NOVEL APPROACHES TO DIAGNOSIS AND PREVENTION OF BOVINE FATTY LIVER

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Abstract

The prevalence of fatty liver in transition dairy cattle has been reported to be as high as 50%. There are a few reliable on-farm diagnostic tools and even fewer methods to effectively prevent fatty liver. Non-alcoholic steatohepatitis, an advanced form of non-alcoholic fatty liver in humans, is accurately diagnosed with a commercial blood test that detects plasma cytokeratin-18 (CK18) fragments released during hepatocyte apoptosis. A study was performed using 89 Holstein cows in early lactation to determine if CK18 could serve as a novel indicator of liver triglyceride (TG) content. Although no previous work has been done with CK18 in bovine plasma, our results indicated that CK18 fragments were present in plasma. However, CK18 concentrations did not correlate with liver TG content or other measures of liver function, suggesting it is not a reliable diagnostic tool. Nevertheless, based on liver TG, plasma non-esterified fatty acid (NEFA), and plasma β-hydroxybutyric acid (BHBA) concentrations, this sample population as a whole was not suffering from severe metabolic problems or fatty liver, making it possible that plasma CK18 fragments are elevated only in the most extreme cases. Currently, there is no widely-adopted preventative strategy for fatty liver. A second study was performed to evaluate if encapsulated niacin (EN) could prevent liver TG accumulation during the transition period. Twenty-four primiparous (n=9) and multiparous (n=13) cows were randomly assigned to receive 0 or 24 g of dietary EN, beginning 3 weeks prior to expected calving until 21 days postpartum. Feeding EN did not influence liver TG content, but decreased plasma NEFA concentrations, suggesting inhibition of lipolysis. Multiparous EN cows also experienced depressed dry matter intake (DMI) in the 4 days prior to calving. However, even when EN reduced DMI, plasma NEFA was still suppressed. A novel finding was the prolonged clearance of caffeine in plasma on day 7 postpartum in EN-treated animals. In contrast to other studies, this dose and delivery method of EN did not result in an increase in plasma NEFA after EN treatment ended. These research projects determined that plasma CK18 is likely not a useful diagnostic tool for mild to moderate bovine fatty liver and that feeding EN can inhibit lipolysis but may influence DMI as well. This is one of the first studies into the metabolic effects of feeding EN, and further research is needed in this field.

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CHAPTER 1 – Literature Review

Fatty Liver: Etiology and Current Therapies

The transition into lactation, defined as the last few weeks of gestation and the first few weeks of lactation, bring about numerous challenges and disorders to the dairy cow. One of the most prevalent, yet overlooked of these transition disorders is hepatic lipidosis, or fatty liver. Genetic selection of dairy cattle based almost solely on milk production has created an animal that cannot consume enough energy to support its requirements in the early lactation phase after parturition. The deficit between energy consumed and energy expended through maintenance and milk production is referred to as negative energy balance (NEB). The NEB results in the mobilization of stored body (lipolysis) fat at a rate higher than the liver can oxidize, resulting in the storage of triglycerides (TG) within the liver cells. Fatty liver can only be diagnosed by microscopic examination of the liver, which is rarely performed, making this a poorly recognized disease. The prevalence of fatty liver has been estimated at over 50% and costs associated with it at greater than $60 million per year in the U.S. (Bobe et al., 2004). These costs are due to decreased milk production, reproductive inefficiency, treatment and veterinary costs, and mortality. This literature review will focus on the etiology and diagnosis of fatty liver along with the use of niacin as a preventative strategy.

Etiology

Risk factors for fatty liver have been grouped several different ways, however, most focus around nutrition (Bobe et al., 2004). The primary risk factors are obesity, decreased peripartum feed intake, high energy prepartum diets and low energy postpartum diets. Secondary

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risk factors are prolonged calving interval, increased dry period and concurrent diseases. One of the newer etiological theories of fatty liver involves an inflammatory process within the liver. Experimentally, fatty liver can be induced by periods of fasting or feed restriction over the period of several days (Drackley et al., 1991) or by overfeeding during the dry period (Osman et al., 2008). Regardless of the inciting cause, fatty liver is associated with negative energy balance and can be exacerbated by a higher prepartum body condition score.

Controlling body condition score of cows in late lactation has become a challenge for most dairy producers. During late lactation and the dry period, most cows return to a positive energy balance and begin to deposit body fat. Since fertility and reproductive efficiency in dairy cattle have declined (Pryce et al., 2004), average lactation length has increased. As a result, cows become overfed in both the late lactation and dry periods due to declining milk production and a subsequent lower energy requirement (Grummer, 1995). This leads to increased weight gains and BCS scores prior to the next parturition. Over-conditioned cows have a more severe decrease in prepartum feed intake compared to cows in normal body condition, resulting in a more severe NEB (van den Top, 1996; Stockdale, 2001). The combination of increased body fat and severe NEB leads to increased lipolysis in obese cows compared to cows with a normal BCS (Rukkwamsuk et al., 1998). This may explain why fatty liver is seen more commonly and more severely in cows with BCS > 4.0 (Rukkwamsuk et al., 1998).

The increased demand for energy in late gestation due to fetal growth has been estimated at 30% of total maternal energy requirement (Bell, 1995). This increase in energy requirement is paralleled by a declining dry matter intake (DMI), with DMI at parturition being approximately 50% lower than during the early dry period (Bertics et al., 1992). As DMI is decreasing, the body compensates by mobilization of fatty acids from stored TG in adipose tissue. Numerous studies

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have found a significant correlation between decreasing DMI and increased nonesterified fatty acids (NEFA) in plasma (Bertics et al., 1992; Grummer, 1995). Although DMI will steadily increase following parturition, the demand for energy increases by 300%, creating a more severe postpartum NEB (Bell, 1995). Bertics and others (1992) demonstrated a correlation between decreased prepartum DMI and increased hepatic TG and plasma NEFA concentrations which suggests that cows with depressed DMI are in a more severe NEB at calving. In contrast, Holcomb and others (2001) showed that restricting intake prepartum resulted in increased DMI and milk production immediately postpartum until almost 30 days in milk. Despite many attempts to improve energy intake prepartum, the relationship between DMI, plasma NEFA and liver TG has not been determined. It is likely that measuring energy intake may be more important than using dry matter as a reference.

One of the more extensively studied areas of the transition dairy cow is the composition of prepartum diets. One common method of increasing energy in prepartum diets is to include additional forms of non-fermentable carbohydrates (NFC), which will also adapt the ruminal microbes to a high-NFC lactation diet and enhance papillae development (Dirksen et al., 1985). However, Holtenius et al. (2003) suggested that high-energy diets prepartum may lead to insulin resistance. This may occur as a result of prolonged periods of increased blood glucose and subsequent elevated insulin concentrations. As a result, postpartum insulin resistance may increase lipolysis and the severity of fatty liver. However, little research currently exists on the effect of diet on insulin resistance in prepartum dairy cattle (Grummer, 2008). The exact correlation between prepartum DMI, lipolysis and liver TG is not fully understood despite extensive research on prepartum diets. Doepel et al. (2002) found that increasing NFC concentration from 24% to 30% of dry matter resulted in a significant decrease in postpartum

hepatic TG. In contrast, Rukkwamsuk et al. (1999) induced fatty liver by offering ad libitum intake of a high energy ration during the dry period. These cows had increased levels of liver TG and NEFA compared to controls which were fed according to their NRC requirements. The dietary treatments were fed for 7 weeks, which resulted in a greater weight gain in the high energy ration cows. Although overfeeding can result in fatty liver, the obesity of the cows may have had more of an influence in inducing fatty liver than the prepartum diet composition itself. It is possible that increasing NFC in prepartum diets may decrease plasma NEFA and hepatic TG, but other non-dietary factors are likely involved in postpartum lipid metabolism.

Another seemingly simple way to increase the energy density of a diet is by adding fat into the ration. In a review by Grummer and Carroll (1991), the majority of studies showed increases in plasma NEFA in fat-supplemented groups compared to controls. In a study by Bertics and Grummer (1999), fatty liver was induced by feeding supplemental fat during a period of feed restriction. They found that supplemental fat resulted in not only higher levels of NEFA, but also increased liver TG (% DM basis). In contrast, Grum et al. (1996) found that cows fed high fat diets throughout the dry period had lower concentrations of liver TG and total hepatic lipid compared to control and high grain treatment groups. This contrast between trials further supports that fat mobilization and liver TG are not solely controlled by diet alone and that there may be several factors that play a role in the development of fatty liver.

Lipid Metabolism

It is widely accepted that elimination of a negative energy balance is not feasible during early lactation given the high milk production expectation of modern dairy cows. Therefore, a certain level of subclinical ketosis and mild fatty liver may be unavoidable. Ketosis occurs when the demand for glucose is greater than the capacity of the liver for gluconeogenesis, which is

common in early lactation (Herdt, 2000). Rukkwamsuk et al. (1999) experimentally induced

fatty liver by overfeeding cows during the dry period. Liver biopsies and serum samples were

collected throughout the transition period. Compared to controls, the overfed group had

increased serum NEFA from weeks 1 to 12 postpartum and higher liver TG content from weeks

1 to 6 postpartum. Also, overfed cows had decreased concentrations of phosphoenolpyruvate

carboxykinase (PEPCK), a hepatic gluconeogenic enzyme. At 1 week prior to parturition, the

overfed cows already had decreased PEPCK levels compared to controls, which continued at 0.5

week and 2 weeks postpartum as well. These data suggest that hepatic gluconeogenesis is also

impaired in cows with fatty liver.

Milk production requires synthesis of large amounts of lactose, which is produced from

glucose primarily supplied by the liver (Herdt, 2000). Adipose tissue, a potent energy source, is

mobilized and concentrations of NEFA begin to rise (Zurek et al., 1995). Milk production also

requires fatty acids, which are supplied by dietary fatty acids or NEFA during times of NEB

(Pullen et al., 1989). Many peripheral tissues are capable of using NEFA for energy during

times of NEB (Boden, 1998). However, up to 25% of NEFA are removed from plasma by the

liver (Bergman, 1971; Reid et al., 1979). Bell (1979) showed that hepatic uptake of NEFA is

concentration dependent, meaning that increased serum concentrations will result in greater

hepatic uptake. The liver is responsible for converting NEFA into TG for packaging and export

as VLDL (very low density lipoprotein) (Katoh, 2002). Ruminants have a poor ability to secrete

TG as VLDL compared to non-ruminants (Pullen et al., 1990). Previously, Pullen and others

(1988) used sheep to show that TG within the hepatocyte that is not immediately exported as

VLDL will remain in an intracellular storage pool. The poor ability of the liver to increase

VLDL export coupled with increased transport of NEFA into the hepatocyte results in accumulation of intracellular TG and subsequent fatty liver.

The production and export of VLDL from the liver seems to be the limiting factor in prevention of fatty liver, as hepatocellular VLDL export does not increase at the same rate as import of NEFA. Therefore, it is of great importance to understand the mechanisms involved with VLDL export to determine why VLDL production does not increase during times of NEB. The purpose of VLDL secretion by the liver is for transport of TG to peripheral tissues like muscle, which use the TG for energy during times of NEB.

A great deal of interest has been given to apolipoprotein B-100, a protein required for stabilizing VLDL particles (Gibbons, 1990). Herdt and others (1983) determined that cows with fatty liver have a reduction in serum VLDL concentrations, suggesting that fatty liver may occur as a result of reduced ability to export VLDL. During early lactation the concentration of apoB-100 is low (Marcos et al., 1990) relative to other stages of lactation. This is surprising, as early lactation is a time when apoB-100 concentrations are expected to be high due to the increased need for VLDL secretion. Interestingly, Gruffat and others (1997) determined that apoB-100 mRNA is not significantly lower during early lactation indicating that there may be a post-transcriptional alteration in apoB-100 concentration. However, in studies by Bernabucci and others (2004 & 2009) both the mRNA abundance and protein concentrations of apoB-100 were decreased in early lactation. Recently, Bernabucci and others (2009) showed that plasma levels of apoB-100 begin decreasing two weeks prior to calving, reached a nadir at 3 days postpartum before beginning to rise. The pattern of decreasing plasma apoB-100 throughout the transition period inversely relates to the accumulation of TG in hepatocytes (Bertics et al., 1992). This finding suggests that apoB-100 may be partially responsible for decreased VLDL export and

subsequent TG accumulation and fatty liver. Recently, there has been some investigation into supplemental choline as a method to reduce liver TG by increasing VLDL export. Yao and Vance (1990) found that choline deficiency in rats caused a six-fold increase in liver TG. This may be due to the fact that choline is a substrate for phosphatidylcholine, which is a component of VLDL (Grummer, 2008). Therefore, if peripartum cows have a choline deficiency, there would be a potential for decreased export of VLDL from hepatocytes. The decreased apoB-100 concentration that occurs around parturition is still unexplained, but current research is pursuing the theory that oxidative damage may cause destruction of newly synthesized apoB-100 (Bernabucci et al., 2009).

Ketosis

One of the most commonly recognized diseases associated with fatty liver is ketosis, likely because their etiologies are related. The incidence of ketosis has been reported as 30% in cows with fatty liver, while only 10% in those without fatty liver (Grohn et al., 1987). Ketosis is defined as increased levels of circulating ketone bodies, which are acetone, acetoacetate and beta-hydroxybutyrate (BHBA) (Duffield, 2000). The prevalence of clinical and subclinical ketosis ranges from 2 to 15% and 8.9 to 34%, respectively (Duffield, 2000). Ketone bodies provide an essential source of energy during times of NEB (Herdt, 2000).

There are three main metabolic fates of NEFA entering the hepatocyte; conversion to TG and export as VLDL, conversion to TG without export (resulting in fatty liver), and mitochondrial metabolism by beta-oxidation (Van den Top et al., 2005). During times of positive energy balance, the mitochondrial pathway is minimal. However, during times of NEB, beta-oxidation is essential in utilizing the energy of stored adipose tissue (Webber et al., 1994). Besides its use as a fuel to other body tissues, BHBA are used in small amounts for milk fat

synthesis (Palmquist et al., 1969). Within the mitochondria of the hepatocyte, NEFA undergo β-oxidation into acetyl-CoA and reducing equivalents used in energy metabolism. Acetyl-CoA can enter the TCA (tricarboxylic acid) cycle and be used for energy production (Katoh, 2002). However, during early lactation when gluconeogenesis is increased, other components of the TCA cycle are depleted and acetyl-CoA cannot enter the TCA cycle and are diverted to ketogenesis (Krebs, 1966).

The underlying causes of ketosis are generally the same as that for fatty liver. Dry period overfeeding coupled with negative energy balance overwhelms the liver with NEFA. Excessive delivery of NEFA to the liver at a time when hepatic gluconeogenesis is limited results in both TG storage in the cytoplasm and increased mitochondrial production of ketone bodies. Veenhuizen et al. (1991) induced fatty liver and ketosis by feed restriction and administration of 1, 3-butanediol. They found that fatty liver occurred following a rise in serum ketones, indicating that fatty liver follows ketosis. However, other reports indicate that fatty infiltration of the liver results in decreased gluconeogenesis (Cadorniga-Valine et al., 1997), which could cause increased lipolysis and subsequent ketosis. This would suggest that ketosis occurs as a result of fatty liver. Although the exact number of mechanisms connecting fatty liver and ketosis is unknown, it is clear that their etiologies are inter-related and should be further explored.

Other Associated Diseases

Fatty liver is frequently associated with left displacement of the abomasum (LDA) (Rehage et al., 1996). One of the early signs of LDA is decreased feed intake, which results in greater mobilization of lipid and subsequent fatty liver (Herdt et al., 1983). A study by Rehage et al. (1996) looked at liver TG, BCS, DMI and serum BHBA and NEFA in 53 cows with LDA on both the day of surgery and 4 days later. They found that 32% and 40% of cows had severe and

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moderate fatty liver at presentation, respectively. Cows with moderate and severe fatty liver had higher BCS compared to those with mild or no fatty liver. They also found that cows with severe fatty liver had lower intakes on the day of surgery and remained low 4 days later as well. This resulted in significantly lower milk production during the post-surgical period. The serum concentrations of NEFA and BHBA in cows with severe fatty liver were significantly increased on both the day of surgery and 4 days later compared to cows mildly or moderately affected. These results indicate that fatty liver is associated with LDA during early lactation, and post-surgical prognosis is related to the severity of fatty liver upon presentation.

Postparturient hypocalcemia (milk fever) has been associated with fatty liver and increased NEFA (Katoh, 2002). Oikawa and Katoh (2002) surveyed cows with milk fever and downer-cow syndrome to determine their association with fatty liver. Downer cows were defined as recumbent cows with normal serum calcium concentrations and no other identifiable disorders. He determined that cows with milk fever had decreased concentrations of serum apoB-100 and increased NEFA, and downer cows had decreased serum apoB-100 as well. In unpublished data from Oikawa, 60-70% of downer cows had fatty liver when investigated postmortem. Fatty liver was defined as greater than 30mg TG/g of liver tissue (wet weight). These data suggest that fatty liver may play a role in the etiology of milk fever and downer cow syndrome.

Inflammation

Fatty liver is associated with many infectious diseases during the postpartum period as well. Hill et al. (1985) investigated the effect of liver TG concentration on elimination of experimentally induced E. coli mastitis. They found that 80% of cows with liver TG less than 20.2% eliminated the E. coli from their udder within 12 hours after inoculation. However, all

cows with liver TG greater than 28.3% had quantitative bacteria for longer than those less than 28.3%. These findings suggest that fatty liver may be associated with immunological depression. Wentink et al. (1997) found that cows with fatty liver had decreased cell-mediated (lymphocytic) and humoral (antibody) responses following vaccination with tetanus toxoid. In a follow-up study by Wentink and others (1999), the lymphocytic response to skin transplantation was compared between feed-restricted and control animals. The feed-restricted animals had significantly higher liver TG levels and lower lymphocyte accumulation around the skin graft. This may establish a cause-effect relationship between hepatic lipidosis and impaired immune responses.

