EFFECTS OF EXOGENOUS PROGESTERONE DURING THE PERI-IMPLANTATION PERIOD OF PREGNANCY ON GROWTH AND DEVELOPMENT OF OVINE CONCEPTUSES

A Thesis

by

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ABSTRACT

This study was conducted to confirm that exogenous P4 administered during the peri-implantation period (Days 1.5 to 8) advances conceptus growth, development, and implantation to Day 12 of gestation. This study determined how exogenous P4 affects enzymes involved in the classical (ornithine decarboxylase 1) and novel pathways (arginine decarboxylase and agmatinase) of conversion of arginine to polyamines. Additionally, expression of mRNAs of nutrient transporters for glucose, arginine, and glycine, as well as progestamedins were analyzed. Suffolk ewes (n=40) were assigned randomly to receive daily intramuscular injections of either 25 mg P4 in 1 ml corn oil (P4, n=18) or 1 ml corn oil alone (CO, n=20 from Day 1.5 through Day 8 of pregnancy. Nine P4-treated ewes and 10 CO-treated ewes were hysterectomized on Day 9 of pregnancy and 9 P4-treated ewes and 10 CO-treated ewes hysterectomized on Day 12 of pregnancy. Conceptuses from D12 P4 ewes were elongated, while all D12 CO conceptuses remained spherical at the time of necropsy. CO-treated ewes necropsied on Day 9 of pregnancy had 3-fold greater expression of ODC1 mRNA in comparison to P4-treated ewes necropsied on either Day 9 or Day 12 of pregnancy, and CO-treated ewes necropsied on Day 12 of pregnancy ($P < 0.01$). Furthermore endometrial expression of FGF7 mRNA increased by approximately 1.5-fold in P4 D9 ewes compared to CO-treated ewes of the same gestation day and D12 ewes in both treatments ($P < 0.05$) and expression of FGF10 mRNA for CO- and P4-treated ewes necropsied on Day 12 of pregnancy was about 2.5-fold greater than for CO-treated D9 ewes ($P <0.01$). Finally, P4-treated ewes necropsied on Day 12 of pregnancy had an approximately 3.5-fold greater expression of glycine transporter (SLC6A9) than D12 CO-treated ewes, D9 P4-treated ewes, or D9 CO-treated ewes ($P < 0.01$).

DEDICATION

To Harless Benthul (Daddad) (1935-2018)

Fightin' Texas Aggie Class of 1957

Here.

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INTRODUCTION AND LITERATURE REVIEW

Introduction

Mammalian implantation is a complex phenomenon involving the temporal and spatial-specific up-regulation or down-regulation of expression of genes encoding for proteins required for the conceptus (embryo and associated membranes) to make contact with the maternal uterine luminal epithelium (LE) in order to begin the process of placentation and vascularization for nutrient exchange. Unfortunately, this intricate process fails often, leading to pregnancy loss in 67% of conceptions during the peri-implantation period for all mammalian species studied [1]. Previous studies in sheep have shown that administering exogenous progesterone, the hormone required to maintain pregnancy, can accelerate molecular mechanisms of implantation and the growth and development of the conceptus by advancing down-regulation of progesterone receptors and up-regulating genes essential for conceptus survival. Further studies of these mechanisms may provide insight into the cellular communication required for successful implantation, leading to potential therapies to decrease embryonic losses in livestock and therefore, more efficient animal production systems. Furthermore, women undergoing assisted reproductive technologies such as *in vitro* fertilization are administered large amounts of steroid treatments in order to hyper-stimulate the ovary to produce more ovulations. Embryos that result as products of this hyper-stimulation often fail to implant in the mother's uterus. The sheep serves as a valuable model for studying the interactions of exogenous steroids with implantation mechanisms, and could potentially contribute novel protocols for use in fertility clinics to reduce the occurrences of implantation failure.

Progesterone and Maternal Recognition of Pregnancy

Progesterone is known as the hormone of pregnancy and its requirement to establish and maintain pregnancy is conserved across all mammalian species studied to date. In the sheep, theca cells and granulosa cells of the dominant follicle form small and large luteal cells of the corpus luteum, respectively, [2]and begin to produce progesterone on approximately Day 3 of pregnancy [3]. Progesterone production by the CL then steadily increases until Day 7 when it reaches maximum concentrations of approximately 4 ng/mL in the maternal plasma [4]. Beginning on Day 60 of pregnancy, ewes can be ovariectomized, but still sustain pregnancy as the placental chorion produces sufficient amounts of progesterone by this time [5].

In order to prevent luteolysis, the structural and functional destruction of the CL, molecular signaling from the conceptus (embryo and associated fetal membranes) must communicate its presence to the mother. In ruminant species, the maternal recognition of pregnancy signal is interferon tau (IFNT) [6-10]. Interferon tau is secreted by the mononuclear trophectoderm cells between Days 10 and 21 of pregnancy and abrogates the luteolytic mechanism responsible for pulsatile release of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) by uterine epithelia. Interferon tau induces expression of interferon regulatory factor 2 (IRF2) which is a transcriptional repressor of estrogen receptors (ESR1) and that prevents the biological actions of estradiol (E2). Without the interaction of E2 ligands with ESR1, oxytocin receptors (OXTR) are not expressed in uterine epithelia to bind oxytocin released from the posterior pituitary and induce luteolytic pulses of $PGF_{2\alpha}$ from uterine epithelial cells [11].

Uterine Histoarchitecture

In order to comprehend the temporal and cell-specific events of implantation, it is important to have a thorough understanding of uterine histology. The uterine histoarchitecture is composed of a myriad of cells with differing functions dependent on temporal or local classification (Figure 1.1). The layers of the uterus consist of the endometrium (mucosa and submucosa), myometrium (muscularis), and perimetrium (serosa). Three categories of epithelial cells exist in the endometrium: luminal epithelia (LE), superficial glandular epithelia (sGE), and deep glandular epithelia. Beneath the uterine epithelia lies the stroma, also known as the lamina propria, which is collagenous connective tissue that houses uterine glands, vasculature, and lymphatics. In the ruminant, the stromal cell-dense lamina propria is called the stratum compactum while the diffuse area of stromal cells is the stratum spongiosum. Additionally, the myometrium is composed of two layers, an inner circular and an outer longitudinal layer of smooth muscle important for uterine contractions prefacing sperm transport, fertilization, and parturition.

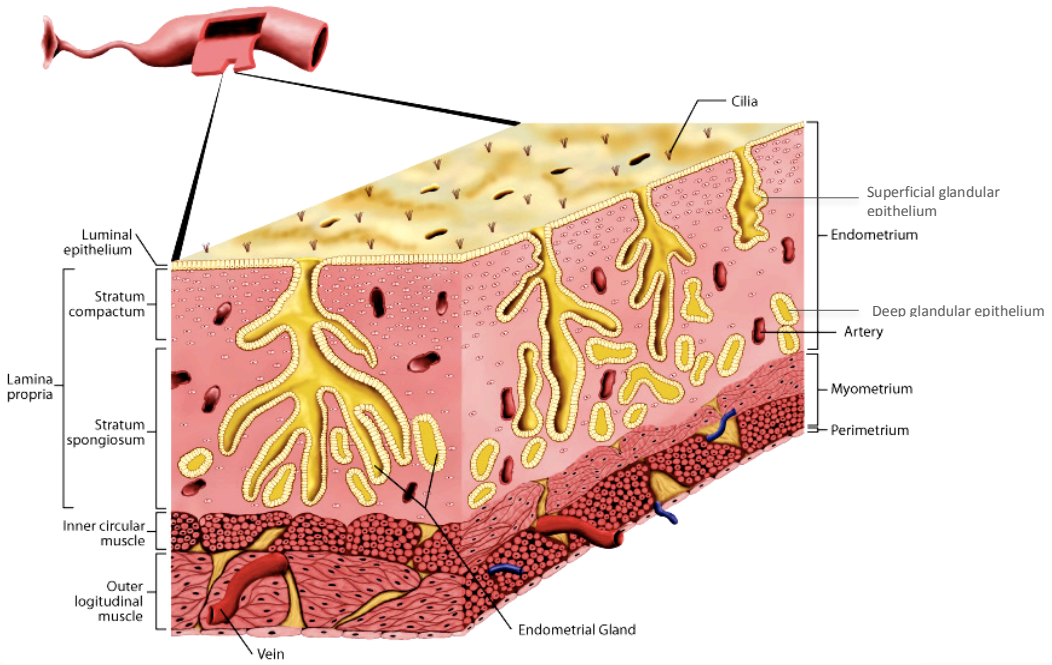


Fig 1.1. Histoarchitecture of the ovine uterus. Figure reprinted with permission and has been modified from Geisert and Bazer (2015) [12].

Implantation of blastocysts in sheep

After ovulation, the oocyte is released from the dominant follicle into the fimbriae of the infundibulum and then travels through the oviduct where it is fertilized at the ampullary-isthmic junction. The embryo then undergoes rapid cell division to enter the uterus as a morula on Day 4 after breeding. By Day 6, the uniform cells of the morula differentiate into the inner cell mass (ICM, precursor to the fetal tissue) and trophectoderm (Tr) cells (precursor to placental tissue) [13]. For all mammalian species studied, the process of implantation of blastocysts can be segregated into four phases: 1) hatching from the zona pellucida 2) pre-contact and orientation of blastocyst to uterine LE; 3) apposition to uterine LE and 4) adhesion to uterine LE [14]. The blastocyst expands and hatches out of the protective glycoprotein membrane called the zona pellucida between Days 8 and 9 of pregnancy. The ovine, porcine, bovine, and murine blastocysts directionally orient the ICM to face away and the Tr cells toward the uterine LE.

Ovine, bovine, and porcine blastocysts undergo elongation as the spherical blastocyst becomes tubular and then a filamentous, floss-like structure predominantly composed of trophectoderm. This phenomenon begins on approximately Day 10 in sheep, as the blastocyst progresses from approximately 200 μm in diameter on Day 8 to between 400 and 900 μm by Day 10, 10-22 mm by Day 12, and 25 cm by Day 17 of pregnancy [13, 15].

Interestingly, although successful pregnancies require constant endogenous P4 production, attachment of the conceptus (embryo and associated placental membranes) to the uterine (LE) for implantation requires a paradoxical down-regulation of progesterone receptors (PGR) in the uterine LE, sGE, and GE. PGR are present in uterine LE between Days 9 and 11, but expression decreases or is absent in those cells after Day 12 of gestation [16]. This down-regulation of the PGR correlates with changes in gene expression essential for implantation including a decrease in anti-adhesive mucin proteins (MUC-1) in the uterine LE allowing for exposure of adhesion proteins for contact and adhesion of the trophectoderm of the elongating blastocyst to the uterine LE [17, 18]. Apposition in the sheep blastocyst consists of transient interdigitaton of microvilli found on the apical surface of uterine LE and sGE and cytoplasmic projections of trophoblast cells [14, 19]. Additionally, ruminant trophoblast cells form finger-like papillae that dip down into the uterine glands of the endometrium between Days 15 and 18 of pregnancy to immobilize the conceptus and facilitate adhesion [14, 20].

MUC-1 undergoes a sharp decline in expression in uterine LE following down-regulation of PGR on Day 12 of pregnancy and this is necessary for adhesion of blastocysts and uterine LE between Days 16 and 18 of gestation [14]. Mononuclear trophoblast cells differentiate into binucleate giant cells (BNC) by Day 16 of pregnancy and they fuse with uterine LE cells to form multi-nucleated syncytial plaques [21, 22]. These syncytial plaques will displace the uterine LE

in aglandular areas of the endometrium, known as caruncles, in order to eventually form placentomes. Accordingly, placentation of the sheep is classified as synepitheliochorial, as inter-caruncular areas do not form a syncytium and have epitheliochorial placentation, while placentomal regions are syncytialized in order to allow for efficient maternal-fetal nutrient transport. It should be noted that previous description of ovine placentation is now controversial due to novel immunofluorescence work depicting Tr BNC cells alone forming multinucleated syncytial plaques. (H. Seo, unpublished results).

An abundance of adhesive proteins are responsible for binding the trophectoderm to the uterine LE. However, integrins are the primary adhesive glycoprotein receptor involved in the implantation cascade. Integrins are a family of transmembrane heterodimers that bind extracellular matric (ECM) proteins such as osteopontin (also known as secreted phosphoprotein 1, SPP1) [23] and intracellularly to adaptor and cytoskeletal proteins allowing them to form strong adhesions during implantation [24, 25]. Integrin trafficking is stimulated not only by internal genetic programing of the blastocyst itself, but also by external signals from the uterine LE. Indeed, results of in vitro studies demonstrated that there is a 2- to 3-day delay in embryonic development in *in vitro* compared to that for *in vivo* experiments [26]. Adhesion of conceptus Tr and uterine LE in the ewe is completed by approximately Days 21 to 23 [14, 15, 27].

