Original Research *Highlight article*

Dietary supplementation with L-citrulline improves placental angiogenesis and embryonic survival in gilts

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Impact statement

Mammals (including humans and swine) have high rates of embryonic mortality. In this study, gilts (nonparous female swine) were used as an animal model to test the hypothesis that dietary supplementation with L-citrulline (Cit) improves placental angiogenesis and embryonic survival during early pregnancy. Our results indicated that dietary supplementation with Cit was effective in increasing L-arginine availability to gilts between Days 14 and 25 of gestation. This nutritional strategy augmented the volumes of allantoic and amniotic fluids, the placental syntheses of nitric oxide and polyamines, the expression of angiogenic factors and angiogenesis in placentae (as indicated by increases in the number of placental blood vessels and their diameters), the placental expression of aquaporins, embryonic survival, as well as fetal-placental growth and development. Our findings indicate that Cit can improve the reproductive performance of swine and have important implications for improving fertility and pregnancy outcomes in women and other mammalian species.

Abstract

This study was conducted with gilts as an animal model to test the hypothesis that dietary supplementation with L-citrulline (Cit) improves placental angiogenesis and embryonic survival. Between Days 14 and 25 of gestation, each gilt was fed a corn- and soybean-meal-based diet (2 kg/day) supplemented with 0.4% Cit or an isonitrogenous amount of L-alanine (Control). On Day 25 of gestation, gilts were hysterectomized to obtain conceptuses. Amniotic and allantoic fluids and placentae were analyzed for NOx [stable oxidation products of nitric oxide (NO)], polyamines, and amino acids (AAs). Placentae were also analyzed for syntheses of NO and polyamines; concentrations of AAs and related metabolites; and the expression of angiogenic factors and aquaporins (AQPs). Compared to the control group, Cit supplementation increased ($P < 0.01$) the number of viable fetuses by 2.0 per litter, the number and diameter of placental blood vessels (21% and 24%, respectively), placental weight (15%), and total allantoic and amniotic fluid volumes (20% and 47%, respectively). Cit supplementation also increased ($P < 0.01$) enzymatic activities of GTP-cyclohydrolase-1 (32%) and ornithine decarboxylase (27%) in placentae; syntheses of NO (29%) and polyamines (26%); concentrations of NOx (19%), tetrahydrobiopterin (28%), polyamines (22%), cAMP (26%), and cGMP (24%) in placentae; total amounts of NOx (22–40%), polyamines (23–40%), AAs (16–255%), glucose (22–44%), and fructose (22–43%) in allantoic and amniotic fluids. Furthermore, Cit supplementation increased ($P < 0.05$) placental mRNA levels for angiogenic factors (*eNOS* [84%], *GTP-CH1* [55%], *PGF* [61%], *VEGFA120* [26%], and *VEGFR2* [137%], as well as AQPs – AQP1 [105%], AQP3 [53%], AQP5 [77%], AQP8 [57%], and AQP9 [31%]). Collectively, dietary Cit supplementation enhanced placental NO and polyamine syntheses as well as angiogenesis to improve conceptus development and survival.

Keywords: L-arginine, L-citrulline, fetus, placenta, angiogenesis, reproduction

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Introduction

Embryonic loss is a significant issue in mammals, including humans[1] and swine.[2] Clark[3] estimated that 27% of human embryos are lost at the time of, or soon after, implantation. Swine lose up to 50% embryos per litter, with 75% of the embryonic loss occurring before Day 25 of pregnancy.[4] Furthermore, there is severe intrauterine growth restriction (IUGR) of fetuses (i.e. 20–25% of newborn pigs) that suffer from high rates of neonatal morbidity and mortality.[5] Successful implantation and placental growth (including vascular growth) are key to embryonic/fetal survival, growth, and development,[6,7] all of which are critically affected by maternal nutrition.[8,9]

We have conducted research to enhance conceptus survival and growth in swine through improving maternal L-arginine (Arg) nutrition.[9–12] Notably, dietary supplementation with 0.4% Arg to gilts between Days 14 and 25 of gestation increased placental growth by 34%,[13] folding at the uterine–placental interface by 28%,[14] placental angiogenesis

by 26% (based on the number of blood vessels[15]), and the number of viable fetuses per litter by about 2.[13] Arg stimulates placental protein synthesis, inhibits placental protein degradation,[16] promotes the placental production of nitric oxide (NO) by tetrahydrobiopterin (BH4)-dependent NO synthase, and increases the expression of angiogenic factors,[15,17–19] thereby enhancing placental growth (including vascular growth) and development.

Arg can be synthesized effectively from L-citrulline (Cit) in mammals, including humans and swine.[12,20] A concern over dietary Arg supplementation is its potential antagonism with basic amino acids (AAs), including Arg, lysine, and histidine.[10] By contrast, Cit is a neutral AA and does not share the same transporters with basic AAs.[20] In addition, on a molar basis, Cit contains 25% less nitrogen than Arg, therefore, introducing a lower nitrogen load to humans and animals while facilitating the removal of ammonia (a highly toxic substance at elevated concentrations greater than 30 μM in the plasma of adult humans).[21] Furthermore, in contrast to Arg, Cit has a favorable taste to humans.[20] Finally, the half-life of Cit in the blood of gestating mammals (e.g. swine and sheep) is about 95% longer than that of Arg,[21] thereby maintaining an increase in the circulating level of Arg for a longer period. Of note, dietary supplementation with Cit (2 g/kg body weight/day) between Days 7 and 21 of gestation enhanced fetal growth in rats fed a low protein (4% casein) diet.[22] However, to date, little is known about effects of dietary Cit supplementation on embryonic survival and growth in mammals. The present study was conducted with gilts (non-parous female swine) as an animal model to determine the role of dietary Cit in improving reproductive health.

