DIETARY ARGININE SUPPLEMENTATION ENHANCES PLACENTAL WATER TRANSPORT AND ANGIOGENESIS IN GESTATING GILTS

A Thesis

by

CASSANDRA MARIE HERRING

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Chair of Committee,	Guoyao Wu
Co-Chair of Committee,	Fuller W. Bazer
Committee Members,	Gregory A. Johnson
	Courtney L. Daigle
Head of Department,	G. Cliff Lamb

August 2018

Major Subject: Physiology of Reproduction

ABSTRACT

This study was conducted to test the hypothesis that dietary supplementation with 0.4% L-arginine between days 14 and 30 of gestation would enhance survival and development of conceptuses (embryo and its extra-embryonic membranes) in gilts. Gilts were bred at the onset of second estrus and fed twice daily 1 kg of a corn- and soybean meal-based diet containing 12% crude protein beginning on day 0 of gestation (the day of breeding). Either 0.4% L-arginine or an isonitrogenous amount of L-alanine (control) was supplemented to the basal diet from days 14 to 30 of gestation. At day 30 of gestation, gilts were hysterectomized and euthanized to obtain uteri, conceptuses and fetal fluids. Placental water transport was determined by using Ussing chambers. Concentrations of AAs in fetal fluids and maternal plasma were determined by HPLC. Total RNA and protein were extracted from the frozen tissues. Quantitative RT-PCR and western blotting were performed to determine the changes in gene expression at mRNA and protein levels. Porcine trophectoderm (PTr2) cells were cultured in medium with different concentrations of arginine to determine water transport and cGMP production.

Compared to the control group, arginine supplementation increased ($P < 0.05$) embryonic survival from 87.3% in the control group to 96.5% in the arginine group. Allantoic fluid volume was increased by 25% and amniotic fluid volume was increased by 48% in the arginine group. The placentae of arginine-supplemented gilts were more vascularized in terms of the number and size of blood vessels. Compared to the control

group, arginine supplementation increased ($P < 0.05$) the number of placental blood vessels; placental expression of angiogenic factors VEGFA120, VEGFR1, VEGFR2, eNOS, PlGF, GTP-CH and FGF-2; and placental transport of water. We conclude that dietary arginine supplementation to pigs between days 14 and 30 of gestation improves survival and development of the conceptuses through stimulating placental water transport and angiogenesis.

ACKNOWLEDGEMENTS

I would like to extend my utmost gratitude to my mentors, Drs. Guoyao Wu and Fuller W. Bazer, for their continuous support and guidance during my graduate study at Texas A&M University. I could not have chosen a better lab group to become a part of. They have made numerous contributions to their fields of study and have been great role models as scientists. They both have a passion for research and teaching and getting the opportunity to learn from them is greatly appreciated. I would also like to thank my committee member Dr. Gregory A. Johnson for his continuous help and support as well. This project could not have been completed without the help of him and his lab. Dr. Johnson's knowledge and research regarding uterine biology has greatly benefited my graduate training. I would also like to think my committee member Dr. Courtney L. Daigle for all of the wonderful advice and assistance she has provided regarding my interest in pursing a career in the zoo industry. Her prior experience has opened many doors for my future career.

I could have not completed this project without the help of my fellow labmates, graduate students and postdoctoral fellows. Specifically I would like to thank Dr. Shengdi Hu, Dr. Heewon Seo, Dr. Gayan Nawaratna and Dr. Mohamed Elmetwally for the extensive lab training and troubleshooting you have provided. I would also like to thank Ms. Avery Kramer, Mr. Bryan McLendon, Ms. Bin Wang, Mr. Wenliang He, Mr. Daniel Long, Mr. Kyler Gilbreath, Ms. Erin Posey, Ms. Katherine Halloran, Ms. Emily Hoskins, Dr. Yasser Lenis, Ms. Ashley Padgett and Mr. Neil Wu for their countless hours of help.

CONTRIBUTORS AND FUNDING SOURCES

This work was supported by a thesis committee consisting of Dr. Guoyao Wu [advisor], Dr. Fuller W. Bazer [co-advisor] and Dr. Courtney L. Daigle, Department of Animal Science, and Dr. Gregory A. Johnson, Department of Veterinary Integrative Biosciences.

The placental water transport experiment was conducted in collaboration with Dr. Shengdi Hu. Quantitative RT-PCR and Western blotting were completed with the help of Dr. Mohamed Elmetwally, Department of Theriogenology, University of Mansoura, and Dr. Heewon Seo, Department of Veterinary Integrative Biosciences. HPLC analysis was conducted with the help of Dr. Gayan Nawaratna. Immunofluorescence was completed by Mr. Bryan McLendon, Department of Veterinary Integrative Biosciences.

All other work conducted for the thesis was completed by the student independently.

This research was supported by Agriculture and Food Research Initiative Competitive Grant No. 2015-67015-23276 from the USDA National Institute of Food and Agriculture.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Introduction

Pigs experience high rates of embryonic mortality, especially during the peri-implantation period of gestation (Bazer et al. 2011; Edwards et al. 2012). Maternal nutrition plays an important role in embryonic survival and development, especially maternal dietary intake of amino acids (AA) (Wu et al. 2017). Both low and high dietary protein intake can adversely affect fetal development and embryonic death due to deficiencies and imbalances of amino acids (Herring et al. 2018; Ji et al. 2017). Specifically, arginine is a "conditionally essential AA" in the diet that is important for optimal embryonic/fetal development and survival by affecting placental growth and development (Hou et al. 2015; Wu et al. 2010). Arginine produces nitric oxide (NO) and polyamines, which are essential for placental angiogenesis (Wu et al. 2010). Angiogenesis, defined as sprouting new blood vessels from existing ones (Chen and Zheng 2014), is essential for increasing utero-placental blood flow from mother to fetus to exchange water, nutrients, and waste (Reynolds et al. 2006). Arginine is also a precursor for synthesis of polyamines, ornithine, creatine, homoarginine and agmatine (Hou et al. 2016; Wu et al. 2013, 2016). These biologically important molecules are essential for growth and survival of the conceptus (Wu et al. 2017).

Maternal dietary protein intake

Both maternal under-nutrition and over-nutrition can be detrimental to the developing fetus. Specifically, insufficient or excessive maternal dietary protein intake can cause lifelong consequences for the neonate due to fetal programming. Fetal programming refers to the heritable changes in gene expression without changes in DNA sequences within the genome (Ji et al. 2017). Malnutrition alters expression of the fetal genome, leading to metabolic disorders, organ dysfunction, hormonal imbalances and cell signaling defects (Ji et al. 2017).

Amino acids (AAs) are essential for synthesis of proteins and other nitrogenous substances such as catecholamines, creatine, dopamine, nitric oxide (NO), polyamines and thyroid hormones (Wu et al. 2017). Additionally, certain AAs are responsible for regulating cell signaling and metabolic pathways. Low maternal intake of dietary protein is linked to intrauterine growth restriction (IUGR) of the conceptus (embryo/fetus and placenta), as well as reduced postnatal growth and feed efficiency (Ji et al. 2017). The placenta requires adequate levels of AAs for proper growth and development to supply nutrients to the fetus (Wu et al. 2017). With low dietary protein intake, the limited supply of AAs to the placenta results in placental insufficiency and consequently IUGR (Herring et al. 2018). High maternal dietary protein intake is also linked to IUGR and can cause fetal or neonatal death due to ammonia toxicity. Like low intake of dietary protein, high intake results in AA excesses during pregnancy (Ji et al. 2017). In all species studied, including swine, cattle and rodents, high concentrations of ammonia in plasma increase embryonic death (Herring et al. 2018). Ammonia is a product of AA

catabolism, and high dietary protein intake leads to toxic levels of ammonia in plasma (Wu 2013). Excess production of other metabolites of AAs, such as hydrogen sulfde, homocysteine and indoles, may also impair embryonic/fetal survival and growth (Wu 2016; Taylor 2016).

There is growing interest in the functional roles of certain AAs in mammalian pregnancies (Palencia et al. 2018; Wu et al. 2017). One of these AAs is L-arginine (Arg), which has been properly recognized as a "conditionally essential AA" in the diet, especially for embryonic growth and survival (Wu et al. 2016). As noted previously, Arg is a precursor to biologically important substances such as polyamines, ornithine, proline, glutamate, agmatine, creatine and NO (Wu 2013). Arg is also required for hepatic urea synthesis to remove ammonia from the liver and blood. Of interest, this AA regulates protein synthesis in skeletal muscle and placenta by activating mechanistic target of rapamycin (mTOR) signaling, stimulating the secretion of growth hormone and insulin, and enhancing anti-oxidative signaling and the cellular redox state (Bazer et al. 2014; Kong et al. 2012; Kim et al. 2013; Wu 2013). Results of recent studies indicate that Arg enhances placental angiogenesis and growth to improve blood flow across the placenta, thereby increasing nutrient transfer from the mother to her fetus (Wu et al. 2017). NO and polyamines are also necessary for implantation (Chwalisz and Garfield 2000), and they are known to regulate steroid hormone synthesis and stimulate cell proliferation and migration in the conceptus (Bazer et al. 2010; Ducsay and Myers 2011; Kim et al. 2013). Because of these beneficial effects of Arg during gestation, dietary supplementation of Arg has been found to improve reproductive performance and

increase embryonic survival and growth in swine, sheep, rats and mice (Palencia et al. 2018; Wu et al. 2013). For dietary supplementation, Arg is administered as the neutral salt Arg-HCl to maintain a physiological acid-base balance (Wu et al. 2016).

Pigs experience high rates of embryonic loss and neonatal deaths, which are greatly influenced by maternal nutrition (Bazer et al. 2012). For example, 14% crude protein (CP) diets are commonly fed to gestating gilts and sows on many farms worldwide; however, 14-18% CP is considered high dietary protein intake and is detrimental to embryonic development (Ji et al. 2017). This level of dietary protein creates a toxic environment for the fetus due to high levels of ammonia and possibly other metabolites in the plasma (Wu et al. 2013). Also, high dietary protein intake reduces skeletal muscle fiber size and number in newborn piglets (Wu et al. 2006; 2010). For this reason, it is recommended that gestating gilts are fed about 50% of their ad libitum feed intake (Kim et al. 2009). When sows are fed greater than 50% of ad libitum feed intake, there is a significant increase in embryonic loss resulting from gaining excessive subcutaneous white adipose tissue (WAT) and oxidative stress (Ji et al. 2017). Fifty percent of ad libitum feed intake equates to 2 to 2.2 kg of feed per day consisting of 12% crude protein (CP). Consequently, specific AAs are deficient in a 12% CP diet (Ji et al. 2017). Interestingly, 24% of newborn piglets from crossbred sows fed a 12% CP diet are considered IUGR, weighing less than 1.1 kg (Ji et al. 2017). IUGR piglets have an extremely high risk of mortality before weaning (Wu et al. 2006).

Arginine is an example of an AA deficient in a 12% CP diet that is important for proper placental growth (Wu et al. 2013). Also, glutamate, glycine and cysteine are

required for the synthesis of glutathione, which is an essential antioxidant (Wu 2013). Since increasing dietary CP above 12% is detrimental, supplementing certain AAs to the basal diet may help to overcome AA deficiencies (Wu et al. 2017). Placental angiogenesis occurs rapidly between days 20 and 40 of gestation in pigs; therefore dietary Arg supplementation during this period could improve the growth and development of the placental vasculature by increasing NO production (Li et al 2014). Li et al. (2014) found that supplementation of a 2 kg corn-soybean meal based diet with 0.4% or 0.8% Arg between days 14 and 25 of gestation increased placental growth, number and diameter of placental blood vessels and number of viable fetuses by 2 per litter. Also, multiple studies conducted in different countries have shown that Arg supplementation between day 30 of gestation and farrowing increases litter size and litter birth weight (Wu et al. 2013; Wu et al. 2017). Arg supplementation during late gestation (days 90 to 114) also increased birth weights of live piglets (Wu et al. 2017). Supplementation with greater than 2% Arg may cause antagonism between Arg and Lys and increase harmful levels of ammonia (Wu 2013), and consequently none of the beneficial effects seen with lower doses of Arg may be observed.

A study conducted by Rehfeldt et al. (2012) indicated the negative impacts that maternal under-nutrition and over-nutrition have on postnatal growth in IUGR piglets (2012). The gestating gilts fed a high protein diet (30% CP) produced piglets with IUGR. At day 83 of age, the piglets had increased brain weights and decreased thymus and bone weights compared to the offspring of the gilts fed the control diet (12.1% CP). As an organ of the immune system, a small thymus gland may be associated with decreased

immune function (Wu et al. 2012). At day 188 of age, these piglets had increased liver weights compared to the control piglets, which may lead to metabolic dysfunction when they are used for breeding (Rehfeldt et al. 2012). The gestating gilts fed a low protein diet (6.5% CP) also produced piglets with IUGR, but those piglets showed compensatory gain that was maintained from day 83 to day 188 of age (Rehfeldt et al. 2012). However, these piglets had a larger proportion of body fat and decreased numbers of skeletal muscle fibers, skeletal muscle mass and total muscular DNA compared to the control piglets (Rehfeldt et al. 2012). Altmann et al. (2013) found that both low and high protein diets fed to gestating sows affected their expression of metabolic genes including glucocorticoid receptor *NR3C1,* peroxisome proliferator-activated receptor alpha (*PPARα*)*,* insulin receptor (*INSR*)*,* PPAR gamma coactivator 1-alpha (*PGC1α*)*,* 3-hydroxy-3-methylglutaryl-CoA reductase (*HMGCR*) and cytochrome P450 2C34 (*CYP2C34*). Fetal expression of *NR3C1* increased as a consequence of increased glucocorticoid activity in sows fed a low protein diet, which also affected expression of *INSR, PPARα, PGC1α* and *HMGCR* in fetuses from sows fed either a high or low protein diet compared to fetuses from control-fed sows, possibly resulting in changes in lipid metabolism in the offspring.