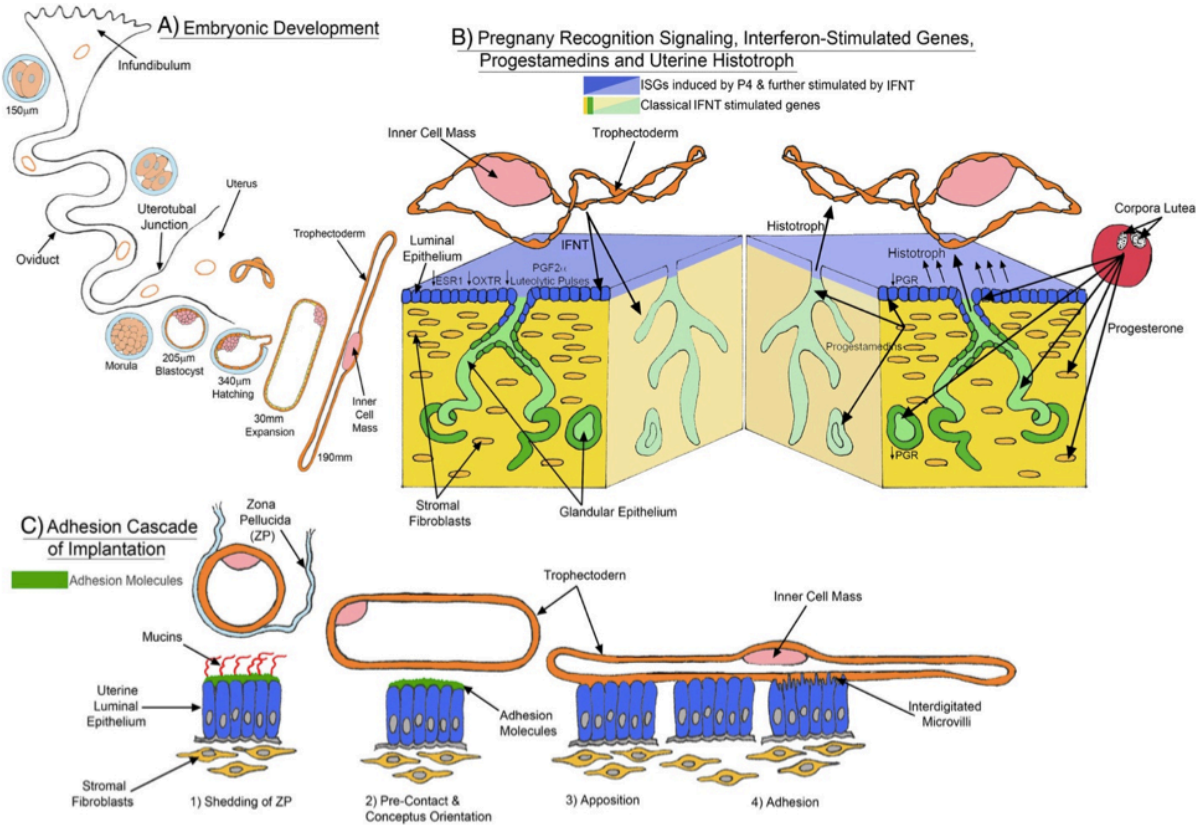


Fig 1.2. Structural, molecular, and temporal changes during the ovine pre- and peri-implantation period. A) Ovine conceptuses undergo rapid elongation from a 200 μm spherical blastocyst on Day 8 to a 25 cm elongated conceptus by Day 17. B) IFNT, the maternal recognition of pregnancy protein, and progesterone, produced by the CL, stimulate expression of genes conducive to pregnancy in addition to abrogating the luteolytic mechanism. C) After hatching from the zona pellucida, the blastocyst is able to expand, form a conceptus, and implant due to down-regulation of anti-adhesive mucins which exposes adhesive proteins. Image reprinted under the terms of the Creative Commons Attribution License from Bazer et al., 2012 [28].

The Importance of Histotroph

Down-regulation of PGR in the uterine LE and sGE presents a biological conundrum, as the mechanistic actions of progesterone binding to its receptors on uterine epithelial cells, in addition to the signaling to those epithelial cells by IFNT, must stimulate the expression of genes associated with transport of proteins and nutrients, cytokines, enzymes, adhesion proteins, and other molecules that accumulate in the uterine lumen to form histotroph. Histotroph is essential for supporting the growth and survival of the conceptus throughout pregnancy, but it is

particularly important during the peri-implantation period of pregnancy [29, 30]. The physiological demand for histotroph has been studied utilizing the uterine-gland-knockout ewe model (UGKO), in which neonatal lambs were exposed to progesterone for the first 56 days of their life to disrupt normal adenogenesis and prevent development of uterine glands. Adult UGKO ewes are unable to experience normal estrous cycles or support development of their conceptuses beyond Day 14 of pregnancy. That is due to the absence of uterine glands and their secretions resulting in pregnancy failure, and not an inability of uterus to respond to IFNT [31-33]. Additionally, while histotroph is vital for the duration of pregnancy for livestock species including sheep and pigs [34-37], uterine adenogenesis in calves can be disrupted when they are given a restricted diet [38], or exposed to a progestin [37], and uterine glands may be essential during early- to mid- pregnancy in primates [39, 40]. The complex composition of histotroph has been thoroughly studied, but novel molecules secreted from the uterine glands that play significant roles in conceptus growth and survival in addition to implantation continue to be discovered.

In order for histotroph to be transported from the uterine glands into the uterine lumen, specific transport mechanisms are utilized. The following are several of the specific transport proteins investigated in this study.

Solute Carrier Family 2 Member 1 (SLC2A1)

SLC2A1 is the gene that encodes for a facilitated glucose transporter. Glucose is metabolized via glycolysis rather than oxidative phosphorylation by the blastocyst at the time of implantation [41]. Concentrations of glucose in the uterine lumen of pregnant ewes increases six-fold between Days 10 and 15 of pregnancy, and SLC2A1 mRNA is expressed by uterine LE, sGE, and the conceptus trophectoderm and endoderm between Days 14 and 20 of pregnancy.

SLC2A1 protein was more abundant in uterine LE and sGE in pregnant ewes than in cyclic ewes [42, 43]. Furthermore, there were increased amounts of total glucose in the uterine flushings of ewes treated with exogenous progesterone during the peri-implantation period of pregnancy [44].

Solute Carrier Family 5 Member 1 (SLC5A1)

The gene SLC5A1 encodes for the sodium dependent glucose transporter. Expression of SLC5A1 mRNA increases between Days 9 and 16 of pregnancy in uterine LE, GE, and sGE between Days 12 and 14 of gestation, but it is localized only to uterine GE between Days 16 and 20 of pregnancy. SLC5A1 protein is abundant on the apical surfaces of uterine LE between Days 12 and 14 of pregnancy indicating its importance for transport during the peri-implantation period of pregnancy, and expression increased in the uterine LE and sGE in ewes treated with exogenous progesterone in early pregnancy [43-45].

Solute Carrier Family 7 Member 1 (SLC7A1)

SLC7A1 is a gene for the System y^+ high affinity cationic amino acid transporter. A cationic amino acid of particular importance for implantation and pregnancy is arginine. Arginine is not only a precursor for the synthesis of proteins, but is also involved in the pathways for angiogenesis as a substrate for production of nitric oxide (vasodilation), and synthesis of polyamines [46, 47], which will be discussed at length later in this review. Additionally, arginine plays a vital role as a nutritional requirement during gestation, without which intrauterine growth restriction and altered genome expression of the fetus occurs [48]. Ewes fed a diet to meet 50% NRC requirements between Days 28 and 78 of pregnancy underwent a 25% decrease in concentrations of arginine and its precursor citrulline in plasma, in addition to polyamines in allantoic fluid and both fetal and maternal plasma. Wu et al., (2004) hypothesized that a deficiency in arginine diminishes the production of nitric oxide, leading to a decrease in maternal

blood transport to the conceptus. [48]. In pregnant ewes, total amounts of arginine and histidine in uterine flushings increase 8- to 25-fold between Days 10 and 16 of pregnancy [42, 49]. Treatment with exogenous progesterone during the peri-implantation period of pregnancy increases amounts of arginine recovered from the uterine lumen on Day 9 of pregnancy [44]. SLC7A1 mRNA is most highly expressed in the uterine LE and sGE between Days 16 and 20 of pregnancy, and in conceptus trophectoderm and endoderm [50].

Solute Carrier Family 6 Member 9 (SLC6A9) and Solute Carrier Family 1 Member 4 (SLC1A4)

The gene SLC6A9 encodes for the sodium and chloride dependent glycine transporter protein while SLC1A4 encodes for an alanine/serine/cysteine/threonine transporter protein. The most abundant amino acid found in uterine flushings from pregnant ewes is glycine, while the second most abundant amino acid is serine [42]. Glycine and serine are interconverted in most animal tissues by serine hydroxymethyltransferase. Serine is the precursor amino acid for glycine which plays important roles in metabolism and protein synthesis, Ca^{2+} modulation, cytokine mediation in the immune system, and serve as a central nervous system neurotransmitter [51]. Expression of SLC6A9 mRNA in the jejunum of piglets after supplementing their diet with glycine [52], and transport of radiolabeled serine and glycine into ovine fetal hepatocytes has been reported [53]. There are no reports of expression of mRNAs for glycine or serine transporters by cells within the uterus of any mammals during the peri-implantation period of pregnancy.

Fructose in Early Pregnancy of the Sheep

The most abundant hexose carbohydrate in conceptuses of ungulate and cetacean species is fructose. Fructose is unable to cross the placenta and enter the maternal circulation; therefore, it is hypothesized that while glucose serves as the main hexose sugar for metabolism by the

fetus, fructose serves that purpose for the placenta. Glucose can be metablized via either the glycolytic pathway, serinogenesis/one-carbon metabolism, the pentose phosphate pathway, or the hexosamine biosynthesis pathway. In contrast, fructose may only be metabolized via the serinogenesis and hexosamine biosynthesis pathways. While past studies have examined concentrations of fructose in fetal plasma and allantoic and amniotic fluids during later pregnancy [54, 55], recent research by Wang et al. [56] demonstrated that fructose plays an essential role in stimulating the proliferation of trophectoderm cells from Day 15 ovine conceptuses by activating the PI3K (phosphatidylinositol 3-kinase) and Akt (Protein Kinase B)-tuberous sclerosis complex 2 (TSC2)-mechanistic target of rapamycin (MTOR) cell signaling pathway via the hexosamine biosynthesis pathway.

Progestamedins

Progesterone receptors are not down-regulated in the uterine stromal cells (SC); therefore, P4 must act through a different mode of action in order to stimulate gene expression associated with the production and secretion of histotroph from uterine epithelial cells. There are two primary theories as to how this phenomenon occurs. First, it is possible that P4 acting via PGR inhibits the IFNT- and P4-induced gene expression by uterine epithelia [57]. Second, P4 may act on uterine epithelia via specialized growth factors, particularly fibroblast growth factor 7 (FGF7), fibroblast growth factor 10 (FGF10), and hepatocyte growth factor (HGF) that are collectively termed progestamedins. These growth factors, secreted by uterine stromal cells in response to P4 binding to stromal PGR, act in a paracrine fashion by binding to receptors on the uterine epithelial cells and stimulating secretion of histotroph and expression of transporters for nutrients [17, 58-60]. In sheep, FGF10 mRNA is expressed abundantly by ovine endometrial

stromal cells, in addition to mesenchymal cells of the chorioallantois, while FGF7 is localized to endometrial and myometrial blood vessels [17]. Expression of mRNA for the high affinity receptor for both FGF7 and FGF10, FGFR2IIIb, was limited to uterine LE, GE, and trophectoderm of the conceptus [17]. While FGF10 is an established progestamedin in the primate endometrium and has been localized to human spiral arteries and stroma [61], FGF7 is not expressed in the ovine stroma directly beneath uterine LE and sGE and was expressed to a much lesser extent than FGF10, suggesting that FGF7 functions independently from FGF10 [17]. HGF is produced by fibroblasts and functions to mediate mesenchymal-epithelial cross-talk [62, 63]. HGF mRNA is localized to ovine uterine stromal cells, and chorioallantois of the conceptus, while mRNA for its receptor, c-*met*, was expressed only by uterine LE and GE and trophectoderm cells [60]. Based on these findings, it is probable that FGF10 and HGF serve as paracrine mediators between uterine stromal cells and uterine LE, sGE, and ovine conceptus trophectoderm to stimulate changes in the genome leading to the production and release of histotroph. Additionally, specific progestamedins and their receptors are up-regulated by exogenous P4 treatment. Expression of mRNAs for FGF10 and the receptor for HGF, c-*met,* increased between Day 9 and Day 12 of pregnancy, while expression of mRNAs for FGF7 and HGF in uterine tissue was unaffected [59].

