ABSTRACT

ALEXANDER, MOLLY. TNF-alpha Production by Corpus Luteum (CL) Macrophages (MAC) and its Control by Progesterone in the Porcine CL. (Under the direction of Dr. Daniel Poole).

Developing a better understanding of the mechanisms that regulate the acquisition of luteolytic sensitivity (LS) is a critical aspect in controlling the reproductive cycle of livestock species. Swine provide a unique model to study this phenomenon, as porcine corpora lutea (CL) remain unresponsive to luteolytic effects of prostaglandin F2a (PGF) until days 12-13 of the estrous cycle. Previous data from our laboratory has demonstrated that luteal macrophages (CL MAC) play an active role in this process through production of proinflammatory cytokines, such as tumor necrosis factor (TNF). Due to the immunomodulatory effects of progesterone (P4), it was hypothesized that suppressing P4 synthesis prior to LS would permit the premature activation of CL MAC enabling these cells to play a key role in regulating luteolytic sensitivity during the estrous cycle in pigs. The first objective of this study was to determine an effective way to reduce P4 concentrations and/or P4's actions, prior to luteolytic sensitivity, in vivo. The second objective was to determine the effects of decreased P4 production/actions on the acquisition of luteolytic sensitivity, as indicated by the expression of PGR, CYP19A1, FOSB and TNF mRNA (CL tissue), and TNF protein (CL MAC). Mature gilts (n=25) that displayed estrus following synchronization with Matrix® were randomly assigned to the following treatments: Control, PGF (25 mg i.m. 10hrs prior to euthanasia), Trilostane, (TRIL, 10 mg/kg per pig/day on days 7, 7.5, 8 and 8.5), RU486 (400 mg i.m./pig/day on days 7 and 8), TRIL+PGF, or RU486+PGF. In order to determine serum P4 concentrations, daily jugular blood samples were taken on days 6 through 9 of the estrous cycle. On day 9 of the estrous cycle, all gilts were euthanized, and the ovaries removed, CL were dissected and ~ 500 mg tissue was immediately frozen in liquid

nitrogen for subsequent P4 analysis, via RIA. Additional luteal tissue collected on day 9 was enzyme-dissociated; cultured for 3 hours at 37^{o}C and 5% CO_2; and analyzed for P4. There was a day by treatment interaction (P=0.0008) for serum P4 concentrations with TRIL and TRIL+PGF suppressing P4 concentrations on days 8 and 9 of the estrous cycle compared with the other treatment groups (Control, PGF, RU486 and RU486+PGF). Luteal P4 concentrations were significantly less in TRIL and TRIL+PGF, 1.9 and 7.6 ng/ml, respectively, compared with controls (20.7 ng/ml; P<0.0001). No difference in P4 concentrations were observed in the control, PGF, RU486 and RU486+PGF treatment groups (P>0.05). Progesterone concentrations from luteal cell media were reduced in TRIL and TRIL+PGF (2.8 ng/mL and 2.9 ng/mL respectively) treated gilts compared with control gilts (5.3 ng/mL; P=0.0556). Cell cultured luteal cells had a tendency to have reduced P4 in TRIL and TRIL + PGF (P=0.0570) compared with the control. No significant differences were observed in PGR or CYP19A1 mRNA expression across treatment groups (P>0.05). Animals treated with PGF, TRIL, TRIL + PGF and RU486 + PGF had a tendency to have decreased mRNA expression of FOSB (P=0.0886) compared with the control, but not RU486. Animals treated with TRIL and TRIL + PGF had a tendency to have increased expression of TNF mRNA compared with PGF, RU486 and RU486 + PGF treated animals (P=0.0887). Isolated MAC populations did not have a significant differences in TNF protein concentrations across treatment groups (P=0.3689). Based on these data, transient reduction in progesterone did not have significant effects on luteal macrophage activation or response to exogenous prostaglandin. Future studies should explore greater P4 reduction sustained for longer period of time prior to analysis.

TNF-alpha Production by Corpus Luteum (CL) Macrophages (MAC) and its Control by Progesterone in the Porcine CL

by
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DEDICATION

I dedicate this thesis in its entirety to, Audrey Kate and Clara Jane Still.

Mud Goblin,

If there's one thing I want you to remember, it's that you are so loved. You are going to do such amazing things in life and I don't mean "amazing things" in a high expectations kind of way. The things you do in this life are going to be amazing because YOU did them. I am infinitely proud of you every single day for just waking up and being unapologetically you. Every fiber of your being was crafted perfectly, don't let anyone make you feel different. From day one, you have kept all of us on our toes and I hope you never stop. You are everything to me. Never lose your independence, fearlessness and sass.

I loved you the moment you were born. I will love you at the end of time. And I will love you in all the spaces between.

Sincerely,
Your biggest fan

My forever baby,

Sweet, sweet Clara, you are everything this world needs, even if it doesn't know it yet. From the day you came into this world, you have radiated all things good. I hope you never lose your pure heart and joy for life. You are the greatest piece this family was missing and I could not imagine life without you.

I swear I couldn't love you more than I do right now, and yet I know I will tomorrow.

Sincerely,
Your favorite Aunt

BIOGRAPHY

Molly Alexander was born to David and Melissa Alexander in Syracuse, NY. Being the youngest of three girls, Molly has always been the life of the party and the first one willing to lend a hand to anyone in need. Molly has always had a big heart for animals and has always known she wanted to work with them as a career.

Molly graduated high school at Holly Springs HS and then attended Wake Technical Community College where she received her Associates of Arts in Accounting and received a certificate in Public Accounting. After receiving her Associates degree, she moved to Ashland, VA to attend Randolph-Macon College. It was there she participated in an undergraduate Schapiro research fellowship where she was researching nickel phosphide nanoparticles as catalysts for the hydrogen evolution reaction. She also began volunteering for a veterinarian, Dr. Steve Escobar at Springfield Veterinary Hospital, who specialized in animal reproduction. After falling in love with veterinary medicine and reproductive specialties, Molly transferred to NC State University in the Animal Science Department. Her love for animals and reproduction grew as she worked at the NCSU Equine Education Unit and volunteering in Dr. Daniel Poole's reproduction laboratory. After completing her Bachelors at NC State, she continued working with Dr. Daniel Poole to get her Master's of Science in Animal Science. Her research concentration is a proof of concept study looking at how immune cells, specifically macrophages, effect cyclicity. After completion of her masters, she plans to transfer to East Carolina University where she will attend the Brody School of Medicine in pursuance of her PhD in Anatomy and Physiology.

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much and I am a better person for knowing you. The M&Ms finally found their nuts and I wouldn't want that to be anyone else but you.

Mariam. Mariam. Mariam. I don't even know where to begin or how to write this without getting emotional. You are incredible. Like actually incredible. I don't think you realize how amazing you are. I think if I logged the number of hours I spent working on my masters, 90% of that time would have been spent with you. You are going to do so many amazing things in this life, and I'm honored to be able to say I was there when you were just starting out. Remember me when you win a Nobel Prize, okay?

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CHAPTER 1

Review of Literature

Introduction

Luteolysis is the degradation and structural reformation of an animals corpora lutea (CL) post ovulation[1]. Understanding the mechanistic pathways by which luteolytic capacity occurs is essential to manipulating estrous cycles in livestock species. Luteolytic sensitivity (LS) is the responsiveness of an animals CL to hormones, naturally occurring or exogenous, involved in inducing luteolysis[2]. In domestic mammalian species, Prostaglandin F2a (PGF2a) is a common, naturally occurring, luteolysin that has been used exogenously to induce luteolysis. Initiation of luteolysis begins with oxytocin stimulating myometrial contractions. This, in turn, causes PGF2a to be released from the uterus, which then disrupts progesterone (P4) synthesis[3] in the CL. While much research has described luteolysis the exact mechanistic pathway involved in acquisition of luteolytic capacity remains unclear, however it is clear immune cell involvement, specifically macrophages (MACs), play a critical role[1,5]. Macrophage secretion of tumor necrosis factor-alpha (TNF) plays a role in P4 suppression by inducing a proinflammatory response within the CL[1]. The goal of this research is to determine the role of luteal MAC cytokine secretion of TNF on the process of inducing luteolysis in mammalian livestock species.

The Ideal Animal Model

Swine pose as an excellent animal model when studying luteolytic sensitivity because of their anatomical, physiological, immunological and endocrine similarities to other livestock species[6]. While, it is known that cattle are unresponsive to the luteolysin, PGF2a, until day 5 post ovulation[6]. This insensitivity can also be seen until day 8 in marmoset monkeys and day 4 in pseudopregnant rats[3,6]. Whereas, swine have a larger window of insensitivity to PGF2a until days 12 to 13 post ovulation[1,2,5]. This extended period of luteal insensitivity to PGF2a observed in this

species makes the pig a particularly useful model in which to investigate physiological events leading to luteolytic sensitivity.

Porcine Estrous Cycle

The normal range for the porcine estrous cycle is 18 to 23 days with the average being 21 days. It involves changes in ovarian structures, regulation of steroidogenic hormones and behavioral changes[7]. Similar to other domestic livestock species, the porcine estrous cycle consists of two phases, the follicular phase and the luteal phase; each with different stages of differing hormones fluctuating the control of follicle formation, ovulation and luteal regression[7].

The follicular phase lasts around 5 to 7 days and begins at the end of the estrous cycle (luteolysis) with a period of proestrus, where elevated concentrations of PGF2a, estrogen and follicle stimulating hormone (FSH), as well as, low pulses of luteinizing hormone (LH) are secreted[8] (Figure 1.1). This proestrus stage lasts between days 17 and 21 of the porcine estrous cycle (day 0 = ovulation) and is the time when corpora lutea (CL) regression and follicle formation (folliculogenesis) occurs[8].

Following the proestrus stage and follicle formation, the estrus stage begins. Estrus begins on ~days 0 to 1 of the porcine estrous cycle and occurs in response to increased estrogen by the pre-ovulatory follicles[7]. Ovulation is when estrogen concentrations increase via, FSH and LH pulses cause the fully formed follicle on the ovary to rupture and release an oocyte. This usually happens 24-48 hours post peak estrogen release[8]. Swine, being a litter bearing species, can have between 15 to 30 follicles develop and rupture, causing the release of multiple oocytes for fertilization[7].

The luteal phase is between days 2 to 18, following the follicular phase. Beginning with metestrus, estrogen, FSH and LH concentrations rapidly decrease and the sow is no longer receptive to the boar[8]. This period of time usually lasts 2 to 4 days, where P4 is being produced at

a greater rate from the corpus hemorhagicum, newly ruptured follicles on the ovary[8]. Due to swine being a litter bearing species, multiple CL are formed on the ovary in place of each ruptured follicle since multiple ova are released.

The longest stage of the estrous cycle, diestrus, is the period occurring between days 5 to 18 of the estrous cycle, when P4 secretion by the CLs is at its maximum, is also known as the luteal phase. It is also during diestrus stage/luteal phase, when the maternal recognition of pregnancy occurs (days 12-14) if the female is inseminated and has a minimum of 4 viable embryos present[9]. Thus in pregnant animals, the CL will continue to produce P4 to maintain the pregnancy and the CL structure will remain on the ovary. If no viable embryos, or less than 4 exists by day 15 of the cycle, PGF2a will act on the CLs to inhibit production of P4 and cause CL regression[8].

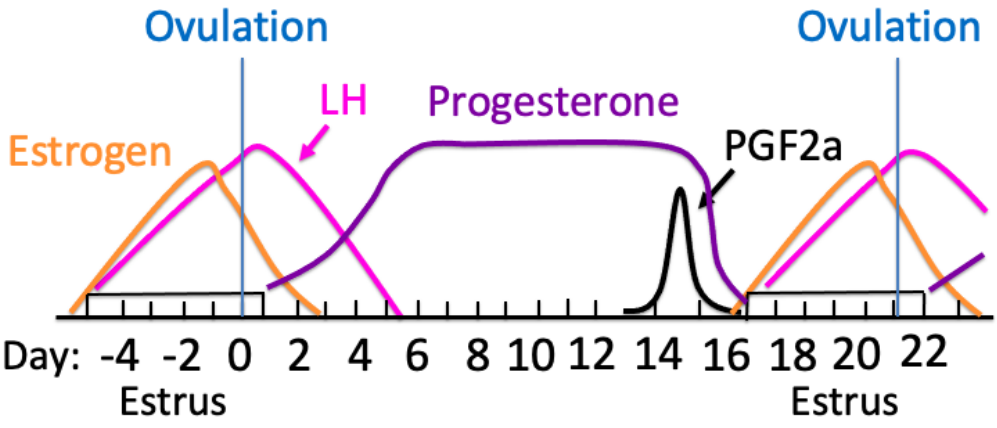

Figure 1.1: Porcine estrous cycle hormone regulation including elevated progesterone (P4) synthesis between days 5-16 and a spike in prostaglandin F2a (PGF2a) around day 14 post ovulation.

The regression of the CL is known as luteolysis. Proposed mechanisms of luteolysis involve increase secretion of uterine PGF2a which acts on the CL to disrupt the production of luteal P4. Disruption of P4 secretion results in functional regression subsequently followed by structural regression of the CL[6].

Corpus Luteum

Corpora lutea (CL) were first discovered in 1573 by physiologist Volcherus Coiter and has long been known to be essential for pregnancy in mammalian livestock species[10]. Later researcher, Marcello Malpighi, would coin the name corpus lutea which means "yellow body"[11]. The first illustrations of the CL were made by Regnier de Graaf from pregnant rabbits, where globular bodies were seen present on the ovary[11]. In 1901, the CL was proven to be essential for pregnancy when Ludwig Frankel removed CL from the ovaries of pregnant rabbits and consequently caused the rabbits to abort[12]. It was then discovered in 1929 that secretions from the CL could replace ovarian function if the ovaries were removed. This newly discovered excretion was named progesterone by combining "progestin" and "luteo-sterone"[11]. From these early stepping stones, research on the CL focused on development, steroid hormone production and the luteal regulation of pregnancy.

Following ovulation, formation of the CL begins following ovulation, when estrogen decreases and P4 increases (luteinization)[13]. The CL develops from the granulosal and thecal cells of the ovulated follicles present on the ovary. Once ovulation occurs, the theca and granulosa cells that makeup the follicle undergo terminal differentiation (luteinization) to transform into the small and large luteal cells, respectively[14]. These newly formed luteal cells are highly steroidogenic cells, and produce primarily P4, which is the major secretion of the CL[15]. In addition to these two important steroidogenic luteal cell types, the CL also comprises vascular endothelial cells, fibroblasts, leukocytes and macrophages[16,17]. Paracrine and autocrine regulation of CL tissue growth is essential for normal luteal function[17].

Luteal Formation

Following ovulation, pulses of luteinizing hormone cause granulosa cells and theca cells within the ovary to differentiate into large and small luteal cells, respectively, that make up the

CL[13]. The cells that make up the CL are small luteal cells, large luteal cells endothelin cells, fibroblasts and immune cells, such as, leukocytes, lymphocytes and macrophages. Combined, large luteal cells and small luteal cells makeup roughly 40% of the cell population within the CL, meaning the immune cells, or non-steroidogenic cells account for over 50%[18,19]. While more abundant, fibroblasts, endothelial cells and other immune cells do not have steroidogenic abilities like the large luteal cells (LLCs) or small luteal cells (SLCs)[18]; however, they do produce various cytokines that act as a steroidogenic inhibitors and vasoconstrictors; such as, endothelin-1 and tumor necrosis factor-alpha (TNF), in response to PGF2a[1].

The steroidogenic cells, such as LLC and SLC produce steroid hormones involved in the regulation of the estrous cycle. Differentiation of LLCs and SLCs cell types can be identified based on morphological and biochemical characteristics[20]. Morphological differentiation identifies SLCs sized as 12 to 22 mm in diameter, while LLCs maintain a diameter of 22 to 35 mm, or larger[21-23]. Structurally, SLCs are generally elongated in shape and contain a nucleus irregular in shape. Small luteal cells also house numerous mitochondria and have an abundance of smooth endoplasmic reticulum (ER), similar to other steroidogenic cells[24]. Large luteal cells also contain numerous mitochondria and smooth ER but, in contrast to SLCs, also contain Golgi complexes, abundant rough ER and membrane-bound secretory granules, similar to protein secreting cells[22,25-26].

Upon formation of the CL, hormone production shifts from producing estradiol to primarily producing P4 (Diaz, 2004). Both SLCs and LLCs produce P4 in some capacity[27]; and although SLCs are more numerous, LLCs produce roughly 10 to 20 fold greater the amount of P4 as SLCs[23]. The biochemical differences between LLCs and SLCs lies in the receptors present, second messenger function and steroidogenic characteristics[27].

Small luteal cells have luteinizing hormone (LH) receptors that are coupled to protein kinase A (PKA) via a second messenger pathway[27]. Small LCs increase P4 production up to 20

fold upon simulation from LH but is regulated by the PKA[23]. In the event PKA is activated, the second messenger pathways inhibits LH-stimulated P4 secretion[28].

In most cases, large luteal cells do not have LH receptors, but rather FSH receptors[23]. This makes them generally unresponsive to LH in terms of P4 secretion[29]. Large LC are able to produce more P4 since to the PKA second messenger pathway is not being inhibited or regulated by LH[27].

Luteal Production of Progesterone

Progesterone has been intensely researched and identified as the intracrine regulator of luteal function[30]. The presence of P4 maintains the CL viability by protecting luteal cells from the damaging effects of luteolytic hormones and immune cells within the CL[1]. Initiation of P4 synthesis via luteotropic hormones is very species specific. In humans, monkeys, cows and other ruminants, P4 synthesis relies heavily on pulses of LH[31]; whereas, in rabbits and rodents, prolactin and estradiol are the essential luteotropic hormones that control P4 stimulation[31]. After stimulation from the necessary luteotropic hormones, luteal cells begin to synthesize P4 from the upstream steroid hormone, cholesterol[31]. Cholesterol can be sequestered and stored in luteal cells in small amounts. However, its main source are the esters in high density lipoproteins (HDL), in rodents and ruminants and endocytosis of low density lipoproteins (LDL), in humans, monkeys and pigs[31]. Once obtained, cholesterol is then transported into the inner membrane of the mitochondria of luteal cells by way of enzymatic translocation. Steroidogenic acute regulatory (StAR) protein is the governing factor in the movement of cholesterol and also serves as the rate limiting step in P4 synthesis[1, 31-32]. Once inside the mitochondrial inner membrane, enzymatic cytochrome P450-cholesterol side-chain cleavage (P450scc) cleaves the carbon side-chain on cholesterol to produce pregnenolone, an intermediary steroid hormone[31]. Pregnenolone is then converted into P4 by enzymatic removal of hydrogen (H) from the oxygen (O) molecule present. The enzyme responsible for this cleavage is 3b-hydroxysteroid dehydrogenase (3b-HSD), and is located in the

smooth endoplasmic reticulum (SER)[31, 33-34]. Figure 1.2 displays the cascade of steroid hormone cleavage via enzymatic alterations from cholesterol to P4. 3b-Hydroxysteroid dehydrogenase is essential to the production of P4 and its subsequent steroid hormones, such as 17a-hydroxyprogesterone and cortisol[34]. Diaz et al (2011) used epostane, a known 3b-HSD inhibitor, and saw significant decreases in P4 concentrations in treated gilts, supporting the enzymatic requirement of 3b-HSD for P4 synthesis by the CL[35]. Progesterone synthesis is essential for maintenance of the CL's integrity; however, suppression of P4 synthesis is essential for the onset of luteolysis to occur. Progesterone suppression can occur with the use of exogenous agonists or antagonists of the P4 receptors.

