EFFECTS OF PROGESTERONE SUPPLEMENTATION ON GROWTH AND DEVELOPMENT OF THE FETAL-PLACENTAL UNIT

A Thesis

by

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ABSTRACT

In a previous study, administration of progesterone (P4) to ewes during the first 9 to 12 days of pregnancy has been shown to accelerate blastocyst development by Day 12 of pregnancy, likely due to P4-induced upregulation of key genes in uterine epithelia responsible for secretion of histotroph. This study determined if exogenous P4-induced acceleration of blastocyst development during the peri-implantation period affects fetal-placental development on Day 125 of pregnancy. Suffolk ewes (n=40) were mated to fertile rams and assigned randomly to receive daily intramuscular injections of either corn oil vehicle (CO, n=20) or 25 mg progesterone in CO (P4, n =20). After breeding (Day 0), treatments began on Day 1.5 and continued through Day 8 of pregnancy. Ewes from each treatment group were hysterectomized on Day 125 of pregnancy. After separating the endometrium from the chorioallantois, sections of placentomes, caruncles, cotyledons, and endometrium were processed to assess expression of mRNAs and proteins. Plasma from maternal and fetal blood was analyzed for concentrations of P4 by RIA. Samples of allantoic and amniotic fluid were obtained for analysis of glucose, fructose, amino acids, and polyamines. Volumes of the fetal fluids were measured. Fetal parameters recorded included weight, crown-rump length, abdominal and chest circumferences, and sex. Placental measurements included weight, length, and number of placentomes. There was no difference in fetal growth nor placental growth due to treatment; however, placentae of single pregnancies were longer ($P<0.0001$) and had more placentomes ($P<0.0001$) than those in twin pregnancies. qPCR analysis showed differential expression of genes between P4 and CO treated ewes in endometrium and placentomes. Low-affinity cationic amino acid transporter *SLC7A2* mRNA increased ($P=0.0022$) in endometria of P4 treated ewes,

while *VEGFA* and *TUB* were increased (P=0.0242) and decreased (P= 0.0089), respectively, in single pregnancies. In placentomes, expression of *SLC2A5* and *SLC2A8* mRNAs increased (P=0.0024) and (P=0.0005), respectively, in P4 treated ewes. Expression of both *ACTB* and *TUB* mRNAs increased (P=0.0019) and (P<0.0001), respectively, in placentomes of P4 treated ewes. *SDHA* and *GAPDH* were used as control genes. There was significantly more fructose than glucose in placental fluids (P > 0.05).

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Introduction

It is estimated that embryonic mortality accounts for 20-40% of pregnancy failures in all mammals, and 66% of these losses occur during the peri-implantation period of pregnancy [1, 2]. This occurs during the time between fertilization of the oocyte, successful implantation of the blastocyst/conceptus (embryo and it's associated extra-embryonic membranes), and placentation, i.e., the establishment of the placenta in the uterus. Because this period of time coincides with maternal recognition of pregnancy, these numbers may be underestimated, as blastocysts frequently fail in their development before the mother's body knows it is pregnant.

Embryonic survival affects all facets of the livestock industry. In sheep, beef cattle, and moderate yielding dairy cattle, unassisted natural breeding results in 85-100% successful fertilization rates—however by the peri-implantation period, over half of all embryos are lost. High yielding dairy cows have lower fertilization and pregnancy rates, likely due to years of selection for high milk yield, while selection for genes associated with fertility are lowly heritable, and therefore, went largely ignored [3]. A female that repeatedly loses pregnancies or is a repeat breeder (requires multiple inseminations in one breeding season) decreases a producer's profitability. Losses are evident even with the use of assisted reproductive technologies (ART). Using ART such as superovulation, embryo transfer, *in vitro* oocyte maturation and fertilization (IVM/IVF), and artificial insemination (AI) alone or in combination yields decreased pregnancy rates compared to natural service. Increasing reproductive efficiency has been a large driving force for livestock-based

reproductive research, as excellent genetics and reliable fertility result in more efficient production of animals. Identifying traits or genes that are correlated with embryonic survival would allow producers to select and/or retain females with high fertility without having to waste a breeding season. While reproductive efficiency is not usually 100% in livestock, it is undoubtedly higher than that of humans [4].

Despite the importance of the reproductive process, it is quite inefficient in humans. In a normal menstrual cycle, the probability of conception is only 30% [5]. Some 10-15% of the human population experience infertility issues, and for those that utilize assisted reproductive technologies, 50-75% of pregnancies fail to be established [6]. Humans have a relatively narrow window of receptivity of 5 days when the blastocyst has a chance to implant in the uterus [7]. While conception and pregnancy rates are relativley low in the healthy population of humans, benign reproductive diseases are common in women who experience fertility issues, and thus have increased difficulties conceiving and maintaining a pregnancy [8]. ART has helped families with low fertility, but implantation and pregnancy rates remain low. A typical procedure used in fertility clinics is controlled ovarian stimulation (COS), which involves steroid hormone treatment for superovulation and induction of endometrial receptivity. However, these exogenous hormones alter the uterine environment, such as affecting steroid hormone receptors and uterine secretions. Individuals utilizing ART have decreased endometrial receptivity, because the natural uterine environment is difficult to emulate in an unnatural setting [9]. *In vitro* studies, no matter how complex and sophisticated in design, cannot recapitulate the functional uterus, which is why reproductive biologists utilize animal models to understand pregnancy.

Sheep have been used as a model for human pregnancies for the past 40 years [10]. While pregnant sheep do not demonstrate a perfect rendition of the human reproductive timeline, they do possess similarities that allow for interpretation of events during pregnancy. The endocrine profiles of sheep and humans are more similar to one another than are the endocrine profiles of rodents and humans, even though mice are a popular model for human pregnancy. This allows reproductive biologists to better understand how steroid hormones affect implantation, pregnancy, and parturition. The peri-implantation period in sheep is extended for a longer period of time than is observed in invasive implanting species, which allows for sampling of tissues and fluids from pregnant ewes over a relatively long period of time to assess how the uterus and conceptus are changing during this process. Sheep conceptuses begin as a spherical structure, but rapidly elongate into a filamentous structure, while at the same time the uterus becomes more edematous, vascularized, and secretory. Although the human conceptus does not undergo these morphological changes, the process of conceptus elongation in sheep allows for reliable visual appraisal of the progression of embryonic development and uterine responses. Sheep do not have invasive implantation as humans do, but interestingly syncytial cells inhabit the unique placentomal anatomy in sheep, and this resembles the syncytial layer of cells that line the placental villi of humans. Sheep have a gestational period of about average 147 days and a term fetal lamb weighs about the same as a human fetus, signifying their advantage for fetal physiology studies [11]. Animal models for pregnancy are essential in this field, as the samples required for study of embryonic/fetal and placental development in humans often cannot be, or are difficult to obtain for obvious ethical reasons.

Much is known, but much remains to be elucidated as to why embryonic death loss is prevalent in mammals and particularly humans. Implantation requires coordinated spatiotemporal signaling from both the conceptus and the uterus—a disconnect between the two may lead to embryonic death. In humans, the short window of receptivity necessitates close synchrony between development of the conceptus and uterus, which may easily be disrupted by hormonal treatments. Ovarian stimulation with estradiol and progesterone alter the endocrine milieu of the female, and thus the receptivity of the uterus (a sensitive organ to steroid hormones). Expression of genes important in the implantation process may be altered in low-fertility females as well. Adhesive molecules that aid in attachment and/or invasion of a conceptus or histotrophic support provided by the uterus are vital for embryonic survival and may be decreased in females experiencing fertility issues. Furthermore, the age of a female may play a role. It is not uncommon for producers to retain an older female if she consistently produces an offspring each year, or for a woman to decide to have a child later in her reproductive lifespan. However, as maternal age increases, the ovarian reserves of viable oocytes are exponentially depleted. By the end of a female's reproductive capabilities, oocytes are likely to have many abnormalities. Or, perhaps implantation is a screening process of the reproductive system to filter out any blastocysts/embryos that would not have survived to term regardless, which may have been due to chromosomal abnormalities or improper development of the zygote. Early termination of such a pregnancy would allow the female to return to cyclicity and have an opportunity to become pregnant again, rather than waste maternal resources on gestating an embryo that would likely die. Ultimately, the mechanisms that cause a pregnancy to fail many and most remain a mystery.

Understanding the physiological, structural, and temporal changes that occur during conceptus development is important as it provides basic insight into key mechanisms responsible for normal fetal and placental development; this may improve our understanding of means to increase fertility and decrease pregnancy losses in both livestock and humans. The aim of this project is to understand how a post-conception exogenous systemic progesterone treatment affects fetal and placental growth in sheep as determined on Day 125 of pregnancy. We are interested in the progesterone-induced modifications of uterine and placental gene expression and composition of uterine secretions. Our hypothesis is that the altered uterine environment that accelerates conceptus development during early pregnancy will result in advanced feto-placental development later in pregnancy.

Progesterone in early pregnancy

Following ovulation, the follicular theca cells and granulosa cells luteinize to form the corpus luteum (CL), which is a temporary ovarian endocrine structure that produces progesterone (P4) [12, 13]. If the oocyte is not fertilized, a luteolytic hormone will be secreted by the uterus in sub-primate mammals to cause structural and functional termination of the CL. In the case of sub-primate species, the luteolytic hormone, prostaglandin F2α (PGF2α), is secreted from the uterine epithelia [14, 15]. PGF2α causes structural and functional termination of the CL, which allows selected follicle(s) on the ovary to mature and become dominant. The female will then come into estrous, ovulate, and the estrous cycle continues. However, if sperm are present to fertilize the oocyte, the resulting blastocyst must produce a signal to alert the uterus of its presence; this is the

pregnancy recognition signal, and it is required for the uterine environment to become receptive to implantation and placentation by the developing conceptus. In ruminants, interferon-tau (IFNT) is an anti-luteolytic protein that serves as the maternal recognition of pregnancy signal. One of the main functions of IFNT is to prevent luteolysis from occurring such that progesterone secretion by the CL is prolonged and pregnancy can be established and maintained [16, 17]. While ovine IFNT is secreted only between Days 10 and 21 of pregnancy [18], the CLs persist for much longer. It isn't until Days 55 to 60 of pregnancy that the placenta begins to secrete enough P4 to maintain pregnancy [14, 19].

Progesterone is classically considered the "hormone of pregnancy", because it performs many functions within the uterus to support growth and development of the conceptus (embryo/fetus and placenta). This includes decreasing myometrial contractility to support implantation and placental development, inhibiting estrus through negative feedback on the hypothalamus and thus GnRH pulsatility, as well as stimulating development and activity of uterine glands [20-22]. However, one of the counter-intuitive aspects of mammalian pregnancy is that the progesterone receptor (PGR) must be down-regulated in the uterine luminal (LE) and superficial glandular (sGE) epithelia in order for successful implantation and placentation to occur [14, 23]. There are two theories as to how progesterone continues to stimulate the LE and sGE, despite an absence of receptors. Progesterone is hypothesized to act through its receptors that remain in the uterine stromal cells to signal the epithelia in a paracrine manner via growth factors. Secretion of these growth factors—FGF7, FGF10, and HGF—from stromal fibroblasts is mediated by progesterone and are thus termed progestamedins [24-26]. Or, PGR itself is inhibitory, and down-regulation allows for the expression of key genes. This communication allows

progesterone to continue to act on the epithelia of the uterus even though receptors are absent in those cells. The uterine histoarchitecture can be viewed in Figure 1.1. Within the uterus, anti-adhesive proteins such as mucins (particularly Muc-1) prevent epithelial surfaces from adhering—a physiologically important characteristic for any tubular type of organ [27, 28].

Early conceptus development and formation of the extra-embryonic membranes

In a cyclic ewe, continuous P4 secretion for 8-10 days down-regulates the progesterone receptors (PGR) in all uterine epithelial cells, but PGR expression is maintained in the stromal cells of the endometrium [29, 30]. By Day 13, estrogen receptors (ESR1) up-regulate, which indirectly causes up-regulation of oxytocin receptors (OXTR) by Day 14 [24, 31]. Binding of OXT to its receptor (OXTR) induces secretion of luteolytic pulses of PGF2α between Days 14 and 16 of the estrous cycle, and by Day 17 the CL is fully regressed and the estrous cycle begins again [24, 32].

However, if sperm are present to fertilize the oocyte, the resulting zygote will undergo cell division and form a morula, which has more than 32 totipotent cells. The morula will enter the uterus on Day 4 and develop into a blastocyst on Day 6; this morphological change can be visually appraised as the morula compacts and differentiates into the inner cell mass (ICM) and the trophectoderm (Tr) [33, 34]. At this point, the cells that make up the blastocyst are pluripotent—the ICM will give rise to the embryo/fetus and the Tr will become the chorion or fetal portion of the placenta. The blastocyst will shed the zona pellucida (ZP) between Days 8 and 9 and is only 100-200 um in diameter (Figure 1.2) [35, 36]. The blastocyst develops into a conceptus, which refers to the embryo/fetus

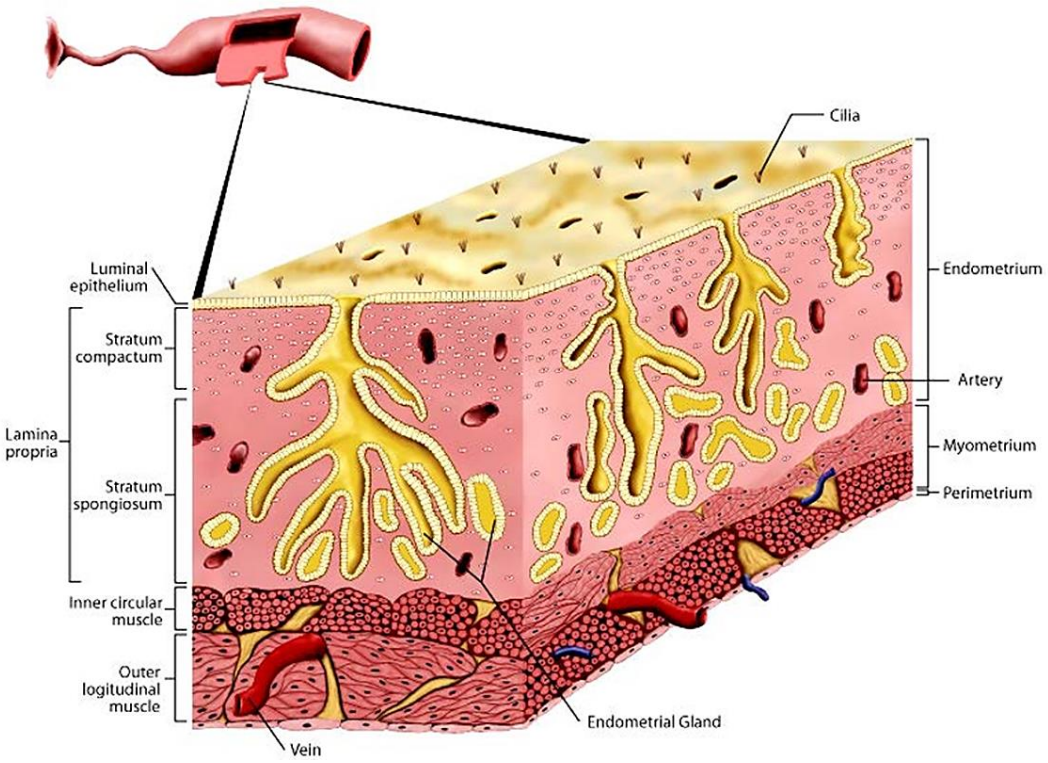

Figure 1.1. Uterine histoarchitecture in livestock species. The luminal epithelia (LE) is a single layer of polarized columnar cells with microvilli that line the lumen of the uterus. Supporting the LE is the lamina propria, which is predominantly composed of stromal fibroblast cells, and can be further divided into the stratum compactum and the stratum spongiosum. Together, the uterine LE, sGE, GE, and lamina propria constitute the endometrium of the uterus. The branched tubular uterine glands secrete histotroph (discussed later) into the uterine lumen that is essential for conceptus development and implantation. The epithelia of these glands can be subdivided into sGE and GE. The areas of the uterus that do not have glands opening to the lumen are the caruncular regions; glandular openings can be found only in the intercaruncular areas. The uterus contains a layer of smooth muscle called the myometrium, which has an inner circular and an outer longitudinal layer that generates uterine contractions. The perimetrium is the outer surface lining of simple squamous epithelium that covers the uterus. Figure 1.1 reprinted with permission and has been modified from Geisert and Bazer, 2015 [37].

and its associated placental membranes. On Day 10, the mononuclear Tr cells secrete IFNT, which abrogates the luteolytic mechanism [18, 38]. This is coincident with rapid elongation of the conceptus, as these morphological changes are necessary to increase surface area of the conceptus, and are required for the production of IFNT [39-41]. The conceptus develops from a spherical (400-900 um) to a tubular from between Days 9 and 13 (10-22 mm), then a filamentous (>100mm) form between Days 13 and 15, and by Days 16 to 18 the extra-embryonic membranes extend into the contralateral uterine horn and the adhesion process is nearly complete [24, 34, 36]. Adhesion of the conceptus to the uterine LE is a protracted process that extends from Days 13 to 17 in the sheep, with full attachment completed between Days 20 to 23 of pregnancy [36, 42, 43]. Between Days 15 to 18 of gestation, the ICM differentiates into the embryo proper, while the trophectoderm and the extra-embryonic membranes also develop as the conceptus elongates [44].

The fetus develops from the primitive ectoderm, endoderm, and mesoderm, while both the amnion and allantois will form from the extra-embryonic ectoderm and mesoderm [44, 45]. The fetus is surrounded by the amnion, which creates a cavity filled with amniotic fluid [45]. This fluid serves to protect the fetus and allows for its symmetrical development [46]. External to the amnion is the allantoic cavity enveloped by the allantoic membrane [45]. Studies in both the pig [47] and the sheep [48] refute the dogma that allantoic fluid is merely a reservoir for waste, as it also allows for the accumulation of nutrients that support development of the embryo/fetus throughout gestation. In livestock species, the allantois (endoderm and mesoderm) and the chorion—which is the differentiated trophectoderm (ectoderm and mesoderm)—fuse to form the chorioallantois, which is what is in contact with the uterine LE [45].

