THE EFFECTS OF THE NITRIC OXIDE (NO) SYSTEM AND NUTRITIONAL PLANE ON OVARIAN FUNCTION

A Dissertation
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By

Casie Shantel Bass

In Partial Fulfillment of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY

Major Department:
Animal Sciences

July 2015

Fargo, North Dakota

North Dakota State University
Graduate School

Title

The Effects of the Nitric Oxide (NO) System and Nutritional Plane on Ovarian Function

By

Casie Shantel Bass

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

DOCTOR OF PHILOSOPHY

SUPERVISORY COMMITTEE:

Anna Grazul-Bilska
Chair

Dale Redmer

Marc Bauer

Kendra Greenlee

Approved:

07-02-15
Date

Dr. Greg Lardy
Department Chair

ABSTRACT

Researchers predict a world population of approximately 9.5 billion people by the year 2050. Current resources, including the amount of meat that is produced, would not be able to sustain that population. Therefore, the current dissertation research was completed to assist in improving reproductive efficiency. A third of pregnancies are "lost", or spontaneously aborted with unknown reason(s). Therefore, research has been directed towards improving reproductive efficiency, especially in regards to proper ovarian function in both pregnant and non-pregnant females.

Numerous factors influence reproduction including hormones, growth and angiogenic factors, nutritional plane, and supplements such as arginine (Arg), a semi-essential amino acid and precursor for proteins, polyamines, and nitric oxide (NO). Nitric oxide is a reactive gas molecule made naturally in the body and is critical for angiogenesis, which is the development of blood vessels, generally capillaries, from pre-existing blood vessels to allow for blood flow to specific tissues. During a female's reproductive cycle, certain reproductive tissues undergo rapid changes including vascular development and regression, and tissue growth and regression. One of those tissues, the corpus luteum (CL), is critical for pregnancy as the tissue produces progesterone (P4), a pregnancy maintenance hormone. Therefore, the objective of this research was to investigate how manipulation of the NO system via Arg supplementation to nutritionally compromised ewes impacts ovarian function and endocrine activity in non-pregnant sheep.

Keywords: Nutrition, Ewe, Progesterone, Nitric Oxide

ACKNOWLEDGMENTS

I cannot express enough appreciation to my advisors, Drs. Anna Grazul-Bilska and Dale Redmer. Thank you both so much for your patience, support, and allowing me to ask lots of questions and grow as a researcher. I would also like to express gratitude to Drs. Marc Bauer and Kendra Greenlee for serving as committee members and professors. Together, this committee has spent a tremendous amount of time with me and focused on this research, for which I am so truly, truly grateful.

I am indebted to Mr. Jim Kirsch, Mr. Bob Weigle, Drs. Jodie Haring and Sheri Dorsam, the ANPC staff, and our undergrad working crew. Thanks for being so peppy, hard-working, and knowledgeable. Each of you were a blast to work with!

Much thanks to my NDSU graduate student family over the last three years. By sharing an office together (Leslie, Beth, Arshi, Mellissa, Dani, Mattia, and Jordan), bon fire parties, working out together, and on and on, each of you have given me so many happy memories and have become a part of my life. I wish each of you a happy future filled with love, curiosity, and a passion for life.

Sami Kaminski became not only a colleague of mine, but also a close buddy. We shared a monumental amount of time together at the barn with our sheep, in the laboratory working, and studying for different courses. I can't imagine having accomplished this work without you.

A big hug to all of my family who have encouraged, pushed, prodded, and cried with me. I love you all so much and hope I've made you proud.

Lastly, to my phenomenal husband, Jeremiah, thank you for whole-heartedly agreeing to be on this adventure with me. I love you much more than you will ever know…let's be done with school now!

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

aa .....Amino Acid

ANGPT .....Angiopoietin

Arg .....Arginine

ART.....Assisted Reproductive Technologies

ASL .....Arginosuccinate Lyase

ASS .....Arginosuccinate Synthase

BBB.....Blood Brain Barrier

BCS .....Body Condition Score

BME .....Bovine Microvascular Endothelial Cell

BSA .....Bovine Serum Albumin

BW .....Body Weight

C .....Control

Ca^{2+} .....Calcium

CaM.....Calmodulin

cAMP .....Cyclic Adenosine Monophosphate

cGMP .....Cyclic Guanosine Monophosphate

CIDR .....Controlled Internal Drug Release

CL .....Corpus Luteum

CS .....Calf Serum

d .....Day

DETA .....Diethylenetriamine

DETA-NONOate .....(Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate

DMEM .....Dulbecco's Modified Eagle Medium

DMI .....Dry Matter Intake

D-NMME .....N(G)-Nitro-D-Arginine-Methyl Ester

E_2 .....Estradiol

EDRF .....Endothelial Derived Relaxing Factor

eNOS .....Endothelial Nitric Oxide Synthase

ER .....Endoplasmic Reticulum

FBS .....Fetal Bovine Serum

FF .....Follicular Fluid

FGF .....Fibroblast Growth Factor

FLT .....FMS-like Tyrosine Kinase

FSH .....Follicle Stimulating Hormone

GH .....Growth Hormone

GLUT 4 .....Glucose 4 Transporter

GnRH .....Gonadotropin Releasing Hormone

GTP .....Guanosine Triphosphate

GUCY1B3 .....Guanylate Cyclase 1, Soluble, Beta 3

HBSS .....Hank's Balanced Salt Solution

HDL .....High Density Lipoproteins

HSPGs .....Heparan Sulfate Proteoglycans

ICM .....Inner Cell Mass

IGF-1 .....Insulin-like Growth Factor 1

IGF-1R .....Insulin-like Growth Factor 1 Receptor

iNOS .....Inducible Nitric Oxide Synthase

IVF .....In Vitro Fertilization

KO .....Knock Out

LDL .....Low Density Lipoproteins

LH .....Luteinizing Hormone

L-NAME .....L-NG-Nitroarginine Methyl Ester

NBF .....Neutral Buffered Formalin

NEFAs .....Non-Esterified Fatty Acids

nNOS .....Neuronal Nitric Oxide Synthase

NO .....Nitric Oxide

NOS .....Nitric Oxide Synthase

NPY .....Neuropeptide Y

O .....Overfed

P4 .....Progesterone

P450scc .....Cholesterol Side-chain Cleavage Enzyme

PGC .....Primordial Germ Cell

PGF .....Placental Growth Factor

PGF2α .....Prostaglandin F2α

PKC .....Protein kinase C

PKG .....Protein Kinase G

RTK .....Receptor Tyrosine Kinase

Sal .....Saline

sGC .....Soluble Guanylyl Cyclase

SMC .....Smooth Muscle Cells

SNO .....S-Nitroso-Proteins

SNP .....Sodium Nitroprusside

SOF .....Synthetic Oviductal Fluid

Tie .....Tyrosine Kinase with Immunoglobulin and Epidermal Growth Factor Homology Domain

U .....Underfed

UPR .....Protein Uptake Response

VEGF .....Vascular Endothelial Growth Factor

VSMC .....Vascular Smooth Muscle Cell

WT .....Wild Type

CHAPTER 1: LITERATURE REVIEW

Nutritional Effects on Reproduction

Nutritional Effects on Metabolic Hormones

Hormones are molecules released by a specific group of cells within an endocrine system that are capable of influencing changes in another group of cells. Hormones, along with the nervous system, control the body's responses to internal and external stimuli and are carried to their target cells in the bloodstream (Nussey and Whitehead, 2001). Hormones have the ability to cause a physiological change within the body by binding at the target cell, which has produced a specific hormone receptor (Neave, 2008). When a hormone binds to its corresponding receptor, it causes a conformational change in the receptor's shape; the shape change allows the receptor to fit with other cell molecules in a way it previously could not, thus initiating new activities in the cell (Neave, 2008). While a hormone reaches the majority of tissues and cells within the body, only some cells produce corresponding receptors, which are then sensitive to a specific hormone's actions.

Insulin is a peptide hormone produced by beta cells within the pancreas and regulates the metabolism of carbohydrates and fats through increasing the absorption of glucose from blood plasma to skeletal muscle; it also inhibits the production of glucose by the liver (Sonksen and Sonksen, 2000). Insulin is produced and stored in the body as a hexamer (a unit composed of six insulin molecules), with the active form being a monomer (Mayer, 2007). The hexamer is an inactive form but has long-term stability, while the biologically active form is a monomer (Mayer, 2007). Specific transporter proteins in myocyte and adipocyte cell membranes allow glucose from the blood to enter a cell; these transporters being directly under the control of

insulin (De Meyts, 2004). Low blood insulin concentrations will prevent glucose from entering the cells, while higher concentrations will allow for cell glucose entry. Activation of insulin receptors causes internal cellular mechanism changes that directly affect the cellular uptake of glucose (De Meyts, 2004). Specifically, when insulin binds the extracellular portion of the alpha subunit of the insulin receptor, it causes a conformational change, activating the kinase domain residing on the beta subunit within the intracellular receptor. The activated kinase domain autophosphorylates tyrosine residues on the C-terminus of the receptor, resulting in an increase in glucose 4 transporters (GLUT 4) present in the cell membrane (Huang et al., 2004). A negative energy balance has been associated with changes in metabolic hormones, including insulin concentrations; specifically, a negative energy balance is associated with hypoinsulinemia (Kiyma et al., 2004; Wade and Jones, 2005; Ferraretto et al., 2014). Oppositely, positive energy balance has been reported to result in increased insulin in ruminant species (Armstrong et al., 2003; Grazul-Bilska et al., 2012; Kaminski et al., 2015), as well as glucose uptake. Insulin is responsible for regulating over 100 genes, most at the level of transcription, in many different organs including the ovary (Poretsky et al., 1999). Insulin receptors are widely expressed on several ovarian cells and tissues, including granulosa, theca, and stromal tissues (Poretsky et al., 1984, 1999; Hernandez et al., 1992; Samoto et al., 1993), leading some researchers to postulate that insulin has a direct effect on ovarian development and/or function (Samoto et al., 1993). Moreover, in vitro studies have demonstrated that insulin stimulates ovarian steroidogenesis by granulosa and theca cells, increasing androgens, estrogens, and progesterone production in tissues from women (Willis et al., 1996; McGee et al., 1996), and

sows (Barbieri et al., 1983, 1994), and that this increase is mainly mediated by changes in insulin receptor expression (Poretsky et al., 1999).

Leptin is a metabolic protein consisting of 167 amino acids (Baratta, 2002) produced predominantly by white adipose tissue, as well as brain (Burguera et al., 2000), skeletal muscle, mammary epithelial cells (Casabiell et al., 1997), bone marrow, and ovaries (Ryan et al., 2002). After leptin is secreted, it circulates in free-form, or with carrier proteins (Sinha et al., 1996), to cross the blood brain barrier (BBB), wherein it binds to the hypothalamic leptin receptors and to neuropeptide Y (NPY) neurons in the arcuate nucleus, providing information about the energy stores within the body (Meister et al., 2000; Sahu et al., 2004). Leptin signaling functions as a feedback mechanism to the brain to regulate food intake and maintain body weight (Pelleymounter et al., 1995). Short-term (12 h) or long-term (two to eight weeks) of overfeeding results in an increase in adipocyte leptin expression, as well as circulating leptin in healthy humans and other species (Kolaczynski et al., 1996; Spicer et al., 2001; Chilliard et al., 2005; Kiani et al., 2013; Kaminski et al., 2015). Leptin is also involved with adaptation to energy deprivation (Klok et al., 2007). Specifically, fasting for three days results in a significant decrease in peripheral leptin concentrations (Kolaczynski et al., 1996; Weigle et al., 1997). Leptin is also involved with pubertal development and reproductive functions (Hamm et al., 2004). Mice with two defective Ob genes, the gene responsible for expressing leptin, not only have metabolic issues such as being obese, but also are infertile, have fewer ovarian follicles (Barash et al., 1996), impaired folliculogenesis, and increased granulosa cell apoptosis (Ryan et al., 2002; Hamm et al., 2004).

Insulin-like growth factor 1 (IGF-1) is a protein consisting of 70 amino acids produced predominately by the liver as an endocrine hormone. Approximately 98% of IGF-1 is bound to one of six proteins, is regulated by insulin, and its primary action is mediated by binding to either its specific receptor (IGF-1R) or the insulin receptor (Mayer, 2007). The IGF-1R is considered the more physiologically active receptor as it binds IGF-1 at a significantly higher affinity (Huang et al., 2004). By binding its corresponding receptor, IGF-1 activates the AKT signaling pathway, stimulating cell growth and proliferation, as well as inhibiting programmed cell death (Huang et al., 2004). It should be noted that IGF-1 is also the primary activator for growth hormone (GH), which is produced form the anterior pituitary gland (Blogowska et al., 2001). Once released into the blood stream, GH stimulates the liver to produce IGF-1, which in turn stimulates body growth. While IGF-1 is produced throughout life, the highest rates are during pubertal growth and the lowest concentrations are produced during infancy and old age (Scarth, 2006). While an increase in protein consumption increases IGF-1 concentrations in humans, it appears to be independent of total caloric consumption (Scarth, 2006). Other factors that influence IGF-1 production include genetics, stress levels, body mass index, nutritional plane (Scarth, 2006), and insulin (Poretsky et al., 1988). Moreover, dietary restriction can rapidly reduce IGF-1 concentrations, and in vivo studies with rats and women demonstrated that hyperinsulinemia upregulates IGF-1R expression in the ovaries (Poretsky et al., 1988; Samoto et al., 1993). On the other hand, overfeeding enhances serum IGF-1 concentrations in several species including sheep (Armstrong et al., 2001, 2003; Ginther et al., 2004, Adamiak et al., 2005; Kaminski et al., 2015). There is a functional link between IGF-1 and ovarian function, as IGF-1R and FSH receptor co-localize in both small and preovulatory follicle granulosa cells (Zhou et al.,

1997). In granulosa cells, IGF-1 enhances FSH actions by mechanisms that are not entirely clear (Orly, 2000). Mice that are not capable of producing IGF-1 or its receptor exhibit severe ovarian follicular growth retardation (Baker et al., 1996; Burks et al., 2000; Richards et al., 2002).

Nutritional Effects on Reproductive Hormones

Nutrition has a significant impact on reproductive hormones, ovarian follicle and corpora lutea (CL) function, oocyte competence, and fertility (McEvoy et al., 1995; Wrenzycki et al., 2000; Kwong et al., 2000; Boland et al., 2001; Armstrong et al., 2001, 2003; Da Silva et al., 2002). An animal, when in a negative energy balance, must use its own body energy stores (as opposed to energy from food intake) in an effort to reverse the energy deficit, resulting in negative effects on both the hypothalamic-pituitary level as well as the reproductive system (Scaramuzzi et al., 2006).

While follicle stimulating hormone (FSH) and luteinizing hormone (LH) are critical signaling hormones for follicular growth, ovulation, and luteinization, their actions are directly dependent upon other signaling pathways, including those of metabolic hormones like insulin (Poretsky et al., 1999; Richards et al., 2002) and leptin (Ryan et al., 2002; Hamm et al., 2004). Tatman et al. (1990) reported that the LH content within the pituitary was lower in ewes with decreased body condition scores (BCS). Moreover, researchers have observed suppressed LH mRNA expression in underfed ewes (Kile et al., 1991). While long-term feed restriction can cause anestrous in cattle due to insufficient circulating LH (Rhodes et al., 1995, 1996), short-term effects cause either decreased preovulatory surges of LH (Kiyma et al., 2004) or no changes to LH concentrations (Abecia et al., 1995). Conversely, researchers have also reported no

differences in LH concentrations in ewes of high and low nutritional plane during the weeks prior to mating (Rhind et al., 1985).

Serum FSH concentrations were greater in chronically underfed heifers compared to controls (Rhodes et al., 1996; Bossis et al., 1999; Mackey et al., 2000). Researchers postulate the different responses of LH and FSH concentrations are due to differences in storage of the hormones (Diskin et al., 2003). Specifically, LH is produced and stored in the pituitary, while very little FSH is stored (McNeilly et al., 1995) and once synthesized, is constitutively secreted (Farnworth et al., 1995). Regardless of hormonal storage, there is little evidence to suggest that negative energy balance has any direct effects on ovine ovarian function that are independent of its effects on the hypothalamo-pituitary axis (Lozana et al., 2003; Kiyma et al., 2004). Several researchers have suggested and agreed on the "ovarian hypothesis" that altered nutrition first influences metabolic hormones, such as insulin or leptin, and secondarily, the ovary by modulating gonadotropin action (Beitz et al., 1985; Downing and Scaramuzzi, 1991; Diskin et a., 2003), having effects on FSH and LH.

Serum concentrations of estradiol (E_2) have been reported to be lower in fasted ewes when compared to control ewes (Adams et al., 1997; Kiyma et al., 2004) and rats (Oukonyong et al., 2000). Grazul-Bilska et al (2012) demonstrated greater serum E_2 concentration in underfed than overfed FSH-treated ewes. The decreased E_2 concentrations following short-term feed deprivation may be due to reduced ovarian follicular development caused by suppressed serum gonadotropin concentrations (Gougeon, 1996). Additionally, Adams et al. (1994) reported not only that undernourished ewes exhibited less E_2 secretion, but a simultaneous reduced metabolic

clearance rate of the hormone, which may explain the overall lower peripheral concentration observed in nutritionally restricted ewes (Adams et al., 1994, 1997).

A direct relationship between nutritional plane and P4 concentrations have been reported in ewes and heifers, with overfed ewes having greater P4 when compared to control or underfed ewes (Kaminski et al., 2015; Armstrong et al., 2006). However, an inverse relationship between nutritional plane and P4 have also been demonstrated, with decreased nutrition resulting in increased P4 concentrations in ewes (Williams and Cumming, 1982; Parr et al., 1982; Rhind et al., 1989; Kiyma et al., 2004), sows (Dyck et al., 1980; Miller et al., 1999), and cows (Donaldson et al., 1970; Jordan and Swanson, 1979; Rabiee et al., 2001; Ferraretto et al., 2014). The effect of decreased nutritional quantity causing increased peripheral P4 concentrations has been hypothesized to be due to multiple reasons including P4 metabolism and clearance rate (Kiyma et al., 2004; Parr et al., 1993; Smith et al., 2006). There are conflicting reports as to whether greater CL number can produce greater P4 concentrations. For example, Amiridis et al. (2002) demonstrated a linear relationship between CL number and P4 concentrations, while Lamond et al. (1972) reported increased CL numbers do not have greater plasma P4 concentrations in sheep. In our studies, a greater number of CL was associated with greater serum P4 concentration (Grazul-Bilska et al. 2007; Kaminski et al. 2015), Furthermore, it has been suggested that animals provided inadequate nutrition may have prolonged P4 catabolism (Kiyma et al., 2004). In addition, nutritional changes have altered hepatic metabolism of steroids, slowing clearing rates in livestock (Parr et al., 1993; as reviewed by Smith et al., 2006). Lastly, the catabolism of adipose tissues releases previously stored P4 into the circulatory system in cows (i.e. animals losing weight, and therefore metabolizing fat tissues, may actually have an increase in peripheral

P4 as it is freed from fat) (Hamudikuwanda et al., 1996). It should be noted that increased P4 concentrations in underfed animals is not likely due to increased adrenal synthesis of P4, as serum concentrations of cortisol from differing nutritional treatments have not been observed (Kiyma et al., 2004).

Positive energy balance due to excess dietary intake also causes changes in production and functions of reproductive hormones. Parr (1992) demonstrated that overfed ewes had lower peripheral P4 concentrations, and attributed the change to an increase in P4 clearance rate rather than to decrease P4 secretion rates. In cows fed increased dry matter intake (DMI), Sangsritavong et al. (2002) observed increased blood flow to the liver, which was associated with increased metabolic clearance of P4 and E_2 and, therefore, decreased peripheral P4.

Nutritional Effects on Ovarian Function

While early studies focused primarily on nutritional effects to the hypothalamic-pituitary axis, more recent investigations have hypothesized that nutritional changes and, therefore, changes to metabolic hormones have a direct effect on the ovary (Coop et al., 1966; Parr, 1992; Gong et al., 2002; Viñoles et al., 2010). In fact, direct effects of nutrition on ovarian function including effects on folliculogenesis (Coop et al., 1966; Diskin et al., 2003; Viñoles et al., 2010), oocyte quality, ovulation rates, luteal function, and fertility in several species have been reported (Coop et al., 1966; Botkin et al., 1988; Dunn and Moss, 1992; O'Callaghan and Boland, 1999; Papadopoulos et al., 2001; Borowczyk et al., 2006; Grazul-Bilska et al., 2012). It should be noted that differences caused by positive or negative energy balances can occur before any detectable changes to the body, such as changes in BW or BCS (Scaramuzzi et al., 2006).

Numerous studies have demonstrated that increased dietary energy and/or protein, BW, and/or BCS resulted in increased ovarian follicular recruitment resulting in enhanced number of visible follicles in sheep (Coop et al., 1966; Xu et al., 1989; Abecia et al., 2006). Conversely, other researchers have demonstrated that overfeeding and underfeeding did not affect the number of both small and large follicles when compared to controls in sheep (Abecia et al., 1995, 1997; Borowczyk et al., 2006; Grazul-Bilska et al., 2012). However, nutrient restriction has been associated with reduced ovulation in FSH-treated and not treated ewes (Lassoued et al., 2004) and in ewes treated with FSH (Yaakub et al., 1997). Nutrient restriction three to six days prior to ovulation has also been associated with reduced dominant follicle size (Murphy et al., 1991) and ovulations for prostaglandin-treated heifers (Mackey et al., 1999).

Researchers have reported specific times during the mature ewe's life wherein nutritional effects are more critical (Robinson et al., 2006). For example, if nutrient restriction occurs when follicles are emerging from the primordial pool and become committed to growth, which occurs six months prior to the breeding season, it results in reduced ovarian follicle numbers, and therefore ovulation, at mating time (Robinson et al., 2006).

Nutritional Effects on Oocyte Quality

The effects of plane of nutrition on oocyte quality have been evaluated in both non-stimulated and FSH-stimulated in sheep and cows. However, nutrient intake effects on oocyte quality are frequently contradictory, demonstrating no effects, or either negative or positive effects in ruminant and other species (McEvoy et al., 1995; O'Callaghan et al., 2000; Boland et al., 2001; Lozano et al., 2003; Peura et al., 2003; Grazul-Bilska et al., 2012).

While high nutritional plane feeding has been reported to increase oocyte quality in spontaneously ovulating ewes, the opposite was true in FSH-treated animals, which have a lower ovulation rate compared with ewes offered diets of twice maintenance energy requirements (Yaakub et al., 1997). Overfeeding FSH-treated ewes resulted in negative effects on oocyte quality as determined by decreased in vitro cleavage rates (Papadopoulos et al., 2001; Lozano et al., 2003; Grazul-Bilska et al., 2012). While reasons for these changes are not well understood, it has been postulated that an excess of rumen degradable components cause elevated ammonia concentrations in follicular fluid (FF), which cause a detrimental environment for the oocyte (Rooke et al., 2004).

Yaakub et al. (1997) reported interchromatin granule detachment from the nucleus in oocytes from non-stimulated underfed ewes, which may account for decreased cleavage rates. Researchers have also reported underfeeding having a negative impact on oocyte quality in superovulated ewes, resulting in lower oocyte cleavage rates after IVF when compared to controls (Yaakub et al., 1997; Snijders et al., 2000; Borowczyk et al., 2006; Grazul-Bilska et al., 2012). In dairy cows, Jorritsma et al. (2004) observed that greater concentrations of non-esterified fatty acids (NEFA), which are elevated in underfed animals, reduced granulosa cell proliferation and delayed oocyte maturation as well. Conversely, other researchers have reported a greater proportion of ova were considered viable in underfed FSH-treated ewes when compared to those derived from overfed ewes (McEvoy et al., 1995; Lozano et al., 2003). Differences in reported results could be due to different species or breeds utilized, plane of nutrition and/or length of nutritional treatments.

Excess or reduction in caloric intake result in changes to endogenous lipid content within the oocyte (Metwally et al., 2007; Robker et al., 2011). Mammalian oocytes have an intracellular lipid reserve that, while varying between species and not completely understood in terms of function, appears to be vital for early embryonic development (Dunning and Robker, 2012). Lipid droplets, which serve as triglyceride storage sites, are composed of a neutral lipid core enclosed by a phospholipid monolayer and an outer coat layer that regulates droplet size (Dunning and Robker, 2012). The accumulation of lipids within the oocyte is thought to provide sustaining energy to a preimplantation embryo (Ferguson and Leese, 2006). Triglyceride stores decrease throughout in vitro oocyte maturation in cows (Ferguson and Leese, 1999) and sows (Sturmey and Leese, 2003). Therefore, it has been postulated that a greater cytoplasmic store of lipids might be advantageous to the oocyte as it undergoes high-energy demanding processes, such as fertilization and cleavage (Castaneda et al., 2013). On the other hand, it has been reported that the accumulation of excess intracellular lipids within cells leads to increased concentrations of free fatty acids, which are subject to both oxidative damage and highly reactive lipid peroxidases. These damages can ultimately lead to intracellular organelle damage, predominantly to the endoplasmic reticulum (ER) and mitochondria (Borradaile et al., 2006). Exposure of the ER to high concentrations of free fatty acids causes alterations in ER function and lead to the production and accumulation of unfolded proteins (Diakogiannaki et al., 2008), which then activates the unfolded protein uptake response (UPR). The UPR includes cell arrest, protein degradation (Rutkowski et al., 2004), and if not corrected, can lead to cell apoptosis (Kaufman et al., 1999). Furthermore, studies in obese female mice have demonstrated that obesity induces slower blastocyst development and an altered trophectoderm to inner cell mass

(ICM) ratio (Minge et al., 2008). These oocytes also contain increased lipid content, mitochondrial activity changes, and show signs of ER stress (Wu et al., 2010). However, it remains unclear as to whether nutritional effects on oocytes is a result that occurs during folliculogenesis or is instigated immediately before conception (Dunning and Robker, 2012).

Nutritional Effects on Early Embryonic Development

Fertilization rates and early embryonic development are affected by the mother's nutritional plane. Nutritional effects may be one of many factors leading to the 20 to 30% of early pregnancy loss observed in ewes (Edey, 1969). For nutrient restricted ewes, an increase in unfertilized ova (Rhind et al., 1989), reduced development to morula and blastocysts after IVF (Rhind et al., 1989; Abecia et al., 1995; Borowczyk et al., 2006; Grazul-Bilska et al., 2012), and increased fetal and neonatal mortality in sheep (Wallace et al., 2004) have been reported. Additionally, others have shown that underfeeding resulted in decreased embryonic development during the first two weeks post-fertilization in sheep (Parr et al., 1987; Abecia et al., 1997).

Overfeeding has also resulted in decreased cleavage rates after IVF in ewes (Papadopoulos et al., 2001; Borowczyk et al., 2006; Grazul-Bilska et al., 2006; Zhu et al., 2010). McEvoy et al. (1995) reported that excessive feeding in superovulated ewes resulted in a reduced number of embryos developing to the blastocyst stage. Furthermore, Abecia et al. (2006) hypothesized that "overfeeding during follicle recruitment and oocyte maturation imparts a legacy of embryonic loss and developmental retardation." While reasons for seemingly contradictory results are not readily evident, inconsistent results discussed here may be due to differences in experimental design, such as duration of over/underfeeding, diet composition, time

of nutritional treatment, breed utilized and/or culture conditions (Botkin et al., 1988; Grazul-Bilska et al., 2012)

Angiogenesis

Angiogenic Process

The organization of vascular network structures has enthralled scientists for many years (Carmeliet, 2000; Conway et al., 2001) and Aristotle first described it as such:

> "…the system of blood vessels in the body may be compared to those of water-courses which are constructed in gardens: they start from one source, or spring, and branch off into numerous channels, and then into still more, and so on progressively, so as to carry a supply to every part of the garden".

The body's vessel formation and function, however, is obviously much more complex. The three main processes that initially form and later remodel blood vessels are termed vasculogenesis, angiogenesis, and arteriogenesis (Carmeliet, 2004). Vasculogenesis describes the *de novo* blood vessel formation throughout embryogenesis, while angiogenesis refers to new capillary branching and remodeling from preexisting vessels (Risau and Flamme, 1995). Arteriogenesis denotes existing arterial reconstruction to increase luminal diameter in response to increased blood flow (Heil et al., 2006). In healthy adults, angiogenesis is infrequent, occurring during tissue repair, some pathological conditions and within the female reproductive system (D'Amore and Klagsbrun, 1989; Reynolds et al., 2002).

Vascular changes are possible due to cell differentiation, proliferation, migration and tissue remodeling to form new blood vessel structures (Folkman and Klagsburn, 1987; Risau, 1997). Angiogenesis (Fig. 1.1) first commences with vasodilation, an expansion of the blood

vessel, due to increased nitric oxide (NO) (Carmeliet, 2000) and an increase in vascular permeability (Distler et al., 2002).

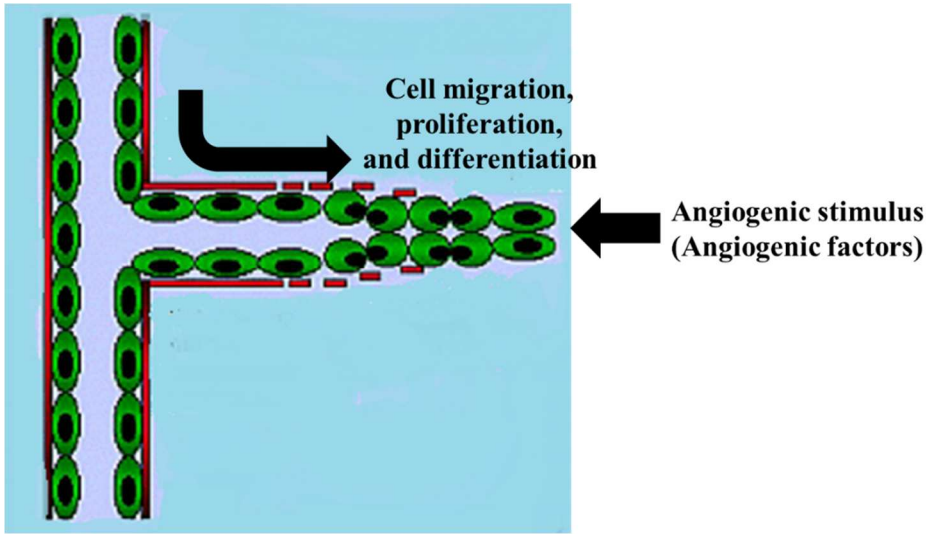


Figure 1.1. Creation of new blood vessels by angiogenesis via endothelial cell migration, proliferation, and creation of new basement membranes.

The increase in NO transcriptionally upregulates vascular endothelial growth factor (VEGF) expression (Kimura et al., 2000). In addition, before endothelial cells can migrate from existing blood vessel, they must be loosened from their vessel matrix. The preexisting vessel's organized destruction is regulated by increased angiopoietin (ANGPT) 2 expression. Similar to ANGPT1, ANGPT2 binds Tie (tyrosine kinase with immunoglobulin and epidermal growth factor homology domain) 2 but, instead of causing phosphorylation, ANGPT2 simply blocks ANGPT1-mediated Tie2 receptor activation. This change is involved in detaching smooth muscle cells (SMC) and loosening the matrix (Maisonpierre et al., 1997; Gale and Yancopoulos, 1999). Enhanced VEGF expression increases blood vessel permeability, allowing extravasation, or leakage, of plasma proteins (Senger et al., 1983; Dvorak et al., 1995), which pass through the blood vessel and are responsible for laying down a basic "scaffold" for the new blood vessel.

However, permeability has to be tightly controlled, as excessive vascular leakage can have negative implications, such as blood vessel collapse (Carmeliet, 2000). The redistribution of platelet endothelial cell adhesion molecule (PECAM)-1 and ANGPT 1 helps modulate the blood vessel wall permeability (Eliceiri et al., 1999; Gale and Yancopoulos, 1999). ANGPT1 is a Tie2 receptor ligand and an inhibitor of vascular permeability, tightening preexisting vessels (Thurston et al., 2000). Several matrix metalloproteinase (MMP) partake in angiogenesis by degrading matrix molecules and by initiating growth factors action within the extracellular matrix (Coussens et al., 1999; reviewed in Nelson et al., 2000). Once the vessel matrix is more permeable and has been infiltrated with plasma proteins, local vessel conditions are favorable for cell proliferation and migration of endothelial cells from the capillary walls (Nguyen et al., 2001; Distler et al., 2002). At this phase, capillary tubules migrate towards angiogenic stimuli (Folkman and Haundenschild, 1979; Gross et al., 1983). Blood vessels are then able to "sprout", or expand, and during further vascular maturation, capillaries can fuse to form larger vessels like arterioles and venules (Karamysheva, 2008).

Ovarian Angiogenesis

Ovarian structures, such as follicles and the corpus luteum (CL), undergo a cyclic growth and regression, and depend upon the continuous hemodynamic changes (Collins et al., 1991; Redmer and Reynolds, 1996; Reynolds et al., 2000; Reynolds and Redmer, 2001).

Follicular development is contingent upon adequate microvasculature (Hirshfield, 1991), as both nutrients and hormones are supplied through diffusion from blood vessels of the thecal layer to the granulosa cell layer, under control of gonadotropins and other factors leading to follicular development. In fact, follicles with reduced DNA synthesis of follicular endothelial

cells are associated with reduced vascular development and atresia (Greenwald, 1989). After the primordial germ cells (PGC) undergo mitosis, they form oogonium and then primary oocytes. While the majority of ovarian follicles recruited into a growing pool will undergo atresia, a few antral follicles (the number varying among species) will continue to develop and become preovulatory follicles. Development and growth of a follicle is dependent upon healthy vascularization. Capillaries of the theca interna form a vast network, known as the capillary wreath, which exists close to the basement membrane. While blood flow decreases at the apex of the Graafian follicle, it actually increases around the base of the follicle (Brannstrom et al., 1998), facilitating follicular rupture and ovulation. As ovulation nears, the wreath capillaries enlarge and form wide sinusoids (Ellinwood et al., 1979; Reynolds et al., 2005). Researchers have postulated that changes in capillary wreath structure allow for permeability of the granulosa cell layer for capillary invasion after ovulation, an event necessary to support quick CL growth (Redmer et al., 2001).

Ovulation is dependent upon the change of blood flow to ovarian follicles. Ovulation has been compared with an inflammatory response, due to the active follicular hyperemia, increase in prostaglandin release, and synthesis of the hyaluronic-rich extracellular matrix that occurs (Richards et al., 2008). Together, the oocyte and surrounding granulosa cells ovulate from the antral follicle.

After ovulation, the vasculature system is rapidly growing in the developing CL (Dharmarajan et al., 1985; Redmer et al., 2001). The angiogenic activity in the developing CL includes destruction of the basement membrane that separated the granulosa and theca cell layers of the Graafian follicle, followed by capillary development into the formerly avascular granulosa

cell layer (Meyer and McGeachie, 1988; Reynolds et al., 2000; Reynolds et al., 2005). This capillary growth is extensive and necessary for providing crucial nutrients to luteal cells, and allowing for efficient outflow of P4 produced and secreted by luteal cells; because of this, the CL receives the majority of the ovarian blood supply (Reynolds et al., 1986; Niswender and Nett, 1988; Reynolds et al., 1994). Maturation of the CL, during the luteal phase, includes stabilization and maturation of blood vessels. During this time, pericytes from the theca capillaries migrate into the granulosa cell area, and multiply so that a great percentage of mature CL vessels contain pericytes (Goede et al., 1998; Reynolds and Redmer, 1999).

The length of angiogenic processes within CL varies among species, but the growth phase lasts eight to ten days in ruminants (Reynolds et al., 2002). The majority, between 50 to 85%, of cellular proliferation that occurs within the developing CL occurs within the microvascular structures (Reynolds et al., 1994, 2002; Christenson and Stouffer, 1997) and the CL pericytes and endothelial cells encompass about 40-70% of the total cell population (Reynolds et al., 2000; Reynolds et al., 2002). Since the majority of the luteal call compostion are vascular cells, the majority of steroidogenic cells in the mature CL are in contact with one or more capillaries (Dharmarajan et al., 1985; Redmer et al., 2001).

Prostaglandin $F_{2\alpha}$ ($PGF_2\alpha$) is the main luteolytic hormone, with the main action being to decrease ovarian blood flow and control luteolysis (Knickerbocker et al., 1988; Niswender et al., 2000). There is evidence that capillary endothelial cells are involved in the luteolytic cascade (Smith and Meidan, 2014) described in more detail in several reviews (Townson, 2006; Skarzynski and Ferreira-Dias, 2008; Miyamoto et al., 2010). $PGF_2\alpha$ enhances permeability of endothelial cell and inhibits cell proliferation within luteal tissues (Davis, 2010). Binding of

$PGF_2\alpha$ to specific membrane receptors on the luteal cell results in rapid activation of phospholipase C, the release of intracellular Ca2+ from the endoplasmic reticulum (ER), and the activation of phosphokinase C (PKC) (Davis and Rueda, 2002). When $PGF_2\alpha$ is administered in vivo, an acute increase in blood flow is observed between 30 minutes and 2 hours post-injection (Acosta et al., 2002), suggesting the initial increase in blood flow induced by $PGF_2\alpha$ injection during mid-luteal cycle commences the luteolytic cascade (Acosta and Miyamoto, 2004). Administration of $PGF_2\alpha$ also induced cyclooxygenase COX-2 expression between one and four hours post-injection in the ewe (Tsai and Wiltbank, 1997), and is hypothesized to be partially responsible for the increase in CL blood flow during mid-cycle $PGF_2\alpha$ release (Acosta and Miyamoto, 2004). Shortly after $PGF_2\alpha$ administration and blood flow increase, capillary alterations appear followed by a decrease in P4 secretion and CL volume (Acosta and Miyamoto et al., 2004).

Regulation of Ovarian Angiogenesis and Angiogenic Factors

For angiogenesis to successfully occur, a complex array of highly regulated angiogenic factors interact with several cell and tissue types (Goede et al., 1998). Angiogenic factors are responsible for stimulating angiogenesis within growing tissues and several are present within the ovary including members of VEGF, ANGPT and FGF families of growth factors (Reynolds et al., 1994; Goede et al., 1998; Neufeld et al., 1999; Grazul-Bilska et al., 2001).

Vascular Endothelial Growth Factor

While there are several molecules known to serve as angiogenesis regulators, not all are specific to vascular endothelial cells and only some are able to directly influence endothelial cells in culture (Karamysheva et al., 2008). One critical signaling cascade for angiogenic

regulation involves vascular endothelial growth factor (VEGF). The homodimeric glycoprotein of 45 kD is a potent mitogen for vascular endothelial cells (Leung et al., 1989; Geva and Jaffe, 2000) and also increases vessel permeability (Senger et al., 1983; Ferrara and Davis-Smith, 1997). Five known VEGF isoforms differ in molecular weight and binding abilities varying amongst isoform. Two isoforms are bound in the extracellular matrix (ECM), while the other three forms are secreted (Park et al., 1993). Growth factors from the VEGF family such as VEGF or PGF exert cellular biological effects by binding to specific receptors, FLT (FMS-like tyrosine kinase) 1, KDR and FLT4 located on endothelial cell membranes (Davis et al., 1996). These receptors belong to the receptor tyrosine kinase (RTK) family of proteins (as reviewed by Karamysheva, 2008).

In vivo studies have demonstrated that VEGF stimulates angiogenesis in the ovaries including the CL (Leung et al., 1989; Redmer et al., 1996, 2001). VEGF expression is not only great during CL vascular development in ovine tissues (Redmer et al., 1996; Redmer et al., 2001), but have also observed that VEGF remains at greater expression during the mid-luteal phase when compared to early-and mid-luteal phases, suggesting VEGF is also a survival factor for newly developed blood vessels (Alon et al., 1995; Moreira et al., 2007). Thecal pericytes expressing VEGF invade the granulosa cell layer within hours of ovulation (Redmer et al., 2001), appearing to be one of the major angiogenic factors in the female reproductive organs (Reynolds et al., 2002). Moreover, thecal-derived perivascular cells may direct the developing vascular system within the early CL due to high VEGF expression (Reynolds et al., 2000; Redmer et al., 2001). In cultured ovine luteal cells, VEGF mRNA expression is increased by 30% after LH treatment (Toutges et al., 1999). In addition, treatment with an antibody to VEGF

neutralizes approximately 65% of the endothelial chemotactic activity of ovine (Reynolds et al., 2000) and primates (Fraser et al., 1999) CL.

Several studies have demonstrated the expression and importance of VEGF within the female reproductive system, for angiogenic and other processes. During follicular recruitment and development, VEGF expression from theca cells in growing follicles is increased by gonadotropins and leads to increased vascular network in theca layer (Mattioli et al., 2001). As folliculogenesis continues, FF, which is a derivative from plasma, accumulates. As theca cell VEGF secretion increases, vascular permeability also increases (Levin et al., 1998), resulting in further accumulation of antral FF (Kamat et al., 1995). Mattioli et al. (2001) observed that follicles with greater E_2 concentrations also had greater VEGF concentrations, and suggested increased vascular permeability induced by VEGF facilitates the delivery of androgen precursors, such as selected lipids, to theca cells. Theca androgens are then aromatized in the avascular granulosa cell layer and form E_2. Further evidence of the importance of VEGF in folliculogenesis and steroidogenesis include the observation that administration of a VEGF antibody to monkey follicles at the periovulatory stage prevents follicle development and E_2 synthesis (Zimmermann et al., 2001). Additionally, reduced follicular vascularity and DNA synthesis are observed in follicles undergoing atresia (Greenwald, 1989) and may actually mediate follicular selection, as dominant follicles have more vascular theca (Zeleznik et al., 1981; Redmer et al., 1996).

Redmer et al. (2001) reported that pericytes produce and secrete VEGF in sheep CL, and CL growth and P4 production depends on proper blood supply stimulated by VEGF (Ferrara et al., 1998). In fact, administering an antibody to VEGF, and thereby neutralizing VEGF activity,

resulted in suppressed luteal endothelial cell proliferation, restricted development of microvasculature, and decreased P4 production in rats (Ferrara et al., 1998) and monkeys (Fraser et al., 1999, 2000).

While VEGF is a potent angiogenic factor, its effects are known to be different in developing versus adult organisms. In fact, the importance of VEGF and its corresponding receptors for vascular development has been well documented in genetically engineered mice lacking these proteins (Fong et al., 1995; Shalaby et al., 1995; Carmeliet et al., 1996). Gerber et al. (1999) demonstrated that VEGF inhibition via administration of mFLT (1-3)-IgG, a soluble VEGF receptor chimeric protein, in newborn mice pups but not in adult mice caused insufficient maturation of blood vessels and significant apoptotic changes. Loss of VEGF dependence may result from development and involvement of pericytes (Gerber et al., 1999). As mentioned previously, VEGF also increases blood vessel permeability (Senger et al., 1983; Dvorak et al., 1995). Under hypoxic conditions, VEGF mRNA expression is increased (Dor et al., 2001), and is also increased by other angiogenic factors, such as fibroblast growth factors (FGF).

Placental Growth Factors

Placental growth factor (PGF) was the second member of the VEGF family discovered and, as the name refers,t was originally cloned from a human placenta cDNA library (Maglione et al., 1991). Similar to other members of the VEGF family, isoforms exist due to alternative splicing (Maglione et al., 1993; Ferrara et al., 2003); it is secreted as a glycosylated homodimer. As for receptor binding, PGF binds VEGFR with almost native affinity (Keyt et al., 1996).

The involvement of PGF in angiogenesis processes has been confirmed through gain-of-function research and was first reported as a pro-angiogenic factor by Ziche et al. (1997). In

transgenic mice overexpressing PGF in epidermal layers, increased number and branching of dermal blood vessels with both a significant increase of smooth muscle surrounding vessels and also vascular leakiness has been obsterved (Odorisio et al., 2002). Gain and loss of function experiments have also demonstrated that PGF stimulates angiogenesis in multiple ways. By acting on growth, migration, and survival of endothelial cells, PGF can stimulate vessel growth directly (Ziche et al., 1997; Carmeliet et al., 2001; Fischer et al., 2007). Placental growth factor also partakes in blood vessel maturation by increasing the proliferation and recruitment of SMC and supporting fibroblast proliferation (Yonekura et al., 1999; Bellik et al., 2005).

Within the adult female reproductive tract, PGF is expressed in the ovary, including granulosa cells, and is present in FF (Hou et al., 2014; Tal et al., 2014). VEGF and PGF concentrations within FF are positively correlated with follicular size, and may mediate ovarian follicle angiogenesis and dominant follicle selection, as well as contribution to oocyte development in women undergoing IVF (Hou et al., 2014). The PGF is expressed in human endothelial cells (Yonekura et al., 1999). In adult PGF knock out (KO) mice, impairment of angiogenesis and arteriogenesis during pathological conditions, such as tumor growth, have been reported (Carmeliet et al., 2001). In a double KO for PGF and endothelial nitric oxide synthase (eNOS), loss of function has also demonstrated the importance of PGF (Papapetropoulos et al., 1997). This enzyme and its product, NO, are a downstream target for VEGF and the mice have hind-limb ischemia and increased death rates. This mouse model represented the first experimental animal model of defective angiogenesis demonstrating the interactions between PGF and eNOS (Gigante et al., 2006). Further, gain and loss of function studies have

demonstrated that PGF promotes angiogenesis by acting directly on endothelial cells, stimulating their growth, migration, and survival (Ziche et al., 1997; Adini et al., 2002; Fischer et al., 2007).