Surprisingly, inflammation itself has been implicated in the etiology of fatty liver. During the postpartum period, additional energy is supplied by lipolysis. In an article by Hotamisligil (2006), a connection between metabolic dysfunction and inflammation was established based on the key role of inflammation in human diabetes. There is evidence that obese animals exhibit what he refers to as "metaflammation", which is metabolically induced inflammation. There is also strong evidence that tumor necrosis factor-alpha (TNF), an acute inflammatory cytokine, plays a role in fatty liver in rodent models (Li et al., 2003). Administration of antibodies against TNF prevents or resolves fatty liver in genetically obese mice (Li et al., 2003). TNF is produced in adipose tissue and is increased in cows with fatty liver (Ohtsuka et al., 2002). Additionally, TNF deficient mice have decreased levels of circulating free fatty acids and do not exhibit obesity or insulin resistance as compared to controls (Uysal et al., 1997). This may provide an additional link between obesity and fatty liver in dairy cows, explaining why obese cows are more likely to have fatty liver.

Bradford et al. (2009) administered recombinant bovine TNF-alpha to late-lactation cows and measured liver lipid accumulation along with abundance of 3 enzymes involved with gluconeogenesis. Treatment with TNF-alpha resulted in accumulation of liver TG along with decreased mRNA abundance of 2 gluconeogenic enzymes, glucose-6-phosphatase and phosphoenolpyruvate carboxykinase. This is consistent with results by Ohtsuka and others (2002) in that TNF is increased in cows with fatty liver, likely causing activation of adipose tissue lipolysis. These results further support inflammation as a component in the etiology of fatty liver.

Treatment

The treatment of fatty liver is similar to the treatment of ketosis. The goals in treating fatty liver are to increase serum glucose concentrations, decrease lipolysis and increase hepatic TG output. As previously discussed, increasing hepatic output is difficult due to the poor ability of ruminants to secrete TG as VLDL (Pullen et al., 1990). Since hypoglycemia is a major drive for lipolysis, increasing glucose concentrations should also decrease lipolysis. There are few studies that have found successful treatments of fatty liver, but many studies focusing on treatment of ketosis have also been found to reduce liver TG accumulation.

Increasing glucose concentration can be accomplished in several ways. Intravenous infusion glucose in cattle has been studied (Gruchy et al., 1963). Although infusion is successful at increasing glucose concentrations, its effects are short-lived, as glucose returns to baseline 80-100 minutes after infusion is stopped (Shaw, 1956). Another method of increasing glucose concentrations is through the use of glucocorticoids such as prednisone and dexamethasone. Shaw (1956) showed that glucocorticoids cause hyperglycemia in non-postparturient cows, but the increase was not as great in ketotic cows. However, the positive effects of glucocorticoids are

increased when glucose is concurrently administered. Shpigel et al. (1996) performed a clinical trial in 127 ketotic cows with urine acetoacetate concentrations greater than 60mg/dl. Treatments consisted of 40 mg of dexamethasone with or without 500ml of 50% glucose intravenously or 5mg flumethasone with or without 500ml of 50% glucose intravenously. Those cows who concurrently received IV glucose had increased plasma glucose concentrations and decreased serum BHBA and urine acetoacetate concentrations following treatments. Although treatment with glucose and gluconeogenic compounds is effective in individual cows, the benefits are usually short-lived and cannot currently be administered easily on a herd basis.

Glucagon may be a more effective treatment for fatty liver. Glucagon improves carbohydrate status by increasing hepatic gluconeogenesis and glycogenolysis (Bobe et al., 2003). Hippen et al. (1999) attempted the first use of glucagon for the alleviation of fatty liver. During days 14 to 42 postpartum, cows were subjected to a protocol to induce fatty liver. Intravenous continuous infusions of glucagon were given from days 21 to 35 postpartum. Immediately following initiation of treatment with glucagon, serum glucose concentration was increased and liver TG concentration was decreased. Even 3 days after treatment ended, liver TG was 4.6% for glucagon treatment compared with 15.7% in control cows. Since intravenous infusions of glucagon are not practical, Bobe and others (2003) tested the hypothesis that subcutaneous glucagon injections would also treat fatty liver based on improved carbohydrate status. Saline, 2.5 mg or 5 mg of glucagon were injected subcutaneously every 8 hours beginning at 8 days postpartum. Glucagon injections increased plasma glucose concentration and decreased plasma NEFA concentration, however only the 5 mg treatment decreased concentrations of hepatic TG. Interestingly, this effect was only seen in cows over 3.5 years of age. The authors

speculated that this may have been due to differences in how gluconeogenic precursors are partitioned in young animals that are still growing.

Oral supplements of glycerol, propylene glycol and sodium propionate have been used to increase glucose concentrations and treat ketosis. Osman et al. (2008) compared treatments of subcutaneous glucagon, oral glycerol or both on altering plasma glucose, insulin, NEFA, BHBA and TG. When used in combination, glucagon and glycerol treatment increased plasma glucose and insulin along with decreasing NEFA and BHBA. Although liver biopsies were not collected during this experiment, combination treatment increased plasma TG concentrations. This suggests that treatment may have caused increased disposal of liver TG through improved export of hepatic VLDL.

Prevention

The practices for preventing fatty liver are similar to those used for treatment (Bobe et al., 2004). Use of glucagon (Nafikov et al., 2006) at 15 mg/day and prednisone (Fürll et al., 1993) at 200 mg/day have been successful at preventing accumulation of liver TG when administered in the early postpartum period. Propylene glycol, a glucogenic precursor, has been successful at preventing ketosis (Emery et al., 1964) and decreasing plasma NEFA (Sauer et al., 1973) in postpartum cows. Studer and others (1993) tested the hypothesis that prepartum administration of propylene glycol could prevent postpartum accumulation of liver TG by increasing plasma glucose. They administered 1 liter of either propylene glycol or water once daily for the last 7 days prior to calving. They found that liver TG accumulation was reduced by 32 and 42% at 1 and 21 days postpartum respectively. It is likely that postpartum liver TG was decreased due to the reduction in prepartum NEFA concentrations in treated animals. The author speculated that the effects of propylene glycol are indirect on decreasing plasma NEFA, and that

likely the stimulation of insulin secretion led to decreased lipolysis and subsequent reduction in hepatic TG. Christensen and others (1997) showed that oral drenching of propylene glycol is more effective at reducing plasma NEFA than when administered as part of the TMR. Pickett and others (2003) found that oral drenching of propylene glycol once daily for 3 days postpartum reduced plasma NEFA and BHBA during the first 21 and 7 days postpartum, respectively.

Undoubtedly, one of the easier methods of preventing fatty liver would be from a dietary additive. Feeding supplemental fat or B-vitamins such as niacin and choline has been extensively studied. Grum et al. (1996) found that feeding supplemental fat from dry-off until 7 days prepartum resulted in decreased liver TG compared to high grain and control diets. The reduction in liver TG continued through 3 weeks postpartum. Although high-fat diet cows had decreased liver TG, prepartum plasma NEFA tended ($P < 0.08$) to be higher compared to high-grain and controls diets. However, the spike of NEFA at parturition was not seen with the high-fat diet, whereas the high-grain and control diets saw the typical increase in NEFA following parturition. The rate of peroxisomal β-oxidation tended ($P < 0.09$) to be higher in high-fat cows, indicating that prepartum increases of β-oxidation may have prevented the increase in liver TG accumulation. However, feeding prepartum fat has not consistently prevented fatty liver (Duske et al., 2009). Further research is needed to understand the role of prepartum dietary fat on postpartum lipid metabolism.

Feeding supplemental niacin has been extensively researched and will be discussed in greater detail later in this review. Supplemental choline has also been examined, as choline is a precursor for VLDL assembly in the liver (Guretzky et al., 2006). In rats, choline deficiency resulted in a six fold increase in hepatic TG accumulation (Yao and Vance, 1990). Ruminants rapidly degrade dietary choline, so a rumen-protected form must be used (Atkins et al., 1988).

Cooke et al. (2007) conducted a series of experiments feeding rumen-protected choline (RPC) and measuring both hepatic TG accumulation during fatty liver induction and hepatic TG clearance following induction of fatty liver. They found that not only does RPC decrease hepatic TG accumulation, it also promotes TG clearance. Previously, Piepenbrink et al. (2003) found no difference in liver TG between cows fed RPC and controls, but hepatic glycogen content increased as the intake of RPC increased. This study fed varying amounts of choline from day 21 prepartum until day 63 postpartum, whereas Cooke et al. fed choline during experimental induction of fatty liver. Guretzky et al., (2006) also fed RPC from days 21 prepartum until day 21 postpartum. They only measured blood metabolites (NEFA, BHBA TG, cholesterol and phospholipids) and found that RPC had no effect on any of the measured parameters. Zahra and others (2006) fed RPC to transition cows from 3 weeks prior to 28 days after parturition. They found that cows with BCS > 4 produced 4.4 kg/day more milk during the first 60 DIM and ate 1.2 kg/day more dry matter from 3 weeks prior to 4 weeks after parturition. The results of these studies may imply that there may be numerous variables during the transition period that play a role in the efficacy of RPC. Despite much research into preventatives for fatty liver, a reliable single preventative has not been identified. Elimination of risk factors is the most reliable strategy for prevention of fatty liver.

Diagnosis

The diagnosis of fatty liver presents a challenge to both producers and veterinarians. Accurate clinical diagnosis is often difficult because signs are both vague and nonspecific and may be masked by concurrent diseases (Herdt et al., 1983). As cows develop moderate fatty liver they often lose a significant amount of body weight (Jorritsma et al., 2001) and have depressed

DMI (Veenhuizen et al, 1991). Currently, the only reliable method of diagnosing fatty liver is by histological or biochemical analysis of liver tissue (Bobe et al., 2004). This method is impractical for on-farm diagnosis because it requires minor surgery, poses a risk of infection and can be lethal if a major blood vessel is punctured (Bobe et al., 2008). Several attempts at non-invasive diagnostics for bovine fatty liver have been investigated. Ideally, diagnosis of fatty liver would be done with a rapid cow-side test that is both sensitive and specific along with being cost-effective to the producer.

In order to diagnose fatty liver histologically or biochemically, a liver biopsy must be taken (Bobe et al., 2008). Grohn and Lindberg (1982) showed the reliability of antemortem percutaneous needle biopsies for microscopic examination of liver tissue. Gaal and Husveth (1983) then determined that both histological liver fat estimation and biochemical analysis of liver TG content are equivocal methods of assessing liver fat infiltration. There are several methods described for processing and analyzing liver biopsies. Typically, the tissue is frozen using liquid nitrogen and stored at -80 degrees Celsius until analysis (Duske et al., 2009). Some methods have utilized a pre-freeze rinse with saline (Cooke et al., 2007) or phosphate buffered saline (Grum et al., 2002). For histological analysis, the tissue can also be placed in 10% formalin for several hours prior to fixing in paraffin wax (Basoglu et al., 2002; Kalaitzakis et al., 2006). The sample can then be sliced and analyzed histologically for degree of fatty infiltration. Histological grading of fatty liver is ultimately subjective, however several grading classifications have been developed (Bernabucci et al., 2009; Kalaitzakis et al., 2007). Bernabucci et al. (2009) described a system based on both micro and macro-vesicular characteristics that depending on both cellular lipid accumulation and location within the hepatic lobule. Kalaitzakis and others (2007) described a scale ranging from 0 = normal to 5= pan-

lobular infiltration of lipid. In this method several locations were graded within the lobule based on individual cell swelling and amount of vacuolization. Biochemically, liver tissue is measured for total lipids or TG content (Kalaitzakis et al., 2007). In a review by Bobe and others (2004), a system was modeled for classifying severities of fatty liver into normal, mild, moderate and severe based on liver TG content. On a wet weight basis, normal liver is <1% TG, mild fatty liver is 1-5%, moderate fatty liver is 5-10% and >10% is severe fatty liver. However, other studies have varied in their exact system for classifying severities of fatty liver. This is likely because changes associated with increasing liver TG are not consistent between cows because liver TG is only an indirect measure of decreasing hepatocyte function (Johannsen et al., 1993). Although there is variability between certain classification systems, histological and biochemical analysis of liver remain the current gold standard and are used to measure the reliability of other diagnostic techniques.

Numerous investigators have studied whether serum biochemical variables can aid in the diagnosis of fatty liver. Several clinical tests performed on serum can aid in the diagnosis of fatty liver, however a reliable combination of tests have not been determined (Kalaitzakis et al., 2007). There are several parameters that can be done on serum to directly or indirectly measure hepatic function, such as total bilirubin (tBIL), NEFA, glucose, cholesterol, albumin, bile acids and ketones. Hepatocyte enzymes that are released into the bloodstream during periods of cellular stress include aspartate aminotransferase (AST), glutamate dehydrogenase (GDH), sorbitol dehydrogenase (SDH), gamma glutamyltransferase (GGT), ornithine carbamoyl transferase (OCT), alkaline phosphatase (ALP), and alanine aminotransferase (ALT) (Kalaitzakis et al., 2007). Tsuchiya et al. (1994) determined that plasma ornithine carbamoyl transferase (OCT) is a reliable indicator of liver necrosis in ruminants. Recently, OCT has been investigated as a

diagnostic tool for the severity of fatty liver. Severity of fatty liver and postsurgical convalescence following LDA surgery can be predicted using plasma OCT activity (Kalaitzakis et al., 2006). In that study, OCT, AST, GDH and tBIL were found to be helpful in diagnosis of fatty liver in cows with LDA, however OCT had higher sensitivity and specificity for differentiating the severity of fatty liver. In a later study by Kalaitzakis et al. (2007), they further agreed that AST, tBIL and OCT have the diagnostic ability to differentiate between mild and severe fatty liver. Since increased liver TG results in varying effects on hepatocytes resulting in varying cellular function and stress, serum parameters alone are not a reliable method of diagnosing fatty liver.

Reid et al. (1984) investigated the relationship between liver fat content and a complete blood count in 369 dairy cows during the 2nd week of lactation. The complete blood count consisted of an erythrocyte count, packed cell volume, hemoglobin concentration and a white blood cell count (WBC). They found that there was a significant ($P < 0.01$) reduction in WBC count in cows with greater than 20% liver fat. However, the increased incidence and severity of low WBC associated with fatty liver may be due to the increased incidence of infectious diseases seen in cows with fatty liver (Fronk et al., 1980; Hill et al,. 1985).

Ultrasonography has been investigated as a means for estimating liver triglyceride content. The use of ultrasound has been effective in diagnosing liver abscesses in cattle (Lechtenberg & Nagaraja, 1991). However, the challenging aspect of ultrasonic diagnosis of fatty liver is that the changes seen in hepatic echogenicity are much less distinct than in a focal hepatic abscesses (Nyland et al., 2002). In cows, fat deposition during fatty liver is generally a diffuse process occurring in all lobes of the liver (Mohamed et al., 2004). The echogenicity of tissue is a result of the number of solid-liquid interfaces. Since fatty liver is a result of increased

TG molecules within the parenchyma of the liver, the resulting image is more echogenic compared to normal (Thijssen et al., 2008). Although severe cases of fatty liver are relatively easy to diagnose using an ultrasonic image (Mohamed et al., 2004), the increasing echogenicity can be difficult to detect in less severe cases. This makes subjective interpretation an unreliable method for determining the severity of fatty liver.

A complicating aspect of ultrasound diagnosis is that the result is ultimately subjective to the experience of the user and the variability between users (Nyland et al., 2002). In a study by Acorda et al. (1995) they found that ultrasonic classification of fatty liver was only 49% accurate when analyzed visually by the user. Bobe et al. (2008) investigated if analysis of ultrasonograms could accurately detect the degree of hepatic TG infiltration. They used 49 liver biopsies and ultrasounds taken during the first two weeks postpartum. A digital analysis was performed using a computer program that calculated several first and second-order parameters on the image. The first order parameters measured the distribution of gray scale levels in the area of interest, while the second-order parameters were based on the difference of gray scale levels between adjacent pixels. Results showed that combining 17 of the parameters correctly classified 82% of the liver samples into normal, mild, moderate and severe. This indicates that there is potential to utilize ultrasound with digital analysis as a reliable estimator of liver TG content. These results are significant because this could provide a rapid, non-invasive on-farm diagnosis and allow for more specific treatment protocols.

Nonalcoholic steatohepatitis (NASH) is a form of nonalcoholic fatty liver disease affecting about 80 million Americans (Feldstein et al., 2009). The first step in the pathogenesis of NASH is the accumulation of lipids in the liver and recent evidence has shown that apoptosis plays a significant role in the progression of NASH (Feldstein and Gores, 2005). During the

apoptotic process, several caspases are activated that aid in the breakdown of several cellular substrates. Caspases-3, 6 and 7 are responsible for cleaving the major intermediate filament protein, cytokeratin-18 (CK18) (Hetz et al., 2007). The fragments of CK18 can be found in the blood of patients with progressing NASH, which is useful for differentiating patients with NASH from simple fatty liver (Wieckowska et al., 2006). A monoclonal antibody test has been developed that recognizes a neoepitope on CK18 (Leers et al., 1999). The exact role of apoptosis in bovine fatty liver has not been investigated to the same degree as in human medicine. Currently, no studies have been published on the role of CK18 in bovine fatty liver. This leaves the possibility for CK18 to be a novel indicator and provide another minimally invasive diagnostic for fatty liver in dairy cattle. Although diagnosis of fatty liver remains a challenge in the field, there are some promising potential alternatives. Further research is needed in the areas of an ultrasound or blood-based diagnosis.

Niacin

One of the more recently studied treatments for fatty liver in dairy cattle is niacin. Niacin has been used in human medicine for over fifty years since it was discovered to have a potent lipid modifying effect (Carlson, 2005). It wasn't until the last thirty years that niacin has been viewed as a potential treatment for fatty liver in transition dairy cows. Research has shown that ruminal microbes have the ability to synthesize niacin, which is positively related to microbial protein synthesis (Shields et al., 1983). This has led nutritionists to believe that dietary intake along with ruminal synthesis is adequate for the needs of a dairy cow. However, deficiencies of B-vitamins may occur in mature dairy cattle when requirements are high, like in high producing

dairy cows (Harmeyer and Kollenkirchen, 1989). This deficiency theory has also been supported by the positive effects of supplementing dairy cows with niacin during the transition period.