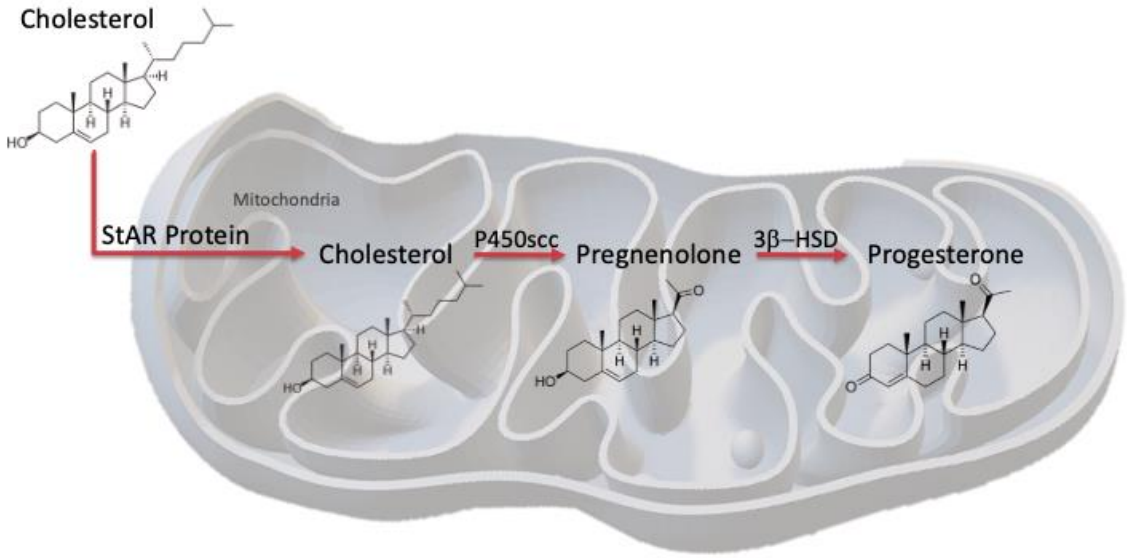

Figure 1.2. Steroid hormone cascade involved in progesterone synthesis. Cholesterol is translocated to the inner membrane of luteal mitochondria via steroidogenic acute regulatory (StAR) protein, then cleaved with cytochrome P450scc into pregnenolone before final cleavage to progesterone via enzymatic function of 3b-hydroxysteroid dehydrogenase (3b-HSD).

Another inhibitor, Trilostane (TRIL), blocks the enzymatic function of 3b-HSD from cleaving pregnenolone to P4. Without the enzyme 3b-HSD, P4 cannot be synthesized or activated to produce a cellular response. Trilostane has been used to study luteal endocrine processes,

including the inhibition of steroid hormone synthesis[36]. Etherton (1989) used TRIL in prepubertal gilts to suppress cortisol, a steroid hormone of P4. Etherton found a significant reduction in cortisol in gilts treated with TRIL, suggesting enzymatic suppression of 3b-HSD successfully restricted P4 synthesis[36].

Regulators of Luteal Function

Regulating luteal function can also be achieved via PGF2a-induced transcriptional regulation of genes essential to luteal function[37]. Two vital genes of interest are FOSB and aromatase (CYP19A1), both of which may be regulated by PGF2a. FOSB is a part of the activating protein-1 (AP-1) family of transcription factors that are responsible for regulating several different physiological responses such as cell death, inflammation and proliferation[37]. The AP-1 proteins have been found localized to the CL in pigs[37-38]. In bovine and porcine luteal cells, PGF2a has been shown to increase AP-1 proteins via protein kinase pathway[39-40]. Studies have also shown that AP-1 proteins differentially regulate the CL depending on if the CL has reached luteolytic capacity or not[13]. For example, aromatase (CYP19A1) is controlled by the AP-1 transcriptional complexes and upregulates CYP19A1 in the presence of PGF2a[35]. Aromatase is the enzyme responsible for luteal estradiol synthesis[41], an essential component for normal regulation of the estrous cycle[41]. When looking for identifying markers for luteal capacity, FOSB and CYP19A1 can be monitored due to their increased transcription after luteolytic sensitivity is reached[35].

Agonist Vs. Antagonist

A hormone's effectiveness is determined by the affinity of the hormone receptors and the abundance of those receptors. With regard to steroid hormones, activation of cells or target tissue is initiated by steroid hormones binding to their respective receptors. Prior to binding, hormone complexes or agonists cannot produce cellular response, and similarly, neither can unbound receptors[42]. Together, the hormone and receptor activate the intended response mechanism to

induce changes in the cells and surrounding tissue. Modulations of the activation of these hormones and their respective receptors can be achieved by hormone receptor agonists (which mimic the actions of the hormone) or agonists (which block or antagonize the actions of the hormone)[42].

Agonists can be naturally occurring or synthetically modified hormones that bind to specific receptors on cell surfaces to produce similar cellular responses[42]. Agonists do eventually inhibit their targeted hormones activation, however, allow for an initial cellular response to occur[42]. Similarly, Antagonists will bind to targeted receptors, but unlike agonists, antagonists prevent cellular response altogether. The chemical suppression of P4 can be done one of two ways: blocking P4 receptors on cell surfaces or reducing P4 synthesis by blocking enzymatic processes[43].

Mifepristone, or Roussel-Uclaf 486 (RU486), is considered a P4 antagonist because it binds to receptors in the nucleus, called the Progesterone Genomic Receptor (PGR) and prevents activation in response to P4 from occurring. There is still P4 being produced, but with no receptors to bind to, it cannot successfully stimulate a biological response. Matthew et al (2010) used RU486 to explore the consequences of pregnancy in a P4 suppressed environment in newly inseminated gilts[44]. Gilts were treated with RU486 on days 3-5 or on days 6 and 7 of gestation. On days 8 or 12 of gestation, uteri and ovaries were removed and examined, The results showed that gilts treated with RU486 failed to maintain pregnancy and had increased endometrial PGF mRNA compared with the control group that were not treated with RU486[44]. These results indicate P4's essentiality for maintenance of pregnancy and suggests that in its absence, PGF is able to synthesize more abundantly to induce physiological change on the CL[44]. However, it should be noted RU486 is also known to have an affinity for glucocorticoid receptors and androgen receptors[45]. This can cause adverse effects by restricting essential anti-inflammatory hormones or estrogens[45].

Immune Cells within Ovaries

Similar to chemical and hormonal regulation of luteal function, immune cells have also been known to induce changes to the CL. The female reproductive tract hosts immune cells in fluctuating quantities throughout the estrous cycle[46]. White blood cells, such as MACs and T-cells, are responsible for regulating apoptosis and phagocytic action on dying or dead cells, as well as, matrix dissolution, tissue remodeling and production and secretion of cytokines, chemokines and growth factors[1,47-48].

Macrophages are derived from bone marrow precursors and once mature, enter the blood stream as monocytes[48]. Circulating in the blood stream as monocytes, the maturation into MACs only occurs once the immune cells infiltrate tissue. This process can be described in three steps: (1) initial attachment of monocytes to leukocytes and endothelin cells in blood circulation; (2) exposure to cytokines and chemokines stimulate monocytes to produce cell surface b2 integrins that ensure strong cell adhesion with intercellular adhesion molecule 1 (ICAM-1) on endothelial cells; and (3) migration of monocytic-endothelin complexes out of blood and into tissue, allowing for the maturation and conversion of monocytes to MACs based on the needs of the surrounding tissue[48].

White blood cells were first discovered in bovine CL in 1968 by Lobel and Levy[49]. They discovered lymphocytes residing in the connective tissue surrounding luteal vasculature on day 14; and on days 15-17 the lymphocytes penetrated the luteal cells. By day 19, Lobel and Levy noted macrophages present in luteal cells to be later discovered to have phagocytic actions on cells and dying cells[1, 49-50]. Continued research on MACs and their possible role in the CL support the findings of Lobel and Levy (1968), and it was observed that MAC infiltration in the CL increased around the time of luteolysis in the cow, human and rabbit[1, 51-55]. A study by Naftalin et al. (1997) observed an influx in MAC concentrations in the CL when estradiol, a luteotropic hormone

responsible for CL formation, was removed in the rabbit[1, 56]. This increase in MAC populations around the time of luteolysis has been hypothesized to be in response to the secretion of monocyte chemoattractant ptotein-1 (MCP-1) in the cow, rat and rabbit CL[1,57-60]. Monocyte chemoattractant protein-1 is a known chemokine that recruits inflammatory immune cells, such as MACs, into tissue throughout the body[60]. Research in the rat by Olson and Townson (2000) also noted that ICAM-1 is also present in the CL in response to a prolactin-induced luteal regression, and subsequently recruits monocytes that mature into MACs to the CL[61]. This suggests that both MCP-1 and ICAM-1 play a role in MAC recruitment and maturation during the time of luteolysis[1, 61]. Once activated, MACs and T lymphocytes secrete cytokines that have either positive or negative effects on endocrine cells[1].

Cytokines: Intracellular Communication

Cytokines are small proteins that are secreted by various cells within the body that bind to specific receptors on cell surfaces to induce an immune response[62-64]. Cellular response to cytokines can be on adjacent cells (paracrine response), on the secreting cell (autocrine response) or on cells throughout the body (endocrine response)[63]. Cytokines are also involved with the regulation of inflammation, leukocyte recruitment, angiogenesis, apoptosis, proliferation and differentiation[17, 48, 65].

Cytokines and their receptors can be grouped into proinflammatory and anti-inflammatory cytokines based on the response they induce. Anti-inflammatory cytokines are responsible for the immunoregulation of proinflammatory cytokine response[64]. Anti-inflammatory cytokines include interleukins (IL) 1 receptor antagonist, IL-4, IL-10, IL-11 and IL-13[62]. Proinflammatory cytokines, such as IL-1b, IL-6 and TNF are responsible for pain response, an adaptive and innate immunological action[62]. Macrophages present in the ovaries have been known to secrete TNF as a proinflammatory cytokine responsible for phagocytic action on the CL[1].

Tumor Necrosis Factor-alpha

Tumor necrosis factor-alpha (TNF) is a proinflammatory cytokine produced and secreted by monocytes and MAC and other immune cells, in response to pain or other immunological signals[62]. Tumor necrosis factor-alpha was first discovered to be a mediator of tumor cell death[17, 66], and has since been found in natural killer cells, follicles, granulosa and thecal cells[17, 67]. Effects of TNF on domestic mammalian species' reproductive organs include anti-steroidogenic processes, induction of apoptosis and phagocytic actions that lead to structural tissue remodeling[1]. Tumor necrosis factor alpha (TNF) has been reportedly found in the CL MAC populations in rabbits[68], cows[69], ovines[70] and pigs[71]; but has also been reportedly found in LLC and granulosa cells[69, 72-74]. Wuttke et al. (1998) observed in the pig that micro-doses of PGF2a stimulated P4 production; but in the presence of TNF, PGF2a suppressed P4 synthesis. Evidence of this suggests TNF is the cytokine that switches PGF2a from a luteotropic hormone to a luteolytic hormone[1, 75].

In the cow and pig CL, the major source of TNF secretion is from MAC immune cells[18] but it has also been reported to be found in large luteal cells or granulosa cells[1, 69]. While mRNA for TNF have been reported to be present consistently throughout its lifespan, bioactivity or action caused by TNF has only been reported to occur once P4 concentrations decrease in bovine and ovine species[1, 70, 76]. Tumor necrosis factor-alpha plays major roles in the induction of luteolysis once P4 activation is inhibited by PGF2a and luteal cells are vulnerable to the effects of TNF[1]. Wuttke et al (1998) reported an increase in P4 concentrations when micro doses of PGF2a were introduce to the porcine CL[75]; however, if TNF was present, PGF2a had luteolytic effects on the CL. This suggests TNF may be the determining factor if the effects of PGF2a are luteotropic or luteolytic. It has also been reported that TNF induces a positive feedback loop on the secretion of luteal PGF2a[1, 77]. Anti-steroidogenic effects of TNF aim to reduce P4 concentrations and increase PGF2a concentrations in order to expose luteal cells to its apoptotic and phagocytic effects.

In addition to the anti-steroidogenic effects, previous research in ovine species has suggested that the induction of apoptosis on luteal cells is evidence of luteal regression[1, 78-79]. Tumor necrosis factor-alpha receptors are also found on endothelin cells, causing endothelial cells to undergo apoptosis, signaling to the CL to endure major structural remodeling[1, 18, 80-81]. The detrimental effects of TNF can only occur in the presence of reduced P4 and increased PGF2a[1]. The mechanistic pathway in which luteolysis occurs has been highly researched and proposed mechanisms have been supported in multiple domestic mammalian species[1-2, 5]. However, synthetic manipulation of the estrous cycle using exogenous agents has reportedly only been successful after reduction of P4 has occurred[1].

Suppression of Progesterone

Natural suppression of P4 around the time of luteolysis is initiated by the secretion of uterine PGF2a[1]. In the absence of viable embryos, uterine PGF2a acts on the CL to limit steroidogenic effects immediately by blocking LH pulses and other luteotropic hormones from stimulating P4 synthesis[1]. It has also been shown in the bovine CL that uterine PGF2a reduces 3b-HSD enzymatic function in further attempts at limiting P4 synthesis[33]. Endothelin cells interact with the uterine PGF2a and produce endothelin-1 (ET-1), which further inhibits steroidogenic processes[82]. After this initial prostaglandin exposure, a decrease in the StAR protein inhibits the transport of cholesterol through the mitochondrial membrane, further preventing P4 synthesis[1, 31, 35]. After the initial decrease in P4 concentrations, uterine PGF2a exhibits a positive feedback loop to stimulate luteal PGF2a synthesis[1]. Luteal PGF2a, in conjunction with endothelin-1, cause vasoconstriction of luteal capillaries and induces apoptosis of endothelial cells[1]. This begins the structural degradation of the CL.

Simultaneously, MACs present in the CL are seen to increase in numbers around the time of luteolysis and secrete the cytokine TNF[1]. Once P4 synthesis is decreased initially by uterine

PGF2a, luteal cells are no longer protected by the luteotropic hormone and are exposed to the apoptotic effects of TNF[1, 27]. Tumor necrosis factor-alpha induces anti-steroidogenic effects that causes a decrease in mRNA for the StAR protein and also induces a positive feedback loop on increased PGF2a synthesis by luteal cells[1]. Reduced P4 concentrations in the CL means luteolysis can now occur and full degradation and CL tissue remodeling take place.

Synthetic suppression of P4 has been reported successful around days 5 and 13 of the bovine and porcine estrous cycle, respectively, with the use of exogenous hormone PGF2a[1, 13, 18, 27]. Prior to this luteolytic sensitivity, the bovine and porcine CL produces elevated P4 concentrations, protecting luteal cells from the luteolytic effects of PGF2a. Once luteolytic capacity is reached and P4 synthesis is reduced, exogenous PGF2a has been reported to induce the natural luteolytic cascade of events that leads to structural degradation and reformation of the CL[13].

TNF Role in Luteolysis

Inducing luteolysis is essential to manipulating mammalian estrous cycles. In order for luteolytic capacity to be achieved, activated P4 concentrations must be reduced so consequent luteal cells are exposed to the luteolytic effects of PGF2a. Progesterone concentrations can be reduced with the use of P4 antagonists that act on P4 receptors to block activation, or by blocking enzymatic function of 3b-HSD to reduce P4 synthesis. Once luteal cells are no longer protected by the luteotropic effects of P4, they become vulnerable to undergoing luteolysis[1, 13].

Previous research from our laboratory evaluated progesterone genomic receptor (PGR) mRNA expression and TNF mRNA expression in mid vs late stage CL, before and after luteolytic capacity is achieved. In the pig, the mid stage of the cycle is considered to be prior to the onset of luteolytic sensitivity to PGF (D8-9), and the late stage of the cycle is considered to be after luteolytic capacity is reached (D13-14). Thus, as shown in figure 1.3[5], PGR mRNA decreased in late stage CL vs mid stage CL and conversely, TNF mRNA concentrations increased in late stage

CL compared to mid stage CL (figure 1.4)[5]. This observation that the PGR mRNA expression was inversely related to TNF mRNA expression might suggest that TNF may play a role in reducing activated P4 actions via PGR and thus inducing the cascade that ultimately leads to luteolysis in the porcine ovary[5].Alternatively these observations can be interpreted to suggest that when PGF levels fall that TNF expression is released from P4/PGR inhibition leading to increased TNF expression. This inverse relationship between P4/PGF and TNF was further confirmed by culturing isolated MACs alone, or co-culturing in the presence of P4 secreting luteal cells. Results of these studies showed that a significant decrease in TNF mRNA expression by MACs occurred in the culture in the presence of luteal cells (Figure 1.5)[5], supporting an inhibitory role for P$ on TNF expression. Furthermore, when isolated MACs were then placed into culture with varying known doses of P4, there was a significant dose dependent inhibition of TNF mRNA expression, in response to P4 (Figure 1.6)[5].

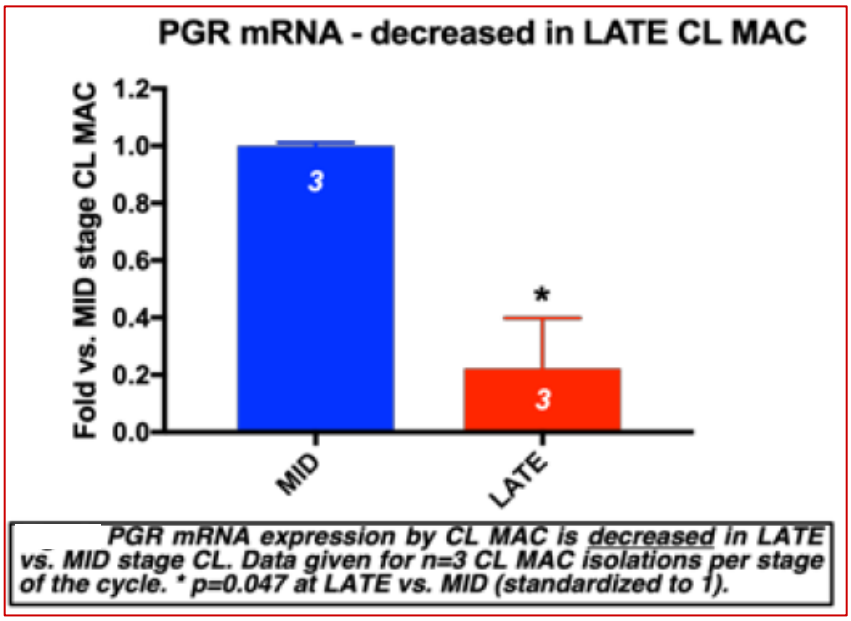

Figure 1.3: Progesterone genomic receptor (PGR) mRNA expression decreases in late-stage corpora lutea (CL) (days 13-14, after luteolytic sensitivity) compared with mid-stage CL (days 8-9, prior to luteolytic sensitivity)[5].