Angiopoietins

While the VEGF signaling system initiates vascular development and remodeling, ANGPT1, 2, and 4 and their corresponding RTK receptors Tie1 and 2 (tyrosine kinase with immunoglobulin and epidermal growth factor homology domains 1 and 2; Dumont et al., 1992; Sato et al., 1993) are involved with capillary maturation and stabilization, permeability, and inflammation (Fukuhara et al., 2008). Specifically, ANGPT1 is expressed in pericytes that cover mature blood vessels (Suri et al., 1996) and ANGPT2 is expressed by endothelial cells and specialized tip cells (leading endothelial cells at the tips of vascular sprouts which coordinate multiple processes during angiogenesis; Siemerink et al., 2013) of sprouting blood vessels (Holash et al., 1999; del Toro et al., 2010). Within endothelial cells, ANGPT2 is stored in secretory granules known as Weibel-Palade bodies. Expression of ANGPT2 is increased in hypoxic conditions or during an inflammatory response (Fiedler et al., 2004). Angiopoietins form unique multimeric structures, and a more complex multimer known as a tetramer, is required for Tie2 activation (Davis et al., 2003). Because of the unique structure of ANGPT, Tie receptor complexes, unlike classic RTK receptors, are believed to be comprised of several receptor units that are activated by multimeric ligands (Davis et al., 2003). When ANGPT1 expression increases, Tie receptors are quickly translocated to cell-cell junctions wherein they arrange to make complexes that are capable of moving across the endothelial cell junctions (Fukuhara et al., 2008; Saharinen et al., 2008). Once activated and in this subcellular location, the ANGPT1-Tie2 complex mediates endothelial cell survival, stabilization, and anti-

inflammatory functions (Fukuhara et al., 2008; Sako et al., 2009). In endothelial cells that are mobile, matrix-bound ANGPT1 activates Tie2 in cell matrices, which mediates changes in adhesion and cell mobility (Fukuhara et al., 2008). As mentioned previously, ANGPT1 is also responsible for controlling vascular permeability and leakage caused by VEGF (Thurston et al., 1999).

Angiopoietin-specific Tie receptors are expressed in the blood and lymphatic endothelium and in some immunological cells, such as Tie2 positive macrophages (TEMs) (Mazzieri et al., 2011). Blood flow and shear stress are considered common regulators of Tie1 expression (Chen-Konak et al., 2003; Porat et al., 2004). Mice offspring deficient in Tie1 receptors die from embryonic day (E) 13.5 to birth; they have edema, hemorrhage, and poor endothelial cell structure (Puri et al., 1995; Sato et al., 1995). Mice offspring lacking Tie2 receptors die at an earlier stage (E10.5), with the most prominent defect being the lack of proper cardiac endothelial lining development and failure of remodeling in capillaries (Dumont et al., 1994).

Chowdhury et al. (2010) reported that the ANGPT proteins are present in both granulosa and theca cells of the ovine ovarian follicle, but and that ANGPT mRNA was consistently expressed at greater concentrations in theca compared to granulosa cells. In addition, ANGPT levels of expression are related to the stage of the estrous cycle and to follicle size (Chowdhury et al., 2010). Vonnahme et al. (2006) observed no change in *ANGPT1* mRNA expression in ovine CL after $PGF_{2\alpha}$-induced regression, while the expression of *ANGPT2* decreased just 8 h post-$PGF_{2\alpha}$ injection. Oppositely, in the bovine CL, researchers have reported *ANGPT1* mRNA decreased and there were no changes in the expression of *ANGPT2* mRNA during luteal

regression. Together, these data demonstrate that the expression pattern of ANGPT in luteal tissues is species specific (Vonnahme et al., 2006).

Local administration of ANGPT to ovarian follicles have demonstrated that ANGPT are critical for both follicular development and ovulation. Administration of anti-ANGPT1 antibody resulted in a delay in rat follicular development (Parborell et al., 2008). Additionally, Xu et al. (2005) demonstrated that delivery of ANGPT2 to the preovulatory follicle not only prevents ovulation, but also terminates the menstrual cycle in rhesus monkeys. Within the CL, ANGPT2 is postulated to be involved in luteal tissue regression, as enhanced expression of *ANGPT2* mRNA occurs in late-luteal tissues when compared to mid-luteal tissues in humans (Sugino et al., 2005).

Basic Fibroblast Growth Factors

Fibroblast growth factors (FGF) were one of the first angiogenic proteins identified (Shing et al., 1984), now having at least 22 factors within the family. Of these known proteins, they are 30 to 70% identical in their primary amino acid sequences (Cross et al., 2001) and are heparin-binding proteins. FGF signaling regulates embryonic development, such as mesenchymal-epithelial signaling (De Moerlooze et al., 2000) and development of organ systems (Yamaguchi et al., 1994), and assists in cell proliferation, differentiation, and survival (Baird et al., 1986). Endothelial cells have been reported to express the FGF-2 gene and synthesize bioactive FGF-2 protein (Schweigerer et al., 1987).

The most studied FGFs, those being FGF-1 (acidic) and FGF-2 (basic), lack cytoplasmic sequences for extracellular secretion, which is in contrast to the majority of growth factors that are capable of being released from producer cells (Gospodarowicz et al., 1974). This varying

system of FGF export has led to varying beliefs for its role in angiogenesis, such as extracellular sequestration and release suggested by Vlodavsky et al. (1991). However, exact export mechanisms of FGF proteins are still not well understood (Lieu et al., 2011). Multiple proteins to which FGFs bind have been identified. First, FGFs bind with high affinity to heparan sulfate proteoglycans (HSPGs), which are located on cellular surfaces and within the extracellular matrix (Vlodavsky et al., 1991). HSPGs modulate FGF effects under in vivo and in vitro conditions (Jaye et al., 1992). Secondly, FGFs bind to four structurally related RTK receptors known as receptor (FGFR)-1, -2, -3, and -4, each with differing functions (Johnson and Williams, 1993). Mice embryos lacking FGFR-1 or -2 expression died prior to gastrulation (Deng et al., 1997; Xu et al., 1998), and FGFR-1 is necessary for both the development and maintenance of embryonic blood vessels (Lee et al., 2000). Disruption of FGFR-3 results in mice offspring with skeletal overgrowth and deafness (Colvin et al., 1996). Lastly, FGFR-4 has been reported to be involved with metabolism and FGFR-4 KO mice have a glucose intolerance (Wu et al., 2009).

While some FGFs are expressed during embryonic development exclusively, for example, FGF-3, 4, 8, 15, and 19, others such as FGF-1, 2, 5-7, 9-14, and 20-23 are expressed in both embryonic and adult tissues (review in Ornitz and Itoh, 2001). Within the ovary, FGF was one of the first angiogenic proteins found (Gospodarowicz et al., 1985) and has been identified in ovaries of livestock species (Stirling et al., 1991; Jablonka-Shariff et al., 1997; Reynolds et al., 2000), and specifically the CL of sheep (Grazul-Bilska et al., 1993; Doraiswamy et al., 1998, Vonnahme et al., 2006). The FGF-2 mRNA pattern of expression in bovine CL closely mimics that of angiogenic activity (Stirling et al., 1991) and FGF-2 mRNA is stimulated by LH, the

major luteotropic hormone in livestock (Redmer et al., 1987, 1988). Moreover, in vitro incubation of luteal conditioned media with an FGF antibody immunoneutralized approximately 80% of angiogenic activity in the cow, sow, and ewe (Reynolds et al., 2000). A study utilizing endothelial cells derived from bovine CL demonstrated that FGF-1 was not expressed in endothelial cells, and that FGF-2 together with VEGF up-regulated both KDR and FLT receptors in vitro (Gabler et al., 2004). Schams et al. (1994) demonstrated that vascular cells express FGF-2 in a phase-dependent manner in the bovine CL, while fibroblasts are known to increase in number in the regressing CL of several species including sheep (Farin et al. 1986, Sawyer et al. 1990). In fact, Vonnhamme et al. (2006) reported that FGF-2 mRNA expression first increased after induction of CL regression and then decreased.

Regulation of Corpora Lutea Function

Corpora Lutea Growth

Before ovulation, the antral follicle consists of a fluid, known as *liquor folliculi*, surrounded by an avascular layer of granulosa cells, a basement membrane and theca layer (as reviewed in Hazzard and Stouffer, 2000). It is evident from many studies that the critical stimulus for luteinization is the luteinizing hormone (LH) surge that initiates ovulation (Kaltenbach et al., 1968; Murphy et al., 2004). Moreover, the LH surge has long-term effects on the steroidogenic capability of luteal cells, including maintenance of cytochrome P450 enzyme (P450scc) and steroidogenic acute regulatory protein (StAR; Juengel et al., 1995; Murphy et al., 2004), both necessary for P4 production.

The CL forms after the oocyte is released from the antrum of the follicle at ovulation, and is the primary source of the gestational-support hormone progesterone (P4) (Diaz et al., 2002).

Luteal formation is essential for early embryonic development, implantation, and viviparity within most mammalian species (Murphy, 2004). This structure is capable of phenomenal growth, rivaling even the fastest growing tumors (Reynolds et al., 2000), due to synergistic changes within vasculature. In fact, during its growth phase, the CL doubles in size and cell number every 60 to 70 hours (Reynolds et al., 1994). The capillaries expand by "sprouting", or penetrating, into the previously avascular granulosa cell layer to form the dense capillary network in the developing luteal tissue, which, within the female reproductive tract, receive some of the highest blood flow rates per unit of tissue and exhibit a high rate of metabolism (Reynolds et al., 1986). The CL is a unique endocrine gland due to its transient nature and is comprised of endothelial, small and large luteal, pericytes, smotth muscle cells, immune and fibroblastic cells (Lei et al., 1991).

Corpora Lutea Differentiation

In livestock species, the granulosa and theca cells contribute to CL morphology (Reynolds et al., 2002), and rapid invasion of these cells, along with thecal-derived perivascular cells (Redmer et al., 2000; Redmer et al., 2001), allow for proper CL vascularization and development. In ewes, small luteal cells are 12 to 20 μm in diameter, believed to be of follicular thecal cell origin, contain LH receptors, respond to both LH or cAMP with a 5 to 15 fold increase in P4 secretion, and often contain lipid droplets (Niswender et al., 2002). Even within the mature CL, theca cells are found in close association with vascular elements and express genes associated with CL differentiation, such as the LH receptor, StAR, and low-density lipoprotein receptors (LDLr). Large luteal cells are greater than 20 μm, are primarily of granulosa cell origin, secrete more P4 than small cells, and while containing LH receptors, are

not responsive to LH or cGMP with increased P4 production (Niswender et al., 2002). There can be as much as a 10-fold increase in granulosa cell volume during luteinization (Smith et al., 1994). Among mammalian species, steroid production (P4) of luteal tissue is two or three times greater than steroid production (E_2) of follicles (Murphy et al., 2004). There are major increases within the functional CL in the expression of proteins that make up both high-density lipoprotein (HDL) and LDL cholesterol importation pathways during luteinization (Plotkin et al., 2002; Niswender et al., 2002), as cholesterol transportation to the inner mitrochondrial membrane is the rate-limiting step in P4 biosynthesis (Murphy et al., 2004).

Corpora Lutea Regression

Prostaglandin $F_2\alpha$ is the primary luteolysin at the late luteal phase of the estrous cycle or at the end of pregnancy in several species (Carlson and Gole, 1978) and is produced by the uterus (as reviewed in McCracken et al., 1999; Diaz et al., 2002). The CL, while refractory to the luteolytic action of $PGF_2\alpha$ in the early luteal phase, becomes responsive to $PGF_2\alpha$ once the CL is mature in several species (Marcinkiewicz et al. 1992; Boiti et al. 1998, 2001; Davis and Rueda, 2002). Activation of luteolysis occurs by $PGF_2\alpha$ binding to its receptor within the large luteal cells (Niswender et al., 2002), which activates phospholipase C, causing the release of intracellular Ca2+ from the endoplasmic reticulum and activating of PKC isoforms (McGuire et al., 1994; Davis and Rueda, 2002). This signaling pathway results in further increase of $PGF_2\alpha$ release from the uterus, and also stimulates the release of oxytocin from the pituitary gland in ruminants (Davis and Rueda, 2002; Sirois et al., 2004). The feedback loops formed between $PGF_2\alpha$ and oxytocin serve as luteolytic signals, with the CL acting as an amplifier (Sirois et al., 2004). Structural luteolysis also results in physical destruction and regression from the ovary

(Davis and Rueda et al., 2002; Bowen-Shauver and Gibori, 2004). This action is stimulated by an acute increase in luteal blood flow as the possible trigger of the luteolytic cascade (Acosta et al., 2002). $PGF_2\alpha$ is released by the uterus and stimulates both NO production and release in peripheral arteriolar vasculature of the mature CL (Friden et al., 2000; Miyamoto et al., 2005). An increase in NO production leads to an increase in blood flow to the CL. This also increases shear stress and $PGF_2\alpha$, the latter increasing angiotensin II secretion from microvilli within the CL (Miyamoto et al., 2005). These changes are partially responsible for later vasoconstriction and regression in the CL.

It should be noted that while $PGF_2\alpha$ is no longer contested as a mammalian luteolysin (Sirois et al., 2004), it remains unclear if it is directly responsible for luteal cell death. In the ewe, luteal cells that produce $PGF_2\alpha$ receptor (FP) are not the first to die during $PGF_2\alpha$-induced luteolysis (Davis and Rueda, 2002). In fact, subluteolytic administration of $PGF_2\alpha$ in ewes can reduce P4 secretion without resulting in a major loss of luteal cells, leading researchers to postulate that the prostaglandin has an indirect effect on luteolysis in certain species (McCracken et al., 1999; Davis et al., 2002).

Arginine

Arginine Effects on Vascular Function

Arginine (Arg) is an amino acid (aa) that was first discovered in seedlings (Schultz and Steiger, 1886) and later in animal proteins (Hedin, 1895). Research commenced in the late 1940's led to the classification of Arg as a semi-essential aa, as dietary Arg was required for growth of the chick (Klose et al., 1938) and rat pup (Wolf and Corley, 1939), but not for healthy adult rats (Borman et al., 1946). Around the same time, researchers also reported that Arg was

required for creatine synthesis (Foster et al., 1939). Arg is a precursor for multiple cell regulators including cell signaling molecules glutamate, agmatine, and NO (Palmer et al., 1998) (Figure 1.2). Polyamines, which are necessary for ion channel function (Williams, 1997), are also derivatives of Arg.

While Arg can be acquired exogenously through the diet, 40% of dietary Arg is catabolized by the small intestine before it can enter the blood stream (Wu and Morris, 1998). The majority of endogenously acquired Arg is derived from the intestinal-renal pathway. Within the small intestine, the enterocyte is responsible for citrulline or arginine synthesis from glutamine and glutamate (Wu and Knabe, 1995). These precursors are catabolized by the small intestine (Windmueller and Spaeth, 1975), both being major precursors for endogenously synthesized Arg (Wu, 1998). The small intestine produces and releases citrulline into the blood circulation wherein it is extracted by the kidneys and stoichiometrically converted to Arg by enzymes arginosuccinate synthase (ASS) and arginosuccinate lyase (ASL) (Dhanakoti et al., 1990). The kidneys synthesize approximately 60% of net Arg in adult mammals (Dhanakoti et al., 1990; Yu et al., 1996). With only 5-15% of endogenous synthesis being *de novo*, the major contributor to Arg flux is whole-body protein turnover, or "protein recycling" (taken from Wu and Morris, 1998). However, evidence supports the theory that Arg synthesized within the urea cycle is not available for hepatic NO synthesis (Pastor et al., 1995) and that Arg from extracellular fluid is the preferred substrate for NO production by endothelial cells (Forstermann et al., 1994; Kurz and Harrison, 1997; Wu et al., 1998).

In Vivo Arginine Supplementation

Arginine supplementation has improved NO-dependent endothelial-stimulated relaxation (Figure 1.2) in patients with high cardiovascular risk factors and common cardiovascular disorders (such as coronary artery disease and heart failure) (Wu and Meininger et al., 2000; Maxwell and Cooke, 2001). Researchers have reported that feeding an Arg- or protein-deficient diet to young rats not only decreases plasma Arg concentrations and also prevents maximal synthesis of NO (Wu et al., 1999). Others have observed that supplementation of rumen-protected Arg to non-pregnant ewes has resulted in increased blood flow to ovaries (Saevre et al., 2011). Recently, however, Kaminski et al. (2015) reported no changes in serum metabolites or hormones in non-pregnant ewes of various nutritional planes treated with Arg.

Supplementation of Arg to pregnant animals (through diet or injected intravenously) has also been investigated, with the goal being to potentially increase NO and, therefore, vasodilation of blood vessels. Sows supplemented with Arg had a 22% increase in the number of live piglets born (Mateo et al., 2007) and by 30% in rats (Zeng et al., 2008). Ewes provided additional Arg during late gestation had increased offspring number (Luther et al., 2009) and lamb birth weights (De Boo et al., 2005) compared to control offspring. Moreover, ewe lambs were 12% heavier at birth when dams were supplemented with Arg i.v. when compared to ewe lambs from untreated dams (McCoard et al., 2013).

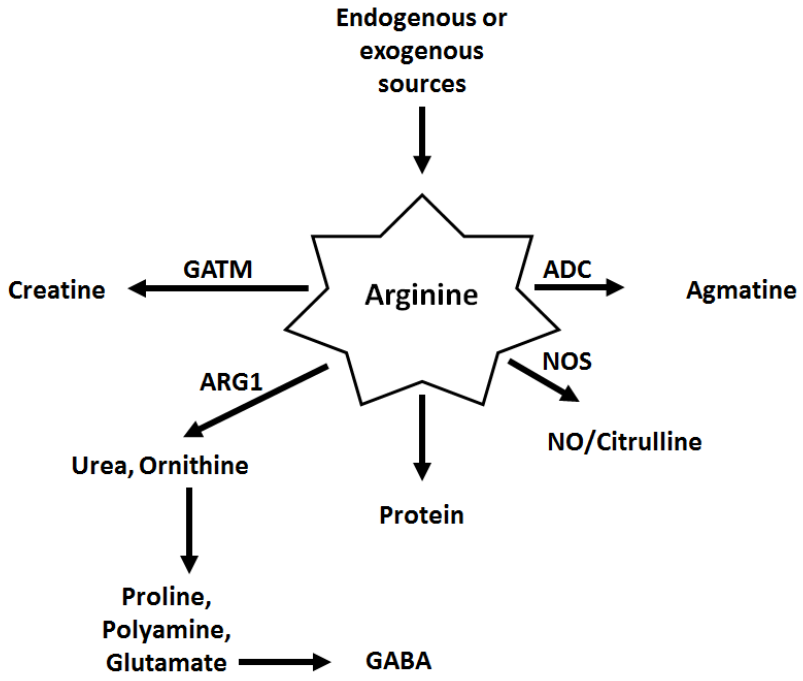


Figure 1.2. Arginine metabolism. Arginine can be derived from exogenous sources (dietary components) or endogenous sources (intestinal-renal pathway) and can be converted to several metabolites including creatine, citrulline, agmatine, and nitric oxide. Arginine can also be utilized within the urea cycle. Conversion of Arg is catalyzed by several enzymes and/or metabolic factors including arginine decarboxylase (ADC), arginase 1 (ARG1), glycine amidinotransferase (GATM), and nitric oxide synthase (NOS). NO, nitric oxide.

When undernourished ewes were provided Arg supplementation, fetal growth restriction was prevented and lamb birth weight was enhanced by 21% (Lassala et al., 2010). However, to our knowledge, the effects of Arg supplementation to non-pregnant animals of different nutritional planes on reproductive processes have not been investigated in detail.

In Vitro Arginine Supplementation

Mouse follicle culture absent of Arg resulted in significantly reduced follicle survival and ovulation, with nearly half the follicles surviving when compared to control cultures containing Arg (Mitchell et al., 2004). Researchers have also observed varying morphological development

when comparing follicles grown with or without Arg, with those grown in the absence of Arg generally having dark granulosa cells, degenerating cells, and/or prematurely releasing a nude oocyte (Mitchell et al., 2004).

However, the addition of Arg to Arg-free medium has resulted in a significantly greater follicle survival rate when compared even to a control, complete medium (Mitchell et al., 2004). Researchers have reported that in vitro Arg supplementation to d 16 ovine conceptuses resulted in a 12-fold increase in proliferation of trophectoderm cells (Kim et al., 2011).

Conversely, elevated Arg inhuman FF has been associated with reduced oocyte retrieval and in vitro fertilization rates (Bodis et al., 2010). Furthermore, researchers have observed attenuated LH-stimualted P4 secretion from granulosa cells obtained from nutritionally compromised ewes (Grazul-Bilska et al., 2015). Together, these studies suggest that there may be an optimal dose of Arg necessary for normal luteal cell function.

Nitric Oxide

Nitric Oxide Production and Function

Nitric oxide is a mediator of many physiological mammalian processes, including reproductive functions (Ignarro et al., 1990; Biswas et al., 1998). Nitric oxide, also known as endothelial-derived relaxing factor (EDRF), was first named by Dr. Robert Furchgott (Furchgott and Zawadski, 1980). Dr. Furchgott, along with other researchers, discovered this free radical gas and some of its various actions; the most researched focusing on vascular smooth muscle cell (VSMC) relaxation. NO is a small biological product of mammalian cells (Nathan, 1992) and has a half-life of less than 5 seconds (Palmer et al., 1987).

There are three isoform enzymes responsible for NO production from Arg. The brain, vascular endothelium, and macrophages were the first areas where these enzymes were discovered to play a specific function, hence the nomenclature (Forstermann et al., 1991; Stuehr and Griffith, 1992). While termed neuronal constitutive NO synthase (NOS I or nNOS), endothelial constitutive NO synthase (NOS III or eNOS), and inducible NO synthase (NOS II or iNOS), these isoforms are distributed across a wide spectrum of cell types (Stuehr and Griffith, 1992). Production of NO induced by iNOS occurs with a delay of approximately six to eight hours after stimulation, yet once induced, iNOS is active for hours or even days and produces NO in 1000-fold larger quantities than the constitutive forms (Huang et al., 1993; Nathan Xie, 1994; Beck et al., 1999; Moncada et al., 2002). Together, nNOS and eNOS are responsible for the continuous basal release of NO and both enzymes require the calcium-calmodulin complex for activation (Griffith and Stuehr, 1995; Snyder, 1995). Researchers have also reported that a cell type can express more than one NOS isoform (Radomski et al., 1990; Suschek et al., 1993).

Increased intracellular calcium (Ca^{2+}) concentrations initiate a cascade of events leading to eNOS or nNOS activation. Intracellular Ca^{2+} both binds calmodulin (CaM) to form calcium-calmodulin complexes and regulates the binding of CaM to target proteins. Therefore, by binding to CaM, Ca^{2+} causes significant conformational changes with the hydrophobic face of the target recognition site (O'Neil and DeGrado, 1990). This allows electron transport from NADPH within the reductase group to the heme-containing active site, facilitating the conversion of O_2 and Arg to NO and citrulline (Abu-Soud et al., 1994). It should be noted that maximal NOS activity is dependent upon substrate availability and co-factors NADPH, flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and tetrahydrobiopterin (THB), and

the availability of these factors determines the cellular rates of NO synthesis (Morris and Billiar, 1994; Davies et al., 1995). These dependencies for NO production implies that metabolic activity pathways either generate or compete for these cofactors (whether during normal physiological or pathological conditions) can be important to determining cellular NO production rates. Additionally, NO can regulate its own activity. Assreuy et al. (1993) demonstrated that NO synthesis is not linear after 20 minutes, suggesting the possibility of a negative feed-back inhibition. Once NO is produced, it can carry out multiple functions in two different ways: 1) by binding and activating soluble guanylyl cyclase (sGC), or 2) through s-nitrosylation (Assreuy et al., 1993).

The short half-life of NO dictates its mediation of many biological functions. NO produced by endothelial cells, diffuses into the blood and binds to the heme group within hemoglobin in the red blood cells, or diffuses to pericytes and SMC (Foster et al., 2009). However, as Foster et al. (2009) explains "…hemes do not generally elicit cellular signaling involving posttranslational modifications of proteins and thus an explanation for most NO-based bioactivity was not apparent." Within smooth muscle cells, NO binds to the heme group of sGC (Stone and Marletta, 1994) (Figure 1.2), which seems to be the most relevant target of NO at low concentrations (Maul et al., 2003). This binding catalyzes dephosphorylation (cleavage of two phosphate groups) of guanosine triphosphate (GTP) to 3′, 5′-cyclic guanosine monophosphate (cGMP), which serves as a second messenger for many important cellular functions and also activates protein kinase G (PKG) (Denninger and Marletta, 1999). Cyclic GMP then induces VSMC relaxation by inhibiting cellular Ca^{2+} entry. Cellular Ca^{2+} entry inhibition by activated cGMP activates K^{+} channels, which leads to VSMC hyperpolarization (Denninger and Marletta,

1999). This activity stimulates a cGMP-dependent kinase, myosin light-chain kinase (MLCK), which dephosphorylates myosin light-chains, as the name implies, and causes muscle cell relaxation and, therefore, dilation of the NO-exposed blood vessel (Denninger and Marletta, 1999) (Figure 1.3). Blood vessel dilation allows for increased amounts of O_2 and nutrients that can be delivered to appropriate tissues. Increased concentrations of cAMP also activates, in turn, three vital signaling pathways, including 1) cGMP-regulated ion channels, 2) cGMP-regulated protein kinases, and 3) cGMP-dependent protein kinases and phosphatases (as reviewed in Beck et al., 1999). Furthermore, NO and its downstream signaling cascades also are responsible for platelet aggregation prevention (Ignarro et al., 1999), increasing endothelial cell proliferation (Morbidelli et al., 1996; Ziche et al., 1997) and migration (Ziche et al., 1994), and act as a neurotransmitter and mediator of the immune system (Bredt et al., 1999) in mammals. Since there is only a limited amount of guanylyl cyclase (GC) enzyme present in one SMC, and once all GC enzyme have been activated, additional NO cannot initiate any further dilation. Instead, unused NO will be appropriated as a nitrosothiol bound to hemoglobin in red blood cells (RBCs) for future usage (Burke, 2002).

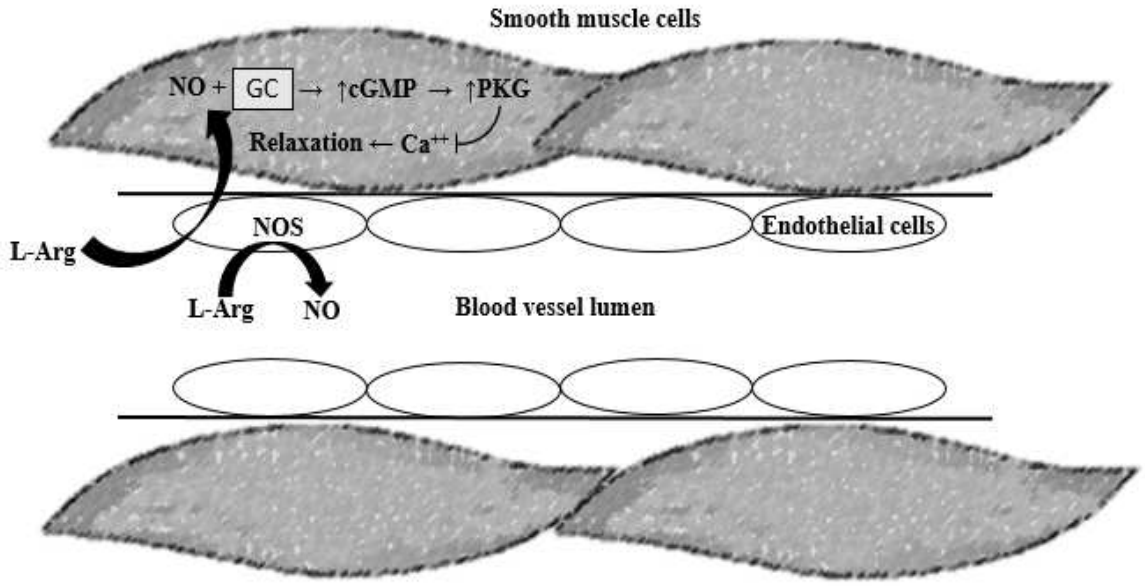


Figure 1.3. Mechanism of blood vessel relaxation induced by NO. L-Arginine (L-Arg) is converted to nitric oxide (NO) via enzyme nitric oxide synthase (NOS) within the endothelial cells. Nitric oxide then diffuses into the smooth muscle cells where it can bind guanylate cyclase (GC), causing an increase in cyclic guanosine monophosphate (cGMP), which then causes an increase in Protein Kinase G (PKG). The increase in PGK reduced intracellular calcium (Ca^{++}) concentrations, allowing for smooth muscle cell relaxation and dilation of the blood vessel.

A second way in which NO can carry out various actions in cell signaling transduction is through s-nitrosylation, through the formation of S-nitroso-proteins, what are collectively termed SNO-proteins. S-nitrosylation is the result of the attachment of NO to a thiol sidechain of, generally, a cysteine (Cyst) residue (Foster et al., 2003; Hess et al., 2005; Sen and Snyder, 2010). Nitric oxide can actually interact with and modify many different cellular molecules including other free radicals, redox regulators (such as glutathionine), and macromolecules (such as DNA and other proteins) and there are over 3,000 SNO-proteins currently known (Benhar et al., 2009). There are many types of post-translational modifications, such as palmitoylation,

phosphorylation, ubiquitation, sumolation, acetylation, and others (Stamler and Meissner, 2001). S-nitrosylation is similar to these in modifications in multiple ways (Stamler and Meissner, 2001); for example, both S-nitrosylation and many of these processes lead to covalent modifications of proteins. However, the post-translational modifications listed above are driven by enzymes, whereas S-nitrosylation is reliant strictly upon the chemical reactivity between the NO molecule and its target. SNO-proteins are often within close proximity to eNOS or nNOS, providing a type of compartmentalization within cells (Lima et al., 2010). While protein S-nitrosylation is vital for many cellular changes, hypo- or hyper-S-nitrosylation of target proteins, and, therefore, alterations in protein function can contribute to many human ailments such as cardiovascular, musculoskeletal, and nervous system disorders (Foster et al., 2009; Gu et al., 2010).

Nitric Oxide and NOS Localization and Expression in Follicles and Corpora Lutea

Detection of both eNOS and/or iNOS in stroma, granulosa, and theca cells of rat, mouse and/or ovine ovarian follicles have been reported (Van Voorhis et al., 1995; Zackrisson et al., 1996; Jablonka-Shariff and Olson, 1997; Grazul-Bilska et al., 2006). Moreover, researchers have reported that eNOS mRNA was expressed in the blood vessels of developing and pre-ovulatory follicles (Grazul-Bilska et al. (2006). A distinct cell-specific eNOS and iNOS expression pattern depends on the stage of the estrous cycle (Zackrisson et al., 1996; Jablonka-Shariff and Olson, 1997; Grazul-Bilska et al., 2006). In sows, eNOS immunoreactivity increased in parallel with follicular size; there were lesser eNOS expression concentrations in smaller follicles and greater expression in larger follicles (Tao et al., 2004). In contrast, no immunoreactivity for iNOS was found in primordial, early antral follicle or the cumulus oocyte

complexes (COC) aspirated from small and medium follicles in porcine ovaries; however, the large follicle-enclosed oocyte had weak immunoreactivity to iNOS (Tao et al., 2004). While most tissues only express iNOS in response to immune stimuli (such as trauma or infection), iNOS expression in mature ovaries has been observed (Zackrisson et al., 1996). Nitric oxide synthase has also been localized in luteal tissues; Vega et al. (1998) detected the expression of eNOS in mid- and early, and to a lesser extent, in late CL. Additionally, an immunohistochemical study in women indicated that both eNOS and iNOS were expressed in mid-luteal CL, eNOS being the more abundant enzyme present. Through immunoreactivity, all NOS isoforms have been observed in luteal cells from early-, mid-, and late-luteal phase bovine CL (Zerani et al., 2013).

Nitric Oxide Functions in Female Reproduction

It should also be noted that one of the major contributors to eNOS expression, and therefore NO production, is VEGF (Ku et al., 1993; Van der Zee et al., 1997; Kroll and Waltenberger, 1998; Duda et al., 2004). Nitric oxide, in turn, assists in VEGF expression regulation and angiogenesis (Chin et al., 1997; Reynolds et al., 2000, 2002; Beckman et al., 2006). An existence of a paracrine loop was postulated, wherein endothelial cells secrete NO, stimulating perivascular VEGF production, which then stimulated endothelial expression of eNOS. Within the ovarian structure, this paracrine loop would provide a "feed-forward" system to increase vasodilation and angiogenesis, which are vital to proper ovarian function (Reynolds et al., 2002).

Researchers have postulated that E_2 is one of the major reproductive hormones regulating NO production in the vasculature and reproductive tract, indicating that E_2 stimulates vascular

NO by upregulating eNOS and/or inhibiting superoxide anion production in ewes (Van Buren et al., 1992) and guinea pigs (Weiner et al., 1994).

Because NO inhibits apoptotic DNA fragmentation in granulosa cells, researchers postulate that NO is an important modulator and vital for follicular development and survival factor (Tilly and Tilly, 1995). Supporting this is the observation that NO concentration within FF increases with follicular development and are correlated with increased E_2 concentrations (Rosselli et al., 1994, 1998).

Studies utilizing NO donors and inhibitors have demonstrated the growth-promoting effects of NO to ovarian follicle granulosa cells since 1) NO donors increase epidermal growth factor receptors on granulosa cells, while 2) NO synthesis inhibitors decrease these same receptors (Hattori et al., 1996). To investigate the role of NO in the ovulatory process, researchers utilized NOS inhibitors N^g-methyl-L-arginine and aminoguanidine, which resulted in a dose-dependent reduction of hCG-induced ovulation in rats (Shukovski and Tsafriri, 1994). The administration of a NO donor reversed the anti-ovulatory effect, suggesting NOS is involved in ovulation. Because NOS inhibition reduced ovulation by approximately 54% in rats, it has been postulated that NO is also involved in the ovulation process.

Nitric oxide regulates oocyte meiotic maturation in rodents (Jablonka-Shariff et al., 1999) and in vitro (Kazuo et al., 2001). Bu et al. (2003) reported data supporting the theory that NO could have dual actions pertaining to meiotic maturation, acting in some instances as a stimulator and in other cases as an inhibitor, depending on NO concentration, and hypothesized that the action of NO on oocyte maturation acts through the cGMP pathway. An oocyte within a Graafian follicle is arrested in the first meiotic division; high concentrations of cAMP and cGMP

in the oocyte are necessary for this arrest phase, and NO is a known cGMP stimulator (Eppig et al., 1991, 2004). An LH surge leads to a systematic decrease in NO concentrations and, therefore, a decrease in cAMP and cGMP, allowing for meiotic resumption (Eppig et al., 2004). When Bu et al. (2003) supplemented medium with sodium nitrioprusside (SNO; a NO donor) polar body extrusion was inhibited. Oppositely, low concentrations of SNP had stimulatory effects on oocytes. This paradox led researchers to postulate that NO can have opposing effects, dependent upon NO concentration, on oocyte maturation. Moreover, the evaluation of nitrite/nitrate concentrations in culture medium revealed that a low concentration of NO (30.69 ± 5.06 μmol) stimulated meiotic maturation of mouse oocytes, while a high concentration (59.88 ± 8.07 μmol) inhibited it. Similar results have been demonstrated in livestock species. The administration of NOS inhibitors during in vitro oocyte pre-maturation and/or at maturation affect embryo development and quality, causing a decrease in bovine (Bilodeau-Goeseels, 2007; Schwarz et al., 2010), porcine (Tao et al., 2004) and caprine (Amale et al., 2013).

Furthermore, Dong et al. (1999) have demonstrated that NO donor diethylenetriamine (DETA-NONOate) increased in a dose-dependent manner P4 secretion by rat ovaries. Moreover, NO was capable of reversing the $PGF_2\alpha$ -induced inhibition of rat ovarian P4 secretion in vitro (Dong et al., 1999), which is contradictory to the findings of other researchers utilizing rat CL (Motta et al., 1999). The latter researchers suggested the involvement of NO in CL regression, reporting that increased NO production increases $PGF_2\alpha$ concentrations during the last two days of luteal development, demonstrating a positive feedback mechanism between the two to ensure CL regression (Motta et al., 1999).

While $PGF_2\alpha$ is considered to be the main luteolytic hormone, several studies examining the direct effects of $PGF_2\alpha$ on bovine populations of steroidogenic luteal cells demonstrate that the addition of $PGF_2\alpha$ alone does not inhibit basal P4 production from large luteal cells (Alila et al., 1988; Meidan et al., 1992; Girsh et al., 1995; Okuda et al., 1998) and actually stimulates P4 secretion from small luteal cells (Okuda et al., 1998; Skarzynski and Okuda, 2000). Due to these results, researchers have suggested that some other biomolecule produced within the CL may be responsible for mediating $PGF_2\alpha$ and CL regression (Meidan et al., 1998; Korzekwa et al., 2006). Therefore, it has been postulated that NO acted as a mediator for $PGF_2\alpha$ in bovine luteal tissue (Jaroszewski et al., 2000), as in vitro NO donors inhibit P4 secretion from bovine luteal cells (Skarzynski and Okuda, 2000; Jaroszewski et al., 2003) and enhances luteolytic activity (Jaroszewski et al., 2000). Supporting the suggestion that NO mediates luteolytic activity are the cumulating results that demonstrate 1) decreased NO concentration via NO inhibitors to luteal cell cultures result in increased P4 concentrations in women (Van Voorhis et al., 1994), rabbits (Gobbetti et al., 1999), and cows (Skarzynski and Okuda, 2000; Jaroszewski et al., 2003), and 2) increased NO concentration within luteal cell culture by administration of NO donors in rats (Motta and Gimeno, 1997), rabbits (Gobbetti et al., 1999) and cows (Jaroszewski et al., 2003). An inverse relationship between treatment of spermine NO complex (an NO donor) to luteal cell cultures and luteal cell viability has been observed in bovine (Jaroszewski et al., 2003). Consequently, P4 inhibition after spermine-NO complex administration may be due to luteal cell viability reduction. Conflicting results regarding NO inhibitors have also been demonstrated; Olson et al. (1996) reported the supplementation of NO synthase inhibitor N^{ω} –nitro-L-arginine methyl ester reduced endogenous NO production, but did not affect P4 production from

luteinized rat ovaries. Differing results reported could be due to different NO donor or inhibitors, culture conditions (e.g., length of culture, different media, different cell concentration and others), and/or species.

Nitric Oxide Synthase Knockout Animals and Reproduction

Female eNOS KO mice lack eNOS within tissues, including lack of immunoreactivity within the ovary (Drazen et al., 1999). Mice treated with L-NAME, (L-NG-nitroarginine methyl ester) a NOS inhibitor, are characterized by shorter estrous cycles (Drazen et al., 1999), impaired follicular and oocyte growth, a decreased follicular cohort, abnormal meiotic maturation, reduced ovulation rates, and increased oocyte death rates when compared to wild type (WT) control mice (Jablonka-Shariff et al., 1999; Drazen et al., 1999; Pallares et al., 2008). Specifically, Jablonka-Shariff (1999) utilized eNOS deficient mice to demonstrate altered steroidogenesis, abnormal estrous cycles, and reduced ovulation in rats. These results, along with other studies demonstrating altered ovarian blood flow after NO inhibition (Jablonka-Shariff et al., 1999), suggest that role of eNOS in the ovulatory process is to enhance ovarian blood flow to the developing follicle (Kassab et al., 1998). Pregnant eNOS KO mice also have narrowed uterine arteries when compared to WT mice (Kulandavelu et al., 2012), demonstrating the importance of NO to arterial structural changes. The absence of functional iNOS through KO has no effect on ovulation in rodents (Hefler and Gregg, 2002). Instead, while expression of iNOS in the rodent ovary is profuse (Hefler and Gregg, 2002), NO derived from iNOS stimulated by interleukin-1β was reported to be a vital mediator of cell death and to stimulate tissue remodeling events during in vivo ovulation (Ellman et al., 1993). Together, these data demonstrate that NO is involved in

the maintenance of ovarian vascular function, as well as the regulation of follicle and luteal tissue angiogenesis (Grazul-Bilska et al., 2006).

References

Abe, L., K. Matsuoka, N. Inoue, M. Taga, T. Kato. 1999. Messenger RNA of neuronal nitric oxide synthase is expressed and possibly functions in mouse oocytes and embryos during preimplantation development. Biomed. Res. 20: 61-5.

Abecia, J. A., J. M. Lozano, F. Forcada, L. Zarazaga. 1997. Effect of level of dietary energy and protein on embryo survival and progesterone production on day eight of pregnancy in Rasa Aragonesa ewes. Anim. Reprod. Sci. 48: 209-18.

Abecia, J. A., S. M. Rhind, T. A. Bramley, S. R. McMillen. 1995. Steroid production and LH receptor concentrations of ovarian follicles and corpora lutea and associated rates of ova wastage in ewes given high and low levels of food intake before and after mating. J. Anim. Sci. 60: 57–62.

Abu-Soud, H. M., L. L. Yoho, D. J. Stuehr DJ. 1994. Calmodulin controls neuronal nitric-oxide synthase by a dual mechanism. J. Biol. Chem. 269: 32047-50.

Acosta, T. J., A. Miyamoto. 2004. Vascular control of ovarian function: ovulation, corpus luteum formation and regression. Anim. Reprod. Sci. 82: 127-40.

Acosta, T. J., N. Yoshizawa, M. Ohtani, A. Miyamoto A. 2002. Local changes in blood flow within the early and midcycle corpus luteum after prostaglandin F2α injection in the cow. Biol. Reprod. 66: 651-8.

Adamiak, S. J., K. Mackie, R. G. Watt, R. Webb, K. D. Sinclair. 2005. Impact of nutrition on oocyte quality: cumulative effects of body composition and diet leading to hyperinsulinemia in cattle. Biol. Reprod. 73:918-26.

Adams, N. R., J. A. Abordi, J. R. Briegel, M. R. Sanders. 1994. Effect of diet on the clearance of estradiol-17-beta in the ewe. Biol. Reprod. 51: 668-74.

Adams, N. R., J. R. Briege, M. R. Sanders, M. A. Blackberry, G. B. Martin. 1997. Level of nutrition modulates the dynamics of oestradiol feedback on plasma FSH in ovariectomized ewes. Anim. Reprod. Sci.47: 59-70.

Adini, A., T. Kornaga, F. Firoozbakht, L. E. Benjamin. 2002. Placental growth factor is a survival factor for tumor endothelial cells and macrophages. Cancer Res. 62: 2749-52.

Amale, M. H., A. Z. Shahne, S. Nasrollahi. 2013. Effects of nitric oxide synthase inhibition on goat oocyte meiotic maturation. Arch. Tierz. 25: 255-63.

Amiridis, G. S., C. A. Rekkas, G. C. Fthenakis, E. Vainas, A. Lymberopoulos, V. Christodoulou, S. Belibasaki. 2002. Progesterone concentration as an indicator of ovarian response to superovulation in Chios ewes. 57:1143-50.

Alila, H. W., J. P. Dowd, R. A. Corradino, W. V. Harris, W. Hansel. 1988. Control of progesterone production in small and large bovine luteal cells separated by flow cytometry. J. Reprod. Fertil. 82: 645-55.

Alon, T., I. Hemo, A. Itin, J. Pe'er, J. Stone, E. Keshet. 1995. Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. Nat. Med. 1: 1024-8.

Armstrong, D. G., J. G. Gong, R. Webb. 2003. Interaction between nutiriton and ovarian activity in cattle: physiological, cellular, and molecular mechanisms. Reprod. Suppl. 61: 403-14.

Armstrong, D. G., T. G. McEvoy, G. Baxter, J. J. Robinson, C. C. Hogg, K. J. Woad, R. kakar, K. D. Sinclair. 2001. Effect of dietary energy and protein on bovine follicular dynamics and embryo production in vitro: associations with the ovarian insulin-like growth factor system. Biol. Reprod. 64;1624-32.

Assreuy, J., F. Q. Cunha, F. Y. Liew, S. Moncada. 1993. Feedback inhibition of nitric oxide synthase activity by nitric oxide. Brit. J. Pharmacol. 108: 833-7.

Baird, A., T. Durkin. 1986. Inhibition of endothelial cell proliferation by type β-transforming growth factor: interactions with acidic and basic fibroblast growth factors. Biochem. Bioph. Res. Co. 138: 476-82.

Baker, J., M. P. Hardy, J. Zhou, C. Bondy, F. Lupu, A. R. Bellve, A. Efstratiadis. 1996. Effects of an Igf1 gene null mutation on mouse reproduction. Mol. Endocrinol. 10: 903-18.

Barash, I. A., C.C Cheung, D.S Weigle, H Ren, E.B Kabigting, J.L Kuijper, D.K Clifton, R.A Steiner. 1996. Leptin is a metabolic signal to the reproductive system. Endocrinol. 137: 3144-7.

Baratta M. 2002. Leptin- From a Signal of Adiposity to a Hormonal Mediator in Peripheral Tissues. Med. Sci. Mon. 8: 282-92.

Barbieri, R. L. 1994. Insulin stimulates androgen accumulation in incubations of minced porcine theca. Gynecol. Obstet. Invest. 37: 265-9.

Barbieri, R. L., A. Makris, K. L. Ryan. 1983. Effects of insulin on steroidogenesis in cultured porcine ovarian theca. Fertil. Steril. 40: 237-41.

Beck, K. F., Eberhardt, W. Frank, S. Huwiler, A. Messmer, U. K. Muhl, J. Pfeilschifter. 1999. Inducible NO synthase: role in cellular signalling. J. Exp. Biol. 202: 645-53.

Beckman, J. D., A. T. Grazul-Bilska, M. L. Johnson, L. P. Reynolds, D. A. Redmer. 2006. Isolation and characterization of ovine luteal pericytes and effects of nitric oxide on pericyte expression of angiogenic factors. Endocrine. 29: 467-76.

Bellik, L., M. C. Vinci, S. Filippi, F. Ledda, A. Parenti. 2005. Intracellular pathways triggered by the selective FLT-1-agonist placental growth factor in vascular smooth muscle cells exposed to hypoxia. Brit. J. Pharmacol. 146: 568-75.

Beitz, D.C. 1985. Physiological and metabolic systems important to growth: an overview. J Anim. Sci. 61: 1-20.

Benhar, M., M. T. Forrester, J. S. Stamler. 2009. Protein denitrosylation: enzymatic mechanisms and cellular functions. Nat. Rev. Mol. Cell. Bio. 10: 721-32.

Bilodeau-Goeseels, S., M. Sasseville, C. Guillemette, F. J. Richard. 2007. Effects of adenosine monophosphate-activated kinase activators on bovine oocyte nuclear maturation in vitro. Mol. Reprod. Dev. 74: 1021-34.

Biswas, S., S. N. Kabir, A. K. Pal. 1998. The role of nitric oxide in the process of implantation in rats. J. Reprod. Fertil. 114: 157-61.