Both low and high maternal dietary protein intake cause an imbalance of AAs, which may lead to embryonic loss or impaired growth and development of the conceptus. Surviving IUGR neonates likely face lifelong consequences as a result of maternal malnutrition. To overcome the harmful consequences of maternal malnutrition, dietary supplementation with Arg and Gln during specific stages of gestation can help

fulfill the requirements of both mother and fetus (Wu et al. 2010). In numerous animal and human studies, dietary Arg supplementation during pregnancy improved embryonic survival and growth by increasing placental angiogenesis and blood flow, promoting embryonic protein synthesis, preventing IUGR, and increasing litter size.

Placental angiogenesis

Development of the placental vasculature is essential for proper exchange of water, nutrients, respiratory gases and waste between the mother and fetus (Chen and Zheng 2014). The placental vasculature develops via angiogenesis, which is defined as the formation of blood vessels from existing ones (Reynolds and Redmer 2001). Angiogenesis and vasodilation in the placenta, as well as in the uterus, are primarily responsible for increasing utero-placental blood flow (Reynolds et al. 2006). The placenta contains many pro-angiogenic and anti-angiogenic factors for regulation of this process. Important pro-angiogenic factors found in the placenta include vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF2), placental growth factor (PlGF), endocrine gland-derived-VEGF, transforming growth factor-β1 (TGFB1), leptin and angiopoietins (Chen and Zheng 2014). The conventional form of VEGF, or VEGFA, is universally found at angiogenic sites where it can release endothelial cells for migration and proliferation by breaking down the extracellular matrix (Chen and Zheng 2014). VEGF also stimulates NO production from the endothelial cells of the placental arteries (Chen and Zheng 2014). It has been found that placental artery endothelial cells also express VEGF receptors 1 (VEGFR1) and 2 (VEGFR2) (Chen and Zheng 2014). VEGFR2 is the primary VEGF-family receptor associated with

angiogenesis (Nieminen et al. 2014). As a receptor tyrosine kinase, VEGFR2 is activated via auto-phosphorylation and stimulates many signaling cascades responsible for cell survival, proliferation and migration (Nieminen et al. 2014). These pathways include the PI3K/Akt pathway, eNOS and the phospholipase Cγ-MAPK pathway (Nieminen et al. 2014). As a member of the VEGF family, PlGF acts by binding VEGFR1 (Sanchis et al. 2015). FGF-2 is also an important stimulator of angiogenesis in the placenta (Reynolds and Redmer 2001). NO is a potent vasodilator and is necessary for placental angiogenesis (Chen and Zheng 2014). Placental NO comes form the conversion of L-arginine to L-citrulline by endothelial nitric oxide synthase (eNOS) (Chen and Zheng 2014). Vasodilation and angiogenesis are critical for sufficient utero-placental blood flow. By stimulating NO production, VEGF and FGF-2 are also regulators of utero-placental blood flow (Reynolds and Redmer 2001). Improper development of the placental vasculature leads to placental insufficiency, or improper nutrient exchange from the mother to the fetus, which may result in IUGR or embryonic/fetal death (Herring et al. 2018).

Placental water transport

Aquaporins (AQP) are plasma membrane proteins that allow for rapid transport of water across membranes, and they are also essential for placental development (Zhu et al. 2015). Water requirements of the fetus are very high due to its exponential rate of growth (Damiano 2011). However, the transport of substances across the placenta is very selective indicating the importance of AQP for placental water transport (Damiano 2011). The maternal-fetal fluid balance is critical for maintaining homeostasis of

amniotic fluid, which is positively correlated with embryonic survival and growth (Zhu et al. 2015). There are 13 isoforms of mRNAs for AQP that have been discovered in mammals, 12 of which are expressed in the female reproductive tract (Zhu et al. 2015). These isoforms can be categorized into three subgroups: classical aquaporins, aquaglyceroporins and super-aquaporins (Damiano 2011). The classical aquaporins (AQP 0, 1, 2, 4, 5, 6 and 8) are highly selective to water transport, but some are also permeable to CO_2, O_2, NO and anions (Zhu et al. 2015). The aquaglyceroporins (AQP 3, 7, 9, and 10) are selective to transport of water, glycerol, urea, ammonia and other small solutes (Zhu et al. 2015). The role of super-aquaporins (AQP 11 and 12) is unclear (Zhu et al. 2015). AQP expression is mediated by multiple signaling pathways, including cAMP, mitogen-activated protein kinases (MAPK), protein kinase C (PKC), and phosphatidylinositide 3-kinases (PI3K)/protein kinase B (Akt)/mechanistic target of rapamycin (mTOR). At present, little is known about effects of AA nutrition on expression of AQPs in placental tissues of any species.

Summary and objective

Regulation of maternal dietary protein intake during pregnancy is essential for proper embryonic survival, growth and development. Specific AAs are required for certain processes involved in pregnancy, including implantation, placental growth and angiogenesis, and the transfer of water and nutrients from mother to fetus (Wu et al. 2006). Understanding the role of maternal dietary protein intake can have great economical benefits in the livestock industry by increasing reproductive success and litter size. Although the timing and dose of arginine supplementation to pregnant gilts

has been studied, the exact mechanism through which it increases embryonic survival is not fully understood. This study was conducted to test the hypothesis that dietary supplementation with 0.4% L-arginine to gilts between days 14 and 30 of gestation would increase embryonic survival and development by stimulating placental angiogenesis and placental transport of water.

CHAPTER II

DIETARY SUPPLEMENTATION OF 0.4% ARGININE BETWEEN DAYS 14 AND 30 OF GESTATION ENHANCES CONCEPTUS SURVIVAL, GROWTH AND DEVELOPMENT IN GILTS

Introduction

Arginine is a "conditionally essential AA" in the diet that has important functions that affect placental growth and development and, therefore, is important for optimal conceptus development (Hou et al. 2015; Wu et al. 2010). Arginine is a precursor for synthesis of polyamines, ornithine, creatine, agmatine and nitric oxide (NO) (Hou et al. 2016; Wu et al. 2013, 2016). NO is essential for placental angiogenesis, which is defined as sprouting new blood vessels from existing ones (Wu et al. 2010; Chen and Zheng 2014). Placental angiogenesis is necessary for increasing utero-placental blood flow from mother to fetus to exchange water, nutrients and waste (Wu et al. 2010).

Previous studies have shown that dietary supplementation with arginine to gilts during specific periods of gestation can decrease embryonic mortality. Mateo et al. (2007) reported that supplementation of 0.83% L-arginine between days 30 and 114 of gestation increased litter size in gilts by 2. Li et al. (2014) found that supplementation of 0.4% and 0.8% L-arginine between days 14 and 25 of gestation also increased litter size in gilts by 2 and the volume of amniotic fluid in conceptuses. However, supplementation with 0.8% Arg from days 0 to 25 impaired embryonic survival showing the importance of timing of arginine supplementation. Supplementation of arginine too early

compromised follicle development on the ovaries, which reduced the number of corpora lutea (CL) and concentrations of progesterone in maternal plasma (Li et al. 2010).

Aquaporins (AQP) also affect exchange of water and nutrients from mother to fetus by allowing for rapid transport of water across membranes (Zhu et al. 2015). There are 13 isoforms of mRNAs for AQP that have been discovered in mammals, and 12 have been discovered in the female reproductive tract (Zhu et al. 2015). AQP expression is regulated by several different signaling pathways, such as cAMP, mitogen-activated protein kinases (MAPK), protein kinase C (PKC), and phosphatidylinositide 3-kinases (PI3K)/protein kinase B (Akt)/mechanistic target of rapamycin (mTOR). Little is known about the effects of AA nutrition on expression of AQPs in placental tissue of any species, but previous studies have found that arginine supplementation increases expression of mTOR (Li et al. 2014).

This study was conducted to test the hypothesis that dietary supplementation with L-arginine between days 14 to 30 of gestation would ameliorate the high rates of embryonic loss that pigs experience during the peri-implantation period by increasing expression of pro-angiogenic factors and AQPs (Edwards et al. 2012).

Materials and methods

Experimental design

Fourteen sexually mature crossbred gilts (Yorkshire X Landrace sows and Duroc X Hampshire boars) were bred at onset of their second estrus and 12 h later. Day of breeding was recorded as day 0 of gestation. Gilts were group-housed and had free access to water throughout the experiment. Following breeding, gilts were assigned

randomly to one of two treatment groups, 0.0% arginine (control) or 0.4% arginine (Ajinomoto Co., Inc., Tokyo, Japan), with 7 gilts in each treatment group. All gilts were fed 1 kg of a corn- and soybean meal-based diet containing 12% crude protein twice-daily beginning on day 0 of gestation. The basal diet contained 0.7% arginine, as analyzed by HPLC (Li et al. 2010). Gilts were fed individually and either 0.4% L-arginine or an isonitrogenous amount of L-alanine was supplemented to the basal diet between days 14 and 30 of gestation. An isonitrogenous amount of 0.83% L-alanine (as L-alanine-HCl, Ajinomoto Co., Inc., Tokyo, Japan) and 0.43% cornstarch was added to the 0.0% and 0.4% arginine diets, respectively, as top dressing.

Hysterectomy and tissue collection

On day 30 of gestation, gilts were fed either L-arginine-HCl or L-alanine, and then euthanized and hysterectomized within 30 min of death. Gilts were anesthetized with an intramuscular injection of 10 mg Telazol (Zoetis, USA) per kg of body weight and then a surgical plane of anesthesia was established and maintained with inhalation of 1-5% isofluorane (Wu et al. 1996). Blood was collected from both uterine vein and artery before euthanasia, and after euthanasia gilts were hysterectomized to obtain uteri and conceptuses. Euthanasia was achieved by intra-cardiac injection of saturated KCl. The number of CL, number of live fetuses, placental weight, fetal body weight, fetal length, fetal liver weight, volumes of amniotic and allantoic fluid, and number and diameter of placental blood vessels was measured as described previously (Wu et al. 1996, 2017). For each variable, the mean of the six placental measurements was calculated to represent the value for the gilt. Samples of placenta, endometrium, CL, fetal liver and

muscle, and maternal tissues was either snap frozen in liquid nitrogen, fixed in 4% paraformaldehyde, or embedded in OCT and snap frozen in liquid nitrogen.

Placental vasculature measurements

Briefly, a picture was taken of each placenta. Three placentae (from the first, middle, and last fetuses within the left uterine horn) and three placentae (from the first, middle, and last fetuses within the right uterine horn) from each gilt were used to count the total number of blood vessels per 1 cm^2, and to measure the diameter of the central blood vessel under a microscope (40×objective).

Determination of placental and PTr2 water transport

Transport of ^3H_2O was measured using Ussing chambers (Physiologic Instruments, San Diego, CA) containing 5 ml of oxygenated (95% O_2/5% CO_2) Krebs buffer, as well as physiological concentrations of amino acids and glucose. Sections of placental (chorioallantois) tissue (1 cm^2) were mounted onto Ussing chambers, followed by the addition of 0.2 μCi ^3H_2O to the "mucosal" side of each chamber. Thereafter, 20 μl aliquots of solution were obtained from the "serosal" side of the chamber at 5, 10 and 15 min to determine transport of ^3H_2O across the placenta. Radioactivity was determined using a liquid scintillation counter (Li et al. 2016).

RNA extraction, reverse transcription and quantitative PCR

Placental tissue was homogenized with TRIzol (Invitrogen, USA), and RNA was extracted with chloroform and precipitated with isopropanol. RNA was washed with 75% ethanol. Total RNA was measured using a NanoDrop ND 1000 spectrophotometer. The cDNA was synthesized using the SuperScript First Strand Synthesis System for RT-

PCR (Invitrogen, USA). RT-qPCR was performed using the SYBR Green and the Applied Biosystems 7900HT Real Time PCR system. Tubulin was used as the housekeeping gene (Steinhauser et al. 2017). The sequences of forward and reverse primers used are provided in Table 2.1. The relative expression values were calculated using the $\Delta\Delta Ct$ method (Fu et al. 2006).

Table 2.1 Sequences of primers used for quantitative RT-PCR.

