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Maternal *N*-Carbamylglutamate Supplementation during Early Pregnancy Enhances Embryonic Survival and Development through Modulation of the Endometrial Proteome in Gilts[1–3]

Jinlong Zhu,[4] Xiangfang Zeng,[4]* Qian Peng,[4] Shenming Zeng,[5] Haiyi Zhao,[6] Hexiao Shen,[6] and Shiyan Qiao[4]

[4]State Key Laboratory of Animal Nutrition, Ministry of Agriculture Feed Industry Center, and [5]Key Laboratory of Animal Genetics, Breeding and Reproduction, College of Animal Science and Technology, China Agricultural University, Beijing, China; and [6]Genecreate Biological Engineering Co., Ltd., National Bio-industry Base, Wuhan, China

Abstract

Background: Early pregnancy loss is a major concern in humans and animals. *N*-carbamylglutamate (NCG) has been found to enhance embryonic survival during early pregnancy in rats. However, little is known about the key factors in the endometrium involved in the improvement of embryonic implantation and development induced by maternal NCG supplementation.

Objectives: Our objectives were to investigate whether NCG supplementation during early gestation enhanced embryonic survival and development in gilts and to uncover the related factors using the approach of endometrium proteome analysis with isobaric tags for relative and absolute quantification (iTRAQ).

Methods: Uteruses and embryos/fetuses were obtained on days 14 and 28 of gestation from gilts fed a basal diet that was or was not supplemented with 0.05% NCG. The iTRAQ-based quantitative proteomics approach was performed to explore the endometrium proteome altered by NCG supplementation.

Results: Maternal NCG supplementation significantly increased the number of total fetuses and live fetuses on day 28 of gestation by 1.32 and 1.29, respectively ($P < 0.05$), with a significant decrease in embryonic mortality ($P < 0.05$). iTRAQ results indicated that a total of 59 proteins showed at least 2-fold differences ($P < 0.05$), including 52 proteins that were present at higher abundances and 7 proteins present at lower abundances in NCG-supplemented gilts. The differentially expressed proteins primarily are involved in cell adhesion, energy metabolism, lipid metabolism, protein metabolism, antioxidative stress, and immune response. On day 14 of gestation, several proteins closely related to embryonic implantation and development, such as integrin-αv, integrin-β3, talin, and endothelial nitric oxide synthase, were upregulated (3.7-, 4.1-, 2.4-, and 5.4-fold increases, respectively) by NCG supplementation.

Conclusion: To our knowledge, our results provide the first evidence that altered abundance of endometrial proteome induced by NCG supplementation is highly associated with the improvement of embryonic survival and development in gilts. *J Nutr* doi: 10.3945/jn.115.216333.

Keywords: *N*-carbamylglutamate, embryonic survival, gilts, Arg, iTRAQ

Introduction

Early pregnancy loss is a common complication that has a profound impact on pregnancy outcome in humans and animals (1). About 15–20% of clinical pregnancies result in pregnancy loss (2). Although assisted reproductive technology has increased the pregnancy rate for infertile women, a considerable incidence of early pregnancy loss still exists (3). For mammals, early pregnancy loss accounts for 30–50% of spontaneous conceptions (4). However, human tissue is difficult to obtain for investigation of the important factors involved in early pregnancy events (5). Based on the high number of genetic similarities between humans and pigs, a porcine model is one of the best models for researchers in the fields of maternal and fetal nutrition (6).

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[3] Supplemental Figures 1 and 2 and Supplemental Tables 1 and 2 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 http://jn.nutrition.org.
* To whom correspondence should be addressed. E-mail: zengxf@cau.edu.cn.

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Pregnancy outcome in mammals is affected by complex factors (7), among which embryonic mortality is a major controlling factor (8, 9). Hence, new knowledge about nutritional support of early embryonic survival and fetal development is essential for humans and animals to enhance reproductive efficiencies and health by reducing early pregnancy loss and improving fetal growth (10).

Arg plays an important role in embryo implantation and conceptus development by activating the mammalian target of rapamycin (mTOR)[7] cell signaling pathway to stimulate protein synthesis in the placenta, uterus, and fetus (10–13). Dietary Arg supplementation can enhance embryonic survival during early gestation (1, 14), improve placental growth (15, 16), and prevent fetal growth restriction (17).

N-carbamylglutamate (NCG) has been reported to regulate the expression of vascular endothelial growth factor A and endothelial NO synthase (eNOS) in the umbilical vein during late gestation in sows (18). Our previous study indicated that dietary NCG supplementation during early pregnancy improved early embryonic implantation in rats (19). Moreover, we found that dietary supplementation with 0.05% NCG between days 0 and 28 of gestation significantly increased litter size in sows (J Zhu, X Zeng, Q Yang, S Wang, H Jia, and S Qiao, unpublished results, 2014).

However, the series of important factors in the endometrium that may be involved in improving early embryonic survival induced by NCG supplementation are largely unknown. We hypothesized that maternal NCG supplementation during early gestation may enhance embryonic survival and development in gilts through modulation of the crucial factors related to embryonic implantation and development in the endometrium. Therefore, the objective of this study was to test this hypothesis and to uncover the related factors using the approach of endometrial proteome analysis by isobaric tags for relative and absolute quantification (iTRAQ).