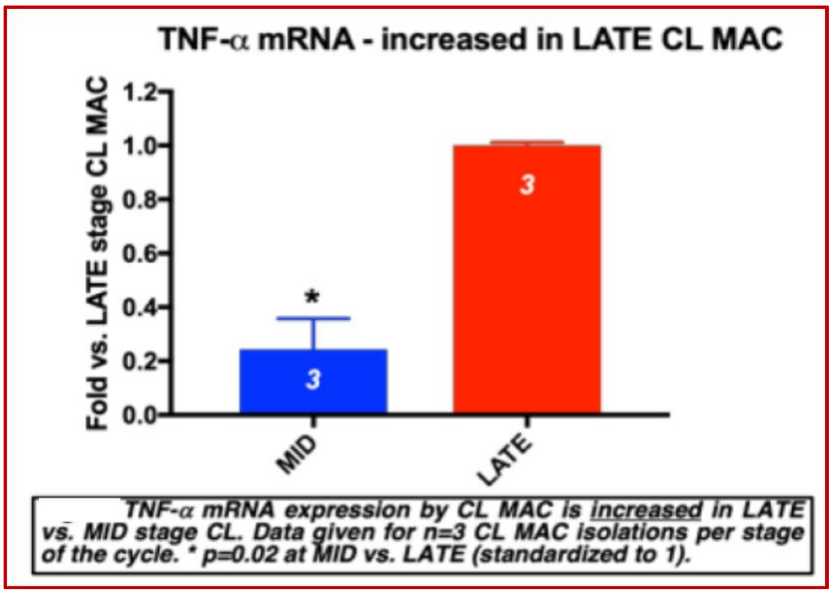

Figure 1.4: Tumor necrosis factor-alpha (TNF) mRNA expression increases in late-stage corpora lutea (CL) (days 13-14, after luteolytic sensitivity) compared with mid-stage CL (days 8-9, prior to luteolytic sensitivity)[5].

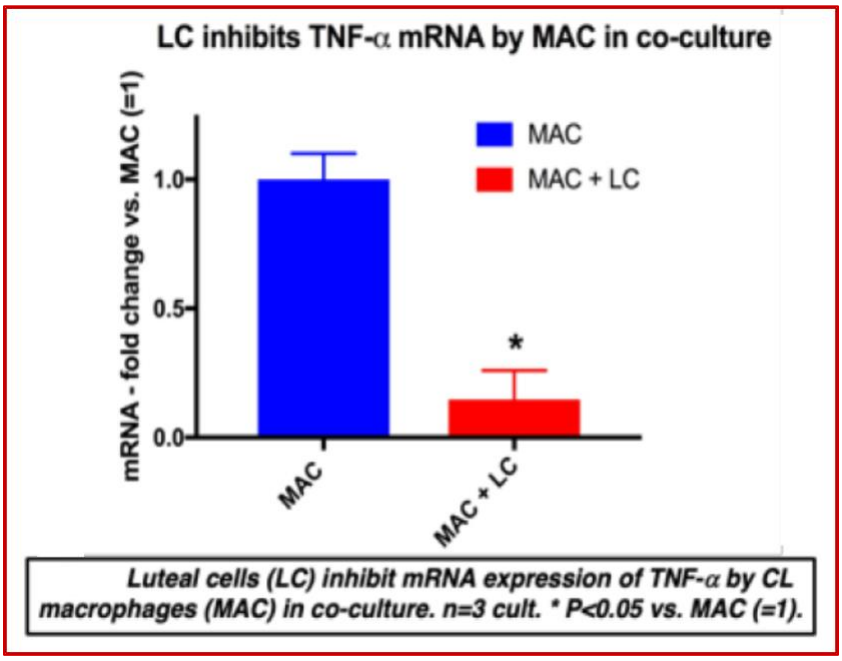

Figure 1.5. Tumor necrosis factor-alpha (TNF) mRNA expression evaluated in cultures with isolated macrophages (MACs) and MACs with luteal cells. Significant decrease in TNF mRNA expression in co-culture with luteal cells present[5].

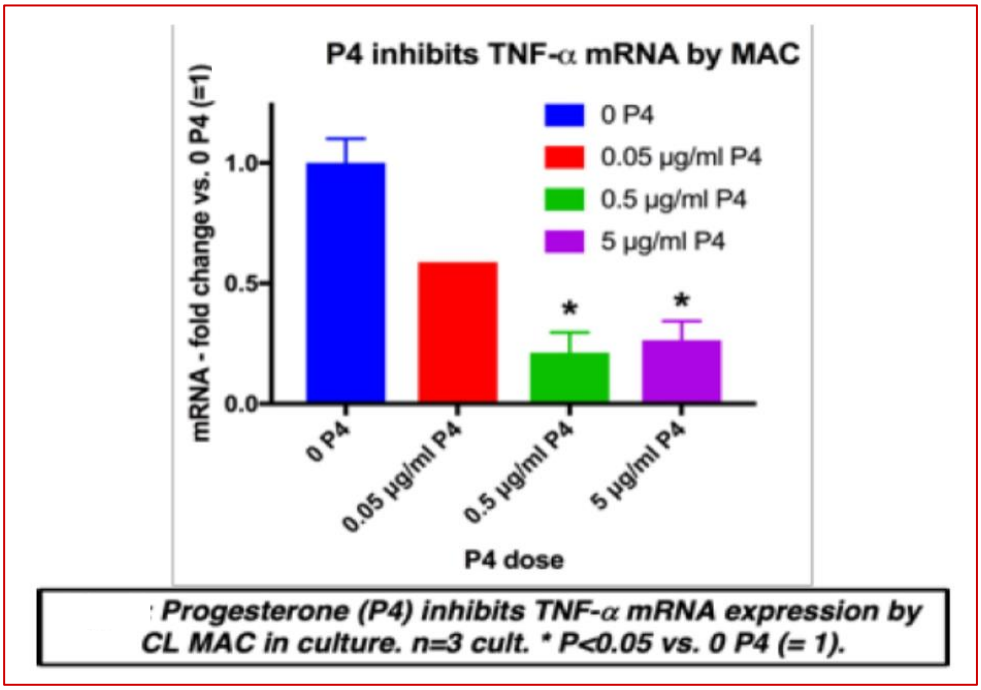

Figure 1.6. Tumor necrosis factor-alpha (TNF) mRNA expression decreases based on dose dependent response to progesterone (P4) present. Cultures treated with 0.5 ug/mL and 5 ug/mL P4 had significant decrease in TNF mRNA expression compared with other treatments and control[5].

Collectively, these observations lead to the development of working hypothesis illustrated in Figure 1.7. Briefly, prior to luteolytic sensitivity, luteal cells are producing greater concentrations of P4 and have greater PGR mRNA expression on the ovary, leading to a reduced expression of TNF mRNA. Subsequently, after luteolytic sensitivity occurs, luteal cells are producing less P4 and the ovary has less expression of PGR mRNA, leading to a greater expression of TNF mRNA. Research goals aim to target the latter endocrine environment, after luteolytic sensitivity occurs, with reduced P4 and PGR mRNA expression and increased TNF mRNA expression since this is when luteolysis can effectively be induced.

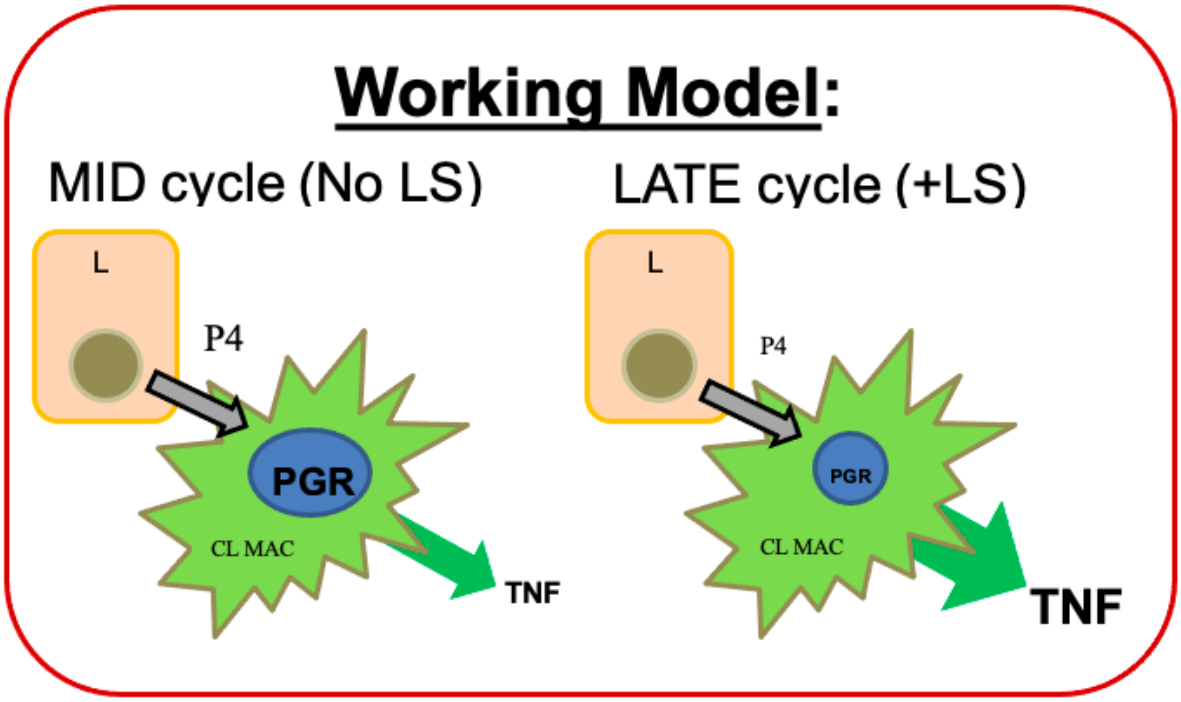

Figure 1.7. Working model explaining the relationship between progesterone (P4) concentrations, progesterone genomic receptor (PGR) expression and the subsequent tumor necrosis factor-alpha (TNF) mRNA expression for mid-cycle (days 8-9, prior to luteolytic sensitivity) verses late-cycle (days 13-14, after luteolytic sensitivity). Mid-cycle corpora lutea (CL) produce greater concentrations of P4 and have increased PGR mRNA expression, with subsequently reduced expression of TNF. Late-cycle CL produce lesser concentrations of P4 and have a decreased expression of PGR mRNA, with greater TNF mRNA expression.

The overall objective of this research was to investigate the effect of either suppressing P4 synthesis, or blocking P4 actions, via its PGR receptors, on MAC cytokine production of TNF, and its subsequent effect on inducing luteolysis/luteolytic capacity in cyclic gilts.

In the investigations described below, we utilized Trilostane (TRIL) to suppress P4 synthesis, or the progestin receptor (PGR) antagonist RU486 to block P4s actions via its receptor.

Trilostane is an inhibitor of the 3bHSD enzyme, and although it has not been previously used for P4 reduction in cycling gilts, it has been used to block CL prog. production in the rhesus monkey during the menstrual cycle. It was however, shown previously to be effective at inhibiting

cortisol secretion in the pig and thus it was chosen for our studies described below, as a means for reducing CL P4 synthesis/secretion in cyclic gilts in vivo.

Furthermore, previous studies have also shown that the PGR receptor antagonist RU486 was effective in blocking pregnancy in the pig, and thus provided support for the use of this inhibitor for our studies examining the effects of blocking P4/PGR actions in the CL in cyclic gilts, in vivo, as described below.

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CHAPTER 2

Trilostane effectively reduces Progesterone Synthesis prior to Acquisition of Luteolytic Capacity in Cyclic Gilts.

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2.1 Abstract

Developing a better understanding of the mechanisms that regulate the acquisition of luteolytic sensitivity (LS) is a critical aspect in controlling the reproductive cycle of livestock species. Swine provide a unique model to study this phenomenon, as porcine corpora lutea (CL) remain unresponsive to luteolytic effects of prostaglandin F2a (PGF) until days 12-13 of the estrous cycle. While the precise mechanism behind LS is undetermined; previous data from our laboratory has demonstrated that luteal macrophages (MACs) play an active role in this process through production of proinflammatory cytokines. Due to the immunomodulatory effects of progesterone, it was hypothesized that suppressing progesterone production prior to LS would permit activation of MACs and initiate LS. The objective of this study was to confirm the efficacy of Trilostane, (TRIL) a commercially available 3β-hydroxysteroid dehydrogenase inhibitor, and determine the optimal dose to reduce luteal and serum progesterone concentrations by a minimum of 80%. , which is critical for future studies addressing LS. Cyclic gilts received Matrix® for 14 days and then checked twice daily for estrus. Upon display of a standing reflex (day 0), gilts were randomly assigned to receive TRIL orally (0, 3, 5, 7.5, or 10 mg/kg, n=2 per treatment) orally, every 12 hours for 36h starting on day 7 of the estrous cycle. Blood samples were taken every 12 hours on days 6 through 9 post estrus to measure the change in progesterone concentrations following treatment. Gilts were euthanized 12 h after the last dose of TRIL (i.e. on day 9), the ovaries were removed; transported on ice to the laboratory, and CL tissue frozen for progesterone analysis.

Progesterone concentrations, luteal and serum, were determined via RIA. Data were analyzed using a MIXED procedure of SAS and statistical significance was determined at $P<0.05$ and a tendency at $0.05<P<0.10$. Serum progesterone concentrations were reduced by 50% by12 hrs post treatment in all TRIL groups compared with control ($P<0.05$). However, only the 10 mg/Kg dose effectively reduced serum progesterone concentrations by 80% compared with control ($P<0.05$) and suppressed progesterone concentration throughout the treatment period. Luteal progesterone concentrations on day 9 of the estrous cycle were reduced by 72.4, 82.4, 87.7, and 88.6% compared with controls ($P<0.05$) in treated with 3, 5, 7.5 and 10 mg/kg of TRIL, respectively. Collectively, these data indicate that oral administration of TRIL effectively reduced both serum and luteal progesterone concentrations, with the optimal dose of 10mg/Kg TRIL chosen for use in future studies addressing LS.

2.2 Introduction

The ability to control luteolysis allows producers to regulate important reproductive events such as estrus and breeding and parturition. Current estrous synchronization protocols in the cattle industry include the use of exogenous hormone, prostaglandin F2a (PGF2a), to induce luteolysis as early as days 5-6 post ovulation[1,2]. Swine are unresponsive to exogenous hormones until days 12-13 of their estrous cycle[1,3-6], and thus induction of luteolysis cannot occur prior to this time. Based on hormone regulation throughout the estrous cycle, progesterone (P4) concentrations are greater during this time of insensitivity in swine[6]. Previous research shows P4 may act to protect the corpus luteum from the induction of luteolysis and therefore methods to reducing P4 are essential to controlling luteolytic sensitivity[6].

Steroidogenesis is the only process by which cells can produce P4[7]. Progesterone synthesis by way of steroidogenesis involves the transport of cholesterol into the inner membrane of the mitochondria of the cell. Cholesterol is able to do this with the help of steroidogenic acute regulatory protein (StAR), a translocation enzyme[7]. Once inside the mitochondrial inner membrane, enzymatic cytochrome P450-cholesterol side-chain cleavage (P450scc) cleaves cholesterol to produce pregnenolone, an intermediary steroid hormone[7]. Pregnenolone is then cleaved into P4 by enzymatic cleavage. The enzyme responsible for this cleavage is 3β-hydroxysteroid dehydrogenase (3β-HSD), located in the smooth endoplasmic reticulum (SER)[7, 8, 9]. 3β-Hydroxysteroid dehydrogenase is essential to the production of progesterone[8], thus inhibiting this enzyme will reduce progesterone synthesis[8].

Research from Diaz et al. (2011) used epostane, a known 3b-HSD inhibitor, to effectively reduce P4 concentrations in cyclic gilts[10]. Due to the high cost and inaccessibility to epostane, similar compounds were sought out for 3b-HSD enzyme reduction in our studies. Etherton et al.

(1989) investigated trilostane's (TRIL) effect on the reduction of cortisol and weight gain in prepubertal gilts[11]. Cortisol is a downstream product of progesterone, and a known immunosuppressant[12], so theoretically the reduction in cortisol can be caused by the reduction of progesterone since the cleavage of pregnenolone to progesterone cannot occur. Trilostane has not been researched as a progesterone suppressant in cycling gilts; however, TRIL and epostane have very similar chemical structures and so treatment of TRIL in cycling gilts was hypothesized to be similar to epostane. Etherton et al. (1989) studied differing doses of TRIL and found the effective dose to be 9.24 mg/kg of body weight[11]. Using these data (Etherton et al., 1989) as a foundation, the objective of this study was to determine the effective dose of TRIL that reduces progesterone synthesis by a minimum of 80% in cyclic gilts.

2.3 Methods and Materials

Research was conducted at North Carolina State University's Swine Education Unit in Raleigh, NC. All procedures involving animals was approved by North Carolina State University Institutional Animal Care and Use Committee (Protocol # 21-289-A).

Animals

Twelve cyclic gilts (147kg-182kg) were synchronized on Matrix® (Merck Animal Health; Patterson, NJ) for 14 days. Estrus detection began following the cease of Matrix® by giving the gilts daily boar exposure and applying pressure to their back, noting ear position, receptiveness, vulva swollenness and vaginal discharge. Estrus was determined by gilts standing still upon receiving pressure on back, ears in an upright and interested position, swollen vulva and vagina discharge present. A total of 9 gilts displayed estrus on average 6 days after the last dose of Matrix® (D0 = display of estrus). Following the display of estrus (day 0), treatment groups were randomly assigned with various doses of Trilostane (TRIL): 0 mg/kg (control n=1), 3 mg/kg (n=2),

5 mg/kg (n=2), 7.5 mg/kg (n=2) and 10 mg/kg (n=2). TRIL treatment were administered orally via a sweet strawberry treat, every 12 hours starting on day 7 post estrus (figure 2.1).

Sample Collection

Gilts were subjected to an initial blood sample on day 6 post estrus and collected every 12 hours through day 9 post estrus (figure 2.1). Blood samples were placed on ice and transported to the laboratory for further processing. In the laboratory, blood samples underwent centrifugation at 2500 G for 20 minutes at 4^{o} Celsius to separate the whole blood. Serum was then transferred to dram bottles for long term storage in -80^{o}C freezer for progesterone analysis.

All gilts were euthanized with sodium pentobarbital (1 mL/10 lbs. IV via the ear (Auricular) vein; in accordance to the AVMA guidelines for the Euthanasia of Animals, 2020) on day 9 of the estrous cycle. The reproductive tracts, specifically ovaries, were removed, transported on ice to the laboratory for further processing. Ovarian structures were recorded and then the corpora lutea were removed from the ovarian stroma. Corpora lutea were separated into several cryotubes, frozen in liquid nitrogen and then placed in the -80^{o}C freezer for subsequent progesterone analysis

Progesterone Radioimmunoassay (RIA)

Progesterone analyses were evaluated using Immuchem Coated Tube Progesterone 125I RIA assays (RIA; MP Biomedicals, LLC., Solon, OH) from blood serum and luteal tissue and were counted for 1 min using the Cobra II Auto Gamma Counter (Packard Instrument Company, Meriden, CT). Serum samples were diluted 1:10 with 0 ng/mL progesterone standard solution from assay kit. Luteal tissue progesterone sample preparation was adapted from (Estill 1995)[13]. One hundred milligrams of CL tissue was homogenized in 10mL of ice cold ETOH. Samples were centrifuged at 500 G for 10 minutes and 500uL aliquots were taken from supernatant and transferred to new tubes. Aliquots were then subjected to a gentle stream of air until all ETOH was

evaporated. Samples were then reconstituted in 1mL of PBS 10x solution and incubated in 4C for 8 hours. Progesterone RIA protocol was then completed using 100uL of the prepared samples per manufacturer's instruction. The intra- and inter-assay coefficients of variation were 6.9 and 4.8%, respectively.

