EFFECTS OF ARGININE ON REPRODUCTIVE PERFORMANCE IN EWES

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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

MASTER OF SCIENCE

ABSTRACT

Saevre, Chelsey Brie, M.S., Department of Animal Sciences, College of Agriculture, Food Systems, and Natural Resources, North Dakota State University, November, 2010. The Effects of Arginine on Reproductive Performance in Ewes. Major Professors: Dr. Christopher Schauer & Dr. Dale Redmer.

Reproductive performance is the largest determinant of income in the livestock industry. In the U.S. sheep industry, embryonic and fetal deaths during pregnancy account for almost half of the total number of fertilized ova and a majority of these losses have been reported to occur before d 18. In study 1, the objective was to determine if arginine supplementation enhances ovarian function and prevents early reproductive losses in sheep. Ewes received L-arginine HCl (equivalent to 27 mg of L-arginine/ kg of BW, ARG, n = 20) or saline (CON, n = 20) i.v. from d 0 (estrus) to d 15. On d 12, serum concentrations of arginine (nmol/ml) were elevated in ARG vs. CON ewes at 0 ($P < 0.001$), 0.5 ($P < 0.001$), 1 ($P < 0.001$), 2 ($P < 0.005$), and 4 h ($P < 0.05$), but were similar ($P > 0.05$) at -0.5, 8 and 24 h. Pulsatility index in the ovarian artery on d 12 was reduced in ARG vs. CON ewes ($P < 0.05$). Despite similarities in the number of corpora lutea (CL) per ewe ($P > 0.05$), ARG ewes had greater P4 concentrations throughout treatment compared to CON ewes. Although pregnancy rate was not influenced (ARG, 55% and CON, 60%; $P > 0.05$), ARG ewes had more embryos per ewe ($P \leq 0.04$) and less CL not represented by embryos ($P \leq 0.03$) compared to CON ewes at d 25 of pregnancy. Ewes treated with ARG gave birth to more lambs when compared to control ewes (ARG, 1.6 ± 0.16 vs. CON, 1.1 ± 0.16 lambs born per ewe). In summary, early reproductive losses can be prevented by treatment with arginine. The objective of Study 2 was to determine if arginine supplementation surrounding the time of maternal recognition of pregnancy enhances ovarian function and minimizes reproductive losses. Ewes received L-arginine HCl (equivalent to 27 mg of L-

arginine/kg of BW, ARG, n= 47) or saline (CON, n = 47) i.v. from d 9 to d 14 following estrus (d 0). On d 10, serum concentrations of arginine (nmol/mL) were elevated in ARG versus CON ewes at 0 ($P < 0.001$), 0.5 ($P < 0.001$), 1 ($P < 0.001$), 2 ($P < 0.001$) and 4 h ($P < 0.001$). Despite similarities in the number of CL per ewe ($P > 0.05$), serum progesterone concentration (ng/mL) was greater in CON compared with ARG on d 9 ($P < 0.02$) and 10 ($P < 0.005$). Treatment with arginine influenced pregnancy rate (ARG, 55% and CON, 30%) throughout the treatment period. Ewes treated with ARG gave birth to similar ($P > 0.05$) number of lambs when compared to CON ewes (ARG, 1.78 ± 0.17 vs. CON, 1.6 ± 0.27 lambs born per ewe). In summary, arginine supplementation surrounding the time of maternal recognition of pregnancy may prevent early reproductive loss or influence vascular resistance and circulating serum progesterone concentration in ewes. In study 3, the objectives were to determine if rumen-protected arginine supplemented to ewes on d 8 to 13 of the estrous cycle affected serum amino acid concentration, ovarian blood flow, and circulating progesterone. Ewes fed 360 mg/kg BW arginine (360 ARG) had greater serum arginine concentration than control (CON), 90 mg/kg BW arginine (90 ARG), and 180 mg/kg BW arginine (180 ARG) on d 11 ($P \leq 0.07$) and d 12 ($P \leq 0.03$). Arginine supplementation increased peak systolic velocity in the CL for 360 ARG and 90 ARG compared to CON ($P \leq 0.04$). Supplemental rumen-protected arginine had no effect on serum concentration of progesterone ($P > 0.50$). Results indicate that rumen-protected arginine supplemented to ewes at the rate of 360 mg/kg BW may increase circulating serum arginine concentration, in addition to increasing ovarian blood flow.

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LIST OF ABBREVIATIONS

ARG.....Arginine

ASL..... Argininosuccinate lyase

ASS..... Argininosuccinate Synthetase

BW..... Body weight

CIDR..... Controlled Internal Drug Release

CL..... Corpus Luteum

CON.....Control

CPS I.....Carbamoylphosphate synthetase-I

d..... Day

E.....Estrogen

EDV..... End Diastolic Velocity

FSH..... Follicle Stimulating Hormone

KG..... Kilogram

LH..... Luteinizing Hormone

MG.....Milligrams

ML..... Milliliter

NMOL.....Nanomole

P4..... Progesterone

OAT..... Ornithine Aminotransferase

OCT .....Ornithine Transcarbamylase

ODC..... Ornithine Decarboxylase

OIFNτ..... Ovine Interferon - tau

P5C..... L-pyrroline-5-carboxylate

PDG ..... Phosphate-Dependent Glutaminase

PI..... Pulsatility Index

PSV..... .... Peak Systolic Velocity

RI..... Resistance Index

SEM.....Standard Error of the Mean

TAMV.....Time Averaged Mean Velocity

VEGF.....Vascular Endothelial Growth Factor

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

Introduction

Reproductive performance is the largest determinant of income in the livestock enterprise. In U.S. sheep industry, embryonic and fetal deaths during pregnancy account for 25 to 50% of the total number of fertilized ova (Knights et al., 2003; Dixon et al., 2007), and can lead to complete pregnancy losses and decreases in dam productivity. Most embryonic loss has been reported to occur before d 18 (Hulet et al., 1956; Moore et al., 1960; Quinlivan, 1966). In multiple pregnancies, the loss of individual embryos or fetuses can occur without a total loss of pregnancy (Rhind et al., 1980; Schrick and Inskeep, 1993). Only a small percentage of embryos are inherently non-viable in the ewe (Wilmut et al., 1986), which would suggest that the majority of early embryonic losses can be prevented.

This thesis focuses on an alternative strategy to enhance reproductive performance in sheep with supplemental arginine. As a precursor for nitric oxide, polyamines, creatine, proteins, and glutamate, the amino acid arginine plays a vital role in metabolism and reproduction (Wu and Morris, 1998). Nitric oxide is the endothelium-derived relaxing factor essential for increasing systemic vasodilation (Ignarro et al., 2001; Martin et al., 2001). Supplemental arginine has been reported to increase the number of live piglets born per sow (Mateo et al., 2007). Furthermore, pregnant rats supplemented with arginine throughout gestation exhibited an increase in embryonic survival and litter size (Zeng et al., 2008). Collectively, these studies would suggest that reproductive efficiency can be enhanced via supplementation of arginine.

The following research focuses on improving reproductive performance in the ewe with supplemental arginine. The development of strategies for enhancing prenatal growth and survival in sheep could have a major economic impact.

Literature Review

Reproduction in Sheep

Many mammalian species have an endogenous rhythm of reproductive activity that is synchronized by annual changes in day length (Woodfill et al., 1991, Barrell et al., 2000, Malpaux et al., 1996). Seasonal patterns of reproductive activity allow for the propagation and survival of offspring. Most breeds of sheep in mid to high latitudes exhibit a seasonal pattern of reproduction with a short day breeding season (November-December; Bryant and Rosa, 2003). The short day breeding season allows for parturition to occur during the spring of the year when the weather is mild and feedstuffs are plentiful. This allows the offspring to grow and develop during the summer months when forage availability is high and weather is optimum for growth and development prior to reaching the winter season. In tropical and sub-tropical environments, ewes have a tendency to breed at all times of the year with quality of forages dictating breeding activity (Bryant and Rosa, 2003). The seasonal breeding season relies on environmental cues such as photoperiod and temperature to initiate sexual activity.

Photoperiod is the main external factor controlling the seasonality of reproduction via neural and humoral processes in sheep (Malpaux et al., 1997). Photoperiod is perceived by the amount of light contacting the retina of the eye. As light enters the retina, presynaptic neurons are stimulated in the suprachiasmatic nucleus, sending signals towards the superior cervical ganglion initiating the firing of the postganglionic neurons (Senger,

2005). The postganglionic neurons synapse with inhibitory neurons connecting with pinealocytes where the message regulates melatonin secretion (Senger, 2005; Malpaux et al., 1996). During short photoperiods, the neural signal transmission is decreased, decreasing the inhibition on the pineal gland and increasing the amount of melatonin released. The duration of melatonin secretion regulates the activity of the hypothalamo-hypophyseal and gonadal axis (Karsch et al., 1989). Melatonin stimulates the release of gonadotropin releasing hormone (GnRH).

Exhibiting multiple estrous cycles throughout a particular reproductive season the ewe is considered to be seasonally polyestrous. On average during the fall of the year, ewes enter an estrous cycle every 17 d. The estrous cycle can be divided into four stages; estrus, metestrus, diestrus, and proestrus. These stages can be grouped into two main phases; the follicular phase and the luteal phase. The follicular phase can be described as the period from regression of the corpora lutea (CL) to ovulation and thus, it contains proestrus and estrus. Whereas, the luteal phase is the period from ovulation until CL regression and thus, it includes metestrus and diestrus.

The follicular phase is recognized by having growing dominant follicles that produce estradiol and is governed by the hypothalamus, anterior pituitary, and ovary (Senger, 2005). This phase begins following luteolysis as a result from the decline in progesterone (P4) concentration. During this phase, proestrus initiates with the decline in P4 and the transition to estrogen (E) dominance as follicles are recruited for ovulation. The transition from P4 to E production by the ovary is regulated by the gonadotropins; follicle stimulating hormone (FSH) and luteinizing hormone (LH). Follicle stimulating hormone and LH are the major signals for follicular recruitment and selection. Primordial follicles

contain a single that is surrounded by a squamous layer of follicular epithelium and are the largest class of follicles in the ovary throughout life (Hansel and Convey, 1983). As these follicles grow, the squamous cells proliferate to form several layers of granulosa cells, which are then termed primary follicles. By a series of mitotic divisions, the primary follicle is transferred into a secondary follicle that contains a multifacet of granulosa and theca cell layers (Greenwald and Roy, 1994). Due to the presence of an antral cavity, a tertiary follicle is derived from the secondary follicle. The tertiary follicle has increased growth and development due to mitotic activity and accumulation of follicular fluid (Greenwald and Roy, 1994).

Two follicular waves occur during the follicular phase. The first takes place from d 1 to 12, in which a large tertiary follicle forms on d 7 to 9 that ultimately becomes atretic (Greenwald and Roy, 1994). The second wave takes place from d 12 to 17, which recruits two large follicles that grow rapidly in size on d 15 following luteolysis on d 14 (Greenwald and Roy, 1994).

During the final follicular phase, estrogen dominance occurs and major physiological changes occur in the female to induce the onset of estrus or 'heat', and aid the reproductive tract in sperm transport. Estrus is the period in which the ewe is sexually responsive to the ram. Estrus lasts approximately 30 h with ovulation occurring at 24-30 h after the onset of estrus.

Initiation of ovulation ends the follicular phase and the ewe enters the beginning of the luteal phase. Metestrus is the period between ovulation and the formation of CL. The luteal phase is characterized by the presence of a CL on the ovary. The CL is an endocrine gland in the adult ovary that forms from the follicle wall after ovulation (Stouffer, 1994)

whose primary function is to secrete P4. The ewe displays a short-lived CL that forms rapidly after ovulation and functions until d 17 postestrus (Stouffer, 1994). If pregnancy occurs, the CL is sustained until the placental-luteal shift in P4 production occurs. However if there is an absence of pregnancy, rapid luteolysis occurs and the estrous cycle is repeated

The hypothalamus is the main control center for the neural regulation of reproductive hormones. Gonadotropin releasing hormone is a neuropeptide released from the hypothalamic surge and tonic centers of the brain, and is responsible for the release of FSH and LH from the anterior lobe of the pituitary (Senger, 2005). As previously mentioned, melatonin stimulates the production of GnRH in the ewe. The tonic center of the hypothalamus releases small pulses of GnRH at a constant, basal level. Whereas the surge center is responsible for the preovulatory release of GnRH, which in turn stimulates the surge of LH that in turn initiates ovulation. As E reaches threshold level, in the absence of P4, GnRH is released from the hypothalamus in a surge-like pattern.

Luteinizing hormone is the major luteotropic hormone released from the anterior lobe of the pituitary in the ewe through its regulatory feedback mechanism on adenylate cyclase and cyclic AMP (cAMP; Hansel and Convey, 1983). Hansel and Convey (1983) described the series of events regarding the feedback mechanism of LH. LH binds to its receptor in the plasma membrane, activating adenylate cyclase and cAMP, initiating protein kinase activation. Phosphorylation of steroidogenic enzymes and increased protein synthesis occur. Bound LH is internalized and degraded, whereby the LH receptors are then recycled via secretory granules and released into the plasma membrane via exocytosis (Hansel and Convey, 1983). The release of LH is triggered by a positive feedback of E from the preovulatory follicle. Increasing concentration of LH stimulates follicular growth

and maturation of follicles. Estrogen released from the follicle and P4 from the CL negatively impact the release of LH. Following luteal regression, LH rises to aid with follicular maturation. LH concentrations increase in peripheral blood following luteal regression, resulting from the negative feedback effect of P4. Increased LH during the preovulatory period is characterized by increased frequency of LH release.

Early in the follicular phase, GnRH pulse frequency is increased due to low levels of P4, initiating release of LH and FSH (Senger, 2005). The presence of LH causes release of E from follicles stimulating the large release of GnRH.

During postestrous FSH levels are released at a greater rate due to decreased LH release. The rise in FSH may be due to removal of the negative feedback of inhibin following ovulation (Hansel and Convey, 1983). As the follicular phase progresses, the follicle begins to secrete inhibin, which causes a negative feedback on FSH from the pituitary (Senger, 2005).

Estrogen increases in venous and peripheral blood during the prevoulatory period, reaching its peak at estrus (Hansel and Convey, 1983). Estrogen, in the absence of P4, acts on the brain to induce estrus-like behavior. The peak in LH causes ovulation, the release of an oocyte from the follicle, following a cascade of events.

Progesterone is responsible for preparing the uterine environment for the maintenance of pregnancy. Progesterone decreases the tonic release of GnRH and LH, ultimately decreasing the onset of ovulation and estrus like behavior. If an embryo is present, the CL will maintain the production of P4 throughout pregnancy until the placental-luteal shift occurs. The CL begins to secrete P4 around d 4 or the beginning of diestrus

while still continuing to grow until reaching maximum size by d 14 (Bazer et al., 1998). In the absence of pregnancy, luteolysis begins on d 15 or 16, thus resuming the estrous cycle.

Produced by the uterus, prostaglandin F2α (PGF2α) is responsible for initiating the regression of the CL via ceasing the production of P4. High progesterone exposure during the latter stages of the luteal phase is critical for the initial production of PGF2α and luteolysis. Progesterone is responsible for increasing phosolipid stores and prostaglandin synthase activity necessary for arachidonic acid conversion to PGF2α (Bazer et al., 1998).

PGF2α is transferred from the uterine vein by counter-current exchange into the ipsilateral ovarian artery where it then reaches the CL causing luteolysis (Hansel and Convey, 1983). This particular anatomical mechanism allows for the delivery of high concentrations of PGF2α to the CL during luteolysis.

Release of PGF2α is regulated by P4, E and oxytocin around d 15 and 16 of the cycle (Bazer et al., 1998). Progesterone and E are primarily responsible for regulating the release of oxytocin through mediating endometrial oxytocin receptor expression in the endothelial lining. The "Progesterone Block" occurs for 10 to 12 d following ovulation, which inhibits the synthesis of oxytocin receptors (Bazer et al., 1998). High levels of P4 exposed to the endometrium exhibits a negative feedback on the number of P4 receptors, causing them to decline. As P4 receptors decline, the epithelium of the endometrium synthesizes and releases E. The release of E up-regulates oxytocin receptor expression in the luminal and glandular portions of the endometrial epithelium (Bazer et al., 1998). The increase in circulating oxytocin then stimulates the pulsatile release of PGF2α .

PGF2α stimulates contractions of smooth muscle in the ovary, forcing the stigma to protrude from the ovary (Senger, 2005). Lysosomes are also released within the granulosa

cells causing connective tissue deterioration at the apex of the follicle (Senger, 2005). Prostaglandin E aids the follicle to remodel itself into a CL following ovulation. This hormone activates plasminogen, which is converted to plasmin by a plasminogen activator (Senger, 2005). Plasmin plays a key role in tissue remodeling by dissolving the coagulum of the corpus hemorrhagicum.

Maternal Recognition of Pregnancy

Communication between the embryo and the maternal system must be established following conception to ensure normal development and differentiation of the embryo. Maternal recognition of pregnancy occurs around d 13 following ovulation. Embryonic migration in sheep has been reported to occur between d 12 to 14 of gestation, with physical attachment of the embryonic trophoblast to the and endometrial epithelium at d 16 to 17 (Nephew et al., 1989). Intrauterine migration of embryos is associated with the synthesis of estradiol and embryonic migration (Nephew et al., 1989).

