ARGININE AND FETAL GROWTH IN OVINE MODELS OF INTRAUTERINE GROWTH RESTRICTION

A Dissertation

by

ARANTZATZU L. LASSALA

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

DOCTOR OF PHILOSOPHY

December 2008

Major Subject: Physiology of Reproduction

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Approved by:

Co-Chairs of Committee,	Thomas E. Spencer
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ABSTRACT

Arginine and Fetal Growth in Ovine Models of Intrauterine Growth Restriction.

(December 2008)

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This research was conducted to test the hypothesis that parenteral arginine supplementation is effective in enhancing birth weights of intrauterine growth restricted (IUGR) fetuses. Underfed and prolific ewes were used as experimental models. The first study characterized the pharmacokinetics of arginine and citrulline and assessed the potential of citrulline to serve as a precursor for enhancing arginine availability in fetal and maternal plasma. Six late pregnant ewes and their fetuses were instrumented to access arterial and venous circulations. Intravenous boluses of 155 μmol of L-arginine-HCl or L-citrulline per kg body weight were administered to each ewe. Administration of citrulline was more effective than arginine in achieving a sustained increase in concentrations of arginine in maternal and fetal blood. Accordingly, the clearance rate of citrulline was lower and its biological half-life in maternal blood greater, when compared with arginine. The second experiment determined if administration of arginine to underfed ewes is effective in ameliorating or preventing IUGR. Ewes were fed either 100% or 50% of the National Research Council recommended nutrient requirements for pregnant sheep. Between Day 60 of pregnancy and parturition control-fed ewes received saline solution and

underfed ewes received either saline solution or L-arginine-HCl solution (155 μmol of arginine/kg body weight) intravenously three times daily (n=5 / treatment group). Birth weights of lambs were lower in saline-infused underfed ewes. There was no difference in birth weights of lambs from control-fed and arginine-treated underfed ewes. The third experiment determined whether administration of arginine could improve survival rates of lambs and enhance fetal growth in ewes carrying multiple fetuses. Between Days 100 and 121 of pregnancy, ewes received an intravenous infusion of either saline solution (n= 14) or L-arginine-HCl solution (345 μmol of arginine/kg body weight, n=20) three times daily. Parenteral administration of arginine increased the percentage of lambs born alive and enhanced the birth weights of quadruplets. Collectively, these results indicate that 1) parenteral administration of arginine improves pregnancy outcomes in underfed and prolific ewes; and 2) the use of arginine or citrulline may have important implications for the design of an effective treatment for preventing or ameliorating IUGR in mammals.

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TABLE OF CONTENTS

CHAPTER Page

CHAPTER Page

LIST OF TABLES

TABLE Page

LIST OF FIGURES

CHAPTER I
INTRODUCTION AND LITERATURE REVIEW

Introduction

Fetal growth is a complex and dynamic process that is characterized by an orchestrated regulation of cell proliferation, organization, and differentiation (Redmer *et al.*, 2004; Ergaz *et al.*, 2005). It depends on the interaction between genes, nutrition and uterine environment which can affect uterine capacity, the maternal–placental–fetal unit, and the hormonal fetal and maternal milieu (Wu *et al.*, 2004a; Eleftheriades *et al.*, 2006; Gicquel & Le Bouc, 2006). When metabolic insults or functional disturbances are present during fetal development, severe alterations in the growth trajectory of the fetus may occur, with consequences that can have an effect throughout the life of the individual (Schroder, 2003; Sizonenko *et al.*, 2006; Wu *et al.*, 2006; Mari & Hanif, 2007). Indeed, many animal studies and human epidemiological findings indicate an association between impaired growth *in utero* and increased perinatal morbidity and mortality, as well as with metabolic and physiological abnormalities later in life (Barker & Clark, 1997; Symonds *et al.*, 2001; Ozanne & Hales, 2002; Ergaz *et al.*, 2005; Fowden *et al.*, 2006b).

Intrauterine growth restriction (IUGR) represents nearly 11% of all live-born infants worldwide and is therefore a condition of immense clinical and economic importance in human health (Ergaz *et al.*, 2005; Murphy *et al.*, 2006). In addition, it is a major concern in animal sciences, where production, efficiency, and performance of domestic species can be severely compromised (Wu *et al.*, 2006). However, the pathophysiological processes underlying this disorder are complex and incompletely understood (Ergaz *et al.*, 2005; Wu *et al.*, 2006).

This dissertation follows the style of *Journal of Physiology*.

Abnormalities of placental development and function result in impaired fetal growth (Ott *et al.*, 1997; Resnik, 2002; Chaddhaa *et al.*, 2004; Wu *et al.*, 2006; Karowicz-Bilinska *et al.*, 2007); and a reduction in fetoplacental blood flow has been associated with IUGR and fetal survival (Reynolds & Redmer, 1995; Sooranna *et al.*, 1995; Sheppard *et al.*, 2001; Lang *et al.*, 2003; Wallace *et al.*, 2005). In effect, the factors that regulate placental vascular formation and function throughout pregnancy impact blood flow, which is critical to transplacental exchange and nutrient transport, thereby influencing growth and development of the fetus (Sheppard *et al.*, 2001; Mayhew, 2002; Kwon *et al.*, 2004b; Reynolds *et al.*, 2006).

Arginine, a nutritionally essential amino acid for the fetus (Wu *et al.*, 2004b), is a precursor for the synthesis of nitric oxide (NO) and polyamines in cells (Wu & Morris, 1998). Nitric oxide is a key mediator of vasodilation and placental angiogenesis, thus exerting an important role in the regulation of fetoplacental blood flow during pregnancy (Reynolds & Redmer, 2001; Kwon *et al.*, 2004b; Vonnahme *et al.*, 2005). In addition, polyamines are key regulators of DNA and protein synthesis, which are crucial to the growth and proliferation of mammalian cells (Wu & Morris, 1998; Igarashi & Kashiwagi, 2000; Zhao *et al.*, 2008). Nitric oxide biosynthesis is augmented during gestation in rats and sheep (Sladek *et al.*, 1997) and increases in NO synthesis by the placenta are associated with an enhanced fetal growth in ewes during late gestation (Kwon *et al.*, 2004b). Interestingly, decreased concentrations of arginine and polyamines occur at day 78 of pregnancy in undernourished sheep (Kwon *et al.*, 2004a) and inhibition of polyamine synthesis reduces placental weight and impairs fetal growth in rats (Ishida *et al.*, 2002). Conversely, there is evidence that short-term fetal arginine infusion in an ovine model of IUGR by placental embolization stimulates protein accretion by the fetus (De Boo *et al.*, 2005). In addition, enteral administration of arginine to gilts between days 30 and 114 of gestation increased the number of live-born piglets per litter (Mateo *et al.*, 2007).

Furthermore, enteral or parenteral administration of arginine improved utero-placental circulation and ameliorated fetal growth restriction in women (Neri *et al.*, 1996; Lampariello *et al.*, 1997; Di Renzo *et al.*, 2005; Xiao & Li, 2005). Hence, modulation of the arginine-NO and polyamine pathways may play an important role in optimizing placental function and fetal growth and development. However, little is known about arginine metabolism in pregnant ewes and fetuses.

Because circulatory and transport systems in the sheep placenta are similar to those in the human placenta, the pregnant sheep offers a valuable model to elucidate the mechanisms underlying IUGR (Schroder, 2003; Wallace *et al.*, 2005). Thus, the objectives of this work were to i) determine the pharmacokinetics of arginine and citrulline (a neutral amino acid that can be utilized for arginine synthesis in cells) (Wu & Morris, 1998) in maternal circulation, as well as arginine availability in the fetus following administration of either amino acid to the ewe; and ii) assess if intravenous administration of arginine during mid- to late-gestation ameliorates or prevents fetal growth retardation in sheep. The knowledge generated from this research will help to lay a framework for studying cellular and molecular mechanisms responsible for the beneficial effects of arginine in regulating growth and development of the conceptus, as well as for the design of therapeutic interventions for the treatment and prevention of IUGR in mammals.

Impact of arginine on homeostasis and pregnancy

Main functions of arginine in whole body homeostasis

Arginine is required by immature mammals for adequate growth (Mertz *et al.*, 1952; Ha *et al.*, 1978; Wu *et al.*, 1997). This amino acid is also needed by all young and adult mammals for maintenance of normal intermediary metabolism, including detoxification of ammonia via hepatic and intestinal urea cycles (Wu &

Morris, 1998). Therefore, arginine plays an important role in preserving whole-body homeostasis in mother and fetus during pregnancy (Wu *et al.*, 2004a). Indeed, arginine is a metabolically versatile amino acid in animals, serving as a substrate for protein synthesis and regulating the metabolism of energy substrates (Beaumier *et al.*, 1996; Wu & Morris, 1998; Wu *et al.*, 1999; Morris, 2002). In addition, arginine can stimulate the secretion of important metabolic hormones such as insulin, GH, prolactin and glucagon, and is also able to activate nutrient-sensitive signaling pathways such as the mammalian target of rapamycin in skeletal muscle and small intestine (McAtee & Trenkle, 1971; Davis, 1972; Kuhara *et al.*, 1991; Morris, 2002; Thureen *et al.*, 2002; Meijer & Dubbelhuis, 2004; Wu *et al.*, 2007a). Moreover, arginine is the precursor of biologically important molecules, such as creatine, agmatine, NO, polyamines, proline, ornithine, citrulline and glutamate (Wu & Morris, 1998).

Arginine can be catabolized by multiple pathways. The enzymes that initiate arginine degradation are arginine:glycine amidinotransferase, arginine decarboxylase, arginases, and nitric oxide synthases (NOS). With the exception of arginine decarboxylase, all these enzymes act on the guanidino group of arginine (Morris, 2004). Arginine:glycine amidinotransferase catalyzes the first and rate-controlling step in the synthesis of creatine (Morris, 2004), which involves the metabolic cooperation of kidney, liver and muscle (Beaumier *et al.*, 1996). Creatine plays a major role in energy metabolism in skeletal muscle and neuronal cells, where it serves as a storage form of high energy phosphate (Beaumier *et al.*, 1996; Wu & Morris, 1998).

Arginine decarboxylase converts arginine to CO_2 and agmatine (Morris, 2002), which occurs in brain and kidney. Agmatine is a non-catecholamine ligand at α_2-adrenergic receptors that may act as a neurotransmitter (Li *et al.*, 1994).

There are two arginase isoforms: cytosolic (type I) and mitochondrial (type II). Type I arginase is present primarily in hepatocytes and to a much

lesser extent in small intestine, mammary tissue, and certain placentae. Type II arginase is expressed in most extrahepatic cells and tissues. Arginases play an important role in glutamate and proline synthesis via ornithine production (Wu & Morris, 1998). Glutamate, which is also synthesized from glutamine, proline and branched-chain amino acids, is an excitatory neurotransmitter that can give rise to yet another cell-signaling molecule: γ-aminobutyric acid (GABA) (Wu & Morris, 1998). Proline plus its derivative hydroxyproline constitutes one third of amino acids in collagen, therefore playing important roles in wound healing, chronic inflammatory diseases, and infection (Beaumier *et al.*, 1996; Morris, 2004). Ornithine serves as the precursor for biosynthesis of polyamines, which are aliphatic cations that can regulate gene expression, signal transduction, ion channel function, DNA and protein synthesis, apoptosis, as well as cell proliferation and differentiation (Thomas & Thomas, 2001; Flynn *et al.*, 2002; Zhao *et al.*, 2008). A rate-controlling step in the synthesis of polyamines from ornithine is catalyzed by ornithine decarboxylase (Zhao *et al.*, 2008). Ornithine is also required for ammonia detoxification via the hepatic urea cycle (Meijer *et al.*, 1990) and proline synthesis via ornithine aminotransferase.

The NOS has three isoforms that were named after the cell type from which they were first isolated. Endothelial NOS and neuronal NOS are constitutively expressed in different cells and their activity is regulated by the Ca^{++}/calmodulin complex. A third NOS isoform is inducible and requires a delay of 6–8 h before the onset of NO production but, once induced, produces large quantities of NO for hours to days in a calcium-independent manner (Wu & Morris, 1998; Beck *et al.*, 1999). Catabolism of arginine by NOS results in the release of NO and citrulline. Nitric oxide is a free radical that functions as a major mediator of numerous biological processes, including vasodilation, immune responses and neurotransmission (Wu & Meininger, 2002; Maul *et al.*, 2003). Citrulline is a key intermediate in the urea cycle and an efficient antioxidant protecting DNA, lipids and proteins from hydroxyl radical-induced

oxidative damage (Akashi *et al.*, 2001). Although citrulline is not a precursor for tissue protein synthesis, it can be effectively recycled to arginine, because of the widespread presence of argininosuccinate synthase and argininosuccinate lyase in tissues (Wu & Morris, 1998).

Arginine requirements in most mammalian cells are met primarily by uptake of extracellular arginine via specific transporters. An important transporter is the high-affinity, Na^+-independent system y+ (Wu & Morris, 1998). Because of its major role in arginine transport, regulation of system-y+ expression or activity represents a potential target for modulating cellular arginine metabolism (Wu & Morris, 1998). Other cationic amino acids and positively charged analogues are effective inhibitors of arginine uptake by system y+ (Wu & Morris, 1998).

Arginine abundance in the conceptus

Arginine is one of the most abundant amino acids deposited in fetal tissue proteins (Meier *et al.*, 1981; Wu *et al.*, 1999). In fact, the relative amounts of arginine in tissue mixed proteins range from 5% to 15% (Silk *et al.*, 1985; Davis *et al.*, 1993), indicating the quantitative importance of arginine in fetal growth. In addition, concentrations of arginine and of other biomolecules linked to arginine metabolism change dynamically in fetal fluids during gestation, which can affect fetal and placental development (Wu *et al.*, 1999; Kwon *et al.*, 2003a; Kwon *et al.*, 2003b; Kwon *et al.*, 2004b). Notably, arginine and citrulline are unusually abundant in porcine and ovine allantoic fluids, respectively, during early gestation (Wu *et al.*, 1995; Wu *et al.*, 1996; Kwon *et al.*, 2003a). Interestingly, arginase is absent from porcine placenta, therefore maximizing the transfer of arginine from maternal to fetal plasma and the abundance of arginine in porcine allantoic fluid. Conversely, arginase activity is present in ovine placenta and allantoic fluid, thus the high concentrations of citrulline could reflect an adaptation of the ovine fetus to maintain an efficient reservoir of an arginine

precursor (Kwon *et al.*, 2003a). Hence, it appears that different strategies can be used by different animal species to conserve arginine during this stage of pregnancy (Kwon *et al.*, 2003a). Moreover, increased activities of argininosuccinate synthase and argininosuccinate lyase at Day 60 of gestation were associated with increased arginine concentration in ovine allantoic fluid between Days 40 and 60 of gestation (Kwon *et al.*, 2003a). In addition, arginase and ornithine decarboxylase activities were elevated on Day 40 of gestation in placental and endometrial tissues of sheep (Kwon *et al.*, 2003b). Accordingly, concentrations of ornithine increased and supported high rates of polyamine synthesis (Kwon *et al.*, 2003b). Notably, all these changes occurred during a stage of maximal placental growth (Alexander, 1964; Reynolds & Redmer, 1995). Furthermore, relatively high levels of polyamine concentrations were present in ovine placental and endometrial tissues in the second half of pregnancy (Kwon *et al.*, 2003b), when there is continued development of the placental vascular bed and increases in total uterine blood flow (Ford, 1995; Reynolds & Redmer, 1995, 2001). This vascular adaptation plays an important role in enhancing the transfer of nutrients and oxygen from maternal to fetal blood to support the phase of most rapid absolute growth of the fetus in late gestation. Moreover, maximal activities of NOS, and highest rate of NO synthesis occurred in ovine placenta and endometrium in the first half of pregnancy (Sooranna *et al.*, 1995; Kwon *et al.*, 2004b), exhibiting a second peak during late gestation (Zheng *et al.*, 2000; Sheppard *et al.*, 2001; Kwon *et al.*, 2004b). Hence, it appears that metabolic coordination through different integrated pathways allows increased arginine availability during crucial stages in growth and development of the conceptus.

Functions of arginine in the establishment and maintenance of pregnancy

Studies in the past fifteen years have linked the arginine-NO and polyamines pathways to crucial events for the establishment and maintenance of

mammalian pregnancy, including early embryo development, implantation, growth and vascularization of the placenta and possibly uterine quiescence and cervical ripening (Henningsson *et al.*, 1983; Sooranna *et al.*, 1995; Gregg, 2003; Kwon *et al.*, 2003b; Kwon *et al.*, 2004b; Wu *et al.*, 2006; Zhao *et al.*, 2008). Translation of this basic knowledge into clinical practice or animal production has received increasing interest.

Early embryonic development and implantation. Studies conducted in murine embryos have shown that normal in vitro development required NO production by the embryo itself, and that an inhibition of NO synthesis, accomplished by addition of NOS inhibitors to the culture media, arrested embryo development (Gouge *et al.*, 1998; Chen *et al.*, 2001). Moreover, impairment of embryo development produced by the lack of NO could be reversed by the addition of an NO donor (Tranguch *et al.*, 2003). However, as an oxidant, a high concentration of NO can be detrimental for the embryo (Tranguch *et al.*, 2003). The presence of mRNA for all three NOS isoforms in preimplantation embryos of rodents provided further evidence for the importance of NO production in the embryo (Gouge *et al.*, 1998; Tranguch *et al.*, 2003). In addition, pre-implantation stage rhesus monkey blastocysts expressed the inducible form of NOS, while cytotrophoblast cells lining the embryonic cavity expressed both endothelial and inducible forms of NOS at distinct stages of placentation (Sengupta *et al.*, 2005). Furthermore, a pilot study with humans showed that increased NO production, measured indirectly through mean nitrite/nitrate concentrations in culture media, was positively associated with the growth potential of a developing embryo (Battaglia *et al.*, 2003).

Although the molecular mechanisms for NO in regulation of embryo development and survival remain unclear, the cGMP pathway was involved in the signal transduction of NO-regulated embryo development but not in embryo apoptosis induced by high concentrations of NO (Chen *et al.*, 2001).

Nitric oxide also participates in modulating vascular changes that accompany trophoblast implantation, which appears to be regulated by an interaction of NO with estrogen and progesterone (Gouge *et al.*, 1998; Chwalisz *et al.*, 1999 ; Sengoku *et al.*, 2001 ; Maul *et al.*, 2003) and mediated by cGMP (Duran-Reyes *et al.*, 1999). High NOS activities have been observed in the connective tissue surrounding spiral arterioles in the endometrium and the implantation site in baboon and mice, respectively, during implantation (Purcell *et al.*, 1999a; Purcell *et al.*, 1999b). Also, invading trophoblast cells co-expressing the endothelial and inducible NOS forms caused dilatation of utero-placental arteries in guinea pigs, suggesting that NO mediates spiral arterial changes occurring during pregnancy (Nanaev *et al.*, 1995).

An additional mode of action of NO in implantation might involve induction of matrix-degrading proteases, resulting in remodeling of the extracellular matrix necessary for trophoblast invasion (Novaro *et al.*, 2001). Production of NO by trophoblasts and endometrial leukocytes may also contribute to the local modulation of maternal immunity at the peri-implantation stage (Dembinska-Kiec *et al.*, 1991; Thaler & Epel, 2003).

Polyamines also participate in embryo development and implantation. Studies in mice show that expression of ornithine decarboxylase in the subluminal uterine stroma was dependent upon the presence of an active blastocyst in implantation sites (Zhao *et al.*, 2008), and homeostasis of uterine polyamines was important for endometrial cell proliferation (Henningsson *et al.*, 1983; Zhao *et al.*, 2008). In addition, an immunosuppressive role for polyamines during early pregnancy has also been proposed (Sooranna *et al.*, 1995). Moreover, polyamines are required at different phases of cell cycle progression, modulating the functions of RNA, DNA, nucleotide triphosphates, proteins and other acidic substances (Igarashi & Kashiwagi, 2000; Thomas & Thomas, 2001). Hence, polyamines seem to be essential for embryo implantation and are critical

for survival and development of the conceptus (Fozard *et al.*, 1980; Henningsson *et al.*, 1983; Ishida *et al.*, 2002; Zhao *et al.*, 2008).

Growth and vascularization of the placenta. Adequate placental growth is essential for adequate fetal growth (Gootwine, 2004; Murphy *et al.*, 2006), and arginine, through NO and polyamines, is involved in placental development and function throughout gestation. Indeed, polyamines regulate cell growth and proliferation in the placenta and fetus (Igarashi & Kashiwagi, 2000; Thomas & Thomas, 2001), whereas NO participates in the modulation of placental vascular growth and thus of placental blood flow (Sladek *et al.*, 1997; Reynolds & Redmer, 2001; Bird *et al.*, 2003). Indeed, a positive correlation exists between placental size and function and birth weight (Resnik, 2002; Luther *et al.*, 2005; Regnault *et al.*, 2005a; Bryan & Hindmarsh, 2006). Hence, remodeling of the placenta, including growth and development of the vascular bed, is essential to normal placental function, therefore impacting the transfer of nutrients and O_2 from the mother to the fetus and supporting fetal growth (Mayhew, 2002; Lang *et al.*, 2003; Reynolds *et al.*, 2005a). Certainly, IUGR has been associated with reduced fetoplacental blood flow (Sooranna *et al.*, 1995; Chaddhaa *et al.*, 2004). Furthermore, uniform restriction of uterine blood flow by the use of adjustable vascular occluders, without primary placental insults, led to changes in the growth pattern of affected fetuses and placentae (Lang *et al.*, 2000). In addition, placental expression of angiogenic factors was reduced in nutrient-restricted and overnourished ewes (Redmer *et al.*, 2005; Reynolds *et al.*, 2005a). Similarly, placental angiogenesis was altered in other ovine models of compromised pregnancy, including thermal stress, hypobaric conditions, and multiple fetuses (Reynolds *et al.*, 2005a; Murphy *et al.*, 2006).