Percent Reduction Calculation

Percent reduction was calculated compared to the control by analyzing each blood sample's P4 concentration per day and dividing it by the respective control animal's P4 concentration for that same time of collection. This ratio of P4 concentration was then subtracted from 1 and multiplied by 100 to give a percentage (Equation 1).

$$Percent\ Reduction\ = (1 - \frac{Progesterone\ conc.\ for\ treatment\ on\ day\ x}{Progesterone\ conc.\ for\ control\ on\ day\ x}) * 100$$

Equation 1: Calculation of percent reduction in progesterone concentrations compared to the control at the same sample collection.

Statistical Analysis

Data were analyzed using repeated measures in PROC MIXED of SAS with least squared means (SAS Institute, 1999). The model included treatment and sample date, and the experimental unit was animal within each treatment. Terms with a significance value of $P > 0.20$ were removed from the complete model in a stepwise manner to derive the final reduced model for each variable. P values of ≤ 0.05 represented significant differences, and P values of $0.05 \leq P \leq 0.10$ were defined as tendencies.

2.4 Results and Discussion

The goal of this study was to determine the efficacy of TRIL on progesterone concentrations in blood serum and luteal tissue and to determine the optimal dosage of TRIL that reduces progesterone concentrations by minimum 80%. Blood serum progesterone concentrations

show no significant difference on day 7 among treatment groups. Twelve hours later (day 7.5) significant decreases in P4 concentrations in TRIL treated groups were observed (Figure 2.2). In blood serum, 10mg/kg was the only treatment group to effectively reduce and maintain P4 concentrations at 80% of those observed in the control group (Figure 2.3). Although P4 concentrations were reduced, treatment groups 3, 5 and 7.5 mg/kg failed to maintain 80% reduction to the end of treatment period (Figure 2.3).

Luteal tissue progesterone analysis from day 9 resulted in treatment groups 5mg/kg, 7.5mg/kg and 10mg/kg effectively reduced progesterone concentrations by 80% (Figure 2.4); however, combination of the serum and tissue P4 analysis was needed to confirm efficacy. Treatment of 10mg/kg of TRIL effectively reduced P4 concentrations in both blood serum and luteal tissue and therefore was deemed the effective dose of TRIL. These results coincide with Etherton (1989) who deemed the effective dose of TRIL in prepubertal gilts to be 9.24mg/kg[11]. These results indicate that TRIL is an effective enzyme 3b-HSD inhibitor, similar to Diaz et al.'s (2011) study on epostane as an effective enzyme inhibitor in cyclic gilts[10].

The administration of TRIL was done so via sweet strawberry treat, and while most gilts consumed the treat willingly, the amount of TRIL in mg/kg resulted in quite a bit of powder. Gilts were reluctant to consume so much powder and loose powder consequently would fall out of the strawberry treat. In an attempt to overcome this challenge, strawberry syrup was drizzled on escaped loose powder in the feed trough to ensure accurate consumption of TRIL dose. Other challenges included the synchronization of gilts using Matrix®. The study began with 12 gilts but only 9 successfully displayed estrus within the timeframe Matrix® suggests its effectiveness. The present study continued with the 9 gilts and the remaining 3 were removed from the study. Kraeling et al. (1981) found Matrix® to be effective at synchronization in normal cycling gilts and sows when used for 14 days consistently[14]. Matrix® was successfully fed for 14 days, the question of

normal cyclicity in the 12 selected gilts was unknown. Matrix® is not known to work on prepubertal gilts at synchronization[14], and this begs the question if the 3 gilts who were unresponsive to Matrix® were prepubertal and had not yet had a normal cycle. Spencer et al. (2010) also used Matrix® for gilt synchronization but treated them with PG600, a drug used to induce estrus containing 400 IU eCG and 200 IU of hCG, 10 days prior to the start of Matrix®[15]. This ensured the gilts had successfully displayed estrus prior to the use of synchronization protocols to increase effectiveness. In future studies, the use of PG600 or other estrus-inducing methods prior to synchronization involving Matrix® should be explored.

2.5 Conclusions

Effectively treating animals with Matrix® is important to successful synchronization because inaccurate dosing can lead to cyst formation on the ovaries[14]. Spencer et al. (2010) found 7% of their gilts to have either cystic ovaries or fluid filled oviducts, consequences unrelated to the PG600[15]. These preliminary results emphasize the importance of accurate dosing of Matrix® and the importance of animals already cycling normally prior to synchronization.

Based on these preliminary studies, the dose of 10mg/kg of TRIL will effectively suppress serum and luteal progesterone concentrations prior to luteolytic sensitivity. Inaccurate dosing of TRIL may lead to insufficient P4 reduction, causing future results to be inaccurate. Proper reduction of P4 by minimum 80% is needed to allow us to examine the role of progesterone in regulating macrophage function.

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2.7 List of Figures

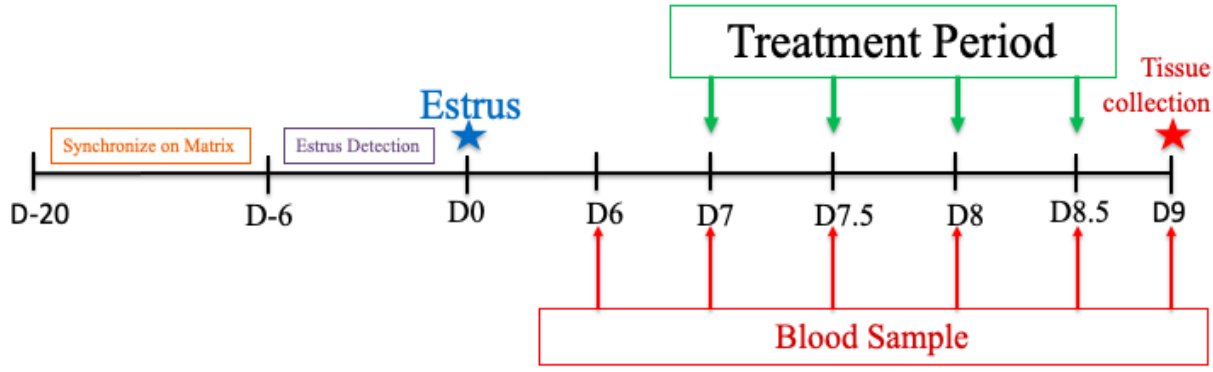

Figure 2.1: Experimental timeline for animal measurements used for gilts. Synchronization of gilts using Matrix® for 14 days followed by estrus detection to determine the display of estrus as day 0 (d=0). Blood samples were taken on days 6, 7, 7.5, 8, 8.5 and 9 (t=6). Treatment of Trilostane was every 12 hrs from day 7 to day 8.5 (t=4). Euthanasia and tissue collection on day 9.

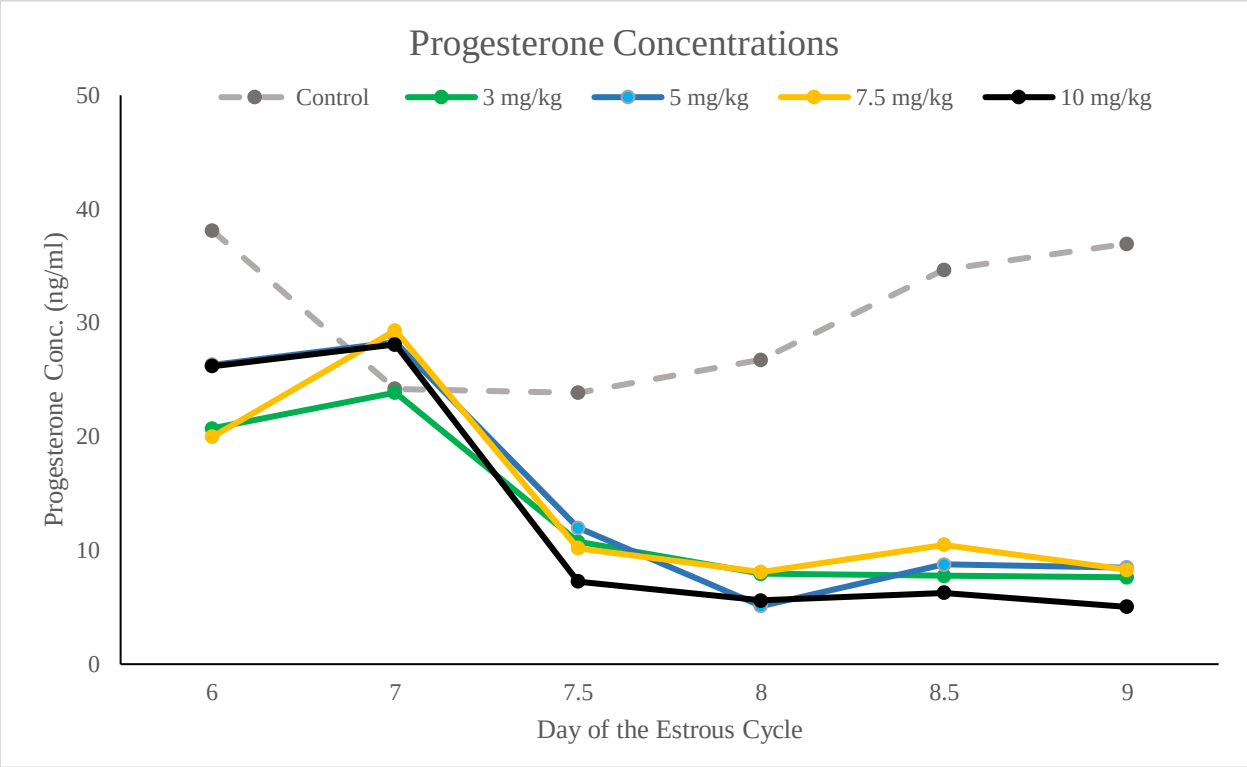

Figure 2.2: Progesterone concentrations (ng/ml) for blood serum between days 6-9 post display of estrus. Treatment groups 3 mg/kg, 5 mg/kg, 7.5 mg/kg and 10 mg/kg effectively reduced progesterone concentrations compared with the control.

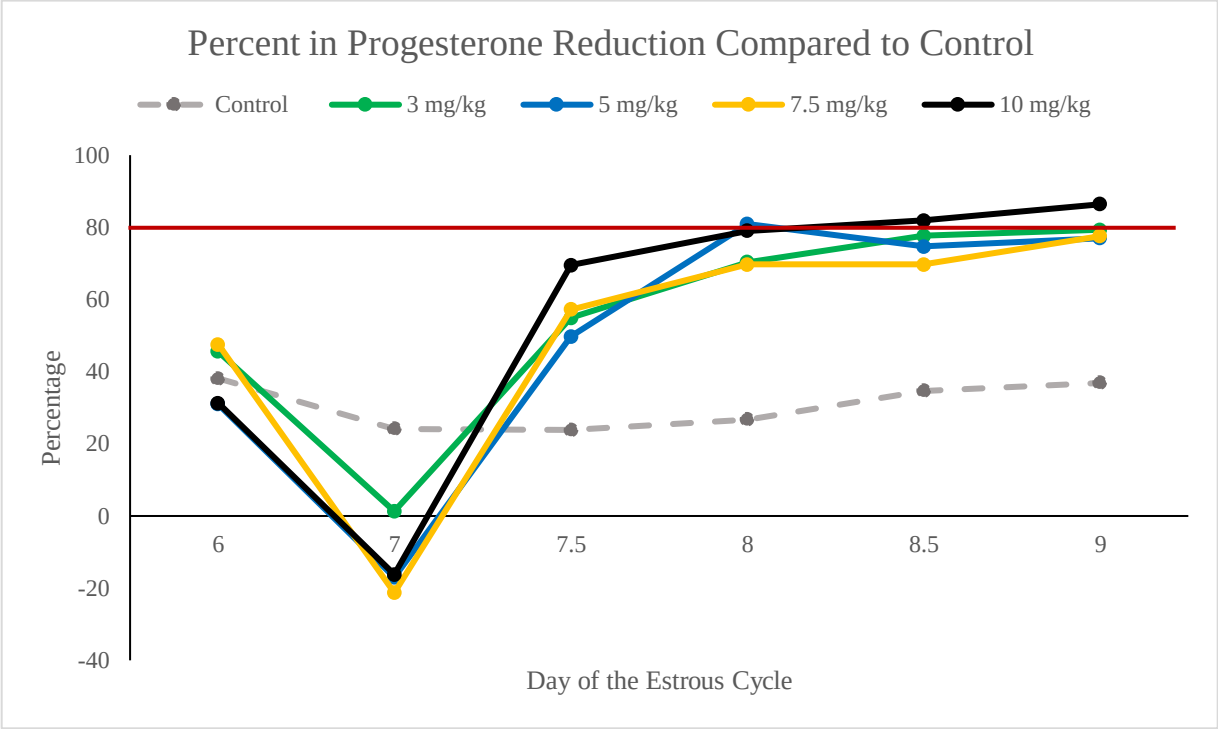

Figure 2.3: Percent reduction in progesterone (P4) concentrations compared with the control. Treatment 10 mg/kg dose of Trilostane effectively reduced P4 concentrations by 80% beginning around day 8.5. Other treatments did not consistently reduce blood serum P4 by minimum 80%. Control represents the actual concentration of P4 present in ng/ml.

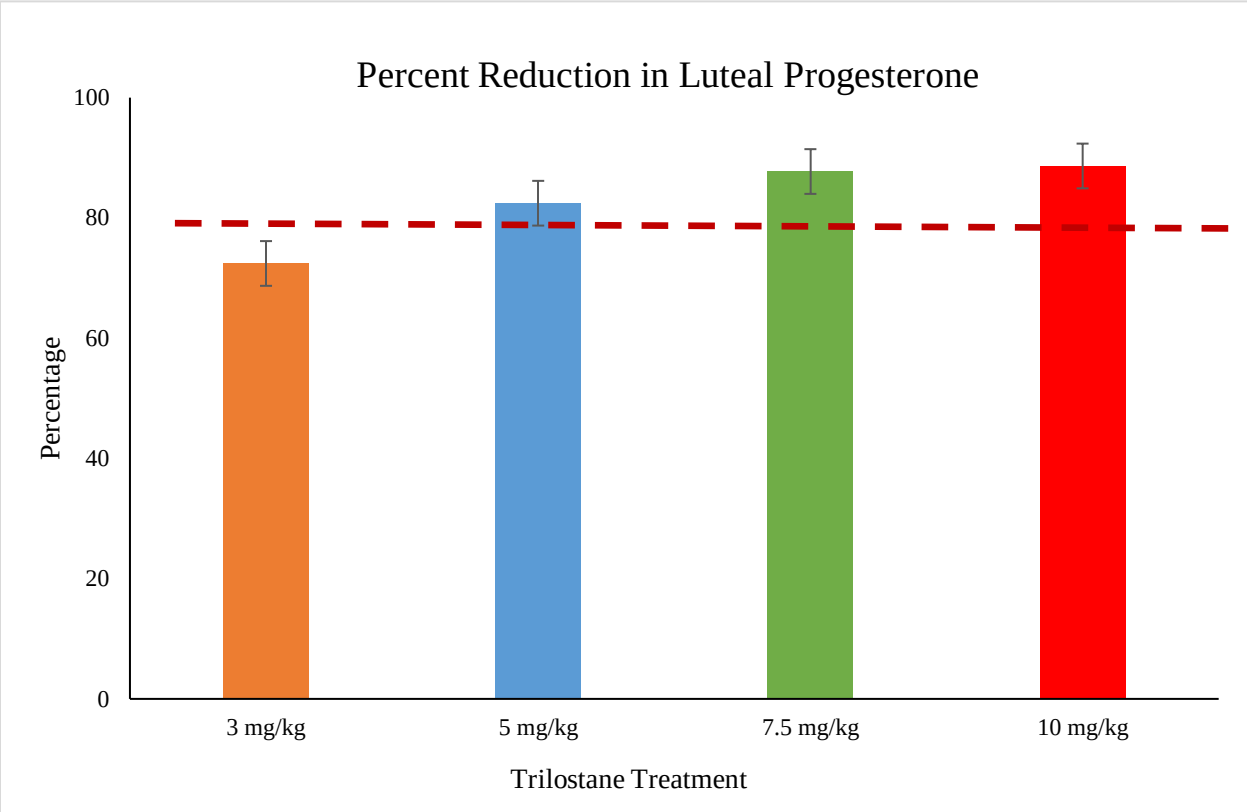

Figure 2.4. Percent progesterone reduction in luteal tissue isolated on day 9. Treatments 5mg/kg, 7.5mg/kg and 10mg/kg effectively reduced progesterone concentrations by minimum 80% compared with the control.

CHAPTER 3

Effect Reduced Progesterone has on Macrophage Production of Cytokine Tumor Necrosis Factor-α.

M.K. Alexander, X. Wang, J.E. Gadsby, and D.H. Poole

3.1 Abstract

Developing a better understanding of the mechanisms that regulate the acquisition of luteolytic sensitivity (LS) is a critical aspect in controlling the reproductive cycle of livestock species. Swine provide a unique model to study this phenomenon, as porcine corpora lutea (CL) remain unresponsive to luteolytic effects of prostaglandin F2a (PGF) until days 12-13 of the estrous cycle. Previous data from our laboratory has demonstrated that luteal macrophages (CL MAC) play an active role in this process through production of proinflammatory cytokines, such as tumor necrosis factor (TNF). Due to the immunomodulatory effects of progesterone (P4), it was hypothesized that suppressing P4 synthesis prior to LS would permit activation of CL MAC to play a key role in regulating luteolytic sensitivity during the estrous cycle in pigs. The objective of this study was to examine the effect of reduction in P4 concentrations on the activation of luteal MACs in the porcine CL and its responsiveness to prostaglandins. Mature gilts (n=25) that displayed estrus following synchronization with Matrix® were randomly assigned to the following treatments: Control, PGF (25 mg i.m. 10hrs prior to euthanasia), Trilostane, (TRIL, 10 mg/kg per pig/day on days 7, 7.5, 8 and 8.5), RU486 (400 mg i.m./pig/day on days 7 and 8), TRIL+PGF, or RU486+PGF. In order to determine serum P4 concentrations, daily jugular blood samples were taken on days 6 through 9 of the estrous cycle. On day 9 of the estrous cycle, all gilts were euthanized, and the ovaries removed, CL were dissected and ~ 500 mg tissue was immediately frozen in liquid nitrogen for subsequent P4 analysis, via RIA. Additional luteal tissue collected on day 9 was dissociated; cultured for 3 hours at 37ºC and 5% CO_2; and analyzed for P4. There was

a day by treatment interaction (P=0.0008) for serum P4 concentrations with TRIL and TRIL+PGF suppressing P4 concentrations on days 8 and 9 of the estrous cycle compared with the other treatment groups (Control, PGF, RU486 and RU486+PGF). Luteal P4 concentrations were significantly less in TRIL and TRIL+PGF, 1.9 and 7.6 ng/ml, respectively, compared with controls (20.7 ng/ml; P<0.0001). No difference in P4 concentrations were observed in the control, PGF, RU486 and RU486+PGF treatment groups (P>0.05). Progesterone concentrations from luteal cell media were reduced in TRIL and TRIL+PGF (2.8 ng/mL and 2.9 ng/mL respectively) treated gilts compared with control gilts (5.3 ng/mL; P=0.0556). Cell cultured luteal cells had a tendency to have reduced P4 in TRIL and TRIL + PGF (P=0.0570) compared with the control. No significant differences were observed in PGR or CYP19A1 mRNA expression across treatment groups (P>0.05). Animals treated with PGF, TRIL, TRIL + PGF and RU486 + PGF had a tendency to have decreased mRNA expression of FOSB (P=0.0886) compared with the control, but not RU486. Animals treated with TRIL and TRIL + PGF had a tendency to have increased expression of TNF mRNA compared with PGF, RU486 and RU486 + PGF treated animals (P=0.0887). Isolated MAC populations did not have a significant differences in TNF protein concentrations across treatment groups (P=0.3689). Based on these data, transient reduction in progesterone did not have significant effects on luteal macrophage activation or response to exogenous prostaglandin. Future studies should explore greater P4 reduction sustained for longer period of time prior to analysis.

