# THE IMPORTANCE OF AMINO ACIDS, POLYAMINES, AND HEXOSE SUGARS FOR CONCEPTUS DEVELOPMENT DURING THE PERI-IMPLANTATION PERIOD OF PREGNANCY IN SHEEP

A Dissertation

by

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ABSTRACT

The mammalian embryo must develop from a single cell (the oocyte fertilized by the sperm) to a multi-cellular fetal-placental unit. In particular, the conceptuses (embryo/fetus and associated placental membranes) of livestock species develop from a spherical structure into an elongated filamentous form. In order to achieve this significant increase in cellular growth and proliferation, the conceptus is entirely reliant upon the nutrients secreted from the uterine epithelia. It has been demonstrated that the secretion of these nutrients is regulated by progesterone (P4; the hormone of pregnancy) and/or interferon tau (IFNT; the signal for maternal recognition of pregnancy in ruminants). Of particular interest, agmatine and polyamines (putrescine, spermidine, and spermine) have important roles in the survival, growth, and development of mammalian conceptuses. The cationic polyamines are derived from arginine metabolism and bind anionic molecules to regulate DNA transcription, RNA translation, and protein expression. However, it is not known if P4 or IFNT regulate the endometrial mRNAs or proteins involved in polyamine biosynthesis or interconversion. While arginine and its products are important for embryonic growth, it is actually serine and glycine that are found in greater abundance in uterine flushings and fetal fluids of sheep. Both serine and glycine are utilized in one carbon (1C) metabolism, which is responsible for the production of formate required for synthesis of purines and thymidine for nucleic acid synthesis. This biochemical pathway is vital for mammalian conceptuses because the developing cells of the conceptus must increase the synthesis of nucleic acids to build DNA strands during cellular division. The findings from the studies described here revealed: 1) how P4 and IFNT work cooperatively

and independently to affect expression of mRNAs and proteins involved in endometrial polyamine metabolism and transport during the peri-implantation period of pregnancy; and 2) the contributions of serine, glycine, glucose, and fructose for 1C metabolism by the ovine conceptus during the peri-implantation period of pregnancy. These novel findings are valuable in advancing our understanding of the importance of specific nutrients in the context of conceptus development during the peri-implantation period of pregnancy in sheep to ultimately improve pregnancy success in livestock species.

# DEDICATION

This dissertation is dedicated to the laboratory equipment utilized for the analyses of this research, for without which, I would not fully appreciate the value of perseverance, humility, and above all, patience.

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NOMENCLATURE

| | |
|---|---|
| 1C | One-carbon |
| ATP | Adenosine triphosphate |
| BNC | Binucleate cell |
| BV | Blood vessel |
| CL | Corpus luteum (or corpora lutea) |
| CX | Control |
| E2 | Estrogen |
| ESR1 | Estrogen receptor |
| GAF | Gamma activation factor |
| GAS | Gamma activation sequence |
| GC | Gas chromatography |
| GE | Glandular epithelium |
| HIF | Hypoxia inducible factor |
| HPLC | High performance liquid chromatography |
| ICM | Inner cell mass |
| IFNAR | Interferon α/β receptor |
| IFNT | Interferon tau |
| IRF | Interferon regulatory factor |
| ISG | Interferon stimulated gene |
| ISGF | Interferon stimulate gene factor |
| ISRE | Interferon stimulated response element |

| | |
|---|---|
| JAK | Janus kinase |
| KHB | Krebs-Henseleit Bicarbonate |
| LE | Luminal epithelium |
| MS | Mass spectrometry |
| Myo | Myometrium |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NO | Nitric oxide |
| OPA | *o*-phthaldialdehyde |
| OXT | Oxytocin |
| OXTR | Oxytocin receptor |
| P4 | Progesterone |
| PGF2α | Prostaglandin F2α |
| PGR | Progesterone receptor |
| SAM | *S*-adenosylmethionine |
| SC | Stratum compactum |
| sGE | Superficial glandular epithelia |
| STAT | Signal transducer and activator of transcription |
| TGC | Trophoblast giant cell |
| Tr | Trophectoderm |
| TYK | Tyrosine kinase |

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# CHAPTER I

# INTRODUCTION

## The estrous cycle in sheep

The predominant purpose of the estrous cycle in sub-primate mammalian species is to allow the female repeated opportunities to achieve pregnancy. In sheep, the estrous cycle occurs every 17 days during each breeding season, which in the United States takes place during the autumnal months of October through December in seasonal breeds of sheep. The estrous cycle is divided into two phases – the luteal phase and the follicular phase – which can be further divided into four stages: proestrus and estrus (follicular phase), and metestrus and diestrus (luteal phase). The phases are named according to the primary structure present on the ovary during that time of the estrous cycle, and the phases can also be classified by the predominant sex steroid hormone that is produced by these structures. The ovarian follicular granulosa cells produce estradiol (E2) during the follicular phase and the ovarian corpus luteum (CL) produces progesterone (P4) in the luteal phase. In sheep (and most other sub-primate species), the day of onset of estrus is considered Day 0 of the estrous cycle and ovulation in sheep occurs about 30 h later. The estrus stage of the follicular phase ends when the metestrus stage of the luteal phase begins. After ovulation of the oocyte from the dominant follicle, the ovarian granulosa and theca cells undergo a structural and functional transformation to large and small luteal cells, respectively. This change occurs gradually over the course of a few days, during metestrus, as the ruptured follicle becomes a corpus hemorrhagicum and soon develops into a CL. Receptors for P4 (PGR) and E2 (ESR1) are expressed by the uterine luminal

(LE), superficial glandular (sGE), and glandular (GE) epithelia, as well as fibroblasts within the uterine stroma and the smooth muscle cells that make up the myometrium [1]. A depiction of ovine uterine histoarchitecture is summarized in Figure 1B.

Arguably, the most important hormone in pregnancy is P4, and it is aptly referred to as the hormone of pregnancy. P4 has a myriad of functions to promote the establishment and maintenance of pregnancy, including, but not limited to increasing the secretion of nutrients from uterine glands and decreasing myometrial contractions [2,3]. E2 is equally important for uterine function, stimulating myometrial contractions and the uterine expression of ESR1, PGR, and oxytocin receptor (OXTR) mRNA [1,3,4]. Overall, E2 functions to prepare the uterus for the actions of P4, while P4 acts to create a quiescent and receptive intra-uterine environment that promotes conceptus elongation and attachment in a tightly coordinated and controlled manner. The uterus needs to be exposed sequentially to E2 and P4, then interferon tau (IFNT; the maternal recognition of pregnancy signal in sheep), and placental lactogen (PL; also known as chorionic somatomammotropin [CSH]) for activation and maintenance of endometrial angiogenesis and remodeling, uterine gland secretion, and gene expression that promotes pregnancy establishment and maintenance [5].

In sheep, the cells of the CL begins producing P4 around three days after ovulation, when the large and small luteal cells gain functional capacity for steroidogenesis [6]. P4 production by the newly formed CL steadily rises through

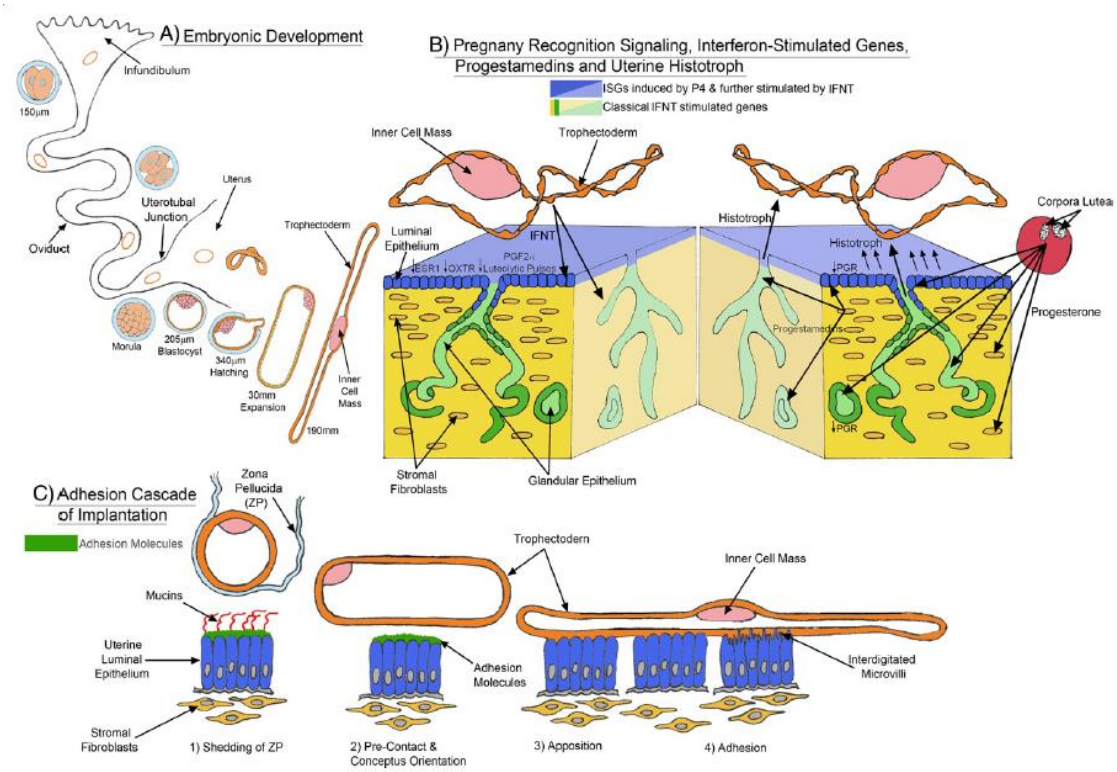


**Figure I-1 Embryonic development, pregnancy recognition signaling, and the adhesion cascade in sheep.** The oocyte is fertilized in the oviduct the embryo and will enter the uterine horn at the morula stage, develop into a spherical blastocyst, then elongate to a tubular then a filamentous form (A). The conceptus secretes interferon tau (IFNT) as the pregnancy recognition signal to silence estradiol receptors (ESR1) and oxytocin receptors (OXTR) to abrogate the luteolytic mechanism and prevent pulsatile release of prostaglandin F2α (PGF2α) (B). Progesterone (P4) is secreted by the corpus luteum and binds to P4 receptors (PGR) and works cooperatively with IFNT from the conceptus to stimulate the endometrial expression of genes important for the establishment of pregnancy (B). After the blastocyst hatches from the zona pellucida and elongates, it must orient itself to oppose the adjacent uterine luminal epithelium before firm attachment to the epithelia (C) [7].

metestrus to reach maximal levels at about Day 8 of the estrous cycle [6], which is when the beginning of the diestrus stage is typically denoted in sheep. During diestrus, waves of ovarian follicular cohorts are subsequently recruited and selected for dominancy, although high levels of P4 prevent any dominant follicles from achieving ovulation, and the oocytes in these cohorts undergo atresia. After eight to ten days, continuous exposure of the reproductive tissues to P4 causes auto-downregulation of PGR in uterine LE and sGE, although PGR remain in deep uterine GE, stromal cells, and myometrium [8,9]. Concurrently, uterine LE and sGE increasingly express receptors ESR1 and OXTR, and uterine GE and stromal cells later increase their expression of these receptors as well [1] (Figure 1B). Reproductive cyclicity is contingent upon the repeated intricate interactions between these receptors and their associated ligands in the appropriate cell types within the uterus. These shifts in gene expression within the uterine epithelial cells dictate the activation of luteolytic mechanism, which in sub-primate species is uterine-dependent as the uterine epithelial cells are the source of prostaglandin F2α (PGF2α; the luteolytic hormone) [8,9].

Towards the end of diestrus, when PGR have downregulated in uterine LE and sGE and are replaced by ESR1 and OXTR, pulsatile release of OXT from the CL and/or posterior pituitary acts on its receptors in uterine epithelia to induce pulsatile release of prostaglandin F2α (PGF2α) required for luteolysis [9]. PGF2α is transported by the local vascular network from the uterine vein to the ovarian artery, where it acts on its receptors on the CL, thus causing the structural and functional termination of the CL (luteolysis) [4]. This marks the end of diestrus and the beginning of proestrus between Days 14 to 16

of the estrous cycle in sheep. In turn, circulating levels of P4 plummet, while levels of E2 begin to steadily rise as follicles, now free from the inhibitory effects of P4, increase in size. During proestrus, one or two follicles (although there may be more in highly prolific sheep breeds) achieve dominancy and produce high levels of E2. Within the hypothalamo-hypophyseal axis, E2 stimulates production and secretion of follicle stimulating hormone from the anterior pituitary, which acts on its receptors on follicular granulosa cells to stimulate follicular growth and additional E2 secretion. This feed-forward mechanism is one of the few examples of positive feedback loops in the endocrine system. Once circulating concentrations of E2 reach a certain peak threshold, this induces a surge of luteinizing hormone from the anterior pituitary, which then binds its receptors on follicular theca cells and induce ovulation, marking the end of proestrus, the beginning of estrus, and the start of a new estrous cycle.

**Early embryonic development and pregnancy recognition signaling**

While the cellular events, placentation types, and hormonal profiles of pregnancy are remarkably unique across eutherian species, the events of early embryonic growth in sheep are fairly similar to those of other mammalian species as summarized in Figure 1A and Figure 2. A single oocyte (the haploid female gamete), protected by an outer glycoprotein membrane called the zona pellucida, is released from a dominant follicle during ovulation and is transported along the oviduct by ciliary movements to the junction at which the ampulla and isthmus meet [8]. If sperm (the haploid male gametes

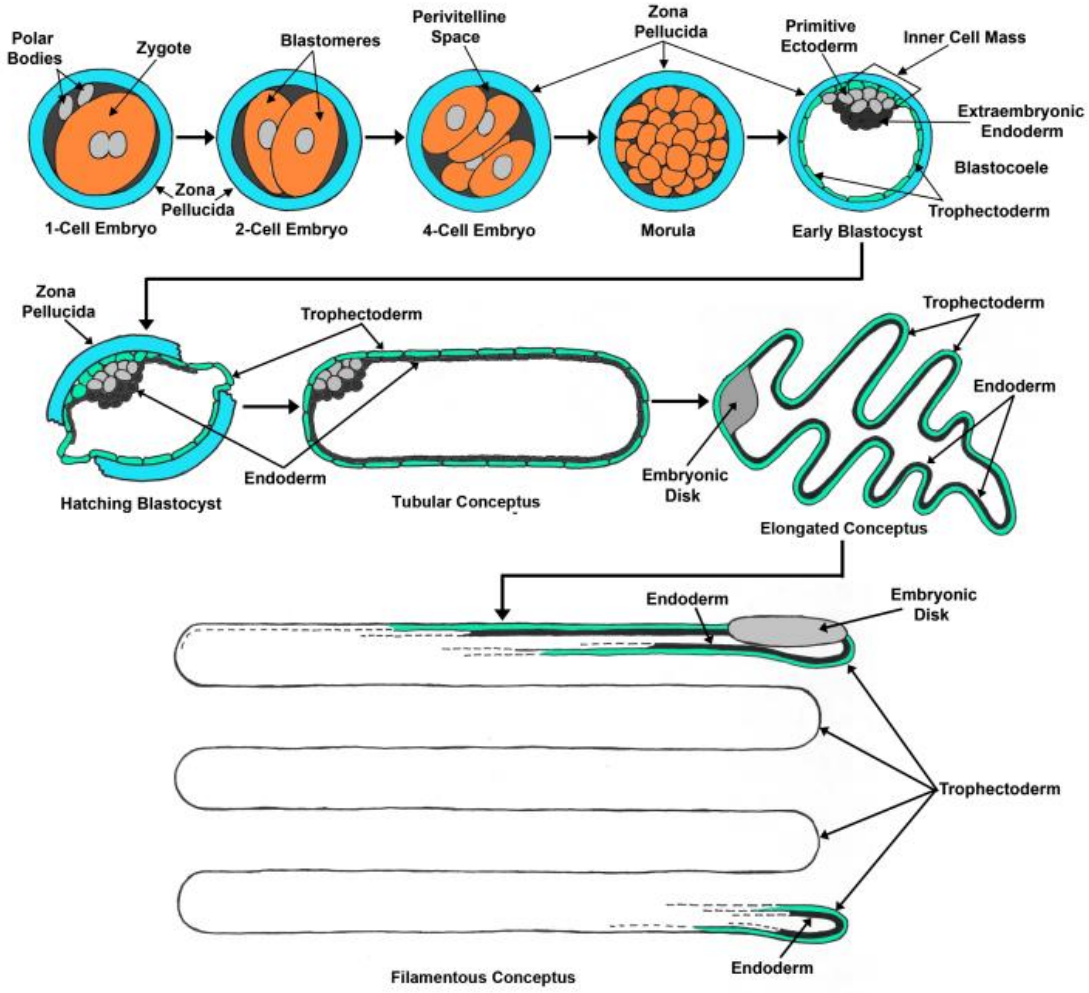


**Figure I-2. Conceptus elongation in sheep.** The oocyte is fertilized by a sperm cell to form a single celled embryo and is surrounded by the protective glycoprotein "shell" called the zona pellucida. The embryo undergoes cellular division to form a multicellular morula. The cells of the morula then differentiate to form the inner cell mass (ICM) and the trophectoderm (Tr) that make up the blastocyst. Compaction of the ICM within the Tr layer forms a blastocoele cavity that fills with fluid, allowing for expansion of the spherical blastocyst. The blastocyst hatches from the zona pellucida and the trophectoderm begins elongation to increase surface area for placentation. The ICM (embryonic disk) remains comparatively small during the peri-implantation period during the rapid proliferation of the trophectoderm [8].

are not present to fertilize the oocyte, the oocyte will degenerate, luteolysis will occur, and the estrous cycle begins anew. If the female is inseminated during estrus, sperm will travel up the reproductive tract to meet the oocyte at the ampullary-isthmus junction, where fertilization occurs. The oocyte fuses with one sperm cell which yields a single-celled zygote that has a diploid set of chromosomes (maternal and paternal) [8]. The zygote undergoes rapid cell division within the zona pellucida to form a 2-cell embryo followed by further cell divisions through the 8- to 16-cell stage when individual cells are no longer easily distinguishable and transcriptome activation occurs. Beyond this point (16- to 32-cell stage) the embryo is referred to as a morula [8]. The outer cells of the morula develop tight junctions, while the inner cells of the morula form gap junctions and sodium pumps and increasing sodium concentrations within the extracellular space cause water to enter the morula by osmosis. Water accumulation within the morula causes the outer cells of the morula to flatten and a fluid-filled cavity (the blastocoele) is formed [8]. This marks one of the first cellular differentiations of a multi-cellular totipotent morula to form the pluripotent blastocyst, comprised of the outer monolayer of trophectoderm cells and the inner cell mass (ICM; also known as the embryonic disk) (Figure 2), which will eventually develop into the placental membranes and the embryo/fetus, respectively [8].

In sheep, the morula leaves the oviduct and enters the uterine horn three to four days after fertilization, and then forms a blastocyst on gestational Day 6 [9]. The increasing fluid accumulation within the blastocyst, as well as continued cellular proliferation, increases the diameter of the blastocyst and it will “hatch” out of the zona pellucida between Days 8 and 9 (200 μM in diameter and only 300 cells) [8]. The spherical

blastocyst increases in size (400-900 μm in diameter and 400-900 cells) before transitioning rapidly into a tubular structure between Days 11 and 12 then elongating further into a filamentous structure between Days 12 to 16, exceeding lengths of 25 cm after Day 17 (Figures 1 and 2) [8]. At this point, in the case of a single ovulation, the extraembryonic membranes extend through the uterine body and into the contralateral uterine horn. However, the conceptus (the embryo/fetus and associated extraembryonic membranes) must signal to the uterus its presence, otherwise the uterus will initiate luteolysis to begin a new estrous cycle. Beginning on about Day 10, the mononuclear cells of the trophectoderm start to produce and secrete interferon tau (IFNT) as the conceptus begins elongation [8]. In sheep, goats, and cattle, IFNT is the maternal recognition of pregnancy signal that serves to prevent luteolysis from occurring in order to maintain the CL [9].

The secretion of IFNT is greatest on Day 16, then gradually declines until Day 21 at which point production stops as the conceptus does not express the IFNT genes after Day 21 [9,10]. In the uterine epithelia, IFNT induces expression of interferon regulatory factor 2 (IRF2) which silences expression of ESR1 and OXTR mRNAs, thus abrogating the oxytocin-dependent pulsatile release of PGF2α [11]. The uterine LE, sGE, GE, and stromal cells express the interferon α/β receptor (IFNAR), which is a cytokine receptor composed of two subunits, IFNAR1 and IFNAR2 [12,13]. As it is secreted by the conceptus into the uterine lumen, IFNT acts in a paracrine manner to exert its effects as the maternal recognition of pregnancy signal [9]. In uterine GE and stromal cells, binding of IFNT to IFNAR induces autophosphorylation of tyrosine kinase 2 (TYK2) and janus

kinase 1 (JAK1), which are constitutively expressed with IFNAR1 and IFNAR2, respectively [14,15]. This activation sequence recruits signal transducer and activator of transcription 1 and 2 (STAT1 and STAT2) [15]. STAT1 can form a homodimer known as a gamma activation sequence (GAF), which induces the transcription of genes containing gamma activation sequence (GAS) elements such as interferon regulatory factor (IRF1) [15,16]. Alternatively, phosphorylation of JAK1 and TYK2 leads to phosphorylation of a STAT1 and STAT2 heterodimer, which then associates with a protein called interferon regulatory factor 9 (IRF9), and this complex is referred to as interferon stimulated gene factor 3 (ISGF3) [16]. Both IRF1 and ISGF3 cooperatively bind and activate the interferon stimulated response element (ISRE) within genes in the nucleus [17,18]. This activation sequence is common amongst other Type 1 interferons, thus stimulates the transcription of classical interferon stimulated genes (ISGs) such as STAT1, STAT2, IRF9, and interferon stimulated gene 15 (ISG15) [17]. Conversely, in uterine LE and sGE, IFNT binding to IFNAR induces interferon regulatory factor 2 (IRF2), which acts as a transcriptional repressor when it binds ISRE [11]. Thus, the transcription of non-classical ISGs occurs in the uterine LE and sGE, such as hypoxia inducible factor 2 α [19], galectin 15 [20], solute carrier family 2 member 1 [21], and solute carrier family 2 member 2 [22]. As STAT1, STAT2, and IRF9 are not present in the uterine LE and sGE, the ISGS transcribed in these cells are activated via STAT-independent pathways [11]. The activation of ISGs in uterine epithelia and stromal cells is detailed in Figure 3.

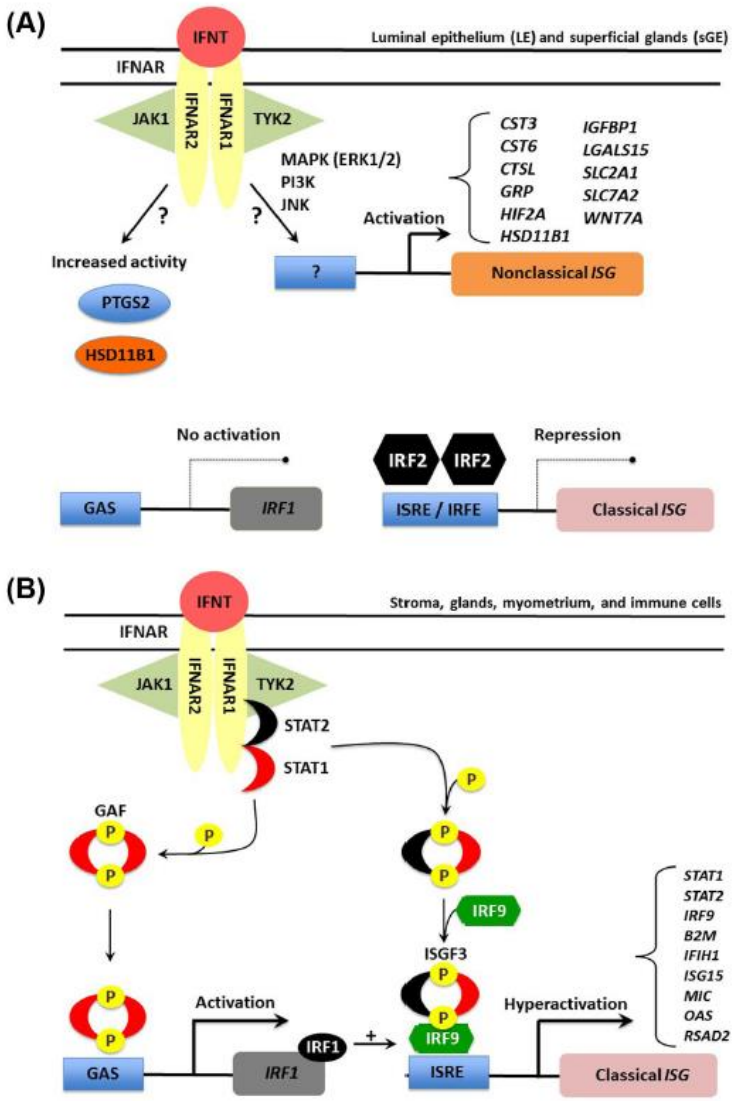


**Figure I-3 Interferon tau (IFNT) signaling in the uterine luminal epithelium (LE), superficial glandular epithelia (sGE), glandular epithelia (GE), stromal cells, and myometrium.** In uterine LE and sGE (a), the presence of interferon regulatory factor 2 (IRF2) prevents classical JAK/STAT signaling, but the mechanism(s) by which IFNT stimulates novel interferon stimulated genes (ISGs) is unknown. In deep glandular epithelia (GE), stromal cells, myometrial cells, and immune cells (b) IFNT activates JAK/STAT signaling in which STAT1 forms a heterodimer (GAF) that binds to gamma activated sequences (GAS) to initiate transcription of interferon regulatory factor 1 (IRF1). JAK1/TYK2 can also activate a STAT1/STAT2 heterodimer which forms a complex with interferon regulatory factor 9 (IRF9) to form interferon stimulated gene factor 3 (ISGF3) that can act via interferon stimulated response elements (ISRE) to stimulate, in concert with IRF1, transcription of classic ISGs [23].

## Implantation cascade and placentation in sheep

The implantation cascade (Figure 1C) refers to a series of events that allows for the physical interaction between the conceptus (a semi-allograft) and the endometrium to prepare for extensive placentation [8]. In domestic farm animals, 'implantation' is typically considered non-invasive and superficial, although recent evidence suggests that implantation and placentation in sheep is more invasive than originally speculated, which will be discussed later in this section.

The first step of the attachment cascade is the shedding of the zona pellucida and elongation of the conceptus (Figure 1 and Figure 2) [8,24]. As the conceptus elongates to maximize surface area, it is considered "free-floating" within the uterine lumen. At this stage, the conceptus is entirely reliant upon molecules transported or secreted by the uterine epithelia into the uterine lumen (referred to as histotroph). The conceptus takes up components of histotroph, which include water, nutrients, hormones, enzymes, adhesion factors, growth factors, and other molecules that are required for conceptus elongation, implantation, and placentation [8,9]. To establish a physical connection, the apical surfaces of the conceptus must attach to the apical surfaces of the uterine LE to begin implantation (Figure 2C). However, the luminal surfaces of the uterine LE express anti-adhesive proteins such as mucins, which act as a protective barrier to prevent adhesion of conceptus trophectoderm the apical surfaces of LE on opposing apical surfaces of the uterus [8,25]. Therefore, a requirement of attachment of the conceptus to the uterine LE is downregulation of these mucins to expose adhesive molecules such as integrins [26].

The second step of the adhesion cascade involves conceptus orientation – which in sub-primate species means that the ICM faces the uterine lumen – and pre-contact of the trophectoderm (Tr) as it stabilizes itself just adjacent to the LE which occurs around Days 14 – 15 in sheep [8,24]. At this time point, the conceptus can still be recovered intact by uterine lavage. In ruminants, temporary outgrowths termed chorionic papillae extend into the openings of the uterine glands, which may aid in immobilizing the conceptus adjacent to the uterine epithelia or may provide anchor points to generate forces for further elongation [27]. After this, the third step of the attachment cascade consists of apposition of the Tr to the uterine LE that is mediated by light attachments involving carbohydrate-lectin (such as glycosylation-dependent cell adhesion molecule-1) interactions by Days 16 – 17 in sheep [8]. The conceptus can still be recovered by uterine lavage at this stage, albeit with some superficial damage to tissues.

