Effects of mid-gestational L-citrulline supplementation to twin-bearing ewes on umbilical blood flow, placental development, and lamb production traits

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ABSTRACT

The interaction between the embryo and fetus with the maternal environment can have both short- and long-term consequences on health and development after birth. In some cases, these changes may be detrimental to the individual, but in other cases these developmental changes may be beneficial and manipulated to produce desired effects. Our interest is to use this concept of fetal programming to improve skeletal muscle development and meat production in ruminants. To achieve this, we targeted the period of gestation when fetal muscle fiber formation occurs. Primary muscle fibers form during embryonic development, and it is this small number of primary muscle fibers that will serve as templates for secondary fiber formation that occurs in the fetus during mid-gestation. Supplementing amino acids that influence blood flow within the reproductive tract is one potential way to provide fetuses with added nutrients during gestation, and this supplementation strategy may be especially useful when the maternal diet is compromised. L-citrulline was chosen for this work because of its long half-life in maternal circulation.

This work utilized twin-bearing ewes with a moderate dietary energy restriction to assess the effects of mid-gestational L-citrulline supplementation on umbilical blood flow, placental function, neonatal lamb size, and lamb performance. We hypothesize that i.v. administration of L-citrulline will increase uterine and placental blood flow in gestating ewes and this will improve fetal growth, development, and overall postnatal performance. Blood flow parameters were not influenced by treatment ($P>0.05$). Circulating levels of progesterone and pregnancy-specific protein B (PSPB) were used as indicators of placental function and were unaffected by treatment administration ($P>0.05$). A treatment by time interaction was detected in both analyses, but no differences between treatments were detected within any time points. There was no effect of treatment on lamb weights or survival to weaning ($P>0.05$). Lamb sex effects are absent with the exception that body weights were greater in ewe lambs ($P>0.05$). There was no effect of treatment on any carcass traits or visceral organ weights assessed, though there was an effect of sex on dressing percentage and pancreas weight with wethers having a greater dressing percentage and heavier pancreases per kg body weight than that of ewes ($P<0.05$). In summary, contrary to our hypothesis L-citrulline supplementation to pregnant ewes under a minor to moderate metabolic challenge had no impact on blood flow and provided no programming benefit to the lambs.

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GENERAL AUDIENCE ABSTRACT

The global population continues to grow, along with the consumption of animal protein. This can be met with increasing the numbers of animals within our food production systems, however, there is also increasing pressure for livestock production systems to produce more while utilizing less space and resources. And simultaneously, we face growing concerns about climate change, its impacts on agriculture, and the role of agriculture in both the cause and any future solution. To combat both these issues, the efficiency of our livestock systems needs to improve with each individual animal becoming much more efficient. This increase in efficiency can occur in many ways including reproductive efficiency, feed efficiency, and in overall producing more meat per individual.

The improvement in efficiency of an animal can begin in the womb. Livestock in meat production spend 35-40% of their life within the uterus being nourished by their mother. The interactions the embryo and fetus have with the maternal environment during this time can have both short- and long-term impacts on health and development after birth. In some cases, these changes may be detrimental to the individual, but in other cases these developmental changes may be beneficial and manipulated to produce the desired effects. Thus, it is important to understand the impact of these fetal-maternal interactions as it directly affects both fetal growth and growth and development after birth. This concept is known as fetal programming.

Our interest is to use this concept to improve skeletal muscle development and meat production in cattle and sheep. To achieve this, we targeted the period of pregnancy when fetal muscle formation occurs. Primary muscle fibers form early in pregnancy, and it is this small number of primary muscle fibers that will serve as templates for secondary fiber formation that occurs in the fetus during mid-pregnancy. Supplementing amino acids that influence blood flow within the reproductive tract is one potential way to provide fetuses with added nutrients during pregnancy, and this supplementation strategy may be especially useful when the maternal diet is compromised. L-citrulline was chosen for this work because of its long half-life in maternal circulation.

This work utilized twin-bearing ewes with a moderate dietary energy restriction to assess the effects of L-citrulline supplementation on blood flow, placental function, newborn lamb size, and lamb performance. We hypothesize that intravenous administration of L-citrulline will increase uterine and placental blood flow in pregnant ewes and this will improve fetal growth, development, and overall postnatal performance. There was no beneficial effect on blood flow to the fetus and on placental function. Additionally, there were very minimal effects on carcass traits or internal organ weights assessed. In summary, contrary to our hypothesis L-citrulline supplementation to pregnant ewes under a moderate metabolic challenge had no impact on blood flow and provided no programming benefit to the lambs. We can conclude that the potential benefit of amino acid supplementation was not realized in our sheep model.

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List of Abbreviations

ADG	Average daily gain
AFC	Antral follicle count
ASL	Arginosuccinate lyase
ASS	Arginosuccinate synthase
BMP	Bone morphogenetic proteins
BNC	Binucleate cell
CAMKII	Calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
Cdx2	Caudal-related homeobox 2
C/EBP	CCAAT/enhancer binding protein
CL	Corpus luteum
COC	Cumulus-oocyte complex
DAG	Diacylglycerol
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DOHAD	Developmental origins of health and disease
EGA	Embryonic genome activation
EMT	Epithelial to mesenchymal transition
ERK	Extracellular receptor kinase
FGF	Fibroblast growth factors
FGFR	Fibroblast growth factor receptor
GH	Growth hormone
GlyCAM1	Glycosylation-dependent cell adhesion molecule-1
GnRH	Gonadotropin releasing hormone
Grb2	Growth factor receptor-bound protein 2
GV	Germinal vesicle
ICM	Inner cell mass
IFNT	Interferon-Tau
IL6	Interleukin-6
IL6R	Interleukin-6 receptor
IP3	Inositol triphosphate
ISG	Interferon-stimulated gene
ITGA4	Integrin alpha 4
IUGR	Intrauterine growth restriction
IVP	In vitro embryo production
JAK	Janus kinase
LE	Luminal epithelium
LH	Luteinizing hormone
MAPK	Mitogen-activated protein kinase
MNC	mononucleate cell
MPF	M-phase promoting factor
MRF	Myogenic regulatory factors
mTOR	Mechanistic target of rapamycin
MUC1	Mucin-1

mRNA	Messenger ribonucleic acid
Myf5	Myogenic factor-5
MyoD	Myogenic differentiation 1
MII	Metaphase II
NCG	N-carbamylglutamate
NO	Nitric oxide
NOS	Nitric oxide synthase
OCT	Ornithine carbamoyltransferase
Oct4	Octamer-4
PAG	Pregnancy-associated glycoproteins
PAX	Paired box gene
PE	Primitive endoderm
PI	Pulsatility index
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PL	Placental lactogen
PLCζ	Phospholipase C zeta
PPARγ	Peroxisome proliferator-activated receptor γ
PRL	Prolactin
PSPB	Pregnancy-specific protein B
P4	Progesterone
RI	Resistance index
RNA	Ribonucleic acid
S/D	Systolic/diastolic ratio
Shh	Sonic hedgehog
STAT	Signal transducer and activator of transcription
TE	Trophectoderm
Tead4	TEA domain transcription factor 4
TEP	Trophectoderm projections
TET	Ten-eleven translocation methylcytosine dioxygenases
TGF-β	Transforming growth factor-beta
TKDP	Trophoblast Kunitz domain protein
VCAM1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
Wnt	Wingless and Int
Yap1	Yes associated protein 1

Chapter 1

Literature Review

Introduction

An inadequate maternal environment can lead to the development of diseases during pregnancy, as well as result in programming of the fetus for disease later in life (Barker and Clark, 1997; Reynolds and Caton, 2012). This early period *in utero* includes embryogenesis where the embryo undergoes major cell lineage specification, implantation, placental development, organogenesis, and fetal development. The events occurring during the early fetal period are impacted by the maternal environment during this period *in utero*. Inadequate conditions can have detrimental consequences on offspring growth and development before and after birth. An example of this is intrauterine growth restriction (IUGR).

Intrauterine growth restriction is an issue in livestock production systems wherein it can compromise production efficiency. Inadequate conditions due to things such as maternal undernutrition caused by poor forage quality or availability, excessive temperatures causing heat stress, or twinning in typically monotocous species can lead to reduced or abnormal fetal growth and predispose the individual to abnormal growth after birth. Overall, this can result in an inability to attain an individual's full genetic potential and result in lost profits. Exact mechanisms leading to IUGR phenotype and therapeutic strategies to counteract the disease remain to be fully elucidated. It is important to gain a better understanding of these early events and how environmental stressors can impair early fetal and placental development and ultimately lead to poor fetal and post-natal outcomes.

Recognizing the impact maternal environment has on fetal and post-natal growth can lead to development of beneficial management schemes. The goal of developmental programming as it pertains to livestock production is to develop interventions that augment placental function, enhance blood flow, and increase nutrient delivery at critical periods during fetal development to prime offspring for optimum performance. Examples of these schemes in livestock include programming of meat production and adipogenesis and schemes that improve blood flow to the reproductive tract to increase nutrient delivery (Du et al., 2010a; Du et al., 2010b; Satterfield et al., 2010). These will be further discussed below in this review. The literature review will also report our current understanding of embryogenesis, development of the placenta, and early fetal growth, and will describe how maternal undernutrition may impact these events.

Key Events in Preimplantation Development

Oocyte Maturation and Fertilization

Oocyte maturation involves nuclear and cytoplasmic alterations that permit the oocyte to become fertilized and support the initial stages of embryonic development. Nuclear maturation involves the resumption and completion of meiosis. In the bovine, oocytes enter into the early stages of meiosis during fetal life and become arrested at prophase I, also called the germinal vesicle (GV) stage (Lonergan and Fair, 2016). This meiotic arrest is maintained by elevated cytoplasmic cAMP levels, which inhibit GV breakdown through blocking M-phase promoting factor (MPF, a heterodimer composed of Cdk1 and cyclin B) activation via activation of protein kinase A and phosphorylation of Cdk1 rendering it inactive (Sirard, 2001; Larose et al., 2019). The cAMP is derived from the granulosa cells that release their contents into the oocyte through gap junctions in the developing follicle (Larose et al., 2019). In vivo, resumption of meiosis, extrusion of the first polar body, and progression to arrest at metaphase II occurs due to the

preovulatory surge of luteinizing hormone (LH) (Lonergan and Fair, 2016). The LH surge stimulates GV breakdown and the weakening of the gap junctions between the oocyte and granulosa cells resulting in reduced cAMP levels and MPF activation, which induce resumption of meiosis I (Sanders and Jones, 2018; Larose et al., 2019). In vitro, removing the oocyte from the follicle causes spontaneous maturation (Sanders and Jones, 2018). After resumption of meiosis, oocytes will arrest meiosis at metaphase II (MII) with high MPF activity sustaining the arrest (Larose et al., 2019).

The cytoplasmic maturation process involves reorganization of organelles and storage of mRNAs, proteins, and transcription factors necessary for development through early embryogenesis. Prior to ovulation the oocyte undergoes an extensive period of transcription, storage, and processing of the mRNAs necessary for growth and development up until embryonic genome activation (EGA) when the embryo begins to direct its own development (for review see, Sirard, 2001). A second aspect of cytoplasmic maturation involves the redistribution of various organelles, including most notably mitochondria and cortical granules, in preparation for fertilization and subsequent development (Lonergan and Fair, 2016). Mitochondria shift from a peripheral distribution to a more clustered, central position in the cell at the MII phase, also in close proximity to lipid droplets, ready to metabolize stored triglycerides to supply the zygote with energy for growth and development up until EGA (Hyttel et al., 1997). Cortical granules are exclusively found in oocytes and are derived from the Golgi complex. They contain an array of proteins, structural molecules, enzymes, and glycosaminoglycans that play an important role in the cortical reaction after fertilization as a mechanism to prevent polyspermy (Ferreira et al., 2009). In the GV stage, these cortical granules are found in clusters throughout the cytoplasm,

but at the MII stage, they are distributed evenly just under the plasma membrane, awaiting to be released upon spermatozoon entry and oocyte activation (Ferreira et al., 2009).

As a dominant follicle develops, increasing concentrations of estradiol are produced and stimulate an increase in GnRH pulse frequency from the hypothalamus. When progesterone is absent, this increased GnRH pulse frequency stimulates an LH surge that acts on the dominant follicle leading to ovulation of the oocyte. Fertilization then occurs at the ampullary-isthmic junction of the oviduct and involves a series of specific interactions between spermatozoa and the oocyte. Prior to fertilization both the oocyte and spermatozoa must obtain the capacity for successful fertilization, involving the correct maturation of the oocyte and sperm capacitation. *In vivo* capacitation involves exposure of spermatozoa to the female tract and is also related to timing of ovulation. Capacitation results in destabilization of the plasma membrane, the removal of the glycoprotein coat, modulation of flagellar activity, and shedding of some of the associated surface proteins, thus allowing for proper recognition and binding to the zona pellucida of the oocyte (Rodriguez-Martinez, 2007).

The zona pellucida is the first portion of the oocyte the sperm encounters, and it is zona protein 3 that will bind to proteins on the spermatozoa membrane. It is this binding of the spermatozoon to the zona pellucida that will initiate the acrosomal reaction. The acrosome reaction accomplishes two things: 1) the release of acrosomal enzymes allowing the spermatozoon to digest its way through the zona pellucida into the perivitelline space, and 2) the modification of the equatorial segment on the sperm head resulting in activation of fusion proteins that enable the sperm and oocyte membranes to fuse (Hirohashi and Yanagimachi, 2018). As evidenced in mice, it is hypothesized that Izumo1, an essential sperm cell-surface protein, binds to Juno, the receptor for Izumo1 on the egg. This Izumo1-Juno interaction is

essential for spermatozoon and oocyte recognition and adhesion directly prior to membrane fusion (Bianchi et al., 2014).

After membrane fusion, the oocyte undergoes an activation process that includes the cortical reaction and resumption of meiosis for final nuclear maturation. Both of these events are triggered by calcium oscillations post-fertilization. The trigger for Ca^{2+} release is thought to be phospholipase C zeta (PLCζ) from the spermatozoon, which cleaves PIP_2 into DAG and IP_3, the latter of which triggers intracellular release of calcium from the endoplasmic reticulum (Saunders et al., 2002). DAG will activate protein kinase C (PKC) leading to the exocytosis of the cortical granules into the perivitelline space and induce changes to the zona proteins that contribute to the prevention of polyspermic fertilization (Sun, 2003). In addition, these calcium oscillations also reactivate the meiotic cycle and result in the extrusion of the second polar body (Lonergan and Fair, 2016; Sanders and Swann, 2016). This meiotic arrest at MII is maintained by high levels of MPF. It is through the activation of calmodulin-dependent protein kinase II (CAMKII) that MPF is inactivated. CAMKII phosphorylates Emi2, the molecule responsible for maintaining high levels of MPF, leading to its degradation and subsequent inactivation of MPF (Sanders and Swann, 2016; Sanders and Jones, 2018).

The end of meiosis resumption is marked by pronuclei formation and entrance into interphase of the first mitotic division of the zygote. On the male side, once the sperm nucleus has entered the cytoplasm of the egg it begins to decondense. This decondensing involves reduction by glutathione of the many disulfide cross-links that were put in place during sperm maturation in the epididymis and the replacement of protamines by histones (Collas and Poccia, 1998). On the female side, the completion of meiosis and entry into interphase of the first mitotic division depends on the decline in MAPK activity, which is triggered by CAMKII activity and

the decline in MPF (Moos et al., 1996). In mammals, the pronuclei do not fuse. They migrate toward each other, come into close contact, lose their nuclear envelope, and the two haploid genomes become united in the center of the zygote and begin the first mitotic division.

Early Cleavage Events

After fertilization, zygotes undergo cell divisions that do not increase the total volume of the embryo. This process, known as cleavage, leads to an increase in the number of cells in the embryo. The first cleavage event occurs at 1 to 2 days after fertilization in the ovine (Crosby et al., 1988). Following this first division, subsequent divisions occur at a greater frequency, around 8 to 12 hours per cell cycle. When a solid ball of cells has formed and individual blastomeres can no longer be identified, the embryo is called a morula.

Epigenetic Changes

The epigenome consists of histone protein or DNA modifications. The interactions between the epigenome, signaling pathways, and transcription factors confer gene expression patterns in a cell. In early development, male and female genomes undergo two waves of epigenetic reprogramming. The first wave occurs shortly after fertilization and involves a near complete demethylation of both the maternal and paternal genomes, leaving imprinted genes untouched (Liu et al., 2016; Larose et al., 2019). The second wave occurs upon specification of the germline to reflect the sex of the developing embryo (Liu et al., 2016; Larose et al., 2019). Both these waves of reprogramming occur through demethylation and methylation of cysteine residues in DNA. These reprogramming events which are well studied in the mouse and assumed true for all mammals will be described in further detail below, however, known differences between mouse and livestock species will be highlighted.

The first reprogramming wave in the embryo occurs right after pronuclear formation and is asymmetric between the maternal and paternal genome. On the paternal side, once the male pronucleus forms it undergoes rapid, active demethylation by TET3, an enzyme involved in the DNA demethylation pathway (Zhou and Dean, 2015; Larose et al., 2019). Imprinted loci and maternal genome are protected from TET3 demethylation by the DNA-binding protein STELLA (Zhou and Dean, 2015; Larose et al., 2019). In addition to active demethylation, the paternal genome is decondensed via the removal and replacement of protamines with maternal histones (Zhou and Dean, 2015). On the maternal side, demethylation is a slower, passive process. TET3 is not active, and it is through downregulation and inactivation of DNA methyltransferase 1 (DNMT1), an enzyme that is responsible for maintenance of methylated sites during DNA replication, that methylation marks are lost as the embryo replicates and divides through the cleavage stage of development (Larose et al., 2019). This widespread reprogramming after fertilization is essential for totipotency of the early embryo. Interestingly, evidence shows that sheep undergo no demethylation at any point during the first cell cycle (Young and Beaujean, 2004).

Along with DNA demethylation during cleavage stage embryogenesis, histone modifications are also involved in the changes seen to the epigenome during early development. In the male, the histones that replace the protamines in paternal DNA are acetylated, which produces a more ‘open’ conformation of chromatin. Maternal DNA, by contrast, maintains histone methylation patterns through early cleavage stages, which produces a more ‘closed’ conformation of chromatin (Zhou and Dean, 2015).

The second reprogramming event occurs during specification of the germline to reflect the sex of the embryo. This occurs later in development, around day 27 in cattle as primordial

germ cells migrate from the hindgut to the gonadal ridge (Wrobel and Süß, 1998). DNA methylation will reach its lowest point, with TET1 and TET2 working to actively remove methyl groups as well as the down regulation of DNMT1 and reorganization of repressive histone marks during this period (Hanna et al., 2018; Larose et al., 2019). This includes erasure of imprinted genes as well (Hanna et al., 2018; Larose et al., 2019). These epigenetic marks are then re-established in a sex-dependent manner to reflect the sex of the embryo during subsequent spermatogenesis or oogenesis (Liu et al., 2016; Hanna et al., 2018; Larose et al., 2019). In males this re-establishment of methylation marks occurs before birth, and in females it occurs after birth (Hanna et al., 2018). DNMT3A and DNMT3B have been identified as essential factors in this process of de novo methylation at imprinted loci in the germline (Larose et al., 2019).

Embryonic Genome Activation

Embryonic genome activation (EGA) is one part of the maternal to embryonic transition that occurs in early embryo development. The other part is the gradual depletion of maternal RNAs and proteins stored in the oocyte through degradation and translation (Graf et al., 2014a). EGA is a gradual transition composed of a minor and major activation. In cattle, the minor activation will occur at the 4-cell stage and beforehand, with activated genes relating to functions in RNA processing, translation, and transport first detected (Graf et al., 2014b). This effectively prepares the embryo for subsequent major EGA when genes from a broader range of functions are activated. The major EGA will occur at the 8- to 16-cell stage in cattle (Graf et al., 2014a). At the 8-cell and 16-cell stage embryo, genes found to be expressed include those related to cell fate decisions, protein synthesis, RNA processing, regulation of glycolysis, ATP biosynthetic process, and negative regulation of transcription, which are all implicated in the maintenance of transcription and translation and the initiation of metabolic processes that occurs at EGA (Graf et

al., 2014b). This transition from maternal to embryonic genome is crucial for embryonic survival as it permits the embryo to respond to environmental stressors that may otherwise hinder development.