3.2 Introduction

Following ovulation, the corpus luteum (CL) is the endocrine gland responsible for the production of progesterone (P4) within the ovary[1]. Regression of this endocrine gland allows nonpregnant animals to cycle normally and undergo luteolysis. Luteolysis has been identified as the reduction of P4 and induction of cell death and structural regression of the CL[2-5]. Prostaglandin F2a (PGF2a) secreted from the uterus in nonpregnant animals is the primary factor in initiating luteolysis[2-4]. Exogenous PGF2a has been successful at inducing luteolysis, however, it is only effective after certain days in the animal's reproductive cycle depending on the species[6-11]. In bovids, PGF2a can be effective at inducing luteolysis as early as days 5-6 of their estrous cycle[12-14]. However, swine are unresponsive to PGF2a until days 12-13 of their estrous cycle[12-14]. Prior to reaching this time when they display luteolytic capacity, pigs are unable undergo luteolysis early with the use of PGF2a even though the CL has abundant receptors for PGF2a[2-3, 14-15]. The mechanisms that prevent and allow luteolysis to occur before and after days 12-13 are unknown, but research has identified specific responses in the CL to PGF-induced luteolysis that do not occur prior to luteolytic capacity being reached[1]. In the pig and cow CL, PGF-induced luteolysis signals for luteal estradiol synthesis, also known as aromatase (CYP19A1), and luteal PGF2a production[1, 16]. The increase in PGF2a causes a reduction of steroidogenesis of P4 only in CL with luteolytic capacity. These changes to the CL, such as CYP19A1 synthesis and luteal PGF2a synthesis, are useful markers for luteolytic capacity in the CL[1, 13].

Subsequent changes to the CL in response to luteal PGF2a production also includes the immune-regulation of cytokine production[12]. Resident macrophages (MACs) present in the ovary throughout the estrous cycle will secrete cytokine tumor necrosis factor-alpha (TNF) in response to the increase in luteal PGF2a and subsequent decrease in P4[12]. Tumor necrosis factor-alpha is an inhibitor of P4 synthesis by interfering with the steroidogenic acute regulatory (StAR) protein,

responsible for the movement of cholesterol into the inner membrane of the mitochondria for P4 synthesis[1,12]. In addition to the anti-steroidogenic effects, previous research in ovine species shows that TNF is also responsible for the induction of apoptosis on luteal cells, which leads to structural degradation and transformation of the CL[12, 18-19]. Lobel and Levy (1968) first observed an increase in MAC concentrations around the time of luteolysis in the cow, human and rabbit and other have reported similar findings to support this observation[12, 21-25] but the damaging effects of TNF can only be effective if P4 is reduced. Similarly, activation of the gene FOSB is associated with cell death in several physiological processes[1, 26]. Previous studies supplemented P4 in pregnant rats and saw a decrease in FOSB expression[1, 27]. This is due to P4 preserving the integrity of the cells that make up the CL in order to maintain pregnancy and in a P4 depressive environment, the CL is vulnerable to the damaging effects of TNF and FOSB[1].

The main steroid hormone responsible for maintenance of pregnancy, mammary gland development and lactation, and reproductive cycles is P4[1]. Extensive research supports the direct role luteal P4 has on the maintenance and regulation of the CL[1, 28-31] in bovine[32-33] and in rats[1, 34-36]. The current study's objective is to explore the onset of luteolytic capacity following either a reduction in progesterone production or through inhibition of PGR. In early stages of the estrous cycle, when P4 is in abundance, P4 receptors (PGR) are also seen in elevated expression, further supporting P4s protective role on the CL[1]. The current study hypothesizes the inhibition of P4 synthesis by luteal cells, or blockade of PGR receptors, will result in increased TNF production by CL MAC and lead to the premature onset of luteolysis, in vivo. Research aims include the investigation of the effect suppressing P4 synthesis and blocking PGR receptor signaling, expression of FOSB and CYP19A1, MAC cytokine production of TNF, both mRNA expression and protein synthesis, and its subsequent effect on inducing luteolysis in cyclic gilts

3.3 Methods and Materials

Research was conducted at North Carolina State University's Swine Education Unit in Raleigh, NC. All procedures involving animals was approved by North Carolina State University Institutional Animal Care and Use Committee (Protocol # 21-289-A).

Animals

Thirty two cyclic gilts (147kg-182kg) were synchronized on Matrix® (Merck Animal Health; Patterson, NJ) for 14 days. Estrus detection began following the cease of Matrix® by subjecting gilts to daily boar exposure and applying pressure to their back, noting ear position, receptiveness, vulva swollenness and vaginal discharge. Estrus was determined by gilts standing still upon receiving pressure on back, ears in an upright and interested position, swollen vulva and vagina discharge present. A total of 25 gilts displayed estrus on average 6 days after the last dose of Matrix® (D0 = display of estrus).

Experimental Design

Upon display of a standing reflex, treatments were randomly assigned as control (n=4), PGF (n=4), TRIL (n=5), TRIL and PGF (n=4), RU486 (n=4), and RU486 and PGF (Lutalyse) (n=4). As outlined in Figure 3.1, the treatment period began 7 days after the display of a standing reflex and continued until 10 hours before euthanasia on day 9. Control pigs were not given any exogenous hormones and fed normally per farm standards. Pigs treated with TRIL were given 10 mg/kg orally on days 7, 7.5, 8 and 8.5. Prostaglandin (Lutalyse) was given as a single 25mg dose intramuscularly 10hrs prior to euthanasia on day 8.5. Pigs treated with RU486 were given 400 mg/pig/day dissolved in 1.5mL of sesame oil, intramuscularly, on days 7 and 8 (figure 3.1).

Gilts were subjected to an initial blood sample on day 6 post estrus and collected every 24 hours through day 9 post estrus (figure 3.1). Blood samples were placed on ice and transported to the laboratory for further processing. In the laboratory, blood samples underwent centrifugation at

2500 G for 20 minutes at 4^{o} Celsius to separate the whole blood. Serum was then transferred to dram bottles for long term storage in -80^{o}C freezer for progesterone analysis.

All gilts were euthanized with sodium pentobarbital (1 mL/10 lbs. IV via the ear (Auricular) vein; in accordance to the AVMA guidelines for the Euthanasia of Animals, 2020) on day 9 of the estrous cycle. The reproductive tracts, specifically ovaries, were removed, transported on ice to the laboratory for further processing. Ovarian structures were recorded and then the corpora lutea were removed from the ovarian stroma.

Tissue Dissociation

Dissociation protocol was adapted from Gadsby et al. (2020)[14]. Corpora lutea were minced until consistency was a fine slurry. Tissue was then washed with plate media and aliquoted samples were separated into cryotubes, frozen in liquid nitrogen and then placed in the -80^{o}C freezer for subsequent luteal progesterone analysis, RNA analysis and protein analysis.

Remaining minced tissue was transferred into a R2D2 spinning flask with 25mL 0.05M collagenase and placed on hot water bath at 37^{o}C for 30 minutes. Tissue was then broken up further with a broken tip pipet and supernatant was transferred to a new centrifuge tube. Twenty five milliliters of 0.05M collagenase was replaced in the flask. This process of being in the hot water bath, breaking up tissue with broken tip pipet trough reverse pipetting and transferring supernatant was repeated until tissue was no longer present in R2D2 flask. The collected supernatants were then spun in the centrifuge at 300 G for 5 minutes to pull all cells into a pellet. Supernatants were discarded and 25mL of RPMI Plate media was added to the first tube, resuspended and transferred to the second tube and repeated to combine all cell pellets into one 50ml conical tube. Pellets were resuspended and cells were counted on hemocytometer. Aliquot samples of 100,000 cells per 1 mL were transferred for cell culture analysis (see below).

Samples were then centrifuged at 400 rpm for 7 minutes and supernatant was transferred to a new tube. Twenty five milliliters of RPMI Plate media was replaced in sample and this process was repeated, centrifuging at 400 rpm for 7 minutes, transferring supernatant and replacing 25mL of RPMI Plate media, four times. Collected supernatants were then centrifuged at 300 G for 5 minutes and supernatants were discarded. 10mL of red blood cell lysis (RBC) buffer was then used to combine all the pellets in each tube and then incubated on ice for 5 minutes. Sample was then centrifuged at 300 G for 5 minutes and supernatant was discarded. Sample was resuspended in 25mL MACs buffer and filtered using 100um, 70um, 40um and 30um filters. Cells were then counted under microscope on hemocytometer and appropriate amount of MACs buffer and CD14 microbeads were calculated and added per manufacturer's instructions for MAC isolation on MAC column.

Luteal Tissue Preparation for Progesterone Analysis

Luteal tissue progesterone sample preparation was adapted from (Estill 1995)[37]. 100mg of CL tissue was homogenized in 10mL of ice cold ETOH. Samples were centrifuged at 500 G for 10 minutes and 500uL aliquots were taken from supernatant and transferred to new tubes. Aliquots were then subjected to a gentle stream of air until all ETOH was evaporated. Samples were then reconstituted in 1mL of PBS 10x solution and incubated in 4C for 8 hours. Progesterone RIA protocol was then completed using 100uL of the prepared samples per manufacturers instruction. The intra-assay coefficient of variation was 20%.

Luteal Cell Culture

Aliquots of each sample were transferred to new tube and diluted to 100,000 cells/mL M199 Plate media for a total volume of 6mLs. Aliquots were transferred to cell culture plate, 1mL per well for 6 wells. Cell culture plates were placed in incubator for 3 hrs at 35^{o}C and 5% CO_2. Samples were then transferred to microcentrifuge tube and centrifuged at 12 G for 10 mins.

Supernatant was isolated from luteal cell pellet and flash frozen with liquid nitrogen before storing in -80^{o}C.

Macrophage Isolation

Once the appropriate amount of MACs buffer and CD14 microbeads were calculated and added per manufacturer's instructions, cells were incubated at 4C for 15 minutes, swirling sample every 5 minutes. Ten milliliters of MACs buffer was added and samples were centrifuged at 300 G for 5 minutes. Supernatant was discarded and 2mL of MACs buffer was added and cells resuspended. Cells were then placed on a MAC column and appropriate manufacturer protocol was followed to isolate CD14- and CD14+ cells.

Progesterone Radioimmunoassay (RIA)

Progesterone analyses were evaluated using Immuchem Coated Tube Progesterone 125I RIA assays (RIA; MP Biomedicals, LLC., Solon, OH) from blood serum and luteal tissue and were counted for 1 min using the Cobra II Auto Gamma Counter (Packard Instrument Company, Meriden, CT). Serum samples were diluted 1:10 with 0 ng/mL progesterone standard solution from assay kit. Luteal tissue progesterone sample preparation was adapted from (Estill 1995)[37]. Luteal tissue (100 mg) was homogenized in 10mL of ice cold ETOH. Samples were centrifuged at 500 G for 10 minutes and 500uL aliquots were taken from supernatant and transferred to new tubes. Aliquots were then subjected to a gentle stream of air until all ETOH was evaporated. Samples were then reconstituted in 1mL of PBS 10x solution and incubated in 4C for 8 hours. Progesterone RIA protocol was then completed using 100uL of the prepared samples per manufacturers instruction. The intra-assay coefficient of variation was 20%.

Percent Reduction Calculation

Percent reduction was calculated compared to the control by analyzing each blood sample's P4 concentration per day and dividing it by the respective control animal's P4 concentration for

that same time of collection. This ratio of P4 concentration was then subtracted from 1 and multiplied by 100 to give a percentage (Equation 1).

$$Percent\ Reduction\ = (1 - \frac{Progesterone\ conc.\ for\ treatment\ on\ day\ x}{Progesterone\ conc.\ for\ control\ on\ day\ x}) * 100$$

Equation 1: Calculation of percent reduction in progesterone concentrations compared to the control at the same sample collection.

TNFα ELISA Assay

Isolated MACs from CL tissue were evaluated with a TNFα ELISA kit from Invitrogen per manufacturer's instructions. Isolated cells (1 mil cells) were resuspended in 500µl of dilutant buffer and vortexed for 1 minute. Plates were prepped with 50µl of incubation buffer and 100µl of samples or standards in each well. Plates were then incubated at room temperature for 3 hours. Wells were aspirated and washed with washing buffer 4 times after incubation. One hundred microliters of Biotin Conjugate solution was added to each well and incubated at room temperature for an additional hour. Following incubations, wells were aspirated and washed 4x with washing buffer. One hundred microliters of 1x SAV HRP solution was added and incubated for 30 minutes at room temperature. Wells were aspirated and washed with washing buffer 4x. One hundred microliters of TMB was added to each well and incubated in the dark for 30mins and then 100µl stop solution was added to each well before reading plate on plate reader. Plates were read at absorbance level of 450nm. The intra- and inter-assay coefficients of variation were 10 and 14.6% respectively.

RNA Isolation

Frozen tissue samples were homogenized in 1mL of TRIzol reagent (Thermo Fisher) twice for 30 seconds and then placed on ice for 20 sec. Samples were then centrifuged for 10

mins at 12,000 G at 4ºC to create a pellet. The supernatant was transferred to a 1.5 mL microcentrifuge tube and mixed with 200 µl of 1-bromo-3-chloropropane by inverting sample for 20 sec. Samples were then incubated at room temperature for 3 min and centrifuged at 12,000 G at 4ºC for 18 mins. Aqueous supernatant was removed and placed into a new tube, mixed with 200 µl of chloroform by inverting and shaking for 20 sec. Samples were then incubated at room temperature for 3 mins and then centrifuges at 21,000 G at 4ºC for 18 mins. 500 µl of supernatant was transferred to new tube and equal parts of 100% ethanol was added and mixed manually. Samples were filtered in columns from the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Columns were then washed with 700 µl of RW1 buffer once and three times with 500 µl of RPE buffer. RNA was eluted with RNase free water. The quantity of total RNA was determined via spectrometry.

Quantitative Real-time PCR

RNA was reverse transcribed into cDNA using the Moloney Murine Leukemia Virus (M-MLV, Thermo Fisher) per manufacturer's instructions. Quantitative RT-PCR (qRT-PCR) was performed using CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and the SsoAdvanced™ Universal SYBR® Green Supermin (BioRad) with oligonucleotide primers synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA) or the SsoAdvanced™ Universal probes (Applied Biosystems). Delta-delta Ct values were calculated using GAPDH control amplification results to obtain relative mRNA levels per sample. Primers provided in Table 3.1.

Statistical Analysis

Data were analyzed using repeated measures in PROC MIXED of SAS with least squared means (SAS Institute, 1999). The model included treatment and sample date, and the

experimental unit was animal within each treatment. For PCR and TNF variables, data were analyzed using a 1 way ANOVA with a Fishers F test. Terms with a significance value of P > 0.20 were removed from the complete model in a stepwise manner to derive the final reduced model for each variable. P values of ≤ 0.05 represented significant differences, and P values of $0.05 \leq P \leq 0.10$ were defined as tendencies.

3.4 Results

Progesterone

Circulating P4 concentrations in blood serum were similar in all groups before treatment of TRIL and RU486 began on day 7 (figure 3.2). Pigs treated with TRIL and TRIL + PGF displayed significantly reduced P4 concentrations in blood serum samples beginning on day 8 compared with other treatments; TRIL contained 1.1 ng/mL and 0.85 ng/mL on days 8 and 9 respectively, and TRIL + PGF contained 0.9 ng/mL and 1.3 ng/mL on days 8 and 9 respectively (p-value = 0.0008; figure 3.2). Pigs treated with PGF, RU486, RU486 + PGF and controls had 5.7 ng/mL, 4.8 ng/mL, 3.7 ng/mL and 4.3 ng/mL on day 9 respectively. There was also a significant difference between day effect of these pigs, meaning day to day, there was a significant decrease in P4 in TRIL treated pigs from 1.1 ng/mL to 0.85 ng/mL (p-value = 0.0394; figure 3.2). As expected, there was no significant difference between circulating P4 concentrations in treatment groups PGF, RU486, and RU486 + PGF compared the control (SEM = +/-0.6). Based on the treatment interaction alone, TRIL (2.1 ng/mL) and TRIL + PGF (1.8 ng/mL) had significantly reduced P4 concentrations in blood serum with the standard error +/- 0.3 (p-value < 0.0001; figure 3.3).

Percent reduction in P4 concentrations in blood serum compared to the control exhibited 80.23% reduction in the TRIL treatment group on day 9 (Figure 3.4). Day 6 to 8 reduction in P4 for TRIL treated animals resulted in 9.09%, 0% and 66.67% reduction, respectively. PGF treated animals had a 12.12%, 8.82%, 27.27% and -32.56% reduction on days 6 to 9 compared to the

control, respectively (Figure 3.4). TRIL + PGF treated animals displayed reductions on days 6 to 9 equaling 21.21%, 32.35%, 72.73% and 69.77%, respectively (Figure 3.4). RU486 treated animals had P4 reduction percentages on days 6 to 9 equaling 48.48%, 17.65%, -18.18 and -11.63%, respectively. RU486 + PGF treated animals displayed 27.27%, 11.76%, 3.03% and 13.95% reduction across days 6 to 9 respectively (Figure 3.4).

Trilostane (1.9 ng/mL) and TRIL + PGF (7.6 ng/mL) treated pigs also had significantly reduced P4 concentrations in luteal tissue compared with control animals and treatment animals, such as PGF, RU486 and RU486 + PGF that had as 20.7 ng/mL, 26.7 ng/mL, 22.2 ng/mL and 22.1 ng/mL, respectively, shown in figure 3.5 (p-value < 0.0001; SEM = +/- 2.7). Similar to blood serum PGF, RU486 and RU486 + PGF treatments, there was no significant difference in P4 concentrations for those treatments compared with the control. Luteal cell cultured samples had a tendency to have reduced P4 concentration in pigs treated with TRIL (2.74 ng/mL) and TRIL + PGF (2.71 ng/mL) (p-value = 0.0570, SEM = +/- 1.08; figure 3.6). No significant differences in luteal cell cultured pigs treated with PGF, RU486, and RU486 + PGF treated pigs compared with the control (5.16 ng/mL), 5.6 ng/mL, 6.29 ng/mL and 7.16 ng/mL respectively.

Gene expression:

No changes were observed in PGR or CYP19A1 mRNA expression across treatments (Figure 7 & 8; P >0.05). PGR mRNA expression for PGF, TRIL, TRIL + PGF, RU486 and RU486 + PGF were compared with the housekeeping gene, GAPDH, and had 0.78, 0.9, 0.54, 0.98 and 0.57 mRNA expression respectively (figure 3.7). CYP19A1 mRNA expression for PGF, TRIL, TRIL + PGF, RU486 and RU486 + PGF were also compared with the housekeeping gene, GAPDH, and had 1.2, 0.56, 0.82, 0.79 and 1.22 mRNA expression respectively (figure 3.8).

Relative mRNA expression of FOSB compared with the housekeeping gene, GAPDH, in luteal tissue tended to be reduced in PGF (0.46), TRIL (0.58), TRIL + PGF (0.47) and RU486 +

PGF (0.49) treated animals compared with control (1), but not RU486 (0.67) (figure 3.9; P =0.0886). Relative TNF mRNA expression compared with the housekeeping gene, GAPDH, in luteal tissue tended to be reduced in PGF (0.41), RU486 (0.6) and RU486 + PGF (0.54) treated animals compared with control (1), but not TRIL (0.76) or TRIL + PGF (0.74) (figure 3.10; p=0.0887).