Methods

Animals and diets. The animal handling procedures were in accordance with the Chinese Guidelines for Animal Welfare and were approved by the China Agricultural University Animal Care and Use Committee (Beijing, China). A total of 32 gilts (F1 crosses of Yorkshire × Landrace sows and Duroc boars) with an initial body weight of 132 ± 2 kg (mean ± SEM) were used in this study. Gilts were housed individually in partially steel-slatted concrete floor pens (2.4 × 1.8 m). All gilts were checked for estrus using fence-line contact with an experienced boar once daily in the morning. At onset of the second estrus, gilts were artificially inseminated 3 times with fresh semen from Landrace boars (10–12 h apart). Immediately after breeding, gilts were assigned randomly to 1 of the 2 treatment groups fed the basal diet (control group) or the basal diet supplemented with 0.05% NCG (NCG group; wt:wt) for 28 d (n = 16). All gilts were fed 1.1 kg of basal diet or NCG diet twice daily with free access to drinking water. Gilts consumed all of the feed offered throughout the experiment; thus, each gilt in the NCG-supplemented group consumed 1100 mg NCG/d. The diets met the recommendations of the NRC for gestating gilts (20) (**Supplemental Table 1**). NCG (purity, 97%) was obtained from the National Feed Engineering Technology Research Center (Beijing, China).

Sample collection. On days 14 and 28 of gestation, blood samples from 8 gilts in each group were collected as described previously after overnight starvation (21). The 8 gilts in each group were killed by electrocution to obtain uteri. Immediately, uteruses were placed on ice for transport to the laboratory. For confirmation of pregnancy on day 14 of gestation, both horns were flushed with 20 mL of PBS and examined for the presence of morphologically normal conceptuses as described previously (22). Strips of epithelial endometrium (1 × 5 cm) were then collected at 3 different attachment sites of each horn (23). On day 28 of gestation, the placenta and endometrium were carefully isolated from the uterus of individual fetuses. Uterine weight, corpora lutea number, total number of fetuses, total number of viable fetuses, fetal weight, placental weight, and volume of amniotic fluid were measured and recorded. All tissue samples were immediately snap frozen in liquid nitrogen and stored at −80°C. One gilt from the control group on day 14 of gestation and one gilt from the NCG group on day 28 of gestation were not pregnant at the time of slaughter and were therefore removed from the experiment.

Analysis of plasma amino acids, hormones, and NO. Plasma amino acids were analyzed via ion-exchange chromatography using the Amino Acid Analyzer S 433D (Sykam) as described previously (9). Serum concentrations of progesterone (cat. no. ab108670; Abcam), estradiol (cat. no. KGE014; R&D Systems), and nitrite and nitrate (cat. no. 23479; Sigma-Aldrich) were analyzed using assay kits according to the manufacturers' protocols.

Endometrial proteomics assays. Three individual endometrial samples were randomly chosen from 3 confirmed pregnant gilts in each group to conduct iTRAQ assays on days 14 and 28 of pregnancy. Proteins were extracted and digested as described previously (24). Peptides from samples were reconstituted in 0.5 mol/L tetraethylammonium bromide and subsequently labeled with iTRAQ 4-plex reagents (AB SCIEX) as follows: tag 114 for the control group on day 14 of gestation, tag 115 for the NCG group on day 14 of gestation, tag 116 for the control group on day 28 of gestation, and tag 117 for the NCG group on day 28 of gestation. Labeled samples were mixed and fractionated into 13 fractions by a strong cation-exchange chromatography system (Thermo Scientific). LC-electrospray ionization-MS/MS analysis was performed with a Triple TOF 5600 plus system (AB SCIEX) as described previously (25). The original MS/MS file data were submitted to ProteinPilot Software version 4.5 (AB SCIEX) for data analysis. For protein identification, the Paragon algorithm integrated into ProteinPilot was used against the Uniprot *Sus scrofa* database. Only unique peptides were contained for iTRAQ labeling quantification, and only data with a false discovery rate of <1% were used for subsequent analysis. Protein quantification was performed using 3 individual iTRAQ assays. Bioinformatics analysis was conducted as described previously (26).

RNA extraction and RT-PCR analysis. Total RNA extraction and real-time qRT-PCR was performed as described previously (27), with a

TABLE 1 Reproductive performance on day 28 of gestation of gilts fed a basal diet that was or was not supplemented with 0.05% NCG from days 0 to 28 of gestation[1]

Variable	Control[2]	NCG[3]
Body weight at breeding, kg	132 ± 2	132 ± 2
Body weight on day 28 of gestation, kg	148 ± 3	153 ± 2
Total fetus, *n*	11.3 ± 0.3	12.6 ± 0.5*
Live fetus, *n*	11.0 ± 0.3	12.3 ± 0.5*
Corpora lutea, *n*	14.3 ± 0.5	13.9 ± 0.6
Embryonic mortality, %	22.9 ± 2.5	11.1 ± 3.0*
Uterine weight, kg	1.30 ± 0.07	1.36 ± 0.04
Total viable fetal weight, g	12.8 ± 0.4	15.1 ± 0.4**
Total placental weight, g	202 ± 5	228 ± 9*
Total amniotic fluid volume, L	2.71 ± 0.15	3.20 ± 0.07*

[1] Values are means ± SEMs, n = 8 (control) or 7 (NCG). *$P < 0.05$, **$P < 0.01$ compared with the control group. NCG, *N*-carbamylglutamate.
[2] Gilts fed the basal diet.
[3] Gilts fed the basal diet supplemented with 0.05% NCG.