Materials and methods

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

Animals and dietary Cit supplementation

Twenty-eight gilts (F1 crosses of Yorkshire × Landrace sows and Duroc × Hampshire boars) with a body weight of 108–125 kg were bred at the onset of their second estrus and 12 h later.[13] The day of breeding was recorded as Day 0 of gestation. Thereafter, gilts were assigned randomly to one of two treatment groups, representing dietary supplementation with 0.0% (control) or 0.4% Cit (Sigma Chemicals, St. Louis, MO). There were 14 gilts in each treatment group. An isonitrogenous amount of 0.61% of L-alanine (Ala; Ajinomoto Co., Inc., Tokyo, Japan) and 0.21% cornstarch were added to the 0.0% and 0.4% Cit diets, respectively. The molecular weights of Cit and Ala are 175.19 and 89.09 Da, respectively. Cit and Ala contain 3 and 1 N atoms per molecule, respectively. Thus, in 100 g diet, 0.4 g Cit provides 6.85 mmol N (i.e. $0.4 \times 1000 \times 3/175.19$), and 0.61 g Ala supplies 6.85 mmol N (i.e. $0.61 \times 1000 \times 1/89.09$). The dose of Cit was chosen because results of our previous study indicated that dietary supplementation with 0.4% Arg to gilts between Days 14 and 25 of gestation enhanced embryonic survival.[13] Each gilt was fed 1 kg of a corn- and soybean meal-based diet containing 12% crude protein twice daily (07:00 and 18:00 h; 2 kg diet/day) beginning on Day 0 of gestation.[13] The basal diet contained 0.70% Arg and no detectable Cit, as analyzed by high-performance liquid chromatography (HPLC) following acid hydrolysis.[23] Either 0.4% Cit + 0.21% cornstarch or 0.61% Ala was supplemented to the basal diet as top dressing between Days 14 and 25 of gestation. Cit supplementation was initiated on Day 14 of gestation because results of our previous study indicated that dietary supplementation with Arg to gilts between Days 0 and 14 of gestation reduced both the number of corpora lutea (CL) in ovaries due to excessive NO production and the concentration of progesterone in the maternal plasma.[12] Dietary Cit supplementation ended on Day 25 of gestation for hysterectomy because most embryonic deaths occur in swine before this day of pregnancy.[4,6] For this reason, we did not determine effects of Cit supplementation on fetal survival in late pregnancy.

Hysterectomy and tissue collection

On Day 25 of gestation, gilts consumed either 8 g Cit + 4.2 g cornstarch or 12.2 g Ala (provided in a plastic bowl) 30 min before they were anesthetized. Cit or Ala was fed to the gilts at this time to determine effects of the supplement on concentrations of AAs in maternal plasma and conceptuses. Anesthesia of pigs was initiated with an intramuscular injection of 10 mg Telazol (Midwest Veterinary Supply, Lakeville, MN, USA) per kg of body weight, followed by inhalation of 1–5% isofluorane to achieve a surgical plane of anesthesia.[13] Blood was collected from the uterine artery before euthanasia was induced by an intracardiac injection of 15 mL Beuthanasia (Merk, Rahway, NJ). After the abdomen was opened and the uterus was removed, the number of CL in ovaries, the number of live fetuses, placental weight, fetal body weight, fetal crown-to-rump length (the distance from the crown of the head to the base of the tail), volumes of amniotic and allantoic fluid in viable conceptuses, and the number and diameter of placental blood vessels were determined, as we described previously.[13,15] Rates of embryonic survival, expressed as a percentage, were calculated as the number of live fetuses divided by the number of CL present on the ovaries at the time of necropsy. Samples of placentae were snap-frozen in liquid nitrogen. For the analyses of metabolites, allantoic fluid or amniotic fluid from each viable conceptus of the same gilt was combined in equal proportions according to sample type.

Determination of NO and polyamine syntheses by placentae

Fresh placental tissues (~200 mg) were rinsed three times with 1 mL of oxygenated (95% O_2/5% CO_2; v/v) custom-made **Dulbecco's-modified Eagle medium** containing physiological concentrations of AAs (including 0.2 mM Arg), 5 mM D-glucose, 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B,[16] preincubated at 37°C for 0.5 h in 4 mL of fresh oxygenated medium, and then incubated at 37°C for 6 h in 1 mL of fresh oxygenated medium that contained 5 mM D-glucose, 0.2 mM Arg, and concentrations of other AAs found in the plasma of gilts.[13] At the end of a 6 h incubation period, the medium was analyzed for nitrite plus nitrate (NOx, stable oxidation products of NO). In all

experiments, the medium incubated without cells was analyzed as the blank. Nitrite and nitrate in culture medium were determined by HPLC, as we described previously.[24]

To determine effects of treatment on polyamine synthesis, placentae were incubated as described above, except that the medium contained 0.5 mM L-[1-^{14}C]ornithine. ^{14}C-Labeled putrescine, spermidine, and spermine were separated by HPLC, and their radioactivities were measured using a liquid scintillation counter, as we described previously.[25] The rates of production of putrescine, spermidine, and spermine were calculated on the basis of their radioactivities and the specific radioactivity of L-[1-^{14}C]ornithine in the incubation medium.

Determination of NOx, polyamines, BH4, cAMP, cGMP, glucose, fructose, and glycerol

For the analysis of NOx (nitrite plus nitrate) and polyamines (the sum of putrescine, spermidine, and spermine), placentae (~50 mg) were homogenized in 1 mL of 1.5 M $HClO_4$, followed by neutralization with 0.5 mL of 2 M K_2CO_3. NOx and polyamines were determined using our established HPLC methods.[24,26] For BH4 analysis, tissues (~50 mg) were homogenized in 0.5 mL of 0.1 M phosphoric acid containing 5 mM dithioerythritol and 60 µL of 2 M trichloacetic acid, and the tissue extract was used for BH4 analysis by HPLC.[27] For the determination of cGMP in placentae, the tissue (~100 mg) was homogenized in 1 mL of 1.5 M $HClO_4$, followed by neutralization with 0.5 mL of 2 M K_2CO_3. The extract was analyzed for 3′-5′-cGMP using the Amersham cGMP Enzyme Immunoassay Biotrak (EIA) System (GE Healthcare, Buckinghamshire, UK). cAMP was analyzed using an HPLC method involving the precolumn derivatization with 2-chloroacetaldehyde and fluorescence detection, as we previously described.[28] Glucose, fructose, and glycerol were determined, as we described previously.[13,15]