TNF Protein Expression

Isolated MACs showed no significant difference in TNF protein concentrations in isolated MACs for all treatment groups compared with the control (P = 0.3689; figure 3.11). Protein concentrations for control, PGF, TRIL, TRIL + PGF, RU486 and RU486 + PGF were 0.097 pg/mL, 0.104 pg/mL, 0.111 pg/mL, 0.085 pg/mL, 0.096 pg/mL and 0.104 pg/mL respectively (figure 3.11).

3.5 Discussion

The goal of the present study was to reduce P4 actions in the body by way of PGR antagonist or via enzymatic inhibition and determine if luteolytic capacity was successfully achieved. Progesterone was reduced significantly in TRIL + TRIL and PGF treated pigs for blood serum and luteal tissue and cell culture samples for TRIL and TRIL + PGF had a tendency to have reduced P4 concentrations. This indicates that the dose of TRIL at 10 mg/kg was effective at reducing P4 and supports a previous study that used this product in swine[41]. Diaz et al. (2011) reported significant decreases in P4 concentration in cyclic gilts treated with epostane, another known 3β-HSD inhibitor[1]. Being that epostane and TRIL have very similar chemical structures, this study confirms the efficacy of TRIL as a 3β-HSD inhibitor.

Uterine PGF2a is thought to begin the luteolytic cascade during luteolysis in pigs[1-2, 38] but in bovine and swine species, the CL is unresponsive to the damaging effects of PGF2a until days

5-6 and 12-13 of the estrous cycle respectively[1, 12-14]. It has been thoroughly researched that steroid hormone, P4, is in high concentrations during this time of luteolytic insensitivity and acts to protect luteal cells from cell death[12]. It has also been reported in the cow that PGR mRNA expression is increased during this time of insensitivity[39], furthering support of P4 acting as a protector of the CL[1, 15, 40]. Identifying markers before and after luteolytic capacity is reached include P4 concentrations and the subsequent expression of PGR mRNA, CYP19A1, FOSB and TNF.

Knowing P4 was effectively reduced in animals treated with TRIL and TRIL + PGF, identifying markers of luteolytic sensitivity can be evaluated. In bovine and swine species, it has been reported that as the CL ages and P4 is decreased, PGR also decreases[1]. The present study evaluated PGR mRNA expression and found no significant changes in expression for any of the treatment groups. This contradicts previous research that found PGR to decrease as P4 also decreases[1, 16]. Reasonings for this contradiction could be that the P4 reduction was not substantial enough to also reduce PGR mRNA expression or that the reduction in P4 did not have enough time to effect mRNA expression. A review by Robinson (1989) compared the efficacy of TRIL and epostane and found that in the gonads, epostane is more consistent at inhibiting 3b-HSD and TRIL's inhibitory function can be variable[42]. This could be why PGR mRNA expression was not decreased like previous research from Diaz et al. (2011). Nevertheless, methodological differences cannot be ruled out to explain this inconsistency.

Aromatase, or CYP19A1, is another luteolytic capacity marker that has been reported to be stimulated by PGF only in animals with luteolytic capacity[1, 16]. Around the time the CL regresses, an upregulation in CYP19A1 mRNA has been reported[43-44]. Results of the present study did not find significant differences between treatments for mRNA expression of CYP19A1. This could be due to inadequate reduction of P4 among treatment groups. Diaz et al (2011) reduced P4 concentrations with epostane and also had a treatment group with epostane + PGF, similar to the

present studies treatment groups. In animals treated with only epostane, Diaz et al (2011) found an ~85% reduction in P4 from luteal tissue compared with the control group, however, in animals treated with epostane + PGF, over 90% reduction in luteal P4 was observed compared with the control[1]. Between the two treatment groups, epostane and epostane + PGF, there was half the concentration of P4 in animals treated with epostane + PGF compared with epostane alone. Subsequently, when looking at PGF2a concentrations across treatments, Diaz et al (2011) did not find significant differences in the epostane treated pigs that had roughly an 85% reduction in luteal P4 compared with the control but in pigs treated with epostane + PGF and had over 90% P4 reduction, PGF2a concentrations were 7.86-fold greater than the control (p-value < 0.05)[1]. This dramatic increase in PGF2a could indicate that the over 90% reduction in P4 was substantial enough to allow for luteal PGF2a to become luteolytic. Similarly, Diaz et al. (2011) looked at mRNA expression of CYP19A1 and did not find any significant differences between animals treated with epostane alone compared with the control; however, did report a significant increase in mRNA expression of CYP19A1 in animals treated with epostane + PGF[1]. The significant increase in CYP19A1 mRNA expression could have been possible due to effective reduction of P4 being over 90%, whereas P4 reduction in the present study was only by 80% on day 9 for TRIL treated animals only. Another possibility is that the difference in consistent 3b-HSD inhibition for TRIL verses epostane in the gonads means epostane will ultimately be more efficient at P4 suppression[42].

Alternative luteolytic capacity markers include activation of the gene, FOSB, which has been known to result in signaling cell death[1]. Gilts treated with PGF, TRIL, TRIL + PGF, and RU486 + PGF had a tendency for decreased mRNA expression compared with the control, but not RU486 alone. This contradicts the research conducted by Diaz et al. (2011) who reported an increase in FOSB in animals treated with epostane + PGF[1]. This is thought to be because the

decrease in P4 signals the cascade of events that ultimately leads to cell death and transformation, so an increase in FOSB is to be expected. In the present study, the lack of FOSB in P4 suppressed animals could mean the luteal cells were not undergoing apoptosis and cell death, thus indicating early luteolysis was not properly achieved. Reasons for this could include methodological inconsistencies or the suppression of P4 was not dramatic enough for cell death to ensue. The goal of this research was to ultimately reduce P4 by a minimum of 80% but that may not have been significant enough for FOSB to upregulate transcription. In the same study mentioned above by Diaz et al (2011), FOSB was also a genetic marker evaluated for luteolytic capacity and similar results to the PGF2a concentrations and CYP19A1 mRNA expression were found. When compared with the control, animals treated with epostane alone (~85% P4 reduction) did not have significant differences in FOSB mRNA, but did have a significant increase in mRNA expression of FOSB in animals treated with epostane + PGF (over 90% P4 reduction)[1]. Being that the treatment groups from the present study were only reducing P4 by 80%, it is likely the discrepancy in FOSB mRNA expression is due to inadequate reduction of P4. This would allow for residual P4 to protect luteal cells from undergoing cell death, thus restricting luteolysis from occurring. It is also a possibility that the amount of time the cells were exposed to the 80% reduction in P4 wasn't long enough. Animals treated with TRIL had an 80% reduction beginning on day 9, the same day as tissue collection. If the study was prolonged to allow the luteal cells to become vulnerable to PGF2a, luteolysis may have occurred.

Quantitative PCR analysis of TNF mRNA expression in luteal tissue samples resulted in a tendency for TRIL and TRIL + PGF treated pigs to have a greater expression of TNF than PGF, RU486 and RU486 + PGF treated pigs. These results correspond to Pate et al (2001) that states in the absence of P4, increased TNF is upregulated[12]. Although not significant, the tendency for TRIL and TRIL + PGF treated animals to have an increase in TNF mRNA than PGF, RU486 and RU486

+ PGF treated animals means the opportunity for an increase in TNF protein concentrations in the CL are plausible. It is possible the mRNA would translate into protein if the time between treatment and tissue collection was increased, allowing for more time for that translation to occur.

It was hypothesized that the reduction in P4 would result in an increase in TNF protein as described by the working model in figure 11. However, isolated MAC populations did not have any significant differences in TNF protein. This conflicts with research by Pate (2001) that states a reduction in P4 leads to an increase in MAC populations in the CL, thus leading to an increase in TNF being produced[12]. Previous research from Gadsby et al. (2020) reported an increase in CL MAC TNF mRNA expression in late stage CL, around days 13-14 of the estrous cycle[14]. This is around the time of the porcine estrous cycle when activated P4 concentrations decrease, resulting in luteolytic capacity being reached. Results of the present study conflict with these data from Gadsby et al (2020) because even in TRIL and TRIL + PGF treated pigs that had significantly decreased P4, there is no significant difference in TNF protein found in the luteal tissue[14]. This could be due to a number of reasons, the first being that MAC populations were lost during the isolation process. The cell dissociation protocol was adapted from Gadsby et al. (2020) but various other protocols have also been successful[14]. Okuda et al. (1992) dissociated luteal cells from bovine tissue using a similar procedure as the present study but did not centrifuge samples as often[45]. Increased centrifugation could have attributed to the loss in MAC populations. Macrophage secretion of TNF also may have been secreted or lost during this isolation process, and thus would not be found in abundance in an ELISA assay. Alternatively, the increased TNF may have been undergoing transcription and still in transcription as confirmed in this study. If increased time between treatment and tissue collection was conducted, the increase in mRNA for TNF may turn into an increase in TNF protein, thus supporting the hypothesis that TNF concentrations increase in the absence of P4. Although the hypothesis cannot be confirmed, the

working model shown in figure 3.11 demonstrates the relation to P4 and TNF before and after luteolytic sensitivity occurs. It is still believed that prior to luteolysis, increased P4 concentrations limit the TNF secretion by CL MACs and after luteolysis, decreased P4 concentrations allow for an increase in TNF production by CL MACs.

3.6 Conclusion

In the present study, P4 was significantly reduced in TRIL and TRIL + PGF treated animals. The question of whether that reduction was adequate for the induction of early luteolysis is still unknown. Previous studies by Diaz et al. (2011) were more successful at inducing luteolysis when P4 was reduced by over 90%[1] but it also cannot be ruled out that the amount of time between when the treatments were administered and the tissue was collected played a factor in these results. It is possible luteolysis would have occurred if luteal cells were given enough time to become vulnerable to the effects of PGF2a and induce cellular responses. It is also possible Diaz et al. (2011) had successes because epostane is a more consistent 3b-HSD inhibitor than TRIL[1, 41]. Animal age and cyclicity also can be considered factors of these differences as Gadsby et al. (2020) conducted the experiments on slaughterhouse ovaries and treated them in vitro[14]. The animal age and cyclic performance is unknown; and the difference between in vitro studies and in vivo studies are apparent.

Future research should explore methods to increase P4 reduction 90-95% for adequate P4 depressive environments to ensure luteal cells are vulnerable to PGF2a and not still protected by P4. The timeline of tissue collection should also be considered, by increasing the time between treatments and tissue collection, luteal cells may present the luteolytic changes necessary to then evaluate TNF content in the CL. The goal of this research was to explore the mechanisms associated with luteolysis by reducing P4 and subsequently evaluating PGR, CYP19A1, FOSB mRNA expression to evaluate whether early luteolysis was achieved, and evaluating MAC

production of cytokine TNF, both mRNA expression and protein secretion in a luteolytic CL. Although the results do not indicate early luteolysis was achieved, P4 was suppressed significantly in TRIL and TRIL + PGF treated animals and tendencies for necessary genetic markers indicate early luteolysis is achievable.

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3.8 List of Figures

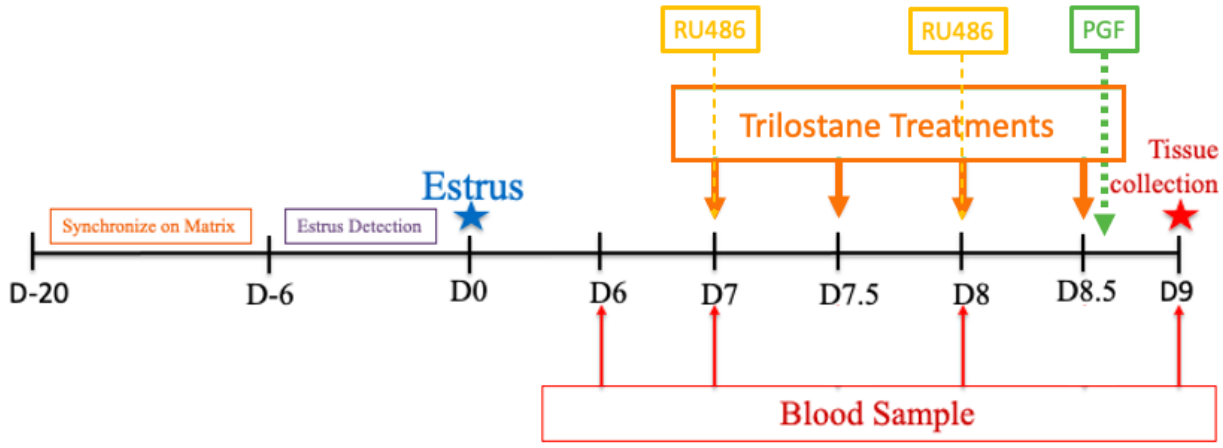

Figure 3.1. Pigs were fed Matrix® for 14 days and then estrus detection began. Display of estrus was on average 6 days post Matrix®. Pigs treated with TRIL were given 10 mg/kg of body weight, a total of 4 TRIL treatments were given orally on days 7, 7.5, 8 and 8.5. Prostaglandin (Lutalyse) was given as a single 25mg dose intramuscularly 10hrs prior to euthanasia. Pigs treated with RU486 were given 400 mg/pig/day, intramuscularly, on days 7 and 8. Euthanasia and tissue collection occurred on day 9.

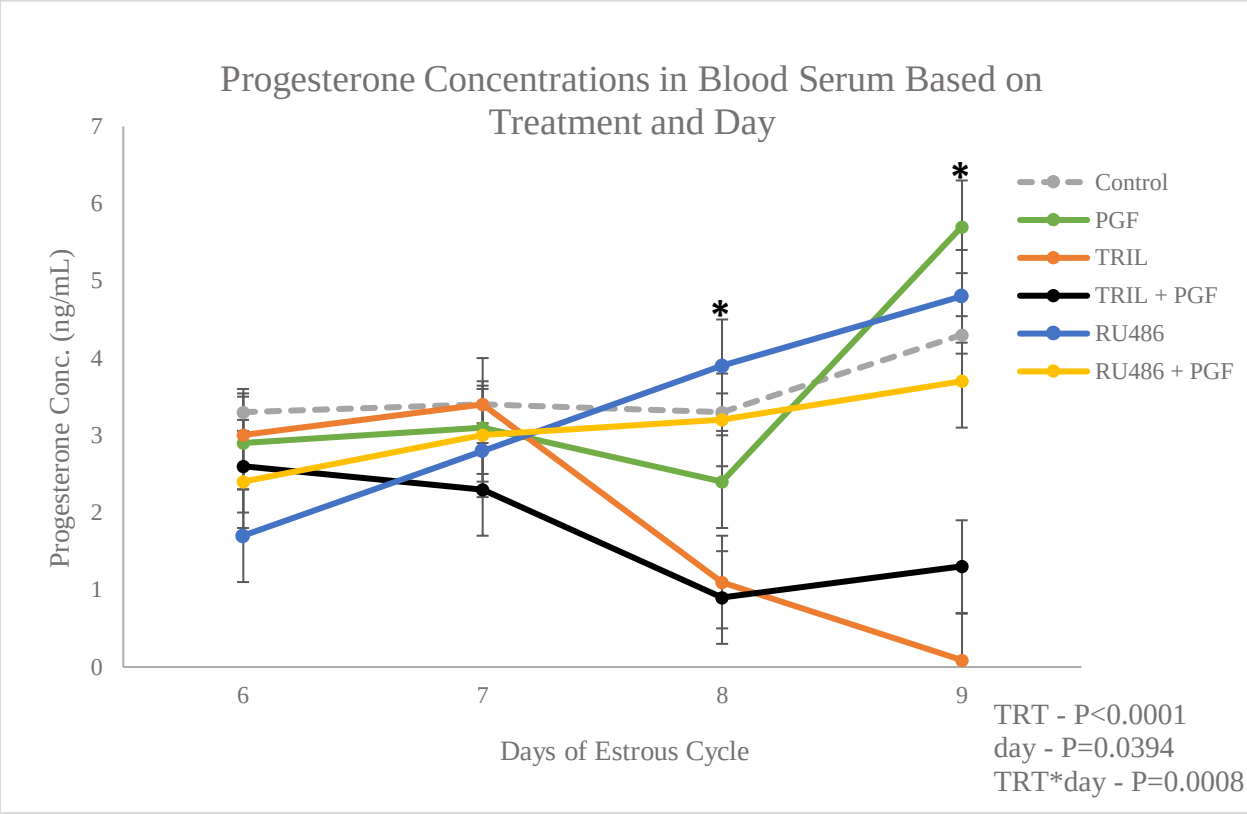

Figure 3.2. Progesterone concentrations (ng/ml) for blood serum between days 6-9 post display of estrus. Treatment groups TRIL and TRIL + PGF had significantly reduced progesterone concentrations compared with other treatment groups (P-value = 0.0008). Treatment effect of these groups were significant that TRIL and TRIL + PGF treatment groups had significantly reduced P4 concentrations (P-value < 0.0001). Day effect of these groups also was also significant (P-value = 0.0394).

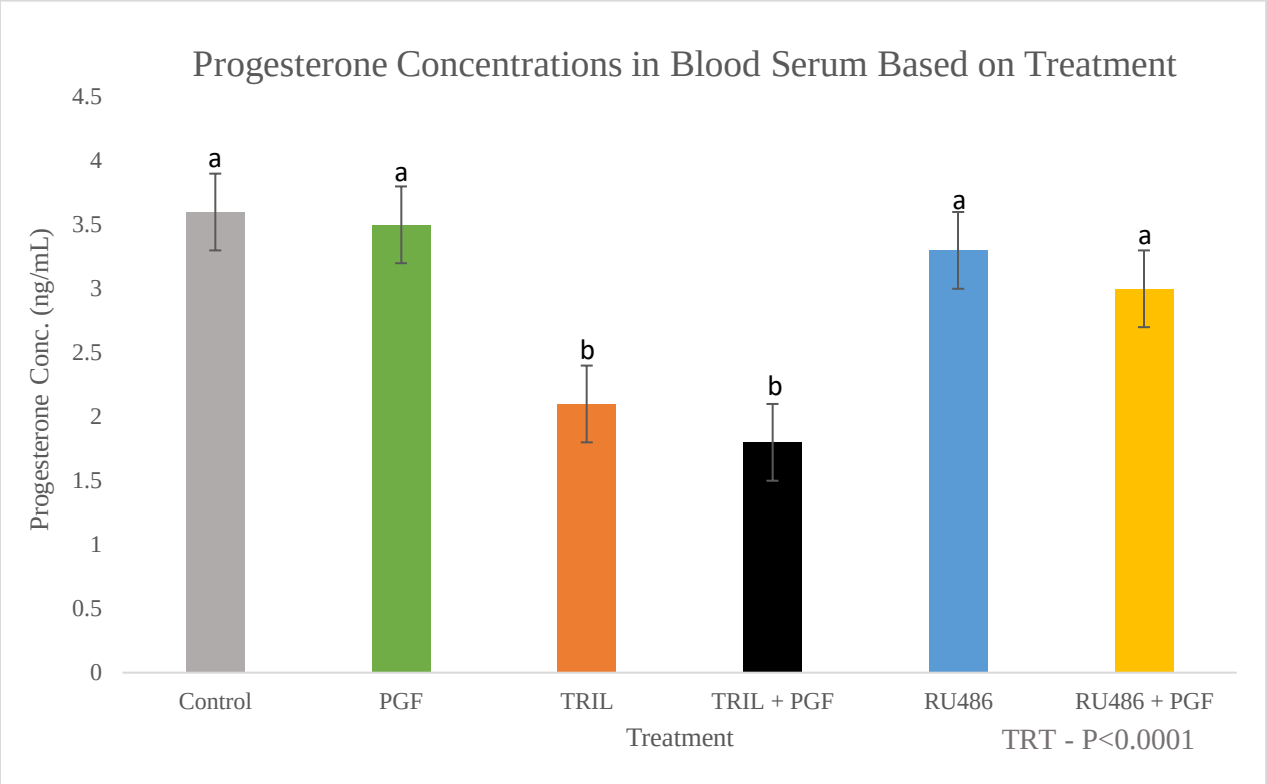

Figure 3.3. Progesterone concentrations (ng/mL) for blood serum based on treatment. Treatment groups TRIL and TRIL + PGF had significantly reduced P4 concentrations compared with the other treatment groups (P-value < 0.0001).