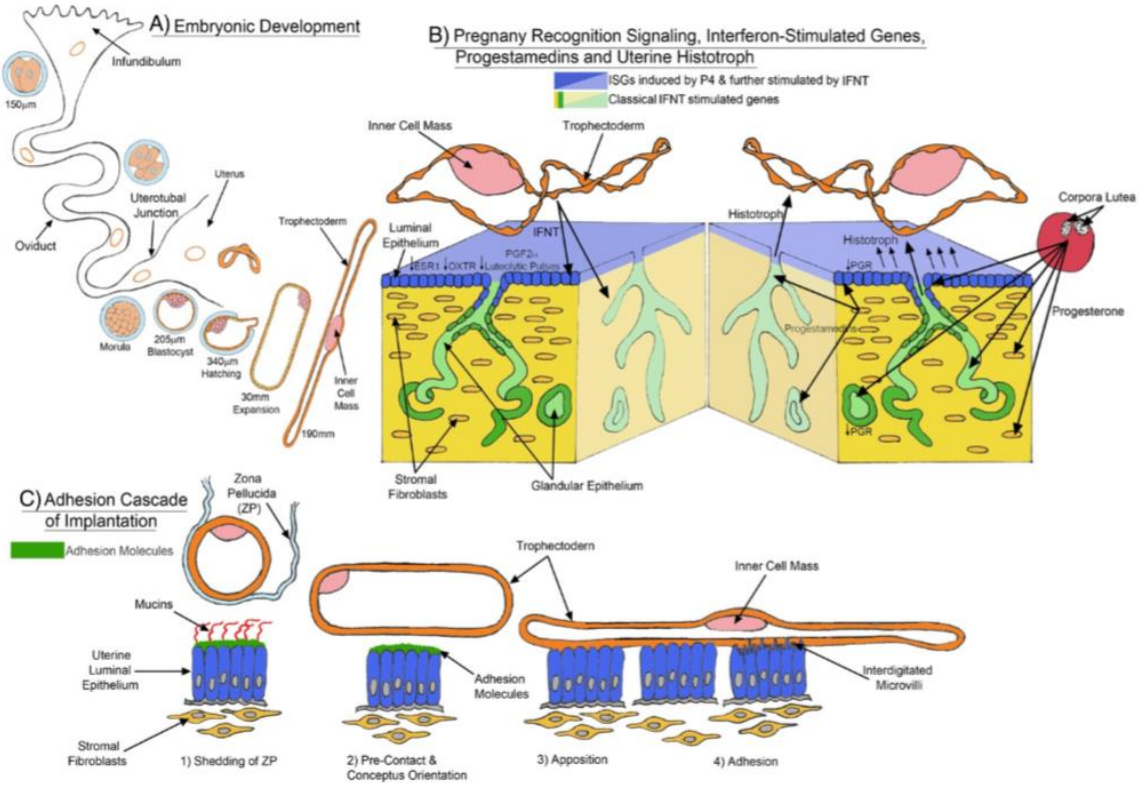

Figure 1.2. Events of conceptus development, pregnancy recognition signaling, and adhesion cascade. Early conceptus development in the sheep is a dynamic process. Once the blastocyst enters the uterus, the implantation cascade is initiated, and the conceptus undergoes massive morphological changes. Although the timing is different, the stages of implantation are similar across all mammals. Most species will finish implanation at Stage 4; in species with hemochorial placentation however, Stage 5 involves invasion of the blastocyst through the uterine LE and into the stroma. Reprinted from [49].

Implantation

The phases of implantation are summarized in Figure 1.2. Phase 1 of the implantation cascade begins with hatching of the blastocyst within the uterine lumen, which occurs at Day 8 in sheep [35, 43]. Anti-adhesive mucins are still present on the LE; therefore, the blastocyst cannot adhere to the uterine LE [28, 50]. In Phase 2, the uterine LE has decreased expression of mucins at the apical surface while the blastocyst has oriented itself with the ICM facing towards the uterine lumen [28, 51, 52]. In sheep, rodents, pigs, and other litter-bearing species, expression of mucins is down-regulated throughout the entire uterus [53, 54]. Apposition occurs in Phase 3, which is the first contact the trophectoderm makes with the uterine LE. A mosaic of adhesive glycoproteins on the apical surface of uterine LE and Tr, such as L-selectin and glycans [27], allow initial, transient attachments that serve to: a) properly orient the Tr in close juxtaposition with the uterine LE; and b) briefly anchor the Tr to provide mechanical force for it to continue to elongate [36, 55]. In sheep and cows, outgrowths of the trophectoderm called chorionic papillae will protrude into uterine glands, which also help immobilize the elongating conceptus, and provide forces to aid in its elongation [36, 56]. In Phase 4, microvilli of the uterine LE begin to interdigitate with the microvilli of the trophectoderm to create maximum surface area between the two membranes [36, 43]. Integrin receptors are transmembrane glycoproteins on both the uterine LE and Tr that bind extra-cellular matrix (ECM) proteins for adhesion [57]. Secreted ECM proteins include but are not limited to secreted phosphoprotein 1 [SPP1, also known as osteopontin (SPP1)], glycosylated cell adhesion molecule (GlyCAM1), and galectin 15 (LSGS-15) [27]. These adhesions at the uterine-placental interface allow for juxtacrine interactions of two adjacent

epithelial surfaces, which is a unique physiological phenomenon, as there is no other place in the body where two apical surfaces of epithelia attach—in a healthy pathophysiologic state [28, 57, 58]. Finally, in Phase 5, mononuclear cells of the trophectoderm fuse to form binucleate cells (BNC), which then migrate and fuse with a uterine LE cell to form feto-maternal trinucleated cells (TNC), and eventually, a multinucleated cells syncytium [43, 55, 59]. In the sheep, firm adhesion and formation of BNC begins on about Day 16 and is complete between Days 20 and 23 of pregnancy [36, 42]. Fusion of more BNCs to TNCs establishes a syncytial plaque (Figure 1.3) [59]. This only occurs in the non-glandular caruncles of the uterus; the LE of the caruncle and the BNCs of the Tr completely fuse and that whole non-glandular area will be syncytialized in the sheep by Days 24 to 25 of pregnancy [59]. Syncytial layers are valuable in the placenta because a) they are adept at transporting molecules, as more nuclei in one cell allows for increased production of transport proteins, and b) a single layer brings fetal and maternal vasculatures closer together compared to two separate layers [59]. In the inter-caruncular glandular areas of the uterus, syncytial plaques do not form and the trophectoderm and uterine LE remain as monolayers [36, 45]. It is paramount that this nidatory cascade occurs in both a spatial (cell specific) and temporal manner for implantation to be successful.

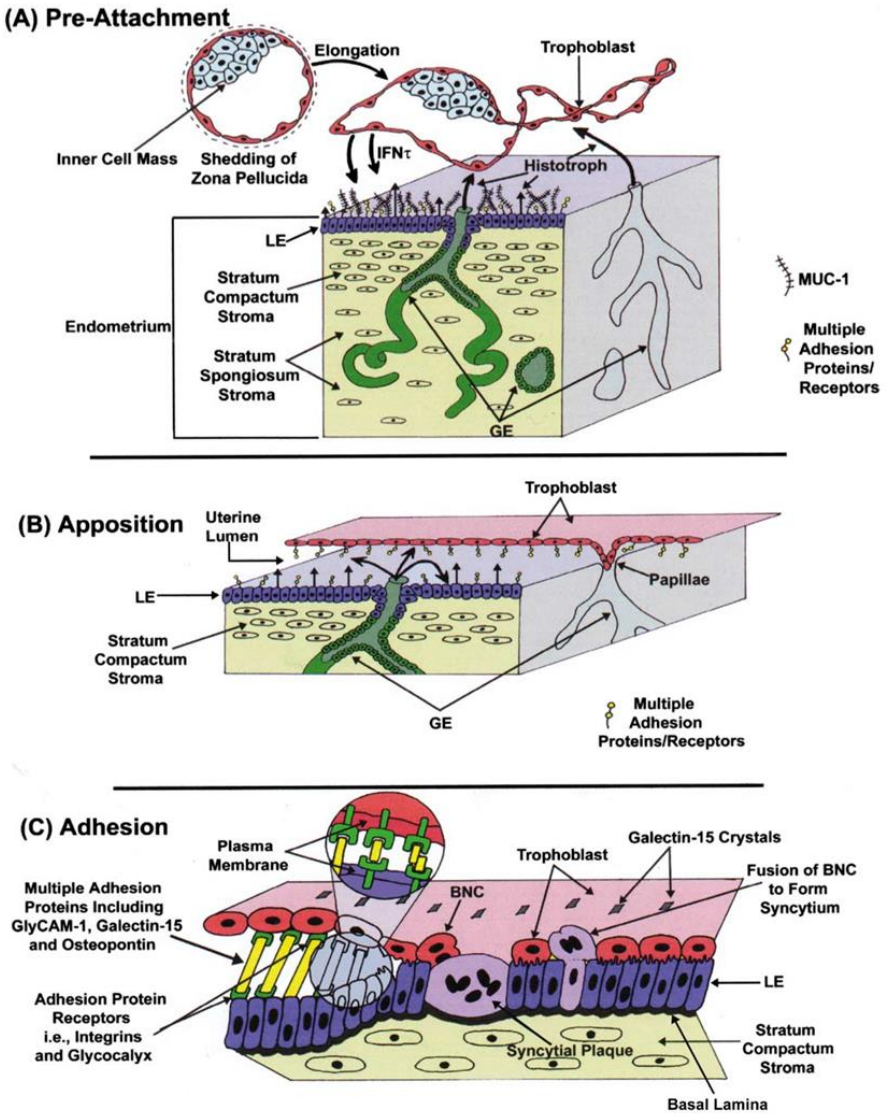

Figure 1.3. Interactions between the uterus and the conceptus. Pre-attachment constitutes the hatching, pre-contact and blastocyst orientation phases of the implantation cascade. Here, interactions between the free-floating conceptus and the endometrium allow for the secretion of histotroph by uterine GE. Histotroph supports the conceptus in early pregnancy and continues to provide nutrients to the fetus until parturition. In the apposition phase shown here, adhesion proteins allow the Tr to stabilize itself in close proximity to the uterine LE that has downregulated expression of the anti-adhesive MUC-1. During adhesion, bridging ligands such as GlyCAM-1, Galectin-15, and osteopontin form between integrin receptors on both the uterine LE and the Tr for a strong attachment of the two epithelial surfaces. The BNC of the trophectoderm begin to migrate and fuse with LE, which form syncytial plaques. Figure reprinted with permission from Spencer et al., 2004 [43].

Placentation and factors indicative of placental function

Sheep and cows have a cotyledonary type of placenta. Specialized regions of the chorioallantois will develop structures called cotyledons [34, 60]. During apposition of the chorioallantois and caruncle, caruncular tissue increases in vascular permeability and becomes edematous between Days 15-18 of pregnancy [42]. The caruncles will develop small folds that deepen into crypts as pregnancy progresses. At the same time, the cotyledons form irregular ridges and cotyledonary villi will invade into the caruncular crypts to form a highly interdigitated structure that brings placental and maternal capillaries into intimate proximity, called a placentome (Figure 1.4). The placentome is the location of syncytial plaque formation, from which stems the classification of synepitheliochorial placentation. A placentome is the discrete structure wherein diffusible molecules and gasses can be exchanged readily between maternal and fetal-placental circulations (hematrophic support) and is thus the functional unit of the placenta [61]. Nevertheless, the remainder of the placental-maternal interface is equally important in supporting the conceptus (embryo/fetus and placenta) throughout gestation. The inter-placentomal regions of the placenta exhibit characteristics of epitheliochorial placentation similar to pigs and horses. Chorionic papillae that initially penetrated into the uterine glands withdraw and undergo morphological changes, resulting in invaginations and folding of the chorioallantois around the mouths of the uterine glands to form a structure called an areolae [34, 60]. These pockets of space accumulate secretions produced by the uterine LE, sGE, and GE as early as Days 20 to 22 in the sheep [60] and serve as small repositories for the accumulation of macromolecules from the uterine glands, particularly nutrients for histotrophic support of conceptus development. Tall columnar epithelial cells

take up and transport macromolecules via fluid phase pinocytosis for release into the vascular system of the conceptus.

Vascularization

The circulatory system of the fetus is connected to the placental vasculature via the umbilical blood vessels. The placental vasculature is housed within the allantois and supplies the interplacentomal chorion and the chorionic villi of the placentome. The placentome is a highly vascularized structure; in sheep, 16% of maternal cardiac output will go to the pregnant uterus, and 80-90% of blood that supplies the uterine artery will flow through the placentomes [62, 63].

Early in pregnancy, vascular growth, or angiogenesis, is a hallmark of the uterus preparing for implantation and developing a vascular bed sufficient to support a developing conceptus [64]. Angiogenesis is not only active early in pregnancy but will contribute to caruncular and cotyledonary vascularization throughout the duration of pregnancy as fetal growth increases. One of the primary proteins involved in angiogenesis is vascular endothelial growth factor (VEGF). VEGF is a growth factor produced and secreted by endothelial cells to stimulate endothelial cell migration and proliferation, vascular permeability, and production of nitric oxide [64, 65]. During early pregnancy, mRNA for VEGF is more highly expressed in fetal and placental tissues than uterine tissues; likewise, this trend continues into late pregnancy as effects of VEGF to increase angiogenesis help regulate uterine blood flow to the placentome [64]. Decreased angiogenesis in early pregnancy may be a contributor to embryonic loss, or later in gestation may be indicative of a high-risk pregnancy due to increased vascular resistance

and reduced blood flow [64, 66, 67]. In the placentomes, angiogenesis results in a distinct vascular morphology within the caruncles and cotyledons. Caruncular vascular beds increase in diameter during gestation, while cotyledonary vascular beds increase in capillary density (number of capillaries per unit surface area) [68, 69]. This is a method whereby the maternal tissues decrease resistance and increase blood flow to the placenta, whereas fetal tissue increases surface area of capillaries for maximum exchange of gasses and molecules within the placenta.

Insulin-like growth factor 2

Insulin-like growth factor 2 (IGF2) is a peptide that is structurally similar to insulin and serves as a growth factor during pregnancy. In sheep, expression of IGF2 is induced by P4 and stimulated by IFNT in uterine LE and sGE during the peri-implantation period, suggesting its importance for fetal and placental growth. Later in pregnancy, it regulates fetal growth as well as nutrient transport across the placenta [70-72]. IGF2 can be found at the interface between caruncular and cotyledonary tissues of the placentome as well as in the chorioallanotis and uterine LE, and potentially increases the abundance of molecules involved in the mechanistic target of rapamycin (mTOR) pathway nutrient sensing pathway that directs cellular events such as growth and proliferation [72-74]. Furthermore, IGF2 may be involved in remodeling cells and altering their migration [74]. This suggests that IGF2 is involved in cell signaling mechanisms critical for development of the conceptus.

Placental lactogen

On Days 15 and 16 of pregnancy, the BNC of the trophectoderm will begin to produce chorionic somatomammotropin (CSH1; also known as placental lactogen). The placenta will continue to secrete CSH1 until parturition [75]. Estradiol, progesterone, IFNT, and CSH1 act consecutively on the uterus to stimulate and maintain the function of endometrial glands [76]. CSH1 is structurally similar to prolactin (PRL) and growth hormone (GH) from the anterior pituitary and activates a second messenger system via a prolactin receptor (PRLR) homodimer or a prolactin receptor and growth hormone receptor (GHR) heterodimer [77]. These receptors are specific to the GE [78], indicating that CSH1 affects the activity of uterine glands. Indeed, CSH1 stimulates proliferation and secretory functions of uterine GE [79], as well as fetal growth and metabolism [80], development of mammary glands [81], and maternal metabolism [82]. Progesterone also plays a role in regulating secretions from uterine GE [83], which indicates that CSH1 and P4 may work in concert to stimulate functional aspects of uterine GE function to support the developing conceptus.

Structural proteins

All cells are supported by an intracellular network of polymeric protein fibers called the cytoskeleton, which is made up of microfilaments, intermediate filaments, and microtubules. The most common microfilament, actin, has numerous functions, which include cell division, cell migration, gap and tight junction formation, vesicle trafficking, and regulation of cell shape [84]. There are multiple isoforms of actin, but β-actin is ubiquitously expressed, and its importance is evident in embryonic lethal knock-out mice

[85]. Microtubules made up of α-tubulin and β-tubulin heterodimers are the largest cytoskeletal components [86]. Functions of tubulin include intracellular transport, organelle positioning, and cell shape [86]. Tubulins are expressed ubiquitously, but found particularly in dividing, differentiating, and secreting cells [87]. Early in pregnancy, as a conceptus develops from spherical, to tubular, and filamentous forms, the actin cytoskeleton of the trophectoderm must quickly modify and reorganize to account for the massive morphological changes. There is a lack of studies on these changes in sheep, but data from swine models are applicable, as pig conceptuses experience similar changes. The actin cytoskeleton rearranges during compaction and elongation with actin bundles near the basal surface of trophectoderm (Tr) cells interacting with myosin fibers to generate force during elongation [88, 89]. Little is known, however, as to the roles of those proteins in late pregnancy. Histological studies of cytoskeletal morphogenesis of the term human placenta show that an extensive microfilament and microtubule network offer support to maintain the complex structure of the villous trees [90]. Actin forms a lattice-like mat at the periphery of the syncytial layer, just beneath the plasma membrane, and may act to increase flexibility and/or facilitate endo- and exocytosis through the syncytium. Tubulin fibers form parallel bands in this area as well and seem to concentrate at structural deviances such as branch points or bends in the villous tree [90]. While other epithelial layers have junctional complexes with adjacent cells to lend structural stability, the syncytial layer receives support from microfilaments and microtubules over a relatively larger area. This may provide insight into the organization of a placentome.

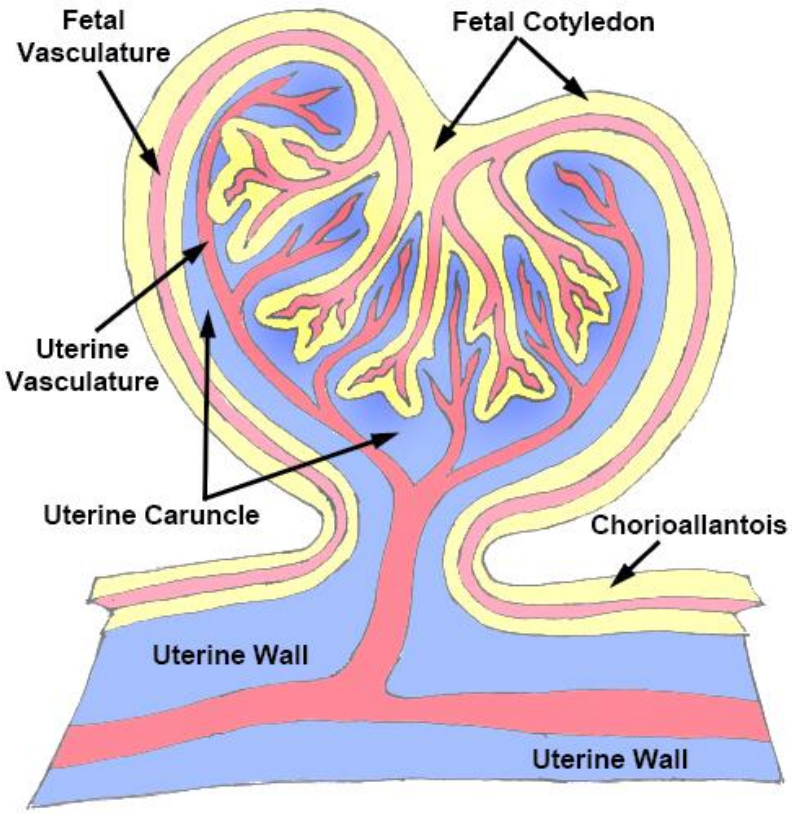

Figure 1.4. Structure of a sheep placentome. The uterine caruncle develops crypts into which cotyledonary villi interdigitate, allowing for maximum surface area for exchange of nutrients and gasses between the two tissues. Figure provided by Dr. Greg A. Johnson.

Components of histotroph

The uterine LE, sGE, and GE are active secretory cells that produce and secrete, as well as transport molecules into the uterine lumen during pregnancy in all mammals. This complex mixture of water, amino acids, hexose sugars, ions, growth factors, hormones, enzymes, cytokines, mitogens, and vitamins is collectively referred to as histotroph [2, 91]. Conceptuses will not continue to develop if the uterus does not increase secretory activity and produce histotroph. This finding is supported by results from studies of the uterine gland knockout model, in which ewes could not maintain pregnancy without uterine glands, and development of conceptuses failed before implantation [22]. In humans, histotrohic support of the conceptus is important in the first trimester until the placenta is dominant and provides hematrophic support, therefore inadequate secretory activity of the uterine glands may contribute to early embryonic loss in humans [92]. In all species with epitheliochorial and synepitheliochorial placentation however, components of histotroph accumulate in allantoic fluid to provide nutrition throughout the duration of pregnancy [93]. The molecules secreted into the uterine lumen, as well as the signaling mechanisms that stimulate them, must be regulated in both a spatial (cell specific) and temporal manner. Namely, the female reproductive tract must be modified with respect to specific cell types and at the proper time to maximize development and survival of conceptuses. Some of the secreted nutrients of interest include glucose, fructose, and amino acids and polyamines.