Niacin is the general term for vitamin B3, which consists of two vitamers, nicotinic acid (NA) and nicotinamide (NAM) (Bender, 1992) that differ by the exchange of a hydroxide ion (NA) for an amine (NAM). Carlson (2005) described niacin as having two faces: one is a vitamin potent in milligram doses; the other is a lipid drug potent in gram doses. Niacin as a vitamin is required in milligram doses in both humans (Carlson, 2005) and cattle (NRC, 2001) for maintenance of cellular metabolism. Niacin is required as a precursor for the coenzymes NAD and NADP (DiPalma and Thayer, 1991), which act as reducing equivalents in cellular metabolism. Most species, including cattle, are able to synthesize niacin from the amino acid tryptophan (Foster and Moat, 1980) and quinolinate (Gholson et al., 1963) to support their requirement. However, microorganisms are able to synthesize quinolinate from aspartate and dihydroxyacetate thus providing ruminants with an additional source of niacin.

The use of niacin as a lipid modifying drug has been extensively studied for over 50 years. In 1955, Rudolf Altshul discovered that gram-sized doses of NA substantially lowered plasma cholesterol in humans (Altshul et al., 1955). It was later discovered that cholesterol was lowered primarily by decreasing LDL cholesterol (Parsons and Flinn, 1959). Nicotinic acid was later shown to prevent a rise in free fatty acids (FFA) following treatment with norepinephrine (Carlson and Oro, 1962). The decrease in FFA was shown to be due to the rapid accumulation of NA on adipose tissue (Carlson and Hanngren, 1964). This was the first sign showing that NA directly acts on adipose tissue to prevent lipolysis. Carlson theorized that reduction in FFA release from adipose tissue would prevent FFA delivery to the liver and subsequent VLDL production. A reduction in plasma triglycerides (VLDL) and decreased LDL concentrations

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would therefore result in decreased cholesterol concentration. Although the exact mechanism is not known, NA is also the most potent HDL-raising drug known (Carlson, 2005). This is an important finding in human medicine, as low LDL and high HDL concentrations likely reduce the risk of coronary heart disease.

Interestingly, the lipid modifying effects of NA are not seen with NAM treatment. Several studies have shown that NAM does not prevent FFA release (Altshul et al., 1955; Parsons and Flinn, 1959; Carlson and Oro, 1962). Lorenzen et al. (2001) discovered a G-protein coupled receptor on adipose tissue with a high-affinity for NA. This receptor was later identified as HM74A (Wise et al., 2003) and has high affinity for NA with only low affinity for NAM. This was the first evidence as to why NAM does not share the same lipid modifying effects as NA. Niacin binds with HM74A causing inhibition of adenyl cyclase activity and a subsequent reduction of intracellular cAMP, leading to suppression of lipolysis. This receptor has been identified in other species as well (Gille et al., 2009) and is referred to as GPR109A. Another ligand for GPR109A is BHBA (Gille et al., 2009), which is of considerable interest, especially in postpartum dairy cows. If BHBA acts on GPR109A to inhibit lipolysis, this may identify a negative feedback loop for lipolysis in cattle during ketosis. Recent work by Bradford and others (2009a) has identified the GPR109A receptor in adipose, liver, muscle and brain of steers. This novel distribution of the niacin receptor may prove to be important in determining the response of niacin in dairy cattle.

There are several reasons why niacin is of interest in dairy cattle. One of the major side effects of NA is humans is the "flush", or reddening of the skin following NA treatment. This is caused by cutaneous vasodilation mediated by prostaglandin release (Kaijser et al., 1979) and is the most common cause for human patients to stop taking NA (Carlson, 2005). Di Costanzo et al.

(1997) investigated whether the vasodilator effects of NA could decrease heat stress in lactating dairy cows. They found that there was decreased cutaneous temperature with NA treatment, but no difference in rectal temperature. They theorized that cows experienced cutaneous vasodilation and therefore had increased evaporative heat loss. Similar results were found by Zimbelman and others (2010) as well. Another interest in niacin for dairy cows is from work done by Carlson et al. (1967) where a 90% reduction of hepatic ketone production was seen in diabetic humans immediately following NA treatment. This suggested that niacin had the potential to decrease the incidence of ketosis in postpartum dairy cows. Finally, the reduction in lipolysis seen with NA treatment could be of tremendous benefit to postpartum dairy cows in preventing the development of fatty liver.

Although oral treatment of NA in humans is beneficial, the data cannot be directly extrapolated to cattle. The rumen is able to synthesize large amounts of both NA and NAM (Huntgate, 1966; Santschi et al., 2005). However, NAM is absorbed from the rumen in higher amounts than NA, but only in small quantities (Erickson et al., 1991). This is because most ruminal niacin is within the microbes and only 3-7% is in the free fluid (Erickson et al., 1991). Most of the absorption occurs in the small intestine (Rerat et al., 1959), making it a challenging task to supplement cattle. The amount of niacin synthesized in the rumen is related to the amount supplemented. Riddell and others (1983) measured total niacin concentration in rumen fluid in vitro 6 hours after incubation. When no niacin was added, there was a 79% increase in total niacin concentration, however, as niacin supplementation increased, the amount of total niacin decreased. If niacin was supplemented at 2ppm and 8ppm, the total niacin concentration decreased by 13% and 20%, respectively. This indicated that synthesis of niacin not only decreases as supplementation increases, but may actually result in increased degradation as well.

Although niacin is delivered to the duodenum, over 90% of is contained in the microbial population and 50% is incorporated into the coenzymes NAD and NADP (Harmeyer and Kollenkirchen, 1989). This means that very little niacin reaches the small intestines in the form of NA or NAM.

The ruminal environment also plays a role in conversion of NA and NAM, as most NAM is converted rapidly to NA (Campbell et al., 1994). Differences between NA and NAM within the rumen are still not fully understood. NAM has been found to increase fiber digestibility in vitro, whereas NA had no effect (Hannah and Stern, 1985). In that study, neither supplement had any effect on microbial protein production. Jaster and Ward (1990) investigated the effects of 6g of either NA or NAM from 2 weeks prior to 12 weeks following parturition in 30 Holstein cows. They found that both NA and NAM decreased BHBA in week 4, while only NAM increased glucose and reduced FFA during week 4. This is in contrast to human medicine, where NAM is ineffective at reducing FFA and therefore may be the result of ruminal conversion of supplemented NAM to NA. In 1994, Campbell and others supplemented 4 cows in a 4X4 Latin square with either 12 g/d NA, 12 g/d NAM, 6 g/d NA and 6g/d NAM, or neither NA or NAM. Although they tested for both NA and NAM in rumen and duodenal fluid, only NA was detected in samples. As expected, all supplemented cows had higher NA concentrations in both ruminal and duodenal fluid than control cows. Interestingly, NAM cows had significantly higher NA in the duodenum than NA supplemented cows, further supporting that additional NAM is converted to NA.

A substantial amount of supplemented niacin is destroyed or used before it reaches the duodenum. Santschi and others (2005) found that 98% of supplemented niacin was unaccounted for in the duodenum. This indicated that niacin was either degraded in the rumen or absorbed in

one or more of the forestomachs or duodenum prior to reaching the duodenal cannula. In that same study they found that 84% of niacin that reached the duodenum was absorbed in the small intestines. The amount of niacin that reaches the duodenum has also been found to vary with the forage to concentrate ratio. In a study by Schwab and others (2006), they found that higher F:C ratio diets resulted in a significant decrease in NAM flow, and tended to reduce NA flow as well. They also found a positive effect of increasing NFC content and apparent ruminal synthesis of niacin. This may be due to altering the microbial population with differing feeds, since most niacin reaching the duodenum is contained within ruminal microbes (Harmeyer and Kollenkirchen, 1989). The lower pH in the abomasum also affects the conversion of NAM to NA, as abomasal supplementation of NAM does not increase duodenal NAM, but instead increases NA (Santschi et al., 2005). Riddell and others (1985) also observed higher niacin flows to the duodenum when supplemented, but fecal excretion was equal indicating that intestinal absorption of NA was likely higher in the supplemented group.

There has been a great deal of research done on the effects of niacin in postpartum dairy cows. Since NA has the ability to suppress lipolysis (Carlson, 2005), it has the potential to decrease NEFA delivery to the liver and possibly reduce or prevent fatty liver. Dufva and others (1983) supplemented cows with 6g of NA daily beginning two weeks before calving and then increased the dose to 12g per day for 4 weeks following parturition. Plasma glucose was higher in the treated group during the week prior to parturition and treated cows had decreased NEFA in the week following parturition. Fronk and Schultz (1979) gave 12 g of NA daily to cows suffering from ketosis, as defined by low blood glucose and high BHBA and NEFA concentrations. They showed that NA increased glucose while reducing BHBA and NEFA concentrations. This same finding was seen by Ghorbani and others (2008) when cows were

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given 0, 6, or 12g of niacin per day. Although some studies have shown positive effects, there has been poor consistency on NA reducing NEFA in peripartum cows. Many studies have seen no changes in BHBA or NEFA following supplementation (Jaster and Ward, 1990; Minor et al., 1998; Christensen et al., 1996; Drackley et al., 1998). Pires and Grummer (2007) investigated if NA could suppress NEFA in feed-restricted cows. NA was given abomasally through a rumen cannula to avoid ruminal degradation as a single dose of 0, 6, 30, or 60 mg/kg after 48 hours of feed-restriction. All treatments resulted in significant reduction of NEFA within the first hour. However, by 2 hours after treatment with 6 mg/kg, NEFA increased to twice that of the control, and by 6 hours NEFA was increased about three times control for the 30 and 60 mg/kg doses. In the same experiment they gave feed restricted cows abomasal infusions of 0, 6, or 10 mg/kg NA hourly for 8 hours. The same rebound in plasma NEFA was seen within 4 hours after treatments ended, and NEFA peaked at four times the control level. Results from these experiments show that the rebound is affected by both dose and duration of NA treatment, with high-dose, longer treatments resulting in a more severe increase in NEFA. Compared to a transition cow study, this was a relatively controlled experiment and gives evidence that although not seen in every transition cow NA study, NA can significantly reduce plasma NEFA.

The recent finding by Taggart and others (2005) found BHBA as a ligand for GPR109A, making BHBA concentration a considerable interest during NA supplementation. Nicotinic acid has been shown to reduce BHBA in postpartum cows fed 6g of top-dressed NA at each feeding (twice daily) (Erickson et al., 1992). As with plasma NEFA concentrations, this reduction is not seen in all studies (Christensen et al., 1996; Drackley et al., 1998; Jaster and Ward, 1990). Although unknown, it is likely that the reduction in BHBA is due to reduction of lipolysis and rate of NEFA delivery to the liver (Erickson et al., 1990). Since NEFA are not consistently

lowered in many studies, it would be unlikely for BHBA to be decreased. Plasma BHBA increases when the rate of hepatic oxidation of fatty acids is decreased or when delivery of NEFA increases (Van den Top et al., 2005). If BHBA concentrations were to be decreased by NA supplementation, it is likely that there would be less accumulation of TG in the liver as well. Until this finding, it was assumed that the rate of ketone body production was determined by the rate of lipolysis and hepatic oxidation. This finding indicates that BHBA may play a vital role in a negative feedback loop for lipolysis. Taggart and others (2005) speculated that this may be a homeostatic mechanism for preservation during starvation and for prevention of ketoacidosis. This agrees with the speculation by Erickson and others (1990) that NA would decrease BHBA production through a mechanism inhibiting lipolysis and NEFA delivery to the liver.

One of the ways to prevent fatty liver and ketosis is to maintain blood glucose concentrations (Studer et al., 1993). Fürll (1989) found that in postpartum dairy cows producing 25kg of fat-corrected milk had 28% higher blood glucose concentrations on days 7 and 14 postpartum when given 5 g NA or NAM daily. Dufva and others (1983) also found increased blood glucose concentrations of cows supplemented with 6 g NA daily beginning 2 weeks before calving. Although blood glucose was numerically higher at each sampling, it was only significant in the final week prepartum. As with BHBA and NEFA, increased blood glucose is neither a common, nor consistent finding. The mechanism by which niacin increased blood glucose is unclear. It is possible that niacin may have reduced the rate of glucose removal from the blood, increased glucose synthesis by the liver, or both (Flachowsky, 1993). Flachowsky and others (1988) found that 0.5g and 1g of NA per day fed to sheep resulted in no increase in ruminal volatile fatty acids (VFA). However, the percentage of propionate increased at the expense of decreased butyrate. Since propionate is the most gluconeogenic VFA, this may be a

possible mechanism for increased blood glucose. However, in many studies that found increased glucose they did not measure VFA production.

Nicotinic acid may also play a role in altering insulin response following parturition. Elevated NEFA is associated with decreased response to insulin (Pires et al., 2007). This occurs in order to prioritize glucose reserves for more vital functions and milk production while increasing the mobilization of NEFA from adipose (Bell and Bauman, 1997). Oikawa and Oetzel (2006) found that insulin resistance in fasted cows was associated with increased plasma NEFA and liver TG. A decreased response to insulin could potentially exacerbate lipolysis, resulting in an increase in metabolic disturbances like ketosis and fatty liver (Pires et al., 2007). Pires and others (2007) found that hourly abomasal infusions of 6mg/kg NA decreased plasma NEFA and insulin while not having an effect on plasma glucose. This implies that there was an increased responsiveness to insulin following treatment with NA. In that same study, the authors performed intravenous glucose tolerance tests following 8 hours of infusions. The NA treated cows had significantly increased glucose clearance along with a more rapid decline in insulin concentration. This implies that high plasma NEFA concentrations are associated with insulin resistance, and reducing NEFA with NA can prevent this. However, due to a lack in current research, the exact mechanism between glucose and insulin resistance in the dairy cow is not fully understood (Grummer, 2008).

Conclusion

The transition period is a challenging time in a dairy cow's production cycle. An unavoidable negative energy balance from an increased demand for milk production coupled with decreased DMI results in lipolysis and NEFA delivery to the liver (Grummer, 2008). Typically this rate of NEFA delivery is less than the liver's capability for oxidation of fatty

acids, resulting in accumulation of TG within hepatocytes. Fatty liver occurs in over 50% of

dairy cattle, costing the industry $60 million annually (Bobe et al., 2004). Diagnosis of fatty liver

is challenging, as a liver biopsy is the only reliable method and is both invasive and rarely

performed (Bobe et al., 2008). Prevention of fatty liver presents another challenging aspect of

fatty liver, as there is no single reliable method. Niacin has proven to be a potent inhibitor of

lipolysis resulting in decreased plasma NEFA (Pires et al., 2007); however results are

inconsistent in transition dairy cattle. These inconsistencies may be due to metabolism of niacin

in the rumen resulting in variations in absorption along with having inconsistent dosages

administered. Future research should aim to find the proper dosage and delivery method of

niacin in order to investigate the ability to prevent or reduce fatty liver in postpartum dairy cows.

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CHAPTER 2 - The Use of Cytokeratin 18 as

a Marker for Fatty Liver in Dairy Cattle

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ABSTRACT

Diagnosis of fatty liver is difficult and can only be accurately performed by microscopic examination which is done in live cattle via an invasive liver biopsy. In humans, non-alcoholic steatohepatitis (NASH) can be diagnosed with an enzyme-linked immunosorbent assay (ELISA) to detect plasma cytokeratin-18 (CK18) fragments. Our aim was to determine if CK18 fragments can be detected in bovine plasma, and if they can predict liver triglyceride (TG) content. A preliminary study determined that the M6/M30 antibody pair in the CK18 ELISA had higher sensitivity for bovine liver homogenate than the M5/M30 antibody pair ($P < 0.01$). Eighty-nine primiparous and multiparous cows between 7 and 18 d in milk were used in a cross-sectional study. Liver biopsies were taken to determine the TG content, and plasma was analyzed for CK18, nonesterified fatty acid (NEFA), β-hydroxybutyrate (BHBA), TG, insulin, glucose, haptoglobin, albumin, bilirubin, lactate dehydrogenase (LDH), aspartate aminotransferase (AST), and gamma glutamyl transferase (GGT) concentrations. Data were analyzed using Pearson's correlations and ANOVA. The mean CK18 concentration was 82.2 U/L with a maximum of 440.7 U/L. Plasma CK18 did not correlate with liver TG; however AST was positively related to CK18 concentrations ($P < 0.02$). Liver TG tended to negatively correlate with LDH ($P < 0.08$) and GGT ($P < 0.07$) and was highly positively correlated with AST ($P < 0.001$). No other significant correlations with liver TG or plasma CK18 were found for other plasma parameters. Based on these results, it appears that plasma CK18 may be associated with hepatocellular damage, but is not a reliable indicator of liver TG content in dairy cows. However, the mean concentrations of NEFA (456 μM) and BHBA (624 μmol/L) indicate that the majority of the cows included in this study were not suffering from severe metabolic problems.

Key Words: fatty liver, cytokeratin-18, liver triglyceride

INTRODUCTION

The diagnosis of fatty liver presents a challenge to both producers and veterinarians. Accurate clinical diagnosis is often difficult because signs are vague, nonspecific and may be masked by concurrent diseases (Herdt et al., 1983). As cows develop moderate fatty liver, they often lose a significant amount of body weight (Jorritsma et al., 2001) and have depressed DMI (Veenhuizen et al, 1991). Currently, the only reliable method of diagnosing fatty liver is by histological or biochemical analysis of liver tissue (Bobe, 2004). This method is impractical for on-farm diagnosis because it requires minor surgery, poses a risk of infection and can be lethal if a major blood vessel is punctured (Bobe et al., 2008).

