

EFFECTS OF INFLAMMATION ON THE TRANSITION DAIRY COW

by

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B.S., Kansas State University, 2007
M.S., Oklahoma State University, 2009

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

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Abstract

The transition into lactation is a period of primary concern to dairy producers because of the tremendous incidence of health disorders observed during this time. Two common disorders that lead to decreases in production and retention within the herd include fatty liver disorder (FL) and ketosis. These two disorders have been commonly associated with negative energy balance, yet recently it has been hypothesized that inflammation is a contributor to the etiology of these disorders. Three individual projects were completed for this dissertation, all involving inflammation. The role of endogenous inflammation was determined by administration of sodium salicylate (SS) to cows for 7 d after parturition, and metabolites and production responses were evaluated. Overall it appears that SS induced hypoglycemic conditions and increased triglyceride accumulation in the liver (while administered), increased lipid mobilization and ketones (2 weeks after administration ended), and increased whole lactation milk production in older cows. A sensitive, specific sandwich ELISA for bovine tumor necrosis factor- α was developed, which provided the ability to measure “normal” circulating levels of this cytokine. The final study involved inducing inflammation by daily injections of the TNF α to the early lactation dairy cow. In this model, cows receiving TNF α had a reduction in dry matter intake, water intake, and decreases in milk production and milk components. Overall, it appears that inflammation is involved in the normal biology of the transition dairy cow and disrupting this can lead to interesting negative effects and some improvements of production; however, when inflammation is much greater it can lead to negative production effects.

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Chapter 1 - Literature Review

The dairy industry in the United States is a large, multi-faceted sector with a direct economic impact of approximately \$31.5 billion in 2010 (U.S. Census Bureau, 2012). From the latest annual review (2010), the US dairy industry has been showing tremendous improvement in milk yields with less cows in production (USDA Agricultural Marketing Service, 2010). This increase in productivity has occurred due to improvements in nutrition, management, and genetics, yet overall disease incidences have been increasing (summarized by Goff, 2010). According to the National Animal Health Monitoring Systems (NAHMS) surveys from the last decade, the percentage of cattle affected with mastitis, lameness, and abomasal displacement were increased by 2, 3.5 and 0.7% respectively (National Animal Health Monitoring System, 1996; National Animal Health Monitoring System, 2007). The only disease that had a decreasing incidence was milk fever, which is probably explained by the nutritional modifications that have occurred over the past decade. It has been hypothesized that the increase in milk yield by individual cows is a driving force in the increase in health disorders; however this has not been proven to be the case. When comparing the average of 61 high-yielding U.S. herds to other “normal-yielding” herds, there was no difference in health disorders, although there was a much larger variation in disorder incidences within the high-yielding herds (Ingvarsten, 2006). This large variation might possibly explain why some studies have identified a correlation between milk yield and mastitis, ketosis, and foot/leg disorders (Ingvarsten et al., 2003).

The transition period is the time when the greatest incidences of diseases are observed on a dairy operation. The first several weeks after parturition accounts for the majority of morbidities observed on dairy operations (Ingvarsten, 2006). Typically, these disorders can be classified as being related to feeding- and management-related or infection-related disorders. The most commonly observed feeding- and management-related transition disorders include fatty liver, ketosis, ruminal acidosis, milk fever, and displaced abomasum (DA) (Ingvarsten, 2006). Mastitis and metritis are also commonly observed during the transition period and are infection-related disorders. Transition disorders cause negative impacts on the whole lactation and cost the producer revenue. Cows suffering from one transition disorder are at greater risk for contracting other health issues (Bobe et al., 2004). Seemingly unrelated conditions like mastitis

and ketosis often occur simultaneously (Goff, 2006). This observation has caused several investigators to study how a disease that is caused by bacterial infection can trigger a metabolic disorder. Several diseases build on each other to generate a complex of metabolic and infectious problems that can impact the longevity and fertility of a cow. Poor fertility, in turn, is the primary reason for involuntary culling of animals in the dairy industry (National Animal Health Monitoring System, 2007).

This review will focus on two key transition disorders that are related, fatty liver and ketosis, including their symptoms, metabolic effects and causes. The two putative causes that will be addressed at length are negative energy balance and inflammation. Finally, this review will discuss the possibility of managing these disorders with the use of non-steroidal anti-inflammatory drugs (NSAIDs).

Overview of fatty liver and ketosis and their effects on reproduction.

Fatty liver disorder

Fatty liver disorder is defined as an abnormal accumulation of lipid in the liver. Fatty liver is diagnosed in the live animal by measurement of triacylglycerol (TAG) content of liver biopsies or histological examination of tissue cross-sections. The gross pathology of fatty liver includes an enlarged, swollen, yellowish liver with rounded edges (Morrow, 1976; Kapp et al., 1979; Bobe et al., 2004). The histological findings include fatty cysts in liver parenchyma, increased volume of hepatocytes, mitochondrial damage, compression and decreased volume and number of organelles (Kapp et al., 1979; Reid and Collins, 1980; Johannsen et al., 1993; Bobe et al., 2004).

It has been estimated that 5-10% of dairy cows have severe fatty liver (>10% TAG in liver) and between 30 and 40% have moderate fatty liver (5-10% TAG) in the first month of lactation (Bobe et al., 2004). Clinical symptoms of fatty liver include lack of appetite and weight loss, and weak and apathetic cows (Radostits et al., 2000). Fatty liver is highly associated with other transition disorders such as DA, ketosis, laminitis, mastitis, metritis, milk fever, and retained placenta, with the two that have the highest association with fatty liver disorder being DA and ketosis (Bobe et al., 2004). As Bobe et al. (2004) summarized, the severity of fatty liver has a very strong positive relationship with urinary ketone concentration and large negative

associations with feed intake, milk production, health status, and reproductive performance. The monetary cost of fatty liver has been estimated to be \$145/case (Guard, 1994). Fatty liver is often reversible, yet it predisposes cows to other conditions and impairs liver function (Bruss, 1993; Grummer, 1993; Drackley, 1999; Rukkwamsuk et al., 1999; Drackley et al., 2001).

Metabolic changes that are seen with fatty liver include elevated blood concentrations of non-esterified fatty acids (NEFA), β -hydroxybutyric acid (BHBA), and acetoacetate (Bobe et al., 2004). Cows with fatty liver also exhibit suppression in lipid transport from the liver. Some specific lipoproteins that are decreased in ketotic cows compared to healthy lactating cows include apolipoproteins B, A-I, A-IV, and C-III (İleri-Büyükoğlu et al., 2009).

Elevated concentrations of NEFA increase lipogenesis and ketogenesis in the liver, while high concentrations of BHBA and acetoacetate decrease rates of β -oxidation, gluconeogenesis, and the citric acid cycle (Bobe et al., 2004). Additionally, some enzyme activity is decreased in the liver with cows that have fatty liver. Two specific enzymes include protein kinase C and carnitine palmytoyltransferase which further impact lipid metabolism in cows with fatty liver (Mizutani et al., 1999; Katoh, 2002; Bobe et al., 2004). These enzymes are involved in post-translational modification of apoprotein A-I, β -oxidation, and ketogenesis (Mizutani et al., 1999; Katoh, 2002). Fatty liver is commonly associated with changes in carbohydrate, lipid, and protein metabolism in the liver, while lipolysis occurs in the adipocyte (Bobe et al., 2004).

Bobe et al. (2004) summarized in a review on fatty liver that there are three categories of risk factors for fatty liver: nutritional, managerial, and genetic. One nutritional risk factor is negative energy balance and the changes in nutrient requirements associated with lactation. However, not all risk factors can be classified into one specific category; for example, overconditioning in the dry period can be caused by overfeeding energy or by allowing the dry period to be too long.

Nutritional risks for fatty liver. The transition from gestation to lactation dramatically increases requirements for energy while a depression in intake simultaneously occurs, which initiates a negative energy balance. Negative energy balance is when intake of energy (i.e. calories) is less than the output of energy. In the dairy cow some of the outputs include milk production and maintenance requirements. Feed intake of healthy cows in early lactation has been reported to be deficient of requirements by 26 and 25% for NE_L and metabolizable protein, respectively (Bell, 1995). The mammary gland is the organ that takes up the largest percentage

of the energy that is consumed, accounting for up to 97% of the NE_L intake (summarized by Drackley, 1999 as recreated from Bell, 1995). Obesity further compounds negative energy balance as obese cows (BCS \geq 4.0 on scale of 1-5) have a greater magnitude of feed intake depression and subsequently more lipolysis (Rukkwamsuk et al., 1998; Stockdale, 2001). Increased adipose tissue mass in obese cows is a result of either greater adipocyte size or increased adipocyte number. Adipocytes are able to secrete hormones and cytokines including leptin and TNF α , both of which depress feed intake and insulin sensitivity and increase hepatic lipogenesis, peripheral tissue catabolism, and inflammation (Drackley, 1999; Ohtsuka et al., 2001; Kushibiki et al., 2003). Therefore, increased adipose tissue mass in the obese cow may contribute to increased cytokine secretion and subsequent impairment in systemic metabolism. However, not all obese cows experience fatty liver disorder, especially if they are able to avoid other disorders and adapt their intake to match milk production (Smith et al., 1997). This alteration in the hypothesis that cows that are obese will have fatty liver indicates that fatty liver disorder must be very complex and multi-factorial in its etiology.

The adaptation of the diet for the transition cow is an important nutritional factor to consider in regards to fatty liver. During the dry period, diets fed to cows are lower in energy and cows must be able to adapt quickly to a higher energy diet intended to offset the depression in intake after parturition. This transition must be done correctly to avoid acidosis, which causes further issues for the transition dairy cow. Another nutritional component that must be accounted for after parturition is calcium. Calcium is in tremendous demand after parturition for synthesis of milk and if its concentration in plasma drops too low, milk fever can result (summarized by Bobe et al., 2004). Milk fever is one of the transition disorders that is associated with fatty liver and as such, managing calcium will be important to managing fatty liver disorder. Additionally, both vitamin A and E (as retinol and α -tocopherol, respectively) are decreased around parturition by 38 to 47%, respectively (Goff and Stabel, 1990). All the above mentioned nutrients are needed to maintain a healthy animal and as such they need to be provided in a correct balance.

Management risks for fatty liver. Management risks include feed issues, incorrect diet management, and facility issues. Certain feeds can promote development of fatty liver. Silage with elevated levels of butyrate could play a role in fatty liver disorder because of a suppression in feed intake and an increase in BHBA production (Stober and Scholz, 1991).

A strong correlation between the amount of concentrate fed and the occurrence of acidosis, fatty liver, ketosis, left DA, and laminitis has been identified through several epidemiological studies (Coppock, 1972; Dougherty et al., 1975; Nagaraja et al., 1978). Acidosis can be a risk factor for fatty liver because it increases ketogenesis and concentrations of endotoxins and proinflammatory cytokines (Nikov et al., 1981; Goff and Horst, 1997; Furll and Leidel, 2002; Kushibiki et al., 2003).

Over-conditioning in the dry period is another management issue that has effects on fatty liver. Cows that are over-conditioned have higher TG content in their liver in the first week of lactation which could be directly caused by elevated NEFA compared to cows that were fed maintenance diets (Rukkwamsuk et al., 1998). Obese cows lose more body weight during the first 14 DIM than normal cows, although in this study the incidence of ketonemia and clinical ketosis was similar regardless of body fat (Smith et al., 1997). Overconditioning in the prepartum period can increase total lipids and triglycerides in the liver while significantly increasing the triglyceride to phospholipid ratio postcalving (Fronk et al., 1980). Total health disorders were numerically higher for cows that were overconditioned, in which the 3 most common disorders were mastitis, milk fever, and ketosis (Fronk et al., 1980). Similarly, in a smaller trial, Reid et al. (1979) observed that fat cows have a higher incidence of disorders at parturition.

Older cows are at a higher risk of developing fatty liver disorder (Reid, 1980; Woltow et al., 1991) as well as most other common transition disorders. This could be due to a multitude of different risk factors that have been observed in older cows. These include excessive adipose tissue at calving that could be related to longer calving intervals, higher milk production, weaker immune responses, or a lower antioxidant status (Stober and Scholz, 1991; Gilbert et al., 1993; Jorritsma et al., 2000; Mehrzad et al., 2002).

Other management issues that can increase the risk of fatty liver in cows include issues such as inadequate space, lack of exercise, poor sanitary conditions, high environmental temperatures, high humidity, and poor air circulation (Stober and Scholz, 1991; Gerloff, 2000). Goff and Horst (1997) suggested that these management issues increase release of catecholamines which increase NEFA mobilization from adipose tissue, decrease feed intake, and increase risk for infections. Catecholamines are hormones produced by the adrenal glands and released during periods of stress. Some major catecholamines involved with the transition

dairy cow include norepinephrine and epinephrine. These hormones coordinate to maintain homeostasis and homeorhesis. Homeorhesis is defined as “the orchestrated or coordinated changes in the metabolism necessary to support a physiological state” (Bauman and Currie, 1980). As summarized by Ingvarlsen (2007), responses to catecholamines are highly tissue specific and have a high tissue responsiveness in late pregnancy and early lactation indicating that these hormones, if stimulated, can lead to lipid mobilization. Additionally, grouping changes after parturition are another source of potential stress as cows have to reestablish a hierarchical order that could possibly induce the release of catecholamines which impact the normal homeorhesis of lactation for the transition dairy cow.

Genetic risk factors for fatty liver. There have not been any genetic factors directly associated with fatty liver, however, there is some heritability that has been associated with other health disorders related to fatty liver. For example, heritability estimates for ketosis are between 0.07 and 0.32 (Duffield, 2000). Displaced abomasum has also been identified as having a heritability of 0.24 (summarized by Bobe et al., 2004). Since ketosis and DA are the two transition health disorders that have the closest association to fatty liver, it is possible that genetic selection against these two issues might be a management decision that will help minimize fatty liver.

Ketosis

Ketosis and fatty liver have been extensively researched and found to correlate strongly with each other (summarized by Ingvarlsen, 2006). Ketosis has been defined as high plasma levels of ketones, such as acetoacetate, BHBA, and acetone (Ingvarlsen, 2006). Ketosis incidence in the United States has been reported to be between 2 and 20% (summarized by Ingvarlsen, 2006). There are several different ways to determine ketotic cows, such as ratios of milk fat to protein (Gantner et al., 2008), urine ketones (Campos et al., 2005), with the gold standard being measured in plasma ketones. There are four classifications of ketosis: primary ketosis (spontaneous or production ketosis), secondary ketosis, butyric acid ketosis, and under-feeding ketosis (Lean et al., 1992).

Primary ketosis typically occurs 3-6 weeks after calving, when lipid mobilization is stimulated because glucose demands exceed the gluconeogenic output of the liver. Because glucogenic substrates are low, it causes an increase in ketogenesis, and as such, there are high

concentrations of ketone bodies in blood, milk, and urine. This disorder is mainly seen in fat cows and cows that have long dry periods (Markusfeld et al., 1997). Secondary ketosis is defined as ketosis that results from another disease. It has been hypothesized that this occurs principally because of depressed feed intake and increased lipid mobilization. Secondary ketosis is also identified by a reduction in plasma glucose concentration and an increase in NEFA and ketone bodies (summarized by Ingvarlsen, 2006). Butyric acid ketosis, like the name implies, is caused by large amounts of butyrate in the feed. High butyrate concentrations can suppress feed intake and increase blood concentrations of BHBA (Adler et al., 1958; Andersson and Lundstrom, 1985; Tveit et al., 1987; Tveit et al., 1992). Some sources of butyric acid and ketogenic feeds include silage with high butyrate, sugar beets, and molasses, because they increase rumen butyrate concentration (Aaes, 1988). Underfeeding ketosis occurs when insufficient nutrients are fed, or the ration has a high fill-value. This type of ketosis appears to have similar causes and symptoms as primary and secondary ketosis.

Type I ketosis. Type I ketosis has been named this due to its similarities to Type I diabetes because there are several similarities in metabolites. Type I ketosis occurs due to a shortage of glucose precursors such as propionate and amino acids. Additionally, low plasma concentrations of insulin and a low insulin to glucagon ratio are found in ketotic cows (Xia et al., 2012). Type I ketosis is identified as hypoglycaemic-hypoinsulinaemic and generally occurs 3-6 weeks after parturition (Holtenius and Holtenius, 1996). In this type of ketosis plasma levels of glucose and insulin are very low, ketone bodies are high, and there are small risks for fat accumulation in the liver. In this type of ketosis glucagon injections cause very limited responses in glucose and insulin (Holtenius, 1992).

Type II ketosis. Characteristics of Type II ketosis includes hyperglycaemia-hyperinsulinaemia that occurs earlier in lactation. One primary cause of this type of ketosis is overfeeding leading to over-conditioning in the dry period. This can lead to disturbances in hormonal adaptation to metabolism at parturition, which can result in increased lipolysis from adipose depots, hepatic lipid synthesis, and development of fatty liver (Holtenius and Holtenius, 1996).

Subclinical ketosis. Subclinical ketosis (SCK) is defined as elevated concentrations of ketones in the absence of clinical signs of ketosis. One issue with this is that many times it remains undetected and can have serious effects on productivity. It has been approximated that

nearly 30% of dairy cows have SCK in the first two weeks after parturition (Duffield et al., 1998; Duffield, 2000). Potential indicators of cows that will be subclinically ketotic during the first week after calving include lower DMI, fewer visits to the feeder, and less time spent at the feeder than healthy animals (Goldhawk et al., 2009). According to these authors, decrease in DMI before parturition predicts the risk of cows having subclinical ketosis in early lactation. Determination of SCK is based on blood levels of BHBA, yet specific thresholds for SCK have not been agreed upon (Goldhawk et al., 2009).

Risk factors for ketosis. Parity has impacts on ketosis in which older cows are at a higher risk of developing any type of ketosis (Kauppinen, 1983; Gröhn et al., 1984). Ketosis in previous lactation increases the risk of ketosis in the following lactation as well as BCS > 3.5 at calving (Andersson and Lundstrom, 1985; Markusfeld and Adler, 1986; Bendixen et al., 1987; Gillund et al., 2001). Over-conditioning at parturition, poor feed quality, and diminished appetite will increase ketogenesis as the plasma concentration of NEFA are elevated. In the ruminant, the endogenous source of ketogenic substrate is primarily long-chained fatty acids (Ingvarsen, 2006).

Production impacts of ketosis. Using a fitted lactation model to analyze the lactation curve shape and magnitude, authors observed that cows that ketosis, mastitis, milk fever, and retained placenta negatively impacted the lactation curve mainly when multiple disorders were combined (Hostens et al., 2012). Milk yield can be significantly decreased in ketotic cows (Miettinen and Setälä, 1993; Gantner et al., 2008; Duffield, 2010; Xia et al., 2012). In contrast to results observed in clinical ketosis, SCK sometimes has no effect on milk production (Goldhawk et al., 2009).

Reproductive issues caused by transition disorders

Pregnancy rates are decreased in cows with fatty liver which could potentially be explained by a couple of factors. Triacylglycerol concentration in the liver is positively associated with the time to first heat and time to conception, possibly indicating that negative energy balance, fatty liver, and ketosis have effects on reproductive success (Kruip et al., 1998; Rukkwamsuk et al., 1999; Jorritsma et al., 2000; Royal et al., 2000; Jorritsma et al., 2003). First, uterine involution may be delayed in cattle that have transition health disorders, particularly with metritis and retained placenta (Haraszti et al., 1982; Higgins and Anderson, 1983; Heinonen et

al., 1987; Sheldon et al., 2002). Uterine involution is impaired by metritis and leads to a reduction in ovarian activity. Metritis usually occurs within 7 d post-partum and can adversely affect fertility in cows (Morrow, 1976; Fonseca et al., 1983; Hussain and Daniel, 1991). Second, ovarian activity can be negatively impacted by transition disorders, potentially via negative energy balance and delayed synthesis of steroidogenic hormones (progesterone and luteinizing hormone) (Herd, 1991; Zhou et al., 1997). Ovarian function is also impaired by low levels of IGF-I, insulin, and lipoproteins, or by high concentrations of ammonia, NEFA, and urea, all changes which occur in the weeks immediately post-partum (Herd, 1991; Comin et al., 2002; Jorritsma et al., 2003). Low insulin levels, in particular, negatively affect follicle development and ovulation (Landau et al., 2000; Gong et al., 2002). It has been indicated that a low energy intake in early lactation is associated with a prolonged interval to first ovulation (Butler et al., 1981; El-Din Zain et al., 1995; Opsomer et al., 2000). Liver triglyceride accumulation decreases fertility in dairy cows regardless of milk production, leading to the conclusion that it is the level of TAG in the liver that impacts reproduction more so than negative energy balance (Jorritsma et al., 2000). A large field trial evaluated fertility, diseases, and liver TAG content during early lactation (6 to 17 DIM) and around breeding time (38 to 50 DIM). Overall the probability of pregnancy was 30% lower for cows with high TAG, the probability of estrus was 35% lower, and as such, the parturition to first heat and parturition to pregnancy intervals were longer (Jorritsma et al., 2000).

Negative energy balance leads to lipid mobilization

Negative energy balance (NEB) has traditionally been identified as the cause of fatty liver and ketosis. While cows are in NEB they mobilize large quantities of fatty acids from adipose tissue deposits which increases plasma concentration of NEFA (Herd, 1988). During the transition period plasma concentration of insulin is low and induces an increase in NEFA release from adipose tissue (Mashek et al., 2001). Some of the fatty acids can be used as a energy source to yield ATP (Sordillo et al., 2009). Non-esterified fatty acids are primarily used in the mammary gland or in the liver. Non-esterified fatty acids can undergo several different processing procedures in the liver to yield new products. The possibilities are that NEFA can be completely oxidized to carbon dioxide, partially oxidized to produce ketone bodies, or reconverted to triglycerides (TG). Energy is provided to the animal by complete oxidation or

ketones that are released into the blood to be used as fuel for other tissues. A portion of the fatty acids taken up by the liver are oxidized either in the mitochondria or peroxisomes. In the bovine liver it has been found *in vitro* that there is a considerable capacity for β -oxidation in the peroxisomes (Grum et al., 1994; Grum et al., 1996; Drackley et al., 2001). The TAG are then either stored in the liver or incorporated into VLDL and released into the blood. When uptake of fatty acids by the liver is greater than the ability of the liver to oxidize them, TAG accumulate in the liver and fatty liver develops. Very low density lipoprotein secretion from the liver is very limited in ruminants compared with other species (Kleppe et al., 1988; Rayssiguier et al., 1988; Pullen et al., 1990) and cows with the highest lipid in the liver have the lowest concentration of lipoprotein in plasma (Rayssiguier et al., 1988; Marcos et al., 1990). Uptake of NEFA by the liver increases around calving, possibly because of increased blood flow to the liver (Emery et al., 1992; Ingvarlsen, 2006). Additionally, blood flow through the liver is proportional to energy intake and is higher in early lactation than in the dry period, further increasing the hepatic delivery of NEFA (Lomax and Baird, 1983).

Inflammation in metabolic syndromes

Inflammation has been hypothesized to be a contributing factor for fatty liver, because in other species inflammation induces symptoms similar to those observed in fatty liver. Chronic low-grade inflammation has been identified to play a role in metabolic disorders. Cytokines can increase body temperature, induce anorexia, promote lipolysis in adipose tissue, stimulate muscle breakdown, and direct several endocrine and metabolic changes (Elsasser et al., 1995). Hotamisligil et al. (1993) found that adipose tissue is capable of producing inflammatory cytokines such as $\text{TNF}\alpha$, and low-grade adipose tissue inflammation can be induced by obesity (Hotamisligil et al., 1993). In obese sheep circulating $\text{TNF}\alpha$ is increased versus control animals (Daniel et al., 2003a). Pro-inflammatory cytokines are released due to the physical activity of parturition itself (Simpson et al., 1998) or in the placenta as observed in women (Hauguel-de Mouzon and Guerre-Millo, 2006). Amniotic fluid contains multiple cytokines such as interleukin 1 (IL-1), $\text{TNF}\alpha$, as well as inhibitory cytokines such as an IL-1 receptor antagonist and interleukin-10 (IL-10) and the chemokine interleukin-8 (IL-8) (Tamatani et al., 1988; Romero et al., 1989; Heyborne et al., 1994; Kelly, 1996; Simpson et al., 1998). Cytokines that

are released from activated liver macrophages stimulate liver hepatocytes to release serum amyloid A (SAA) and haptoglobin (Hp).

Endotoxin has been proposed by several investigators to play a role in fatty liver, DA, and laminitis (Dougherty et al., 1975; Aiumlamai et al., 1992; Anderson et al., 1994). However, the precise role of endotoxin in these important disorders of dairy cattle has not been determined. Inflammation was summarized by Ametaj (2005) to be caused by endotoxin being released from the rumen, due to the rumen microbial population shifting from cellulytic microbes to starch-digesting microbes. These starch digesting microbes are primarily gram negative bacteria in which the lipopolysaccharide wall has endotoxin which induces an immune response. This immune response has been hypothesized to be localized to the liver in which the resident macrophages take up the endotoxin, stimulating cytokine production. The primary pro-inflammatory cytokines produced are $\text{TNF}\alpha$, IL-1, and IL-6. Endotoxin can induce a $\text{TNF}\alpha$ response, and research shows that $\text{TNF}\alpha$ administration decreases appetite and feed intake while increasing blood NEFA concentration (Kushibiki et al., 2000). The three cytokines aforementioned also play roles in production of Hp and SAA in the liver (Kushner, 1988; Edbrooke et al., 1993; Ametaj, 2005). Haptoglobin is also generated in an endotoxin-induced immune response. This compound is released for two reasons in this instance. One function is its antibacterial capabilities and the other is that Hp binds hemoglobin which binds iron (Wassell, 2000). Iron is needed for bacterial growth and function so decreasing this mineral suppresses bacterial growth. One potential issue with elevated levels of haptoglobin is that it can bind apolipoprotein A-I which interferes with high density lipoprotein (HDL) receptor binding (Kato, 2002). Serum amyloid A is also produced in the liver during an endotoxin-induced immune response. In this process SAA associates with HDL and endotoxin and this complex is then removed from circulation by liver cells (Hoffman and Benditt, 1982; Harris et al., 2002).

The acute phase response has been associated with changes in lipid and glucose metabolism which include increased lipolysis and plasma NEFA concentration (Hardardottir et al., 1994). From a glucose metabolism standpoint, during the early stage of the acute phase response there is an increase in plasma glucose which is then followed by hypoglycemia (Kushibiki et al., 2000). Several of the effects observed with acute phase proteins are observed in the early lactation cow and it has been reported that Hp and SAA are significantly increased

during the early postpartum period compared with cows in later postparturient periods (Kovac et al., 2009).