The increase in utero-placental blood flows with advancing pregnancy results from vasodilation and angiogenesis as the placenta continues to remodel (Reynolds & Redmer, 2001; Sheppard *et al.*, 2001; Lang *et al.*, 2003; Vonnahme

et al., 2005). Both placental and uterine tissues from pregnant sheep produce NO as well as polyamines in patterns that coincide with the periods of most rapid placental and fetal growth (Zheng *et al.*, 2000; Kwon *et al.*, 2003b; Kwon *et al.*, 2004b). Moreover, endothelial NOS expression is increased in uterine arteries during late pregnancy in sheep (Zheng *et al.*, 2000; Sheppard *et al.*, 2001), and inhibition of NOS resulted in the ipsilateral reduction of uterine blood flow without altering flow in the contralateral uterine artery or changing maternal systemic blood pressure (Miller *et al.*, 1999). These results suggest that endogenously produced NO in the uterine vasculature of late-pregnant ewes contributes to basal maternal vasodilator tone. In support of this view, intramuscular administration of sildenafil citrate, a vasodilator that enhances the effect of NO by inhibiting phosphodiesterase type 5, from gestational days 28 to 112 was effective to increase fetal growth in both underfed and adequately nourished ewes (Satterfield *et al.*, 2007). In effect, impaired placental syntheses of NO and polyamines has been proposed as a unified explanation for IUGR in response to nutritional problems such as under- or over- feeding (Wu *et al.*, 2004a).

Exogenous administration of arginine is an effective means of increasing endogenous NO synthesis (Wu and Meininger, 2002). In fact, arginine supplementation has been shown to improve uteroplacental circulation and infant weight at birth in women presenting IUGR (Lampariello *et al.*, 1997; Di Renzo *et al.*, 2005; Xiao & Li, 2005). In addition, dietary arginine provision has resulted in enhanced litter size in gilts (Mateo *et al.*, 2007) and rats (Zeng *et al.*, 2008).

Mediation of angiogenesis by NO may occur by triggering and transducing cell growth and differentiation via NOS activation, cGMP elevation, mitogen activated kinase (MAPK) activation, and fibroblast growth factor-2 (FGF-2) expression (Ziche & Morbidelli, 2000; Zheng *et al.*, 2006). In addition, NO can regulate the expression of other angiogenic factors such as VEGF (Reynolds & Redmer, 2001; Maul *et al.*, 2003). Furthermore, in the

endometrium, NO may interact with platelet-activating factor, a potent inflammatory lipid mediator that is known to increase vascular permeability and vasodilatation (Ahmed *et al.*, 1998). Regulation of expression of metalloproteases and their tissue inhibitors by NO also contributes to the degradative capacity of endothelial cells that correlates with matrix invasion during angiogenesis (Ziche & Morbidelli, 2000).

Uterine quiescence and cervical ripening. Some studies suggest that NO may have a role in the complex control system that maintains uterine quiescence during pregnancy (Izumi *et al.*, 1993; Ramsay *et al.*, 1996; Sladek *et al.*, 1997; Norman *et al.*, 1999; Gregg, 2003); as well as in cervical ripening at term (Maul *et al.*, 2003; De Pace *et al.*, 2007). Indeed, all three NOS isoforms have been found in myometrial and cervical tissues from various species, including humans (Izumi *et al.*, 1993; Sladek *et al.*, 1997; Norman *et al.*, 1999), and inhibition of NO synthesis causes preterm delivery in the mouse (Tiboni & Giampietro, 2000). Notably, while the NO system seems to be up-regulated in the myometrium during pregnancy, the opposite occurs in the cervix, and these conditions are reversed at term (Buhimschi *et al.*, 1996; Maul *et al.*, 2003). In addition, the myometrium and cervix have a differential temporo-spatial expression of the NOS isoforms (Bansal *et al.*, 1997; Dong *et al.*, 1998; Norman *et al.*, 1999; Maul *et al.*, 2003).

Despite functional and molecular studies, the exact role of the NO system in the control of myometrial function during pregnancy has not yet been clearly defined (Tiboni & Giampietro, 2000). Nonetheless, it appears likely that NO acts in concert with progesterone affecting the cGMP pathway, as well as other systems such as direct effects on ion handling by the muscle (Buhimschi *et al.*, 1996; Chwalisz & Garfield, 1998; Maul *et al.*, 2003). As for cervical ripening, NO may act in cooperation with other biomolecules of the inflammatory cascade, such as prostaglandins and COX, to induce local vasodilation and increase

vascular permeability and leukocyte infiltration (Chwalisz & Garfield, 1998; Maul *et al.*, 2003). In addition, NO may regulate metalloproteases that allow for the rearrangement of collagen fibers (Chwalisz & Garfield, 1998; Maul *et al.*, 2003). However, further investigation is needed to fully understand the biochemical processes involved (Sladek *et al.*, 1997; Norman *et al.*, 1999; Maul *et al.*, 2003).

Intrauterine growth restriction

Growth of the fetus depends on multiple biological and environmental factors (Wu *et al.*, 2004a; Eleftheriades *et al.*, 2006; Gicquel & Le Bouc, 2006). Intrauterine growth restriction can be defined as a deviation from an expected pattern of fetal growth that leads to attenuation of fetal growth potential due to an insult that has occurred in utero (Ergaz *et al.*, 2005; Wu *et al.*, 2006). Further, IUGR may be classified clinically on the basis of a birth weight below the tenth percentile for gestational age in infants, relative to the reference population (McMillen *et al.*, 2001). In fact, assessment of fetal growth currently constitutes an essential issue for prenatal surveillance in human medicine (Gardosi, 1997). Certainly, growth aberration is one of the main manifestations of abnormal intrauterine development (Barr *et al.*, 1994). Moreover, the growth trajectory of the fetus and its adaptive responses to the prenatal and postnatal environment may be determined beginning from the period around conception and continuing throughout gestation (Ergaz *et al.*, 2005; Myatt, 2006; Valsamakis *et al.*, 2006). In fact, an IUGR fetus can be further classified as symmetric or asymmetric according to the onset of the causative fetal insult (Resnik, 2002; Monk & Moore, 2004; Ergaz *et al.*, 2005). Symmetric IUGR fetuses are often exposed to intrauterine insults early in gestation, with a generalized decrease in cell division and cell growth that results in a proportionate reduction of fetal measurements. Conversely, asymmetric IUGR fetuses suffer an adverse environment later in intrauterine life, undergoing a disproportionate growth because normal growth of

vital organs such as the brain is usually maintained (Resnik, 2002; Valsamakis *et al.*, 2006).

The etiology of IUGR could be multifactorial, involving both genetic and environmental factors to a varying degree (Monk & Moore, 2004). Several maternal, fetal and uteroplacental factors can be related with perturbation of fetal growth. These factors include maternal age, nutrient intake, hypoxia, health, smoking and alcohol or drug abuse; fetal disruption of gene expression, chromosomal abnormalities, as well as fetal number; and placental hormone production, nutrient transport capacity and vascularity (Bajoria *et al.*, 2001; Cetin, 2003; Monk & Moore, 2004; Dunger *et al.*, 2006; Fowden *et al.*, 2006a; Murphy *et al.*, 2006; Wu *et al.*, 2006; Gootwine *et al.*, 2007). However, the specific pathophysiological processes underlying IUGR are complex and incompletely understood (Ergaz *et al.*, 2005; Wu *et al.*, 2006). Nevertheless, growing evidence suggests that, independent of the predisposing factor(s), impaired fetal growth is frequently associated with abnormalities of placental development and function (Lang *et al.*, 2003; Lea *et al.*, 2005; Luther *et al.*, 2005; Reynolds *et al.*, 2005a; Wallace *et al.*, 2005; Murphy *et al.*, 2006; Wu *et al.*, 2006). Indeed, both genetic and environmental factors can disrupt placental development, having an impact on placental vascularity and uteroplacental blood flows, which ultimately determines placental sufficiency (Vonnahme *et al.*, 2003; Redmer *et al.*, 2004; Wu *et al.*, 2004a; Regnault *et al.*, 2005b; Fowden *et al.*, 2006a; Gluckman & Hanson, 2006; Wu *et al.*, 2006).

Focal aspects of normal placental vascular development

In mammalian species, placental growth occurs predominantly in the first half of pregnancy, but continues to undergo morphological, structural and biochemical changes throughout gestation to ensure that placental function parallels fetal growth (Mellor, 1983; Owens, 1991; Ford, 1995; Reynolds *et al.*, 2006). In fact, disruption of the normal pattern of placental development will

lead to a placenta with an altered function (Chaddha *et al.*, 2004; Myatt, 2006). One of the factors that plays a central role in adequate placental function is nutrient transfer capacity of the placenta, which directly influences the growth trajectory of the fetus and hence weight at birth (Reynolds & Redmer, 1995; Redmer *et al.*, 2004). Transplacental exchange is largely dependent upon uterine and umbilical blood flows, and these blood flows are in turn dependent on adequate vascularization of the placenta (Lang *et al.*, 2000; Myatt, 2006; Reynolds *et al.*, 2006; Luther *et al.*, 2007). Indeed, utero-placental vasculature undergoes adaptive changes throughout gestation that include vasculogenesis, angiogenesis and vasodilation, as well as an increase in vascular permeability (Reynolds & Redmer, 1995; Myatt, 2006). Vasculogenesis is the process of forming a primitive vascular network through endothelial progenitor cells, and angiogenesis represents the development of new vessels from pre-existing ones (Zygmunt *et al.*, 2003). Actually, vasculogenesis can first be observed at the stage of early tertiary villi in human placentae (Asan *et al.*, 1999), and morphological studies using microscopical sections at different periods of gestation confirmed that angiogenesis is linked to villous growth and maturation (Mayhew, 2002).

Placental vascular growth begins as early as 21 days after conception and continues throughout gestation in humans (Asan *et al.*, 1999). Certainly, dramatic changes in the vascular content and arrangement, characterized by disproportionate growth and expansion of villous arborizations, occur between the end of the first trimester (10-13 weeks) and the last month of pregnancy (38-41 weeks) (Mayhew, 2002). In sheep, vascular volume of the endometrium exhibits a two-fold increase in the gravid uterine horn by day 24 after mating (Reynolds & Redmer, 1992). In addition, capillary area density of both maternal caruncular and fetal cotyledonary tissues increased exponentially from day 50 through day 140 after mating (Reynolds *et al.*, 2005b). Accordingly, the increase of uterine and umbilical blood flows is most remarkable from mid to late

gestation (Rosenfeld *et al.*, 1974; Fuller *et al.*, 1975), coinciding with the period of maximal fetal growth (Redmer *et al.*, 2004; Gootwine *et al.*, 2007). In fact, it is estimated that by the end of gestation, 20% of the maternal cardiac output is targeted to the uterus to support fetal growth in sheep (Gootwine *et al.*, 2007).

The placenta is a rich source of angiogenic factors that may play an important role in the regulation of placental vessel formation, as well as in maternal vascular adaptation to pregnancy (Reynolds & Redmer, 2001; Zygmunt *et al.*, 2003). Expression of VEGF, FGF, angiopoietins and their receptors, as well as endothelial NO synthase, has been described in different mammalian species, including humans (Geva & Jaffe, 2000; Zheng *et al.*, 2000; Reynolds & Redmer, 2001; Wulff *et al.*, 2003; Reynolds *et al.*, 2005b; Vonnahme *et al.*, 2005; Borowicz *et al.*, 2007; Luther *et al.*, 2007). Interestingly, expression of angiogenic factors can be altered in compromised pregnancies, including those resulting from maternal undernutrition or overnutrition and pre-eclampsia (Rutherford *et al.*, 1995; Cooper *et al.*, 1996; Brennecke *et al.*, 1997; Wu *et al.*, 1998a; Redmer *et al.*, 2005; Reynolds *et al.*, 2005b; Luther *et al.*, 2007).

Fetal growth restriction, physiologic adaptations and postnatal development

Low birth weight is associated with an increased risk of neonatal mortality and morbidity (Mellor, 1983; McIntire *et al.*, 1999; Resnik, 2002; Van Rens *et al.*, 2005). Findings from both animal and human studies indicate that IUGR neonates frequently present complications such as hypoxia, hypoglycemia and oxidative stress, have a diminished capacity to withstand thermoregulatory challenges soon after birth, and are likely to develop pulmonary, digestive and circulatory disorders (Resnik, 2002; Greenwood & Bell, 2003; Monk & Moore, 2004; Wu *et al.*, 2004a). Furthermore, there is a higher incidence of premature deliveries among fetuses that are growth restricted (Cook *et al.*, 1988; Fliegner, 1989; Bryan & Hindmarsh, 2006; Siddiqui & McEwan, 2007), with approximately

5% of all IUGR infants being born before term (Bryan & Hindmarsh, 2006). Consequently, IUGR is associated with considerable financial and emotional costs both in medical and agricultural sectors (Ergaz *et al.*, 2005; Luther *et al.*, 2005; Wu *et al.*, 2006).

Experimentally, the metabolic, physiological and functional abnormalities seen in individuals that suffered IUGR may be linked to the physiological adaptations that the growth-retarded fetus suffers in response to a suboptimal intrauterine environment, which can be of critical importance not only for perinatal health and survival but also for longer term health outcome (Barker & Clark, 1997; McMillen *et al.*, 2001; Luther *et al.*, 2005). Indeed, the timing, nature and intensity of the uterine insults can differentially impact the individual, affecting development and function of organs, tissues and systems with varying severity (McMillen *et al.*, 2001; Resnik, 2002; Greenwood & Bell, 2003). For instance, premature birth of IUGR fetuses has been associated with an accelerated maturation of the hypothalamic-pituitary-adrenal (HPA) axis, that may result in altered concentrations of ACTH and glucocorticoids during late pregnancy (Economides *et al.*, 1988; Phillips *et al.*, 1996; Edwards & McMillen, 2002; Bloomfield *et al.*, 2004; Kumarasamy *et al.*, 2005). In fact, concentrations of glucocorticoids in the growth-restricted fetus may be partly modulated by the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD), because restriction of placental growth resulted in a twofold increase in 11β-HSD mRNA expression in the liver of IUGR sheep fetuses in late gestation (McMillen *et al.*, 2000). Further, the increase in intrahepatic 11β-HSD may play a role in glucocorticoid-mediated increases in glycogen deposition and gluconeogenesis in the liver that occur immediately before birth and which could be critical for perinatal survival after a period of substrate deprivation (McMillen *et al.*, 2001). Additionally, it has been proposed that elevated or decreased cortisol concentrations in adults that were once IUGR fetuses may occur as a consequence of in utero adaptations of the HPA axis, possibly altering neuroendocrine response to stressors throughout

lifetime (Edwards & McMillen, 2002; Kajantie *et al.*, 2002; Eleftheriades *et al.*, 2006).

Other metabolic and/or endocrine systems that alter functions such as growth, reproduction and energy metabolism may also be disturbed by environmental disruptions during fetal life (McMillen *et al.*, 2001; Greenwood & Bell, 2003; Gardner *et al.*, 2005; Eleftheriades *et al.*, 2006; Sizonenko *et al.*, 2006). For example, IUGR has been associated with changes in fetal amino acid metabolism, where urea production is reduced in the fetus during late gestation, increasing the risk of toxic hyperammonemia after birth (Greenwood & Bell, 2003; De Boo *et al.*, 2007). There is also evidence that fetal plasma concentrations of anabolic hormones such as IGF-I, insulin, prolactin and thyroid hormones are decreased near term in an ovine model of IUGR due to placental restriction (Symonds *et al.*, 2001). Free thyroxine and triiodothyronine concentrations in plasma are also reduced in IUGR human fetuses when compared with gestationally-matched normal fetuses (Kilby *et al.*, 1998). Moreover, abundance of mRNA for the long form of the prolactin receptor in adipose tissue and for prolactin in the fetal pituitary are decreased in growth-restricted ovine fetuses (Phillips *et al.*, 2001; Symonds *et al.*, 2001). In addition, IUGR induced by maternal protein restriction throughout pregnancy and lactation in rats resulted in the development of diabetes, hyperinsulinemia and tissue insulin resistance of the offspring in adulthood (Fernandez-Twinn *et al.*, 2003; Ozanne *et al.*, 2003). Further, the capacity of the fetal adrenal medulla to synthesize and secrete catecholamines is potentially impaired from mid-gestation in placentally restricted ovine fetuses (Coulter *et al.*, 1998), and IUGR rat-pups have impaired adrenaline secretory responses to acute hypoxia after birth (Shaul *et al.*, 1989).

It has been postulated that the adaptive responses that occur in the growth restricted fetus could result from an asymmetric redistribution of blood flow that distinctly influences the developmental trajectory of organs and tissues,

thus affecting and probably resetting their functional characteristics (Desai *et al.*, 1996; Hoet & Hanson, 1999; Tekay & Jouppila, 2000; Gluckman & Hanson, 2006; Malamitsi-Puchner *et al.*, 2006). The sequelae of such alterations may be immediate or may not be clinically apparent until later in life, therefore impacting long term health and the onset of diseases such as type 2 diabetes, cardiovascular disease and hypertension (Barker & Clark, 1997; Symonds *et al.*, 2001; Osgerby *et al.*, 2002; Ingelfinger, 2004 ; Gardner *et al.*, 2007). Indeed, animal models of IUGR induced by undernutrition or by placental insufficiency indicate that most of the fetal circulating nutrients and energy are directed to growth maintenance of vital organs, such as the heart and brain at the expense of other tissues, including liver, pancreas, muscle, bone and fat, which have decreased sizes and weights in growth restricted fetuses (Desai *et al.*, 1996; McMillen *et al.*, 2001; Osgerby *et al.*, 2002; Satterfield *et al.*, 2007). In fact, evidence now suggests that even organs of primary importance such as the brain, which may not have an evident reduction in relative fetal mass, can be functionally compromised by IUGR, leading to an increased risk of neurological and behavioral disorders due to reduced number of neurons and long-term alterations in brain structure formation and function (Kilby *et al.*, 2000; Mallard *et al.*, 2000; Duncan *et al.*, 2004; Rehn *et al.*, 2004).

Epigenetic processes may also modify the physiological structure and function of organs and systems in IUGR fetuses, by altering gene expression (Wu *et al.*, 2006; Gluckman *et al.*, 2007). Specifically, DNA methylation and covalent modification of histones seem to be susceptible to environmental influences and are involved in the induction of an altered phenotype by nutritional constraint in early life (Burdge *et al.*, 2007a). Nutrition affects methylation patterns because components and cofactors needed for methylation reactions come from dietary methyl donors such as methionine, choline, folic acid and vitamin B_{12} (Wu *et al.*, 2006). Indeed, feeding a protein-restricted diet to pregnant rats altered gene expression by altering methylation and histone

modifications of the hepatic peroxisomal proliferator-activated receptor and the hepatic glucocorticoid receptor promoters (Lillycrop *et al*., 2005; Lillycrop *et al*., 2007). Further, the differential changes in the methylation of individual CpG dinucleotides in the hepatic peroxisomal proliferator-activated receptor alpha promoter that occur in juvenile rats persist in adults (Lillycrop *et al*., 2008). Moreover, epigenetic alterations resulting from intrauterine fetal adaptation to nutritional constraint can be passed to subsequent generations (Burdge *et al*., 2007b; Godfrey *et al*., 2007).

In sum, IUGR is a significant problem in both medicine and animal production. Available evidence suggests that environmental insults (e.g., maternal undernutrition) may cause IUGR, which is associated with impairment of structural and functional development of the placenta and fetus. Identifying effective means to prevent or ameliorate IUGR has important implications for both medicine and animal sciences. One of such approaches may be exogenous administration of arginine, a nutritionally essential amino acid for the fetus that has important roles in metabolism and physiology, including vascular homeostasis, angiogenesis and placental growth.

Objectives

The main goal of this dissertation research was to determine if parenteral administration of arginine during mid- to late gestation to ovine models of IUGR would ameliorate or prevent fetal growth retardation. In the first experiment, we hypothesized that intravenous administration of citrulline to the pregnant ewe would be more effective than arginine for enhancing arginine availability to the fetus. The second experiment was conducted to determine if long-term maternal intravenous administration of arginine during pregnancy would positively affect weights at birth of lambs from undernourished ewes. Finally, the third experiment was designed to assess if administration of arginine to late pregnant

mothers would enhance fetal survival and increase lamb birth weights in ewes carrying multiple fetuses.

CHAPTER II
INTRAVENOUS ADMINISTRATION OF CITRULLINE TO PREGNANT EWES IS MORE EFFECTIVE THAN ARGININE TO ENHANCE ARGININE AVAILABILITY IN THE FETUS

Introduction

There is increasing evidence that arginine plays an important role in mammalian pregnancy (Henningsson *et al.*, 1983; Sooranna *et al.*, 1995; Kwon *et al.*, 2003b; Kwon *et al.*, 2004b; Wu *et al.*, 2006; Zhao *et al.*, 2008). Of particular interest, nitric oxide (NO) and polyamines (products of arginine catabolism) are involved in crucial events such as implantation, embryogenesis, and uterine quiescence throughout gestation, and regulate vascularization, growth, and development of the placenta (Neri *et al.*, 1995; Reynolds & Redmer, 2001; Ishida *et al.*, 2002; Bird *et al.*, 2003; Maul *et al.*, 2003; Zhao *et al.*, 2008). As a major vasodilator, NO modulates utero-placental blood flow and the transfer of nutrients from mother to fetus (Bird *et al.*, 2003). Indeed, administration of arginine improved uteroplacental circulation and ameliorated fetal growth restriction in women (Lampariello *et al.*, 1997; Di Renzo *et al.*, 2005; Xiao & Li, 2005) and dietary arginine supplementation enhanced fetal survival and growth in gilts (Mateo *et al.*, 2007).