The exact mechanism for EGA is unknown, but it is characterized by changes in chromatin structure, transcriptional machinery, and the gradual degradation of maternal transcripts (Memili and First, 2000). Nuclear reprogramming, though, is one requirement of EGA. Through altering chromatin structure via DNA methylation and histone modification described above, accessibility to regulatory elements can be controlled (Graf et al., 2014a). Thus, the timing of EGA and the selection of genes to be first transcribed is a result of the balance between chromatin-based repressive mechanisms and the establishment of a more open state of chromatin permissive to transcription.

Formation of the Blastocyst

After compaction of the morula at about the 32-cell stage on day 4 after mating, the ovine embryo becomes a blastocyst on day 6 (Spencer et al., 2004). Morphological blastocyst formation, or cavitation, occurs concurrently with trophectoderm differentiation. Cavitation involves gene families including the E-cadherin-catenin cell adhesion family, tight junction gene family, the Na/K-ATPase gene family, and the aquaporin gene family. The current hypothesis is that cavitation is driven by an ion gradient established by Na/K-ATPase across the trophectoderm (TE), which promotes the movement of water through aquaporins across the epithelium to form the blastocoel in the extracellular space of the blastocyst (Watson and Barcroft, 2001). The formation of a tight junction permeability seal is the necessary first step to cavitation. The E-cadherin-B-catenin adherens junction established during compaction provides the foundation for tight junction formation (Watson and Barcroft, 2001). After compaction, zona

occludens 1 and the transmembrane occludin and claudins-1,3 physically associate to form mature tight junctions between cells (Eckert and Fleming, 2008). This tight junction permeability seal between the outer epithelium and inner cells regulates leakage of blastocoel fluid paracellularly during blastocoel formation.

The blastocoel fluid is comprised mostly of water and its production relies on the ion transport systems of the TE. The basis for this hypothesis comes from ionic analysis of murine blastocyst fluid, where studies showed that many ions, including Na^+, Cl^-, K^+, Ca^{2+}, and Mg^{2+}, were concentrated in the blastocoel fluid (Borland et al., 1977; Powers and Tupper, 1977; Van Winkle et al., 1988; Manejwala et al., 1989). In brief, transport of sodium likely occurs via apically located sodium channels and various sodium cotransporters (Na^+/H^+ exchanger, Na^+/glucose cotransporter) and completed by the action of the basolaterally located Na/K-ATPase (Watson and Barcroft, 2001). This ion gradient would result in the movement of water across the gradient and lead to gradual accumulation of fluid within the blastocoel cavity. Additionally, aquaporins have been shown to be expressed during preimplantation development in mammals including the bovine, and it is suggested that the transport of water during cavitation is mediated by these aquaporin transport mechanisms (Watson and Barcroft, 2001).

Transcriptional Control of ICM and TE Formation

The first cell-fate decision is the differentiation of the trophectoderm (TE) from the inner cell mass (ICM). Upon compaction of the mouse morula, outer cells of the embryo begin to express caudal-related homeobox 2 (*Cdx2)*, establishing TE commitment, while the inner cells of the embryo express octamer-4 (*Oct4*) to maintain a state of pluripotency in the future ICM (Cockburn and Rossant, 2010). This pattern of expression, beginning with upregulation of *Cdx2*, is reinforced by reciprocal repression of *Cdx2* by *Oct4* in the ICM and *Oct4* by *Cdx2* in the TE

(Cockburn and Rossant, 2010). *Cdx2* null mouse embryos do make it to the blastocyst stage, but failure to maintain the TE results in implantation failure (Strumpf et al., 2005). Additionally, without *Cdx2*, *Oct4* and *Nanog* remain active in the outer cells of the blastocyst (Cockburn and Rossant, 2010). Murine embryos lacking *Oct4* are unable to form a proper epiblast, a cell type derived from the ICM (Le Bin et al., 2014).

In the bovid, contrary to what is seen in the mouse, the reciprocal repression between *OCT4* and *CDX2* is absent (Berg et al., 2011; Simmet et al., 2018). In fact, *OCT4* is expressed by both ICM and TE until about day 11 in the cow (Berg et al., 2011). In *OCT4* knockout bovine embryos development to the blastocyst stage was decreased, though total cell numbers and allocation of cells to ICM or TE were unchanged compared to controls (Simmet et al., 2018). Ablation of *CDX2* in bovine embryos does not prevent formation of the blastocyst (Berg et al., 2011; Goissis and Cibelli, 2014), but does result in loss of epithelial integrity (Goissis and Cibelli, 2014) and failure to elongate (Berg et al., 2011) indicating a role in downstream blastocyst development.

In the mouse, working in conjunction with *Cdx2* and *Oct4* is TEA domain transcription factor 4 (*Tead4*). *Tead4* itself is not restricted to just outer cells of the embryo, and it is Yap1 localization in the outer cells of the embryo that provides localized activation of *Tead4*. In inner cells of the embryo, Yap1 (Yes associated protein 1) is phosphorylated by Lats, a hippo signaling pathway component, and is excluded from the nucleus. Consequently, *Tead4* remains inactive and inner cells adopt an ICM fate (Nishioka et al., 2009). Conversely, in outside cells the lower level of Yap1 phosphorylation allows for nuclear accumulation of Yap1 and activation of *Tead4*. Activated *Tead4* then induces trophoblast genes including *Cdx2* (Nishioka et al., 2009; Cockburn and Rossant, 2010). *Tead4* null embryos do not express *Cdx2* and fail to implant (Yagi et al.,

2007). In the bovine, *TEAD4* appears to have TE-specific expression, though its exact role in the bovine blastocyst remain unclear due to mixed findings from *TEAD4* knockdown studies (Sakurai et al., 2017; Akizawa et al., 2018).

Transcriptional Control of Epiblast and Hypoblast Differentiation

The second fate specification step in embryo development is the ICM segregating into the epiblast and the primitive endoderm (PE). The PE forms a monolayer of cells between the epiblast and the blastocoel, and the epiblast remains a mass of cells between the PE and the TE (Cockburn and Rossant, 2010). The formation of either epiblast or PE is directed by the interaction of fibroblast growth factor 4 (*FGF4*) and its receptor (*FGFr2*), activation of the MAPK pathway, and transcription factors Gata6 and Nanog (Lanner and Rossant, 2010).

The current model of ICM lineage specification has been well studied in the mouse and involves a complex gene regulatory network of *Nanog-Gata6* and FGF signaling pathways. Activation of the FGF4 signaling pathway is necessary for the generation of PE, and the inhibition of the FGF4 pathway is essential for development of the epiblast (Lanner and Rossant, 2010; Gasperowicz and Natale, 2011). The expression of *Gata6* or *Nanog* is regulated by FGF4 interaction with FGFr2 (Lanner and Rossant, 2010). In PE precursor cells, binding of FGF4 to FGFr2 activates Grb2 (growth factor receptor-bound protein 2) and subsequently the MAPK pathway, which leads to *Gata6* expression and downregulation of *Nanog,* a factor that maintains pluripotency. In epiblast precursor cells, FGFr2 is absent, the MAPK pathway is not activated, and *Nanog* is transcribed, thus maintaining pluripotency (Lanner and Rossant, 2010; Morris et al., 2010). *Gata6* null embryos initially form PE but fail to form visceral endoderm (Morrisey et al., 1998; Koutsourakis et al., 1999). *Nanog* inhibited embryos do not produce epiblast cells, and additionally lack development of the PE (Mitsui et al., 2003; Cockburn and Rossant, 2010).

In the bovine, *NANOG* and *GATA6* are genetic markers of epiblast and PE respectively, uniquely expressed in each cell type (Guo et al., 2010; Kuijk et al., 2012), but the mechanism of activation for both factors differs slightly from what is described above in the mouse. Inhibition of MAPK activation by a MEK1/2 inhibitor demonstrated the necessity for MAPK activation for *GATA6* transcription in successful PE differentiation in mouse embryos (Yamanaka et al., 2010), however, in the bovine MAPK pathway inhibition does not completely block PE differentiation though still results in more *NANOG* positive cells than GATA6 positive cells (Kuijk et al., 2012; Brinkhof et al., 2015). In addition, FGF4 treated embryos formed ICMs composed completely of hypoblast cells, with no effect on total cell numbers in the ICM (Kuijk et al., 2012). This suggests that *GATA6* expression in the bovine is not completely regulated by FGF signaling. It is likely that the FGF4 signaling is involved in the regulation of epiblast and hypoblast development in the bovine as was described above in the mouse, however, there is potential for an unknown pathway that also regulates *GATA6* and *NANOG* transcription.

Embryo Hatching

The first observation of the embryo hatching or exiting the confines of the zona pellucida was described by Lewis and Gregory observing rabbit embryos (1929). The zona pellucida has multiple functions in early development, but embryo hatching from the zona pellucida is a critical prerequisite for implantation, increasing embryo-maternal communication, and pregnancy recognition and establishment (Seshagiri et al., 2016). Timing of hatching is species dependent with hatching in the mouse occurring at day 4 to 4.5 (Cole, 1967; Sawada et al., 1990), hamster at day 3 to 4 (Gonzales and Bavister, 1995), rabbit at day 4 to 5 (Lewis and Gregory, 1929), human at day 5.5 to 6.5 (Cohen et al., 1990), and bovine at day 8 to 10 (Berg and Menino, 1992; Negrón-Pérez and Hansen, 2017).

The exact molecular network regulating hatching remains unclear and there is variation in importance of hatching mechanisms amongst species, however, three mechanisms have been implicated in embryo hatching: 1) mechanical or hydrostatic forces exerted on the zona pellucida by the increasingly expanding blastocyst thought to be controlled by ionic pumping systems within the embryo (Cole, 1967; Gonzales and Bavister, 1995), 2) release of proteolytic enzymes from the embryo to subsequently weaken the zona pellucida to allow for escape (Sawada et al., 1990; Berg and Menino, 1992; Seshagiri et al., 2016), and 3) the protrusions of trophectoderm cells, called TEPs, that penetrate the zona pellucida and are often found at the point of zona lysis (Gonzales and Bavister, 1995; Seshagiri et al., 2016). Though, the functional significance of these TEPs during hatching is unknown (Seshagiri et al., 2016).

Conceptus Development

After the blastocyst stage, the bovine embryo undergoes extensive morphological changes that result in an elongated, filamentous embryo with three germ layers present in the embryonic disc. This filamentous embryo can now be termed a "conceptus". The set of events that occur post-hatching to conceptus formation are the loss of Rauber's layer, formation of the embryonic disc, formation of the primitive streak, and establishment of three germ layers. Rauber's layer is defined as the set of cells that make up the polar trophectoderm or the TE that overlays the ICM/epiblast. The polar TE in eutherian mammals is either lost via apoptosis, such as in the cow, sheep, rabbit, and others, thus exposing the underlying epiblast to the uterine environment (Williams and Biggers, 1990; Guillomot et al., 2004), or it is maintained and proliferated such as in the mouse (Williams and Biggers, 1990). In addition, to maintain the permeability seal of the blastocyst, prior to the loss of Rauber's layer the outermost layer of epiblast cells form tight junctions with each other and the edge mural TE (Williams and Biggers,

1990). After loss of this layer, the epiblast and hypoblast can be referred to as the embryonic disc. The embryonic disc then elongates becoming more ovoid in shape and formation of the primitive streak begins. This is first observed around day 14-15 in cattle when a crescent shaped thickening of the embryonic disc becomes visible in the posterior end of the embryonic disc (Vejlsted et al., 2006). The primitive streak will progressively extend from the posterior to the anterior pole during gastrulation, and it is from the primitive streak that ingression of epiblast cells will occur to establish the three germ layers: endoderm, mesoderm, and ectoderm.

The ingression of epiblast cells through the primitive streak is the first epithelial-to-mesenchymal transitions (EMT) in the developing embryo and is critical for migration of cells during gastrulation. At the onset of gastrulation, prospective mesodermal and endodermal cells reside in the epiblast as epithelium and characterized by close contacts with neighboring cells through various junctions and cell adhesion molecules (i.e. gap junctions, tight junctions, cadherins; Thiery et al., 2009). The EMT process entails disassembly of these epithelial junctions, downregulation of cell adhesion molecules, and acquisition of migratory and invasive properties (Thiery et al., 2009). After EMT the mesendodermal cells will "fall" through the primitive streak in a movement termed ingression and migrate to their final positions. Prospective mesodermal cells will invade the space between the epiblast and the primitive endoderm, and prospective endodermal cells will move to replace primitive endoderm establishing an epiblast derived definitive endoderm (Solnica-krezel and Sepich, 2012). Mesoderm will give rise to cardiovascular, urogenital, and musculature systems, while the endoderm gives rise to the gut, respiratory system, and related organs (Oestrup et al., 2009). The population of cells that remain in the epiblast are termed the ectoderm and will eventually

differentiate into neural and surface ectoderm, later making up the epidermis and all neural tissues (Oestrup et al., 2009).

Much work has been done elucidating mechanisms of cell movement and the EMT during gastrulation in vertebrates. The major pathways involved include the Wnt/β-catenin pathway, Nodal (TGF-β family member), bone morphogenetic protein (BMP), and FGF signaling pathways (Heisenberg and Solnica-Krezel, 2008). Nodal, at high levels, is involved in specifying endoderm cell fate from mesendoderm precursors (Thiery et al., 2009). Within the endoderm lineage Nodal promotes expression of transcription factors including Mix-like proteins, Foxa2, Sox17, Eomesodermin, and Gata 4 – 6, which function to commit cells to an endoderm specification and direct future differentiation (Zorn and Wells, 2009). Nodal is involved in the formation of the other two germ layers during gastrulation as well. Low levels of Nodal and primarily FGFs are involved in fate induction of prospective mesoderm cells, and also involved in upregulation of *Brachyury*, the first marker of mesoderm formation (Thiery et al., 2009). In cattle though, contrary to what is seen in the mouse and other species, Brachyury expression appears prior to formation of the primitive streak, indicating mesoderm formation prior to gastrulation and ingression of mesendoderm cells through the primitive streak (Hue et al., 2001). Absence of nodal, coupled with the repression of mesoderm or endoderm fates through TGF-β signaling pathway inhibition by various molecules specifies ectoderm (Heisenberg and Solnica-Krezel, 2008; Reich and Weinstein, 2019).

Elongation

Cows, pigs, and other ungulates exhibit a prolonged period after hatching, prior to implantation where the embryo undergoes elongation (Blomberg et al., 2008; Negrón-Pérez and Hansen, 2017). In cattle, the elongation process is characterized by about a 1,000-fold increase

in conceptus size over about 10 days prior to maternal recognition of pregnancy, attachment, and implantation (Maddox-hyttel et al., 2003). Elongation is initiated between days 12 to 14, and after rapid growth of the TE, on day 19 the now filamentous conceptus can reach a length of 20 cm or more and extend through much of the two uterine horns. This drastically increases the area for maternal-embryo crosstalk, nutrient exchange, and area that can contact the endometrium (Blomberg et al., 2008; Brooks et al., 2014). These morphological changes involve a rapid increase in both length and weight of the TE, and in ruminants these changes are driven by cell proliferation rather than geometric changes in cell shape (Wang et al., 2009). Additionally, there is a strong correlation between conceptus length and amounts of interferon-tau (IFNT) produced, an important molecule for maternal recognition of pregnancy in cattle (Mann and Lamming, 2001). Consequently, improper elongation can lead to failure to establish pregnancy due to insufficient IFNT production.

As established in uterine gland knockout ewes, elongation is maternally driven and dependent upon the uterine histotroph, which contains growth factors, hormones, cytokines, and many other substances (Gray et al., 2001). Recent global transcript and protein profiling studies have described gene and protein expression in the endometrium, conceptus, and uterine flushes during the period of elongation to identify elongation markers and elucidate mechanisms driving this process. In general, early elongation of the conceptus is characterized by cell multiplication, growth, and remodeling as well as general metabolism and protein trafficking, as evidenced by a clear increase in cell numbers between and confirmed by molecular approaches as well (Hue et al., 2012). Specifically, the trophoblast Kunitz domain protein (TKDP) and pregnancy-associated glycoprotein (PAG) gene families have commonly been found to be the most upregulated gene families during elongation, however, their precise role in the elongation process is still unknown

(Ushizawa et al., 2004; Hue et al., 2007; Clemente et al., 2011). Cellular interactions, cell signaling, and cell adhesion become more predominant towards the later stage of elongation, likely prepping for subsequent implantation (Ushizawa et al., 2004; Hue et al., 2007; Hue et al., 2012).

It is known that adequate levels of progesterone (P4) on the maternal side and adequate levels of IFNT on the conceptus side play a large role in the initiation and maintenance of elongation. IFNT is produced by mononuclear cells of the TE and it functions in signaling pregnancy recognition, driving the continued secretion of P4 from the corpus luteum (CL), stimulating endometrial functions necessary for growth and survival of the conceptus, and suppressing the immune rejection of the conceptus during implantation (Bazer et al., 1997; Ushizawa et al., 2004; Hue et al., 2012). IFNT performs these functions via inhibiting the transcription of the oxytocin receptor in the endometrium, thus preventing the pulsatile release of prostaglandin-F2α and sustaining the life of the CL and P4 production and through stimulating ISGs, a group of interferon stimulated genes that regulate uterine receptivity, conceptus elongation, and implantation (Dorniak et al., 2013). Adequate levels of P4 are necessary for proper elongation, as evidenced by enhanced conceptus elongation *in vivo* when P4 is supplemented to cattle (Garrett et al., 1988; Carter et al., 2008) and increased conceptus length on day 14 when embryos are transferred to a primed P4 environment on day 7 (Clemente et al., 2009). This is an indirect effect with P4 induced changes in the endometrial environment as the driver of enhanced elongation, specifically the modification of endometrial gene expression resulting in modification of the uterine fluid composition to which the embryo is exposed (Clemente et al., 2009). In summary, both IFNT and P4 work to regulate expression of genes related to elongation and implantation in the endometrium that govern conceptus survival,

elongation, and implantation through effects on TE proliferation, migration, attachment, and adhesion (Dorniak et al., 2013; Lonergan et al., 2016).

Attachment

Elongation can be considered the first step of the attachment and implantation process where embryo-maternal communication via IFNT and P4 establish uterine and embryo receptivity and promote the necessary growth of the conceptus. After elongation, the initial attachment or apposition occurs (Wooding and Wathes, 1980; Guillomot, 1995). This requires proper uterine receptivity and attainment of attachment competency in the embryo. Embryo attachment competency includes hatching from the zona pellucida and expression of various adhesion-promoting proteins on the surface of the TE, and uterine receptivity has been characterized by changes in gene expression via IFNT and P4 as described above, as well as loss of MUC1 (Constantinou et al., 2015). MUC1 is a transmembrane mucin that's been identified in the uterine epithelia of multiple species, including the cow, that blocks cell to cell adhesion (Spencer and Bazer, 2004; Constantinou et al., 2015).

Initial contact or apposition is characterized by loose cell contact between the developing TE and endometrial epithelium beginning in the embryonic zone and spreading out towards the ends of the conceptus. As described in sheep, small villi develop over the TE and begin to interdigitate with microvilli over the luminal epithelium (LE) and the openings of the uterine glands (Guillomot, 1995). These microvilli are short lived, disappearing around day 20, and are thought to anchor the periattchment conceptus and absorb uterine histotroph (Spencer and Hansen, 2015). Similar events are described in the cow (Wooding and Wathes, 1980). This TE and LE microvilli interdigitation provides the initial unstable adhesion between the conceptus

and the endometrium prior to more stable cell adhesion brought about in the next stage of implantation.