Błogowska, A., B. Krzyzanowska-Swiniarska, S. Zółtowski, I. Rzepka-Górska. 2001. Growth hormone, insulin, IGF-1 and estradiol levels during maturation of internal sex organs before and at menarche. Pol. Gynecol. 72: 1555-9.

Boiti, C., C. Canali, M. Zerani, A. Gobbetti. 1998. Changes in refractoriness of rabbit corpora lutea to a prostaglandin F 2α analogue, alfaprostol, during pseudopregnancy. Prostag. Oth. Lipid. M. 56: 255-64.

Boiti, C., D. Zampini, M. Zerani, G. Guelfi, A. Gobbetti. 2001. Prostaglandin receptors and role of G protein-activated pathways on corpora lutea of pseudopregnant rabbit in vitro. J. Endocrinol. 168: 141-51.

Boland, M. P., P. Lonergon, D. O'Callaghan. 2001. Effect of nutrition on endocrine parameters, ovarian physiology, and oocyte and embryo development. Theriogenology. 55: 1323-40.

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, A. T. Grazul-Bilska. 2006. Effects of plane of nutrition on in vitro fertilization and early embryonic development in sheep. J. Anim. Sci. 84: 1593-9.

Borman, A., T. R. Wood, H. C. Black, E. G. Anderson, M. J. Oesterling, M. Womack, W. C. Rose. 1946. The role of arginine in growth with some observations on the effects of argininic acid. J. Biol. Chem. 166: 585.

Borradaile, N. M., X. Han, J. D. Harp, S. E. Gale, D. S. Ory, J. E. Schaffer. 2006. Disruption of endoplasmic reticulum structure and integrity in lipotoxic cell death. J. Lipid Res. 47: 2726-37.

Bossis, I., R. P. Wettleman, S. D. Welty, J. A. Vizcarra, L. J. Spicer, G. Diskin. 1999. Nutritionally induced anovulation in beef heifers: Ovarian and endocrine function preceding cessation of ovulation. J. Anim. Sci. 77: 1536-46.

Botkin, M.P., R.A. Field, C.L. Johnson. 1988. Sheep and Wool-Science, Production and Management. Prentice Hall, NJ.

Bowen-Shauver, J. M., G. Gibori. 2004. The corpus luteum of pregnancy. *The Ovary*, 201-230.

Brännström, M., U. Zackrisson, H. G. Hagström, B. Josefsson, P. Hellberg, S. Granber, W. P. Collins, T. Bourne. 1998. Preovulatory changes of blood flow in the different regions of the human follicle. Fertil. Steril. 69: 435-42.

Bredt, D. S. 1999. Knocking signalling out of the dystrophin complex. Nat. Cell. Biol. 1: 89-91.

Bu, S., G. Xia, Y. Tao, L. Lei, B. Zhou. 2003. Dual effects of nitric oxide on meiotic maturation of mouse cumulus cell-enclosed oocytes in vitro. Mol. Cell. Endocrinol. 207: 21-30.

Burguera, B., M. E. Couce, G. L. Curran. 2000 Obesity is associated with a decreased of leptin transport across the blood brain barrier in rats. Diabetes. 49: 1219-23.

Burks, D. J., J. F. de Mora, M. Schubert, D. J. Withers, M. G. Myers, H. H. Towery, S. L. Altamuro, C. L. Flint, M. F. White. 2000. IRS-2 pathways integrate female reproduction and energy homeostasis. Nature 407: 377-82.

Burke, T. 2002. Nitric oxide and its role in health and diabetes. Diabetes in Control. Com Newsletter. 76 Part 4.

Carlson, J. C., J. W. D. Gole. 1978. CL regression in the pseudopregnant rabbit and the effects of treatment with prostaglandin F-2a and arachidonic acid. J. Reprod. Fert. 53: 381-7.

Carmeliet, P. 2000. Mechanisms of angiogenesis and arteriogenesis. Nat. Med. 6: 389-96.

Carmeliet, P. 2004. Manipulating angiogenesis in medicine. J. Intern. Med. 255: 538-61.

Carmeliet, P., V. Ferreira, G. Breier, S. Pollefeyt, L. Kieckens, M. Gertsenstein, A. Nagy. 1996. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature. 380: 435-9.

Carmeliet, P., L. Moons, A. Luttun, V. Vincenti, V. Compernolle, M. De Mol, M. Persico. 2001. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. Nat. Med. 7: 575-83.

Casabiell, X., V. Pineiro, M. A. Tome, R. Peino, C. Dieguez, F. F. Casanueva. 1997. Presence of leptin in colostrum and/or breast milk from lactating mothers: a potential role in the regulation of neonatal food intake. J. Clin. Endocrinol. Metab. 82: 4270-3.

Castaneda, C. A., P. Kaye, M. Pantaleon, N. Phillips, R. Fry, M. J. D'Occhio. 2013. Circulating concentrations of leptin, ovarian follicle number, and oocyte lipid content and active mitochondria, in Zebu crossbred cows maintained on standard or improved nutrition. Anim. Reprod. Sci. 140: 7-13.

Chen-Konak, L., Y. Guetta-Shubin, H. Yahav, A. Shay-Salit, M. Zilberman, O. Binah, N. Resnick. 2003. Transcriptional and post-translation regulation of the Tie1 receptor by fluid shear stress changes in vascular endothelial cells. Faseb. J. 17:2121-3.

Chilliard, Y., C. Delavaud, M. Bonnet. Leptin expression in ruminants: Nutritional and physiological regulations in relation with energy metabolism. 2005. Domest. Anim. Endocrinol. 29: 3–22.

Chin, K., Y. Kurashima, T. Ogura, H. Tajiri, S. Yoshida, H. Esumi. 1997. Induction of vascular endothelial growth factor by nitric oxide in human glioblastoma and hepatocellular carcinoma cells. Oncogene. 15: 437-42.

Chowdhury, M. W., R. J. Scaramuzzi, C. P. D. Wheeler-Jones, M. Khalid. 2010. The expression of angiogenic growth factors and their receptors in ovarian follicles throughout the estrous cycle in the ewe. Theriogenology. 2010: 856-72.

Christenson, L. K., R. L. Stouffer. 1997. Follicle-Stimulating Hormone and Luteinizing Hormone/Chorionic Gonadotropin Stimulation of Vascular Endothelial Growth Factor Production by Macaque Granulosa Cells from Pre-and Periovulatory Follicles. J. Clin. Endoc. Metab. 82: 2135-42.

Collins, W. P., D. Jurcovic, T. H. Bourne. 1991. Ovarian morphology, endocrine function and intrafollicular blood flow during the periovulatory period. Hum. Reprod. 6: 319-24.

Colvin, J. S., B. A. Bohne, G. W. Harding, D. G. McEwen, D. M. Ornitz. 1996. Skeletal overgrowth and deafness in mice lacking fibroblast growth factor receptor 3. Nat. Genetics. 12: 390-7.

Conway, K. A., J. C. Rochet, R. M. Bieganski, P. T. Lansbury. 2001. Kinetic stabilization of the α-synuclein protofibril by a dopamine-α-synuclein adduct. Science. 294: 1346-9.

Coop, I. E. 1996. Effect of flushing on reproductive performance of ewes. J. Agr. Sci. 67: 305-23.

Coussens, L. M., W. W. Raymond, G. Bergers, M. Laig-Webster, D. Hanahan. 1999. Inflammatory mast cells up-regulate angiogenesis during squamous epithelial carcinogenesis. Gene. Dev. 13: 1382-97.

Cross, M. J., L. Claesson-Wels. 2001. FGF and VEGF function in angiogenesis: signalling pathways, biological responses and therapeutic inhibition. Trends. Pharmocol. Sci. 22: 201-7.

D'Amore, P. A., M. Klagsbrun. 1989. Angiogenesis: Factors and Mechanisms. In: The Pathobiology of Neoplasia. Ed: A.E. Sirica: New York: Plenum. 513-31.

Dhanakoti, S. N., J. T. Brosnan, G. R. Herzberg, M. E. Brosman. 1990. Renal arginine synthesis: studies in vitro and in vivo. Am. J. Physiol. 259: E437–42.

Da Silva, P., R. P. Aitken, S. M. Rhind, P. A. Racey, J. M. Wallace. 2002. Impact of maternal nutrition during pregnancy on pituitary gonadotrophin gene expression and ovarian development in growth-restricted and normally grown late gestation sheep fetuses. Reprod. 123: 769-77.

Dharmarajan, A. M., N. W. Bruce, G. T. Meyer. 1985. Quantitative ultrastructural characteristics relating to transport between luteal cell cytoplasm and blood in the corpus luteum of the pregnant rat. Am. J. Anat. 172: 87-99.

Davies, M. G., G. J. Fulton, P. O. Hagen. 1995. Clinical biology of nitric oxide. Brit. J. Surg. 82: 1598-1610.

Davis, J. S., B. R. Rueda. 2002. The corpus luteum: an ovarian structure with maternal instincts and suicidal tendencies. Front. Biosci. 7: 1949-78.

Davis, S., N. Papadopoulos, T. H. Aldrich, P. C. Maisonpierre, T. Huang, L. Kovac, A. Xu. 2003. Angiopoietins have distinct modular domains essential for receptor binding, dimerization and superclustering. Nat. Struct. Mol. Biol. 10: 38-44.

Davis, S., T. H. Aldrich, P. F. Jones, A. Acheson, D. L. Compton, V. Jain, G. D. Yancopoulos. 1996. Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. Cell. 87: 1161-9.

Davis, T. L., R. C. Bott, T. L. Slough, J. E. Bruemmer, G. D. Niswender. 2010. Progesterone inhibits oxytocin-and prostaglandin F2alpha-stimulated increases in intracellular calcium concentrations in small and large ovine luteal cells. Biol. Reprod. 82: 282-8.

De Boo, H. A., P. L. Van Zijl, D. E. Smith, W. Kulik, H. N. Lafeber, 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. 52: 270-7.

del Toro, R., C. Prahst, T. Mathivet, G. Siegfried, J. S. Kaminker, B. Larrivee, C. Breant. 2010. Identification and functional analysis of endothelial tip cell–enriched genes. Blood 116: 4025-33.

De Meyts, P. 2004. Insulin and its receptor: structure, function and evolution. Bioessays. 26: 1351-1362.

De Moerlooze, L., B. Spencer-Dene, J. Revest, M. Hajihosseini, I. Rosewell, C. Dickson. 2000. An important role for the IIIb isoform of fibroblast growth factor receptor 2 (FGFR2) in mesenchymal-epithelial signalling during mouse organogenesis. Development. 127. 483-92.

Deng, C., M. Bedford, C. Li, X. Xu, X. Yang, J. Dunmore, P. Leder. 1997. Fibroblast growth factor receptor-1 (FGFR-1) is essential for normal neural tube and limb development. Devel. Biol. 185: 42-54.

Denninger, J. W., M. A. Marletta. 1999. Guanylate cyclase and the NO/cGMP signaling pathway. Biochim. Biophys. Acta. 1411; 334-50.

Diakogiannaki, E., H. J. Welters, N. G. Morgan. 2008. Differential regulation of the endoplasmic reticulum stress response in pancreatic β-cells exposed to long-chain saturated and monounsaturated fatty acids. J. Endocrinol. 197: 553-63.

Diaz, F. J., L. E. Anderson, Y. L. Wu, A. Rabot, S. J. Tsai, M. C. Wiltbank. 2002. Regulation of progesterone and prostaglandin F 2α production in the CL. Mol. Cell. Endocrinol. 191: 65-80.

Diskin, M. G., D. R. Mackey, J. F. Roche, and J. M. Sreenan. 2003. Effects of nutrition and metabolic status on circulating hormones and ovarian follicle development in cattle. Anim. Reprod. Sci. 78: 345-70.

Distler, O., A. Del Rosso, R. Giacomelli, P. Cipriani, M. L. Conforti, S. Guiducci, M. Matucci-Cerinic, M. 2002. Angiogenic and angiostatic factors in systemic sclerosis: increased levels of vascular endothelial growth factor are a feature of the earliest disease stages and are associated with the absence of fingertip ulcers. Arthritis. Res. 4: R11.

Donaldson, L. E., J. M. Bassett, G. D. Thorburn. 1970. Peripheral plasma progesterone concentration of cows during puberty, oestrous cycles, pregnancy and lactation, and the effects of under-nutrition or exogenous oxytocin on progesterone concentration. J. Endocrinol. 48: 599-614.

Dong, Y. L., P. R. Gangula, L. Fang, C. Yallampalli. 1999. Nitric oxide reverses prostaglandin-induced inhibition in ovarian progesterone secretion in rats. Hum. Reprod. 14: 27-32.

Dor, Y., T. D. Camenisch, A. Itin, G. I. Fishman, J. A. McDonald, P. Carmeliet, E. Keshet. 2001. A novel role for VEGF in endocardial cushion formation and its potential contribution to congenital heart defects. Development. 128: 1531-8.

Doraiswamy, V. Y., D. L. Knutson, A. T. Grazul-Bilska, D. A. Redmer, L. P. Reynolds. 1998. Fibroblast growth factor receptor (FGFR)-1 and-2 in the ovine corpus luteum throughout the estrous cycle. Growth Factors. 16: 125-35.

Downing, J. A., R. J. Scaramuzzi. 1991. Nutrient effects on ovulation rate, ovarian function and the secretion of gonadotrophic and metabolic hormones in sheep. J. Reprod. Fertil. Supp. 43: 209-27.

Drazen, D. L., S. L. Klein, A. L. Burnett, E. E. Wallach, J. K. Crone, P. L. Huang, R. J. Nelson. 1999. Reproductive function in female mice lacking the gene for endothelial nitric oxide synthase. Nitric oxide. 3: 366-74.

Duda, D. G., D. Fukumura, R. K. Jain. 2004. Role of eNOS in neovascularization: NO for endothelial progenitor cells. Trends. Mol. Med. 10: 143-5.

Dumont, D. J., G. Gradwohl, G. H. Fong, M. C. Puri, M. Gertsenstein, A. Auerbach, M. L. Breitman. 1994. Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesis of the embryo. Gene. Dev., 8: 1897-909.

Dumont, D. J., T. P. Yamaguchi, R. A. Conlon, J. Rossant, M. L. Breitman. 1992. A novel tyrosine kinase gene located on mouse chromosome 4, is expressed in endothelial cells and their presumptive precursors. Oncogene. 7: 1471-80.

Dunn, T.G., G.E. Moss. 1992. Effects of nutrient deficiencies and excesses on reproductive efficiency of livestock. J. Anim. Sci. 70: 1580-93.

Dunning, K.R., R. L. Robker. 2012. Promoting lipid utilization with l-carnitine to improve oocyte quality. Anim. Reprod. Sci. 134: 69-75.

Dvorak, H. F., L. F. Brown, M. Detmar, A. M. Dvorak. 1995. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. Am. J. Pathol. 46: 1029.

Dyck, G.W., W.M. Palmer, S. Simaraks. 1980. Progesterone and luteinizing hormone concentration in serum of pregnant gilts on different levels of feed consumption. Can. J. Anim. Sci. 60: 877-84.

Edey, T.N. 1969. Prenatal mortality in sheep; a review. Anim. Breed. Abstr. 37: 43-58.

Eliceiri, B. P., R. Paul, P. L. Schwartzberg, J. D. Hood, J. Leng, D. A. Cheresh. 1999. Selective requirement for Src kinases during VEGF-induced angiogenesis and vascular permeability. Mol. Cell. 4: 915-24.

Ellinwood, W. E., T. M. Nett, G. D. Niswender. 1979. Maintenance of the corpus luteum of early pregnancy in the ewe. I. Luteotropic properties of embryonic homogenates. Biol. Reprod. 21: 281-8.

Ellman, C., J. A. Corbett, T. P. Misko, M. McDaniel, K. P. Beckerman. 1993. Nitric oxide mediates interleukin-1-induced cellular cytotoxicity in the rat ovary. A potential role for nitric oxide in the ovulatory process. J. Clin. Invest. 92: 3053.

Eppig, J. J. 1991. Intercommunication between mammalian oocytes and companion somatic cells. Bioessays. 13: 569-74.

Eppig, J. J., M. M. Vivieros, C. Marin-Bivens, R. De La Fuente. 2004. Regulation of mammalian oocyte maturation. In: Leung, PCK.; Adashi, EY., editors. The Ovary. 113-29.

Farin, C. E., C. L. Moeller, H. R. Sawyer, F. Gamboni, G. D. Niswender. 1986. Morphometric analysis of cell types in the ovine corpus luteum throughout the estrous cycle. Biol. Reprod. 35: 1299-308.

Farnworth, P. G. 1995. Gonadotrophin secretion revisited. How many ways can FSH leave a gonadotroph? J. Endocrinol. 145: 387-95.

Ferguson, E. M., H. J. Leese. 1999. Triglyceride content of bovine oocytes and early embryos. J. Reprod. Fertil. 116: 373-8.

Ferguson, E.M., H. J. Leese. 2006. A potential role for triglyceride as an energy source during bovine oocyte maturation and early embryo development. Mol. Reprod. Dev. 73:1195-201.

Ferrara, N., H. Chen, T. Davis-Smyth, H. Gerber, T. Nguyen, D. Peers, V. Chisholm, K. J. Hillan, R. H. Schwall. 1998. Vascular endothelial growth factor is essential for corpus luteum angiogenesis. Nat. Med. 4: 336-40.

Ferrara, N., H. P. Gerber, J. LeCouter. 2003. The biology of VEGF and its receptors. Nat. Med. 9: 669-76.

Ferrara, N., T. Davis-Smyth. 1997. The biology of vascular endothelial growth factor. Endocr. Rev. 18: 4-25.

Ferraretto, L. F., H. Gencoglu, K. S. Hackbar, A. B. Nascimento, F. Dalla Costa, R. W. Bender, J. N. Guenther, M. C. Wiltbank. 2014. Effect of feed restriction on reproductive and metabolic hormones in dairy cows. J. Dairy Sci. 97: 754-63.

Fiedler, U., M. Scharpfenecker, S. Koidl, A. Hegen, V. Grunow, J. M. Schmidt, W. Kriz, G. Thurston, H. G. Augustin. 2004. The Tie-2 ligand angiopoietin-2 is stored in and rapidly released upon stimulation from endothelial cell Weibel-Palade bodies. Blood. 103: 4150-6.

Fischer, C., B. Jonckx, M. Mazzone, S. Zacchigna, S. Loges, L. Pattarini, P. Carmeliet. 2007. Anti-PlGF inhibits growth of VEGF (R)-inhibitor-resistant tumors without affecting healthy vessels. Cell. 131: 463-75.

Folkman, J., C.C. Haundenschild, B.R. Zetter. 1979. Long-term culture of capillary endothelial cells. Proc. Natl. Acad. Sci. 5217-21.

Folkman, J., M. Klagsbrun. 1987. Angiogenic factors. Science. 235: 442-7.

Fong, G. H., J. Rossant, M. Gertsenstein, M. L. Breitman. 1995. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature. 376: 66-70.

Foster, G. L., R. Schoenheimer, D. Ritenberg. 1939. Studies in protein metabolism. V. The utilization of ammonia for amino acid and creatine formation in animals. J. Biol. Chem. 127: 319-27.

Foster, M. W., J. S. Stamler. 2009. Protein S-nitrosylation in health and disease: a current perspective. Trends Mol. Med. 15: 391-404.

Förstermann, U., E. I. Closs, J. S. Pollock, M. Nakane, P. Schwarz, I. Gath, H. Kleinert. 1994. Nitric oxide synthase isoenzymes, characterization, purification, molecular cloning, and functions. Hypertension. 23: 1121-31.

Förstermann, U., J. S. Pollock, H. H. Schmidt, M. Heller, F. Murad. 1991. Calmodulin-dependent endothelium-derived relaxing factor/nitric oxide synthase activity is present in the particulate and cytosolic fractions of bovine aortic endothelial cells. P. Natl. Acad. Sci. 88: 1788-92.

Fraser, H. M., S. F. Lunn, D. Harrison, J. B. Kerr. 1999. Luteal regression in the primate: different forms of cell death during naturaland gonadotropin-releasing hormone antagonist or prostaglandin analogue-induced luteolysis. Biol. Repro. 61: 1468-79.

Friden, B. E., E. Runesson, M. Hahlin. 2000. Evidence for nitric oxide acting as a luteolytic factor in the human corpus luteum. Mol. Hum. Reprod. 6: 397.

Fukuhara, S., K. Sako, T. Minami, K. Noda, H. Z. Kim, T. Kodama, N. Mochizuki. 2008. Differential function of Tie2 at cell–cell contacts and cell–substratum contacts regulated by angiopoietin-1. Nat. Cell. Biol. 10: 513-26.

Furchgott, R. F., J. Zawadzki. 1980. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature. 288: 373-6.

Gabler, C., A. Plath-Gabler, G. J. Killian, B. Berisha, D. Schams. 2004. Expression Pattern of Fibroblast Growth Factor (FGF) and Vascular Endothelial Growth Factor (VEGF) System Members in Bovine Corpus Luteum Endothelial Cells During Treatment with FGF-2, VEGF or Oestradiol. Reprod. Domest. Anim. 321-7.

Gale, N. W., G. D. Yancopoulos. 1999. Growth factors acting via endothelial cell-specific receptor tyrosine kinases: VEGFs, angiopoietins, and ephrins in vascular development. Gene. Dev. 13: 1055-66.

Gerber, H. P., T. H. Vu, A. M. Ryan, J. Kowalski, Z. Werb, N. Ferrara. 1999. VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nat. Med. 5: 623-8.

Geva, E., R. B. Jaffe. 2000. Role of vascular endothelial growth factor in ovarian physiology and pathology. Fertil. Steril. 74: 429-38.

Gigante, D. P., E. C. D. Moura, L. M. V. Sardinha. 2009. Prevalence of overweight and obesity and associated factors, Brazil, 2006. Revista de Saúde Pública. 43: 83-9.

Ginther, O. J., D. R. Bergfelt, M. A. Beg, C. Meira, K. Kot. 2004. In vivo effects of an intrafollicular injection of insulin-like growth factor 1 on the mechanism of follicle deviation in heifers and mares. Biol. Reprod. 70: 99-105.

Girsh, E., Y. Greber, R. Meidan. 1995. Luteotrophic and luteolytic interactions between bovine small and large luteal-like cells and endothelial cells. Biol. Reprod. 52: 954-62.

Gobbetti, A., C. Boiti, C. Canali, M. Zerani. 1999. Nitric oxide synthase acutely regulates progesterone production by in vitro cultured rabbit corpora lutea. J. Endocrinol. 160: 275-83.

Goede, V., T. Schmidt, S. Kimmina, D. Kozian, H. G. Augustin. 1998. Analysis of blood vessel maturation processes during cyclic ovarian angiogenesis. Lab. Invest. 78: 1385-94.

Gong, J.G. 2002. Influence of metabolic hormones and nutrition on ovarian follicle development in cattle: practical implications. Domest. Anim. Endocrin. 23: 229-41.

Gougeon, A. 1996. Regulation of ovarian follicular development in primates: facts and hypotheses. Endocr. Rev. 17: 121-55.

Gospodarowicz, D., J. Cheng, G. M. Lui, A. Baird, F. Esch, P. Bohlen. 1985. Corpus Luteum Angiogenic Factor Is Related to Fibroblast Growth Factor. Endocrinology. 117: 2383-91.

Gospodarowicz, D. 1974. Localisation of a fibroblast growth factor and its effect alone and with hydrocortisone on 3T3 cell growth. Nature. 249 123-7.

Grazul-Bilska, A. T., D. A. Redmer, L. P. Reynolds. 2001. Growth factors during ovarian angiogenesis. In Vascular Morphogenesis in the Female Reproductive System. New York: Birkhauser Boston. 267:131. (Abstr.).

Grazul-Bilska, A. T., D. A. Redmer, S. D. Killilea, J. Zheng, L. P. Reynolds. 1993. Initial characterization of endothelial mitogens produced by bovine corpora lutea from the estrous cycle. Biochem. Cell. Biol. 71: 270-7.

Grazul-Bilska, A. T., E. Borowczyk, J. J. Bilski, L. P. Reynolds, D. A. Redmer, J. S. Caton, K. A. Vonnahme. 2012. Overfeeding and underfeeding have detrimental effects on oocyte quality measured by in vitro fertilization and early embryonic development in sheep. Domest. Anim. Endocrin. 43: 289-98.

Grazul-Bilska, A. T., E. Borowczyk, W. Arndt, J. Evoniuk, M. O'neil, J. J. Bilski, R. M. Weigl, J. D. Kirsch, K. C. Kraft, K. A. Vonnahme, D. A. Redmer, L. P. Reynolds, and J. S. Caton. 2006. Effects of overnutrition and undernutritionn on in vitro fertilization (IVF) and early embryonic development in sheep. Biol. Reprod. 4: 579-87.

Grazul-Bilska, A. T., J. D. Kirsch, J. J. Bilski, K. C. Kraft, E. J. Windorski, J. S. Luther, K. A. Vonnahme, L. P. Reynolds, D. A. Redmer. 2007. Superovulation in sheep: number and weight of the corpora lutea and serum progesterone. Sheep and Goat Research Journal. 22: 26-31.

Greenwald, G. S. 1989. Temporal and topographic changes in DNA synthesis after induced follicular atresia. Biol. Reprod. 41: 175-81.

Griffith, O. W., D. J. Stuehr. 1995. Nitric oxide synthases: properties and catalytic mechanism. Annu. Rev. Physiol. 57: 707-34.

Gross, J. L., D. Moscatelli, D. B. Rifkin. 1983. Increased capillary endothelial cell protease activity in response to angiogenic stimuli in vitro. P. Natl. Acad. Sci. 2623-7.

Gu, Z., T. Nakamura, S. A. Lipton. 2010. Redox reactions induced by nitrosative stress mediate protein misfolding and mitochondrial dysfunction in neurodegenerative diseases. Mol. Neurobiol. 41: 55-72.

Hamm, M. L., G. K. Bhat, W. E. Thompson, D. R. Mann. 2004 Folliculogenesis is impaired and granulosa cell apoptosis is increased in leptin-deficient mice. Biol Reprod. 71: 66-72.

Hamudikuwanda, H., G. Gallo, E. Block, and B. R. Downey. 1996. Adipose tissue progesterone concentrations in dairy cows during late pregnancy and early lactation. Anim. Reprod. Sci. 43:15– 23.

Hattori, K., B. Heissig, Y. Wu, S. Dias, R. Tejada, B. Ferris, S. Rafii. 2002. Placental growth factor reconstitutes hematopoiesis by recruiting VEGFR1+ stem cells from bone-marrow microenvironment. Nat. Med. 81: 841-9.

Hattori, M., K. Sakamoto, N. Fujihara, I. Kojima. 1996. Nitric oxide: a modulator for the epidermal growth factor receptor expression in developing ovarian granulosa cells. Am. J. Physiol. Cell. Ph. 270: C812-8.

Hazzard, T. M., R. L. Stouffer. 2000. Angiogenesis in ovarian follicular and luteal development. Best. Pract. Res. Cl. Ob.14: 883-900.

Hedin, S. G. 1895. Über die Bildung von Arginin aus Proteinkörper. Z. Physiol. Chem.21: 155 68.

Hefler, L. A., A. R. Gregg. 2002. Inducible and endothelial nitric oxide synthase: genetic background affects ovulation in mice. Fertil. Steril. 77. 147-51.

Heil M. 2006. Arteriogenesis versus angiogenesis: similarities and differences. J. Cell. Mol. Med. 10: 45-55.

Hernandez, E.R., A. Hurwitz, A. Vera, A. Pellicer, E. Y. Adashi, D. LeRoith. 1992 Expression of the genes encoding the insulin-like growth factors and their receptors in the human ovary. J. Clin. Endocrinol. Metab. 74: 419-25.

Hess, D. T., A. Matsumoto, S. O. Kim, H. E. Marshall, J. S. Stamler. 2005. Protein S nitrosylation: purview and parameters. Nat. Rev. Mol. Cell. Biol. 6: 150-66.

Hirshfield, A. 1991. Development of follicles in the mammalian ovary. Int. Rev. Cytol. 124: 43 101.

Holash, J. M. P. C., P. C. Maisonpierre, D. Compton, P. Boland, C. R. Alexander, D. Zagzag, S. J. Wiegand. 1999. Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. Science. 284: 1994-8.

Huang, C. C., C. C. Lee, K. S. Hsu. 2004. An investigation into signal transduction mechanisms involved in insulin-induced long-term depression in the CA1 region of the hippocampus. J Neurochem 89: 217-31.

Huang, P. L., T. M. Dawson, D. S. Bredt, S. H. Snyder, M. C. Fishman. 1993. Targeted disruption of the neuronal nitric oxide synthase gene. Cell. 75: 1273-86.

Ignarro, L. J., G. Cirino, A. Casini, C. Napoli. 1999. Nitric oxide as a signaling molecule in the vascular system: an overview. J. Cardiovasc. Pharmacol. 34: 879-86.

Ignarro, L. J., P. A. Bush, G. M. Buga, K. S. Wood, J. M. Fukuto, J. Rajfer. 1990. Nitric oxide and cyclic GMP formation upon electrical field stimulation cause relaxation of corpus cavernosum smooth muscle. Biochem. Biophy. Res. Comm. 170: 843-50.

Jablonka-Shariff, A., L. M. Olson. 1997. Hormonal regulation of nitric oxide synthases and their cell-specific expression during follicular development in the rat ovary. Endocrinol. 138: 460-8.

Jablonka-Shariff A., S. Ravi, N. A. Beltsos. 1999. Abnormal estrous cyclicity after disruption of endothelial and inducible nitric oxide synthase in mice. Biol. Reprod. 61: 171.

Jaroszewski, J. J., D. J. Skarzynski, R. M. Blair, W. Hansel. 2003. Influence of nitric oxide on the secretory function of the bovine corpus luteum: dependence on cell composition and cell-to-cell communication. Exp. Biol. M. 228: 741-8.

Jaroszewski, J. J., W. Hansel. 2000. Intraluteal administration of a nitric oxide synthase blocker stimulates progesterone and oxytocin secretion and prolongs the life span of the bovine corpus luteum. P. Soc. Exp. Biol. Med. 224: 50-5.

Jaye, M., J. Schlessinger, C. A. Dionne. 1992. Fibroblast growth factor receptor tyrosine kinases: molecular analysis and signal transduction. Biochimica et Biophysica Acta (BBA) Molecular Cell Research, 1135: 185-99.

Johnson, D. E., L. T. Williams. 1993. Structural and functional diversity in the FGF receptor multigene family. Adv. Cancer. Res. 1.

Jordan, E. R., L. V. Swanson. 1979. Effect of crude protein on reproductive efficiency, serum total protein and albumin in the high-producing dairy cow. J. Dairy Sci. 62: 58.

Jorritsma, R., M. L. César, J. T. Hermans, C. L. J. J. Krintwagen, P. L. A. M. Vos, T. A. M. Kruip. 2004. Effects of non-esterified fatty acids on bovine granulosa cells and developmental potential of oocytes in vitro. Anim. Reprod. Sci. 81: 225-35.

José-Alfonso, A., C. Sosa, F. Forcada, A. Meikle. 2006. The effect of undernutrition on the establishment of pregnancy in the ewe. Reproduction Nutrition Development, EDP Sciences. 46: 367-78.

Juengel, J. L., B. M. Meberg, A. M. Turzillo, T. M. Nett, G. D. Niswender. 1995. Hormonal regulation of messenger ribonucleic acid encoding steroidogenic acute regulatory protein in ovine corpora lutea. Endocrinology. 136: 5423-9.

Kamat, B. R., L. F. Brown, E. J. Manseau, D. R. Senger, H. F. Dvorak. 1995. Expression of vascular permeability factor/vascular endothelial growth factor by human granulosa and theca lutein cells. Role in corpus luteum development. Am. J. Pathol. 146: 157.

Kaltenbach, C. C., J. W. Graber, G. D. Niswender, A.V. Nalbandov. 1968. Luteotrophic properties of some pituitary hormones in nonpregnant or pregnant hypophysectomized ewes. Endocrinology. 82: 818-24.

Kaminski, S. L. , D. A. Redmer, C. S. Bass, D. H. Keisler, L. S. Carlson, K. A. Vonnahme, S. T. Dorsam, A. T. Grazul-Bilska. 2015. The effects of diet and arginine treatment on serum metabolites and selected hormones during the estrous cycle in sheep. Theriogenology. 83: 808-16.

Karamysheva, A. F. 2008. Mechanisms of angiogenesis. Biochemistry- Moscow. 73: 751-62.

Kassab, S., M. T. Miller, R. Hester, J. Novak, J. P. Granger. 1998. Systemic hemodynamics and regional blood flow during chronic nitric oxide synthesis inhibition in pregnant rats. Hypertension. 31: 315-20.

Kaufman, R. J. 1999. Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. Gene. Dev. 13: 1211-33.

Kazuo, S.K., H. K. Michiharu, T. Keiko, K. Harumi, S. Dinara, T. Kenichi, I. Mutsuo. 2001. Requirement of nitric oxide for murine oocyte maturation, embryo development, and trophoblast outgrowth in vitro. Mol. Reprod. Dev. 58: 262-8.

Keyt, B. A., H. V. Nguyen, L. T. Berleau, C. M. Duarte, J. Park, H. Chen, N. Ferrara. 1996. Identification of vascular endothelial growth factor determinants for binding KDR and FLT-1 receptors Generation of receptor-selective VEGF variants by site-directed mutagenesis. J. Biol. Chem. 271: 5638-46.

Kiani, A. 2013. Temporal changes in plasma concentration of leptin, IGF-1, insulin and metabolites under extended fasting and refeeding conditions in growing lambs. International J. Endocr. Metab. 11: 34-40.

Kile, J., B. Alexander, G. Moss, D. Hallford and T.Nett. 1991. Gonadotropin-releasing hormone overrides the negative effect of reduced dietary energy on gonadotropin synthesis and secretion in ewes. Endocrinology. 128: 843-9.

Kim, J. Y., R. C. Burghardt, G. Wu, G. A. Johnson, T. E. Spencer, F. W. Bazer. 2011. Select nutrients in the ovine uterine lumen. VII. Effects of arginine, leucine, glutamine, and glucose on trophectoderm cell signaling, proliferation, and migration. Biol. Reprod. 84: 62-9.

Kiyma Z, B. M. Alexander, E. A. Van Kirk, W. J. Murdoch, D. M. Hallford, G. E. Moss. Effects of feed restriction on reproductive and metabolic hormones in ewes. J Anim Sci 2004, 82: 2548-57.

Klok, M. D., S. Jakobsdottir, M. L. Drent. 2007. The role of leptin and ghrelin in the regulation of food intake and body weight in humans: a review. Obes. Rev. 8: 21-34.

Klose, A. A., E. L. R. Stockstad, H. J. Almquist. 1938. J. Biol. Chem. 123: 691-8.

Knickerbocker, J. J., M. C. Wiltbank, G. D. Niswender. 1988. Mechanisms of luteolysis in domestic livestock. Dom. Anim. Endocrin. 5: 91-107.

Kolaczynski, J. W., J. P. Ohannesian, R. V. Considine, C. C. Marco, J. F. Caro1996. Response of leptin to short-term and prolonged overfeeding in humans. J. Clin. Endocrinol. Metab. 81: 4162-5.

Korzekwa, A. J., K. Okuda, I. Woclawek-Potocka, S. Murakami, D. J. Skarzynski. 2006. Nitric oxide induces apoptosis in bovine luteal cells. J. Reprod. Dev. 52: 353-61.

Kroll, J., J. Waltenberger. 1998. VEGF-A induces expression of eNOS and iNOS in endothelial cells via VEGF receptor-2 (KDR). Biochem. Biophys. Res. Comm. 252: 743-6.

Ku, D. D., J. K. Zaleski, S. Liu, T. A. Brock. 1993. Vascular endothelial growth factor induces EDRF-dependent relaxation in coronary arteries. Am. J. Physiol. Heart. C. 265: H586-92.

Kulandavelu, S., K. J. Whiteley, D. Qu, J. Mu, S. A. Bainbridge, S. L. Adamson. 2012. Endothelial nitric oxide synthase deficiency reduces uterine blood flow, spiral artery elongation, and placental oxygenation in pregnant mice. Hypertension. 60: 231-8.

Kurz, S., D. G. Harrison. 1997. Insulin and the arginine paradox. J. Clin. Invest. 99: 369–70.

Kwong, W. Y., A. E. Wild, P. Roberts, A. C. Willis, T. P. Fleming. 2000. Maternal undernutrition during the preimplantation period of rat development causes blastocyst abnormalities and programming of postnatal hypertension. Devlop.127: 4195-202.

Lamond, D. R., R. G. Gaddy, S. W. Kennedy. 1972. Influence of season and nutrition on luteal Plasma progesterone in Rambouillet ewes. J. Anim. Sci. 34: 626-9.

Lassala, A., F. W. Bazer, T. A. Cudd, S. D., D. H. Keisler, M. C. Satterfield, T. E. Spencer, G. Wu. 2010. Parenteral administration of L-arginine prevents fetal growth restriction in undernourished ewes. J. Nutr. 140: 1242-8.

Lassoued, N., M. Rekik, M. Mahouachi, B. Hamouda. 2004. The effect of nutrition prior to and during mating on ovulation rate, reproductive wastage, and lambing rate in three sheep breeds. Small. Ruminant. Res. 52: 117-25.

Lee, S. H., D. J. Schloss, J. L. Swain. 2000. Maintenance of vascular integrity in the embryo requires signaling through the fibroblast growth factor receptor. J. Biol. Chem. 275: 33679-87.

Lei, Z. M., N. Chegini, C. V. Rao. 1991. Quantitative cell composition of human and bovine corpora lutea from various reproductive states. Biol. Reprod. 44: 1148-56.

Leung, D. W., G. Cachianes, W. J. Kuang, D. V. Goeddel, N. Ferrara. 1989. Vascular endothelial growth factor is a secreted angiogenic mitogen. Science. 246: 1306-9.

Levin, E. R., G. F. Rosen, D. L. Cassidenti, B. Yee, D. Meldrum, A. Wisot, A. Pedram. 1998. Role of vascular endothelial cell growth factor in Ovarian Hyperstimulation Syndrome. J. Clin. Invest. 102: 1978.

Lieu, C., J. Heymach, M. Overman, H. Tran, S. Kopetz. 2011. Beyond VEGF: inhibition of the fibroblast growth factor pathway and antiangiogenesis. Clin. Cancer Res. 17: 6130-9.

Lima, B., M. T. Forrester, D. T. Hess, J. S. Stamler. 2010. S-nitrosylation in cardiovascular signaling. Circ. Res. 106: 633-46.

Lozano, J. M., P. Lonergan, M. P. Boland, D. O'Callaghan. 2003. Influence of nutrition on the effectiveness of superovulation programmes in ewes: effect on oocyte quality and post-fertilization development. Reproduction. 125: 543-53.

Luther, J. S., E. J. Windorski, J. S. Caton, G. Wu, J. D. Kirsch, K. A. Vonnahme, L. P. Reynolds, C. S. Schauer. 2009. Effects of arginine supplementation on reproductive performance in Rambouillet ewes. Sheep Res. Rep. 50: 11-3.

Mackey, D. R., A .R. G. Wylie, J. M. Sreenan, J. F. Roche, and M. G. Diskin. 2000. The effect of acute nutritional change on follicle wave turnover, gonadotropin, and steroid concentration in beef heifers. J. Anim. Sci. 78 :429-42.

Mackey, D. R., J. M. Screenan, J. F. Roche, and M. G. Diskin. 1999. Effect of acute nutritional restriction on the incidence of anovulation and periovulatory estradiol and gonadotropin concentrations in beef heifers. Biol. Reprod. 61: 1601-7.

Maglione, D., V. Guerriero, G. Viglietto, M. Grazia Ferraro, O. Aprelikova, K. Alitalo, S. Del Vecchio, K. J. Lei, J. Yang Chou, and M. G. Persico. 1993. Two alternative mRNAs coding for the angiogenic factor, placenta growth factor (PlGF), are transcribed from a single gene of chromosome 14.Oncogene. 8: 925-31.

Maglione, D., V. Guerriero, G. Viglietto, P. Delli-Bovi, M. G. Persico. 1991. Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor. P. Nat. A. Sci. 88: 9267-71.

Maisonpierre, P. C., C. Suri, P. F. Jones, S. Bartunkova, S. J. Wiegand, C. Radziejewski, G. D. Yancopoulos. 1997. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science. 277: 55-60.

Marcinkiewicz, J. L., E. S. Moy, J. M. Bahr. 1992. Change in responsiveness of rabbit corpus luteum to prostaglandin F-2α during pregnancy and pseudopregnancy. J. Reprod. Fertil. 94: 305-10.

Mateo, R. D., G. Wu, F. W. Bazer, J. C. Park, I. Shinzato, S. W. Kim. 2007. Dietary L-arginine supplementation enhances the reproductive performance of gilts. J. Nutr. 137: 652-6.

Mattioli, M., B. Barboni, M. Turriani, G. Galeati, A. Zannoni, G. Castellani, P. A. Scapolo. 2001. Follicle activation involves vascular endothelial growth factor production and increased blood vessel extension. Biol. Reprod. 65: 1014-9.

Maul, H., M. Longo, G. R. Saade, R. E. Garfield. 2003. Nitric oxide and its role during pregnancy: from ovulation to delivery. Curr. Pharm. Design. 9: 359-80.

Maxwell, A. J., J. P. Cooke. 2001. L-Arginine: its role in cardiovascular therapy. In: Loscalzo J, Vita JA, editors, Nitric oxide and the cardiovascular system. Totowa (NJ): Humana Press. 547-85.

Mayer, J.P. 2007. Insulin structure and function. Biopolymers. 88: 687-713.

Mazzieri, R., F. Pucci, D. Moi, E. Zonari, A. Ranghetti, A. Berti, M. De Palma. 2011. Targeting the ANG2/TIE2 axis inhibits tumor growth and metastasis by impairing angiogenesis and disabling rebounds of proangiogenic myeloid cells. Cancer. Cell. 19: 512-6.

McCoard, S., F. Sales, N. Wards, Q. Sciascia, M. Oliver, J. Koolaard, D. van der Linden. 2013. Parenteral administration of twin-bearing ewes with L-arginine enhances the birth weight and brown fat stores in sheep. Springer Plus. 2: 684.

McCracken, J. A., E. E. Custer, J. C. Lamsa. 1999. Luteolysis: a neuroendocrine-mediated event. Physiol. Rev. 79: 263-323.

McEvoy, T. G., J. J. Robinson, R. P. Aitken, P. A. Findlay, R. M. Palmer, I. S. Robertson. 1995. Dietary induced suppression of pre-ovulatory progesterone concentrations in superovulated ewes impairs the subsequent in vivo and in vitro development of their ova. Anim. Reprod. Sci. 38; 89-107.

McGee, E. A., C. Sawetawan, I. Bird, W. E. Rainey, B. R. Carr. 1996. The effect of insulin and insulin-like growth factors on the expression of steroidogenic enzymes in a human ovarian thecal-like tumor cell model. Fertil. Steril. 65: 87-93.

McGuire, W. J., J. L. Juengel, G. D. Niswender. 1994. Protein kinase C second messenger system mediates the antisteroidogenic effects of prostaglandin F2 alpha in the ovine corpus luteum in vivo. Biol. Reprod. 51: 800-6.

McNeilly, A. S., J. Brooks, J. R. McNeilly, P. Brown. 1995. Synthesis and release of FSH. J Reprod Fert. Abstract Series. Abstract #S2: p2.

Meidan, R., R. A. Milvae, S. Weiss, N. Levy, A. Friedman. 1998. Intraovarian regulation of luteolysis. J. Reprod. Fertil. Supp. 54: 217-28.

Meidan, R., E. Aberdam, L. Aflalo. 1992. Steroidogenic enzyme content and progesterone induction by cyclic adenosine 3', 5'-monophosphate-generating agents and prostaglandin F2 alpha in bovine theca and granulosa cells luteinized in vitro. Biol. Reprod. 46: 786-92.

Meister, B. 2000. Control of food intake via leptin receptors in the hypothalamus. Vitam. Horm. 59: 265-304.

Metwally, M., T. C. Li, W. L. Ledger. 2007. The impact of obesity on female reproductive function. Obes. Rev. 8: 515-23.

Meyer, G. T., J. K. McGeachie. 1988. Angiogenesis in the developing corpus luteum of pregnant rats: a stereologic and autoradiographic study. The Anatomical Record. 222: 18-25.

Miller, H. M. 1999. The Effects of Feed Intake and Body Fatness on Progesterone Metabolism in Ovariectomized Gilts. J. Anim. Sci, 77: 3253-61.

Minge, C. E., B. D. Bennett, R. J. Norman, R. L. Robker. 2008. Peroxisome proliferator activated receptor-γ agonist rosiglitazone reverses the adverse effects of diet-induced obesity on oocyte quality. Endocrinology. 149: 2646-56.

Mitchell, L. M., C. R. Kennedy, G. M. Hartshorne. 2004. Expression of nitric oxide synthase and effect of substrate manipulation of nitric oxide pathway in mouse ovarian follicles. Hum. Reprod. 19: 1-11.

Miyamoto A., K. Shirasuna, M. P. Wijayagunawardane, S. Watanabe, M. Hayashi, D. Yamamoto, M. Matsui, T. J. Acosta. 2005. Blood flow: a key regulatory component of corpus luteum function in the cow. Domest. Anim. Endocrinol. 29: 329–39.

Miyamoto, K., E. F. Sato, E. Kasahara, M. Jikumaru, K. Hiramoto, H. Tabata, M. Inoue. 2010. Effect of oxidative stress during repeated ovulation on the structure and functions of the ovary, oocytes, and their mitochondria. Free. Radical. Bio. Med. 49: 674-81.

Moncada, S., J. D. Erusalimsky. 2002. Does nitric oxide modulate mitochondrial energy generation and apoptosis? Nat. Rev. Mol. Cell. Bio. 3: 214-20.

Morbidelli, L., C. H. Chang, J. G. Douglas, H. J. Granger, F. Ledda, M. Ziche. 1996. Nitric oxide mediates mitogenic effect of VEGF on coronary venular endothelium. Am. J. Physiol. 270: H411-5.

Moreira, I. S., P. A. Fernandes, M. J. Ramos. 2007. Vascular endothelial growth factor (VEGF) inhibition-a critical review. Anti-Canc. Res. 7: 223-45.

