

DISSERTATION

ENDOCRINE ACTIONS OF IFNT DURING EARLY RUMINANT PREGNANCY

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ABSTRACT

ENDOCRINE ACTIONS OF IFNT DURING EARLY RUMINANT PREGNANCY

The mechanisms of how conceptus-derived interferon tau (IFNT) induces interferon stimulated genes (ISGs) and cell survival genes through endocrine action that contributes to resistance of the Corpus Lutum (CL) to prostaglandin F₂ α (PGF) and the maintenance of early pregnancy in sheep were examined. Using microarray screens, several genes were identified in the CL to be significantly up-regulated [ISG15 and myxovirus (influenza virus) Resistance 1; MX1], maintained [interleukin 6; IL-6, Pentraxin, long 3; PTX3, luteinizing hormone receptor; LHR, and vascular endothelial growth factor; VEGF] or down-regulated [serpin peptidase inhibitor, clade E; SERPINE1, thrombospondin 1; THBS1] in response to pregnancy and luteolysis. These studies in the CL were expanded to other tissues (endometrium, liver, uterine vein tissue) and bodily fluids (blood and histotroph) to identify other endocrine actions of IFNT and other conceptus secretory products that could be used to explain mechanisms of establishment and maintenance of pregnancy and to be used as blood markers for pregnancy status. The extensive examination of key genes described herein that were differentially regulated during Days 12-15 of the estrous cycle and pregnancy in ewes is novel. The experiments are the first to examine extensive temporal regulation of key genes associated with luteolysis such as estrogen receptor (ESR1), oxytocin receptor (OXTR), prostaglandin transporter (SCLO2A1) and caspase 3 (CASP3); and luteotrophic-cell survival genes such as Phosphoinositide-3-Kinase/V-Akt Murine Thymoma Viral Oncogene (PI3K/AKT) and ISGS. To identify novel markers of pregnancy status and establishment of pregnancy, global mass spectroscopy was used

for analysis of the proteome and metabolome. From this extensive analysis, 14 proteins/metabolites were more extensively examined: Acetyl-carnitine, Carnitine, Ecdysteroids, N-acetyldileucine, Valine, Collagen, Type I, Alpha 1 (COL1A1), Collagen, Type I, Alpha2, (COL1A2) Annexin A1, Annexin A2, Annexin A5, IFNT, Trophoblast Kunitz Domain Protein 1 (TDKP1) Profilin 1 (PFN1) and S100 Calcium-Binding Protein A11 (S100a11). The critical roles for these metabolites and proteins are discussed in context of the establishment and survival of a pregnancy. To confirm that IFNT has an endocrine role during early pregnancy, a highly specific [no cross-reaction with Interferon alpha, beta and gamma (IFNA, IFNB or IFNG)] and sensitive (23.95 pg/ml in serum) IFNT radioimmunoassay was validated herein. IFNT could be detected using this refined assay in ewes from Days 13-16 of pregnancy in histotroph, in uterine vein serum by Day 15-16 of pregnancy and as early as Day 19 of pregnancy in tail blood from pregnant dairy cows. Through these studies it was determined that Day 14 is a very pivotal day for the establishment of pregnancy in ewes. Detection of IFNT in tail blood of pregnant cows on Day 19 provides further evidence for the endocrine action and systemic release of IFNT during pregnancy in ruminants.

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CHAPTER 1
LITERATURE REVIEW
REPRODUCTION IN SHEEP

Environmental and Hormonal Signaling

Environmental cues such as changes in the length of the photoperiod during the day are vital for many mammals that reproduce seasonally. Sheep depend on the length of the daily photoperiod as a physiological signal for the proper time to breed. In the ovine species photoperiod plays an important role in inducing puberty and reproduction. In natural breeding conditions the long days of the summer provide an important photoperiod stimulus to lambs born in the spring, preparing them for sexual maturity in the upcoming fall season (in the northern hemisphere). Sheep are what is known as short day breeders, this means that they are in estrus or reproductively receptive in the autumn season. The hormone that produces the stimulatory and inhibitory signal via day length is melatonin. Melatonin is a hormone that is released from the pineal gland in a circadian fashion during the night (Rollag and Niswender 1976; Bittman and Karsch 1984; Cardinali and Vacas 1987). Once the ewe's body recognizes the environmental/ hormonal cues she will begin estrous cycles. The ovine estrous cycle is typically 16-17 days (Anderson LL 1987). An adult ewe contains approximately 12,000 to 86,000 primordial follicles per ovary (Driancourt, Gibson et al. 1985). During each estrous cycle a group of primordial follicles are recruited due to stimulation from gonadotropins and released via signaling from the hypothalamo-pituitary axis. Typically a ewe will only ovulate one follicle per estrous cycle; however it is not uncommon to see double or triple ovulations in sheep (Driancourt, Gibson et al.

1985; Bowen RA 1989). During those 17 days the ewe will enter estrus or be sexually receptive for roughly 24-36 hours (Anderson LL 1987). Estrus in a ewe is rather inconspicuous and is typically determined by behavioral changes and/ or by the presence of a ram (Anderson LL 1987; Bowen RA 1989). Some ewes in estrus will seek out the male; however, most ewes are generally passive (Bowen RA 1989).

INTRODUCTION TO THE CORPUS LUTEUM

The Corpus Luteum

During the estrous cycle, the corpus luteum (CL) a unique endocrine gland develops. The CL plays an intricate role in female reproduction and has the ability to undergo rapid proliferation, differentiation and regression. Growth of the CL is rapid and rivals the rate of many tumors. The CL is formed post-ovulation after the collapse of the follicle (Anderson LL 1987). The CL is composed of several cell types; such as, endothelial, fibroblasts, immune, and steroidogenic cells (Niswender, Juengel et al. 2000). There are two types of steroidogenic cells in the CL; large and small luteal cells (LLC; SLC). Both steroidogenic cell types produce progesterone. The CL primary function is producing progesterone, which acts on the uterus to prepare it for sustaining a conceptus. Progesterone production results in the maintenance of pregnancy in ruminants.

History of the Corpus Luteum

Marcello Malpighi (1628-1694) is given credit with naming the corpora (bodies) lutea (yellow) and Regnier De Graaf (1641-1673) was credited with precisely describing the (CL) that he observed on rabbit ovaries (Smith, McIntush et al. 1994; Niswender, Juengel et al. 2000). However; it was Prenant (1898) that concluded the CL was a

secretory gland and hypothesized that the CL might produce substances dealing with pregnancy regulation (Smith, McIntush et al. 1994). The discovery of the CL by these early scientists (Malpighi, De Graaf and Prenant) has led to many more questions than answers, several of which we are still trying to answer today.

Structure of the Corpus Luteum

The CL arises after the preovulatory surge of gonadotropins that leads to the release of the oocyte from the ovulating follicle (Moor and Rowson 1966). Rupture of the ovulating follicle results in the formation of a corpus hemorrhagicum, prior to the development of the CL (Niswender, Juengel et al. 2000). After ovulation, differentiation of residual follicular granulosa and theca cells occurs to form the CL. The granulosa cells give rise to the LLC and the theca cells give rise to the SLC; (Alila and Hansel 1984). The LLC have a polyhedral shape and are approximately 25-50 μ m in diameter (O'Shea, Cran et al. 1979). The SLC have an irregular shape and are seldom larger than 15 μ m in diameter. In the non-regressing CL (Farin, Moeller et al. 1986) there is a fivefold increase in the number of SLC from Day 4 to Day 16 when compared with LLC. Growth in the CL is immense and rapid, LLC populations remain relatively constant ($\sim 15 \times 10^6$) but the cells double in size as the estrous cycle advances (Niswender, Juengel et al. 2000). The SLC population increases 5 fold (~ 10 to 50×10^6), fibroblast populations double (~ 21 to 50×10^6) and endothelial cells increase 6.5 fold [18 to 120×10^6] (Farin, Moeller et al. 1986; Niswender, Juengel et al. 2000). Follicular granulosa and theca cells are luteinized as a direct result of the preovulatory luteinizing hormone (LH) surge. Lutenization causes the steroidogenic pathway to be altered, resulting in progesterone being the primary steroid hormone produced by the CL. There

are two main steroidogenic changes that occur during lutenization of granulosa and theca cells; the first results in decreased expression of enzymes responsible for converting progesterone to estrogens; such as, 17 α -hydroxylase cytochrome P-450 and aromatase cytochrome P-450 (Niswender, Juengel et al. 2000). The second step is increased expression of genes responsible for the production of progesterone; such as, cholesterol side-chain cleavage enzyme, P450_{scc} (*CYP11A*) and 3 β -hydroxysteroid dehydrogenase/ Δ^5 , Δ^4 isomerase (HSD3B). The mechanisms controlling luteinization are not well defined due to the complexity of the process, but intracellular signaling pathways, cell adhesion factors, intracellular cholesterol, oxysterols and possibly the paracrine or intracrine action of progesterone itself are all believed to be involved (Murphy 2000).

MECHANISMS OF ACTION IN THE CORPUS LUTEUM

Steroidogenesis

Progesterone production by the CL is activated in a cell specific manner; SLC are stimulated by LH binding to its receptor and the steroidogenic pathway in LLC is constitutively activated [(Niswender, Juengel et al. 2000; Bogan and Niswender 2007; Davis, Bott et al. 2010) Reviewed by Niswender]. Several studies have been done demonstrating that both cell types of the CL contain LH receptors [LHR (Fitz, Mayan et al. 1982; Harrison, Kenny et al. 1987; Chegini, Lei et al. 1991)]. Without stimulation LLC secrete progesterone at a rate that is approximately 20 fold greater than SLC, this accounts in the ovine CL for more than 80% of the secreted progesterone (Fitz, Mayan et al. 1982; Hoyer, Fitz et al. 1984). SLC are much more responsive to LH stimulation than LLC. When luteal cells are stimulated with LH, LLC will secrete one tenth of the

progesterone released by SLC. LH binding to the LHR in SLC correlates with an increase in intracellular Cyclic Adenosine Monophosphate (cAMP); cAMP activates the protein kinase A (PKA) system leading to increased phosphorylation of Steroid Acute Regulatory Protein (STAR), and increased biosynthesis and secretion of progesterone (Pon and Orme-Johnson 1986; Epstein and Orme-Johnson 1991; Arakane, King et al. 1997). Phosphorylation of STAR appears to cause greater transport of cholesterol across mitochondrial membranes leading to the 5- to 20-fold increase seen when SLC are stimulated with LH (Wiltbank, Belfiore et al. 1993). In LLC it is believed that PKA is constitutively activated since it is vastly insensitive to LH and LH mimetics or agents that cause increased intracellular cAMP (Fitz, Mayan et al. 1982). Therefore the downstream signaling by LH as seen in SLC to increase progesterone production is not effective in LLC since PKA is constitutively activated.

Progesterone is synthesized through a series of enzymatic reactions beginning with the substrate cholesterol (Niswender, Juengel et al. 2000). Cholesterol enters the CL via receptor mediated endocytosis from the blood stream in the form of high density lipoprotein (HDL) and low density lipoprotein (LDL). Once in the cell, lysosomes fuse with the endosome resulting in dissociation of LDL from its receptor and LDL breakdown into cholesterol. HDL binds to a plasma membrane bound HDL binding protein; however cholesterol entry from HDL into the cell is unknown currently (Lestavel and Fruchart 1994; Niswender, Juengel et al. 2000). Once in the cell cholesterol is transported to the mitochondrial membrane by Sterol Carrier Protein (SCP-2). Cholesterol transport from the outer mitochondrial membrane to the inner membrane is facilitated by the STAR / peripheral benzodiazepine receptor (PBR) complex. P450 side-chain cleavage

(P450scc) cleaves the side chain from cholesterol forming pregnenolone, completing the first step of steroidogenesis (Farkash, Timberg et al. 1986; Stocco 2001).

Formally, it was believed that P450scc was the rate limiting step in steroidogenesis; however, it was discovered that P450scc is active in unstimulated cells (Tuckey and Atkinson 1989; Tuckey 1992). The true rate limiting step is facilitated by STAR; cholesterol is hydrophobic and unable to cross the aqueous intermembraneous space of the mitochondria (Farkash, Timberg et al. 1986; Black, Harikrishna et al. 1994). The expression of STAR is confined only to steroidogenic tissues (Stocco 2001). STAR was discovered by Orme-Johnson and colleagues as an ACTH-induced phosphoprotein in mouse adrenocortical cells and LH-induced protein in rat CL and mouse leydig cells (Krueger and Orme-Johnson 1983; Pon, Epstein et al. 1986; Pon, Hartigan et al. 1986; Pon and Orme-Johnson 1986; Pon and Orme-Johnson 1988; Alberta, Epstein et al. 1989; Epstein and Orme-Johnson 1991; Epstein and Orme-Johnson 1991). In order to demonstrate the direct link of STAR and its functionality in steroidogenesis the protein was purified, cloned and sequenced in 1994 (Clark, Wells et al. 1994). Transient transfection of the clone and mutational studies helped to solidify that STAR was necessary for steroidogenesis (Lin, Sugawara et al. 1995; Sugawara, Lin et al. 1995; Stocco and Clark 1996). The expression of STAR is induced/inhibited by several compounds. Increased expression of STAR can be induced by LH (Juengel, Larrick et al. 1998; Lejeune, Sanchez et al. 1998; Luo, Chen et al. 1998; Wang, Walsh et al. 1999), chorionic gonadotropin (Lin, Wang et al. 1998; Kerban, Boerboom et al. 1999) FSH, (Balasubramanian, Lavoie et al. 1997; Pescador, Houde et al. 1997; LaVoie, Garmey et al. 1999) ACTH (Nishikawa, Sasano et al. 1996; Fleury, Ducharme

et al. 1998; Lehoux, Fleury et al. 1998), cAMP analogs (Balasubramanian, Lavoie et al. 1997; Pescador, Houde et al. 1997; Wang, Walsh et al. 1999) and calcium ions (Cherradi, Rossier et al. 1997; Cherradi, Brandenburger et al. 1998; Manna, Pakarinen et al. 1999) to name a few. PGF (Chung, Sandhoff et al. 1998) (Juengel, Haworth et al. 2000) heat shock (Liu and Stocco 1997), interferon γ (Lin, Hu et al. 1998), transforming growth factor β (Mauduit, Gasnier et al. 1998; Budnik, Jahner et al. 1999; Chen, Feng et al. 1999) and tumor necrosis factor α (Mauduit, Gasnier et al. 1998; Budnik, Jahner et al. 1999) are some of the proteins involved in the down regulation of STAR expression. In addition to STAR, PBR has been shown to be necessary for transport of cholesterol across mitochondrial membranes (Papadopoulos and Brown 1995). PBR is present in the mitochondrial membranes of steroid producing cells. The targeted deletion of the PBR gene from cells that constitutively produce steroids caused a drastic decrease in steroid secretion (Papadopoulos, Amri et al. 1997). The effects from deleting the PBR gene were reversed when the gene was reintroduced into the cells. It is likely that STAR and PBR interact in the transport of cholesterol across mitochondrial membranes (Papadopoulos, Amri et al. 1997). Upon entry of cholesterol into the mitochondria the side chain must be removed to form pregnenolone by P450_{scc} (Farkash, Timberg et al. 1986; Stocco 2001). Pregnenolone is then transported to the smooth endoplasmic reticulum where HSD3B will convert pregnenolone to progesterone (Caffrey, Nett et al. 1979). It is believed that progesterone then diffuse out of the cell and is not stored (Niswender, Juengel et al. 2000).

Continued Progesterone exposure changes gene expression in the Uterus

Continuous progesterone exposure to the uterus for 8 to 10 Days causes decreased expression of the progesterone receptor in the endometrium, this allows for estradiol to bind to estrogen receptor (ESR1), thus resulting in the synthesis and insertion of the Oxytocin Receptor (OXTR) in the endometrium (Leavitt, Okulicz et al. 1985; Spencer, Burghardt et al. 2004). Oxytocin (OXT) binds to its endometrial receptor activating the synthesis and pulsatile release of Prostaglandin F₂ α (PGF) into the uterine ovarian vein (Moore, Choy et al. 1986; Allison Gray, Bartol et al. 2000). PGF crosses over into the ovarian artery from the ovarian vein via a counter current exchange mechanism (McCracken, Carlson et al. 1972; Banu, Arosh et al. 2005). Once in the ovarian artery this allows PGF delivery to the CL. The local effects of PGF results in the demise of the CL; leading to a new estrous cycle if she is not pregnant (Niswender, Juengel et al. 2000; Spencer, Burghardt et al. 2004).

LUTEOLYSIS

Demise of the Corpus Luteum

Luteolysis is the functional/ structural degradation of the CL, affecting its progesterone production (Niswender, Juengel et al. 2000; Spencer, Burghardt et al. 2004). In 1970 McCracken indicated that PGF is the luteolytic agent and will cause luteolysis when delivered to the CL (McCracken, Glew et al. 1970). Luteolysis occurs in two phases the first of the two is functional regression which is strongly associated with the decrease in progesterone production (Stocco, Telleria et al. 2007), the second phase deals with the structural changes that occur after the initial drop in progesterone concentrations.

Functional Luteolysis

Functional regression in the CL occurs prior to any distinguishable morphological changes and compromised integrity of luteal cells is observable. PGF has been shown to signal through a seven trans-membrane Gq- coupled receptor in several mammalian species (Davis and Rueda 2002). The MAPK signaling cascade has been shown to be activated by PGF. Within this cascade ERK1/2 phosphorylation has been shown to occur within 15 minutes of administration of a luteolytic dose of PGF in cattle (Arvisais, Hou et al.). A study with luteinized rat cells showed that PGF activates the MAPK pathway by increasing free intracellular calcium in a calmodulin-dependent manner (Stocco, Lau et al. 2002). In rats PGF has also been shown to decrease SCP-2 (Colles, Woodford et al. 1995) and STAR expression (Sandhoff and McLean 1996; Sandhoff and McLean 1999; Nackley, Shea-Eaton et al. 2002). The inhibition of STAR by PGF is due to PGF activating dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1 (DAX-1) which reduces STARs responsiveness to cAMP (Sandhoff and McLean 1999). Another protein that has implication in PGF action is HSP70. HSP70 is increased in ewe and rat luteal cells after administration of PGF precluding steroid depletion and apoptotic death of luteal cells (McPherson, Van Kirk et al. 1993; Khanna, Aten et al. 1995).

Structural luteolysis

During structural luteolysis the CL undergoes significant remodeling leading to changes in vascularization, steroidogenic capacity, decreased size and weight. These changes result in a gland composed essentially of connective tissue known as the corpus albicans [CA; (Stocco, Telleria et al. 2007)]. In order for a CA to form the CL

must undergo programmed cell death via apoptosis. There are two major apoptotic signaling pathways; the extrinsic and intrinsic pathway. Both of these pathways lead to activation of executioner caspases 3, 6, and 7 (Sartorius, Schmitz et al. 2001; Gupta 2003). Activation of executioner caspases result in the cleavage of many substrates leading to characteristic morphological and biochemical indicators of apoptosis such as nuclear condensation, genomic DNA fragmentation and phosphatidylserine exposure. Lamkanfi 2010 caspases-7. The BCL-2 family tightly regulates apoptosis by either inhibition (Bcl-2, Bcl-xl, Mcl-1) or promotion (Bad, Bax, Bak, Bid, Puma) of cell death. The Bcl-2 family regulates apoptosis by regulating the permeability of mitochondria (Borner 2003). In the absence of Bcl-2-like survival factors, Bax oligomerizes in the mitochondrial outer membrane and interacts with voltage- dependent anion channel/ adenine nucleotide transporter (VDAC/ANT) to form a channel that allows for the release of caspase-dependent (cytochrome c) and caspase- independent (Serine Proteinase Omi, Endonuclease G, and Apoptosis Inducing Factor) death signals (Borner 2003). Matrix Metalloproteinase activation by PGF is another protein involved with the demise of the CL resulting in the degradation of the extracellular matrix (Ricke, Smith et al. 2002) expression and activity following prostaglandin induced luteolysis

Discovery of Uterine PGF source

Over the years several studies have resulted in the knowledge that the local immediacy of the uterus and ovary in the ewe is required for typical cycle length and luteolysis (Inskeep and Butcher 1966; Goding, Harrison et al. 1967; McCracken, Glew et al. 1970). Hysterectomy between Days 13.5-15 of the estrous cycle prolongs the life

of the CL in ewes to approximately the length of gestation, which is close to 148 Days (Inskeep and Butcher 1966).

Inskeep and collaborators in 1966 discovered that the removal of the ipsilateral or contralateral uterine horns revealed an extended lifespan of the CL. If the ipsilateral uterine horn was removed the CL would have an extended lifespan out to 35 Days. If the contralateral uterine horn was removed a normal lifespan of 15-17 Days would be observed. Collectively, these studies helped to identify the local effects that the uterus has on the lifespan of the CL. Proximity of the uterus to the CL for proper luteolysis was further determined, by ovarian auto-transplantation from the body cavity into the neck (Goding, Harrison et al. 1967; McCracken, Glew et al. 1970). These studies demonstrated that only when the ovary and ipsilateral uterine horn were transplanted in a ewe's neck would normal luteal regression take place. This implies that the luteolytic agent (PGF) first originates in the uterus and that it has local, nonsystemic effects.

In 1988, Zarco demonstrated that PGF is secreted in non-pregnant ewes in a pulsatile manner; while in pregnant ewes, PGF release increases in a basal secretory pattern. (Zarco, Stabenfeldt et al. 1988) In the CL of pregnant ewes the enzyme responsible for metabolizing PGF, Prostaglandin Dehydrogenase (PGDH), is elevated when compared to Day 13 of the cycling ewes, thus indicating higher metabolism of PGF (Silvia and Niswender 1984; Silva, Juengel et al. 2000). PGF synthesis was thought to only be of uterine origin; however it has been shown to be produced by the CL in multiple species such as; rats, cows, pigs, sheep, horses and primates (Patek and Watson 1976; Shutt, Clarke et al. 1976; Swanston, McNatty et al. 1977; Guthrie, Rexroad et al. 1979; Rexroad and Guthrie 1979; Watson, Shepherd et al. 1979; Milvae

and Hansel 1983; Pate 1988; Watson and Sertich 1990; Olofsson, Norjavaara et al. 1992). Binding of uterine derived PGF to the CL induces several downstream effects in both large and small luteal cells. In LLCs, PGF binding to its receptor results in a suicidal loop of PGF being produced from the CL through activation of the PTGS-2 (COX-2; Prostaglandin Synthase-2) pathway (Wiltbank and Ottobre 2003; Spencer, Burghardt et al. 2004; Niswender, Davis et al. 2007) PGF also induces calcium intake into the cell leading to apoptosis, activates the Protein Kinase C (PKC) pathway which blocks the synthesis of progesterone and causes the production of OXT [Fig. 1.1.(Wiltbank, Guthrie et al. 1989; Niswender, Davis et al. 2007)]. In 1986 Moore, demonstrated that PGF release in ewes increases prior to OXT and oxytocin-associated neurophysin, indicating that PGF initiates release of OXT (Moore, Choy et al. 1986). The OXT produced from LLC binds to the OXTR on the SLC causing the release of calcium and activation of the PKC pathway, both which will lead to cell death via apoptosis [Fig. 1.1. (Vinatier, Dufour et al. 1996; Niswender, Davis et al. 2007)].

In 1976, Nett and collaborators determined cell specific effects of PGF in the ovine CL (Nett, McClellan et al. 1976). They demonstrated that PGF causes reduced blood flow to the ovary containing the CL, as well as reduced systemic progesterone concentrations. Ewes treated with PGF had CL with reduced volume formerly occupied by endothelial cells, red blood cells and capillary lumen. However; PGF injection did not affect the volume of the CL occupied by theca-lutein cells, white blood cells, eosinophils, fibroblasts, and connective tissue, revealing that PGF is very cell specific. LLCs have the majority of PTGFR in the CL and this could very well be why an effect of

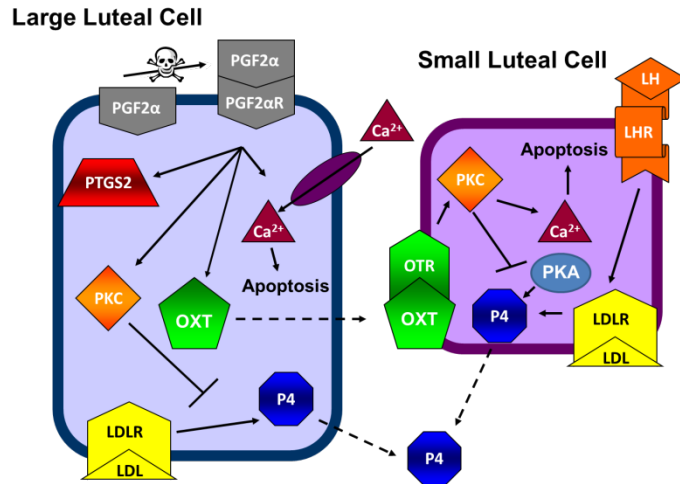


FIGURE 1.1.

PGF pathways activated in Large and Small cells during luteolysis (Day 16) in ewes adapted from (Niswender, Juengel et al. 2000). For a pregnancy to be maintained, the CL must not undergo luteolysis. Luteolysis occurs when PGF binds to the PTGFR on LLCs; this binding results in a suicidal loop of PGF production through the COX-2 pathway. PGF binding causes an influx of calcium (Ca^{2+}) resulting in apoptosis, activation of the PKC pathway, which is inhibitory to progesterone production and synthesis of OXT. OXT will bind to the OXTR on the SLC also activating the PKC pathway and causing influx of Ca^{2+} resulting in apoptosis.

PGF was not seen on the SLC [theca-lutein (Nett, McClellan et al. 1976; Fitz, Mayan et al. 1982)]. Dwyer was also able to show that injections of PGF cause significantly reduced amounts of progesterone, and that this treatment with PGF resulted in decreased activity of HSD3B and cholesterol esterase in hysterectomized guinea pigs. Hawkins's revealed that one hour after a second injection of PGF, HSD3B mRNA is reduced by 80 % (Hawkins, Belfiore et al. 1993). PGF may decrease the rate of synthesis and increase the rate of degradation of HSD3B. Juengel demonstrated the effects of PGF on steroidogenesis indicating that PGF administration reduces STAR mRNA in ovine CL (Juengel, Meberg et al. 1995). DNA fragmentation appears in the CL within a few hours after impaired steroidogenesis begins due to PGF exposure (Hoyer 1998). PGF exposure combined with reduced progesterone production leads to a drastic decrease in ovarian blood flow (Niswender, Moore et al. 1975). In the non-regressing CL, the most abundant cell types are endothelial and pericytes; however endothelial cells are the first to demonstrate signs of apoptosis during luteolysis (Farin, Moeller et al. 1986; Sawyer, Niswender et al. 1990). PGF induced luteolysis appears to have three mechanisms of action; the first is inducing PGF production in the CL, second is decreasing steroidogenesis; and third is reducing blood flow to the CL.

Synthesis of PGF

PGF is synthesized through a series of enzymatic reactions (Arosh, Banu et al. 2004). The primary precursor of prostaglandins is arachidonic acid (AA), which is an essential fatty acid found in membrane phospholipids. Phospholipase A2 (major source), and phospholipases C and D (lesser degree) generate free AA from membrane phospholipids (Godkin, Bazer et al. 1982; Dennis, Rhee et al. 1991; Olofsson and

Leung 1994). AA is converted into Prostaglandin H₂ (PGH₂) by Cyclooxygenase, 1 or 2 (COX-1/ COX-2) which are also known as Prostaglandin H Synthases [PTGHS-1/ PTGHS-2(Smith 1992)]. The conversion of AA to PGH₂ is a two-step process: the first step is conversion of AA to PGG₂ and the second step is a reduction reaction of the 15-hydroperoxyl group on PGG₂ to PGH₂. Either of the cyclooxygenase enzymes can convert AA to PGH₂. PGH₂ is a predecessor for many prostaglandins such as PGF and Prostaglandin E (PGE). PGH₂ is converted into PGF by PGF synthase. PTGS-1 is constitutively expressed while PTGS-2 up-regulation is reliant upon a stimulus (Masferrer, Reddy et al. 1994) such as LH. Conversion of AA to PGG₂/ PGH₂ by PTGS-1/2 is the rate limiting step in PGF synthesis (Olofsson and Leung 1994). PGF can also be produced by the conversion of PGE₂ into PGF by PGE 9-ketoreductase or from PGH₂ or PGE₂ by PGH 9-11-endoperoxide reductase (Leslie and Levine 1973; Watson, Shepherd et al. 1979) .

Metabolism of prostaglandins

There have been several studies determining the metabolism of prostaglandins. The majority of prostaglandin metabolism occurs in the lungs. In 1967, Ferreira determined that 90-95% of infused prostaglandins are metabolized and removed from the venous system with one pass through the pulmonary vasculature (Ferreira and Vane 1967). These infusion studies were performed in rabbits, cats and dogs. The enzyme responsible for the first step in metabolism of prostaglandins in the lungs is 15-hydroxyprostaglandin dehydrogenase [PGDH(Piper, Vane et al. 1970)]. Several authors have isolated PGDH from bovine, swine and guinea pig lung (Piper, Vane et al. 1970; Saeed and Roy 1972). Prostaglandin delta 13-reductase further breaks down

PGF by removing a hydrogen thus forming 13, 14-dihydro-15-keto-PGF₂ (Barcikowski, Carlson et al. 1974). 13, 14-dihydro-15-keto-PGF₂ is further reduced in the final step of PGF metabolism to 13, 14-dihydro-PGF₂. PGF avoids this rapid metabolism in the lungs by crossing into the ovarian artery from the ovarian vein via a counter current exchange mechanism (McCracken, Carlson et al. 1972; Banu, Arosh et al. 2005). Once in the ovarian artery this allows delivery of PGF to the CL. The local effects of PGF₂a results in the structural demise of the CL (Niswender, Juengel et al. 2000; Spencer, Burghardt et al. 2004).

MATERNAL RECOGNITION OF PREGNANCY IN RUMINANTS

The mechanism through which pregnancy is recognized and established varies substantially among mammals. Interferon Tau (IFNT) is the maternal recognition signal in ruminants that indicates a viable embryos presence to the mother, resulting in the blockage of luteolysis and continued progesterone production (Austin, Ward et al. 1996; Teixeira, Austin et al. 1997; Johnson, Austin et al. 1998; Staggs, Austin et al. 1998; Johnson, Stewart et al. 2001; Spencer, Burghardt et al. 2004). The conceptus must be present from Day 12 through Day 17 in the ovine mother for a successful pregnancy to be recognized and maintained (Moor and Rowson 1964; Moor and Rowson 1966; Moor and Rowson 1966; Hansen, Anthony et al. 1985). IFNT is released by the ovine conceptus beginning on Day 10 through Day 25, with the greatest concentrations released between Days 14-17 (Harrison, Kenny et al. 1987; Ashworth and Bazer 1989; Roberts 1989; Spencer, Burghardt et al. 2004). IFNT was initially named protein X after its discovery on Day 13 of pregnancy as the major conceptus secretory protein (Godkin, Bazer et al. 1982) and further renamed to trophoblast protein-1 prior to being renamed

as IFNT (Bazer and Roberts 1983; Bazer, Thatcher et al. 1991; Roberts, Cross et al. 1992). IFNT silences *ESR1* transcription and consequently inhibits the production of OXTR and insertion into the endometrium, furthermore disrupting the pulsatile release of PGF (Spencer, Becker et al. 1995; Spencer, Burghardt et al. 2004; Bott, Ashley et al. 2010). IFNT also acts through a paracrine anti-luteolytic action on the endometrium protecting the CL from demise (Bott, Ashley et al. 2010). The paracrine action of IFNT has been shown to reduce PGF pulsatility in the ewe (Zarco, Stabenfeldt et al. 1988; Zarco, Stabenfeldt et al. 1988; Bott, Ashley et al. 2010) and reduce PGF concentrations in the cow [(Danet-Desnoyers, Wetzels et al. 1994; Meyer, Desnoyers et al. 1996; Godkin, Smith et al. 1997) reviewed in (Niswender, Juengel et al. 2000)] the luteolytic pulses of PGF from the endometrium. PGF is released in pulses in non-pregnant ewes, but in pregnant ewes its release has been shown to be more of a constant release that steadily increases (Peterson, Tervit et al. 1976; Zarco, Stabenfeldt et al. 1988). Maternal recognition of pregnancy is clearly a paracrine mechanism, but could also be sustained through endocrine induction of luteal resistance through interferon-stimulated genes (ISGs). Even though the mechanism for maternal recognition of pregnancy varies among mammals, the up-regulation of Interferon Stimulated Gene 15 (ISG15) in the endometrium appears to be a universal response to the presence of an embryo; this has been seen in humans, baboons, cows, sheep, swine and mice (Austin, Carr et al. 2004). IFNT has been shown to result in the production of ISGs in the glandular epithelium (Johnson, Stewart et al. 2001). Several of these ISGs have been identified, such as, ISG15 (Naivar, Ward et al. 1995; Austin, Ward et al. 1996; Johnson, Spencer et al. 1999), myxovirus (influenza virus) resistance (MX1) (Ott, Yin et al. 1998) and 2',

5'-oligoadenylate synthetase (OAS) (Mirando, Short et al. 1991; Schmitt, Geisert et al. 1993; Johnson, Stewart et al. 2001). One of these ISGs could potentially serve as a marker for pregnancy. IFNT and possibly ISGs enter the blood stream and condition T-cells and macrophages potentially activating a first line of defense against viruses possibly preventing early embryonic mortality or persistent infection of the embryo (Hansen, Henkes et al. 2010). Recently the evidence of IFNT having an endocrine action on the CL has expanded.

