DISSERTATION

# THE ROLE OF INTERFERON-TAU (IFNT) IN LUTEAL GENE EXPRESSION, STEROIDOGENESIS, AND LUTEAL LIFESPAN IN THE EWE

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY REBECCA CLARK BOTT ENTITLED THE ROLE OF INTERFERON-TAU (IFNT) IN LUTEAL GENE EXPRESSION, STEROIDOGENESIS, AND LUTEAL LIFESPAN IN THE EWE BE ACCEPTED AS FULLFILING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

THE ROLE OF INTERFERON-TAU (IFNT) IN LUTEAL GENE EXPRESSION, STEROIDOGENESIS, AND LUTEAL LIFESPAN IN THE EWE

Interferon-tau (IFNT) was evaluated for endocrine actions on the corpus luteum (CL). The hypothesis was that infusion of IFNT would increase luteal expression of interferon-stimulated gene (ISG)-15, and the length of time for ewes to return to estrus. Osmotic pumps containing 200 μg IFNT or BSA (n=12 each) were connected to the uterine vein of non-pregnant ewes 10 days post-estrus. Messenger RNA encoding ISG15 was elevated in CL from pregnant and IFNT-infused ewes ($P<0.05$) compared to non-pregnant and BSA-treated ewes, respectively. Luteal mRNA encoding ISG15 from ewes treated with IFNT was greater than in ewes treated with BSA ($P<0.05$). Serum concentrations of progesterone were not different in ewes that received infusions of BSA or IFNT. Progesterone decreased by six hours ($P<0.05$) in ewes that received BSA+PGF or IFNT+PGF, but did not differ in ewes that received infusions of IFNT +/- PGF at 8, 10, or 12 hours after PGF. There were no differences in prostaglandin E synthase (PGES) or prostaglandin F synthase (PGFS), or in prostaglandin dehydrogenase (PGDH), steroidogenic acute regulatory protein (StAR), peripheral type benzodiazepine receptor (PBR), cytochrome P450 side chain cleavage enzyme (CYP-11A), or 3β-hydroxysteroid dehydrogenase (3β-HSD). Seven day infusion of IFNT during the time frame of maternal recognition of pregnancy resulted in 20% of IFNT-treated ewes returning to estrus by d19

compared to 100% of BSA-treated ewes ($P<0.01$). In conclusion IFNT acts systemically, alters gene expression in the corpus luteum, and decreases the number of ewes returning to estrus by d19.

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## Dedication

This dissertation is dedicated to my loving family and in memory of Alice Bullock who is celebrating the completion of my Ph.D. from a little further away. Thank you for your support, strength and love along the way.

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# Chapter 1

# Literature Review

## Introduction to the Corpus Luteum

### *History of the Study of the Corpus Luteum*

The corpus luteum (CL) is a transient ovarian endocrine gland named by Marcello Malpighi (reviewed in [1] and [2]) because it looked like a yellow (luteal) globular body (corpus) on the surface of ovaries. In 1672, Renier de Graaf provided the first accurate and detailed description of corpora lutea in French. This document was later translated in English [3] and revealed that de Graaf correlated the number of corpora lutea with the number of embryos. Mossman and Duke [4] wrote a review detailing evidence that corpora lutea are derived from follicular cells during a tissue remodeling process that takes place surrounding the time of ovulation. The development of corpora lutea from follicles is a process called luteinization. Structural remodeling and altered patterns of steroidogenesis are hallmarks of this process.

### *Structure of the Corpus Luteum*

In ruminants, thecal cells, fibroblasts and endothelial cells become intimately associated with granulosal cells during the breakdown of the follicular wall which occurs during ovulation [5]. Small steroidogenic luteal cells (SLC) arise from thecal cells [6]. Small luteal cells are 12-18μm in diameter and have an irregular shape to their nuclei [7]. Large luteal cells (LLC), which arise from granulosal cell precursors [6] are 25-40μm in

diameter with smooth nuclei, rough endoplasmic reticulum, abundant mitochondria and secretory granules [7, 8]. Both cell types contain lipid droplets, and smooth endoplasmic reticulum [7]. Development of the CL includes a five fold increase in number of SLC, and an increase in number of non-steroidogenic cell types [9]. Large luteal cells did not increase in number, but did undergo hypertrophy, achieving a three fold increase in volume by mid-luteal cycle [9].

*Steroidogenesis in Luteal Cells*

Steroidogenesis is differentially regulated in LLC and SLC. Both LLC and SLC express receptors for LH [10-12]. Treatment of SLC with LH in culture yields a 3-15 fold increase in production of progesterone [11, 13, 14]. LH activates protein kinase A (PKA) through the adenylate cyclase messenger system [14] (reviewed by Davis [15]) which, in turn, phosphorylates steroidogenic acute regulatory protein (StAR) and stimulates cholesterol transport across the mitochondrial membrane [16-18]. Large luteal cells do not respond to LH with a significant increase in production of progesterone [11, 13, 14]. Yet, LLC produce upwards of 85% of total progesterone in the corpus luteum [19-21]. Regardless of the cell type, progesterone is synthesized through a series of intracellular events.

This first and rate-limiting step in the synthesis of progesterone is the transport of cholesterol to the inner mitochondrial membrane where it can be converted to precursors for progesterone [22-24]. Transfer of cholesterol across the mitochondrial membrane where P450-SCC is regulated by three proteins including endozapine, its receptor,

peripheral type benzodiazepine receptor (PBR) [25] and StAR. The amino-terminus of StAR targets the protein and cholesterol cargo to the mitochondrial membrane where the carboxy-terminal tail allows for transport across the membrane through unknown mechanisms [26]. Researchers have postulated that aggregates of PBR form a four to six molecule pore or channel in the mitochondrial membrane to allow transport of cholesterol [27, 28]. Aggregations of PBR in the mitochondrial membrane of ovine luteal cells was confirmed, yet no direct interactions between StAR and PBR have been identified [29].

Once inside the inner mitochondrial membrane, cholesterol is processed to yield pregnenelone by the P450 side chain cleavage (SCC), or CYP-11A enzyme [30] (reviewed in [31]). Pregnenelone exits the mitochondria where it is converted to progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD) in the endoplasmic reticulum (reviewed in [32]). Ewes reach maximal production of progesterone on day 7 of the estrous cycle and progesterone are maintained in ruminants during pregnancy or until a luteolytic signal causes a decrease in steroidogenesis [33].

## Review of Luteolysis

A functional CL is necessary for maintenance of pregnancy. In the event that an animal does not become pregnant, luteolysis must occur in order for the female to have another chance to conceive. Lifespan of the CL in a normally cycling, non-mated ewe is 16-17 days [34]. Many experiments have been conducted over the years in an attempt to define the numerous factors involved in luteolysis.

*Involvement of the Uterus*

Wiltbank and Casida [35] hysterectomized ewes and watched for signs that the animals had returned to estrus, finding that hysterectomized ewes did not return to estrus for more than 100 days. Furthermore, the original CL was maintained until the time of necropsy, with minimal follicular activity. Additionally, if a total hysterectomy was performed on d13.5-15 the CL has a lifespan of approximately 148 days, similar to the length of gestation [36, 37]. The lifespan of the CL varies when one uterine horn is removed at a time, and is dependent on proximity of the removed horn to the CL [36, 37]. If the uterine horn ipsilateral to the ovary with the CL was removed, the lifespan of the CL was 35 days. However if the uterine horn contralateral to the CL was removed, the CL regressed in 15-17 days, similar to the normal cycle. These studies were among the first to demonstrate a local effect of the uterus on luteal lifespan.

Once the uterus was implicated in regulating luteal lifespan, additional experiments were performed, revealing that local proximity of the uterus to the CL was necessary for luteolysis. Goding and colleagues [38] developed an ovarian autotransplantation technique where the jugular vein and carotid artery of a ewe were encapsulated in a pouch of skin from the neck. The ovary and its vascular pedicel were transplanted to the neck and connected to the venous and arterial blood supply. These experiments were initially planned to study steroid hormone concentrations leaving the ovary. They yielded important data over the next few years about spatial relationship between the ovary and uterus and how it influenced the length of cycle in a ewe. During this initial

study of ewes with ovarian autotransplants little data on cycle length were collected. The authors stated that the "teaser" ram only detected one of the ewes returning to estrus during the season and suggested that this was due to sexual inexperience [38]. Several studies that followed also reported infrequent display of estrus in ewes with ovarian autotransplants.

Removal of the uterine horn which is ipsilateral to the CL containing ovary extends luteal lifespan [39]. Autotranslpantation of the ovary in six ewes in a subsequent study resulted in only one animal demonstrating estrual behavior [40]. Following estrus this ewe produced high concentrations of ovarian progesterone and failed to exhibit signs of estrus for over 160 days, similar to the duration of gestation. McCracken and Baird also reported that estrus was detected at irregular intervals in ewes with transplanted ovaries ranging from 25-79 days compared to every 17 days in control ewes [41]. When one uterine horn, the uterine body, cervix and the anterior vagina were transplanted to the neck of a six year old ewe she failed to demonstrate estrous during the following breeding season, maintained high circulating concentrations of progesterone and had 2 large CL present 31 days after treatment with pregnant mare serum gonadotropin [40]. Normal luteal regression occurred if both the ovary and local uterine horn were transplanted to a ewe's neck [42]. Therefore, local proximity of the uterus to the ovary is critical for normal cycle length and luteal regression in ewes. These experiments were among the first to implicate a luteolysin of uterine origin and that the luteolysin functions in a local fashion.

*Prostaglandin as Luteolysin*

Prostaglandin $F_{2\alpha}$ a has been implicated as the initiator of luteolysis in many species including the ovine [42-44]. Goding [45] set criteria that must be met for PGF to be considered 'the' luteolysin in sheep. He claimed that hysterectomy should prolong the estrous cycle, PGF should be able to shorten the cycle, PGF should be found in uterine venous blood, there should be a physical configuration that would allow transfer of PGF from the uterine venous flow to the ovarian artery and that the criteria must be measurable.

With evidence that hysterectomy prolongs the luteal lifespan and consequently cycle length, several investigators tested the hypothesis that PGF would cause luteolysis if injected into a ewe. In one study, ovarian arterial infusions of PGF [42, 46] in ewes with ovarian autotransplants decreased concentration of progesterones and animals exhibited estrous behavior. McCracken [42] reported a transient increase in both blood flow and production of progesterone following infusion of PGF, followed by a decline in both prior to the ewe returning to estrus. Thornburn and Nicol [47] demonstrated that synthesis of progesterone could be recovered when they infused 120 μg PGF over three hours and noted an initial decline in concentrations of progesterone recovered over time. Nett and colleagues [48] demonstrated that ovarian blood flow and production of progesterone decreased from treatment with 5mg PGF and that PGF directly affected the structure of luteal vasculature.

Prostaglandin from the uterus acts as a luteolytic agent. In a study where the vascular drainage from the uterus of a donor ewe on d15 of the estrous cycle was connected to the ovarian blood supply in the recipient ewe with a CL on an auto-transplanted ovary, production of progesterone in the recipients was reduced to less than 50% and two out of five ewes returned to estrus [44]. When the same experiment was conducted using ovaries from a donor ewe on d2, 6, 10, or 13 of the estrous cycle the recipient ewe experienced only a 20% reduction in progesterone and did not experience luteal regression or subsequent return to estrus. Similarly, when the vasculature source leading to the recipient from the donor was a peripheral vein instead of the vasculature draining the uterus on d15, the recipients did not experience significant decreases in progesterone and did not exhibit luteal regression or estrous behavior. The investigators calculated that 42 ng/ml PGF was released by the uterus at a flow of 15 ml/min. Therefore, they injected 25 μg/hr for three hours into the uterine vein of ewes on d6, 10 or 14 of the estrous cycle. Production of progesterone decreased in these animals and luteal regression was induced. When PGF was infused systemically for six hours a concentration of 50 μg/hr was necessary to induce luteal regression [49]. Thus, the uterine luteolysin, PGF, elicits its effects locally, and is only effective during a specific time during the estrous cycle.

In 1971, [46] a mechanism was postulated in which uterine PGF could reach the CL through an interaction between the ovarian artery and the utero-ovarian vein. Ginther [50] reviewed the vascular architecture of the ovarian artery and the vein which drains both the uterus and ovary. In several species including the ewe, there is close juxtaposition of the ovarian arterial and venous networks. He reported that the walls of

the vessels are fully intact where they come into contact with one another, but that there is potential for transport of certain molecules between the two. His group performed unilateral hemi-hysterectomies ipsilateral to the ovary containing a CL and connected the uterine vein, or the ovarian artery from the intact side into the corresponding vasculature on the hysterectomized side [51], finding that either method caused luteolysis as compared to controls. When no connection was made between the vasculature on the intact side of the reproductive tract to that on the hysterectomized side the CL did not regress [51, 52]. When the ovary containing the CL was ipsilateral to the intact uterine horn but the uterine vein from the intact side was disconnected above the branch point that comes to contact with the ovarian vein, the CL did not regress [52]. Luteolysis occurred in sheep if 2 mg PGF was administered into the uterus, but a larger dose (6-8 mg) was necessary to have the same effect when given intramuscularly [53]. These studies provided strong evidence that the uterine PGF can act locally on the CL to induce luteolysis.

*Initiators of Prostaglandin Production*

Prostaglandin $F_{2\alpha}$ is released from the endometrium in a pulsatile fashion [54, 55]. The early PGF peaks begin at d13 and increase on d15-16 [56]. Pulse amplitude and frequency increase towards the end of the estrous cycle. Prostaglandin pulses associated with luteolysis occur every 7-8 hours in the ewe [57]. Synthesis and release of prostaglandin is hormonally regulated.

Progesterone regulates expression of many receptors involved in coordinating the estrous cycle and secretion of prostaglandin. Progesterone prevents expression of the estrogen receptor (ER) and the oxytocin receptor (OTR) in the endometrium, which generally precede PGF release. As concentration of progesterone decreases, expression of these receptors increases [58]. Therefore, towards the end of the luteal phase progesterone no longer prevents formation of OTR. This allows increased pulse amplitude and frequency of PGF secretion. Continuous exposure to progesterone in cycling ewes causes a down-regulation of the endometrial progesterone receptor (PR) by d11 [59]. Down-regulation of the endometrial PR is followed by an increase in ER and OTR expression and PGF release on d13, 14 and 14-16, respectively [59-63]. The onset of OT and PGF pulses is concomitant with an increase in endometrial OT receptors, suggesting that PGF synthesis and release can be driven by OT signaling [61, 64].

*Anti-steroidogenic and Luteolytic Properties of PGF*

Prostaglandin $F_{2\alpha}$ has anti-steroidogenic and luteolytic effects on the CL. Prior to luteolysis, the CL rapidly loses its capacity to synthesize progesterone [48]. Within five to six hours of administering PGF to a ewe in mid-cycle, production of progesterone and blood flow to the ovary containing the CL decline dramatically [48, 65]. Loss of production of progesterone can be attributed to PGF induced decreases in 3β-HSD, StAR and cholesterol esterase, all of which are important in steroidogenesis [66-71]. Following the decline in steroidogenesis the process of luteolysis begins. Contrary to the literal translation, luteolysis occurs through an organized pattern of cell death rather than an actual lysis of cells [72]. DNA fragmentation, an indicator of apoptosis, is first detected

several hours after the anti-steroidogenic actions of PGF are evident [73]. Oligonucleosomes are detected in the ovine CL 12 hours after exposure to PGF [65]. Steroidogenic and vascular cell types are both affected during luteolyis.

Blood flow and capillary density decrease in CL which are exposed to PGF concomitant with reduced production of progesterone [74]. Like the steroidogenic cell types, endothelial cells which line capillaries and other blood vessels have PGF receptors and can be directly impacted by the luteolytic agent [1]. Even though the LLC lose their steroidogenic capacity early after PGF exposure it is the endothelial cells which are the first to show signs of apoptotic death in the CL [75]. Endothelial cells are the most abundant cell type in the ovine CL [9]. Demise of this cell type and the blood vessels would deprive the CL of nutrients, oxygen and other endocrine stimuli, further contributing to luteolysis.

*Contribution of Luteal PGF to Luteolysis*

Historically PGF has been thought to be solely of uterine origin. However, Production of PGF occurs within luteal tissue of rats [76], cattle [77], swine [78, 79], sheep [80] and primates [81, 82]. Recent evidence for auto-regulation of luteolysis by the CL in sheep leads one to speculate that the CL may also have an intricate self-preserving mechanism to prolong its lifespan in the event that pregnancy occurs. Ovine LLC have the ability to produce PGF [83]. These cells also have PGFR on their surface and when stimulated with PGF respond by an increase in enzymes necessary for PGF synthesis and subsequent Production of PGF [83]. LLC also contain a cytosolic enzyme, PGDH, the enzyme

responsible for metabolizing PGF [84]. This enzyme is elevated 13-fold in the CL of d13 pregnant vs. cycling ewes [85], indicating that it might be important for the prevention of luteolysis in pregnant animals.

More recently there have been shifts in the traditional understanding of luteolysis in ruminants as data implicating luteal production of PGF and its role in luteolysis of the ewe have become available [80, 86-89]. When ovine luteal cells are treated with PGF analog cloprostenol in vivo, cultured luteal slices produce more PGF than non-treated tissues [80]. PGF-stimulated Production of PGF in luteal tissue has been described as an auto-amplification loop [88].

Several experiments have been conducted to elucidate the role of luteal PGF in luteolysis. Indomethacin is an inhibitor of prostaglandin synthesis [90]. Injecting indomethacin (20 mg) into the uterine horn of ewes between d12-17 maintains concentration of progesterones and luteal weights [91]. Niswender [89] inserted 0, 1, or 10 mg indomethacin into the CL of d11 ewes. The serum concentration of progesterone was greater in the ewes treated with 10 mg indomethacin from d13-16, but all groups had minimal progesterone by d17. CL were collected from all ewes on d18. Luteal weights were greater in both indomethacin treated groups than the controls and the group receiving 10 mg had the largest remaining CL. This study provided evidence that intra-luteal PGF is necessary for luteal regression. Because the indomethacin implants were local to the CL and because blood flow leaving the ovary does not circulate directly back to the uterus, it is likely that only PGF of luteal origin were inhibited. Based on these

results, PGF from the uterus appears to act on the CL to decrease steroidogenesis and to stimulate synthesis of intra-luteal PGF. Consequently, the role of luteal PGF appears to be structural demise of the CL through an auto-regulatory mechanism (Figure 1).

The theory that uterine PGF decreases production of progesterone and stimulates intra-luteal production of PGF which causes luteolysis is supported by the aforementioned delay in time between decreased steroidogenesis and DNA fragmentation of the luteal cells [65]. Concentration of progesterones within the CL are upwards of 17-30 μg/ml tissue during diestrus [89]. The percentage of LLC in vitro which respond to PGF in the presence of 30 μg/ml progesterone is significantly less than those responding to PGF in the presence of 0, 1 or 10 mg/ml progesterone [48]. Therefore, PGF first appears to act to decrease progesterone and then to induce apoptosis of the CL.

## Biochemical Regulators of Prostaglandin

### *Prostaglandin Synthesis*

Prostaglandin is synthesized from lipid precursors through a series of enzymatic events (Figure 2). Arachidonic acid (AA), precursor to PGF, is synthesized from cellular phosphoglycerides [92]. When PGF synthesizing cells are stimulated with thromboxanes, concentrations of phosphatidyl inositol transiently decrease, giving rise to AA [93]. Thus, cells which produce PGF are capable of responding to stimuli, quickly making AA available for production of PGF. Progesterone appears to be a regulator of the availability of PGF precursors as the number of lipid droplets in the ovine uterus are

highest on d10 of the estrous cycle, remain high through estrus and are lowest on d4-6 [94]. Lipid droplet accumulation in the ovine corpus luteum begins to occur between d13 and 14 which is slightly delayed to the uterus [95] and supports the hypothesis that uterine Production of PGF stimulates production of PGF in the CL.