[7] Abbreviations used: EF1A, elongation factor-1α; eNOS, endothelial NO synthase; *ITGAV*, integrin-αv; *ITGB3*, integrin-β3; iTRAQ, isobaric tags for relative and absolute quantification; mTOR, mammalian target of rapamycin; NCG, *N*-carbamylglutamate; *NOS3*, NO synthase 3; PRX2, peroxiredoxin 2; PRX5, peroxiredoxin 5; PRX6, peroxiredoxin 6; *TLN1*, talin.

TABLE 2 Concentrations of free amino acids in the plasma on days 14 and 28 of gestation of gilts fed a basal diet that was or was not supplemented with 0.05% NCG from days 0 to 28 of gestation[1]

Amino acid	Control[2]	NCG[3]
Day 14 of gestation		
Glutamate	172 ± 7	201 ± 8*
Ornithine	90 ± 6	129 ± 4**
Arg	192 ± 7	250 ± 13**
Pro	277 ± 13	317 ± 11*
Day 28 of gestation		
Glutamate	153 ± 9	216 ± 21*
Ornithine	109 ± 6	135 ± 3**
Arg	195 ± 7	271 ± 20**
Pro	343 ± 16	402 ± 18*

[1] Values (expressed as μmol/L) are means ± SEMs, $n = 7$ (control) or 8 (NCG) on day 14; $n = 8$ (control) or 7 (NCG) on day 28. *$P < 0.05$, **$P < 0.01$ compared with the control group. NCG, *N*-carbamylglutamate.
[2] Gilts fed the basal diet.
[3] Gilts fed the basal diet supplemented with 0.05% NCG.

modified PCR system consisting of 5 μL of SYBR Premix Ex Taq (Takara), 0.3 μL each of forward and reverse primers (10 mM), 0.2 μL of ROX reference dye (Takara), 1 μL of cDNA, and 3.2 μL of double-distilled water. Primers for the selected genes were designed using Primer Premier 6.0 software (Premier; **Supplemental Table 2**).

Western blot analysis. Western blot analysis in the endometrium samples on day 14 of gestation was conducted as described previously (13). Primary antibodies (1 μg/mL) against integrin-αv, integrin-β3, talin, and eNOS (Santa Cruz Biotechnology) were used. Blots were stripped and reprobed with anti–β-actin antibody (Cell Signaling Technology) to demonstrate equal loading.

Statistical analysis. For results of reproductive performance, plasma biochemical indexes, qRT-PCR, and Western blot, data were analyzed using the Student's *t* test procedures of SAS software (version 9.0; SAS Institute). Each gilt was considered the experimental unit for analysis. Data on embryonic mortality were analyzed using the χ^2-test of SAS. A value of $P < 0.05$ was considered statistically significant. For analysis of proteomic results, within each iTRAQ assay, differentially expressed proteins were determined based on the ratios of differently labeled proteins and *P* values provided by ProteinPilot. Fold changes of proteins from samples on days 14 and 28 of gestation were calculated as the average ratio of tag 115/114 and 117/116, respectively. Compared with the control group, proteins with a threshold of >2- or <0.5-fold and values of $P < 0.05$ were considered differentially expressed proteins. All values are presented as means ± SEMs.

Results

Reproductive performance of gilts on day 28 of gestation. Compared with the control group, dietary NCG supplementation during early gestation increased the number of total fetuses per litter and viable fetuses per litter by 1.32 ($P < 0.05$) and 1.29 ($P < 0.05$), respectively, and decreased embryonic mortality by 11.8% ($P < 0.05$; **Table 1**). The litter weight of viable fetuses was 18% higher ($P < 0.05$) for the NCG group than for the control group. Total placental weight and total amniotic fluid volume were increased by 13% and 18% ($P < 0.05$; Table 1) in the NCG group compared with the control group. However, the body weight of gilts, uterine weight, and the number of corpora lutea did not differ between the NCG and the control groups (Table 1).

Plasma concentration of amino acids, hormones, and NO. Plasma concentrations of glutamate, ornithine, Arg, and Pro were 17%, 43%, 30%, and 14% higher, respectively, in gilts fed the NCG-supplemented diet than in gilts fed the control diet on day 14 of gestation ($P < 0.05$; **Table 2**). Similarly, plasma concentrations of glutamate, ornithine, Arg, and Pro were 41%, 24%, 39%, and 17% higher, respectively, in gilts fed the NCG-supplemented diet than in gilts fed the control diet on day 28 of gestation ($P < 0.05$; Table 2). Concentrations of plasma estradiol and progesterone on both day 14 and day 28 of gestation did not differ between the NCG and control groups (**Table 3**). Concentrations of plasma NO on days 14 and 28 of gestation were increased by 49% and 55%, respectively, in the NCG group compared with the control group ($P < 0.05$; Table 3).