Glucose

Glucose is an important source of energy for the fetus and placenta, but fetal and placental tissues cannot produce it themselves, and thus must rely on glucose from the dam [94, 95]. Glucose trafficking across membranes requires SLC2 (solute carrier family 2) facilitated nutrient transporters. The many isoforms of SLC2 transporters allows for differential movement of glucose, but SLC2A1 (solute carrier family 2, member 1) and SLC2A3 (solute carrier family 2, member 3) are of notable importance during pregnancy. SLC2A1 is ubiquitously expressed in maternal, fetal, and placental tissues, and is considered to be the primary glucose transporter in both humans and sheep [96-99]. In both pregnant and cyclic ewes, SLC2A1 expression is most abundant in uterine LE/sGE, but expression was greater and increased at Day 10 in pregnant ewes [100]. SLC2A1 expression in the sheep uterus increases in early to mid-gestation to peak at D120 [96, 101], and SLC2A1 is expressed highly in human placentae throughout gestation [102, 103]. There are few reports as to where these transporters are localized in the placentome, but there is a report of SLC2A1 localized predominantly in the syncytial plaques [97]. In early pregnancy, SLC2A3 is present in the trophectoderm of sheep conceptuses but not endometrial tissue [100]. This illustrates tissue and cell-specific roles in glucose transport during pregnancy. SLC2A3 expression increases throughout gestation and peaks at term in the placentome [96, 101], but there appears to be conflicting evidence as to the expression and localization pattern in human placentae [98, 103]. Currie et al (1997) suggested that SLC2A3 expression discrepancies between species may be due to distinct placental morphology, and consequently differential concentrations in glucose affecting transport across syncytial layers [101]. Concentrations of glucose in allantoic fluid range from 0.1

to 0.3 mg/mL and remain relatively constant throughout pregnancy in sheep. The concentrations of glucose in amniotic fluid are similar and do not change throughout pregnancy [104].

Fructose

While glucose may be an important sugar for fetal and placental growth, fructose is the most abundant hexose sugar in the fetal fluids and blood of ungulates and cetaceans [105-107]. Studies with sheep show that the placenta can convert glucose to fructose before transfer to the fetus, as well as allow for fetal-to-maternal transfer of glucose but not fructose, indicating that fructose is a sugar that is sequestered within placental and fetal tissue [108-110]. However, until recently, fructose has been largely ignored as a primary nutrient involved in pregnancy as it is not involved in metabolism via the Krebs cycle or glycolysis [111]. SLC2A1 transporters bring glucose from maternal circulation and into the placental vasculature, where it is converted to sorbitol and then fructose via the polyol pathway. From here, fructose is transported across the placenta by SLC2A5 (solute carrier family 2, member 5) and SLC2A8 (solute carrier family 2, member 8) [112]. SLC2A5 is a low affinity, high capacity transporter of fructose only, and SLC2A8 has high affinity for glucose but can also transport fructose [113]. Fructose has multiple fates once inside the trophectoderm cell: it may enter the hexosamine synthesis pathway to produce glycosaminoglycans such as hyaluronic acid and UDP-N-acetylglucosamine, or it may be metabolized and utilized in serinogenesis to produce one carbon units [110, 114]. There is one report of SLC2A8 expression and localization in the sheep placentome. At D35 of pregnancy SLC2A8 was present in chorionic epithelium and syncytial plaques, and by

D135 expression of SLC2A8 increased and could be localized in most cells of the placentome [115]. In pigs, both SLC2A8 and SLC2A5 are expressed in both the uterine LE and GE, as well as the chorion throughout pregnancy [116]. In pigs, the free-floating conceptuses are supported by fructose synthesized by the uterus, but after implantation, the chorion becomes self-sufficient for fructose synthesis and transport. This may provide insightful as to the function of these transporters in the inter-placentomal region of the ovine placenta. Concentrations of fructose in allantoic fluid vary between 1 and 6 mg/ml throughout gestation, but total fructose increases between Day 25 and Day 120 of gestation and then decreases to term [1].

Amino acids and polyamines

Amino acids are important building blocks for synthesis of peptides and protein-based molecules (such as signaling molecules, hormones, and nucleic acids). With regards to a pregnant mammal, amino acids play an important role in placental development and embryonic/fetal growth [117]. Amino acids either accumulate in both the allantoic and amniotic fluid of sheep and pigs over the course of gestation [48] or are transported directly into the fetal vasculature in the placentome to work in concert with IGFs, VEGF, other growth factors, and polyamines to mediate angiogenesis, embryogenesis, placental growth, and increases in blood flow [118]. The amino acids and their derivatives of particular interest are serine, glycine, arginine, and polyamines (putrescine, spermine, and spermidine), as well as glutamate and glutamine.

Arginine is a nutritionally essential amino acid for conceptus development. It can be used to either generate nitric oxide (NO)—an important molecule for vasodilation and

angiogenesis in the uterus—or synthesize polyamines (which may be used for nucleic acid and protein synthesis), play a role in cell communication and proliferation, and act as antioxidants [119]. Polyamine synthesis from arginine typically follows a "classic" pathway via the conversion of arginine to ornithine by arginase and then ornithine to putrescine via ornithine decarboxylase (ODC1) [120]. However, it has been reported that conceptuses were able to activate an alternative pathway for polyamine synthesis if ODC1 was not present [121]. This pathway involves conversion of arginine to agmatine via arginine decarboxylase (ADC), then conversion of agmatine to polyamines via agmatinase (AGMAT). Conceptuses that were able to activate this alternative pathway were functionally and morphologically normal, while those unable to activate that pathway sufficiently were abnormal. This highlights the importance of polyamine synthesis for proper conceptus development. Solute carrier family 7 members 1 and 2 (SLC7A1, SLC7A2) are y+ member cationic amino acid transporters that have a high affinity for arginine.

Both serine and glycine serve as carbon units for DNA synthesis and methylation, and are involved in metabolic pathways [48, 122]. Glycine may be utilized for synthesis of purines, serine, heme, act as an antioxidant, and has anti-inflammatory properties. Serine is used in gluconeogenesis and participates in protein phosphorylation [120]. Glycine is transported by solute carrier family 6 (sodium and chlorine dependent glycine transporter) member 9 [SLC6A9] and serine is transported by solute carrier family 1 (sodium dependent neutral amino acid transporter for serine) member 4 [SLC1A4].

As described by Kwon et al. (2003), concentrations of amino acids change in allantoic fluid throughout gestation. Arginine increases between Days 60 and 100, then

remains constant between Days 100 and 140. Arginine is the second most abundant alpha amino acid in allantoic fluid of lambs on Day 140 of gestation [48]. Serine and glycine are the most abundant amino acids in placental fluids throughout gestation [48].

Supplementation of progesterone manipulates reproductive cyclicity

It is common knowledge that recipient and donor estrous cycles need to be in close synchrony when performing embryo transfer. Moore and Shelton (1964) showed that embryonic survival was highest in recipients with estrous cycles within 12-hour synchrony with the donor, and that once the recipient was ±48 hours out of synchrony with the donor ewe, survivability of conceptuses plummeted [123]. Thus, the uterus has the ability to accept and maintain a 'younger' or 'older' embryo. Asynchronous embryo transfer studies show that embryos placed in 'older' uteri (those that were ahead in their pregnancy by 1-2 days) experienced rapid elongation, likely in response to the more advanced uterine environment [124]. It was theorized that because giving exogenous progesterone early in the estrous cycle resulted in short estrous cycles [125, 126], that giving post-ovulatory exogenous P4 may alter the functional state on the uterus [127]. In cattle, a delayed increase in circulating concentrations of progesterone hinders conceptus development and reduces or delays IFNT secretion. Progesterone supplementation during the post-ovulatory increase in progesterone enhances conceptus development and secretion of IFNT [127-129]. This indicates that progesterone may regulate early conceptus growth by modifying the timing of gene expression by cells of the uterine endometrium and uterine secretory activity. However, it is the clearly early increase in concentrations of progesterone in

maternal plasma, rather than the concentrations of progesterone in maternal plasma, that is the element influencing accelerated development of the conceptus [129].

In previous studies, progesterone was administered to ewes 36 hours after the onset of estrus and mating to observe the effects of progesterone on development of the pre-implantation conceptus [25, 130, 131]. In those studies, exogenous progesterone administration advanced conceptus development likely due to increased nutrient trafficking into the uterine lumen; however, little is known as to how those effects translate to the uterine environment and conceptus development in late gestation. There have been some reports of enhanced fetoplacental growth when ewes were administered progesterone during the first three days of pregnancy. The ewes receiving progesterone supplementation experienced an altered post-ovulation endocrine milieu, and thus it could be proposed that the uterine physiological timeline was accelerated. Those studies showed that conceptus exposure to a progesterone-primed uterus influenced fetal growth at Day 76 of pregnancy. These conceptuses exhibited an increase in fetal crown-rump lengths and organ weights compared to fetuses from untreated ewes [132, 133]. It is clear that perturbations in the uterine environment caused by hormonal manipulation affect development of conceptuses and subsequent fetal growth, but the underlying mechanisms are unclear.

CHAPTER II

EFFECTS OF EXOGENOUS PROGESTERONE ADMINISTERED TO EWES DURING THE PRE-IMPLANTATION PERIOD OF PREGNANCY ON FETAL AND PLACENTAL GROWTH IN LATE GESTATION

Introduction

Pig and ruminant conceptuses undergo massive morphological changes during the peri-implantation period. In sheep, the conceptus will enter the uterus on Day 4, develop from a spherical to a tubular form between Days 9 and 13, become filamentous between Days 13-15, and extra-embryonic membranes will extend into the contralateral uterine horn between Days 16-20, when implantation is established and placentation begins [36, 43]. Elongation of the conceptus is key for the production of a maternal recognition of pregnancy signal, which is secreted by the conceptus to alert the uterus of its presence. In ruminants, this pregnancy recognition signaling molecule is interferon-tau (IFNT). IFNT and progesterone work in concert to alter the uterine environment to support implantation and placental development.

These uterine alterations are mediated by complex signaling mechanisms in order to up- and down-regulate genes in specific endometrial cell types. The coordinated actions of IFNT and P4 increase synthesis and secretion of histotroph by uterine GE, which not only supports the free-floating conceptus before placentation occurs, but also provides a source of nutrients throughout the duration of pregnancy. As pregnancy progresses, the endometrium becomes more vascularized to be able to adequately supply the developing placenta as well as the fetus. Both the conceptus and the uterus undergo a complex series

of changes, and any perturbations to the dam during this peri-implantation period may have lasting effects on either the fetus or the placenta.

In previous studies, progesterone administered to ewes 36 hours after the onset of estrus and mating resulted in advanced conceptus development [130, 131, 134]. It was suggested that the accelerated conceptus growth was due to advanced increases in the uterine secretory response. However, it is unclear as to how modifications to the uterine environment in early pregnancy affect subsequent fetal and placental growth. Some reports demonstrated enhanced fetoplacental growth in ewes administered progesterone during the first three days of pregnancy. Those studies showed that conceptuses exposed to a uterus altered by progesterone supplementation resulted in fetuses with longer crown-rump lengths and heavier organ weights than their untreated counterparts at Day 76 of pregnancy [132, 133]. Factors affecting the post-ovulatory endocrine profile of females, and thereby the uterine environment, have effects on fetal growth, but the underlying mechanisms are not clear.

This study was performed to assess the effects of exogenous progesterone administered to ewes during the pre-implantation period of pregnancy on fetal and placental development in late gestation. To determine this, we measured fetal and placental phenotypic parameters indicative of growth. We also analyzed differences in mRNA and protein expression to observe any difference in gene expression that may have affected fetal or placental performance. A more robust placenta is one that has the capacity to transport more nutrients and molecules that maximizes fetal growth, and we thus analyzed the composition of allantoic and amniotic fluid, as well as fetal plasma. We

hypothesized that progesterone-induced accelerated blastocyst development in early pregnancy would result in increased fetal and/or placental growth at Day 125 of pregnancy.

Materials and methods

Animals

Mature Suffolk-type ewes (Ovis aries) were observed for estrus (designated as Day 0) in the presence of a vasectomized ram and used in experiments only after exhibiting at least two estrous cycles of normal duration (16–18 days). All 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.

Experimental Design

Ewes were mated at estrus (Day 0) and assigned randomly to receive daily intramuscular injections of either corn oil vehicle (CO; n = 20) or 25 mg progesterone dissolved in ethanol in corn oil vehicle (P4; n = 20) from Day 1.5 through Day 8 of pregnancy. At Day 125 of pregnancy ewes were euthanized and then hysterectomized after collecting blood via jugular venipuncture. After the uteri were weighed, the chorioallantois was separated from the endometrium to expose the fetus and placental membranes. Samples of allantoic and amniotic fluids were collected, and volumes were determined. Sections of endometrium, whole placentomes, and separated caruncles and cotyledons were collected and either: a) frozen in liquid nitrogen and stored at -80^{o}C; b) fixed in 4% paraformaldehyde and dehydrated in 70% ethanol for 48 hours prior to

embedding in Parrafin wax; or c) frozen in optimal cutting temperature (OCT) gel. Fetal blood was collected from the heart. After separating the fetus and placenta, placental length and weight was measured, and the number of cotyledons was determined. In the case of a twin pregnancy, the fetuses were designated as either Fetus 1 or Fetus 2. The fetuses were also weighed and measurements indicative of growth (crown-rump length, abdominal circumference, and chest circumference) were taken.

Radioimmunoassay analysis of progesterone

Blood samples were stored on ice until processing. Plasma was collected following centrifugation (8000 x g for 7 min at room temperature) and stored at -20^{o}C for analysis. Concentrations of progesterone in maternal plasma were determined following a modified protocol validated by the laboratories of G. Williams, C. Cardoso, and T. Welsh (J. Scarpa, unpublished data) using a Progesterone Coated Tube RIA Kit (07-270102, MP Diagnostics, Santa Ana, CA, United States).

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

RNA extraction, cDNA synthesis, and quantitative real-time PCR analysis was performed as described previously [121]. Briefly, total RNA was isolated from ovine endometria, caruncles, cotyledons, and placentomes using Trizol (Invitrogen, Carlsbad, CA, United States) according to manufacturer's instructions. The quantity and quality of total RNA were determined by spectrometry (wavelength = 230 nm) and by denaturing agarose gel electrophoresis, respectively. Total RNA samples were digested with RQ1 RNase-Free DNase (Promega, Madison, WI, United States) and further purified using a

RNeasy Mini Kit (Qiagen, Hilden, Germany). The levels of expression of mRNAs encoding for genes of interest were determined by quantitative real-time polymerase chain reaction. Primer sequences of genes of interest are listed in Table 2.1. Primer specificity and efficiency were determined by the addition of a dissociation curve step to the reverse transcriptase (RT) reaction and through the generation of a standard curve using known quantities of cDNA, respectively. Only those primers amplifying a single product and determined to be between 97.5% and 102.5% efficient were utilized for quantitative analyses. First-strand cDNAs were synthesized from 5 µg of total RNA using oligo (deoxythymidine) primers and SuperScript II Reverse Transcriptase. Quantitative PCR was performed using the ABI prism 7900HT system (Applied Biosystems, Foster City, CA, United States) with Power SYBR Green PCR Master Mix (Applied Biosystems) as specified by the manufacturer. Each individual sample was run in triplicate using the following conditions: 50^{o}C for 2 min, 95^{o}C for 10 min, followed by 40 cycles of 95^{o}C for 15 sec and 60^{o}C for 30 sec. *ADC* and *AGMAT* were preamplified using a Thermocycler (Eppendorf AG) with 5 µg of total RNA and SYBR Green PCR Master Mix for 15 cycles, and 1 µL of preamplicon template was used for qPCR. A dissociation curve was generated to determine amplification of a single product. The threshold line was set at the linear region of the plots above the baseline noise, and threshold cycle (CT) values were determined at the cycle number at which the threshold line crossed the amplification curve. SDHA and GAPDH were used as reference genes for endometrial tissue and placental tissue, respectivley. The abundance of mRNAs for genes of interest were calculated using the comparative Ct method [25].

Table 2.1 Primers used for real-time qPCR

Gene	Forward/reverse primers (5'3')	GenBank accession no.
VEGFA	CACCAAAGCCAGCACATAGG	AF071015.1
	GCCTCGGCTTGTCACATTTTT	
IGF2	GGACACCCTCCAGTTTGTCTGT	NM_001009311.1
	CGGTTTATGCGGCTGGAT	
CSH1	CAGGCACAGCATCCACCATA	M31660.1
	GTTAGCCACCGTTGTTGCTC	
ACTB	AGTACTCCGTGTGGATTGGC	NM_001009784.2
	AGGGTGTAACGCAGCTAACAG	
TUB	GGTCTTCAAGGCTTCTTGGT	AF251146.1
	CATAATCGACAGAGAGGCGT	
SLC2A1	TGGGAAAGTCCTTTGAGATGC	NM_174602.2
	GGTCAGGCCGCAGTACACA	
SLC2A3	AAATTAGGGCCATGGGGACCA	NM_174603.3
	TTTTATGATCGCCTCAGGAGCA	
SLC2A5	GGTGGGAATATGTGCAGGTC	NM_001009451.1
	CAGTCAATCCGAGGAGGATGG	
SLC2A8	CGTCCTCACCAACTGGTTCA	NM_201528.1
	CCCTTTGGTCTCAGGGACAC	
SLC7A1	CCTAGCGCTCCTGGTCATCA	AF212146
	GGGCGTCCTTGCCAAGTA	
SLC7A2	GCAGAGCAGCGCTGTCTTT	XM_010820288.3
	ACTGTCCAGAGTGACGATTTTCC	
ODC1	GCACATCCAAAGGCCAAGTT	M92441.1
	GGCGACAGACTGCTTTGGAA	
ADC	TCCCTGCCTCTAGAAGAAGCTCACT	NM_001038510
	ATCGTTTCCACTCCGGATAGA	
AGMAT	AGACTGCTGGCTGAAGCTAC	XM_002694143
	GTAAGCAGGGTCCAAGCCAT	
SDHA	CATCCACTACATGACGGAGCA	AY970969.1
	ATCTTGCCATCTTCAGTTCTGCTA	
GAPDH	GGGCAGCCCAGAACATCAT	NM_001009451.1
	CCAGTGAGCTTCCCGTTCAG	

Analyses for glucose in fetal and placental fluids

Allantoic and amniotic fluid, as well as fetal plasma, were analyzed for concentrations of glucose using a Glucose Assay Kit (STA-680, Cell Biolabs, Inc., San Diego, CA, United States), as per manufacturer's instructions. Allantoic fluid, amniotic fluid, and fetal plasma were diluted 1:5, 1:2, and 1:80, respectively, with 1X Assay Buffer. Samples and reaction mixture were pipetted in duplicate onto a 96 well plate and incubated for 40 min at 37^{o}C protected from light. The plate was read on a spectrophotometric plate reader (nm= 540 nm) within 5 min after removing from incubation. Kit standards were used to generate a standard curve, and the concentration of glucose in samples were calculated by comparing the sample OD to the standard curve. Allantoic and amniotic fluid data are expressed as total glucose [volume of fluid (mL) x concentration of glucose (µmol/L)] in the respective fluids. Total glucose in fetal plasma was not calculated as blood volumes among fetuses were assumed to be homogenous.

Analysis for fructose in fetal and placental fluids

Allantoic fluid, amniotic fluid, and fetal plasma were analyzed for concentrations of fructose using Fructose Assay Kit (EFRU-100; BioAssay Systems, Hayward, CA, United States), as per manufacturer's instructions. Allantoic fluid, amniotic fluid, and fetal plasma were diluted 1:8,1:15, 1:10, respectively, with double distilled water. Samples and reaction mixture were pipetted in duplicate onto a 96 well plate in a dark room and the plate was incubated for 60 min at room temperature. The plate was read on a spectrophotometric plate reader (nm= 565) within 5 min after removing from incubation. Kit standards were used to generate a standard curve, and the concentration of fructose in

samples were calculated by comparing the sample OD to the standard curve. Allantoic and amniotic fluid data are expressed as total fructose [volume of fluid (mL) x concentration of fructose μmol/L] in the respective fluids. Total fructose in fetal plasma was not calculated as blood volume among fetuses was assumed to be homogenous.