Morris, Jr., S. M., T. R. Billiar. 1994. New insights into the regulation of induced nitric oxide synthesis. Am. J. Physiol. 266: E829-39.

Motta, A. B., A. Estevez, M. F. de Gimeno. 1999. The involvement of nitric oxide in corpus luteum regression in the rat: feedback mechanism between prostaglandin F2α and nitric oxide. Mol. Hum. Reprod. 5: 1011-6.

Motta, A. B., M. A. F. Gimeno. 1997. Nitric oxide participates in the corpus luteum regression in ovaries isolated from pseudopregnant rats. Can. J. Physiol. Pharm. 75: 1335-9.

Murphy, B.B. 2004. Luteinization. In *The Ovary*. E. Y. Adashi, Eds. 185-99. Elsevier Academic Press, San Diego.

Murphy, M. G., W. J. Enngth, M. A. Crow, K. McConnel, L. J. Spicer, M. P. Boland, J. E. Roche. 1991. Effect of dietary intake on pattern of growth of dominant follicles during the oestrous cycle in beef heifers. J. Reprod. Fertil. 92: 333-8.

Nathan, C. 1992. Nitric oxide as a secretory product of mammalian cells. FASEB J. 6: 3051-64.

Nathan, C., Q. W. Xie. 1994. Nitric oxide synthases: roles, tolls, and controls. Cell. 78: 915-8.

Neave, N. 2008. Hormones and behaviour: a psychological approach. Cambridge: Cambridge Univ. Press.

Nelson, A. R., B. Fingleton, M. L. Rothenberg, L. M. Matrisian. 2000. Matrix metalloproteinases: biologic activity and clinical implications. J. Clin. Oncol. 18: 1135.

Neufeld, G., T. Cohen, S. Gengrinovitch, Z. Poltorak. 1999. Vascular endothelial growth factor (VEGF) and its receptors. The FASEB Journal. 13: 9-22.

Nguyen, M., J. Arkell, C. J. Jackson. 2001. Human endothelial gelatinases and angiogenesis. The international journal of biochemistry & cell biology. 33: 960-70.

Niswender, G. D. 2002. Molecular control of luteal secretion of progesterone. Reproduction. 123: 333-9.

Niswender, G. D., J. L. Juengel, P. J. Silva, M. K. Rollyson, E. W. McIntush. 2000. Mechanisms controlling the function and life span of the corpus luteum. Physiol. Rev. 80: 1-29.

Niswender, G. D., T. M. Nett. 1988. The corpus luteum and its control. In: *The Physiology of Reproduction*, Volume 1, Knobi, E. et al. (eds) New York: Raven Press. 489-525.

Nussey, S., S. Whitehead. 2001. Endocrinology: an integrated approach. Oxford: Bios Scientific Publ.

O'Callaghan, D., M. P. Boland. 1999. Nutritional effects on ovulation, embryo development and the establishment of pregnancy in ruminants. Anim. Sci. 68: 299-314.

Odorisio, T., C. Schietroma, M. L. Zaccaria, F. Cianfarani, C. Tiveron, L. Tatangelo, G. Zambruno. 2002. Mice overexpressing placenta growth factor exhibit increased vascularization and vessel permeability. J. Cell Sci. 115: 2559-67.

Okuda, Y., S. Sakoda, H. Fujimura, T. Yanagihara. 1998. Aminoguanidine, a selective inhibitor of the inducible nitric oxide synthase, has different effects on experimental allergic encephalomyelitis in the induction and progression phase. J. Neuroimmunol. 81: 201-10.

Olson, L. M., C. M. Jones-Burton, A. Jablonka-Shariff. 1996. Nitric oxide decreases estradiol synthesis of rat luteinized ovarian cells: possible role for nitric oxide in functional luteal regression. Endocrinology. 137: 3531-9.

O' Neil, K. T., W. F. DeGrado. 1990. A thermodynamic scale for the helix-forming tendencies of the commonly occurring amino acids. Science. 250: 646-65.

Orly, J. 2000. Molecular events defining follicular developments and steroidogenesis in the ovary. In Shupnik MA, ed. Gene Engineering in Endocrinology. Totowa, NJ: Humana Press. 239-75.

Ornitz, D. M., N. Itoh. 2001. Fibroblast growth factors. Genome Biol. 2: 1-12.

Oukonyong, E. E., F. Okutani, S. Takahashi, T. Murata, N. Morioka, H. Kaba, T. Higuchi. 2000. Effect of food deprivation and leptin repletion on the plasma levels of estrogen (E2) and NADPH-d reactivity in the ventromedial and arcuate nuclei of hypothalamus in the female rats. Brain. Res. 887: 70-9.

Pallares, P., R. A. Garcia-Fernandez, L. M. Criado, C. A. Letelier, D. Esteban, J. M. Fernandez Toro, J. M. Flores, A. Gonzalez-Bulnes. 2008. Disruption of the endothelial nitric oxide synthase gene affects ovulation, fertilization and early embryo survival in a knockout mouse model. Reproduction. 136: 573-9.

Palmer, R. M. J., A. G. Ferrige, S. Moncada. 1987. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature. 327: 524-6.

Palmer, R. M. J., D. S. A. Ashton, S. Moncada. 1998. Vascular endothelial cells synthesize nitric oxide from L-arginine. Nat. 33: 664-8.

Papadopoulos, S., P. Lonergan, V. Gath, K. M. Quinn, A. C. O. Evans, D. O'Callaghan, M. P. Boland. 2001. Effect of diet quantity and urea supplementation on oocyte and embryo quality in sheep. Theriogenology. 55: 1059-69.

Papapetropoulos, A., G. Garcia-Cardena, J. A. Madri, W. C. Sessa. 1997. Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. J. Clin. Invest.100: 3131–9.

Parborell, F., D. Abramovich, M. Tesone. 2008. Intrabursal administration of the antiangiopoietin 1 antibody produces a delay in rat follicular development associated with an increase in ovarian apoptosis mediated by changes in the expression of BCL2 related genes. Biol. Reprod. 78: 506-13.

Park, J. E., G. A. Keller, N. Ferrara. 1993. The vascular endothelial growth factor (VEGF) isoforms: differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF. Mol. Bio.Cell. 4: 1317-26.

Parr, R. A. 1992. Nutrition–progesterone interactions during early preg-nancy in sheep. Reprod. Fertil. Dev. 4: 297-300.

Parr, R.A., I. A. Cumming, I. J. Clarke. 1982. Effects of maternal nutrition and plasma progesterone concentrations on survival and growth of the sheep embryo in early gestation. J. Agrie. Sci., Camb. 98: 39-46.

Parr, R. A., I. F. Davis, M. A. Miles, T. J. Squires. 1993. Liver blood flow and metabolic clearance rate of progesterone in sheep. Res. Vet. Sci. 55: 311-6.

Pastor, C. M., M. R. Losser, D. Payen. 1995. Nitric oxide donor prevents hepatic and systemic perfusion decrease induced by endotoxin in anesthetized rabbits. Hepatology. 22: 1547-53.

Pelleymounter, M. A., M. J. Cullen, M. B. Baker, R. Hecht, D. Winters, T. Boone, F. Collins. 1995. Effects of the obese gene product on body weight regulation in ob/ob mice. Science. 269: 540-3.

Pepper, M. S., N. Ferrara, L. Orci, R. Montesano. 1992. Potent synergism between vascular endothelial growth factor and basic fibroblast growth factor in the induction of angiogenesis in vitro. Biochem. Biophys. Rev. Co. 189: 824-31.

Peura, T. T., D. O. Kleemann, S. R. Rudiger, G. S. Nattrass, C. J. McLaughlan, and S. K. Walker. 2003. Effect of nutrition of oocyte donor on the outcomes of somatic cell nuclear transfer in the sheep. Biol. Reprod. 68: 45-50.

Plotkin, D., S. Miller, S. Nakajima, E. Peskin, R. Burkman, D. Richardson, Y. Mitchel. 2002. Lowering low density lipoprotein cholesterol with simvastatin, a hydroxy-3-methylglutaryl-coenzyme a reductase inhibitor, does not affect luteal function in premenopausal women. J. Clin. Endocr. Metab. 87: 3155-61.

Porat, R. M., M. Grunewald, A. Globerman, A. Itin, G. Barshtein, L. Alhonen, K. Alitalo, E. Keshet. 2004. Specific induction of tie1 promoter by disturbed flow in atherosclerosis-prone vascular niches and flow-obstructing pathologies. Circul. Res. 94: 394-401.

Poretsky, L., D. Smith, M. Seibel, A. Pazianos, A. C. Moses, J. S. Flier. 1984. Specific insulin binding sites in the human ovary. J. Clin. Endocrinol. Metab. 59: 809-11.

Poretsky, L., G. Bhargava, M. F. Kalin, S. A. Wolf. 1988. Regulation of insulin receptors in the human ovary: in vitro studies. J. Clin. Endocrinol. Metab. 67: 774-8.

Puri, M. C., J. Rossant, K. Alitalo, A. Bernstein, J. Partanen. 1995. The receptor tyrosine kinase TIE is required for integrity and survival of vascular endothelial cells. The EMBO Journal. 14: 5884.

Rabiee, A. R., K. L. Macmillan, F. Schwarzenberger, P. J. Wright. 2001. Effects of level of feeding and progesterone dose on plasma and faecal progesterone in ovariectomised cows Anim. Reprod. Sci. 73: 185-95.

Radomski, M. W., R. M. J. Palmer, S. Moncada. 1990. An L-arginine to nitric oxide pathway in human platelets regulates aggregation. P. Natl. Acad. Sci. USA. 87: 5193-7.

Redmer, D. A., A. T. Grazul, J. D. Kirsch, L. P. Reynolds. 1988. Angiogenic activity of bovine corpora lutea at several stages of luteal development. J. Reprod. Fert. 82: 627-34.

Redmer, D. A., L. P. Reynolds. 1996. Angiogenesis in the ovary. Rev. Reprod. 1: 182-92.

Redmer, D. A., J. D. Kirsch, A. T. Grazul. 1987. In vitro production of angiotropic factor by bovine corpus luteum: partial characterization of activities that are chemotactic and mitogenic for endometrial cells. In *Regulation of Ovarian and Testicular Function.* 683-8. Springer US.

Redmer, D. A., V. Doraiswamy, B. J. Bortnem, K. Fisher, A. Jablonka-Shariff, A. T. Grazul Bilska, L. P. Reynolds. 2001. Evidence for a role of capillary pericytes in vascular growth of the developing ovine corpus luteum. Biol. Repro. 65: 879-89.

Reynolds, L. P. 1986. Utero-ovarian interactions during early pregnancy: role of conceptus induced vasodilation. J. Anim. Sci. 62, (Suppl. 2): 47-61.

Reynolds, L. P., A. T. Grazul-Bilska, D. A. Redmer. 2000. Angiogensis in the corpus luteum. Endocr. Rev. 12: 1-9.

Reynolds, L. P., A. T. Grazul-Bilska, D. A. Redmer. 2002. Angiogenesis in the female reproductive system: pathological implications. Review Article. Int. J. Exp. Pathol. 83: 151-63.

Reynolds, L. P., D. A. Redmer. 2001. Minireview: Angiogenesis in the placenta, Biol Reprod. 64: 1033-40.

Reynolds, L. P., D. A. Redmer. 1999. Growth and development of the corpus luteum. J. Reprod. Fertil. Suppl. 54: 181-91.

Reynolds, L. P., D. A. Redmer, A. T. Grazul-Bilska. 2005. The Microvasculature of the Ovary. In: *Microvascular Research: Biology and Pathology* (PA D'Amore et al., eds. [D Shepro, Editor-in-Chief]), Elsevier Academic Press, NY (ISBN 0- 12-639510-1).

Reynolds, L. P., A. T. Grazul-Bilska, S. D. Killilea, D. A. Redmer. 1994. Mitogenic factors of corpora lutea. Prog. Growth Factor Res. 5: 159-75.

Rhind, S. M., G. B. Martin, S. McMillen, C. G. Tsonis, A. S. McNeilly. 1989. Effect of level of food intake of ewes on the secretion of LH and FSH and on the pituitary response to gonadotrophin-releasing hormone in ovariectomized ewes. J. Endocr. 121: 325-30.

Rhind, S. M., I. D. Leslie, R. G. Gunn, J. M. Doney, 1985. Plasma FSH, LH, prolactin and progesterone profiles of Cheviot ewes with different levels of intake before and after mating, and associated effects on reproductive performance. Anim. Reprod. Sci. 8: 301-13.

Rhodes , F. M., K. W. Entwistle, J. E. Kinder. 1996. Changes in ovarian function and gonadotrophin secretion preceding the onset of nutritionally induced anoestrus in Bos indicus heifers. Biol Reprod. 55: 1437-43.

Rhodes, F. M., L. A. Fitzpatrick, K. W. Entwistle, and G. De'ath. 1995. Sequential changes in ovarian follicular dynamics in Bos indicus heifers before and after nutritional anoestrus. J. Reprod. Fertil. 104: 41-9.

Richards, J. S., S. C. Sharma, A. E. Falender, Y. H. Lo. 2002. Expression of FKHR, FKHRL1, and AFX genes in the rodent ovary: evidence for regulation by IGF-I, estrogen, and the gonadotropins. Mol. Endocrinol. 16: 580-99.

Richards, J. S., Z. Liu, M. Shimada. 2008. Immune-like mechanisms in ovulation. Trends. Endocrin. Met. 19: 191-6.

Risau, W. 1997. Mechanisms of angiogenesis. Nature. 386: 671-4.

Risau, W., I. Flamme. 1995. Vasculogenesis. Annu. Rev. Cell. Dev. Biol. 11: 73-91.

Robinson, J. J., C. J. Ashworth, J. A. Rooke, L. M. Mitchell, T. G. McEvoy. 2006. Nutrition and fertility in ruminant livestock. Anim. Feed. Sci. Tech. 126: 259-76.

Robker, R. L., L. L. Wu, X. Yang. 2011. Inflammatory pathways linking obesity and ovarian dysfunction. J. Reprod. Immunol. 88: 142-8.

Rooke, J. A., M. Ewen, K. Mackie, M. E. Staine, T. G. McEvoy, K. D. Sinclair. 2004. Effect of ammonium chloride on the growth and metabolism of bovine ovarian granulosa cells and the development of ovine oocytes matured in the presence of bovine granulosa cells previously exposed to ammonium chloride. Anim. Reprod. Sci.1: 53-71.

Rosselli, M., B. Imthurn, E. Macas. 1994. Circulating nitrite/nitrate levels increase with follicular development: indirect evidence for estradiolmediated NO release. Biochem. Biophys. Res. Comm. 212: 1543-52.

Rosselli, M., P. J. Keller, R. K. Dubey. 1998. Role of nitric oxide in the biology, physiology and pathophysiology of reproduction. Hum. Reprod. Update. 4: 3-24.

Rosselli, M., R. K. Dubey, M. A. Rosselli, E. Macas, D. Fink, U. Lauper, P. J. Keller, B. Imthurn. 1996. Identification of nitric oxide synthase in human and bovine oviduct. Mol. Hum. Reprod. 2: 607-12.

Rutkowski, D. T., R. J. Kaufman. 2004. A trip to the ER: coping with stress. Trends. Cell. Biol. 14: 20-8.

Ryan, N. K., C. M. Woodhouse, K. H. Van der Hoek, R. B. Gilchrist, D. T. Armstrong, R. J. Norman. 2002. Expression of leptin and its receptor in the murine ovary: possible role in the regulation of oocyte maturation. Biol Reprod 66: 1548- 54.

Saharinen, P., L. Eklund, J. Miettinen, R. Wirkkala, A. Anisimov, M. Winderlich, A. Nottebaum. 2008. Angiopoietins assemble distinct Tie2 signalling complexes in endothelial cell–cell and cell–matrix contacts. Nature. Cell, Biol. 10: 527-37.

Sahu A. 2004. Leptin signaling in the hypothalamus: emphasis on energy homeostasis and leptin resistance. Front Neuroendocrinol. 24: 225-3.

Sako, K., S. Fukuhara, T. Minami, T. Hamakubo, H. Song, T. Kodama, N. Mochizuki. 2009. Angiopoietin-1 induces Krüppel-like factor 2 expression through a phosphoinositide 3-kinase/AKT-dependent activation of myocyte enhancer factor 2. J. Biol. Chem. 284: 5592-601.

Samoto, T., T. Maruo, H. Matsuo, K. Katayama, E. R. Barnea, M. Mochizuki. 1993. Altered expression of insulin and insulin-like growth factor-I receptors in follicular and stromal compartments of polycystic ovaries. Endocr. J. 40: 413-24.

Sangsritavong, S., D. K. Combs, R. Sartori, and M. C. Wiltbank. 2002. High feed intake increases blood flow and metabolism of progesterone and estradiol-17β in dairy cattle. J. Dairy Sci. 85: 2831-42.

Sato, T. N., Y. Tozawa, U. Deutsch, K. Wolburg-Buchholz, Y. Fujiwara, M. Gendron-Maguire, Y. Qin. 1995. Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. Nature. 376: 70-4.

Sato, T. N., Y. Qin, C. A. Kozak, K. L. Audus. 1993. Tie-1 and tie-2 define another class of putative receptor tyrosine kinase genes expressed in early embryonic vascular system. P. Natl. Acad. Sci. 90: 9355-8.

Sawyer, H. R., K. D. Niswender, T. D. Braden, G. D. Niswender. 1990. Nuclear changes in ovine luteal cells in response to PGF 2α. Domest. Anim. Endocrin. 7: 229-37.

Scaramuzzi, R. J., B. K. Campbell, J. A. Downing, N. R. Kendall, M. Khalid, M. Munoz Gutierrez, A. Somchit. 2006. A review of the effects of supplementary nutrition in the ewe on the concentrations of reproductive and metabolic hormones and the mechanisms that regulate folliculogenesis and ovulation rate. Reprod. Nutr. Dev. 46: 339-54.

Scarth, J. P. 2006. Modulation of the growth hormone-insulin-like growth factor (GH-IGF) axis by pharmaceutical, nutraceutical and environmental xenobiotics: an emerging role for xenobiotic-metabolizing enzymes and the transcription factors regulating their expression. A review. Xenobiotica. 36: 119-218.

Schams, D., B. Berisha. 2004. Regulation of corpus luteum function in cattle–an overview. Reprod. Domest. Ani. 39: 241-51.

Schwarz, K. R. L., P. R. L. Pires, T. H. C. de Bem, P. R. Adona, C. L. V. Leal. 2010. Consequences of nitric oxide synthase inhibition during bovine oocyte maturation on meiosis and embryo development. Reprod. Dom. Anim. 45: 75-80.

Schweigerer, L., G. Neufeld, J. Friedman, J. A. Abraham, J. C. Fiddes, D. Gospodarowicz. 1987. Capillary endothelial cells express basic fibroblast growth factor, a mitogen that promotes their own growth. Nature. 325: 257-9.

Sen, N., S. H. Snyder. 2010. Protein modifications involved in neurotransmitter and gasotransmitter signaling. Trends. Neurosci. 33: 493-502.

Senger, D. R., S. J. Galli, A. M. Dvorak, C. A. Perruzzi, V. S. Harvey, H. F. Dvorak. 1983. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science. 219: 983-5.

Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC. 1995. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature. 376;62-6.

Shing, Y., Folkman, J., Sullivan, R., Butterfield, C., Murray, J., & Klagsbrun, M. (1984). Heparin affinity: purification of a tumor-derived capillary endothelial cell growth factor. Science. 223: 1296-9.

Shukovski, L., A. Tsafriri. 1994. The involvement of nitric oxide in the ovulatory process in the rat. Endo. 135: 2287.

Sinha, M. K., J. Sturis, J. Ohannesian, S. Magosin, T. Stephens, M. L. Heiman, K. S. Polonsky, J. F. Caro. 1996 Ultradian oscillations of leptin secretion in humans. Biochem Biophys Res Commun 228: 733-8.

Siemerink, M. J., I. Klaassen, C. J. Van Noorden, R. O. Schlingemann. 2013. Endothelial tip cells in ocular angiogenesis potential target for anti-angiogenesis therapy. J. Histochem. Cytochem. 61: 101-15.

Sirois, J., K. Sayasith, K. A. Brown, A. E. Stock, N. Bouchard, M. Doré. 2004. Cyclooxygenase 2 and its role in ovulation: a 2004 account. Hum. Reprod. Update. 10: 373-85.

Skarzynski, D. J., G. Ferreira-Dias, K. Okuda. 2008. Regulation of luteal function and corpus luteum regression in cows: hormonal control, immune mechanisms and intercellular communication. Reprod. Domest. Anim. 43: 57-65.

Skarzynski, D. J., K. Okuda. 2000. Different actions of noradrenaline and nitric oxide on the output of prostaglandins and progesterone in cultured bovine luteal cells. Prost. Oth. Lipid. M. 35-47.

Smith, D. L., B. M. Stinefelt, K. P. Blemings, M. E. Wilson. 2006. Diet-induced alterations in progesterone clearance appear to be mediated by insulin signaling in hepatocytes. J. Anim. Sci. 84: 1102-9.

Smith, G. W., R. Meidan. 2014. Ever-changing cell interactions during the life span of the corpus luteum: Relevance to luteal regression. Reprod. Biol. 14: 75-82.

Smith, M. F., E. W. McIntush, G. W. Smith. 1994. Mechanisms associated with corpus luteum development. J. Anim. Sci. 72: 1857-72.

Snijders, S. E., P. Dillon, D. O'Callaghan, M. P. Boland. 2000. Effect of genetic merit, milk yield, body condition and lactation number on in vitro oocyte development in dairy cows. Theriogenology. 53: 981-9.

Snyder, S.H. 1995. NO endothelial NO. Nature. 377: 196-7.

Sonksen, P., J. Sonksen. 2000. Insulin: understanding its action in health and disease. Br. J. Anaesth. 85: 69-79.

Spicer, L. J., J. Alonzo, C. S. Chamberlain. 2001. Effects of thyroid hormones on bovine granulosa and thecal cell function in vitro: dependence on insulin and gonadotropins. J. Dairy Sci. 84: 1069-76.

Stamler, J. S., G. Meissner. 2001. Physiology of nitric oxide in skeletal muscle. Physiol. Rev. 81: 209-37.

Stirling, D., M. R. Waterman, E. R. Simpson. 1991. Expression of mRNA encoding basic fibroblast growth factor, bFGF, in bovine corpora lutea and cultured luteal cells J. Reprod. Fertil. 91: 1-8.

Stone, J. R., M. A. Marletta. 1994. Soluble guanylyl cyclase from bovine lung; activation with nitric oxide and carbon monoxide and spectral characterization of the ferrous and ferric states. Biochem. 33: 5636-40.

Stuehr, D. J., O. W. Griffith. 2006. Mammalian nitric oxide synthases. Adv. Enzymol. Ramb. 287-346.

Sturmey, R. G., H. J. Leese. 2003. Energy metabolism in pig oocytes and early embryos. Reproduction. 126: 197-204.

Sugino, N., T. Suzuki, A. Sakata, I. Miwa, H. Asada, T. Taketani, Y. Yamagamata, H. Tamura. 2005. Angiogenesis in the human corpus luteum: changes in expression of angiopoietins in the corpus luteum throughout the menstrual cycle and in early pregnancy. J. Clin. Endocrin. Metab. 90: 6141-8.

Suri, C., J. McClain, G. Thurston, D. M. McDonald, H. Zhou, E. H. Oldmixon, G. D. Yancopoulos. 1998. Increased vascularization in mice overexpressing angiopoietin-1. Science: 282: 468-71.

Suschek C, Rothe H, Fehsel K, Enczmann J, Kolb BV. 1993. Induction of a macrophage-like nitric oxide synthase in cultured rat aortic endothelial cells. IL-1b mediated induction regulated by tumor necrosis factora and IFN-g. J. Immunol. 151;3283–3291.

Tal, R., D. B. Seifer, R. V. Grazi, H. E. Malter. 2014. Follicular fluid placental growth factor is increased in polycystic ovarian syndrome: correlation with ovarian stimulation. Reprod Biol Endocrinol. 20: 12-82.

Tao, Y., Z. Fu, M. Zhang, G. Xia, J. Yang, H. Xie. 2004. Immunohistochemical localization of inducible and endothelial nitric oxide synthase in porcine ovaries and effects of NO on antrum formation and oocyte meiotic maturation. Mol. Cell. Endocrin. 222: 93-103.

Tatman, W.R., Judkins, M.B., Dunn, T.G., Moss, G.E. 1990. Luteinizing hormone in nutrient restricted ovariectomized ewes. J. Anim. Sci. 68: 1097-102.

Thurston, G., C. Suri, K. Smith, J. McClain, T. N. Sato, G. D. Yancopoulos. D. M. McDonald. 1999. Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1. Science. 286: 2511-4.

Thurston, G., J. S. Rudge, E. Ioffe, H. Zhou, L. Ross, S. D. Croll, G. D. Yancopoulos. 2000. Angiopoietin-1 protects the adult vasculature against plasma leakage. Nature Med. 6: 460-3.

Tilly, J. L., K. I. Tilly. 1995. Inhibitors of oxidative stress mimic the ability of follicle Stimulating hormone to suppress apoptosis in cultured rat ovarian follicles. Endocrinology. 136: 242-52.

Toutges, M. J., A. T. Grazul-Bilska, J. D. Kirsch, L. P. Reynolds, D. A. Redmer. 1999. Effects of uteinizing hormone (LH) and oxygen (O2) levels on vascular endothelial growth factor (VEGF) mRNA expression by ovine luteal cells in culture. Biol. Reprod. 60: 280.

Townson, D. H. 2006. Immune cell–endothelial cell interactions in the bovine corpus luteum. Integr. Comp. Bio. 46: 1055-9.

Tsai, S. J., M. C. Wiltbank. 1997. Prostaglandin F2alpha induces expression of prostaglandin G/H synthase-2 in the ovine corpus luteum: a potential positive feedback loop during luteolysis. Biol. Reprod. 57: 1016-22.

Van Buren, G. A., D. S. Yang, K. E. Clark. 1992. Estrogen-induced uterine vasodilatation is antagonized by L-nitroarginine methyl ester, an inhibitor of nitric oxide synthesis. A. J. Obstet Gynecol. 167: 828-33.

van der Zee, R., T. Murohara, Z. Luo, F. Zollmann, J. Passeri, C. Lekutat, J. M. Isner. 1997. Vascular endothelial growth factor/vascular permeability factor augments nitric oxide release from quiescent rabbit and human vascular endothelium. Circulation. 95: 1030-7.

Van Voorhis, B. J., K. Moore, P. J. Strijbos, S. Nelson, S. A. Baylis, D. Grzybicki, C. P. Weiner. 1995. Expression and localization of inducible and endothelial nitric oxide synthase in the rat ovary. Effects of gonadotropin stimulation in vivo. J. Clin. Invest. 96: 2719.

Van Voorhis, B. J., M. S. Dunn, G. D. Snyder, C. P. Weiner. 1994. Nitric oxide: an autocrine regulator of human granulosa cell steroidogenesis. Endocrinol. 135: 1799-1806.

Vega, M., M. C. Johnson, H. A. Diaz, L. R. Urrutia, J. L. Troncoso, L. Devoto. 1998. Regulation of human luteal steroidogenesis in vitro by nitric oxide. Endocinol. 8: 185-91.

Viana, K. S., M.C. Caldas-Bussiere, S. G. C. Matta, M. R. Faes, C. P. de Carvalho, C. R. Quirino. 2007. Effect of sodium nitroprusside, a nitric oxide donor, on the in vitro maturation of bovine oocytes. Anim. Reprod. Sci. 102: 217-27.

Viñoles, C., B. Paganoni, K. M. M. Glover, J. T. B. Milton, D. Blache, M. A. Blackberry, G. B. Martin. 2010. The use of a 'first wave' model to study the effect of nutrition on ovarian follicular dynamics and ovulation rate in the female sheep. Reproduction. 140: 865-74.

Vlodavsky, I., R. Ishai-Michaeli, P. Bashkin, E. Levi, G. Korner, R. Bar-Shavit, Z. Fuks, M. Klagsbrun. 1991. Extracellular matrix-resident basic fibroblast growth factor: Implication for the control of angiogenesis. J. Cell. Bioch. 45: 167-76.

Vonnahme, K. A., D. A. Redmer, E. Borowczyk, J. J. Bilski, J. S. Luther, M. L. Johnson, L. P. Reynolds, A. T. Grazul-Bilska. 2006. Vascular composition, apoptosis, and expression of angiogenic factors in the corpus luteum during prostaglandin F2α-induced regression in sheep. Reproduction. 131: 1115-26.

Wade, G. N., J. E. Jones. 2005. Neuroendocrinology of nutritional infertility. Am. J. Physiol. Regul. Integr. Comp. Physiol. 287: R1277-96.

Wallace, J. M., J. S. Milne, R. P. Aitken. 2004. Maternal growth hormone treatment from day 35 to 80 of gestation alters nutrient partitioning in favor of uteroplacental growth in the overnourished adolescent sheep. Biol. Reprod. 70: 1277-85.

Weigle, D. S., P. B. Duell, W. E. Connor, R. A. Steiner, M. R. Soules, J. L. Kuijper. 1997. Effect of fasting, refeeding, and dietary fat restriction on plasma leptin levels. J. Clin. Endocrinol. Metab. 82: 561-5.

Weiner, C. P., I. Lizasoain, S. A. Baylis, R. G. Knowles, I. G. Charles, S. Moncada. 1994. Induction of calcium-dependent nitric oxide synthases by sex hormones. P. Nat. Acad. Sci. 91: 5212-6.

Williams, A. H., I. A. Cumming. 1982. Inverse relation¬ ship between concentration of progesterone and nutrition in ewes. J. Agri. Sci. Camb. 98: 517-22.

Williams, K. 1997. Interactions of polyamines with ion channels. Biochem. J. 325: 289-97.

Willis, D., H. Mason, C. Gilling-Smith, S. Franks. 1996. Modulation by insulin of follicle stimulating hormone and luteinizing hormone actions in human granulosa cells of normal and polycystic ovaries. J. Clin. Endocrinol. Metab. 81: 302-9.

Windmueller, H. G., A. E. Spaeth. 1975. Intestinal metabolism of glutamine and glutamate from the lumen as compared to glutamine from blood. Arch. Biochem. Biophys. 171: 662-72.

Wolf, P. A., R. C. Corley. 1939. Significance of amino acids for the maintenance of nitrogen balance in the adult white rat. Am. J. Physiol. 127: 589-96.

Wrenzycki, C., P. De Sousa, E. W. Overstrom, R. T. Duby, D. Herrmann, A. J. Watson, H. Niemann H, D. O'Callaghan, M. P. Boland. 2000. Effects of superovulated heifer diet type and quantity on relative mRNA abundances and pyruvate metabolism in recovered embryos. J. Reprod. Fertil. 118: 69-78.

Wu, G. 1998. Intestinal mucosal amino acid catabolism. J. Nutr. 128: 1249-52.

Wu, G., C.J. Meininger. 2000. Arginine Nutrition and Cardiovascular Function. J. Nutri. 130: 2626-9.

Wu, G., D. A. Knabe. 1995. Arginine synthesis in enterocytes of neonatal pigs. Am. J. Physiol. 269: R621-9.

Wu, G., N. E. Flynn, S. P. Flynn, C. A. Jolly, P. K. Davis. 1999. Dietary protein or arginine deficiency impairs constitutive and inducible nitric oxide synthesis by young rats. J. Nutr. 129: 1347-54.

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

Wu, L. L. Y., Dunning, K. R., Yang, X., Russell, D. L., Lane, M., Norman, R. J., R. L. Robker. 2010. High-fat diet causes lipotoxicity responses in cumulus-oocyte complexes and decreased fertilization rates. Endocrinology. 151: 5438-45.

Wu, X., H. Ge, B. Lemon, J. Weiszmann, J. Gupte, N. Hawkins, X. Li, J. Tang, R. Lindberg, Y. Li. 2009. Selective activation of FGFR4 by an FGF19 variant does not improve glucose metabolism in ob/ob mice. P. Nat. Acad. Sci. 1064: 14379-84.

Xu, F., R. L. Stouffer. 2005. Local delivery of angiopoietin-2 into the preovulatory follicle terminates the menstrual cycle in rhesus monkeys. Biol. Reprod. 72: 1352-8.

Xu, Z. Z., M. F. McDonald, S. N. McCutcheon. 1989. The effects of nutritionally-induced liveweight differences on follicle development, ovulation rate, oestrous activity and plasma follicle-stimulating hormone levels in the ewe. Anim. Reprod. Sci. 19: 67-78.

Yaakub, H., D. O'Callaghan, J. O'Doherty, P. Hyttel. 1997. Effect of dietary intake on follicle numbers and oocyte morphology in unsuperovulated and superovulated ewes. Theriogenology. 47: 182.

Yamaguchi, T. P., K. Harpal, M. Henkemeyer, J. Rossant. 1994. Fgfr-1 is required for embryonic growth and mesodermal patterning during mouse gastrulation. Genes. Devel. 8: 3032-44.

Yonekura, H., S. Sakura, X. Liu, H. Migita, H. Wang, S. I. Yamagishi, H. Yamamoto. 1999. Placenta growth factor and vascular endothelial growth factor B and C expression in microvascular endothelial cells and pericytes implication in autocrine and paracrine regulation of angiogenesis. J. Bio. Chem. 274: 35172-8.

Yu, Y. M., R. L. Sheridan, J. F. Burke, T. E. Chapman, R. G. Tompkins, V. R. Young. 1996. Kinetics of plasma arginine and leucine in pediatric burn patients. Am. J. Clin. Nutr. 4: 60-6.

Zackrisson, U., M. Mikuni, A. Wallin, D. Delbro, L. Hedin, M. Brannstrom. 1996. Cell-specific localization of nitric oxide synthases (NOS) in the rat ovary during follicular development, ovulation, and luteal formation. Hum. Reprod. 11: 2267-73.

Zeliznik, A. J. 1981. Premature Elevation of Systemic Estradiol Reduces Serum Levels of Follicle-Stimulating Hormone and Lengthens the Follicular Phase of the Menstrual Cycle in Rhesus Monkeys. Endocrinology. 109: 352-5.

Zerani, M., G. Catone, G. Betti, F. Parillo. 2013. Immunopresence and functional activity of prostaglandin-endoperoxide synthases and nitric oxide synthases in bovine corpora lutea during diestrus. Folia. Morphol.-Warsz. 72: 36-40.

Zheng, Y., I. Appleton, P. F. Smith, C. L. Darlington. 2000. Subregional variation in the effects of unilateral vestibular deafferentation on nitric oxide synthase activity and nitrite formation in the guinea pig hippocampus. Neurosci. Res. Commun. 27: 109-16.

Zhou, J., T. R. Kumar, M. M. Matzuk, C. Bondy. 1997. Insulin-like growth factor I regulates gonadotropin responsiveness in the murine ovary. Mol. Endocrinol. 11: 1924-33.

Zhu, M. J., Y. Ma, N. M. Long, M. Du, S. P. Ford. 2010. Maternal obesity markedly increases placental fatty acid transporter expression and fetal blood triglycerides at midgestation in the ewe. Am. J. Physiol. Regul. Integr. Comp. Physiol. 299: R1224-31.

Ziche, M., L. Morbidelli, R. Choudhuri, H. T. Zhang, S. Donnini, H. J. Granger, R. Bicknell. 1997. Nitric oxide synthase lies downstream from vascular endothelial growth factor-induced but not basic fibroblast growth factor-induced angiogenesis. J. Clin. Invest. 1: 2625-34.

Ziche, M., L. E. Morbidelli, S. Masini, H. J. Amerini, C. A. Granger, P. Maggi, Geppetti, F. Ledda. 1994. Nitric oxide mediates angiogenesis in vivo and endothelial cell growth and migration in vitro promoted by substance P. J. Clin. Invest. 94: 2036-E. 44.

Zimmermann, R. C., E. Xiao, N. Husami, M. V. Sauer, R. Lobo, J. Kitajewski, M. Ferin. 2001. Short-Term Administration of Antivascular Endothelial Growth Factor Antibody in the Late Follicular Phase Delays Follicular Development in the Rhesus Monkey 1. J. Clin. Endocr. Metab. 86: 768-72.

CHAPTER 2: LUTEAL FUNCTION DURING THE ESTROUS CYCLE IN ARGININE-TREATED EWES FED DIFFERENT PLANES OF NUTRITION: CELL PROLIFERATION, VASCULARIZATION, AND EXPRESSION OF ANGIOGENIC FACTORS

Abstract

Numerous factors influence corpus luteum (CL; luteal tissue) function and includes hormones, growth and angiogenic factors, nutritional plane, and supplements such as arginine (Arg), a semi-essential amino acid and precursor for proteins, polyamines, and nitric oxide (NO). During the estrous cycle, the CL undergoes rapid changes including vascular development and regression, and tissue growth and regression. The aim of this study was to determine if Arg supplementation to nutritionally compromised ewes impacts 1) progesterone (P4) concentrations in serum and luteal tissue, 2) luteal vascularity, cell proliferation, endothelial nitric oxide synthase (eNOS), and receptor soluble guanylate cyclase (sGCβ) protein and mRNA expression, and 3) luteal mRNA expression for selected angiogenic factors during the estrous cycle. Ewes were categorized by weight and randomly assigned to one of three different nutritional planes: C (2.14 Mcal/kg; n=37), O (2xC; n=37), and U (60% of C; n=37) beginning 60 days prior to Arg-treatment. After estrus synchronization, ewes from each diet were assigned randomly to one of two treatments; Arg (L-Arg-HCl; 155 μmol Arg/kg BW) or saline (~10 ml). Serum and CL were collected at the early, mid and late luteal phases. The present study demonstrates that 1) nutritional plane affects ovulation rates, serum P4 concentrations, and *KDR* mRNA expression, 2) Arg affects luteal vascularity and the NO system and 3) luteal vascularity, cell proliferation, and the VEGF systemdepends on the stage of the estrous cycle, and 3). These data indicate that

plane of nutrition and Arg supplementation can alter vascularization and expression of related angiogenic factors in luteal tissue during the estrous cycle. In addition, our data further support previous observations that the VEGF and NO systems are involved in the regulation of luteal function throughout the estrous cycle in sheep.

Introduction

The CL forms after ovulation and is the primary source of the gestational-support hormone P4 (Corner and Allen, 1929; Diaz et al., 2002). During its growth phase, the CL doubles in size and cell number every 60-70 hours and is capable of tremendous growth due to coordinated changes within vasculature (Reynolds et al., 1994). The CL receive some of the highest blood flow rates per unit of tissue, exhibit a high rate of metabolism, and contain parenchymal (e.g., steroidogenic) and non-parenchymal cells (e,g., fibroblasts, immune cells and vascular cells such as pericytes, smooth muscle cells and endothelial cell) (Reynolds et al., 2000; Redmer et al., 2001; Grazul-Bilska et al., 2001). Vascular cells comprise 40-70% of the total cell population within the mature CL, and the majority of steroidogenic cells within the CL are in direct contact with one or more capillaries (Reynolds et al., 1992, 2000).

Nutritional plane, including either undernutrition or overnutrition, impacts several female reproductive functions including follicular growth (Diskin et al., 2003; Webb et al., 2004, 2007), oocyte quality (Rhind et al., 1989: Grazul-Bilska et al., 2012), ovulation (O'Callaghan and Boland, 1999), luteal function and hormone production (Abecia et al., 1997; Parr et al., 1987; Armstrong et al., 2003; Diskin et al., 2003; Kaminski et al., 2015). Additional studies regarding inadequate diet effects on ovarian function in ewes is important as the nutrient uptake of grazing ewes in the western United States is often less than 50% of the National Research Council's

(NRC) recommendation (NRC, 1985) (Thomas and Kott, 1995). Moreover, the ewe is a seasonal breeder, becoming pregnant in late fall or early winter in the US; this results in the majority of gestation coinciding with winter, when grazing forages are of lower quality (Hoaglund et al., 1992).

Arginine is a semi-essential amino acid obtained through exogenous (via diet; Mertz et al., 1952; Wu and Morris, 1998) and endogenous (via intestinal-renal pathway; Windmueller and Spaeth, 1981; Wakabayashi and Jones, 1983; Wu and Knabe, 1995) sources. While Arg is a precursor for proteins and polyamines, it is also a physiological nitrogen donor for nitric oxide synthase (NOS) that catalyzes NO formation. In addition, whether through competitive inhibition of Arg (Morris and Billiar, 1994; Carter et al., 2004) or through a low-Arg diet (Wu et al., 1999; Wu and Meininger, 2002), reduced systemic Arg concentration impairs NO production. Moreover, several studies have demonstrated that NO production catalyzed by eNOS is primarily determined by extracellular Arg bioavailability (Hallemeesch et al., 2002; Hardy et al., 2002; Rajapakse et al., 2009; Shin et al., 2011).

It has been clearly demonstrated that NO is one of the major regulators of vascular development and angiogenesis within the ovary (Reynolds et al., 2002), and has been implicated in the regulation of luteal function (Motta et al., 1999, 2001). For several species including cows and sheep, effectors of the NO system have direct effects on steroidogenesis in luteal tissues (Vega et al., 1998; Mitsube et al., 1999; Dong et al., 1999; Skarzynski et al., 2000; Jaroszewski et al., 2001, 2003; Hurwitz et al., 2002). Three NOS isoforms, endothelial (eNOS), neuronal (nNOS), and/or inducible (iNOS) have been detected in the luteal cells during early-, mid-, and late-luteal phases in several species (Skarzynski et al., 2003; Rosiansky-Sultan et al., 2006;

Grazul-Bilska et al., 2006; Zerani et al., 2013). Hurwitz et al. (2002) reported that iNOS-mediated NO production is involved in regulation of prostaglandin synthesis in rat luteal cells, which may alter P4 synthesis.

One of the major regulators of eNOS expression, and therefore NO production, is vascular endothelial growth factor (VEGF) (Ku et al., 1993; Van der Zee et al., 1997; Kroll and Waltenberger, 1999; Duda et al., 2004). Nitric oxide, in turn, is involved in regulation of VEGF expression and thus angiogenesis (Chin et al., 1997; Reynolds et al., 2000, 2002; Beckman et al., 2006). The existence of a paracrine loop, wherein endothelial cells secrete NO, stimulating perivascular VEGF production, which then stimulate endothelial expression of eNOS has been proposed for reproductive tissues (Reynolds et al., 2002). Within the ovarian structure, this paracrine loop would provide a "feed-forward" system to increase vasodilation and angiogenesis, which are vital to proper ovarian function (Reynolds et al., 2002).

The CL growth and regression, as well as vascularization, is regulated by numerous factors including members of the VEGF, angiopoietins (ANGPT), and fibroblast growth factor (FGF) families and the NO system (Friden et al., 2000; Boiti et al., 2002; Miyamoto et al., 2005; Vonnahme et al., 2006; Beckman et al., 2006). The VEGF family of proteins including VEGF, placental growth factor (PGF) and receptors FLT and KDR is considered to be one of the major regulators of angiogenesis and vascular function (Ferrara, 1999; Geva and Jaffe et al., 2000; Hoeben et al., 2004). Another importan regulators of angiogenesis, angiopoietins (ANGPT)-1 and -2 are produced exclusively by endothelial cells, are responsible for blood vessel maturation or destabilization, respectively, and bind receptor Tie (tyrosine kinase with immunoglobulin and epidermal growth factor homology domains) 1 and 2 (Dumont et al., 1992; Sato et al., 1993).

Basic fibroblast growth factor (FGF2) is one of many fibroblast growth factors and is involved in regulation of angiogenesis and tissue growth (Baird et al., 1985; Kottakis et al., 2011). FGF2 binds its receptor FGFR2 (Johnson and Williams, 1993).

Objectives and Hypotheses

We hypothesized that inadequate nutrition (either diet excess or restriction) would result in negative effects on CL function, while Arg would restore function as evaluated by serum and luteal P4 concentrations, vascularization, cell proliferation, expression of eNOS and NO receptor sGC proteins, and mRNA expression of selected angiogenic factors in CL. Therefore, the aim of this study was to evaluate the effects of nutritional plane and Arg on 1) CL number and weight, 2) serum P4 concentration and P4 content in CL, 3) CL vascularization and cell proliferation as measured by protein expression of CD31 and Ki67, respectively, 4) luteal eNOS and sGC-beta protein expression, and 5) luteal mRNA expression of *eNOS*, *GUCY1B3*, *VEGF*, *PGF, FLT*, *KDR*, *ANGPT1*, *ANGPT2*, *TIE2*, *FGF2 and FGF2R* during the estrous cycle.

Materials and Methods

All animal procedures performed were approved by the North Dakota State University Institutional Animal Care and Use Committee (#A12013).

Animals and Experimental Design

Animal treatments, diet composition and experimental design are described in detail by Kaminski et al. (2015). Briefly, non-pregnant, non-lactating Rambouillet ewes (n = 111, between 3 to 5 years of age and of similar genetic background) were penned individually at the Animal Nutrition and Physiology Center on the NDSU campus. Ewes were stratified by weight and randomly assigned to one of three nutritional plane treatments: control (C; n = 37) received a

maintenance diet (760 g/kg BW/day), overfed (O; n = 37) received 2 x C, and underfed (U; n = 37) received 0.6 x C 60 days prior to the onset of the estrous cycle (day 0). Ewes were fed their individual diets twice daily at 0800 h and 1500 h. Ewes were weighed and BCS was evaluated on a 5-point scale (1 = extremely thin and 5 = obese) by the same individual once weekly. Diets were adjusted weekly to ensure the proper BW. Body condition score was achieved by day 0 and maintained throughout the estrous cycle until completion of experiment on day 15 (late-luteal phase of the first estrous cycle), or day 21 or day 26 (early-luteal phase or mid-luteal phase of the second estrous cycle, respectively) of Arg. Estrus was synchronized by insertion of a controlled internal drug release device for 14 days. Based on previous results (Grazul-Bilska et al., unpublished), approximately 36 h after CIDR removal, ewes were in estrus, and treated as day 0 of the estrous cycle. At day 0, ewes were randomly assigned to Arg or saline (Sal) groups, and Arg or Sal injections were initiated at 0700 h.