Gene	Primer sequence	Accession number
eNOS3	Forward: 5'- ATCTTCAGCCCCAAACGGAG -3'	NM_214295.1
	Reverse: 5'- TTTCCACCGAGAGGACCGTA -3'	
VEGF120	Forward: 5'- AAGGCCAGCACATAGGAGAG -3'	KJ729036
	Reverse: 5'- CCTCGGCTTGTCACATTTTT-3'	
VEGF164	Forward: 5'- GAGGCAAGAAAATCCCTGTG -3'	NM214084
	Reverse: 5'- TCACATCTGCAAGTACGTTCG- 3'	
VEGFR1	Forward: 5'- CACCCCGGAAATCTATCAGATC -3'	EU714325.1
	Reverse: 5'- GAGTACGTGAAGCCGCTGTTG -3'	
VEGFR2	Forward: 5'- GAAATGGCTTCATCCTCCAA -3'	AF513909.1
	Reverse: 5'- CAAGGAAGACTTGGCTCAGG -3'	
GTP-CH-1	Forward: 5'- AGTTCTTGGCCTCAGCAAAC -3'	XM_021102249.1
	Reverse: 5' TGCTTCAACCACTACTCCGAC -3'	
PlGF	Forward: 5'- CATCGTGTCTGTGTACCCCA -3'	FJ177137.1
	Reverse: 5'- TGACATTGACCGTCTCCACG -3'	
FGF-2	Forward: 5'- GTGCAAACCGTTACCTTGCT -3'	NM_001001855.2
	Reverse: 5'- ACTGCCCAGTTCGTTTCAGT -3'	
AQP1	Forward: 5'- TTGGGCTGAGCATTGCCACGC -3'	(68)
	Reverse: 5'- CAGCGAGTTCAGGCCAAGGGAGTT -3'	
AQP2	Forward: 5'- TCAACCCTGCCGTGACTGTAG -3'	EU636238.1
	Reverse: 5'- GTTGTTGCTGAGGGCATTGAC -3'	
AQP3	Forward: 5'- ACCCTTATCCTCGTGATGTTT -3'	HQ888860.1
	Reverse: 5'- CATTCGCATCTACTCCTTGTG -3'	
AQP4	Forward: 5'- TCTGGCTATGCTTATCTTTGTCC -3'	NM_001110423.1
	Reverse: 5'- CGATGCTAATCTTCCTGGTGC -3'	
AQP5	Forward: 5'- TGAGTCCGAGGAGGATTGGG -3'	NM_001110424.1
	Reverse: 5'- GAGGCTTCGCTGTCATCTGTTT -3'	
AQP6	Forward: 5'- TCTGGATGACTGTCAGCAAAGC -3'	NM_001128467.1
	Reverse: 5'- TCTCTCGGATGTCCTCAGGTATG -3'	
AQP7	Forward: 5'- ATAAGGCACTTCAGCAGACATC -3'	NM_001113438.1
	Reverse: 5'- AAACTTCTTCCAGGACATTCG -3'	
AQP8	Forward: 5'- GGTGCCATCAACAAGAAGACG -3'	EU220426.1
	Reverse: 5'- CCGATAAAGAACCTGATGAGCC -3'	

Table 2.1 continued

Gene	Primer sequence	Accession number
AQP9	Forward: 5'- TTTGCTGATGGAAAACTGCTC -3'	NM_001112684.1
	Reverse: 5'- CTCTGGTTTGTCCTCCGATTGT -3'	
AQP10	Forward: 5'- TGGGCGTTATACTAGCCATCTAC -3'	EU582021
	Reverse: 5'- GGTTGGGCACAGTTTACTTCCT -3'	
AQP11	Forward: 5'- CGTCTTGGAGTTTCTGGCTACC -3'	EU220425
	Reverse: 5'- CCTGTCCCTGACGTGATACTTG -3'	

Western blot analyses

Frozen placentae (approximately 200 mg) were homogenized at 4^{o}C with a model PRO200 homogenizer (PRO Scientific, Oxford, CT) in 1.5 ml of lysis TM buffer (pH 7.9) containing $MgCl_2$, KCl, EDTA, sucrose, glycerol, sodium deoxycholate, NP-40, sodium orthovanadate, and a protease inhibitor cocktail (Set I, Calbiochem, La Jolla, CA). The lysates were centrifuged at 10,000 x g and 4°C for 5 min. The supernatant fluid was transferred to 1.5 ml tubes and centrifuged at 12,000 x g and 4°C for 5 min. The supernatant fluid was used for protein assay and western blot analyses. Protein concentrations were measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc., USA) with bovine serum albumin as the standard. Proteins were denatured in 2X Laemmli Sample Buffer (Bio-Rad, Hercules, CA) with 10% mercaptoethanol. Denatured proteins (30 μg) were loaded into Any kD™ Mini-PROTEAN® TGX™ Precast Gels (Bio-Rad, USA). Electrophoresis was conducted at 120V for 70 min in Electrophoresis Buffer (25mM Tris, 192 mM glycine, and 10% SDS). Proteins were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA) in Transfer Buffer (25 mM Tris, 192 mM glycine, and 20% methanol) at 100V for 75 min using the Bio-Rad Transblot apparatus (Hercules, CA). Membranes were blocked in 5% nonfat dry milk or 5% BSA, which were dissolved in Tris-buffered saline-Tween solution (TBST; 20 mM Tris, 150 mM NaCl, pH 7.6, and 0.1% Tween-20) for 1 h at room temperature. The membranes were incubated with primary antibodies (Table 2.2) overnight at 4°C with gentle rocking. After being washed three times with TBST, the membranes were incubated at room temperature for 1 h with a secondary antibody (1.0

mg/mL Goat Anti-Mouse IgG or Goat Anti-Rabbit IgG, KPL, Gaithersburg, MD) at 1:10,000. Finally, the membranes were washed three times with TBS, followed by development using Supersignal West Dura Extended Duration Substrate according to the manufacturer's instructions (Thermo Fisher Scientific Inc., USA). Western blots were quantified by measuring the intensity of target protein bands using a ChemiDoc XRS system and Quantity One software (Bio-Rad, Hercules, CA). Images for β-actin were used as a loading control to normalize the relative abundance of target proteins.

Table 2.2 Information on antibodies used for western blotting.

Protein	Company	Catalog No.	Dilution	Molecular Weight (kDa)
PlGF	abcam	ab74778	1:1000	25
VEGFR2	abcam	ab2349	1:2000	152
eNOS	Bioss	13074R	1:2000	140
p-eNOS	Cell Signaling Technology	9571S	1:2000	140
GTP-CH-1	Abbiotec	250680	1:2000	32
FGF-2	abcam	ab8880	1:2000	18
p-VEGFR2	Cell Signaling Technology	24785	1:1000	152
VEGFR1				151
AQP1	EMD Millipore	AB2219	1:2000	28, 35-60
AQP2	Santa Cruz Biotechnology	sc515770	1:2000	29, 35-45
AQP4	ThermoFisher	PA5-36521	1:200	35
AQP5	Sigma	A115544	1:2000	42
AQP8	Sigma	SAB1403559	1:2000	54
β-actin	GeneTex	GTX26276	1:2000	43

Immunofluorescence

Paraffin embedded sections (5 μm) of the uterine-placental interface were adhered to slides, deparaffinized and rehydrated in CitriSolv, ethanol, and water. For antigen unmasking, the sections were brought to a boil in a 10mM Sodium Citrate Buffer solution. The sections were then washed in PBS 3 times for 5 min each, blocked with 10% normal goat serum, and then the sections were incubated with rabbit anti-AQP1 (5 μg/ml) or mouse anti-AQP8 (5 μg/ml) overnight at 4°C and detected with fluorescein-conjugated goat anti-rabbit IgG (1:250) for one hour (Chemicon International). Slides were then overlaid with Prolong Gold Anti-fade mounting reagent containing DAPI (Molecular Probes) and a coverslip. Images were taken using an Axioplan 2 microscope (Carl Zeiss, Thornwood, NY) interfaced with a Axioplan HR digital camera. Photographic plates were assembled using Adobe Photoshop (version 6.0, Adobe Systems Inc., San Jose, CA).

Analysis of amino acids, fructose, glucose and glycerol

Amino acids in plasma from uterine arterial plasma and amniotic and allantoic fluids were analyzed by HPLC methods involving precolumn derivatization with o-phthaldialdehyde (Li et al. 2010). Glucose and glycerol in the allantoic fluid, amniotic fluid and maternal plasma from the uterine artery was determined using enzymatic fluorometric methods (Wu et al. 2005; Tekwe et al. 2013). Fructose in the allantoic fluid, amniotic fluid and maternal plasma from the uterine artery was determined using EnzyChrom™ Fructose Assay Kit (BioAssay Systems, Hayward, CA).

pTr2 cell culture

An established porcine trophectoderm (pTr2) cell line was cultured in 75-cm^2 flasks with 15 ml of DMEM/F-12 containing 5% fetal bovine serum (FBS), 1% P/S, 0.05% insulin and 5 ng/mL epidermal growth factor (Kong et al. 2012). The medium was changed every 2 days, and when cells reach confluence they were collected using 0.125% trypsin. Cells were then used for determination of cGMP production.

Determination of cGMP production in pTr2 cells

The pTr2 cells were seeded onto a 96-well plate in Arg-free DMEM at between 10^4-10^6 cells/ml and incubated overnight at 37°C. Then, 0, 0.1, 0.25 or 0.5 mM Arg was added to the medium and cultured for 24 h at 37°C. cGMP production was determined using the Amersham cGMP Enzymeimmunoassay Biotrak (EIA) System (GE Healthcare, Buckinghamshire, UK).

Statistical analyses

Embryonic survival was compared between the two groups using Chi-square analysis (Steel and Torrie 1980). Parameters of reproductive efficiency, concentrations of amino acids, glucose, fructose and glycerol and placental water transport were compared between the two groups, and gene expression and protein expression changes were analyzed using the unpaired t-test (Steel and Torrie 1980). Water transport by PTr2 cells and cGMP production by PTr2 cells were analyzed using one-way ANOVA (Steel and Torrie 1980). Probability values ≤ 0.05 were considered statistically significant.

Results

Reproductive performance of gilts

After supplementation of 0% (control) or 0.4% arginine from days 14 to 30 of gestation, gilts were euthanized and hysterectomized to assess reproductive performance. Maternal body weight, uterine weight, maternal liver weight, number of CL and relative weights of fetal livers were measured at the time of necropsy and **were** not significantly different between the 0.4% arginine-treated and control gilts. Embryonic survival rates were calculated as number of live fetuses per number of CL present on the ovaries at the time of necropsy. Embryonic survival for gilts in the 0.4% arginine-supplemented group was 9.2% greater than that for the control gilts ($P < 0.05$) (Table 2.3). The average allantoic fluid volume (ALF) for gilts in the 0.4% arginine-supplemented group was 25.3% greater than that for gilts in the control group ($P < 0.05$) (Table 2.3). The average amniotic fluid volume (AMF) for gilts in the 0.4% arginine-supplemented group was 48.1% greater than that for gilts in the control group ($P < 0.05$) (Table 2.3).

Table 2.3 Reproductive performance and placental angiogenesis of gilts fed diets supplemented with 0% (control) or 0.4% L-arginine from days 14 to 30 of gestation.

Variable	Control	0.4% Arginine	P-value
No. of corpora lutea	15 ± 0.82	13.4 ± 0.90	0.222
No. of live fetuses	13.2 ± 0.98	12.6 ± 0.64	0.609
Embryonic survival rate, %	87.3 ± 0.04	96.5 ± 0.02*	<0.001
Total volume of allantoic fluid, ml	187.4 ± 10.1	234.8 ± 17.3*	0.045
Total volume of amniotic fluid, ml	1.16 ± 0.07	1.71 ± 0.12*	0.003
Average number of placental blood vessels per cm^2	9.40 ± 0.52	11.7 ± 0.59*	0.015
Average diameter of placental blood vessels, mm	7.15 ± 0.26	8.26 ± 0.32*	0.023

Data are mean values ± SEM, n=6 (control) and n=7 (0.4% arginine). The asterisks indicate that effects of treatment were significant ($P < 0.05$). Embryonic survival was calculated as number of live fetuses per number of corpora lutea present on the ovaries at the time of necropsy on day 30 of gestation.

Placental angiogenesis

Angiogenesis in the placenta was determined by counting the number of blood vessels present and measuring their diameter. The average number of blood vessels per cm^2 of

placentae in the 0.4% arginine-supplemented group was 24.5% greater than that for placentae of gilts in the control group ($P < 0.05$) (Table 2.3). The average diameter of the blood vessels in placentae of the 0.4% arginine-supplemented group was 16% greater than that for placentae of gilts in the control group ($P < 0.05$) (Table 2.3). As shown in Figure 2.1, the placental blood vessels for 0.4% arginine-supplemented gilts (B) were more developed and more abundant than blood vessels in placentae from control gilts (A).

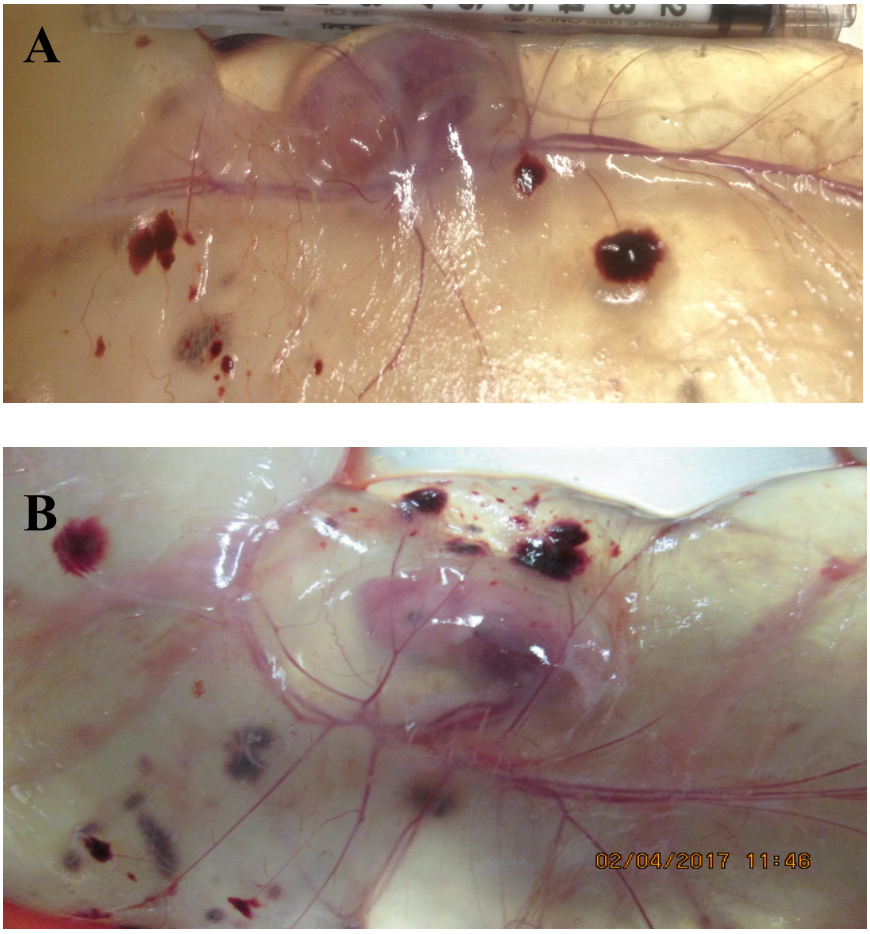


Fig. 2.1 Placental blood vessels at day 30 of gestation in gilts fed diets supplemented with either: (A) 0% (control) or (B) 0.4% arginine.

