The Journal of Nutrition
Nutrient Physiology, Metabolism, and Nutrient-Nutrient Interactions

Parenteral Administration of L-Arginine Enhances Fetal Survival and Growth in Sheep Carrying Multiple Fetuses[1–3]

Arantzatzu Lassala,[4] Fuller W. Bazer,[4] Timothy A. Cudd,[5] Sujay Datta,[6] Duane H. Keisler,[7] M. Carey Satterfield,[4] Thomas E. Spencer,[4] and Guoyao Wu[4]*

[4]Department of Animal Science, [5]Department of Veterinary Physiology and Pharmacology, and [6]Department of Statistics, Texas A&M University, College Station, TX 77843; and [7]Department of Animal Science, University of Missouri, Columbia, MO 65211

Abstract

The frequency of multiple fetuses has increased in human pregnancies due to assisted reproductive technologies. This translates into a greater proportion of premature and low-birth weight infants in the United States and worldwide. In addition, improvements in sheep breeding have resulted in new breeds with increased litter size but reduced fetal survival and birth weight. Currently, there are no treatments for preventing fetal growth restriction in humans or sheep (an established model for studying human fetal physiology) carrying multiple fetuses. In this work, Booroola Rambouillet ewes (FecB+/−) with 2–4 fetuses were fed a diet providing 100% of NRC-recommended nutrient requirements. Between d 100 and 121 of gestation, ewes received an i.v. bolus injection of either saline solution or 345 μmol arginine-HCl/kg body weight 3 times daily. The arginine treatment reduced ($P < 0.05$) the percentage of lambs born dead by 23% while increasing ($P = 0.05$) the percentage of lambs born alive by 59%. The i.v. administration of arginine enhanced ($P < 0.05$) the birth weights of quadruplets by 23% without affecting maternal body weight. The improved pregnancy outcome was associated with an increase in maternal plasma concentrations of arginine, ornithine, cysteine, and proline, as well as a decrease in circulating levels of ammonia and β-hydroxybutyrate. These novel results indicate that parenteral administration of arginine to prolific ewes ameliorated fetal mortality and growth retardation. Our findings provide support for experiments to assess the clinical use of arginine to enhance fetal growth and survival in women gestating multiple fetuses. J. Nutr. 141: 849–855, 2011.

Introduction

Uterine capacity is a major factor limiting fetal survival and growth in mammals (1,2). 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 (3). Indeed, an inverse relationship between the number of fetuses and birth weight has been described for humans (4,5) and other mammals (2,6,7).

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 (5). This translates into a greater proportion of premature and low-birth weight infants in the United States and elsewhere (8,9). In addition, genetic selection and breeding in sheep has resulted in new breeds with increased litter size (up to 6 fetuses/ewe) but greatly reduced birth weights and survival of lambs (2). However, there are no current treatments for preventing intrauterine growth restriction (IUGR)[8] in humans or sheep gestating multiple fetuses. We have suggested that IUGR may be ameliorated by modulation of the placental NO and polyamine synthetic pathways, thus affecting utero-placental blood flow and perhaps exerting direct actions on the fetus (10). In support of this notion, Mateo et al. (11) recently reported that supplementing arginine (a common physiological substrate for the synthesis of NO 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 carrying 2 or more fetuses.

[1] Supported by grants from the NIH (1R21 HD049449) and National Research Initiative Competitive Grants (2006-35203-17283, 2008-35203-19120, 2008-35206-18764, and 2009-35206-05211) of the USDA National Institute of Food and Agriculture.
[2] Author disclosures: A. Lassala, F. W. Bazer, T. A. Cudd, S. Datta, D. H. Keisler, M. C. Satterfield, T. E. Spencer, and G. Wu, no conflicts of interest.
[3] Supplemental Tables 1–3 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at jn.nutrition.org.
* To whom correspondence should be addressed. E-mail: g-wu@tamu.edu.

[8] Abbreviations used: BHB, β-hydroxybutyrate; GH, growth hormone; IUGR, intrauterine growth restriction.

Manuscript received January 10, 2011. Initial review completed February 04, 2011. Revision accepted February 28, 2011.
First published online March 23, 2011; doi:10.3945/jn.111.138172.

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. To minimize paternal genetic effects on size and weight of the fetuses, the same 2 rams were used throughout the breeding period. Pregnancy diagnosis and initial fetal counts were conducted by transabdominal ultrasonography at d 35 post mating (7.5-MHz probe, Aloka console) and subsequently reassessed at d 45. During the first two-thirds of pregnancy, ewes were individually housed in covered pens with cement floors. In the last one-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) to meet 100% of the NRC-recommended maintenance requirements for pregnant ewes gestating multiple fetuses (12). The dietary composition is shown in **Table 1**. The content of amino acids in the diet was analyzed after base or acid hydrolysis, as described by Li et al. (13). The amounts of feed intake over the experimental period are summarized in (**Supplemental Table 1**). This study was approved by the Texas A&M University Institutional Animal Care and Use Committee.