Prostaglandin H-synthase (PGHS) converts AA to PGH2 which occurs in a series of two reactions and is considered the rate limiting step in the PGF biosynthesis pathway. Cyclooxygenase (COX/PGHS) catalyzes the formation of PGG2 [92, 96]. COX-1 is a constitutively expressed isoform and COX-2 is up-regulated in a tissue and stimulus dependent pattern [97]. COX-2 is produced by endometrial cells and in LLC [87, 98]. The products of PGHS enzyme activity serve as precursors for PGF synthesis.

Prostaglandin F2alpha can be synthesized from PGH2, or PGE2 by PGH 9-,11-endoperoxide reductase, or PGE 9-ketoreductase, respectively. PGE 9-keto-reductase has been demonstrated to convert PGE to PGF in several species [99, 100]. This reaction may be reversible, also functioning to convert PGF to PGE [99, 100]. Patek [78] added COX inhibitor, Indomethacin, with the PGE 9-keto-reductase enzyme to the follicle, and CL of both pregnant and cycling sows and found no difference in resulting PGF or PGE concentrations from incubations without indomethacin. Taken together, these data provide evidence for PGE 9-ketoreductase in two-way conversions between PGE and PGF. PGD 11-ketoreductase catalyzes PGD2 to 9alpha-, 11beta-PGF2 (not PGF) and can also convert PGH2 to PGF, but is incapable of reducing PGE to PGF [101]. In summary, PGF can be synthesized directly from PGH2 or PGE. The rate limiting step in

PGF synthesis is the conversion of AA to PGH2 by PGHS/COX2. There is preliminary evidence that PGF may be converted to PGE by PGE 9-keto-reductase.

*Prostaglandin Metabolism*

Prostaglandin metabolism is an important regulatory mechanism in many physiological systems. PGE and PGF are metabolized by 15-hydroxprostaglandin dehydrogenase (PGDH) which oxidizes the 15-hydroxyl group [102]. Two forms of the enzyme have been identified. Type I PGDH is NAD+ dependent while type II utilizes NADP+ as its cofactor. Both are cytosolic enzymes yielding the same ketone group at position 15 [92]. The lungs of ruminants have an abundance of PGDH [103] to rapidly metabolize and inactivate PGF before it returns to general circulation. Prostaglandin metabolism in the lungs is not specific to ruminants. Piper and colleagues [104] found that expression of PGDH in the lung of guinea pigs could metabolize up to 90% of infused PGF in one pass. The half life of PGDH is approximately 45 minutes [105]. While type I and II PGDH have roughly the same Michaelis constant (Km) for PGF, type I PGDH has a lower Km for PGF than does type II, potentially making it more physiologically relevant [92, 106]. Furthermore, NAD+ is present in cells predominantly in the oxidized state while NADP+ is present mostly in the reduced form which further implicates a dominant physiological role for type I PGDH [63]. Once the 15-hydroxyl group of PGF has been oxidized by PGDH, prostaglandin delta 13-reductase removes a single hydrogen molecule, yielding 13, 14-dihydro-15-keto-PGF2. Carbonyl reductase catalyzes the final step in PGF metabolism by once again reducing the 15-keto group resulting in 13, 14-dihydro-PGF2.

Prostaglandin metabolism is elevated in various tissues of pregnant animals. Type I PGDH is elevated 20 fold in the lungs of pregnant vs. non-pregnant rabbits [107-109]. Elevated PGDH has also been found in human and rat placenta during specific times in pregnancy [110, 111]. Sufficient evidence exists to implicate a critical role for PGDH in pregnancy in a variety of animal models including humans.

## Maintenance of Pregnancy

*Reduced Effects of PGF on Luteal Cells During Pregnancy*

The CL of a pregnant animal is resistant to the luteolytic effects of PGF [85]. One hypothesis for the reduced effects of PGF on luteal cells during pregnancy was that pregnant ewes would have fewer PGF receptors (PGFR) in the CL. However, concentrations of PGFR, based on radio-receptor assays, in the corpus luteum are not different in pregnant and non-pregnant ewes except at d15 when receptor concentration is greater in pregnant ewes, coinciding with a reduction in total weight of the non-pregnant CL [112]. Therefore, the CL might be protecting itself through auto-regulation of PGF.

Alterations in expression of PGF degrading and synthesizing enzymes have also been evaluated in the endometrium and CL. The manner in which PGF is secreted is dependent upon an animal's physiological status. Pregnant and cycling ewes have very different patterns of PGF release [56, 57]. While peak production of PGF occurs at d14-15, regardless of pregnancy status, cycling ewes secrete PGF in a pulsatile fashion while pregnant ewes have a more constant, slowly increasing pattern in the release of PGF

metabolite [113, 114]. More PGF is found exiting the uterus through the uterine vein in d13 pregnant vs cycling ewes [115]. The change in PGF peaks during pregnancy may be due to an increase in its metabolism by PGDH within the CL. Also, the presence of PGF in the uterine vein during pregnancy may necessitate a self-preservation mechanism for the CL to make PGDH. PGDH mRNA is elevated in CL on d4 of the estrous cycle and d13 of pregnancy when compared to d13 of the estrous cycle in ewes [116]. Additionally, enzyme activity assay was employed to determine the PGDH activity on d4 and 13 of the estrous cycle and d13 of pregnancy. The results were indicative that production of PGFM was greater on d4 in CL from cycling ewes and d13 in CL from pregnant ewes compared to CL from non-pregnant ewes 13 days post estrus [72]. Cyclooxygenase-2 mRNA did not differ in CL from pregnant and cycling animals at d 12 or 13 post estrus [116, 117]. Silva and colleagues [72] reported a significant increase in PGDH mRNA and enzyme activity in d13 pregnant ewes compared to d13 non-pregnant ewes while Costine et al., [73] did not detect these differences on d12. Aside from a change in synthesis or degradation of PGF or numbers of available receptors on luteal cells there are other potential mechanisms by which the CL may become resistant to PGF during critical times in pregnancy. Therefore, the CL might be protecting itself through auto-regulation of PGF. While there have been no reports confirming changes in protein production it seems likely that a change in synthesis or degradation of PGF by the CL is critical for maintaining pregnancy. As with most physiological systems, there are probably redundant mechanisms in place to maintain the CL during pregnancy.

*Contribution of Embryo to Lifespan of the CL*

Critical to the maintenance of pregnancy is the contribution of embryonic proteins and substances which act directly on the uterus and potentially at the level of the CL. The embryo must signal its presence and stimulate anti-luteolytic mechanisms to sustain the CL. Timing of the embryonic signal is critical for maintenance of the corpus luteum and pregnancy. If embryos are transferred into a recipient ewe later than d12 post-estrus the chances of the fetuses surviving to term are reduced [118, 119]. Therefore, the uterus is programmed to receive an embryonic signal until d12, after which it becomes unresponsive, or unable to maintain pregnancy. In addition to timing, local exposure of the CL to embryonic signals by means of connecting vasculature is also important.

When the gravid and non-gravid horns in a pregnant ewe are separated via ligation, the importance of transfer of luteolytic and embryonic anti-luteolytic agents between the CL and uterus are revealed. In double-ovulating ewes only the CL on the gravid side of the reproductive tract remains, while the CL on the non-gravid side regresses. Anastomoses of the uterine vein from the pregnant horn to the non-pregnant horn up-stream of where it meets the ovarian artery, will prevent regression of the CL on the non-gravid side [120]. This is suggestive that release of PGF from the gravid horn is altered to protect the CL, or that there is a substance, perhaps of embryonic origin, exiting the uterus through the uterine vein that helps maintain luteal function. Yet, if an anastomoses is performed through redirecting the uterine vein from the non-gravid horn to the gravid side, both CL remain [120]. Similar experiments where the ovarian artery from the gravid side is connected via anastomoses to the non-gravid side will result in regression of the corpus

luteum near the gravid horn, and maintenance of the CL on the non-gravid side [121]. Therefore, whatever anti-luteolytic substance is leaving the pregnant uterus through the uterine vein must travel through the ovarian artery to maintain the CL.

Embryonic signaling and maintenance of the CL may be accomplished by any number of mechanisms including a reduction in PGF synthesis, enhanced PGF metabolism, conversion to PGE and/or down-regulation of PGFR. Previous studies have demonstrated that there is no reduction in luteal concentrations of PGFR in pregnant ewes [112]. Embryonic production of signals which prevent luteolysis in ruminants is called embryo recognition of pregnancy which causes the dam to maintain her CL rather than initiating luteolysis and resumption of cyclicity. A specific embryonic trophectoderm derived protein is responsible for prolonged luteal lifespan which is critical for maintenance of pregnancy.

Interferon~tau (IFNT), originally described as protein X, ovine tropoblastin (oTP) or bovine trophoblast protein (bTP) is an anti-luteolytic protein secreted by the elongating embryonic trophectoderm beginning at d10 and d15 in the sheep and cow, respectively [34, 122-125]. Proteins produced by d15-16 sheep conceptuses extend luteal maintenance and progesterone levels when infused into the uterine lumen of cyclic ewes [34]. Wiltbank and colleagues [126] demonstrated that PGF inhibited lipoprotein stimulated LLC production of progesterone in vitro. When IFNT isolated from d15 sheep embryos was cultured in that system production of progesterone was not recovered, however when embryo secreted proteins without IFNT were added to PGF treated LLC

production of progesterone increased [79]. While IFNT did not directly block the actions of PGF on LLC steroidogenesis, an indirect mechanism could still be in place. It is not implausible to consider that some of the conceptus secretory proteins which protect the CL could be up-regulated by IFNT. Mechanisms for IFNT mediated extension of luteal lifespan have been identified in the uterus.

Infusion or injection of IFNT [127] or recombinant IFNT [128] into the ovine uterus extended luteal lifespan. Similarly, infusion of IFNT into non-pregnant bovine uteri between d15.5-21 extended the interestrous interval [129]. Peak IFNT secretion (100 μg/day) was achieved between d15-17 in the ovine conceptus [124]. Interferon~tau abrogated transcription of ER [130] and OTR [130, 131] in the endometrium. The ER promoter region contains IFNT regulatory factor elements (IRFE) which appear to down regulate transcription of the ER through IFNT induced actions of IRF-2 [132]. Decreased transcription of the OTR is likely due to the decrease in ER caused by IFNT, because the OTR are not directly regulated by IFNT [133]. The result of suppressed ER and subsequent OTR expression in the endometrium is the inhibition of luteolytic PGF pulses. Suppression of estradiol receptor was also demonstrated when IFNT was infused into the sheep uterus [134]. Cycle length in non-pregnant ewes which have received exogenous IFNT is extended compared to controls [131] indicating that down regulation of the ER and OTR prevent luteolysis and resumption of the estrous cycle.

*Interferon-stimulated Genes*

Interferon tau elicits its actions through the type one interferon receptor which shares two subunits IFNAR1 and IFNAR2. These subunits are expressed in the luminal epithelium, sub-glandular epithelium, and stroma of the ovine uterus in d14-15 cycling and pregnant ewes [135]. In ovine endometrial cells, IFNT causes tyrosine phosphorylation and nuclear translocation of signal transduction and activator of transcription (STAT) -1, 2, 3, 5 and 6 as well as increased transcription of STAT 1 and 2 [136]. Interferon-stimulated gene factor (ISGF) -3 and STAT 1 form homodimers and bind to IFNT stimulated response elements (ISRE) and gamma activated sequences (GAS) to drive expression of interferon-stimulated genes (ISG) [136]. In cycling ewes STAT 1, IRF 2, and IRF 9 decrease on d11-15, while STAT 1, 2, IRF 1, IRF 2, and IRF 9 increase during d11-15 of pregnancy [137]. Differential expression of STAT and IRF genes occurs in the endometrium of cycling vs. pregnant ewes.

Induction of ISG is predominantly localized to the stroma and glandular epithelium, however, IFNT causes down regulation of ER in the luminal epithelium. The ability of IFNT to stimulate ISG expression is therefore regulated by physiological status and tissue type.

Several interferon-stimulated genes in addition to STAT and IRF have been identified in the ruminant uterus. These ISG include but are not limited to 2', 5' -oligoadenylate synthetase (OAS) [138-140], Mx [141], and ubiquitin cross reactive protein (UCRP) [142, 143], later referred to as IFNT stimulated gene 15 (ISG15) [144]. ISG15 can be found in its free 15kDa form and conjugated to target proteins in the uteri of pregnant

cows on d17-45 [145]. ISG15 protein was localized to the glandular epithelium with light staining in the luminal epithelium and stroma during the time frame of peak IFNT expression around d18 of pregnancy [145]. IFN response factor (IRF) -2 is a repressor of ISG and is present in the LE and sub-glandular epithelium, thus restricting the ability of IFNT to increase ISG in the LE, but not in stroma and glandular epithelium regions of the ovine uterus [137].

The ISG15 protein contains two internal ubiquitin -like domains and shares the carboxy-terminal Leu-Arg-Gly-Gly sequence with ubiquitin [146]. For these reasons and because ISG15 is known to conjugate to target proteins in a process called ISGylation, it was hypothesized that ISG15 may form covalent conjugations to other proteins through the use of the E1 activating, and the E2 and E3 conjugating enzymes like ubiquitin. Further studies [147] revealed that while ubiquitin and ISG15 conjugation pathways are similar, different machinery and mechanisms were used. The ubiquitin-activating E1-like (UBE1L) protein was identified as the ISG15 activating enzyme in humans [148] and has been characterized in the bovine uterus during pregnancy [149]. UBE1L is present in the bovine endometrium on d17-21 and is localized to the GE and LE with minimal staining in the S [149]. Type 1 interferon inducible proteins Ubc8 [150], and Herc5 [151] have been implicated as the ISG15 E2 and E3 conjugating enzymes, respectively.

While ISG15 conjugation does not necessitate protein degradation, it seems that the complicated conjugation process would not take place unless ISG15 protein conjugation was somehow vital to certain physiological processes such as pregnancy or anti-viral

responses. To date, a few hundred ISG15 conjugates have been identified. Research efforts are currently being made to identify targets for ISG15 conjugation and explanations for physiological relevance.

*IFNT and the CL*

In sheep, IFNT is the embryonic trophoblastic protein which signals maternal recognition of pregnancy. Changes in endometrial gene and receptor expression during pregnancy have been well documented. Until recently, the actions of IFNT during maternal recognition of pregnancy were studied exclusively in the uterus. In 2006, Chen and colleagues [131] reported that luteal expression of OTR was not different between ewes that had been treated with exogenous IFNT or normally cycling ewes, but that luteal expression of PGHS was decreased in ewes that received an intrauterine dose of IFNT. These data are suggestive that prostaglandin synthesis in the CL of IFNT-treated ewes may be inhibited.

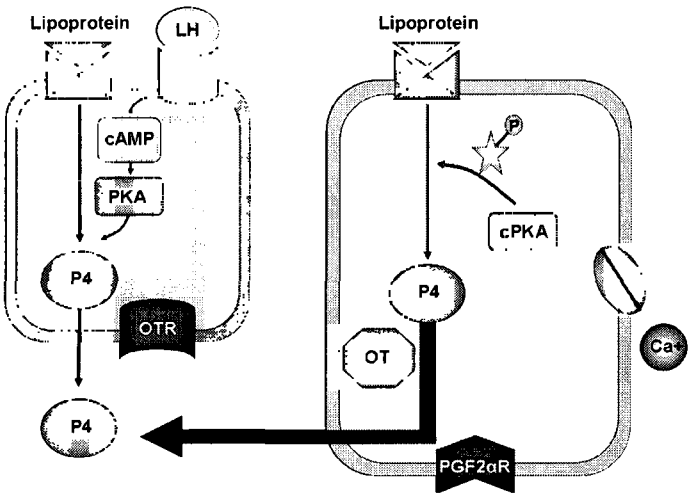
Recently Oliveira and colleagues [152] found that mRNA encoding ISG15 and OAS-1 are up-regulated in endometrial, luteal, and peripheral blood samples from pregnant vs. non-pregnant ewes. This group went on to characterize ISG15 and to confirm that expression was due to pregnancy and more specifically conceptus-derived IFNT. Corpora lutea from pregnant ewes had increased free and conjugated ISG15 protein expression which was refined to LLC and some SLC. Luteal cells that were collected from non-pregnant ewes and treated with IFNT had increased ISG15 mRNA and protein. While IFNT luteal receptors have not been found directly, the fact that IFNT can alter

gene expression in isolated luteal cell populations is highly indicative that receptors do exist and can therefore regulate expression of other luteal genes. Expression of ISG15 in the CL of pregnant ewes appeared to be induced by IFNT.

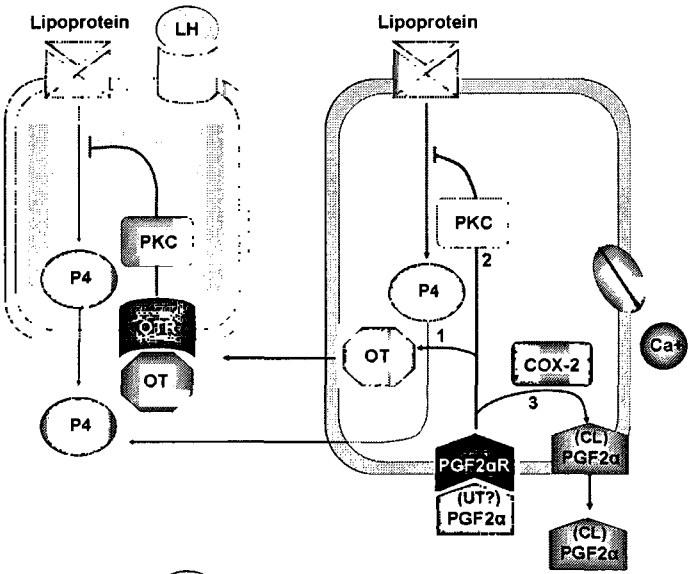
The first evidence that IFN left the uterus was obtained by means of an anti-viral IFN protection assay [152]. Blood plasma was collected from the jugular vein, uterine artery, and uterine vein from day 15 pregnant and non-pregnant ewes. MDBK cells were plated and grown to confluence. Cells were treated with dilutions of blood plasma collected from the peripheral and uterine samples. Next, the cells were challenged with exposure to vesicular stomatitis virus (VSV). The concept behind the protections assay was that cells that were exposed to blood plasma with high levels of IFN, which has anti-viral properties, would be able to survive viral challenge, while cells that received minimal or no treatment with IFN containing plasma would die in response to viral challenge. There was no difference in the ability of blood samples from non-pregnant jugular vein or uterine artery samples from pregnant ewes to provide an anti-viral protection. However, uterine vein samples from pregnant ewes were able to significantly prevent MDBK cells from dying when challenged with VSV. The anti-viral protection from uterine vein blood was equivalent to 10 ng/ml IFN. This is equivalent to approximately 250μg/day based on uterine blood flow. Taken together, these data are indicative of a mechanism by which IFN leaves the uterine vein and acts on extrauterine tissues such as the corpus luteum. Furthermore, ISG15 can be used as a biological marker for IFN activity in extra-uterine tissues.

Figure 1: Autoregulation of luteolysis by the CL. Progesterone is produced by SLC (left) and LLC (right) on d5-12 of the estrous cycle. On d12-14 uterine PGF binds to its receptor on the surface of LLC. In turn, luteal PGF synthesis is initiated and steroidogenesis begins to decline. PGF stimulates release of OT from LLC which act on SLC to induce apoptosis, while PGF itself induced apoptosis in LLC.

