each week postpartum.
[5]linear score = -3.643586 + (3.321928 × ln (SCC/1000)/2.302)

Table 4-2 (Continued)

[6]FCM = (0.4324 × kg of milk) + (16.216 × kg of milk fat).
[7]ECM = [(0.323 × kg of milk) + (12.82 × kg of fat) + (7.13 × kg of protein)].

total solids, 3.5% FCM, or ECM. Cows fed Cr-Pro tended to have slightly higher concentrations of MUN (11.2 vs. 10.6 mg/dL; *P* = 0.08) and linear SCC (2.6 vs. 2.0; *P* = 0.12). Body weight and BCS were not affected by treatment during either the prepartum or postpartum periods.

Results for plasma metabolites, insulin, and haptoglobin along with concentrations of glycogen in blood neutrophils are provided in Table 4-3. For plasma variables, data were analyzed separately by prepartum and postpartum periods. Cows fed Cr-Pro tended to have lower concentrations of plasma NEFA during the prepartum period (184 vs. 211 μEq/L; P = 0.08); however, differences during the postpartum period were not significant. Plasma concentrations of BHBA were not affected by treatment during either the prepartum or postpartum period. Supplementation with Cr-Pro did not affect overall concentrations of glucose in plasma during either period studied; however, a trend for a treatment by time interaction existed for glucose concentrations during the postpartum period such that concentrations were lower in cows fed Cr-Pro on d 1 postpartum and similar for the rest of the study period (*P* = 0.002; Figure 4-1). Plasma concentrations of insulin were not affected by treatment during either the prepartum or postpartum period. Plasma concentrations of haptoglobin, analyzed either from weekly postpartum samples or during only the first week postpartum, were not affected by treatment. Glycogen content of neutrophils harvested from whole blood on d 7 postpartum and on one day between d 40 and 60 postpartum was not affected by treatment (Table 4-3).

Results for endometrial cytology conducted on d 7 postpartum (1st lavage) and on one day during d 40 to 60 postpartum (2nd lavage) are presented in Table 4-4. Six

Table 4-3. Plasma non-esterified fatty acid (NEFA), beta-hydroxybutyric acid (BHBA), glucose, insulin, and haptoglobin (Hp), and blood polymorphonuclear neutrophils (PMN) glycogen concentrations in cows fed control or chromium propionate (Cr-Pro) during the periparturient period and early lactation.

Item	Treatment		SEM[1]	*P*-value		
	Control	Cr-Pro		Trt[2]	Wk[3]	Trt x Wk
NEFA, µEq/l						
Prepartum period[4]	210.7	183.8	10.5	0.08	< 0.01	0.78
Postpartum period[5]	684.3	705.7	45.0	0.74	< 0.01	0.49
BHBA, mg/dl						
Prepartum period[4]	4.23	4.41	0.15	0.38	0.49	0.27
Postpartum period[5]	6.64	7.73	0.62	0.22	0.02	0.17
Glucose, mg/dl						
Prepartum period[4]	63.43	63.77	0.68	0.73	0.47	0.99
Postpartum period[5]	56.51	56.63	0.78	0.91	< 0.01	0.05
Insulin, ng/ml						
Prepartum period[4]	0.59	0.63	0.02	0.23	< 0.01	0.75
Postpartum period[5]	0.26	0.24	0.02	0.52	< 0.01	0.39
Hp, mg/mL						
Postpartum period[6]	0.69	0.70	0.05	0.90	< 0.01	0.37
1 wk postpartum[7]	0.97	0.96	0.10	0.94	-	-
Glycogen, µg/10^6 PMN						
7 d postpartum[8]	2.64	3.08	0.44	0.46	-	-
40-60 d postpartum[9]	4.64	4.81	0.44	0.77	-	-

[1]SEM = standard error of the mean.
[2]Trt = treatment.
[3]Wk = week.
[4]Represents plasma samples collected 3 times per week from 3 wk prepartum through 1 wk prepartum.
[5]Represents plasma samples collected 3 times per week from 1 wk postpartum through 8 wk postpartum.
[6]Represents plasma samples collected weekly from 1 wk postpartum through 8 wk postpartum.
[7]Represents plasma samples collected from 1 wk postpartum only.
[8]Represents PMN samples isolated at 7 d postpartum.
[9]Represents PMN samples isolated on one day between 40 and 60 d postpartum.

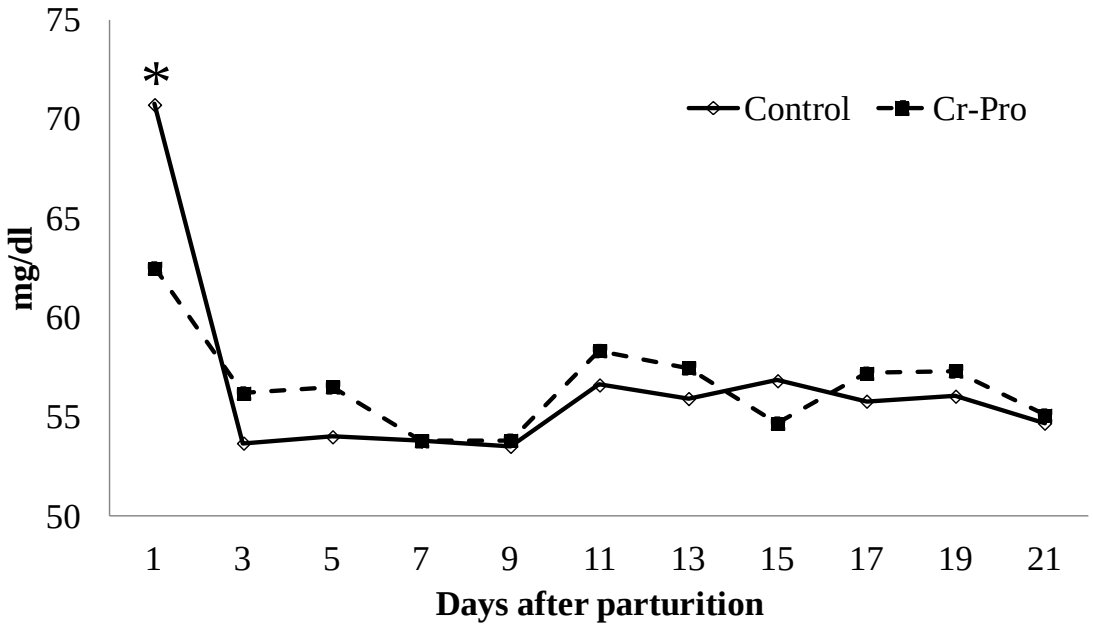

Figure 4-1. Postpartum plasma glucose concentration in cows fed control or chromium propionate (Cr-Pro) during the periparturient period and early lactation. Values are least squares means; SEM averaged 1.84 mg/dl; the *P*-value for the interaction of treatment × week was 0.06; At 1 day after parturition, plasma glucose was lower in cows fed Cr-Pro than control (*$P = 0.002$).

Table 4-4. Percentage of neutrophils in 1st uterine lavage and incidence of cytological endometritis (CE) in 2nd uterine lavage from cows fed control or chromium propionate (Cr-Pro) during the periparturient period and early lactation

Item	Treatment		SEM[1]	*P*-value
	Control	Cr-Pro		Trt[2]
% of neutrophils[3]				
1st lavage[4]	32.8	41.1	4.1	0.15
2nd lavage[5]	14.3	11.5	2.4	0.41
Cows with CE[6]	16	8	-	0.02
Cows without CE	11	20		

[1]SEM = standard error of the mean.
[2]Trt = treatment.
[3]Percentage of polymorphonuclear neutrophils (PMN) in total 200 cells, excluding erythrocytes.
[4]Lavage at 7 d postpartum.
[5]Lavage at one day between 40 and 60 d postpartum.
[6]Number of cows that developed CE (cytological endometritis diagnosed positive if more than 10% PMN were counted in 2nd lavage).

cows from control were not used for analysis for 1st lavage (one due to aforementioned removal from dataset due to health problem, one due to difficulty to retrieve lavage, and four due to difficulty to distinguish PMN from other cells). Two cows from Cr-Pro were not used for analysis for 1st lavage due to difficulty to distinguish PMN from other cells. Four cows from control (2 cows due to aforementioned removal from dataset and two cows due to difficulty to retrieve lavage) were not used to analyze for 2nd lavage. Two cows from Cr-Pro were not used for analysis for 2nd lavage due to aforementioned removal from dataset. Cows fed Cr-Pro tended to have increased neutrophil infiltration of the uterus at 7 d postpartum (41.1 vs. 32.8% of cells; $P = 0.15$); however, the percentage of PMN was not affected by treatment at 40 to 60 postpartum. Cows fed Cr-Pro had about 50% reduction in the incidence of cytological endometritis as assessed using data from the 2nd lavage.

Analyses of the intervals from calving to first ovulation (within 56 d postpartum) and calving to conception (within 150 d postpartum) are depicted in Figures 4-2 and 4-3, respectively. Supplementation with Cr-Pro did not affect either interval studied.

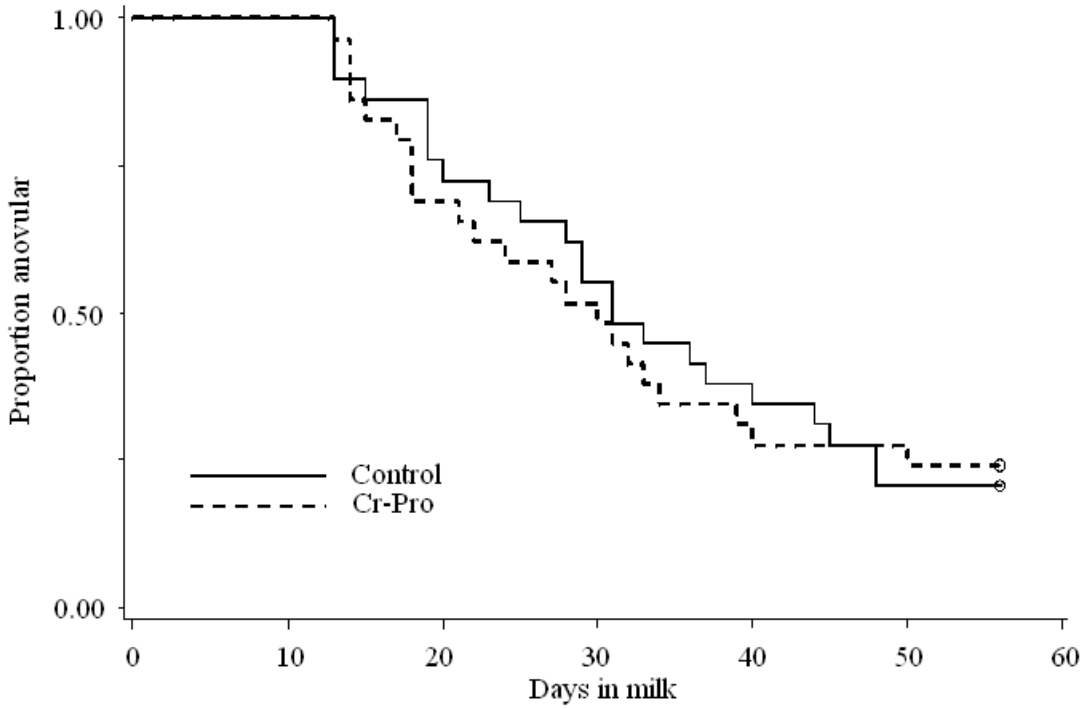

Figure 4-2. Kaplan-Meier survival analysis for treatments effects on calving to 1st ovulation within 56 days in milk for cows fed control or chromium propionate (Cr-Pro) during the periparturient period and early lactation. Circles show cows, which had not ovulated by 56 days in milk. Treatments did not affect calving to ovulation interval ($P = 0.64$).

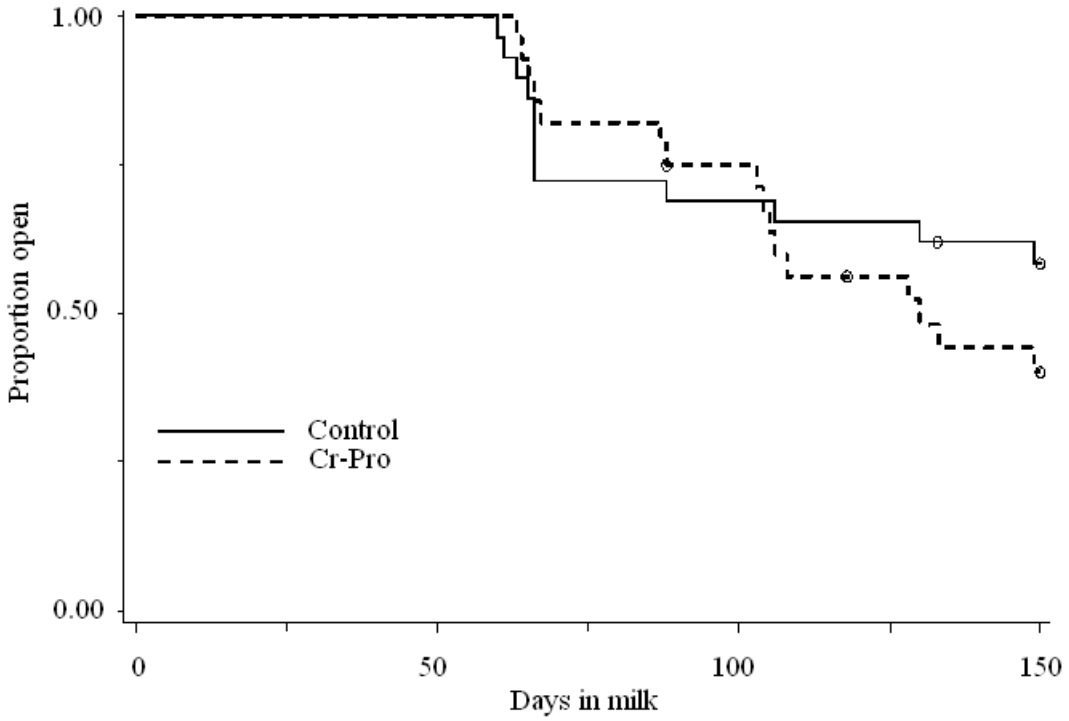

Figure 4-3. Kaplan-Meier survival analysis for treatments effects on calving to conception within 150 days in milk for cows fed control or chromium propionate (Cr-Pro) during the periparturient period and early lactation. Circles show censored cows, which came off from the reproduction record or had not been pregnant within 150 days in milk. Treatments did not affect calving to conception interval (P = 0.57).

DISCUSSION

Chromium plays a role in potentiating the effect of insulin on tissues (NRC, 2001); therefore, Cr status can affect metabolism or productive performance in dairy cows. Although the amount of Cr required in the diet for optimal performance is unclear (NRC, 2001), supplemental Cr in dairy cows during the periparturient period consistently has increased milk yield of cows during early lactation (Hayirli et al., 2001; McNamara and Valdez, 2005; Smith et al., 2005). In the current study, however, milk production was not affected by feeding Cr-Pro (Table 4-2). Despite the lack of response of milk yield to Cr-Pro supplementation, cows fed Cr-Pro tended to have higher DMI during the prepartum period (Table 4-2). This finding is in agreement with Hayirli et al. (2001). Although several studies show that DMI during the postpartum period was also increased with Cr supplementation (Hayirli et al., 2001; Smith et al., 2005; Soltan, 2010), postpartum DMI was not affected by treatment in the current study. McNamara and Valdez (2005) and Soltan (2010) suggested that the effect of Cr supplementation on feed intake preceded the effect on milk production. Therefore, Cr may have direct effect on feed intake, although the mechanism of the effect remains unclear. Attention has recently turned to chromodulin from glucose tolerance factor (GTF), which was used be considered as an active form of Cr, because it has been proposed that GTF is merely a decomposition product of chromodulin (Pechova and Pavlata, 2007). Chromodulin seems to amplify insulin signaling (Vincent, 2004). Further, there is possibility that a therapeutic agent that mimics chromodulin's action can be used in treating insulin insensitivity (Vincent, 2004);

therefore, Cr could enhance insulin sensitivity through function of the biomolecule and thereby may decrease NEFA concentrations, which results in increased DMI and increased milk yield (Smith et al., 2008). Indeed, McNamara and Valdez (2005) suggested Cr-Pro supplementation may reduce net lipolysis and hence increase DMI. In addition, feeding organic Cr markedly decreased serum cortisol in stressed calves (Mowat et al., 1993); therefore, feeding Cr may lead to increasing feed intake by mitigating stress responses in dairy cows. In the current study, the effect of Cr-Pro on feed consumption appears to have been confined to the prepartum period and did not persist into lactation.

Overall effects of treatment on percentages and yields of major milk components (e.g., fat, true protein, lactose, total solids) were not significant (Table 4-2). There were tendencies for increased concentrations of MUN and increased linear SCC in cows fed Cr-Pro. In general, the literature suggests minimal effects of Cr supplementation on milk composition. Sadri et al. (2009) reported that Cr supplementation decreased milk protein content and McNamara and Valdez (2005) indicated that cows fed Cr-Pro had decreased concentrations of milk fat; however, other investigators reported no effects of Cr supplementation on milk component percentages (Hayirli et al., 2001; Smith et al., 2005; Soltan, 2010). Yield responses of milk fat and lactose to Cr supplementation reported by Hayirli et al. (2001) followed milk yield responses.

Body weight was not affected by treatment during either the prepartum or postpartum periods, which is consistent with other experiments (Hayirli et al., 2001; McNamara and Valdez, 2005; Sadri et al., 2009). Smith et al. (2005) reported that

administering increasing amounts of Cr linearly increased postpartum BW; however, effect of Cr on prepartum BW was not significant and calculated postpartum energy balance was not affected by Cr treatment (Smith et al., 2005). Furthermore, there were no effects of treatment on BCS in the current study. McNamara and Valdez (2005) also reported a lack of effect of Cr-Pro on BCS, but another study showed that prepartum BCS significantly increased with increasing Cr supplementation (Smith et al., 2005). Again, effect of Cr on energy balance during the prepartum period was not significant (Smith et al., 2005). Overall, feeding Cr has caused inconsistent results for BW and BCS, which suggests that other nutritional or metabolic factors may be involved.