Synthesis of Polyamines

Polyamines play an instrumental role in mammalian embryogenesis, implantation, and placentation [64-66]. Polyamines (putrescine, spermidine, and spermine) are essential for many cellular functions including proliferation [67], gene transcription and translation [68, 69], and angiogenesis, [70], and are known to reduce reactive oxygen species [71]. Polyamines are

derived from arginine, a highly abundant amino acid within tissues and fluids of uterus, fetal fluids, and placenta during the peri-implantation and placentation periods of pregnancy [42, 48] via the enzymes arginase and ornithine decarboxylase (ODC1) [69]. Ovine conceptuses express the greatest amounts of polyamines, arginine, and ornithine on Days 15 and 16 of pregnancy and polyamines steadily decrease in the uterine lumen between Days 12 and 16 of pregnancy [72]. Interestingly, this corresponds with the differentiation of mononuclear trophoblast cells into binucleate cells, extensive elongation of the conceptus, and the preparation for adhesion of Tr cells to uterine LE (see previous section on ovine implantation). Correspondingly, arginine administered to ovine Tr cells stimulates extensive cell proliferation and IFNT production by activating nitric oxide and the tuberous sclerosis complex 2 (TSC2)-mechanistic target of rapamycin (MTOR) cell signaling pathway [49]. Additionally, our laboratory knocked-down ODC1 mRNA translation in ovine conceptuses [72, 73] and confirmed a secondary pathway (Figure 1.3) for converting arginine to polyamines via arginine decarboxylase (ADC) and agmatinase (AGMAT) enzymes.

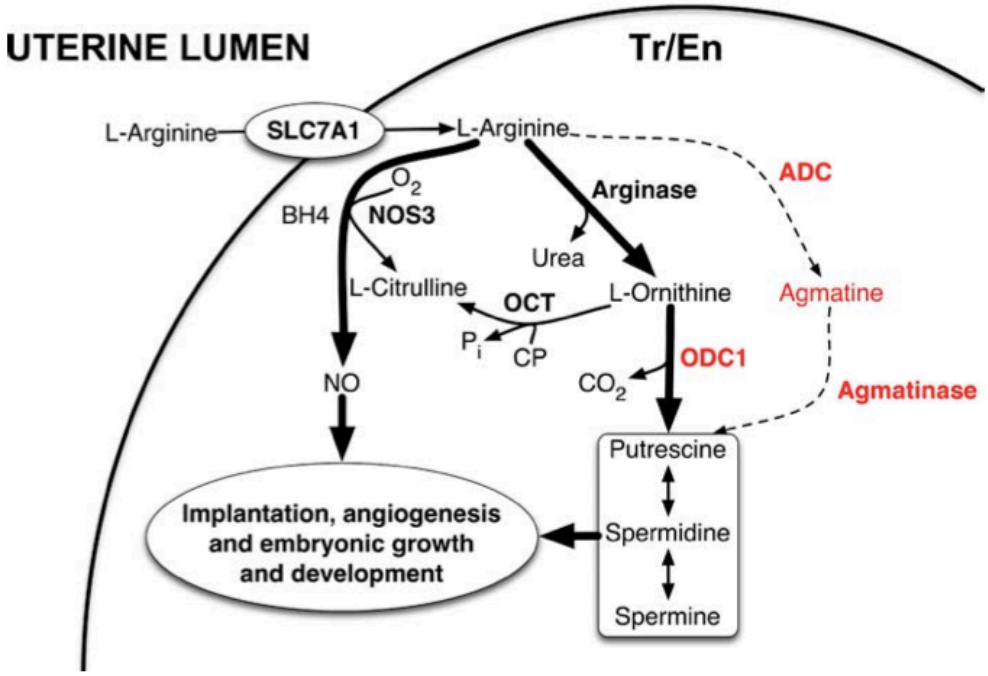


Fig 1.3. Schematic representation of classical and novel pathways for synthesis of polyamines. L-arginine is transported into the trophectoderm and extraembryonic endoderm of the conceptus (Tr/En) from the uterine lumen via Solute Carrier Family 7 Member 1 (SLC7A1) transporter. Polyamines (putrescine, spermidine, and spermine) can be synthesized via the classical pathway in which L-arginine is converted to L-ornithine via arginase, which is then converted to polyamines by ornithine decarboxylase (ODC1). Additionally, production of polyamines can occur through a novel pathway in which L-arginine is converted to agmatine by arginine decarboxylase (ADC), which is then converted to putrescine via agmatinase (AGMAT) Figure reprinted with permission from Wang et al., 2014 [72].

One-half of the conceptuses of ewes in which ODC1 mRNA was knocked-down produced conceptuses that had normal, elongated morphology, while the other one-half of the ewes produced conceptuses with abnormal, fragmented, and under-developed morphology. Polyamines were less abundant in uterine flushings of ewes in which translation of ODC1 mRNA was knocked-down compared to control ewes. There were no differences in the abundance of polyamines between conceptuses with normal morphology from ODC1 knocked-down ewes and control ewes; however, abnormally developed conceptuses from ODC1 knocked-

down ewes have decreased polyamines compared to normally developed and control conceptuses. Furthermore, there were greater amounts of ornithine in the uterine flushings from ODC1 knocked-down ewes than control ewes. It was, therefore, hypothesized by Wang et al. [72] that an alternative mechanism was responsible for "rescuing" the conceptuses that maintain normal morphology. Indeed, expression of ADC and AGMAT mRNAs increased in conceptuses from ODC1 knocked-down ewes as compared to control ewes. Normally developed conceptuses from ODC1-knocked down ewes had a two-fold increase in AGMAT protein in the uterine LE, sGE, and GE compared to abnormally developed conceptuses from ODC1 knocked-down ewes [72].

Progesterone Therapy

Up to two-thirds of pregnancy losses in mammals occur during the peri-implantation period of pregnancy [1], suggesting that significant physiological events essential for maintaining pregnancy must occur during this time-frame. For this reason, much of the research regarding embryonic mortality has focused on molecular therapies to positively influence the process of implantation and placentation. The administration of exogenous P4 during early pregnancy in cattle results in increased blastocyst size [74, 75], and increased rates of embryonic survival [76, 77]. Cattle administered 100 mg of P4 on Days 1 to 4 of pregnancy have conceptuses with advanced development compared to control cattle [78]. Additionally, exogenous P4 treatment administered during the peri-implantation period accelerates growth and development of ovine conceptuses [79, 80]. The mechanisms through which this process occurs are still being investigated; however, it is probable that early administration of exogenous P4 causes an early down-regulation of PGR in uterine LE and GE to accelerate changes in the

uterine gene expression, and the subsequent abundance of histotroph [81]. Indeed, early administration of progesterone results in increased expression of genes for uterine transport proteins responsible for transporting nutrients into the uterine lumen that likely support accelerated growth and development of ovine and bovine conceptuses[44, 82]. Specifically, expression of mRNA for the glucose transporter SLC2A1 is P4-induced and IFNT stimulated while expression of SLC5A1 mRNA increased due to P4 alone [43] and are both increased in uterine LE and sGE [44]. Ovariectomized ewes treated with P4 for 20 days have increased expression in uterine LE, GE, and stromal cells of SLC7A1 mRNA [50]. Furthermore, exogenous progesterone treatment up-regulates genes involved in the growth and adhesion of conceptuses such as galectin 15 (LGALS15), gastrin-releasing peptide (GRP), cathepsin-L (CTSL), and down-regulates proteins responsible for the formation of tight and adherens junctions between the uterine LE [79, 83-86]. It is also clear that the determining factor in this acceleration of conceptus development is not the dose of exogenous progesterone, but the early treatment, as increases in physiological levels of progesterone due to the administration of exogenous progesterone during the post-ovulatory period is sufficient [87]while a delay in administration of exogenous progesterone can lead to stunted conceptus development[88].

Human applications

There is much conflict currently in the literature regarding ovarian stimulation prior to *in vitro* fertilization. Women undergoing *in vitro* fertilization also undergo controlled ovarian hyperstimulation (COH) in order to have more ovulations to provide multiple embryo transfer opportunities, as well as delay the ovulatory surge of luteinizing hormone (LH) so it can be timed appropriately. The protocol generally consists of administration of exogenous

gonadotropin releasing hormone (GnRH) agonist (long protocol) or antagonist (short protocol) in addition to gonadotropins [89]. Mild cases of treatment induced ovarian hyperstimulation syndrome (OHSS) in approximately 33% of women, in which an increase of vascular endothelial growth factor (VEGF) production by follicles leads to increased vascular permeability and extravascular fluid accumulation. Severe, potentially fatal, cases can occur in up to 8% of COH protocols [90, 91]. Newer protocols and preventative measures have been implemented in order to decrease these occurrences, but each new protocol must overcome a similar barrier: lower stimulation leads to fewer follicles, fewer collected oocytes, and therefore fewer chances for embryo transfer and implantation. Although meta-analysis has suggested that these milder ovarian stimulation protocols do not result in more implantation failures than conventional COH [92], pregnancy rates remain extremely low at 20-30% [93, 94]. Perhaps the focus in the field should be shifted from increasing chances of implantation by performing more and more embryo transfers, to that of the implantation mechanisms of the uterus. Uterine receptivity to implantation is stimulated by subsequent exposure to ovarian steroids: estrogen from the dominant follicle and progesterone from the corpus luteum. Although the use of these hormones is well established in human fertility treatments, the dosage and timing required to synchronize the receptive uterus with embryo transfer is not. Studies have shown that steroid receptor profiles in the women undergoing COH is abnormal, but these results are somewhat conflicting. Papanikolaou et al. [95]demonstrated that there is greater expression of PGR protein in the endometrium during late follicular phase of treated compared to un-treated women. In contrast, Bourgain et al. [96]found there to be far less expression of PGR protein in both GE and stroma on the day of ovulation in treated women than those untreated. Chai et al.[97]reported that PGR protein was lower than controls in endometrial glands, but higher than controls in women

undergoing IVF treatment. Nevertheless, these authors agree that COH treatment with exogenous steroids leads to unbalanced, aberrant steroid receptor expression in the endometrium [98].

While there is no ideal animal model for research on human implantation and pregnancy besides humans themselves, the sheep is a valuable *in vivo* model for further consideration. Non-human primates, while far more closely related to humans, are incredibly expensive to care for, house, and provide appropriate enrichment. Additionally, many of the great apes [99, 100], which share the closest reproductive physiology with humans, are endangered, and thus can only be utilized in greatly restricted areas of research. Implantation is documented as the rate-limiting step for advancements in *in vitro* fertilization and other assisted reproductive techniques [101]. While the present experiment with ewes was not designed to mimic COH, changes in ovine gene expression, acceleration of development of the uterine environment, and effects on cell-specific expression of steroid receptor proteins should be considered when moving forward with the challenges of human implantation research.

CHAPTER II

EFFECTS OF EXOGENOUS PROGESTERONE DURING THE PERI-IMPLANTATION PERIOD OF PREGNANCY ON GROWTH AND DEVELOPMENT OF OVINE CONCEPTUSES

Introduction

Pregnancy loss during the peri-implantation period constitutes approximately two-thirds of all mammalian embryonic mortality [1]. These losses decrease efficiency in livestock production, as well as result in financial and emotional burdens involved in human assisted reproductive techniques. High concentrations of circulating progesterone (P4) are required for successful establishment of pregnancy; however there must also be a temporal- and cell-specific auto down-regulation of progesterone receptors (PGR) in the endometrial luminal (LE), superficial glandular (sGE) epithelia [16]. This down-regulation corresponds with the expression of genes by uterine epithelia that encode for secreted proteins and nutrient transporters responsible for accumulation of molecules, nutrients, cytokines, and enzymes known as histotroph [29, 30]. Previous research has demonstrated that exogenous progesterone therapy administered during the peri-implantation period can accelerate the growth and development of bovine and ovine conceptuses [74, 75, 78-80]by potentially affecting gene expression involving signaling molecules, nutrients and their transporters, and enzymes vital for the process of implantation. These signaling molecules include progestamedins, progesterone mediated fibroblast-derived growth factors, which bind to their receptors on uterine LE, sGE, and the conceptus in order to exert the effects of P4 [17, 59]. Gene expression for nutrient transporters for glucose and arginine are up-regulated by P4 exposure in the endometrium. Additionally, total glucose and arginine in uterine flushes of ewes increases during pregnancy [42-44]. Lastly,

polyamines, essential molecules required for DNA and protein synthesis, angiogenesis, cell proliferation, and reduction of reactive oxygen species [67-70] are found in high amounts in the conceptus, fetal fluids, and uterine flushings from early pregnant ewes [72]. This study explored the effects of exogenous P4 administered to ewes during the peri-implantation period on enzymes involved in the synthesis of polyamines. Those enzymes include arginase and ornithine decarboxylase (ODC1) as well as enzymes arginine decarboxylase (ADC) and agmatinase (AGMAT) in a novel pathway for synthesis of polyamines recently discovered in the trophectoderm of sheep conceptuses during early pregnancy [72].