Endocrine Release of IFNT into the Uterine Vein.

In order to explain why an up-regulation of ISGs in peripheral blood mononuclear cells (PBMC) had been seen and to possibly discover the mechanism that allows the release of IFNT from the uterus, the lymph nodes that drain the uterus (iliac) and the head (submandibular) from Day 15 pregnant ewes were examined (Oliveira, Henkes et al. 2008). This examination revealed that there was no difference in ISG gene expression in the iliac and submandibular lymphnodes; suggesting that IFNT is not inducing PBMC ISGs by being released into uterine lymphatic drainage. To further delineate the mechanism by which IFNT is released from the uterus, antiviral activity was evaluated in uterine vein blood from Day 15 pregnant sheep. Significant amounts of IFNT (~200µg/24h) are released on Day 15 of pregnancy in to the uterine vein (Oliveira et al 2008). This study has recently been repeated through two additional breeding seasons where plasma (2007) and serum (2008) from uterine vein blood was examined. Pre-adsorption of uterine vein blood from Day 15 pregnant ewes with a highly specific monoclonal antibody against roIFNT was shown to significantly reduce antiviral activity

(Bott, Ashley et al. 2010). From these studies it's been concluded that IFNT is released into the uterine vein on Day 15 of pregnancy.

Endometrial and Systemic induction of ISGs during Early Pregnancy

In addition to well-documented paracrine action of IFNT on the endometrium, it has been concluded from the aforementioned studies that IFNT also has an endocrine action on extra-uterine tissues such as PBMC and the CL. Antoniazzi and co-workers (unpublished data) confirmed the temporal induction of ISGs in endometrial tissue and the CL, as well as the suppression of *ESR1* and *OXTR* in the endometrium. Using the serum progesterone profile seen in non-pregnant and pregnant ewes between Days 12-15 the daily changes that occur in endometrial *ISG15*, *ESR1* and *OXTR* mRNA concentrations as well as corpus luteum and liver *ISG15* mRNA concentrations were examined during those exact Days (See Fig. 1.2, 1.3). Serum progesterone concentrations were maintained in pregnant ewes, but declined in non-pregnant ewes, by Day 14. This suggests that luteolysis might be initiated between 13 and 14 Days in these non-pregnant ewes. In this 12-15 Day profile, concentrations of *ISG15* mRNA in the endometrium were the first to increase as seen on Day 13 in pregnant ewes. *ISG15* mRNA concentrations were also significantly up-regulated in the corpus luteum and liver by Day 14 of pregnancy. On Day 14 *ESR1* and *OXTR* mRNA concentrations increased in non-pregnant ewes by Day 14. This increase in *ESR1* and *OXTR* mRNA concentrations appears to have been blocked in pregnant ewes by Day 14 (Fig. 1.2). The aforementioned studies are the first describing gene expression in the endometrium CL and liver due to the paracrine and endocrine actions induced by pregnancy. These studies were the first to suggest that endocrine induction of ISGs in the CL is occurring

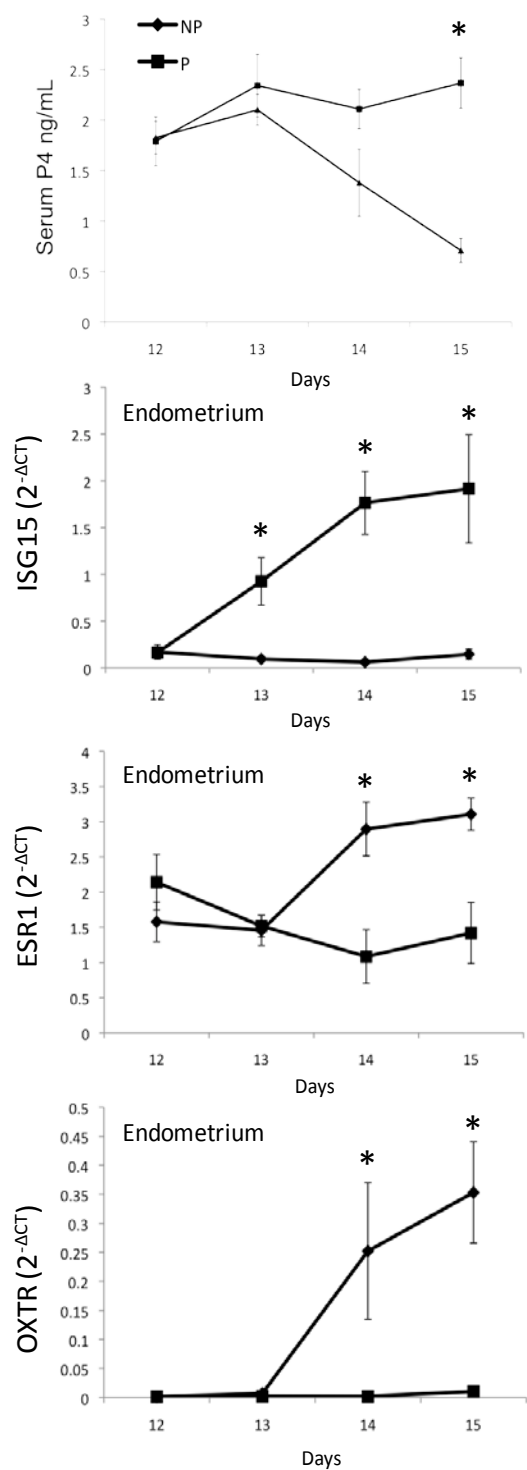


FIGURE 1.2.

Serum progesterone profile and paracrine action of IFNT on endometrial production of *ISG15*, *ESR1*, and *OXTR* during pregnancy (P) compared to the estrous cycle [(non-pregnant; (P < 0.05))].

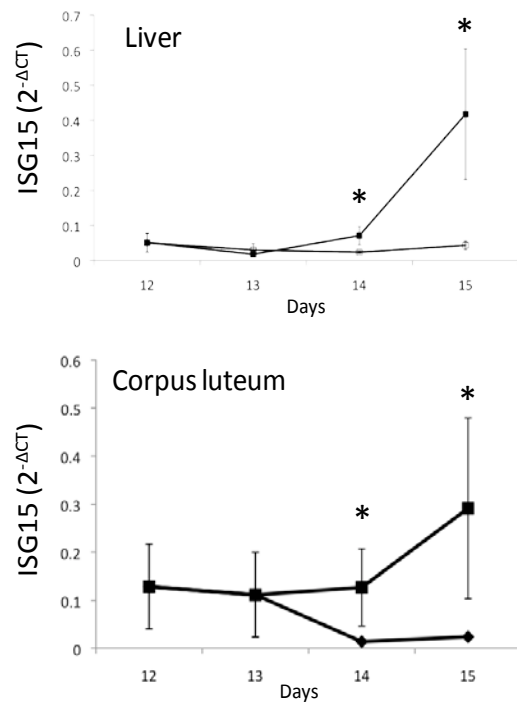


FIGURE 1.3.

ISG15 mRNA concentrations are significantly up-regulated in the Liver and maintained in the CL by Day 14 of pregnancy (P) compared to the estrous cycle (non-pregnant) due to IFNT endocrine delivery ($P < 0.05$). This data suggests that the concentrations of IFNT that are released in an endocrine fashion are great enough in the blood by Day 14 of Pregnancy to induce/maintain *ISG15* expression in the liver and CL.

at a similar time as paracrine inhibition of *ESR1* and *OXTR* up-regulation in the endometrium. Seven Day Endocrine Delivery of 200 µg roIFNT/d into the uterine vein delayed return to estrus

Endocrine actions induced by IFNT were further studied by miniosmotic pump infusion studies infusing IFNT for seven days into the uterine vein, Utilizing the average weight of 60 kg and blood volume of 58ml/kg for ewes the blood volume was estimated to be 3.48 L. We previously estimated that the release of IFNT into the uterine vein on Day 15 is approximately ~ 200 µg/d (Oliveira, Henkes et al. 2008). Based on these data, osmotic pumps were loaded to deliver 200 µg/d, which results in a release of 8.3 µg/h into the uterine vein. Employing the estimated blood volume calculated above, systemic levels of IFNT in circulation would stabilize around 2.4 ng/ml/h. The systemic level of IFNT in circulation is biologically relevant in the context of a dissociation constant (K_d) for the receptor of 3.7×10^{-10} M (Li and Roberts 1994) and estimated 50% occupancy of the receptor at 6.3 ng IFNT/ml. Although, based on the concept of spare receptors, only 1% of IFNT receptors need to be occupied to elicit a biological response, which would reflect physiological levels of IFNT in the blood as low as 63 pg IFNT/ml. To achieve endocrine delivery of 200 µg/d of recombinant interferon tau (roIFNT), osmotic pumps were surgically installed into the abdominal cavity to infuse roIFNT via a catheter into the uterine vein on Day 10 of the estrous cycle in sheep.

This seven Day infusion study resulted in eighty percent (4 out of 5 ewes) (Bott, Ashley et al. 2010) of ewes having extended estrous cycles. The ewe that did not respond to IFNT infusion had declining serum progesterone concentrations at the time of pump installation. This is the first experiment demonstrating that endocrine delivery of low

concentrations of IFNT systemically can induce a significant (long-term) delay in returning to a normal estrous cycle.

Twelve hour Endocrine Delivery of roIFNT into the Uterine Vein Provides Luteal Resistance to PGF

Osmotic pumps loaded to deliver 200 µg roIFNT or bovine serum albumin (BSA)/d were implanted to the abdominal cavity on Day 10 of the estrous cycle to infuse via a catheter into the uterine vein. Ewes were challenged with an injection of PGF 12 h later. Serum was collected from the ewes every 2 h for an additional 12h up until the time of necropsy when the CL was collected. In both treatment groups (roIFNT- vs. BSA) serum progesterone concentrations did not differ in the ewes during the last 12h of infusion. Ewes infused with BSA and challenged with PGF (4 mg/58 kg. i.m.; (Silvia and Niswender 1984; Silvia and Niswender 1986; Silva, Juengel et al. 2000; Bott, Ashley et al. 2010) had serum progesterone concentrations decrease ($P < 0.05$; a, b) by 6h and this decline was maintained 12h later. Ewes that were infused with roIFNT for 12h and then challenged with PGF had serum progesterone concentration that initially declined, but then returned to levels similar as the controls within 8-12h later. It was concluded from these results that IFNT is able to prevent a sustained decrease in serum progesterone concentrations when challenged with PGF after a 12h exposure of systemic roIFNT (200µg/d). Because the recovery of serum progesterone in ewes treated with roIFNT+PGF was subtle in this 24 h infusion study the study was further expanded to a 72 hour study. Recombinant oIFNT was delivered via canulation into the uterine vein, jugular vein, or subcutaneously into the neck for 24h starting on Day 10 of the estrous cycle in to the uterine vein, jugular vein or subcutaneously into the neck.

These ewes were then challenged with PGF 24 h after infusion began and blood samples were collected for two additional days to test direct luteo-protective action of IFNT on the CL. The expansion from a 12 to 72 h design allowed for more time for rolIFNT to further induce luteoprotective mechanisms. Endocrine delivery of 200 µg/d IFNT for 24h prior to PGF challenge into the uterine vein was able to induced significant concentrations of *ISG15* mRNA in ipsilateral and contralateral CL relative to the osmotic pump as well as in liver and endometrium. Due to the effectiveness of this dosage, for future delivery and usage in the agricultural community the jugular and subcutaneous delivery with dosages of 200 and 20µg/d was tested.

RATIONAL AND SIGNIFICANCE

The emotional and economic cost of miscarriage to humans is immeasurable; miscarriage occurs at an approximate rate of 11-27% in all clinically recognized pregnancies (Ellish, Saboda et al. 1996; Blohm, Friden et al. 2008; Herbert, Lucke et al. 2009). Human miscarriages can be associated with chromosomal abnormality of the conceptus, parental age, maternal obesity, maternal infection, diabetes mellitus and insufficient production of progesterone by the CL (Temmerman, Lopita et al. 1992; de la Rochebrochard and Thonneau 2002; Vorsanova, Kolotii et al. 2005; Metwally, Ong et al. 2008; Khaskheli, Baloch et al. 2010; Rocchetti, Marconi et al. 2010; Nigro, Mazzocco et al. 2011). Reasons behind why some embryos live and others die, especially when excluding chromosomal abnormalities, are not known across mammalian species, but probably involves impaired communication/signaling between the conceptus and the mother. Embryonic mortality rates have an occurrence of 28-43% in dairy cows, 33-38% in beef cows and 12-26% in sheep (Humblot 2001; Diskin, Murphy et al. 2006; Diskin

and Morris 2008). In dairy cattle most embryo loss occurs before Day 16 following breeding (Berg, van Leeuwen et al.). Farm revenue from large animal agricultural industries (beef, dairy and sheep) is approximately 63 billion annually. The economic impact in the United States of America is estimated to be close to \$332.5 billion per year (W2112 Reproduction Performance in Domestic Ruminants). Production and processing of these animals employs roughly 2.3 million people (Otto D 2002; Cryan 2004; Shiflett 2011). Revenue losses to cattle producers were estimated in 2005, to be greater than \$1.2 billion dollars, due to early embryonic mortality (Geary 2005). The value of 1.2 billion dollars was estimated only for the beef cattle industry, this did not include the cost to dairy producers where calving rates are between 40-55% after a 90% conception rate when using semen of superior quality (Diskin, Murphy et al. 2006). In order to understand why some embryos fail we need a better understanding of the mechanisms behind luteolysis. Previous scientists (Moor and Rowson and Mapletoft) believed that a systemic role for IFNT in maternal recognition of pregnancy was not plausible or supported by methods at that time (Moor and Rowson 1966; Mapletoft, Lapin et al. 1976) . However, it has been shown that IFNT is released into the uterine vein and initiates a peripheral antiviral response thru the induction of ISGs protecting the pregnancy from maternal viral infection (Bott, Ashley et al. 2010; Hansen, Henkes et al.). Increased knowledge of maternal recognition of pregnancy could allow possible rescue of at risk embryos by extrapolating the endocrine actions of IFNT and examining how it induces luteal resistance to PGF and long-term survival of the conceptus, CL and maintenance of pregnancy (Oliveira, Henkes et al. 2008; Hansen, Henkes et al.). These

advances in the understanding of IFN and maternal recognition of pregnancy could lead to reduced occurrence of miscarriage in society and our agricultural food industries.

HYPOTHESIS AND SPECIFIC AIMS

HYPOTHESIS:

Conceptus-derived IFNT induces ISGs and cell survival genes through endocrine action that contributes to resistance of the CL to PGF and the maintenance of early pregnancy in sheep.

Specific Aim 1: Identify genes that are significantly up and down regulated due to pregnancy and luteolysis in the Corpus Luteum.

The CL from Day 12 and Day 14 non-pregnant and pregnant ewes will be analyzed by microarray to identify specific genes that are affected by pregnancy status or by early stages of luteolysis.

Specific Aim 2: Determine how IFNT mediates luteal resistance to PGF.

The actions of IFNT on endometrium and CL have previously been studied by the Hansen lab and several collaborators (Hansen, Austin et al. 1999; Perry, Austin et al. 1999; Binelli, Guzeloglu et al. 2000; Binelli, Subramaniam et al. 2001; Pru, Rueda et al. 2001; Guzeloglu, Binelli et al. 2004). A profile of key gene expression over Days 12-15 of the estrous cycle and pregnancy has not been completed before. Previously identified genes that have been associated with luteolysis (ESR1, OXTR, SCLO2A1 and CASP3) and luteotrophic-cell survival (PI3K/AKT and ISGs) will be examined by RT-PCR and later followed up with Western blots.

Specific Aim 3: Detection of IFNT and identification of novel conceptus secretory products and components of uterine flushings during early pregnancy using mass spectroscopy approaches.

Using global mass spectrometry we hypothesize novel metabolites and proteins will be identified in uterine flushings. This will be done in collaboration with Dr. Prenni, who is the Director of the CSU Proteomics Core Facility. Presence of proteins will be validated by Western blot and other antibody-based approaches such as RIA. Metabolomic analysis will allow for better understanding of the ongoing cellular processes that occur in response to pregnancy. Small molecules such as PGF, Prostaglandin E2 (PGE2) and estradiol may be shown to be differentially expressed in uterine flushings from pregnant compared to non-pregnant sheep.

Specific Aim 4: Determine if IFNT is detectable in uterine vein blood /tail blood (from sheep/Dairy Cows) using a newly developed radioimmunoassay (RIA) for IFNT.

IFNT is expected to be detected in uterine flushings, uterine vein blood and possibly tail blood. Until recently, IFNT was thought to act exclusively through paracrine disruption of release of PGF from the endometrium. However, indirect evidence has surfaced to suggest that IFNT is released into the uterine vein and has endocrine action on the CL. Analysis, through RIA and mass spectroscopy of uterine flushings and uterine vein blood from Day 12-16 sheep for IFNT will confirm presence and allow better understanding of the temporal nature of up-regulation of IFNT during early pregnancy and maternal recognition of pregnancy. Uterine vein blood and uterine flushings will be analyzed using a highly specific RIA for IFNT. The IFNT RIA developed at the ARBL has a sensitivity of limit of detection of 24pg, which reflects 50% binding at 0.4 ng/ml

under binding kinetics with a primary antibody dilution of 1:60,000. In the uterine vein blood samples, IFNT concentrations are expected to increase and to be high by Day 15 of pregnancy. A similar pattern is expected in the uterine vein flushes but the pattern may be shifted a Day or two earlier.

CHAPTER 2

*PREGNANCY-ASSOCIATED GENES CONTRIBUTE TO ANTILUTEOLYTIC MECHANISMS IN OVINE CORPUS LUTEUM

CHAPTER SUMMARY

The hypothesis that ovine luteal gene expression in pregnancy compared to non-pregnant status differs according to pregnancy status and by Day of estrous cycle was tested. RNA was isolated from corpora lutea (CL) on Days 12 and 14 of the estrous cycle (NP) or pregnancy (P) and analyzed using the Affymetrix bovine microarray. RNA also was isolated from luteal cells on Day 10 of estrous cycle that were cultured for 24 h with luteolytic (OXT and PGF) hormones and secretory products of the conceptus (IFNT and PGE₂). Differential gene expression (>1.5 fold, $P < 0.05$) was confirmed using semi-quantitative real time PCR (RT-PCR). Serum progesterone concentrations decreased from Day 12 to Day 15 in NP ewes ($P < 0.05$) reflecting luteolysis; and remained > 1.7 ng/ml in P ewes reflecting rescue of the CL. Early luteolysis (Days 12 to 14) was associated with differential expression of 683 genes in the CL, including up-regulation of *SERPINE1* and *THBS1*. Pregnancy on Day 12 (55 genes) and 14 (734 genes) also was associated with differential expression of genes in the CL, many of which were ISGs (i.e., *ISG15*, *MX1*) that were induced when culturing luteal cells with IFNT, but not PGE₂. Finally, many genes, such as *PTX3*, *IL-6*, *VEGF* and *LHR* were unaltered during pregnancy and down-regulated during the estrous cycle and in

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response to culture of luteal cells with luteolytic hormones. In conclusion, pregnancy circumvents luteolytic pathways, and activates or stabilizes genes associated with interferon, chemokine, cell adhesion, cytoskeletal, and angiogenic pathways in the CL.

INTRODUCTION

A better understanding of the mechanisms underlying establishment and loss of pregnancy may be applied to reduce the economic impact of embryo mortality on the agricultural community. For example, early embryonic mortality rates are as high as 28-43% in dairy cows, 33-38% in beef cows, and 12-26% in sheep (Diskin and Morris 2008). The consequences of embryo mortality in the beef cattle industry alone were estimated to be a loss of \$1.2 billion dollars in 2005 (Geary 2005). Causes of early embryo mortality may entail impaired signaling between the conceptus and mother. This “communication” is through conceptus secretory signals such as interferon tau (IFNT) that act directly on the endometrium and possibly through endocrine action on the corpus luteum (CL). The CL functions primarily to produce progesterone, which is critical in preparing the uterus for sustaining the early developing conceptus.

Prostaglandin F₂ alpha (PGF) causes luteolysis, which is the structural and functional (loss of serum progesterone) demise of the CL (Niswender, Juengel et al. 2000). Binding of PGF to its receptor [prostaglandin F receptor, (PTGFR)] on large luteal cells (LLC) induces several downstream apoptotic pathways in both LLC and small luteal cells (SLC). These include: i) induction of a suicidal loop of PGF being produced by LLC through the prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase/cyclooxygenase, (PTGS2)) pathway (Spencer, Burghardt et al. 2004; Niswender, Davis et al. 2007); ii) induction of calcium influx into LLC; iii) activation of the

protein kinase C (PKC) pathway, which blocks the synthesis of progesterone and causes the production of oxytocin (OXT) (Wiltbank, Guthrie et al. 1989; Niswender, Davis et al. 2007); and iv) binding of OXT secreted by the LLC to the oxytocin receptor (OXTR) on the SLC, which causes an influx of calcium, activation of the PKC pathway and lysis of the SLC (Vinatier, Dufour et al. 1996; Niswender, Davis et al. 2007).

The sheep conceptus signals its presence by releasing IFNT. IFNT binds receptors in the endometrium and activates anti-luteolytic responses, which permit continued production of progesterone from the CL. IFNT is released by the ovine conceptus on Days 10 through 25, with the greatest concentrations released between Days 14-16 (Spencer, Burghardt et al. 2004). For a successful pregnancy to be recognized and maintained in the ewe the conceptus must be present from Day 12 through Day 17 (Moor and Rowson 1966; Hansen, Anthony et al. 1985). The anti-luteolytic actions of IFNT in the endometrium are mediated by silencing the up-regulation of the estrogen receptor (ESR1), which normally occurs during the estrous cycle. Consequently, inhibition of ESR1 inhibits production of the endometrial OXTR, thereby disrupting pulsatile release of PGF (Spencer, Becker et al. 1995; Spencer, Ing et al. 1995; Spencer, Burghardt et al. 2004). This paracrine action of conceptus-derived IFNT on the endometrium indirectly protects the CL of pregnancy.

A direct action of pregnancy on the ovine CL also has been suggested because the CL of pregnant animals is more resistant to the lytic effects of PGF (Inskeep, Smutny et al. 1975; Mapletoft, Del Campo et al. 1976; Pratt, Butcher et al. 1977; Silvia and Niswender 1984). More recent evidence to support this concept is based on detection of IFNT in uterine vein blood and demonstration that IFNT has action on the

CL through induction of IFN stimulated genes (ISGs), such as IFN stimulated gene 15 (*ISG15*) (Oliveira, Henkes et al. 2008; Bott, Ashley et al. 2010; Hansen, Henkes et al. 2010).

It is hypothesized herein that genes induced in the CL in response to early pregnancy counter the activation of genes involved in the demise of the CL in response to PGF. Objectives were to test this hypothesis by screening mRNA isolated from CL on Days 12 and 14 of the estrous cycle [non-pregnant (NP)] and pregnancy (P) in ewes using the bovine Affymetrix microarray, determining major activated pathways in response to pregnancy and early luteolysis, and comparing luteal gene expression during the estrous cycle and early pregnancy with responses induced by PGF and OXT as well as IFNT and PGE2 in cultured luteal cells.

MATERIALS AND METHODS

Animal Care, Collection of Corpora Lutea and Blood Samples

All experiments using sheep were reviewed and approved by the Colorado State University Animal Care and Use Committee. Western range ewes purchased from local producer were either exposed to a vasectomized ram (NP group, no semen exposure) or mated to a fertile ram (P group) to generate CL derived from the estrous cycle or pregnancy, respectively (Day 0 = Day of estrus). CL were collected during necropsy: Day 12 (n = 4 NP & 4 P), Day 13 (n = 5 NP & 5 P); Day 14 (n = 5 NP & 6 P), Day 15 (n = 6 NP & 10 P), and Day 16 (n = 5 P). The CL had regressed by Day 16 of the estrous cycle and for this reason was not examined. Presence of a conceptus was confirmed by visual identification following flushing the uterine lumen with sterile saline solution at necropsy.

Progesterone Assay

Blood samples were collected two times per Day starting on Day 12, processed to yield serum and then analyzed for progesterone concentrations using radioimmunoassay (Niswender 1973). The sensitivity of the assay was 15 pg/ml. The mean intra-assay coefficient of variation was 5.83%. Three quality controls were examined in duplicate for each assay. The CVs were calculated for each standard used in the assay and presented as an average CV for the assay.

RNA Isolation

Total RNA was extracted from CL using TRIzol Reagent (MRC; Cincinnati, OH) protocol. RNase-free DNase and RNeasy MinElute Cleanup Kits (Qiagen, USA) were used to digest DNA and purify RNA. RNA was quantified using a NanoDrop (NanoDrop Technologies, Inc. Valencia, CA). Purity of RNA was determined using A260/280 and A260/230 ratios. Proper ratios were between 1.75-2.0. RNA integrity was determined using an Agilent 2100 Bioanalyzer.

Analysis of Gene Microarray data

Microarray analysis was completed at the Microarray Core Facility at the University of Nebraska Medical Center (Dr. Xiaoying Hou). The cDNA probes were synthesized from 200 ng of CL mRNA representing Day 12 and 14 of the estrous cycle or pregnancy (n=3 ewes for each Day and pregnancy status) and were used to screen 24,000 targets by using the bovine microarray from Affymetrix (Santa Clara, CA). The microarray data were preprocessed using RMA algorithm for background correction, quartile normalization and gene-level probe set summation (Irizarry, Hobbs et al. 2003). Differential expression ($P < 0.05$) was determined using the LIMMA method (Smyth

2004). These data were further analyzed using the Metacore pathway analysis program from GeneGo (Carlsbad, CA) to identify signal transduction pathways and genes that are impacted by main effects of Day and pregnancy status. Genes with a fold changes >1.5 and $P < 0.05$ were determined to be differentially expressed and included in this analysis.

Semi-quantitative Real Time Reverse Transcriptase PCR (RTPCR)

Single-stranded cDNA was synthesized from 1 µg of RNA using the iScript cDNA Synthesis Kit (Bio-Rad Life Science; Hercules, CA). The synthesized cDNA was used as a template for RTPCR using iQ SYBR Green Supermix (Bio-Rad Life Science). The cDNA samples were amplified in a 384-well plate with oligonucleotide primers specific to the targets (Table 2.1). Oligonucleotide primers were designed with an annealing temperature of 61°C, single-product melting curves, and consistent amplification efficiencies (Table 2.1). Amplification of PCR products was performed at 95°C for 30 sec, 61°C for 30 sec, 72°C for 15 sec and repeated over 40 cycles. Amplification of cDNA was normalized using the geometric mean of *GAPDH*, *RNA polymerase II* (*POLR2A*), *Ribosomal Protein L19* (*RPL19*) and 18s ribosomal RNA (*RN18s*) as internal standards. CT values were analyzed, whereas relative expression of RTPCR products were plotted using mean $2^{-\Delta CT}$; RTPCR amplification efficiencies were between 1.8 and 2.2 (Schmittgen and Livak 2008). Amplicon size was verified through PCR amplification and gel electrophoresis; all amplicons were sequenced to confirm identity with targeted genes.

TABLE 2.1. Oligonucleotide Primer sequences and efficiency of amplification by using semi-quantitative RTPCR

Gene Target	Forward Primer	Reverse Primer	RTPCR Efficiency (1.8-2.2)
SERPINE1	5'TCATGCCCAACTTCTTCAGG3'	5'TTGACGATGAACCTGGCTCT3'	2.13
THBS1	5'ACTGGGTTGTACGCCATCAG3'	5'CACGGCGTTAAATTCGTCAT3'	2.18
ISG15	5'GGTATCCGAGCTGAAGCAGTT3'	5'ACCTCCCTGCTGTCAAGGT3'	1.96
MX1	5'TCTGCAAATGGAGTGCTGTG3'	5'TTCACAAACCCTGGCAACTC3'	2.00
IL-6	5'CTGCAGTTCAGCCTGAGAG3'	5'CCCAGTGGACAGGTTTCTGA3'	2.04
PTX3	5'TTGGGTCAAAGCCACAGAAG3'	5'CCACCCACCACAAGCATTAT3'	2.00
VEGF	5' TCTGCTCTCTTGGGTGCA3'	5'TCACTTCATGGGGTTTCTGC3'	2.06
LHR	5'GTGCAACCTCTCCTTTGCAG3'	5'CTGCCAGTCTATGGCATGGT3'	1.92
GAPDH	5'TGACCCCTCATTGACCTTC3'	5'GGTTCCTGCCTTGACTGTG3'	1.95
POLR2A	5'AGTCCAACATGCTGAAGGACATGA3'	5'AGCCAAGTGCCGGTAATTGACGTA3'	2.04
RPL19	5'TCGCCGGAAGGGCAGGCATA3'	5'GGCTGTGATACATGTGGGGGT C3'	2.20
RN18S	5'GAGGCCCTGTAATTAGAATGAG3'	5'GCAGCAACTTTAATATACGCTATTGG3'	2.20

Culture of Isolated Small, Large and Mixed Luteal Cells

Luteal cells were isolated from CL collected from adult western range ewes on Day 10 of the estrous cycle; SLC and LLC were separated by elutriation (Fitz, Mayan et al. 1982). Cells were cultured in 6-well plates at the following concentrations: SLC- 2×10^6 /ml, LLC- 5×10^5 /ml and MLC- 1×10^6 /ml luteal cells. Isolated luteal cell populations were cultured for 24 h at 37° C and 5% CO₂ in M199 media supplemented with 10% FBS and 1% penicillin/streptomycin. After 24 h incubation, the media was replaced with serum free media and luteal cells were not treated (control) or treated with (i) rolFNT (1 ng/ml; 10^8 U/mg; from Dr. Fuller Bazer; Texas A&M University) or (ii) prostaglandin E2 (PGE2; 3.5 ng/ml, Sigma Aldrich; Milwaukee, Wisconsin). In addition to rolFNT and PGE2, SLC were also treated with OXT [10 µg/ml; Sigma Aldrich; St. Louis, Missouri, (Davis, Bott et al. 2010)] and LLC were treated with PGF (Davis, Bott et al. 2010) (3.5 ng/ml; Fisher Scientific, Houston, Texas). All cells were treated with IFNT and PGE2 to study genes that were up-regulated based on microarray in CL from pregnant ewes. PGE2 was tested in these experiments because it has been described as a luteotrophic agent (Henderson, Scaramuzzi et al. 1977; Pratt, Butcher et al. 1977; Magness, Huie et al. 1981; Reynolds, Stigler et al. 1981). PGF binds receptors on LLC and OXT binds receptors on SLC to induce luteolysis (Niswender, Davis et al. 2007), thus these luteolytic hormones were tested so that genes regulated in response to luteolysis could be examined. Luteal cell mRNA was isolated following 24 h culture with treatments using Trizol Reagent. Concentrations of OXT, PGE2, and PGF were selected based on previous reports of effectiveness in inducing an *in vitro* response (Davis, Bott et al. 2010). Amount of rolFNT (1 ng/ml), added to luteal cells, was determined using a

concentration dependent induction of ISG15 described by Antoniazzi et al. (Antoniazzi, Webb et al. 2013) and represented the lowest concentration required to induce a maximal ISG15 response.

Data Analysis of RTPCR

Analysis of RTPCR data was completed on gene targets reflecting major impacted signal transduction pathways by use of two-way ANOVA for unequal sample size with Day (12-15) and pregnancy status as main effects using SAS software version 9.3 (SAS Institute Inc., Cary, North Carolina). Type I error within the two-way ANOVA was corrected by using a Tukey adjustment. CL collected on Day 16 of pregnancy were compared to CL collected on other Days of pregnancy using ANOVA rather than being included in two way ANOVA because of lack of a viable CL for comparison on Day 16 of the estrous cycle. Note that ewe sample size was larger for analysis of CL by RTPCR: Day 12 (n = 4 NP & 4 P), Day 13 (n = 5 NP & 5 P); Day 14 (n = 5 NP & 6 P), Day 15 (n = 6 NP & 10 P), and Day 16 (n = 5 P) when compared to microarray analysis. Differences between treatments in cell culture were tested using ANOVA with a Tukey adjustment.