Responses of plasma metabolites, insulin, haptoglobin, and the glycogen content of blood neutrophils to Cr-Pro supplementation are reported in Table 4-3. Consistent with other studies (Hayirli et al., 2001; Smith et al., 2008; Soltan, 2010), Cr-Pro supplementation during the prepartum period tended to decrease plasma NEFA concentrations in this experiment. Given that DMI tended to be increased concurrently with the trend for reduced NEFA, decreased NEFA might be as a result of increased energy intake. Hayirli et al. (2001) reported that increased serum insulin concentrations were associated with decreased plasma NEFA concentrations in cows supplemented with Cr; however, insulin concentrations were not affected by treatment in our experiment. McNamara and Valdez (2005) reported that Cr-Pro supplementation reduced net lipolysis and that Cr might have specific actions on adipose tissue metabolism that would be consistent with increased responses of adipose tissue to circulating insulin. Although NEFA tended to be decreased during

the prepartum period by Cr-Pro supplementation, effects on postpartum NEFA and BHBA were not significant. Plasma BHBA was not affected by supplemental Cr, which is consistent with several studies (Hayirli et al., 2001; Bryan et al., 2004; Smith et al., 2008). However, other studies have shown that cows fed Cr had decreased postpartum BHBA (Yang et al., 1996; Besong, 1996). When including effect on NEFA, it reveals that supplemental Cr reduced both BHBA and NEFA (Yang et al., 1996), only BHBA (Besong, 1996; Sadri et al., 2012), only NEFA (Hayirli et al., 2001; Bryan et al., 2004), and none of them (Smith et al., 2008), which suggests that other factors may influence the effect of Cr on those metabolites. In fact, Smith et al. (2008) reported that the lack of response of those metabolites did not reflect productive outcome, which showed increased milk yield and DMI in cows fed Cr (Smith et al., 2005). A study of periparturient gene expression revealed that placental cytokines and hepatic proinflammatory cytokine were positively correlated with lipid mobilization from adipose tissue and fatty acid oxidation (Loor et al., 2005). Those cytokines may influence effect of Cr on lipid mobilization and fatty acid oxidation. Although overall effects of Cr-Pro on plasma glucose was not significant during either the prepartum or postpartum periods, plasma glucose was lower than controls at 1 wk postpartum (Figure 4-1). Besong (1996) showed that plasma glucose level decreased after parturition regardless of feeding Cr or not; however, no study so far has shown that supplemental Cr reduced plasma glucose right after parturition. Postpartum plasma glucose concentrations decreased linearly ($P = 0.19$) by administering increasing amounts of Cr (Smith et al., 2008), whereas milk yield was increased by supplemental Cr in the same experiment (Smith et al., 2005); therefore, supplemental

Cr-Pro might increase glucose uptake by mammary gland, resulting in low blood glucose. Alternatively, because secretion of the cortisol is increased at parturition (Overton and Waldron, 2004) and act as an insulin antagonist through increasing blood glucose concentration (Pechova and Pavlata, 2007), supplemental Cr-Pro might decrease blood cortisol level as shown in stressed calves fed Cr (Mowat et al., 1993), which might lead to decreased plasma glucose level immediately after parturition.

Several studies have shown that Cr has immunomodulatory effects in dairy cows (Burton et al., 1993; Burton et al., 1996; Chang et al., 1996); however, no studies to date have evaluated effects of Cr on acute phase proteins (APP), which increase in concentration following stimulus conditions such as infection, inflammation or trauma (Eckersall, 2000). Among APP, Hp is a major APP in ruminants because it has a negligible circulating level in normal animals, but increases over 100-fold on stimulation (Eckersall, 2000). Burton et al. (1996) reported that dairy cows fed Cr had decreased *in vitro* concentrations of IFN-γ, IL-2, and TNF-α following mitogen stimulation of mononuclear cells collected during the peripartum period. Among the cytokines, TNF-α is known to be one of pro-inflammatory cytokines, which stimulate hepatic production of APP (Eckersall, 2000). Therefore, it was expected that feeding Cr-Pro might suppress Hp production through an immunomodulatory effect; however, concentration of Hp was not affected by feeding Cr-Pro. Further, increased circulating Hp concentrations within a week postcalving have been associated with uterine diseases such as metritis and CE in dairy cows (Huzzey et al., 2009; Chan et al., 2010; Dubuc et al., 2010). The lack of response of plasma Hp to Cr-Pro supplementation suggests similar inflammatory states between cows fed the two treatments.

The PMN found in peripheral blood accumulate glycogen intracellularly for use as reserve energy to meet requirements for their activity, especially phagocytosis (Naidu and Newbould, 1973; Weisdorf et al., 1982). Therefore, as Galvão et al. (2010) suggested, low glucose levels observed during the transition to lactation could lead to decreased PMN glycogen stores, thereby impairing phagocytic and killing capability. Also, impaired PMN function in diabetic rats was abolished with insulin treatment even with no marked change in glycemia, which indicates insulin may have a direct effect on neutrophil metabolism and function (Alba-Loureiro et al., 2006). Therefore, our hypothesis was that feeding Cr-Pro would improve glycogen level in neutrophil by enhancing insulin function. Our results showed that glycogen content of PMN was not affected by Cr-Pro supplementation at either d 7 postpartum or when sampled on 1 d between d 40 and 60 postpartum (Table 4-3). Overall, these results are consistent with the lack of response of other variables related to glucose metabolism in this experiment.

The results of endometrial cytology conducted on d 7 postpartum and on 1 d between d 40 and 60 postpartum are shown in Table 4-4. In this experiment, cows fed Cr-Pro tended to have increased percentages of neutrophils in the uterine lavage at 7 d postpartum and had an approximate reduction of CE of 50% when characterized between d 40 and 40 postpartum. We chose the range (40 to 60 d postpartum) as the sampling time point for 2nd lavage because a prevalence of CE, which is diagnosed by percentage of PMN in uterine lavage, decreases over time after calving and remains nearly unaffected after around 35 d postpartum (Sheldon et al., 2009) and it was found that CE diagnosed late in the voluntary waiting period (40 to 60 d postpartum) was

highly prevalent in commercial herds in New York (Gilbert et al., 2005; Cheong et al., 2011). Gilbert et al. (2007) reported that cows with the greatest influx of PMN to the uterus on the day of calving had the lowest rates of positive bacterial culture and prevalence of CE later in lactation; therefore, early infiltration of PMN into the uterus postcalving may be an important factor in reducing subsequent CE. In spite of evidence of immunomodulatory effects of Cr in other studies, the underlying mechanism remains unknown (Pechova and Pavlata, 2007). Galvão et al. (2011) showed that expression of the TNF-α gene in uterine tissue was decreased in cows with endometritis compared to control cows at calving and 1 wk postpartum, suggesting that a lower local level of expression of pro-inflammatory cytokines in the endometrium right after calving might impair activation of inflammation and clearance of bacteria, and lead to development of endometritis. As discussed above, Burton et al. (1996) reported lower TNF-α production *in vitro* from mitogen-stimulated mononuclear cells collected during the peripartum period in cows supplemented with Cr. If Cr has the same effect on local TNF-α production, feeding Cr may exacerbate the impaired inflammation response around the periparturient period. Alternatively, Cr may selectively exert immunomodulatory effects through cytokines and endocrine hormones. Human and animal studies suggest that Cr affects various components of the immune system and may result in immunostimulation or immunosuppression (Shrivastava et al., 2002). The further research is needed to clarify the mechanism of Cr on immune system in dairy cows. Although the incidence of CE was affected, effect of supplemental Cr-Pro on the percentage of PMN at 40 to 60 postpartum was not significant, which may suggest that the distribution of the

percentage of PMN in cows with CE in Cr-Pro treatment was different from those with CE in control, resulting in the same least squre means between treatments.

Because CE has detrimental effects on subsequent reproductive performance (Gilbert et al., 2005) and our results showed lower incidence of CE with Cr-Pro, we would hypothesize that supplemental Cr-Pro would improve reproductive performance. In this experiment, neither the calving to 1st ovulation within 56 DIM nor the calving to conception within 150 DIM was affected by treatments (Figure 4-2 and 4-3). We recognize that our numbers of animals per treatment to detect differences in reproductive outcomes is was too small; therefore, results from this study suggest the need for studies with larger numbers of animals to evaluate reproductive performance of cows fed supplemental Cr. In intensively grazed dairy cows, greater percentages of cows supplemented with Cr were observed to be anestrus by dairy personnel; however, Cr supplementation tended to increase the percentage of cows pregnant in the first 28 d of the mating season (Bryan et al., 2004). Yang et al. (1996) showed that effects of Cr supplementation on days to 1st estrus and days open were not significant, but recent study showed that a lower proportion of non-cycling cows was observed in cows fed Cr along with a higher 28-d pregnancy rate compared with the control group (Soltan, 2010). Future research should evaluate comprehensively the effects of Cr-Pro supplementation during the periparturient period and early lactation on reproduction.

CONCLUSIONS

Supplementation of cows with Cr-Pro during the periparturient period and early lactation in this study resulted in trends for higher DMI and lower NEFA during the prepartum period, but no effects on postpartum DMI, milk yield, or milk composition. Furthermore, effects of Cr-Pro on postpartum energy metabolism and inflammation were not significant. However, cows fed Cr-Pro during the periparturient period and early lactation had decreased incidence of CE during early lactation, which suggests potentially positive effects on reproductive function. Future research should evaluate comprehensively the effects of Cr-Pro supplementation on reproductive performance using larger numbers of cows.

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CHAPTER FIVE: THE EFFECTS OF MONENSIN AND STARCH LEVEL IN FRESH COW DIETS ON ENDOMETRITIS, INDICES OF IMMUNE FUNCTION, AND REPRODUCTIVE PERFORMANCE

ABSTRACT

Primiparous and multiparous Holstein cows (n=70) were used to determine the effects of monensin supplementation throughout the periparturient period and early lactation and starch level in the early postcalving diet (wk 1 to 3 postcalving) on cytological endometritis (CE), immune function, and reproductive performance. Monensin was supplemented within a topdress pellet at 400 mg/d for 3 wk precalving and at 450 mg/d for 9 wk of lactation. In addition, cows that were fed either 0 or 400 mg/d of monensin during the prepartum period were assigned randomly (within monensin treatment) to one of two dietary starch levels beginning at calving. Therefore, postcalving dietary treatments were: 1) High starch (HS), no monensin; 2) HS, monensin; 3) Low starch (LS), no monensin; 4) LS, monensin. On d 22 of lactation, all cows were fed the HS diet with or without monensin as appropriate. Endometrial cytology as characterized by low volume uterine lavage at 7 d postcalving (7 d) and on one day between 40 and 60 d postcalving (40-60 d) was not affected by treatment. In samples collected at d 7 postpartum, cows fed high starch diets tended to have increased positive percentage (%) and had greater index [positive % x MFI (mean fluorescence intensity)] in monocytes conducting phagocytosis compared to cows fed the low starch diet. Furthermore, cows fed monensin tended to have higher MFI in monocytes conducting phagocytosis. Effects

of starch level and monensin treatments on oxidative burst in cells collected at 7 d postpartum were generally not significant, except for a trend for increased monocytes index following stimulation with phorbol 12-myristate 13-acetate (PMA) in cows fed high starch diet. At 40-60 d, overall effects of starch and monensin were not significant for phagocytosis activity; however, for oxidative burst activity, monensin treatment increased the positive % of polymorphonuclear neutrophils (PMN) and monocytes and the monocytes index stimulated by *E. coli* and tended to increase the positive % of PMN and monocytes stimulated by phorbol myristate acetate. Blood PMN glycogen content at 7 d postpartum was not affected by any treatment; however, blood PMN from cows previously fed high starch diets had higher glycogen content at 40-60 d. Reproductive performance determined as calving to conception within 150 days in milk was not affected by treatment. Although postpartum starch level and supplemental monensin did not affect uterine immune status and reproductive performance, the results suggests that aspects of phagocyte function in early lactation were affected by treatment.

INTRODUCTION

Most immune functions are decreased during the transition period (Mallard et al., 1998; Waller, 2000; Rainard and Riollet, 2006) and as a result dairy cows are susceptible to infection, which contributes to comparatively high incidence of diease around parturition (Waller, 2000; Ingvartsen et al., 2003; Rainard and Riollet, 2006). Because PMN are critical for the initial defense against invading microbial pathogens (Burton et al., 2005; Rinaldi et al., 2008), the high incidence of some periparturient

diseases can be associated with impairment of PMN function. Indeed, PMN functional competences such as cell adhesion molecules expression, chemotaxis activity, and oxidative burst activity are known to be reduced around the periparturient period (Detilleux et al., 1995; Burvenich et al., 2003; Rainard and Riollet, 2006).

The mobilization of active PMN is a key factor in the defense against infectious disease in dairy cows. For example, the severity of intramammary infection with *E. coli* is determined by the speed of PMN influx into the udder tissue rather than *E. coli* pathogenicity (Burvenich et al., 2003). Likewise, uterine defenses also rely on innate immunity rather than adaptive immunity at the initial stage (Wathes et al., 2009). Gilbert et al. (2007) reported that proportion of PMN sampled from endometrial cytology at calving was negatively correlated to the extent of bacterial infection at calving and the proportion of PMN at 49 d postpartum and blood PMN functions were significantly impaired during the periparturient period for cows that were diagnosed with puerperal metritis and cytological endometritis (CE) compared to cows with normal uterine health (Hammon et al., 2006). Therefore, the speed of PMN influx into uterus and the adequate PMN functions are crucial to counter bacterial invasion during the early postcalving period and decrease risk for subsequent uterine disease.

Cytological endometritis is defined by the proportion of PMN in endometrial cytology samples in the absence of clinical endometritis (Sheldon et al., 2006), is prevalent in high-producing dairy cows (Galvão et al., 2009), and results in low reproductive performance such as low pregnancy rate (Kasimanickam et al., 2004; Gilbert et al., 2005) and increased interval between calving and pregnancy (Gilbert et

al., 2005; Galvão et al., 2009; Cheong et al., 2011). Negative energy balance (EB) and rate of mobilization of body reserves during early lactation are known to be related to the postpartum interval to first ovulation and lower conception rate (Butler, 2003) and metabolites such as BHBA and NEFA that relate to negative energy balance rather than negative EB per se are considered to be responsible for the deteriorating reproductive function in high yielding dairy animals (Leroy et al., 2008; Friggens et al., 2010). However, evidence suggests that incidence of CE is associated with negative EB (Galvão et al., 2010; Cheong et al., 2011) and therefore it is possible that CE is directly related to poor reproductive performance. Because the extent of negative EB is also negatively correlated with function (Hammon et al., 2006) and energy status (Galvão et al., 2010) of PMN, improving energy status in fresh cows may have positive effects on innate immunity, incidence of CE, and reproduction.

Negative EB begins a few days before calving and usually reaches the most negative level (nadir) about 2 wk after parturition (Butler, 2003). In addition, a recent study showed that propionate induces degranulation of PMN in dairy cows (Carretta et al., 2013), suggesting that a condition to increase propionate production could be favorable condition for PMN function. Therefore, application of high starch diet immediately postpartum could mitigate negative EB, resulting in improved immune status. However, Allen et al. (2009) suggested a hypothesis that feeding highly fermentable starch diets in early lactation period causes satiety and decrease dry matter intake because propionate is likely a potential satiety signal during the period. Hence, investigation of postpartum starch level could help determine approaches to optimize not only EB, but also immune status during the transition period.

Monensin changes the products of ruminal fermentation; it decreases methanogenesis and increases propionate production by propionate producing bacteria, which are tolerant to monensin (Russell and Strobel, 1989). As a result, monensin increases the ratio of propionic to acetic acid production (Russell and Strobel, 1989), which leads to increased hepatic gluconeogenesis and improved energy metabolism (Bergen and Bates, 1984; Duffield et al., 2008a). Therefore, it can be speculated that feeding monensin during the transition period mitigates metabolic disorders resulting from negative energy balance. Indeed, monensin administration has been shown to reduce negative EB related metabolites (Duffield et al., 2008a) and incidence of ketosis (Duffield et al., 2002; Duffield et al., 2008b; Duffield, 2010). The decreased incidence of retained placenta (Duffield et al., 2002) and mastitis (Duffield et al., 2008b; Duffield, 2010) in cows fed monensin suggests that it can affect immune status in dairy cows.

We hypothesized that feeding increasing starch level or supplemental monensin or both to dairy cows in early lactation will decrease the incidence of CE, enhance related immune function, and improve reproductive performance through improvement of energy metabolism without a detrimental effect on DMI.

MATERIALS AND METHODS

Experimental Animals, Treatments, and Procedures

A total of 80 (primiparous and multiparous) Holstein cows from the Cornell University Teaching and Research Center Dairy were enrolled in the experiment as they became available at 21 d before expected calving. Multiparous cows had been

dried off no later than 60 d prepartum, and blocks were formed using dry cows/heifers that were within 40 to 35 d prepartum. Cows were blocked by parity and assigned to one of the following postpartum dietary treatments in a completely randomized design with randomization restricted to balance for expected calving date and previous lactation 305-d mature-equivalent milk production (multiparous cows only): 1) high starch (HS; 28% starch, dry matter (DM) basis) without monensin (contained 176 g/kg of monensin sodium, Elanco Animal Health, Greenfield, IN); 2) HS with 450 mg/d monensin; 3) low starch (LS; 21%, DM basis) without monensin; and 4) LS with 450 mg/d monensin (Figure 5-1). Thus, treatments were in a 2 (high starch and low starch) × 2 (0 or 450 mg/d monensin) factorial arrangement. Cows assigned to postpartum dietary treatments containing monensin were fed a prepartum controlled energy ration and received 400 mg/d of monensin during the prepartum period. Similarly, cows assigned to postpartum dietary treatments without monensin were fed the same prepartum controlled energy ration without monensin. Postpartum treatment rations were fed for ad libitum intake for 21 d, after which all cows were fed a high starch ration with or without monensin topdress (continuing with fresh period monensin treatment assignment) for ad libitum intake until termination of the experiment at 63 days in milk. Topdress pellets were formulated to contain either 0 g/metric ton (control) or 441 g/metric ton of monensin and fed to meet corresponding amount of monensin for each treatment of each period.

