Etiology and Prevention of Fatty Liver and Ketosis in Dairy Cattle

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Introduction

According to the most recent National Animal Health Monitoring System for dairy cattle (National Animal Health Monitoring System, 2008), leading causes of morbidity in dairy cattle are clinical mastitis, lameness, infertility, retained placenta, milk fever, reproductive problems, and displaced abomasum. Of cows removed from herds, about 53% leave for one or more of the above reasons. Additionally, the rate of mortality of cows in U.S. dairy herds is nearly 6%, with 43% of these related to periparturient health issues, and likely a large portion of those classified as "unknown" (25%) occurring as a result of complications from the above. Overall, 16.2% of the cows that are permanently removed from a dairy herd are removed before 50 days in milk (**DIM**). These cows represent losses before the most profitable period of lactation. The relationship of the above disorders to excess prepartal body condition score (**BCS**) has been documented by numerous researchers and extensively reviewed (Bewley and Schultz, 2008). Briefly, cows with excessive body condition at calving, or excessive weight loss after calving, demonstrate overall decreased reproductive performance and increased likelihood of dystocia, retained placenta, metritis, milk fever, cystic ovaries, lameness, and mastitis as well as metabolic disorders, fatty liver, and ketosis. An epidemiological study by Gillund et al. (2001) found 20% of cows in surveyed herds to experience ketosis, and cows with BCS > 3.5 at calving to be 2.5 times more likely to become ketotic. Cows that became ketotic lost more body weight (**BW**) during early lactation, and the likelihood of conceiving at first insemination was decreased by 37%. Thus, the relationship of BCS, fatty liver, and ketosis has enormous economic impact on health and reproductive performance of dairy cows.

Fatty liver (i.e., hepatic lipidosis) is recognized as a major metabolic disease of dairy cows. We define *fatty liver* as the percentage of triacylglycerol (**TAG**) that causes detrimental effects to the health, well-being, productivity, or reproductive success of a cow (Bobe et al., 2004). On the basis of its effects, we categorize fatty liver into three classes: *clinical fatty liver* as 10% TAG and greater (wet weight basis), *moderate fatty liver* as between 5 and 10% TAG, *mild fatty liver* as between 1 to 5%, and *normal liver* as < 1% TAG; however, these TAG percentages are not absolute. As many as 50% of the cows in many dairy herds has fatty liver (Gonzalez et al., 2011). Fatty liver exacerbates the outcome of other metabolic diseases, in particular displaced abomasum and ketosis, because fatty liver decreases glucose availability for peripheral tissues (Veenhuizen et al., 1991). Furthermore, similar to excess body condition, increased liver TAG concentrations are associated with increased incidences and

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severity of laminitis, mastitis, milk fever, retained placenta, and metritis (Herdt, 1991). In the long-term, increased TAG concentrations are associated with decreased reproductive success and milk production in dairy cows. Given these facts, it is clear that a preventive for fatty liver would improve health, well-being, productivity, reproduction, and average lifetime of cows, which would result in major savings for U.S. dairy farmers.

Hypothesis for Our Research

On the basis of previous findings, we hypothesized that glucagon will prevent accumulation of TAG in liver of early lactation dairy cows by increasing glucose and lipid availability for peripheral tissues and by decreasing lipolysis in adipose tissue, thereby counteracting the effects of proinflammatory cytokines, in particular tumor necrosis factor α (**TNFα**). This hypothesis is based on results that demonstrate that fatty liver is preceded by and highly correlated with (r = 0.96) increased prepartal plasma TNFα concentrations and that fatty liver can be prevented by glucagon injection (Nafikov et al., 2006).

Research Goal and Summary

Veterinarians and herd managers have expressed a strong need for "tools" to prevent fatty liver and the array of prepartal diseases and disorders. Our *long-term goal* is to develop an effective and practical on-farm preventive for fatty liver that will decrease the incidence and severity of fatty liver and other periparturient diseases, thereby improving health, well-being, productivity, reproduction, and average lifetime of dairy cows. We expect future research to lead to such a tool. Our previous research has helped define the etiology and metabolic physiological consequences of ketosis and fatty liver (Hippen et al., 1999; Nafikov et al., 2006) and has led to an improved nutritional protocol that causes fatty liver for experimentation and to the development of glucagon as a treatment/preventive of clinical fatty liver. Glucagon has no known harmful effects on cattle, and it is a relatively small peptide that can be produced by recombinant or chemical means, which makes it economically feasible as a slow-release subcutaneous implant for use in the dairy industry.