Total lipids in the liver have been shown to positively correlate with plasma SAA (Ametaj et al., 2005b). After parturition liver TG was correlated positively with plasma SAA, Hp, and negatively with plasma prostaglandin E₂, total cholesterol, and glucose (Ametaj et al., 2005b). Another way that lipids can enhance the immune and inflammatory response is through biosynthesis of lipid mediators such as eicosanoids, lysophospholipids, phosphatidic acid, and ceramide (Serhan et al., 2008). The eicosanoid family has been recognized as a key regulator of both chronic and acute inflammatory reactions and depending on the timing and magnitude of expression, can be either an enhancer or a resolver of the inflammatory response (summarized by Sordillo et al., 2009).

Nuclear factor κ B (NF κ B) is an important transcription factor for inflammatory processes. Activation of NF κ B leads to expression of genes that mediate cell proliferation and release of antimicrobial molecules and cytokines that activate the immune response (Hayden and Ghosh, 2008). This signaling pathway has been demonstrated to be involved in metabolic diseases in the liver, adipose tissue, and central nervous system (Baker et al., 2011). Nuclear factor kappa-B is found in the cytoplasm of cells in the inactive form, associated with I κ B- α . When I κ B- α is phosphorylated, ubiquitinated, and degraded by a proteasome-dependent pathway, NF κ B is released to translocate to the nucleus (summarized from Pierce et al., 1996). NF κ B can be activated by cytokines and pathogen-associated molecular patterns (PAMP) on the cell surface. The NF κ B pathway is one potential unifying pathway between inflammation and metabolic diseases, and as such can be a source of novel treatment strategies (Baker et al., 2011). Salicylates have been found to directly target IKK β within the NF κ B pathway and were shown to inhibit fat-induced insulin resistance in skeletal muscle (Yin et al., 1998; Kim et al., 2001; Yuan et al., 2001).

Several cell-intrinsic and cell-extrinsic mechanisms work in coordination to regulate the inflammatory response. Some examples include a short half-life of pro-inflammatory cytokines, counteractive anti-inflammatory cytokines such as IL-10, and removal of dead cells to prevent tissue necrosis (Baker et al., 2011). During periods of chronic inflammation these cell-extrinsic and -intrinsic mechanisms are dysfunctional, leading to continuous increases in inflammatory

cytokines. Chronic inflammation in rodent and human models has roles in the metabolic disorders of obesity, insulin resistance, and atherosclerosis (summarized by Baker et al., 2011).

Immune system changes at the time of parturition

The transition from gestation to lactation impacts the immune system, changing the killing and infiltration abilities of immune cells. The week prior to and immediately following parturition is associated with neutrophilia, eosinopenia, lymphopenia, and monocytosis (Meglia et al., 2001; Meglia et al., 2005). Even with the increase in some white blood cells, there is actually a decrease in phagocytosis and oxidative burst activity (Meglia et al., 2001; Meglia et al., 2005). Neutrophil and lymphocyte function, as measured by iodination and blastogenesis, is impaired during the weeks immediately before and after parturition (Goff and Horst, 1997). This suppression in activity may be what is leading to chronic infections such as mastitis that recur through the entire lactation. Cows with fatty liver have lower cellular cytotoxicity and reactive oxygen species generated by polymorphonuclear cells potentially indicating a suppression in phagocytic ability (Zerbe et al., 2000). Body condition score at parturition can impact the haematological profile. Specifically, white cell count was found to be lower in fat cows (BCS \approx 4.0) versus thin cows (BCS \approx 2.5) in the first week of lactation (Reid et al., 1986).

Leukopenia has been associated with ketosis and fatty liver and provides justification for interactions between the immune system and various metabolites that are characteristic of ketosis (Morrow, 1976; Morrow et al., 1979; Reid et al., 1984; Reid et al., 1986; Franklin et al., 1991). In an *in vitro* study where cultured bovine lymphocytes were incubated with different metabolites found in ketosis, it was observed that butyrate concentrations greater than that seen in bovine plasma and acetate at normal levels inhibited proliferation of lymphocytes (Franklin et al., 1991). Depending on the type of activation for lymphocyte proliferation, low glucose media had differing results. In one activation scenario lymphocyte proliferation was increased in low glucose media, however when adding acetate to the low glucose media it inhibited lymphocyte proliferation (Franklin et al., 1991). In another instance, low glucose suppressed lymphocyte proliferation with acetate, β -hydroxybutyrate, and acetoacetate (Franklin et al., 1991). However, in this study these authors concluded that at concentrations that are found in normal/healthy cows *in vivo*, ketones, butyrate, and glucose have minimal effects on lymphocyte proliferation, but at

concentrations of acetate typically found in ketotic cows suppress lymphocyte function (Franklin et al., 1991). In a skin allotransplant model on cows with either fatty liver or not, it was found that in “normal cows” that there was a greater number of invading lymphocytes than those that had fatty liver (Wentink et al., 1999). Bovine leukocytes cultured with BHBA show a suppression in respiratory burst activity, and the authors suggest the mode of action for this is a reduction in generation of superoxide anions (Hoeben et al., 1997). As summarized by Suriyasathaporn et al. (2000), killing capacity of leukocytes is impaired *in vitro* and *in vivo* by ketones.

Signaling molecules are released by activated macrophages and mast cells. Some of the molecules promote local inflammation and increased blood flow. Inflammatory cytokines play a key role in stimulating systemic inflammatory responses, including increased body temperature, increased heart rate, and decreased feed intake (Dantzer and Kelley, 2007). Mammary and uterine infections result in both local and systemic inflammation. Coliform mastitis releases endotoxin into the bloodstream and increased plasma concentrations of cytokines and acute phase proteins (Hoeben et al., 2000). Plasma HP is elevated prior to clinical signs of metritis in transition dairy cows (Huzzey et al., 2009). Primiparous cows have been shown to have a higher viability of blood and milk polymorphonuclear leukocytes (PMN) one month before and immediately following parturition, and even though a depression in total PMN was identified around parturition, primiparous cows had a quicker recovery rate than their pluriparous counterparts (Mehrzhad et al., 2002).

Metabolic disorders in transition dairy cows could be attributed to circulating cytokines. Certain cytokines facilitate the breakdown of fat stores through decreased feed intake (Kushibiki et al., 2003), decreased insulin sensitivity, and direct stimulation of lipolysis (Kushibiki et al., 2001a; Kushibiki et al., 2001b). Lipid mobilization and impaired insulin sensitivity is associated with ketosis and fatty liver in dairy cattle (Ingvarsen, 2006). Cytokines have further been confirmed in the peripartum period to induce effects that relate to fatty liver, including anorexia, catabolic conditions, fever (with an increase of energy wastage), adipose mobilization, and altered liver synthesis activity (Klasing and Leshchinsky, 2000). One specific cytokine, TNF α has been demonstrated to decrease liver glucose production (Kettelhut et al., 1987b) and promotes triglyceride accumulation once mobilized NEFA reach the liver (Garcia-Ruiz et al., 2006). Serum TNF α activity is increased in cows that have moderate to severe fatty liver

(Ohtsuka et al., 2001). Monocytes become more responsive to inflammatory cytokines during the transition period resulting in a greater secretion of inflammatory cytokines (Sordillo et al., 1995). Fatty liver could further be induced by endotoxin-induced mastitis which alters expression of metabolic genes in the liver, including decreased expression of genes that are important in glucose production (Jiang et al., 2008).

Controlled studies with tumor necrosis factor alpha in the bovine show similar results as that in the transition period. Tumor necrosis factor- α is a cytokine that has been researched extensively in rodents and humans and has been the focus of some bovine models to determine this cytokine's effects on metabolism and inflammation. In an ovine model, obesity was found to increase levels of circulating TNF α and a very strong positive correlation was observed between TNF α and fat thickness (Daniel et al., 2003a). In another study where recombinant bovine TNF α (rbTNF α ; 2.5 μ g/kg/d) was injected s.c. for seven days, DMI was suppressed, Hp was elevated, NEFA was increased, and cortisol levels were increased (Kushibiki et al., 2003). Milk yield was decreased, milk fat percentage was increased, and milk protein was decreased while rbTNF α was injected (Kushibiki et al., 2003). In a long term scenario where rbTNF α (2.5 μ g/kg/d) was administered for 12 days in steers, it induced insulin resistance without hypoglycemia (Kushibiki et al., 2001a). Daily administration of rbTNF α for 9 days caused metabolic changes such as hyperglycemia, hyperlipidemia, hyperinsulinemia, and hypoglucagonemia (Kushibiki et al., 2001b). All these results indicate in the ruminant model that insulin resistance occurs with TNF α but other results are not as consistent as those observed in other species.

Treatment of Inflammation

Inflammation is a key component of the immune system's attempt to fight an invading pathogen. Modulating the inflammatory response is beneficial because if it is uncontrolled it can lead to coliform mastitis and septic shock (Hill, 1981; Burvenich et al., 2007). On the other hand, if inflammation is suppressed too much, it might increase the risk of infectious disorders by blunting the immune response. Five NSAIDs tested in vitro suppressed phagocytosis by neutrophils isolated from milk (Paape et al., 1991; Bas et al., 1998). However, not all NSAIDs have the same suppressive effects on immune response. For example, aspirin was found to increase phagocytosis by bovine neutrophils in vitro (Paape et al., 1991). The NSAIDs that

caused a suppression in phagocytosis include ibuprofen, benzydamine, acetaminophen, indomethacin, and phenylbutazone (Paape et al., 1991). Bas and coworkers (1998) also observed an increase in microbicidal activity in response to low levels of aspirin. If a compound such as salicylate can inhibit metabolic inflammation without suppressing immune responses, short-term NSAID treatment may provide an effective means to prevent metabolic disorders in the transition period.

Non-steroidal anti-inflammatory drugs are classified into 5 broad subclasses: (1) salicylic acid and its derivatives, (2) propionic acid and derivatives, (3) pyrazole derivatives, (4) aniline derivatives, and (5) oxicam derivatives (Gallo et al., 2008). Each class functions slightly differently. The drugs discussed in this review are classified as followed: carprofen and ketoprofen (propionic acid), meloxicam (oxicam), flunixin (aniline), and salicylate (salicylic acid). Since each subclass has a slightly different mode of action, they likely offer different potencies for combating acute and chronic inflammation. Not surprisingly, different results have been observed in regard to transition disorders.

Several NSAIDs in the propionic acid and oxicam subclasses have been used effectively in treating mastitis. In one study, carprofen had limited ability to suppress inflammation, but was shown to partially alleviate the decrease in ruminal contractions during mastitis (Vangroenweghe et al., 2005), which could help prevent a subsequent displaced abomasum. Ketoprofen, a similar compound, promoted ruminal contractions and was also effective at decreasing inflammatory responses to mastitis (Banting et al., 2008). Meloxicam treatment lowered somatic cell count and a reduced the number of cows removed (culled) from the herd after mastitis (McDougall et al., 2009).

Flunixin meglumine in dairy cows. One of the most common NSAIDs that has been tested on the transition dairy cow is flunixin meglumine, with varying results. One study indicated that uterine involution was accelerated by flunixin meglumine treatment for metritis (Amiridis et al., 2001b), but another showed no beneficial effects, either systemically or in the reproductive tract (Drillich et al., 2007b). Schwartz and colleagues (2009) showed no benefit to administration of flunixin meglumine for the first 3 days of lactation. In fact, flunixin meglumine administered in the first 3 days of lactation depressed feed intake and milk yield over the first week of lactation (Schwartz et al., 2009), and in a separate study a significant increase in risk of retained placenta and metritis was observed (Duffield et al., 2009). This negative finding

may be due to the importance of inflammatory pathways for expulsion of the placenta; regardless, at this point it appears that flunixin is not a promising candidate for treatment of transition cows. In contrast, one study from Greece showed that flunixin actually shortened the mean calving-to-first-detected-estrus interval and increased the percentage of cows with complete uterine involution in both an acute and subacute metritis incidence (Amiridis et al., 2001a). Metritis incidences in this study were identified on days 5-8 of lactation and had a red to brownish, watery or purulent fetid uterine discharge, fever, reduced appetite, reduced ruminal movements and reduced milk production. Acute metritis was defined as animals that were febrile (40.5-41.5°C), completely off-feed, with similar symptoms as previously described. Subacute metritis was identified as cows that were moderately pyrexia (39.5-40.4°C) with reduced appetite, ruminal movements, and milk production (Amiridis et al., 2001a).

Salicylates and their modes of action. Salicylic acids have been utilized for therapeutic purposes for almost 2000 years (extracts of willow bark) and purified salicylic acid was first used as a therapeutic in rheumatic disease nearly 120 years ago (Amann and Peskar, 2002). It has been well documented that this class of NSAID can alleviate pain and inflammation in humans and several times has been identified as having anti-diabetic and insulin sensitizing effects. An improvement in glucose tolerance was a by-product of administration of aspirin and other salicylates when given to patients with rheumatic disease (Williamson, 1901). When aspirin (acetylsalicylic acid) is taken orally, about 50% is de-acetylated to salicylate during and immediately after absorption. Plasma half-life of aspirin is about 15 minutes, whereas salicylate half-life is between 2 and 30 hours depending on concentration (Amann and Peskar, 2002). Nearly 80% of the circulating salicylate is bound to plasma proteins (Needs and Brooks, 1985).

One salicylate derivative, salsalate, was administered to adults with a body mass index greater than 30 kg/m² and insulin action was analyzed as rate of glucose disposal in a euglycemic-hyperinsulinemic clamp and a glucose tolerance test. Salsalate had a glucose-lowering effect which the authors attributed to insulin concentration, rather than an improvement in insulin action (Koska et al., 2009). In humans acetylsalicylate decreases concentrations of plasma free fatty acids (Carlson and Ostman, 1965; Vane, 1971).

Salicylate is an NSAID that inhibits both cyclooxygenase (COX) isoforms and can inhibit nuclear factor κ B (NF κ B) and subsequently decrease new synthesis of inflammatory cytokines like TNF α and IL-1 (Kutuk and Basaga, 2004; Kim et al., 2005; Mortaz et al., 2005). Vane in

1971 demonstrated the “inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs”. Inhibition of prostaglandin formation by aspirin-like drugs occurs through inhibition of cyclooxygenase that converts arachidonic acid to prostaglandin H₂. There are two isoforms of the cyclooxygenase enzyme. Cyclooxygenase-1 (COX-1) is considered the constitutive isoform (Mitchell et al., 1993), while cyclooxygenase-2 (COX-2) is the major isoform responsible for prostaglandin biosynthesis in inflamed tissue (Herschman, 1996; Smith and Dewitt, 1996) and can be activated by some cytokines, mitogens, and endotoxin (Mitchell et al., 1993). However, COX-2 has been found to be constitutively expressed in several tissues including the central nervous system, kidney, and possibly arterial endothelial cells (Yamagata et al., 1993; Harris et al., 1994; Topper et al., 1996; Amann and Peskar, 2002). Since aspirin is readily converted to salicylate in the body, it is of interest to understand how salicylate operates within the body. Some reports claim that there is moderate COX-1 sensitivity to salicylate, whereas other studies suggest that salicylate has preferential COX-2 inhibition (Mitchell et al., 1993). Additionally, in intact cells salicylates appears to inhibit prostaglandin biosynthesis by a mechanism that is separate from COX activity (Whitehouse and Graham, 1996; Hinz et al., 2000).

When looking at using an NSAID to manage inflammation, understanding what organs and/or organ systems can be affected is important. For example, prostaglandin production is important in the uterus for maintaining the fetus and subsequent expulsion of the fetus at parturition. The prostaglandins are also important in the estrus cycle to initiate reproductive success. For example, prostaglandins -F_{2α} (PGF_{2α}) and -E₂ (PGE₂) are generated by COX-2 (Parent et al., 2003), which is often a target of NSAIDs, and as such, knowing the inhibited pathways is of importance. Long-term NSAID administration can lead to suppression in gut mobility and in turn can lead to ulcers and other gastric disorders. One issue that is associated with COX-1 selectivity by NSAIDs is that it can lead to gastrointestinal issues as evidenced in humans (Warner et al., 1999). However, in experimental models, sodium salicylate does not cause ulcerogenicity and can prevent gastric mucosal damage by drugs such as indomethacin, aspirin, and ethanol (Ezer et al., 1976; Glenn et al., 1979; Whittle et al., 1980; Robert, 1981; Ezer et al., 1984).

Some prostaglandins are anti-inflammatory and are involved in resolving inflammatory issues. Some specific fatty acid metabolites include the prostaglandins 15 deoxy-Δ¹²⁻¹⁴

prostaglandin J2 (15d-PGJ2) which has been found to inhibit NF κ B by reacting with I κ B kinase and the DNA-binding domain of p65 (Straus et al., 2000; Ghisletti et al., 2007). 15d-PGJ2 has also been identified as a ligand for PPAR γ (Forman et al., 1995). PPAR γ is a member of the nuclear receptor superfamily that binds to *cis*-acting elements and provides a promoter for its target genes. Other classes of anti-inflammatory fatty acid metabolites include resolvins and protectins, which have been found to directly correlate with the resolution phase of the inflammatory response (Ariel and Serhan, 2007). They have been found to exert anti-inflammatory effects, but according to one source, their cellular action remains unclear (Olefsky and Glass, 2010). Omega 3 fatty acids such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) inhibit effects of LPS and TNF α (Lee et al., 2001; Lee et al., 2003; Song et al., 2006; Solinas et al., 2007) and inhibit proinflammatory effects of saturated fatty acids (Solinas et al., 2007).

Another inhibitory mode of action of salicylate is its ability to interfere with kinases. Aspirin and sodium salicylate disrupt signal transduction by inhibiting the inhibitor of NF κ B (I κ B) kinase β , preventing translocation of NF κ B to the nucleus (summarized by Cronstein et al., 1999). Put simply, the transcription factor NF κ B is inhibited by salicylate (Amann and Peskar, 2002). Nuclear factor kappa-B is regarded as one of the primary elements involved in inflammatory stimuli. Kopp and Ghosh (1994) inhibited the NF κ B pathway with aspirin and salicylate in the human Jurkat T cell line and the mouse pre-B cell line, PD31. The concentrations used in this study were relatively high, especially in relation to normal *in vivo* levels (Kopp and Ghosh, 1994). In mice that are deficient in p105 (precursor of the p50 component of NF κ B), aspirin and sodium salicylate retained their anti-inflammatory efficacy (Cronstein et al., 1999). Lipolysis in fat cells is regulated by cyclic 3',5'-AMP (Robison et al., 1968) and sodium salicylate may potentially have antilipolytic effects through effects on the adenyl cyclase system (Schonhofer et al., 1973). Specifically, changes observed in response to sodium salicylate in fat cells include a reduction in ATP levels, a reduction in the accumulation of cyclic 3',5'-AMP, inhibition of phosphodiesterase, and a reduction in binding of cyclic 3',5'-AMP to cyclic 3',5'-AMP-dependent protein kinase, all of which might lead to inhibition of lipolysis (Schonhofer et al., 1973). Cyclic AMP (cAMP) is involved in multiple cellular processes that can be activated or deactivated by several different metabolic processes, including those associated with glucose metabolism, ketogenesis, lipolysis, and protein metabolism

(Robison et al., 1968). Another mode of action for salicylates is the uncoupling of oxidative phosphorylation, leading to ATP catabolism (Cronstein et al., 1994; Cronstein et al., 1999).

Aspirin (acetylsalicylic acid) effects in cattle. Aspirin is an antiprostaglandin agent used as an analgesic, antipyretic, and anti-inflammatory. Prostaglandins mediate inflammation, fever, pain, anaphylaxis, and platelet aggregation. Cyclooxygenase forms prostaglandin from arachadonic acid derived from activated cellular lipases or cell-membrane phospholipases. Oral administration of acetylsalicylic acid results in a slow absorption time (half-life of absorption 2.91 hrs) and a rapid elimination with a biologic half-life of 32 minutes for salicylates (Gingerich et al., 1975). However, even with a short half-life it has been found that an oral dosage of 100 mg/kg every 12 hours was enough to maintain serum salicylate concentrations above 30 µg/mL which is the therapeutic dosage (Gingerich et al., 1975). Literature states that the bioavailability of salicylate in commercial aspirin (acetylsalicylate: ASA) tablet is 70% (Gingerich et al., 1975).

Salicylates have been evaluated for use in the treatment of mastitis, and in general they are effective at reducing body temperatures, but do not appear to decrease the severity of the infection (Morkoc et al., 1993a). However, this class of NSAIDs shows some promise in regard to metabolic inflammation. Cows treated with lysine acetyl-salicylate (LAS) for the first 5 days of lactation had significantly lower plasma concentrations of acute phase proteins and tended to have greater peak milk production than controls (Bertoni et al., 2004). They also had improved fertility traits; more cows pregnant after 1st insemination, fewer services per conception, a slight reduction in days open, and fewer cows culled for infertility. Additionally, LAS had favorable effects on inflammatory status: higher blood Zn and lower plasma HP, ceruloplasmin, total proteins and globulins. Improvement in liver protein synthesis (albumin, lipoproteins, total cholesterol, and retinol-binding protein) was also seen with cows that received LAS. Some of the negative effects observed with LAS treatment were a more pronounced energy deficiency, as supported by higher levels of NEFA and a marked and prolonged reduction of BCS. Additionally, plasma glucose concentrations were reduced on day 3 of injections with LAS. Metritis was also increased with cows that were given LAS i.m. (Bertoni et al., 2004).

In a separate but similar study, aspirin treatment for 5 days postpartum improved milk yield in the first 2 months of lactation and improved first service conception rates (Trevisi and Bertoni, 2008). A relatively small number of cows was included in the study (23/treatment); however, ketosis incidence appeared to decrease with aspirin treatment (4.4% vs. 22.7%) while

metritis incidence appeared to increase (30.4% vs. 13.6%). Trevisi and Berton (2008) concluded that utilizing a five day intramuscular injection of aspirin immediately after calving accelerates the recovery from the inflammatory consequences of calving.

Feeding Brahman cows aspirin every 12 hours at a rate of 100 mg/kg of body weight between 7 and 14 d postpartum led to a lower pregnancy rate, increased incidence of abnormal estrous cycles, and a decline in the presence of corpora lutea (Stahringer et al., 1999). However, these results were not the same between pluriparous and primiparous cows (Stahringer et al., 1999), indicating that the number of lactations or age of cows impact either the metabolism of aspirin or the ability of the drug to have impacts on the cow.

Sodium salicylate in dairy cows. An early trial by Anderson et al. (1979) utilizing sodium salicylate (SS) looked at the different biochemical effects of oral SS in healthy non-lactating adult Holstein cows. Three cows were given twice daily doses of SS for 2 days at a dose of 124 mg/kg body weight. Serum and urine salicylate levels, urine and rumen pH, and hemotologic and biochemical profiles were determined periodically for 84 hours. At this dosage, therapeutic serum levels were achieved 3 hours after the first dose and remained above the level until 14 hours after the last dose. The cows receiving SS had high concentrations of salicylates in the urine and both urine and rumen pH were lower in SS cows versus control cows.

Even though SS is rapidly removed from the blood, the twice a day dosing technique allowed the cows to maintain therapeutic levels (Anderson et al., 1979). Sodium salicylate has a pKa of 3.5, affecting its ionizing ability. The nonionized form of SS is what is absorbed from the gastrointestinal tract. At low rumen pH, more SS is in the ionized form, thus it should reduce the diffusible SS concentration. Anderson et al. (1979) suggested that cows that have a lower rumen pH and alkaline urine would require more salicylate to maintain therapeutic levels in the bloodstream. In a different study using both goats and cattle, SS was administered in an i.v. dose of 44 mg/kg of body weight; 51.77% was found to be excreted in the urine and nearly 90% of the drug was excreted within 12 hours after dosing (Short et al., 1990). However, the amount excreted in urine was much lower after an oral dose, at the same concentration as i.v. (6.72% vs 51.77%) although again, nearly 90% was excreted by 24 hours (Short et al., 1990). Salicylate is cleared through conversion to several different metabolites such as salicyuric acid and salicylglycyronide, which can be measured in urine (Davis and Westfall, 1972).

Conclusion

Given that transition cow management is of tremendous importance to the dairy producer, coming up with ways to manage this complex situation will provide areas of research for many years. Fatty liver disorder and ketosis are common in transition dairy cows and affected cows have depression in production and length of time retained in the herd. If inflammation is involved in fatty liver disorder, then modulating this by use of an NSAID like sodium salicylate should be less damaging to the animal and might help with the transition into lactation. Understanding the different modes of action for the different classes of NSAIDs is also very important as some may contribute to metabolic disorders. However, to be the best stewards, one should be cognizant of dietary, reproduction, and facility management practices to best minimize issues associated with the transition period.

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Chapter 2 - Sodium salicylate in early lactation increases milk yield and fat yield through the lactation in dairy cows

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ABSTRACT

Inflammation has been proposed as a contributor to fatty liver disease, and this disorder has negative impacts on milk production in dairy cattle. Our objective was to determine if administration of sodium salicylate (SS), a non-steroidal anti-inflammatory drug (NSAID), in the first week of lactation changes whole-lactation productivity and retention in the herd. At calving, 78 cows ($n = 39$ primiparous [1P]; $n = 24$ 2nd lactation [2P]; $n = 15 \geq 3$ lactations [3P]) were alternately assigned to either control (CON) or SS treatment for 7 d postpartum. SS treatment was administered via individual water bowls at a concentration of 1.95 g/L, delivering a mean of 123.3 ± 5.5 g salicylate/d during the 7 d of treatment. For the first 21 days in milk dry matter intake, water intake, milk yield, and health were monitored daily and milk samples collected twice weekly for milk component analysis. Additionally, cows were followed through the lactation by monthly milk yield and component testing, and the effects of treatment on the risk of leaving the herd and on 305-day milk, fat, and protein yields were assessed. Sodium salicylate did not alter water or dry matter intake during the treatment period. Milk fat yield was increased (12.5%, $P = 0.02$) and energy corrected milk tended to be increased (8.4%, $P = 0.06$) in the 3rd week of lactation in SS cows. During the 1st 21 DIM there were no differences in health disorder incidence, except for increased risk of metritis in 3P SS cows. Treatment by parity interactions were detected for both 305-day milk and fat yields. Milk yield was $1,988 \pm 704$ kg greater over the lactation in 3P SS cows compared to 3P controls (17% increase, $P = 0.05$); no effects were detected in 1P or 2P cows. Furthermore, 3P SS cows produced 144 ± 25 kg more milk fat over the lactation (34% increase, $P < 0.001$). Again, no effects were detected for 1P or 2P. Treatments tended to increase 305-day milk protein yield ($P = 0.06$) in 3P cows. A treatment by parity interaction was observed for the risk of leaving the herd. First parity cows treated with SS cows tended to have greater risk of leaving the herd than controls (30% vs. 6% risk, $P < 0.10$). However, treatment did not alter herd retention in 2P or 3P groups, and SS had

no effect on the risk of leaving the herd overall ($P = 0.59$). Results indicate that sodium salicylate has long term effects on lactation of aged cows, particularly on fat metabolism, but has potential negative effects for primiparous cows.