Jugular vein cannulations were conducted 5-7 days prior to day 0 of the estrous cycle. Neck wool was removed by shearing, the area was disinfected with betadine, and wiped with sterile gauze and 70% ethyl alcohol. Approximately 10 minutes before cannulation, a local anesthetic (lidocaine HCl, 2%, sterile, Phoenix Scientific Inc., St. Joseph, MO) was injected subcutaneously into two sites (approximately 1 mL/site) along the jugular vein. The sheep were restrained manually, the skin was incised, and a sterile cannulation needle (12-gauge, Popper & Sons Inc., New Hyde Park, N.Y.) was inserted into the jugular vein, parallel to the trachea near the middle of the neck. Immediately after inserting the needle, a sterile cannula (outer diameter [O.D.] 2.16 mm, inner diameter [I.D.] 1.02 mm, Silastic Laboratory Tubing, DOW Corning Corporation, Midland, MI) was inserted through the needle into the jugular vein and the needle

was carefully removed. The length of the cannula was 25 cm (20 cm was inserted inside the jugular vein and 5 cm was allowed to remain outside). The cannula was flushed with sterile saline solution to make sure it was functional. The cannula was then filled with sterile heparin (sodium salt from porcine intestinal mucosa; Sigma, St. Louis, MO) in saline (300 IU of heparin/mL) solution and sealed with a sterile stopper. The outside portion of the cannula was attached to the skin via surgical tape, staples, and silicone glue. The skin around the cannula was washed to with sterile water, wiped with sterile gauze, and rinsed with 70% ethyl alcohol to remove traces of blood.

Arginine or Saline Treatment

Ewes in the Sal group received 5-10 mL three times daily of sterile saline solution, while ewes in the Arg group received a dose of 155 μmol Arg/kg BW three times daily (Sigma, St. Louis, MO) as previously described by Kaminski et al. (2015) in 5-10 mL via the jugular cannula. Ewes received Arg or Sal for 15, 21, or 26 days. Arg or Sal injections were initiated on day 0 of the estrous cycle and occurred three times daily (0700, 1400, 2100 h) until the end of experiment. After each injection, 1 cc of heparin solution was placed into the cannula to prevent blood from clotting within the tubing.

Tissue and Blood Collection

Ovaries and blood samples were collected at the early- or mid-luteal phase of the second estrous cycle, or late-luteal phase of the first estrous cycle. These luteal stages correspond to day 21, 26, or 15 of Arg or Sal. Blood samples were centrifuged, and serum was stored at -20˚C until P4 analysis. The number of CL was recorded, and CL were dissected from the ovary and weighed. Corpora lutea from each ewe was divided into four portions: the first portion was fixed

in 10% neutral buffered formalin (NBF) for immunohistochemistry, the second portion was fixed in Carnoy's solution for immunohistochemistry, the third portion (approximately 20-50 mg) was immediately frozen on dry ice and stored at -80 C until homogenization in PBS (100 mg/1 ml), and the fourth portion (20-50 mg) was placed in 600 uL buffer RLT (Qiagen, Valencia, CA) in a nuclease-free 2.0 mL tube and homogenized using a 7 mm generator on a Polytron homogenizer for 10-15 seconds, then frozen on dry ice and stored at -80˚C prior to RNA extraction.

Progesterone Analysis

Progesterone concentrations from serum and homogenized luteal tissue were determined using a chemiluminescence immunoassay (Immulite 1000, Siemens), as previously described (Kaminski et al., 2015). Briefly, a progesterone kit utilizes a solid phase, competitive immunoassay using enzyme-labeled chemiluminescent technology. Each sample was run as 50 µL duplicates. Beads coated with polyclonal rabbit anti-P4 antibodies are utilized for the initial solid phase. Alkaline phosphatase conjugated to P4 is used for the liquid phase. Samples and reagents are mixed and incubated for thirty minutes, during which sample P4 competes with enzyme-conjugated P4 in reagent for limited antibodies on the bead. Centrifugal washes remove unbound sample and enzyme conjugate. Lastly, chemiluminescent substrate is added and the luminescent signal that is generated is indicative of P4 concentration. The intra-assay CV was 6.9% for serum P4 and 5.2% for luteal P4.

Immunohistochemistry

All tissues were sectioned using a Leica rotary microtome (Leica RM 2255, Nussloch, Germany) and mounted on poly-L-lysine treated slides (Fisher Scientific, Pittsburg, PA). Tissue sections were deparaffinized in xylene and rehydrated in decreasing concentrations of alcohol in

water and then washed in distilled water. Tissue sections underwent antigen recovery (2100 Retriever, Prestige Medical, Lancashire, England) for 20 minutes in a 10 mM sodium citrate buffer with 0.05 Tween (pH 6). Slides were allowed to cool at room temperature for 20 minutes and then washed in TBST (0.1% Tween 20) twice for 5 minutes each. Tissue sections were blocked with 10% normal goat serum for 20 minutes at room temperature. Afterwards, tissue sections were incubated with a specific primary antibody followed by incubation with a secondary antibody. Table 2.1 presents fixatives used, tissue thickness, and source and dilution of primary and secondary antibodies for all antigens immunodetected in this experiment. Tissue sections were then washed twice for five minutes in distilled water at room temperature and cover slipped using Prolonged Gold with DAPI (Life Technologies, Eugene, OR).

Image Generation and Analysis

Photomicrographs were taken with a Zeiss Imager M2 epifluorescence microscope equipped with Zeiss piezo automated stage and AxioCam HRm camera (Zeiss Inc., Thornwood, NY). All images were taken where distribution of steroidogenic and accessory cells is relatively uniform; areas with connective tissue tracts were avoided. The percentage of area that exhibited positive fluorescent staining for CD31, Ki67, eNOS, or sGC was evaluated quantitatively with an image analysis system (Image Pro-Plus, Media Cybernetics, Silver Spring, MD) as described previously (Grazul-Bilska et al., 2006; Vonnahme et al., 2006). For each CL, three randomly chosen fields were evaluated in each tissue section.

Table 2.1. Tissue fixation, tissue section thickness, and antibodies used for immunohistochemistry to detect Ki67, CD31, eNOS, and sGC proteins in the CL.

ANTIGEN	PURPOSE	FIXATIVE	TISSUE THICKNESS	1^0 ANTIBODY, DILUTION, INCUBATION TIME	2^0 ANTIBODY, DILUTION, INCUBATION TIME	REFERENCES
CD31	Endothelial cell marker	NBF	15 µm	CD31 rabbit polyclonal, item # ab28364, Abcam Biotech Company, San Fransisco, CA; 1:100; overnight	CF 633 goat anti-rabbit, item # 20122-0.5mL, Biotium, San Fransisco, CA; 1:100; 1 hour	Redmer et al., 2013
Ki67	Cell proliferation marker	NBF	5 µm	Ki67 mouse monoclonal, item # VP-k452, Vector Labs, Burlingame, CA; 1:100; overnight	Alexa 568 goat anti-mouse, item # A110040, Life Technologies, Carlsbad, CA; 1:200; 1 hour	Grazul-Bilska et al., 2013; 2014
eNOS	Endothelial nitric oxide synthase marker	Carnoy's	5 µm	Anti-eNOS/NOS III purified mouse, item # 610297, BD Biosciences, San Jose, CA; 1:250; overnight	Alexa 647 goat anti-mouse, item # A21235, Invitrogen, Eugene, OR; 1:200: 1 hour	Grazul-Bilska et al., 2006
sGC	Soluble guanylate cyclase marker	NBF	5 µm	Guanylate cyclase β rabbit polyclonal, item # 160897, Cayman Chemical, Ann Arbor, MI; 1:500; 1 hour	CF 633 goat anti-rabbit, item # 20122-0.5mL, Biotium, San Fransisco, CA; 1:100; 1 hour	

Background fluorescence was minimal and was adjusted to the same level for each section by the image analysis system. Data were expressed as percentage of positive staining out of tissue area within each field.

Quantitative Polymerase Chain Reaction (qPCR)

TaqMan probe-primer sets (Applied Biosystems, Life Technologies Grand Island, NY) for 11 ovine genes (*eNOS3*, *GUCY1B3*, *VEGF*, *PGF, FLT*, *KDR*, *ANGPT-1*, *ANGPT-2, Tie-2*, *FGF2 and FGFR*) were used as previously described (Redmer et al., 2005; Grazul-Bilska et al., 2013). Quantifications of total RNA were completed via reading absorbance on a Synergy H1 Microplate Reader (Bioteck, Winooski, VT). Reverse transcription was utilized to produce cDNA (Quantitect RT Kit, Qiagen, Valencia, CA), from 1 μg of RNA. Gene expression was determined (7500 Fast Applied Biosystem Instrument, Life Technologies, Grand Island, NY) and data was analyzed using the comparative CT method using 18s rRNA as the normalizing gene (Livak and Schmittgen, 2001).

Statistical Analysis

Data was analyzed as a 3x3x2 factorial, with interactions evaluated. Data for BW, BCS, P4 concentration in serum and luteal tissues, CL weights, area of positive staining, and luteal angiogenic factor mRNA expression were analyzed using general linear model (GLM) procedures of SAS (SAS Institute Inc. 2011, Base SAS® 9.3 Procedures Guide. Cary, NC: SAS Institute Inc.). When an F-test was significant, differences between specific means were evaluated by using least significant difference test (Kirk, 1982). Means were considered different when $P<0.05$. Data are expressed as mean±SEM.

Results

A summary of observed results are presented in Table 2.2.

During nutritional and Arg treatments, C maintained BW and BCS, O gained 4.1±1.3 kg, and U lost 15.5±0.6 kg; for O, BCS increased by 1.1±0.1 and U decreased by 0.5±0.1.

The CL number, and thus the ovulation rate, was greater ($P<0.05$) in O and C ewes when compared to U ewes and was not affected by Arg, and therefore combined data are presented (Fig. 2.1).

Table 2.2. Summary of effects of control (C), overfed (O), or underfed (U) plane of nutrition, arginine (Arg) or saline (Sal), early-(early), mid-(mid), or late-(late) estrous

phase, and their interactions on several measurements of reproductive function.

Treatments	Measurements*	Observed change
Nutritional Plane	Ovulation rate	Greater in O and C vs U
	Serum P4 concentrations (tendency)	Greater in O vs C or U
	CD31 protein (tendency)	Greater in C vs O or U
Arginine	CD31 protein	Greater in Sal vs Arg
	GUCY1B3 mRNA (tendency)	Greater in Arg vs Sal
Stage of the estrous cycle	CL weight	Greater in mid and late vs early
	Serum and luteal P4	Greater in mid vs early or late
	CD31 protein	Greater in early vs mid or late
	Ki67 protein	Greater in early vs mid vs late
	eNOS protein	Greater in mid vs early or late
	VEGF mRNA	Greater in early and mid vs late
	ANGPT2 mRNA	Greater at mid vs early or late
Interactions	Ki67 (nutrition by Arg by stage of luteal phase)	Greatest in U and least in O in early treated with Arg
	eNOS mRNA (Arg by stage of luteal phase)	Greater in Arg in mid in all nutrition groups
	KDR mRNA (nutrition by Arg by stage of luteal phase)	Greatest in C and U vs O in mid treated with Arg

*Measurements that were significantly ($P \leq 0.05$) or tended ($P \leq 0.1$) to be significantly affected are listed.

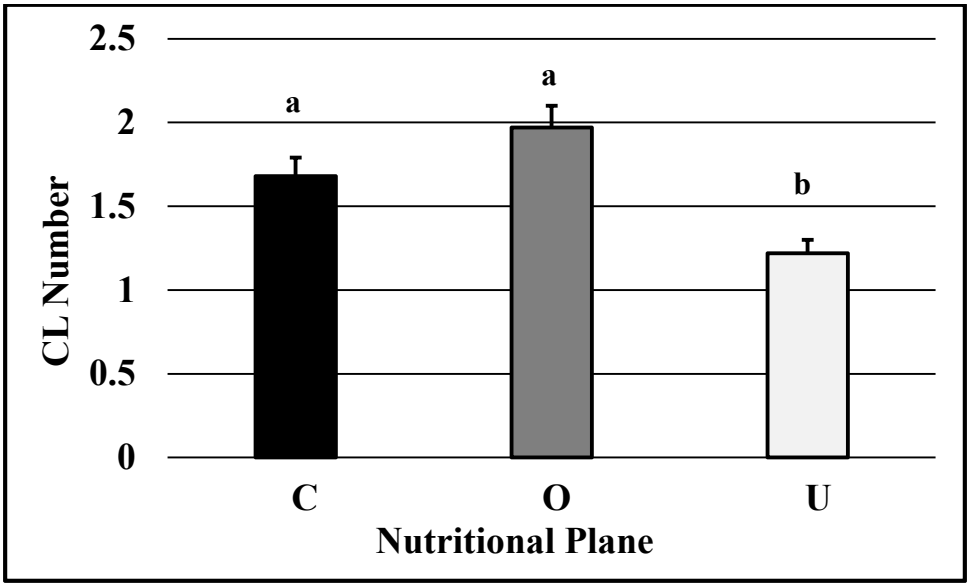


Figure 2.1. The number of CL in control (C), overfed (O), or underfed (U) ewes. [a,b] P<0.05, means ±SEM with different superscripts differ.

The CL weight was greater (P<0.0001) at mid- and late-luteal phase when compared to early-luteal phase, and was not affected by nutritional plane or Arg, and therefore combined data are presented (Fig. 2.2). Serum P4 concentration was greater (P<0.0001) at mid-luteal phase when compared to early- or late-luteal phases (Fig. 2.3), tended to be greater (P=0.18) in O compared to U (1.7±0.2, vs. 1.4±0.2, respectively), and was intermediate in C (1.5±0.2).

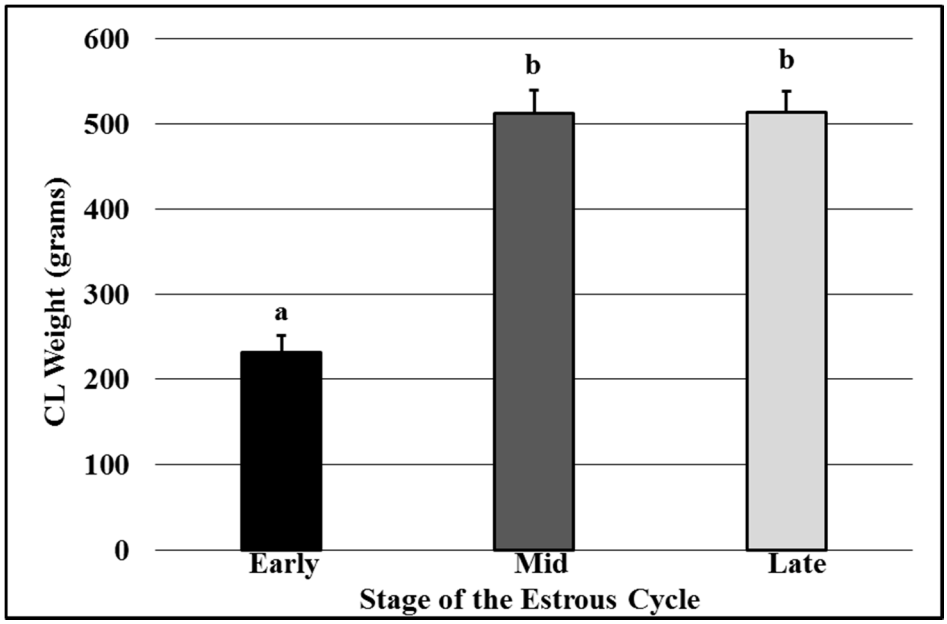


Figure 2.2. The weight of CL at early-, mid-, or late-luteal phases of the estrous cycle. [a,b] P<0.05, means ±SEM with different superscripts differ.

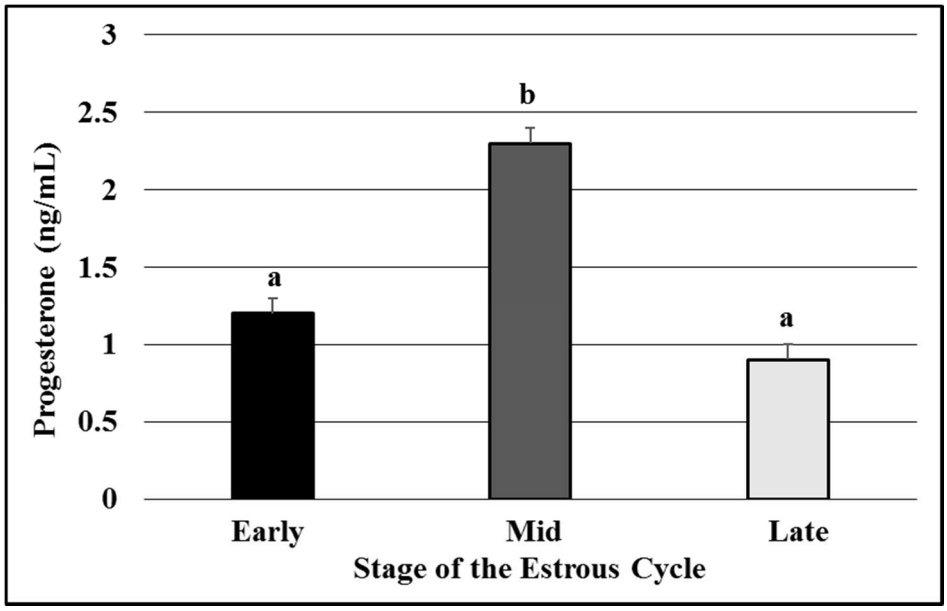


Figure 2.3. The concentration (ng/ml) of progesterone in serum at early-, mid-, or late-luteal phases of the estrous cycle. [a,b] $P<0.05$, means ±SEM with different superscripts differ.

Concentration of P4 in luteal tissues was greater ($P=0.001$) in mid- than early- or late-luteal phase (Fig. 2.4). Serum and luteal tissue P4 concentrations were not affected by Arg.

CD31 protein was immunodetected in luteal tissues and was localized to blood vessels throughout the estrous cycle (Fig. 2.5A, B). CD31 protein expression was greater ($P<0.0001$) in early- compared to mid- or late-luteal phase (Fig. 2.5C). Overall, compared to Sal, Arg decreased ($P<0.02$) CD31 expression and thus luteal vascularity; and CD31 expression tended ($P=0.1$) to be greater in C than U. A tendency ($P=0.09$) for Arg, nutritional plane, and stage of the estrous cycle interactions demonstrated less CD31 expression in Arg ewes at the early-luteal phase in C ewes.

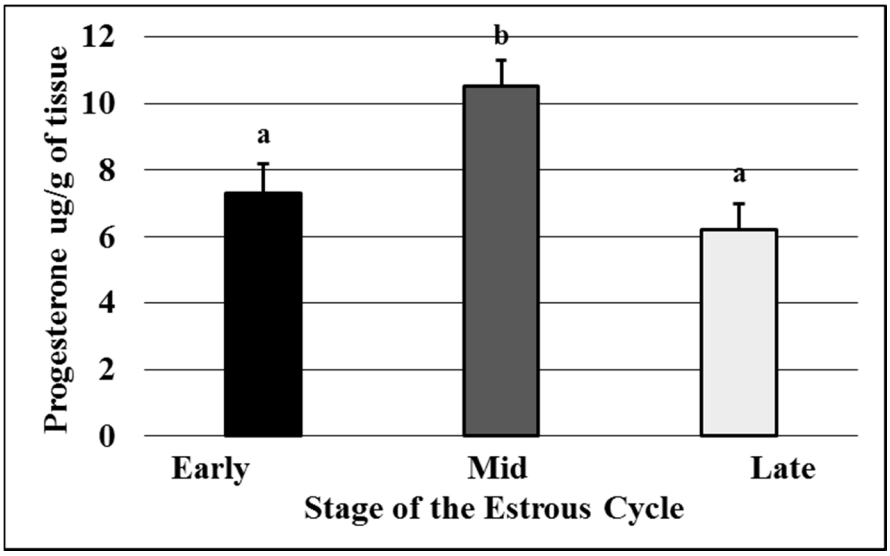
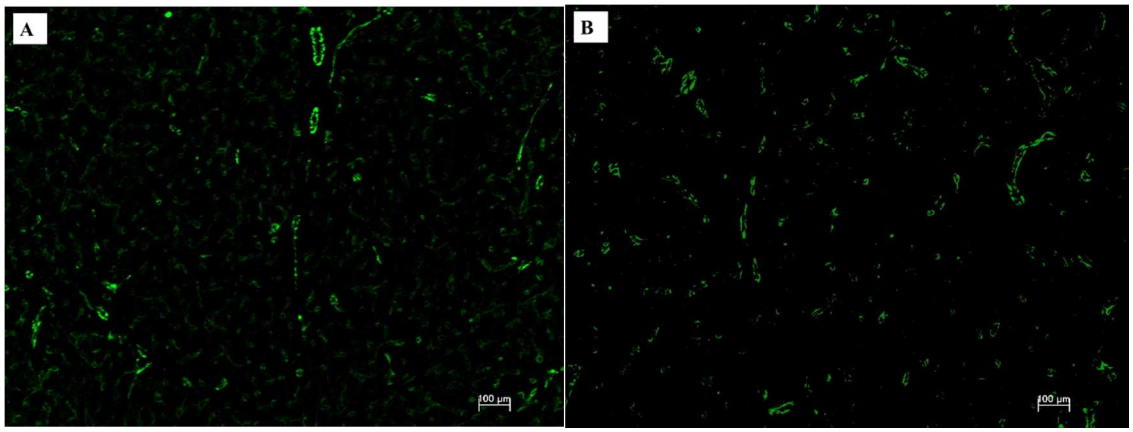


Figure 2.4. The concentration (µg/g of tissue) of progesterone in CL at early-, mid-, or late-luteal phases of the estrous cycle. [a,b] $P<0.05$, means ±SEM with different superscripts differ.



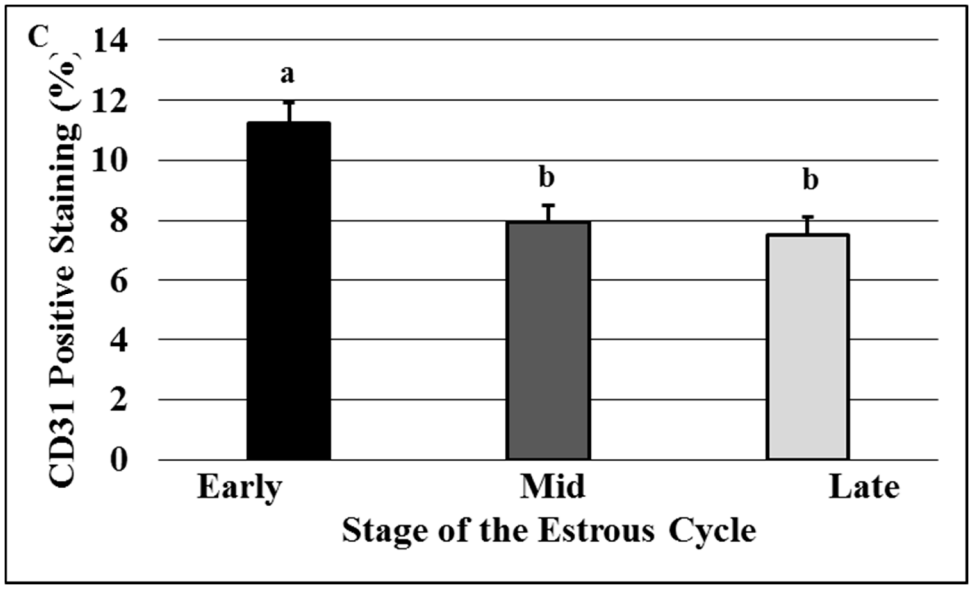
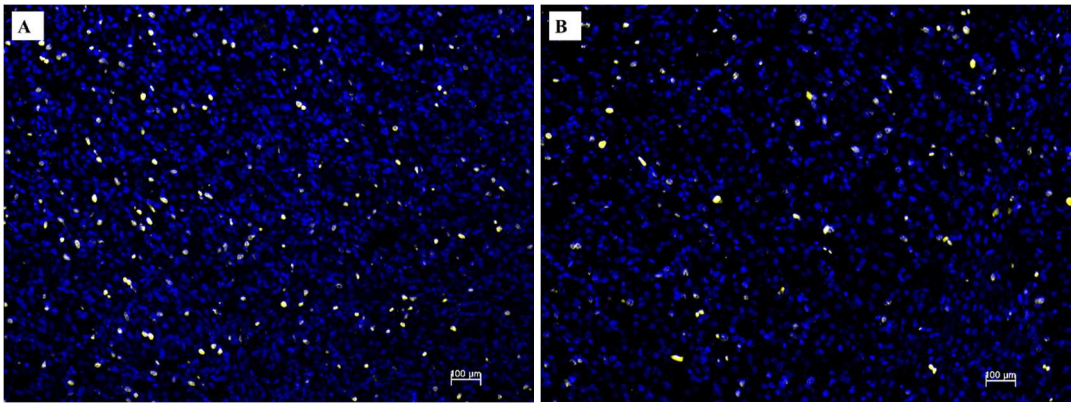


Figure 2.5. Representative images of CD31 (marker of vascularization) positive staining (purple) in an early- (A) and mid-luteal (B) phase CL, and expression of CD31 in CL from ewes at early, mid-, or late-luteal phases of the estrous cycle (C). ^{a,b} $P<0.05$, means ± SEM with different superscripts differ.

Ki67 protein was immunodetected in the nuclei of luteal cells throughout the estrous cycle (Fig. 2.6A, B, C). Luteal cell proliferation was greatest ($P<0.0001$) during the early-luteal phase, less at mid-luteal phase, and least at the late-luteal phase (Fig. 2.6D). A tendency ($P=0.07$) for Arg and stage of the estrous cycle interactions demonstrated greater cell proliferation in Arg ewes in the early-luteal phase compared to similarly treated ewes in the mid- or late-luteal phase of the estrous cycle in all nutritional groups.

The eNOS protein was immunodetected and localized to blood vessels of the luteal tissue throughout the estrous cycle (Fig. 2.7A, B). Expression of eNOS was greater ($P<0.01$) at mid-luteal phase compared to at early or late-luteal phases, and was not affected by nutritional plane or Arg, therefore combined data are presented (Fig. 2.7C). For eNOS mRNA, Arg by stage of

the estrous cycle interaction (P<0.04) demonstrated greater expression in Arg than Sal-treated ewes during the mid-luteal phase.



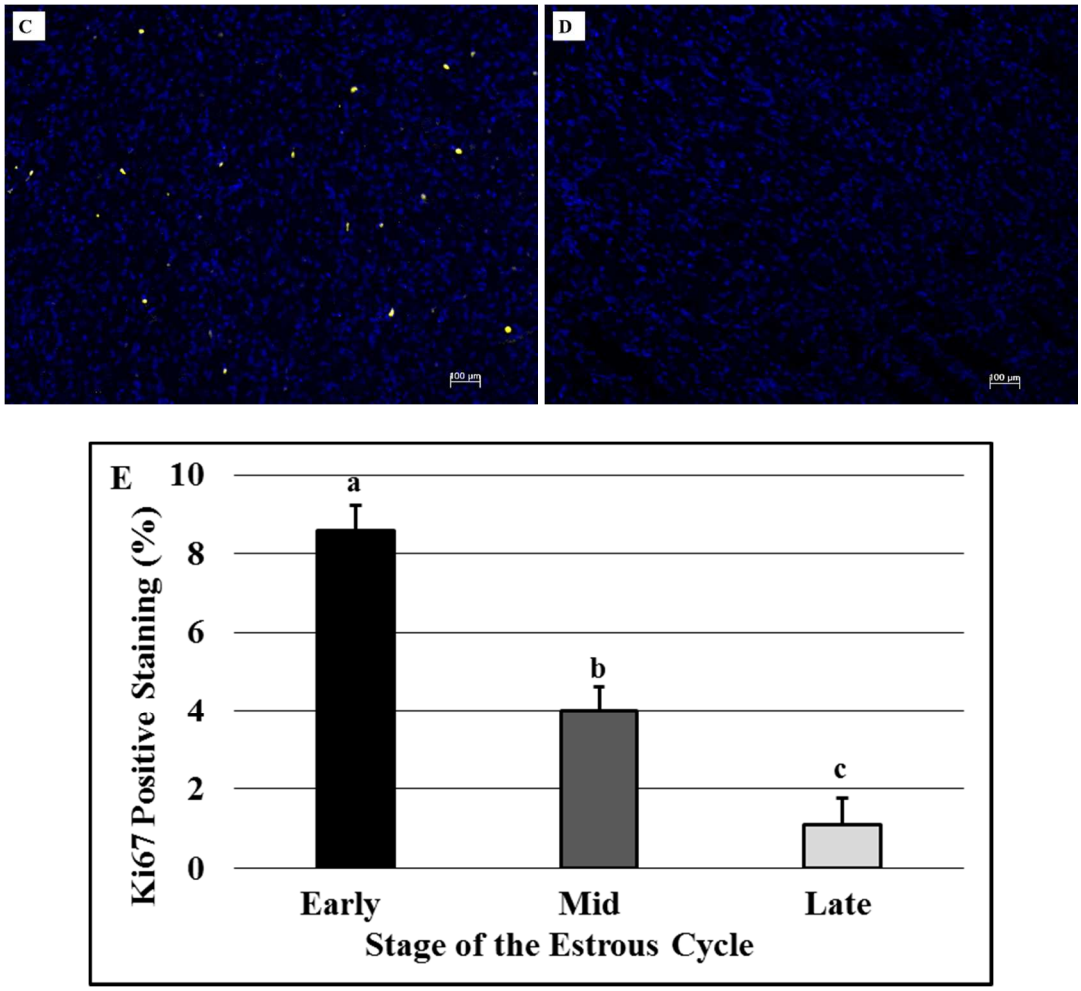


Figure 2.6. Representative images of Ki67 (marker of vascularization) positive staining (yellow) in early- (A), mid- (B), and late-luteal (C) phase CL, control (D). Non-proliferating cells stained purple (DAPI). Labeling index (graph; percentage of Ki67 positive cells out of total cells/tissue area) in CL at early-, mid-, or late-luteal phases of the estrous cycle (E). ^{a,b,c} $P<0.05$, means ± SEM with different superscripts differ.

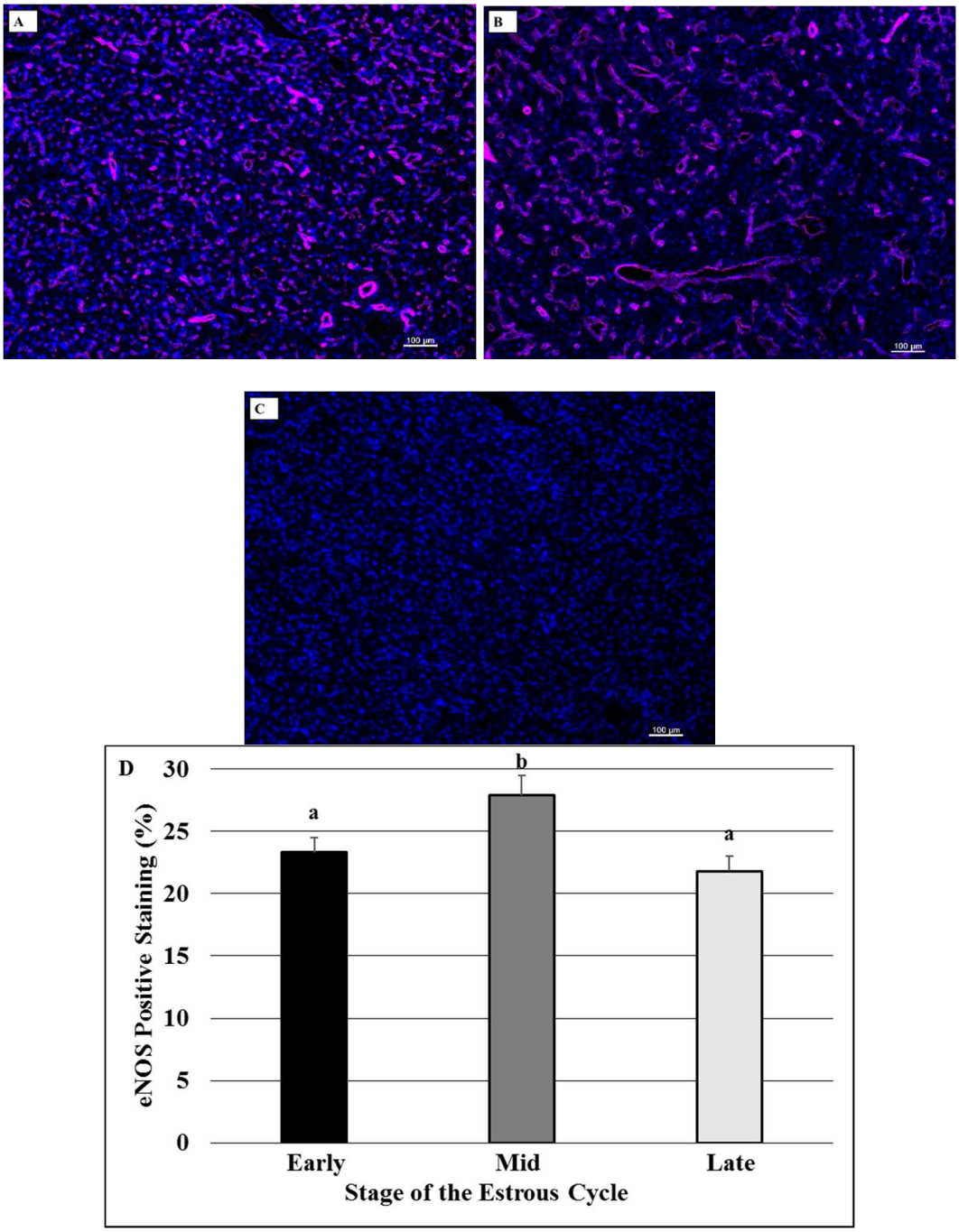


Figure 2.7. Representative images of endothelial nitric oxide synthase (eNOS) positive (pink) staining in early- (A) and mid-luteal (B) phase CL. Non-stained cells (blue; DAPI). Control slide (C). eNOS protein staining (graph; % positive staining out of total tissue area) in the ovine CL at early- mid- or late-luteal phases of the estrous cycle. $^{a,\ b}P<0.05$, means ±SEM with different superscripts differ.

The presence of sGC protein was immunodetected in luteal blood vessels (Fig. 2.8) and expression was similar in all groups (data not shown) throughout the estrous cycle. *GUCY1B3* mRNA expression tended (P=0.09) to be greater in Arg ewes, but was not affected by nutritional plane or stage of the estrous cycle. In addition, interactions (P<0.02) between Arg and stage of the estrous cycle demonstrated an increased *GUCY1B3* mRNA expression in luteal tissue from Arg compared to Sal ewes at the mid-luteal phase.

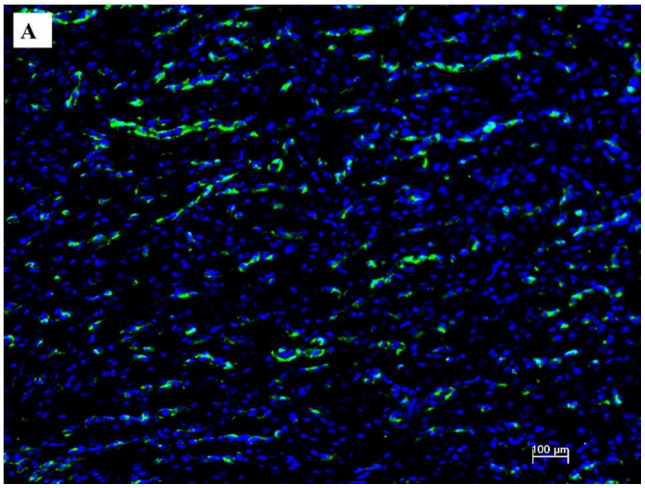


Figure 2.8. Representative image of soluble guanylyl cyclase (sGC) positive staining (pink) in early-, mid-, and late-luteal phase CL (A).

Expression of *VEGF* mRNA was greater (P=0.02) at early- and mid- compared to late-luteal phase (Fig. 2.9) and was not affected by Arg or nutritional plane. Expression of KDR and ANGPT2 mRNA was greater (P< 0.02) at the mid-luteal phase than at early or late-luteal phases and was not affected by Arg or nutritional plane therefore combined data are presented. For *KDR* mRNA, nutritional plane by Arg by luteal phase interactions (P=0.05) demonstrated that in mid-luteal stage, expression was greater in 1) Arg than Sal C ewes, and 2) Arg C and U compared to O ewes treated with Arg (Fig. 2.10). Expression of *ANGPT2* mRNA was greater (P=0.01) in mid- compared to early- and late-luteal tissues (Fig. 2.11).

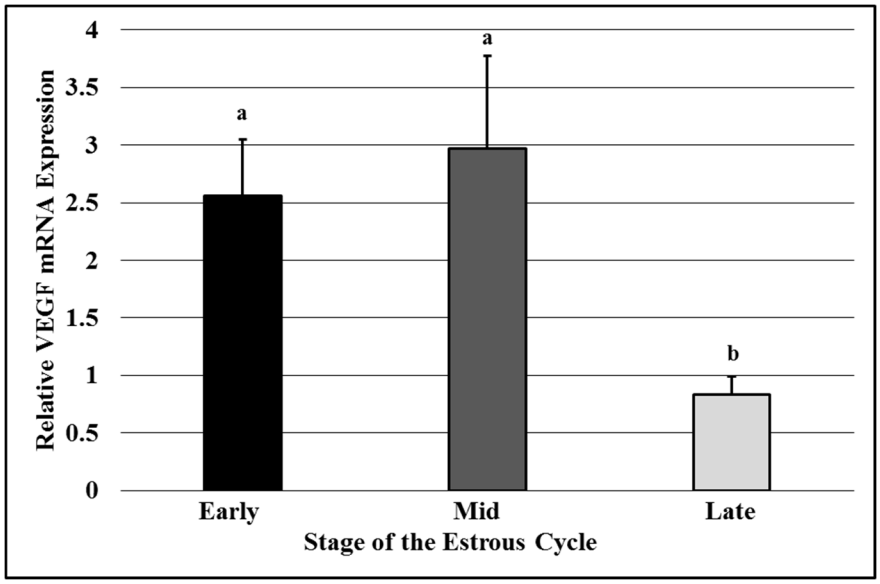


Figure 2.9. Vascular endothelial growth factor (VEGF) mRNA expression in CL at early-, mid-, or late-luteal phases of the estrous cycle. [a,b] P<0.05, means ± SEM with different superscripts differ.

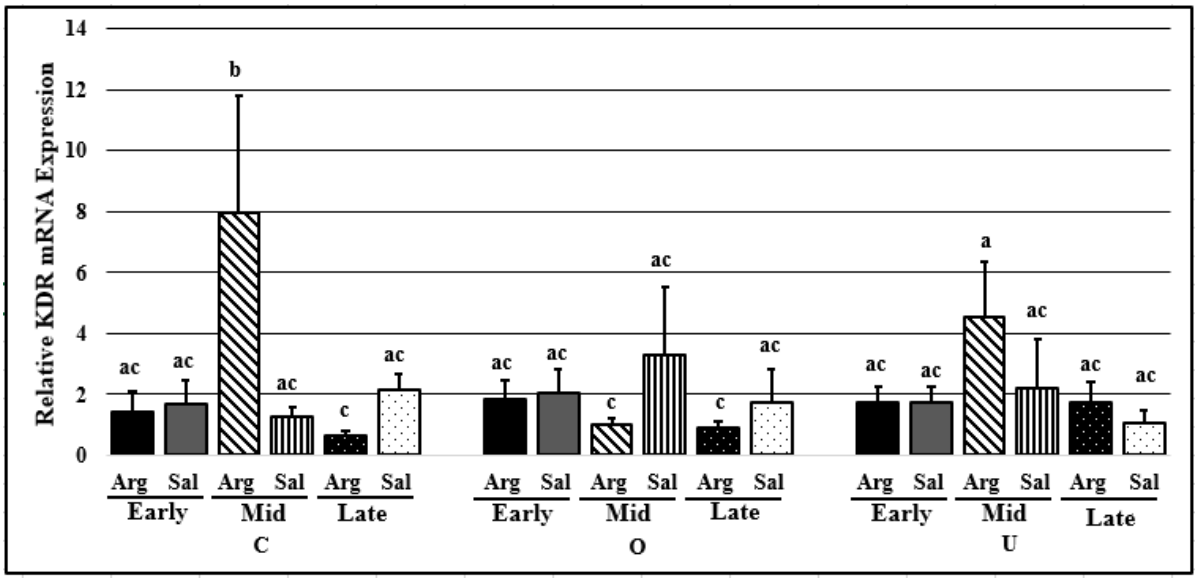


Figure 2.10. KDR mRNA expression in CL at early-, mid-, and late-luteal phases of the estrous cycle in ewes treated with arginine (Arg) or saline (Sal) on control (C), overfed (O) or underfed (U) nutritional planes. [a, b, c] P<0.05, means ± SEM with different superscripts differ.

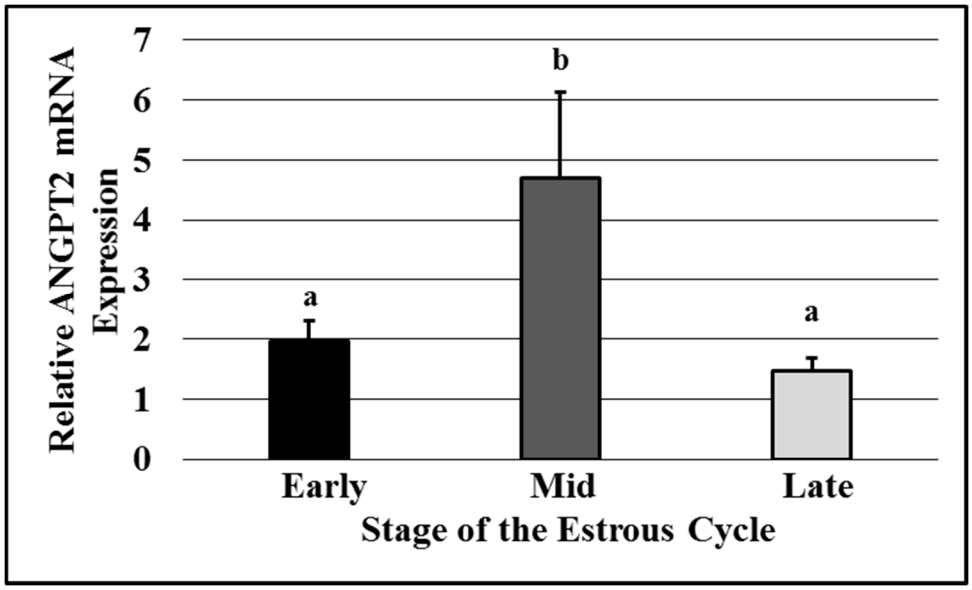


Figure 2.11. Angiopoietin-2 (ANGPT-2) mRNA expression in CL at early-, mid-, or late-luteal phases of the estrous cycle. [a,b] $P<0.05$, means ± SEM with different superscripts differ.

Expression of *ANGPT1*, *TIE2*, *FGF2*, *FGF2R*, *PGF*, and *FLT* mRNA in luteal tissues was similar in all treatment groups and stages of the estrous cycle (data not shown).

Discussion

The present study demonstrates that nutritional plane and/or Arg affects several measurements of luteal function including ovulation rates, serum P4 concentrations, luteal vascularity and cell proliferation, and mRNA expression of *eNOS* and *KDR*.

Ovulation rates were greater in O and C compared to U ewes. The association between nutritional plane and ovulation rate has been previously described by several researchers. Prior studies have reported a positive correlation between higher BCS and ovulations rates (Gunn and Doney, 1975; Smith, 1995; Rhind et al., 1989; Xu et al., 1989; Abecia et al., 1997), and that lower BCS lead to low ovulation rates in the ewe (Keisler and Buckrell, 1997; Lassoued et al., 2004). When an animal is in a negative energy balance, the animal must use its own energy stores in an effort to reverse the energy deficit, resulting in negative effects on both the

hypothalamic-pituitary axis as well as the reproductive system (Scaramuzzi et al., 2006). Additionally, restricted nutritional planes lead to a multitude of hormonal imbalances including, but not limited to, elevated plasma growth hormone (GH), the inhibition of gonadotropin-releasing hormone (GnRH), reduced ovulation rates, and even caused anovulation (Scaramuzzi et al., 2006). Follicular populations and ovulation rates are reported to be very sensitive to nutritional plane alterations in the ewe (Scaramuzzi et al., 2006). Therefore, adequate nutrition should be provided to an animal to achieve normal ovulation rate, and thus, lambing rates. Although our results concerning nutritional effects on ovulation rates in ewes are reflective of previous reports (Coop, 1966; Boland et al., 1988; O'Callaghan and Boland, 1999), our study demonstrated that ovulation rate was not affected by Arg supplementation. We hypothesize, that supplements Arg is not involved in the regulation of ovulation rate in non-pregnant sheep. Further studies should be undertaken to elucidate the mechanism(s) of interactions between nutritional plane and ovulation rate.

In this study, CL weight, serum P4 concentration, P4 luteal content, eNOS protein expression, and *VEGF* mRNA expression were greatest at the mid-luteal phase indicating normal luteal development and function (Stabenfeldt et al., 1969; Niswender and Nett, 1988; Grazul-Bilska et al., 2006). CL weight was not affected by nutritional plane or Arg, but serum P4 was greater in O than U ewes. Similarly, Kaminski et al., (2015) observed increased serum P4 throughout the estrous cycle in O ewes. In contrast, increased serum P4 concentrations during the first estrous cycle on reduced nutrient intake in cows (Donaldson et al., 1970; Jordan and Swanson, 1979) and ewes (Cumming et al., 1971) have been reported. However, if cows remain on a restricted nutritional intake, peripheral P4 concentrations are significantly reduced in later,

consecutive estrous cycles (Gombe and Hansel, 1973; Beal et al., 1978; Rodrigues et al., 2011). This subsequent decrease in P4 may be attributed to a further depletion in adipose tissue, and therefore adipose-stored P4. It has been demonstrated that a considerable amount of P4 can be stored in adipose tissues and released into circulation if the fat is metabolized (Hamudikuwanda et al., 1996). These findings could explain why the present study demonstrated a tendency for serum P4 to be lower in underfed ewes when compared to overfed ewes. These ewes lost 15% of their initial BW, and thus fat storage was depleted, as reflected by decreased BW and BCS. Scaramuzzi et al. (2006) describes "negative and positive energy balances" to highlight the differences that occur between nutritional plane and reproduction. When a ewe's caloric requirement is greater than her intake, she is considered to be in a "negative energy balance", while one that is consuming an excess of calories is considered to have a "positive energy balance". These changes to an animal's metabolic state, and the underlying homeostasis, also affect reproductive performance.