Key Events from Implantation and Beyond

Trophectoderm Differentiation

The earliest formation of the trophectoderm (TE) is an epithelial layer in the early blastocyst that surrounds the inner cell mass (ICM). The two main cell types the TE will differentiate into are mononucleate cells (MNC) and binucleate cells (BNC). The major morphological difference between these two cell types is the number of nuclei present and that BNCs contain a large number of granules, but both cell types function uniquely in conceptus nutrition, survival, and placentation. BNC formation and function will be discussed in further detail in the next section, but MNCs will be further discussed below.

Mononucleate cells form the majority of the maternal-fetal interface constituting around 80% of the TE population, and are involved in nutrient exchange (Wooding and Wathes, 1980; Igwebuike, 2006). These cells exhibit the typical features of epithelial cells. They are mononuclear cuboidal to columnar cells located on a basement membrane and connected by tight junctions and desmosomes (Dent, 1973). On their apical surface there are villi processes that extend out and interdigitate with villi from the maternal endometrial epithelium. This apposition remains in intercaruncular areas throughout gestation, but in the caruncular areas placentomes will develop through extensive proliferation into elaborate villous structures (Assis Neto et al., 2010).

Mononucleate cells in both placentomal and interplacentomal areas function in the transport of many maternal derived nutrients, ions, and other molecules. Hemotrophic exchange occurs in placentomal areas and histotrophic transfer occurs in interplacentomal areas

(Igwebuike, 2006). Histotroph producing uterine glands are located only in interplacentomal areas and will continue to function throughout pregnancy even after placental formation (Spencer and Bazer, 2004). Over top of the opening of each uterine gland, MNCs will form placental areolae and act as specialized areas for absorption and transport of histotroph to the fetus (Schlafer et al., 2000; Spencer and Bazer, 2004). In placentomal areas, there are several glucose and amino acid transport proteins localized to the placentome throughout pregnancy, with stage of pregnancy, nutritional status, and other regulatory molecules impacting the abundance of these transporters and thus overall transport capacity of the placentome (Batistel et al., 2017). Similar to what is seen in the areolae (Schlafer et al., 2000), there is a second population of phagocytic MNCs in the 'arcade zone,' which is located towards the fetal side of the placentomes. These particular cells are involved in phagocytosis of macromolecules (Schlafer et al., 2000).

Binucleate Cell Formation and Function

Binucleate cells were first described by Assheton in the sheep placenta and later extensively described by Wooding in both sheep and cattle (Assheton, 1906; Wooding and Wathes, 1980; Wooding, 1984; Wooding, 1992). These cells are unique to ruminants and are one component that designates the ruminant placenta as synepitheliochorial. They are not uniformly distributed, tending to occur in small clusters in both placentomal and interplacentomal areas (Igwebuike, 2006). They are multinucleated cells that arise from MNCs through acytokinetic mitosis (Wooding, 1984; Attiger et al., 2018). Recent gene expression analysis has revealed that BNC differentiation regulates genes that enable TE cells to interact with their environment (Polei et al., 2020). Specifically, molecular functions and biological processes related to extracellular matrix and its interactions with cellular receptors and cell migration are differentially regulated between BNCs and MNCs (Polei et al., 2020). This makes sense as BNCs have a unique

migratory function. In their early stages, BNCs are located deep within the TE, however as these cells mature they begin to migrate towards and fuse with maternal epithelial cells forming trinucleate cells of both fetal and maternal origin (Igwebuike, 2006). Also, as they mature large membrane bound granules develop, eventually occupying more than 50% of the cell volume (Wooding, 1992).

The migration of BNCs, fusion to form a trinucleate cell and then exocytosis of granular contents into the maternal stroma is key to performing BNC's endocrine function. Upon fusion with maternal epithelial cells the granules will be released into the maternal stroma, delivering contents close to maternal blood circulation (Wooding and Wathes, 1980; Wooding, 1992). Not much is known about the fusion of BNCs with uterine epithelial cells, but it has been found to be promoted by syncytins. These are genes of retroviral origin that are expressed in the placenta and function to promote cell fusion (Carter, 2019). *Syncytin-Rum1* was identified in ruminants and facilitates fusion of BNCs with maternal epithelium (Cornelis et al., 2013). A second syncytin gene *Fematrin-1* is expressed by BNCs in cattle and induces fusion between BNCs and endometrial cells *in vitro* at a higher level than *Syncytin-Rum1* (Nakaya et al., 2013). Both these syncytin genes and likely others work together to induce fusion in the formation of trinucleate feto-maternal cells. After the release of fetal derived molecules, the transient trinucleate cells are reabsorbed by the TE (Wooding and Wathes, 1980; Wooding, 1992).

Placental hormones produced and released by BNCs include PAGs, P4 and estrogens, placental lactogens, prolactin-like proteins, and placental growth hormone (Igwebuike, 2006; Carter, 2019). All PAG genes are originally duplications of the Pepsin-F gene, which encodes for an aspartic proteinase, though there are two groups of PAGs classified based on their evolutionary upbringing. The group of PAGs that likely retain the active site of the proteinase

and are more evolutionarily ancient are termed ancient PAGs, and the group that likely lack the active site and came about more recently are called modern PAGs (Telugu et al., 2009; Telugu et al., 2010). Interestingly, modern PAGs are mainly transcribed in the cotyledon and ancient PAGs mainly in the intercotyledonary areas (Touzard et al., 2013). The exact function of PAGs remains unclear and future studies are required to determine the role of PAGs at the feto-maternal interface. However, due to the fact that BNCs are continuously releasing their contents into the maternal stroma over most of gestation, their contents are able to accumulate in maternal circulation. These contents have been identified as a potential means of early pregnancy detection in ruminants, specifically PAG1 (Sasser et al., 1986; Zoli et al., 1992). Immunoreactivity assays have been developed, and while these tests can reliably detect pregnancy by the end of the first month of gestation, a crucial disadvantage is that PAG1 concentrations rise markedly at term, and due to the molecules long half-life, it can be detected for up to 80 to 100 days postpartum (Sasser et al., 1986; Zoli et al., 1992; Kiracofe et al., 1993; Green et al., 2005). Interestingly, this issue has not been noted in sheep though, where levels of PAG1 immediately decline after pregnancy (Ranilla et al., 1994). Even with this potential for false-positives early in the postpartum period for cattle, the accuracy of these PAG tests for detecting bovine PAGs in maternal blood and milk range from 93 to 96% (Silva et al., 2007; Pohler et al., 2013).

Though the level of steroid contribution varies among species, in ruminants BNCs have been found to produce both P4 and estrogens. In cattle, throughout most of gestation the placenta only contributes a small portion of P4 to maternal circulation, however, in late gestation placental P4 is for the most part able to maintain pregnancy and it is thought that this increase in placental P4 may be important for local interactions at the feto-maternal interface that may be

dose-dependent (Estergreen et al., 1967; Hansen, 1998). In addition to P4 synthesis, the ruminant placenta produces significant amounts of estrogens, estrone being the major estrogen produced (Hoffmann et al., 1997). The role of placental estrogens is predominantly in preparing the female reproductive tract for parturition and mammary gland development in late gestation, however, in the cow there is growing evidence for a potential role in placental differentiation and function (Eley et al., 1979). Up-regulation of estrogen receptor 2 (ESR2) and steroidogenic enzymes in trophoblast cells during BNC differentiation, and the high expression of steroid sulfatase in the maternal caruncle, which acts to produce local bioactive estrogens in the placentome, indicate potential roles in placental differentiation (Schuler et al., 2005; Schuler et al., 2008; Polei et al., 2014).

Lastly, placental lactogens (PL), which have evolved due to gene duplication of the *growth hormone* (GH) or *prolactin* (PRL) gene in different orders of mammals, work to modify maternal physiology in ways to maximize growth of the fetus. In ruminants, *GH* genes do not play a large role. In fact, in cattle, there is a single *GH* gene and it is not expressed in the placenta (Ishiwata et al., 2003). However, duplication of the *GH* gene did occur in the sheep and goat (Wallis et al., 1998). Placentally expressed ovine GH is required alongside IFNT and PL for uterine gland hyperplasia and histotrophic secretion (Noel et al., 2003). Both bovine and ovine PL are derived from PRL gene duplications. PL production is restricted to BNCs, and after release into the maternal compartment PL is thought to work in a paracrine manner to stimulate secretion of uterine histotroph, as PL is barely detectable in maternal plasma (Byatt et al., 1987; Noel et al., 2003). It is suggested that PLs also stimulate fetal growth by promoting repartitioning of maternal nutrients to the fetus and also by stimulating the fetus to increase uptake and utilize

substrates for growth (Spencer et al., 1999; Noel et al., 2003; Song et al., 2006). These effects on fetal growth appear to be mediated by IGF-II in the cow and sheep (Byatt et al., 1992).

Implantation

Firm adherence of the trophoblast to the LE of the endometrium is characterized by the formation of syncytial plaques, arrest of IFNT expression from the mononuclear cells of the TE, and establishment of more permanent cell adhesion via several adhesion molecules and their respective receptors (Guillomot, 1995; Spencer et al., 2004). During implantation BNCs will migrate through the TE and then fuse with the endometrial LE, forming syncytia of trinucleate cells replacing the endometrial LE (Wooding, 1984). Through continued migration and fusion larger and larger syncytial plaques will form and later aid in formation of the placentome. In the cow, the synctyial plaques that form during implantation are later replaced by regrowth of the uterine epithelium by day 40, and any further BNC migration and fusion throughout the remainder of gestation produces only transient trinucleate cells (Peter, 2013). The establishment of more permanent cell adhesion occurs through multiple adhesion molecules that are secreted by the glandular epithelium of the endometrium and interact with receptors on the apical surfaces of the TE and the LE (Spencer et al., 2004). These molecules and receptors vary by species and are continuing to be elucidated in many species, but in the sheep evidence indicates that adhesion molecules include *glycosylation-dependent cell adhesion molecule-1* (*GlyCAM1*), *Galectin-15*, and *osteopontin*, and their receptors found on both the TE and LE include L-selectin, glycoconjugates, and multiple integrin receptors respectively (Spencer et al., 2004). In the cow, *vascular cell adhesion molecule 1* (*VCAM1*), rather than *GlyCAM1*, and *VCAM1 receptor integrin alpha 4* (*ITGA4*), are both heavily upregulated days 20 and 22, and appear to play a large role in bovine implantation (Bai et al., 2014). The binding of adhesion molecules to

receptors on both the LE and the TE form the tight adhesion essential for implantation and subsequent placentation.

Placentation

The placenta is an organ for maternal-fetal exchange functioning to supply the growing fetus with an adequate nutrient supply. It also is a site of waste excretion from the fetus to the mother, acts as a barrier against pathogens and the maternal immune system, and anchors the fetus in the uterus. These functions are similar among all mammals, though placental structure between species varies greatly at both the macroscopic and microscopic level leading to multiple placental types.

There are multiple ways to categorize placental types including based on the extraembryonic tissue that contributes to the placenta (choriovitelline or chorioallantoic), based on the surface structure and maternal interactions (diffuse, cotyledonary, zonary, discoid), based on number of tissue layers separating fetal and maternal circulation (epitheliochorial, endotheliochorial, hemochorial), and based on after birth (deciduate, adeciduate). The ruminant placenta is categorized as chorioallantoic, cotyledonary, synepitheliochorial, and adeciduate on the basis of its macroscopic and microscopic features that are established at around day 40 to 50 of pregnancy (Igwebuike, 2006; Peter, 2013). This means the placenta will form cotyledons, or tufts of villous projections of the outer cell layer formed by fusion of the chorion and allantois into what is termed chorioallantois, opposite endometrial folds/crypts called caruncles (Wooding, 1992). Together the cotyledon and caruncle make up the placentome, and all together the placentomes make up the sites of hemotrophic exchange between fetus and mother as well as attachment points to maintain position of the placenta. The subsections below will further detail placentome development and vascularization of the placenta.

Placentome Development

After successful implantation in cattle the placenta is apposed via microvillus interdigitation of the TE and uterine luminal epithelium in both the caruncular and intercaruncular areas. This apposition remains in intercaruncular areas throughout the remainder of gestation, but in the caruncular areas placentomes will subsequently develop (Assis Neto et al., 2010). Placentome development entails the interdigitation of TE and endometrial villi, subsequent increase in number of fetal cotyledons opposite maternal caruncles, and then growth in size and weight of placentomes throughout gestation.

Cotyledon and caruncle formation both involve extensive proliferation of microvilli into final villous tree and crypt structures that interlock in a fully developed placentome. The cotyledon initially begins as an area of chorioallantoic membrane that proliferates into a villous form (Peter, 2013). These villi are lined with TE cells, while below this layer stromal tissue makes up the villous core and provides support to the fetal vasculature. This histological layering of tissues remains constant throughout gestation. Maturation of these villi involves the formation of elaborate, heavily vascularized villous trees composed of a villus stem, then secondary and tertiary branching (Estrella et al., 2017). This drastically increases the surface area for nutrient and gaseous transfer leading to a highly efficient site of exchange between the fetus and mother. On the maternal side, four rows of nodules that protrude into the uterine lumen and lack endometrial glands can be observed running the length of each horn in both pregnant and non-pregnant cows (Peter, 2013). These nodules will proliferate and form equally elaborate crypts that will interlock with chorionic villi to form the placentome (Atkinson et al., 1984).

Placentome number, size, weight, and shape is highly variable both within and between species. In the cow, the total number of placentomes present is variable with some finding a

range of 100 to 140 in number (Haeger et al., 2016) and others finding lower ranges of 40 to 90 in number (Laven and Peters, 2001), though all confirm that the number does not change significantly throughout gestation after day 70 and the distribution is skewed towards the pregnant horn. In the sheep, total placentome number has been found to range from 40 – 100 in number (Vatnick et al., 1991; Clarke et al., 1998; Gardner et al., 2002; Dwyer et al., 2005; Vonnahme et al., 2008) and increases with twins or triplets compared to singleton pregnancies (Dwyer et al., 2005; Vonnahme et al., 2008). Overall, total placentome weight and size will significantly increase between days 60 and 190 of gestation but level off after day 190, though in the sheep placentome growth ceases around day 70 (Doizé et al., 1997; Laven and Peters, 2001). In the cow, two shapes have been identified: normal convex, mushroom-like shape and flat (Laven and Peters, 2001). In the sheep, inverted or concave (Type A), flat (Types B and C), and everted (Type D) placentome types are seen (Vatnick et al., 1991). Interestingly, the distribution of placentome type does not change significantly over gestation in the cow (Laven and Peters, 2001), but in the sheep placentome shape can vary throughout gestation (Vatnick et al., 1991), potentially acting as an adaptive mechanism to deal with increasing fetal demands and varying dam nutrition. More recently it has been found that in maternal undernutrition dams increase the types C and D placentomes in the uterus, leading to the idea that these types may be more functional and efficient (Vonnahme et al., 2006). Potentially, understanding the development to the most efficient placentomes can lead to efficient placental programming schemes that can maximize *in utero* fetal growth.

Factors regulating placentome growth and formation though remain unknown, however there is evidence that some may be present. When assessing the proportion of fetal and maternal tissue within a placentome over the course of gestation, the growth of the two tissues is different

in character. Thus, it can be assumed to be differentially regulated by tissue, and additionally throughout gestation as the rate of change in the proportion was not consistent through gestation (Laven and Peters, 2001). Interestingly, in sheep a non-terminal procedure that removes a placentome has been developed as a means to obtain placental tissue for genetic and histological analysis during pregnancy without compromising fetal and placental development (Lambo et al., 2020). The validation and optimization of this procedure will drastically advance our understanding of factors necessary for early placental development, how the placenta and fetus can respond to insults over the course of gestation and link any differences to performance postnatally.

Placental Vascularization

Vascularization of the placenta occurs concurrent to placentome development and is especially important in the last half of gestation as nutrient demand increases due to increasing fetal growth. The development of blood vessels occurs through two processes: angiogenesis and vasculogenesis, and placental vascularization involves a coordinated process of the two. Angiogenesis is defined as the growth of new blood vessels from existing blood vessels, whereas vasculogenesis is the formation of blood vessels from differentiating endothelial cells. This section will overview the general structure of the vasculature within placentomes as well as the molecular networks and factors that are known to aid in the process.

Vascular growth begins in early pregnancy and continues throughout gestation in both maternal and fetal tissues (Borowicz et al., 2007). As an overview of the final vascular structure seen in placentomes, vessels in the core or primary villi/crypt structures are stem arteries and veins, that subsequently branches into arterioles and venules of the secondary structure. These secondary structures supply blood to or from the tertiary structures, which are extensive

networks of capillary beds found in the tertiary branching of the villi or crypts (Pfarrer et al., 2001). As the villi and crypt structure progressively branch throughout gestation so too does the branching of the fetal and maternal vessels occupying them. With this in mind, when observing the vascular structure during early months of gestation extensive capillary beds and tertiary branching is absent, though exchange of nutrients and gases is not compromised. During this time, the stem and intermediate vessels of the caruncle and cotyledon meet in a countercurrent fashion throughout the primary and secondary structure and provide efficient exchange in the absence of capillary beds (Pfarrer et al., 2001). This countercurrent exchange mechanism remains intact but becomes less important as tertiary villi, crypts, and capillary beds form and take over the majority of exchange (Pfarrer et al., 2001).

The regulatory networks that are responsible for the development of the vascular network within villi and crypt structures are not completely elucidated, however FGFs, specifically FGF2 and vascular endothelial growth factor (VEGF), have been shown to be two major angiogenic factors in the processes of neovascularization and vascular permeability in the placenta (Reynolds and Redmer, 2001). The actions of FGF2 are mediated through its receptors FGFR1-4, which are tyrosine kinase receptors that after dimerization can activate ERK and PI3K signaling cascades (Campos et al., 2010). VEGF is a well-known regulator of migration and proliferation of endothelial cells and vascular permeability, and the actions of VEGF are mediated through two receptors: VEGFR1 (or Flt1), and VEGFR2 (Pfarrer et al., 2006; Campos et al., 2010). Both these systems have been identified in bovine and ovine placental tissue.

Placental Blood Flow

Blood flow and vascular development within the placenta are essential for its function and for fetal growth. In fact, in many models of compromised pregnancy fetal growth or

placental growth are impaired and blood flow to the reproductive tract and fetus is reduced (Hill et al., 2000; Wallace et al., 2002; Konje et al., 2003; Redmer et al., 2004; Miglino et al., 2007; Grazul-bilska et al., 2013; Camacho et al., 2018). Uterine blood flow represents the circulation to the maternal portion of the placenta with the uterine artery branching into the stem arteries of the caruncular crypts and umbilical blood flow represent the circulation to the fetal portion of the placenta with the umbilical artery branching into the stem arteries of the cotyledonary villi (Borowicz et al., 2007). Across species, blood flow will increase throughout gestation, as evidenced by decreased resistance indices, increased vessel diameter, and increased velocity measurements when assessing uterine artery hemodynamics (Konje et al., 2001; Bollwein et al., 2002; Panarace et al., 2006; Beltrame et al., 2017). This increase in blood flow is important for the increase in placental uptake that meets the increasing metabolic demands of the fetus during late gestation. Placental uptake can be defined as: *Uptake = blood flow X [A-V],* where [A-V] represents the arteriovenous concentration difference of a molecule (Reynolds and Redmer, 1995). This, however, does not downplay the importance of increased transporter abundance that occurs over gestation as well.

Angiogenesis is responsible for a large portion of the increase in blood flow over the course of gestation. The current model proposed for vascular growth and remodeling in placentomes by Borowicz and colleagues (2007) shows two different patterns in the caruncle and cotyledonary tissue. Caruncular capillary beds primarily grow by an increase in capillary size with only a small increase in capillary number, and thus produces a slower moving system that is more designed for delivery of nutrients (Borowicz et al., 2007). On the fetal side, cotyledonary capillary beds grow primarily by branching. This results in a large increase in capillary density paired with a decrease in capillary size, and thus creates a large surface area for exchange and

faster moving velocities suitable for nutrient uptake and transport (Borowicz et al., 2007). The mechanisms by which this angiogenesis occurs are described in the above section on vascularization.