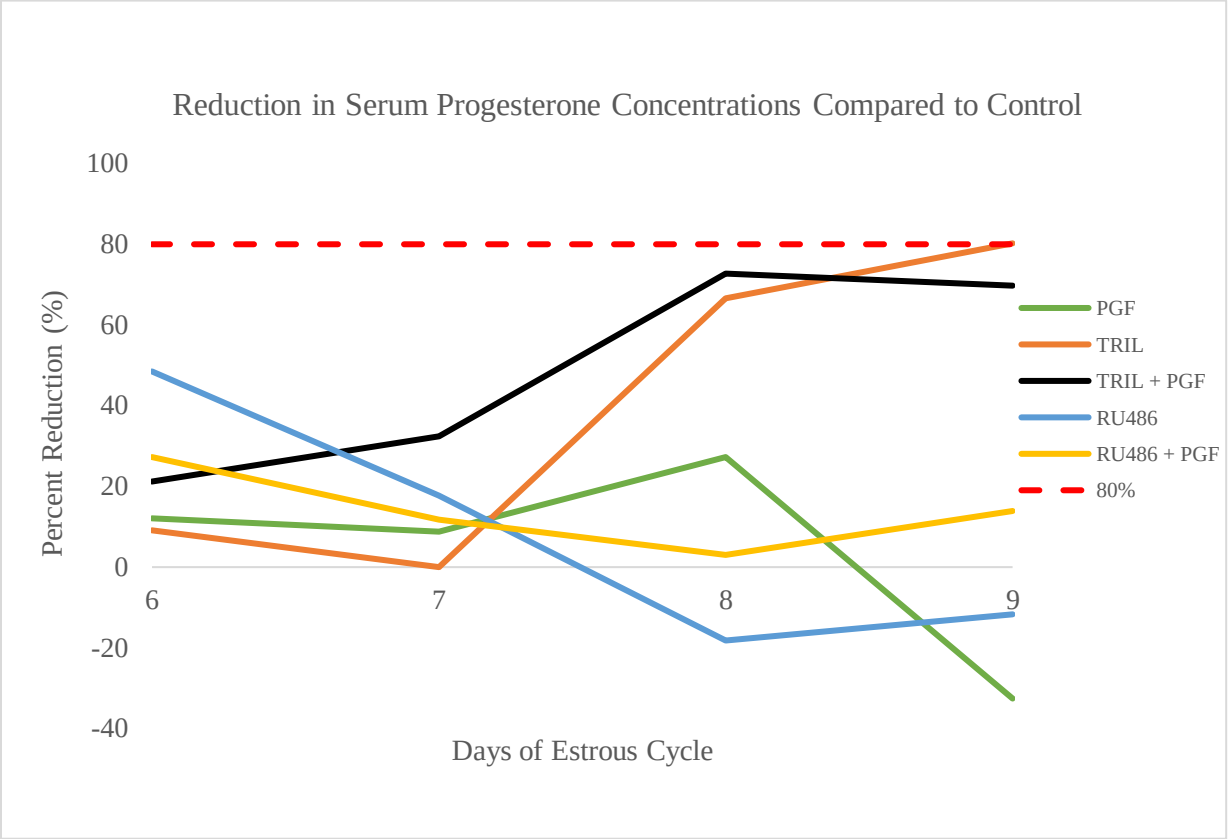

Figure 3.4: Percent reduction in progesterone compared with the control. TRIL treated animals were the only group to reach 80% reduction on day 9.

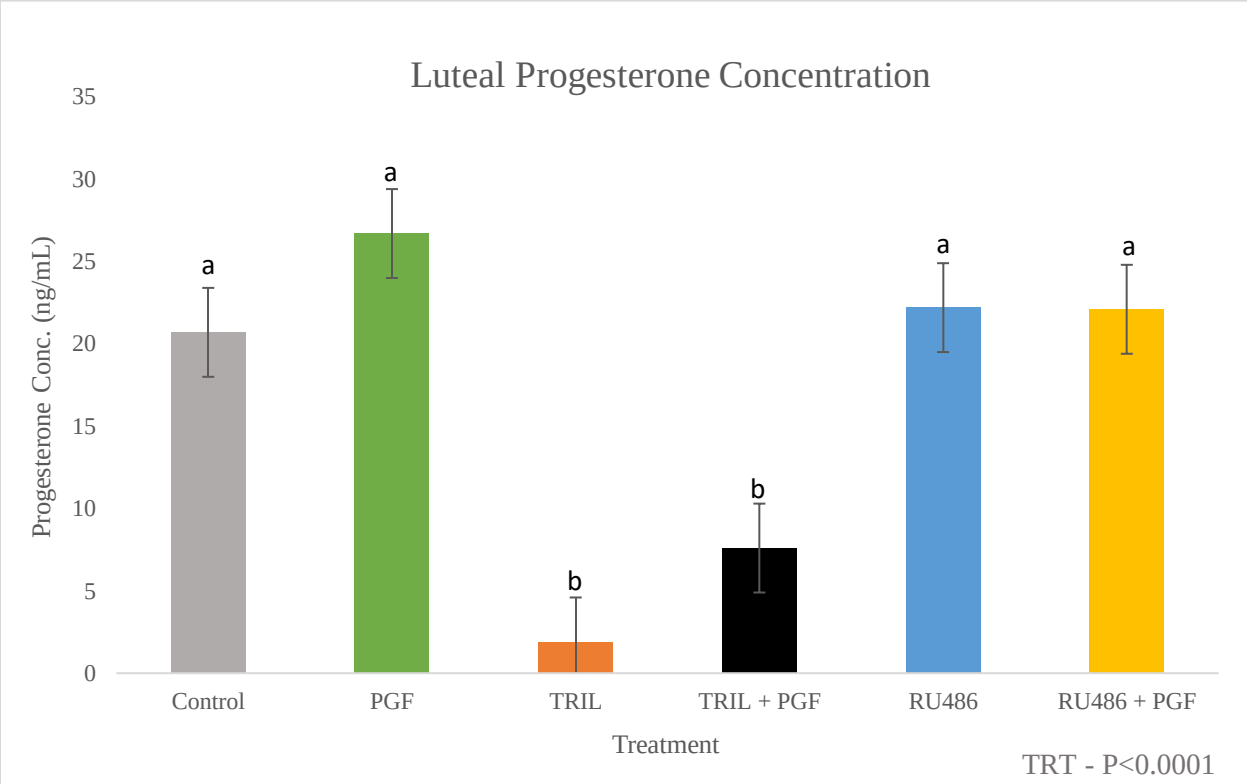

Figure 3.5. Luteal P4 concentrations on day 9. Treatments TRIL and TRIL + PGF significantly reduced P4 concentrations compared with other treatment groups (P-value < 0.0001).

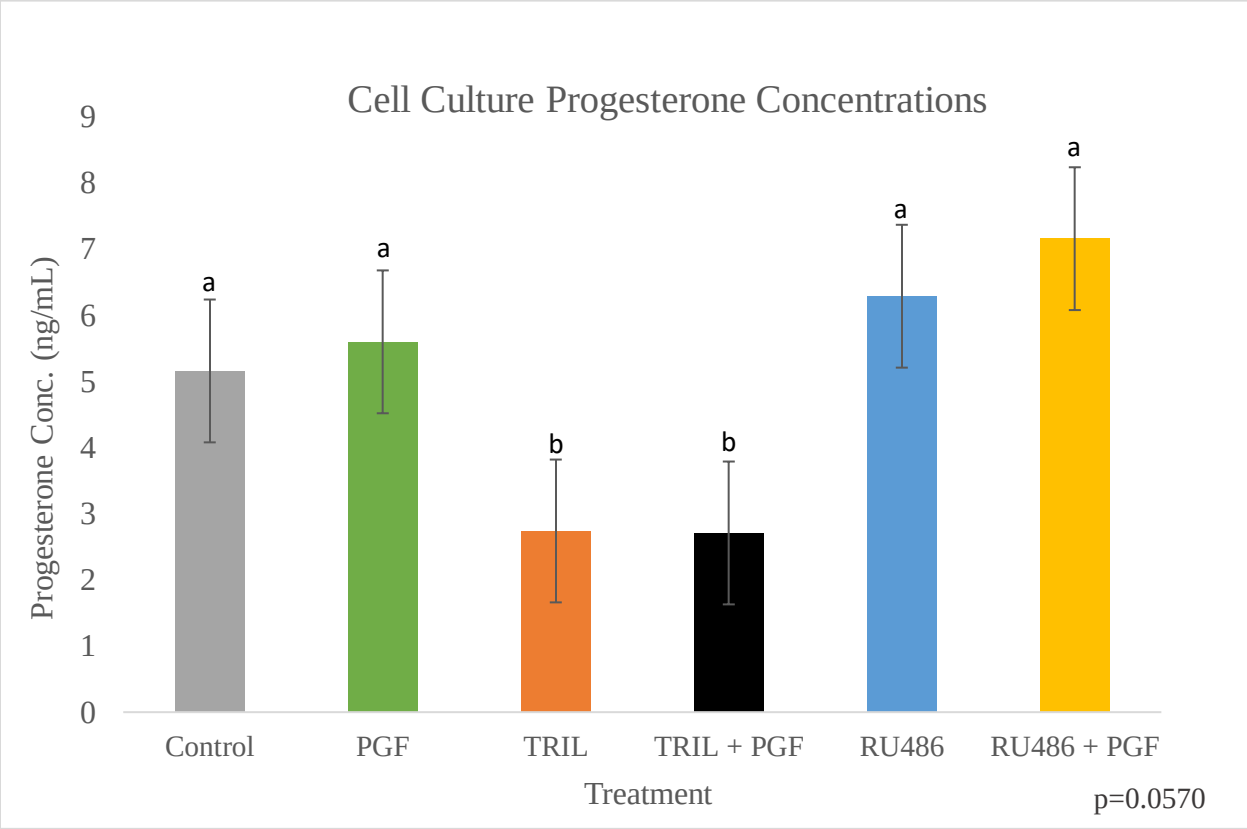

Figure 3.6. Progesterone concentrations from cell culture beginning with 100,000 luteal cells in M199 for 3 hrs at 35ºC and 5% CO_2. (P-value = 0.0570)

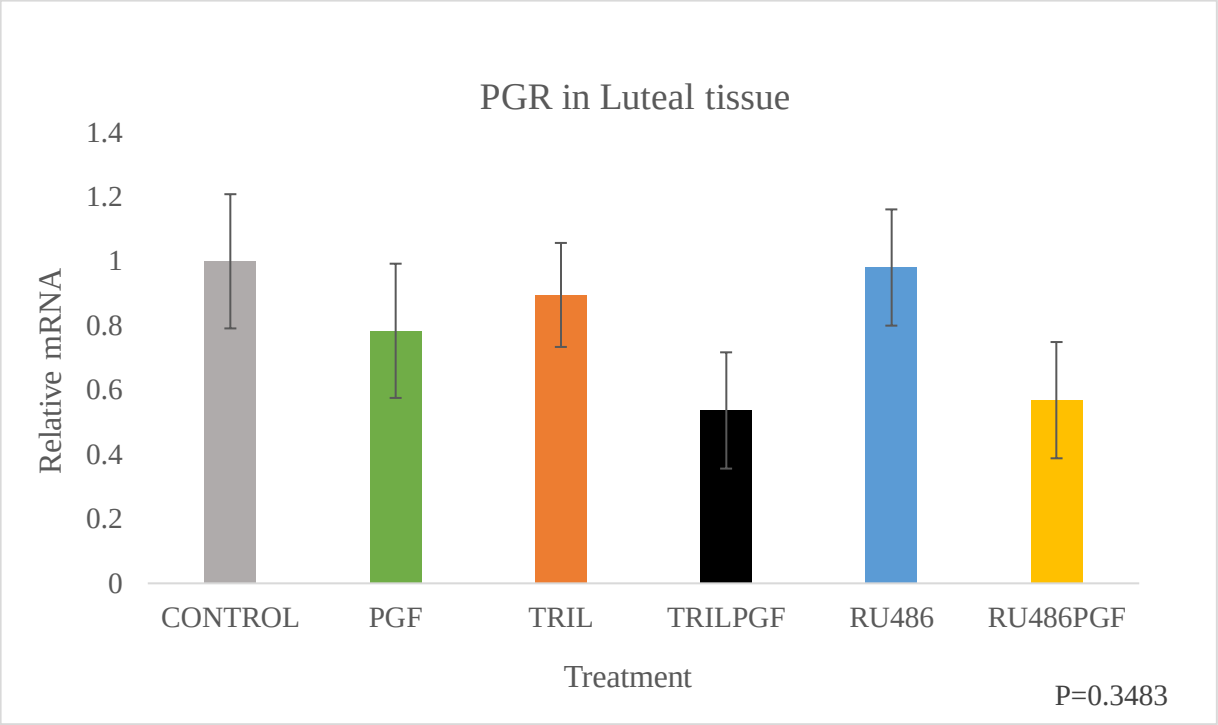

Figure 3.7. Relative mRNA expression of progesterone receptors (PGR) in luteal tissue collected on day 9. No significant differences between control or treatments is observed. P-value = 0.3483.

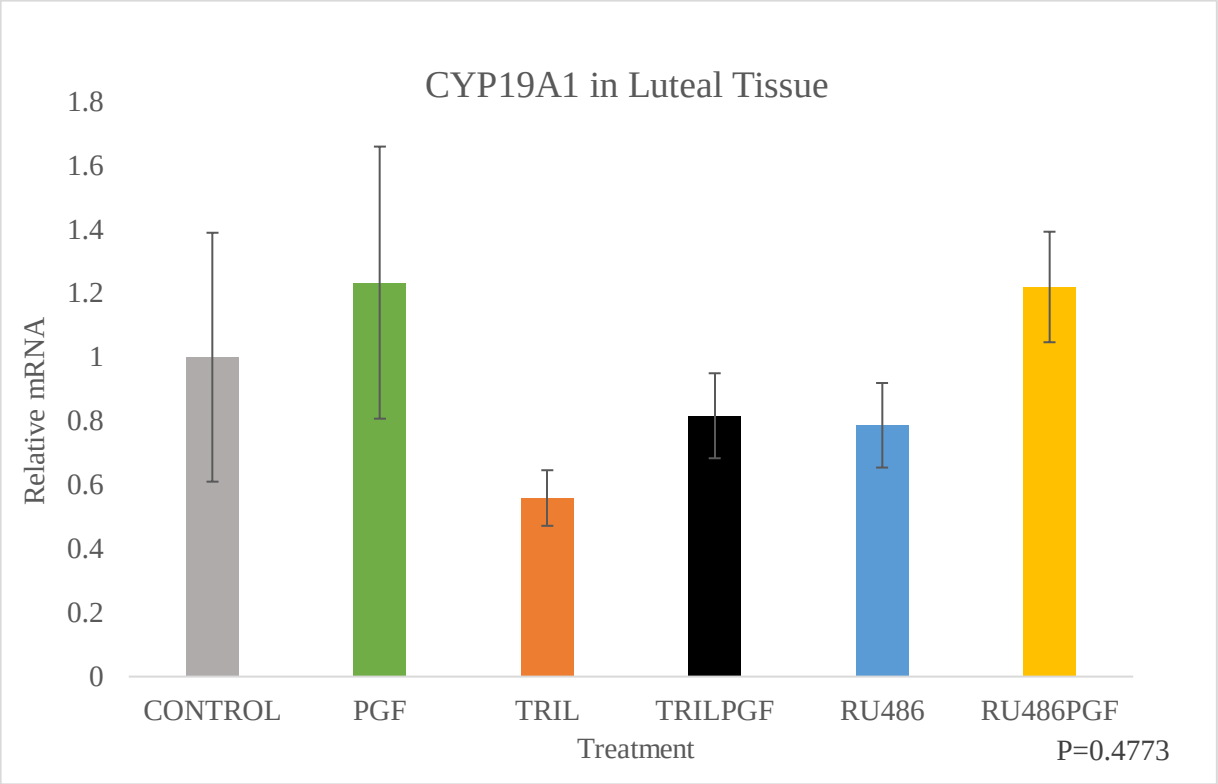

Figure 3.8. Relative mRNA expression of Aromatase (CYP19A1) in luteal tissue collected on day 9. No significant differences between control or treatments is observed. P-value = 0.4773.

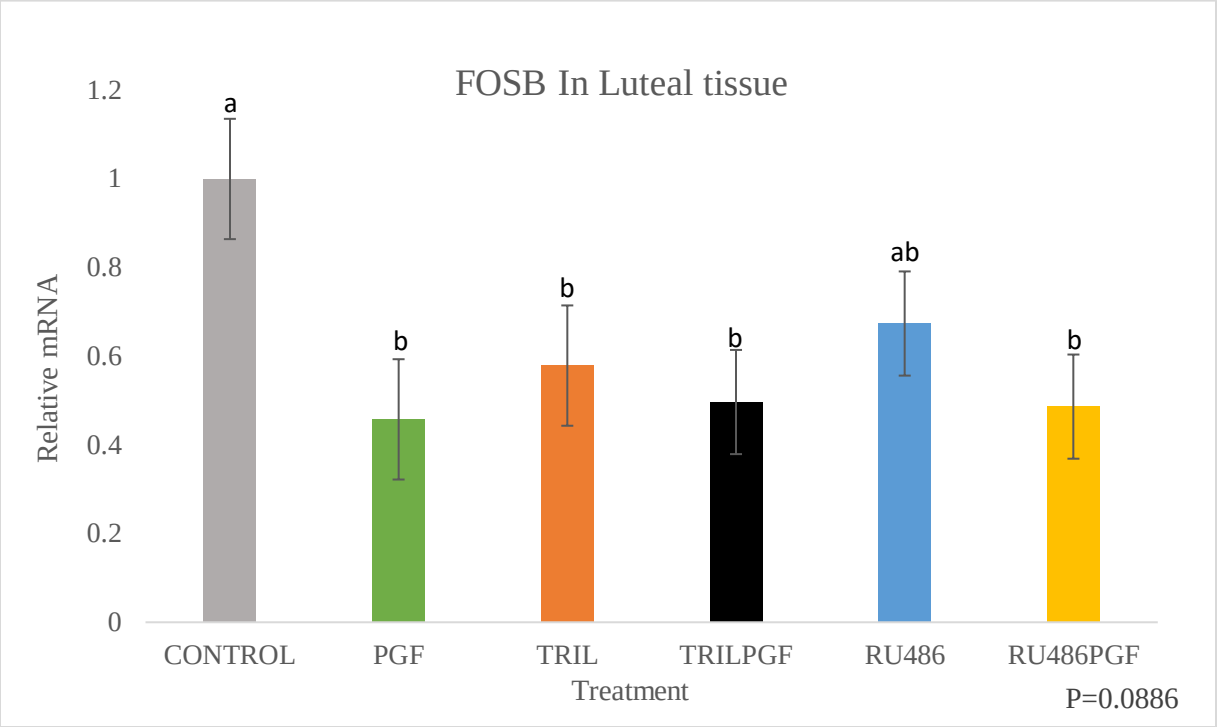

Figure 3.9. Relative mRNA expression of FOSB in luteal tissue collected on day 9. Control pigs had a tendency to have greater FOSB mRNA expression compared with treatment groups PGF, TRIL, TRIL + PGF and RU486 + PGF. Treatment group RU486 had a tendency to be similar to both control and other treatment groups when compared. P-value = 0.0886.