The present study demonstrated that CD31 expression, and therefore vascularity of the ovine luteal tissue was greatest at the early-luteal phase. Similar data of luteal vascularity were previously reported for cow (Miyamoto et al., 2005) and primate (Fraser and Lunn, 2001). In fact, 50 to 85% of the cells that are present as the growing CL are endothelial cells and pericytes (Christenson and Stouffer, 1996; Reynolds et al., 2000; Redmer et al., 2001). During the early luteal phase, angiogenesis is quickly progressing in order to establish a blood vessel network (Reynolds et al., 2000). The rapid microvasculature growth rate is critical for proper CL development and function, and inadequate ovarian blood supply has been associated with abnormal, decreased luteal function (Hazzard et al., 2002; Reynolds et al., 2000). Furthermore,

decreased vascularity is characteristic for luteal regression during the late luteal phase (Knickerbocker et al., 1988; Reynolds et al., 2000; Vonnahme et al., 2006).

In our study, Arg reduced vascularity at the early-luteal phase in C ewes. However, studies regarding the cardiovascular system have demonstrated improvements of endothelial-dependent vasodilation or blood pressure reduction after Arg-treatment to animal models or patients with cardiovascular disease (Girerd et al., 1990; Creager et al., 1992; Cooke et al., 1992; Clarkson et al., 1996; Palloshi et al., 2004; Dong et al., 2011). Since luteal vascularity was decreased in Arg C ewes at the early-luteal phase, endothelial cells may have prematurely senesced due to increased Arg concentration. In fact, it has been reported that Arg could cause senescence of human endothelial cell in vitro (Scalera et al., 2009; Xiong et al., 2014). However, decreased vascularity in Arg-treated ewes was not associated with changes in P4 secretion in this study, thus Arg did not affect the major luteal function. Additional research is required to elucidate the association between Arg and endothelial cell function in the ovary.

In the present study, cell proliferation was greatest in the early-, less at the mid-, and least at the late-luteal phase, which corresponds to luteal growth, differentiation and regression, respectively (Jablonka-Shariff et al., 1993). In addition, pattern of cell proliferation was similar to vascular development during the estrous cycle. The CL is one of the fastest growing tissues (Jablonka-Shariff et al., 1993) and within just a few days post-ovulation, the ovine CL can weigh more than 500 mg (Farin et al., 1986; Jablonka-Shariff et al., 1993).

Similar to previous observations, eNOS protein was localized within luteal tissue vasculature in ewes in this study (Al-Gubory et al., 2005, Grazul-Bilska et al., 2006). The current data demonstrate that eNOS protein expression was greatest at the mid-luteal phase when

compared to early- or late-luteal phase. In contrast to our results, previous studies have reported greater eNOS protein expression in the CL from the early-luteal phase in sheep (Al-Gubory et al., 2005; Grazul-Bilska et al., 2006). These differences are likely due to methodological differences including antibody sensitivity, detection technique (fluorescence vs colorimetric), and image analysis program. Although eNOS expression was localized to luteal blood vessels, pattern of expression of CD31 (marker of vascularization) and eNOS differed. We hypothesize that CD31 marks presence of the vascular bed and may not play any additional function in regulation of blood vessel function in the CL. In addition, since eNOS plays a regulatory function in the CL, its expression depends not only on the number of vascular cells but also on regulatory role.

In the current study, Arg affected luteal vascularity, as well as *eNOS* and *GUCY1B3* mRNA expression, depending on the stage of the estrous cycle. Since Arg is a precursor for many components, including NO, supplementing Arg in excess could enhance NO production. In fact, several previous studies have demonstrated that NO production is primarily determined by extracellular Arg bioavailability in bovine (Hardy et al., 2002) and human (Xiong et al., 2014) endothelial cells, and that Arg administration for just 30 minutes results in an increase of NO production (Xiong et al., 2014).

Luteal expression of *GUCY1B3* mRNA but not sGC protein tended to be affected by Arg in our study. However, neither was affected by plane of nutrition or stage of the estrous cycle. Grazul-Bilska et al. (2006) reported similar results, as GUCY1B3 mRNA expression was unchanged in luteal tissue throughout the estrous cycle. It can be speculated that, since sGC is

the main receptor for NO, as serum Arg increased, the receptor gene expression increased as well.

In the present study, *VEGF* mRNA expression was greatest at the early- and mid-luteal phase and VEGF receptor *KDR* mRNA expression was influenced by nutritional plane and Arg depending on the stage of the estrous cycle. VEGF is an important regulator of blood vessel permeability, protease production, endothelial cell proliferation and migration to initiate angiogenesis, survival of endothelial cells, and normal blood vessel function (Alon et al., 1995; Ferrara and Davis-Smyth, 1997; Gerber et al., 1998). We postulate that during the early-luteal phase specific concentration of VEGF expression is required to regulate angiogenesis, and during the mid-luteal phase VEGF may be required to control blood vessel permeability and endothelial cell function. Our findings are similar to a previous studies, wherein VEGF and/or receptor KDR were detected in luteal tissues of women (Sugino et al., 2000), sow (Kaczmarek et al., 2006), water buffalo (Papa et al., 2006), cow (Berisha et al., 2000), mare (Al-zi′abi et al., 2003; Müller et al., 2009), monkey (Ravindranath et al., 1992; Fisher et al., 2013), and ewe (Redmer et al., 1996; Vonnahme et al., 2006). Furthermore, increased *VEGF* mRNA expression during the mid-luteal phase was observed in women and mares (Al-zi′abi et al., 2003; Papa et al., 2006; Müller et al., 2009, respectively). Inhibition of VEGF clearly demonstrated critical importance of the VEGF system for luteal function in several species (Ferrara et al., 1998; Kuhnert et al., 2008; Fraser et al., 2000; Zimmermann et al., 2001; Hazzard et al., 2002). In fact, VEGF inhibition resulted in decreased luteal vasularization, angiogenesis, endothelial cell proliferation and/or decreased P4 secretion in rats, mice and primates (Ferrara et al., 1998; Kuhnert et al., 2008; Fraser et al., 2000; Zimmermann et al., 2001; Hazzard et al., 2002).

In the present study, *ANGPT2* mRNA expression was greatest at mid-luteal phase when compared to the early- or late-luteal phase, and expression of ANGPT1 mRNA was similar at all stages of luteal development. Similarly, no change in *ANGPT1* mRNA expression, but increased *ANGPT2* mRNA expression was observed in ovine and bovine CL after PGF2α administration (Vonnahme et al., 2006; Berisha et al., 2010).

Our hypothesis was based on previous observations for pregnant animals, demonstrating that Arg potentially benefitted placental function and increased the number of offspring born (Mateo et al., 2007; Zeng et al., 2008; Luther et al., 2009) and offspring body weights (de Boo et al., 2005; Lassala et al., 2010) in sheep and rats. However, Arg to non-pregnant ewes, while changing luteal vascularity, demonstrated no change in ovulation rate, CL weight, or P4 production. The limited effects of Arg on ovarian function in the present study may be due to the fact that Arg is a semi-essential amino acid in non-pregnant animals. Further research is required to determine potential Arg benefits in non-pregnant females.

In summary, we have demonstrated that 1) nutritional plane affects ovulation rates, serum P4 concentrations, and *KDR* mRNA expression, 2) P4 concentrations, luteal tissue vascularity, cell proliferation, eNOS protein, and the VEGF system change during the estrous cycle, and 3) Arg affects luteal vascularity and the NO system during the estrous cycle. These data indicate that plane of nutrition and/or Arg can alter P4 secretion, vascularization and expression of selected angiogenic factors in the CL during the estrous cycle. In addition, our data further support previous observations that the VEGF and NO systems are involved in luteal function throughout the estrous cycle in sheep.

References

Abecia, J. A., J. M. Lozano, F. Forcada, and L. Zarazaga. 1997. Effect of level of dietary energy and protein on embryo survival and progesterone production on day eight of pregnancy in Rasa Aragonesa ewes. Anim. Reprod. Sci. 48:209-18.

Al-Gubory, K. H., I. Ceballos-Picot, A. Nicole, P. Bolifraud, G. Germain, M. Michaud, C. Mayeur, and F. Blachier. 2005. Changes in activities of superoxide dismutase, nitric oxide synthase, glutathione-dependent enzymes and the incidence of apoptosis in sheep corpus luteum during the estrous cycle. Biochim. Biophys. Acta. 1725:348-57.

Alon, T., I. Hemo , A. Itin, J. Pe'er, J. Stone, and E. Keshet. 1995. Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. Nat. Med. 1:1024-8.

Al-zi´abi, M. O., E. D.Watson, and H. M. Fraser. 2003. Angiogenesis and vascular endothelial growth factor expression in the equine corpus luteum. Reproduction. 125:259-70.

Armstrong, D. G., J. G. Gong, and R. Webb. 2003. Interactions between nutrition and ovarian activity in cattle: Physiological, cellular and molecular mechanisms. Reproduction. Suppl. 61:403–414.

Baird, A., F. Esch, D. Gospodarowicz, and R. Guillemin. 1985. Retina- and eye-derived endothelial cell growth factor: Partial molecular characterization and identity with acidic and basic fibroblast growth factors. Biochemistry. 24:7855-9.

Beal, W.E., Short R.E., Staigmiller R.B., Bellows R.A., Kaltenbach C.C., and Dunn T.G. 1978. Influence ofdietary energy intake on bovine pituitary and luteal function. J. Anim. Sci. 46:181-8.

Beckman JD, Grazul-Bilska AT, Johnson ML, Reynolds LP, and Redmer DA. 2006. Isolation and characterization of ovine luteal pericytes and effects of nitric oxide on pericyte expression of angiogenic factors. Endocrine. 29:467-76.

Berisha, B., D. Schams, M. Kosmann, W. Amselgruber, and R. Einspanier. 2000. Expression and tissue concentration of vascular endothelial growth factor, its receptors, and localization in the bovine corpus luteum during estrous cycle and pregnancy. Biol. Reprod.63:1106-14.

Boiti, C., D. Zampini, G. Guelfi, F. Paolocci, M. Zerani, and A. Gobbetti. 2002. Expression patterns of endothelial and inducible nitric oxide synthase isoforms in corpora lutea of pseudopregnant rabbits at different luteal stages. Journal of Endocrinology. 173:285-96.

Carter, B. W. Jr, L. G. Chicoine, and L. D. Nelin. 2004. L-lysine decreases nitric oxide production and increases vascular resistance in lungs isolated from lipopolysaccharide-treated neonatal pigs. Pediatric. Res. 55:979-87.

Chen, J., P. Kuhlencord, F. Urano, H. Ichinose, J. Astern, and P. L. Huang. 2003. Effects of chronic treatment with L-arginine on atherosclerosis in apoE knockout and apoE/inducible NO synthase double-knockout mice. Arterioscler. Thromb. Vasc. Biol. 23:97-103.

Chin, K., Y. Kurashima, T. Ogura, H. Tajiri, S. Yoshida, and H. Esumi. 1997. Induction of vascular endothelial growth factor by nitric oxide in human glioblastoma and n hepatocellular carcinoma cells. Oncogene. 15:437-42.

Christenson, L. K. and R. L. Stouffer. 1996. Proliferation of microvascular endothelial cells in the primate corpus luteum during the menstrual cycle and simulated early pregnancy. Endocrinology. 137:367-74.

Clarkson, P., M. R. Adams, and A. J. Powe. 1996. Oral L-arginine improves endothelium-dependent dilation in hypercholesterolemic young adults. J Clin Invest. 97:1989-94.

Cooke, J. P., A. H. Singer, P. Tsao, P. Zera, R. A. Rowan, and M. E. Billingham. 1992. Antiatherogenic effects of L-arginine in the hypercholesterolemic rabbit. J. Clin. Invest. 90:1168-72.

Corner, G. W., and W. M. Allen. 1929. Physiology of the corpus luteum, II; production of a special uterine reaction (progestational proliferation) by extract of the corpus luteum. Am. J. Physiol. 88:326-99.

Creager, M. A., S. J. Gallagher, X. J. Girerd, S. M. Coleman, V. J. Dzau, and J. P. Cooke. 1992. L-arginine improves endothelium-dependent vasodilation in hypercholesterolemic humans. J. Clin. Invest. 90:1248-53.

Cumming, I. A., J. A. Brown, M. A. Blockley, C. G. Winfield, R. Baxter, and J. R. Goding. 1971. Constancy of interval between luteinizing hormone release and ovulation in the ewe. J. Reprod. Fertil. 24:134-5.

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.

Diaz, F. J., L. E. Anderson, Y. L. Wu, A. Rabot, S. J. Tsai, and M. C. Wiltbank. 2002. Regulation of progesterone and prostaglandin F-2 alpha production in the CL. Mol. Cell. Endocrinol. 191:65-80.

Diskin, M. G., Mackey, D. R., Roche, J. F. & Sreenan, J. M. 2003. Effects of nutrition metabolic status on circulating hormones and ovarian follicle development in cattle. Anim. Reprod. Sci. 78: 345-70.

Donaldson, L. E., J. M. Basset, and G. D. Thorburn. 1970. Peripheral plasma progesterone concentration of cows during puberty, oestrous cycles, pregnancy and lactation, and the effects of under nutrition or exogenous oxytocin on progesterone concentration. J Endocrinology. 48:599-614.

Dong, Y. L., R. R. Gangula, L. Tang, and C. Yallampalli. 1999. Nitric oxide reverses prostaglandin induced inhibition in ovarian progesterone secretion in rats. Hum. Reprod. 14:27-32.

Dong, J. Y., L. Q. Qin. Z. Zhang, Y. Zhao, J. Wang, F. Arigoni, and W. Zhang. 2011. Effect of arginine supplementation on blood pressure: a meta-analysis of randomized, double-blind, placebo-controlled trials. Am. Heart. J. 162:959-65.

Duda, D. G., D. Fukumura, and R. K. Jain. 2004. Role of eNOS in neovascularization: NO for endothelial progenitor cells. Trends. Mol. Med. 10:143-5.

Dumont, D. J., T. P. Yamaguchi, R. A. Conlon, J. Rossant, and M. L. Breitman. 1992. Oncogene. 7:1471-80.

Farin, C. E., C. L. Moeller, H. R. Sawyer, F. Gamboni, and G. D. Niswender. 1986. Morphometric analysis of cell types in the ovine corpus luteum throughout the estrous cycle. Biol. Reprod. 35:1299–1308.

Ferrara, N. 1999. Molecular and biological properties of vascular endothelial growth factor. J. Mol. Med. (Berl). 77:527-43.

Ferrara, N., H. Chen, T. Davis-Smyth, H. P. Gerber, T. N. Nguyen, D. Peers, V. Chisholm, K. J. Hillan, and R. H. Schwall. 1998. Vascular endothelial growth factor is essential for corpus luteum angiogenesis. Nat. Med. 4:336-40.

Ferrara, N., and T. Davis-Smyth. 1997. The biology of vascular endothelial growth factor. Endocr. Rev.18:4-25.

Fisher, T. E., T. A. Molskness, A. Villeda, M. B. Zelinski, R. L. Stouffer, and J. Xu. 2013. Vascular endothelial growth factor and angiopoietin production by primate follicles during culture is a function of growth rate, gonadotrophin exposure and oxygen milieu. Hum. Reprod. 28:3263-70.

Fraser, H. M., S. E. Dickson, S. F. Lunn, C. Wulff, K. D. Morris, V. A. Carroll, and R. Bicknell. 2000. Suppression of luteal angiogenesis in the primate after neutralization of vascular endothelial growth factor. Endocrinology. 141:995-1000.

Friden, E. B., E. Runessen, and M. Hahlin. 2000. Evidence for nitric oxide acting as a luteolytic factor in the human corpus luteum. Mol. Hum. Reprod. 6:397-403.

Gerber, H. P., A. McMurtrey, J. Kowalski, M. Yan, B. A. Keyt, V. Dixit, and N. Ferrara. 1998. Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. J. Biol. Chem. 273:30336-43.

Girerd, X. J., A. T. Hirsch, J. P. Cooke, V. J. Dzau, and M. A. Creager. 1990. L-arginine augments endothelial dependent vasodilation in cholesterol-fed rabbits. Circul. Res. 67:1301-8.

Geva, E., and R. B. Jaffe. 2000. Role of vascular endothelial growth factor in ovarian physiology and pathology. Fertil. Steril. 74:429-38.

Gombe, S., and W. Hansel. 1973. Plasma luteinizing hormone (LH) and progesterone levels in heifers on restricted energy intakes. J. Anim. Sci. 37:728-33.

Grazul-Bilska, A. T., E. Borowczyk, J. J. Bilski, L. P. Reynolds, D. A. Redmer, J. S. Caton, and K. A. Vonnahme. 2012. Overfeeding and underfeeding have detrimental effects on oocyte quality measured by in vitro fertilization and early embryonic development in sheep. Dom. Anim. Endo. 43:289-98.

Grazul-Bilska, A. T., C. Navanukraw, M. L. Johnson, D. A. Arnold, L. P. Reynolds, and D. A. Redmer. 2006. Expression of endothelial nitric oxide synthase (eNOS) in the ovine ovary throughout the estrous cycle. Reproduction. 132:579-87.

Grazul-Bilska, A. T., D. A. Redmer, and L. P. Reynolds. 2001. Growth factors during ovarian angiogenesis. In: Vascular Morphogenesis in the Female Reproductive System. Eds: HG Augustin, ML Iruela-Arispe, PAW Rogers, SK Smith. Springer Verlag. pp. 131-47.

Gunn, R. G., and J. M. Doney. 1975. The interaction of nutrition and body condition score at mating on ovulation rate and early embryo mortality in Scottish Blackface ewes. J. Agri. Sci. 85:465-70.

Hallemeesch, M. M., W. H. Lamers, and N. E. Deutz. 2002. Reduced arginine availability and nitric oxide production. Clin. Nutr. 21:273-9.

Hamudikuwanda, H., G. Gallo, E. Block, and B E. Downey. 1996. Adipose tissue progesterone concentrations in dairy cows during late pregnancy and early lactation. Anim. Reprod. Sci. 43:15–23.

Hardy, T. A. and J. M. May. 2002. Coordinate regulation of L-arginine uptake and nitric oxide synthase activity in cultured endothelial cells. Free. Rad. Res. Commun. 32:122-31.

Hazzard, T. M., F. H. Xu, and R. L. Stouffer. 2002. Injection of soluble vascular endothelial growth factor receptor 1 into the preovulatory follicle disrupts ovulation and subsequent luteal function in rhesus monkeys. Biol. Reprod. 67:1305-12.

Hoaglund, C. M., V. M. Thomas, M. K. Petersen, and R. W. Kott. 1992. Effects of supplemental protein source and metabolizable energy intake on nutritional status in pregnant ewes. J. Anim. Sci. 70:273.

Hoeben, A., B. Landuyt, M. S. Highley, H. Wildiers, A. T. Van Oosterom, and E. A. De Bruijn. 2004. Vascular endothelial growth factor and angiogenesis. Pharmacol. Rev. 56:549-80.

Hurwitz, A., Z. Finci-Yeheskel, A. Milwidsky, and M. Mayer. 2002. Regulation of cyclooxygenase activity and progesterone production in the rat corpus luteum by inducible nitric oxide synthase. Reproduction. 123:663-9.

Jaroszewski, J. J., M. Bogacki, and D. J. Skarzynski. 2003. Progesterone production in bovine luteal cells treated with drugs that modulate nitric oxide production. Reproduction. 125:389-95.

Jaroszewski, J. J., D. J. Skarzynski, and K. Okuda. 2001. Nitric oxide as a local regulator in the mammalian ovary. In: Reproductive Biotechnology: Reproductive Biotechnology Update and Its Related Physiology. H Miyamoto and N Manabe. Elsevier, Tokyo. Pp. 105-12.

Jablonka-Shariff, A., A. T. Grazul-Bilska, D. A. Redmer, and L. P. Reynolds. 1993. Growth and cellular proliferation of ovine corpora lutea throughout the ovine estrous cycle Endocrinology. 133:1871–9.

Johnson, D. E., and L. T. Williams. 1993. Structural and functional diversity in the FGF receptor multigene family. Adv. Canc. Res. 60:1-41.

Jordan, E. R., and L. V. Swanson. 1979. Serum progesterone and luteinizing hormone in dairy cattle fed varying levels of crude protein. J. Anim. Sci. 48:1154-8.

Kaczmarek, M. M., A. E. Kowalczyk, A. Waclawik, D. Schams, and A. J. Ziecik. 2007. Expression of vascular endothelial growth factor and its receptors in the porcine corpus luteum during the estrous cycle and early pregnancy. Mol. Reprod. Dev.74:730-9.

Kaminski, S. L., D. A. Redmer, C. S. Bass, D. H. Keisler, K. A. Vonnahme, S. T. Dorsam, and A. T. Grazul-Bilska. 2015. Metabolic and reproductive hormones, and selected metabolites in serum of overfed or underfed ewes treated with arginine during the estrous cycle. Theriogenology. (in press).

Keisler, D. H., and B. C. Buckrell. 1997. Breeding Strategies. In: Youngquist RS. Current Therapy in Large Animal Theriogenology. WB Saunders & Co. Philadelphia. 603-11.

Kirk, R. E. 1982. Experimental design: Procedures for the behavioral sciences (2nd ed.). Monterey, CA: Brooks/Cole.

Knickerbocker, J. J., M. C. Wiltbank, and G. D. Niswender.1988. Mechanisms of luteolysis in domestic livestock. Domest. Anim. Endo. 5:91–107.

Korzekwa, J. A., K. Okuda, I. Woclavek-Potocka, S. Murakami, and D. J. Skarzynski. 2006. Nitric oxide induces apoptosis in bovine luteal cells. J. Reprod. Dev. 52:351-61.

Kottakis, F., C. Polytarchou, P. Foltopoulou, I. Sanidas, S. C. Kampranis, and P. N. Tsichlis. 2011. FGF-2 regulates cell proliferation, migration, and angiogenesis through an NDY1/KDM2B-miR- 101-EZH2 pathway. Mol. Cell. 43:285-98.

Kroll, J., and J. Waltenberger. 1999. A novel function of VEGF receptor-2 (KDR): rapid release of nitric oxide in response to VEGF-A stimulation in endothelial cells. Biochem. Biophys. Res. Comm. 265:636-9.

Ku, D. D., J. K. Zaleski, S. Liu, and T. A. Brock. 1993. Vascular endothelial growth factor induces EDRF dependent relaxation in coronary arteries. Am. J. Physiol. 265:586–92.

Kuhnert, F., B. Y. Tam, B. Sennino, J. T. Gray, J. Yuan, A. Jocson, N. R. Nayak, R. C. Mulligan D. M. McDonald, and C. J. Kuo. Soluble receptor-mediated selective inhibition of VEGFR and PDGFRbeta signaling during physiologic and tumor angiogenesis. Proc. Natl. Acad. Sci. 105; 10185-90**.**

Lassala, A., F. W. Bazer, T. A. Cudd, S. Datta, D. H. Keisler, M. C. Satterfield, T. E. Spencer, and G. Wu. 2010. Parenteral administration of L-arginine prevents fetal growth restriction in undernourished ewes. J. Nutr. 140:1242-8.

Lassoued, N., M. Rekik, M. Mahouachi, and M. Ben Hamouda. 2004. The effect of nutrition prior to and during mating on ovulation rate, reproductive wastage, and lambing rate in three sheep breeds. Small. Rum. Res. 52:117-25.

Luther, J.S., E. J. Windorski, J. S. Caton, G. Wu, J. D. Kirsch, K. A. Vonnahme, L. P. Reynolds, and C. S. Schauer. 2009. Effects of arginine supplementation on reproductive performance in Rambouillet ewes. http://www.ag.ndsu.edu/HettingerREC/sheep/2009-Sheep-Report.pdf.

Maisonpierre, P. C., C. Suri, P. F. Jones, S. Bartunkova, S. J. Wiegand, C. Radziejewski, D. Compton, J. McClain, T. H. Aldrich, N. Papadopoulos, T. J. Daly, S. Davis, T. N. Sato, G. D. Yancopoulos. 1997. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science. 277:55-60.

Mateo, R. D., G. Wu, and F. W. Bazer. 2006. Dietary L-arginine supplementation enhances the reproductive performance of gilts. J. Nutr.137:652-6.

Mertz, E.T., W. M. Beeson, and H. D. Jackson. 1952. Classification of essential amino acids for the weanling pig. Arch. Biochem. Biophys. 38:121-8.

Mitsube, K., M. Mikuni, M. Matouse, and M. Brännström. 1999. Effects of a nitric oxide donor and nitric oxide synthase inhibitors on luteinizing hormone-induced ovulation in the ex-vivo perfused rat ovary. Hum. Reprod. 14:2537-43.

Miyamoto, A., K. Shirasuna, M. P. Wijayagunawardane, S. Watanabe, M. Hayashi, D. Yamamoto, M. Matsui, and T. J. Acosta. 2005. Blood flow: a key regulatory component of corpus luteum function in the cow. Domest. Anim. Endocrinol. 29:329-39.

Morris, Jr. S. M., and T. R. Billiar. 1994. New insights into the regulation of induced nitric oxide synthesis. Am. J. Physiol. 266:E829-39.

Motta, A. B., A. Estevez, M. F. de Gimeno. 1999. The involvement of nitric oxide in corpus luteum regression in the rat: feedback mechanism between prostaglandin F2αand nitric oxide. Mol. Hum. Reprod. 5:1011-6.

Motta, A. B., A. Estevez, M. Tognetti, M. F. de Gimeno, and A. M. Franchi. 2001. Dual effects of nitric oxide in functional and regressing rat corpus luteum. Mol. Hum. Reprod. 7:43-7.

Müller, K., C. Ellenberger, and H. A. Schoon. 2009. Histomorphological and immunohistochemical study of angiogenesis and angiogenic factors in the ovary of the mare. Res. Vet. Sci. 87:421-31.

Niswender, G. D., and T. M. Nett. 1988: The corpus luteum and its control. In: Knobil E, Neill J, Ewing LL, Greenwald GS, Markert CL, Pfaff DW, The Physiology of Reproduction. Raven Press, New York. pp. 489–525.

NRC. 1985. Nutrient requirements of sheep. Natl. Acad. Sci., Washington, D.C.

O'Callaghan, D., and M. P. Boland. 1999. Nutritional effects on ovulation, embryo development and the establishment of pregnancy in ruminants. Anim. Sci. 68:299-314.

Palmer, R. M. J., D. D. Rees, D. S. Ashton, and S. Moncada. 1988. L-arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation. Biochem. Biophys. Res. Comm. 153:1251-6.

Papa, P. C., C. E. Moura, L. P. Artoni, L. A. Fatima, D. B. Campos, J. E. Marques, P. S. Baruselli Jr, M. Binelli, C. Pfarrer, and R. Leiser. 2006. VEGF system expression in different stages of estrous cycle in the corpus luteum of non-treated and superovulated water buffalo. Domest. Anim. Endocrinol. 29:329-39.

Parr, R. A., I. F. Davis, R. J. Fairclough, and M. A. Miles. 1987. Overfeeding during early pregnancy reduces peripheral progesterone concentration and pregnancy rate in sheep. J. Reprod. Fert. 80:317-320.

Palloshi, A., G. Fragasso, P. Piatti, L. D. Monti, E. Setola, G. Valsecchi, E. Galluccio, S. L. Chierchia, and A. Margonato. 2004. Effect of oral L-arginine on blood pressure and symptoms and endothelial function in patients with systemic hypertension, positive exercise tests, and normal coronary arteries. Am. J. Cardiol. 93:933-5.

Rajapakse, N. W. and D. L. Mattson. 2009. Role of L-arginine in nitric oxide production in health and hypertension. Clin Exp Pharmacol Physiol. 36:249-55.

Redmer, D. A., Y. Dai, J. Li, D. S. Charnock-Jones, S. K. Smith, L. P. Reynolds, and R. M. Moor. 1996. Characterization and expression of vascular endothelial growth factor (VEGF) in the ovine corpora luteum. Journal of Reproduction and Fertility. 108:157-165.

Redmer, D. A., R. P. Aitken, J. S. Milne, L. P. Reynolds, and J. M. Wallace. 2005. Influence of maternal nutrition on messenger RNA expression of placental angiogenic factors and their receptors at midgestation in adolescent sheep. Biol. Reprod. 72:1004-9.

Redmer, D. A., V. Doaiswamy, B. J. Bortnem, K. Fisher, A. Jablonka-Shariff, A. T. Grazul-Bilska, and L. P. Reynolds. 2001. Evidence for a role of capillary pericytes in vascular growth of the developing ovine corpus luteum. Biol. Reprod. 65:879-89.

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

Reynolds, L. P., A. T. Grazul-Bilska, and D. A. Redmer. 2002. Angiogenesis in the female reproductive system: pathological implications. Review Article. Int. J. Exp. Pathol. 83:151-63.

Reynolds, L. P., A. T. Grazul-Bilska, S. D. Killilea, and D. A. Redmer. 1994. Mitogenic factors of corpora lutea. Prog. Growth. Factor. Res. 5:159-75.

Reynolds, L. P., S. D. Killilea, and D. A. Redmer. 1992. Angiogenesis in the female reproductive system. FASEB J. 6:886-92.

Rhind, S. M., G. B. Martin, S. McMillen, C. G. Tsonis, and A. S. McNeilly. 1989. Effect of level of food intake of ewes on the secretion of LH and FSH and on the pituitary response to gonadotrophin-releasing hormone in ovariectomized ewes. J. Endocr.121:325-30.

Rodrigues, R. O., C. Trevisanuto, R. F. Cooke, and J. L. Vasconcelos. 2011. Effects of body weight loss on serum progesterone concentrations of non-lactating dairy cows. Theriogenology. 75:131-7.

Rosiansky-Sultan, M., E. Klipper, K. Spanel-Borowski, R. Meidan. 2006. Inverse relationship between nitric oxide synthases and endothelin-1 synthesis in bovine corpus luteum: interactions at the level of luteal endothelial cell. Endocrinology. 147:5228-35.

Rosselli, M. 1997. Nitric oxide and reproduction. Molecular Human Reproduction. 3:639-41.

Sato, T. N., Y. Qin, C. A. Kozak, and K. L. Audus. 1993. Tie-1 and tie-2 define another class of putative receptor tyrosine kinase genes expressed in early embryonic vascular system. Proc. Natl. Acad Sci. 90:9355-8.

Scalera, F., E. I. Closs, E. Flick, J. Martens-Lobenhoffer, J. P. Boissel, U. Lendeckel, A. Heimburg, and S. M. Bode-Böger. 2009. Paradoxical effect of L-arginine: acceleration of endothelial cell senescence. Biochem. Biophys. Res. Commun. 386:650-5.

Scaramuzzi, R. J., B. K. Campbell, J. A. Downing, N. R. Kendall, M. Khalid, M. Munoz-Gutierrez, and A. Somchit. 2006. A review of the effects of supplementary nutrition in the ewe on the concentrations of reproductive and metabolic hormones and the mechanisms that regulate folliculogenesis and ovulation rate. Reprod. Nutr. Dev. 46:339-54.

Shin, S., S. Mohan, and H. L. Fung. 2011. Intracellular L-arginine concentration does not determine NO production in endothelial cells: implications on the "L-arginine paradox". Biochem. Biophys. Res. Comm. 414:660-3.

Skarzynski, D. J., J. J. Jaroskewski, M.M. Bah, K. M. Deptula, B. Barszczewska, B. Gawronska, and W. Hansel. 2003. Administration of a nitric oxide synthase inhibitor counteracts prostaglandin F_2-induced luteolysis in cattle. Bio. Reprod. 68:1674-81.

Skarzynski, D. J., S. Kobayashi, and K. Okuda. 2000. Influence of nitric oxide and noradrenaline on prostaglandin PGF2-induced oxytocin secretion and intracellular calcium mobilization in cultured bovine luteal cells. Bio. Reprod. 63:1000–5.

Smith, J. F. 1995. Protein, energy, and ovulation rate. In: Land RB, Robinson DW (Eds.), Genetics of Reproduction in Sheep. Butterworth, London, p 349-59.

Stabenfeldt, G. H., J. A. Holt, and L. L. Ewing. 1969. Peripheral plasma progesterone levels during the ovine estrous cycle. Endocrinology. 85:11-5.

Sugino, N., S. Takiguchi, S. Kashida, A. Karube, Y. Nakamura, and H. Kato. 2000. Superoxide dismutase expression in the human corpus luteum during the menstrual cycle and in early pregnancy. Mol. Hum. Reprod. 6:19-25.

Suzuki, T., H. Sasano, R. Takaya, T. Fukaya, A. Yajima, and H. Nagura. 1998. Cyclic changes of vasculature and vascular phenotypes in normal human ovaries. Hum. Reprod. 13:953 9.

van der Zee, R., T. Murohara, Z. Luo, F. Zollmann, J. Passeri, C. Lekutat, and J. M. Isner. 1997. Vascular endothelial growth factor/vascular permeability factor augments nitric oxide release from quiescent rabbit and human vascular endothelium. Circulation. 18:1030-7.

Vega, M., M. C. Johnson, H. A. Díaz, L. R. Urrutia, J. L. Troncoso, and L. Devoto. 1998. Regulation of human luteal steroidogenesis in vitro by nitric oxide. Endocrine. 8:185-91.

Vonnahme, K. A., D.A. Redmer, E. Borowczyk, J. J. Bilski, J. S. Luther, M. L. Johnson, L. P. Reynolds, and A. T.Grazul-Bilska. 2006. Vascular composition, apoptosis, and expression of angiogenic factors in the corpus luteum during prostaglandin F2alpha-induced regression in sheep. Reproduction. 131:1115-26.

Wakabayashi, Y., and M. E. Jones. 1983. Pyrroline-5-carboxylate synthesis from glutamateby rat intestinal mucosa. J. Biol. Chem. 258:3865-72.

Webb R., P. C. Garnsworthy, B. K. Campbell, M.G. Hunter. Intra-ovarian regulation of follicular development and oocyte competence in farm animals. Theriogenology. 2007; 68 Suppl 1:S22-29.

Webb R., P. C. Garnsworthy, J. G. Gong, D. G. Armstrong. Control of follicular growth: local interactions and nutritional influences. J. Anim. Sci. 2004; 82 E-Suppl:E63-74.

Wilson, A. M., R. Harada, N. Nair, N. Balasubramanian, and J. P. Cooke. 2007. L-arginine supplementation in peripheral arterial disease: no benefit and possible harm. Circulation.116:188-95.

Windmueller, H. G., and A. E. Spaeth. 1981. Am J Physiol. 241:E473-80.

Wu, G., N. E. Flynn, S. P. Flynn, C. A. Jolly, and P. K. Davis.1999. Dietary protein or arginine deficiency impairs constitutive and inducible nitric oxide synthesis by young rats. J. Nutr. 129:1347-54.

Wu, G., and D. A. Knabe. 1995. Arginine synthesis in enterocytes of neonatal pigs. Am. J. Physiol. 269:621-9.

Wu, G., and C. J. Meininger. 2002. Arginine metabolism: nitric oxide and beyond. Ann. Rev. Nutr. 22:61-86.

Wu, G., and S. M. Morris Jr. Arginine metabolism: nitric oxide and beyond. 1998. Biochemisty. J. 15:336:1-17.

Xiong, Y., M.F. Fru, Y. Yu, J. P. Montani, X. F. Ming, and Z. Yang. 2014. Long term exposure to L-arginine accelerates endothelial cell senescence through arginase-II and S6K1 signaling. Aging. 6:369-79.

Xu, Z., M. F. McDonald, and S. N. McCutheon. 1989. The effects of nutritionally-induced liveweight differences on follicular development, ovulation rate, oestrus activity, and plasma follicle stimulating hormone levels in ewe. Anim. Reprod. Sci. 19:67-78.

Zackrisson, U., M. Mikuni, and A. Wallin. 1996. Cell-specific localization of nitric oxide synthase (NOS) in the rat ovary during follicular development, ovulation and luteal formation. Hum. Reprod. 11:2667-74.

Zeng, X. F., F. L. Wang, X. Fan, W. J. Yang, B. Zhou, P. F. Li, Y. L. Yin, G. Wu, and J. J. Wang. 2008. Dietary arginine supplementation during early pregnancy enhances embryonic survival in rats. J. Nutr. 138:1421-5.

Zerani, M., G. Catone, G. Betti, and F. Parillo. 2013. Immunopresence and functional activity of prostaglandin-endoperoxide synthases and nitric oxide synthases in bovine corpora lutea during diestrus. Folia Morphol (Warsz). 72:36-40.

Zimmermann, R. C., E. Xiao, N. Husami, M. V. Sauer, R. Lobo, J. Kitajewski, and M. Ferin. 2001. Short-term administration of antivascular endothelial growth factor antibody in the late follicular phase delays follicular development in the rhesus monkey. J. Clin. Endocrinol. Metab. 86:768-72.

CHAPTER 3: ARGININE (ARG) EFFECTS ON EARLY EMBRYONIC DEVELOPMENT AND OOCYTE LIPID DROPLET CONTENT IN OVERFED AND UNDERFED SHEEP

Abstract

Plane of nutrition may affect reproductive function, including oocyte quality. We have recently demonstrated that nutritional plane and/or in vivo arginine (Arg) supplementation (a precursor for proteins, nitric oxide and polyamines) affected selected ovarian functions such as ovulation rate and luteal function. However, the effects of nutritional plane and in vitro Arg on ovine oocyte lipid droplet accumulation and ability to be fertilized have not been studied. We hypothesized that high concentrations of Arg in maturation, fertilization, and culture media would affect function of oocytes collected from nutritionally compromised ewes. Ewes (n = 43) were stratified by weight and randomly assigned to one of three nutritional groups: control (C, maintenance control), overfed (O, 2xC) or underfed (U, 0.6xC). Control ewes were fed to maintain BW and offered 760 g/kg BW/day. Each ewe was fed half her total pelleted diet individually twice daily. Diets contained 2.4 Mcal of ME/kg and 13% crude protein (dry matter basis). Estrus was synchronized by a 14-day treatment with CIDRs and estrus (day 0) was estimated to be approximately 36 h after CIDR removal. Follicular development was induced by twice daily injections of follicle stimulating hormone (FSH) on days 13 and 14 of the estrous cycle. On day 15 of the estrous cycle, the number of visible follicles was determined and oocytes were collected. Cumulus oocyte complexes (COC) from two ewes of the same nutritional treatment were pooled (n=7-9 pools for each nutritional treatment) for in vitro maturation (IVM) and fertilization (IVF). Half of the oocytes (n=16-30) from each pool were

incubated with low (0.004-0.247 mM) or high (2 mM) Arg concentrations in maturation, fertilization, and culture media. During the ten week nutritional treatments, C maintained BW, O gained 13.2 ± 1.2 kg, and U lost 7.3 ± 0.7 kg. Increases in BCS were observed by 0.5 ± 0.1 and 1.2 ± 0.1 for C and O ewes, respectively, but decreased by 0.3 ± 0.2 for U ewes. The number of visible follicles was similar in all treatment groups. On day four after IVM, accumulation of lipid droplets in unfertilized oocytes was determined using BODIPY staining followed by image analysis. After IVF, developing embryos were evaluated throughout a six-day culture period. Lipid droplet accumulation was the greatest ($P<0.0001$) in O, less in C, and least in U (23.2 ± 1.0, 17.0 ± 1.3 and 11.8 ± 0.7%, respectively), and high Arg tended ($P<0.1$) to increase (17.8 ± 1.3 vs. 16.2 ± 1.3%) lipid droplet quantity regardless of nutritional plane. Cleavage rate was less ($P<0.03$) in high than low Arg (53.2 ± 5.9 vs. 35.8 ± 5.1%), and was not affected by nutritional plane. Morula formation rate was affected by a nutritional plane x high Arg interaction. These data demonstrate that nutritional plane did not affect follicular development measured by the number of visible follicles, but affected accumulation of lipid droplets in oocytes, and morula formation rate. Further, high Arg in medium decreases the rate of cleavage. Thus, plane of nutrition affects oocyte function and quality, possibly through lipid droplet accumulation. Also, high Arg during in vitro maturation, fertilization and early embryonic development has detrimental effects on oocyte quality. The mechanism of regulation of oocyte function and quality by diet and/or Arg remains to be elucidated.

Introduction

Assisted reproductive technologies (ART) over the last 20 years, have found many applications in agriculture (Grazul-Bilska, 2003). Continuous progress has been made in modern

embryology in order to improve production of good quality oocytes and embryos. The major ways leading to the improvements of in vitro embryo production are through improvements of culture conditions and/or quality of gametes through diet manipulation of donor animals. Thus, numerous experiments have been performed to improve in vitro culture conditions for embryo production (Thompson, 1998; Guler et al., 2000; Rizos et al., 2002; Borowczyk et al., 2006; Grazul-Bilska et al., 2006). In addition, numerous studies have been performed to evaluate the effects of specific plane of nutrition on reproductive performance to maintain high reproductive efficiency for several species including sheep and cows (O'Callaghan et al., 2000; Lozano et al., 2003; Peura et al., 2003; Yaakub et al., 1999; Armstrong et al., 2001; Webb et al., 2004; Scaramuzzi et al., 2006; Grazul-Bilska et al., 2012).

Proper oocyte function and quality, and early embryonic development are critical for embryonic survival (Krisher et al., 2003). These processes are regulated by several intrinsic (e.g., hormones) and extrinsic (e.g., nutrition and other environmental factors) factors (Boland et al., 2001; Burdge et al., 2007; Wu et al., 2010; Grazul-Bilska et al., 2012; Castaneda et al., 2013). Nutrition during the periconception period has been demonstrated to influence reproductive outcomes such as changes in hormone production, oocyte lipid content, and fertilization rates (Robinson et al., 1990; Webb et al., 2004; Kakar et al., 2005; Borowczyk et al., 2006; Castaneda et al., 2013; Grazul-Bilska et al., 2012). However, contradictory results have been reported for the effects of a high or low nutritional plane on oocyte quality (Papadopoulos et al., 2001; Lozano et al., 2003; Borowczyk et al., 2006) and early embryonic development in livestock species (Mantovani et al., 1993; Creed et al., 1994; Nolan et al., 1998; Lozano et al., 2003; Kakar et al., 2005; Grazul-Bilska et al., 2012; Rattay et al., 1980; Smith et al., 1983). In

fact, researchers have reported positive, negative and no effect of plane of nutrition on oocyte quality and early embryonic development and differing results likely depended on duration of nutritional treatment, hormones used, animal breed, and/or culture conditions (McEvoy et al., 1995; O'Callaghan et al., 2000; Boland et al., 2001; Lozano et al., 2003; Peura et al., 2003; Grazul-Bilska et al., 2012).

Arginine, which can be derived exogenously through the diet or endogenously via the intestinal-renal pathway, is a precursor for nitric oxide (NO), proteins, and polyamines (Wu et al., 2009, 2013). The NO system is involved in the regulation of oocyte development, angiogenesis, proper vascular function, ovulation, and luteolysis (Rosselli et al., 1998; Gregg et al., 2003; Wu et al., 2009; Zeng et al., 2013). A few studies utilizing swine oocytes have demonstrated that Arg in media used for in vitro maturation, fertilization and/or embryo culture enhance oocyte developmental competence and/or early embryonic development (Hong and Lee, 2007; Bauer et al., 2011).

The associations between nutrition, lipid content, and function of the oocyte have been identified for several species (Ferguson and Leese, 2006; Wu et al., 2010; Yang et al., 2012; Castaneda et al., 2013; Auclair et al., 2013; Crocco et al., 2013; Hiraga et al., 2013). Nutritional plane affects lipid droplet accumulation or depletion within cells, including the mouse oocyte (Wu et al., 2010). Lipids appear to be an important source of energy during oocyte growth, maturation and fertilization (Barcelo-Fimbres and Seidel, 2011; Jungheim et al., 2011; Aardema et al., 2013; Auclair et al., 2013; Castadena et al., 2013; Hiraga et al., 2013). Furthermore, lipid accumulation in oocytes has been associated with developmental competence of oocytes in cows

and sows (Castaneda et al., 2013; Hiraga et al., 2013). Lipid storage is reduced during the fertilization stage to formation of a two-cell embryo (Ferguson and Leese, 1999).

Objectives and Hypothesis

We hypothesized that high concentrations of Arg in maturation, fertilization and culture media will affect function of oocytes collected from ewes fed adequate (maintenance control), excess or restricted diets. Therefore, the objective of this study was to evaluate the in vitro effects of Arg on oocyte lipid droplets accumulation, in vitro fertilization and early embryonic development in overfed to underfed FSH-treated ewes.

Materials and Methods

Animals and Experimental Design

All procedures performed were approved by the North Dakota State University Institutional Animal Care and Use Committee (#A12013). The study commenced in August and ended in November, during the natural breeding season for sheep in the northern hemisphere. Non-pregnant, non-lactating Rambouillet ewes (n=43) between three to five years of age and of similar genetic background were individually penned at the Animal Nutrition and Physiology Center. Ewes were stratified by weight and randomly assigned into one of three dietary treatments: control (C; n = 14) ewes received a maintenance diet (100% NRC requirements), overfed (O; n = 13) ewes received 2.0 x C, and underfed (U; n = 16) ewes were fed 60.6 x C 60 days prior to the onset of the estrous cycle (day 0). Ewes were fed half their individual diet twice daily (at 0800 h and 1500) and had ad libitum access to water. For the duration of the experiment, body weights (BW) and body condition scores (BCS) were determined weekly. Diets were adjusted weekly to ensure the proper BW and BCS was achieved by day 0, and

maintained until completion of experiment on day 15 of the estrous cycle. Estrus was synchronized by vaginal insertion of a controlled internal drug release (CIDR) device for 14 days. Based on previous results (Grazul-Bilska et al., unpublished), 36 h after CIDR removal, ewes were considered in estrus and treated as day 0 (d 0) of the estrous cycle. All ewes were injected twice daily (morning and evening) with FSH (FSH-P; Sioux Biochemical, Sioux Center, IA, USA) on days 13 (5 mg/injection) and 14 (4 mg/injection) following estrus as described previously (Grazul-Bilska et al., 2012). On day 15 of the estrous cycle, ovaries were collected, immersed in phosphate-buffered saline (PBS), and transported to the laboratory in an incubator (37-39°C).