The ingredient composition of the diets fed during the experiment is shown in Table 5-1. All formulated diets were typical of the Northeastern US. The prepartum diet that was fed to all cows was a controlled energy ration based on corn silage, wheat

	d 21 prepartum to parturition	Parturition to d 21 postpartum	d 22 to d 63 postpartum
HN	PR[1]	HS[2]	HS
HY	PR + 400 mg of monensin/d	HS + 450 mg of monensin/d	HS + 450 mg of monensin/d
LN	PR	LS[3]	HS
LY	PR + 400 mg of monensin/d	LS + 450 mg of monensin/d	HS + 450 mg of monensin/d

[1]Prepartum controlled energy ration
[2]High starch diet including 29% starch, dry matter (DM) basis.
[3]Low starch diet including 21% starch, dry matter (DM) basis.

Figure 5-1. Prepartum and postpartum treatments: HN (high starch, control), HY (high starch + monensin), LN (low starch, control), and LY (low starch + monensin).

Table 5-1. Ingredient composition of diets including topdress pellets (% of DM).

	Prepartum	Postpartum[1]	
		H	L
Corn silage, processed	39.38	–	–
BMR corn silage[2]		36.90	36.91
Wheat straw	20.33	11.06	11.10
Legume silage		9.22	9.22
Shelled corn, finely ground	4.00	20.10	9.87
Citrus pulp	6.76	0.97	6.85
Corn germ meal	–	2.42	5.33
Soybean hulls	6.62	–	3.43
Wheat middlings	2.18	1.38	1.38
Soybean meal	7.13	7.02	5.10
Canola meal	6.50	4.00	3.37
Blood meal	0.98	1.86	1.85
Amino Plus[3]	1.66	1.63	2.25
Energy Booster 100[4]		0.74	0.92
Calcium carbonate	1.43	1.07	0.78
Sodium bicarbonate	–	0.83	0.82
Soy Chlor[5]	1.25	–	–
Salt	0.15	0.40	0.40
Calcium sulfate	0.72	0.16	0.17
Magnesium Oxide	0.20	0.14	0.14
Magnesium sulfate, 9.9%	0.61	–	–
Selenium 0.06%	0.04	0.05	0.04
Mono dicalcium phosphate	–	0.02	0.07
Trace mineral premix[6]	0.02	0.03	0.03
Vitamin A, D, E premix[7]	0.05	0.02	0.02
Vitamin E premix[8]	0.005	–	–
Zinc sulfate	0.002	–	–
Copper sulfate	0.0003	0.001	0.001

[1]H = high starch diet and L = low starch diet.
[2] BMR= brown midrib corn silage.
[3] Rumen undegradable protein supplement; AGP Inc., Omaha, NE.
[4] Milk Specialties Global; Carpentersville, IL.
[5] Anionic feed supplement; West Central, Ralston, IA.
[6] Contained 30,317 mg/kg of Cu, 136,466 mg/kg of Mn, 3,393 mg/kg of Cot, 3,040 mg/kg of I, and 153,916 mg/kg of Zn.
[7] Contained 30,464 IU/kg of Vitamin A, 5,862 IU/kg of Vitamin D, and 93,784 IU/kg of Vitamin E.
[8] Contained 510,750 IU/kg of Vitamin E

straw, and a concentrate mix. Precalving, cows also received 0.9 kg/d of either control or monensin topdress pellets depending on postpartum treatment assignment. Postpartum diets were formulated with BMR corn silage that was the predominant forage along with smaller amounts of wheat straw and haylage. The concentrate portion of the high starch diet was based on corn grain (20.1% of diet DM). For the low starch diet, corn grain (9.8% of diet DM) was partially replaced with citrus pulp (6.5% of diet DM) and soy hulls (3.4% of diet DM). Cows received 1.2 kg/d of either control or monensin topdress pellets post calving depending on treatment assignment. Mean composition (with standard deviations) of the TMR analyses is presented in Table 5-2. The composition of the topdress pellet (Table 5-2) was analyzed as a single composite sample and standard deviations are not given. 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 0800 h and water was made available at all times. The weekly samples of forages, concentrate mixtures, and TMR were composited into 4-wk composite samples and submitted to a commercial laboratory for Cornell Net Carbohydrate and Protein System/Cornell-Penn-Miner profiles by a commercial laboratory (Cumberland Valley Analytical Services, Hagerstown, MD). Samples were analyzed for DM (method 930.15; AOAC, 2000), CP (method 990.03; AOAC, 2000), ADF (method 973.18; AOAC, 2000), NDF (Van Soest et al., 1991), NDFD (Van Amburgh et al., 2003), sugar (DuBois et al., 1956), starch (Hall, 2009), ether extract (method 2003.05; AOAC, 2006), ash (method

Table 5-2. Chemical composition of experimental diets (± SD).

	Prepartum	Postpartum		Topdress pellet	
Item		High Starch	Low Starch	Control[1]	Monensin[2]
DM, %	50.7 (2.4)	48.3 (2.7)	48.0 (3.2)	93.2 (1.0)	93.7 (1.2)
CP, %	13.0 (0.8)	15.5 (1.2)	15.4 (0.8)	37.5	37.0
ADF, %	28.2 (1.2)	22.7 (1.2)	25.2 (1.2)	11.1	12.9
NDF, %	42.9 (2.0)	34.3 (1.5)	36.9 (1.5)	22.6	21.3
30 h NDFD, %	–	18.9 (1.2)	20.7 (1.1)	–	–
30 h NDFD, % of NDF	–	55.1 (2.0)	56.1 (1.4)	–	–
Sugar, %	4.9 (0.8)	3.5 (0.6)	4.5 (0.4)	–	–
Starch, %	17.4 (1.2)	26.2 (1.2)	21.5 (1.0)	–	–
Fat, %	2.6 (0.2)	4.0 (0.2)	2.2 (0.6)	–	–
Calcium, %	1.28 (0.16)	0.94 (0.09)	1.01 (0.04)	0.51	0.6
Phosphorous, %	0.30 (0.02)	0.34 (0.02)	0.34 (0.02)	0.97	0.99
Magnesium, %	0.41 (0.04)	0.28 (0.02)	0.3 (0.03)	0.48	0.48
Potassium, %	1.12 (0.13)	1.12 (0.09)	1.18 (0.08)	1.67	1.70
Sulfur, %	0.37 (0.04)	0.21 (0.09)	0.22 (0.01)	–	–
Sodium, %	0.12 (0.02)	0.47 (0.08)	0.46 (0.05)	0.07	0.07
Chloride, %	0.37 (0.01)	0.44 (0.04)	0.44 (0.03)	–	–
NE_L[3], Mcal/kg	1.48 (0.03)	1.64 (0.02)	1.56 (0.03)	1.74	1.72

[1]Contained 0 g/metric ton monensin.

[2]Contained 441 g/metric ton monensin.

[3]Net energy for lactation

942.05; AOAC, 2000), minerals (method 985.01; AOAC, 2000), and net energy lactation (NRC, 2001; Linn, 2003).

Endometrial Cytology

Evaluation of endometrial cytology by low volume lavage (Gilbert et al., 2005) was determined on all cows at 7 d postcalving (1st lavage) and on one day between 40 and 60 d (2nd lavage) postcalving as previously described (Cheong et al., 2011). The perineum of the cow was cleansed and a 64-cm Flex Tip sterile plastic infusion pipette (Exodus Breeders Corp., York, PA) was manipulated through the cervix into the uterus. Sterile saline solution (20 mL) was injected into the uterus and agitated gently per rectum; then a sample of the fluid was aspirated. The recovered fluid was centrifuged using a cytocentrifuge directly onto a glass slide. After drying, the slides were fixed and stained using a rapid Romanowsky-type staining procedure and examined under 400× magnification. Two hundred cells were counted from each slide, and results were expressed as the percentage of neutrophils (polymorphonuclear neutrophils; PMN) in total cells (excluding erythrocytes). All the slides were read masked to treatment by the same investigator (TY). The percentage of PMN was continuously compared among treatments for 1st lavage. For 2nd lavage, incidence of CE diagnosed with cut-off point of 10% PMN (Cheong et al., 2011) was dichotomously analyzed.

Phagocytosis, Oxidative Burst, and Flow Cytometory Analyses

Samples of blood were obtained from each cow via coccygeal blood vessel puncture at 7 d postcalving and on one day between 40 and 60 d postcalving. Phagocytosis was analyzed using a commercial kit (Phagotest, Glycotope

Biotechnology, Heidelberg, Germany). Briefly, heparinized and mixed whole blood (100 μL per test) was pipetted into the bottom of two 12 x 75 mm test tubes (Becton Dickinson Biosciences, San Jose´, CA). Fluorescein isothiocyanate-labeled *Escherichia coli* (20 μL per test) was added to the whole blood samples. After vortexing gently, a control sample was remained on ice. A test sample was incubated for 10 min at 37.0°C in a water bath. Precisely at the end of the incubation time, all samples together on one rack simultaneously were taken out of the water bath and placed on ice in order to stop phagocytosis. Quenching solution (100 μL) was added to each of the samples, followed by mixing the samples. The samples were washed twice with 3 ml of washing solution and centrifuged at 250 × g for 10 minutes at 4°C after each washing. The supernatant was discarded. The cells in samples were lysed and fixed with 2 ml of lysing solution for 20 min at room temperature. After centrifuging the samples and discarding the supernatant, the samples were washed once more and centrifuged. After discarding the supernatant, DNA staining solution (200 μL) was added, followed by incubation for 10 min on ice (light protected in the ice bath). Flow cytometry was performed within 60 min after the incubation.

Oxidative burst activity was analyzed using a commercial kit (Phagoburst, Glycotope Biotechnology, Heidelberg, Germany). Briefly, heparinized and mixed whole blood (100 μL per test) was pipetted into the bottom of four 12 × 75 mm test tubes (Becton Dickinson Biosciences, San Jose, CA). For activation, the following solutions (20 μL per tube) were added in tubes: washing solution as a negative control (tube #1); opsonized (non-labeled) *E. coli* (tube #2); chemotactic peptide N-formyl-MetLuePhe solution as a low physiological stimulus (tube #3); and phorbol 12-

myristate 13-acetate (PMA) solution as a high stimulus (tube #4). All tubes were mixed and incubated for 10 min at 37.0 °C in a water bath. After the 10 min incubation, substrate solution (20 μL) was added. The samples were vortexed thoroughly and incubated again for 10 min at 37.0 °C in the water bath. The cells in samples were lysed and fixed with 2 ml of lysing solution for 20 min at room temperature. After centrifuging the samples (5 min, 250 × g, 4°C) and discarding the supernatant, the samples were washed with 3 ml of washing solution and centrifuged. After discarding the supernatant, DNA staining solution (200 μL) was added, followed by incubation for 10 min on ice (light protected in the ice bath). Flow cytometry was performed within 30 min after the incubation.

Flow cytometric analyses were conducted as previously described (Flaminio et al., 2002). The whole blood samples were analyzed for phagocytosis and oxidative burst on a flowcytometer, BD FACS Calibur flowcytometer (Becton Dickinson Biosciences, San Jose, CA), equipped with a 488 μm argon laser, 7600 Power Macintosh computer and Cell Quest Analysis software (Becton Dickinson Biosciences, San Jose, CA). Ten thousand leukocytes per sample were collected. Leukocyte subpopulation (PMN and monocytes) were displayed in a dot plot and gated according to size (forward light scatter) and granularity (90-degree side light scatter). Percentage fluorescence-positive events (positive %) and mean fluorescence intensity (MFI), the latter being correlated with the mean number of bacteria ingested by single phagocyte or the mean oxidative burst activity of single phagocyte (Moya et al., 2008), were recorded for *E. coli* and PMA activations in each gated area (PMN or monocyte). An index of overall phagocytic or oxidative burst activity was also

calculated by multiplying the percentage of responding cells by the corresponding MFI: [index = (positive %) × (MFI)] (Canning et al., 1991; Clapperton et al., 2005; Moya et al., 2008). Higher values for the index reflected higher phagocytic or oxidative burst activities (Canning et al., 1991). The control sample in each test was used to set a marker as a relevant leukocyte population, where less than 10% of the population was positive.

Blood Neutrophil Glycogen

Samples of blood were obtained from each cow via coccygeal blood vessel puncture at 7 d postcalving and on one day between 40-60 d postcalving. The PMN were isolated as previously described (Flaminio et al., 2002) and frozen for later glycogen determination. Glycogen in PMN was determined as described previously (Galvão et al., 2010). Briefly, glycogen was hydrolyzed to glucose using amyloglucosidase; available glucose was determined by reacting 50 μL of supernatant with a 1-mL mixture of 1 mM ATP, 0.9 mM NADP, 5 μg of glucose-6-phosphate dehydrogenase, 0.3 M triethanolamine, and 4 mM MgSO4 and recording the appearance of NADPH after the addition of 5 μL of hexokinase (2 mg/mL) as change in optical density at 340 nm on a spectrophotometer. This change in optical density was compared with a standard curve of glycogen assayed in similar fashion, and results were expressed as micrograms of glycogen/10^6 PMN. Spectrophotometric measurements were conducted using a Versamax tunable microplate reader (Molecular Devices, Sunnyvale, CA).

Reproductive performance

Calving to conception within 150 days in milk (DIM) was analyzed as an outcome variable for reproductive performance. DairyComp 305 software (Valley Agricultural Software, Tulare, CA) was used to collect information of days to pregnancy for each cow.

Statistical Analyses

Non-normally distributed data were transformed to reduce skewness prior to analysis: logarithmic transformation was conducted for all oxidative burst variables in monocytes stimulated by *E. coli* at 7 d and one day between 40 and 60 d (40-60 d) postcalving, oxidative burst positive % in monocytes stimulated by PMA at 7 d and 40-60 d postcalving, phagocytosis index in monocytes at 40-60 d postcalving, oxidative burst MFI and index in PMN stimulated by PMA at 40-60 d postcalving, and oxidative burst index in monocytes stimulated by PMA at 40-60 d postcalving. Square-root transformation was conducted for phagocytosis positive % in PMA and oxidative burst in PMN stimulated by PMA at 7 d and 40-60 d postcalving.

Data for PMN percentage at 1st lavage, phagocytosis, oxidative burst, and PMN glycogen were analyzed by MIXED procedure of SAS (SAS Institute, Cary, NC) for a randomized completely block design with a 2 x 2 factorial arrangement of treatment. Model terms were parity (primiparous or multiparous), starch (high or low), monensin (control or monensin), and the interaction of starch and monensin, with cow as random effect. The method of Kenward-Rogers was used for calculation of denominator degrees of freedom. Aforementioned transformed data were transformed

back to original scales for presentation. Least squares means and standard error of the mean are reported.

Effects of parity, starch, and monensin on incidence of CE at 2nd lavage were analyzed by chi-square for each effect using FREQ procedure of SAS and by multivariate Poisson regression with an adjustment for overdispersion using GENMOD procedure of SAS (Ospina et al., 2012). The model terms were parity, starch, and monensin. Kaplan-Meier survival analysis (Kaplan and Meier, 1958) was conducted to produce survival curves of effects of parity, starch, and monensin on calving to conception within 150 DIM using LIFETEST procedure of SAS. Effects of parity, starch, and monensin on calving to conception within 150 DIM were also evaluated with a semiparametric proportional hazards model (Cox, 1972) using PHREG procedure of SAS. The model terms were parity, starch, monensin, and all 2-way interactions.

Incidences of health disorders and ketosis were analyzed by chi-square test and Fisher's exact test respectively, using FREQ procedure of SAS.

Statistical significance was declared at $P < 0.05$ and trends were discussed at $0.05 < P \leq 0.10$.

RESULTS

A total of 10 cows were removed from the experiment for reasons not related to experimental treatments (6 did not have the 2-wk period required on dry period treatment prior to calving, 3 calved with twins, 1 was removed from data set for poor milk production). Therefore, the final number of cows enrolled in each treatment was

18 (primiparous n = 5, multiparous n = 13), 18 (primiparous n = 5, multiparous n = 13), 19 (primiparous n = 6, multiparous n = 13), and 15 (primiparous n = 5, multiparous n = 10) for HS without monensin, HS with monensin, LS without monensin, and LS with monensin, respectively.

The results of endometrial cytology in cows fed varying levels of starch in the fresh diet and either control or monensin topdress are shown in Tables 5-3 and 5-4. At 1st lavage, percentages of PMN were not affected by treatments (Table 5-3). One primiparous cow and one multiparous cow fed low starch with monensin treatment were not used for analyses at 2nd lavage due to difficulty to retrieve lavage; therefore, total number of cows was 68 for chi-square analyses. Effects of treatment on incidence of CE were not significant at 2nd lavage (Table 5-3). Relative risks to develop CE (Table 5-4) were 0.800, 1.363, and 0.781 for primiparous cows (vs. multiparous), low starch (vs. high), and control treatment (vs. monensin treatment), respectively; however, none of these were different from 1.

The results of phagocytosis and oxidative burst activities for cows fed varying levels of starch in the fresh diet and either control or monensin topdress are shown in Tables 5-5 and 5-6, respectively. In samples collected at d 7 postpartum, cows fed high starch diets tended to have increased percentage of monocytes conducting phagocytosis and greater monocyte index compared to cows fed the low starch diet (P = 0.06 and 0.05; Table 5-5). Furthermore, cows fed monensin tended (P = 0.10) to have higher MFI in monocytes conducting phagocytosis (Table 5-5). Effects of starch level and monensin treatments on oxidative burst in cells collected at 7 d postpartum were generally not significant, with the exception of a trend (P = 0.07) for increased

Table 5-3. Uterine cytology and incidence of cytological endometritis (CE) for cows fed either high or low starch diets from weeks 1 to 3 and either control or monensin treatments beginning during the prepartum period and continuing through early lactation.

	Parity[1]			Starch[2]			Monensin[3]			*P* value			
Item	PP	MP	SEM[4]	H	L	SEM	N	Y	SEM	Parity	Starch	Monensin	Starch X monensin
% of Neutrophils[5]													
1st lavage[6]	35.4	33.3	5.5	31.7	37.0	4.5	35.3	33.3	4.5	0.76	0.37	0.74	0.47
2nd lavage[7]	8.5	14.0	3.4	13.6	8.9	2.8	11.1	11.4	2.9	0.17	0.20	0.93	0.25
Cows with CE[8]	6	18		11	13		12	12		0.56	0.39	0.47	-
Cows without CE	14	30		25	19		26	18					

[1]Parity: PP = primiparous and MP = multiparous.
[2]Starch: H = high starch diet and L = low starch diet during wk 1 to 3 postcalving.
[3]Monensin: N = control and Y = monensin treatment.
[4]SEM = standard error of the mean.
[5]Percentage of polymorphonuclear neutrophils (PMN) in total 200 cells, excluding erythrocytes.
[6]Lavage at 7 d postpartum.
[7]Lavage at one day between 40 and 60 d postpartum.
[8]Number of cows that developed CE (cytological endometritis diagnosed positive if more than 10% PMN were counted in 2nd lavage).