Materials and Methods

Animals

Eighty Suffolk and Suffolk-crossed mature ewes (*Ovis aries)* that were observed to have had a minimum of two normal estrous cycles in the presence of a vasectomized ram were bred to Suffolk rams of proven fertility once estrus (Day 0) was detected. All experimental and surgical procedures were in compliance with Texas A&M University's Guide for the Care and Use of Agriculture Animals In Research and Teaching.

Experimental Design and Tissue Collection

Bred ewes (n=40) were assigned randomly to receive daily intramuscular injections of either 25 mg progesterone (P4, n=18) in 1 ml corn oil vehicle or 1 ml corn alone (CO, n=20) from Day 1.5 through Day 8 of pregnancy as described previously [79] Nine P4-treated ewes and 10 CO-treated ewes were hysterectomized on Day 9 of pregnancy and 9 P4-treated ewes and 10 CO-treated ewes hysterectomized on Day 12 of pregnancy. At the time of euthanasia, blood

samples were taken from ewes via jugular venipuncture. Uterine flushes and blastocysts were collected for analyses by flushing uteri with 20 ml of sterile phosphate buffered saline into a grid dish (pH=7.2). Uterine flush volume was recorded at time of collection. Endometrial tissue from the uterine horn ipsilateral to the CL was collected and snap frozen in liquid nitrogen for storage at -80°C for qRT-PCR analysis and adjacent tissue was fixed in OCT and frozen for immunofluorescence analyses. Tissues were also fixed in 4% paraformaldehyde for 24 h, transferred to 70% ethanol for 24 h, and then dehydrated through a graded series of alcohol to xylene and embedded in paraffin wax. Photomicroscopy was with a Nikon SMZ18 camera to capture images of all blastocysts and conceptuses in the grid dishes for morphological analyses and volume measurements before storage in 4% paraformaldehyde at room temperature. Uterine flushes were centrifuged, (5,000 x *g* for 15 min at 4°C) aliquoted into 1.5 ml eppendorf tubes, and stored at -20° C. Blood from ewes was stored on ice and centrifuged (10,000 x *g* for 7 min) in order to obtain plasma for radioimmunoassay (RIA).

Radioimmunoassay (RIA) Analysis for Concentrations of Progesterone in Plasma

In order to confirm the efficacy of the progesterone injections, concentrations of P4 in plasma were determined by RIA according to the manufacturer's instructions (MP Diagnostics ImmuChem Progesterone Coated Tube radioimmunoassay, 07-270102, Santa Ana, California) following the modified protocol validated in the Reproductive Neuroendocrinology joint laboratory of Drs. Gary Willams, Rodolfo Cardoso, and Thomas Welsh (Scarpa et al., unpublished manuscript).

RNA Isolation and Quantitative Real-Time qPCR Analyses

Total RNA was isolated from endometria from the ipsilateral uterine horn of pregnant ewes with respect to the corpus luteum (CL) using Trizol (Invitrogen, Waltham, MA) according to manufacturer's instructions. Total RNA samples were cleaned using a RNase-Free DNase Set (Quiagen, 79254). Total RNA quantity and quality were determined by spectrometry and bioanalysis (Agilent 2100 Bioanalyzer, Santa Clara, CA). Synthesis of cDNA from 5 ug total RNA was performed using Invitrogen™ SuperScript™ First-Strand Synthesis System for RT-PCR, 1904-018. Gene expression was analyzed via ABI PRISM 7700 (Applied Biosystems, Foster, CA) with detector SYBR Green PCR Master Mix (Applied Biosystems, 4309155, Foster City, CA) as described previously [72]. All primers were designed utilizing NCBI Primer-Blast software and are presented in Table 2.1. *ADC* and *AGMAT* were pre-amplified with SYBR green for 15 cycles in thermocycler (Eppendorf AG; 22331, Hamburg, Germany) and 1 ul of pre-amplicon template was used for RT-qPCR. Tubulin was used as a reference gene and abundances of all mRNAs were calculated via the comparative Ct method.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
SLC2A1	TGGGAAAGTCCTTTGAGATGC	GGTCAGGCCGCAGTACACA
SLC5A1	GCTGGAGCCTGCGTAACA	TGAATGTCCTCGTCTTCTGCAT
SLC7A1	CCTAGCGCTCCTGGTCATCA	GGGCGTCCTTGCCAAGTA
FGF10	GGAAAACGGATACAATACCTATGCA	TTTCCATTCAATGCCACATACATT
FGF7	AGTTTGCTCCACAGATCATGCTT	TGCTCTGGAGTCATGTCATTGC
HGF	ACAGCTTTTTGCCTTCGAGC	AACTCTCCCCATTGCAGGTC
ODC1	TGCCTTCTATGTTGCGGACC	TGACGGCATAAAAGGGGGTG
ADC	TCCCTGCCTCTAGAAGCTCACT	ATCGTTTCCACTCCGGATAGAC
AGMAT	TCTCTTCAAGCTGACCCACCAT	TCACGCCTTCACTGCAGATT
Tubulin	GGTCTTCAAGGCTTCTTGGT	CATAATCGACAGAGAGGCGT

Table 2.1. Primer sequences designed for RT-qPCR analyses.

Immunohistochemistry

Immunohistochemistry was performed to allow cell-specific localization of ODC1, ADC, and AGMAT in endometrium. Tissue processing and sectioning were completed as described previously[102]. Tissues were sectioned at approximately 7 μm in width. A universal rabbit and mouse Vectastain kit (Fisher Sci; NC9365554, Waltham, MA) was used for all proteins visualized in accordance with manufacturer instructions. ODC1 protein was detected using a primary rabbit polyclonal to ODC1 (Abcam; ab97395, Cambridge, UK) at a final dilution of 1:500. ADC protein was detected using a primary rabbit polyclonal antibody (Abcam; ab192771, Cambridge, UK) at a final dilution of 1:500. AGMAT protein was detected using a primary rabbit polyclonal to AGMAT (Abcam; ab231894, Cambridge, UK) at a final dilution of 1:250. Antigen retrieval was performed using boiling citrate for ODC1 and AGMAT, and protease (0.5 mg/mL in phosphate buffered saline, PBS) for ADC. Primary antibodies were replaced with

mouse IgG – Isotype (Abcam; ab37355, Cambridge, UK) as controls. Sections were stained with chromagen 3,3'- diaminobenzidine tetra-hyrdochloride (Sigma) for visualization and counterstained with Harris hematoxylin (Sigma, St. Louis, MO).

Spectrophotometric Assay

Spectrophotometric assays (Fisher Scientific Cell Biolabs Inc Glucose Assay Color 500 Assay; 50-109-8803, Waltham, MA and Bioassay Systems Enzychrom Fructose Assay Kit; 50-489-269, Hayward, CA) were used to determine concentrations of glucose and fructose (nmol) in uterine flushings. Uterine flushes were diluted 1:2 for glucose and analyzed without dilution for the fructose assay. Total recoverable glucose and fructose in uterine flushings was calculated by multiplying volume (mL) of uterine flush by concentration of glucose or fructose (nmol/mL) as described previously [103].

HPLC Analyses

Concentrations of amino acids and polyamines in uterine flushes were determined via a high-performance liquid chromatography method as previously described by Wu et al., [104, 105]. Uterine flush samples were de-proteinized using equal volumes of 1.5 M $HClO_4$, followed by adding 0.25 ml of 2 M K_2CO_3 as described by Satterfield et al. [44].

Statistical Analysis

Data from the radioimmunoassay were analyzed via a two-way analysis of variance (ANOVA) with ewe, treatment, and day as main effects and day by treatment as the interaction. Relative expression of mRNAs from RT-qPCR analyses were analyzed via Proc GLM procedure in SAS with data expressed as least square means (LSM) with standard errors of means (SEM). Probability of survival of blastocysts was determined by using the GLIMMX procedure in SAS with a maximum likelihood estimation technique. Data are represented as LSM ± SEM, with significant differences denoted by a different superscript letter. Statistical trends significance ($P < 0.1$) are represented in graphs with black bracket between bars.

Results

Concentration of Progesterone in Maternal Plasma

Maternal plasma samples from P4- and CO-treated ewes were analyzed by RIA (Figure 2.1) P4-treated ewes on Day 9 of pregnancy had higher concentrations of P4 in plasma than D9 CO-treated ewes ($P < 0.01$). Additionally, P4-treated ewes on Day 9 of pregnancy had greater concentrations of P4 in plasma than P4-treated ewes on D12 of pregnancy ($P < 0.05$). There was an interaction of day x treatment ($P < 0.03$). There were no differences in concentrations of P4 in plasma between P4 D12, CO D12, and CO D9 ewes or between P4 D9 and CO D12 ewes.

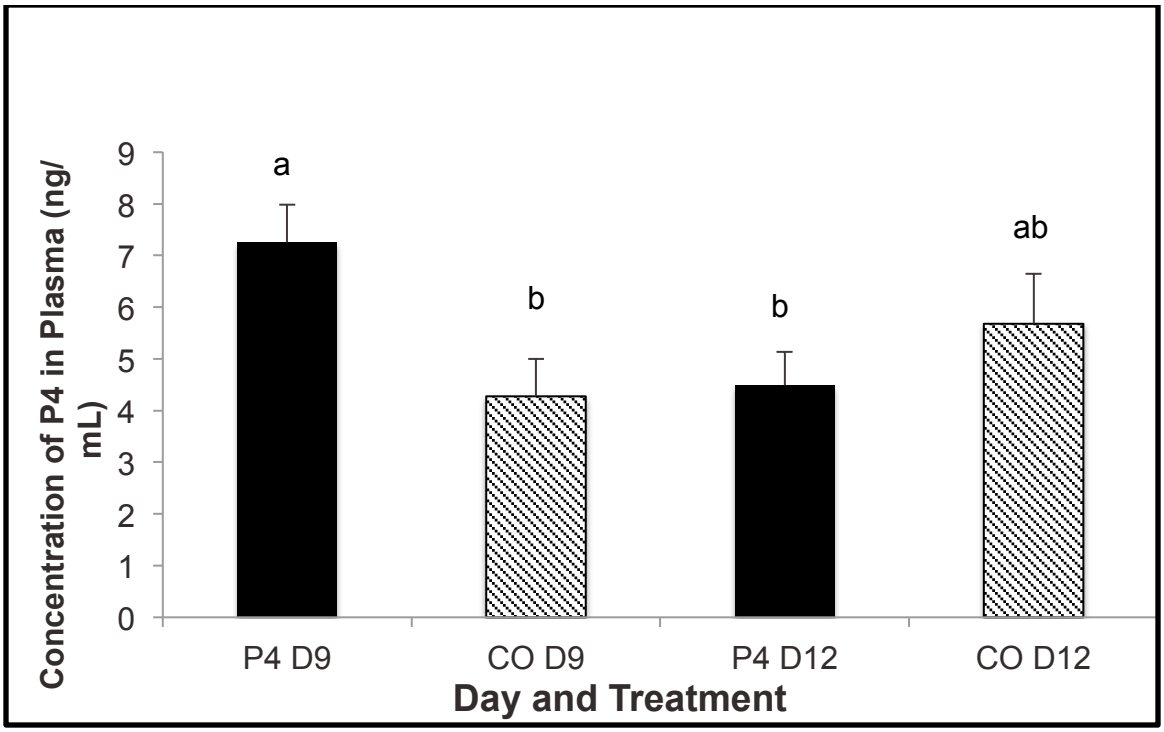
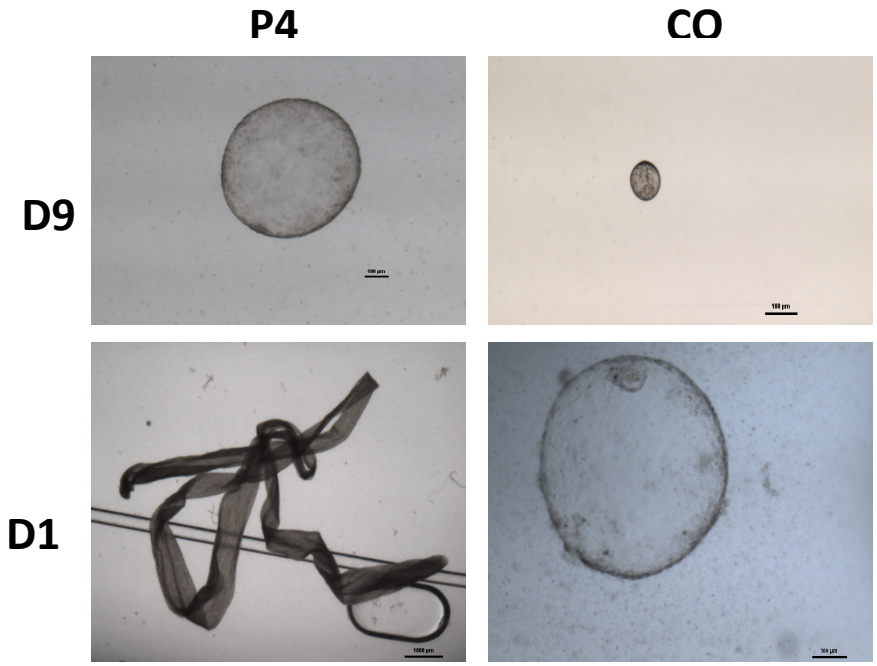


Figure 2.1. Concentration of P4 in maternal plasma (ng/mL). Ewes necropsied on Day 9 of pregnancy and treated with P4 had higher concentrations of P4 in plasma than CO-treated ewes ($P < 0.01$) but on D12 concentrations of P4 in plasma were not different ($P > 0.05$). There was a day x treatment interaction ($P < 0.05$). Data are presented as LSM±SEM.