Differentially expressed proteins in the endometrium affected by NCG supplementation. A total of 28,125 peptides and 26,678 unique peptides from trypsin-digested proteins were identified. In total, our approach allowed the identification and quantification of 1528 proteins by searching the database of Uniprot *Sus scrofa*. Among the identified proteins, a total of 59 proteins showed >2-fold changes between the control and the NCG group ($P < 0.05$), including 32 upregulated proteins and 4 downregulated proteins on day 14 of gestation (**Table 4**), as well as 34 upregulated proteins and 4 downregulated proteins on day 28 of gestation (**Table 5**). The differentially expressed proteins were assigned to 21 biological processes, 8 cellular components, and 6 molecular functions on the basis of gene ontology enrichment analysis (**Supplemental Figure 1**).

Compared with the control group, a total of 52 differentially expressed proteins showed >2-fold increases in the NCG group ($P < 0.05$), which primarily are involved in carbohydrate and energy metabolism, amino acid and protein metabolism, lipid transport and metabolism, cell adhesion, immune response, antioxidative stress, cell cytoskeleton and mobility, biological regulation, and signal transduction. Particularly, 7 proteins primarily involved in cell adhesion on day 14 of gestation showed higher abundance in the NCG group ($P < 0.05$), including integrin-αv, integrin-β3, talin, E-cadherin, calreticulin, dipeptidyl peptidase 4, and hyaluronan and proteoglycan link protein 1. A total of 7 proteins showed less abundance in NCG-supplemented gilts than in the control gilts, including

TABLE 3 Concentrations of NO and hormones in the plasma on days 14 and 28 of gestation of gilts fed a basal diet that was or was not supplemented with 0.05% NCG from days 0 to 28 of gestation[1]

Variable	Control[2]	NCG[3]
Day 14 of gestation		
NO, μmol/L	19.0 ± 2.0	28.3 ± 1.8*
Estradiol, pg/mL	135 ± 9	131 ± 9
Progesterone, μg/L	29.2 ± 2.4	28.1 ± 2.0
Day 28 of gestation		
NO, μmol/L	17.9 ± 2.1	27.8 ± 2.7*
Estradiol, pg/mL	103 ± 12	109 ± 14
Progesterone, μg/L	26.4 ± 1.2	27.9 ± 1.5

[1] Values are means ± SEMs, $n = 7$ (control) or 8 (NCG) on day 14; $n = 8$ (control) or 7 (NCG) on day 28. *$P < 0.05$ compared with the control group. NCG, *N*-carbamylglutamate.
[2] Gilts fed the basal diet.
[3] Gilts fed the basal diet supplemented with 0.05% NCG.

TABLE 4 Differentially expressed proteins in the endometrium on day 14 of gestation of gilts fed a basal diet that was or was not supplemented with 0.05% NCG[1]

Accession no.	Protein name	Fold change
Carbohydrate and energy metabolism		
trIK9IVM5	GTPase IMAP family member 4	4.20 ± 0.40
trIW5TZE9	Guanylate-binding protein 1	3.88 ± 0.18
trII3LP41	Malate dehydrogenase	3.21 ± 0.62
trID0G7F6	Triosephosphate isomerase	0.47 ± 0.03
Amino acid and protein metabolism		
trID0G0C6	Asn synthetase	4.98 ± 0.36
trIQ0PY11	EF1A	2.65 ± 0.30
trIQ5MJE5	Cathepsin D protein (fragment)	0.42 ± 0.03
trIF1SEN2	Glutamate dehydrogenase 1, mitochondrial	3.80 ± 0.66
trIB0LY44	Mitochondrial ornithine aminotransferase	0.47 ± 0.01
Lipid transport and metabolism		
trIQ95JG9	Carnitine palmitoyltransferase I	4.06 ± 0.15
trIF1SCY4	Lipase	0.46 ± 0.04
Cell adhesion		
trIB5B2Z3	αv-Integrin subunit	3.72 ± 0.27
spIP28491ICALR	Calreticulin	2.78 ± 0.13
trIQ95JH1	Integrin-β3	4.12 ± 0.44
trIK9IWI4	Talin 1	2.37 ± 0.10
trIC6EVT4	E-cadherin	4.29 ± 0.21
spIP22411IDPP4	Dipeptidyl peptidase 4	6.23 ± 0.08
spIP10859IHPLN1	Hyaluronan and proteoglycan link protein 1	3.32 ± 0.22
Immune response		
spIP80015ICAP7	Azurocidin	11.0 ± 0.7
trIC3S7K4	Calcium-binding protein A12	4.21 ± 0.28
trIQ6YT39	Lactotransferrin	7.95 ± 0.67
spIQ64L94IPSME1	Proteasome activator complex subunit 1	2.62 ± 0.20
Antioxidative stress		
trIQ9GLW8	PRX5	5.81 ± 0.14
spIQ9TSX9IPRDX6	PRX6	3.26 ± 0.10
trIF1RGJ3	Stress-70 protein, mitochondrial	2.49 ± 0.32
Cell cytoskeleton and mobility		
trIK9J6J2	Erythrocyte band 7 integral membrane protein isoform a	4.27 ± 0.11
spIQ9TV36IFBN1	Fibrillin	2.16 ± 0.08
Biological regulation and signal transduction		
trIV5KX18	Cytochrome c oxidase subunit 2	9.35 ± 0.47
trID0G6S0	Cytochrome P450, family 51, subfamily A, polypeptide 1	3.40 ± 0.21
trIF8TEL5	Eukaryotic translation initiation factor-4γ1	2.48 ± 0.16
trIK9IWF9	Ras GTPase–activating-like protein	2.55 ± 0.27
trIQ95MF8	Sulfotransferase	2.11 ± 0.07
trIQ28969	NOS3	5.36 ± 0.38
Other biological processes		
trII3LR69	Ferritin	2.59 ± 0.26
trIF1RIP3	Ferritin	3.29 ± 0.06
trID0G6Y1	Hydroxysteroid (17-β) dehydrogenase 2	8.31 ± 1.11