Determination of enzymatic activities

Fresh placental tissue (~100 mg) was used to prepare the cytosolic fraction for the assay of ornithine decarboxylase (ODC) activity with the use of 2 mM L-[1-^{14}C]ornithine (2500 dpm/nmol), as we described previously.[9] The activities of constitutive NO synthase (cNOS) and inducible NO synthase (iNOS) in frozen placental tissue were determined using L-[U-^{14}C] Arg plus 0.1 mM Arg.[27] The activity of GTP-cyclohydrolase-1 (GTP-CH1; the key enzyme for BH4 synthesis) in frozen placental tissue was determined using desalted tissue extract and 2 mM GTP.[27]

RNA extraction, reverse transcription, and quantitative polymerase chain reaction

Placental tissue (~100 mg) was homogenized with 1 mL of TRIzol (Invitrogen, USA), and RNA was extracted with chloroform and precipitated with isopropanol.[18] RNA was washed with 75% ethanol. Total RNA was measured using a NanoDrop ND 1000 spectrophotometer. cDNA was synthesized using the SuperScript First Strand Synthesis System for RT-PCR (Invitrogen, USA). Real-time quantitative polymerase chain reaction (RT-qPCR) was performed using SYBR Green and the Applied Biosystems 7900HT RT-qPCR.[18] Sequences of primers used for the quantitative RT-qPCR were the same as we described previously.[15] Tubulin was used as the housekeeping gene.[15] Data for target genes were normalized on the basis of the tubulin mRNA levels.

Statistical analysis

All data, except for embryonic survival rates, were analyzed statistically using the unpaired t test.[29] Embryonic survival rates were compared using the χ^2 analysis.[29] Probability values ≤ 0.05 were considered statistically significant.

Results

Reproductive performance of gilts

The body weights of gilts at Days 0 and 25 of gestation did not differ ($P > 0.05$) between the control and Cit-supplemented groups (Table 1). Dietary supplementation with Cit did not affect ($P > 0.05$) maternal weight gain, uterine weight, or the number of CL. Compared with the control group, dietary supplementation with 0.4% Cit reduced ($P < 0.05$) embryonic mortality from 25% to 11% and enhanced ($P < 0.01$) the number of viable fetuses by 2.0 per litter. Compared with control gilts, Cit supplementation increased ($P < 0.01$) total placental weight (15%), total allantoic fluid volume (20%), and total amniotic fluid volume (47%) (Table 1), but did not influence ($P > 0.05$) the weight of total viable fetuses or the fetal crown-to-rump length on Day 25 of gestation.

Concentrations and total amounts of AAs, glucose, fructose, and glycerol in maternal plasma, placenta, and fetal fluids of gilts

Compared to control gilts, dietary supplementation with Cit increased concentrations of Cit (70%, $P < 0.01$), Arg (17%, $P < 0.01$), ornithine (11%, $P < 0.01$), and proline (12%, $P < 0.05$) in maternal uterine arterial plasma and in placentae (26%, 13%, 15%, and 12% for these four AAs, respectively; $P < 0.01$), but had no effect ($P > 0.05$) on concentrations of other AAs, glucose, fructose, or glycerol (Table 2). Concentrations of alanine in maternal plasma and placentae were approximately 10% greater ($P < 0.05$) in control gilts than in Cit-supplemented gilts because Ala was added to the diet of the former as the isonitrogenous control. Compared to control gilts, Cit supplementation increased concentrations of Cit (175%, $P < 0.01$), Arg (19%, $P < 0.01$), ornithine (13%, $P < 0.05$), and proline (11%, $P < 0.05$) in both allantoic and amniotic fluids (67% [$P < 0.01$], 16% [$P < 0.01$], 11% [$P < 0.05$], and 12% [$P < 0.05$], respectively), but did not affect concentrations of other AAs or glucose and fructose (Table 2). Concentrations of glycerol in allantoic and amniotic fluids were 18% and 21% lower ($P < 0.01$), respectively, compared with values for control gilts (Table 2). Due to increased volumes of allantoic and amniotic fluids, total amounts of all AAs (16–255%), glucose (22–44%), and fructose (22–43%) in the fetal fluids were greater ($P < 0.01$ for all the metabolites with the exception of His, Thr, and Lys in allantoic fluid [$P < 0.05$]) in Cit-supplemented than in control gilts (Table 3). Total amounts of glycerol in allantoic and amniotic fluids of gilts did not differ ($P > 0.05$) due to treatment (Table 3).

Table 1. Reproductive performance and placental angiogenesis of gilts fed diets supplemented with either 0% (control) or 0.4% L-citrulline (Cit) between Days 14 and 25 of gestation.

Variable	Control	0.4% Cit
Maternal body weight at breeding (Day 0), kg	115.3 ± 1.7	117.1 ± 1.6
Maternal body weight at Day 25 of gestation, kg	116.4 ± 1.8	118.3 ± 1.6
Maternal body weight gain during 25 days, kg	1.1 ± 0.3	1.2 ± 0.2
Uterine weight, kg	2.55 ± 0.06	2.67 ± 0.08
Number of corpora lutea, n	14.3 ± 0.56	14.1 ± 0.43
Total number of fetuses/litter, n	12.2 ± 0.43	13.4 ± 0.45†
Total number of live fetuses/litter, n	10.6 ± 0.37	12.6 ± 0.34*
Embryonic mortality rate, %	25.3 ± 1.1	10.9 ± 1.2†
Weight of total viable fetuses/litter, g	5.78 ± 0.11	5.91 ± 0.15
Fetal crown-to-rump length (mm)	1.81 ± 0.05	1.80 ± 0.04
Weight of total placentae/litter, g	94.5 ± 2.9	108.4 ± 3.7*
Total volume of allantoic fluid/litter, mL	975 ± 37	1175 ± 49*
Total volume of amniotic fluid/litter, mL	2.68 ± 0.06	3.93 ± 0.14*
Average number of placental blood vessels per cm^2	27.1 ± 1.3	32.8 ± 1.4*
Average diameter of placental blood vessels, mm	1.36 ± 0.04	1.69 ± 0.05*

Data are means ± the standard error of the mean (SEM), n = 14. Embryonic survival rate was calculated as the number of live fetuses divided by the number of corpora lutea present on the ovaries at the time of necropsy on Day 30 of gestation.
†$P < 0.05$ vs the control group.
*$P < 0.01$ vs the control group.