Initially, the embryo depends upon the maternal and paternal genes to illustrate proper development. The conceptus elongates from a blastocyst to a filamentous form, which produces interferon tau (IFNτ) that is responsible for preventing the development of the endometrial luteolytic mechanism (Spencer and Bazer, 2002). The antiluteolytic signal responsible for the recognition of pregnancy is interferon tau. The presence of interferon tau allows for maintenance of the CL, which is the primary structure responsible for progesterone production during early pregnancy in sheep. Interferon tau is produced by embryonic trophectoderm cells which stimulates a paracrine, antiluteotropic effect on the endometrium to inhibit production of pulsatile PGF2α (Bazer et al., 1998). In sheep, IFNτ is a major protein secreted by the trophectoderm of the peri-implantation conceptus and is

secreted between d 10 and 21 of pregnancy (Bazer et al., 1998). As the conceptus develops from a spherical to filamentous shape, the concentration of IFNτ increases around d 12 or 13, suggesting that IFNτ is important for the development and recognition of pregnancy. Progesterone is also critically important for the maintenance of pregnancy due to its ability to suppress expression of PGF2α.

During the peri-implantation period, the endometrium secretes histotroph to nourish the conceptus. Progesterone is necessary for the secretion of histotroph by uterine glands during early pregnancy (Spencer et al., 2004), which is important for early embryonic growth and development. Histotroph contains a unique collection of enzymes, growth factors, cytokines, lymphokines, hormones, and transport proteins (Spencer et. al., 2004). All of these factors have been shown to ensure proper embryonic growth and survival in humans, (Burton et. al., 2002), primates (Bazer et. al., 1979; Roberts & Bazer, 1988; Carson et. al., 2000; Gray et. al., 2001a) and sheep (Lawson et. al., 1983; Flechon et. al., 1986; Gray et. al., 2001b; and Gray et. al., 2002).

Reproductive Loss in the Ewe

Reproductive performance is the largest determinant of income in the livestock enterprise. In the sheep industry, approximately 30% of the total number of fertilized ova are not represented by live births (Knights et al., 2003; Bolet, 1986; Dixon et al., 2007). This loss can lead to complete pregnancy losses and decreases in dam productivity resulting in a frequent, yet unrecognized economic loss. Most embryonic loss has been reported to occur before d 18 of gestation (Hulet et al., 1956; Moore et al., 1960; and Quinlivan, 1966). In pregnancies with multiple fetuses, the loss of individual embryos or fetuses can occur without a total loss of pregnancy (Rhind et al., 1980; Schrick and Inskeep, 1993).

Approximately 54% of ewes with twin ovulations had only one embryo by day 18 of gestation, while 3.9% of ewes had evidence of complete loss of all embryos (Qunilivan, 1966). Cross (2001) concluded that the majority of loss in the ewe occurs during the critical period of maternal recognition of pregnancy, attachment and initial placental development. Late embryonic and fetal mortality after day 18 of gestation has been estimated at 9.4% (Hulet et al., 1956).

Only a small percentage of embryos are inherently non-viable in the ewe (Wilmut et al., 1986), which would suggest that the majority of early embryonic losses may be prevented. Embryonic losses in sheep have been associated with numerous environmental and physiological factors. More recently, abnormal circulating concentrations of progesterone, estrogen, and vascular endothelial growth factor in the ewe have been identified as factors to prenatal losses (Dixon et al., 2007). These factors are important for optimizing the uterine environment and ensuring proper development of the placenta during early pregnancy (Nephew et al., 1991; Spencer et al., 2004). External environmental factors such as temperature, nutrition and disease may also influence embryonic survival.

Progesterone (P4) is secreted from the CL and placenta in the ewe and is required throughout gestation for the maintenance of pregnancy (Casida and Warwick, 1945). Recently, low circulating concentrations of steroids have been identified as a critical factor to prenatal loss (Dixon et al., 2007). Embryonic survival tended to be lower in ewes with lower concentrations of P4 during the luteal phase (Wilmut et al., 1986). Three and six days after mating, cattle with normal embryos had greater concentrations of peripheral P4 than cattle with degenerating embryos (Maurer and Echternkamp, 1982). Pregnancy rates have

been correlated to circulating levels of peripheral P4 prior to maternal recognition of pregnancy (Henricks et al., 1971; Lamming et al., 1989).

Decreased concentrations of P4 have been associated with nutritional status of the dam early in gestation. Reduced concentrations of P4 due to poor nutrition decreased embryonic survival during early pregnancy (Rhind et al., 1989). Similarly, overfeeding ewes reduced embryonic survival (Cumming et al., 1975). Decreased concentrations of peripheral P4 may be due to increased metabolism of P4 from an increase in energy fed in the diet (Rabiee et al., 2000). Increases in metabolism resulted in a high clearance rate of P4 due to an increase in blood flow to the liver from increase feed intake (Bensadoun and Reid, 1967). Therefore, the nutritional status of the ewes may have a significant effect upon P4 in plasma based upon an altered metabolic rate rather than secretion of P4 from the CL (Rabiee et al, 2000).

Estrogen remains lower during the early luteal phase in pregnant than in non-pregnant animals. In cattle, high levels of estrogen in ovarian follicles during maternal recognition of pregnancy may be detrimental to the maintenance of pregnancy (Thatcher et al., 1989). Decreases in conception rates in cattle were correlated with the increase in serum estrogen concentration (Pritchard et al., 1994).

Vascular endothelial growth factor (VEGF) is a stimulator of vascular endothelial cellular proliferation, angiogenesis, endothelial migration, and vascular permeability (Reynolds et al., 1992; Reynolds et al., 2000). Angiogenesis, or the growth of new blood vessels from existing blood vessels, is critically important during embryonic development and placentation (Reynolds et al., 1987; Reynolds and Redmer, 1988).

Ambient temperature plays a critical role in the survivability of the embryo. Reduced fertility in females mated with heat stressed males can result in 1) fertilization failure (Dutt & Simpson, 1957), 2) normal fertilization, but an increase in embryonic death (Horwarth, 1969), or 3) a failure in fertilization or an increase in embryonic death (Rathorne, 1970). One factor known to cause reproductive failure early in the breeding season is lowered fertility of rams (Dutt, 1954). In 1992, Mieusset et al. addressed the impact of fertilization rate and embryonic mortality when ewes were bred to rams exposed to high scrotal temperature. There was no difference in fertilization rates; however embryonic loss was significantly increased with ewes inseminated from frozen-thawed semen that had been previously exposed to high temperatures (Mieusset et al., 1992). Fertility in rams is improved early in the season when rams are kept at cooler temperatures during the summer months (Dutt and Simpson, 1957). Dutt (1954) has also shown fertility in the ewe is decreased early in the breeding season due to the frequent occurrence of morphologically abnormal ova.

Shorn and unshorn ewes were exposed to elevated temperature and the effects on estrus, fertility rate, morphology of ova, and embryo survival in ewes exposed to heat before or after breeding has been examined. Overall fertilization rate for ewes exposed to elevated air temperature on the 12^{th} day of the cycle before breeding was significantly lower than shorn ewes held at the ambient temperature (Dutt et al., 1957). Furthermore, heat treatment was shown to increase percentage of abnormal ova and increase embryonic loss (Dutt et al., 1957).

Spermatogenesis in mammals is susceptible to damage if the testicular temperature is higher than normal (Mieusset et al., 1992). Southdown rams were placed in a heated

chamber either shorn or unshorn during January. Pulse and respiration rate were elevated in unshorn rams located in the heated chamber. Unshorn rams placed in the heated chamber had the lowest percentage of motile cells with a high incidence of abnormal cells with a decreased sperm cell concentration (Hamm and Dutt, 1957).

Nutrition plays a critical role on reproductive physiology, including hormonal production, oocyte quality, fertilization, and embryonic development. Nutrition has been correlated with embryo survival and is a key factor influencing the efficiency in assisted reproductive technologies (Borowczyk et al., 2006). Borowczyk et al, evaluated the effects of nutritional plane (control vs. underfed) on follicular development, in vitro fertilization and early embryonic development in FSH-treated ewes (Borowczyk et al., 2006; Dutt et al., 1957). Experimental conditions of underfeeding ewes (60% of control diet) resulted in lower BCS and fertilization rates (Borowczyk et al., 2006). They also reported a decreased yield in poorer quality oocytes with fewer blastocysts in response to lower BCS, yet no effect on follicle number or oocyte maturation (Borowczyk et al., 2006). Ad libitum feeding of ewes for approximately 3 weeks enhanced BCS, lowered superovulation responses, lowered number of good quality oocytes and embryos and increased percentage of poorly developed embryos through the morula stage (Lozano et al., 2003).

Arginine

History of Arginine. The amino acid arginine is a versatile amino acid in animal cells that plays a critical role in serving as a precursor for proteins, nitric oxide and polyamines; all of which are all essential for proper development of the embryo and placenta (Wu and Morris, 1998). Arginine (2-amino-5-guanidinovaleric acid) was first isolated from lupin seedlings in 1886 (Wu and Morris, 1998). By 1895 it was identified as a component of

animal proteins and the structure was determined in 1897. Thirty years following the identification of arginine, W.C. Rose examined the mammalian synthesis using the nutritional studies in 1930. As reviewed by Wu and Morris (1998), following the discovery of the Urea cycle in 1932 (Krebs and Henseleit, 1932) the importance of arginine in physiology and metabolic pathways was reported. In the late 1930's and early 1940's, arginine was discovered to be a requirement for the synthesis of creatine. During the 1950's, 60's and 70's the initial classification of arginine was a non-essential amino acid for adult humans, however an essential amino acid for youth, growing mammals and carnivores (Wu and Morris, 1998). As reviewed by Wu and Morris (1998), Windmueller and Spaeth (1981) reported that the small intestine was the major source of circulating citrulline for endogenous synthesis of arginine in the adult rat. By 1987 arginine was identified as the precursor for mammalian nitrite/nitrate synthesis and that nitric oxide was the endothelium-derived relaxing factor; one year later nitric oxide was identified as the active intermediate of the arginine/nitric oxide pathway in macrophages and endothelial cells (Wu and Morris, 1998).

Arginine Synthesis. Endogenous arginine synthesis varies with species, nutritional status and developmental stage. Synthesis in mammals involves the intestinal-renal axis in which the citrulline released by the small intestine is transferred into arginine in the kidney via argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL; Wu and Knabe, 1995). It is important to recognize that the requirements for arginine are high in the neonate and are necessary to support rapid postnatal growth and development; however, as development progresses the need for arginine substantially decreases.

Intestine. There are intestinal differences in the synthesis of arginine from glutamine based upon the stage of development. At birth, the small intestine is the major site of arginine synthesis, however as development progresses the small intestine becomes the site of citrulline production as intestinal arginase expression increases (Wu and Morris, 1998). This transition is due to the kidneys ability to synthesize arginine from citrulline. Wu demonstrated that net arginine synthesis from glutamine by enterocytes was greater than net citrulline production at birth in piglets; however, by d 14 arginine and citrulline had declined (Wu, 1997). Similarly, arginine synthesis from glutamine decreased in 7-day-old pigs compared with 0- to 2-day-old pig enterocytes (Wu and Knabe, 1995). Wu and Knabe also noticed a decrease in the metabolism of glutamine to citrulline and the conversion of citrulline to arginine based upon the decrease in activities of key regulatory enzymes (Wu and Knabe, 1995). The decrease of glutamine to citrulline was recognized with a decrease in the enzymes; PDG, P-5-C synthase and CPS I (Wu and Knabe, 1995). A decreased conversion of citrulline to arginine was elicited with a decrease of ASS and ASL production (Wu and Knabe, 1995).

Enteral glutamine and glutamate and plasma glutamine are catabolized by the small intestine and serve as major precursors for intestinal synthesis of arginine and citrulline (Figure 1.1; Wu and Morris, 1998). Glutamine serves as a major fuel source for neonatal pig enterocytes and also it provides all carbon and nitrogen molecules for arginine synthesis in the enterocytes (Wu and Knabe, 1995). Arginine synthesis from glutamine involves two portions of the enterocyte cells; mitochondria and the cytosol. The synthesis of arginine from glutamine involves phosphate-dependent glutaminase (PDG), pyrroline-5-carboxylate

synthase (P-5-C), ornithine aminotransferase (OAT), ornithine carbamoyltransferase (OCT), carbamoyl phosphate synthase I (CPS I), ASS and ASL (Wu and Knabe, 1995). PDG, P-5-C synthase, OAT, OCT, and CPS I catalyze citrulline formation from glutamine in the mitochondria, whereas ASS and ASL convert citrulline to arginine in the cytosol (Wu and Knabe, 1995). Of all mammals, the pig is the only species that releases endogenously synthesized arginine into venous circulation (Wu and Morris, 1998).

Kidney. Endogenous arginine synthesis is regulated by the intestinal-renal axis. The ability for the kidney to produce arginine develops in late fetal stages and increases after birth. Approximately 60% of net arginine synthesis in adult mammals occurs in the kidney within the proximal convoluted tubules whereby citrulline is transferred into arginine via ASS and ASL (Wu and Morris, 1998). In the adult rat, the glutamine-derived citrulline is released by the small intestine into circulation and converted into arginine in the kidneys (Wu and Knabe, 1995). Arginine synthesis in the kidney is limited by the amount of citrulline produced by the small intestine.

Arginine Catabolism. Catabolism of arginine is a complex, diverse process due to the compartmentalization of cell types and enzymes. Argininase is a critical enzyme that is required for the regulatory availability of arginine for the synthesis of nitric oxide, polyamines, proline and glutamate. There are two distinct argininase enzymes that control the catabolic fate of arginine.

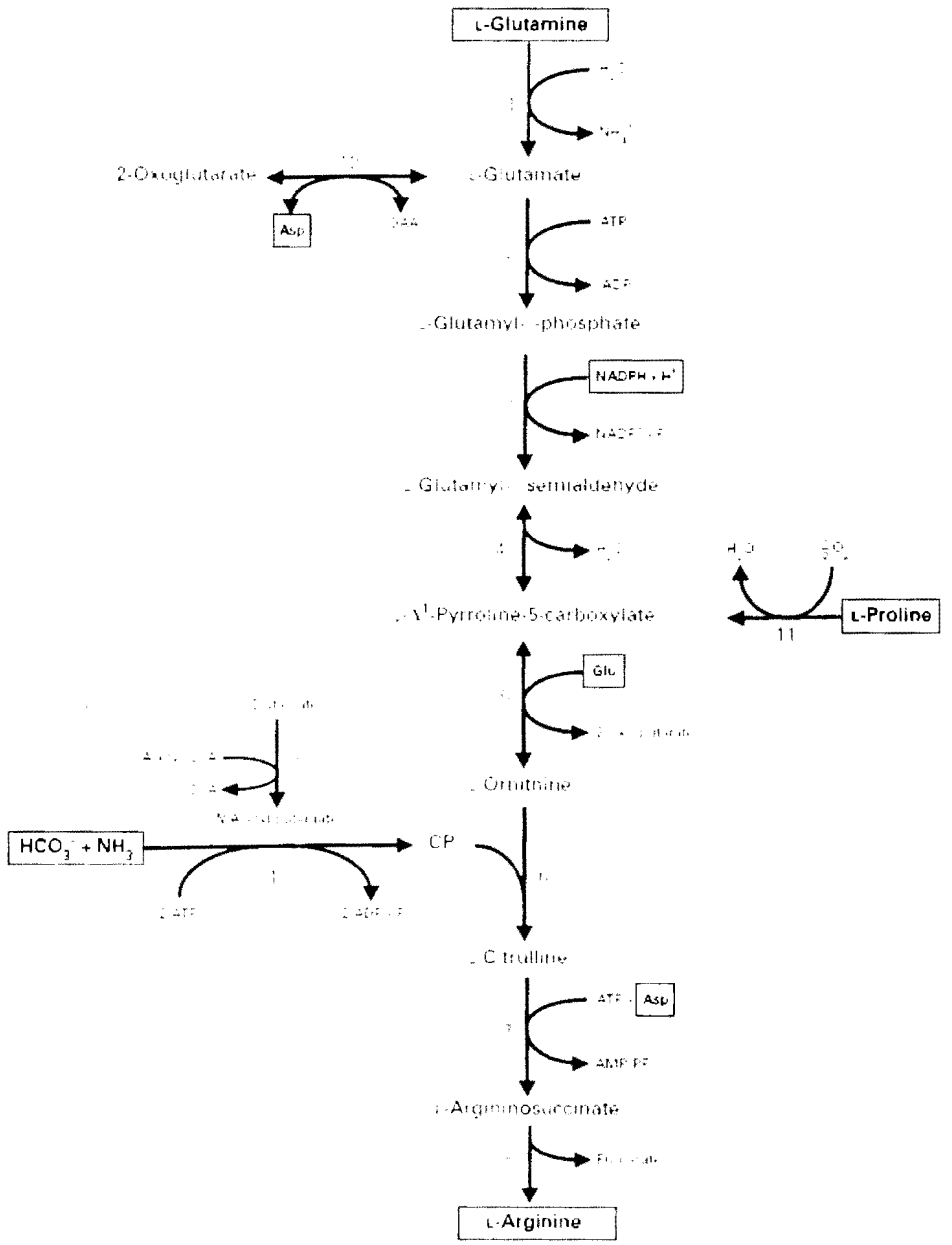

Figure 1.1. Pathways of arginine synthesis. Enzymes involved are; 1. phosphate-dependent glutaminase; 2 and 3. P5C sythetase; 4. spontaneous non-enzymatic reaction; 5. Ornithine aminotransferase; 6. Ornithine carbamoyltransferase; 7. Argininosuccinate synthase; 8. Argininosuccinate lyase; 9. N-acetylglutamate sythase; 10. Carbamoyl-phosphate synthase I; 11. Proline oxidase; 12. Aspartate aminotransferase. Reactions 1-6 and 9-11 occur in mitochondria and reactions 7 and 8 occur in the cytosol. Reaction 12 can occur in both mitochondria and the cytosol. Adapted from Wu and Morris, 1998.