Statistical analysis

Data were subjected to least squares analysis of variance using the general linear model procedures of the Statistical Analysis System (SAS version 9.4). Each fetus/placenta was treated as an experimental unit. Significance ($P < 0.05$) was determined by probability differences of least squares means. Data are presented as least-squares means with overall SE.

Results

Early progesterone supplementation advances conceptus development

Injections of P4 to ewes beginning on Day 1.5 after mating advanced conceptus development, and by Day 12 of pregnancy the P4 treated ewes, but not CO ewes, had elongated conceptuses [25, 130, 131]. This was confirmed in a concurrent study in our laboratory (E. Hoskins, unpublished results).

Circulating concentrations of progesterone

Concentrations of progesterone in maternal plasma were determined by radioimmunoassay (RIA) of plasma from jugular venous blood. There was no difference ($P > 0.05$) in concentrations of progesterone by Day 125 of pregnancy between P4-treated

and CO-treated ewes. Administration of exogenous P4 beginning on Day 1.5 of pregnancy increased concentrations of progesterone in maternal blood of P4-treated ewes on Day 9 of pregnancy, but concentrations were not different by Day 12 of pregnancy (E. Hoskins, unpublished results).

Fetal and placental development

Measurements of fetal weight, crown-rump length, abdominal circumference, and chest circumference were taken as indicators of fetal growth and are summarized in Table 2.2. Fetal weight (Figure 2.1) was not significantly different due to treatment ($P > 0.05$), litter size ($P > 0.05$), or their interaction ($P > 0.05$). Fetal crown-rump length (Figure 2.2) was not significantly different due to treatment ($P > 0.05$), litter size ($P > 0.05$), or their interaction ($P > 0.05$). Fetal abdominal circumference (Figure 2.3) was not significantly different due to treatment ($P > 0.05$), litter size ($P > 0.05$), or their interaction ($P > 0.05$). Fetal chest circumference (Figure 2.4) was not significantly different due to treatment ($P > 0.05$), litter size ($P > 0.05$), or their interaction ($P > 0.05$).

Placenta weight, length, number of placentomes, and volumes of allanotic and amniotic fluid were measured as indicators of placental development and are summarized in Table 2.2. Placenta weight (Figure 2.5) was not affected by treatment ($P > 0.05$), litter size, ($P > 0.05$), or their interaction ($P > 0.05$). Placenta length (Figure 2.6) was significantly longer in single fetus pregnancies than twin fetus pregnancies ($P < 0.01$), but was not affected by treatment ($P > 0.05$). There were more placentomes (Figure 2.7) per placentae from single fetus pregnancies than those from twin fetus pregnancies ($P < 0.01$) but numbers of placentomes were not affected by treatment ($P > 0.05$). Volumes of

allantoic fluid (Figure 2.8) were not different due to treatment ($P > 0.05$), litter size ($P > 0.05$), or their interaction ($P > 0.05$). Volumes of amniotic fluid (Figure 2.9) were not significantly different due to treatment ($P > 0.05$), litter size ($P > 0.05$), nor their interaction ($P > 0.05$).

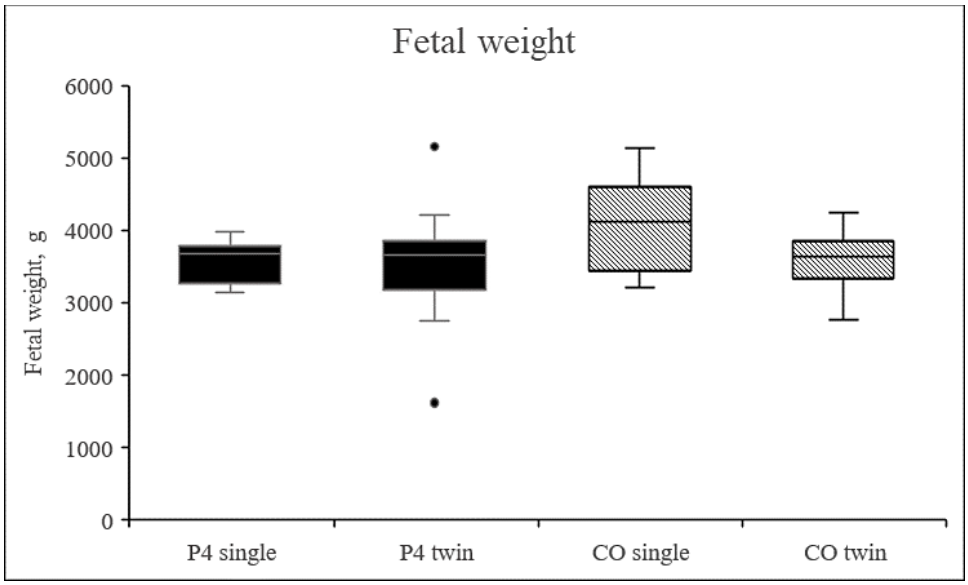

Figure 2.1. Effects of exogenous progesterone on fetal weight. Administration of exogenous progesterone from Day 1.5 through Day 8 of pregnancy did not have an effect on fetal weight ($P > 0.05$). Outliers are indicated by dots above and below the corresponding box and whisker.

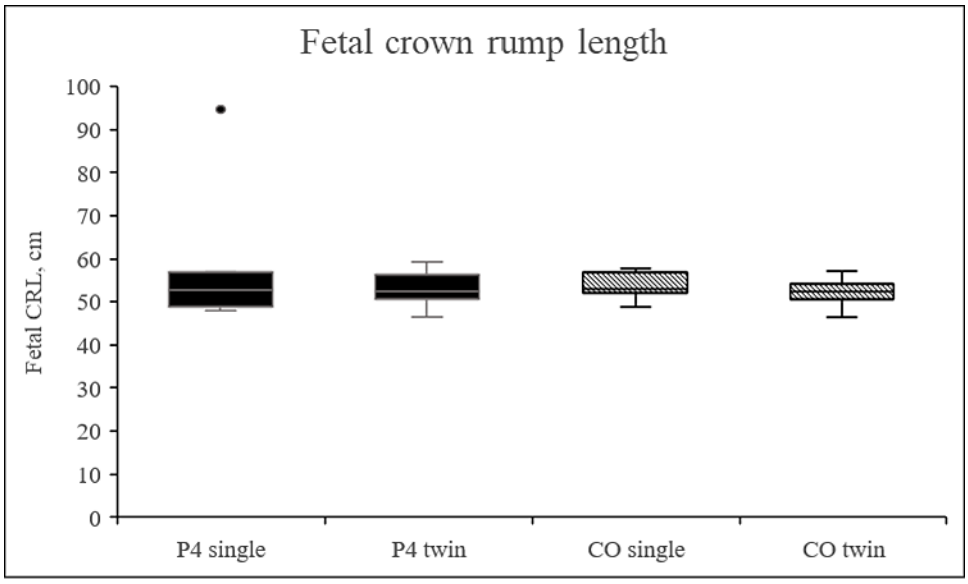

Figure 2.2. Effects of exogenous progesterone on fetal crown rump length. Administration of exogenous progesterone from Day 1.5 through Day 8 of pregnancy did not have an effect on fetal crown rump length ($P > 0.05$). Outliers are indicated by dots above and below the corresponding box and whisker.

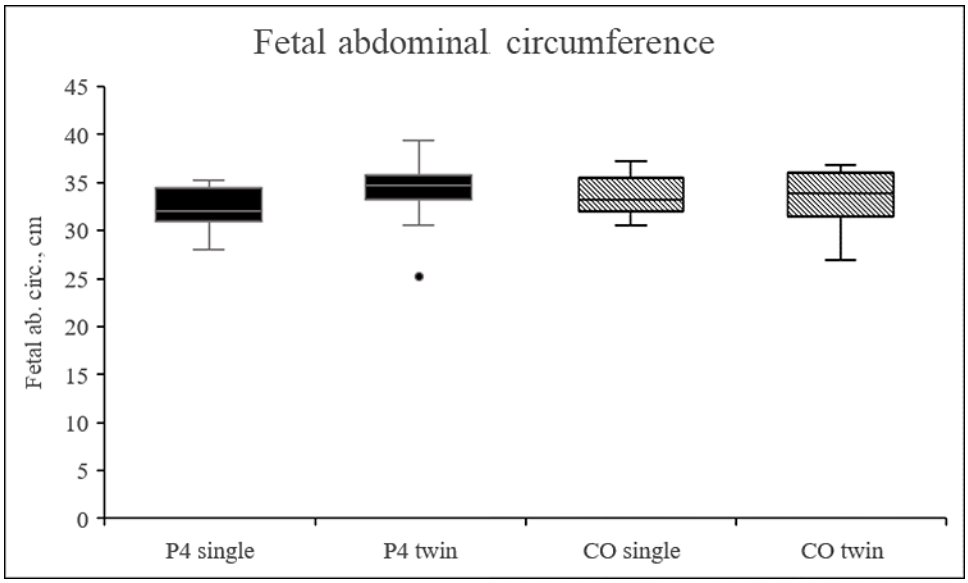

Figure 2.3. Effects of exogenous progesterone on fetal abdominal circumference. Administration of exogenous progesterone from Day 1.5 through Day 8 of pregnancy did not have an effect on fetal abdominal circumference ($P > 0.05$). Outliers are indicated by dots above and below the corresponding box and whisker.

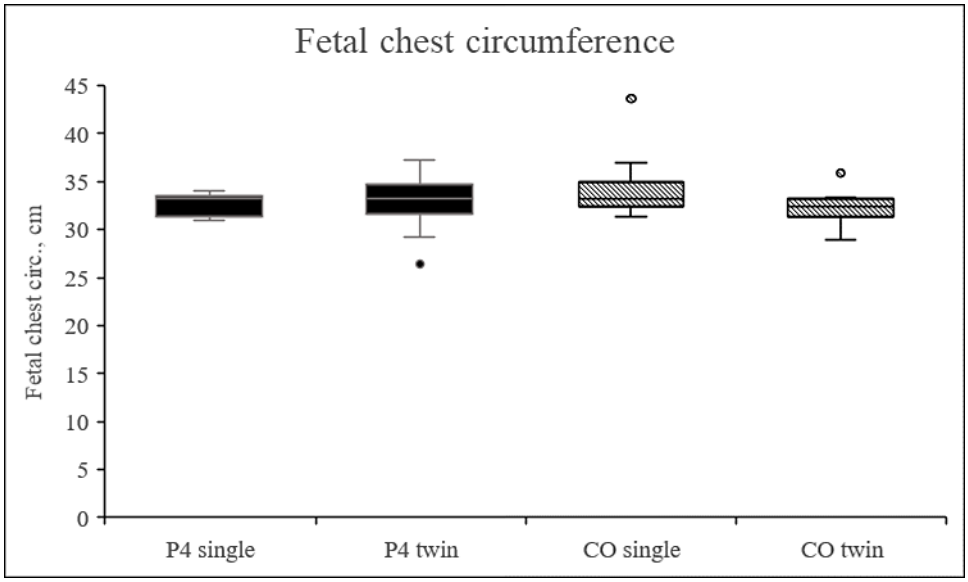

Figure 2.4. Effects of exogenous progesterone on fetal chest circumference. Administration of exogenous progesterone from Day 1.5 through Day 8 of pregnancy did not have an effect on fetal chest circumference ($P > 0.05$). Outliers are indicated by dots above and below the corresponding box and whisker.

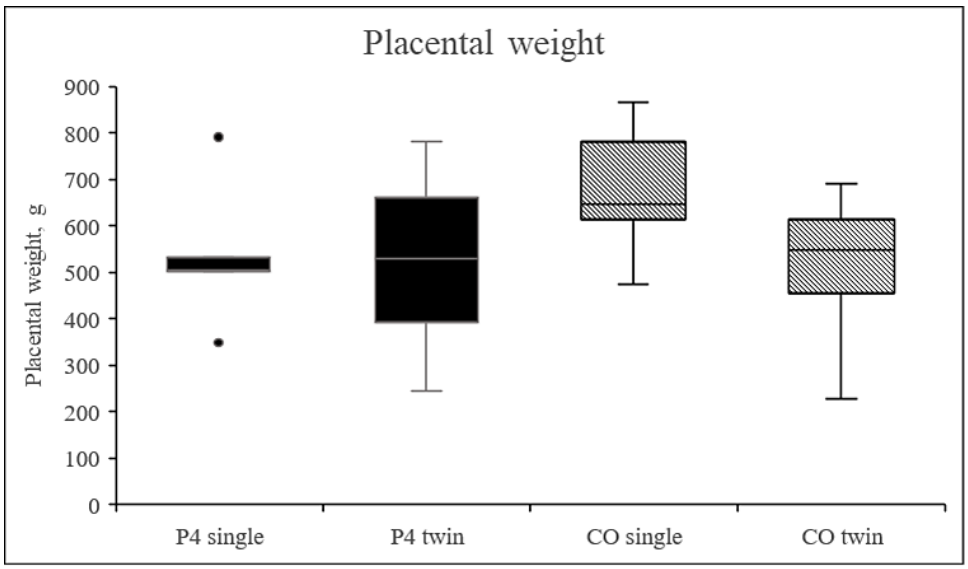

Figure 2.5. Effects of exogenous progesterone on placental weight. Administration of exogenous progesterone from Day 1.5 through Day 8 of pregnancy did not have an effect on placental weight ($P > 0.05$). Outliers are indicated by dots above and below the corresponding box and whisker.

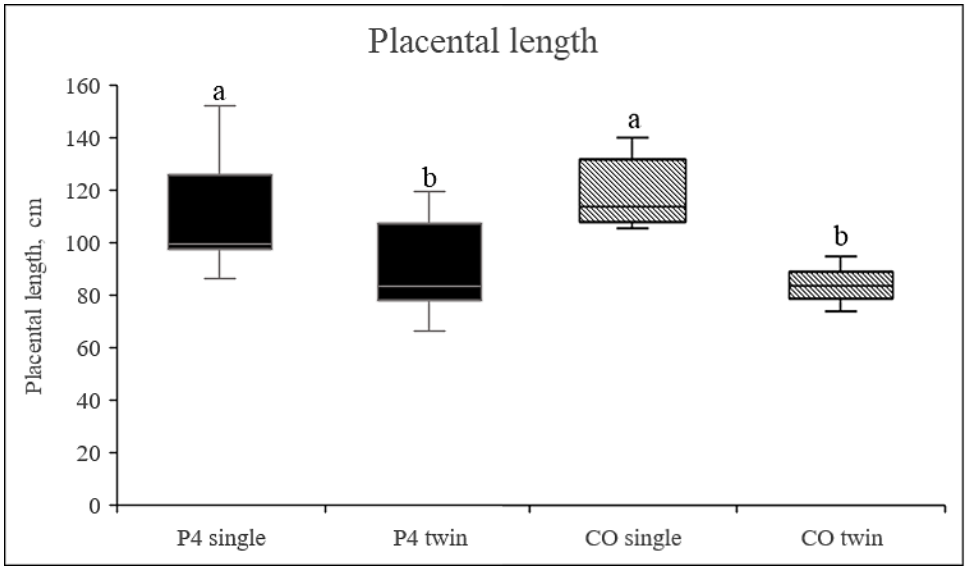

Figure 2.6. Effects of exogenous progesterone on placental length. Administration of exogenous progesterone from Day 1.5 through Day 8 of pregnancy did not have an effect on placental length ($P > 0.05$). Placentae were longer in single pregnancies than twin pregnancies ($P < 0.01$.

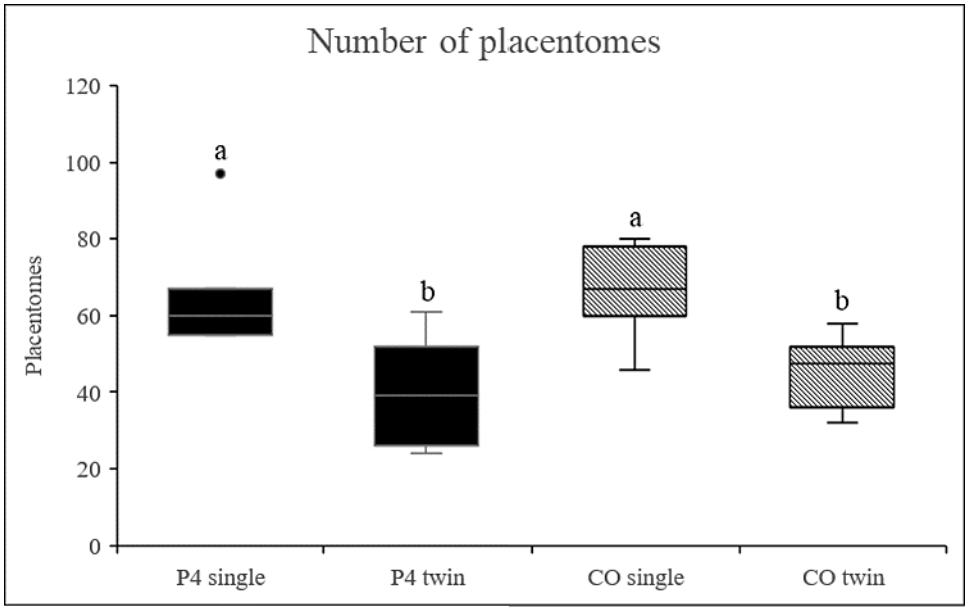

Figure 2.7. Effects of exogenous progesterone on the number of placentomes per placentae. Administration of exogenous progesterone from Day 1.5 through Day 8 of pregnancy did not have an effect on the number of placentomes per placentae ($P > 0.05$). There were more placentomes in single pregnancies than twin pregnancies ($P < 0.01$). Outliers are indicated by dots above and below the corresponding box and whisker.

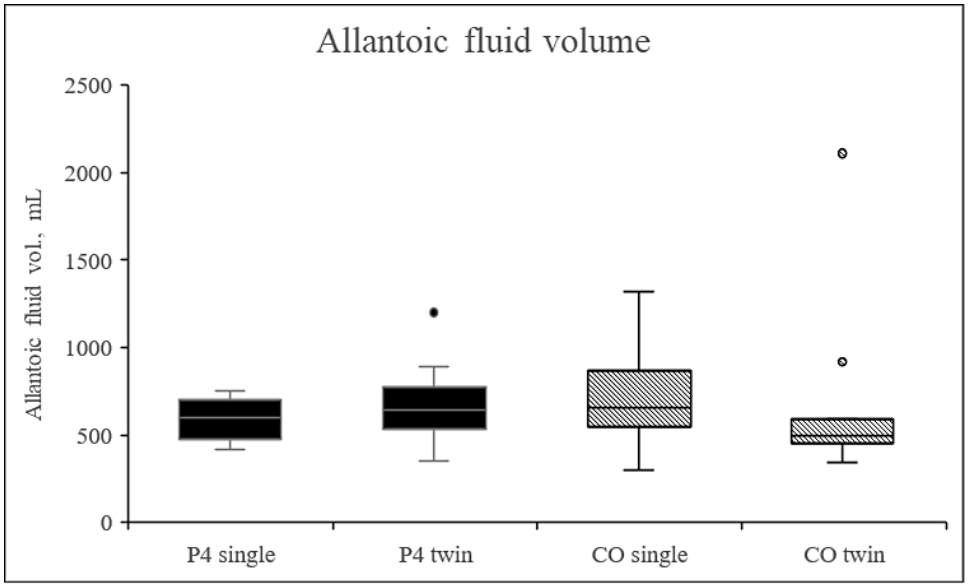

Figure 2.8. Effects of exogenous progesterone on allantoic fluid volume. Administration of exogenous progesterone from Day 1.5 through Day 8 of pregnancy did not have an effect on allantoic fluid volume ($P > 0.05$). Outliers are indicated by dots above and below the corresponding box and whisker.