Arginine is alkaline in physiological solutions, and therefore, to prevent an acid-base imbalance, its hydrochloride (HCl) salt is generally used for intravenous administration into animals and humans (Wu & Meininger, 2000). However, there are concerns over the effect of chronic provision of chloride on the metabolism and health of both mother and fetus. Furthermore, the biological half-life of arginine in mammals is relatively short (e.g., 45 min in ewes on Day 105 of gestation) (Wu *et al.*, 2007a) due to arginase that degrades arginine in cells and tissues, including the ovine placenta (Wu & Morris, 1998; Morris, 2002; Kwon *et al.*, 2004b). An alternative approach is to use citrulline (Wu &

Meininger, 2000), a neutral amino acid that can be utilized for arginine synthesis in cells (Wu & Morris, 1998). In addition, limited degradation of citrulline in the placenta would maximize its transfer from mother to fetus (Kwon *et al.*, 2003a; Wu *et al.*, 2008).

We hypothesized that intravenous administration of citrulline to pregnant dams would be more effective than arginine for enhancing arginine availability to the fetus. This hypothesis was tested in gestating ewes, an established valuable model for studying human fetal growth (Bird *et al.*, 2003; Schroder, 2003), by determining the pharmacokinetics of arginine and citrullline in maternal plasma, as well as arginine availability in the fetus, in response to intravenous administration of either amino acid to the ewe.

Materials and methods

Ewes

Six multiparous Suffolk crossbred ewes with a body weight of 62.4 ± 3.2 kg (mean ± SEM) were used for catheterization of maternal and fetal blood vessels. Animals were sheared, washed and confined in individual pens indoors with free access to drinking water and fed at 0700h and 1800h to meet 100% of the NRC (National Research Council, 1985) nutrient requirements for pregnant sheep. A complete pelleted diet (Table 2.1) was purchased from Producers Cooperative Association (Bryan, TX, USA). Ewes consumed all of the feed (22 g/kg body weight) provided daily. This study was approved by the Texas A&M University Institutional Animal Care and Use Committee.

Table 2.1. Exp. 1/ Composition of the diet (as-fed basis)[1]

Ingredients	Content
Wheat midds	37.79%
Corn	20%
Dehydrated alfalfa	15%
Soybean hulls	12%
Soybean meal	5.25%
Rice bran	5%
Liquid binder	2.5%
Ground limestone	1.42%
Ammonium chloride	0.50%
Mineral mixture	0.30%
Vitamin mixture	0.05%

[1]Provided the following in the diet: 15% crude protein; 4.09% crude fat; 11.81% crude fiber; 1.0% calcium; 0.55% phosphorus; 0.60% chlorine; 0.20% sodium; 1.13% potassium; 0.18% sulfur; 0.27% magnesium; 174 ppm manganese; 187 ppm iron; 13.3 ppm copper; 0.31 ppm cobalt; 150 ppm zinc; 1.01 ppm iodine; 0.56 ppm selenium; 1.00 ppm molybdenum; 13,451 IU/kg vitamin A; 15.6 IU/kg vitamin E; 1.76 mg/kg thiamin; and 0.27 mg/kg vitamin K.

Surgical instrumentation and experimental design

After a 7-day period of acclimation to their confinement conditions and coinciding with gestational day 130 ± 1 (mean ± SEM), ewes were instrumented with catheters to access maternal and fetal vessels as previously described (Cudd *et al.*, 2001). Briefly, anesthesia was induced by intravenous administration of diazepam (0.2 mg/kg, Abbott Laboratories, North Chicago, IL, USA) and ketamine (4 mg/kg, Fort Dodge, IA, USA). A surgical plane of anesthesia was maintained using isofluorane (0.5%-2.5%, Abbott Laboratories, North Chicago, IL, USA) after endotracheal intubation. The ewes were positioned in dorsal recumbency and a ventral midline laparotomy was performed to expose uterus and fetal membranes which were incised. After exteriorizing a hind leg of the fetus, a polyvinyl chloride catheter (0.08 cm inner diameter, 0.13 cm outer diameter) was passed from the cranial tibial artery into the femoral artery. The procedure was repeated for the alternate leg, after

which the fetus was returned to the amniotic sac, and the uterus was closed with sutures prior to closing the maternal midline incision. The ewes were then instrumented to access arterial and venous circulation by advancing polyvinyl chloride catheters (0.13 cm inner diameter, 0.23 cm outer diameter) into the maternal aorta and vena cava via the femoral artery and vein, respectively. Fetal and maternal catheters were passed through the abdominal wall in the flank of the ewe and secured in a pouch attached to the skin.

Following post-surgical recovery for 5 days, all ewes received a sterile intravenous bolus dose of L-arginine-HCl (Sigma-Aldrich Corp.) equivalent to 155 μmol L-arginine/kg body weight on the first day of sampling (Day 1) or the same dose of L-citrulline (Sigma-Aldrich Corp.) on the subsequent day (Day 2). A previous study from our laboratory showed that arginine was completely cleared from the circulation at 5 h after its intravenous administration to pregnant ewes (Wu *et al*., 2007a). Infused solutions were prepared at a concentration of 1.5 g of arginine per 5 ml or 1.5 g of citrulline per 20 ml, using sterile physiologic saline (0.9% sodium chloride, Hospira Inc., Lake Forest, IL, USA). After adjusting the pH to 7.0 with 1 M NaOH, solutions were filtered through a 0.22-μm cellulose acetate filter (Corning Inc., NY, USA) into sterile glass containers fitted with sterile rubber caps. The total volumes of the arginine-HCl or citrulline solution administered to a ewe were approximately 6.0 and 22.5 ml, respectively. On both treatment days, maternal and fetal arterial blood samples (1 ml) were obtained simultaneously at -120, -60, 0, 5, 15, 30, 60, 120, 180 and 240 min from the time of delivery of the amino acid solution and placed into vials containing 2 μl of 0.3 M EDTA. Blood samples were carefully inverted to allow mixing with the anticoagulant and immediately centrifuged at 10,000 X g for 1 min. Plasma was separated and stored at -80℃ until assayed for amino acids.

Determination of amino acids

Amino acids were determined using fluorometric HPLC methods involving precolumn derivatization with *o*-phthaldialdehyde as previously described (Wu *et al.*, 1997). High-performance liquid chromatography (HPLC)-grade water and methanol used for the analysis were obtained from Fisher Scientific (Fair Lawn, NJ, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Plasma (40 μl) was deproteinated with 40 μl of 1.5 M $HClO_4$, followed by addition of 20 μl of 2 M K_2CO_3 and 900 μl of HPLC water. Amino acids in samples were quantified on the basis of authentic standards (Sigma-Aldrich Corp., St. Louis, MO) using Millenium-32 Software (Waters, Milford, MA) (Wu & Meininger, 2008). Concentrations of total amino acids at each time point were a mathematical sum of individual amino acids for the corresponding sampling.

Calculations and statistical analyses

Pharmacokinetics of the intravenous bolus of arginine or citrulline were analyzed after subtraction of baseline concentrations of plasma arginine and citrulline, using the single exponential model, plasma amino acid = $a \times e^{(-b \times t)}$, where "a" is maximum concentration in plasma and "b" is the elimination rate, as described previously (Wu *et al.*, 2007a). The internal exposure to exogenous arginine or citrulline was estimated by calculating the area under the concentration-time curve (AUC; a/b), with total clearance (CL) = amino acid dose/AUC. The maximum concentration of arginine or citrulline (C_{max}) in plasma was calculated by back-extrapolation of the elimination curve to time zero. The biological half-life ($T_{1/2}$) of the infused amino acid was determined from the elimination curve (Wu *et al.*, 2007a).

Differences in pharmacokinetic parameters between arginine and citrulline following administration to ewes were determined by one-way ANOVA. Data on concentrations of amino acids in plasma among different time points

after arginine or citrulline infusion were analyzed by ANOVA for repeated measures, using the mixed model procedure of SAS (version 9.1, SAS Institute, Cary, NC, USA). In addition, the linear regression procedure was used to compare slopes of amino acids throughout the sampling periods. A *P* value of equal or less than 0.05 was designated as an indication of a statistical effect of treatment.

Results

Concentrations of amino acids in maternal or fetal plasma obtained at -120, -60 and 0 min did not differ on either sampling day ($P > 0.05$). Hence, averaged values for the -120, -60 and 0 min time points were used to represent baseline values as time 0 min. In addition, baseline concentrations of all amino acids in maternal or fetal plasma were not different between sampling days ($P > 0.05$), indicating a lack of carry-over effects of arginine administration on the subsequent day of sampling.

Amino acids in maternal plasma

A rapid increase in arginine and citrulline in maternal plasma was observed within 30 min after intravenous bolus administration of the two amino acids to ewes (Fig 2.1). The concentration of arginine in maternal plasma peaked at 5 min after arginine infusion, with circulating levels remaining above ($P < 0.01$) the baseline for up to 120 min post-injection (Table 2.2, $P < 0.01$). Similar results were obtained for concentrations of citrulline after citrulline administration, except that the value at 240 min continued to be greater (Table 2.3, $P < 0.01$) than the baseline level.

Administration of arginine to the ewes did not ($P > 0.10$) increase concentrations of citrulline in maternal plasma at any time point post-injection (Fig. 2.1). However, concentrations of arginine in maternal plasma increased ($P < 0.05$) between 5 and 240 min after citrulline infusion (Fig. 2.1). Compared with the baseline, concentrations of ornithine in maternal plasma increased ($P <$

0.01) 94% between 15 and 30 min after exogenous arginine infusion and remained elevated ($P < 0.05$) up to 120 min post-injection (Table 2.2 and Fig. 2.2). In contrast, there were no changes ($P > 0.10$) in concentrations of ornithine in maternal plasma at any time point after citrulline administration (Table 2.3 and Fig. 2.2).

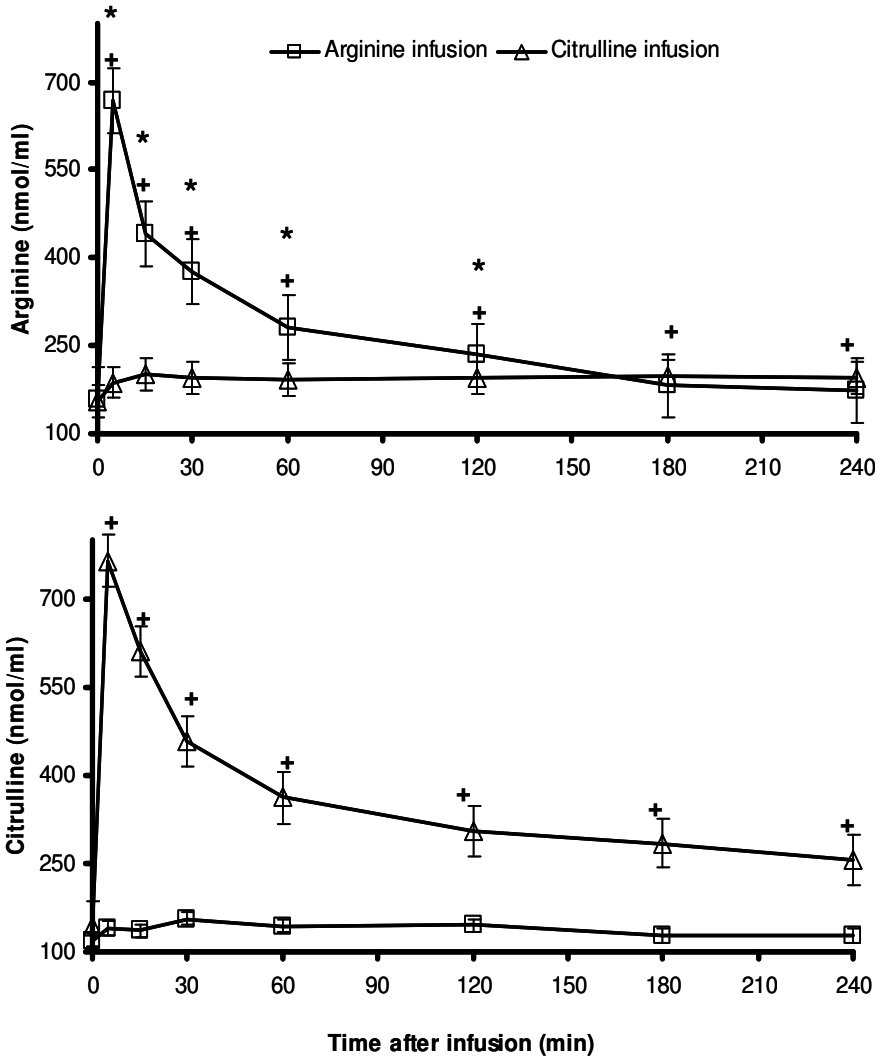

Fig 2.1. Concentrations of arginine and citrulline in maternal plasma after a single intravenous bolus injection of 155 μmol/kg body weight of either arginine-HCl or citrulline on Day 135 ± 1 of gestation (*,+ $P < 0.05$ for arginine and citrulline infusion, respectively).

Table 2.2. Concentrations of arginine, citrulline and ornithine in maternal and fetal plasma after a single intravenous bolus injection of arginine-HCl (155 µmol L-arginine/kg body weight) to late pregnant ewes

Amino acid	Time after bolus injection (min)								SEM	*P* value
	0	5	15	30	60	120	180	240		
	µmol/L									
Maternal plasma:										
Arginine	159^{d}	668^{a}	441^{b}	377^{b}	281^{c}	234^{ce}	181de	174de	55	<0.0001
Citrulline	119	140	136	157	144	145	128	127	11	0.086
Ornithine	56^{d}	88^{bc}	96^{ab}	108^{a}	93^{bc}	82^{c}	64^{d}	58^{d}	10	<0.0001
Fetal plasma:										
Arginine	125^{c}	128^{c}	161^{b}	190^{a}	141^{bc}	135^{c}	128^{c}	134^{c}	12	<0.0001
Citrulline	119	114	119	118	114	115	115	126	6	0.335
Ornithine	35^{c}	39^{bc}	46^{ab}	49^{a}	47^{ab}	41^{bc}	38^{c}	40^{bc}	4	0.007

Data are means with pooled SEM, n = 6. Values sharing different superscripts within a row differ, as analyzed by one-way ANOVA for repeated measures and the Student-Newman-Keuls multiple comparisons test.

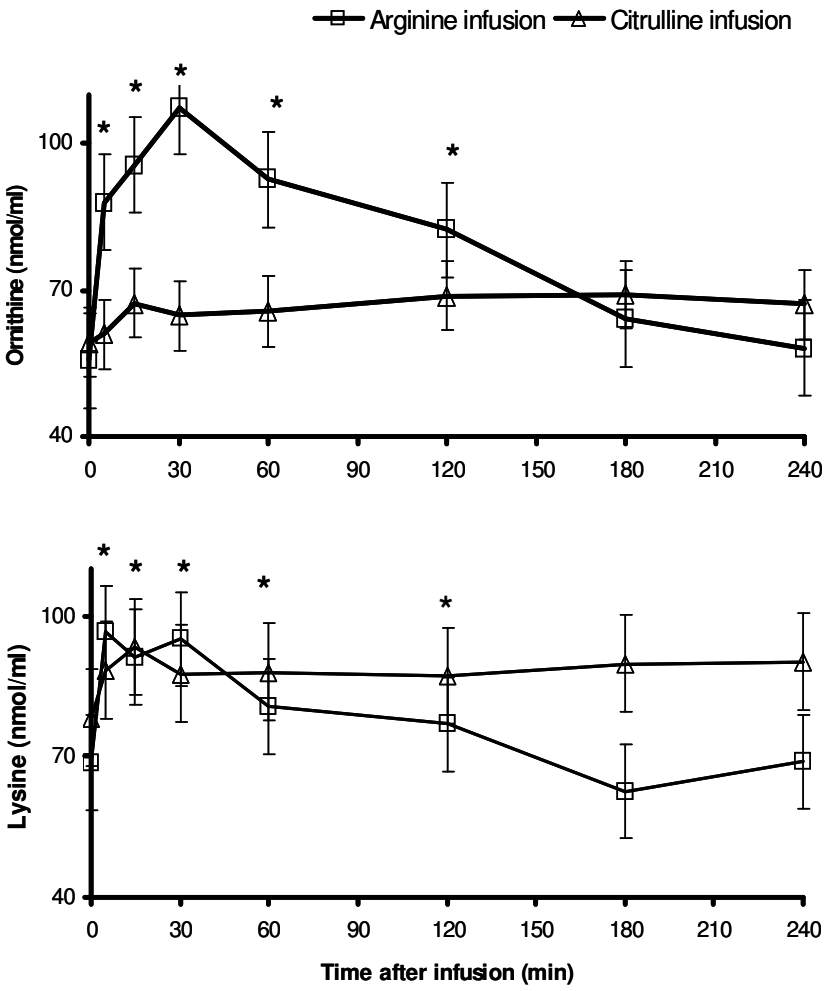

Fig 2.2. Concentrations of ornithine and lysine in maternal plasma after a single intravenous bolus injection of 155 μmol/kg body weight of either arginine-HCl or citrulline on day 135 ± 1 of gestation (∗,+ $P < 0.05$ for arginine and citrulline infusion, respectively).

When compared with time 0, concentrations of aspartate, glutamate, asparagine, threonine, and leucine in maternal plasma decreased ($P < 0.05$) and concentrations of glutamine, taurine, methionine and lysine increased ($P < 0.05$) at various time points after bolus intravenous administration of arginine to ewes (Table 2.4). Particularly, the concentration of aspartate in plasma decreased ($P < 0.01$) by 50% at 180 min, whereas the concentration of lysine increased ($P < 0.01$) by 41% at 5 min (Fig 2.2). In addition, concentrations of proline in maternal plasma were higher than the baseline between 5 and 180 min after arginine injection (Table 2.4). Concentrations of other amino acids were not affected ($P > 0.10$) by the arginine treatment.

Concentrations of aspartate in maternal plasma decreased ($P < 0.01$) between 30 and 240 min, while concentrations of proline increased between 30 and 120 min after citrulline infusion. However, concentrations of other amino acids did not change ($P > 0.10$) at any time point after citrulline injection to ewes (Table 2.5).

Amino acids in fetal plasma

Compared with baseline values, concentrations of arginine in fetal plasma were 50% and 29% greater ($P < 0.01$) at 30 min and 60 min after intravenous administration of arginine and citrulline to ewes, respectively (Fig. 2.3). Circulating levels of arginine in the fetus remained higher ($P < 0.05$) than baseline values between 30 and 240 min after citrulline administration, but only

Table 2.3. Concentrations of arginine, citrulline and ornithine in maternal and fetal plasma after a single intravenous bolus injection of citrulline (155 µmol/kg body weight) to late pregnant ewes

Amino acid	Time after bolus injection (min)								SEM	*P* value
	0	5	15	30	60	120	180	240		
	µmol/L									
Maternal plasma										
Arginine	156^{b}	187^{a}	202^{a}	195^{a}	192^{a}	194^{a}	198^{a}	196^{a}	28	0.014
Citrulline	143^{f}	765^{a}	610^{b}	459^{c}	362^{d}	306de	285^{e}	256^{e}	43	<0.0001
Ornithine	59	61	67	65	66	69	69	67	7	0.134
Fetal plasma										
Arginine	131^{c}	134^{bc}	138^{bc}	148^{b}	169^{a}	166^{a}	172^{a}	164^{a}	9	<0.0001
Citrulline	112^{d}	121^{d}	131^{c}	145^{b}	162^{a}	168^{a}	170^{a}	167^{a}	5	<0.0001
Ornithine	35^{bc}	33^{c}	36^{bc}	44^{ab}	49^{a}	47^{a}	53^{a}	52^{a}	8	0.0007

Data are means with pooled SEM, n = 6. Values sharing different superscripts within a row differ, as analyzed by one-way ANOVA for repeated measures and the Student-Newman-Keuls multiple comparisons test.