The first is type I argininase, a cytosolic enzyme that is expressed in high concentrations in the liver as part of the urea cycle. The other, type II argininase, is a mitochondrial enzyme that is expressed in the kidney, brain, small intestine, mammary gland and macrophages (Wu and Morris, 1998). Type I argininase, specifically located in the liver, is a critically important enzyme within the urea cycle for detoxifying waste nitrogen.

Argininase competes for arginine for the synthesis of nitric oxide. High levels of argininase can limit the amount arginine needed for nitric oxide synthesis produced by cells. Argininase has also proved to be very important in the wound healing process. The wound healing process is very complex beginning with an early burst of nitric oxide synthesis at the site, decreasing amounts of arginine, and a significant rise in ornithine and proline synthesis (Wu and Morris, 1998). First, argininase is critically important for removing arginine, the critical substrate of nitric oxide synthesis. Secondly, the generation of ornithine for synthesis of proline from argininase activity, which is required for collagen synthesis needed for tissue repair. In wounds, extracellular fluid has exhibited high amounts of ornithine and low amounts of arginine, suggesting high amounts of argininase activity are present (Wu and Morris, 1998).

Polyamines are critical for cellular proliferation and differentiation (Wu and Morris, 1998). Polyamines are synthesized from ornithine via ornithine decarboxylase (ODC) and argininase (Figure 1.2). Cells with decreased amount of argininase activity cannot proliferate. In new born mammals, the level of argininase activity is low in the enterocytes resulting in a decreased amount of proliferation from polyamine synthesis in the small intestine. As the newborn animal ages the amount of argininase and ODC activity in the

enterocyte begins to rise; therefore, increasing polyamine synthesis. The increased levels of argininase and ODC are due in part to glucocorticoid introduction (Wu and Morris, 1998).

Proline is the major product of arginine catabolism in the enterocyte of post-weaning pigs (Wu et al., 1997). Intestinal synthesis of proline from arginine varies with species and age. In newborn piglets, arginine derived from proline synthesis is not detectable in the enterocyte, however increasing amounts of arginine are present at weaning (Wu et al., 1997). This data suggests the importance of proline as an essential dietary amino acid for the neonatal piglet and this requirement decreases with age and development.

Proline synthesis by the lactating mammary gland is regulated in part by argininase activity. The enzymes required for the synthesis of proline from arginine (argininase, OAT and P-5-C reductase) are present in the mammary gland (Figure 1.2; Wu and Morris, 1998). The output of proline in the milk of many mammals exceeds the uptake of proline by the lactating mammary gland. However, arginine uptake in the plasma of the lactating mammary gland exceeds the output of arginine in the milk (Wu and Morris, 1998). Therefore, the proline produced by the mammary gland is arginine-dependent, rather than glutamate, due to the absence of P-5-C synthase (Wu and Morris, 1998). Proline synthesis during lactation, along with the presence of argininase, provides a substrate for glutamate synthesis.

Arginine Decarboxylase. Arginine decarboxylase has been identified in the brain, liver, kidney, adrenal gland, macrophages and small intestine. This enzyme is located within the mitochondria and produces CO_2 and agmatine from arginine (Wu and Morris, 1998).

Nitric Oxide Synthase. Nitric oxide plays an important role in steroidogenesis, ovulation, embryo implantation, and the maintenance of pregnancy (Gouge et. al., 1998; Manser et. al., 2004). Nitric oxide is important for relaxing vascular smooth muscle and is produced by endothelial cells (Moncada and Higgs, 1993). Nitric oxide is synthesized from the oxidation of arginine to citrulline by nitric oxide synthase (Figure 1.2). Nitric oxide synthase is an important enzyme regulating the role of arginine degradation to nitric oxide. These enzymes are regulated by a Ca2+/calmoduline feedback mechanism (Wu and Morris, 1998). Nitric oxide production by endothelial cells that contains the properties of the endothelium-derived relaxing factor which is important for relaxing vascular smooth muscle (Moncada and Higgs, 1993).

Through activation of cyclic guanosine monophosphate pathway, nitric oxide causes relaxation of vascular smooth muscle (Moncada and Higgs,1993). Furthermore, during pregnancy an inhibition of nitric oxide synthase expressed a greater incidence for embryonic mortality in the rat (Chwalisz et. al., 1999).

Arginine and Animal Reproduction

Arginine supplementation has been investigated within monogastric species due to the catabolic fate of arginine within the rumen. Traditionally, pig and rat have been the model of choice for arginine supplementation research.

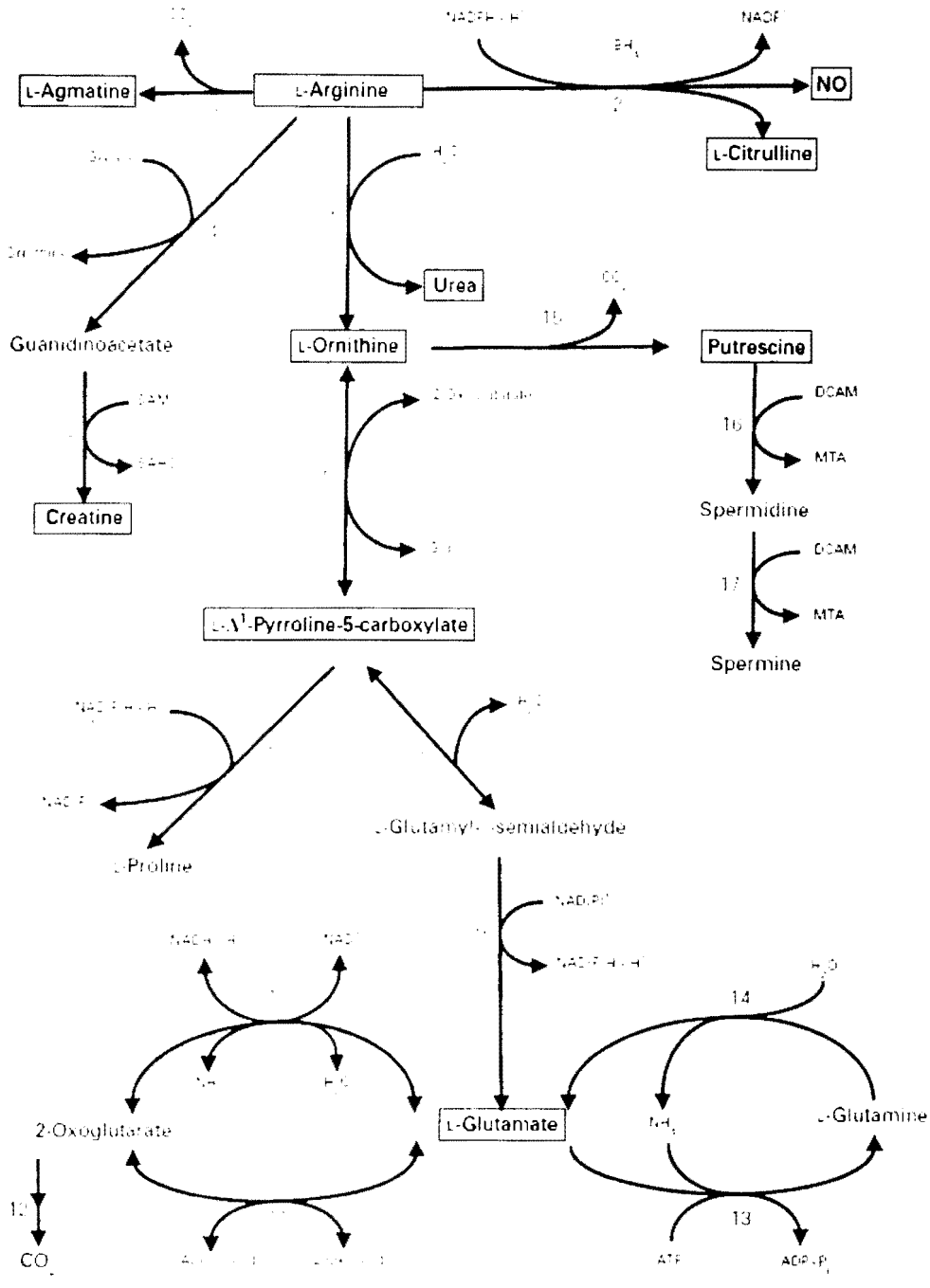

Figure 1.2. Pathways of arginine catabolism. Enzymes involved are; 1. argininase; 2. nitric oxide synthase; 3. arginine decarboxylase; 4. arginine:glycine aminotransferase; 5. guanidinoacetate N-methyltransferase; 6. OAT; 7. P5C reductase; 8. spontaneous, non-enzymatic reaction; 9. P5C dehydrogenase; 10. glutamate dehydrogenase; 11. alainine aminotransferase; 12. aspartate aminotransferase; 13. glutamine sythetase; 14. glutaminase; 15. ODC; 16. sperimidine synthase; 17. spermine synthase. Adapted from Wu and Morris, 1998.

Recently, gestating sows supplemented with arginine achieved a 22% increase in live piglets born when compared to nonsupplemented sows (11.4 vs. 9.4, $P < 0.03$, respectively; Mateo et al., 2007). Furthermore, pregnant rats supplemented with 1.3% Arginine-HCl throughout pregnancy or between d 1 and 7 of gestation had increased embryonic survival and litter size (30% increase; Zeng et al., 2008). In addition to these beneficial effects on prenatal survival, arginine treatment during late pregnancy increased transport of nutrients to the unborn lamb (Thureen et al., 2002) and enhanced fetal protein accretion in sheep, ultimately increasing lamb birth weight (De Boo et al., 2005).

Arginine also plays an essential role in polyamine synthesis, which is essential for cellular growth and differentiation particularly for spermatogenesis (Table 1.1). Concentrations of polyamines are high in boar seminal fluid and when supplemented with 1% arginine-HCL to sexually active boars for 30 d. There was no effect on semen volume, however enhanced concentrations of arginine and polyamines were exhibited in seminal fluid by 43 and 63% respectively (Wu et al., 2009). In addition, arginine supplementation increased sperm counts by 18% and motility by 8% (Wu et al., 2009).

Arginine has been shown to play an important role in fetal survival and growth (Table 1.1). Feeding arginine free diets to pregnant rats resulted in increased fetal reabsorptions, intrauterine growth retardation, increased embryonic loss and decreased number of live fetuses (Greenburg et al., 1997). Dietary arginine supplementation in drinking water prevented hypoxia-induced fetal growth retardation in rats (Vosatka et al., 1998).

Table 1.1. Roles of arginine on reproduction. Adapted from Wu et al., 2009.

Roles of arginine on reproduction	Effect	Mediators
Embryo implantation, survival and growth	Increase	Nitric oxide, polyamines, and protein synthesis
Fetal survival, growth and health	Increase	Nitric oxide, polyamines, and protein synthesis
Ovulation, ovarian steroidogenesis and oocyte quality	Increase	Nitric oxide and polyamines
Placental angiogenesis, growth and function	Increase	Nitric oxide, polyamines, and protein synthesis
Spermatogenesis, sperm quality, and male fertility	Increase	Nitric oxide, polyamines, and protein synthesis
Uterine contractility and preterm labor	Decrease	Nitric oxide
Erectile dysfunction	Decrease	Nitric oxide
Preeclampsia in human pregnancy and animal models	Decrease	Nitric oxide

The amino acid arginine is a versatile amino acid in animal cells that plays a critical role in serving as a precursor for proteins, nitric oxide and polyamines; all of which are all essential for proper development of the embryo and placenta (Wu and Morris, 1998). Supplemental protocols with arginine during pregnancy may improve reproductive performance of the dam and improve the uterine environment for the maintenance of pregnancy.

Ultrasonography

The use of ultrasonography provides a noninvasive opportunity to evaluate the physiological status of reproductive organs. In livestock, most ultrasound imaging occurs utilizing the B-mode (brightness modality) type ultrasonography. B-mode ultrasonography was first utilized in bovine reproduction in the 1980's, which allowed tremendous advantages in research and clinical practice to visualize the reproductive tract (Bollwein and

Herzog, 2007). Currently, B-mode ultrasonography is used to identify and measure structures in a two-dimensional format during different physiological states.

Ultrasonography is derived from crystals housed in the transducer that contain piezoelectric properties, which are activated by an electrical charge (Bollwein and Herzog, 2007). The crystals expand and contract when activated electrically, which emits a sound wave. These sound waves penetrate the body and are reflected by tissue surfaces depending upon their density (Bollwein and Herzog, 2007). The resistance offered by a tissue to sound transmission is known as its acoustic impedance (Maulik, 2005). Most soft tissues contain a minor impedance; however, bones have a significantly greater impedance rate. The impedance in tissues allow for echoes to occur, which gives rise for the basis of ultrasonography and Doppler. The transducer serves as the initiator of sending the sound waves and also receiving the sound echoes.

In most large livestock species (cattle, swine and sheep), transrectal imagining is the most common way to analyze the reproductive tract in the non-pregnant state. Transrectal imaging allows for an image that is in close proximity to the tract through the rectal wall. In the ewe, as gestation advances past d 25 or 30, the use of transrectal imaging is decreased due to the morphological changes of the reproductive tract. Therefore transabdominal scanning becomes more popular after day 25 of pregnancy. Transabdominal imaging is obtained by placing the transducer on the rear flank angling towards the rectum of the female.

Although B-mode ultrasonography can depict organ morphology, it cannot provide information regarding vessel hemodynamics. During the last ten years, color Doppler

ultrasonography has been utilized to evaluate the changes in vascular perfusion during a particular physiological state.

Doppler Ultrasonography

The use of Doppler ultrasonography is an emerging technology based upon Doppler shifts. Doppler ultrasound adds blood flow information in various tissues as seen with the B-mode ultrasonography. The Doppler ultrasound measures the frequency of echoes from moving red cells that increase or decrease in speed as they move toward or away from the transducer.

The Doppler effect is named after Johann Christian Andreas Doppler, an Austrian scientist who described the changing color of stars as they move closer or farther away from the Earth (Abramowicz and Sheiner, 2008). The Doppler effect is based upon the changes in frequency of energy wave transmission. The change in the frequency is known as the Doppler shift. The Doppler shift can be obtained with the following formula:

$$Fd = ft - fr$$

where Fd is the Doppler shift, Ft is the transmitted frequency and fr is the received frequency. The shift in frequency is proportional to the speed of movement between the source and the receiver (Maulik, 2005). As the source and observer move closer, the wavelength decreases and the frequency increases. Similarly, as the source and observe move farther apart, the wavelength increases and the frequency decreases. With regards to blood flow, the Doppler effect can be described as the frequency of moving red blood cells moving towards or away from the transducer.

The Doppler ultrasonography utilizes B-mode imaging to visualize the morphology of a particular organ while the color-flow mode of the Doppler measures arterial pulses,

which are displayed quantitatively within each cardiac waveform. Large vessels are measured in laminar flow mode while small, tortuous vessels are measured in spectral flow mode.

In laminar flow, blood cells moving close to the vessel wall have a lower velocity compared with cells moving along the center of the vessel lumen (Ginther and Utt, 2004). When the sample gate cursor encompasses the entire width of the vessel, this is referred to as laminar flow. The angle at which the ultrasound beams intersect with the lumen of the blood vessel is called the angle of insonation (Ginther and Utt, 2004). The velocity of blood flow is proportional to the degree of the Doppler-shift frequency and to the cosine of the Doppler angle as calculated (Ginther and Utt, 2004):

Velocity = Doppler-shift frequency / Cosine of Doppler angle

When sampling small vessels in which the Doppler angle is difficult to obtain, Doppler indices are used as an alternative. Indices are ratios of velocity measurements and are independent of the Doppler angle (Ginther and Utt, 2004). The sampled area measured with Doppler indices can be inferred with the correlation of vascular perfusion within the particular vessel. Doppler indices must be taken in spectral mode. Measurements are obtained by placing the sample gate cursor into the B-mode image of the lumen of a targeted vessel to acquire three cardiac cycle waveforms. From these wave forms the resistance and pulsatility indices are calculated as well as peak systolic velocity, end diastolic velocity and time-averaged maximum velocity.