The final step of the attachment cascade in domestic livestock species is adhesion of the apical surfaces of the Tr to the uterine LE via strong integrin-ligand interactions. Integrins are transmembrane glycoprotein receptors with α and β subunits that bind extracellular ligands to promote cell-to-cell and cell-to-extracellular matrix adhesion [25]. A key ligand that binds integrins at the maternal-fetal interface in sheep is secreted phosphoprotein 1, although there are other integrin-ligand interactions that aid in adhesion of the conceptus to the uterine epithelia [8,28] . While integrins are constitutively expressed by both the uterine LE and the conceptus Tr during the peri-implantation period of pregnancy, downregulation of MUCs is required for integrin interaction between the two tissue types [8]. Additionally, microvilli on the apical surfaces of the LE and Tr

interdigitate (Figure 2C) to contribute to a strong connection by Day 18 of pregnancy [8]. An attempt to remove the conceptus from the uterine lumen by uterine lavage would result in significant structural damage to the conceptus at this point. In species such as rodents and primates, there is one more step in the attachment cascade: invasion [9]. This stage results in the conceptus cells migrating past the basement membrane of the uterine LE and into the underlying decidua, either by penetration between the LE as in primates or by causing apoptosis of LE as in rodents [9].

In sheep, the mononucleate cells of the Tr begin to differentiate by nuclear division without cytokinesis to form binucleate cells (BNCs) [29,30], and by Day 18 BNCs account for 15-20% of the Tr cells attached to the uterine LE [31,32]. The BNCs contribute to syncytialization within the placentome, and will produce and secrete pregnancy associated glycoproteins and placental lactogen (also known as chorionic somatomammotropin), both of which are detectable in maternal blood from around Days 30 to 50, respectively, as well as P4 [27]. Based on Wooding's hypothesis, the BNCs that differentiate from the mononuclear cells of the trophectoderm fuse with individual uterine LE cells, forming a trinucleate fetal-maternal hybrid cell [33,34]. Further BNC fusion with LE and the growing fetal-maternal syncytial layer creates syncytial plaques in placentomes; therefore, the syncytialized layer of cells between maternal caruncular stroma and placental allantoic stroma are composed of both maternal and conceptus cells [33,34]. The purpose of a syncytialized layer of cells is to reduce the distance between maternal and placental vasculatures for exchange of nutrients, gasses, and waste products.

Interestingly, compelling evidence suggests that the sheep conceptus is more invasive than originally speculated [35]. Seo and coauthors [35] demonstrated that cells containing more than two nuclei are derived from the mononuclear trophectoderm cells, and characterized these as trophoblast giant cells (TGCs). Furthermore, uterine LE in close apposition to the conceptus Tr lost polarity and began to express apoptotic markers, before being engulfed by the TGCs. Gaps in the uterine LE were observed as TGCs appeared to take the engulfed LE into the underlying stroma for elimination by immune cells, and these gaps were replaced by more TGCs. Lateral fusion of TGCs resulted in large syncytial plaques, exclusively composed of trophectoderm cells. It should be noted that this only occurs within the placentomes of sheep, as the inter-placentomal regions of the apical surfaces of the chorioallantoic epithelium remain attached to the apical surfaces of the uterine LE.

Sheep and cattle have synepitheliochorial placenta in which the placentomes that form along the length of the placenta bear syncytial plaques, while the inter-placentomal regions of the placenta remain epitheliochorial, similar to the pig [27]. Placentomes develop when placental villous folds, called cotyledons, form around Day 30 and these villi protrude into crypts within maternal caruncles (aglandular regions of the endometrium), resulting in considerable interdigitation of placental and maternal tissues by about Day 40 (Figure 4) [8]. However, migration of TGCs begins as early as Day 20 [35]. Therefore, syncytialization occurs in concert with placentome formation, and these

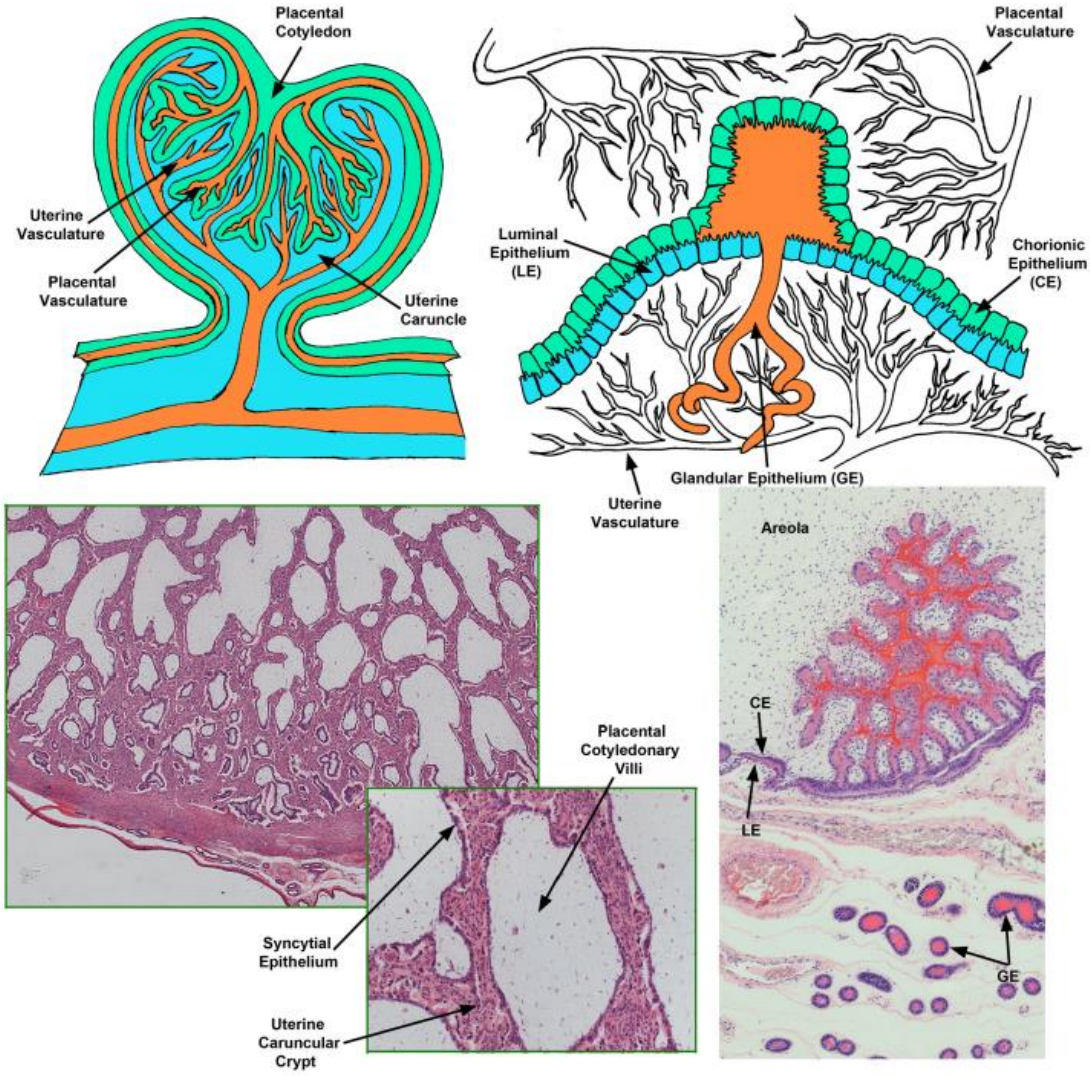


**Figure I-4 The structure of an ovine placentome and areolae.** Sheep have a typically concave placentome structure consisting of the maternal caruncle and the placental cotyledon. Cotyledonary villi grow into caruncular crypts so the placental and maternal tissue are intimately connected. The inter-placentomal regions of the placenta retain epitheliochorial placentation, and the chorionic epithelium (CE) will form a pocket around the opening of the uterine gland to form an areolae, which serves as a repository for the histotrophic secretions from the uterine glandular epithelia (GE) [8].

structures continue to develop, increasing in size and vascularity to provide hemotrophic support for the fetus. It's important to note that not all placentomes are equal in size and shape, and the total number of placentomes per placenta varies among species and even among animals within a species [27]. Placentomes can be classified as types (A – D) depending on their structure. Type A placentomes are concave and have cotyledons predominantly surrounded by caruncular tissue, whereas type B placentomes have cotyledons that are somewhat convex and protrude out over the caruncle [36,37]. Type C placentomes appear to have both cotyledonary and caruncular tissues present in equivalent amounts and are relatively flat, whereas type D placentomes are completely convex and are predominantly comprised of cotyledonary tissue surrounding caruncular tissue, similar to a bovine placentome [36,37]. All placentome types may be found on a sheep placenta, although Type A placentomes are typically observed for sheep [36].

In contrast, the inter-placentomal regions of the placenta remain epitheliochorial [8]. While the chorioallantois is attached along the apical surfaces of the uterine LE, pockets of space form around the openings of the uterine glands due to increased secretion of histotroph, and these structures are called areolae (Figure 4) [8]. Nutrients from areolae can be taken up directly by columnar epithelia of the areolae and transported across that cell layer for release into the placental vasculature, and will either be utilized or cleared through the kidney into bladder and then into the allantoic fluid via the urachus. So, while fetal wastes enter and accumulate in the allantoic fluid, the nutrients taken up by the placenta also enter the allantoic fluid that serves as a repository of nutrients to support conceptus development. Nutrients can be further transported from the allantoic fluid,

across the amniotic membrane, and into the amniotic fluid. The amniotic sac immediately surrounds the fetus and contains amniotic fluid that maintains buoyancy to allow for symmetrical fetal growth, prevents the fetal skin from adhering to the amniotic membrane, and provides nutrients that are directly swallowed by the fetus that promote gut maturation and nourish the fetus [9,38]. Therefore, in mid- to late gestation, when the conceptus is primarily supported by the nutrients acquired directly by the placental vasculature, the secretion of histotroph from the uterine glands and into the areolae serve as another means by which the placenta takes up nutrients from the mother.

## Amino acids, polyamines, and hexose sugars are important for conceptus development

As mentioned previously, the conceptus is entirely reliant upon the nutrients and other molecules supplied in histotroph during the peri-implantation period of pregnancy. Even once the hemotrophic placenta is established, these molecules in histotroph continue to support conceptus development as secretions from the uterine glands accumulate in the areolae and are transported across the chorioallantoic epithelium and into the underlying placental vasculature. The secretions from the uterine glands are so crucial, in fact, that the conceptus will not develop past the blastocyst stage if uterine glands are absent [39]. Of the molecules secreted or transported by the uterine epithelia, the nutritional composition of histotroph is of particular interest.

Amino acids are the building blocks for the synthesis of peptide and protein-based molecules, and the products of amino acid metabolism may also be utilized in other

biosynthetic reactions. Analyses of the amino acids in uterine flushings across the estrous cycle and throughout the peri-implantation period of pregnancy, as well as throughout pregnancy in sheep, revealed dynamic changes in the abundance of amino acids in response to pregnancy status or day of gestation [2,40,41]. Collectively, this not only suggests that these amino acids are important for conceptus development, but they are also regulated by signaling factors associated with pregnancy. For example, the expression of solute carrier family 7 member 2 (SLC7A2, a cationic amino acid transporter responsible for transporting arginine) is induced by P4 and further stimulated by IFNT, implicating arginine as important for conceptus development in sheep [22].

Arginine, while technically considered non-essential as it can be synthesized within the body, is nutritionally essential for gestating mammals, as arginine and its metabolic products have a variety of beneficial roles in the reproductive system [42]. Additionally, while arginine may not be the most abundant amino acid in uterine flushings during the peri-implantation period of pregnancy, the rate by which arginine increases in the uterine lumen is substantial [2]. Arginine is used to synthesize proteins, but the metabolism of arginine also yields nitric oxide (NO), agmatine, polyamines (putrescine, spermidine, and spermine), and creatine [42]. NO stimulates vasodilation of blood vessels, angiogenesis, and induces expression of hypoxia inducible factors (HIF), which in turn stimulate the transcription of genes involved in vasculogenesis and angiogenesis, thus contributing to the development of new blood vessels in the endometrium and later the placenta [43–45]. Agmatine and polyamines are also implicated in the regulation of angiogenesis [46,47] as well as mammalian embryogenesis and trophectoderm cell

functions [48–50]. Polyamines are cationic molecules that are fully protonated at physiological pH and will bind to anionic molecules such as DNA, RNA, and proteins to modulate gene transcription, mRNA translation, and protein expression, thereby participating in cellular functions that are crucial for growth, proliferation, and differentiation [51,52]. While arginine is classically considered the precursor for polyamines, methionine is also used for polyamine synthesis as *S*-adenosylmethionine (SAM; an intermediate of methionine metabolism) is decarboxylated and then utilized to synthesize putrescine [53]. In sheep, the knock-down of expression of enzymes involved in polyamine biosynthesis inhibits elongation and conceptuses that are morphologically and histologically abnormal [50]. However, conceptuses that activate other pathways for polyamine synthesis will develop normally, suggesting that polyamines are crucial for conceptus development past the elongation stage in sheep [50].

In addition, serine and glycine, two of the most abundant amino acids in uterine flushings from both cyclic and pregnant ewes, and are found in greater amounts in pregnant than cyclic ewes [2]. Both serine and glycine are highly abundant in umbilical venous plasma, allantoic fluid, and amniotic fluid throughout gestation [40]. While serine, like arginine, is considered a non-essential amino acid, this should not diminish its importance in mammalian reproduction. Serine synthesis from glucose is a pivotal branching point from glycolysis, as 3-phosphoglycerate, instead of being used to generate pyruvate and acetyl-CoA for entrance into the Krebs cycle, is converted to serine by sequential enzymatic conversions via phosphoglycerate dehydrogenase, phosphoserine aminotransferase, and phosphoserine phosphatase [53]. Then, serine can be used as a

building block for the production of proteins, or it may be used directly in other metabolic processes. The most well-known pathway for serine utilization is the one-carbon (1C) metabolism pathway. The 1C metabolism pathway links the folate cycle and the methionine cycle, which ultimately serves to synthesize and transfer single carbon groups (i.e., methyl groups) [54,55]. This pathway, particularly the folate cycle, produces formate used to synthesize adenine, guanidine, and thymidine nucleotides [56]. Thus, serine is the precursor required for the building of DNA and RNA strands. Serine and glycine are regularly interconverted by serine hydroxymethyltransferase (SHMT). This enzyme has two isoforms: SHMT1 is cytosolic and SHMT2 is mitochondrial [57]. Thus, SHMT may be utilized as an indicator of DNA synthesis and cell proliferation [58]. Moreover, the methionine cycle regenerates SAM, as this is the primary methylating agent during DNA methylation reactions [59]. The production of SAM is required for epigenetic modifications that regulate gene expression that occurs throughout gestation [59]. Epigenetic modifications do not alter the DNA sequence; rather, these modifications include methylation and acetylation to turn on and off specific regions of a DNA sequence. Importantly, while many of these epigenetic modifications are normal occurrences and required for normal embryonic development, sometimes external factors, such as poor maternal nutrition or environmental chemicals, can induce epigenetic modifications resulting in an altered expression of genes and proteins detrimental to development of conceptuses [59].

In most eukaryotic cells, glucose is the primary substrate for adenosine triphosphate (ATP) production via glycolysis and the Krebs cycle, but it can also be

utilized via the pentose phosphate pathway to produce reduced nicotinamide adenine dinucleotide phosphate (NADPH) and ribose. NADPH can be utilized for synthesis of fatty acids and steroid hormones, production of fructose via the sorbitol pathway, and synthesis of nitric oxide from arginine, while ribose provides structural units for ribonucleotide synthesis [60]. While glucose is an important metabolic substrate for the conceptus, fructose is particularly abundant in placental fluids and fetal plasma of ungulate species [61,62]. Studies in both sheep and pigs have demonstrated that the placenta converts maternal glucose to fructose before transfer to the placental vasculature and fluids, and the placenta allows for the fetal-to-maternal exchange of glucose. However, unlike glucose, fructose cannot be transferred from fetal blood to maternal blood, illustrating that fructose is a sequestered sugar to be used exclusively by the fetal-placental unit [63–65]. Fructose can then enter multiple biosynthetic pathways: 1) the hexosamine pathway to produce glycosaminoglycans such as hyaluronic acid and UDP-N-acetylglucosamine, 2) serinogenesis to produce serine that is utilized in 1C metabolism, and 3) the pentose cycle to produce ribose sugars and NADPH [65–67]. Fructose has long been ignored as a substrate for conceptus metabolism, as it was generally assumed that fructose could not be utilized for generation of ATP via the Krebs cycle. Unpublished findings from our laboratory suggest that while fructose is undetectable in uterine flushings from ewes at Day 12 of pregnancy (just after initiation of IFNT secretion), the abundance of fructose increases to detectable levels by Day 15 and rises steadily through Day 18 of pregnancy. While differing pathways are used for the metabolism and utilization

of amino acids and hexose sugars, these pathways are intimately linked, and it is essential to improve understanding of the interactions between these nutrients during pregnancy.

The dialogue between the conceptus and the uterus is complex and requires cooperation from each tissue type to properly establish and maintain pregnancy. Failure to do so results in embryonic losses. There is significant variation across mammalian species in the rate of embryonic loss. It is estimated to be between 20-40% in mammalian species, with most of these losses occurring during the peri-implantation period of pregnancy. After implantation and placentation, the likelihood of embryonic loss is decreased, but never zero. Thus, it is crucial to understanding mechanisms by which mammalian conceptuses thrive or perish, which ultimately provides a foundational knowledge by which to implement strategies to improve pregnancy success and outcomes in livestock and other mammalian species.

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CHAPTER II

# PROGESTERONE AND INTERFERON TAU REGULATE EXPRESSION OF POLYAMINE ENZUMES DURING THE OVINE PERI-IMPLANTATION PERIOD[1]

## Introduction

Successful establishment of pregnancy requires complex signaling between both the conceptus and the endometrium to ensure normal conceptus development and secretion of interferon tau (IFNT), the signal for maternal recognition of pregnancy in ruminants. In turn, IFNT acts in an antiluteolytic manner to prevent the pulsatile release of prostaglandin $F_{2\alpha}$ (PGF) that would result in the structural and functional termination of the corpus luteum (CL). P4 produced by the CL is unequivocally required for the maintenance of pregnancy [1]. IFNT is produced by the mononuclear cells of the elongating trophectoderm in sheep and is secreted from Days 11 to 21 with maximal production on Day 16 of gestation in sheep [2]. Not only do interactions between the endometrium and conceptus prevent luteolysis to maintain pregnancy, but they also signal to increase uterine blood flow, endometrial remodeling, and secretion of histotroph (a mixture of nutrients, enzymes, growth factors, cytokines, electrolytes, vitamins, minerals, and water), all of which are necessary for conceptus development and establishment of pregnancy [3].

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Amino acids provide building blocks for the synthesis of peptides and proteins, which include molecules that initiate cell signaling cascades, serve as enzymes for metabolic reactions, or regulate the transport of nutrients [4]. The abundance of certain amino acids, including arginine, citrulline, and ornithine, is greater in uterine flushings of pregnant than cyclic ewes during mid- to late-diestrus [5]. Additionally, the unusually high abundance of arginine and ornithine increases 8- and 5-fold, respectively, in the uterine flushings of pregnant ewes between Days 10 to 16 of gestation [5]. Importantly, these dynamic changes in the abundance of amino acids during pregnancy are coordinate with rapid elongation of the ovine conceptus and its production of IFNT.

Amino acids are not only used to build complex molecules, but products of amino acid catabolism are also utilized in a variety of biological pathways important for the establishment and maintenance of pregnancy [4]. In particular, arginine can be catabolized to generate nitric oxide (NO), creatine, agmatine, and polyamines (putrescine, spermidine, and spermine) [4]. Similar to NO, agmatine and polyamines are involved in angiogenesis and contribute to trophectoderm cell function and mammalian embryogenesis [6–9]. Under physiological conditions, polyamines are fully protonated and are considered ‘supercations’ that interact with anionic molecules, including nucleic acids and proteins, which affect key cellular functions required for cell growth, proliferation, migration, and differentiation, including gene transcription, mRNA translation, protein synthesis, and protein functions [10–12]. As polyamines are involved in many cellular regulatory pathways, and an over-abundance of polyamines is generally associated with cellular hyperproliferation [13]; therefore, the abundance of these molecules is tightly regulated at

the levels of enzymatic biosynthesis, interconversion, degradation, and transport across cell membranes [14].

Polyamines are critical for development of ovine conceptuses, as those that are unable to generate polyamines do not develop properly and are physiologically and morphologically abnormal [15,16]. Additionally, conceptuses that fail to synthesize polyamines produce significantly less IFNT compared to conceptuses that develop normally, which illustrates a loss of functional capacity and decreased ability to signal pregnancy recognition to the uterus [15], so that such pregnancies are likely to fail. However, it is unknown how this crosstalk between the endometrium and the conceptus during the peri-implantation period of pregnancy contributes to endometrial synthesis of amino acids, agmatine, and polyamines that are critical for survival of the conceptus and thus success of the pregnancy. As embryonic mortality in mammals is estimated to be between 20-40%, with two-thirds of these losses in the peri-implantation period of pregnancy [17], it is imperative to understand how the uterus responds to endocrine (P4) and paracrine (IFNT) signals to support conceptus development.

While the previous reports described above have demonstrated that the abundance of amino acids are likely induced by conceptus-associated signaling molecules such as IFNT [18], the independent and/or synergistic effects of P4 and IFNT on the abundance of amino acids, as well as polyamines, in histotroph on Day 16 of pregnancy have not been elucidated. Furthermore, P4, independently of IFNT, increases the mRNA levels of cationic amino acid transporter solute carrier family 7 member 1 (*SLC7A1*) in the luminal (LE) and glandular (GE) epithelia of ewes after long-term treatment with both P4 and

IFNT, while mRNA expression of the cationic amino acid transporter solute carrier family 7 member 2 (*SLC7A2*) is induced by P4 and stimulated by IFNT after short term P4 and IFNT treatment [19], which suggests increased transport of arginine into the uterine lumen in response to both maternal and conceptus-derived signals. However, is it unknown how these independent and/or synergistic effects of P4 and IFNT affect the endometrial transporters for polyamines themselves, rather than their precursors. Therefore, the aim of this study was to determine if genes required for the synthesis, catabolism, secretion, and/or transport of agmatine and polyamines are induced by P4 and/or further stimulated by IFNT.

## Materials and Methods

### *Animals*

Estrous cycles of mature Rambouillet ewes (*Ovis aries*, n=24) were synchronized using a progesterone intravaginal insert (CIDR, Zoetis) for 12 days followed by an intramuscular injection of prostaglandin F2α (20 mg Lutalyse, Zoetis) upon removal of the CIDR. Ewes were observed for estrus (designated as Day 0) in the presence of a vasectomized ram. All ewes had exhibited at least two estrous cycles of normal duration (16-18 days) prior to synchronization of estrus. Experimental procedures followed the Guide for the Care and Use of Agriculture Animals in Research and Teaching and approved by the Institutional Animal Care and Use Committee of Texas A&M University (AUP IACUC 2018-0173).

*Surgical procedures and catheter placement*

On Day 7 of the estrous cycle, ewes were surgically fitted with bilateral intrauterine catheters as described previously [20]. Briefly, ewes were fasted for 12 h prior to surgery, but water was available ad libitum. Anesthesia was induced and maintained with Isoflurane. After a surgical plane of anesthesia was achieved, the reproductive tract was exposed via a mid-ventral laparotomy and catheters were inserted into the lumen of the respective uterine horn 2 cm below the utero-tubal junction. Catheters had two sets of cuffs that allowed the catheters to be secured to the oviduct and the intercornual ligament near the uterine body. The catheters were exteriorized through the flank, and the external portion of the catheters were kept in a plastic zip-lock bag that was stored in a cloth pouch sutured to the skin. Catheters were cleared with saline and capped with a sterile nail when not in use. Each animal was monitored daily for overall health and the catheters were monitored and cleaned daily to ensure that they were maintained properly.

*Preparation of proteins*

Recombinant ovine interferon tau (IFNT) was produced as described previously [21]. IFNT ($1 \times 10^8$ antiviral units) was prepared for delivery into the uterine lumen via the intrauterine catheters by diluting stock IFNT (1.5 mg/mL) such that 12.5 µg IFNT in a volume of 1 mL per uterine horn was delivered twice daily (total of 25 µg/uterine horn/day). Control serum proteins were isolated from a singular jugular blood sample from a mature ram. Blood was allowed to clot, and serum was collected by centrifugation at 3000 x *g* for 30 min at 4°C, filtered (0.45 µm), and stored at -20°C until used. A Bradford

assay (Biorad) was used to determine concentrations of protein in serum. Serum proteins were diluted in saline to 12.5 µg/mL and 1 mL was delivered into each uterine horn twice daily (total of 25 µg/horn/day). Control and IFNT proteins were stored at -20°C and brought to room temperature before intrauterine injections.

*Experimental design*

Beginning on Day 8 of the estrous cycle, ewes were assigned randomly to receive daily (0700h) intramuscular injections of 50 mg progesterone (P4) (P8783 Sigma-Aldrich), dissolved in ethanol and suspended in corn oil vehicle, or 50 mg P4 and 75 mg progesterone receptor antagonist (mifepristone, also known as RU486; M8046 Sigma-Aldrich) (RU486+P4), which was dissolved in ethanol and suspended in corn oil vehicle. Starting on Day 11 of the estrous cycle, ewes received twice daily (0700h and 1700h) intrauterine injections via the intrauterine catheters of either control serum proteins (25 µg/uterine horn/day [CX]) or IFNT (25 µg/uterine horn/day [IFNT]). This resulted in four treatment groups: 1) P4+CX (n=6); 2) P4+IFNT (n=6), 3) RU486+P4+CX (n=6); and 4) RU486+P4+IFNT (n=6). Injections of proteins were followed by 50 mg of ampicillin (Gibco) in 0.1 mL of saline and then 0.9 mL saline to clear the catheter.

On Day 16 of the estrous cycle, ewes were euthanized and then hysterectomized after collecting blood via jugular venipuncture into an EDTA-coated vacutainer tube. One ewe in the RU486+P4+CX treatment group did not have a corpus luteum present and was thus removed from the study. The uteri were flushed with 10 mL phosphate buffered saline (PBS, pH 7.2), and uterine flushings were centrifuged at 10,000 x *g* for 15 min at 4°C and

the supernatant was stored at -20°C until further analyses. Sections of uteri and endometria were collected and either frozen in liquid nitrogen and stored at -80°C or fixed in 4% paraformaldehyde and dehydrated in 70% ethanol for 48 h prior to embedding in Paraffin wax. Blood was centrifuged at 8,000 x *g* for 10 min at 4°C to obtain plasma. Plasma was stored at -20°C until analyzed.