Table 5-4. Relative risk for development of cytological endometritis for cows fed high or low starch diets during the fresh period and either control or monensin treatments during the prepartum period and early lactation.

Item	Relative risk	95% CI of RR[1]	*P* value
Parity[2]	0.800	0.374 - 1.712	0.56
Starch[3]	1.363	0.703 - 2.643	0.36
Monensin[4]	0.781	0.403 - 1.510	0.46

[1]95% confidence interval of relative risk.
[2]Parity: primiparous vs. multiparous.
[3]Starch: low starch diet vs. high starch diet.
[4]Monensin: control vs. monensin treatment.

Table 5-5. Phagocytosis at 7d postpartum for cows fed varying levels of starch and monensin during the periparturient period.

	Parity[1]			Starch[2]			Monensin[3]			*P* value			
Item	PP	MP	SEM[4]	H	L	SEM	N	Y	SEM	Parity	Starch	Monensin	Starch x monensin
Neutrophils													
Positive %[5]	89.3	89.5	1.7	89.9	88.9	1.4	88.6	90.2	1.5	0.90	0.59	0.37	0.68
MFI[6]	145	154	8	150	149	6	144	154	7	0.32	0.90	0.25	0.57
Index[7]	12296	13596	902	13205	12688	743	12446	13446	766	0.23	0.61	0.32	0.56
Monocytes													
Positive %	39.0	43.6	1.5	43.0	39.6	1.3	41.3	41.4	1.3	0.02	0.06	0.97	0.58
MFI	66.8	71.9	3.3	72.3	66.4	2.7	66.3	72.4	2.8	0.20	0.11	0.10	0.12
Index	2650	3132	209	3124	2657	173	2799	2982	181	0.06	0.05	0.44	0.66

[1]Parity: PP = primiparous and MP = multiparous.
[2]Starch: H = high starch diet and L = low starch diet during wk 1 to 3 postcalving.
[3]Monensin: N = control and Y = monensin treatment.
[4]SEM = standard error of the mean.
[5]Positive % = percentage fluorescence-positive events.
[6]MFI = mean fluorescence intensity.
[7]Index = positive % × MFI.

Table 5-6. Oxidative burst at 7d postpartum for cows fed varying levels of starch and monensin during the periparturient period.

	Parity[1]			Starch[2]			Monensin[3]			*P* value			
Item	PP	MP	SEM[4]	H	L	SEM	N	Y	SEM	Parity	Starch	Monensin	Starch x monensin
E coli stimulation													
Neutrophils													
Positive %[5]	81.5	85.6	2.3	83.7	83.4	1.9	83.9	83.2	2.0	0.14	0.93	0.78	0.95
MFI[6]	17.9	20.6	1.2	18.7	19.9	1.0	19.3	19.2	1.0	0.08	0.40	0.91	0.53
Index[7]	1437	1768	128	1557	1648	106	1603	1602	108	0.04	0.53	0.99	0.43
Monocytes													
Positive %	6.5	7.8	1.7	8.5	6.0	1.7	7.5	6.8	1.5	0.51	0.15	0.66	0.35
MFI	8.1	8.3	0.6	7.9	8.5	0.5	8.0	8.4	0.5	0.74	0.32	0.49	0.70
Index	48.0	67.1	12.0	68.0	47.3	13.6	57.9	55.6	11.8	0.22	0.15	0.87	0.51
PMA stimulation													
Neutrophils													
Positive %	85.5	89.3	2.5	87.0	87.8	2.0	86.1	88.7	2.1	0.20	0.77	0.32	0.81
MFI	36.0	49.0	4.0	45.1	39.9	3.4	42.8	42.2	3.5	0.01	0.27	0.91	0.99
Index	2983	4560	446	4090	3453	372	3876	3667	387	0.01	0.21	0.68	0.69
Monocytes													
Positive %	20.4	25.1	4.6	26.6	19.2	4.6	22.8	22.4	4.1	0.40	0.15	0.94	0.32
MFI	27.4	31.1	2.7	28.6	29.9	2.2	28.0	30.5	2.3	0.25	0.65	0.40	0.85
Index	563	824	117	815	572	98	640	747	102	0.07	0.07	0.42	0.50

[1]Parity: PP = primiparous and MP = multiparous.
[2]Starch: H = high starch diet and L = low starch diet during wk 1 to 3 postcalving.
[3]Monensin: N = control and Y = monensin treatment.
[4]SEM = standard error of the mean.
[5]Positive % = percentage fluorescence-positive events.
[6]MFI = mean fluorescence intensity.
[7]Index = positive % × MFI.

monocytes index following stimulation with PMA in cows fed high starch diet (Table 5-6).

For phagocytosis activity at 40 to 60 d postpartum, overall effects of starch and monensin were not significant (Table 5-7); however, MFI in PMN tended ($P = 0.09$) to have an interaction of starch and monensin (Figure 5-2) such that cows that were previously fed the low starch diet and were concurrently being fed monensin tended to have greater MFI than cows in either previous high starch and concurrent monensin treatment or previous low starch and concurrent control treatment. In addition, index in PMN tended ($P = 0.06$) to have an interaction of starch and monensin (Figures 5-3) such that cows that were previously fed the low starch diet and were concurrently being fed monensin had higher PMN index than cows in previous low starch and concurrent control treatment. For oxidative burst activity at 40 to 60 d postpartum, overall effects of starch were not significant; however, monensin treatment increased the percentage of PMN and monocytes ($P = 0.02$ and 0.03) and the monocyte index ($P = 0.05$) stimulated by *E. coli* and tended to increase the percentages of PMN and monocytes ($P = 0.06$ and 0.07) stimulated by PMA (Table 5-8). Furthermore, a trend ($P = 0.08$) existed for an interaction of starch and monensin (Figure 5-4) such that cows that were previously fed the low starch diet and were concurrently being fed control had higher MFI in PMN stimulated by *E. coli* than cows in previous high starch and concurrent control treatment.

Blood PMN glycogen content at 7 d postpartum was not affected by treatments (Table 5-9); however, at 40 to 60 d postpartum the effect of starch was significant ($P = 0.02$) such that cows previously fed high starch had higher glycogen content (Table 5-

Table 5-7. Phagocytosis at 40 to 60 d postpartum for cows fed either high or low starch diets during the first 3 weeks postcalving and control or monensin treatments during the periparturient period and early lactation.

	Parity[1]			Starch[2]			Monensin[3]			*P* value			
Item	PP	MP	SEM[4]	H	L	SEM	N	Y	SEM	Parity	Starch	Monensin	Starch x monensin
Neutrophils													
Positive %[5]	87.2	86.8	1.9	88.5	85.5	1.5	86.2	87.8	1.6	0.88	0.15	0.44	0.24
MFI[6]	152	150	8	155	147	7	146	156	7	0.88	0.44	0.25	0.09
Index[7]	13245	12984	972	13547	12682	793	12384	13845	825	0.82	0.42	0.18	0.06
Monocytes													
Positive %	37.4	43.9	1.8	40.4	40.9	1.4	39.6	41.6	1.5	0.00	0.80	0.30	0.45
MFI	67.1	70.2	4.2	67.5	69.9	3.5	66.7	70.6	3.6	0.54	0.60	0.40	0.96
Index	2418	2953	222	2640	2705	199	2501	2854	221	0.07	0.80	0.18	0.43

[1]Parity: PP = primiparous and MP = multiparous.
[2]Starch: H = high starch diet and L = low starch diet during wk 1 to 3 postcalving.
[3]Monensin: N = control and Y = monensin treatment.
[4]SEM = standard error of the mean.
[5]Positive % = percentage fluorescence-positive events.
[6]MFI = mean fluorescence intensity.
[7]Index = positive % × MFI.

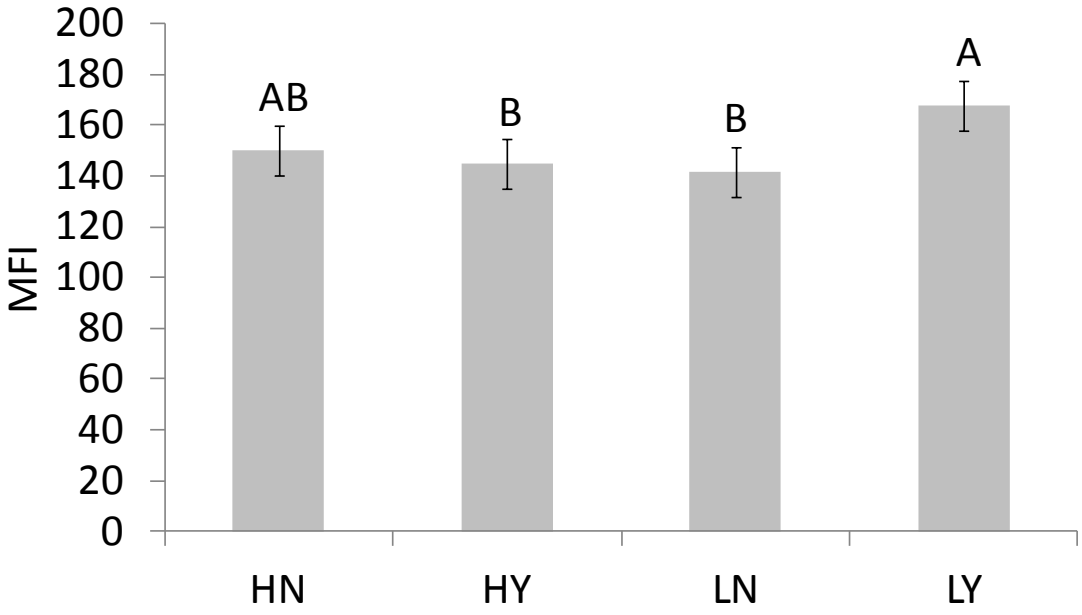

Figure 5-2. Mean fluorescence intensity (MFI) in neutrophils phagocytosis at 40 to 60 d postpartum in cows fed HN (high starch, control), HY (high starch + monensin), LN (low starch, control), and LY (low starch + monensin). Starch x monensin ($P = 0.09$) and values with different upper-case superscript ($P < 0.10$). SEM = 9.9.

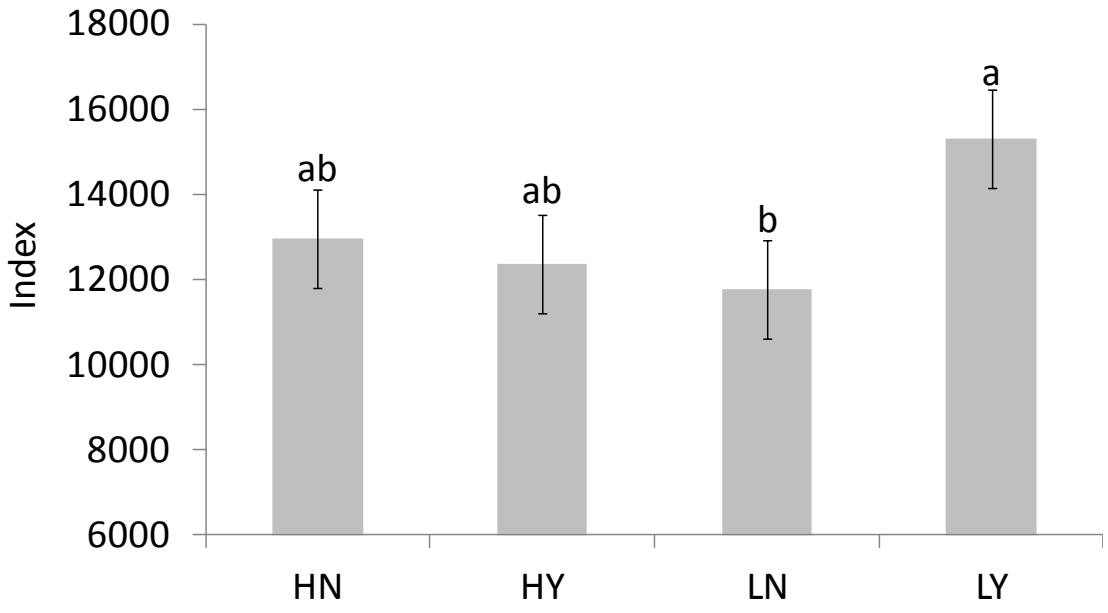

Figure 5-3. Index in neutrophils phagocytosis at 40 to 60 d postpartum in cows fed HN (high starch, control), HY (high starch + monensin), LN (low starch, control), and LY (low starch + monensin). Starch x monensin ($P = 0.06$) and values with different lower-case superscript ($P < 0.05$). SEM = 1162.

Table 5-8. Oxidative burst at 40 to 60 d postpartum for cows fed either high or low starch diets during the first 3 weeks postcalving and control or monensin treatments during the periparturient period and early lactation.

	Parity[1]			Starch[2]			Monensin[3]			*P* value			
Item	PP	MP	SEM[4]	H	L	SEM	N	Y	SEM	Parity	Starch	Monensin	Starch x monensin
E coli stimulation													
Neutrophils													
Positive %[5]	85.6	83.3	1.8	84.1	84.8	1.5	82.2	86.8	1.5	0.29	0.72	0.02	0.43
MFI[6]	18.1	17.3	1.0	18.5	16.9	0.8	17.4	18.0	0.8	0.47	0.14	0.55	0.08
Index[7]	1558	1469	114	1540	1487	92	1460	1566	94	0.52	0.67	0.39	0.68
Monocytes													
Positive %	4.4	4.3	1.0	4.5	4.2	0.8	3.4	5.6	1.0	0.95	0.79	0.03	0.77
MFI	7.5	7.4	0.4	7.5	7.3	0.4	7.4	7.4	0.4	0.81	0.62	0.99	0.49
Index	33.1	36.7	8.0	34.2	35.5	6.8	27.4	44.3	8.9	0.69	0.87	0.05	0.90
PMA stimulation													
Neutrophils													
Positive %	94.2	92.0	1.5	94.4	91.9	1.2	91.5	94.7	1.3	0.22	0.14	0.06	0.55
MFI	33.2	34.0	3.1	35.3	31.9	2.7	33.1	34.0	2.6	0.82	0.30	0.80	0.30
Index	3269	3132	366	3469	2952	314	3190	3209	302	0.74	0.17	0.96	0.76
Monocytes													
Positive %	12.4	14.0	2.6	12.6	13.9	2.3	10.9	16.0	2.8	0.60	0.65	0.07	0.70
MFI	25.2	25.0	2.3	24.6	25.6	1.9	23.7	26.5	1.9	0.92	0.67	0.27	0.43
Index	311	333	67	313	331	56	273	379	68	0.77	0.79	0.13	0.76

[1]Parity: PP = primiparous and MP = multiparous.
[2]Starch: H = high starch diet and L = low starch diet during wk 1 to 3 postcalving.
[3]Monensin: N = control and Y = monensin treatment.
[4]SEM = standard error of the mean.
[5]Positive % = percentage fluorescence-positive events.
[6]MFI = mean fluorescence intensity.
[7]Index = positive % × MFI.

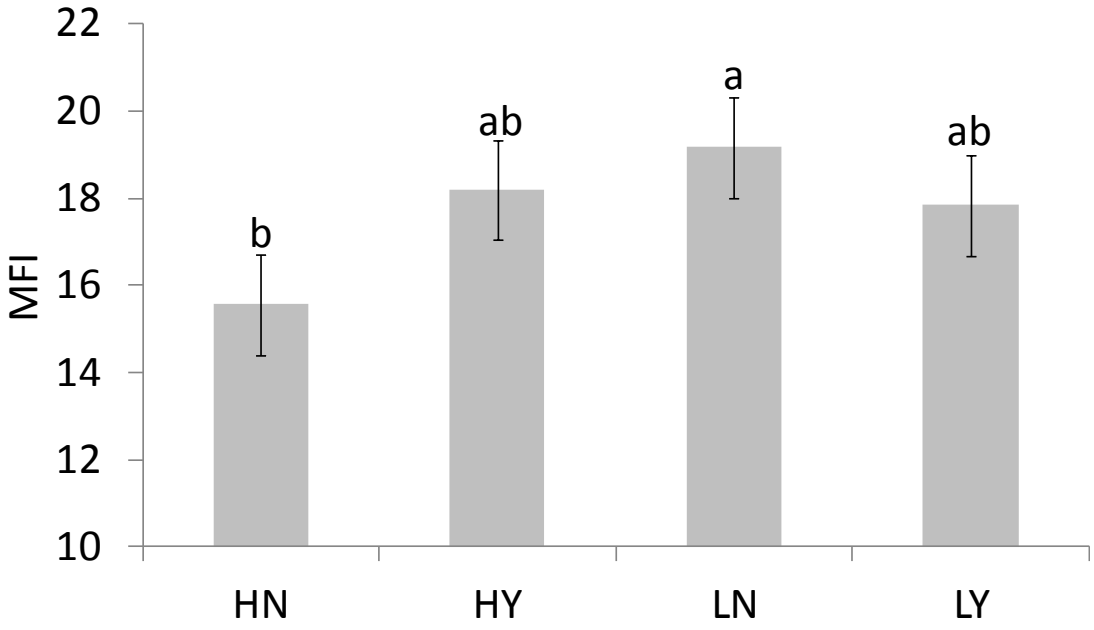

Figure 5-4. Mean fluorescence intensity (MFI) in *E. coli* stimulated neutrophils oxidative burst at 40 to 60 d postpartum in cows fed HN (high starch, control), HY (high starch + monensin), LN (low starch, control), and LY (low starch + monensin). Starch x monensin ($P = 0.08$) and values with different lower-case superscript ($P < 0.05$). SEM = 1.2.

Table 5-9. Glycogen concentrations (μg) per 10^6 neutrophils isolated from cows fed either high or low starch diets during the first 3 weeks postcalving and control or monensin treatments during the periparturient period and early lactation.