Concentrations of amino acids in maternal uterine arterial plasma on day 30 of gestation

Concentrations of glutamate, asparagine, serine, threonine, alanine, tyrosine, methionine and valine were lower ($P < 0.05$) in gilts supplemented with 0.4% arginine compared to the control group (Table 2.4). Concentration of arginine was higher ($P < 0.05$) in gilts supplemented with 0.4% arginine compared to the control gilts. All other amino acids measured did not differ between treatment groups.

Concentrations of amino acids in amniotic fluid on day 30 of gestation

Concentration of taurine was less ($P < 0.05$) and concentration of tryptophan was greater ($P < 0.05$) in amniotic fluid from gilts supplemented with 0.4% arginine compared to the control gilts (Table 2.5). Concentrations of all other amino acids measured did not differ between treatment groups.

Concentrations of amino acids in allantoic fluid on day 30 of gestation

Concentration of arginine and tryptophan was greater ($P < 0.05$) and that for methionine was less ($P < 0.05$) in allantoic fluid from gilts supplemented with 0.4% arginine compared to the control gilts (Table 2.6). Concentrations of all other amino acids did not differ between treatment groups.

Concentrations of glucose, fructose and glycerol on day 30 of gestation

Concentrations of glucose and fructose in AMF did not differ between treatment groups, but concentrations of glycerol were less ($P < 0.05$) in AMF from gilts supplemented with 0.4% arginine compared to the control group (Table 2.7). Concentrations of

glucose, fructose and glycerol in ALF and maternal uterine arterial plasma did not differ between treatment groups.

Total amounts of amino acids in amniotic fluid and allantoic fluid on day 30 of gestation

Total amounts of aspartate, glutamate, glycine, threonine, β-alanine, tryptophan, methionine, phenylalanine and leucine were greater ($P < 0.05$) in the amniotic fluid from gilts supplemented with 0.4% arginine, compared to control gilts (Table 2.8). Total amounts of aspartate, asparagine, serine, glutamine, histidine, glycine, threonine, arginine, tyrosine, tryptophan, valine and lysine were greater ($P < 0.05$) in the allantoic fluid from gilts supplemented with 0.4% arginine, compared to control gilts (Table 2.9).

Table 2.4 Concentrations of amino acids (nmol/ml) in uterine arterial plasma on day 30 of gestation for gilts fed a diet supplemented with 0 or 0.4% L-arginine (Arg) between days 14 and 30 of gestation.

Amino Acid	Control	0.4% Arg	P-value
Asp	15.9 ± 1.9	11.9 ± 1.5	0.116
Glu	213 ± 6.9	165 ± 14	0.014
Asn	44.1 ± 3.0	30.4 ± 2.2	0.003
Ser	108 ± 5.6	79.8 ± 4.1	0.002
Gln	376 ± 30	309 ± 21	0.09
His	68.3 ± 2.9	57.9 ± 3.6	0.051
Gly	750 ± 58	631 ± 26	0.071
Thr	99.9 ± 5.6	67.4 ± 4.4	0.001
Cit	71.5 ± 6.2	63.5 ± 4.1	0.291
Arg	140 ± 5.3	170 ± 9.6	0.021
β-Ala	7.21 ± 0.6	7.00 ± 0.7	0.816
Tau	129 ± 22	128 ± 48	0.995
Ala	852 ± 160	269 ± 21	0.002
Tyr	71.1 ± 4.4	54.8 ± 3.4	0.013
Trp	53.4 ± 5.0	60.3 ± 1.2	0.175
Met	50.7 ± 1.3	45.8 ± 1.2	0.015
Val	213 ± 8.0	176 ± 6.8	0.005
Phe	57.0 ± 1.9	50.6 ± 2.2	0.056
Ile	90.6 ± 3.1	77.9 ± 5.0	0.061
Leu	191 ± 2.1	187 ± 4.6	0.406
Orn	87.3 ± 8.8	88.3 ± 10	0.946
Lys	140 ± 11	122 ± 8.9	0.237

Values are means plus SEM; n=6 (Control) or n=7 (0.4% Arg). P-values < 0.05 were considered significant.

Table 2.5 Concentrations of amino acids (nmol/ml) in amniotic fluid on day 30 of gestation for gilts fed a diet supplemented with 0 or 0.4% L-arginine (Arg) between days 14 and 30 of gestation.

Amino Acid	Control	0.4% Arg	P-value
Asp	20.6 ± 1.2	17.6 ± 2.2	0.272
Glu	227 ± 19	201 ± 28	0.459
Asn	59.5 ± 4.3	54.6 ± 5.0	0.478
Ser	420 ± 14	358 ± 25	0.067
Gln	831 ± 52	693 ± 72	0.163
His	58.3 ± 3.4	48.8 ± 5.2	0.163
Gly	298 ± 14	269 ± 13	0.156
Thr	250 ± 22	224 ± 20	0.386
Cit	10.4 ± 1.6	7.79 ± 1.0	0.178
Arg	175 ± 5.7	158 ± 17	0.4
β-Ala	7.79 ± 0.7	7.75 ± 0.1	0.965
Tau	265 ± 29	176 ± 13	0.015
Ala	254 ± 8.4	229 ± 14	0.161
Tyr	52.7 ± 5.2	52.2 ± 4.6	0.944
Trp	14.5 ± 1.2	36.0 ± 1.2	0.0001
Met	72.5 ± 0.9	67.8 ± 3.2	0.206
Val	194 ± 17	185 ± 17	0.718
Phe	68.0 ± 4.6	70.7 ± 6.0	0.733
Ile	56.1 ± 6.1	50.5 ± 5.4	0.499
Leu	155 ± 5.2	162 ± 6.2	0.426
Orn	102 ± 7.2	88.6 ± 11	0.301
Lys	223 ± 15	188 ± 20	0.218

Values are means plus SEM; n=6 (Control) or n=7 (0.4% Arg). P-values < 0.05 were considered significant.

Table 2.6 Concentrations (nmol/ml) of amino acids in allantoic fluid on day 30 of gestation for gilts fed a diet supplemented with 0 or 0.4% L-arginine (Arg) between days 14 and 30 of gestation.

Amino Acid	Control	0.4% Arg	P-value
Asp	3.38 ± 0.5	4.92 ± 0.7	0.107
Glu	52.3 ± 13	71.5 ± 12	0.304
Asn	29.0 ± 5.0	40.3 ± 4.5	0.122
Ser	230 ± 38	314 ± 23	0.085
Gln	245 ± 42	333 ± 39	0.158
His	42.1 ± 6.5	51.9 ± 5.4	0.271
Gly	367 ± 47	486 ± 31	0.055
Thr	85.3 ± 10	121 ± 14	0.066
Cit	7.85 ± 1.5	9.25 ± 1.0	0.446
Arg	152 ± 19	227 ± 16	0.014
β-Ala	20.0 ± 2.1	21.0 ± 1.4	0.695
Tau	399 ± 13	417 ± 34	0.656
Ala	105 ± 22	119 ± 12	0.558
Tyr	24.3 ± 4.0	26.3 ± 1.6	0.618
Trp	9.55 ± 2.5	33.2 ± 1.9	0.0001
Met	32.6 ± 2.1	21.9 ± 3.8	0.041
Val	48.0 ± 7.6	55.5 ± 5.7	0.44
Phe	17.8 ± 3.6	20.3 ± 2.3	0.55
Ile	15.2 ± 2.6	13.9 ± 1.8	0.692
Leu	119 ± 3.8	122 ± 4.4	0.562
Orn	119 ± 19	110 ± 14	0.687
Lys	276 ± 30	274 ± 50	0.976

Values are means plus SEM; n=6 (Control) or n=7 (0.4% Arg). P-values< 0.05 were considered significant.

Table 2.7 Concentrations of glucose, fructose and glycerol in allantoic fluid, amniotic fluid and maternal uterine arterial plasma on day 30 of gestation for gilts fed a diet supplemented with 0 or 0.4% L-arginine (Arg) from days 14 to 30 of gestation.

Variable	Control	0.4% Arg	P-value
Concentrations in ALF			
Glucose, *nmol/ml*	1319 ± 845	1635 ± 373	0.389
Fructose, *nmol/ml*	2435 ± 245	2234 ± 117	0.477
Glycerol, *nmol/ml*	221 ± 35	155 ± 20	0.114
Concentrations in AMF			
Glucose, *nmol/ml*	1448 ± 83	1392 ± 82	0.642
Fructose, *nmol/ml*	2436 ± 307	1954 ± 70	0.157
Glycerol, *nmol/ml*	129 ± 10	82.1 ± 6.1	0.002
Concentrations in plasma			
Glucose, *nmol/ml*	5472 ± 106	5170 ± 81	0.273
Fructose, *nmol/ml*	500 ± 59	503 ± 51	0.973
Glycerol, *nmol/ml*	101 ± 11	124 ± 12	0.190

Values are means plus SEM; n=6 (Control) or n=7 (0.4% Arg). P-values < 0.05 were considered significant.

Table 2.8 Total amounts (nmol/amniotic fluid) of amino acids in amniotic fluid on day 30 of gestation for gilts fed a diet supplemented with 0 or 0.4% L-arginine (Arg) from days 14 to 30 of gestation.

Amino Acid	Control	0.4% Arg	P-value
Asp	23.9 ± 2.0	34.8 ± 4.2	0.049
Glu	242 ± 11	310 ± 37	< 0.0001
Asn	76.3 ± 4.8	93.0 ± 9.9	0.179
Ser	516 ± 15	609 ± 47	0.107
Gln	1050 ± 79	1070 ± 120	0.896
His	67.9 ± 6.3	76.8 ± 11	0.517
Gly	343 ± 22	429 ± 26	0.031
Thr	250 ± 20	383 ± 50	0.041
Cit	11.8 ± 0.8	13.5 ± 2.2	0.511
Arg	211 ± 7.7	236 ± 23	0.357
β-Ala	8.90 ± 0.7	11.9 ± 1.0	0.037
Tau	333 ± 28	277 ± 19	0.118
Ala	317 ± 14	367 ± 21	0.083
Tyr	67.3 ± 5.9	89.0 ± 9.1	0.081
Trp	16.7 ± 1.9	57.6 ± 3.2	< 0.0001
Met	89.1 ± 2.3	149 ± 5.3	< 0.0001
Val	254 ± 11	292 ± 31	0.303
Phe	86.3 ± 4.8	121 ± 12	0.029
Ile	73.9 ± 2.9	86.2 ± 11	0.337
Leu	194 ± 7.9	274 ± 10	0.0001
Orn	118 ± 12	133 ± 16	0.482
Lys	274 ± 20	291 ± 33	0.681

Values are means plus SEM; n=6 (Control) or n=7 (0.4% Arg). P-values< 0.05 were considered significant.

Table 2.9 Total amounts (μmol/allantoic fluid) of amino acids in allantoic fluid on day 30 of gestation for gilts fed a diet supplemented with 0 or 0.4% L-arginine (Arg) from days 14 to 30 of gestation.

Amino Acid	Control	0.4% Arg	P-value
Asp	0.56 ± 0.04	1.16 ± 0.11	0.001
Glu	9.80 ± 2.0	12.3 ± 1.1	0.278
Asn	5.44 ± 0.72	9.54 ± 0.38	0.0003
Ser	38.6 ± 4.5	69.9 ± 4.2	0.0004
Gln	46.2 ± 6.4	74.0 ± 7.1	0.015
His	7.94 ± 1.0	11.5 ± 1.0	0.029
Gly	75.5 ± 3.6	108 ± 9.5	0.012
Thr	16.2 ± 1.5	29.1 ± 1.6	0.0001
Cit	1.66 ± 0.2	2.13 ± 0.3	0.235
Arg	29.8 ± 2.7	56.7 ± 1.8	<0.0001
β-Ala	3.86 ± 0.4	4.72 ± 0.4	0.159
Tau	76.9 ± 5.4	95.8 ± 12	0.203
Ala	22.2 ± 2.9	26.2 ± 1.5	0.227
Tyr	4.57 ± 0.6	5.88 ± 0.3	0.066
Trp	2.07 ± 0.4	7.46 ± 0.6	<0.0001
Met	6.25 ± 0.4	5.09 ± 1.0	0.334
Val	9.08 ± 1.1	12.3 ± 1.0	0.05
Phe	3.33 ± 0.5	4.48 ± 0.4	0.096
Ile	2.88 ± 0.4	3.07 ± 0.3	0.706
Leu	23.0 ± 1.4	27.5 ± 1.7	0.071
Orn	19.4 ± 1.7	25.1 ± 3.7	0.231
Lys	47.4 ± 1.4	72.7 ± 6.4	0.004

Values are means plus SEM; n=6 (Control) or n=7 (0.4% Arg). P-values< 0.05 were considered significant.

