LIPOIC ACID SUPPLEMENTATION

IN THE OVARIECTOMIZED EWE

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

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ABSTRACT

Mottet, Rachel Susan, M.S., Department of Animal Sciences, College of Agriculture, Food Systems, and Natural Resources, North Dakota State University, July 2011. Lipoic Acid Supplementation in the Ovariectomized Ewe. Major Professor: Dr. Erika L. Berg.

Inadequate concentrations of progesterone during gestation can result in impaired embryonic growth and losses. These losses may be attributed to an overactive mechanism of progesterone catabolism or improper luteal function, which results in low concentration of progesterone. Progesterone catabolism occurs to the greatest extent by the liver, which holds a vast supply of cytochrome P450 enzymes and aldo-keto reductases that are involved in steroid inactivation. Insulin is a hormone produced by the pancreas that is involved in glucose uptake and metabolism. Progesterone catabolism is decreased in the presence of elevated insulin levels. Lipoic acid is a naturally occurring antioxidant and multienzyme cofactor which has been shown to increase insulin sensitivity and enhance glucose uptake in a number of species. The objectives of the current experiments were to 1) determine if administering a racemic mixture of lipoic acid by gavage at a dose of 32 mg/kg BW would increase peripheral progesterone concentrations, decrease progesterone clearance rates, or modulate cytochrome P450 2C (CYP2C), cytochrome P450 3A (CYP3A), or aldo-keto reductase 1C (AKR1C) hepatic enzyme activity, and 2) determine if dosing lipoic acid directly into the rumen at 32 mg/kg BW or 64 mg/kg BW would increase progesterone in the blood, decrease progesterone clearance rates, or modulate insulin. In the first trial, Katahdin cross ovariectomized ewes were randomly assigned to a control or a lipoic acid treatment group. In this experiment, a controlled internal drug release (CIDR) device was inserted in all ewes and serum samples were collected daily for five days to determine progesterone. Liver biopsies were performed on day 10 to measure

CYP2C, CYP3A, and AKR1C activity. Following liver biopsies, CIDRs were removed and an intensive blood sampling was performed to measure progesterone decay from peripheral circulation. We found that while lipoic acid does not have an effect on peripheral progesterone concentrations or hepatic enzyme activity, lipoic acid supplemented ewes have decreased progesterone clearance rates compared to control ewes. In the second trial, ovariectomized Katahdin cross ewes were randomly assigned to a control, low lipoic acid (32 mg/kg BW), or a high lipoic acid (64 mg/kg BW) treatment group. A CIDR was inserted in all ewes and blood samples were taken daily for 4 days. Following CIDR removal on day 11, an intensive blood sampling was performed to measure progesterone decay from peripheral circulation. One week following CIDR removal, ewes underwent an intravenous glucose tolerance test. It was found that lipoic acid supplementation did not affect progesterone concentrations, progesterone clearance, or insulin area under the curve. There was a treatment effect such that high lipoic acid dosed ewes had higher area under the curve for glucose when compared to control and low lipoic acid dosed ewes. Although no differences in progesterone concentrations were seen in the second trial, we speculate that the administration method rather than the efficacy of lipoic acid may account for the lack of differences observed. This theory is based on evidence from our first trial that oral lipoic acid supplementation did in fact reduce progesterone catabolism, as well as published data demonstrating that ruminally dosed lipoic acid is less effective than the equivalent oral dose.

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Lipoic Acid

Introduction

Lipoic acid is a naturally occurring compound found in most prokaryotic and eukaryotic cells in the body (Fuchs et al., 1997). The compound plays a role in energy formation while acting as a cofactor in various multienzyme complexes (Fuchs et al., 1997). Lipoic acid was originally classified as a vitamin until it was discovered to be synthesized *de novo* by plants and animals (Fuchs et al., 1997). In addition to being synthesized within the body, lipoic acid is consumed from various dietary sources including potatoes, carrots, spinach, and meat, with the highest tissue concentrations in the heart and liver (Fuchs et al., 1997; Packer et al., 2001).

Lipoic acid first gained attention in the 1930s when it was discovered by Snell et al. (1937) and referred to as "potato growth factor." Snell and coworkers (1937) observed the ability of an unknown growth factor to replace the growth stimulating activity of acetate in certain lactic acid bacteria. O'Kane and Gunsalus (1947) reported on a similar unknown growth factor that was required for pyruvate oxidation and dismutation by *Streptococcus faccalis*. The unknown growth factor in both cases was eventually found to be lipoic acid.

The molecular characteristics of lipoic acid posed a number of challenges for early researchers working towards isolating the compound. Lipoic acid is not extractable from tissue by a lipid solvent or hot water, but rather through hydrolysis with acid, alkali, or crude proteolytic enzymes (Fuchs et al., 1997). The compound was first isolated and identified in 1951 by Reed and coworkers when they extracted 30 mg of the yellow crystalline substance from 100 kg of acid-hydrolyzed liver residue (Reed et al., 1951).

Since its discovery in the 1930s, lipoic acid has been classified by various trivial names including acetate replacing factor, pyruvate oxidation factor, and protogen A (Fuchs et al., 1997). The isolation by Reed and coworkers in 1951, along with the elucidation of lipoic acid's molecular structure, led to the compound being classified as 1,2-dithiolane-3-pentanoic acid, 1,2-dithiolane-3-valeric acid, and thioctic acid. Due to its characteristic lipophilicity and acidity, the compound has been given the common name alpha-lipoic acid (Fuchs et al., 1997). In the scientific literature, lipoic acid is most commonly identified as α-lipoic acid, LA, or ALA. The acronym 'LA' will be used for the remainder of this thesis.

Structure

Octanoate is the precursor for LA's fatty acid chain and the amino acid cysteine is the source of sulfur (Breslow and Skolnik, 1966; Dupre et al., 1980). The molecular structure of LA includes a 1,2-dithiolane moiety which contains a five-membered ring system (Figure 1). The carbon ring contains two sulfur atoms attached by a disulfide bond. Lipoic acid has a chiral center at the C_6 carbon which is naturally found in the R configuration (Carlson et al., 2007).

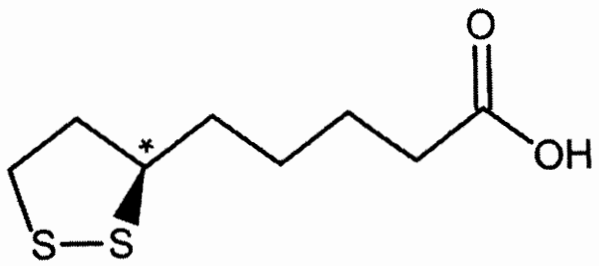

Figure 1. Molecular structure of LA (Packer et al., 2001). Asterisk represents chiral center.

Lipoic acid can be produced synthetically as an R or S enantiomer or as a racemic mixture. Of these two enantiomers, the R configuration is the only one found in nature and will bind preferentially over the S configuration to dihydrolipoamide dehydrogenase which is a component of mitochondrial enzyme complexes that utilize lipoic acid (Raddatz and Bisswanger, 1997). Differences in physiological effectiveness between the R and S enantiomers have been examined and reported in a variety of studies. A study by Packer et al. (2001) indicated that R-LA is more effective in insulin mediated glucose transport, reduction of free fatty acids in circulation, and reduction of plasma insulin levels. A study by Zimmer et al. (1995) reported that R- over S-LA improved aortic blood flow during reoxygenation and increased mitochondrial ATP synthesis in a working rat heart in an environment of oxidative stress. Further, insulin stimulated 2-deoxy-D-glucose uptake was increased 65% by R-LA whereas S-LA improved 2-deoxy-D-glucose uptake by only 29% in rat skeletal muscle (Streeper et al., 1997).

While it has been demonstrated that the R-LA is the most potent supplemented form of LA, a racemic mixture retains a portion of that potency. The racemic mixture is widely used in scientific trials due to a lack of evidence indicating the S-LA has any harmful effects on patients and due to the high cost of R-LA. According to MTC Industries Inc. (Edgewood, NY) in 2011, the price of R-LA was $580/kg, versus racemic LA which was $180/kg. Racemic LA has demonstrated its effectiveness at increasing insulin sensitivity in human patients, preventing oxidative stress, and having an antihypertensive effect (Jacob et al., 1999; Maddux et al., 2001; Midaoui and Champlain, 2002). Furthermore, the racemic mixture has been successfully used as a therapeutic agent for many years and is known to have potent antioxidant qualities (Ametov et al., 2003).

Synthesis

While the biosynthetic pathway of LA is not well understood, it is accepted that LA is a disulfide derivative of octanoic acid which is synthesized in the mitochondria of cells (Packer et al., 2001). Carreau et al. (1979) speculated that unsaturated fatty acids are the preferential precursors for LA synthesis. A study by Wada et al. (1997) focused on determining the role of mitochondrial synthesized fatty acids and their involvement in LA formation in plant eukaryotes. This study found a notable product of fatty acid synthesis in the mitochondria to be octanoic acid attached to the H protein of glycine decarboxylase (Wada et al., 1997). These findings are significant because LA is a derivative of octanoic acid and is an essential cofactor for H protein in glycine decarboxylase in chicken liver (Fujiwara et al., 1979). These researchers concluded a role of the mitochondria and fatty acid biosynthesis in the formation of LA.

Although the *de novo* production of LA in eukaryotes is not well defined, LA synthesis has been studied extensively in cultures of *E. coli* (Morris et al., 1995; Boom et al., 1991; Reed and Cronan, 1993). In these studies researchers have found that LA is synthesized from an octanoyl acyl carrier protein by the insertion of two sulfur atoms into an octanoyl group which results in a dithiolane ring (Morris et al., 1995; Boom et al., 1991; Reed and Cronan, 1993). In addition, an in vitro study by Fujiwara et al. (1990) indicated that H protein is lipoylated in the mitochondria and it was hypothesized that this is where LA is synthesized in eukaryotes. Further research is necessary to fully understand the eukaryotic biosynthetic pathway of LA.

Bioavailability and Metabolism

Bioavailability of free LA is important as it is likely to play the biggest role in the therapeutic effects of the compound (Gleiter et al., 1996; Teichert et al., 2003; Breithaupt-Grogler et al., 1999; Biewenga et al., 1997). Oral and intravenous administration of isolated LA in the form of R-LA or a racemic mixture has been studied extensively in human and animal models, and has been found to yield highly bioavailable LA (Podda et al., 1994; Peindo et al., 1989; Teichert and Preiβ, 1995; Breithaupt-Grogler et al., 1999). When lipoic acid is ingested from a natural dietary source such as meat, spinach, or potatoes, it enters the body bound to lysine originating from a multienzyme complex and is not available as free LA (Mattulat, 1992).

A study by Teichert et al (1998) showed a mean bioavailability of 29 ± 10.3% after a single oral ingestion of a 200 mg tablet of racemic LA in human patients. In a similar study, a single 600 mg tablet of LA dosed to diabetic patients yielded a bioavailability of 20.3 ± 4.5% (600 mg is considered to be the therapeutic dosage for diabetes patients). Biewenga et al. (1997) found the absolute bioavailability in human patients dosed with LA to be between 20% and 38%. A study by Harrison and McCormick found that when LA was orally administered to rats or incubated *in vitro* with rat liver systems, the bioavailability was approximately 20-40% (1974).

The limited bioavailability of LA is due to a high first pass metabolism and high hepatic extraction (Teichert et al., 2003). When ingested, approximately 93% of LA is rapidly absorbed and has a mean t_{max} of ~0.8 hours (Teichert et al., 2003). Rapid transport of LA is followed by rapid clearance and uptake into tissues including the liver, brain, heart, and skeletal muscle (Harrison and McCormick, 1973; Smith et al., 2004). Following

uptake into tissues, LA is quickly absorbed and metabolically altered and released by cells in its reduced form, dihydrolipoic acid (DHLA) (Bustamante et al., 1998).