*Analyses of amino acids, agmatine, and polyamines*

Concentrations of amino acids, polyamines, and agmatine were determined in uterine flushings and plasma by high performance liquid chromatography (HPLC) as described previously [15,22] with minor modifications [23]. Briefly, each sample (100 µL) was deproteinized by the addition of 100 µL 1.5 M $HClO_4$ followed by 50 µL 2 M $K_2CO_3$, then centrifuged at 10,000 x *g* for 3 min. The neutralized supernatant was used for analysis using an HPLC method involving precolumn derivatization with *o*-phthaldialdehyde (OPA) reagent I or II. OPA reagent I (for agmatine and polyamines) was prepared by dissolving 50 mg of OPA (P0657 Sigma-Alrich) and 50 mg N-acetyl-cysteine (A9165 Sigma-Aldrich) in 1.25 mL of HPLC-grade methanol (Fisher Scientific) followed by the addition of 11.2 mL 0.04 M sodium borate (pH 9.5), and 0.5 mL of Brij-23 (B4184 Sigma-Aldrich). OPA reagent II (for amino acids) was prepared by dissolving 50 mg OPA in 1.25 mL HPLC-grade methanol, followed by the addition of 11.2 mL sodium borate (pH 9.5), 50 µL 2-mercaptoethanol (M3148 Sigma-Aldrich), and 0.5 mL of Brij-23 (B4184 Sigma-Aldrich). The assay mixture contained 1.4 mL of HPLC-grade water (Fisher Scientific), 100 µL of 1.2% benzoic acid (in 40 mM sodium borate, pH 9.5),

and 100 µL of sample. The assay mixture was derivatized in an autosampler (model 712 WISP, Waters) with 30 mmol/L OPA reagent 1 or II, and 15 µL of the derivatized mixture was injected into a Supelco 3-µm-reversed-phase C18 column (150 x 4.6 mm inner diameter, Sigma-Aldrich). Amino acids, polyamines, and agmatine were separated using a solvent gradient comprised of solution A (0.1 M sodium acetate, 18% methanol, and 1% tetrahydrofuran, pH 7.2) and solution B (HPLC-grade methanol). Amino acids, polyamines, and agmatine in the samples were quantified relative to authentic standards using Millenium-32 Software (Waters). Total amounts of amino acids, polyamines, and agmatine were calculated by multiplying the concentration by the volume of recovered uterine flush.

*RNA extraction, cDNA synthesis, and quantitative real-time PCR analysis*

Total RNA was extracted from ovine endometria using Trizol (15596018 Invitrogen) as per manufacturer's instructions and treated with RNase-Free DNase (79254 Qiagen) before further purification using the RNeasy Mini Kit (74104 Qiagen) as per the manufacturer's instructions. The RNA was quantified using a NanoDrop (ND-1000 Spectrophotometer), and only those samples with a 260/280 value > 2 were used. First-strand cDNAs were synthesized from 1 µg of total RNA using oligo (deoxythymidine) primers and SuperScript II Reverse Transcriptase (11904018 Invitrogen) as per the manufacturer's instructions. Negative controls without reverse transcriptase were included to verify a lack of genomic contamination.

Quantitative polymerase chain reaction (qPCR) was performed using the ABI prism 7900HT system (Applied Biosystems) with Power SYBR Green PCR Master Mix (4309155 Applied Biosystems) as specified by the manufacturer to determine the level of expression of mRNAs encoding genes of interest. Primer sequences are listed in Supplementary Table 1. Primer specificity and efficiency were tested by the inclusion of a dissociation curve for the qPCR reaction and by generating a standard curve from pooled cDNA, respectively. Standard curves were prepared by serial dilution (1:2 to 1:256) of pooled cDNA in nuclease-free water. All primer sets used amplified a single product as determined by a dissociation curve with a single peak and had an efficiency of between 95% and 105%. Each well contained 10% cDNA, 30% nuclease-free water, 10% primer, and 50% SYBR Green reaction mix in a 10 µL reaction volume. All reactions were performed at an annealing temperature of 60°C. For primers of interest with low expression (i.e., Cq values above 30; *ADC* and *AGMAT*), 1 µL of cDNA was used in a modified pre-amplification step [23,24] using a Thermocycler (Eppendorf AG). Briefly, cDNA, nuclease-free water, forward and reverse primers, and SYBR were combined as described above in a 20 µL volume. The reaction was performed with the following conditions for 15 cycles: 94°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec, and the pre-amplified product was used for qPCR analyses. The reference genes tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ)* and TATA-box binding protein (*TBP)* were determined to have stable expression in endometria using geNORM software (M value<1.5) after testing for effects of hormone,

protein, and their combination ($P$>0.1), and were used to normalize expression of mRNAs of interest. The abundances of mRNAs were quantified using the ΔΔCq method.

*Immunohistochemical localization of proteins*

A summary of antibody information is listed in Supplementary Table 2. Paraffin-embedded sections of endometria were cut (5 µm) then deparaffinized and rehydrated through Citrisolv (1601 Decon Laboratories Inc) and decreasing concentrations of ethanol to double distilled water. Antigen retrieval was performed using either 0.1 M boiling citrate buffer (pH 6), Tris-based boiling buffer (pH 9) (H-3301-250 Vector), or 0.5 mg/mL Protease (P5147 Sigma-Aldrich) in 1x PBS (Supplementary Table 2). To block endogenous peroxide activity, slides were incubated for 15 min at room temperature in 0.3% hydrogen peroxide in methanol. Sections were washed with 1x PBS and then incubated for 1 h at room temperature in normal horse serum (PK-6200 Vectastain Elite Universal ABV Kit, Vector Laboratories) to block non-specific binding sites. Sections were incubated overnight in primary antibodies for ADC (1.02 mg/mL, Abcam, ab192771), AGMAT (1 mg/mL, Abcam, ab231894), AMD1 (160 µg/mL, Proteintech 11052-1-AP), ARG2 (393 µg/mL, Proteintech, 14825-1-AP), MAT2B (360 µg/mL, Proteintech, 1592-1-AP), ODC1 (0.92 mg/mL, Abcam, ab97395), PAOX (160 µg/mL, Proteintech, 18972-1-AP), SAT1 (173 µg/mL, Proteintech, 10708-1-AP), CAT1 (0.4 mg/mL, Abcam, ab37588), SMS (133 µg/mL, Proteintech, 15979-1-AP), SMOX (473 µg/mL, Proteintech, 15052-1-AP), and SRM (333 µg/mL, Proteintech, 19858-1-AP) (Supplementary Table 2) at 4°C in a humidified chamber. Rabbit immunoglobulin G (IgG)

(I-1000-5 Vector Laboratories) at the same concentration and dilution for each primary antibody was used as a negative control. After overnight incubation, sections were washed in 1x PBS and incubated at 37°C for 1 h in a humidified chamber with biotinylated anti-rabbit IgG secondary antibody (0.005 mg/ml secondary antibody and 1.5% normal horse serum diluted in 1x PBS, Vector Laboratories). Sections were then incubated in a humidified chamber at 37°C for 30 min in ABC Reagent (Vectastain Elite Universal ABC Kit). Slides were washed in 0.05 M Tris-HCl (pH 8), and immunoreactive protein was visualized using 3,3'-diaminobenzidine tetrahydrochloride (D5637 Sigma) in 0.05 M Tris-HCl with hydrogen peroxide. Sections were counterstained with hematoxylin and dehydrated through increasing concentrations of ethanol to Citrisolv before affixing coverslips with Permount mounting medium (SP15-100 Fisher Scientific). Images of representative fields were captured using a Nikon Eclipse Ni microscope and NIS Elements Software (Nikon).

### *Statistical analysis*

Statistical analyses were performed using either SAS (Version 9.4) or Genstat. Normality of the distribution of data was assessed and a P value of $\leq 0.05$ indicated that the data were not normally distributed. The ROUT test for outliers was performed to identify data points for exclusion. Normality was reassessed after exclusion of the outliers. Transformations were carried out if necessary to achieve a Gaussian distribution. ANOVA with a post-hoc Tukey test was performed on data with a normal distribution. If data were not normally distributed, Kruskal-Wallis and Mann Whitney tests were performed. P

values less than 0.05 were considered significant and those less than 0.1 were considered to indicate a trend towards significance.

## Results

### *Abundance of the amino acids, agmatine, and polyamines in plasma and uterine flushings*

To determine if P4 and/or IFNT altered the abundances of amino acids, agmatine, and polyamines in either plasma or histotroph, HPLC analyses were conducted. All HPLC data for uterine flushings are summarized in Supplementary Table 4, and all HPLC data for plasma are summarized in Supplementary Table 5.

In uterine flushings, recoverable amounts of arginine, citrulline, glycine, ornithine, agmatine, and putrescine were affected by treatment ($P<0.05$) (Table 1), while glutamine and leucine tended to be different ($P<0.10$) (Supplementary Table 4). Arginine ($P<0.05$) and citrulline ($P<0.05$) were less abundant in uterine flushings from P4+CX treated than RU486+P4+CX treated ewes. Glutamine ($P<0.10$) tended to be less abundant in uterine flushings from ewes treated with P4+IFNT compared to RU486+P4+IFNT treated ewes (Supplementary Table 4). Conversely, the abundance of agmatine was greater ($P<0.05$) in uterine flushings from ewes that received P4+IFNT compared to ewes treated with RU486+P4+IFNT. There was more glycine ($P<0.05$) in uterine flushings from P4+IFNT treated compared to P4+CX treated ewes, but less glycine in uterine flushings from RU486+P4+IFNT than RU486+P4+CX treated ewes, suggesting a synergistic effect of P4 and IFNT on transport of glycine into the uterine lumen. There was less ornithine in

uterine flushings from ewes treated with P4+CX compared to ewes treated with RU486+P4+CX ($P<0.05$). Intriguingly, there was more putrescine in uterine flushings of ewes treated with RU486+P4+IFNT compared to those treated with RU486+P4+CX ($P<0.05$), suggesting a paracrine-induced effect of IFNT on its accumulation in the uterine lumen, irrespective of P4. The concentration of putrescine in plasma was greater ($P<0.05$) in ewes treated with RU486+P4+IFNT compared to the other treatment groups (Supplementary Table 5).

**Table II-1 Total amounts (concentration x volume) of amino acids (mg) and polyamines (µg) in uterine flushings from ewes treated with P4+CX (n=6), P4+IFNT (n=6), RU486+P4+CX (n=5), or RU486+P4+IFNT (n=6) that were significantly affected by treatment. Values represent means ± SEM. Different superscripts across treatment columns were different (P<0.05) if bolded or tended to be different (P<0.1) if italicized.**

| | Treatment | | | |
|---|---|---|---|---|
| Nutrient | P4+CX | P4+IFNT | RU486+P4+CX | RU486+P4+IFNT |
| Polyamines | | | | |
| Agmatine | $0.15 \pm 0.04^{a}$ | $0.11 \pm 0.04^{a}$ | $0.07 \pm 0.04^{ab}$ | $0.06 \pm 0.03^{b}$ |
| Putrescine | $0.85 \pm 0.13^{ab}$ | $0.68 \pm 0.15^{ab}$ | $0.54 \pm 0.10^{a}$ | $1.1 \pm 0.44^{b}$ |
| Spermidine | $0.67 \pm 0.04$ | $0.69 \pm 0.07$ | $0.61 \pm 0.1$ | $0.60 \pm 0.04$ |
| Spermine | $11.8 \pm 0.8$ | $13.1 \pm 1.1$ | $13.3 \pm 1.7$ | $9.7 \pm 1.2$ |
| Amino Acids | | | | |
| Arginine | $16.4 \pm 2.7^{a}$ | $12.03 \pm 3.5^{a}$ | $26.9 \pm 6.5^{b}$ | $21.3 \pm 3.2^{ab}$ |
| Citrulline | $9.7 \pm 1.2^{a}$ | $8.9 \pm 2.5^{a}$ | $18.3 \pm 3.3^{b}$ | $13.9 \pm 2.9^{ab}$ |
| Glycine | $122.5 \pm 7.6^{a}$ | $158.1 \pm 15.7^{b}$ | $142.4 \pm 18.0^{ab}$ | $109.7 \pm 7.6^{a}$ |
| Ornithine | $0.96 \pm 0.37^{a}$ | $1.3 \pm 0.58^{ab}$ | $3.1 \pm 1.3^{b}$ | $2.7 \pm 0.93^{b}$ |

*Expression of mRNAs in endometria*

The expression of mRNAs for interferon stimulated gene 15 (*ISG15*) and signal transducer and activator of transcription 1 (*STAT1*), and progesterone receptor (*PGR*) validated expected effects of IFNT and P4, respectively, on the ovine uterine endometrium (Figure 1). The expression of mRNAs involved in polyamine metabolism and transport were quantified to determine effects of P4 and IFNT on the endometrium, including (see Figure 1 and Supplementary Table 3): arginine decarboxylase (*ADC*), agmatinase (*AGMAT*), *S*-adenosylmethionine (SAM) decarboxylase (*AMD1*), arginase 2 (*ARG2*), argininosuccinate lyase (*ASL*), methionine adenosyltransferase 2B (*MAT2B*), ornithine aminotransferase (*OAT*), ornithine decarboxylase (*ODC1*), polyamine oxidase (*PAOX*), spermine/spermidine acetyltranseferase 1 (*SAT1*), spermine oxidase (*SMOX*), spermine synthase (*SMS*), spermidine synthase (*SRM*), solute carrier family 3 member 2 (*SLC3A2*), solute carrier family 7 member 1 (*SLC7A1*), solute carrier family 7 member 2 (*SLC7A2*), solute carrier family 12 member 8 (*SLC12A8*), solute carrier family 22 member 2 (*SLC22A2*), solute carrier family 22 member 3 (*SLC22A3*), and solute carrier family 47 member 1 (*SLC47A1*).

The expression of *ISG15* ($P<0.001$) and *STAT1* ($P<0.001$) mRNAs, both of which are classically stimulated by IFNT [25], was up-regulated in endometria of ewes treated with IFNT compared to control protein as expected (Figure 1a and 1b). Additionally, the expression of *PGR* mRNA was less ($P<0.001$) in endometria of ewes treated with P4 compared to ewes treated with the PGR antagonist RU486 as expected (Figure 1c).

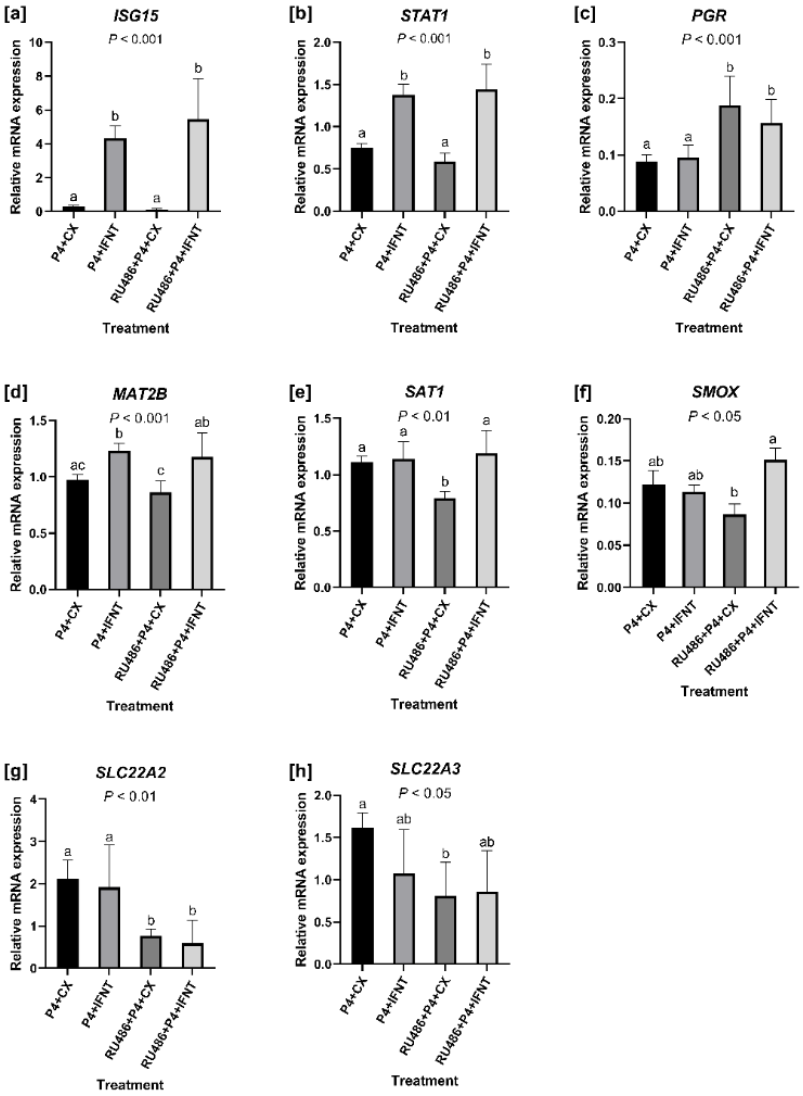


**Figure II-1. Expression of mRNAs required for metabolism and transport of polyamines affected by progesterone (P4) and interferon tau (IFNT).** To validate the effects of both interferon tau (IFNT) and progesterone (P4), the expression of mRNAs for selected genes were quantified via qPCR. ISG15 [a] and *STAT1* [b] are two genes classically stimulated by IFNT, and the expression of both their mRNAs was up-regulated only in ewes treated with IFNT. The expression of progesterone receptor (*PGR*) mRNA [c] was down-regulated in ewes treated with P4 only, confirming auto-downregulation of *PGR* by P4. The expression of *MAT2B* mRNA [d] was up-regulated (P<0.001) by IFNT treatment. The expression of both *SAT1* [e] (P<0.01) and *SMOX* [f] (P<0.05) mRNAs were up-regulated by IFNT, independent of P4. The expression of *SLC22A2* [g] (P<0.01) and *SLC22A3* [h] (P<0.05) mRNAs increased in response to progesterone. Mean relative values and SEM are presented. n=5-6 samples per treatment group.

Of the key enzymes involved in polyamine metabolism, expression of *MAT2B*, *SAT1*, and *SMOX* mRNAs in endometria were affected by treatment (Figure1d, 1e, and 1f). Expression of *MAT2B* mRNA was greater ($P<0.001$) (Figure 1d) and expression of *AGMAT* mRNA tended to be greater ($P<0.1$) (Supplementary Table 3) in endometria of ewes treated with IFNT compared to control proteins, which suggests a direct effect of IFNT on two pathways leading to the synthesis of polyamines – one from arginine (via agmatine and AGMAT; agmatine provides the 4-carbon skeleton for putrescine) and one from methionine (via MAT2B; decarboxylated s-adenosylmethionine, a product of methionine catabolism, provides a 3-carbon amine group for the synthesis of spermidine and spermine).

The expression of mRNAs for *SAT1* ($P<0.01$) and *SMOX* ($P<0.05$) was greater in endometria of ewes treated with RU486+P4+IFNT compared to RU486+P4+CX treated sheep (Figure 1e and 1f), again suggesting an IFNT-induced effect. The expression of *SLC22A2* mRNA was greater ($P<0.01$) in endometria of ewes in both groups treated with P4 compared to ewes that received RU486+P4 (Figure 1g), while the expression of SLC22A3 mRNA was greater in endometria ($P<0.05$) in ewes treated with P4+CX compared to all other treatment groups (Figure 1h).

### *Cell-specific immunolocalization of proteins in endometria*

Representative images of cell-specific localization of proteins involved in polyamine metabolism are shown in Figures 2-6.

MAT2B protein is responsible for converting methionine to SAM and was localized to uterine LE, sGE, and GE as well stratum compactum (SC), myometrium (Myo), and blood vessels (BV) in the endometrium of ewes in of all treatment groups (Figures 2 a-d and f-i). MAT2B protein was more abundant in uterine LE, sGE, and SC of ewes treated with IFNT (Figures 2a-d).

AGMAT converts agmatine to putrescine, and it also localized to uterine LE, sGE, and GE, BV, and Myo (Figures 3 a-d and f-i). ARG2 converts arginine to ornithine that is then converted to putrescine by ODC1. ARG2 localized to uterine sGE, SC, BV, and Myo, but not uterine GE, in all treatment groups (Figures 3 k-n and p-s). Taken together, these results suggest that agmatine is used to produce putrescine in all secretory epithelial subtypes in the endometrium, while putrescine derived from ornithine is produced primarily in uterine sGE.

AMD1 converts SAM to decarboxylated SAM, which is required sequentially by SRM and SMS to convert putrescine to spermidine and spermidine to spermine, respectively. AMD1 protein localized to uterine LE, sGE, some deep GE, and Myo in all treatment groups (Figures 4 a-d and f-i), whereas SRM protein localized to uterine sGE, SC, BV, and Myo in all treatment groups (Figure 4k-s). In ewes treated with IFNT, however, SRM protein was more abundant in uterine LE and GE compared to ewes treated with CX proteins (Figures 4 l,q and n,s).

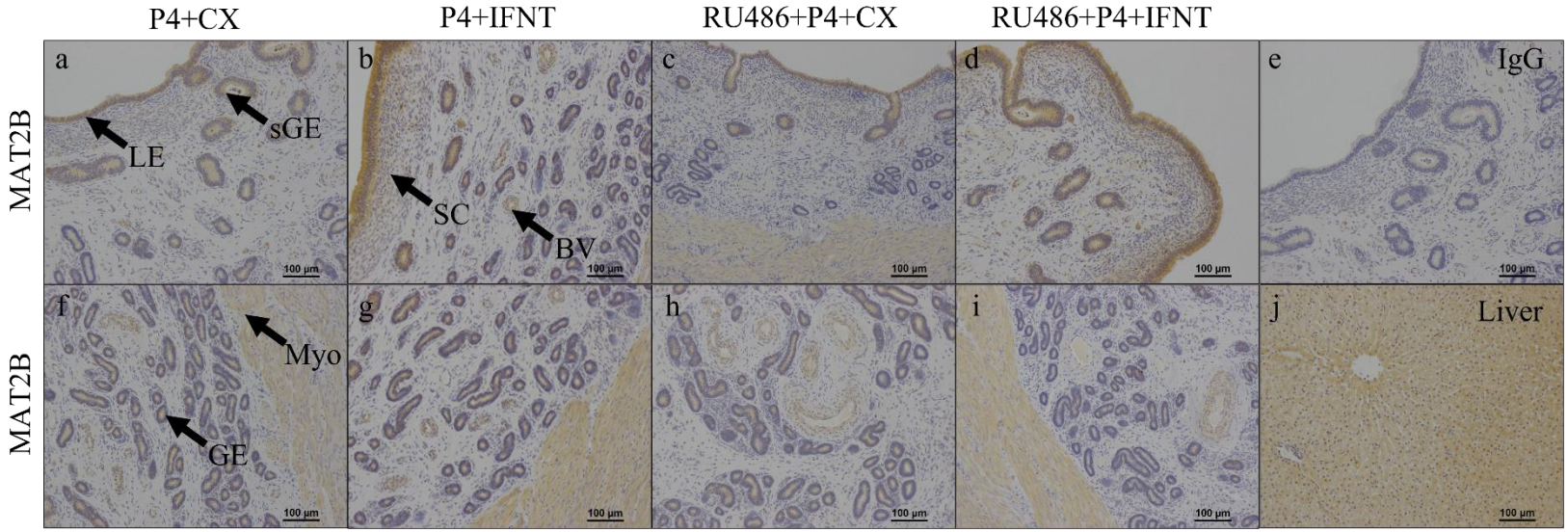


**Figure II-2: Representative images of the cell-specific localization of methionine adenosyltransferase 2B (MAT2B) protein in endometria of ewes treated with either progesterone (P4) or RU486 and P4 (RU486+P4), and either control serum proteins (CX) or interferon tau (IFNT).** MAT2B protein localized to uterine luminal (LE) and superficial glandular (sGE) epithelia, as well as the myometrium (Myo) and blood vessels (BV) in all treatment groups (a-d and f-i). In ewes treated with IFNT (b,g and d,i), MAT2B protein was greatest in the uterine LE and stromal stratum compactum (SC). Negative (e) and positive (j) controls are shown.

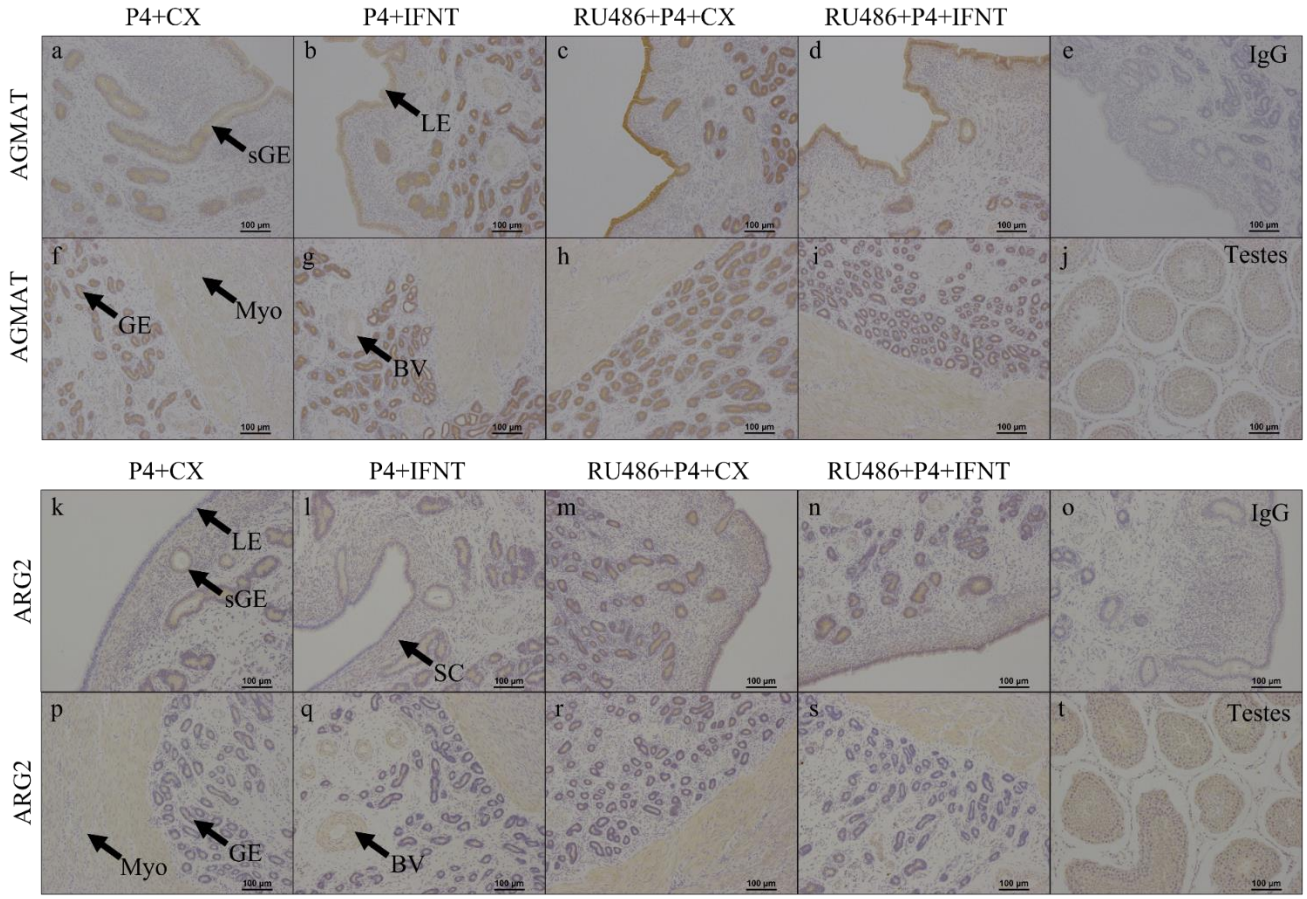


**Figure II-3 Representative images of the cell-specific localization of agmatinase (AGMAT) (a-j) and arginase 2 (ARG2) (k-t) proteins in endometria of ewes treated with either progesterone (P4) or RU486 and P4 (RU486+P4), and either control serum proteins (CX) or interferon tau (IFNT).** AGMAT protein localized to uterine luminal (LE), superficial glandular (sGE) and glandular (GE) epithelia, as well as the myometrium (Myo) (a-d and f-i). ARG2 protein localized to sGE and blood vessels (BV) (k-n and p-s). Negative (e and o) and positive (j and t) controls are shown.