Experimental design. At d 100 of pregnancy, ewes were assigned randomly to receive an i.v. bolus injection of either sterile saline (control group, n = 14; ~15 mL of 0.9% sodium chloride, Hospira) or the same volume of sterile L-arginine-HCl-saline (arginine group, n = 20; Sigma-Aldrich; 345 μmol L-arginine/kg body weight) 3 times/d until d 121 of pregnancy. The dose of arginine was chosen on the basis of its pharmacokinetics in pregnant ewes (14) to increase the maternal circulating levels of arginine by ~200% at 1 h post administration. The gestational period (d 100–121) was selected for arginine administration mainly because of 2 reasons. First, in sheep, placental growth is completed by d 100 of gestation and most of fetal growth occurs thereafter (15). Second, the relative growth rate of fetal lambs (based on body weight gain per day) on d 100–121 of pregnancy is higher than that at d 121–147 (6.6%/d vs 3.9%/d) (16). The period of arginine administration was based on fetal growth, because it was the major end point of measurement in the present study.

TABLE 1 Composition of the diet[1]

Ingredients	Content
	g/100 g (as-fed)
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[2]	0.50
Vitamin mixture[3]	0.10

[1]Provided the following (% of diet): dry matter, 90.3; crude protein, 12.5; crude fat, 5.5; crude fiber, 15; calcium, 0.49; phosphorus, 0.36; chlorine, 0.91; sodium, 0.91; potassium, 1.02; sulfur, 0.17; magnesium, 0.22. The composition of amino acids in the diet (%; as-fed basis) is as follows: alanine, 0.88; arginine, 0.74; asparagine, 0.60; aspartate, 0.72; cysteine, 0.24; glutamate, 0.97; glutamine 1.19; glycine, 0.61; histidine, 0.34; isoleucine, 0.53; leucine, 1.12; lysine, 0.67; methionine, 0.21; phenylalanine, 0.61; proline, 1.05; serine, 0.60; threonine, 0.49; tryptophan, 0.15; tyrosine, 0.53; and valine, 0.63.
[2] Provided the following (mg/kg of the complete diet): manganese, 140; iron, 118; copper, 12.4; cobalt, 0.28; zinc, 137; iodine, 1.01; selenium, 0.16; and molybdenum, 1.00.
[3] Provided the following (mg/kg of the complete diet): retinyl acetate, 1.72; D-α-tocopherol acetate, 11.1; thiamin, 1.76; menadione sodium bisulfate, 0.27; cholecalciferol, 11.4.

The L-arginine-HCl solution was prepared 3 times/wk using sterile physiologic saline (0.9% sodium chloride, Hospira) with a final concentration of 1.5 g arginine/5 mL. The pH was adjusted to 7.0 with 1 mol/L NaOH and the solution passed through 0.22-μm cellulose acetate filters (Corning) 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. A bacteriological culture of randomly selected saline and arginine solutions was performed by the Texas Veterinary Medical Diagnostic Laboratory on 2 occasions to verify sterility. To administer the saline and L-arginine-HCl solutions, sterile 21 G × 3/4 inches (0.80 × 19 mm) disposable winged infusion sets (SURFLO, Terumo Medical) were used.

At d 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 d 121, samples were drawn at 1 h after the solutions were administered to determine changes in circulating levels of hormones, amino acids, and other metabolites in response to either injection. Anticoagulant-free, sterile, 10-mL vacuum tubes (Vacutainer, Becton Dickinson) were used for blood collection. Blood samples were placed on ice and immediately centrifuged at 3000 × 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. A live birth was defined as the complete expulsion of a product of conception that, after separation from its mother, could breathe or show any other evidence of life, including pulsation of the umbilical cord, beating of the heart, or any definite movement of voluntary muscles. Lambs that were not alive at birth were classified as born dead.

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 FFA, TG, insulin, and growth hormone (GH). Amino acids were determined by fluorometric HPLC methods involving precolumn derivatization with *o*-phthaldialdehyde, as previously described (11). BHB was measured enzymatically by a spectrophotometric method using 3-hydroxybutyrate dehydrogenase (17). FFA were quantified by an enzymatic colorimetric method using the NEFA-HR assay kit from Waco Chemicals. Glucose was enzymatically determined using a spectrophotometric method involving hexokinase and glucose-6-phosphate dehydrogenase (18). Glycerol and L-lactate were quantified using enzymatic fluorometric methods as described (18). TG were enzymatically determined using the Infinity assay kit from Thermo Electron. Ammonia and urea were determined by fluorometric methods involving glutamate dehydrogenase and urease (19,20). Insulin was analyzed using the ovine insulin ELISA microplate kit from Mercodia, whereas GH was quantified by RIA (21,22) validated for ovine serum.