**Day 5-12**




**Day 12-14**




**Day 16**

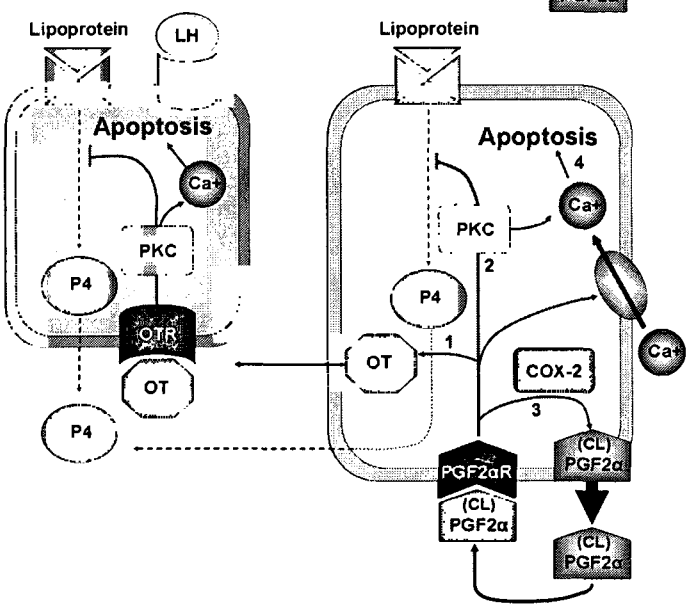
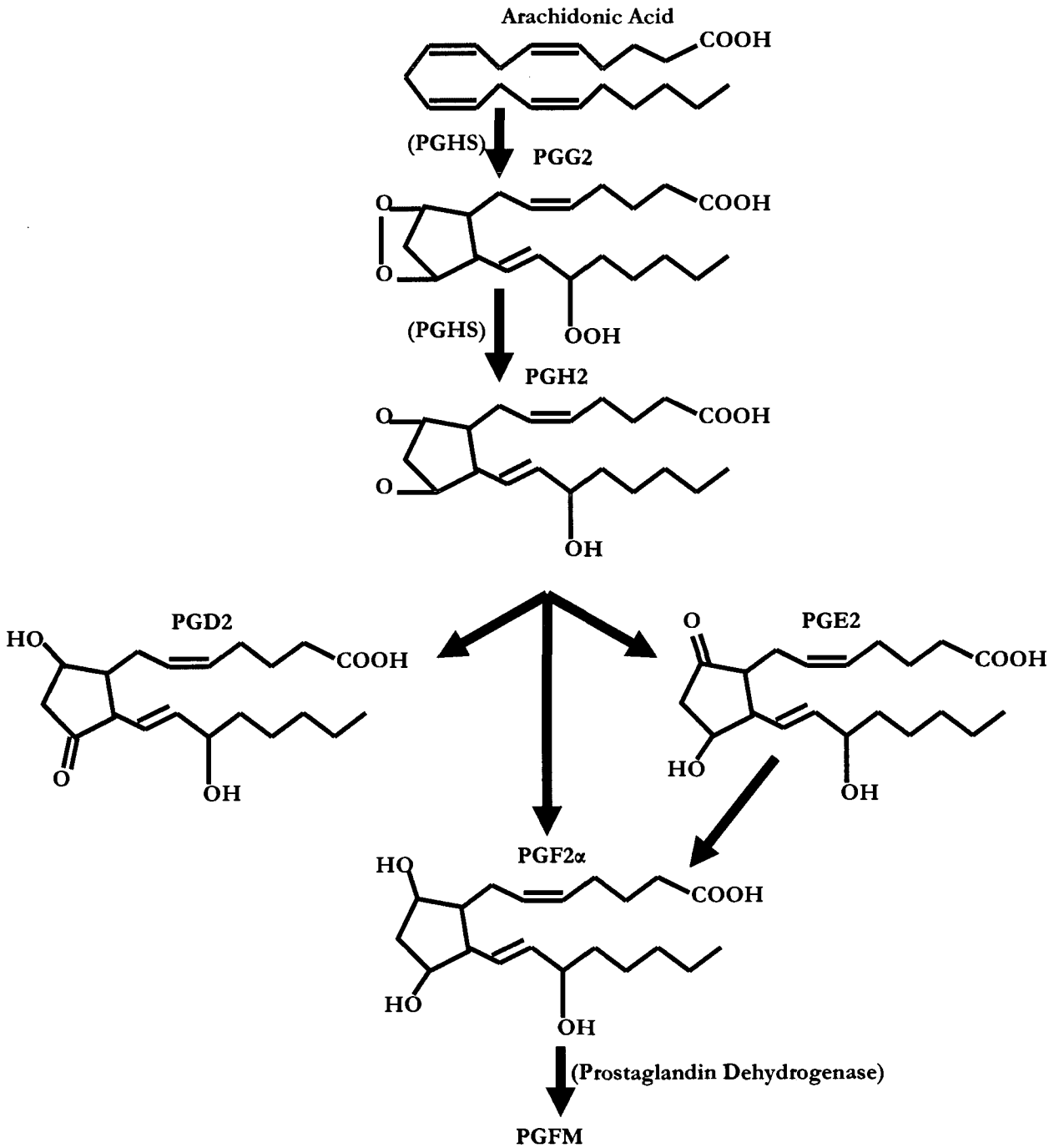


Figure 2: Prostaglandin synthesis and degradation enzymatic pathways. Metabolism of PGF renders it physiologically inactive.


Arachidonic Acid
COOH
(PGHS)
PGG2
O
O
COOH
OOH
(PGHS)
PGH2
O
O
COOH
OH
PGD2
HO
COOH
O
OH
PGE2
O
COOH
HO
OH
PGF2α
HO
COOH
HO
OH
(Prostaglandin Dehydrogenase)
PGFM

## Chapter 2

## Uterine vein infusion of IFNT stimulates luteal gene expression, prevents anti-steroidogenic actions of PGF and extends luteal lifespan in ewes


### Abstract

Interferon-tau (IFNT) was evaluated for endocrine actions on the corpus luteum (CL). The hypothesis was that infusion of IFNT would increase luteal expression of interferon-stimulated gene (ISG)-15, and the length of time for ewes to return to estrus. Osmotic pumps containing 200 μg IFNT or BSA (n=12 each) were connected to the uterine vein of non-pregnant ewes 10 days post-estrus. Messenger RNA encoding ISG15 was elevated in CL from pregnant and IFNT-infused ewes ($P<0.05$) compared to non-pregnant and BSA-treated ewes, respectively. Luteal mRNA encoding ISG15 from ewes treated with IFNT was greater than in ewes treated with BSA ($P<0.05$). Serum concentrations of progesterone were not different in ewes that received infusions of BSA or IFNT. Progesterone decreased by six hours ($P<0.05$) in ewes that received BSA+PGF or IFNT+PGF, but did not differ in ewes that received infusions of IFNT +/- PGF at 8, 10, or 12 hours after PGF. There were no differences in prostaglandin E synthase (PGES) or prostaglandin F synthase (PGFS), or in prostaglandin dehydrogenase (PGDH), steroidogenic acute regulatory protein (StAR), peripheral type benzodiazepine receptor (PBR), cytochrome P450 side chain cleavage enzyme (CYP-11A), or 3β-hydroxysteroid dehydrogenase (3β-HSD). Seven day infusion of IFNT during the time frame of maternal recognition of pregnancy resulted in 20% of IFNT-treated ewes returning to estrus by d19 compared to 100% of BSA-treated ewes ($P<0.01$). In conclusion IFNT acts systemically,

alters gene expression in the corpus luteum, and decreases the number of ewes returning to estrus by d19.

## Introduction

Critical to the maintenance of pregnancy is the contribution of conceptus proteins and substances which act directly on the uterus and potentially at the level of the CL. Timing of the embryonic signal is critical for maintenance of the CL and pregnancy. Transferring an embryo into the uterus of a cycling ewe five days post estrus extends luteal lifespan [153]. If embryos are transferred into a recipient ewe later than d12 post-estrus the chances of the fetuses surviving to term are drastically reduced [118, 119]. Therefore, the uterus is programmed to receive an embryonic signal until d12, after which it becomes unresponsive, or unable to maintain pregnancy.

The major protein product of ovine embryos which are cultured on d13 is of small molecular weight and was initially referred to as protein X [154]. This protein, which is secreted by the elongating embryo beginning at d10-12 in the sheep was renamed ovine trophoblast protein-1 (oTP-1), and later interferon tau (IFNT) [34, 122-125]. Peak secretion of IFNT from the ovine conceptus is achieved between d15-17 [124, 155, 156].

Infusion or injection of IFNT [127] or recombinant IFNT [128, 157] into the uterus of sheep extends luteal lifespan from 22-27 days post-estrus. These injections or infusions ranged from twice daily uterine infusions of IFNT for two [157] or eight days [127, 128]. Increased cycle length is thought to be regulated by the actions of IFNT on the uterine endometrium. IFNT abrogates transcription of ER [130, 158] and OTR [130, 131] in the endometrium. Decreased transcription of the OTR is likely due to the decrease in ER caused by IFNT, because the OTR are not directly regulated by IFNT [133]. Suppressed ER and OTR expression in the endometrium causes alteration (ewe) or attenuation (cow) of luteolytic PGF pulses.

IFNT acts through the type I interferon receptor which shares two subunits IFNAR1 and IFNAR2. These subunits are expressed in the luminal epithelium, sub-glandular epithelium, and stroma of the ovine uterus in d14-15 cycling and pregnant ewes [135]. Several interferon-stimulated genes (ISG) have been identified in the ruminant uterus. These ISG include but are not limited to 2', 5' -oligoadenylate synthetase (OAS) [138-140], Mx [141], and ubiquitin cross-reactive protein (UCRP) [142, 143], later referred to as IFN-stimulated gene 15 (ISG15) [144]. The ISG15 protein contains two internal ubiquitin-like domains and shares the carboxy-terminal Leu-Arg-Gly-Gly sequence with ubiquitin which allows ISG15 to conjugate to target proteins in a process called ISGylation [146]. ISG15 can be found in its free 15kDa form and conjugated to target proteins in the uteri of pregnant cows on d17-45 [145].

The first evidence that IFNT was able to elicit extra-uterine effects was reported in 2004 [159]. Infusing IFNT into the uterine vein does not influence luteal expression of PGHS or PGFS but do increase PGES in the cow [159]. More recently, Chen and colleagues [131] reported that luteal expression of OTR was not different between ewes that had been treated with IFNT or normally cycling ewes, but that PGHS was decreased in ewes that received an intrauterine dose of INF. These data are suggestive that prostaglandin synthesis in the CL of IFNT-treated ewes may be inhibited. Oliveira and colleagues [152] found that ISG15 and OAS-1 are up-regulated in endometrial, luteal, and peripheral blood samples from pregnant vs. non-pregnant ewes. CL from day 15 pregnant ewes had increased free and conjugated ISG15 protein expression which was localized to large luteal cells (LLC) and in some small luteal cells (SLC) [152].

The first report that IFNT exited the uterus was obtained by means of an anti-viral IFNT protection assay [152]. Uterine vein samples from pregnant ewes had anti-viral activity. The anti-viral protection is correlated with the concentration of IFNT leaving the uterus through the uterine vein (250 μg/day).

Taken together, these data are indicative of a mechanism by which IFNT leaves the uterus through the uterine vein and acts on additional tissues such as the CL. Furthermore, ISG15 can be used as a biological marker for IFNT activity in extra-uterine tissues. Therefore, we hypothesized that IFNT maintains production of progesterone and lifespan of the CL through direct actions on the CL. Four experiments were conducted to test this hypothesis in vivo. The first experiment was developed to determine if mRNA

encoding ISG15, PGHS, PGES, PGFS, StAR, PBR, CYP-11A, and 3β-HSD were elevated in CL from day 15 pregnant and cycling ewes. Experiment two was conducted to develop a method of IFNT delivery either into the ovarian artery, or into the uterine vein, creating a model to compare the endocrine actions of IFNT to pregnant animals. The third experiment was designed to determine if uterine vein infusion of IFNT increases expression of ISG15 in the CL of non-pregnant ewes. The final experiment was performed to determine if IFNT had direct anti-luteolytic effects on the CL including increasing the length of time before a ewe would return to estrus.

## Materials and Methods

*Animals*

All experimental procedures using animals were reviewed and approved by the Colorado State University Animal Care and Use Committee. Estrus was synchronized in mature, white faced, western range ewes during mid-luteal phase with two injections of PGF (Lutalyse; Pfizer, New York, NY; 5 mg, i.m.) administered four hours apart. Upon detection of standing estrus (d0) with a caudectomized ram, ewes were placed into experimental groups. Experiments 1 and 3 were performed in two replicates in the fall seasons of 2007 and 2008. Experiments 2 and 4 were conducted entirely in the fall of 2008.

*Experiment 1: Luteal gene expression in d15 pregnant and cycling ewes*

Six ewes were selected and bred to an intact ram on the day of estrus. Six additional ewes were selected as non-mated controls. Fifteen days after estrus, endometrial, luteal,

jugular venous, uterine venous, and ovarian arterial blood samples were collected via necropsy. Pregnancy was confirmed by the presence of embryos. IFNT production by embryos was confirmed by western blot analysis (data not shown). Jugular vein blood samples were collected using Vacutainer (BD, Franklin Lakes, NJ) collection tubes. Ewes were anesthetized with 20 mg/kg sodium pentobarbital (i.v.). A mid-ventral incision was made to expose the reproductive tract and uterine venous and ovarian arterial blood samples were collected. All blood samples were stored at 4°C for 12 hours to clot. Serum was separated via centrifugation, collected and frozen at -20°C until analysis. Ovario-hysterectomies were performed and luteal and endometrial samples collected. Tissues were fixed in 4% paraformaldehyde or snap frozen until analysis of mRNA and protein. Ewes were euthanized by exsanguinations while under anesthesia.

*Experiment 2: Development of a Method for IFNT Delivery*

The first group of ewes was treated with a single ovarian arterial injection containing 200 μg BSA (n=2) or IFNT (n=2) over 60 seconds. The concentration of 200 μg IFNT was chosen based on previous data [152] regarding the concentration of IFNT leaving the uterus through the uterine vein on a daily basis. To study the extra-uterine effects of IFNT on the CL a method for delivery of IFNT into the uterine vein was developed. Ewes were anesthetized with sodium pentobarbital (Sigma, St. Louis, MO) and maintained on isofluorane (Minrad, Inc., Buffalo, NY) on d10 of the estrous cycle. A 0.94 mm ID catheter (Duret Corporation, Cupertino, CA) connected the pump to the uterine vein upstream of the close apposition with the ovarian artery (Figure 1). The catheter and pump were anchored to the reproductive tract with cyanoacrylate (SUPER

GLUE, Bentonville, AR). The mini-osmotic pumps are designed to release their contents over twenty-four hours. In ewes that had a CL on each ovary, the CL were noted as being ipsilateral or contralateral to the side of the reproductive tract on which the pump was placed. Two additional ewes receive min-osmotic pumps which were collected via necropsy 12 hours post-surgery to confirm that pump contents were being released evenly over the 24 hour infusion period.

*Experiment 3: Short term uterine vein infusion of IFNT in vivo*

Alzet osmotic pumps (Durect Corporation, Cupertino, CA) containing 200 μg BSA (n=12), or IFNT (n=12) were surgically implanted. Half of the ewes in the BSA and IFNT groups (n=6 each) received a single 5 mg IM injection of Lutalyse twelve hours after surgery. Six additional sheep underwent sham surgeries (pumps weren't inserted) to serve as procedural controls. Blood samples were collected from the jugular vein at the time of surgery, 12, 14, 16, 18, 20, 22, and 24 hours after surgery. All ewes were euthanized twenty-four hours after surgery. Luteal, endometrial, and uterine venous blood samples were collected as described for experiment one at the time of necropsy.

*Experiment 4: Long term uterine infusion of IFNT in vivo*

The design for final experiment was similar to experiment three. However, 200 μg BSA (n=6), or IFNT (n=6) was infused daily for seven days. Pumps were surgically inserted on d10 of the estrous cycle. CL were marked with charcoal at the time of surgery. Jugular vein blood samples were collected once daily from d10-13. From d14-17 samples were taken at 12 hour intervals and were pooled within ewe to prepare a daily

sample. Caudectomized rams were utilized twice daily to detect standing estrus in ewes from d13-17. Ewes were euthanized upon detection of estrus, or on d32 post estrus and tissues were collected as described in experiment three.

*Quantitative RT-PCR Analysis*

Total RNA was extracted from luteal and endometrial samples using the TRIzol Reagent (Invitrogen, Carlsbad, CA) and protocol. The RNase-free DNase Set (Qiagen, Valencia, CA) and RNeasy MinElute Cleanup Kits (Qiagen, Valencia, CA) were used to digest DNA and purify RNA. Quantification of the RNA was performed using the NanoDrop (NanoDrop Technologies, Inc., Wilmington, DE). Single stranded cDNA was synthesized from 1 μg RNA using the iScript cDNA Synthesis Kit (Biorad, Hercules, CA). This cDNA was used as a template for semi-quantitative real-time PCR amplification using iQ SYBR Green Supermix (BioRad, Hercules, CA). Primers for GAPDH and ISG15 were used as previously described [160]. Primers targeting PGDH, PGHS, PGFS, PGES, Caspase-3, StAR, 3β-HSD, CYP-11A, and PBR were designed for the current experiment (Figure 2). All primers were designed to have an annealing temperature of 61°C and all sequences were verified. Amplification was performed at 95°C for 30 seconds, 61°C for 30 seconds and 72°C for 15 seconds repeated over 40 cycles. Targets were normalized to GAPDH and statistical analysis was performed using ΔCt values [161]. Data are represented by graphing the ΔΔCt values calculated for each group compared to sham controls, or as fold changes compared to SHAM controls.

*Western Blot Analysis*

Protein was extracted using a RIPA lysis buffer with PMSF (Sigma-Aldrich, St. Louis, MO), sodium metavanadate (Sigma-Aldrich, St. Louis, MO), and benzamidine (Sigma-Aldrich, St. Louis, MO). Protein quantification was performed using the BCA Protein Assay Kit (ThermoScientific, Rockford, IL). Protein lysates (50 μg/lane) were separated by SDS-PAGE using 12-15% gels and transferred to a nitrocellulose membrane. ISG15 content was analyzed using monoclonal ISG15 (5F10) antibody [145] at a concentration of 1:5,000 and a secondary goat anti-mouse IgG-HRP (Santa Cruz Biotech, Santa Cruz, CA) at 1:2000. All samples were analyzed for actin (Santa Cruz Biotech, Santa Cruz, CA; 1:2000) expression to confirm equal loading of proteins after quantification with the BCA Kit. Positive bands were detected by chemiluminescence using the Amersham ECL Plus Western Blotting Detection System (GE Healthcare, Piscataway, NJ).

*Interferon protection assay*

The interferon anti-viral assay was performed as previously described [152, 162] with a few modifications. Briefly, Madin-Darby bovine kidney (MDBK) cells were plated in MEM with 5% FBS (v/v) at a concentration of $3x10^4$ cells/well in 96 well plates. When cells reached confluence they were treated with ovine plasma using 1:4-1:32 serial dilutions and cultured for 24 hours. Serial dilutions of Universal Type 1 IFN (Biomedical Laboratories, Piscataway, NJ) were utilized to create an IFN standard. Preadsorption of uterine vein blood on day 15 of pregnancy with antibody against IFNT (kindly provided by Dr. Fuller Bazer and Dr. Thomas Spencer, Texas A&M University) was completed to verify that anti-viral activity represented IFNT. Plasma samples from

uterine vein blood on day 15 of pregnancy and with known high anti-viral activity were pre-adsorbed with 1:20 dilution of rabbit anti-IFNT antibody, or normal rabbit serum (NRS) for 24 hours before treating cells. Cells were then challenged with vesicular stomatitis virus (50-100 PFU) for an additional 42 hours, stained with 0.5% crystal violet in methanol, and plates were analyzed for optical density. Estimation of the IFNT bioactivity index was performed by comparison with the anti-viral activity of the appropriate dilution of IFN standard.