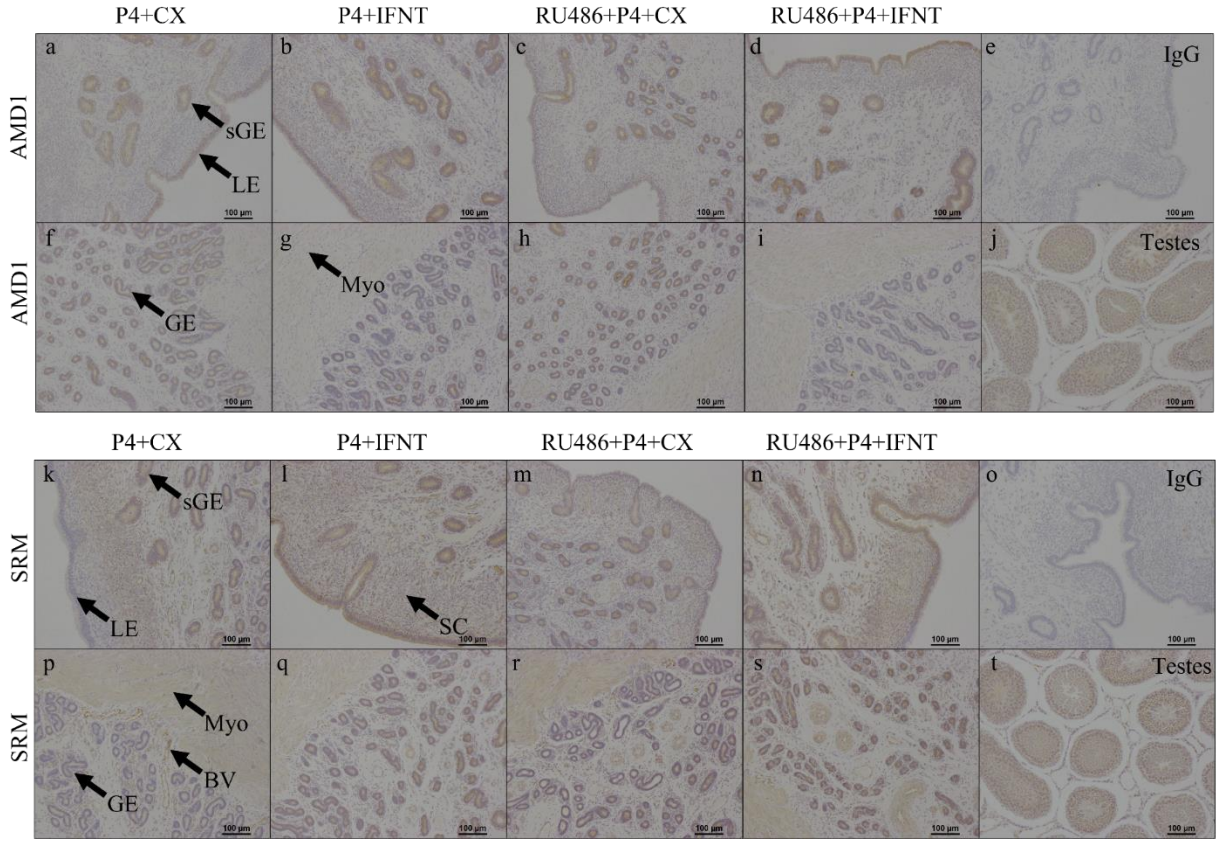


**Figure II-4 Representative images of cell-specific localization of s-adenosylmethionine decarboxylase 1 (AMD1) (a-j) and spermidine synthase (SRM) (k-t) proteins in endometria of ewes treated with either progesterone (P4) or RU486 and P4 (RU486+P4), and either control serum proteins (CX) or interferon tau (IFNT).** AMD1 protein localized to uterine luminal (LE) and superficial glandular (sGE), and the myometrium (Myo) (a-d and f-i). SRM localized to uterine sGE and stromal stratum compactum (SC) (k-n and p-s), but protein expression increased in the uterine LE and deep glandular epithelia (GE) in ewes treated with IFNT (l,q and n,s). SRM also localized to blood vessels (BV) and Myo (k-n and p-s). Negative (e and o) and positive (j and t) controls are shown.

SMS converts decarboxylated SAM and spermidine to spermine, then SMOX oxidizes spermine to spermidine. Both enzymes were immunolocalized to the uterine LE and sGE (Figures 5a-d and k-n), and SMS protein was also detected in deeper uterine GE (Figure 5f-i). SMOX protein was expressed in some, but not all, uterine GE (Figure 5p-s).

Both PAOX and SAT1 are responsible for polyamine interconversions, and both proteins immunolocalized to uterine LE and sGE, but not GE (Figure 6). Similarly, ADC and ODC1, which are responsible for the catabolism of arginine to agmatine and ornithine to putrescine, respectively, were both expressed by the uterine LE and sGE, but not GE (Supplementary Figure 1).

SLC7A1, a cationic amino acid transporter which can transport both arginine and ornithine, was expressed by uterine LE, sGE, and BV, as well as deep uterine GE of ewes treated with P4 (Supplementary Figure 2).

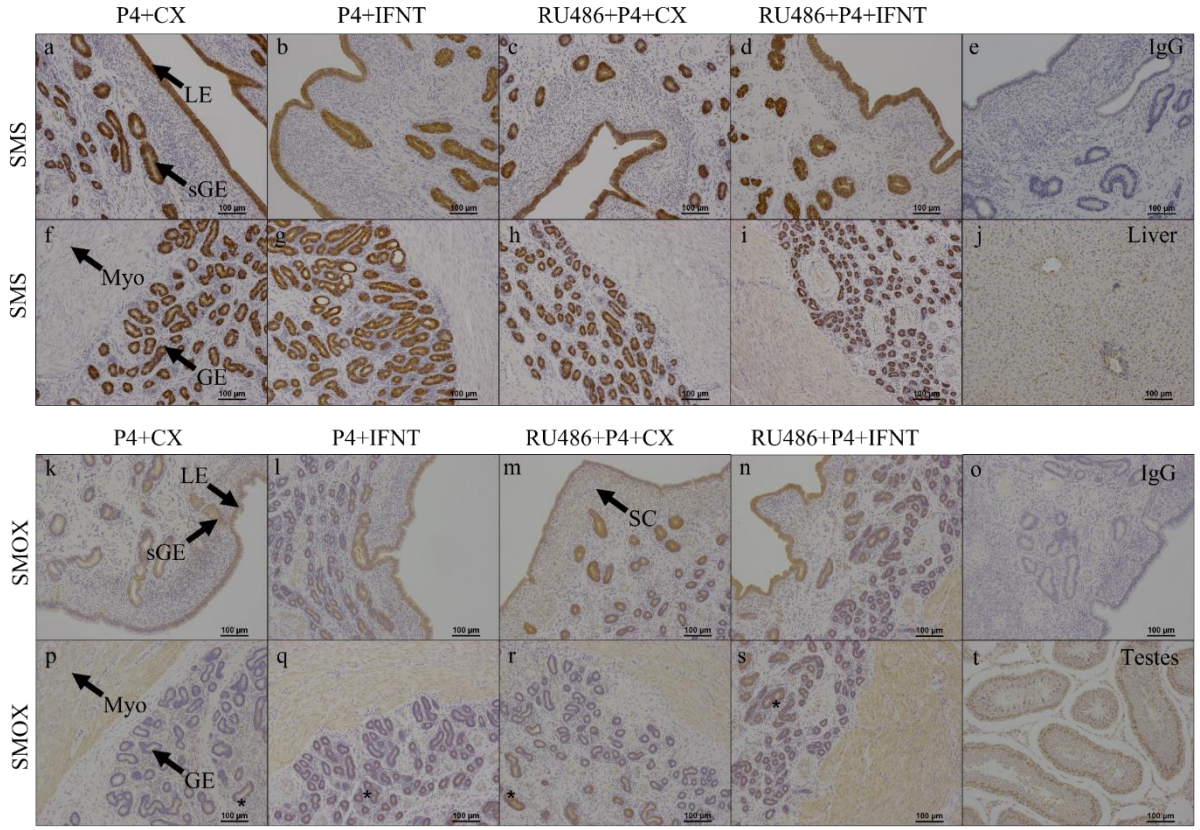


**Figure II-5 Representative images of cell-specific localization of spermine oxidase (SMOX) (a-j) and spermine synthase (SMS) (k-t) proteins in endometria of ewes treated with either progesterone (P4) or RU486 and P4 (RU486+P4), and either control serum proteins (CX) or interferon tau (IFNT).** SMOX protein localized to uterine luminal (LE) and superficial glandular (sGE) epithelia in all treatment groups (a-d), with a gradual decrease in expression in the deeper glandular epithelia (GE) (f-i). Positive GE are indicated by asterisks. SMOX protein also localized to the stromal stratum compactum (SC) (a-d) and myometrium (Myo) (f-i). SMS protein localized to uterine LE, sGE, and GE (k-n and p-s). Negative (e and o) and positive (j and t) controls are shown.

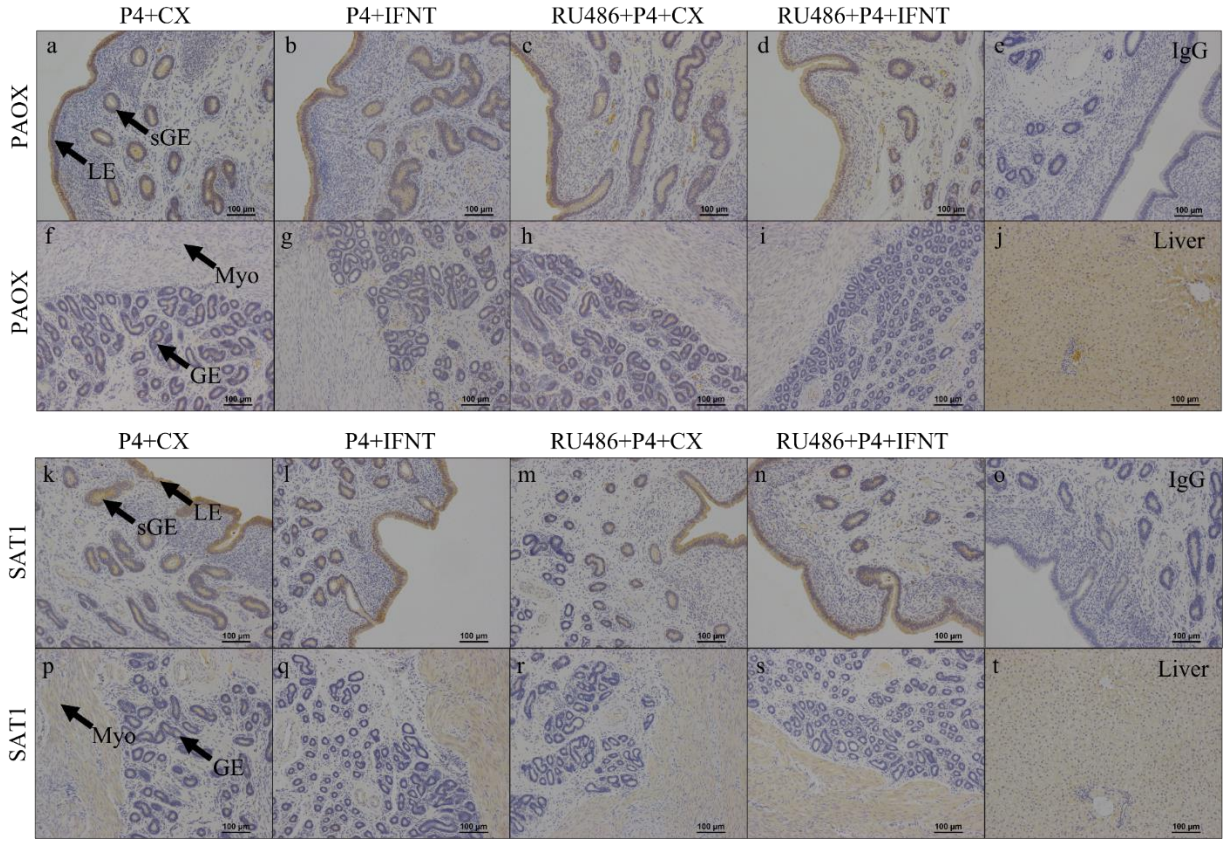


**Figure II-6 Representative images of cell-specific localization of polyamine oxidase (PAOX) (a-j) and N1-spermine/spermidine acetyltransferase 1 (SAT1) (k-t) proteins in endometria of ewes treated with either progesterone (P4) or RU486 and P4 (RU486+P4), and either control serum proteins (CX) or interferon tau (IFNT).** PAOX protein localized to uterine luminal (LE) and superficial glandular (sGE) epithelia, but not deeper glandular epithelia (GE) in all treatment groups. Similarly, SAT1 protein localized to the uterine LE and sGE, but not GE. Negative (e and o) and positive (j and t) controls are shown.

## Discussion

Polyamines are expressed ubiquitously in most eukaryotic cells and are present in the histotroph of livestock species [10,26,27]. While the specific functions and regulatory pathways of these compounds in various organs of the reproductive system are still being identified, some reviews are available [9,28]. In this study, it was hypothesized that P4 and/or IFNT stimulate the endometrial expression of enzymes involved in synthesis, metabolism, and transport of polyamines by cells of the ovine uterus. The novel results of this study revealed that: 1) the abundance of arginine, citrulline, ornithine, and agmatine in uterine flushings is likely regulated by P4; 2) putrescine in uterine flushings may be regulated by IFNT signaling independent of P4; 3) the mRNA expression of *MAT2B*, *SAT1*, and *SMOX*, genes that code for enzymes in polyamine biosynthesis and interconversion, are affected by IFNT independent of P4; 4) cell-specific endometrial expression of MAT2B and SRM proteins are altered by IFNT; and 5) the mRNA expression of *SLC22A2* and *SLC22A3*, genes that code for transporters of agmatine and polyamines, affected regulated by P4. Collectively, these results significantly advance understanding of the complex endocrine and paracrine regulation of polyamine synthesis, interconversion, and transport in the ovine endometrium. In addition, results of the present study suggest that P4 and IFNT, crucial signals for the maintenance of pregnancy, may also regulate the composition of polyamines in histotroph that are vital for conceptus survival and development during the peri-implantation period of pregnancy. This study is, to our knowledge, the first to describe effects of P4 and IFNT alone or in combination, as well as in the presence of a PGR antagonist, on the expression and cell-specific

localization of enzymes involved in polyamine biosynthesis and interconversion, as well as transport, in uteri of ewes.

In sheep, exposure of the uterus to P4 for 8-10 days results in down-regulation of PGR in uterine LE and sGE, while PGR remain detectable in deep uterine GE, stroma, and myometrium; this physiological phenomenon of P4 auto-downregulation of PGR is required for successful implantation and placentation of conceptuses [29]. RU486, a potent PGR antagonist, was used in this study to delineate the effects of P4-PGR signaling as it has been used as a PGR antagonist in a variety of species including sheep [30,31], pigs [32], rodents (reviewed by [33]), and primates [34]. Treatment of ewes with RU486 prevents P4-induced downregulation of PGR by binding to PGR with high affinity and preventing transcription of genes regulated by P4-PGR signaling [35,36].

ODC1 is typically regarded as the rate-limiting enzyme for polyamine synthesis as it is responsible for generating putrescine. However, AMD1 might also be considered rate-limiting as it is required for production of decarboxylated SAM, which interacts with either putrescine and SRM or spermidine and SMS to produce spermidine and spermine, respectively. It has been demonstrated in multiple rodent models that sex steroids regulate the expression of genes that code for rate-limiting enzymes for polyamine biosynthesis. Estrogen (E2) stimulates ODC1 enzymatic activity [37] and mRNA expression [38] in the endometrium, while E2 antagonists reduce ODC1 enzymatic activity [39,40] and mRNA expression [38] in rodents. The enzymatic activity of AMD1 (also known as SAMD or SAMDC) is also increased by E2 [41,42] in rats. Importantly, expression of *Odc*, *Srm*, *Amd1*, *Smox*, *Sat1*, and *Paox* mRNAs increases only at implantation sites on gestational

Day 5 in the mouse, implicating the production of polyamines as important players during implantation and placentation [38]. Furthermore, in a mouse model of delayed implantation, *Odc* mRNA is not expressed in the uterus until after embryo reactivation by E2, at which point expression of *Odc*, *Srm*, *Amd1*, *Sat1*, *Smox*, and *Paox* mRNAs increase significantly [38]. In ovariectomized mice, both P4 and E2 stimulate the expression of *Odc* mRNA, although expression increases to a much greater extent in animals treated with E2 compared to P4, while treatment with each steroid hormone and their respective antagonist ablates any hormone-induced increase in *Odc*.

In pigs, P4, rather than E2, appears to affect the endometrial expression of *SAT1* mRNA during the peri-implantation period of pregnancy. Treatment of gilts with either P4, E2, or P4+E2 from 4 days after onset of estrus and ovariectomy to Day 11 (notably, the period of conceptus elongation) results in increased expression of *SAT1* mRNA in gilts treated with P4 compared to those treated with E2 alone [43]. Intriguingly, when endometrial explants were exposed to medium conditioned by Day 12 filamentous conceptuses, a there was a significant increase in the expression of *SAT1* mRNA when compared to explants exposed to medium supplemented with E2, P4, or E2+P4, suggesting that *SAT1* mRNA expression is also associated with conceptus-secreted factors in pigs [43]. Although one of the primary conceptus-secreted factors in pigs is indeed E2, these results suggest that nonsteroidal conceptus-derived factors may mediate the expression of *SAT1* in the absence of systemic steroid hormones in porcine endometria. Leukemia inhibitory factor (LIF), a growth factor secreted by the endometrium of pigs during the peri-implantation period of pregnancy, has also been associated with up-

regulation of expression of *SAT1* mRNA in porcine endometrial explant cultures [44]. While LIF is not of conceptus origin, other factors secreted by the conceptus such as interferon gamma, interferon delta, and interleukin 1 beta 2 [45] may work in concert to induce the production of growth factors, including LIF, from the endometrium [46]. Endometrial-derived products may not only enhance growth and development of the conceptus, but may also act in a paracrine and/or autocrine manner to regulate the endometrial expression of enzymes in the polyamine synthesis pathway. This complex signaling highlights the importance of crosstalk at the conceptus-maternal interface during the peri-implantation period of pregnancy, which is crucial for the initiation of implantation and the establishment of a functional placenta. In sheep, leukemia inhibiting factor receptor (*LIFR*) mRNA is upregulated by IFNT primarily in uterine LE and sGE between Days 10 and 16 of pregnancy [47]. It is interesting to speculate that LIF in sheep may also be involved in the regulation of polyamine metabolism, similar to its effects in the pig. Interestingly, in this study, the expression of *SAT1* mRNA in endometria was up-regulated by IFNT in the absence of P4, as ewes treated with RU486+P4+IFNT had greater expression of *SAT1* mRNAs than RU486+P4+CX treated ewes. Moreover, *SAT1* mRNA expression was up-regulated by P4 alone, as P4+CX treated animals had increased expression of *SAT1* compared to RU486+P4+CX treated ewes. However, these effects appear to be independent of each other, as the expression of *SAT1* mRNA was not different between the P4+CX, P4+IFNT, and RU486+P4+IFNT treated ewes, suggesting that *SAT1* expression is not P4-induced and IFNT-stimulated. Rather, it appears that P4 upregulates the expression of *SAT1* mRNA and IFNT does not further stimulate this expression, but

IFNT in the absence of P4 may be sufficient to stimulate the endometrial expression of *SAT1* mRNA. When endometrial explants from cattle were exposed to recombinant ovine IFNT or elongated conceptuses, *SAT1* mRNA expression was up-regulated in an IFNT-dependent manner [48], supporting the hypothesis that IFNT influences *SAT1* mRNA expression in ruminants. Interestingly, *SAT1* mRNA expression was not affected by ovine IFNT in either isolated bovine endometrial epithelial cells or stromal cells in co-culture using a transwell insert [49], although this may highlight the importance of intimate contact between uterine epithelial and stromal cells to facilitate necessary signaling and up-regulation of key genes.

The endometrial expression of *SMOX* mRNAs was also up-regulated in response to IFNT in the absence of P4, whereas P4 alone was not sufficient to up-regulate expression of this gene. Collectively, it appears that IFNT is important in stimulating the endometrium to catabolize polyamines by either oxidation or acetylation. Acetylation of spermine and spermidine results in $N^1$-acetylspermine and $N^1$-acetylspermidine, respectively, and this reduces the abundance of intracellular polyamines, as high intracellular levels of polyamines are implicated in tumor growth [50], although rapid turnover of this enzyme may explain the difference between effects of IFNT on mRNA and protein expression [51]. However, it is not known if IFNT is acting on behalf of the conceptus, i.e., to increase polyamine catabolism and turnover for secretion into the uterine lumen for the conceptus to utilize, or rather acting in an altruistic manner, i.e., to stimulate polyamine catabolism to protect the endometrium from excessive accumulation of intracellular polyamines.

In this study, IFNT alone significantly increased endometrial expression of *MAT2B* mRNA and the abundance of MAT2B protein in uterine LE of ewes treated with IFNT. In cattle, *MAT2B* mRNA expression also appears to be regulated by IFNT, as *MAT2B* is up-regulated in endometrial explants exposed to either ovine recombinant IFNT or elongated conceptuses [48], although this IFNT effect was not reproduced in bovine epithelial and stromal cell co-culture [49]. In this study, while the endometrial expression of *AGMAT* mRNA was not significantly affected by either P4 or IFNT, there was a tendency for IFNT to increase expression of *AGMAT* mRNA expression. Taken together, these results suggest that IFNT, a conceptus-derived signal, can increase polyamine production from amino acids via agmatine formation from arginine and SAM generation from methionine in the ovine endometrium.

The total amounts of amino acids and polyamines in the uterine flushings are a direct indicator of the quantity and quality of histotroph [52,53]. This study was designed to ascertain the effects of P4 and IFNT in the presence or absence of functional PGR on the composition of amino acids and polyamines in histotroph. An advantage of the experimental design utilized for this study is that the question of whether nutrients taken up or produced by the conceptus was excluded. Interestingly, ewes treated with P4+CX had lower abundances of arginine in uterine flushings compared to ewes treated with RU486+P4+CX, but there were no significant differences in abundances of arginine in uterine luminal fluids between P4+CX and P4+IFNT treated ewes. This was surprising because total recoverable arginine has been reported to be greater in uterine flushings from pregnant than cyclic ewes on Day 16 after the onset of estrus [5]. Similarly, in this study,

abundances of citrulline and ornithine in uterine flushings were less in ewes treated with P4 compared to RU486+P4, despite previous reports of differences between pregnant and cyclic ewes on Day 16 [5]. Nevertheless, the findings of the present study lay the foundation for the hypothesis that conceptus-secreted factors affect abundances of amino acids in the uterine lumen. It has been demonstrated that bovine conceptuses secrete proteins, other than IFNT, during the time of maternal recognition of pregnancy, which are hypothesized to aid in communication with the endometrium to aid establishment of pregnancy [54,55]. Such factors in the sheep were not examined in this study, but warrant investigation.

Though the hormone and protein treatments did not affect expression of *SLC7A1* or *SLC7A2* mRNAs for transport of arginine and ornithine, there was less arginine and ornithine in uterine flushings from P4+CX treated compared to RU486+P4+CX treated ewes. A previous report indicated that that *SLC7A1* mRNA was not affected by either P4 or IFNT, but that expression of *SLC7A2* mRNA was induced by P4 and further stimulated by IFNT [5]. It is not known why *SLC7A2* mRNA expression in this study did not recapitulate the effects of IFNT as reported by Gao et al. [5], although that study utilized the antiprogestin ZK 136-317 rather than RU486. It has been suggested that ZK 136-317 has less anti-glucocorticoid receptor effects compared to RU486 and this may contribute to differences in results [56].

Uterine flushings from P4+CX and P4+IFNT treated ewes had greater amounts of agmatine compared to those from ewes treated with RU486+P4+IFNT, suggesting that P4 increases the abundance of agmatine in uterine secretions. Further, ewes treated with

RU486+P4+IFNT had greater amounts of putrescine in uterine flushings compared to ewes treated with RU486+P4+CX, suggesting that IFNT stimulation is sufficient to increase the abundance of putrescine in uterine secretions independent of P4. These results suggest that P4 and IFNT can work independently to stimulate the endometrium and alter the amounts of agmatine and putrescine in the uterus. Although there was only a tendency for expression of *AGMAT* mRNA to be up-regulated by IFNT, we speculate that this is responsible for the differences in amounts of agmatine and putrescine in the uterine lumen. However, P4 upregulated the expression of both *SLC22A2* and *SLC22A3* mRNAs, which are transporters for agmatine and polyamines across cell membranes. Collectively, these findings suggest that maternally-derived P4 may regulate the transport of agmatine and polyamines into the uterine lumen for utilization by the ovine conceptus whereas conceptus-derived IFNT may alter the metabolism of agmatine and polyamines within the ovine uterus during the peri-implantation period of pregnancy.

Localization of the enzymes in the polyamine pathway revealed compelling physiological evidence for cell-specific metabolism of polyamines in the ovine endometrium. Both ADC and ARG2 localized to uterine LE and sGE suggesting that only those cells utilize arginine for the production of agmatine and ornithine, respectively, while GE do not utilize arginine to produce agmatine and ornithine. Further, ODC1 protein localized to uterine LE and sGE as well, suggesting that those cells can utilize both arginine and ornithine to generate polyamines. Conversely, AGMAT protein is expressed in uterine LE, sGE, and GE, indicating that all three subtypes of secretory epithelial cells utilize agmatine to produce putrescine. This suggests that while uterine LE and sGE, the

two secretory epithelial subtypes in the endometrium adjacent to the uterine lumen—and, in pregnancy, the conceptus trophectoderm—utilize arginine, agmatine, and ornithine to produce putrescine, it is the deeper uterine GE that utilize agmatine exclusively to produce putrescine. Sheep and other ruminants have particularly high circulating levels of agmatine in plasma [21, Bazer and Wu unpublished observations]; therefore, uterine GE in the endometrium of ruminants may utilize agmatine directly from the systemic circulation to produce putrescine, thus bypassing the need for arginine and ornithine for the production of putrescine.

The results of this study revealed that cells in the endometrium can synthesize SAM from methionine. The expression of both MAT2B and AMD1 proteins by uterine LE, sGE, and GE to produce SAM and decarboxylated SAM, respectively, indicates that this pathway for production of polyamines is functional in uterine epithelia in sheep. Upregulation of MAT2B in uterine LE and sGE suggests that while all subtypes of secretory epithelial cells in the endometrium generate SAM from methionine, conceptus derived IFNT stimulates the expression of MAT2B in cells adjacent to the uterine lumen, and the conceptus trophectoderm in pregnant ewes. This reveals cooperation among different cell types for polyamine synthesis in the ruminant endometrium.

Putrescine and decarboxylated SAM are converted via SRM to produce spermidine. The localization of SRM protein suggests that the uterine LE in ewes are not primarily responsible for the production of spermidine unless under the influence of IFNT, further supporting the hypothesis that IFNT stimulates polyamine metabolism in cell types adjacent to the uterine lumen. In addition, IFNT increased SRM protein in deep GE,

suggesting that conceptus-derived factors may increase GE production of spermidine. Strong expression of SMS protein in all secretory epithelial cell types of the endometrium suggests that the endometrium is very active in the production of spermine. Further, SMOX appears to be the primary regulator of intracellular spermine content in deep GE, as neither SAT1 nor PAOX protein was expressed by the deep uterine GE. This suggests that spermine produced in deep GE must either be secreted or oxidized by SMOX to maintain appropriate intracellular levels of polyamines and may explain the high abundance of spermine in the uterine lumen observed in this study. Spermine is considered the most bioactive polyamine based on its electrical charge, thus it may be the preferred polyamine by the ruminant conceptus [57,58].

Collectively, results of the present study suggest that uterine epithelia adjacent to the uterine lumen, i.e., uterine LE and sGE that are adjacent to the developing conceptus in a pregnant ewe, express most of the enzymes important for fine-tuned control of polyamine metabolism. In addition, these cell types are responsive to conceptus derived signaling factors, such as IFNT. While molecules produced or transported by the uterus are generally considered to benefit the developing conceptus, it must also be taken into consideration that these molecules potentially act in an autocrine fashion to regulate the function of the endometrium itself for establishment and maintenance of pregnancy.