In summary, our research has shown that subcutaneous injections of glucagon prepartally prevents fatty liver by increasing glucose and lipid availability for peripheral tissues through increasing hepatic gluconeogenesis and lipoprotein secretion and decreasing adipocyte lipolysis, thereby counteracting the detrimental effects of inflammatory factors such as TNFα. Application of our results is expected to decrease the incidence of fatty liver and related periparturient diseases and to increase the health, productivity, reproduction, lifetime, and well-being of dairy cows, which will improve the profitability of dairying in the U.S. Practical implementation of the validated approach will require from us further development of (1) noninvasive techniques, such as ultrasonography (Bobe et al., 2008; Weijers et al., 2012), for diagnosis of fatty liver and (2) a slow-release formulation for the delivery of glucagon (similar to commercial bovine somatotropin) for on-farm application.

Pathology of Fatty Liver

The pathology of bovine fatty liver (i.e., hepatic lipidosis) is well known. Up to 50% of all dairy cows accumulate excessive TAG in cells of liver and other organs (i.e., kidney and skeletal and cardiac muscles) during the peripartal and early postpartal period (Gerloff et al., 1986). If fatty liver is left untreated, cows often display signs of hepatic encephalopathy; symptoms progress from loss of appetite to general depression, to ataxia, to recumbency, to coma, and finally to death of the cow because of failure of affected organs. In fatty liver, accumulation of TAG in parenchymal cytoplasm is accompanied by disturbances in hepatic structure, including fatty cysts in liver parenchyma, increased cell volume, compression of sinusoids, decreased volume of rough endoplasmic reticulum, and mitochondrial damage, and by disturbances in metabolism, including decreased gluconeogenesis, because of decreased phosphoenolpyruvate carboxykinase (**PEPCK**) synthesis and activity, ketogenesis, ureagenesis, and lipoprotein synthesis and secretion, and increased lipid peroxidation (Bobe et al., 2004).   In cows with hepatic steatosis, damages in hepatic structure progress into liver necrosis and cirrhosis, and metabolic disturbances progress into 'steep' decreases in hepatic gluconeogenesis, oxidative processes, and ureagenesis, causing toxic concentrations of ammonia (Veenhuizen et al., 1991).

Fatty liver is associated with the duration and severity of other diseases (Gerloff et al., 1986) and decreased milk production and reproductive success. The damaging effects of excessive accumulation of TAG on the structure and metabolism of affected organs together with the increased concentrations of ammonia, ketone bodies, and nonesterified fatty acids (**NEFA**) in plasma and the decreased peripheral glucose, lipid, and amino acid availability explain why hepatic TAG concentrations determine the outcome of a myriad of additional disorders (Rehage et al., 1986). For example, cows with clinical fatty liver retained viable bacteria in the mammary gland much longer after experimental mastitis than did cows with slight fatty infiltration of liver (Hill et al., 1985). Cows with fatty livers are leukopenic, with decreases in neutrophils, eosinophils, and lymphocytes (Herdt et al., 1986). In both the peripartal and early postpartal period, fatty liver is associated strongly with ketosis left displaced abomasum, decreased immune response, increased susceptibility for retained placentas and parturient paresis, infectious diseases such as lameness, mastitis, and metritis , and fertility problems (Bobe et al., 2004). Cows that are obese at calving almost invariably have fatty liver and are highly susceptible to a complex of metabolic and infectious diseases known as "fat-cow syndrome". In one high-incidence herd, morbidity was 82% and mortality was 25% (Morrow et al., 1979). Postpartal serum NEFA concentrations are associated with the risk of developing displaced abomasum, clinical ketosis, metritis, and retained placenta during the first 30 days of lactation (Ospina et al., 2010).

Etiology of Fatty Liver

Deposition of TAG in liver is the consequence of mobilization of NEFA from adipose tissue exceeding capabilities of liver for oxidation and secretion of lipids (Gross et al., 2013). Cows with fatty liver have greater adipose stores and mobilize more TAG,

which leads to greater plasma NEFA concentrations, because adipose tissue from cows with fatty <u>liver is less responsive to lipogenic substances and more responsive to lipolytic substances.</u> Furthermore, cows with fatty liver have decreased fatty acid oxidation, hepatic apolipoprotein synthesis and lipid secretion, as indicated by decreased plasma apolipoprotein and lipid concentrations and decreased serum lecithin: cholesterol acyltransferase (**LCAT**) activity (Bobe et al., 2004). Besides disturbances in lipid metabolism, cows with fatty liver also have disturbances in glucose metabolism: Cows with fatty liver are either hyperinsulinemic-hyperglycemic or hypoinsulinemic-hypoglycemic (Holtenius, 1991), because either peripheral glucose uptake is decreased, indicating insulin resistance, or insulin and glucagon secretion and, therefore, hepatic gluconeogenesis are decreased. Furthermore, plasma amino acids are decreased. In summary, the availability of glucose, amino acids, and lipids for peripheral tissues is decreased in cows with fatty liver.