*Radioimmunoassay for progesterone*

Progesterone was extracted from serum by adding 5 ml petroleum ether (Mallinckrodt Baker, Inc., Phillipsburg, NJ) to 1 ml serum and mixing on a shaker for 5 minutes. Samples were incubated at room temperature for 5 minutes and then placed in a methanol and dry ice bath. The solvent portions containing petroleum ether and steroids were transferred to a new tube and dried on a heating block. The procedure was performed a second time on the serum sample and the products form both extractions were combined. Concentrations of progesterone were then determined via radioimmunoassay as previously described [163]. Each sample was run in duplicate and production of progesterone (ng) was reported. Assay sensitivity was 2 picograms, and the mean intra- and inter-assay coefficients of variation were 10.98% and 20.61%, respectively (n=3).

*Statistical Analysis*

T-tests were used to analyze differences in luteal mRNA from non-pregnant vs pregnant ewes and to determine differences in the number of ewes returning to estrus by d19.

Differences in protein and mRNA expression from the infusions studies were analyzed by performing a one-way ANOVA followed by a Bonferroni's post-test to evaluate differences between the means of treatment groups. If significant differences were not detected additional analysis was performed using a t-test. A two-way ANOVA using repeated measures was used to evaluate differences in serum progesterone concentrations after transforming data so that the concentration of progesterone at the time of PGF treatment was equal to 100%. All differences ($P<0.05$) were considered significant unless otherwise stated.

## Results

*IFNT is the active cytokine in uterine vein blood contributing to antiviral activity*

Uterine vein blood from d15 pregnant sheep had greater antiviral activity ($P<0.05$) compared to uterine vein blood from d15 cycling sheep. This antiviral activity in d15 uterine vein blood from pregnant ewes was diminished 56.3±13.03% ($P<0.05$, Figure 3) following preadsorption of uterine vein blood with antibody against ovIFNT.

*ISG15 mRNA is induced in CL from d15 pregnant ewes*

Luteal concentrations of ISG15 mRNA are greater ($P<0.05$) in d15 pregnant vs d15 non-pregnant ewes which is consistent with previous findings [152]. No differences in mRNA expression were detected for PGHS, PGFS, PGES, PGDH, StAR, PBR, CYP-11A, or 3β-HSD (data not shown).

*Development of a Method for IFNT Delivery*

Two methods of IFNT delivery were examined for their ability to stimulate ISG15 expression in the CL. Interferon was either injected into the ovarian artery for one minute or infused into the uterine vein for 24 hours. Interferon injection into the ovarian artery over one minute elicited increased ISG15 mRNA expression compared with BSA injection ($P<0.05$). However, the induction of ISG15 following IFNT infusion was significantly higher than infusion with BSA ($P<0.05$) or IFNT injections ($P<0.05$; Figure 4). Two ewes that were treated with IFNT mini-osmotic pumps were necropsied 12 hours after surgery to monitor flow rate of the delivery device. One half (100 μl) of the IFNT volume remained in the pumps at necropsy, indicating that 200 μg IFNT was being evenly dispersed throughout the lifespan of the 24 hour pump. The infusion method of IFNT or BSA delivery was used for the remaining experiments where half of the ewes in each treatment group were challenged with a 5mg injection of PGF 12 hours after surgical implantation of the pumps.

*Infusion of IFNT into the uterine vein induces ISG15 expression and maintains production of progesterone*

Luteal mRNA encoding ISG15 was greater in ewes that received treatment with IFNT vs. BSA ($P<0.05$) and in ewes receiving treatment with IFNT+PGF vs. BSA+PGF ($P<0.05$ Figure 5). Free ISG15 protein tended ($P=0.1$) to be higher in CL from IFNT vs BSA-treated ewes and was significantly higher in CL from ewes treated with IFNT+PGF vs BSA+PGF ($P<0.05$; Figure 6). Isgylation (conjugated ISG15) also increased in CL from

ewes treated with IFNT+PGF compared to those treated with BSA+PGF, and tended to be greater in IFNT vs BSA-treated ewes (P=0.07).

Messenger RNA encoding ISG15 was also evaluated in the endometrium and liver of pregnant ewes and from ewes treated with IFNT- and BSA-infusions. Messenger RNA encoding ISG15 was greater in the endometrium, but not in liver of d15 pregnant vs non-pregnant animals (P<0.05). IFNT infused animals had significantly greater ISG15 expression in the liver compared to the BSA (P<0.05). Endometrial expression of ISG15 mRNA was higher in IFNT-infused vs BSA-infused ewes (P<0.01; Figure 7).

Concentration of progesterone was similar in BSA- and IFNT-treated ewes throughout the experiment (Figure 8). Progesterone did decrease in ewes treated with BSA+PGF compared to animals treated with BSA or IFNT (P<0.05) beginning 6 hours after treatment with PGF and remained lower through the collection period. Ewes treated with IFNT+PGF had decreased progesterone (P<0.05) compared to those treated with BSA or IFNT 6 hours after PGF treatment. Concentration of progesterones did not differ 8, 10, or 12 hours after treatment in ewes treated with IFNT+PGF, BSA, or IFNT.

Maintenance of progesterone in ewes treated with IFNT does not appear to be a result of changes in mRNA encoding PGF synthesis or degradation enzymes. No changes were found in luteal PGDH, PGHS, PGES, or PGFS expression in any groups (Figure 9). Messenger RNA encoding StAR and PBR was reduced with treatment of PGF (P<0.05; Figure 9). Messenger RNA encoding side chain cleavage protein CYP-11A tended to

remain elevated in IFNT-treated animals that were challenged with prostaglandin in contrast to ewes treated with BSA+PGF ($P<0.1$). Finally, mRNA encoding 3β-HSD was decreased in CL from ewes treated with BSA+PGF, IFNT, and IFNT+PGF compared to ewes treated with BSA ($P<0.05$).

*Long term uterine vein infusion of IFNT in vivo*

Ewes that received seven day uterine venous infusions of BSA returned to estrus 18.2±0.31 days post-estrus. One IFNT-treated ewe returned to heat 19 days post estrus and the remaining four ewes treated with IFNT did not display signs of estrus at any time before necropsy on d32 post estrus ($P<0.01$, Figure 10). Original CL as indicated by charcoal markings were present in the IFNT infused ewes necropsied on d32 post-estrus (Figure 11). Serum concentrations of progesterone remained elevated through the time of necropsy on d32 in the 4 IFNT-treated ewes that did not return to estrus (Figure 12).

## Discussion

Maternal recognition of pregnancy in ruminants is regulated in part by paracrine uterine responses to IFNT produced by the conceptus. IFNT alters PGF pulsatility by abrogating expression of ER [130], and subsequently OTR [130, 131]. The actions of IFNT are elicited through the type I IFN receptor that contains two subunits which are localized to the luminal epithelium, sub-glandular epithelium, and stroma of the ovine uterus on d14-15 of the estrous cycle and pregnancy [135]. Interferon-stimulated genes including OAS [138-140], Mx [141], and ISG15 [142-144] are expressed in the uteri of pregnant or intrauterine IFNT infused ruminants.

In the current study, mRNA encoding ISG15 was present in the endometrium from both non-pregnant and pregnant ewes, but was significantly higher in endometrium from pregnant ewes. Expression of ISG15 in the uterus occurs concomitantly with IFNT expression and is localized predominantly to the glandular epithelium with additional expression in the luminal epithelium and stroma [145]. When these data are considered together, it seems likely that IFNT elicits its actions entirely within the uterus. It was not until recently that direct evidence in the form of anti-viral activity was found for IFN leaving the uterus [152].

Oliveira and colleagues [152] demonstrated that type 1 IFN was present in the uterine vein of pregnant, but not in non-pregnant ewes using an anti-viral assay. The antiviral activity was not present in blood samples from the jugular vein or even the uterine artery from pregnant ewes [152]. Any of the type 1 IFN could be responsible for eliciting an anti-viral activity. Therefore, in order to accurately attribute ISG expression in the CL to endocrine actions of IFNT, it was necessary to confirm that the anti-viral activity is due specifically to IFNT. In the current study, plasma samples from the uterine vein of pregnant ewes were preadsorbed with an antibody specific to IFNT. When the samples were preadsorbed the antiviral activity was reduced below background levels. Therefore, the antiviral activity observed in the uterine vein is due specifically to IFNT.

In order for IFNT to be present in the uterine vein there must be a mechanism for IFNT to get from the endometrium where is acts to suppress ER and OTR and to attenuate

expression of ISG such as ISG15. Many changes take place in the uterus during peri-implantation. As IFNT is secreted by the conceptus, ISG expression in the uterus arises. One member of the WNT (wingless-type mmtv integration site) family, WNT7A, is expressed in the luminal and sub glandular epithelium of the ovine uterus in response to IFNT [164] and is correlated with embryo elongation and trophoblastic giant cell differentiation, and proliferation of the uterine endometrium [165]. Among the many functions of members of the WNT family, maintenance of tight junctions and adheren is crucial for tissue development and remodeling such as that which takes place in the ruminant uterus during early pregnancy. Satterfield and colleagues [166] described the d12 ovine uterus as having reduced tight junctions allowing for a leaky architecture conducive to transport of small molecules. In 2008, this group reported a decrease in expression of WNT7A and other members of the WNT family at the time of increased uterine stromal permeability [167], indicating that IFNT and progesterone can temporarily decrease expression of molecules that maintain tight junction and adheren complexes. In this model, nutrients would more easily reach the developing embryo. The leaky structure of the uterus at this time could also serve to transport IFNT capillary beds where it could enter the venous blood stream leaving the uterus. Once IFNT exits the uterus through the uterine vein it may be picked up by the ovarian artery to stimulate luteal expression of ISG, although molecules of this size are not known to cross over the utero-ovario vasculature. An alternate model is that IFNT is released from the uterine vein and acts systemically to induce ISG in extrauterine tissues such as the CL and peripheral mononuclear cells.

Expression of ISG15 in CL during d15 of pregnancy was recently reported [152]. We confirmed that mRNA for ISG15 is elevated in the CL of d15 pregnant vs cycling ewes. We did not detect changes in mRNA for PGHS, PGES, PGFS, PGDH, StAR, or CYP-11A in CL collected from non-pregnant and pregnant ewes. Peak production of PGF occurs on day 14-15 in both cycling and pregnant ewes [113, 114]. If enzymes in the PGF synthesis pathway are working at peak in the CL during d15 we may not be able to distinguish differences in PGHS, PGES, or PGFS based on pregnancy status alone. Silva [116] reported that PGDH mRNA and enzyme activity are elevated in CL from pregnant ewes on d13 compared to d4 or 13 of the estrous cycle. We did not see a difference in PGDH expression on d15, however this does not exclude the possibility that the PGDH mRNA is elevated prior to the peak in production of PGF and that it may decline by d15 once translation has occurred.

Two methods of delivering IFT to the ovary were examined. The first entailed injecting IFNT into the ovarian artery of day 10 cycling ewes and resulted in increased expression of ISG15 in the CL. The second approach entailed surgical installation of mini-osmotic pumps that delivered 200 μg IFNT over a 24 h period into the uterine vein. Delivery of IFN through the uterine vein resulted in much greater ISG15 mRNA expression in the CL. Because the mini-osmotic pumps reflected estimated IFN released from the uterine vein, provided an even flow of IFNT during the 24 hour or seven day infusions and elicited a greater ovarian response, this mode of IFNT delivery was selected for all subsequent studies.

Uterine vein infusion of IFNT for 24h caused an increase in CL *ISG15* mRNA and protein compared to infusion of BSA. We also detected greater *ISG15* mRNA in the endometrium and liver from IFNT infused ewes. While there were no significant differences in the expression of *ISG15* in CL that were ipsilateral or contralateral to the side of the uterus to which the pump was implanted, there was a trend for greater expression in the ipsilateral CL (data not shown). Taken together, these data provide evidence that IFNT is eliciting a systemic effect of IFNT, but the possibility that IFNT could be also traveling locally through the utero-ovarian blood exchange also exists.

Messenger RNA encoding ISG15 was elevated in the endometrium from both d15 pregnant and IFNT-infused ewes. Expression of ISG15 in the liver was elevated in response to IFNT-infusions, although it was not elevated in liver from d15 pregnant ewes. Ewes that are 15 days pregnant will have been exposed to IFNT for several days so ISG15 may have already increased and decreased in liver. In the infusion model, ewes received an acute exposure to IFNT and respond with elevated mRNA encoding ISG15 in peripheral tissues including the CL, endometrium, and liver. There were no changes in PGDH, PGHS, PGFS, PGES, StAR, PBR, CYP-11A, or 3β-HSD. This could be due to the fact that comparisons of luteal gene expression in pregnant and non-pregnant ewes were compared on one day only. Trends in gene expression may be changing earlier or later than d15.

The endocrine role of IFNT was studied through implementing 24 h infusion into the uterine vein. Ewes treated with BSA and IFN alone did not differ in serum P4

concentrations during the last 12 h of infusion, which remained high. Thus, IFNT is not luteotrophic. Treatment with PGF at 12 following insertion of the pumps resulted in a decline in P4 by 6 h after PGF to levels that were lower than BSA and IFNT controls and remained lower through 12 h. In ewes treated with IFNT, PGF caused a decrease in concentration of progesterone by 6 hours after treatment that recovered by 8 hours and were not different from control BSA and IFNT ewes for the remainder of the experiment. Temporary decline in production of progesterone production is reported in ewes treated with doses of PGF equal to or less than 3 mg per 60 kg body weight [168], whereas higher doses chronically decreased progesterone. In the present experiments, we used 5 mg per 60 kg body weight which elicited a decrease in P4 by 6 h that was maintained through 1h following injection of PGF.

Similar to previous studies [168], concentrations of serum progesterone in ewes treated with IFNT decreased initially after treatment with PGF, but recovered over time compared to ewes treated with IFNT alone. These comparisons would present a case for a role for IFNT preventing the anti-steroidogenic actions of PGF. This potential recovery of progesterone seen in ewes treated with IFNT+PGF is very subtle in the present studies. However, these data are suggestive for a delayed, but definite recovery of progesterone in ewes treated with IFNT. . Because serum P4 appeared to start to recover in the IFNT and PGF treated ewes by 6 h following PGF, we initiated 7-day infusion studies to test the hypothesis that longer-term infusion of IFNT into the uterine vein would extend the estrous cycle and stabilize P4 in response to endogenous PGF release.

To the best of our knowledge, there is no direct evidence for a pro-steroidogenic role for IFNT. IFNγ, however, has been reported to decrease activity of both StAR and P450-SCC in various systems [169]. IFNT stimulated expression in StAR or CYP-11A was not observed. Costine [170] reported that there were no differences in PGDH between cycling and pregnant ewes on d12-13, and we found no differences on d15. If tissues were collected again on d16 and analyzed there may be decreases in PGDH in cycling animals compared to that of pregnant. Messenger RNA encoding StAR and PBR was reduced with treatment of PGF, regardless of treatment with IFNT. The fact that treatment with IFNT did not increase StAR, PBR, CYP11A, or 3β-HSD means that IFNT is not pro-steroidogenic. IFNT does not stimulate genes that are important regulators in steroidogenesis. Instead, IFNT does act through an unknown mechanism to prevent the anti-steroidogenic actions of PGF. Regardless of the mechanism, uterine vein infusion of IFNT seemd to recover the initial decrease in progesterone compared to animals treated with BSA+PGF.

To test the hypothesis that the length of the estrous cycle could be extended by extra-uterine infusions with IFNT, seven day infusions of IFNT were given to d10 ewes. Uterine infusion of IFNT extends luteal lifespan to at least d22-27 post estrus [127, 128, 171]. In the current study, four out of five ewes treated with a seven day infusion of IFNT did not exhibited signs of estrus by necropsy on d32 post estrus. Analysis of daily samples of progesterone yielded evidence that CL in these ewes remained functioning the entire duration. Upon necropsy discovery of the original CL were confirmed with charcoal markings.

Original data described in this manuscript offer evidence to support a novel method for delivery of IFNT over time. Infusion of IFNT was successful in stimulating luteal expression of ISG15 and preventing the anti-luteolytic actions of PGF. Furthermore, infusions of IFNT outside of the uterus provide protection to the CL, extending both inter-estrus interval and steroidogenic capacity. IFNT does not appear to enhance steroidogenesis, but does prevent a decreased steroidogenesis and luteolysis. Further investigation is necessary to fully elucidate mechanisms involved in IFNT mediated luteal function.

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Figure 1: Anatomy of utero-ovarian vascular system. Mini-osmotic pumps were anchored to the uterus with a catheter leading into the uterine vein upstream to the direct contact between the uterine vein and ovarian artery.