Statistical analyses. Data on lamb birth weight were statistically analyzed by 1-way ANOVA, with ewe as the experimental unit and litter size nested within ewe in treatment (23). Data on concentrations of amino acids, other metabolites, and hormones in serum on d 100, 121, and 140 of pregnancy in control ewes were analyzed by 2-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 d 121 of pregnancy in the arginine group were compared by 2-way ANOVA considering the effects of treatment, litter size, and their interaction. In ANOVA, differences in treatment means were determined by the Student-Newman-Keuls multiple comparison test. Log transformation of variables was performed when the variance of data were 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). P-values ≤ 0.05 were considered significant.

Results

Feed intake and body weight of ewes. Feed intake did not differ between control and arginine-treated ewes throughout the entire gestation (**Supplemental Table 2**). There were no interactions between amino acid treatment and feed intake. Maternal body weight increased ($P < 0.05$) progressively between wk 10 and 19 of gestation but did not differ between saline- and arginine-treated ewes during the experimental period (Supplemental Table 2). There were no interactions between amino acid treatment and maternal body weight. Body weights of saline- and arginine-treated ewes were also similar immediately after parturition (70.1 ± 3.3 and 69.2 ± 2.4 kg, respectively).

Percentages of lambs born alive. Gestational age did not differ between the control and arginine-treated ewes, and was 143.6 ± 1.2 d for the 34 ewes that gave birth. There were twin, triplet, and quadruplet pregnancies in both control and arginine-treated ewes. The control ewes had 3 twin, 7 triplet, and 4 quadruplet pregnancies, and the arginine ewes had 7 twin, 5 triplet, and 8 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 50, 38, and 6%, respectively, in control ewes carrying 2, 3, and 4 fetuses, whereas the values were 100, 40, and 22%, respectively, in arginine-treated ewes carrying 2, 3, and 4 fetuses (**Table 2**). Compared with the control group, i.v. administration of arginine enhanced ($P < 0.05$) fetal survival in twin and quadruplet pregnancies. The overall percentages of lambs born alive were 27.9 and 44.3%, respectively, for control and arginine-treated ewes with different litter sizes (Table 2). Administration of arginine did not affect the total number of lambs born per ewe. However, the arginine treatment reduced ($P < 0.05$) the overall percentage of lambs born dead by 23%, while enhancing ($P = 0.05$) the overall percentage of lambs born alive by 59% (Table 2).

After birth, all live-born lambs were nursed by their mothers. We did not determine postnatal growth of lambs or postpartum changes in maternal body weight. However, we observed that 50 and 26% of live-born lambs from control and arginine-treated ewes, respectively, died within 1 mo after birth. Triplets and quadruplets contributed to ~80% of neonatal deaths in lambs from the ewes.

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 3**). In addition, newborn triplets were heavier than quadruplets in saline-infused ewes ($P = 0.038$) but not in arginine-treated dams ($P = 0.175$). There were no differences in birth weights of twin or triplet lambs between control and arginine-treated ewes (Table 3). However, arginine administration increased ($P < 0.05$) the birth weights of quadruplet lambs by 23% ($P < 0.05$) compared with their counterparts born to control ewes (Table 3). There were no interactions between amino acid treatment and litter size.

TABLE 2 Numbers of lambs born alive and dead from control and arginine-treated ewes carrying multiple fetuses[1]

Treatment	Status at birth	Litter size: Twins	Triplets	Quadruplets	Total	% of total lambs born
Arginine						
Ewes, *n*		7	5	8	20	
	Alive	14	6	7	27	44.3*
	Dead	0	9	25	34	55.7**
	Total	14	15	32	61	
Control						
Ewes, *n*		3	7	4	14	
	Alive	3	8	1	12	27.9
	Dead	3	13	15	31	72.1
	Total	6	21	16	43	

[1] Values are the number of lambs or ewes. Asterisks indicate different from control: *$P = 0.05$, **$P < 0.05$.

TABLE 3 Birth weights of lambs from twin, triplet, and quadruplet pregnancies in 14 control ewes and 20 arginine-treated ewes[1]

Group	Litter size: Twins	Triplets	Quadruplets
Control			
Ewes, *n*	3	7	4
Lambs, *n*	6	21	16
Birthweight, *kg*	4.30 ± 0.38[a]	3.06 ± 0.17[b]	2.47 ± 0.22[c]
Arginine			
Ewes, *n*	7	5	8
Lambs, *n*	14	15	32
Birthweight, *kg*	4.11 ± 0.21[a]	3.39 ± 0.22[b]	3.03 ± 0.14[b*]

[1] Data are means ± SEM. Means in a row with superscripts without a common letter differ, $P < 0.05$. *Different from corresponding control group, $P < 0.05$.