Table 2.4. Concentrations of amino acids in maternal plasma after a single intravenous bolus injection of arginine-HCl (155 µmol L-arginine/kg body weight) to late pregnant ewes

Amino acid	Time after bolus injection (min)								SEM	*P* value
	0	5	15	30	60	120	180	240		
	µmol/L									
Aspartate	12^{a}	10^{ab}	8.4^{bc}	9.9^{ab}	7.6^{bc}	8.1^{bc}	6.0^{c}	7.3^{bc}	1.8	0.006
Glutamate	73^{ab}	69^{ab}	70^{ab}	78^{a}	69^{ab}	78^{a}	64^{b}	62^{b}	11	0.047
Asparagine	22^{a}	23^{a}	22^{a}	24^{a}	22^{a}	22^{a}	17^{b}	18^{b}	3	0.005
Serine	82	78	71	80	65	69	59	66	14	0.081
Glutamine	179^{b}	205^{ab}	206^{ab}	224^{a}	203^{ab}	209^{ab}	179^{b}	184^{b}	38	0.023
Histidine	31^{ab}	38^{a}	35^{a}	35^{a}	33^{ab}	33^{ab}	29^{b}	29^{b}	4	0.040
Glycine	346	406	381	414	385	415	381	364	55	0.398
Threonine	67^{ab}	70^{a}	68^{a}	75^{a}	66^{ab}	69^{a}	57^{c}	58^{bc}	10	0.008
β-Alanine	22	22	22	24	22	24	21	22	6	0.432
Taurine	62^{bc}	79^{a}	74^{ab}	76^{ab}	64^{bc}	67^{abc}	52^{c}	56^{cd}	9	0.002
Alanine	112^{b}	117^{ab}	113^{b}	126^{a}	112^{b}	118^{ab}	99^{c}	98^{c}	15	0.0009
Tyrosine	43	47	45	50	45	47	40	42	6	0.087
Tryptophan	31	31	29	32	29	31	27	29	5	0.627
Methionine	18^{bc}	21^{a}	20^{abc}	23^{a}	21^{ab}	21^{ab}	18^{c}	19^{bc}	4	0.020
Valine	92	83	63	89	81	84	70	72	13	0.251
Phenylalanine	42	52	54	59	51	54	47	51	18	0.188
Isoleucine	48	49	48	54	47	50	42	44	5	0.117
Leucine	89^{ab}	92^{ab}	89^{ab}	97^{a}	87^{abc}	89^{ab}	75^{c}	81^{bc}	11	0.055
Lysine	69^{d}	97^{a}	91^{a}	95^{a}	81^{b}	77^{bc}	63^{d}	69^{cd}	10	<0.0001
Cysteine	165	167	165	166	165	165	166	166	9	0.995
Proline	96^{e}	109^{d}	118^{c}	130^{b}	147^{a}	126^{bc}	109^{d}	99^{e}	4	<0.0001
Total AA	1701^{bc}	1864^{ba}	1792^{bc}	1960^{a}	1804^{abd}	1856^{ab}	1620^{cd}	1634^{cd}	169	0.020

Data are means with pooled SEM, n = 6. Values sharing different superscripts within a row differ, as analyzed by one-way ANOVA for repeated measures and the Student-Newman-Keuls multiple comparisons test. AA, amino acids.

Table 2.5. Concentrations of amino acids in maternal plasma after a single intravenous bolus injection of citrulline (155 µmol/kg body weight) to late pregnant ewes

Amino acid	Time after bolus injection (min)								SEM	*P* value
	0	5	15	30	60	120	180	240		
	µmol/L									
Aspartate	9.6[a]	8.6[abc]	8.8[ab]	7.6[bc]	7.6[bc]	7.4[bc]	7.0[c]	7.3[bc]	1	0.040
Glutamate	69	72	76	69	70	68	71	69	8	0.750
Asparagine	24	25	31	24	24	22	23	23	3	0.258
Serine	70	66	68	64	63	64	64	64	10	0.543
Glutamine	203	234	244	228	222	220	223	224	39	0.432
Histidine	35	41	42	38	36	35	37	36	5	0.250
Glycine	326	380	399	375	358	369	374	385	52	0.309
Threonine	69	71	75	70	69	67	72	73	10	0.940
β-Alanine	21	22	22	21	21	21	21	21	5	0.384
Taurine	69	79	83	78	80	71	65	67	8	0.286
Alanine	106	113	117	107	106	99	102	99	15	0.226
Tyrosine	44	53	55	52	53	50	53	53	6	0.232
Tryptophan	31	35	36	34	33	33	35	34	5	0.176
Methionine	19	20	20	19	19	18	19	19	2	0.885
Valine	100	117	120	110	112	105	112	112	13	0.431
Phenylalanine	45	42	43	40	40	38	40	40	7	0.937
Isoleucine	53	60	64	54	43	53	54	54	6	0.344
Leucine	93	102	104	95	94	88	91	93	11	0.554
Lysine	78	88	93	88	88	87	90	90	10	0.633
Cysteine	168	165	164	164	172	167	166	162	9	0.193
Proline	93[d]	93[d]	96[cd]	110[b]	121[a]	100[c]	97[cd]	93[d]	4	<0.0001
Total AA	1725	1886	1959	1848	1833	1782	1806	1818	149	0.590

Data are means with pooled SEM, n = 6. Values sharing different superscripts within a row differ, as analyzed by one-way ANOVA for repeated measures and the Student-Newman-Keuls multiple comparisons test. AA, amino acids.

between 15 and 30 min after arginine administration (Fig. 2.3 and Tables 2.2 and 2.3). In contrast, concentrations of citrulline in fetal plasma increased ($P < 0.01$) between 15 and 240 min after intravenous infusion of citrulline to ewes, but were not altered ($P > 0.10$) at any time point after administration of arginine (Fig. 2.3 and Tables 2.2 and 2.3).

Concentrations of ornithine in fetal plasma increased ($P < 0.01$) between 15 and 30 min after bolus intravenous administration of arginine to ewes, but did not change ($P > 0.10$) at other time points post-injection, when compared with the baseline value (Fig. 2.4). Interestingly, in response to citrulline infusion, concentrations of ornithine in fetal plasma increased substantially ($P < 0.05$) at 30 min and remained elevated throughout the sampling period for up to 240 min ($P < 0.05$).

Arginine infusion decreased ($P < 0.05$) concentrations of leucine in fetal plasma between 15 and 120 min, and increased concentrations of proline between 15 and 180 min post-administration, but had no effect on other amino acids (Table 2.6). In contrast, citrulline administration increased ($P < 0.05$) concentrations of lysine in fetal plasma between 120 and 240 min and concentrations of proline between 15 and 240 min, but did not affect ($P > 0.10$) other amino acids at any time point (Table 2.7).

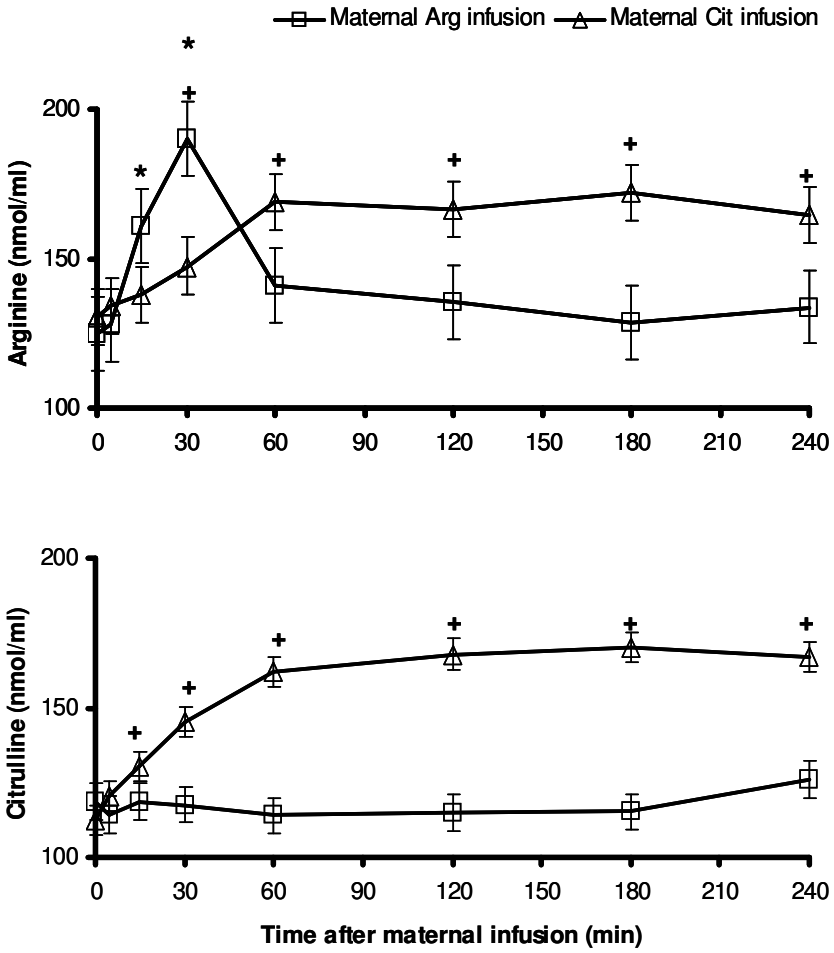

Fig 2.3. Concentrations of arginine and citrulline in fetal plasma after a single intravenous bolus injection of 155 μmol/kg body weight of either arginine-HCl or citrulline to ewes on Day 135 ± 1 of gestation (*,+ $P < 0.05$ for arginine and citrulline infusion, respectively).

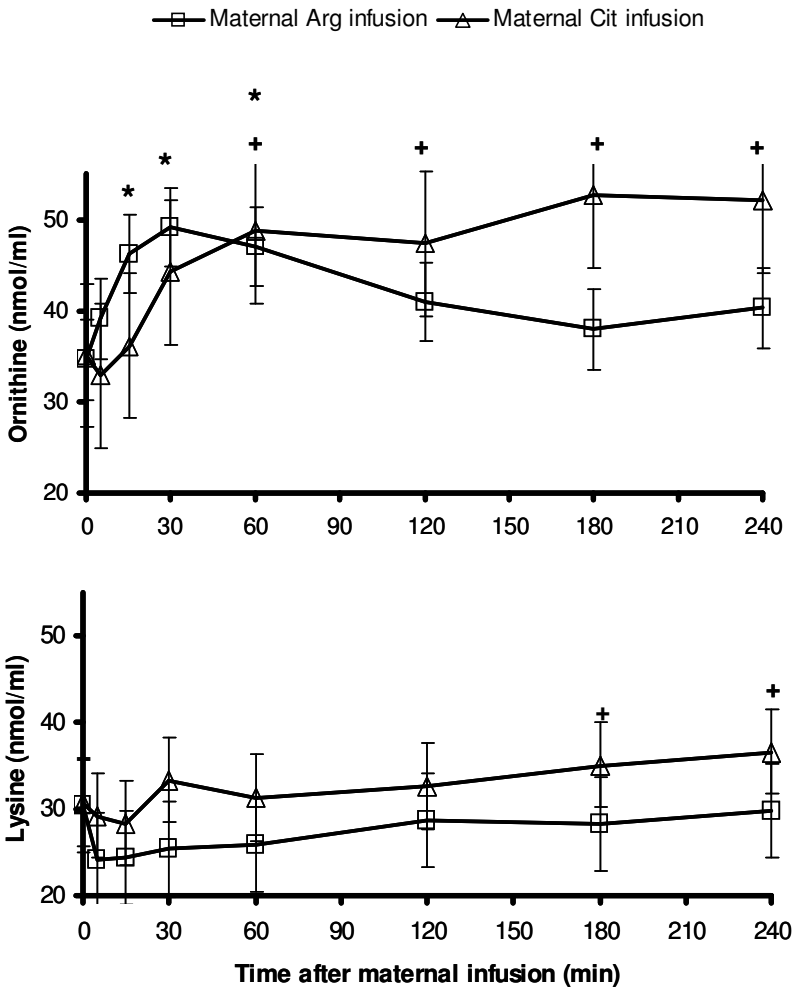

Fig 2.4. Concentrations of ornithine and lysine in fetal plasma after a single intravenous bolus injection of 155 μmol/kg body weight of either arginine-HCl or citrulline to ewes on Day 135 ± 1 of gestation (∗,+ $P < 0.05$ for arginine and citrulline infusion, respectively).

Table 2. 6. Concentrations of amino acids in fetal plasma after a single intravenous bolus injection of arginine-HCl (155 µmol /kg body weight) to late pregnant ewes

Amino acid	Time after bolus injection (min)								SEM	*P* value
	0	5	15	30	60	120	180	240		
	Mmol/L									
Asp	4.7	5.0	5.8	5.8	4.2	4.6	3.6	4.3	0.7	0.169
Glu	34	29	32	32	29	28	28	31	3	0.418
Asn	24	22	19	20	21	21	22	23	2	0.267
Ser	292	336	260	270	277	292	303	309	58	0.536
Gln	210	191	188	183	188	180	217	189	17	0.683
His	30	26	25	26	23	26	29	32	3	0.082
Gly	278	267	300	288	260	270	289	317	29	0.082
Thr	145	155	124	154	128	134	133	139	25	0.703
β-Ala	151	152	138	142	143	148	147	155	46	0.523
Tau	94	103	79	86	90	89	89	103	17	0.508
Ala	154	142	132	137	140	144	146	153	19	0.202
Tyr	61	59	55	57	55	55	56	59	12	0.689
Trp	76	68	62	62	71	70	71	73	28	0.786
Met	24	23	21	21	22	23	23	24	3	0.316
Val	108	103	94	98	101	103	102	130	21	0.262
Phe	73	67	67	68	67	68	68	57	23	0.891
Ile	32	30	29	29	30	32	28	33	5	0.583
Leu	70^{a}	63^{ab}	61^{b}	61^{b}	62^{b}	61^{b}	63^{ab}	71^{a}	8	0.040
Lys	30	24	24	25	26	29	28	30	5	0.326
Cys	176	171	173	174	175	174	171	176	9	0.748
Pro	97^{d}	99^{de}	106^{c}	110^{bc}	122^{a}	115^{b}	105^{ce}	99^{de}	5	<0.0001
Total AA	2108	2134	1990	2028	2048	2030	2121	2135	170	0.434

Data are means with pooled SEM, n = 6. Values sharing different superscripts within a row differ, as analyzed by one-way ANOVA for repeated measures and the Student-Newman-Keuls multiple comparisons test. AA, amino acids.

Table 2.7. Concentrations of amino acids in fetal plasma after a single intravenous bolus injection of citrulline (155 µmol/kg body weight) to late pregnant ewes

Amino acid	Time after bolus injection (min)								SEM	*P* value
	0	5	15	30	60	120	180	240		
	µmol/L									
Asp	3.8	4.6	4.4	3.7	3.4	3.6	3.9	3.8	0.4	0.325
Glu	25	24	23	25	23	23	24	26	2	0.756
Asn	22	21	20	22	21	21	23	23	3	0.801
Ser	260	254	243	266	261	249	270	273	42	0.320
Gln	182	170	169	175	163	157	168	173	16	0.283
His	26	26	24	25	25	24	26	29	2	0.367
Gly	231	242	228	239	233	220	233	250	28	0.475
Thr	116	106	100	117	109	106	118	116	9	0.302
β-Ala	92	85	87	96	84	84	91	96	15	0.356
Tau	113	108	110	112	112	111	118	119	28	0.615
Ala	135	129	125	145	138	134	149	159	21	0.216
Tyr	56	52	50	57	56	54	59	63	12	0.291
Trp	132	129	123	146	147	131	145	122	64	0.938
Met	17	16	16	16	17	16	18	18	1	0.309
Val	121	109	107	115	110	107	118	112	8	0.653
Phe	46	44	43	46	47	44	49	50	4	0.295
Ile	33	29	29	29	31	31	34	30	3	0.699
Leu	61	56	58	61	59	57	63	62	4	0.601
Lys	31^{cd}	29^{cd}	28^{d}	33^{abc}	31^{bc}	33^{abc}	35^{ab}	37^{a}	5	0.021
Cys	179	176	174	169	175	175	173	170	10	0.280
Pro	103^{d}	108^{cd}	110^{c}	119^{b}	128^{a}	123^{ab}	121^{b}	120^{b}	10	<0.0001
Total AA	1984	1981	1970	2027	1973	1983	2039	2050	167	0.510

Data are means with pooled SEM, n = 6. Values sharing different superscripts within a row differ, as analyzed by one-way ANOVA for repeated measures and the Student-Newman-Keuls multiple comparisons test. AA, amino acids.

Pharmacokinetics of arginine and citrulline in ewes

The AUC values were greater ($P < 0.05$) in maternal plasma after bolus intravenous administration of citrulline compared to arginine (Table 2.8). Accordingly, CL values were lower ($P < 0.05$) and the $T_{1/2}$ greater after citrulline injection. Maximum concentrations (C_{max}) of arginine and citrulline in maternal plasma did not differ ($P > 0.10$) after their intravenous infusion into ewes (Table 2.8).

Table 2.8. Pharmacokinetics of arginine and citrulline in late pregnant ewes receiving a single intravenous bolus injection of L-arginine or L-citrulline (155 μmol/kg body weight)

Parameters	Amino acid administered		*P* value
	Arginine	Citrulline	
AUC, (μmol • min)/mL	29.0 ± 6.3	70.8 ± 13.3	0.017
CL, mL/(min • kg)	6.60 ± 1.41	2.64 ± 0.6	0.031
$T_{1/2}$, min	45.3 ± 7.0	89.2 ± 11.6	0.009
C_{max}, μmol/L	453.4 ± 78.1	530.9 ± 40.1	0.398

Data are means ± SEM, n=6, and analyzed by paired t-tests.
AUC, area under the concentration-time curve; CL, total clearance; $T_{1/2}$, biological half life of amino acid; C_{max}, maximal concentration.

Discussion

Arginine is a nutritionally essential amino acid for optimal growth and development of the fetus (Wu *et al.*, 2004a) due to underdevelopment of the intestinal-renal axis for endogenous arginine synthesis from glutamine/glutamate and proline (Wu *et al.*, 2004b). The fetus obtains arginine directly from maternal circulation via utero-placental blood flow, as well as indirectly from citrulline via reactions catalyzed by argininosuccinate synthase and argininosuccinate lyase (Wu *et al.*, 2008). Because citrulline is a neutral amino acid and its administration does not disturb acid-base balance (Wu & Meininger, 2000), there is increasing interest in the use of citrulline as a precursor for arginine in non-pregnant animals and humans (Morris, 2006; Wu *et al.*, 2007b; Moinard *et al.*,

2008); however, there have been no reports of studies to determine the efficacy of exogenous citrulline for arginine synthesis in the mother or fetus. The major finding of the present study is that intravenous administration of citrulline was more effective than that of arginine to sustain a prolonged increase in concentrations of arginine in fetal circulation (Tables 2.2 and 2.3). Consistent with this observation, the biological half-life of citrulline in maternal plasma of ewes was twice that of arginine (Table 2.8). These results can be explained by higher activities for arginase than for argininosuccinate synthase and argininosuccinate lyase (the enzymes that are responsible for citrulline catabolism) in extrahepatic tissues of adult mammals (Wu & Morris, 1998). To our knowledge, this is the first report on citrulline metabolism in pregnant mammals.

There is a high rate of arginine turnover in pregnant ewes, due to its rapid clearance from maternal plasma (Wu *et al.*, 2007a). To confirm and extend this observation, we found that concentrations of arginine and ornithine, a product of arginine metabolism, in plasma of ewes on Day 135 ± 1 of gestation rose to peak values within 5 min after a bolus intravenous administration of arginine and declined rapidly thereafter to baseline values (Table 2.2). Because NO production is quantitatively a minor pathway of arginine catabolism in healthy mammals (Wu & Morris, 1998), arginine administration had no effect on concentrations of citrulline (a co-product of NO synthase) in maternal plasma (Table 2.2). In contrast, intravenous infusion of citrulline into ewes markedly increased concentrations of both citrulline and arginine, but not ornithine, in maternal plasma (Table 2.3). Notably, concentrations of citrulline and arginine in maternal plasma remained elevated throughout a 4 h period after administration of a single bolus of citrulline into the maternal venous circulation. Furthermore, even at 4 h, the concentrations of citrulline and arginine in the fetus were 49% and 25% greater, respectively, than the baseline values (Table 2.3).

Results of the present study suggest that citrulline is readily converted into arginine in maternal tissues. In support of this view, aspartate (a substrate of argininosuccinate synthase in the pathway of arginine synthesis from citrulline) was the only amino acid whose concentrations in maternal plasma were reduced due to its extensive utilization for arginine formation in response to citrulline administration (Table 2.5). The findings of the present study also suggest that the citrulline-derived arginine is not substantially degraded to generate ornithine via arginase in pregnant ewes. This further substantiates evidence that there is complex metabolism of arginine via its compartmentalized pathways in animals (Wu & Morris, 1998). Whether intravenous administration of arginine or citrulline into pregnant ewes is capable of increasing arginine availability in the fetus must be determined experimentally.

A substantial rise in concentrations of amino acids (including arginine) in maternal blood can result in an increase in uptake into the fetal circulation (Battaglia, 2002; Thureen *et al.*, 2002; Wilkes *et al.*, 2003). However, the rate of delivery of an amino acid from the uterus to the fetus depends not only on its transport from maternal circulation to the placenta but also on its utilization and metabolism by placental cells and subsequent efflux from the placenta into the fetus (Battaglia & Regnault, 2001; Wu *et al.*, 2004a). These critical events involve placental transport systems at both maternal and fetal surfaces of the placenta (Battaglia & Regnault, 2001). In sheep, the y^+ and y^+L cationic amino acid transport systems on both surfaces of the placenta are responsible for net transport of arginine to the fetus (Wu & Morris, 1998; Regnault *et al.*, 2005a). A high activity of arginase in ovine placentomes rapidly hydrolyzes arginine into urea and ornithine (Kwon *et al.*, 2003b). Thus, although peak concentrations of arginine in maternal plasma within 5 min of its intravenous administration to ewes was 320% greater than baseline values (Table 2.2), concentrations of arginine in fetal plasma increased only 52% at 30 min and returned to the baseline values at 60 min (Table 2.5). In contrast, intravenous administration of

citrulline to ewes resulted in progressive increases in concentrations of both citrulline and arginine in fetal plasma between 5 and 60 min and, importantly, these elevated levels were sustained throughout the remainder of the sampling period. Indeed, concentrations of citrulline, arginine, ornithine and proline in fetal plasma at 4 h were 49%, 25%, 48% and 17% greater than baseline values, respectively. Collectively, these results indicate for the first time, the prolonged effects of intravenous citrulline administration into ewes in enhancing arginine availability in the fetus.