A study by Schupke et al. (2001) confirms that β-oxidation is the major metabolic pathway *in vivo*, along with the oxidation of the dithiolane ring. The majority of LA breakdown occurs in the liver, which is supported by a study looking at radioactivity of various tissues after intraperitoneal and oral administration of radiolabelled *dl*-[^{14}C]-LA in the rat (Harrison and McCormick, 1976). In this study researchers found that the highest level of radioactivity was in the liver 4 hours after LA dose administration. Sufficient radioactivity was also observed in the intestinal contents and muscle in the same study (Harrison and McCormick, 1976).

Bustamante and coworkers (1998) found urinary excretion of LA is maximal within 3-6 hours after administration. Radiolabeled LA fed or administered via intraperitoneal injection to rodents was excreted at a rate of 80% in the urine within 24 hours of administration (Schupke et al., 2001). Studies in humans and rats show that LA is predominantly excreted in an altered form and at least 12 metabolites have been recovered from plasma and urine (Schupke et al., 2001; Gal and Razevska, 1960; Harrison and McCormick, 1976; Teichert et al., 2003). Reported metabolites include 4,6-bismethylthio-hexanoic acid, bisnorlipoic acid, and 2,4-bismethylthio-butanoic acid, among various others (Teichert et al., 2003).

Cofactor in Multienzyme Complexes

Lipoic acid is a major component in reactions acting as a cofactor tightly bound to protein in multienzyme complexes (Fuchs et al., 1997; Fujiwara et al., 1995; Morris et al.,

1995; Bustamante et al., 1997). Lipoic acid is involved in the function of a number of enzymes including 2-oxoglutarate dehydrogenase, pyruvate dehydrogenase, the branched chain 2-oxoacid dehydrogenases, and as part of the glycine cleavage system (Reed and Hackert, 1990). These enzymes and respective complexes regulate oxidative metabolism via pyruvate oxidation, the citric acid cycle, and amino acid biosynthesis and degradation (Bustamante et al., 1997).

The formally mentioned enzyme complexes are located in the mitochondrial matrix and share similarities in structural components (Bustamante et al., 1997). Each complex includes multiple copies of three common enzymes: E_1 or the α-keto dehydrogenase component (pyruvate 2-oxoglutarate or 2-oxo-osivalerate dehydrogenase); E_2 or the dihydrolipoyl acyltransferase component; and E_3 or the dihydrolipoyl dehydrogenase component (Figure 2) (Bustamante et al., 1997).

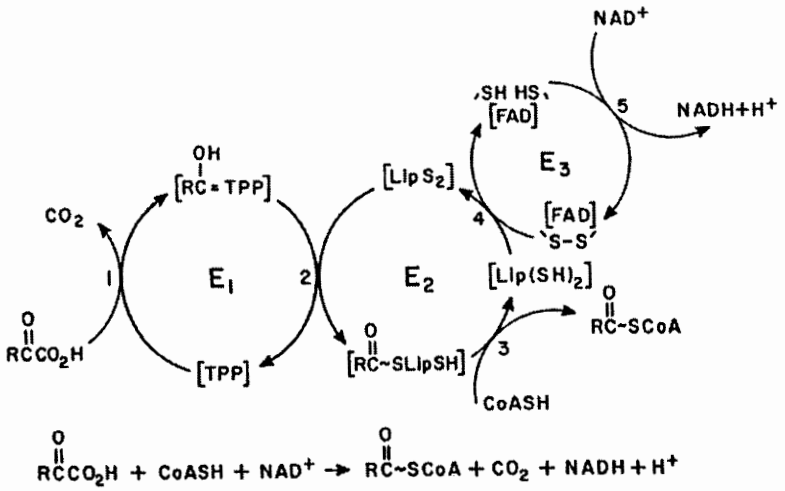

Figure 2. Reaction for the oxidative decarboxylation in 2-oxo acid dehydrogenase complexes (Yeaman, 1989).

While acting as a cofactor, LA is covalently bound to the ε-amino group of a lysine residue in a linkage referred to as a lipoyl group (Zhao et al., 2003). As part of the lipoyl

domain, LA will bind an acyl group from one enzyme complex and transfer it to another (Biewenga et al., 1997). In the process of transferring an acyl group, LA is reduced to dihydrolipoic acid (DHLA) (Biewenga et al., 1997).

Lipoic acid is also involved in the glycine cleavage system. The function of this system is to catalyze the oxidation of glycine to carbon dioxide and ammonia, forming NADH and (N^5, N^{10})-methylenetetrahydrofolate (Bustamante et al., 1997). There are four proteins in this complex termed P-, T-, L-, and H- protein (Bustamante et al., 1997). In this system, LA is covalently bound to the lysine in the H protein. During catalysis, carbon dioxide is released from glycine via catalysis by the P-protein causing a transfer of a methylamine moiety to the lipoyl group on the H protein. The lipoyl group is transferred to the H- protein, effectively reducing the LA group (Bustamante et al., 1998). Thus, LA acts similarly in this system as it does in other enzyme complexes.

Antioxidant Properties

Lipoic acid and its reduced form DHLA are known as powerful biological antioxidants (Packer et al., 1995; Moini et al., 2002). An antioxidant may be considered as any substance that when present at low concentrations compared with those of an oxidizable substrate significantly delays or prevents oxidation of that substrate (Halliwell, 1995). Without this oxidation, the oxidant excess can potentially lead to oxidative stress (Henriksen et al., 2010). Oxidative stress occurs when there is an imbalance such that oxidant production exceeds antioxidant production in cells and plasma (Henriksen et al., 2010). One role of an antioxidant is removal of reactive oxygen species to avoid damage to tissues prone to oxidative stress.

A potential antioxidant will have few or many of the following characteristics: capacity to quench free radicals, metal chelating ability, amphiphilic character, ability to interact with other antioxidants, an active role in repair systems, capacity to regenerate endogenous antioxidants, and the ability to affect gene expression (Packer et al., 1995; Biewenga et al., 1997). Other important qualities of antioxidants include absorption and bioavailability, concentration in tissues, and location (aqueous or membrane domains) (Henriksen et al., 2010). While a compound does not necessarily need to meet all criteria to be considered an antioxidant, LA in its reduced form DHLA does meet all the criteria making it an ideal antioxidant (Packer et al., 1995)

Free radicals which are scavenged by antioxidants have the capacity to cause damage to cellular proteins, membrane lipids, and nucleic acid, which eventually cause cell death (Maritim et al., 2002). The main source of free radicals in the body is believed to be from glucose oxidation (Maritim et al., 2002). In this process, glucose is oxidized to an enediol radical anion which is converted into reactive ketoaldehydes and superoxide anion radicals (Maritim et al., 2002). These radicals undergo dismutation to hydrogen peroxide, which in the absence of glutathione peroxidase, can lead to extremely reactive hydroxyl radicals (Wolff and Dean, 1987).

Lipoic acid will scavenge hydroxyl radicals, hypochlorus acid, peroxynitrite, and singlet oxygen among a broad array of reactive oxygen and nitrogen species (Packer et al., 2001). While the oxidized form of LA is efficient in inactivation of free radicals, LA in its reduced form, DHLA, is a stronger antioxidant able to interact with reactive oxygen species to scavenge superoxide and peroxyl radicals (Packer et al., 1995). DHLA is also a reductant which enables regeneration of oxidized agents including ascorbate, glutathione, coenzyme

Q and vitamin E (Packer et al., 2001). The regeneration quality of DHLA is beneficial to the body because it enables a readily available supply of antioxidants to scavenge potentially damaging free radicals. The interaction between DHLA and regeneration of these other antioxidants is known as the antioxidant network (Packer et al., 2001).

Glucose Metabolism

Maintenance of normal blood glucose levels in mammalian species is an essential physiological function that is maintained through the coordinated efforts of various organs. Organs involved in glucoregulation include the endocrine pancreas, liver, adipose tissue, and specific hypothalamic neurons (Henriksen et al., 2010). Glucose transport and uptake occurs via the glucose transport system which is activated by insulin that is synthesized and secreted from β cells of the pancreas (Henriksen et al., 2010) The liver assists in glucoregulation by mediating alterations in hepatic glucose production and output based on physiologically perceived circulating glucose (Henriksen et al., 2010). Adipose tissue and skeletal muscle act as sites of insulin dependent glucose disposal, while the hypothalamus provides neuroregulation for all of these organs (Henriksen et al., 2010).

Physiologic events necessary for glucose homeostasis are achieved via the glucose transport system (Shepherd and Kahn, 1999; Zierath et al., 2000). This system is regulated by insulin through the engagement of intracellular proteins. These intracellular proteins are part of insulin mediated receptors which enable glucose uptake (Shepherd and Kahn, 1999; Zierath et al., 2000; Schmidt and Hickey, 2009). Insulin binds to the α-subunit on an insulin receptor which increases the tyrosine kinase activity of the β-subunit. This in turn phosphorylates insulin receptor substrates (Henriksen et al., 2010). The phosphorylated

products then interact with insulin receptor substrate proteins and activate the phosphatidylinositol 3-kinase pathway (Henriksen et al., 2010; Schmidt and Hickey, 2009). The phosphoinositide moieties formed then activate the 3-phosphoinositide-dependent kinases, which will then phosphorylate AKt, a serine/threonine kinase (Henriksen et al., 2010). One result of this phosphorylation is the modulation of the glucose transporter protein GLUT-4 through AKt acting on AS160 which contains a domain associated with the GLUT-4 transporter (Cartee and Wojtaszewski, 2007; Sano et al., 2003). This activity will cause the translocation of the GLUT-4 transporter protein to the sarcolemma membrane, at which point glucose transport can occur through facilitative diffusion (Cartee and Wojtaszewski, 2007; Sano et al., 2003). Overall, the activation of the insulin receptor substrate phosphatidylinositol-3 kinase pathway leads to events involved in glucose transport, glycogen synthesis, protein synthesis, nitric oxide synthesis, and anti-apoptosis (Schmidt and Hickey, 2009).

Therapeutic intervention is necessary in patients with an impaired glucose transport system or those with impaired insulin sensitivity in the form of hyperglycemia or insulin resistance. A general definition for insulin resistance is the inability of insulin to demonstrate an adequate response in clearing blood glucose (Treiber et al., 2006). Impaired insulin synthesis and action are common consequences of type I and II diabetes mellitus (Schmidt and Hickey, 2009). Type I diabetes is characterized as a condition in which pancreatic β-cell destruction leads to insulin deficiency (Daneman, 2006). While exogenous insulin administration may control various aspects of type I diabetes, there are numerous complications associated with the illness that affect the vascular system, retina, lens, peripheral nerves, kidney, and skin (Maritim et al., 2002).

Type II diabetes is acquired due to genetic predisposition and behavioral and environmental risk factors including sedentary lifestyle and obesity (Tuomiletho et al., 2001). Patients with type II diabetes will have some degree of impaired glucose tolerance which leads to insulin resistance, decreased insulin stimulated glucose uptake by skeletal muscle, and impaired suppression of glucose output by the liver (Biddinger and Kahn, 2006). These complications can be costly in terms of quality of life and longevity in type II diabetic patients (Maritim et al., 2002).

Oxidative stress is an early event in the pathology of diabetes mellitus that is a major factor in vascular and neurological complications in patients (Borcea et al., 1999). Risks associated with oxidative stress in diabetic patients result from a reduced antioxidant reserve and excessive production of reactive oxygen species, especially the superoxide anion (O_2^-) (Midaoui and Champlain, 2002). As mentioned earlier, LA aids in alleviating oxidative stress by scavenging free radicals and by replenishing other antioxidants in its reduced form, which is one reason it is widely used as a therapeutic agent for patients suffering from diabetes.

Patients with type II diabetes are often initially diagnosed with hyperinsulinemia; therefore insulin treatment is not always a necessity (Jacob et al., 1999). In these cases, therapeutic intervention should focus on clearing blood glucose through enhancing insulin sensitivity through diet and exercise (Jacob et al., 1999). If diet and exercise fail to treat insulin resistance, pharmaceutical intervention is necessary.