Table 2. Concentrations of amino acids, glucose, fructose, and glycerol in plasma from the maternal uterine artery, placentae, allantoic fluid, and amniotic fluid of gilts fed diets supplemented with either 0% (Control) or 0.4% L-citrulline (Cit) between Days 14 and 25 of gestation.

AA	Maternal uterine plasma		Allantoic fluid		Amniotic fluid		Placentae	
	Control	0.4% Cit	Control	0.4% Cit	Control	0.4% Cit	Control	0.4% Cit
Asp	17 ± 0.9	18 ± 0.8	7.8 ± 0.3	8.0 ± 0.2	25 ± 1.2	24 ± 1.3	378 ± 12	382 ± 14
Glu	155 ± 8.6	152 ± 9.4	66 ± 2.0	67 ± 2.4	271 ± 9.1	267 ± 12	746 ± 24	740 ± 26
Arg	163 ± 5.2	191 ± 6.4*	110 ± 4.2	131 ± 5.0*	119 ± 4.1	138 ± 5.3*	293 ± 9.1	332 ± 10*
Ala	469 ± 14	427 ± 10†	207 ± 6.8	203 ± 5.2	355 ± 10	351 ± 13	501 ± 17	453 ± 13†
Orn	71 ± 1.7	79 ± 1.9*	141 ± 5.2	159 ± 6.0†	82 ± 2.2	91 ± 2.6†	106 ± 3.8	122 ± 4.2*
Asn	84 ± 2.4	82 ± 3.4	90 ± 2.8	92 ± 3.3	102 ± 5.2	105 ± 5.7	178 ± 4.9	171 ± 5.7
Ser	135 ± 5.9	132 ± 5.4	537 ± 13	543 ± 14	525 ± 20	522 ± 18	507 ± 15	502 ± 17
Gln	506 ± 11	503 ± 9.8	712 ± 19	703 ± 21	1187 ± 49	1207 ± 42	2186 ± 64	2204 ± 75
His	92 ± 2.8	94 ± 3.3	98 ± 4.7	97 ± 4.3	71 ± 3.0	70 ± 2.5	219 ± 7.0	225 ± 9.1
Gly	737 ± 17	740 ± 14	482 ± 12	479 ± 13	428 ± 13	423 ± 10	964 ± 42	958 ± 46
Thr	194 ± 6.5	192 ± 6.0	217 ± 8.4	209 ± 8.2	220 ± 11	216 ± 9.2	408 ± 18	412 ± 20
Cit	69 ± 2.3	117 ± 6.7*	12 ± 0.4	33 ± 2.0*	21 ± 0.8	35 ± 1.4*	23 ± 1.1	29 ± 1.3*
β-Ala	17 ± 0.7	16 ± 0.6	30 ± 1.2	30 ± 1.1	26 ± 0.8	25 ± 1.0	3.8 ± 0.2	3.7 ± 0.2
Tau	72 ± 2.8	74 ± 2.5	430 ± 12	427 ± 10	217 ± 7.5	220 ± 9.3	987 ± 36	994 ± 39
Tyr	102 ± 4.8	98 ± 3.7	83 ± 2.8	82 ± 3.1	120 ± 5.1	124 ± 6.6	187 ± 5.7	192 ± 6.9
Trp	60 ± 2.4	59 ± 2.1	12 ± 0.5	12 ± 0.5	17 ± 0.6	18 ± 0.7	53 ± 1.9	51 ± 2.2
Met	41 ± 1.3	40 ± 1.0	16 ± 0.5	17 ± 0.9	56 ± 2.0	57 ± 1.7	82 ± 3.3	85 ± 3.6
Val	287 ± 6.9	283 ± 5.4	91 ± 2.9	92 ± 3.6	224 ± 9.3	219 ± 10	324 ± 10	331 ± 12
Phe	78 ± 2.9	76 ± 2.5	35 ± 1.0	35 ± 1.3	83 ± 2.8	81 ± 3.2	146 ± 6.9	140 ± 6.3
Ile	121 ± 4.9	118 ± 5.5	26 ± 0.8	27 ± 0.9	61 ± 2.2	62 ± 2.6	117 ± 4.2	121 ± 5.8
Leu	209 ± 8.4	207 ± 7.3	53 ± 1.2	54 ± 1.4	153 ± 4.5	149 ± 5.7	236 ± 7.8	230 ± 9.2
Lys	219 ± 9.0	215 ± 7.8	307 ± 9.4	302 ± 8.2	170 ± 5.1	166 ± 5.6	406 ± 11	397 ± 13
Pro	262 ± 7.6	293 ± 8.4†	250 ± 7.8	277 ± 8.6†	208 ± 6.8	233 ± 7.4†	272 ± 9.3	304 ± 9.7*
Cys[a]	216 ± 8.7	208 ± 7.9	45 ± 1.4	46 ± 1.5	27 ± 0.8	28 ± 1.0	253 ± 9.8	258 ± 11
Hyp	20 ± 0.8	21 ± 0.7	64 ± 2.6	65 ± 2.4	41 ± 1.4	40 ± 1.3	43 ± 1.2	41 ± 1.4
Gluc	5121 ± 81	5104 ± 60	3671 ± 115	3706 ± 130	2893 ± 84	2842 ± 92	308 ± 13	312 ± 14
Fruc	323 ± 19	311 ± 22	6164 ± 241	6203 ± 216	4592 ± 137	4470 ± 112	77 ± 4.2	76 ± 5.0
Glyc	114 ± 5.3	109 ± 6.2	174 ± 7.9	143 ± 5.8*	107 ± 4.9	84 ± 4.1*	64 ± 2.8	62 ± 3.0

Data are expressed as nmol/mL for uterine arterial plasma, allantoic fluid, and amniotic fluid, and as nmol/g tissue, and are means ± the standard error of the mean (SEM), n = 14.
AA: amino acid; β-Ala: β-alanine; Fruc: D-fructose; Gluc: D-glucose; Glyc: D-glycerol; Hyp: 4-hydroxyproline; Tau: taurine; Orn: ornithine.
[a]Cysteine + ½ cystine.
†$P < 0.05$ vs the control group.
*$P < 0.01$ vs the control group.