Peak systolic velocity (PSV) represents the maximum point in the traced outline of the waveform or the maximum Doppler-shift frequency. Similarly, the lowest point on the traced waveform prior to the next systolic increase is the end diastolic velocity (EDV). The

average of the maximum values over the time of a cardiac cycle is called the time-averaged maximum velocity (TAMV; Ginther and Utt, 2004).

Resistance index (RI) is used to relate the negative relationship between the extent of resistance in the tissues and the extent of vascular perfusion (Ginther and Utt, 2004). The higher the RI value, the lower the vascular perfusion. The formula to obtain RI is as follows

$$RI = PSV - EDV / PSV$$

Similar to RI, pulsatility index (PI) is an expression of the extent of the difference between the PSV and EDV of the blood pulse in the vessel at the level of vessel examination (Ginther and Utt, 2004). PI is determined as follows:

$$PI = PSV - EDV / TAMV$$

The assessment of the physiological status of reproductive organs has been further enhanced with the usage of Doppler ultrasonography techniques. Doppler imaging details not only the morphology of the reproductive organ, but also the hemodynamic changes associated with the organ. As a result, the use of Doppler has proven to be an effective noninvasive method to evaluate the changes of the reproductive tract not only for clinical practitioners but also research scientists.

In conclusion, the economic importance of embryonic loss in the ewe is an area of great concern for the U.S. sheep industry. Many factors have been identified as key regulators of embryonic loss in the ewe, most of which may be prevented by ensuring a more suitable uterine environment for the maintenance of pregnancy throughout gestation. The following studies have been designed to examine and possibly maximize reproductive efficiency in the ewe through arginine supplementation.

CHAPTER 2. IMPACTS OF L-ARGININE ON OVARIAN FUNCTION AND REPRODUCTIVE PERFORMANCE IN EWES

Abstract

The objective was to determine if arginine supplementation enhances ovarian function and prevents early reproductive losses in sheep. Rambouillet ewes of a similar BW (68 ± 1.8 kg) and age (4.7 ± 0.32 yr) received L-arginine HCl (equivalent to 27 mg of L-arginine/kg of BW; ARG; n = 20) or saline (CON; n = 20) i.v. once daily from d 0 (estrus) to d 15 postestrus. Daily blood samples were obtained from 5 ewes/group immediately after treatment (0 h) to assess progesterone (P4) concentrations and at -0.5, 0, 0.5, 1, 2, 4, 8, and 24 h on d 12 to determine serum concentrations of arginine. Ovarian hemodynamics (d 12) and reproductive losses (d 25, 45, and 65) were determined with color-Doppler and B-mode ultrasonography, respectively. On d 12, serum concentrations of arginine (nmol/ml) were elevated in ARG vs. CON ewes at 0 (5043 ± 1015 vs. 355 ± 18, $P < 0.001$), 0.5 (896 ± 76 vs. 290 ± 16, $P < 0.001$), 1 (474 ± 30 vs. 231 ± 12, $P < 0.001$), 2 (471 ± 33 vs. 272 ± 30, $P < 0.005$), and 4 h (358 ± 26 vs. 279 ± 23, $P < 0.05$), but were similar ($P > 0.05$) at -0.5, 8 and 24 h. Pulsatility index in the ovarian hilus on d 12 was reduced in ARG vs. CON ewes (0.56 ± 0.014 vs. 0.75 ± 0.080, $P < 0.05$). Despite similarities in the number of corpora lutea (CL) per ewe (ARG, 1.8 ± 0.20 and CON, 1.8 ± 0.20; $P > 0.05$), ARG ewes had greater P4 concentrations throughout treatment compared to CON ewes (53.6 ± 1.66 vs. 41.3 ± 2.65 AUC, respectively, $P < 0.004$). Although pregnancy rate was not influenced (ARG, 55% and CON, 60%; $P > 0.05$), ARG ewes had more embryos per ewe (1.6 ± 0.15 vs. 1.3 ± 0.13, $P \leq 0.04$) and less CL not represented by embryos (0.18 ± 0.122 vs. 0.58 ± 0.155, $P \leq 0.03$) compared to CON ewes at d 25 of pregnancy. As pregnancy progressed to d 45, the difference in the number of CL not represented by embryos was even greater ($P \leq 0.03$) in

CON (0.75 ± 0.227) vs. ARG ewes (0.18 ± 0.122). Ewes treated with ARG gave birth to more lambs when compared to control ewes (ARG, 1.6 ± 0.16 vs. CON, 1.1 ± 0.16 lambs born per ewe). However, average lamb birth weights did not differ (ARG, 4.85 ± 0.076 and CON, 4.78 ± 0.145 kg). In summary, early reproductive losses can be prevented, at least in part, by treatment with arginine. Decreased ovarian vascular resistance and increased concentrations of P4 may result in a more ideal environment for early embryonic survival.

Key Words: arginine, embryo survival, sheep

Introduction

In the ewe, embryonic and fetal deaths during pregnancy account for 25 to 50% of the total number of fertilized ova (Knights et al., 2003; Dixon et al., 2007). Most embryonic loss has been reported to occur before d 18 (Hulet et al., 1956; Moore et al., 1960; Quinlivan, 1966). In multiple pregnancies, loss of individual embryos or fetuses can occur without a total loss of pregnancy (Rhind et al., 1980; Schrick and Inskeep, 1993).

A small percentage of embryos are inherently non-viable in the ewe (Wilmut et al., 1986), which would suggest that the majority of early embryonic losses can be prevented. Embryonic losses in sheep have been associated with numerous environmental and physiological factors. Recently, low circulating concentrations of progesterone, estrogen, and vascular endothelial growth factor in the ewe have been identified as factors to prenatal losses (Dixon et al., 2007). These factors are important for optimizing the uterine environment and ensuring proper development of the placenta during early pregnancy (Nephew et al., 1991; Spencer et al., 2004).

The amino acid L-arginine is important for the synthesis of polyamines and nitric oxide, both of which are essential for proper development of the embryo and placenta. Gestating sows supplemented with arginine achieved a 22% increase in live piglets born (11.4 vs. 9.4, $P < 0.03$, respectively; Mateo et al., 2007). Furthermore, pregnant rats supplemented with 1.3% Arginine-HCl throughout pregnancy or between d 1 and 7 of gestation had increased embryonic survival and litter size by 30% (Zeng et al., 2008). It is reasonable to hypothesize that supplementation with arginine would have beneficial impacts on optimizing the uterine environment for ensuring early embryonic survival.

The objective of the current study was to determine the effects of arginine supplementation on ovarian function, early reproductive loss and lamb birth weight in Rambouillet ewes.

Materials and Methods

All animal procedures were approved by the North Dakota State University Institutional Animal Care and Use Committee.

Animals and Experimental Design

Rambouillet ewes of a similar BW (68 ±1.8kg) and age (4.7 ± 0.32 yr) at the North Dakota State University Hettinger Research Extension Center (46°N) were randomly assigned to one of two groups: control (**CON**; n = 20) and L-arginine (**ARG**; n = 20). All ewes received a vaginally inserted controlled internal drug release (CIDR-G; 300 mg P4, Pharmacia & Upjohn Limited Co., Auckland, New Zealand) device for 12 d. Following CIDR removal a single injection of 400 IU equine Chorionic Gondotropin (eCG; Novormon 5000, Syntex S.A., Buenos Aires, Argentina) was given to help initiate follicular

development. Thereafter ewes were exposed to fertile rams at a ratio of 1 ram : 2 ewes. From d 0 (estrus) to d 15 postestrus ewes received L-arginine HCl (Ajinomoto AminoScience, LLC, Raleigh, North Carolina; equivalent to 27 mg of L-arginine/kg of BW) or saline (CON) intravenous once daily. Daily blood samples were obtained from the contralateral jugular vein (n = 5 ewes/group) immediately after treatment (0 h) to assess progesterone (P4) concentrations and at -0.5, 0, 0.5, 1, 2, 4, 8, and 24 h on d 12 to determine circulating concentration of arginine in response to treatment. Blood samples were refrigerated and allowed to coagulate for 4 h, thereafter samples were centrifuged at 2,750 x *g* for 20 min at 4°C. Serum was removed and stored at -20°C for further amino acid and progesterone analyses.

Ultrasonography

Ovarian hemodynamics (d 12; n = 5) and reproductive losses (d 25, 45 and 65; n = 40) were determined with color Doppler and B-mode ultrasonography techniques respectively. Transrectal ultrasonography was performed using an Aloka SSD 3500 (Corometrics Medical Systems, Wallingofrd, CT, USA) fitted with a 7.5 MHz linear-array transrectal transducer. Hemodynamics surrounding the ovary and hilus were analyzed on d 12 using cardiac cycles generated from the ovarian vasculature. Three cardiac cycles were chosen from a single scan and the average was used for the measurement of resistance index (**RI**; RI = peak systolic velocity (**PSV**) – end diastolic velocity (**EDV**) / PSV). On day 25, 45, and 65 of pregnancy the total number of luteal structures and embryos were counted utilizing B-mode ultrasonography as described by Schrick and Inskeep (1993).

Chemical Analyses

Progesterone was analyzed using hormonal chemiluminescence technology (IMMULITE, Siemens, Los Angeles, CA). Serum samples from each collection time were assayed in duplicate form. Amino acids in serum were analyzed by HPLC methods as described by Wu (1997).

Statistical Analyses

Ewe serum and ovarian hemodynamic data were analyzed as a complete random design using the PROC MIXED procedure of Statistical Analysis Systems (SAS Inst. Inc., Cary, NC) with treatment as a fixed effect. Reproductive performance data were analyzed as a complete random design using the PROC GENMOD procedure of SAS with pregnancy rate as a fixed effect. Individual ewes were considered experimental units. Means were separated using LSD and were considered significant when $P \leq 0.05$.

Results

Serum ARG Concentrations in Ewes

On d 12 of pregnancy, serum concentrations of arginine (nmol/mL) were elevated in ARG vs. CON ewes at 0 ($P < 0.001$), 0.5 ($P < 0.001$), 1 ($P < 0.001$), 2 ($P < 0.005$), and 4 h ($P \leq 0.05$), but were similar ($P > 0.05$) at -0.5, 8, and 24 h (Figure 2.1). Serum ARG concentrations returned to baseline values within 5 h after administering arginine.

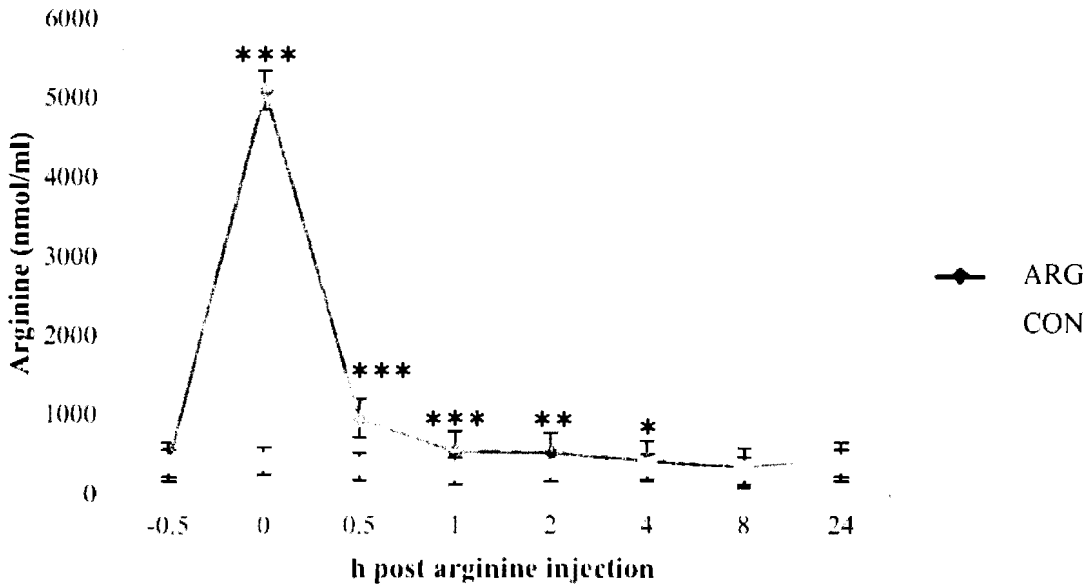

Figure 2.1. Effects of injectable L-arginine on serum arginine concentration (nmol/mL) on d 12 in Rambouillet ewes (***$P = 0.001$; **$P = 0.005$; *$P \leq 0.05$) from d 0 to 15 of the estrous cycle.

Ultrasonography

Vascular pulsatility index in the ovarian hilus was reduced in ARG vs. CON ewes (0.56 ± 0.014 vs. 0.75 ± 0.080; $P \leq 0.05$; data not shown) on d 12 at approximately 4 h after treatment. Arginine treatment had no treatment differences on resistance index in the vasculature surrounding the CL and the ovarian hilus ($P \geq 0.06$; data not shown).

Progesterone

Despite similarities in the number of CL in those ewes that were blood sampled (ARG, 1.8 ± 0.20 and CON, 1.8 ± 0.20 CL/ewe; $P \geq 0.95$), ARG ewes had greater P4 concentrations compared to CON ewes on d 3 (ARG, 1.65 ± 0.23 vs. CON, 1.10 ± 0.07 $P \leq$ 0.05), d 4 (ARG, 2.83 ± 0.40 vs. CON, 1.67 ± 0.05; $P \leq 0.02$), d 5 (ARG, 4.52 ± 0.33 vs.

CON, 1.67 ± 0.05; $P \leq 0.001$) and d 6 (ARG, 4.16 ± 0.21 vs. CON, 3.17 ± 0.30; $P \leq 0.03$), but were similar ($P \geq 0.07$) for the remaining treatment periods (Figure 2.2).

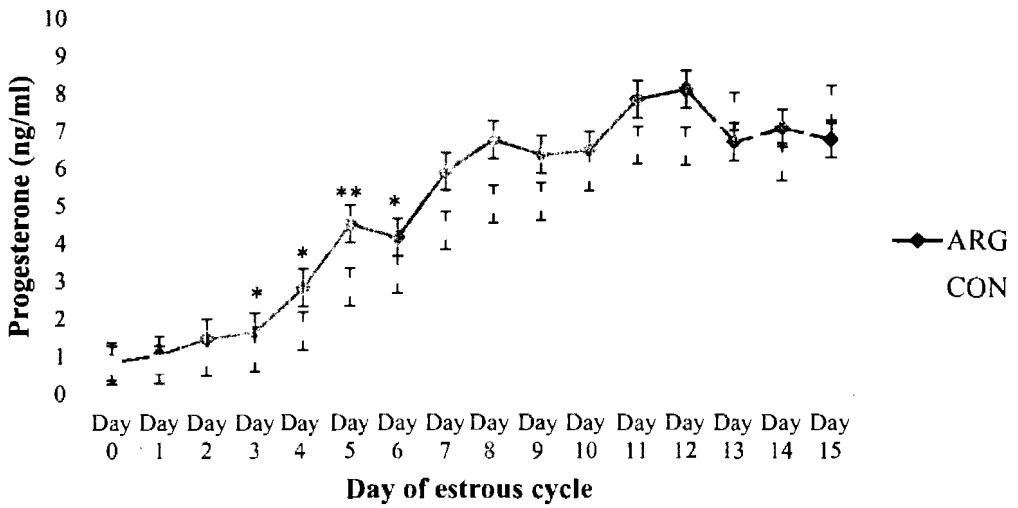

Figure 2.2. Effects of injectable L-arginine on serum progesterone concentration (ng/mL) in Rambouillet ewes (**$P = 0.001$; *$P \leq 0.05$) from d 0 to 15 of the estrous cycle.

Reproductive Loss

Treatment with L-arginine did not influence pregnancy rate (ARG, 55% and CON, 60%; $P \leq 0.70$) or the number of CL among all ewes studied (Table 2.1). However, ARG ewes had more embryos per ewe and fewer CL not represented by embryos compared to CON ewes at d 25 of pregnancy ($P = 0.95$; Table 2.1). As pregnancy progressed to d 45, ARG ewes continued to have more embryos present compared to CON ewes ($P = 0.01$), and the difference in the number of CL not represented by embryos was even greater in CON vs. ARG ewes ($P = 0.03$; Table 2.1). By d 65 of pregnancy, CON ewes maintained number of embryos present whereas the ARG treated ewes lost embryos (Table 2.1). The overall proportion of ewes conceiving, but then exhibiting embryonic loss by d 45 of pregnancy

was reduced in ARG vs. CON ewes (18% vs. 58% respectively; $P \leq 0.04$) and again reduced at day 65 in ARG vs. CON ewes (27% vs. 58%, respectfully; $P \leq 0.12$).

Table 2.1. Effects of injectable L-arginine on number of corpora lutea and embryos

	Arginine[a]			Control[b]			
Item	Mean	SEM	n	Mean	SEM	n	*P*- value[c]
No. Corpora lutea	1.80	0.200	11	1.80	0.120	12	0.95
No. Embryos d 25	1.64	0.152	11	1.25	0.125	12	0.04
No. Embryos d 45	1.64	0.152	11	1.08	0.155	12	0.01
No. Embryos d 65	1.55	0.157	11	1.08	0.155	12	0.03

[a] Arginine, 27 mg/kg BW injectable arginine.
[b] Control, 27 mg/kg BW injectable saline.
[c] *P*-value for F-test for treatment.