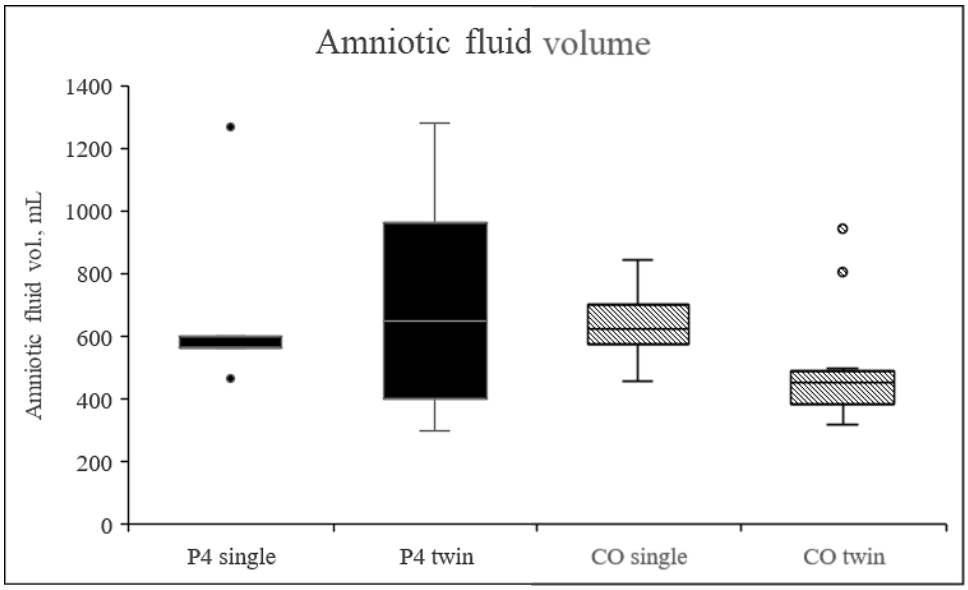

Figure 2.9. Effects of exogenous progesterone on amniotic fluid volume. Administration of exogenous progesterone from Day 1.5 through Day 8 of pregnancy did not have an effect on amniotic fluid volume ($P > 0.05$). Outliers are indicated by dots above and below the corresponding box and whisker.

Table 2.2. The effects of progesterone on fetal and placental measurements

	Pregnancy Type			
Measurement	P4 single	P4 twin	CO single	CO twin
Fetal weight (g)	3575 ± 304	3520 ± 215	4087 ± 227	3607 ± 215
Fetal CRL (cm)	60.2 ± 3.5	53.2 ± 2.5	54.1 ± 2.6	52.3 ± 2.5
Fetal ab. circ. (cm)	32.1 ± 1.4	33.9 ± 1	33.7 ± 1.1	33.4 ± 1
Fetal chest circ. (cm)	32.6 ± 1.3	32.8 ± 0.9	34.8 ± 1	32.3 ± 0.9
Placenta weight (g)	536 ± 70	516 ± 50	683 ± 52	529 ± 50
Placental length (g)	112.3 ± 7.3^{a}	91.3 ± 5.8^{b}	119.1 ± 5.4^{a}	84.6 ± 5.1^{b}
Number of cotyledons	66.8 ± 5.7^{a}	39.9 ± 4.1^{b}	67.1 ± 4.3^{a}	45.1 ± 4.1^{b}
Allantoic fluid vol. (mL)	589 ± 159	680 ± 112	703 ± 118	689 ± 112
Amniotic fluid vol. (mL)	693 ± 117	696 ± 83	642 ± 88	510 ± 83

Values are least squares means ± SEM

Means within rows with different superscripts are significantly different ($P < 0.01$)

Gene expression in ovine endometrium

Quantitative real-time PCR (qPCR) was used to measure expression of mRNAs in ovine endometrial tissue. qPCR analyses indicated that expression of *SLC2A1* mRNA (Figure 2.10) was not affected by treatment ($P > 0.05$), litter size ($P > 0.05$), or their interaction of treatment by litter size ($P > 0.05$). Expression of *SLC7A1* mRNA (Figure 2.11) was not affected by treatment ($P > 0.05$), litter size ($P > 0.05$), or their interaction ($P > 0.05$). Expression of *SLC7A2* mRNA (Figure 2.12) was increased in P4-treated ewes ($P < 0.05$), but there was no difference due to litter size ($P > 0.05$), or their interaction ($P > 0.05$). Expression of *ODC1* mRNA (Figure 2.13) was not different due to treatment ($P > 0.05$), litter size ($P > 0.05$), or their interaction ($P > 0.05$). Expression of *ADC* mRNA (Figure 2.14) was not different due to treatment ($P > 0.05$), litter size ($P > 0.05$), or their interaction ($P > 0.05$). Expression of *AGMAT* mRNA (Figure 2.15) was not different due to treatment ($P > 0.05$), litter size ($P > 0.05$), or their interaction ($P > 0.05$). Expression of *VEGFA* mRNA (Figure 2.16) was increased in single pregnancies ($P < 0.05$) compared to twin pregnancies, but not treatment ($P > 0.05$) or their interaction ($P > 0.05$). Expression of *IGF2* mRNA (Figure 2.17) was not different due to treatment ($P > 0.05$), litter size ($P > 0.05$), or their interaction ($P > 0.05$). Expression of *ACTB* mRNA (Figure 2.18) was not different due to treatment ($P > 0.05$), litter size, ($P > 0.05$), or their interaction ($P > 0.05$). Expression of *TUB* mRNA (Figure 2.19) was greater for twin fetus pregnancies ($P < 0.05$) compared to single fetus pregnancies, but not treatment ($P > 0.05$) or their interaction ($P > 0.05$).

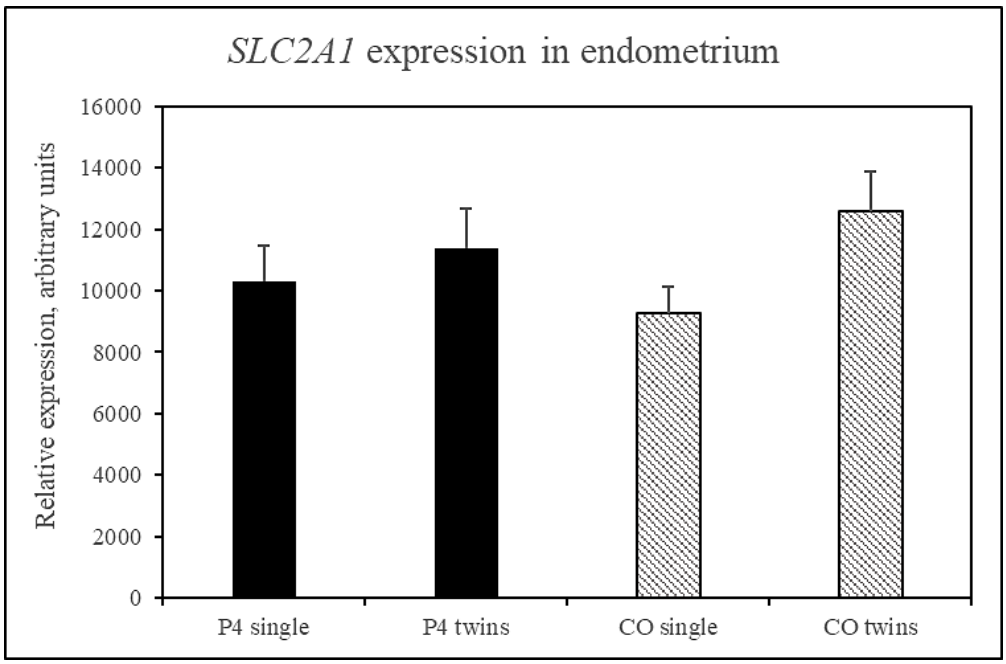

Figure 2.10. Effects of exogenous progesterone on expression of facilitated glucose transporter 1 mRNA endometrial tissue. Administration of exogenous progesterone from Day 1.5 through Day 8 of pregnancy did not affect expression ($P > 0.05$).

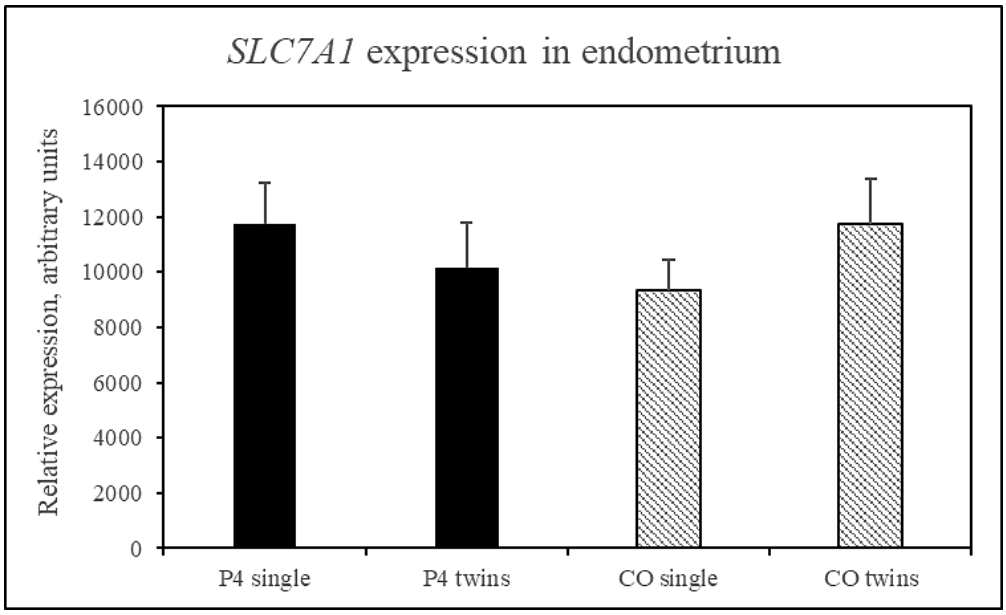

Figure 2.11. Effects of exogenous progesterone on expression of high affinity cationic amino acid transporter 1 mRNA in endometrial tissue. Administration of exogenous progesterone from Day 1.5 through Day 8 of pregnancy did not affect expression ($P > 0.05$).

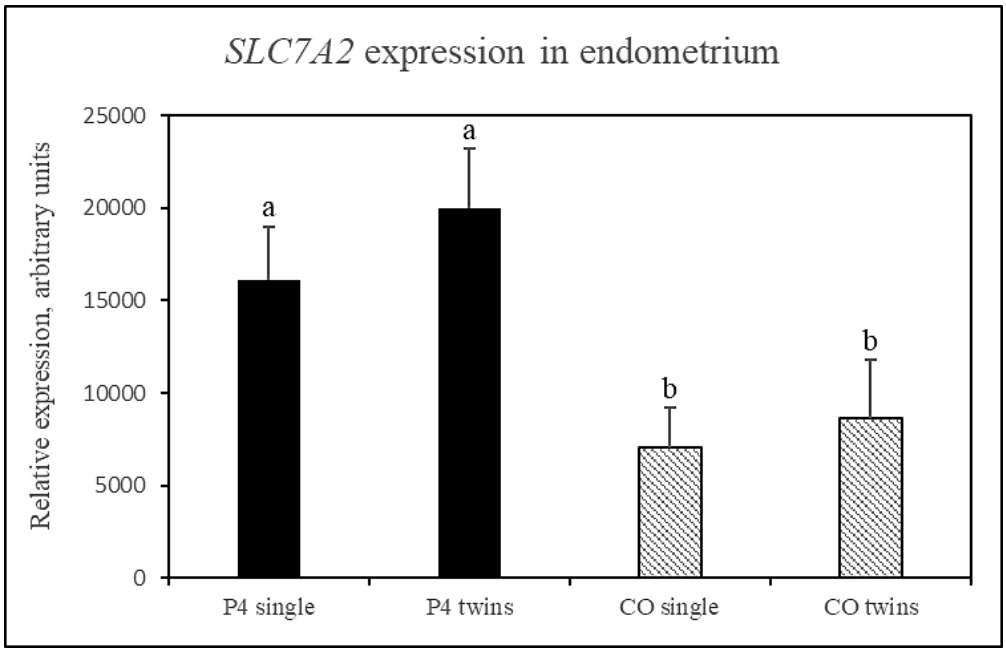

Figure 2.12. Effects of exogenous progesterone on expression of low affinity cationic amino acid transporter 2 mRNA in endometrial tissue. Administration of exogenous progesterone from Day 1.5 through Day 8 of pregnancy increased expression in P4 treated ewes ($P < 0.05$).

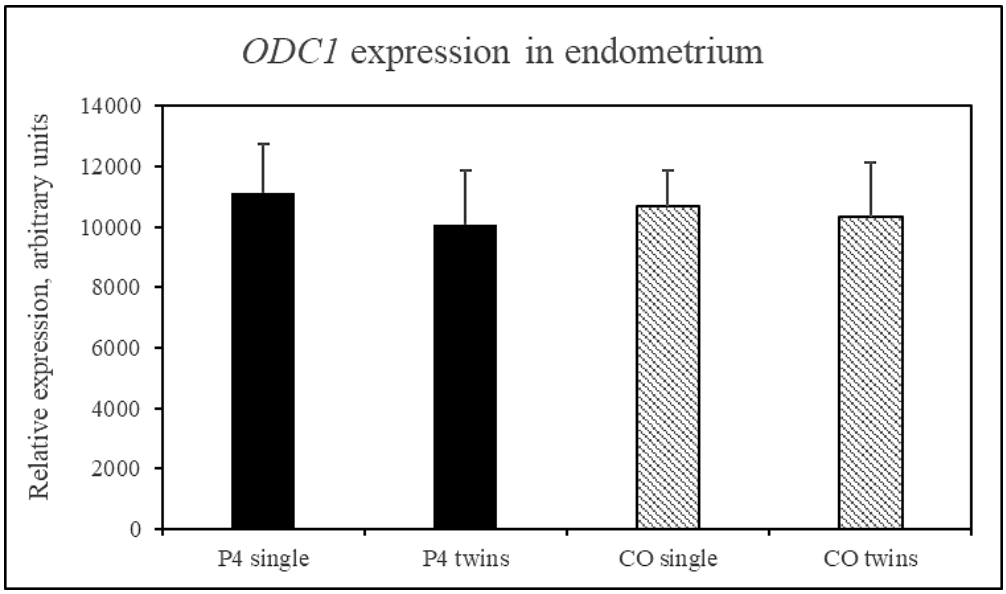

Figure 2.13. Effects of exogenous progesterone on expression of ornithine decarboxylase 1 mRNA in endometrial tissue. Administration of exogenous progesterone from Day 1.5 through Day 8 of pregnancy did not affect expression ($P > 0.05$).

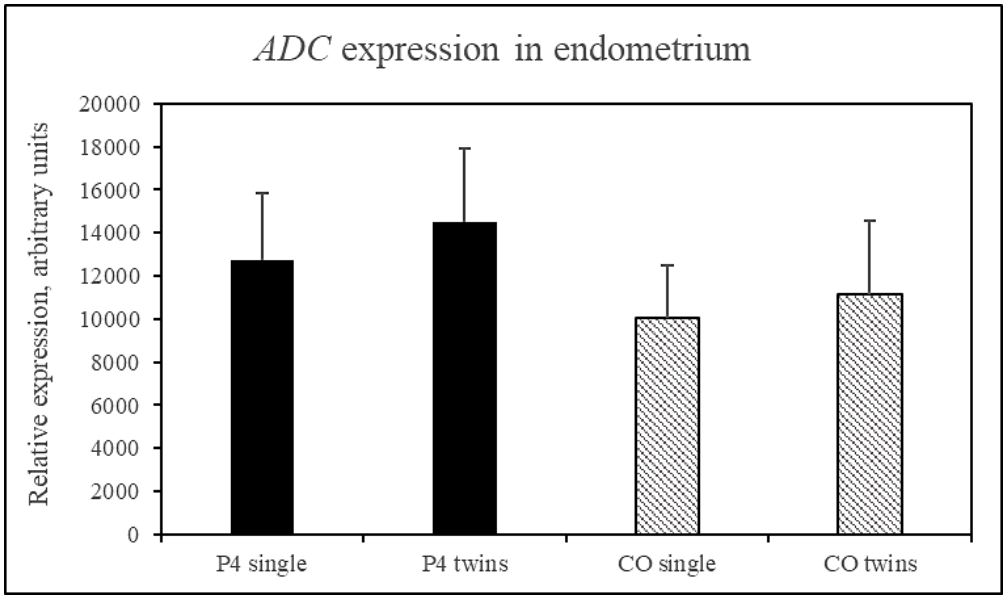

Figure 2.14. Effects of exogenous progesterone on expression of arginine decarboxylase mRNA in endometrial tissue. Administration of exogenous progesterone from Day 1.5 through Day 8 of pregnancy did not affect expression ($P > 0.05$).

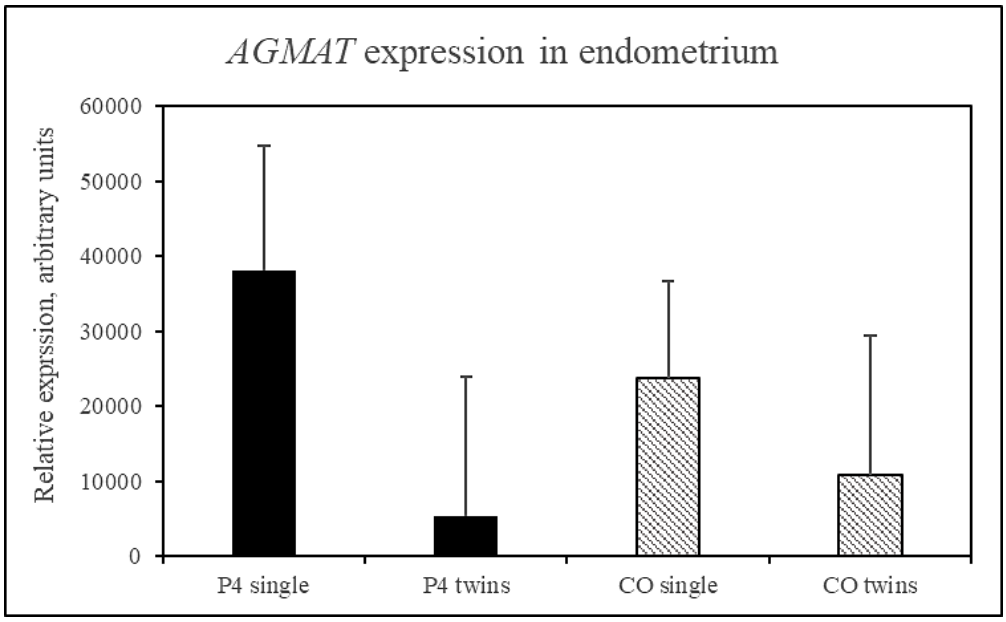

Figure 2.15. Effects of exogenous progesterone on expression of agmatinase mRNA in endometrial tissue. Administration of exogenous progesterone from Day 1.5 through Day 8 of pregnancy did not have affect expression ($P > 0.05$).