Table 3. Total amounts of amino acids, glucose, fructose, and glycerol in allantoic and amniotic fluids of gilts fed diets supplemented with either 0% (Control) or 0.4% L-citrulline (Cit) between Days 14 and 25 of gestation.

AA	Allantoic fluid, μmol		Amniotic fluid, nmol	
	Control	0.4% Cit	Control	0.4% Cit
Asp	7.6 ± 0.4	9.4 ± 0.5*	67 ± 3.5	96 ± 6.0*
Glu	64 ± 3.4	79 ± 4.1*	724 ± 27	1050 ± 58*
Arg	107 ± 4.5	151 ± 9.2*	332 ± 12	544 ± 30*
Ala	203 ± 12	239 ± 12*	953 ± 36	1379 ± 71*
Orn	136 ± 4.6	185 ± 7.9*	220 ± 8.0	354 ± 13*
Asn	89 ± 5.0	108 ± 5.4*	276 ± 17	409 ± 23*
Ser	526 ± 27	637 ± 31*	1411 ± 70	2049 ± 99*
Gln	692 ± 28	825 ± 41*	3192 ± 163	4763 ± 272*
His	95 ± 4.9	114 ± 6.5†	190 ± 10	275 ± 16*
Gly	468 ± 17	562 ± 27*	1144 ± 39	1659 ± 66*
Thr	210 ± 8.9	244 ± 11†	590 ± 31	844 ± 39*
Cit	11 ± 0.5	39 ± 2.9*	55 ± 2.1	138 ± 8.1*
β-Ala	29 ± 1.0	35 ± 1.1*	70 ± 2.8	98 ± 5.1*
Tau	419 ± 19	503 ± 26*	582 ± 23	872 ± 56*
Tyr	80 ± 2.7	95 ± 3.9*	322 ± 13	485 ± 30*
Trp	12 ± 0.5	14 ± 0.5*	46 ± 2.0	71 ± 3.5*
Met	15 ± 0.5	19 ± 0.7*	149 ± 6.0	224 ± 9.6*
Val	89 ± 5.4	107 ± 5.1*	598 ± 23	868 ± 55*
Phe	34 ± 1.3	40 ± 1.1*	220 ± 6.6	318 ± 17*
Ile	25 ± 0.8	31 ± 0.9*	164 ± 8.2	241 ± 11*
Leu	52 ± 2.2	64 ± 3.7*	410 ± 15	579 ± 19*
Lys	299 ± 13	353 ± 15†	457 ± 18	654 ± 32*
Pro	241 ± 6.6	321 ± 9.3*	555 ± 22	914 ± 43*
Cys[a]	43 ± 1.7	54 ± 2.0*	72 ± 2.2	109 ± 5.8*
Hyp	62 ± 4.1	76 ± 4.5†	110 ± 4.2	154 ± 4.4*
Gluc	3598 ± 206	4374 ± 251†	7738 ± 251	11,150 ± 470*
Fruc	5995 ± 318	7318 ± 415†	12,331 ± 508	17,654 ± 952*
Glyc	172 ± 13	166 ± 6.9	288 ± 16	327 ± 20

Data are means ± the standard error of the mean (SEM), n = 14.
AA: amino acid; β-Ala: β-alanine; Fruc: D-fructose; Gluc: D-glucose; Glyc: D-glycerol; Hyp: 4-hydroxyproline; Tau: taurine; Orn: ornithine.
[a]Cysteine + ½ cystine.
†$P < 0.05$ vs the control group.
*$P < 0.01$ vs the control group.

Concentrations and total amounts of NOx and polyamines in allantoic and amniotic fluids

Concentrations of NOx and polyamines in allantoic and amniotic fluids did not differ ($P > 0.05$) between the control and Cit groups of gilts (Table 4). Compared with the control group, dietary supplementation with 0.4% Cit increased ($P < 0.01$) total amounts of NOx (22–40%) and polyamines (23–40%) in allantoic and amniotic fluids of gilts (Table 4). Total amounts of the individual polyamines (putrescine, spermidine, and spermine) in allantoic and amniotic fluids also increased ($P < 0.01$) in response to Cit supplementation.

Concentrations of metabolites (NOx, polyamines, BH4, cAMP, and cGMP), NO and polyamine syntheses, and activities of related enzymes in placentae

These data are summarized in Table 5. Compared with control gilts, dietary supplementation with 0.4% Cit increased ($P < 0.01$) concentrations of NOx (19%), total polyamines (putrescine + spermidine + spermine; 22%), BH4 (28%), cAMP (26%), and cGMP (24%) in placentae. The rates of placental syntheses of NO and polyamines were 29% and 26% greater ($P < 0.01$), respectively, in Cit-supplemented gilts than in control gilts. The rates of syntheses of the individual polyamines (putrescine, spermidine, and spermine) and total polyamines in placentae also increased ($P < 0.05$) in response to Cit supplementation. Compared with control gilts, dietary supplementation with 0.4% Cit increased ($P < 0.01$) the enzymatic activities of GTP-CH1 (32%) and ODC (27%) in placentae but did not affect ($P > 0.05$) the enzymatic activities of cNOS and iNOS.