Ewe Fertility and Lamb Birth Weight

A total of 17 (n = 20) and 13 (n = 20) lambs were born from ewes exposed to rams on synchronized estrous in CON vs. ARG, respectively. Arginine treatment had no effect on the number of lambs born per ewe exposed when compared with CON ewes ($P = 0.27$; Table 2.2). Ewes treated with ARG gave birth to more lambs per pregnant ewe when compared to control ewes ($P = 0.03$; Table 2.2). However, average lamb birth weights did not differ ($P = 0.71$; Table 2.2).

Table 2.2. Effects of L-arginine on lambing performance

	Arginine[a]		Control[b]		
Item	Mean	SEM[c]	Mean	SEM[d]	*P*- value[e]
Lambs born/exposed ewe[f]	0.85	0.20	0.65	0.26	0.27
Lambs born/pregnant ewe[g]	1.6	0.16	1.1	0.16	0.03
Lamb birth wt, kg	4.9	0.08	4.8	0.15	0.71
Estimated Loss[h]	0.3	0.24	0.9	0.14	0.02

[a] Arginine; 27 mg/kg BW injectable arginine.
[b] Control; 27 mg/kg BW injectable saline.
[c] n=17 lambs.
[d] n=13 lambs.
[e] *P*-value for F-test for treatment.
[f] Arginine and Control; n= 20 respectively.
[g] Arginine; n=11 and Control; n=11.
[h] Estimated Loss = No. CL – no. fetuses.

Discussion

This is the first study we are aware of to demonstrate that prenatal losses can actually be prevented in sheep with supplementation of the amino acid arginine during early pregnancy. Ewes receiving arginine from the time of standing estrus to d 15 of pregnancy lost fewer embryos during early pregnancy and ultimately gave birth to more lambs per pregnant ewe indicating a higher incidence of twinning rate. Mateo et al. (2007) also observed favorable results when gestating sows were supplemented with arginine from d 30 to 114 of gestation. Sows supplemented with arginine achieved a 22% increase in live piglets born when compared to non-supplemented sows (11.4 vs. 9.4, respectively; $P <$ 0.03). Furthermore, pregnant rats supplemented with 1.3% Arginine-HCl throughout the entire length of pregnancy or between d 1 and 7 of gestation had increased embryonic survival and litter size by 30% (Zeng et al., 2008). Collectively, these studies would suggest that reproductive efficiency and ultimately, productivity of female farm animal species, can be enhanced via supplementation with supranutritional levels of the amino acid arginine.

Treatment with arginine may have enhanced the early uterine environment, making it more ideal for embryonic survival. Maternal recognition of pregnancy in the ewe occurs at d 13 following ovulation. During this critical period, the conceptus elongates from a blastocyst to a filamentous form and produces interferon tau, which is responsible for preventing the development of the endometrial luteolytic mechanism (Spencer and Bazer, 2002). The presence of interferon tau allows for maintenance of the CL, which is the primary structure responsible for progesterone production during early pregnancy in sheep.

Progesterone is necessary for the secretion of histotroph by uterine glands during early pregnancy (Spencer et al., 2004), which is important for early embryonic growth and development. Histotroph contains a unique collection of enzymes, growth factors, cytokines, lymphokines, hormones, and transport proteins (Spencer et. al., 2004). All of these factors have been reported to ensure proper embryonic growth and survival in humans, (Burton et. al., 2002), primates (Bazer et. al., 1979; Roberts and Bazer, 1988; Carson et. al., 2000; and Gray et. al., 2001a) and sheep (Lawson et. al., 1983; Flechon et. al.; 1986, Gray et. al., 2001b; and Gray et. al., 2002). In the present study, ewes treated with arginine had greater concentrations of progesterone relative to control ewes. Several studies have reported that low levels of progesterone can lead to a greater incidence of embryonic loss in sheep, and ultimately decreased ewe productivity (Casida and Warwick, 1945; Dixon et. al., 2007). Supplementation with exogenous progesterone on d 1 to 3 of pregnancy enhanced litter size and fetal growth in prolific ewes (Kleeman et al. 1994).

Increases in ovarian blood flow and/or vascular perfusion of the CL probably resulted in the higher concentrations of progesterone in ewes treated with arginine. In cyclic cattle, blood flow to the ovary was highest when progesterone concentrations were high and decreased during luteal regression (Wise et. al., 1982). Recent research has indicated that treatment with arginine orally not only significantly improves CL blood flow and luteal function in humans, but also led to a positive correlation between serum progesterone concentration and CL blood flow (Takasaki, 2009). In the present study, vascular resistance index in the ovarian artery was reduced on d 12 at approximately 4 h after treatment in ARG vs. CON ewes, while serum progesterone concentration was greater in ARG relative to CON ewes. These data indicate that supplementation of injectable

arginine may enhance progesterone concentration and ovarian hemodynamics, both of which play a key role in survivability of the embryo.

Arginine is important for many biological functions, including the synthesis of nitric oxide (NO), a chemical important for dilating blood vessels and increasing tissue blood flow. Nitric oxide plays an important role in steroidogenesis, ovulation, embryo implantation, and the maintenance of pregnancy (Gouge et. al., 1998; Manser et. al., 2004). Nitric oxide is synthesized from the conversion of arginine to citrulline by nitric oxide synthase. Through activation of the cyclic guanosine monophosphate pathway, nitric oxide causes relaxation of vascular smooth muscle (Moncada and Higgs,1993). Inhibition of nitric oxide synthase during pregnancy led to a higher incidence for embryonic mortality in the rat (Chwalisz et. al., 1999). Therefore, it is reasonable to hypothesize that injectable arginine administered to pregnant ewes may decrease embryonic mortality by increasing the availability of nitric oxide.

In the present study, as pregnancy progressed past the treatment period from d 25 to 45, the injectable arginine induced changes in embryo development and maturation. Arginine plays an important role as a precursor in the synthesis of polyamines. Polyamines and nitric oxide alike are responsible for cellular proliferation and differentiation (Wu and Morris, 1998). Arginine's primary catabolic enzyme, argininase, serves as a regulator of polyamines by consuming arginine to produce ornithine. Ornithine serves as a substrate for ornithine decarboxylase (the enzyme responsible for the synthesis of the polyamines), putrescine, spermidine and spermine. It is reasonable to hypothesize that supplementation of injectable arginine may increase the synthesis of polyamines, which may aid in cellular proliferation, expression, and differentiation for embryonic growth, maturation and survival.

In summary, early reproductive losses can be prevented, at least in part, by injectable treatment with arginine. The mechanisms responsible for the positive results observed in the current study could be two-fold: 1) decreased ovarian vascular resistance resulting in increased concentrations of progesterone may result in a more ideal environment for early embryonic survival in the pregnant ewe; and 2) elevated concentrations of arginine may have directly enhanced embryonic cellular proliferation, expression and differentiation to ensure proper embryonic growth and survival.

Implications

Although a more suitable delivery method must be developed, the current results imply that embryonic survival in sheep can be enhanced when L-arginine is supplemented during early pregnancy.

CHAPTER 3. IMPACTS OF L-ARGININE ON OVARIAN FUNCTION AND REPRODUCTIVE PERFORMANCE AT THE TIME OF MATERNAL RECOGNITION OF PREGNANCY IN EWES

Abstract

Objectives were to determine if arginine supplementation surrounding the time of maternal recognition of pregnancy enhances ovarian function and minimizes early reproductive losses. Ewes received L-arginine HCL (equivalent to 27 mg of L-arginine/kg of BW; ARG; n= 47) or saline (CON; n = 47) i.v. once daily from d 9 to d 14 following estrus (d 0). Daily blood samples were obtained from a subset of 10 ewes/group to assess progesterone (P4) concentrations and at -0.5, 0, 0.5, 1, 2, 4, 6, and 8 h following treatment on d 10 to determine serum amino acid concentrations. Reproductive losses were determined with B-mode ultrasonography on d 25, 45, and 65 of gestation. On d 10, serum concentrations of arginine (nmol/mL) were elevated in ARG vs. CON ewes at 0, 0.5, 1, 2, and 4 h ($P < 0.001$), but were similar ($P \geq 0.70$) at -0.5, 6, and 8 h. Despite similarities in the number of corpora lutea (CL) per ewe (ARG, 1.69 ± 0.12 and CON, 1.67 ± 0.16; $P > 0.05$), serum progesterone concentration (ng/mL) was greater in this subset of CON compared with ARG ewes on d 9 ($P < 0.02$) and 10 ($P < 0.005$), but similar for the remaining treatment period ($P \geq 0.06$). On d 12, there were no differences in pulsatility index and resistance index in those ewes treated with arginine in the ovarian hilus or the CL ($P > 0.05$). Treatment with arginine increased overall pregnancy rate at d 25 (ARG, 55% and CON, 30%). Pregnant ewes contained similarities in CL number per ewe (ARG, 1.69 ± 0.12 vs. CON, 1.67 ± 0.13; $P > 0.05$) and embryo number (ARG, 1.62 ± 0.12 vs. CON, 1.53 ± 0.13; $P > 0.05$) also at d 25 of gestation. As pregnancy progressed to d 45, similar ($P > 0.05$) number of embryos per ewe were observed in pregnant ARG ewes (1.45 ± 0.14) vs.

pregnant CON (1.50 ± 0.15) with overall pregnancy rate remaining greater ($P \leq 0.02$) in ARG (47%) compared with CON (26%). Number of lambs born per ewe and lamb birth weights were similar ($P > 0.05$) between ARG vs. CON. In summary, treatment with arginine surrounding the time of maternal recognition of pregnancy may have prevented pregnancy losses, but did not enhance ovarian hemodynamics or progesterone concentration.

Key words: arginine, ovarian hemodynamics, sheep

Introduction

Reproductive performance is the largest determinant of income in the livestock enterprise. In sheep, embryonic and fetal deaths during pregnancy account for 25 to 50% of the total number of fertilized ova (Knights et al., 2003; Dixon et al., 2007). Most embryonic loss has been reported to occur before d 18 (Hulet et al., 1956; Moore et al., 1960; Quinlivan, 1966). Only a small percentage of embryos are inherently non-viable in the ewe (Wilmut et al., 1986), which would suggest that the majority of early embryonic losses can be prevented.

The amino acid L-arginine is important for the synthesis of polyamines and nitric oxide, both of which are essential for proper development of the embryo and placenta. In Chapter 2, we observed increased ovarian blood flow, serum progesterone, and fetal number, despite similar ovulation rate. Furthermore, supplemental arginine decreased embryonic death and increased litter size in rats (Zeng et al., 2008), and increased the number of piglets born alive in gilts (Mateo et al., 2007).

Communication between the embryo and the maternal system must be established following conception to ensure normal development and differentiation of the embryo.

Maternal recognition of pregnancy in sheep occurs around d 13 following ovulation. During this critical period, the conceptus elongates from a blastocyst to a filamentous form, which produces interferon tau that is responsible for preventing the development of the endometrial luteolytic mechanism (Spencer and Bazer, 2002). The presence of interferon tau allows for maintenance of the CL, which is the primary structure responsible for progesterone production during early pregnancy in sheep.

The objective of this study was to determine the effects of arginine supplementation surrounding the time of maternal recognition of pregnancy on ovarian hemodynamics, early reproductive loss and lamb birth weight in Rambouillet ewes in a larger-scale study.

Materials and Methods

All animal procedures were approved by the North Dakota State University Institutional Animal Care and Use Committee.

Animals and Experimental Design

Rambouillet ewes of a similar BW (60 ± 0.70 kg) and age (6.1 ± 0.08 yr) at the North Dakota State University Hettinger Research Extension Center (46°N) were randomly assigned to one of two groups: control (**CON**; n = 47) and L-arginine (**ARG**; n = 47). All ewes received a controlled internal drug release (CIDR-G; 300 mg P4, Pharmacia & Upjohn Limited Co., Auckland, New Zealand) device for 12 d. Following CIDR removal a single injection of PG-600 (PG-600; Intervet, Millsboro, DE) was given to help initiate follicular development and ensure ovulation. Thereafter ewes were exposed to fertile rams. From d 9 to d 14 postestrus ewes received L-arginine HCl (Ajinomoto AminoScience, LLC, Raleigh, North Carolina; equivalent to 27 mg of L-arginine/kg of BW) or saline (CON) intravenously

once daily. Daily blood samples were obtained from the contralateral jugular vein (n = 10 ewes/subgroup) immediately after treatment (0 h) to assess progesterone (P4) concentrations and at -0.5, 0, 0.5, 1, 2, 4, 6, and 8 h on d 10 to determine circulating concentration of arginine in response to treatment. Blood samples were refrigerated and allowed to coagulate for 2 h, thereafter samples were centrifuged at 2,750 x *g* for 20 min at 4°C. Serum was removed and stored at -20°C for further amino acid and progesterone analyses.

Ultrasonography

Ovarian hemodynamics (d 12) was determined with color Doppler ultrasonography (n = 10 ewes/subgroup) and reproductive losses (d 25, 45, and 65; n = 94) were determined using B-mode ultrasonography techniques. Transrectal ultrasonography was performed using an Aloka SSD 3500 (Corometrics Medical Systems, Wallingford, CT, USA) fitted with a 7.5 MHz linear-array transrectal transducer. Hemodynamics surrounding the ovary and hilus were analyzed on d 12 using cardiac cycles generated from the ovarian vasculature. Three cardiac cycles were chosen from a single scan and the average was used for the measurement of resistance index [(Peak systolic velocity – End diastolic velocity) / Peak systolic velocity], pulsatility index [(Peak systolic velocity – End diastolic velocity) / Time-averaged maximum velocity], peak systolic velocity, end diastolic velocity, mean velocity, and flow time. On day 25, 45, and 65 of pregnancy the total number of luteal structures and embryos were counted utilizing B-mode ultrasonography as described by Schrick and Inskeep (1993). Carotid blood flow was measured with Doppler ultrasonography on d 12 utilizing similar measurements as described for the ovarian hemodynamics.

Chemical Analyses

Serum was analyzed for progesterone concentration using a solid-phase, competitive, chemiluminescent enzyme immunoassay (Immulite 1000, Diagnostics Products Corp. Diagnostic Products Corp., Los Angeles, CA). All samples were run on a single assay in duplicate form with an intraassay CV of 9.1%. Amino acid concentration (20 AA, citrulline, and ornithine) was determined using the HPLC procedures of Wu et al. (1997). Blood samples were refrigerated and allowed to coagulate for 2 h; thereafter, samples were centrifuged at 2,750 x *g* for 20 min at 4°C. Serum was removed and stored at -20°C for further amino acid and progesterone analyses.

Statistical Analyses

Ewe serum and ovarian hemodynamic data were analyzed as a complete random design using the PROC MIXED procedure of Statistical Analysis Systems (SAS Inst. Inc., Cary, NC) with treatment as the fixed effect. Reproductive performance data were analyzed as a complete random design using the PROC GENMOD procedure of SAS with pregnancy rate as a fixed effect. Individual ewe was considered an experimental unit. Means were separated using LSD and were considered significant when $P \leq 0.05$.

Results

Serum Amino Acid Concentrations

On d 10 of pregnancy, serum concentrations of arginine (nmol/mL) were elevated in a subset of ewes (n=10 ewes/treatment group) ewes injected with arginine vs. CON ewes at 0 (2238 ± 508 vs. 313 ± 11 respectively; $P < 0.001$), 0.5 (706 ± 82 vs. 302 ± 11 respectively; $P < 0.001$), 1 (564 ± 70 vs. 322 ± 17 respectively; $P < 0.001$), 2 (464 ± 26 vs.

289 ± 12 respectively; $P < 0.001$), and 4 h (400 ± 17 vs. 287 ± 12 respectively; $P < 0.001$), but were similar ($P \geq 0.70$) at -0.5, 6, and 8 h (Figure 3.1). Serum ARG concentrations returned to baseline values within 5 h after administering arginine. Arginine treatment had no effect on serum total amino acid ($P \geq 0.09$) or total essential amino acid concentration ($P \geq 0.12$). Metabolites of arginine (ornithine and citrulline) were measured. On d 10, ornithine levels were elevated in ARG vs. CON ewes at 0.5 (148 ± 17 vs. 87 ± 6 respectively; $P < 0.03$), 1 (157 ± 20 vs. 82 ± 6 respectively; $P < 0.001$), 2 (168 ± 11 vs. 80 ± 5 respectively; $P < 0.001$), 4 (121 ± 7 vs. 72 ± 3 respectively; $P < 0.001$), 6 h (97 ± 6 vs. 69 ± 4 respectively; $P < 0.0011$), and 8 h (79 ± 5 vs. 62 ± 5 respectively; $P < 0.02$). However there was no effect on circulating serum citrulline concentration ($P \geq 0.09$; data not shown).