Nonalcoholic steatohepatitis (NASH) is an advanced form of nonalcoholic fatty liver disease affecting about 80 million Americans (Feldstein et al., 2009). The first step in the pathogenesis of NASH is the accumulation of lipids in the liver, and recent evidence has shown that apoptosis plays a significant role in the progression of NASH (Feldstein et al., 2005). During the apoptotic process, several caspases are activated that aid in the breakdown of cellular substrates. Caspases-3, 6 and 7 are responsible for cleaving the major intermediate filament protein, cytokeratin-18 (CK18; Hetz et al., 2007), which then diffuses into serum (Hetz et al., 2007). Fragments of CK18 can be found in the blood of patients with progressing NASH, which is useful in differentiating patients with NASH from simple fatty liver (Wieckowska et al., 2006). A monoclonal antibody test has been developed that recognizes a neoepitope on CK18 that is exposed only after caspase cleavage (Leers et al., 1999). The exact role of apoptosis in bovine fatty liver has not been investigated to the same degree as in human medicine. Currently, no studies have been published on the role of CK18 in bovine fatty liver. It is possible that CK18

could serve as a novel, minimally-invasive diagnostic tool for fatty liver in dairy cattle. The objective of this study was to determine if plasma CK18 concentration is a specific and sensitive predictor of liver triglyceride (TG) content in peripartum dairy cows.

MATERIALS AND METHODS

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

Assay Validation

Two sandwich ELISA kits are available for measuring CK18 levels in plasma; one relies on an M5/M30 antibody pair and the other an M6/M30 pair. A preliminary study was performed in order to assess the reactivity of the CK18 ELISA in bovine liver. Liver samples were taken from four steers immediately post-mortem. Approximately 12 g of tissue was collected from each animal and split into 2 samples. A 6-g sample was incubated for 2-3 h at 37°C in a mixture of 15 mL Hepatozyme-SFM (Invitrogen, Carlsbad, CA, USA) and 3 µL of 1 mM staurosporine (200 nM final concentration). Staurosporine inhibits intracellular kinase proteins, thereby inducing apoptosis in hepatocytes *in vitro* (Kihlmark et al., 2001). A 6-g control sample was immediately frozen in liquid nitrogen and stored at -80°C. After 2-3 h, the control sample was then added to Hepatozyme-SFM and all samples were homogenized, vortexed, and a 1 mL volume was centrifuged at $12,000 \times g$ for 10 min. Approximately 0.7 mL of the supernatant was removed and re-centrifuged at $12,000 \times g$ for an additional 10 min. Finally, 0.5 mL of the supernatant was removed and stored at -20° C until analysis. Quantification of CK18 fragments

was carried out using the M5/M30 and M6/M30-Apoptosense® ELISA kits (Peviva AB, Bromma, Sweden). These ELISAs use monoclonal antibodies recognizing an epitope on the 238–396 fragment of CK18 as a catcher and horseradish peroxidase-conjugated M30 antibody as a detector.

Study Design

The main experiment was an observational cross-sectional study of commercial dairy cows between 7 and 18 days in milk. Eighty cows (reference) were randomly selected between 7 and 18 days in milk from two commercial dairy farms in north-central Kansas over 4 days during the period from August 1, 2007 to October 30, 2007. An additional 9 cows (clinical) were voluntarily enrolled by their owners at the Kansas State University Veterinary Medical Teaching Hospital (KSU-VMTH) after presentation for left displaced abomasum (LDA) from August 2007 to November 2007. Liver biopsies and blood samples were collected from all cows. Liver samples from reference cows were measured for TG content and blood was analyzed for CK18 concentration along with other plasma parameters.

Data and Sample Collection

During the main experiment, reference cows were individually removed from their pens to a portable chute for liver and blood collection. Approximately 14 mL of blood was collected from the coccygeal vein, centrifuged, and plasma was stored at -20°C. Liver samples were collected using a 14-gauge $\times$ 15cm biopsy needle (SABD-1415-15-T, US Biopsy, Franklin, IN). Liver was collected between the 10th and 11th ribs, 5 cm dorsal to a line between the olecranon and tuber coxae. The area was shaved, aseptically prepared and anesthetized with 2 mL of

subcutaneous lidocaine hydrochloride. Anesthesia was assessed by cutaneous response after 5 min and a #11 Bard Parker blade was used to make a stab incision into the body wall. The biopsy needle was inserted cranioventrally towards the liver and approximately 100 mg of tissue was collected (total of 5 biopsies), snap-frozen in liquid nitrogen and stored at -80° C until analysis. Body condition score (BCS) was recorded by a trained investigator at the time of biopsy collection on a 1 to 5 scale according to Wildman et al. (1982). All reference animals were immediately given 4.4 mg/kg Ceftiofur crystalline free acid (Excede, Pfizer Animal Health, USA). Animals were evaluated 24 and 48 h following sampling for signs of infection and illness.

Clinical cows had blood collected from the coccygeal vein prior to LDA surgery. Blood samples were processed as described above. Post-operative care for clinical cows was determined by the attending veterinarian at KSU-VMTH.

Liver and Plasma Analyses

Approximately 20 mg of liver was placed into 0.3 mL of phosphate-buffered saline (pH 7.4) and homogenized. The homogenate was centrifuged at $2000 \times g$ for 10 min at 4° C. The supernatant was then removed for TG and total protein content. Triglyceride concentration was determined by an enzymatic method (TR0100, Sigma-Aldrich, St. Louis, MO, USA) and total protein by a coomassie-binding, colorimetric method (kit #23236, Thermo Scientific, Rockford, IL, USA).

Plasma was analyzed for CK18 using M6/M30-Apoptosense® ELISA (Peviva AB: Bromma, Sweden), NEFA using an enzymatic colorimetric procedure (NEFA-HR, Wako Chemicals USA, Richmond, VA, USA), glucose by colorimetric procedure (kit #439-90901, Wako Chemicals USA, Richmond, VA, USA), insulin by radioimmunoassay (DSL-1600,

Diagnostic Systems Laboratories, Inc., Webster, TX, USA), haptoglobin by bovine-specific

ELISA (#2410-7, Life Diagnostics, West Chester, PA, USA), BHBA by enzymatic reaction

(Pointe Scientific, Inc., Canton, MI, USA), albumin by a dye-binding method (Pointe Scientific,

Inc., Canton, MI, USA), direct bilirubin by colorimetric reaction (Pointe Scientific, Inc., Canton,

MI, USA), and plasma TG by enzymatic method (TR0100, Sigma-Aldrich, St. Louis, MO,

USA). Plasma concentrations of hepatobiliary enzymes were also measured. Lactate

dehydrogenase, aspartate aminotransferase, and gamma glutamyl transferase were determined by

individual kinetic methods (Pointe Scientific, Inc., Canton, MI, USA).

Statistical Analysis

Data were analyzed using the ANOVA procedure of JMP (version 8.0, SAS Institute,

Cary, NC). Pearson correlations among variables were performed on the entire dataset.

Correlations were declared significant at $P < 0.05$; comparisons with $P < 0.10$ are discussed as

trends.

RESULTS AND DISCUSSION

Assay Validation

Concentrations of CK18 in these tissue lysates are shown in Figure 3.1. Results indicate

that control samples had higher levels of CK18 than apoptotic samples ($P < 0.01$); this may be

due to the protocol used to induce apoptosis. Control samples were frozen prior to

homogenizing, whereas the apoptotic samples were incubated at room temperature in the

staurosporine mixture. It is possible that the freezing and thawing process itself induced

apoptosis, perhaps even more dramatically than the staurosporine did. Use of the M6 antibody resulted in significantly higher CK18 values in the tissue lysates ($P < 0.01$). The range of values for the M6 kit was 252 - 1394 U/L, whereas use of the M5 antibody resulted in values ranging from 34 - 291 U/L. Based on the concentrations from the liver tissue samples, it was concluded that the M6 antibody likely binds bovine CK18 more effectively than the M5 antibody. However, the higher concentrations of CK18 measured in control samples after freezing compared to staurosporine incubation was an unexpected finding which we are unable to explain. Based on these preliminary findings, the M6/M30 antibody pair was used for analysis of CK18 in the main experiment.

Liver Triglyceride

At the time of biopsy collection, the BCS of the 80 commercial cows was 2.93 ± 0.27. The range of liver TG content ranged from 0.007 to 3.63 mg/mg protein. We cannot directly compare our results to previous studies due to our methods of quantifying TG. We determined liver TG concentration by correcting mg of TG by mg of protein, which has not been published by other authors. Fronk and others (1980) found that hepatic protein content is consistent at approximately 3.6 % $\pm$ 1.2 % (wet weight). The mean TG content in our study was 0.55 mg/mg protein with a standard deviation of 0.77. Assuming hepatic protein is constant, our mean TG concentration was 2.09% (wet weight) with a standard deviation of 2.7% and the maximum TG concentration correlates with 12.96% TG (wet weight). Commonly, fatty liver is classified based on liver TG concentration (wet weight) with mild (1-5%), moderate (5-10%) and severe (>10%) (Bobe, 2004). Most cows in this study suffered from mild fatty liver, with only a few cows experiencing severe fatty liver. Since the purpose of this study was to determine if CK18 can

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predict liver TG content, this group of transition cows may not have been a good population to use.

The relationship between hepatic TG content and plasma parameters is shown in Table 3.1. NEFA tended to positively correlate with liver TG content ($P < 0.09$), which has been demonstrated by other authors (Bertics et al., 1992; Rukkwamsuk et al., 1999). This is expected, as increased delivery of NEFA to the liver is part of the etiology of fatty liver (Bobe, 2004). Concentrations of plasma NEFA in cows without fatty liver has ranged from 270-540 μM (Kalaitzakis et al., 2007; Ohtsuka et al., 2002; Veenhuizen et al., 1991; Rukkwamsuk et al., 1998), and can exceed 1,000 μM in moderate to severe fatty liver. Seifi and others (2010) found that cows with NEFA greater than 1,000 μM are 4.6 times more likely to be culled. It has also been shown that NEFA greater than 720 μM will result in decreased conception within 70 days postpartum (Ospina et al., 2010). Cows in our study had a mean NEFA concentration of 456 μM, with only 4 out of 80 cows having NEFA concentrations greater than 1,000 μM. This suggests that the prevalence of metabolic problems was low in this cross section of cows. This may have been due in part to the fact that these cows were greater than 7 days in milk. Therefore, they may not have been in a severe negative energy balance as seen in cows during the first week of lactation. During liver collection, we also found that one of the producers gave oral drenches of propylene glycol to all cows for several days after parturition. Propylene glycol suppresses plasma NEFA in postpartum cows (Osman et al., 2008), which could potentially reduce liver TG.

Serum BHBA concentration is a useful predictor of subclinical ketosis (Duffield et al., 1998) and ketosis has a close association with fatty liver (Grohn et al., 1987). Plasma concentrations of BHBA that have been used to define subclinical ketosis have ranged from 1000 to 1400 μmol/L (Duffield et al., 2000), and the mean concentration in our study was 624 μmol/L,

with a 75th percentile of 720 μmol/L and maximum of 1,927 μmol/L. These results indicate that most of the cows in this study were not suffering from ketosis, even at a subclinical level. Several authors have shown significant relationships between liver TG and NEFA (Rehage et al., 1996), BHBA (Mills et al., 1986; Rehage et al., 1996), and AST (Kalaitzakis et al., 2007). The fact that liver TG does not correlate with many of the parameters in this study is not surprising since the range of liver TG concentrations measured in this study was relatively narrow.

An unexpected negative relationship between liver TG and all three hepatic enzymes was found. Liver TG was negatively related with LDH ($P < 0.08$) and GGT ($P < 0.07$) and was highly correlated with AST ($P < 0.001$). This is interesting because these enzymes are expected to increase with hepatobiliary disease in cattle (Stockham & Scott, 2002). Kalaitzakis and others (2007) found that AST is increased with moderate to severe fatty liver compared control cows ($P < 0.05$). The AST of healthy cows in that study averaged 42.5 U/L and ranged from 29.6–71.9 U/L (2.5 to 97.5 percentiles). Values in our study were similar, averaging 42.0 U/L with a range of 10.7 - 85.2 U/L (2.5 to 97.5 percentiles). In the same study by Kalaitzakis and others, GGT did not increase as liver TG increased, and their mean GGT was similar to ours, 17 and 18.9 U/L, respectively. This is an expected finding, as GGT primarily originates from biliary epithelial cells (Stockham & Scott, 2002), and bovine fatty liver has not been shown to involve the biliary system. However, Ohtsuka and others (2002) found increased GGT concentrations in cows with mild, moderate and severe fatty liver. Based on our data, AST, GGT and LDH are not accurate predictors of fatty liver; however, our sample population included only a small proportion of cows with severe fatty liver.

CK18

The correlation between CK18 and plasma parameters is shown in Table 3.1. The only significant correlation with CK18 was with plasma AST ($P < 0.02$). Contrary to the negative correlation with liver TG concentration, the relationship of AST with CK18 is positive. No other significant relationship between any of the remaining parameters and CK18 was found. The scatter plot illustrating the relationship between liver TG and CK18 is shown in Figure 3.2. No correlation between liver TG and CK18 was found in this study ($P = 0.19$).

The statistics for plasma CK18 from the two groups of cows can be seen in the box and whisker plot in Figure 3.3. In the reference cows, CK18 values ranged from 13.4 to 440.7 U/L with a mean of 81.9 U/L. This was not significantly different from the clinical group, which ranged from 15.9 to 291.3 U/L with a mean of 85.7 U/L. Based on the previous discussion, most of the reference cows did not have moderate to severe fatty liver, indicating that this was a group of healthy transition cows. The relationship between fatty liver and displaced abomasum has been established (Wada et al., 1995; Rehage et al., 1996). Rehage and others (1996) found that at the time of displaced abomasum, 40% and 32% of cows had moderate or severe fatty liver, respectively. It was hypothesized that cows in our study with LDA would have increased liver TG and plasma CK18; however, our results do not support this hypothesis. Although we did not measure liver TG content in clinical cows, the relationship shown by Rehage and others (1996) would indicate that they had a 72% chance of having at least moderate fatty liver.

The M30 ELISA used in this experiment has been used in humans to successfully differentiate healthy patients from those with liver disease (Hetz et al., 2007). The mean CK18 concentration for healthy controls in that experiment was 66.8 ± 29.1 U/L, while means for patients with acute liver failure patients and cirrhosis were $1,993.6 \pm 247.7$ U/L, and $673.6 \pm$

86.5 U/L, respectively. Mean CK18 in this experiment was 82.2 U/L with a maximum 440.7 U/L, which is not consistent with concentrations seen in humans with liver dysfunction. Wieckowska and others (2006) determined that a cutoff value of 395 U/L accurately differentiated normal patients from those with nonalcoholic steatohepatitis with 99.9% specificity and 85.7% sensitivity. It appears unlikely that the M6/M30 ELISA can accurately predict liver TG content in cattle. However, this ELISA is not bovine-specific, and no other research has been done to assess cross-species reactivity. It is possible that similar fragments are released during bovine hepatocyte apoptosis, but the M6 antibody is not specific enough to differentiate between diseased and healthy liver. It is also possible that only the most severe forms of bovine fatty liver induce apoptosis of hepatocytes, and our population included very few of these cases.

CONCLUSIONS

Prior to this work, no research had been conducted to investigate the role of apoptosis in bovine fatty liver. We determined that the M6/M30 Apoptosense ELISA is more sensitive to bovine liver than the M5/M30. Plasma CK18 concentration was not an accurate predictor of liver TG in this study. However, few cows in this cross-section of fresh cows were suffering from severe fatty liver or ketosis; the possibility remains that plasma CK18 could be elevated in the most severe cases.

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Figure 2.1 Comparison of the M5 and M6 ELISA on liver homogenate from slaughterhouse samples. Samples were frozen, thawed and homogenized (cont), or were incubated in a staurosporine mixture (200 nM) to induce apoptosis and homogenized (Apop). Control samples had higher levels of CK18 than the apoptotic samples ($P < 0.01$). The M6 antibody resulted in significantly higher CK18 values in the tissue lysates ($P < 0.01$).
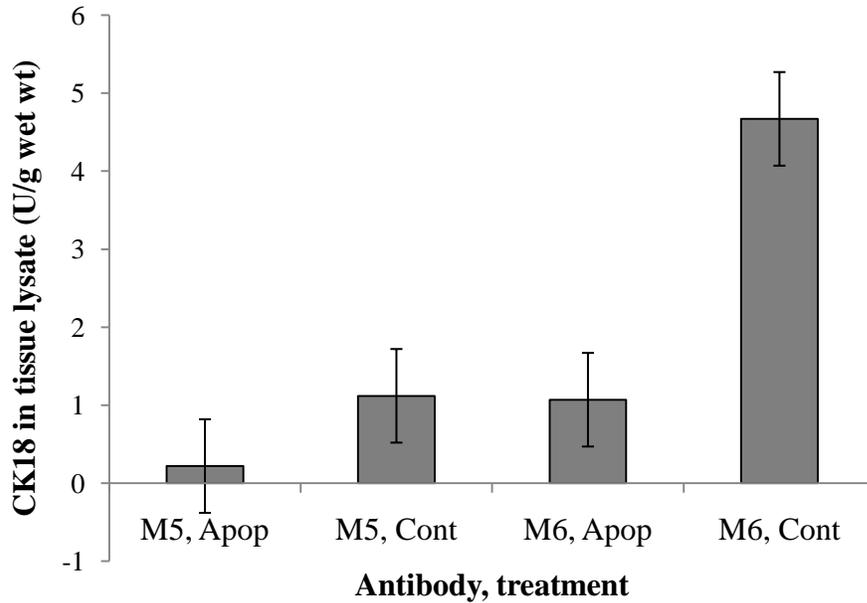
Table 2.1 Comparisons of liver TG and plasma CK18 with other plasma parameters.