Lipoic acid supplementation has a beneficial effect on glucose metabolism through enhanced glucose uptake into muscle cells in human and animal models (Jacob et al., 1996; Yaworsky et al., 2000; Busse et al., 1992). Jacob et al. (1996) have shown that dosing

obese Zucker rates with a racemic mixture of LA significantly enhances insulin action in skeletal muscle by increasing oxidative and nonoxidative glucose metabolism (Jacob et al., 1996). In a similar study, LA treated Zucker rats showed an increase in tissue levels of glutathione, which is a compound that improves insulin sensitivity in insulin-resistant and type II diabetes patients (Busse et al., 1992; Evans et al., 2003). A study by Yaworsky et al (2000) showed LA rapidly stimulated glucose uptake in cell cultures by activating the insulin-signaling pathway. A study in human patients with type II diabetes treated with racemic LA demonstrated improved body glucose disposal indicated by hyperinsulinemic-isoglycemic clamp technique (Jacob et al., 1995; Jacob et al., 1996).

These data provide substantial evidence indicating that LA supplementation can improve insulin sensitivity and reduce the occurrence of oxidative stress when used as treatment in diabetic patients. While the precise mechanism of action for LA on the insulin signaling pathway has yet to be elucidated, it is thought that LA acts on the insulin receptor resulting in enhanced glucose uptake (Moini et al., 2002).

Therapeutic Uses

The therapeutic uses of LA have shown promise for several decades, especially in patients with diabetes as mentioned previously. Lipoic acid has been administered to patients as early as the 1960s with liver cirrhosis, mushroom poisoning, and heavy metal intoxication (Biewenga et al., 1997). In cases of metal intoxication, LA can render compounds such as arsenate As^{3+} inactive by forming a complex in the 2-oxo dehydrogenases (Biewenga et al., 1997). Lipoic acid is also considered a potential therapeutic agent in pathologies associated with imbalance of the oxidoreductive cellular

status (Bustamante et al., 1997). This occurs in the case of ischemia-reperfusion, AIDS, neurodegeneration, polyneuropathy, and hepatic disorder status (Packer et al., 1995). In addition, supplementation with LA can be beneficial in the prevention of hypertension, insulin resistance and oxidative stress (Midaoui and Champlain, 2002).

Progesterone

Introduction

Progesterone (Figure 3) is classified as a steroid hormone and is therefore a derivative of cholesterol (Miller and Auchus, 2011). The molecular structure of the hormone includes a cyclopentanoperhydrophenanthrene nucleus along with three six membered rings and one five membered ring designated as A, B, C, and D respectively (Senger, 2005). Steroid hormones encompass a large class of small-sized lipophilic molecules which are synthesized by steroidogenic tissues and act on respective target tissues (You, 2004).

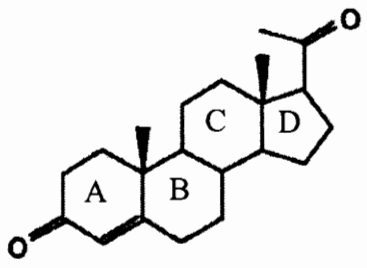

Figure 3. The molecular structure of progesterone (Penning, 2000).

Progesterone is produced primarily by ovarian thecal cells as well as the placenta during gestation (Senger 2005). Target tissues include the uterine endometrium, mammary gland, myometrium, and the hypothalamus (You, 2004; Senger 2005). Progesterone has

various physiological functions including promotion of endometrial secretions that aid in embryonic and fetal development, inhibition of gonadotropin releasing hormone which is involved with cyclicity, inhibition of reproductive behavior, and maintenance of pregnancy (Senger, 2005).

Biosynthesis

The first step in steroid hormone biosynthesis is endocytosis of cholesterol into the mitochondria (Praseetha et al., 2009). The cholesterol derivative for steroidogenesis can come from cholesterol stores in intracellular lipid vesicles, stores within the cell which are synthesized from acetate, or through uptake from lipoproteins (Praseetha et al., 2009). Cholesterol is obtained by cells in ruminants through the selective uptake of cholesterol esters from high density lipoprotein (Christenson and Devoto, 2003). Steroidogenic acute regulatory protein is involved in transporting cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane where steroidogenesis can occur (Miller and Auchus, 2011; Praseetha et al., 2009).

Once in the inner mitochondrial membrane, cholesterol undergoes catalysis by P450 side chain cleavage to create pregnenolone (Christenson and Devoto, 2003). The second conversion is pregnenolone to progesterone catalyzed by 3β-hydroxysteroid dehydrogenase which comes from the smooth endoplasmic reticulum (Christenson and Devoto, 2003). After this step, progesterone is available to act on its specific target tissues in the body.

Progesterone release in the ruminant occurs at the end of estrus and is dependent on a surge of luteinizing hormone acting through the cAMP/protein kinase A pathway (Niswender et al., 2000). Following the luteinizing hormone surge, ovulation will occur

and a corpus luteum will form on the ovary in place of a recently ovulated follicle. Progesterone is then produced by highly specific ovarian thecal cells and is secreted by the corpus luteum. In most eutherian mammals, progesterone from the placenta will take over progesterone production for the developing fetus; in the ewe specifically this occurs after day 50 of gestation (Senger, 2005).

Metabolism

Sheep produce roughly 15 to 30 mg of progesterone per day (Stormshak et al., 1963). Stormshak et al. (1963) reported that the sheep corpora lutea have an average turnover rate of two minutes for progesterone. The turnover rate is the length of time required to release an amount of hormone equal to what is already present in the gland (Stormshak et al., 1963). Stormshak et al. (1963) analyzed ewe ovarian venous blood, and detected average progesterone concentrations of 1.58, 1.82, and 1.10 μg/ml in the early, mid, and late luteal phase respectively. Weems et al. (1989) reported progesterone levels in the uterine venous plasma to be 5 ng/ml compared to 2 ng/ml in the jugular plasma on 9 days post-estrus.

Metabolic clearance rate can be defined as the "amount of blood which is completely and irreversibly cleared of steroid in unit time" (Bedford et al., 1974). Metabolic clearance rates of progesterone in sheep have been reported by Bedford et al. (1972; 1974) to be approximately 3.5 to 5.7 l/min. Bedford et al. (1974) have demonstrated that splanchnic progesterone clearance from abdominal organs accounted for approximately 30% of peripherally administered progesterone while the liver was responsible for 70 - 90% of progesterone clearance (Bedford et al., 1974). A study by

Gurpide (1975) indicated that hepatic extraction of progesterone in pregnant ewes was approximately 70%, while a study by Parr et al. (1993) indicated that progesterone entering the gut and liver regions was metabolized at a rate of 96% by these tissues (Parr et al., 1993).

Progesterone is inactivated by the liver through hydroxylation of the steroid nucleus. This process yields 21-hydroxprogesterone or 6β-hydroxyprogesterone metabolites in sheep and is largely due to the action of cytochrome P450 2C (CYP2C) and cytochrome P450 3A (CYP3A) (Murray, 1991; 1992). Cytochrome P450 is a generic name for a superfamily of oxidative enzymes which contain roughly 500 amino acids and a single a heme cofactor (Miller and Auchus, 2011). The enzymes are involved in various pathways including vitamin D3 activation, metabolism of all major steroid hormones, and the metabolism of cholesterol to bile acids (Waxman et al., 1991). Using their heme cofactor, P450 enzymes activate molecular oxygen and add electrons from the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) (Miller and Auchus, 2011). Murray et al. (1991; 1992) reported CYP2C and CYP3A in the sheep liver metabolized progesterone at a rate of 270 pmol/min/mg microsomal protein and 430 pmol/min/mg microsomal protein, respectively. In sheep, contribution of CYP2C to progesterone inactivation is estimated to be 25% and for CYP3A, 55% (Murray, 1991; 1992).

Authors have also suggested a role of aldo-keto reductase 1C in progesterone inactivation (Penning et al., 2000). The aldo-keto reductase family of enzymes are involved in the reduction of glucose, generation of bile acids, prostaglandin metabolism, and reduction of steroids contacting aldehyde or ketone groups (Penning et al., 2000; Barski et

al., 2008; Kabututu et al., 2009). Aldo-keto reductase 1C is involved in converting progesterone to 3α-hydroxyprogesterone or 20α-hydroxyprogesterone metabolites in humans and rodents (Penning et al., 2000).

It has been demonstrated that increased dry matter intake (DMI) in sheep results in decreased peripheral plasma progesterone concentrations (Williams and Cumming, 1982; Parr et al., 1993). The relationship in dairy cattle between DMI and metabolic clearance of progesterone can be explained by the fact that increased hepatic blood flow results in increased progesterone catabolism, which is presumably through an increase substrate delivery to the liver (Sangsritavong et al., 2002). Burrin et al. (1989) found that increased DMI resulted in increased liver oxygen consumption and increased liver blood flow. Similar relationships have also been reported in dairy cattle and swine (Symonds and Prime, 1989; Rabiee et al., 2001).

Progesterone and Pregnancy

Progesterone is crucial for a successful pregnancy in mammalian species. Miller and Moore (1976) demonstrated in ewes that exposure to progesterone was critical to embryonic survival, and the absence thereof would result in embryonic death. Progesterone is also important in pregnancy as it inhibits myometrial contraction (Leonhardt and Edwards, 2002). Studies examining differences in myometrial contractility show that progesterone leads to a relaxed and quiescent uterine environment and inhibits the release of hormones that can be harmful to the developing embryo (Mesiano, 2007). Studies have also indicated that when the progesterone source is removed during pregnancy the embryo or fetus is aborted (McDonald et al., 1952; Csapo, 1956).

Ruminants undergo spontaneous seasonal ovulation through a uterine-dependent estrous cycle until a pregnancy is established (Spencer and Bazer, 2005). The ruminant estrous cycle, establishment of pregnancy, and maintenance of pregnancy, require endocrine and paracrine signals from the ovary, conceptus, and uterus itself (Spencer et al., 1995). Steroid sex hormones are involved in coordinating reproductive processes by regulating gene expression through specific receptor proteins within target cells (Ing and Tornesi, 1997).

The action of progesterone is mediated by the progesterone receptor (Spencer and Bazer, 2002). Progesterone receptors belong to a family of nuclear receptors that function as ligand-activated transcription factors which in turn regulate the expression of specific sets of target genes (Leonhardt and Edwards, 2002). A major function of these receptors is to autoregulate the expression of their own and other genes (Schmidt and Meyer, 1994). During early pregnancy, progesterone will inhibit estrogen receptor expression (Leonhardt and Edwards, 2002) to prevent cyclic activity and luteolysis.

Responsiveness of the uterus to progesterone then provides an environment that is crucial for embryonic development (Thatcher et al., 1994). In order to maintain a successful pregnancy synchrony must exist between the needs of a developing embryo and hormonal secretions of the uterus (Pope, 1988). In ruminants, the release of interferon-tau signals the presence of an embryo to the mother and elicits physiological responses due to maternal recognition of pregnancy (Spencer and Bazer, 2005). This event will have an antiluteolytic effect by preventing the release of prostaglandin $F_{2\alpha}$ (Spencer and Bazer, 2005). This event will also enable a functional corpus luteum to secrete progesterone to support the embryo (Spencer and Bazer, 2005).

After maternal recognition of pregnancy and prior to implantation in the uterus, the ovary, endometrium and embryo interact to establish pregnancy. The conceptus secretes steroids, prostaglandins, cytokines, and growth factors which vary by species to assist in preventing the secretion of embryo-toxic concentrations of prostaglandin $F_{2\alpha}$ (Goff 2002). Progesterone and estradiol are then synthesized and secreted from the ovary to act on the endometrium to produce proteins that nourish the developing embryo (Goff, 2002).

Progesterone, Insulin and Cytochrome P450 Enzymes

It has been suggested by Dean and Stock (1975) that a mechanism exists to down regulate cytochrome P450 activity to ensure adequate progesterone levels to maintain a pregnancy. A relationship between cytochrome P450 enzymes and insulin was first observed by Barnnett et al. (1990) and Shimojo et al. (1993) who showed that insulin dependent diabetic rats had enhanced expression of CYP3A, which was alleviated by insulin therapy. Later work by Sidhu and Omiecinski (1999) cultured rat hepatocytes in the absence or presence of 1 μM insulin and exposed cultures to varying concentrations of phenobarbital. These researchers found that in the absence of insulin, mRNA expression for cytochrome P450 enzymes was increased 1.5 to 2-fold.