INTRODUCTION

Fatty liver (FL) is a metabolic disease in transition dairy cows that increases health disorders and expenses and subsequently decreases productivity. Fatty liver is characterized by a buildup of fat in the liver which gives it a yellowish color and a sponge-like appearance under the microscope. Cows that have FL often have other diseases associated with this disorder, including ketosis, milk fever, mastitis, metritis, displaced abomasum, and retained placenta (Ametaj, 2005).

The transition from late pregnancy to lactation causes stress for the dairy cow. The high demand for energy by the mammary gland and the parallel reduction in dry matter intake lead to a negative energy balance (NEB) in dairy cows (Grummer, 1993; Drackley, 1999; Bobe et al., 2004). While the cows are in NEB they mobilize large quantities of fatty acids from fat deposits which increase the concentration of plasma non-esterified fatty acids (Herdt, 1988). Non-esterified fatty acids (NEFAs) are either used as an energy source in the mammary gland, muscle, or in the liver. Triglycerides can either be stored in the liver or incorporated and released into the blood as very low density lipoprotein (VLDL). When uptake of fatty acids in the liver is greater than the ability of the liver to oxidize fatty acids, TGs accumulate in the liver and fatty liver can develop. Compared to other species, the ruminant liver is unable to efficiently export fatty acids as components of VLDL (Kleppe et al., 1988).

Traditionally the etiology of FL has been largely attributed to the NEB that dairy cows have following parturition; however, lately researchers have been pointing to inflammation as a potential contributor to FL. Inflammation may link FL to disorders such as mastitis, metritis, etc (Bertoni et al., 2004; Bobe et al., 2004; Ametaj et al., 2005b; Bradford et al., 2009). Animals with excessive adiposity exhibit a low-grade inflammation (Hotamisligil, 2006). Inflammatory cytokines cause endocrine-metabolic changes in dairy cattle, such as anorexia, lipomobilization, impaired insulin sensitivity, reduction of milk yield, and impaired reproductive function (Kushibiki et al., 2001a; Kushibiki et al., 2003; Bertoni et al., 2004), all of which are similar to symptoms associated with FL.

Based on effects observed with inflammation, a possible method to prevent these negative effects could be the use of non-steroidal anti-inflammatory drugs (NSAIDs). Non-steroidal anti-inflammatory drugs are classified into 5 broad categories with slightly different modes of action (Gallo et al., 2008) and different results have been observed in their effects on transition disorders. Prolonged use of certain NSAIDs can have negative effects such as gastrointestinal bleeding, intestinal ulceration, aplastic anaemia, and inhibition of platelet aggregation (summarized by Gallo et al., 2008) potentially due to suppression of both isoforms of cyclooxygenase (COX-1 and COX-2). Sodium salicylate is a weak inhibitor of COX-1 and COX-2 (Mitchell et al., 1993) and its probable mode of action is that it inhibits phosphorylation of I κ B- α which impairs the activation of nuclear factor kappa-B (NF κ B), a common mediator of inflammation (Kopp and Ghosh, 1994; Pierce et al., 1996). The transcription factor NF κ B can be activated by inflammatory stimuli and in turn produces proinflammatory cytokines such as TNF α , interleukin (IL)-1 β , IL-6, and interferon β (Shoelson et al., 2003). In addition to inhibiting this inflammatory pathway, sodium salicylate has demonstrated antilipolytic effects in normal and diabetic patients (Schonhofer et al., 1973).

Based on the responses to exogenous cytokine administration, it was hypothesized that elevated plasma concentrations of NEFA and cytokines cause an inflammatory response in the liver of transition dairy cows, resulting in partitioning of fatty acids toward triglyceride synthesis, depressed gluconeogenesis, and continued adipose tissue mobilization. The specific objective of this study was to determine the effects of sodium salicylate (SS) in the first week of lactation on production and health through the entire lactation.

MATERIALS AND METHODS

All experimental procedures were approved by the Kansas State University Institutional Animal Care and Use Committee #2880.

Design and Treatments

A total of 78 cows [n = 39 primiparous (1P), n = 24 2nd parity (2P), and n = 15 \geq 3rd parity (3P)] from the Kansas State University Dairy Teaching and Research Facility were blocked by parity and alternately assigned to treatment at parturition. Cows that were enrolled were considered to be “healthy”, had a dystocia score less than 2 (1-4 scale), and structurally

sound. Treatment consisted of administration of sodium salicylate (SS; 1.95 g/L) in a molasses carrier (0.14 g/L) via individual water bowls for 7 d postpartum. Control cows received water with the same molasses concentration for 7 d (CON). On d 8 postpartum all cows were given non-medicated water for the remainder of the experiment. Cows were housed in a tie-stall facility in randomly assigned stalls through d 21 postpartum. Cattle were milked 3 times daily (0600, 1000, and 1800 h) and fed ad libitum twice daily (0630 and 1800 h) with a diet formulated to meet all nutrient requirements (Table 2.1). Once cows were removed from the tie-stall facility, they returned to the general herd free-stall facility and were managed similarly.

Data and sample collection

Throughout the 21-d trial, feed and water intake and milk yield were recorded daily. During these 21 d, milk samples were collected at each milking on twice a week for analysis of milk components. For 305-d analyses, milk yields were recorded and samples were collected and analyzed once monthly.

Milk Analyses

Milk samples were analyzed by Heart of America DHIA (Manhattan, KS) to determine concentrations of fat, true protein, lactose (B-2000 Infrared Analyzer, Bentley Instruments), urea nitrogen (MUN spectrophotometer, Bentley Instruments), and somatic cell count (SCC). Energy-corrected milk (ECM) was calculated as: $ECM = 0.327 \times \text{milk yield} + 12.86 \times \text{fat yield} + 7.65 \times \text{protein yield}$ (Dairy Record Management Systems, 2009). Somatic cell score (SCS) was calculated according to Shook (1993): $\log_2(\text{SCC}/100) + 3$.

Disease Incidence

Cows were assessed daily for health status by trained personnel where rectal temperature and urine ketones (ReliOn ketone test strips, Bayer Healthcare LLC., Mishawaka, IN) were recorded. Ketosis, left displaced abomasum, retained placenta, metritis, milk fever, lameness, and other abnormalities were recorded and treated per farm protocol. Ketosis was defined as the detection of urine ketone concentrations exceeding 80 mg/dL on any 1 d, or exceeding 40 mg/dL for 2 consecutive days. Metritis was identified as a cow that had a fever ($>103^\circ\text{F}$) and uterine discharge that was reddish brown and had an odor. Cows that had a displaced abomasums (DA) during the trial were removed on the day of surgery. Data obtained before removal for DA cows

were included in all analyses. Clinical cases of mastitis were recorded as cows that had unusual milk consistency as determined by trained milking personnel and were subsequently treated with an antibiotic. Disorders not described above were identified based on definitions provided by Kelton et al. (1998).

Behavioral data

Each tie stall was equipped with individual feed bunks suspended on load cells and bunk weight was monitored continuously by computer. Feeding activity including meal length and size were recorded electronically. Behavior data was analyzed as described previously (Mullins et al., 2012). Briefly, intermeal intervals were no less than 12 minutes and meals < 0.2 kg DM were excluded. Meal number, meal length, intermeal interval, and meal size (DM basis) data were analyzed for treatment effects.

Retrospective analyses

Herd management data and DHIA production records were used for a retrospective analysis of whole-lactation production and risk of leaving the herd. Cows that did not remain in the herd for a subsequent calving were considered to have left the herd, and the reason entered was recorded. Predicted 305-d yields of milk, fat, and protein generated by DHIA (VanRaden, 1997) were used for all animals for which a value was generated. Predictions were not available for 3 animals that left the herd prior to 95 DIM.

Statistical analysis

Statistical analyses for DMI, water intake, milk yield, and ECM over the first 21 d postpartum were carried out using the Mixed Procedure of SAS (version 9.2; SAS Institute Inc., Cary, NC) to assess the fixed effects of parity, time, treatment, and all 2- and 3-way interactions; cow was included as a random effect. Repeated measures over time were modeled with autoregressive covariance structures, and denominator degrees of freedom were estimated using the Kenward-Rogers method. Values were deemed outliers and omitted from analysis when Studentized residuals were $>|3.5|$. Interactions were investigated when $P < 0.15$ using the slice option, and slices declared significant at $P < 0.05$. Health disorder incidence and risk of leaving the herd were analyzed by Fisher's exact test using JMP (version 8.0; SAS Institute Inc. Cary, NC). To test for randomization bias in milk production, treatment effects on predicted

transmitting ability (PTA) for milk, fat, and protein yield were tested, and no significant differences between treatment groups were observed (all $P > 0.50$). An additional potential confounding factor was the enrollment of 27 of the cows ($n = 14$ control, $n = 13$ SS) in a nutrition study following the completion of the 21 d salicylate study. The study (Vargas et al., 2012) was a Latin square design with treatments that failed to influence any measured outcome. Therefore, the simple effect of enrollment in the study was included in the model. Statistical analysis of 305-d milk, fat, and protein yields were carried out using JMP (version 8.0) to estimate the fixed effects of treatment, parity, treatment by parity interaction, and subsequent study enrollment. Additionally, the PTA for the variable of interest was included as a covariate to account for genetic contributions to variance.

RESULTS

DMI and water intake

Multiparous cows (both 2P and 3P) had higher DMI than 1P cows ($P < 0.001$) and a time effect ($P < 0.001$) was observed, reflecting an increase in DMI from d1 to 21. Additionally, a significant 3-way interaction ($P < 0.001$) was observed where 2P and 3P cows had a number of days with significantly different intakes between treatments. Control 2P cows had greater DMI than SS 2P cows on numerous days through the first 20 DIM (Figure 2.1A), whereas 3P SS cows had greater DMI than 3P CON cows on several days (Figure 2.1B).

Water intake followed a pattern similar to DIM; older cows had higher water intake than primiparous cows ($P < 0.001$) with increasing consumption of water as time progressed ($P < 0.001$). There was a significant treatment by day interaction ($P < 0.001$) where on d 1 CON cows tended ($P < 0.10$) to have a greater water intake, but then the remainder of the differences were observed after treatment ended (after d 8, Figure 2.1C).

Milk production

During the first 21 DIM, daily milk yield was higher for multiparous cows than primiparous cows ($P < 0.001$; 39.51, 38.06, 29.00 \pm 1.04 kg/d for 3P, 2P, and 1P). Daily milk yield increased in the first three weeks ($P < 0.001$) regardless of treatment. There was a significant treatment \times day \times parity interaction for milk yield ($P < 0.001$) where all the observed differences were in 3P cows (Figure 2.2A). Fat yield was increased by 12.5% ($P = 0.02$, Figure

2.2B) in week 3 with SS treatment. Older cows had higher milk fat percentage and milk fat yield than primiparous cows, resulting in a parity effect ($P < 0.01$) for these variables. A treatment \times week interaction ($P < 0.01$) was observed for milk protein content, where in the first week of lactation (while SS was administered), SS cows had higher milk protein content compared to CON cows (4.00 vs. $3.78 \pm 0.05\%$, Figure 2.2C). There were no treatment effects during the following 2 weeks, and overall protein content and yield decreased through the first 3 wk ($P < 0.001$). Multiparous cows had higher protein concentrations than primiparous cows (3.33 , 3.51 , 3.29 ± 0.06 for 3P, 2P, and 1P; $P = 0.01$). There was no treatment ($P = 0.75$) effect during the first 3 weeks for lactose yield, but both lactose content and yield increased through the first 3 wk ($P < 0.001$). A treatment \times parity \times week interaction ($P = 0.01$) was also observed for lactose yield. Salicylate treatment tended to increase ECM yield by 8.4% in week 3 ($P = 0.06$, Figure 2.2D).

Milk urea nitrogen (MUN) concentration was decreased ($P = 0.03$) with SS treatment (Table 2.2) during the first 3 wk. There was also a treatment \times parity interaction ($P = 0.13$) where 3P SS cows had lower MUN versus their CON counterparts (11.51 vs. 14.17 ± 0.80 mg/dL; $P = 0.02$). Somatic cell score was not impacted by treatment ($P = 0.63$), but a parity effect ($P < 0.01$) was observed where 1P cows had the highest SCS followed by 3P and 2P cows (3.39 ± 0.40 , 2.73 ± 0.67 , 1.90 ± 0.52 , respectively).

There was no treatment effect ($P = 0.16$) for 305-d milk yield, but there was a treatment \times parity interaction ($P < 0.001$). Salicylate administration increased ($P = 0.05$) 305-d milk yield in 3P cows by $1,988 \pm 704$ kg (17%; Figure 2.4A). A parity effect was observed ($P < 0.01$) where 3P cows had the highest 305-d milk yield and 1P cows had the lowest ($12,852$, $12,160$, $11,258 \pm 364$ kg for 3P, 2P, and 1P). Estimated 305-d milk fat yield was increased 13% in SS cows ($P < 0.001$). Parity also affected 305-d fat yield ($P < 0.001$), with 3P cows again having greatest 305-d fat yield and 1P cows the lowest (495 , 423 , 392 ± 13 kg for 3P, 2P, and 1P). A treatment \times parity effect was also observed, where 3P SS cows had 144 ± 25 kg more fat yield over the lactation than 3P CON (34% increase, $P < 0.001$; Figure 2.4B). There was no overall treatment effect for 305-d protein yield, but there was a significant parity effect where 1P cows had the lowest protein yield and multiparous cows had higher yields (335 ± 7.5 vs. 370 ± 11.6 kg). There was also a treatment \times parity interaction for 305-d protein yield, where 3P SS cows tended to have greater protein yield than 3P CON (14% increase, Figure 2.4C).

Energy balance

A tendency for a treatment effect ($P = 0.09$) was observed for energy balance where SS cows had a more severe negative energy balance than CON (-13.9 vs. -12.4 ± 0.86 Mcal/d). A parity effect ($P < 0.001$) was observed where the oldest cows (3P) had the most negative energy balance and the 1P cows had the least ($-19.7, -13.4, -10.5 \pm 1.0$ Mcal/d for 3P, 2P, 1P). An interesting interaction for treatment \times week ($P = 0.11$) was observed, where in weeks 2 and 3 SS cows had a more severe energy balance (Figure 2.2E); this response occurred after treatment had ended.

Disease incidence and herd retention

Overall, health disorders were not impacted by SS treatment in the first 21 DIM. However, within the 3P block, SS cows had a higher incidence of metritis than CON ($P = 0.03$, Table 2.3).

A treatment \times parity interaction was observed ($P < 0.05$) where 1P SS cows tended ($P < 0.10$) to have a greater risk of leaving the herd than controls (Table 3). There was no difference observed in 2P and 3P cows. There were no significant differences between treatments ($P > 0.10$) in herd removal risk for specific reasons, but the most prevalent causes for cows leaving the herd were reproductive issues and chronic mastitis (Table 2.6).

Feeding behavior

Feeding behavior results are listed in Table 2.4. For meals per day, there was a 3-way interaction ($P = 0.06$) where all differences were observed in 2P and 3P cows (Figures 2.3A and 2.3B). Among 2P cows, SS increased the number of meals on d 3 postpartum but decreased the number of meals on d 17 postpartum. In contrast, among 3P cows SS tended to decrease the number of meals on d 3 and 5 postpartum but increased the number of meals on d 17 postpartum. For meal length a treatment \times day interaction was observed; SS tended to increase meal length on d 2 postpartum but significantly decreased meal length on d 18 (Figure 2.3C). A treatment \times day \times parity interaction was observed for intermeal interval, where most of the significant differences were observed in 3P cows (Figure 2.3D). In this block, intermeal interval was shorter for SS cows compared to CON on d 1, 11, 17, and 18 postpartum (all $P < 0.10$). A significant parity effect ($P < 0.001$) was observed for meal size where 2P and 3P cows had larger meal sizes than 1P cows ($2.95, 2.83, 2.32 \pm 0.08$ kg DM/meal for 3P, 2P, and 1P). A treatment

× day interaction was also observed with decreased meal sizes for SS on d 1, 14, and 18 postpartum but increased meal size on d 8 (Figure 2.3E).

DISCUSSION

Milk and milk fat yields were increased in cows treated with sodium salicylate, with the greatest differences observed in 3rd or greater lactation cows. Although no treatment effects on milk yield were detected during the 7-d treatment period, milk yields began diverging toward the end of the intensive 21-d monitoring period in the oldest cows. This trend extended into the rest of the lactation, resulting in significantly higher 305-d milk yield for 3P SS cows compared to 3P CON. This finding is similar to results observed by Bertoni et al. (2004), who administered lysine acetylsalicylate (aspirin) by daily injection for the first 5 d postpartum. In that study, aspirin tended to increase milk yield compared to control, primarily at peak lactation. In a separate but similar study, aspirin treatment for 5 days postpartum increased milk yield in the first 2 months of lactation (Trevisi and Bertoni, 2008). Additionally Trevisi et al. (2003) observed increased milk yield compared to a placebo group in cows receiving lysine acetylsalicylate.

However, in an endotoxin-induced mastitis model where SS was used in an attempt to minimize the negative effects on production, the authors observed a slower return to pre-challenge production levels (Morkoc et al., 1993b). The quantity of SS administered to these cows daily was much greater than in the current study (plasma salicylate concentrations of 236 vs. 34 ± 15.0 µg/mL in this study), and the negative production effects may have been related to the high dose of SS. In a study where a different NSAID was used, flunixin meglumine decreased milk production in an endotoxin-induced mastitis model (Anderson et al., 1986). However, in a transition cow model, flunixin meglumine administered i.v. for the first 3 days after parturition had no effect on milk yield, 3.5% fat-corrected milk yield, energy-corrected milk yield, milk fat, protein or lactose percentages (Shwartz et al., 2009). Flunixin meglumine administration also failed to significantly alter milk yield when administered (following ceftiofur treatment) to cows diagnosed with metritis in early lactation (Drillich et al., 2007a). However, in this study milk yield was measured only over the 6 days following treatment, so impacts on the whole lactation were not assessed. In another study where flunixin meglumine was administered to cows with toxic mastitis, the authors did not observe any differences in milk production in the

10 weeks following the development of mastitis (Dascanio et al., 1995). Looking at a third class of NSAID, ketoprofen had no effects on milk yield when given by intramuscular injection for 2 days after parturition (Richards et al., 2009). However, in this study the only evaluation of milk yield was a single measurement obtained from the first individual milk reading of the lactation. Overall, it appears that the different classes of NSAIDs have different effects as evidenced by differences observed in milk production.

Even though increases in milk yield were observed in older cows treated with SS, mechanisms for this increase in the dairy cow have not been identified. In an isolated mammary tissue model from lactating rats, incubation with SS in the media was found to increase in Ca^{2+} (mmol/kg cell H_2O) (Shennan, 1992). An efflux of K^+ is also observed in response to SS in lactating rat mammary tissue, and as such the authors concluded that salicylates activate a calcium-dependent K^+ efflux channel and/or a calcium-dependent non-selective cation channel (Shennan, 1992). The authors speculated that there are 3 possible explanations for the increase in calcium uptake. First, salicylate can complex with Ca^{2+} . Salicylate rapidly crosses biological membranes (Dalmark and Wieth, 1972) and if complexed with Ca^{2+} , can increase intracellular Ca^{2+} levels. Another possible explanation is that salicylate might act like an ionophore to form Ca^{2+} channels. The final potential mechanism the authors suggested was that salicylate may activate quiescent endogenous Ca^{2+} channels. Calcium is important in mammary cell survival (Delbecchi et al., 2005) and milk protein synthesis (Duncan and Burgoyne, 1996) and as such, increasing calcium uptake by the mammary gland might explain why salicylate increased milk yield in this trial.

Early lactation cows have increased transcription of genes in the mammary gland that are involved in lipid transport and metabolism. Specifically, lipoprotein lipase (LPL) and very low density lipoprotein receptor (VLDL-R2) are significantly upregulated (Finucane et al., 2008) in early lactation and are important in fatty acid uptake. These fatty acids are primarily utilized for milk TG synthesis (Bell, 1995). Sodium salicylate may potentially impact the expression of these genes, leading to the increased milk fat observed by week 3 of lactation, and might have a programming role in the expression of lipoprotein receptors extending through the entire lactation.

Glucose utilization for milk synthesis might be another potential mechanism to explain the increase in milk yield that was observed. In early lactation glucose transporters are

upregulated in the mammary gland compared to the dry period (Finucane et al., 2008). In healthy adults receiving salicylates, there is a depression in plasma glucose concentration (Hecht and Goldner, 1959; Carlson and Ostman, 1965; Richardson et al., 1986; Gilbert et al., 1993), which several authors have attributed to an increase in tissue uptake of glucose. Plasma glucose concentration was decreased on d 7 by SS in the current study (Chapter 3). This suppression in plasma glucose might indicate greater use of glucose by tissues, especially the mammary gland, which might partially explain the increased milk yield observed with cows that were given SS. There may be longer-term cellular alterations that are due to SS where glucose transporters or ion pores might be altered to lead to increased milk production.

Administration of SS did not alter incidence of health disorders during the first 21 DIM, except that older cows treated with SS had a greater incidence of metritis. Similarly, a group out of Italy observed an increase in metritis (30% vs. 18% for control) in transition dairy cows given daily injections of aspirin (Bertoni et al., 2004). In a separate study by this group, once again metritis incidence was observed to increase with aspirin treatment (30.4% vs 13.6% in control cows) (Trevisi and Bertoni, 2008). However, in this study, the authors also observed decreases in mastitis and lameness incidences with aspirin treatment (statistical analyses were not conducted).

The increase in milk yield observed in response to SS in the current study could have occurred through a suppression of subclinical health disorders in early lactation. It has been well documented that many health disorders that occur in early lactation decrease whole lactation milk yield (Goff and Horst, 1997; Drackley, 1999; Bobe et al., 2004; Goff, 2006); SS may have improved production by minimizing the “hidden” health disorders that are common in early lactation cows. Even though there were few differences observed in diagnosed health disorders, improving health through prevention of subclinical conditions could be beneficial to production.

Another interesting observation was that there was a greater risk of primiparous cows being removed from the herd if they were given SS during the first week of lactation. There was no consistent reason why these cows were culled, although the most common reasons for leaving the herd consisted of reproductive issues and chronic mastitis. The reproduction issues with primiparous cows could partially be explained by differences in metabolism of SS based on parity. In Brahman cows that were administered aspirin, plasma concentrations of salicylate differed by parity; primiparous cows had higher plasma salicylate concentrations than

multiparous cows (Stahringer et al., 1999). Additionally, the aspirin-treated cows used in this study had lower pregnancy rates, increases in abnormal estrous cycles, and reduced formation of corpora lutea after estrus. Primiparous aspirin-treated cows had a longer postpartum return to estrus interval, providing further evidence of detrimental effects of aspirin administration on postpartum reproduction. When parity was not a factor, as in the Bertoni et al. (2004) trial (only multiparous cows were used), an increase in fertility was observed with aspirin treatment. Specifically, more cows were pregnant after 1st insemination (50 vs. 27%), there were fewer services per conception (2.11 vs 2.33), and a slight reduction in days open (126 vs. 133 d) observed in aspirin-treated cows.

Prostaglandin production is important in reproduction, but also has impacts on inflammation. Prostaglandin $F_{2\alpha}$ is important immediately post-partum for uterine involution and ovarian activity. Suppression of prostaglandin synthesis has been reported to alter postpartum reproductive traits in Brown Swiss cows (Guilbault et al., 1987). A prostaglandin synthase inhibitor administered to cows twice daily for 6 days postpartum reduced ovarian activity and altered progesterone waves after calving. Prostaglandin $F_{2\alpha}$ can decrease the postpartum return to estrus interval and increase follicle size in ovaries (Tolleson and Randel, 1988; Villeneuve et al., 1988; White and Dobson, 1990). Administration of salicylate derivatives suppresses prostaglandin production, which includes both prostaglandin $F_{2\alpha}$ and E_2 . Prostaglandin E_2 has been well documented to induce fever, influence contraction and relaxation of smooth muscle, and affect dilation and constriction of blood vessels (Kelly, 1996). Suppressing prostaglandin production can be an effective way to manage inflammation, however, when suppressing the inflammatory prostaglandin E_2 , it is possible to suppress $PGF_{2\alpha}$ as well, which has negative reproductive effects and may promote retained placenta. Sodium salicylate should not directly suppress prostaglandin production, because the mode of action has been attributed to inhibition of NF κ B activity, not COX activity. However, NF κ B is a transcriptional regulator of COX enzymes, so suppressing its activity may indirectly inhibit prostaglandin synthesis (Shoelsen et al., 2006).

CONCLUSION

Sodium salicylate administered in drinking water during the first 7 d of lactation alters milk production in the dairy cow. The greatest increases in production were observed in older

cows (≥ 3 lactations). Treatment of cows with SS did not significantly alter overall incidence of health disorders. However, metritis was increased in older cows that were given SS, further identifying a need to understand the differences in effects of salicylates in different parities. Another negative effect was observed in primiparous cows, which had a greater risk of leaving the herd when they were given SS during the first week of lactation. Overall, it appears that SS has some benefits in regards to production and potentially profit in 3rd or greater lactation cows, but is not a beneficial management practice for primiparous cows, as it increases their probability of being removed from the herd with no increases in milk production. It is important to note that SS is not approved for commercial use for these purposes. Interesting results were observed when SS was administered during the first week of lactation, however, all the improvements in milk production were observed once cows were not receiving the drug anymore. Further research into potential modes of action for this response, including alterations in mammary gland “programming”, may help clarify the results observed in this study.

Although veterinary forms of aspirin are marketed with label indications for pain, fever, and inflammation, in the U. S. this drug has not been approved for use by the Food and Drug Administration Center for Veterinary Medicine (FDA CVM). Consequently, there are no established withdrawal times in food-producing animals. With this in mind, the only use of aspirin or salicylates is as an extra-label use in cooperation with veterinarians according to AMDUCA (Animal Medicinal Drug Use Clarification Act).