	Parity[1]			Starch[2]			Monensin[3]			*P* value			
Sampling	PP	MP	SEM[4]	H	L	SEM	N	Y	SEM	Parity	Starch	Monensin	Starch x monensin
7 d postcalving	7.57	7.59	0.92	7.37	7.79	0.78	6.81	8.35	0.82	0.99	0.70	0.16	0.58
40 to 60 d postcalving	8.77	11.30	1.30	8.34	11.74	1.17	10.05	10.03	1.15	0.09	0.02	0.99	0.64

[1]Parity: PP = primiparous and MP = multiparous.
[2]Starch: H = high starch diet and L = low starch diet during wk 1 to 3 postcalving.
[3]Monensin: N = control and Y = monensin treatment.
[4]SEM = standard error of the mean.

9).

There were several items affected by parity alone such that multiparous cows always had or tended to have greater values than primiparous cows: in phagocytosis and oxidative burst at 7d postpartum, positive percentage and index in monocytes conducting phagocytosis (P = 0.02 and 0.06; Table 5-5), MFI and index in neutrophils stimulated by *E.coli* (P = 0.08 and 0.04; Table 5-6), MFI and index in neutrophils stimulated by PMA (P = 0.01 and 0.01; Table 5-6), and index in monocytes stimulated by PMA (P = 0.07; Table 5-6); in phagocytosis and oxidative burst at 40 to 60 d postpartum, positive percentage and index in monocytes conducting phagocytosis (P = 0.00 and 0.07; Table 5-7); and at 40 to 60 d postpartum, PMN glycogen content (P = 0.09; Table 5-9).

Kaplan-Meier survival analysis showed the interval between calving and conception during 150 DIM was not significantly affected by any treatment of starch level or monensin supplementation (Figures 5-5 and 5-6). There was a tendency such that primiparous cows took less time to become pregnant than multiparous cows (P = 0.06; Figure 5-7). Because none of effects of 2-way interactions were significant, the result for semiparametric proportional hazards model was shown only for main effects; consistent with the result of Kaplan-Meier survival analysis, all of effects were not significant, except for a tendency for parity such that primiparous cows were 1.72 times more likely to get pregnant than multiparous cows during 150 DIM (P = 0.08; Table 5-10).

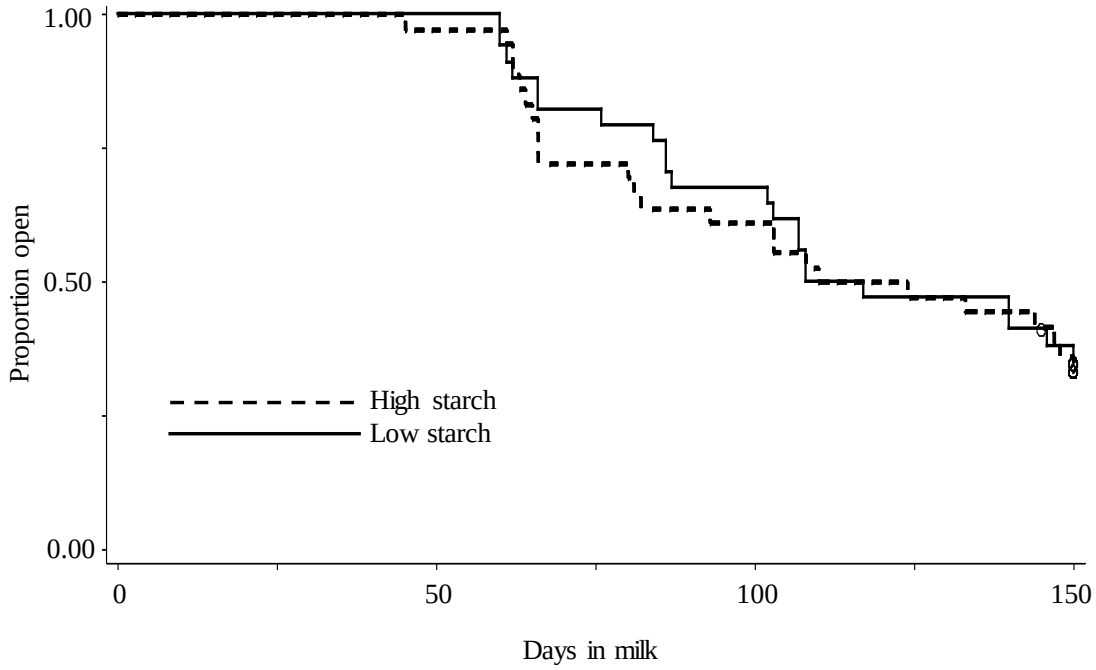

Figure 5-5. Kaplan-Meier survival analysis for effect of feeding high or low starch diets during the first 3 weeks postcalving on calving to conception within 150 days in milk. Circles show censored cows, which came off from the reproduction record or had not been pregnant within 150 days in milk. Treatment did not affect calving to conception interval ($P = 0.73$).

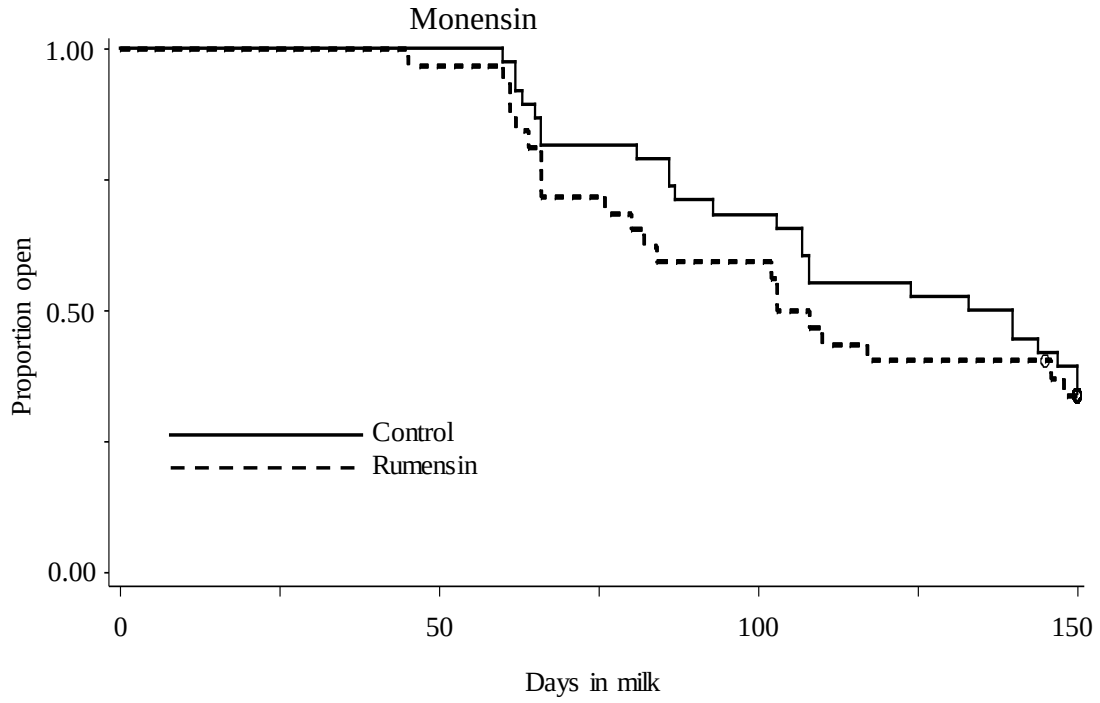

Figure 5-6. Kaplan-Meier survival analysis for effect of monensin supplementation beginning during the prepartum period and continuing through early lactation on calving to conception within 150 days in milk. Circles show censored cows, which came off from the reproduction record or had not been pregnant within 150 days in milk. Treatment did not affect calving to conception interval ($P = 0.37$).

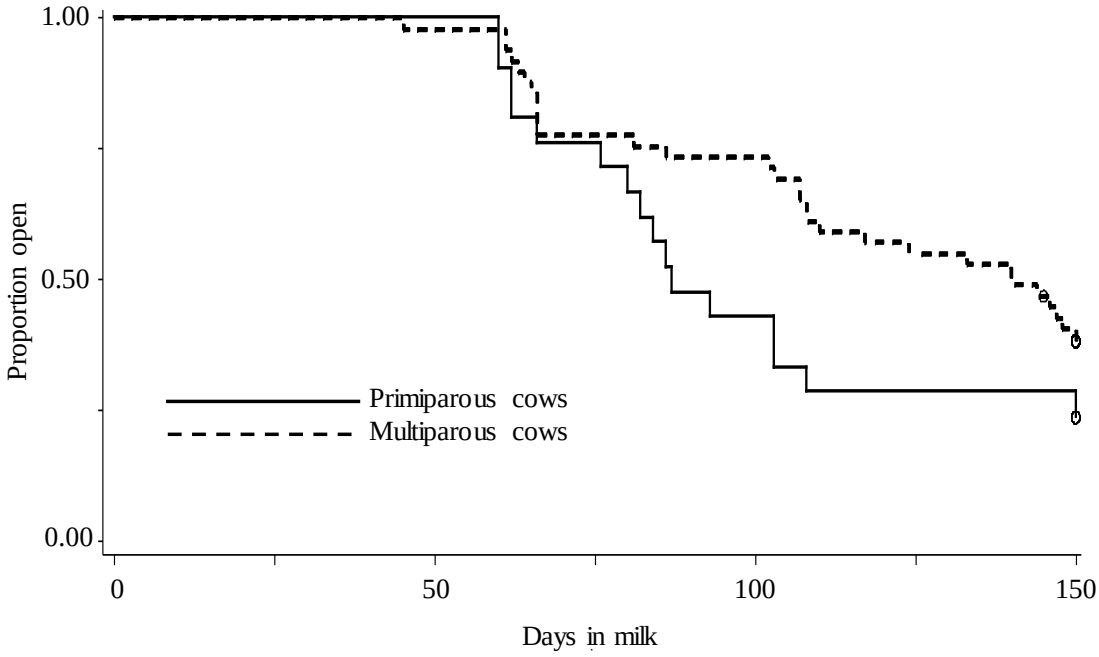

Figure 5-7. Kaplan-Meier survival analysis for effect of parity on calving to conception within 150 days in milk. Circles show censored cows, which came off from the reproduction record or had not been pregnant within 150 days in milk. Treatments tended to affect calving to conception interval ($P = 0.06$).

Table 5-10. Cox proportional hazards model for the effect of varying levels of starch during wk 1 to 3 postcalving, monensin supplementation during the periparturient period, and parity on calving to conception within 150 days in milk.

Variable	Parameter estimate	SE[1]	Hazard ratio	*P* value
Parity[2]	0.54	0.31	1.72	0.08
Starch[3]	-0.06	0.30	0.95	0.85
Monensin[4]	-0.19	0.30	0.83	0.52

[1]SE = standard error.
[2]Parity: primiparous vs. multiparous.
[3]Starch: low starch diet vs. high starch diet.
[4]Monensin: control vs. monensin treatment.

The frequency of occurrence of various health disorders by postpartum dietary treatments is reported in Table 5-11. The incidences of total health disorder did not differ among dietary treatments; however, there was a tendency for increased incidence of ketosis in cows fed either HN or LN ($P = 0.06$; Table 5-12).

Table 5-11. The incidences of health disorders for cows that remained in the final data set.

	Treatments[1]			
Item	HN	HY	LN	LY
Milk fever	1	1	0	0
Ketosis	4	0	5	1
Displaced abomasum	0	0	1	0
Retained placenta	0	2	0	1
Metritis	–	–	–	–
Dystocia	0	2	0	1
Mastitis	3	2	1	2
Total health disorders	8	7	7	5

[1]Treatments: HN (high starch, control), HY (high starch + monensin), LN (low starch, control), and LY (low starch + monensin).

Table 5-12. Analysis of the incidences of health disorders and ketosis for cows that remained in the final data set.

	Treatments[1]				
Item	HN	HY	LN	LY	*P* value
Cows with health disorders[2]	8	7	7	5	0.93
Cows without health disorders	10	11	12	10	
Cows with ketosis[3]	4	0	5	1	0.06
Cows without ketosis	14	18	19	15	

[1]Treatments: HN (high starch, control), HY (high starch + monensin), LN (low starch, control), and LY (low starch + monensin)
[2]Number of cows that developed health disorders.
[3]Number of cows that developed ketosis.

DISCUSSION

The high and low starch diets were formulated to contain 28.0 and 21.0% starch, respectively whereas the analyzed starch content of the high and low starch diets were 26.2 and 21.5% (Table 5-2). The analyzed starch content of the high starch diet was lower than expected; however, the difference between the two diets was considered still large enough to meet the objects of the study.

The percentages of PMN in both the first and second uterine lavage were not affected by postpartum starch level or monensin treatment (Table 5-3). Likewise, both incidence and relative risk of CE were not affected by treatments (Tables 5-3 and 5-4). We chose the range (40 to 60 d postpartum) as the sampling time point for 2nd lavage because a prevalence of CE, which is diagnosed by percentage of PMN in uterine lavage, decreases over time after calving and remains nearly unaffected after around 35 d postpartum (Sheldon et al., 2009) and it was found that CE diagnosed late in the voluntary waiting period (40 to 60 d postpartum) was highly prevalent in commercial herds in New York (Gilbert et al., 2005; Cheong et al., 2011). Polymorphonuclear neutrophils are the main leukocyte type involved in bacterial clearance after uterine infection (Galvão et al., 2011) and it is reported that cows with the greatest influx of PMN to the uterus on the day of calving had the lowest rates of positive bacterial culture, proportion of PMN in uterine lavage, and prevalence of CE later in lactation (Gilbert et al., 2007). Therefore, it was expected that the percentage of PMN at 7 d postpartum was negatively associated with the percentage of PMN at 40 to 60 postpartum and incidence of CE at 40 to 60 d postpartum. Furthermore, more severe

postpartum negative EB is known to be associated with more severe or prolonged uterine inflammation (LeBlanc, 2012). Indeed, Cheong et al. (2011) showed that the odds of having CE were 3.9 times higher in cows with ketosis and Galvão et al. (2010) reported that cows that developed uterine disease such as metritis and CE experienced a greater degree of negative EB when evaluating indicators of EB (NEFA and BHBA). Therefore, approaches maximizing energy intake during the transition period could be one of strategies to improve uterine immune status. We expected varying postpartum starch level to affect uterine defense system through changing energy metabolism.

According to Allen et al. (2009), feeding diets that would promote higher levels of propionate production (e.g., high in fermentable starch) during early lactation could decrease DMI because propionate is main fuel for liver and can be signal to brain to reduce intake. If this theory is true, postpartum low starch diet could result in increased PMN influx to uterus immediately after parturition. However, optimum level of starch to mitigate negative energy balance has been and is being investigated. When feeding either glucogenice diet (26.6% starch; 6.88 MJ/kg NEL) or lipogenic diet (10.4%; 6.81 MJ/kg) from 3 wk before calving through 9 wk postpartum, calculated energy balance was less negative in cows fed the glucogenic diet compared with cows fed lipogenic diet and, during the postpartum period, the glucogenic diet treatment decreased plasma NEFA and BHBA and liver triglyceride concentrations and increased insulin concentration (van Knegsel et al., 2007b). In other research conducted to evaluate effects of dietary treatment for the first 50 d postcalving, plasma insulin and the proportion of cows ovulating within 50 d of calving were increased in cows fed high starch diet (26% starch) compared to isoenergetic control

diet (10%), which suggests improving energy status by feeding high starch diet, although milk production was not affected by treatment (Gong et al., 2002). Nelson et al. (2011) showed that serum BHBA and NEFA for 21 d postpartum period were not different between high (25.5% starch; 1.68 Mcal/kg NEL) and low (21%; 1.65 Mcal/kg) starch diet, which were fed during 3 wk after calving, although negative response of DMI and milk yield was recognized in cows fed high starch diet (Nelson et al., 2011). In addition, investigation of productive performance in cows used in the current study showed that cows fed high starch diet for 3 wk postcalving had higher DMI in wk 2 and 3 postcalving along with a greater increase in milk yield during wk 2 and 3 postcalving and a smaller decrease in BCS during wk 1 to 3 postcalving compared to cows fed low starch diet (McCarthy et al., 2013). Overall, high starch treatment seems to be a favor more positive energy status during early lactation; however, this did not translate to responses in uterine health in the present study.

Monensin is thought to selectively inhibit rumen bacteria through changing cellular ion balance, which leads to higher maintenance energy requirements for certain bacteria (Russell and Strobel, 1989). As a result, feeding monensin changes the microbial populations in the rumen that results in changes in the ratio of volatile fatty acids in the rumen, increasing propionic acid production and reducing the molar percentages of butyric and acetic, which result in increased hepatic gluconeogenic flux (Duffield et al., 2008a). The prevalence and incidence of subclinical ketosis, which results from negative energy balance in dairy cows (Baird, 1982), were reduced (~50% reduction) by monensin treatment (Duffield et al., 1998) and meta-analysis of 59 studies indicated that monensin can reduce BHBA in early lactation (Duffield et al.,

2008a). Supplemental monensin in the current study also showed suppressive effect on the incidence of ketosis (Table 5-12). Therefore, administration of monenisn containing monensin sodium was expected to improve uterine immune response through improving energy metabolism. The lack of response from monensin treatment in the current study is consistent with the result of a meta-analysis from 16 papers (Duffield et al., 2008b), where no significant effects of monensin were found for metritis and retained placenta. The mechanism of the lack of response of monensin on uterine health status is unknown; however, further investigation of the meta-analysis revealed that delivery method of monensin influenced the incidence of retained placenta and metritis, with risk being lower with controlled release capsule treatment compared with delivery in either topdress or in a total mixed ration (Duffield et al., 2008b). In addition, it was suggested that exposure to prolonged treatment in the dry period with monensin may increase the risk of dystocia and retained placenta (Duffield et al., 2008b). The current study did not show any positive or negative effect of monensin on percentage of PMN and incidence of CE. Because few studies have been conducted to evaluate effect of monensin on uterine immune status, further research is needed, considering how long to be fed during the prepartum period and which form of monensin to be used.