Oocyte Collection and Processing

Oocyte collection, processing, fertilization, and culture have been described in detail by Grazul-Bilska et al. (2003, 2006, 2012). Briefly, the number of visible small (≤ 3mm) and large (>3mm) follicles on each ovary was determined and oocytes were isolated by opening each visible follicle with a sterile scalpel blade and flushing it two to three times with oocyte collection medium with heparin (Sigma, St. Louis, MO). Under a stereomicroscope, oocytes were recovered from each dish and transferred to a petri dish containing fresh collection medium without heparin. Oocytes were then evaluated and categorized as healthy or atretic based on their morphology (Grazul-Bilska et al., 2006, 2012). Oocytes from two ewes of the same nutritional plane were combined and divided into two groups. One group of oocytes was randomly allotted to incubation in maturation, fertilization and culture media with low Arg concentration (247.02, 4.41 and 49.59 μM, respectively), while the other oocyte group underwent the same procedures but in media with high Arg concentration (2-2.3 mM). All

oocytes were then washed three times in maturation medium (TCM-199 containing 10% fetal bovine serum, ovine FSH [5 μg/ml; oFSH-RP-1; NIAMDD-NIH, Bethesda, MD], ovine LH [5 μg/ml; oLH-26; NIADDK-NIH], estradiol [1 μg/ml; Sigma St. Louis, MO], glutamine [2 mM; Sigma], sodium pyruvate [0.25 mM; Sigma], epidermal growth factor [10 ng/ml; Sigma] and penicillin/streptomycin [100 units/ml penicillin and 100 μg/ml streptomycin]).

In Vitro Maturation

Oocytes were matured in vitro in maturation medium for 24 h at 39°C in 5% CO_2 and 95% air followed by cumulus cell removal using a solution of 1% (wt/vol) hyaluronidase (Type I; Sigma) in PBS. The oocytes were again evaluated for health based on morphology. Oocytes classified as healthy were used for IVF and were transferred to fertilization medium consisting of synthetic oviductal fluid (SOF) prepared in our laboratory and 2% heat-inactivated sheep serum collected from sheep on day 0-1 of the estrous cycle.

In Vitro Fertilization and Embryo Culture

Frozen capacitated semen from one Hampshire ram was thawed, and viable and unviable sperm were separated using the swim up technique. Sperm ($0.5\text{-}1.0\text{x}10^6$ sperm/ml) were added to the IVF medium containing healthy oocytes and incubated for 18 h at 39ºC, 5% O_2, 5% CO_2 and 90% N_2. The presumptive zygotes were washed three times with culture medium without glucose (SOF supplemented with BSA, glutamine, MEM amino acids, BME amino acids [Sigma] and penicillin/streptomycin) and cultured in fresh culture medium for 24 h at 39ºC, 5% O_2, 5% CO_2 and 90% N_2. The number of cleaved oocytes in each treatment group was determined and the embryos were transferred to culture medium containing glucose (1.5 mM). After 48 h, the developmental stage was evaluated and embryos were transferred to the fresh

culture medium with glucose. The rate of cleavage (number of cleaved vs. non-cleaved oocytes), and the rate of early embryonic development (time and percentage reaching stage of morula) were evaluated every second day during a total of six days of culture. Data was compared among treatment groups.

Staining of Lipid Droplets and Image Analysis

Three days after IVF, all non-cleaved oocytes (n=1-7/culture) were separated from embryos. Sperm was removed from oocytes by repeated pipetting with a 150 μm diameter micropipette. Unfertilized, naked oocytes fixed in 10% formalin solution were stained with BODIPY (1 μg/ml; Molecular Probes; Eugene, OR) followed by staining with DAPI present in mounting media (ProLong; Life Technologies, Grand Island, NY). Lipid droplets were visualized with BODIPY 493/503 using 488 nm excitation and 503 nm emission wavelengths. Images of oocytes were generated using a Zeiss Axiovision microscope (Carl Zeiss, Thornwood, NY). Then, percentage of area occupied by lipid droplets out of the total oocyte area was determined by image analysis (ImagePro-Plus ver. 5.0 count module; Media Cybernetics, Rockville, MD).

Statistical Analysis

Data was analyzed as a 3x3x2 factorial, with interactions evaluated. All data are reported as mean ± SEM. Data for BW and BCS, number of follicles and oocytes, and oocyte quality parameters (e.g., the rates of cleavage and morula formation) for control, overfed and underfed ewes were analyzed statistically by using the general linear models program of the Statistical Analysis System (SAS User's Guide, 2014). For all other parameters, the model included nutritional plane and Arg. When F-tests were significant, differences between specific means

were evaluated by using Least Significant Difference test (Kirk, 1982). In addition, data for the percentage of oocytes cleaved, and the rate of morula and blastocyst formation (%) were analyzed by using Chi-square analysis (SAS 2014).

Results

During the 10-week nutritional treatments, C ewes maintained BW and BCS, while O ewes gained and U ewes lost BW and BCS (Table 3.1). Plane of nutrition did not affect ($P=0.15$) the number of visible ovarian follicles (Table 3.1). Cleavage rate was decreased ($P<0.03$) by high Arg, but was not affected by plane of nutrition (Table 3.2). Morula formation tended ($P=0.14$) to be affected by plane of nutrition, being greater ($P=0.14$) in C and U than O group and was not affected by Arg (Table 3.2). Accumulation of lipid droplets was affected ($P<0.0001$) by plane of nutrition, being greatest in O, less in C, and least in U ewes (Table 3.2, Figure 3.1) and was not affected by Arg ($P=0.16$).

Table 3.1. Efects of nutritional plane on ovarian follicle number, and body weight (BW) and body condition score (BCS) for ewes.

Measurement	Control	Overfed	Underfed	P value
Number of ewes	14	13	16	
Total follicles (n)	30.9 ± 2.8	26.4 ± 2.0	24.2 ± 2.4	0.150
Initial BW (kg)	53.9 ± 1.4	55.0 ± 1.0	52.5 ± 2.0	0.552
Final BW (kg)	55.9 ± 1.5^{a}	68.2 ± 1.3^{b}	45.2 ± 2.0^{c}	0.0001
Difference in BW (kg)**	1.9 ± 0.8^{a}	13.2 ± 1.2^{b}	-7.3 ± 0.7^{c}	0.0001
ADG (kg)	0.03 ± 0.01^{a}	0.18 ± 0.02^{b}	-0.10 ± 0.01^{c}	0.0001
Initial BCS	2.7 ± 0.1	2.7 ± 0.1	2.6 ± 0.1	0.959
Final BCS	3.1 ± 0.1^{a}	3.9 ± 0.1^{b}	2.3 ± 0.2^{c}	0.0001
Difference in BCS**	0.5 ± 0.1^{a}	1.2 ± 0.1^{b}	-0.3 ± 0.2^{c}	0.0001

All values (mean ± SEM) are expressed per ewe.
**Difference between initial and final BW or BCS.

Table 3.2. Effects of nutritional plane and arginine (Arg) or saline (Sal) treatment on cleavage and morula rates, and liquid droplet content in oocytes.

Measurement	Control[3]	Control+Arg	Overfed3	Overfed+Arg	Underfed	Underfed+Arg	Diet	Arg	Diet x Arg
Number of cultures[4]	7	7	8	8	9	9			
Oocytes for IVF (n)	29.8 ± 4.4	30.4 ± 3.4	18.1 ± 2.4	19.0 ± 2.8	16.8 ± 3.2	18.4 ± 2.9	0.001	0.690	0.984
Percent cleaved (%)	60.4 ± 8.0^{a}	32.8 ± 9.5^{bc}	45.3 ± 8.8^{ac}	31.5 ± 9.8^{bc}	51.8 ± 10.7^{ac}	41.9 ± 8.5^{ac}	0.592	0.030	0.637
Percent morula (%)	36.0 ± 9.9^{a}	22.2 ± 8.7^{abc}	19.1 ± 12.0^{abc}	10.9 ± 4.4^{b}	32.7 ± 10.2^{ac}	33.8 ± 10.3^{ac}	0.140	0.386	0.742
Lipid droplet content (%)	15.7 ± 1.1^{a}	17.0 ± 2.1^{ab}	22.4 ± 1.7^{c}	23.9 ± 1.2^{c}	11.0 ± 0.8^{d}	12.4 ± 1.1^{bd}	0.0001	0.157	0.954

*All values (mean ± SEM) are expressed per culture.

[1]Ewes were fed a maintenance (control), overfed (2 x control), or underfed (0.6 x control) diet.

[2]To determine lipid droplet content, unfertilized oocytes (n=1-7/culture) were fixed in formalin solution on day 3 after fertilization, and stained with BODIPY (1 µg/ml) followed by image analysis to determine percentage of positively stained area/oocyte.

[3]Concentration of arginine in maturation, fertilization, and culture media was 247.02, 4.41, and 49.59 µM, respectively. For Arg-treatment groups, 2 mM of Arg (Sigma) were added. Average concentration of Arg in follicular fluid was 97.52 ± 22.4 µM.

[4]For one culture, oocytes from two ewes of the same nutritional treatment were pooled.

$^{a-d}$means±SEM; values with different superscripts differ within a row.

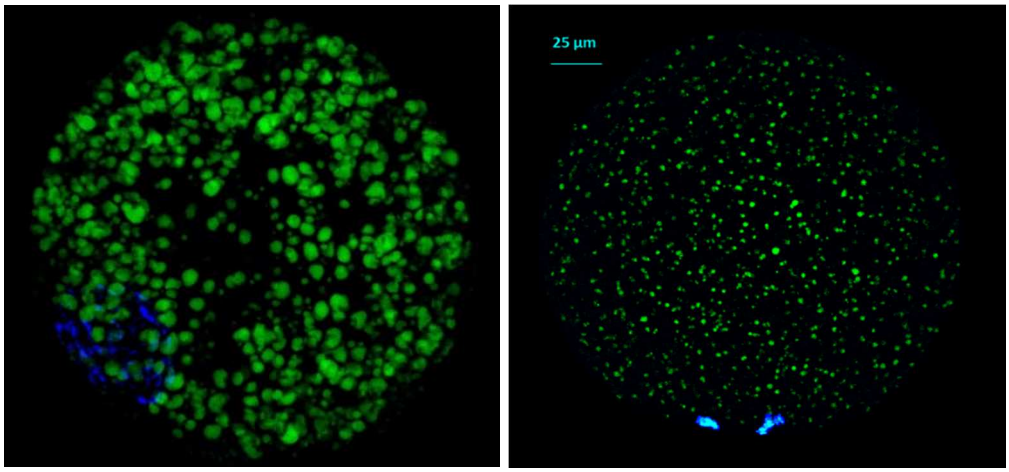


Figure 3.1. Representative image of BODIPY staining of lipid droplets (green) in a non-mature (GV stage; left) and a mature (MII stage; right) oocyte from a control ewe. Oocyte DNA is stained with DAPI (blue).

Discussion

The present study demonstrated that nutritional plane affected morula formation and lipid droplet accumulation in oocytes, while high Arg affected cleavage rate. Furthermore, plane of nutrition did not affect the number of visible follicles. Other researchers have also demonstrated that overfeeding and underfeeding ewes did not affect number of small or large follicles when compared to control fed ewes (Abecia et al., 1995, 1997; Peura et al., 2003; Borowczyk et al., 2006; Grazul-Bilska et al., 2012). Additionally, the number of follicles was similar in lactating dairy cows (not treated with FSH) that received a low or high energy diet for approximately 25 weeks postpartum (Kendrick et al., 1999) and in yearling beef heifers (not treated with FSH) fed ad libitum or 0.75 x ad libitum for 100 days (Tripp et al., 2000). Conversely, the association between nutritional plane and changes to follicular development has been previously described in ewes (Coop et al., 1966; Yaakub et al., 1997; O'Callaghan et al., 2000; Viñoles et al., 2010). In addition, excess nutrition caused decreased follicle numbers recruited during follicular wave turnover in cattle (Stagg et al., 1995). Researchers have postulated that overnutrition can cause

these changes due to altered endocrine profiles of FSH and E_2, since a positive energy balance often results in abnormally high FSH secretions and reduced E_2 concentrations (Scaramuzzi et al., 2006). Abecia et al. (2006) hypothesized, "overfeeding during follicle recruitment and oocyte maturation imparts a legacy of embryonic loss and developmental retardation." Undernutrition has also been associated with lower follicle numbers in cattle (Rhodes et al., 1996; Robinson et al., 2006), as well as decreased ovulation rates in sheep (Lassoued et al., 2004). A negative energy balance causes inhibition of GnRH secretion from the hypothalamus, prevents FSH increases, and inhibits luteinizing hormone (LH) pulsatility (Scaramuzzi et al., 2006). While long-term feed restriction can cause anestrous in cattle due to insufficient circulating LH (Rhodes et al., 1995, 1996), short-term effects cause either decreased preovulatory surges of LH (Kiyma et al., 2004) or no changes to LH concentrations (Abecia et al., 1995). Varying results of nutritional plane on follicle number reported could be due to dissimilarities in experimental design, such as different breeds, nutritional treatment lengths, and/or dietary compositions (Botkin et al., 1988; Grazul-Bilska et al., 2012).

In the present study, the high Arg concentration in medium decreased the rate of cleavage. Silva et al. (2014) recently demonstrated similar detrimental effects of high Arg in IVF medium; a significant reduction in cleavage and blastocyst rates was observed when high Arg (50 mM) was added to medium. Negative Arg effects on oocyte and cleavage rates may occur through increased NO production, as NO concentrations are primarily determined by extracellular Arg bioavailability (Hallemeesch et al., 2002; Hardy et al., 2002; Rajapakse et al., 2009). Previous studies have demonstrated the detrimental effects Arg can have on other cell types; for example, additional Arg to human endothelial cells for only 30 minutes increases NO

production (as detected by increased staining of 4,5-diaminofluorescein-2 diacetate [DAF-2DA], a marker of NO) and stimulates cell senescence (Xiong et al., 2014). These high concentrations of NO can also lead to detrimental effects on the oocyte. For example, high concentrations of sodium nitroprusside (SNP; a NO donor) in maturation medium have inhibited mouse oocyte meiotic development (Bu et al., 2003; Viana et al., 2006). While high NO concentrations are detrimental to cells, others have demonstrated that the presence of NO can be beneficial or non-harming to oocytes. For example, low concentrations of SNP have had no stimulatory effects on bovine meiotic maturation (Viana et al., 2006) or stimulatory effects on mouse oocyte meiotic maturation (Bu et al., 2003). Still yet, Goud et al. (2008) reported that NO actually extends the oocyte temporal window for fertilization while L-NAME decreased fertility, demonstrating the importance of NO presence in oocytes. Similarly, the presence of NOS inhibitors during in vitro oocyte maturation reduced embryo development and quality in cows (Bilodeau-Goeseels, 2007; Schwarz et al., 2010), sows (Tao et al., 2004) and goat does (Amale et al., 2013). These results have led researchers to hypothesize that NO can have opposing effects, dependent upon NO concentration; Bu et al. (2003) reported that NO at a concentration 30.69±5.06 μmol stimulated meiotic maturation of mouse oocytes, while a concentration of 59.88±8.07 μmol was inhibitory.

Morula formation was affected by nutritional plane but not by Arg in the present study, with greater morula formation in C and U than O. Previous researchers have reported similar results, with the proportion of developing morula being less (Grazul-Bilska et al., 2012) or tending to be less (Borowczyk et al., 2006) in O and U ewe compared to C ewes. In fact, several studies indicate that changes to nutritional plane, and therefore BCS, are associated with effects on early embryonic development (Snijders et al., 2000; Lozano et al., 2003).

In our study, lipid droplet accumulation within unfertilized oocytes was affected by nutritional plane, similar to observations in cattle (Ferguson and Leese, 1999; Leroy et al., 2005), swine (Sturmey and Leese, 2003; Prates et al., 2013, 2014), mice (Wu et al., 2010), and women (Yang et al., 2012). It has been suggested that a specific lipid composition within the oocyte is vital for proper preimplantation development (Sturmey and Leese, 2003; Lapa et al., 2011; Prates et al., 2014). Lipid droplets are believed to be produced within the endoplasmic reticulum (ER), deposited within the ER membrane, and then join together to form a globule wherein they are covered by a cytoplasmic phospholipid monolayer (Suzuki et al., 2011). Because lipid droplets are found in close proximity with organelles associated with cellular metabolism, for example mitochondria, ER, and endosomes (Ambruosi et al., 2009; Sturmey et al., 2006; Thiele and Spandl, 2008; Walther and Farese, 2009;), researchers have suggested that lipid droplets are used as an energy source for the oocyte (Sturmey and Leese, 2003; Sturmey et al., 2006; Gilchrist et al., 2008). The amount of lipid droplets contained within the oocyte is believed to be critical to the changing metabolic pathways utilized by the oocyte and throughout early embryonic development (Ferguson and Leese, 1999; Sturmey and Leese, 2003; Prates et al., 2013). During cleavage, the pentose phosphate pathway (PPP) is utilized; however, succeeding embryonic development preferentially uses glycolysis and β-oxidation, with the activity increasing during compaction and blastocyst formation (Sturmey and Leese, 2003; Gandhi et al., 2001; Swain et al., 2001). Moreover, the varying lipid contents (higher in O and lower in U ewe oocytes compared to C ewe oocytes) observed in the present study and in previous studies (cited above) may account for the previously reported link between nutrition plane, cleavage rates, and early embryonic development (Grazul-Bilska et al., 2012).

The present study demonstrated that 1) nutritional plane does not alter visible follicle number but can alter morula formation and lipid droplet accumulation in oocytes, and 2) high Arg concentration in medium decreased cleavage rates. This data indicated that nutritional management is important to donor animals providing oocytes for assisted reproductive technologies (ART), as nutrition can impact both fertilization rates as well as early embryonic development, as demonstrated here. Lastly, high Arg concentrations in medium are detrimental to fertilization rates and early embryonic development in ewes. Future investigations addressing nutrition and/or specific medium supplements should provide insight into the underlying mechanisms associated with alterations in the efficiency of in vitro fertilization and early embryonic development.

References

Aardema, H., F. Lolicato, C. H. A. van de Lest, J. F. Brouwers, A. B. Vaandrager, H. T. A van Tol, B. A. J. Roelen, P. Vos, J. B. Helms, B. M. Gadella. 2013. Bovine cumulus cells protect maturing oocytes from increased fatty acid levels by massive intracellular lipid storage. Biol. Repro. 88: 164.

Abecia, J. A., C. Sosa, F. Forcada, A. Meikle. 2006. The effect of undernutrition on the establishment of pregnancy in the ewe. Reprod. Nutr. Dev. 46: 367-78.

Abecia, J. A., S. M. Rhind, T. A. Bramley, S. R. McMillen. 1995. Steroid production and LH receptor concentrations of ovarian follicles and corpora lutea and associated rates of ova wastage in ewes given high and low levels of food intake before and after mating. J. Anim. Sci. 60: 57-62.

Abecia, J. A., J. M. Lozano, F. Forcada, L. Zarazaga. 1997. Effect of level of dietary energy and protein on embryo survival and progesterone production on day eight of pregnancy in Rasa Aragonesa ewes. Anim. Reprod. Sci. 48: 209-18.

Amale, M. H., A. Z. Shahne, S. Nasrollahi. 2013. Effects of nitric oxide synthase inhibition on goat oocyte meiotic maturation. Arch. Tierz. 25: 255-63.

Ambruosi, B., G. M. Lacalandra, A. I. Iorga, T. De Santis, S. Mugnier, R. Matarrese, G. Goudet,

and M. E. Dell'Aquila. 2009. Cytoplasmic lipid droplets and mitochondrial distribution in equine oocytes: implications on oocyte maturation, fertilization and developmental competence after ICSI." Theriogenology. 71: 1093-104.

Armstrong, D. G., T. G. McEvoy, G. Baxter, J. J. Robinson, C. C. Hogg, K. J. Woad, R. kakar, K. D. Sinclair. 2001. Effect of dietary energy and protein on bovine follicular dynamics and embryo production in vitro: associations with the ovarian insulin-like growth factor system. Biol. Reprod. 64; 1624-32.

Auclair, S., R. Uzbekov, S. Elis, L. Sanchez, I. Kireev, L. Lardic, R. Dalbies-Tran, S. Uzbekova. 2013. Absence of cumulus cells during in vitro maturation affects lipid metabolism in bovine oocytes. Am. J. Pphysiol-Endoc. M. 304: E599-613.

Barceló-Fimbres, M., G. E. Seidel. 2011. Cross-validation of techniques for measuring lipid content of bovine oocytes and blastocysts. Theriogenology. 75: 434-44.

Bauer, B. K., L. D. Spate, C. N. Murphy, and R. S. Prather. 2011. Arginine Supplementation in vitro increases porcine embryo development and affects mRNA transcript expression. Reprod. Fert. Devel. 23: 107.

Bilodeau-Goeseels, S., M. Sasseville, C. Guillemette, F. J. Richard. 2007. Effects of adenosine monophosphate-activated kinase activators on bovine oocyte nuclear maturation in vitro. Mol. Reprod. Dev. 74: 1021-34.

Boland, M. P., P. Lonergon, D. O'Callaghan. 2001. Effect of nutrition on endocrine parameters, ovarian physiology, and oocyte and embryo development. Theriogenology. 55: 1323-40.

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, A. T. Grazul-Bilska. 2006. Effects of plane of nutrition on in vitro fertilization and early embryonic development in sheep. J. Anim. Sci. 84: 1593-9.

Bu, S., G. Xia, Y. Tao, L. Lei, B. Zhou. 2003. Dual effects of nitric oxide on meiotic maturation of mouse cumulus cell-enclosed oocytes in vitro. Mol. Cell. Endocrinol. 207: 21-30.

Burdge, G. C., M. A. Hanson, J. L. Slater-Jefferies, K. A. Lillycrop. 2007. Epigenetic regulation of transcription: a mechanism for inducing variations in phenotype (fetal programming) by differences in nutrition during early life. Brit. J. Nutr. 97: 1036-46.

Castaneda, C. A., P. Kaye, M. Pantaleon, N. Phillips, S. Norman, R. Fry, J. D. Michael. 2013. Lipid content, active mitochondria and brilliant cresyl blue staining in bovine oocytes. Theriogenology. 79: 417-22.

Coop, I. E. 1996. Effect of flushing on reproductive performance of ewes. J. Agr. Sci. 67: 305-23.

Creed, J., T. G. McEvoy, J. J. Robinson, R. P. Aitken, R. M. Palmer, and I. Robertson. 1994. The effect of preovulatory nutrition on the subsequent development of superovulated sheep ova in an in vitro culture system. Anim. Prod. 58: 447.

Crocco, M. C., Kelmansky, D. M., & Mariano, M. I. 2013. Does serum cause lipid-droplet accumulation in bovine embryos produced in vitro, during developmental days 1 to 4. J. Assist. Reprod. Gen. 30: 1377-88.

Farese, R. V., T. C. Walther. 2009. Lipid droplets finally get a little RESPECT. Cell. 139: 855 60.

Ferguson, E.M., H. J. Leese. 2006. A potential role for triglyceride as an energy source during bovine oocyte maturation and early embryo development. Mol. Reprod. Dev. 73:1195-201.

Gandhi, A. P., M. Lane, D. K. Gardner, and R. L. Krisher. 2001. Substrate utilization in porcine embryos cultured in NCSU23 and G1. 2/G2. 2 sequential culture media. Mol. Reprod. Dev. 58: 269-75.

Gilchrist, R. B., M. Lane, J. G. Thompson. 2008. Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality. Hum. Reprod. Update. 14: 159-77.

Goud, P. T., P. Anuradha, M. P. Goud, P. Diamond, B. Gonik, H. M. Abu-Soud. 2008. Nitric oxide extends the oocyte temporal window for optimal fertilization. Free. Rad. Biol. Med. 45: 453-9.

Grazul-Bilska, A. T., E. Borowczyk, J. J. Bilski, L. P. Reynolds, D. A. Redmer, J. S. Caton, K. A. Vonnahme. 2012. Overfeeding and underfeeding have detrimental effects on oocyte quality measured by in vitro fertilization and early embryonic development in sheep. Domest. Anim. Endocrin. 43: 289-98.

Grazul-Bilska, A. T., J. T. Choi, J. J. Bilski, R. M. Weigl, J. D. Kirsch, K. C. Kraft, L. P. Reynolds, and D. A. Redmer. 2003. Effects of epidermal growth factor on early embryonic development after in vitro fertilization of oocytes collected from ewes treated with follicle stimulating hormone. Theriogenology. 59: 1449-57.

Grazul-Bilska, A. T., E. Borowczyk, W. Arndt, J. Evoniuk, M. O'neil, J. J. Bilski, R. M. Weigl, J. D. Kirsch, K. C. Kraft, K. A. Vonnahme, D. A. Redmer, L. P. Reynolds, and J. S. Caton. 2006. Effects of overnutrition and undernutritionn on in vitro fertilization (IVF) and early embryonic development in sheep. Biol. Reprod. 4: 579-87.

Gregg, A. R. 2003. Mouse models and the role of nitric oxide in reproduction. Curr. Pharm. Design. 9: 391-8.

Guler, A., N. Poulin, P. Mermillod, M. Terqui, and Y. Cognie. 2000. Effect of growth factors, EGF and IGF-I, and estradiol on in vitro maturation of sheep oocytes. Theriogenology. 54: 209-18.

Hallemeesch, M. M., W. H. Lamers, and N. E. P. Deutz. 2002. Reduced arginine availability and nitric oxide production. Clin. Nutr. 21: 273-9.

Hardy, T. A., J. M. May. 2002. Coordinate regulation of L-arginine uptake and nitric oxide synthase activity in cultured endothelial cells. Free. Rad. Biol. Med. 32: 122-31.

Hiraga, K., Y. Hoshino, K. Tanemura, E. Sato. 2013. Selection of in vitro-matured porcine oocytes based on localization patterns of lipid droplets to evaluate developmental competence. J. Reprod. Develop. 59: 405.

Jungheim, E. S., G. A. Macones, R. R. Odem, B. W. Patterson, S. E. Lanzendorf, V. S. Ratts, K. H. Moley. 2011. Associations between free fatty acids, cumulus oocyte complex morphology and ovarian function during in vitro fertilization. Fertil. Steril. 95: 1970-4.

Kakar, M. A., S. Maddocks, M. F. Lorimer, D. O. Kleemann, S. R. Rudiger, K. M. Hartwich, and S. K. Walker. 2005. The effect of peri-conception nutrition on embryo quality in the superovulated ewe. Theriogenology. 64: 1090-103.

Kendrick, K. W., T. L. Bailey, A. S. Garst, A. W. Pryor, A. Ahmadzadeh, R. M. Akers, W. E. Eyestone, R. E. Pearson, and F. C. Gwazdauskas. 1999. Effects of energy balance on hormones, ovarian activity, and recovered oocytes in lactating Holstein cows using transvaginal follicular aspiration. J. Dairy. Sci. 82: 1731-41.

Kiyma Z, B. M. Alexander, E. A. Van Kirk, W. J. Murdoch, D. M. Hallford, G. E. Moss. Effects of feed restriction on reproductive and metabolic hormones in ewes. J Anim Sci 2004, 82: 2548-57.

Krisher, R. L. 2003. The effect of oocyte quality on development. J. Anim. Sci. 82: E14-23.

Lapa, M., C. C. Marques, S. P. Alves, M. I. Vasques, M. C. Baptista, I. Carvalhais, M. Silva Pereira, A. E. M. Horta, R. J. B. Bessa, and R. M. Pereira. 2011. Effect of trans-10 cis-12 conjugated linoleic acid on Bovine Oocyte Competence and Fatty Acid Composition." Reprod. Domest. Anim. 46: 904-10.

Leroy, J. L. M. R., Tom Vanholder, Bart Mateusen, Armand Christophe, Geert Opsomer, Aart de Kruif, Gaëtan Genicot, and Ann Van Soom. 2005. Non-esterified fatty acids in follicular fluid of dairy cows and their effect on developmental capacity of bovine oocytes in vitro. Reproduction. 130: 485-95.

Lozano, J. M., P. Lonergan, M. P. Boland, D. O'Callaghan. 2003. Influence of nutrition on the effectiveness of superovulation programmes in ewes: effect on oocyte quality and post-fertilization development. Reproduction. 125: 543-53.

McEvoy, T. G., J. J. Robinson, R. P. Aitken, P. A. Findlay, R. M. Palmer, I. S. Robertson. 1995. Dietary induced suppression of pre-ovulatory progesterone concentrations in superovulated ewes impairs the subsequent in vivo and in vitro development of their ova. Anim. Reprod. Sci. 38; 89-107.

Nolan, R., D. O'callaghan, R. T. Duby, P. Lonergan, and M. P. Boland. 1998. The influence of short-term nutrient changes on follicle growth and embryo production following superovulation in beef heifers. Theriogenology. 50: 1263-74.

O'Callaghan, D., M. P. Boland. 1999. Nutritional effects on ovulation, embryo development and the establishment of pregnancy in ruminants. Anim. Sci. 68: 299-314.

O'callaghan, D., H. Yaakub, P. Hyttel, L. J. Spicer, and M. P. Boland. 2000. Effect of nutrition and superovulation on oocyte morphology, follicular fluid composition and systemic hormone concentrations in ewes. J. Reprod. Fertil. 118: 303-13.

Papadopoulos, S., P. Lonergan, V. Gath, K. M. Quinn, A. C. O. Evans, D. O'Callaghan, M. P. Boland. 2001. Effect of diet quantity and urea supplementation on oocyte and embryo quality in sheep. Theriogenology. 55: 1059-69.

Peura, T. T., D. O. Kleemann, S. R. Rudiger, G. S. Nattrass, C. J. McLaughlan, and S. K. Walker. 2003. Effect of nutrition of oocyte donor on the outcomes of somatic cell nuclear transfer in the sheep. Biol. Reprod. 68: 45-50.

Prates, E. G., S. P. Alves, C. C. Marques, M. C. Baptista, A. E. M. Horta, R. J. B. Bessa, R. M. Pereira. 2013. Fatty acid composition of porcine cumulus oocyte complexes (COC) during maturation: effect of the lipid modulators trans-10, cis-12 conjugated linoleic acid (t10, c12 CLA) and forskolin. In. Vitro. Cell. Dev.-An. 49: 335-45.

Prates, E. G., J. T. Nunes, R. M. Pereira. 2014. A role of lipid metabolism during cumulus oocyte complex maturation: impact of lipid modulators to improve embryo production.2014. Mediators. Inflamm. 2014.

Rajapakse, N. W., D. L. Mattson. 2009. Role of L-arginine in nitric oxide production in health and hypertension. Clin. Exp. Pharm. Phys. 36: 249-55.

Rhodes , F. M., K. W. Entwistle, J. E. Kinder. 1996. Changes in ovarian function and gonadotrophin secretion preceding the onset of nutritionally induced anoestrus in Bos indicus heifers. Biol Reprod. 55: 1437-43.

Rhodes, F. M., L. A. Fitzpatrick, K. W. Entwistle, and G. De'ath. 1995. Sequential changes in ovarian follicular dynamics in Bos indicus heifers before and after nutritional anoestrus. J. Reprod. Fertil. 104: 41-9.

Rizos, D., P. Lonergan, M. P. Boland, R. Arroyo-Garcia, B. Pintado, J. De La Fuente, A. Gutierrez-Adan. 2002. Analysis of differential messenger RNA expression between bovine blastocysts produced in different culture systems: implications for blastocyst quality. Biol. Reprod. 66: 589-95.

Robinson, J. J. 1990. Nutrition in the reproduction of farm animals. Nutr. Res. Rev. 3: 253-276.

Robinson, J. J., C. J. Ashworth, J. A. Rooke, L. M. Mitchell. T. G. McEvoy. 2006. Nutrition and fertility in ruminant livestock. Anim. Feed. Sci. Tech. 126: 259-76.

Rosselli, Marinella, R. J. Keller, Raghvendra, K. Dubey. 1998. Role of nitric oxide in the biology, physiology and pathophysiology of reproduction. Hum. Reprod. Update. 4: 3-24.

Scaramuzzi, R. J., B. K. Campbell, J. A. Downing, N. R. Kendall, M. Khalid, M. Munoz Gutierrez, A. Somchit. 2006. A review of the effects of supplementary nutrition in the ewe on the concentrations of reproductive and metabolic hormones and the mechanisms that regulate folliculogenesis and ovulation rate. Reprod. Nutr. Dev. 46: 339-54.

Schwarz, K. R. L., P. R. L. Pires, T. H. C. de Bem, P. R. Adona, C. L. V. Leal. 2010. Consequences of nitric oxide synthase inhibition during bovine oocyte maturation on meiosis and embryo development. Reprod. Dom. Anim. 45: 75-80.

Snijders, S. E., P. Dillon, D. O'Callaghan, M. P. Boland. 2000. Effect of genetic merit, milk yield, body condition and lactation number on in vitro oocyte development in dairy cows. Theriogenology. 53: 981-9.

Stagg, K., M. G. Diskin, J. M. Sreenan, and J. F. Roche. 1995. Follicular development in long term anoestrous suckler beef cows fed two levels of energy postpartum. Anim. Reprod. Sci. 38: 49-61.

Sturmey, R. G., H. J. Leese. 2003. Energy metabolism in pig oocytes and early embryos. Reproduction. 126: 197-204.

Suzuki, M., Y. Shinohara, Y. Ohsaki, T. Fujimoto. 2011. Lipid droplets: size matters. J. Electron. Microsc. 60: S101-16.

Swain, J. E., C. L. Bormann, R. L. Krisher. 2001. Development and viability of in vitro derived porcine blastocysts cultured in NCSU23 and G1. 2/G2. 2 sequential medium. Theriogenology. 56: 459-69.

Tao, Y., Z. Fu, M. Zhang, G. Xia, J. Yang, H. Xie. 2004. Immunohistochemical localization of inducible and endothelial nitric oxide synthase in porcine ovaries and effects of NO on antrum formation and oocyte meiotic maturation. Mol. Cell. Endocrin. 222: 93-103.

Thiele, C., J. Spandl. 2008. Cell biology of lipid droplets. Curr. Opin. Cell. Biol. 20: 378-85.

Thompson, J. G., N. W. Allen, L. T. McGowan, A. C. S. Bell, M. G. Lambert, and H. R. Tervit. 1998. Effect of delayed supplementation of fetal calf serum to culture medium on bovine embryo development in vitro and following transfer. Theriogenology. 49: 1239-49.

Tripp, M. W., J. C. Ju, T. A. Hoagland, J. W. Riesen, X. Yang, and S. A. Zinn. 2000. Influence of somatotropin and nutrition on bovine oocyte retrieval and in vitro development. Theriogenology. 53: 1581-90.

Viana, K. S., M.C. Caldas-Bussiere, S. G. C. Matta, M. R. Faes, C. P. de Carvalho, C. R. Quirino. 2007. Effect of sodium nitroprusside, a nitric oxide donor, on the in vitro maturation of bovine oocytes. Anim. Reprod. Sci. 102: 217-27.

Webb, R., P. C. Garnsworthy, J. G. Gong, D. G. Armstrong. 2004. Control of follicular growth: Local interactions and nutritional influences1, 2. J Anim Sci. 82: E63-74.

Wu, G., F. W. Bazer, M. C. Satterfield, X. L., X. Wang, G. A. Johnson, R. C. Burghardt, Z. Dai, J. Wang, Z. Wu. 2013. Impacts of arginine nutrition on embryonic and fetal development in mammals. Amino Acids. 45: 241-56.

Wu, L. L. Y., Dunning, K. R., Yang, X., Russell, D. L., Lane, M., Norman, R. J., R. L. Robker. 2010. High-fat diet causes lipotoxicity responses in cumulus–oocyte complexes and decreased fertilization rates. Endocrinology. 151: 5438-45.

Wu, X., H. Ge, B. Lemon, J. Weiszmann, J. Gupte, N. Hawkins, X. Li, J. Tang, R. Lindberg, Y. Li. 2009. Selective activation of FGFR4 by an FGF19 variant does not improve glucose metabolism in ob/ob mice. P. Nat. Acad. Sci. 1064: 14379-84.

Xiong, Y., M. F. Fru, Y. Yu, J. P. Montani, X. F. Ming, Z. Yang. 2014. Long term exposure to L-arginine accelerates endothelial cell senescence through arginase-II and S6K1 signaling. Aging. Albany, NY. 6: 369.

Yaakub, H., D. O'Callaghan, M. P. Boland. 1999. Effect of type and quantity of concentrates on superovulation and embryo yield in beef heifers. Theriogenology. 51: 1259-66.

Yang, X., L. L. Wu, L. R. Chura, X. Liang, M. Lane, R. J. Norman, R. L. Robker. 2012. Exposure to lipid-rich follicular fluid is associated with endoplasmic reticulum stress and impaired oocyte maturation in cumulus-oocyte complexes. Fertil. Steril. 97: 1438-43.

Zeng, X., X. Mao, Z. Huang, F. Wang, G. Wu, S. Qiao. 2013. Arginine enhances embryo implantation in rats through PI3K/PKB/mTOR/NO signaling pathway during early pregnancy. Reproduction. 145: 1-7.

CHAPTER 4: PROGESTERONE SECRETION BY OVINE LUTEAL CELLS: EFFECTS OF NITRIC OXIDE, SELECTED METABOLIC HORMONES, AND PLANE OF NUTRITION

Abstract

The aim of this study was to investigate the role of the nitric oxide (NO) system and metabolic hormones on in vitro progesterone (P4) secretion by luteal cells from early and mid-luteal phases of the estrous cycle from overfed and underfed ewes. Ewes were randomly assigned to one of three nutritional groups: control (C; 760 g/kg BW/d; 100% NRC requirements), overfed (O; 2.0 x C), or underfed (U; 0.6 x C). Superovulation was induced by follicle stimulating hormone (FSH) injections. On d 5 or d 10 of the estrous cycle, corpora lutea (CL) were collected counted, dissected from ovaries, and luteal cells isolated enzymatically. Then, luteal cells were cultured with or without DETA-NONOate (NO donor), L-NAME (NO synthase [NOS] inhibitor), arginine (Arg), adiponectin, and leptin for 24 h in the presence or absence of LH. The number of CL tended ($P=0.09$) to be greater in C and O ewes when compared to U ewes. For experiment 1, 1.0 and 0.1 mM of DETA-NONOate inhibited ($P<0.05$), 1.0 mM L-NAME+LH stimulated, and Arg, adiponectin, and leptin did not have any effects on basal P4 secretion by luteal cells from mid-luteal phase C ewes. In experiment 2, we observed that 1) DETA-NONOate decreased basal and LH-stimulated P4 secretion by luteal cells, 2) L-NAME increased basal and/or LH-stimulated P4 secretion by luteal cells depends on stage of luteal cells and nutritional plane, 3) Arg and adiponectin did not affect basal or LH-stimulated P4 secretion in early luteal cells, and 4) leptin increased basal P4 secretion by luteal cells from d 5 from C and U ewes, but did not affect LH-stimulated P4 secretion. In summary, this study

demonstrated that nutritional plane affects ovulation rate in FSH-treated ewes, DETA-NONOate decreases P4 secretion, L-NAME increases P4 secretion, and that leptin but not Arg or adiponectin are involved in the regulation of P4 secretion by luteal cells during the estrous cycle in ewes fed different nutritional planes. This study provides novel information since the effects of the NO system, Arg, and metabolic hormones on luteal cell function have not been studied in association with nutritional plane in any species.

Introduction

The CL forms after the oocyte is released from the antral cavity of the ovarian follicle during ovulation. Besides being the main source for P4, proper formation of the CL is essential for pregnancy establishment and support, early embryonic development, and implantation in most mammalian species (Hodgen et al., 1988; Diaz et al., 2002; Murphy, 2004). Throughout the duration of each non-pregnant estrous cycle, luteal tissues exhibit both structural and functional changes, such as changes in weight and P4 production (Jablonka-Shariff et al., 1993; Niswender and Nett, 1994; Smith et al., 1994; Grazul-Bilska et al., 1998; Reynolds et al., 2006). Both intrinsic (e.g., endocrine system, immune system, growth factors) and extrinsic (e.g., environment including nutrition) factors can influence CL function and production of P4 in livestock (Jablonka-Shariff et al., 1993; Niswender and Nett, 1994; O'Callaghan and Boland, 1999; Grazul-Bilska et al., 2012; Kaminski et al., 2015).

Nutritional plane can affect serum concentrations of both metabolic hormones (for example leptin and adiponectin) and reproductive hormones (such as P4), as well as ovulation rates in non-pregnant animals (Flier et al., 2000; Vasselli et al., 2013; Kaminski et al., 2015; O'Callaghan and Boland, 1999; Abecia et al., 1997; Parr et al., 1987; Armstrong et al., 2003;

Diskin et al., 2003). In turn, altered metabolic and reproductive hormone concentration(s) may affect follicular and luteal functions (Armstrong et al., 2003; Scaramuzzi et al., 2006; Grazul-Bilska et al., 2012).

Nitric oxide, a reactive free radical generated from Arg via NOS, acts as a regulator for several physiological systems (Moncada et al., 1991) including reproduction (Jaroszewski and Hansel, 2000; Skarzynski et al., 2000; Jaroszewski et al., 2003; Korzekwa et al., 2006). For several species, including cattle and sheep, it has been demonstrated that effectors of the NO system have direct effects on steroidogenesis in luteal tissues (Van Voorhtis et a., 1994; Vega et al., 1998; Mitsube et al., 1999; Dong et al., 1999; Skarzynski et al., 2000; Jaroszewski et al., 2001, 2003; Hurwitz et al., 2002). Bovine luteal cells from the mid-luteal phase treated with NO donors inhibited P4 production, while treatment with NOS inhibitors increased P4 production (Jaroszewski et al., 2001; Skarzynski and Okuda, 2000). Similarly, treatment with an NO donor decreased and NOS inhibitor increased P4 production by granulosa cells from ewes fed different nutritional planes (Grazul-Bilska et al., 2015). However, the role of the NO system, and metabolic hormones (adiponectin and leptin) in regulation of P4 secretion by luteal cells from ewes fed different nutritional planes has yet to be investigated in detail.

Objectives and Hypothesis

We hypothesized that effectors of the NO system, adiponectin, and leptin would affect P4 production by luteal cells from the early- and mid-luteal phases of the estrous cycle from nutritionally compromised ewes. Therefore, the objective of this study was to determine the effects of a NO donor (DETA-NONOate), a NOS inhibitor (L-NAME), Arg (precursor for NO), and metabolic hormones leptin and adiponectin, on basal and luteinizing hormone (LH)-

stimulated P4 secretion by luteal cells from early- and mid-luteal phases obtained from nutritionally compromised, FSH-treated ewes.

Materials and Methods

Animals and Experimental Design

All animal procedures performed were approved by the North Dakota State University's Institutional Animal Care and Use Committee (#A12013). The study was initiated during the normal breeding season in August and finished in December.

Non-pregnant, non-lactating Rambouillet ewes (n = 31) between 3-5 years of age and of similar genetic background, were individually penned at the Animal Nutrition and Physiology Center on the NDSU campus. Ewes were stratified by weight and randomly assigned into one of three dietary treatments: control (C; n = 9) received a maintenance diet (100% NRC requirements), overfed (O; n = 12) received 200% NRC requirements, and underfed (U; n = 10) received 60% NRC requirements 60 days prior to the onset of the estrous cycle (d 0). Ewes were fed half of their individual diet daily at 0800 and the remaining half at 1500. Ewes were weighed once weekly for the duration of the experiment. Diets were adjusted weekly for each ewe to ensure the proper nutritional plane was achieved at d 0, and maintained throughout the estrous cycle until completion of experiment on d 5 or d 10 of the second estrous cycle. The nutritional treatment is described in detail by Kaminski et al. (2015).

Estrus was synchronized by insertion of a controlled internal drug release (CIDR) device for 14 days. Based on previous results (Grazul-Bilska et al., unpublished), approximately 36 h after removal of the CIDR ewes were considered in estrus and treated as d0 of the estrous cycle. Ewes were injected twice daily (morning and evening) with follicle stimulating hormone (FSH-

P; Sioux Biochemical, Sioux Center, IA, USA) on d 13, d 14, and d 15 of the estrous cycle (5 mg/injection, 4 mg/injection, or 3 mg/injection, respectively) (Grazul-Bilska et al., 2012).

Tissue and Blood Collection

On d 5 or d 10 of the estrous cycle ovaries were collected, immersed in PBS and transported to the laboratory. Then, CL were counted and dissected from ovaries, weighed, and minced for dissociation with collagenase (Sigma, St. Louis, MO) as previously described (Grazul-Bilska et al., 1991, 2001). Blood samples were also collected on d 5 or d 10, centrifuged (20 min at 1,500*g*), and serum was stored at -20˚C until P4 analysis.

Dissociation of Luteal Cells

Minced luteal tissues were incubated in dissociation medium containing Hank's balanced salt solution (HBSS; Gibco, Grand Island, NY) and collagenase (0.01% wt/vol), bovine serum albumin (BSA; 2%, wt/vol; Sigma, St. Louis, MO), penicillin (100 U/mL; Gibco, Grand Island, NY), and streptomycin (100 µgmL; Gibco, Grand Island, NY) in a shaking (100 cycles/min) water bath at 37 C in a capped 50-mL Erlenmeyer flasks. After initial 15 min incubation, medium containing dispersed luteal cells was aspirated, and 2-3 mL of fresh dissociation medium was added to the remaining tissue. After this initial collection and replacement, medium was aspirated and replaced every 7-10 minutes for ~10 incubations. This process was continued for approximately 3 h, or until luteal tissues were completely dissociated. At the end of collection, medium containing dispersed luteal cells was centrifuged (600 x *g*) for 5 min. The pellet was washed three times with HBSS containing antibiotics (100 U penicillin and 100 µg streptomycin/mL) and then resuspended in the same medium via trituration with a siliconized Pasteur pipette. The cell suspension was filtered through a sterile nylon filter with 70 µm pores

(Tetko, New York, New York) to remove pieces of tissue and then centrifuged (300 x *g*; 10 min). Cells were resuspended in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY) plating medium containing 10% (vol/vol) calf serum (CS; Gibco, Grand Island, NY), 10% fetal bovine serum (FBS; Gibco, Grand Island, NY) and antibiotics (100 U penicillin and 100 µg streptomycin/mL). Luteal cells were counted using a hemocytometer and viability was evaluated by trypan blue (Gibco, Grand Island, NY) exclusion. The viability of freshly dispersed luteal cells was 87.1±2.4% and the proportion of large luteal cells in the total steroidogenic cell population was 8.25±1.6% for early and mid-luteal phases for all nutrition treatments.