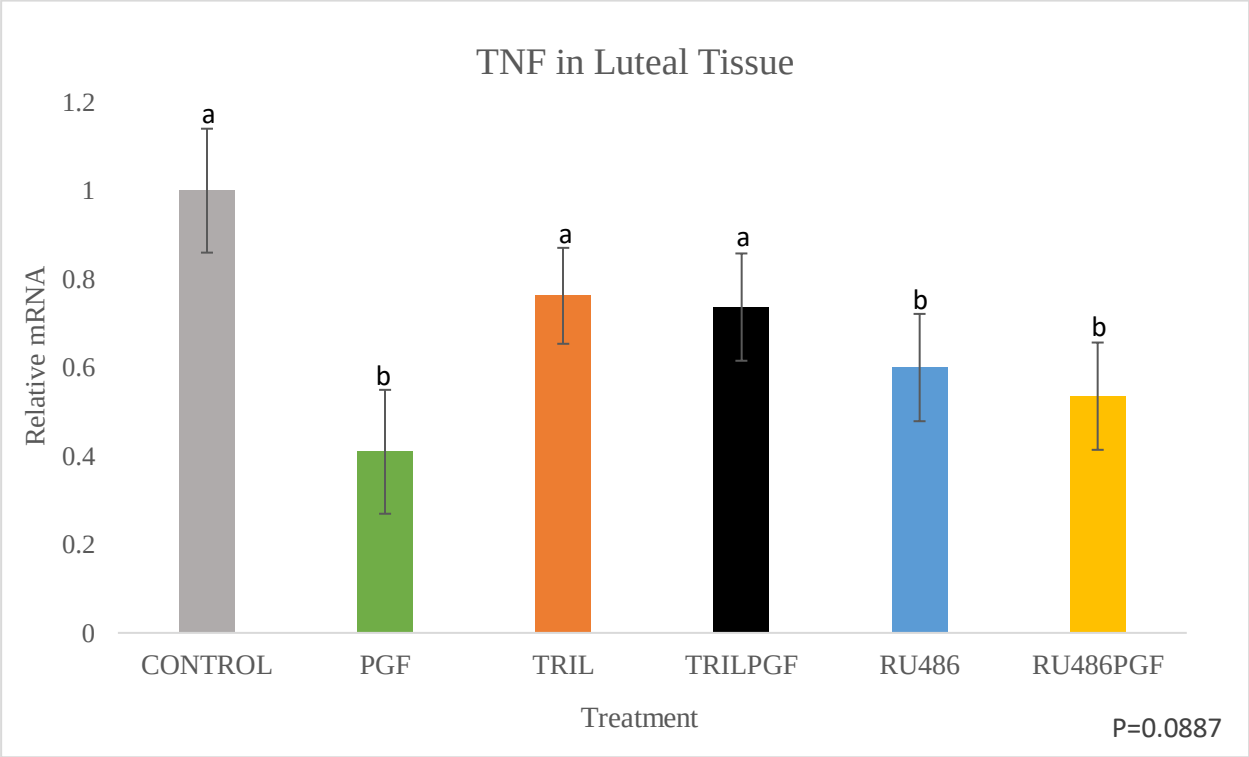

Figure 3.10. qPCR analysis on TNF mRNA expression in luteal tissue extracted from ovariectomized ovaries on day 9. Pigs treated with PGF, RU486 and RU486 + PGF and had a tendency to have lesser TNF mRNA expression. P-value = 0.0887.

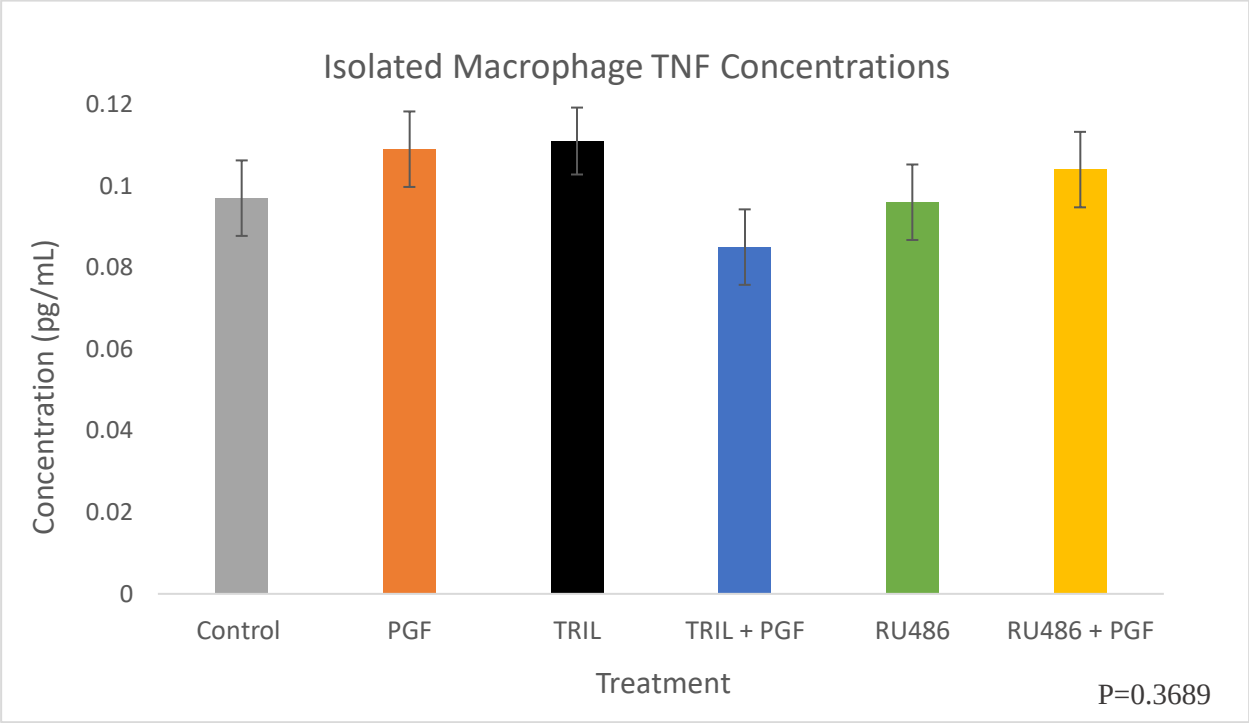

Figure 3.11. Isolated MAC TNF concentrations. No significant difference in concentration between treatments. P-value = 0.3689.

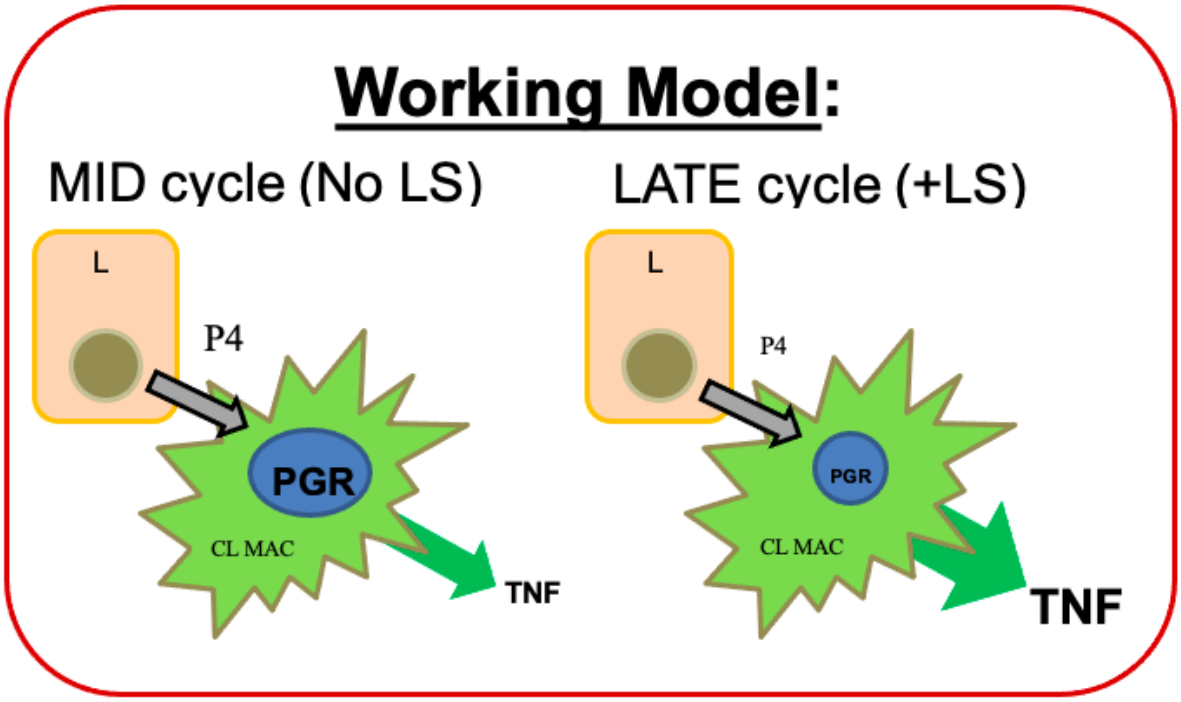

Figure 3.12. Working model explaining the relationship between P4 concentrations, PGR expression and the subsequent TNF mRNA expression for mid cycle (days 8-9, prior to LS) verses late cycle (days 13-14, after LS). Mid cycle CL produce greater concentrations of P4 and have increased PGR mRNA expression, with subsequently reduced expression of TNF. Late cycle CL produce lesser concentrations of P4 and have a decreased expression of PGR mRNA, with greater TNF mRNA expression.

3.9 List of Tables

Table 3.1: Summary of primer sequence for quantitative PCR.

Gene name	Primer	Primer Sequence (5'—3')	Amplicon Length (bp)	Reference
GAPDH	Forward	TCAAGAAGGTGGTGAAGCAG	214	Gadsby et al. (2010)
	Reverse	TTGACGAAGTGGTCGTTGAG		
RPL4	Forward	AGCGAATGAGAGCTGGTAAAG	250	Gadsby et al. (2010)
	Reverse	TTACGCCAAGTGCCATAGAG		
TNF-α	Forward	CTACTGCACTTCGAGGTTATC	114	Gadsby et al. (2010)
	Reverse	GGGCTTATCTGAGGTTTGAG		
PGR	Forward	TTCCACCCAGGGAGTATT	98	Gadsby et al. (2010)
	Reverse	CCCTGGATAGCACTTTATGG		
FOSB	Forward	CCGGGCATGAGTGGCTACAG	129	Diaz et al. (2011)
	Reverse	CGTCTCCTCTCGGGGTCTCCT		
CYP19A1	Forward	TTCCATCACCAAGCACCTGGAC	358	Diaz et al. (2011)
	Reverse	GGCCAAAATCAACTCAGTGGCG		

CHAPTER 4

Interpretive Summary and Future Directions

The focus of these studies was to inhibit progesterone (P4) synthesis by luteal cells, or block progesterone receptors (PGR), and determine the effect of cytokine, tumor necrosis factor-alpha (TNF), secreted by corpora lutea (CL) macrophages (MAC), and its subsequent effect on inducing early luteolysis. The initial study presented in this thesis, outlined in chapter 2, was to determine the correct dose of Trilostane (TRIL), a 3-beta-hydroxysteroid dehydrogenase (3b-HSD) inhibitor, that would reduce P4 concentrations by a minimum of 80%. Results of this preliminary study found the effective dose to be 10mg/kg, similar to results found by Etherton et al. (1989). Challenges to this initial study were that out of the original 12 gilts synchronized on Matrix®, only 9 displayed estrus and were able to continue with treatment. This meant only 2 animals were assigned per treatment, and subsequently, one control. The reduction in P4 could have been skewed if this one control animal had variable P4 concentrations compared to a larger sample size. Nevertheless, the full study was conducted using this dose to effectively reduce P4 concentrations. As described in chapter 3, results of this study confirm TRILs efficacy at reducing P4. In both TRIL and TRIL + PGF treated animals, P4 was significantly suppressed compared with the other treatment groups. Other luteolytic markers were then analyzed to determine if early luteolysis was achieved. Analysis of PGR mRNA should have decreased in expression in treatments with a significant reduction in P4 (Diaz et al 2011), however, results of the present study did not find any significant differences in PGR mRNA expression across treatments. Similarly, previous studies reported an increase in aromatase (CYP19A1) expression in response to PGF2a (Diaz 2011, Gregoraszczuk 1997, Gregoraszczuk 1999), as well as, an increase in mRNA expression of FOSB (Diaz 2011). Results of the present study did not have any significant decreases in CYP19A1 across all treatments. The expression of FOSB mRNA had a tendency to

be lesser in animals treated with PGF, TRIL, TRIL + PGF and RU486 + PGF, contradictory to results from Diaz et al. (2011). These data signify that luteolysis was not successfully induced prematurely as hypothesized it would be. Furthering analysis of MAC production of TNF protein was not significantly different across all treatments groups and controls. Tumor necrosis factor-alpha mRNA expression from luteal tissue did present a tendency to be greater in animals treated with TRIL and TRIL + PGF, the same treatments with significant decreases in P4. If the amount of time between administering treatments and tissue collection was increased, it may give the mRNA a chance to translate into TNF protein in luteal tissue. The review by Pate et al. (2001) states that a reduction in P4 leads to increase MAC secretion of TNF, and while we can deduce the TNF mRNA would have translated into protein, the differences across treatments were not significant. While methodological differences could explain these contradicting results, there are several other factors to consider. The percent of P4 reduction, shown in figure 4.1, shows TRIL treated animals reaching 80% reduced right at day 9, the same day as tissue collection. It could be that this reduction isn't large enough to produce large cellular responses. A study by Diaz et al. (2011) reduced P4 concentrations 85 to 90% in animals treated with a 3b-HSD inhibitor and 3-bHSD inhibitor + PGF, respectively. An 80% reduction in P4 from the present study may not be sufficient enough to render the luteal cells vulnerable enough to the damaging effects of exogenous PGF2a. Alternatively, the time between treatment and tissue collection where this P4 reduction occurs may not be sufficient enough to allow the luteal cells to fully respond to the damaging effects of PGF, and therefore did not undergo luteolysis. Diaz et al (2011)'s study resulted in the epostane treated animals experiencing a 36hr P4 deficiency, whereas gilts from the present full study only experienced 24 hours of reduced P4. This major difference in P4 reduction could be due to the initial study only having one control animal being compared to the TRIL treated animals. If that one control animal was an outlier, 10mg/kg dose of TRIL may not be effective at reducing

P4 by 80% in a larger sample size. Solutions to this should include increasing the TRIL dose to greater than 10mg/kg. Alternatively, methodologies for TRIL administrations should be reconsidered. The TRIL powder was fed orally to the gilts and many of the gilts did not like the taste. Ensuring each gilt received the complete dose was difficult, so converting the mode of delivery to an injectable should be considered. Future directions should aim to reduce P4 to more than 90%. It is also worth considering beginning treatment prior to day 7 or collecting tissue after day 9 so the luteal cells within the CL have more time to undergo cellular change in response to adequate reduction in P4. It is essential to reduce P4 adequately and for a substantial amount of time in order to induce premature luteolysis prior to day 13 of the estrous cycle for subsequent luteal MAC cytokine production analysis.

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4.3: List of Figures

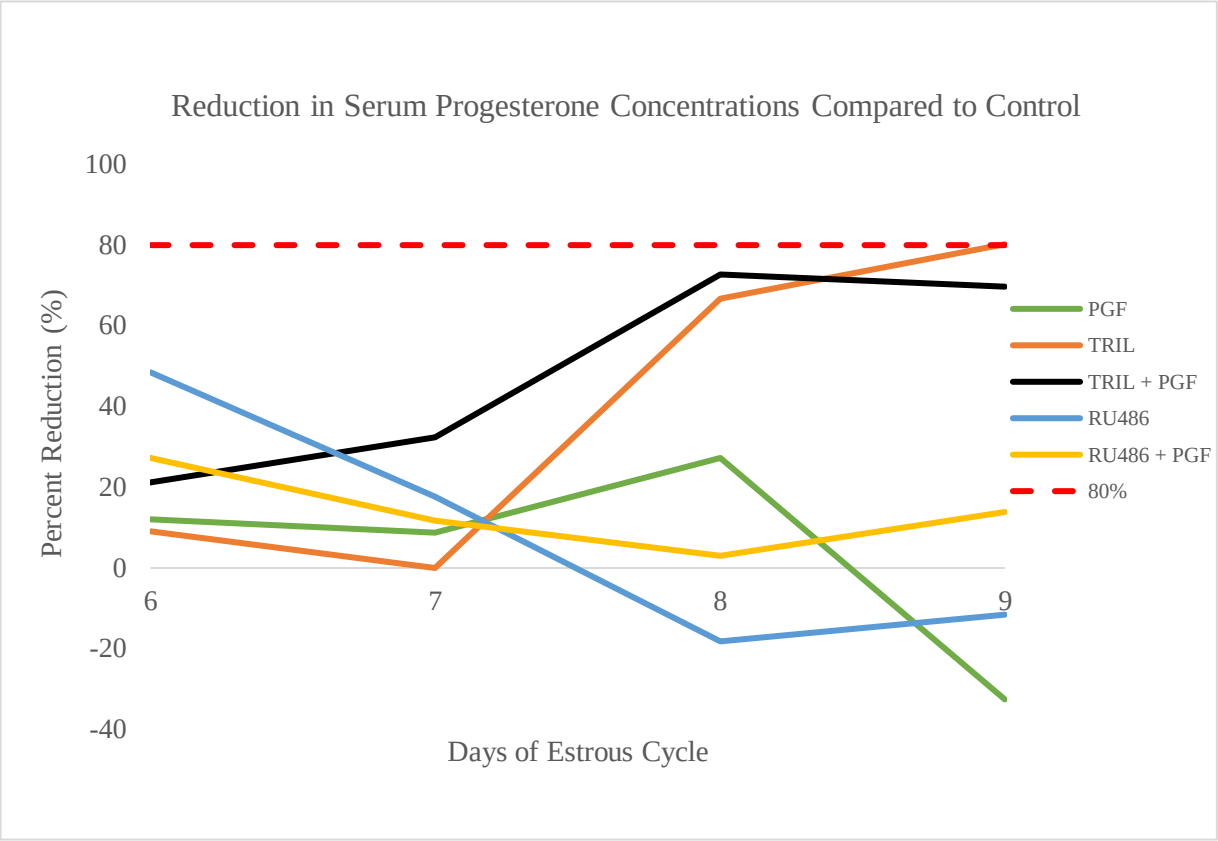

Figure 4.1: Percent reduction in progesterone compared with the control. TRIL treated animals were the only group to reach 80% reduction on day 9.