Concentrations of amino acids in maternal serum. Concentrations of amino acids in maternal serum at 1 h after arginine or saline administration on d 121 of pregnancy are summarized in **Table 4**. Circulating levels of arginine, ornithine, cysteine, and proline increased ($P < 0.01$) by 183, 286, 32, and 44%, respectively, in arginine-treated ewes 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-treated than in saline-infused ewes. Litter size affected concentrations of glycine, threonine, and citrulline in maternal serum on d 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 between twin and quadruplet pregnancies (Table 4).

When data from control ewes were analyzed (**Supplemental Table 3**), concentrations of cysteine and proline in serum decreased ($P < 0.001$) 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 compared with triplet pregnancies. Litter size did not affect concentrations of other amino acids in the control group of ewes (Supplemental Table 3).

Concentrations of several amino acids in serum from control ewes changed throughout gestation. Circulating levels of glutamate increased between d 100 and 121 of pregnancy but then fell abruptly at d 140 ($P < 0.01$; Supplemental Table 3). Conversely, concentrations of asparagine decreased ($P < 0.05$) between d 100 and 121, whereas no differences were found between d 121 and 140 (Supplemental Table 3). Glutamine also decreased ($P < 0.05$) between d 100 and 121 of gestation but increased ($P < 0.05$) at d 140 to values similar to those found at d 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 d 121 and 140

TABLE 4 Concentrations of amino acids in the serum of control and arginine-treated ewes at 1 h after administration on d 121 of pregnancy[1]

	Treatment group			Litter size			
Amino acid	Arginine, n = 20	Control, n = 14	P-value	Twins, n = 10	Triplets, n = 12	Quadruplets, n = 12	P-value
	μmol/L				μmol/L		
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	29 ± 2	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	149 ± 30	<0.01	273 ± 37	279 ± 31	304 ± 33	0.79
β-Alanine	8.3 ± 0.7	12 ± 0.9	<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	157 ± 12	0.03	133 ± 14	162 ± 12	124 ± 13	0.10
Tyrosine	41 ± 5	70 ± 6	<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	15 ± 1	<0.01	12 ± 2	12 ± 1	13 ± 1	0.84
Valine	71 ± 8	97 ± 10	0.04	90 ± 12	75 ± 10	87 ± 11	0.61
Phenylalanine	27 ± 2	37 ± 3	<0.01	33 ± 3	28 ± 3	34 ± 3	0.29
Isoleucine	46 ± 4	68 ± 5	<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	43 ± 15	<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	87 ± 5	<0.01	109 ± 6	99 ± 5	96 ± 5	0.24
Proline	143 ± 3	99 ± 3	<0.01	142 ± 4^{a}	117 ± 4^{b}	105 ± 4^{b}	<0.01

[1] Data are means ± SEM. Means in a row with superscripts without a common letter differ, $P < 0.05$.

of gestation compared with d 100 (Supplemental Table 3). In contrast, concentrations of threonine and ornithine were lower ($P < 0.05$) at d 140 compared with d 100 or 121 of pregnancy. Serum levels of lysine and proline decreased ($P < 0.05$) with the advancement of pregnancy (Supplemental Table 3). There were no interactions between amino acid treatment and litter size on concentrations of all measured amino acids in maternal serum.

Concentrations of other metabolites and hormones in maternal serum. Concentrations of insulin and GH as well as metabolites other than amino acids in maternal serum differed ($P < 0.05$) between control and arginine-treated ewes at d 121 of pregnancy (**Table 5**). The i.v. administration of arginine to prolific ewes reduced ($P \leq 0.05$) the concentrations of BHB and ammonia but had no effect on FFA, glycerol, TG, 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 2 to 4/ewe. Litter size had no effect on concentrations of glucose, glycerol, TG, urea, insulin, or GH in prolific ewes (Table 5).

Circulating levels of metabolites in control ewes differed ($P < 0.05$) between d 100 and 140 of gestation (**Table 6**). Concentrations of BHB ($P = 0.05$), FFA ($P < 0.01$), and glycerol ($P < 0.01$) were greater ($P < 0.01$) at d 140 than d 100 of pregnancy, whereas lactate was lower ($P < 0.01$) at d 121. There was no effect of day of pregnancy on concentrations of glucose in serum of the control ewes (Table 6). Interestingly, concentrations of GH increased ($P < 0.05$) gradually, whereas those of insulin decreased ($P < 0.01$) progressively between d 100 and 140 of gestation. Levels of TG, urea, and ammonia did not differ during this 40-d period of late pregnancy. Between d 100 and 140 of gestation, litter size did not affect concentrations of glycerol, lactate, TG, urea, insulin, or GH in serum of control ewes. However, concentrations of BHB ($P = 0.05$) and FFA ($P < 0.01$) increased, but concentrations of glucose decreased ($P < 0.01$), in ewes carrying quadruplets compared with ewes with twins or triplets. In addition, serum levels of ammonia increased progressively ($P < 0.01$) with increasing litter size (Table 6). There were no interactions between amino acid treatment and litter size on concentrations of BHB, FFA, glycerol, TG, glucose, lactate, urea, insulin, or GH.