Placental angiogenesis

Dietary supplementation with 0.4% Cit increased ($P < 0.01$) the number of blood vessels per cm^2 and their diameter in the placentae by 21% and 24%, respectively, compared with control gilts (Table 1). As shown in Figure 1, blood vessels in the allantois of placentae from conceptuses of gilts receiving 0.4% Cit supplementation (Figure 1(b)) were more developed

Table 4. Effects of dietary supplementation with either 0% (Control) or 0.4% L-citrulline (Cit) to gilts between Days 14 and 25 of gestation on concentrations and total amounts of NOx and polyamines in allantoic and amniotic fluids.

Variable	Concentrations, nmol/mL		Total amounts, nmol	
	Control	0.4% Cit	Control	0.4% Cit
Allantoic fluid				
NO_X	63.8 ± 2.9	64.6 ± 2.7	61,789 ± 3082	75,203 ± 3519*
Putrescine	1.08 ± 0.05	1.12 ± 0.06	1049 ± 50	1305 ± 70*
Spermidine	1.64 ± 0.07	1.71 ± 0.08	1583 ± 65	1977 ± 78*
Spermine	1.72 ± 0.06	1.74 ± 0.07	1679 ± 84	2031 ± 106*
Total polyamines	4.45 ± 0.16	4.50 ± 0.18	4324 ± 202	5315 ± 238*
Amniotic fluid				
NOx	29.4 ± 1.3	28.8 ± 1.7	78.6 ± 3.5	110.4 ± 4.2*
Putrescine	0.32 ± 0.02	0.31 ± 0.02	0.85 ± 0.04	1.20 ± 0.05*
Spermidine	0.58 ± 0.03	0.56 ± 0.04	1.54 ± 0.07	2.14 ± 0.12*
Spermine	0.56 ± 0.04	0.54 ± 0.05	1.48 ± 0.09	2.09 ± 0.16*
Total polyamines	1.46 ± 0.08	1.41 ± 0.07	3.88 ± 0.17	5.43 ± 0.25*

Values are means ± the standard error of the mean (SEM), n = 14. Total amounts of metabolites were calculated as concentrations in fetal fluid × volume of fetal fluid. Total amounts of the metabolites in the Cit group were greater than those in the Control group due to greater volumes of allantoic and amniotic fluids in the former (Table 1).
NOx: oxidation end products (nitrite plus nitrate) of NO.
†$P < 0.05$ vs the control group.
*$P < 0.01$ vs the control group.

and more abundant than those in the allantois of placentae from conceptuses of control gilts (Figure 1(a)).

Expression of angiogenic factors and aquaporins in placentae

Data for the mRNA abundances of angiogenic factors and aquaporins (AQPs) in placentae are summarized in Table 6. Dietary supplementation with 0.4% Cit increased placental mRNA levels for endothelial NO synthase (*eNOS*, 84%), *GTP-CH1* (55%), placental growth factor (*PGF*, 61%), vascular endothelial growth factor A-120 (*VEGFA120*, 26%), vascular endothelial growth factor A (*VEGFR2*, 137%), *AQP1* (105%), *AQP3* (53%), *AQP5* (77%), and *AQP8* (57%) (all at $P < 0.01$), as well as *AQP9* (31%, $P < 0.05$). However, placental mRNA levels for fibroblast growth factor 2 (*FGF-2*), vascular endothelial growth factor A-164 (*VEGFA164*), vascular endothelial growth factor receptor 1 (*VEGFR1*), *AQP2*, *AQP4*, and *AQP 11* did not differ ($P > 0.05$) between control and Cit-supplemented gilts.

Discussion

Arg is required for the synthesis of molecules with enormous physiological importance in humans and other members of the animal kingdom, including NO, polyamines, creatine, homoarginine, and agmatine.[30] Both NO and polyamines are required for the syntheses of DNAs and proteins, as well as the proliferation of vascular endothelial cells essential for angiogenesis (the growth of blood vessels from existing ones).[27,31] Arg plays important roles in regulating cell signaling (including the mechanistic target of rapamycin), gene expression, vasodilation, blood flow, and nutrient metabolism in animals (including pregnant dams) to improve cardiovascular function, immunity, antioxidative responses, and metabolic health, as well as embryonic survival and fetal growth.[21,30,32–35]

Table 5. Effects of dietary supplementation with 0.4% L-citrulline (Cit) to gilts between Days 14 and 25 of gestation on concentrations of NOx and polyamines, as well as syntheses of NO and polyamines and activities of related enzymes in placentae.

Variable	Control	0.4% Cit
Placental concentrations		
NOx, nmol/g tissue	33.4 ± 1.6	39.7 ± 1.8*
Putrescine, nmol/g tissue	50.1 ± 2.2	59.6 ± 2.8*
Spermidine, nmol/g tissue	87.7 ± 4.8	110 ± 6.0*
Spermine, nmol/g tissue	89.6 ± 4.0	108 ± 4.8*
Total PAs, nmol/g tissue	227 ± 11	278 ± 12*
BH4, pmol/g tissue	287 ± 14	368 ± 17*
cAMP, pmol/g tissue	182 ± 9.0	229 ± 11*
cGMP, pmol/g tissue	13.9 ± 0.7	17.2 ± 0.9*
Placental synthesis		
NO, nmol/g tissue/h	9.79 ± 0.39	12.6 ± 0.50*
Putrescine, nmol/g tissue/h	0.51 ± 0.03	0.63 ± 0.04†
Spermidine, nmol/g tissue/h	0.81 ± 0.05	1.02 ± 0.06†
Spermine, nmol/g tissue/h	0.78 ± 0.06	0.99 ± 0.07†
Total PAs, nmol/g tissue/h	2.10 ± 0.14	2.64 ± 0.15†
Placental enzyme activity		
cNOS, nmol/g tissue/h	1.15 ± 0.07	1.10 ± 0.06
iNOS, nmol/g tissue/h	1.20 ± 0.08	1.25 ± 0.09
GTP-CH1, nmol/g tissue/h	1.06 ± 0.06	1.40 ± 0.08*
ODC1, nmol/g tissue/h	7.47 ± 0.26	9.46 ± 0.31*

Values are means ± the standard error of the mean (SEM), n = 14.
cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; cNOS: constitutive NO synthase; GTP-CH1: GTP-cyclohydrolase-I; iNOS: inducible NO synthase; NOx: oxidation end products (nitrite plus nitrate) of NO; ODC1: ornithine decarboxylase; PAs, polyamines (putrescine + spermidine plus spermine).
†$P < 0.05$ vs the control group.
*$P < 0.01$ vs the control group.