## Conclusion

To our knowledge, there are no previous reports that: a) characterize the expression of genes and proteins responsible for the metabolism and transport of polyamines in uteri

of sheep; or b) provide mechanistic insight into the maternal and/or conceptus-derived factors that regulate polyamine metabolism in a cell-specific manner. This study provides compelling evidence for the endocrine and paracrine regulation of enzymes related to polyamine synthesis and catabolism in endometria of sheep during the peri-implantation period of pregnancy, highlighting the importance of this pathway in the establishment and maintenance of pregnancy. Understanding how the endometrium responds to different signaling molecules (derived from either the maternal system or the conceptus) is imperative, as this can be used to develop strategies to improve pregnancy outcomes in mammalian species.

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Author Contributions: The experiments were planned by FWB, GW, and GAJ and executed by KH with assistance from CS, RM, and HS. The first draft of the manuscript was written by KH and edited by FWB, GW, GAJ, CS, HS and RM. The final version of the manuscript was prepared by KH and FWB. All authors read and approved the final manuscript.

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## Supplementary Tables

**Supplementary Table II-1Primer sequences used for qPCR.**

| Gene Abbrev. | Gene Name | Forward Primer | Reverse Primer | Product Size | GenBank accession no. |
|---|---|---|---|---|---|
| ADC | Arginine decarboxylase | GCTGTGGTGTGGACATCCTT | ACTGAGGTGGTACACGATGC | 161 | NM_001293722.1 |
| AGMAT | Agmatinase | GATGGCTTGGACCCTGCTTA | CCTTGACAACCCCGGATGAT | 98 | XM_004013796.4 |
| AMD1 | S-Adenosylmethionine decarboxylase 1 | TTCGCACCATCCCAAGATCC | CGTCTCTTGGAGACAAACATGC | 119 | XM_004014358.4 |
| ARG2 | Arginase 2 | ACACGGACAGCCAGTTTCAT | TTCAGGAGGGTCCACGTCTC | 139 | XM_004010737.3 |
| ISG15 | Interferon stimulated gene 15 | GAATCTACGTCTGCGGGGTG | TATTCACTACGCTGCATGGGG | 76 | NM_001009735.1 |
| ASL | Argininosuccinate lyase | GACTTTGTGGCCGAGTTCCT | GCTGCACAAAGCTGAACTCC | 109 | XM_027961934.1 |
| MAT2B | Methionine adenosyltransferase 2B | TTTCAGCCCCATGTCATCGTA | TTCCCAGAAGCATCCACGTT | 104 | XM_004009048.4 |
| OAT | Ornithine aminotransferase | TTGGACCATTTATGCCGGGTT | ATGAATGCGGCAACGTTTGG | 94 | XM_027960152.1 |
| ODC1 | Ornithine decarboxylase 1 | TGCCTTCTATGTTGCGGACC | TGACGGCATAAAAGGGGGTG | 92 | XM_015094220.2 |
| SAT1 | Spermidine/Spermine acetyltransferase 1 | GAGTGAGGAGTGCTGGTGTA | TGCAACAACGCCACTGGTAAT | 144 | XM_004021966.4 |

| | | | | | |
|---|---|---|---|---|---|
| SMOX | Spermine oxidase | TCCAAGACAGCGCAAGGAAG | AACAGCACCTGCATAGGCTT | 119 | XM_004014358.4 |
| SMS | Spermine synthase | TGAAGACTCACCTTACCAGAACAT | TACGCCAAATCACTTTCCGC | 105 | XM_027963693.1 |
| SRM | Spermidine synthase | AGTGCGAGATTGACGAGGATG | AAGGCGTCCTGGTTCTGTTT | 136 | XM_027975643.1 |
| PGR | Progesterone receptor | AGTTGTCCCTAGCTCACAGC | AGAGGTTTCACCATCCCTGC | 198 | XM_015100878.3 |
| PAOX | Polyamine oxidase | GGGTCAGCTCCCCATTTCTC | ACATTTTTATGTGCCACCGACG | 127 | XM_027960288.2 |
| SLC3A2 | Solute carrier family 3 member 2 | CCCTCTACCGCATTGGAGAC | GGGCCCAACACAATACCCTT | 121 | XM_004019620.3 |
| SLC7A1 | Solute carrier family 7 member 1 | CCTAGCGCTCCTGGTCATCA | GGGCGTCCTTGCCAAGTA | 156 | XM_042254841.1 |
| SLC7A2 | Solute carrier family 7 member 2 | GCAGAGCAGCGCTGTCTTT | ACTGTCCAGAGTGACGATTTTCC | 162 | XM_015104573.3 |
| SLC12A8 | Solute carrier family 12 member 8 | AGTTGCTGTGCTTAAATGGGTA | TGATTTCCCAGAGGGCTGAA | 142 | XM_015092475.1 |
| SLC22A2 | Solute carrier family 22 member 2 | TGAGTCTCCTCGATGGCTGA | CTGAAGAGATCCGGGCAAGG | 106 | XM_012127204.2 |
| SLC22A3 | Solute carrier family 22 member 3 | GCCGGTAAAAGTGGAGCCAA | TCAATGCTGATGCGCTTTTAAGG | 135 | XM_012183210.2 |
| SLC47A1 | Solute carrier family 47 member 1 | TTCCCATCGGGATCTCCCTC | TTCCAGTTTAGCCGGGCAAT | 127 | XM_012185792.2 |
| STAT1 | Signal transducer and activator of transcription 1 | ATGCCACCGAACTTACCCAG | AGCTGATCCAGGCAAGCATT | 112 | NM_001166203.1 |
| TBP | TATA-box binding protein | CCACCGTTTGGTGTCCA | CTAGAGCATCCCCAGCACAC | 79 | XM_012166509.2 |

| | | | | | |
|---|---|---|---|---|---|
| YWHAZ | Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta | GACTGGGTCTGGCCCTTAAC | GAGAGCAGGCTTTCTCAGGG | 72 | NM_001267887.1 |

**.Supplementary Table II-2 Antibody information for immunohistochemistry**

| Antibody target | Supplier | Product number | Research Resource Identifier | Species, clonality, isotype | Concentration and dilution used | Antigen retrieval |
|---|---|---|---|---|---|---|
| ADC | Abcam | ab192771 | Not available | Rabbit, polyclonal, IgG | 1.02 mg/ml, 1:350 (endometrium) and 1:500 (liver) | 0.5 mg/ml Protease in 1x PBS @ 37°C (8 min) |
| AGMAT | Abcam | ab231894 | Not available | Rabbit, polyclonal, IgG | 1 mg/ml, 1:250 (endometrium and testes) | Tris-based boiling buffer, pH 9 (10 min) |
| AMD1 | Proteintech | 11052-1-AP | AB_2226430 | Rabbit, polyclonal, IgG | 160 ug/ml, 1:225 (endometrium) and 1:200 (testes) | Tris-based boiling buffer, pH 9 (10 min) |
| ARG2 | Proteintech | 14825-1-AP | AB_2058977 | Rabbit, polyclonal, IgG | 393 ug/ml, 1:700 (endometrium) and 1:500 (testes) | Tris-based boiling buffer, pH 9 (10 min) |
| MAT2B | Proteintech | 15952-1-AP | AB_10637268 | Rabbit, polyclonal, IgG | 360 ug/ml, 1:350 (endometrium) and 1:250 (liver) | 0.1 mol/L boiling citrate buffer, pH 6 (10 min) |
| ODC1 | Abcam | ab97395 | AB_10679334 | Rabbit, polyclonal, IgG | 0.92 mg/ml, 1:450 (endometrium) and 1:500 (liver) | 0.1 mol/L boiling citrate buffer, pH 6 (10 min) |
| PAOX | Proteintech | 18972-1-AP | AB_2159196 | Rabbit, polyclonal, IgG | 160 ug/ml, 1:350 (endometrium) and 1:250 (liver) | 0.1 mol/L boiling citrate buffer, pH 6 (10 min) |

| | | | | | | |
|---|---|---|---|---|---|---|
| SAT1 | Proteintech | 10708-1-AP | AB_2877739 | Rabbit, polyclonal, IgG | 173 ug/ml, 1:100 (endometrium) and 1:250 (liver) | 0.1 mol/L boiling citrate buffer, pH 6 (10 min) |
| SLC7A1 (CAT1) | Abcam | ab37588 | AB_2190720 | Rabbit, polyclonal, IgG | 0.4 mg/ml, 1:300 (endometrium) and 1:350 (liver) | 0.1 mol/L boiling citrate buffer, pH 6 (10 min) |
| SMS | Proteintech | 15979-1-AP | AB_2270820 | Rabbit, polyclonal, IgG | 133 ug/ml, 1:100 (endometrium) and 1:250 (testes) | 0.1 mol/L boiling citrate buffer, pH 6 (10 min) |
| SMOX | Proteintech | 15052-1-AP | AB_2239689 | Rabbit, polyclonal, IgG | 473 ug/ml, 1:300 (endometrium) and 1:300 (testes) | Tris-based boiling buffer, ph 9 (10 min) |
| SRM | Proteintech | 19858-1-AP | AB_10665555 | Rabbit, polyclonal, IgG | 333 ug/ml, 1:600 (endometrium) and 1:750 (testes) | Tris-based boiling buffer, ph 9 (10 min) |

**Supplementary Table II-3 Expression of mRNAs required for metabolism and transport of polyamines not affected by progesterone (P4) and interferon tau (IFNT). Mean relative values and SEM are presented. n=5-6 samples per treatment group.**

| | Relative mRNA expression | | | | |
|---|---|---|---|---|---|
| Gene | P4+CX | P4+IFNT | RU486+P4+CX | RU486+P4+CX | P value |
| *ADC* | 0.88 ± 0.16 | 0.98 ± 0.13 | 1.23 ± 0.22 | 0.91 ± 0.20 | 0.5387 |
| *AGMAT* | 0.76 ± 0.11 | 1.20 ± 0.08 | 0.81 ± 0.10 | 1.09 ± 0.16 | 0.0783 |
| *AMD1* | 1.13 ± 0.10 | 1.24 ± 0.12 | 1.23 ± 0.19 | 1.22 ± 0.09 | 0.9192 |
| *ARG2* | 0.87 ± 0.08 | 0.1 ± 0.08 | 0.99 ± 0.07 | 0.94 ± 0.09 | 0.7666 |
| *ASL* | 0.36 ± 0.03 | 0.39 ± 0.03 | 0.28 ± 0.02 | 0.35 ± 0.05 | 0.1791 |
| *OAT* | 0.14 ± 0.03 | 0.10 ± 0.02 | 0.15 ± 0.02 | 0.14 ± 0.02 | 0.4543 |
| *ODC1* | 0.93 ± 0.12 | 0.83 ± 0.12 | 1.22 ± 0.13 | 1.07 ± 0.21 | 0.3541 |
| *PAOX* | 1.35 ± 0.5 | 1.25 ± 0.19 | 0.98 ± 0.15 | 1.42 ± 0.22 | 0.7949 |
| *SMS* | 1.00 ± 0.09 | 1.03 ± 0.05 | 1.19 ± 0.14 | 1.08 ± 0.13 | 0.6449 |
| *SRM* | 1.06 ± 0.09 | 1.29 ± 0.16 | 0.14 ± 0.07 | 1.37 ± 0.24 | 0.6888 |
| *SLC3A2* | 0.99 ± 0.06 | 0.92 ± 0.06 | 0.10 ± 0.06 | 1.02 ± 0.08 | 0.7331 |
| *SLC7A1* | 0.99 ± 0.11 | 0.94 ± 0.05 | 1.19 ± 0.1 | 1.19 ± 0.11 | 0.1647 |
| *SLC7A2* | 1.14 ± 0.15 | 1.04 ± 0.07 | 0.94 ± 0.12 | 1.14 ± 0.17 | 0.717 |
| *SLC12A8* | 0.98 ± 0.19 | 1.33 ± 0.14 | 0.96 ± 0.19 | 1.02 ± 0.20 | 0.4577 |
| *SLC47A1* | 0.14 ± 0.03 | 0.10 ± 0.03 | 0.22 ± 0.04 | 0.136 ± 0.04 | 0.1498 |

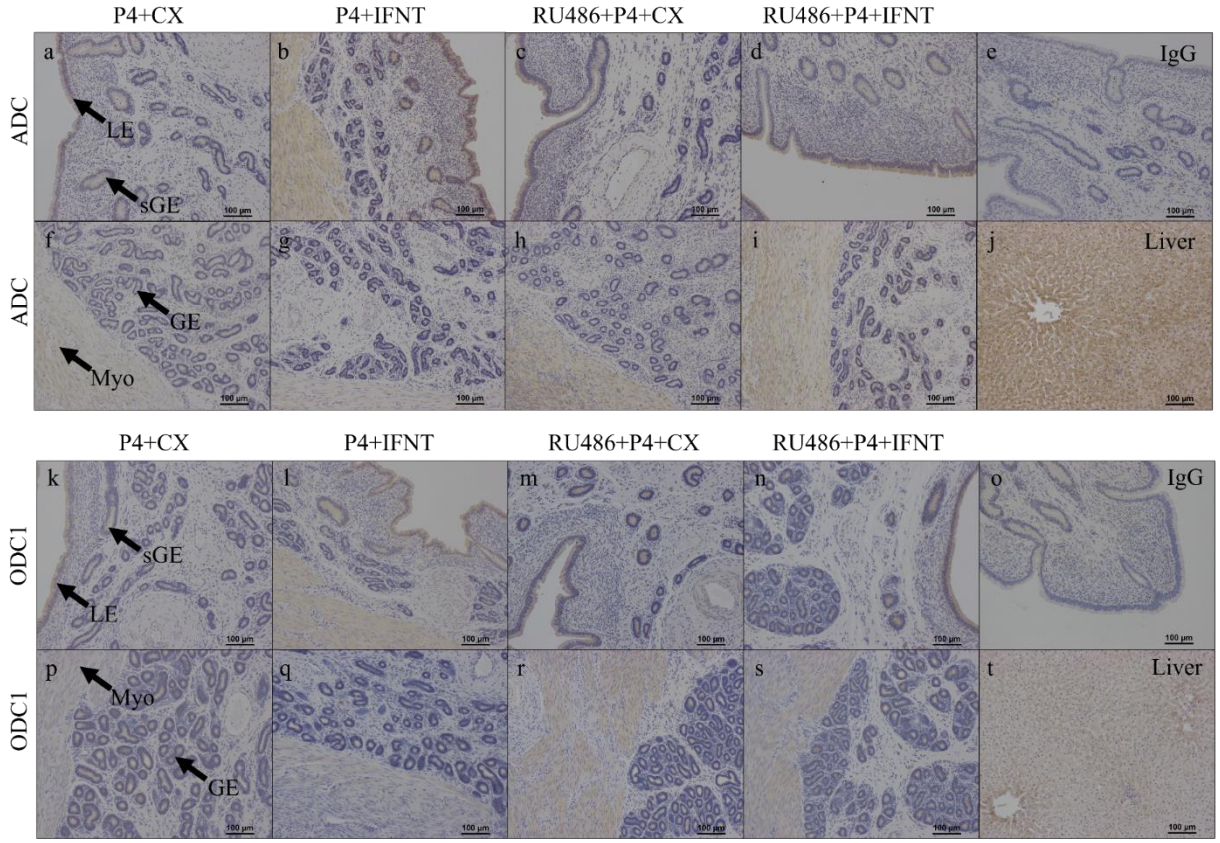
**Supplementary Table II-4 Total amounts (concentration x volume) of amino acids (mg) and polyamines (µg) in uterine flushings from ewes treated with P4+CX (n=6), P4+IFNT (n=6), RU486+P4+CX (n=5), or RU486+P4+IFNT (n=6). Values represent means ± SEM. Different superscripts across treatment columns were different (P<0.05) if bolded or tended to be different (P<0.1) if italicized.**

| | Treatment | | | |
|---|---|---|---|---|
| Nutrient | P4+CX | P4+IFNT | RU486+P4+CX | RU486+P4+IFNT |
| Amino Acids | | | | |
| Alanine | 29.3 ± 5.1 | 29.5 ± 5.6 | 31.9 ± 9.6 | 26.8 ± 4.5 |
| β-Alanine | 1.8 ± 0.5 | 1.6 ± 0.4 | 2.2 ± 0.7 | 1.5 ± 0.3 |
| Arginine | **16.4 ± 2.7$^{a}$** | **12.03 ± 3.5$^{a}$** | **26.9 ± 6.5$^{b}$** | **21.3 ± 3.2$^{ab}$** |
| Asparagine | 4.9 ± 1.4 | 4.4 ± 1.2 | 7.7 ± 2.1 | 5.8 ± 0.64 |
| Aspartate | 6.7 ± 1.7 | 6.5 ± 1.7 | 6.7 ± 1.8 | 8.7 ± 1.9 |
| Citrulline | **9.7 ± 1.2$^{a}$** | **8.9 ± 2.5$^{a}$** | **18.3 ± 3.3$^{b}$** | **13.9 ± 2.9$^{ab}$** |
| Glutamate | 63.2 ± 10.02 | 66.3 ± 16.7 | 50.4 ± 15.1 | 60.5 ± 11.7 |
| *Glutamine* | *12.1 ± 1.9$^{a}$* | *10.7 ± 2.7$^{a}$* | *16.0 ± 4.5$^{ab}$* | *17.0 ± 1.2$^{b}$* |
| Glycine | **122.5 ± 7.6$^{a}$** | **158.1 ± 15.7$^{b}$** | **142.4 ± 18.0$^{ab}$** | **109.7 ± 7.6$^{a}$** |
| Histidine | 3.3 ± 1.02 | 5.4 ± 1.6 | 5.6 ± 1.04 | 6.5 ± 1.3 |
| Isoleucine | 3.6 ± 0.82 | 3.9 ± 0.82 | 7.09 ± 2.3 | 5.6 ± 1.07 |
| *Leucine* | *10.3 ± 1.5$^{a}$* | *10.3 ± 1.3$^{a}$* | *17.3 ± 4.7$^{b}$* | *14.2 ± 2.2$^{b}$* |
| Lysine | 9.4 ± 2.7 | 9.3 ± 3.9 | 16.04 ± 6.6 | 15.7 ± 4.3 |
| Methionine | 2.2 ± 0.8 | 2.2 ± 0.6 | 3.2 ± 1.3 | 3.4 ± 0.5 |
| Ornithine | **0.96 ± 0.37$^{a}$** | **1.3 ± 0.58$^{ab}$** | **3.1 ± 1.3$^{b}$** | **2.7 ± 0.93$^{b}$** |
| Phenylalanine | 3.3 ± 1.2 | 4.08 ± 1.2 | 6.1 ± 2.1 | 6.02 ± 1.2 |
| Serine | 24.3 ± 6.3 | 44.6 ± 18.1 | 18.5 ± 7.6 | 20.8 ± 3.6 |
| Taurine | 37.6 ± 8.7 | 50.02 ± 10.2 | 32.4 ± 6.9 | 31.5 ± 9.6 |
| Threonine | 5.1 ± 0.8 | 5.7 ± 1.01 | 7.3 ± 1.7 | 6.3 ± 0.9 |
| Tryptophan | 1.6 ± 0.6 | 2.3 ± 0.8 | 3.0 ± 1.2 | 2.7 ± 0.8 |
| Tyrosine | 7.8 ± 1.2 | 8.8 ± 1.5 | 11.2 ± 2.6 | 9.5 ± 1.4 |
| Valine | 8.6 ± 1.6 | 9.3 ± 2.6 | 16.04 ± 3.3 | 11.2 ± 2.2 |
| Polyamines | | | | |
| Agmatine | **0.15 ± 0.04$^{a}$** | **0.11 ± 0.04$^{a}$** | **0.07 ± 0.04$^{ab}$** | **0.06 ± 0.03$^{b}$** |
| Putrescine | **0.85 ± 0.13$^{ab}$** | **0.68 ± 0.15$^{ab}$** | **0.54 ± 0.10$^{a}$** | **1.1 ± 0.44$^{b}$** |
| Spermidine | 0.67 ± 0.04 | 0.69 ± 0.07 | 0.61 ± 0.1 | 0.60 ± 0.04 |
| Spermine | 11.8 ± 0.8 | 13.1 ± 1.1 | 13.3 ± 1.7 | 9.7 ± 1.2 |

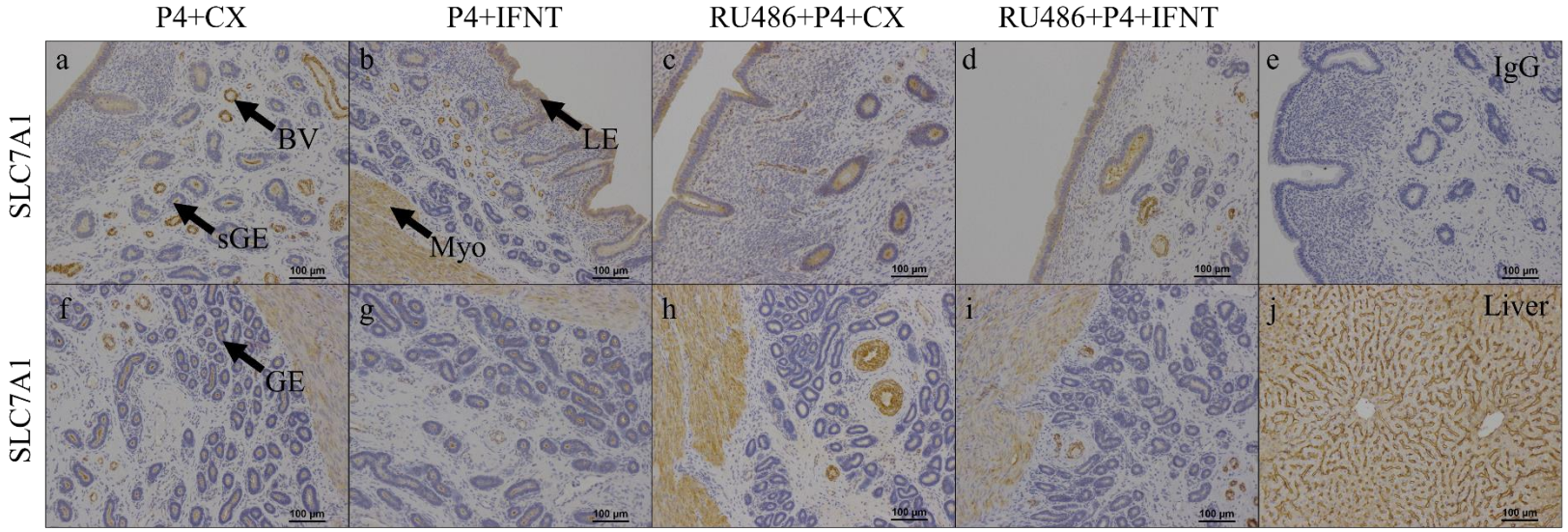
**Supplementary Table II-5 Concentrations (nmol/ml) of amino acids and polyamines in plasma from ewes treated with P4+CX (n=6), P4+IFNT (n=6), RU486+P4+CX (n=5), or RU486+P4+IFNT (n=6). Values represent means ± SEM. Different superscripts across treatment columns were different (P<0.05) if bolded or tended to be different (P<0.1) if italicized.**

| | Treatment | | | |
|---|---|---|---|---|
| Nutrient | P4+CX | P4+IFNT | RU486+P4+CX | RU486+P4+IFNT |
| Amino Acids | | | | |
| Alanine | 175.3 ± 13.0 | 163.8 ± 15.7 | 180.4 ± 19.5 | 162.4 ± 10.6 |
| β-Alanine | 8.9 ± 2.4 | 8.8 ± 2.4 | 12.1 ± 3.1 | 8 ± 2.9 |
| Arginine | 149.8 ± 10.2 | 145.4 ± 19.3 | 160.9 ± 27 | 137.9 ± 23.8 |
| Asparagine | 23.9 ± 2.4 | 29.9 ± 4.3 | 38.2 ± 9.8 | 35.1 ± 4.3 |
| Aspartate | 1.6 ± 0.7 | 2 ± 0.5 | 1.5 ± 0.1 | 1.7 ± 0.4 |
| Citrulline | 256.7 ± 59.7 | 243 ± 44 | 235.7 ± 23.5 | 265.6 ± 17.54 |
| Glutamate | 88.4 ± 5.6 | 86.6 ± 9.2 | 98.1 ± 13.3 | 74.8 ± 5.4 |
| Glutamine | 222.8 ± 11.7 | 247.5 ± 19 | 252.9 ± 19.3 | 238.6 ± 22.37 |
| Glycine | 268.5 ± 58.3 | 280.6 ± 38.2 | 324.6 ± 52.74 | 298.1 ± 39.3 |
| Histidine | 45.23 ± 1.95 | 48.72 ± 2.61 | 56.75 ± 4.69 | 44.35 ± 7.55 |
| Isoleucine | 79.1 ± 2.5 | 73.8 ± 5.6 | 80.9 ± 8.2 | 74.5 ± 9.0 |
| Leucine | 151.9 ± 13.7 | 144.8 ± 6.2 | 148.4 ± 11.2 | 147 ± 9.9 |
| Lysine | 102.9 ± 4.9 | 130.4 ± 14.1 | 108.5 ± 28.1 | 128.3 ± 11.4 |
| Methionine | 28.9 ± 2.1 | 29.1 ± 2 | 33.5 ± 4.3 | 30.6 ± 1.4 |
| Ornithine | 78.9 ± 12 | 104.5 ± 15.8 | 74.8 ± 7.5 | 88.6 ± 9.1 |
| *Phenylalanine* | *64.9 ± 6.1$^{a}$* | *67 ± 3.1$^{ab}$* | *55.6 ± 4.6$^{a}$* | *65.6 ± 3.1$^{b}$* |
| Serine | 69.2 ± 14.6 | 61.9 ± 10.2 | 82.2 ± 15.1 | 75.4 ± 10.9 |
| Taurine | 79.5 ± 12.4 | 65 ± 12.6 | 83.1 ± 9.4 | 77.7 ± 10.5 |
| Threonine | 109.8 ± 25.1 | 103 ± 18.8 | 153.5 ± 25.8 | 98.2 ± 13.7 |
| Tryptophan | 47.2 ± 7.5 | 42.8 ± 3.2 | 43.4 ± 4.2 | 43.2 ± 2.6 |
| *Tyrosine* | *56.8 ± 4.2$^{a}$* | *70.3 ± 9.4$^{ab}$* | *57.4 ± 12.8$^{ab}$* | *74 ± 6.5$^{b}$* |
| Valine | 234.6 ± 14.4 | 237 ± 17.6 | 233.4 ± 13.7 | 228.8 ± 20.1 |
| Polyamines | | | | |
| Agmatine | 41.8 ± 5.6 | 37.6 ± 1.6 | 38.3 ± 3.7 | 37.8 ± 0.92 |
| **Putrescine** | **0.37 ± 0.03$^{a}$** | **0.40 ± 0.05$^{a}$** | **0.41 ± 0.04$^{a}$** | **0.58 ± 0.08$^{b}$** |
| Spermidine | 3.4 ± 0.19 | 3.5 ± 0.30 | 3.6 ± 0.40 | 3.01 ± 0.32 |
| Spermine | 7.45 ± 0.19 | 6.70 ± 1.09 | 5.9 ± 1.5 | 6.62 ± 1.0 |

## Supplementary Figures




**Supplementary Figure II-1. Representative images of cell-specific localization of arginine decarboxylase (ADC) (a-j) and ornithine decarboxylase (ODC1) (k-t) proteins in endometria of ewes treated with either progesterone (P4) or RU486 and P4 (RU486+P4), and either control serum proteins (CX) or interferon tau (IFNT).** ADC protein was expressed weakly by uterine luminal (LE) and superficial glandular (sGE) epithelia (a-d), and appeared to be stimulated in the presence of both P4 and IFNT (b). ADC protein was also expressed in the myometrium (Myo) (f-i). ODC1 protein was expressed by the uterine LE and sGE, but not GE (k-n and p-s). Negative (e and o) and positive (j and t) controls are shown.




**Supplementary Figure II-2 Representative images of the cell-specific localization of solute carrier family 7 member 1 (SLC7A1) proteins in endometria of ewes treated with either progesterone (P4) or RU486 and P4 (RU486+P4), and either control serum proteins (CX) or interferon tau (IFNT).** SLC7A1 protein localized to uterine luminal (LE) and superficial glandular (sGE) epithelia (a-d) but not the deeper glandular epithelia (GE) (f-i). SLC7A1 protein also localized to the myometrium (Myo) and blood vessels (BV). Negative (e) and positive (j) controls are shown.