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Table 2.1 Diet composition

Diet composition	
Ingredient (% of DM)	
Corn silage	22.2
WCGF ¹	30.3
Alfalfa hay	20.4
Cottonseed	4.9
Corn grain	9.2
Sorghum grain	4.0
Micronutrient premix ²	9.0
Nutrient (% of DM)	
DM (% as-fed)	53.2
CP	18.6
ADF	18.0
NDF	30.5
NFC	38.1
Ether extract	5.2
Ash	7.9
NE _L ³ (Mcal/kg)	1.72

¹Wet corn gluten feed (Sweet Bran; Cargill Inc., Blair, NE).

²Premix consisted of 54.9% SoyBest, 14.3% limestone, 1.6% salt; 10.2% sodium bicarbonate, 2.0% magnesium oxide, 0.6% 4-plex, 0.3 selenium (0.06%), 0.10% of Vitamin A and D, 1.6% Vitamin E, 0.1% Rumensin 90, 2.6% Diamond V XP, 10.0% MegalacR, 1.6% potassium carbonate, 0.1% Zinpro 100.

³Estimated according to NRC (2001).

Table 2.2 Energetics and milk components in the first 21 DIM

Item	CON ¹	SS ²	SEM	<i>P</i> -value ³		
				Trt	Par	Wk
DMI ^{**,***,††}	17.28	17.41	0.66	0.87	< 0.001	< 0.001
Water intake (L/d) ^{**}	85.41	87.57	2.32	0.51	< 0.001	< 0.001
Energy balance, Mcal/d	-12.44	-13.93	0.86	0.23	< 0.001	< 0.01
Milk yield, kg/d ^{†,***}	35.04	36.00	1.36	0.56	< 0.001	< 0.001
Fat (%)	4.94	5.18	0.11	0.15	< 0.01	< 0.001
Protein (%) ^{*,†}	3.37	3.43	0.04	0.35	< 0.001	< 0.001
Lactose (%)	4.67	4.63	0.03	0.31	0.22	< 0.001
ECM, kg/d	42.31	44.39	1.38	0.29	< 0.001	< 0.001
Yield, kg/d						
Milk Fat [*]	1.70	1.83	0.06	0.16	< 0.001	< 0.001
Milk Protein	1.17	1.20	0.04	0.66	< 0.001	< 0.001
Milk Lactose ^{***}	1.70	1.72	0.05	0.75	< 0.001	< 0.001
MUN, mg/dL ⁴	12.80	11.66	0.37	0.04	0.35	< 0.01
SCS ⁵	2.57	2.78	0.31	0.63	< 0.01	< 0.01

¹ CON; control treatment

² SS; sodium salicylate treatment

³ *P*-value: Trt; treatment effect (CON vs SS); Par; parity effect (1P, 2P, 3P); Wk; week effect

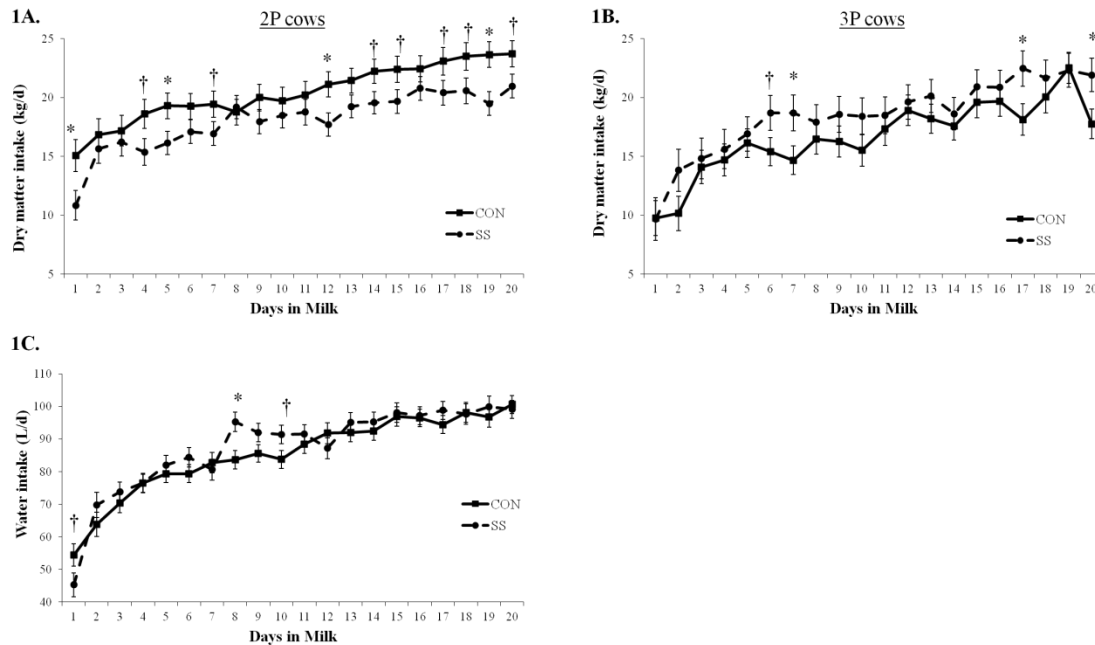
⁴ MUN; milk urea nitrogen

⁵ SCS; somatic cell score: $\log_2(\text{SCC}/100) + 3$ (Shook, 1993)

^{*}Parity \times week ($P < 0.05$); ^{**}Treatment \times week ($P < 0.05$); ^{***}Treatment \times parity \times week ($P < 0.05$); [†]Treatment \times week ($P < 0.10$), ^{††}Parity \times week ($P < 0.10$).

Values reported are least square means (LSM)

Figure 2.1 Dry matter intake and water intake during the first 21 DIM



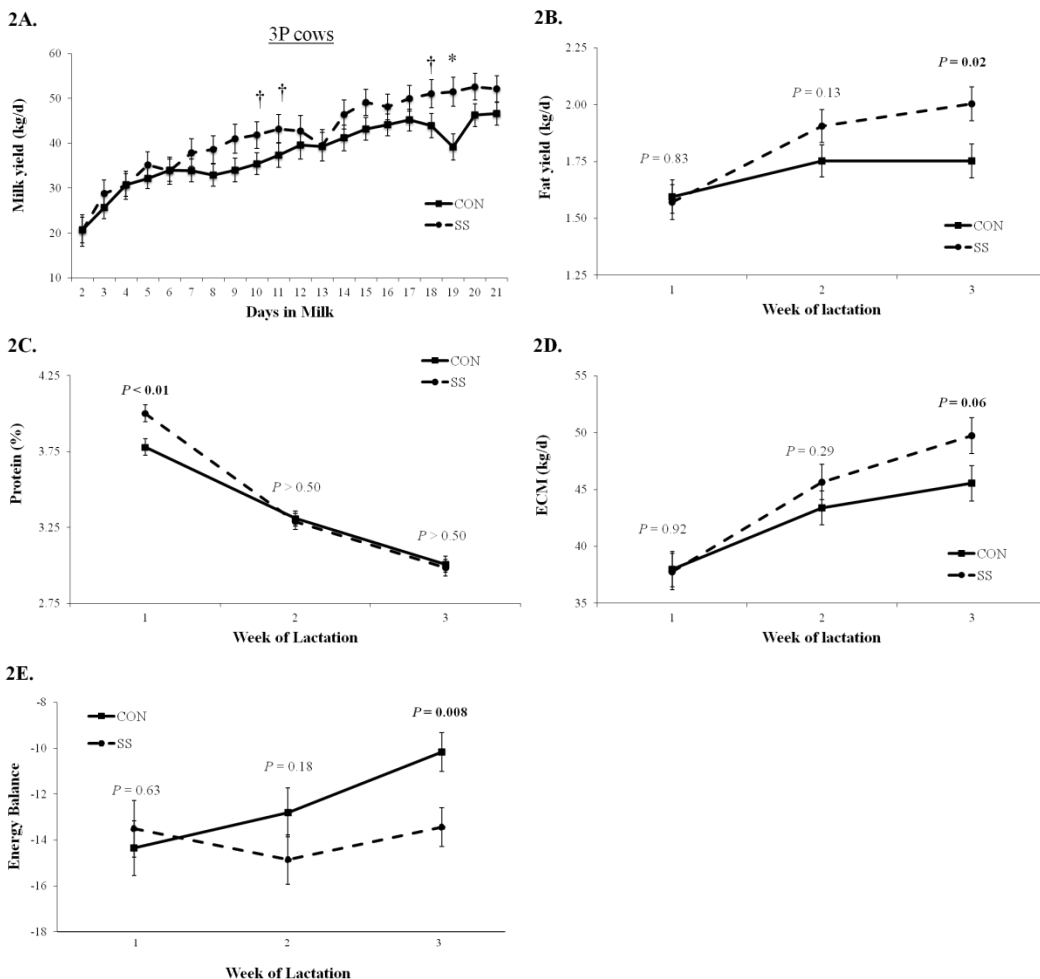
A time effect (day or week) was observed ($P < 0.05$) for DMI and water intake where all increased over time. A treatment \times parity \times day interaction was observed ($P < 0.001$) for feed intake where all the differences were observed in 2P (Panel A) and 3P cows (Panel B). There were a greater number of days where CON 2P cows had a greater DMI, however 3P SS cows had a greater number of days with a greater DMI. Panel C. A significant treatment \times day interaction ($P < 0.001$) was observed for daily water intake. A tendency was observed on d1 where CON cows had greater intake, and all the remainder of differences occurred once treatment was removed (after d 8).

All values are least squares means \pm SE.

* $P < 0.05$

† $P < 0.10$

Figure 2.2 Milk production and energetic changes observed during the first 21 DIM



A significant time (DIM or week) was observed ($P < 0.05$) for all variables shown. Panel A. A treatment \times day \times parity interaction ($P < 0.001$) was observed for daily milk yield where all the differences were associated with 3P cows, shown here. Panel B. There was a significant treatment \times week interaction ($P = 0.04$) for fat yield. SS treated cows had significantly higher fat yield in the third week of lactation. Panel C. A treatment \times week interaction ($P < 0.01$) was observed for protein concentration where in the first week, while SS was administered, SS treated cows had higher milk protein concentration. Panel D. A treatment \times week interaction ($P = 0.11$) for ECM was observed where there was a tendency for SS cows to have a higher ECM in week 3. Panel E. A treatment \times day interaction was observed ($P = 0.05$) where SS treated cows had a more pronounced negative energy balance in week 3. All values are least squares means \pm SE.

* $P < 0.05$

† $P < 0.10$

Table 2.3 Health disorder incidences in first 21 DIM

Disorder	Incidence (%) ¹	CON (n) ²	SS (n) ³	P-value
Ketosis	32	12	13	NS
Lameness	5	3	1	NS
Temperature ⁴	28	11	11	NS
Metritis	23	8	10	⁵
Displaced abomasum	8	2	4	NS
Diarrhea	8	4	2	NS
Retained Placenta	10	4	4	NS
Mastitis	19	7	8	NS
Other ⁶	12	4	6	NS
Multiple ⁷	45	16	19	NS

¹ Total diagnosed and treated incidence of each specific disorder out of all 78 cows.

² Count of control cows (out of 39) diagnosed with the specific disorder.

³ Count of salicylate treated cows (out of 39) diagnosed with the specific disorder.

⁴ Cows had a fever >104°F but no other symptoms, so no specific disease diagnosis.

⁵ Parity x treatment interaction; treatment effect was significant for 3P block ($P = 0.03$).

⁶ Cows that were diagnosed with something other than that listed above (i.e. hardware).

⁷ Cows that were diagnosed and treated as having more than one disorder during the trial.

Table 2.4 Feeding behavior during the first 21 DIM

Item	CON ¹	SS ²	SEM ³	P-value		
				Trt ⁴	Par ⁵	Day ⁶
Meal frequency (d ⁻¹) ^{**}	15.17	15.27	0.46	0.88	0.69	<0.01
Meal length (min) [*]	12.33	11.87	0.55	0.54	0.07	<0.01
Intermeal interval (min) ^{**}	73.49	72.29	2.46	0.73	0.85	<0.01
Meal size (kg of DM) [*]	2.72	2.68	0.06	0.67	<0.01	<0.01

¹ CON; control treatment

² SS; sodium salicylate treatment

³ SEM; standard error of means

⁴ Trt; treatment effect (CON vs. SS)

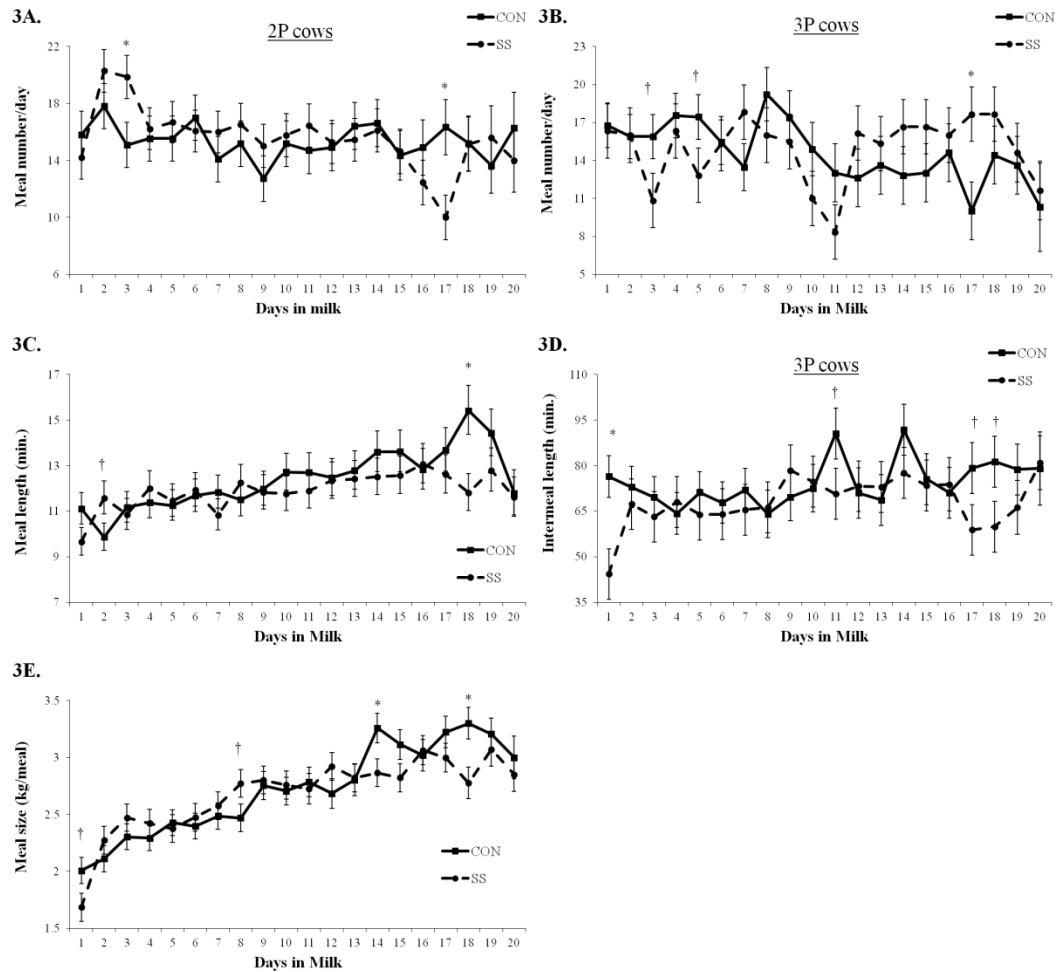
⁵ Par; parity effect (1P, 2P, 3P)

⁶ Day; day effect

^{*}Treatment × day ($P < 0.05$); ^{**} Treatment × parity × day ($P < 0.05$).

Values reported are least square means (LSM)

Figure 2.3 Feeding behavior changes during the first 21 DIM



There was a tendency for a treatment \times day \times parity interaction ($P = 0.06$) for meals per day, where the majority of the differences were for 2P (Panel A) and 3P (Panel B) cows. Panel C. A treatment \times day interaction ($P = 0.02$) was observed for meal length. Panel D. A treatment \times parity \times day interaction ($P = 0.03$) was observed for intermeal length where the greatest number of days with significant differences were in 3P cows as illustrated in Panel D. SS cows had a number of days with a shorter intermeal interval. Panel E. A treatment \times day interaction ($P < 0.01$) was observed for meal size where SS cows had a number of days with smaller meal sizes in the 3rd week, but larger meal size on d 8. All variables shown had a significant day effect ($P < 0.05$).

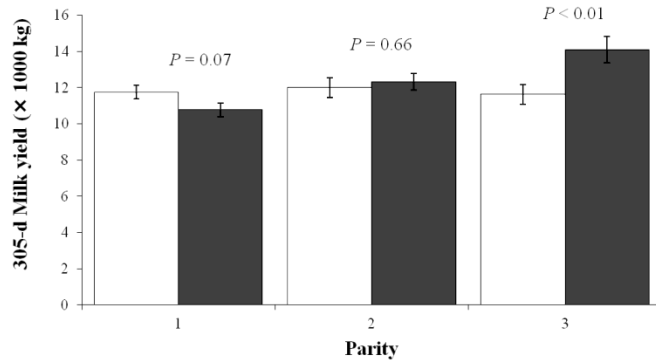
All values are least squares means \pm SE.

* $P < 0.05$

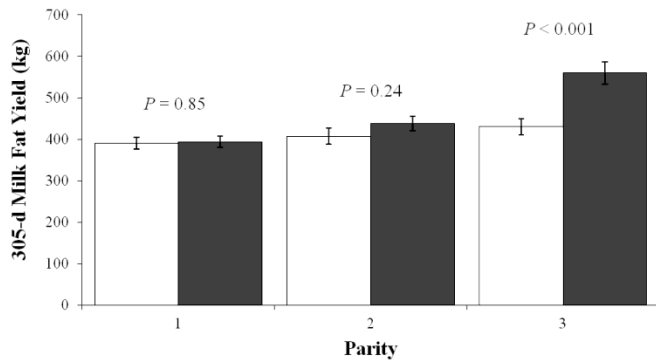
† $P < 0.10$

Figure 2.4 Whole lactation data for cows treated during the first week of lactation

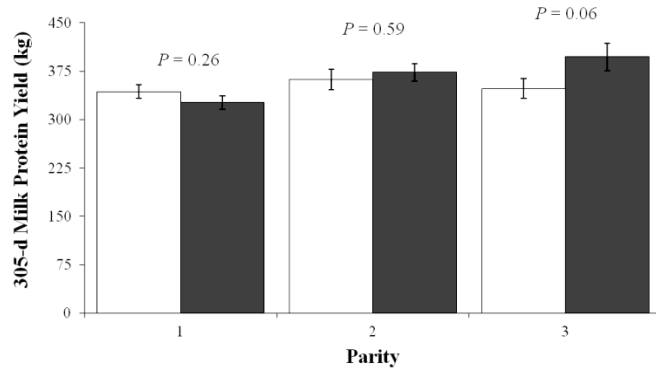
4A.



4B.



4C.



Whole lactation data for cows treated during the first week of lactation with sodium salicylate (SS ■) or control (CON □). X-axis labels are parity (1=1P, 2=2P, 3=3P). Values are 305-d estimates and are least square means \pm SEM. Panel A. There was a treatment \times parity interaction ($P < 0.01$) for 305-d milk yield, with 3P SS cows having higher milk yield ($P < 0.01$), but also a tendency ($P = 0.07$) for 1P CON cows to have higher milk yield. Panel B. A treatment \times parity effect ($P < 0.01$) was observed for 305-d milk fat yield. Panel C. 305-d milk protein yield had a tendency ($P = 0.08$) for a treatment \times parity effect with a tendency for 3P SS cows to have higher protein yield.

Table 2.5 Reasons cows left herd¹

Reason	1P²		2P³		3P⁴		Total
	CON⁵	SS⁶	CON	SS	CON	SS	
Foot/Leg	--	--	--	--	1	--	1
Low Production	--	1	1	--	--	--	2
Reproduction	1	3	1	2	1	1	9
Injury	--	--	--	--	--	1	1
Died	--	--	1	1	1	--	3
Mastitis	--	2	2	2	1	--	7
Udder	--	--	1	--	--	--	1
Total	1	6	6	5	4	2	24

¹All values are numerical counts based on parity and treatment for each specific reason cows were removed from the herd.

² 1P; First parity cows

³ 2P; 2nd lactation cows

⁴ 3P; 3rd or greater lactation cows

⁵ CON; control

⁶ SS; sodium salicylate treatment

Chapter 3 - Inflammatory pathways contribute to the metabolic adaptations to lactation in dairy cattle

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ABSTRACT

Inflammation has been proposed as a contributor to metabolic disorders in dairy cows during the transition from gestation to lactation. The purpose of this experiment was to determine if a non-steroidal anti-inflammatory drug, sodium salicylate (SS), alters metabolism of transition dairy cows. At calving, cows were alternately assigned, based on parity, to either control (CON) or SS treatment delivered in drinking water for 7 d, and metabolic responses were monitored for the first 21 d of lactation. Hepatic inflammation, as assessed by mRNA abundance of TNF α , was decreased by SS during treatment. Plasma glucose concentration and expression of the gluconeogenic gene glucose-6-phosphatase were decreased in SS treated cows, especially among cows in their 3rd lactation or greater. Although no effects on lipolysis or ketogenesis were detected during treatment, both non-esterified fatty acid and beta-hydroxybutyrate concentrations were elevated in SS-treated cows in the 2 weeks after treatments ended. Liver triglyceride concentration was increased during SS administration but by d 21 there were no differences between treatment groups. Because the most dramatic responses were observed in aged cows, plasma eicosanoids were analyzed for these cows. No effects of treatment on plasma eicosanoids were observed at the end of the treatment period; however, SS cows had elevated concentrations of several inflammatory eicosanoids, including thromboxane B₂, on d 14 of lactation. Post-treatment metabolic responses to SS therefore may have been due to altered metabolic programming or to post-treatment increases in inflammatory signals. In summary, SS suppressed liver inflammation but decreased plasma glucose, increased plasma ketones, and contributed to liver triglyceride accumulation. These findings suggest that interrupting inflammation during the first week after parturition disrupts the metabolic adaptations to lactation.

INTRODUCTION

Fatty liver (FL) is a common metabolic disease in dairy cows during the transition from gestation to lactation. Like non-alcoholic FL in humans, bovine FL is characterized by hepatocellular accumulation of triglycerides and other lipids. The etiologies of these conditions, however, are quite different. Human fatty liver disease is closely associated with obesity and typically develops over many years (Farrell and Larter, 2006). Bovine FL, on the other hand, commonly develops over the course of just 1 or 2 weeks during a period of dramatic weight loss (Drackley, 1999).

The transition from late pregnancy to lactation is a time of great physiological stress for the dairy cow. The decline in feed intake that accompanies parturition, coupled with the rapid increase in energy requirements during lactogenesis, generates a negative energy balance (NEB) (Grummer, 1993; Drackley, 1999; Bobe et al., 2004). Cows in NEB mobilize large quantities of fatty acids from adipose tissue stores, increasing plasma non-esterified fatty acid (NEFA) concentrations by as much as 10-fold within a few days after parturition (Herdt, 1988). Although some NEFA are used for mammary gland lipogenesis, a proportion is taken up by the liver. When uptake of fatty acids by the liver is greater than the ability of the liver to oxidize fatty acids, TGs accumulate in the liver and fatty liver develops. Compared to other species, the ruminant liver is inefficient at exporting triglycerides in VLDL (Kleppe et al., 1988). Nearly 90% of all metabolic diseases in dairy cattle occur during the first 4 wk of the 305-d lactation (Ingvarsen, 2006).

Bovine FL has often been seen as a natural, if unfortunate, consequence of the NEB that occurs in early lactation. However, several observations suggest that this may not be the case. Although lipolysis is certainly an expected response to NEB, early lactation cows had 7-fold greater increases in plasma NEFA concentration than mid-lactation cows subjected to a similar NEB (Gross et al., 2011), suggesting that the magnitude of lipolysis in early lactation may be unnecessary. Similarly, dramatic lipomobilization occurs during early lactation in numerous species, perhaps most obviously in aquatic mammals such as the Northern elephant seal, which can lose more than 50% of its adipose mass over a 4-week lactation (Costa et al., 1986; Houser et al., 2007) without experiencing obvious metabolic disease.

Despite their reliance on mobilized lipid as an energy source, dairy cattle entering lactation with greater adipose mass are at greatest risk of developing metabolic diseases

(Morrow, 1976). It has become clear in the past decade that animals with excessive adiposity exhibit a low-grade inflammation (Hotamisligil, 2006), suggesting that perhaps inflammation underlies the metabolic disturbances in obese dairy cows. In support of this hypothesis, cows with moderate or severe FL have increased levels of the inflammatory cytokine tumor necrosis factor- α (TNF α) (Ohtsuka et al., 2001). Inflammatory cytokines cause myriad metabolic changes in dairy cattle, including anorexia, lipomobilization, impaired insulin sensitivity, and reduced milk yield (Kushibiki et al., 2001a; Kushibiki et al., 2003; Bertoni et al., 2004), all of which are associated with FL. Furthermore, daily injection of TNF α for 7 d increased liver TG content independent of effects on feed intake, and this effect was accompanied by changes in hepatic gene expression consistent with both inflammation and a shift from fatty acid oxidation to TG synthesis (Bradford et al., 2009).

These recent findings suggest that exogenous inflammatory agents are sufficient to induce FL. It remains unclear, however, whether inflammation is a necessary causative factor in the natural progression of bovine FL. To test this broad question, use of a non-steroidal anti-inflammatory drug (NSAID) sodium salicylate (SS). Sodium salicylate is a weak inhibitor of cyclooxygenase (COX)-1 and COX-2 (Mitchell et al., 1993), and its probable mode of action is that it inhibits phosphorylation of the NF- κ B inhibitor I κ B- α (Yin et al., 1998). Phosphorylation of I κ B results in its degradation, allowing NF- κ B to be released for translocation into the nucleus and subsequent activation of an inflammatory transcription program (Baker et al., 2011).

The specific hypothesis for this study was that SS, by interrupting inflammatory signals in the liver during the first 7 d of lactation, would limit accumulation of TG, promote gluconeogenesis, and limit metabolic disease in transition dairy cows. In contrast, our findings suggest that inflammatory signals may be necessary for metabolic adaptations to lactation, especially increased gluconeogenesis necessary to support lactogenesis, and modulation of lipolysis and ketogenesis as animals return to positive energy balance.

METHODS AND MATERIALS

All experimental procedures were approved by the Kansas State University Institutional Animal Care and Use Committee #2880.