Although Arg is truly a functional AA,[21] its use as a nutritional supplement may present challenges under some pathological, nutritional, and physiological conditions for certain populations. For example, for people and animals

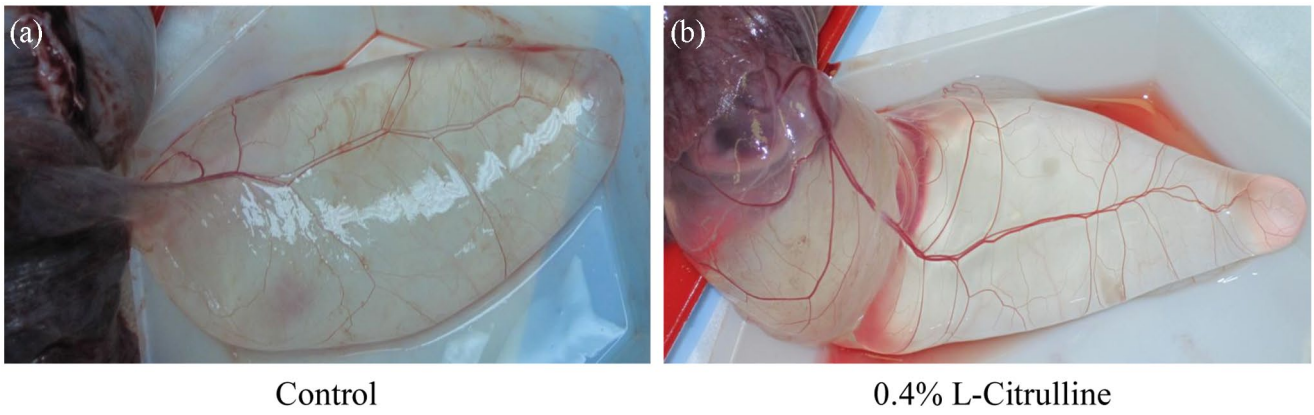

Figure 1. Placental blood vessels in the allantois on Day 25 of gestation in gilts supplemented with either 0 (control) or 0.4% L-citrulline. Placental blood vessels in the allantois of gilts without L-citrulline supplementation (control; (a)) and gilts receiving dietary supplementation with 0.4% L-citrulline (b) are shown. Calcium deposits were detected in the chorioallantois of some gilts in the control group but not in the Citrulline group.

Table 6. Relative expression of mRNAs for angiogenic factors and AQPs in placentae of gilts fed a diet supplemented with either 0% (Control) or 0.4% L-citrulline (Cit) between Days 14 and 25 of gestation.

Gene	Control	0.4% Cit
Angiogenic factors		
eNOS	1.00 ± 0.09	1.84 ± 0.20*
FGF-2	1.00 ± 0.08	1.03 ± 0.06
GTP-CH1	1.00 ± 0.06	1.55 ± 0.14*
PGF	1.00 ± 0.07	1.61 ± 0.16*
VEGFA120	1.00 ± 0.04	1.26 ± 0.07*
VEGFA164	1.00 ± 0.10	1.04 ± 0.12
VEGFR1	1.00 ± 0.07	1.08 ± 0.09
VEGFR2	1.00 ± 0.10	2.37 ± 0.25*
AQPs		
AQP1	1.00 ± 0.07	2.05 ± 0.18*
AQP2	1.00 ± 0.08	1.06 ± 0.11
AQP3	1.00 ± 0.07	1.53 ± 0.13*
AQP4	1.00 ± 0.06	1.06 ± 0.08
AQP5	1.00 ± 0.08	1.77 ± 0.22*
AQP8	1.00 ± 0.05	1.57 ± 0.15*
AQP9	1.00 ± 0.05	1.31 ± 0.10†
AQP11	1.00 ± 0.09	0.95 ± 0.08

Values are means ± the standard error of the mean (SEM), n = 10.
AQP: aquaporin; eNOS: endothelial nitric oxide synthase; FGF-2: fibroblast growth factor 2 (also known as basic fibroblast growth factor and FGF-β); GTP-CH1: GTP-cyclohydrolase-1; PGF: placental growth factor; VEGFA: vascular endothelial growth factor A; VEGFR: vascular endothelial growth factor receptor.
†$P < 0.05$ vs the control group.
*$P < 0.01$ vs the control group.

with defects (e.g. lysinuric protein intolerance) in the intestinal absorption of Arg and its renal reabsorption,[36] it is not feasible to increase Arg availability through its dietary intake. Likewise, for persons who do not tolerate the taste of the Arg base or Arg salts,[21] dietary Arg supplementation will be of limited value. A solution to these practical problems is the intake of Cit, which neither shares transmembrane transporters with basic AAs (including Arg and Lys) nor antagonizes those nutrients in nutrition and metabolism.[20] Rather, Cit is readily absorbed by the small intestine via transporters for neutral AAs, bypasses the liver, and is effectively used by extrahepatic tissues (primarily the kidneys) and cells (e.g. endothelial cells and macrophages) for Arg synthesis.[20] Swine can effectively use dietary Cit to produce Arg via argininosuccinate synthase and lyse in all cell types and tissues, while catabolizing Arg to various metabolites via multiple pathways, including arginase, NO synthase, and arginine-glycine amidinotransferase.[12] Arginase-generated ornithine is readily converted into proline via ornithine aminotransferase and pyrroline-5-carboxylate reductase.[20] Thus, Cit supplementation to gilts between Days 14 and 25 of gestation increased concentrations of Arg, ornithine, proline, NOx, and polyamines in conceptuses, as well as the synthesis of NO and polyamines in placentae (Tables 2 to 5), thereby improving placental growth (including vascular growth) and embryonic survival during early pregnancy (Table 1).