At 7 d postpartum, varying starch level affected phagocytosis and oxidative burst in monocytes such that cows in high starch treatment tended to have greater indices for both phagocytosis and PMA stimulated oxidative burst, although effect of supplemental monensin on any immune functions was not significant (Tables 5-5 and 5-6). We speculate that feeding higher starch diets resulted in more available glucose

to activate monocyte function; however, the reason for lack of response of neutrophils is not clear. Energy for phagocytosis of PMN is dependent primarily upon intercellular glycogen (Naidu and Newbould, 1973; Weisdorf et al., 1982). The level of intracellular glycogen in PMN may be determined by dietary treatment during the prepartum period rather than immediately postpartum dietary treatment (Galvão et al., 2010). Alternatively, effect of starch on both phagocytosis and oxidative burst in PMN during the transition period might have been eliminated by several confounding factors such as low calcium level (Martinez et al., 2012) and antioxidant status (LeBlanc et al., 2004). On the other hand, there is evidence to suggest that excessive glucose may decrease PMN phagocytic activity (Sanchez et al., 1973). Oral administration of 100 g of carbohydrate from glucose, fructose, sucrose, honey, or orange juice all significantly decreased the capacity of human neutrophils to engulf bacteria, whereas starch ingestion did not have this effect (Sanchez et al., 1973). Phagocytosis and PMA-stimulated hydroxyl peroxide production were decreased in neutrophils from diabetic rats (Alba-Loureiro et al., 2006). The treatment with insulin abolished the changes induced by the diabetic state even with no marked change in glycemia, which indicates insulin may have a direct effect on neutrophil metabolism and function (Alba-Loureiro et al., 2006). In another study, however, the adhesion to human aortic endothelial cells cultured in the condition of elevated glucose for 7 to 10 d was significantly increased in monocytes, but not in neutrophils (Kim et al., 1994), which suggests that different response to increased carbohydrate treatment in different immune function can be occurred between PMN and monocytes. Further research is needed to investigate response of PMN to starch level in transition dairy cows,

considering possible effects of insulin on PMN. For oxidative burst at 7d postpartum, the reason why starch level affected only PMA stimulated monocytes is also unknown; however, numerical increases ($P = 0.15$) of positive percentage and index were also noted in *E. coli* stimulated monocytes in cows fed high starch diet compared to those fed low starch diet, which was the same pattern as in PMA stimulated monocytes. Because it is known that PMA can activate NADPH oxidase, which is a primary enzyme of the oxidative burst cascade, both in the plasma membrane and in the granule membrane (Karlsson et al., 2000), PMA stimulation may result in producing oxygen radicals both in the plasma membrane and the granule membrane, while *E. coli* stimulation might have resulted in the production only in one membrane, which might have led to less index. Indeed, the different response of oxidative burst between *E. coli* and PMA stimulations can occur under the same treatment or condition (Volk et al., 2000). Also, it is reported that oxidative burst at calving in dairy cows was impaired only in extracellular but not intracellular production of oxygen radicals (Rinaldi et al., 2008). Therefore, a stimulant, which mainly activates extracellular oxidative burst, may not be appropriate to see treatment effects right after calving.

Few studies have evaluated the effects of monensin on immune function; this was the first study to investigate effect of monensin on phagocytosis and oxidative bust of PMN and monocytes. Stephenson et al. (1996) reported that PMN migration towards the chemoattractant was greater in the plasma of monensin treated cows than that of untreated cows during early lactation. However, in the present experiment monensin did not affect immune function studied at 7 d after calving. Crawford et al.

(2005) evaluated effects of monensin on serum haptoglobin (Hp) level, and determined that monensin treatment increased Hp level at wk 1 postcalving in clinically normal heifers and cows, increased the Hp level in diseased heifers when analyzed only for heifers, and did not affect the Hp level in either normal or diseased cows when analyzed only for cows, which suggests there were several confounding factors other than disease and parity. One of the possible factors is periparturient calcium status because it is reported that hypocalcemia, which is common in periparturient dairy cows, is associated with decreased intracellular calcium stores in peripheral mononuclear cells (Kimura et al., 2006). Therefore, consideration of factors such as calcium status around parturition may be needed when investigating immune function immediately after parturition.

At 40 to 60 d postpartum, overall effects of starch level during the 3 wk postcalving on phagocytosis and oxidative burst were not significant (Tables 5-7 and 5-8); however, there were interactions between the previous starch level during 3 wk postpartum and monensin treatment such that PMN phagocytic activities were increased by monensin treatment under the condition of previous low starch treatment (Figures 5-1 and 5-2) and that PMN oxidative burst activity stimulated by *E. coli* was greater in cows fed previous low starch treatment than those fed previous high starch treatment under the condition of control treatment (Figure 5-3). Collectively, previous low starch treatment seems to favor PMN activities at 40 to 60 d postpartum, although the mechanism is not clear. Galvão et al. (2010) reported that cows that developed CE tended to have greater indicators of negative energy balance (plasma NEFA and BHBA) than healthy cows around calving and had lower PMN glycogen content than

healthy cows at 7, 28, and 42 days in milk (Galvão et al., 2010); therefore, improving energy balance around calving can affect PMN glycogen content after 40 d postpartum, which may result in enhanced immune function in PMN. Because feeding diets that would promote higher levels of propionate production (e.g., high in fermentable starch) during early lactation could decrease DMI (Allen et al., 2009) if the theory that propionate is oxidized as fuel for liver is true, low starch treatment for 3 wk postcalving in the current research might have led to improving energy balance, which might cause those positive response in PMN. However, research results are inconsistent as mentioned above: improved postpartum energy balance by not low, but high starch diet (Gong et al., 2002; van Knegsel et al., 2007b); and no difference for indicators of energy status after calving by neither low nor high starch diet (Nelson et al., 2011). Indeed, results of production examined in cows used in the current research showed increased DMI and milk yield along with less losing BCS in high starch treatment (McCarthy et al., 2013). Further research is needed to investigate effect of postpartum starch level right after calving on subsequent immune function during early lactation.

At 40 to 60 d postpartum, effects of monensin on phagocytosis was not significant; however, monensin treatment caused clear changes in oxidative burst activities in both phagocytes: significant increases in positive percent in PMN and monocyte stimulated by *E. coli*; significant increase in index monocyte stimulated by *E. coli*; and tendencies in increases in positive percent in PMN and monocyte stimulated by PMA. Collectively, effects of monensin were more prominent at 40 to 60 d than 7 d postcalving. Granulocytes including PMN are known to depend on

anaerobic glycolysis for the energy required for chemotaxis, phagocytosis, and microbial killing and the sources of needed glucose are extracellular glucose and intracellular glycogen (Weisdorf et al., 1982). In patients with glycogen storage disease type Ib, who have an inability to produce glucose by either gluconeogenesis or glycogenolysis and are dependent on dietary carbohydrate to maintain adequate blood glucose levels, oxidative burst acitivity in both PMN and monocytes are lower than normal range (Ueno et al., 1986; Gitzelmann and Bosshard, 1993) and production of hydrogen peroxide, which is produced during oxidative burst, in human peripheral blood monocytes were abolished in the glucose-absent media (Musset et al., 2012); therefore, monocytes in dairy cows also probably depend on glucose for energy to conduct oxidative burst and the needed glucose presumably depends on extracellular source. Improvement of oxidative burst activity in both PMN and monocytes at 40 to 60 d postpartum in the current research suggests that monensin treatment increases available glucose for the phagocytic activity by increased hepatic gluconeogenic flux during early lactation. Although there is possibility that monensin treatment favored the conditions for PMN phagocytosis (Figures 5-1 and 5-2), the reason for the lack of a corresponding response in monocytes is unknown. Differences in energy metabolism for phagocytosis between monocytes and PMN may result in some of the variation in response. Originally, oxygen consumption, oxidative burst, and phagocytosis in stimulated monocytes are constitutively lower than stimulated PMN (Steigbigel et al., 1974; Reiss and Roos, 1978; Dale et al., 2008). In stimulated monocytes, 30% of consumed oxygen is used for production of reactive oxygen species, while 70% of consumed oxygen is used for oxidative phosphorylation in mitochondria (Reiss and

Roos, 1978). Furthermore, phagocytosis in monocytes, but not PMN, is dependent on the mitochondrial oxidative phosphorylation (Reiss and Roos, 1978), which indicates that monocytes can only function in well-oxygenated areas, whereas PMN are able to do so in a more anaerobic environment, such as synovial fluid (Reiss and Roos, 1978). Therefore, oxygen level or other related metabolism may be dominant factor to influence constitutively low phagocytosis in monocytes rather than energy intake per se.

Improving energy balance around calving can affect PMN intercellular glycogen content after 40 d postpartum (Galvão et al., 2010); therefore, immediately postpartum starch level may affect PMN phagocytosis activity at the time point because energy source of the activity is depend on the glycogen (Naidu and Newbould, 1973; Weisdorf et al., 1982). However, energy source of monocyte immune function is depend on extracellular glucose as mentioned above, the immune function may be responsive to the current monensin treatment rather than previous starch treatment. This can explain why both phagocytosis and oxidative burst activities at 40 to 60 d postcalving in monocytes were not affected by the previous starch treatment.

Glycogen content in PMN at 7 d postpartum was not affected by either postpartum starch level or monensin treatment. The reason for lack of response is unknown. Intracellular glycogen reserves in PMN may be more reflective of prepartum glucose status than current blood glucose level (Galvão et al., 2010) because low blood glucose concentrations preceded low PMN glycogen concentrations (Galvão et al., 2010); therefore, postpartum starch treatment might not have affected blood glucose status and thereby PMN glycogen content at 7 d

postpartum in the current experiment. Numerical increases in the glycogen content in cows fed monensin at 7 d postpartum (P = 0.16; Table 5-9) might reflect effect of monensin on glucose status during tha prepartum period. In addition, a study showed that blood glucose concentration was positively associated with PMN glycogen concentration during the postpartum period (Galvão et al., 2010). Therefore, it is possible that approaches, which increase blood glucose level before and after parturition, affect PMN glycogen content. However, those approaches have not shown consistent responses on blood glucose level during the transition period. Feeding either glucogenic diet (26.6% starch; 6.88 MJ/kg NE_L) or lipogenic diet (10.4%; 6.81 MJ/kg) from 3 wk before calving through 9 wk postpartum did not affect both prepartum (3 wk before calving) and postpartum (9 wk postpartum) plasma glucose concentrations (van Knegsel et al., 2007b). Oral administration of propylene glycol (a glucogenic precursor) for 3 wk before and after calving also did not affect prepartum or postpartum plasma glucose level (Castañeda-Gutiérrez et al., 2009). Therefore, factors other than glucose status may influence PMN glycogen concentration right after calving. Insulin may have a direct effect on metabolism and function in neutrophils (Alba-Loureiro et al., 2006); therefore, it may also affect PMN glycogen content. It was reported that high starch diet during the postpartum or peripartum period increased postpartum insulin level in dairy cows (Gong et al., 2002; van Knegsel et al., 2007a); however, insulin concentrations were not different in cows diagnosed with uterine disease compared to normal cows (Galvão et al., 2010), although the glycogen content was decreased in cows with uterine diseases or negative

energy balance (Galvão et al., 2010). Further research is needed to evaluate whether there are other possible factors that affect PMN glycogen content right after parturition.

Cows fed high starch diet for the first 3 wk after calving had greater glycogen concentrations in peripheral blood PMN at 40 to 60 d postpartum, although effects of monensin were not signifcant (Table 5-9). Because energy status around parturition is positively associated with PMN glycogen content even at 42 d postpartum (Galvão et al., 2010), it could be interpreted that feeding the higher starch diet for 3 wk postpartum improved energy status, which led to higher PMN glycogen content at 40 to 60 d. On the other hand, previous low starch treatment may favor PMN phagocytosis or oxidative burst activity at 40 to 60 d postpartum (Figures 5-1, 5-2, and 5-3); therefore this may show that PMN at 40 to 60 d postpatum was more active in the previous low starch treatment, which might have resulted in more glycogen consumed than under the previous high starch condition. Further research is needed to examine associations of postpartum starch level with subsequent PMN glycogen.

In the current study, neither postpartum starch level nor monensin supplementation affected calving to conception within 150 DIM. This result is consistent with the lack of response of endometrial cytology to treatment in this study as postpartum uterine disease is associated with impaired reproductive performance (LeBlanc et al., 2002; Gilbert et al., 2005; Cheong et al., 2011). However, it was also expected that high level of fermentable starch treatment improved reproductive outcome independent of uterine immune status because negative EB can affect reproductive functions directly such as LH secretion (Butler, 2003) or indirectly such as ovarian function through increased NEFA (Vanholder et al., 2006; Van Hoeck et

al., 2011) and because examination of productive performance in cows used in the current research showed positive response on DMI and energy status (less losing BCS) during early lactation in cows fed high starch diet (McCarthy et al., 2013). A study supports the current result related to starch level. Compared with lipogenic diet (starch 18%), feeding a glucogenic diet (starch 27%) from 3 wk prepartum to 9 wk postpartum did not affect reproductive variables except for a tendency to decrease the number of days until first milk progesterone rise in multiparous cows (not in primiparous cows) (van Knegsel et al., 2007b). The lack of monensin treatment on reproduction in the current study is also consistent with results from a large field study (n = 908; Beckett et al., 1998) and a meta-analysis study based upon 16 publications (Duffield et al., 2008b). The mechanism of unresponsiveness on reproductive variables in high starch level or monensin treatment is not clear. Insulin level may affect effect of the treatment on reproduction because insulin is known to be critical for ovarian follicular function such as upregulating LH receptor on follicles, recruiting small follicles, and facilitating follicle growth (Butler, 2003) and both plasma glucose and insulin are decreased in cows with negative EB (Butler, 2000). However, reproductive variables were not affected by starch level, although feeding the glucogenic diet did increase postpartum blood insulin concentrations (van Knegsel et al., 2007b). Furthermore, monensin treatment did not affect blood insulin level during the transition period through early lactation in a meta-analysis study (Duffield et al., 2008a). Because sample size may also account for variation of reproductive outcomes, large-scale studies may be needed to evaluate effects of starch level and supplemental monensin.

As mentioned in above, interactions of parity and starch level or monensin treatment were not significant; however, a trend existed such that primiparous cows conceived faster than multiparous cows during the first 150 DIM. Because the metabolic demands of high milk production and associated negative EB are linked with diminished quality of oocytes and capability for embryo development (Butler, 2003), it can be speculated that high milk production in multiparous cows, which contributes to overall greater negative EB, negatively affect reproductive functions more in multiparous than in primiparous cows. Indeed, the result in the current study is consistent with some studies, which showed that primiparous cows had shorter days open and higher conception rate at first service compared to cows in second or later lactation (Barton et al., 1996) and that multiparous cows had lower conception rate than primiparous cows when evaluated in 3161 cows (Chebel et al., 2004). However, other studies showed opposite results such that primiparous cows had lower odds ratio for pregnancy at first service compared to multiparous cows in data from 23 farms (Loeffler et al., 1999) and that primiparous cows had longer intervals to first ovulation (Lucy et al., 1992). Lucy (2001) suggestedt that first-lactation cows have lower energy balance than multiparous cows because they eat less and have energy requirements for growth in addition to lactation and that the lower energy balance may be associated with compromised reproductive outcomes. On the other hand, some studies reported no difference of reproductive variables between primiparous and multiparous cows such as interval to pregnancy (Lucy et al., 1992), pregnancy loss (Chebel et al., 2004), and first-service conception rate (Cheong et al., 2011). Because calving season and postpartum diseases are reported to be more important factors than milk yield for

hazard ratio on conception (Gröhn and Rajala-Schultz, 2000), difference of reproductive performance by parity should to be evaluated considering several factors.

In the current study, multiparous cows generally had or tended to have greater values related to immune function than primiparous cows: at 7d postpartum, phagocytosis in monocytes and oxidative burst in both neutrophils and monocytes; at 40 to 60 d postpartum, phagocytosis in monocytes and PMN glycogen content. In contrast to our findings, studies have suggested that primiparous cows are more immune-competent than multiparous cows. Gilbert et al. (1993) determined that cows in the fourth or greater lactation suffered more profound periparturient impairment of PMN function than younger cows and Mehrzad et al. (2002) reported that PMN oxidative burst and viability were increased in primiparous cows compared to multiparous cows during the transition period. In a study using experimental *E. coli* intramammary infections, second-lactation cows had more circulating peripheral leukocytes before infection and less severe outcomes (bacterial counts in infected quarter and milk production in uninfected quarters) after infection than fourth or fifth lactation cows (Werven et al., 1997); however, the immune functions were not consistent with the number of leukocyts and the outcomes such that expression of one of integrins, CD11b/CD18, was lower in second parity cows, while expression of another integrin, CD11c/ CD18, was higher in second parity cows than older (4+ parity) cows, along with no difference for phagocytosis, oxidative burst, and chemotaxis among parity (Werven et al., 1997). Those mixed reactions may be associated with maturity of PMN (Glasser and Fiederlein, 1987) or genetic variation in these immunological traits (Detilleux et al., 1995). On the other hand, the

immunocompromising effect of parturition can be boosted by lactogenesis (Mehrzad et al., 2002) and higher milk production in multiparous cows could be related to more severe negative energy balance followed by immune suppression than primiparous cows. However, there is possibility that high milk yield is not correlated with unfavorable immune response during the transition period (Detilleux et al., 1995) and a decline in neutrophil function in periparturient is not prevented in mastectomized cows (Kimura et al., 1999), which suggests there may be several factors related to endocrine system and metabolism to cause different immune responses among parity. Indeed, fluctuation of metabolic related hormones is different between primiparous and multiparous cows such that IGF-1 plays a major role for partitioning of nutrients in primiparous cows while insulin does in multiparous cows (Wathes et al., 2007). Furthermore, the relationship between milk yield in early lactation and uterine disease status indicates the complexity. For primiparous cows, a study showed that greater milk yield in healthy cows or cows with CE than cows with metritis (Galvão et al., 2010), but another showed that greater milk yield in cows with high risk of CE than cows with low risk (Cheong et al., 2011). For multiparous cows, the former showed that greater milk yield in cows with metritis than healthy cows or cows with CE (Galvão et al., 2010), but the latter showed that greater milk yield in cows with low risk of CE than cows with high risk (Cheong et al., 2011). Further investigation would be useful to control herd immunity during the transition period through early lactation.