The metabolic effects in the etiology of fatty liver may in part be explained by direct or indirect actions of TNFα, a proinflammatory cytokine synthesized by macrophages, lymphocytes, and primarily adipose tissue (Bradford et al., 2009; Trevisi et al., 2012). Infections, trauma, and also parturition induce TNFα secretion, which mediates inflammatory responses that use great amounts of glucose, amino acids, and lipids. Tumor necrosis factor α induces the secretion of acute phase proteins from liver such as haptoglobin and serum amyloid A (**SAA**) and causes cell apoptosis and necrosis. Tumor necrosis factor α injections increased lipolysis, evidenced by increased plasma NEFA concentrations, decreased lipoprotein secretion, and decreased plasma lipid concentrations and induced a biphasic short-term hyperinsulinemic-hyperglycemic and long-term hypoinsulinemic-hypoglycemic response and insulin resistance in dairy cattle (Holtenius, 1991). The main supporting evidence was revealed when Ohtsuka et al. (2001) linked fatty liver in dairy cows with elevated serum TNFα concentrations and Bradford et al. (2009) showed that the cytokine promoted TAG deposition in liver. Cell culture and *in vivo* rat studies support the link between fatty liver and TNFα, because TNFα induces hepatic lipidosis, increases lipolysis in adipocytes, inhibits pancreatic insulin and glucagon secretion and glucose utilization, induces cell apoptosis and necrosis, decreases hepatic gluconeogenesis, lipoprotein synthesis, and ketogenesis, increases hepatic lipid peroxidation, and interferes with insulin and glucagon signal transduction. These metabolic actions of TNFα have stimulated our research team to conclude that TNFα is a major factor in the etiology of bovine fatty liver.

Tumor necrosis factor α production is increased in adipocytes from obese individuals and seems to contribute to basal lipolysis in obesity (Bradford et al., 2009). Moreover, TNFα-mediated increase in lipolysis seems mediated by changes in perilipin concentrations. Intracellular TAG of adipocytes is hydrolyzed primarily by adipose triglyceride lipase (desnutrin, **ATGL**) to diacylglycerol (**DAG**; Ducharme and Bickel, 2008). The DAG is hydrolyzed primarily by hormone-sensitive lipase (**HSL**) to monoacylglycerol, which then is hydrolyzed by another lipase to glycerol and another fatty acid. Perilipin in the phosphorylated form (protein kinase A-catalyzed synthesis) facilitates HSL interaction with the lipid droplet. Preliminary data from a colleague (Koltes and Spurlock, 2011) suggest that increased phosphorylation and not concentration of perilipin in bovine

adipose tissue is associated positively with NEFA mobilization from adipose tissue. Therefore, perilipin phosphorylation seems to be a major contributor to increased NEFA mobilization for fatty liver. We believe glucagon counteracts these actions.

Glucagon

Glucagon is a 29 amino acid long hyperglycemic hormone secreted from alpha cells of the pancreas and is highly conserved between species.   Glucagon increases plasma glucose by stimulating hepatic gluconeogenesis, glycogenolysis, amino acid uptake, and ureagenesis (Bobe et al., 2009). Glucagon increases lipolysis *in vitro* but not *in vivo* because of its hyperinsulinemic and hyperglycemic effects. Glucagon decreases hepatic TAG synthesis, increases TAG oxidation in bovine liver, and increases lipoprotein synthesis.   Effects of glucagon on metabolic pathways are mediated by cAMP, which binds to nuclear factors to increase mRNA expression and stability of key enzymes of metabolic pathways such as pyruvate carboxylase (**PC**) and phosphoenol pyruvate carboxykinase (**PEPCK**). Infusions of glucagon have increased k-casein and as-casein and decreased αs1-casein and α-lactalbumin in milk of dairy cows. Importantly, we have shown that intravenous infusions of glucagon for 14 d beginning at 2 d postpartum prevent fatty liver (Nafikov et al., 2006).