RESULTS

Serum Progesterone Concentrations

Serum progesterone concentrations did not differ in Day 12 or 13 NP and P ewes (Fig. 2.1). In NP ewes, serum progesterone concentrations started to decline on Day 14 and then continued to decline by Day 15 to levels below 1 ng/ml indicating that the CL was regressing in these ewes. In contrast, serum progesterone concentrations remained unchanged (~ 1.7 ng/ml) from Days 12-15 in P ewes.

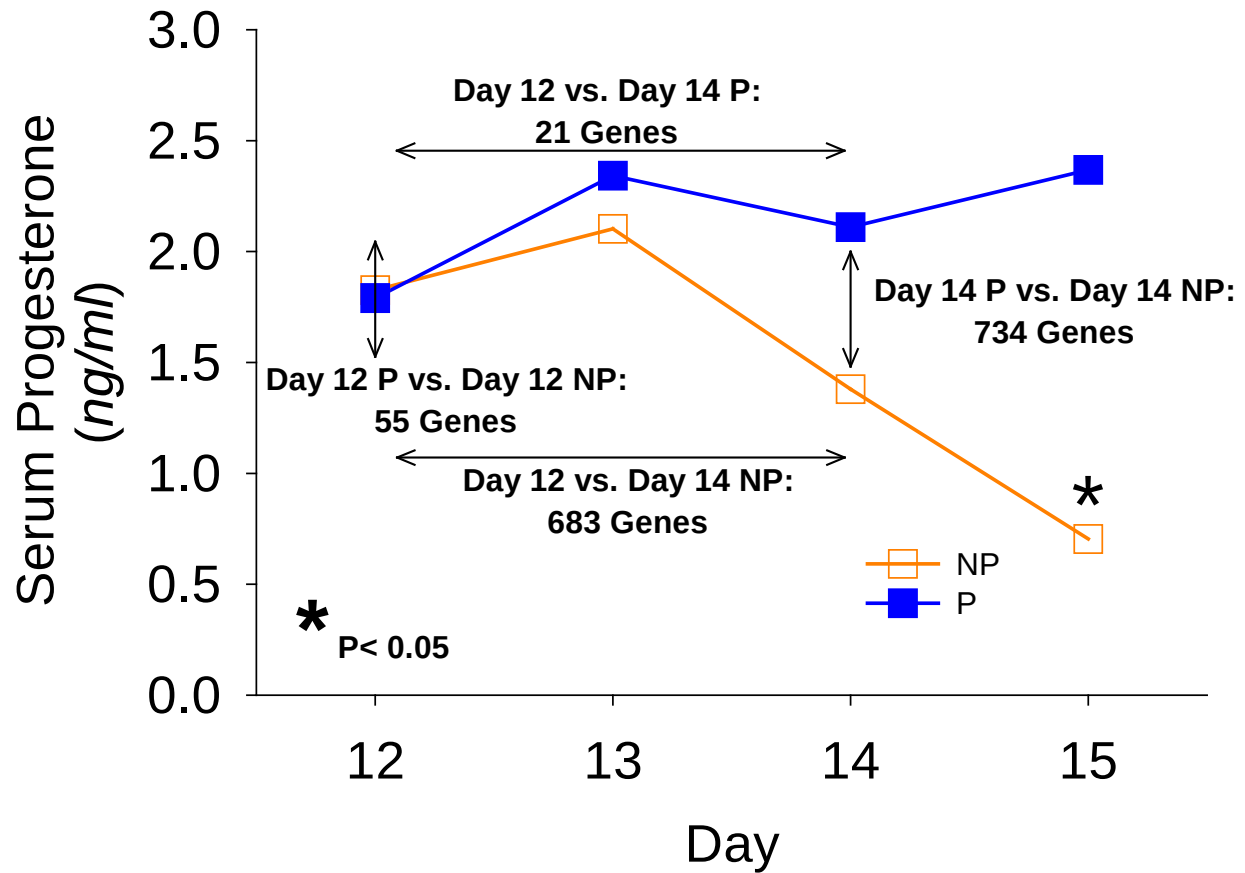


FIGURE 2.1.

Serum progesterone concentrations and differential gene expression in CL collected on Days 12 and 14 of pregnancy (P) and the estrous cycle (NP). Serum progesterone was maintained from Day 12 to 15 of pregnancy, whereas it declined in a manner consistent with luteolysis from Day 12 to 15 of the estrous cycle. Number of genes differentially expressed (>1.5 fold; $P < 0.05$) was determined via microarray and is shown in context of serum progesterone profiles as a function of Day or pregnancy status.* - Indicates a significant difference ($P < 0.05$) between serum progesterone from non-pregnant and pregnant ewes on Day 15. Values represent mean $\pm$ standard error.

CL Microarray Analysis

Numbers of genes differentially expressed 1.5 fold or greater ($P < 0.05$) following microarray analysis are presented in Fig. 2.1 in context of serum progesterone profiles in Day 12 or 14 NP and P ewes. On Day 12, 55 genes were differentially expressed in CL collected from NP compared to P ewes. As the estrous cycle progressed from Day 12 to 14, which also corresponded with the onset of luteolysis in response to endogenous PGF, there were 683 differentially expressed genes. As pregnancy progressed from Day 12 to 14, there were 21 differentially expressed genes in P ewes. On Day 14, there were 734 differentially expressed genes in CL from P compared to NP ewes.

Pathway Analysis

Because there were only 55 differentially expressed genes on Day 12 in NP compared to P ewes and 21 differentially expressed genes as pregnancy progressed from Day 12 to 14, pathway analysis was limited due to low numbers of genes identified per pathway, but implicated the pregnancy-associated immune response and IFN alpha/beta signaling as well as steroid biosynthesis and cytoskeletal remodeling in the CL (Tables 2.2 and 2.3). Key pathways identified in CL from Day 14 P compared to NP ewes were: cell adhesion, chemokines (interleukin 8; IL-8), cytoskeletal remodeling and transforming growth factor (TGF)-beta signaling ($P < 0.0001$; 8-21 genes; Table 2.4). Genes differentially expressed as the CL entered early stages of luteolysis from Day 12 to 14 NP belonged to cell cycle, adhesion, chemokine (IL-8), TGF-beta, and cytoskeleton pathways ($P < 0.0001$; 10-21 genes; Table 2.5). Selection of the

TABLE 2.2. The top pathways in CL on Day 12 of the estrous cycle compared to pregnancy in ewes.

	Pathway 12np/12p	P-Value	Genes in Pathway
1.	Delta 508-CFTR traffic/ ER-to Golgi in CF	0.02	2/13
2.	Normal wtCFTR traffic/ ER-to-Golgi	0.02	2/13
3.	Cell cycle Initiation of mitosis	0.07	2/25
4.	Cytoskelton remodeling Fibronectin-binding integrins in cell motility	0.08	2/28

CFTR, cystic fibrosis transmembrane conductance regulator; CF cystic fibrosis

TABLE 2.3. The top pathways in CL from Days 12 to 14 of pregnancy in ewes.

	Pathway 14p/12p	P-Value	Genes in Pathway
1.	Androstenedione and testosterone biosynthesis and metabolism p.2	0.03	2/17
2.	Androstenedione and testosterone biosynthesis and metabolism p.2 rodent version	0.03	2/18

TABLE 2.4. The top 10 pathways in CL on Day 14 of the estrous cycle compared to pregnancy in ewes.

	Pathway 14np/14p	P-Value	Genes in Pathway
1.	Cell adhesion Chemokines and adhesion	1×10^{-6}	20/93
2.	Cytoskeleton remodeling	2×10^{-6}	20/96
3.	TGF, WNT and cytoskeletal remodeling	4×10^{-6}	21/107
4.	Development TGF-beta-dependent induction of EMT via MAPK	4×10^{-6}	13/46
5.	Cell adhesion Plasmin signaling	6×10^{-6}	11/34
6.	Development TGF-beta- dependent induction of EMT via Smads	5×10^{-5}	10/35
7.	Cell Adhesion ECM remodeling	8×10^{-5}	12/51
8.	Development Role of IL-8 in Angiogenesis	2×10^{-4}	11/47
9.	Development Regulation of epithelial-to-mesenchymal transition (EMT)	2×10^{-4}	13/63
10.	Cytoskeleton remodeling. Fibronectin-binding integrins in cell motility	3×10^{-4}	8/28

TGF, transforming growth factor; WNT, wingless; EMT, Epithelial-mesenchymal transition

TABLE 2.5. The top ten pathways in CL during early luteolysis on Days 12 to 14 of the estrous cycle in ewes.

	Pathway14np/12np	P-Value	Genes in Pathway
1.	Cell cycle Spindle assembly and chromosome separation	3×10^{-6}	11/32
2.	Cell adhesion Chemokines and adhesion	5×10^{-6}	19/93
3.	Development TGF-beta-dependent induction of EMT via SMADs	7×10^{-6}	11/35
4.	Cytoskeleton remodeling	8×10^{-6}	19/96
5.	TGF, WNT and cytoskeletal remodeling	1×10^{-6}	20/107
6.	Cell cycle Chromosome condensation in prometaphase	2×10^{-5}	8/20
7.	Development TGF-beta-dependent induction of EMT via MAPK	2×10^{-5}	12/46
8.	Development Role of IL-8 in angiogenesis	3×10^{-5}	12/47
9.	Cytoskeleton remodeling Fibronectin-binding integrins in cell motility	4×10^{-5}	9/28
10.	Development Regulation of epithelial-to-mesenchymal transition (EMT)	1×10^{-4}	13/63

TGF, transforming growth factor; WNT, wingless; EMT, Epithelial-mesenchymal transition

differentially expressed genes for further study was based on representation in the pathway analysis, but also on significance and fold change from the microarray analysis data (Table 2.6). The eight genes selected for further analysis Selection of the differentially expressed genes for further study was based on representation in the pathway analysis, in addition to significance ($P < 0.05$) and fold change (fold change > 1.5) from the microarray analysis data (Table 2. 6). The eight genes selected for further analysis were: serpine peptidase inhibitor (SERPINE1), thrombospondin 1 (THBS1), ISG15, myxovirus (influenza virus) resistance 1 (MX1), interleukin 6 (IL-6), pentraxin 3 long (PTX3), vascular endothelial growth factor A (VEGF), and luteinizing hormone/ choriogonadotropin receptor (LHR). We also examined genes that have been implicated previously in the CL in processes such as steroidogenesis (STAR, PBR, CYP11A1, and HSD3B), and prostaglandin biosynthesis and action (HPGD, PTGS2, PGFS, and PTGES) to determine if they were differentially expressed in the CL in response to pregnancy or luteolysis based on microarray analysis (Table 2.6). STAR, CYP11A1 and *HSD3B* mRNA concentrations were down-regulated reflecting decline in production of progesterone from Days 12 to 14 of the estrous cycle. These steroidogenic proteins also were down-regulated on Day 14 of the estrous cycle compared to pregnancy (Table 2.6). PBR and the prostaglandin biosynthesis enzyme mRNA concentrations did not change during the estrous cycle or pregnancy.

Microarray and RTPCR Validation of Gene Targets

Genes up-regulated in the CL during early stages of luteolysis based on microarray data were *SERPINE* and *THBS1* (Fig. 2.2). *SERPINE1* ($P < 0.05$) and *THBS1* ($P < 0.01$) were affected by pregnancy in the model and increased in CL from NP

TABLE 2.6. Genes selected from pathway analysis that were differentially expressed in the CL in response to of the estrous cycle or pregnancy or in response to pregnancy status

Gene Targets	Day 12 P vs. NP		Day 14P vs. 12P		Day 14NP vs.12 NP		Day 14 P vs. NP	
	Fold	P-value	Fold	P-value	Fold	P-value	Fold	P-value
SERPINE1	-	NS		NS	21.3	5x10 ⁻¹¹	-16.8	1x10 ⁻¹⁰
THBS1	-	NS		NS	4.09	2x10 ⁻⁶	-4.06	3x10 ⁻⁶
ISG15	-	NS	2.3	0.022	-	NS	3.1	0.004
MX1	1.5	0.035	1.35	0.096	-	NS	2.2	0.0005
IL-6	-	NS	-	NS	-6.8	0.0007	4.4	0.0045
PTX3	-	NS	-	NS	-4.1	0.0008	3.6	0.0016
VEGF	-	NS	-	NS	-2.7	3.x10 ⁻⁵	3.4	4x10 ⁻⁶
LHCGR	-	NS	-	NS	-2.2	0.0002	2.1	0.0004
STAR	-	NS	-	NS	-1.62	0.0001	1.7	5x10 ⁻⁵
PBR	NA	NA	NA	NA	NA	NA	NA	NA
CYP11A1	-	NS	-	NS	-1.38	0.015	1.4	0.001
HSD3B	-	NS	-	NS	-1.7	0.0009	1.7	0.001
HPGD	NA	NA	NA	NA	NA	NA	NA	NA
PTGS2	NA	NA	NA	NA	NA	NA	NA	NA
PGFS	NA	NA	NA	NA	NA	NA	NA	NA
PTGES	NA	NA	NA	NA	NA	NA	NA	NA
TGFB	-	NS	-	NS	1.69	4x10 ⁻⁵	-1.68	4x10 ⁻⁵
IL-8	-1.27	0.013	-	NS	1.19	0.06	-1.48	0.0004

¹NA represents non-applicable genes because they were not shown to be differentially expressed in the microarray data. ²NS represents non-significant genes that appeared in the microarray data.

Gene, Function Pathway, Fold Change

RTPCR

Serpine peptidase inhibitor Member 1, SERPINE1

- ✓ Inhibits tissue plasminogen activator and urokinase plasminogen activator. Involved in extracellular remodeling.
- ✓ 14 vs. 12 NP: 21.3 fold, $P < 0.0001$
- ✓ 14 P vs. NP: -16.8 fold, $P < 0.0001$
- ✓ Cytoskeleton remodeling pathway

Thrombospondin1, THBS1

- ✓ Plays a role in cell to cell adhesion and platelet aggregation.
- ✓ 14 vs. 12 NP: 2.1 fold, $P < 0.0001$
- ✓ 14 P vs. NP: -1.9 fold, $P < 0.001$
- ✓ Chemokine and Adhesion pathways

Corpora Lutea

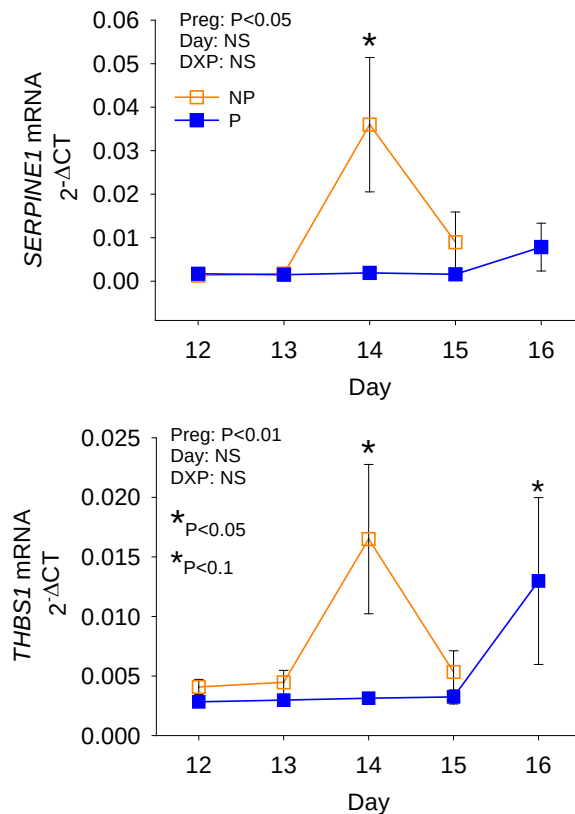


FIGURE 2.2.

Genes transiently up-regulated in response to luteolysis based on microarray and RTPCR. Two genes, *SERPINE1* and *THBS1*, out of 683 genes differentially expressed in response to luteolysis, were more extensively examined in the CL. General function, fold changes in *SERPINE1* and *THBS1* mRNA concentrations based on microarray analysis, and implicated pathways are described in left panels, Factorial analysis of RTPCR for these mRNAs over days of the estrous cycle (NP) and pregnancy (P) are shown in right panels. * - Represents differences across pregnancy status on the same day; $P < 0.05$. * - Represents tendency for difference across pregnancy status on the same day; $P < 0.10$. Values represent mean $\pm$ standard error.

compared to P ewes on Day 14. Neither *SERPINE1* nor *THBS1* mRNA concentrations changed very much from Days 12-15 of pregnancy. However, after Day 15, *THSB1* mRNA concentrations tended ($P<0.10$) to increase.

Genes up-regulated in response to pregnancy based on microarray analysis were *ISG15* and *MX1* (Fig. 2.3). *ISG15* and *MX1* mRNA concentrations did not change during the estrous cycle. However, by Day 15 of pregnancy, mRNA concentrations increased and continued to increase through Day 16 of pregnancy. Genes stabilized during pregnancy and down-regulated in response to luteolysis were *IL-6* ($P<0.05$), *PTX3* ($P<0.001$), *LHR* ($P=0.05$) and *VEGF* ($P<0.05$; Fig. 2.4 A&B). *IL-6* and *PTX3* mRNA concentrations were down-regulated as early as Day 14 and remained down-regulated through Day 15 of the estrous cycle. *LHR* and *VEGF* were down-regulated by Day 15 of the estrous cycle. All of these genes were stabilized during pregnancy and in one case, *LHR*, there was a tendency for up-regulation in mRNA concentrations by Day 16 of pregnancy.

Culture of Small, Large and Mixed Luteal Cells

SERPINE1 mRNA concentrations increase transiently during luteolysis (Fig. 2.2) and for this reason it was examined in cultured luteal cells (Fig. 2.5A). Isolated SLC and LLC had similar *SERPINE1* mRNA concentrations regardless of treatments in vitro. While IFNT had no effect, PGE₂, PGF and OXT tended ($P<0.10$) to decrease *SERPINE1* mRNA concentrations when cultured with MLC. Culture of SLC, LLC and MLC with IFNT caused massive induction of *ISG15* (Fig. 2.5B), which was consistent with the response of the CL to pregnancy based on microarray and RTPCR data.

Gene, Function Pathway, Fold Change

Interferon Stimulated Gene 15, ISG15

- ✓ Conjugates to proteins through pathway similar to ubiquitin, possibly modifying or stabilizing function of proteins
- ✓ 14P vs. 12 P: 2.3 Fold, $P < 0.05$
- ✓ 14 P vs. NP: 3.1 Fold, $P < 0.05$
- ✓ Immune response/ IFN alpha/ beta signaling pathway

Myxovirus Resistance 1, MX1

- ✓ Mx1 inhibits a wide range of viruses composed of RNA and DNA genomes
- ✓ 12 P vs. NP: 1.5 fold, $P < 0.05$
- ✓ 14 P vs. NP: 2.2 fold, $P < 0.001$
- ✓ Immune response/ IFN alpha/ beta signaling pathway

RTPCR

Corpora Lutea

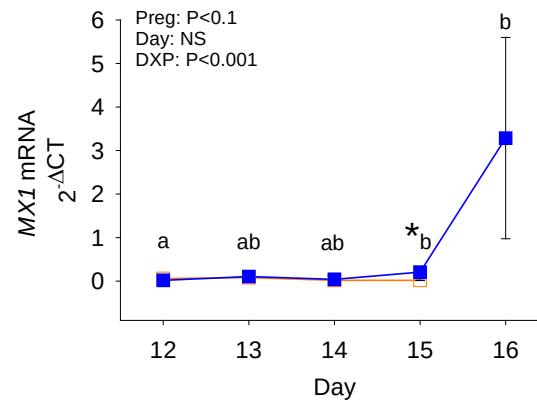
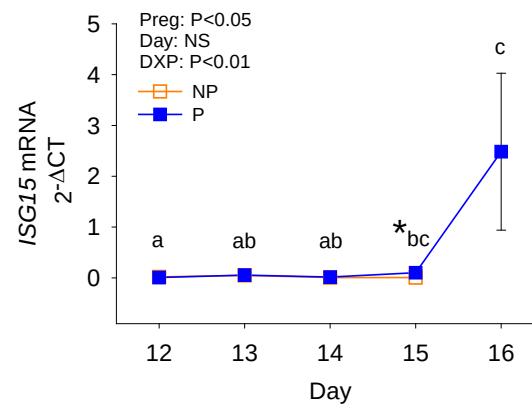


FIGURE 2.3.

Genes up-regulated in the CL during pregnancy. Two genes, *ISG15* and *MX1*, out of 21 differentially expressed in response to pregnancy on Day 12, 21 genes from Day 12 to 14 of pregnancy and 734 genes in response to pregnancy on Day 14, were more extensively examined. General function, fold changes in *ISG15* and *MX1* mRNA concentrations based on microarray analysis, and implicated pathways are described in left panels. Factorial analysis of RTPCR for these mRNAs over days of the estrous cycle (NP) and pregnancy (P) are shown in right panels. Means marked with different letters differ across days of pregnancy ($P < 0.05$). * - Represents differences across pregnancy status on the same day; $P < 0.05$.

Gene, Function Pathway, Fold Change

Interleukin 6, IL6

- ✓ Maturation of B cells
- ✓ 14 vs. 12 NP: -6.8 fold, $P < 0.001$
- ✓ 14 P vs. NP: 4.4 fold, $P < 0.05$
- ✓ Immune response IFN alpha/beta signaling pathway

Pentraxin, 3 long, PTX3

- ✓ Provide resistance to neurodegeneration.
- ✓ 14 P vs. NP: 3.61 fold, $P < 0.001$
- ✓ 14 vs. 12 NP: -4.077 fold, $P < 0.05$
- ✓ Immune response pathway

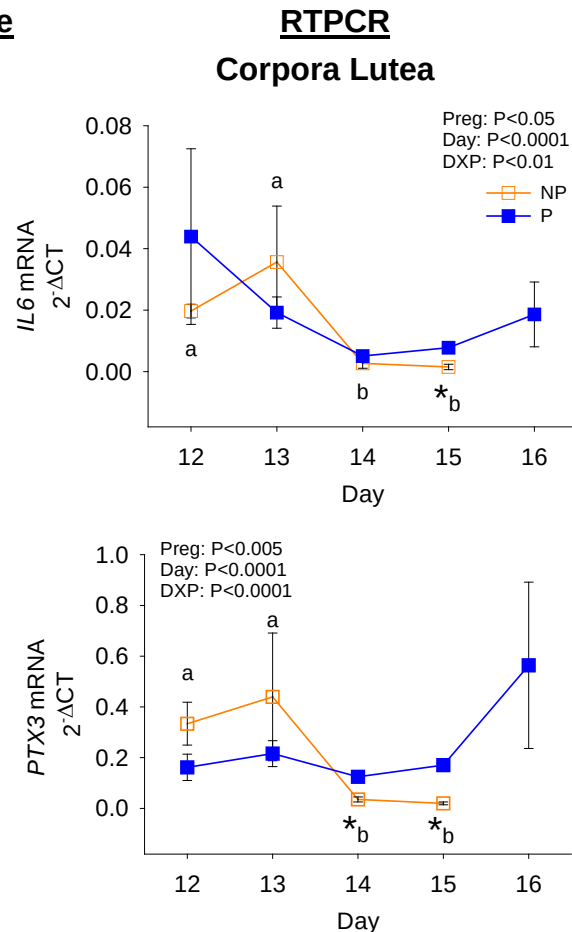


FIGURE 2.4A.

Genes stabilized during pregnancy and down-regulated in the CL during luteolysis. Selected genes out of 734 differentially expressed in CL on Day 14 of pregnancy (P) compared to the estrous cycle (NP) included *IL-6* and *PTX3* (A); and *LHR* and *VEGF* (B). General function, fold changes in mRNA concentrations based on microarray analysis, and implicated pathways are described in left panels. Factorial analysis of RTPCR for these mRNAs over days of the estrous cycle (NP) and pregnancy (P) are shown in right panels. Means represented with different letters differ across days of the estrous cycle ($P < 0.05$). * - Represents differences ($P < 0.05$) across pregnancy status on the same day. * - Represents tendency ($P < 0.10$) in differences across pregnancy status on the same day. Values represent mean $\pm$ standard error.

Gene, Function Pathway, Fold Change

Luteinizing Hormone Receptor, LHR

- ✓ Binding of LH to its receptor is required for normal CL function and formation.
- ✓ 14 vs. 12 NP: 2.2 fold, $P < 0.001$
- ✓ 14 P vs. NP: 2.1 fold, $P < 0.0001$
- ✓ Hormone biosynthesis pathway
- ✓

Vascular endothelial growth factor, VEGF

- ✓ Necessary for follicular growth, enhancing micro vascular permeability, and inhibiting apoptosis.
- ✓ 14 vs. 12 NP: -1.4 fold, $P < 0.05$
- ✓ 14 P vs. NP: 1.9 fold, $P < 0.001$
- ✓ Angiogenesis pathway

RTPCR Corpora Lutea

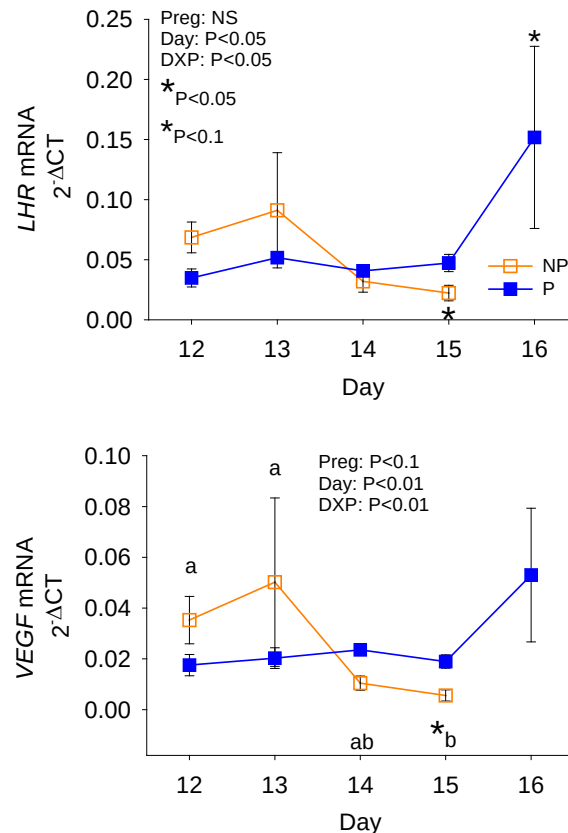


FIGURE 2.4B.

Genes stabilized during pregnancy and down-regulated in the CL during luteolysis. Selected genes out of 734 differentially expressed in CL on Day 14 of pregnancy (P) compared to the estrous cycle (NP) included *IL-6* and *PTX3* (A); and *LHR* and *VEGF* (B). General function, fold changes in mRNA concentrations based on microarray analysis, and implicated pathways are described in left panels. Factorial analysis of RTPCR for these mRNAs over days of the estrous cycle (NP) and pregnancy (P) are shown in right panels. Means represented with different letters differ across days of the estrous cycle ($P < 0.05$). * - Represents differences ($P < 0.05$) across pregnancy status on the same day. * - Represents tendency ($P < 0.10$) in differences across pregnancy status on the same day. Values represent mean $\pm$ standard error.

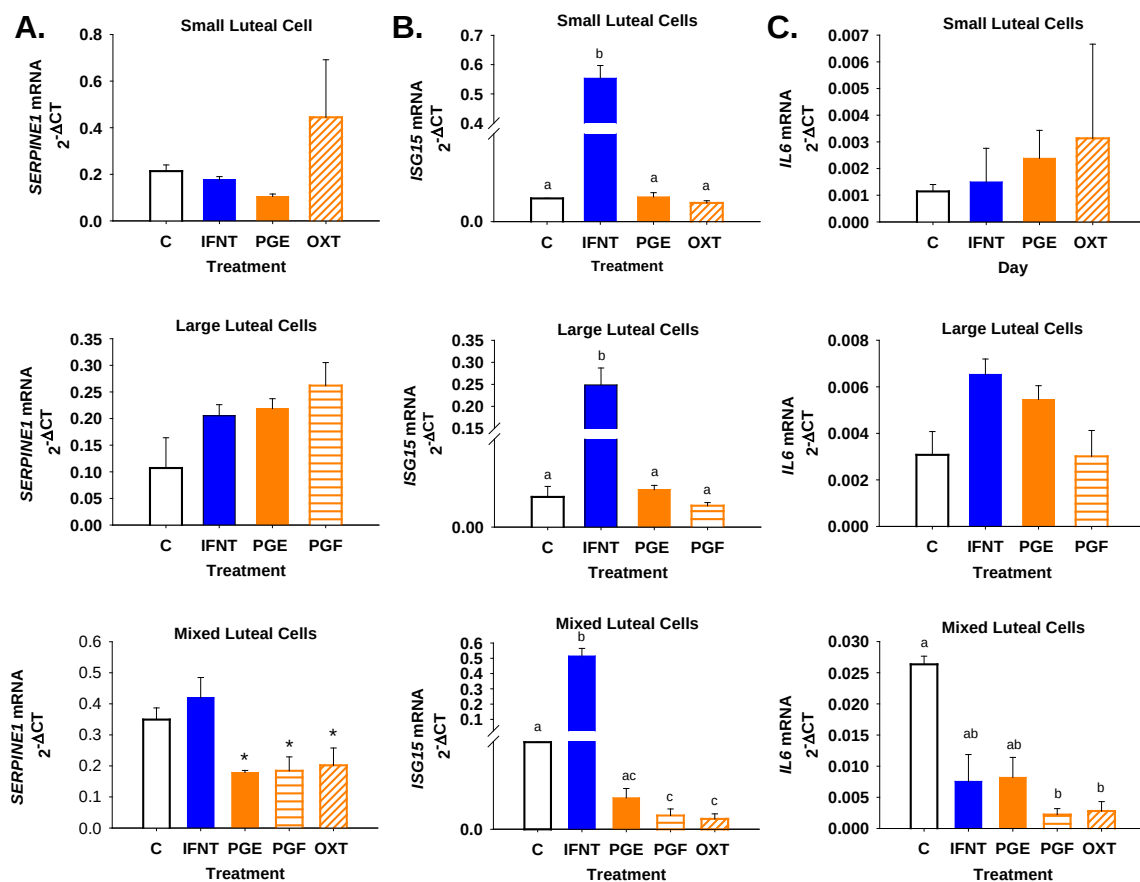


FIGURE 2.5.

SERPINE1 (A), *ISG15* (B) and *IL-6* (C) mRNA concentration in cultured small, large or mixed luteal cells. Luteal cells were cultured for 24 h with either 0, or 1 ng/ml IFNT, 3.5 ng/ml PGE2 (PGE), 3.5 ng/ml PGF or 10 μ M OXT. Means marked with different superscript letters differ ($P < 0.05$). * - Represents a tendency ($P < 0.10$). Values represent mean $\pm$ standard error.

Culture of luteal cells with PGE2 had no impact on ISG15 mRNA concentrations. Likewise, culture of SLC and LLC with PGF and OXT had no effect on ISG15 mRNA concentrations. In contrast, culture of MLC luteal cells with PGF and OXT caused down-regulation of ISG15 mRNA concentrations when compared to control cultures. IL-6 mRNA concentrations did not change in cultured isolated SLC and LLC (Fig. 2.5C). In MLC, PGF and OXT caused down-regulation of IL-6, whereas culture with IFNT and PGF had no effect. Interestingly, this same general trend in down-regulation by culture with PGF and OXT in MLC with no effect in isolated SLC and LLC was the same for PTX3 (Fig. 2.6A) and VEGF (Fig. 2.6C) mRNA concentrations. A tendency ($P<0.10$) for down-regulation of LHR mRNA concentrations following culture of SLC with OXT, and LLC with PGF was supported by significant ($P<0.05$) down-regulation of LHR mRNA concentrations following culture of MLC with PGF and OXT (Fig. 2.6B). Interestingly, culture of only LLC with IFNT caused an up-regulation of LHR and a tendency ($P<0.08$) for up-regulation of VEGF mRNA concentrations, whereas there was no effect of IFNT in cultured SLC or MLC.