Experiment 1

For the dose-response experiment, freshly dissociated luteal cells from d10 of the estrous cycle from control ewes (n = 4) were plated at a concentration of 250,000/well/ml with DMEM containing 10% FBS and 10 % CS at 37^0 C in 5% CO_2 in humidified air for 24 h. After plating, media were changed to serum-free DMEM with penicillin/streptomycin (0.5 ml/well) and incubated for 24 h at 37^0 C in 5% CO_2 in humidified air in the presence or absence of the following treatments: DETA-NONOate (0.01, 0.1, or 1.0 mM; Cayman, Ann Arbor, MI, USA), L-NAME (0.1 or 1.0 mM; Cayman), Arg (1 or 2 mM; Sigma, St. Louis, MO), adiponectin (1, 3, or 10 µg/mL; BioVendor, Ashville, NC), and leptin (1.0, 10, or 100 ng/mL; RayBiotech Inc., Norcross, GA). In addition, cells were incubated in the presence of LH (100 ng/mL; oLH-26; NIAKKK-NIH, USA) in media containing DETA-NONOate (0.01 mM), L-NAME (1 mM), Arg (1 or 2 mM), adiponectin (3 µg/mL), and leptin (10 ng/mL). Treatment with dbcAMP (1 mM; Sigma) was used as a positive control. As additional controls, DMSO (less than 1%; diluting factor for DETA-NONOate), DETA (1 mM; analog of the DETA-NONOate; Dow Chemical

Company, Midland, MI, USA), and D-NMME (1 mM; analog of L-NAME; Cayman) were used. Media were then collected for P4 analysis.

Experiment 2

Isolated luteal cells from d 5 and d 10 of the estrous cycle from C, O and U ewes were plated at a concentration of 250,000/well/ml with DMEM containing 10% FBS and 10 % CS at 37^0 C in 5% CO_2 in humidified air for 24 h. Media then were changed to serum-free DMEM with penicillin/streptomycin (0.5 ml/well) and cells were incubated for 24 h at 37^0 C in 5% CO_2 in humidified air with or without DETA-NONOate (1.0 mM), L-NAME (1.0 mM), arginine (1, mM), leptin (10 ng/mL), and adiponectin (3µg/mL), in the presence or absence of LH (100 ng/ml). Additional controls were used as described for experiment 1 above. Media were then collected for P4 analysis.

Progesterone Analysis

Progesterone concentrations from serum and media were determined using a solid phase chemiluminescence, competitive binding immunoassay (Immulite 1000, Siemens), as previously described (Grazul-Bilska et al., 2014; Kaminski et al., 2015). Each sample was run as 50 µL duplicates. Beads coated with polyclonal rabbit anti-P4 antibodies were utilized for the initial solid phase. Alkaline phosphatase conjugated to P4 is used for the liquid phase. Samples and reagents are mixed and incubated for thirty minutes, during which time sample P4 competes with reagent enzyme-conjugated P4. Centrifugal washes remove unbound sample and enzyme conjugate. Lastly, chemiluminescent substrate is added and the signal that is generated is indicative of P4 concentration. The intra-assay CV for P4 in media was 14.3%.

Statistical Analysis

Data were analyzed using the GLM program of SAS. When the F-test was significant ($P \leq 0.05$), means were separated by using least significant differences.

Results

Initial BW and BCS were similar for all groups (56.3±1 kg and 2.8±0.05, respectively). During study duration, C maintained BW and BCS, O ewes gained ($P<0.001$) 15.8±1 kg and BCS increased to 3.2±1, and U ewes lost ($P<0.001$) 11.2±1 kg and BCS decreased to 2±0.1, similar to our previous studies (Borowczyk et al., 2006; Grazul-Bilska et al., 2012; Kaminski et al., 2015). The number of CL tended ($P=0.09$) to be greater in C and O ewes when compared to U ewes (12.5±1.8, 12.3±2.1, 7.3±0.6, respectively). Serum P4 was greater ($P<0.05$) in d10 compared to d5 luteal cells (14.0±2.8 ng/mL and 7.0±1.2 ng/mL, respectively), but was not affected by nutritional plane.

Experiment 1

The dose-response experiment demonstrated that 1.0 mM and 0.1 mM doses of DETA-NONOate were inhibitory, while 0.01 mM of DETA-NONOate or LNAME (either 1.0 or 0.1 mM doses) did not affect basal P4 secretion (Fig. 4.1). P4 secretion by luteal cells was increased ~2-fold in the presence of LNAME+LH. In addition, neither Arg, leptin nor adiponectin affected basal or LH-stimulated P4 secretion by luteal cells (data not shown).

Experiment 2

Basal P4 secretion by luteal cells was greater ($P<0.0001$) on d10 when compared to d5 (18.3±2.4 ng/mL and 4.6±1.0 ng/mL, respectively; Fig. 4.2). However, plane of nutrition did not affect basal P4 concentrations. DETA-NONOate decreased ($P<0.0001$) basal and LH-stimulated

P4 secretion by luteal cells from d 5 and d 10 of the estrous cycle from all nutritional groups (range 0.42-fold -0.74-fold; Fig. 4.3), whereas, L-NAME increased ($P<0.0001$) basal P4 secretion by luteal cells from d 5 and d 10 of the estrous cycle from all nutritional groups with the exception of cells from d 5 in C and d 10 in O ewes (1.18 ± 0.06 and 1.28 ± 0.04, respectively; Fig. 4.3). In addition, L-NAME enhanced ($P<0.05$) LH-stimulated P4 secretion by luteal cells from d5 and d10 of the estrous cycle in C, and d 5 in U ewes (Fig. 4.3). Arg and adiponectin did not affect basal or LH-stimulated P4 secretion by luteal cells from any day of the estrous cycle in any nutritional group (Fig. 4.3). Leptin increased basal P4 secretion by luteal cells from d 5 from C and U ewes, but did not affect LH-stimulated P4 secretion by luteal cells from any day of the estrous cycle in any nutritional group. Secretion of P4 by luteal cells from d5 and d10 of the estrous cycle was increased ($P<0.0001$) by dbcAMP (range 1.6-1.8-fold) in all nutritional groups (Fig. 4.3).

Discussion

This study demonstrated that nutritional plane affected ovulation rates but did not have any effects on in vitro P4 secretion by luteal cells from early and mid-luteal phases of the estrous cycle. The number of CL, and thus ovulation rates, tended to be greater in C and O compared to U ewes after FSH-induced superovulation in our study.

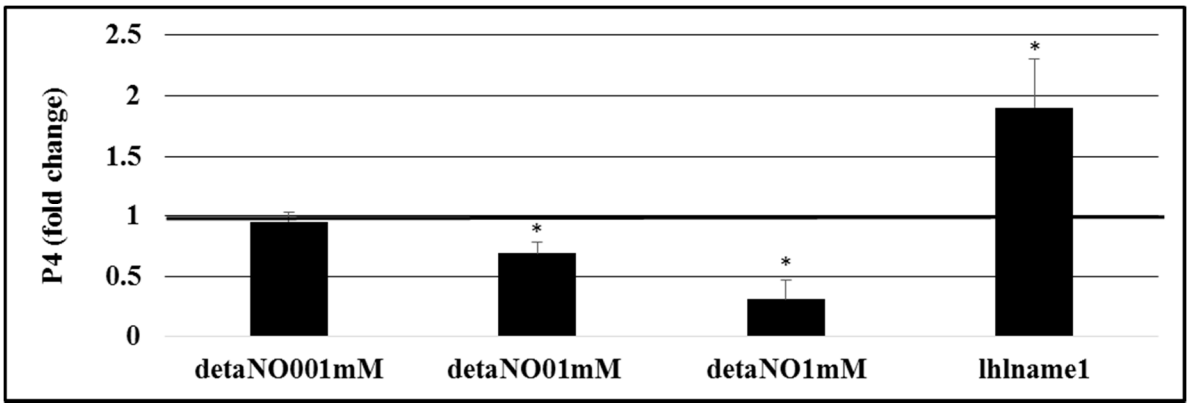


Figure 4.1. The effects of DETA-NONOate (1.0, 0.1, or 0.01 mM) and LH + L-NAME (1.0 mM) dose response on P4 secretion from d 5 luteal cells collected from control ewes. * indicates P<0.05 compared to 1 (bold line).

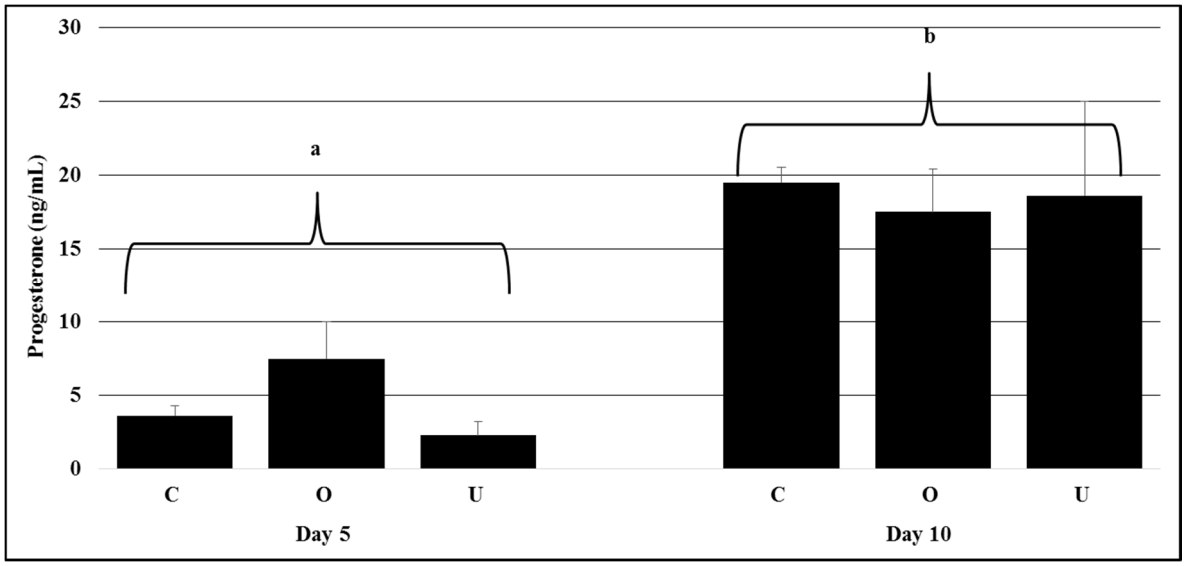


Figure 4.2. Basal progesterone (ng/mL) secretion from d 5 compared to d 10 luteal cells for control (C), overfed (O), and underfed (U) ewes. The basal P4 concentration in media was 4.6±1.0 ng/mL and 18.3±2.4 ng/mL on d5 and d10 of the estrous cycle, respectively. Data are combined for C, O, and U ewes because diet did not affect basal P4 secretion at any day.

Day 5

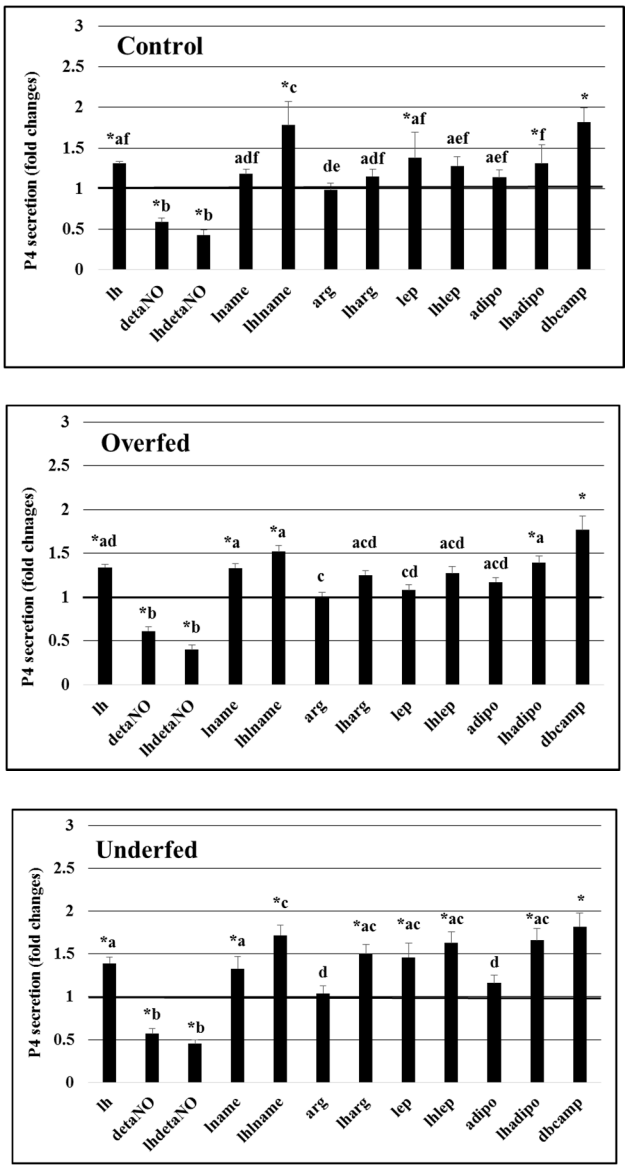


Figure 4.3. The effects of DETA-NONOate (detaNO; NO donor), L-NAME (lname; NOS inhibitor), arginine (arg), leptin (lep) and adiponectin (adipo) on basal and LH (lh)-stimulated P4 secretion by luteal cells from d 5 of the estrous cycle from control, overfed and underfed ewes treated with follicle stimulating hormone (FSH). Dibutyryl cyclic adenosine monophosphate (dbcAMP), a factor that stimulates P4 secretion by luteal cells was used as a positive control, and DETA and D-NMME were used as controls for DETA-NONOate and L-NAME, respectively. Data are expressed as fold-change compared to cultures with no treatment (no treatment arbitrarily set as 1, and marked as a bold line). Basal P4 concentration in media was 4.6±1.0 ng/mL and 18.3±2.4 ng/mL on d 5 of the estrous cycle, respectively. $^{a\text{-}f}P<0.05$; values with different superscripts differ. * indicates $P<0.05$ compared to control set as 1 (bold line).

Day 10

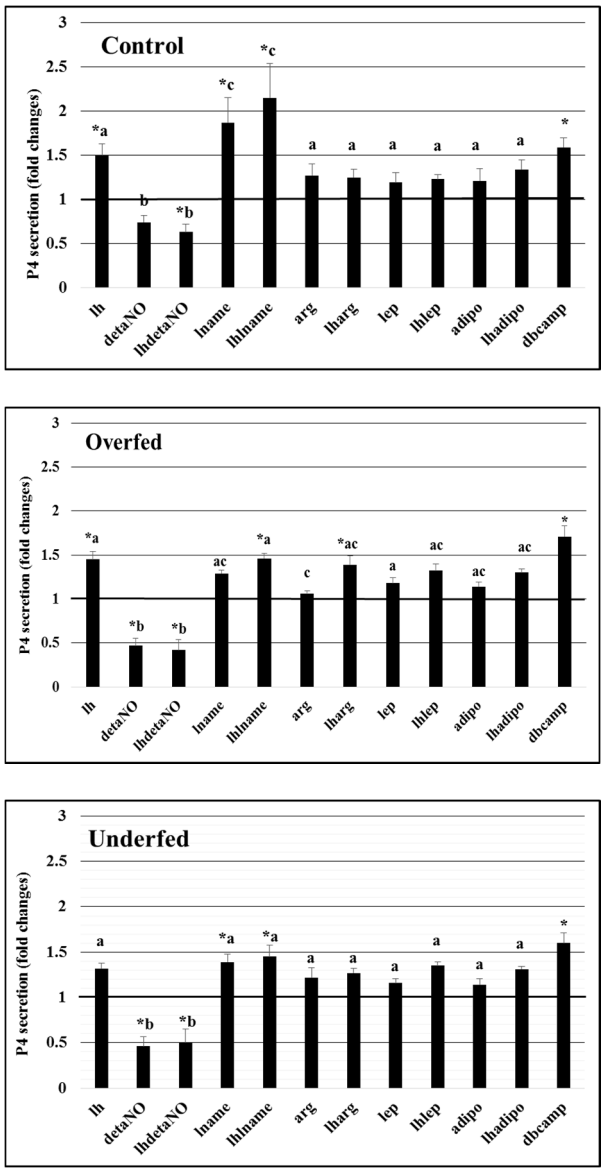


Figure 4.4. The effects of DETA-NONOate (detaNO; NO donor), L-NAME (lname; NOS inhibitor), arginine (arg), leptin (lep) and adiponectin (adipo) on basal and LH (lh)-stimulated P4 secretion by luteal cells from d 10 of the estrous cycle from control, overfed and underfed ewes treated with follicle stimulating hormone (FSH). Dibutyryl cyclic adenosine monophosphate (dbcAMP), a factor that stimulates P4 secretion by luteal cells was used as a positive control, and DETA and D-NMME were used as controls for DETA-NONOate and L-NAME, respectively. Data are expressed as fold-change compared to cultures with no treatment (no treatment arbitrarily set as 1, and marked as a bold line). Basal P4 concentration in media was 4.6±1.0 ng/mL and 18.3±2.4 ng/mL on d 10 of the estrous cycle, respectively. $^{a\text{-}f}P<0.05$; values with different superscripts differ. * indicates $P<0.05$ compared to control set as 1 (bold line).

In non-stimulated ewes, we have also observed lower ovulation rates in U than C and O ewes (see Chapter 2). The association between nutritional plane and ovulation rate have also been reported for ewes by other researchers; for example higher BCS correlated positively with ovulations rates (Gunn and Doney, 1975; Smith, 1995; Rhind et al., 1989; Xu et al., 1989; Abecia et al., 1997), and lower BCS lead to low ovulation rates in the ewe (Keisler and Buckrell, 1997; Lassoued et al., 2004). Scaramuzzi et al. (2006) explained that when an animal is in a negative energy balance, the animal must use its own energy stores in an effort to reverse the energy deficit, resulting in negative effects on both the hypothalamic-pituitary level as well as the reproductive system leading to decreased ovulation rates. Additionally, restricted nutritional planes lead to a multitude of hormonal imbalances including, but not limited to, elevated plasma growth hormone (GH), the inhibition of gonadotropin-releasing hormone (GnRH), reduced ovulation rates, and even anovulation (Scaramuzzi et al., 2006). Follicular populations and ovulation rates are reported to be very sensitive to nutritional plane alterations in the ewe (Scaramuzzi et al., 2006). Thus, optimal nutritional plane should be considered to achieve normal ovulation rates, and therefore, lambing rates.

The current study supports previous reports that both serum and basal P4 secretion by ovine luteal cells were greater at d10 when compared to d5 of the estrous cycle (Borowczyk et al., 2007). The CL growth phase lasts eight to ten days in ewes and is parallel to increasing P4 production (Reynolds et al., 2002; Murphy et al., 2004). There are major changes within the functional CL in the expression of proteins that make up both high-density lipoprotein (HDL), and LDL cholesterol importation pathways during luteinization (Plotkin et al., 2002; Niswender et al., 2002). In fact, cholesterol transportation to the inner mitochondrial membrane is the rate-

limiting step in P4 biosynthesis (Murphy et al., 2004). These synergistic changes, along with alterations in other processes within the CL, allow for high secretion of P4 by luteal cells in the mid-luteal phase.

The present study demonstrates that the NO donor DETA-NONOate decreased both basal and LH-stimulated P4 secretion by ovine luteal cells in ewes from early and mid-luteal phase in all nutrition groups. Similar, the in vitro treatment with NO donors (sodium nitroprusside, S-nitroso-N-acetyl-DL-penicillamine, or spermine NONOate) of bovine luteal cells from the mid-luteal phase resulted in inhibition of P4 production (Skarzynski and Okuda, 2000; Jaroszewski et al., 2001). In addition, we have recently demonstrated that DETA-NONOate (NO donor) decreased P4 production by ovine granulosa cells in vitro (Grazul-Bilska et al., 2015). Our findings support previous reports that NO donors decrease P4 secretion by luteal cells, and in addition, demonstrate that nutritional plane does not affect NO inhibitory effects on luteal function during the estrous cycle.

Our present experiment confirmed results from previous in vitro studies demonstrating the inhibition of NO production via a nonselective NOS inhibitor, L-NAME results in increased basal P4 secretion by luteal cells from rabbits (Gobbetti et al., 1999) and cows (Skarzynski and Okuda, 2000; Jaroszewski et al., 2003; Korzekwa et al., 2004). Similar, L-NAME enhanced in vitro P4 secretion by granulosa cells from ewes fed different nutritional planes (Grazul-Bilska et al., 2015). Researchers utilizing NOS inhibitors in vivo reported that in vivo infusion of a NOS inhibitor at mid- and late-luteal phase in cows not only increased P4 secretion, but that it also increased the functional life of the CL (Jaroszewski et al., 2000; Skarzynski et al., 2003). It has been postulated that the administration of $PGF_2\alpha$ can cause an acute increase in blood flow in the

CL (Acosta et al., 2002), enhancing NO production from luteal endothelial cells (Korzekwa et al., 2004). The increase in NO production from the mid- and late-luteal phase CL after the first few hours post- $PGF_2\alpha$ may induce functional luteolysis including further blood flow changes to the CL and a decrease in P4 production (Skarzynski et al., 2003; Jaroszewski et al., 2000, 2003).

Arg did not affect P4 production by luteal cells at any day of the estrous cycle and any nutritional group. While Arg can be acquired exogenously through the diet, 40% of dietary Arg is catabolized by the small intestine before it can enter the blood stream (Wu and Morris, 1998). The majority of endogenously acquired Arg is derived from the intestinal-renal pathway. Within the small intestine, the enterocyte is responsible for citrulline or arginine synthesis from glutamine and glutamate (Wu and Knabe, 1995); these precursors are then catabolized by the small intestine (Windmueller and Spaeth, 1975), both being major precursors for endogenously synthesized Arg (Wu, 1998). While Arg is a precursor for NO, it is also necessary for polyamines, creatine, and agmatine production, all of which have enormous biological importance (Wu et al., 2009; Lefèvre et al., 2011). Further studies should be undertaken to determine if Arg and their derivatives may have additional effects on luteal function.

We observed that leptin increased basal P4 secretion by luteal cells from the early luteal phase in C and U ewes, but did not affect LH-stimulated P4 secretion by luteal cells from any stage or nutritional group. In rabbits, leptin decreased basal P4 secretion by luteal cells and it was suggested that leptin has a luteolytic role (Zerani et al. 2004). Others have shown that leptin influence ovarian function including luteal function in women (Löffer et al., 2001), sows (Ruiz-Cortés et al., 2003), and cows (Nicklin et al., 2007). In fact, leptin may act as the critical link between adipose tissue and the reproductive system, indicating whether adequate energy reserves

are present for normal reproductive function (Moschos et al. 2002). The leptin receptor is expressed on the bovine CL and leptin affected in vitro P4 secretion in bovine and equine luteal cells (Nicklin et al., 2007; Galvão et al., 2014).

Changes in plasma leptin concentration has been associated with changes in P4 concentration, reaching its peak during the luteal phase in women (Lugwig et al., 2000). However, in O ewes, leptin concentration was lower during the mid-luteal phase when plasma P4 concentration is high (Kaminski et al. (2015). In addition, Kaminski et al. (2015) demonstrated that in O ewes leptin and P4 concentrations were greater that in C or U ewes. While it is evident that metabolic hormones can influence the production of steroidogenic hormones, further research is needed to explicate the relationship between metabolic state and luteal function.

In the current study, adiponectin did not affect basal and LH-stimulated P4 secretion by ovine luteal cells. It has been demonstrated that the adiponectin receptor 1 protein expression is greatest in early CL and that adiponectin plasma concentrations were significantly higher throughout the luteal phase than in the follicular phase, demonstrated its potential involvement in regulation of CL function in sows (Maleszka et al., 2014a). Furthermore, inhibitory effects of adiponection on P4 secretion by porcine luteal cells in vitro have been reported (Maleszka et al., 2014b). Adiponectin is known to influence female reproduction including folliculogenesis, implantation (Dos Santos et al., 2012) and periovulatory changes (Palin et al., 2012). However, the role of adiponectin in regulation of luteal function is rather unclear due to limited research in this area. Our current results suggest that adiponectin is not involved in the regulation of P4 secretion by luteal cells during the estrous cycle in ewes.

In summary, this study demonstrated that 1) nutritional plane affects ovulation rate in FSH-treated ewes; 2) DETA-NONOate decreased basal and LH-stimulated P4 secretion by luteal cells, 3) L-NAME increased basal and/or LH-stimulated P4 secretion by luteal cells and depends on stage of estrous cycle and nutritional plane, 4) Arg and adiponectin did not affect basal or LH-stimulated P4 secretion, and 5) leptin increased basal P4 secretion by luteal cells from d5 from C and U ewes, but did not affect LH-stimulated P4 secretion. Thus, we have supported previous reports that nutritional plane is critical for increasing ovulation rates, and have also demonstrated that the NO system and metabolic hormones play a role in modulating P4 secretion by ovine luteal cells during the estrous cycle in ewes fed different nutritional planes. This study provides novel information since the effects of the NO system, Arg, and metabolic hormones on luteal cell function have yet to be studied in association with nutritional plane in any species. The molecular pathway(s) of regulation in P4 secretion from luteal cells still requires further research.

References

Abecia, J. A., J. M. Lozano, F. Forcada, L. Zarazaga. 1997. Effect of level of dietary energy and protein on embryo survival and progesterone production on day eight of pregnancy in Rasa Aragonesa ewes. Anim. Reprod. Sci. 48: 209-18.

Acosta, T. J., N. Yoshizawa, M. Ohtani, A. Miyamoto A. 2002. Local changes in blood flow within the early and midcycle corpus luteum after prostaglandin F2α injection in the cow. Biol. Reprod. 66: 651-8.

Armstrong, D. G., J. G. Gong, R. Webb. 2003. Interaction between nutiriton and ovarian activity in cattle: physiological, cellular, and molecular mechanisms. Reprod. Suppl. 61: 403-14.

Diaz, F. J., L. E. Anderson, Y. L. Wu, A. Rabot, S. J. Tsai, M. C. Wiltbank. 2002. Regulation of progesterone and prostaglandin F 2α production in the CL. Mol. Cell. Endocrinol. 191: 65-80.

Diskin, M. G., D. R. Mackey, J. F. Roche, and J. M. Sreenan. 2003. Effects of nutrition and metabolic status on circulating hormones and ovarian follicle development in cattle. Anim. Reprod. Sci. 78: 345-70.

Dong, Y. L., P. R. Gangula, L. Fang, C. Yallampalli. 1999. Nitric oxide reverses prostaglandin-induced inhibition in ovarian progesterone secretion in rats. Hum. Reprod. 14: 27-32.

Galvão, A., A. Tramontano, M. R. Rebordão, A. Amaral, P. P. Bravo, A. Szóstek, D. Skarzynski, A. Mollo, G Ferreira-Dias. 2014. Opposing Roles of Leptin and Ghrelin in the Equine Corpus Luteum Regulation: An In Vitro Study. Med. Inflamm. 2014.

Girsh, E., Y. Greber, R. Meidan. 1995. Luteotrophic and luteolytic interactions between bovine small and large luteal-like cells and endothelial cells. Biol. Reprod. 52: 954-62.

Gobbetti, A., C. Boiti, C. Canali, M. Zerani. 1999. Nitric oxide synthase acutely regulates progesterone production by in vitro cultured rabbit corpora lutea. J. Endocrinol. 160: 275-83.

Grazul-Bilska, A. T., C. S. Bass, S. L. Kaminski, G. A. Perry, D. A. Redmer. 2015. Progesterone secretion by ovine granulosa cells: effects of nitric oxide and plane of nutrition. Can.J. Physiol. Pharm.

Grazul-Bilska, A. T., D. A. Redmer, A. Jablonka-Shariff, M. E. Biondini, and L. P. Reynolds. 1995. Proliferation and progesterone production of ovine luteal cells from several stages of the estrous cycle: effects of fibroblast growth factors and luteinizing hormone. Can. J. Physiol. Pharm. 73: 491-500.

Grazul-Bilska, A. T., E. Borowczyk, J. J. Bilski, L. P. Reynolds, D. A. Redmer, J. S. Caton, K. A. Vonnahme. 2012. Overfeeding and underfeeding have detrimental effects on oocyte quality measured by in vitro fertilization and early embryonic development in sheep. Domest. Anim. Endocrin. 43: 289-98.

Grazul-Bilska, A. T., Redmer, D. A., L. P. Reynolds. 1991. Secretion of angiogenic activity and progesterone by ovine luteal cell types in vitro. J. Anim. Sci, 69: 2099-107.

Gunn, R. G., J. M. Doney. 1975. The interaction of nutrition and body condition at mating on ovulation rate and early embryo mortality in Scottish Blackface ewes. J. Agri. Sci. 85: 465-70.

Hodgen, G. D., J. Itskovitz. 1988. Recognition and maintenance of pregnancy. The Physiology of Reproduction. 2: 1995-2021.

Hurwitz, A., Z. Finci-Yeheskel, A. Milwidsky, M. Mayer. 2002. Regulation of cyclooxygenase activity and progesterone production in the rat corpus luteum by inducible nitric oxide synthase. Reproduction. 123: 663-9.

Keisler, D. H., B. C. Buckrell. 1997. Breeding strategies. Current therapy in large animal theriogenology. Philadelphia, USA. WB Saunders Co. 603-11.

Korzekwa, A. J., K. Okuda, I. Woclawek-Potocka, S. Murakami, D. J. Skarzynski. 2006. Nitric oxide induces apoptosis in bovine luteal cells. J. Reprod. Devel. 52: 353-61.

Jablonka-Shariff, A., A. T. Grazul-Bilska, D. A. Redmer, and L. P. Reynolds. 1993. Growth and cellular proliferation of ovine corpora lutea throughout the estrous cycle. Endocrinology. 133: 1871-9.

Jaroszewski, J. J., W. Hansel. 2000. Intraluteal administration of a nitric oxide synthase blocker stimulates progesterone and oxytocin secretion and prolongs the life span of the bovine corpus luteum. P. Soc. Exp. Biol. Med. 224: 50-5.

Jaroszewski, J. J., Skarzynski, D. J., K. Okuda. 2001. Nitric oxide as a local regulator in the mammalian ovary. Miyamoto H., Manabe n., Eds. Reproductive Biotechnology: Reproductive Biotechnology Update and Its Related Physiology. Tokyo: Elsevier Scientific Publishers. 105-12.

Jaroszewski, J. J., D. J. Skarzynski, R. M. Blair, W. Hansel. 2003. Influence of nitric oxide on the secretory function of the bovine corpus luteum: dependence on cell composition and cell-to-cell communication. Exp. Biol. M. 228: 741-8.

Kaminski, S. L. , D. A. Redmer, C. S. Bass, D. H. Keisler, L. S. Carlson, K. A. Vonnahme, S. T. Dorsam, A. T. Grazul-Bilska. 2015. The effects of diet and arginine treatment on serum metabolites and selected hormones during the estrous cycle in sheep. Theriogenology. 83: 808-16.

Knickerbocker, J. J., M. C. Wiltbank, G. D. Niswender. 1988. Mechanisms of luteolysis in domestic livestock. Dom. Anim. Endocrin. 5: 91-107.

Lassoued, N., M. Rekik, M. Mahouachi, B. Hamouda. 2004. The effect of nutrition prior to and during mating on ovulation rate, reproductive wastage, and lambing rate in three sheep breeds. Small. Ruminant. Res. 52: 117-25.

Löffler, Sabine, Gabriela Aust, Uwe Köhler, and Katharina Spanel-Borowski. 2001. Evidence of leptin expression in normal and polycystic human ovaries. Mol. Hum. Reprod. 7: 1143-9.

Ludwig, M., H. H. Klein, K. Diedrich, O. Ortmann. 2000. Serum leptin concentrations throughout the menstrual cycle. Arch. Gynecol. Obstet. 263: 99-101.

Maleszka, A., N. Smolinska, A. Nitkiewicz, M. Kiezun, K. Chojnowska, K. Dobrzyn, H. Szwaczek, T. Kaminski. 2014. Adiponectin expression in the porcine ovary during the oestrous cycle and its effect on ovarian steroidogenesis. Int. J. Endo. 2014: Article ID 957076.

Maleszka, A., N. Smolinska, A. Nitkiewicz, M. Kiezun, K. Dobrzyn, J. Czerwinska, K. Szeszko, T. Kaminski. 2014. Expression of adiponectin receptors 1 and 2 in the ovary and concentration of plasma adiponectin during the oestrous cycle of the pig. Acta. Veterinaria. Hungarica. 62: 386-96.

Mitsube, K., Mikuni, M., Matousek, M., M. Brännström. 1999. Effects of a nitric oxide donor and nitric oxide synthase inhibitors on luteinizing hormone-induced ovulation in the ex-vivo perfused rat ovary. Hum. Reprod. 14: 2537-43.

Moschos S, Chan JL, Mantzoros CS. 2002. Leptin and reproduction: a review. Fertil. Steril. 77: 433-44.

Moncada, S. R. M. J., R. M. L. Palmer, E. A. Higgs. 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharm. Rev. 43: 109-42.

Murphy, B.B. 2004. Luteinization. In *The Ovary*. E. Y. Adashi, Eds. 185-99. Elsevier Academic Press, San Diego.

Nicklin, L. T., R. S. Robinson, P. Marsters, B. K. Campbell, G. E. Mann, M. G. Hunter. 2007. Leptin in the bovine corpus luteum: receptor expression and effects on progesterone production. Mol. Reprod. Dev. 74: 724-9.

Niswender, G. D. 2002. Molecular control of luteal secretion of progesterone. Reproduction. 123: 333-9.

Niswender, G. D., T. M. Nett. 1994. Corpus luteum and its control in infraprimate species. The physiology of reproduction. 1: 781-816.

Niswender, G. D., J. L. Juengel, P. J. Silva, M. K. Rollyson, E. W. McIntush. 2000. Mechanisms controlling the function and life span of the corpus luteum. Physiol. Rev. 80: 1-29.

O'Callaghan, D., M. P. Boland. 1999. Nutritional effects on ovulation, embryo development and the establishment of pregnancy in ruminants. Anim. Sci. 68: 299-314.

Okuda, Y., S. Sakoda, H. Fujimura, T. Yanagihara. 1998. Aminoguanidine, a selective inhibitor of the inducible nitric oxide synthase, has different effects on experimental allergic encephalomyelitis in the induction and progression phase. J. Neuroimmunol. 81: 201-10.

Parr, R. A., I. F. Davis, R. J. Fairclough, M. A. Miles.1987. Overfeeding during early pregnancy reduces peripheral progesterone concentration and pregnancy rate in sheep. J. Reprod. Fertil. 80: 317-20.

Plotkin, D., S. Miller, S. Nakajima, E. Peskin, R. Burkman, D. Richardson, Y. Mitchel. 2002. Lowering low density lipoprotein cholesterol with simvastatin, a hydroxy-3-methylglutaryl-coenzyme a reductase inhibitor, does not affect luteal function in premenopausal women. J. Clin. Endocr. Metab. 87: 3155-61.

Reynolds, L. P., A. T. Grazul-Bilska, D. A. Redmer. 2002. Angiogenesis in the female reproductive system: pathological implications. Review Article. Int. J. Exp. Pathol. 83: 151-63.

Rhind, S. M., G. B. Martin, S. McMillen, C. G. Tsonis, A. S. McNeilly. 1989. Effect of level of food intake of ewes on the secretion of LH and FSH and on the pituitary response to gonadotrophin-releasing hormone in ovariectomized ewes. J. Endocr. 121: 325-30.

Ruiz-Cortés, Z. T., Y. Martel-Kennes, N. Y. Gévry, B. R. Downey, M. F. Palin, B. D. Murphy. 2003. Biphasic effects of leptin in porcine granulosa cells. Biol. Reprod. 68: 789-96.

Scaramuzzi, R. J., B. K. Campbell, J. A. Downing, N. R. Kendall, M. Khalid, M. Munoz Gutierrez, A. Somchit. 2006. A review of the effects of supplementary nutrition in the ewe on the concentrations of reproductive and metabolic hormones and the mechanisms that regulate folliculogenesis and ovulation rate. Reprod. Nutr. Dev. 46: 339-54.

Skarzynski, D. J., K. Okuda. 2000. Different actions of noradrenaline and nitric oxide on the output of prostaglandins and progesterone in cultured bovine luteal cells. Prost. Oth. Lipid. M. 35-47.

Smith, M. F., E. W. McIntush, G. W. Smith. 1994. Mechanisms associated with corpus luteum development. J. Anim. Sci. 72: 1857-72.

Van Voorhis, B. J., M. S. Dunn, G. D. Snyder, C. P. Weiner. 1994. Nitric oxide: an autocrine regulator of human granulosa cell steroidogenesis. Endocrinol. 135: 1799-1806.

Vasselli, J. R., P. J. Scarpace, R. B. S. Harris, W. A. Banks. 2013. Dietary components in the development of leptin resistance. Adv. Nutr.4: 164-75.

Vega, M., M. C. Johnson, H. A. Diaz, L. R. Urrutia, J. L. Troncoso, L. Devoto. 1998. Regulation of human luteal steroidogenesis in vitro by nitric oxide. Endocinoly. 8: 185-91.

Xu, Z. Z., M. F. McDonald, S. N. McCutcheon. 1989. The effects of nutritionally-induced liveweight differences on follicle development, ovulation rate, oestrous activity and plasma follicle-stimulating hormone levels in the ewe. Anim. Reprod. Sci. 19: 67-78.

Zerani, M., C. Boiti, D. Zampini, G. Brecchia, C. Dall'Aglio, P. Ceccarelli, A. Gobbetti. 2004. Ob receptor in rabbit ovary and leptin in vitro regulation of corpora lutea. J. Endocrinol. 183: 279-88.

CHAPTER 5: GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

Proper ovarian function is critical for reproductive performance in pregnant and non-pregnant females. However, while nutrition (McEvoy et al., 1995; Wrenzycki et al., 2000; Kwong et al., 2000; Boland et al., 2001; Armstrong et al., 2001; Lozano et al., 2003; Grazul-Bilska et al., 2012) and the NO system (Okuda et al., 1998; Skarzynski and Okuda, 2000; Bu et al., 2003; Tao et al., 2004) are reported to influence reproduction, very limited information exists regarding the associations between nutritional plane, the NO system, and their collective influences on ovarian function in sheep.

Feeding non-pregnant sheep to achieve different planes of nutrition (i.e. overfeeding or underfeeding) for a prolonged period of time resulted in different body conditions, ovulation rates in both non-stimulated and FSH-treated ewes, angiogenic factor expression in luteal tissues, serum P4 concentrations, and oocyte function when compared to control fed ewes in our study. Overfeeding and underfeeding may be negatively influencing reproduction in females through fat accumulation or depletion, as we also observed altered lipid droplet accumulation in oocytes. Based on this information, further research is warranted to determine the underlying molecular mechanisms associated with varying plane of nutrition and reported changes of lipid content in oocytes. In addition, research is needed to determine if lipid content is altered in earlier-stage oocytes (such as primordial, primary, and secondary oocytes) and, therefore has long-term effects on female reproduction, as we only investigated mature oocytes in the current study.

It has been reported that Arg supplementation to pregnant animals (through diet or injected intravenously) resulted in an increase in the number of live offspring born (Mateo et al., 2007; Zeng et al., 2008; Luther et al., 2009), heavier lamb birth weights (De Boo et al., 2005;

McCoard et al., 2013), and prevents fetal growth restriction when supplemented to underfed mothers (Lassala et al., 2010). Our current study was performed using non-pregnant animals, and therefore it provides novel information regarding the effects of Arg supplementation on ovarian function in animals fed different nutritional plane. In vivo Arg supplementation to non-pregnant ewes resulted in increased luteal vascularity and selected angiogenic factor expression in luteal tissue, but did not affect ovulation rate, CL weight, and P4 production. Additional research regarding Arg and the NO system is necessary to determine additional potential benefits in non-pregnant females. Because Arg supplementation was associated with increased vascularization (as noted by increased endothelial cell marker CD31), but did not lead to changes in production of P4, future research is needed to determine factors that may influence P4 production and could, therefore, be utilized to increase reproductive efficiency.

Supplementation of Arg during in vitro maturation and fertilization resulted in increased lipid droplet accumulation in oocytes and decreased cleavage rates after in vitro fertilization, respectively. Future research explaining how the oocyte, as well as reproductive tissues, utilize(s) Arg may assist in elucidating why Arg supplementation to pregnant mothers is beneficial, while supplementation to non-pregnant females has limited effects.

In Chapter 4, we reported that the NO system and metabolic hormones play a role in modulating P4 secretion by ovine luteal cells during the estrous cycle in ewes fed different nutritional planes. The data provide novel information since the effects of the NO system, Arg, and metabolic hormones on luteal cell function have yet to be studied in association with nutritional plane in any species. However, supplemental research regarding the molecular pathway(s) of regulation in P4 secretion from luteal cells is still required.

In summary, these studies contribute to the knowledge of nutritional plane and the NO system and how they influence reproduction in female livestock. The results obtained provide beneficial knowledge for future research regarding the improvement of reproductive efficiency in females, which could additionally assist producers considering therapeutic supplements for reproductively-sound livestock.

References

Armstrong, D. G., T. G. McEvoy, G. Baxter, J. J. Robinson, C. C. Hogg, K. J. Woad, R. kakar, K. D. Sinclair. 2001. Effect of dietary energy and protein on bovine follicular dynamics and embryo production in vitro: associations with the ovarian insulin-like growth factor system. Biol. Reprod. 64;1624-32.

Boland, M. P., P. Lonergon, D. O'Callaghan. 2001. Effect of nutrition on endocrine parameters, ovarian physiology, and oocyte and embryo development. Theriogenology. 55: 1323-40.

Bu, S., G. Xia, Y. Tao, L. Lei, B. Zhou. 2003. Dual effects of nitric oxide on meiotic maturation of mouse cumulus cell-enclosed oocytes in vitro. Mol. Cell. Endocrinol. 207: 21-30.

De Boo, H. A., P. L. Van Zijl, D. E. Smith, W. Kulik, H. N. Lafeber, 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. 52: 270-7.

Grazul-Bilska, A. T., E. Borowczyk, J. J. Bilski, L. P. Reynolds, D. A. Redmer, J. S. Caton, K. A. Vonnahme. 2012. Overfeeding and underfeeding have detrimental effects on oocyte quality measured by in vitro fertilization and early embryonic development in sheep. Domest. Anim. Endocrin. 43: 289-98.

Kwong, W. Y., A. E. Wild, P. Roberts, A. C. Willis, T. P. Fleming. 2000. Maternal undernutrition during the preimplantation period of rat development causes blastocyst abnormalities and programming of postnatal hypertension. Devlop.127: 4195-202.

Lassala, A., F. W. Bazer, T. A. Cudd, S. D., D. H. Keisler, M. C. Satterfield, T. E. Spencer, G.Wu. 2010. Parenteral administration of L-arginine prevents fetal growth restriction in undernourished ewes. J. Nutr. 140: 1242-8.

Luther, J. S., E. J. Windorski, J. S. Caton, G. Wu, J. D. Kirsch, K. A. Vonnahme, L. P. Reynolds, C. S. Schauer. 2009. Effects of arginine supplementation on reproductive performance in Rambouillet ewes. Sheep Res. Rep. 50: 11-3.

Lozano, J. M., P. Lonergan, M. P. Boland, D. O'Callaghan. 2003. Influence of nutrition on the effectiveness of superovulation programmes in ewes: effect on oocyte quality and post-fertilization development. Reproduction. 125: 543-53.

Mateo, R. D., G. Wu, F. W. Bazer, J. C. Park, I. Shinzato, S. W. Kim. 2007. Dietary L-arginine supplementation enhances the reproductive performance of gilts. J. Nutr. 137: 652-6.

McCoard, S., F. Sales, N. Wards, Q. Sciascia, M. Oliver, J. Koolaard, D. van der Linden. 2013. Parenteral administration of twin-bearing ewes with L-arginine enhances the birth weight and brown fat stores in sheep. Springer Plus. 2: 684.

McEvoy, T. G., J. J. Robinson, R. P. Aitken, P. A. Findlay, R. M. Palmer, I. S. Robertson. 1995. Dietary induced suppression of pre-ovulatory progesterone concentrations in superovulated ewes impairs the subsequent in vivo and in vitro development of their ova. Anim. Reprod. Sci. 38; 89-107.

Okuda, Y., S. Sakoda, H. Fujimura, T. Yanagihara. 1998. Aminoguanidine, a selective inhibitor of the inducible nitric oxide synthase, has different effects on experimental allergic encephalomyelitis in the induction and progression phase. J. Neuroimmunol. 81: 201-10.

Skarzynski, D. J., K. Okuda. 2000. Different actions of noradrenaline and nitric oxide on the output of prostaglandins and progesterone in cultured bovine luteal cells. Prost. Oth. Lipid. M. 35-47.

Tao, Y., Z. Fu, M. Zhang, G. Xia, J. Yang, H. Xie. 2004. Immunohistochemical localization of inducible and endothelial nitric oxide synthase in porcine ovaries and effects of NO on antrum formation and oocyte meiotic maturation. Mol. Cell. Endocrin. 222: 93-103.

Wrenzycki, C., P. De Sousa, E. W. Overstrom, R. T. Duby, D. Herrmann, A. J. Watson, H. Niemann H, D. O'Callaghan, M. P. Boland. 2000. Effects of superovulated heifer diet type and quantity on relative mRNA abundances and pyruvate metabolism in recovered embryos. J. Reprod. Fertil. 118: 69-78.

Zeng, X. F., F. L. Wang, X. Fan, W. J. Yang, B. Zhou, P. F. Li, Y. L. Yin, G. Wu, and J. J. Wang. 2008. Dietary arginine supplementation during early pregnancy enhances embryonic survival in rats. J. Nutr. 138:1421–5.