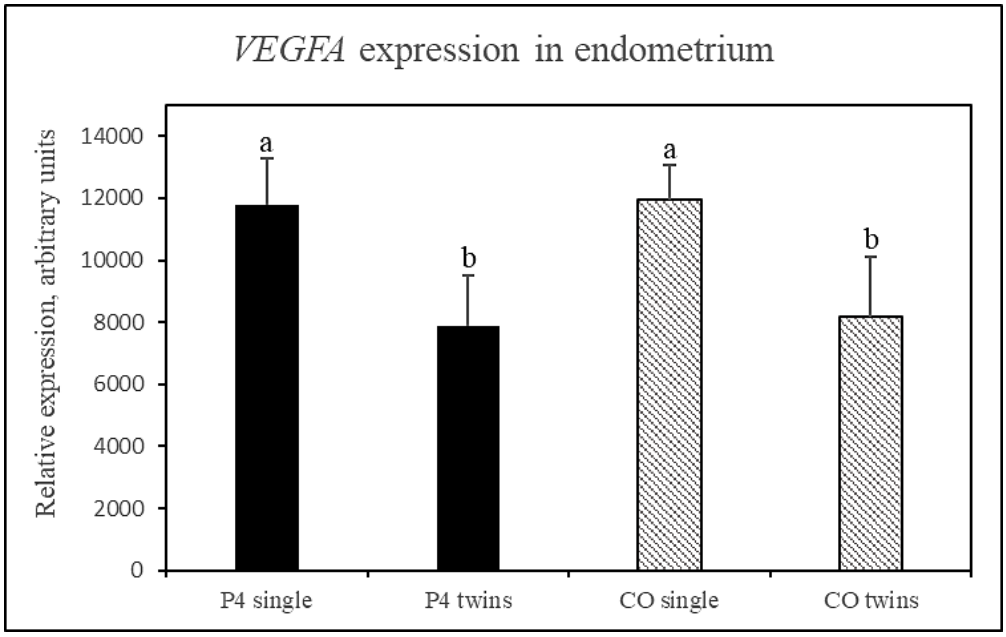

Figure 2.16. Effects of exogenous progesterone on expression of vascular endothelial growth factor A mRNA in endometrial tissue. Expression was increased in single fetus pregnancies compared to twin pregnancies ($P < 0.05$).

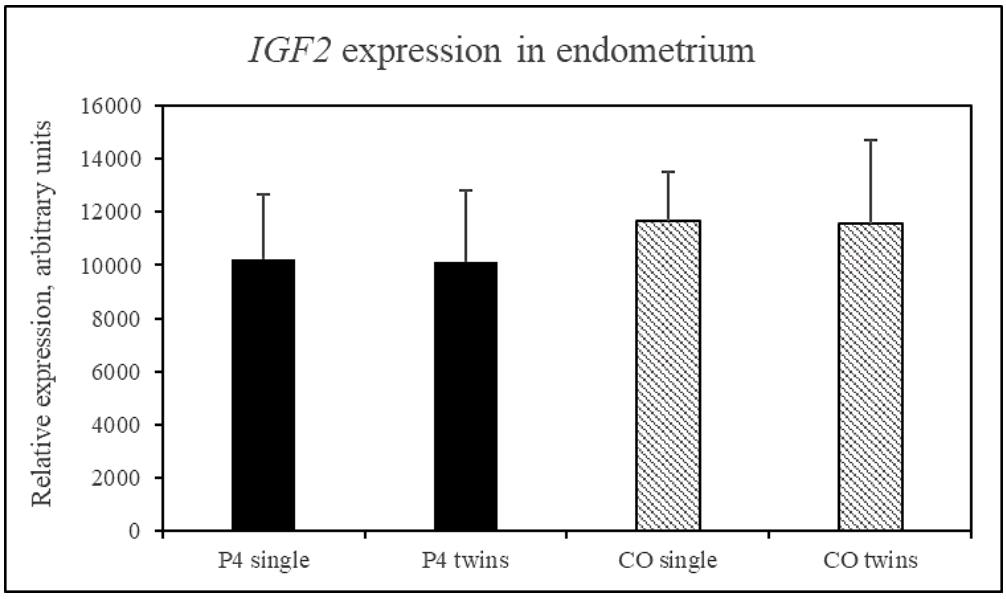

Figure 2.17. Effects of exogenous progesterone on expression of insulin-like growth factor 2 mRNA in endometrial tissue. Administration of exogenous progesterone from Day 1.5 through Day 8 of pregnancy did not affect expression ($P > 0.05$).

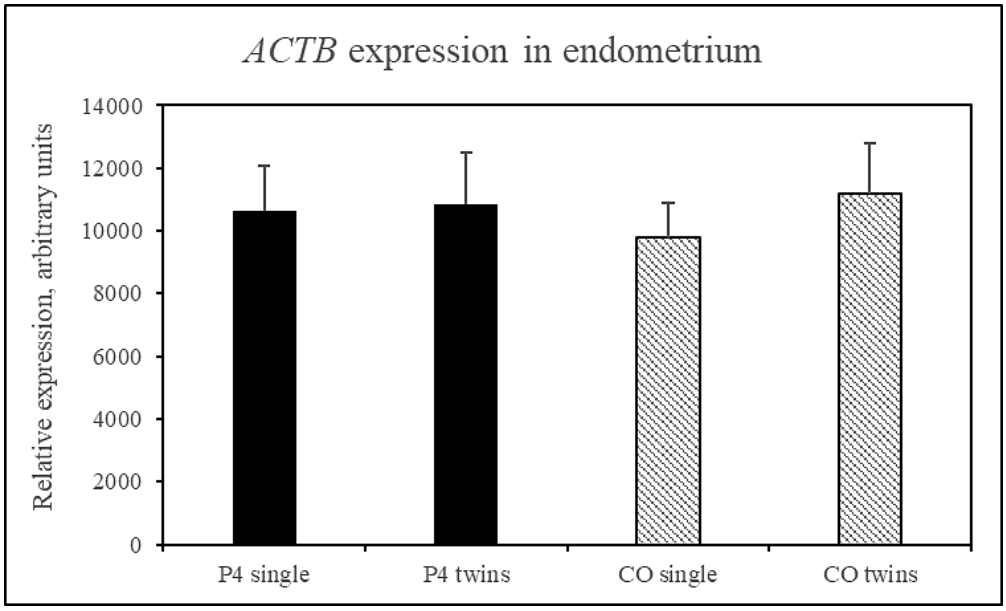

Figure 2.18. Effects of exogenous progesterone on expression of beta-actin mRNA in endometrial tissue. Administration of exogenous progesterone from Day 1.5 through Day 8 of pregnancy did not affect expression ($P > 0.05$).

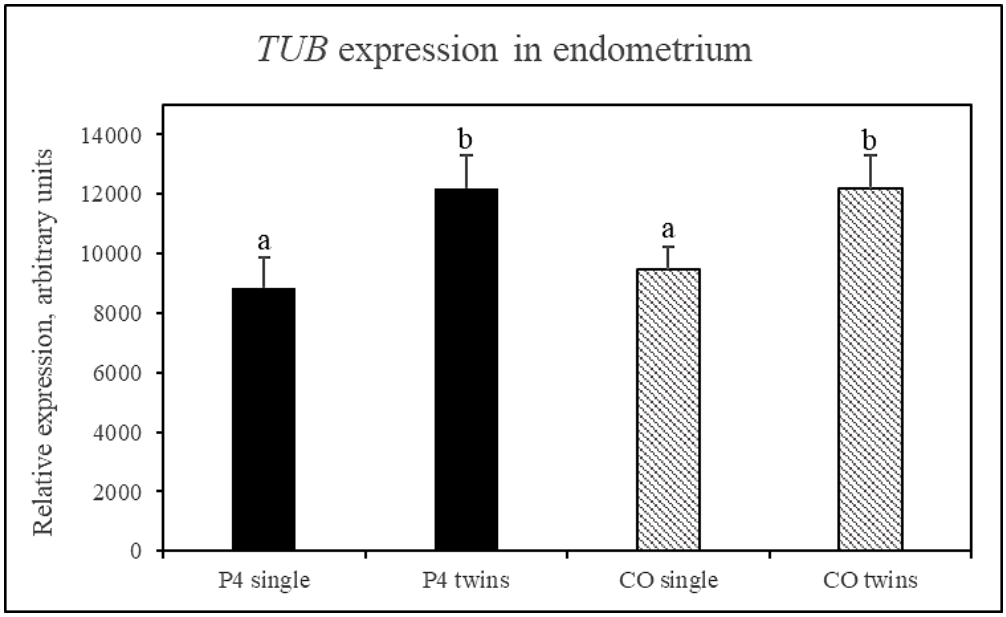

Figure 2.19. Effects of exogenous progesterone on expression of tubulin mRNA in endometrial tissue. Expression was increased in twin fetus pregnancies compared to single fetus pregnancies ($P < 0.05$).

Gene expression in ovine placentomes

Quantitative real-time PCR (qPCR) was used to determine expression of mRNAs in ovine placentomes (Figure 2.4). Expression of *SLC2A1* mRNA (Figure 2.20) was greater in CO single fetus pregnancies compared to CO twin fetus ($P < 0.01$), P4 single fetus ($P < 0.01$) and P4 twin fetus ($P < 0.01$) pregnancies, but all other pregnancy types were not different compared to each other ($P > 0.05$). Similarly, expression of *SLC2A3* mRNA (Figure 2.21) was greater for CO single fetus pregnancies compared to CO twin fetus ($P < 0.05$), P4 single fetus ($P < 0.05$), and P4 twin fetus ($P < 0.01$) pregnancies, but all other pregnancy types were not different compared to each other ($P > 0.05$). Expression of *SLC2A5* mRNA (Figure 2.22) was greater for P4 treated compared to CO treated ewes ($P < 0.01$), but was not affected by litter size ($P > 0.05$). Expression of *SLC2A8* mRNA (Figure 2.23) was greater for P4 treated compared to CO treated ewes ($P < 0.01$), but was not affected by litter size ($P > 0.05$). Expression of *SLC7A1* mRNA (Figure 2.24) was not affected by treatment ($P > 0.05$), litter size ($P > 0.05$), or their interaction ($P > 0.05$). Expression of *SLC7A2* mRNA (Figure 2.25) was not different due to treatment ($P > 0.05$) or litter size ($P > 0.05$), but there was a significant interaction ($P < 0.05$). Expression of *ODC1* mRNA (Figure 2.26) was greater for CO single fetus compared to P4 twin fetus pregnancies ($P < 0.05$), but all other pregnancy types were not different compared to each other ($P > 0.05$). Expression of *ADC* mRNA (Figure 2.27) was not affected by treatment ($P > 0.05$), litter size ($P > 0.05$), or their interaction ($P > 0.05$). Expression of *AGMAT* mRNA (Figure 2.28) was not affected by treatment ($P > 0.05$), litter size ($P > 0.05$), or their interaction ($P > 0.05$). Expression of *VEGFA* mRNA (Figure 2.29) was not affected by treatment ($P > 0.05$), litter size ($P > 0.05$), or their interaction ($P > 0.05$). Expression of

CSH1 mRNA (Figure 2.30) was not different due to treatment ($P > 0.05$), litter size ($P > 0.05$), or their interaction ($P > 0.05$). Expression of *IGF2* mRNA (Figure 2.31) was not significantly due to treatment ($P > 0.05$), litter size ($P > 0.05$), or their interaction ($P > 0.05$). Expression of *ACTB* mRNA (Figure 2.32) was greater for P4 treated ewes ($P < 0.01$), but was not affected due to litter size ($P > 0.05$). Expression of *TUB* mRNA (Figure 2.33) was greater for P4 treated pregnancies ($P < 0.01$), but was not affected due to litter size ($P > 0.05$). Expression of *SLC6A9* mRNA (Figure 2.29) was not affected by treatment ($P > 0.05$), litter size ($P > 0.05$), or their interaction ($P > 0.05$).

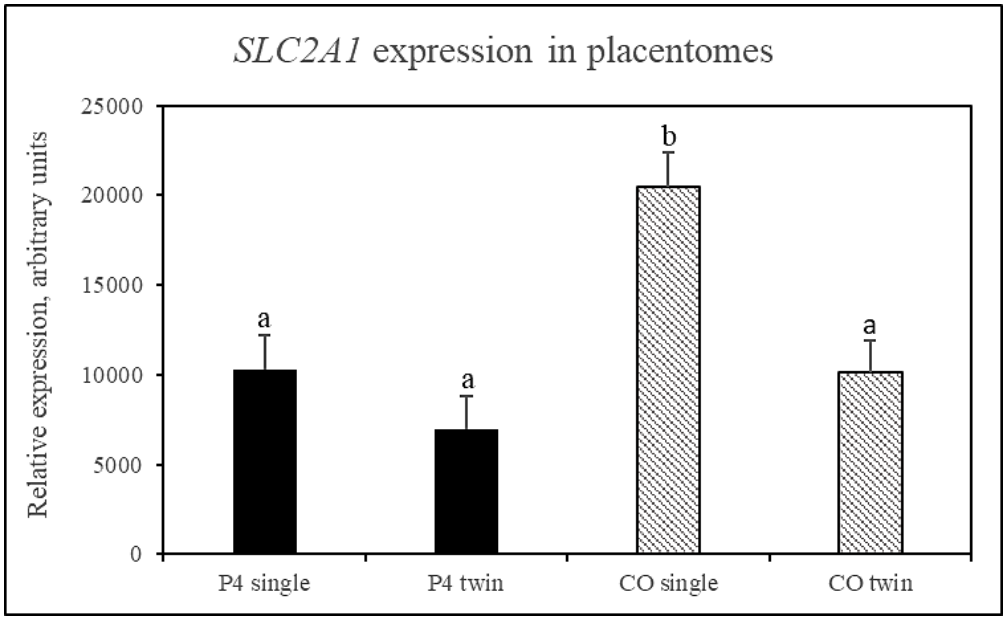

Figure 2.20. Effects of exogenous progesterone on expression of facilitated glucose transporter 1 mRNA in placentomes. Expression was increased in CO treated ewes with single pregnancies compared to the other pregnancy types ($P < 0.05$).

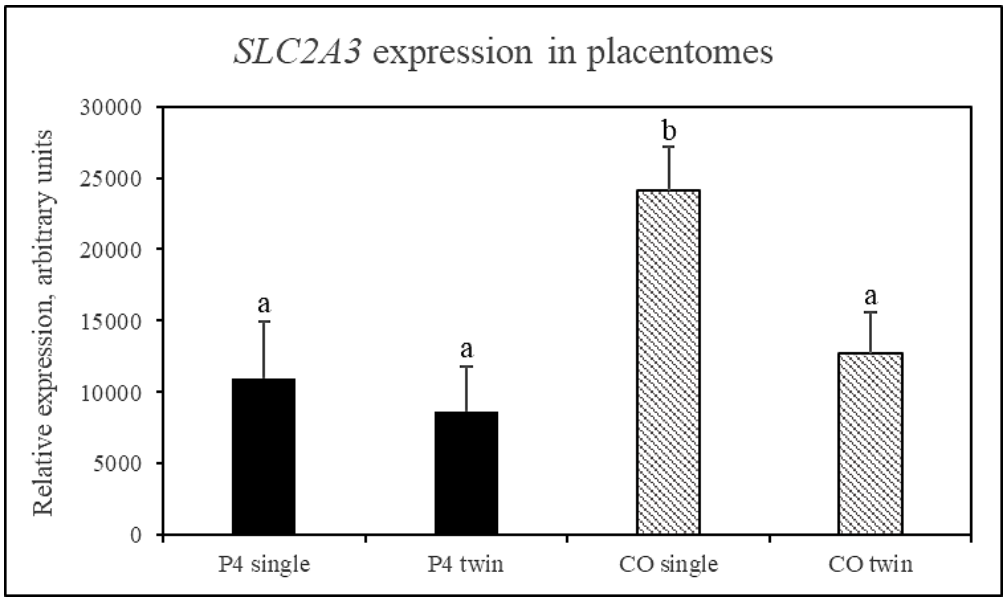

Figure 2.21. Effects of exogenous progesterone on expression of high affinity glucose transporter 3 mRNA in placentomes. Expression was increased in CO treated ewes with single pregnancies compared to the other pregnancy types ($P < 0.05$).

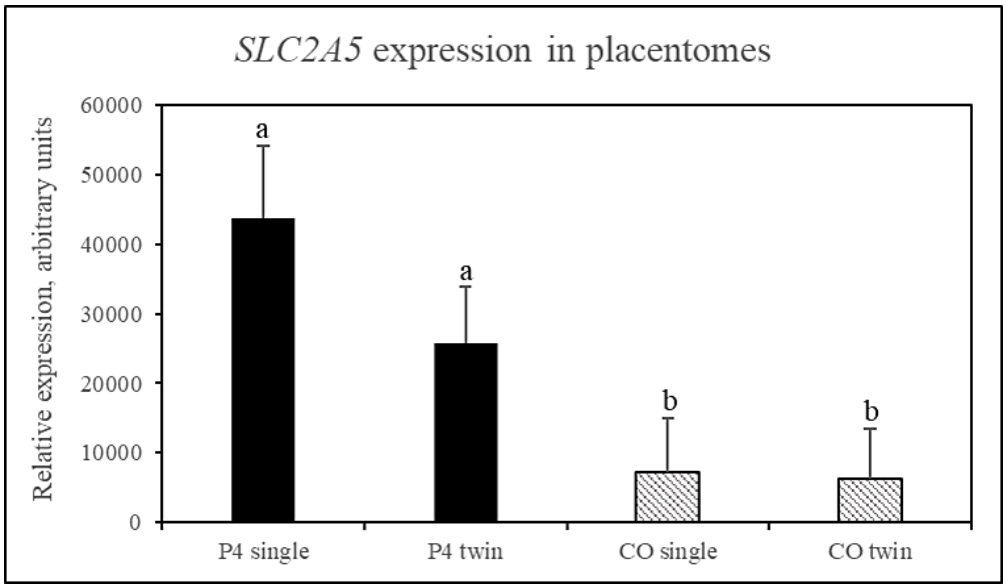

Figure 2.22. Effects of exogenous progesterone on expression of high affinity fructose transporter 5 mRNA in placentomes. Administration of exogenous progesterone from Day 1.5 through Day 8 of pregnancy increased expression in P4 treated ewes ($P < 0.05$).

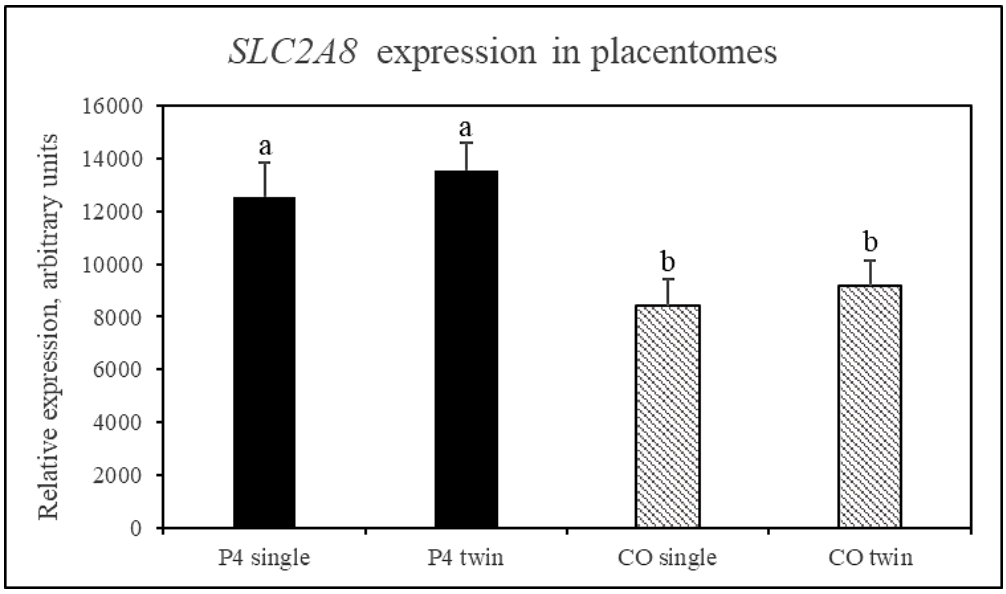

Figure 2.23. Effects of exogenous progesterone on expression of facilitated fructose and glucose transporter 8 mRNA in placentomes. Administration of exogenous progesterone from Day 1.5 through Day 8 of pregnancy increased expression in P4 treated ewes ($P < 0.05$).