Other studies strengthen the notion that there may be a relationship between insulin, cytochrome P450 enzymes, and progesterone clearance (Smith et al. 2006; Lemley et al. 2008). Smith et al. (2006) found that gavaging ewes with sodium propionate, a gluconeogenic substrate, resulted in elevated insulin concentrations and decreased progesterone clearance rates compared to ewes dosed with isocaloric sodium acetate. A study by Lemley et al. (2008) examined hepatic CYP2C and CYP3A activity in

ovariectomized ewes dosed with sodium propionate and found treated ewes had elevated insulin concentrations and a 50% reduction in hepatic CYP2C and CYP3A activity when compared to ewes dosed an isocaloric level of sodium acetate. These studies suggest that cytochrome P450 activity can be modulated by increased levels of insulin or a gluconeogenic substrate, which leads to decreased progesterone catabolism.

Statement of the Problem

Over one billion dollars is lost annually in the beef and dairy industries as a result of pregnancy loss (Bellows et al., 2002). Early embryonic losses account for approximately 30% of all pregnancy losses and in sheep these losses are the highest within the first three weeks of gestation (Moore at al., 1985). Factors that contribute to these losses can include inadequate luteal function, low levels of circulating progesterone, an overactive mechanism of progesterone catabolism, or a combination of these factors (Inskeep and Dailey, 2005). Due to the variability in success with use of exogenous progesterone sources to improve conception rates and pregnancy, our research focused on using a dietary supplement to down regulate progesterone catabolism. Elevated insulin concentrations have been shown to result in decreased progesterone catabolism and lipoic acid is a dietary supplement that has been found to affect both insulin and glucose concentrations. As a result, we supplemented lipoic acid with the long term goal of creating an environment with sufficient progesterone levels post conception, ultimately increasing pregnancy maintenance and reducing financial loss to producers.

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CHAPTER 2. LIPOIC ACID DECREASES PROGESTERONE CLEARANCE IN THE OVARIECTOMIZED EWE

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Abstract

Lipoic acid is a naturally occurring compound that has been shown to modulate insulin sensitivity when supplemented to the diet. Elevated blood insulin concentrations have been shown to decrease progesterone catabolism in several species by modulating metabolic liver enzyme activity and expression. We[1] hypothesized that lipoic acid supplementation would decrease progesterone ($\mathbf{P_4}$) catabolism by the liver. Eight ovariectomized ewes were fed an alfalfa-grass ration at 95% of ad libitum for the duration of the experiment. Ewes were randomly assigned to a control group [an empty bolus administered by gavage (n = 4; **CON1**)], or lipoic acid group [supplemented at 32 mg/kg BW administered by gavage (n = 4; **LA**)]. Progesterone was administered via CIDR devices on d 5 to all ewes. Blood samples were collected daily from d 6 to 9. On d 10, liver biopsies were obtained from each ewe to determine CYP2C, CYP3A, and AKR1C activity. On d 11, serial blood samples were collected after CIDR removal to determine P_4 clearance. Ewes treated with LA had a lower rate constant ($P < 0.03$) for P_4 clearance compared to CON1 ewes; however no difference ($P = 0.20$) in hepatic enzyme activity was

[1] This project was a collaborative effort by the authors listed above. The contribution of Rachel Mottet includes experimental planning and organization, data collection, data analysis, and written documentation of the experimental procedures and outcomes. Co-authors Dr. Caleb Lemley, Dr. Erika Berg, and Dr. Kimberly Vonnahme contributed the novel experimental proposal, design, and editing as needed of written material by Rachel Mottet pertaining to the trial.

found and there were no differences of treatment ($P = 0.13$) on circulating P_4 in CON1 ewes compared to LA ewes. We conclude that while lipoic acid decreased P_4 clearance in the blood, it did so without affecting hepatic enzyme activity; therefore the mechanism of action is yet to be elucidated.

Introduction

Embryonic death in sheep is the greatest within the first 3 weeks of gestation and contributes greatly to economic losses suffered by producers (Moore, 1985). Over $1 billion is lost annually as a result of reproductive disease, infertility, and pregnancy conditions causing abortions, stillbirths, or dystocia in beef and dairy cattle (Bellows et al., 2002). Progesterone plays a critical role in the maintenance of pregnancy in mammals (Csapo, 1956); therefore factors contributing to early pregnancy losses may include low initial levels of circulating P_4 during gestation, an overactive mechanism of P_4 catabolism, improper luteal function, or a combination of these factors (Inskeep and Dailey, 2005). Progesterone is critical during early gestation as it influences the production of endometrial secretions that aid in early embryonic development (Nephew et al., 1991). Consequently, low concentrations of P_4 can lead to poor embryonic development (Nephew et al., 1994) and may enable an increase in embryo-toxic hormones (Inskeep, 2004).

Catabolism of P_4 occurs predominantly by the liver where there is an abundance of hepatic cytochrome P450 enzymes involved in steroid inactivation (Parr et al., 1993). Those contributing the greatest to P_4 catabolism include cytochrome P450 2C (**CYP2C**), cytochrome P450 3A (**CYP3A**) and aldo-keto reductase 1C (**AKR1C**) (Murray et al. 1991; 1992; Penning et al., 2000). Decreased P_4 catabolism has been shown to be positively

correlated with elevated insulin levels in ewes administered a gluconeogenic substrate (Smith et al., 2006; Lemley et al., 2008). In addition, rat hepatocyte cell lines challenged with increasing physiological concentrations of insulin showed a dose-dependent decrease in CYP2C and CYP3A activity (Lemley et al., 2009).

Lipoic acid is a naturally occurring compound that has been found to increase insulin sensitivity and glucose uptake when supplemented in human and animal models (Jacob et al., 1996; Moini et al., 2002; Yaworsky et al., 2000). Jacob et al. (1996) have shown that dosing obese Zucker rates with a racemic mixture of lipoic acid significantly enhances insulin action in skeletal muscle by increasing oxidative and nonoxidative glucose metabolism. A study by Yaworsky et al (2000) showed lipoic acid rapidly stimulates glucose uptake in cell cultures by activating the insulin-signaling pathway. Consequently, we hypothesized that lipoic acid would decrease P_4 catabolism and clearance rates by modulating hepatic steroid metabolic enzyme activity.

Materials and Methods

Animals and Diets

Animal care and use was approved by the Institutional Animal Care and Use Committee at North Dakota State University (Fargo, USA). Eight mature Katahdin-cross ovariectomized ewes were housed at the Animal Nutrition and Physiology Center in Fargo. Ewes were penned individually in 0.91 x 1.20-m pens in a temperature controlled (12°C), well-ventilated facility with ad libitum access to fresh water. Lighting was automatically timed to mimic the natural photo period during the fall season in North Dakota. Ewes were weighed, marked, individually penned, and fed an ad libitum diet of alfalfa-grass mix hay

for 1 wk prior to start date (d 0). Intake was determined by measuring weight of initial feed offered subtracted by weight of residual feed after a 24 h period and averaged for the 1 wk period. Subsequently, intake was restricted to 95% of intake from d -14 to -7 with a 1 wk acclimation period to this diet. From d -7 until completion of the trial ewes continued to receive 95% of intake.

Ewes were randomly assigned to one of two groups; control (CON1; empty bolus; n = 4), or lipoic acid (LA; 32 mg/kg BW; n = 4; racemic mixture of lipoic acid, MTC Industries, Inc. Edgewood, NY). Ewes were weighed on d -2 and d -1 and BWs averaged to determine lipoic acid dosages. Lipoic acid was measured into clear gelatin capsules that held a maximum of 1 g (Torpac, Inc., Fairfield, NJ). After ort collection and prior to feeding, LA ewes were dosed with lipoic acid at 0730 on d 0. Lipoic acid was administered to LA ewes by gavage using a plastic tube with a wooden rod for capsule projection to encourage swallowing. Similarly, CON1 ewes were gavaged with empty capsules. The number of capsules administered to the control group was based on the weight of a full capsule of lipoic acid and BW of control ewes.

Sample Collection

Prior to feeding and lipoic acid supplementation on d 5, serum samples were collected via jugular venipuncture (BD Vacutainer, Franklin Lakes, NJ). Blood samples were immediately put on ice and cooled for 2 h before centrifugation. Samples were centrifuged (Beckman Coulter, Fullerton, CA) at 2500 x g for 20 min, serum was pipetted and stored at -20°C until further analysis. Following d 5 blood sampling, a controlled internal drug release [CIDR; EAZI-BREED CIDR, 0.3 g progesterone (P4), Pfizer Animal

Health] was inserted vaginally into ewes. From d 6 to 9 blood samples were collected daily at 0730 and handled as described previously.

Liver Biopsy Procedure

On d 10, liver biopsies were performed 1 h post gavage and feeding to determine hepatic enzyme activity. Ewes were placed in restraining pens and wool was removed from the animals' right side over the rib cage. Ultrasonography was performed on the first ewe to determine a location devoid of major branches of the hepatic portal vein and hepatic artery. The point of insertion was at the 10^{th} intercostal space intersected by a line drawn from the point of the elbow to the point of the hip. The area was cleaned three times with betadine scrub and sprayed with 70% ethanol. Ewes were administered 10 cc of 2% lidocaine hydrochloride subcutaneously and intramuscularly at the point of insertion. The skin was punctured using a scalpel and liver samples were collected using a biopsy needle machined at the West Virginia University Physics Department (Morgantown, USA) following the specifications of Swanson et al. (2000). The biopsy tool was guided to the liver and a 0.5 to1 g sample was collected. Approximately 200 mg of liver was submerged in 100 mM potassium phosphate buffer containing 1 mM EDTA to assess CYP2C and CYP3A activity in fresh liver samples. The remaining portion of the liver was snap frozen in liquid nitrogen and stored at -80 °C for later analysis of AKR1C activity. Upon completion of the liver biopsy procedure, the incision site was closed with a sterile surgical staple and sprayed with Blu-Kote (H. W. Naylor Company Inc., Morris, NY).

Progesterone Clearance

On d 11 CIDRs were removed and serial blood samples were obtained via jugular catheters to determine P_4 clearance. The catheterization site was prepared, cleaned with alcohol and injected with 1 mL of lidocaine prior to insertion of a 14 gauge catheter needle and catheter (I-CATH, Charter Med Inc., Winston-Salem, NC). After placement was confirmed, catheters were flushed with 3 mL of heparin saline, stitched into place and covered with co-flex vet wrap. Serial blood samples were collected through jugular catheters at 0, 2, 5, 10, 15, 30, 60, 120, and 360 min post CIDR removal. Gavage of treatment and feeding was performed coinciding with the 0 min sample. At each sample time, the first1 mL of blood was discarded, followed by a 3 to 4 mL blood sample which was placed into a vacutainer for serum collection (Red Tops, BD Diagnostics, Franklin Lakes, NJ) that was immediately placed on ice. Following each sample collection, the catheter was flushed with 2 mL of heparin saline. Catheters were removed after the 120 min sample and jugular venipuncture was used for the 360 min sample. Blood samples were cooled for 2 h followed by centrifuge to obtain serum as described previously.

Hepatic Enzyme Activity

Activity of CYP2C and CYP3A was determined in fresh liver samples following the protocol by Lemley et al. (2008). Briefly, liver samples were submerged in phosphate buffer and homogenized using a Polytron homogenizer. Microsomes were collected and concentrated using differential centrifugation techniques. Fresh homogenized tissue was spun for 10 min at 10,000 × g. The pellets were discarded and the supernatants were centrifuged at 100 000 × g for 60 min. The microsomal pellets were resuspended in

phosphate buffer, and the activity of cytochrome c reductase (product number CY0100; Sigma Chemical Co.) was used to standardize CYP2C and CYP3A activities. CYP2C was measured as the non-ketoconazole-inhibitable, omeprazole-dependent oxidation of NADPH. CYP2C enzymatic reactions contained CYP3A-inhibited microsomes, 2.5 mM omeprazole, and 250 μM NADPH. CYP3A activity was measured as the nifedipine-dependent oxidation of NADPH. CYP3A enzymatic reactions contained fresh microsomes, 200 μM nifedipine, and 250 μM NADPH.