[1] Values are means ± SEMs, $n = 3$. Fold changes of proteins were calculated as the average ratio of tag 115/114. Compared with the control group, proteins with a threshold of >2- or <0.5-fold and $P < 0.05$ were considered differentially expressed proteins. EF1A, elongation factor 1α; IMAP, immunity-associated protein; NCG, *N*-carbamylglutamate; NOS3, NO synthase 3; PRX5, peroxiredoxin 5; PRX6, peroxiredoxin 6.

4 proteins on day 14 of gestation (triosephosphate isomerase, cathepsin D protein, mitochondrial ornithine aminotransferase, and lipase) and 4 proteins on day 28 of gestation (creatine kinase, cathepsin D protein, catalase, and proliferating cell nuclear antigen).

A total of 15 proteins were differentially expressed on both day 14 and day 28 of gestation (**Supplemental Figure 2**). Among these, 14 were upregulated and 1 was downregulated by NCG supplementation. These proteins primarily were involved in amino acid and protein metabolism [Asn synthetase, elongation factor-1α (EF1A), and cathepsin D protein], cell adhesion (integrin-αv, calreticulin, and integrin-β3), immune response (azurocidin and calcium-binding protein A12), antioxidation and stress response [peroxiredoxin 6 (PRX6) and stress-70 protein], cell cytoskeleton and mobility (erythrocyte band 7 integral membrane protein and fibrillin-1), and biological regulation and signal transduction (Ras GTPase-activating–like protein, sulfotransferase and NO synthase).

TABLE 5 Differentially expressed proteins in the endometrium on day 28 of gestation of gilts fed a basal diet that was or was not supplemented with 0.05% NCG[1]

Accession no.	Protein name	Fold change
Carbohydrate and energy metabolism		
trIF1SFF8	α-1,4-Glucan phosphorylase	6.36 ± 0.07
trII7HD36	Sodium/potassium-transporting ATPase subunit-α-1 (fragment)	3.18 ± 0.15
trII3LPB5	Creatine kinase B-type (Fragment)	0.33 ± 0.02
Amino acid and protein metabolism		
trID0G0C6	Asn synthetase	10.2 ± 0.2
spIA5GFY8ISERA	D-3-Phosphoglycerate dehydrogenase	10.3 ± 0.6
trIQ0PY11	EF1A	2.38 ± 0.10
trIQ5MJE5	Cathepsin D protein (fragment)	0.48 ± 0.04
Lipid transport and metabolism		
spIP79274IACADL	Long-chain specific acyl-CoA dehydrogenase, mitochondrial	3.51 ± 0.18
Cell adhesion		
trIB5B2Z3	αv-Integrin subunit	4.57 ± 0.12
spIP28491ICALR	Calreticulin	5.20 ± 0.15
trIQ95JH1	Integrin-β3	3.65 ± 0.16
spIP26234IVINC	Vinculin	2.51 ± 0.14
Immune response		
spIP80015ICAP7	Azurocidin	14.4 ± 0.7
trIC3S7K4	Calcium-binding protein A12	11.0 ± 1.2
trIF1SJB5	Annexin 1	5.35 ± 0.35
Antioxidative stress		
trIF1SDX9	PRX2	4.63 ± 0.28
spIQ9TSX9IPRDX6	PRX6	4.24 ± 0.11
trIF1RGJ3	Stress-70 protein, mitochondrial	2.38 ± 0.14
trIF1SGS9	Catalase	0.39 ± 0.04
Cell cytoskeleton and mobility		
spIQ9XSD9IPGS2	Decorin	4.66 ± 0.16
trIK9J6J2	Erythrocyte band 7 integral membrane protein isoform a	2.24 ± 0.12
spIQ9TV36IFBN1	Fibrillin	2.85 ± 0.24
trIF2Z584	Histone H2B	7.06 ± 0.73
trIF1RLQ2	Prelamin-A/C	13.6 ± 1.7
trIK9IWG6	Spectrin beta chain, brain 1 (fragment)	3.07 ± 0.16
spIP02543IVIME	Vimentin	8.18 ± 0.36
Biological regulation and signal transduction		
trIK7GMQ7	Cytochrome b-245 heavy chain	2.53 ± 0.32
trII3L813	Proliferating cell nuclear antigen	0.29 ± 0.03
trIK9IWF9	Ras GTPase-activating–like protein	9.04 ± 0.89
trIQ3S3F7	Sulfotransferase	10.2 ± 0.2
trIQ95MF8	Sulfotransferase	9.46 ± 0.40
trIQ28969	NOS3	5.03 ± 0.31
Other biological processes		
trIF5XVC2	von Willebrand factor	2.68 ± 0.15
trIF1S073	Annexin 2	4.81 ± 0.29
trID0G0C5	Annexin 4	6.03 ± 0.41
trIF2Z5C1	Annexin 5	5.75 ± 0.27
spIP79381IHYEP	Epoxide hydrolase 1	2.31 ± 0.12
spIQ95250IPGRC1	Membrane-associated progesterone receptor component 1	11.1 ± 0.8