Design and Treatments

A total of 78 cows [n = 39 primiparous (1P), n = 24 2nd parity (2P), and n = 15 \geq 3rd parity (3P)] from the Kansas State University Dairy Teaching and Research Facility were randomly assigned to treatment at parturition. Treatment consisted of administration of sodium salicylate (SS; 1.95 g/L) via individual water bowls for 7 d postpartum with a molasses carrier (0.14 g/L). Control cows received the same molasses additive for 7 d (CON). On the 8th day postpartum all cows were given non-treated water for the remainder of the 21 days. Cows were housed in a tie-stall facility in randomly assigned stalls. Cattle were milked 3 times daily (0600, 1000, and 1800 h) and fed ad libitum twice daily (0630 and 1800 h).

Data and sample collection

Throughout the 21-d trial, feed and water intake and milk yield were recorded daily. Health checks were conducted by trained personnel once daily (0630 h), where urine ketone and rectal temperature were collected and recorded. Milk samples were collected at all 3 milkings, twice weekly, for analysis of milk components. Blood samples were collected from coccygeal vessels on days 1, 7, 14, and 21 post-partum and processed as described previously (Morey et al., 2011). Briefly, approximately 6 mL of blood was collected into a tube containing EDTA and another 6 mL was collected into a tube containing potassium oxalate with sodium fluoride as a glycolytic inhibitor (Vacutainer; Becton, Dickinson and Co., Franklin Lakes, NJ). Blood samples were centrifuged at $2,000 \times g$ for 10 min immediately after sample collection, plasma was harvested and frozen at -20°C until analysis. Liver samples were taken on days 4 and 21 as described previously (Morey et al., 2011). Briefly, liver samples were taken using a 14-gauge \times 15 cm biopsy needle (SABD-1415-15-T; US Biopsy, Franklin, IN) between the 10th and 11th ribs, 5 cm dorsal to a line between the olecranon and tuber coxae. The area was shaved, scrubbed, and a local anesthetic (lidocaine hydrochloride; Agri Laboratories Ltd., St. Joseph, MO) applied before making a small incision in the skin with a #10 blade (Feather Safety Razor Co. Ltd., Kita-Ku, Osaka, Japan). The biopsy needle was inserted cranioventrally toward the liver and ~200 mg of tissue was collected (a total of 10 biopsies per collection day). The liver sample was immediately frozen in liquid nitrogen and stored in a -80°C freezer until used for analysis. Body condition score (BCS, scale 1-5 with 1=emaciated and 5=severely obese) was evaluated by 3 trained personnel on d 1, 7, 14, and 21.

Plasma analyses

Metabolites. Non-esterified fatty acids were analyzed using an enzymatic colorimetric procedure (NEFA-HR; Wako Chemicals USA Inc., Richmond, VA), glucose by a colorimetric kit (kit #439-90901; Wako Chemicals USA Inc.), BHBA using an enzymatic reaction (kit #H7587-58; Pointe Scientific Inc., Canton, MI), and insulin by a bovine-specific sandwich ELISA (#10-1201-01; Mercodia AB, Uppsala, Sweden). Plasma fibroblast growth factor-21 (FGF-21) was evaluated on d 7 with a bovine FGF-21 ELISA kit (#CSB-EL008627BO; Cusabio Biotech Co., Ltd., Wuhan, China). Absorbance was read on a spectrophotometer (PowerWave XS; BioTek Instruments Inc., Winooski, VT) and calculations were performed using Gen5 software (BioTeck Instruments Inc.).

Eicosanoid panel. Eicosanoid concentrations were determined in the lipid fraction of plasma samples using liquid chromatography and mass spectrometry (LC-MS). Plasma samples (500 μ L) from 3P cows were mixed with 500 μ L of HPLC grade water, 100 μ L methanol, 4 μ L antioxidant/reducing agent containing ethylenediaminetetraacetic acid (EDTA), butylhydroxy toluene (BHT), triphenylphosphine (TPP), and indomethacin (4 μ L/mL), 100 μ L of a mixture of internal standards, and 1 μ L of formic acid. The internal standards mixture contained the following deuterated eicosanoids (0.1 ng/ μ L, 10 ng total): LTB₄-d₄, TxB₂-d₄, PGF_{2 α} -d₄, PGE₂-d₄, PGD₂-d₄, 13(S)-HODE-d₄, 6-keto PGF_{1 α} -d₄, 9(S)-HODE-d₄, LTD₄-d₅, 12(S)-HETE-d₈, 15(S)-HETE-d₈. Sample mixtures were centrifuged at 3200 $\times g$ for 10 min at 4°C. Lipids were isolated from the sample supernatant by solid phase extraction using a Phenomenex Strata-X 33 μ m Polymeric Reversed Phase 60 mg/3 mL columns, catalog # 8B-S100-UBJ SPE (Phenomenex, Torrance, CA). Columns were first conditioned with 3 mL methanol (MeOH) then 3 mL water. The samples were passed through the columns; 3 mL of 40% MeOH was passed through afterward as the wash. After a 4 min vacuum drying step, samples were eluted in 2 mL MeOH/acetonitrile (50:50 v/v), dried in a Savant SVD121P SpeedVac (Thermo Scientific, Waltham, MA), and resuspended in 100 μ L acetonitrile/water/formic acid (37:63:0.02 v/v/v).

Eicosanoids were analyzed using two distinct LC-MS methods. Both utilized reverse-phase LC on a Waters ACQUITY UPLC® BEH C18 1.7 μ m column (2.1 \times 100mm) at a flow rate of 0.6 mL/min at 35°C and a quadrupole mass spectrometer (Waters ACQUITY SQD H-Class) in electrospray negative ionization mode. The electrospray voltage was -3 kV and the turbo ion spray source temperature was 450°C. Nitrogen was used as the drying gas. For each method, 10

μL samples were injected in triplicate. An isocratic mobile phase consisting of acetonitrile:water:0.1% formic acid (45:55:10; v/v/v) with an analysis time of 15 min was used to analyze LTB₄, PGE₂, PGD₂, 5(S),6(R)-Lipoxin A₄, PGF_{2α}, TxB₂, 6-keto PGF_{1α}, resolvin D₁, and resolvin D₂. The second method utilized an isocratic mobile phase of acetonitrile:methanol:water:0.1% formic acid (47.4:15.8:26.8:10; v/v/v/v) and an analysis time of 10 min to analyze 9(S)-HODE, 13(S)-HODE, 15-deoxy-^Δ_{12,14}-PGJ₂, 15-oxoETE, 5-oxoETE, 5(S)-HETE, 12(S)-HETE, 15(S)-HETE, 7(S)-maresin 1, 7(S),17(S)-dihydroxy-8(E),10(Z),13(Z),15(E), 19(Z)-docosapentaenoic acid, and LTD₄. Eicosanoids were identified in samples by matching their deprotonated (i.e., [M-H]⁻) m/z values and LC retention times with those of a pure standard. Extraction and ionization efficiencies were measured for the internal standards by comparing the intensity of the samples with the ion intensity of the extraction controls. The efficiency range was between 18% to 84%.

Salicylate Analyses. Plasma salicylate concentrations were determined with liquid chromatography (Shimadzu Prominence, Shimadzu Scientific Instruments) and triple quadrupole mass spectrometry (API 2000, Applied Biosystems). The ions monitored were: salicylate (m/z 136.91→93.00) and the internal standard acetaminophen (m/z 152.17→110.0). The mobile phase consisted of A: acetonitrile and B: 0.1% formic acid. The mobile phase gradient was 75% B from 0 - 0.5 min, 75% B to 50% B from 0.5 - 4.0 min, and 50% B to 75% B from 4.0 - 5.5 min with a total run time of 6.5 min. A C8 column (Supelco Discovery, 2.1×150 mm, 5 μM, Sigma-Aldrich, St. Louis, MO, USA) achieved separation. Sample processing consisted of adding 0.1 mL plasma to 0.4 mL methanol with 0.1% formic acid containing 1 μg/mL acetaminophen. The samples were vortexed, centrifuged for 5 min at 15,000 ×g, the supernatant transferred to an injection vial and 0.01 mL was the injection volume. The accuracy of the assay was 100, 96, 104, 103, 105 and 97% at 1, 5, 10, 25, 50, and 100 μg/mL on replicates of 3 for each concentration, respectively. The coefficients of variation were 5, 9, 2, 10, 1, and 12% at 1, 5, 10, 25, 50, and 100 μg/mL on replicates of 3 for each concentration, respectively.

Liver Analyses

Triglyceride. Approximately 20 mg of liver was placed in 500 μL of chilled PBS and homogenized, centrifuged at 2,000 × g for 10 min at 4°C, and 100 μL of the supernatant was then removed for free glycerol and total protein analysis. Triglyceride content was measured as

described previously (Morey et al., 2011). Briefly, some liver homogenate was incubated with 100 μ L of lipase (porcine pancreatic lipase, MP Biomedicals, Solon, OH) and glycerol content was then determined by an enzymatic glycerol phosphate oxidase method (#F6428 Sigma-Aldrich Co., St. Louis, MO). Triglyceride amount was calculated by the difference between glycerol concentrations before and after lipase digestion. Total protein content of original homogenate was analyzed by a Coomassie blue (Bradford, 1976) colorimetric method (kit #23236; Thermo Scientific, Pierce, Rockford, IL). Variation in moisture content of liver samples was accounted for by normalizing with protein concentration of the sample (Fronk et al., 1980).

Gene expression. Transcript abundance of genes of interest, involved in glucose and lipid metabolism and inflammation, were determined by real-time PCR. Total RNA was isolated from liver samples using a commercial kit (RNeasy Lipid Tissue Mini Kit; Qiagen Inc., Valencia, CA) and spectroscopy was used to quantify RNA (Nanodrop 1000; NanoDrop Technologies Inc., Wilmington, DE). Complementary DNA was synthesized from 2 μ g of total RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed in duplicate utilizing synthesized cDNA in the presence of 200 nmol/L gene-specific forward and reverse primers with SYBR green fluorescent detection (7500 Fast Real-Time PCR System, Applied Biosystems). Control genes β -actin, GAPDH, and RPS9 were used in a geometric means model to determine the respective abundance differences in genes. All genes, primers, and amplification region are identified in Table 1.

Western blot. Protein abundance of c-Jun and Ikk-B and their respective phosphorylated forms were determined by Western blot essentially as described (Titgemeyer et al., 2011). Antibodies used were I κ B- α (catalog #sc-847, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), phosphor-I κ B- α (catalog #sc-8404, Santa Cruz Biotechnology), c-Jun (catalog #sc-166540, Santa Cruz Biotechnology), and phosphorylated c-JUN (catalog #sc-166540, Santa Cruz Biotechnology). Briefly, ~20 mg of liver tissue was homogenized at 4°C in RIPA lysis buffer with PhosphoStop (Santa Cruz Biotechnology) containing a broad-spectrum protease inhibitor cocktail (Protease Inhibitor Cocktail I; Calbiochem, Gibbstown, NJ). The homogenate was centrifuged and the supernatant was measured by the Bradford method (1976). Forty micrograms of total protein was diluted in Laemmli sample buffer, heated at 90°C for 5 min, cooled, vortexed, separated by SDS-PAGE on a 4 to 12% Tris-HCl gel, and dry-transferred onto

nitrocellulose membranes (iBlot; Invitrogen, Carlsbad, CA). Membranes were blocked in Tris buffer with 5% dry milk powder for 2 h at room temperature with antibodies (1:500) of interest from Santa Cruz Biotechnology. After washing, a secondary antibody (anti-rabbit sc2313 1:10,000; Santa Cruz Biotechnology) was incubated for 1 h at room temperature.

Immunodetection was performed by chemiluminescence (West-Dura; Thermo Scientific, Waltham, MA) and bands quantified by scanning densitometry (ChemiDoc-It Imaging System; UVP Inc., Upland, CA). ImageJ software from NIH website was used to calculate densitometry bands.

Nuclear extract of NFkB. Nuclear extracts were collected from bovine liver samples according to manufacture guidelines (kit #SK-0001, Signosis Inc., Sunnyvale, CA). Briefly, 20 mg of liver was homogenized using lysis buffer, centrifuged, and the pellet was reconstituted with a second lysis buffer, centrifuged, and supernatant collected. This supernatant was the nuclear extract. Total protein was measured using the Bradford method (1976) and a total of 0.7 mg of nuclear extract was used in the collection plate for evaluation of NFkB content. Extract was stored at -20°C until used in a NFkB filter plate assay (#FA-0001; Signosis). The extract is incubated with a NFkB probe for 30 min at 18°C then washed through a filter plate in which NFkB-bound probes are retained. Bound NFkB was eluted, denatured, and hybridized to a hybridization plate overnight at 45°C. The captured DNA was then detected with streptavidin-HRP and measured using luminescence on a microplate luminometer.

Statistical analysis

Statistical analyses were carried out using the Mixed Procedure of SAS (version 9.2; SAS institute Inc., Cary, NC) to assess the fixed effects of parity, time, treatment, and all 2- and 3-way interactions; cow was included as a random effect. Repeated measures over time were modeled with autoregressive covariance structures, and denominator degrees of freedom were estimated using the Kenward-Rogers method. Values were deemed outliers and omitted from analysis when Studentized residuals were $>|3.5|$. Interactions were investigated when $P < 0.15$ using the slice option, and slices declared significant at $P < 0.05$.

RESULTS AND DISCUSSION

Sodium salicylate inhibits inflammatory cascades in the liver

Salicylate has a half-life of approximately 30 min in cattle (Gingerich et al., 1975). Therefore, to deliver a therapeutic dose and to maintain relatively consistent plasma concentrations throughout the d, SS was delivered in drinking water. A preliminary study indicated that SS decreased water consumption by cows, but this problem was avoided when the flavor of SS was masked with 6 ppm molasses. Based on analysis of water bowl samples and measured individual water intakes, total daily intake of salicylate was 123.3 ± 5.5 g/cow, and plasma salicylate concentrations on d 7 were 34.4 ± 15.0 μ g/mL, slightly above the therapeutic target of 30 μ g/mL (Gingerich et al., 1975; Anderson et al., 1979; Coetzee et al., 2007). Plasma SS concentration did not differ by parity ($P > 0.50$). There was no effect of treatment on daily water intake during the 7-d treatment period ($P = 0.81$; 72.4 vs. 73.2 ± 3.5 L/d for CON and SS, respectively), suggesting that the inclusion of molasses in water for both treatments prevented any aversion to consumption of SS in drinking water.

To assess whether SS treatment effectively inhibited inflammatory signaling in the liver, the relative phosphorylation of I κ B- α was determined. Western blot analyses were evaluated on the liver samples from 3P cows for measurement proteins of I κ B- α (total and phosphorylated) and c-Jun and densitromitry was done to quantify protein expression. Total I κ B- α tended ($P = 0.07$) to be lower for SS cows on d 4 but were significantly higher ($P = 0.04$) on d 21. Total phosphorylated I κ B- α was not different between treatment groups, however, when analyzing the percentage of I κ B- α that was phosphorylated, CON cows had a higher percentage ($P = 0.04$) phosphorylated on d 21. Phosphorylated c-JUN was not impacted by treatment ($P = 0.71$) or day ($P = 0.26$).

Inflammation was decreased (28%) in SS treated cows as evidenced by a decrease in tumor necrosis factor- α (TNF α) expression in the liver on d 4, then on d 21 had equivalent expression of this gene (Figure 3.1A). However, no effect ($P = 0.30$) on nuclear NF κ B abundance was observed between treatment groups.

Removal of salicylate results in elevated inflammatory eicosanoids in plasma

Eicosanoids such as prostaglandins, prostacyclins, leukotrienes, lipoxines, and thromboxanes have been identified as key regulators of both acute and chronic inflammatory reactions (Sordillo et al., 2009). Fatty acids can be used as precursors for inflammatory eicosanoids and due to the increase in NEFAs observed in early lactation may impact the

duration and magnitude of inflammation (Sordillo et al., 2009). Since a majority of the production responses were observed in 3P cows (Chapter 2), the plasma samples for these cows were analyzed for 15 different eicosanoids. Significant treatment \times day interactions ($P < 0.05$) for eicosanoids show a significant increase on d 14 (Figure 3.2 panels A-D). This trend was similar when looking at a pro-inflammatory index comprised of 11 pro-inflammatory eicosanoids (Figure 3.2E).

Sodium salicylate promotes liver TG accumulation and causes hypoglycemia in aged cows

Previously it was reported that low-grade inflammation induced by 7-d administration of the inflammatory cytokine TNF α promoted liver triglyceride (TG) accumulation (Bradford et al., 2009), which led us to hypothesize that blocking endogenous inflammation during the transition period would decrease liver TG content. In contrast to our hypothesis, liver triglyceride (TG) concentrations were increased 29% by SS on d 4 of lactation ($P < 0.01$), although by d 21 no differences were observed ($P = 0.47$, Figure 3.3E). Glucose metabolism was likewise altered, as evidenced by decreased ($P < 0.05$) plasma glucose concentration on d 7 (Fig. 3.3A).

Interestingly, SS induced hypoglycemia on d 7 in 2P (45.4 vs. 53.5 ± 2.4 mg/dL, $P = 0.05$) and 3P (36.0 vs. 50.2 ± 3.5 mg/dL, $P < 0.01$) cows, but not in 1P (55.5 vs. 53.5 ± 2.1 mg/dL for SS vs. CON, $P = 0.50$). Similar to our results, dairy cows injected with lysine acetyl-salicylate for the first 5 d of lactation had decreased plasma glucose on d 3 of lactation (Bertoni et al., 2004).

Transcript abundance for key rate-determining enzymes in hepatic fatty acid oxidation and gluconeogenesis was measured to assess potential mechanisms underlying these responses. Carnitine palmitoyl transferase 1A, which catalyzes the rate-determining step in mitochondrial fatty acid oxidation, was not altered by treatment ($P = 0.60$, Fig. 3.3C). Although SS decreased hepatic expression of the gluconeogenic gene glucose-6-phosphatase (G6P) by 49% on d 4 ($P = 0.03$, Figure 3.3C), it also tended to increase abundance of cytosolic phosphoenolpyruvate carboxykinase (PCK1) by 81% ($P = 0.10$) and had no effect on pyruvate carboxylase (Figure 3.3C). Targeted analysis of hepatic transcripts failed to identify a clear shift in transcriptional programs underlying the increased liver TG content and hypoglycemia.

Milk lactose synthesis requires as much as 70% of total glucose supply in lactating dairy cattle (Reynolds et al., 1988). Milk lactose yield was greater in multiparous than primiparous cows (Chapter 2), but treatment did not influence lactose yield in any parity group ($P > 0.30$).

Therefore, although greater glucose demands for lactose synthesis may have made multiparous cows more susceptible to hypoglycemia, this does not explain the treatment effects on plasma glucose concentration. Likewise, feed intake was not affected during treatment (Chapter 2), indicating that altered supply of gluconeogenic precursors is an unlikely explanation for the effect on plasma glucose.

A key adaptation to lactation in the ruminant animal is insulin resistance, leading to decreased utilization of glucose by muscle and adipose tissue (Olefsky and Glass, 2010), thus sparing glucose for use by the mammary gland (Bell and Bauman, 1997). Additionally, a depression in liver mRNA expression of the insulin receptor in ketotic cows and those diagnosed with fatty liver, has been observed which might further induce insulin resistance (Liu et al., 2010). The ability of inflammatory mediators to induce insulin resistance is now well established, and SS increases glucose uptake in cultured adipocytes (Ostman, 1965). Studies in mice (Nixon et al., 2012) and humans (Goldfine et al., 2008) have suggested that salicylate can improve insulin sensitivity, resulting in increased glucose utilization rates and decreased fasting glucose concentrations. Therefore, SS may have interrupted the homeorhetic induction of peripheral insulin resistance early in lactation. Plasma insulin concentrations tended to be decreased on d 7 in SS treated cows (Figure 3.3B), consistent with the observed hypoglycemia.

Fibroblast growth factor 21 (FGF-21) is an endocrine factor produced primarily in the liver which is critical to liver and adipose tissue adaptations to negative energy balance; not surprisingly, FGF-21 production is dramatically enhanced during the transition to lactation (Schoenberg et al., 2011). Analysis of hepatic FGF-21 gene expression demonstrated a treatment by time interaction ($P = 0.05$), with SS-treated cows showing elevated FGF-21 transcript abundance on d 4 of lactation and decreased abundance on d 21 (Fig. 3.3D). Plasma FGF-21 had an interesting treatment \times parity interaction on d 7 ($P = 0.11$). Plasma FGF21 was higher in 3P cows ($P < 0.01$; 53.94, 5.87, 5.37 ± 13.52 pg/mL). Additionally, 3P SS cows had greater plasma concentration of FGF21 compared to 3P CON ($P = 0.04$; 198.33 vs. 14.67 ± 116.69 pg/mL). In adipose tissue, FGF-21 induces expression of the GLUT-1 glucose transporter (Ge et al., 2011) and promotes systemic glucose uptake (Xu et al., 2009). The altered temporal pattern of liver FGF-21 expression with SS may have contributed to the hypoglycemia on d 7.

Sodium salicylate causes a delayed elevation in plasma BHBA and NEFA concentrations

Beta hydroxybutyric acid (BHBA) is a ketone produced after fatty acid oxidation and can be used as a source of energy by animals in energy deficit. Although BHBA is a key energy substrate in early lactation, sustained or excessive elevations in BHBA concentrations are associated with poor health and production outcomes in dairy cattle (Bobe et al., 2004). Surprisingly, although SS did not alter plasma BHBA concentration during treatment (d 7), it increased BHBA levels on d 14 and 21 of lactation, well after treatments ended on d 7 (Figure 3.4A). Non-esterified fatty acid concentrations were increased by SS, but again, this response was observed only on d 21, 2 wk after treatment ended (Figure 3.4B). As expected for cows in negative energy balance, body condition score decreased through the 3 wk trial ($P < 0.01$). Consistent with treatment effects on measures of lipolysis and ketogenesis, SS increased the rate of BCS loss in wk 2 of lactation ($P = 0.003$, Fig. 3.4C).

CONCLUSION

Inflammation plays critical roles in the early lactation dairy cow. An alteration of normal inflammation by use of the NSAID sodium salicylate leads to significant changes in lipid and glucose metabolism. In general, SS administered during the first week of lactation leads to decreased plasma glucose, and increased plasma BHBA and NEFA. The changes in glucose metabolism were not accompanied by consistent alterations in gluconeogenic gene expression. Potentially, there is an increase in glucose utilization by other organs, such as muscle and adipose tissue. This theory stems from work by Kim and others (2001) where in male mice subjected to hyperinsulinemic-euglycemic clamp, salicylate overcame lipid-mediated insulin resistance and maintained similar glucose uptake as control mice (Kim et al., 2001).

More research needs to be completed to understand how SS impacts multiple tissues. Such research may better clarify how inflammatory signals contribute to the homeorhetic adaptations necessary to sustain lactation.

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Table 3.1 Primers used for quantitative real-time PCR detection of transcripts in liver tissue

Gene ¹	Accession #	Forward Primer (5' – 3') Reverse Primer (5' – 3')
B-actin	NM_173979.3	ACGACATGGAGAAGATCTGG ATCTGGGTCATCTTCTCACG
GAPDH	NW_0031039231.1	TCAACGGGAAGCTCACTGG CCCCAGCATCGAAGGTAGA
RPS9*	DT860044.1	GAACAAACGTGAGGTCTGGAGG ATTACCTTCGAACAGACGCCG
TNF α *	NM_173966.1	AAGTAACAAGCCGGTAGCCCA CTTCCAGCTTCACACCGTTG
FGF21	NW_003104466.1	GCCAGGCGTCATTGAGATCT GAAAGCTGCAGGCTTTGGG
G6PC	NM_001076124.1	TGAGGATGGAGAAGGGAATG AACCAAATGGGGAAGAGGAC
cPEPCK*	NM_174737.2	CGAGAGCAAAGAGATACGGTGC TGACATACATGGTGCGACCCT
CPT1a*	DV820520.1	CTTCCCATTCCGCACTTTC CCATGTCCTTGTAATGAGCCA
PC*	NM_177946.2	CTTCAAGGACTTCACTGCCACC GCCAAGGCTTTGATGTGCA

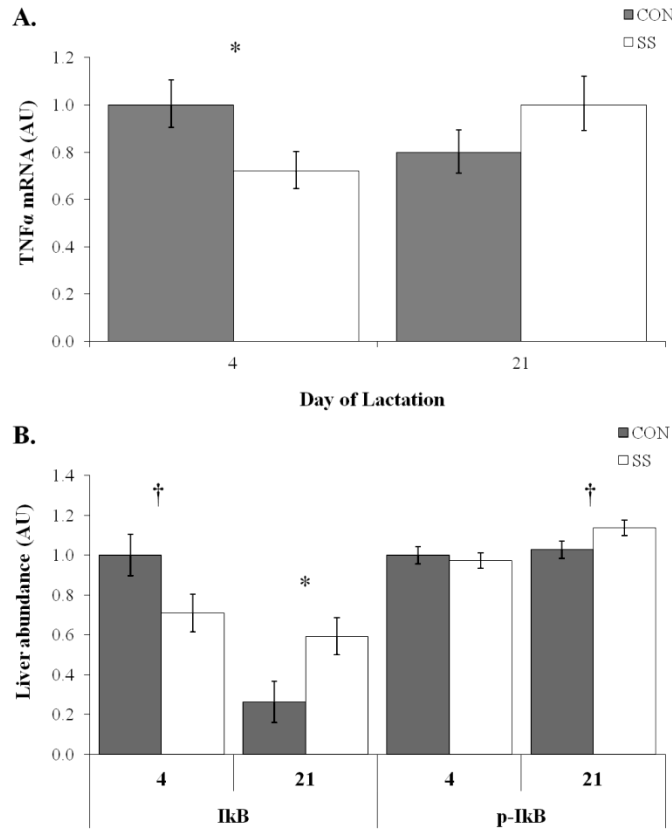
¹*GAPDH* = glyceraldehydes 3-phosphate dehydrogenase; *RPS9* = ribosomal protein subunit 9; *TNF α* = tumor necrosis factor- α ; *FGF21* = fibroblast growth factor-21; *G6PC* = catalytic subunit of glucose-6-phosphatase; *cPEPCK* = cytosolic phosphoenolpyruvate carboxykinase; *CPT1a* = mitochondrial carnitine palmitoyltransferase 1A; *PC* = pyruvate carboxylase;

²From NCBI Entrez Nucleotide Database

(<http://www.ncbi.nlm.gov/sites/entrez?db=nucleotide>).

* Amplicons span an exon-exon boundary, as predicted by aligning the specific sequence to the bovine genome using Splign (<http://www.ncbi.nlm.nih.gov/sutils/splign>).