	Liver TG		CK18	
	r	*P*-value	r	*P*-value
CK18	-0.148	0.19	-	-
NEFA	0.189	0.09**	0.130	0.24
Glucose	-0.086	0.44	-0.008	0.94
Insulin	-0.084	0.46	0.012	0.91
Haptoglobin	0.126	0.26	-0.066	0.56
BHBA	-0.031	0.74	0.011	0.92
LDH	-0.195	0.08**	0.046	0.69
AST	-0.401	< 0.001*	0.252	0.02*
GGT	-0.200	0.07**	-0.001	0.99
Albumin	-0.186	0.10	-0.085	0.45
Bilirubin	-0.071	0.52	-0.048	0.67
Plasma TG	0.045	0.69	-0.004	0.97
Liver TG	-	-	-0.148	0.19

*Indicates a significant relationship ($P < 0.05$)
**Indicates a trend ($P < 0.10$)

Figure 2.2 Scatterplot illustrating the relationship between liver TG and plasma CK18. No significant relationship exists ($P < 0.19$).
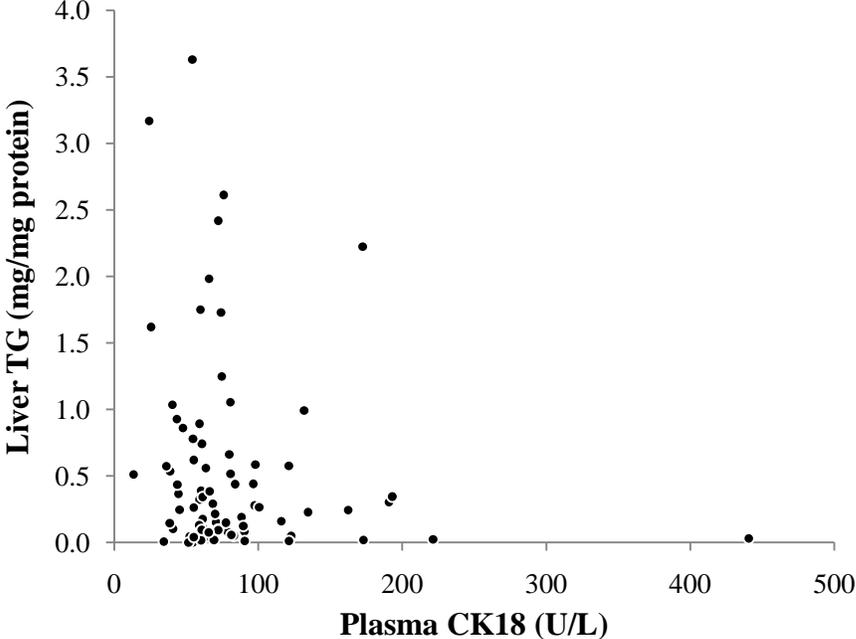
Figure 2.3 Box and whisker plot of the distribution of plasma CK18 concentrations of cross-sectional study cows (reference) and cows presenting with LDA (clinical). Mean values are represented with an X.
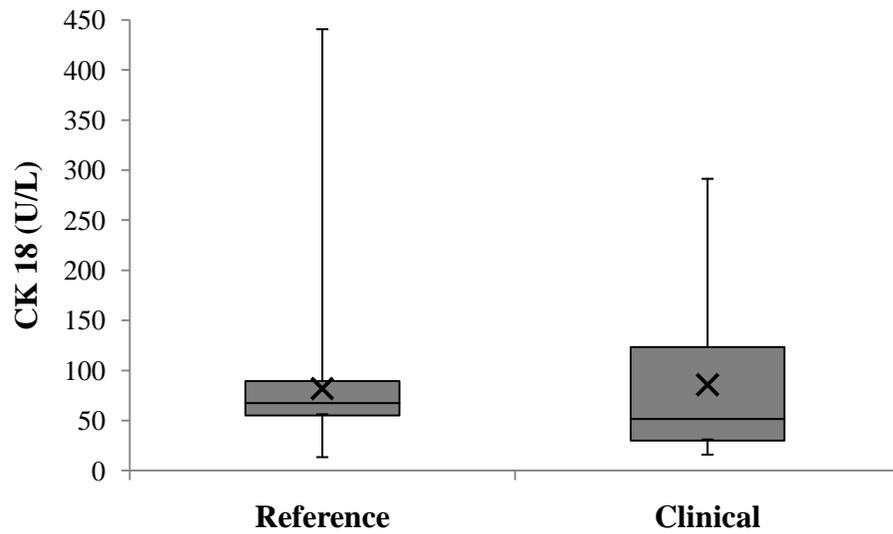
CHAPTER 3 – Effects of Encapsulated Niacin on Metabolism and Production of Periparturient Dairy Cows

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ABSTRACT

Nicotinic acid (niacin) can suppress lipolysis, but responses to dietary niacin have been inconsistent in cattle. Our aim was to determine if a relatively high dose (24 g/d) of encapsulated niacin (EN) alters lipid metabolism and productivity of transition cows. Primiparous (n=9) and multiparous (n=13) cows (BCS 3.63 ± 0.08) were included in the study beginning 21 d prior to expected calving and were sequentially assigned within parity to EN (12 g provided with ration twice daily) or control treatments through 21 d postpartum. Liver biopsies were collected on days -21, -4, 1, 7, and 21 relative to parturition for analysis of triglyceride (TG) content and mRNA abundance of the niacin receptor GPR109A. Blood samples were collected on days -21, -14, -7, -4, 1, 4, 7, 14 and 21 relative to parturition for nonesterified fatty acid (NEFA), β-hydroxybutyrate (BHBA), glucose, insulin, haptoglobin, nicotinic acid, and nicotinamide analyses. On d 7 postpartum, a caffeine clearance test was performed to assess liver function, and on d 21-23 postpartum, blood samples were collected every 8 h to monitor post-treatment NEFA responses. Data were analyzed using mixed models with repeated measures over time. There was a treatment × time × parity effect on prepartum dry matter intake (DMI, $P < 0.07$) caused by a 4 kg/d decrease in DMI of EN-treated cows compared to control cows during the final 4 d prepartum. There was a significant increase in nicotinamide (NAM) concentration at d 7 prior to parturition. There was a decrease in prepartum glucose concentration in treated animals ($P < 0.04$), with no difference in plasma insulin concentration. Treatment × time × parity effects were detected for NEFA ($P = 0.09$) and BHBA concentrations ($P < 0.02$) during the postpartum period. Plasma NEFA peaked at 1467 ± 160 μM for control animals, compared with 835 ± 154 μM for EN-treated animals ($P < 0.01$). The EN treated cows tended to have prolonged half-life of caffeine on d 7 postpartum compared to controls ($P = 0.06$; 130.7 vs. 97.6 ± 12.1 min,

respectively). After treatments ended on d 21, there was a treatment $\times$ time $\times$ parity interaction ($P < 0.09$) on plasma NEFA; however, treatment means showed a continued suppression of plasma NEFA by EN in cows, with no evidence of a rebound in either parity group. No treatment effects were observed for liver TG concentration, BCS, BW, or milk or milk component production. These results indicate that a high dose of EN can decrease postpartum plasma NEFA, but may also decrease prepartum DMI and postpartum caffeine clearance.

Key Words: niacin, transition, ketosis

INTRODUCTION

Fatty liver affects up to 50% of postpartum dairy cattle, which is costly due to milk production losses and secondary diseases, such as ketosis (Bobe et al., 2004). Fatty liver occurs when cattle enter a negative energy balance (NEB), usually during the first 2 wk of lactation (Grummer, 1993). Lipolysis occurs as a response to the NEB and results in the liver being overwhelmed by high concentrations of plasma nonesterified fatty acids (NEFA; Ingvartsen and Andersen, 2000). The high influx of NEFA to the liver is usually greater than the oxidative capacity, resulting in storage of NEFA as triglyceride (TG) within the hepatocytes (Drackley, 2001).

Use of niacin in dairy cattle is widely studied; however, results have been inconclusive or contradictory. Niacin is a B vitamin which is required in very small amounts to maintain cellular metabolism (NRC, 2001). At much higher inclusion rates, niacin (NA) also has the ability to suppress the release of fat stores (Pires et al., 2007). As a widely used commercial feed additive, NA is claimed to reduce heat stress (DiCostanzo et al., 1997) and decrease postpartum plasma NEFA concentration (Pires and Grummer, 2007). Experimentally, NA has been shown to have anti-lipolytic effects, causing an immediate reduction in plasma NEFA when given post-ruminally (Pires and Grummer, 2007).

Supplemented niacin has poor stability in the rumen and it is estimated that only 5% is bioavailable, making supplementation inefficient (Santschi et al., 2005). A rumen-protected form of NA (encapsulated niacin: **EN**) is now commercially available, providing a more effective option for dietary supplementation of niacin. This product (Niashure, Balchem Corp., New Hampton, NY) is in the form of small pellets which include a core of NA surrounded by several

layers of lipids. Since these lipids are relatively insoluble in the rumen, the majority of the

pellets exit the rumen intact, largely preventing microbial degradation of the encapsulated niacin.

The EN product has an estimated 40% bioavailable NA by weight.

Recently, the G-protein coupled receptor GPR109A was identified as having high affinity

for NA (Wise et al., 2003). Niacin binds to GPR109A, causing inhibition of adenyl cyclase

activity and a subsequent reduction of intracellular cAMP, leading to suppression of lipolysis.

This receptor also has a high affinity for β-hydroxybutyric acid (BHBA), a ketone which is of

considerable interest in postpartum dairy cows due to the high prevalence of ketosis. Recent

work by Bradford and others (2009) has identified the GPR109A receptor in adipose, liver,

muscle and brain of steers.

Until this experiment, no known studies have been conducted to explore the metabolic

and production responses to EN in peripartum dairy cows. The purpose of this study was to

determine if 24 g/d of dietary EN could suppress lipolysis enough to control plasma NEFA in

postpartum dairy cattle, thereby preventing or reducing the severity of fatty liver.

MATERIALS AND METHODS

All experimental procedures were approved by the Kansas State University Animal Care

and Use Committee.

Design and Treatments

Primiparous (n = 9) and multiparous (n = 13) Holstein cows from the Kansas State

University Dairy Teaching and Research Facility were randomly assigned within parity to

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receive either 24 grams/d EN or none (control) beginning 21 d before expected calving date and continuing until 21 d postpartum. This dose was based on a typical human dose of 1-4 g per day (Carlson, 2005), which can be extrapolated to a dose of approximately 10-40 g for an average Holstein cow. The EN product used has an estimated 40% bioavailable NA by weight, which would result in supplementation of 9.6 g/d. Cows entered the study from June 2008 to August 2008. Dry matter intake and milk production were measured daily until d 21 postpartum. Cattle were housed in a tie-stall facility in randomly assigned stalls, milked 3 times daily (0400, 1100, and 2100 h), and fed twice daily (0700 and 1500 h) according to the previous day's intake. Prepartum and postpartum diets were formulated to meet requirements (NRC, 2001), as shown in Table 3.1. All cows were fed similarly, except treated cows received 12 g EN (Niashure, Balchem Corp., New Hampton, NY, USA) mixed by hand into the top 10% of the ration. Cows were fed twice daily at 110% of expected intake.

Data and Sample Collection

Feed ingredient and TMR samples were taken every 2 wk and corn silage DM was determined twice weekly and adjusted in ration formulation. Milk yields were recorded at each milking and milk was sampled at every milking beginning at 4 DIM until cows exited the study.

Liver biopsies were taken on d -21, -4, 1, 7, and 21 relative to parturition. Blood was collected on days -21, -14, -7, -4, 1, 4, 7, 14, and 21. On each collection day, liver biopsies and blood samples were taken at 1300 h. Approximately 14 mL of blood was collected from the coccygeal vein and immediately emptied into 2 tubes, one containing potassium EDTA and the other containing potassium oxalate with sodium fluoride as a glycolytic inhibitor (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA). Both were centrifuged at 2000 x *g* for 15 min

immediately after sample collection, and plasma was harvested and frozen at -20°C until analysis. Liver samples were collected using a 14-gauge x 15cm biopsy needle (SABD-1415-15-T, US Biopsy, Franklin, IN, USA). Liver was collected between the 10th and 11th ribs, 5 cm dorsal to a line between the olecranon and tuber coxae. The area was shaved, aseptically prepared and anesthetized with 2 mL of subcutaneous lidocaine hydrochloride. Anesthesia was assessed by cutaneous response after 5 min and a #11 Bard Parker blade was used to make a stab incision into the body wall. The biopsy needle was inserted cranioventrally towards the liver and approximately 100 mg of tissue was collected (total of 5 biopsies), snap-frozen in liquid nitrogen and stored at -80°C until analysis. Body condition score (BCS) was recorded by 3 trained investigators on d -21, -4, 1, 7, and 21 on a 1 to 5 scale according to Wildman et al. (1982). Body weight (BW) was measured on d 1, 7, 14, and 21 at 1300 h.

On d 7 postpartum, a caffeine clearance test was performed to assess liver function, following the protocol of Lakritz and others (2006). Jugular catheters (#1411, Mila International, Erlanger, KY, USA) were placed and caffeine was administered intravenously (2 mg/kg BW) as caffeine and sodium benzoate (C4144, Sigma-Aldrich Co., St. Louis, MO) in a sterile pyrogen-free normal saline solution (50 mg of caffeine/mL of solution). Blood was collected into K_3 EDTA-containing tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA) every 30 min for 180 min, centrifuged, and the plasma was removed and stored at -20°C until analysis. Catheters were maintained by flushing with 6 mL of sterile 3.5% sodium citrate solution following each collection. On d 21 postpartum, jugular catheters were placed for 48 h. Blood was collected as above every 8 h to assess if a post-treatment increase in plasma NEFA occurred.

Liver and Plasma Analysis

Approximately 20 mg of liver was placed into 0.3 mL of phosphate-buffered saline (pH 7.4) and homogenized. The homogenate was centrifuged at 2000 x g for 10 min at 4° C. The supernatant was then removed for triglyceride and total protein analysis. Triglyceride concentration was determined by an enzymatic glycerol phosphate oxidase method (# T7532-120, Pointe Scientific Inc., Canton, MI, USA) and total protein by a coomassie-binding, colorimetric method (kit #23236, Thermo Fisher Scientific, Rockford, IL, USA).

The mRNA abundance of GPR109A in liver tissue was determined by real-time PCR as described by Bradford and others (2009). Briefly, RNA was extracted from tissue homogenate using QIAGEN Rneasy Lipid Tissue Mini Kit (QIAGEN Inc., Valencia, CA). Coding DNA was then synthesized from 2 µg total RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed in triplicate with 1/20 of the cDNA product in the presence of 200 nmol/L gene-specific forward and reverse primers with real-time SYBR green fluorescent detection (7500 Fast Real-Time PCR System, Applied Biosystems). Abundance of β-actin mRNA was also determined, and GPR109A abundance expressed relative to this internal control gene. Primers for β-actin were designed from sequence NM_173979.3 (National Center for Biotechnology Information); forward = ACGACATGGAGAAGATCTGG, reverse = ATCTGGGTCATCTTCTCACG.

Plasma was analyzed for NEFA using an enzymatic colorimetric procedure (NEFA-HR, Wako Chemicals USA, Richmond, VA), glucose by a colorimetric kit (Autokit Glucose; Wako Chemicals USA), insulin by a bovine-specific sandwich ELISA (#10-1201-01, Mercodia AB, Uppsala, Sweden), haptoglobin by a bovine-specific ELISA (kit # 2410-7, Life Diagnostics,

West Chester, PA, USA), and BHBA using an enzymatic reaction (kit #H7587-58, Pointe Scientific, Inc.).

Plasma concentrations of nicotinic acid and nicotinamide were determined by high performance liquid chromatography (HPLC) with a method adapted from Lahély and others (1999). A Discovery BIO Wide Pore C18 column (25 cm x 4.6 mm, 5 µm particle size; Supelco # 568222-U, Sigma-Aldrich, St. Louis, MO, USA) and Discovery BIO Wide Pore C18 guard column (2 cm x 4 mm, 5 µm particle size; Supelco #568272-U, Sigma-Aldrich, St. Louis, MO, USA) were used for all analyses. The photochemical reaction was carried out in a mobile phase consisting of 0.07 M KH_2PO_4, 0.075 M H_2O_2 and 5.1 µM $CuSO_4$ at a pH of 4.25 in a PTFE tube (10 m x 0.5 mm) wound around a black light (300-400 nm). Detection was carried out with a scanning fluorescence detector (HP 1046A, Hewlett-Packard) that operated at excitation and emission wavelengths of 322 nm and 380 nm, respectively. The injection volume was 20 µL and the flow rate was 0.8 mL/min.

Caffeine was analyzed using HPLC as described by Lakritz and others (2006). Briefly, 250 µL of plasma was added to 250 µL of 0.8 M perchloric acid and centrifuged at 14,000 x g for 20 min at $21^{o}C$. A 200-µL aliquot of clarified supernatant was transferred into an autosampler vial containing 10 µL of 4 M NaOH. Vials were capped and 50 µL was injected. The column and guard column were the same as described above, and results were read at 273 nM using an Acutect 500 UV/Vis detector (#06-653-5, Thermo Fisher Scientific) at flow rate of 1 mL/min.

Feed and Milk Analysis

Diet ingredients and TMR samples were dried in a 55°C forced-air oven for 72 h. Feed ingredients were analyzed for DM concentration, ground to pass through a 1-mm screen using a Wiley mill (Arthur H. Thomas, Philadelphia, PA), and composited by feed type on an equal mass basis. Ash concentration was determined after 5 h of oxidation at 500°C in a muffle furnace. Concentration of NDF was determined (Van Soest et al., 1991) using an Ankom Fiber Analyzer (ANKOM Technology, Fairport, NY). Crude protein was determined by oxidation and detection of N_2 (Leco Analyzer, Leco Corp, St. Joseph, MI). Crude fat was determined by ether extraction (AOAC, 2000: method 920.9). Starch was determined by α-amylase and glucoamylase digestion, followed by colorimetric glucose quantification using a commercial kit (Autokit Glucose; Wako Chemicals USA). Concentrations of all nutrients except for DM were expressed as percentages of DM determined by drying at 105°C in a forced-air oven for 16 h. All analyses were performed in duplicate.

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, Chaska, MN), urea nitrogen (MUN spectrophotometer, Bentley Instruments), and somatic cells (SCC 500, Bentley Instruments). Energy-corrected milk (ECM; 0.327 x milk yield + 12.86 x fat yield + 7.65 x protein yield; DHI glossary, Dairy Record Management Systems, 2009) and solids-corrected milk (SCM) yield were calculated (Tyrrell and Reid, 1965).

Disease Incidence

Cows were assessed daily for health by rectal thermometer, stethoscope and physical examination. Diseases recorded included displaced abomasum, ketosis, metritis, lameness, and "other digestive disorder".