Activity of AKR1C was measured in cytosolic cellular fractions using the specific substrate 1-acenapthenol (Lemley et al., 2010). Briefly, AKR1C enzymatic reactions contained 150-160 μg of cytosolic protein, 250 μM 1-acenapthenol and 500 μM NADP. The 1-acenapthenol-dependant reduction of NADP was standardized using cytosolic protein concentrations. Solutions were added to 96-well plates (PGC Scientifics, Frederick, MD, USA) and the oxidation of NADPH (CYP2C and CYP3A) or reduction of NADP (AKR1C) was determined by measuring the amount of light absorbed at 340 nm for 5 min at 37°C. The rate of oxidized NADPH or reduced NADP was determined to be linear over the 5-min period. The extinction coefficient for NADPH (6220 l/mol*cm) was used to calculate oxidized NADPH or reduced NADP per unit time.

Progesterone Analysis

Serum progesterone concentrations were determined as described by Galbreath et al. (2008) using solid-phase, competitive chemiluminescent enzyme immunoassay (Immulite 1000, Diagnostic Products Corp., Los Angeles, CA, USA). Fifty μl of serum

were assayed in duplicate. Within each assay, low, medium, and high P_4 pools were run in duplicate. The intraassay coefficient of variance was 4.7%.

Calculations and Statistical Analysis

Statistical analysis of treatment differences and P4 clearance were conducted using the MIXED and GLM procedures of SAS (SAS Inst. Inc., Cary, NC). Comparisons of means with a $P < 0.05$ were considered statistically significant and data are reported as least squares means. The fractional rate constant of P_4 clearance at time points 0, 2, 5, 10, 15, 30, 60, 120, and 360 following CIDR removal was determined using the equation: $N(t)=N0e^{-kt}$ where N is the reactant (P4) at time t, N0 is the initial value, k is the first-order fractional rate constant, t is time, and e is the base of natural logarithms. The fractional rate constant (k) and progesterone intercept (N0) were calculated for each individual animal using Sigma Plot (Systat Software Inc., San Jose, California).

Results

There were no significant differences in DMI (P = 0.19) or BW (P = 0.62) between treatment groups throughout the trial. Mean P_4 concentrations did not differ (P = 0.30) on d 6 through 9 in CON1 ewes (3.66 ± 0.18 kg) when compared to LA ewes (3.17 ± 0.20 kg), nor was there a differences in P_4 concentrations over time between CON1 and LA ewes (P = 0.33) (Figure 4).

On d 10 mean serum concentrations of P_4 were lower ($P < 0.03$) in LA supplemented ewes (3.33±0.17 ng/ml) compared to CON1 ewes (4.07±0.17 ng/ml), however there was no difference (P = 0.30) over time between CON1 and LA ewes

(Figure 5). On d 11 there was a trt × time interaction ($P < 0.02$) for P4 samples obtained after CIDR removal to 360 min such that P_4 concentrations were significantly higher in CON1 ewes when compared to LA ewes for 10 minutes following CIDR removal (Figure 6).

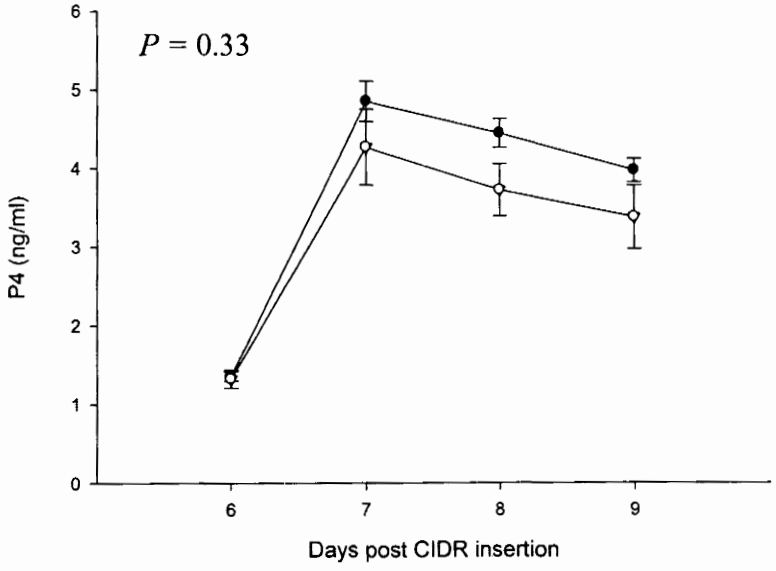

Figure 4. Serum concentrations of progesterone in ewes supplemented with 32 mg/kg lipoic acid (LA, ○) or with a control bolus (CON1, ●) from individual d 6 through 9 blood samples drawn prior to feeding at 0730.

The fractional rate constant for P_4 clearance on d 11 was higher ($P < 0.03$) in LA treated ewes than CON1 ewes (Figure 7). There was no difference ($P = 0.15$) in the average intercept for P_4 clearance between CON1 and LA supplemented ewes (Figure 8). No differences ($P = 0.20$) were found in CYP hepatic enzyme activity obtained by liver biopsy. There was a trend ($P < 0.08$) for lower AKR1C in CON1 ewes (Table 1).

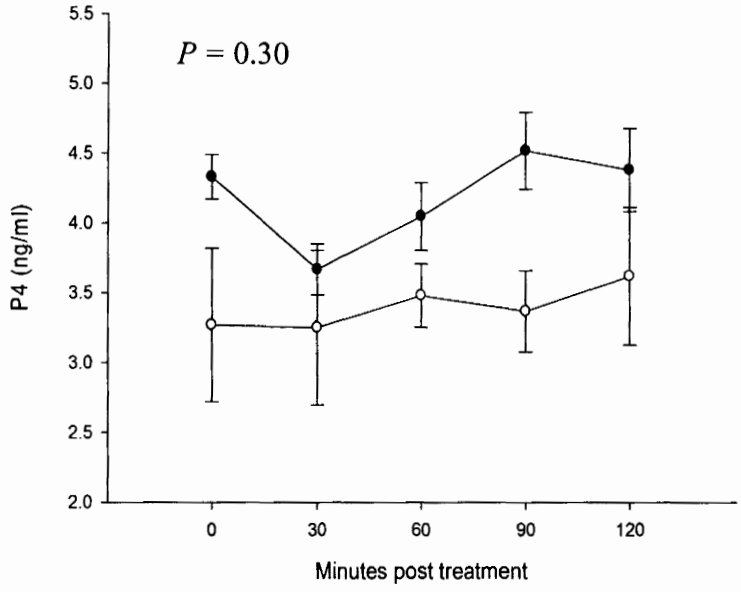

Figure 5. Serum concentrations of progesterone in ewes supplemented with 32 mg/kg lipoic acid (LA, ○) or with a control bolus (CON1, ●) from d 10 samples drawn with treatment at 0730 and at 30, 60, 90, and 120 minutes post treatment.

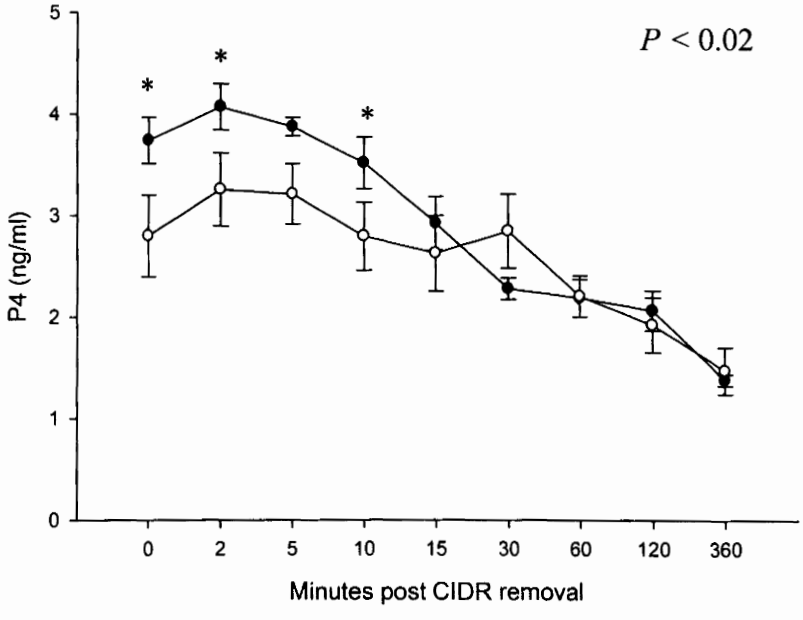

Figure 6. Serum concentrations of progesterone in ewes supplemented with 32 mg/kg lipoic acid (LA, ○) or with a control bolus (CON1, ●) from d 11 samples drawn post at 0, 2, 5, 10, 15, 30, 60, 90, 120, and 360 min post treatment and CIDR removal. Statistically significant time interactions can be seen at 0, 2, and 10 minutes post CIDR removal. Statistical significance of $P < 0.05$ is denoted by *.

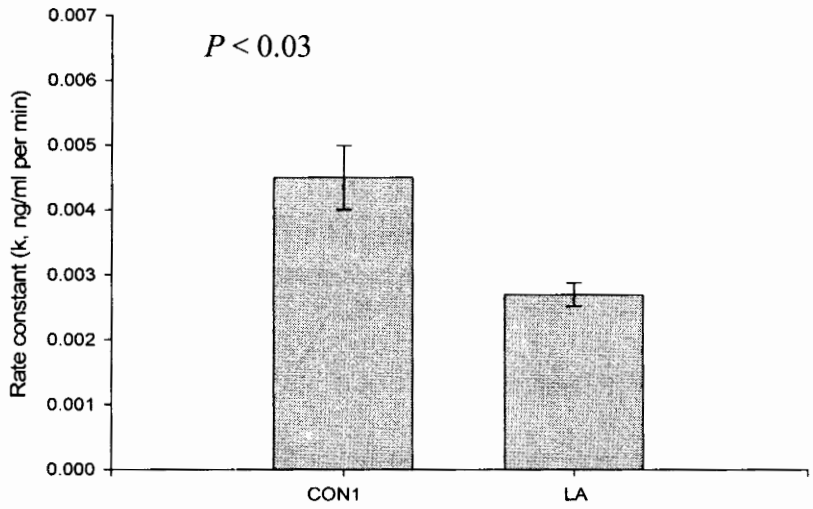

Figure 7. Average fractional rate constant of progesterone decay in CON1 and LA supplemented ovariectomized ewes. Rate constants were calculated from blood samples drawn at 0, 2, 5, 10, 15, 30, 60, 90, 120, and 360 post CIDR removal on d 11.

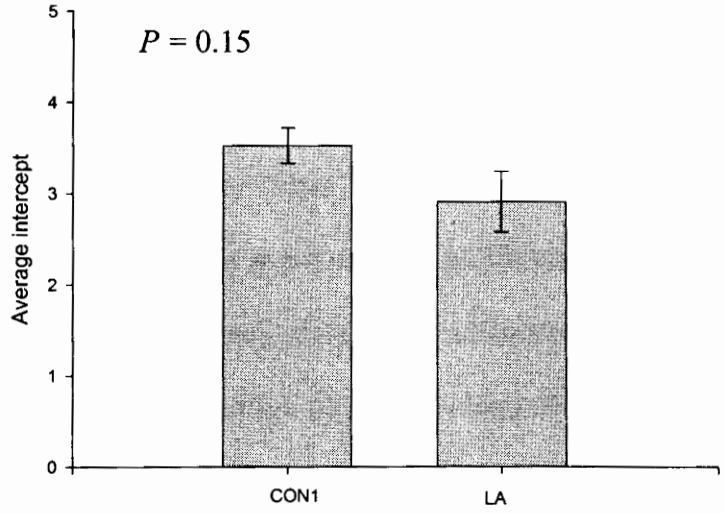

Figure 8. Average intercept for progesterone was calculated by analyzing progesterone decay curves from blood samples drawn at 0, 2, 5, 10, 15, 30, 60, 90, 120, and 360 post CIDR removal on d 11.