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# CHAPTER III

# THE OVINE CONCEPTUS UTILIZES EXTRACELLULAR SERINE, GLUCOSE, AND FRUCTOSE TO GENERATE FORMATE VIA THE ONE CARBON METABOLISM PATHWAY

## Introduction

In sheep, the blastocyst hatches from the zona pellucida about eight days after fertilization (200 um in diameter with around 300 total cells), and will increase in size to a spherical blastocyst (0.4 to 0.9 mm in diameter with around 400 – 900 total cells) through Day 10 [1]. The spherical conceptus (embryo/fetus and placental membranes) develops into a tubular form and then elongates rapidly to become a long filamentous structure between Days 11-12 (10 to 22 mm in length) through Day 14 (10 cm in length), and exceeds lengths of 25 cm by Day 17 when the conceptus is composed primarily of extraembryonic membranes [1]. To achieve this incredible increase in cellular hyperplasia, the developing cells of the conceptus must increase the synthesis of nucleic acids to build DNA strands during cellular division. This is associated with increased demand of substrates for metabolic reactions that not only generate nucleic acids, but also other vital pathways such as those that generate ATP, reducing agents, or intermediates for subsequent reactions. However, for survival during the peri-implantation period of pregnancy, the conceptus is entirely reliant upon the histotroph (comprised of water, hormones, minerals, cytokines, growth factors, adhesion molecules, and nutrients such as amino acids and hexose sugars) that is secreted and/or transported by uterine epithelia into the uterine lumen [1]. Of particular interest, serine is the second most abundant amino acid

(following glycine) in uterine flushings from both cyclic and pregnant ewes [2]. Further, more serine is present in uterine flushings of pregnant ewes compared to cyclic ewes between Days 10 to 16 of pregnancy [2]. The total abundance of serine in uterine flushings increases 6.2-fold between Day 10 to 16 [2] indicating that serine is important for conceptus development during the peri-implantation period of pregnancy. Moreover, as gestation advances, serine continues to be one of the most abundant amino acids in umbilical venous plasma and allantoic fluid of sheep [3], further implicating serine as a key amino acid for conceptus development. Serine can also be synthesized from glucose and/or fructose by serinogenesis, as 3-phosphoglycerate (3PG, a glycolytic intermediate) can be converted to serine by the sequential enzymatic conversions that include phosphoglycerate dehydrogenase (PHGDH), phosphoserine aminotransferase 1 (PSAT1), and phosphoserine phosphatase (PSPH) [4].

The one carbon (1C) metabolism pathway utilizes serine as a substrate for the processes of utilizing, synthesizing, and/or transferring 1C units (i.e., methyl groups) among carrier molecules which links together the folate cycle (folate itself acts as the carrier of 1C unit) and the methionine cycle (responsible for recycling components of the folate cycle) [5,6]. Serine is a large contributor, but glycine and methionine also donate segments of their carbon skeletons through 1C metabolism. The production of 1C units though the folate cycle is important for the production of formate utilized to build adenine, guanidine, and thymidine nucleotides [7]. Additionally, 1C metabolism is important for the generation of *S*-adenosylmethionine (SAM) through the methionine cycle and SAM is required for methylation of nucleic acids [8,9], thereby participating in important

epigenetic modifications that occur during gestation [8]. Reducing agents such as nicotinamide adenine dinucleotide phosphate (NADPH) are also generated via 1C metabolism, implicating this pathway in mitochondrial redox control, particularly under hypoxic conditions [10]. It is important to note that although 1C metabolism functionally links the folate and methionine cycles, these pathways may not be spatially linked – as homocysteine is recycled back to methionine via betaine-homocysteine S-methyltransferase only in hepatocytes, this portion of the methionine cycle is confined to the liver [9,11], whereas methionine synthase catalyzes the same reaction using vitamin $B_{12}$ as a cofactor primarily in the liver and, to a lesser extent, in most other eukaryotic cells [9]. Therefore, when considering fundamental demands for non-essential amino acids, maternally derived nutrients in histotroph are critical as demands of conceptuses far exceed the limited capacity of the conceptus to synthesize amino acids de novo to meet demands for growth and development.

1C metabolism is known to be a crucial focal point for cancer metabolism, as this indispensable biochemical cycle bridges other metabolic pathways for the synthesis of proteins, lipids, and nucleic acids in cells with a high demand for nutrients [5,12,13]. Notably, cancer cells are highly proliferative under hypoxic conditions, not unlike cells of the developing conceptus during the peri-implantation period of pregnancy, and a mechanism whereby cancer cells maintain a proliferative state while oxygen deprived is by upregulating serine catabolism and 1C metabolism [10,14]. Therefore, conceptuses of livestock species undergoing extensive cellular proliferation and rapid elongation likely utilize similar metabolic pathways, such as 1C metabolism, but must rely on extracellular

nutrients secreted and/or transported from maternal blood to the uterine lumen. Thus, we hypothesized that ovine conceptuses utilize extracellular serine or glucose and/or fructose for production of formate during the peri-implantation period of pregnancy.

## MATERIALS AND METHODS

### *Animals, experimental design, and sample collection*

The estrous cycles of mature Suffolk ewes (*Ovis aries*, n=8) were synchronized using a progesterone intravaginal insert (CIDR, Zoetis) for 12 days followed by an intramuscular injection of prostaglandin F2α (20 mg Lutalyse, Zoetis) upon removal of the CIDR. Ewes were observed for in the presence of a vasectomized ram. All ewes had exhibited at least two estrous cycles of normal duration (16-18 days) prior to synchronization of estrus. Experimental procedures followed the Guide for the Care and Use of Agriculture Animals in Research and Teaching and approved by the Institutional Animal Care and Use Committee of Texas A&M University (AUP IACUC 2017-0419).

Upon detection of estrus (designated as Day 0), ewes were mated to rams of proven fertility, then every 12 h thereafter for the duration of estrus. On Day 17 of pregnancy, ewes were euthanized and hysterectomized. The uteri were flushed with 20 mL of phosphate buffered saline (pH 7.2) into a square grid dish to recover conceptuses. Conceptuses from 5 ewes were stored in a 15 mL tube containing RPMI 1640 medium (11875093 Gibco Inc., Billings, MT, U.S.A.) containing 1 mM glutamate for transport back to the laboratory, while conceptuses from 3 ewes were snap frozen in liquid nitrogen. The uteri were opened using surgical scissors to expose the endometrium, and the

endometrium was dissected from the myometrium and snap frozen in liquid nitrogen before storage at -80°C. The uterine flushings were collected, centrifuged at 10,000 x g for 15 min, and the supernatant was stored at -20°C until analyzed.

*Preparation of culture media and in vitro culture of conceptuses*

All solutions were prepared in oxygenated (95% $O_2$/5% $CO_2$) Krebs-Henseleit bicarbonate (KHB) buffer (119 mM NaCl, 4.8 KCl, 2.5 $CaCl_2H_2O$, 1.2 mM $MgSO_4$, 1.2 mM $KH_2PO_4$, 25 mM $NaHCO_3$) containing 20 mM HEPES [15]. At the laboratory, conceptuses were washed three times with KHB buffer to remove the RPMI 1640 media. Approximately 20 mg of conceptus tissue was weighed and transferred to a 15 mL polypropylene culture tube (17x120 mm, 352097 Corning Life Sciences, Corning, New York, U.S.A.). Conceptuses were cultured in one of six conditions: 1) 4 mM D-glucose + 2 mM [U-$^{13}$C]serine (CLM-1574-H, Cambridge Isotope Laboratories Inc, Massachusetts, U.S.A) 2) 6 mM glycine + 4 mM D-glucose + 2 mM [U-$^{13}$C]serine; 3) 4 mM D-fructose + 2 mM [U-$^{13}$C]serine; 4) 6 mM glycine + 4 mM D-fructose + 2 mM [U-$^{13}$C]serine; 5) 4 mM D-glucose + 4 mM D-fructose + 2 mM [U-$^{13}$C]serine; or 6) 6 mM glycine + 4 mM D-glucose + 4 mM D-fructose + 2 mM [U-$^{13}$C]serine, in duplicate for each animal. No unlabeled serine (i.e., $^{12}$C-serine) was added to any tubes, therefore the isotopic enrichment of [U-$^{13}$C]serine was about 100%. Blank tubes containing medium with each of the six culture conditions, but without conceptus tissue, were also prepared, including blank tubes containing KHB buffer + 2 mM [U-$^{13}$C]serine and KHB buffer only (without 2 mM [U-$^{13}$C]serine). Each tube was gassed for 15 sec with 95% $O_2$/5% $CO_2$. Tubes were

capped and placed in a shaking water bath for 2 h at 37°C. After the 2 h incubation, 0.2 mL of 1.5 M $HClO_4$ and 0.1 mL of 2 M $K_2CO_3$ [16] were injected through the cap and into the incubation medium to terminate the reaction for one set of the duplicates (n=5 conceptuses per treatment), which was to be used for detection of $^{13}C$-formate by gas chromatography after homogenization of conceptus tissue and culture medium. The second set of duplicates (n=3 conceptuses per treatment) was centrifuged at 1000 x g for 30 s. The supernatant (culture medium) and pellet (conceptus tissue) were separated and transferred to 1.5 mL Eppendorf tubes. The conceptus pellets were snap frozen in liquid nitrogen, and both conceptus and medium samples were stored at -80°C until analyzed.

*Mass spectrometry analysis of $^{13}C$-formate and $^{12}C$-formate*

The $^{13}C$-formate and $^{12}C$-formate were measured as described previously [17] using $^{18}O_2$-formate as an internal standard instead of $^{13}C$-acetate. The samples were processed simultaneously with a standard curve ranging from 0 to 500 µM. The GC-MS system consisted of an Agilent gas chromatograph (model 7890B) interfaced with a single quadrupole mass selective detector (MSD 5977B). The chromatographic conditions were as described previously [17]. Peak detection and integration were performed using MassHunter (Version B07.01 SP2, Agilent, California, U.S.A). Blank values were subtracted from sample values.

*qPCR analysis of candidate genes*

Total RNA was extracted from Day 17 ovine endometria (n=5) using Trizol (15590018 Invitrogen, California, U.S.A.) as per manufacturer's instructions and treated with RNase-free DNase (79254 Qiagen, Germantown, MD, U.S.A.) before RNA purification using the RNeasy Mini Kit (74104 Qiagen) as per manufacturer's instructions. RNA was extracted from conceptuses that were snap frozen after the in vitro culture experiment (n=3 per culture condition) and untreated conceptus tissue (n=3) using RLT buffer in the RNeasy Mini Kit as per the manufacturer's instructions. A NanoDrop (ND-1000 Spectrophotometer) was used to quantify the RNA, and samples with a 260/280 value $\geq 2$ were used. First strand cDNAs were synthesized from 1 μg of total RNA using the SuperScript II First-Strand Synthesis System for RT-PCR kit (11904018 Invitrogen Corp., Carlsbad, CA, U.S.A.) using oligo (deoxythymidine) primers as per the manufacturer's instructions. Negative controls without reverse transcriptase were included to verify a lack of genomic contamination.

Quantitative polymerase chain reaction (qPCR) was performed using an ABI Prism 7900HT (Applied Biosystems, Bedford, MA, U.S.A.) with Power SYBR Green PCR Master Mix (4309155 Applied Biosystems) to quantify the level of expression of mRNAs encoding genes of interest. Primer sequences are listed in Supplementary Table 1. First, the efficiency and specificity of the selected primer sets were tested by generating a standard curve from a serial dilution (1:2 to 1:256) of pooled cDNA in nuclease free water, and by the inclusion of a dissociation curve for the qPCR reaction, respectively. Primer sets that were used for this study had an efficiency of 95% - 105% and only

amplified a single product (as determined by a dissociation curve with a single peak). Each well contained 10% cDNA, 30% nuclease free water, 5% forward and 5% reverse primers, and 50% SYBR Green reaction mix in a 10 µL reaction volume at an annealing temperature of 60°C. *MTHFD2L* in conceptus tissue was lowly expressed (i.e., Cq values above 30), therefore 1 µL of cDNA was combined with nuclease free water, forward and reverse primer, and SYBR as described above in a 20 µL reaction volume for a modified pre-amplification step [18] using a Thermocycler (Eppendorf AG, Hamburg, Germany). The reaction was performed for 15 cycles with the following conditions: 94°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec, and the pre-amplified products were used for qPCR analysis. The reference genes beta-actin (*ACTB*) and hydroxymethlbilane synthase (*HMBS*) in endometria, and TATA-box binding protein (*TBP*) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*) in conceptuses were determined to have stable expression using geNORM software (M value<1.5) before testing for effect of treatment (P>0.1). Thus, the average CT values for *ACTB* and *HMBS* in endometria and *TBP* and *YWHAZ* in conceptus tissue were used to normalize the expression of mRNAs of interest in each tissue. The expression of mRNAs of interest was quantified using the ΔΔCq method.

### *HPLC analysis of amino acids in culture media*

Concentrations of amino acids were determined in culture medium by high performance liquid chromatography (HPLC) as described previously [18,19]. Briefly, each sample (100 µL) was deproteinized by the addition of 100 µL 1.5 M $HClO_4$ followed

by 50 µL 2 M $K_2CO_3$, and then centrifuged at 10,000 x *g* for 3 min. The neutralized supernatant was used for analysis using an HPLC method involving precolumn derivatization with *o*-phthaldialdehyde (OPA) reagent. OPA reagent was prepared by dissolving 50 mg OPA in 1.25 mL HPLC-grade methanol, followed by the addition of 11.2 mL sodium borate (pH 9.5), 50 µL 2-mercaptoethanol (M3148 Sigma-Aldrich, St. Louis, MO, U.S.A.), and 0.5 mL of Brij-23 (B4184 Sigma-Aldrich). The assay mixture contained 1.4 mL of HPLC-grade water (Fisher Scientific, Waltham, MA, U.S.A.), 100 µL of 1.2% benzoic acid (in 40 mM sodium borate, pH 9.5), and 100 µL of sample. The assay mixture was derivatized in an autosampler (model 712 WISP, Waters) with 30 mmol/L OPA reagent, and 15 µL of the derivatized mixture was injected into a Supelco 3-µm-reversed-phase C18 column (150 x 4.6 mm inner diameter, Sigma-Aldrich). Amino acids were separated using a solvent gradient comprised of solution A (0.1 M sodium acetate, 18% methanol, and 1% tetrahydrofuran, pH 7.2) and solution B (HPLC-grade methanol). Amino acid concentrations were quantified relative to authentic standards using Millenium-32 Software (Waters), and data are presented as concentrations of amino acid present in the homogenate per gram of conceptus tissue.

### *Analysis of glucose and fructose in uterine flushings*

Uterine flushings were analyzed for concentrations of glucose using a glucose assay kit (STA-680, Cell Biolabs, Inc., San Diego, CA, U.S.A.), as per manufacturer's instructions. All samples were assayed on a single plate and read with a spectrophotometric plate reader (SynergyH1, BioTek, Winooski, VA, U.S.A.) (nm=540)

after incubation (30 min at 37°C). Kit standards were used to generate a standard curve from 0-100 µM, and samples were diluted 1:5 with 1x Assay Buffer to ensure all sample concentrations were within the limits of the standard curve. The limit of detection of the assay was 6.25 umol/L. The intra-assay coefficient of variation was 2.12%.

Uterine flushings were analyzed for concentrations of fructose using a fructose assay kit (EFRU-100, Bioassay Systems, Hayward, CA, U.S.A.) as per manufacturer's instructions. All samples were assayed on a single plate and read with a spectrophotometric plate reader (SynergyH1, Biotek) (nm=565) after incubation (60 min at room temperature). Kit standards were diluted to generate a standard curve from 0 to 1000 µM, and samples were diluted 1:2 to ensure concentrations were within the limits of the standard curve. The limit of detection of the assay was 21 uM and the intra-assay coefficient of variation was 2.26%.

Total amounts of glucose and fructose (g) were calculated by multiplying their respective concentrations (µM) by the volume of uterine flushings recovered at necropsy (mL) and accounting for the molecular weight of glucose and fructose (180.156 g/mol).

### *Immunofluorescence analysis*

Immunoreactive phosphoglyceride dehydrogenase (PHGDH) and phosphoserine phosphatase (PSPH) proteins were localized in paraffin-embedded sections from Day 18 pregnant ewes using immunofluorescence microscopy. Antigen retrieval was performed using boiling citrate. Sections were then blocked in 10% normal goat serum for 1 h at room temperature. These sections were incubated overnight at 4°C with the following

primary antibodies: rabbit anti-PHGDH polyclonal antibody (Sigma-Aldrich, St. Louis, MO; HPA021241; 1:200), and mouse anti-PSPH monoclonal antibody (Lifespan Biosciences, Seattle, WA; LS-B2935; 1:100). Each antibody was used at a dilution optimized for that antibody. Normal rabbit (EMD Millipore; Billerica, MA; 12-370) or mouse (EMD Millipore; 12-371) IgG, at a concentration equal to that for the primary IgG, was used as the negative control. Immunoreactive proteins were detected using the appropriate Alexa Fluor 488- or Alexa Fluor 594-conjugated secondary antibodies (Life Technologies, Grand Island, NY) for 1 h at room temperature at a dilution of 1:250. Tissue sections were then washed three times for 5 min/wash in PBS. Slides were counterstained with Prolong Gold Antifade reagent containing DAPI (Life Technologies) and coverslipped. Images were taken using an Axioplan 2 microscope (Carl Zeiss, Thornwood, NY) interfaced with an Axioplan HR digital camera.

### *Statistical analysis*

Statistical analyses were performed using GraphPad Prism 9. Normality of the dataset was assessed using either the Anderson-Darling or Shapiro-Wilk test and when a P value $\leq 0.05$ indicated that the data were not normally distributed, the ROUT test was performed to identify outliers as potential data points for exclusion. Normality was reassessed after exclusion of the outliers to confirm a Gaussian distribution. If the data did not contain outliers, logarithmic transformations of the data were performed if necessary to achieve a Gaussian distribution. Either orthogonal contrasts or paired t-tests were performed to assess the differences between treatment groups. P values $< 0.05$ were

considered significant, and those < 0.1 were considered to indicate a trend towards significance.

## Results

### *The ovine endometrium expresses mRNA transcripts required for the production of serine from glucose and/or fructose, and for transport of these nutrients into the uterine lumen*

Analyses by qPCR demonstrated that Day 17 ovine endometria expresses mRNAs for solute carrier family 2 member 1 (*SLC2A1*; glucose transporter), solute carrier family 2 member 8 (*SLC2A8*; glucose and fructose transporter), solute carrier family 6 member 9 (*SLC6A9*; glycine transporter), and solute carrier family 1 members 4 and 5 (*SLC1A4* and *SLC1A5*; serine transporters) (Figure 1a) indicating that the endometrium can transport these nutrients into the uterine lumen on Day 17 of pregnancy. Additionally, the endometrium expresses mRNAs for phosphoglycerate dehydrogenase (*PHGDH*), phosphoserine aminotransferase 1 (*PSAT1*), and phosphoserine phosphatase (*PSPH*) mRNAs (Figure 1a) indicating that the endometrium expresses the enzymes required for synthesis of serine from 3-phosphoglycerate (3PG), an intermediate product of glycolysis. On Day 17 of gestation, the amounts of glucose and fructose in uterine flushings were 0.24 ± 0.03 g and 2.85 ± 0.60 g, respectively. Therefore, fructose was 11.4-times more abundant than glucose in ovine uterine flushings on Day 17 of pregnancy ($P<0.01$).

*Day 17 ovine conceptus tissue expresses transcripts necessary for the uptake of serine, glycine, glucose, and fructose, as well as for serinogenesis and one carbon metabolism*

Analysis by qPCR demonstrated that ovine conceptuses express mRNAs for the uptake of extracellular serine (*SLC1A4* and *SLC1A5*), glucose and fructose (*SLC2A1*, *SLC2A3*, *SLC2A5* [solute carrier family 2 member 5; fructose transporter] and *SLC2A8*), glycine (*SLC6A9*), as well as sideroflexin-1 (*SFXN1*; mitochondrial serine transporter) (Figure 1b). These results indicate that ovine conceptuses can transport and take-up these nutrients (serine, glycine, glucose, and fructose) from the uterine lumen and that serine can be transported into the mitochondria. Conceptuses also expressed *PHGDH*, *PSAT1*, and *PSPH* mRNAs indicating that they have the capacity to convert glucose and fructose to serine de novo, as well as mRNAs that code for enzymes involved in 1C metabolism: serine hydroxymethyltransferase 1 (*SHMT1*), serine hydroxyemethyltransferse 2 (*SHMT2*), methylenetetrahydrofolate dehydrogenase 1 (*MTHFD1*), methylenetetrahydrofolate dehydrogenase 2 (*MTHFD2*), methylenetetrahydrofolate dehydrogenase 1 like (*MTHFD1L*), and methylenetetrahydrofolate dehydrogenase 2 like (*MTHFD2L*) (Figure 1b).

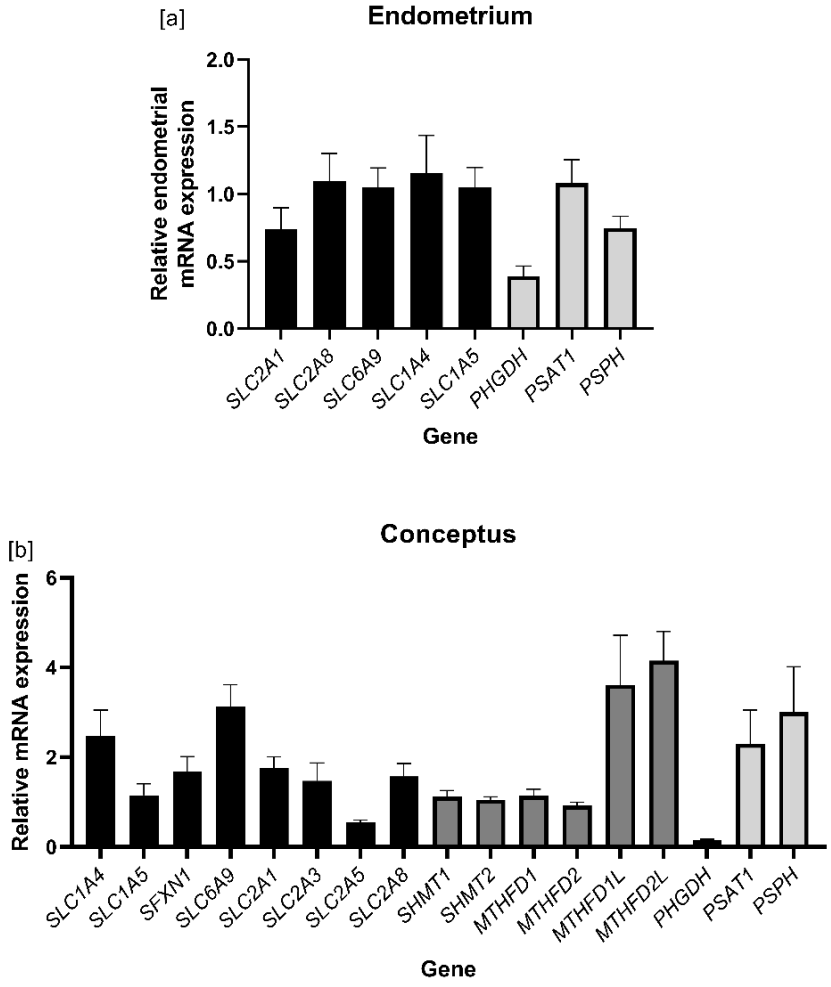


**Figure III-1 Expression of mRNAs required for nutrient transport and serinogenesis in (a) endometrial tissue from Day 17 of gestation and (b) the expression of mRNAs required for nutrient transport, serinogenesis, and one carbon metabolism in conceptus tissue (uncultured) on Day 17 of gestation.** Data are expressed as relative expression of mRNAs, means ± SEM. n=5 endometrial samples and n=3 conceptus samples.

Additionally, conceptuses that were cultured with fructose + [U-$^{13}$C]serine had greater expression of *SHMT1* (P<0.01), *SHMT2* (P<0.1), *MTHFD2* (P<0.01), *SLC1A4* (P<0.01), *SFXN1* (P<0.05), *SLC2A5* (P<0.05), and *SLC2A8* (P<0.05) mRNAs compared to conceptuses cultured in glucose + [U-$^{13}$C]serine (Figure 2 and Supplementary Figure 1), suggesting that fructose alone may stimulate the expression of these genes involved in 1C metabolism, serine transport, and fructose transport compared to glucose alone. However, conceptuses cultured in glycine + fructose + [U-$^{13}$C]serine had lower expression of *SLC6A9* (P<0.1), *SLC2A1* (P<0.05), and *PSAT1* (P<0.05) mRNAs compared to those cultured in glycine + glucose + [U-$^{13}$C]serine (Supplementary Figures 1 and 2). Finally, there was greater expression of MTHFD1L (P<0.1) in conceptuses cultured with glucose + fructose + [U-$^{13}$C]serine compared to those only cultured in glucose + [U-$^{13}$C]serine.

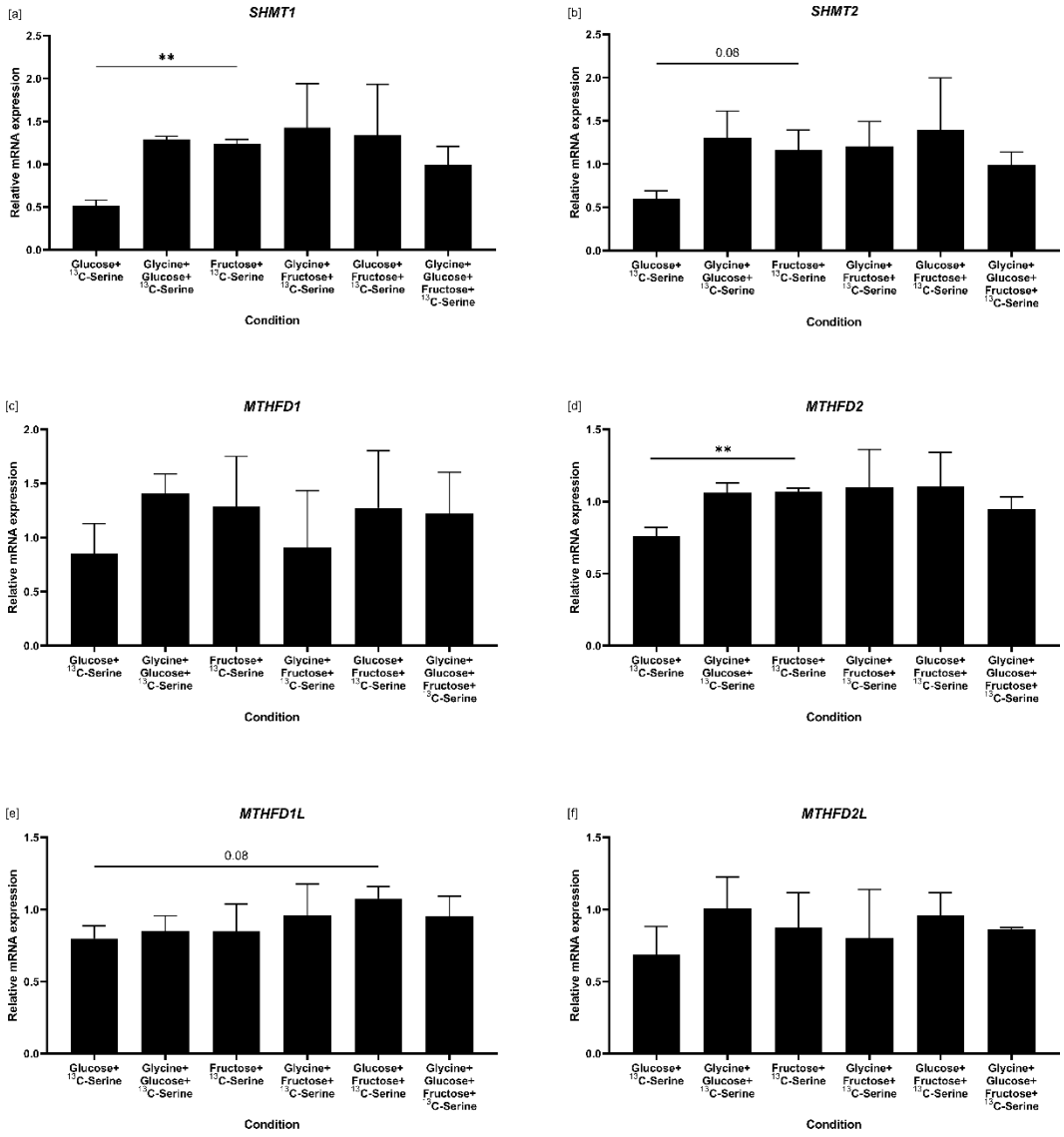


**Figure III-2 Expression of mRNAs required for one carbon metabolism in conceptus tissue from Day 17 of gestation after a 2 h incubation.** Data are expressed as relative expression of mRNAs means ± SEM. n=3 samples per group.