Effects of Exogenous P4 on Blastocyst Morphology, Volume, and Survival

All conceptuses recovered from D12 P4-treated ewes were elongated and filamentous while conceptuses from CO-treated ewes were spherical (Figure 2.2A). There were no differences in volume between blastocysts from D9 P4-treated ewes and D9 CO-treated ewes ($P>0.05$) (Figure 2.2B). Ewes in which blastocysts were unable to be recovered after three separate individuals had inspected the grid dish, or ewes that had malformed blastocysts, or unfertilized oocytes were deemed non-pregnant.

[A]



[B]

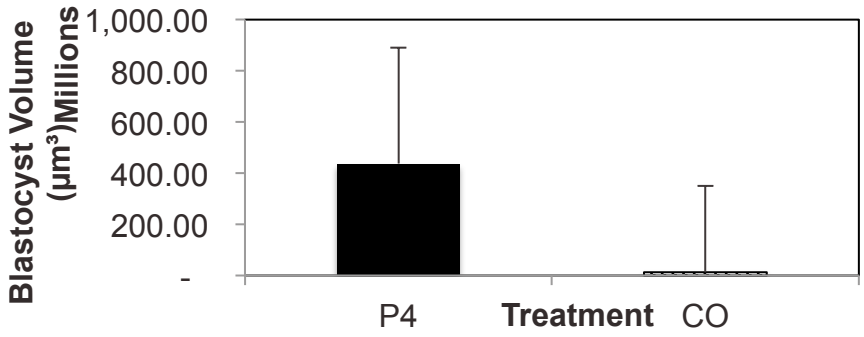


Figure 2.2. Morphology and volume of conceptuses. [A] All conceptuses from D12 P4- treated ewes were filamentous while blastocysts from D12 CO-treated ewes were spherical. The scale bar represents 100 µm for spherical blastocysts and 1,000 µm for filamentous conceptuses. All blastocysts were photographed on site at the time of necropsy. [B]. There was no difference in volume among blastocysts from D9 P4-treated ewes and from D9 CO-treated ewes due to the large amount of variation (*P>0.05)*. Data are represented as LSM±SEM.

The final number of ewes in each treatment group were six D9 P4, five D9 CO, four D12 P4, and nine D12 CO. In order to investigate correlations between survival and treatment, a

Maximum Likelihood test was performed in SAS by comparing number of blastocysts recovered to number of CL present on the ovaries at time of necropsy (Figure 2.3). Blastocysts from D12 CO ewes had a greater chance of surviving than those from D12 P4 ewes ($P < 0.05$). Blastocysts from D12 CO-treated ewes were more likely to survive than blastocysts from CO-treated D9 ewes ($P < 0.05$). There were no differences in survival probability for blastocysts from D9 P4-treated ewes, D9 CO-treated ewes, and D12 CO-treated ewes ($P > 0.05$). D12 P4-treated ewes were more likely to lose pregnancies than D12 CO-treated ewes. There was a treatment x day interaction ($P < 0.05$)

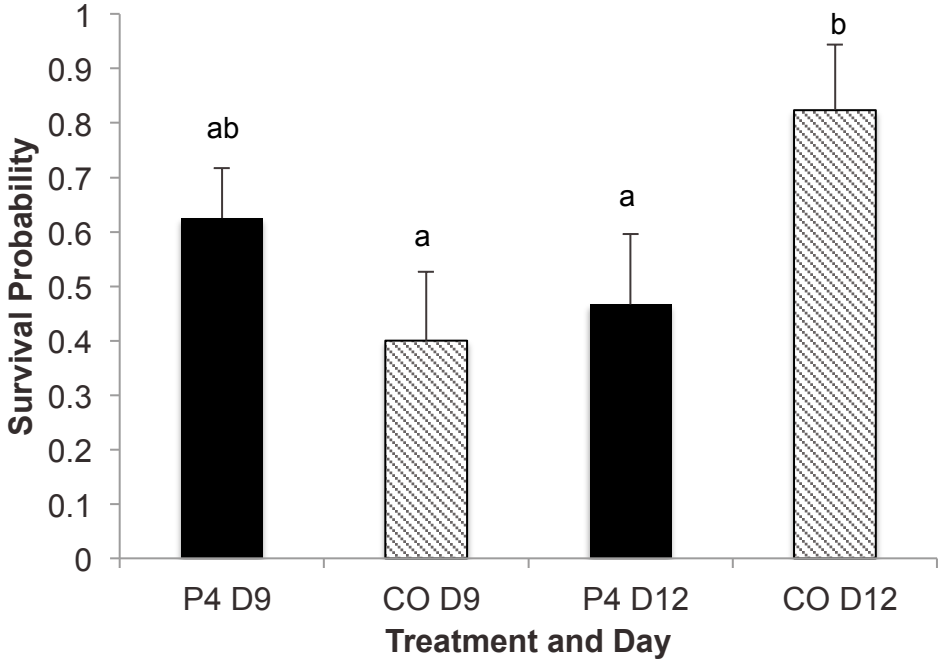
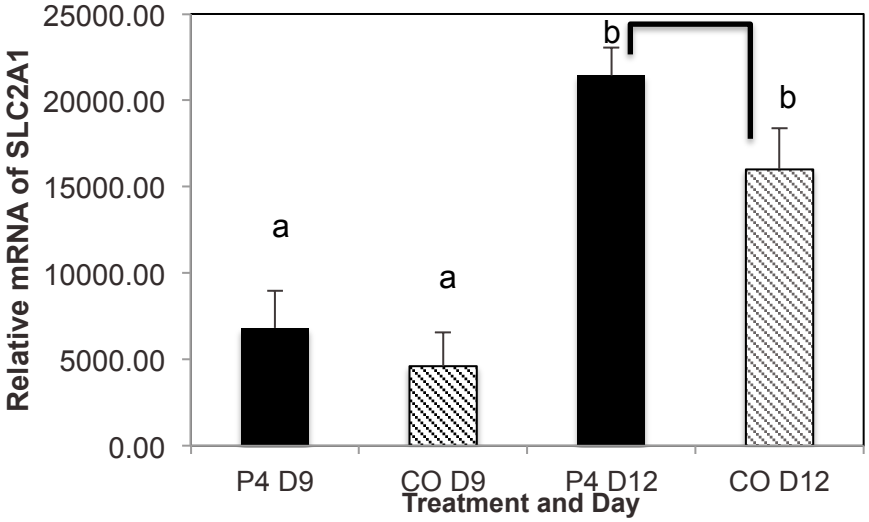


Figure 2.3. Probability of survival of blastocysts from CO- and P4-treated ewes. The maximum likelihood test was used to determine the probability of blastocyst survival based on number of blastocysts obtained and number of corpora lutea present. Blastocysts from CO-treated D12 ewes were more likely to survive than blastocysts from CO-treated D9 ewes ($P < 0.05$). Blastocysts from D12 CO-treated ewes were more likely to survive than blastocysts from P4-treated D12 ewes ($P < 0.05$). There was a treatment x day interaction ($P < 0.05$). There were no differences in blastocyst survival among P4 D9, CO D9, and P4 D12 ewes ($P > 0.05$). Data are presented as LSM±SEM.

Effects of Exogenous P4 on Expression of Genes for Nutrient Transporters

Steady-state levels of SLC2A1 mRNA were greater in endometria of ewes necropsied on Day 12 of pregnancy (facilitated glucose transporter) (Figure 2.4A). There was a significant effect of day ($P < 0.01$) and a tendency for an effect of treatment ($P < 0.1$), but the day x treatment interaction was not significant ($P > 0.05$). Expression of SLC2A1 mRNA in endometria was increased by approximately 3-fold in D12 CO ewes compared to D9 ewes of both treatments ($P < 0.01$). The endometria from D12 P4 ewes had about a 4-fold greater expression of SLC2A1 than D9 ewes of both treatments ($P < 0.01$) and tended to have greater expression than D12 CO ewes ($P < 0.1$).

[A]



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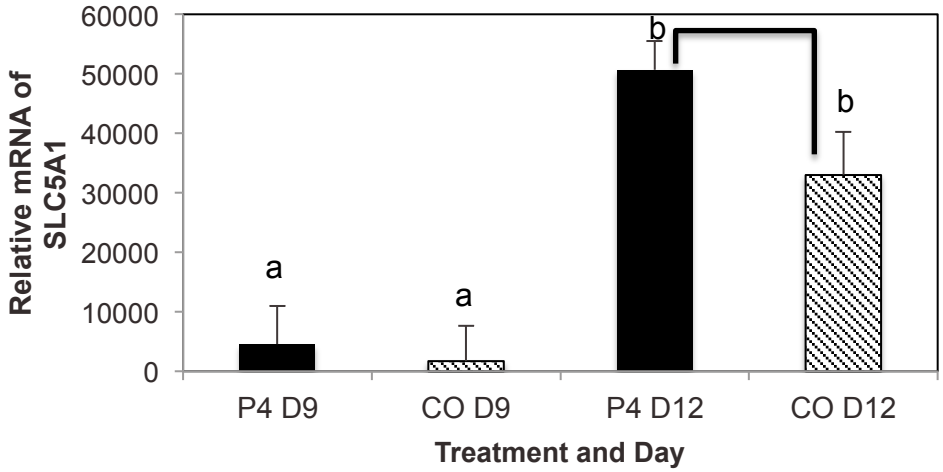


Figure 2.4. Relative expression of SLC2A1 and SLC5A1 mRNAs [A]. The expression of SLC2A1 mRNA increased 3-fold in endometrial tissue of CO D12 ewes and 4-fold in D12 P4 ewes compared to ewes treated with P4 through Day 9 ($P < 0.01$). P4 D12 ewes tended to have greater expression of SLC2A1 mRNA in endometria than CO D12 ewes ($P < 0.1$). [B]. There was an effect of day for expression of SLC5A1 mRNA ($P < 0.01$) Expression of SLC5A1 mRNA increased by 11-fold in D12 P4-treated ewes compared to D9 P4-treated and CO-treated ewes, and increased about 7-fold in P4 D12 ewes in comparison to P4 D9 and CO D9 ewes ($P < 0.01$). CO D12 ewes tended to have lower expression of SLC5A1 mRNA than P4 D12 ewes ($P < 0.1$) [B]. Data are represented as LSM±SEM.

Expression of SLC5A1 mRNA (glucose and sodium co-transporter) was not affected by treatment or treatment x day interaction ($P > 0.05$) but there was an effect of day of pregnancy ($P < 0.01$) (Figure 2.4B). Expression of SLC5A1 in endometria of CO-treated ewes necropsied on Day 12 of pregnancy was approximately 7-fold greater than for both CO- and P4-treated ewes on D9 of pregnancy ($P < 0.01$). P4-treated ewes necropsied on Day 12 of pregnancy had nearly an 11-fold greater expression of SLC5A1 than P4 D9 and CO D9 ewes ($P < 0.01$) and there was a trend towards significance in comparison to CO D12 ewes ($P < 0.1$). Additionally, expression of SLC7A1 mRNA (cationic amino acid transporter) increased approximately 2-fold in endometrial tissue of ewes necropsied on Day 12 compared to Day 9 of pregnancy ($P < 0.01$) (Figure 2.5) and there was an effect of day ($P < 0.01$) but not an effect of either treatment or treatment x day ($P > 0.05$).