Discussion

An increase in the number of gestating fetuses has an adverse effect on intrauterine growth and survival in mammals, including humans and sheep (2,5). Surprisingly, there are currently no effective methods to prevent or treat IUGR in human medicine or animal agriculture. To our knowledge, the present study is the first to determine the impact of litter size on concentrations of amino acids and other metabolites in maternal serum and the effect of i.v. 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 (24) and effectively increase circulating levels of arginine. Such an intervention is also applicable to pregnant women with persis-

TABLE 5 Concentrations of metabolites and hormones in the serum of control and arginine-treated ewes at 1 h after administration on d 121 of pregnancy[1]

	Treatment group			Litter size			
Variables	Arginine, $n = 20$	Control, $n = 14$	P-values	Twins, $n = 10$	Triplets, $n = 12$	Quadruplets, $n = 12$	P-value
BHB, *mmol/L*	0.57 ± 0.11	0.96 ± 0.15	0.05	0.68 ± 0.19^{ab}	0.50 ± 0.13^{b}	1.09 ± 0.16^{a}	0.03
FFA, *μmol/L*	369 ± 47	433 ± 61	0.42	224 ± 71^{c}	430 ± 61^{b}	549 ± 68^{a}	0.01
Glucose, *mmol/L*	2.60 ± 0.12	2.32 ± 0.16	0.17	2.59 ± 0.19	2.55 ± 0.16	2.23 ± 0.17	0.28
Glycerol, *μmol/L*	34.1 ± 3.4	36.7 ± 4.2	0.63	26.4 ± 5.2	36.6 ± 4.3	43.2 ± 4.5	0.07
Lactate, *mmol/L*	1.47 ± 0.18	1.09 ± 0.23	0.20	0.71 ± 0.28^{b}	0.93 ± 0.23^{b}	2.18 ± 0.24^{a}	<0.01
TG, *μmol/L*	330 ± 32	292 ± 39	0.45	370 ± 48	262 ± 41	302 ± 42	0.24
Urea, *mmol/L*	6.57 ± 0.30	6.53 ± 0.37	0.93	6.72 ± 0.45	7.02 ± 0.38	5.90 ± 0.40	0.14
Ammonia, *μmol/L*	97.3 ± 2.6	108 ± 3.2	0.01	82.9 ± 3.9^{c}	99.9 ± 3.3^{b}	126 ± 3.5^{a}	<0.01
Insulin, *pmol/L*	116 ± 18	97.4 ± 23	0.53	136 ± 28	110 ± 24	75.5 ± 25	0.28
GH, *μg/L*	2.97 ± 0.21	3.13 ± 0.23	0.60	3.11 ± 0.28	3.02 ± 0.29	3.03 ± 0.26	0.97

[1] Data are means ± SEM. Means in a row with superscripts without a common letter differ, $P < 0.05$.

tent severe nausea and vomiting. There are 3 major findings from the current study. First, concentrations of BHB, FFA, ammonia, cysteine, and proline in maternal serum were markedly altered by the number of fetuses in the litter. Second, i.v. infusion of arginine between d 100 and 121 of gestation reduced fetal death and improved fetal survival in ewes carrying 2 and 4 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 6), 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 (Supplemental Table 3) 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 (25). 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 (Supplemental Table 3). 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 (26). Additionally, because cysteine is the most limiting amino acid for the synthesis of glutathione (an antioxidant) (10), 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 uteroplacental blood flow in dams carrying multiple fetuses.

Arginine serves as a common precursor for the synthesis of 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 (24,27–29). Thus, alterations in the arginine-NO and polyamine pathways could contribute to impaired uteroplacental blood flow and IUGR both in animal models and humans (1,30,31). Using the prolific sheep as an animal model, we observed that i.v. administration of arginine enhanced fetal growth in ewes carrying quadruplets, which corresponded with the most severe IUGR under the present experimental conditions (Table 3).

TABLE 6 Concentrations of metabolites and hormones in serum on d 100, 121, and 140 of pregnancy in control ewes with different litter sizes[1]