*Enzymes for serine biosynthesis are expressed by the uterine luminal epithelia and the conceptus trophectoderm*

Serine biosynthesis from 3-phosphoglycerate (3-PG), an intermediate of glycolysis, is catalyzed by the successive actions of the enzymes phosphoglycerate dehydrogenase (PHGDH), phophoserine aminotransferase (PSAT), and phosphoserine phosphatase (PSPH). PHGDH converts 3-PG to 3-phosphohydoypyruvate (PHP) which is the committed step in serine biosynthesis. PSAT next converts PHP to 3-phosphoserine (P-Ser), and PSPH removes the phosphate group to yield serine. Immunofluorescence microscopy on cells at the uterine-conceptus interface on Day 18 of gestation using antibodies to PHGDH and PSPH revealed that these enzymes are predominantly expressed by the uterine LE, with weaker but detectable expression in the conceptus trophectoderm (Figure 3).

*Ovine conceptuses utilize extracellular serine, glucose, and fructose to produce formate*

Mass spectrometry of the supernatant was utilized to determine the extent of [U-$^{13}$C]serine incorporation into $^{13}$C-formate or $^{12}$C-formate after a 2 h incubation and homogenization of conceptus tissue and medium. $^{13}$C-formate was detectable in the supernatant indicating that conceptuses used the extracellular [U-$^{13}$C]serine to produce formate. $^{12}$C-formate was also detectable in supernatant, indicating that this formate was produced from sources other than [U-$^{13}$C]serine, i.e. glucose, fructose, and glycine. The average amount of $^{13}$C-formate produced was 1.01 ± 0.21, 0.89 ± 0.23, 1.10 ± 0.25, 0.88 ± 0.38, 0.89 ± 0.10, and 0.59 ± 0.10 nmol/mg tissue, and the average amount of $^{12}$C-

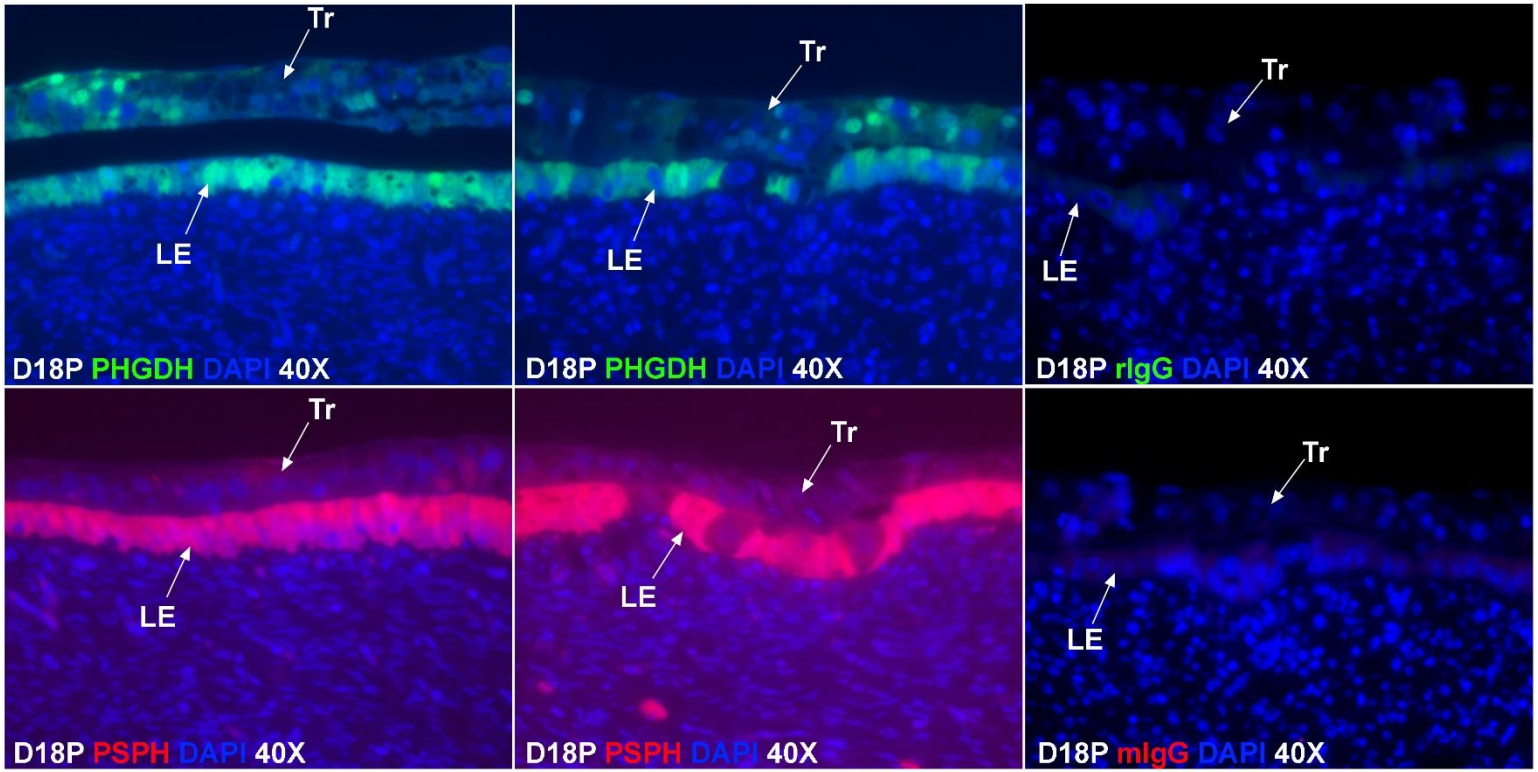


**Figure III-3. Localization of enzymes for serine biosynthesis at implantation sites of sheep.** (A) Immunofluorescence staining for PHGDH (Top Row; green) and PSPH (Bottom Row, red) at implantation sites. PHGDH and PSPH, enzymes involved in serine biosynthesis, are expressed by uterine LE and conceptus Tr in sheep. Tr, trophectoderm cells; LE, luminal epithelium; D, Day; P, pregnancy. The width of field for the microscopic image captured at 40X is 220 μm.

formate produced was 10.97 ± 2.21, 9.44 ± 1.46, 10.80 ± 2.74, 10.73 ± 4.08, 8.66 ± 1.11, and 12.16 ± 7.80 (Figure 4a) for conditions 1-6 (Listed in Materials and Methods). Conceptuses cultured in glycine + glucose + fructose + [U-$^{13}$C]serine produced less $^{13}$C-formate compared to conceptuses cultured in glucose + fructose + [U-$^{13}$C]serine ($P<0.01$) or conceptuses cultured in fructose + serine ($P<0.1$) (Figure 4a). There were no differences in the amount of $^{12}$C-formate produced between treatment groups (Figure 4a). The ratio of $^{12}$C-formate:$^{13}$C-formate was significantly greater for conceptuses cultured in glycine + glucose + fructose + [U-$^{13}$C]serine compared to the other treatment groups ($P<0.05$) (Figure 4b).

Interestingly, at the end of the incubation period, there was less serine present in culture homogenates from the glycine + glucose + fructose + [U-$^{13}$C]serine condition compared to the glucose + [U-$^{13}$C]serine and fructose + [U-$^{13}$C]serine conditions ($P<0.05$) (Table 1), and there was less serine present in culture homogenates when conceptuses were cultured in glycine + fructose + [U-$^{13}$C]serine or glucose + fructose + [U-$^{13}$C]serine compared to glucose + [U-$^{13}$C]serine ($P<0.05$) (Table 1). The amount of glycine ($P<0.01$) and citrulline ($P<0.001$) in homogenates were greater when culture medium contained glycine (glycine + glucose + [U-$^{13}$C]serine, glycine + fructose + [U-$^{13}$C]serine, and glycine + glucose + fructose + [U-$^{13}$C]serine) compared to the values for homogenates of cultured tissue in medium without glycine (glucose + [U-$^{13}$C]serine, fructose + [U-$^{13}$C]serine, and glucose + fructose + [U-$^{13}$C]serine) (Table 1). Finally, there was more aspartate in homogenates when the culture medium contained fructose + [U-$^{13}$C]serine

compared to the other conditions, except for glycine + fructose + [U-$^{13}$C]serine ($P<0.01$) (Table 1).

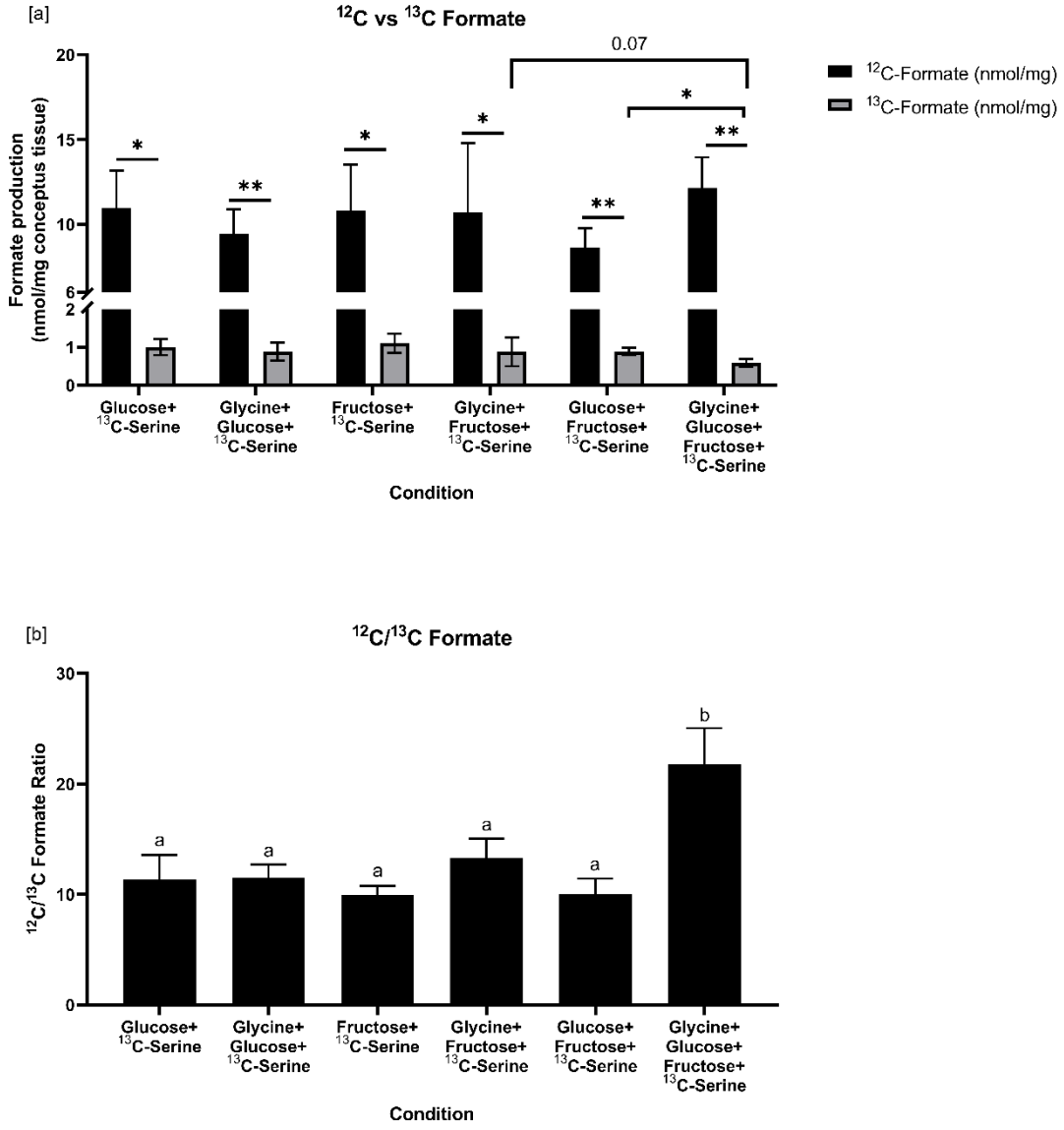


**Figure III-4 Production of $^{12}$C-Formate and $^{13}$C-Formate by the ovine conceptuses cultured with [U-$^{13}$C]serine (a), and the ratio of 12C- to 13C-fromate produced by ovine conceptuses cultured with [U-$^{13}$C]serine (b).** Data are expressed as mean $^{12}$C- and $^{13}$C-formate produced per milligram conceptus tissue (nmol/mg) ± SEM. n=4 samples per treatment group.

**Table III-1 Concentrations of amino acids in supernatants after homogenization of conceptus tissue in the medium (nmol/mg tissue).** Concentrations of amino acids in the supernatants following homogenization of tissue in medium and centrifugation after a 2h period of incubation (nmol/g). The data are expressed as means ± SEM. n= 5 samples per group. Capital letters (A, B) represent significant differences (P<0.05) with an overall significant P value, whereas lowercase letters (a,b) represent significant differences among groups when the overall P value was not significant (P>0.05).

| | Condition | | | | | | |
|---|---|---|---|---|---|---|---|
| Amino Acid | Glucose+ [U-$^{13}$C]serine | Glycine+ Glucose+ [U-$^{13}$C]serine | Fructose+ [U-$^{13}$C]serine | Glycine+ Fructose+ [U-$^{13}$C]serine | Glucose+ Fructose+ [U-$^{13}$C]serine | Glycine+ Glucose+ Fructose+ [U-$^{13}$C]serine | P value |
| Aspartate | **0.60 ± 0.10$^{A}$** | **0.58 ± 0.10$^{A}$** | **1.20 ± 0.13$^{B}$** | **0.94 ± 0.11$^{AB}$** | **0.66 ± 0.12$^{A}$** | **0.66 ± 0.02$^{A}$** | **0.0142** |
| Glutamate | 4.35 ± 0.84$^{ab}$ | 4.80 ± 0.55$^{ab}$ | 6.17 ± 0.53$^{a}$ | 5.12 ± 0.58$^{ab}$ | 5.29 ± 0.55$^{ab}$ | 4.73 ± 0.18$^{b}$ | 0.3419 |
| Asparagine | 0.66 ± 0.14 | 0.54 ± 0.12 | 0.72 ± 0.07 | 0.69 ± 0.09 | 0.69 ± 0.12 | 0.76 ± 0.09 | 0.7756 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Serine | **125 ± 5.28$^{A}$** | **105 ± 9.39$^{ABC}$** | **122 ± 11.40$^{AB}$** | **91.62 ± 8.93$^{BC}$** | **102 ± 8.12$^{BC}$** | **87.98 ± 5.66$^{C}$** | **0.0198** |
| Glutamine | 1.06 ± 0.24 | 0.98 ± 0.26 | 1.00 ± 0.12 | 0.98 ± 0.16 | 1.39 ± 0.23 | 1.24 ± 0.10 | 0.5744 |
| Histidine | 0.28 ± 0.07$^{ab}$ | 0.22 ± 0.04$^{a}$ | 0.37 ± 0.02$^{b}$ | 0.34 ± 0.04$^{ab}$ | 0.37 ± 0.06$^{ab}$ | 0.39 ± 0.05$^{b}$ | 0.1397 |
| Glycine | **3.36 ± 0.79$^{A}$** | **303 ± 34.83$^{B}$** | **3.29 ± 0.22$^{A}$** | **270 ± 30.36$^{B}$** | **3.26 ± 0.34$^{A}$** | **317 ± 20.12$^{B}$** | **0.0040** |
| Threonine | 0.96 ± 0.19 | 1.48 ± 0.46 | 1.08 ± 0.11 | 1.13 ± 0.32 | 1.01 ± 0.15 | 1.86 ± 0.88 | 0.9617 |
| Citrulline | **0.06 ± 0.10$^{A}$** | **0.22 ± 0.03$^{B}$** | **0.05 ± 0.01$^{A}$** | **0.21 ± 0.03$^{B}$** | **0.04 ± 0.01$^{A}$** | **0.20 ± 0.03$^{B}$** | **0.0005** |
| Arginine | 0.49 ± 0.06 | 0.45 ± 0.08 | 0.52 ± 0.03 | 0.55 ± 0.04 | 0.53 ± 0.06 | 0.66 ± 0.07 | 0.2407 |
| b-Alanine | 0.11 ± 0.04 | 0.14 ± 0.03 | 0.16 ± 0.05 | 0.15 ± 0.04 | 0.12 ± 0.04 | 0.13 ± 0.03 | 0.9463 |
| Taurine | 0.21 ± 0.06 | 0.30 ± 0.06 | 0.31 ± 0.06 | 0.24 ± 0.09 | 0.28 ± 0.05 | 0.19 ± 0.05 | 0.7136 |
| Alanine | 2.78 ± 0.44 | 1.90 ± 0.53 | 2.26 ± 0.17 | 2.14 ± 0.23 | 2.65 ± 0.46 | 2.77 ± 0.32 | 0.4726 |
| Tyrosine | 0.48 ± 0.09 | 0.43 ± 0.09 | 0.49 ± 0.03 | 0.52 ± 0.07 | 0.43 ± 0.07 | 0.54 ± 0.05 | 0.8548 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Tryptophan | 0.07 ± 0.03 | 0.02 ± 0.001 | 0.06 ± 0.03 | 0.04 ± 0.001 | 0.05 ± 0.02 | 0.07 ± 0.03 | 0.6943 |
| Methionine | 0.26 ± 0.05 | 0.16 ± 0.04 | 0.24 ± 0.04 | 0.23 ± 0.02 | 0.20 ± 0.03 | 0.24 ± 0.03 | 0.5306 |
| Valine | 0.86 ± 0.20 | 0.63 ± 0.18 | 0.94 ± 0.09 | 0.80 ± 0.09 | 0.79 ± 0.14 | 0.93 ± 0.11 | 0.6610 |
| Phenylalanine | 0.48 ± 0.10$^{ab}$ | 0.34 ± 0.07$^{a}$ | 0.54 ± 0.03$^{b}$ | 0.49 ± 0.06$^{ab}$ | 0.48 ± 0.09$^{ab}$ | 0.45 ± 0.11$^{ab}$ | 0.6640 |
| Isoleucine | 0.58 ± 0.12 | 0.43 ± 0.10 | 0.58 ± 0.04 | 0.54 ± 0.09 | 0.58 ± 0.10 | 0.49 ± 0.12 | 0.8604 |
| Leucine | 0.98 ± 0.21 | 0.80 ± 0.21 | 1.10 ± 0.11 | 1.00 ± 0.13 | 0.95 ± 0.14 | 1.04 ± 0.07 | 0.8244 |
| Ornithine | 0.34 ± 0.10 | 0.32 ± 0.11 | 0.44 ± 0.09 | 0.47 ± 0.14 | 0.29 ± 0.07 | 0.35 ± 0.08 | 0.6858 |
| Lysine | 1.01 ± 0.21 | 0.85 ± 0.22 | 1.08 ± 0.11 | 1.01 ± 0.16 | 1.01 ± 0.16 | 1.14 ± 0.12 | 0.8901 |

## Discussion

One carbon (1C) metabolism is a metabolic pathway that bridges the folate cycle and the methionine cycle to ultimately provide the substrates needed for essential cellular processes, including formate for the production of purines and thymidine and 1C groups utilized in methylation reactions. We speculated that conceptuses employ 1C metabolism to generate these products vital for cell proliferation. The results of this novel study demonstrated that: 1) the ovine conceptus indeed utilizes extracellular serine to produce formate; 2) the ovine conceptus also utilizes glucose and fructose to produce formate; 3) the ovine conceptus utilized the extracellular substrates to produce formate in a 2 h incubation, indicating that both the 1C metabolism and serinogenesis pathways are highly active in the Day 17 ovine conceptus; and 4) the ovine conceptus utilizes glucose and fructose to a greater extent than serine for the production of formate.

1C metabolism, specifically the folate cycle, is compartmentalized within the cytosol and the mitochondria [20]. In each compartment, related enzymes are used to generate and recycle the same metabolic intermediates – SHMT1 and MTHFD1 are located in the cytosol, while SHMT2, MTHFD1L, MTHFD2, and MTHFD2L localize to the mitochondria [20]. In the folate cycle, the folate intermediates themselves are not modified, but merely serve as carriers of the carbon units. As a result of the cellular compartmentation, the mitochondrial enzymes are responsible for the generation of formate from serine and glycine, while the cytosolic enzymes sequentially generate and incorporate formate into pathways for the synthesis of adenine, guanidine, and thymidine [11]. Additionally, glucose and fructose are important for purine and pyrimidine

nucleotide synthesis via generation of ribose-5-phosphate (R5P) through the pentose cycle [21]. The de novo synthesis of DNA is one of the rate-limiting factors of cellular proliferation, thus the ability of a cell to increase production of nucleic acids is crucial in highly proliferative tissues.

Conceptuses of livestock species undergo extensive proliferation and elongation during the peri-implantation period of pregnancy to maximize the surface area for uptake of nutrients from histotroph during the peri-implantation period [1]. This translates to maximal surface area of the placenta after implantation and placentation for exchange of nutrients, gasses, and waste products. The peri-implantation period, however, is only a short window of time during gestation wherein a recently fertilized oocyte develops from a 1-cell zygote to a multicellular conceptus that must maximize its ability to produce DNA for every cell that is proliferating.

Certain amino acids were traditionally classified as nutritionally non-essential because eukaryotic cells have the ability to synthesize them from other sources including other amino acids and nutrients in their diet [11]. However, in the context of demands for gestation, the conceptus is entirely reliant upon nutrients provided in sufficient amounts by the mother for survival. Therefore, it should not be a surprise that the nutrients consumed by the pregnant mother directly influence development of the conceptus. In late gestation, serine flux in the sheep fetus is incredibly high, and most of the serine in fetal blood is derived from glycine synthesized by the fetal liver [22]. The ovine placenta takes up large quantities of serine from the maternal circulation, but it then converts the serine to glycine and releases it into fetal blood such that serine and glycine are present in

equimolar amounts [22–24], and the fetal liver converts the glycine to serine [22]. Collectively, this suggests that the fetal-placental unit in sheep has a high demand for methylating precursors during late pregnancy [25]. Additionally, high levels of formate in ovine fetal plasma (191 μM) and amniotic fluid (296 μM) compared to maternal plasma (33 μM) in late gestation suggest that the mother also provides formate directly to the fetus [26] and further demonstrating the importance of formate for fetal growth. However, the authors of that study emphasized that the precursors of the formate in the sheep fetus are yet to be experimentally determined, although they speculated that formate was generated from serine, glycine, choline and choline oxidative metabolites, and vitamin $B_{12}$ [26]. The utilization of serine by the peri-implantation ovine conceptus has not been investigated previously, thus the novel results of the present study here indicate that the peri-implantation ovine conceptus utilizes serine to generate formate. Additionally, this study demonstrated that the ovine conceptus also utilizes glucose and fructose to produce serine, which was then used to generate formate.

Serine and glycine are the two most abundant amino acids in histotroph during the peri-implantation period of pregnancy [2]. Serine can enter multiple metabolic pathways, including those for the biosynthesis of other amino acids and the production of phospholipids [11]. Glycine, the simplest amino acid, is not only used within 1C metabolism, but can be utilized to generate glutathione, creatine, and heme [11]. While glucose is classically considered the predominant hexose sugar utilized to generate ATP, fructose is more abundant in uterine flushings during the peri-implantation period of pregnancy. Fructose is not detectable in uterine flushings on Day 12 of pregnancy [27],

but clearly increases significantly in the uterine lumen between Day 12 (onset of conceptus elongation in sheep) and Day 17 of gestation, as demonstrated here. In this study, equimolar amounts of glucose and fructose were used as this was determined to be the optimal concentration for proliferation of ovine trophectoderm cells in culture [28]. Fructose, by way of hexokinase and ketohexokinase, can contribute to the glycolytic intermediates fructose-6-phosphate, fructose-1-phosphate, and glyceraldehyde-3-phosphate, respectively [4]. Both glucose-6-phosphate and fructose-6-phosphate can contribute to the production of ribose-5-phosphate for nucleotide synthesis through the pentose cycle [11]. Our results also indicate that fructose increased the utilization of serine and the production of aspartate (another substrate for purine and pyrimidine syntheses) by the ovine conceptus. The underlying mechanisms remain to be elucidated.

Results of this study demonstrate that the ovine uterine endometrium expresses mRNAs important for the transport of vital nutrients into the uterine lumen, including those for glucose and/or fructose (*SLC2A1*, *SLC2A5*, and *SLC2A8*), glycine (*SLC6A9*), and serine (*SLC1A4* and *SLC1A5*) at Day 17 of pregnancy. Further, *SLC2A1*, *SLC1A4*, and *SLC1A5* mRNAs are expressed by the ovine endometrium throughout the peri-implantation period of pregnancy (Days 10-20) [29,30], while *SLC6A9* [27] and *SLC2A5* [31,32] are expressed on Days 12 and 14, respectively. This is the first report of the expression of *SLC2A8* mRNAs in the ovine endometrium during the peri-implantation period of pregnancy. Additionally, the conceptus expresses mRNAs for transporters of glucose and/or fructose (*SLC2A1*, *SLC2A3*, *SLC2A5*, *SLC2A8*), glycine (*SLC6A9*), and serine (*SLC1A4*, *SLC1A5*, and *SFXN1*) for uptake by cells of the conceptus. During the peri-implantation period of

pregnancy, *SLC2A1*, *SLC2A3*, *SLC1A4*, *SLC1A5* mRNAs localize to the extra-embryonic endoderm and trophectoderm cells of the ovine conceptus, and the temporal alterations in intensity of expression among these transporters suggest coordinated transport of their respective nutrients into conceptuses [29,30]. This report is also the first to demonstrate expression of *SLC2A5*, *SLC2A8*, and *SLC6A9* mRNAs in ovine conceptuses during the peri-implantation period of pregnancy. The expression of mRNAs involved in serinogenesis (*PHGDH*, *PSAT1*, and *PSPH*) in both the endometrium and conceptus indicate that both of these tissues can convert glucose to serine. Because the conceptuses in this study produced 12C-formate, this indicates that the serinogenesis pathway is very active in this tissue. Additionally, ovine conceptus tissue expresses mRNAs to generate formate from serine in both the cytoplasmic (*SHMT1* and *MTHFD1*) and mitochondria compartments (*SHMT2*, *MTHFD2*, *MTHFD1L*, and *MTHFD2L*).

Interestingly, in this study, decreased production of $^{13}$C-formate by the conceptuses cultured in glycine + glucose + fructose + [U-$^{13}$C]serine may suggest that (a) glycine may competitively reduce the uptake of serine by cells; and (b) the conceptus may use glucose and fructose via serinogenesis (therefore, less labeled formate is detectable from labeled serine) compared to the conceptuses cultured in glucose + fructose + [U-$^{13}$C]serine and the fructose + [U-$^{13}$C]serine conditions. Indeed, greater amounts of $^{12}$C-formate was produced than $^{13}$C-formate, suggesting a previously unappreciated potential preferential utilization of hexose sugars for formate production, instead of serine. Interestingly, we found that, in the presence of both glucose and fructose, glycine stimulated the production of formate by the ovine conceptus. Additionally, as the

expression of *SHMT1*, *SHMT2*, and *MTHFD2*, as well as *SLC1A4*, *SFXN1*, *SLC2A5*, and *SLC2A8*, mRNAS was greater for conceptuses incubated in fructose compared to glucose, this may suggest a possible preference for fructose utilized via 1C metabolism, and that glucose may be favored for other pathways such as glycolysis, the Krebs cycle, or the pentose cycle. Rapid changes in mRNA levels for these genes within a relatively short period (2h) may be characteristics of the rapid growth and development of the conceptus on Day 17 of gestation.