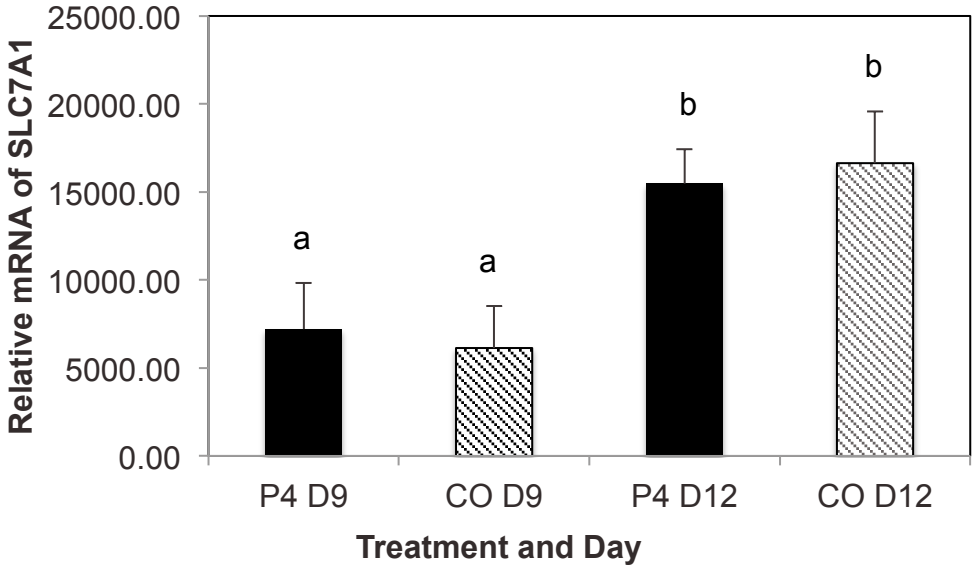


Figure 2.5. Relative expression of SLC7A1 mRNA in endometrial tissue. For both P4 and CO treatments, expression was greater by about 2-fold in D12 compared to D9 ewes. There was an effect of day ($P < 0.01$) but not treatment or treatment x day interaction ($P > 0.05$). Data are represented as LSM±SEM.

Effects of Exogenous P4 on Total Glucose and Fructose in Uterine Flushings

Total glucose in uterine flushings (µg) was affected by treatment ($P < 0.05$) and day ($P < 0.01$), but not their interaction ($P > 0.05$) (Figure 2.6). CO–treated ewes necropsied on Day 12 of pregnancy had about a 3.5-fold more total glucose in comparison to CO-treated ewes necropsied on Day 9 of pregnancy ($P < 0.05$). Total glucose in uterine flushings from P4-treated ewes necropsied on Day 12 of pregnancy was about 5-fold greater than that for CO-treated ewes necropsied on Day 9 of pregnancy ($P < 0.01$). Furthermore, P4-treated ewes necropsied on Day 9 of pregnancy tended to have greater amounts of total glucose in uterine flushings than CO-treated ewes necropsied on Day 9 of gestation ($P < 0.1$). Fructose was not detectable in uterine flushings from Day 9 or Day 12 of pregnancy.

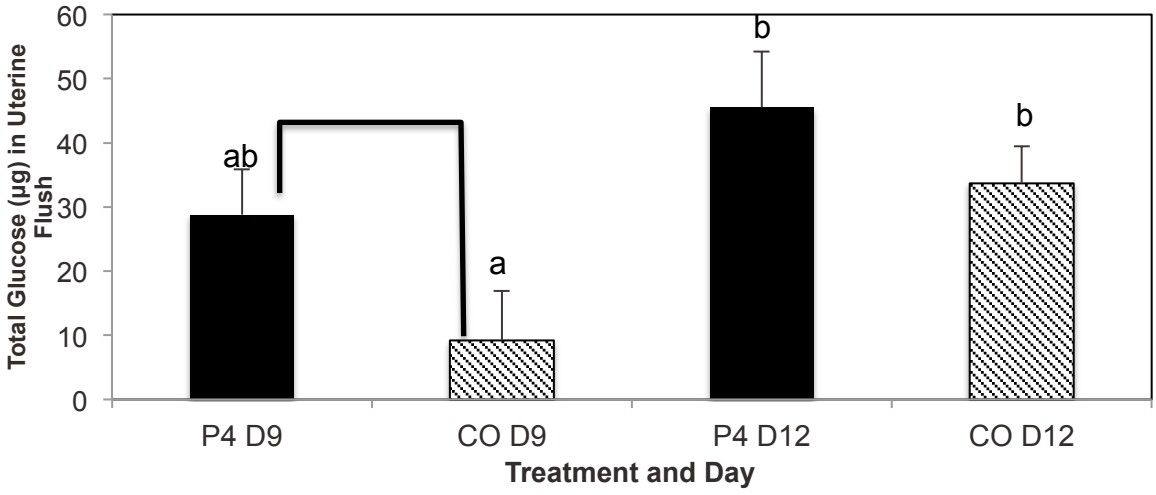
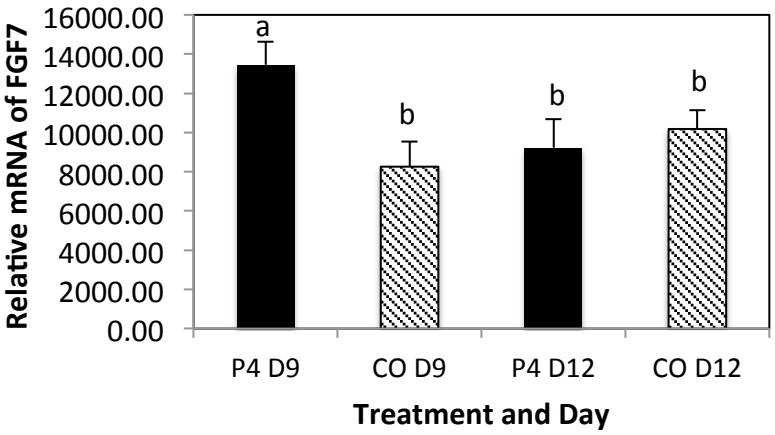


Figure 2.6. Total glucose (µg) in uterine flush. Total glucose in uterine flushing from P4- and CO-treated ewes were affected by treatment ($P < 0.05$) and day ($P < 0.01$), but not their interaction ($P > 0.05$). Total glucose in uterine flushings from CO D12 ewes was 3.5-fold greater than for CO D9 ewes ($P < 0.05$). P4 D12 ewes had 5-fold greater amounts of glucose than D9 CO ewes ($P < 0.01$). P4 D9 ewes tended to have greater amounts of glucose in uterine flushings than that for CO D9 ewes. Data are represented as LSM±SEM.

Effects of Exogenous P4 on Gene Expression of Progestamedins

Day effect on steady state levels of FGF7 was not significant ($P > 0.05$) however, treatment x day was significant ($P < 0.05$) and there was a trend for an effect of treatment ($P < 0.1$). Endometrial expression of FGF7 mRNA increased by approximately 1.5-fold in P4 D9 ewes compared to CO-treated ewes of the same gestation day and D12 ewes in both treatments ($P < 0.05$). There were effects of treatment ($P < 0.01$) and treatment x day interaction ($P < 0.01$), as well as a trend for an effect of day ($P < 0.1$) on endometrial expression of FGF10. Expression of FGF10 mRNA for CO- and P4-treated ewes necropsied on Day 12 of pregnancy was about 2.5-fold greater than for CO-treated D9 ewes ($P <0.01$). Additionally, P4-treated ewes necropsied on Day 9 of pregnancy had a 2.5-fold greater expression of FGF10 in comparison to D9 CO-treated ewes at the same gestation day ($P < 0.01$). The endometrial expression of HGF was not affected by treatment, day, or their interaction ($P > 0.05$).

[A]



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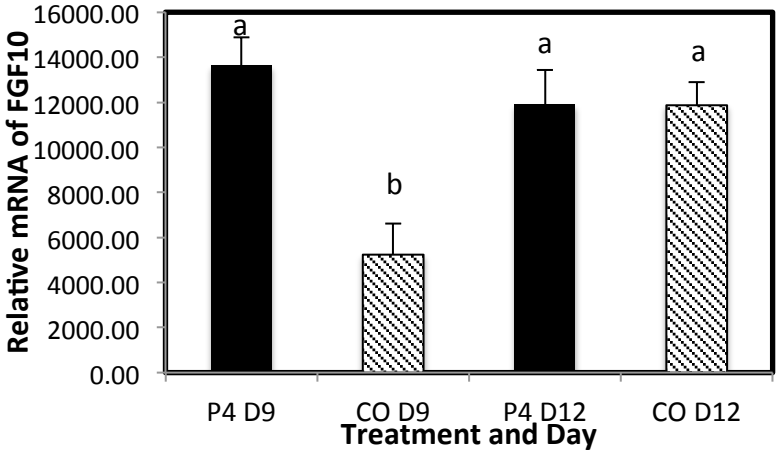


Figure 2.7. Relative expression of FGF7 and FGF10 mRNAs in endometrial tissue. [A] Endometrial expression of FGF7 mRNA was greater for D9 P4-treated ewes compared to CO D9, P4 D12, and CO D12 ewes ($P < 0.05$). [B] Steady state levels of FGF10 mRNA was about 2.5-fold greater in endometria from P4 D9, P4 D12, and CO D12 ewes in comparison to CO D9 ewes ($P < 0.01$). Data are represented as LSM±SEM.

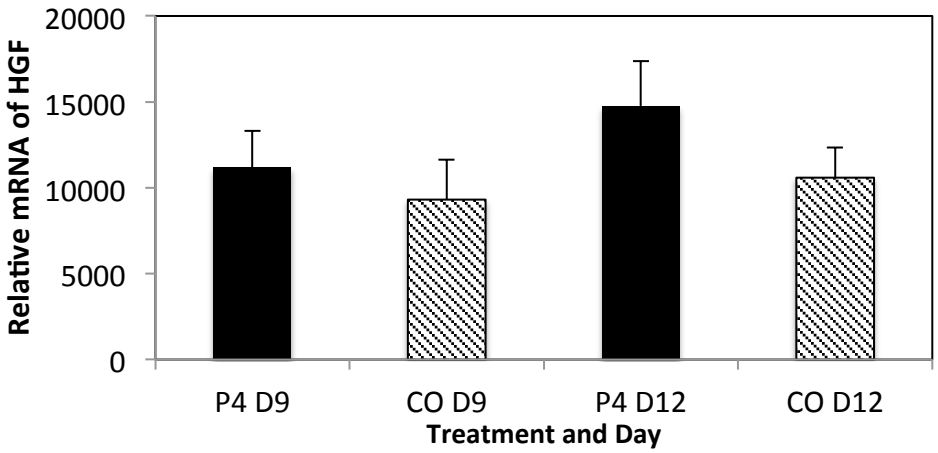


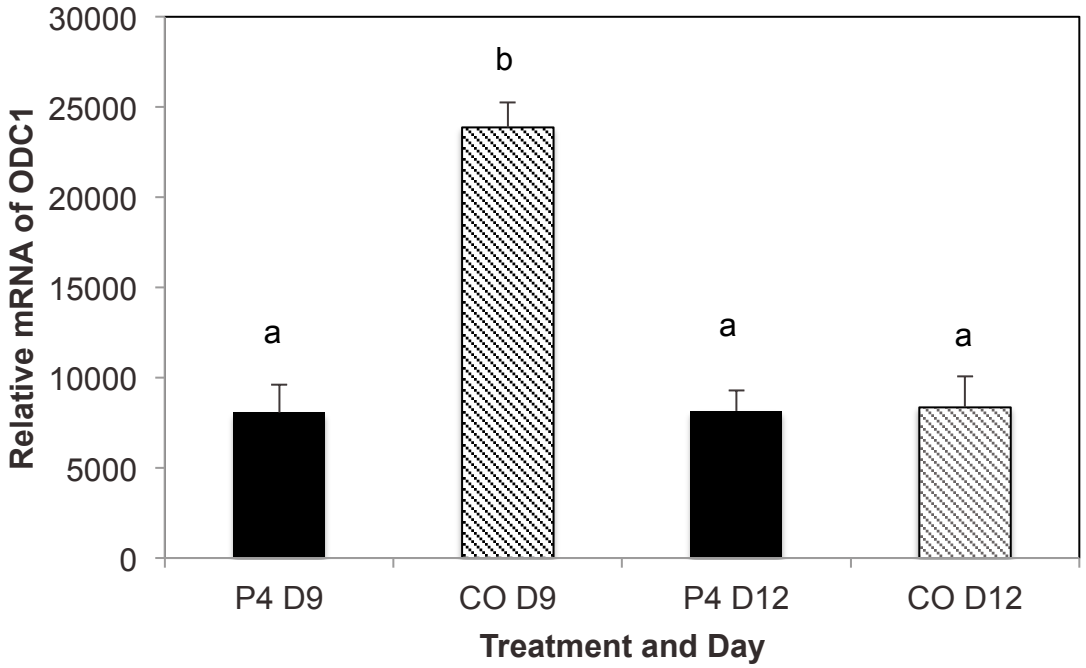
Figure 2.8. Relative expression of HGF mRNA in endometrial tissue. Steady state levels of endometrial HGF mRNA were not different due to effects of treatment, day, or their interaction ($P > 0.05$). Data are represented as LSM±SEM.

Effects of Exogenous P4 on Polyamine Synthesis Enzymes

Steady state levels of endometrial ODC1 mRNA were affected ($P < 0.01$) by treatment, day, and treatment x day interaction (Figure 2.9A). CO-treated ewes necropsied on Day 9 of pregnancy had 3-fold greater expression of ODC1 mRNA in comparison to P4-treated ewes necropsied on either Day 9 or Day 12 of pregnancy, and CO-treated ewes necropsied on Day 12 of pregnancy ($P < 0.01$). ODC1 protein was localized to uterine LE, sGE, and some stromal cells immediately beneath uterine LE cells. Similar to results from RT-qPCR, ODC1 protein was more abundant in endometria from D9 CO than D9 P4 ewes (Figure 2.9B).