	Day of pregnancy				Litter size			
Variables	100, $n = 14$	121, $n = 14$	140, $n = 14$	P-value	Twins, $n = 4$	Triplets, $n = 7$	Quadruplets, $n = 3$	P-value
BHB, *mmol/L*	0.49 ± 0.15^{b}	0.94 ± 0.16^{ab}	1.16 ± 0.17^{a}	0.05	0.54 ± 0.06^{b}	0.53 ± 0.03^{b}	1.52 ± 0.05^{a}	0.05
FFA, *μmol/L*	350 ± 50^{b}	434 ± 53^{b}	774 ± 74^{a}	<0.01	342 ± 76^{b}	468 ± 52^{b}	749 ± 81^{a}	<0.01
Glucose, *mmol/L*	2.33 ± 0.14	2.32 ± 0.14	1.77 ± 0.21	0.07	2.39 ± 0.21^{a}	2.44 ± 0.14^{a}	1.58 ± 0.22^{b}	<0.01
Glycerol, *μmol/L*	35.6 ± 7.0^{b}	36.7 ± 7.0^{b}	87.6 ± 11^{a}	<0.01	43.3 ± 10	46.2 ± 6.6	70.4 ± 11	0.14
Lactate, *mmol/L*	2.07 ± 0.23^{a}	1.09 ± 0.22^{b}	1.37 ± 0.34^{b}	<0.01	1.67 ± 0.34	1.34 ± 0.23	1.55 ± 0.36	0.74
TG, *μmol/L*	301 ± 28	292 ± 28	254 ± 43	0.63	342 ± 40	265 ± 27	240 ± 43	0.21
Urea, *mmol/L*	6.99 ± 0.41	6.53 ± 1.41	6.38 ± 0.61	0.69	6.84 ± 0.37	6.67 ± 0.29	6.40 ± 0.47	0.78
Ammonia, *μmol/L*	109 ± 2.3	108 ± 2.3	109 ± 3.4	0.96	83.6 ± 2.2^{c}	114 ± 1.6^{b}	129 ± 2.7^{a}	<0.01
Insulin, *pmol/L*	110 ± 19^{a}	122 ± 21^{a}	60.9 ± 24^{b}	0.03	107 ± 37	97.1 ± 24	89.0 ± 35	0.94
GH, *μg/L*	2.65 ± 0.17^{c}	3.14 ± 0.17^{b}	3.89 ± 0.26^{a}	<0.01	3.02 ± 0.23	3.27 ± 0.16	3.38 ± 0.26	0.57

[1] Data are means ± SEM. Means in a row with superscripts without a common letter differ, $P < 0.05$.

Interestingly, i.v. administration of arginine to underfed ewes with singletons also stimulated fetal growth (32). These results further support the notion that metabolic regulation can be an effective means to ameliorate or prevent IUGR (1,33). In multiple fetuses, a 23% increase in birth weight would translate into a significant benefit in terms of growth and survival of neonates (5). In contrast to ewes with quadruplets, arginine treatment did not affect fetal growth in ewes carrying twins or triplets (Table 3). 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.

Multiple pregnancies increase risk for fetal and neonatal death in both humans (8,9) and ewes (1,2). Thus, in the control group of ewes carrying multiple pregnancies, only 28% of lambs were born alive (Table 2) and 50% of live-born lambs died within 1 mo after birth. This is comparable to our observations in the past decade that in the breed of ewes used in the present study, the survival rates of singletons, twins, triplets, and quadruplets within 1 mo after birth in Texas are ~95, 85, 50, and 30%, respectively. Notably, i.v. administration of arginine for only 21 d in the 3rd trimester enhanced the percentage of live-born lambs to 44% (an ~60% increase over the control group), perhaps by improving the intrauterine environment, including metabolic status and oxygen supply, for fetal development. Although this enhanced rate of live birth in multiple pregnancies remains suboptimal, our study represents the first step toward improving fetal survival and growth under such a compromised condition. Another benefit of arginine administration during gestation is a substantial reduction in neonatal deaths of lambs from arginine-treated prolific ewes compared with the control group (26 vs. 50%).

At present, it is unknown how arginine promotes 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 d 121 of pregnancy (Table 4) as well as a decrease in circulating levels of ammonia and BHB (Table 5). As noted above, cysteine may enhance antioxidant capacity through the synthesis of glutathione (34). Additionally, ornithine would facilitate the detoxification of ammonia, a highly toxic substance at elevated levels, via the urea cycle (27). Also, proline regulates intracellular redox state and placental function (26). Further, hepatic ketogenesis, which contributes to metabolic acidosis, is under the control of insulin (35). Although serum levels of insulin did not differ between saline- and arginine-treated ewes in this study (Table 5), a nearly 3-fold increase in maternal concentrations of arginine in arginine-infused ewes can markedly augment systemic NO synthesis (36). Because physiological levels of NO increase tissue sensitivity to insulin (37), 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 5). This may be an important factor contributing to the enhanced survival of fetal lambs born to ewes carrying multiple fetuses (Table 2).