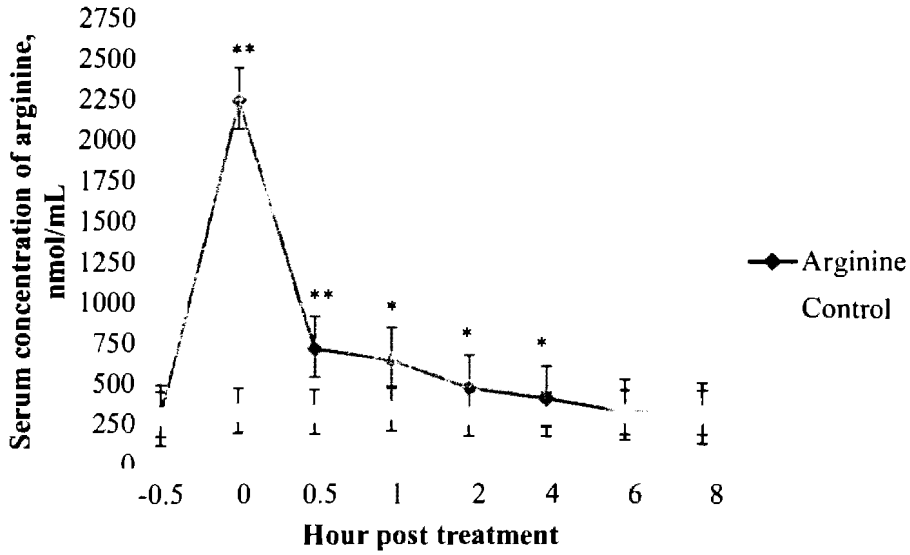

Figure 3.1. Effects of injectable L-arginine on serum arginine concentration (nmol/mL) on Day 10 in Rambouillet ewes (**$P = 0.001$; *$P < 0.001$) from d 9 to 14 of the estrous cycle.

Serum Progesterone Concentration and Ultrasonography

Carotid artery and ovarian hemodynamics were measured on d 12 with Doppler ultrasonography on a subset of ewes (n=10 ewes/treatment group; Table 3.1). There were no

differences ($P = 0.49$) in pulsatility index in those ewes treated with arginine vs. control in the ovarian hilus. When measuring the vasculature surrounding the CL, there was no effect of arginine treatment compared with control ($P = 0.51$) on puslatility index. Similar to the pulsatility index, resistance index was also not influenced ($P \geq 0.46$) with arginine treatment in the ovarian hilus or in the CL. Unlike the ovary, arginine treatment had a slight effect on pulsatility index ($P = 0.08$) and resistance index ($P = 0.05$) in the carotid artery (Table 3.1).

Table 3.1. Effects of L-arginine on ovarian and carotid hemodynamics in Rambouillet ewes injected intravenously from d 0 to 15 of the estrous cycle

	Control[a]		Arginine[b]		
Item	Mean	SEM[c]	Mean	SEM[c]	*P*- value[d]
Hilus					
Peak systolic velocity, cm/s	30.6	1.7	28.9	2.2	0.57
Pulsatility index[e]	0.962	0.08	0.887	0.06	0.49
Resistance index[f]	0.590	0.03	0.558	0.03	0.46
Mean velocity, cm/s	19.16	1.2	18.50	1.1	0.66
Flow time, ms	627	38	605	60	0.75
Corpus Luteum					
Peak systolic velocity, cm/s	28.7	2.0	29.7	1.4	0.65
Pulsatility index[e]	0.322	0.02	0.339	0.02	0.51
Resistance index[f]	0.270	0.01	0.281	0.02	0.58
Mean velocity, cm/s	24.2	1.7	24.9	1.3	0.70
Flow time, ms	646	35	661	62	0.83

[a] Control; 27 mg/kg BW injectable saline.
[b] Arginine; 27 mg/kg BW injectable arginine.
[c] n = 10.
[d] *P*-value for F-test for treatment.
[e] Pulsatility index = (Peak systolic velocity – End diastolic velocity)/Time-averaged maximum velocity.
[f] Resistance index = (Peak systolic velocity – End diastolic velocity)/Peak systolic velocity.

Despite similarities in CL in the subset of ewes blood sampled (ARG; 1.69 ± 0.12 and CON; 1.67 ± 0.16 CL/ewe; $P = 0.42$; Table 3.2), CON ewes had greater serum progesterone concentration (ng/mL) compared with ARG on d 9 (6.11 ± 0.27 vs. 5.30 ±

0.15 respectively; $P < 0.02$) and 10 (6.50 ± 0.40 vs. 5.06 ± 0.21 respectively; $P < 0.005$) but similar for the remaining treatment period ($P \geq 0.06$; data not shown) .

Reproductive Loss

Treatment with arginine influenced pregnancy rates (ARG, 55%, n=47; and CON, 30%, n=47; $P \leq 0.02$) as determined by ultrasound on d 25 despite treatment similarities in number of CL and number of embryos/pregnant ewe (Table 3.2). Similar to day 25, on d 45 and d 65 number of embryos in ARG and CON ewes were similar, with pregnancy rates remaining greater in ARG (47% vs. 26%, $P \leq 0.03$ for d 45; 47% vs. 23%, $P \leq 0.02$ for d 65).

Table 3.2. Effects of L-arginine on number of corpora lutea and embryos/ewe

	Arginine[a]			Control[b]			
Item/ewe	Mean	SEM	n[c]	Mean	SEM	n[c]	*P*- value[d]
No. Corpora lutea	1.69	0.12	26	1.67	0.13	15	0.42
No. Embryos d 25	1.62	0.12	26	1.53	0.16	15	0.33
No. Embryos d 45	1.45	0.14	22	1.50	0.15	12	0.41
No. Embryos d 65	1.50	0.14	22	1.45	0.16	11	0.42

[a] Arginine, 27 mg/kg BW injectable arginine.
[b] Control, 27 mg/kg BW injectable saline.
[c] Decreased from d 25 to d 65 due to pregnancy loss.
[d] *P*-value for F-test for treatment.

Ewe Fertility and Lamb Birth Weight

A total of 32 and 16 lambs were born from ewes exposed to rams on synchronized estrous in ARG (n=47) vs. CON (n=47) ewes, respectively. Arginine treatment had no effect on the number of lambs born per ewe exposed when compared with CON ewes (P = 0.15; Table 3.3). Ewes treated with ARG gave birth to a similar number of lambs/ewe when compared with CON (P = 0.58; Table 3.3). Average lamb birth weights did not differ (P = 0.24) between treatment groups (Table 3.3).

Table 3.3. Effects of L-arginine on lambing performance

	Arginine[a]		Control[b]		
Item	Mean	SEM[c]	Mean	SEM[d]	*P*- value[e]
Lambs born/exposed ewe[f]	0.68	0.23	0.34	0.24	0.15
Lambs born/pregnant ewe[g]	1.78	0.17	1.60	0.27	0.58
Lamb birth wt, kg	3.8	0.20	4.0	0.26	0.24
Estimated Loss[h]	0.2	0.10	0.2	0.13	0.89

[a] Arginine; 27 mg/kg BW injectable arginine.
[b] Control; 27 mg/kg BW injectable saline.
[c] n=32 lambs.
[d] n=16 lambs.
[e] *P*-value for F-test for treatment.
[f] Arginine and Control; n=47 respectively.
[g] Arginine; n=18 and Control; n=10.
[h] Estimated Loss = No. CL – no. fetuses.

Discussion

In the present study, pregnancy rate was greater in those ewes treated with injectable arginine when compared to control ewes. It is important to note that this study was conducted out of season, and only ewes bred by a fertile ram to the synchronized estrus were used for this study and overall pregnancy rates reported are reflected and typical for this flock as compared to pregnancy rates in a non-synchronized flock during the breeding season. A total of 32 and 16 lambs were born from ewes exposed to rams on synchronized estrous in ARG vs. CON ewes, respectively. Arginine treatment had no effect on the number of lambs born per ewe exposed or the number of lambs per pregnant ewe when compared with CON.

In the current study, ewes treated with injectable L-arginine surrounding the time of maternal recognition of pregnancy (d 9 to 14) enhanced pregnancy rates, increased circulating serum arginine and ornithine concentration, and elevated vascular resistance in peripheral blood flow. Arginine is important for many biological functions, including the synthesis of nitric oxide (Gouge et. al., 1998; Manser et. al., 2004). Nitric oxide is

synthesized from the oxidation of arginine to citrulline by nitric oxide synthase. Released by endothelial cells, nitric oxide, activates guanylyl cyclase in smooth muscle cells thereby causing smooth muscle relaxation. In the present study ewes treated with arginine had greater circulating serum arginine concentrations at 0, 1, 2, and 4 h proceeding treatment. These findings were similar to Chapter 2 which reported arginine concentrations reached basal levels around 6 h following treatment. Similarly, Wu et al. (2005) reported a return to baseline by 5 h following injection.

Interferon-τ (IFN-τ) is produced by mononuclear cells of the embryonic trophectoderm and acts locally on the endometrial cells of the uterus to block the production of oxytocin receptors to prevent the pattern of prostaglandinF2α (PGF2α) secretion. In pregnant and nonpregnant ewes, PGF2α is secreted from the uterus beginning on d 12 to 13. In those ewes that are not pregnant, luteolysis is initiated at this time and secretion of progesterone begins to decline. Ewes treated with arginine in the current study had greater pregnancy rates throughout the entire experiment when compared with control ewes. It may be reasonable to hypothesize that treatment with arginine at or slightly before the time of maternal recognition of pregnancy in the ewe may have enhanced the survival of the embryo during early embryogenesis through its role in polyamine and nitric oxide synthesis. Nitric oxide and polyamines may have directly enhanced embryonic cellular proliferation and differentiation to ensure proper embryonic survival. The rescuing of early embryos may have resulted in a strong signal for the synthesis of IFN-τ ultimately leading to a more suitable uterine environment for embryonic maintenance and survival.

Polyamines regulate gene expression and are important for cellular proliferation and differentiation. Specifically in reproduction, they are critically important for early

embryogenesis, embryonic growth, and placental growth and angiogenesis (Kwon et al., 2003). Arginine is an important precursor for polyamine synthesis. Arginine is catabolized into ornithine via argininase. Ornithine decarboxylase is a rate-controlling enzyme for polyamine synthesis. It converts ornithine into putrescine, which is converted to spermidine and spermine (Flynn et al., 2002). In the present study, ewes treated with injectable L-arginine had greater ornithine concentrations at 0.5, 1, 2, 4, 6, and 8 h following treatment. It is reasonable to hypothesize that elevated levels of ornithine increased polyamine synthesis allowing for an enhanced uterine environment for embryonic survival in arginine treated ewes.

Progesterone is important for histotropic nutrition of the early embryo and suppression of the luteolytic mechanism (Lambing et al., 1989). In the present study, ewes treated with arginine had lower concentrations of progesterone relative to control ewes on d 9 and 10 of gestation, but were similar for the remaining treatment period. In our previous study (Chapter 2), vascular resistance index in the ovarian artery was reduced on d 12 at approximately 4 h after treatment in ARG vs. CON ewes, while serum progesterone concentration was higher in ARG relative to CON ewes. Ewes in that study were injected with arginine from d 0 to 15, indicating that arginine may have not only improved CL blood flow and luteal function, but also led to an increase in serum progesterone concentrations. In the present study, the lower levels of progesterone in arginine treated ewes may be due to an increase in metabolic clearance rate of steroids within the liver. Nitric oxide is produced when the enzyme nitric oxide synthase catalyzes the oxidation of L-arginine to L-citrulline (Gouge et al., 1998). Nitric oxide has been demonstrated to stimulate vasodilation of blood vessels increasing blood flow to various organs throughout the body. Given that the liver is

the major site of progesterone metabolism (Sangsritavong et al., 2002), it may be logical that an increase in liver blood flow may elevate liver oxygen consumption leading to an increase in metabolic clearance rate of circulating progesterone. It may also be reasonable to hypothesize that ewes treated with arginine in the present study may have had an increase in peripheral blood flow, resulting in an increase in blood flow to organs such as the liver. The increase in blood flow may have elevated steroid metabolism, which may have ultimately resulted in the observed lower circulating concentration of progesterone noticed in the current study. Several studies have shown that low levels of progesterone can lead to a greater incidence of embryonic loss in sheep, and ultimately result in decreased ewe productivity (Casida and Warwick, 1945; Dixon et. al., 2007). However, this finding was not observed in the current study. In fact, ARG treated ewes actually had greater pregnancy rates resulting in a greater amount of lambs born per ewe exposed when compared to CON ewes.

Implications

In summary, treatment with arginine surrounding the time of maternal recognition of pregnancy may have prevented pregnancy loss in some ewes. Despite similarities in total CL and embryo numbers, overall pregnancy rate was increased in ewes treated with arginine. The enhanced pregnancy rate may have been due to arginine supplementation creating a more ideal uterine environment for the maintenance of embryos. During the early stages of embryogenesis, the supplemental arginine could have rescued weaker embryos entering the early stages of regression through its role in nitric oxide and polyamine synthesis. Nitric oxide and polyamines may have directly enhanced embryonic cellular proliferation and differentiation to ensure and promote proper embryonic survival.

CHAPTER 4. EFFECTS OF RUMEN-PROTECTED ARGININE SUPPLEMENTATION ON EWE SERUM AMINO ACID CONCENTRATION, CIRCULATING PROGESTERONE, AND OVARIAN BLOOD FLOW

Abstract

Objectives were to determine if rumen-protected arginine supplemented to ewes on d 8 to 13 of the estrous cycle affected serum amino acid concentration, ovarian blood flow, and circulating progesterone. Nineteen multiparous Dorset ewes (63.8 ± 1.1 kg initial BW) were individually housed and randomly allocated to 1 of 4 rumen-protected arginine treatments: 0 (CON; n = 5), 90 (90 ARG; n = 4), 180 (180 ARG; n = 5), or 360 mg/kg BW supplemental arginine (360 ARG; n = 5). Following estrous synchronization, ewes were individually fed rumen-protected arginine blended into 150 g ground corn, which was immediately followed with 650 g of a pelleted diet (2.40 Mcal ME/kg and 12.9% CP; DM basis) on d 8 to 12 of the estrous cycle. Jugular blood samples were taken for amino acid and progesterone analysis. On d 12, color Doppler ultrasonography was used to determine ovarian hemodynamics. Ewes fed 360 ARG generally had greater serum arginine concentration than CON, 90 ARG, and 180 ARG on d 11 (175.5 vs. 153.2, 132.3, and 145.4 ± 8.6 nmol/mL, respectively; $P \leq 0.07$) and d 12 (166.4 vs. 142.7, 121.7, and 128.2 ± 7.4 nmol/mL, respectively; $P \leq 0.03$). On d 11, arginine as a percent of total amino acid concentration was greater in 360 ARG compared with CON and 90 ARG (7.16 vs. 6.19, 5.70 ± 0.34 nmol/mL, respectively; $P \leq 0.05$). Total essential amino acid concentration was elevated in 360 ARG compared with 90 ARG and 180 ARG ($P \leq 0.03$) on d 12. Arginine supplementation increased peak systolic velocity in the corpus luteum (CL) for 360 ARG and 90 ARG compared to CON (30.53 and 32.59 vs. 22.63 ± 2.48 cm/s, respectively; $P \leq 0.04$). Flow time (milliseconds) in the ovarian hilus was increased and CL was generally

increased in 360 ARG compared to all other treatments ($P \leq 0.04$ and $P \leq 0.09$, respectively). Supplemental rumen-protected arginine had no effect on serum concentration of progesterone ($P > 0.50$). Results indicate that rumen-protected arginine supplemented to ewes at the rate of 360 mg/kg BW may increase circulating serum arginine concentration, in addition to increasing ovarian blood flow.

Key words: arginine, ovarian hemodynamics, sheep

Introduction

As a precursor for nitric oxide, polyamines, creatine, proteins, and glutamate, the amino acid arginine plays a vital role in metabolism and reproduction (Wu and Morris, 1998). Nitric oxide is the endothelium-derived relaxing factor essential for increasing systemic vasodilation (Ignarro et al., 2001; Martin et al., 2001).

Supplemental arginine has been reported to increase the number of live piglets born per sow (Mateo et al., 2007). Furthermore, pregnant rats supplemented with arginine throughout gestation exhibited an increase in embryonic survival and litter size (Zeng et al., 2008). Recently, we observed in a separate study (see Chapter 2) increased ovarian blood flow, serum progesterone, and fetal number, despite similarities in ovulation rate, in ewes injected with L-arginine during the first 15 d post-breeding. Collectively, these studies suggest that reproductive efficiency can be enhanced via supplementation of supranutritional levels of arginine.

In previous studies, arginine supplementation has been investigated within monogastric species due to the catabolic fate of arginine within the rumen. To protect arginine from ruminal degradation, the amino acid is encapsulated in a ruminal-protected product to partially escape the rumen only to be catabolized in the small intestine for absorption. Due

to the lack of available rumen-protected arginine, research in ruminants has been limited. We hypothesize that feeding rumen-protected arginine will increase circulating levels of arginine, in addition to increasing systemic blood flow through its role in nitric oxide synthesis. Our specific objectives were to determine the effects of feeding rumen-protected arginine on serum amino acids, ovarian hemodynamics, and serum progesterone.

Materials and Methods

All animal procedures were approved by the North Dakota State University Institutional Animal Care and Use Committee.