Statistical Analysis

Data were analyzed using mixed models with repeated measures over time (SAS 9.1, SAS Institute, Cary, NC). Spatial power covariance structures were used to model repeated measures over time within cow. Fixed effects were treatment, parity, day, treatment × parity, treatment × day, and treatment × parity × day. Individual cows were treated as a random effect. Plasma insulin and haptoglobin data also included d -21 values as a covariate because of group differences prior to treatment initiation; outliers were removed when studentized residuals were > |3.0|. Liver GPR109A mRNA and plasma NA and nicotinamide data were log-transformed prior to analysis to achieve normal residual distributions. Prepartum and postpartum measures were analyzed separately for DMI as well as plasma glucose, NEFA and BHBA concentrations because of relatively discontinuous data at calving and an adequate number of data points pre- and post-partum. Treatment and two-way interactions were declared significant at $P < 0.05$ and trends are discussed at $P < 0.10$. Three-way interactions were considered significant at $P < 0.10$.

Caffeine elimination half-lives were determined by using the slope of the regression line for post-infusion concentrations for each animal and the equation: half-life = ln 2/slope (Lakritz et al., 2006). One outlier from each treatment group was removed based on studentized residuals > |2.5|. Half-life and distribution volume (VD) data were analyzed using fixed effects of treatment, parity, and their interaction, and the random effect of cow. Disease incidence was

analyzed using Fishers exact test, including 2 additional categories: having at least one disorder and having at least 2 disorders.

RESULTS

Diet Analysis and Dry Matter Intake

Composition and nutrient analysis of prepartum and postpartum diets are shown in Table 3.1. Daily DMI is shown in Figure 3.1. Cows had higher intakes than heifers ($P < 0.01$) and there was a time effect ($P < 0.01$) with all groups experiencing an increase in DMI from d 1 to d 21. There was also a treatment $\times$ parity $\times$ time interaction ($P < 0.07$) during the prepartum period. This was due to a decreased DMI of EN-treated cows compared to control cows ($P < 0.02$) by 4.1 kg/d during the final 4 d before calving. There were no treatment effects on postpartum DMI.

Body Weight, Body Condition and Milk Production

Body condition and BW throughout the study are shown in Figures 3.2 and 3.3, respectively. On average, all animals lost one BCS (3.63 to 2.64) during the experiment; however, no differences were observed between treatments. Milk production and components are shown in Table 3.2. As expected, multiparous cows produced higher yields of milk ($P < 0.01$), fat ($P < 0.01$), protein ($P < 0.001$), and lactose ($P < 0.01$) than primiparous cows. Treatment had no effect on milk production or milk components.

Liver Tissue

Liver TG content throughout the experiment is shown in Figure 3.4; there were no treatment or parity differences. All cows experienced an increase in liver TG following parturition ($P < 0.001$). Abundance of GPR109A mRNA is shown in Figure 3.5. There were no treatment, parity or time effects on mRNA abundance of GPR109A in liver tissue.

Plasma Parameters

Plasma concentrations of BHBA and NEFA are displayed in Figure 3.6. There was a prepartum parity effect ($P < 0.02$) due to heifers having increased NEFA compared to cows. During the postpartum period, a treatment $\times$ time $\times$ parity interaction ($P < 0.09$) for plasma NEFA was detected. NEFA peaked at 1467 ± 160 µM for control cows compared with 835 ± 154 µM for EN-treated cows ($P < 0.01$). There was a treatment $\times$ time $\times$ parity interaction ($P < 0.03$) detected for plasma BHBA during the postpartum period. Although treatment did not significantly alter peak postpartum BHBA, EN-treated primiparous cows had lower BHBA concentrations than controls on d 7 postpartum (937 vs. 1793 ± 261 µM; $P < 0.02$).

Concentrations of glucose and insulin are shown in Figure 3.7. There were no treatment effects on plasma glucose postpartum; however, EN treatment resulted in lower prepartum glucose ($P < 0.04$) compared to control. There was both a parity effect prepartum ($P < 0.01$) and a trend postpartum ($P < 0.07$) for cows to have lower glucose than heifers. There was a treatment $\times$ parity $\times$ time interaction ($P < 0.001$) for plasma insulin. On day -7 prepartum, EN-treated multiparous cows had higher plasma insulin than control multiparous cows ($P < 0.02$), and on day -4 prepartum, EN-treated primiparous cows were higher than control primiparous cows ($P <$

0.02). There was also a time effect ($P < 0.001$) for plasma insulin caused by a decreasing concentration during the prepartum period that reached a nadir at day 4 postpartum.

Plasma NA and NAM concentrations throughout the study are shown in Figure 3.8. Plasma NA was unaffected by treatment, but heifers had higher plasma NA than cows ($P < 0.02$), and NA increased for all animals over the course of the study ($P < 0.001$), most obviously after calving. Treatment significantly increased plasma NAM ($P < 0.001$), and when individual days were tested, EN-treated cows were higher on days -7 and 21 relative to parturition ($P < 0.001$). Plasma NAM tended to remain elevated for EN on d 23 ($P < 0.07$), 50 h after the final EN treatment.

Plasma NEFA concentrations were measured every 8 h for 50 h following the first feeding without EN added. Treatment cows received EN at the morning feeding, and blood was first drawn at 1400 h that day, which was just prior to the next feeding. Results for post-treatment NEFA concentrations are shown in Figure 3.9. There was a time effect ($P < 0.01$) but no treatment or parity effects.

Liver Indices and Disease Incidence

Results of the caffeine challenge test are shown in Table 3.3. Encapsulated niacin tended to increase the half-life of caffeine ($P = 0.06$). The volume of distribution (VD) of caffeine was also calculated to ensure that there were no errors in administering the caffeine dose. This was confirmed, as d 7 BW was well correlated with VD ($r = 0.72$, $P < 0.001$). The VD of multiparous cows was higher than that of primiparous cows ($P < 0.001$), which is expected due to their larger BW.

Haptoglobin results are shown in Figure 3.10; a parity effect ($P < 0.01$) was detected with primiparous cows having higher haptoglobin than multiparous cows (308 vs. 179 ± 35 µg/mL). A time effect ($P < 0.01$) was also detected for haptoglobin as a result of an increase in the early postpartum period. Caffeine half-lives were tested against other liver indices to determine if any relationships existed. The relationships between liver indices and caffeine half-lives are shown in Table 3.4. No significant correlations were found. Incidence of disease is displayed in Table 3.5, and no differences were found between treatment or parity groups.

DISCUSSION

As expected, both groups began the study with similar NAM concentrations. The increase in NA and NAM following parturition may have been from increased dietary niacin in the postpartum diet or increased production by ruminal microbes as intake of fermentable organic matter increased. As expected, within 50 h following the end of treatment, NAM concentrations in treated cows decreased, although they remained slightly higher than in control cows ($P < 0.07$). Plasma NAM concentrations were about 100 times higher than NA concentrations throughout the experiment. Several authors have reported that the acidic environment in the abomasum favors NA (Santschi et al., 2005; Campbell et al., 1994). However, most NA is absorbed from the duodenum and is rapidly converted to NAD and then hydrolyzed to NAM, which is the main transport form of niacin in the blood (Henderson, 1983). Therefore, we would expect that most of the NA we supplemented should have been converted to NAM after absorption. The difference in NA and NAM concentrations in this study can also be explained by the affinity for the GPR109A receptor. Nicotinic acid has a very high affinity for the GPR109A

receptor, and is bound in adipose tissue within 5 min after intravenous injection in mice (Carlson and Hanngren, 1964). Nicotinamide, on the other hand, has a very low affinity for the GPR109A receptor (Gille et al., 2009). Therefore, since plasma NA is short-lived, finding increased plasma concentrations of NA would be unlikely. It may also be possible that some of the NAM produced (Huntgate, 1966) and absorbed from the rumen (Erickson et al., 1991) resulted in increased NAM concentrations as well, especially for differences between parities and over time.

Recent work by Bradford and others (2009) found GPR109A in brain and liver of steers. Prior to this, it was believed that GPR109A was only in adipose tissue and immune cells. This novel finding allows us to examine the effects of niacin from a different perspective. Activation of GPR109A receptors in the brain may have resulted in the decreased DMI in treated cows. However, it is unknown if the GPR109A receptor is involved with the satiety center of the brain. Allen et al. (2009) discussed the hepatic oxidation theory for hypophagia in transition cows caused by increased fatty acid (FA) oxidation. It is believed that increased hepatic FA oxidation results in hepatic signaling to the brain causing hypophagia. The EN-treated cows may have had depressed DMI as a result of increasing FA oxidation because NEFA and BHBA were rising prepartum. If this was the case, however, we would have expected control cows to have reduced DMI as well. However, the role of GPR109A in the liver-brain-hunger axis is unknown.

Peak plasma NEFA concentrations were lower with EN-treated cows compared to controls. Increased NEFA occurs from increased lipolysis (Zurek et al., 1995), and extent of lipolysis depends on the severity of NEB (Jorritsma et al., 2001; Drackley et al., 1992) and BCS of the animal at parturition (Rukkwamsuk et al., 1998). Since there were no treatment differences in BCS during this study, it is likely that niacin inhibited lipolysis. Niacin has been shown to reduce NEFA in cattle (Pires and Grummer, 2007), while also reducing plasma very-low density

lipoprotein (VLDL) in humans (Carlson, 2005). The lowering of VLDL in humans with niacin is believed to be a result of decreased FA delivery to the liver (Carlson, 2005). In postpartum dairy cows, both increased plasma NEFA and decreased VLDL synthesis play a role in the development of fatty liver. Ruminants have a poor ability to secrete TG as VLDL compared to non-ruminants (Pullen et al., 1990), which is believed to be a result of decreased synthesis of apolipoprotein B-100, a major constituent of VLDL particles (Bertics et al., 1992). Plasma VLDL was not measured in this study, and because cattle differ from humans both in their propensity to secrete VLDL and in the presence of the GPR109A receptor in liver, it is unclear what effect EN may have had on VLDL concentrations.

Although EN treatment decreased postpartum plasma NEFA, it did not suppress peak BHBA concentrations in either heifers or cows, and it only had a significant effect on plasma BHBA concentrations of primiparous cows on d 7 postpartum. Non-esterified fatty acids are passively taken up by the liver, and uptake is concentration dependent (Bell, 1979). Excessive delivery of NEFA to the liver results in both TG storage in the cytoplasm and increased mitochondrial production of ketone bodies (Veenhuizen et al., 1991). It is unclear why the significant decrease in NEFA caused by EN did not have more dramatic effects on plasma BHBA concentration, but it is possible that hepatic metabolism of NEFA was also influenced by EN, potentially by shifting a greater proportion of NEFA to the ketogenic pathway.

There were no treatment differences in liver TG content throughout the study. This was unexpected since we hypothesized that niacin would reduce liver TG due to its ability to inhibit lipolysis (Carlson, 1963). However, even with a reduced prepartum DMI, the EN-treated cows did not have increased liver TG compared to controls. Depressed prepartum intake increases the

risk of fatty liver postpartum (Bobe et al., 2004), so the EN-treated cows were at a somewhat greater risk for elevated liver TG content.

The tendency for increased elimination half-life of caffeine in treated cows was unexpected. This was the opposite of our hypothesis, which was that niacin would reduce liver TG, and therefore improve liver function. Caffeine elimination half-life is highly correlated with liver function (Lakritz et al., 2006), as it is metabolized through the P-450 cytochrome oxidase system (CYP-450; DeGraves et al., 1995). There have been reports of half-life elimination in lactating dairy cattle ranging from 2.6 to 6.9 hours (DeGraves et al., 1995). Half-lives in this study were less than that reported by DeGraves and others (1995); however, no work has been reported on transition cows, which may differ metabolically from cows later in lactation. Although the GPR109A receptor has been found in liver tissue (Bradford et al., 2009), its pathways are not fully understood. Therefore, treatment with EN may have affected the CYP-450 pathway or may have reduced the liver's ability to function properly via GPR109A signaling. Although not reported in cattle, there have been reports of hepatotoxicity following sustained-release niacin treatment in humans (Dalton and Barry, 1992; Lawrence, 1993). Caffeine half-lives were not correlated with concentrations of liver TG, plasma BHBA or haptoglobin. This indicates that liver function may not be influenced by these parameters or the CYP-450 pathway was unaffected by the severity of metabolic disorders in this study.

The decreased prepartum glucose in treated animals with only occasional increases in plasma insulin was another unexpected finding. Niacin treatment has been shown to reduce plasma insulin without affecting glucose concentrations (Pires et al., 2007). Increased plasma NEFA is associated with increased insulin concentration and insulin resistance (Pires et al., 2007). It may be possible that the lower prepartum glucose in treated cows was due to an

increased sensitivity to insulin. Also, insulin resistance in adipose tissue results in enhanced

lipolysis and NEFA release (Pires et al., 2007). This is also supported by the fact that control

animals had increased NEFA postpartum, which may have been caused in part by greater insulin

resistance at parturition.

An interesting finding in this study was the 68% incidence of ketosis during the

postpartum period. The incidence of ketosis has been reported as 30% in cows with fatty liver,

and only 10% in those without fatty liver (Grohn et al., 1987). Ketone bodies provide an

essential source of energy during times of NEB (Herdt, 2000), and are produced when NEFA

delivery to the liver is high (Drackley et al., 2001). A total of 7 cows (32%) in this experiment

experienced LDA, which is relatively high compared to normal incidence rates of 1% to 5%

(Shaver, 1997). This may have been a result of the high ketosis rate and depressed intake, which

reduces rumen fill and forestomach motility, predisposing to LDA (Cameron et al., 1998).

However, due to the number of cows in our study, we had little statistical power to assess

differences in LDA incidence between treatments.

It was surprising to find that EN-treated cows did not have increased NEFA following

treatment removal. This differs from work by Pires and Grummer (2007), which found a post-

treatment rebound in plasma NEFA as high as 4 times the control NEFA concentration. This

occurred within 6 h following a single 6 mg/kg post-ruminal dose in feed-restricted cows,

whereas our dose should have been absorbed more slowly since it has to move through the

rumen. We conclude that including EN in feed, administering it for 42 d, and/or removing it

from animals in positive energy balance may have eliminated the post-treatment rebound in

lipolysis. We expected that by 21 d postpartum, lipolysis would not be severe enough to

significantly change plasma NEFA concentrations, and our data supports this. The time effect for

plasma NEFA following treatment removal is likely due to pre-prandial increases in lipolysis during the 48 h period (Bradford and Allen, 2008). The feedings occurred at 2, 18, 26, and 42 h following treatment removal.

Haptoglobin is an acute phase protein which is released by the liver during periods of inflammation (Hachenberg et al., 2007) and has been found to be increased in cows with fatty liver (Bobe et al., 2004). The increased haptoglobin in primiparous cows indicates that they were suffering from a higher degree of hepatic inflammation than multiparous cows. Baseline values of plasma haptoglobin in transition cows have been established (Hachenberg et al., 2007; Uchida et al., 1993) and are consistent with cows in our study. In those studies, haptoglobin increased in the first few days postpartum, a finding seen in this experiment as well. However, the other studies saw peak means of 1500 μg/mL during the peripartum period, whereas means in our experiment did not exceed 517 μg/mL. Therefore, it is unlikely that either parity group experienced excessive inflammation during our experiment, despite the high incidence of transition disorders in this study.

CONCLUSIONS

In this study, we found that 24 g/d of dietary EN inhibited lipolysis in postpartum cows, which was demonstrated by a decrease in postpartum plasma NEFA concentrations. Depression of prepartum DMI in multiparous cows is a novel finding and is difficult to explain. We found that depressed DMI in EN-treated multiparous cows followed a significantly increased plasma NAM concentration compared to controls. We cannot explain why treated heifers did not

experience the same depression in DMI as treated cows. There is still much to learn about the GPR109A distribution within the body and how it affects feed intake. However, we found that even when EN reduced DMI in multiparous cows, it still suppressed plasma NEFA after calving. Although significant alterations in plasma lipid metabolism were detected after EN treatment, this did not result in decreased liver TG content. In fact, EN treatment resulted in prolonged caffeine half-life clearance, another finding that is difficult to interpret. In contrast to other studies, we found that our NA dose and delivery method did not result in a post-treatment rebound of NEFA. In summary, 24 g/d of EN can decrease postpartum plasma NEFA. Further research is needed to understand the effects of niacin on prepartum DMI and mechanisms involving postpartum liver metabolism.