[1] Values are means ± SEM, $n = 3$. Fold changes of proteins were calculated as the average ratio of tag 117/116. Compared with the control group, proteins with a threshold of >2- or <0.5-fold and values of $P < 0.05$ were considered differentially expressed proteins. EF1A, elongation factor-1α; NCG, *N*-carbamylglutamate; NOS3, NO synthase 3; PRX2, peroxiredoxin 2; PRX6, peroxiredoxin 6.

RT-PCR and Western blot analysis. Western blot analyses of 4 differentially expressed proteins were conducted to validate iTRAQ results. RT-PCR analyses were conducted to evaluate the mRNA expression of the 4 corresponding genes. We focused on the pathways involved in the process of embryonic implantation and survival during early gestation; therefore, proteins (integrin-αv, integrin-β3, talin, and eNOS) closely related to cell adhesion and embryonic/fetal development, as well as the corresponding genes [*ITGAV*, *ITGB3*, NO synthase 3 (*NOS3*), and *TLN1*], were selected.

In samples from gilts on day 14 of gestation, the 4 genes (*ITGAV*, *ITGB3*, *NOS3*, and *TLN1*) displayed similar expression patterns to their protein abundances of iTRAQ results (**Figure 1**A). In samples from gilts on day 28 of gestation, the mRNA expression of *ITGAV*, *NOS3*, and *TLN1* was consistent with their protein abundances from iTRAQ results, whereas the mRNA

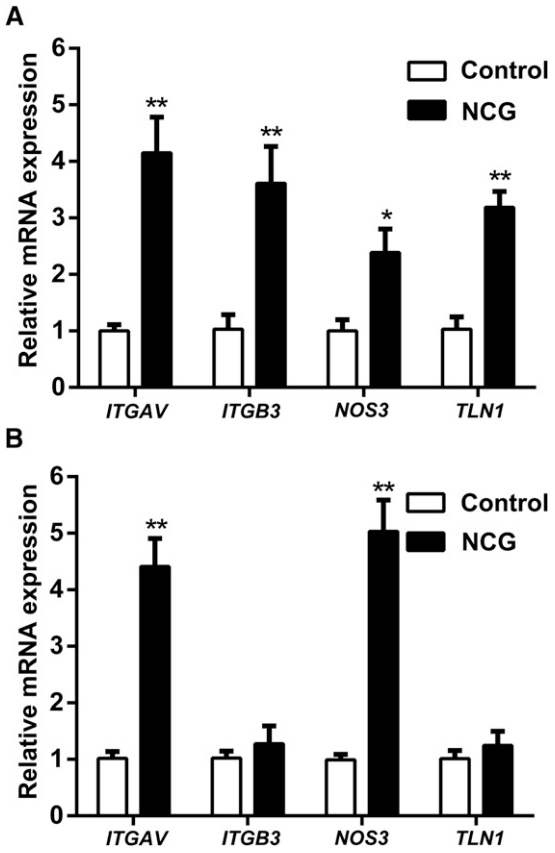

FIGURE 1 The relative mRNA expression in the endometrium tissue on days 14 (A) and 28 (B) of gestating gilts fed a basal diet that was or was not supplemented with 0.05% NCG from days 0 to 28 of gestation. Gilts in the control group were fed the basal diet; gilts in the NCG group were fed the basal diet supplemented with 0.05% NCG. Values are means ± SEMs, $n = 6$. $^*P < 0.05$, $^{**}P < 0.01$ compared with the control group. *ITGAV*, integrin-αv; *ITGB3*, integrin-β3; NCG, *N*-carbamylglutamate; *NOS3*, NO synthase 3; *TLN1*, talin.

expression of *ITGB3* showed poor agreement with protein expression abundance (Figure 1B). The Western blot results of integrin-αv, integrin-β3, talin, and eNOS were consistent with the findings from the iTRAQ analysis (**Figure 2**).