Figure 3.1 Inflammation is suppressed while sodium salicylate is administered

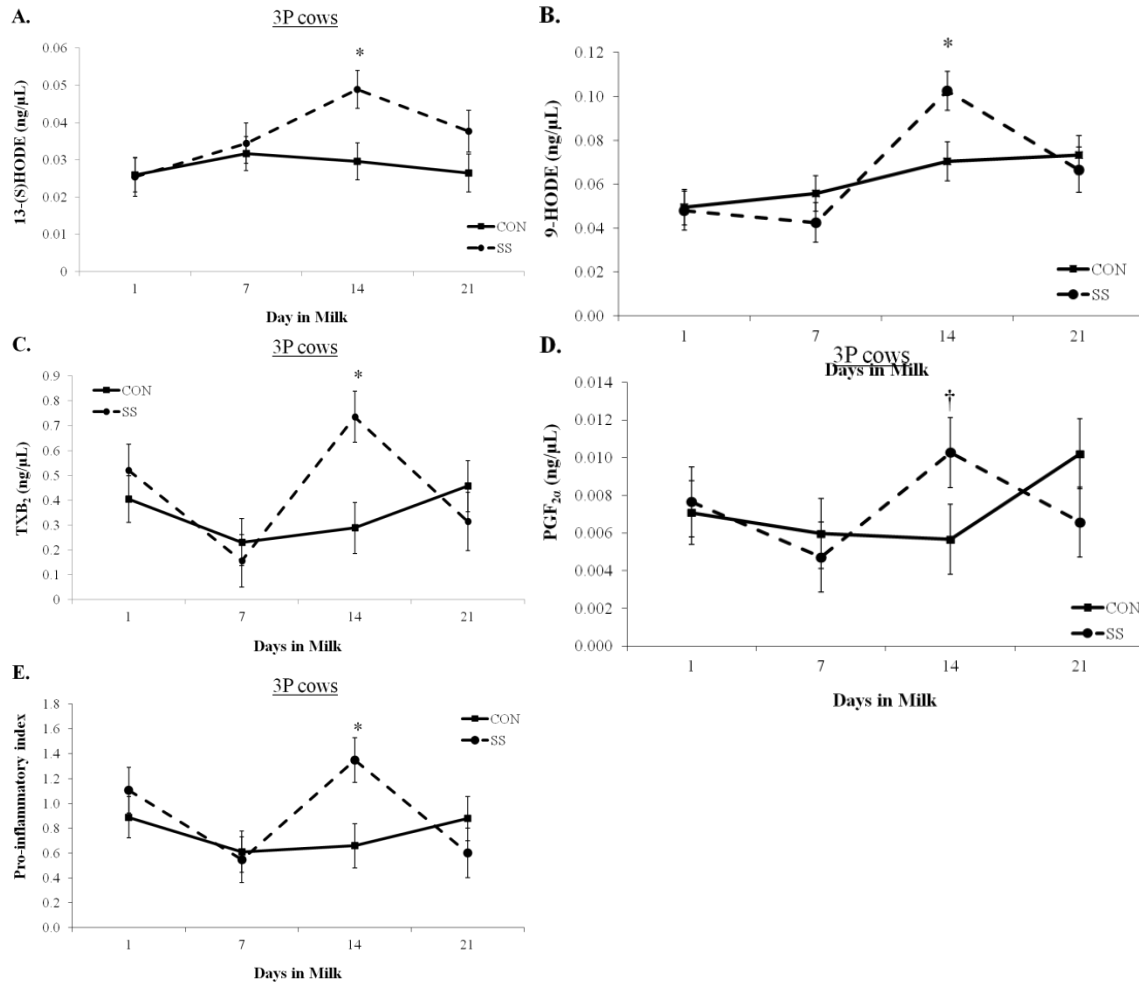


Liver biopsies were collected on days 4 and 21 of lactation from control (CON■) and sodium salicylate (SS□). Panel A. qRT-PCR was completed for the inflammatory cytokine tumor necrosis factor- α (TNF α). Values are arbitrary units with standard error of means. mRNA abundance of tumor necrosis factor- α (TNF α) was measured in the liver on d 4 and 21. There was no overall treatment effect on TNF α ($P > 0.50$) but there was a significant ($P < 0.01$) treatment \times day interaction where TNF α was significantly lower on d 4, but then returned to similar levels by d 21. Panel B. There was no treatment effect ($P = 0.70$) for liver I κ B, with a decrease on d 21 ($P = 0.01$). A significant treatment \times day interaction ($P = 0.05$) was observed where SS tended to decrease total I κ B on d 4 ($P = 0.07$) and on d 21 increased ($P = 0.04$). Phosphorylated I κ B (p-I κ B) had no treatment ($P = 0.30$) effect yet tended to increase on d 21 ($P = 0.07$). The treatment \times day interaction ($P = 0.17$) observed a tendency ($P = 0.08$) for SS cows to have higher p-I κ B on d 21.

* P - value < 0.05

† $0.05 < P$ - value < 0.10

Figure 3.2 Removal of sodium salicylate increases pro-inflammatory eicosanoids in plasma

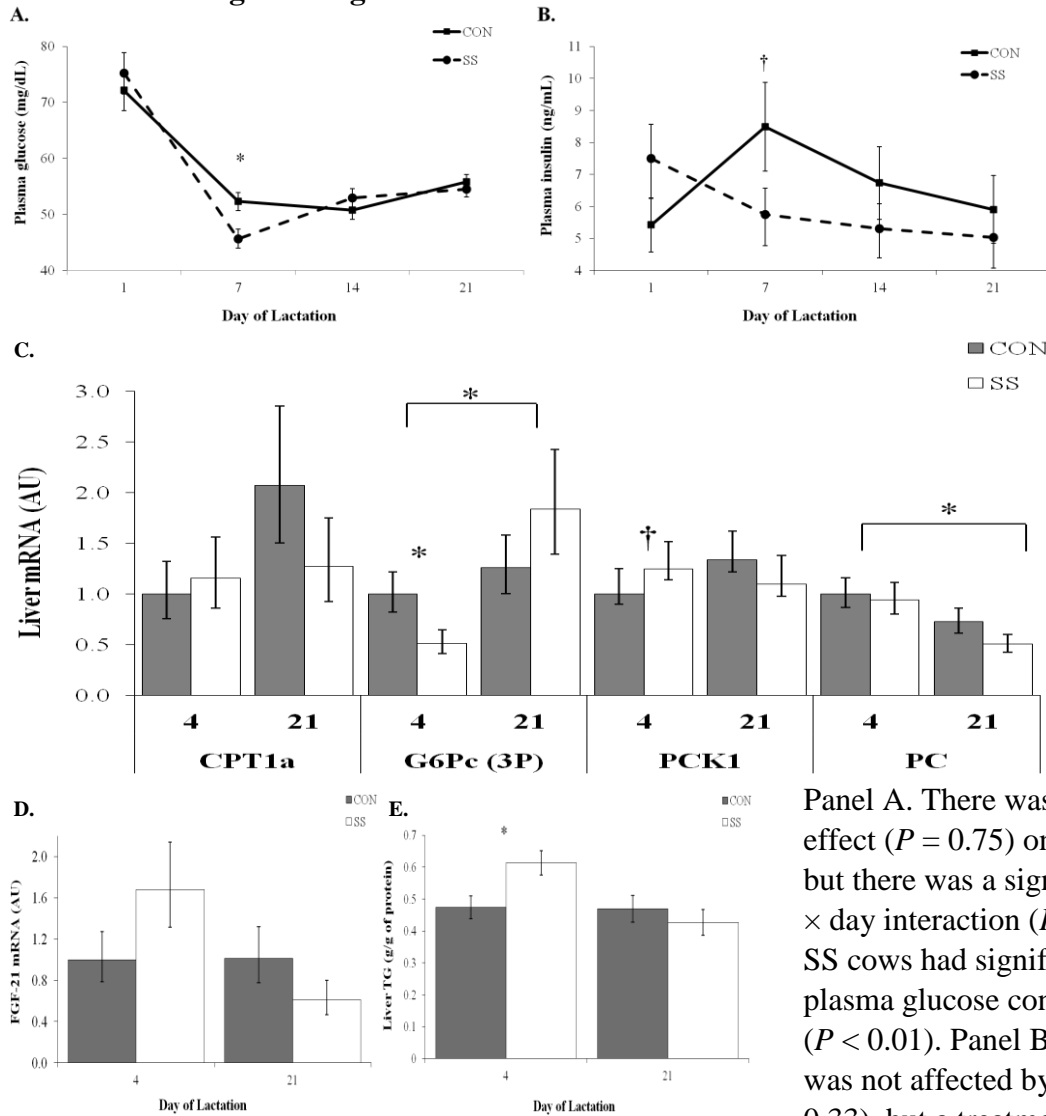


Plasma samples were collected on days 1, 7, 14, and 21 of lactation and the 3rd parity or greater cows were analyzed by a panel of 15 eicosanoids using a Solid Phase Lipid extractions and liquid chromatography–mass spectrometry. The panel consisted of both pro-inflammatory and resolving eicosanoids. There was no treatment effect ($P > 0.15$) observed for any of the eicosanoids evaluated. Eicosanoids with a treatment \times day ($P < 0.05$) interactions are shown (Panels A-D). Control (CON) cows are identified with the solid (■) and sodium salicylate (SS) cows are identified by the black dashed (●) line. Hydroxyoctadecadienoic acid [9(S)-HODE, Panel A; 13(S)-HODE, Panel B], thromboxane B₂ (TXB₂, Panel C), and prostaglandin F_{2α} (PGF_{2α}, Panel D) are shown. Panel E. An index comprised of 11 pro-inflammatory eicosanoid index showed an elevation in pro-inflammatory eicosanoids after SS was removed.

* P -value < 0.05

† $0.05 < P$ -value < 0.10

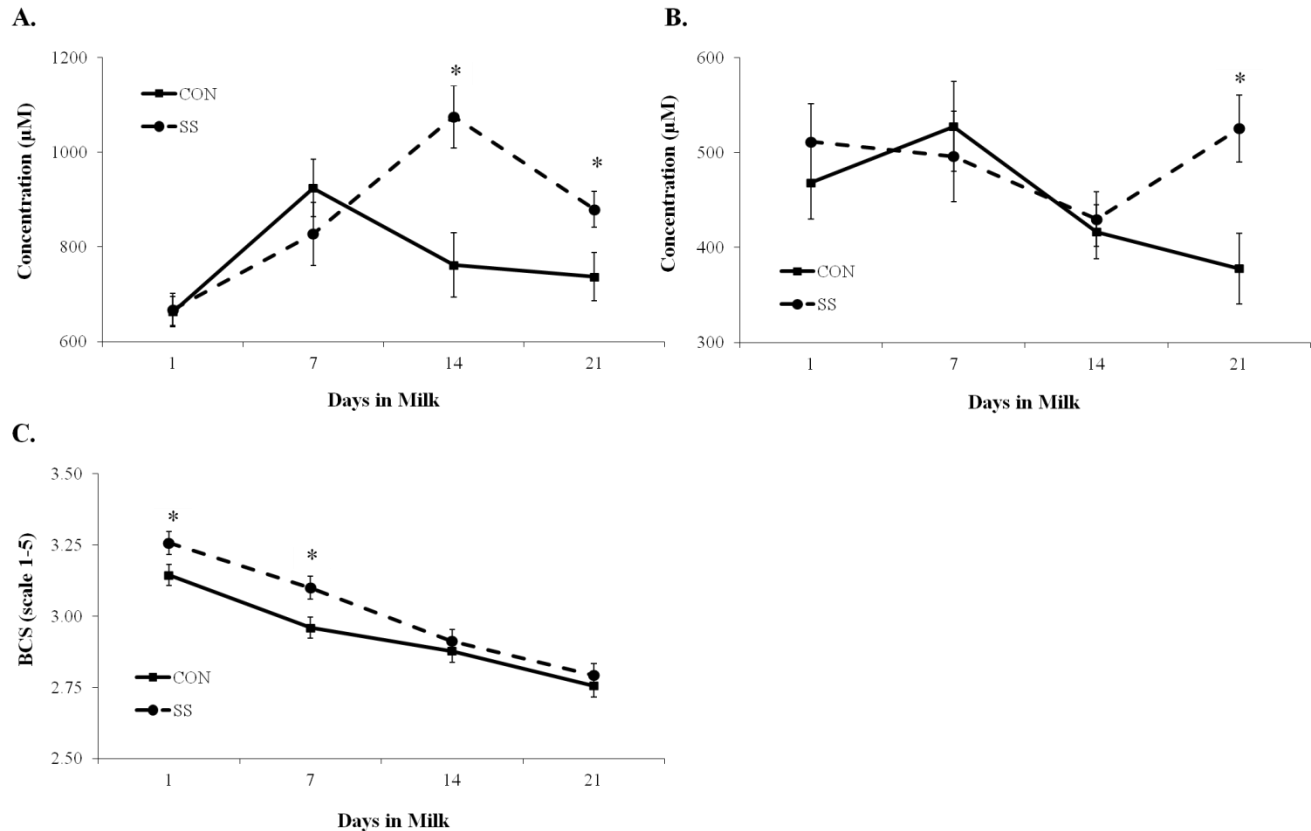
Figure 3.3 Glucose metabolism is altered by SS administration, but not through an alteration in gluconeogenesis



Panel A. There was no treatment effect ($P = 0.75$) on plasma glucose, but there was a significant treatment \times day interaction ($P < 0.01$) where SS cows had significantly lower plasma glucose concentrations on d 7 ($P < 0.01$). Panel B. Plasma insulin was not affected by treatment ($P = 0.33$), but a treatment \times day

interaction ($P = 0.09$) was observed where SS depressed insulin on d 7 ($P = 0.07$). Panel C. mRNA abundance for genes associated with gluconeogenesis and fatty acid metabolism. The four genes of interest were carnitine palmitoyl transferase 1A (CPT1a), catalytic subunit of glucose-6-phosphatase (G6P), cytosolic phosphoenolpyruvate carboxykinase (PCK1), and pyruvate carboxylase (PC). No treatment effect ($P > 0.23$) was observed for genes shown. A treatment \times parity \times day interaction ($P = 0.11$) for G6P where 3P SS cows had lower abundance d 4 ($P = 0.03$) with no differences observed on d 21 ($P > 0.50$). There was a treatment \times day ($P = 0.03$) interaction for PCK1 where on d 4 SS cows had higher abundance ($P = 0.10$). Panel D. Fibroblast growth factor-21 (FGF-21) mRNA abundance was not affected by treatment ($P = 0.98$), there was a treatment \times day interaction ($P = 0.05$) where on d 4 SS tended ($P = 0.13$) to increase liver FGF-21 expression, but on d 21 there was no difference ($P = 0.18$). Panel E. There was no treatment effect ($P = 0.24$) for TG concentration, but there was a significant treatment \times day interaction ($P = 0.02$) where on d 4, SS cows had a higher concentration of fat in their liver, with no difference on d 21.

Figure 3.4 Sodium salicylate causes a delayed elevation of plasma β -hydroxybutyric acid and non-esterified fatty acids



Panels A. Plasma ketones in the form of BHBA showed a tendency ($P = 0.07$) for plasma BHBA to be elevated in SS treated cows and there was a significant treatment \times day interaction ($P = 0.002$) where SS cows had significantly higher BHBA on d 14 and 21. Panel B. Fatty acid mobilization was quantified using NEFA. There was no treatment effect ($P = 0.23$) no plasma NEFA; however a significant treatment \times day interaction was observed ($P = 0.05$; where SS cows had higher NEFAs on d 21. Panel C. There was not treatment effect ($P > 0.50$) on body condition score, but there was a significant treatment \times day interaction ($P = 0.04$) where on days 1 and 7, SS cow had a higher BCS. Sodium salicylate increased the rate of BCS loss in week 2 of lactation ($P < 0.01$) which corresponds with the greater magnitude of negative energy balance observed in weeks 2 (between d 7 and d 14) and 3 (between d 14 and d21) with SS administration ($P = 0.08$ and $P = 0.02$, respectively).

* P -value < 0.05 .

Chapter 4 - Technical note: TNF α ELISA for bovine plasma

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Technical Note: Validation of an ELISA for measurement of tumor necrosis factor alpha in bovine plasma – Published in Journal of Dairy Science 2011 94:3504-3509.

ABSTRACT

Tumor necrosis factor alpha (TNF α) is an inflammatory cytokine that is involved in immune function and is proposed to play a role in metabolic disorders. While some bovine-specific methods have been published recently, assays used for determining plasma TNF α concentration in bovine disease models often do not offer acceptable precision for measurement of basal concentrations in healthy animals. The objective of this work was to develop an effective, low-cost sandwich enzyme-linked immunosorbent assay (ELISA) procedure with improved sensitivity. A protocol developed for use with cell culture supernatant was modified for use with bovine plasma and serum by optimizing antibody concentrations, incubation times and temperatures, and standard diluents. The coating antibody concentration was decreased from 10 $\mu\text{g/mL}$ to 6.8 $\mu\text{g/mL}$, while the detection antibody concentration remained 2.5 $\mu\text{g/mL}$. Sample incubation was increased from 1 h at room temperature to an overnight incubation at 4°C, which increased the sensitivity of the assay. Multiple matrices were tested for dilution of standards and were assessed by determining recovery of bovine TNF α spiked into bovine serum and plasma. Recoveries were acceptable in both bovine serum and plasma (71-103%) when quantified with standards diluted in human serum or phosphate-buffered saline. The modified bovine TNF α ELISA offers a detection range of 2 to 250 pg/mL. This detection limit is at least an order of magnitude lower than previously reported, and will allow for greater precision in determining basal TNF α concentrations in bovine plasma. The improved sensitivity of this ELISA will be critical to assessing current hypotheses concerning the metabolic effects of moderately elevated TNF α concentrations.

MANUSCRIPT

In recent years, the role of cytokines in immunity, inflammation, and metabolism have attracted increasing attention in the animal sciences. Tumor necrosis factor alpha (TNF α), in particular, is of interest because it represents one potential mediator linking infectious and metabolic diseases. Infectious diseases such as mastitis and metritis (Sordillo et al., 2009) can cause systemic increases in TNF α , which may in turn promote the development of fatty liver and associated metabolic problems (Bradford et al., 2009).

Research on the role of TNF α in bovine physiology has been slowed by a lack of commercially-available analytical reagents. Although human and bovine forms of TNF α share ~80% sequence homology, numerous anti-human TNF α antibodies failed to detect bovine TNF α (Dernfalk et al., 2004). Kenison and colleagues (1990) generated anti-bovine TNF α serum for use in a competitive radioimmunoassay, and this assay has subsequently been used in a number of key studies (Daniel et al., 2003b; Elsasser et al., 2005). However, broad use of this approach is difficult because the reagents are not commercially available and because it requires radioimmunoassay capabilities, including facilities for protein iodination.

Bovine-specific reagents for measurement of TNF α have become commercially available (bTNF α ELISA; Bovine TNF α Screening Set, Thermo Fisher Scientific Inc., Rockford, IL); however, the protocols published are for cell culture supernatants, not bovine serum or plasma. Additionally, TNF α in bovine plasma was measured using a human ELISA kit (hTNF α ELISA; catalog #589201, Cayman Chemical, Ann Arbor, MI) to relate its concentration in periparturient cows with incidence of fatty liver (Ametaj et al., 2005a). However, this kit was not validated for use with bovine samples. Our goal in this work was to evaluate the validity of both ELISA kits for measurement of bTNF α in plasma. An additional goal was to develop a low cost, precise method for measurement of TNF α in bovine serum and plasma with adequate sensitivity to detect basal levels of TNF α . All experimental procedures were approved by the Kansas State University Institutional Animal Care and Use Committee.

To produce a protein standard for evaluation of ELISA assays, a recombinant expression vector encoding the secreted form of bTNF α (Entrez Protein Accession AAB84086.1, region 77 - 233) was expressed in *E. coli* and purified by a commercial laboratory. Purity and biological activity of the isolated recombinant bTNF α were verified by SDS-PAGE and a WEHI 13Var-164-based assay, respectively. There are a number of commercial sources of recombinant

bTNF α , and it is expected that all such standards would work equally well for the purpose of this ELISA assay; evaluating these standards was outside the scope of this study.

The specificity of the polyclonal rabbit anti-bovine TNF α antibody was initially evaluated by Western blot analysis of bovine plasma proteins. Plasma samples (1 μ L) collected from cows receiving continuous low-dose infusions of recombinant bTNF α (concentrations ranging from ~5 to 35 pg/mL) were diluted with 19 μ L Laemmli sample buffer (Bio-Rad, Richmond, CA). The homogenate was heated at 90°C for 5 min, cooled, vortexed, and loaded onto a 4-20% Tris-HCl gel for electrophoresis. Samples were separated by SDS-PAGE and dry-transferred onto nitrocellulose membranes (iBlot, Invitrogen, Carlsbad, CA). Membranes were blocked for 2 h in blocking buffer (5% dry milk in Tris-HCl buffer, pH 7.5, with 0.05 % Tween 20). After incubation with blocking buffer, the membranes were washed 3 times for 5 min each with washing buffer (phosphate-buffered saline, pH 7.5, containing 0.05% Tween 20), then incubated for 1 h with the biotin-conjugated polyclonal rabbit antibody (detection antibody, PBOTNFABI, Thermo-Fisher Scientific Inc.) diluted 20,000-fold in blocking buffer. After incubation, membranes were rinsed 3 times with washing buffer and then incubated for 1 h with horseradish peroxidase-labeled streptavidin diluted 50,000-fold in blocking buffer. Immunodetection was performed by chemiluminescence (West-Dura; Thermo Scientific, Waltham, MA).

Western blot results (Fig. 1A) showed that the detection antibody used in the bTNF α ELISA detected a single band in plasma with the expected molecular weight of bTNF α (~17 kDa). The lack of other bands suggests that cross-reactivity of the antibody with other common proteins found in bovine plasma is likely to be minimal. The product literature notes that ~ 2% cross-reactivity with bovine interleukin-6 (IL-6) was detected with the bTNF α ELISA, but no other bovine cross-reactivities were reported. We conclude that the specificity of the bTNF α ELISA antibodies are sufficient for use in plasma samples.

The bTNF α ELISA kit originally included suggested protocols for determination of bovine TNF α in cell culture media (screening kit protocol no longer available online), and did not include information regarding precision of the assay for concentrations typically found in bovine plasma (\leq 300 pg/mL; Kenison et al., 1991b). We therefore tested a variety of primary and secondary antibody concentrations to determine the optimal protocol for determination of basal TNF α concentrations in bovine plasma, with the goal of achieving a low detection limit

and an assay range of at least 2 orders of magnitude. The optimal concentration of coating antibody was determined to be 6.8 µg/mL while the optimal concentration of detection antibody was 2.5 µg/mL (data not shown).

The final protocol was as follows. First, a 96-well plate (Corning Costar high binding 96 well plate, Thermo Fisher Scientific Inc.) was coated with 100 µL of coating antibody (polyclonal rabbit anti-bovine TNFα, PBOTNFAI, Thermo Fisher Scientific Inc.) at a concentration of 6.8 µg/mL diluted in 0.2 M sodium-bicarbonate buffer (pH 9.4; 0.2 µm filtered). The plate was then sealed and incubated overnight at room temperature. On the next day, the wells were aspirated and then washed 5 times with wash buffer (0.05% Tween 20 and 0.1% sodium azide in PBS). Following the wash steps, 300 µL of blocking buffer (4% bovine serum albumin, 5% sucrose in PBS, 0.2 µm filtered) was added to the wells and incubated for 2 h at room temperature. The wells were aspirated and again washed with wash buffer 5 times, and then samples (100 µL) and standards (100 µL at concentrations 200 pg/mL, 50 pg/mL, 12.5 pg/mL, and 3.125 pg/mL in human serum) were added to the wells. Human serum was added to non-specific binding and 0 pg/mL standard wells. The samples were allowed to incubate overnight at 4°C. On the third day of the procedure, the wells were washed 5 times with wash buffer followed by addition of 100 µL of detection antibody (biotin labeled polyclonal rabbit anti-bovine TNFα, PBOTNFABI, Thermo Fisher Scientific Inc.) at a concentration of 2.5 µg/mL, then allowed to incubate for 1 h. After the incubation period, the plates were again washed 5 times, then 100 µL of horseradish peroxidase-labeled streptavidin (diluted 1:500 in reagent diluent; HRP-conjugated streptavidin, Thermo Fisher Scientific Inc.) was added to each well and incubated at room temperature for 30 min, followed by another round of 5 washes with wash buffer. Finally, 100 µL of 3,3',5,5'-tetramethylbenzidine substrate solution (TMB Solution; Thermo Fisher Scientific Inc.) was added to each well and allowed to incubate for 20 min at room temperature, followed by the addition of 100 µL of stop solution (0.18 M sulfuric acid). Optical density was measured at 450 and 550 nm using a plate reader (PowerWaveXS, BioTek, Winooski, VT). Gen5 software (BioTek Instruments, Inc.) was used to calculate the concentration of TNFα in the samples by calculating the difference between the A450 and A550. A450-A550 values were corrected by subtracting the mean value for non-specific binding wells from all other values. The corrected difference values were fit to the standard curve by point to point regression.

With this protocol, we were able to detect bTNF α concentrations in a range from 2 to 250 pg/mL. After reviewing the literature there was only one protocol that had a similar detection limit in bovine serum/plasma; the sandwich ELISA protocol described by Ellis et al. (1993) had a detection limit of 35 pg/mL in bovine serum. Other protocols in previous bTNF α ELISA studies reported basal plasma concentrations that were likely inflated by poor sensitivity of the assays (133 – 15,630 pg/mL: Hisaeda et al., 2001; Røntved et al., 2005; Burciaga-Robles et al., 2010).

One challenge in developing ELISA protocols is to find a diluent for standards that mimics the matrix effect of plasma or serum. For example, Dernfalk et al. (2004) observed that the apparent recovery of recombinant hTNF α added to bovine serum was only 36.4% of the apparent recovery when recombinant hTNF α was added to a buffer solution. Therefore, standards were prepared in a variety of diluents, and standard curves were generated. These were then used to quantify known concentrations of recombinant bTNF α that were spiked into serum and plasma from healthy cattle. Table 1 shows the different diluents utilized and the apparent recovery of the spiked in recombinant bTNF α . Based on these results, we suggest that PBS and human serum can serve as valid standard diluents for both bovine serum and plasma analyses. We also tested the difference in TNF α concentrations in plasma and serum collected at the same time from 8 cows with detectable TNF α to determine if the concentrations were similar. We observed no significant difference ($P > 0.10$) between bovine serum and plasma samples (means: 3.59 vs. 4.92 ± 0.88 pg/mL in plasma vs. serum, $n=8$). Thus, the procedure appears to be equally useful for either sample type. However, the type of sample collected could influence the choice of standard curve diluent (Table 1).