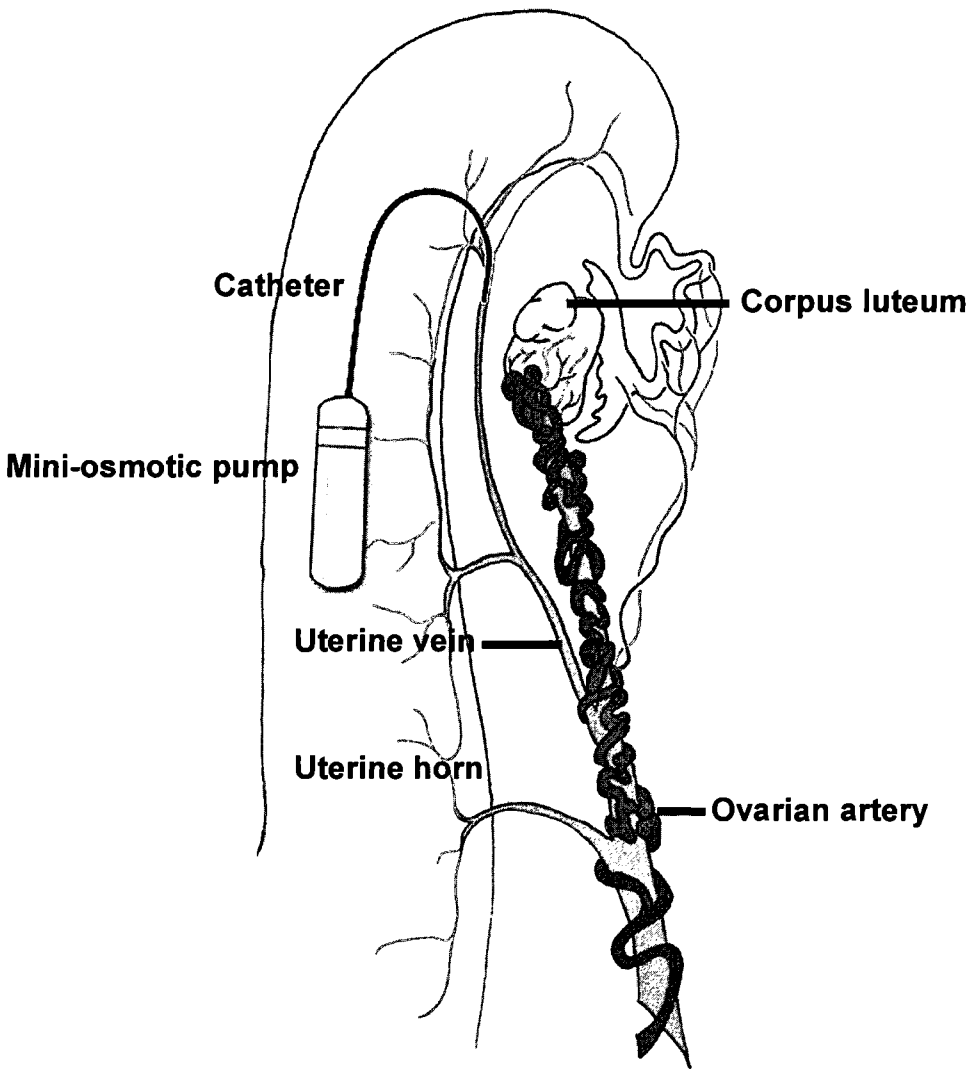

Catheter
Corpus luteum
Mini-osmotic pump
Uterine vein
Uterine horn
Ovarian artery

Figure 2: Real time PCR primer sequences.

| | Target | Sequence | Sequenced | Accesion # |
|---|---|---|---|---|
| Steroidogenesis | StAR | SP GAGTGGAACCCCAGTGTCAA | 1/13/2008 | NM_001009243.1 |
| | | ASP GGGGCATCTCCTCGTAGAGT | | S80098.1 |
| | PBR | SP GGCTTCTCAAAGGAGGCAGT | 1/13/2008 | NM_001009747.1 |
| | | ASP AGGCCAGGTACGGGTACAGT | | |
| | CYP11A | SP CAAGAATCTGGGGTCATGGA | 1/13/2008 | S65754.1 |
| | | ASP AGCCCCGTCATTTCAGTTCT | | |
| | HSD-3B | SP CCCAAAAAGGTCCCAAACAT | 11/19/2008 | NM_001135932.1 |
| | | ASP AGAAGCCCCATTCTTTGCTC | | |
| Prostaglandin Cascade | PGDH | SP AACCTACCTGGGCTTGGATT | 1/13/2008 | NM_001034419.1 |
| | | ASP TAAACAGGCTGCTGTGCAAC | | DQ231564.1 |
| | PGHS | SP TCTGCGGTGCAGCAAATCCTT | 1/15/2008 | NM_001009432.1 |
| | | ASP TTTTCACCATAGAATCCTGTTCGGG | | |
| | PGFS | SP ATGATGGCCACTTCATTCCTGTCC | 1/13/2008 | AF257738.1 |
| | | ASP TGGCGGAACCCAACCTCTATAGC | | |
| | PGES | SP AGGACGCTCAGAGACATGGA | 8/11/2008 | NM_174443.2 |
| | | ASP GTTCGGTCCGAGGAAAGAGT | | |
| ISG | ISG15 | SP GGTATCCGAGCTGAAGCAGTT | Han et al., 2006 | NM_174336 |
| | | ASP ACCTCCCTGCTGTCAAGGT | | |
| Housekeeping | GAPDH | SP GATTGTCAGCAATGCCTCCT | Han et al., 2006 | BC102589 |
| | | ASP GGTCATAAGTCCCTCCACGA | | |
| Apoptosis | Caspase3 | SP ATGGAAGCAAATCAATGGACTCTGG | 1/13/2008 | AF068837.1 |
| | | ASP CCATGCCAGTATTTTCGTGGAAGTT | | |

Figure 3: Antiviral indices detected in plasma from d15 ewes incubated with normal rabbit serum (NRS) (n=2), preadsorbed with IFNT antibody (n=2), d15 open ewe (n=1), or d15 open ewe spiked with IFNT (n=1). Values are represented as mean ± SE. Significant differences (*P<0.05) between treatments were detected with t-tests.

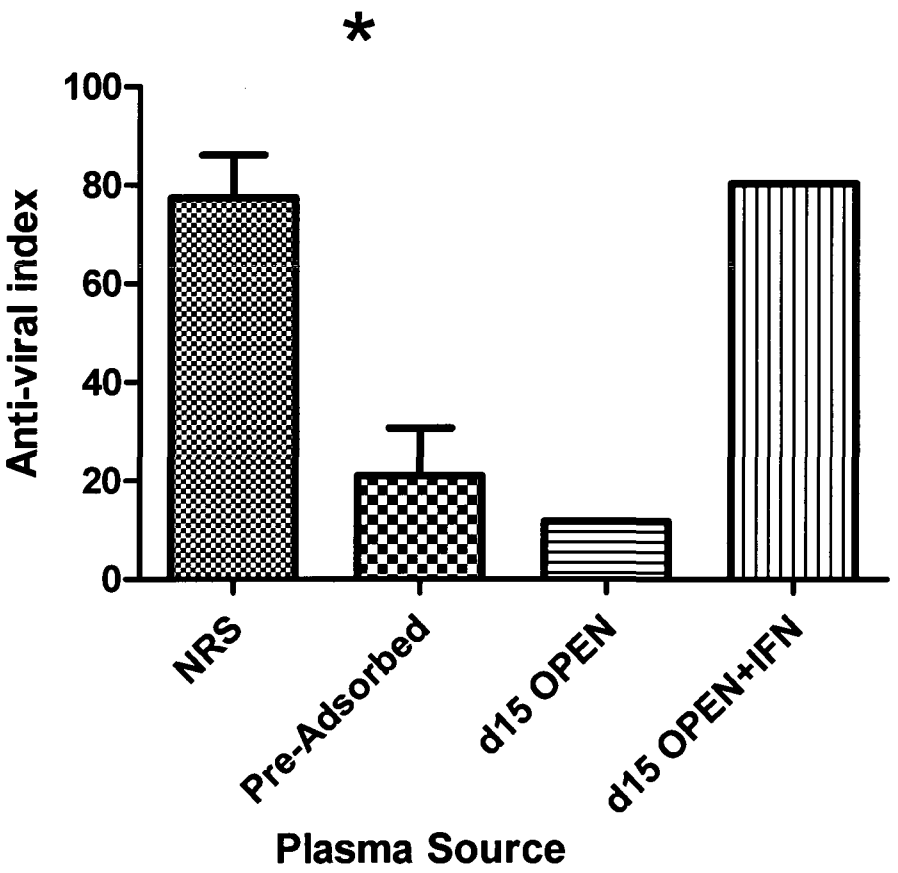

*
100
80
60
40
20
0
Anti-viral index
NRS
Pre-Adsorbed
d15 OPEN
d15 OPEN+IFN
Plasma Source

Figure 4: Relative quantities of luteal mRNA encoding ISG15 from d10 cyclic ewes that received ovarian artery injections of BSA (OA BSA, n=2), or IFNT (OA IFNT, n=2), or 24 hour infusions of BSA (BSA, n=2), or IFNT (IFNT, n=3). ΔΔ Ct values are presented for each treatment group compared to BSA controls. Data presented as mean ± SE with significant differences ($P<0.05$) between treatments denoted by different letters.

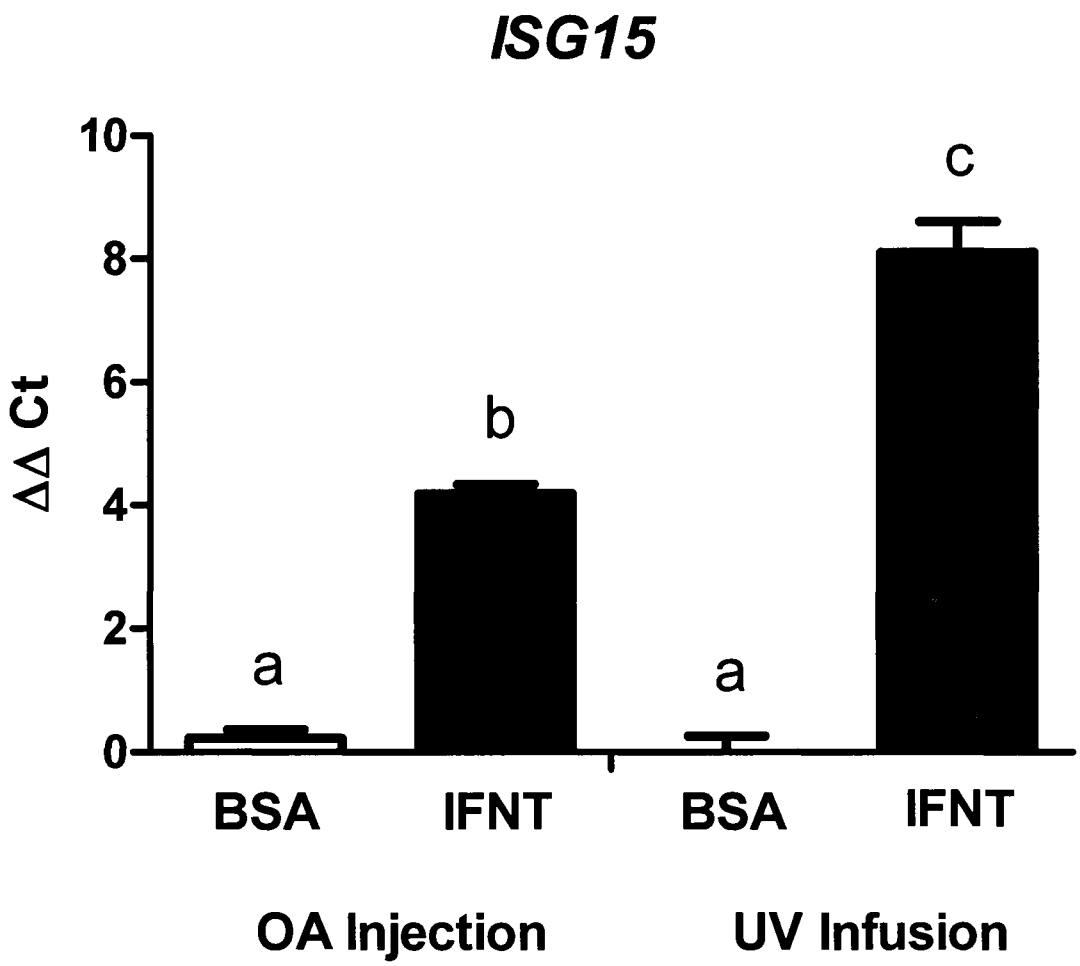

ISG15
10
8
6
4
2
0
ΔΔ Ct
a
b
a
c
BSA
IFNT
BSA
IFNT
OA Injection
UV Infusion

Figure 5: Relative quantities of luteal mRNA encoding ISG15 in d10 cyclic ewes treated with BSA, BSA+PGF, IFNT, or IFNT+PGF. ΔΔ Ct values are presented for each treatment group (n=6) compared to sham controls. Data are presented as mean ± SE with significant differences ($P<0.05$) between treatments represented as different letters.

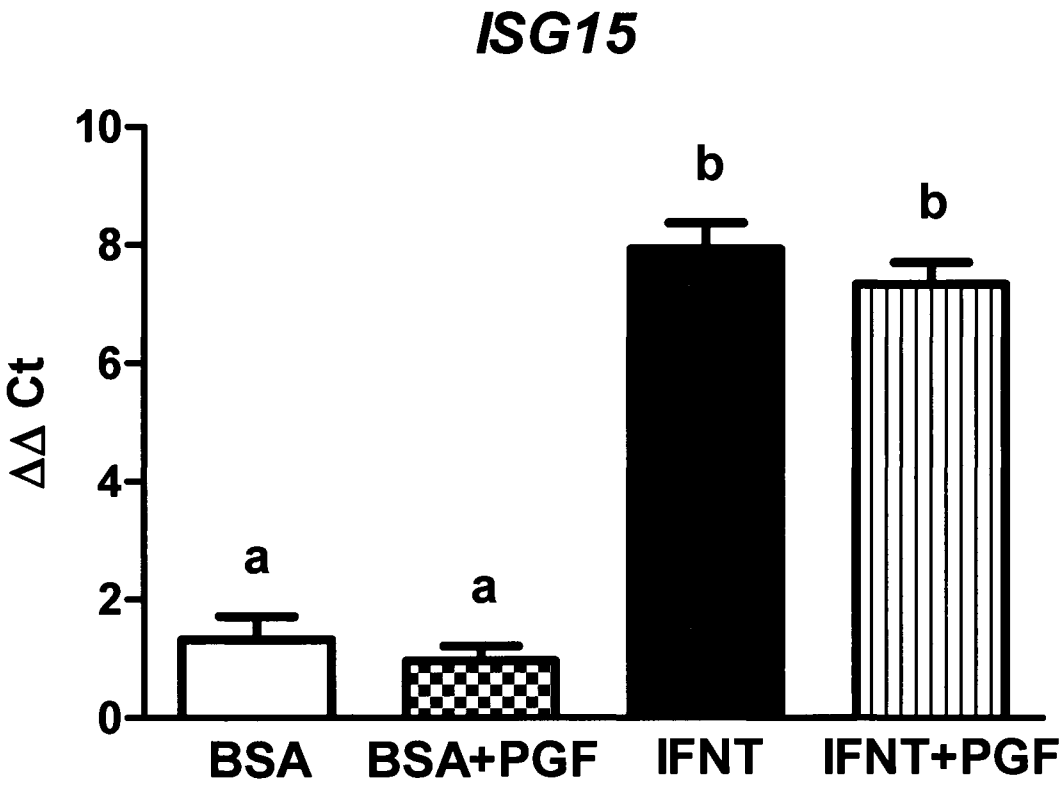

ISG15
ΔΔ Ct
10
8
6
4
2
0
a
a
b
b
BSA
BSA+PGF
IFNT
IFNT+PGF

Figure 6: Western blot detection of ISG15 protein from recombinant ovine ISG15 (+), and in CL from SHAM, BSA, BSA+PGF, IFNT, and IFNT+PGF-treated ewes (A) (n=6 per group). Optical density of the protein bands for free (B) and conjugated (C) ISG15 was calculated and represented as mean ± SE with significant differences ($P<0.05$) between treatments represented as different letters.

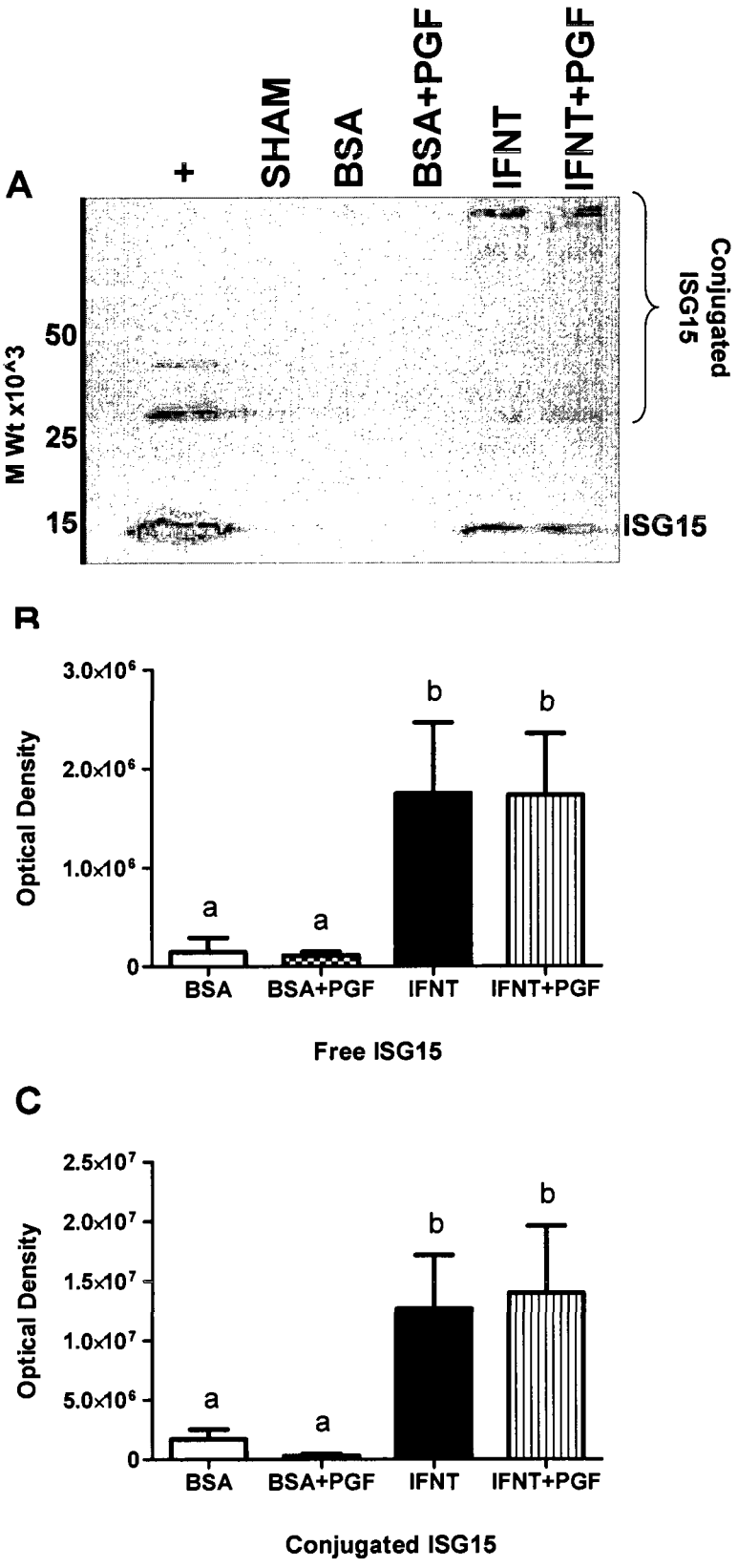

A
+
SHAM
BSA
BSA+PGF
IFNT
IFNT+PGF
M Wt x10^3
50
25
15
Conjugated ISG15
ISG15
B
Optical Density
3.0x10^6
2.0x10^6
1.0x10^6
0
a
a
b
b
BSA
BSA+PGF
IFNT
IFNT+PGF
Free ISG15
C
Optical Density
2.5x10^7
2.0x10^7
1.5x10^7
1.0x10^7
5.0x10^6
0
a
a
b
b
BSA
BSA+PGF
IFNT
IFNT+PGF
Conjugated ISG15

Figure 7: Relative quantities of messenger RNA encoding ISG15 in the endometrium (A, n=6) and liver (B, n=6) from d15 non-pregnant (NP) and pregnant (P) ewes, and in endometrium (C, n=6) and liver (D, n=3) of ewes treated with BSA, BSA+PGF, IFNT, or IFNT+PGF-infusions. $\Delta\Delta$ Ct values are presented for each treatment group compared to sham controls. Data are represented as mean ± SE with significant differences ($P<0.05$) between treatments or physiological status represented as different letters.