DISCUSSION

Establishment of early pregnancy in sheep is mediated through conceptus-derived IFNT and paracrine inhibition of up-regulation of ESR1 and OXTR that occurs in the endometrium during the estrous cycle (Spencer, Becker et al. 1995). In addition to this well described paracrine action, an endocrine role for IFNT has been suggested based on detection of antiviral activity in uterine vein blood (Oliveira, Henkes et al. 2008). Confirmation that IFNT is present in uterine vein blood was based on inhibition of antiviral activity following pre-adsorption with antibody against IFNT

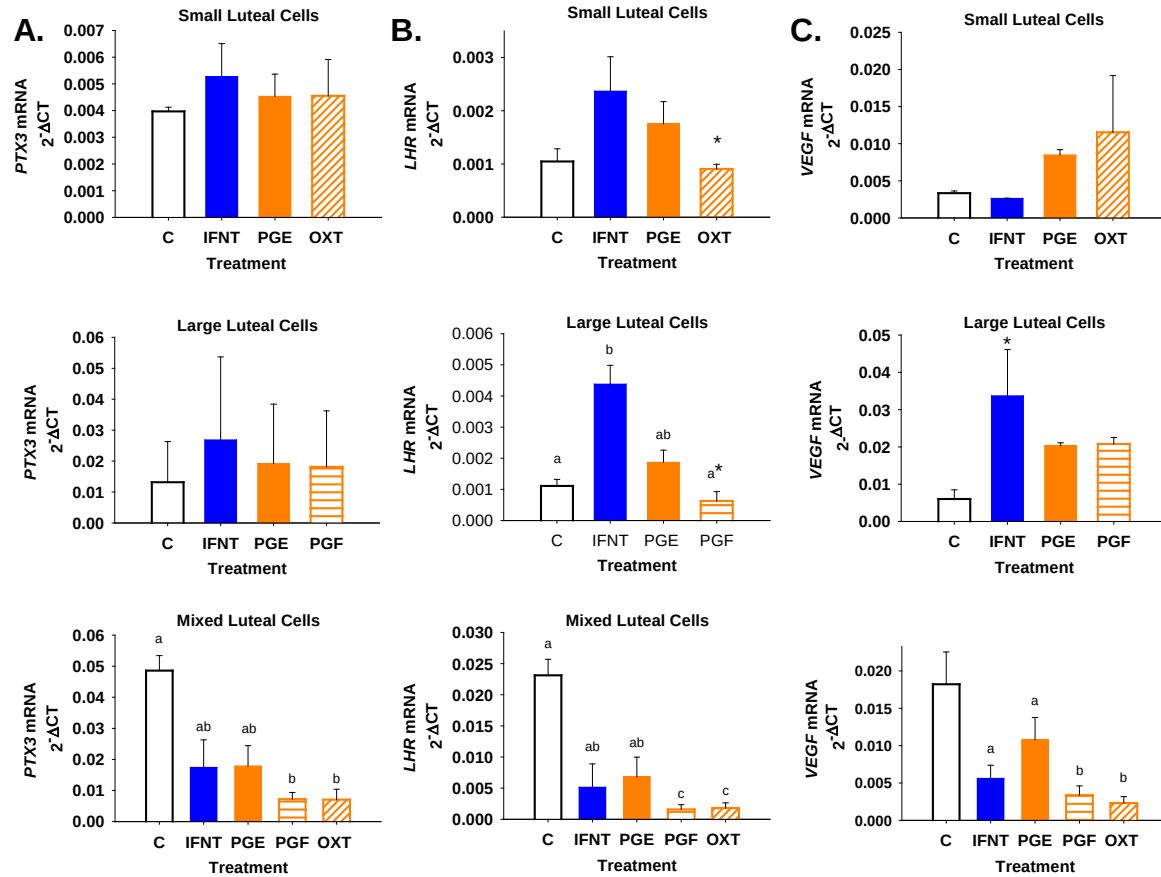


FIGURE 2.6. *PTX3* (A), *LHR* (B) and *VEGF* (C) mRNA concentration in cultured small, large or mixed luteal cells. Luteal cells were cultured for 24 h with either 0, 1 ng/ml IFNT, 3.5ng/ml PGE₂ (PGE), 3.5 ng/ml PGF or 10 μ M OXT. Means marked with different superscript letters differ ($P < 0.05$). *- Represents a tendency ($P < 0.10$). Values represent mean $\pm$ standard error.

(Bott, Ashley et al. 2010) and detection of IFNT by using radioimmunoassay (Antoniazzi, Webb et al. 2013) and mass spectroscopy approaches (Romero J.J. and Hansen T.R., unpublished results). Indirect evidence to support endocrine action of IFNT provided herein and reported previously (Oliveira, Henkes et al. 2008; Bott, Ashley et al. 2010) is based on up-regulation of ISGs in the CL in response to pregnancy and IFNT. Likewise, systemic infusion of rolFNT for 24 h protects the CL against a subluteolytic challenge with PGF (Antoniazzi, Webb et al. 2013) when administered on Day 10 of the estrous cycle. However, to our knowledge, IFNT has never been detected in systemic blood during pregnancy in ruminants. The present experiments studied systemic impact of early pregnancy in sheep on the CL and further examined the hypothesis that pregnancy induces genes, including ISGs that contribute to survival of the CL and resistance of the CL to luteolysis. Differential CL gene expression in response to early stages of luteolysis also was examined.

Validation of Animal Model

Serum progesterone concentrations were the same regardless of pregnancy status on Days 12 and 13. For this reason, collection and analysis of CL on these Days provides an excellent reference point in context of representing a functional CL that is producing progesterone. By Day 14 of the estrous cycle, serum progesterone concentrations were declining and by Day 15 of the estrous cycle serum progesterone had reached concentrations that were significantly lower and representative of luteolysis when compared to Days 12 and 13 of the estrous cycle and Days 14-15 of pregnancy. Rather than focusing on Day 15, which represented endpoint responses of the CL to luteolysis, CL were collected on Day 14 of the estrous cycle and pregnancy to focus on

early stages of luteolysis and maternal recognition of pregnancy. However, by also implementing RTPCR, a larger sample (ewe) size and Days 12, 13, 14 and 15 of the estrous cycle and pregnancy, a more temporal representation of gene expression in the CL was possible.

Early Mediators of Luteolysis

One of the proteins that is believed to be involved with the extensive extracellular matrix remodeling of the CL during its formation and regression is SERPINE1 (PAI-1) (Smith, Gentry et al. 1997). *SERPINE1* mRNA concentrations have not been shown to change in CL during the ovine estrous cycle on Days 3, 7, 10, 13 and 16 (Smith, Gentry et al. 1997). However, in the present studies, *SERPINE1* mRNA concentrations increased transiently from Days 13 to 14 and then declined by Day 15. This transient increase in SERPINE1 also was described by others in the ovine CL within 6 h following in vivo treatment with PGF (Smith, Gentry et al. 1997). Exactly why *SERPINE1* mRNA concentrations increase transiently during the late estrous cycle and in response to PGF in vivo is unknown, but might be related to preparing the extracellular matrix of the CL for luteolysis. A balance of remodeling of extracellular matrix may be achieved through this transient increase in this inhibitor of plasminogen activator. For example, in the bovine CL, SERPINE1 and other plasminogen activators such as uPA, uPAR and tPA have been shown to be up-regulated in response to PGF-mediated luteolysis (Kliem, Welter et al. 2007). Through regulation of ECM, SERPINE and PA may regulate invasion of immune cells as well as inhibition of the synthesis of progesterone. The transient increase in *SERPINE1* mRNA concentrations during the estrous cycle was not observed during pregnancy.

When examined in cultured luteal cells, *SERPINE1* mRNA concentrations did not change in isolated SLC and LLC. However, in MLC there was a tendency for down-regulation of *SERPINE1* mRNA concentrations following 24 h culture with PGE₂, PGF and OXT. Smith and co-workers (Smith, Gentry et al. 1997) described a transient increase in *SERPINE1* mRNA concentrations within 6 h following in vivo treatment with PGF. Whether this early rapid increase in *SERPINE* mRNA concentrations also occurs following treatment of MLC with PGF in vitro remains to be determined. Also, the reason for tendency in *SERPINE1* mRNA concentrations to be suppressed following treatments in vitro, while concentrations are transiently increased on Day 14 of the estrous cycle, remains to be determined.

This transient increase in CL *SERPINE1* mRNA concentrations on Day 14 of the estrous cycle was very similar to the profile of *THBS1* mRNA concentrations in the CL during the estrous cycle. *THBS1* increased as early luteolysis progressed in CL from Day 12 to 14 of the estrous cycle. THBS1 is a secreted extracellular matrix glycoprotein that has been shown to be involved in platelet activation, cell adhesion, cell to cell and cell to matrix communication, promotion and inhibition of angiogenesis and tissue healing (Lawler 2000; Esemuede, Lee et al. 2004; McLaughlin, Mazzoni et al. 2005). THBS1 induces TGFB (Ren, Yee et al. 2006), which also was up-regulated 1.7 fold ($P < 0.05$) in the CL during the estrous cycle (see supplemental microarray files). Previous studies in the cow demonstrate that PGF induces luteal expression of TGFB (Hou, Arvisais et al. 2008; Mondal, Schilling et al. 2011), which may contribute to functional and structural regression of the CL (Maroni and Davis 2011). TGFB induces

SERPINE1 gene expression (Zhu, Yin et al. 2012). Thus, THBS1 may cause up-regulation of SERPINE1 through the TGF β pathway.

The anti-angiogenic properties of THBS1 have been shown in several studies (Lawler 2000; Mirochnik, Kwiatek et al. 2008). Zalman (Zalman, Klipper et al. 2012) demonstrated that endothelial and steroidogenic cells of the CL express abundant concentrations of *THBS1* mRNA. THBS1 has been shown to bind as well as sequester pro-angiogenic factors such as VEGF, which was stabilized in the present studies in CL during pregnancy and might be associated with luteal resistance (Gupta, Gupta et al. 1999; Margosio, Marchetti et al. 2003). THBS1 promotes the internalization of VEGF by low density lipoprotein receptor-related protein-1 in non-endothelial cells and partially suppresses VEGF expression in those cells (Greenaway, Lawler et al. 2007). The effects of THBS1 on endothelial cells result in cell cycle arrest, repressed motility, chemotaxis and increased apoptosis (Guo, Kruttsch et al. 1997; Jimenez, Volpert et al. 2000; Armstrong, Bjorkblom et al. 2002; Ren, Yee et al. 2006). In the bovine CL, Zalman also demonstrated that PGF induced *THBS1* in MLC (Zalman, Klipper et al. 2012) and suggested that it may be involved in luteolysis. THBS1 has been shown to cause apoptosis in endothelial cells; thus it may act in a similar manner in the CL.

THBS1 may cause apoptosis by activation of the CD36-p59^{fyn}-caspase 3-p38MAPK cascade in endothelial cells. Antibodies against THBS1 can block apoptotic activation through neutralizing THBS1 or preventing access of THBS1 to CD36 (Jimenez, Volpert et al. 2000). THBS1 action can also be blocked by compounds that inhibit p38MAPK or caspase3-like proteases (Jimenez, Volpert et al. 2000). THBS1 up-regulates the cytokines FasL and TNF (Rege, Stewart et al. 2009) and Bax (Nor, Mitra

et al. 2000), which are known mediators of apoptosis. In this regard, TNF induces apoptosis of bovine endothelial cells (Pru, Lynch et al. 2003; Henkes, Sullivan et al. 2008). Because *THBS1* is up-regulated in CL on Day 14 of the estrous cycle, is induced by PGF, has anti-angiogenic and pro-apoptotic properties, it may have a critical role in the demise of the CL.

Up-regulation of Pregnancy-Associated Genes in the CL: Interferon Stimulated Genes

Pregnancy induces several ISGs in the endometrium and CL (Hansen, Henkes et al. 2010). Microarray analysis demonstrated up-regulation of several of these ISGs in the CL such as: *ISG15* (Fig. 2.3), *MX1* (Fig. 2.3), *MX2*, *IRF6*, *IRF9*, *CCL2*, *CCL8*, *IFI44* and *OAS* in response to pregnancy. *ISG15* has been shown to be induced by IFNT in several reproductive tissues, as well as peripheral blood cells (Austin, Ward et al. 1996; Johnson, Spencer et al. 1999; Spencer, Stagg et al. 1999; Yankey, Hicks et al. 2001; Austin, Carr et al. 2004; Han, Austin et al. 2006; Gifford, Racicot et al. 2007) and in luteal cells cultured with roIFNT (Fig. 2.5). While the exact functions of *ISG15* in the CL are not known, *ISG15* is able to conjugate to and regulate proteins through an enzymatic pathway similar to that described for ubiquitin utilizing the ubiquitin-activating enzyme 1-like protein (Rempel, Francis et al. 2005). This IFNT-induced regulation of intracellular proteins by *ISG15* may help provide resistance of the CL to lysis by PGF and is the focus of future experiments.

Culture of SLC, LLC and MLC with roIFNT caused an up-regulation of *ISG15* mRNA concentrations, which is consistent with earlier reports (Oliveira, Henkes et al. 2008; Bott, Ashley et al. 2010; Hansen, Henkes et al. 2010; Antoniazzi, Webb et al. 2013). The addition of PGE2 had no impact on *ISG15* mRNA concentrations in cultured

luteal cells. This is interpreted to suggest that luteotrophic action of PGE₂ is not mediated through the ISGs. The suppression of *ISG15* mRNA concentrations in MLC after culture with PGF or OXT might reflect damaging effects of these lytic hormones.

MX1 gene expression is induced in the endometrium by pregnancy, progesterone and IFNs (α , β and τ) (Gray, Abbey et al. 2006; Pletneva, Haller et al. 2006). *MX1* is up-regulated in the ovine glandular epithelium in response to IFNT released from the conceptus during early pregnancy in ruminants and following in vitro culture of endometrial cells with IFNT (Charleston and Stewart 1993; Toyokawa, Carling et al. 2007; Toyokawa, Leite et al. 2007). *MX1* protein concentrations have also been shown to increase in uterine flushings from pregnant ewes after Day 15 (Toyokawa, Carling et al. 2007; Toyokawa, Leite et al. 2007).

In the present experiments, *MX1* mRNA concentrations were up-regulated in the CL in response to pregnancy as early as Day 12 and remained up-regulated through Day 14 of pregnancy based on the microarray analysis. RTPCR demonstrated that *MX1* mRNA concentrations were significantly greater in the CL on Days 15 and 16 in P ewes. Thus, *MX1* mRNA concentrations remained elevated in the CL up to and possibly beyond Day 16 of pregnancy. *MX1* is a GTPase that mediates antiviral responses (Horisberger, Staeheli et al. 1983) through a functional GTP binding motif (Flohr, Schneider-Schaulies et al. 1999). *MX1* may also facilitate “non-traditional” secretion of proteins (Nickel 2003; Toyokawa, Carling et al. 2007) that are distinct from known classical secretion mechanisms via the endoplasmic reticulum and Golgi (Palade 1975). The function of *MX1* in the CL during early pregnancy is unknown but might entail

mediating acute immune responses and intracellular GTP-driven mechanisms such as nontraditional release of proteins.

Pregnancy Stabilizes Genes that are Down-Regulated in the CL during the Estrous Cycle

IL-6, *PTX3*, *LHR* and *VEGF* mRNA concentrations were stabilized in the CL over Days 12, 13, 14, 15 and 16 of pregnancy, with a tendency for an increase in *LHR* mRNA concentrations on Day 16 of pregnancy. All of these genes were down-regulated by Day 14 (*IL-6* and *PTX3*) or 15 (*LHR* and *VEGF*) of the estrous cycle, which corresponds to the decline in serum progesterone concentrations and luteolysis. In cultured MLC, PGF and OXT caused down-regulation of each of these genes when compared to controls. This is consistent with in vivo data showing down-regulation of these genes in the CL as the late estrous cycle progressed. Culture of SLC and MLC with IFNT had no effect on mRNA concentrations for these genes. Interestingly, the MLC model tended to provide greater responses to treatments applied in vitro. This might be explained through interactions in MLC cultures between SLC and LLC, but also with cells in the CL such as endothelial and immune cells. However, in LLC, IFNT up-regulated *LHR* and tended to up-regulate *VEGF* mRNA concentrations. These data are interpreted to mean that pregnancy (i.e., IFNT) stabilizes expression of these genes, which would otherwise become down-regulated in response to luteolysis during the estrous cycle. Stabilization of these genes during early pregnancy may contribute to resistance of the CL to luteolysis.

IL-6, also known as IFN beta 2, is pro- and anti- inflammatory, pyrogenic and activates B- and T-cells (McWaters, Hurst et al. 2000). Over-expression of *IL-6* is

associated with several diseases such as rheumatoid arthritis and postmenopausal osteoporosis (Stein and Yang 1995; Muller-Newen, Kuster et al. 1998). IL-6 has been described in Day 15 cultured luteal cells; however treatment of these cells with progesterone silenced expression of IL-6 (Telleria, Ou et al. 1998) and IL-6 gene expression appears to be silenced in the CL during pregnancy. Pregnancy-associated signals, such as IFNT, may help stabilize IL-6 expression in the ovine CL, if progesterone does indeed have inhibitory action. In MLC described herein, culture with PGF and OXT reduced *IL-6* mRNA concentrations. Stabilization of basal levels of IL-6 might be necessary in the CL during early pregnancy. One benefit for continued action of IL-6 in the CL is the indirect induction of angiogenesis through inducing VEGF expression (Cohen, Nahari et al. 1996). VEGF may have a functional role in luteal resistance; therefore IL-6 expression could be functioning in luteal resistance and maintenance of the CL by inducing VEGF (see later discussion). It also could be acting synergistically with IFNT to modulate immune responses in the CL during early pregnancy.

Pentraxins are a superfamily of multifunctional proteins that are highly conserved from arthropods to mammals and expressed by several cell types (Deban, Jaillon et al. 2010; Braunschweig and Jozsi 2011). PTX3 has been shown to be present in follicular fluid and plasma (Garlanda, Maina et al. 2008). PTX3 expression is up-regulated in human stromal cells by progesterone and by trophoblast conditioned medium or trophoblast explants (Garlanda, Maina et al. 2008). It also is up-regulated in follicular theca and granulosa cells in response to LH (Christenson, Gunewardena et al. 2013). PTX3 expression has been shown to provide resistance to neurodegeneration possibly

rescuing neurons from irreversible damage (Ravizza, Moneta et al. 2001; Deban, Jaillon et al. 2010). PTX3 double knockout mice have increased myocardial damage suggesting that PTX3 plays a cardio-protective role (Salio, Chimenti et al. 2008). These two findings indicate that PTX3 may play a protective role in cells undergoing stress and may contribute to the maintenance of pregnancy by protecting the CL. Stabilization of PTX3 in the CL during pregnancy may be relevant in context of cell survival responses designed for protection against apoptosis and autoimmune responses such as those mounted against cell remnants from antigen-presenting cells (Rovere, Peri et al. 2000). The idea that PTX3 is stabilized in the CL of pregnancy is further supported by the fact that *PTX3* is down-regulated when MLC are cultured with PGF and OXT for 24 h. These data are different than those described by Zalman et al. (Zalman, Klipper et al. 2012) where PTX3 mRNA concentrations were shown to increase following 4 h culture of MLC with 100 ng/ml PGF. Two primary differences in the design of these experiments were a longer culture period (24 h) and use of lower concentrations of PGF (3.5 ng/ml) in the present in vitro experiments. The enclosed experiments also examine CL at several stages of the late estrous cycle.

LH is required for normal CL function and formation and for the maintenance of the mature CL (Kaltenbach, Graber et al. 1968). Infusion of LH has been shown to prolong luteal life (Karsch, Roche et al. 1971). Ovine luteal weight, luteal concentration of progesterone, total number of LH receptors and the number of receptors occupied by LH do not change 7.5 h after injection of PGF (Diekman, O'Callaghan et al. 1978). However, all of these parameters were impacted negatively by 22 h following injection of PGF, which is consistent with decline in LHR mRNA concentrations by Day 15 of the

estrous cycle in the present studies. The number of occupied, unoccupied and affinity of LHR also do not change on Days 12, 16 and 20 of pregnancy. This was later confirmed by Stormshak and co-workers (Zelinski, Selivonchick et al. 1988) when comparing Days 13 to 16 of the estrous cycle to pregnancy (Diekman, O'Callaghan et al. 1978). However, Smith and co-workers demonstrated that *LHR* mRNA concentrations were greater during the mid-luteal phase on Days 10-13 compared to earlier or later Days of the estrous cycle in sheep CL (Smith, Gentry et al. 1996). A decrease in *LHR* within 6 hours after injection of PGF also was described. This concurs with the decline in *LHR* mRNA concentrations in CL during the late estrous cycle when analyzed herein using microarray and RTPCR approaches. It also is consistent with the down-regulation of LHR mRNA concentrations when MLC were cultured herein for 24 h with PGF and OXT.

The primary difference in the present studies compared to those previously reported for LHR in ovine CL during pregnancy is the tendency for up-regulation of LHR mRNA concentrations on Day 16 of pregnancy and the apparent increase in LHR mRNA concentrations in response to culture of MLC with roIFNT. An explanation for this discrepancy might be provided by slight differences in actual Day of pregnancy and degree of development of the conceptus across these studies. For example, in the present experiments, Day 16 of pregnancy might represent a slightly more advanced conceptus and secretory protein (i.e., IFNT) producing capacity.

IFNT has been implicated in lymphangiogenesis in bovine CL through induction of VEGF (Nitta, Shirasuna et al. 2011). VEGF is a multifunctional cytokine that is necessary for follicular growth, enhancing micro-vascular permeability, angiogenesis,

ovulation and development and function of the CL (Otani, Minami et al. 1999; Berisha, Schams et al. 2000) VEGF protein concentrations steadily decrease from early to late stages of luteolysis (Otani, Minami et al. 1999; Berisha, Schams et al. 2000). This is probably caused through direct action of PGF because Day 11 luteal cells cultured with PGF have decreased *VEGF* mRNA concentrations when compared to control, untreated cells (Zalman, Klipper et al. 2012). This also is consistent with enclosed down-regulation of *VEGF* mRNA concentrations following culture of MLC with luteolytic PGF and OXT. In microarray and RTPCR results described herein, *VEGF* mRNA concentrations decreased on Day 14 and 15 during the estrous cycle suggesting that PGF causes a decrease in *VEGF* mRNA concentrations. *VEGF* gene expression is tightly associated with other genes that were identified in our microarray such as *IL-6* and *THBS1*. *IL-6* mRNA concentrations were associated with pregnancy and correlated with up-regulation of *VEGF* in the CL. *THBS1* was associated with luteolysis and with a down-regulation in *VEGF*. VEGF mRNA concentrations in the ovine CL appear to be associated with pregnancy status, are stabilized in the CL during early pregnancy and tend to be induced, at least in LLC, following culture with IFNT.

CONCLUSIONS

In the absence of a conceptus, spontaneous regression of the CL occurs as a consequence of differential expression of at least 683 genes that include cell adhesion, chemokines, cytoskeletal remodeling and apoptotic pathways. Two of these genes, *SERPINE1* and *THBS1*, were selected for further study and found to be transiently up-regulated during the latter part of the estrous cycle and during early luteolysis. These genes may play a role in regulation of the extracellular matrix (Khan and Falcone 1997;

Huang, Border et al. 2006) to facilitate invasion of immune cells and inhibition of the synthesis of progesterone. Future experiments may focus on the TGFB pathway, which had 19 genes differentially expressed during luteolysis and may provide a link between SERPINE1 and THBS1 action in the early regressing CL.

Until recently, early pregnancy was described to be maintained through exclusive paracrine action of the conceptus on the endometrium in ruminants. Based on enclosed microarray data describing differential expression of 55 genes in CL between P and NP ewes on Day 12, 21 genes between Days 12 and 14 of pregnancy, and 734 genes between P and NP ewes on Day 14, it is concluded that pregnancy-associated gene expression occurs in the CL. Several conceptus secretory products might be driving this differential gene expression in the CL, but a primary candidate based on induction of ISGs by pregnancy and through culture of isolated luteal cells is IFNT. This conclusion is supported by up-regulation of several ISGs in the CL based on microarray data, two of which, ISG15 and MX1 were more extensively examined in the CL by using RTPCR approaches. Neither of these ISGs was impacted by culture of SLC, LLC or MLC with PGE2, which is interpreted to suggest that luteotrophic action of PGE2 is not mediated via ISGs.

The maintenance of a healthy conceptus is a complicated process that involves cell proliferation, differentiation and continued activation of the steroidogenic pathway for continued production of progesterone (IL-6, VEGFA and LHR) as well as regulation of immune responses and activation of interferon signaling (PTX3, ISG15 and MX1). Pregnancy stabilizes ISG15, MX1, IL-6, PTX3, LHR and VEGFA, whereas luteolysis causes down-regulation of these genes. Conceptus- derived IFNT maintains/induces

IL-6, *VEGFA*, *LHR*, *ISG15*, *MX1* and *PTX3* in vivo. In vitro, PGF and OXT suppressed all of these genes in MLC. In addition to providing protection or resistance of the CL to lytic action of PGF, the endocrine actions of IFNT may prime the maternal innate immune system for more rapid and robust antiviral responses to protect the embryo and early developing fetus from disease or infection

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CHAPTER 3

TEMPORAL RELEASE, PARACRINE AND ENDOCRINE ACTION OF CONCEPTUS-DERIVED INTERFERON-TAU DURING EARLY PREGNANCY IN EWES

CHAPTER SUMMARY

Sheep have increased antiviral activity in uterine vein serum (UVS) during early pregnancy. Antiviral activity is blocked in Day 15 UVS of pregnancy when preadsorbed with anti-interferon-tau (IFNT) antibodies. For these reasons, it was hypothesized that IFNT induces gene expression in the endometrium and enters peripheral circulation inducing genes in extrauterine tissues such as the corpus luteum (CL) and liver. Blood was collected from ewes on Days 12-15 of the estrous cycle (non-pregnant, NP) or pregnancy and on Day 16 of pregnancy. Serum progesterone concentrations remained >1.7 ng/ml in pregnant (P) ewes and in NP ewes on Days 12-13 of the estrous cycle, but declined to concentrations <0.6 ng/ml by Day 15 of the estrous cycle. A highly specific (no cross-reaction with IFN α , IFN β or IFN γ) and sensitive (71.25pg/ml in uterine flushings; 58.7pg/ml in serum) IFNT radioimmunoassay (RIA) was validated herein and used to demonstrate that IFNT was not detected in NP ewes but could be detected from Days 13-16 of pregnancy in uterine flushings and detected in UVS on Days 15-16 . IFNT detection in uterine flushing correlated with paracrine induction of ISGs in endometrium and preceded blocking up-regulation of endometrial ESR1 and OXTR by Day 14. The induction of ISG mRNAs in jugular vein white blood cells, liver and CL occurred by Day 14, prior to detection of IFNT in UVS on Day 15 of pregnancy. To confirm activation of IFNT signal transduction in CL, mRNA concentrations of IFN signal transducers and ISGs were determined to be greater in CL from Day 14 P compared to

NP ewes. It is concluded that paracrine action of IFNT coincides with IFNT detection in uterine flushings. Endocrine action of IFN ensues through induction of ISGs in peripheral blood. Lack of detection of IFNT in UVB at time of induction of ISGs might be related to sensitivity of the IFNT RIA in this study.

INTRODUCTION

Maternal recognition of pregnancy in ruminants requires elongation of the conceptus coinciding with production of interferon-tau (IFNT) (Godkin, Bazer et al. 1984; Hansen, Austin et al. 1999; Bazer, Wu et al. 2010). The ovine conceptus secretes IFNT from Days 10 to 21-25 with greatest release occurring on Days 14 to 16 of pregnancy (reviewed in (Roberts, Ealy et al. 1999)), although the precise pattern of secretion of IFNT and activation of interferon stimulated genes (ISGs) has not been described. IFNT is a major product of ovine and bovine conceptuses before attachment that functions to prevent the return to estrous cycles (Roberts, Chen et al. 2008). IFNT acts in a paracrine manner to prevent up-regulation of ESR1 and OXTR in the endometrial luminal epithelium and superficial glandular epithelium, thereby preventing the release of PGF (Spencer and Bazer 1996). In addition, IFNT has recently been reported to function through endocrine action in the ovine CL (Oliveira, Henkes et al. 2008).

IFNT binds type 1 receptors (IFNR1 and IFNR2) and activates the Janus kinase-signal transducer and activator of transcription (JAK/STAT) pathway (Binelli, Subramaniam et al. 2001). The JAK/STAT pathway includes downstream mediators such as the STATS (1 and 2), IRFs and IFN-stimulated genes (ISGs). A hypothesized mechanism of how IFNT mediates maternal recognition of pregnancy, is through the increased expression of several (ISGs) in the uterus (Mirando, Short et al. 1991; Rueda,

Naivar et al. 1993; Naivar, Ward et al. 1995), such as ISG15 (Austin, Ward et al. 1996), *IFIH5* (Song, Bazer et al. 2007) and *DDX58* (Song, Fleming et al. 2011) . Additionally, pregnancy induces expression of ISGs in several ovine extra uterine tissues, such as the CL (Oliveira, Henkes et al. 2008; Bott, Ashley et al. 2010). ISG15, first termed ubiquitin cross-reactive protein because of its cross-reactivity with antibody against ubiquitin (Haas, Ahrens et al. 1987; Austin, Ward et al. 1996) is increased in mouse (Austin, Bany et al. 2003) and human (Bebington, Doherty et al. 1999) endometrium in response to pregnancy. ISG15 is induced by type I IFN and becomes conjugated to intracellular proteins (Loeb and Haas 1992) in a mechanism parallel, but different to that described for ubiquitin (Narasimhan, Potter et al. 1996).

In a recent study, we evaluated expression of ISGs in extra uterine tissues during early pregnancy in sheep (Oliveira, Henkes et al. 2008). Expression of ISG15 was greater in LLC from Day 15 pregnant sheep compared to LLC from Day 15 non-pregnant (NP) cyclic sheep using semi-quantitative Real Time PCR, western blot analysis and immunohistochemistry approaches. Also, an antiviral assay was used to evaluate levels of IFNs in serum from the uterine vein, uterine artery and jugular vein. Higher levels of type I IFN bioactivity were found in the serum from the uterine vein in Day 15 pregnant compared to NP ewes (Oliveira, Henkes et al. 2008). Uterine vein serum samples from Day 15 of pregnancy, which had been pre-adsorbed with anti-IFNT antibody, had diminished antiviral activity. This was interpreted to mean that IFNT was the type I IFN released into the uterine vein on Day 15 of pregnancy (Bott, Ashley et al. 2010).

Based on our previous data describing antiviral activity in the uterine vein serum on Day 15 of pregnancy being blocked by pre-adsorption using anti-IFNT antibody (Bott, Ashley et al. 2010) and a higher expression of ISG15 in CL from a Day 15 pregnant (P) ewe, it is hypothesized herein that in ruminants endocrine IFNT signaling occurs during maternal recognition of pregnancy possibly as early as Day 14 of pregnancy in sheep. To test this hypothesis, a sensitive and specific radioimmunoassay was developed and used to examine IFNT levels in uterine flushings and uterine vein blood.

Because IFNT was detected in uterine flushing and uterine vein blood, these studies also examined temporal events following detection of IFNT in uterine flushings in context of regulation of endometrial, corpus luteum and liver gene expression. Some of the well-characterized endometrial responses to IFNT also were examined in the CL such as preventing up-regulation and transcription of *ESR1* and *OXTR* mRNA (Spencer, Ott et al. 1996) in the CL. Several studies have examined changes in mRNA for ISGs, *ESR1* and *OXTR* in ovine endometrium in response to early pregnancy (Spencer, Becker et al. 1995; Johnson, Spencer et al. 1999; Joyce, White et al. 2005), but these studies have not examined consecutive Days of pregnancy in context of uterine flushing and uterine vein concentrations of IFNT. None of these previously published experiments have focused on temporal relationships between paracrine effects of the conceptus on the endometrium, development of antiluteolytic responses and endocrine induction of genes in the CL on Days 12, 13, 14, and 15. This represents a period in which critical responses mediate maternal recognition of pregnancy in the ewe. The aims of this experiment were to develop a specific and sensitive radioimmunoassay for IFNT to detect IFNT in uterine flushings and uterine vein blood.

The temporal relationships of IFNT in blood and regulation of ISG15, ESR1 and OXTR in the endometrium, and IFN signaling in the CL were examined.

MATERIALS AND METHODS

Experimental Design: Day of Estrous Cycle and Early Pregnancy

The Colorado State University Animal Care and Use Committee approved all experimental procedures with animals. Mature crossbred ewes were observed daily for estrus using a caudoepididectomized ram. On the day of standing estrus (Day 0), half of the ewes were bred with an intact ram. NP ewes were not exposed to a ram. Groups were assigned according to pregnancy status (NP and P) and Days after detection of estrus (12, 13, 14 and 15). Three to six ewes per day and per pregnancy status were used (12NP=5; 12P=4, 13NP=5; 13P=5, 14NP=5; 14P=3, 15NP=6; 15P=4). On Days 12, 13, 14 and 15 of either the estrous cycle or pregnancy, ewes were euthanized and jugular and uterine vein blood, lymph nodes (iliac and submandibular), CL, endometrium, uterine vein (tissue) and liver were collected. Tissues were snap frozen in liquid nitrogen and kept at -80°C for later processing. Pregnancy was confirmed by the presence of a conceptus.