Animals and Experimental Design

Nineteen mature, multiparous Dorset ewes (63.8 ± 1.1 kg initial BW) were randomly allocated to 1 of 4 rumen-protected arginine treatments: 0 (CON; n = 5), 90 (90 ARG; n = 4), 180 (180 ARG; n = 5), or 360 mg/kg BW supplemental arginine (360 ARG; n = 5). Rumen-protected arginine (ARG 60; Eurhema Srl., Carviago, Itlay) was a 60% Arginine HCL product, calculated to have a minimum intestinal availability of 50%. Calculation of these doses used this assumption and that 40% of arginine reaching the small intestine is catabolized in this tissue (Wu and Morris, 1998), resulting in 30% of the rumen-protected arginine consumed reaching circulation. The 90 ARG treatment was estimated to deliver 27 mg arginine/kg BW to circulation, which was the injected dose we used in previous studies (see Chapters 2 and 3)

For estrous synchronization, all ewes received a vaginally inserted controlled internal drug release (CIDR-G®; 300 mg progesterone; Pharmacia & Upjohn Limited Co., Auckland, New Zealand) device for 12 d. Following CIDR removal, a single injection of 400 IU equine chorionic gondotropin (eCG®; Novormon 5000, Syntex S.A., Buenos Aires,

Argentina) was given to initiate follicular development and ensure ovulation. After synchronization, ewes were moved into the Animal Nutrition and Physiology Center at NDSU (approximately 46.9° latitude and 96.8° longitude) where they were individually housed in 0.91 x 1.2-m pens. The facility was temperature controlled (12 to 21°C) and ventilated with lighting automatically timed to mimic daylight patterns.

Diet

Ewes were allowed a 7-d acclimation period to the facility and diet before beginning rumen-protected arginine supplementation on d 8 of the estrous cycle (d 0 = estrus). For 5 d, ewes were fed rumen-protected arginine blended into 150 g of ground corn, which was immediately followed with 650 g of a pelleted diet (44.9% beet pulp, 25.0% alfalfa meal, 19.7% soyhulls, 6.7% corn, 3.7% soybean meal; pelleted diet: 2.23 Mcal ME/kg and 13.6% CP, DM basis; total diet: 2.40 Mcal ME/kg and 12.9% CP, DM basis).

Ovarian Hemodynamics

On d 12 of the estrous cycle, color Doppler ultrasonography (Aloka SSD 3500, Tokyo, Japan) was used to determine ovarian hilus and luteal resistance index [(Peak systolic velocity – End diastolic velocity) / Peak systolic velocity], pulsatility index [(Peak systolic velocity – End diastolic velocity) / Time-averaged maximum velocity], peak systolic velocity, end diastolic velocity, mean velocity, and flow time.

Serum Analyses

Blood samples were collected via jugular venipuncture every 12 h from d 8 to 13 of the estrous cycle. Serum was analyzed for progesterone concentration using a solid-phase, competitive, chemiluminescent enzyme immunoassay (Immulite 1000, Diagnostics Products Corp. Diagnostic Products Corp., Los Angeles, CA). All samples were analyzed

as a single assay in duplicate form with the intraassay CV 9.1%. Amino acid concentration (20 AA, citrulline, and ornithine) was determined using the HPLC procedures of Wu et al. (1997). Blood samples were refrigerated and allowed to coagulate for 2 h; thereafter, samples were centrifuged at 2,750 x *g* for 20 min at 4°C. Serum was removed and stored at -20°C for further amino acid and progesterone analyses.

Statistical Analyses

Ewe serum and ovarian hemodynamic data were analyzed as a randomized complete block design using the MIXED procedure of SAS (SAS Institute, Inc., Cary, NC) with arginine treatment as the fixed effect. Means were separated using LSD and were considered significant when $P \leq 0.10$.

Results

Serum Arginine Concentration

Ewes fed 360 ARG had generally greater serum arginine concentration than CON, 90 ARG, and 180 ARG on d 11 (175.5 vs. 153.2, 132.3, and 145.4 ± 8.6 nmol/mL, respectively; $P \leq 0.07$; Figure 4.1) and greater serum arginine concentration on d 12 (166.4 vs. 142.7, 121.7, and 128.2 ± 7.4 nmol/mL, respectively; $P \leq 0.03$).

On d 11, arginine as a percent of total amino acid concentration was greater in 360 ARG compared with CON and 90 ARG (7.16 vs. 6.19, 5.70 ± 0.34 nmol/mL, respectively; $P \leq 0.05$; Table 4.1). Total essential amino acid concentration was elevated in 360 ARG compared with 90 ARG and 180 ARG ($P \leq 0.03$) on d 12. Supplemental rumen-protected arginine had no effect on citrulline or ornthine levels throughout the treatment period (data not reported; $P > 0.15$).

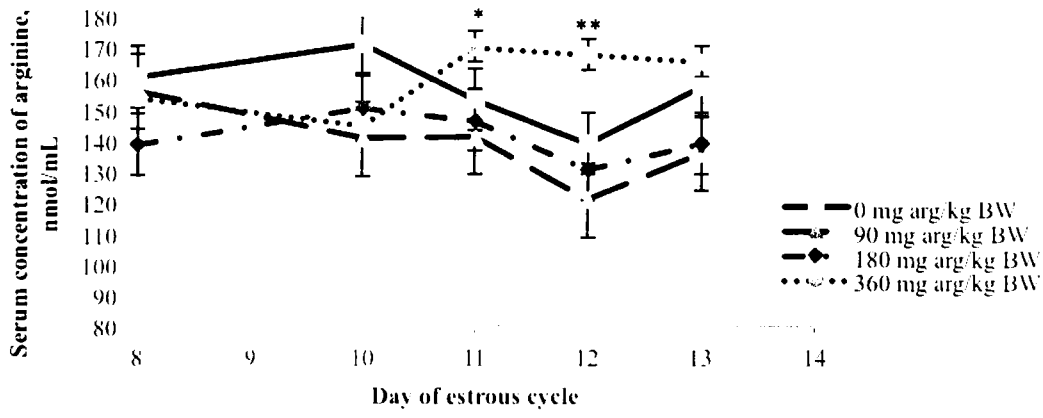

Figure 4.1. Effects of feeding different levels of rumen-protected arginine on serum arginine concentration (nmol/mL) in Dorset ewes (*$P = 0.01$; **$P = 0.002$) from d 8 to 12 of the estrous cycle.

Ovarian Hemodynamics

Arginine supplementation increased peak systolic velocity in the CL for 360 ARG and 90 ARG compared to CON (30.53 and 32.59 vs. 22.63 ± 2.48 cm/s, respectively; $P \leq 0.04$; Table 4.2). Flow time (milliseconds) in the ovarian hilus and corpus luteum was increased in 360 ARG compared to all other treatments ($P \leq 0.04$ and $P \leq 0.09$, respectively). Pulsatility index and resistance index did not differ among treatments for the CL and ovarian hilus ($P \geq 0.18$).

Circulating Serum Progesterone

Supplemental rumen-protected arginine had no effect on serum concentration of progesterone (CON, 6.17 ± 0.24; 90 ARG 6.14 ± 0.31; 180 ARG 5.93 ± 0.39 and 360 ARG 5.41 ± 0.44; $P \geq 0.50$).

Table 4.1. Effects of rumen-protected arginine on serum amino acid concentration

	Treatment[1]					
Item	0	90	180	360	SEM[2]	*P*-value[3]
Day 8[4]						
Total essential AA, nmol/mL	1,030	915	938	949	82	0.78
Total AA, nmol/mL	2,447	2,196	2,264	2,360	163	0.72
Arginine, % of total essential AA	15.5	15.1	15.0	16.4	1.3	0.86
Arginine, % of total AA	6.43	6.32	6.08	6.55	0.49	0.92
Day 10						
Total essential AA, nmol/mL	1,085	920	973	1,081	79	0.39
Total AA, nmol/mL	2,600	2,323	2,422	2,599	187	0.66
Arginine, % of total essential AA	15.6	14.4	15.6	15.0	1.1	0.83
Arginine, % of total AA	6.47	5.70	6.13	6.27	0.39	0.54
Day 11						
Total essential AA, nmol/mL	987	932	895	1,055	63	0.34
Total AA, nmol/mL	2,502	2,345	2,260	2,464	125	0.51
Arginine, % of total essential AA	15.7	14.3	16.4	16.8	0.9	0.29
Arginine, % of total AA	6.19[a]	5.70[a]	6.44[ab]	7.16[b]	0.34	0.04
Day 12						
Total essential AA, nmol/mL	936[ab]	828[a]	809[a]	1,014[b]	58	0.08
Total AA, nmol/mL	2,320	2,057	2,037	2,378	118	0.12
Arginine, % of total essential AA	15.9	15.0	16.1	16.6	0.9	0.62
Arginine, % of total AA	6.21	5.99	6.34	7.02	0.32	0.15
Day 13						
Total essential AA, nmol/mL	963	943	885	1,028	95	0.77
Total AA, nmol/mL	2,430	2,384	2,260	2,443	185	0.89
Arginine, % of total essential AA	16.1	15.9	16.5	17.1	0.9	0.80
Arginine, % of total AA	6.34	6.19	6.37	7.14	0.35	0.26

[a, b]Means with different superscripts differ ($P \leq 0.10$) for each treatement.

[1]Treatments: 0, 90, 180, and 360 mg/kg BW of rumen-protected arginine supplemented from d 8 to 12 of the estrous cycle (n = 5, 4, 5 and 5 respectively).

[2]Standard error of mean.

[3]*P*-value for F-test for treatment.

[4]Day refers to day of estrous cycle (day 0 = estrus). An initial sample taken on Day 8 prior to rumen-protected arginine supplementation.

Table 4.2. Effects of rumen-protected arginine on ovarian hemodynamics

	Treatment[1]					
	0	90	180	360	SEM[2]	*P*-value[3]
Corpus Luteum						
Peak systolic velocity, cm/s	22.6[a]	32.5[b]	28.4[ab]	30.5[b]	2.4	0.07
Pulsatility index[4]	0.32	0.39	0.30	0.33	0.04	0.48
Resistance index[5]	0.26	0.32	0.25	0.28	0.03	0.42
Mean velocity, cm/s	20.1[a]	26.7[b]	24.4[ab]	25.7[b]	1.9	0.13
Flow time, ms	566[a]	596[a]	489[a]	753[b]	61	0.06
Hilus						
Peak systolic velocity, cm/s	31.3	22.3	31.9	29.0	3.3	0.21
Pulsatility index[4]	0.40	0.51	0.40	0.47	0.046	0.30
Resistance index[5]	0.32	0.39	0.31	0.37	0.027	0.18
Mean velocity, cm/s	25.1[b]	17.0[a]	25.8[b]	22.5[ab]	2.5	0.12
Flow time, ms	579[a]	595[a]	514[a]	736[b]	43	0.02

[a, b]Means with different superscripts differ ($P \leq 0.10$) for each treatment.
[1]Treatments: 0, 90, 180, and 360 mg/kg BW of rumen-protected arginine supplemented from d 8 to 12 of the estrous cycle (n = 5, 4, 5 and 5 respectively).
[2]Standard error of mean.
[3]*P*-value for F-tests for treatment.
[4]Pulsatility index = (Peak systolic velocity – End diastolic velocity) / Time-averaged maximum velocity.
[5]Resistance index = (Peak systolic velocity – End diastolic velocity) / Peak systolic velocity.

Discussion

Arginine supplementation has primarily been evaluated in non-ruminant species. Limited research investigating arginine supplementation in ruminants has been conducted because of the high degree of ruminal arginine catabolism in the rumen and lack of rumen-protected products. Research in pigs (Wu et al., 1997) and sheep (Chapter 2) has indicated that intravenous injection of arginine at the rate of 27 mg of arginine/kg BW increased serum arginine within one hour of injection. Data published herein provides seminal information on the effects of rumen-protected arginine on serum arginine concentrations and ovarian hemodynamics in sheep. The 90 ARG treatment used in this study was estimated to deliver 27 mg arginine/kg BW to circulation over a 24 h period. This is in

contrast to other studies (Wu et al., 1997; Chapter 2; Chapter 3), which used intravenously injected arginine. In the current study, only ewes supplemented with the largest dose (360 ARG) had greater serum arginine concentrations, which occurred on d 11 and 12 after 3 and 4 d of supplementation, respectively.

Nitric oxide is produced when the enzyme nitric oxide synthase catalyzes the oxidation of L-arginine to L-citrulline and is considered the endothelium-derived relaxing factor essential for increasing systemic vasodilation (Ignarro et al., 2001; Martin et al., 2001; Gouge et al., 1998). Increased vascular permeability at the site of blastocyst attachment has demonstrated to be a requirement for implantation in many species (Gouge et al., 1998). Nitric oxide is an important factor involved in the initiation implantation due to its ability to increase blood flow (Gouge et al., 1998). Nitric oxide is produced in preimplantation embryos and its production is required for normal embryonic development (Gouge et al., 1998). In addition to nitric oxide's ability to regulate embryonic development, the embryo may also produce nitric oxide as a signal to the uterus to stimulate local vasodilation and capillary permeability required for successful implantation (Gouge et al., 1998). In the present study, rumen-protected arginine supplementation increased peak systolic velocity in the CL for 360 ARG and 90 ARG compared to CON on d 12 of the estrous cycle. These findings are similar to our previous report (Chapter 2), in which vascular resistance in the ovarian artery was reduced on d 12 following L-arginine injection.

Polyamines and nitric oxide are important for placental growth and angiogenesis. More specifically, they are essential for cellular proliferation and differentiation (Wu and Morris, 1998). The enzyme arginase regulates the availability of arginine for the synthesis of

ornithine. Polyamines are synthesized from ornithine via ornithine decarboxylase (ODC) and arginase. In the current study, there were no differences seen in circulating serum ornithine concentration.

Several studies have reported that low levels of progesterone can lead to a greater incidence of embryonic loss in sheep and ultimately result in decreased ewe productivity (Casida and Warwick, 1945; Dixon et. al., 2007). Although rumen-protected arginine in the present study exhibited stimulatory effects on ovarian hemodynamics, it did not affect serum progesterone concentrations, which is in contrast to our previous data on intravenous arginine supplementation (Chapter 2).

Implications

Results of this study indicate that rumen-protected arginine supplemented to ewes may increase circulating serum arginine concentration in addition to increasing ovarian blood flow. These preliminary data suggest that biological responses to rumen-protected arginine may be obtained without changing circulating arginine concentration. Additional research is needed to determine the potential of rumen-protected arginine as a component of strategic supplementation programs. Moreover, the ability of rumen-protected arginine to successfully reach the small intestine and enter circulation needs to be determined *in vivo*.

CHAPTER 5. SUMMARY AND CONCLUSIONS

As one of the largest factors determining income in the livestock industry, reproductive performance of the dam is critically important for a profitable enterprise for the livestock producer. Embryonic and fetal deaths during pregnancy in the U.S. sheep industry account for 25 to 50% of the total number of fertilized ova (Knights et al., 2003; Dixon et al., 2007). Most of this embryonic loss has been reported to occur before d 18 (Hulet et al., 1956; Moore et al., 1960; Quinlivan, 1966). Only a small percentage of embryos are inherently non-viable in the ewe (Wilmut et al., 1986), which would suggest that the majority of early embryonic losses can be prevented.

Arginine serves as a versatile amino acid in mammals as it plays an important regulatory role the synthesis of proteins, nitric oxide and polyamines, all of which are critically important for enhancing the uterine environment for the maintenance of the pregnancy. The studies presented in this Thesis indicate early reproductive losses can be prevented, at least in part, by injectable treatment with arginine. The mechanisms responsible for the positive results observed might be due to decreased ovarian vascular resistance resulting in increased concentrations of progesterone, which ultimately results in a more ideal environment for early embryonic survival in the ewe. Elevated concentrations of arginine may have also enhanced embryonic cellular proliferation, expression and differentiation to ensure proper embryonic growth and survival through its role in synthesis of ornithine.

Due to the increased labor of injectable arginine protocols, a feed grade product may be more suitable for utilizing the positive effects of arginine for sheep producers. Rumen-protected arginine supplemented to ewes increased circulating serum arginine

concentration in addition to increasing ovarian blood flow. Additional research is needed for determining the potential of rumen-protected arginine as a component of strategic supplementation programs for sheep producers. Moreover, the ability of rumen-protected arginine to successfully reach the small intestine and enter circulation needs further evaluation *in vivo*.

Collectively, our studies would suggest that reproductive efficiency can be enhanced via supplementation of arginine. The development of strategies for enhancing prenatal growth and survival in sheep could have a positive economic impact on the sheep industry.

LITERATURE CITED

Abramowicz J. S., and E. Sheiner. 2008. Ultrasound of the placenta: a systematic approach. Part II: functional assessment (Doppler). Placenta. 29:(921-929).

Barrell, G. K., L. A. Thrun, M. E. Brown, C Viguie, and F. J. Karsch. 2000. Importance of photoperiodic signal quality to entrainment of the circannual reproductive rhythm of the ewe. Biol. Reprod. 63:769-774.

Bazer F. W., R. M. Roberts, and W. W. Thatcher. 1979. Actions of hormones on the uterus and effect on conceptus development. J. Anim. Sci. 49:35-45.

Bazer F. W., T. L. Ott, and T. E. Spencer. 1998. Maternal recognition of pregnancy: comparative aspects. Tropho. Res. 12:375-386.