Discussion

The reproduction process in humans is relatively inefficient compared with other mammals (28). It has been estimated that ~20–50% of spontaneous conceptions are lost during early pregnancy (29). Likewise, the incidence of embryonic mortality is extremely high in pigs by the 25th day of pregnancy (8). Unfortunately, there are few effective methods to reduce such a high loss of embryos during early pregnancy (30). Therefore, strategies to alleviate early pregnancy loss are of great significance for the improvement of reproductive efficiency and health in humans and animals. To our knowledge, the present study provides the first evidence that maternal NCG supplementation during early pregnancy enhances embryonic survival and development through modulation of the endometrial proteome, which is of critical importance for embryonic implantation and development.

Protein expression differences between days 14 and 28 of gestation. During the early stages of gestation, the 2 critical processes in mammalian reproduction are embryonic implantation and placentation (31, 32), both of which are regulated by a series of different pregnancy-related proteins. The initiation of embryonic attachment to the endometrium occurs on day 14 of gestation in pigs (33). The major physiologic developmental event that occurs after attachment is the initial growth and development of the placenta. Consistent with these views, our results showed different expression patterns of the endometrial proteome on days 14 and 28 of gestation. A total of 21 and 23 proteins were exclusively differentially expressed on days 14 and 28 of gestation, respectively. On day 14 of gestation, differentially expressed proteins primarily were involved in embryonic implantation and maternal preparation for a receptive uterus, and on day 28 of gestation, differentially expressed proteins were primarily involved in placental growth and nutrient transport from the mother to the fetus. Therefore, we focused on different points for the following discussion of the proteomics results on days 14 and 28 of gestation.

Cell adhesion proteins. Among the 32 upregulated proteins in the endometrium on day 14 of gestation, 5 participated in the cell adhesion pathway mediated by integrin, including integrin-αv, β3-subunits, talin, calreticulin, and dipeptidyl peptidase IV. Integrin-mediated adhesion plays major roles in mammalian reproduction as modulators of cellular function through both attachment and signal transduction (34). The binding of talin to integrin-β cytoplasmic domains is the final step in integrin activation (35). Calreticulin, a high-affinity Ca^{2+}-binding protein, is essential for integrin-mediated cell adhesion to extracellular matrix by associating with the cytoplasmic domains of integrin-α subunits (36). Dipeptidyl peptidase IV has been reported to be associated with integrin-dependent adhesion by regulating MAPK-dependent phosphorylation of integrins (37). Our results indicate that NCG may enhance embryonic implantation via the integrin-mediated signaling pathway, thus decreasing the rate of embryonic losses during early pregnancy. However, the mechanisms involved need further investigation. E-cadherin is also a primary adhesion molecule implicated in embryonic implantation (33). As a marker of the receptive endometrium, E-cadherin plays a central role during implantation by maintaining cell-cell linkages and signaling (38). The upregulated expression of cadherin in the NCG group may indicate a more receptive uterus, which likely enhances embryonic implantation and survival.

Proteins related to carbohydrate, lipid, and protein metabolism. A total of 13 differentially expressed proteins participate in the metabolism of carbohydrates (e.g., malate dehydrogenase), lipids (e.g., carnitine palmitoyltransferase I), and proteins (e.g., EF1A). Malate dehydrogenase is the crucial enzyme that catalyzes the reversible interconversion of malate and malate-oxaloacetic acid (39). Carnitine palmitoyltransferase I esterifies long-chain FAs to carnitine, thereby initiating mitochondrial import (40). The increased expression of these proteins in the NCG group probably indicated an enhanced endometrial glucose and lipid metabolism. EF1A is one of the most important factors participating in protein biosynthesis (41). Large increases in mRNA levels for EF1A were observed in rapidly proliferating cells and embryos, which indicate that the level of expression of EF1A correlates with the rate of cell growth and proliferation (42). Previous studies have shown that NCG supplementation enhances protein synthesis in the skeletal muscle of piglets (43) and improves the intestinal absorptive function in weaned piglets by increasing the expression of amino acid transporters in the intestine (44). An adequate endometrial nutrient metabolism is an essential part of endometrial differentiation and decidualization