Progesterone Assay

Concentration of progesterone in serum was determined by radioimmunoassay as previously described (Niswender 1973). All samples were analyzed in one assay. The sensitivity was 6pg/ml and the intra-assay coefficient of variation was 4.85%.

IFNT Radioimmunoassay

Radioiodination of recombinant ovine (ro)IFNT with ¹²⁵I was completed using chloramine T procedure and purified using column chromatography (Sephadex G25;

GE Health Care). These methods have been generally described previously by Niswender et al., (Niswender, Reichert et al. 1969). Briefly, uterine flushing samples were diluted 1:50 in 0.1% PBS gel for analysis in the RIA. If the samples were undetectable in the first run, they were reanalyzed un-diluted. Anti-rolFNT antibody (1:60,000 dilution) from Dr. Fuller Bazer (Texas A&M University) was added to uterine flushing samples, vortexed and incubated at 4°C for 24 hours. Radioactive ¹²⁵I-labeled rolFNT was added (100 µl 50,000 counts), vortexed and incubated for 24 hours at 4°C. This was followed with an incubation at 4°C for 72 hours with secondary anti-rabbit gamma globulin (1: 25 dilution; generated at Colorado State University). The assay was terminated by addition of 3 ml of cold PBS and centrifugation at 2800 rpm for 30 minutes. The supernatant was removed and the radioactivity in the pellet was determined utilizing a gamma counter (Apex automatic gamma counter, ICN Micromedic Systems). This RIA was optimized for detection of rolFNT in uterine flushing samples at a sensitivity of 0.1 ng/ml and a range of detection of 0.1 to 13 ng/ml. The intra-assay coefficient of variation was 6.2% and the inter-assay coefficient of variation was 4.0%.

Total RNA Isolation and Semi Quantitative Real Time PCR (RTPCR)

Extraction of RNA from tissues (endometrium, uterine vein, lymph nodes, CL and liver) and blood were performed using TRIZOL and TRI BD reagent (MRC, Cincinnati, OH), respectively. After extraction, RNA was treated with DNase I (Qiagen, Valencia, CA) and total RNA was purified using RNeasy MinElute Cleanup Kit (QIAGEN, Valencia, CA). Single stranded cDNA was synthesized from 1 µg of total RNA using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Reactions were completed using a

final volume of 10 µl per well using iQ Supermix Bio-Rad with cDNA (2 µl) as template. Amplification was performed at 95°C for 30 sec, 62°C for 30 sec, and 72°C for 15 sec for 40 cycles. All targets were normalized to the geometric mean of the following housekeeping genes: *GAPDH*, *POLR2A*, *RPL19* and *RNA18s* for mRNA expression. The primers used in this study for the target genes are listed in Table 3.1. All primers were designed to have single product melting curves, and consistent amplification efficiencies. All amplicons were verified by sequencing.

Statistical Analysis

All statistical analyses were performed using SAS software package (SAS Institute Inc., Cary, NC, USA). Data are presented as mean ± SEM, unless otherwise stated. A probability value of $P \leq 0.05$ was considered significant. Main effects of treatment Day, pregnancy status and their interaction were analyzed. Continuous data were submitted to 2-way ANOVA using the General Linear Models (GLM). The geometric mean of *GAPDH*, *POLR2A*, *RPL19* and *RNA18s* was used to normalize each targeted mRNA amplification by using the ΔCT values (Livak and Schmittgen 2001; Schmittgen and Livak 2008). CT values were analyzed, and relative expression of RTPCR products were graphed using mean $2^{-\Delta CT}$ values calculated for each group.

RESULTS

Progesterone Profile of Mid-late Estrous Cycle and Early Pregnancy

Concentration of serum progesterone was not affected by pregnancy status on Days 12 and 13. Serum progesterone declined from Day 13 to 14 and by Day 15 reached levels less than 1 ng/ml in NP ewes; whereas in P ewes, concentrations of serum progesterone stabilized to > 2 ng/ml (Fig. 3.1).

TABLE 3.1 Oligonucleotide Primer sequences used for semi-quantitative RTPCR

Target	Accession	Primer sequences
IFNAR1	NM_001009748.1	F ctcagattggtccccagac R cttctgttccaggggagag
IFNAR2	NM_001009342.1	F tcccaggataccaagtgaagaa R atttgggggtgatgcctctt
ISG15	NM_174336	F ggtatccgagctgaagcagtt R acctccctgctgtcaaggt
ESR1	AY033393	F gttgcatggctgtagcagaa R caaattgaaggggcaggtg
OXTR	AF101724	F ggcagaatttacggctcaag Rgatgagcttgacgttgctga
SLCO2A1	DQ026455	F cccctctacatgatggtgct R ggtgaggccatagagagctg
PTGFR	OAU73798	F ctggtctgtgccactttt R aaaagcacaccccactcaac
PTGER2	AJ876410	F gtgcacactacttcgcttct R actcggcgctggtagaagta
PTGER3	U37148	F tgctggcgtaggtcagtat R agaagcgaagaaaacgttgc
PTGER4	AJ617580	F gtgaccatcgccacctactt R ctcacgcacagatgatgct
AKT	AJ617580	F gtgaccatcgccacctactt R ctcacgcacagatgatgct
XIAP	AJ617580	F gtgaccatcgccacctactt R ctcacgcacagatgatgct
CASPASE 3	AJ617580	F gtgaccatcgccacctactt R ctcacgcacagatgatgct
GAPDH	AV610889	F tgacccttcattgaccttc R cgttctctgccttgactgtg
POLR2A,	XM_004013289.1	F agtccaacatgctgaaggacatga R agccaagtgccggttaattgacgta
RPL19	XM_004012837.1	F tcgccggaagggcaggcata R ggctgtgatacatgtgggggtc
RNA18s	AY753190.1	F gaggccctgtaattagaatgag R gcagcaactttaataacgctattgg
STAT1	NM_001166203.1	F ccaccgaacttaccagaa R accaatttgcagctgatcc
STAT2	XM_004006580.1	F agaaccttgacagcccgtttt R caagtgtgtcgaacatcca
IRF3	NM_001029845.2	F ggcttgtgatggtcaaggtt R tgcaggctgacagtggtctc
IRF7	NM_001105040.1	F gcctcctggaaaaccaactt R ccttatgagggtcggtaggg
IRF9	XM_004010311.1	F ctgggtggtagagcaagtgg R gccttctgagttcccctct
IFIH1	XM_004004655.1	Fcttgcttctccatcgtttg R ctgcaaccagaagcttgctc
DDX58	XM_004005323.1	F cagcaagatcctggacccta R ttttctcggcctgaatacg

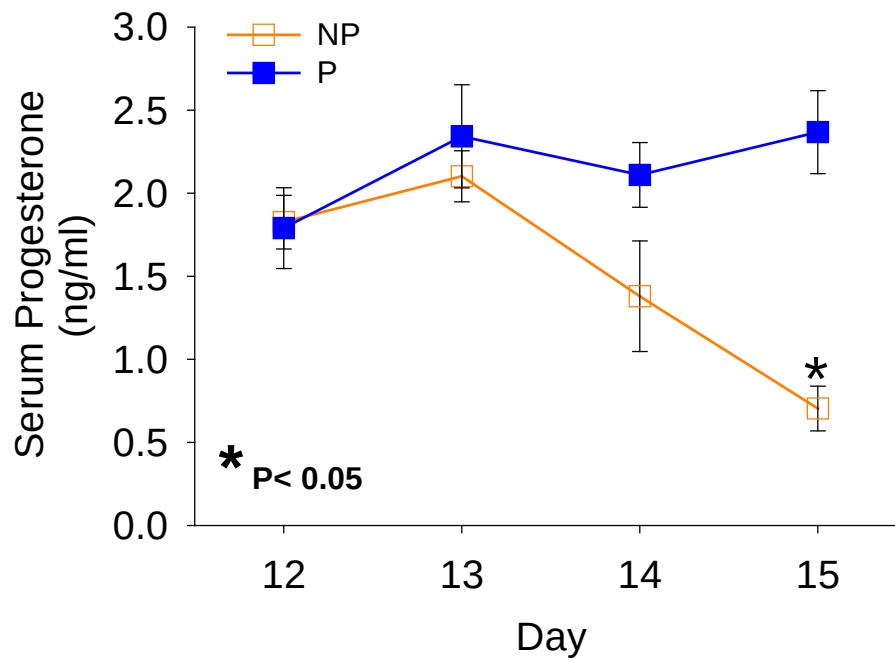


FIGURE 3.1.
Serum progesterone concentrations and intrauterine response to pregnancy.
Concentration of serum progesterone (A) in non-pregnant and pregnant ewes of estrous cycle or early pregnancy. * indicates means differ ($P < 0.05$).

RIA detection of IFNT in uterine flushing samples and uterine vein serum.

Specificity of the IFNT RIA was tested against other IFNs such as IFN α , β , and γ ; revealing no cross reactivity of the anti-IFNT antibody with similar (type I) or distinct (type II) IFNs (Fig. 3.2A). Sensitivity of the assay was optimized utilizing various primary antibody dilutions. The 1:60,000 dilution was identified as optimal (Fig. 3.2B). Uterine flushings were collected from NP ewes on Days 12, 13, 14 and 15. These samples had non-detectable concentrations of IFNT serving as a negative control for this study. Three standard dose response curves were run within each assay to serve as positive controls. IFNT was detectable as early as Day 13 of pregnancy in uterine flushings and by Day 15 in uterine vein blood, concentrations increased thereafter on each Day of pregnancy up to Day 16 (Fig 3.3). The sensitivity of these assays was from 11pg/ml to 23ng/ml. The limit of detection in uterine flushings was 0.077ng /ml and in uterine vein serum it was 0.06ng /ml. The intraassay coefficients of variation for the 3 assays ranged from 7.25% to 14.7% and the interssay coefficient of variation was 10.1%.

mRNA Profiles of ISG15, ESR1 and OXTR in the Endometrium

Endometrial *ISG15* mRNA concentrations were very low in endometrium from NP ewes, but increased in P versus NP ewes by Day 13 and remained greater in P ewes through Day 15 (Fig. 3.4A). Endometrial *ESR1* (Fig. 3.4B) and *OXTR* (Fig. 3.4C) mRNA concentrations were greater in NP compared to P ewes by Day 14. The *ESR1* mRNA concentrations increased dramatically from Days 13 to 14 and remained greater on Day 15 in NP compared to P ewes. *OXTR* mRNA concentrations were up-regulated in NP compared to P ewes on Day 14, but the increase noted for *ESR1* on Days 13

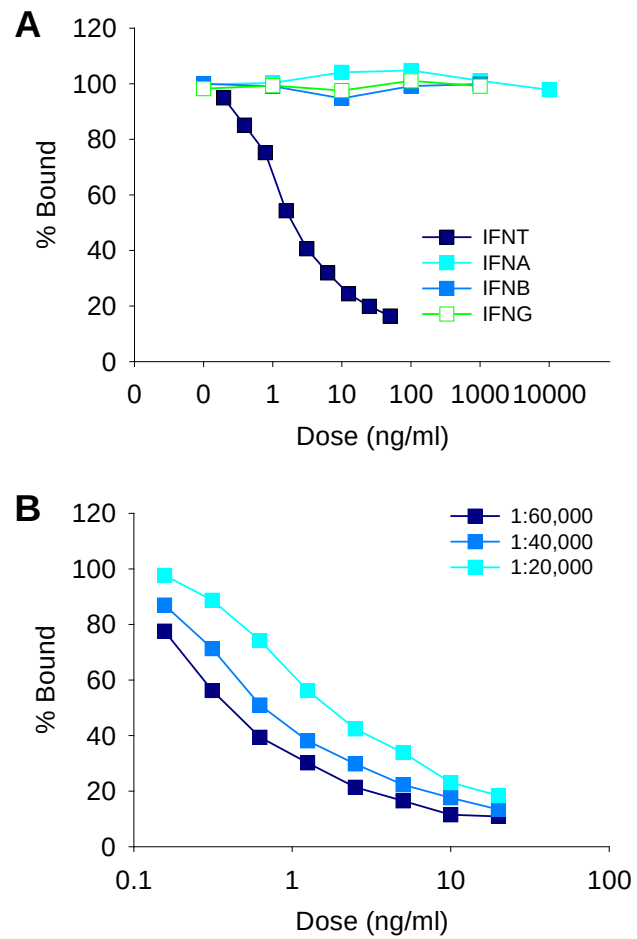


FIGURE 3.2.
 IFNT Radioimmunoassay. (A) Specificity: competitive radioimmunoassay testing IFNT against other type I (alpha and beta) and type II (gamma) interferons. (B) Sensitivity: radioimmunoassay testing different antibody concentrations. * indicates means differ ($P < 0.05$).

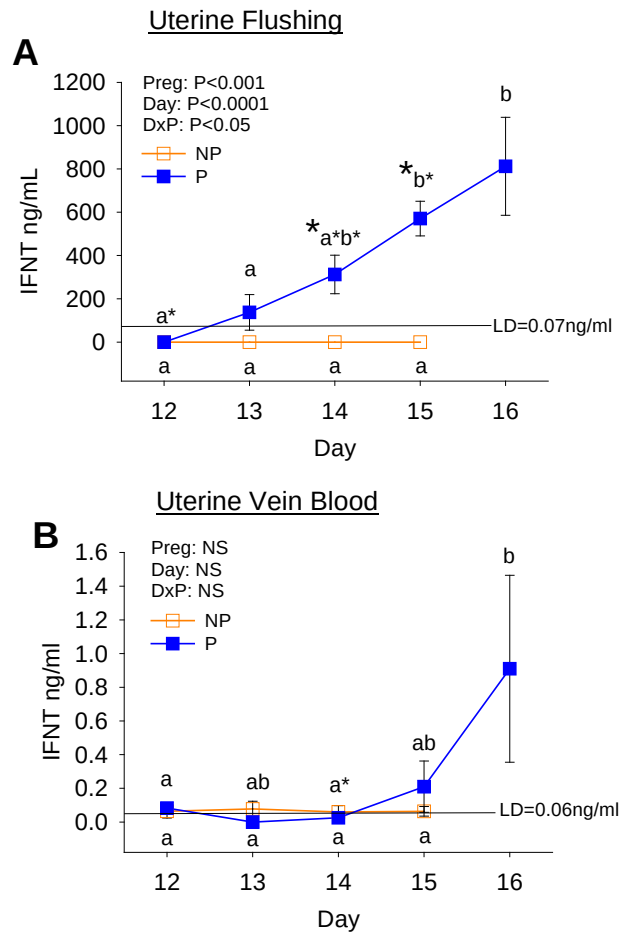


FIGURE 3.3.
Detection of IFNT in Uterine Flushing and Uterine Vein using RIA. (A) Detection of IFNT in uterine flushings from Day 12-15 non-pregnant and pregnant ewes. (B) Detection of IFNT in uterine vein serum from Days 12-15 non-pregnant and pregnant ewes. * indicates means differ ($P < 0.05$).

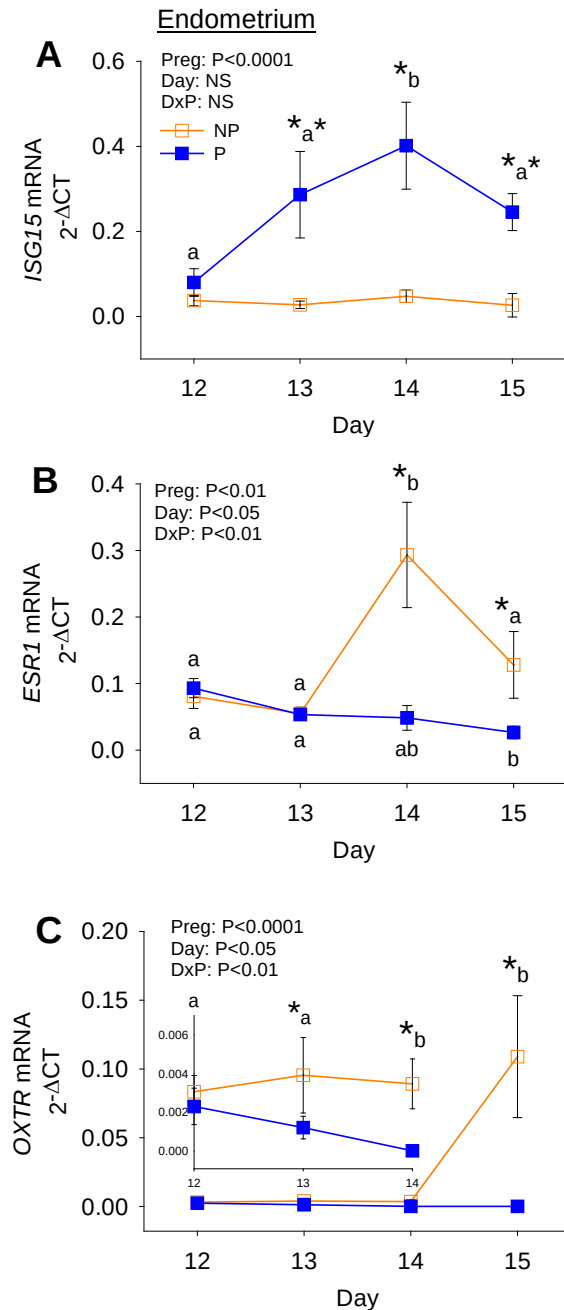


FIGURE 3.4.
ISG15, *ESR1* and *OXTR* Expression in the Endometrium during the Estrous and Early Pregnancy. Relative expression ($2^{-\Delta CT}$) of mRNA levels of *ISG15* (A), *ESR1* (B) and *OXTR* (C; amplified inlay of Days 12-14 for better visualization) in the endometrium, on Days 12, 13, 14 and 15 of the estrous cycle or early pregnancy in sheep relative to geometric mean of housekeeping genes. Data are presented as mean $\pm$ SEM. * indicates means differ ($P < 0.05$).

to 14 of the estrous cycle was delayed one Day for OXTR mRNA concentrations, which occurred on Days 14 to 15 of the estrous cycle. *OXTR* mRNA concentrations in NP ewes were greatest on Day 15 when compared to earlier stages of the estrous cycle and any stage of pregnancy.

Uterine and Jugular Vein Blood ISG15 mRNA Profile during Days of Estrous Cycle and Early Pregnancy

In both uterine and jugular vein PBMCs, *ISG15* mRNA levels were not affected by pregnancy status from Days 12-14 (Fig. 3.5A and 3.5B). No change was seen in *ISG15* in uterine vein PBMC's (Fig. 3.5B). However, *ISG15* mRNA concentrations increased in jugular PBMCs from P compared to NP ewes (Fig. 3.5A).

Uterine Vein Tissue, Lymph Nodes, Liver ISG15 mRNA Profile during Estrous Cycle or Early Pregnancy

ISG15 mRNA concentrations were examined in uterine vein tissue collected upstream of the utero-ovarian plexus, liver and CL (described below) on Days 12 to 15. In the uterine vein tissue, there was no change in *ISG15* expression. (Fig. 3.6A). *ISG15* mRNA and protein were present in lymph nodes collected on Day 15, but did not differ in concentration by pregnancy status (NP vs. P) or by anatomic location (iliac vs. submandibular; data not shown). Liver *ISG15* mRNA levels were similar on Days 12 and 13 and were not affected by pregnancy status (Fig. 3.6B). However, by Day 14 of pregnancy, liver *ISG15* mRNA levels increased and continued to increase by Day 15, while *ISG15* mRNA levels remained low during this period in NP ewes.

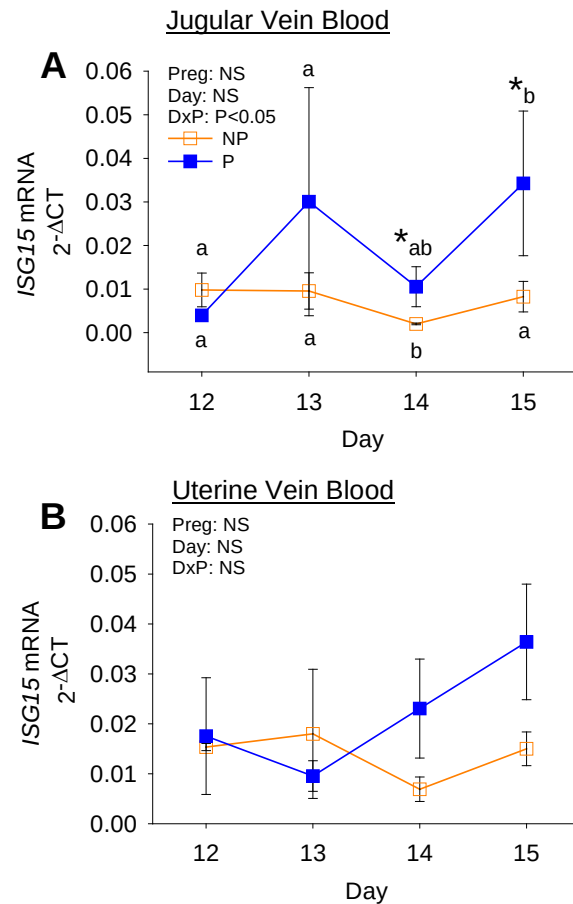


FIGURE 3.5.

ISG15 Expression in PBMCs isolated from Uterine and Jugular Vein Blood. Relative expression ($2^{-\Delta CT}$) of mRNA levels of *ISG15* in Jugular Vein Blood (A) and Uterine Vein Blood (B) on Days 12, 13, 14 and 15 of the estrous cycle or early pregnancy in sheep relative to geometric mean of housekeeping genes. Data are presented as mean $\pm$ SEM. * indicates means differ ($P < 0.05$).

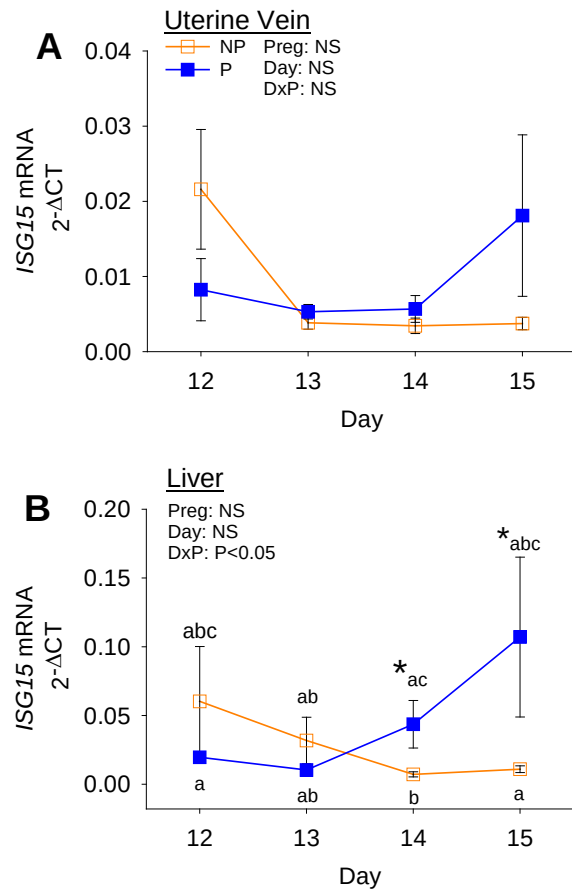


FIGURE 3.6.
ISG15 mRNA Levels in Response to Early Pregnancy in Extra Uterine Tissues (Uterine Vein and Liver). Relative expression ($2^{-\Delta CT}$) of mRNA levels for *ISG15* relative to geometric mean of housekeeping genes. Uterine vein (A) and liver (B). *ISG15* mRNA expression on Days 12, 13, 14 and 15 of the estrous cycle or early pregnancy in sheep. Data are presented as mean $\pm$ SEM. * indicates means differ ($P < 0.05$).

Endocrine Actions of IFNT in the Corpus Luteum

Interferon (Alpha, Beta and Omega) Receptor 1 (IFNAR1) and *Interferon (Alpha, Beta and Omega) Receptor 2 (IFNAR2)* mRNAs were present in CL during the estrous cycle and early pregnancy (data not shown). However, both mRNA encoding subunits of the IFNAR had similar patterns of expression in the CL and were not affected by pregnancy status or Day. However, signaling downstream from the IFN receptors was up-regulated in the CL during pregnancy, despite no difference in IFN receptor mRNA. *STAT1* and *STAT2* mRNA concentrations were both up-regulated on Day 14 of pregnancy and *STAT1* mRNA concentration remained elevated on Day 15 (Fig. 3.7A). *IRF3* mRNA concentration was significantly up-regulated in both NP and P ewes on Day 15 when compared to earlier Days (Fig. 3.7B). The pattern of *IRF7* and *IRF9* mRNA concentrations were similar to pattern observed for *STAT1* concentrations and were up-regulated on Day 14 and 15 of pregnancy (Fig. 3.7B). ISGs were also up-regulated in response to pregnancy (Fig. 3.7C). *DDX58*, *IFH1* and *ISG15* mRNA concentrations were all greater in CL from P compared to NP ewes by Day 14 of pregnancy and this increase in ISGs continued through Day 15 of pregnancy (Fig. 3.7C).

mRNA Profiles of Luteotrophic, Luteolytic, Cell Survival and Cell Death Genes in the Corpus Luteum

ESR1, *OXTR*, *PTGFR*, *PTGER2*, *PTGER3*, *PTGER4*, *AKT*, *Casp3* and *XIAP* mRNAs were examined in the CL and no differences were found for Day or pregnancy status (data not shown).

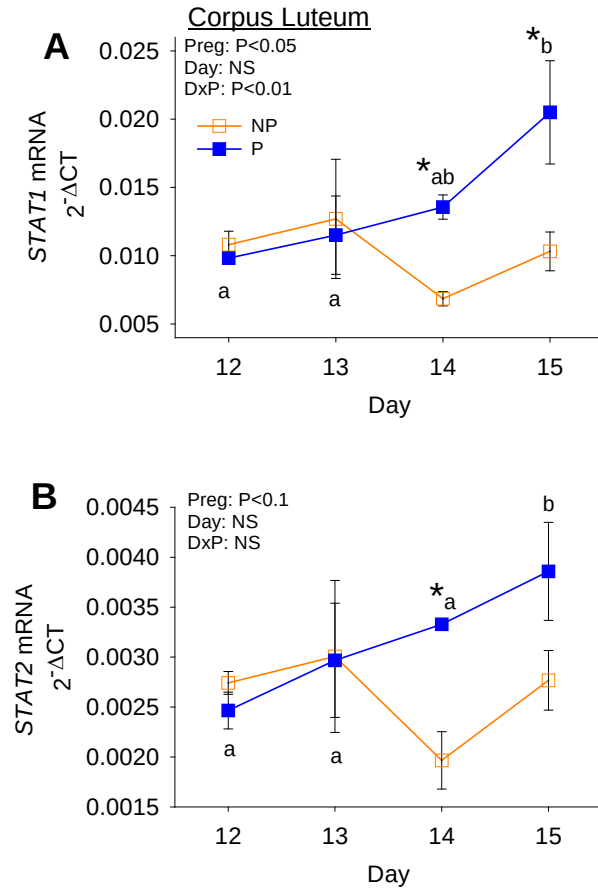


FIGURE 3.7A.
IFN Signaling in the CL. Relative expression to geometric mean ($2^{-\Delta CT}$) of mRNA levels of *STAT1* (A) and *STAT2* (B) on Days 12, 13, 14 and 15 of the estrous cycle or early pregnancy in sheep. Data are presented as mean ($\pm$ SEM). * indicates means differ (P<0.05).

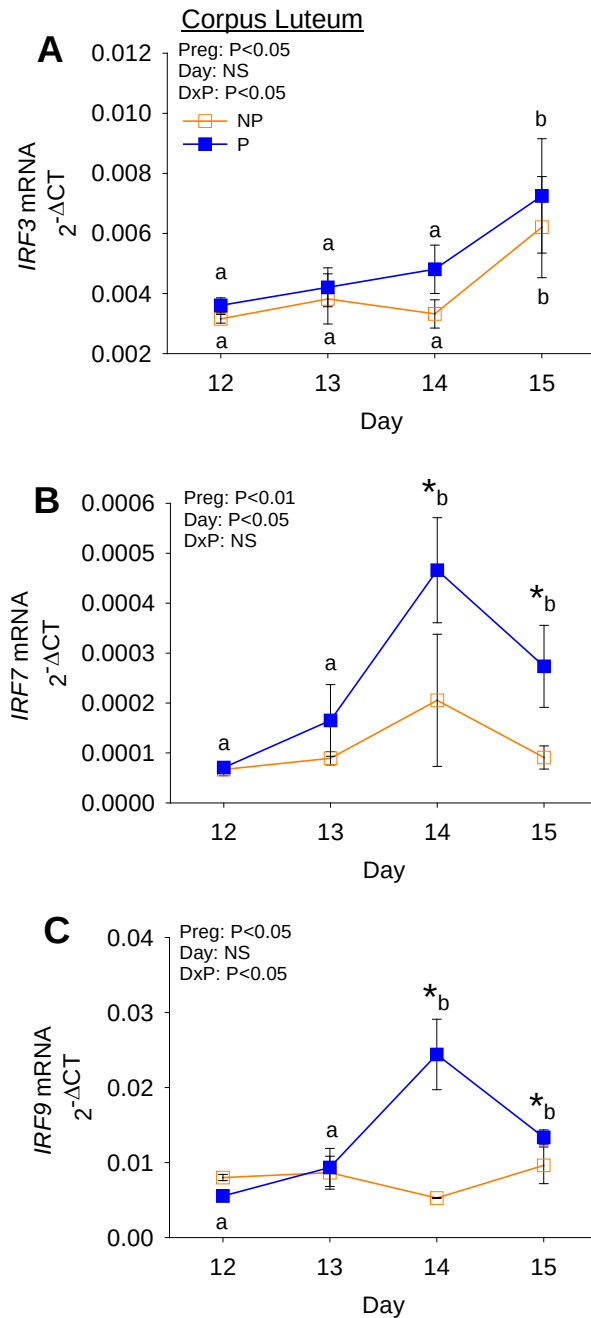


FIGURE 3.7B.
IFN Signaling in the CL. Relative expression to geometric mean ($2^{-\Delta CT}$) of mRNA levels of *IRF3* (A), *IRF7* (B) and *IRF9* (C) on Days 12, 13, 14 and 15 of the estrous cycle or early pregnancy in sheep. Data are presented as mean ($\pm$ SEM). * indicates means differ ($P < 0.05$).

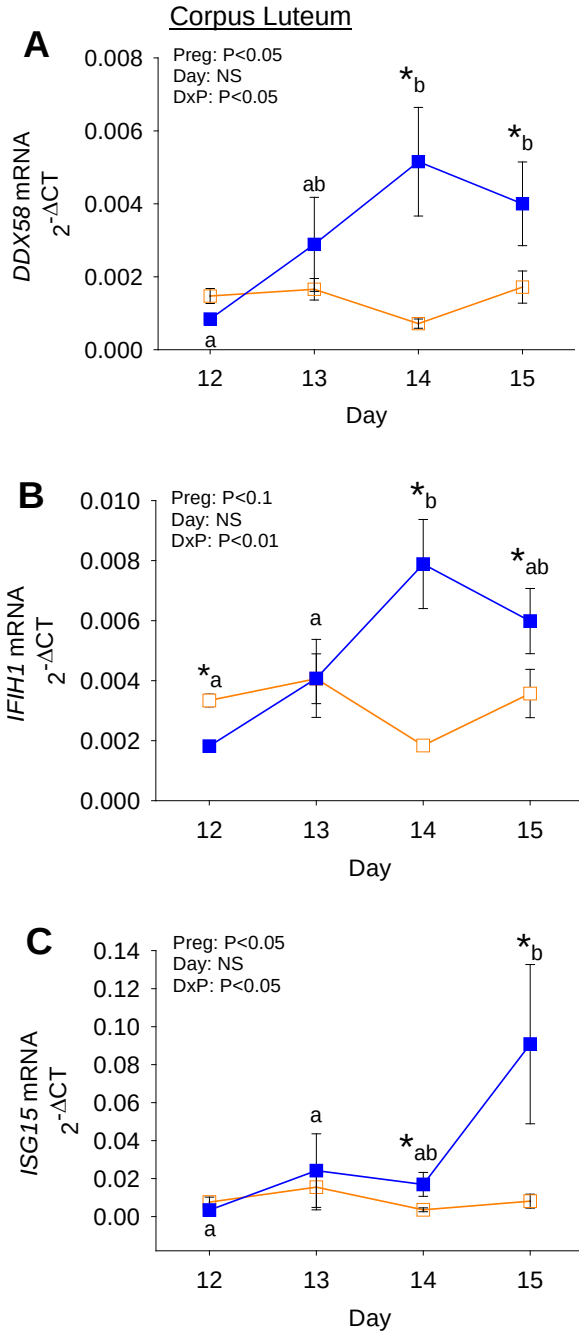


FIGURE 3.7C.