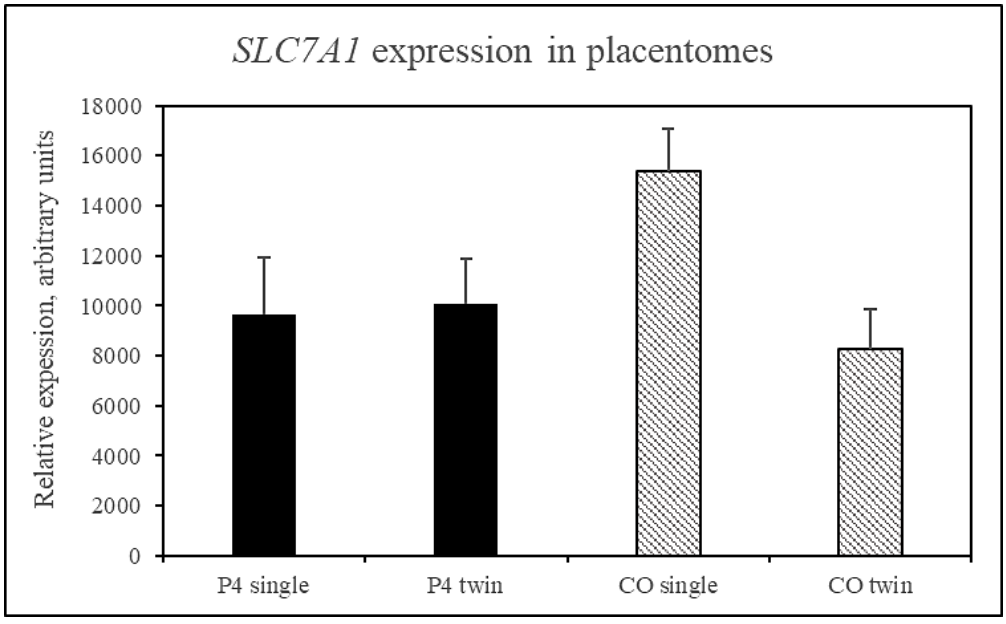

Figure 2.24. Effects of exogenous progesterone on expression of high affinity cationic amino acid transporter 1 mRNA in placentomes. Due to a significant interaction term ($P < 0.05$), it cannot be determined if either treatment or litter size affected expression.

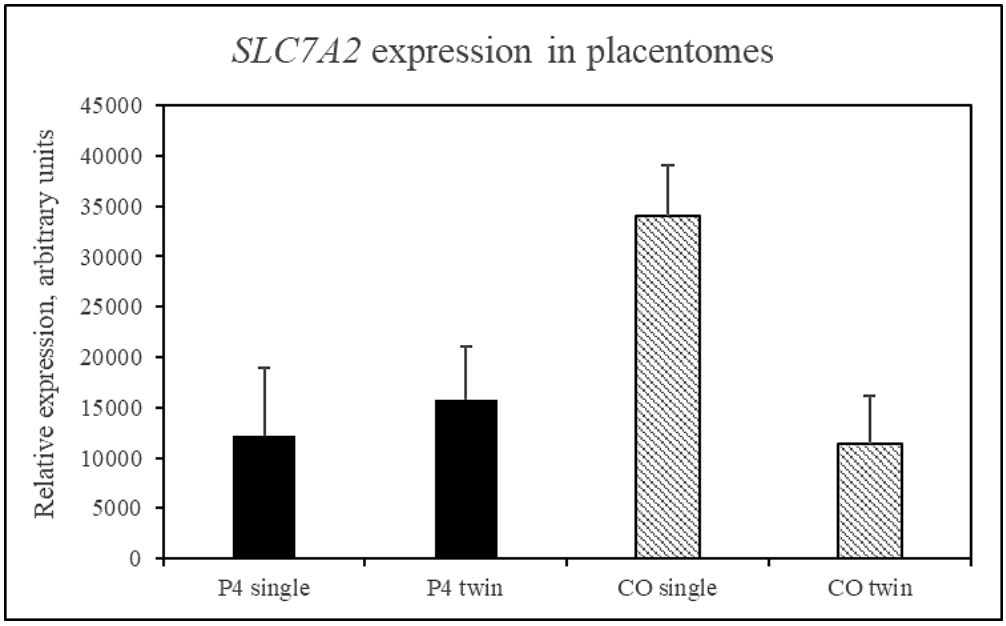

Figure 2.25. Effects of exogenous progesterone on expression of low affinity cationic amino acid transporter 2 mRNA in placentomes. Due to a significant interaction term ($P < 0.05$), it cannot be determined if either treatment or litter size affected expression.

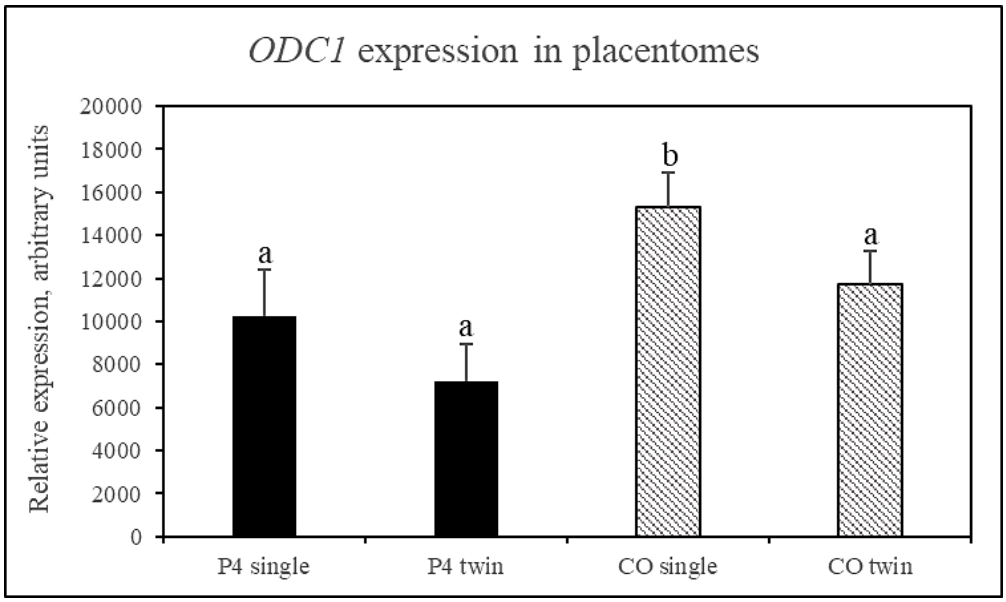

Figure 2.26. Effects of exogenous progesterone on expression of ornithine decarboxylase 1 mRNA in placentomes. Expression was increased in CO treated ewes with single pregnancies compared to the other pregnancy types ($P < 0.05$).

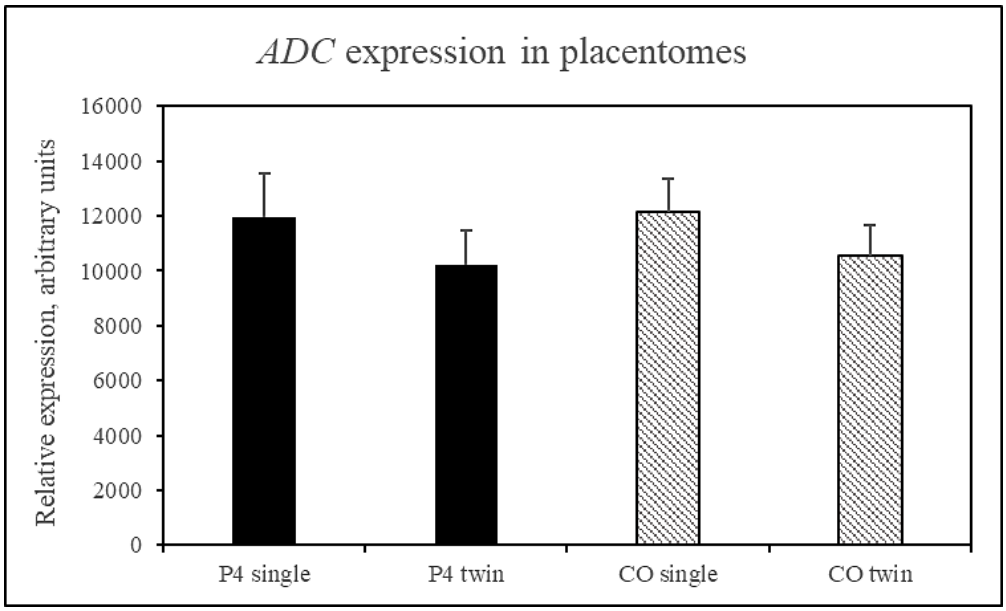

Figure 2.27. Effects of exogenous progesterone on expression of arginine decarboxylase mRNA in placentomes. Administration of exogenous progesterone from Day 1.5 through Day 8 of pregnancy did not have an effect on expression ($P > 0.05$).

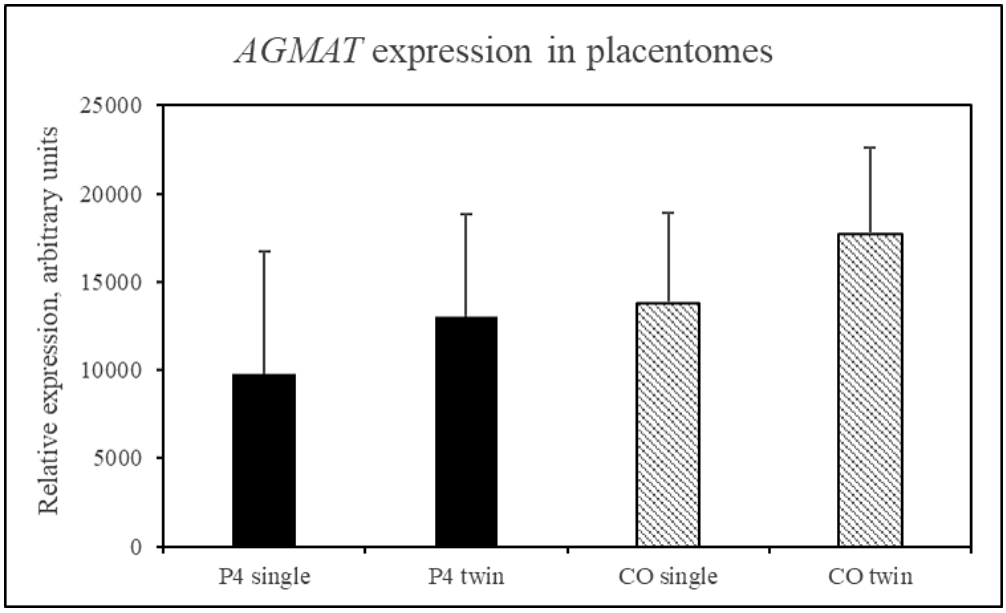

Figure 2.28. Effects of exogenous progesterone on expression of agmatinase mRNA in placentomes. Administration of exogenous progesterone from Day 1.5 through Day 8 of pregnancy did not have an effect on expression ($P > 0.05$).

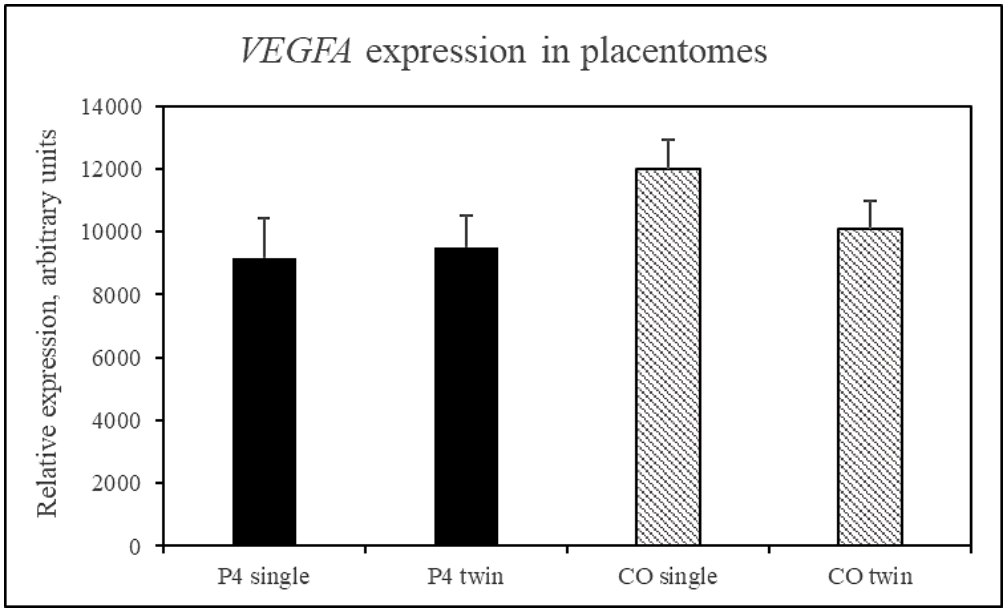

Figure 2.29. Effects of exogenous progesterone on expression of vascular endothelial growth factor A mRNA in placentomes. Administration of exogenous progesterone from Day 1.5 through Day 8 of pregnancy did not have an effect on expression ($P > 0.05$).

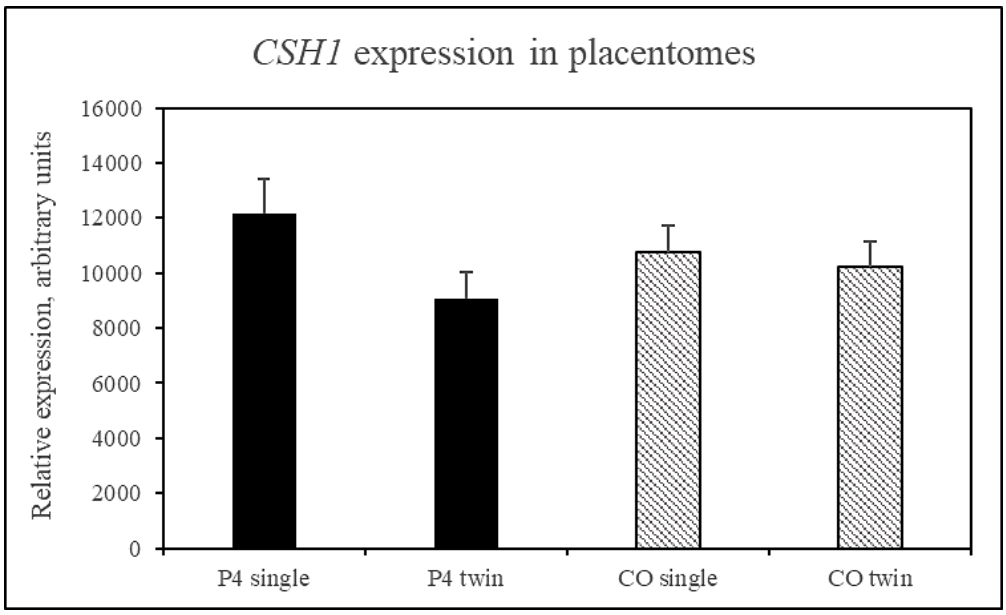

Figure 2.30. Effects of exogenous progesterone on expression of chorionic somatomammotropin hormone 1 mRNA in placentomes. Administration of exogenous progesterone from Day 1.5 through Day 8 of pregnancy did not have an effect on expression ($P > 0.05$).

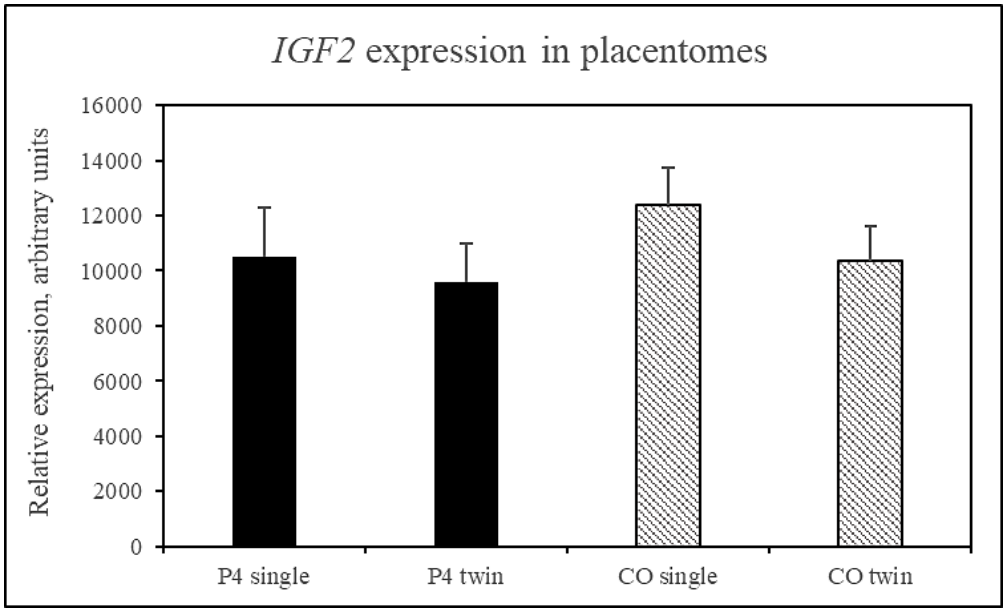

Figure 2.31. Effects of exogenous progesterone on expression of insulin-like growth factor 2 mRNA in placentomes. Administration of exogenous progesterone from Day 1.5 through Day 8 of pregnancy did not have an effect on expression ($P > 0.05$).

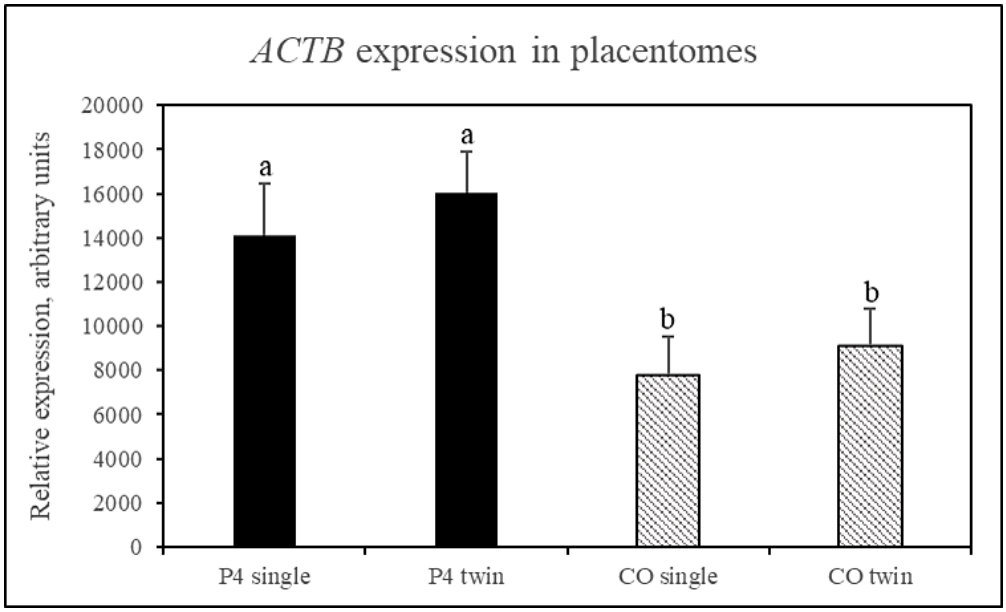

Figure 2.32. Effects of exogenous progesterone on expression of beta-actin mRNA in placentomes. Administration of exogenous progesterone from Day 1.5 through Day 8 of pregnancy increased expression in P4 treated ewes ($P < 0.05$).