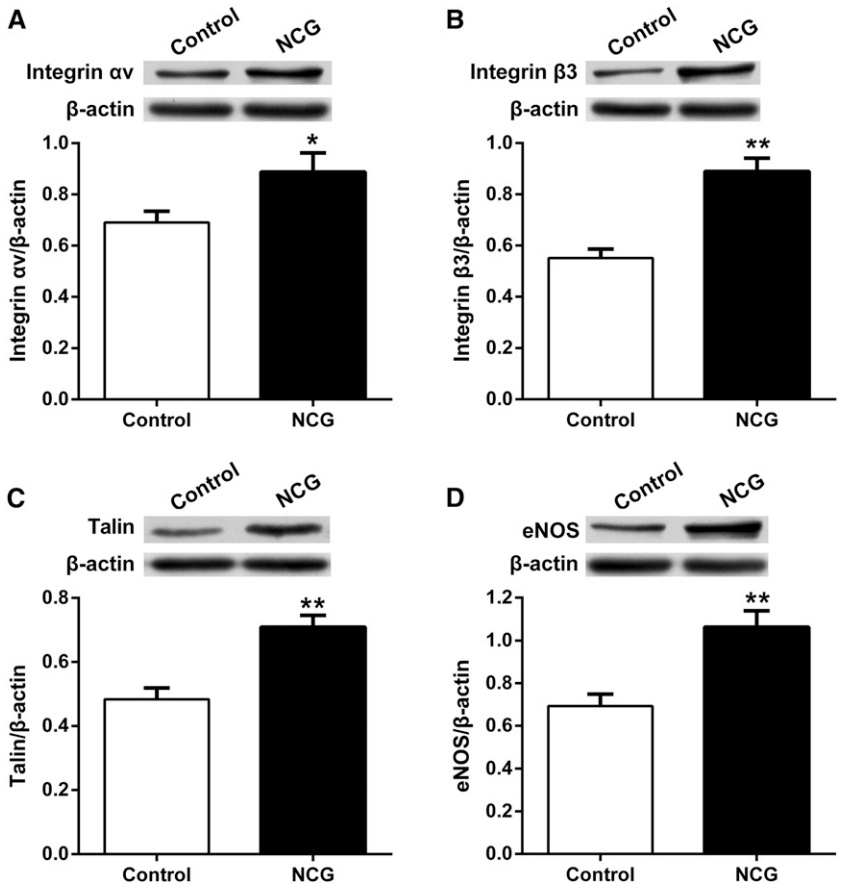

FIGURE 2 Western blot analysis of integrin-αv (A), integrin-β3 (B), talin (C), and eNOS (D) protein abundance in the endometrium tissue on day 14 of gestating gilts fed a basal diet that was or was not supplemented with 0.05% NCG from days 0 to 14 of gestation. Representative Western blots are shown. Gilts in the control group were fed the basal diet; gilts in the NCG group were fed the basal diet supplemented with 0.05% NCG. Values are means ± SEMs, $n = 6$. $^*P < 0.05$, $^{**}P < 0.01$ compared with the control group. eNOS, endothelial NO synthase; NCG, *N*-carbamylglutamate.

to provide a nutritional and receptive environment (45). Therefore, enhanced endometrial metabolism of carbohydrates, lipids, and proteins likely promoted early embryonic survival in NCG-supplemented gilts and positively affected fetal survival and growth in subsequent stages of gestation.

Proteins related to antioxidative stress and immune response. This study identified 10 endometrial proteins that were involved in antioxidative stress and immune response, such as stress-70 protein, azurocidin, and peroxiredoxins. The stress-70 protein, a member of the heat shock protein family, responds to heat or other stresses and is induced for maintaining cellular homeostasis (46). The increased abundance of stress-70 protein in the NCG-supplemented group indicated an enhanced ability to protect gilts against stress. It was reported that azurocidin might act to alarm the immune system and thus serve as an important mediator during the initiation of the immune response (47). Dietary NCG supplementation has been reported to benefit intestinal mucosal immunity in *Escherichia coli*–challenged piglets (48). Our results in the present study showed that 3 members of the peroxiredoxin family were upregulated by NCG supplementation, including peroxiredoxin 2 (PRX2), peroxiredoxin 5 (PRX5), and PRX6. PRX2 contributes to the protection against hydrogen peroxide–induced cell damage and apoptosis (49). Likewise, PRX6 acts as an antioxidant protecting cells and tissues against peroxide or hydroxide-mediated oxidative stress (50). Similar results of upregulated expression of PRX2 and PRX6 were reported in pregnant pigs (51). Antioxidant supplementation may be a potential strategy to overcome reproductive disorders associated with infertility (52). The changes in these proteins could enhance antioxidative reactions and immune response, which may result in a better uterine environment for embryonic and fetal survival.

Other proteins closely related to embryonic/fetal development. Our results showed significantly increased abundances in several members of the annexin family, including annexin A1, A2, A4, and A5, in the endometrium on day 28 of gestation. Annexins are a family of calcium- and phospholipid-binding proteins that are essential for the survival and growth of a developing embryo/fetus (53). The changes in annexins may promote embryonic/fetal survival in NCG-supplemented gilts by attenuating inflammatory response in the uterus (54) and enhancing blood circulation in both the endometrium and the placenta (55). Consistent with a previous study (56), eNOS was upregulated in response to NCG supplementation. NO is a powerful vasodilator that may promote embryonic implantation and fetal development (57). Another finding of the present study is that NCG supplementation enhanced placental growth in gestating gilts. Similarly, enhancement of placental growth and function was observed in Arg-supplemented gilts, which indicated that maternal Arg supplementation provided an effective solution to improve embryonic survival and fetal development (58). Collectively, NCG supplementation may enhance embryonic survival and growth by regulating the activity of eNOS and enhancing placental development.

In conclusion, our data demonstrated that NCG supplementation during early pregnancy enhanced embryonic survival and development in gilts and, ultimately, increased the total number of fetuses, live fetuses, and total viable fetus weight. To our knowledge, our results provide the first evidence for an alteration of the endometrial proteome in response to NCG supplementation in

gilts, which are primarily involved in cell adhesion, energy metabolism, lipid metabolism, protein metabolism, antioxidative stress response, and immune response. Collectively, these findings may be the major mechanisms responsible for improvement in early embryonic survival, as well as fetal and placental development induced by maternal NCG supplementation. These findings have profound implications for NCG application in boosting embryonic survival and development in humans and animals.

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