Bensadoun, A., and J.T. Reid. 1967. Estimation of a rate of portal blood flow in ruminants; effects of feeding fasting, and anesthesia. J. Dairy Sci. 45:540-543.

Bolet, G. 1986. Timing and extent of embryonic mortality in pigs, sheep and goats: Genetic variability in Embryonic Mortality in Farm Animals. J.M. Sreenan and M.G. Diskin, eds. Boston, MA:Dordrect. 12-43.

Bollwein H., and K. Herzog. 2007. Application of Doppler ultrasonography in cattle reproduction. Repro. Dom. Ani. 42:51-58.

Borowczyk, E., J. S. Caton, D. A. Redmer, J. J. Bilski, R. M. Weigl, K. A. Vonnahme, P. P. Borowicz, J. D. Kirsch, K. C. Kraft, L. P. Reynolds, and A. T. Grazul-Bilska. 2006. Effects of plane of nutrition on in vitro fertilization and early embryonic development in sheep. J. Anim. Sci. 84:1593-1599.

Bryant, M. J., and H. J. D. Rosa. 2003. Seasonality of reproduction in sheep. Small Rum. Res. 48:155-171.

Burton G. J., A. I. Watson, J. Hempstock, J. N. Skepper, and E. Jauniaux. 2002. Uterine glands provide histotrophic nutrition for the human fetus during the first trimester of pregnancy. J. Clinical Endo. and Met. 87:2954-2959.

Carson D. D., I. Bagchi, S. K. Dey, A. C. Enders, A. T. Fazlebas, B. A. Lessey, and K. Yoshinaga. 2000. Embryo implantation. Developmental Biology 223:217-237.

Casida, L. E., and E. J. Warwick. 1945. The necessity of the corpus luteum for maintenance of pregnancy in the ewe. J. Anim. Sci. 4:34-38.

Chwalisz, K., E., Winterhager, T. Thienel, and R.E. Garfield. 1999. Synergistic role of nitric oxide and progesterone during the establishment of pregnancy in the rat. Hum. Reprod. 14:542-552.

Cross, J. C. 2001. Genes regulating embryonic and fetal survival. Theriogeneology. 55:193-207.

Cumming, I. A., M. A. Blockey, C. G. Winfield, R. A. Parr, and A. H. Williams. 1975. A study of the relationships of breed, time of mating, level of nutrition, live weight, body condition, and face color, to embryo survival in ewes. J. Agric. Sci. Camb. 84:559-565.

De Boo, H. A., P. L. van Zijl, D. E. Smith, W. Kulik, H. N. Lafeber, and J. E. Harding. 2005. Arginine and mixed amino acids increase protein accretion in the growth-restricted and normal ovine fetus by different mechanisms. Pediatr. Res. 58:270-7.

Dixon, A. B., M. Knights, J. L. Winkler, D. J. Marsh, J. L. Pate, M. E. Wilson, R. A. Dailey, G. Seidel, and E. K. Inskeep. 2007. Patterns of late embryonic and fetal mortality and association with several factors in sheep. J. Anim. Sci. 85:1274-1284.

Dutt, R. H. 1954. Fertility rate and embryonic death loss in ewes early in the breeding season. J. Anim. Sci. 13:464-473.

Dutt, R. H., and E. C. Simpson. 1957. Environmental temperature and fertility of southdown rams early in the breeding season. J. Anim. Sci. 16:136-143.

Dutt, R. H., E. F. Ellington, and W. W. Carlton. 1957. Fertilization rate and early embryo survival in sheared and unsheared ewes following exposure to elevated air temperature. J. Anim. Sci. 16:1308-1318.

Flechon J. E., M. Guilomot, M. Chrlier, B. Flechon, and J. Martal. 1986. Experimental studies on the elongation of the ewe blastocyst. Reproduction, Nutrition, Development. 26:1017-1024.

Flynn, N. E., C. J. Meininger, T. E. Haynes, and G. Wu. 2002. Th metabolic basis of arginine nutrition and pharmacotherapy. Biomed. & Pharm. 56:427-438.

Ginther, O. J., and M. D. Utt. 2004. Doppler ultrasound in equine reproduction: principles, techniques and potential. J. Equine Vet. Sci. 24:516-526.

Gouge, R. C., P. Marshburn, B. E. Gordon, W. Nunley, and Y. M. Huet-Hudson. 1998. Nitric oxide as a regulator of embryonic development. Biol. Reprod. 58:875-879.

Gray C. A., F. F. Bartol, B. J. Tarleton, A. A. Wiley, G. A. Johnson, F. W. Bazer, and T. E. Spencer. 2001a. Developmental biology of uterine glands. Biol. Repro. 65:1311-1323.

Gray C. A., K. M. Taylor, W. S. Ramsey, J. R. Hill, F. W. Bazer, F. F. Bartol, and T. E. Spencer. 2001b. Endometrial glands are required for preimplantation conceptus elongation and survival. Biol. Repro. 64:1608-1613.

Gray C. A., R. C. Burghardt, G. A. Johnson, F. W. Bazer, and T. E. Spencer. 2002. Evidence that absence of endometrial gland secretions in uterine gland knockout ewes compromises conceptus survival and elongation. Reproduction. 124:289-300.

Greenberg, S. S., J. R. Lancaster, J. Xie, T. G. Sarphie, X Zhao, L. Hua, T. Freeman, D. R. Kapusta, T. D. Giles, and D. R. Powers. 1997. Effects of NO synthase inhibitors, arginine-deficient diet, and amiloride in pregnant rats. Am. J. Physiol Regul Intergr Comp Physiol. 273:(1031-1045).

Greenwald, G. S., and S. K.Roy.1994. Follicular development and its control. In: Knobil E., Neill J. (eds) The Physiology of Reproduction. Raven Press, New York. 629-724.

Hamm P. T., and R. H. Dutt. 1957. Effect of exposure to high environmental temperature and shearing on semen production of rams in winter. J. Anim. Sci. 16:328-334.

Hansel W., and E. M. Convey. 1983. Physiology of the estrous cycle. J. Anim Sci. 57:404-424.

Henricks, D. M., D. R. Lamond, J. R. Hill, and J. F. Dickey. 1971. Plasma progesterone concentrations before mating in early pregnancy in the beef heifer. J. Anim. Sci. 33:450-454.

Horwath, B. 1969. Fertility in the ram following exposure to elevated ambient temperature and humidity. J. Reprod. Fertil. 19:179-183.

Hulet, C. V., H. P. Voigtlander, A. L. Pope, and L. E.Casida. 1956. The nature of early-season infertility in sheep. J. Anim. Sci. 15:607-616.

Ignarro, L. J., G. M. Buga, L. H. Wei, P. M. Bauer, G. Wu, and P. Soldato. 2001. Role of the Arginine-Nitric Oxide Pathway in the Regulation of Vascular Smooth Muscle Cell Proliferation. Proc. Natl. Academy of Sci. 98:4202-4208.

Karsh F. J., J. E. Robinson, C. J. Woodfill, and M. B Brown. 1989. Circannual cycles of luteinizing hormone and prolactin secretion in ewes during prolonged exposure to a fixed photoperiod: evidence for an endogenous reproductive rhythm. Biol. Repro. 41:1034-1046.

Kleeman D. O., S. K. Walker, and R. F. Seamark.1994. Enhanced fetal growth in sheep administered progesterone during the first three days of pregnancy. J. Reprod. and Fert. 102:411-417.

Knights, M., Q. S. Baptiste, A . B. Dixon, J. L. Pate, D. J. Marsh, E. K. Inskeep and P. E. Lewis. 2003. Effects of dosage of FSH, vehicle and time of treatment on ovulation rate and prolificacy in ewes during the anestrous season. Small Rum. Res. 50:1-9.

Krebs, H. A., and K. Henseleit. 1932. Studies on urea formation in the animal organism. Physiol. Chem. 210:33-66.

Kwon, H., G. Wu, F. W. Bazer, and T. E. Spencer. 2003. Developmental changes in polyamine levels and synthesis in the ovine conceptus. Biol. Reprod.. 69:1626-1634.

Lamming, G. E., A. O. Darwash, and H. L. Back. 1989. Corpus luteum function in dairy cows and embryonic mortality. J. Reprod. Fert. 37:245-252.

Lawson R. A., R. A. Parr, and L. P. Cahill. 1983. Evidence for maternal control of blastocyst growth after asynchronous transfer of embryos to the uterus of the ewe. J. Reprod. and Fert. 67:477-483.

Lozano, J. M., P. Lonergan, M. P. Boland, and D. O'Callaghan. 2003. Influence of nutrition on the effectiveness of superovulation programs in ewes: effect on oocyte quality and post-fertilization development. J. Reprod. and Fert. 125:543-553.

Malpaux B., C Viguie, D. C. Skinner, J. C. Thiery, and P. Chemineau. 1997. Control of the circannual rhythm of reproduction by melatonin in the ewe. Brain Res. Bulletin. 44:431-438.

Malpaux B., C. Viguie, D. C. Skinner, J. C. Thiery, J. Pelletier, and P. Chemineau. 1996. Seasonal breeding in sheep: Mechanism of action of melatonin. Ani. Repro. Sci. 42:109-117.

Manser, R. C., H. J. Leese, F. D. Houghton. 2004. Effect of inhibiting nitric oxide production on mouse preimplantation embryo development and metabolism. Biol. Reprod. 71:528-533.

Martin, M. J., M. D. Jimenez, and V. Motilva. 2001. New issues about nitric oxide and its effects on the gastrointestinal tract. Current Pharmaceutical Design. 7:881-908.

Mateo, R. D., G. Wu, F. W. Bazer, J. C. Park, I. Shinzaro, and S. W. Kim. 2007. Dietary L-arginine supplementation enhances the reproductive performance of gilts. J. Nutr. 137:652-656.

Maulik, D. 2005. Physical principles of Doppler ultrasonography. In: Doppler ultrasound in obstetrics and gynecology. D. Maulik (ed). Springer, New York. 9-17.

Maurer, R. R., and S. E. Echternkamp. 1982. Hormonal asynchrony and embryonic development. Theriogenology. 17:11-22.

Mieusset R., P. Quintana Casares, L. G. Sanchez Partida, S. F. Sowerbutts, J. L. Zupp, and B. P. Setchell. 1992. J. Reprod. and Fert. 94:337-343.

Moncada S., and E. A. Higgs. 1993. The L-arginine-nitric oxide pathway. N. Engl. J Med. 329:2002-2012.

Moore, N. W., L. E. Rowson, and R. V. Short. 1960. Egg transfer in sheep. Factors affecting the survival and development of transferred eggs. J. Reprod. and Fertil.. 1:322-349.

Nephew, K. P., K. E. McClure, and W. F. Pople. 1989. Embryonic migration relative to maternal recognition of pregnancy in sheep. J. Anim. Sci. 69:999-1005

Nephew, K. P., K. E. McClure, T. L. Ott, D. H. Dubois, F. W. Bazer, and E. F. Pope. 1991. Relationship between variation in conceptus development and differences in estrous cycle duration in ewes. Biol. Repro. 44:536-539.

Pritchard, J. Y., F. N Schrick, and E. K Inskeep. 1994. Relationship of pregnancy rate to peripheral concentrations of progesterone and estradiol in beef cows. Theriogenology. 42:247-259.

Quinlivan, T. D. 1966. Estimates of pre- and preinatal mortality in the New Zealand Romney Marsh ewe. J. Reprod. Fertil. 11:379-390.

Rabiee, A. R., K. L. MacMillian, and F. Scharzenberger. 2000. Effect of level of feed intake on plasma progesterone concentration in desolrelin-implanted dairy cows treated with a CIDR device. J. Anim. Sci. 78:221.

Rathorne, A. K. 1970. Fertility of rams heated for 1,2,3 and 4 days, mated to superovulated ewes. Aust. J. Agric. Res. 21:355-358.

Reynolds, L. P., D. S. Millway, J. D. Kirsch, J. E. Infeld, and D. A. Redmer. 1987. Angiogenic activity of placental tissues of cows. J. Reprod. Fertil. 81:233-240.

Reynolds, L. P., and D. A. Redmer. 1988. Secretion of angiogenic activities by placental tissues of cows at several stages of gestation. J. Reprod. Fertil. 83:497-502.

Reynolds, L. P., S. D. Killilea, and D. A. Redmer. 1992. Angiogenesis in the female reproductive system. FASEB J. 6:886-892.

Reynolds, L. P., A. T. Grazul-Bilska, and D. A. Redmer. 2000. Angiogenesis in the corpus luteum. Endocrine. 12(1):1-9.

Rhind, S. M., J. J. Robinson, and I. McDonald. 1980. Relationships among uterine and placental factors in prolific ewes and their relevance to variations in fetal weight. Anim. Prod. 30:115-124.

Rhind, S. M., W. A. C. McKelvey, S. R. McMillian, R. G. Gunn, and D. A. Elston. 1989. Effect of restricted food intake bfore and/or after mating, on the reproductive performance of Greyface ewes. Anim. Prod. 48:149-155.

Roberts, R. M., and F. W. Bazer. 1988. The functions of uterine secretions. J. Reprod. and Fert. 82:875-892.

Sangsritavong, S., D. K. Combs, R. Sartori, L. E. Armentano, and M. C. Wiltbank. 2002. High feed intake increases liver blood flow and metabolism of progesterone and estradiol-17β in dairy cattle. J. Dairy Sci. 85:2831-2834.

Schrick, F. N., and E.K. Inskeep. 1993. Determination of early pregnancy in ewes utilizing transrectal ultrasonography. Theriogenology. 40:295-306.

Senger, P. L., 2005. Pathways to Pregnancy and Parturition. 2nd Ed. Pullman, WA: Current Conceptions.

Spencer, T. E., and F. W. Bazer. 2002. Biology of progesterone action during pregnancy recognition and maintenance of pregnancy. Front. Biosci. 7:1879-1898.

Spencer, T. E., G. A. Johnson, R. C. Burghardt, and F. W. Bazer. 2004. Progesterone and placental hormone actions on the uterus: insights from domestic animals. Biol. Reprod. 71:2-10.

Stouffer, R. L. 1994. Structure, Function and Regulation of the Corpus Luteum. In: Knobil E., Neill J. (eds) The Physiology of Reproduction. Raven Press, New York. 425-503.

Takasaki, A. T. Hiroshi, K. Taniguchi, H. Asada, T. Taketani, A. Matsuoka, Y. Yamagata, K. Shimamura, H. Morioka, and N. Sugino. 2009. Luteal blood flow and luteal function. J. Ovar. Res. 2:1-6.

Thatcher, W. W., K. L. MacMillian, P. J. Hansen, and M. Dorst. 1989. Conceptus for regulation of corpus luteum function by the conceptus and ovarian follicles to improve fertility. Theriogenology. 31:149-164.

Thureen P. J., K. A. Baron, P. V. Fennessey, and W. W. Hay Jr. 2002. Ovine placental and fetal arginine metabolism at normal and increased maternal plasma arginine concentrations. Pediatr Res. 51:464–71.

Vosatka, R. J., P. M. Hassoun, and K. B. Harvey Wilkes. 1998. Dietary l-arginine prefects fetal growth restriction in rats. Am. J. Obstet. Gyn. 178:242-246.

Wilmut, I, D. I. Sales, and C. J. Ashworth. 1986. Maternal and embryonic factors associated with prenatal loss in mammals. J. Reprod. Fertil. 76(2):851-864.

Windmueller, H. G., and A. E. Spaeth. 1981. Source and fate of circulating citrulline. Am. J. Physiol. 241:E473-E480.

Wise, T. H., D. Caton, W. W. Thatcher, D. H. Barron, and M. J. Fields. 1982. Ovarian function during the estrous cycle of the cow: ovarian blood flow and progesterone release rate. J. Anim. Sci. 55:627-637.

Woodfill, C. J., J. E. Robinson, B. Malpaux, and F. J. Karsch. 1991. Synchronization of the circannual reproductive rhythm of the ewe by discrete photoperiodic signals. Biol. Reprod. 45:110-121.

Wu, G., and D. A. Knabe. 1995.Arginine synthesis in enterocytes of neonatal pigs. Am. J. Physiol. 269:R621-R629.

Wu, G. 1997. Synthesis of citrulline and arginine from proline in enterocytes of postnatal pigs. Am. J. Phys. G1382–G1390.

Wu, G., P. K. Davis, N. E. Flynn, D. A. Knabe, and J. T. Davidson. 1997. Endogenous Synthesis of Arginine Plays an Important Role in Maintaining Arginine Homeostasis in Postweaning Growing Pigs. J. Nutr. 127:2342-2349.

Wu, G., and S. M. Morris, Jr. 1998. Arginine metabolism: Nitric oxide and beyond. J. Biochem. 336:1-17.

Wu G., F. W. Bazer, T. A. Davis, S. W. Kim, P. Li, J. M. Rhoads, M. C. Satterfield, S. B. Smith, T. E. Spencer, and Y. Yin. 2009. Arginine metabolism and nutrition in growth, health, and disease. Amino Acids. 37:153-168.

Zeng, X., F. Wang, X. Fan, W. Yang, B. Zhou, P. Li, Y. Yin, G. Wu, and J. Wang. 2008. Dietary arginine supplementation during early pregnancy enhances embryonic survival in rats. J. Nutr. 138:1304-1309.