IFN Signaling in the CL. Relative expression to geometric mean ($2^{-\Delta CT}$) of mRNA levels of *RIGI* (A), *MDA5* (B) and *ISG15* (C) on Days 12, 13, 14 and 15 of the estrous cycle or early pregnancy in sheep. Data are presented as mean ($\pm$ SEM). * indicates means differ ($P < 0.05$).

DISCUSSION

IFNT is produced by the ruminant conceptus and for the last three decades was thought to have only paracrine function through binding to receptors on the maternal endometrium of the uterus (Godkin, Bazer et al. 1984; Bazer, Vallet et al. 1986). The primary paracrine role of IFNT during early pregnancy is antiluteolytic and mediated through disruption of prostaglandin F₂-alpha release from the uterus. This restriction of detection and action of IFNT within the uterine lumen was based on lack of detection of antiviral activity in peripheral blood, utilizing a bioassay for IFN, and more recently was based on lack of detection of IFNT in blood using antibody-based detection methods. Increased antiviral activity caused by pregnancy has not been observed in systemic blood collected from ruminants during early pregnancy. However, one report in 1991 (Schalue-Francis, Farin et al. 1991) described an antiviral assay with a sensitivity of ~1 unit/ml that could not detect IFNT in jugular vein blood, but was efficacious in detecting 58 U/ml in uterine vein serum from Day 15 pregnant sheep. 58 U/ml is equivalent to 7.25 ng/ml based on the 8×10^8 U/mg IFN standard used in this study. The conclusion that IFNT was not detectable in jugular vein blood also was based on antibody-based detection of IFNT in ELISA and RIA. For example, an RIA for IFNT was developed by others with sensitivity of detecting 6.1-7.8 ng IFNT/ml (Takahashi, Takahashi et al. 2005). These authors reported a sensitivity of 6.1 ng/ml, but the lowest standard used in the assay was 7.8 ng with binding of ~95%. Use of this RIA by these authors revealed detection of IFNT in uterine flushing representing Day 16 of bovine pregnancy. There was no report of attempting to detect IFNT in blood by these authors.

A more recent study by our group also found detectable antiviral activity in uterine vein blood from Day 15 pregnant sheep, with no detection of antiviral activity in systemic blood (Oliveira, Henkes et al. 2008). We demonstrated that the antiviral activity was attributed specifically to IFNT and not other type I IFNs, through blocking antiviral activity in uterine vein blood using preadsorption with an antibody against IFNT (Bott, Ashley et al. 2010). The amount of IFNT in uterine vein blood on Day 15 of pregnancy was ~500-1,000 U/ml, which we estimated to be 5-10 ng/ml using a standard having 1×10^8 U/mg IFN standard (Oliveira, Henkes et al. 2008).

Indirect evidence that IFNT might be released from the uterine vein and has a systemic-endocrine role during pregnancy was demonstrated by up-regulation of interferon-stimulated genes (ISGs) in peripheral blood cells [see: (Han, Austin et al. 2006; Gifford, Racicot et al. 2007; Oliveira, Henkes et al. 2008)]. Based on these studies, it was concluded that IFNT was produced by the conceptus during early stages of pregnancy, attenuated PGF release from the endometrium and was released into the uterine vein in high enough concentrations to possibly have a functional and biological effect on peripheral tissues such as blood cells, the corpus luteum and liver (Oliveira, Henkes et al. 2008; Bott, Ashley et al. 2010; Antoniazzi, Webb et al. 2013). However, until the experiments described herein were completed, no one had been able to directly detect IFNT circulating in the blood during early pregnancy in ruminants.

A double antibody radioimmunoassay (RIA) for IFNT is described herein using recombinant ovine IFNT and anti-roIFNT antibody provided through collaboration with Dr. Fuller Bazer (Texas A&M University) using methods described previously by Niswender et al., (Niswender, Reichert et al. 1969). This RIA was confirmed to be

specific for IFNT because the anti-IFNT antibody did not cross-react with other type I IFN such as alpha and beta or with type II IFN such as IFN gamma. After specificity of the IFNT RIA for IFNT was demonstrated by lack of competition by up to 10 µg/ml of related, but not identical type I and II IFN. Sensitivity of this IFNT RIA was improved through increasing dilutions of the primary antibody. The amount of ligand required to displace 50% binding decreased from ~1.5 ng to 0.4 ng with increasing dilutions of primary anti-IFNT antibody to 1:60,000. While this assay had improved sensitivity, and was very useful when detecting IFNT in ovine uterine flushings from Days 13-16 of pregnancy and in uterine vein blood from Days 15 to 16 of pregnancy, it was not able to detect IFNT in jugular vein blood in sheep or in tail vein blood from similar stages of early pregnancy in cattle (data not shown). The sensitivity of the assays in ovine uterine vein flushings was about 0.07 ng/ml. The sensitivity of the assays for IFNT in serum was 0.06 ng/ml; which was significantly improved over other assays described for IFNT, but possibly not enough to allow detection of IFNT in the blood. The sensitivity of the IFNT RIA using serum may have been impacted by factors in serum that are not present in uterine flushing which interfered with the assay. We are completing experiments to test this hypothesis and to improve sensitivity further for possible detection of IFNT in jugular or tail vein blood from sheep and cattle, respectively.

IFNT was first discovered in 1982 by Godkin et al. (Godkin, Bazer et al. 1982) and they were able to quantify concentrations (50-100µg) of IFNT derived from the trophoblast cells of Day 14 and 16 conceptuses using a Lowry assay. Since the initial discovery, there have been several attempts to improve IFNT detection. In 1988 the first RIA for IFNT was validated and was used to quantify IFNT in culture media as well as uterine

flushings (Vallet, Bazer et al. 1988). Utilizing this assay Davis and collaborators (Davis, Ott et al. 1992) were able to determine IFNT in uterine flushings, at concentrations of 117-164 µg/conceptus, for Day 16 Pregnant ewes. More recently in 2005 Takahashi et al. developed a new RIA for IFNT and were able to detect bovine IFNT at a concentration of 11.65-15.73µg in Day 16 uterine flushings (Takahashi, Takahashi et al. 2005). By increasing the primary antibody dilution relative to previous dilutions used in the aforementioned studies an increased sensitivity of 0.07ng/ml in uterine flushings and 0.06g/ml in serum was achieved. Herein we have demonstrated the initial Day of detection (within constraints of this assay) analyzing samples in a temporal fashion. The detection of IFNT in uterine flushings on Day 14 corresponds with changes in gene expression due to pregnancy and the estrous cycle.

During early pregnancy, the first response to conceptus release of IFNT is induction of ISGs, specifically ISG15 in the endometrium. This paracrine action of the conceptus occurs between Days 12 and 13 of pregnancy and is one Day prior to the decline in serum progesterone observed between Days 13 and 14 in NP ewes (Romero, Antoniazzi et al. 2013) reviewed in (McCracken, Custer et al. 1999)). Paracrine action coincides with first detection by RIA of IFNT on Day 13 in uterine flushing and in uterine vein serum of Day 15 and 16. It also corresponds with an up-regulation of *ESR1* and *OXTR* mRNAs, which results in luteolytic pulses of PGF. Up-regulation of *ISG15* mRNA levels in response to pregnancy has been described in sheep and bovine models (Johnson, Spencer et al. 1999; Austin, Carr et al. 2004). Austin et al. (Austin, Carr et al. 2004) indicated that endometrial ISG15 expression occurs as early as Day 17 and is maintained until Day 45 in pregnant cows. On Day 11 of pregnancy in sheep, *ISG15*

mRNA abundance (gene expression) was low, followed by a significant increase by Day 15. However, no time points were analyzed between Days 11 and 15 to clarify when this response to IFNT occurred (Joyce, White et al. 2005). In the present study, we analyzed *ISG15* mRNA expression daily from Days 12-15, and were able to determine when *ISG15* mRNA is induced in endometrium during early pregnancy. Greater expression of *ISG15* mRNA in P compared to NP ewes occurred in endometrium between Days 12 and 13, which is consistent with the greatest temporal release of IFNT from the ovine conceptus (Hansen, Imakawa et al. 1988).

Endometrial expression of *ESR1* is high on Day 1, decreases on Days 2-11 and then increases between Days 11 and 15 of the estrous cycle (Spencer, Becker et al. 1995). In pregnant ewes, up-regulation of *ESR1* was blocked between Days 11 and 15 (Spencer and Bazer 1995), and this was confirmed in our experiments: decreased expression of *ESR1* from Days 12-15 during pregnancy was detected. Lower concentrations of *ESR1* and *OXTR* in ovine endometrium in Day 15 pregnant compared to cyclic ewes was described previously by Spencer et al. (Spencer and Bazer 1996). Spencer and colleagues reported that IFNT suppressed the transcription of the *ESR1* and *OXTR* genes in the ovine endometrium, and in altered the pulsatile pattern of PGF release, thus abrogating luteolysis (Spencer and Bazer 1996).

Recently ISGs have been shown to be expressed in other tissues in response to early pregnancy, such as *Mx* (Yankey, Hicks et al. 2001) and *ISG15* (Han, Austin et al. 2006) expression in PBMCs. Temporal expression of *Mx* mRNA in PBMCs from jugular blood was characterized from Days 0-30 in NP vs. P ewes. *Mx* mRNA levels were not different on Days 0, 9 and 12, but increased beginning on Day 15 of pregnancy

compared to the estrous cycle (Yankey, Hicks et al. 2001). Han and collaborators (Han, Austin et al. 2006) examined blood *ISG15* mRNA levels associated with progesterone to predict pregnancy status in cows. *ISG15* mRNA expression was examined in blood from Days 15-21, 25 and 32 in NP and P cows. Analysis of *ISG15* mRNA in PBMCs allowed these investigators to predict 100% of NP cows on Day 32, which was confirmed with ultrasound (Han, Austin et al. 2006). In the present experiments, *ISG15* mRNA expression was examined in jugular and uterine vein blood from NP and P ewes from Days 12-15. *ISG15* expression was not different in PBMCs isolated from the Uterine vein, but *ISG15* was significantly greater by Day 14 and 15 of pregnancy.

ISGs are expressed in extrauterine tissues besides blood, such as in the liver and CL on Days 14 and 15 of pregnancy. In the present experiments, we examined *ISG15* mRNA in extrauterine tissues during several Days of the estrous cycle and pregnancy. Uterine vein was examined as a target for IFNT action because it represents the first tissue exposed to IFNT upon its exit from the uterus. *ISG15* mRNA levels were not affected by pregnancy status, suggesting that the uterine vein tissue might not be a target for IFNT signaling during early pregnancy. However the CL is a target for IFNT signaling. *IFNAR1* and *IFNAR2* mRNA were not affected but several genes downstream of these receptors were up-regulated due to Day and/ or pregnancy status. Upon phosphorylation STAT1: STAT2 heterodimers release from IFN receptors and associate with IRF9, which is a DNA binding protein. This complex is thus known as ISG factor-3 (ISGF3) and is able to translocate to the nucleus where it is able to initiate transcription of genes by binding to IFN-stimulated response elements (ISRE) (Samuel 2001; Bekisz, Schmeisser et al. 2004). One of the genes induced by ISGF3 binding to the ISRE is

IRF-7. Following de novo synthesis IRF-7 is phosphorylated and has been shown to activate IFN α / β transcription (Taniguchi and Takaoka 2002) thus possibly preparing the mother to defend against any viral infection that could cause a loss of pregnancy. IRF3 is constitutively expressed and only active when phosphorylated, which might explain no change in mRNA expression levels. Follow up experiments with phosphorylated protein expression are required to determine if it is also activated during pregnancy. The binding of ISGF3 to ISRE results in transcription of ISGs such as ISG15. *ISG15* mRNA levels increased in the liver beginning on Day 14 and *MDA5*, *RIGI* and *ISG15* were greater in the CL on Day 14 and/or 15 of pregnancy. To our knowledge, this is the first temporal description of induction of IFN signaling in extrauterine tissues with specific emphasis on the CL and liver during early pregnancy in sheep.

Glass and co-workers (Glass, Fitz et al. 1984) reported that ovine luteal *ESR1* levels were low on Day 4, increased significantly by Day 6, reached maximum levels by Day 8, decreased by Day 12 and then increased by Day 16 of the estrous cycle. OXT treatment inhibited LH-stimulated secretion of progesterone from SLC, suggesting presence of OXTR (Mayan & Niswender, unpublished data). Since IFNT controls the transcription of endometrial *ESR1* and *OXTR* genes during maternal recognition of pregnancy in ruminants (Spencer and Bazer 1996), we tested whether pregnancy would have similar effect on *ESR1* and *OXTR* mRNA concentrations in the CL. We observed no difference for *ESR1* or *OXTR* mRNA in the CL during the Days examined for the estrous cycle or early pregnancy and conclude that these genes are not regulated by pregnancy in the CL within this time period. The first paracrine endometrial response to IFNT is an increase in *ISG15* mRNA levels on Day 13. One Day later, on Day 14, *ESR1*

and *OXTR* mRNAs are up-regulated in endometrium from NP ewes, but this up-regulation is blocked in P ewes. IFNT signaling in hepatic and luteal tissues were up-regulated one day later than the endometrium, indicating that the endocrine action of IFNT might take approximately one Day longer than paracrine signaling.

In conclusion, these results show that IFNT response reflected in *ISG15* mRNA expression in the endometrium (paracrine action) occurs as early as Day 13 of pregnancy and in extra uterine tissues (endocrine action) on Day 14. The direct action of IFNT on the CL may be involved with luteal protection and resistance against PGF luteolytic pulses. This direct action of IFNT also may control antiapoptotic and cell survival genes to assure luteal cell differentiation that will prolong luteal life span. Ultimately, luteal survival may be driven by ISG15 action, possibly altering luteolysis/antiluteolytic/cell survival signaling pathways by conjugating to intracellular proteins. These hypotheses are subjects of ongoing experiments.

Declaration of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported

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CHAPTER 4

IDENTIFICATION OF PREGNANCY-ASSOCIATED METABOLITES AND PROTEINS IN OVINE UTERINE FLUSHINGS BY MASS SPECTROSCOPY

CHAPTER 4 SUMMARY

The ovine conceptus is free-floating in the uterine lumen during early maternal recognition of pregnancy, which is mediated through release of interferon tau (IFNT) by Day 14. The conceptus actually becomes associated with the uterine epithelium by Day 15 (apposition) and then develops very superficial adhesion complexes by Day 17 of pregnancy, but does not fully develop placental exchange of nutrients until much later during pregnancy. During the early establishment of pregnancy, the conceptus receives nutrients from maternal histotroph, which is derived from progesterone-induced and cytokine-mediated endometrial glandular secretions of proteins and metabolites. This fluid environment has been examined by many groups, but not in context of both the proteome and the metabolome. By using mass spectroscopy approaches, we have identified previous (i.e., IFNT) and new proteins and metabolites that may contribute to survival of the conceptus and establishment of pregnancy with the longer-term goal of testing for the presence of new markers for a viable conceptus in uterine vein and jugular vein blood by use of antibody, gas chromatography or mass spectroscopy approaches. Functional relevance of metabolites and protein identified herein include lipid transport, cell survival, cell motility, prostaglandin synthesis and immune regulation. These functional pathways and attributes are considered in context of establishment and maintenance of pregnancy; and why some embryos live why others die.

INTRODUCTION

Eutherian mammals are relatively new in the world's history and because of their recent evolution there is high variability in implantation and placental types (Spencer, Johnson et al. 2004). In all eutherian mammals the conceptus must signal its presence to the mother; ruminants such as the ovine and bovine species do this by releasing a protein known as interferon tau (IFNT) (Godkin, Bazer et al. 1982). Other species have similar mechanisms. For example, human concepti release human chorionic gonadotropin and rodents release prolactin (Roberts, Xie et al. 1996) to signal early establishment of pregnancy. Within the time of pregnancy recognition by the mother the initial events between the trophoblast and maternal uterine endometrial epithelium are similar across species. Likewise, embryonic mortality/failure is a problem that faces most all mammalian species.

Reasons associated with failed maintenance of pregnancy can be contributed with chromosomal abnormality of the conceptus, parental age, maternal obesity, maternal infection, diabetes mellitus and insufficient production of progesterone by the corpus luteum (CL) (Temmerman, Lopita et al. 1992; de la Rochebrochard and Thonneau 2002; Vorsanova, Kolotii et al. 2005; Metwally, Ong et al. 2008; Khaskheli, Baloch et al. 2010; Rocchetti, Marconi et al. 2010; Nigro, Mazzocco et al. 2011). Outside of chromosomal abnormalities a key explanation behind why some embryos die and others survive is not known across mammalian species, but it is hypothesized that it likely involves impaired communication/signaling between the conceptus and the mother.

Embryonic mortality rates in ruminants (beef cows, dairy cows and sheep) are observed at similar if not at a greater occurrence of 12-43% when compared with humans (Humblot 2001; Diskin, Murphy et al. 2006; Diskin and Morris 2008). Most embryo losses in dairy cattle occurs prior to Day 16 following breeding (Berg, van Leeuwen et al. 2010). The economic impact of large animal agriculture in the United States of America is estimated to be close to \$332.5 billion per year (W2112 Reproduction Performance in Domestic Ruminants). The estimated revenue loss in 2005, due to embryonic mortality in the beef industry alone was estimated to be greater than \$1.2 billion dollars (Geary 2005). Studying embryonic mortality in ruminants provides an opportunity to make significant discoveries that can reduce the severe economic burden placed on agricultural producers due to embryonic mortality, in addition to providing a model that allows for the discovery of potential impairments in human pregnancy as well. Humans also suffer from infertility. The inability to conceive after 12 months of regular, unprotected sexual intercourse is defined as infertility in humans (Bromer and Seli 2008). The emotional and economic costs associated with infertility and especially embryo mortality, which is called miscarriage in humans is immeasurable. Miscarriage occurs at an approximate rate of 11-27% in all clinically recognized pregnancies (Elish, Saboda et al. 1996; Blohm, Friden et al. 2008; Herbert, Lucke et al. 2009).

Proteomic and metabolomic methods provide avenues that allow for the analysis of the second largest (100,000) and smallest (6,000) group of compounds; respectively (Heazell, Brown et al. 2010). Histotroph consists of secretions from uterine epithelia and molecules transported into the uterine lumen, it consists of a mixture of enzymes,

growth factors, cytokines, lymphokines, hormones, transport proteins, amino acids and other nutrients (Gao, Wu et al. 2009). Previously, proteomic analysis limited to Day 16 of the estrous cycle or pregnancy has been performed on ovine uterine flushings (Koch, Ramadoss et al. 2010); however interferon tau (IFNT), the major secretory protein released during maternal recognition by the conceptus, was not detected in this study. Also mass spectroscopy technology has advanced exponentially over the past couple years. For these reasons, more extensive mass spectroscopy analysis of uterine flushings during maternal recognition of pregnancy is justified. We hypothesize that proteomic and metabolomic analysis of uterine flushing from Day 12 of the estrous cycle (EC) compared to Days 12, 14 and 16 pregnant ewes (PREG) will provide insight on the function and profile of compounds that are being released from the uterus and conceptus.

METHODS

Animal Care and Uterine Flush Collection

The highest standards of animal husbandry and care were used with all sheep involved in this study. Approval for experiments involving sheep was obtained from the Colorado State University Animal Care and Use Committee. Western range ewes (n = 19) were either exposed to a vasectomized ram or mated to a fertile ram on Day 0, to generate uterine flushes derived from the estrous cycle or pregnancy. Uterine flushings were collected following necropsy of ewes on Days 12, 14 and 16: Day 12 (n = 5EC & 4 P), Day 14 (n = 5 P) and Day 16 (n = 5) by using 20 ml of sterile saline solution. Serum progesterone is not different on Day 12 of pregnancy or the estrous cycle (Romero, Antoniazzi et al. 2013) reviewed in (McCracken, Custer et al. 1999)). Day 12 NP uterine

flushings serves as a negative control for comparisons to uterine flushings from pregnant ewes. Presence of a conceptus was confirmed in these uterine flushings when designating ewes as pregnant.

Sample Preparation

Uterine flush samples were centrifuged for at 4°C for 5 minutes at 10,000 x g to reduce cellular debris. Uterine flushes were then precipitated with 80% methanol at 20°C for one hour, centrifuged at 10,000 x g for 5 minutes at 4°C and the supernatant was transferred to a new tube for removal of the solvent using a speed vac. The solute was re-suspended in 100 µl of HPLC-MS grade water and transferred to an auto-sampler vial with a 150 µl glass insert.

UPLC-MS

From the auto-sampler vial, 1µl injections were analyzed on a Waters Acquity UPLC system. Sample separation was performed using a Waters Acquity UPLC T3 column (1.8 µM, 1.0 x 100 mm), using a gradient from solvent A (water, 0.1% formic acid) to solvent B (Acetonitrile, 0.1% formic acid). Injections were made in 100% A. Solvent A was held for 1 minute, and was then transferred to 95% B over a 12 minute linear gradient. Gradient B was held at 95% for 3 minutes then the system was returned to starting conditions over 0.05 minutes, and allowed to equilibrate at for 3.95 minutes. A constant flow rate of 200 µl/ minute was used for the duration of the run. Samples were held at 5°C and the column was held at 50°C. Column eluent was infused into a Waters Xevo G2 Q-ToF MS fitted with an electrospray source. Data were collected in positive ion mode, scanning from 50-1200 at a rate of 0.2 seconds per scan, alternating between MS and MS^E mode. Collision energy was set to 6 V for MS mode,

and ramped from 15-30 V for MS^E mode. Calibration was performed prior to sample analysis via infusion of sodium formate solution, with mass accuracy within 1 ppm. The capillary voltage was held at 2200V, the source temp at 150°C, and the desolvation temperature at 350°C at a nitrogen desolvation gas flow rate of 800 L/hr.

Data Processing and analysis

Raw data files were converted to .cdf format, and feature detection and alignment was performed using XCMS in R (statistical software). Raw peak areas were normalized to total ion signal in R, and the normalized dataset was subjected to principal component analysis PCA and analysis of variance (ANOVA) in R using the pca Methods package, and the aov function, respectively.

Metabolomic Overview of data analysis workflow: Detection of peaks

Each mass signal which comes off the column with a roughly normal peak shape is detected, and the mass and retention time information are coupled to designate the name of that feature. After all features are detected (100's to 1000's of them), the dataset is normalized to the total ion signal, and submitted for Principle Component Analysis – an unbiased method for looking at sample-to-sample variability. Data are then analyzed using ANOVA to look for features changing due to the effect of treatment.

idMS/MS spectral reconstruction:

XCMS (software) peak detection was performed on both the low and high collision energy channels (mass spectroscopy and exponential mass spectroscopy (MS and MSe)). The datasets were separated following alignment, and the isotope dilution MS/MS workflow was applied for generation of indiscriminant MS/MS spectra for library searching and compound identification.

Principle component analysis

PCA takes into account all the features in each sample, fits a model to the data which generates a single value for each sample – this value is called the PC1 score for each sample. The remaining variation is again fitted to a model, and a new score is generated for each sample, the PC2 score. Plotting these scores for each sample as a scatterplot, allows for determination of the largest source of variation in the data set. PCA is an unbiased visualization tool which allows visualization of the overall structure of the data. Principle component analysis was conducted in R using the *pca* methods package, with pareto scaling, on total ion signal normalized data. Confidence intervals for PCA figures are calculated using the *simpleEllipse* function in *PCA Methods*, at a 95% confidence interval. Analysis of variance was performed in R, using a model containing pregnancy status, and Day.

Protein Digestion and Liquid Chromatography (LC)-MS/MS

To reduce cellular debris uterine flush samples were centrifuged for at 4°C for 5 minutes at 10,000g. Protein concentrations were determined via Bradford Assay (Thermo Scientific, Rockford, IL) and 30 µg of each sample underwent in-solution digestion using Protease Max (Promega, Madison, WI). Samples were solubilized in 8 M urea, 0.2% protease max, then reduced, alkylated, and digested with 1% protease max and trypsin at 37°C for 3 hours. Samples were dried in a Speed Vac® vacuum centrifuge, desalted using Pierce PepClean C18 spin columns (Pierce, Rockford, IL), dried and resuspended in 30 µl 3% ACN, 0.1% formic acid. All solvents, water, and acid were LC-MS/MS grade from Sigma (St. Louis, MO).

Each digested sample was analyzed in duplicate via LC-MS/MS. Peptides (0.5 μ l injection) were purified and concentrated using an on-line enrichment column (Thermo Scientific 5 μ m, 100 μ m ID x 2 cm C18 column). Subsequent chromatographic separation was performed on a reverse phase nanospray column (Thermo Scientific EASYnano-LC, 3 μ m, 75 μ m ID x 100 mm C18 column) using a 90 minute linear gradient from 10%-30% buffer B (100% ACN, 0.1% formic acid) at a flow rate of 400 nanoliters/min. Peptides were eluted directly into the mass spectrometer (Thermo Scientific Orbitrap Velos Pro) and spectra were collected over a m/z range of 400-2000 Da using a dynamic exclusion limit of 2 MS/MS spectra of a given peptide mass for 30 s (exclusion duration of 90 s). Compound lists of the resulting spectra were generated using Xcalibur 2.2 software (Thermo Scientific) with a S/N threshold of 1.5 and 1 scan/group. MS/MS spectra were searched against a Uniprot *Bovidae* database concatenated to a reverse database (126,284 entries) using the Mascot database search engine (Matrix Science, version 2.3.02) and SEQUEST (version v.27, rev. 11, Sorcerer, Sage-N Research). The following search parameters were used in Mascot: monoisotopic mass, parent mass tolerance of 20 ppm, fragment ion mass tolerance of 0.8 Da, complete tryptic digestion allowing two missed cleavages, variable modification of methionine oxidation, and a fixed modification of cysteine carbamidomethylation. Sequest search parameters were the same except for a fragment ion mass tolerance of 1.0 Da and a parent ion tolerance of 0.0120 Da. Peptide identifications from both of the search engines were combined using protein identification algorithms in Scaffold 4 (Keller, Nesvizhskii et al. 2002) (Searle, Turner et al. 2008) (Version 4.0.3, Proteome Software, Portland, OR) with protein clustering enabled. All data files for each biological

sample were then combined using the “mudpit” option in Scaffold 4 generating a composite listing for all proteins identified in each sample. Thresholds were set to 99% protein probability, 95% peptide probability, and a 2 unique peptide minimum was required. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins were annotated with GO terms from gene_association.goa_uniprot (downloaded Mar 14, 2013). (Ashburner, Ball et al. 2000). The false discovery rate (FDR) was 0% after manual validation of a subset of proteins identified by 2 unique peptides. (Kall, Storey et al. 2008) Criteria for manual validation included the following: 1) a minimum of at least 5 theoretical y or b ions in consecutive order that are peaks greater than 5% of the maximum intensity; 2) an absence of prominent unassigned peaks greater than 5% of the maximum intensity; and 3) indicative residue specific fragmentation, such as intense ions N-terminal to proline and immediately C-terminal to aspartate and glutamate.

Label-free Protein Quantitation

Relative quantitation was determined by a combination of spectral counting and average MS/MS total ion current (MS^2 TIC) (Liu, Sadygov et al. 2004) (Freund and Prenni 2013). Unweighted spectrum counts and MS^2 TIC values were exported from Scaffold 4. Data was normalized by summing the total spectral counts or MS^2 TIC values for each biological sample and then dividing each spectral count or MS^2 TIC value for that biological sample by the total. For proteins present in all samples, anova analysis was performed in SAS to determine significant changes. To be considered “present” proteins must be present in a minimum of 3 out of 4 biological replicates for a given state and the total normalized spectral counts for a given state must be > 10.

Proteins not detected across all of the samples were characterized as “present” or “absent”.

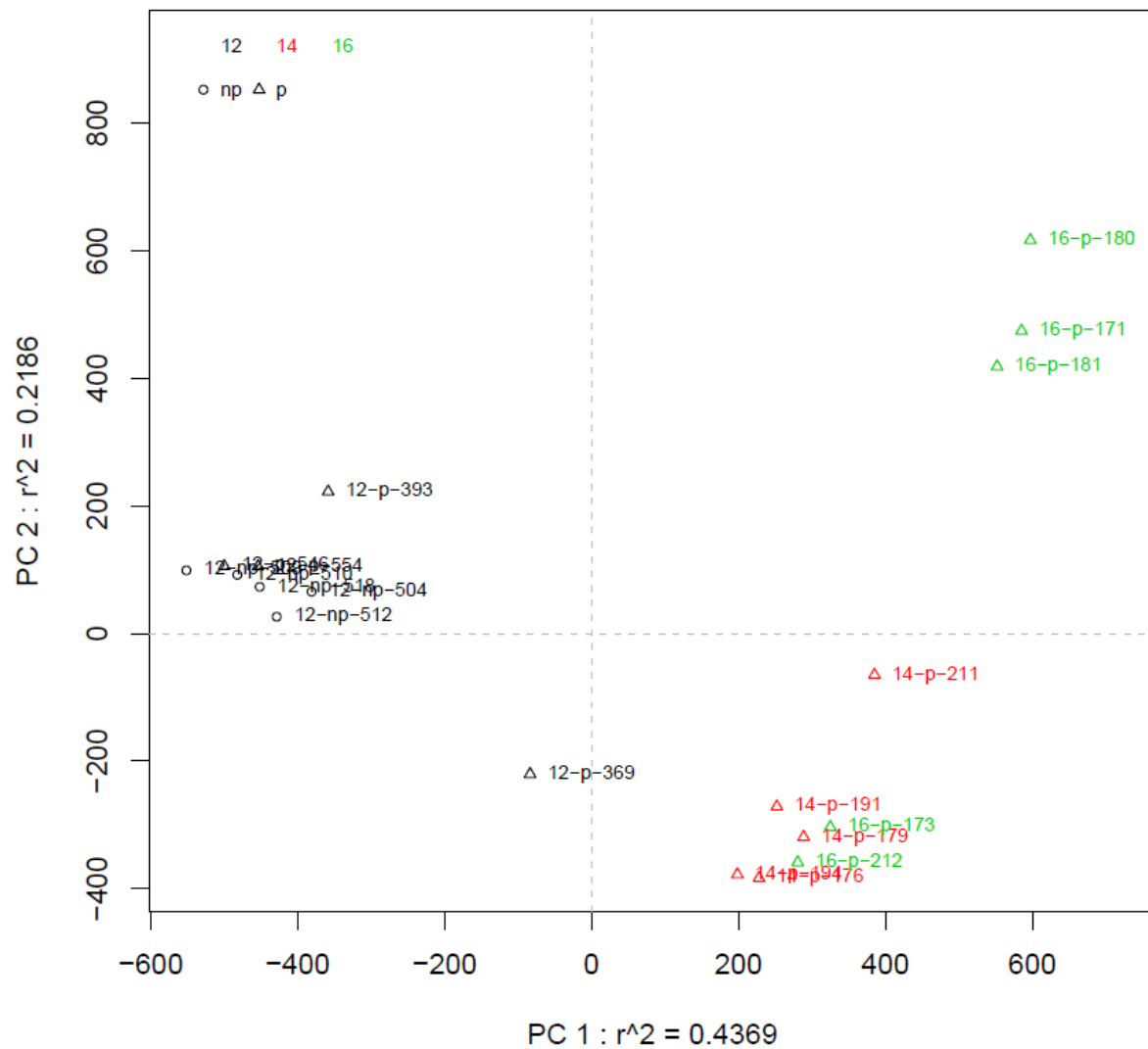
Pregnancy associated metabolites

A total of 8510 mass/retention time signals were identified in the initial metabolomic identification process. Preliminary analysis of metabolites in uterine flushings revealed that ewe metabolite profiles are very similar on Day 12 and that most distinctions between non-pregnant and pregnant ewes appear on Days 14 and 16 of pregnancy (Fig. 4.1). Metabolites were selected based on fold change >3 and P value <0.05 (Table 4.1). UPLC-MS analysis identified 5 key metabolites that are associated with pregnancy (Fig. 4.2). Acetylcarnitine, carnitine, ecdysteroids, N-acetyldileucine and valine, were not different on Day 12, regardless of pregnancy status, however, all were significantly greater on Days 14 and 16 of pregnancy compared to the Day 12 uterine flushings.