In sheep, the vascular density of the fetal placental tissue (cotyledons) remains relatively constant between d 40 and 80 of gestation and increases exponentially thereafter (15). In contrast, the vascular density of the maternal placental tissue (caruncules) increases substantially from d 40 until mid-gestation and then more slowly thereafter (38). Through NO signaling, dietary supplementation with arginine stimulates porcine placental angiogenesis (39). Thus, on the basis of our current knowledge about the regulation of NO synthesis in endothelial cells (36), increasing concentrations of arginine in maternal plasma of prolific ewes are expected to increase placental angiogenesis (15) and uteroplacental blood flow (40). This, in turn, would enhance the transfer of oxygen and nutrients from maternal to fetal circulations. Elevation of uteroplacental blood flow through i.m. administration of sildenafil citrate to underfed ewes between d 28 and 112 of gestation has been reported to enhance concentrations of nutrients in fetal plasma and fluids as well as fetal growth (41). Sildenafil citrate, which acts to enhance intracellular cGMP availability by inhibiting phosphodiesterase-5 (an enzyme that hydrolyzes cGMP to GMP), increased uteroplacental blood flow via the protein kinase G-signaling pathway (42). Because catheterization of fetal vessels for blood sampling could affect fetal growth, we chose not to perform this invasive procedure or determine the rate of uteroplacental blood flow in the present study. Therefore, precise changes in concentrations of nutrients in fetal circulation, as well as amniotic and allantoic fluids, due to i.v. 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. Furthermore, the experimental design of the current study did not allow us to measure systemic NO synthesis or placental growth in ewes. We hope that findings from this work will guide our future research.

In conclusion, parenteral administration of arginine between d 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.

Acknowledgments

A.L., F.W.B, T.E.S., T.A.C., M.C.S., D.H. K., and G.W. designed and conducted research; A.L., S.D., and G.W. analyzed data; A.L., F.W.B., T.E.S., and G.W. wrote the paper; and G.W. had primary responsibility for final content. All authors read and approved the final manuscript.

Literature Cited

1. Wu G, Bazer FW, Wallace JM, Spencer TE. Board-invited review: intrauterine growth retardation: implications for the animal sciences. J Anim Sci. 2006;84:2316–37.
2. Gootwine E, Spencer TE, Bazer FW. Litter-size-dependent intrauterine growth restriction in sheep. Animal. 2007;1:547–64.
3. Gluckman PD, Hanson MA. Maternal constraint of fetal growth and its consequences. Semin Fetal Neonatal Med. 2004;9:419–25.
4. Blickstein I, Kalish RB. Birthweight discordance in multiple pregnancy. Twin Res. 2003;6:526–31.
5. Blickstein I. Growth aberration in multiple pregnancy. Obstet Gynecol Clin North Am. 2005;32:39–54.
6. Freetly HC, Leymaster KA. Relationship between litter birth weight and litter size in six breeds of sheep. J Anim Sci. 2004;82:612–8.
7. Wootton R, Flecknell PA, Royston JP, John M. Intrauterine growth retardation detected in several species by non-normal birth weight distributions. J Reprod Fertil. 1983;69:659–63.
8. Fliegner JR. When do perinatal deaths in multiple pregnancies occur? Aust N Z J Obstet Gynaecol. 1989;29:371–4.
9. Siddiqui F, McEwan A. Twins. Obstetrics, Gynaecol Reprod Med. 2007;17:289–95.