Rationale to Study the Role of Glucagon on Fatty Liver

To summarize our research that led us to test the hypothesis that glucagon would control fatty liver development and/or treatment, we determined that cows in early lactation are approximately 500 g/d deficient in glucose. On the basis of those results, we developed a preventive for fatty liver consisting of intraduodenal infusion of 500 g glucose per day for 14 d; we discontinued this approach because several cows showed signs of hyperexcitability during the last days of glucose infusion. We switched our research emphasis to use glucagon to increase blood glucose concentrations. Glucagon injections of 0.5 mg gave significantly increased plasma glucose concentrations for 2 h, which led us to suggest that administration of glucagon might be beneficial in alleviating or preventing the subsequent development of clinical ketosis/fatty liver by supplying more blood glucose (Veenhuizen et al., 1991. Because glucagon has a physiological half-life of 5 min, we found that continuous, 14-d infusions would cure cows of fatty liver (Hippen et al., 1999). We then developed the more practical preventive for fatty liver by using subcutaneous injections of glucagon every 8 h for 14 d starting on d 2 postpartum (Nafikov et al., 2006). The glucagon injections actually led to an acute decrease in plasma NEFA concentrations, which suggest antilipolytic effects (via perilipin phosphorylation?) in adipose tissue.

Prevention of Fatty Liver

We determined that intervention with glucagon as a treatment/prevention of fatty liver is most effective within 14 days after parturition. The results demonstrated that subcutaneous injections of glucagon of 7.5 and 15 mg/d starting at 2 d postpartum are

sufficient for fatty liver prevention (Figure 1); however, some cows developed fatty liver already at d 2 postpartum.

We have confirmed our previous results (Osman et al., 2008) showing that prepartally and subcutaneously injected glucagon will decrease markedly the accumulation of lipid in the liver of the postparturient dairy cow. Daily administration of the same amount (15 mg/day) of glucagon for several days prepartally in a limited number of cows was effective in preventing fatty liver during the early postparturient period. Figure 2 summarizes our proposed mechanism of action of glucagon on prevention and alleviation of fatty liver in dairy cows by subcutaneous administration of glucagon.

Summary of Our Research Contributions

Our research group has studied ketosis and fatty liver in ruminants for over 40 years. We developed techniques and models to investigate the effects of ketosis and fatty liver. The results of our work has:

- improved technique for collection of hepatic tissue and showed that liver biopsies provide representative samples of liver (Hippen et al., 1999).
- developed and improved laboratory techniques to study metabolic effects of fatty liver in vitro in bovine liver and adipose tissue (Veenhuizen et al., 1991).
- established and improved a nutritional model to induce fatty liver (Hippen et al., 1999).
- developed the first reliable method to diagnose fatty liver by using ultrasonography with a 3.5 MHz probe, which enables us to categorize normal and fatty liver cows with an accuracy > 90% (Bobe et al., 2008).
- demonstrated that glucagon administration treats (Hippen et al., 1999) and prevents (Nafikov et al., 2006) fatty liver in post-parturient dairy cows.

We helped elucidate the etiology and pathology of fatty liver as summarized in our review article (Bobe et al., 2004). We proved that fatty liver is preceded by hormonal imbalances as indicated by decreased plasma insulin and glucagon concentrations and increased growth hormone concentrations, which increase lipolysis in adipose tissue. During fatty liver, pancreatic insulin and glucagon secretion and peripheral glucose uptake are inhibited. We proved that fatty liver is preceded by metabolic disturbances in lipid metabolism as indicated by increased lipolysis in adipocytes, leading to increased plasma NEFA and ketone body concentrations. During fatty liver, the metabolic imbalances worsen as demonstrated by steep increases in adipocyte lipolysis, even stronger in the presence of lipolytic hormones, and increased plasma and liver NEFA and ketone concentrations, despite decreased hepatic fatty acid ketogenesis and oxidation. We also demonstrated that fatty liver is preceded by metabolic disturbances in glucose metabolism, as indicated by decreased plasma insulin, glucagon, and glucose concentrations and decreased liver glycogen concentrations. During fatty liver, the metabolic imbalances worsen because hepatic ketogenesis, oxidative processes, and gluconeogenesis from different gluconeogenic precursors are decreased strongly. The

latter changes are stimulated by decreased concentrations of gluconeogenic precursors, such as plasma amino acids, as well as by decreased PEPCK mRNA synthesis and PEPCK activity, as further indicated by increased hepatic phosphoenol pyruvate concentrations. Pancreatic insulin and glucagon secretion and peripheral glucose uptake seemed inhibited in cows with fatty liver (Veenhuizen, et al., 1991; Hippen et al., 1999; Nafikov et al., 2006; Osman et al., 2008).