	CON1	LA	SEM	*P*-value
CYP2C pmol/min*mU	25.59	24.13	4.82	0.84
CYP3A pmol/min*mU	10.12	6.27	2.78	0.37
AKR1C pmol/min*mg	169.26	309.48	46.42	0.08

Table 1. Hepatic CYP2C, CYP3A, and AKR1C activity on d 10. No differences ($P = 0.20$) were found in hepatic enzyme activity.

Discussion

In the current study, ovariectomized ewes were used as a model to determine P_4 clearance and hepatic enzyme activity after oral supplementation with lipoic acid. No differences in P_4 concentrations on d 6 through 9 were observed; however we did observe a main effect of treatment on peripheral P_4 concentrations on d 10. While lower P_4 concentrations were observed in the lipoic acid supplemented ewes, the mean concentrations were within normal range for ewes in similar studies (Ainsworth and Downey, 1986). Additionally, it has been suggested that the rate of P_4 catabolism may have a greater effect on pregnancy maintenance than circulating P_4 concentrations (Arndt et al., 2009). Ewes treated with lipoic acid showed a lower rate of P_4 clearance compared to control ewes suggesting an effect on P_4 catabolism. No differences in hepatic enzyme activity were seen when compared to control ewes; therefore the mechanism of action requires further investigation.

Previous studies have shown a reduction in CYP2C and CYP3A activity in the presence of elevated insulin or a gluconeogenic substrate (Smith et al., 2006; Lemley et al., 2008). Smith et al. (2006) found that gavaging ewes with sodium propionate resulted in elevated insulin concentrations and decreased P_4 catabolism compared to ewes dosed with

isocaloric sodium acetate. A study by Lemley et al. (2008) examined hepatic CYP2C and CYP3A activity in ovariectomized ewes dosed with sodium propionate, a gluconeogenic substrate, and found treatment ewes had elevated insulin concentrations and a 50% reduction in hepatic CYP2C and CYP3A activity when compared to ewes dosed an isocaloric level of sodium acetate. Although lipoic acid is not known to be a gluconeogenic substrate, it does modulate the action of insulin which is what led us to speculate it would have a similar effect on P_4 clearance as would a gluconeogenic substance (Jacob et al., 1996; Moini et al., 2002; Yaworsky et al., 2000).

While we did not measure insulin or glucose concentrations in the current study, we speculate that lipoic acid may have altered these substrates based on studies indicating that lipoic acid modulates the action of insulin in human and animal models (Packer et al., 2001; Jacob et al., 1995). In a study by Jacob et al. (1996), it was indicated that treatment with lipoic acid to obese Zucker rats improved glycogen synthesis, insulin-stimulated glucose oxidation, and resulted in greater muscle glycogen concentration when compared to control group. In this same study, a long term adaptation of the body to lipoic acid treatment decreased plasma insulin levels 15-17% compared to control group (Jacob et al., 1996). A study by Yaworsky et al. (2000) found that treatment of lipoic on 3T3-L1 adipocytes resulted in an increase in GLUT1 and GLUT4 to the plasma membrane, an increase in insulin receptor substrate-1 and antiphosphotyrosine-associated activity, and activation of kinases in target cells which can result in increased glucose uptake. These studies indicate that lipoic acid does have an effect on the action of insulin in glucose uptake, although the exact mechanism of action is still unknown. Because we found no differences in hepatic enzyme activity it is possible that the rate of P_4 clearance in our study

was impacted by enhanced insulin effectiveness rather than alterations in hepatic catabolic enzyme activity. Measuring insulin and glucose concentrations would have provided further insight into the mechanistic action of lipoic acid as to whether it mimics the action of insulin or simply enhances the effect of insulin to take up glucose.

A further limitation to our study that may have impacted P_4 concentrations was that we did not assess body fat on the ovariectomized ewes prior to the study. While there were no significant differences in body weight, casual visual appraisal of sheep used for this trial indicated that many of the sheep were overweight. A study by Zomzely et al. (1959) demonstrated that obese animals retain more steroid hormones than non-obese animals by a function of the increased adipose tissue. Thus the possibility exists that P_4 may have been sequestered into adipose tissue by some of the overweight ewes, resulting in differences in serum P_4 concentrations.

Exploring methods of decreasing the rate of P_4 clearance is a worthwhile area of study as this will likely decreased early embryonic losses by maintaining adequate P_4 concentration during the critical early embryonic period. While our data does not indicate lipoic acid alters hepatic P_4 catabolic enzymes, it does suggest that supplementing with lipoic acid results in a decreased rate of P_4 clearance in the ovariectomized ewe; however, the mechanism of action of lipoic acid has is yet to be determined.

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CHAPTER 3. THE EFFECTS OF LIPOIC ACID SUPPLEMENTATION ON PROGESTERONE, INSULIN AND GLUCOSE IN THE OVARIECTOMIZED EWE

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Abstract

Early embryonic and fetal losses are often associated with low concentrations of progesterone in the blood. Increased catabolism of progesterone (**P_4**) by the liver reduces circulating P_4 concentrations, contributing to these early losses. Recent studies suggest P_4 catabolism can be down regulated under physiological conditions of elevated blood insulin. Lipoic acid is a naturally occurring compound that has been shown to modulate insulin sensitivity when supplemented to the diet. We[2] hypothesized that dosing lipoic acid directly into the rumen at a low and high experimental concentration would decrease P_4 catabolism. Fourteen ovariectomized ewes were fed an alfalfa-grass ration at 95% of ad libitum for the duration of the experiment. Ewes were randomly assigned to a control group [12 mL of ethanol placed directly into the rumen through a rumen cannula (n = 4; **CON2**)], a low lipoic acid group [supplemented at 32 mg/kg BW dissolved in ethanol to reach a total volume of 12 mL (n = 5; **LowLA**)], or a high lipoic acid group [supplemented at 64 mg/kg BW dissolved in ethanol to reach a total volume of 12 mL (n = 5; **HighLA**)]. Progesterone was administered via CIDR devices on d 7 to all ewes. Daily blood samples were collected

[2] This project was a collaborative effort by the authors listed above. The contribution of Rachel Mottet includes experimental planning and organization, data collection, data analysis, and written documentation of the experimental procedures and outcomes. Co-authors Dr. Caleb Lemley, Dr. Erika Berg, and Dr. Kimberly Vonnahme contributed the novel experimental proposal, design, and editing as needed of written material by Rachel Mottet pertaining to the trial.

from d 7 to 11. On d 12, serial blood samples were collected after CIDR removal to determine P_4 clearance from the blood stream. On d 19 ewes underwent an intravenous glucose tolerance test (**IVGTT**) to determine insulin and glucose area under the curve. There were no significant differences between treatment groups in P_4 levels on d 7 to11 ($P = 0.20$), no differences in P_4 clearance after CIDR removal from an intensive sampling period ($P = 0.95$), and no differences in insulin area under the curve ($P = 0.77$); however, there were significant differences in glucose area under the curve ($P = 0.0007$) between treatment groups. We conclude that administering lipoic acid directly into the rumen did not alter P_4 catabolism. We speculate that delivering lipoic acid via the rumen may not be an efficient dosing method for lipoic acid due to differences in P_4 clearance rates compared to our previous studies.

Introduction

Progesterone plays a role in maintenance of pregnancy and influences the production of endometrial secretions that aid in early embryonic development (Nephew et al., 1991). Early embryonic losses account for approximately 30% of all pregnancy loss in mammals (Wilmut et al., 1986). In sheep, pregnancy losses are the greatest within the first three weeks of gestation (Moore, 1985). This may be due in part to low concentrations of P_4 which can result in poor embryonic development (Nephew et al., 1994) and may enable an increase in hormones that cause embryonic death (Inskeep, 2004). Mechanisms by which P_4 concentrations are reduced may be related to overactive P_4 catabolism by the liver, improper luteal function, or a combination of these factors (Inskeep and Dailey, 2005). Progesterone is catabolized to the greatest extent in the liver, which contains an

abundance of cytochrome P450 enzymes that are involved in steroid inactivation (Murray et al., 1991:1992).

A positive correlation between reduced P_4 catabolism and elevated insulin levels have been demonstrated in the ewe and the dairy cow (Smith et al., 2006; Lemley et al., 2008). Lipoic acid is a naturally occurring compound that has been shown to increase insulin sensitivity when supplemented in human and animal models (Jacob et al., 1999; Jacob et al., 1996; Yaworsky et al., 2000). Previous unpublished data from our lab has shown that oral dosing with lipoic acid at 32 mg/kg BW in ovariectomized ewes results in a lower rate of P_4 decay. In this study, we hypothesized that dosing lipoic acid directly into the rumen at 32 mg/kg BW and at 64 mg/kg BW would decrease P_4 clearance rates.

Materials and Methods

Animals and Diets

Animal care and use was approved by the Institutional Animal Care and Use Committee at North Dakota State University (Fargo, USA). Fourteen Katahdin cross ovariectomized ewes were housed at the Animal Research and Physiology Center in Fargo. Ewes were penned individually in 0.91 x 1.20-m pens in a temperature controlled (12°C) and well-ventilated facility. Lighting was automatically timed to mimic the natural photo period. Ewes were given ad libitum access to fresh water and individual feeders.

Ewes were randomly assigned to 1 of 3 groups: CON2 (0 mg/kg BW lipoic acid; n = 4); LowLA (32 mg/kg BW lipoic acid; n = 5); HighLA (64 mg/kg BW lipoic acid; n = 5). On d -15, ewes were weighed, marked for identification, individually penned, and randomly divided into three groups staggered by one day to facilitate sampling. Groups

were comprised as follows: group 1 (n = 4; CON2 n = 2; HighLa n = 2); group 2 (n = 5; CON2 n = 1; LowLa n = 2; HighLa n = 2); group 3 (n=5; CON2 n = 2; LowLa n = 2; HighLa n = 1). Ewes were individually fed an ad libitum diet of alfalfa hay from d -12 through d -8. Intake was determined by measuring weight of initial feed offered subtracted by weight of residual feed after a 24 h period and averaged for the 1 wk period following d 0. Intake was restricted to 95% from d -8 to d 19 with a 1 wk acclimation period to adjust to this diet from day -8 to 0.

On d -14 and d -15, ewes 1-9 and 10-16 respectively underwent a rumen fistula procedure to facilitate dosing of lipoic acid directly into the rumen. Twenty-four hours prior to surgery, ewes were taken off feed and water. The surgical site was close clipped and cleaned with betadine scrub followed by a rinse with water and repeated three times. The site was then sprayed with 70% alcohol followed by betadine solution.

Ewes were anesthetized with intramuscular doses of xylazine (Rompun; Phoenix Pharmaceutical Inc.) at 0.9 mg/kg BW, followed by ketamine hydrochloride (Ketaject; Phoenix Pharmaceutical Inc.) at 2.2 mg/kg BW. If additional anesthesia was necessary, 0.5 ml of ketamine hydrochloride was administered intramuscularly.

A vertical incision was made in the middle of the paralumbar fossa extending beginning 3 to 5 cm ventral of the lumbar vertebrae transverse processes. The external and internal abdominal oblique and transverse abdominal muscles were separated by blunt dissection. Warmed potassium penicillin and sterile saline was poured into the peritoneal cavity. To gain access to the rumen, tissue forceps were used to grasp the peritoneum and the peritoneal cavity was incised. The rumen was anchored to the skin with a number 1 polyamid suture. A vertical incision was made in the anchored rumen wall and a 1-cm o.d.

polyvinyl chloride cannula was inserted. The rumen was sutured to the skin using a mattress suture. The cannula was fitted and held in place by a simple suture pattern. The exterior of the animal was rinsed and a topical antibiotic (Nolvasan ointment, Fort Dodge Animal Health, Fort Dodge, IA) was applied.