Proteomic analysis identified 783 potential protein targets. The top 40 proteins were further sorted by presence or absence during the estrous cycle or pregnancy (Table 4.2). Three proteins were identified as being present only on Day 12 of the estrous cycle and pregnancy, but were absent on Day 14 and 16 of pregnancy. Eleven proteins were present on Days 12, 14 and 16 and absent on Day 12 NP. Twenty-six proteins were up-regulated on Days 14 and 16 of pregnancy, but absent on Day 12 of pregnancy and the estrous cycle. MS analysis of uterine flushes identified 9 key proteins differentially expressed during estrous or pregnancy (Fig. 4.3-4.5). The first of these proteins is up-regulated during the estrous cycle. Type I collagen (composed of

TABLE 4.1. Metabolites Identified from Uterine Flushings of Pregnant Ewes

Metabolite	Fold Change	P –Value
Acetylcarnitine	5≥	0.05
Carnitine	5≥	0.05
Ecdysteroid	5<3≥	0.05
N-acetyldileucine	5≥	0.05
Valine	5≥	0.05



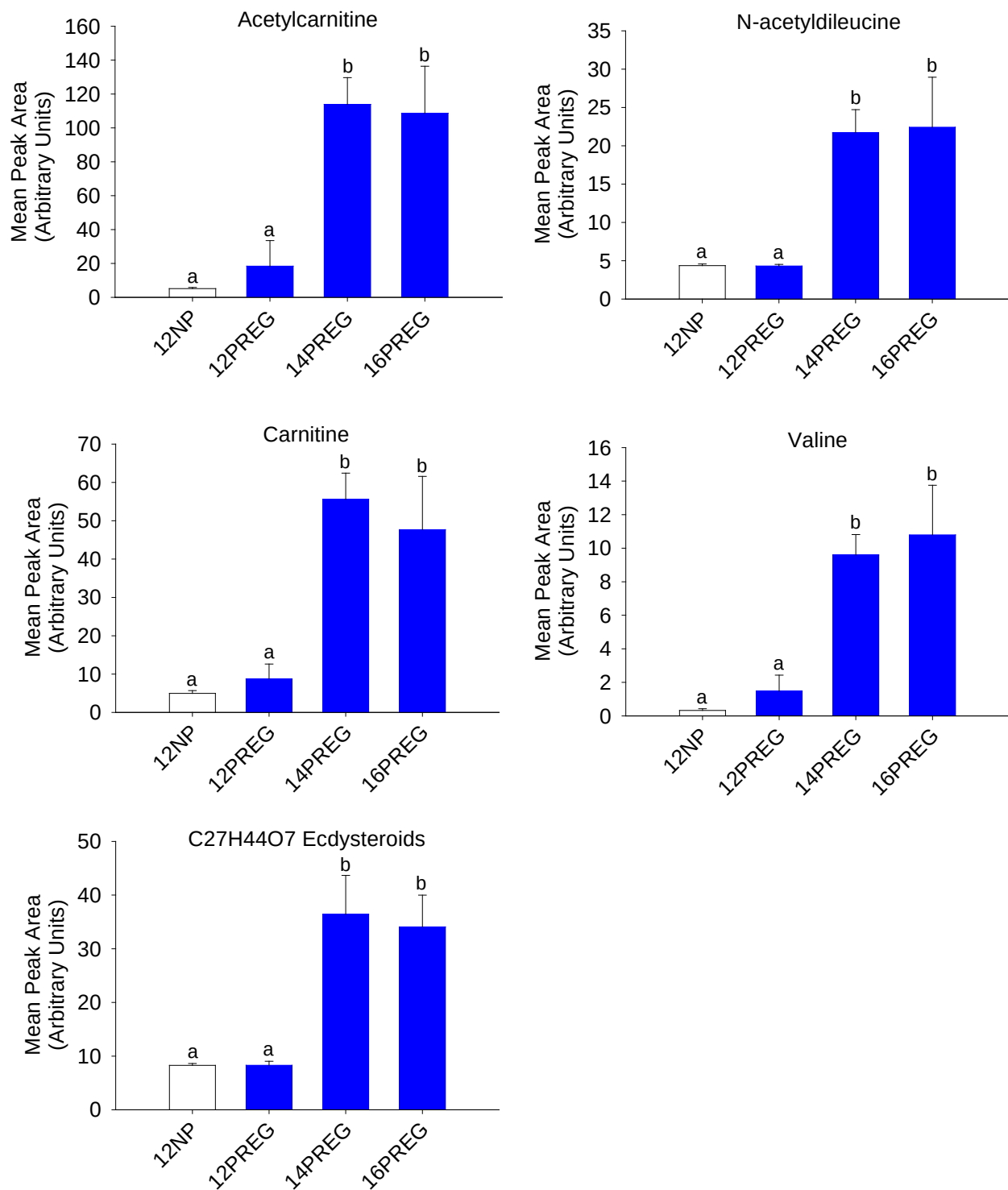


FIGURE 4.2.
Differentially expressed metabolites in uterine flushing due to pregnancy status.
Differential expression is indicated by letters ($P < 0.05$) and * indicates tendencies ($P < 0.10$).

TABLE 4.2. Top 40 Proteins Selected from Proteomic Analysis of Uterine Flushings

[illegible]

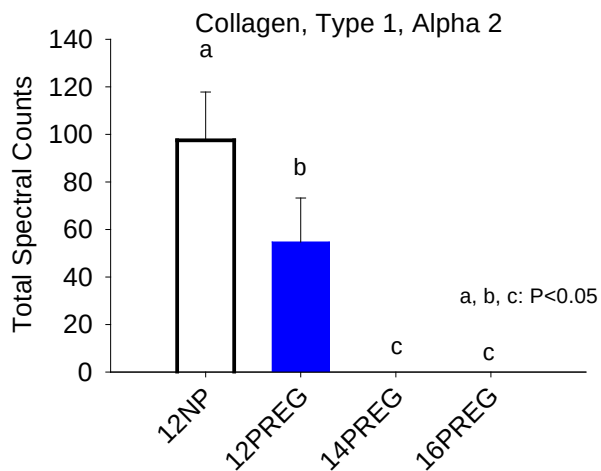
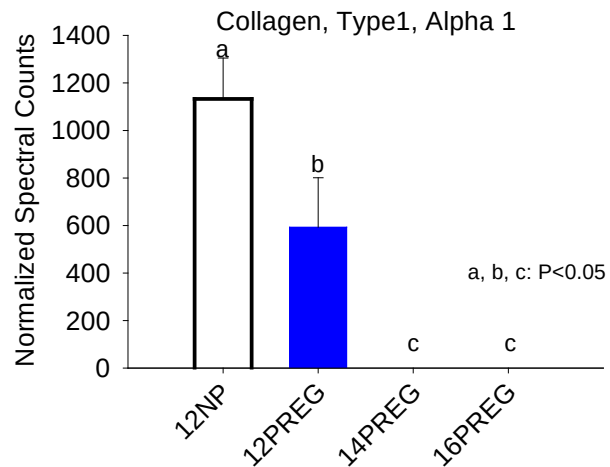


FIGURE 4.3.

Differential expression of Type 1 Collagen in uterine flushing due to pregnancy status. COL1A1 and COL1A2 are differentially up-regulated in NP ewes when compared with P ewes on Days 12, 14 and 16. Differential expression is indicated by letters ($P < 0.05$) and * indicates tendencies ($P < 0.10$).

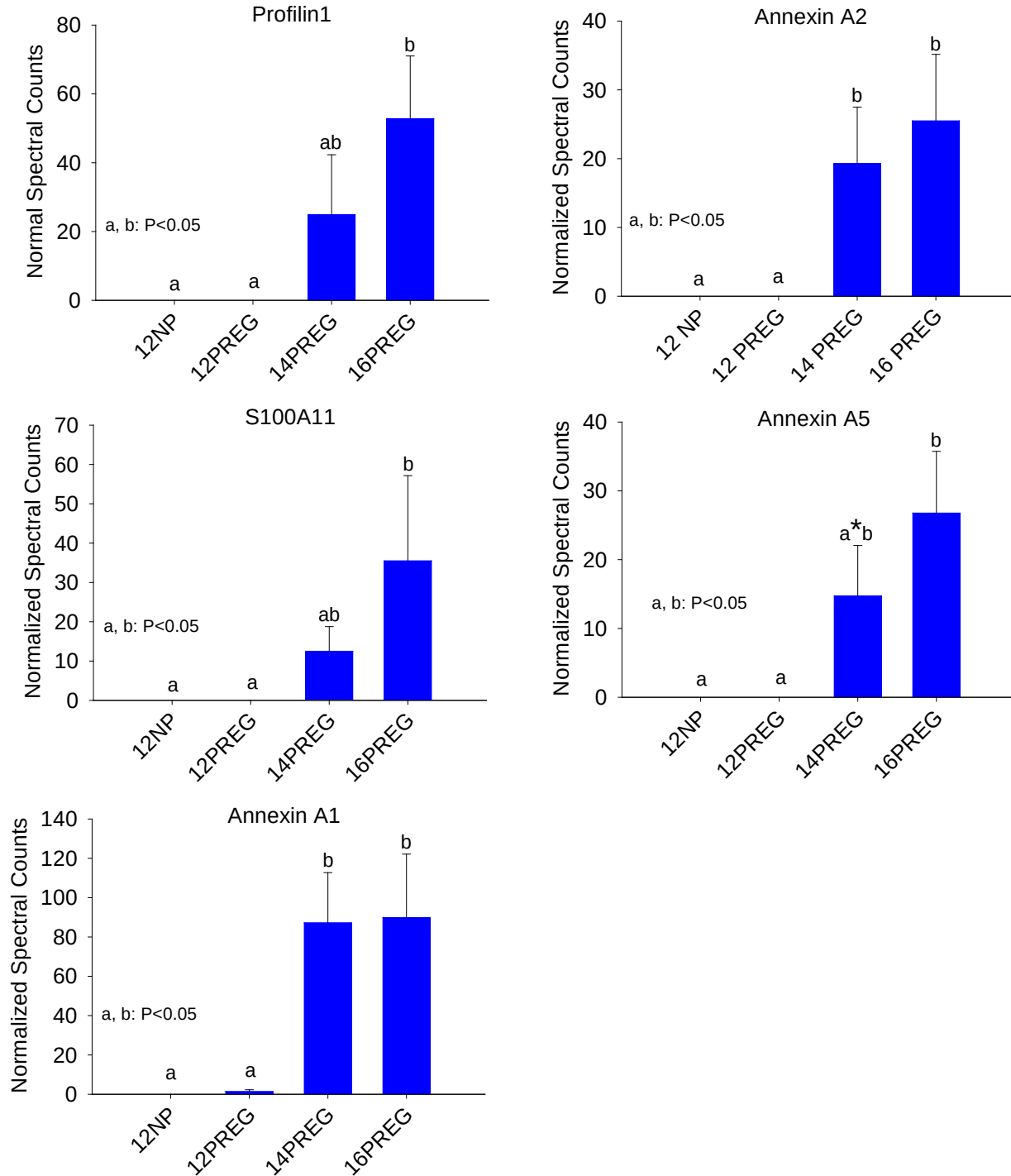


FIGURE 4.4.
Differentially expressed proteins in uterine flushing due to pregnancy status. Annexin A1 and Annexin A2 are significantly up-regulated due to pregnancy on Day 14 and 16. Profilin and S100A11 were significantly greater on Day 16 of pregnancy. Differential expression is indicated by letters ($P < 0.05$) and * indicates tendencies ($P < 0.10$).

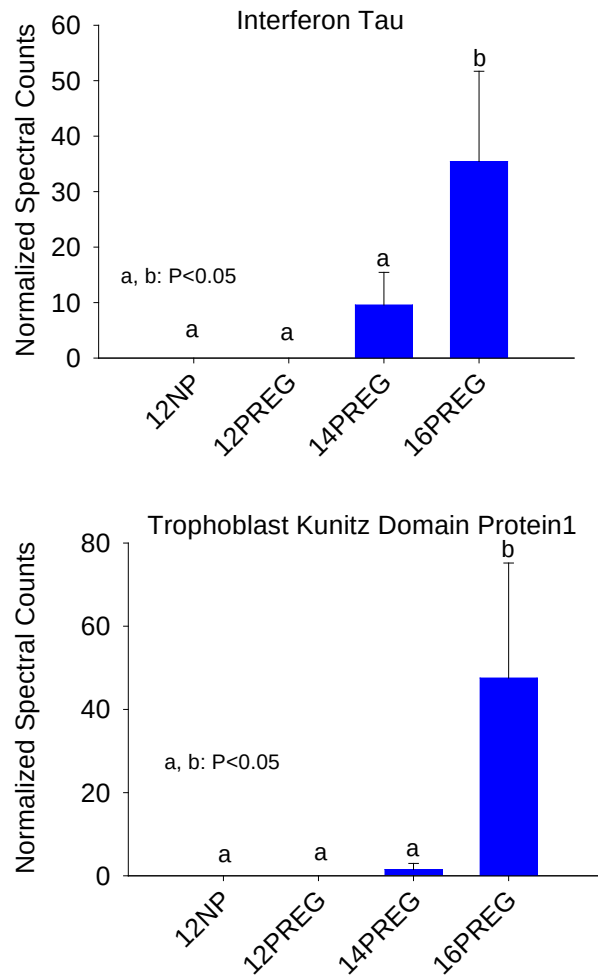


FIGURE 4.5.
Differentially expressed trophoblast proteins in uterine flushing due to pregnancy status. IFNT and TKDP1 were significantly greater on Day 16 of pregnancy. Differential expression is indicated by letters (P<0.05) and * indicates tendencies (P<0.10).

COL1A1 and COL1A2) was significantly greater on Day 12 of the estrous cycle and down-regulated during pregnancy on Days 12, 14, and 16 (Fig 4.3). This estrous cycle associated protein may serve as one of the earliest known diagnostic markers for non-pregnancy in ruminants. The next three proteins are part of the Annexin family, annexin A1, annexin A2 and annexin A5. Both annexins A1 and A2 had significantly greater spectral counts on Day 14 and 16 of pregnancy and annexin A5 tended to be greater on Day 14 and were greater on Day 16 of pregnancy. IFNT the known maternal recognition signal of pregnancy in ruminants as well as TDKP1 (Trophoblast Kunitz domain protein 1), Profilin 1 (PFN1) and S100A11 were differentially expressed in uterine flushes on Day 16 of pregnancy. IFNT tended to be different from Day 14 to 16 of pregnancy and PFN1 tended to be greater on Day 14 when compared with Day 12 despite pregnancy status.

DISCUSSION

Analysis of the metabolome in uterine flushings is a novel approach and only a handful of papers have been published on proteomic analysis of uterine flushings in ruminants (3 on the bovine species(Faulkner, Elia et al. ; Gomez, Caamano et al. ; Munoz, Corrales et al.) and 1 on the ovine species (Koch, Ramadoss et al. 2010). However, none of the previous papers have completed a comprehensive temporal analysis during the establishment of pregnancy time period. The paper by Koch et al. analyzed uterine flushings from Day 16 ewes of the estrous cycle and of pregnancy. When we compared proteins found herein with the Koch study there were some similarities in protein expression. The proteins that were similar between the studies were apolipoprotein A1, BCL2-like 15 and transgelin. New analysis herein identified

several metabolites and additional proteins that may play a role in the establishment of pregnancy.

METABOLOME IN UTERINE FLUSHINGS

Fatty acid transport in the cell

Carnitine, was first detected in the early 1900's and was largely ignored by scientists until its role in fatty acid metabolism was later established (Bremer 1983). Carnitine can be reversibly acetylated by acetyl coenzyme A and because of this fact it was suggested that carnitine transports activated fatty acids (Bremer 1983). Presently, carnitine is known to be a carrier of activated fatty acyl groups across the inner mitochondrial membrane (Indiveri, Iacobazzi et al. 2011). Fatty acids are transferred from acyl-CoAs to carnitine and cross the outer membrane of the mitochondria, which is permeable to small molecules (Zeth and Thein). From there, fatty acids are translocated through the inner membrane of the mitochondria by the carnitine/acylcarnitine carrier in exchange for free intramitochondrial carnitine (Indiveri, Iacobazzi et al. 2011). Transport of fatty acids is important during pregnancy in the context of progesterone production by the corpus luteum. In 1983 a study done by Tialoska et al. (Tialowska, Klimek et al. 1983) demonstrated that carnitine in addition to oleic acid, bovine serum albumin, CoA, ATP and malate are supportive of progesterone production in human term placental mitochondria. This increase in fatty acids may also contribute to production of prostaglandins. For example, Arosh et al (Lee, McCracken et al. 2012) suggest that PGE2 is released into the uterine vein leaves the uterine vein via prostaglandin transporter and then has luteotrophic action on the CL. Also Spencer and coworkers (Spencer, Forde et al. 2013) suggest that prostaglandins are produced

by the conceptus during early pregnancy, on Day 13, and contributes to regulating gene expression in the endometrium. However, there is evidence indicating that a major metabolic pathway for prostaglandins (β oxidation) is dependent on the presence of carnitine (Johnson, Davison et al. 1972). *Thus the interaction of carnitine and prostaglandin synthesis during pregnancy needs to be further delineated.*

Exotic steroids affect mammalian pregnancy

Ecdysteroids represent the most widespread steroid hormones on earth, it is the steroid hormone of insects, crayfish, spiders and other arthropods (Lehmann and Koolman 1989). Ecdysteroids have not been thought of as products of mammalian metabolism (Dinan and Lafont 2006). They have been detected in mammalian tissues and fluids due to dietary intake of ecdysteroid-containing plants such as spinach and quinoa or by consumption of insects. However, most crop plants species do not contain phyto-ecdysteroids. Consequently, it is highly unlikely that ecdysteroids are actually present in uterine flushings from pregnant ewes when compared to non-pregnant ewes especially since ewes were on the same dietary regiment. The actual mammalian steroid is identical to ecdysteroids, at least as determined by mass in global mass spectroscopy analysis. In this case, the ecdysteroid may be a unique mammalian steroid metabolite. Regardless of actual nomenclature or name, physiologically the detection of ecdysteroids is logical in pregnant animals since ecdysteroids have been shown to have anti-apoptotic effects on mammalian cells (Oehme, Bosser et al. 2006). ecdysteroids were shown to inhibit Caspase-8 activation and cause up-regulation of anti-apoptotic Bcl-2 family member Bcl-xl. Muristerone A an ecdysteroid mimetic was shown in an additional study to be able to activate the same survival pathway, the

PI3K/AKT signaling pathway, by increasing AKT-Ser473 phosphorylation (Osaki, Oshimura et al. 2004). Previously PGF the luteolytic signal, has been shown in vivo to suppress AKT phosphorylation in the Corpus Luteum (Arvisais, Hou et al. 2010) which produces progesterone resulting in maintenance of early pregnancy in ruminants. Thus, ecdysteroids could be providing a luteoprotective role during early pregnancy by stimulating phosphorylation of AKT and production of anti-apoptotic protein Bcl-xl. Additionally the amino acid leucine, which is another known activator of AKT was also found within this study.

Amino acids in uterine flushes

Two amino acids, N-acetyldileucine (leucine substructure) and valine were identified in the present study as being up-regulated in uterine flushings on Day 14 and 16 of pregnancy when compared with Day 12 of pregnancy and cyclicity. Amino acids are a vital part of the histotroph that provides nutrients to the growing conceptus and serve as essential precursors for many biologically active substances (Kim, Burghardt et al. 2011). Leucine and valine have previously been identified in uterine flushes in a study done by Gao et al. (Gao, Wu et al. 2009). This study revealed that leucine is up-regulated between Days 10-16 and valine is up-regulated on Days 13-16 of pregnancy when compared to cycling ewes. Primary ovine trophectoderm isolated from Day 15 conceptuses stimulated with leucine has been shown by Kim et al. 2011 (Kim, Burghardt et al. 2011) to also stimulate cell proliferation and migration of conceptus trophectoderm during peri-implantation via activation of the AKT1-MTOR/FRAP1-RPS6K-RPS6 cell signaling cascade. Additionally Kim et al. 2013 identified leucine in greater concentrations in uterine luminal fluids of pregnant gilts on Days 12 and 15 but

not in cyclic gilts (Kim, Song et al. 2013). This same study stimulated proliferation of porcine trophectoderm cells from day 12 conceptuses through activation of the MTORRPS6K-RPS6-EIF4EBP1 signaling transduction pathway. Additionally valine has been shown to have increased expression in bovine uterine fluid (Hugentobler, Sreenan et al.) due to progesterone stimulation which is vital to maintenance of pregnancy. The combination of these studies helps to reveal the cross species importance of amino acids in the histotroph.

Pregnancy markers in the proteome

COL1A1 (2 chains) and COL1A2 (1 chain) from monomeric type 1 collagen, which is the most abundant vertebrate protein (Okuyama 2008). Of the markers for pregnancy status detected in uterine flushing, COL1A1 and COL1A2 are expressed at the earliest time, Day 12 compared to all other markers. COL1A1 and COL1A2 were expressed in higher amounts on Day 12 of the estrous cycle compared to Days 12, 14, or 16 of pregnancy. Herein Type I collagen is described as a marker for a non-fertilized ewe not as a marker for failed fertilization. The endometrium undergoes significant functional changes and remodeling during the estrous cycle and pregnancy (Bauersachs, Mitko et al. 2008). The endometrium of ruminant unlike humans does not undergo sloughing (Senger 2003), however is still remodeled in the absence or presence of a conceptus. In the case of this study we hypothesize that the absence of Type 1 collagen on Days 14 and 16 of pregnancy occurs after pregnancy specific remodeling of the endometrium has occurred. It is postulated that Type 1 collagen is absent in failed pregnancies, however this requires further analysis of endometrium from pregnant ruminants with lost embryos to conclude with any conviction that Type 1

collagen is a marker of failed fertilization. Yamada et al. identified a change in the distribution of bovine type 1 Collagen by Day 14 when comparing the estrous cycle and pregnancy (Yamada, Todoroki et al. 2002). They observed the endometrial extracellular matrix from Day 0 to 30 and described type 1 Collagen fibers in pregnant animals as having a thinner consistency than those of the estrous cycle and having a thicker intertwinement within the epithelium during the implantation period. If this relationship is truly conserved across species further development with this protein could be elaborated into a test to determine if a dairy cow is open 2 weeks prior to ultrasound and allow the cow to be set up for artificial insemination/breeding, reducing costs to the producer.

Interconnectivity of Profilin 1, S100a11, the Annexin family and IFNT expressed in the uterine flushings of pregnant ewes

PFN1 is G-actin binding protein that is crucial for normal cell proliferation and cell differentiation (Jockusch, Murk et al. 2007). PFN1 is involved in actin based cell motility, cytokinesis, neuronal differentiation and regulation of membrane trafficking and nuclear transport (Yarmola and Bubb 2009). Menkhorst et al. demonstrated that decidualized culture media up-regulated PFN1 levels in human extravillous trophoblast condition media, suggesting that decidualized culture media induced PFN1 secretion (Menkhorst, Lane et al. 2012). Menkhorst et al. identified another protein Annexin A2 that will be further discussed below.

Women with failed pregnancies have been found to have significantly lower concentrations of S100A11 (Liu, Ding et al. 2012). Knockdown of endometrial S100A11 in mice resulted in reduced embryo implantation rates; adverse expression of factors

related to receptivity of the endometrium and in human endometrial cells caused an immune response. S100A11 plays a critical role in epidermal growth factor stimulated adhesion, receptivity of the endometrium and immunotolerance by regulating intracellular calcium uptake and release. Calcium signaling plays a key role in the regulation of many cellular processes and the Annexins are of class of proteins that are regulated by calcium (Gerke, Creutz et al. 2005). Annexins have long been associated with the S100 protein family (Rintala-Dempsey, Rezvanpour et al. 2008). Annexins are only able to bind to S100 protein family via the N-terminal region after calcium binding. Annexins have been associated with a wide range of actions. Annexin A2 plays a role in membrane organization and calcium dependent organization of lipid rafts (Babiychuk and Draeger 2000). Annexin A1 is able to modulate the release of arachidonic acid (Flower and Rothwell 1994) (an essential fatty acid found in membrane phospholipids) by regulating phospholipase A2 activity (Flower 1988). Arachidonic acid conversion to PGG₂/PGH₂ is the rate limiting step in prostaglandin production (Olofsson and Leung 1994). Regulation of prostaglandins is extremely important during pregnancy since prostaglandin F₂ α (PGF) is the luteolytic signal and prostaglandin E₂ is thought to be the luteotropic signal (Nett, McClellan et al. 1976; Huie, Magness et al. 1981; Magness, Huie et al. 1981; Levasseur 1983). Therefore release of arachidonic acid by Annexin A1 is extremely important. It was long believed that Annexins were intracellular proteins; however in the last decade it has been shown that Annexins (Annexin A1 and A2) can be transported out of the cell in unconventional secretory mechanisms (Faure, Migne et al. 2002; Chapman, Epton et al. 2003; Danielsen, van Deurs et al. 2003). Surface and secreted Annexin A2 interacts with proteases and cell matrix to regulate adhesion and

cell migration (Paradela, Bravo et al. 2005; Sharma, Ownbey et al. 2010; Yan, Ding et al. 2011). Annexin A2 was identified in in vivo syncytiotrophoblasts previously (Kaczan-Bourgois, Salles et al. 1996; Paradela, Bravo et al. 2005) and since it has been shown to previously be required for invasion and metastasis in cancer it might have a role in conceptus invasion (Zheng, Foley et al. ; Sharma, Ownbey et al. 2010). During inflammation Annexin A1 has long been known to occur extracellularly (Gerke, Creutz et al. 2005). Exogenous administration of Annexin A1 in both in vitro and in vivo models has both inhibited neutrophil extravasation thereby limiting the degree of inflammation (Flower and Rothwell 1994; Perretti and Gavins 2003; Perretti and Flower 2004). Another member of the Annexin family that perpetuates the idea of immunotolerance/ protection is Annexin A5. Annexin A5 has previously been isolated from human placenta (Tait, Sakata et al. 1988) and shown to be necessary for maintaining placental integrity (Wang, Campos et al. 1999). Annexin A5 forms an anticoagulant (Rand 2000) protective shield on the apical surfaces (Mosser, Ravanat et al. 1991; Krikun, Lockwood et al. 1994) of the microvilli of syncytiotrophoblasts (Krikun, Lockwood et al. 1994) by binding phospholipid surfaces and forming two-dimensional crystallin arrays (Mosser, Ravanat et al. 1991; Voges, Berendes et al. 1994). Shu et al. indicated that there is decreased expression of Annexin A5 on trophoblasts of preeclamptic placentas, and that this decrease correlates with a rise in markers for blood coagulation (Shu, Sugimura et al. 2000). Overall this would facilitate the tolerance of the immunologically 'foreign' conceptus while allowing for activation of the immune system to defend against microbial and viral infections. Recently the comparisons of bovine endometrial gene expression during pregnancy (IFNT) and in response to intrauterine application of

human IFNA2 were compared to determine the effects of IFN (Bauersachs, Ulbrich et al. 2012). This study suggests during pregnancy that a fine-tuned regulation of immune cells occurs rather than a general suppression of the immune system (Bauersachs and Wolf 2013). Therefore the immune-regulatory role of Annexin A1 and A5 makes sense during pregnancy.

Another protein that induces immunological tolerance in several animal models and might have applications treating obesity and type 2 diabetes due to its anti-inflammatory properties is IFNT (Bazer, Kim et al. 2012). IFNT is the maternal recognition signal in ruminants (Hansen, Anthony et al. 1985; Austin, Ward et al. 1996; Johnson, Austin et al. 1998; Staggs, Austin et al. 1998; Johnson, Stewart et al. 2001; Spencer, Burghardt et al. 2004). IFNT released from the conceptus (Harrison, Kenny et al. 1987; Ashworth and Bazer 1989; Roberts 1989; Spencer, Burghardt et al. 2004) silences transcription of estrogen receptor alpha (ESR1) in uterine luminal and superficial glandular epithelia (Bazer, Wu et al. 2010). Silencing of ESR1 blocks ESR1-dependent transcription of the oxytocin receptor, thereby abrogating oxytocin induced luteolytic endometrial pulses of PGF. IFNT causes the up-regulation of several interferon stimulated genes during pregnancy (Austin, Ward et al. 1996; Johnson, Austin et al. 1998; Ott, Yin et al. 1998; Johnson, Austin et al. 1999; Johnson, Spencer et al. 1999; Johnson, Spencer et al. 2000; Johnson, Stewart et al. 2001; Bott, Ashley et al. 2010). The mechanism for maternal recognition of pregnancy varies among mammals, but the up-regulation of Interferon Stimulated Gene 15 (ISG15) in the endometrium appears to be a universal response to the presence of an embryo; as seen in humans, baboons, cows, sheep, swine and mice (Austin, Carr et al. 2004). The exact actions of

these ISGs is still to be determined however, ISG15 can conjugate to and regulate proteins through an enzymatic pathway similar to that described as ubiquitin, utilizing the ubiquitin-activating enzyme 1-like protein (Rempel, Francis et al. 2005). Another trophoblast specific protein was identified herein this study Trophoblast Kunits Domain Protein 1 (TKDP1).

TKDP1 is a highly expressed placenta-specific protein that shares an almost identical expression pattern with IFNT (Roberts et al 2007). TKDP1 is a protease inhibitor, but its function in placental development is abstract currently, MacLean suggested that TKDPs may modulate ion channels (MacLean, Roberts et al. 2004).

CONCLUSIONS

For pregnancy to be recognized and maintained, the uterine environment must be in synchrony with embryo development. This occurs through primary signaling from the conceptus which cooperates with many uterine responses to pregnancy that are primed by exposure to progesterone. Many interesting metabolites and proteins were described in histotroph that may play key roles in the establishment and early detection of pregnancy identified. Further examination of these metabolites and proteins may provide insight on the proper uterine environment for successful maintenance of pregnancy. Future directions for the research will be to confirm presence of these metabolites by gas chromatography and presence of the proteins with antibody-based detection (western blot or RIA) or mass spectroscopy detection in the endometrium and conceptus. Also, on Day 16 of pregnancy and Day 12 of the estrous cycle, new samples of uterine vein blood and jugular vein blood will be examined in future studies to determine if pregnancy or estrous cycle associate products are release from the uterine

lumen into the uterine vein and are detectable in jugular vein blood to provide insights and blood serum diagnostics for the presence or absence of a viable embryo.

CHAPTER 5

DETECTION OF INTERFERON-TAU IN BLOOD FROM PREGNANT DAIRY COWS USING RADIOIMMUNOASSAY

CHAPTER SUMMARY

A sensitive radioimmunoassay (RIA) for bovine and ovine interferon tau (IFNT) has been developed. Potentially this assay has application for use in all ruminant species. IFNT is released by the ruminant conceptus and historically was thought to be sequestered in the uterine lumen with no release into the systemic circulation in detectable amounts. IFNT is produced only by the trophoblast cells of the ruminant embryo. For this reason it is a very specific marker for presence of a conceptus (embryo proper and extraembryonic trophoblast). Recently, antiviral activity was described to be greater in uterine vein blood from Day 15 pregnant compared to non-pregnant sheep. Preabsorption of uterine vein blood from Day 15 pregnant sheep with antibody against IFNT ablated antiviral activity leading to the conclusion that the active antiviral cytokine in uterine vein blood was IFNT. Detection of IFNT in uterine vein blood by Day 16 of pregnancy in sheep using RIA and by mass spectroscopy approaches confirmed this conclusion. To date, IFNT has not been found in peripheral blood in sheep or cattle during early pregnancy. The present experiments tested the hypothesis that lack of detection of IFNT in peripheral blood is because of sensitivity issues in the assays used to detect IFN, rather than not being present. Through optimizing primary anti-IFNT antibody dilution, reducing background in the assay serum protein interference and using freshly collected serum, we demonstrate detection of IFNT in blood from cattle by Day 18 of pregnancy. Detection of IFNT in tail vein or jugular vein blood is novel and

significant in context of application for identify cattle carrying a conceptus. However, because embryo mortality in dairy cows is >43%, the greatest utility is identification of cattle that are not carrying an embryo and which facilitates immediate management to induce estrus and ovulation.