To better understand the etiology and pathology of fatty liver, we added a new aspect to our investigations and analyzed samples of 4 cows with fatty liver and 4 normal cows for inflammatory responses. Our results show that lipid accumulation in the liver is preceded by increased prepartal TNFα concentrations in plasma. During lipid accumulation, concentrations of the acute phase proteins haptoglobin and SAA in plasma are increased, indicating an inflammatory response, plasma NEFA concentrations are increased, indicating increased lipolysis, and plasma glucose and lactate concentrations are decreased, indicating a shortage of gluconeogenic precursors. During the progression of fatty liver, plasma NEFA concentrations remained elevated, indicating increased lipolysis, and plasma cholesterol concentrations are decreased, indicating decreased liver lipid secretion. The correlation between liver lipid and plasma cholesterol on d 6 postpartum was r = -0.86. The inflammatory response, as shown by plasma TNFα, haptoglobin, and SAA concentrations, decreases in parallel to the decrease in liver lipid concentration. Concentrations of plasma haptoglobin on d 2 and liver lipid concentration on d 9 postpartum are highly correlated (r = 0.71); concentrations of plasma SAA on d 2 and liver lipid concentration on d 9 postpartum are even more highly correlated (r = 0.86). Even more remarkable is the correlation of 0.96 between the prepartal plasma TNFα concentration and the liver lipid concentration at d 9 postpartum (Ametaj et al.,2005).

Future Research

Our long-term goal is to develop an effective and practical on-farm preventive for fatty liver that will decrease the incidence and severity of fatty liver and other fatty liver-related periparturient diseases, thereby improving health, well-being, productivity, and average lifetime of dairy cows. If fatty liver could be either prevented or controlled in its earliest stages, millions of dollars in losses to U.S. dairy farmers could be avoided. Estimated annual economic losses range from $62,640,000 to $150,000,000 (Bobe et al., 2004). Currently, there is no practical preventive available for fatty liver. In future research, we wish to test the hypothesis that prepartal administration of glucagon will precondition the liver so that postpartum fatty liver development is less likely to occur. The rationale for testing subcutaneous glucagon injections is that (1) early postpartal glucagon injections have been proven to be effective in preventing fatty liver in dairy cows and (2) glucagon administration has proven to be safe for the cows when given on a repeated basis. Because subcutaneous glucagon injections increase gluconeogenesis and decreases lipolysis, we believe that these two processes will, in part, explain a preconditioning of the liver by prepartal glucagon administration to decrease postpartal fatty liver development. Finally, glucagon is a relatively small peptide that can be produced by recombinant or chemical means, making it likely that

glucagon can become economically feasible as a slow-release subcutaneous implant for use in the dairy industry. The rationale for focusing on the effects of glucagon on adipose lipolysis, hepatic gluconeogenesis, and lipoprotein secretion is that results from our laboratory and from refereed literature clearly indicate that a major problem in the etiology of fatty liver and other fatty liver-related periparturient diseases is deficiencies in glucose and lipid availability for animal tissues. Therefore, our future research will test the metabolic processes that have (1) a direct impact on the glucose, amino acids, and lipid availability and (2) are known to be affected by fatty liver. Our rationale for also focusing on TNFα is based on the intriguing fact that infectious and nutrition-related periparturient diseases are associated so strongly (Bradford et al., 2009).