Estrogen as well as nitric oxide (NO) can be seen as important regulators of blood flow to the uterus in addition to VEGF and FGF2. Various estrogens are known to cause a dilation of the uterine vasculature with estradiol-17β promoting the strongest response (Resnik et al., 1974; Johnson et al., 2006). The angiogenic effects appear to be mediated by VEGF and FGF2 primarily (Johnson et al., 2006). In addition to the angiogenic response mediated by VEGF and FGF2, both molecules are also thought to stimulate NO production by increasing endothelial nitric oxide synthase (NOS3) expression, and it is then NO that mediates changes in vasodilation (Johnson et al., 2006). NO is produced by NOS3 is endothelial cells and results in the dilation of blood vessels through activation of the enzyme soluble guanylate cyclase, which catalyzes the conversion of GTP to cGMP (Condorelli and George, 2002).

There is a close relationship between fetal weight, final placental size, and uterine and umbilical blood flow, thus the formation of functional fetal and maternal vascular systems early in gestation is of utmost importance for fetal growth and thereby postnatal survival and growth (Borowicz et al., 2007). In compromised pregnancies uteroplacental blood flow is often altered, and this can be seen at the molecular level as well with altered VEGF, FGF2, and NO levels or altered steroid production and metabolism with potential impacts on blood flow down the line (Vonnahme et al., 2007; Collier et al., 2009; Grazul-bilska et al., 2014). Thus, if therapeutic targets can be identified we can utilize them in an effort to intervene early to improve blood flow during gestation and overall growth and survival.

Fetal Skeletal Muscle Development

Skeletal muscle development involves myogenesis, adipogenesis, and fibrogenesis. Myogenesis is the formation of muscle tissue that occurs during fetal development. A portion of somite cells commit to the myogenic lineage upon receiving signals such as Wingless and Int (Wnt) and Sonic hedgehog (SHH) that induce expression of paired box 3 (Pax3) and paired box 7 (Pax7; Kassar-Duchossoy et al., 2005), which then initiate expression of myogenic regulatory factors (MRF) including *myogenic factor-5* (*Myf5*), *myogenic differentiation 1* (*MyoD*), *myogenin*, and *MRF-4* (Relaix et al., 2005). These myoblasts undergo extensive proliferation and subsequent differentiation. Differentiation entails withdrawal from the cell cycle and expression of muscle-specific genes. The various MRFs cooperate to regulate fusion of myoblasts into multinucleated myotubes and maturation into myofibers (Keren et al., 2006; Kollias and McDermott, 2008). The fetal stage is crucial to skeletal muscle development as the full complement of muscle fibers is set at birth no increase in muscle fiber number occurs after birth.

Myogenesis occurs in two waves of development. Primary myofibers form during embryonic development, and it is this small number of primary muscle fibers that will serve as templates for secondary myofiber formation that occurs during mid-gestation (Du et al., 2010a). In cattle, primary muscle fibers form within the first 2 months of gestation, and the more abundant secondary fibers form in the second wave of development between 2 and 7 months of gestation (Russell and Oteruelo, 1981). In sheep, primary muscle fibers form shortly before 50 days of gestation, and secondary fibers form shortly after and increase in number and size throughout gestation (Ashmore et al., 1972). It is the secondary muscle fibers that form the majority of muscle fibers.

Ensuring adequate nutrients during this period of myoblast proliferation and myofiber formation is critical for skeletal muscle development. Evidence from porcine and ovine studies show that maternal nutrition impacts fetal skeletal muscle development, specifically, a decreased number of secondary myofibers that permanently reduces muscle mass and negatively impacts animal performance when a nutrient restriction to 50% of NRC requirements is imposed upon the dam (Dwyer et al., 1994; Zhu et al., 2004; Quigley et al., 2005). After day 105 of gestation in sheep, nutrient restriction does not have a major impact on myofiber number, rather restriction during this period reduces myofiber size (Greenwood et al., 1999). Additionally, when comparing fetal muscle growth in single and twin bearing ewes, competition for nutrient between littermates during late gestation was found to affect fetal skeletal muscle mass, though only affecting muscle fiber size and not number (McCoard et al., 2000). Though, in recognizing the detrimental effects of nutrient restriction, providing nutrient supplementation during this vulnerable period can be done in an effort to rescue normal development.

Adipogenesis

Intramuscular fat, or marbling, is a crucial component in the palatability of meat because marbling contributes to both the flavor and juiciness of meat. The formation of intramuscular fat cells, or adipocytes, is called adipogenesis (Du et al., 2010b). The amount of intramuscular fat is determined by the number and size of intramuscular adipocytes (Du et al., 2010b). Adipocytes are also generated from mesenchymal stem cells. The transcription factors C/EBP (CCAAT/enhancer binding protein) and PPARγ (peroxisome proliferator-activated receptor γ) regulate the process of adipogenesis. Absence of Wnt and SHH signaling as well as the presence of BMP-2 and -4 promote expression of C/EBPα (Shang et al., 2007; Fontaine et al., 2008; Tseng et al., 2008). C/EBPα will bind directly to the promoter of PPARγ and induce its

expression, which positively feedback to reinforce the expression of C/EBPα (Clarke et al., 1997). In sheep, the process of adipogenesis begins in mid-gestation (Gnanalingham et al., 2005)

Post-natal Muscle Growth

After birth muscle fiber number remains constant and growth is due to an increase in muscle fiber size, or hypertrophic growth. Protein deposition (hypertrophy and growth in fiber length) in muscles is the difference between the rates of protein synthesis and protein degradation. Muscle fiber hypertrophy is regulated by a complex system of factors. Nutrition, amino acid availability, growth factor levels such as insulin, IGF-1, and myostatin, and others influence skeletal muscle hypertrophy working most commonly through the mechanistic target of rapamycin (mTOR) and TGF-β signaling pathways (Vandenburgh et al., 1991; McPherron et al., 1997; Bodine et al., 2001; Long et al., 2005).

Satellite cells are a population of myogenic and progenitor cells found in skeletal muscle that are located beneath the basement membrane and outside the cell membrane of muscle fibers (Mauro, 1961; Allen et al., 1979). As myofiber nuclei are mitotically inactive, these cells are responsible for providing nuclei necessary for hypertrophy of myofibers in post-natal muscle growth (Moss and Leblond, 1971). Satellite cells have the ability to differentiate and fuse with nearby muscle fibers as well as to self-renew in order to achieve this function (Kuang et al., 2007). Essentially, increased DNA content reflects an increased translational capacity of muscle to synthesize and maintain muscle protein for protein synthesis and muscle hypertrophy (Allen et al., 1979).

Improving Placental and Fetal Development

Placental and Fetal Programming

A classic example of fetal programming, and one that led to the development of this programming concept, came from observations made after the 1944 Dutch famine. This was a period of 5-6 months of extreme food shortage in the Netherlands that occurred during World War II. Follow up of people whose mothers were pregnant around this time has revealed that *in utero* exposure to maternal starvation has lasting effects on health through to adult life, with effects seen dependent on the time of gestation during which the exposure occurred (Ravelli et al., 1998; Ravelli et al., 1999; Lopuhaä et al., 2000; Roseboom et al., 2000b; Roseboom et al., 2000a). This concept was termed 'developmental origins of health and disease' or 'developmental programming.'

In mammals, the major determinant of fetal growth is placental nutrient supply. Livestock bred for meat production spend 35 - 40% of their life within the uterus being nourished by the placenta. Thus, because profitability of meat production depends on the efficiency of growth and development after birth, it is important to understand the impact of placental development as it directly affects fetal and postnatal health, growth, and development. Therefore, the goal of fetal programming when it pertains to livestock production is to develop interventions to augment or rescue placental function and enhance blood flow and nutrient delivery at critical periods during fetal development, priming offspring for optimum performance (Du et al., 2010a; Reynolds and Caton, 2012).

Intrauterine Growth Restriction in Ruminants

Intrauterine growth restriction (IUGR) is another type of fetal programming prevalent in a number of species. In ruminants most cases of IUGR are derived from maternal undernutrition, excessive temperatures causing heat stress, twinning in typically monotocous species, or inadequate maternal-fetal circulation. Often these causes are linked or overlapping and contain the common theme of placental insufficiency. These incidences of IUGR provide great models to study the mechanisms behind the process and develop therapeutic interventions (Galan et al., 1999; Luther et al., 2007a; Vonnahme et al., 2008; Long et al., 2009; Redmer et al., 2012).

Nutrient restriction will commonly lead to IUGR and is one of the most commonly studied models of IUGR and placental insufficiency. Undernutrition during pregnancy will commonly result in low birth weight, impaired placental function, asynchronous organ development, and altered growth rates and carcass quality in postnatal life (Long et al., 2009). These characteristics are replicated in controlled nutrient restriction studies in both cows and sheep where animals are typically restricted fed to 50% of NRC requirements (Vonnahme et al., 2003; Ford et al., 2007; Long et al., 2012). Additionally, the dam will progressively deplete maternal body reserves in an effort to sustain herself and the pregnancy and see increased vascularity in both caruncular and cotyledonary tissue (Zhu et al., 2007a; Zhu et al., 2007b; Ma et al., 2010). It is placental adaptations during restriction that may lead to negative consequences later in life as well, such as predisposition to obesity and altered growth rates, type II diabetes, decreased progesterone levels and fertility, and impaired testicular development in male offspring (Bielli et al., 2002; Ford et al., 2007; Long et al., 2009; Long et al., 2013). Molecularly MAPK and PI3K/Akt signaling, which play a key role in angiogenesis via cell proliferation, protein synthesis, cell survival, and cell migration, are increased in nutrient restricted

cotyledonary tissue, as well as higher *VEGF* expression and nutrient transporter production in cotyledonary tissues (Zhu et al., 2007b; Zhu et al., 2007a; Ma et al., 2010). This increase in growth signaling in endothelial cells of cotyledonary tissue is consistent with the increase in angiogenesis and vascularity that would be expected to enhance nutrient transfer from mother to fetus in the face of nutrient restriction.

Citrulline/Arginine Metabolism and the Impacts of Nitric Oxide

Citrulline is a non-protein amino acid that initially was only known as an intermediate in the urea cycle, however, research into citrulline has grown because of its specific metabolism and use as a precursor to arginine and nitric oxide (NO) synthesis. NO is a potent vasodilator and angiogenic factor that can increase blood flow (Condorelli and George, 2002). Additionally, arginine can be metabolized into polyamines. These molecules can be used for embryonic and fetal gene expression, protein synthesis, and cell proliferation (Wu et al., 2009; Lenis et al., 2017).

In circulation, citrulline, unlike arginine, will bypass intestinal and liver metabolism (Bahri et al., 2012). There are four main enzymes important for citrulline metabolism: ornithine carbamoyltransferase (OCT), nitric oxide synthase (NOS), arginosuccinate synthase (ASS), and arginosuccinate lyase (ASL) (Curis et al., 2005). OCT catalyzes the conversion of ornithine into citrulline primarily in the intestine and is the source of endogenous citrulline (Curis et al., 2005). If citrulline is not used in NO metabolism it is largely metabolized by the kidney, where it is converted into arginine by ASS and ASL then released back into circulation (Levillain et al., 1990). Outside of the kidney, citrulline is converted to arginine in nearly all cell types (Wu and Morris, 1998). The enzymes arginase and NOS will then compete for arginine. Arginase will work to convert arginine into polyamines for future protein synthesis and cell proliferation (Wu

et al., 2009), whereas NOS3, the NOS enzyme found in endothelial cells, will catalyze the conversion of arginine into citrulline and NO, with NO the primary product of this reaction (Curis et al., 2005).

Nitric oxide (NO) is an important regulator of blood flow and vessel development during pregnancy. NO is an intracellular messenger that is produced from arginine by NOS3 and results in vasodilation of blood vessels (Condorelli and George, 2002). The importance of NO is demonstrated by the fact that arginine free diets or inhibition of NO synthesis in rats results in increased fetal resorptions, IUGR, increased perinatal mortality, and decreased number of live fetuses (Greenberg et al., 1997), and that in compromised pregnancies, levels of NO as well as arginine are decreased (Casanello and Sobrevia, 2002; Kim et al., 2006). Thus, NO donors or substrates like arginine and citrulline have been targeted as potential therapeutics for IUGR, preeclampsia, and other ailments of the NO cycle.

One extensively studied example of a supplemented substrate for nitric oxide synthesis is arginine supplementation in swine. Oral supplementation schemes have been developed for arginine to improve reproductive capacity in pigs. Many have found that arginine supplementation can enhance placental growth, reduce embryonic mortality, and enhance overall reproductive performance in swine (Mateo et al., 2007; Gao et al., 2012; Li et al., 2014). Molecularly, it has been seen that arginine supplementation increases VEGF and NOS3 mRNA expression in placental surface vessels, thus indicating that NO may be responsible for these beneficial effects (X. Wu et al., 2012; Zeng et al., 2013). Additionally, these effects appear to be influenced by gestational period of supplementation (Li et al., 2010; Bass et al., 2017). Potentially this same scheme can be used in other livestock as well.

Citrulline, and N-carbamylglutamate (NCG) supplementation studies have been done in sheep and human pregnancies as well, with similar results to arginine supplementation in pigs. NCG is a metabolically stable analogue of N-acetylglutamate, which is an activator of the enzymes pyrroline-5-carboxylate synthase and carbamoylphosphate synthase-I within the endogenous citrulline synthesis pathway (Wu et al., 2004). Both intravenous and oral supplementation of arginine and NCG improves placental growth and development as well as ameliorate the effects of IUGR resulting in higher birth weights (van der Linden et al., 2015; Zhang et al., 2016a; Zhang et al., 2016b). In pregnant women supplementation of arginine and citrulline demonstrated enhanced maternal and fetal hemodynamics, prevention of pre-eclampsia, and improved birth weight (Weckman et al., 2019; Rylewski et al., 2006). Similarly, all these effects are thought to be mediated by the manipulation of the NO-system.

Summary and Implication

Embryonic and fetal development is becoming more recognized as important to survival, pre- and post-natal growth, and overall health. Various environmental stressors, such as IUGR, can have both short- and long-term impacts on the development and health of the individual. Stressors during embryonic and early fetal development can result in high embryonic loss, altered placentation, and fetal growth restriction. Through mid-gestation environmental stressors can impact growth and vascularization of the placenta as well as affect initial muscle and adipose tissue formation. Overall, developmental programming is a process that works to prepare the fetus for survival in the environment that it will be born. This means *in utero* stressors result in programming of the individual for these same stressors in post-natal life. However, when these stressors disappear in adult life, this programming can lead to disease and perturbed growth. The following work has focused on evaluating the effectiveness and benefit of maternal

supplementation schemes on programming offspring for optimal development even in times of maternal stress.

As we understand the mechanisms of fetal programming, we can begin to utilize them to benefit individual health and development. One method of doing this is through alteration of blood flow and angiogenesis via nitric oxide precursors. The benefit of nitric oxide precursors, such as arginine, N-carbamylglutamate, and citrulline, have been explored in monogastrics (pigs, rats), but knowledge is more limited in ruminants. This work will provide us with an assessment of L-citrulline benefit in ruminants. Additionally, the majority of work in this area has focused on examining outcomes in either early embryonic development and implantation or during late gestation. The absence of work focusing on mid-gestational fetal development is surprising as this is a critical period for fetal muscle and initial fat development occurs as well as extensive reorganization of the placental vasculature that optimizes nutrient transfer and gives rise to drastic increases in blood flow during the third trimester when nutrient demand is at its highest. The current work isolates mid-gestational impacts of L-citrulline supplementation and aims to identify the validity of L-citrulline supplementation as a programming scheme to maximize fetal and post-natal growth, and as a therapeutic intervention in the face of maternal environmental stressors.

Chapter 2

Effects of mid-gestational L-citrulline supplementation to twin-bearing ewes on umbilical blood flow, placental development, and lamb production traits

Introduction

The environment a fetus encounters *in utero* can impact physiological function and risk of disease after birth and into adulthood. This phenomenon is termed the developmental origins of health and disease (DOHAD). It often is also referred to as fetal programming. Various fetal programming events have been described to date, and each is founded on the concept that the embryo and fetus respond to the maternal environment to alter fetal development and epigenetic traits in ways that improve progeny survivability in the anticipated environment (Reynolds and Caton, 2012). This interaction between the embryo/fetus with the maternal environment can have short-term and long-term consequences on health and development after birth. In some cases, these changes may be detrimental to the individual. For example, in livestock both undernutrition and overnutrition can alter birth weight, alter organ development, impair placental function, and influence growth rates in postnatal life (Long et al., 2009; Ford and Long, 2011; McCoski et al., 2018). In other cases, however, these developmental changes may be beneficial and even be manipulated to produce desired effects. For example, increasing the plane of nutrition to cows in late gestation can improve daughter fertility (Martin et al., 2007; Cushman et al., 2014).

Our interest is to use fetal programming to improve skeletal muscle development, gain, and meat production in ruminants. To do this, we specifically targeted our programming scheme to the period of gestation when muscle fiber formation occurs. Myogenesis is key to fetal skeletal muscle development. Primary muscle fibers form during embryonic development, and it is this

small number of primary muscle fibers that will serve as templates for secondary fiber formation that occurs during mid-gestation (Du et al., 2010a). It is the secondary muscle fibers that form the majority of muscle fibers. No postnatal muscle fiber hyperplasia occurs. Rather, the full complement of muscle fibers is set at birth. Thus, this period of mid-gestation is critical period for skeletal muscle development. Nutrient restriction and other environmental and metabolic stressors occurring during this period reduces muscle fiber numbers in several livestock species (Dwyer et al., 1994; Zhu et al., 2006; Gonzalez et al., 2013).

We propose that uterine blood flow plays a role in supplying adequate nutrients during this period of myogenesis. In fact, many models where pregnancies are compromised by reduced uterine blood flow or inadequate vascularization of the placenta, reduced fetal growth and post-natal development is observed (Reynolds et al., 2005; Reynolds et al., 2006).

The use of L-arginine supplementation will modulate blood flow and vascularization to improve reproductive outcomes and ameliorate intrauterine growth restriction (IUGR) in some species. In pigs, dietary L-arginine supplementation in gestating sows and gilts improves placental growth, piglet birth weight, and embryonic survival (Mateo et al., 2007; Gao et al., 2012; Wu et al., 2013; Li et al., 2014). In sheep, intravenous L-arginine delivery enhances placental growth and improves lamb birth weight (Lassala et al., 2010; van der Linden et al., 2015). In humans, administration of L-arginine improves uterine blood flow, ovarian response to gonadotropin, endometrial receptivity, and pregnancy rate in women who responded poorly to *in vitro* fertilization (Battaglia et al., 1999). There is also evidence that arginine therapy in women exhibiting IUGR improves fetal growth and increases birth weight at term (Xiao and Li, 2005; Shen and Hua, 2011) and that arginine treatment reduces diastolic pressure in preeclamptic women (Gui et al., 2014).

L-citrulline, a precursor to L-arginine, will be utilized to improve blood flow. Both citrulline and arginine are metabolized to nitric oxide throughout the body (Wu and Morris, 1998; Wu et al., 2013). Nitric oxide (NO) is a vasodilator, angiogenic factor, and smooth muscle relaxer that is critical for female reproductive functions involved in early embryonic development, implantation, placental perfusion, and cervical ripening at parturition (Maul et al., 2003). Additionally, increasing uterine blood flow with a vasodilator (Sildenafil citrate; Viagra) increases fetal size in sheep (Satterfield et al., 2010). Citrulline and arginine can also be metabolized into ornithine and proline, which are substrates for polyamine synthesis (Wu et al., 2013). Polyamines are key players in embryonic and fetal gene expression, protein synthesis, and angiogenesis (Lenis et al., 2017). Furthermore, L-citrulline supplementation has the potential to increase brown adipose tissue development in ovine fetuses (Z. Wu et al., 2012). The proposed benefit for utilizing L-citrulline rather than L-arginine is due to citrulline having a longer half-life in circulation when compared with arginine, largely due to its ability to bypasses liver metabolism (Curis et al., 2005; Wu et al., 2007).