This ELISA procedure was used across 10 plates to test the precision of the assay when using human serum as the standard diluent. The intra-assay CV values at specific concentrations are shown in Table 2, and a typical standard curve is shown in Figure 1B. Pooled samples were included on each plate to determine inter-assay CVs, which were 13.9% and 10.4% for serum and plasma, respectively. Linearity of the assay was tested using plasma samples ($n = 10$) with relatively high concentrations of TNF α (605 ± 253 pg/mL, mean \pm SD). Samples were plated in duplicate at each of 3 volumes (20 μ L, 10 μ L, and 5 μ L per well). To ease data interpretation, concentrations within sample were divided by the result for the highest sample volume (20 μ L =

100%). The slope of the resulting regression equation (1.06, Figure 1C) suggests that the assay produces unbiased results across the range of the assay (14 – 199 pg/mL for these samples).

Additionally, we tested the ability of this assay to detect physiological changes in TNF α concentrations in a model mimicking a disease scenario. To accomplish this, plasma samples from cows that were injected i.v. with rbTNF α (5 μ g/kg BW) were used in this assay. Samples (n = 8 per time point) collected immediately before and at 1, 2, and 3 h post-infusion (diluted with human serum when necessary) were assayed for TNF α . The assay was able to detect a highly significant increase in plasma TNF α concentrations ($P < 0.001$; Figure 2) in spite of the fact that the mean concentration at 1 h post-infusion remained below the detection limit for most bovine TNF α assays.

We were interested in determining if bTNF α is detectable by human TNF α antibodies, especially because of previously-reported associations between fatty liver disease and bTNF α determined using an hTNF α ELISA (Ametaj et al., 2005). We used the same kit as Ametaj et al. (2005; Catalog #589201, Cayman Chemical, Ann Arbor, MI) to determine whether the anti-hTNF α antibodies employed in the kit would detect recombinant bTNF α . We added human and bovine TNF α to the kit's standard diluent and carried out the assay according to the manufacturer's directions. The results showed that the recombinant bTNF α protein did not cross react with the human antibodies (Figure 3A). Another paper reported similar findings when human TNF α antibodies were used in an attempt to detect recombinant bTNF α and recombinant ovine TNF α in bovine milk and serum samples (Dernfalk et al., 2004). The authors observed no cross-reactivity with recombinant bTNF α , and recombinant ovine TNF α was only detectable at concentrations greater than 2.5 ng/mL.

Although we were unable to detect recombinant bTNF α with the hTNF α ELISA kit, the original report (Ametaj et al., 2005) demonstrated differences across time, and we also clearly detected some immunoreactive protein in bovine plasma using this assay. To further assess whether the hTNF α ELISA kit was detecting bTNF α , the detection antibody from the human TNF α kit was used in a Western blot. Serum samples (1 μ L) collected from early lactation dairy cows (concentrations ranging from ~2 to 9 pg/mL) and a 1- μ L aliquot of recombinant bTNF α (0.58 μ g/ml) were diluted with Laemmli sample buffer (Bio-Rad, Richmond, CA) and prepared as described above. The Western blot was carried out according to the same procedure described above, but used the TNF α AChE:Fab' antibody (TNF α acetylcholinesterase:Fab; hTNF α EIA Kit,

Cayman Chemical) diluted 10,000-fold in blocking buffer in the overnight incubation. The results (Fig. 3B) showed strong bands between 50-60 kDa, indicating that the detection antibody in the kit is able to detect some protein in bovine plasma. However, only very faint bands were observed at 17 kDa, even for the purified protein, suggesting that this antibody is a poor probe for bovine TNF α . With the findings from this series of experiments, it seems unlikely that the protein detected by Ametaj et al. (2005) was TNF α , and the previous results therefor need to be re-evaluated.

In conclusion, the procedure described herein provides a useful tool to determine basal concentrations of bovine TNF α , which are often below 10 pg/mL. This improved sensitivity should aid in determining the roles of TNF α in bovine metabolism and chronic diseases.

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Table 4.1 Apparent recovery of recombinant bTNF α spiked into bovine serum or plasma as assessed using various standard curve diluents¹

Diluent type	Regression Equation Serum	Regression Equation Plasma
Dialyzed FBS ²	$y = 0.28x + 1.01$	$y = 0.42x + 0.82$
FBS	$y = 0.49x + 1.93$	$y = 0.70x - 2.53$
Human plasma	$y = 1.02x - 22.65$	$y = 1.54x - 21.57$
Human serum	$y = 0.71x + 2.41$	$y = 0.96x + 4.98$
Lyophilized bovine plasma	$y = 1.89x - 14.54$	$y = 1.97x + 9.30$
Lyophilized human plasma	$y = 0.99x - 21.96$	$y = 1.60x - 25.54$
Lyophilized human serum	$y = 0.88x - 0.75$	$y = 1.19x + 0.66$
Phosphate-buffered saline	$y = 0.85x + 2.91$	$y = 1.02x + 13.61$

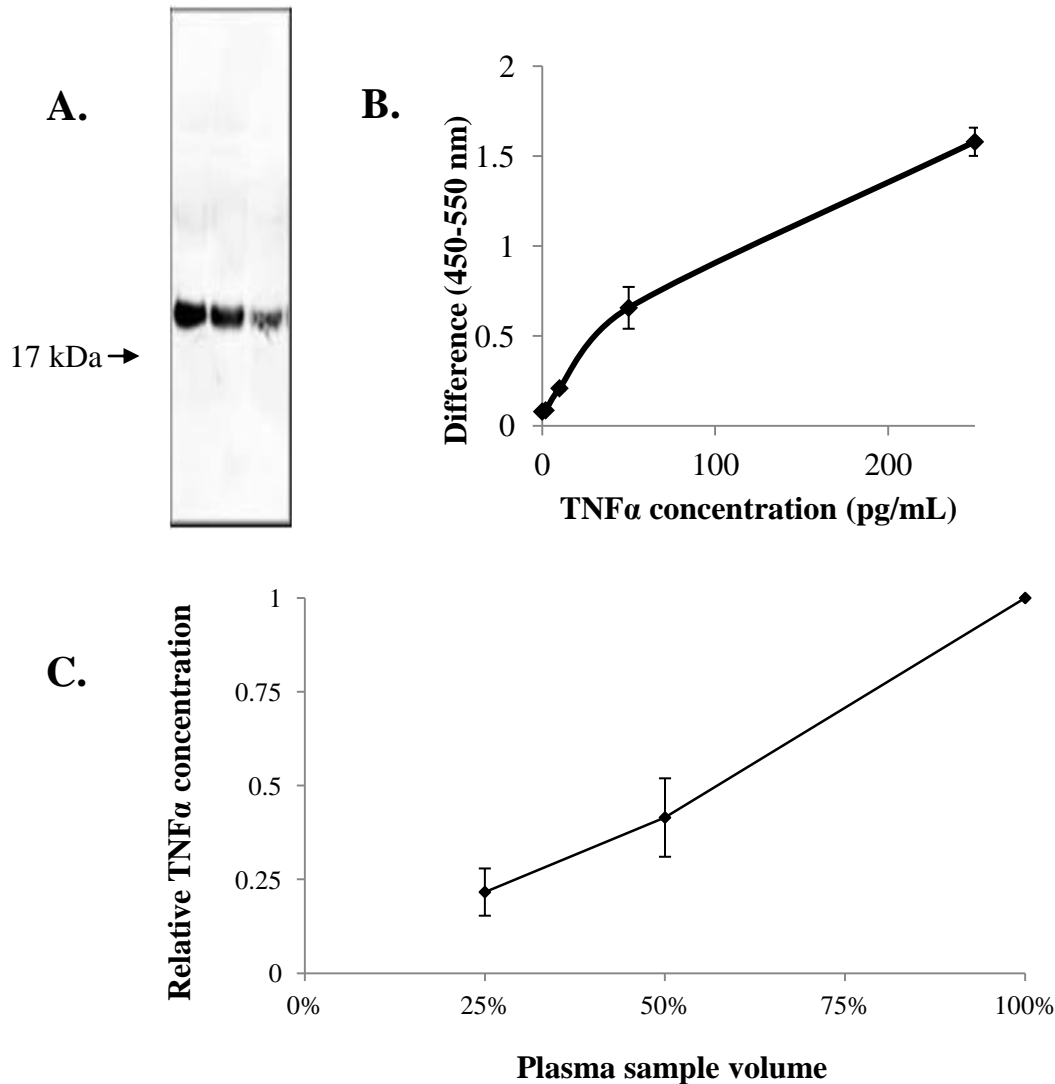
¹ Equations derived from plots of spiked rbTNF α (x) against observed TNF α concentrations (y), both in pg/mL.

² FBS – fetal bovine serum

Table 4.2 Average intra-assay coefficient of variation from 10 plates

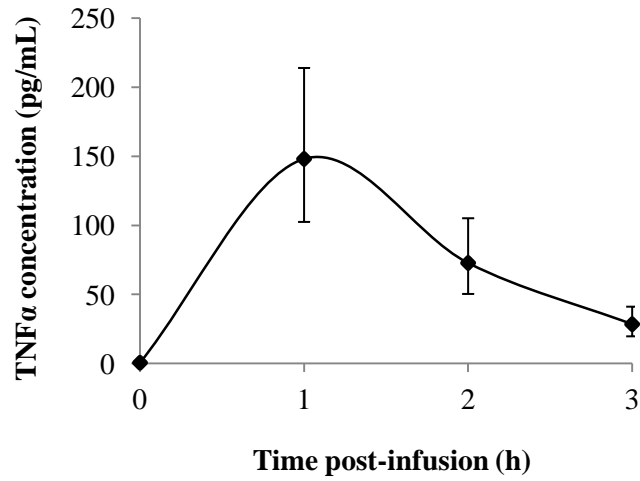
Concentration	CV(%)
200 pg/mL	2.58
50 pg/mL	12.66
12.5 pg/mL	13.00
3.125 pg/mL	23.61

Figure 4.1 Characteristics of the bovine-specific ELISA assay.



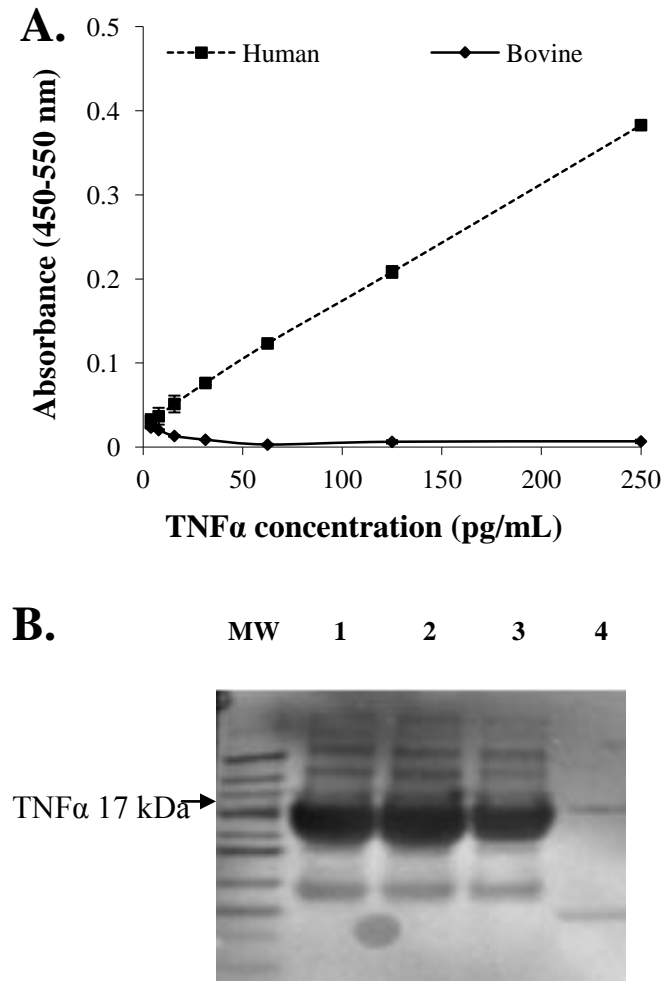
A) Western blot analysis of TNF α protein in bovine plasma (1 μ L per lane) using the bovine-specific ELISA detection antibody. The Western blot image shows bands corresponding to bTNF α (~17 kDa), with no other proteins apparently detected. B) A typical standard curve utilizing bovine-specific antibodies and recombinant bTNF α diluted in human serum is shown. Values are means \pm SD of standards plated in triplicate. C) Linearity of the assay was assessed by linearly decreasing sample volume. Bovine plasma samples ($n = 10$) were plated in duplicate at each of 3 sample volumes (20 μ L, 10 μ L, and 5 μ L per well). Within sample, concentrations of the 20 μ L samples were scaled to 1, and the lower volume concentrations scaled accordingly. Points represent the mean \pm SD; a linear regression of the plot generated the equation: $y = 1.063x - 0.076$.

Figure 4.2 Changes in bovine plasma TNF α in response to rbTNF α injection.



Lactating cows were administered an i.v. bolus of rbTNF α (5 μ g/kg BW) immediately after a blood sample was collected (time 0 h). Blood samples were collected at 1, 2, and 3 h post-infusion. Plasma samples were analyzed using the bovine TNF α ELISA. Values are means \pm SEM, n = 8; values were log-transformed for analysis and means shown are back-transformed. The effect of time was significant (P < 0.001).

Figure 4.3 Human TNF α ELISA does not detect bovine TNF α .



A) Recombinant bovine TNF α was used to test cross-reactivity of a human TNF α ELISA kit (Cayman Chemical Company, Ann Arbor, MI). The assay was performed according to the manufacturer's instructions, and bovine standards were diluted to match those of the human TNF α standard curve. Values are means \pm SD (error bars are too small to be seen for most points). B) Western blot analysis of TNF α protein in bovine serum using the anti-human TNF α detection antibody. Lanes are as follows: MW, protein ladder (8 μ L, Novex Sharp Protein Standard, Invitrogen, CA, standards at 260, 160, 110, 80, 60, 50, 40, 30, 20, 15 and 10 kDa); lanes 1-3, bovine serum (1 μ L diluted with 19 μ L Laemmli buffer); lane 4, 0.58 ng recombinant bovine TNF α . The results demonstrated that the antibody did not detect the bovine form of TNF α with reasonable specificity.

Chapter 5 - Daily injection of tumor necrosis factor alpha in the first week of lactation decreases milk production and promotes health disorders in Holstein dairy cows

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ABSTRACT

Inflammation may be a contributing factor to several transition disorders, so the objective of this study was to determine the production effects observed when an inflammatory cytokine, TNF α , is injected in the first week of lactation to Holstein dairy cows. At calving, 33 cows (n=9 primiparous [PP]; n=24 multiparous [MP]) were alternately assigned to either control (CON; 0 μ g TNF α /kg BW), low dose (LOW; 1.5 μ g TNF α /kg BW), or high dose (HIGH; 3.0 μ g TNF α /kg BW) injections daily for 7 d. Daily data collected included DMI, water intake, and health status; plasma samples were also collected daily for metabolite analyses. Data were analyzed using mixed models with repeated measures over time and significance was declared at $P < 0.05$ and tendencies at $P < 0.10$. Preplanned contrasts evaluating CON vs. TNF α treatment and LOW vs. HIGH were evaluated. Plasma TNF α concentrations tended to be increased in cows receiving TNF α injections (64% increase), but there were no differences observed between HIGH and LOW treatments. DMI was significantly reduced in cows receiving TNF α injections (18% decrease). Similarly, water intake was decreased (13%) with TNF α . Milk production was reduced in TNF α treated cows as evidenced by 15 to 18% decreases in yields of milk, milk fat, milk protein, milk lactose, energy-corrected milk, and solids-corrected milk. Milk fat yield and energy-corrected milk tended to be further depressed in HIGH cows, but no other differences were observed between LOW and HIGH for any other milk variable. No treatment differences were observed for plasma glucose, β -hydroxybutyrate (BHBA), non-esterified fatty acids (NEFA), or triglyceride concentrations. Daily injection of TNF α increased ($P = 0.04$) diagnosed health disorders in the first week of lactation, due in part to a tendency for an increased incidence of ketosis in cows injected with TNF α . The results of this study indicate that low grade inflammation induced by daily injection of TNF α negatively impacts milk production and increases physiological stress on the transition dairy cow as evidenced by increased health disorders.

Keywords: Inflammation, anorexia, metabolism

INTRODUCTION

There is growing evidence in multiple species that inflammation not only affects the immune system, but also affects metabolism (Hotamisligil, 2006). Metabolic adaptations during the transition period are of primary concern to the dairy industry as this impacts production and sustainability. The dairy cow must undergo important metabolic adaptations during late pregnancy and early lactation, and these homeorhetic adaptations target a variety of tissues and homeostatic signals (Bauman and Currie, 1980; Bell, 1995). Inadequate adaptation to lactation can lead to health disorders such as fatty liver and ketosis (Drackley, 1999; Bobe et al., 2004; Ingvarsen, 2006) while negatively impacting overall milk production (Micinski et al., 2009).

Energy requirements increase in early lactation (Bell and Bauman, 1997) and during this time cows have a depression in feed intake (Bobe et al., 2004) which can lead to severe negative nutrient balance. To compensate for the reduction in energy intake cows mobilize adipose tissue triglycerides, increase uptake of fatty acids by the liver, and increase hepatic triglyceride (TG) synthesis (Bobe et al., 2004). The increase in TG synthesis coupled with an inefficient export of TG leads to an accumulation of fat in the liver. This accumulation of fat in the liver has been reported in approximately 50% of all cows in early lactation (Jorritsma et al., 2000; Jorritsma et al., 2003).

Tumor necrosis factor- α has been researched extensively in rodents and humans and has been the focus of some bovine research to determine effects of inflammation on metabolism. In obese mice, TNF α is highly expressed in adipose tissue and has been proposed as one link between obesity, diabetes, and chronic inflammation (Hotamisligil et al., 1993). Tumor necrosis factor- α has several effects such as a depression in feed intake and insulin sensitivity, as well as increased hepatic lipogenesis, muscle and adipose catabolism, and further increases in inflammation (Drackley, 1999; Ohtsuka et al., 2001; Kushibiki et al., 2003). In a bovine model, recombinant bovine TNF α (rbTNF α) decreases appetite and feed intake and can increase blood NEFA concentrations (Kushibiki et al., 2000). Additionally, TNF α has been demonstrated to decrease liver glucose production (Kettelhut et al., 1987a) and promote triglyceride accumulation once mobilized NEFA reach the liver (Garcia-Ruiz et al., 2006).

Serum TNF α activity was increased in cows with moderate to severe fatty liver (Ohtsuka et al., 2001) possibly providing a link between inflammation and metabolic disorders. However, such results demonstrate correlation, not necessarily cause and effect relationships. To evaluate the ability of TNF α to disrupt hepatic metabolism, rbTNF α was injected daily for 7 d in late-lactation Holstein cows, and the authors observed a higher liver triglyceride content, with no effects on plasma NEFA concentration (Bradford et al., 2009).

Inflammatory cytokines are known to increase body temperature, induce anorexia, and promote catabolism of adipose tissue and muscle (Elsasser et al., 1995), all conditions which are commonly observed in transition dairy cows. Since transition cow health and management is important, it is critical to understand how elevated systemic inflammation alters productivity and wellness of dairy cows during this period. The objective of this study was to determine if adding an inflammatory stressor in early lactation compounds transition issues and alters milk production.

MATERIALS AND METHODS

All experimental procedures were approved by the Kansas State University Institutional Animal Care and Use Committee #3075.

Design and Treatments

Recombinant bovine TNF α was acquired in the same fashion as described in Bradford et al. (2009). To briefly summarize, a recombinant expression vector encoding for the secreted form of bovine TNF α (Entrez Protein Accession AAB84086.1, region 72-233) was expressed in *Escherichia coli*, purified by a commercial laboratory, and tested by SDS-PAGE for purity. Thirty-three Holstein cows [n=9 primiparous (PP), n=24 multiparous (MP)] from the Kansas State University Dairy Teaching and Research Facility were randomly assigned to treatment at parturition. Treatments were balanced within parity and were administered s.c. daily for 7 d. Treatments were control (CON; 10% glycerol in PBS), low dose (LOW; 1.5 μ g rbTNF α /kg BW in 10% glycerol in PBS), and high dose (HIGH; 3.0 μ g rbTNF α /kg BW in 10% glycerol in PBS). Injections were given in the neck at 1600 h daily, and were administered on alternate sides to minimize local tissue inflammation at injection sites. After calving, cows were housed in a tie-stall facility for 8 d before returning to free-stall housing. Cows were milked 3 times daily

(0800, 1200, and 2000 h) and fed twice daily (0700 and 1600 h) for ad libitum intake of a diet formulated to meet all nutrient requirements (Table 5.1).

Data and sample collection

During the 7-d trial, feed and water intake and milk yield were recorded daily. Milk samples were collected from d 4 to 7 of treatment at each of the 3 milkings for analysis of milk components. Samples were analyzed for concentrations of fat, true protein, lactose (B-2000 Infrared Analyzer; Bentley Instruments Inc., Chaska, MN), urea nitrogen (MUN spectrophotometer, Bentley Instruments Inc.), and somatic cell (SCC 500, Bentley Instruments Inc., Heart of America DHIA, Manhattan, KS). Energy corrected milk (ECM) was calculated: $0.327 \times \text{milk yield} + 12.86 \times \text{fat yield} + 7.65 \times \text{protein yield}$ (Dairy Records Management Systems, 2010). Solids corrected milk (SCM) was calculated as described by Tyrrell and Reid (1965): $12.3 \times \text{fat yield} + 6.56 \times \text{SNF yield} - 0.0752 \times \text{milk yield}$. Somatic cell score was calculated according to Shook (1993): $\log_2(\text{SCC}/100) + 3$.

Blood samples were collected from coccygeal vessels daily in 2 tubes, one containing potassium EDTA and the other containing potassium oxalate with sodium fluoride as a glycolytic inhibitor (Vacutainer; Becton, Dickinson and Co., Franklin Lakes, NJ). Blood was centrifuged at $2,000 \times g$ for 10 min immediately after sample collection, and plasma was frozen at -20°C until used for analysis of BHBA, NEFA, glucose, TG, and TNF α concentration. Non-esterified fatty acids were analyzed using an enzymatic colorimetric procedure (NEFA-HR; Wako Chemicals USA Inc., Richmond, VA), glucose by a colorimetric kit (kit #439-90901; Wako Chemicals USA Inc.), and BHBA using an enzymatic reaction (kit #H7587-58; Pointe Scientific Inc., Canton, MI). Plasma TG was analyzed by enzymatic reaction (kit #T7532-120; Pointe Scientific Inc.). Plasma TNF α was evaluated by sandwich ELISA according to the protocol described by Farney et al. (2011). Absorbance was read on a spectrophotometer (PowerWave XS; BioTek Instruments Inc., Winooski, VT) and calculations were performed using Gen5 software (BioTeck Instruments Inc.).

Disease Incidence

Cows were assessed daily for health by trained personnel where rectal temperature and urine ketones (ReliOn ketone test strips, Bayer Healthcare LLC., Mishawaka, IN) were recorded. Ketosis, left displaced abomasum, retained placenta, metritis, milk fever, lameness, and other

abnormalities were recorded. Any cow identified as displaying signs of a health disorder was treated according to on-site standard operating procedures. Ketosis was defined as the detection of urine ketone concentrations exceeding 80 mg/dL on any 1 d, or exceeding 40 mg/dL for 2 consecutive days. Metritis was defined by the combination of a fever ($>39.4^{\circ}\text{C}$), a uterine discharge that was reddish brown, and an odor. Cows with a rectal temperature greater 39.4°C but with no other symptoms to suggest an infectious disorder were classified as fever cases. Disorders not described above were identified based on definitions defined by Kelton et al. (1998).

Statistical analysis

Statistical analyses were carried out using the Mixed Procedure of SAS (version 9.2; SAS Institute Inc., Cary, NC) to assess the fixed effects of parity, time, treatment, and all 2- and 3-way interactions; cow was included as a random effect. Repeated measures over time were modeled with either autoregressive or autoregressive heterogeneous covariance structures, depending on which analysis had the lowest Bayesian Information Criterion value. Denominator degrees of freedom were estimated using the Kenward-Rogers method. Values were deemed outliers and omitted from analysis when Studentized residuals were $>|3.5|$. Interactions were investigated when $P < 0.15$ using the slice option, and slices declared significant at $P < 0.05$. Interactions with a P -value greater than 0.30 were removed from the model. Preplanned contrasts were evaluated to compare CON vs. both $\text{TNF}\alpha$ treatments and LOW vs. HIGH. Health disorders were analyzed using JMP (version 8.0; SAS Institute Inc. Cary, NC). A nominal logistic analysis was first run for each health disorder to assess the fixed effects of treatment, parity, and treatment \times parity interaction. For those disorders with an interaction P value < 0.10 , Fisher's exact test was used to assess CON vs. $\text{TNF}\alpha$ and HIGH vs. LOW contrasts within each parity group. For all other disorders, Fisher's exact test was used to test these contrasts across all animals.

RESULTS

Feed and water intake and energy balance

Dry matter intake was decreased in cows injected with $\text{rbTNF}\alpha$ ($P = 0.02$, Table 5.2). There was no difference detected between LOW and HIGH treatments ($P = 0.15$). Multiparous

cows had greater DMI than PP cows ($P < 0.001$; 14.10 ± 0.48 vs. 10.09 ± 0.79 kg DM/d). Water intake was decreased with both TNF α treated groups versus CON ($P = 0.04$, Table 5.2) with no difference between HIGH and LOW ($P = 0.34$). There was a tendency for a 3-way interaction ($P = 0.07$) where all the differences were observed in MP cows (Figure 5.1). Energy balance was not affected by treatment, day, parity, or interactions ($P > 0.10$) and all values were negative, indicating that cows were in a negative energy balance through the 7 d trial.

Milk yield and milk components

Milk production data is shown in Table 5.2. Milk yield was decreased when TNF α was injected ($P = 0.03$) with no differences observed between LOW and HIGH ($P = 0.76$). Milk yield increased daily over the 7-d treatment period ($P < 0.001$) and MP cows had higher milk yield than PP cows (34.3 ± 1.1 kg/d vs. 26.6 ± 1.7 kg/d). Additionally, a 3-way interaction was observed ($P = 0.03$; Figure 5.2A) where the differences were primarily observed in MP cows.