ACKNOWLEDGMENTS

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Figure 3.1 Dry matter intakes during the experimental period. Multiparous cows had higher DMI than primiparous cows ($P < 0.01$). There was a treatment × parity × time interaction ($P < 0.07$) caused by a decreased DMI for EN-treated multiparous cows by 4.1 kg/d during the final 4 d prepartum ($P < 0.02$). SEM = 1.4 kg. EN animals received 24 g/d of dietary encapsulated niacin.
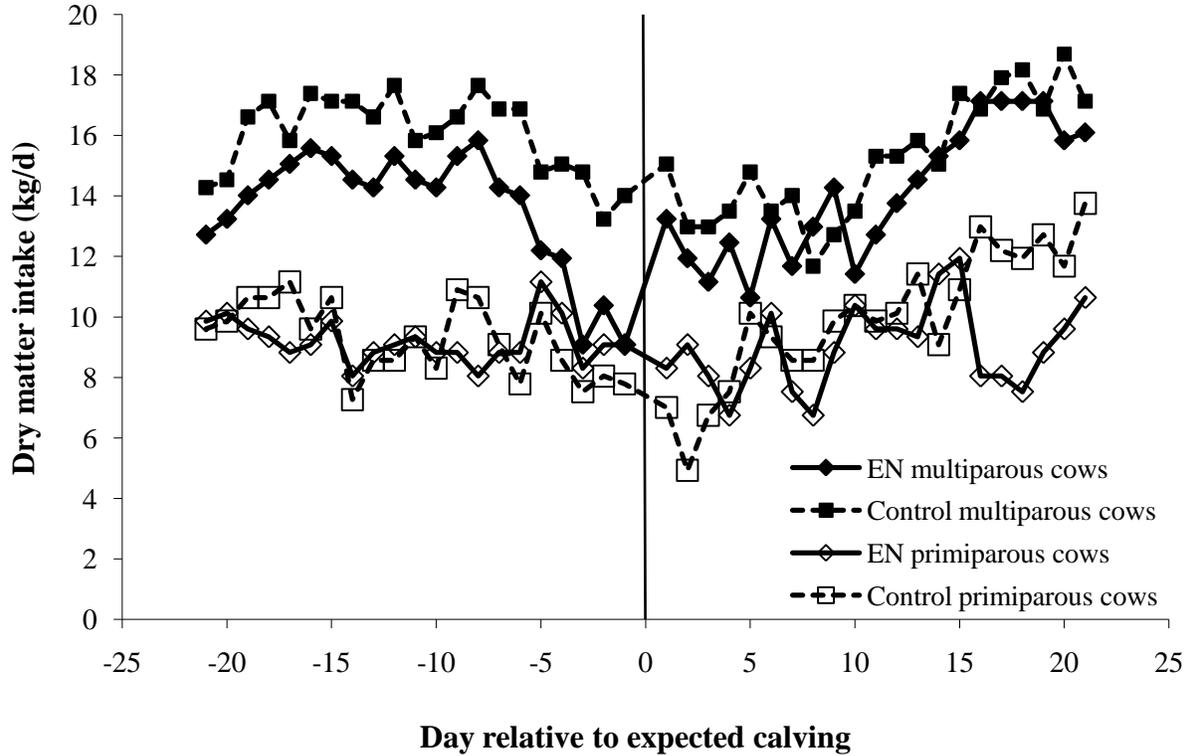
Figure 3.2 Body condition score during the experimental period. There were no significant differences between treatment or parity groups, and all groups had a decrease in body condition score during the experimental period ($P < 0.001$). SEM = 0.17. EN animals received 24 g/d of dietary encapsulated niacin.
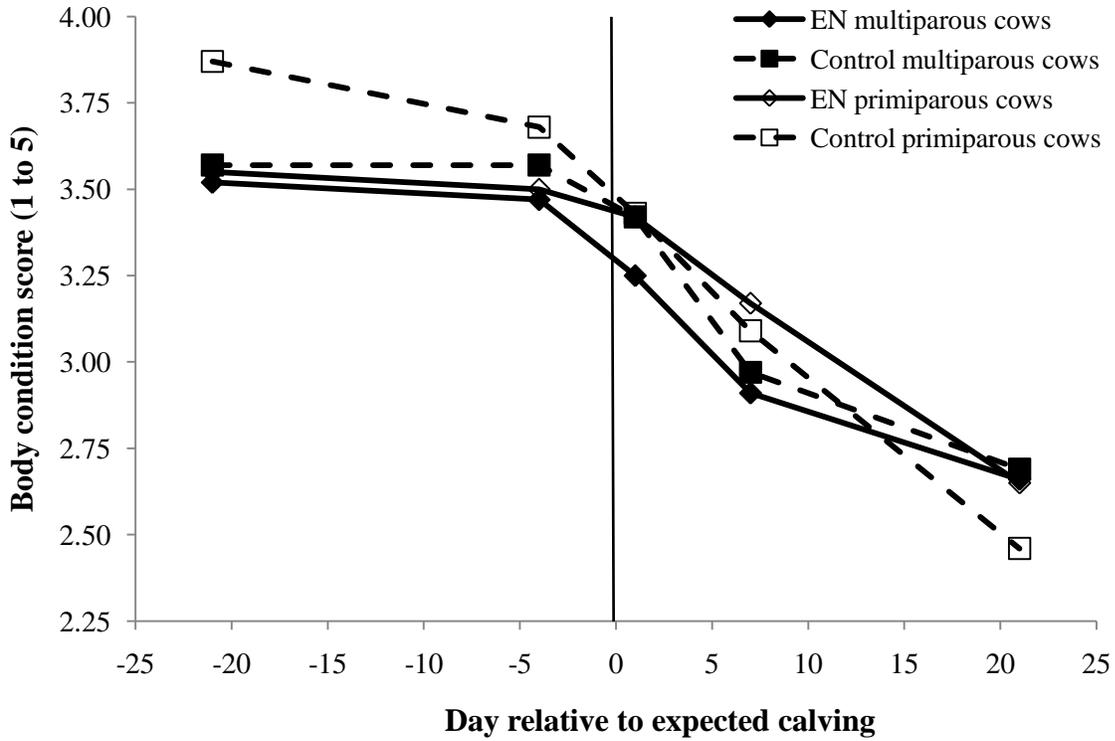
Figure 3.3 Body weight during the postpartum experimental period. Multiparous cows were heavier than primiparous cows at all time points ($P < 0.001$), and all groups lost weight during the experimental period ($P < 0.001$). SEM = 26.1 kg. EN animals received 24 g/d of dietary encapsulated niacin.
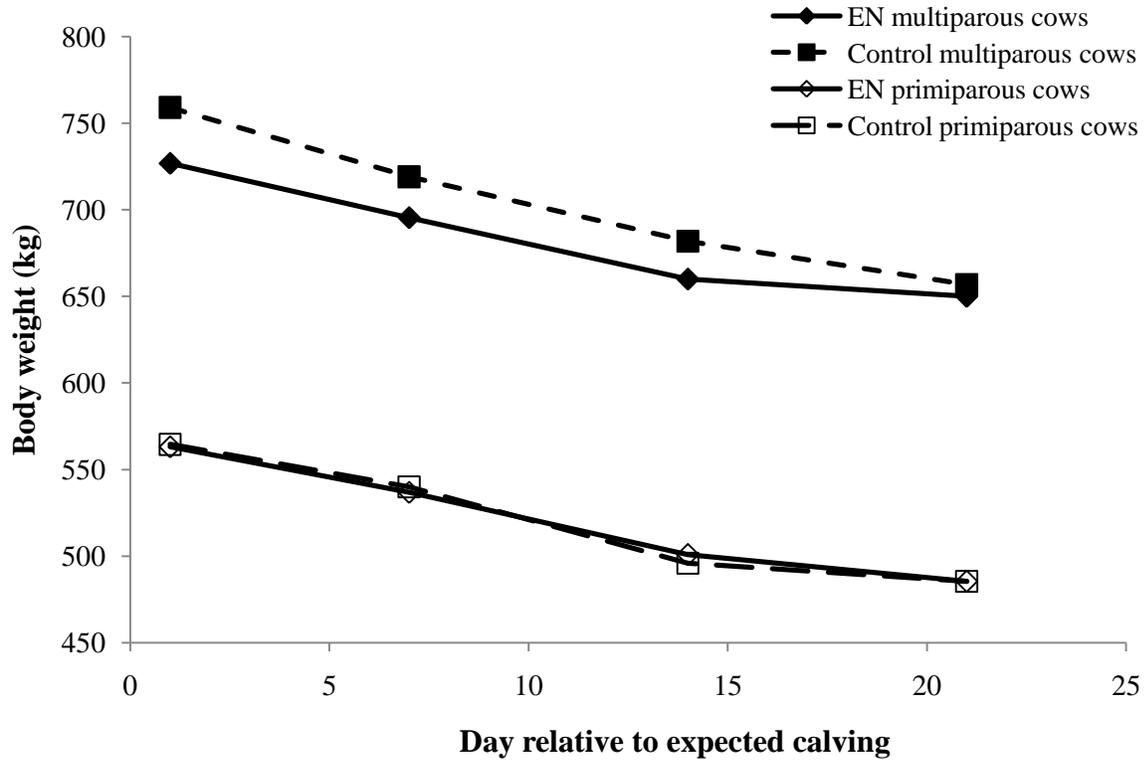
Figure 3.4 Liver triglyceride concentrations during the experimental period. No significant differences were found between treatment or parity groups; however there was a time effect ($P <$ 0.001). SEM = 22.8 mg/g. EN animals received 24 g/d of dietary encapsulated niacin.
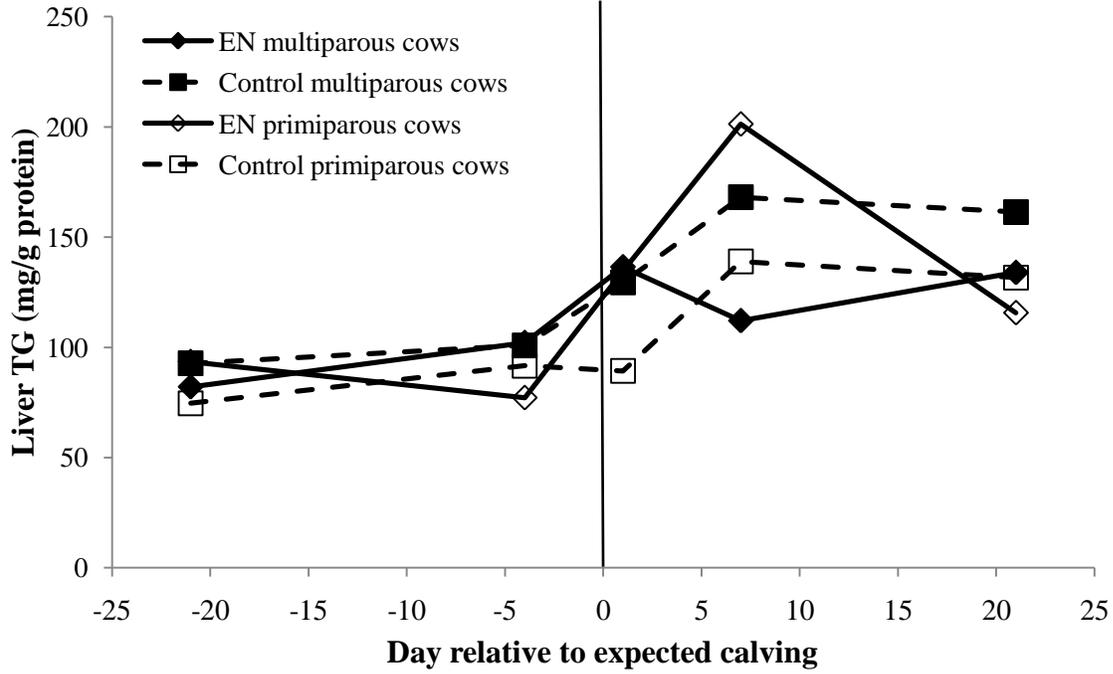
Figure 3.5 Abundance of the niacin receptor GPR109A mRNA in liver tissue during the experimental period. No treatment, parity or time differences were detected. SEM = 0.13. EN animals received 24 g/d of dietary encapsulated niacin.
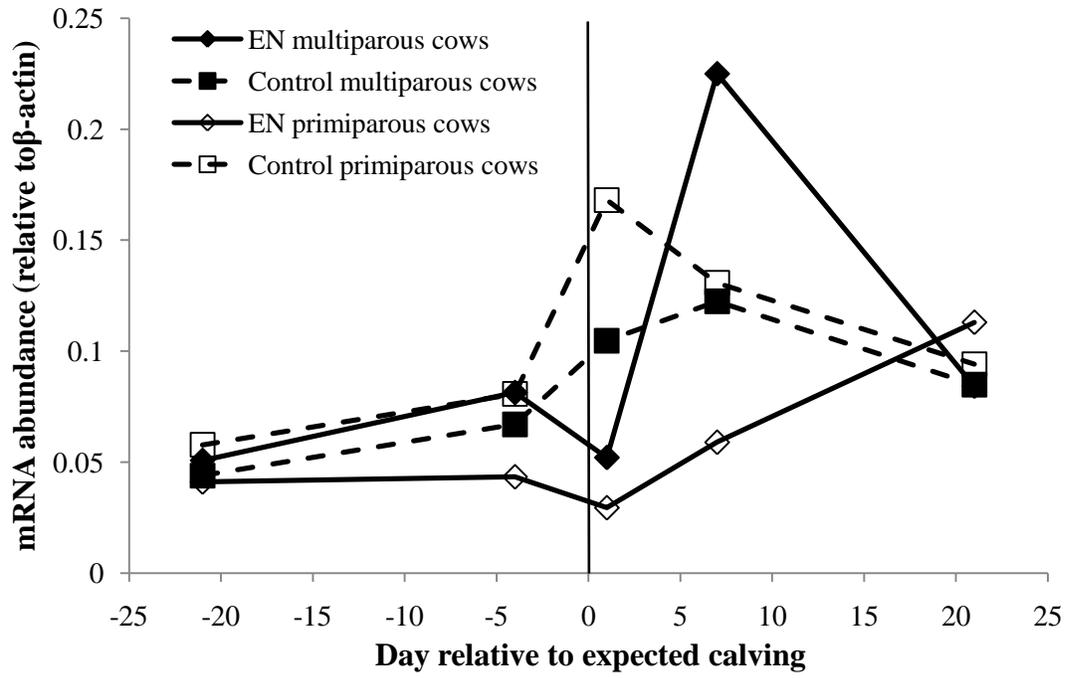
Figure 3.6 Plasma concentrations of nonesterified fatty acids (A) and beta-hydroxybutyrate (B) during the experimental period. A. A treatment × time × parity interaction was detected ($P = 0.09$) after calving. SEM = 82 µM-Pre, 216 µM-Post. **B.** A treatment × time × parity interaction was detected ($P < 0.02$) after calving. SEM = 97 µM -Pre, 234 µM -Post. ***Indicates a** treatment effect for primiparous cows ($P < 0.05$). **Indicates a treatment effect for multiparous cows ($P < 0.06$). EN animals received 24 g/d of dietary encapsulated niacin.
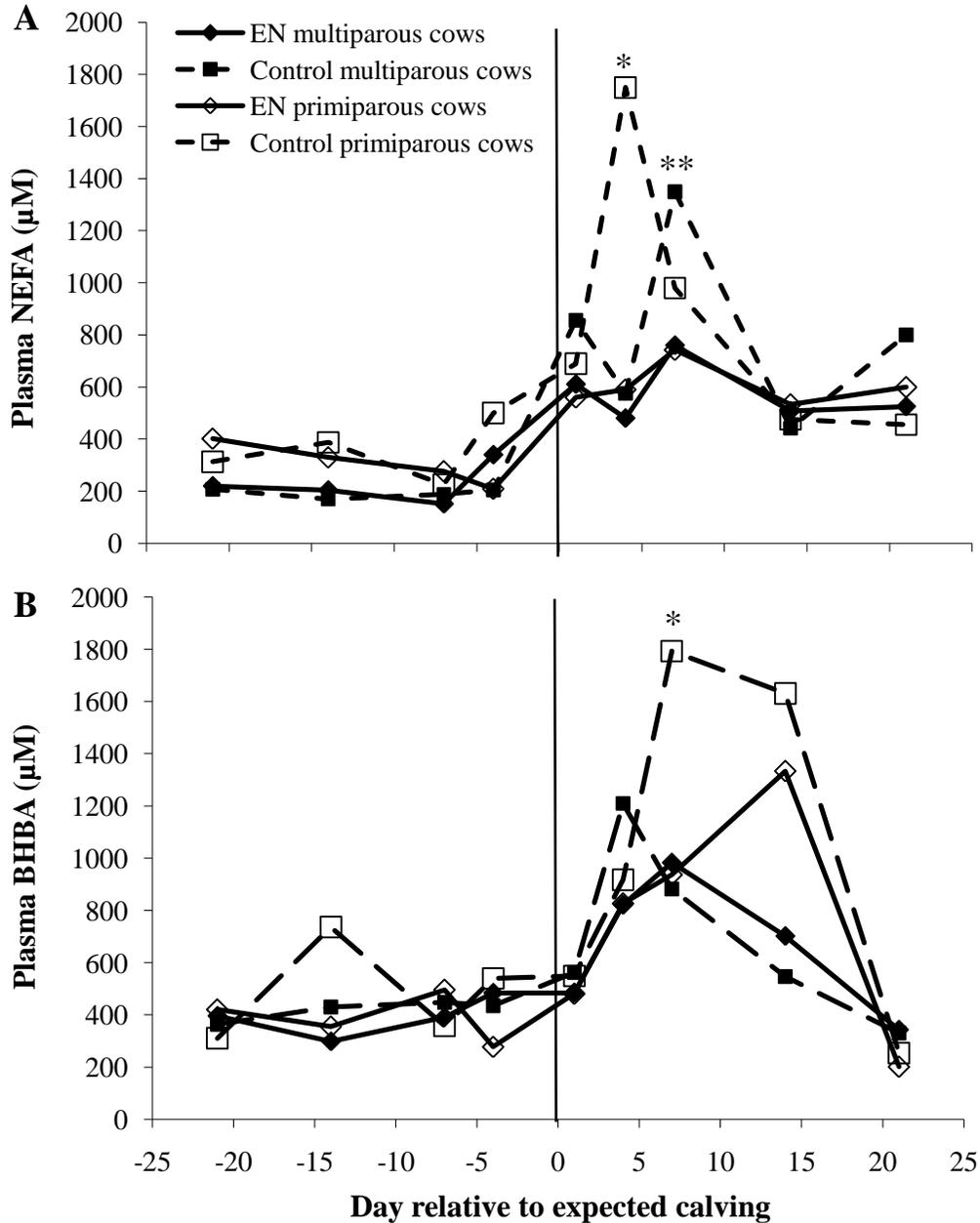
Figure 3.7 Plasma concentrations of glucose (A) and insulin (B) during the experimental period. A. There were treatment ($P < 0.04$) and parity ($P < 0.009$) effects prepartum and a parity trend postpartum ($P < 0.07$). SEM = 3.73 mg/dl-Pre, 2.70 mg/dl-Post. **B.** A treatment × parity × time effect was detected ($P < 0.001$) along with a time effect ($P < 0.001$). SEM = 0.61 ng/mL. *****Indicates a treatment effect for primiparous cows, ******Indicates a treatment effect for multiparous cows. EN animals received 24 g/d of dietary encapsulated niacin.
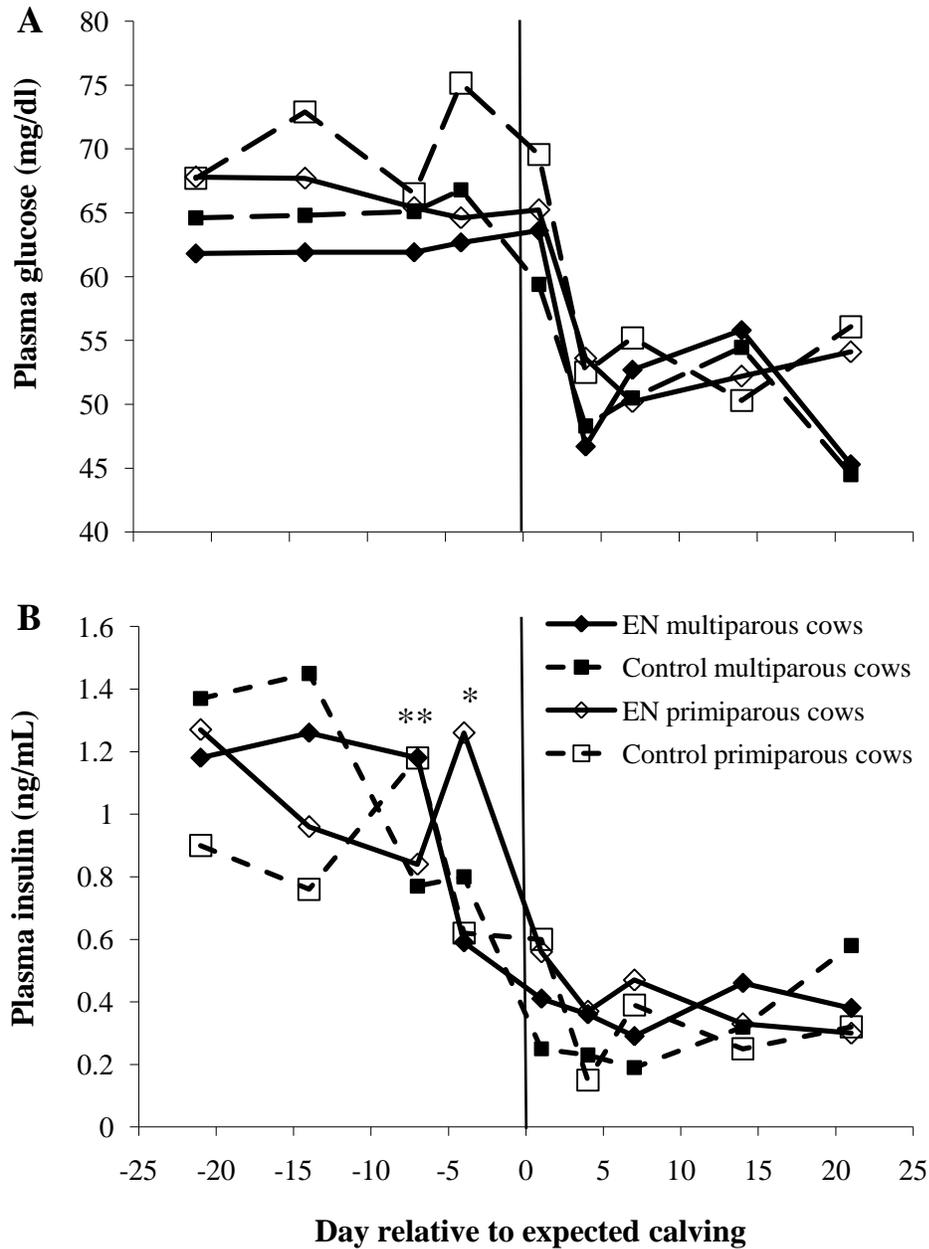
Figure 3.8 Plasma concentrations of NA (A) and NAM (B) during the experimental period.
A. NA was unaffected by treatment, however there was a parity effect ($P < 0.02$) from heifers having higher NA concentrations than cows and a time effect ($P < 0.001$). SEM = 0.01 µg/mL. **B**. Treatment with NA raised plasma NAM ($P < 0.001$) compared to control. This was caused by significant ($P < 0.001$) differences on days -7 and 21. **Indicates significant ($P < 0.001$) differences between treatments. † Tendency for a treatment effect ($P < 0.07$). The last time point is 50 h following treatment removal. SEM = 0.093 µg/mL. EN animals received 24 g/d of dietary encapsulated niacin.
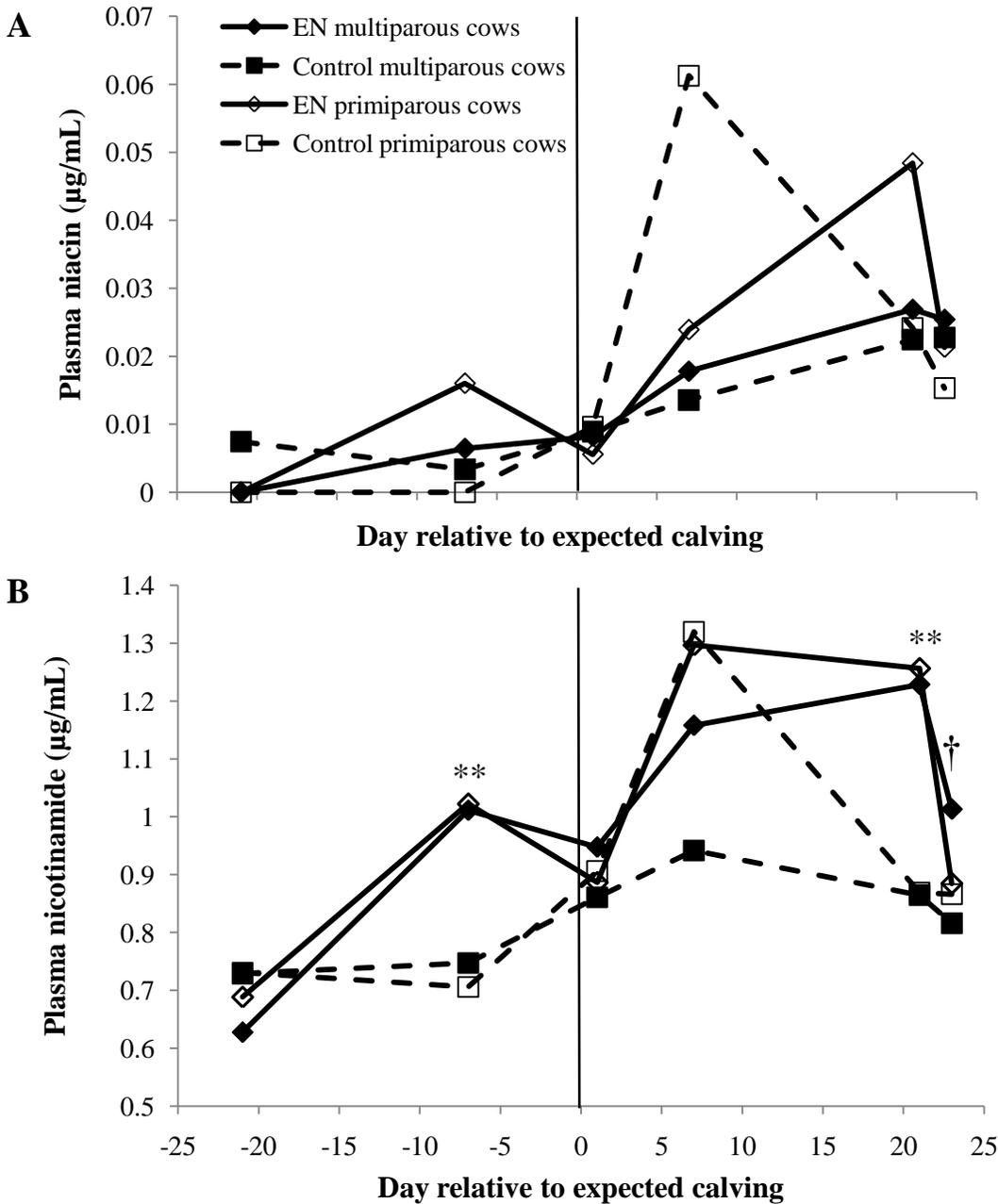
Figure 3.9 Plasma concentrations of non-esterified fatty acids during the post-treatment period. There was a time effect ($P < 0.01$) but no treatment or parity effects. SEM = 73 µM. EN animals received 24 g/d of dietary encapsulated niacin.
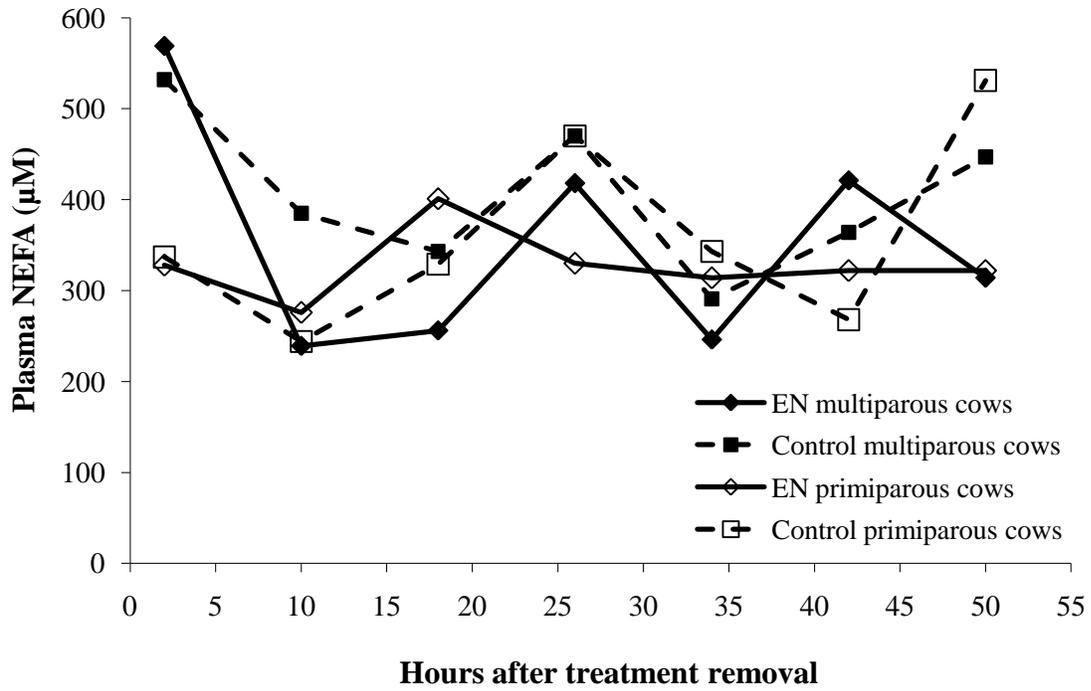
Figure 3.10 Plasma concentrations of haptoglobin during the experimental period. A parity effect ($P < 0.02$) was detected with primiparous cows having higher haptoglobin than multiparous cows (308 vs. 179 ± 35 µg/mL). A time effect was detected as well ($P < 0.01$). SEM =102 µg/mL. EN animals received 24 g/d of dietary encapsulated niacin.