CONCLUSIONS

Effect of postpartum starch level or monensin treatment on percentage of PMN in the uterine lavage conducted at 7d postpartum and incidence of CE at 40 to 60 d postpartum were not significant. Investigation of immune functions in phagocytes showed that phagocytosis and oxidative burst activities at 7d postpartum were enhanced only in monocytes and primarily by feeding higher starch diets for 3 wk postpartum period, whereas those activities at 40 to 60 d postpartum in both PMN and monocyte were affected mainly by monensin treatment. Blood PMN glycogen content at 7 d postpartum was not affected by treatments; however, the glycogen at 40 to 60 d postpartum was increased by high starch diet previously fed for 3 wk postpartum period. Parturition to conception within 150 DIM was not affected by postpartum starch level or monensin treatment.

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CHAPTER SIX: ASSOCIATIONS OF ENDOMETRITIS WITH ENERGY METABOLISM AND INFLAMMATION DURING THE PERIPARTURIENT PERIOD AND EARLY LACTATION IN DAIRY COWS

ABSTRACT

Multiparous Holstein cows (n=108) were used to determine the associations of cytological endometritis (CE) with plasma nonesterified fatty acids (NEFA) and beta-hydroxybutyrate (BHBA) as markers of energy metabolism, calculated energy balance (EB), and plasma haptoglobin (Hp) as a marker of inflammation during the periparturient period and early lactation. Evaluation of endometrial cytology by low volume uterine lavage was conducted on one day between 40 and 60 d postcalving. The incidence of CE among cows sampled was 40%. Area under the curve (AUC) was calculated for both NEFA and BHBA using data collected from 3 wk before to 3 wk after (wk -3 to wk +3) parturition. Overall, cows that subsequently developed CE tended to have higher AUC for NEFA and BHBA. Further stratification into prepartum (wk -3 to parturition) and postpartum (parturition to wk +3) revealed that prepartum AUC for both NEFA and BHBA were not associated with subsequent CE, whereas postpartum AUC for NEFA tended to be increased and BHBA was increased for cows that developed CE. Consistent with the results for plasma NEFA and BHBA, calculated EB during the prepartum period was not different in cows that did or did not develop CE; however, cows with CE had lower EB during the 6-wk postpartum period compared to cows without CE. Analysis of EB by week (wk -3 to -1 before calving and wk +1 to +6 postcalving) indicated that EB in cows with CE was lower at

wk +1, wk +2, and wk +3 and tended to be lower at wk +6 than cows without CE. Plasma Hp concentrations were analyzed from wk +1 to +8 of lactation; concentrations of Hp were not different during either wk +1 or the entire postpartum period between cows that did or did not develop CE. These results suggest that decreased energy status, particularly during the first 3 wk postpartum, predisposes dairy cows to subsequent CE.

INTRODUCTION

The demand for energy in dairy cows, which have been selected for high milk production, is highest in early and peak lactation (Butler and Smith, 1989) and even unusual postparturient increases in voluntary intake cannot satisfy this increased nutrient demand (Bell, 1995). Therefore, dairy cattle do not maintain positive energy balance (EB) during early lactation and hence mobilize body reserves (Butler and Smith, 1989). Furthermore, immunocompetence is decreased during the transition period (Mallard et al., 1998) and the incidence of mastitis, ketosis, digestive disorders, and laminitis is highest around parturition (Ingvartsen et al., 2003). There is possibility that immunosuppression results from changes in hormones around parturition (Goff, 2008), which may contribute to the high incidence of diseases (Mallard et al., 1998). However, those hormones are known to have different effects on immune system (Ingvartsen et al., 2003) that can be categorized as either negative (estrogen, progesterone, and cortisol) or positive (growth hormone (GH), prolaction, insulin, and insulin-like growth factor (IGF)-1). Also, each of hormones has different pattern of change during the transition period (Bell, 1995; Ingvartsen and Andersen, 2000) such

that estrogen, cortisol, GH, and prolactin are increased, while progesterone, insulin, and IGF-1 are decreased (Bell, 1995; Ingvartsen and Andersen, 2000). Therefore, it is difficult to clarify the cause and effect of immune suppression resulting from changes in periparturient hormones.

In addition to the potential effects of hormones, metabolites related to energy metabolism such as nonesterified fatty acids (NEFA) and beta-hydroxybutyrate (BHBA) are also associated with immunocompetence during the periparturient period. Indeed, it has been reported that negative EB status affect PMN function (Hammon et al., 2006) and polymorphonuclear neutrophils (PMN) energy status (Galvão et al., 2010) in dairy cows. Several studies have suggested association of uterine diseases with negative EB status (Carson, 2009; Dubuc et al., 2010; Giuliodori et al., 2013a). Among uterine diseases, cytological endometritis (CE) is defined by proportion of PMN in samples collected by flushing the uterine lumen or by endometrial cytobrush in the absence of clinical endometritis about 5 wk postpartum (Sheldon et al., 2009). Study conducted in 5 commercial farms showed that CE was highly prevalent and exerted a profoundly detrimental effect on subsequent reproductive performance such as longer days open (Gilbert et al., 2005), which was also confirmed in a larger scale research with 38 herds (Cheong et al., 2011). Development or incidence of CE was also related to EB presented as NEFA and BHBA in blood (Hammon et al., 2006; Galvão et al., 2010) and development of postpartum ketosis (Cheong et al., 2011); however, association of CE with NEFA, BHBA, and net EB calculated from productive performance data has not yet been elucidated.

Upon stimulation by the release of proinflammatory cytokines such as IL-1, IL-6 and TNF-α from macrophages and monocytes at the site of inflammatory lesions or infection, hepatic acute phase proteins (APP) are produced and released (Eckersall, 2000). An increase in the circulation of the cytokines stimulates further hepatic APP production (Eckersall, 2000). Haptoglobin (Hp) is a major APP in ruminants and is present in negligible circulating levels in normal animals, but increases over 100-fold on stimulation (Eckersall, 2000). Studies have suggested that increased blood Hp is related to uterine diseases such as metritis (Huzzey et al., 2009; Chan et al., 2010; Dubuc et al., 2010) and CE (Dubuc et al., 2010). Fatty liver was reported to induce production of Hp (Murata et al., 2004); however, it has also been suggested that fatty liver may suppress the secretion of important immune proteins, especially APP (Ingvartsen et al., 2003). Collectively, production of Hp appears to be affected by inflammation and EB status during the periparturient period.

The purpose of the current study was to clarify associations of energy metabolism and inflammation with CE during the transition period and early lactation. We hypothesized that development of CE was associated with severely negative EB and increased inflammatory response and that EB status was correlated with inflammatory responsiveness.

MATERIALS AND METHODS

Experimental Animals and Procedures

Data from multiparous Holstein cows (n = 108) that had been subjected to uterine cytology on one day between d 40 and 60 postpartum for use in diagnosis of

CE in two studies (Yasui et al., 2012a,b), were pooled to analyze the association in question. Incidence of CE had been determined using a cut-off point of 10% PMN (Cheong et al., 2011) in the two studies. Plasma samples obtained weekly from cows in one study (Yasui et al., 2012b) and 3 times per week from cows in another study (Yasui et al., 2012a) from 3 wk prepartum through 3 wk postpartum (wk -3 through wk +3) were used to analyze NEFA and BHBA. Plasma concentrations of NEFA and BHBA were analyzed by enzymatic analyses (NEFA-C; Wako Pure Chemical Industries, Osaka, Japan; and BHBA dehydrogenase; kit no. 310, Sigma Chemical). Energy balance was calculated using NRC (2001) equations from wk -3 through wk +6 as follows:

Prepartum EB = (DMI × NE_L diet) - [($BW^{0.75}$ × 0.08) + (((2 × 0.00159 × Days Pregnant - 0.0353) × (Calf BW/45)/0.14) × 0.64)]

Postpartum EB = (DMI × NE_L diet) - [($BW^{0.75}$ × 0.08) + ((0.0929 × Milk fat % + 0.0563 × Milk true protein % + 0.0395 × Milk lactose %) × milk yield)].

Plasma samples collected from wk +1 through wk +8 also were analyzed for concentrations of Hp. Plasma concentrations of Hp were measured by enzymatic analysis (Phase Range Haptoglobin Assay, Tridelta Diagnostics Ltd., Morris Plains, NJ). All spectrophotometric measurements were conducted using a Versamax tunable microplate reader (Molecular Devices, Sunnyvale, CA).

Statistical Analyses

Area under the curve (AUC) was calculated for both NEFA and BHBA using the EXPAND procedure of SAS version 9.1 (SAS Institute, Cary, NC) by using cubic spline interpolation and the trapezoidal rule. The AUC of NEFA and BHBA was

subjected to ANOVA using the MIXED procedure of SAS. EB and plasma Hp were subjected to ANOVA by using theMIXED procedure of SAS with the REPEATED statement used in analysis of repeated measures, where four covariance structures were tested: compound symmetry, heterogeneous compound symmetry, first-order autoregressive, and heterogeneous first-order autoregressive. The covariance structure that resulted in the smallest Akaike's Information Criterion was used. Degrees of freedom for the MIXED procedure were estimated using the Kenward-Roger option in the model statement. Fixed effects included CE (Yes or No), time (week or day only for REPEATED analysis), and the 2-way interaction (only for REPEATED analysis). The random effect was cow or cow nested within CE only for REPEATED analysis. Least squares means and standard error of the mean were reported. Statistical significance was declared at $P < 0.05$ and trends were discussed at $0.05 < P < 0.15$.

RESULTS AND DISCUSSION

The incidence of CE among cows analyzed was 40%. This is slightly higher than the level in a review by Sheldon et al. (2009), who reported that about 30% of cattle have CE diagnosed at about 5 wk postpartum; however, the prevalence of CE among herds was reported to range from 37 to 74% in 5 herds with an average of 53% (Gilbert et al., 2005) and 4.8 to 52.6% in 38 herds with 25.9% as average (Cheong et al., 2011). The incidence of CE in the current study appears to have been typical of that existing in commercial dairy farms.

Overall, cows that developed CE tended to have higher AUC than those who did not develop CE for NEFA (7759 vs. 6602 µEq/L x d; $P = 0.08$) and BHBA (126.9

vs. 112.6 mg/dL x d; *P* = 0.08) as shown in Table 6-1 and Panels A and C in Figure 6-1. Further stratification into prepartum (wk -3 to wk -1) and postpartum (wk +1 to wk +3) revealed that prepartum AUC was not associated with subsequent CE for both NEFA (*P* = 0.21) and BHBA (*P* = 0.81); however, postpartum AUC for NEFA (5391 vs. 4427; *P* = 0.11) tended to be increased and postpartum AUC for BHBA was increased (72.0 vs. 58.9; *P* = 0.049) for cows that subsequently developed CE (Table 6-1 and Panels B and D in Figure 6-1). Consistent with these results, prepartum EB was not associated with subsequent development of CE (*P* = 0.61); however, cows that developed CE had lower (*P* = 0.02) postpartum EB than those that did not develop CE (-3.8 vs -1.9 Mcal/d; Table 6-2). Analysis of EB by week showed that EB in cows with CE was lower at wk +1 (-8.1 vs. -4.9 Mcal/d; *P* = 0.01), wk +2 (-7.9 vs. -5.5 Mcal/d; *P* = 0.04), and wk +3 (-4.9 vs. -2.7 Mcal/d; *P* = 0.048) and tended to be lower at wk +6 (0.9 vs. 2.5 Mcal/d; *P* = 0.10) than cows without CE (Figure 6-2).

Our findings were consistent with other studies, which showed that elevated plasma NEFA or BHBA during early lactation was positively associated with development of CE (Hammon et al., 2006; Dubuc et al., 2010; Galvão et al., 2010); however, Hammon et al. (2006) showed that the increased NEFA begun from 2 wk prior to parturition and Kaufmann et al. (2010) reported that higher concentrations of NEFA at one week prepartum were associated with increased odds of CE. Also, cows with CE showed decreased DMI for both prepartum and postpartum periods (Hammon et al., 2006), which was consistent with the increased NEFA.

The period of decreased DMI in Hammon et al. (2006) study is not consistent with our result showing that EB was associated with the outcome of CE only after

Table 6-1. Calculated area under the curve (AUC) for plasma NEFA and BHBA during the periparturient period for cows categorized as either negative or positive for cytological endometritis (CE).

	CE[1]			*P* value
Item	-	+	SEM[2]	CE
NEFA, AUC				
Wk -3 through wk +3[3]	6602	7759	507	0.08
Wk -3 to wk -1[4]	1374	1518	90	0.21
Wk +1 to wk +3[5]	4427	5391	463	0.11
BHBA, AUC				
Wk -3 through wk +3	113	127	6	0.08
Wk -3 to wk -1	38	39	2	0.81
Wk +1 to wk +3	59	72	5	0.05

[1]CE- = cows without CE and CE+ = cows with CE.
[2] SEM = standard error of the mean.
[3] AUC for 3 wk before and after parturition.
[4] AUC for 3 wk before parturition.
[5] AUC for 3 wk after parturition.

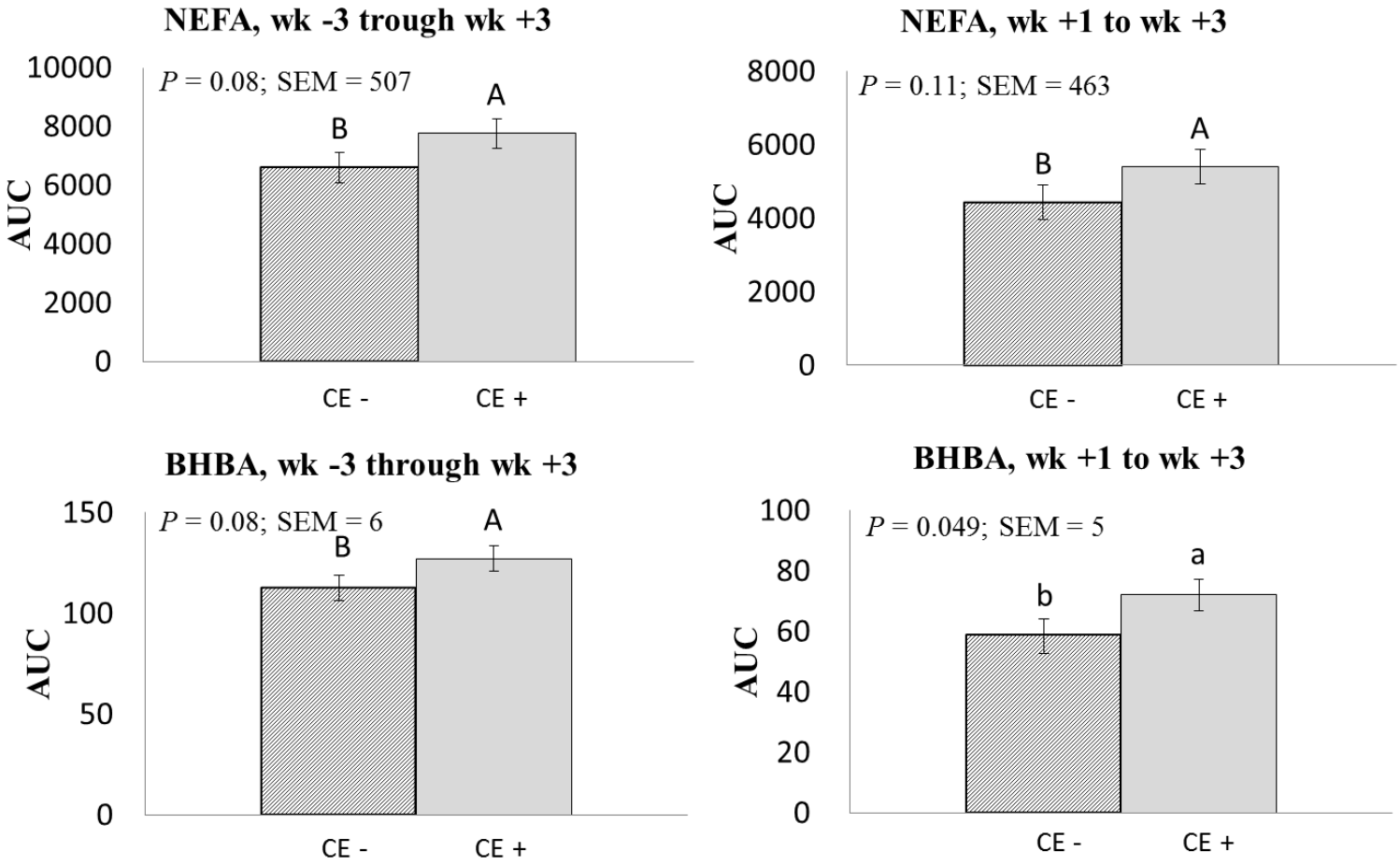

Figure 6-1. Calculated area under the curve (AUC) for plasma NEFA and BHBA during the periparturient period for cows categorized as either negative or positive for cytological endometritis (CE). Wk -3 through wk +3 and wk +1 to wk +3 represent AUC for 3 wk before and after parturition and 3 wk after parturition, respectively. P value and standard error of the mean (SEM) is presented in each graph. CE- = cows without CE and CE+ = cows with CE. Values with different upper-case superscript in Panels A, B, and C differ at P < 0.15 and values with different lower-case superscript in Panel D differ at P < 0.05.

Table 6-2. Energy balance (EB) during 3 wk prepartum period and 3 wk postpartum period for cows categorized as either negative or positive for cytological endometritis (CE).

	CE[1]			*P* value		
Item	-	+	SEM[2]	CE	Wk[3]	CE x wk
EB, Mcal/d						
Wk -3 to wk -1[4]	8.6	9.0	0.6	0.61	<0.001	0.60
Wk +1 to wk +3[5]	-1.9	-3.8	0.6	0.02	<0.001	0.77

[1]CE- = cows without CE and CE+ = cows with CE.
[2] SEM = standard error of the mean.
[3] Wk = week effect.
[4] EB duirng 3 wk before parturition. Calculated according to NRC (2001): EB = (DMI × NE_L diet) - [($BW^{0.75}$ × 0.08) + (((2 × 0.00159 × Days Pregnant - 0.0353) × (Calf BW/45)/0.14) × 0.64)].
[5]EB during 3 wk after parturition. Calculated according to NRC (2001): EB = (DMI × NE_L diet) - [($BW^{0.75}$ × 0.08) + ((0.0929 × Milk fat % + 0.0563 × Milk true protein % + 0.0395 × Milk lactose %) × milk yield)].