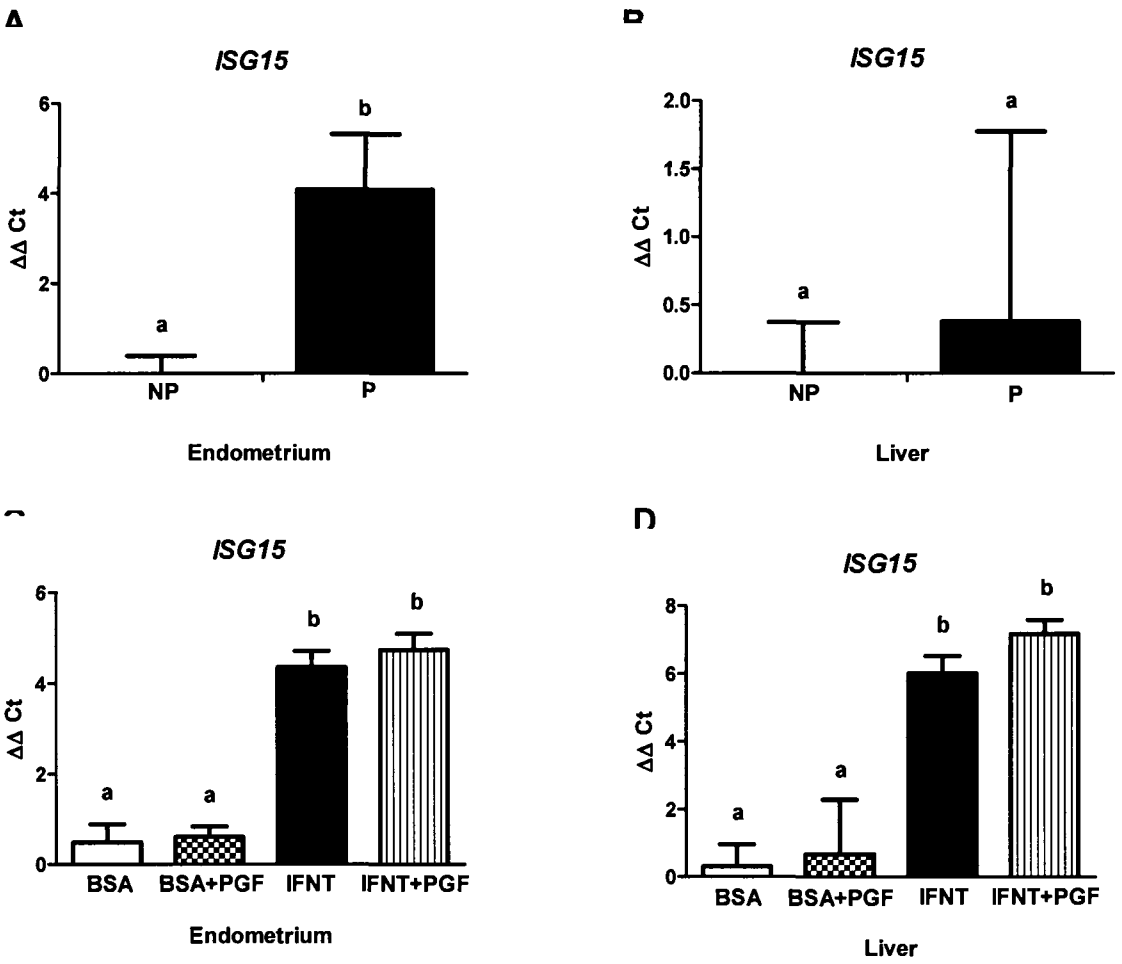

A
ISG15
ΔΔ Ct
6
4
2
0
a
b
NP
P
Endometrium
B
ISG15
ΔΔ Ct
2.0
1.5
1.0
0.5
0.0
a
a
NP
P
Liver
C
ISG15
ΔΔ Ct
6
4
2
0
a
a
b
b
BSA
BSA+PGF
IFNT
IFNT+PGF
Endometrium
D
ISG15
ΔΔ Ct
8
6
4
2
0
a
a
b
b
BSA
BSA+PGF
IFNT
IFNT+PGF
Liver

Figure 8: Serum concentration of progesterone (ng/ml) in ewes that received 24 hour uterine vein infusions of BSA, BSA+PGF, IFNT, or IFNT+PGF. Data are presented as mean of the replicates (n=6 per treatment) ± SE. Significant differences ($P<0.05$) in treatments and time are denoted with different letters.

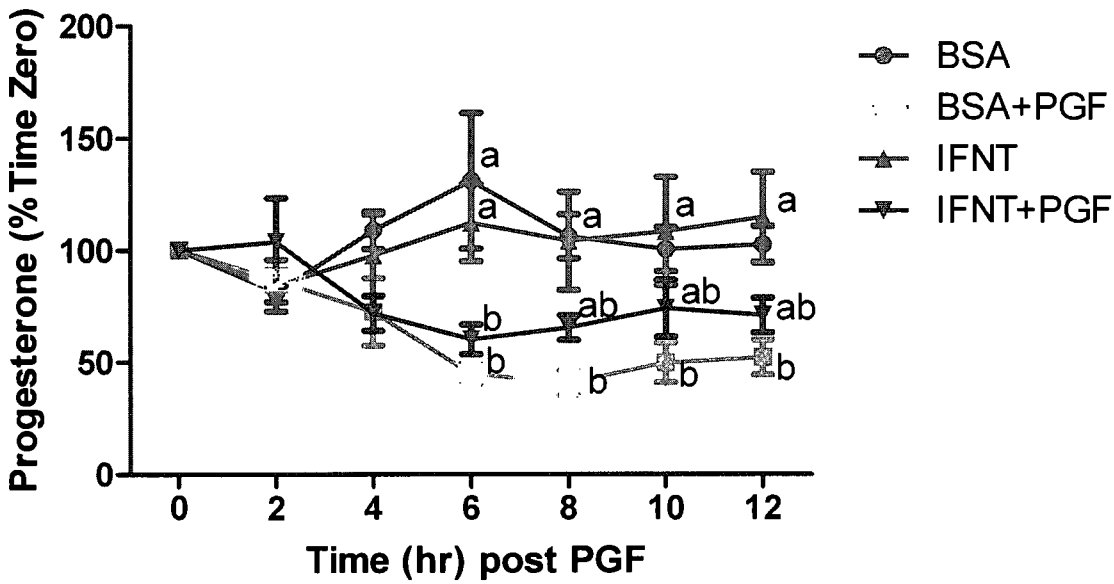
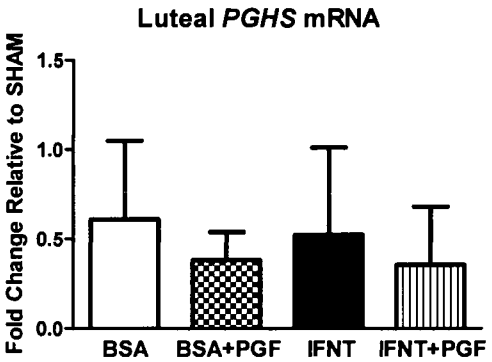
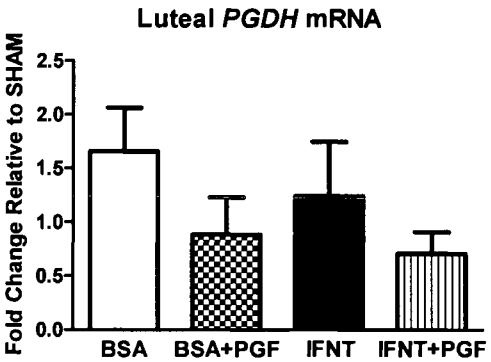
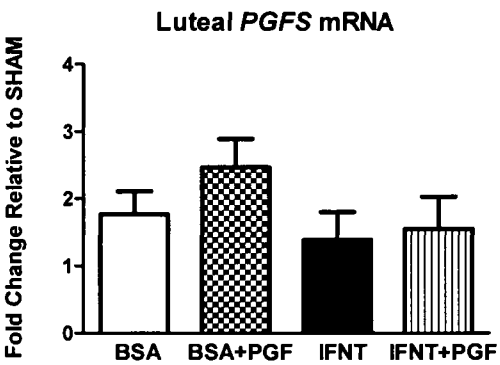
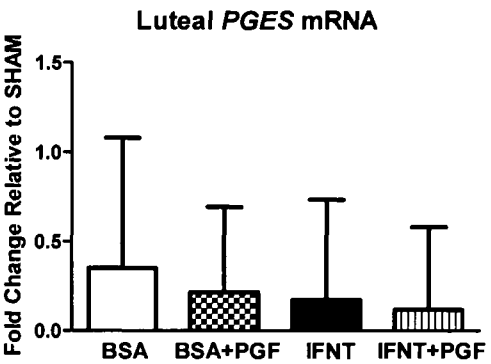

Progesterone (% Time Zero)
200
150
100
50
0
0
2
4
6
8
10
12
Time (hr) post PGF
a
a
a
a
a
b
ab
ab
ab
b
b
b
BSA
BSA+PGF
IFNT
IFNT+PGF

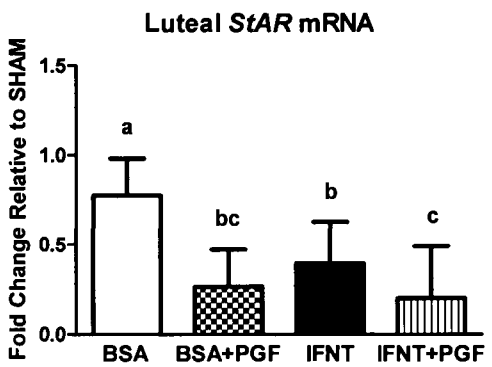
Figure 9: Relative quantities of luteal mRNA encoding PGDH, PGHS, PGFS, PGES, StAR, PBR, CYP-11A, and 3β-hsd (n=6). Fold changes relative to SHAM controls are presented for each treatment group. Data are represented as mean ± SE with significant differences ($P<0.05$) between treatments or physiological status represented as different letters.

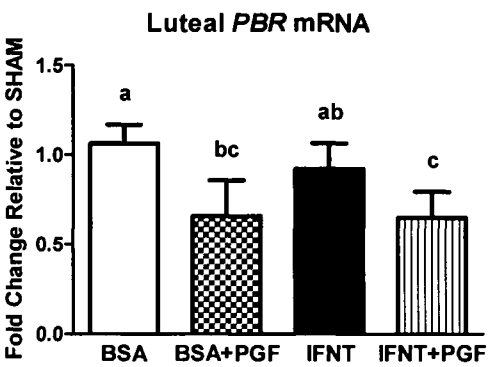

Luteal PGHS mRNA
Fold Change Relative to SHAM
1.5
1.0
0.5
0.0
BSA
BSA+PGF
IFNT
IFNT+PGF

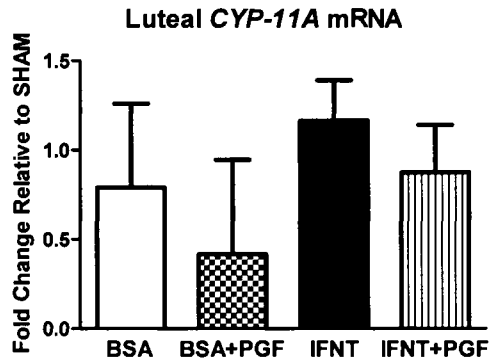

Luteal PGDH mRNA
Fold Change Relative to SHAM
2.5
2.0
1.5
1.0
0.5
0.0
BSA
BSA+PGF
IFNT
IFNT+PGF


Luteal PGFS mRNA
Fold Change Relative to SHAM
4
3
2
1
0
BSA
BSA+PGF
IFNT
IFNT+PGF


Luteal PGES mRNA
Fold Change Relative to SHAM
1.5
1.0
0.5
0.0
BSA
BSA+PGF
IFNT
IFNT+PGF


Luteal StAR mRNA
Fold Change Relative to SHAM
1.5
1.0
0.5
0.0
a
bc
b
c
BSA
BSA+PGF
IFNT
IFNT+PGF


Luteal PBR mRNA
Fold Change Relative to SHAM
1.5
1.0
0.5
0.0
a
bc
ab
c
BSA
BSA+PGF
IFNT
IFNT+PGF


Luteal CYP-11A mRNA
Fold Change Relative to SHAM
1.5
1.0
0.5
0.0
BSA
BSA+PGF
IFNT
IFNT+PGF

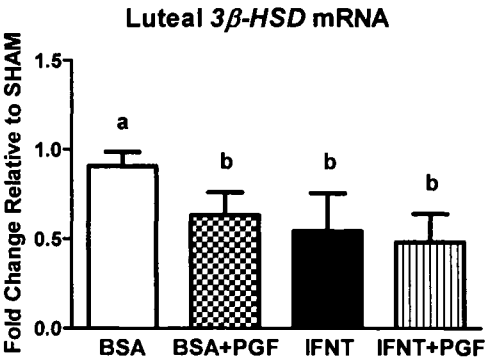

Luteal 3β-HSD mRNA
Fold Change Relative to SHAM
1.5
1.0
0.5
0.0
a
b
b
b
BSA
BSA+PGF
IFNT
IFNT+PGF

Figure 10: Percent of ewes failing to return to estrus by d32 after a seven day infusion of BSA or IFNT. All ewes were necropsied upon detection of heat or on d32 post estrus if estrus was not observed before. Data are presented as mean ± SE (*P<0.01).

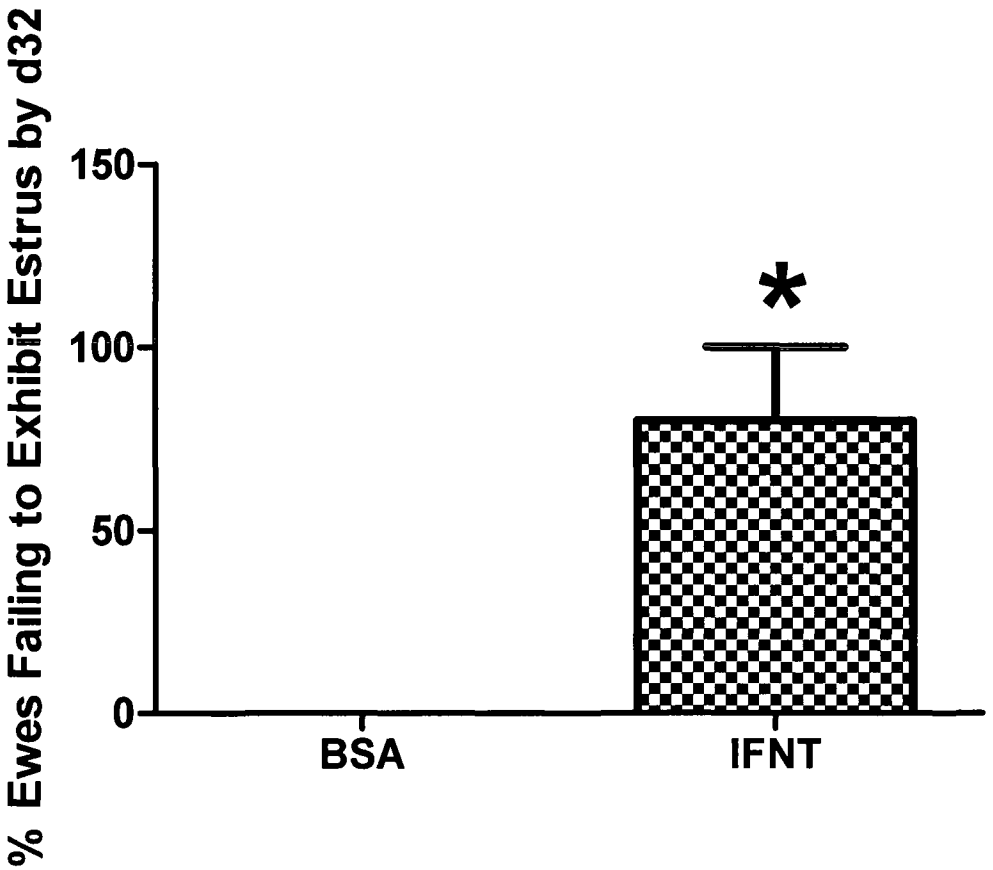

% Ewes Failing to Exhibit Estrus by d32
150
100
50
0
*
BSA
IFNT

Figure 11: Original CL were present d32 post estrus. These CL (A) were marked with charcoal (Arrows) at the time of surgery. The uterine vein catheter (arrow head) was still in the original location (B), as well as the mini-osmotic pump (notch arrow, C).

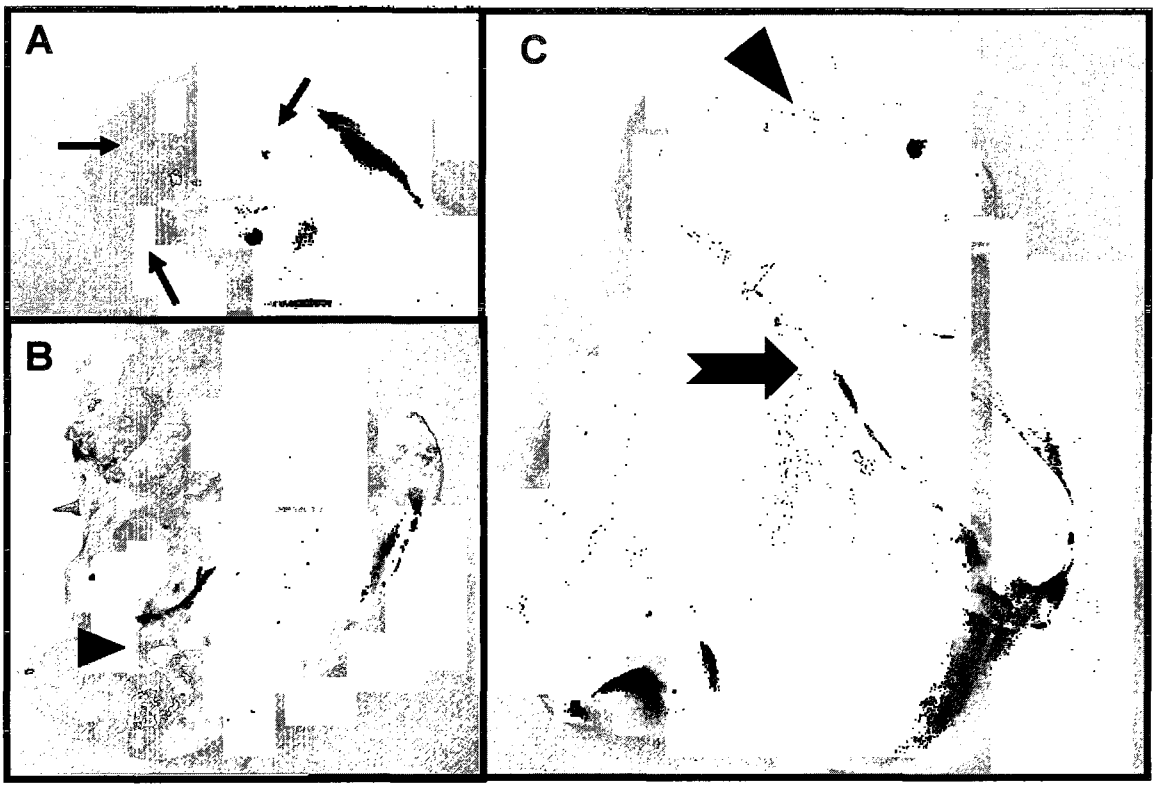
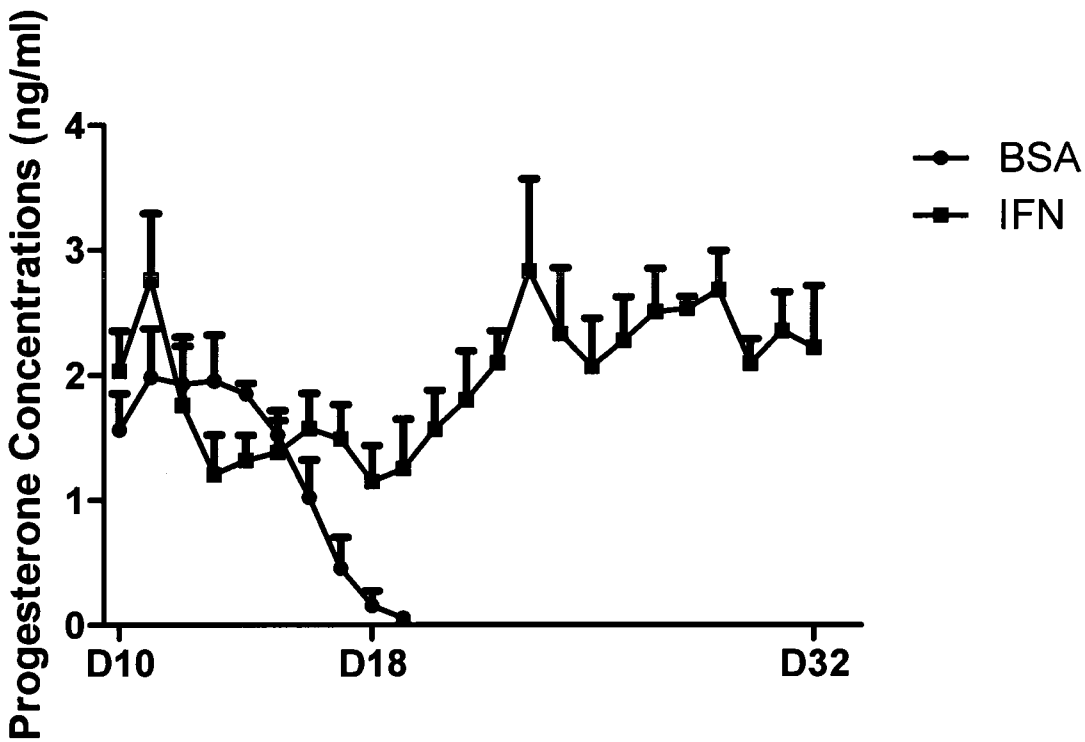

A
B
C

Figure 12: Serum concentrations of progesterone (ng/ml) in ewes that received seven day uterine vein infusions of BSA or IFNT. Data are presented as mean of the replicates (n=6 BSA, n=5 IFNT) + SE.