INTRODUCTION

Interferon tau (IFNT) is a cytokine released from the ruminant conceptus early in establishment of pregnancy. Bovine IFNT exists as a glycoprotein with two primary molecular weight classes (22,000 and 24,000 Da) and 4-5 isoforms: pI = 5.6-6.6 (Plante, Hansen et al. 1990), or pI=6.3-6.8 (Imakawa, Hansen et al. 1989); whereas ovine IFNT is not glycosylated, has an estimated molecular weight of 18,000 and a pI of 5.5 to 5.8 (Imakawa, Anthony et al. 1987). The gene encoding IFNT is not found in humans, pigs, horses, or non-ruminant species. For this reason, IFNT is a very specific marker for ruminant embryos. The primary paracrine role of IFNT during early pregnancy is antiluteolytic and mediated through disruption of prostaglandin F₂-alpha release from the uterus. For the last three decades IFNT was thought to have only paracrine function through binding to receptors on the maternal endometrium of the uterus (Godkin, Bazer et al. 1984; Bazer, Vallet et al. 1986). This restriction of detection and action of IFNT within the uterine lumen was based on lack of detection of antiviral activity in peripheral blood, which is a bioassay for IFN, and more recently was based on lack of detection of IFNT in blood using antibody-based detection methods (i.e., RIA; See Chapter 3).

However, ISG mRNAs are up-regulated in peripheral blood mononuclear cells in response to pregnancy in both sheep (Yankey, Hicks et al. 2001) and cattle (Han,

Austin et al. 2006; Gifford, Racicot et al. 2007). For example, at least 674 genes were up-regulated and 721 genes were down-regulated in blood cells in response to pregnancy on day 18 in cattle (Hansen, Henkes et al. 2010). The induction of ISGs in blood cells was intriguing, especially in light of opinion that the conceptus acted locally in paracrine manner as an anti-luteolysin. To explain up-regulated ISGs in PBMC by IFNT, lymph nodes draining the uterus (iliac) and the head (submandibular) from Day 15 pregnant ewes were examined and found not to differ in ISG expression, suggesting that IFNT was not released into the uterine lymphatics and this was not a pathway through which IFNT induced ISGs in PBMC. Uterine vein blood from pregnant or non-mated, non-pregnant sheep was examined for antiviral activity to evaluate the possibility that IFNT was released systemically from the uterus. Interestingly, on Day 15 pregnant ewes have higher antiviral activity in uterine vein blood; this was equivalent to $\sim 200 \mu\text{g}$ ($2 \times 10^7 \text{ U}$)/24 h (Oliveira, Henkes et al. 2008). Because antiviral assays are general to IFN, pre-adsorption with antibody against IFNT was used to confirm that this antiviral activity was caused by IFNT and not some other Type I or II IFN. More recently, IFNT has been detected in uterine vein blood using RIA (Chapter 3; Fig. 5.1) and mass spectroscopy approaches. For these reasons, the present experiments tested the hypothesis that lack of detection of IFNT in peripheral blood is because of sensitivity issues in the assays used to detect IFN, rather than IFNT not being present in peripheral blood.

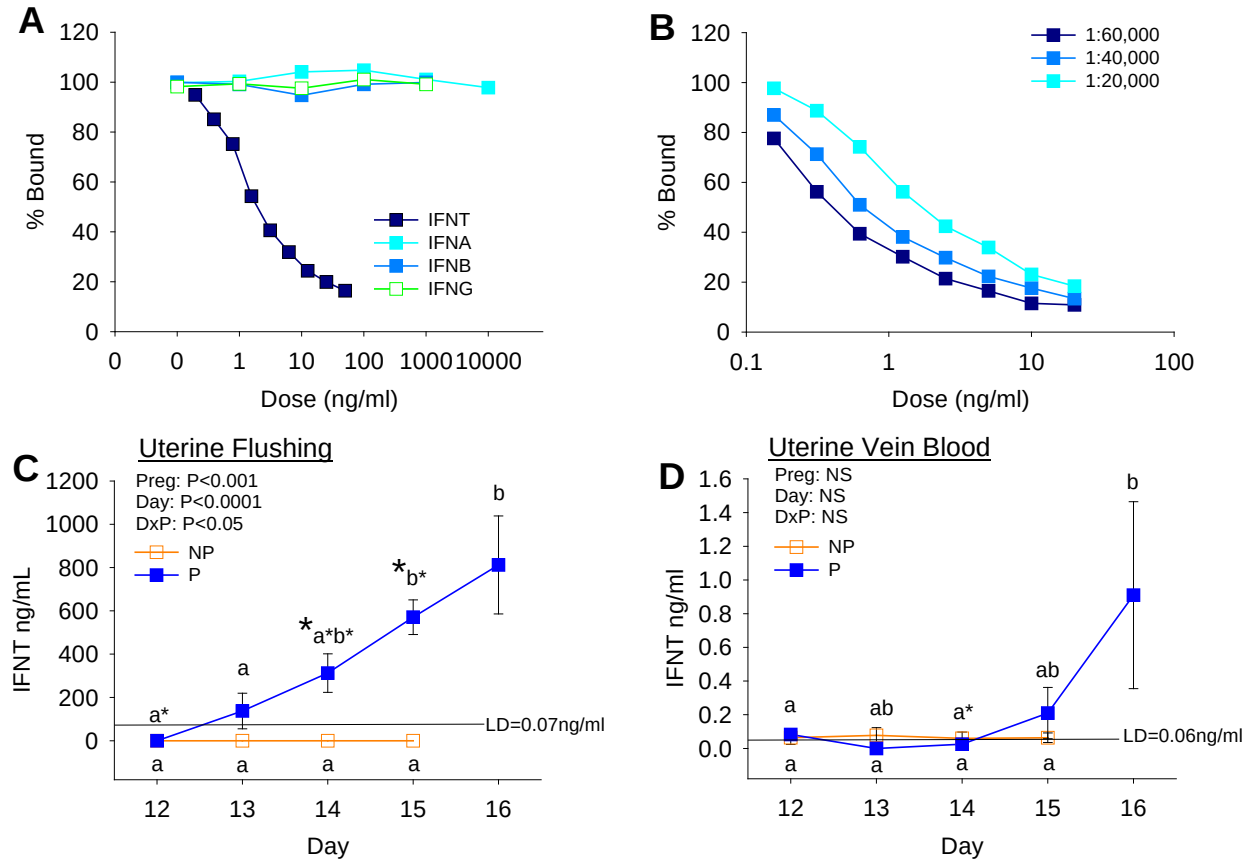


FIGURE 5.1.

Validation of IFNT RIA for use in detecting IFNT in uterine flushing and uterine vein serum. Increasing concentration of IFNT competed with radiolabeled IFNT (A). Notice that addition of other type I (alpha or beta) or type II (gamma) IFN had no cross-reactivity with the anti-IFNT antibody even at 1 microgram protein. Increasing dilution of primary anti-IFNT antibody improved sensitivity of the assay, although the range of detection was compromised to achieve this sensitivity (B). IFNT was detected in uterine flushings at concentration of ~140 ng/ml to 800 ng/ml from Days 13-16 of pregnancy and was not detected in uterine flushings from non-pregnant ewes (C). IFNT was first detected in uterine vein blood by Day 15 and increased to about 0.9 ng/ml by Day 16 of pregnancy (D).

METHODS

Radioimmunoassay for IFNT

A double antibody radioimmunoassay (RIA) for recombinant ovine (ro) IFNT was developed at Colorado State University (Antoniazzi et al., unpublished results) by using roIFNT protein and anti-roIFNT polyclonal antibody (1:60,000) from Dr. Fuller Bazer (Texas A&M University). Second antibody was anti-rabbit gamma globulin (generated at Colorado State University), which was diluted 1:25. Briefly, roIFNT was radioiodinated with ^{125}I using chloramine T procedure and purified using column chromatography (Sephadex G25; GE Health Care) using methods generally described previously by Niswender et al., (Niswender, Reichert et al. 1969). Standards in the assay were spiked with 200 μL of ram serum to increase sensitivity. Anti-roIFNT antibody was added to concentrated serum samples, vortexed and incubated at 4°C for 24 hours. Radioactive roIFNT was added, vortexed and incubated for 24 hours at 4°C followed by incubation with secondary anti-rabbit gamma globulin antibody at 4°C for 72 hour. The assay was then terminated with 3 ml of cold PBS and centrifugation at 2800 rpm for 30 minutes. Supernatant was removed and radioactivity of the pellet was quantified using a gamma counter.

IFNT Peptide and Glycosylation Identification after Trypsin Digest

To determine IFNT peptides that are conserved across the ovine and bovine species after trypsin digestion, the proper sequence were located utilizing UNIPROT (www.uniprot.org) and placed into ExPASy peptide cutter program (http://web.expasy.org/peptide_cutter/). From there the peptides were placed into ExPASy glycomod program (<http://web.expasy.org/glycomod/>) to identify glycosylated

amino acids in the bovine amino acid sequence that would increase the mass of the protein. From there the amino acids were compared for conservation between the species and an absence of glycosylation.

RESULTS

Radioimmunoassay Identification of IFNT in Peripheral Blood

Utilizing the assay with serum added to the standard we were able with 100% accuracy to determine if a dairy cow was pregnant by measuring IFNT in Tail blood samples collected on Day 19 after artificial insemination (Fig 5.2). In cows that were bred but did not calve we determined that 3 were not pregnant and 2 appeared to be pregnant at Day 19 but later lost the conceptus (Fig 5.2). In aims to improve detection methods, reduce cost and analysis time due to analysis with RIA, protein enrichment, mass spectroscopy and gas chromatography methods are being developed.

Identification of 3 Conserved IFNT Peptides that are not Glycosylated

Utilizing the ExPASy modeling software we were able to trypsin digest ovine and bovine IFNT and determine conserved amino acid sequences between the two (Table 5.1). This software was also used to determine amino acids that have the potential to be glycosylated in bovine IFNT and change the molecular mass of the peptide (Table 5.2). Utilizing these approaches 3 unique and conserved peptide sequences were identified for bovine and ovine IFNT that can be utilized to detect IFNT using mass spectroscopy and gas chromatography identification in serum (Table 5.3).

Protein Enrichment Prior to Identification

The acidic isoelectric point of IFNT allows for it to be enriched from serum through binding to a strong anion exchanger at pH of 8.2. IFNT was first purified from

Serum IFNT (pg/ml)			
Non-Pregnant		Pregnant	
Cow	IFNT	Cow	IFNT
358	37.15	709	40.49
2132	16.12/ ¹ ND	247	38.25
3216	9.46/ND	2824	77.15
234	13.59/ ¹ ND	499	29.56
240	45.15	1193	46.84
Ave	24.29	Ave	46.46

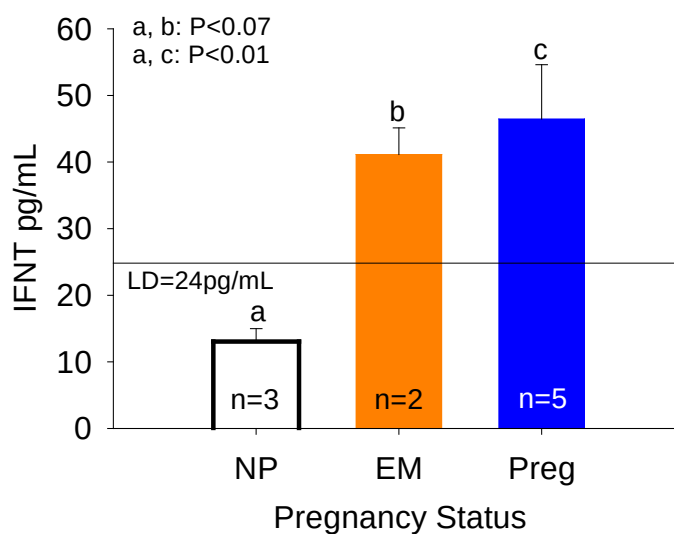


FIGURE 5.2.

Pregnancy status of Day 19 dairy cows identified by blood test for IFNT. Concentration of IFNT (pg/mL) in Day 19 tail blood from NP (Non-pregnant) cows exposed to semen, cows that were NP to term but had detectable limits at Day 19, and possibly had a fetus but lost it (Embryonic Mortality; EM) and P (Pregnant) cows that calved. LD is the limit of detection for this assay. Letters signify $P < 0.05$ and * is $P < 0.1$.

TABLE 5.1. Conserved IFNT Peptide Amino Acid Sequences

Sheep		Bovine	
AA sequence	Mass (Da)	AA sequence	Mass (Da)
ENLR	530.581	ENLR	530.581
LLDR	515.610	LLAR	471.600
MNRPSPHSCLQDR	1540.735	MNR	419.449 (1584.781)
		LSPHPCLQDR	1165.332
K	146.189	K	146.189
MDPIVTVK	902.117	MGPIITVK	858.107
YFQGIHDYLQEK	1540.695	YFQGIHVYLK	1267.493
VEMMR	664.836	VEMMR	664.836
ALTSSTTLK	921.059	ALSSSTTLQK	1035.162

TABLE 5.2. Glycosylated Amino Acids in Conserved IFNT Sequences

Bovine	
AA sequence	Mass (Da)
ENLR	530.581
LLAR	471.600
MNR	419.449
LSPHPCLQDR	1165.332
K	146.189
MGPILTVK	858.107
YFQGIHVYLK	1267.493
VEMMR	664.836
AL SS STTLQK	1035.162

TABLE 5.3. Non-glycosylated Conserved Peptide Sequences for Mass Spectroscopy and Gas Chromatography Identification of IFNT

Sheep		Bovine	
AA sequence	Mass (Da)	AA sequence	Mass (Da)
ENLR	530.581	ENLR	530.581
K	146.189	K	146.189
VEMMR	664.836	VEMMR	664.836

conceptus secretory proteins by using similar DEAE anion exchange chromatography (Godkin, Bazer et al. 1982). In order to achieve a 5-fold enrichment (assuming 100% recovery), 1 ml serum from jugular blood was diluted in 14 ml Tris buffer (pH 8.1) in order to obtain a final pH of 8.2 for binding to the anion exchange matrix (Fig 5.3A). This buffered and diluted bovine serum was pre-filtered using a 0.45 μ m filter (Millipore) and then loaded and centrifuged (500x g; 5 min) through the Pierce Strong Ion Exchange columns (anion exchange) spin column (Fig 5.3B). Proteins with no affinity to the matrix were removed through washing with 10 ml of Tris pH 8.2 and then the columns were loaded with 10 mL of 0.025 M Tris pH 8.2/0.15 M NaCl and spun again. This first salt cut was predicated to not appreciably impact amount of IFNT bound to the column and if this is the case, then future studies will entail loading and washing columns with 0.15 M NaCl. The flow through was collected and the columns were then loaded with 0.025 M Tris pH 8.2/0.3M NaCL and the flow through was collected again, which is predicted to contain IFNT. Finally, the columns were loaded with 0.025 M Tris pH 8.2/2M NaCL and the flow through was collected to ensure complete elution of IFNT. Fractions were then desalted using 3,000 Da columns from Millipore (Amicon ultracentrifuge filter) and resuspended in 200 μ l RIA buffer. All collection fractions are stored frozen in preparation for analysis in the IFNT RIA.

DISCUSSION

Increased antiviral activity caused by pregnancy has not been observed in systemic blood collected from ruminants during early pregnancy until the present experiments. However, one report in 1991 (Schalue-Francis, Farin et al. 1991) described an antiviral assay with a sensitivity of ~1 unit/ml that could not detect IFNT in

detection of IFNT in

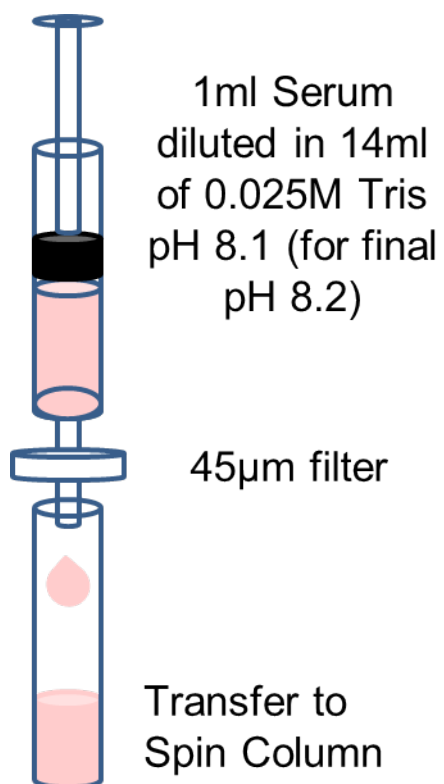


FIGURE 5.3A.

Methods for Enrichment of IFNT from Serum based on Molecular Properties of IFNT

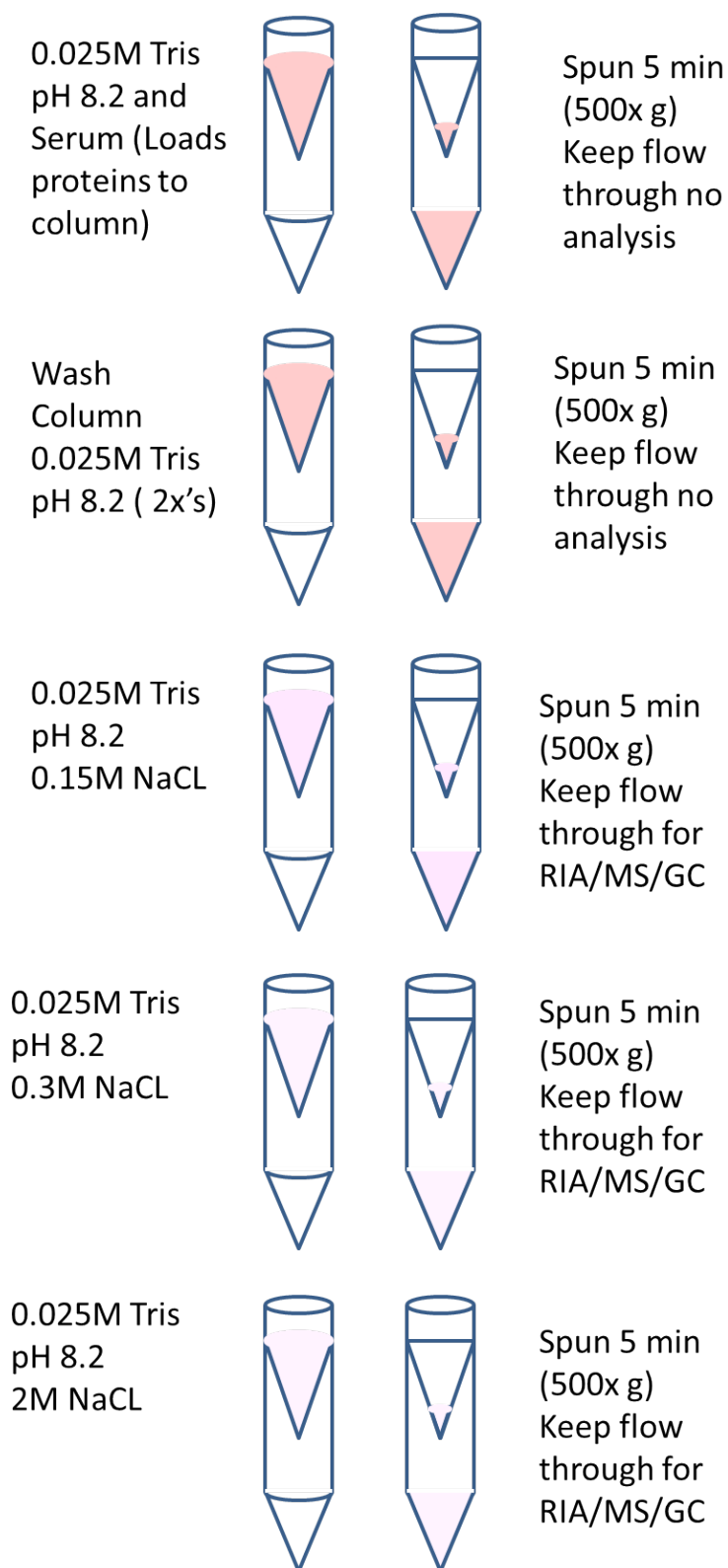


FIGURE. 5.3B.
Methods for enrichment of IFNT from serum based on molecular properties of IFNT

jugular vein blood, but was efficacious in detecting 58 U/ml uterine vein serum from Day 15 pregnant sheep. The 58 U/ml is equivalent to 7.25 ng/ml based on the 8×10^8 U/mg IFN standard used in this study. The conclusion that IFNT was not detectable in jugular vein blood also was based on antibody-based ELISA and RIA.

For example, an RIA for IFNT was developed by others with sensitivity of detecting 6.1-7.8 ng IFNT/ml (Takahashi, Takahashi et al. 2005). In this study, a sensitivity of 6.1 ng/ml was reported, but the lowest standard used in the assay was 7.8 ng/ μ l with binding of ~95%. Use of this RIA by these authors revealed detection of IFNT in uterine flushings representing Day 16 of bovine pregnancy. There was no report of attempting to detect IFNT in bovine blood by these authors.

A more recent study by our group also found detectable antiviral activity in uterine vein blood from Day 16 pregnant sheep, with no detection of antiviral activity in systemic blood (Oliveira, Henkes et al. 2008). By blocking antiviral activity in uterine vein blood by preadsorption with antibody against IFNT (Bott, Ashley et al. 2010). we demonstrated that the antiviral activity was attributed specifically to IFNT and not other type I IFN through The amount of IFNT in uterine vein blood on Day 16 of pregnancy was ~500-1,000 U/ml, which we estimated to be 5-10 ng/ml using a 1×10^8 U/mg IFN standard (Oliveira, Henkes et al. 2008). Indirect evidence that IFNT is released uterine vein and has a systemic-endocrine role during pregnancy was demonstrated by up-regulation of interferon-stimulated genes (ISGs) in peripheral blood cells [see: (Han, Austin et al. 2006; Gifford, Racicot et al. 2007; Oliveira, Henkes et al. 2008)]. Based on these studies, it was concluded that IFNT was produced by the conceptus during early stages of pregnancy, attenuated PGF release from the endometrium, but also was

released into the uterine vein in high enough concentrations to possibly have a functional and biological effect on peripheral tissues such as blood cells, the corpus luteum and liver (Oliveira, Henkes et al. 2008; Bott, Ashley et al. 2010; Antoniazzi, Webb et al. 2013).

Data to suggest an endocrine role for IFNT (Oliveira, Henkes et al. 2008; Bott, Ashley et al. 2010) includes an induction of ISG15 and other ISGs in CL from Day 15 pregnant ewes when compared to non-pregnant ewes. Likewise, ISG15 protein and its ISGylated protein targets also were up-regulated in CL in response to pregnancy. Finally, based on microarray data we have demonstrated that many ISGs are up regulated in ovine CL in response to pregnancy (Romero, Antoniazzi et al. 2013)

Specificity of the assay was determine the (see Chapter 3) by first increasing concentrations of IFNT competing for binding with radiolabeled IFNT. Secondly specificity was confirmed with the addition of other type I (alpha and beta) or type II (gamma) IFN, resulting in an absence of cross reaction with the antibody used in this assay, even at a concentration of 1 microgram of protein (Reprinted for convenience from Chapter 3 here; Fig 5.1A). The sensitivity of the assay was fine-tuned by increasing the dilution of primary antibody (Fig 5.1B) which improved sensitivity ~10-fold. This RIA detected IFNT in uterine flushings at a concentration ranging from ~140 ng/ml to 800 ng/ml only in pregnant ewes on Days 13-16 (Fig 5.1C) and in uterine vein blood from pregnant ewes on Day 15 and increasing to a concentration of ~0.9 ng/ml on Day 16 in pregnant ewes (Fig 5.1D). Until the present experiments, IFNT has not been detected in peripheral blood during early pregnancy in ruminants.

This level of detection was improved to 24 pg/ml by adding serum from rams to the buffered standards used in this RIA. By reducing “matrix effect”, this resulted in improving sensitivity of detection such that IFNT was detected in 5/5 (100%) tail vein serum samples from pregnant cows on Day 18 of pregnancy. IFNT was not detected in tail blood in 60% of cows bled on Day 18 following AI and found to be open on Day 32 via ultrasound. These cattle probably never developed an embryo and were truly non-pregnant (or open). In 40% of cattle bled on Day 18 and found to be open on Day 32 via ultrasound, IFNT was detected in amounts greater than the threshold of this assay (24 pg/ml). These cattle may have developed an embryo with detectable IFNT in serum by Day 18 that was dead, dying or about to die. This 40% detection of IFNT on Day 18, with no detection of pregnancy by Day 32 is similar to general embryo mortality rates in cattle. Ongoing studies will significantly expand sample size to include ~130 cows that will be bled to generate serum on Days 17, 18, and 21 of pregnancy in order to confirm utility of the IFN RIA to identify open cows that can then be managed immediately (by Day 18) for return to estrus and ovulation rather than waiting another 14 Days to manage these cattle on Day 32 of pregnancy.

Detection of IFNT in tail vein or jugular vein blood is a novel finding and is a novel biomarker for a viable embryo as well as a pregnant animal. Lack of detection of IFNT is key in managing reproductive cycles, because this is indicative of a non-pregnant animal. Detection of pregnancy in cattle is typically done by transrectal ultrasonography. This technique is applicable in the field setting and should be able to determine pregnancies in cattle between Days 25-28 (Kahn, Fraunholz et al. 1990). Detection of pregnancy by ultrasonography prior to this time point is not reliably

accurate enough for large scale practice. The current gold standard for determining pregnancy status is through rectal ultrasound on Day 32 of pregnancy. Mechanisms allowing for earlier detection of pregnancy would allow for improved reproductive efficiency by reducing the interval between artificial insemination (Fricke 2002).

Determination of pregnancy by Days 17-18 in cattle following exposure to semen from a bull or following artificial insemination, via lack of detection of IFNT allows producers to make re-breeding decisions for non-pregnant (open) cows 14 Days earlier than waiting until Day 32 ultrasound. A blood test for Interferon-tau is ideal for earlier detection of pregnancy. Diagnosis of pregnancy status earlier than the industry standard of Day 32 of pregnancy would have great economic benefit for dairy and some beef cattle operations.

One freeze thaw of IFNT can impact protein structure(Pikal-Cleland, Rodriguez-Hornedo et al. 2000), biological activity (antiviral activity) and possible detection with antibody. Some of our serum samples have been frozen and thawed several times. For this reason, we are in the process of bleeding dairy cows on Days 0, 14, 18, 21 and 32 following AI. All cattle will be ultrasounded 32 Days following AI. We also are collecting serum from about 20 cows on Day 18 of the estrous cycle that were not exposed to semen to serve as true negative controls. All serum will be assayed in the IFNT RIA.

To increase sensitivity of the IFNT RIA, we are working on improvements to the assay. In addition to adding serum to the standards, some preliminary enrichment (purification) approaches are being evaluated. One approach to increase the effectiveness of this RIA would be partial purification of serum immediately following

collection from the cow, but prior to RIA. After considering biochemical properties of IFNT such as the acidic isoelectric point, we initiated studies to enrich IFNT from serum through binding to a strong anion exchanger at pH of 8.2. IFNT was first purified from conceptus secretory proteins by using similar DEAE anion exchange chromatography (Godkin, Bazer et al. 1982). These experiments are ongoing.

Other ongoing experiments include ELISA and RIA, lateral flow devices, microfluidics and mass spectroscopy approaches to detect IFNT in blood. Bovine IFNT is glycosylated and this property can be exploited in context of an alternative approach towards enriching serum for IFNT. Serum can be loaded onto a concanavalin A column and then all glycoproteins bound to this column in addition to IFNT can be eluted and then introduced into the RIA as described above. This approach might be considered in the event that the anion exchange approach becomes problematic. Other more crude approaches to slightly enrich serum for IFNT might entail simple ammonium sulfate precipitation or possibly acetone precipitation.

Likewise, a mass spectroscopy approach will be evaluated for direct analysis of IFNT peptides in serum from ruminants. For this approach to be feasible, it must be able to detect and quantify peptides from the bovine glycoprotein [(MW ranging from 22 to 24 kDa because of variation in sugar residues (Anthony, Helmer et al. 1988)] at circulating serum concentrations ranging from 30 to 100 pg/ml. Based on previous analysis using selected reaction monitoring (SRM) of peptides in complex biological matrices, sensitivities in the range of .001-1 femtomol/mL are feasible. Ultimate sensitivity will depend on peptide ionizability (sequence dependent) and matrix effects at time of chromatographic elution.

The novel detection of IFNT in tail blood as a biomarker for a viable embryo/ pregnancy has the ability to revolutionize the detection of pregnancy in the agricultural industry. This technique can be used to detect pregnancy in cattle up to 14 Days earlier than ultrasonography depending on detection method. Protein enrichment of serum may permit detection of pregnancy even earlier than Day 18.

Ultimately the development of approaches to enrich IFNT in blood samples by using ion exchange for example (Fig. 5.3A-B.) or different methods of detection such as mass spectroscopy or gas chromatography will allow for a rapid (1-2 Day) Ultimately further development of this technology will save producers money allowing them to cull cows from the herd that are not pregnant and set them up for another round of natural or artificial insemination earlier than previous detection methods allowed, thus increasing the efficiency of our dairy and beef cattle industries.

CHAPTER 6

OVERALL SUMMARY AND FUTURE DIRECTIONS

Several key discoveries related to determination of pregnancy as well as advancements in the knowledge of the proper uterine environment for establishment and maintenance of pregnancy are described herein. Genes involved with early spontaneous regression of the CL that occurs during the late luteal phase of the estrous cycle were identified. Microarray analysis of CL gene expression corresponding with this time period revealed differential expression of at least 683 genes that encode proteins with function in cell adhesion, chemokine signaling, cytokine action, cytoskeletal remodeling, apoptotic and cell life pathways. *SERPINE1* and *THBS1* genes were selected for additional study and were found to be transiently up-regulated during early luteolysis. *SERPINE1* and *THBS1* may regulate the extracellular matrix (Khan and Falcone 1997; Huang, Border et al. 2006) aiding in the invasion of immune cells into the CL as well as inhibition of progesterone synthesis. Future studies on how the CL regresses will focus on the TGFB pathway, primarily because *SERPINE1* and *THBS1* have both been linked to the TGFB pathway and the TGFB pathway has 19 genes that are differentially expressed during luteolysis.

Historically it was believed that early pregnancy was maintained exclusively through the paracrine action of the conceptus on the ruminant endometrium. However; microarray analysis also revealed differential expression of 55 genes in CL between P and NP ewes on Day 12, 21 genes between Days 12 and 14 of pregnancy, and 734 genes between P and NP ewes on Day 14. Many of these genes were ISGs. Pregnancy/ conceptus- derived IFNT maintains/induces *IL-6*, *VEGFA*, *LHR*, *ISG15*,

MX1 and *PTX3*, whereas luteolysis causes down-regulation of these genes (*ISG15*, *MX1*, *IL-6*, *PTX3*, *LHR* and *VEGFA*). From these data it is concluded that pregnancy-associated gene expression occurs in the CL.

The maintenance of a healthy conceptus is a complicated process that involves cell proliferation, differentiation and activation of the steroidogenic pathway for continued progesterone production as well as immune regulation and activation of interferon signaling. There are several conceptus secretory products that might drive differential gene expression in the CL, however IFNT is the primary candidate based on induction of ISGs in the CL during pregnancy and following culture of isolated luteal cells with IFNT.

The paracrine actions of IFNT in the endometrium temporally involve induction of *ISG15* mRNA expression as early as Day 13 of pregnancy and in extra-uterine tissues (endocrine action) on Day 14. Direct action of IFNT on the CL may be involved with luteal protection/ resistance against luteolytic pulses of PGF. Prolong luteal life span by activation of antiapoptotic and cell survival genes may induced by IFNT. Ultimately, luteal survival may be driven by ISG action. For example, *ISG15* may alter luteolysis/antiluteolytic/cell survival signaling pathways by conjugating to intracellular proteins. These hypotheses are subjects of ongoing and future experiments.

Several advancements on the work completed herein have the potential for developing a diagnostic for early pregnancy in ruminants at a much earlier time point than is possible using current ultrasound technology. Specifically, mass spectroscopy analysis of uterine flushings revealed several key metabolites and proteins of interest. Further examination of these metabolites and proteins may provide insight on the

proper uterine environment for successful maintenance of pregnancy as well as serving as possible markers for determining pregnancy status. Confirmation of these metabolites by gas chromatography and presence of proteins in uterine flushing, uterine vein blood and systemic (i.e., jugular or tail vein) blood with antibody-based detection (western blot or RIA) is currently ongoing. Future studies include identification of these metabolites and proteins in serum.

Ultimately the best biomarker for a viable embryo/pregnancy would be IFNT, and the novel detection of IFNT by RIA in tail blood is very encouraging. The use of IFNT as a biomarker has the potential to detect pregnancy up to 14 days earlier than ultrasonography depending on detection method. Either method ultimately saves producers money by allowing them to separate cows from the herd that are not pregnant and set them up for another round of insemination, prior to Day 32, thus increasing the efficiency for of our dairies and beef cattle producers. We believe that the experiments included in this dissertation can lead to the development of several new diagnostics and possible therapeutic approaches for managing reproduction in large animals and will also provide additional general knowledge of pregnancy.

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