There is evidence that some citrulline may escape rumen digestion after feeding (Gilbreath et al., 2020a; Gilbreath et al., 2020b). Others have utilized encapsulated, rumen protection schemes to feed arginine to ruminants (Ruiz de Chávez et al., 2015; Zhang et al., 2016a; Sun et al., 2017; Peine et al., 2018). Our work utilized an injection scheme to ensure that adequate increases in circulating L-citrulline concentrations were generated for an extended period each day. Twin bearing ewes with a moderate dietary energy restriction were used to test the hypothesis that L-citrulline administration during mid-gestation will increase uterine and placental blood flow and enhance placental development, fetal growth, and lamb performance.

Materials and Methods

Animal Use

All animal work was completed in compliance and with the approval of the Virginia Tech Institutional Animal Care and Use Committee (IACUC; #18-102). Purebred Suffolk or crossbred (Suffolk x Dorset) sheep used in this work were provided by the Virginia Tech Sheep Center (Blacksburg, VA).

Treatment Preparation and Administration

Treatments were prepared daily in warmed USP grade sterile saline (sodium chloride 0.9%, Vet One, Boise, Idaho) to a final concentration of 2 g / 15 ml for L-citrulline and 1.6 g / 15 ml for L-alanine. This concentration permitted injection of 155 µmol / kg BW of citrulline and 465 µmol / kg BW of alanine within a 13 to 32 ml bolus. Treatments were isonitrogenous. Treatments were administered within 1.5 hours of preparation into the jugular vein after cleaning the neck with 70% [v/v] ethanol. A topical analgesic and anti-inflammatory agent (Surpass®, Boehringer Ingelheim Vetmedica, Inc., St Joseph, Missouri, USA) was applied as needed to limit hematoma formation. The location of injection sites varied each day, and different sides of the neck were used each week.

Experimental Design

An initial pharmacokinetic pilot study was completed. Mature, non-pregnant ewes (n=3/treatment) received L-citrulline or L-alanine on a single day. Blood samples were taken immediately before and 0.5, 1, 2, 4, 8, and 24 hours after treatment. Blood samples were maintained on ice until plasma was isolated via centrifugation (1500 g x 15 minutes) and stored at – 20 °C. A complete free amino acid profile was completed in plasma samples by the

University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA). Treatments in subsequent studies were adjusted if inadequate increases in concentration of supplemented amino acids in maternal plasma were not seen in study one.

The second study utilized mature, twin-bearing ewes (n=6 ewes/treatment). Ewes chosen for this work were taken from a large group of ewes (n=60 ewes) maintained on pasture at the Virginia Tech Sheep Center. Estrous cycles were synched using a 14-day CIDR synch. CIDRs (EAZI-BREED CIDR Sheep Insert, Zoetis Inc., Kalamazoo, MI, USA) were inserted for 14 days and removed before turnout with rams (Abecia et al., 2012). Ewes were exposed to 2 crossbred (Dorset x Suffolk) and 2 Dorset rams for 4 days upon CIDR removal. Pregnancy was diagnosed by transabdominal ultrasonography on days 36-39 of gestation (day 0 = day of breeding). Body weights were recorded on this day and weekly throughout the treatment period. Ewes were assigned randomly to treatments after blocking based on age and body weight. Beginning at day 38-41 of gestation, all ewes were comingled in one dry-lot pen with free access to water and a trace mineral salts (Sweetlix Mineral with Bovatech, Sweetlix, Mankato, MN, USA). Daily L-citrulline or L-alanine intravenous injections occurred for 39 days from days 42-45 to 81-84 of gestation. One ewe receiving citrulline aborted during the study. None of the data collected from this ewe was included in the analyses.

At the end of the treatment period, body weights were taken each month until lambing. Ewes lambed indoors and transitioned to shelters containing pasture access after 7 days. Lamb body weight and wither heights were taken at birth, day 7, and at weaning. Body weights were taken on days 124-128 and 176-180 days of age. Weaning occurred at 63-67 days of age. Average daily gain (ADG) from birth to weaning and from weaning to slaughter were calculated.

Animal Diets and Feed Analyses

Ewes were fed at 85% of the NRC requirements for ME for gestating ewes (NRC, 2007) by limit-feeding ewes 0.9 kg tall fescue hay/head/day (Table 2-1 and 2-2). All hay was consumed each day. At the end of the treatment period, ewes were maintained on pasture with 1 kg/head/day cracked corn (Table 2-3). The lamb pre-weaning diet consisted of two types of creep feed. Post-weaning lambs were maintained on pasture (Table 2-3).

Biweekly samples were collected from hay, pasture, and grains and processed for nutrient analysis during the ewe treatment period, post-treatment period, and both pre- and post-weaning lamb periods. Feed samples were thawed for 24 hours and dried in a forced-air oven for 72 hours at 55°C in order to determine dry matter (DM) content. Dried samples were ground in a hammer-miller (Willey Mill) to pass through a 1-mm sieve, composited within period and feed type, and stored at room temperature for posterior nutrient analysis. Composite samples were analyzed for DM, crude protein (CP), neutral detergent fiber (aNDF), acid detergent fiber (ADF), non-fiber carbohydrates (NFC), and metabolizable energy (ME) content in a commercial laboratory (Dairy One Forage Laboratory, Ithaca, NY, USA).

Doppler Ultrasonography

Transrectal color doppler ultrasonography was performed one week before the end of the treatment period (gestational day 71 – 74) and about 2 weeks prior to lambing (gestational day 134 – 137) by a single operator. Ewes were maintained upright and blood flow assessment was performed by transrectal doppler ultrasonography. The device (ExaPad Mini; IMV Imaging, Rochester, MN, USA) was equipped with a 5-mHz sector probe. Blood flow parameters assessed included pulsatility index (PI), resistance index (RI), and systolic/diastolic ratio (S/D) in the umbilical artery and in one placentome of one of the twin lambs.

Blood analyses

Blood samples were collected from ewes from the jugular vein weekly throughout the treatment period and monthly thereafter until lambing. Samples were maintained on ice until plasma was isolated via centrifugation (1500 g x 15 minutes) and stored at – 20 ºC. Plasma progesterone concentrations were determined using the IMMULITE 2000 XPi Immunoassay system (Siemens Medical Solutions Diagnostics, Tarrytown, NY). Plasma pregnancy-specific protein B (PSPB) concentrations were completed by BioTracking Inc (Moscow, ID, USA) (Thompson et al., 2013). Intra-assay CV was 4.82%.

Lamb Carcass Data

Lambs were slaughtered at the Virginia Tech Meat Laboratory (Virginia Tech, Blacksburg, VA) at 179-183 days of age. Carcasses were weighed prior to chilling and were evaluated after 24 h at 2°C. Backfat thickness was measured perpendicular to the longissimus dorsi, and body wall thickness was measured 12.5 cm from the midline between the 12th and 13th ribs. Yield and Quality Grades and leg conformation score were assigned according to USDA standards (USDA, 1992). Dressing percentage was calculated as a ratio of hot carcass weight to live weight [DP = (HCW/LW) X 100].

Visceral organs were harvested after removal from the carcass and wet weights were recorded. Reproductive tracts were collected in the ewe lambs. Connective tissue was trimmed away, vagina was trimmed off just after the cervix, and the remaining tract including the ovaries was weighed. Antral follicle counts were also determined. All visible follicles were counted by one person and confirmed by a second.

Statistical analysis

All statistical analyses were performed using the SAS software package (version 9.4, SAS Institute Inc., Cary, NC). Each ewe was an experimental unit in analysis of ewe body weights, amino acid pharmacokinetic data, PSPB, and progesterone concentrations. Analysis of data collected on each individual lamb used each lamb as an experimental unit, as lambs were considered independent of each other and lamb parameters exhibited variability within twin pairs.

Analysis of doppler data, ADG of lambs, organ weights, and carcass traits were performed by least-squares ANOVA using the general linear model procedure. Organ weights were expressed relative to body weight as g/kg lamb body weight at slaughter. The statistical model used for these analyses included the main effects of treatment as well as sex in the ADG, organ and carcass trait analyses. Differences between individual means were compared using pairwise comparisons (PDIFF [probability of difference] option in SAS).

Analysis of ewe body weights, lamb body weights, and lamb wither heights were performed by mixed ANOVA with repeated measures. Main effects included treatment, day, and treatment by day interactions. Differences between individual means were compared using within day ANOVAs and pairwise comparisons (PDIFF [probability of difference] option in SAS).

Amino acid pharmacokinetic, progesterone, and PSPB datasets were analyzed by using the Proc Mixed model for ANOVA with repeated measures. Main effects in the model included treatment, day of pregnancy or time after administration, and their interactions. Post-hoc pairwise comparisons were completed using the probability of difference (PDIFF) option is SAS.

Lamb survival analysis was performed using Chi-squared in the Genmod procedure. The statistical model used for this analysis included the main effect of treatment and sex.

All data is presented as least square means ± standard error of the mean and adjusted in accordance with the Tukey Kramer test. A P-value ≤ 0.05 was considered statistically significant and $0.05 <$ P-value ≤ 0.10 was considered a tendency.

Results

Pharmacokinetic Pilot Study

Intravenous injection of L-citrulline increased ($P < 0.05$) circulating citrulline concentrations from 0.5 h to 8 h post-injection, returning to control values by 24 h post-injection (Figure 2-1, panel A). Arginine concentration differed between ewes given L-alanine and L-citrulline treatments ($P < 0.05$) but were unchanged ($P > 0.05$) from the pre-treatment value at time 0 in L-citrulline injected ewes (Figure 2-1, panel B). Injection of L-alanine did not affect citrulline or arginine concentration, but increased circulating alanine concentrations from 0.5 to 1 h before returning to pre-injection concentrations (Figure 2-1, panel C). Neither treatment affected blood ornithine, proline, urea, and other free amino acid concentrations (Figure 2-1). Treatment scheme was unaltered for study two.

Pregnant Ewe Body Weights

Initial body weights of ewes were 71.62 ± 2.00 kg for alanine ewes and 71.80 ± 2.20 kg for citrulline ewes. Ewe body weight decreased over the treatment period by 8.3% and 12.8% for alanine and citrulline treated ewes, respectively (Figure 2-2). There was no effect of treatment on ewe body weights over gestation but there was an effect of time ($P < 0.0001$). An overall

treatment by time interaction was detected (P = 0.001), but no differences between treatments were detected within any time points.

Circulating Progesterone, PSPB Concentrations and Umbilical Artery Blood Flow

There was no effect of treatment on progesterone concentrations over the treatment period (Figure 2-3). There was an increase in progesterone concentrations over time with a decrease in concentrations right before lambing (P < 0.0001). There was a treatment by time interaction detected, but no differences between treatments were detected within any time points.

There was no effect of treatment on PSPB concentrations over gestation (Figure 2-4). There was an effect of time on PSPB concentrations (P = 0.001). A treatment by time interaction was detected (P = 0.02), however within day analyses failed to detect treatment differences.

Blood flow parameters were not influenced by treatment during and after the treatment period (Table 2-4).

Lamb Body Weights

There was no difference in lamb survival to weaning due to maternal L-citrulline supplementation (Table 2-5). Additionally, there was no effect of sex on survival (P > 0.05) and assessment of treatment by sex interactions was not done as the lamb sex distribution within treatments did not allow for accurate comparisons. There was no effect of treatment on lamb body weights over their lifetime (Figure 2-5). There was an effect of time (P < 0.0001) but no treatment by time interaction. Within day analyses failed to identify any effects of treatment on body weights. Lamb sex effects are absent with the exception that body weights were greater (P = 0.04) in ewe lambs regardless of treatment. There was no effect of treatment or sex on ADG from birth to weaning (P = 0.79 and 0.49, respectively). There was no effect of treatment on

ADG from weaning to slaughter (P = 0.90), however, there was an effect of sex present (P = 0.01) with ewe lambs gaining more weight per day post-weaning when compared to wethers (0.25 ± 0.01 kg/d vs. 0.19 ± 0.015 kg/d).

There was no effect of treatment on lamb wither heights (Figure 2-5). There was an effect of time (P < 0.0001) but no treatment by time interaction. Within day analyses did not identify any effect of treatment on wither heights. Also, no effect of sex on wither height was observed on days 0 and 7. At weaning, there was an effect of sex on wither height (P = 0.01) with wethers being taller than ewe lambs. Assessment of treatment by sex interactions was not done as the lamb sex distribution within treatments did not allow for accurate comparisons.

Lamb Carcass Data

There was no effect of treatment on any carcass traits assessed (Table 2-6). There was an effect of sex on dressing percentage (P = 0.02), with wethers having a greater dressing percentage than that of ewes (52.77 ± 1.72 % and 47.01 ± 1.19 % respectively). Assessment of treatment by sex interactions was not done as the lamb sex distribution within treatments did not allow for accurate comparisons.

Lamb Organ Data

L-citrulline treatment did not affect heart, kidney, liver, lung, and pancreas weights at the time of slaughter (Table 2-7). However, regardless of treatment, wethers had heavier pancreases per kg body weight (P = 0.04) and tended (P = 0.09) to have heavier lungs per kg body weight than ewe lambs (pancreas: 0.98 ± 0.10 g/kg BW vs. 0.68 ± 0.08 g/kg BW; lungs: 23.23 ± 2.53 g/kg BW vs 17.29 ± 1.76 g/kg BW). There was no treatment effect on overall reproductive tract

weight, but there was a tendency (P = 0.1) for ewe lambs from citrulline-treated ewes to have greater antral follicle counts (Table 2-8).

Discussion

The importance of fetal growth and development for maximum progeny performance has long been recognized, but relatively few maternal dietary supplementation schemes are being used to modulate blood flow in order to improve progeny performance. Previous work in swine supplementing L-arginine has shown improvements in piglet birth weight, enhanced placental growth, and increased embryonic survival (Mateo et al., 2007; Gao et al., 2012; Wu et al., 2013; Li et al., 2014). Work in sheep is minimal and there are varied results with some L-arginine supplementation schemes showing improved lamb birth weight, greater internal organ mass, and enhanced placental growth (Lassala et al., 2010; van der Linden et al., 2015; Zhang et al., 2016a; Peine et al., 2018), and others recently showing no effects (Gootwine et al., 2020).

A citrulline supplementation scheme was developed to provide an arginine precursor for nitric oxide and polyamine synthesis. We utilized L-citrulline rather than L-arginine namely because L-citrulline exhibits lower liver catabolism than arginine in mammals (Curis et al., 2005). Also, in one study it was more effective at increasing circulating arginine levels than L-arginine itself in sheep (Lassala et al., 2009). An injection scheme was chosen to ensure an adequate citrulline increase in the blood for an extended period each day.

The first study revealed that the citrulline supplementation scheme was successful at increasing circulating citrulline concentrations in maternal plasma. Additionally, arginine was greater in citrulline ewes when compared to alanine treated ewes over 24 hours, though within treatment arginine levels did not differ from zero seeming to indicate that citrulline was not readily converted to arginine in maternal tissues. This finding is different from previous work

that found arginine levels increased between 5 and 240 minutes after citrulline infusion (Lassala et al., 2009). It is known that pregnant and non-pregnant ewes metabolize amino acids differently (Wu et al., 2007), also lending one potential reason for contradicting results as we utilized non-pregnant ewes for our work, while late pregnant ewes were utilized in the previous study (Lassala et al., 2009). Citrulline can be metabolized into ornithine and proline, and further metabolized into polyamines, which are key molecules in fetal gene expression, protein synthesis, and angiogenesis (Wu et al., 2013; Lenis et al., 2017). Although, plasma ornithine and proline concentrations did not differ between treatments or change due to treatment over time. Also, as indicated by unchanged plasma urea concentrations in both supplementation schemes due to treatment, suggesting that ewes maintained normal protein metabolism and kidney function after supplementing these amino acids (McWilliam and Macnab, 2009). Lastly, it is noteworthy to mention that ewes given an L-alanine bolus injection exhibited an increase in plasma alanine concentrations for 1 to 2 h post-injection but that citrulline, arginine, and other amino acids remain unchanged.

Over the 6-week treatment period, ewe body weight decreased slightly, demonstrating that restricting ME was effectively putting ewes in a metabolically compromised state. We also choose to work with twin-bearing ewes, in part to have a greater metabolic burden placed on the ewes compared to singletons, though the combination of bearing twins and nutrient restriction appears to only be a minor challenge. However, contrary to our hypothesis, blood flow was unaltered by treatment administration. The levels of blood flow parameters assessed appeared normal for the period of gestation assessed and follow the pattern of decreasing resistance indices (RI, PI, S/D) over the course of gestation, resulting in increased blood flow over gestation (Carr et al., 2016; Yilmaz et al., 2017). Interestingly, uterine blood flow was reduced

due to IUGR in singletons but was unaffected in twin bearing ewes exposed to IUGR (Newnham et al., 1991). Perhaps, twin bearing ewes do not exhibit the same hemodynamics and adaptive capacity as ewes carrying singletons. Potentially, with a more severe metabolic restriction and measurement in both twins, an effect would be detected.

Indices indicative of placental function did not change due to treatment as well. One way that placental function was assessed was by examining circulating PSPB concentrations. This molecule is produced exclusively by the placenta. It is one member of the pregnancy associated glycoprotein (PAG) group of molecules produced by trophoblast cells of the ruminant placenta (Telugu et al., 2009). PAG concentrations in ruminants with compromised placentas are known to be altered, specifically lower in animals more likely to abort the pregnancy, and can thus serve as a useful marker of placental function (Wallace et al., 1997; Wallace et al., 2015). In sheep PAG concentrations begin to rise in maternal blood during the 4th week of pregnancy then exhibit a biphasic pattern of secretion containing two peaks over the course of pregnancy, with one peak occurring around the end of the first trimester and the second peak at term (Wallace et al., 1997; Egen et al., 2009; Roberts et al., 2017). PSPB concentrations were unaffected by citrulline administration, meaning that the placenta was not modified by citrulline supplementation, and more specifically supplemented citrulline does not increase the number of PSPB producing trophoblast cells.

Placental function was also assessed by examining circulating progesterone, which is produced primarily by the placenta in ewes from day 50 onward (Wooding et al., 1996; Al-Gubory et al., 2000). In a normal pregnancy, progesterone concentrations will steadily increase throughout gestation and declines can be associated with either luteal or placental insufficiency depending on the timing of insult (Wallace et al., 1997). During the treatment period plasma

progesterone concentrations increased to approximately 25 ng/ml for both treatment groups (Figure 2-3). The IMMULITE has been validated by our lab for use in cattle, however, it has not yet been validated for use in sheep. Our results appear slightly high when compared to other studies assessing progesterone over gestation in sheep (Ranilla et al., 1994; Wallace et al., 1997; Luther et al., 2007b; Rosales-Nieto et al., 2021). However, nutrient restriction (Luther et al., 2007b; Vonnahme et al., 2013; Reynolds et al., 2018; Rosales-Nieto et al., 2021) as well as an increased fetal number (Butler et al., 1981; Rosales-Nieto et al., 2021) has been shown to increase circulating progesterone concentrations. Rerunning these samples collected with an ELISA that is validated for use in sheep would be recommended prior to drawing final conclusions about our data. As is, a lack of an effect of citrulline supplementation on progesterone concentrations shows that there was no increase in steroidogenic capacity of the placenta due to modification by citrulline supplementation. This means citrulline administration had no impact on the production of progesterone by the placenta, levels of available cholesterol for placental steroidogenesis, or metabolic clearance rates of progesterone in the ewe.

Most parameters of lamb production traits were unchanged between treatments. Skeletal muscle is a lower priority in nutrient partitioning compared with the individual's organ systems making fetal muscle fiber formation especially vulnerable to nutrient deficiency (Bauman et al., 1982; Close and Pettigrew, 1990). A decrease in nutrient availability to the dam during fetal muscle fiber formation can result in a reduced number of muscle fibers, reduced muscle mass at birth, and restrict future performance. However, lamb birth and weaning weights and heights did not differ due to treatment in our study (Figure 2-5), and ADG did not differ due to treatment in both the pre-weaning and post-weaning time period. This is consistent with what is found in IUGR studies through mid-gestation in ewes (Daniel et al., 2007; Sen et al., 2016), indicating

minimal impact during mid-gestation. Though in ewe and swine arginine supplementation studies during IUGR, lambs born from supplemented ewes had a higher birth weight (Mateo et al., 2007; Lassala et al., 2010). However, differences seen here in birth weight seen are likely due to supplementation encompassing late gestation when majority of fetal growth occurs (Lassala et al., 2011). This indicates the importance of timing on any maternal dietary restriction or supplementation scheme on subsequent lamb birth weights, with late gestation and hypertrophy of muscle fibers being of the utmost importance.