Changes in concentrations of amino acids other than arginine, citrulline, ornithine and aspartate in maternal and fetal plasma after intravenous administration of arginine or citrulline deserve comments. Basic amino acids share the same transport systems in plasma membranes (Christensen, 1990). Moreover, the systems $b^{o,+}$, $B^{o,+}$, and $y^{+}L$ can transport both basic and large neutral amino acids into cells (Palacin *et al.*, 1998). Thus, with a rise in concentrations of arginine in plasma of ewes, uptakes of lysine (a basic amino acid) as well as proline and methionine (large neutral amino acids) by maternal tissues would be reduced, leading to transient increases in concentrations of these amino acids in the maternal circulation (Table 2.5). An increase in concentrations of glutamine in maternal plasma after arginine infusion is likely due to the formation of glutamine via the pathway involving arginase, ornithine aminotransferase, and glutamine synthetase (Wu & Morris, 1998). Interestingly, intravenous administration of arginine or citrulline increased concentrations of proline in fetal plasma, but had no significant or prolonged effects on concentrations of other neutral amino acids (Tables 2.6 and 2.7). This finding is exciting, because proline has been recently suggested to have an important role in conceptus metabolism and development (Wu *et al.*, 2008). Also, it indicates that the dosages of arginine or citrulline administered to pregnant ewes do not compromise the availability of all amino acids in the fetus, therefore, remaining accessible to promote fetal growth.

In summary, results of this pharmacokinetic study revealed that the biological half-life of citrulline in plasma of pregnant ewes is approximately twice that for arginine. Therefore, intravenous administration of citrulline into ewes is more effective than arginine in maintaining prolonged increases in available arginine in both maternal and fetal circulations, without reducing concentrations of all amino acids in the fetus. These novel findings should aid in the design of an effective arginine or citrulline therapeutic solution to ameliorate intrauterine growth restriction, a significant problem in both human medicine and animal production (Wallace *et al.*, 2005; Wu *et al.*, 2006).

CHAPTER III
PARENTERAL ADMINISTRATION OF ARGININE PREVENTS FETAL GROWTH RESTRICTION IN UNDERNOURISHED EWES

Introduction

Intrauterine growth restriction (IUGR) is a major health problem worldwide, representing 11% of all newborns in developing countries and a large number of all newborns in developed nations, *e.g.* 2-5% in the U.S. (Murphy *et al.*, 2006). Maternal undernutrition, which occurs under conditions such as inadequate supply of food or severe nausea and vomiting in pregnant women, is an important factor that adversely impacts fetal growth (Attard *et al.*, 2002; Wu *et al.*, 2004a). Intrauterine growth restriction results in emotional stress and contributes to extremely high costs of health care due to perinatal and life-long medical complications (Mari & Hanif, 2007). For example, ~50% of non-malformed stillbirths result from IUGR and 5% of premature deliveries are due to poor growth of fetuses *in utero* (Bryan & Hindmarsh, 2006; Pallotto & Kilbride, 2006). Also, infants who weigh < 2.5 kg at birth have 5- to 30-times higher rates of perinatal mortality than newborns who have average birth weights, and these rates are 70- to 100-times higher for infants weighing < 1.5 kg at birth (Pallotto & Kilbride, 2006). Furthermore, surviving infants with IUGR are at increased risk for neurological, respiratory, intestinal, and circulatory disorders (Gluckman & Hanson, 2006). To date, there is no therapeutic means for preventing or ameliorating IUGR, the current management being empirical and primarily aimed at selecting a safe time for delivery (Resnik, 2002; Mari & Hanif, 2007).

Arginine, a nutritionally essential amino acid for the fetus (Wu *et al.*, 2004b), is a precursor for synthesis of nitric oxide (NO) and polyamines in cells (Wu & Morris, 1998). Nitric oxide is a major endothelium-derived vasodilator, whereas polyamines are key regulators of DNA and protein synthesis (Wu and Morris, 1998). Consequently, arginine may play a critical role in placental

growth (including vascular growth), utero-placental blood flow, and hence the transfer of nutrients from mother to fetus (Wu *et al.*, 2006). We found that maternal undernutrition reduced arginine concentrations in maternal and fetal plasma of ewes (Kwon *et al.*, 2004a) and decreased the availability of arginine, polyamines and NO synthesis in the conceptus (fetus and associated membranes) (Wu *et al.*, 1998a; Wu *et al.*, 1998b; Kwon *et al.*, 2004a). Interestingly, direct infusion of arginine into the fetal femoral vein for 3 to 4 h increased fetal whole-body protein accretion in an ovine model of IUGR induced by placental insufficiency (De Boo *et al.*, 2005).

We hypothesized that parenteral administration of arginine to underfed ewes would ameliorate or prevent fetal growth retardation. This hypothesis was tested using an ovine model of IUGR, which is an established and valuable animal experimental approach for studying fetal growth in humans (Schroder, 2003; Luther *et al.*, 2005).

Materials and methods

Ewes

Fifteen multiparous Suffolk crossbred ewes weighing 76.7 ± 2.8 kg (mean ± SEM) were mated to a single fertile Suffolk ram when detected in estrus (Day 0) and 12 h later, to minimize paternal genetic effects on size and weight of the fetuses. At Day 21 post-mating, ewes were transported to the Texas A&M Animal Science Teaching, Research and Extension Center (ASTREC), where they were individually housed in outdoor covered pens with free access to drinking water and allowed a 7-day period of acclimation. Transabdominal ultrasonography (7.5 MHz probe, Aloka Inc. console) was used to confirm pregnancy. Ewes were fed a wheat, cottonseed, rice mill and alfalfa-based diet (Table 3.1; Producers Cooperative Association, Bryan, TX, USA) to meet 100% of the NRC (National Research Council, 1985) nutrient requirements for pregnant sheep. Ewes were fed once daily between 0700 h and 0800 h. This

study was approved by the Texas A&M University Institutional Animal Care and Use Committee.

Table 3.1. Exp. 2/ Composition of the diet (as-fed basis)[1]

Ingredients	Content
Wheat midds	42.25%
Cottonseed hulls	27.4%
Rice mill feed	15%
Dehydrated alfalfa	10%
Liquid binder	2.5%
Ground limestone	1.76%
Sodium bicarbonate	0.50%
Mineral mixture	0.50%
Vitamin mixture	0.09%

[1]Provided the following in the diet: 10.2% crude protein; 0.60% arginine; 3.4% crude fat; 22.3% crude fiber; 1.0% calcium; 0.50% phosphorus; 1.01% salt; 0.94% potassium; 0.18% sulfur; 0.25% magnesium; 226 ppm manganese; 228 ppm iron; 11.3 ppm copper; 0.35 ppm cobalt; 146 ppm zinc; 1.00 ppm iodine; 0.53 ppm selenium; 1.00 ppm molybdenum; 8,110 IU/kg vitamin A; 13.2 IU/kg vitamin E; and 455 IU/kg vitamin D.

Experimental design

At Day 28 of pregnancy, ewes were assigned randomly to be fed either 100% (n=5) or 50% (n=10) of the daily NRC nutrient requirements for pregnant sheep (NRC, 1985). The diet and daily feeding schedule were the same as provided during the acclimation period. Ewes were weighed weekly, before feeding, and diets were adjusted on an individual basis according to recorded live-weight changes. The 50% level of underfeeding was adopted because it has been shown to reduce placental and fetal growth in sheep (Vonnahme *et al.*, 2003; Kwon *et al.*, 2004a; Satterfield *et al.*, 2007).

One to three days before Day 60 of gestation, a 16 G X 13 cm polyurethane peripheral catheter (Milicath, MILA international, Inc., Florence, KY, USA) was placed into the jugular vein of the ewes and fixed with suture

points to the skin of the neck. Prior to its insertion, wool at the catheterization site was clipped (number 40 blade, Oster, McMinville, TN, USA), and the skin was scrubbed with an iodine-soap solution (Povidine-iodine, Vedadine Surgical Scrub, VEDCO St Joseph, MO, USA) and wiped with 70% ethanol. Once fixed, the catheter was fitted with a 30.5 cm microbore extension (Hospira Inc., Lake Forest, IL) which was screwed, by means of a hub, onto the catheter at one end, sutured to the skin at the joint site with the catheter, then at half-length on the back of the neck, and again at the far end close to the base of the head adjacent to the occipital region, thus allowing access from the back of the ewe. The extension was capped with an intermittent infusion plug (Kendall Argyle, Tyco Healthcare Retail Group, Inc., King of Prussia, PA, USA) that permitted repeated insertion of regular disposable needles. After the catheter and extension were positioned and fixed, a covering elastic cotton stocking was placed around the neck of the ewe, in an effort to maintain a clean and protected area. Initially, Vetrap bandaging tape was used (3M Animal Care Products, St. Paul, MN, USA) for this purpose, but it quickly contracted due to continuous movement of the neck; therefore, an elastic cotton was chosen to replace the tape to avoid development of edema or other associated problems. The intermittent infusion plugs that capped the catheter extensions were periodically changed throughout the experimental period. Extensions and catheters were only replaced when damaged or pulled out. During this study, 10 ml disposable syringes (latex-free, luer-lok tip, Becton Dickinson, Franklin Lakes, NJ, USA) and 21 G X 3.8 cm needles (Precision glide, Becton Dickinson, Franklin Lakes, NJ, USA) were used for delivery of solutions.

Between Day 60 of pregnancy and parturition, control-fed ewes (Group 100% NRC; n = 5) received 10 ml of sterile saline solution as a bolus injection (sodium chloride 0.9%, Hospira Inc. Lake Forest, IL) through the jugular catheter three times daily, and underfed ewes were randomly divided in two groups, to receive either 10 ml of sterile saline solution (Group 50% NRC; n = 5) or sterile

L-arginine-HCl solution (155 µmol arginine/kg body weight; Sigma-Aldrich Co. St Louis MO, USA) (Group 50% + Arginine; n = 5) three times daily (0800, 1500, and 2200 h). The arginine-HCl solution was prepared twice per week, using sterile physiological saline (sodium chloride 0.9%, Hospira Inc., Lake Forest, IL, USA) with a final concentration of 1.5 g arginine per 5 ml. The pH was adjusted to 7.0 with 1 M NaOH and the solution filtered through a 0.22 µm cellulose acetate filter (Corning Inc, NY, USA) into re-usable sterile glass containers fitted with adjustable-sealing sterile rubber caps. The prepared L-arginine-HCl solution was kept at -20°C and thawed at 4°C the night before being used. Disposable 10 ml syringes (latex free, luer-lok tip, Becton Dickinson, Franklin Lakes, NJ, USA) were marked with ewe identification numbers and filled up in the laboratory with either saline or arginine-HCl in saline solution before every infusion. The arginine-HCl saline solution was used throughout the day, with the rubber cap on the bottle being cleaned with 70% ethanol before insertion of the disposable needle. A bacteriological culture of randomly selected saline and arginine solutions was performed by the Texas Medical Diagnostic Laboratory (College Station, TX) on two occasions to verify sterility. Between infusions, catheters were flushed and filled with 0.75 ml heparin solution (40 units/ml, American Pharmaceutical Partners, Inc., Schaumburg, IL) to prevent clotting and maintain patency.

Every 10 days from Day 60 of pregnancy until parturition, approximately 7 ml of maternal blood were obtained from all ewes from the contralateral jugular vein to the catheterized jugular vein, using anticoagulant-free, sterile vacuum tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) and 20 G X 3.8 cm blood collection needles (Vacutainer, Becton Dickinson, Franklin Lakes, NJ). Blood was drawn in the morning immediately before the first infusion of either saline or arginine-HCl saline solution. Samples were placed on ice and immediately centrifuged at 3,000 x g for 15 min. Serum was separated and stored at -80°C until analyzed for amino acids, metabolites and hormones.

At parturition, a portable scale was used to record birth weights of lambs. Care was taken to weigh the newborns immediately after birth.

Determination of amino acids, other metabolites and hormones in serum

Deproteinated serum was used for the analyses for amino acids, ammonia, urea, glucose, lactate, glycerol, and β-hydroxybutyrate (BHB), whereas whole serum was assayed for free fatty acids (FFA), triglycerides, insulin and growth hormone (GH). Amino acids were determined by fluorometric HPLC methods involving precolumn derivatization with *o*-phthaldialdehyde as described (Wu *et al.*, 1997). β-Hydroxybutyrate was measured enzymatically by a spectrophotometric method using 3-hydroxybutyrate dehydrogenase (Wu *et al.*, 1991). Free fatty acids were quantified by an enzymatic colorimetric method using the assay kit from Waco Chemicals Inc (Richmond, VA, USA). Glucose was determined enzymatically using a spectrophotometric method involving hexokinase and glucose-6-phosphate dehydrogenase (Fu *et al.*, 2005). Glycerol and L-lactate were quantified using enzymatic fluorometric methods as described by Fu *et al.* (2005) and Wu *et al.* (1991), respectively. Triglycerides were determined enzymatically using the Infinity^{TM} assay kit from Thermo Electron Corporation (Pittsburgh, PA, USA). Ammonia and urea were determined using fluorometric methods involving glutamate dehydrogenase and urease (Wu, 1995; Wu *et al.*, 1995). Insulin was analyzed using the ovine insulin ELISA microplate kit from Mercodia AB (Uppsala, Sweden), whereas growth hormone was quantified by RIA (Powell & Keisler, 1995; Lalman *et al.*, 2000) validated for ovine serum.

Statistical analyses

Data on lamb birth weight were statistically analyzed by one-way ANOVA, using a variance-covariance matrix that considered the effects of number of lambs born (singles or twins) (Steel *et al.*, 1997). Data on concentrations of

amino acids, other metabolites, and hormones in serum were analyzed by two-way ANOVA for repeated measures to determine the effects of day of pregnancy, treatment, and day of pregnancy x treatment interactions (Steel *et al.*, 1997). Where there was a statistically significant day x treatment interaction, effects of day within nutritional treatment were analyzed by one-way ANOVA. Log transformation of variables was performed when variance of data was not homogenous among treatment groups, as assessed using the Levene's test. *P* values ≤ 0.05 were taken to indicate statistical significance.

Results

Body weights of ewes and newborn lambs

Feed intake did not differ between saline- and arginine-infused underfed ewes throughout the experimental period. The underfed ewes consumed 50% less feed than the control-fed ewes (Table 3.2). On the day of onset of nutrient restriction (Day 28), the live weight of the ewes did not differ between control-fed (100% NRC-requirement), underfed (50% NRC-requirement), and arginine-treated underfed (50% NRC + Arg) groups (Table 3.3). At the end of pregnancy, control-fed ewes gained 13% in body weight over that at Day 28, whereas the underfed ewes receiving either intravenous saline or arginine lost 6.7% and 2.1% of their body weight, respectively (Table 3.3). The length of gestation was 142.3 ± 1.2 days (n=15) and did not differ among the three groups of ewes.

There were singleton and twin lambs born to ewes in all groups. Both the control-fed and the 50% NRC + Arg groups had three singleton and two twin lambs each, while the 50% NRC group had two singleton and three twin lambs. Birth weights for single lambs were greater ($P < 0.01$) than those for individual lambs born as twins (4.03 ± 0.21 vs 3.17 ± 0.14 kg, respectively). Thus, fetal number per ewe was included in the statistical analysis as a covariate to assess differences in weight at birth between groups.

Table 3.2. Feed intake (g/kg body weight/day) over the experimental period in control-fed (100%NRC) and underfed ewes (50%NRC) with or without intravenous arginine treatment

Week of pregnancy	Feed intake		
	100% NRC	50% NRC	50% NRC + Arg
5	22.5 ± 0.53 [a]	11.0 ± 0.22 [b]	11.1 ± 0.32 [b]
6	22.6 ± 0.57 [a]	11.3 ± 0.26 [b]	11.4 ± 0.41 [b]
7	22.6 ± 0.62 [a]	11.2 ± 0.31 [b]	11.4 ± 0.41 [b]
8	22.6 ± 0.62 [a]	11.2 ± 0.24 [b]	11.3 ± 0.41 [b]
9	22.6 ± 0.54 [a]	11.3 ± 0.36 [b]	11.4 ± 0.43 [b]
10	22.7 ± 0.62 [a]	11.3 ± 0.07 [b]	11.3 ± 0.44 [b]
11	22.3 ± 0.71 [a]	11.3 ± 0.33 [b]	11.3 ± 0.48 [b]
12	22.2 ± 0.67 [a]	11.3 ± 0.33 [b]	11.3 ± 0.44 [b]
13	22.3 ± 0.66 [a]	11.3 ± 0.32 [b]	11.3 ± 0.45 [b]
14	21.9 ± 0.66 [a]	11.3 ± 0.17 [b]	11.2 ± 0.39 [b]
15	22.0 ± 0.73 [a]	11.2 ± 0.13 [b]	11.1 ± 0.39 [b]
16	22.0 ± 0.76 [a]	11.3 ± 0.29 [b]	11.3 ± 0.41 [b]
17	22.0 ± 0.68 [a]	11.4 ± 0.31 [b]	11.1 ± 0.33 [b]
18	22.0 ± 0.70 [a]	11.5 ± 0.28 [b]	11.2 ± 0.37 [b]
19	22.0 ± 0.66 [a]	11.3 ± 0.09 [b]	11.2 ± 0.42 [b]
20	21.5 ± 0.37 [a]	11.7 ± 0.24 [b]	11.4 ± 0.43 [b]
21	21.8 ± 0.35 [a]	11.2 ± 0.15 [b]	11.1 ± 0.41 [b]

Data are means±SEM, n = 5.
a-b: Means sharing different superscripts within a row differ ($P < 0.01$).

The birth weights of lambs from saline-infused undernourished ewes were 23% lower ($P < 0.01$) than those for lambs born to control-fed dams (Table 3.3). Arginine infusion to underfed ewes increased ($P < 0.01$) birth weight of lambs by 21%, when compared with underfed ewes receiving saline infusion (Table 3.3). There was no difference ($P > 0.10$) in birth weights between control-fed and 50% NRC + Arg groups.

Table 3.3. Body weights of control and underfed ewes on Days 28 and 140 of pregnancy and weights of lambs at birth

Group	Body weight of ewes (kg)			Birth weight of Lambs (kg)
	Day of pregnancy		Weight change	
	Day 28	Day 140	(Day 140 - Day 28)	
Control-fed	71.1 ± 4.7	80.2 ± 4.7	9.1 ± 3.2[a]	3.99 ± 0.24[a]
Underfed	79.5 ± 2.7	74.2 ± 1.8	-8.4 ± 3.9[b]	3.06 ± 0.16[b]
Underfed + Arginine	80.3 ± 5.7	78.6 ± 6.3	-4.1 ± 1.6[b]	3.70 ± 0.21[a]

Data are means ± SEM, n = 5. a-b: Means sharing different superscripts within a column differ ($P < 0.01$).

Concentrations of amino acids in maternal serum

Concentrations of amino acids in serum of control-fed ewes and underfed ewes with or without arginine are summarized in Table 3.4. Levels of glutamate, glutamine, glycine and β-alanine in maternal serum were not affected by maternal underfeeding. However, concentrations of all other amino acids were lower ($P < 0.05$) in saline-infused undernourished ewes, compared to control-fed ewes. Concentrations of asparagine, histidine, threonine, citrulline, taurine, tyrosine, tryptophan, valine, phenylalanine and lysine were lower ($P < 0.05$) in the 50% NRC + Arg group than in control ewes, but did not differ from values for ewes in the 50% NRC group. Concentrations of methionine, isoleucine, leucine,

ornithine, cysteine and proline in maternal serum were 19-55% greater in the 50% NRC + Arg group ($P < 0.01$) than in the 50% NRC ewes, and 15-40% lower ($P < 0.01$) than for the control-fed ewes. Notably, concentrations of arginine in maternal serum increased ($P < 0.01$) by 69% in the 50% NRC + Arg ewes compared with the 50% NRC ewes and were similar to values for 100% NRC ewes (Table 3.4). Concentrations of aspartate, serine and alanine in serum also increased ($P < 0.05$) in the 50% NRC + Arg group to concentrations comparable to those for control ewes (Table 3.4).

Except for asparagine, serine, histidine, β-alanine, alanine and methionine, concentrations of amino acids in maternal serum varied with day of pregnancy (Table 3.4). The most striking changes were observed for glutamate and threonine, whose concentrations progressively decreased ($P < 0.01$) by 71% and 48%, respectively, between Days 60 and 140 of gestation. In contrast, concentrations of glutamine, isoleucine, taurine and lysine increased ($P < 0.05$) by 95%, 63%, 138%, and 65%, respectively in maternal serum, between Days 60 and 140 of gestation. Levels of cysteine in maternal serum also increased between Days 60 and 80 of pregnancy, but subsequently decreased gradually until Day 140 of gestation. In addition, there was a treatment x day interaction ($P < 0.05$) for aspartate, citrulline, arginine, alanine, valine, isoleucine, lysine, cysteine and proline in that concentrations in serum at Day 60 of gestation were lower ($P < 0.05$) in both the 50% NRC and the 50% NRC + Arg ewes compared to values for control-fed ewes. At Day 140 of pregnancy, concentrations of aspartate, arginine, valine, isoleucine, lysine and cysteine in serum of the 50% NRC +Arg ewes were comparable to those in control-fed ewes (Table 3.5).

Concentrations of other metabolites and hormones in maternal serum

Undernourished ewes (with and without arginine infusion) had lower (P < 0.01) concentrations of glucose in serum than control-fed ewes (Table 3.6), but there was no effect of day of pregnancy (Table 3.6). Interestingly, levels of triglycerides in serum were 34% lower (P < 0.05) and those of free fatty acids were greater (P < 0.05) in 50% NRC + Arg ewes than in both the 50% NRC and 100% NRC ewes (Table 3.6). Intravenous infusion of arginine reduced (P < 0.05) concentrations of ammonia in serum from underfed ewes, but had no effect on those of β-hydroxybutyrate, glucose, lactate, urea, insulin, or growth hormone.

Concentrations of β-hydroxybutyrate, free fatty acids, glycerol, triglycerides, and growth hormone increased (P < 0.01), while concentrations of insulin decreased (P < 0.01), in ewes between Days 60 and 140 of gestation (Table 3.6). Day of pregnancy had no effect on concentrations of glucose, urea, or ammonia in maternal serum. Among all metabolites and hormones measured, only glycerol and growth hormone were affected by a treatment X day interaction (P = 0.05, Tables 3.4 and 3.5).