Milk fat % was similar in CON and HIGH (5.41 ± 0.25 , $5.18 \pm 0.24\%$, respectively; Table 5.2), and the overall effect of TNF α was not significant. LOW cows, however, had significantly higher milk fat concentration than HIGH ($P = 0.02$). Additionally, there was a treatment \times parity \times day interaction ($P < 0.05$) where on d 6 PP LOW cows had higher milk fat than PP HIGH, with tendencies for the same observation to occur on d 6 and d 7 in MP cows (Figures 5.2B and 5.2C). Milk fat yield was decreased in cows treated with TNF α ($P = 0.01$) and there was a tendency for the HIGH group to have a more pronounced decrease in milk fat yield than LOW ($P = 0.07$; Table 5.2). Multiparous cows had higher milk fat yield than PP cows ($P < 0.001$; 2.18 ± 0.06 vs. 1.51 ± 0.11 kg/d).

Milk protein concentration was not impacted by treatment ($P > 0.10$; Table 5.2); however there was a difference by parity ($P < 0.001$) where MP cows had higher milk protein concentration than PP (3.70 ± 0.07 vs. $3.26 \pm 0.11\%$). Milk protein yield was decreased with TNF α administration ($P < 0.01$). Multiparous cows had significantly ($P < 0.001$) higher protein yield than PP cows (1.35 ± 0.03 vs. 0.92 ± 0.05 kg/d).

There was no effect of treatment on milk lactose concentration ($P > 0.10$), nor an effect of parity ($P > 0.10$). There was a significant day effect ($P = 0.03$) where milk lactose concentration increased from d 4 to 7. A significant treatment \times day interaction was observed for lactose concentration, where treatments tended to separate on d 6 and 7 (Figure 5.2D). Milk

lactose yield was decreased when TNF α was administered ($P < 0.001$, Table 5.2). Similar to other milk component yields, milk lactose was greater in MP cows compared to PP cows (1.67 vs. 1.24 ± 0.06 kg/d; $P < 0.001$). Milk urea nitrogen (MUN) concentration was not impacted by treatment or parity ($P > 0.10$), but MUN tended to decrease ($P = 0.07$) as days in milk increased, regardless of treatment.

Energy corrected milk yield was decreased by TNF α administration ($P < 0.01$, Table 5.2) with no differences observed between LOW and HIGH treatments ($P = 0.13$). Multiparous cows had higher ECM (50.9 ± 1.3 kg/d) than PP cows (35.8 ± 2.1 kg/d; $P < 0.001$). Solids corrected milk yield was decreased in cows that were treated with TNF α ($P < 0.01$). Additionally, the HIGH treatment group tended ($P = 0.10$) to have lower SCM than the LOW treatment. Multiparous cows had higher SCM than PP cows (46.4 vs. 32.2 ± 1.9 kg/d; $P < 0.001$).

Plasma metabolites

Plasma concentrations of TNF α , BHBA, glucose, NEFA, and triglyceride (TG) are displayed in Table 5.3. There was a tendency for TNF α injection to increase plasma TNF α concentrations over controls ($P = 0.09$), but there was no difference observed between LOW and HIGH treatment groups ($P = 0.19$). Plasma BHBA was not affected ($P > 0.10$) by treatment, parity, or any interaction. A day effect for BHBA was observed ($P < 0.001$), where plasma BHBA concentrations increased through the treatment period (Figure 5.3). Similar results were observed for plasma glucose, where treatment, parity, and interaction effects were not significant ($P > 0.10$; Table 5.3). A day effect was observed ($P < 0.001$) where plasma glucose concentrations decreased through the trial (Figure 5.3). Plasma NEFA concentration was not affected by treatment, parity, day, or any interaction between these ($P > 0.10$; Table 5.3). Plasma triglyceride (TG) was not affected by treatment or day ($P > 0.10$) but there was a tendency ($P = 0.10$) for a treatment \times day interaction with no specific differences observed by slice evaluation. There was a parity effect ($P = 0.03$) reflecting higher concentrations of plasma TG in PP cows than MP (14.51 ± 1.16 vs. 11.29 ± 0.71 mg/dL).

Health disorders

Health disorder incidences are shown in Table 5.4. At the site of injection in most cows, TNF α produced some local heat and swelling; this response was not considered a health disorder. A treatment effect was detected ($P = 0.02$) for the proportion of cows with 1 or more health

disorders, where cows receiving TNF α had a greater number of health disorders ($P = 0.04$), with a tendency ($P = 0.08$) for HIGH cows to have greater incidence of health disorders than LOW (Table 5.4). Ketosis was the most common disorder observed, but treatment contrasts for specific disorders were not significant.

DISCUSSION

Fatty liver disease is associated with decreased milk production and increased costs to dairy producers (Bobe et al., 2004). Clinical symptoms of fatty liver include depressed appetite, weight loss, and weak and apathetic cows (Radostits et al., 2000). Fat cows have a higher risk of developing fatty liver as it has been found that obese cows lose more body weight (Smith et al., 1997) and have higher plasma NEFA concentration and liver TG content (Rukkwamsuk et al., 1998). The extra fat in these cows can be due to both increased number and size of adipocytes, so there are additional fatty acids available for release as a source of energy. Additionally, because adipose tissue is metabolically active, producing cytokines and other hormones that alter carbohydrate and protein metabolism, this tissue may play a tremendous role in etiology of fatty liver (Hotamisligil, 2006). In an obese ruminant model, as well as in rodents and humans, circulating concentrations of plasma TNF α are increased as adiposity increased (Hotamisligil et al., 1993; Corica et al., 1999; Tsukui et al., 2000; Ohtsuka et al., 2001; Daniel et al., 2003a). In the study of Ohtsuka et al. (2001), serum TNF activity was associated with fatty liver in dairy cows.

Tumor necrosis factor- α in ruminants can cause severe damage to the animal as indicated in a calf trial where prolonged treatment of up to 5 $\mu\text{g/kg}$ BW induced inflammation in the liver, primarily in the portal region (Bielefeldt-Ohmann et al., 1989). Additional damage observed in the liver included infiltration of mononuclear leukocytes, fibrous tissues, fatty degeneration, and necrosis of hepatocytes. The kidney were also affected by high dose rbTNF α , characterized as interstitial infiltration of mononuclear cells and degenerative changes in glomeruli and medullary edema (Bielefeldt-Ohmann et al., 1989).

Appetite was decreased in cows that received a daily injection of rbTNF α in this study. Similarly, in a study using late lactation non-pregnant Holstein cows injected with 2.5 μg rbTNF α /kg BW per day for 7 d, the authors observed a decrease in DMI (Kushibiki et al., 2003). However, in Bradford et al. (2009), no significant differences were observed in the DMI of late

lactation cows given daily injections of rbTNF α . Several other studies have observed reductions in DMI in response to other inflammatory cytokines (Ingvarsen and Andersen, 2000; Johnson and Finck, 2001; Trevisi et al., 2002). Such responses are especially problematic in the 1st month of lactation, when suppressed DMI results in a more pronounced negative energy balance (Trevisi et al., 2002).

A significant reduction in milk yield was observed in this study, regardless of dosage, when cows received rbTNF α . This milk yield reduction might be partially explained by the reduction in DMI and water intake that was observed in rbTNF α treated cows. A direct correlation between intake of feed and water and milk production is typically observed (Murphy, 1992). Kushibiki and colleagues (2003) observed a decrease in milk production in cows injected with similar concentrations of rbTNF α . In this study there was an increase in milk fat percentage for LOW compared to HIGH treatment, yet the combination of the two TNF α treatments was not different than controls. The increase in milk fat percentage for LOW is similar to results observed by Kushibiki et al. (2003). However, despite the higher values observed in milk fat percentage, the significant decrease in milk production led to a reduction in milk fat yield in cows treated with rbTNF α , with HIGH treated cows having the most pronounced reduction in yield. In contrast to the decrease in milk protein concentration observed by Kushibiki et al. (2003), this study did not observe any differences. However, a decrease in total milk protein yield occurred, and once again this difference could be attributed to the suppression in DMI.

Effects of TNF α injections on plasma glucose concentration have been variable. Bradford and colleagues (2009) did not observe a difference in plasma glucose in response to daily injection of TNF α , similar to that observed in this trial. In a steer model where 2.5 μ g rbTNF α /kg BW was injected for 9 days, the authors observed an increase in plasma glucose concentration (Kushibiki et al., 2001b) which might be explained by a reduction in glucose uptake by the animal (Kushibiki et al., 2000). Similarly, plasma glucose concentration was higher in cows with fatty liver, when serum TNF α activity was also elevated (Ohtsuka et al., 2001). Decreased glucose disposal rate is a measure of the metabolic syndrome in patients with type I diabetes. In Ohtsuka et al. (2001), the authors observed an inverse correlation between serum TNF ($P < 0.05$) and glucose disposal rate, demonstrating that cows with lower levels of TNF α had higher glucose disposal rates.

Lipid mobilization often occurs in cattle receiving TNF α , as evidenced by elevated concentrations of NEFA (Kushibiki et al., 2003). It is unclear though, whether this was due to decreased DMI or alteration of lipid metabolism by this cytokine. No difference in plasma NEFA concentration was detected in late lactation cows injected with rbTNF α (Bradford et al., 2009). The authors did, however, observe differences in hepatic mRNA abundance of genes that relate to lipid metabolism, primarily those that can lead to increased accumulation of fat in the liver. Increased abundance of a transcript involved in NEFA uptake (fatty acid translocase; CD36) was observed, along with a decrease in a key transcript contributing to fatty acid oxidation (carnitine palmitoyltransferase 1A; CPT1a), and an increase in a TG synthesis transcript (1-acylglycerol-3-phosphate I-acyltransferase 1). In this study cows that received rbTNF α had higher TG content in the liver, which might be explained by the observations of gene alterations described above (Bradford et al., 2009). An increase in uptake of NEFA might explain why there were no differences observed in plasma NEFA concentration. Even though liver TG was not evaluated in the current paper, it is possible that the same mechanisms were occurring in this trial in regards to lipid metabolism. To further complicate the effects of TNF α , Kenison and colleagues (1991) did not observe differences in plasma TG or NEFA in bull calves injected with TNF α . All these studies evaluated responses in cattle in different stages of maturity, lactation status, and gender, and as such, different responses suggest that the impact of TNF α may depend on the metabolic state of the animal. Interestingly, in the early lactation model, where plasma NEFA concentrations are already elevated, TNF α administration did not further increase this marker of lipolysis. One hypothesis is that there is a threshold of NEFA that is available to be released, and once this threshold is met, a “resistant” state for catabolism might be induced.

Early lactation cows that received daily injections of rbTNF α had increased risk for health disorders, suggesting that inflammation is a potential contributor to transition disorders. In an early study using young Holstein steers, rbTNF α administration of 10-20 μ g/kg BW twice daily induced substantial weight loss, such that calves were diagnosed as being clinically emaciated, depressed, and lethargic, with intermittent watery diarrhea and light coughing (Bielefeldt-Ohmann et al., 1989). The authors also observed a decrease in blood lymphocytes, with unclear functional responses, as some tests showed improved immune function while others showed a suppression. Additionally, these calves that received TNF α injections had

significantly lower plasma levels of iron and copper, perhaps in response to acute phase protein sequestration of these minerals. The combination of hepatic tissue damage, a reduction in circulating immune cells, and depletion of plasma concentrations of important minerals associated with immunity observed in the calf trial (Bielefeldt-Ohmann et al., 1989) might help to explain why TNF α increased health disorders in the current study. Although the doses used in the current study were an order of magnitude lower than those given in the calf study, similar but more subtle responses may have occurred.

CONCLUSION

Daily s.c. injection of a low dose inflammatory cytokine decreased milk production, likely through reductions in DMI and water consumption. In addition to this costly loss in productivity, clinical disorders were also increased when TNF α was administered. There were minimal production differences observed between the two levels of TNF α administered in this study, possibly indicating that the low dose is biologically effective enough to negatively impact performance, but the higher dose tended to increase the incidence of disorders relative to the lower dose. In contrast to many studies in non-ruminant species where TNF α induced lipid mobilization and impacted glucose metabolism primarily through insulin resistance, this study did not show the same responses. Determining the cellular mechanisms that contributed to the suppression in DMI that was observed would be important as the reduction in DMI was apparently not caused by pyrexia. Additionally, the longer-term effects of inflammatory cytokine administration on lactation persistency and reproductive efficiency are worthy of continued research.

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Table 5.1 Ingredient and nutrient composition of diets

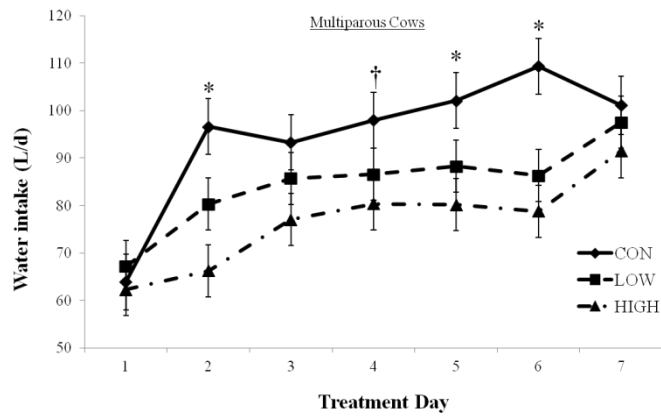
Diet composition	
Ingredient (% of DM)	
Corn silage	22.2
WCGF ¹	30.3
Alfalfa hay	20.4
Cottonseed	4.9
Corn grain	9.2
Sorghum grain	4.0
Micronutrient premix ²	9.0
Nutrient (% of DM)	
DM (% as-fed)	57.2
CP	19.0
ADF	20.0
NDF	33.0
NFC	35.5
Ether extract	5.1
Ash	7.9
NE _L ³ (Mcal/kg)	1.72

¹Wet corn gluten feed (Sweet Bran; Cargill Inc., Blair, NE).

²Premix consisted of 54.9% SoyBest, 14.3% limestone, 1.6% salt; 10.2% sodium bicarbonate, 2.0% magnesium oxide, 0.6% 4-plex, 0.3 selenium (0.06%), 0.10% of Vitamin A and D, 1.6% Vitamin E, 0.1% Rumensin 90, 2.6% Diamond V XP, 10.0% MegalacR, 1.6% potassium carbonate, 0.1% Zinpro 100.

³Estimated according to NRC (2001).

Figure 5.1 Daily water intake



Daily water intake was decreased ($P = 0.04$) for cows treated with $\text{rbTNF}\alpha$, with no difference ($P = 0.34$) between LOW and HIGH treatment groups. There was a tendency for a 3-way interaction ($P = 0.07$) where all the differences were observed in multiparous cows and are illustrated in the figure above. Values are least square means with standard error bars.

* $P < 0.05$ for day effect

† $P < 0.10$ for day effect

Table 5.2 Intake, milk production and composition, and energy balance responses

Item	Treatment ¹			SEM	<i>P</i> - value	
	CON	LOW	HIGH		C vs. T ²	L vs. H ³
DMI, kg/d	13.7	12.1	10.4	0.80	0.02	0.15
Water intake, L/d ^{**}	79.8	71.9	66.5	3.9	0.04	0.34
Milk yield, kg/d [*]	33.7	29.1	28.4	1.7	0.03	0.76
Milk fat ⁴ , % [*]	5.41	6.00	5.18	0.24	0.55	0.02
Milk protein ⁴ , %	3.51	3.57	3.37	0.11	0.75	0.19
Milk lactose ⁴ , %	4.52	4.52	4.45	0.05	0.65	0.33
SCS ⁴ , × 10 ³ cells/mL	3.27	2.49	3.13	0.51	0.50	0.39
MUN ⁴ , mg/dL	11.1	12.7	12.3	0.75	0.13	0.75
ECM ⁴ , kg/d	49.1	42.8	38.2	2.1	< 0.01	0.13
SCM ⁴ , kg/d	44.4	38.8	34.2	1.9	< 0.01	0.10
Yield, kg/d						
Milk fat ⁴	2.08	1.87	1.59	0.11	0.01	0.07
Milk protein ⁴	1.29	1.11	1.01	0.05	< 0.01	0.19
Milk lactose ⁴	1.65	1.39	1.32	0.08	< 0.01	0.55
EB ⁴ , Mcal/d	-23.6	-21.6	-22.0	1.6	0.36	0.89

¹Treatments: CON = No TNF α ; LOW = 1.5 μ g/kg BW TNF α ; HIGH = 3.0 μ g/kg BW TNF α .

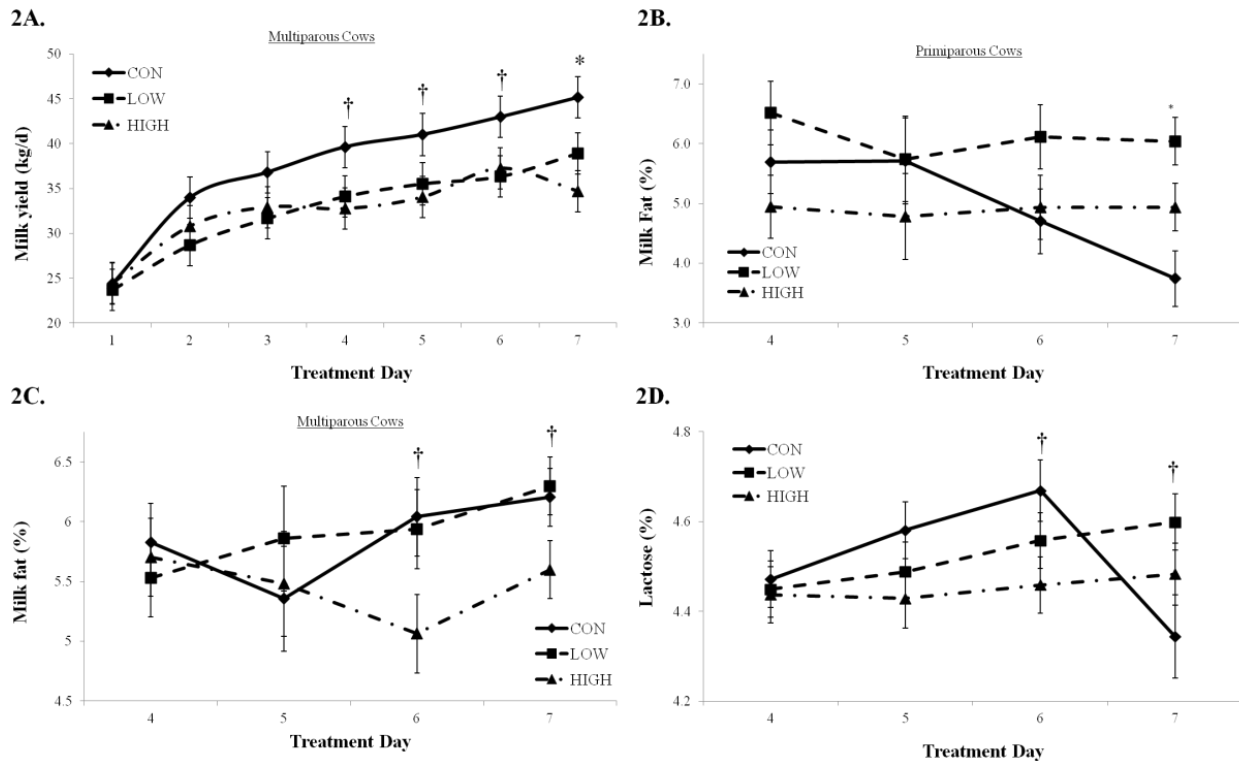
² *P*-value for preplanned contrast between CON and TNF injection (Low and High).

³ *P*-value for preplanned contrast between LOW and HIGH treatment.

⁴ Data represent d 4 to d 7 for these variables.

^{*}Treatment × parity × day (*P* < 0.05); ^{**}Treatment × parity × day (*P* < 0.10).

Figure 5.2 Milk responses by day



Milk yield was decreased ($P = 0.03$) when cows were injected with rbTNF α , with no differences observed between HIGH and LOW treatment groups ($P = 0.76$). Milk yield increased daily ($P < 0.001$) and a 3-way interaction was observed ($P = 0.03$) where all differences were observed in multiparous cows (Panel A). Milk fat % was significantly lower ($P = 0.02$) for LOW treatment. A 3-way interaction was observed ($P < 0.05$) where both primiparous cows (Panel B) and multiparous (Panel C) cows had treatment \times day interactions for milk fat %. There was no overall effect of treatment for milk lactose % ($P = 0.56$), but there was a significant treatment \times day ($P = 0.04$) interaction (Panel D) where values tended to differ on days 6 and 7 of treatment. Values are least square means with standard error bars.

* $P < 0.05$ for day effect

† $P < 0.10$ for day effect

Table 5.3 Plasma metabolites

Item	Treatment ¹			SEM	<i>P</i> – value	
	CON	LOW	HIGH		C vs. T ²	L vs. H ³
BHBA, μ M	996	1108	1186	154.3	0.40	0.72
Glucose, mg/dL	50.8	51.9	51.7	2.1	0.68	0.94
NEFA, μ M	857	854	757	83	0.62	0.41
TG*, mg/dL	13.5	12.9	12.4	1.1	0.52	0.73

¹Treatments: CON = No TNF α ; LOW = 1.5 μ g/kg BW TNF α ; HIGH = 3.0 μ g/kg BW TNF α .

² *P*-value for preplanned contrast between CON and TNF injection (Low and High).

³ *P*-value for preplanned contrast between LOW and HIGH treatment.

*Treatment \times day (*P* = 0.10).

Figure 5.3 Plasma BHBA and glucose by day

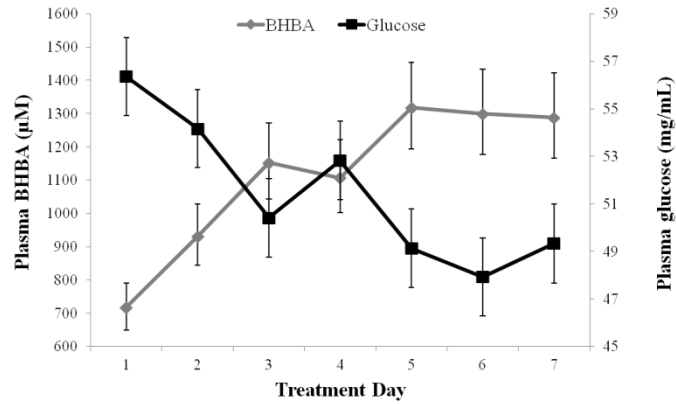


Figure 3: Daily plasma BHBA and glucose.

There were no treatment, parity, or treatment \times parity for plasma BHBA and glucose ($P > 0.10$). There was a significant day effect ($P < 0.001$) were BHBA increased over time, but plasma glucose decreased. Values are least square means with standard error bars.

Table 5.4 Health disorders

Disorder	Treatment ¹			Total by disorder
	CON	LOW	HIGH	
Ketosis ^{**}	1	3	3	7
Respiratory	0	0	2	2
Metritis	1	0	0	1
Milk fever	0	0	1	1
Fever	0	0	2	2
≥ 1 event ^{2,3,4}	1	3	7	

¹Treatments: CON = No TNF α ; LOW = 1.5 μ g/kg BW TNF α ; HIGH = 3.0 μ g/kg BW TNF α ; n=11 per treatment.

² Cows diagnosed with one or more health disorders

³ Preplanned contrast between CON and TNF injection (Low and High), $P = 0.05$.

⁴ Preplanned contrast between LOW and HIGH treatment, $P = 0.09$.

^{**}Treatment \times parity ($P < 0.10$).

Appendix A - Bovine ELISA Detailed Protocol

1. Add 100 μ L of reconstituted coating antibody diluted in carbonate-bicarbonate buffer at a concentration of **6.8 μ g/mL**. Seal the plate and incubate overnight at room temperature.
 - a. Carbonate-Bicarbonate Buffer: 0.2M sodium carbonate-bicarbonate buffer, pH 9.4, 0.2 μ m filtered. Dilute the Coating Antibody 1:75 in buffer. Calculation for 1 plate: 75 μ L Coating Antibody in 10.925 mL carbonate-bicarbonate buffer.
2. Aspirate the wells and wash **five times** with 300 μ L Wash Buffer in each well.
 - a. Wash Buffer: 0.05% Tween 20 and 0.1% Sodium Ixide in PBS.
 - b. NOTE: after adding wash buffer, gently agitate for 30 seconds before removing liquid at each wash cycle. Completely remove wash buffer before next step.
3. Add 300 μ L blocking buffer (4% BSA, 5% Sucrose in PBS) to each well. Seal the plate and incubate for **2 hours** at room temperature.
 - a. 4% BSA, 5% Sucrose in PBS, 0.2 μ m filtered
 - b. Calculation for 200 mL: weigh 8 g BSA and add to 192 mL PBS, weight 5 g sucrose and add to 95 mL 4% BSA solution and filter using 0.2 μ m. Filter remaining 4% BSA solution to use as reagent diluent.
 - c. NOTE: 4% BSA in PBS (filtered 0.2 μ m) will use as reagent diluent.
4. Aspirate the wells and wash **five times** with 300 μ L wash buffer in each well.
5. Add 100 μ L of each standard (example: 250, 125, 62.5, 31.25, 15.625, 7.8, 3.9 pg/mL diluted in human serum) or 100 μ L of each sample to each well (**for NSB and 0 standard, 100 μ L of human serum was used**). Seal the plate and **incubate overnight at 4^o C**.
 - a. Make standards the day of use
 - b. Samples should be in triplicate with the standard replicates being spread out through the plate (ex. Row A, Row E. and Row H)
6. Aspirate the wells and wash **five times** with 300 μ L wash buffer in each well.
7. Add 100 μ L of detection antibody in reagent diluent at a concentration of **2.5 μ g/mL** to each well **EXCEPT** for NSB wells, use 100 μ L reagent diluent. Seal the plate and incubate for **1 hour** at room temperature.
 - a. Dilute the Detection Antibody 1:100 in Reagent Diluent by adding 110 μ L of Detection Antibody 10.89 mL Reagent Diluent (Calculation for 1 plate).
8. Aspirate the wells and wash **five times** with 300 μ L wash buffer in each well.
9. Add 100 μ L diluted SA-HRP reagent to each well. Seal the plate and incubate for **30 minutes** at room temperature.
 - a. Dilute SA-HRP 1:500 in Reagent Diluent by adding 24 μ L SA-HRP to 12 mL of Reagent Diluent (Calculation for 1 plate).
10. Aspirate the wells and wash **five times** with 300 μ L wash buffer in each well.
11. Add 100 μ L Substrate Solution to each well. Seal the plate and incubate in the dark for **10 minutes** at room temperature.
 - a. Substrate Solution is ready to use 3,3',5,5'- tetramethyl benzidine (TMB).
12. Stop the reaction by adding 100 μ L of stop solution to each well
 - a. Stop Solution - 0.18M Sulfuric Acid
13. Measure the absorbance at A₄₅₀ minus A₅₅₀.