Cit-derived Arg stimulates the expression of GTP-CH1 and ODC1 in mammalian cells, which are key enzymes in the synthesis of BH4 (and therefore NO) and polyamines, respectively.[12] Both ornithine and proline serve as substrates for the synthesis of putrescine in placentae, which is subsequently converted into spermidine and spermine by *S*-adenosylmethionine-dependent spermidine synthase and spermine synthase, respectively.[25] Thus, Cit supplementation to pregnant gilts enhanced the syntheses and concentrations of NO and polyamines in placentae (Table 5), as well as concentrations and total amounts of NOx and polyamines in allantoic and amniotic fluids (Table 4). Polyamines stabilize DNAs for transcription to mRNAs for protein synthesis in animal cells.[31] As a signaling molecule, NO stimulates guanylate cyclase to enhance the production of cGMP from GTP and activate adenylate cyclase to promote the generation of cAMP from adenosine triphosphate (ATP) in mammalian cells, resulting in protein phosphorylation and physiological responses.[37] Consistent with this notion, dietary supplementation with Cit to pregnant gilts increased the concentrations of both cGMP and cAMP in placentae (Table 5). Furthermore, Cit supplementation to gilts enhanced the expression of angiogenic factors, including eNOS, VEGFA120, VEGFR2, PGF, and GTP-CH1 (Table 6). VEGFA (the conventional form of VEGF) acts on endothelial cells to induce their migration and proliferation via an NO-dependent mechanism.[27] VEGFA120 and VEGFA164, which are splice variants of VEGFA in porcine placentae,[38] bind to VEGF receptors 1 and 2 and act in

synergy with PGF to increase the proliferation of endothelial cells and vascular permeability.[39,40] Placental angiogenesis is crucial for the growth and development of the placental vasculature, which is responsible for the delivery of nutrients and gases for exchange across the utero-placental interface between mother and fetus, as well as for the removal of fetal metabolic wastes.[8] Collectively, dietary Cit supports conceptus survival and growth in mammals.

Growth and development of the conceptus are accompanied with the rapid accumulation of water, as well as changes in the concentrations of nutrients in placentae, allantoic fluid, and amniotic fluid.[41] This necessitates the placental transport of water from mother to fetus. We reported that porcine placentae expressed AQPs 1, 2, 3, 4, 5, 8, 9, and 11, but not AQP 10, on Day 25 of gestation[15,42,43] and that AQPs 1, 5, 8, and 10 were localized to specific cell types within both the endometrium and placenta.[44] Thus, pigs appear to use AQPs 1, 5, 8, and 9 to transport water from maternal blood across the utero-placental interface into the allantoic sac as allantoic fluid. AQPs (e.g. AQPs 1 and 5) are cGMP-gated transmembrane channels[45] and AQPs are activated by cAMP-dependent protein kinase A.[19,46] Therefore, both cGMP and cAMP cell signaling upregulate water transport across the placenta. Consequently, as reported for dietary Arg supplementation,[15] supplementation with Cit to gilts increased the volumes of both allantoic and amniotic fluids during early gestation (Table 1), as well as the expression of genes for AQPs 1, 3, 5, 8, and 9 in the placentae (Table 6). A rapid expansion of these fetal fluids is essential to force the chorioallantoic into direct and close apposition to the uterine luminal epithelia and, therefore, positively correlates with embryonic growth and survival in mammals, including pigs.[41] This explains, in part, the greater embryonic survival in Cit-supplemented gilts (Table 1).

Findings from the present study have important implications for human nutrition and pregnancy. Although there are differences in pregnancy between swine and humans, which include the timing of implantation, the type of placentation, the length of gestation, and the number of offspring,[6,7] both swine and humans are monogastric omnivores with similarities in anatomy, physiology, nutrition, and metabolism.[47,48] As the fertility rate of women in the United States has declined gradually over the past 60 years from 3.55 births/woman in 1960 to only 1.64 births/woman in 2020,[49] due to a plethora of factors (including nutrition and obesity),[50] dietary supplementation with synthetic Cit or a Cit-rich food may provide a means for alleviating such a health issue. In this regard, it is noteworthy that a naturally abundant source of Cit for humans is watermelon,[51] as 1 L of watermelon juice provides 2.65 g Cit.[52,53] Oral consumption of watermelon juice has been reported to reduce circulating levels of soluble vascular cell adhesion molecule-1 (sVCAM-1, a biomarker of inflammatory endothelial activation) in obese postmenopausal women,[53] peripheral arterial stiffness in healthy young women,[54] and blood pressure in hypertensive postmenopausal women.[55] Cit and watermelon juice hold promise for improving reproductive health[30] as well as vascular and metabolic health[56,57] in humans.

Conclusions

Dietary supplementation with Cit was effective in increasing Arg availability to gilts and conceptuses between Days 14 and 25 of gestation. This nutritional strategy augmented the volumes of allantoic and amniotic fluids, the placental syntheses of NO and polyamines, the expression of angiogenic factors and angiogenesis in placentae (as indicated by increases in the number of placental blood vessels and their diameters), and the placental expression of AQPs for water transport, as well as fetal-placental growth and development. Thus, maternal Cit supplementation beneficially increased embryonic survival. Our findings clearly indicate that Cit can enhance the reproductive performance of swine and have important implications for improving fertility and pregnancy outcomes in women and other mammalian species.

AUTHORS' CONTRIBUTIONS

GW, FBW, and GAJ designed and supervised the study. XL, FBW, GAJ, RCB, and GW performed the experiment. XL and GW statistically analyzed and summarized results. XL and GW wrote the manuscript. FBW, GAJ, and RCB contributed to data interpretation and manuscript revisions. All authors read and approved the final manuscript.

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