Table 3.4. Concentrations (μM) of amino acids in maternal serum on Days 60, 80, 110 and 140 of pregnancy in control-fed (100% NRC), and underfed ewes (50% NRC) with or without arginine infusion (155 μmol/kg body weight, three times daily)

Amino acid	Treatment (T)			Day (D)				SEM	*P* values		
	100% NRC	50% NRC	50% NRC+ Arg	60	80	110	140		T	D	T*D
Aspartate	7.9[a]	4.6[b]	6.0[ab]	5.6[b]	7.3[a]	5.6[b]	6.1[ab]	0.9	0.014	0.044	0.029
Glutamate	120	93	98	177[a]	106[b]	81[c]	51[d]	13	0.086	<0.0001	0.494
Asparagine	30[a]	18[b]	23[b]	25	25	24	20	4	0.001	0.331	0.685
Serine	79[a]	58[b]	71[ab]	72	76	69	61	8	0.049	0.101	0.186
Glutamine	232	173	193	125[b]	202[a]	226[a]	244[a]	22	0.070	<0.0001	0.099
Histidine	43[a]	27[b]	34[b]	35	37	31	35	4	0.003	0.282	0.804
Glycine	394	381	442	362[b]	379[b]	409[ab]	472[a]	58	0.548	0.042	0.451
Threonine	105[a]	28[b]	36[b]	71[a]	65[a]	53[ab]	37[b]	14	<0.0001	0.008	0.097
Citrulline	261[a]	159[b]	190[b]	182[b]	267[a]	186[b]	179[b]	24	0.0009	0.0002	0.039
Arginine	295[a]	178[b]	300[a]	204[c]	301[a]	265[b]	262[b]	26	0.0005	0.0001	0.004
β-Alanine	13	7.9	9.4	9.8	9.9	9.8	11	2	0.057	0.704	0.526
Taurine	142[a]	77[b]	92[b]	48[c]	144[a]	110[b]	113[b]	17	0.0002	<0.0001	0.759
Alanine	178[a]	118[b]	149[ab]	148	163	133	149	16	0.010	0.055	0.015
Tyrosine	52[a]	27[b]	33[b]	33[b]	41[a]	36[ab]	40[a]	4	0.0001	0.013	0.537
Tryptophan	40[a]	22[b]	26[b]	30[b]	38[a]	27[b]	21[c]	3	<0.0001	<0.0001	0.177
Methionine	17[a]	7.5[c]	10[b]	11	11	12	12	2	<0.0001	0.413	0.468
Valine	162[a]	77[b]	106[b]	117[a]	140[b]	101[a]	102[a]	15	0.0002	0.001	0.013
Phenylalanine	46[a]	28[b]	36[b]	28[b]	38[a]	38[a]	44[a]	4	0.002	0.0003	0.650
Isoleucine	81[a]	51[c]	70[b]	51[c]	65[b]	70[b]	84[a]	6	0.0006	<0.0001	0.033
Leucine	126[a]	75[c]	102[b]	82[c]	115[a]	98[b]	109[ab]	11	0.002	0.0008	0.451
Ornithine	108[a]	42[c]	65[b]	64[b]	102[a]	66[b]	55[b]	11	0.0006	<0.0001	0.261
Lysine	127[a]	69[b]	89[b]	62[b]	113[a]	101[a]	103[a]	31	0.0005	<0.0001	0.028
Cysteine	167[a]	112[c]	133[b]	121[c]	180[a]	135[b]	113[d]	6	<0.0001	<0.0001	<0.0001
Proline	145[a]	95[c]	123[b]	115[b]	122[a]	122[a]	125[a]	6	<0.0001	0.006	<0.0001

Data are means with pooled SEM, n = 15 observation per day (5 ewes x 3 treatments) and n = 20 observations per treatment (5 ewes x 4 days). Values sharing different superscripts within a row differ due to effects of treatment (T), day (D) or treatment x day (T*D) interaction based on two-way ANOVA for repeated measures and the Student-Newman-Keuls multiple comparisons test.

Table 3.5. Concentrations of amino acids, glycerol and GH in serum of control-fed (100% NRC) and underfed ewes (50% NRC) with or without intravenous arginine infusion (155 µmol/Kg body weight, three times daily)

AA and Metab	Day 60				Day 80				Day 110				Day 140			
	100% NRC	50% NRC	50% NRC+ Arg	SEM	100% NRC	50% NRC	50% NRC+ Arg	SEM	100% NRC	50% NRC	50% NRC+ Arg	SEM	100% NRC	50% NRC	50% NRC+ Arg	SEM
Asp (µM)	7.8[a]	4.7[b]	4.3[b]	0.7	9.6	7.6	7.0	1	5.9	6.0	5.5	0.9	8.8[a]	3.1[b]	7.2[a]	1.1
Cit (µM)	284[a]	127[b]	129[b]	18	362[a]	197[b]	241[b]	28	225	148	180	20	160	155	203	37
Arg (µM)	287[a]	150[b]	181[b]	26	327[a]	186[b]	368[a]	23	274[a]	212[b]	322[c]	12	259[a]	175[b]	327[a]	30
Ala (µM)	195[a]	120[b]	127[b]	13	158	167	183	23	154	126	133	11	198[a]	106[b]	144[b]	11
Val (µM)	187[a]	74[b]	88[b]	13	200	105	123	25	142[a]	63[b]	94[b]	10	132[a]	69[b]	115[a]	14
Ile (µM)	73[a]	40[b]	41[b]	5	70[a]	47[b]	75[a]	6	81[a]	56[b]	73[a]	4	99[a]	63[b]	92[a]	7
Lys (µM)	110[a]	35[b]	44[b]	7	154	94	100	19	128[a]	74[c]	103[b]	8	127[a]	72[b]	115[a]	13
Cys (µM)	161[a]	105[b]	96[b]	6	218[a]	150[c]	173[b]	7	162[a]	105[c]	139[b]	7	127[a]	88[b]	124[a]	4
Pro (µM)	147[a]	96[b]	102[b]	7	142[a]	95[b]	128[a]	5	142[a]	92[b]	132[a]	5	149[a]	97[c]	130[b]	5
Glycerol (µM)	22	33	38	8	18	28	38	8	28	25	48	7	14[a]	3.6[c]	8.6[b]	1.6
GH (ng/ml)	3.0	2.8	2.4	0.4	2.8	2.7	3.0	0.5	3.1	3.1	4.1	0.6	7.0	12,	5.7	1.8

Data are means ± pooled SEM for 5 ewes per treatment group. On each Day of gestation, values sharing different superscripts within a row differ ($P < 0.05$), as analyzed by one-way ANOVA and the Student-Newman-Keuls multiple comparisons test.
AA = amino acids, Metab = other metabolites, GH = Growth Hormone

Table 3.6. Concentrations of metabolites and hormones in serum on Days 60, 80, 110 and 140 of pregnancy in control fed (100% NRC), and underfed ewes (50% NRC) with or without arginine infusion (155 µmol/kg body weight, three times daily)

Metabolite	Treatment (T) means			Day (D) means				SEM	*P* values		
	100% NRC	50% NRC	50% NRC+ Arg	60	80	110	140		T	D	T*D
BHB (mM)	1.1	1.4	1.1	0.7^{b}	0.9^{b}	1.5^{a}	1.5^{a}	0.3	0.323	0.0007	0.202
FFA (µM)	319^{b}	411^{b}	589^{a}	236^{c}	340^{bc}	404^{b}	777^{a}	114	0.038	<0.0001	0.413
Glucose (mM)	2.5^{a}	2.1^{b}	1.9^{b}	2.3	2.2	2.0	2.1	0.2	0.010	0.285	0.559
Glycerol (µM)	49	29	54	30^{b}	28^{b}	34^{b}	84^{a}	14	0.093	0.0002	0.046
Lactate (mM)	1.5	1.0	1.0	1.9^{a}	1.2^{b}	0.8^{b}	0.9^{b}	0.3	0.130	<0.0001	0.151
Triglycerides (µM)	161^{a}	157^{a}	106^{b}	135^{b}	113^{b}	140^{b}	178^{a}	21	0.030	0.004	0.466
Urea (mM)	5.8	4.7	4.6	5.5	5.0	4.6	5.0	0.6	0.098	0.158	0.119
Ammonia (µM)	91^{ab}	103^{a}	85^{b}	96	92	91	93	6.0	0.067	0.353	0.317
Insulin (ng/L)	528	322	533	669^{a}	564^{a}	333^{b}	277^{b}	134	0.267	0.003	0.884
GH (ng/ml)	3.8	5.0	3.6	2.7^{b}	2.9^{b}	3.4^{b}	7.5^{a}	1.0	0.213	<0.0001	0.051

Data are means with pooled SEM, n = 15 observation per day (5 ewes x 3 treatments) and n = 20 observations per treatment (5 ewes x 4 days). Values sharing different superscripts within a row differ due to effects of treatment (T), day (D) or T*D interaction, as analyzed by two-way ANOVA for repeated measures and the Student-Newman-Keuls multiple comparisons test.
BHB= β-Hydroxybutyrate, FFA= Free Fatty Acids, GH= Growth Hormone

Discussion

Despite advanced technologies for prenatal care of both mothers and fetuses, IUGR remains a global health problem that causes significant perinatal complications and may contribute to adult-onset diseases (Murphy *et al.*, 2006). Since there is currently a lack of therapeutic treatment for IUGR in humans (Mari & Hanif, 2007), animal models are used to test novel hypotheses in research that will provide the bases for development of effective therapies (Luther *et al.*, 2005). Although there are several basic differences in pregnancy between sheep and humans, including time of implantation, type of placentation, and gestational length (Leiser & Kaufmann, 1994), the ewe is a well-established animal model for studying human placental-transfer of nutrients to the fetus for several reasons (Wallace *et al.*, 2005): First, the number of offspring and regulation of nutrient transfer from mother to fetus are similar between ewes and women (Battaglia & Regnault, 2001). Second, ewes can be conveniently managed for easy access to blood and other sample collection. Third, pregnant ewes are tolerant of surgical procedures that include placement of catheters into maternal and fetal blood vessels without aborting (Caton *et al.*, 1983). Fourth, there is a large database in the literature on placental and fetal development in normal and underfed ewes (Mellor, 1983; Reynolds & Redmer, 1995).

Among intrauterine environmental factors, nutrition plays the most decisive role in influencing placental and fetal growth (Wu *et al.*, 2004a). In fact, well-controlled animal studies have consistently demonstrated that maternal undernutrition during a critical period of pregnancy substantially reduces birth weight at term (Osgerby *et al.*, 2002). Although enteral re-feeding to 100% of NRC-recommended nutrient requirements is potentially effective to reverse IUGR caused by undernutrition (Kwon *et al.*, 2004a), this means of intervention is not applicable under clinical conditions such as hyperemesis gravidarum, which is characterized by severe nausea and vomiting in gestating women (Snell *et al.*, 1998). This life-threatening disorder occurs in 1-2% of all pregnancies and

generally extends beyond the 16th week of gestation (Attard *et al.*, 2002). Therefore, parenteral nutrition must be explored to improve pregnancy outcome in these women. Through bypassing intestinal catabolism (Wu, 1995), direct intravenous infusion of arginine into dams may effectively increase its concentrations in maternal and fetal blood of underfed ewes. Consistent with this proposition, maternal and fetal concentrations of arginine in serum were increased by 170% and 40%, respectively after an infusion of a large dose of arginine (695 μmol/kg body weight over 280 min) into the femoral vein of well-fed sheep (Thureen *et al.*, 2002). Further, in an ovine model of IUGR induced by placental embolization, infusion of arginine into the fetal femoral vein increased fetal whole-body protein accretion (De Boo *et al.*, 2005). However, these studies involved short-term (3 to 4 h) infusion of arginine into ewes or fetus, so the effects of arginine on fetal growth could not be evaluated.

Birth weight is one of the most sensitive and important measures of fetal growth (Redmer *et al.*, 2004). Consistent with previous reports (Vonnahme *et al.*, 2003; Kwon *et al.*, 2004a; Satterfield *et al.*, 2007), results from the current study indicated that global nutrient restriction (50% of NRC requirements) beginning on Day 28 of gestation resulted in IUGR (Table 3.3). In addition, marked reductions in concentrations of most amino acids (Table 3.4) and glucose (Table 3.6) were detected in maternal serum. Thus, ewes subjected to severe malnutrition were not able to maintain homeostasis of amino acids for normal fetal growth. A novel and important finding of the present study is that intravenous infusion of arginine to underfed ewes (155 μmol arginine-HCl per kg body weight) three times daily between Day 60 of pregnancy and parturition effectively prevented IUGR, without affecting maternal body weight (Table 3.3). This indicates that the arginine treatment enhanced the availability of nutrients to the conceptus to support fetal growth. Indeed, concentrations of several essential and conditionally essential amino acids (methionine, isoleucine, leucine and cysteine) were higher in serum of arginine-treated than in saline-

infused underfed ewes (Table 3.4). Moreover, concentrations of some non-essential amino acids (aspartate, serine and alanine) in serum of arginine-treated underfed ewes were comparable to those for control-fed ewes (Table 3.4). In addition, concentrations of proline, a neutral amino acid that has been recently suggested to have an important role in conceptus metabolism and development (Wu *et al.*, 2008), were 29% higher in arginine-treated than in saline-underfed ewes. Because all underfed ewes had the same intake of nutrients, including protein (Table 3.2), the higher concentrations of amino acids in serum from arginine-treated ewes compared to the saline-infused underfed ewes may have resulted from alterations in maternal nitrogen metabolism. In support of this view, there is evidence that NO reduces the urea cycle activity and the oxidation of amino acids in hepatocytes (Jobgen *et al.*, 2006).

Increasing concentrations of arginine in maternal plasma by 69% can enhance NO synthesis by endothelial cells (Wu & Meininger, 2002) and utero-placental blood flow (Neri *et al.*, 1995). This, in turn, would be expected to promote the transfer of oxygen and nutrients from maternal to fetal circulations. Consistent with this theory, intramuscular administration of Sildenafil citrate to underfed ewes between Days 28 and 112 of gestation increased concentrations of most amino acids and polyamines in fetal plasma and fluids, as well as fetal growth (Satterfield *et al.*, 2007). Sildenafil citrate, which acts through enhancing intracellular cGMP availability by inhibiting phosphodiesterase-5 (an enzyme that hydrolyzes cGMP), may have augmented utero-placental blood flow via the protein kinase G signaling pathway (Wareing *et al.*, 2005). Because catheterization of fetal vessels for blood sampling could affect fetal growth, we chose not to perform this invasive procedure in the present study. Therefore, precise changes in concentrations of nutrients in fetal circulation as well as amniotic and allantoic fluids due to intravenous infusion of arginine into ewes were not determined. Additional studies are warranted to test the hypothesis

that parenteral administration of arginine may increase utero-placental blood flow and, thus, the supply of nutrients from mother to fetus.

The changes in maternal serum concentrations of hormones and metabolites observed with advanced gestation and arginine infusion in this study deserve comments (Table 3.6). In contrast to a large pharmacological bolus of arginine (2 mmol/kg body weight) (Davis, 1972), intravenous infusion of a physiological dosage of arginine (155 μmol/kg body weight three times daily) had no effect on circulating levels of insulin or growth hormone (Table 3.6). However, there were progressive increases in concentrations of free fatty acids, glycerol and β-hydroxybutyrate in maternal serum during late gestation (Table 3.6). These results indicate mobilization of maternal fat stores to provide energy for maternal and fetal metabolism and are consistent with the progressive decrease in concentrations of insulin (an anti-lipolytic hormone) (Owens, 1991) and the progressive increase in concentrations of growth hormone (a lipolysis-enhancing hormone) (Owens, 1991) in maternal serum between Days 60 and 140 of gestation (Table 3.6). In addition, concentrations of triglycerides were lower, but concentrations of free fatty acids were higher, in serum of arginine-treated ewes than saline-infused underfed or control-fed ewes (Table 3.6). Of particular interest, physiological levels of NO stimulate the hydrolysis of triglycerides in adipose tissue (Fu *et al.*, 2005), thereby increasing the availability of circulating free fatty acids for oxidation by maternal tissues (e.g., skeletal muscle) as metabolic fuels (Jobgen *et al.*, 2006). This, in turn, can spare the oxidation of amino acids, which may have contributed to elevated levels of some amino acids in maternal serum of arginine-treated underfed ewes (Table 3.4). In support of this suggestion, concentrations of ammonia were lower in serum of underfed ewes in response to intravenous infusion of arginine (Table 3.6).

In conclusion, parenteral administration of arginine to underfed ewes increased concentrations of arginine and related amino acids in maternal serum and prevented fetal growth restriction. These novel findings provide an

experimental basis for the clinical use of arginine to eliminate or ameliorate IUGR in humans. The results also provide a new framework for studies of molecular mechanisms responsible for beneficial effects of arginine in regulating conceptus growth and development.

CHAPTER IV
PARENTERAL ADMINISTRATION OF ARGININE TO EWES CARRYING MULTIPLE FETUSES ENHANCES FETAL SURVIVAL AND BIRTH WEIGHTS OF QUADRUPLETS

Introduction

Uterine capacity is a major factor limiting fetal survival and growth in mammals (Wu *et al.*, 2006; Gootwine *et al.*, 2007). This maternal constraint is particularly evident in women or ewes carrying multiple fetuses, where demand for nutrients and space to nurture all fetuses cannot be adequately met (Gluckman & Hanson, 2004). Indeed, an inverse relationship between the number of fetuses and birth weight has been described for humans (Blickstein & Kalish, 2003; Blickstein, 2005) and other mammals (Wootton *et al.*, 1983; Freetly & Leymaster, 2004; Gootwine *et al.*, 2007).

With the advancement of assisted reproductive technologies, the frequency of twins and higher-order multiple fetuses has markedly increased in human pregnancies over the past 2 decades (Blickstein, 2005). This translates into a greater proportion of premature and low birthweight infants in the United States of America and elsewhere (Fliegner, 1989; Siddiqui & McEwan, 2007). In addition, genetic selection and breeding in sheep has resulted in new breeds with increased litter size (up to 6 fetuses per ewe), but greatly reduced birth weights and survival of lambs (Gootwine *et al.*, 2007). However, there are no current treatments for preventing fetal growth restriction (IUGR) in humans or sheep gestating multiple fetuses. We have suggested that this problem may be ameliorated by modulation of the placental nitric oxide- and polyamine-synthetic pathways, thus impacting utero-placental blood flow and perhaps direct actions on the fetus (Wu *et al.*, 2004a). In support of this notion, Mateo *et al.* (2007) recently reported that supplementing arginine (a common substrate for the synthesis of nitric oxide and polyamines) to the gestation diet for gilts (a litter-

bearing species) increased the number and litter weight of live-born piglets by 22% and 24%, respectively. On the basis of these findings, we hypothesized that parenteral administration of arginine may enhance fetal survival and growth in pregnancies with multiple fetuses. This hypothesis was tested with prolific Booroola Rambouillet ewes.

Materials and methods

Ewes

Multiparous Booroola Rambouillet ewes (FecB+/-) (n=41) with a body weight of 68.4 ± 1.4 kg (mean ± SEM) were bred to fertile Booroola Rambouillet rams (FecB+/-) fitted with marking harnesses. In order to minimize paternal genetic effects on size and weight of the fetuses, the same two rams were used throughout the breeding period. Breeding dates were determined by daily inspection of crayon marks on lumbar region of the ewes. The color of the marking crayon was changed every 15 days to detect return to estrus. The day of first observation of a color mark on the dam was considered as the first day of estrus and of pregnancy (Day 0). Pregnancy diagnosis and initial fetal counts were conducted by transabdominal ultrasonography at Day 35 post-mating (7.5 MHz probe, Aloka Inc. console). Ewes that were confirmed pregnant and carrying twins, triplets or quadruplets were sheared and housed in outdoor pens from Day 40 until parturition. To confirm that ewes were gestating multiple fetuses and to reassess initial counts of fetal numbers, a second transabdominal ultrasonography was performed at Day 45. During the first two thirds of pregnancy, ewes were individually housed in covered pens with cement floors. In the last third of gestation, collective partially-covered dirt-floor pens were used. Throughout pregnancy, ewes had free access to drinking water and were fed a corn, soybean, rice and alfalfa-based diet (Producers Cooperative Association, Bryan, TX, USA) to meet 100% of the National Research Council

(NRC)-recommended maintenance requirements for pregnant ewes gestating multiple fetuses (NRC, 1985). The dietary composition is shown in Table 4.1.

Table 4.1. Exp. 3/ Composition of the diet (as-fed basis)[1]

Ingredients	Content
Corn	37.45%
Soybean hulls	32.65%
Soybean meal	10%
Rice bran	5%
Dehydrated alfalfa	5%
Rice mill feed	4.25%
Liquid binder	2.5%
Soy oil	1.8%
Ground limestone	0.25%
Sodium bicarbonate	0.50%
Mineral mixture	0.50%
Vitamin mixture	0.10%

[1]Nutrients provided in the diet were: 12.5% crude protein; 0.74% arginine; 5.5% crude fat; 15% crude fiber; 0.91% sodium; 0.49% calcium; 0.36% phosphorus; 1.02% potassium; 0.91% chloride; 0.17% sulfur; 0.22% magnesium; 140.09 ppm manganese; 117.76 ppm iron; 12.4 ppm copper; 0.28 ppm cobalt; 136.75 ppm zinc; 1.01 ppm iodine; 0.16 ppm selenium; 1.00 ppm molybdenum; 5,698 IU/kg vitamin A; 11.1 IU/kg vitamin E; and 454.5 IU/kg vitamin D.