In the past decade there has been a significant increase in the interest in 1C metabolism as it relates to tumorigenesis. Not only does 1C metabolism generate nucleotides for DNA and RNA synthesis, but other outputs of 1C metabolism include macromolecules such as proteins, lipids, reducing agents such as NADPH [33]. Although eukaryotic cells have the ability to synthesize non-essential amino acids needed for metabolic reactions, such as serine and glycine, cancer cells are reliant on an exogenous supply of these amino acids to maintain a high level of proliferative activity. Cancer cells also synthesize serine from glucose and glutamate via serinogenesis [34]. In addition to a dependency on these extracellular components, cancer cells undergo metabolic switching to enhance glycolysis to produce lactate even in the presence of oxygen (Warburg effect) [35–37]. In this way, cancer cells maintain high levels of proliferation in 'unfavorable' extracellular conditions (such as low pH and low $O_2$), even after hijacking nutrients and oxygen from their surrounding vascular network while reducing the amounts of reactive oxygen species (ROS) that could lead to cell death [21]. Notably, characteristics of conceptus development overlaps with these characteristics of cancer cell proliferation. In this study,

conceptus tissue was exposed to oxygenated conditions before the incubation period to allow for optimal conditions for cell growth [28]. Future research should determine how hypoxic conditions affect the entrance of serine, glucose, and fructose into 1C metabolism and formate production in ovine conceptus tissue. Understanding how conceptuses develop in pregnancy not only has implications for potentially improving pregnancy rates in mammalian species, but there is also the benefit of translational knowledge gained that lays the foundation for future research on 1C metabolism outside the realm of reproductive physiology.

## Conclusion

The processes of elongation and implantation of conceptuses of livestock species are protracted and complex, and require substantial amounts of nutrients provided by the uterus in histotroph. Serine, along with glucose and fructose, are either transported from maternal blood or synthesized by cells of the conceptus in large quantities during the peri-implantation period of pregnancy to support key metabolic pathways such as 1C metabolism as described in this study. The results of this study demonstrate the ovine conceptus utilizes extracellular serine for the production of formate required for 1C metabolism and the production of purines, thymidine, and S-adenosylmethionine. Intriguingly, this study also demonstrated that not only can glucose and fructose contribute to formate production, but also that the ovine conceptus may preferentially utilize glucose and fructose, rather than serine, to produce formate. As embryonic mortality rates are greatest during the peri-implantation period of pregnancy, understanding the importance

of specific molecules in the key metabolic pathways critical for cellular functions is key to the knowledge that lay the foundation for strategies aimed at improving reproductive efficiency in mammalian species.

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Conflicts of interest: The authors have no conflict of interest to declare.

Author Contributions: The experiments were planned by FWB, GW, and GAJ. Animal work and sample collection was executed by KMH, CS, RMM, NS, HS, and FWB. The experiments were executed by KMH, CS, ACK, and SGL, and analysis performed by KMH, SGL, GW, and FWB. The first draft of the manuscript was written by KH and FWB, and edited by CS, RMM, ACK, NS, SGL, GAJ, and GW. The final version of the manuscript was prepared by KH and FWB and approved by all authors.

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## Supplementary Tables

**Supplementary Table III-1 Primer sequences for qPCR.**

| Gene Symbol | Gene Name | Accession Number | Primer Sequence (5'→ 3') | | Tm (ºC) | Amplicon Size |
|---|---|---|---|---|---|---|
| *ACTB* | Beta-actin | NM_001009784.3 | Fwd | CCACCGCAAATGCTTCTAGG | 60 | 79 |
| | | | Rev | CGTTTTCTGCGCAAGTTAGG | | |
| *HMBS* | Hydroxymethylbilane synthase | XM_004016091.5 | Fwd | CCGACACCGGAGGACATTG | 60 | 98 |
| | | | Rev | GGTGTCTTCTTCCGCCGTTG | | |
| *MTHFD1* | Methylenetetrahydrofolate dehydrogenase, cyclohydrolase and | XM_004010715.4 | Fwd | ATTCACGGACGGCTCAGTTT | 60 | 70 |
| | | | Rev | GAACTGGTGAGAGCCAGGAC | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | formyltetrahydrofolate synthetase 1 | | | | | |
| *MTHFD2* | Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2, methenyltetrahydrofolate cyclohydrolase | XM_004006088.4 | Fwd | AGATGGCCTCCTTGTTCAGC | 60 | 71 |
| | | | Rev | GAGAAACGGCATTGCAGACC | | |
| *MTHFD1L* | Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1 like | XM_004011411.3 | Fwd | AATTTGCCCATCTGCATGGC | 60 | 74 |
| | | | Rev | CCCTTGGGTACGCCTTTCTT | | |

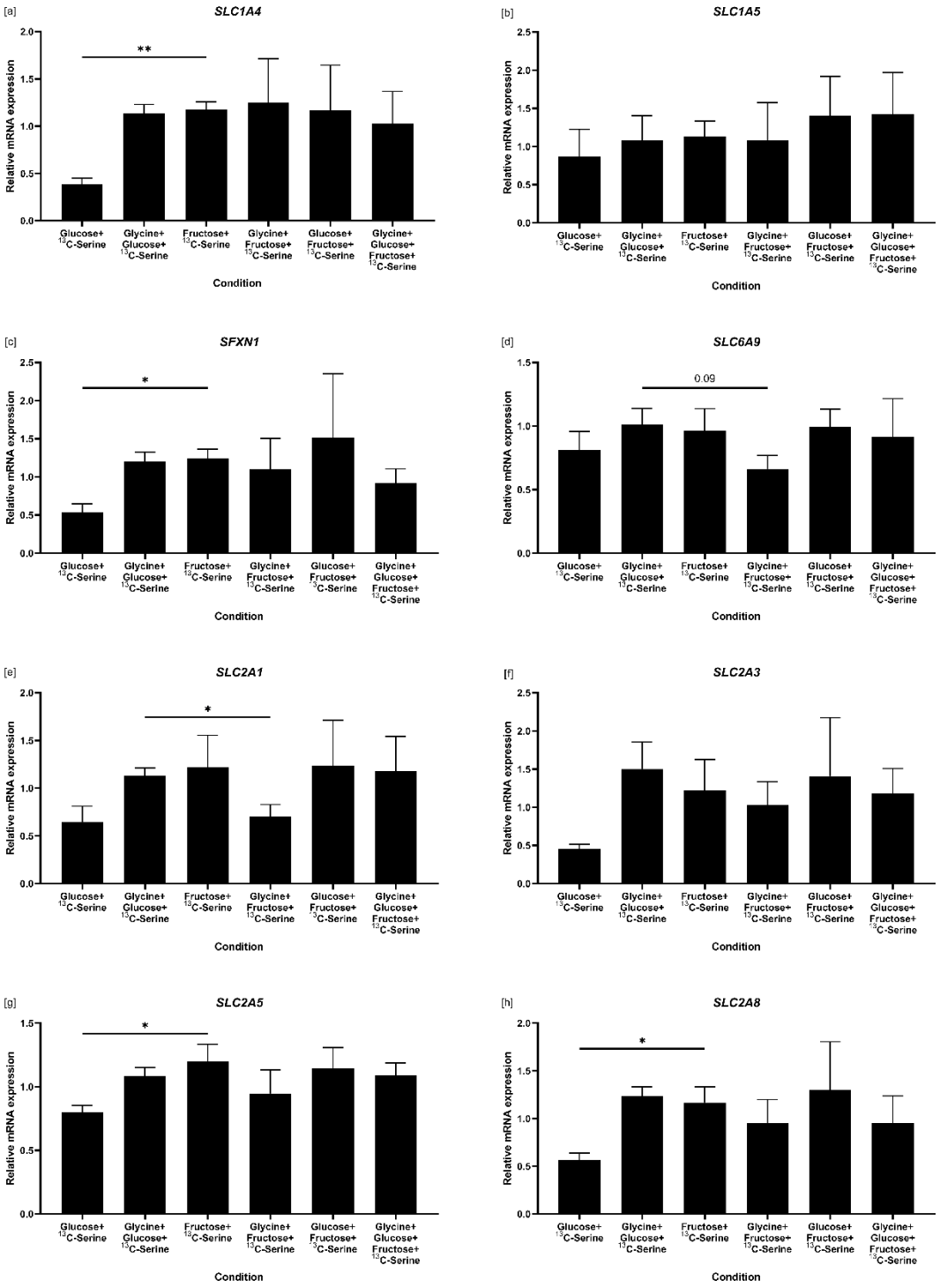
| | | | | | | |
|---|---|---|---|---|---|---|
| *MTHFD2 L* | Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2 like | XM_015096566.2 | Fwd | AGCTTTATCACTCCAGTTCCCG | 60 | 78 |
| | | | Rev | AGCTGCCAAAAGGGTGTTCTT | | |
| *PHGDH* | Phosphoglycerate dehydrogenase | XM_004002389.3 | Fwd | CGGATGCAGTCCTTTGGGAT | 60 | 107 |
| | | | Rev | GGTGTGTGCACAGTGACGA | | |
| *PSAT1* | Phosphoserine aminotransferase 1 | XM_004004296.4 | Fwd | TGGCAACACCAAAGGAGACG | 60 | 141 |
| | | | Rev | ACGTCTTCGACAGTGACAGC | | |
| *PSPH* | Phosphoserine phosphatase | XM_012103912.2 | Fwd | AAGGGAGCTAGTAAGTCGCC | 60 | 190 |
| | | | Rev | TTCAGCTGTTGGCTGAGTCT | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| *SHMT1* | Serine hydroxymethyltransferase 1 | NM_001009469.1 | Fwd | TCCCGATACCGGCTACATCA | 60 | 89 |
| | | | Rev | AGCAGCTAGTCCCTGCAATG | | |
| *SHMT2* | Serine hydroxymethyltransferase 2 | XM_004006554.3 | Fwd | GCAAGACCACCAAACTCCAG | 60 | 78 |
| | | | Rev | TAAGGTCGGCCAGCTGATGA | | |
| *SFXN1* | Sideroflexin 1 | NM_001126350.1 | Fwd | TAGTTGGCTTTTGCTTGGTG | 60 | 80 |
| | | | Rev | GCTTGTCACAGACATGGAAC | | |
| *SLC1A4* | Solute carrier family 1 member 4 | XM_012168282.4 | Fwd | CCGGACGTATGCAACTGACT | 60 | 90 |
| | | | Rev | ATTTCGGTGCCAATGGGGAT | | |

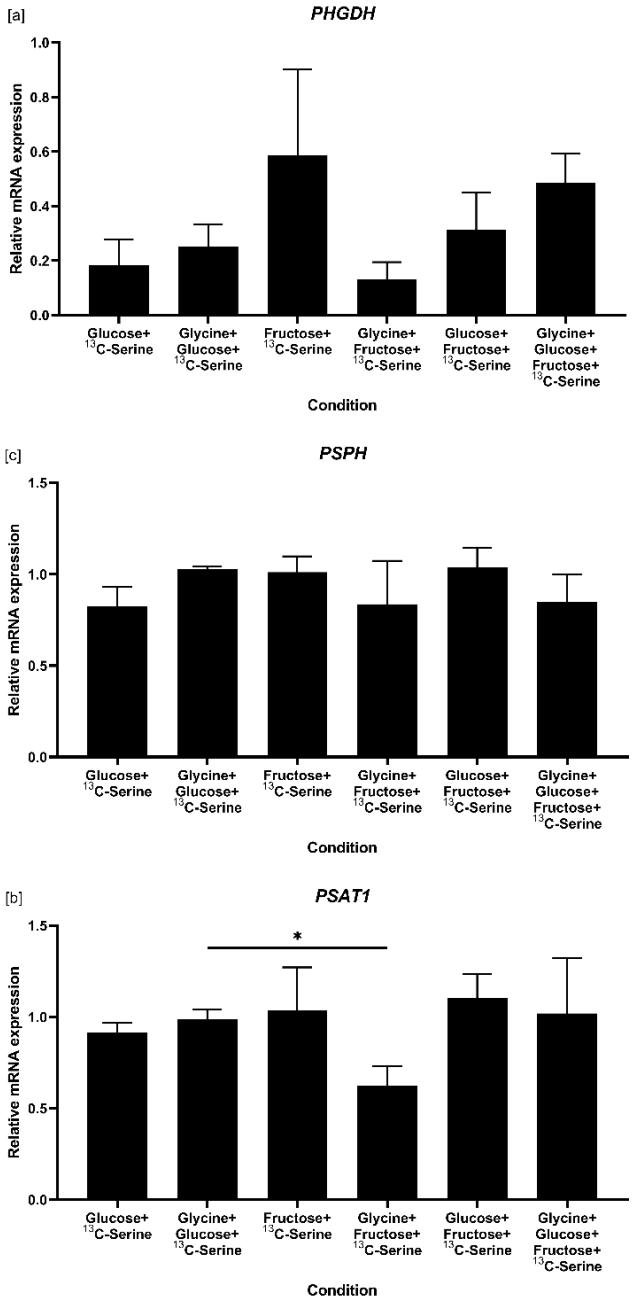
| | | | | | | |
|---|---|---|---|---|---|---|
| *SLC1A5* | Solute carrier family 1 member 5 | XM_02797852 5.2 | Fwd | ATCTTACCGCTGGTGGTGTG | 60 | 100 |
| | | | Rev | CAAGGAAAAAGAGCAGCGCC | | |
| *SLC2A1* | Solute carrier family 2 member 1 | XM_02796862 8.2 | Fwd | TGGGAAAGTCCTTTGAGATGC | 60 | 81 |
| | | | Rev | GGTCAGGCCGCAGTACACA | | |
| *SLC2A3* | Solute carrier family 2 member 3 | NM_00100977 0.1 | Fwd | AAATTAGGGCCATGGGGACCA | 60 | 128 |
| | | | Rev | TTTTATGATCGCCTCAGGAGCA | | |
| *SLC2A5* | Solute carrier family 2 member 5 | NM_00100945 1.1 | Fwd | GGTGGGAATATGTGCAGGTC | 60 | 197 |
| | | | Rev | CAGTCAATCCGAGGAGGATGG | | |
| *SLC2A8* | Solute carrier family 2 member 8 | A XM_04224568 4.1 | Fwd | CGTCCTCACCAACTGGTTCA | 60 | 154 |
| | | | Rev | CCCTTTGGTCTCAGGGACAC | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| *SLC6A9* | Solute carrier family 6 member 9 | XM_042239773.1 | Fwd | CGCTTCGTCTCACCAGCTAT | 60 | 126 |
| | | | Rev | CAGAGCCATGAGGAAGCCAA | | |
| *TBP* | TATA-box binding protein | XM_012166509.2 | Fwd | ACCACCGTTTCTTGGTGTCC | 60 | 147 |
| | | | Rev | TGACGTTCACCTTCTTGGCA | | |
| *YWHAZ* | Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta | NM_001267887.1 | Fwd | GACTGGGTCTGGCCCTTAAC | 60 | 72 |
| | | | Rev | GACTGGGTCTGGCCCTTAAC | | |

## Supplementary Figures




**Supplementary Figure III-1 Expression of mRNAs required for nutrient transport in conceptus tissue on Day 17 of gestation after a 2 h incubation with [U-$^{13}$C]serine.** Data are expressed as relative expression of mRNAs, means ± SEM. n=3 samples per group.




**Supplementary Figure III-2 Expression of mRNAs required for serinogenesis in conceptus tissue from Day 17 of gestation after a 2 h incubation with [U-$^{13}$C]serine.** Data are expressed as relative expression of mRNAs, means ± SEM. n=3 samples per group.

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# CHAPTER IV

# GENERAL DISCUSSION AND CONCLUSIONS

## General Discussion

Sheep are a relevant species to utilize for the study of reproductive physiology. First, sheep are beneficial for the animal agriculture industry, as they are utilized for the production of meat (lamb/mutton), wool, leather, dairy, other byproducts, and for management of vegetation. While sheep are an agriculturally relevant species within their own right, they also provide researchers with a model for ruminant species that is more economical compared to cattle. Sheep cost less to purchase and maintain, so the inferences drawn from research utilizing sheep may be used broadly to describe ruminant physiology or may lay the foundational knowledge for future studies utilizing cattle and other ruminants. Additionally, the length of gestation for sheep is shorter than that of cattle, so sample collection and analysis is expedited.

Sheep are a relevant and accepted animal model for the study of human pregnancy. For obvious ethical reasons, studying pregnancy in women has its limitations. Thus, scientists must rely on animal models to test their hypotheses. Many animal models exist to provide a means by which scientists can answer these questions, including non-human primate species, laboratory rodents, and large animal livestock species. Even though the utilization of eutherian (placenta-bearing) mammals could theoretically provide a model by which to study translational reproductive physiology, the reproductive system is one of the most diverse organ systems across eutherian species. Therefore, it is important to choose an appropriate animal model to test a hypothesis. While the sheep placenta has

more cell layers between maternal and placental vasculatures, the villous tree of the cotyledon mirrors that of the human placenta [1]. The growth rate of the sheep fetus and the maturity level of the neonate closely mirrors that of the human fetus, unlike the litter-bearing rodent species that give birth to precocious neonates. Additionally, the relatively protracted length of the peri-implantation period of pregnancy in sheep, accompanied by predictable changes in conceptus morphology, allows researchers a wide window of opportunity to study conceptus development. While no animal model can truly represent a human pregnancy, sheep serve as a valuable substitute for research to address human conditions during pregnancy [2].

There is an incredible amount of tightly regulated communication that must occur between the conceptus and the uterus to achieve a successful pregnancy. The primary signaling factors that contribute to the establishment and maintenance of pregnancy are well characterized [3]. However, it is important to note that no single signal is sufficient for the establishment of a successful pregnancy – it is the coordinated actions of signals and subsequent responses from both the mother and the conceptus that are required for the establishment and maintenance of a successful pregnancy. Perhaps one of the most important signaling factors to come from the sheep conceptus initially is interferon tau (IFNT), the maternal recognition of pregnancy signal. On the maternal side, progesterone (P4) is a required hormone for the establishment and maintenance of pregnancy. During the peri-implantation period, P4 secreted from the corpus luteum binds to progesterone receptors that localize to uterine epithelia and stromal cells in the endometrium to upregulate the expression of genes and proteins that promote conceptus development, such

as those that code for amino acid and glucose transporters [4]. IFNT also acts on the cells of the endometrium to further stimulate gene and protein expression, some of which were first induced by P4 [4]. The secretions from the uterine luminal (LE) and glandular (GE) epithelia contain a myriad of molecules, and the nutrients (amino acids and hexose sugars) that support the metabolism of the conceptus before the placenta is established are of particular interest. It is estimated that 20-40% of pregnancies in mammalian species are lost, with two-thirds of these losses occurring during the peri-implantation period of pregnancy, some of which may have been due to failed communication between the conceptus and the endometrium [5–7]. Therefore, understanding the importance of these signals and how they induce changes in the endometrium, such as increasing the transport of specific nutrients and how these nutrients may be utilized by the conceptus, is crucial for understanding what makes a successful pregnancy. This knowledge benefits researchers by providing a foundation for development of strategies to improve conception rates, fetal growth, and overall pregnancy success in mammalian species.

Arginine has been extensively studied because the products of arginine metabolism, including nitric oxide (NO), agmatine and polyamines, and creatine are important for conceptus development, implantation, placentation, and angiogenesis [8]. Increases in endometrial angiogenesis mediated by NO prepares a favorable environment in which the conceptus can attach and initiate placentation, and later NO stimulates placental angiogenesis, therefore increasing uterine and placental blood flows and nutrient transfer to the fetus [9,10]. Additionally, polyamines promote conceptus development in rodents [11], pigs [12,13], and sheep [14]. In ruminants, however, arginine is degraded

rapidly by ruminal microbes such that little arginine reaches the small intestine [15], which means that supplementation of arginine in the diet of ruminants with the hopes of increasing reproductive success will have little benefit. Therefore, dietary supplementation of citrulline, as the precursor for arginine, may provide an indirect way to supplement arginine for ruminants during pregnancy as citrulline escapes ruminal degradation by bacteria and can be converted to arginine by the enterocytes of the small intestine [16,17]. This highlights the importance of understanding specie-specific physiology with the aim for improving reproductive success.

Recently, studies have demonstrated that the mRNAs or proteins involved in the pathway for converting arginine to polyamines in the ovine endometrium on Days 9, 12, and 125 of may be regulated by P4 [18,19]. Additionally, it has been suggested that polyamines are involved in the regulation of conceptus elongation in sheep, as conceptuses which are unable to synthesize adequate amounts of polyamines do not elongate or secrete sufficient amounts of IFNT for the establishment of pregnancy [14]. However, it was unknown how two important signaling factors during the peri-implantation period of pregnancy (P4 and IFNT) affect the synthesis and secretion of polyamines by the ovine endometrium. The results of Chapter 2 demonstrate that P4 and/or IFNT affect the endometrial expression of mRNAs and enzymes involved in polyamine metabolism, as well as transporters for the transport of polyamines into the uterine lumen. Previously published reports demonstrated a clear increase arginine in the uterine lumen between Days 3 to 14 of the estrous cycle, suggesting P4-regulated increases in transport [20]. Further, the abundance of arginine and ornithine is greater in the uterine flushings of

pregnant than cyclic ewes, implicating IFNT as a contributor to these increases in amino acids [20]. Interestingly, the results presented in Chapter 2 suggest that there may be other maternal- or conceptus-derived factors that play a role in the endometrial secretion of amino acids and polyamines. It was expected that the animals treated with P4 and/or IFNT would have greater concentrations of amino acids such as arginine, ornithine, and citrulline, but the results did not confirm this hypothesis. Proteomics data from bovine uterine flushings identified conceptus-derived signaling factors, other than IFNT, that stimulate the endometrium and aid in the establishment of pregnancy [21,22]. It would be interesting to perform the same type of analysis on the uterine flushings collected in this study and compare them to uterine flushings from a normally pregnant or cyclic ewe on Day 16 of gestation or the estrous cycle, respectively.

In sheep, glycine and serine are the first and second most abundant amino acids in the uterine flushings during the peri-implantation period of pregnancy [20], and serine is the most abundant amino acid in amniotic fluid and allantoic fluid across pregnancy [23], implicating both of these amino acids as important mediators for conceptus growth. Serine can be utilized for the biosynthesis of other molecules, such as phospholipids, but one of the most well-studied roles of serine metabolism is through one-carbon (1C) metabolism [24]. This pathway links the folate cycle and the methionine cycle, which together generate formate (via the folate cycle) required for nucleotide biosynthesis [25] and synthesis of s-adenosylmethionine (SAM; via the methionine cycle), which serves as an important methylating agent [26]. Serine is required as a donor of carbons into the folate cycle, as is glycine, while methionine donates carbons through the methionine cycle [25].

Results from the study described in Chapter 3 demonstrate, for the first time, that the ovine conceptus utilizes extracellular serine, as well as glucose and fructose, to generate formate. Interestingly, this study also demonstrated that the ovine conceptus utilizes significantly more glucose and fructose, rather than serine, to produce formate. The abundance of glucose in uterine flushings is greater in pregnant than cyclic ewes, and increases in abundance between Days 10 to 16 of the peri-implantation period [20]. Fructose is undetectable in uterine flushings of pregnant ewes on Day 12 of pregnancy [19], but results in Chapter 3 show that fructose is detectable by Day 17 of pregnancy and 11.4 time more abundant than glucose, indicating a sharp increase in the synthesis of fructose by conceptus trophectoderm during the peri-implantation period of pregnancy. As fructose is a sequestered sugar within the placenta and fetal fluids of livestock species and other ungulates [18,27–29], there has been a growing interest in the potential roles of fructose during conceptus growth. It has been suggested that fructose is utilized for the production of nucleic acids in the fetal pig [30], but this has not been experimentally demonstrated for conceptus tissue in sheep. The results from Chapter 3 indicate that both fructose and glucose are utilized in equivalent amounts for the production of formate, which is a precursor for nucleic acid synthesis. It was interesting to discover that extracellular serine was not utilized as extensively as glucose or fructose to produce formate, suggesting that serine may be preferentially utilized in other metabolic pathways.

The study described in Chapter 3 was intended to include data from Day 15 of pregnancy when one could have demonstrated any potential increases in formate production by conceptuses between Days 15 and 17 of pregnancy. Unfortunately, due to

poor pregnancy rates, we were unable to collect enough conceptus tissue to have enough statistical power to perform the analyses. Therefore, it would be interesting to expand the results from Chapter 3 to include conceptus tissue from other gestational days to determine how the rate of formate synthesis changes over time. Additionally, it would be interesting to determine the rate of formate synthesis by the ovine conceptus cultured in the presence of physiological concentrations of glucose and fructose, as well as in hypoxic environments.

Polyamine metabolism and 1C metabolism are linked to each other by their shared intermediate, SAM. Through polyamine metabolism, SAM is decarboxylated and then donates a propylamine group to putrescine to form spermidine (via spermidine synthase) or to spermidine to form spermine (via spermine synthase), which generates methylthioadenosine (MTA) as a byproduct [31]. MTA carries the 1C unit removed during the methionine cycle and is converted by MTA-phosphorylase to methylthioribose-1-phosphate and adenine, which are recycled through the methionine and purine salvage pathways, respectively [32]. Thus, as DNA and RNA strands are built from the products of 1C metabolism, the transcription or translation of relevant genes may be, in turn, regulated by putrescine, spermidine, or spermine produced through polyamine metabolism.

Current studies in the laboratory are yielding data not included in this dissertation but aim at further understanding the importance of polyamines in the ovine uterus across gestation. Previous studies have demonstrated the importance of polyamines for conceptus development in sheep, but it is unknown how each individual polyamine affects genes in

the endometrium that are important for implantation and placentation. Thus, an experiment was performed with endometrial explants cultured with P4 and IFNT, in addition to either agmatine, putrescine, spermidine, and spermine alone, as well as their combination. qPCR analyses for genes of interest (those involving cell proliferation and apoptosis, angiogenesis, cytoskeletal remodeling, cell-to-cell adhesion, and immune regulation) are being performed, and HPLC analyses are revealing concentrations of polyamines in culture medium. Additionally, given the emerging evidence for roles for P4 and IFNT in the regulation of the expression of mRNAs and proteins regulating polyamine synthesis and secretion, it was hypothesized that other molecules with important roles in the regulation of conceptus growth may regulate expression of those genes. Dr. Russell V. Anthony at Colorado State University has a well-established sheep model of reduced placental lactogen (also known as chorionic somatomammotropin, CSH), which leads to both intrauterine growth restricted (IUGR) and non-IUGR phenotypes. It was hypothesized that the CSH knockdown-induced decreases in blood flow may affect the concentrations of polyamines in fetal plasma or the expression of genes involved in polyamine metabolism in the placenta. Dr. Anthony has generously donated maternal and fetal serum samples, as well as samples of caruncles and cotyledons to expand our investigations of regulators of polyamine metabolism.

## Summary and Conclusions

The findings from the studies described in this dissertation revealed: 1) how progesterone (P4) and interferon tau (IFNT) work cooperatively and independently to affect expression of mRNAs, enzymes and transporters involved in polyamine metabolism in the uterus and transport of polyamines into the uterine lumen during the peri-implantation period of pregnancy; and 2) the relative contributions of serine, glycine, glucose, and fructose for one-carbon (1C) metabolism by the ovine conceptus during the peri-implantation period of pregnancy. These novel findings are valuable in advancing understanding of the importance of specific nutrients in the context of conceptus development during the peri-implantation period of pregnancy in sheep and improving pregnancy success in livestock species

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