Endometrial expression of ADC was not different due to effects of treatment, day, or their interaction ($P > 0.05$) (Figure 2.10A). ADC protein was localized to uterine LE, sGE, and stromal cells (Figure 2.10B). Steady state levels of AGMAT mRNA were not affected by day or treatment x day interaction ($P > 0.05$) but there was a trend for an effect of treatment ($P< 0.1$). CO-treated ewes necropsied on Day 9 of pregnancy had a 6-fold greater expression of AGMAT mRNA than P4-treated ewes necropsied on Day 12 of pregnancy ($P < 0.05$). AGMAT protein was localized to uterine LE and sGE (Figure 2.11B).

[A]



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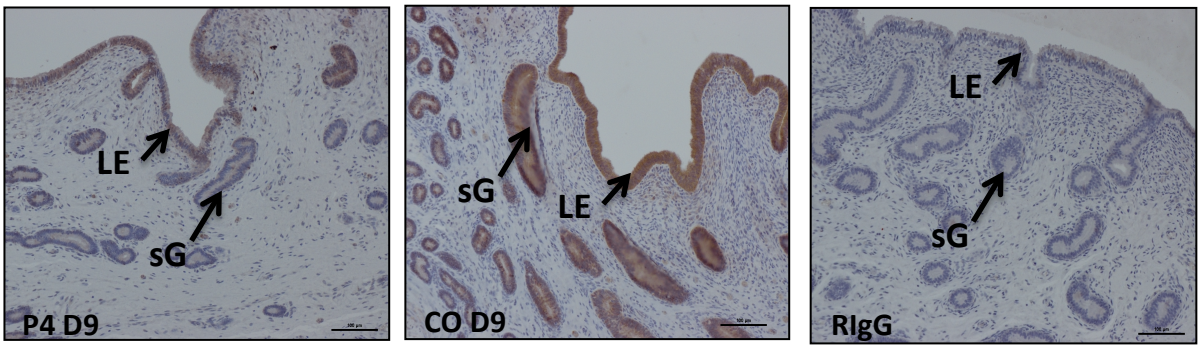
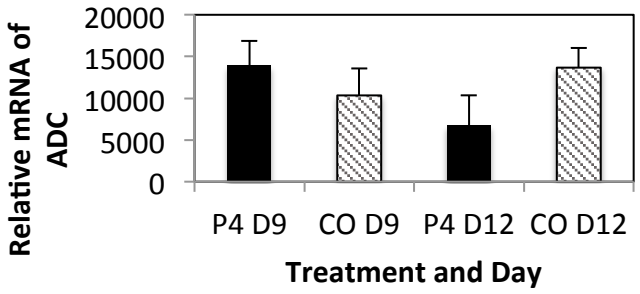


Figure 2.9. Relative expression of ODC1 mRNA and localization of protein. [A]. Endometrial mRNA expression of ODC1 mRNA was affected by treatment, day, and their interaction ($P < 0.01$). CO D9 ewes had a 3-fold greater expression of ODC1 mRNA in comparison to P4 D9, P4 D12, and CO 12 ewes ($P < 0.01$). Data are represented as LSM±SEM.
[B]. ODC1 protein was localized to uterine LE, sGE, and stromal cells adjacent to uterine LE. ODC1 protein was more abundant in D9 CO than D9 P4 endometria. Scale bar represents 100 μm.

[A]



[B]

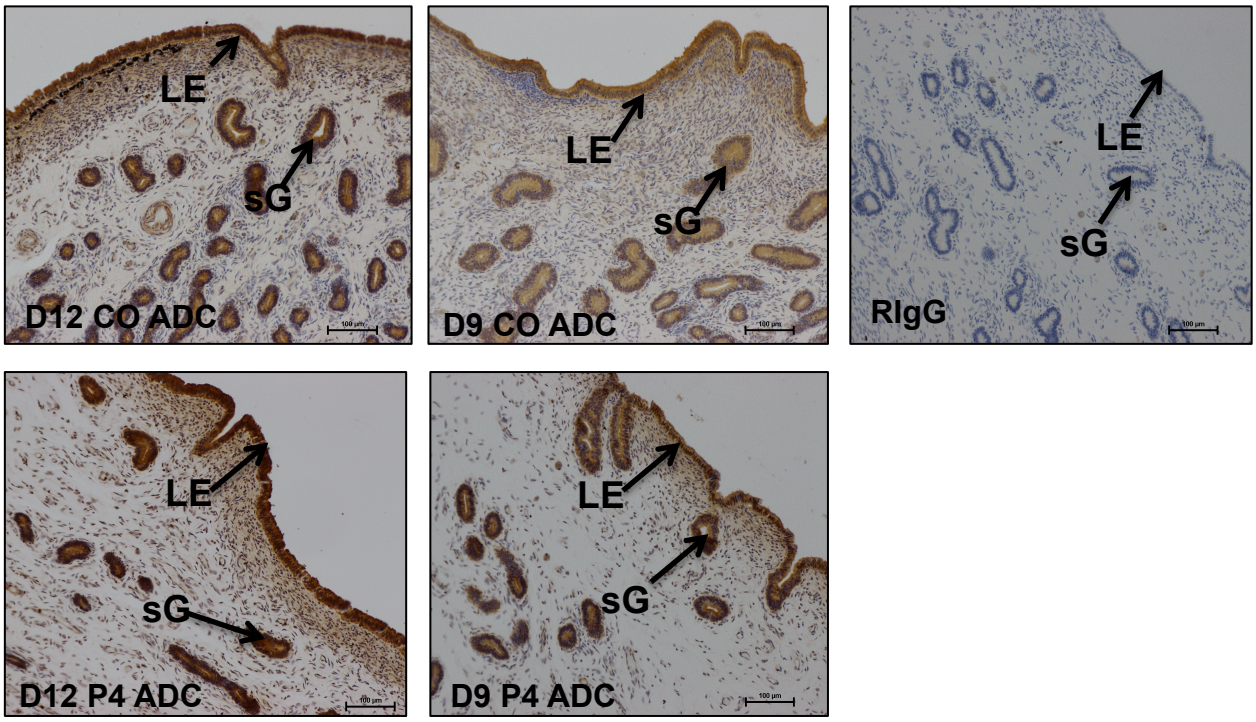
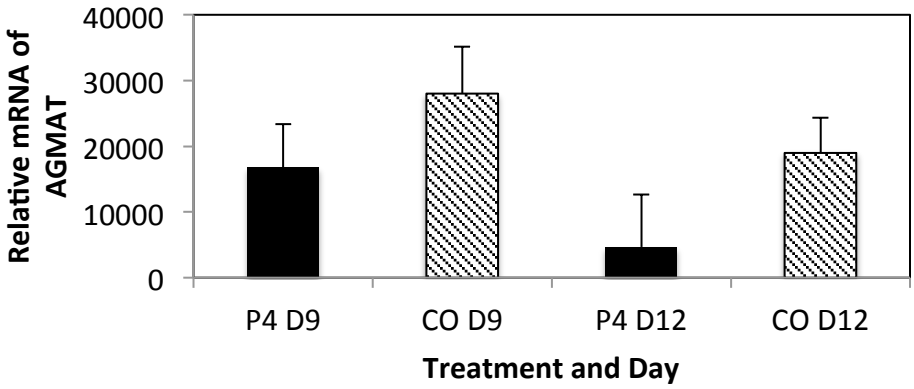


Figure 2.10. Relative expression of ADC mRNA and localization of protein. [A]. Steady state levels of endometrial ADC mRNA were not affected treatment, day, or their interaction ($P > 0.05$) Data are represented as LSM±SEM.
[B]. ADC protein was localized to uterine LE, sGE, and stromal cells. Scale bar represents 100 µm.

[A]



[B]

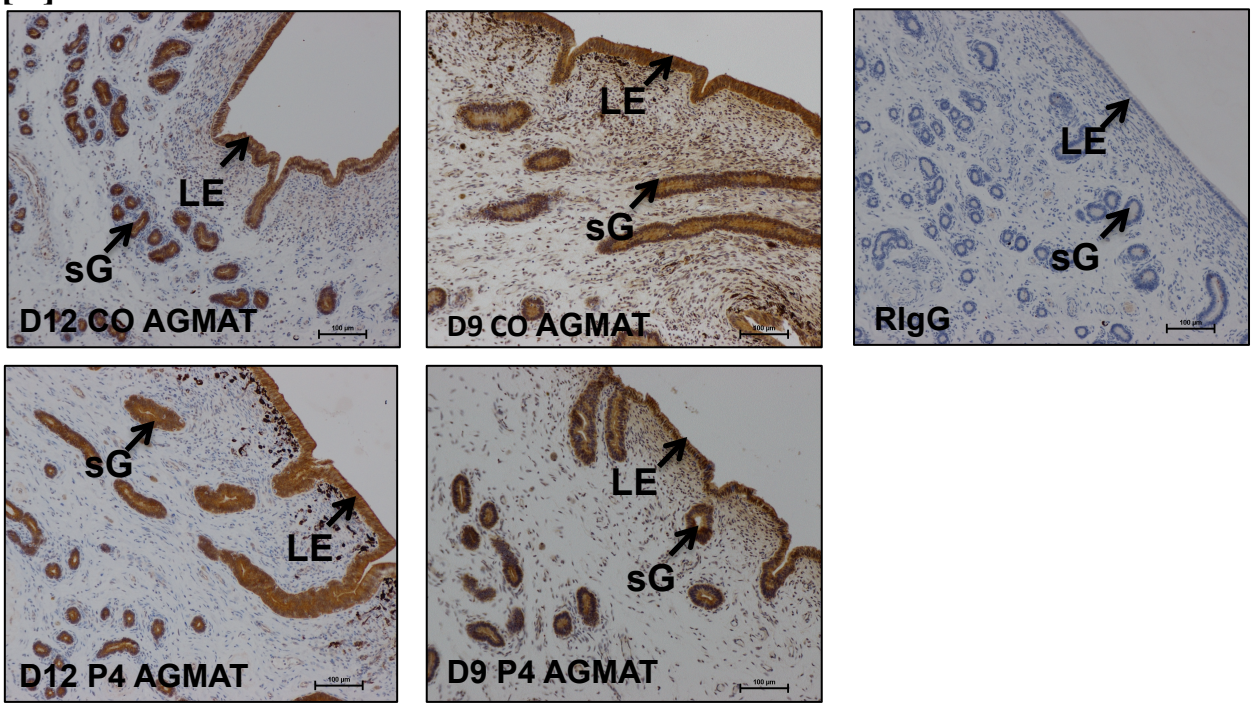
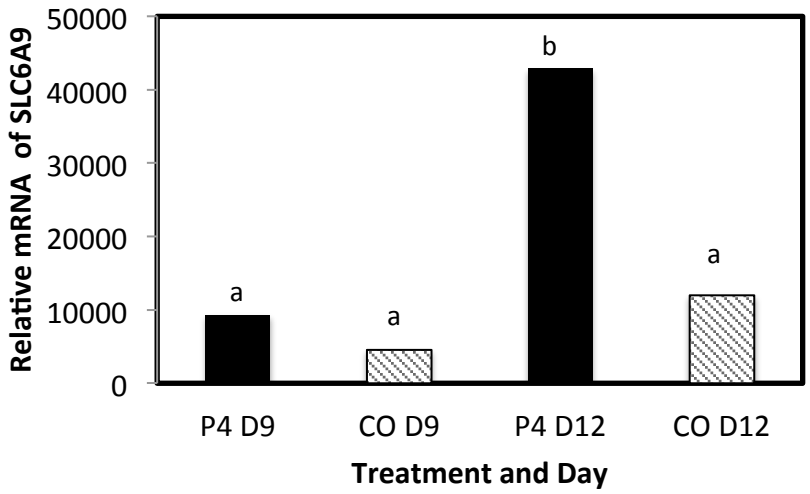


Figure 2.11. Relative expression of AGMAT mRNA and localization of protein. [A]. Expression of AGMAT mRNA was not affected by day, or treatment x day interaction ($P > 0.05$) but there was a trend for an effect of treatment ($P < 0.1$). Endometrial AGMAT mRNA in CO D9 ewes was 6-fold greater than that for D12 P4 ewes ($P < 0.05$). Data are represented as LSM±SEM. [B]. AGMAT protein was localized to uterine LE and sGE for D12 and to LE, sGE, and stroma for D9. Scale bar represents 100 μm.