Additionally, our data shows progeny carcass and organ characteristics at harvest were unchanged by treatment and correspond to what we would expect from crossbred lambs at this age (USDA, 1992; Daniel et al., 2007; Sen et al., 2016). This was contrary to our expectations, as we anticipated citrulline supplementation providing increased blood flow during mid-gestation, providing increased nutrients for muscle formation during *in utero* muscle development. Thinking a programming benefit would exhibit itself, we also expected benefits for lamb gain, organ characteristics corresponding to a more efficient lamb, and improved carcass qualities. It is likely that the effect of citrulline on fetal growth and development depends on many factors (i.e. severity of nutritional challenge, IUGR, timing/length of administration, postnatal diet, and compensatory growth) other than the circulating levels of citrulline and arginine during gestation, and since we saw no increase in blood flow during gestation and no differences in lamb weights or gain this corresponds to our other findings of no effect in this study.

Interestingly, antral follicle counts (AFC) of ewe lambs from ewes given daily citrulline tended to be higher (Table 2-8). In ruminants, AFCs can be used as a marker of the ovarian reserve, and multiple studies have linked this measure to reproductive features and to predicted overall fertility (Ireland et al., 2008; Jimenez-Krassel et al., 2009; Torres-Rovira et al., 2014;

McNeel and Cushman, 2015; Pinto et al., 2018; Santa Cruz et al., 2018). Ovarian reserve is established before birth, and it is the processes of oogenesis and folliculogenesis that are responsible for changes in germ cell number before and after birth (Monniaux et al., 2014). In ewe lambs exposed to undernutrition during various periods of gestation reduced folliculogenesis is present (Rae et al., 2001; Rae et al., 2002). Perhaps citrulline supplementation during this time reversed this effect of undernutrition. Further data on maternal productivity of these ewe lambs, including longevity in the flock, lambing rates, and lamb birth and weaning weights would be needed to determine if this tendency would translate into a realized increase in fertility. Additionally, utilizing this data as preliminary inputs for power and sample size analyses determined there was low power for determining significance and that an increased sample size to an n of at least 11 ewes per group would be necessary to reveal a statistic difference if one is present.

To conclude, the collective findings of our work indicates that L-citrulline supplementation to pregnant ewes under a minor to moderate metabolic challenge had no impact on blood flow to the uterus and placenta during gestation and provided no programming benefit to the lambs. The data obtained from this sheep study provides a challenge to existing data showing benefit of supplementation in swine, however, it corroborates recent findings in sheep that find minimal to no benefit in sheep. Perhaps in a more challenged state this supplementation scheme may provide benefit. Future work should utilize a wider range of metabolic challenges, and also assess muscle fiber distribution at harvest to tease apart potential implications of higher dressing percentages in wethers born from ewes supplemented L-citrulline that was unable to be assessed here due to lamb sex distribution within treatments not allowing for accurate comparisons.

Acknowledgements

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Table 2-1. Amino acid quantification in feed ingredients offered to ewes during the treatment period

Amino Acid	g/100 g (as fed)
Taurine	0.15
Hydroxyproline	0.03
Aspartic Acid	0.54
Threonine	0.27
Serine	0.23
Glutamic Acid	0.63
Proline	0.32
Lanthionine	0.00
Glycine	0.34
Alanine	0.38
Cysteine	0.08
Valine	0.36
Methionine	0.10
Isoleucine	0.27
Leucine	0.50
Tyrosine	0.16
Phenylalanine	0.33
Hydroxylysine	0.05
Ornithine	0.01
Lysine	0.25
Histidine	0.08
Arginine	0.26
Tryptophan	0.04

Table 2-2. Nutritional characterization of feed ingredients offered to ewes during the treatment period

Chemical Composition	Content
Dry matter, %	88.3
Crude protein, %	8.3
Neutral detergent fiber, %	73.9
Acid detergent fiber, %	47.6
Non-fiber carbohydrates, %	7.8
Metabolizable energy, Mcal/kg	1.92

Table 2-3. Nutritional characterization of feed ingredients offered to ewes in late gestation and lambs pre- and post-weaning

	Ewe		Lamb	
	Late Gestation		Pre-weaning	Post-weaning
Chemical Composition	Hay	Cracked corn	Creep feed	Pasture
Dry matter, %	70.4	90.4	91.0	26.4
Crude protein, %	11.6	8.3	19.7	17.8
Neutral detergent fiber, %	68.9	7.0	11.4	57.1
Acid detergent fiber, %	43.7	2.5	5.6	32.6
Non-fiber carbohydrates, %	9.6	79.1	57.7	14.3
Metabolizable energy, Mcal/kg	2.02	3.44	3.39	2.38

Table 2-4. Blood flow indices in pregnant ewes during (d71) and after (d134) treatment period[1]

		Umbilical Vessels			Placentome		
		RI	PI	S/D	RI	PI	S/D
d71	Alanine	0.59 ± 0.08	1.08 ± 0.15	4.04 ± 1.56	0.43 ± 0.13	1.01 ± 0.45	2.07 ± 1.10
	Citrulline	0.74 ± 0.09	1.39 ± 0.16	5.18 ± 1.71	0.54 ± 0.15	0.97 ± 0.51	5.24 ± 1.42
	p-value	0.22	0.19	0.64	0.61	0.95	0.13
d134	Alanine	0.54 ± 0.06	0.87 ± 0.16	2.53 ± 0.48	0.54 ± 0.08	0.90 ± 0.24	2.23 ± 0.85
	Citrulline	0.64 ± 0.07	1.06 ± 0.18	3.15 ± 0.52	0.53 ± 0.09	1.03 ± 0.29	3.52 ± 1.05
	p-value	0.33	0.44	0.40	0.96	0.74	0.36

[1]Values are means with individual SEM. RI = resistance index, PI = pulsatility index, S/D = systolic/diastolic ratio.

Table 2-5. Lamb treatment, sex distribution, and survival from birth to weaning

Treatment	Sex	Lambing (d0)		d7	Weaning (d65)	Survival (%)	P-value
		Alive	Stillborn				
Alanine	Ewe	5	0	4*	4	9/12 (75)	0.91
	Ram	6	1	6	5*		
Citrulline	Ewe	7	1	7	7	8/10 (80)	
	Ram	2	0	1*	1		

*Indicates lamb in that group did not survive the time since the previous time lambs were observed. Lambs that did not survive died within the first 2 weeks of life.

Table 2-6. Means for carcass traits for lambs from ewes given daily i.v. injection of L-citrulline or L-alanine over mid-gestation[1]

	Treatment		
Variable	Alanine	Citrulline	P-value
No. of lambs	9	8	-
Live weight, kg *	47.11 ± 1.24	46.88 ± 1.53	0.91
Hot carcass weight, kg	23.07 ± 1.03	23.75 ± 1.27	0.69
Dressing percentage, % *	48.84 ± 1.29	50.95 ± 1.59	0.33
Backfat thickness, mm	3.48 ± 0.43	4.20 ± 0.53	0.32
Body wall thickness, mm	14.15 ± 1.51	17.54 ± 1.86	0.19
Adjusted fat thickness, mm	3.50 ± 0.54	4.71 ± 0.67	0.19
Loin muscle area, mm^2	16.12 ± 0.51	16.40 ± 0.63	0.73
Leg score[a]	11.98 ± 0.23	12.23 ± 0.28	0.52
Quality grade[a]	10.76 ± 0.24	11.13 ± 0.30	0.35
Yield grade	1.78 ± 0.21	2.25 ± 0.26	0.19
Percentage boneless closely trimmed retail cuts (%BCTRC), %	49.19 ± 0.36	48.58 ± 0.44	0.31

[1]Values are least squares means with SEM.
* asterisk indicates that differences between the sexes were found ($p<0.05$).
[a]Leg conformation score and quality grade based on a numeric score of 10 = low choice, 11 = average choice, 12 = high choice.

Table 2-7. Visceral organ weights of lambs from ewes given daily i.v. injection of L-citrulline or L-alanine over mid-gestation[1]

	Treatment		
Organ (g / kg bw)	Alanine	Citrulline	P-value
Heart	3.26 ± 0.15	3.30 ± 0.19	0.88
Kidney (with peri-renal fat)	6.52 ± 0.74	7.93 ± 0.92	0.26
Kidney (without peri-renal fat)	2.79 ± 0.10	2.76 ± 0.12	0.83
Liver	17.54 ± 0.65	18.64 ± 0.80	0.31
Lungs	20.96 ± 1.90	19.55 ± 2.34	0.65
Pancreas	0.79 ± 0.08	0.87 ± 0.10	0.55

[1]Values are means with individual SEM.

Table 2-8. Reproductive characteristics of ewe lambs from ewes given daily i.v. injection of L-citrulline or L-alanine over mid-gestation[1]

	Treatment		
Variable	Alanine	Citrulline	P-value
Tract Weight (g/kg bw)	0.51 ± 0.06	0.52 ± 0.04	0.85
AFC	30.00 ±7.18	46.43 ± 5.42	0.10

[1]Values are least squares means with SEM.

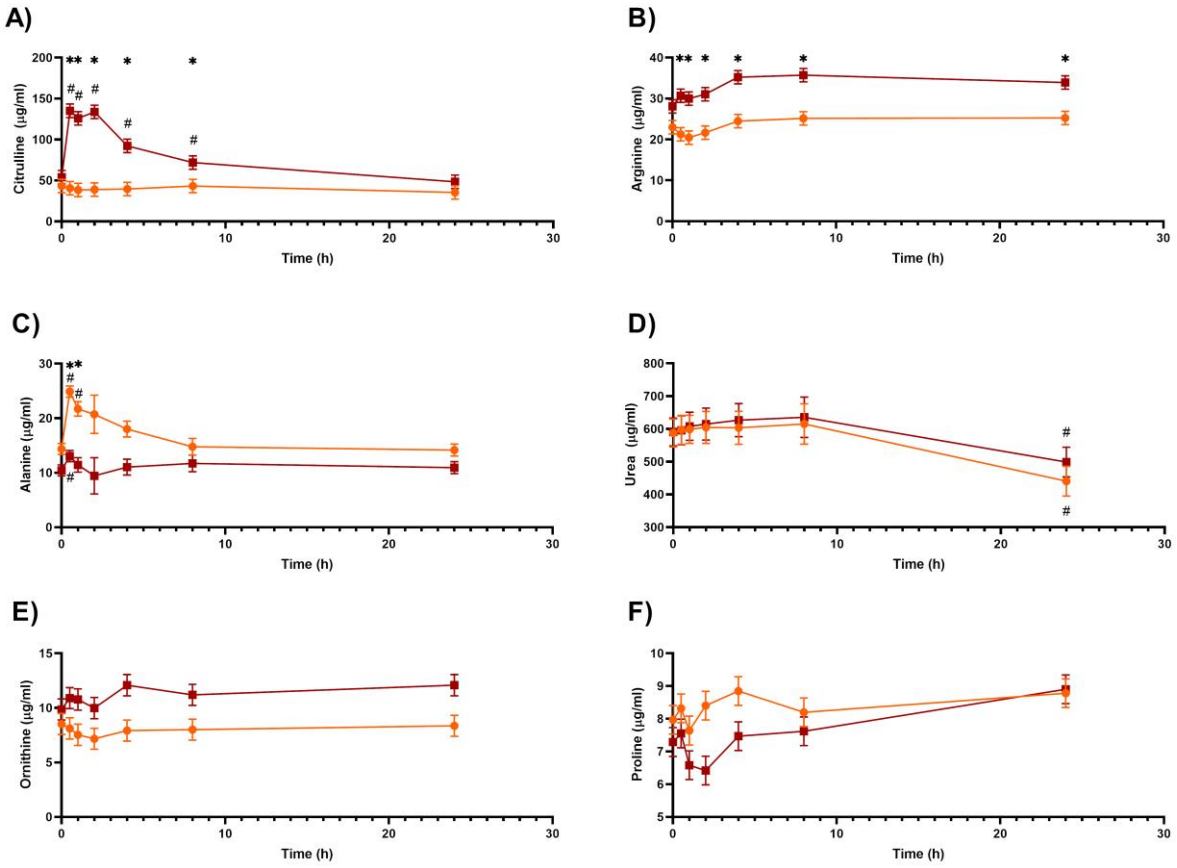

Figure 2-1. Concentrations of arginine, citrulline, ornithine, urea, and alanine in maternal plasma after a single bolus i.v. injection of L-citrulline or L-alanine to non-pregnant ewes. Blood samples were collected immediately before and 0.5, 1, 2, 4, 8, and 24 hours after treatment. Repeated measures analysis showed an effect of treatment on maternal alanine, citrulline, and arginine concentrations ($P<0.05$). There was an effect of time in all amino acids assessed ($P<0.05$), and a treatment by time interaction present for maternal alanine and citrulline concentrations ($P<0.0001$).

Data is shown as least squares means and SEM. Alanine is shown in orange and citrulline is shown in maroon. * indicates means between treatments at that time point differ. # indicates means differ from zero value within their respective treatment.

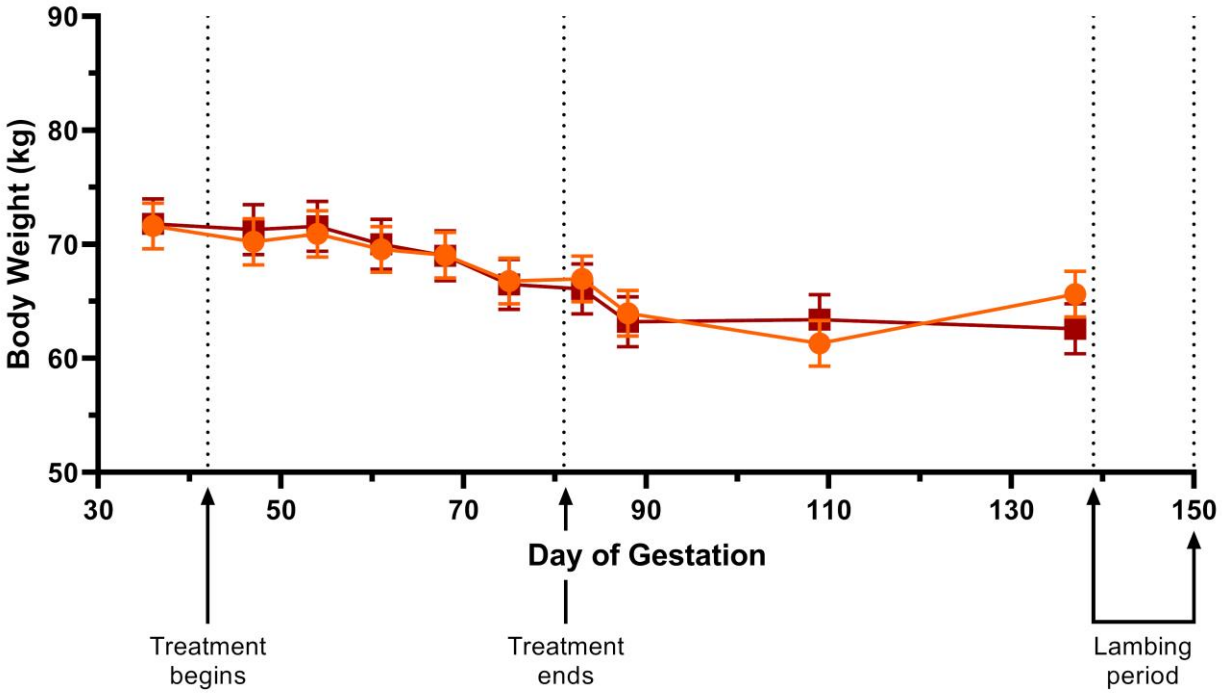

Figure 2-2. Ewe body weights obtained before the treatment period, taken at intervals of 1 week during the treatment period and monthly in the post-treatment period until lambing. Repeated measures was used to examine ewe body weights over all of gestation. Least squares means and SEM are depicted, with alanine shown in orange and citrulline shown in maroon. No effect of treatment was found. There was an effect of time ($P<0.0001$) and a treatment by time interaction ($P=0.001$). Additionally, within day analyses showed no difference in body weight due to treatment.

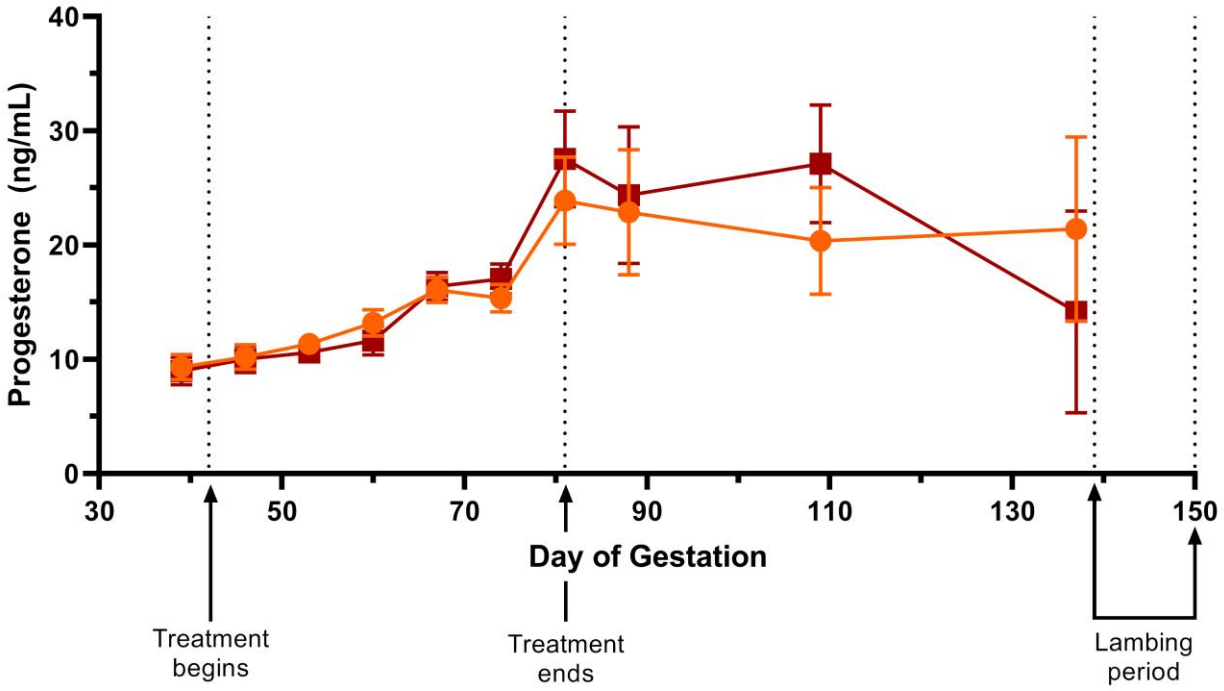

Figure 2-3. Maternal plasma progesterone concentrations over the treatment period. Blood samples were collected weekly through the treatment period. Data is shown as least squares means and SEM, with alanine shown in orange and citrulline shown in maroon. Repeated measures analysis showed no effect of treatment on progesterone concentrations (P=0.86), but an effect of time (P<0.0001) was present. Treatment by time interactions were present (P<0.0001), however, within day analyses showed no difference in progesterone concentration due to treatment.