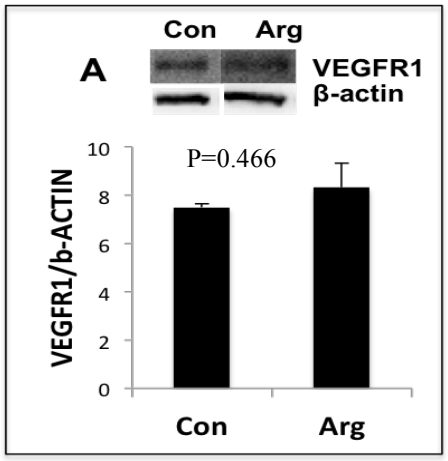
Expression of pro-angiogenic factors in the placenta

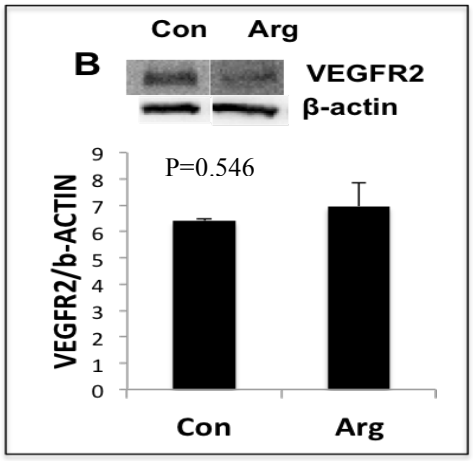
qPCR was performed on placental tissue from gilts at day 30 of gestation supplemented with either 0% (control) or 0.4% arginine in their diet from day 14 to day 30 of gestation to analyze factors associated with angiogenesis (Table 2.10). Vascular endothelial growth factor A 120 (VEGFA120) and VEGFA164 are two isoforms of VEGF measured in this study. Expression of VEGFA120 mRNA was greater ($P < 0.05$) in placentae from the 0.4% arginine-supplemented compared to control gilts. Expression of mRNAs for VEGF receptors 1 and 2 were also more abundant in placentae from the 0.4% arginine-supplemented gilts than those from gilts in the control group. Expression of mRNA for GTP cyclohydrolase-1 (GTP-CH-1), an enzyme responsible for synthesis of BH4, was more highly expressed in placentae of gilts supplemented with 0.4% arginine, compared to placentae from control gilts, however, mRNA expression of endothelial nitric oxide synthase (eNOS) did not differ between treatment groups. Another angiogenic-related growth factor, placental growth factor (PlGF), was also more highly expressed in placentae of gilts supplemented with 0.4% arginine, compared to placentae from control gilts. mRNA expression of fibroblast growth factor-2 (FGF-2) did not differ between treatment groups. Protein abundance of VEGFR1, VEGFR2, p-VEGFR2, eNOS, p-eNOS, GTP-CH-1, and PlGF did not differ between treatment groups (Figure 2.2).

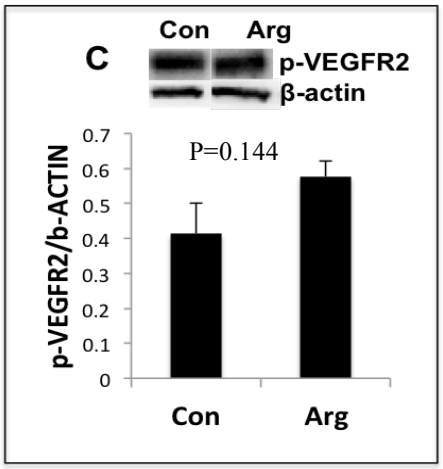
Table 2.10 Relative expression of mRNAs for pro-angiogenic genes in the placentae of gilts fed a diet supplemented with 0.4% arginine versus 0% arginine.

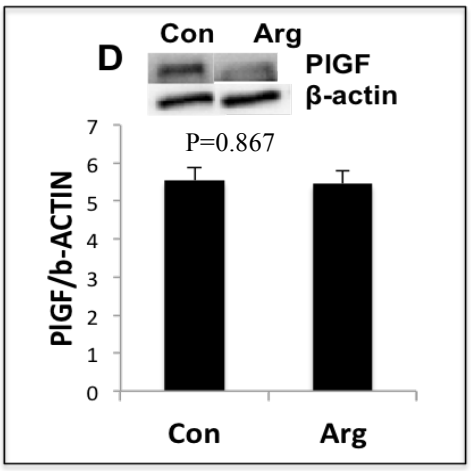
Gene	Fold change	P-value
VEGFA120	1.17	0.031
VEGFA164	0.99	0.948
VEGFR1	4.45	0.008
VEGFR2	3.73	0.0003
eNOS	1.14	0.145
PlGF	1.97	< 0.0001
GTP-CH-1	1.26	0.001
FGF-2	0.96	0.144

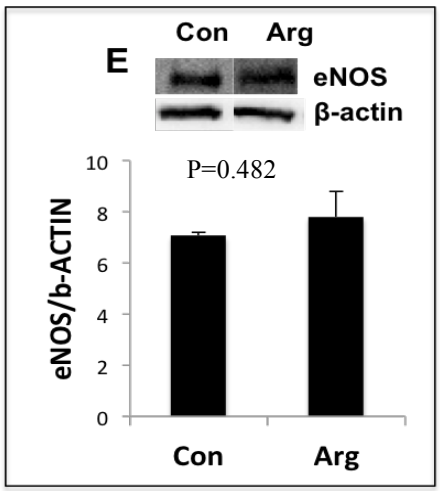
Relative expression of genes for VEGFA120, VEGFA164, VEGF-R1, VEGF-R2, eNOS3, PIGF, GTP-CH-1, and FGF-2 in placentae of gilts supplemented with 0.4% arginine compared to gilts supplemented with 0% arginine (control) between days 14 and 30 of gestation. Differences in expression of VEGFA120, VEGF-R1, VEGF-R2, PIGF, and GTP-CH-1 were greater for gilts in the 0.4% arginine group compared to the control group. Expression of VEGFA164, eNOS and FGF-2 were not affected by treatment. The abundances of mRNAs was measured by qPCR using SYBR Green. Data are expressed as means ± SEM, n=7 (control) and n=9 (0.4% arginine), by the ΔΔCt method. P-values < 0.05 were considered significant.

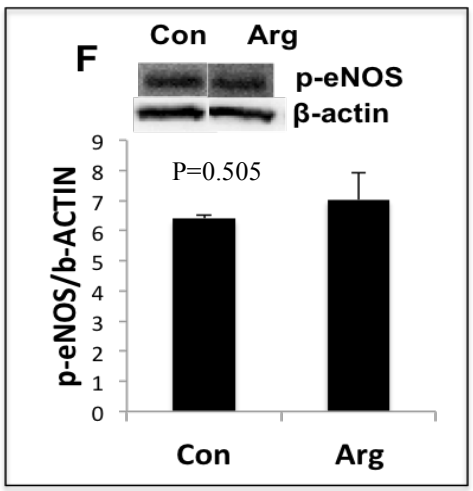

Con
Arg
A
VEGFR1
β-actin
P=0.466
VEGFR1/b-ACTIN
10
8
6
4
2
0
Con
Arg


Con
Arg
B
VEGFR2
β-actin
P=0.546
VEGFR2/b-ACTIN
9
8
7
6
5
4
3
2
1
0
Con
Arg


Con
Arg
C
p-VEGFR2
β-actin
P=0.144
p-VEGFR2/b-ACTIN
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Con
Arg


Con
Arg
D
PlGF
β-actin
P=0.867
PlGF/b-ACTIN
7
6
5
4
3
2
1
0
Con
Arg


Con
Arg
E
eNOS
β-actin
P=0.482
eNOS/b-ACTIN
10
8
6
4
2
0
Con
Arg


Con
Arg
F
p-eNOS
β-actin
P=0.505
p-eNOS/b-ACTIN
9
8
7
6
5
4
3
2
1
0
Con
Arg

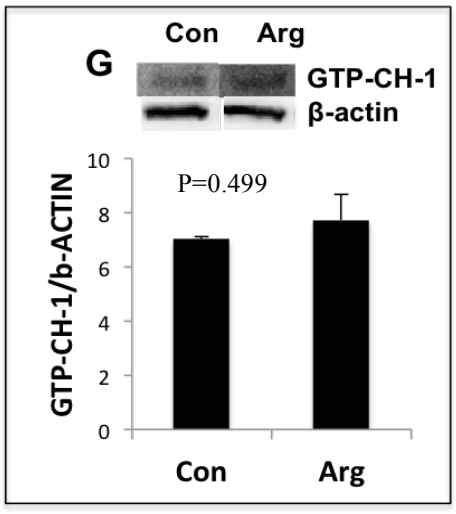


Fig. 2.2 Relative abundances of pro-angiogenic proteins in placentae of gilts supplemented with 0% (Control) or 0.4% arginine (Arg) between days 14 and 30 of gestation. The abundance of β-actin protein was used to normalize values for the relative abundance of target proteins. (A) VEGFR1; (B) VEGFR2; (C) p-VEGFR2; (D) PlGF; (E) eNOS; (F) p-eNOS; (G) GTP-CH-1.

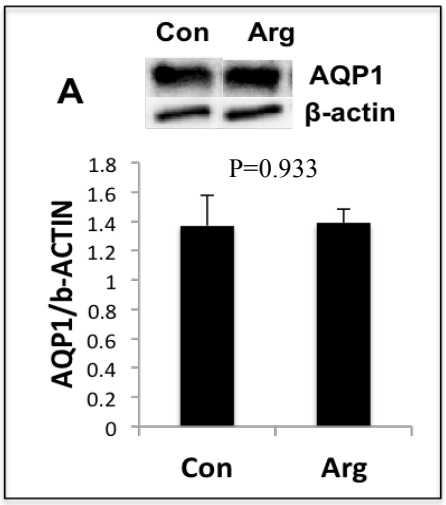
Expression of aquaporins for transport of water in the placenta

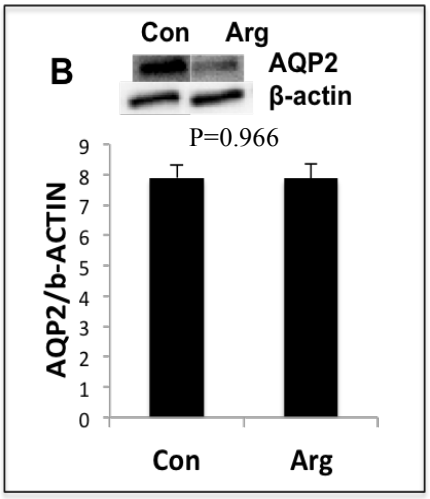
Expression of mRNAs for aquaporins by placentae from gilts supplemented with either 0% (control) or 0.4% arginine from day 14 to day 30 of gestation (Table 2.11). Expression of mRNAs for AQPs 1, 2, 3, 4, 5, 8, 9, and 11 were detected in placentae of gilts on Day 30 of gestation. Expression of mRNAs for AQP1, AQP3, AQP5, AQP8 and AQP9 was greater for placentae from gilts in the 0.4% arginine-supplemented group compared to placentae from gilts in the control group ($P < 0.05$). Expression of mRNA for AQP2 was lower for placentae from gilts in the 0.4% arginine-supplemented group compared to placentae from gilts in the control group. The abundance of AQP5 protein was more abundant ($P < 0.05$) in placentae from arginine-supplemented, compared with the control gilts (2.02 ± 0.23 vs 1.33 ± 0.1 AU) (Figure 2.3).

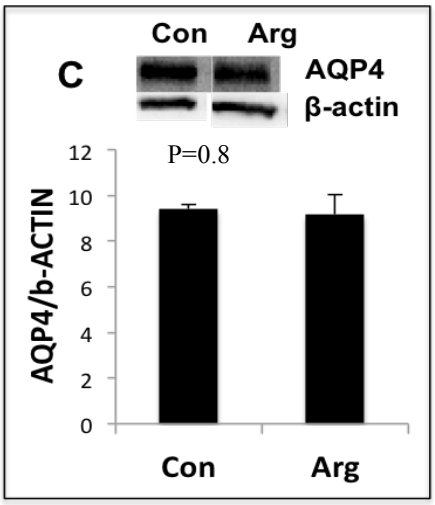
Table 2.11 Relative fold-change in expression of mRNAs for aquaporins (AQPs) in placentae of gilts supplemented with 0.4% versus 0% arginine.

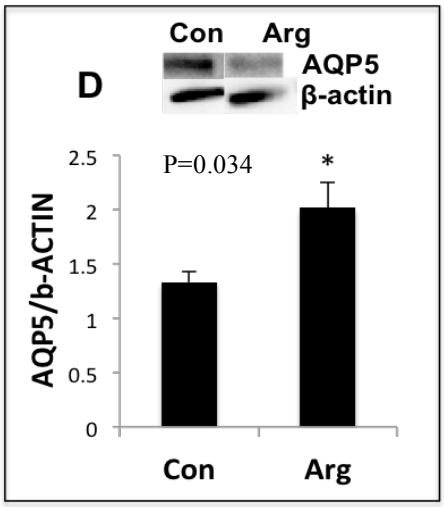
Gene	Fold Change	P-value
AQP1	2.8	0.002
AQP2	0.83	0.0002
AQP3	1.37	0.05
AQP4	8.37	0.131
AQP5	4.86	0.05
AQP8	1.65	0.004
AQP9	1.27	0.02
AQP11	1.14	0.806

Effects of treatment on fold-change in expression of genes for AQP 1, 2, 3, 4, 5, 8, 9, and 11in placentae of gilts supplemented with 0.4% or 0% arginine between days 14 and 30 of gestation. The fold-change in expression of mRNAs for AQP1, AQP2, AQP3, AQP5, AQP8 and AQP9 were significant, but fold-changes in expression of mRNAs for AQP4 and AQP11 were not affected by treatment. The abundances of expression of mRNAs were measured by qPCR using SYBR Green. Data are expressed as means ± SEM, n=7 (control) and n=9 (0.4% arginine), by the ΔΔCt method. P-values < 0.05 were considered significant.