During the first two thirds of pregnancy, the given amount of feed was provided in two equal rations at 0700 h and 1500 h and adjusted on an individual basis weekly, according to live-weight changes of ewes. In the last third of gestation (from week 15), the feed was provided *ad libitum* to ensure adequate intake of nutrients. The amounts of feed intake over the experimental period are summarized in Table 4.2. This study was approved by the Texas A&M University Institutional Animal Care and Use Committee.

Table 4.2. Feed intake (g/kg body weight per day) over the experimental period in control or arginine-HCl infused ewes

Week of pregnancy	Feed intake	
	Control	Arginine
6	17.3 ± 0.22^{c}	17.5 ± 0.37^{e}
7	17.5 ± 0.30^{c}	17.5 ± 0.30^{e}
8	17.6 ± 0.37^{c}	17.5 ± 0.35^{e}
9	17.2 ± 0.31^{c}	17.4 ± 0.33^{e}
10	17.2 ± 0.29^{c}	17.1 ± 0.30^{fe}
11	16.7 ± 0.26^{dc}	16.7 ± 0.27^{fg}
12	16.5 ± 0.26^{d}	16.8 ± 0.28^{fg}
13	16.5 ± 0.22^{d}	16.5 ± 0.25^{g}
14	16.1 ± 0.24^{d}	16.2 ± 0.26^{g}
15	25.8 ± 0.43^{a}	26.2 ± 0.57^{a}
16	25.7 ± 0.46^{a}	25.2 ± 0.44^{b}
17	24.8 ± 0.34^{b}	24.6 ± 0.35^{c}
18	24.8 ± 0.50^{b}	24.3 ± 0.40^{cd}
19	24.8 ± 0.49^{b}	24.6 ± 0.44^{c}
20	24.1 ± 0.55^{b}	24.2 ± 0.52^{cd}
21	24.8 ± 0.71^{b}	23.9 ± 0.52^{d}

Data are means ± SEM. Values sharing different superscripts within a column differ ($P < 0.05$).

Experimental design

At Day 100 of pregnancy, ewes were assigned randomly to receive an intravenous infusion of either sterile saline (control group, n=14; approximately 15 ml of 0.9% sodium chloride, Hospira Inc. Lake Forest, IL) or the same volume of sterile L-arginine-HCl-saline (arginine group, n=20; Sigma-Aldrich Co., St Louis MO, USA; 345 µmol arginine/kg body weight) three times per day until Day 121 of pregnancy. The L-arginine-HCl solution was prepared three times per week, using sterile physiologic saline (0.9% sodium chloride, Hospira Inc, Lake Forest, IL, USA) with a final concentration of 1.5 g arginine per 5 ml. The pH was adjusted to 7.0 with 1 M NaOH and the solution passed through 0.22 µm cellulose acetate filters (Corning Inc, NY, USA) into reusable sterile glass containers fitted with adjustable sealing sterile rubber caps. The prepared L-

arginine-HCl solution was stored at -20°C and thawed at 4°C the night before being used. Disposable 20 ml syringes (latex free, luer-lok tip, Becton Dickinson, Franklin Lakes, NJ, USA) were marked with ewe identification numbers and filled in the laboratory with either saline solution or L-arginine-HCl solution before each infusion. A bacteriological culture of randomly selected saline and arginine solutions was performed by the Texas Veterinary Medical Diagnostic Laboratory (College Station, TX) on two occasions to verify sterility. To administer the saline and L-arginine-HCl solutions, sterile 21 G X ¾" (0.80 X 19 mm) disposable winged infusion sets (SURFLO®, Terumo Medical Corporation, Elkton, MD, USA) were used. An extra 0.5 ml volume of solution was considered when calculating the total volume to be infused, allowing for losses remaining inside the infusion set tubing after injection. To minimize damage to tissues due to frequent insertion of the infusion set needles that was required for repeated daily administration of solutions, care was taken to alternate jugular veins and to change sites of penetration.

At Days 100, 115 and 140 of pregnancy, blood samples were collected, immediately before the administration of saline or L-arginine-HCL solutions, to measure concentrations of amino acids, other metabolites, and hormones in maternal serum. On Day 121, samples were drawn at 1 h after the solutions were infused to determine changes in circulating levels of hormones, amino acids, and other metabolites in response to either infusion. Anticoagulant-free, sterile, 10 ml vacuum tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) were used for blood collection. Blood samples were placed on ice and immediately centrifuged at 3,000 x g for 15 min. Serum was obtained and stored at -80°C until analysis.

At parturition, a portable scale was used to obtain and record the weight of each lamb at birth. Care was taken to weigh the lambs immediately after birth.

Determination of concentrations of amino acids, other metabolites, and hormones in maternal serum

Deproteinated serum was used for the analysis of amino acids, ammonia, urea, glucose, lactate, glycerol, and β-hydroxybutyrate (BHB), whereas whole serum was assayed for free fatty acids (FFA), triglycerides, insulin and growth hormone (GH). Amino acids were determined by fluorometric HPLC methods involving precolumn derivatization with *o*-phthaldialdehyde as previously described (Wu *et al.*, 1997). β-Hydroxybutyrate was measured enzymatically by a spectrophotometric method using 3-hydroxybutyrate dehydrogenase (Wu *et al.*, 1991). Free fatty acids were quantified by an enzymatic colorimetric method using the NEFA-HR (2) assay kit from Waco Chemicals Inc. (Richmond, VA, USA). Glucose was determined enzymatically using a spectrophotometric method involving hexokinase and glucose-6-phosphate dehydrogenase (Fu *et al.*, 2005). Glycerol and L-lactate were quantified using enzymatic fluorometric methods as described (Wu *et al.*, 1991; Fu *et al.*, 2005). Triglycerides were determined enzymatically using the Infinity^{TM} assay kit from Thermo Electron Corporation (Pittsburgh, PA, USA). Ammonia and urea were determined by fluorometric methods involving glutamate dehydrogenase and urease (Wu, 1995; Wu *et al.*, 1995). Insulin was analyzed using the ovine insulin ELISA microplate kit from Mercodia AB (Uppsala, Sweden), whereas GH was quantified by RIA (Powell & Keisler, 1995; Lalman *et al.*, 2000) validated for ovine serum.

Statistical analyses

Data on lamb birthweight were statistically analyzed by one-way ANOVA, with litter size nested within treatment (Steel *et al.*, 1997). Data on concentrations of amino acids, other metabolites, and hormones in serum on Days 100, 121 and 140 of pregnancy in control ewes was analyzed by two-way ANOVA for repeated measures to determine the effects of day, litter size, and

their interaction. Concentrations of amino acids, other metabolites, and hormones in maternal serum on Day 121 of pregnancy in the arginine group were compared by two-way ANOVA considering the effects of treatment, litter size and their interaction. Log transformation of variables was performed when the variance of data was not homogenous among treatment groups, as assessed by the Levene's test. Differences in numbers of live and dead fetuses between treatment groups were evaluated by Chi-square analysis. All analyses were performed using the statistical package SAS (version 9.1, SAS Institute, Cary, NC, USA). *P* values ≤ 0.05 were taken to indicate statistical significance.

Results

Body weight of ewes

Maternal body weight increased ($P < 0.05$) progressively between weeks 10 and 19 of gestation, but did not differ ($P > 0.05$) between saline- and arginine-infused ewes during the experimental period (Table 4.3). Body weights of saline- and arginine-treated ewes were also similar ($P > 0.05$) immediately after parturition (70.1 ± 3.3 and 69.2 ± 2.4 kg, respectively).

Percentage of lambs born alive

There were twin, triplet and quadruplet pregnancies in both control and arginine-treated ewes. The control ewes had three twin, seven triplet and four quadruplet pregnancies, while the arginine ewes had seven twin, five triplet and eight quadruplet pregnancies. A number of ewes in this study presented clinical signs of ketosis and delivered stillborn lambs. The percentages of lambs born alive were 27.9% and 44.3%, respectively, for control and arginine-treated ewes (Table 4.4). Infusion of arginine did not affect the total number of lambs born per ewe. However, the arginine treatment reduced ($P < 0.05$) the percentage of lambs born dead by 23%, while enhancing ($P = 0.05$) the percentage of lambs born alive by 59% (Table 4.4).

Table 4.3. Body weights of control and arginine-infused ewes from weeks 6 to 21 of pregnancy

Week of pregnancy	Body weight of ewes	
	Control	arginine
6	68.2 ± 1.5 ^{k}	68.8 ± 2.2^{k}
7	67.0 ± 1.7^{k}	66.5 ± 2.0^{k}
8	67.8 ± 1.9^{k}	67.2 ± 2.0^{jk}
9	69.0 ± 1.8^{jk}	68.6 ± 2.0^{ij}
10	70.8 ± 1.7^{ij}	70.6 ± 1.9^{hi}
11	72.5 ± 1.9^{hi}	72.6 ± 2.0^{gh}
12	74.1 ± 1.8^{gh}	73.7 ± 1.9^{fg}
13	75.6 ± 1.8^{fg}	75.2 ± 1.9^{ef}
14	76.5 ± 1.6^{ef}	76.5 ± 1.9^{de}
15	77.8 ± 1.5^{e}	78.1 ± 1.9^{d}
16	81.1 ± 1.7^{d}	81.6 ± 2.0^{c}
17	83.5 ± 1.6^{c}	84.3 ± 2.1^{b}
18	83.4 ± 2.1^{c}	85.2 ± 2.3^{b}
19	85.6 ± 2.2^{bc}	87.6 ± 2.5^{a}
20	86.9 ± 3.0^{ab}	87.7 ± 2.9^{a}
21	86.8 ± 3.8^{ab}	89.9 ± 3.5^{a}

Data are means ± SEM. Values sharing different superscripts within a column differ ($P < 0.05$).

Table 4.4. Number of lambs born alive and dead from control and arginine-infused ewes

Treatment	Status at birth	Litter size				% of total lambs born per group
		twins	triplets	quadruplets	Total	
Arginine	alive	14	6	7	27	44.3 †
	dead	0	9	25	34	55.7 ‡
	total	14	15	32	61	
Control	alive	3	8	1	12	27.9
	dead	3	13	15	31	72.1
	total	6	21	16	43	

† $P = 0.05$ and ‡ $P < 0.05$ *vs* the control group.

Birth weights of lambs

The birth weights of twins were greater ($P < 0.02$) than those of triplets and quadruplets within each treatment group (Table 4.5). In addition, newborn triplets were heavier than quadruplets in saline-infused ewes ($P = 0.038$), but not in arginine-infused dams ($P = 0.175$). There were no differences ($P > 0.05$) in birth weights of twin or triplet lambs between control and arginine-treated ewes (Table 4.5). However, arginine infusion increased ($P < 0.05$) the birth weights of quadruplet lambs by 23% ($P < 0.05$), when compared with counterparts born to control ewes (Table 4.5).

Table 4.5. Birth weights of lambs from twin, triplet and quadruplet pregnancies from control and arginine-infused ewes

Group	Birth weight of lambs (kg)		
	Twins	triplets	quadruplets
Control	4.30 ± 0.38 [a]	3.06 ± 0.17 [b]	2.47 ± 0.22 [c]
Arginine infused	4.11 ± 0.21 [a]	3.39 ± 0.22 [b]	3.03 ± 0.14 [b]

Data are means ± SEM. a-b: Means sharing different superscripts within a row or a column differ ($P < 0.05$).

Concentrations of amino acids in maternal serum

Concentrations of amino acids in maternal serum at 1 h after arginine or saline infusions on Day 121 of pregnancy are summarized in Table 4.6. Circulating levels of arginine, ornithine, cysteine and proline increased ($P < 0.01$) by 183%, 286%, 32% and 44% respectively, in arginine-infused ewes, when compared with saline-infused dams. In contrast, concentrations of asparagine, β-alanine, alanine, tyrosine, methionine, valine, phenylalanine and isoleucine were lower ($P < 0.05$) in arginine-infused than in saline-infused ewes. Litter size affected concentrations of glycine, threonine and citrulline in maternal serum on

Table 4.6. Concentrations of amino acids in serum in arginine-treated (345 µmol/kg body weight) and control ewes at one hour after infusion on Day 121 of pregnancy

Amino acid	Treatment group			Litter size			
	Arginine	Control	*P* values	Twins	Triplets	Quadruplets	*P* value
Aspartate	5.1 ± 0.4	4.3 ± 0.5	0.18	5.3 ± 0.6	4.3 ± 0.5	4.4 ± 0.5	0.34
Glutamate	108 ± 4	108 ± 5	0.96	112 ± 6	108 ± 5	104 ± 6	0.64
Asparagine	22 ± 2 [b]	29 ± 2 [a]	0.03	26 ± 3	25 ± 2	26 ± 2	0.98
Serine	67 ± 3	66 ± 4	0.87	71 ± 5	68 ± 4	62 ± 4	0.39
Glutamine	135 ± 5	136 ± 7	0.96	140 ± 8	136 ± 7	130 ± 7	0.64
Histidine	31 ± 2	37 ± 3	0.13	38 ± 4	28 ± 3	37 ± 3	0.06
Glycine	339 ± 21	342 ± 26	0.93	408 ± 31 [a]	320 ± 26 [b]	294 ± 28 [b]	0.03
Threonine	54 ± 7	71 ± 9	0.13	85 ± 11 [a]	42 ± 9 [b]	60 ± 9 [ab]	0.02
Citrulline	103 ± 10	114 ± 13	0.52	142 ± 15 [a]	93 ±13 [b]	90 ± 14 [b]	0.03
Arginine	422 ± 24 [a]	149 ± 30 [b]	<0.01	273 ± 37	279 ± 31	304 ± 33	0.79
β-Alanine	8.3 ± 0.7 [b]	12 ± 0.9 [a]	<0.01	9.9 ± 1	9.5 ± 0.9	12 ± 1	0.29
Taurine	110 ± 9	99 ± 11	0.41	102 ± 13	100 ± 11	112 ± 12	0.73
Alanine	122 ± 10 [b]	157 ± 12 [a]	0.03	133 ± 14	162 ± 12	124 ± 13	0.10
Tyrosine	41 ± 5 [b]	70 ± 6 [a]	<0.01	48 ± 8	68 ± 7	50 ± 7	0.08
Tryptophan	24 ± 2	28 ± 3	0.23	28 ± 3	26 ± 3	24 ± 3	0.61
Methionine	10 ± 1 [b]	15 ± 1 [a]	<0.01	12 ± 2	12 ± 1	13 ± 1	0.84
Valine	71 ± 8 [b]	97 ± 10 [a]	0.04	90 ± 12	75 ± 10	87 ± 11	0.61
Phenylalanine	27 ± 2 [b]	37 ± 3 [a]	<0.01	33 ± 3	28 ± 3	34 ± 3	0.29
Isoleucine	46 ± 4 [b]	68 ± 5 [a]	<0.01	51 ± 6	58 ± 5	61 ± 6	0.52
Leucine	74 ± 7	92 ± 9	0.10	82 ± 10	73 ± 9	95 ± 9	0.23
Ornithine	165 ± 12 [a]	43 ± 15 [b]	<0.01	130 ± 19	84 ± 16	98 ± 16	0.18
Lysine	88 ± 7	89 ± 9	0.91	88 ± 10	80 ± 9	98 ± 9	0.38
Cysteine	115 ± 4 [a]	87 ± 5 [b]	<0.01	109 ± 6	99 ± 5	96 ± 5	0.24
Proline	143 ± 3 [a]	99 ± 3 [b]	<0.01	142 ± 4	117 ± 4	105 ± 4	<0.01

Data are means ± SEM. Values sharing different superscripts within a row differ, as analyzed by two-way ANOVA and the Student-Newman-Keuls multiple comparisons test.

Table 4.7. Concentrations of amino acids in serum on Days 100, 121 and 140 of pregnancy in control ewes with different litter sizes

Amino acid	Day of pregnancy				Litter size			
	100	121	140	*P* value	Twins	Triplets	Quadruplets	*P* value
Aspartate	4.8 ± 0.6	4.3 ±0.6	4.5 ± 0.8	0.57	5.1 ± 1	4.8 ± 0.7	3.8 ± 1	0.62
Glutamate	84 ± 4 ^{b}	108 ± 4 ^{a}	47 ± 6 ^{c}	<0.01	89 ± 6	81 ± 4	68 ± 6	0.07
Asparagine	39 ± 3 ^{a}	29 ± 3 ^{b}	29 ± 4 ^{ab}	0.04	34 ± 5	34 ± 3	28 ± 5	0.57
Serine	65 ± 5	66 ± 4	54 ± 6	0.14	61 ± 7	67 ± 5	58± 7	0.58
Glutamine	189 ± 10 ^{a}	136 ±10 ^{b}	221 ± 15 ^{a}	<0.01	194 ± 13	189 ± 9	163 ± 15	0.26
Histidine	36 ± 3	37 ± 3	39 ± 3	0.61	36 ± 5	35 ± 3	41 ± 4	0.62
Glycine	369 ± 23	342 ± 23	343 ± 37	0.50	391 ± 42	292 ± 26	372 ± 38	0.10
Threonine	76 ± 9 ^{a}	71 ± 8 ^{a}	42 ± 11 ^{b}	0.03	77 ± 15	47 ± 10	65 ± 14	0.27
Citrulline	114 ± 12	114 ± 11	88 ± 15	0.20	128 ± 20	98 ± 13	90 ± 19	0.37
Arginine	144 ± 8	149 ± 8	157 ± 9	0.17	154 ± 15	139 ± 10	157 ± 13	0.49
β-Alanine	10 ± 1 ^{b}	12 ± 1 ^{a}	14 ± 1 ^{a}	<0.01	11 ± 2	12 ± 1	13 ± 2	0.76
Taurine	133 ± 9 ^{a}	99 ± 9 ^{b}	95 ± 12 ^{b}	<0.01	110 ± 15	97 ± 10	120 ± 14	0.42
Alanine	159 ± 17	157 ± 17	153 ± 22	0.94	147 ± 31 ^{ab}	211 ± 21 ^{a}	111 ± 29 ^{b}	0.04
Tyrosine	67 ± 9	70 ± 9	60 ± 13	0.75	52 ± 16 ^{b}	96 ± 11 ^{a}	50 ± 15 ^{b}	0.04
Tryptophan	28 ± 3	28 ± 3	24 ± 4	0.47	29 ± 5	29 ± 3	22 ± 4	0.48
Methionine	14 ± 2	15 ± 1	14 ± 2	0.73	13 ± 3	16 ± 2	15 ± 2	0.67
Valine	94 ± 10	98 ± 10	93 ± 12	0.88	83 ± 18	107 ± 12	93 ± 16	0.51
Phenylalanine	34 ± 3	37 ± 3	36 ± 3	0.37	33 ± 5	38 ± 3	36 ± 5	0.73
Isoleucine	57 ± 7	68 ± 6	76 ± 9	0.24	51 ± 8 ^{b}	87 ± 5 ^{a}	63 ± 8 ^{b}	0.03
Leucine	77 ± 8 ^{b}	92 ± 8 ^{a}	103 ± 10 ^{a}	0.04	77 ± 14	99 ± 9	96 ± 13	0.44
Ornithine	50 ± 5 ^{a}	43 ± 5 ^{a}	28 ± 7 ^{b}	0.02	48 ± 9	34 ± 6	39 ± 8	0.46
Lysine	100 ± 9 ^{a}	89 ± 9 ^{ab}	71 ± 12 ^{b}	0.04	84 ± 17	83 ± 11	93 ± 15	0.87
Cysteine	93 ± 2 ^{a}	87 ± 2 ^{b}	82 ± 3 ^{b}	<0.01	101 ± 4 ^{a}	86 ± 3 ^{b}	75 ± 4 ^{c}	<0.01
Proline	109 ± 2 ^{a}	99 ± 2 ^{b}	88 ± 2 ^{c}	<0.01	124 ± 4 ^{a}	94 ± 3 ^{b}	78 ± 4 ^{c}	<0.01

Data are means ± SEM. Values sharing different superscripts within a row differ, as analyzed by two-way ANOVA for repeated measures and the Student-Newman-Keuls multiple comparisons test.

Day 121 of pregnancy. Serum levels of glycine and citrulline decreased ($P < 0.05$) in triplet and quadruplet pregnancies, whereas threonine was lower in triplets, but did not differ ($P > 0.05$) between twin and quadruplet pregnancies (Table 4.6).

When data from control ewes were analyzed (Table 4.7), concentrations of cysteine and proline in serum decreased ($P \leq 0.001$) with increasing litter size, whereas levels of tyrosine and isoleucine were greater ($P < 0.05$) in the serum of ewes carrying triplets than in ewes carrying twins or quadruplets. In addition, concentrations of alanine were lower ($P < 0.05$) in ewes carrying quadruplets, when compared with triplet pregnancies. Litter size had no effect on concentrations of other amino acids in saline-infused ewes (Table 4.7).

Concentrations of several amino acids in serum from control ewes changed throughout gestation. Circulating levels of glutamate increased between Days 100 and 121 of pregnancy, but then fell abruptly at Day 140 ($P < 0.01$; Table 4.7). Conversely, concentrations of asparagine decreased ($P < 0.05$) between Days 100 and 121, while no differences were found between Days 121 and 140 (Table 4.7). Glutamine also decreased ($P < 0.05$) between Days 100 and 121 of gestation, but increased ($P < 0.05$) at Day 140 to values similar to those found at Day 100. Serum levels of leucine and β-alanine were greater ($P < 0.01$), but those of taurine and cysteine were lower ($P < 0.01$), on Days 121 and 140 of gestation, when compared with Day 100 (Table 4.7). In contrast, concentrations of threonine and ornithine were lower ($P < 0.05$) at Day 140, when compared with Day 100 or Day 121 of pregnancy. Serum levels of lysine and proline decreased ($P < 0.05$) with the advancement of pregnancy (Table 4.7).