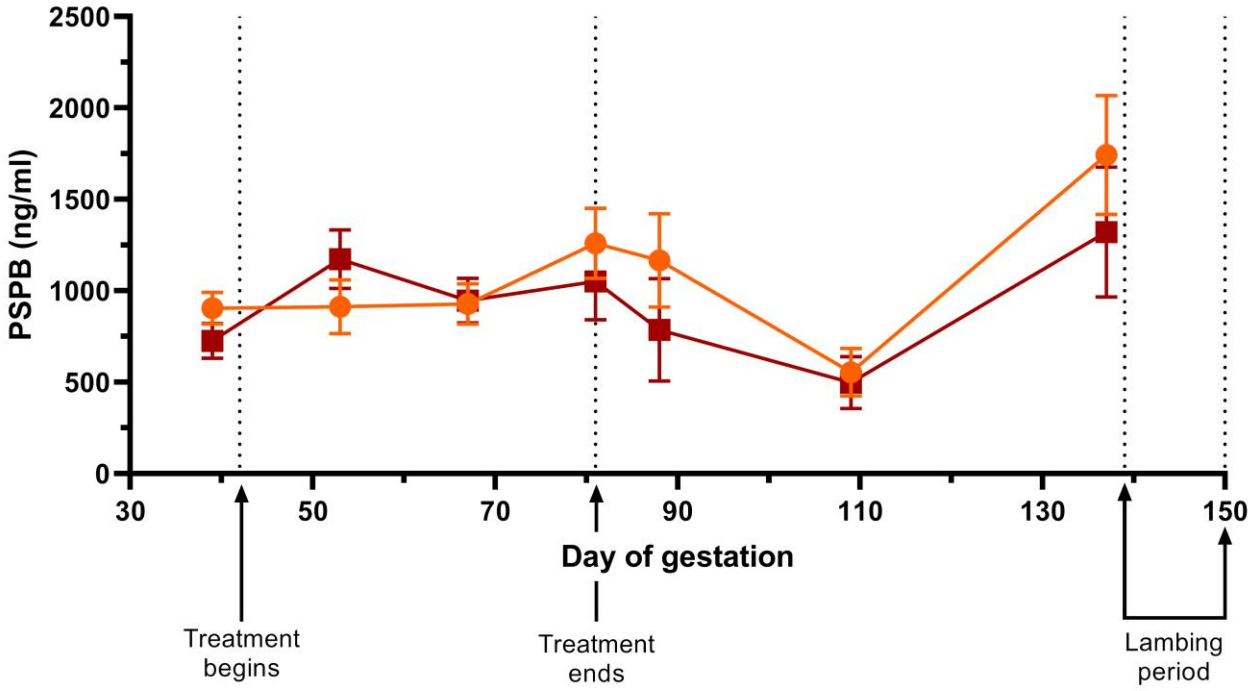

Figure 2-4. Maternal plasma pregnancy-specific protein B (PSPB) concentrations over gestation. Blood samples were collected every other week through the treatment period and monthly after the treatment period. Data is shown as least squares means and SEM, with alanine shown in orange and citrulline shown in maroon. Repeated measures analysis showed no effect of treatment on PSPB concentrations (P=0.44), but an effect of time (P=0.001) and treatment by time interaction (P=0.02) was present. However, within day analyses showed no difference in PSPB concentration due to treatment.

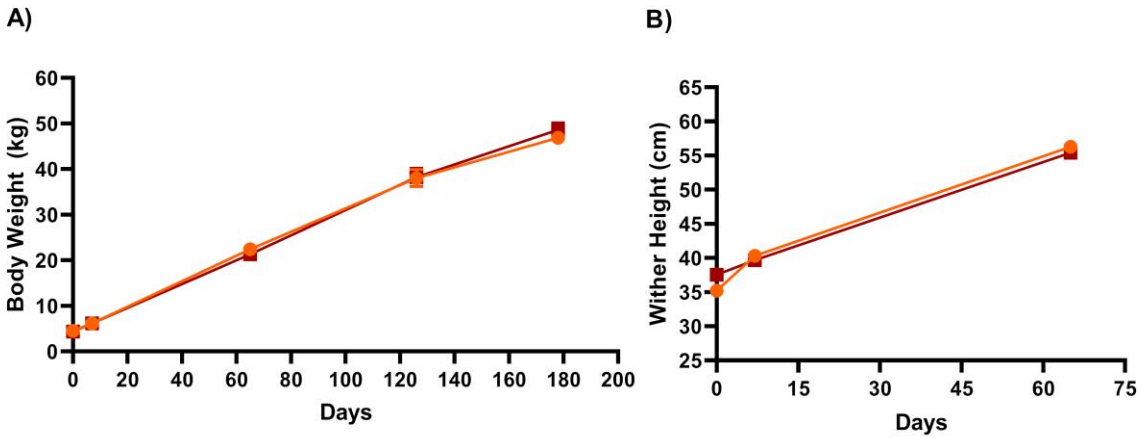

Figure 2-5. Lamb body weights and wither heights over lifetime. Data is shown as least squares means and SEM, with alanine shown in orange and citrulline shown in maroon. *Panel A:* Lamb body weights were taken at birth, day 7, weaning (day 65), day 126 and three days before slaughter (day 178). Repeated measures analysis shows no effect of treatment or treatment by time interactions, but effect of time was significant ($P<0.0001$). *Panel B:* Lamb wither heights were taken at birth, day 7, and weaning (day 65). Repeated measures analysis showed no effect of treatment or treatment by time interactions, but an effect of time ($P<0.0001$) on wither heights.

Interpretive Summary

In summary, contrary to our hypothesis, administering L-citrulline supplementation to moderately challenged, pregnant ewes during mid-gestation had no impact on blood flow and provided no programming benefit to the lambs. The objective of this work was to determine if L-citrulline supplementation to ewes can provide an alternative to L-arginine supplementation in ruminants and provide programming benefits to the developing lamb by increasing blood flow to the fetus during gestation.

It was interesting to see that our supplementation scheme had no effect on blood flow. The dose of L-citrulline we gave is comparable to amounts of L-arginine or L-citrulline used in sheep studies (Lassala et al., 2009), and is about twice the amount consumed by humans for performance based results (86 mg/kg BW of citrulline malate). However, it is more than half as much as the amount of L-arginine used in another study (van der Linden et al., 2015). From the results from our pharmacokinetic study, perhaps our scheme was ineffective at increasing arginine concentrations and subsequent NO concentrations. To have ensured an increase in NO and likely an increase in blood flow we could have measured circulating NO concentrations as well. Additionally, it would have been interesting to assess maternal blood flow in both fetuses and in other blood vessels outside of the reproductive tract as well.

Even though we did not see any gestational effects, we continued collecting lamb performance data to see if post-natal growth and metabolism was affected. Potentially fetal muscle development was unaltered, but the lamb's organs and metabolism were programmed in a way for enhanced growth after birth. L-citrulline supplementation has the potential to increase brown adipose tissue development in ovine fetuses (Z. Wu et al., 2012). The primary site of brown adipose tissue accumulation is in the peri-renal region, thus we measured kidneys with

and without peri-renal fat deposits. Additionally, we assessed pancreas and liver to observe potential differences in glucose metabolism, and lungs to observe potential differences in O2 availability. Though, we did not see any differences in organ weights due to treatment.

Overall, we can conclude that the benefit of arginine supplementation seen in swine cannot be easily reproduced in the sheep. Some differences between the two species that may lead to limitations of reproducing beneficial effects in sheep include pigs are polytocous while sheep are monotocous or twin-bearing, differing placenta types (diffuse vs. cotyledonary), and differing digestive systems (monogastric vs. ruminant). The differences in digestive systems can be especially limiting in translating oral supplementation schemes, due to the fact that in ruminants much of what is eaten is modified by the rumen environment prior to absorption. Additionally, when working to modify placental efficiency we must take into account placenta type differences and that transport across the placenta will vary by species, and that nutrient partitioning may vary between litter bearing and monotocous species as well. Even with these limitations and the injection scheme we developed rather than oral supplementation, I believe the root cause of finding no differences is that our treatment scheme was ineffective.

From these results, I do not believe the supplementation strategy we developed is worth considering in a production setting right now. Further work should examine the effects of citrulline supplementation at different time points, different routes of supplementation, and in ewes with a larger metabolic challenge before being totally discounted. Perhaps, in a more challenged state this supplementation scheme may have provided more benefit. Effects seen in rodents showing enhanced implantation would be interesting to examine in cattle, potentially supplementing citrulline for a period prior to and after embryo transfer of *in vivo* and *in vitro* derived embryos. Additionally, citrulline supplementation in late gestation during the major

period of fetal growth would be of interest to examine. Altogether, this work provides insight into the effects, or lack thereof, of citrulline supplementation to gestating ewes in mid-gestation. It is likely that the effect of citrulline on fetal and post-natal growth and development depends on many factors (i.e. severity of nutritional challenge, IUGR, timing/length of administration, postnatal diet, and compensatory growth) other than the circulating levels of citrulline and arginine during gestation.

Appendix A

Vero cell conditioned media enhances bovine trophectoderm development

Introduction

Blastocyst development is a complex process involving multiple steps from immature oocyte to a blastocyst capable of elongation and implantation. Blastocyst development is characterized by two cell lineage specification events. The first is segregation of cells into trophectoderm (TE) and the inner cell mass (ICM). This event begins during blastomere compaction at the morula stage in cattle, day 5/6 post-fertilization (Betteridge and Fléchon, 1988) and is determined primarily by differential Hippo-based signaling in the outer and inner cells leading to CDX2 expression by the TE and lack thereof in the inner cells resulting in maintenance of a pluripotent ICM (Nishioka et al., 2009). The second cell lineage specification event is the formation of the epiblast and hypoblast. The epiblast will give rise to the embryo proper and the allantoic sac, and the hypoblast will contribute to both embryonic and extraembryonic tissues.

Blastocyst development can occur *in vitro* in a process termed *in vitro* embryo production (IVP), a three-step process involving *in vitro* maturation, fertilization, and culture. Current IVP culture conditions allow for the production of transferable embryos capable of establishing pregnancy and producing a calf post-transfer, however, conditions remain suboptimal. IVP embryos exhibit lower blastocyst yields, lower cryotolerance, altered gene expression patterns and epigenetic changes, altered ICM:TE cell ratios, and lower pregnancy rates compared to their *in vivo* counterparts (Maillo et al., 2016). In cattle, only about 27% of IVP embryo recipients will produce a live calf, which is about 25% lower than pregnancy rates for embryos generated by superovulation (Ealy et al., 2019).

Interleukin-6 (IL6) has been identified as an important mediator of bovine embryo development. IL6 is a pleiotropic cytokine involved in a wide variety of biological functions and is mostly regarded as a pro-inflammatory cytokine with functions in inflammation and the infection response, but is also implicated in cancer, metabolism, placental development, and implantation (Scheller et al., 2011; Sharma et al., 2016; Yoo et al., 2017). IL6 will bind to the IL6 receptor (IL6R), dimerize with the transmembrane signal transducer protein (gp130/IL6ST), leading to triggering of the janus kinase/signal transducer and activator of transcription 3 (JAK/STAT3) and ERK1/2 signaling pathways (Scheller et al., 2011; Eulenfeld et al., 2012; Garbers and Scheller, 2013). Specifically, in the bovine blastocyst, it has been found that IL6 induced increases in ICM cell number occurs through a STAT3 dependent pathway (Wooldridge et al., 2019; Wooldridge and Ealy, 2019).

This work sought to addresses the question of if IL6 is a sole driver of ICM development or just one of multiple factors that can affect STAT3 activity and ICM development. A Vero cell conditioned media system was utilized to model the uterine secretome, hypothesizing that a more complex culture milieu more accurately reproduces the *in vivo* environment *in vitro*. In addition, these Vero cells are known to secrete a few known embryokines produced by the reproductive tract that modulate embryonic development, including LIF, IL6, and PDGF (Kauma and Matt, 1995; Carnegie et al., 1997; Desai and Goldfarb, 1998). Two studies were completed to test the hypothesis that the addition of Vero cell conditioned media during embryo culture will increase ICM cell numbers and that IL6 is one of many factors acting through STAT3 necessary for ICM development and maintenance.

Methods

In vitro embryo production

Bovine embryos were produced by *in vitro* maturation, fertilization, and embryo culture (Fields et al., 2011; Wooldridge and Ealy, 2019). Cumulus-oocyte complexes (COCs) were harvested from ovaries purchased from Brown Packing Company (Gaffney, SC, USA) and incubated overnight for 21 to 24 hours at 38.5°C in 5% CO2 in groups of 20 – 35 in 500 µl TCM-199 containing Earle's salts and supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Flowery Branch, Georgia, USA), 25 µg/ml bovine follicle stimulating hormone (Bioniche Animal Health Canada Inc., Belleville, Ontario, Canada), 2 µg/ml estradiol (Sigma-Aldrich, St. Louis, MO, USA), 22 µg/ml sodium pyruvate, 1 mM L-alanyl-L-glutamine (Glutamax), and 25 µg/ml gentamicin sulfate. For fertilization, COCs were washed in HEPES-SOF and placed in groups of 150 – 200 in 3 ml SOF-FERT covered by paraffin oil (Ovoil; Vitrolife, Göteborg, Sweden). Frozen semen pooled from four Holstein bulls (donation from Select Sires, Plain City, OH, USA) was thawed and spermatozoa were isolated through a biphasic (40 and 80%) Bovipure™ density gradient (Nidacon; Spectrum Technologies Healdsburg, CA, USA) before addition to fertilization media at a concentration of 1 million sperm/ml media. The day of fertilization was designated as day 0. After incubation for 14 to 18 hours at 38.5°C in 5% CO2, presumptive zygotes were denuded by repeat pipetting, washed in HEPES-SOF, and placed in groups of 20 – 30 in 50 µl droplets of SOF-BE1 covered by paraffin oil and incubated at 38.5°C in 5% CO2, 5% O2, and 90% N2 in humidified air.

Vero cell conditioned medium and treatment administration

Vero cells were purchased from the ATCC (ATCC CCL-81; ATCC, Manassas, VA, USA) and maintained in culture at 37°C in 5% CO2 throughout all the described studies. Conditioned medium was prepared fresh from maintained Vero cells for each treatment time. To produce conditioned medium used for treatments, Vero cells were seeded in a T25 flask at a

density of 750,000 and 1,000,000 cells and incubated for 24 hours at 37°C in 5% CO2. After 24 hours, confluency in the flasks were recorded and the flask with the most morphologically normal cells and a confluency within the range of 30 – 70% was utilized for subsequent conditioning. Vero cell growth media in the chosen flask was aspirated, cells were washed with SOF-BE1, and then replaced by fresh SOF-BE1. Additionally, a flask with SOF-BE1 with no Vero cells present was prepared to serve as a negative control. Both control flask and Vero cell conditioning flask were incubated at 37°C in 5% CO2 for 48 hours. After 48 hours, conditioned and non-conditioned media were mixed 1:1 with warmed, fresh SOF-BE1 and sterile filtered through a 0.2 µm nylon membrane syringe filter.

In the study with treatment administration on days 1, 3, and 5, embryo culture plates with 50 µl drops of Vero cell conditioned and non-conditioned SOF-BE1 covered by paraffin oil were made and presumptive zygotes were transferred directly to droplets. Subsequent treatments on day 3 and day 5 were administered via exchange of 25 µl of Vero cell conditioned or non-conditioned media from each droplet with fresh Vero cell conditioned and non-conditioned media. This method was utilized to reduce the amount of handling each embryo experienced to ultimately reduce culture stress and improve overall blastocyst development. In the study with treatment administration on day 5, embryo culture plates with 50 µl drops of Vero cell conditioned and non-conditioned SOF-BE1 covered by paraffin oil were made and healthy embryos with >4 cells were harvested on day 5 post-fertilization and transferred to fresh 50 µl droplets.

Embryo assessment and differential immunofluorescent staining

Embryo development was assessed on day 3, 5, 7, and 8. Cleavage rates were examined on day 3 and calculated as the number of embryos that have cleaved (>2-cells) over the total

number of embryos present and multiply this by 100. Morula were examined on day 5 and classified as early or compact morula, and morula rates were calculated as the number of morulae over the total number of cleaved embryos and multiply this by 100. Blastocysts were examined on day 7 and day 8 and classified as early blastocyst, regular, expanded, or hatched. Blastocyst rates were calculated as the number of blastocysts over the number of total cleaved embryos present and multiply by 100.

At day 8 post-fertilization, inner cell mass and trophectoderm cell numbers were determined in a representative subset of blastocysts. All blastocysts were utilized in replicates where blastocyst numbers were low (<8/treatment group/replicate), and in replicates where blastocyst numbers were high a representative subset of the types (regular, expanded, or hatched) present in each treatment group were sampled (up to 15 blastocysts per treatment). Sometimes abnormal blastocysts were identified after staining (<64 total cells). These blastocysts were excluded from analysis and considered outliers because their total cell counts were abnormally lower than others and because they were abnormally lower than the expected total cell numbers in a bovine blastocyst, which begins blastulation after the 64-cell stage (Hosseini et al., 2015).

Embryos were fixed in 4% paraformaldehyde for 15 minutes at room temperature, permeabilized in 0.25% Triton-X for 20 minutes, and blocked with 10% horse serum for 1 hour at room temperature. Embryos were then incubated overnight at 4ºC with anti-CDX2 primary antibody (Biogenex, AM392-5 M, sold ready to use). The next morning embryos were washed and incubated for 1 hour at room temperature with either donkey anti-mouse FITC or Alexa Fluor 647 (Invitrogen, A16018 or A131571) at a 1:200 dilution for either. Embryo DNA was then stained with DAPI (1 µg/ml) for 5 minutes at room temperature. Embryos were then placed in PBS-PVP and imaged immediately by flattening on a glass slide lined with a thin layer of

petroleum jelly. Immunoreactive complexes and embryo DNA were visualized by using an Eclipse Ti-E inverted microscope equipped with an X-cite 120 epifluorescence illumination system. Images were obtained with a DS-L3 digital camera and assembled with NIS-Elements Software (Nikon Instruments, Melville, NY, USA). The program FIJI was used to label and record individually stained nuclei. By using the cell counter plug-in, nuclei stained for CDX2 and DAPI were counted. CDX2+ cells indicated TE, and CDX2- and DAPI+ cells indicated ICM.

Statistical analyses

All analyses were completed by least-squares ANOVA using the general linear model of the Statistical Analysis System (Proc GLM; SAS for Windows, version 9.4; SAS Institute Inc., Cary, NC, USA). IVP replicate was used as the experimental unit in embryo development analysis and individual embryos were used as the experimental unit in differential staining analysis. Main effects in the model included treatment and replicate. Pairwise comparisons were completed using the Probability of Difference (PDIFF) option in SAS. Blastocyst formation rates were arcsine transformed before analysis, but are presented as non-transformed means and SEM.

Results

Study a: Conditioned media treatment at day 5 post-fertilization

In this first study, embryos were transferred from SOF droplets to Vero cell conditioned media droplets on day 5. The day 5 time point was chosen because it corresponds to the initiation of blastomere compaction and ICM and TE specification in bovine embryos, as well as corresponding to the day that the bovine embryo would first enter the uterus in vivo (Betteridge and Fléchon, 1988). Both day 7 and day 8 blastocyst formation was enhanced with Vero cell conditioned media treatment (Figure A-1a). This effect also resulted in increased percentages of

blastocysts reaching the expanded blastocyst stage at day 8 post-fertilization in Vero cell conditioned media treated embryo groups (Figure A-1b). Conditioned media treatment had no effect on ICM cell numbers ($P>0.05$), though increased total cell numbers ($P=0.0002$) through increasing TE cell numbers ($P<0.0001$) (Figure A-2). This also resulted in a decreased ICM:TE cell ratio in treated blastocysts ($P=0.0006$).