Con
Arg
A
AQP1
β-actin
P=0.933
AQP1/b-ACTIN
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Con
Arg


Con
Arg
B
AQP2
β-actin
P=0.966
AQP2/b-ACTIN
9
8
7
6
5
4
3
2
1
0
Con
Arg


Con
Arg
C
AQP4
β-actin
P=0.8
AQP4/b-ACTIN
12
10
8
6
4
2
0
Con
Arg


Con
Arg
D
AQP5
β-actin
P=0.034
*
AQP5/b-ACTIN
2.5
2
1.5
1
0.5
0
Con
Arg

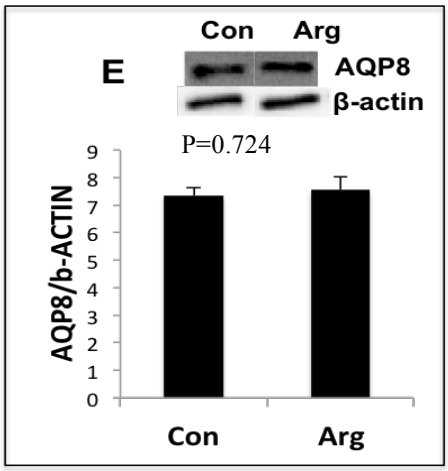

Con
Arg
E
AQP8
β-actin
P=0.724
AQP8/b-ACTIN
9
8
7
6
5
4
3
2
1
0
Con
Arg

Fig. 2.3 Relative abundances of AQP proteins in placentae of gilts supplemented with 0% (Control) or 0.4% arginine (Arg) between days 14 and 30 of gestation. The abundance of β-actin protein was used to normalize the relative abundances of target proteins. (A) AQP1; (B) AQP2; (C) AQP4; (D) AQP5; (E) AQP8.

Localization of AQPs in placentae of gilts on days 15, 20, 25, 40 and 90 of gestation

AQP1 protein is highly expressed in all endothelial cells of both the uterine and placental vasculatures from day 15 to day 90 of pregnancy (Figure 2.4). AQP1 expression was also observed in RBCs and the smooth muscle cells of the myometrium. Interestingly, only the smooth muscle cells in the myometrium showed expression of AQP1, as there was no expression of AQP1 in the smooth muscle cells of the tunica media of blood vessels. The AQP8 protein is expressed in the tunica media of both uterine and placental blood vessels throughout pregnancy, and expression in smooth muscle cells is limited to the tunica media and is not observed in the myometrium (Figure 2.5). AQP8 is also expressed in the trophectoderm on day 15 and abundance decreases between days 15 and 25 of gestation. AQP8 protein is expressed within the chorionic cells of areolae and stromal cells in the allantois on day 40, with expression increasing through day 90 of gestation.

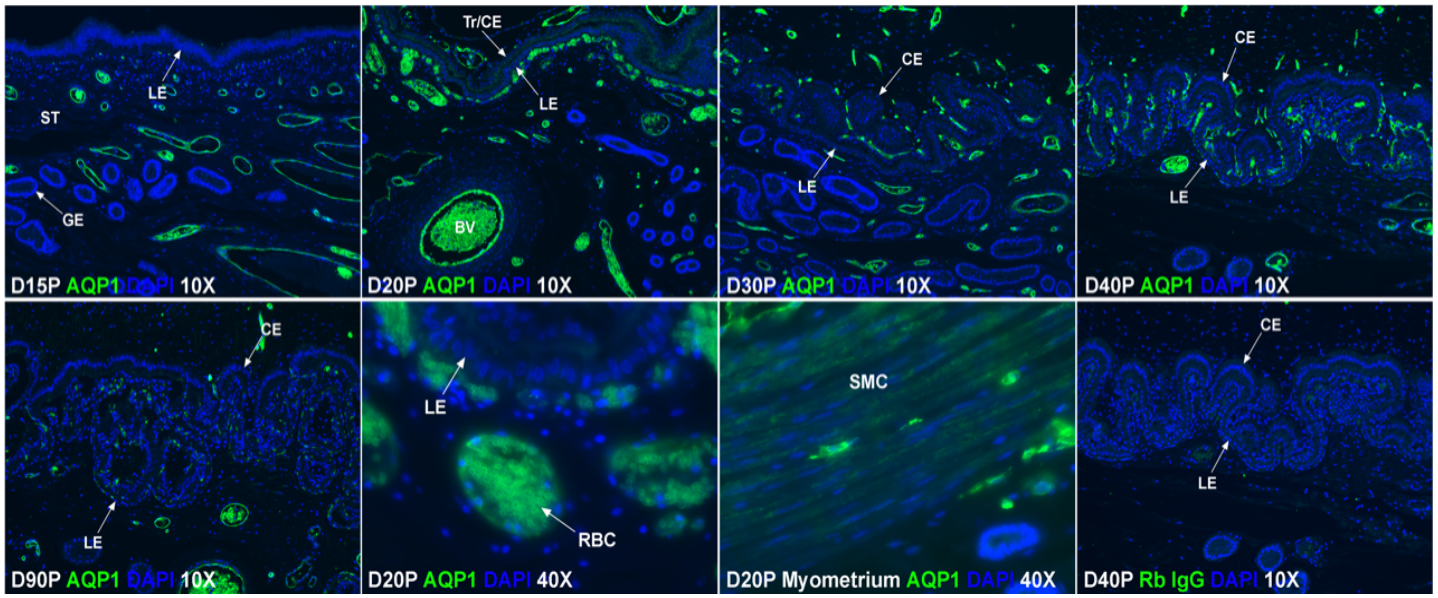

LE
ST
GE
D15P AQP1 DAPI 10X
Tr/CE
LE
BV
D20P AQP1 DAPI 10X
CE
LE
D30P AQP1 DAPI 10X
CE
LE
D40P AQP1 DAPI 10X
CE
LE
D90P AQP1 DAPI 10X
LE
RBC
D20P AQP1 DAPI 40X
SMC
D20P Myometrium AQP1 DAPI 40X
CE
LE
D40P Rb IgG DAPI 10X

Fig. 2.4 Immunofluorescence microscopy for aquaporin 1 (AQP1; green) at the uterine-placental interface of gilts on days 15 (D15), 20, 30, 40, and 90 of pregnancy. AQP1 protein is localized to all endothelial cells within both uterine and placental tissues, to the myometrium and within red blood cells. Nuclei are stained with DAPI for histologic reference. The D40 rabbit IgG (Rb IgG) panel serves as the negative control. Width of fields for microscopic images captured at 10X is 940 μm. Width of fields for microscopic images captured at 40X is 230 μm. Legend: LE, luminal epithelium; GE, glandular epithelium; ST, stroma; BV, blood vessel; Tr, trophectoderm; CE, chorionic epithelium; RBC, red blood cells; SMC, smooth muscle cells.

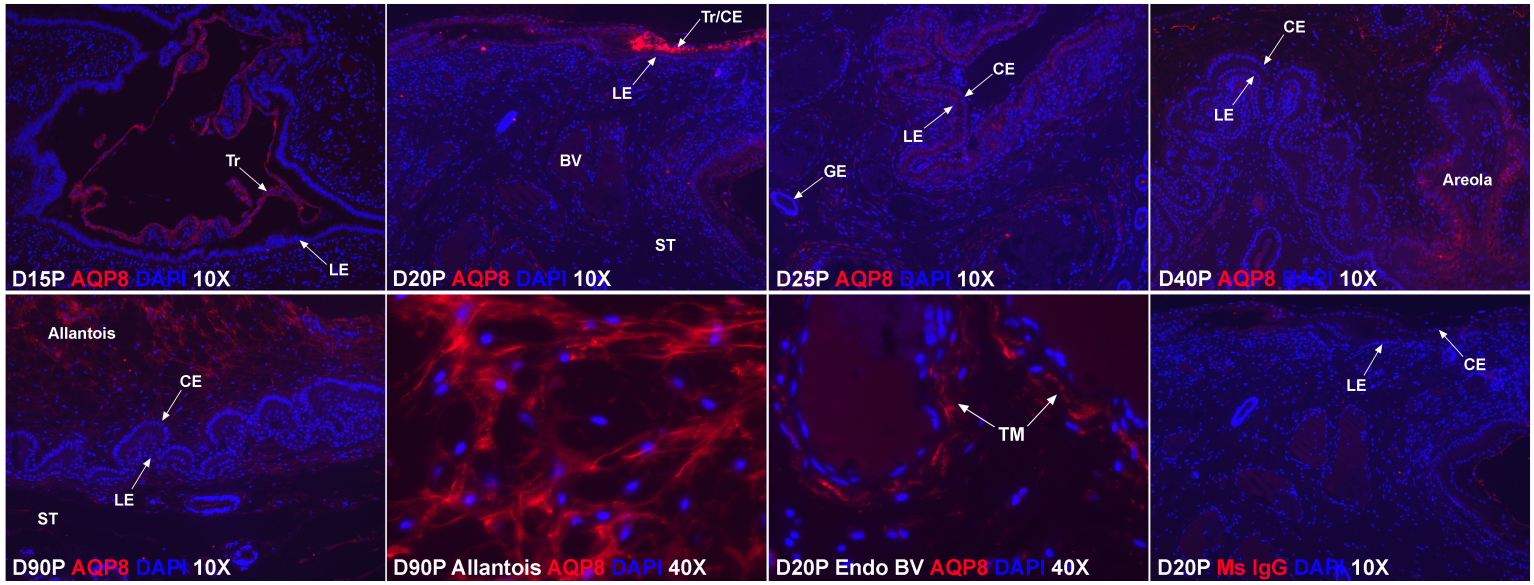

Tr
LE
D15P AQP8 DAPI 10X
Tr/CE
LE
BV
ST
D20P AQP8 DAPI 10X
CE
LE
GE
D25P AQP8 DAPI 10X
CE
LE
Areola
D40P AQP8 DAPI 10X
Allantois
CE
LE
ST
D90P AQP8 DAPI 10X
D90P Allantois AQP8 DAPI 40X
TM
D20P Endo BV AQP8 DAPI 40X
LE
CE
D20P Ms IgG DAPI 10X

Fig. 2.5 Immunofluorescence microscopy for aquaporin 8 (AQP8; red) at the uterine-placental interface of gilts on days 15 (D15), 20, 30, 40, and 90 of pregnancy. AQP8 protein is localized to the trophectoderm on days 15 and 20, the tunica media of blood vessels within both uterine and placental tissues, to placental areolae, and cells within the allantois on days 40 and 90 of gestation. Nuclei are stained with DAPI for histological reference. The day 20 mouse IgG (Ms IgG) panel serves as the negative control. Width of fields for microscopic images captured at 10X is 940 μm. Width of fields for microscopic images captured at 40X is 230 μm. Legend: LE, luminal epithelium; GE, glandular epithelium; ST, stroma; BV, blood vessel; Tr, trophectoderm; CE, chorionic epithelium; TM, tunica media.

Placental transport of ^3H_2O

Tritiated water (^3H_2O) was readily transported from the "mucosal" to the "serosal" side of placentae in Ussing chambers. The rates of net water transfer by placentae in the arginine group were 34.6% greater than for placentae from gilts in the control group ($P < 0.05$) (Table 2.12).

Table 2.12 Rates of net water transport by placentae from gilts fed a diet supplemented with 0% (control) or 0.4% L-arginine from days 14 to 30 of gestation.

Time (min)	Control	0.4% Arginine	P-value
5	0.370 ± 0.025	0.498 ± 0.036*	0.018
10	0.373 ± 0.021	0.502 ± 0.033*	0.009
15	0.368 ± 0.027	0.496 ± 0.030*	0.010

Data, µl/mg wet tissue/min, are mean values ± SEM, n=6 (control) and n=7 (0.4% arginine). Water transport was measured using ^3H_2O in Ussing chambers to assess movement of ^3H_2O from the mucosal to the serosal side of the placental tissue.

cGMP production by PTr2 cells

The production of cGMP by PTr2 cells was very low in all treatment groups (Table 2.13). However, cGMP production by PTr2 cells cultured in medium containing 0.5 mM arginine was less ($P < 0.05$) than for PTr2 cells cultured in medium containing 0.0 mM, 0.1 mM and 0.25 mM arginine.

Table 2.13 Effects of arginine on cGMP concentration and NO synthesis in pTr2 cells.

Variable	Arginine Concentration		
	0.1 mM	0.25 mM	0.5 mM
cGMP in tissue, *fmol/mg protein*	55.0 ± 3.9^{c}	142 ± 10^{b}	196 ± 12^{a}
NO synthesis, *pmol/h/10^6 cells*	15.8 ± 1.0^{c}	72.2 ± 4.1^{b}	94.6 ± 5.3^{a}

Values are means ± SEM, n = 8. a-b: Values with different superscript letters within a row are different ($P < 0.05$), as analyzed by one-way ANOVA and the Student-Newman-Keuls multiple comparison test.