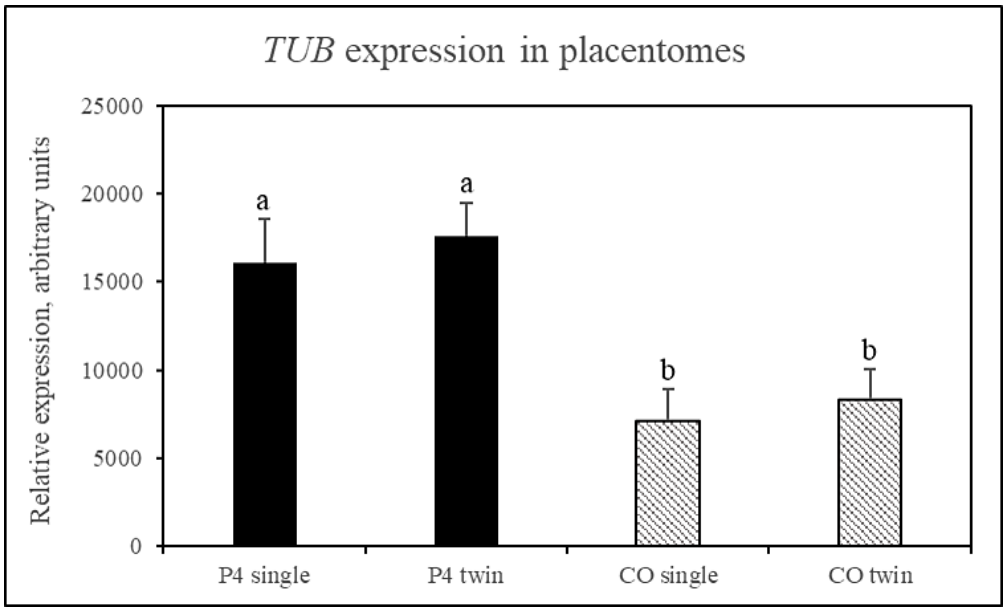

Figure 2.33. Effects of exogenous progesterone on expression of tubulin mRNA in placentomes. Administration of exogenous progesterone from Day 1.5 through Day 8 of pregnancy increased expression in P4 treated ewes ($P < 0.05$).

Hexose sugars in fetal and placental fluids

Concentrations of glucose were determined in allantoic fluid, amniotic fluid, and fetal plasma before calculating total glucose in each fluid. Total glucose in fetal plasma was not calculated as total volume of fetal blood was assumed to be similar among fetuses. Concentrations of glucose in allantoic fluid were greater in single compared to twin pregnancies ($P < 0.05$), but was not affected due to treatment or their interaction ($P > 0.05$). Concentrations of glucose in amniotic fluid were not affected by treatment, litter size, nor their interaction ($P > 0.05$). Concentration of glucose in fetal plasma was not affected due to treatment, litter size, or their interaction ($P > 0.05$) (Figure 2.34). After multiplying the concentration by the volume of fluid collected, total glucose in allantoic fluid was not different due to treatment or litter size ($P > 0.05$). Total glucose in amniotic

fluid did not differ due to treatment or litter size ($P > 0.05$). Data are summarized in Table 2.3.

Concentrations of fructose were determined in allantoic fluid, amniotic fluid, and fetal plasma before calculating total fructose in each of the fetal fluids. Total fructose in fetal plasma was not calculated as total volume of fetal blood was assumed to be similar among fetuses. Concentrations of fructose in allantoic fluid were not different due to treatment, litter size, or their interaction ($P > 0.05$). Concentrations of fructose in amniotic fluid were not affected by treatment, litter size, or their interaction ($P > 0.05$). Concentrations of fructose in fetal plasma were greatest in single fetus pregnancies of P4 treated ewes ($P < 0.05$), but was not different in other pregnancy types ($P > 0.05$) (Figure 2.35). After multiplying the concentration by the volume of fluid collected, total fructose in allantoic fluid was not different due to treatment or litter size ($P > 0.05$). Total fructose in amniotic fluid did not differ due to treatment or litter size ($P > 0.05$). There was more total fructose in allantoic and amniotic fluid than glucose, regardless of pregnancy type ($P < 0.05$). Data are summarized in Table 2.4.

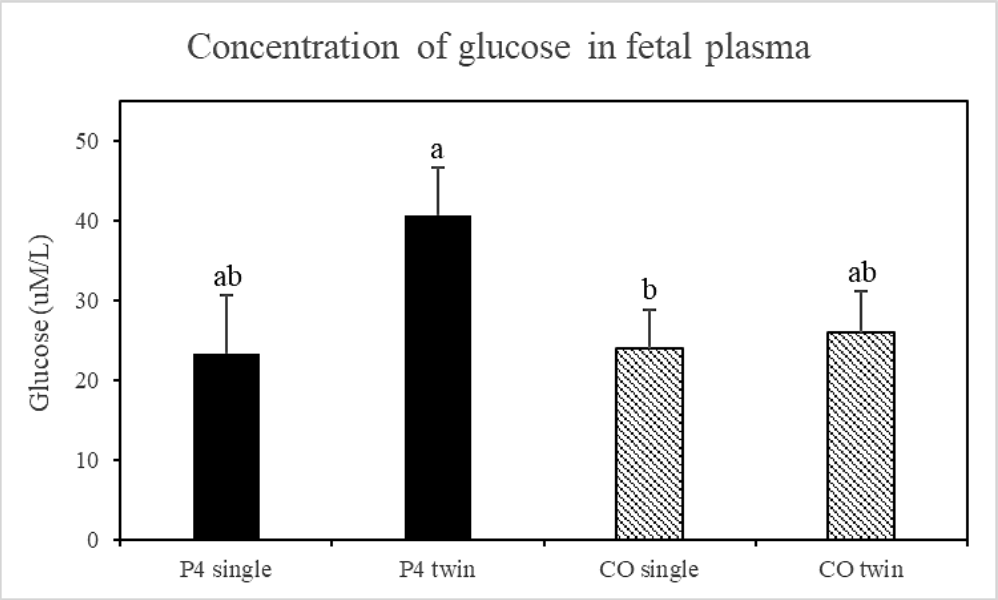

Figure 2.34. Concentration of glucose in fetal plasma. Fetuses from P4 treated ewes with twin pregnancies had more glucose in plasma than fetuses from CO single fetus pregnancies ($P < 0.05$), and were not different compared to twin fetuses in CO treated ewes and P4 treated ewes with single fetuses ($0.10 < P > 0.05$).

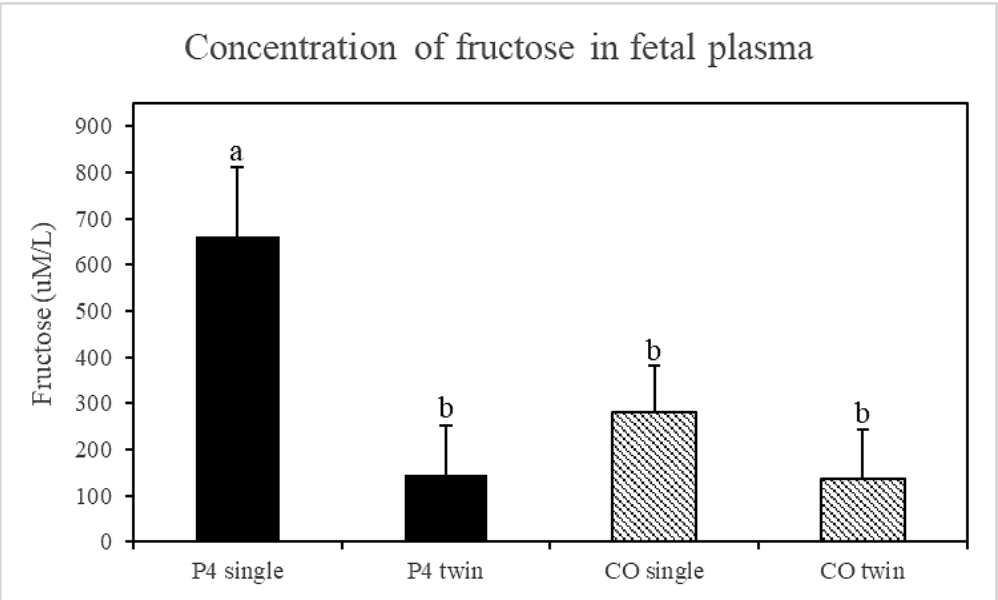

Figure 2.35. Concentrations of fructose in fetal plasma. Fetuses from P4 treated ewes with single fetuses had more fructose in plasma than for the other pregnancy types ($P < 0.05$).

Table 2.3. Total glucose (mg; concentration x volume) in placental fluids

	Pregnancy type			
Fluid	P4 single	P4 twin	CO single	CO twin
Allantoic fluid	2.1 +/- 1	2.2 +/- 0.9	2.8 +/- 1	2.1 +/- 1.7
Amniotic fluid	3.3 +/- 2.8	2.3 +/- 1	4.1 +/- 2.1	2.5 +/- 2.3

Values are treatment means ± SEM

Table 2.4. Total fructose (mg; concentration x volume) in placental fluids

	Pregnancy type			
Fluid	P4 single	P4 twin	CO single	CO twin
Allantoic fluid	64.1 +/- 33.7	78.8 +/- 35.3	59.2 +/- 26.1	89.9 +/- 97.1
Amniotic fluid	54.4 +/- 57.3	33.6 +/- 18.8	49.8 +/- 25.8	36.5 +/- 25.5

Values are treatment means ± SEM

Discussion

In the present study, we hypothesized that exogenous P4 supplementation in early pregnancy would result in advanced fetal and/or placental development in late pregnancy. Results of our study show that exogenous P4 administered to ewes from Day 1.5 through Day 9 of pregnancy did not result in phenotypic differences in fetal or placental growth on Day 125 of pregnancy. P4 treatment did not affect size of the fetuses or the placentae, despite temporarily advancing conceptus elongation (E. Hoskins, unpublished results); the only significant effects were attributed to the number of fetuses per pregnancy. These results conflict with results that P4 supplementation increases fetal growth [132, 133]. However, those studies collected tissues at Day 76 of pregnancy, which is approximately the mid-point of gestation in sheep. We collected tissues at Day 125, which is about 20 days before natural parturition. A possible explanation could be that previous studies observed changes in fetoplacental growth because at that point in pregnancy, the fetus is beginning exponential growth [104], and in our study the fetuses had reached a plateau in their growth curves. While the advanced development of conceptuses observed in early pregnancy did not result in morphological differences in late pregnancy, changes in endometrial and placental gene expression indicates differential functions of these tissues between treatments.

The expression of *SLC2A1* mRNA is induced by P4 and stimulated by IFNT [100]. Cattle with higher post-ovulatory circulating levels of P4 do not show differences in transcript abundance for glucose transporters or concentrations of glucose in uterine flushings on Day 7 of pregnancy compared to cows with low post-ovulatory circulating levels of P4 [135]. Exogenous P4 administered to ewes from Day 1.5 through Day 9 of

pregnancy increases expression of *SLC2A1* mRNAs in endometrium, as well as total glucose in the uterine lumen, while treatment from Day 1.5 through Day 12 of pregnancy does not result in more total glucose in the uterine lumen despite an increase in *SLC2A1* mRNA expression [131]. Based on those results, P4 not only regulates the expression of glucose transporters, but advanced conceptus development observed at this time may be attributed to exogenous P4-induced increases in expression of *SLC2A1* mRNA and increased glucose in histotroph at Day 9 of pregnancy [131]. In our study, *SLC2A1* was not differentially expressed in the endometrium, which would indicate that there was no difference in the amount of glucose in placental fluids. Indeed, we did not see any difference in total glucose in either allanotic or amniotic fluid due to treatment of ewes with P4. Theoretically then, an early rise in P4 does not affect expression of glucose transporters in the endometrium in late pregnancy; this may be due to the time that elapsed between P4 treatment and tissue collection. In placentomes, expression of *SLC2A1* and *SLC2A3* mRNAs were greater only in CO treated ewes with a single fetus. It is interesting that P4 treated ewes with single fetuses had relatively less expression of *SLC2A1* and *SLC2A3* mRNAs compared to CO treated ewes with a single fetus, but there was no difference in expression of those mRNAs between P4 treated and CO treated ewes carrying twin fetuses. This may have been a compensatory decrease, as fructose transporters were increased in placentomes of P4 treated ewes. A difference in fructose transport into placental and fetal vasculature would affect the concentration gradient of hexose sugars in these tissues, and thus cause the placentomes to down-regulate glucose-only transporters.

SLC2A8 and *SLC2A5* are both transporters for fructose. Both genes were upregulated in placentomes of P4 treated ewes, which leads us to believe that P4 regulates

fructose transport. Increased expression of mRNAs for *SLC2A8* and *SLC2A5* in placentomes may indicate that more fructose was being transported into the fetal vasculature in P4 treated ewes. Interestingly, there was a higher concentration of fructose in fetal plasma from P4 treated ewes with single pregnancies than CO treated ewes with single pregnancies. These fetuses had more fructose availability than their untreated counterparts, and thus more fructose could be used as a substrate in one-carbon metabolism pathway, hexosamine biosysnthesis, or mTOR nutrient sensing pathway [110]. During mid- to late-pregnancy in pigs, *SLC2A8* transports glucose from the maternal vasculature into the chorion, where it can either be transported directly into the placental vasculature via *SLCA8* or converted to fructose first and then transported via *SLC2A5*/8 [116]. This may be what occurs in the inter-placentomal regions of a ruminant synepitheliochorial placenta.

SLC7A1 (high affinity) and *SLC7A2* (low affinity, but high capacity) are both cationic amino acid transporters highly expressed by the ovine endometrium and weakly expressed by the conceptus [136]. These transporters have a high affinity for arginine, a essential amino acid for pregnant animals [119]. Both *SLC7A1* and *SLC7A2* are induced in uterine LE, GE, and stromal cells and the uterine LE and sGE, respectively, by long-term treatment of ovariectomized ewes with P4 [136]. Exogenous supplementation of P4 from Day 1.5 through Day 12 of pregnancy increased expression of *SLC7A2* and is thus associated with increases in arginine and lysine in the uterine flushes at that time [131]. In the present study, expression of *SLC7A2* in endometria, but not placentomes, was increased in P4 treated ewes. It may be possible that *SLC7A2* is induced by P4 to increase amino acid transport across the interplacentomal uterine LE or secretion by uterine GE.

This would indicate that fetuses from P4 treated ewes had increased amino acid delivery than fetuses from CO treated ewes. *SLC7A2* also increase the amounts of amino acids, particularly arginine and lysine, in placental fluids. Amino acids in amniotic fluid, when swallowed, provide nutrients for the developing fetus and play a role in gut maturation [48]. Placental fluids and fetal plasma will need to be analyzed to determine amino acid profiles and thus functionality of these transporters.

Arginine is a nutritionally essential amino acid for conceptuses in early pregnancy and may also be particularly important during late pregnancy when the fetus is growing exponentially and has reached a large size. Arginine is important as it is the precursor for nitric oxide (NO) as well as polyamines—putrescine, spermidine, and spermine. Polyamines are important because they are building blocks for DNA and protein synthesis, are involved in angiogenesis, and help mediate cell proliferation [117]. Synthesis of polyamines typically follows a "classic" pathway, which involves ornithine decarboxylase (ODC1). However, if ODC1 mRNA translation is blocked, sheep conceptuses have the ability to activate an alternative pathway for polyamine synthesis via arginine decarboxylase (ADC) and agmatinase (AGMAT) [121]. The ADC/AGMAT pathway can serve as a compensatory pathway for the production of polyamines. Interestingly, in this study, *ODC1* mRNA expression showed a similar pattern to those for *SLC2A1* and *SLC2A3* in the placentomes; *ODC1* mRNA expression was greater in CO treated ewes with a single fetus than for any other pregnancy type. These results, along with the fact that there was no differences in *ADC* and *AGMAT* mRNA expression, indicates that these pathways are not influenced by P4.

It is interesting that both *ACTB* and *TUB* mRNA were up-regulated in placentomes of P4 treated ewes. Both of these genes are ubiquitously expressed and are thus typically used as control or reference genes when performing analyses such as qPCR. However, as both of these genes were significantly up-regulated in ewes treated with P4, they are likely regulated by steroid hormones. Perhaps progesterone treatment in early pregnancy modifies the structure of uterine caruncles and the resulting placentomes are more interdigitated than placentomes from untreated ewes, thus the maternal and placental vasculature are in closer proximity. There is one report gamma-tubulin in binucleate trophoblast giant cells (BNC) of bovine placentomes [137]. Gamma-tubulin localized to the centrosomes of BNCs, which indicate that they are involved in nuclear duplication. It is possible that the conceptuses of P4 treated ewes in the present study developed BNC faster than control conceptuses, resulting in structural differences among placentomes. Localization of these proteins in placentomes will be useful to determine whether these genes are up-regulated in maternal or placental tissue.

CHAPTER III

CONCLUSION

The uterus is not just an idle structure in which the conceptus develops—it is a dynamic organ that undergoes structural as well as temporal and cell-specific changes in gene expression that function to maximize support of fetal growth. Proper uterine-conceptus signaling during the peri-implantation period of pregnancy is imperative for conceptus survival. Estrogen (E2) and progesterone (P4) are steroid hormones that set up the uterus to receive signals from the conceptus and in ruminants, this pregnancy recognition signal is interferon tau (IFNT) [76]. P4 and IFNT work in concert to fine tune the uterus to suit the needs of the conceptus (embryo/fetus and its associated placental membranes). This includes upregulating key genes for the production of histotroph, a complex mixture of nutrients, growth factors, hormones, and other molecules that support development of the conceptus before placentation occurs [2]. Conceptuses fail to develop without the presence of histotroph, as evidenced by the uterine gland knockout model in sheep [22]. Advancing conceptus development by administering exogenous P4 has been described in both cattle [127, 129] and sheep [25, 130, 131], and is likely due to advancing the time of secretion of histotroph. It has also been shown that asynchronous embryo transfer in sheep results in increased fetal growth on Day 76 of pregnancy for those conceptuses that were transferred into more advanced uteri [132, 133]. The uterus is a sensitive organ that can respond to a multitude of hormones; the timing being regulated as it receives signals from endocrine organs—the ovaries (E2, P4), the conceptus (IFNT), the placenta (CSH1), or the anterior pituitary gland (prolactin); all being crucial for its function and thus pregnancy success. However, the effects of an asynchronous uterine environment

on near-term fetal and placental development have not been demonstrated. Therefore, our objective was to gain insight as to how alteration of the maternal endocrine profile that results in a shift of the uterine environment in early pregnancy affects fetal and placental growth in late pregnancy.

Treatment of ewes with exogenous P4 during the pre-implantation period of pregnancy did not result in enhanced fetal or placental development. There was however, a genotypic difference in endometrial and placental gene expression between P4 treated and CO treated pregnancies. The expression of mRNAs for specific nutrient transporters, such as *SLC2A1* and *SLC2A3*, occurred in placentae of CO treated ewes. In placentomes, increased expression of *SLC2A5* and *SLC2A8* (transporters for fructose) resulted in increased total fructose in fetal plasma. While these nutrients may have not been utilized in metabolism to increase growth, they may have been involved in other metabolic pathways that may have affected post-partum growth. Another interesting aspect of this study was the differences in structural proteins within placentomes. Further studies will be needed to asses the morphometry of these placentomes, as well as the surface densities of caruncular crypts and cotyledonary villi. This would help determine if P4 results in increased interdigitation of placentomes, which would indicate an increase in functional capacity for transport across the syncytium.

The use of hormones to artificially manipulate the uterine environment early in pregnancy does not appear to influence overall fetal or placental growth during late gestation. Based on these results, exogenous hormones could be used without compromising the pregnancy once it has been established. It is unknown how these fetuses would perform post-partum. It would be valuable to examine the repercussions of this type

of treatment on neonatal growth and attainment of puberty. Ultimately, these results help elucidate what aspects of fetal and placental growth are regulated by exogenous progesterone administered in early pregnancy.

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