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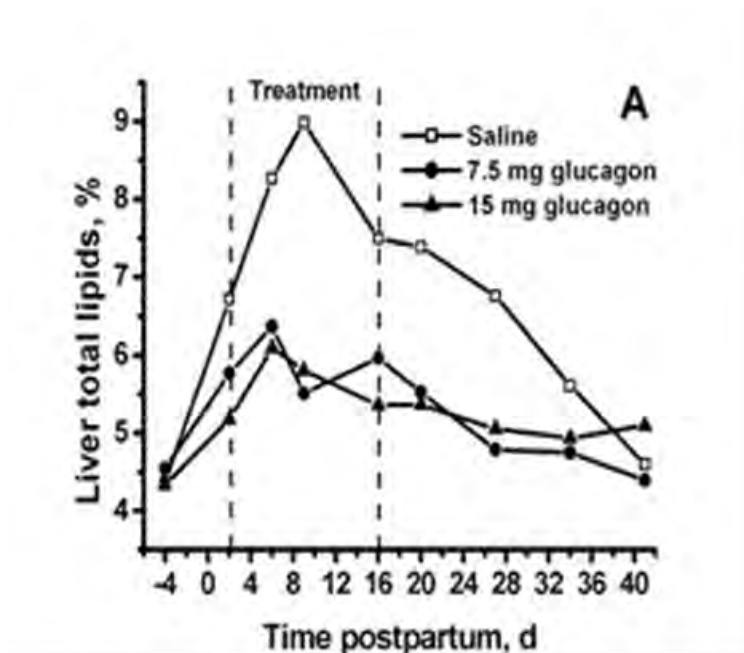
Figure 1. Effect of subcutaneous glucagon injections over 14 days beginning at d 2 (fatty liver prevention study) on liver lipid concentrations (SEM = 0.6) in dairy cows (n=8 in each group). These data were presented in (Nafikov et al., 2006).
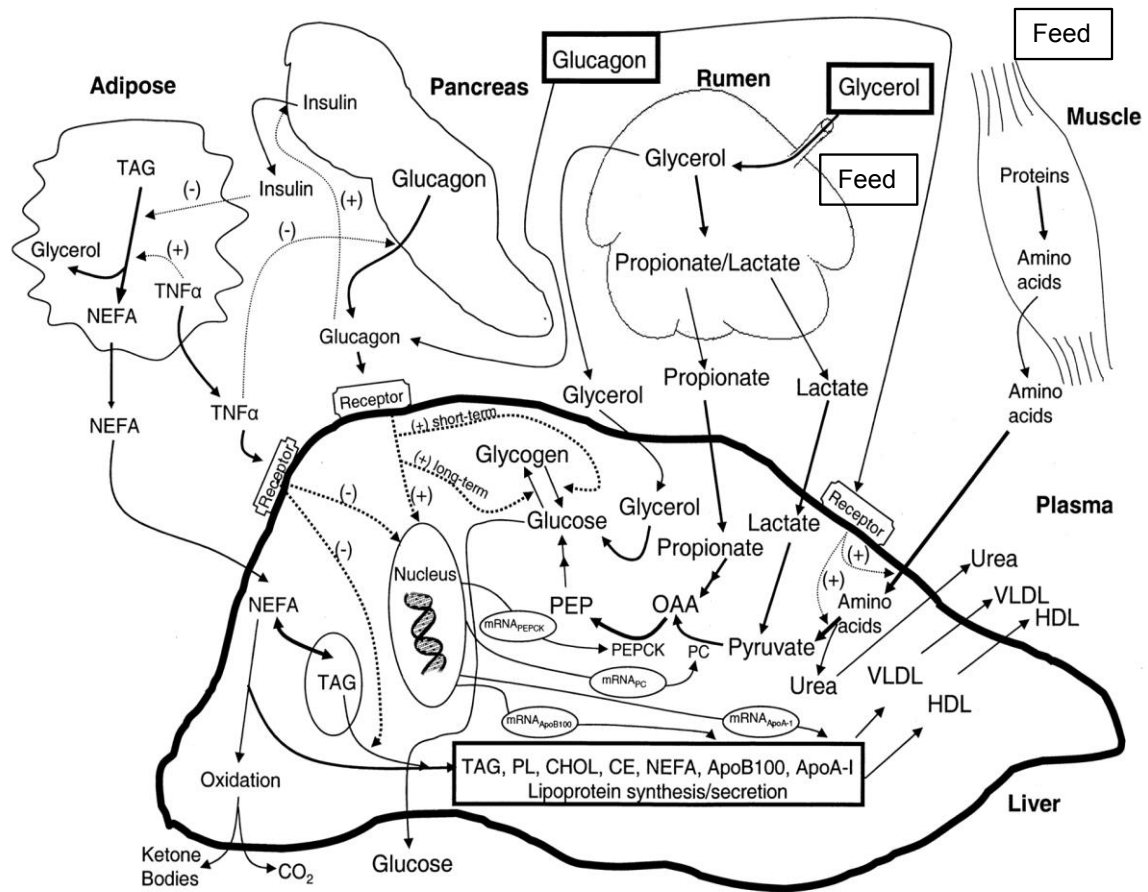
Figure 2. Expected cellular mechanisms by which consecutive subcutaneous injections of glucagon prevent fatty liver in dairy cows. Dashed arrows indicate regulatory functions.

SESSION NOTES