CHAPTER III

CONCLUSIONS

Because swine experience high embryonic mortality during early gestation, a management practice to ameliorate such loss would be highly beneficial to both the swine industry and researchers. A corn- and soybean meal-based diet containing 12% crude protein is considered optimal to provide essential amino acids while preventing hyperammonemia and associated embryonic deaths in gestating pigs (Wu et al., 2017). However, a gestation diet containing 12% crude protein does not meet dietary requirements for arginine (Wu et al. 2017). Thus, supplementing the maternal diet with this deficient amino acid is an effective way to enhance growth and development of the conceptus without detrimental effects of high amounts of crude protein in the diet (Wu et al. 2013). Most embryonic loss in pigs occurs before day 30 of gestation, making this time period an appropriate target for improvement of reproductive performance (Edwards et al. 2012). However, Li et al. discovered that 0.8% arginine supplementation from days 0 to 25 interfered with follicle development and this decreased number of CL, resulting in a decrease in litter size (2010). Excessive intake of L-arginine too early in gestation led to reduced concentrations of progesterone in maternal plasma (Li et al. 2010). In a subsequent study, Li et al. (2014) reported that 0.4% and 0.8% arginine supplementation from days 14 to 25 of gestation increased litter size by two piglets and increased volumes of AMF and the concentration of arginine in the ALF and AMF, compared to the control gilts. The dose of arginine supplementation is important to prevent an amino acid imbalance. Therefore, total dietary arginine should be less than

2% so that the ratio of arginine to lysine is less than three to prevent competition for transport into cells between these two amino acids (Wu et al. 2013). We used 0.4% arginine in the present study, because this dose is sufficient for enhancing survival and development of conceptuses in gestating gilts (Li et al. 2014).

Arginine is a nutritionally essential amino acid especially for gestating mammals, as it is the precursor for NO, ornithine, polyamines, creatine and agmatine (Wu et al. 2013). NO is a potent vasodilator and stimulator of angiogenesis that increases in the placenta during pregnancy to increase utero-placental blood flow and placental exchange of nutrients and gases (Chen and Zheng, 2014). Specifically, NO enhances blood flow through dilation of the blood vessels and an increase in placental angiogenesis (Wu et al. 2013, 2017). The placental vasculature is responsible for nutrient and gas exchange between mother and fetus, as well as removal of fetal metabolic waste (Chen and Zheng 2014). Our results suggest that arginine can increase placental angiogenesis by increasing expression of pro-angiogenic factors, such as VEGFA120, VEGFR1, VEGFR2, eNOS, PlGF, GTP-CH-1 and FGF2. VEGFA is the conventional form of the VEGF family that acts on endothelial cells to allow their migration and proliferation along with increasing endothelial production of NO (Chen and Zheng 2014). VEGFA120 and VEGFA164 are splice variants of VEGFA that are expressed in the porcine placenta to increase vascular permeability (Vonnahme et al. 2001). VEGFA binds to VEGF receptors 1 and 2 (Roskoski Jr. 2008). PlGF is also part of the VEGF family that acts in synergy with VEGF to promote angiogenesis (Sanchis et al. 2015). eNOS converts arginine to NO in endothelial cells (Wu et al. 2013). GTP-CH-1 is

involved in the production of BH4, which, as an essential cofactor for eNOS, is necessary for conversion of arginine to NO (Jobgen et al. 2006). Arginine can increase the bioavailability and synthesis of BH4, thereby increasing production of NO (Wu et al. 2013). In addition to NO, arginine supplementation increased the activity of ODC1 and the synthesis of polyamines in the placenta, which also stimulates angiogenesis (Wu et al. 2004). Augmenting the expression of these pro-angiogenic factors increases angiogenic activity in the placenta, resulting in a more highly developed placental vasculature. Therefore, more water and nutrients can be transported by the placenta to increase growth, development and survival of the conceptus.

The twelve AQPs (AQP 1-12) expressed in the female reproductive tract can be classified into three different subgroups. AQPs 1, 2, 4, 5, 6 and 8 are classical aquaporins that are highly selective for transport of water. AQPs 3, 7, 9 and 10 are aquaglyceroporins that transport urea, glycerol and other small solutes in addition to water. AQPs 11 and 12 are superaquaporins for which a function has not been established. Zhu et al. (2015) discovered that AQPs 1, 3, 4, 5, 6, 7, 8, 9 and 11 are expressed in the porcine placentae on day 25 of gestation. In the present study, AQPs 1, 2, 3, 4, 5, 8, 9, and 11 were expressed in the placentae of gilts on day 30 of gestation. To our knowledge, this is the first report of AQP 2 being expressed in the porcine placenta on day 30 of gestation. These results suggest that arginine supplementation can increase placental water transport by increasing expression of AQPs 1, 3, 4, and 5. Of note, dietary supplementation with arginine enhanced both the abundance of AQP1 in the placenta and placental water transport. AQPs are essential for maintaining the balance

between production of amniotic fluid and its reabsorption for optimal development of the embryo/fetus (Zhu et al. 2015). These findings were consistent with our observation that volumes of both AMF and ALF increased in response to dietary supplementation with 0.4% L-arginine to gestating gilts (Li et al. 2014).

In 1986, while examining human erythrocytes, Benga et al. localized the first water channel protein (WCP). It was not until 1992, however, that its water transport property was identified by the Agre group, leading to the name aquaporin 1 (AQP1) (Benga 2012). There are many hypotheses about the function of AQP1 in the membranes of red blood cells (RBCs), one of which states that AQP1 is responsible for undulations or "flickering" of the RBC membrane, which helps in moving the RBCs through capillaries. The second states that the high permeability of RBC membranes allows for concomitant displacement of water molecules when rapid exchange of ions and solutes occurs (Benga 2012). Mobasheri and Marples (2004) later studied the expression of AQP1 using tissue microarray technology and found that AQP1 is expressed in endothelial cells throughout the human body. This expression is expected because AQP1 is responsible for the high water permeability of the endothelium, which helps with maintaining water and ion homeostasis for numerous functions including cell differentiation, proliferation, secretion, and apoptosis (Mobasheri and Marples 2004). These are all crucial functions for uterine stromal cells and cells located in the conceptus, supporting the need for AQP1 to be expressed in the uterine vasculature and within the developing conceptus. Lastly, AQP1 expression in the myometrium of the uterus has been shown by Lindsay and Murphy in rats (2004). This expression is

believed to play a role in decreasing the size of the uterine lumen to assist in positioning of the blastocyst and implantation. In rodents, the uterine lumen closes down to form an implantation chamber and previous reports have speculated that AQP1 in the myometrium could allow water into the cells, leading to swelling of the muscle and closing of the lumen (Gannon et al. 2000). It has also been suggested that increased expression of AQP1 in the mesometrial muscle rather than the antimesometrial myometrium could initiate contraction or cause swelling which could contribute to the antimesometrial location of the implanting blastocyst (Lindsay and Murphy 2004). Along with the data in the rat, Skowronska et al. showed that AQP1 mRNA and protein were expressed in the myometrium of pigs in explant cultures by real-time PCR and Western Blot analyses, respectively (2015). This localization in the smooth muscle cells of the myometrium is novel and we believe the presence of AQP1 in these cells assists in readying the uterus for pregnancy.

Aquaporin 8 (AQP8), like AQP1, is a water transporter, but AQP8 also transports hydrogen peroxide through multiple membranes, including the plasma membranes of cells (Bertolotti et al. 2013). In low concentrations, hydrogen peroxide is important for normal growth factor signaling, suggesting multiple other potential functions for AQP8 in trophectoderm cells of the elongating and implanting conceptus, the tunica media of blood vessels, the chorionic epithelium of areolae, and stromal cells of the allantois (Bae et al. 1997). When ovariectomized rats were administered both estrogen and corn oil (control), AQP8 protein was expressed in the stromal cells of the endometrium and in the myometrium (Jablonski et al. 2003). Based on quantitative real-time PCR data, sheep

conceptuses express AQP8 mRNA beginning on day 27 and expression is maintained throughout the remainder of gestation (Liu et al., 2004). Klein et al. (2013) also reported expression of AQP8 mRNA in uteri of mares on day 14 of pregnancy. However, the present results are the first to reveal AQP8 localization in uteri and conceptuses of pigs. Jablonski et al. (2003) proposed that AQP8 in the rat uterus is responsible for shuttling water between the myometrium and stroma of the endometrium, and Liu et al. (2004) proposed that AQP8 in the sheep conceptus was partially responsible for the high water permeability of the placenta. The present results suggest that AQP8 in the pig uterus, specifically the smooth muscle cells of the tunica media in blood vessels, the trophectoderm cells of the early conceptus, placental areolae, and allantois, may play a similar role to AQP8 in the rat uterus and sheep conceptus in the transport of water from the maternal circulation to the chorioallantois during pregnancy. This is supported by our AQP1 results indicating that AQP1 protein is expressed in erythrocytes and endothelial cells of the uterine and placental vasculatures. Therefore, using AQP1 and AQP8 alone, cells can potentially transport water from the uterine vasculature, through the tunica intima and tunica media, across the chorion and either through the tunica media and tunica intima of placental blood vessels or into the allantois for utilization within the stromal compartment of the placenta. Indeed, the reverse is also possible, and may explain the changing volumes of allantoic fluid and hydration of placental connective tissues during pregnancy in pigs (Knight et al. 1977). To date, a water transporter has not been localized to the endometrial LE, but it is expected that uterine LE express one or more water transporters. The localization of AQP8 protein in epithelial cells of the

amnion and allantois of canines has been suggested to mediate fluid transfer across fetal membranes (Aralla et al. 2012). Knight et al. (1977) showed that there are dynamic changes in allantoic fluid volume in pigs throughout pregnancy and that those changes are responsible for expanding the chorioallantoic membranes and allowing them to establish intimate contact with a maximum amount of endometrial surface area. This agrees with our immunofluorescence results showing AQP8 protein in the allantois of pigs later in pregnancy and suggests that it is involved in water transport within the placenta. Lastly, localization of AQP8 protein in the placental areolae associated with the opening of the uterine glands in the pig endometrium is novel and important because there is significant transport of water and other nutrients through the areolae throughout pregnancy (Bazer et al. 2012). We suggest that AQP8 is at least partly responsible for the transport of water through the chorionic epithelium of the areolae.

AQP expression is associated with the exchange of fluids between mother and conceptus and homeostasis of amniotic fluid (Zhu et al. 2015). Increased angiogenic activity in response to 0.4% L-arginine supplementation increases utero-placental blood flow to supply water and nutrients to the utero-placental interface where AQPs are present to increase water and solute transport resulting in increases in volumes of amniotic and allantoic fluid. Total volumes of AMF and ALF are positively correlated with placental development that is a prerequisite for conceptus survival and growth during later stages of gestation (Bazer and Johnson, 2014; Zhu et al. 2015).

Concentrations of most amino acids in the ALF and AMF did not differ between the control and the 0.4% arginine supplemented gilts, which was similarly reported in

previous studies (Li et al. 2010; 2014). However, in the plasma obtained from the uterine artery, glutamate, asparagine, serine, threonine, alanine, methionine and valine were lower in the 0.4% arginine supplemented compared with the control gilts. It is possible that rates of synthesis of these amino acids were greater or rates of degradation were lower in the whole body of control gilts that received isonitrogenous amounts of alanine compared to the gilts supplemented with 0.4% arginine. Embryonic survival was still greater in gilts supplemented with 0.4% arginine than control gilts even though circulating levels of these amino acids were lower in the 0.4% arginine supplemented gilts compared to control gilts. Arginine supplementation increased the concentration of arginine in the maternal uterine arterial plasma and the allantoic fluid. Glucose and fructose concentrations in the ALF, AMF and plasma did not differ between treatment groups, but glycerol concentrations were greater in the plasma of the 0.4% arginine supplemented gilts. This finding may indicate that more glycerol was being converted to glucose for utilization by the arginine-supplemented gilts.

On the other hand, total amounts of many amino acids were greater in the amniotic and allantoic fluid from gilts supplemented with 0.4% arginine compared to control gilts. Specifically, in the amniotic fluid, aspartate, glutamate, glycine, threonine, β-alanine, tryptophan, methionine, phenylalanine and leucine were greater ($P < 0.05$) in the arginine-supplemented gilts compared to the control. Also, in the allantoic fluid, aspartate, asparagine, serine, glutamine, histidine, glycine, threonine, arginine, tryptophan, valine and lysine were greater in the arginine-supplemented group compared to the controls. Higher amounts of these amino acids in the fetal fluids may result from

increases in their transport across the placenta and possibly contribute to increased embryonic/fetal survival and growth.

In conclusion, results of the present study revealed that 0.4% L-arginine supplementation to gilts between days 14 and 30 of gestation increased embryonic survival by 28%, and increased amniotic and allantoic fluid volumes by 48.1% and 25.3%, respectively. Also, 0.4% L-arginine supplementation increased synthesis of NO and polyamines, expression of pro-angiogenic factors VEGF120, VEGFR1, VEGFR2, eNOS, PlGF, FGF-2 and GTP-CH-1 and AQP 1, 3, 4 and 5, as well as numbers of blood vessels and water transport by the placenta. Those effects of dietary arginine elucidated the multitude of mechanisms whereby dietary arginine supplementation significantly enhances embryonic survival during the peri-implantation period of pregnancy, as well as subsequent growth and development of the conceptus during the later stages of gestation (Figure 2.6). The findings have important nutritional implications for increasing reproductive performance in swine and other mammalian species by increasing birth weights and survivability of newborn during the neonatal period of life.