Concentrations of other metabolites and hormones in maternal serum

Table 4.8 summarizes concentrations of insulin and GH, as well as metabolites other than amino acids in maternal serum of both control and

arginine-infused ewes at Day 121 of pregnancy. Intravenous infusion of arginine to prolific ewes reduced ($P \leq 0.05$) the concentrations of BHB and ammonia, but had no effect on FFA, glycerol, triglycerides, glucose, lactate, urea, insulin or GH. Concentrations of BHB were greater ($P < 0.05$) in mothers carrying quadruplets than in those with triplets ($P < 0.05$), whereas concentrations of FFA and ammonia increased ($P < 0.05$) with increasing litter size. Similarly, concentrations of lactate increased ($P < 0.01$) by 207% as the number of fetuses increased from two to four per ewe. Litter size had no effect on concentrations of glucose, glycerol, triglycerides, urea, insulin, or GH in prolific ewes.

Data from control ewes between Days 100 and 140 of gestation are summarized in Table 4.9. Concentrations of BHB ($P = 0.57$), FFA ($P < 0.01$) and glycerol ($P < 0.01$) were greater ($P < 0.01$) at Day 140 than Day 100 of pregnancy; whereas lactate was lower ($P < 0.01$) at Day 121. There was no effect of day of pregnancy on concentrations of glucose in serum of saline-infused ewes (Table 4.9). Interestingly, concentrations of insulin increased ($P < 0.05$) gradually, while those of GH decreased ($P < 0.01$) progressively between Days 100 and 140 of gestation. Levels of triglycerides, urea or ammonia did not differ ($P > 0.1$) during this 40-day period of late pregnancy. Between Days 100 and 140 of gestation, litter size did not affect ($P > 0.05$) concentrations of glycerol, lactate, triglycerides, urea, insulin or GH in serum of control ewes. However, concentrations of BHB ($P = 0.057$) and FFA ($P < 0.01$) increased, but concentrations of glucose decreased, in ewes carrying quadruplets when compared with ewes with twins or triplets. In addition, serum levels of ammonia increased progressively ($P < 0.01$) with increasing litter size (Table 4.9).

Table 4.8. Concentrations of metabolites and hormones in serum in arginine-treated (345 µmol/Kg body weight) and control ewes at one hour after injection on Day 121 of pregnancy

Metabolites and Hormones	Treatment goup			Litter size			
	Arginine	Control	*P* values	Twins	Triplets	Quadruplets	*P* value
BHB (mM)	0.6 ± 0.1 [b]	1 ± 0.2 [a]	0.05	0.7 ± 0.2 [ab]	0.5 ± 0.1 [b]	1 ± 0.2 [a]	0.03
FFA (µM)	369 ± 47	433 ± 61	0.42	224 ± 71 [c]	430 ± 61 [b]	549 ± 68 [a]	0.01
Glucose (mM)	2.6 ± 0.1	2.3 ± 0.2	0.17	2.6 ± 0.2	2.6 ± 0.2	2.2 ± 0.2	0.28
Glycerol (µM)	34 ± 3	37 ± 4	0.63	26 ± 5	37 ± 4	43 ± 5	0.07
Lactate (mM)	1.5 ± 0.2	1 ± 0.2	0.20	0.7 ± 0.3 [b]	0.9 ± 0.2 [b]	2.2 ± 0.2 [a]	<0.01
Triglycerides (µM)	330 ± 32	292 ± 39	0.45	370 ± 48	262 ± 41	302 ± 42	0.24
Urea (mM)	6.6 ± 0.3	6.5 ± 0.4	0.93	6.7 ± 0.5	7.0 ± 0.4	5.9 ± 0.4	0.14
Ammonia (µM)	97 ± 2.6 [b]	108 ± 3.2 [a]	0.01	83 ± 3.9 [c]	100 ± 3.3 [b]	126 ± 3.5 [a]	<0.01
Insulin (ng/L)	675 ± 108	565 ± 134	0.53	788 ± 164	636 ± 139	436 ± 145	0.28
GH (ng/ml)	3.0 ± 0.2	3.1 ± 0.2	0.60	3.1 ± 0.3	3.0 ± 0.3	3.0 ± 0.3	0.97

Data are means ± SEM. Values sharing different superscripts within a row differ, as analyzed by two-way ANOVA and the Student-Newman-Keuls multiple comparisons test.
BHB= β-Hydroxybutyrate, FFA= Free Fatty Acids, GH= Growth Hormone

Table 4.9. Concentrations of metabolites and hormones in serum on Days 100, 121 and 140 of pregnancy in control ewes with different litter sizes

Metabolites and Hormones	Day of pregnancy				Litter size			
	100	121	140	*P* value	Twins	Triplets	Quadruplets	*P* value
BHB (mM)	0.5 ± 0.2 ^{b}	0.9 ± 0.2 ^{ab}	1.2 ± 0.2 ^{a}	0.05	0.5 ±0.1 ^{b}	0.5± 0.03^{b}	1.5 ± 0.05^{a}	0.05
FFA (µM)	350 ± 50 ^{b}	434 ± 53 ^{b}	774 ± 74 ^{a}	<0.01	342 ± 76 ^{b}	468 ± 52 ^{b}	749 ± 81^{a}	<0.01
Glucose (mM)	2.3 ± 0.1	2.3 ± 0.1	1.8 ± 0.2	0.07	2.4 ± 0.2 ^{a}	2.4 ± 0.1 ^{a}	1.6 ± 0.2 ^{b}	0.01
Glycerol (µM)	36 ± 7 ^{b}	37 ± 7 ^{b}	88 ± 11 ^{a}	<0.01	43 ± 10	46 ± 7	70 ± 11	0.14
Lactate (mM)	2.1 ± 0.2 ^{a}	1.1 ± 0.2 ^{b}	1.4 ± 0.3 ^{b}	<0.01	1.7 ± 0.3	1.3 ± 0.2	1.6 ± 0.4	0.74
Triglycerides (µM)	301 ± 28	292 ± 28	254 ± 43	0.63	342 ± 40	265 ± 27	240 ± 43	0.21
Urea (mM)	7.0 ± 0.4	6.5 ± 1.4	6.4 ± 0.6	0.69	6.8 ± 0.4	6.7 ± 0.3	6.4 ± 0.5	0.78
Ammonia (µM)	109 ± 2.3	108 ± 2.3	109 ± 3.4	0.96	84 ± 2.2 ^{c}	114 ± 1.6 ^{b}	129 ± 2.7 ^{a}	<0.01
Insulin (ng/L)	639 ± 114 ^{a}	707 ± 127 ^{b}	356 ± 141^{c}	0.03	621 ± 218	563 ± 140	518 ± 205	0.94
GH (ng/ml)	2.7 ± 0.2 ^{c}	3.1 ± 0.2 ^{b}	3.9 ± 0.3 ^{a}	<0.01	3.0 ± 0.2	3.3 ± 0.2	3.4 ± 0.3	0.57

Data are means ± SEM. Values sharing different superscripts within a row differ, as analyzed by two-way ANOVA for repeated measures and the Student-Newman-Keuls multiple comparisons test.
BHB= β-Hydroxybutyrate, FFA= Free Fatty Acids, GH= Growth Hormone

Discussion

An increase in the number of gestating fetuses has an adverse effect on intrauterine growth and survival in mammals, including humans and sheep (Blickstein, 2005; Gootwine *et al.*, 2007). Surprisingly, there are currently no effective methods of prevention or treatment for IUGR in human medicine or animal agriculture. To our knowledge, the present study is the first to determine: 1) the impact of litter size on concentrations of amino acids and other metabolites in maternal serum; and 2) the effect of intravenous arginine infusion during late gestation (a period of rapid fetal growth) on pregnancy outcomes in prolific ewes. Parenteral administration of arginine was adopted to avoid extensive catabolism of arginine in the small intestine (Wu *et al.*, 2007a) and effectively increase circulating levels of arginine. Such an intervention is also applicable to pregnant women with persistent severe nausea and vomiting. There are three major findings from the current study. First, concentrations of β-hydroxybutyrate, FFA, ammonia, cysteine, and proline in maternal serum were altered markedly by the number of fetuses in the litter. Second, intravenous infusion of arginine between Days 100 and 121 of gestation reduced fetal death and improved fetal survival in ewes carrying multiple fetuses. Third, arginine intervention greatly enhanced fetal growth of quadruplets, without affecting maternal body weight. These novel findings not only provide a new approach to enhance sheep production, but also have important implications for human medicine.

Concentrations of ammonia, fatty acids and BHB in maternal serum increased substantially with litter size in control ewes (Table 4.9), suggesting that ewes carrying multiple fetuses adapted to increasing fetal demands for nutrients by mobilizing maternal protein and fat stores. Interestingly, homeostasis of most amino acids (Table 4.7) in maternal serum was maintained, independent of the number of fetuses, indicating that rates of dietary provision and endogenous synthesis were closely matched by rates of utilization. These

results are important because they suggest that any alterations in uterine and placental uptakes of amino acids in prolific ewes are not likely attributable to changes in their concentrations in maternal serum, but rather result from changes in utero-placental blood flow and nutrient delivery. Similarly, there is evidence that reduced activities of placental transport, rather than changes in circulating concentrations, are primarily responsible for reduced transfer of amino acids from mother to fetus (Bajoria *et al.*, 2001). Notably, in the control ewes, cysteine and proline were the only amino acids that exhibited a progressive decrease in maternal plasma independent of litter size (Table 4.7). As a substrate for the synthesis of pyrroline-5-carboxylate (a regulator of cellular redox state) and polyamines, proline is now known to play an important role in conceptus growth and development (Wu *et al.*, 2008). Additionally, because cysteine is the most limiting amino acid for the synthesis of glutathione (an antioxidant) (Wu *et al.*, 2004a), our findings raised the question of whether increased oxidative stress in ewes with multiple fetuses is a significant factor contributing to impaired uteroplacental blood flow and fetal growth. The ewe will provide a useful model to examine placental vasculature and quantify utero-placetal blood flow in dams carrying multiple fetuses.

Arginine serves as a common precursor for the synthesis of nitric oxide (NO, a vasodilator and a signaling molecule) and polyamines (key regulators of DNA and protein synthesis) that are crucial for placental angiogenesis and growth in mammals (Wu & Morris, 1998; Sheppard *et al.*, 2001; Ishikawa *et al.*, 2007; Wu *et al.*, 2007a). Thus, alterations in the arginine-NO and polyamine pathways could contribute to impaired uteroplacental blood flow and IUGR both in animal models and in humans (Sooranna *et al.*, 1995; Kwon *et al.*, 2003b; Wu *et al.*, 2006). Using sheep as a model, we observed that intravenous infusion of arginine enhanced fetal growth in ewes carrying quadruplets, which corresponded with the most severe IUGR under the present experimental conditions (Table 4.5). The arginine treatment also increased the percentage of

live-born lambs by approximately 60% (Table 4.4), perhaps by improving the intrauterine environment (including metabolic status and oxygen supply) for fetal development. Interestingly, intravenous infusion of arginine into underfed ewes with singletons also stimulated fetal growth (Chapter III). These results further support the notion that metabolic regulation can be an effective means to prevent IUGR (Wu *et al.*, 2004a). In multiple fetuses, a 23% increase in birth weight would translate into a significant benefit in terms of growth and survival of neonates (Blickstein, 2005). In contrast to ewes with quadruplets, arginine treatment did not affect fetal growth in ewes carrying twins or triplets (Table 4.5). Thus, it is likely that the effect of arginine on fetal growth and development depends on factors (e.g., the relative severity of uterine crowding, IUGR, and ketosis) other than circulating levels of arginine.

At present, it is unknown how arginine promoted fetal growth in ewes carrying quadruplets. However, the improved pregnancy outcome was associated with an increase in concentrations of arginine, cysteine, ornithine, and proline in maternal serum on Day 121 of pregnancy (Table 4.6), as well as a decrease in circulating levels of ammonia and BHB (Table 4.8). As noted above, cysteine may enhance anti-oxidant capacity through the synthesis of glutathione. Additionally, ornithine would facilitate the detoxification of ammonia (a highly toxic substance at elevated levels) via the urea cycle (Wu & Morris, 1998). Also, proline regulates intracellular redox state and placental function (Wu *et al.*, 2008). Further, hepatic ketogenesis, which contributes to metabolic acidosis, is under the control of insulin (Foster & McGarry, 1983). Although serum levels of insulin did not differ between saline- and arginine-infused ewes in this study (Table 4.8), a nearly three-fold increase in maternal concentrations of arginine in arginine-treated ewes can markedly augment systemic NO synthesis (Wu & Meininger, 2002). Because physiological levels of NO increase tissue sensitivity to insulin (Jobgen *et al.*, 2006), parenteral administration of arginine may be capable of reducing hepatic ketogenesis, which was reflected

by a 41% decrease in concentrations of BHB in maternal serum (Table 4.8). This may be an important factor contributing to the enhanced survival of fetal lambs born to ewes carrying multiple fetuses (Table 4.4).

On the basis of our current knowledge about the regulation of NO synthesis in endothelial cells (Wu & Meininger, 2002), increasing concentrations of arginine in maternal plasma of prolific ewes are expected to increase utero-placental blood flow (Neri *et al.*, 1995). This, in turn, would enhance the transfer of oxygen and nutrients from maternal to fetal circulations. Elevation of utero-placental blood flow through intramuscular administration of Sildenafil citrate to underfed ewes between Days 28 and 112 of gestation has been reported to enhance concentrations of nutrients in fetal plasma and fluids, as well as fetal growth (Satterfield *et al.*, 2007). Sildenafil citrate, which acts to enhance intracellular cGMP availability by inhibiting phosphodiesterase-5 (an enzyme that hydrolyzes cGMP to GMP), increased utero-placental blood flow via the protein kinase G signaling pathway (Wareing *et al.*, 2005). Because catheterization of fetal vessels for blood sampling could affect fetal growth, we chose not to perform this invasive procedure in the present study. Therefore, precise changes in concentrations of nutrients in fetal circulation, as well as amniotic and allantoic fluids, due to intravenous infusion of arginine into prolific ewes were not determined. Additional studies are warranted to test the hypothesis that parenteral administration of arginine increases uteroplacental blood flow and, thus, the supply of nutrients transferred across the placenta from mother to fetus.

In conclusion, parenteral administration of arginine between Days 100 and 121 of pregnancy improved the survival rate of lambs born to ewes gestating multiple fetuses and increased the birth weights of quadruplets. These results have important implications for both human medicine and sheep production. Particularly, the findings provide a basis for the use of arginine as a therapeutic intervention to improve pregnancy outcomes, under conditions of a

severely stressed uterine environment, in women and ewes gestating multiple fetuses.

CHAPTER V
SUMMARY AND DIRECTION OF FUTURE RESEARCH

Intrauterine growth restriction (IUGR) accounts for a large incidence of infant mortality and morbidity worldwide and may be associated with an increased risk of metabolic and physiological disorders later in life (Barker & Clark, 1997; Ozanne & Hales, 2002; Bryan & Hindmarsh, 2006; Eleftheriades *et al.*, 2006). An effective therapeutic strategy to ameliorate or prevent this condition is still lacking, which results in a vast emotional, clinical and economic toll (Ergaz *et al.*, 2005; Gluckman & Hanson, 2006; Mari & Hanif, 2007). Furthermore, IUGR is also a major concern in animal agriculture where it severely impacts animal survival and health, as well as general production and performance efficiencies (Wu *et al.*, 2006).

This dissertation research tested the hypothesis that parenteral administration of arginine during mid- to late gestation is effective in enhancing fetal growth and hence weight at birth of IUGR lambs. Undernourished or prolific ewes were used as experimentally-induced and naturally-occurring ovine models of IUGR, respectively. Parenteral administration of arginine was adopted in this work to avoid extensive catabolism of arginine in the rumen and small intestine (Wu *et al.*, 2007a), hence effectively increasing circulating levels of arginine. However, it is important to indicate that intravenous administration of any substance, particularly if long-term catheterization of peripheral vessels is used, can cause unfavorable complications (*e.g.*, phlebitis and thrombosis), which predispose to catheter-associated bacteremias. Catheter complications often arise during the experimental period in livestock, increasing the number of animals necessary to complete a study (Barraza *et al.*, 2006), and must always be considered when contemplating protocols involving central venous access. In addition, although arginine is generally well-tolerated when administered via the intravenous route, local irritation and phlebitis may occur due to the high

osmolality of the solution, and accidental extravasation may lead to local tissue injury (Baker & Franklin, 1991; Boger & Bode-Boger, 2001). This problem is ameliorated when low concentrations of arginine are infused intravenously into animals over a prolonged period of time. Thus, mode and interval of administration of arginine must be considered in the experimental design.

Because arginine has an alkaline property, its hydrochloride salt is generally used for intravenous administration (Wu & Meininger, 2000). However solutions of arginine hydrochloride used for infusion have a high chloride content that could affect the metabolism and health of mother and fetus and may be hazardous in individuals with an electrolyte imbalance (Boger & Bode-Boger, 2001). In addition, the biological half-life of arginine is relatively short due to its degradation by arginase in virtually all cells (Wu & Morris, 1998; Wu *et al.*, 2007a). Hence, the initial experiment was designed to establish if parenteral administration of citrulline to late pregnant ewes is an effective alternative approach to enhance circulating concentrations of available arginine in maternal and fetal plasma. Additionally, the pharmacokinetics of citrulline and arginine in maternal plasma in response to administration of an intravenous bolus of either amino acid to ewes were determined (Chapter II). A prolonged increase in concentrations of arginine and citrulline was maintained in maternal and fetal plasma after citrulline administration to ewes, whereas the increase in concentrations of arginine in fetal and maternal plasma declined more rapidly after arginine infusion. This was consistent with pharmacokinetic results indicating that the half-life of citrulline was twice that of arginine in ewes. These findings revealed that intravenous administration of citrulline to pregnant ewes is more effective than that of arginine in maintaining high concentrations of available arginine in maternal and fetal circulations, and provide a basis for the design of alternative therapeutic strategies to ameliorate IUGR in mammals.

The second experiment was conducted to determine if intravenous administration of arginine to underfed ewes prevented fetal growth restriction

(Chapter III). Global nutrient restriction of 50% of the National Research Council (1985) maintenance requirements for pregnant sheep leads to IUGR (Vonnahme *et al.*, 2003; Kwon *et al.*, 2004a; Satterfield *et al.*, 2007). Results from this study indicated that parenteral administration of arginine to underfed ewes enhanced birth weights of lambs by 21% compared with saline-infused underfed ewes. Moreover, there was no difference in birth weights of lambs between the control-fed and the arginine-infused underfed ewes. In addition, intravenous administration of arginine to underfed ewes increased concentrations of arginine (69%), ornithine (55%), proline (29%), methionine (37%), leucine (36%), isoleucine (35%), cysteine (19%), and free fatty acids (43%) in maternal serum, and decreased levels of ammonia (18%) and triglycerides (32%). Under these experimental conditions, administration of arginine to underfed ewes prevented fetal growth restriction without affecting maternal body weight, indicating that arginine treatment enhanced the availability of nutrients to support fetal growth, as well as overall efficiency of nutrient utilization by the dam.

The third experiment was designed to assess the effects of administration of arginine to late pregnant mothers on fetal survival and birth weights of lambs born to ewes with multiple fetuses. The frequency of multiple fetuses has increased in human pregnancies due to assisted reproductive technologies, consequently increasing the incidence of premature and low birth-weight infants (Blickstein, 2005; Siddiqui & McEwan, 2007). Furthermore, improvements in sheep breeding have resulted in new breeds with increased litter size, but reduced fetal survival and birth weight (Gootwine *et al.*, 2007). Intravenous administration of arginine three times daily from Day 100 to Day 121 of pregnancy to ewes carrying multiple fetuses improved survival rate of lambs by 59%. In addition, arginine treatment enhanced fetal growth of quadruplets, which was reflected by a 23% increase in birth weights, without affecting maternal body weight. The improved pregnancy outcome was associated with an increase in concentrations of arginine, ornithine, cysteine and proline, as well

as a decrease in circulating levels of ammonia and β-hydroxybutyrate in maternal plasma. These results indicate that parenteral administration of arginine to prolific ewes improved pregnancy outcome and ameliorated fetal growth restriction under conditions of a severely stressed uterine environment, perhaps by modulating metabolic pathways and improving intrauterine nutrient availability for fetal development.

Collectively, results of research for this dissertation indicate that arginine treatment improves pregnancy outcome and enhances birth weights of lambs born to undernourished and prolific ewes that served as ovine models of IUGR. In addition, the results provide support for the study of the clinical use of arginine as a therapeutic strategy to prevent or ameliorate IUGR in mammals and highlight the need for additional studies to investigate cellular and molecular mechanisms that could be responsible for the beneficial effects of arginine in regulating fetal growth and development. Additionally, future research should focus on a role for arginine in placental development, as well as uteroplacental blood flow and the supply of nutrients from mother to fetus. Furthermore, detailed studies to investigate single or multiple interventions at specific stages of gestation or even the periconceptual period could clarify potential mechanisms whereby arginine influences growth and development of the conceptus, as well as the relative contributions of altered substrate supply on fetal growth and on the physiological adaptations that impact neonatal survival and predispose to adult onset disease. Moreover, the use of functional genomics to focus on arginine metabolism at specific stages of pregnancy in small and eventually large animal models should facilitate understanding the mechanisms for fetal adaptation to perturbations of the intrauterine environment, and identify similarities and differences among species. Finally, alternative approaches such as the use of citrulline supplementation in IUGR animal models should be evaluated to develop therapeutic strategies.

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