Following surgery, ewes were placed in individual pens and monitored every 6 h for 24 h and every 12 h for 6 d thereafter. Rectal temperature was taken for 3 d post-surgery to detect any signs of systemic infection. Ewes were given an intramuscular injection of 10,000 units/kg BW benzathine/procaine penicillin daily through 3 d post-surgery. Observation and daily cleaning of ruminal cannulas was done as needed.

Ewes were weighed on d -8 and began a 1 wk acclimation to the 95% of actual intake diet. Lipoic acid doses were measured for individual ewes based on BW obtained at d -8 and treatment began on d 0. Lipoic acid was delivered to ewes in a liquid form directly into the rumen through a fistula. Ewes received a calculated dose of lipoic acid based on treatment group and BW. To liquefy lipoic acid, 1 g of lipoic acid was dissolved into 2.16 mL ethanol. All ewes received a dose with a total volume of 12 mL. Ewes were dosed daily from d 0 to 19 at 0730, prior to feeding.

Sample Collection

Prior to lipoic acid supplementation and feeding on d 7 to11, serum samples were drawn for P_4 analysis via jugular venipuncture (BD Vacutainer, Franklin Lakes, NJ). Blood samples were immediately put on ice and cooled for 2 h before centrifugation. Samples were centrifuged (Beckman Coulter, Fullerton, CA) at 2500 × g for 20 min, and serum was collected and stored in two 2-mL vials at -20°C until further analysis. Ewes received a

controlled internal drug release insert on d 7 [CIDR; EAZI-BREED CIDR, 0.3 g P_4, Pfizer Animal Health]. From d 7 to 11 blood samples were collected and handled as described above.

Progesterone Clearance

To determine P_4 clearance, serial blood samples were taken on d 12. Catheters (I-CATH, Charter Med Inc. Winston-Salem, NC) were placed in the jugular vein after administration of 1 mL of lidocaine and cleaning with an alcohol spray to prepare the area followed by insertion of a 14 gauge catheter needle and catheter. After placement was confirmed, catheters were flushed with 3 mL of heparin saline. Catheters were stitched into place and subsequently covered with co-flex vet wrap. Blood samples were collected at -5, 0, 2, 5, 10, 15, 30, 60, 120, and 360 min relative to CIDR removal. Blood samples were put on ice and handled as described above.

Intravenous Glucose Tolerance Test

On d 19 an intravenous glucose tolerance test (**IVGTT**) was performed following procedures similar to Vonnahme et al. (2010). Ewes were removed from feed 24 h prior to the IVGTT. Jugular catheters were placed in the vein opposite the P_4 sampling catheter (I-CATH, Charter Med Inc., Winston-Salem, NC). A minimum of 30 minutes was allowed between catheterization and blood sampling. Two pre-dose samples were obtained for a baseline measure of glucose and insulin levels at -5 and 0 min relative to glucose bolus infusion. Glucose was infused at time 0 (0.25 g glucose/kg BW; 50% glucose solution in 0.9% saline) and blood samples were obtained at 5, 10, 15, 30, 45, 60, 90, 120, 150, 180,

and 240 min. Following each sample collection, catheters were flushed with approximately 2 ml of 0.9% heparin saline. Five mL of blood were obtained per sample and placed in a serum separator tube (BD Vacutainer, Franklin Lakes, NJ). . Blood was immediately put on ice and handled as described above. Samples were stored in 2 vials at -20 °C until insulin and glucose analysis was performed.

Hormone and Metabolite Analysis

Progesterone samples were assayed in duplicate as described by Galbreath et al. (2008) by a chemiluminescense immunoassay method using an Immulite 1000 (Siemens, Los Angelos, CA). Within each assay, low medium, and high P_4 pools were run in duplicate. The inter- and intra- assay CVs were 10.7% and 4.3% respectively.

Insulin concentrations were determined via a chemiluminescence assay with samples run in duplicate (Immulite 1000; Siemens, Los Angelos, CA). Low and high pools were run with each assay. The inter- and intra- assay CVs were 4.8% and 8.4% respectively.

Glucose analysis was conducted using a colorimetric enzyme end point assay kit (Thermo Electron Corporation, Melbourne, Australia) described by Vonnahmne et al. (2010) and Lekatz et al. (2010). Five microliters of serum sample, 5 μL of each standard (8 standards total, ranging from 17.65 to 300.00 μg/dL), 5 μL of Accutrol serum (used for control; Sigma, St. Louis, MO), and 5 μL of 18 MΩ water (used for blank) were pipetted in duplicate into a 96-well plate (Costar EIA/RIA plate #3369, Corning Inc., Corning, NY). Infinity Glucose Reagent (TR15421, Thermo Electron Corporation, Pittsburgh, PA) was then pipetted into each well at 250 μL. Each plate was incubated and read in a plate reader

at 37 °C for 20 min. Absorbance was measured at 340 nm (Spectra Max 340, Fullerton, CA). The intraassay CV was 5.2%.

Calculations and Statistical Analysis

Statistical analysis of treatment differences and P_4 clearance were conducted using the MIXED and GLM procedures of SAS (SAS Inst. Inc., Cary, NC). Comparisons of means with $P < 0.05$ were considered statistically significant. The fractional rate constant of P4 clearance at time points 0, 2, 5, 10, 15, 30, 60, 120, and 360 following CIDR removal was determined using the equation: $N(t)=N0e^{-kt}$ where N is the reactant (P4) at time t, NO is the initial value, k is the first-order fractional rate constant, t is time, and e is the base of natural logarithms. The fractional rate constant (k) and progesterone intercept (N0) were calculated for each individual animal using Sigma Plot (Systat Software Inc., San Jose, California). Area under the curve (**AUC**) was determined for insulin and glucose using the trapezoidal rule with Sigma Plot software (SPSS Inc., Chicago, IL). Least squares means ± standard error are presented.

Results

There were differences in DMI ($P < 0.04$) between CON2 (1813.36 ± 147.25 g), LowLA (1755.17 ± 131.70 g), and HighLA (1295.99 ± 131.70 g) treatment groups such thatHighLa DMI was lower than CON2 ($P < 0.02$) and LowLA ($P < 0.03$) treatment groups. There were no differences in BW ($P = 0.45$) among groups (CON2, 174.51 ± 10.95 kg; LowLA, 162.49 ± 9.79 kg; HighLA 155.39 ± 9.79 kg).

Progesterone concentrations from blood drawn on d 7-11 were not different (P = 0.20) in CON2, LowLa or HighLa groups (Figure 9). On d 12 following CIDR removal there were no differences over time in P_4 between groups (P = 0.60) (Figure 10). The fractional rate constant for P_4 clearance prior to CIDR removal through 360 min following CIDR removal on d 12 was not different (P = 0.95) among groups based on treatment (Figure 11). There was no difference (P = 0.64) in the average intercept for P_4 clearance in CON2, LowLA, and HighLA supplemented ewes (Figure 12).

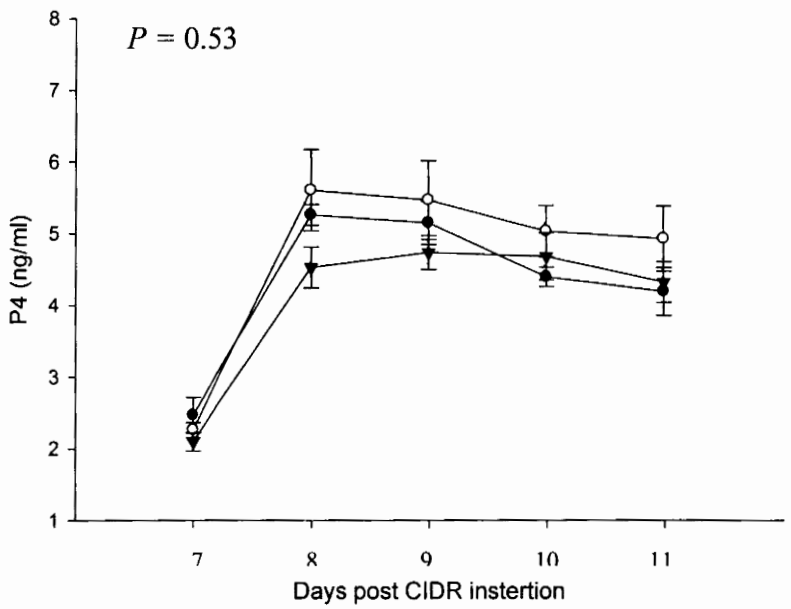

Figure 9. Serum concentrations of progesterone on d 7 to11 in ewes supplemented with 32 mg/kg BW lipoic acid (LowLA, ○), 64 mg/kg BW lipoic acid (HighLA, ▼) or with a control bolus (CON2, ●). Blood samples were drawn prior to feeding at 0730.

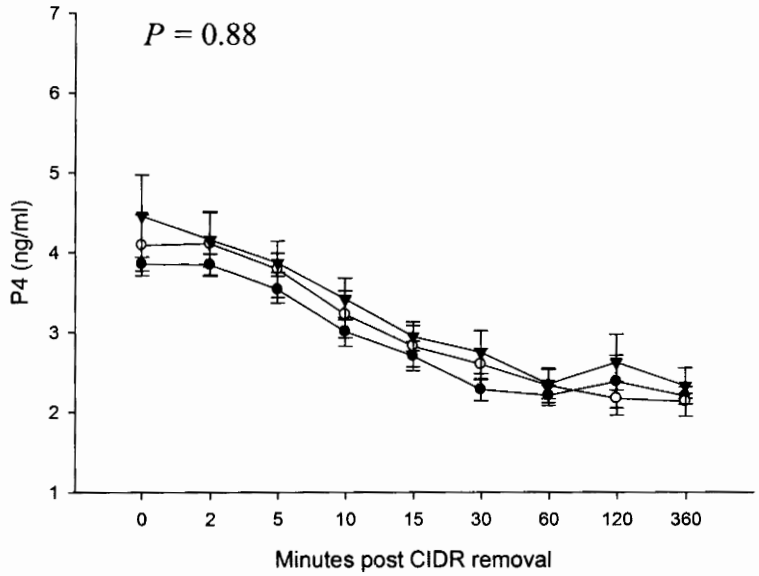

Figure 10. Serum concentrations of progesterone at 0, 2, 5, 10, 15, 30, 60, 90, 120, and 360 min post treatment and CIDR removal on d 12 in ewes supplemented with 32 mg/kg BW lipoic acid (LowLA, ○), 64 mg/kg BW lipoic acid (HighLA, ▼) or with a control bolus (CON2, ●).

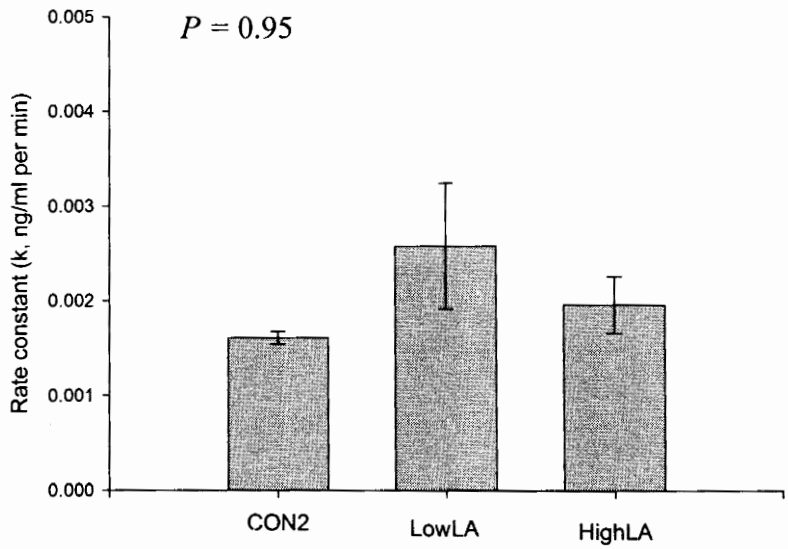

Figure 11. Average fractional rate constant for progesterone clearance in control (CON2), ewes receiving 32 mg/kg BW of LA (LowLA), and ewes receiving 64 mg/kg BW of LA (HighLA). Rate constant were calculated from blood samples drawn at 0, 2, 5, 10, 15, 30, 60, 90, 120, and 360 post CIDR removal on d 12. No differences were found ($P = 0.95$).