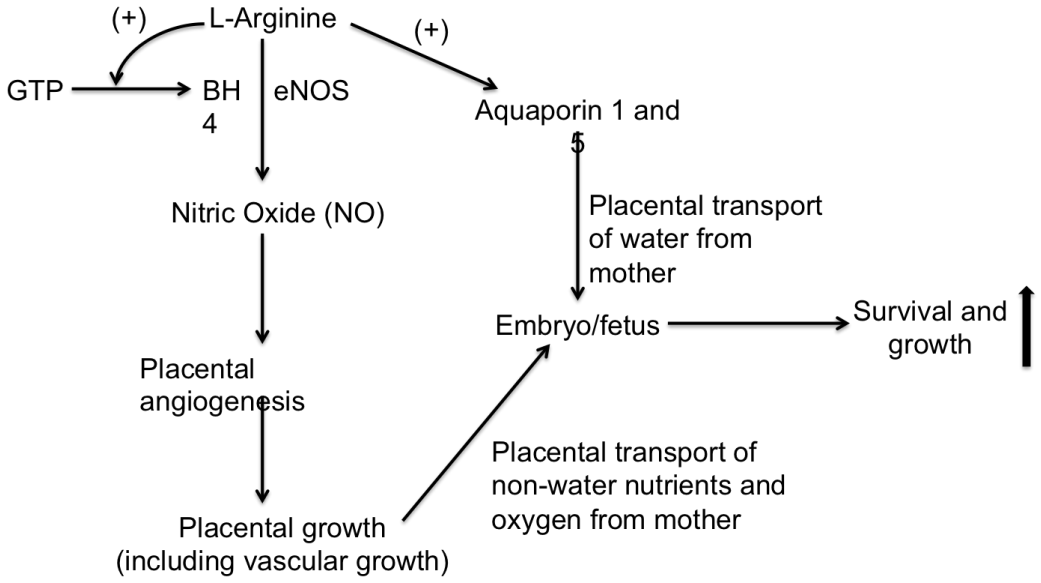


Figure 2.6 Proposed mechanisms whereby dietary arginine supplementation improves survival and growth of conceptuses in gestating swine.

REFERENCES

Altmann S, Murani E, Schwerin M, Metges CC, Wimmers K, Ponsuksili S (2013) Dietary protein restriction and excess of pregnant German Landrace sows induce changes in hepatic gene expression and promoter methylation of key metabolic genes in the offspring. J Nutr Biochem 24:484-495

Aralla M, Mobasheri A, Groppetti D, Cremonesi F, Arrighi S (2012) Expression of aquaporin water channels in canine fetal adnexa in respect to the regulation of amniotic fluid production and absorption. Placenta 33(6): 502-510

Bae YS, Kang SW, Seo MS, Baines IC, Tekle E, Chock PB, Rhee SG (1997) Epidermal growth factor (EGF)-induced generation of hydrogen peroxide: Role in EGF receptor-mediated tyrosine phosphorylation. J Biol Chem 272:217–221

Bazer FW, Wu GY, Johnson GA, Wang XQ (2014) Environmental factors affecting pregnancy: endocrine disrupters, nutrients and metabolic pathways. Molec Cell Endocrin 398:53-68

Bazer FW, Song G, Kim J, Dunlap KA, Satterfield MC, Johnson GA, Burghardt RC, Wu G (2012) Uterine biology in pigs and sheep. Journal Anim Sci Biotech 3:23

Bazer FW, Wu G, Spencer TE, Johnson GA, Burghardt RC, Bayless K (2010) Novel pathways for implantation and establishment and maintenance of pregnancy in mammals. Molec Human Reprod 16:135-152

Benga G (2012) The first discovered water channel protein, later called aquaporin 1: Molecular characteristics, functions and medical implications. Mol Aspects Med 33: 518-534

Bertolotti M, Bestetti S, García-Manteiga JM, Medraño-Fernandez I, Dal Mas A, Malosio ML, Sitia R (2013) Tyrosine Kinase Signal Modulation: A Matter of H2O2 Membrane Permeability? Antioxid Redox Signal 19(13):1447–1451

Charles A Ducsay, Dean A Myers (2011) eNOS activation and NO function: Differential control of steroidogenesis by nitric oxide and its adaptation with hypoxia. J Endocrin 210:259

Chen D, Zheng J (2014) Regulation of Placental Angiogenesis. Microcirc 21:15-25

Chwalisz K, Garfield RE (2000) Role of nitric oxide in implantation and menstruation. Human Reprod (Oxford, England) 15 Suppl 3:96-111

Damiano AE (2011) Review: Water channel proteins in the human placenta and fetal

membranes. Placenta 32:S211

Edwards A, Wessels J, Kerr A, Tayade C (2012) An Overview of Molecular and Cellular Mechanisms Associated with Porcine Pregnancy Success or Failure. Reprod Domest Anim 47:394-401

Feras Hatahet, Lloyd W Ruddock (2007) MINIREVIEW. The FEBS Journal 274:5223.

Fu WJ, Hu J, Spencer T, Carroll R, Wu G (2006) Statistical models in assessing fold change of gene expression in real-time RT-PCR experiments. Comput Biol Chem 30:21-26

Gannon BJ, Warnes GM, Carati CJ, Verco CJ (2000) Aquaporin-1 expression in visceral smooth muscle cells of female rat reproductive tract. J Smooth Muscle Res 36: 155–167

Herring CM, Bazer FW, Johnson GA, Wu G (2018) Impacts of maternal dietary protein intake on fetal survival, growth, and development. Exp Biol Med 243:525-533

Hou Y, Hu S, Jia S, Nawaratna G, Che D, Wang F, Bazer F, Wu G (2016) Whole-body synthesis of l-homoarginine in pigs and rats supplemented with l-arginine. Amino Acids 48:993-1001

Hou Y, Yin Y, Wu G (2015) Dietary essentiality of "nutritionally non-essential amino acids" for animals and humans. Exp Biol Med 240:997-1007

Jablonski EM, McConnell NA, Hughes FM, Huet-Hudson YM (2003) Estrogen Regulation of Aquaporins in the Mouse Uterus: Potential Roles in Uterine Water Movement. Biol Reprod 69(5):1481–1487

Ji Y, Wu ZL, Dai ZL, Wang XL, Li J, Wang BG, Wu G (2017) Fetal and neonatal programming of postnatal growth and feed efficiency in swine. J Anim Sci Biotech 8:5

Kim J, Song G, Wu G, Gao H, Johnson GA, Bazer FW (2013) Arginine, leucine, and glutamine stimulate proliferation of porcine trophectoderm cells through the MTOR-RPS6K-RPS6-EIF4EBP1 signal transduction pathway. Biol Reprod 88:113

Kim SW, Hurley WL, Wu G, Ji F (2009) Ideal amino acid balance for sows during gestation and lactation. J Anim Sci 87:E132

Klein C, Troedsson M, Rutllant J (2013) Expression of Aquaporin Water Channels in

Equine Endometrium is Differentially Regulated During the Oestrous Cycle and Early Pregnancy. Reprod Domest Anim 48: 529-537

Knight JW, Bazer FW, Thatcher WW, Franke DE, Wallace HD (1977) Conceptus development in intact and unilaterally hysterectomized-ovariectomized gilts: interrelations among hormonal status, placental development, fetal fluids and fetal growth. J Anim Sci 44:620-637

Kong X, Tan B, Yin Y, Gao H, Li X, Jaeger LA, Bazer FW, Wu G (2012) l-Arginine stimulates the mTOR signaling pathway and protein synthesis in porcine trophectoderm cells. J Nutr Biochem 23:1178-1183

Kong X, Wang X, Yin Y, Li X, Gao H, Bazer FW, Wu G (2014) Putrescine stimulates the mTOR signaling pathway and protein synthesis in porcine trophectoderm cells. Biol Reprod 91:106

Reynolds LP, Caton JS, Redmer DA, Grazul-Bilska AT, Vonnahme KA, Borowicz PP, Luther JS, Wallace JM, Wu G, Spencer TE (2006) Evidence for altered placental blood flow and vascularity in compromised pregnancies. J Physiol 572:51-58

Li H, Meininger C, Bazer F, Wu G (2016) Intracellular sources of ornithine for polyamine synthesis in endothelial cells. Amino Acids 48:2401-2410

Li X, Bazer FW, Johnson GA, Burghardt RC, Erikson DW, Frank JW, Spencer TE, Shinzato I, Wu G (2010) Dietary supplementation with 0.8% L-arginine between days 0 and 25 of gestation reduces litter size in gilts. J Nutri 140:1111-1116

Li X, Bazer F, Johnson G, Burghardt R, Frank J, Dai Z, Wang J, Wu Z, Shinzato I, Wu G (2014) Dietary supplementation with l-arginine between days 14 and 25 of gestation enhances embryonic development and survival in gilts. Amino Acids 46:375-384

Lindsay LA, Murphy CR (2004) Aquaporin-1 increases in the rat myometrium during early pregnancy. J Mol Histol 35: 75–79

Liu H, Koukoulas I, Ross MC, Wang S, Wintour EM (2004) Quantitative Comparison of Placental Expression of Three Aquaporin Genes. Placenta 25(6): 475-478

Mateo RD, Wu GY, Bazer FW, Park JC, Shinzato I, Kim SW (2007) Dietary L-arginine supplementation enhances the reproductive performance of gilts. J Nutr 137:652-656

Mobasher A, Marples D (2004) Expression of the AQP-1 water channel in normal

human tissues: a semiquantitative study using tissue microarray technology. Am J Physiol Cell Physiol 286: C529–C537

Nieminen T, Toivanen PI, Rintanen N, Heikura T, Jauhiainen S, Airenne KJ, Alitalo K, Marjomäki V, Ylä-Herttuala S (2014) The impact of the receptor binding profiles of the vascular endothelial growth factors on their angiogenic features. Biochimica et Biophysica Acta 1840:454

Palencia JYP, Lemes MAG, Garbossa CAP, Abreu MLT, Pereira LJ, Zangeronimo MG (2018) Arginine for gestating sows and foetal development: A systematic review. J Anim Physiol Anim Nutr 102:204-213

Rehfeldt C, Stabenow B, Pfuhl R, Block J, Nürnberg G, Otten W, Metges CC, Kalbe C (2012) Effects of limited and excess protein intakes of pregnant gilts on carcass quality and cellular properties of skeletal muscle and subcutaneous adipose tissue in fattening pigs. J Anim Sci 90:184-196

Sanchis EG, Cristofolini AL, Merkis CI (2015) Porcine placental immunoexpression of vascular endothelial growth factor, placenta growth factor, Flt-1 and Flk-1. Biotechnic & Histochemistry : official publication of the Biological Stain Commission 90:486-494

Skowronska A, Mlotkowska P, Nielsen S, Skowronski MT (2015) Difference in expression between AQP1 and AQP5 in porcine endometrium and myometrium in response to steroid hormones, oxytocin, arachidonic acid, forskolin and cAMP during the mid-luteal phase of the estrous cycle and luteolysis. Reprod Biol Endocrinol 13: 131

Steel, RGD, Torrie, JH (1980) Principles and Procedures of Statistics. New York, McGraw-Hill

Steinhauser CB, Wing TT, Gao H, Li X, Burghardt RC, Wu G, Bazer FW, Johnson GA (2017) Identification of appropriate reference genes for qPCR analyses of placental expression of SLC7A3 and induction of SLC5A1 in porcine endometrium. Placenta 52:1-9

Taylor CE (2016) A novel treatment for “morning sickness”: Nausea of pregnancy could induced by excess sulfite which molybdenum can help alleviate. Med Hypotheses 95:31-33

Tekwe CD, Lei J, Yao K, Rezaei R, Li X, Dahanayaka S, Carroll RJ, Meininger CJ,

Bazer FW, Wu G (2013) Oral administration of interferon tau enhances oxidation of energy substrates and reduces adiposity in Zucker diabetic fatty rats. BioFactors 39:552-563

Wu G (2013) Amino acids: biochemistry and nutrition. Boca Raton, USA, CRC Press

Wu G, Bazer FW, Hu J, Johnson GA, Spencer TE (2005) Polyamine Synthesis from Proline in the Developing Porcine Placenta. Biol Reprod 72:842-850

Wu G, Bazer FW, Burghardt RC, Johnson GA, Kim SW, Li XL, Satterfield MC, Spencer TE (2010) Impacts of amino acid nutrition on pregnancy outcome in pigs: mechanisms and implications for swine production. J Anim Sci 88:E204

Wu G, Bazer FW, Wallace JM, Spencer TE (2006) BOARD-INVITED REVIEW: Intrauterine growth retardation: Implications for the animal sciences. J Anim Sci 84:2316

Wu G (2016) Dietary protein intake and human health. Food & Function 7:1251-1265

Wu G, Bazer FW, Johnson GA, Herring C, Seo H, Dai Z, Wang J, Wu Z, Wang X (2017) Functional amino acids in the development of the pig placenta. Molec Reprod Dev 84:870-882

Wu G, Bazer FW, Tuo W, Flynn SP (1996) Unusual Abundance of Arginine and Ornithine in Porcine Allantoic Fluid. Biol Reprod 54:1261-1265

Wu G, Bazer F, Satterfield M, Li X, Wang X, Johnson G, Burghardt R, Dai Z, Wang J, Wu Z (2013) Impacts of arginine nutrition on embryonic and fetal development in mammals. Amino Acids 45:241-256

Wu G, Imhoff-Kunsch B, Girard AW (2012) Biological Mechanisms for Nutritional Regulation of Maternal Health and Fetal Development. Paediatr Perinat Epidemiol 26:4-26

Wu Z, Hou Y, Hu S, Bazer F, Meininger C, McNeal C, Wu G (2016) Catabolism and safety of supplemental l-arginine in animals. Amino Acids 48:1541-1552

Zhanqian Yu, Anu R. Sawkar, Jeffery W. Kelly (2007) Minireview. FEBS J. 274:4944

Zhu C, Jiang Z, Bazer FW, Johnson GA, Burghardt RC, Wu G (2015) Aquaporins in the female reproductive system of mammals. Front Biosci (Landmark edition) 20:838