Progesterone Concentrations (ng/ml)
4
3
2
1
0
D10
D18
D32
BSA
IFN

## Chapter 3

**Recombinant equine luteinizing hormone (reLH) stimulates production of progesterone from murine Leydig, equine granulosal, and ovine small luteal cells.**

### Abstract

Recombinant equine luteinizing hormone (reLH) was evaluated for its ability to stimulate production of progesterone in cell lines from three species including murine Leydig tumor (MA-10) cells, equine granulosal, and ovine small luteal cells (SLC). The response to reLH was compared to that of human chorionic gonadotropin (hCG), equine chorionic gonadotropin (eCG), ovine luteinizing hormone (oLH), and equine luteinizing hormone (eLH). Cell lines were treated with vehicle control, or 1, 10, or 100ng/ml hormone for one hour. Media were collected hourly for 6 hours and assayed for progesterone content via radioimmunoassay. In MA-10 cells, production of progesterone was stimulated above baseline by eLH, reLH, and hCG ($P<0.05$). Ovine SLC responded to treatment with eLH, reLH, oLH, and hCG by increasing production of progesterone above that stimulated by vehicle control ($P<0.05$). Production of progesterone in equine granulosal cells was maximally stimulated by treatment with hCG ($P<0.05$), followed by reLH and eLH ($P<0.05$). Total areas under the curve measurements were highest for reLH and hCG in MA-10 cells, for hCG in equine granulosal cells, and for eLH, reLH and hCG in ovine SLC ($P<0.05$), consistent with total production of progesterone. In conclusion, reLH elicited a progesterone response similar to eLH and hCG in the MA-10 cells line. Concentrations of progesterone were elevated in ovine SLC by eLH, reLH,

oLH and hCG. In equine granulosal cells, production of progesterone was stimulated by hCG, eLH, and reLH. Based on these data, reLH stimulates the production of progesterone in cell lines from three species.

Keywords

Recombinant equine luteinizing hormone; production of progesterone; equine granulosal cells; MA-10 cells; ovine small luteal cells

## Introduction

Gonadotropic hormones are often utilized in assisted reproductive technology protocols. Human chorionic gonadotropin (hCG) is commonly used to induce ovulation in the equine [172]. However, several studies have shown that horses become less responsive to hCG during the course of a breeding season [173] apparently due to production of antibodies to the human protein [174] [175]. A hormone, that induces ovulation but does not activate the antibody response, would provide a desirable alternative to hCG. A new single chain recombinant equine luteinizing hormone (reLH), with a linker protein between the α and β subunits, was recently developed [176]. Further characterization of the cellular actions of reLH is critical for understanding how to integrate its use into current reproductive protocols. The objective of the current study was to evaluate the ability of reLH to stimulate production of progesterone from cells from three species and to compare the response to that induced by other well characterized glycoprotein hormones including equine chroionic gonadotropin (eCG), equine LH (eLH), ovine LH

(oLH), and human chorionic gonadotropins (hCG). We hypothesized that production of progesterone from cells treated with reLH would resemble the pattern of production of progesterone stimulated by hCG [177] increasing upon treatment and remaining elevated throughout the six hour culture period, while production of progesterone in cells treated with oLH, eLH, and eCG would increase transiently followed by a rapid return to baseline. This hypothesis was based on previous results obtained with oLH and hCG in MA-10 cells [178] and ovine small steroidogenic luteal cells [6].

## Materials and Methods

*Animals*

Western range ewes of mixed breeding and age were treated with two 5mg injections of Lutalyse (Pfizer, New York, NY) four hours apart during the mid-luteal phase of the estrous cycle. Equine Chorionic gonadotropin (1500IU) was administered (IP) at the time of the second Lutalyse injection, followed by 50μg GnRH (Vedco, St. Joseph, MO) 48 hours later to synchronize ovulations. Corpora lutea were collected from ewes on day 10 (day 0 = estrus) of the following estrous cycle. To obtain equine granulosal cells mares of mixed breeding and ages were utilized during the breeding season. Upon detection of a 35mm follicle and uterine edema they received 1.5mg Deslorelin (Franck's Pharmacy, Ocala, FL) followed by 2000IU Chorulon (Intervet, Millsboro, DE) four hours later. Granulosa cells were collected via follicular aspiration 20 hours after Chorulon injection. All animal protocols were approved by the Colorado State University Animal Care and Use Committee.

*Cell Collections and Preparation*

MA-10 cells were retrieved from frozen stocks. Populations of ovine small luteal cells were purified from the CL as previously described [177]. Briefly, luteal tissues were dissociated in a collagenase solution and elutriated via centrifugation to yield large and small luteal cell populations. Ovine SLC in each experiment were pools of cells from at least two ewes.

*Equine Granulosal Freezing Protocol*

Because aspirations of mare follicles on any given day did not yield sufficient granulosa cells to conduct a full experimental replicate, it was necessary to develop a protocol to freeze granulosal cells. Equine granulosal cells were collected via trans-vaginal aspiration from pre-ovulatory follicles. Follicular fluid containing granulosa cells was diluted 1.5 fold in DMEM containing 0.05% (50mg/100mls) collagenase A (Boehringer Manheim Co., Indianapolis, IN). The granulosa cells were vortexed, incubated in a 37°C water bath for 20 minutes, and centrifuged for 20 minutes at 1250rpm. The medium was replaced with an equivalent volume of collagenase free medium and cells were dispersed by repetitive pipetting. DMSO (Malinckrodt Baker, Inc., Paris, KY) was added for a final concentration of 5% (v/v) and 1ml aliquots were placed in cryovials (CryoTube, Roskilde, Danmark), and cells frozen using a Kryo10 liquid nitrogen freezing apparatus (Pacific Science Inc., Torrance, CA). Eight cryoprotectants and three freezing protocols were used in combination to develop an efficacious freezing protocol. Equine granulosal cells were mixed in 50% (v/v) E-Z Freezin Lactose EDTA or E-Z Freezin Modified

French semen extenders (Animal Reproduction Systems, Chino, CA) or in DMSO (Malinckrodt Baker, Inc., Paris, KY), DMF (Sigma-Aldrich, St. Louis, MO), or Glycerol (Viampac, Inc., River Falls, WI), at 1% or 5% final concentration. The freezing protocols began at 20°C and brought the chamber to a final temperature of -120°C at different rates. The first protocol reduced chamber temperature to 4°C by one quarter of a degree per minute. Then the chamber was cooled at a rate of 10°C/minute to -15°C and finally to -120°C at a rate of 15°C/minute. The second protocol decreased the temperature to -15°C at a rate of 1°C/minute, then to -80°C by decreasing 13°C/minute and decreasing to -120°C at a rate of 15°C/minute. The final protocol was developed at Colorado State University to freeze stallion semen. The cells were cooled to -10°C at a rate of 10°C/minute and then were cooled to -120°C at 15°C/minute. Upon completion of the freezing protocols cells were transferred to liquid nitrogen for storage. Cells were thawed in a 37°C water bath, plated at a concentration of 1,000,000 cells/ 35mm well, and incubated overnight. The following morning a 0.2% (v/v) solution of Trypan Blue (Malinckrodt Baker, Inc., Paris, KY) was used to differentiate between live and dead cells. Each equine granulosal cell experimental replicate was conducted using a pool of granulosa cells containing cells collected from mares on at least three different days.

*Cell Culture*

MA-10, ovine SLC, and equine granulosal cells were plated at a concentration of $5x10^5$, $2x10^5$ and $1x10^6$ cells per 35mm well, respectively, in filter sterilized DMEM (Cellgro, Herndon, VA) with 10% (v/v) heat inactivated fetal bovine serum (Atlanta Biologicals, Norcross, GA) and 0.1% (v/v) PennStrep (Cellgro, Herndon, VA). Cells were incubated

at 37°C overnight. There were a total of three replicates for MA-10 and equine granulosal cells and two replicates for ovine SLC.

*Hormone Treatments*

After cells were incubated overnight the media were changed four times over a one-hour period to remove progesterone that had been produced in culture and to establish a stable base-line. The fourth rinse was used to establish basal concentrations of production of progesterone. Cells were then treated for one hour with 1, 10 or 100ng/ml of hormone or gelatin phosphate buffered saline (gel PBS) as a vehicle control. Hormone treatments included eCG (Dr. Papkoff, UCLA), eLH, oLH, hCG (National Hormone and Peptide Program), or reLH (Apsen Biotech) diluted in gel PBS. Hormone treatment lasted for one hour followed by hourly media collection and replacement for an additional five hours. Samples were snap frozen in an acetone dry ice bath and stored at -20°C.

*Radioimmunoassy*

Progesterone content was determined via radioimmunoassay as previously described [163]. Each sample was run in duplicate and production of progesterone (fg/cell/hour) was calculated. Assay sensitivity was 3.41 picograms, and the mean intra- and inter-assay coefficients of variation were 7.26 and 18.26, respectively (n=7).

*Statistical Analysis*

Peak production of progesterone, interval to peak and area under the curve (AUC) were analyzed using the proc mixed procedure in a complete randomized design. Treatment

was considered the only source of variation in this model. Initial analysis revealed no significant interactions between treatment and dose, so the dose effect was removed from each model. Treatment comparisons among cell lines were analyzed by transforming data so that vehicle control was set to 100% and the progesterone response in each treatment group was expressed as percent of vehicle control. Complete randomized design with the proc mixed procedure was used to analyze differences among cell lines. Cell line and treatment were sources of variation in this model. Comparisons with $P<0.05$ were considered significant.

## Results

*Freezing Equine Granulosal Cells*

Twenty-four combinations of freezing rates and cryoprotectant were tested to determine a successful freezing protocol that yielded live equine granulosal cells. The only process yielding live cells as determined by the Trypan Blue cell exclusion procedure was freezing using 5% DMSO and cooled to -15°C at a rate of -1°C/minute, then to -80°C at -13°C/minute and finally to -120°C at a rate of -15°C/ minutes.

*Production of progesterone*

The parameters analyzed for the production of progesterone included peak concentration of progesterones, time to peak production and total production as determined by total area under the curve. When production of progesterone was plotted over time (Figure 1) distinct patterns within and between cells lines became detectable. In MA-10 cells,

progesterone increases and decreases rapidly. Recombinant eLH was the only treatment that maintained production of progesterone over vehicle control six hours after treatment ($P<0.05$). Production of progesterone remained high at six hours in cells treated with eLH, reLH, and hCG compared to vehicle controls in ovine SLC and equine granulosal cells ($P<0.05$).

Base-line progesterone (fg/cell) did not differ within cell lines prior to treatment. Progesterone did not increase in vehicle treated MA-10 or ovine SLC, but did increase in equine granulosal cells after treatment with the vehicle control ($P<0.05$). Production of progesterone in MA-10 cells was significantly increased by treatment with eLH, reLH, and hCG ($P<0.05$). Equine LH, reLH, oLH, and hCG induced elevated production of progesterone in ovine SLC compared to eCG, or vehicle control ($P<0.05$). Human chorionic gonadotropin elicited maximal production of progesterone in equine granulosal cells ($P<0.05$). Production of progesterone in these cells was also increased by eLH, and reLH treatments compared to vehicle control ($P<0.05$).

Maximum (peak) progesterone in MA-10 cells was 25 fg/cell, while baseline production in equine granulosal cells was 3 fg/cell with peak production induced by hCG reaching 326 fg/cell. Ovine SLC responded to hormone production more potently than the other cells lines, reaching a peak of 7,650 fg/cell. The average interval from initial treatment to time of peak production of progesterone was 3.6, 2.9, and 4.3 hours in MA-10, ovine SLC, and equine granulosal, respectively. The interval did not differ among treatments within MA-10 or equine granulosal cells, but was higher in ovine SLC treated with hCG

(4.3 hours) compared to the other treatments ($P<0.05$). Peak progesterone (fg/cell) was no different between treatments in MA-10 cells (Figure 2A). The progesterone peak in ovine SLC was higher in eLH, reLH, oLH, and hCG treated cells compared to those treated with eCG or vehicle control (Figure 2B; $P<0.05$). Finally, hCG yielded a higher peak progesterone concentrations in equine granulosal cells than any other treatment (Figure 2C; $P<0.05$).

Areas under the curve (AUC) were also calculated for each treatment within each cell line (Figure 3) as a measure of total production of progesterone. The AUC for reLH was greater than all other treatments in MA-10 cells with exception of hCG which was the only other hormone to yield an AUC greater than vehicle control ($P<0.05$). In ovine SLC, eLH, reLH, and hCG had larger AUC measurements than other treatments ($P<0.05$). In equine granulosal cells, hCG was the only treatment that differed from others in AUC ($P<0.05$).

*Progesterone Responses Among Cell Lines*

Both MA-10 and ovine SLC responded to treatment with eLH, reLH, and hCG by producing higher progesterone as a percent of vehicle control than equine granulosal cells (Figure 4; $P<0.05$). Recombinant eLH also stimulated a significant increase in progesterone response from MA-10 compared to ovine SLC ($P<0.05$). There were no differences between cell lines in their ability to respond to treatment with eCG. Ovine SLC responded more potently to treatment with oLH than either MA-10 or equine granulosal cells ($P<0.05$).

## Discussion

The gonadotropic hormones used in this study bind to LH receptors and elicit production of progesterone. The MA-10 [179], equine granulosal [180]; [181] and ovine SLC [182] cells all express functional LH receptors. Several previous experiments were conducted on MA-10 and ovine SLC to evaluate and compare the actions of oLH and hCG on cell responses [177, 178]. These studies provided a foundation for the current objectives and anticipated results.

MA-10 cells respond to a ten minute pulse of hCG and oLH with elevated production of progesterone [178]. The steroidogenic response to hCG differed from oLH in the duration of elevated production of progesterone. Progesterone increased in response to hCG and remained elevated throughout the experiment. In contrast, progesterone increased transiently in response to oLH. In the current study progesterone was stimulated by eLH, reLH and hCG in MA-10 cells. However, eLH and hCG stimulation was transient compared to progesterone productin in cells treated with reLH. The internalization rates of bound oLH and hCG are similar in the MA-10 cell line [183], therefore the ability of reLH to maintain production of progesterone is unlikely due to a varying rate of internalization. Instead, reLH may have a different dissociation rate allowing it to sustain cellular responses over time.

Ovine SLC respond to a one hour pulse of hCG with increased production of progesterone that is maintained over time while oLH causes an increase in steroidogenesis that declines within six hours [177]. Bound oLH is internalized more

quickly than bound hCG in SLC [184]. Differences in cellular responses to these hormones could be influenced by differential hormone receptor complex internalization. In the current study, production of progesterone was elevated by eLH, reLH, oLH, and hCG within an hour of hormone treatment. Production of progesterone was significantly higher at the end of the 6 hour experiment in cells treated with eLH, reLH, and hCG, while progesterone in oLH treated cells had returned to baseline. These findings support data presented by Bourdage and colleagues [177] who reported that hCG maintained progesterone responses longer than oLH in vitro. Equine CG did not stimulate production of progesterone. While eCG and eLH share identical β subunit sequences [185], eCG may elicit FSH-like responses [186, 187], which is why increased production of progesterone was not observed in our model. Ovine SLC demonstrated the broadest range of response to hormone treatment in terms of production of progesterone which could have implications for use in vivo.

While the current study is the first to investigate the ability of reLH to stimulate production of progesterone in MA-10, equine granulosal and ovine SLC, the effects of reLH on cultured Leydig cells from the stallion have been studied in vivo. Testosterone production is increased by cultured equine Leydig cells, and in stallions treated with reLH [176]. In the mare reLH can be used to induce ovulation within 48 hours similar to hCG, yet does not change inter-ovulatory timing [188]. Considering data from both applied studies and in vitro culture systems will enhance our understanding of the effects and actions of reLH in a variety of models. Basal production of progesterone in the equine granulosal cells was extremely high compared to the other two cells lines in the

current study. The high baseline production of progesterone observed in these cells lines is likely caused by hCG treatment of the mares prior to follicular aspiration. Even if mares had not been treated with hCG to induce ovulation, LH is normally increasing leading up to the time of ovulation and for a short time afterwards. Collecting granulosa cells much earlier in the mare's reproductive cycle could yield cells with a lower content of LH/CG receptors [180] and ability to respond to treatment in culture. Equine LH, reLH, and most significantly, hCG were able to stimulate production of progesterone beyond the high baseline concentrations in this experiment. All three of these hormones maintained a progesterone response in equine granulosal cells above baseline 6 hours after treatment. However, hCG was the only treatment yielding a higher total production of progesterone than vehicle control as calculate by AUC.

A granulosal cell freezing protocol was developed in the course of this study. This protocol will allow pools of equine cells to be available for use in research laboratories so that experiments can be conducted outside of the duration of the breeding season. The granulosa cells that had been frozen and thawed demonstrated the ability to be stimulated by hormone treatment in culture.

The objective of the study was to examine the ability of reLH compared to other commonly used gonadotropins to elicit steroidogenic responses in cell lines from three species. Recombinant eLH can elicit production of progesterone in MA-10, ovine SLC, and equine granulosal cells. Steroidogenic response in these three cell lines was maintained over the 6 hour culture experiment in response to reLH. Given these results,

it is possible that reLH can be used in vivo to elicit endogenous production of progesterone during early pregnancy and to prevent early embryonic wastage. An additional aim of the experiment was to determine if the biological actions of reLH resembled those of hCG or other commonly used gonadotropins. The actions of reLH appeared to closely resemble those of hCG in the current models. There was also a likeness in cellular responses to reLH and eLH. The ability of reLH to elicit a significant progesterone response that lasts through the end of six hour sampling in cells from three species indicates that this hormone is highly effective. The current experiments focused on the ability of reLH to stimulate production of progesterone, however, reLH may be a likely candidate for additional uses. Further experiments are necessary to fully elucidate potential role for reLH as a substitute for hCG during the induction of ovulation without causing resistance with repeated use.

**Acknowledgements**

I would like to thank my co-authors Shevin Schwartzenberger, Dr. Bruemmer, Dr. Arreguin-Arrevelo, and Dr. Niswender for their assistance and efforts. The authors sincerely thank Dr. J. Roser for supplying reLH, Dr. H. Papkoff for providing eCG, Dr. E. Carnevale for supplying the equine granulosal cells, and to M. Gallegos, and R. Brandes for help with animal care. We thank J. Walker for statistical assistance, R. Bogan for advice on cell culture systems, H. Mayan and P. Moffet for advice on cell freezing protocols, and to Dr. T. Hansen and Dr. R. Ashley for critical advice.