Effects of Exogenous P4 on Glycine and Serine Transporters

Steady state levels of endometrial SLC6A9 mRNA were affected ($P < 0.01$) by treatment, day, and their interaction ($P < 0.05$) (Figure 2.12A). P4-treated ewes necropsied on Day 12 of pregnancy had an approximately 3.5-fold greater expression of SLCA9 than D12 CO-treated ewes, D9 P4-treated ewes, or D9 CO-treated ewes. Expression of SLC1A4 mRNA was affected by day and treatment x day interaction ($P > 0.05$) but not by treatment ($P > 0.05$). P4- and CO-treated ewes necropsied on Day 9 of gestation and CO-treated ewes necropsied on Day 12 of pregnancy had at least a 1.5-fold greater expression of SLC1A4 mRNA than CO-treated ewes necropsied on Day 12 of pregnancy.

[A]



[B]

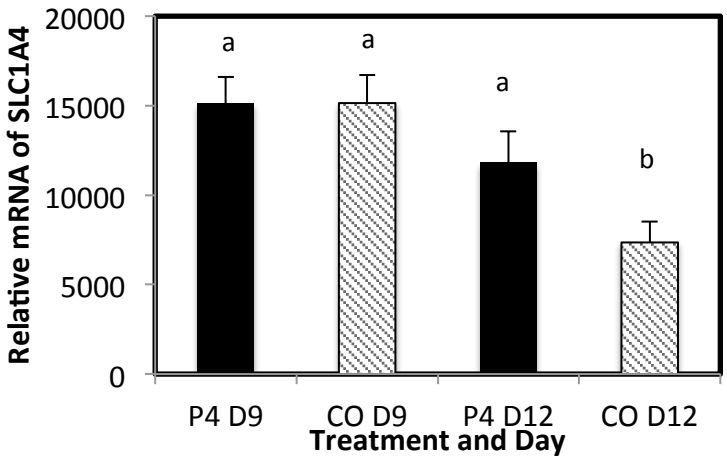


Figure 2.12. Relative expression of endometrial SLC6A9 and SLC1A4 mRNAs. [A]. Steady state levels of SLC6A9 endometrial mRNA were affected by treatment and day ($P < 0.01$) and day x treatment interaction ($P < 0.05$). P4-treated D12 ewes had greater expression of SLC6A9 mRNA than any other ewe. [B]. Expression of endometrial SLC1A4 mRNA was affected by day and treatment x day interaction ($P > 0.05$) but not by treatment ($P > 0.05$). CO D12 ewes had less expression of SLC1A4 than all other ewes. Data are represented as LSM±SEM.

Discussion

Previous research has demonstrated that administration of exogenous P4 during the peri-implantation period of pregnancy can advance the growth and development of bovine [74, 75, 78] and ovine conceptuses [79, 80]. In the present study, exogenous P4 given to pregnant ewes from Days 1.5-8 of pregnancy resulted in elongated, filamentous conceptuses while control ewes only administered corn oil had spherical blastocysts on Day 12 of pregnancy. These results confirm previous findings and clearly demonstrate the physiological capacity of P4 to cause drastic genomic and structural changes in the conceptus and its uterine environment. The acceleration in development of D9 blastocysts reported by Satterfield et al. (2006) was not detected in the present study, as P4-treated and control D9 blastocysts had similar volumes that were highly variable among blastocysts. Reasons for this variation are numerous, but could be due to differing times for fertilization of oocytes. Although ewes were mated to two separate rams within a few minutes of each other left overnight with a third ram, some blastocysts could result from earlier or later fertilization of oocytes resulting in large variability in volumes among blastocysts on Day 9 of pregnancy. Results of the present study should not be interpreted to negate the findings of Satterfield et al. (2006).

Results of RIA confirmed that P4-treated ewes necropsied on Day 9 of pregnancy had higher concentrations of P4 in maternal plasma than ewes administered corn oil. This difference was not detected in the D12 ewes because intramuscular P4 has a biological half-life of approximately 12 hours, with concentrations within plasma returning to normal values by about 72 hours [106]. Ewes necropsied on Day 12 of pregnancy had not been administered exogenous progesterone since Day 8 of pregnancy. Interestingly, blastocysts from D12 P4-treated ewes were less likely to survive in comparison to blastocysts from D12 CO-treated ewes, but

blastocysts from D9 P4-treated ewes were just as likely to survive as blastocyst from D9 CO-treated ewes. Exogenous P4 treatment has traditionally been shown to increase embryonic survival rates [76, 77, 107], but it has also been argued that the control animals in earlier studies had below average reproductive performance which exaggerated treatment-effects [108]. More likely, the low survival of blastocysts from D12 P4-treated ewes may reflect the decrease in circulating concentrations of P4 approximately 24 hours before necropsy. In previous studies in which ewes were treated with either P4 or CO and necropsied on Day 12 of pregnancy, the injections of P4 were from Day 1.5 through the morning of Day 12 and just prior to necropsy [79]. In the present experiment, ewes involved in a concurrent experiment in our laboratory were necropsied on Day 125 of pregnancy, but all ewes were treated the same during the pre-implantation period of pregnancy. In order to compare results from the present study and the concurrent study of effects of P4 treatment on placental and fetal development (Halloran, unpublished results), it was necessary to treat the ewes uniformly. Otherwise, it could not have been stated with confidence that any fetal or placental changes in late pregnancy were caused by accelerated conceptus development during the pre-implantation period of pregnancy.

All nutrient transporters examined, SLC2A1, SLC5A1, and SLC7A1, underwent extensive changes in expression due to day but not treatment. As both glucose transporters (SLC2A1 and SLC5A1) trended towards a treatment based difference in expression at Day 12 of pregnancy, it is possible that a larger sample size of pregnant P4-treated ewes necropsied on Day 12 of pregnancy been larger (endometrium from non-pregnant ewes was not analyzed) would have allowed detection of a treatment effect in D12 ewes. Indeed, Satterfield et al. (2006) demonstrated that expression of both SLC2A1 and SLC5A1 mRNA was greater in uterine LE and sGE in P4-treated ewes in comparison to CO-treated ewes on both Day 9 and D12 of

pregnancy. Furthermore, steady state levels of SLC2A1 increase in the endometrium between Days 10 and 14 of pregnancy and remain elevated and SLC5A1 increased between Days 10 and 18 of pregnancy before decreasing until Day 20 [43]. Total glucose in the uterine flushings differed due to effects of day and treatment. Satterfield et al. (2010) observed that D9 P4-treated ewes tended to have greater amounts of total glucose in uterine flushings than D9-CO ewes, but there was no difference between P4- and CO-treated ewes on Day 12 [44]. This is consistent with results of the present study, indicating that P4-treated Day 9 ewes tended to have more total glucose in uterine flushing than CO-treated D9 ewes. Although total glucose in uterine flushing increased with advancing stages of pregnancy as reported previously [42] there was no treatment effect by D12 of pregnancy.

Steady state levels of endometrial cationic amino acid transporter SLC7A1 changed due to effects of day. The two-fold increase in SLC7A1 for both P4-treated and CO-treated ewes by Day 12 of pregnancy correlates with previous findings stating that SLC7A1 increases 4-fold between Days 10 and 14 of pregnancy [50]; however Satterfield et al. (2006) reported an increase in the basic amino acid transporter SLC7A2B in endometria of P4-treated D12 ewes, but there was no difference between D9 CO-treated and D9 P4-treated ewes [44]. It is unclear why there was no effect of treatment on expression of SLC7A1 in the present study, but it could be because SLC7A1 requires long–term treatment of P4 (20 days) to induce expression of mRNA in uterine LE, GE, and stroma while expression of SLC7A2 mRNA increased about 4-fold after short-term P4 treatment (10 days) and further increased in response to IFNT [50]. Potentially, the present study did not allow enough time for exposure of the uterus to P4 in order to increase expression of SLC7A1 mRNA. Enzymes involved in the synthesis of polyamines from arginine in endometria of ewes were affected by treatment of the ewes with exogenous P4.

For CO-treated D9 ewes, there was a 3-fold increase in expression of ODC1 mRNA in comparison to P4-treated D9 ewes. This effect of treatment was not detected on Day 12 of pregnancy. Interestingly, results from a concurrent study revealed that CO-treated ewes with a single fetus pregnancy expressed more ODC1 mRNA than P4-treated ewes with a single fetus pregnancy (Halloran, unpublished data). Mechanisms pertaining to these results are unknown and can only be speculative at this point. When α−difluoromethylornithine (DFMO) was administered to cycling adult female mice to inhibit ODC1 enzyme, steroidogenesis, luteogenesis, vascularization of the corpus luteum, and concentrations of P4 in plasma decreased due to decreases in expression of key ovarian enzymes responsible for the production of P4: cytochrome cholesterol side chain cleavage enzyme, steroidogenic factor 1, and steroidogenic acute regulatory protein [109]. Furthermore, an increase in expression of ODC1 in uteri of hamsters is correlated with increasing concentrations of P4 in blood of hamsters. Pregnancy was arrested once hamsters were ovariectomized or luteolysis was induced to inhibit expression of ODC1, but the pregnancy was able to recover in hamsters treated with exogenous P4 [110]. Our results are somewhat elusive as P4 is clearly positively correlated with expression of ODC1. A potential hypothesis is that the additional exogenous P4 in plasma resulted in a negative feedback on luteal steroidogenensis related enzymes, including ODC1. However, it should be noted that while expression of ODC1 mRNA was less for P4-treated D9 ewes, expression was not low enough to induce greater expression of enzymes involved in the alternate ADC/AGMAT pathway to produce polyamines, which are up-regulated when translation of ODC1 mRNA is entirely knocked out [72].

Results of this study reaffirmed current knowledge of progestamedins, but additionally provided some novel insight. Although previous research reported no correlation between

expression of endometrial FGF7 and exogenous P4 treatment [59], we observed that P4-treated D9 ewes had higher steady state levels of both FGF7 and FGF10 mRNAs than CO-treated D9 ewes. This suggests that although FGF7 is not as highly expressed in ovine stromal cells as FGF10 [17], it may serve as an important role in molecular signaling between the stromal fibroblasts and uterine LE, GE, and conceptus trophectoderm as does FGF10.

This study is the first to report expression of mRNAs for glycine and serine transporters within the endometrium of the sheep during the peri-implantation period of pregnancy. Our observations indicate that transporters for both glycine and serine are not affected by exogenous P4 treatment until Day 12 of gestation. Furthermore, P4-treated ewes necropsied on Day 12 of pregnancy had greater expression of both glycine and serine transporters. These results are concurrent with the pattern this study observed in glucose transporters, in which SLC2A1 and SLC5A1 tended to differ by treatment on Day 12 but not Day 9 of pregnancy. Glycine is interconvertible with serine through methyltransferase reactions, a key mechanism in one carbon metabolism. Briefly, one carbon metabolism utilizes folate, methionine, histidine, and serine to transfer one carbon-units in order to synthesize purines, DNA, and facilitate methylation. Although previous research has demonstrated the importance of one carbon metabolism for the growth and development of the ovine fetus and placenta in late pregnancy, [111-113] the roles of these mechanisms during the peri-implantation period have yet to be investigated. This apparent abundance of glycine, serine, and their respective transporters during early pregnancy should encourage future studies that focus specifically on the biological functions of these amino acids during the dynamic process of conceptus growth, elongation, and implantation and their potential role in early embryonic mortality.

CHAPTER III

SUMMARY AND CONCLUSION

This study confirmed and extended key discoveries regarding the effects of exogenous P4 treatment on molecular mechanisms of ovine implantation. Acceleration of conceptus growth and development due to administration of P4 during the peri-implantation period has now been demonstrated in multiple species and experiments as an established physiological effect of the treatment. Exogenous P4 treatment also resulted in increased expression of progestamedins FGF7 and FGF10. This up-regulation could result in increased molecular signaling by endometrial stromal cells to uterine LE, sGE, and conceptus resulting in changes in gene expression responsible for the production and secretion of histotroph. It is probable that this increase in histotroph production could elicit and expedite the process of conceptus elongation and implantation. Additionally, we observed that expression of ODC1 mRNA and protein, an enzyme responsible for the synthesis of biologically essential polyamines, is increased in CO-treated ewes on Day 9 of pregnancy. This was an unexpected result, as previous studies have demonstrated that P4 induces expression of ODC1 mRNA, but could provide new insight into the roles of this enzyme during the peri-implantation period of pregnancy. Finally, this study was the first to demonstrate that endometrial mRNA of both glycine and serine transporters are up-regulated in P4-treated ewes necropsied on Day 12 of gestation, indicating possible roles of one carbon metabolism during the peri-implantation period of pregnancy. Future studies investigating these mechanisms should focus on changes in gene expression on Day 9 or Day 12 of pregnancy exclusively, as there appears to be dynamic molecular changes during this time. Furthermore, analyzing in conjunction with Day 12 of pregnancy resulted in day x treatment interactions, making it difficult to state with confidence whether physiological effects were due

to treatment or due to natural advancement of pregnancy. Nevertheless, these results should be considered when investigating not only reproductive inefficiency of the livestock industry, but also the effects of controlled ovarian hyperstimulation on women undergoing *in vitro* fertilization, how this could potentially affect molecular mechanisms required for implantation, and furthermore how these mechanisms could lead to defects in placental establishment, and therefore, pregnancy loss.

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