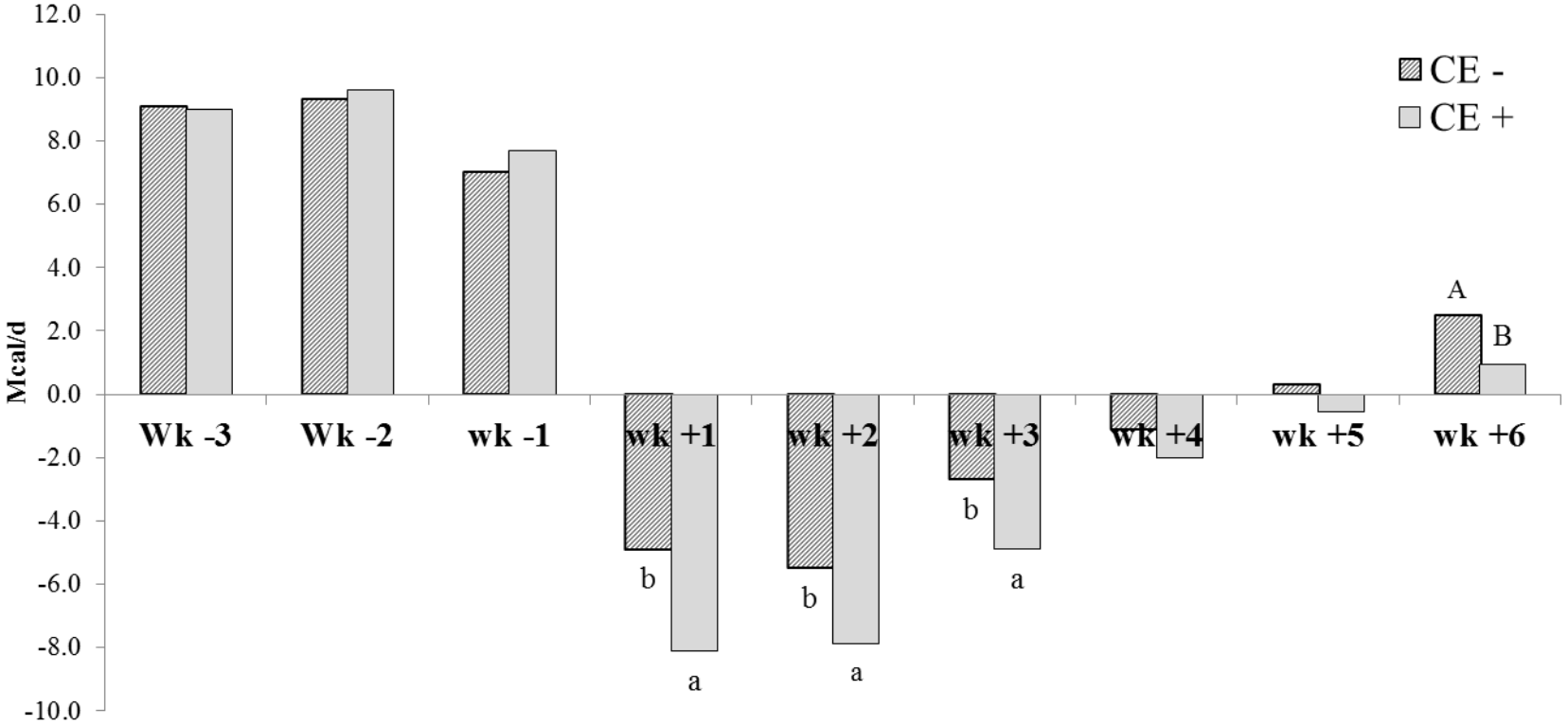

Figure 6-2. Energy balance (EB) from 3 wk prepartum through 6 wk postpartum for cows diagnosed with cytological endometritis (CE) between d 40 and 60 postpartum. Prepartum EB was calculated according to NRC (2001): EB = (DMI × NE_L diet) - [($BW^{0.75}$ × 0.08) + (((2 × 0.00159 × Days Pregnant - 0.0353) × (Calf BW/45)/0.14) × 0.64)]. Postpartum EB was calculated according to NRC (2001): EB = (DMI × NE_L diet) - [(0.08 × $BW^{0.75}$) + ((0.0929 × Fat + 0.0563 × Protein + 0.0395 × Lactose) × milk yield)]. Standard error of the mean was 0.8 and 1.0 for prepartum EB and postpartum EB, respectively. CE- = cows without CE and CE+ = cows with CE. Values with different lower-case superscript and different upper-case superscript differ at $P < 0.05$ and $P < 0.15$, respectively.

parturition. Associations of the stage of negative EB status with other uterine diseases have also been inconsistent. Carson (2009) and Dubuc et al. (2010) determined that elevated prepartum NEFA concentration was positively associated with development of metritis whereas the concentration of postpartum NEFA was not. A recent study also reported that increased prepartum NEFA and postpartum BHBA, but not NEFA during the immediate postpartum period, were associated with development of clinical endometritis (Giuliodori et al., 2013a). However, Giuliodori et al. (2013b) reported that cows with metritis had higher blood BHBA concentrations than healthy cows immediately after parturition, but prepartum BHBA and NEFA and immediately postpartum NEFA were not associated with the development of metritis. Hammon et al. (2006) reported that cows with metritis had increased NEFA for both prepartum and postpartum periods and had increased BHBA after parturition when compared to cows with healthy uterus. Cheong et al. (2010) found that cows that developed postpartum ketosis had 3.8 times higher odds ratio to have CE than cows that did not. Homeorhetic adaptation, which causes decreased insulin responsiveness in adipose tissue during the late pregnancy (Petterson et al., 1994), and negative EB, which begins a few days before parturition (Butler, 2003), contribute to elevated circulating levels of NEFA and ketones during the late pregnancy (Bell, 1995). However, prepartum negative EB is typically less of a concern (Huzzey et al., 2011) and negative EB usually reaches its nadir about 2 wk after parturition (Butler, 2003). The reason why several studies have shown associations of uterine diseases only with prepartum EB status rather than the nadir of EB is unknown. Because increased liver triglyceride in the first and second week after calving is associated with decreased

functional capacities of PMN derived from blood and uterus (Zerbe et al., 2000), the extent of body fat deposition before calving may be predominant factor over EB per se. This may explain why cows that are too fat at calving have a higher incidence of infectious diseases such as endometritis (Zerbe et al., 2000). However, Reist et al. (2000) showed that cows with the same moderate BCS before and at calving had different metabolic responses such that serum and milk BHBA concentrations during the first 6 wk of lactation, but not before calving, were higher in cows with late-onset ovulation than in cows with early-onset ovulation, suggesting that other metabolic or endocrine factors can cause higher secretion of energy-related metabolites after calving. BHBA is known to suppress function and proliferation of leukocyte (Hoeben et al., 1997; Hoeben et al., 1999). Sheldon et al. (2009) suggested that postpartum reductions in neutrophil function are most marked in high producing dairy cows that have compromised energy metabolism after parturition, which may predispose cows to the establishment of uterine disease. Nevertheless, it is reported that cows with metritis and mastitis had lower PMN function during the prepartum period (Cai et al., 1994). Although EB status varies among individuals due to different ability of feed ingestion and milk production (Villa-Godoy et al., 1988; Butler, 2003), it would be too simple to attribute the association with uterine diseases to individual differences. On the other hand, Ospina et al. (2010) reported that the concentration of both prepartum and postpartum NEFA in serum were negatively associated with risk of conception. Cytological endometritis, of which the prevalence is high in herds (Gilbert et al., 2005; Cheong et al., 2011), is known to be associated with poor reproductive performance such as lower percentage of pregnancy to first service (Gilbert et al., 2005) and longer

median days open (Gilbert et al., 2005; Galvão et al., 2009; Cheong et al., 2011) compared to cows without CE. Although our findings support the idea that CE, negative EB, and deteriorated reproductive performance are linked each other, further studies are needed to elucidate how the stage, duration, and severity of negative EB affects subsequent development of CE.

Plasma Hp concentrations were during the first 8 wk postpartum were not associated with subsequent development of CE (Table 6-3). Analysis of samples at 1 wk postpartum also found no relationship between subsequent CE and the concentration of plasma Hp (Table 6-3). Increased Hp is known to be a marker of inflammation in the week after calving in dairy cows (Humblet et al., 2006). Association of Hp with uterine diseases also has been reported. Chan et al. (2010) showed that cows that developed metritis had higher concentration of blood Hp for 6 months postpartum than healthy cows. Huzzey et al. (2009) and Dubuc et al. (2010) reported that high levels of Hp in blood within the first week postpartum were associated with development of metritis and Dubuc et al. (2010) further reported associations of Hp during the immediate postpartum period with CE. Therefore, we expected that plasma Hp would be higher during early lactation or right after parturition in cows that developed CE than cows that did not. Other production diseases during the transition period such as ruminal acidosis, which causes inflammatory response (Mulligan and Doherty, 2008), might have been confounding factors for values of plasma Hp because blood Hp is known to be increased in ruminal acidosis conditions (Jacobsen et al., 2004; Khafipour et al., 2009). Further, each APP has different extent of response to the same inflammatory stimulation (Jacobsen et al.,

Table 6-3. Plasma haptoglobin (Hp) during 8 wk postpartum period for cows categorized as either negative or positive for cytological endometritis (CE).

	CE[1]			*P* value		
Item	-	+	SEM[2]	CE	Wk[3]	CE x wk
Hp, mg/ml						
Wk +1 to wk +8[4]	0.78	0.73	0.05	0.38	<.001	0.97
Wk +1 only[5]	0.98	0.99	0.09	0.95	-	-

[1]CE- = cows without CE and CE+ = cows with CE.
[2]SEM = standard error of the mean.
[3]Wk = week effect.
[4]Represents plasma samples collected weekly from 1 wk postpartum through 8 wk postpartum.
[5]Represents plasma samples collected from 1 wk postpartum only.

2004) and has different pattern of response according to the stage of inflammation (Humblet et al., 2006). Therefore, consideration of other health problems and various acute phase proteins may be needed to investigate association of CE with inflammatory response,. On the other hand, Hp is known to be induced in cows with fatty liver syndrome (Murata et al., 2004; Guzelbektes et al., 2010). Uchida et al. (1993) reported that the detection rate of serum HP was significantly higher at parturition compared to before and after parturition, and the detection was in concurrence with higher cortisol and NEFA in blood. Also, Hiss et al. (2009) showed that blood NEFA sampled at 2 wk postpartum and blood BHBA sampled at 1 wk prepartum were associated with greater concentration of Hp in serum and milk during the prepartum through early lactation period. However, our analysis did not find postpartum concurrent change of plasma Hp with plasma NEFA or BHBA. Huzzey et al. (2011) reported that prepartum NEFA and HP in plasma was positively correlated, but the correlation coefficient was low. The authors pointed out that NEFA, cortisol, and Hp are interrelated but these relationships are complex and vary depending on the physiological status of the cow (Huzzey et al., 2011). Therefore, the non-association of Hp with energy metabolism in the current analysis may result from the correlations among energy metabolism, endocrine system, and inflammatory response.

CONCLUSIONS

Cows that developed CE tended to have higher AUC for NEFA and BHBA in plasma from wk -3 to wk +3 relative to parturition. Prepartum AUC for both NEFA and BHBA were not associated with subsequent CE; however, postpartum AUC for

NEFA tended to be increased and BHBA was increased for cows that developed CE. Consistent with the results for plasma NEFA and BHBA, calculated EB during the prepartum period was not different in cows that did or did not develop CE; however, cows with CE had lower EB during the 6-wk postpartum period compared to cows without CE. Analysis of EB by week indicated that EB in cows with CE was lower at wk +1, wk +2, and wk +3 and tended to be lower at wk +6 than cows without CE. Concentrations of plasma Hp were not different during either wk +1 or the first 8-wk lactation period between cows that did or did not develop CE. These results suggest that decreased energy status, particularly during the first 3 wk postpartum, predisposes dairy cows to subsequent CE.

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CHAPTER SEVEN: INTEGRATED SUMMARY AND CONCULUSION

The factors related to reduction of immune function during the transition period can include oxidative stress, metabolic dysfunction, and energy status. However, the cause and effect is still not clear among those factors, and the exact mechanism between immune suppression and any of each factor remains uncertain. Because these factors also affect both productive and reproductive performance, studies that clarify the associations among immunity, production, and reproduction and identify approaches that optimize productivity, health, and reproduction in transition dairy cows will contribute to longstanding progress in this area.

The first objective of this work was to examine the effect of source of trace minerals on production, oxidative metabolism, and profile of endometrial cytology. Supplementation of hydroxyl trace minerals (HTM), a newly available source of trace minerals, resulted in improvement of milk production compared to both inorganic form (ITM) and a blend of inorganic and organic sources (ITM/OTM). Feeding HTM modulated markers related to oxidative metabolism during the periparturient period; cows fed HTM had lower plasma total antioxidant capacity than those in ITM along with a tendency for decreased plasma concentrations of thiobarbituric acid reactive substances (TBARS) in cows fed HTM compared to ITM. Furthermore, plasma haptoglobin (Hp) concentrations were lower in cows fed HTM at 1 wk postpartum than those fed ITM/OTM. Although feeding HTM resulted in indications of improved performance and decreased oxidative stress, the lack of response of endometrial

cytology to dietary trace mineral source suggests that uterine immunity is more complex than oxidative status.

The second objective of this work was to investigate effects of chromium propionate (Cr-Pro) supplementation during the transition period and early lactation on production, metabolism, and uterine endometrial cytology. Supplementation of Cr-Pro resulted in a trend for increased prepartum DMI and lower plasma NEFA during the prepartum period. These results would be consistent with improved insulin action in cows fed Cr-Pro. Lower plasma glucose immediately postpartum in cows fed Cr-Pro may also implicate enhanced insulin-related metabolism. Cows fed Cr-Pro tended to had higher percentage of uterine polymorphonuclear neutrophils (PMN) right after parturition, and had lower incidence of cytological endometritis (CE) assessed between 40 and 40 DIM, which suggests that feeding Cr-Pro can facilitate PMN influx into uterus right after parturition. However, plasma Hp was not affected by feeding Cr-Pro. This suggests that feeding Cr-Pro may not affect systemic stress status, cytokine production, and liver function related to production of acute phase proteins. Effects of supplemental Cr-Pro on reproductive parameters were not significant, although sample size in this experiment was insufficient to assess reproductive endpoints.

The third objective of this work was to examine effects of supplemental monensin and varied postpartum dietary starch level on incidence of CE and related immune functions. Neither PMN % in cells from uterine lavage at 7d postpartum nor incidence of CE at 40 to 60 d postpartum was affected by monensin treatment or the starch level. On contrary to effect of Cr-Pro, these treatments that likely increased gluconeogenic substrate supply did not affect uterine PMN influx. It implies that other

dominant factors exist to control PMN mobilization beyond energy status. Consistent with the result, days to pregnancy was not affected by starch level or monensin treatment. However, innate immune functions of peripheral blood cells were affected by treatment. At 7 d postpartum, monocyte phagocytosis and oxidative burst activity were enhanced mainly by feeding higher dietary starch concentrations during this timeframe. Although monocytes are proportionally not the major component of innate immunity, it suggests that increased fermentable starch can enhance immune status right after parturition. At 40 to 60 d postpartum phagocytosis and oxidative burst activities in PMN and monocytes were affected mainly by monensin treatment. At this time point, negative energy balance (EB) may not be severe compared to the transition period. Nevertheless, it suggests that monensin can improve immune function presumably through increasing energy source for immune cells. However, blood PMN glycogen content was not affected at 7 d postpartum by any treatment, but was increased during d 40 to 60 postcalving in cows preiviously fed a high starch diet for the first 3 wk postpartum period. It suggests that the glycogen content may not reflect the current energy status; rather, it may be influenced by the previous or long-term energy status.

The final objective of this work was to clarify the association of CE with energy metabolism and inflammation status. Cows that developed CE tended to have higher area under the curve (AUC) for NEFA and BHBA in plasma for 3 wk before and after parturition. Prepartum AUC for both NEFA and BHBA were not associated with subsequent CE; however, postpartum AUC for NEFA tended to be increased, and BHBA was increased for cows that developed CE. These data suggest that cows that

developed CE mobilized more fatty acid from adipose tissue after parturition than cows that did not. Consistent with the results for plasma NEFA and BHBA, calculated EB during the prepartum period was not different in cows that did or did not develop CE; however, cows with CE had lower EB during the 6-wk postpartum period compared to cows without CE. Analysis of EB by week indicated that EB in cows with CE was lower at wk 1, 2, and 3 postpartum and tended to be lower at 6 wk postpartum than cows without CE. These results suggest that decreased energy status, particularly during the first 3 wk postpartum, predisposes dairy cows to subsequent CE. Concentrations of postpartum plasma Hp were not different between cows that did or did not develop CE. Although common cytokines can affect the mobilization of acute phase proteins and the innate immunity, the severity of inflammation may be a key to influence those proteins. The degree of inflammation during CE is likely less than that during other uterine diseases such as metritis and clinical endometritis.

In conclusion, the source of dietary trace minerals can improve productive performance, modify oxidative metabolism, and reduce inflammatory response. These changes were not associated with incidence of CE. There are further opportunities to investigate effect of source of trace minerals on other immune functions such as phagocytice activities and cytokine kinetics. Also, increased amount of supplemental minerals or fed in combination with other antioxidants could provide more prominent immune responses. Feeding Cr-Pro improved periparturient metabolism, increased postpartum uterine PMN influx, and decreased incidence of CE. Given these favorable results, further research should employ larger numbers of cows to evaluate the effects of feeding Cr-Pro on reproductive performance. Research conducted as

part of this program indicated that supplemental monensin and postpartum dietary starch level can modulate immune cell function during the transition period and early lactation. Examination of incidence of other infectious diseases such as mastitis and metritis can clarify the impact of those immune responses on farm level. Considering different production process among acute phase proteins, studying association of negative EB with other acute phase proteins would provide opportunity to investigate relationship between energy status and inflammatory response. Overall, this work showed evidences that control of three factors, namely oxidative stress, metabolism, and energy status, can be critical to optimize immune status and productive performance in transition dairy cows, and that those factors are likely interrelated with one another.