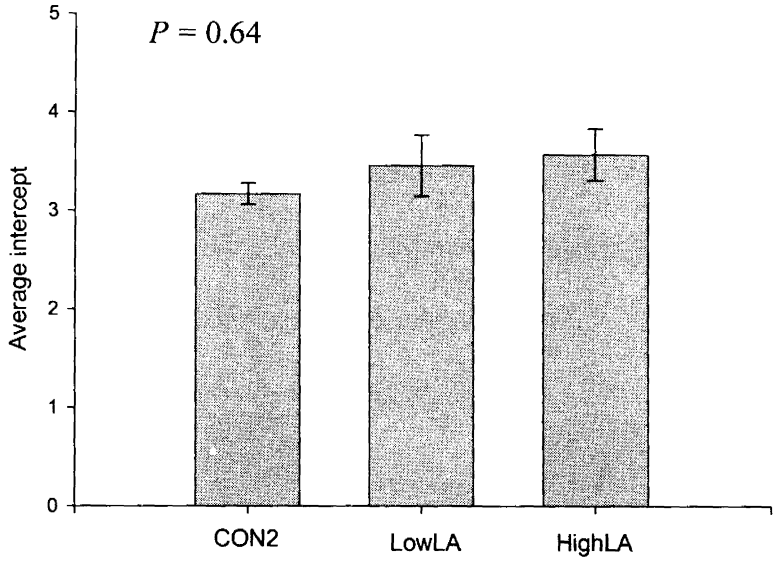

Figure 12. Average intercept for progesterone was calculated by analyzing progesterone decay curves from blood samples taken at 0, 2, 5, 10, 15, 30, 60, 90, 120, and 360 post CIDR removal on d 12 in control (CON2), ewes receiving 32 mg/kg BW of LA (LowLA), and ewes receiving 64 mg/kg BW of LA (HighLA). No differences were found ($P = 0.64$).

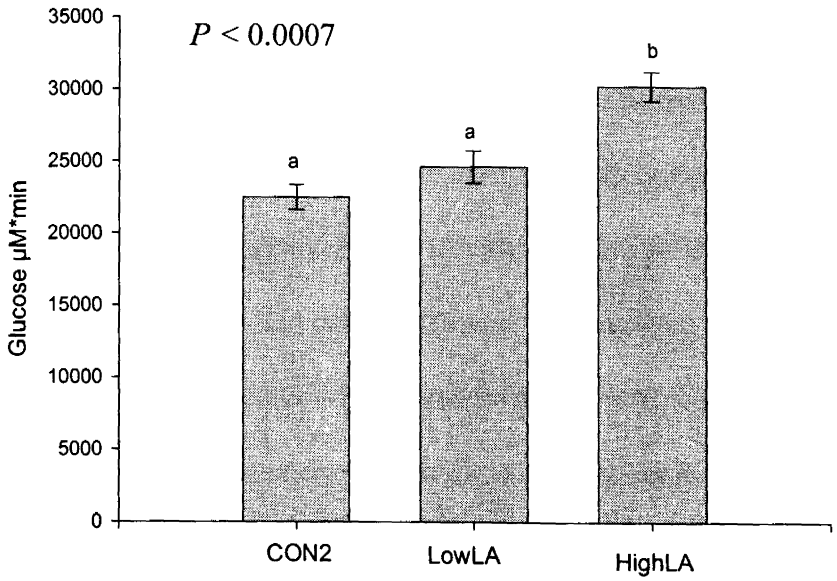

Figure 13. Glucose area under the curve for control (CON2) ewes, ewes receiving 32 mg/kg BW of LA (LowLA), and ewes receiving 64 mg/kg BW of LA (HighLA) during d 19 IVGTT. Means with different letters (a-b) differ ($P < 0.002$).

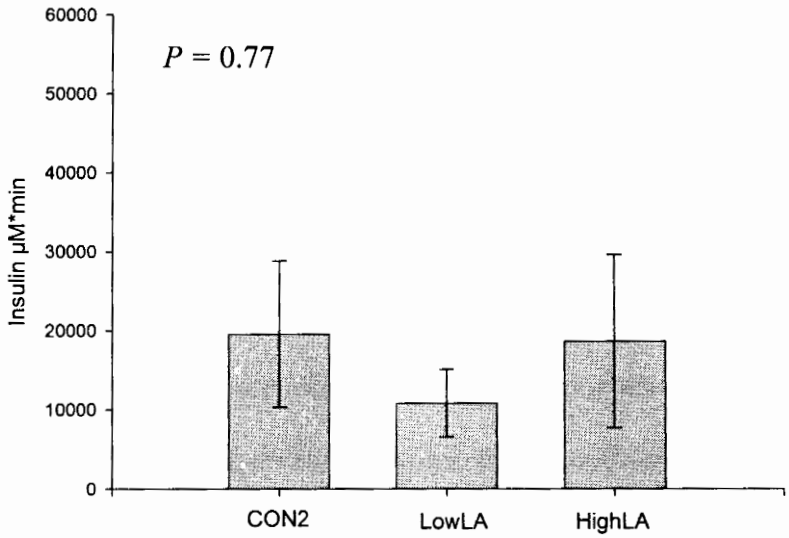

Figure 14. Insulin area under the curve for control (CON2), ewes receiving 32 mg/kg BW of LA (LowLA), and ewes receiving 64 mg/kg BW of LA (HighLA) during d 19 IVGTT. No differences were found ($P = 0.77$).

Discussion

In the current study, there were differences in DMI such that CON2 and LowLA ewes had a significantly higher intake than HighLA ewes. In previous studies it has been reported in that high DMI after mating results in reduced P_4 concentration and has been linked to embryonic mortality (Parr et al., 1987; Brien et al., 1977). These findings have been reported in sheep, swine, and cattle (Williams & Cumming, 1982; Dunne et al., 1999; Ashworth et al., 1999). This relationship is due to increased blood flow to the liver resulting in increased steroid hormone catabolism (Bensadoun & Reid, 1962; Bedford et al., 1974). In contrast, data from this study showed that P_4 concentrations from 5 daily blood samples and from serial blood samples drawn post CIDR removal were not different

among treatment groups even though DMI differed among groups. Measuring blood flow to the liver would have been beneficial to determine whether a relationship between DMI and hepatic blood flow was evident in this study.

No differences were observed at any time during the study in P_4 concentrations, rate constant indicating progesterone clearance, or average intercept as a result of lipoic acid supplementation. This is in contrast to previously unpublished data from our lab that indicated lipoic acid does decrease progesterone clearance rates in sheep. We speculate that the ruminal dosing method used in this study may have impacted the effectiveness of lipoic acid at the chosen dosages. Schmidt (2004) reported a 6,000-fold increase in blood serum concentrations of lipoic acid 3 h after oral dosing of lipoic acid in sheep, compared to only an 80-fold increase when lipoic acid was placed directly into the rumen of cannulated steers. Schmidt and coworkers (2004) speculated that due to lipoic acid's small size, oral dosing resulted in a much more rapid increase in blood levels of lipoic acid due to absorption of lipoic acid in the mouth and esophagus of orally dosed animals, as opposed to rumen absorption.

It has been reported that a relationship exists between P_4 clearance and insulin through a mechanism of modulating hepatic progesterone catabolizing enzymes. Progesterone catabolism occurs to the greatest extent in the liver by cytochrome P450 enzymes, specifically cytochrome P450 2C (CYP2C) and cytochrome P450 3A (CYP3A) (Murray 1991; 1992). It has been hypothesized by Dean and Stock (1975) that a mechanism exists to down regulate cytochrome P450 enzymes during pregnancy to maintain elevated P_4 concentrations. A study by Lemley et al. (2008a) demonstrated that infusing 1.0 μg of insulin/kg BW in dairy cows resulted in a reduction of CYP2C and

CYP3A mRNA by 88% and 45% respectively. Other studies have further explored this mechanism and have found that elevated insulin decreases P_4 clearance (Lemley et al., 2008b; Smith et al., 2006). This mechanism may also impact fertility based on a study looking at pregnancy in cattle which demonstrated that breeding cows injected with insulin had heightened plasma P_4 concentrations and had a 24% higher overall conception rate (Selvaraju et al., 2002).

Although previous unpublished data from our lab indicates that lipoic acid does not affect CYP3A and CYP2C activity, it is known that lipoic acid modulates insulin activity. Lipoic acid is a substance that is known to enhance insulin sensitivity and increase glucose uptake in animal and human models (Jacob et al., 1996; Yaworsky et al., 2000; Busse et al., 1995). A relationship between a gluconeogenic substrate and elevated insulin concentrations in decreasing P_4 clearance in sheep has been reported (Lemley et al., 2008b; Smith et al., 2006). Smith et al. (2006) demonstrated that dosing ewes with sodium propionate, a gluconeogenic substrate, resulted in increased peripheral glucagon and insulin concentrations when compared to ewes dosed with sodium acetate. In the same study, researchers looked at cultured hepatocytes and their response to insulin treatment; in which they found P_4 clearance was lower in response to 1.0 and 10 n*M* of insulin. A study by Lemley et al. (2008b) indicated that sodium propionate supplemented ewes had a three times greater increase in insulin concentrations when compared to acetate supplemented ewes at 15 and 30 minutes after feeding.

Although lipoic acid is not considered a gluconeogenic substrate, we hypothesized that based on the role of lipoic acid in impacting the glucose transport system that it would have similar effects as an environment of elevated insulin. Furthermore, we hypothesized

that the lipoic acid dosed ewes would have lower insulin and glucose area under the curve compared to control ewes based on the previously mentioned relationship. However, our glucose data indicates that the HighLa ewes had the greatest glucose area under the curve that was significantly different from the other two groups, and also that there was no differences in insulin area under the curve. Thus, our findings were opposite of what we had expected. A possible explanation for this would be due to the route of administration which was dosed directly into the rumen for the present study versus oral gavage.

Progesterone concentrations and early embryonic losses remain a major area of focus for decreasing economic losses faced by producers. Our previous data indicates that lipoic acid as a dietary supplement may be beneficial in maintaining adequate P_4 levels in the body for a longer duration of time. Further investigation is necessary to determine the optimal delivery method, mechanism of action, and dosage of lipoic acid needed to positively affect progesterone concentrations.

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CHAPTER 4. CONCLUSIONS AND IMPLICATIONS

Pregnancy loss continues to be a major area of research as it causes a tremendous negative economic impact on producers. Since a majority of pregnancy losses occur within the early embryonic period and adequate progesterone during this time is crucial for embryonic vitality, we focused on examining whether lipoic acid could potentially decrease progesterone clearance rates. The justification for choosing lipoic acid as the substrate to be tested was based on the fact that elevated insulin concentrations have been linked to reduced progesterone clearance and that lipoic acid has been shown to modulate insulin sensitivity. Because there is currently no published research on the effect of lipoic acid supplementation on progesterone concentrations, these studies provide novel information on these interactions as well as the ground work for future research.

Specifically, examining the relationship between lipoic acid supplementation, dry matter intake and hepatic blood flow may provide insight in to the mechanism of action of lipoic acid. While it is known that the majority of lipoic acid and progesterone are broken down by the liver, our first study indicated that lipoic acid does not affect the hepatic enzymes that are predominantly responsible for progesterone catabolism. Thus, looking at liver blood flow could indicate if lipoic acid is affected by an increase in blood flow which would indicate if the compound is actually impacting hepatic enzymes other than those we explored.

Another direction for future research would be investigation into the most efficacious dosing method. In our first trial we found that orally dosing with lipoic acid results in reduced progesterone clearance rates while our second trial revealed no effects on progesterone from lipoic acid when dosing directly into the rumen. Exploring the best

method of dosing would be valuable to determine if using lipoic acid is practical in a livestock production operation. Further, while we demonstrated that lipoic acid can impact progesterone clearance, we cannot explain this phenomena: therefore, elucidating lipoic acid's precise mechanism of action as it relates to insulin, glucose and progesterone dynamics is important to determine if this relationship may impact fertility and/or pregnancy losses.