Figure 1: Production of progesterone (fg/cell/hour) over time in MA-10 cells (A), ovine SLC (B), and equine granulosal cells (C). Cells were exposed to treatment for one hour beginning at time zero (0).

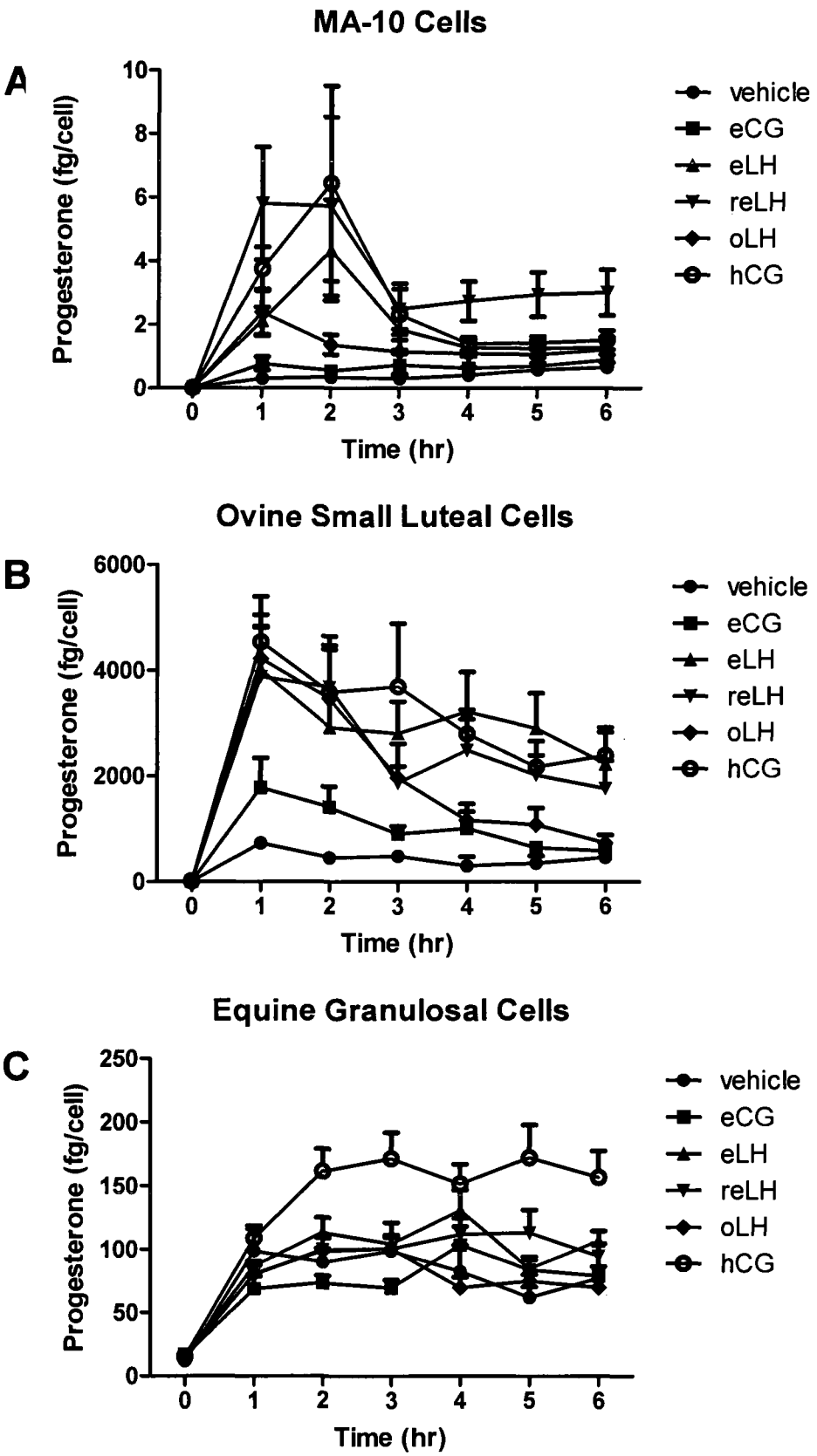
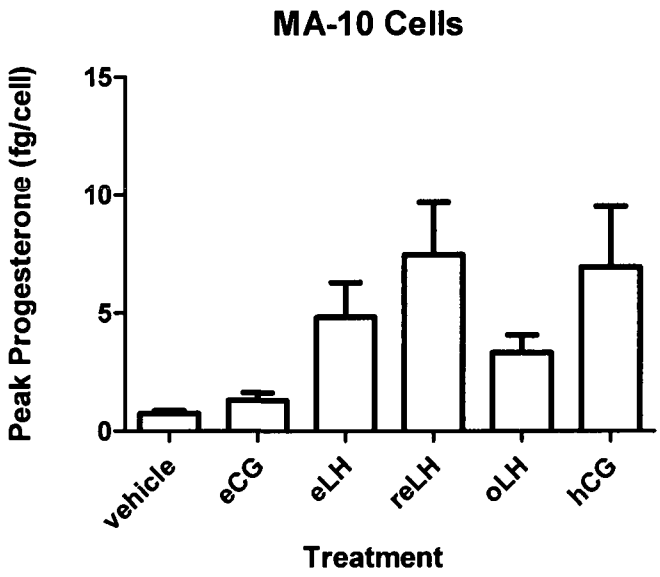
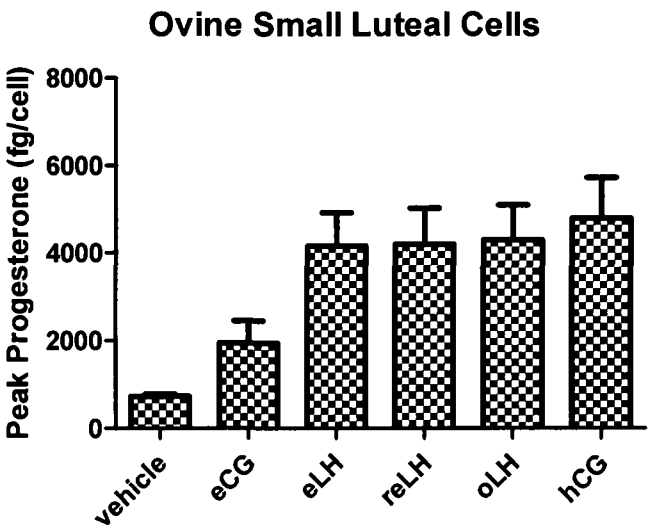

MA-10 Cells
A
Progesterone (fg/cell)
10
8
6
4
2
0
0
1
2
3
4
5
6
Time (hr)
vehicle
eCG
eLH
reLH
oLH
hCG
Ovine Small Luteal Cells
B
Progesterone (fg/cell)
6000
4000
2000
0
0
1
2
3
4
5
6
Time (hr)
vehicle
eCG
eLH
reLH
oLH
hCG
Equine Granulosal Cells
C
Progesterone (fg/cell)
250
200
150
100
50
0
0
1
2
3
4
5
6
Time (hr)
vehicle
eCG
eLH
reLH
oLH
hCG

Figure 2: Peak concentration of progesterone in MA-10 cells (A), ovine SLC (B), and equine granulosal cells (C).

A


MA-10 Cells
Peak Progesterone (fg/cell)
15
10
5
0
vehicle
eCG
eLH
reLH
oLH
hCG
Treatment

B


Ovine Small Luteal Cells
Peak Progesterone (fg/cell)
8000
6000
4000
2000
0
vehicle
eCG
eLH
reLH
oLH
hCG

C

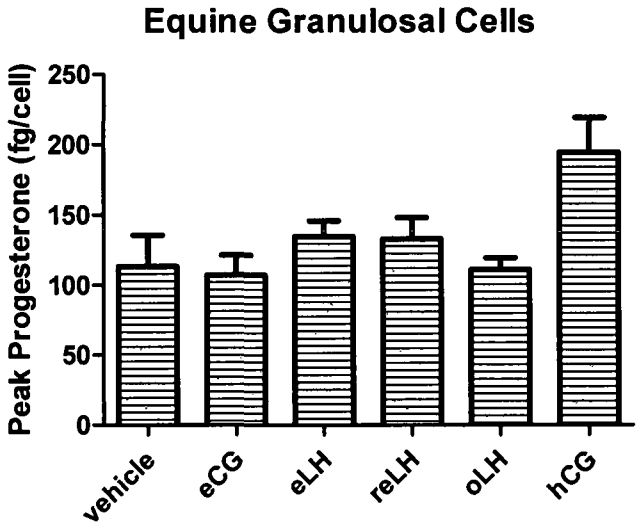

Equine Granulosal Cells
Peak Progesterone (fg/cell)
250
200
150
100
50
0
vehicle
eCG
eLH
reLH
oLH
hCG

Figure 3: Total concentrations of progesterone presented as area under the curve in MA-10 cells (A), ovine SLC (B), and equine granulosal cells (C).

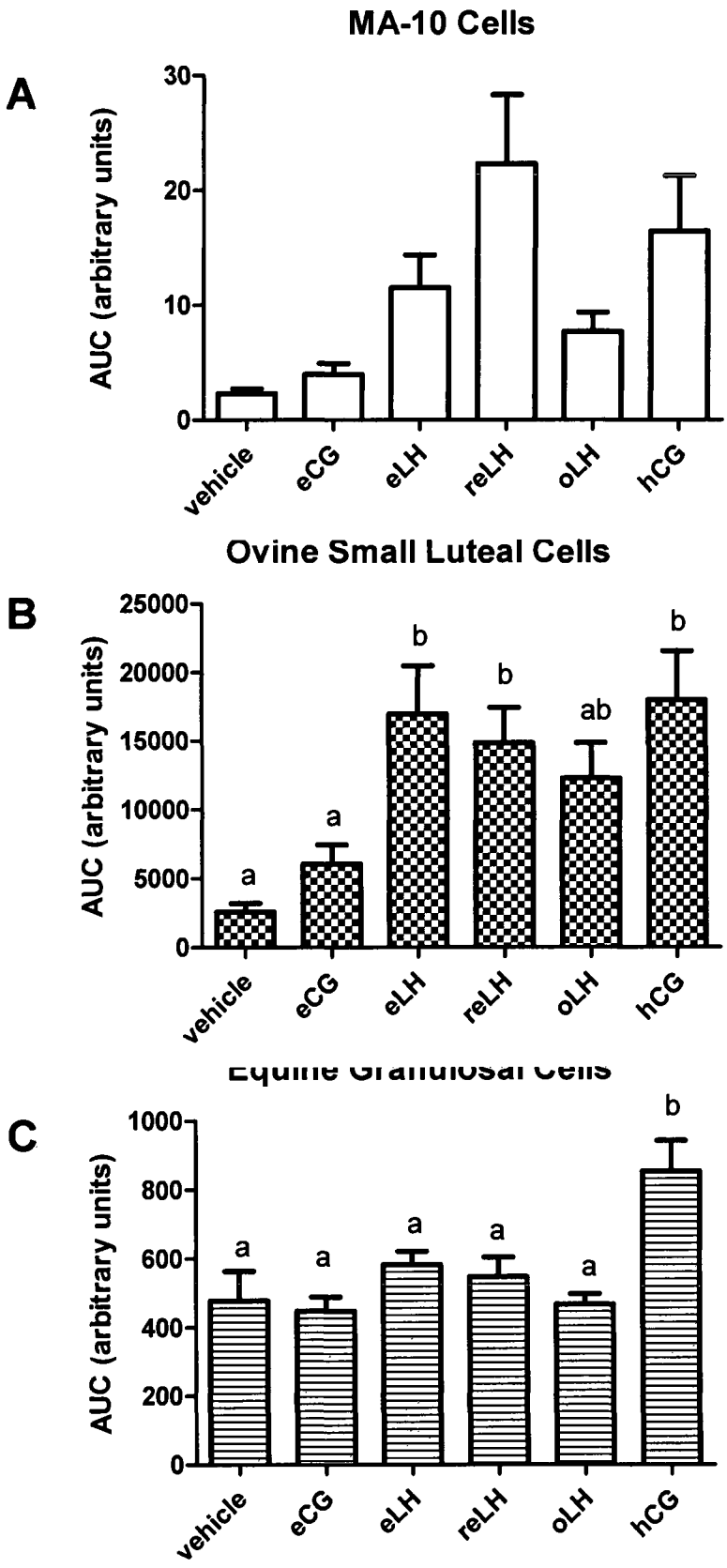
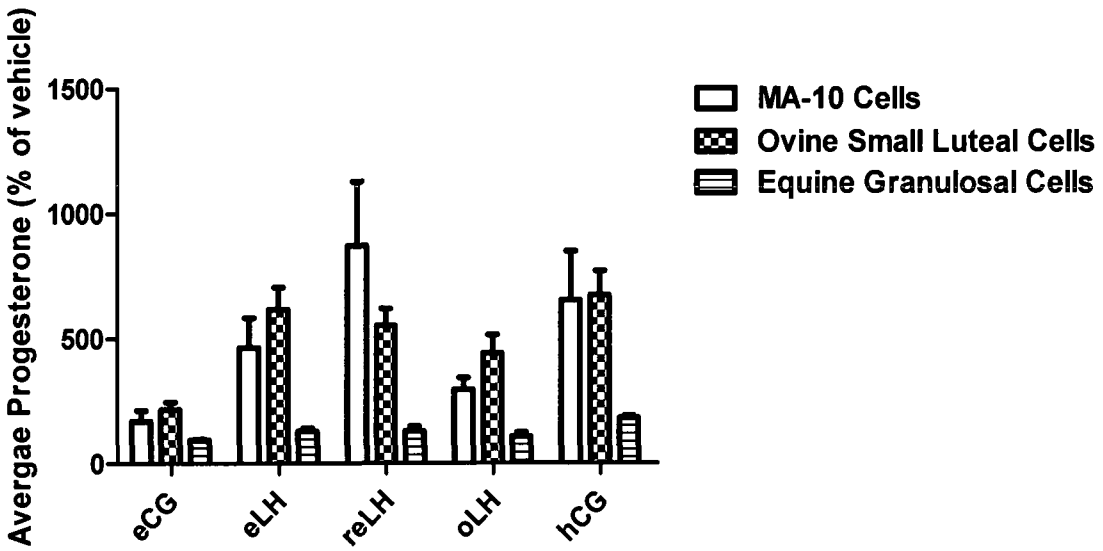

MA-10 Cells
A
AUC (arbitrary units)
30
20
10
0
vehicle
eCG
eLH
reLH
oLH
hCG
Ovine Small Luteal Cells
B
AUC (arbitrary units)
25000
20000
15000
10000
5000
0
a
a
b
b
ab
b
vehicle
eCG
eLH
reLH
oLH
hCG
C
AUC (arbitrary units)
1000
800
600
400
200
0
a
a
a
a
a
b
vehicle
eCG
eLH
reLH
oLH
hCG

Figure 4: Comparison of production of progesterone among cell lines.


Avergae Progesterone (% of vehicle)
1500
1000
500
0
eCG
eLH
reLH
oLH
hCG
MA-10 Cells
Ovine Small Luteal Cells
Equine Granulosal Cells

# Chapter 4

## Conclusions and Future Directions

The Hansen laboratory members have published data that were indicative of IFNT bioactivity in the uterine vein. These findings were significant in the field because they were the first to demonstrate that IFNT may leave the uterus to elicit endocrine actions elsewhere. In the same study, ISG15 was up-regulated in the CL from d15 pregnant ewes, which was indicative that the CL was a potential target for IFNT leaving the uterine vein. This prompted a collaborative effort with the Bruemmer, Niswender, and Hansen laboratories. Dr. Niswender recently published a review article which articulated his belief that the CL, while impacted by the uterus, was a self-regulating transient endocrine gland that aided in its own demise once stimulated by uterine PGF.

My predecessor in the laboratory, Rick Silva focused on the potential importance of PGDH during pregnancy in sheep. He described how this enzyme was responsible for degrading PGF into a non-active metabolite which could help prevent luteolysis during pregnancy. In a conversation with Dr. Bruemmer, he mentioned his belief that this enzyme could be important in context of our story regarding the endocrine actions of IFNT. When I began this experiment I was hoping to find that PGDH was up-regulated by IFNT. Instead, I did not find mRNA encoding PGDH to be differentially regulated in CL from ewes treated with BSA or IFNT. I still think that PGDH may be an important factor in luteal response to IFNT. PGDH could be post-transcriptionally regulated in this system and an enzyme activity assay should be performed to test this hypothesis.

During the course of the experiments we demonstrated that the anti-viral activity detected in previous experiments was inhibited when preadsorbed with an antibody specific to IFNT. These were very exciting data because they confirmed that IFNT was leaving the uterus which meant that we were truly investigating the endocrine actions of IFNT. To pursue this investigation we developed a novel model using mini-osmotic pumps to study the role of IFNT while trying to exclude uterine actions. IFNT was infused directly into the uterine vein where it could potentially reach the CL through a local mechanism involving the utero-ovarian blood supply, or by traveling through systemic circulation. It is my belief that the application of this technique was a wonderful success and will be adopted in many experiments to come.

ISG15 mRNA was elevated in response to IFNT infusions, mirroring the increase seen in CL from pregnant ewes. While IFNT did not stimulate an increase in synthesis of progesterone, infusion of IFNT did appear to prevent the anti-steroidogenic actions of PGF. We evaluated expression of genes involved in steroidogenesis and found no differences in StAR, PBR, CYP-11A, or HSD-3B that were induced by IFNT. This further confirmed that IFNT does not induce steroidogenesis. Instead, IFNT somehow managed to maintain production of progesterone in the face of a challenge with PGF.

In a follow up experiment we tested the role of IFNT infusion on luteal lifespan. Five animals were treated with a seven day infusion with IFNT. One ewe returned to estrus 19 days post-estrus. The remaining four animals did not display any signs of estrus and still

had original CL present at the time of necropsy 32 days post-estrus. To date, there are no other data that IFNT-infusions could prolong luteal lifespan for this length of time. The ewes displayed a slight reduction in concentration of progesterone around d16-19 post estrus when steroidogenesis would normally decline and luteolysis would occur in non-pregnant animals. This slight decrease was transient, and then concentration of progesterone remained steady until the time of necropsy.

If I were to continue my investigations in this topic I would first try to understand the underlying mechanisms which allow IFNT to recover production of progesterone and luteal lifespan. My original plans for the long-term infusion study were to necropsy the ewes on d17 to analyze the CL, uterus, and blood for IFNT induced genes. We made the better decision to carry this experiment out to d32 post-estrus so that the effect of IFNT on luteal lifespan could be analyzed. Knowing what we do now, I would perform the experiment in its original design and would analyze the CL for changes in genes related to apoptosis, survival, and cell signaling pathways that are influenced by IFNT.

Traditionally, IFNT has been described as acting through the type-1 interferon receptor, stimulating the JAK/STAT pathway. Data from the Bazer and Spencer laboratories are indicative that IFNT can also stimulate other signaling intermediates such as mTOR. Type-1 interferon signaling has also co-stimulated the mTOR and PI3 kinase/AKT pathways [189]. I would investigate IFNT infusion-induced intermediates in these signaling cascades. The PI3K/AKT pathway is known to be involved in cell proliferation

and survival. I believe that IFNT could be promoting luteal cell survival through induction of these pathways.

The data presented in this dissertation are very exciting to me. Significant strides have been made in the development of the story about the endocrine actions of IFNT. However, the intricate mechanisms of IFNT actions have not yet been fully elucidated. A collaborator, Dr. Antoniazzi has been investigating the expression of PGFR in our infusion model at the suggestion of Dr. Bazer. Because I did not find any difference in the expression of enzymes involved in PGF synthesis, I will not be surprised if Dr. Antoniazzi finds that IFNT prevents luteolysis in part by reducing the ability of the CL to respond to PGF.

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