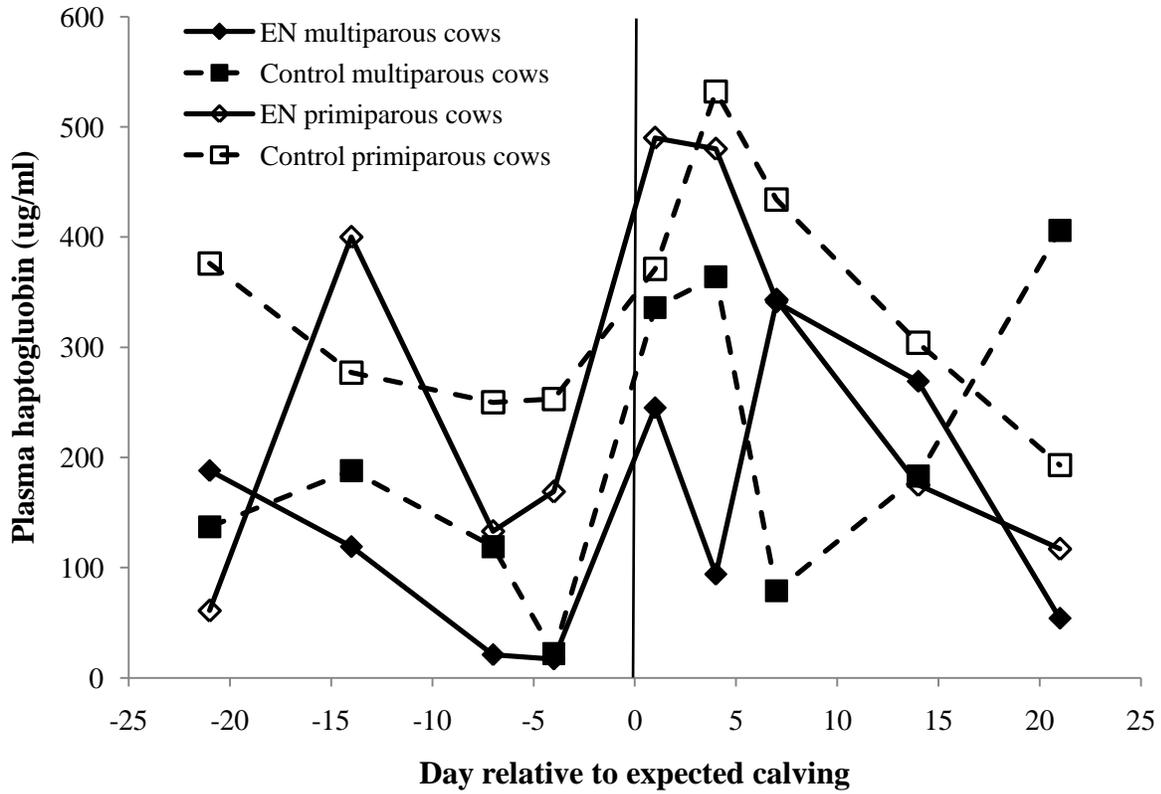**Table 3.1 Composition and nutrient analysis of dietary treatments during the experimental period.**

		Ration	
		Prepartum	Postpartum
% DM	Corn silage	24.2	26.7
	Prairie hay	27.7	-
	Alfalfa hay	-	14.5
	WCGF[1]	34.6	35.3
	Cracked corn	0.0	14.5
	Ground corn	9.99	-
	SoyBest[2]	0.0	6.4
	Soybean meal 48%	3.24	-
	Salt-white	0.08	-
	Limestone	-	1.7
	Trace mineral salt	-	0.14
	Sodium bicarbonate	-	0.41
	MFP-Mintrex[3]	-	0.08
	Magnesium oxide	-	0.08
	Vitamin/mineral pre-mix[4]	0.07	0.16
Nutrients[5]			
	DM, % as-fed	63.8	60.9
	CP	13.8	18.9
	NDF	41.3	27.9
	Starch	17.9	22.7
	NFC[6]	35.5	42.0
	Ether extract	2.4	2.9
	Ash	7.0	8.2

[1]Wet corn gluten feed; Sweet Bran, Cargill, Inc.
[2]SoyBest, Grain States Soya, West Point, NE.
[3]MFP-Mintrex, Novus International.
[4]Composed of Vitamins A, D, E, Selenium, 4-Plex (Zinpro Corp.), and organic iodine salt.
[5]Nutrients other than DM expressed as a percentage of diet DM.
[6]Calculated as $DM - (CP + NDF + EE + ash)$.

Table 3.2 Milk production and composition during the experimental period.

	EN[1]		Control			P value	
	Cows	Heifers	Cows	Heifers	SEM	Treatment	Parity
Milk yield k/d	31.2	21.8	33.9	25.1	2.8	0.29	**0.003**
Milk fat, %*	4.68	5.43	4.89	5.11	0.14	0.72	**0.001**
Milk protein, %	3.29	3.14	3.17	2.91	0.10	0.13	0.09
SCC, 10^3 cells/mL**	115	365	623	190	285	0.59	0.77
MUN, mg/dL	12.56	10.77	12.31	9.92	1.53	0.72	0.19
ECM, kg/d	29.2	21.7	31.4	23.1	2.0	0.38	**0.001**
SCM, kg/d	35.5	26.0	38.3	28.0	3.1	0.44	**0.004**
Yield, kg/d							
Milk fat	1.53	1.21	1.73	1.29	0.56	0.34	**0.01**
Milk protein	1.09	0.71	1.10	0.74	0.09	0.81	**0.001**
Milk lactose	1.59	1.08	1.66	1.22	0.15	0.49	**0.003**

[1]Animals received 24 g/d of dietary encapsulated niacin. Bolded P values are considered significant.

*Indicates a treatment by parity trend ($P = 0.06$)

**Indicates a treatment by parity by DIM interaction ($P = 0.06$)

Table 3.3 Results of day 7 caffeine clearance test[1]

	Treatment					Parity			
	EN[2]	Control	SEM	*P*-value		Cows	Heifers	SEM	*P*-value
[3]Half-life (min)	130.7	97.6	12.1	0.06		109.9	118.3	12.2	0.63
VD (L)	414.7	418.3	31.7	0.93		469.8	330.1	23.2	0.001

[1] Treatment × parity interactions were not significant.
[2] Animals received 24 g/d of dietary encapsulated niacin.
[3] Two outliers removed

Table 3.4. Correlations between day 7 caffeine clearance half-life and liver indices.

	Half-life	
	r^2	*P*-value
Haptoglobin	<0.01	0.95
BHBA	0.04	0.40
Liver TG	<0.01	0.92

Table 3.5 Incidence of diseases during the experimental period.

Disorder	EN[1]		Control	
	Multiparous (n=6)	Primiparous (n=5)	Multiparous (n=7)	Primiparous (n=4)
Ketosis	4	3	5	3
Metritis	0	1	0	1
Lameness	2	0	2	2
Displaced abomasum	2	2	1	2
Other digestive disorder	2	0	1	1
At least 1disorder	4	3	6	3
At least 2 disorders	3	2	3	3

[1] Animals received 24 g/d of dietary encapsulated niacin. No differences exist between treatment or parity groups.