10. Wu G, Bazer FW, Cudd TA, Meininger CJ, Spencer TE. Maternal nutrition and fetal development. J Nutr. 2004;134:2169–72.
11. Mateo RD, Wu G, Bazer FW, Park JC, Shinzato I, Kim SW. Dietary L-arginine supplementation enhances the reproductive performance of gilts. J Nutr. 2007;137:652–6.
12. NRC. Nutrient requirements of sheep. Washington, DC: National Academy of Sciences; 1985.
13. Li XL, Rezaei R, Li P, Wu G. Composition of amino acids in feed ingredients for animal diets. Amino Acids. 2011;40:1159–68.
14. Lassala A, Bazer FW, Cudd TA, Li P, Li XL, Satterfield MC, Spencer TE, Wu G. Intravenous administration of L-citrulline to pregnant ewes is more effective than L-arginine for increasing arginine availability in the fetus. J Nutr. 2009;139:660–5.
15. Reynolds LP, Borowicz PP, Caton JS, Vonnahme KA, Luther JS, Buchanan DS, Hafez SA, Grazul-Bilska AT, Redmer DA. Uteroplacental vascular development and placental function: an update. Int J Dev Biol. 2010;54:355–66.
16. Kwon H, Spencer TE, Bazer FW, Wu G. Developmental changes of amino acids in ovine fetal fluids. Biol Reprod. 2003;68:1813–20.
17. Wu G, Gunasekara A, Brunengraber H, Marliss EB. Effects of extracellular pH, CO2, and HCO3 on ketogenesis in perfused rat liver. Am J Physiol. 1991;261:E221–6.
18. Fu WJ, Haynes TE, Kohli R, Hu J, Shi W, Spencer TE, Carroll RJ, Meininger CJ, Wu G. Dietary L-arginine supplementation reduces fat mass in Zucker diabetic fatty rats. J Nutr. 2005;135:714–21.
19. Li P, Kim SW, Li XL, Datta S, Pond WG, Wu G. Dietary supplementation with cholesterol and docosahexaenoic acid affects concentrations of amino acids in tissues of young pigs. Amino Acids. 2009; 37:709–16.
20. Wu G. Urea synthesis in enterocytes of developing pigs. Biochem J. 1995;312:717–23.
21. Lalman DL, Williams JE, Hess BW, Thomas MG, Keisler DH. Effect of dietary energy on milk production and metabolic hormones in thin, primiparous beef heifers. J Anim Sci. 2000;78:530–8.
22. Powell MR, Keisler DH. A Potential strategy for decreasing milk production in the ewe at weaning using a growth hormone release blocker. J Anim Sci. 1995;73:1901–5.
23. Steel RGD, Torrie JH, Dickey DA, editors. Principles and procedures of statistics: a biometrical approach. New York: McGraw-Hill; 1997.
24. Wu G, Bazer FW, Cudd TA, Jobgen WS, Kim SW, Lassala A, Li P, Matis JH, Meininger CJ, et al. Pharmacokinetics and safety of arginine supplementation in animals. J Nutr. 2007;137:S1673–80.
25. Bajoria R, Sooranna SR, Ward S, D'Souza S, Hancock M. Placental transport rather than maternal concentration of amino acids regulates fetal growth in monochorionic twins: implications for fetal origin hypothesis. Am J Obstet Gynecol. 2001;185:1239–46.
26. Wu G, Bazer FW, Datta S, Johnson GA, Li P, Satterfield MC, Spencer TE. Proline metabolism in the conceptus: implications for fetal growth and development. Amino Acids. 2008;35:691–702.
27. Wu G, Morris SM Jr. Arginine metabolism: nitric oxide and beyond. Biochem J. 1998;336:1–17.
28. Ishikawa T, Harada T, Koi H, Kubota T, Azuma H, Aso T. Identification of arginase in human placental villi. Placenta. 2007;28:133–8.
29. Sheppard C, Shaw CE, Li Y, Bird IM, Magness RR. Endothelium-derived nitric oxide synthase protein expression in ovine placental arteries. Biol Reprod. 2001;64:1494–9.
30. Kwon H, Wu G, Bazer FW, Spencer TE. Developmental changes in polyamine levels and synthesis in the ovine conceptus. Biol Reprod. 2003;69:1626–34.
31. Sooranna SR, Morris NH, Steer PJ. Placental nitric oxide metabolism. Reprod Fertil Dev. 1995;7:1525–31.
32. Lassala A, Bazer FW, Cudd TA, Datta S, Keisler DH, Satterfield MC, Spencer TE, Wu G. Parenteral administration of L-arginine prevents fetal growth restriction in undernourished ewes. J Nutr. 2010;140:1242–8.
33. Wu G, Bazer FW, Davis TA, Kim SW, Li P, Rhoads JM, Satterfield MC, Smith SB, Spencer TE, et al. Arginine metabolism and nutrition in growth, health and disease. Amino Acids. 2009;37:153–68.
34. Wu G. Amino acids: metabolism, functions, and nutrition. Amino Acids. 2009;37:1–17.
35. Foster DW, McGarry JD. The metabolic derangements and treatment of diabetic ketoacidosis. N Engl J Med. 1983;309:159–69.
36. Wu G, Meininger CJ. Regulation of nitric oxide synthesis by dietary factors. Annu Rev Nutr. 2002;22:61–86.
37. Jobgen WS, Fried SK, Fu WJ, Meininger CJ, Wu G. Regulatory role for the arginine-nitric oxide pathway in metabolism of energy-substrates. J Nutr Biochem. 2006;17:571–88.
38. Reynolds LP, Borowicz PP, Vonnahme KA, Johnson ML, Grazul-Bilska AT, Redmer DA, Caton JS. Placental angiogenesis in sheep models of compromised pregnancy. J Physiol. 2005;565:43–58.
39. Li X, Bazer FA, Johnson GA, Burghardt RC, Erikson DW, Frank JW, Spencer TE, Shinzato I, Wu G. Dietary supplementation with 0.8% L-arginine between days 0 and 25 of gestation reduces litter size in gilts. J Nutr. 2010;140:1111–6.
40. Neri I, Di Renzo GC, Caserta G, Gallinelli A, Facchinetti F. Impact of the L-arginine/nitric oxide system in pregnancy. Obstet Gynecol Surv. 1995;50:851–8.
41. Satterfield MC, Bazer FW, Spencer TE, Wu G. Sildenafil citrate treatment enhances amino acid availability in the conceptus and fetal growth in an ovine model of intrauterine growth restriction. J Nutr. 2010;140:251–8.
42. Wareing M, Myers JE, O'Hara M, Baker PN. Sildenafil citrate (Viagra) enhances vasodilatation in fetal growth restriction. J Clin Endocrinol Metab. 2005;90:2550–5.