Study b: Conditioned media treatment at day 1, 3, and 5 post-fertilization

This follow up study began conditioned media treatment day 1 post-fertilization (beginning of embryo culture) to test the effect of Vero cell conditioned media on cleavage rates at day 3, as well as treatment at day 3 post-fertilization to assess if supplementation beginning earlier in development could additionally enhance blastocyst formation and/or ICM and TE cell numbers as the embryonic genome is being activated (8 to 16 cell stage, or about day 3 in cattle) (Graf et al., 2014a). Both day 7 and 8 blastocyst formation were enhanced by Vero cell conditioned media supplementation on days 1, 3, and 5 (Figure A-3a). An increase in percentages of blastocysts reaching the expanded blastocyst and hatched blastocyst stages was seen numerically, though statistically insignificant (Figure A-3b). When compared to day 7 and 8 blastocyst rates in the day 5 study it was found that both day 7 and day 8 blastocyst formation was greater in the day 5 study ($P=0.005$). The distribution of blastocyst stage on day 8 was the same between the two treatments. Conditioned media treatment at day 1, 3, and 5 had no effect on total cell number or ICM cell numbers. There was a tendency for conditioned media treatment to increase cell numbers ($P=0.09$) which had a significant effect on ICM:TE cell ratios ($P=0.03$) with decreased ratios in treated blastocysts (Figure A-4). When compared to ICM, TE, and total cell numbers obtained in the day 5 study no differences were found between the two studies ($P>0.05$).

Discussion

More IVP embryos are being produced and transferred each year, however, it is well known that *in vitro* produced embryos are of sub-par quality and have higher rates of losses post-transfer (Ealy et al., 2019). Use of embryokine supplementation to embryo culture has been growing in interest, as it more accurately recapitulates the *in vivo* environment. Of particular interest, is IL6. IL6 is unique and that when it is supplemented during embryo culture will result in blastocysts with increased number of ICM cells, working through a STAT3 dependent pathway (Wooldridge et al., 2019; Wooldridge and Ealy, 2019). This work sought to establish if IL6 is the sole driver of improved ICM development or if IL6 is one of multiple factors that can induce STAT3 activity and improve ICM development.

We hypothesized that the addition of Vero cell conditioned media during embryo culture will increase ICM cell numbers and that IL6 is one of many factors acting through STAT3 to improve ICM development and maintenance. The Vero cell line was established from the kidney of a normal adult African green monkey and has the morphology of an epithelial cell type (Ammerman et al., 2008). These cells are known to secrete known embryokines produced by the reproductive tract epithelium, including LIF, IL6, and PDGF (Kauma and Matt, 1995; Carnegie et al., 1997; Desai and Goldfarb, 1998). Thus, we chose to utilize a Vero cell conditioned media system to model the uterine secretome *in vitro.*

Opposite of the hypothesized effect, an increase in TE cell numbers as well as an improvement in blastocyst formation on both day 7 and day 8, rather than an increase in ICM cell numbers was observed. While the untreated control group had similar ICM and TE cell numbers and blastocyst formation rates observed when compared to Wooldridge and Ealy (2019), the IL6 treated group and Vero cell conditioned groups exhibited opposite effects on the

ICM and TE. Additionally, blastocyst rates observed in this study were drastically improved with Vero cell conditioned media, while improvements in blastocyst formation were not observed in Wooldridge and Ealy (2019). However, blastocyst formation rates were calculated differently between the two studies and could lend to inaccurate comparisons. This could be due to a much more complex culture milieu provided by Vero cell conditioned media. Factors other than IL6 are present and likely having an effect on embryo development during the culture period, and likely rather than overall impacting ICM development the group of embryokines present are impacting TE development. From the present data it is unknown what molecules are present providing this effect on the TE, and if they are impacting TE differentiation, TE proliferation, or preventing TE apoptosis, however, future studies should tease apart what is occurring here.

Building from these results future studies should work to assess what molecules are present in Vero cell conditioned media, tease apart how the trophectoderm is being impacted by conditioned media and evaluate conditioned media impacts past day 8. Further work could begin with a proteomic profiling of the Vero cell secretome. Following identification of a group of potential embryokines, antibody capture studies could be performed to assess impacts of conditioned media with one or a group of potential embryokines knocked down in order to tease apart individual molecule impacts on bovine trophectoderm development. Additionally, to assess the impact of Vero cell conditioned media past day 8 of embryo development a cow trophoblast cell line (CT1) could be used. Vero cells could condition CT1 growth media. Proliferation and apoptosis assays could then be performed. Alternatively, trophectoderm outgrowths could be performed. Outgrowth formation and diameter could be assessed to determine the influence of conditioned media on bovine trophectoderm maintenance and growth past day 8. Lastly, eventual transfers into sheep and subsequent flushing to assess elongation capacity could be performed.

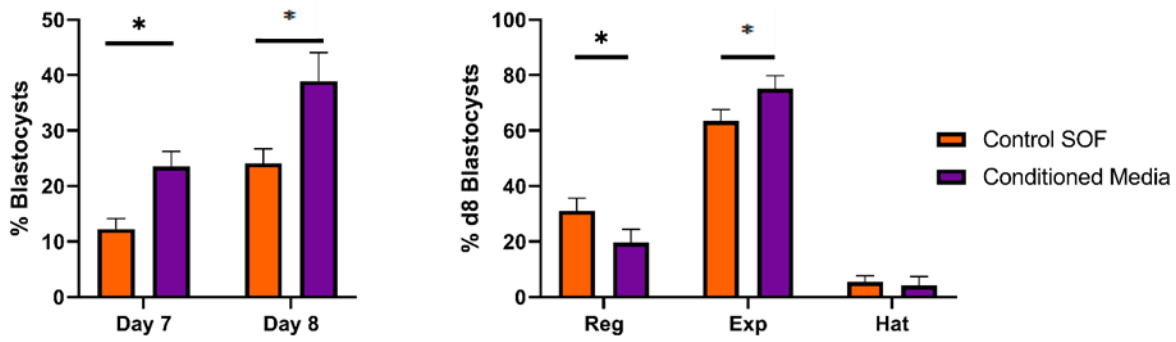

Figure A-1. Effect of Vero cell conditioned media treatment on day 5 post-fertilization on embryo development. *Panel A:* Percent blastocysts on day 7 and day 8 post-fertilization. *Panel B*: Distribution of blastocyst stages on day 8 post-fertilization. Asterisk (*) indicates an effect of treatment ($P < 0.05$).

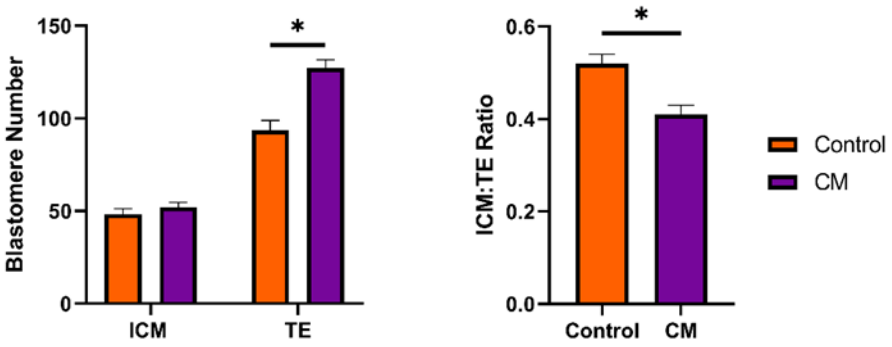

Figure A-2. Effect of Vero cell conditioned media treatment on day 5 post-fertilization on blastomere cell numbers and cell type distribution in blastocysts collected at day 8 post-fertilization. *Panel A*: ICM and TE cell numbers in blastocysts harvested at day 8. *Panel B*: ICM:TE cell ratio. Asterisk (*) indicates an effect of treatment ($P < 0.05$).

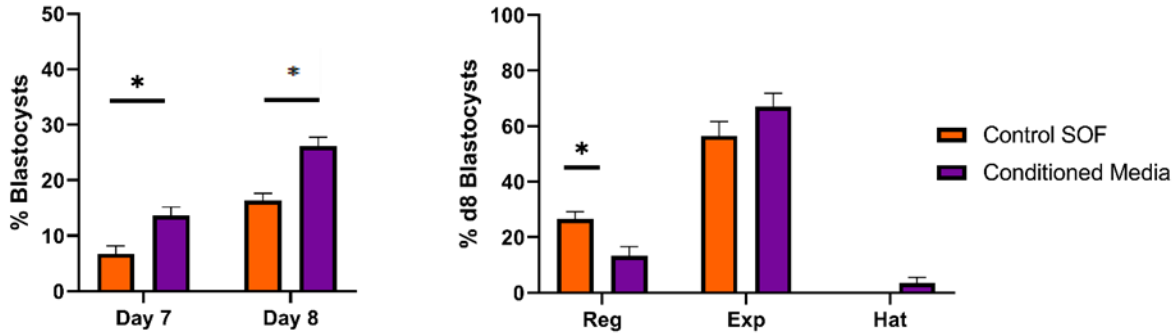

Figure A-3. Effect of Vero cell conditioned media treatment on days 1, 3, and 5 post-fertilization on embryo development. *Panel A*: Percent blastocysts on day 7 and day 8 post-fertilization. *Panel B*: Distribution of blastocyst stages on day 8 post-fertilization. Asterisk (*) indicates an effect of treatment ($P < 0.05$).

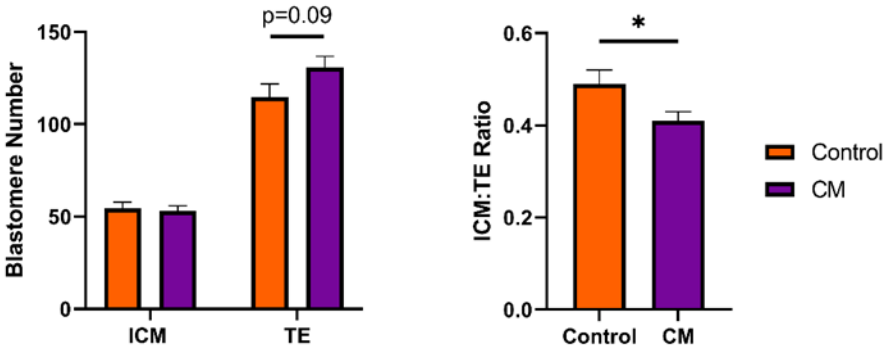

Figure A-4. Effect of Vero cell conditioned media treatment on days 1, 3, and 5 post-fertilization on blastomere cell numbers and cell type distribution in blastocysts collected at day 8 post-fertilization. *Panel A*: ICM and TE cell numbers in blastocysts harvested at day 8. *Panel B*: ICM:TE cell ratio. Asterisk (*) indicates an effect of treatment ($P < 0.05$).

Appendix B

Vero cell culture growth, maintenance, and conditioned media treatment preparation protocol

Propagation of Vero cell culture from frozen stocks

Materials:

- Vero cell stock, frozen in liquid nitrogen or at – 80 ºC
- DMEM + 10% FBS
- 25 cm^2 tissue culture flasks with vented caps
- 10 ml serological pipette
- Pasteur pipette

Procedure:

1. Add 8 mL mL DMEM + 10% FBS to 25 cm2 tissue culture flask.
2. Quickly thaw cryovial of Vero cells by gently swirling in 37 ºC water bath. Will take about 1-2 minutes. *Make sure to keep cap of the cryovial out of the water to reduce potential contamination*
3. Decontaminate vial with ethanol before bringing it into the laminar flow hood.
4. Using Pasteur pipette add about 1 mL DMEM + 10% FBS to cryovial of Vero cells. Then, transfer Vero cell suspension to tissue culture flask.
5. Label side of flask: [initials – date – vero – passage#]
    a. Passage number is the same number as was on the vial that was thawed.
6. Incubate flasks in 37 ºC incubator with 5% CO2.
7. Observe cells daily or every other day. Change media every 2 – 3 days. When cells reach a >90% confluent monolayer, passage cells into new tissue culture flasks.

NOTE: Vero cells recover slowly after freezing, therefore, it may take a week or more before the cells are ready to be passaged. It may take 2 or 3 passages before the Vero cells reach their normal growth rate.

Maintenance of Vero cell culture

Materials:

- Vero cells grown to a confluent monolayer
- DPBS without calcium or magnesium
- 1X trypsin-EDTA in DPBS without calcium of magnesium
- DMEM + 10% FBS
- 75 cm^2 tissue culture flasks with vented caps
- Serological pipettes

Procedure:

1. Remove growth medium from confluent monolayer of Vero cells.
2. Wash cells twice with 10 mL 1X DPBS by gently rocking the flask to allow the solution to wash all cells. When adding DPBS try to minimize number of cells disturbed.
3. Add 3 mL of 1X trypsin-EDTA and incubate cells at 37 ºC for 2-3 minutes, until cells start to streak as they detach from the flask. Tapping or gently shaking the flask may help facilitate cell detachment.
4. Add 7 mL of DMEM + 10% FBS to inactivate the trypsin-EDTA.
5. Wash down cells in media by gently pipetting to break up any clumps of cells.
6. Prepare desired dilution of cells in a total of 10 mL DMEM + 10% FBS and add to 75 cm2 cell culture flasks with vented caps.

a. For a 1:2 dilution: add 5 mL DMEM + 10% FBS to each new culture flask. Then add 5 mL of cell suspension to each flask.
    i. Likely passage the next day
b. For a 1:5 dilution: add 8 mL DMEM + 10% FBS to each new culture flask. Then add 2 mL of cell suspension to each flask.
    i. Likely passage in 2 days
c. For a 1:10 dilution: add 9 mL DMEM + 10% FBS to each new culture flask. Then add 1 mL of cell suspension to each flask
    i. Likely passage in 3 days

7. Incubate flasks in 37 ºC incubator with 5% CO2.
8. Observe cells daily or every other day. Change media every 2 - 3 days. When cells reach a >90% confluent monolayer, passage cells into new tissue culture flasks.

Preparation of frozen stocks of Vero cells

Materials:

- Vero cells grown to a confluent monolayer
- DPBS without calcium or magnesium
- 1X trypsin-EDTA in DPBS without calcium or magnesium
- DMEM + 10% PBS
- DMSO
- Cryovials
- 2, 15 mL conical tubes
- Serological pipette
- Pasteur pipette

Procedure:

1. In a 15 mL conical tube add 0.5 mL DMSO to 10 mL DMEM + 10% FBS.
    a. Want 5% DMSO solution.
2. Remove growth medium from confluent monolayer of Vero cells.
3. Wash cells twice with 10 mL 1X DPBS by gently rocking the flask to allow the solution to wash all cells.
4. Add 3 mL of 1X trypsin-EDTA and incubate cells at 37 ºC for 2-3 minutes, until cells start to streak as they detach from the flask. Tapping or gently shaking the flask may help facilitate cell detachment.
5. Add 7 mL DMEM + 10% FBS to inactivate the trypsin-EDTA.
6. Wash down cells in media by gently pipetting to break up any clumps of cells.
7. Remove cell suspension from flask and transfer to a sterile 15 mL conical tube.
8. Centrifuge at 200 x g (0.200 rcf) for 5 minutes at room temperature.
9. Remove and discard supernatant. Resuspend (well mixed) cells in DMEM + 10% PBS and DMSO that was prepared in step 1.
    a. If doing a 1:4 dilution, add 4 mL of DMEM + 10% PBS + 5% DMSO
10. Aliquot 1 mL of resuspended cells into each labelled cryovial. Label should include: [cell line – passage - date cryopreserved - initials].
11. Freeze cells slowly to -80 ºC, then continue to store cells at -80 ºC or in liquid nitrogen.
    a. Put vials in Styrofoam holder and place in -80 ºC. Leave in for 2-6 hours (or overnight), then transfer into liquid nitrogen storage.

NOTE: It is ideal to freeze the cells with the temperature decreasing at a rate of -1 ºC per minute. Alternatively, the cells can be put at 4 ºC for several hours, then at -20 ºC overnight, then at -80 ºC overnight, and then at -80 ºC or transferred into liquid nitrogen storage.

Conditioned medium treatment preparation

Medium Conditioning:

1. Remove growth medium from monolayer of Vero cells.
2. Wash cells twice with 10 mL 1X DPBS by gently rocking the flask to allow the solution to wash all cells.
3. Add 2.5 mL of 1X trypsin-EDTA and incubate cells at 37 ºC for 2-3 minutes, until cells start to streak as they detach from the flask. Tapping or gently shaking the flask may help facilitate cell detachment.
4. Add 7.5 mL of DMEM + 10% FBS to inactivate the trypsin-EDTA.
5. Wash down cells in media by gently pipetting to break up any clumps of cells.
6. Transfer cell suspension to a 15 mL conical tube.
7. Centrifuge at 200 x g (0.2 rcf) for 5 minutes at room temperature (23ºC).
8. Remove and discard supernatant. Aim to have 250-500 µL left in conical tube.
9. Resuspend cells and determine total cell number using a hemocytometer:
    a. Prepare hemocytometer
        i. Clean surface of hemocytometer slide and coverslip with 70% alcohol.
    b. Mix remaining cells.
    c. Remove 2 µL of cell suspension and combine with 18 µL DMEM in a small tube.
    d. Add 10 µL of cell-DMEM mixture to the hemocytometer

e. Place hemocytometer under microscope and count cells in 5 cells within the blue-shaded region in the diagram below.
    i. If cells are not evenly distributed, wash and reload the hemocytometer.
f. Calculate total cell number
    i. Determine cells per milliliter using the following equation:
        1. Cells/mL = (cell count) * (500,000)
        2. Cells/mL = (cell count in large square) * (df)*(10^4)
            a. Df = dilution factor = (final volume) / (initial volume) where final volume = aliquot volume + diluent volume
            b. 10^4 is the volume correction factor for the hemocytometer
    ii. Multiply cell concentration calculated above by the volume left in the conical tube to obtain total cell number.

10. Dilute cell suspension with 2 mL DMEM media to achieve a seeding density of 3,000,000 cells and use this diluted cell suspension to seed a T25 flask.
    a. Use the following equation to determine how many microliters of cell suspension to add to the 2 mL of DMEM:
        i. Vol = (3,000,000 * volume cell suspension in conical tube) / (total cell number)
11. Incubate flask for 24 hours in 37 ºC incubator with 5% CO2.
12. After 24 hours (day 3), record confluency. Want to be in the range of 30-60% confluent.
13. Remove DMEM from flask and wash with 1 mL SOF-BE1. Then, add 2 mL of SOF-BE1. Also add 2 mL SOF-BE1 to a T25 flask that does not contain cells to obtain a negative control.

14. Incubate both flasks for 48 hours in 37 ºC incubator with 5% CO2.
15. After 48 hours, sterile filter Vero cell conditioned SOF-BE1 and non-conditioned SOF-BE1 using a 10 mL Luer Lock syringe that lacks a rubber stopper and a 0.20 µm, 25 mm syringe filter.
    a. Transfer condition media and SOF control media to two different round dishes. Mix 1:1 with fresh SOF-BE1 before sterile filtering.
    b. Add 3-5 mL of air to the syringe before pulling up the medium. When you pass the medium through the filter this air will help to remove any residual liquid remaining in the filter.
    c. Directly pull up medium with the syringe, attaching a needle may break up any debris making it easier for it to pass through the filter.
16. Freeze any leftover conditioned media in 0.5 mL aliquots. Store in -80 ºC freezer.

<u>Preparing SOF drops and adding embryos:</u>

1. Prepare SOF drops:
    a. Pipette 50 µL drops of SOF-BE1 into 4-well NUNC plates.
        i. Use 1 drop per well of 4-well NUNC plate
        ii. For each plate, 2 wells contain drops with a 1:1 mix of Vero cell conditioned and fresh SOF-BE1 media, and 2 wells contain drops with a 1:1 mix of SOF-BE1 from control flask and fresh SOF-BE1.
    b. Flood wells with mineral oil so that SOF drop is completely covered.
    c. Place well plate(s) into incubator to warm up at 38.5 °C for 1-2 hours before use.
2. Transfer of embryos into SOF drops:

a. Using a Wiretrol or Captrol, remove embryos from one drop and transfer to new conditioned media drop.
    i. Aim to have 20-30 embryos per drop
    ii. Work with one plate at a time so that SOF drops are not exposed to atmospheric air any longer than is necessary.

3. Place SOF plates in Tri-gas chamber. Flush with Tri-gas for 3-5 minutes, close valves and seal chamber, and then place chamber back into incubator at 38.5 °C.

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